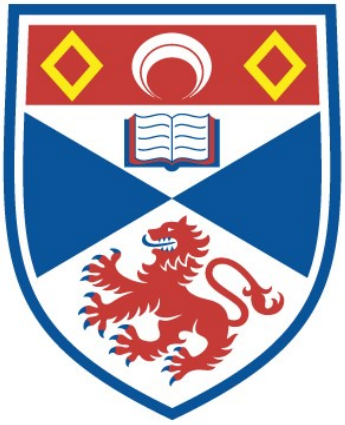
TRANSCRIPTIONAL ACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 BY NF-κB

James Robert Matthews

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1993

Transcriptional activation of human immunodeficiency virus type 1 by NF-κB

by

James Robert Matthews

A Thesis Presented for the Degree of

Doctor of Philosophy

in

The Faculty of Science

at

The University of St.Andrews

Division of Cell and Molecular Biology

Irvine Building

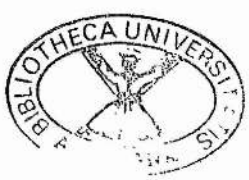
North Street

St. Andrews

Fife

KY16 9AL

September 1992



DECLARATION FOR THE DEGREE OF Ph.D.

(a) I James Robert Matthews hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed..... **Date**...1/10/92.....

(b) I was admitted to the Faculty of Science of the University of St.Andrews under Ordinance General No. 12 on 1st October 1988 and as a candidate for the degree of Ph.D. on 1st October 1988.

Signed..... **Date**...1/10/92.....

(c) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor.....**Date**...15/12/92.....

(d) In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and and supplied to any *bona fide* library or research worker.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I am very grateful to Dr. Ronald T. Hay for his supervision and guidance during the course of these studies. Thanks are also due to other members of Lab 28 for their help and useful comments over the last four years. Especial thanks are due to John Nicholson for his efforts with HPLC and tissue culture, to Bill Blyth for his excellent photographic support, and to Ian Armitt for provision of synthetic oligonucleotides. Thanks are also due to members of my family for their unwavering encouragement during this period.

During the course of these studies, the author was a recipient of a Medical Research Council AIDS Directed Programme Research Studentship for postgraduate training.

SUMMARY

This study has analysed some of the mechanisms involved in the transcriptional activation of the human immunodeficiency virus type 1 (HIV-1) by transcriptional modulator proteins of the NF-κB/rel family.

Initial attempts to purify a single NF-κB (p50-p65) heterodimer species from HeLa cells suggested that a family of proteins might contribute to κB motif DNA binding activity. HeLa cell κB motif DNA binding proteins were shown to be modified by glycosylation. Using circularly permuted DNA probes carrying a κB motif, it was shown that κB binding proteins induced significant DNA bending upon binding, while studies of the effects of the polyamine spermidine on purified HeLa cell κB motif DNA binding proteins showed it greatly stimulated their DNA binding activity.

The DNA binding activity of native κB motif DNA binding proteins was also greatly stimulated by the reducing agent dithiothreitol. Using a cDNA encoding the p105 precursor to the NF-κB p50 subunit, the wild type DNA binding and dimerisation region (aa35-381) of p50, and three cysteine to serine mutants at cysteine residues (aa62, 119, and 273) conserved throughout the NF-κB/rel/dorsal family, were expressed in bacteria. The dissociation constant of the aa62 p50 mutant for the κB motif was 10-fold higher than that of the wild type p50. Also, dissociation rate constants for the aa62 mutant-κB motif complex in both the presence and absence of spermidine were anomalously high. The above changes suggested a different DNA binding specificity for the aa62 mutant - this was confirmed by oligonucleotide competition studies. Oligonucleotide protection experiments suggested the presence of a cysteine residue in the p50 DNA binding

site - substrate protection experiments showed that this was cysteine 62, and that this residue is involved in redox regulation of p50 DNA binding activity.

ABBREVIATIONS

A	adenine
A	absorbance
APS	ammonium persulphate
ATP	adenosine-5'triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
CNBr	cyanogen bromide
cpm	counts per minute
dATP	2'-deoxyadenosine-5'triphosphate
dCTP	2'-deoxycytidine-5'triphosphate
dGTP	2'-deoxyguanosine-5'triphosphate
dNTP	2'-deoxynucleoside-5'triphosphate
dTTP	2'-deoxythymidine-5'triphosphate
DEAE	diethylaminoethyl
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide

G	guanine
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HTLV-I	human T-cell leukaemia virus type I
HTLV-II	human T-cell leukaemia virus type II
KOAc	potassium acetate
LTR	long terminal repeat
mRNA	messenger ribonucleic acid
NaOAc	sodium acetate
n-OGP	n-octylglucopyranoside
NP40	nonidet p40
PMSF	phenylmethylsulphonylfluoride
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV40	simian virus 40
T	thymine
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol, (tris)
UV	ultraviolet
v/v	volume to volume ratio
WGA	wheat germ agglutinin
w/v	weight to volume ratio

INTRODUCTION.

1. Natural history of human immunodeficiency virus type 1.

The causative agents of the acquired immunodeficiency syndrome (AIDS) have been identified as the human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) (reviewed in Vaishnav and Wong-Staal, 1991; Haseltine, 1991) - these are retroviruses - RNA-containing viruses which replicate via a DNA intermediate by use of a virally-encoded RNA-dependent DNA polymerase, also known as reverse transcriptase. The retrovirus family can be divided into three subfamilies:- oncoviridae including all oncogenic retroviruses and many closely related non-oncogenic viruses; lentiviridae including the "slow" viruses such as visna-maedi virus, equine infectious anaemia virus (EIAV), and the various immunodeficiency viruses; and the spumaviridae comprising the "foamy " viruses which induce persistent infections with vacuolisation of cultured cells but without causing clinical disease.

This short survey will concentrate on the better characterised and more pathogenic human immunodeficiency virus type 1 (HIV-1). This is a complex retrovirus - in addition to genes encoding the virus particle and replicative enzymes common to all retroviruses, HIV-1 encodes at least six additional proteins which regulate the viral life cycle. The virus particle contains an inner core which contains the diploid, single-stranded RNA genome, as well as viral enzymes and other factors needed for early replication events. This inner core is, in turn, surrounded by capsid proteins, and the capsid is surrounded by a lipid bilayer envelope. A viral matrix protein is inserted into the inner surface of the lipid bilayer envelope, and a viral integral membrane protein, the envelope

glycoprotein, protrudes through the membrane and forms the outer surface of the virion.

Infection begins when an HIV-1 virus particle or a cell producing virus particles encounters a cell with a high affinity receptor for the virus - a specific high affinity binding reaction occurs between the viral envelope glycoprotein and a target cell CD4 molecule (Dalgleish et al., 1984). The CD4 surface glycoprotein molecule is found at low concentration on monocytes, macrophages, and antigen-presenting dendritic cells, but is present at high levels on the surface of immature T-cells, as well as mature $CD4^+$ T helper cells. There have been suggestions that HIV-1 may enter cells which lack the CD4 molecule via a low efficiency membrane fusion reaction (Clapham et al., 1989).

After the high affinity binding reaction, viral entry to the cell occurs by fusion of viral and cell membranes. The fusion reaction is mediated by the viral envelope glycoprotein, the external subunit of which specifies binding to CD4, while the transmembrane subunit is responsible for the membrane fusion event. Viral envelope glycoprotein-mediated reactions are cytopathic for cells carrying large numbers of CD4 molecules - by fusion reactions beween budding virus and cell surface CD4 which destroy the integrity of the plasma membrane (Popovich et al., 1984), and by fusion reactions with uninfected $CD4^+$ cells to generate non-viable, giant multinucleated cells.

The HIV-1 viral genomic RNA is converted into DNA by the action of virally-encoded RNA-dependent DNA polymerase and ribonuclease H activities present within the viral core. This results in the conversion of the diploid single-stranded RNA viral genome into a double-stranded DNA copy within the

first six hours of infection (Farnet and Haseltine, 1990), this reverse transcription process occurring in the cell cytoplasm. The next event in the viral life cycle is the insertion of the linear double-stranded viral DNA into the host genome, this is mediated by a virally-encoded integrase - also packaged within the virus particle. Preintegration complexes of double-stranded viral DNA and integrase protein can be detected in the cytoplasm. The viral integrase protein has three activities:- it trims the ends of the linear double-stranded DNA provirus prior to the recombination reaction; it cleaves the host cell chromosomal DNA; finally the integrase covalently joins the freshly cut ends of the viral and host DNA, followed by repair of the site of insertion by host cell enzymes (Bushman and Craigie, 1991). All the above integration reactions occur in the nucleus, leading to the generation of a proviral DNA which resembles a cellular gene.

Once integrated, the proviral HIV-1 DNA seems to remain permanently associated with the host cell genetic material, where it remains quiescent until the host cell is activated in some way (see Introduction section 2.1). Upon host cell activation, HIV-1 viral expression begins with the synthesis of a full-length RNA copy of the proviral DNA, transcription initiates within the 5' terminally redundant long terminal repeat (LTR) flanking the coding sequences of the provirus.

The initial rate of transcription initiation from the HIV-1 proviral DNA is determined by the activity of a large range of host cell transcription modulatory proteins - including importantly, host cell proteins of the NF-κB/rel family binding to two κB motifs located between nucleotides 80 and 105 upstream of the transcriptional start site (see Results section). The same range of host cell

transcription modulatory proteins is also involved in the transcriptional control of host cell genes - some of these transcription modulator proteins such as the NF-κB/rel family seem to be inactive in most cell types until the cell receives some sort of activation signal (Tong-Starksen et al., 1987).

Thus, proviral DNA is not transcribed into RNA in resting T-cells and as a consequence little HIV-1 virus is produced by this population of infected cells. However, specific or non-specific mitogenic stimulation of the $CD4^+$ T-cell population results in active transcription from the HIV-1 LTR and virus replication. This ability of the HIV-1 provirus to persist in the host cell without viral replication is in part responsible for the long latent period of HIV-1 infections. In addition to the cell-specified transcription modulators giving rise to transcriptional activation from the HIV-1 LTR upon host cell activation, a virally-encoded transactivator (tat) protein can, by binding to a stem-loop structure (the TAR region) at the 5' end of the nascent RNA increase the steady-state level of full-length viral RNA (Laspia et al., 1989) - however this process is dependent on the initial transcription from the LTR mediated by cellular factors, and translation to generate the tat protein.

The full-length viral RNA transcript can be multiply spliced to generate mRNAs whose translation products include the tat protein, and two regulatory proteins rev and nef. The rev protein profoundly affects the fate of primary RNA transcripts within the nucleus - in the absence of rev only small multiply spliced transcripts accumulate in the cytoplasm, whereas in the presence of rev, full-length and singly-spliced viral messenger RNAs (mRNAs) can accumulate in the cytoplasm (Malim et al., 1990). The action of rev is mediated by its binding to a rev

responsive element (RRE), thus in the absence of rev only regulatory proteins made from multiply spliced transcripts are made, while in the presence of rev, unspliced and singly-spliced RNAs are exported and translated to yield the viral capsid protein and envelope glycoprotein (Heaphy et al., 1990).

The full-length viral RNA is translated to yield a capsid polyprotein precursor and a fusion protein of capsid polyprotein precursor with the replicative enzymes, different but overlapping reading frames specify the capsid precursor and replicative enzymes. A ribosomal frameshift event occurs near the 3' end of the capsid genes to allow at low frequency (approximately 5%) the generation of the capsid replication enzyme fusion protein (Jacks et al., 1988).

The capsid precursor polyprotein and the capsid replication enzyme fusion protein assemble at the inner surface of the plasma membrane and bind to the plasma membrane by virtue of the myristylated amino terminal residue of the capsid precursor protein (Veronese et al., 1988). The capsid precursor also binds to a specific packaging sequence near the 5' end of the viral genomic RNA. A complex of capsid precursor protein, capsid precursor replication enzyme fusion protein, and viral RNA then assembles into a closed spherical particle which buds through the plasma membrane (Gelderblom et al., 1987) incorporating the viral envelope glycoprotein as it does so. Interestingly, the newly-budded particle seems to remain attached to the cell surface by a tether before release.

The viral envelope protein is translated from the singly spliced viral mRNA and is cotranslationally translocated at the rough endoplasmic reticulum, where it assembles as a dimer, and where the lumenal part of the envelope protein is heavily modified by approximately twenty complex oligosaccharide chains. The

envelope glycoprotein then enters the Golgi apparatus where it is cleaved into two subunits - the amino terminal region destined to become the external gp120 component, and the carboxyl terminal region which becomes the integral membrane protein gp41 (Allen et al., 1985). The complex carbohydrates are modified during passage through the Golgi apparatus before transport of the gp120/gp41 complex to the cell surface and concentration in the plasma membrane regions of virus budding.

Other virally-encoded regulatory proteins include the vif protein (which may act as a protease to clip the carboxyl terminus of gp41) - the lack of vif function results in progeny HIV-1 being only poorly infectious (Strebel et al., 1987). The vpu protein which facilitates the export of virus particles from the cell (Klimkait et al., 1990), the vpr protein which is packaged within the virus itself and may function in transactivation of viral and cellular promoters (Ogawa et al., 1989), and finally, the nef protein which is expressed soon after viral infection but whose role is unclear (Kim et al., 1989). The late maturation events for the HIV-1 virion include activation of the viral protease to cleave the capsid polyprotein precursors into their mature forms, and the release by an unknown mechanism of the mature HIV-1 virus particle from its tether to the plasma membrane.

2. The NF-κB/rel/dorsal transcription factor family.

2.1. NF-κB as an inducible transcriptional activator

The sequence-specific DNA binding activity termed NF-κB was first identified as a protein which bound to a sequence in the immunoglobulin κ light chain gene major (J-C) intron transcriptional enhancer (Sen and Baltimore, 1986a). This factor initially appeared to be not only tissue-specific and limited to cells of the B

lymphoid lineage, but also seemed to be stage-specific within that lineage - its DNA binding activity could not be detected in several standard pre-B-cell lines, but was detectable in several mouse and human B-cell and myeloma cell lines. The nuclear factor bound around an 11bp sequence - GGGGACTTTCC, termed the B site, in the κ chain gene major intron enhancer - and hence was named NF-κB. In the cell lines examined, there was a striking correlation between the apparent presence of this NF-κB activity and immunoglobulin κ light chain gene expression.

Such behaviour tended to suggest that the NF-κB factor was only expressed in mature B-cells and plasma cells, however one discrepancy in this scheme was noted in the above study - that in a pre-B-cell line PD which undergoes κ chain gene rearrangement in culture did show nuclear NF-κB activity. The behaviour of the PD cell line could be rationalised following reports that NF-κB DNA binding activity could be induced in the 70Z/3 pre-B-cell line by treatment with bacterial lipopolysaccharide (LPS), with evidence that induction might involve a posttranslational activation - the combination of LPS and the protein translation inhibitor cycloheximide caused a superinduction of NF-κB activity (Sen and Baltimore, 1986b). Most interestingly, the use of either cycloheximide or the translation inhibitor anisomycin alone resulted in significant induction of NF-κB DNA binding activity - indicating that protein synthesis was not required for induction of NF-κB DNA binding activity, and hence implying that NF-κB existed in some inactive, possibly labile, precursor form in pre-B-cells.

Evidence for such an inhibited form of NF-κB already existed - studies of the 70Z/3 pre-B-cell line had indicated that after treating cells with cycloheximide or

anisomycin, κ gene transcription still occurred following LPS stimulation (Nelson et al., 1985; Wall et al., 1986). Wall et al. also reported that κ gene expression could be induced in the presence of cycloheximide alone - leading them to propose that κ gene expression in 70Z/3 cells was inhibited by one or more labile regulatory proteins that blocked transcription.

Further interesting points to emerge from the study by Sen and Baltimore (1986b) were that the active phorbol ester phorbol 12-myristyl 13-acetate (PMA) caused the induction of NF-κB activity in the 70Z/3 pre-B-cell line, and that PMA treatment also caused the induction of NF-κB in the Jurkat T-cell line and in the HeLa epithelial cell line. The observation that PMA caused a much more rapid induction of NF-κB activity than did LPS was consistent with previous models that LPS binding to a receptor results in activation of phosphatidylinositol-specific phospholipase C with the concomitant generation of inositol 1,4,5-trisphosphate and diacylglycerol - the latter being the physiological activator of protein kinase C (Wightman and Raetz, 1984). As PMA can activate protein kinase C directly, NF-κB activation by this route is much more rapid. The involvement of protein kinase C in this activation process led to suggestions that the critical event might be a phosphorylation of the pre-NF-κB. In such a model, a labile repressor protein could be either a phosphatase or a molecule which blocked the site of phosphorylation by directly binding to the pre-NF-κB, alternatively protein kinase C could phosphorylate the inhibitor, releasing it from its interaction in the pre-NF-κB complex (Sen and Baltimore, 1986b). The other most significant observation from the above study was that neither the NF-κB factor, nor the inhibitor protein, was confined to the B-cell lineage since NF-κB activity could be

induced in T-cell and epithelial cell lines - thus forcing a reformulation of the tissue and stage-specific regulatory problem as one of activating NF-κB from its seemingly ubiquitous precursor.

Although the correlation between the constitutive and inducible activity of NF-κB and the transcription of the κ light chain gene was suggestive of a link, more direct evidence confirming a causal link came from genomic methylation protection studies of the (NF-κB binding) κB site of the κ chain gene large intron enhancer - studies of the 70Z/3 cell line before and after induction of κ chain transcription by LPS treatment gave direct evidence for protein interaction with the κB site in those cells expressing the κ chain gene, similarly in two fully differentiated κ chain expressing lines the protein interaction with the κB site was maintained (Hromas et al., 1988). Another line of evidence indicating a direct causal link between the induction of κB DNA-binding activity and the activation of κ gene transcription emerged from studies of three mutant lines of the 70Z/3 pre-B-cell line (Briskin et al., 1990). All three mutant 70Z/3 lines failed to activate NF-κB after treatment with LPS or PMA, however by deoxycholate/NP40 detergent treatment of cytoplasmic extracts, all three mutants were shown to possess functional NF-κB. Further, cycloheximide treatment of two of the mutants induced activation of NF-κB binding and κ gene transcription to levels comparable to those induced in wild type 70Z/3 cells - confirming that activation of NF-κB binding is a late and critical event in activating κ gene transcription.

The functional role of NF-κB was demonstated by a mutational analysis of the κ gene large intron enhancer (Lenardo et al., 1987) where the B motif, the binding

site for NF-κB, seemed to be the key site as its mutation resulted in little or no enhancer activity in transfected S194 myeloma cells, and abolished inducibility by LPS treatment in transfected PD31 pre-B-cells. Subsequent studies demonstrated directly that oligonucleotides bearing the κB motif when inserted into test plasmids carrying the chloramphenicol acetyltransferase (CAT) reporter gene could act independently as enhancer elements (Pierce et al., 1988). In transient transfection studies of lymphoid and non-lymphoid cells, insertion of a single copy of the κB motif (at -71bp relative to the transcriptional start site) could act as a tissue-specific upstream activating element - giving 24-fold higher levels of CAT transcription in S194 myeloma cells compared to a 3-fold increase in 3T3 cells (which may have had a low level of endogenous active NF-κB). Interestingly, insertion of a dimer of the κB motif at the same upstream site gave a further 10-fold increase in CAT activity and it was also demonstrated that the dimer of the κB site could act as an enhancer element when located 2.5kbp downstream of the transcriptional start site. The inducibility of this enhancer element was confirmed by transfection of the pre-B-cell line 38B9 and induction with LPS - in fact the κB motif dimer proved more active than the entire κ gene enhancer while plasmid constructs bearing a dimer of a mutant non-binding κB motif at the 2.5kb downstream position showed no increase in CAT activity over the parental plasmid. A most interesting observation from the above study was that the presence of two widely separated κB motifs (-71bp and +2.5kbp) was not enough to generate enhancer activity, although the precise spacing requirement for the functional interaction of the two sites was not determined. The reciprocal experiment - that the κB motif enhancer element could also function as a

promoter element was demonstrated via transfection studies using a mouse ß-globin gene and TATA-box with and without the insertion of a κB site 26bp upstream of the TATA-box : transfection into the S194 mouse myeloma and 3T3 fibroblast cell lines revealed that the globin gene was transcribed only in lymphoid cells and was dependent on the presence of the κB site (Wirth and Baltimore, 1988). Moving the site of insertion of the κB motif to approximately 200bp upstream of the TATA-box resulted in transcription levels falling to baseline values - suggesting that the κB site positioned 26bp upstream of the TATA-box was supplying promoter rather than enhancer function.

The implication of these early results was that the induction of NF-κB activity might be the key event for the activation of immunoglobulin light chain gene expression. However more recent studies have suggested a more complex picture. Firstly, more recent studies identifying immunglobulin λ light chain gene enhancer regions (Hagman et al., 1990) have suggested that in the mouse λ locus there are two active transcriptional enhancers, 90% similar, both lacking functional κB sites.

Other studies of κ light chain gene expression demonstrated that while the activation and binding of NF-κB to κB sites was necessary for LPS induced κ gene transcription it was not sufficient as treatment of 70Z/3 pre-B-cells with transforming growth factor-ß (TGF-ß), a potent inhibitor of B-cell lymphopoiesis, inhibited LPS-induced κ gene transcription but not the activation and in vitro binding activity of NF-κB (Briskin et al., 1988). Most interestingly, the induction of 70Z/3 cell κ gene transcription by interferon-γ (IFN-γ) was not inhibited by TGF-ß, nor was NF-κB activated during interferon-γ induction -

results which were confirmed by another group who noted that while IFN-γ was completely inactive in mobilising NF-κB activity, it was a potent activator of protein kinase C in 70Z/3 pre-B-cells (Bomsztyk et al., 1991).

Further evidence indicating the complexity of the control of κ light chain gene transcription was the observation that the endogenous κ light chain gene could still be expressed at high levels in certain myeloma cell lines which posessed little active NF-κB (Atchison and Perry, 1987). Thus the κ gene large intron enhancer was found to be inoperative (and NF-κB activity absent) in both the plasmacytoma S107 cell line and the pre-B-cell line 3-1 - whereas the S107 plasmacytoma produced normal levels of κ gene mRNA, despite its inactive enhancer. Another interesting observation from this study was that transfection and stable integration of exogenous κ genes into the plasmacytoma cell lines S194 (which shows high levels of NF-κB activity) and S107 (which shows little or no active NF-κB) showed that these exogenous κ genes were expressed only in the S194 cells. These results led to suggestions that while establishment of κ gene large intron enhancer activity was absolutely dependent on the presence of active NF-κB, the requirement was only transient - either κ gene large intron enhancer function was only required transiently for establishment of κ gene transcription, or that the enhancer continued to function but with no requirement for NF-κB activity.

These rather perplexing results were supported by studies of a cell line which continued to produce immunoglobulin transcripts after a spontaneous deletion of the heavy chain enhancer (Klein et al., 1985), leading to two possible explanations - either an as yet unidentified enhancer element substituted for the deleted heavy chain enhancer, or that the enhancer somehow organised the DNA into a stable

transcription complex, after which it was no longer required - the authors favouring the latter choice. The mechanisms by which this transcriptionally active state might be maintained were unclear but might involve possibilities such as the demethylation of 5-methylcytosine bases or the establishment of a more open chromatin configuration.

However, there were several pieces of evidence against this postulated transient requirement for the enhancer - for example NF-κB activity was only rarely absent in mature B-cells (Sen and Baltimore, 1986a). Further, a study of μ heavy chain immunoglobulin gene transcription (Grosschedl and Marx, 1988) demonstrated that the μ heavy chain gene enhancer (normally located in the intron between the rearranged VDJ variable and Cμ constant region) was necessary for the establishment of an active transcriptional state during normal B-cell differentiation. However, a μ gene construct containing this enhancer, flanked by D and JH recombination signals, when transfected and stably integrated at various chromosomal locations in the murine pre-B-cell line PD31, then allowed to undergo site-specific deletion of the μ enhancer showed that those cell clones which had deleted the enhancer from the μ gene construct no longer transcribed the μ gene construct. Further, the deletion inactivation occurred even though the μ gene had undergone hypomethylation before enhancer deletion - leading to the conclusion that the enhancer did not confer any sort of memory to the μ gene of its previously transcriptionally active state. Hence for the μ heavy chain gene this enhancer sequence was necessary both for the establishment and maintenance of μ gene transcription.

The above confusion about the roles of the immunoglobulin κ light chain and μ

heavy chain gene enhancers was at least partially resolved by the discovery that both the κ light chain and μ heavy chain genes had additional enhancer elements at large distances downstream of the constant regions. Thus the κ light chain gene was shown to have a second enhancer region, located approximately 4kbp 3' of the previously characterised enhancer in the large (Jκ-Cκ) intron. Further, this enhancer element was significantly more active than the relatively weak κ gene intronic enhancer - 7-fold stronger in transient transfection assays of MPC11 myeloma cells (Meyer and Neuberger, 1989).

The omission of this 3' enhancer led to 20-40-fold lower expression of κ transgenes, while transfection experiments showed that the 3' enhancer, like the κ intronic enhancer, could be induced by treating the 70Z/3 pre-B-cell line with LPS. While LPS induction of intronic enhancer activity was mediated via the induction of NF-κB activity, deletion mapping studies of the LPS-responsive region in the 3' enhancer identified a 50bp region lacking any κB sites - supporting the observation that the 3' enhancer allowed κ gene transcription in the absence of active NF-κB (Meyer et al., 1990). It follows that both the κ intronic and 3' enhancers can be induced by the same agent, in cells at the same stage of differentiation, but by distinct pathways. A similar situation was demonstrated in the rat immunoglobulin heavy chain (IgH) locus - this led to the identification of a strong B-cell-specific enhancer at the 3' end of the rat IgH locus (Pettersson et al., 1990).

Although NF-κB was initially characterised as being constitutively present in the B-cell nucleus, there may be variations in the degree of activation of the factor. Thus, in studies of human ß-interferon promoter regulation (Visvanathan

and Goodbourn, 1989) whole cell extracts of the Daudi B-cell line (which transcribe the κ light chain gene) contained detectable NF-κB DNA binding activity as would be expected, however, deoxycholate/NP40 detergent treatment of these extracts resulted in the release of significantly greater amounts of NF-κB activity. Similarly, in a study of resting murine primary B-cells (Liu et al., 1991) it was shown that although nuclear extracts contained significant amounts of κB-binding activity, the treatment of cytosolic fractions with deoxycholate/NP40 revealed the presence of comparable amounts of κB-binding activity. Most interestingly, κB binding activity was induced after the cross-linking of surface immunoglobulin receptor, and this anti-Ig-induced activity was superinduced in the presence of cycloheximide - indicating that induction of NF-κB activity is a normal result of B-cell stimulation and may play a role in mature B-cell function in addition to its postulated role in κ light chain gene expression in B-cell development.

Factors binding to κB-like motifs have been implicated in the transcriptional activation of a variety of other genes - including :- MHC class I and ß2 microglobulin genes (Yano et al., 1987; Baldwin and Sharp, 1988), genes encoding MHC class II and its invariant chain Ii (Blanar et al., 1989; Pessara and Koch, 1990; Zhu and Jones, 1990). Such observations suggest that the role of NF-κB in B-cells may be as a wide-ranging activator of gene transcription responding to a range of extracellular signals. However, two major questions are posed by the study of NF-κB in B-cells - firstly, if NF-κB can induce the transcription of many different genes in different cell lineages, what mechanism restricts its transcriptional activation to only a subset of those genes in B-cells,

secondly, what mechanism allows the constitutive activity of NF-κB in mature B-cells, but not in (most) other cell types.

One potential mechanism to restrict the response of certain genes to NF-κB to B-cells only was revealed by a study of the immunoglobulin κ light chain gene intronic enhancer (Pierce et al., 1991) this suggested that part of this specificity for the κ chain gene to be expressed only in B-cells lay in an orientation and distance-independent silencer element located in a 232bp restriction fragment 5' of the intronic enhancer κB site. The addition of this silencer element upstream of an active κB site in reporter plasmids transfected into activated Jurkat T-cells caused a 10-fold decrease in transcription relative to a construct with a κB site alone, whereas the presence of this sequence had no effect on levels of transcription in the S194 myeloma cell line.

The idea that constitutive NF-κB activity is found in mature B-cells but not in other cell types may not be entirely true as some evidence exists that NF-κB activity is constitutively present in some T-cell lines (Baeuerle et al., 1988; Böhnlein et al., 1988; Leung and Nabel, 1988; Cross et al., 1989; Lin et al., 1990) and also seems to be present constitutively in mature monocytes and macrophages (Griffin et al., 1989). The mechanism giving rise to constitutive NF-κB activity in any particular cell lineage is still uncertain. However, one obvious possibility for ensuring constitutive NF-κB activity would be a modulation of the activity of a labile inhibitor protein, either by control of the level of its expression, or by control of the rate of its inactivation.

The versatility of the role of NF-κB in inducible gene transcription is illustrated by the perplexingly large number of agents which can upregulate levels of NF-κB

DNA binding activity in the nucleus. Unlike transcription factors such as steroid receptors which respond to a limited number of inducers, NF-κB DNA binding activity can be activated by agents such as bacterial lipopolysaccharide (Sen and Baltimore, 1986b), protein synthesis inhibitors such as cycloheximide and anisomycin (Sen and Baltimore, 1986b; Liu et al., 1991), active phorbol esters (Sen and Baltimore, 1986b; Liu et al., 1991; Griffin et al., 1989), anti-IgM antibodies (Liu et al., 1991), the Human T-cell Leukaemia Virus Type-I (HTLV-I) tax protein (Mauxion et al., 1991; Leung and Nabel, 1988) and by double stranded RNA (Visvanathan and Goodbourn, 1989).

Other agents which can induce active NF-κB include tumour necrosis factor-α (Griffin et al., 1989; Pessara and Koch, 1990; Osborn et al., 1989), tumour necrosis factor-ß (lymphotoxin) (Hohmann et al., 1990), interleukin-1 (Osborn et al., 1989; Zhang et al., 1990), the mitogenic lectin phytohaemagglutinin (Sen and Baltimore, 1986b; Libermann and Baltimore, 1990), anti-CD3 antibody (Jamieson et al., 1991), antigen (Kang et al., 1992; Jamieson et al., 1991), cyclic adenosine 5'-3' monophosphate (Shirakawa et al., 1989), ultraviolet light (Stein et al., 1989), hydrogen peroxide (Schreck et al., 1991), serum (Baldwin et al., 1991), okadaic acid (Thevenin et al., 1990), hepatitis B virus (Faktor and Shaul, 1990), and human herpes virus-6 (Ensoli et al., 1989) - the above list of NF-κB-inducing agents is by no means exhaustive.

The number of different signals and the different sites of their action in the cell suggests that the induction of NF-κB DNA binding activity may play some central integrating role in determining the state of cellular activation. While all of the above inducing agents cause the appearance of NF-κB DNA binding activity, there

are significant differences in the time courses of the inductions - thus early studies demonstrated that induction of 70Z/3 cells by PMA caused maximum κB-binding activity to appear by 30 minutes (Sen and Baltimore, 1986b) with significant induction being present much earlier, and the κB-binding activity decaying relatively quickly after 2-3 hours. In contrast, induction of 70Z/3 cells by LPS caused maximum activity to appear at 2 hours post-treatment, and significant activity still to be present 8 hours after LPS addition. Later studies of induction of NF-κB DNA binding activity by tumour necrosis factors-α and -ß (TNF-α, -ß) showed NF-κB activity detectable within 2 minutes and maximal after 10-15 minutes (Hohmann et al., 1990). Further, studies of 70Z/3 cells demonstrated that NF-κB activity could be detected in nuclear extracts within 15 minutes after treatment with IL-1 (Shirakawa et al., 1989).

The rapid response to stimuli mediated by NF-κB seems to be due to the lack of any requirement for new protein synthesis in the generation of the active transcription factor, coupled with the probable involvement of cellular kinases in inactivating labile inhibitor proteins. However, it should be noted that these control features may not be unique to NF-κB (and related) proteins - for example a labile inhibitor protein is responsible for the cytoplasmic retention of the c-fos (and possibly the c-jun) protein in serum-starved fibroblasts (Roux et al., 1990) - a situation with intriguing parallels to NF-κB activation.

Although the actual mechanisms involved in the activation of NF-κB by the large number of inducers listed previously are uncertain, it seems possible that many, or all of them, may act through a common mechanism involving the generation of reactive oxygen species such as hydrogen peroxide, superoxide

anion, and hydroxyl radicals. Such a possibility was initially raised by observations that the treatment of cells with the antioxidant scavenger molecule N-acetyl-L-cysteine (NAC) resulted in potent inhibition of PMA- and TNF-α-induced transcriptional activation from the HIV-1 long terminal repeat (LTR) (Roederer et al., 1990), further, that this effect was mediated specifically through blocking the activation of NF-κB (Staal et al., 1990).

Subsequent studies demonstrated that micromolar concentrations of hydrogen peroxide could induce transcriptional activation of the HIV-1 LTR in the human Jurkat T-cell line (Schreck et al., 1991) - this effect being mediated by NF-κB induction. It was further shown that by pre-incubating several cell lines with 30mM NAC, the activation of NF-κB by a wide range of inducers (such as hydrogen peroxide, tumour necrosis factor α, interleukin-1, double-stranded RNA, LPS, PMA, cycloheximide, and calcium ionophore) could be blocked. These studies were recently extended to the activation of NF-κB by truncated forms of the hepatitis B virus middle surface antigen and the hepatitis B virus X protein - both of these protein species were shown to mobilise NF-κB and induce κB-directed gene expression by a mechanism which depended on the prooxidant state of the cell. In both cases this activation process could be blocked by antioxidant species such as NAC and pyrrolidine dithiocarbamate (Meyer et al., 1992).

As the hepatitis B virus X protein is known to transactivate genes via cis-acting elements binding inducible transcription factors such as NF-κB, AP-1, AP-2, CREB/ATF and C/EBP it is tempting to speculate that some or all of these inducible factors may be regulated in a similar manner to NF-κB. Some evidence

in favour of such a scheme is provided by observations from HeLa cells that the activity of factors binding to κB and AP-1 sites is inducible by ultraviolet irradiation (Stein et al., 1989). Although it should be noted that the study by Schreck et al. (1991) did not detect any induction of AP-1-specific DNA binding activity in Jurkat T-cells treated with hydrogen peroxide, in contrast to the clear induction observed for κB-specific DNA binding activity - whether this reflects some cell-type specific difference is as yet unclear. While nothing is known about the nature of the steps following the generation of the prooxidant state by the various inducers of κB-specific DNA binding activity, one attractive proposal (Schreck et al., 1991) was the stimulation of protease activity by reactive oxygen species resulting in the release of NF-κB from its labile inhibitor protein.

Another interesting recent development is of the possibility that protein modifications such as methylation and isoprenylation may prove to be important events in the induction of NF-κB DNA-binding activity by LPS but not for induction by PMA (Law et al., 1992). This study demonstrated that treatment of 70Z/3 pre-B-cells with the S-adenosylmethionine-dependent methylation inhibitor 5'-methylthioadenosine blocked the LPS induced activation of κB DNA binding activity and immunoglobulin κ light chain gene expression. The same two events were also blocked by treatment with the protein isoprenylation inhibitor mevinolin - while neither of these treatments significantly inhibited activation of κB DNA binding activity by PMA.

The implication that carboxyl methylated and/or isoprenylated proteins play some essential role in the LPS-mediated NF-κB activation pathway is paralleled by a number of other examples of proteins involved in cellular responses to

extracellular signals being modified by lipidation and methyl esterification. Thus, the protooncogene product $p21^{ras}$ and other low molecular weight guanosine 5' triphosphate (GTP)-binding proteins, the nuclear lamins, and the γ subunit of the heterotrimeric G proteins are all known to be prenylated species (Reviewed in Sinensky and Lutz, 1992). Interestingly, κB DNA-binding activity in crude nuclear extracts of murine pre-B and human T-cells has been shown to be dramatically stimulated by the addition of GTP to 3mM (ATP addition was much less effective as a stimulant) (Lenardo et al., 1988), a similar but much less dramatic stimulation was seen with GTP addition to affinity purified κB-binding species isolated from HeLa cells (Clark et al., 1990). The meaning of the above observations is unclear at the moment but one possibility might be association at, or close to, the plasma membrane of a prenylated protein with NF-κB - all known prenylated proteins are at least partially localised to cell membranes.

The preceding sections have concentrated on the activation of NF-κB and subsequent transcriptional activation - however, some mechanisms for attenuating the response of NF-κB-induced gene transcription must exist. Such mechanisms include purely passive events such as dissociation of NF-κB from its cognate binding site due to the inherent "off-rate" of the DNA-protein complex. However, even events such as this may be under some degree of control - although the preformed complex of native, affinity purified NF-κB and its cognate κB site has been shown to be relatively stable towards challenge with unlabelled κB motif oligonucleotide, with a half-life of approximately 45 minutes (Zabel and Baeuerle, 1990) it is clear that dissociation rate constants for the DNA-protein complex are much larger in the presence of the naturally-occurring polyamine spermidine

(Results, Chapter 3). As the concentration of spermidine and other cellular polyamines are tightly regulated to the eukaryotic cell cycle (Reviewed in Tabor and Tabor, 1984), this mechanism offers obvious possibilities for the control of binding of transcription factors. Once NF-κB is no longer bound to its recognition site, it is possible that intranuclear inhibitor proteins could form complexes with the NF-κB to prevent it re-binding to κB motifs. A more active role for inhibitor proteins has been suggested by studies demonstrating that two forms of the NF-κB inhibitor protein IκB (Baeuerle and Baltimore, 1988a,b) (IκB-α, and -ß) could reduce the half-life of native (p50-p65) NF-κB-κB motif DNA complex with respect to dissociation to 3 and 7 minutes respectively, by a mechanism involving more that one IκB molecule (Zabel and Baeuerle, 1990).

2.2. Cellular genes activated by NF-κB

The target cellular genes which NF-κB activates seem to share one property in that they are all quickly transcriptionally induced in response to extracellular stimuli. As discussed before, a very wide range of agents can cause the induction of NF-κB DNA-binding activity, hence the role of NF-κB may simply be to transmit a rapid signal to the nucleus that the cell is undergoing some inflammatory, immune, or acute phase challenge. Although NF-κB seems to be ubiquitously present in all cell types its behaviour has been most thoroughly characterised in the cells of the immune system - notably lymphocytes, macrophages and monocytes. The majority of the target genes for NF-κB transcriptional activation can be divided into three classes :- (i) genes encoding immunomodulatory cytokines such as TNF-α, ß-interferon, and interleukin-6

(IL-6); (ii) genes encoding immunoregulatory cell surface receptors such as MHC class I antigens, the interleukin-2 (IL-2) cytokine receptor, and the non-polymorphic MHC subunits; (iii) genes encoding acute phase proteins such as angiotensin and serum amyloid A precursor.

One very important subset within the large range of genes which are transcriptionally controlled by NF-κB are those induced during the T-cell activation process. Over 70 genes are transcribed in T lymphocytes upon interaction of the T lymphocyte antigen receptor with antigen in the proper histocompatibility context (Reviewed in Crabtree, 1989). NF-κB in cooperation with other transcriptional regulator proteins (both activators and repressors) seems to be involved in the expression of many genes - especially those induced during the early phases of the response to T-cell receptor stimulation (Jamieson et al., 1991; Lenardo and Baltimore, 1989). Thus for example, virtually all of the cytokine genes whose expression is induced by T-cell activation are detected in early stages of activation - i.e. requiring protein synthesis, but being expressed before cellular DNA replication and cell division (Crabtree, 1989).

Cytokine gene expression can provide an easily assayable model system for studies of the role of NF-κB in early T-cell activation processes. The most thoroughly studied examples of T-cell cytokine gene regulation, the genes encoding IL-2 and the α chain of the IL-2 receptor demonstrates that NF-κB plays only one part in the transcriptional activation process.

In the case of the IL-2 gene, the 300bp promoter/enhancer region immediately upstream of the transcriptional start site has a strong response towards T-cell activation signals (Fujita et al., 1986; Novak et al., 1990). This promoter/enhancer

region contains binding sites for NF-κB, and other transcription factors such as NF-AT, the octamer proteins, the fos-jun AP-1 complex, and in human cells, a CD-28-responsive complex (Lenardo et al., 1988; Hoyos et al., 1989; Durand et al., 1988). Deletion studies have suggested that several of the trans-acting factor binding sites must be occupied to allow the full activity of the IL-2 gene promoter/enhancer (Durand et al., 1988; Hoyos et al., 1989) - thus mutation of the κB motif in the IL-2 promoter (located at positions -206 to -195) resulted in only a partial inhibition of mitogen and HTLV-1 tax protein-mediated transcriptional activation. While certain of the trans-acting factors involved in binding to the IL-2 promoter/enhancer are tissue-specific (NF-AT), others such as NF-κB and AP-1 are not - hence it seems likely that a variety of transcription factors are simultaneously necessary for transcriptional activation of the IL-2 gene.

Some studies have suggested rather paradoxically that in certain cell lines the IL-2 promoter/enhancer κB motif may function in a tissue-specific manner (Radler-Pohl et al., 1990; Briegel et al., 1991). As has been hinted up to this point the p50-p65 heterodimeric NF-κB is but one member of a family of proteins - these all share a highly conserved N-terminal region which is responsible for DNA-binding and dimerisation functions (Kieran et al., 1990; Ghosh et al 1990; Nolan et al., 1991) - as will be discussed later, this presents a wide range of possibilities for transcriptional control by binding to κB motifs. An interesting recent study of non-transformed murine T-cell clones has suggested that more than one protein species of the NF-κB family is important in binding to the IL-2 gene κB site (Kang et al., 1992). The authors proposed that DNA-protein complexes involving a p50 subunit homodimer with the IL-2 gene κB site were

present in unstimulated, nontransformed $CD4^+$ T-cells, and that this p50 homodimer bound more tightly to the IL-2 gene κB motif than to the immunoglobulin/human immunodeficiency virus type-1 enhancer type of κB motif. Interestingly, although the amount of DNA-protein complex in nontransformed $CD4^+$ T-cells involving the NF-κB p50-p65 heterodimer was increased by the addition of antigen presenting cells (APC), by antigen plus APC, or the immunosuppressant cyclosporin A, the amount of DNA-protein complex involving the p50 homodimer was only decreased after full antigenic stimulation. Further, those activation signals which led to the loss of the p50 homodimer complex resulted in IL-2 gene transcription, whereas treatments which resulted in increases in the activities of the p50-p65 NF-κB heterodimer or NF-AT in the nucleus but did not result in loss of the p50 homodimer-IL-2 gene κB motif complex failed to induce IL-2 gene expression.

It was proposed that this loss of DNA-protein complex corresponding to the p50 homodimer might be due to the formation of a complex of p50 homodimer within the nucleus with a newly synthesised inhibitory protein whose synthesis was tightly linked to the process of antigen stimulation, and whose synthesis was blocked by cyclosporin A treatment (Kang et al., 1992). These proposals allow a satisfying explanation of some puzzling aspects of T-cell activation - in this scheme the p50-p65 NF-κB heterodimer can be activated in nontransformed T-cells in response to a variety of signals such as APC, antigen plus APC, cyclosporin A, the protein synthesis inhibitor anisomycin, ionomycin, and ionomycin plus PMA - but not by PMA alone. In contrast the amount of p50 homodimer-IL-2 gene κB motif complex can only be decreased after full antigenic stimulation of the

nontransformed T-cell.

The above model can thus account for the fact that stimulation of nontransformed T-cells with anti-CD3ε antibody does not activate transcription via the IL-2 κB motif (Jain et al., 1992). While the induction of NF-κB DNA-binding activity is only weakly blocked by cyclosporin A at physiological doses, it is possible that other mitogen-induced complexes binding to κB sites may be sensitive to this drug (Randak et al., 1990; Schmidt et al., 1990).

Studies of the transcriptional activation of the gene encoding the α chain of the IL-2 receptor (IL-2R) have revealed the importance of a κB motif in its promoter also. The inducible cell surface expression of the 55kD α chain is critical for the formation (as a complex with the constitutively expressed ß chain) of the high affinity form of the IL-2 receptor - allowing T-cells to respond to this growth factor during their response to antigen.

Several groups have demonstrated that the p50-p65 NF-κB heterodimer or a closely related species is responsible for transducing T-cell activation signals into increased transcriptional activity from the IL-2R promoter (Leung and Nabel, 1988; Böhnlein et al., 1988; Cross et al., 1989; Lin et al., 1990). The activation of the IL2R promoter by the tax transactivator protein of HTLV-I or TNF-α has also been shown to be mediated via NF-κB (Cross et al., 1989; Siekevitz et al., 1987a; Ruben et al., 1988; Lowenthal et al., 1989). In contrast, phorbol ester inducibility of the IL-2R promoter is mediated by another sequence which does not involve the κB binding site (Ballard et al., 1989; Lin et al., 1990). The κB site in the IL-2R promoter is a relatively low affinity one, and shows a poorer response in transactivation experiments than do other κB motifs (Shibuya et al.,

1989). However, recent observations that the activity of the κB motif in T-cells is mediated by cooperation between NF-κB and serum response factor (SRF) and other trans-acting factors such as Sp1 (Ballard et al., 1989; Pomerantz et al., 1989; Roman et al., 1990) may well compensate for the lower affinity of NF-κB for the IL-2R κB motif.

In addition to the above two examples, a variety of cytokines produced by activated T cells appear to involve NF-κB in their transcriptional control :- interleukin-6 (IL-6) - which stimulates B cells to proliferate, differentiate, and secrete antibody (Libermann and Baltimore, 1990); granulocyte-macrophage colony stimulating factor (GM-CSF) - which sustains the proliferation of bone marrow precursor cells for macrophages and granulocytes (Schreck and Baeuerle, 1990); the inflammatory mediators tumour necrosis factors-α and -ß (TNF-α and -ß) (Collart et al., 1990; Drouet et al., 1991), and the neuropeptide precursor proenkephalin (Rattner et al., 1991). In the case of TNF-α and -ß these cytokines are also potent inducers of NF-κB DNA binding activity - leading to proposals that some type of feedback amplification might be initiated in response to antigen.

As indicated before, κB-binding trans-acting factors are also involved in the transcriptional activation of a number of genes expressed in inflammatory and acute phase processes. Certain cytokines which are primarily derived from non-lymphocytic leukocytes are also transcriptionally controlled by NF-κB - these include TNF-α and IL-6 which are expressed primarily in monocytes but also by T-cells (Collart et al., 1990; Libermann and Baltimore, 1990) and the chemotactic factor IL-8 which is responsible for neutrophil recruitment into inflammatory sites (Mukaida et al., 1990). The involvement of NF-κB in ß-interferon gene

expression was initially reported by Visvanathan and Goodbourn (1989), with the observation that transfection of cell lines with double stranded RNA could induce a DNA-binding activity with identical characteristics to those of NF-κB - this raised the possibility that multiple protein kinases, including the double stranded RNA stimulated DI-kinase might release NF-κB from IκB and IκB-like inhibitor proteins. As the ß-interferon gene can be induced in virtually all cell types, it may be that the involvement of NF-κB in gene transcription regulation is more widespread than previously suspected.

It seems that another inflammatory response involving NF-κB-mediated gene transcription is the acute phase of hepatocytes (Edbrooke et al., 1989). During this process, various cytokines (IL-1, IL-6 and TNF-α) acting at the liver cell membrane and steroid hormones result in changes in the expression of particular genes - NF-κB is the mediator for the elevated expression of the genes encoding serum amyloids A2ß and A1 - serum amyloid A2ß being a precursor to the amyloid A subunits of amyloid fibrils. Other acute phase genes such as those encoding angiotensinogen - a precursor to the hormone angiotensin II - and a1-acid glycoprotein - a non-specific immunosuppressant - are also transcriptionally controlled by NF-κB or a related κB-binding species (Ron et al., 1990; Brasier et al., 1990). Further hepatocyte genes apparently regulated by NF-κB, and which can be loosely grouped with immune/inflammatory genes, are those for complement factor 4 (Yu et al., 1989) and the factor B protease (Nonaka and Huang, 1990).

Although only peripheral to the above discussion, evidence is accumulating of a direct involvement of NF-κB in the control of cell growth - it has been proposed

that by binding to a site in the first exon of the c-myc gene transcript, NF-κB could relieve a transcriptional block by interrupting a stable stem-loop structure in this region of the transcript which seems to halt transcriptional elongation by RNA polymerase II (Duyao et al., 1990). Another example is the induction of a binding complex resembling NF-κB in BALB/c 3T3 cells following serum stimulation (Baldwin et al., 1991). Taken together, these reports suggest that NF-κB may play some central regulatory role in genes involved in cell growth in both lymphocytes and non-lymphoid cells.

2.3. NF-κB and viruses

In addition to the important role κB-binding proteins play in regulating the transcription of cellular genes, they also have important roles in the transcriptional regulation of many viruses. Most attention has been focused on two retroviruses which can infect human T-cells - the human immunodeficiency viruses (HIV-1, HIV-2) and human T-cell leukaemia virus type I (HTLV-I). However, NF-κB is also important for the transcriptional control of a number of other virus types:- adenoviruses, simian virus 40 (SV40), herpesvirus types 1 and 6, cytomegalovirus, and hepatitis B virus.

The role of NF-κB in controlling transcription from the long terminal repeat (LTR) of the integrated, proviral form of human immunodeficiency virus type 1 (HIV-1) was intially established by Nabel and Baltimore (1987) with the demonstration of a correlation between increases in NF-κB DNA-binding activity and the transcriptional activity of the HIV-1 LTR in transient transfection experiments following T-cell activation. Deletion analysis of the HIV-1 LTR suggested that a strong transcriptional enhancer within the LTR was localised at a

nucleotide sequence containing two direct repeats of the immunoglobulin κ chain gene κB site (Muesing et al., 1987). Most isolates of HIV-1 have two functional κB sites (as does simian immunodeficiency virus), in contrast in HIV-2 only one functional κB site remains in the LTR (Dewhurst et al., 1990; Anderson and Clements, 1991).

One characteristic feature of infection by lentiviruses such as HIV-1 and -2 is the slow pattern of disease progression (Cullen and Greene, 1989) with symptoms appearing many months or even years after the initial infection. This delay in frank viral pathogenesis may be due to the infection of only a small population of T-cells, with the HIV provirus remaining in a quiescent state. Upon activation of latently infected T-cells by full antigenic stimulation, the increased transcriptional activity of the LTR as a consequence of activation of NF-κB/rel family members (presumably the p50-p65 NF-κB heterodimer - Kang et al.,1992) could result in the generation of viral transcripts and production of progeny virus (Nabel and Baltimore, 1987; Tong-Starksen et al., 1989). Other inducers of active NF-κB, such as TNF-α and IL-1 may also be able to increase virus production (Folks et al., 1989; Osborn et al., 1989).

The experimentally-observed correlation between the induction of NF-κB DNA binding activity and transcriptional activation from the LTR has received much support - many studies have shown that the induction of active NF-κB in T-cells or monocytes resulted in increased transcriptional activity from the LTR (Nabel and Baltimore, 1987; Garcia et al., 1987; Franza et al., 1987; Griffin et al., 1989; Berkhout and Jeang, 1992). Further, the ability to activate transcription from the LTR is not restricted to cell types such as $CD4^+$ T-cells and monocytes which

normally exhibit productive infection but seems to be dependent on the availability of NF-κB or NF-κB-like transcriptional activators (Toyama et al., 1992). Thus if the LTR is tested in B-cells, NF-κB activity results in strong transcriptional activation, and B-cells artificially expressing the CD4 viral receptor allow reasonably efficient viral replication (Calman et al., 1988). In contrast, T-cells which do not express the NF-κB p50-p65 heterodimer have restricted HIV expression (Raziuddin et al., 1991).

Many of the above studies on transcriptional control of the HIV LTR were performed using transient transfections with reporter gene plasmid constructs - such systems may differ in their behaviour compared to the chromatin context of the integrated provirus. Hence it is also necessary to assay the replicative ability of HIV after mutation of various protein-binding motifs in the LTR to allow a true assessment of their importance in the virus life cycle *in vivo*.

Studies of HIV replication in response to cytokine treatment have shown an association between the induction of NF-κB binding activity and transcriptional activation of the provirus. One such *in vivo* study showed that viruses with deletions of both κB motifs were still able to replicate in MT-4 cells (Leonard et al., 1989). Further studies showed that the two NF-κB sites and three Sp1 sites were functionally redundant and not all required for viral infectivity, interestingly, mutation of these sites seemed to alter the cell types which were permissive for viral replication, as well as changing the replicative and cytopathic properties of HIV-1 (Ross et al., 1991; Englund et al., 1991; Parrott et al., 1991). These functional redundancies contrast with the complete loss of viral replication activity seen with mutations of the TAR region (Leonard et al., 1989). The

situation is further complicated by observations that some treatments which act as specific inducers of NF-κB activity can only induce viral replication in certain cell types - thus TNF-α can induce viral replication in some T-cell lines, but not in others. While LPS treatment can stimulate HIV production in monocyte/macrophage cell lines but not in T-cells (Matsuyama et al., 1989; Pomerantz et al., 1990).

Recent studies of transcriptional control of the HIV LTR have identified transcriptional activation mechanisms which are independent of the NF-κB p50-p65 heterodimer. An 86kD protein (HIVEN 86A) has been identified as interacting strongly with the HIV enhancer - this species is now believed to be the c-rel member of the NF-κB/rel/dorsal transcriptional modulator family (Franza et al., 1987; Ballard et al., 1990, 1992) - recent studies have suggested that the c-rel protein may transcriptionally activate the LTR (Muchardt et al., 1992). Further, transcription from the LTR also seems to depend on factors binding to sequences outside the -80 to -105 enhancer region - such as the three Sp1 sites and the NF-AT site (Jones et al., 1986). Enhancer-independent inducible transcription from the HIV LTR has recently been suggested to involve other proteins binding in close proximity to the TATA box (Jones, 1989; Sakaguchi et al., 1991).

Other recent findings tend to reinforce a primary role for NF-κB in determining whether HIV enters a latent state or becomes transcriptionally active upon infecting a host cell (Nabel, 1991). Recent studies have demonstrated that HIV infection causes the induction of NF-κB DNA binding activity in monocytic cell lines which typically only have very low levels of NF-κB activity (Bachelerie et al., 1991; Suzan et al., 1991; Roulston et al., 1992). Such results tend to suggest

that NF-κB is involved in a positive feedback loop which may contribute to maintaining a productive infection. One potential mechanism for mediating such an amplification was revealed by studies showing that the virally encoded protease could cleave the 105kD p50 precursor to generate the active DNA-binding p50 subunit of NF-κB (Riviere et al., 1991). Hence HIV infection, by causing the induction of NF-κB DNA binding activity, seems to be able to alter the cellular environment to make it more favourable for viral replication.

The other major intensively studied retrovirus capable of infecting human T-cells with a role for NF-κB in its transcriptional control is human T-cell leukaemia virus type I (HTLV-I) - the causative agent of adult T-cell leukaemia (Wong-Staal et al., 1983). Although the molecular basis of oncogenesis by HTLV-I is not completely understood, one of the three proteins encoded by the pX region of the viral genome - the viral Tax protein - has been strongly implicated in this process (Nerenberg et al., 1987; Pozzatti et al., 1990). The HTLV-I Tax protein is a transcriptional trans-activator but it does not bind DNA or stimulate the activity of cis-acting regulatory motifs directly. The effects of Tax seem to be independent of new protein synthesis (Jeang et al., 1988), and its activation signals to be transmitted through other transcription factors including NF-κB (Ballard et al., 1988; Leung and Nabel, 1988; Ruben et al., 1988; Mauxion et al., 1991).

The Tax protein seems to be the only component of HTLV-I needed to induce NF-κB DNA binding activity (Arima et al., 1991; Lacoste et al., 1991). Possibly by activating factors such as NF-κB to allow transcription of genes involved in T-cell proliferation, HTLV-I infected cells would be more likely to undergo

mutational events eventually causing outright transformation (Yoshida and Seiki, 1987). Consistent with this hypothesis, the Tax protein of HTLV-I (and the transactivator protein of HTLV-II) activates genes involved in T-cell growth (Greene et al., 1986; Wano et al., 1988). Thus the transcription of both the IL-2 and IL-2 receptor α chain genes are stimulated by Tax-induced NF-κB (Inoue et al., 1986; Ballard et al., 1988; Leung and Nabel, 1988; Hoyos et al, 1989). Other genes which are characteristically expressed in T-cells following antigen stimulation and which are also thought to be upregulated by Tax-induced NF-κB include c-*fos* (Fujii et al., 1988), GM-CSF (Schreck and Baeuerle, 1990), interleukin-3 (Miyatake et al., 1988), ß-interferon (Leblanc et al., 1990), and lymphotoxin (tumour necrosis factor-ß) (Paul et al., 1990) - these products then being able to contribute to the progress of neoplasia.

As might be expected, the HTLV-I Tax protein is a potent transactivator of the HTLV-I LTR (Sodroski et al., 1984; Felber et al., 1985; Seiki et al., 1986), but it also strongly transactivates the HIV-1 and SV40 viral enhancers (Böhnlein et al., 1988; Böhnlein et al., 1989; Marriott et al., 1990; Miyatake et al., 1988; Zimmerman et al., 1991) - hence the importance of HTLV-I infection as a cofactor in accelerating the progression of HIV infections towards AIDS. Interestingly, Tax transactivation of the HTLV-I LTR is dependent on a promoter region which binds factors such as CREB and jun in addition to NF-κB (Yoshimura et al., 1990; Xu et al., 1990; Numata et al., 1991).

Recent progress has been made in understanding the mechanism of action of the Tax protein (Meyer et al., 1992). Initial studies of Tax localisation showed that it was primarily a nuclear protein (Goh et al., 1985; Kiyokawa et al., 1985).

Unexpectedly, soluble Tax protein can be taken up by tissue culture cells, such that the protein is found in both nuclear and cytoplasmic compartments, and can induce NF-κB DNA binding activity (Lindholm et al., 1990; 1992). The mechanism by which Tax protein increases NF-κB activity is still unclear, but it seems likely to involve to involve a prooxidant state-mediated release of NF-κB from IκB/ankyrin repeat inhibitor proteins (Meyer et al., 1992). This in turn is presumably followed by NF-κB binding to and upregulating the transcription of the gene encoding the p105 precursor to the p50 NF-κB subunit (Ten et al., 1992) and conceivably, the genes encoding other NF-κB/rel family proteins.

Other viruses making use of NF-κB for stimulating their own transcription include cytomegalovirus, the cytomegalovirus α (immediate-early) promoter-enhancer NF-κB sites were found to be important for transcription of the viral immediate early gene 1 (IE 1) (Nelson and Groudine, 1986; Ghazal et al., 1988). Interestingly, the protein encoded by the IE 1 gene has been shown to activate the binding of NF-κB, and so raises the possibility of the IE 1 protein regulating the expression of its own gene (Sambucetti et al., 1989). While the cytomegalovirus IE 1 protein shares certain similarities with the HTLV-I Tax protein, in that it is a nuclear phosphoprotein, it is unclear whether it activates NF-κB by the same route.

Other members of the herpes virus family such as herpes simplex virus type 1 and human herpes virus 6 also seem to be able to induce NF-κB DNA binding activity through virally encoded trans-activator proteins, and may be transcriptionally regulated by NF-κB (Gimble et al., 1988; Ensoli et al., 1989; Martin et al., 1991). Again, coinfection with these viruses is likely to result in an

acceleration of HIV infections towards AIDS through their ability to mobilise NF-κB.

Several other viral types employ activation of NF-κB to activate their transcriptional units - thus adenovirus type 5 transformed cells have high levels of nuclear NF-κB and show high levels of expression of ß-interferon and MHC class I genes (Nielsch et al., 1991), further, binding of NF-κB has been implicated in the lymphoid-specific transcription of the adenovirus E3 promoter (Williams et al., 1990). NF-κB has also been implicated in lymphoid-specific transcription of the simian virus 40 (SV40) core enhancer (Kanno et al., 1989; Phares and Herr, 1991). Other NF-κB-like factors identified as binding to viral transcriptional regulatory sequences include the HeLa cell protein EBP1, this was initially identified as an activity binding to the 'core' region of the SV40 enhancer, affinity purification of this protein yielded a species of 60kD size (Clark et al., 1988). Extensive analysis of the DNA contacts made by the purified EBP1 protein indicated that it made base and backbone contacts over one complete turn of the DNA double helix (Clark et al., 1989). The EBP1 methylation interference pattern on its binding site in the SV40 enhancer was clearly distinct from that of other factors such as H2TF1 (Baldwin and Sharp, 1988) and KBF1 (Israel et al., 1987). KBF1 is now known to be identical to the NF-κB p50 subunit (Kieran et al., 1990) , while EBP1 seems likely from its methylation interference and binding site competition behaviour to represent the p50-p65 form of NF-κB (Clark et al., 1988).

A final most interesting example of viral NF-κB mobilisation, bearing in mind its known relationship to retroviruses (Miller and Robinson, 1986), is that by the

hepatitis B virus - the virally-encoded X protein transactivator has been shown to stimulate transcription of the HIV LTR, the SV40 enhancer, and cellular genes such as MHC class I (Siddiqui et al., 1989; Twu and Robinson, 1989; Faktor and Shaul, 1990; Lucito and Schneider, 1992). Most interestingly, truncated forms of hepatitis B virus surface antigen proteins have also been shown to be able to function as NF-κB mobilising trans-activators (Meyer et al., 1992).

2.4. Identification and characterisation of NF-κB as a member of a multiprotein family

The inducibility of NF-κB DNA binding activity detected in early studies after pre-B-cell treatment with agents such as active phorbol esters and mitogenic lectins, and the superinducibility in the presence of protein synthesis inhibitors has already been noted. The question of the nature of these different forms of NF-κB was clarified by the description of a protein species, termed IκB, which acted as a highly specific and reversible inhibitor of NF-κB DNA binding activity (Baeuerle and Baltimore, 1988a,b; Baeuerle et al., 1988). The noncovalent association of a possibly labile inhibitor protein with NF-κB in a cytoplasmic complex which could be dissociated by treatment with agents such as deoxycholate or formamide fitted with the observations of a superinduction of DNA binding activity in the presence of protein synthesis inhibitors. The observation that PMA induction reduced the amount of the cytoplasmic inactive form of NF-κB, and increased the amount of the active nuclear form led to the concept that inactive NF-κB complexed with IκB in the cytoplasm was initially freed from the inhibitor, then transported into the nucleus (Baeuerle and Baltimore, 1988b).

The initial attempts at purifying NF-κB suggested that the DNA binding activity

was associated with proteins in the size range of 40 to 60kD (Kawakami et al., 1988; Lenardo et al., 1988). The above studies also demonstrated that NF-κB was a positive regulator of *in vitro* transcription, and that its DNA binding activity could be stimulated by nucleoside triphosphates (with GTP showing the highest stimulatory activity). More definitive biochemical analysis revealed that the factor giving rise to the NF-κB gel mobility shift complex actually contained two distinct polypeptide subunits of 50kD and 65kD molecular weight - termed p50 and p65 respectively (Baeuerle and Baltimore, 1989). The p50 NF-κB subunit was initially identified as being the DNA-binding subunit of the complex, with p65 lacking DNA-binding activity - leading to the proposal that NF-κB comprised a heterotetramer, with two p50 subunits contacting each half site of the κB motif, with the p65 subunits performing auxiliary roles.

However, later studies demonstrated that the p65 subunit did indeed possess DNA-binding activity, allowing the reformulation of NF-κB as a p50-p65 heterodimer (Urban and Baeuerle, 1990; Nolan et al., 1991; Urban et al., 1991). These studies demonstrated the ability of the p65 subunit to influence the DNA binding specificity of the p50-p65 heterodimer such that it preferentially bound to the immunoglobulin/HIV enhancer κB motif rather than the more symmetrical types of κB motif such as found in the H2 gene.

Significantly, both the p65 and p50 subunits can interact with, and be inhibited by, different species of IκB/ankyrin repeat proteins - allowing the possibility of differential regulation of the DNA binding activity of related κB motif binding complexes (Kerr et al., 1991; Inoue et al., 1992). As the p50-p65 NF-κB complex obtained from the nuclei of stimulated cells seemed to be biochemically

identical to the complex obtained from the cytoplasm of unstimulated cells (Baeuerle et al., 1988) - for example complexes from either source could have their DNA-binding activity inhibited by partially purified IκB. This last observation tended to suggest that the induction of NF-κB activity was the result of some change in the IκB protein(s). Evidence in favour of this came from studies showing that treatment of cytoplasmic extracts with a variety of purified protein kinases - such as the cyclic AMP-dependent protein kinase, protein kinase C, and the haem-regulated eIF-2 kinase - resulted in the induction of NF-κB DNA-binding activity (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Further, the same studies showed that phosphorylation of purified IκB protein(s) with either protein kinase C or haem-regulated eIF-2 kinase resulted in the loss of all NF-κB inhibitory activity. However, treatment of purified IκB with cyclic AMP-dependent protein kinase had no effect on its inhibitory activity - suggesting that this kinase exerted its effects through an indirect mechanism (Ghosh and Baltimore, 1990). In any case it seems clear that no single kinase activity is responsible for the induction of NF-κB DNA binding activity (see Introduction Chapter 2, Section 1). Although studies quoted up to this point have suggested that the inactivation of IκB inhibitory activity is mediated by phosphorylation, recent studies have suggested that dephosphorylation of (the ß-form of) IκB may also stimulate its release from NF-κB (Hohmann et al., 1992; Link et al., 1992). Such results need not necessarily invalidate the model of phosphorylation of IκB causing its release from NF-κB - for example such events could be mediated through different sites.

The initial biochemical characterisation of IκB isolated from human placental

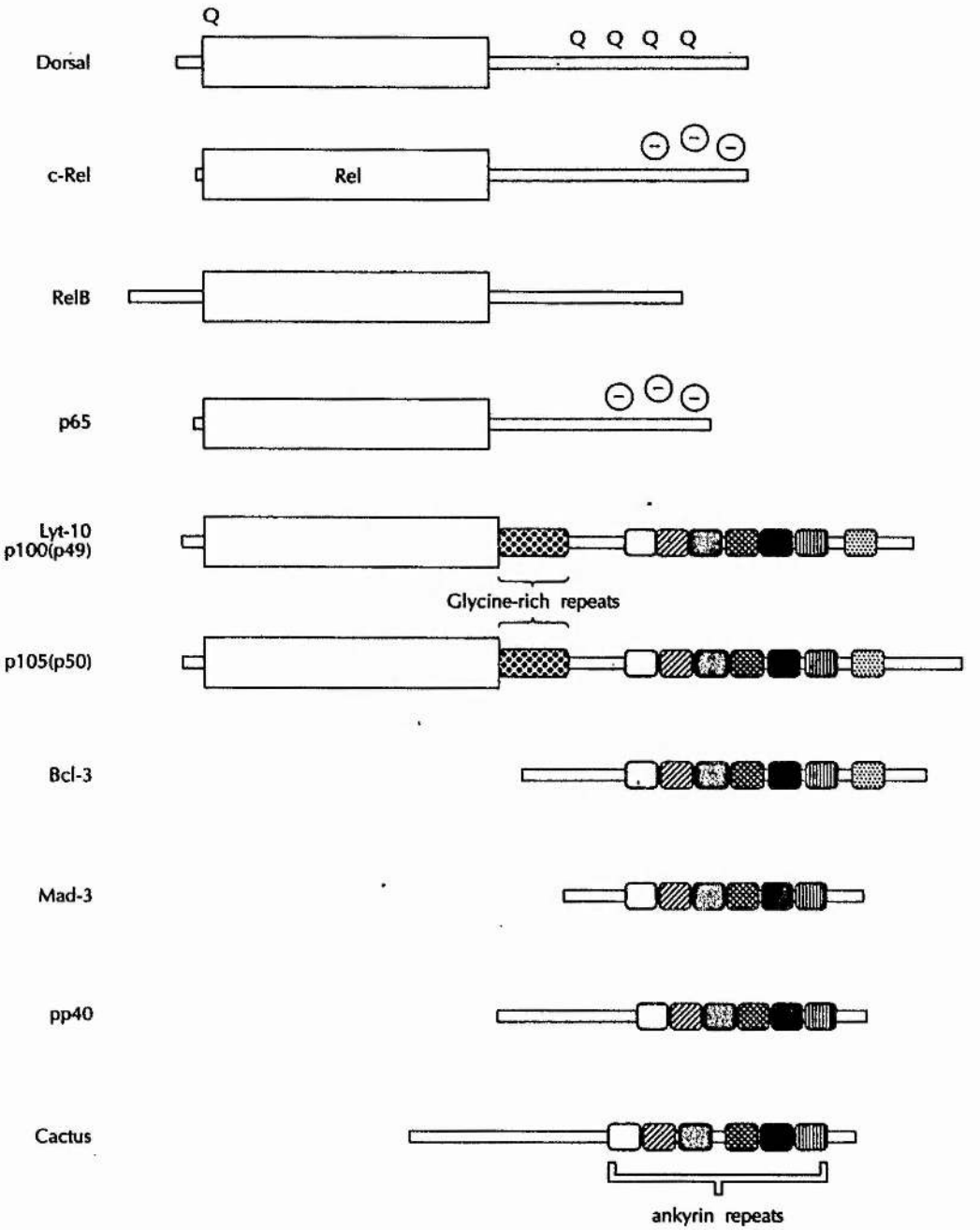
cytoplasmic extracts suggested that NF-κB inhibitory activity existed in two chromatographically separable forms:- IκBα, a 35-37kD polypeptide (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990), and a less abundant form IκBß, a 43kD polypeptide (Zabel and Baeuerle, 1990), which both seemed to interact with the p65 rather than the p50 NF-κB subunit. Later studies demonstrated that the IκBß form could also bind to and inhibit the DNA binding activity of the related c-rel protein (Kerr et al., 1991), IκBα and IκBß can interact with p65 and c-rel polypeptides (but not with p50 and hence cannot inhibit the binding of the p50 homodimer).

While the inhibitory activity of both the IκBα and IκBß forms can be inactivated by phosphorylation, the IκBß form can be inactivated by treatment with cyclic AMP-dependent protein kinase and also by protein kinase C, while IκBα inhibitory activity seems only to be inactivated by protein kinase C treatment (Kerr et al., 1991). Studies have clearly demonstrated the ability of both IκBα and IκBß to dissociate preformed NF-κB-κB motif complexes (Zabel and Baeuerle, 1990) *in vitro* , however it is unclear whether these species can be translocated to the nucleus.

The recent purification of the p50 and p65 subunits of NF-κB and the subsequent molecular cloning of the corresponding cDNAs has allowed rapid progress in studies of these transcription factors (Kieran et al., 1990; Ghosh et al., 1990; Bours et al., 1990, 1991; Nolan et al., 1991; Meyer et al., 1991; Ruben et al., 1991). Comparison of the predicted protein sequences of these cDNAs has revealed that they share a highly conserved N-terminal DNA binding and dimerisation region with a number of other cellular and virally-encoded proteins.

Figure 1.

Schematic diagram of some members of the NF-κB/rel/dorsal family of transcriptional modulator proteins, and of some members of the IκB/ankyrin repeat inhibitor protein family. (taken from Nolan and Baltimore, Current Opinion in Genetics and Development 2, 211-220)



Thus the p50 and p65 subunits are members of a family of proteins related to the protein product of the cellular *rel* protooncogene, the v-rel oncoprotein encoded by an avian erythroblastosis virus, and the *Drosophila* morphogen dorsal. Similar rapid progress has been made in characterising IκB-like proteins with inhibitory activity towards the NF-κB/rel family, following the identification of the inhibitory role of the p105 C-terminal ankyrin repeat region on DNA binding activity (Figure 1).

The initial isolation of cDNAs encoding the NF-κB p50 subunit followed intensive efforts by several groups to purify sufficient protein to allow peptide microsequencing (Kieran et al., 1990; Ghosh et al., 1990; Bours et al., 1990; Meyer et al., 1991). Intriguingly, the p50 subunit is initially translated as a 105kD precursor which is unable to bind DNA, the generation of the mature p50 subunit requiring a proteolytic cleavage event near a glycine-rich 'hinge' region in the centre of the p105 sequence. Cleavage has been reported to involve an ATP-dependent process which leads to the generation of the (N-terminal) mature p50 subunit and the rapid degradation of the C-terminal ankyrin repeat region (Fan and Maniatis, 1991). Comparison of the predicted amino acid sequence of the p50 subunit revealed a region of approximately 360 amino acids located towards the N-terminus which was very similar to equivalent regions in the c-*rel* and v-*rel* gene products, and the *Drosophila* morphogen dorsal - deletion studies with *in vitro* translated p50 derivatives demonstrated that this region was essential for DNA binding and dimerisation functions. The dimerisation function of this so-called rel homologous region allows the p50 subunit to form homodimers or to form heterodimers with other members of the NF-κB/rel/dorsal family (Kieran et

al., 1990; Ghosh et al., 1990).

Initial characterisation of the DNA binding specificity of the p50 homodimer suggested that it was likely to be identical to the constitutively active nuclear factor KBF-1 which bound the more symmetrical type of κB motif found in the H2 gene (Israël et al., 1987, 1989a,b; Baldwin and Sharp, 1987; Kieran et al., 1990). Initial transfection assays suggested that p50 homodimers could not act as transcriptional activators (Kieran et al., 1990), however in *in vitro* transcription assays using baculovirus-expressed p50, the p50 NF-κB subunit proved capable of transcriptional activation (Fujita et al., 1992). In contrast, recent studies of the activation of nontransformed primary T-cell clones have suggested that p50 homodimer binding to κB motifs can suppress the transcriptional activation of those sites by p50-p65 heterodimers (Kang et al., 1992) - the reason for these discrepancies is unclear but might reflect different transcriptional activation mechanisms. Recent studies from other groups have identified a novel method for the regulation of p50 homodimer binding activity - the Bcl-3 ankyrin repeat protein has been shown to inhibit the DNA binding activity of NF-κB p50 homodimers *in vitro* (Hatada et al., 1992) and also *in vivo* by transfection studies (Franzoso et al., 1992). The latter study also demonstrated that the occupancy of κB motifs changed from predominantly p50 homodimers to p50-p65 heterodimers upon activation of resting peripheral blood T-cells and U937 cells, and suggested a role for Bcl-3 in reducing the amount of DNA-protein complex containing NF-κB p50 homodimer.

The p105 precursor to p50 is encoded by a 4.0kb mRNA which is transcribed in many tissues, the amount of this transcript can be increased by many of the

treatments which promote the induction of NF-κB DNA-binding activity (Bours et al., 1990; Meyer et al., 1991) - this effect probably being mediated by a functional κB motif in the promoter of the gene encoding p105 (Ten et al., 1992). Thus active phorbol ester treatment of HL60 or HeLa cells, or TNFα stimulation of HL60 cells greatly increased the level of p105 mRNA, while in primary T-cells, treatments with agents such as PMA, phytohaemagglutinin, or calcium ionophore also increased the level of p105 mRNA (Bours et al., 1990). Intriguingly, cell treatment with cyclosporin A inhibited p105 mRNA induction by calcium ionophore, but had only a small effect on p105 mRNA induction following PMA and phytohaemagglutinin/PMA treatment (Bours et al., 1990).

The C-terminal ankyrin repeat region of p105 may serve both to maintain the p50 region in a non DNA-binding form, and to prevent its translocation to the nucleus (Kieran et al., 1990; Ghosh et al., 1990; Bours et al., 1990; Meyer et al., 1991; Henkel et al., 1992). Recent studies have suggested that the separately expressed C-terminal ankyrin repeat region is a potent inhibitor of p50 DNA binding, but not of p65 homodimers (Liou et al., 1992). Immunofluorescence and immunoprecipitation experiments to determine the subcellular localisation of the p105 protein suggested that the precursor is found only in the cytoplasm, whereas the mature p50 form is found in both nuclear and cytoplasmic compartments (Blank et al., 1991). Although the eight repeated ankyrin-like domains in the p105 C-terminus are very similar to each other, they may not be functionally equivalent - thus the sixth ankyrin repeat and the adjacent acidic region of the p105 precursor were shown to be required for cytoplasmic localisation, while the loss of the seventh ankyrin repeat or the loss of the acidic region between ankyrin

repeats six and seven results in a p105 species able to bind DNA (Blank et al., 1991). Intriguingly, recent transfection studies of *Saccharomyces cerevisiae* with Gal4-p105 fusion protein constructs have suggested that the C-terminal ankyrin repeat region of mouse and human p105 contains a potent transcriptional activation region located around ankyrin repeat numbers six and seven (Morin and Gilmore, 1992).

The isolation of cDNAs encoding the p65 subunit of NF-κB (Nolan et al., 1991; Ruben et al., 1991) again revealed extensive similarities to the DNA binding and dimerisation region of the NF-κB/rel/dorsal protein family (with the p65 subunit being more closely related to the rel family than to the p50 NF-κB sunbunit), a 2.6kb mRNA encoding p65 seemed to be widely expressed and was present in all tissues tested. In contrast to the situation with the p50 NF-κB subunit, p65 did not require cleavage from a larger precursor, and efficiently formed heterodimers with p50 to generate native NF-κB.

While the p65 NF-κB subunit was initially characterised as a non-DNA-binding species (Baeuerle and Baltimore, 1989) on the basis of the inability of electrophoretically gel purified and renatured p65 to bind DNA, this may simply have reflected inefficient renaturation of this subunit. It is now clear that p65 is a DNA-binding subunit in its own right which can also modulate the DNA binding specificity of the p50 subunit (Urban and Baeuerle, 1990; Urban et al., 1991; Zabel et al., 1991). The p65 subunit has also been shown to possess a C-terminal transcriptional activation region (Nolan et al., 1991; Ballard et al., 1990) which is likely to result in the p50-p65 NF-κB heterodimer being a much stronger transcriptional activator than the p50 homodimer. As might be expected, the p65

homodimer also has transcriptional activator properties (Nolan et al., 1991; Schmitz and Baeuerle, 1991).

Suggestions that the p65 subunit and c-rel proteins are closely related and the observation that p65 contained a C-terminal region corresponding to the putative transcriptional activation domain of c-rel (Nolan et al., 1991; Schmid et al., 1991; Schmitz and Baeuerle, 1991; Ruben et al., 1991, 1992a) led to the identification of a C-terminal region of p65 running from amino acids 435 to 459 which was functionally important for transcriptional activation (Ruben et al., 1992a). This activation domain contained a leucine zipper-like motif and an overall net negative charge - leading to the suggestion that the ability of p65 to activate transcription may occur through a leucine zipper type interaction with other regulatory factors. The above study also identified a naturally-occurring derivative of p65, lacking amino acid residues 222 to 231, which was unable to dimerise - suggesting a critical role of this region for dimerisation function.

A recent development has been the identification of cDNAs encoding an NF-κB subunit very closely related to p50 - this species has been termed p49 (Schmid et al., 1991) or p50B (Bours et al., 1992) and analogously to p50/p105, represents the N-terminal half of a longer, non DNA-binding, (p100/p97) protein containing ankyrin/cell cycle repeat domains. As with the p105 precursor, the p100/p97 precursor must be cleaved to a species of approximately 50kD to be able to bind to κB motifs. The mature p49/p50B can form heterodimers with p65 and other rel family proteins to generate species with high affinity for κB motifs. Cotransfection studies have demonstrated that the p49/p50B protein in combination with p65 can give efficient transcription from a κB site and from the

HIV-1 LTR, however the relative amount of transcriptional stimulation compared to that obtained with the NF-κB p50-p65 heterodimer remains controversial (Schmid et al., 1991; Bours et al., 1992).

The cloning of cDNAs encoding the p50 subunit of NF-κB (Kieran et al., 1990; Ghosh et al., 1990; Bours et al., 1990; Meyer et al., 1991) allowed their identification as members of a family including the retroviral v-rel oncoprotein, its cellular counterpart c-rel and a *Drosophila* maternal effect morphogen protein dorsal. The v-*rel* oncogene was isolated from a highly transforming defective avian retrovirus, Rev-T, which causes rapid and fatal lymphoma in young birds. The Rev-T genome encodes a 59kD phosphoprotein - $p59^{v\text{-}rel}$ (Gilmore and Temin, 1986; Herzog and Bose, 1986; Garson et al., 1990) - whose intracellular location is mainly cytoplasmic in transformed spleen cells, but whose location is nuclear in non-transformed chick embryo fibroblasts (Gilmore and Temin, 1986).

The potential involvement of rel proteins in transcriptional control was suggested by early studies demonstrating that the v-rel protein could act as a transcriptional transactivator of the polyomavirus late promoter (Gélinas and Temin, 1988; Hannink and Temin, 1989). Later transfection studies with yeast and mammalian cells demonstrated that the c-rel protein possesses an N-terminal regulatory domain and a C-terminal transcriptional activation domain (Bull et al., 1990). More recent studies have directly demonstrated that the v-rel oncoprotein is capable of binding to κB motifs, but is not capable of stimulating transcription of reporter genes through those motifs (Ballard et al., 1990; Inoue et al., 1991). While the protein encoded by the c-*rel* protooncogene, $p85^{c\text{-}rel}$, also binds with high affinity to κB sites, it in contrast has been shown to be a functional

transcriptional activator (Inoue et al., 1991). The above observations led to proposals that the transforming effect of the v-rel oncoprotein might be due to competition of v-rel and NF-κB factors for DNA binding sites, or a trans-dominant effect in which v-rel inhibited NF-κB via a protein-protein interaction sequestering one of the NF-κB subunits (Ballard et al., 1990; Inoue et al., 1991).

The turkey v-*rel* cDNA (Wilhelmsen et al., 1984), chicken and turkey c-*rel* cDNAs (Hannink and Temin, 1989), mouse c-*rel* cDNA (Bull et al., 1990), human c-*rel* cDNA (Brownell et al., 1989) and a c-*rel*-related cDNA from *Xenopus laevis* (Kao and Hopwood, 1991) have been cloned. Although the chicken c-*rel* gene seems to be expressed at low levels in many cell types, high level expression occurs only in hematopoietic tissues (Moore and Bose, 1989).

Recent studies have demonstrated that the c-*rel* promoter contains a putative κB site and a binding site for the transcription factor HIP-1, and is a relatively weak promoter (Hannink and Temin, 1990). Co-transfection of the c-*rel* promoter with a retroviral vector expressing the c-rel protein resulted in a decrease in expression from the c-*rel* promoter - suggesting that the expression of the c-*rel* proto-oncogene is tightly regulated both at the level of basal promoter activity, and by autoregulation by the c-rel protein product.

The cloning of c-*rel* cDNAs has revealed that the v-*rel* cDNA encodes a truncated version of the c-rel protein $p85^{c\text{-}rel}$, the v-rel $p59^{v\text{-}rel}$ protein has substituted the two amino terminal amino acids of c-rel with a sequence of amino acids derived from the REV-A viral *env* gene. Further, the v-rel protein has lost 118 carboxyl terminal amino acids which may include the c-rel protein

transcriptional activation domain (Bull et al., 1990; Inoue et al., 1991).

Strikingly, v-rel protein isolated from cellular extracts has been shown by several groups to be associated in a complex of over 400kD with at least five different cellular proteins in v-*rel* transformed cells (Morrison et al., 1989; Davis et al., 1990; Kochel et al., 1991). The cellular proteins complexed with the v-rel protein have been shown to have apparent masses of 124, 115, 70, 68 and 40kD by sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis. The 70, 68, and 40kD proteins have been identified as a constitutive avian 70kD heat shock protein, the c-rel protein, and the ß-form of IκB respectively (Lim et al., 1990; Davis et al., 1991; Kerr et al., 1991). More recently, the 124kD protein has been identified as the p105 precursor to the NF-κB p50 subunit (Capobianco et al., 1992), with the proposal that the 115kD protein may represent the nonprocessed form of the related p49/p100 protein.

The family of rel-related proteins has recently been extended with two further members :- Rel B, which cannot bind with high affinity to κB sites, but which can form high affinity heterodimers with the NF-κB p50 subunit and transcriptionally activate promoters containing κB sites (Ryseck et al., 1992); and the very closely related I-Rel protein which does not interact with DNA, and which does not have a carboxy terminal transcriptional activation domain but which can form heterodimers with p50 - which have greatly reduced DNA-binding activity (Ruben et al., 1992b). Intriguingly, both the Rel B and I-Rel proteins contain leucine zipper-like motifs (Landschulz et al., 1988) in their extreme N-termini - between residues 22 and 51 and residues 40 and 68 respectively - hinting at the possibility of interactions with other leucine zipper motifs on proteins within the rel family

and possibly outside the rel family.

A more distantly related member of this NF-κB/rel/dorsal protein family is the *Drosophila* maternal effect morphogen protein dorsal - one of a group of gene products required to establish normal dorsal-ventral polarity in the embryo. The *dorsal* gene was found to be highly similar to the turkey proto-oncogene c-*rel*, and the oncogene v-*rel* - with 47% identity in the amino-terminal amino acid sequence (Steward, 1987). As might be expected the behaviour of the dorsal morphogen as a transcriptional modulator shows many parallels with NF-κB and rel family proteins. Thus dorsal protein function is regulated initially by transport from the cytoplasm to the nucleus (Rushlow et al., 1989). The amino terminal region of dorsal which is highly conserved with rel and NF-κB proteins has been shown to be responsible for the NF-κB-like DNA binding specificity of dorsal (Ip et al., 1991). Interestingly, dorsal seems to be able to behave as a transcriptional activator or repressor depending on the promoter context - thus dorsal binding in the context of the *twi* gene promoter acts as a transcriptional activator, while dorsal binding at the *zen* promoter results in transcriptional repression (Jiang et al., 1992). Analogously to the situation with NF-κB, nuclear uptake of dorsal is prevented by the product of an inhibitor gene (cactus) - further, a deletion of 80 carboxy-terminal amino acids resulted in dorsal losing the ability to activate transcription of mesodermal genes, larger deletions affected the transcriptional repressor function of dorsal (Isoda et al., 1992).

An important mechanism for controlling the DNA binding activity of all members of the NF-κB/rel/dorsal transcriptional modulator family is their potential for interaction with various members of the IκB/ankyrin repeat protein

family. As described previously, initial studies of the inhibitory IκB proteins isolated from the inactive cytoplasmic NF-κB-IκB complex (Baeuerle and Baltimore, 1988a,b; Baeuerle et al., 1988) suggested the existence of two forms of IκB:- a 36kD form denoted IκBα; and a less abundant 43kD IκBß form (Zabel and Baeuerle, 1990). Recent studies have identified a predominantly lymphoid IκBγ of 70kD which is identical to the C-terminal ankyrin repeat containing region of the p105 precursor to the NF-κB p50 subunit (Inoue et al., 1992a).

Rapid progress in this area has been made possible by the identification of the ankyrin repeat structure in the C-terminus of the p105 precursor to the NF-κB p50 subunit as having an inhibitory effect on DNA binding (Kieran et al., 1990; Ghosh et al., 1990). Isolation of a cDNA clone encoding five or six ankyrin repeats, termed MAD-3, from mRNAs induced upon monocyte adherence, and the observation that the encoded 36-38kD MAD-3 protein specifically inhibited the DNA binding activity of p50-p65 NF-κB, but not the p50 homodimer suggested that this was an IκB-like protein (Haskill et al., 1991). Comparison of the MAD-3 predicted amino acid sequence with tryptic peptide amino acid sequences from purified rabbit IκBα suggested that MAD-3 was identical to IκBα (Davis et al., 1991).

Subsequent studies have demonstrated that the pp40 phosphoprotein associated with the c-rel protein can inhibit the DNA binding activity of p50-p65 NF-κB, p50-c-rel heterodimers, and c-rel homodimers. Further, that pp40 was not related to IκBα (which has no effect on the DNA binding activity of the c-rel protein), but was likely to be identical to IκBß, intriguingly, only phosphorylated pp40 could inhibit the DNA binding activity of c-rel, while both unphosphorylated and

hyperphosphorylated pp40 forms were unable to inhibit c-rel binding (Kerr et al., 1991; Davis et al., 1991). The identification of pp40 as IκBß, together with previous observations of the presence of pp40 in both the nucleus and cytoplasm (Morrison et al., 1989; Davis et al., 1990; Kochel et al., 1991) gives rise to the possibility that the repression and activation of DNA binding activity of proteins of the NF-κB/rel/dorsal family may be able to take place in both the nuclear and cytoplasmic compartments.

A third form of IκB has recently been identified - the predominantly lymphoid IκBγ which is identical to the ankyrin repeat-containing C-terminus of the p105 precursor of the NF-κB p50 subunit (Inoue et al., 1992a). The product is generated by either alternative splicing of the p105 gene transcript or different promoter usage to give a 2.6kb mRNA species distinct from the 4.0kb p105-encoding mRNA. This 70kD IκBγ protein can inhibit the DNA binding activity of the p50 homodimer, the p50-p65 NF-κB heterodimer, and c-rel proteins (Inoue et al., 1992a).

The putative proto-oncogene product Bcl-3 has also been shown to be an ankyrin repeat protein with the ability to act as a specific inhibitor of the DNA binding activity of the p50 homodimer. It was proposed that this might be due to the high similarity between the p105 C-terminal ankyrin repeat region and the ankyrin repeat region of Bcl-3 - both contain seven or eight ankyrin repeats compared to the five or six ankyrin repeats of the MAD-3 protein (Hatada et al., 1992). Intriguingly, the above study also demonstrated that the C-terminal ankyrin repeat region of p105 could rapidly dissociate DNA-protein complexes involving either p50 homodimers or native NF-κB. However, other workers have suggested

that while the p105 C-terminal region can abolish the κB-specific DNA binding activity of p50 homodimers, it had only a minimal effect on the binding activity of NF-κB (and p65 homodimers) (Liou et al., 1992). Other studies have demonstrated that two Bcl-3 molecules bind per p50 dimer without dissociating the p50 dimer, and contact part of the p50 protein involved in dimerisation, and that Bcl-3 can act as a p50 subunit-specific inhibitor of p50-p65 NF-κB DNA binding activity (Wulczyn et al., 1992).

The function of the ankyrin repeat region is not entirely clear, an early suggestion was that it might mask a nuclear localisation signal or in some way tether the rel family protein to some cytoskeletal or membrane component (Ghosh et al., 1990). However, the identification of the ankyrin repeat pp40 (IκBß) protein in both nuclear and cytoplasmic compartments tends to suggest that the primary function of the ankyrin repeat region may be to inhibit DNA binding activity, and that cytoplasmic localisation may occur secondarily, possibly via other domains of IκB/ankyrin repeat proteins.

The general aim of this study was to make use of the initially available p105/p50 NF-κB subunit cDNA (kindly supplied by Dr.A.Israel) to study a minimal p50 DNA binding and dimerisation region as a simplified system to try to identify amino acid residues important for DNA binding activity. And hence to try to gain some insights into the mechanisms used by NF-κB to bind to its κB motif recognition site.

MATERIALS AND METHODS.

1. Cell lines and tissue culture procedures.

Suspension-adapted HeLa S3 cells were grown in Glasgow-modified minimal essential medium (Gibco-BRL) supplemented with 50 units/ml penicillin (Glaxo), 50ug/ml streptomycin (Evans Medical), 2.2g/l sodium bicarbonate, and 5% (v/v) new born calf serum (Sera-lab). Jurkat T-cell lymphoma cells were grown in RPMI1640 medium (Gibco-BRL) supplemented as above with penicillin, streptomycin, and sodium bicarbonate - but with the addition of 10% foetal calf serum (Sera-lab). Cell suspensions were grown in 5% CO_2, split in a 1 to 3 ratio on average every 3-4 days, and harvested at a cell density of approximately $5x10^8$ to 10^9 cells/l.

2. Bacterial strains.

All plasmid propagation and recombinant protein expression was carried out in the *E.coli* K12 strain JM101 (*supE, thi, Δ(lac-proAB), [F', traD36, proAB, lacI^{q}ZΔM15]*), (Yanisch-Perron et al., 1985).

3. Bacterial culture.

Liquid bacterial cultures were grown in Luria broth (10g/l bacto-tryptone (Difco), 5g/l yeast extract (Difco), 10mM NaCl, pH 7.5), supplemented with 100ug/ml ampicillin (Sigma) when necessary. Solid media contained Luria broth supplemented with 1.5% agar (Difco) and 10mM $MgSO_4$, and supplemented as above with ampicillin (and tetracycline) when necessary.

4. Synthesis of synthetic oligonucleotides.

Synthetic oligonucleotides were made using an Applied Biosystems 381A DNA

synthesiser and ß-cyanoethyl phosphoramidite chemistry. In this system, the growing DNA chain remains covalently attached to an insoluble matrix - controlled pore glass (CPG). CPG is easily derivatised with adenosine, guanosine, thymidine, or cytosine phosphoramidites. An organic linker is attached to the surface of the CPG and the support is then derivatised by covalent attachment of a nucleoside 3'-hydroxyl to the linker via an ester bond. Following derivatisation, all free amino groups are capped.

To begin the synthesis, the DNA synthesiser is loaded with a column containing one of four support-bound nucleosides, DNA synthesis then proceeds in a 3' to 5' direction. The first step in phosphoramidite oligonucleotide synthesis chemistry is the removal of the acid-labile dimethoxytrityl (DMTO) group by TCA treatment - this yields a reactive 5' hydroxyl which can react with a phosphoramidite during the following coupling or addition step. ß-Cyanoethyl phosphoramidites are chemically modified nucleosides which contain a disopropylamine group on a 3' trivalent phosphorus moiety. A ß-cyanoethyl protecting group is present on the 3' phosphorus group of the nucleoside with a DMTO protecting group on the 5' hydroxyl.

In the coupling step, tetrazole and the phosphoramidite are mixed as they enter the reaction chamber generating a highly reactive species which reacts with the free 5' hydroxyl group of the support-bound nucleotide. Tetrazole protonates the nitrogen of the diisopropyl group on the 3' phosphorus which converts the amine into a good leaving group upon nucleophilic attack by the 5' hydroxyl group - thus leading to a 5' to 3' internucleotide linkage.

During the coupling step, a small fraction of support bound oligonucleotides

will fail to undergo addition, these truncated or failure sequences are chemically modified to prevent their participation in subsequent coupling steps. Acetic anhydride mixed with dimethylaminopyridine forms a powerful acetylating agent which terminates or caps any unreacted chains from the coupling step.

Immediately after capping, the labile trivalent phosphorus linkage formed during the coupling step is oxidised to the stable pentavalent phosphorus linkage of natural DNA using iodine as a mild oxidant. Following oxidation, another cycle of nucleotide addition is initiated by the removal of the DMTO group at the 5' terminus of the oligomer, the process being repeated until the desired length of oligonucleotide has been synthesised.

Upon completion of oligonucleotide synthesis, the chain is cleaved from the CPG support, and the ß-cyanoethyl protecting groups on the phosphates removed by four 15 minute washes with concentrated ammonium hydroxide. Base protecting groups are then removed by heating to 55°C in ammonium hydroxide for 15 hours.

Following the cleavage and deprotection steps, ammonia is removed by evaporation under vacuum, and the oligonucleotide DNA pellet dissolved in water and then ethanol precipitated, finally the DNA pellet is dried and taken up in water, and the DNA concentration determined by UV absorbance measurements at 260nm (an absorbance of 1.0 at 260nm is taken to be equivalent to 40ug/ml).

5. Sequences of synthetic oligonucleotides used in these studies.

All double-stranded oligonucleotides used in this study are given in Table 1.

6. Preparation of plasmid DNA.

Small scale plasmid purifications employed a variant of the STET protocol :-

TABLE 1

Oligo	Nucleotide Sequence
16bp WTκB	5' CTG **GGGACTTTCC** AGG 3' 3' GAC **CCCTGAAAGG** TCC 5'
HIV-L	5' GATCTA **GGGACTTTCC** GCG 3' 3' AT **CCCTGAAAGG** CGCCTAG 5'
HIV-R	5' GATCTG **GGGACTTTCC** AGG 3' 3' AC **CCCTGAAAGG** TCCCTAG 5'
IRE	5' GATCAAAGT **GGGAAATTCC** TCTG 3' 3' TTTCA **CCCTTTAAGG** AGACCTAG 5'
H2TF1	5' GATCT **GGGGATTCCCC** AG 3' 3' A **CCCCTAAGGGG** TCCTAG 5'
EBP'cons'	5' GATCATG **GGGAATTTCCC** CAG 3' 3' TAC **CCCTTAAAGGG** GTCCTAG 5'
SVUP	5' GATCTGAGGC **GGAAAGAACC** AGCTG 3' 3' ACTCCG **CCTTTCTTGG** TCGACCTAG 5'
SV1-M1	5' GATCTAGGGTGT **CCAAAGTCCC** G 3' 3' ATCCCACA **GGTTTCAGGG** CCTAG 5'
SV1-M2	5' GATCTAGGGTGT **GGAATGTCCC** G 3' 3' ATCCCACA **CCTTACAGGG** CCTAG 5'
SV1-M3	5' GATCTAGGGTGT **GGAAAGTGGC** CG 3' 3' ATCCCACA **CCTTTCACCG** GCCTAG 5'

cells were harvested from 3ml of bacterial overnight culture by brief centrifugation in a microcentrifuge and the supernatant discarded. Bacterial pellets were resuspended in 350ul STET buffer (sucrose 8% w/v, Triton X-100 5% v/v, 5mM EDTA,pH8.0, 50mM Tris.HCl,pH8.0), then 25ul of a 10mg/ml lysozyme (Sigma) in STET buffer solution added, and the mixture boiled immediately for 45 seconds. The lysed bacterial suspensions were then centrifuged at 13000 rpm in a microcentrifuge at 4°C for 15 minutes and the pellets discarded. Next, 40ul of 3M sodium acetate was added to the supernatants, followed by 400ul of isopropanol, and the mixtures centrifuged at 13000 rpm in a microcentrifuge at 4°C for 5 minutes. The supernatants were discarded, and the precipitates washed with 400ul of 70% ethanol, before being dried under vacuum, and resuspended in 100ul of sterile water.

Large scale plasmid preparations were by a variant of the alkali method :- 500ml overnight cultures of bacterial transformants in Luria broth,100ug/ml ampicillin were centrifuged using a Beckman JA14 rotor at 8000 rpm for 10 minutes at 4°C, the supernatant discarded, and the bacterial pellet resuspended in 9ml of lysis buffer (50mM glucose, 25mM Tris.HCl, pH8.0, 10mM EDTA, pH8.0) and 1ml of a freshly prepared 20mg/ml lysozyme (Sigma), 0.2M Tris.HCl, pH8.0 solution added. The suspension was mixed and incubated at room temperature for 5 minutes before the addition of 20ml of a freshly made 0.2M NaOH, 1% SDS solution, the suspension was mixed again and placed on ice for 5 minutes, followed by the addition of 10ml of an ice-cold solution 3M with respect to potassium and 5M with respect to acetate anion (pH4.8). This mixture was then

centrifuged in a JA14 rotor at 10000 rpm for 15 minutes at 4°C, the supernatant removed carefully, treated with 16.5ml of isopropanol, mixed and allowed to stand for 15 minutes at 20°C. Nucleic acids were recovered by centrifugation using a Beckman JA20 rotor at 10000 rpm for 15 minutes at 20°C, the supernatant was discarded and the nucleic acid pellet washed with ice cold 70% ethanol before drying under vacuum.

The nucleic acid pellet was dissolved in 2ml of TE buffer (10mM Tris.HCl, pH8.0, 1mM EDTA, pH8.0), divided between two 1.5ml Eppendorf tubes, 1.15g CsCl added to each tube and dissolved, followed by addition of 50ul of 10mg/ml ethidium bromide (Bio-Rad). This mixture was allowed to stand at room temperature for 5 minutes then centrifuged at 13000 rpm for 3 minutes in a microcentrifuge, and the supernatant transferred to Beckman TL100 ultracentrifuge quick-seal tubes.

The sealed TL100 tubes were then centrifuged at 80000 rpm overnight at 20°C, the plasmid band removed by syringe, passed over a 10ml Sepharose 4B column (Pharmacia) equilibrated with TEN_{100} buffer (10mM Tris.HCl, pH7.5, 1mM EDTA, pH8.0, 100mM NaCl), and the void volume collected. This fraction was then passed over a 0.5ml Dowex Dg-50WX8 column (Bio-Rad) equilibrated with TEN_{100}, washed through with TEN_{100}, and fractions containing DNA isopropanol precipitated. This DNA pellet was redissolved in 500ul of TE buffer, RNase A added to a concentration of 50ug/ml, and incubated for 30 minutes at 37°C. This solution was extracted with an equal volume of fresh phenol, followed by extraction with an equal volume of chloroform. The plasmid DNA in the

aqueous phase was precipitated by the addition of 3 volumes (1.5ml) of ice-cold 100% ethanol and 0.1 volume (50ul) of 3M sodium acetate (pH5.2), and collected by centrifugation at 13000 rpm at 4°C for 5 minutes in a microcentrifuge. The precipitate was then washed briefly with 200ul of ice-cold 70% ethanol, dried under vacuum, redissolved in sterile TE buffer and stored at -20°C.

7. Plasmid species used in these studies.

The plasmid species used for the bacterial expression of NF-κB p50 protein derivatives was the expression vector pGEX-2T (Smith and Johnson, 1988), while the parental plasmid used for the generation of the circularly permuted κB binding site plasmid p2xAT/HIV-R was the pAT153 (Twigg and Sherratt, 1980) derivative of pBR322.

8. Generation of circularly permuted κB binding site plasmid.

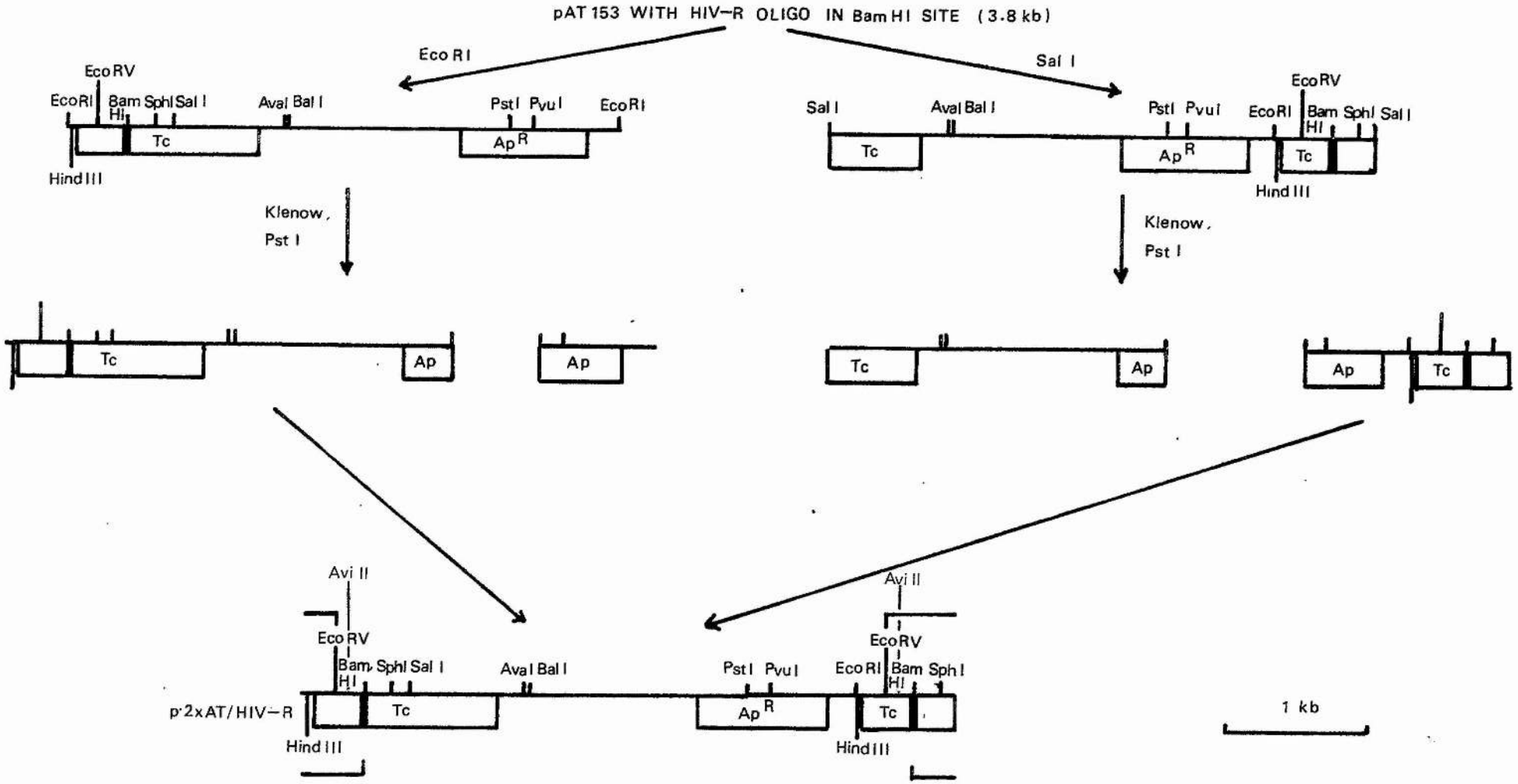
The construction of the cicularly permuted κB binding site plasmid p2xAT/HIV-R is given in Figure 1.

9. Bacterial transformation procedure.

For the transformation of *E.coli*, a one-step method was used (Chung et al., 1989) - generating competent *E.coli* in a transformation and storage solution (TSS). A 100ml culture of *E.coli* strain JM101 in Luria broth was grown at 37°C to a density equivalent to A_{600} = 0.3-0.4, the culture was then centrifuged in a Beckman JA14 rotor at 2500 rpm at 4°C for 10 minutes, the supernatant discarded, and the bacterial pellet resuspended in 10ml of ice cold TSS solution (Luria broth, pH6.5, 10% w/v polyethyleneglycol 6000 (BDH), 5% v/v dimethylsulphoxide, 50mM $MgCl_2$). Aliquots (0.1ml) of the suspension were

Figure 1.

Construction of p2xAT/HIV-R circular permutation κB binding site plasmid



transferred to cold 1.5ml Eppendorf tubes, mixed with 5ul of plasmid DNA solution, and incubated at 4°C for 30 minutes. Finally, 0.9ml of Luria broth, 20mM glucose was added to each tube, the cells shaken at 37°C for 1 hour, and 0.1ml aliquots of bacterial suspension spread onto Luria-agar plates (supplemented with the appropriate antibiotics) and incubated at 37°C overnight.

10. Preparation of oligonucleotide affinity resin.

The scheme for preparing the oligonucleotide affinity resin was based on that of Kadonaga and Tjian (Kadonaga and Tjian, 1986) - to generate the HIV enhancer oligonucleotide affinity resin, 4mg each of the single-stranded amino linked (with a primary amine group coupled to its 5' terminus) HIV-1 oligonucleotide 5' GATCAAGGGACTTTCCGCTGGGGACTTTCCAGG 3', and the single-stranded HIV-2 oligonucleotide 5' GATCCCTGGAAAGTCCCCAGCGGAAAGTCCCTT 3' were hybridised in approximately 1.6ml oligonucleotide hybridisation buffer (10mM Tris.HCl,pH8.0, 1mM EDTA,pH8.0, 100mM NaCl) by heating to 100°C for approximately 2 minutes, then allowing to cool to room temperature over 2-3 hours, the double stranded HIV oligonucleotide was then stored at 4°C. In the following reactions coupling the hybridised HIV oligonucleotide to the Sepharose affinity matrix, all solutions used were ice cold. For the coupling reaction, 3g of CNBr-activated Sepharose 4B (Sigma) was swollen in 15ml of 1mM HCl for 1-2 hours, then washed in a sintered glass funnel with 400ml of 1mM HCl, followed by 500ml of ultrapure water. The resin slurry was then washed with 400ml of 10mM sodium phosphate buffer,pH8.0, virtually all of the slurry supernatant discarded, and the

resin transferred to a 50ml tube. The 8mg of double stranded HIV enhancer oligonucleotide was then adjusted to 10mM sodium phosphate,pH8.0, added to the Sepharose resin slurry, and allowed to react overnight at room temperature with rotation.

After the overnight coupling reaction, the Sepharose resin suspension was centrifuged at low speed, and the supernatant removed, the resin was then washed with 5ml of 10mM sodium phosphate,pH8.0, centrifuged briefly and the supernatant removed. Finally, unreacted sites remaining on the CNBr-activated Sepharose were blocked by washing the slurry with 100ml of 1M ethanolamine,pH8.0 and rotating for 4 hours at room temperature, the resin was then washed with 100ml of 10mM sodium phosphate buffer,pH8.0, 0.1M NaCl, followed by washing with 100ml 1M NaCl, and 100ml TEN_{300} buffer supplemented with 0.02% NaN_3 (10mM Tris.HCl,pH7.5, 1mM EDTA,pH8.0, 300mM NaCl, 0.02% NaN_3). The HIV-Sepharose 4B affinity resin was then stored as a slurry in TEN_{300},0.02% NaN_3 at 4°C.

11. Preparation of calf thymus double stranded DNA affinity resin.

For the preparation of the calf thymus DNA affinity resin, essentially the same reaction procedure was followed using double stranded, sheared calf thymus DNA (Sigma) with CNBr-activated Sepharose 4B (Sigma).

12. Generation of cellular protein extracts.

Cellular protein extracts were prepared by different routes depending on the numbers of cells to be extracted. For small scale cell extractions of Jurkat T-cells, 1ml aliquots of cell suspension (at approximately $5x10^8$ to 10^9 cells/l) were

centrifuged briefly at 6500 rpm in a microfuge, the cell pellet resuspended and washed in ice-cold PBS, the cells pelleted again, and the pellet freeze-thawed in 50ul. of ice-cold lysis buffer (20mM Hepes,pH7.5, 1mM DTT, 1mM EDTA,pH8.0, 1mM sodium metabisulphite, 1mM PMSF (added just before use), 0.5% NP40). After vortexing, the nuclei were collected by brief centrifugation and the supernatants snap-frozen in liquid nitrogen as cytoplasmic extracts. The pelleted nuclei were then resuspended in 25ul of ice-cold extraction buffer (20mM Hepes,pH7.5, 1mM DTT, 1mM EDTA,pH8.0, 1mM sodium metabisulphite, 1mM PMSF, 0.35M NaCl), allowed to stand on ice for 30 minutes, then clarified by centrifugation and the supernatants snap-frozen as nuclear extracts.

For large scale HeLa cell extractions, only nuclear extracts were prepared since the S3 HeLa cell line used has constitutive nuclear NF-κB activity, with virtually no active or IκB-shielded NF-κB DNA binding activity in cytoplasmic extracts. The method used for preparing large scale nuclear extracts was a variant of that of Dignam (Dignam et al., 1983) - a 10ml pellet of liquid nitrogen snap-frozen HeLa S3 cells was thawed on ice, resuspended in 40ml of buffer A (10mM Hepes,pH7.5, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT) and allowed to swell for 30 minutes on ice. The hypotonically swollen cells were then lysed in a Dounce homogeniser (10 strokes with a B-type pestle), a sample checked microscopically for cell lysis, and the homogenate centrifuged at 2500 rpm at 4°C for 10 minutes in a benchtop centrifuge. The supernatant was discarded, and the remaining sedimented crude nuclei (7ml) resuspended with 36ml of ice-cold buffer B (25mM Hepes-NaOH, pH7.5, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT, 0.2M sucrose, 1mM PMSF (added just before use), 0.35M NaCl) by 10 strokes with a B-type

pestle in a Dounce homogeniser (the resulting mixture being 0.25M with respect to NaCl), then stirred gently on ice for 30 minutes. After this extraction stage, the suspension was centrifuged in a Beckman 42.1 rotor at 30000 rpm (100000G) at 4°C for 30 minutes. The supernatant was removed, then centrifuged again in a type 42.1 rotor under the same conditions as before, and the supernatant recovered as the nuclear extract - this was either aliquoted into smaller volumes, snap-frozen in liquid nitrogen, and stored at -70°C, or used directly in attempts to affinity purify NF-κB.

13. Purification of κB binding proteins.

To purify NF-κB from HeLa cells, 50g of cells were fractionated into nuclei and cytoplasm and the nuclei extracted with 0.25M NaCl as described (Clark et al., 1990). All operations were carried out at 4°C, and all buffer solutions kept on ice. 120ml of 0.25M NaCl HeLa cell nuclear extract was supplemented with spermidine (trihydrochloride - Sigma) to 3mM, EDTA,pH8.0 to 2mM, 0.5mg poly dAdT.dAdT/dGdC.dGdC carrier DNA (Pharmacia), and n-octylglucopyranoside (Sigma) to 3mM and applied to a 1.4ml HIV-Sepharose affinity column at a flow rate of 40ml per hour. The column was washed with 10 column volumes of buffer C (25mM Hepes-NaOH,pH7.5, 1mM DTT, 20% w/v glycerol, 0.01% NP-40 (BDH), 10mM EDTA,pH8.0, 3mM spermidine, 3mM n-octylglucopyranoside), supplemented with 0.25M NaCl and bound proteins eluted from the affinity matrix with 3.3ml of buffer C containing 0.6M NaCl: 1 column volume of the high salt buffer C was allowed to pass through before the column was blocked off for 1 hour, and then the remaining eluant allowed to pass through - the whole process taking 1.5 hours.

The sodium chloride concentration of the eluted protein solution was then reduced to 0.1M by the addition of 16.5ml of 50mM Tris.HCl,pH7.5 buffer, the mixture centrifuged in a Beckman 42.1 rotor at 30000 rpm at 4°C for 30 minutes, and the supernatant recovered. This supernatant was then applied to a Mono-Q FPLC chromatography column (Pharmacia-LKB) pre-equilibrated with 50mM Tris.HCl,pH7.5 buffer, and eluted from the Mono-Q column with a salt gradient using 50mM Tris.HCl,pH7.5 and 50mM Tris.HCl,pH7.5, 5M NaCl. Those early eluting Mono-Q fractions (total volume 8.5ml) showing κB DNA binding activity were pooled, and their sodium chloride concentration adjusted to 0.35M by addition of buffer C, 0.5M NaCl prior to loading on a 2ml *E.coli* DNA-Sepharose column equilibrated with buffer C, 0.35M NaCl over approximately 1.5 hours. The combination of 0.35M sodium chloride and non-specific *E.coli* DNA resulted in virtually all of the κB site binding protein remaining in the flowthrough fraction, rather than binding to the column. In contrast, contaminating proteins with high affinity for non-specific DNA sequences bound to the *E.coli* DNA affinity resin under these conditions.

The flowthrough from the non-specific *E.coli* DNA column (29ml) was collected, the salt concentration reduced to 0.25M by addition of 11.6ml of buffer C, and 50ug of poly dAdT.dAdT/dGdC.dGdC carrier DNA added prior to loading onto the final 1ml HIV-Sepharose affinity column over approximately 1 hour. The column washed with 10ml of buffer C,0.25M NaCl over approximately 1 hour and bound proteins eluted with 4ml of buffer C,0.8M NaCl: 1 column volume of high salt buffer was allowed to flow through the column, then the column was blocked off and allowed to stand for 1 hour, before the remainder of the eluant

passed through the column - the whole process taking 2 hours. The eluted protein and the other fractions were snap-frozen in liquid nitrogen before being stored at -70°C.

14. Lectin affinity resin purification of native κB binding proteins.

Following a report of the purification of the transcription factor Sp1 using wheat germ agglutinin lectin - agarose (Jackson and Tjian, 1989), a similar purification scheme was attempted for NF-κB proteins from HeLa S3 cell line nuclear extracts. In this purification scheme, all operations were carried out at 4°C, and all buffer solutions kept on ice. Thus, frozen HeLa cells(0.86g) were resuspended in 5 volumes (4.3ml) of ice-cold PBS, then centrifuged at 2000 rpm at 4°C for 10 minutes in a bench top centrifuge, and the supernatant discarded. The pellet was then resuspended in 5 packed cell pellet volumes (4.3ml) of buffer D (10mM Hepes,pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT), then allowed to stand 10 minutes, before centrifugation at 2000 rpm at 4°C for 10 minutes in a bench top centrifuge, and the supernatant discarded. This cell pellet was resuspended in 2 packed cell pellet volumes (1.7ml) of buffer D, and then lysed in a Dounce homogeniser by 10 strokes with a B-type pestle. The homogenate was checked for cell lysis, then centrifuged at 4000 rpm in a Beckman 42.1 rotor at 4°C for 10 minutes, and the supernatant discarded. The pellet was then recentrifuged in a 42.1 rotor at 15500 rpm at 4°C for 20 minutes and any residual cytoplasmic material removed. The crude nuclear pellet was then resuspended in 3.0ml of buffer E (50mM Tris.HCl,pH7.5, 0.42M KCl, 20% v/v glycerol, 10%

w/v sucrose, 5mM $MgCl_2$, 0.1mM EDTA,pH8.0, 1mM PMSF (added just before use), 1mM sodium metabisulphite, 2mM DTT) by 10 strokes with a B-type pestle in a Dounce homogeniser. The resulting suspension was then stirred gently on ice for 30 minutes before centrifugation in a 42.1 rotor at 28000 rpm at 4°C for 60 minutes, and the supernatant (2.9ml) collected.

This nuclear extract was applied to a 2ml wheat germ agglutinin (WGA)-agarose (Vector) column (pre-equilibrated with buffer E) over approximately 1.5 hours and the column washed successively with 10ml of buffer E, and 20ml of the buffer F (25mM Hepes-KOH, pH7.6, 12.5mM $MgCl_2$, 20% glycerol, 0.1% v/v NP40, 10uM $ZnSO_4$, 1mM DTT, 0.1M KCl). Finally, the 2ml WGA-agarose column was eluted over 1 hour with 10ml of buffer F supplemented with 0.3M N-acetylglucosamine (Sigma) - the eluate being collected as 10x1ml fractions before being snap-frozen in liquid nitrogen, and stored at -70°C. In some experiments N-acetylglucosamine (0.1M) was added to the resuspended crude nuclear pellet before the 30 minute nuclear extraction stage.

15. Generation of cDNA encoding NF-κB p50 amino acids 35-381.

Making use of a pBluescript (Stratagene) plasmid with a cDNA insert encoding the p105 precursor to p50 (kindly provided by Dr.A.Israël), a region of cDNA corresponding to the roughly-defined DNA binding and dimerisation region (Kieran et al., 1990; Ghosh et al., 1990) was amplified by polymerase chain reaction (PCR). The two oligonucleotide PCR primers used had sequences 5' GATGAAGGATCCAATATGGCACTGCCAACAGCAGATGGC 3' for the N-terminal coding sequence (making use of the internal methionine-35 codon does

not affect DNA binding activity), and 5'

TATAGCGAATTCCTAGGCACCACTACCACCGCCGAAACT 3' for the C-terminal coding sequence (with a termination codon after amino acid 381). The PCR amplification would be expected to yield a product of about 1065bp with Bam HI and Eco RI restriction enzyme cleavage sites just beyond the N- and C-termini respectively of the coding sequence.

PCR amplification was carried out in a 100ul volume containing 1uM of each primer, 100ng of the p105 plasmid, 0.2mM of each dNTP, 1xPCR buffer (Perkin Elmer Cetus), 2 Units of Amplitaq (Perkin Elmer Cetus), and 10^{-4}M tetramethylammonium chloride (Sigma). Reaction mixtures were overlaid with mineral oil and subjected to the following thermal cycling program (Techne PHC-1) :- denature at 94°C / 1.5 minutes, anneal at 55°C / 1.5 minutes, extend at 72°C / 2 minutes, repeated for 25 cycles with a final 10 minute extension cycle. Immediately after the completion of the thermal cycling program, the DNA product was phenol, and then chloroform extracted, and 5ul of the DNA product assayed for homogeneity and expected size by gel electrophoresis on a 0.8% agarose gel. The PCR-generated DNA was then precipitated by addition of 0.1 volume of 3M sodium acetate (pH5.2) and 0.6 volumes of isopropanol, standing at room temperature 5 minutes, then centrifuged at 13000 rpm at room temperature for 5 minutes. After washing with 70% ethanol and drying the DNA pellet under vacuum, the PCR product was digested with 10 units each of Bam HI and Eco RI restriction enzymes and 1ul of the correct 10x restriction enzyme buffer made up to a total volume of 10ul.

The Bam HI/Eco RI digested PCR product was then made up to a volume of

100ul with TEN_{100} and again extracted with phenol and chloroform before the addition of 20ug of phenol-extracted yeast tRNA (Sigma) and the mixture then isopropanol precipitated as described before. The resulting pellet was then redissolved and ligated at room temperature overnight into 0.1ug of Bam HI/Eco RI digested pGEX-2T bacterial expression vector (Smith and Johnson, 1988) in a total volume of 20ul - the ligation reaction conditions were as described above. The overnight ligation mixture was used to transform *E.coli* JM101 by the one-step TSS transformation protocol, the bacterial suspension was plated out on Luria-agar/ampicillin (100ug/ml) plates, and ampicillin resistant transformants identified after overnight incubation at 37°C.

16. Generation of cDNA encoding cysteine to serine mutants of NF-κB p50.

To generate cDNA molecules encoding mutant p50 proteins, a PCR mutagenesis technique was employed (Ho et al., 1989) - PCR amplification conditions were as described above but with two internal mutagenising primers and the two external primers described previously to generate two partial length mutant cDNA products (Figure 2.3). Thus for mutation of the amino acid 62 cysteine codon (TGT) to a serine codon (TCC), the two partial length mutant cDNA molecules were made by PCR amplification as described above - but using the N-terminal external primer with the mutation primer 5' ATGGGATGGGCCTTCGGATACATAACGGAAACG 3' to generate a 120bp N-terminal encoding cDNA, and using the C-terminal external primer with the mutation primer 5' CGTTTCCGTTATGTATCCGAAGGCCCATCCCAT 3' to generate a 940bp C-terminal encoding cDNA.

To generate the amino acid 119 cysteine codon (TGT) to serine codon (TCC) mutation, partial length mutant cDNAs were made using the N-terminal external primer with the mutation primer 5'

GATCCCATCCTC**GGA**GTGTTTTCCCACCAG 3' to generate a 270bp N-terminal encoding cDNA, and using the C-terminal primer with the mutation primer 5' CTGGTGGGAAAACAC**TCC**GAGGATGGGATC 3' to generate a 790bp C-terminal encoding cDNA. While for the amino acid 273 cysteine codon (TGT) to serine codon mutation (TCC), the partial length mutant cDNA molecules were made using the N-terminal external primer with the mutation primer 5' CTGAACTTTGTC**GGA**AAGAAGATAAATTTCCTCCCC 3' to generate a 730bp N-terminal encoding cDNA, and using the C-terminal primer with the mutation primer 5' GGGGAGGAAATTTATCTTCTTT**CC**GACAAAGTTCAG 3' to generate a 330bp C-terminal encoding cDNA molecule.

The partial length PCR amplification products were then directly purified by agarose gel electrophoresis in gels ranging in concentration from 0.8% to 3% agarose (Sigma) as appropriate, made up with, and running in, 1xTBE buffer / 0.2ug/ml ethidium bromide. Agarose gel bands corresponding to the correct size partial length PCR products were cut out and 1-2ul of both gel bands used to provide the two partial length templates for each final amplification reaction. The conditions for the second round of PCR amplification were as described previously - but using 1-2ul of each partial length template DNA gel band, with the N-terminal and C-terminal encoding primers. In addition, the thermal cycling program was slightly altered to give longer annealing and extension times of 2 and 2.9 minutes respectively. Samples of the final mutant PCR amplification products

were analysed by agarose gel electrophoresis on a 0.8% gel as described above - showing essentially homogeneous bands of the expected 1065bp size. The DNA products were then isopropanol precipitated, Eco RI and Bam HI digested, and ligated into similarly digested pGEX-2T expression vector as described above. Finally, the ligation mixtures were used to transform *E.coli* JM101, the bacterial suspensions plated out on Luria-agar/ampicillin plates as above, and ampicillin resistant transformants isolated.

17. Sequencing of cDNA encoding cysteine to serine mutants of NF-κB p50.

To confirm that the PCR mutagenesis scheme had yielded the expected products, the inserted sequences of the recombinant pGEX-2T plasmids encoding the p50 mutants were sequenced directly using a double stranded DNA cycle sequencing system (Gibco BRL). Primer labelling for the sequencing reactions employed 2pmoles of primer, 10uCi γ-^{32}P ATP, 1 unit T4 polynucleotide kinase, 0.5ul 10xT4 kinase buffer (0.5M Tris.HCl,pH7.6, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine.HCl, 1mM EDTA,pH8.0), in a total volume of 5ul, the mixture was then incubated at 37°C for 30 minutes, the reaction then stopped by heating the mixture to 55°C for 5 minutes, and the labelled primers stored at 4°C. The template DNA for the sequencing reactions was prepared directly from single bacterial colonies - 1-2mm diameter single colonies were transferred to 0.5ml Eppendorf tubes containing 12ul of single colony lysis solution (10mM Tris.HCl,pH7.5, 1mM EDTA, 50ug/ml Proteinase K (Sigma)), the tubes were vortexed briefly, then incubated at 55°C for 15 minutes. Finally, the Proteinase K

was inactivated by heating to 80°C for 15 minutes, the tubes placed on ice for 1 minute, then centrifuged at 13000 rpm in a microcentrifuge at room temperature for 3 minutes, and 9ul of supernatant carefully removed (as two 4.5ul aliquots) to provide the template for sequencing both strands of the mutant DNA. The sequencing reactions used specific dideoxy termination mixtures for each base type. Pre-reaction mixtures with a total volume of 32ul - containing 4.5ul template supernatant, 2 Units Taq DNA polymerase (Perkin Elmer Cetus) were mixed, centrifuged, and placed on ice. To each of four 0.5ml Eppendorf tubes was added 2ul of one of the dideoxy termination mixes, followed by 8ul from the pre-reaction mixture, these were mixed and overlaid with 10ul of mineral oil. The tubes were then transferred to a thermal cycling block (preheated to 95°C), and PCR amplification carried out using the following regime - 30 seconds denaturing at 95 °C, 30 seconds annealing at 55°C, followed by 60 seconds extension at 70°C - repeated for 20 cycles, with a final 2.5 minute / 70°C extension step, and the reaction terminated by the addition of 5ul of stop solution (Gibco BRL) to each tube. The PCR sequencing reaction products were then stored at -20°C before being analysed by denaturing gel electrophoresis within 24 hours.

18. pGEX-2T bacterial fusion protein expression system.

The bacterial expression of recombinant NF-κB p50 protein derivatives made use of the pGEX-2T expression vector developed by Smith and Johnson (Smith and Johnson, 1988) - in this system the foreign protein is expressed after induction with isopropyl-ß-D-thiogalactopyranoside (IPTG) (Sigma) as a fusion with the C-terminus of a 26kD glutathione S-transferase from a parasitic helminth. This

system has the advantage that a large proportion of such fusion proteins prove soluble in aqueous solution, and can be affinity purified by passage over glutathione-agarose matrices. Further, the vector has been engineered such that the linker region between the C-terminus of the glutathione S-transferase and the mutiple cloning site encodes a recognition sequence for the protease thrombin, allowing the fusion protein to be cleaved and the foreign protein to be isolated. In addition, the pGEX-2T plasmid also encodes an ampicillin resistance gene - allowing the selection of bacterial transformants.

As described previously, Eco RI and Bam HI-digested pGEX-2T vector was ligated with similarly digested p50-encoding PCR products, the ligation mixtures used to transform *E.coli* JM101, and ampicillin-resistant transformants isolated. To confirm that these ampicillin resistant colonies represented the expected recombinant plasmids, 10ml cultures were grown up to an A_{600} of 0.6 in Luria broth and 100ug/ml ampicillin, then induced with the addition of 0.5mM IPTG for 4 hours at 25°C. Samples of the induced bacterial cultures (1.5ml) were centrifuged briefly in a microcentrifuge, the supernatant discarded, and the pellet typically resuspended by vortexing in 50ul of 4x concentrated SDS gel sample buffer and 150ul of ultrapure water. The suspension was heated to 100°C for 3 minutes and quenched on ice, vortexed extensively to shear cellular DNA and 10ul analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis on a 10% polyacrylamide gel under reducing conditions. Those bacterial transformants expressing a correct size recombinant fusion protein were then isolated for further study.

19. Affinity purification of bacterially expressed p50 proteins.

For the large scale purification of bacterially-expressed NF-κB p50 protein, a two-stage purification was used - an initial glutathione-agarose purification of the intact fusion protein, followed by cleavage of the essentially pure fusion protein by incubation with thrombin, and a calf thymus double stranded DNA-Sepharose affinity purification step. Using the appropriate *E.coli* JM101 transformants, 500ml Luria broth/100ug/ml ampicillin cultures were grown at 37°C until A_{600} was approximately 0.6, IPTG was added to 0.5mM and the cultures induced for 4 hours at 25°C. Bacterial cells were collected by centrifugation in a Beckman JA14 rotor at 2500 rpm at 4°C for 10 minutes and the growth media discarded. Except where indicated, all further operations were carried out at 4°C with ice-cold buffer solutions. The bacterial pellets were resuspended in 10ml of high salt buffer (20mM sodium phosphate,pH7.0, 0.5M NaCl, 1mM DTT), then disrupted by sonication on ice (4x20 seconds, MSE Soniprep 150). After the addition of Triton X-100 detergent to the bacterial lysates to 1%, the lysates were clarified by centrifugation in a Beckman JA20 rotor at 14000 rpm at 4°C for 1 hour, and the supernatants recovered and passed over 1ml glutathione-agarose (Sigma) columns (pre-equilibrated with high salt buffer). The 1ml glutathione-agarose columns were then washed extensively with 10ml of high salt buffer, then eluted with reduced glutathione/high salt buffer (10mM reduced glutathione (Sigma), 50mM Tris.HCl,pH8.0, 0.5M NaCl) and 0.5ml fractions collected. Fractions containing fusion protein were identified by SDS polyacrylamide gel electrophoresis under reducing conditions, pooled and the fusion proteins cleaved by incubation with 6 units of human thrombin (Sigma) per mg of fusion protein at 20°C for 3 hours.

For the second purification stage, the concentration of sodium chloride in the cleaved fusion protein solutions was lowered to 150mM by dilution with 10mM sodium phosphate buffer,pH7.4, 2mM DTT - and applied to 1ml calf thymus double stranded DNA-Sepharose columns (pre-equilibrated in PBS, 2mM DTT). The calf thymus DNA-Sepharose columns were then washed extensively with 10ml of PBS, 2mM DTT and bound protein eluted by raising the NaCl concentration to 0.6M. 0.5ml fractions were collected and assayed for protein concentration by the Bradford reagent dye binding method (Bradford, 1976) and for homogeneity by SDS-polyacrylamide gel electrophoresis before being pooled, aliquoted in 50ul fractions, and snap-frozen in liquid nitrogen before storage at -70°C.

20. Radiolabelling of DNA restriction fragments and oligonucleotides.

To label the 16bp blunt-ended double stranded κB motif oligonucleotide with ^{32}P, 10 pmoles of single stranded 16-mer κB motif oligonucleotide were incubated in 20ul of (50mM Tris.HCl,pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine.HCl, 0.1mM EDTA,pH8.0) buffer with 5 Units of T4 polynucleotide kinase (New England Biolabs), and 29 pmoles of $\gamma^{32}P$ ATP (Amersham, specific activity 3000Ci/mmol) at 37°C for 30 minutes in a 1.5ml screw-cap tube. The reaction was then terminated by heating to 100°C for 2 minutes, and 16 pmoles (3.2ul) of complementary strand 16-mer κB motif oligonucleotide added, before allowing the mixture to cool gradually to room temperature over 2-3 hours. Radiolabelled, double stranded 16bp κB motif oligonucleotide was separated from unincorporated $\gamma^{32}P$ ATP and excess complementary single strand unlabelled

oligonucleotide by electrophoresis on a non-denaturing 12% polyacrylamide gel. Radioactivity in the wet gel was visualised by autoradiography with X-ray film (Fuji RX), and the polyacrylamide gel slice containing the double stranded oligonucleotide recovered. The double stranded, radiolabelled oligonucleotide was then passively eluted from the gel slice by addition of 400ul of TEN_{100} buffer, and eluting overnight at room temperature, before storage at 4°C.

For the radiolabelling of other double stranded oligonucleotides (and some restriction fragments) with overhanging 5' ends, the Klenow fragment of *E.coli* DNA polymerase I was used with $\alpha^{32}P$ dATP to incorporate radioactivity into the double stranded DNA. The double stranded HIV-L oligonucleotide was radiolabelled with the following mixture - 10ul of 50mM Tris.HCl,pH7.5, 10mM $MgSO_4$, 0.1mM DTT, 50ug/ml BSA containing 100ng of HIV-L double stranded oligonucleotide, 2 nmoles each dCTP, dTTP, dGTP, 0.5ug gelatin, 25uCi $\alpha^{32}P$ dATP, and 2 Units of the Klenow fragment of *E.coli* DNA polymerase I - was incubated at room temperature for 15 minutes, then 4 nmoles of dATP added, and the mixture incubated at room temperature for a further 20 minutes. Finally, the reaction was stopped by addition of 3ul of DNA dyes (20% glycerol, 50mM EDTA,pH8.0, trace bromophenol blue), and radiolabelled double stranded HIV-L oligonucleotide separated from unincorporated nucleotide by electrophoresis on a non-denaturing 8% polyacrylamide gel. The gel slice containing the double stranded oligonucleotide was isolated as described above, and was passively eluted overnight at room temperature in 200ul of TEN_{100} buffer, before storage at 4°C.

To generate the various circularly permuted κB site restriction fragments for

DNA bending studies, the p2xAT/HIV-R plasmid construct carrying the HIV-R oligonucleotide (with an identical κB motif to that in the HIV-L oligonucleotide) was digested with one of the following restriction enzymes - Hind III, Sph I, Bam HI, Avi II, or Eco RV in 10ul of the appropriate restriction enzyme buffer (50mM NaCl for Hind III and Sph I, 100mM NaCl for Bam HI and Avi II, 150mM NaCl for Eco RV) containing 1ug gelatin, 1.8ug of p2xAT/HIV-R plasmid was digested with 10units of restriction enzyme for 2 hours at 37°C. To the 10ul restriction digest mixtures, 1.5ul of 10x T4 DNA polymerase buffer was added (0.33M Tris-acetate,pH8.0, 0.66M potassium acetate, 0.1M magnesium acetate, 5mM DTT, 1mg/ml BSA), followed by 2units of T4 DNA polymerase, and made up to 15ul with ultrapure water. This mixture was incubated at 37°C for 7 minutes in the absence of deoxynucleoside triphosphates, then 6nmoles each of dCTP, dGTP, and dTTP added, followed by 15uCi α^{32}P dATP and 0.2nmoles dATP, and the mixture incubated at 37°C for 15 minutes. Finally, 4nmoles dATP was added and the reaction incubated again at 37°C for 15 minutes. Nucleotide incorporation was stopped by the addition of SDS to 0.15% followed by addition of 4.5ul of DNA dyes, the radiolabelled restriction fragments were then separated from unincorporated radiolabelled nucleotide by electrophoresis on a non-denaturing 5% polyacrylamide gel. The gel slices containing the 600bp circularly permuted κB site restriction fragment were identified as described above, and the slices then electroeluted (Schleicher and Schuell, Biotrap) in 200ul of 1/20xTBE buffer, after elution sodium chloride was added to 100mM and the cicularly permuted binding site probes stored at 4°C.

21. Determination of protein concentration.

For the estimation of protein concentrations, a microscale variant of Bradford's dye binding method was used (Bradford, 1976), 5-10ul of protein sample was added to 1ml of Bradford reagent (0.1mg/ml Coomassie brilliant blue G250, 5% v/v ethanol, 10% v/v orthophosphoric acid), mixed and allowed to stand for 10 minutes before measurement of A_{595}. In each case absorbance measurements were performed against blanks containing an equal volume of the appropriate buffer in 1ml Bradford reagent. These absorbance measurements were then correlated with a freshly-generated calibration curve (0-25ug of bovine serum albumin in 1ml Bradford reagent) to estimate the protein concentration of the unknown sample.

22. Gel electrophoresis.

For gel electrophoresis DNA binding assays, non-denaturing polyacrylamide gels (6% polyacrylamide for assays using oligonucleotide probes, and 5% polyacrylamide for the 600bp circularly permuted restriction fragment probes) were prepared using a 44 : 0.8 ratio of acrylamide : N,N'-methylene-bis acrylamide. Thus, 50ml of 0.5xTBE buffer supplemented with the desired overall acrylamide concentration (using a 44 : 0.8 ratio 40% acrylamide stock solution) was polymerised with 200ul of 25% w/v ammonium persulphate solution and 20ul of TEMED, and the mixture cast into 1.5mm thick gels (BRL V15-17). Gel plate assemblies were then mounted in BRL V15-17 apparatus and buffer tanks filled with 0.5xTBE prior to sample loading and electrophoresis at 200V for typically 40 minutes. After electrophoresis, radioactive gels were either quantitated directly by Cerenkov counting wet gel slices, alternatively polyacrylamide gels were dried

under vacuum (Bio-Rad) onto DEAE paper (Whatman DE81) and radioactivity visualised by autoradiography.

For the purification of radiolabelled oligonucleotides and restriction fragments, non-denaturing polyacrylamide gels (ranging from 12% polyacrylamide for small oligonucleotides to 5% polyacrylamide for 600bp restriction fragments) were prepared using a 29 : 1 ratio of acrylamide : N,N'-methylene-bis acrylamide. Thus 25ml of 1xTBE buffer supplemented with the necessary overall acrylamide concentration (using a 29 : 1 ratio 30% acrylamide stock solution) was treated with 100ul of 25% w/v ammonium persulphate solution and 10ul of TEMED, and the polymerising mixture cast into 0.75mm thick gels (BRL V15-17). Gel plate assemblies were mounted in BRL V15-17 apparatus and buffer tanks filled with 1xTBE buffer prior to sample loading and electrophoresis at 200V for typically 1 hour. After electrophoresis, the gel bands corresponding to the DNA molecules of interest were identified by autoradiography of the wet gel, the gel bands cut out, and the DNA eluted.

For protein characterisation, denaturing SDS polyacrylamide gel electrophoresis based on the method of Laemmli (Laemmli, 1970) was carried out in a mini gel system (Bio-Rad Mini Protean) under reducing and non-reducing conditions.

23. Reagents and buffer solutions.

All reagents used in these studies were Analar grade or its equivalent.

The following buffer solutions were used routinely during these studies:-

TE (10mM Tris.HCl, pH7.5, 1mM EDTA, pH8.0)

TEN_{100} (10mM Tris.HCl, pH7.5, 1mM EDTA, pH8.0, 100mM NaCl)

TEN300 (10mM Tris.HCl, pH7.5, 1mM EDTA, pH8.0, 300mM NaCl)

TBE (100mM Tris.Borate, 1mM EDTA, pH8.3)

SDS Gel Running Buffer (25mM Tris.HCl, 200mM glycine, 0.1% SDS, pH8.3)

24. DNA Binding assays.

Protein DNA-binding activity was determined using gel electrophoresis on 6% (5% for large restriction fragment-derived probes) non-denaturing polyacrylamide (44 : 0.8 acrylamide : bis acrylamide) gels. Typically, 1ul of protein solution (fully reduced by the addition of DTT to 25mM and incubating on ice for 15 minutes) was added to 18ul gel mobility shift binding buffer (85mM NaCl, 8.5% v/v glycerol, 22mM Hepes,pH7.5, 1.3mg/ml BSA, 0.17% NP40, 3.6mM spermidine (except when specifically omitted), 0.85mM DTT, 0.85mM EDTA,pH8.0, 6.1mM $MgCl_2$) and incubated on ice for 15 minutes. Finally, 1-2ul of radiolabelled DNA probe was added and the solution incubated at 20°C for 15 minutes before the addition of 4ul of mobility shift gel dyes, and loading onto the gel. After electrophoresis in 0.5xTBE buffer at 200V for typically 40 minutes, radioactivity in gels was either determined directly by Cerenkov counting, or by drying the polyacrylamide gel under vacuum onto DEAE-cellulose paper (Whatman, DE81), followed by autoradiography and scintillation counting of dried gel slices.

25. Use of cysteine-reactive reagents.

For the inactivation of p50 protein DNA binding activity, p50 protein/gel mobility shift binding buffer mixtures were treated with N-ethylmaleimide (NEM) or iodoacetate before the addition of radiolabelled probe DNA. For NEM inactivation of DNA binding activity, 80mM NEM stock solution was added to

give a 10mM NEM concentration in the p50 protein/binding buffer mixture, and the mixture incubated at 20°C for 10 minutes. While for iodoacetate inactivation of DNA binding activity, 250mM neutral iodoacetate, 30mM Tris.HCl,pH8.0 stock solution was added to give a final concentration of 25mM iodoacetate in the p50 protein/binding buffer mixture and incubated at 20°C for 15 minutes before the addition of the radiolabelled probe. In those experiments demonstrating the differing dose-response curves for iodoacetate inactivation of the p50 protein mutants, the amount of iodoacetate stock solution added was varied accordingly. Similarly, in the experiment demonstrating the ability of the bound oligonucleotide to protect p50 protein against iodoacetate inactivation, the oligonucleotide probe was added before and after treatment with varying amounts of 250mM neutral iodoacetate stock solution and its incubation at 20°C for 15 minutes.

For the cross-linking of appropriately positioned cysteine residues in the various species of p50 protein aa35-381 dimer, p50 proteins were reduced by addition of DTT to 25mM and incubation on ice for 15 minutes, before the addition of 100mM diazenedicarboxylic acid *bis* [N,N'-dimethylamide] (diamide, Sigma), 0.5M NaCl stock solution to give a final diamide concentration of 33mM, and incubation at 37°C for 1 hour. After the formation of disulphide bonds had been induced by diamide treatment (Kosower et al., 1969), the reactions were quenched by the addition of 250mM neutral iodoacetate stock solution to give a final iodoacetate concentration of 60mM and then incubated at 20°C for 30 minutes.

26. Radioactive counting.

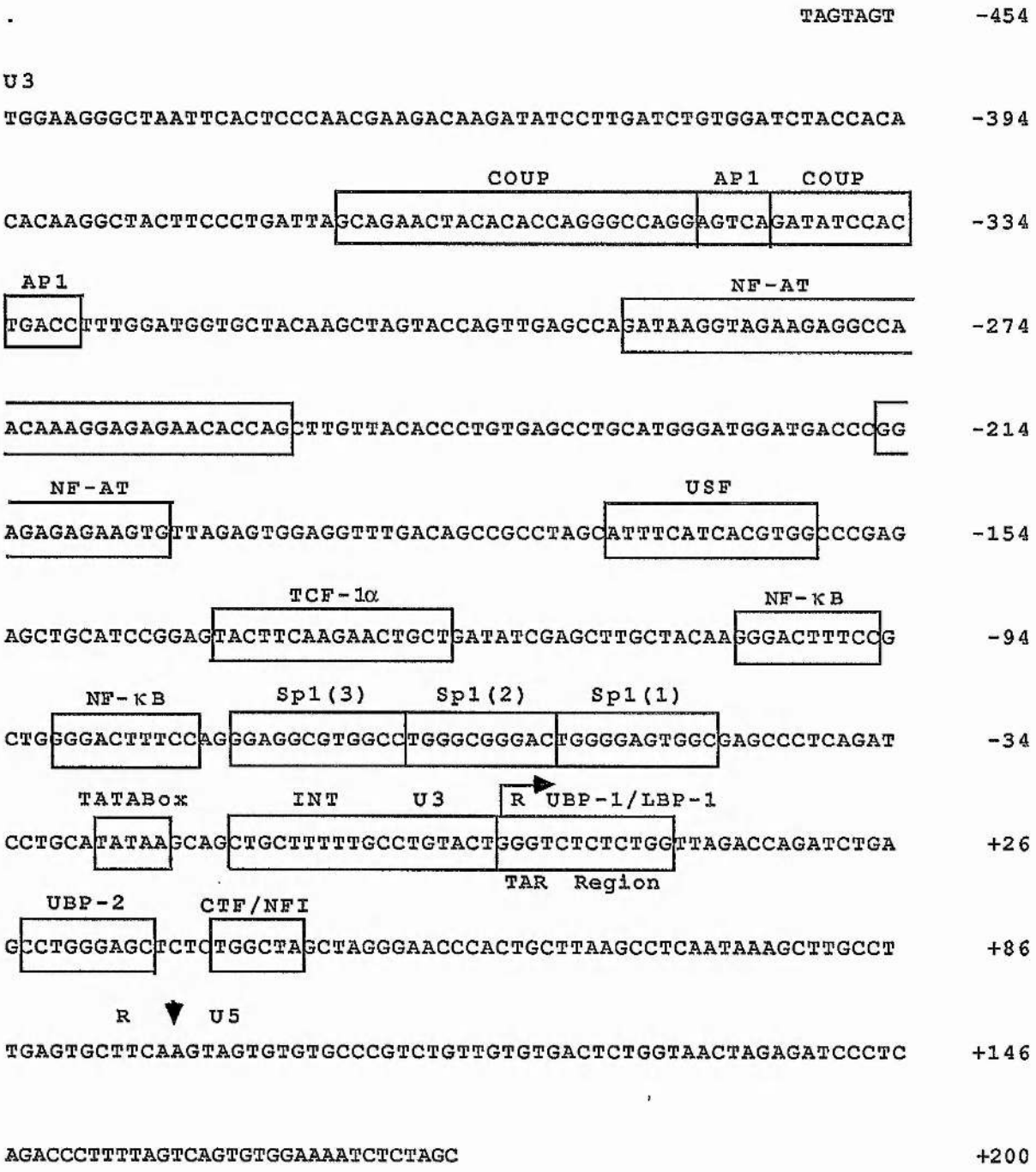
For Cerenkov counting of radioactivity in wet polyacrylamide gels, gel slices were counted in 10ml glass vials using the $^{3}H/^{14}C$ channel of a liquid scintillation spectrometer (Intertechnique SL30), while for liquid scintillation counting of ^{32}P samples, samples were suspended in 4ml of Ecoscint A (National Diagnostics) in 5ml polypropylene pony vials (Packard)

27. Radiolabelling of the p50 DNA binding site.

For the ^{14}C iodoacetate radiolabelling of the NF-κB p50 subunit aa35-381 protein, 100ug of p50 protein in 400ul of PBS, 20mM DTT, was incubated on ice with 200ul of HIV oligonucleotide-Sepharose affinity resin for 3 hours, the mixture centrifuged briefly in a microcentrifuge, and the supernatant removed. The HIV-Sepharose resin was then washed with 2ml of 0.1M NaCl, 10mM sodium phosphate buffer,pH7.5, 2mM DTT and the supernatant removed. Neutral 250mM iodoacetate solution (see above) was added to give a final iodoacetate concentration of 10mM, and the mixture incubated for 15 minutes at 20°C. The affinity resin was then washed with 2ml of 0.1M NaCl, 10mM sodium phosphate buffer,pH7.5, 2mM DTT, the supernatant removed and bound p50 protein eluted with 1ml 0.6M NaCl, 10mM sodium phosphate,pH7.5, 2mM DTT. The 1ml of eluate was concentrated to 300ul by centrifugation through a Centricon-30 membrane, and neutral ^{14}C iodoacetate solution added to give a ^{14}C iodoacetate concentration of 10mM, before incubation for 25 minutes at 20°C. Finally, the reaction was quenched by addition of DTT to 50mM and incubating for 25 minutes at 20°C.

28. Isolation and sequencing of a radiolabelled cysteine-containing peptide.

The quenched radiolabelled p50 subunit aa35-381 protein was then precipitated by addition of TCA to 10%, and centrifuged in a Beckman TL100 rotor at 50000 rpm at 4°C for 20 minutes. The supernatant was removed, and precipitated protein washed twice briefly with ice-cold acetone, before air drying on ice. The dried p50 protein pellet was then redissolved in 50ul of 8M urea (Sigma), 0.4M ammonium bicarbonate buffer, the urea concentration reduced to 2M by dilution with water, and the p50 protein digested with 3ug of bovine trypsin (Sigma) at 37°C for 24 hours. The trypsin digested p50 protein was then analysed by reverse phase chromatography using a Waters HPLC system with a Delta-Pak C18 column and a 0 to 80% acetonitrile gradient in 0.06% v/v trifluoroacetic acid (Sigma) run at a flow rate of 0.5ml per minute. Peptides eluted from the C18 column were collected over 180 x 0.25ml fractions and 10ul aliquots from each fraction spotted onto 5mm x 5mm squares of Whatman 3MM chromatography paper, dried, and the chromatography paper immersed in 5ml Ecoscint A and scintillation counted. Peak radioactivity HPLC fractions were identified, and the corresponding peptides subjected to N-terminal amino acid sequencing (kindly performed by Dr.W.Kaszubska, Glaxo IMB, Geneva).


TAGTAGT -454
U3
TGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACA -394
COUP
AP1
COUP
CACAAGGCTACTTCCCTGATTAGCAGAACTACACACCAGGGCCAGGAGTCAGATATCCAC -334
AP1
NF-AT
TGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCA -274
ACAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCGG -214
NF-AT
USF
AGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGAG -154
TCF-1α
NF-κB
AGCTGCATCCGGAGTACTTCAAGAACTGCTGATATCGAGCTTGCTACAAGGGACTTTCCG -94
NF-κB
Sp1(3)
Sp1(2)
Sp1(1)
CTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTCAGAT -34
TATABox
INT
U3
R
UBP-1/LBP-1
CCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGA +26
TAR Region
UBP-2
CTF/NFI
GCCTGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCT +86
R
U5
TGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTC +146
AGACCCTTTTAGTCAGTGTGGAAAATCTCTAGC +200

RESULTS.

Chapter 1. Purification and characterisation of HeLa cell NF-κB.

1.1 Purification of HeLa cell NF-κB protein

In common with other retroviruses, the integrated proviral form of HIV-1 is flanked by two long terminal repeats (LTRs), these LTR sequences contain binding motifs for multiple endogenous transcription factors. Alterations in the activities of several of these cellular factors in response to cell activation or differentiation ensure that in the infected cell, the initial transcription of the HIV-1 provirus responds to many of the same regulatory mechanisms controlling the expression of cellular genes. In addition to the complex pattern of binding sites for cellular factors in the HIV-1 LTR (extending from -454 to +184 relative to the proviral transcriptional start site), regulatory regions in DNA and RNA forms of the LTR act as targets for virally-encoded transactivator proteins (Figure 1.1).

As noted above, the HIV-1 LTR extends from -454 to +184 relative to the transcriptional start site (Ratner et al., 1985; Wain-Hobson et al., 1985), and can be conveniently classified into three main regulatory regions - a modulatory element extending from -454 to -78, a core promoter element extending from -78 to -1, and the trans-activating response (TAR) element from +1 to +60. Within this modulatory element, there are at least six well-defined motifs which bind cellular transcription factors, in addition to several poorly-characterised motifs. Interestingly, the modulatory element motifs are a mixture of positive and negative regulatory elements. Thus, as discussed in the Introduction, the two κB sites of the proviral enhancer region (-105 to -80) have been shown to be critical for increased gene expression from the HIV-1 LTR in response to T-cell

Figure 1.1 Location of trans-acting factor binding sites on the HIV-1 LTR.

The regions of the HIV-1 LTR (-454 to +184) which bind cellular trans-acting factors are indicated by boxed sequences, the following abbreviations were used :- COUP - chicken ovalbumin upstream promoter, AP-1 - activator protein 1, NF-AT - nuclear factor of activated T-cells, USF - upstream stimulatory factor, TCF-1α - T-cell factor-1α, NF-κB - nuclear factor κB, Sp1- Sp1 transcription factor, TATA - TATA binding protein, INT - initiator element, UBP-1 - untranslated binding protein-1, LBP-1 - leader binding protein-1, UBP-2 - untranslated binding protein-2, CTF/NFI - CCAAT transcription factor/nuclear factor I. The U3, R, and U5 regions of the LTR are indicated, and the transcriptional start site at +1 is indicated by a horizontal arrow.

activation signals (Tong-Starksen et al., 1987; Nabel and Baltimore, 1987). Recent *in vivo* footprinting studies (Demarchi et al., 1992) have confirmed that in a productively HIV-1-infected H-9 T-cell line, major protein footprints are observed over the -105 to -80 enhancer region as predicted from numerous *in vitro* studies.

Other positive or potentially positive factor binding motifs include the -138 to -123 region which binds the 55kD cellular transcription factor TCF-1α (Waterman and Jones, 1990; Waterman et al., 1991) - a T-cell-specific transcription factor which can bind to pyrimidine-rich sequences in both the T-cell receptor Cα gene and the HIV-1 LTR. This TCF-1α protein shares a 68 amino acid conserved so-called high mobility group (HMG) motif with a number of other transcription factor proteins - including the HMG proteins (Wen et al., 1989), the RNA polymerase I enhancer binding protein hUBF (Jantzen et al., 1990), and the gender-determining testis-specific factor SRY (Gubbay et al., 1990). The observation that primary isolates of HIV-1 containing duplications of TCF-1α binding sites show increased growth rates in T-lymphocytes compared to wild type isolates (Golub et al., 1990) suggests that binding of the TCF-1α transcription factor may be involved in positive regulation of HIV-1 growth.

Still further upstream from the transcriptional start site lies the so-called NF-AT region - within this region are two purine-rich regulatory sequences which bind cellular factors which some workers have proposed to have positive effects on HIV gene expression. One region extending from -292 to -255 binds the factor NF-AT - a factor present in low levels in unstimulated T-cells and other cell lines whose binding activity increases dramatically upon cell activation (Shaw

et al., 1988), while another region extending from -216 to -203 is homologous to the NF-AT binding site of the IL-2 gene enhancer.

A final potentially positive type of regulatory sequence comprises binding sites for the cellular inducible transcription factor AP-1 - this heterodimeric complex of *c-fos* and *c-jun* gene family products has been shown to be important in the expression of a number of cellular genes (Angel et al., 1988; Landschulz et al., 1988). Within the LTR, there are two AP-1 recognition sites - running from -347 to -343, and -333 to -329 inclusively (it is interesting to note that the intragenic transcriptional enhancer identified in the HIV-1 *pol* gene contains three functional AP-1 sites (van Lint et al., 1991)).

Binding of the AP-1 factor to its recognition site in promoter elements, for example in the simian virus SV40 promoter, mediates increases in the transcription rate of the promoter in response to stimuli such as treatment with the active phorbol ester phorbol myristyl acetate (PMA), serum growth factors such as epidermal growth factor (EGF), tumour necrosis factor α, and certain transforming oncoproteins - for example polyomavirus middle T antigen. However, it is also possible that the individual fos and jun protein subunits, by interacting (via their leucine zipper structures) with certain other nuclear and transcription factor proteins could generate new protein species with negative effects on gene expression (Landschulz et al., 1988).

Upstream from the TCF-1α site is a negative regulatory element (NRE) (initially thought to be between residues -185 and -340, Rosen et al., 1985), this comprises binding sites for the upstream stimulatory factor (USF) - a cellular protein initially identified as a factor increasing the activity of the adenovirus

major late promoter (Carthew et al., 1985) - located between residues -173 and -160, and a binding site for the chicken ovalbumin upstream promoter transcription factor (COUP-TF). It seems likely that the USF binding site acts as a negative regulatory element in the HIV LTR, since HIV-1 constructs containing deletions of this region show increased gene expression and growth kinetics (Lu et al., 1989). However, it may also be that USF (a helix-loop-helix motif protein) may be able to heterodimerise with other (potentially positive regulatory) helix-loop-helix motif proteins - which may themselves be able to bind as homodimers to the USF motif (Murre et al., 1989).

The COUP-TF binding site in the NRE extends from -371 to -334, and contains a palindromic recognition sequence which is recognised by the 68kD form of COUP-TF - this factor being abundant in T-lymphocytes. The mutation of COUP-TF binding sites in the HIV-1 LTR gives rise to a two- to three-fold increase in HIV-1 gene expression - evidence for a negative regulatory role in the context of the LTR (Orchard et al., 1990; Cooney et al., 1991). However, more complex regulatory possibilities also exist - for example, one of the AP-1 binding sites of the LTR overlaps with the COUP-TF element, allowing the possibility of one factor preventing the binding of the other by direct competition. Further, since COUP-TF proteins belong to the steroid/thyroid hormone receptor superfamily, and the fos and jun proteins may be able to form complexes with such receptor proteins and antagonise their transcriptional activity (Schüle et al., 1990), the possibility may exist for both the AP-1 and COUP-TF binding sites to mediate positive and negative effects on gene expression from the LTR.

The second major region of the HIV-1 LTR is the core promoter element -

extending from -78 to -1 relative to the transcriptional start site - within this region are binding sites for three cellular, positive regulatory factors - the transcription factor Sp1, the TATA-binding general transcription factor TFIID, and initiator binding proteins.

Core promoter element sequences extending from -78 to -46 have been shown by mutagenesis studies to contain three binding sites for the cellular transcription factor Sp1 (Jones et al., 1986), the mutation of all three Sp1 binding sites was shown to eliminate the ability of added exogenous Sp1 protein to stimulate *in vitro* transcription directed by the HIV-1 LTR. Similarly, transfection studies of HIV-1 constructs have demonstrated that constructs with mutations of one or two Sp1 binding sites showed only small decreases in basal and tat-induced gene expression, while constructs with mutations of all three Sp1 binding sites showed severe decreases in basal and tat-induced gene expression (Harrich et al., 1989).

The second positive core promoter element sequence is a consensus TATA box running from -28 to -24, representing the binding site for the TATA binding protein TFIID. Mutagenesis studies have shown that the TATA box has a critical role in the activation of HIV-1 gene expression - by tat, and by other viral transactivator proteins (Garcia et al., 1989). Similarly to the Sp1 case, mutation of the TATA element dramatically reduces *in vitro* transcription activity directed by the HIV-1 LTR (Jones et al., 1988). Again, transfections of HIV-1 LTR plasmids bearing mutations in the TATA element showed marked decreases in basal and tat-induced gene expression (Garcia et al., 1989). An interesting observation from DNase I footprinting experiments in the above study suggested the binding of cellular factors over both the TATA element and flanking sequences - hence the

flanking sequences in addition to the TATA box may be critical determinants of LTR function.

The final type of element in the core promoter are two potential initiator elements - a type of motif initially identified by Smale and Baltimore (Smale and Baltimore, 1989) as a sequence surrounding the RNA start site in the terminal deoxynucleotidyl transferase (TdT) gene which helped to determine the position of RNA initiation in this TATA-less gene - in the HIV-1 LTR, these possible initiator elements extend from -18 to +1.

The third, and final, region of the HIV-1 LTR is the trans-activating response (TAR) element which extends from +1 to +60 relative to the transcriptional start site. This region shows an interesting duality - both the TAR regions in the proviral DNA and the transcribed RNA bind several protein factors, with the TAR RNA also binding the virally-encoded trans-activator protein tat.

Studies of the TAR region DNA using techniques such as DNase I footprinting and gel electrophoresis mobility shifts have shown that several cellular proteins bind to this region - these include the cellular factors UBP-1/LBP-1, UBP-2 and CTF/NFI. The UBP-1/LBP-1 factor binds to a high affinity site extending from -16 to +27 (and also to a low affinity site extending from -38 to -16) (Kato et al., 1991). *In vitro* transcription experiments reported in the above study with purified UBP-1/LBP-1 suggested that binding to the low affinity site inhibited HIV-1 LTR gene expression by preventing TFIID from binding to the TATA element - however, if the promoter was preincubated with TFIID then no such inhibition was seen. The role of the high affinity UBP-1/LBP-1 site is less clear - it did not cause repression of transcription in the above study. However, in the

study by Kato et al. no convincing evidence could be obtained for the proposed positive role (Jones et al., 1988) of the high affinity UBP-1/LBP-1 site. The role of both the cellular UBP-2 factor (Garcia et al., 1989) which binds to TAR DNA sequences +28 to +36, and the cellular CTF/NFI factor (Garcia et al., 1989; Jones et al., 1988) binding TAR DNA sequences from +41 to +46 remains as yet unclear.

In transcribed HIV-1 RNA, the TAR region is also able to interact with a variety of protein factors - both cellular and virally-encoded, by virtue of its ability to form a stable stem-loop structure extending from nucleotide +1 to +60. Within this region of the viral RNA, there is very extensive self-complementarity, allowing the formation of a 24 base pair stem structure - whose only significant interruption is a non-paired bulge on one side of the stem incorporating nucleotides +23 to +25, and the loop structure comprising nucleotides +30 to +35. The TAR RNA region between +18 and +44 is critical for the function of the viral tat transactivator protein (Garcia et al., 1989; Feng and Holland, 1988; Roy et al., 1990), and within this several elements (the bulge, the loop, and the stem structure) shown to be needed for complete tat activation.

The +23 to +25 bulge in the TAR RNA stem-loop structure serves as a high affinity binding site for the viral tat protein, although tat binding is also modulated by sequences in the loop region (Roy et al., 1990; Dingwall et al., 1990). In contrast, the TAR RNA loop sequence contains binding sites for cellular proteins which may cooperate with tat protein in amplifying gene expression. Two cellular factors - p68 and TRP-185 - have been identified (Marciniak et al., 1990; Wu et al., 1991) which bind to the TAR RNA loop sequences. Binding of the

TRP-185 factor requires both wild type loop sequences and an intact bulge structure (unlike tat, the nature of the bulge sequence seems to be unimportant), and increases transcription from the HIV-1 LTR *in vitro* - it seems unclear at present whether TRP-185 stimulates transcriptional initiation or elongation (Wu et al., 1991).

The question of the mechanism of action of the tat transactivator protein remains unresolved, early studies suggested that tat exerted its function on the TAR RNA - fusion proteins of tat with sequence-specific RNA binding proteins were able to bind and activate gene expression from the HIV-1 LTR in constructs where the TAR region had been substituted by the appropriate regulatory element (Selby and Peterlin, 1990; Southgate et al., 1990). However, similar fusion protein experiments have been performed linking tat to the DNA binding domains of sequence-specific DNA binding proteins such as the transcription factor jun (Berkhout et al., 1990), with similar activation of gene expression from the HIV-1 LTR. In any case it is clear that in the presence of tat, steady-state levels of transcription from the HIV-1 LTR are increased by 20- to 50-fold - likely representing contributions from tat protein stimulating transcriptional initiation and increasing the efficiency of the transcriptional elongation process (Laspia et al., 1989).

Since the general initial aim of this project was to investigate the role of the transcription factor NF-κB in the initial transcriptional activation process from the HIV-1 LTR, a strategy of purifying the heterodimeric p50-p65 NF-κB factor to homogeneity was decided upon. It was envisaged that amino acid sequence information from the purified polypeptides could then be used to isolate cDNA

clones encoding the NF-κB subunits, and the proteins overexpressed for use in *in vitro* studies of transcriptional activation.

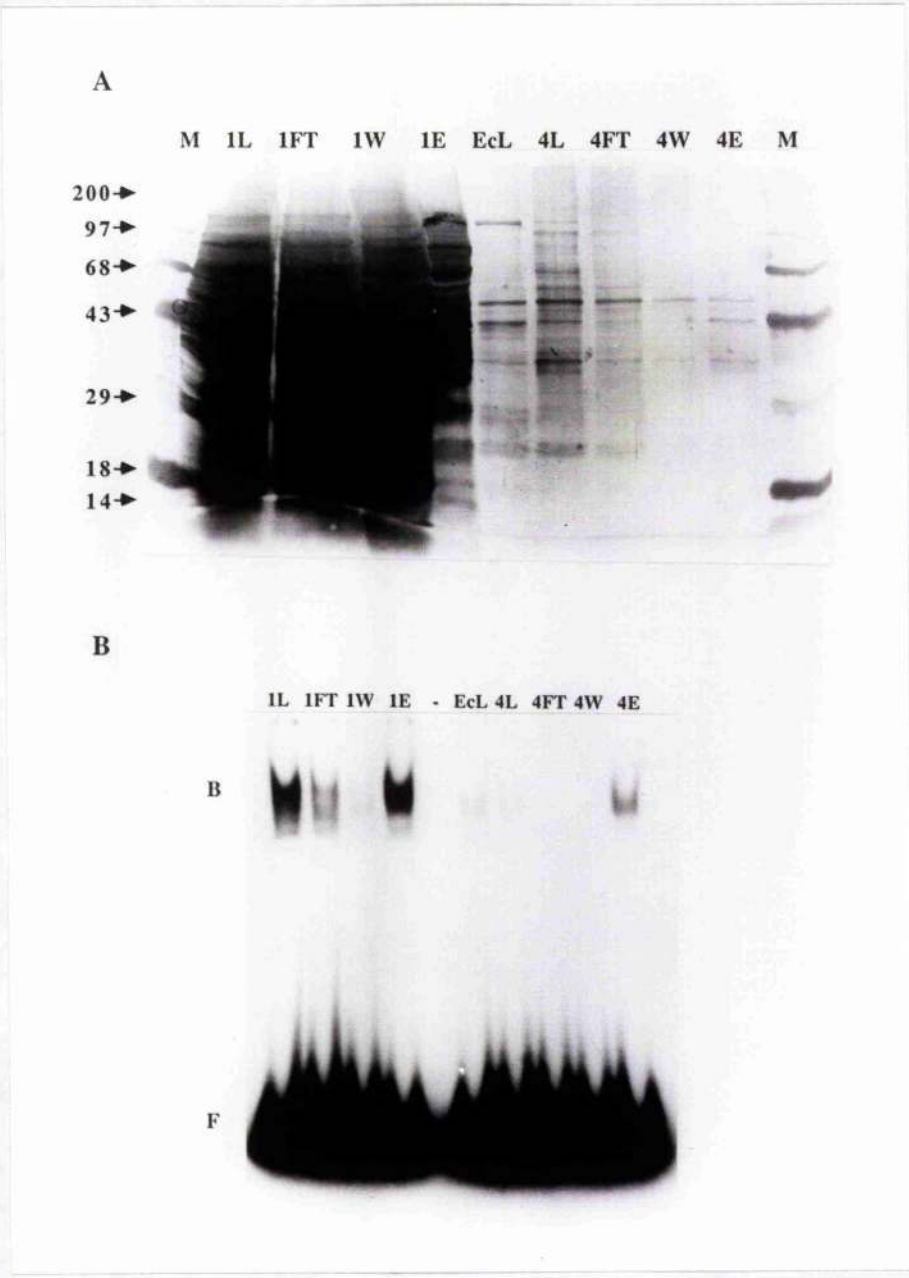
Following several unsuccessful attempts to purify the p50-p65 NF-κB heterodimer to homogeneity by multiple rounds of HIV enhancer oligonucleotide-Sepharose affinity chromatography - a technique significantly improved by Tjian and coworkers (Kadonaga and Tjian, 1986) - a more sophisticated purification strategy was adopted. As described in the Materials and Methods section, this purification scheme involved an initial HIV enhancer oligonucleotide-Sepharose column affinity chromatography step, followed by FPLC chromatography on a Pharmacia-LKB Mono-Q (diethylaminoethyl equivalent) column - and use of the very early eluting minor peak of NF-κB DNA binding activity from this chromatography step. This material was then applied to a non-specific *E.coli* DNA-Sepharose column under non-binding conditions, the flowthrough fraction collected and used in a final round of HIV enhancer oligonucleotide affinity chromatography.

The rationale behind this scheme was prompted by the observation that multiple rounds of HIV enhancer oligonucleotide affinity chromatography yielded a significant initial purification step, but subsequent stages did not give any further purification - presumably the contaminating polypeptide species were proteins with non-specific DNA binding activity for the affinity resin (although large amounts of synthetic poly dAdT.dAdT/dGdC.dGdC DNA were included in the load material applied to the HIV oligonucleotide affinity resin columns, this simple sequence DNA might not be effective at competing away all non-specific binding). Thus the material eluted from the first HIV enhancer oligonucleotide

Figure 1.2 Purification of native NF-κB protein.

A. Protein fractions from the HIV oligo / Mono-Q / E.coli DNA / HIV oligo column chromatography purification analysed by SDS-PAGE under reducing conditions, followed by silver staining (BioRad) of the gel. For gel analysis, 200ul aliquots of the various protein fractions were TCA precipitated, and then resuspended in 6ul of 1x sample buffer (see Materials and Methods) before boiling, quenching on ice, and loading onto the gel. The following abbreviations were used for the sample tracks:- M, molecular weight standards; 1L, crude HeLa cell nuclear extract load for first HIV enhancer oligonucleotide-Sepharose affinity chromatography stage; 1FT, flowthrough fraction from the first chromatography stage; 1W, wash fraction from the first chromatography stage; 1E, eluate from the first chromatography stage; EcL, Mono-Q-derived load for E.coli DNA-Sepharose chromatography stage; 4L, load for the fourth chromatography stage (HIV oligonucleotide-Sepharose chromatography); 4FT, flowthrough fraction from the fourth chromatography stage; 4W, wash fraction from the fourth chromatography stage; 4E, eluate from the fourth (final) chromatography stage. The positions of the various molecular weight standard marker proteins are indicated.

B. Protein fractions from the above 4 column purification procedure assayed for κB-DNA binding activity by a gel electrophoresis DNA binding assay in which the standard incubation mixture (see Materials and Methods) had been supplemented with 0.06 ug/ul poly dAdT.dAdT/dGdC.dGdC carrier DNA - in each case 2ul of protein fractions were used in the assays. The abbreviations for the sample tracks are as in panel A, with no material loaded in the track marked '-', the positions of the DNA-protein complex and the free HIV-L oligonucleotide probe are indicated are indicated by B and F respectively.


A
M 1L 1FT 1W 1E EcL 4L 4FT 4W 4E M
200
97
68
43
29
18
14
B
1L 1FT 1W 1E - EcL 4L 4FT 4W 4E
B
F

affinity purification round (Figure 1.2, Panel A) was still grossly impure (although the bulk of the contaminating protein was found in the flowthrough and wash fractions). This material was then loaded onto the Mono-Q FPLC column and proteins eluted with a 0 to 5M NaCl gradient - it had been noted in earlier studies (Clark, 1989) that if a HeLa cell nuclear extract was loaded onto a DEAE-Sepharose column, a significant fraction of NF-κB activity would fail to bind to the column, while the remainder would bind and could be eluted with a salt gradient.

Elution of κB binding proteins from the Mono-Q column yielded a moderate amount of NF-κB DNA binding activity which eluted from the column before the salt gradient was applied (Figure 1.3, Panels A and B). The κB DNA binding activity of the various fractions was monitored using the standard gel elctrophoresis DNA binding assay (Figure 1.3, Panel B) and fractions 9 to 14 - which were found to be largely free of contaminating protein (Figure 1.3, Panel A) - pooled, and the NaCl concentration adjusted to 0.35M before passing over the *E.coli* DNA-Sepharose column. Comparison of the polypeptide species in the *E.coli* DNA column load and the final HIV enhancer oligonucleotide affinity column load (Figure 1.2, Panel A) showed the selective removal of some proteins after passage over the *E.coli* DNA column - most notably a species of approximately 120 kD molecular weight. Passage of this *E.coli* DNA column flowthrough material over a final round of HIV enhancer oligonucleotide affinity chromatography yielded a final eluate (Figure 1.2, Panel A - Fraction 4E) which contained 6 polypeptide species visible after silver staining of the SDS-PAGE gel - relatively minor amounts of proteins of approximately 140, 48, 36 and 34 kD

Figure 1.3 FPLC Mono-Q column chromatography of NF-κB protein.

A. The UV absorbance output trace at 280nm is shown (solid line) for the elution of the FPLC Mono-Q column with a 0 to 5M NaCl salt gradient (dashed line), the positions of the fractions collected from the gradient are indicated along the base.

B. Fractions 1-27 eluted from the Mono-Q column assayed (3ul of each fraction) for NF-κB DNA binding activity by gel electrophoresis DNA binding assay with the standard incubation mixture supplemented with 0.06 ug/ul poly dAdT.dAdT/dGdC.dGdC carrier DNA and using the HIV-L oligonucleotide probe. The amounts of radioactivity in the DNA-protein complexes were determined by direct Cerenkov counting of wet polyacrylamide gel slices.

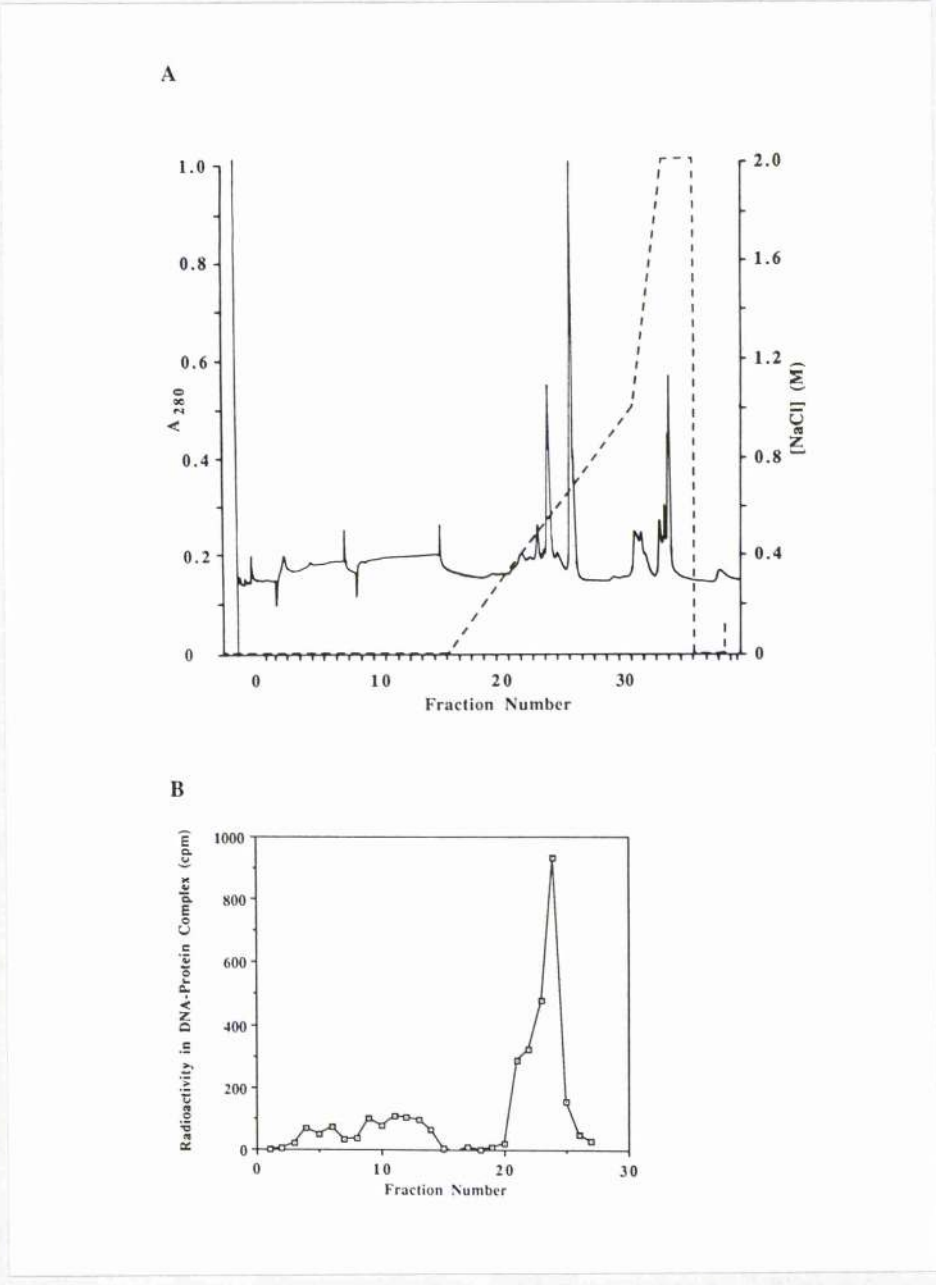


Table 1.1 Recovery of native NF-κB protein.

Fraction	Total DNA binding activity (vol. x 20min. count of GRA complex band)	Cumulative % yield	%Yield over that stage
120ml. 1LOAD	2.6×10^6	100%	-
120ml. 1FT	1.2×10^6	47%	-
15ml. 1WASH	5.2×10^4	2%	-
20ml. 1ELUATE	4.0×10^5	15%	15%
28ml. E.coli LOAD	1.0×10^5	3.9%	25%
40ml. 4LOAD	9.6×10^4	3.7%	95%
40ml. 4FT	1.1×10^4	0.4%	-
10ml. 4WASH	0.4×10^4	0.2%	-
3.7ml. 4ELUATE	3.8×10^4	1.5%	39%

molecular weight, and two major species of approximately 55 and 43 kD molecular weight - the 43 kD component being noticeably concentrated in the final eluate compared with the preceding flowthrough and wash fractions.

This final eluate fraction although highly purified was still heterogeneous and hence likely to be unsuitable for amino acid sequence analysis, the overall recovery of NF-κB DNA binding activity was also very low (Table I). From the gel electrophoresis DNA binding assays (Figure 1.2, Panel B, Figure 1.3, Panel B) it can be seen that large amounts of NF-κB DNA binding activity (approximately 47%) were lost to the flowthrough fraction from the first HIV oligonucleotide affinity chromatography round - whether this was due to overloading of the affinity column by competition from other DNA-binding proteins in the crude extract, or due to the addition of excessive amounts of non-specific carrier DNA to the crude extract is unknown. Further losses occurred inevitably in the FPLC Mono-Q chromatography stage since only a small fraction (approximately 25%) of NF-κB DNA binding activity appeared in the early eluting fractions which were largely free of contaminating proteins - whether the proportion of early eluting activity could be increased by use of different sources of crude extract, or cell physiological state is not known.

1.2 Lectin affinity column characterisation of HeLa cell NF-κB protein

Following reports that a number of nuclear proteins had been found to carry O-glycosidically linked N-acetylglucosamine residues (Holt and Hart, 1986), it was demonstrated by Tjian and coworkers that this sugar group was present on a variety of RNA polymerase II transcription factors (Jackson and Tjian, 1988) -

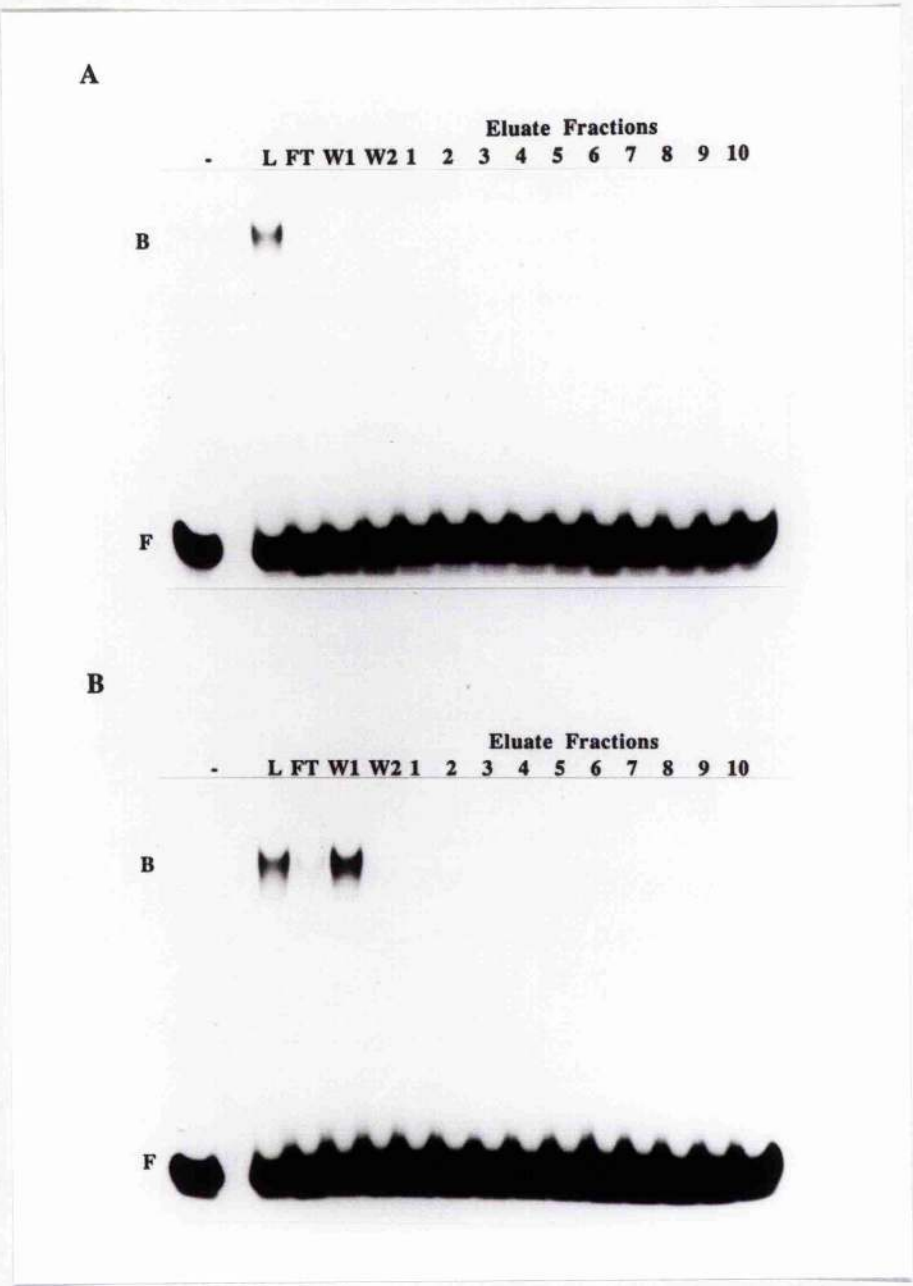
such as human Sp1, AP-1, AP-2, AP-4, CTF/NFI, *Drosophila* Zeste, GAGA and Adf-1 (although only some members of the CTF/NFI and AP-1 families seemed to carry this modification), but was not present on a limited sample of RNA polymerase I and III transcription factors. Jackson and Tjian made use of this property (Jackson and Tjian, 1989) to purify human Sp1 transcription factor by a rapid and high yielding affinity purification scheme involving passing crude HeLa cell nuclear extracts over a column of agarose-conjugated wheat germ agglutinin (WGA) - a lectin with binding specificity for N-acetylglucosamine and a variety of sialic acid residue species, eluting the bound material and passing the N-acetylglucosamine-bearing proteins over a conventional Sp1 recognition site oligonucleotide affinity column. In the above study, the WGA-agarose affinity column step gave an approximately 200-fold purification of Sp1, with a recovery of over 90% of the initial Sp1 activity - suggesting that this technique might be applicable to NF-κB protein purification.

The protocol used for the WGA-agarose affinity purification of NF-κB proteins was virtually identical to that of Jackson and Tjian (1989) - however it was found that once the crude HeLa cell nuclear extract was applied to the WGA-agarose column it proved impossible to elute the NF-κB DNA binding activity from the column (Figure 1.4, Panel A). That this effect was not unique to NF-κB proteins was confirmed by carrying out parallel gel electrophoresis DNA binding assays with a radiolabelled restriction fragment probe encoding multiple binding sites for the Sp1 transcription factor - this showed identical behaviour, the Sp1 binding activity could not be eluted from the WGA-agarose column with 0.1M KCl buffer containing 0.3M N-acetylglucosamine (data not shown).

Figure 1.4 Attempted lectin affinity chromatography of NF-κB protein.

A. Protein fractions from the attempted wheat germ agglutinin-agarose affinity purification of NF-κB DNA binding proteins following the method of Jackson and Tjian (1989) were analysed by gel electrophoresis DNA binding assay with the standard incubation mixture supplemented with 0.06 ug/ul poly dAdT.dAdT/dGdC.dGdC carrier DNA. Assays used radiolabelled HIV-R oligonucleotide as the probe, 2ul of protein fractions were used in each assay. The following abbreviations were used for the sample tracks :- L, high salt HeLa cell nuclear extract load to WGA-agarose column; FT, flowthrough fraction from the WGA-agarose column; W1, high salt wash fraction; W2, low salt wash fraction; 1-10, sequential 1ml eluate fractions. The DNA-protein complex and free oligonucleotide bands are indicated by B and F respectively.

B. Protein fractions from the attempted wheat germ agglutinin-agarose affinity purification of NF-κB DNA binding proteins using the modified protocol involving addition of N-acetylglucosamine to 0.1M to the nuclear extraction buffer. Gel electrophoresis DNA binding assay conditions and figure legend abbreviations were as in Panel A above.


A
Eluate Fractions
- L FT W1 W2 1 2 3 4 5 6 7 8 9 10
B
F
B
Eluate Fractions
- L FT W1 W2 1 2 3 4 5 6 7 8 9 10
B
F

Numerous variations of the WGA-agarose chromatography procedure failed to elute NF-κB DNA binding activity from the column - including raising the N-acetylglucosamine concentration in the eluant to 0.7M. Compare this with the observation that while 0.3M N-acetylglucosamine buffer was used in the elution protocol of Jackson and Tjian (1989) to elute Sp1, the Sp1 protein could still be eluted efficiently by 20mM N-acetylglucosamine buffer (S.P.Jackson pers. comm.).

Despite the observation of identical behaviour by the transcription factor Sp1, it might be possible to argue that passage over the WGA-agarose column had in some way inactivated the NF-κB DNA binding protein. From the behaviour of NF-κB and Sp1 in binding seemingly irreversibly to the WGA affinity column, it seemed possible that polyvalent binding of the proteins to the affinity matrix might be occurring - it having already been demonstrated in one study (Jackson and Tjian, 1988) that Sp1 protomers carried approximately eight N-acetylglucosamine residues. Since one method of overcoming irreversible binding due to polyvalent interactions with lectin columns in cell fractionation experiments has been to include the cognate simple sugar in the binding mixture applied to the column, a similar approach was used here, with N-acetylglucosamine being added to 0.1M in the high salt buffer used for the nuclear extraction. This approach was at least partially successful - in that NF-κB (and Sp1) DNA binding activity could be eluted from the column with virtually 100% recovery, however the binding activity was present in the 0.42M KCl high salt buffer wash fraction (Figure 1.4, Panel B) - since this fraction still contains approximately 50% of the total protein applied to the WGA-agarose affinity resin,

it did not represent a useful purification step. Although several attempts were made with modified protocols to elute the NF-κB DNA binding activity after the contaminating non-binding proteins, none of these were successful, and any attempts to use WGA-agarose affinity chromatography as a purification step abandoned. However, the results obtained here do at least suggest that all NF-κB DNA binding proteins present in nuclear extracts of the S3 HeLa cell line used in these studies are modified with multiple sugar residues capable of binding to wheat germ agglutinin - most probably, N-acetylglucosamine residues.

1.3 DNA Bending of crude and affinity purified native HeLa cell NF-κB

Prompted by many reports of the importance of DNA bending in the assembly of large nucleoprotein structures involved in the control of transcription, DNA replication and site-specific recombination processes (Schultz et al., 1991; Zorbas et al., 1989; Thompson and Landy, 1988), and of observations that binding of NF-κB to its recognition site seemed to distort the structure of the DNA with indications of disruptions in base pairing (Clark et al., 1990), the DNA bending properties of NF-κB were studied using gel electrophoresis DNA binding assays with restriction fragment probes carrying the HIV-R κB motif in circularly-permuted locations. This technique, originally developed by Wu and Crothers (Wu and Crothers, 1984) will detect DNA bending induced by protein binding to a specific recognition site by the position-dependent variation in electrophoretic mobility of the DNA-protein complexes. In this type of analysis, the DNA with the highest mobility has the bend-inducing protein bound near the end of the DNA fragment, and the DNA with the lowest mobility has the protein

bound at the middle of the fragment. Identifying those fragments with the highest and lowest mobilities allows the mapping of the bend centre, while an estimate of the angle of bending can be derived from the ratio of the electrophoretic mobilities of complexes with the protein bound at the end of the DNA and at the centre of the DNA fragment (Thompson and Landy, 1988).

The circular permutation bending analysis in these studies used the p2xAT/HIV-R plasmid, this carries a duplication of a region containing the HIV-R κB binding motif and several unique restriction endonuclease cleavage sites - such that upon digestion with the restriction enzymes Hind III, Sph I, Bam HI, Avi II and Eco RV, an approximately 600bp fragment is released from the parental DNA. The various 600bp restriction fragments were ^{32}P radiolabelled and used in gel electrophoresis DNA binding assays, these were essentially identical to the standard assay - but used a 5% non-denaturing (44:0.8) polyacrylamide gel, and binding incubation mixtures supplemented with 0.16 ug/ul poly dAdT.dAdT/dGdC.dGdC carrier DNA and an additional 1% NP40.

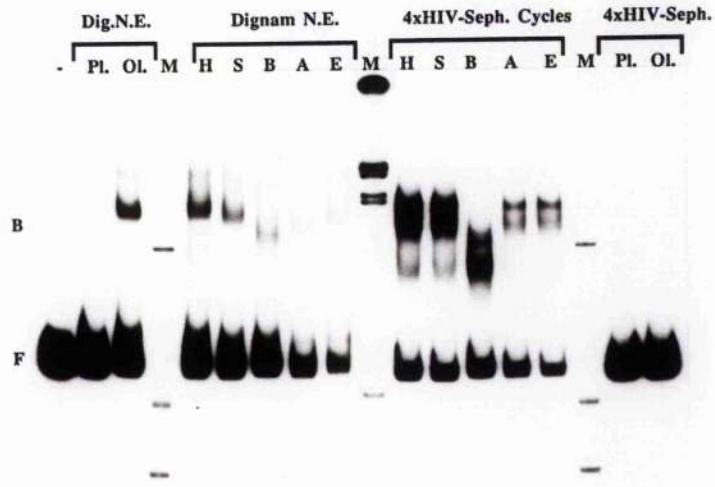
The results of these studies showed an interesting pattern (Figure 1.5, Panel A) - with crude HeLa cell nuclear extracts prepared according to the method developed by Dignam (Dignam et al., 1983) (Figure 1.5, Panel A) the mobility pattern of the various permuted binding site complexes shows the smooth V-shape (with complexes with the κB binding site at the end of the (Bam HI) restriction fragment showing the highest mobility) seen in many other circular permutation studies, and there also seems to be only one complex band. Further, the pre-addition of HIV-R κB site oligonucleotide and p2xAT/HIV-R plasmid showed an unexpected variation in competition behaviour - although pre-addition of the

Figure 1.5 DNA bending induced by binding of NF-κB proteins.

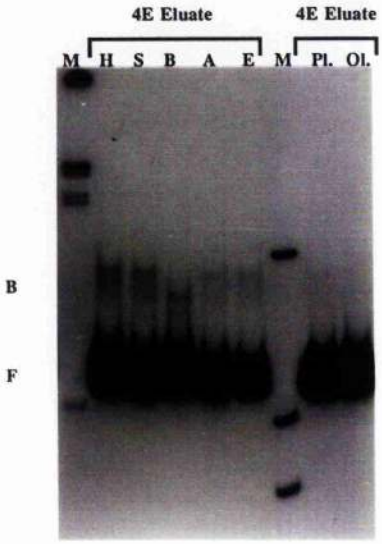
A. DNA bending and competition studies using Dignam HeLa cell nuclear extract and NF-κB proteins purified by four cycles of HIV oligonucleotide affinity chromatography. Gel electrophoresis DNA binding assay conditions followed the standard procedure but with the addition of poly dAdT.dAdT/dGdC.dGdC carrier DNA to 0.16 ug/ul, and addition of an extra 1% NP40 to the binding incubation mixture, gel electrophoresis was carried out on a 5% (44:0.8) non-denaturing polyacrylamide gel. The following abbreviations were used :- M, DNA restriction digest size markers (l/Hind III and pUC13/Hinf I); H, Hind III digest generated 600bp probe; S, Sph I digest generated probe; B, Bam HI generated probe; A, Avi II generated probe; E, Eco RV generated probe. Pl. indicates precompetition by 3.4ug of undigested p2xAT/HIV-R plasmid, Ol. indicates precompetition by 1ug of HIV-R κB oligonucleotide, the DNA-protein complex and free restriction fragment probe are indicated by B and F respectively. For those experiments using Dignam nuclear extract, 0.2ul of extract were used in each case, while for those experiments using the four cycle HIV oligo affinity purified protein, 1ul of final eluate was used in each case.

B. DNA bending and competition studies using NF-κB proteins purified by HIV enhancer oligonucleotide-Sepharose affinity chromatography, followed by FPLC Mono-Q, E.coli DNA-Sepharose, and a final HIV enhancer oligonucleotide-Sepharose affinity purification stage. The experimental details and abbreviations were identical to the previous experiment. For each experiment, 4ul of the four-stage purified eluate was used as the source of NF-κB DNA binding activity.

A


Dig.N.E.
Dignam N.E.
4xHIV-Seph. Cycles
4xHIV-Seph.
- Pl. Ol. M H S B A E M H S B A E M Pl. Ol.
B
F

B


4E Eluate
4E Eluate
M H S B A E M Pl. Ol.
B
F

undigested p2xAT/HIV-R plasmid could compete away binding of the NF-κB binding proteins in the Dignam nuclear extract, the addition of a much greater molar excess (1ug) of the HIV-R oligonucleotide could not compete away formation of the radiolabelled complex. Finally, examination of the mobilities of the free restriction fragments used as the probes in these experiments indicates that they all show identical mobilities - thus there are no inherent bends in these DNA sequences which could be spuriously interpreted as bending in the DNA-protein complexes.

In contrast, NF-κB binding proteins which had been partially purified by four cycles of HIV enhancer oligonucleotide-Sepharose affinity chromatography showed both a different pattern of complex mobilities and competition behaviour - each restriction fragment probe species now yielded three complexes of differing mobilities (Figure 1.5, Panel A). Interestingly, the complexes involving Hind III, Sph I, Avi II, and Eco RV-generated restriction fragments all seemed to show identical mobilities across these four tracks, while the three complexes involving the Bam HI-generated fragment all showed a much higher mobility. One possibility might be that these partially purified NF-κB binding proteins might no longer bend the DNA, or might bend it over a smaller angle upon binding to their recognition site. Further, all three of these radiolabelled complex species could now be competed by pre-addition of HIV-R κB site oligonucleotide (as well as by the undigested p2xAT/HIV-R plasmid).

Bearing the above behaviour in mind, some of the final eluate from the HIV oligo affinity / FPLC Mono-Q / *E.coli* DNA / HIV oligo affinity chromatography purification scheme described in section 1.1 was studied by the same technique.

This revealed (Figure 1.5, Panel B) the presence of essentially only one DNA-protein complex in each lane - with the same pattern of relative mobilities seen with protein purified by four rounds of HIV enhancer oligonucleotide-Sepharose affinity chromatography. Interestingly, the actual mobilities of the complexes seemed to be very similar to those of the highest mobility species in the above experiment using NF-κB proteins purified by four rounds of HIV enhancer oligonucleotide-Sepharose affinity chromatography.

From the above considerations, an analysis of the DNA bending angle and location of the bend centre induced by binding of NF-κB proteins can only be applied to the crude HeLa cell nuclear extract. Even here, although the pattern of the DNA-protein complexes seems homogeneous, the proteins giving rise to this must be a heterogeneous mixture.

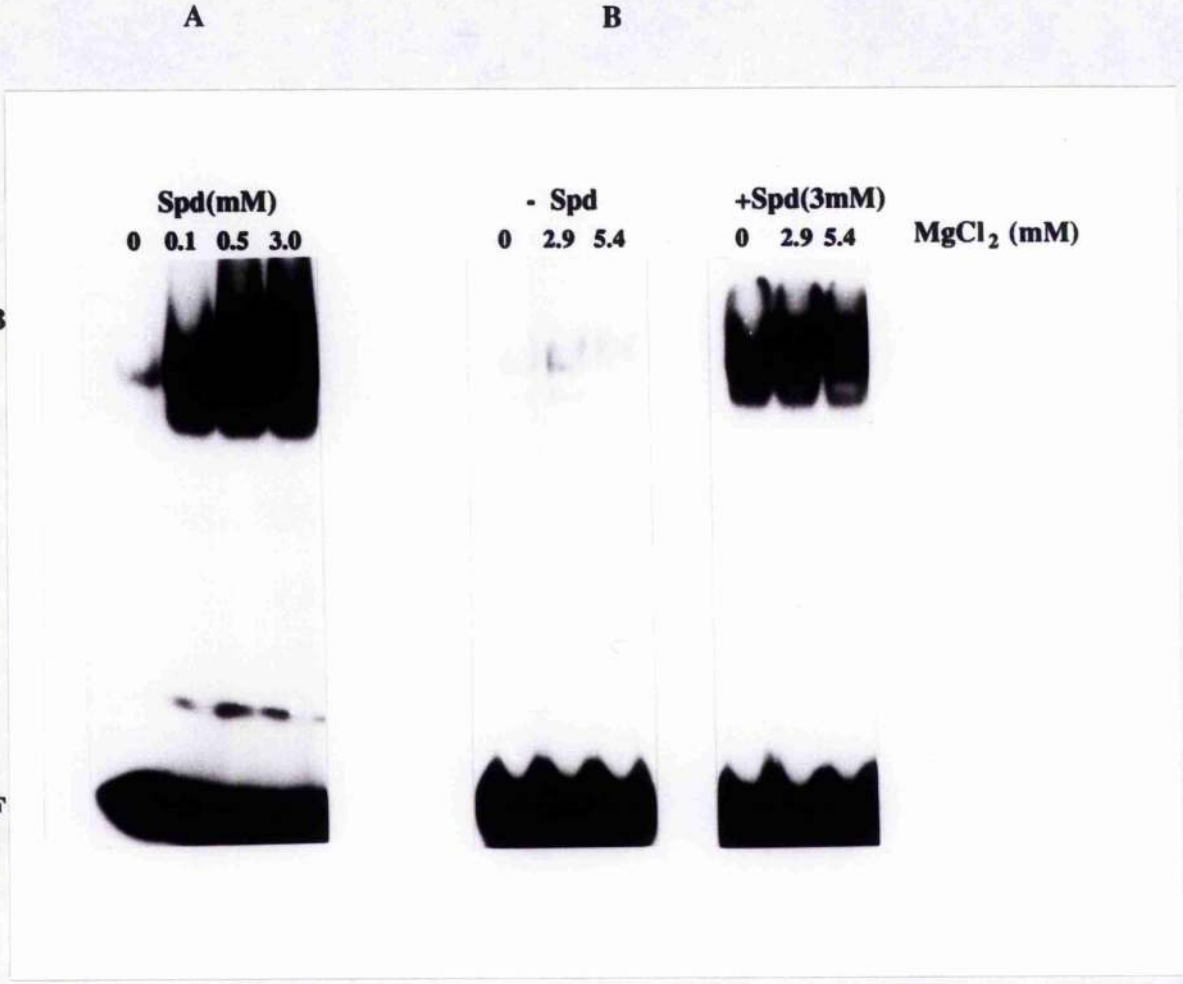
1.4 Stimulation of NF-κB DNA binding activity by the polyamine spermidine

In view of the known ability of entities such as the polyamine spermidine and magnesium chloride to stimulate *in vitro* transcription reactions, these components were tested for any effect on the binding activity of HIV enhancer oligonucleotide affinity-purified NF-κB proteins by the standard gel electrophoresis DNA binding assay (initially the binding assay incubation mixture contained neither $MgCl_2$ nor spermidine). Supplementing the incubation mixture with increasing amounts of spermidine (Figure 1.6, Panel A) caused a dramatic increase in the amount of DNA -protein complex formed, with titration experiments indicating that maximal stimulation was achieved by 3mM spermidine. This stimulation of NF-κB DNA binding activity by spermidine was also apparent with crude nuclear extracts of

Figure 1.6 Stimulation of the DNA binding activity of affinity purified NF-κB by the addition of the polyamine spermidine.

A. Stimulation of DNA binding activity of HIV enhancer oligonucleotide-Sepharose affinity purified NF-κB proteins by addition of spermidine to the standard binding incubation mixture (but lacking spermidine and $MgCl_2$). Standard gel electrophoresis DNA binding assay conditions, ^{32}P radiolabelled HIV-L oligonucleotide used as the κB binding site probe, all experiments used 2ul of purified protein eluate. Positions of the DNA-protein complex and free oligonucleotide probe are indicated by B and F respectively.

B. Effect of the addition of $MgCl_2$ on DNA binding activity and complex mobility in the presence and absence of 3mM spermidine. DNA binding assay conditions as in the experiment in Panel A.



active phorbol ester-treated Jurkat T-cells (data not shown). The addition of $MgCl_2$ to the binding assay incubation mixture did not yield any significant increase in the amount of complex formed in either the presence or absence of 3mM spermidine, however $MgCl_2$ addition did cause the DNA-protein complex to show decreased electrophoretic mobility (Figure 1.6, Panel B). Hence all other gel electrophoresis DNA binding assays were performed with the presence of 3.6mM spermidine in the binding mixture (and 6.1mM $MgCl_2$) - except where specifically omitted.

Chapter 2. Identification of conserved cysteines in the NF-κB p50 aa35-381 construct and the production of individual cysteine to serine mutant proteins.

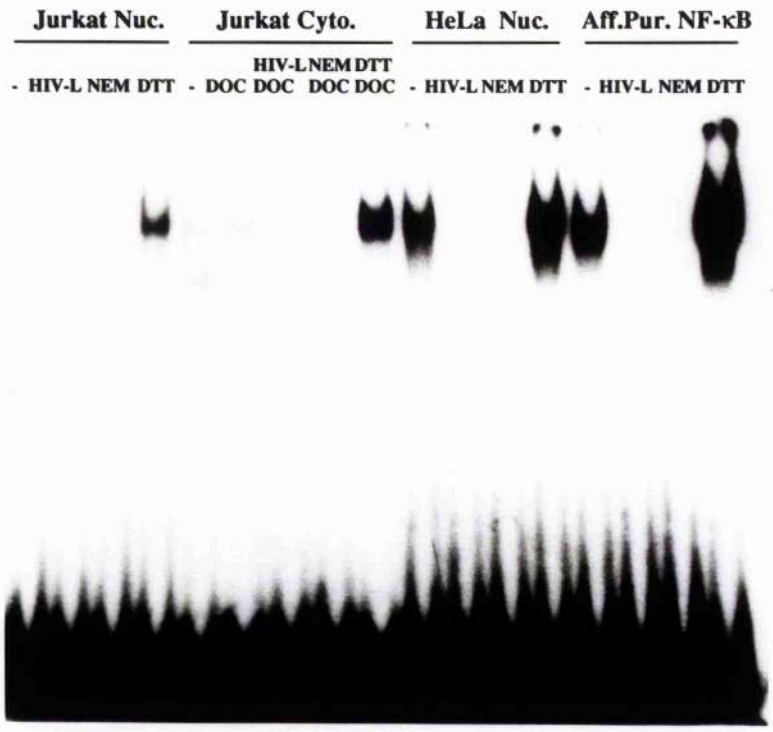
2.1 Stimulation of NF-κB DNA binding activity by dithiothreitol

Following a report that the DNA binding activity of the heterodimeric AP-1 (fos-jun) transcription factor was modulated by the oxidation-reduction state of a single conserved cysteine residue in each subunit (Abate et al., 1990) and that mutation of these residues to serine enhanced the DNA binding activity of the mutant, but abolished modulation of DNA binding activity by oxidation-reduction state, the influence of oxidation-reduction state on the DNA binding activity of NF-κB proteins was investigated.

As an initial experiment to determine if native NF-κB proteins could have their DNA binding activity modulated in a similar way, a variety of sources of native NF-κB proteins - nuclear and cytoplasmic extracts of Jurkat T-cells, Dignam HeLa cell nuclear extract, and affinity purified NF-κB proteins were studied in the standard gel electrophoresis DNA binding assay (with the binding incubation

Figure 2.1 Stimulation of the DNA binding activity of various sources of NF-κB proteins by treatment with DTT.

Various sources of NF-κB DNA binding proteins were used in the gel electrophoresis DNA binding assay (standard procedure but without addition of DTT to 25mM to the protein samples, and with addition of poly dAdT.dAdT/dGdC.dGdC carrier DNA to 0.06 ug/ul to the binding incubation mixture). Jurkat nuclear extract experiments used 2.5ul of nuclear extract, Jurkat cytoplasmic extract experiments used 2.5ul of cytoplasmic extract followed by addition of sodium deoxycholate to 1%, incubating 5 minutes at room temperature, then addition of NP40 to 1.5% and incubating again for 5 minutes at room temperature to liberate NF-κB from the IκB inhibitor protein (Baeuerle and Baltimore, 1988a). Dignam HeLa cell nuclear extract experiments used 1ul of extract, while affinity purified NF-κB protein experiments used 2ul of protein eluate. In tracks marked 'HIV-L', specific binding was competed out by addition of 1ug of unlabelled HIV-L κB site oligonucleotide after the formation of the DNA-protein complex. In tracks marked 'NEM', DNA binding activity was inactivated by addition of N-ethylmaleimide to 10mM and incubating 10 minutes at room temperature prior to quenching by addition of DTT to 25mM, incubating on ice 10 minutes, and adding the radiolabelled HIV-L oligonucleotide. In tracks marked 'DTT', NF-κB DNA binding activity was stimulated by the addition of DTT to 25mM and incubating 10 minutes on ice prior to the addition of the radiolabelled HIV-L oligonucleotide probe.


Jurkat Nuc.
Jurkat Cyto.
HeLa Nuc.
Aff.Pur. NF-κB
- HIV-L NEM DTT
- DOC HIV-L DOC NEM DOC DTT
- HIV-L NEM DTT
- HIV-L NEM DTT

mixture supplemented with 0.06 ug/ul poly dAdT.dAdT/dGdC.dGdC carrier DNA). From this experiment it was apparent that all of these sources of NF-κB proteins formed specific DNA-protein complexes with the radiolabelled HIV-L κB motif oligonucleotide probe (Figure 2.1), that the DNA binding activity of all of these protein sources increased after addition of the reducing agent dithiothreitol (DTT) to 25mM and incubating on ice for 10 minutes, and that all DNA binding activity could be abolished by addition of N-ethylmaleimide (NEM) to 10mM and incubating at room temperature for 10 minutes prior to quenching the reaction with DTT and addition of the HIV-L κB motif radiolabelled probe. Since these results suggested that DNA binding activity might be modulated by the oxidation-reduction state of one or more cysteine residues, it would be essential to identify those cysteine residues for further study of this behaviour.

2.2 Conservation of rel region in NF-κB p50, and identification of candidate cysteine residues for mutagenesis

With the timely publication of cDNA sequences encoding the p50 subunit of p50-p65 NF-κB (Kieran et al., 1990; Ghosh et al., 1990) it was now possible to study a single overexpressed protein species. Analysis of *in vitro* transcribed/translated proteins derived from the above cDNA sequences indicated that the p50 protein is initially translated as a 105kD non-DNA binding precursor - only after a proteolytic cleavage event to remove roughly the C-terminal half can the p50 protein bind to its κB recognition motif. In addition, the region of the p50 protein found to be responsible for its DNA binding and dimerisation functions was located roughly between amino acids 19 and 399 of the human p50 NF-κB subunit (Kieran et al., 1990) - it was decided to generate a p50 protein

Figure 2.2 Comparison of the amino acid sequence of human NF-κB p50 with related proteins.

The deduced amino acid sequence of the human p50 NF-κB subunit (h-p50) (Kieran et al., 1990) compared with the deduced amino acid sequences of the murine p65 NF-κB subunit (m-p65) (Nolan et al., 1991), the Drosophila morphogen dorsal (dorsal) (Steward, 1987), the mouse c-rel protein (mc-rel) (Grumont and Gerondakis, 1989), rel B (rel B) (Ryseck et al., 1992), and the REV-T v-rel protein (v-rel) (Wilhelmsen et al., 1984). Sequence numbering is relative to the methionine start codon of relB, while dots indicate spaces introduced to optimise sequence identity during alignment.

```
          1                                                  50
mc-rel    .......... .......... .......... .......... ..........
 v-rel    .......... .......... .......... .......... ..........
 m-p65    .......... .......... .......... .......... ..........
  relB    MPSRRAARES APELGALGSS DLSSLSLTVS RTTDELEIID EYIKENGFGL
dorsal    .......... .......... .......... .......... ..........
 h-p50    .......... .......... .......... .......... ..........

          51                                                 100
mc-rel    .......... .......... .......... .......... ..MASS...G
 v-rel    .......... .......... .......... ....MDFLTN LRFTEG...I
 m-p65    .......... .......... .......... ....MDDLFP LIFPSEPAQA
  relB    VGTQLSEMPR LVPRGPASLS SVTLGPAAPP PPATPSWSCT LGRLVSPGPC
dorsal    ......MFPN QNNGAAPGQG PAVDGQQSLN YNGLPAQQQQ QLAQSTKNVR
 h-p50    .......... MAEDDPYLGR PEQMFHLDPS LTHTIFNPEV FQPQMALPTA

          101                                                150
mc-rel    YNPYVEIIEQ PRQRGMRFRY KCEGRSAGSI PGERSTDNNR TYPSVQIMNY
 v-rel    SEPYIEIFEQ PRQRGTRFRY KCEGRSAGSI PGEHSTDNNK TFPSIQILNY
 m-p65    SGPYVEIIEQ PKQRGMRFRY KCEGRSAGSI PGERSTDTTK THPTIKINGY
  relB    PRPYLVITEQ PKQRGMRFRY ECEGRSAGSI LGESSTEASK TQPAIELRDC
dorsal    KKPYVKITEQ PAGKALRFRY ECEGRSAGSI PGVNSTPENK TYPTIEIVGY
 h-p50    DGPYLQILEQ PKQRGFRFRY VCEGPSHGGL PGASSEKNKK SYPQVKICNY

          151                                                200
mc-rel    YGKGKIRIT. .LVTKNDPYK PHPHDLVGK. DCRDGYYEAE FGPERRP.LF
 v-rel    FGKVKIRTT. .LVTKNEPYK PHPHDLVGK. GCRDGYYEAE FGPERQV.LS
 m-p65    TGPGTVRIS. .LVTKDPPHR PHPHELVGK. DCRDGYYEAD LCPDRSI.HS
  relB    GGLREVEVTA CLVWKDWPHR VHPHSLVGK. DCTDGVCRVR LRPHVSPRHS
dorsal    KGRAVVVVSC ..VTKDTPYR PHPHNLVGKE GCKKGVCTLE INSE.TMRAV
 h-p50    VGPAKVIVQ. .LVTNGKNIH LHAHSLVGK. HCEDGICTVT AGP.KDMVVG

          201                                                250
mc-rel    FQNLGIRCVK KKEVKEAIIL RI.SAGINPF N......... ..........
 v-rel    FQNLGIQCVK KKDLKESISL RI.SKKINPF N......... ..........
 m-p65    FQNLGIQCVK KRDLEQAISQ RI.QTNNNPF H......... ..........
  relB    FNNLGIQCVR KKEIEAAIER KI.QLGIDPY N......... ..........
dorsal    FSNLGIQCVK KKDIEAALKA RE.EIRVDPF K......... ..........
 h-p50    FANLGILHVT KKKVFETLEA RMTEACIRGY NPGLLVHPDL AYLQAEGGGD

          251                                                300
mc-rel    .......... VPEQQLLDIE DCDLNVVRLC FQVFL.PDEH GNFTTALPPI
 v-rel    .......... VPEEQLHNID EYDLNVVRLC FQAFL.PDEH GNYTLALPPL
 m-p65    .......... VPIEE..QRG DYDLNAVRLC FQVTV.RDPA GR.PLLLTPV
  relB    .......... ..AGSLKNHQ EVDMNVVRIC FQASY.RDQQ GHLHR.MDPI
dorsal    .......... TGFSHRFQPS SIDLNSVRLC FQVFMESEQK GRFTSPLPPV
 h-p50    RQLGDREKEL IRQAALQQTK EMDLSVVRLM FTAFL.PDST GSFTRRLEPV

          301                                                350
mc-rel    VSNPIYDNRA PNTAELRICR VNKNCGSVRG GDEIFLLCDK VQKDDIEVRF
 v-rel    ISNPIYDNRA PNTAELRICR VNKNCGSVKG GDEIFLLCDK VQKDDIEVRF
 m-p65    LSHPIFDNRA PNTAELKICR VNRNSGSCLG GDEIFLLCDK VQKEDIEVYF
  relB    LSEPVYDKKS TNTSELRICR INKESGPCTG GEELYLLCDK VQKEDISVVF
dorsal    VSEPIFDKKA ..MSDLVICR LCSCSATVFG NTQIILLCEK VAKEDISVRF
 h-p50    VSDAIYDSKA PNASNLKIVR MDRTAGCVTG GEEIYLLCDK VQKDDIQIRF

          351                                                400
mc-rel    .....VLNDW EARGVFSQAD VHRQVAIVFK TPPYCKAILE .PVTVKMQLR
 v-rel    .....VLGNW EAKGSFSQAD VHRQVAIVFR TPPFLGDITE .PITVKMQLR
 m-p65    .....TGPGW EARGSFSQAD VHRQVAIVFR TPPYADPSLQ APVRVSMQLR
  relB    .....STASW EGRADFSQAD VHRQIAIVFK TPPYEDLEIS EPVTVNVFLQ
dorsal    FEEKNGQSVW EAFGDFQHTD VHKQTAITFK TPRYHTLDIT EPAKVFIQLR
 h-p50    YEEEENGGVW EGFGDFSPTD VHRQFAIVFK TPKYKDINIT KPASVFVQLR

          401                                                450
mc-rel    RPSDQEVSES MDFRYLPDEK DAYGNKSKKQ KTTLI.FQKL LQDCG.....
 v-rel    RPSDQAVSEP VDFRYLPDEE DPSGNKAKRQ RSTLA.WQKP IQDCGS....
 m-p65    RPSDRELSEP MEFQYLPDTD DRHRIEEKRK RTYET.FKSI MKKSPF....
  relB    RLTDGVCSEP LPFTYLPRDH DSYGVDKKRK RGLPD.VLGE LSSSDP....
dorsal    RPSDGVTSEA LPFEYVPMDS DPAHLRRKRQ KTGGDPMHLL LQQQQK....
 h-p50    RKSDLETSEP KPFLYYPEIK DKEEVQRKRQ KLMPNFSDSF GGGSGAGAGG

          451                                                500
mc-rel    .......... .......... .......... ......HFTE KPRTA....P
 v-rel    .......... .......... .......... ......AVTE RPKAA....P
 m-p65    .......... .......... .......... ......NGPT EPRPPTRRIA
  relB    .......... .......... .......... ......HGIE SKRRKKKPVF
dorsal    .......... .......... .......... ......QQLQ NDHQDGRQTN
 h-p50    GGMFGSGGGG GGTGSTGPGY SFPHYGFPTY GGITFHPGTT KSNAGMKHGT

          501                                                550
mc-rel    LG...STGEG RFIKKESNLF SHGTVL.... .....PEMPR SSGVPGQAEP
 v-rel    IP...TVNPE GKLKKEPNMF SPTLML.... .....PGL.. ..........
 m-p65    VP...TRNST SVPKPAPQPY TFPASLSTIN FDEFSPMLLP SGQISNQALA
  relB    LDHFLPGHSS GLFLPPSALQ PADSDFFPAS ISLPGLEPPG GPDLLDDGFA
dorsal    MNCWNTQNIP PIKTEPRDTS PQPLGFLIGR LLSSHPRRSR CRHRATTTTT
 h-p50    MDTESKKDPE GCDKSDDK.. .NTVNLFGKV IETTEQDQEP SEATVGNGEV
```

construct running from amino acid 35 (translation from this third internal methionine codon having been shown to give p50 proteins still capable of binding DNA - A.Israël, pers. comm.) to amino acid 381 (just before the glycine-rich 'hinge' region).

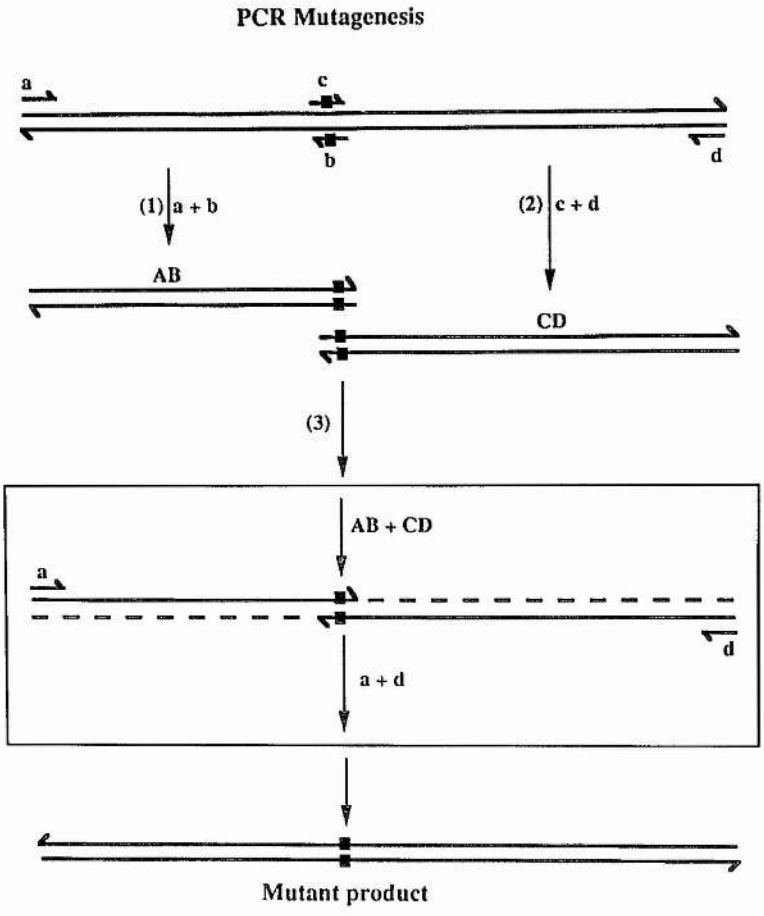
Comparison of the predicted amino acid sequence of this DNA binding and dimerisation region of the p50 protein with other reported amino acid sequences revealed a high degree of similarity to a region in the turkey oncoprotein v-rel and a similar region in the *Drosophila* maternal effect morphogen protein dorsal (Kieran et al., 1990; Ghosh et al., 1990). Further, within the p50 amino acid 35 to 381 region selected for this study, were seven cysteine residues - most interestingly, three of these were absolutely conserved among the various amino acid sequences of this family (Figure 2.2). These three cysteines occurred at positions 62, 119 and 273 in the p50 amino acid sequence and were obvious candidates for individual site-directed mutagenesis to serine residues.

2.3 Generation of cDNAs encoding wild type and cysteine to serine mutant NF-κB p50 aa35-381 proteins

For the generation of a cDNA encoding wild type NF-κB p50 protein amino acids 35 to 381, the appropriate region of DNA was amplified by the polymerase chain reaction (PCR) using the appropriate oligonucleotide primers and a pBluescript plasmid containing the cDNA encoding the p105 precursor to p50 (kindly provided by A.Israël) as the template source. As described in the Materials and Methods section, 25 cycles of DNA amplification yielded an essentially homogeneous product with the predicted size of approximately 1065bp upon 0.8% agarose gel electrophoresis (result not shown).

Figure 2.3 Schematic of PCR site-directed mutagenesis.

The first stage of the PCR mutagenesis scheme used two external primers (a and d) encoding recognition sites for Bam HI and Eco RI restriction enzymes, and two internal mutagenising primers (b and c) to generate two partial length mutant cDNA products - AB and CD. These were then gel purified and mixed together to provide the template for a second PCR amplification stage using only the external primers (a and d) to generate the full length mutant cDNA product.

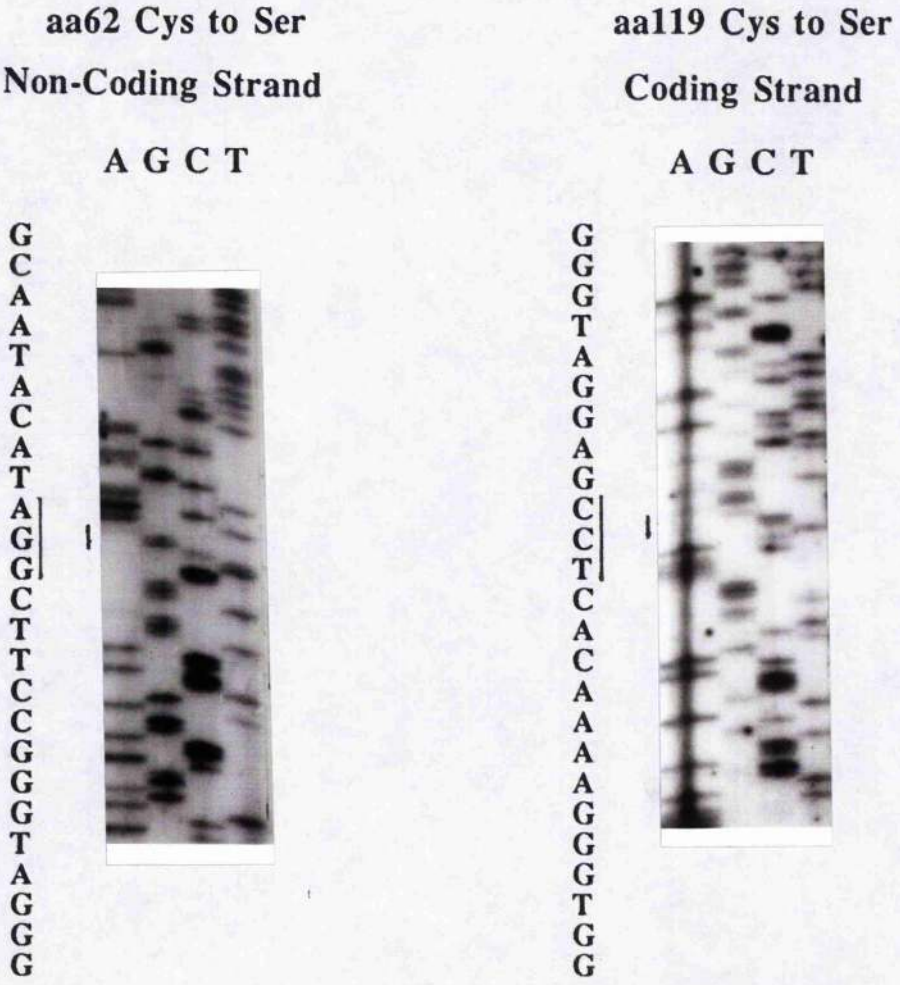


For the generation of the three mutant cDNA species encoding the cysteine to serine mutations at amino acid positions 62, 119, and 273, a rapid PCR mutagenesis method was used (Ho et al., 1989) - as described in the Materials and Methods section, this involved the use of two internal mutagenising primers and the two external PCR primers to generate two partial length mutant cDNA products. The partial length cDNA products were essentially homogeneous and of the expected sizes when analysed by agarose gel electrophoresis (data not shown). The remainder of the partial length products were agarose gel purified to free them from contaminating primers and nucleotides, and 1-2ul of each gel band used to provide the template DNA for a second round of 25 cycles of PCR amplification as described in the Materials and Methods section. Analysis of the three final mutant products by 0.8% agarose gel electrophoresis revealed that all had the expected size of approximately 1065bp and were essentially homogeneous (results not shown), a schematic of this mutagenesis strategy is shown in Figure 2.3.

After the ligation of the Bam HI / Eco RI-digested cDNA products into the similarly digested pGEX-2T bacterial expression vector and the isolation of the appropriate ampicillin-resistant *E.coli* transformants (see Materials and Methods section), the cDNA surrounding the cysteine to serine mutation sites of the three mutants was sequenced using a PCR-based double stranded DNA cycle sequencing kit (Gibco-BRL, see Materials and Methods) allowing the direct sequencing of plasmid DNA isolated from lysed bacterial single colonies. The sequence data from the two mutant cDNA species indicated (Figure 2.4) that the mutagenesis procedure had generated the expected products, and that over the region of

Figure 2.4 Sequence confirmation of cysteine to serine mutations.

The PCR sequencing reaction products for the non-coding DNA strand around the amino acid 62 cysteine to serine (TGT to TCC) mutation and the coding DNA strand around the amino acid 119 cysteine to serine (TGT to TCC) mutation are shown.



readable sequence (≈150 nucleotides surrounding the mutation site) no inadvertent mutations had been generated.

2.4 Expression of wild type and cysteine to serine mutant NF-κB p50 aa35-381 proteins in *E.coli*

For the expression of the proteins encoded by the above wild type and mutant cDNAs, it was decided to use the *E.coli* / pGEX-2T system, the bacterial pGEX-2T expression vector had already been demonstrated to give efficient expression and easy purification of a large number of eukaryotic proteins (Smith and Johnson, 1988). Furthermore, the same expression vector had already been used successfully to express the full length p105 precursor to NF-κB p50 in *E.coli* (Kieran et al., 1990).

As described previously (Materials and Methods) NF-κB p50 cDNA / pGEX-2T ligation mixtures were transformed into *E.coli* JM101 and ampicillin resistant colonies isolated, these were then screened directly by SDS-PAGE under reducing conditions for the overexpression of a ≈65 kD fusion protein upon induction of overnight cultures with IPTG. Following the identification of the various clones expressing wild type and aa62, 119, and 273 cysteine to serine mutant p50 aa35-381 fusion proteins, 500ml *E.coli* cultures were grown to the appropriate density at 37°C before the induction of fusion protein expression. Purification of the individual p50 aa35-381 fusion proteins by glutathione-agarose affinity chromatography, was followed by thrombin cleavage of the fusion protein and calf thymus DNA-Sepharose affinity chromatography to recover the p50 aa35-381 DNA-binding proteins (Materials and Methods). The glutathione-agarose affinity chromatography yielded virtually pure glutathione S-transferase-NF-κB p50

Figure 2.5 Glutathione-agarose affinity purification of GST-NF-κB p50 aa35-381 fusion proteins.

Coomassie brilliant blue R-250 stained 10% polyacrylamide SDS-PAGE (Materials and Methods) gels of glutathione-agarose affinity purification of glutathione S-transferase-NF-κB p50 aa35-381 fusion proteins. All sample tracks used 20ul of protein solution, with the addition of 7ul of 4x sample buffer, before boiling samples, quenching on ice and loading. The following abbreviations were used for the various tracks :- M, protein molecular weight standards; L, crude bacterial load to glutathione-agarose columns; FT, flowthrough from columns; W, 0.5M NaCl buffer wash through columns; E, 10mM reduced glutathione / 0.5M NaCl buffer eluate from glutathione-agarose affinity columns. The positions of the molecular weight standard marker proteins are indicated.

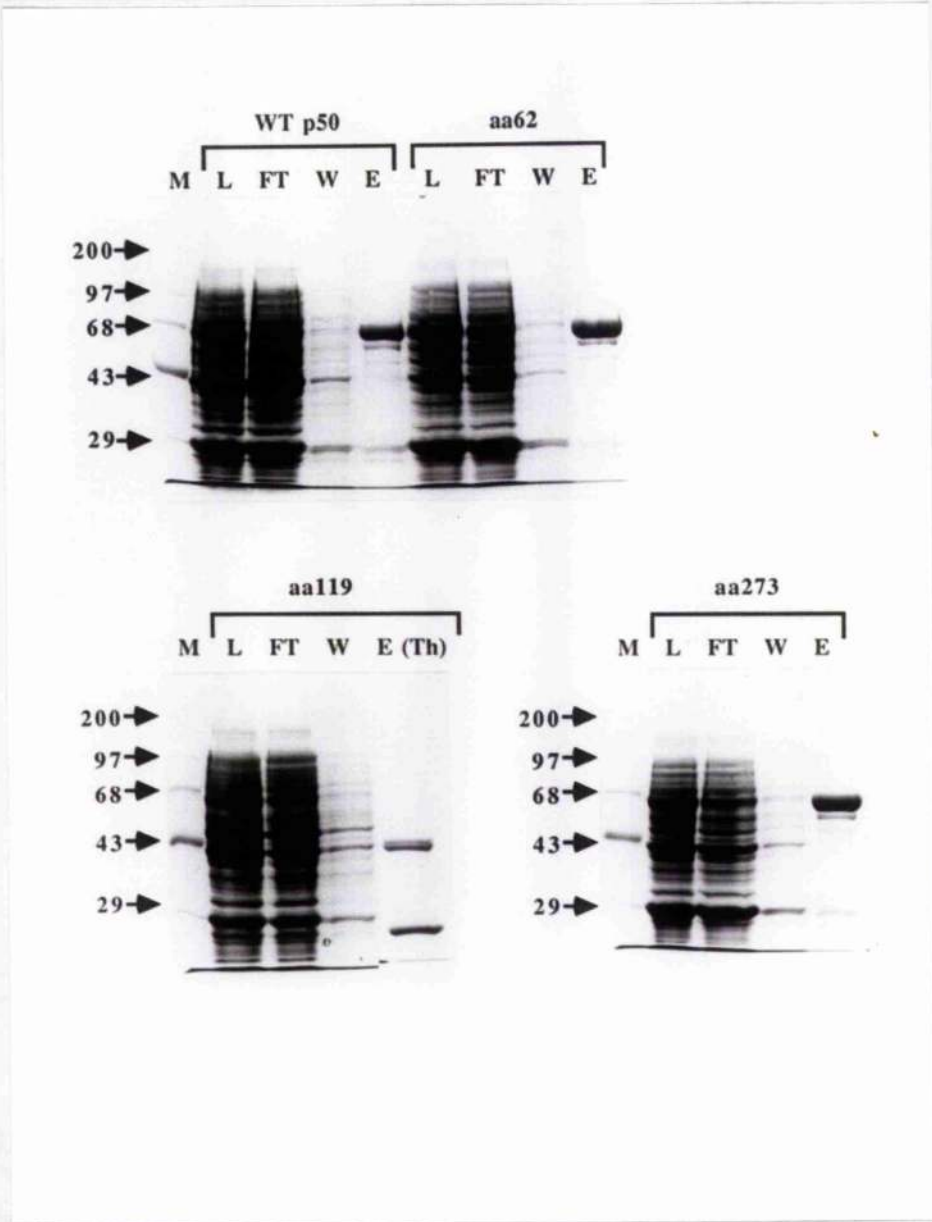


Figure 2.6 Double stranded calf thymus DNA-Sepharose affinity purification of NF-κB p50 aa35-381 fusion proteins.

Coomassie brilliant blue R-250 stained 10% polyacrylamide SDS-PAGE (Materials and Methods) gels of double stranded calf thymus DNA-Sepharose affinity purification of NF-κB p50 aa35-381 proteins. All sample tracks used 20ul of protein solution, with the addition of 7ul of 4x sample buffer, before boiling samples, quenching on ice and loading. The following abbreviations were used for the various tracks :- M, protein molecular weight standards; L, 150mM NaCl load to double stranded DNA-Sepharose affinity columns; FT, flowthrough from columns; W, 150mM NaCl buffer wash through columns; 0.6, 0.6M NaCl buffer eluate from DNA-Sepharose affinity columns. DBD and GST indicate the positions of the NF-κB p50 aa35-381 and glutathione S-transferase proteins respectively. The two protein molecular weight marker bands represent 43 and 29kD.

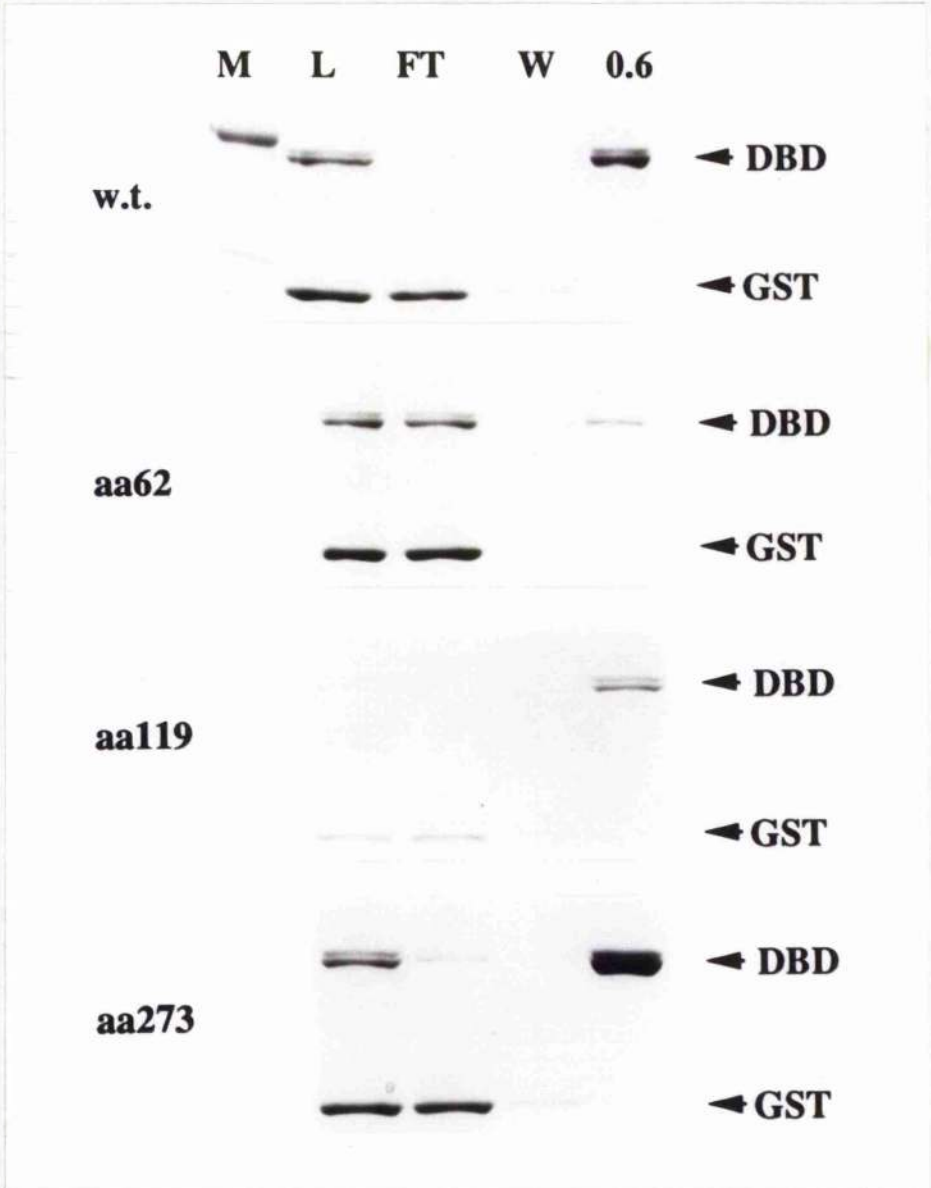


Table 2.1 Protein yields for the four p50 species from 500ml E.coli culture

	WT p50 aa35-381	aa62	aa119	aa273
GST-p50 Fusion protein yield	1.3mg	2.1mg	0.6mg	2.0mg
Purified p50 aa35-381 protein yield	0.17mg	0.062mg	0.084mg	0.36mg

aa35-381 fusion proteins in the various eluates (Figure 2.5). In the case of the aa119 cysteine to serine mutant, the fusion protein was thrombin-cleaved before SDS-PAGE analysis.

To purify the thrombin-cleaved p50 aa35-381 proteins from the glutathione S-transferase and other contaminating proteins, the thrombin cleaved proteins in 150mM NaCl were applied to double stranded calf thymus DNA-Sepharose columns, washed, and eluted with 0.6M NaCl buffer. This second DNA affinity chromatography stage had the additional advantages that all of the protein which bound to and eluted from the column should be active in DNA binding and also be free from non-specifically bound *E.coli* DNA. SDS-PAGE analysis of the various protein fractions from the DNA affinity chromatography stage revealed (Figure 2.6) that all of the p50 amino acid 35-381 species were freed of all contaminating proteins by this purification stage. However, some other features were also apparent - all the purified species were present as doublet bands - this proteolytic clipping event has been localised to the C-terminus of the wild type p50 aa35-381 species (data not shown), and presumably occurs in the same location in the mutant species. It was also noticeable that the aa62 cysteine to serine mutant seemed to bind only poorly to the DNA affinity column - most of the p50 protein remaining in the flowthrough fraction, the reason for this remains unclear. The yields of the various proteins from the purification of the bacterially expressed material are given in Table 2.1.

Chapter 3. Characterisation of the κB-specific binding activity of the four NF-κB p50 aa35-381 proteins.

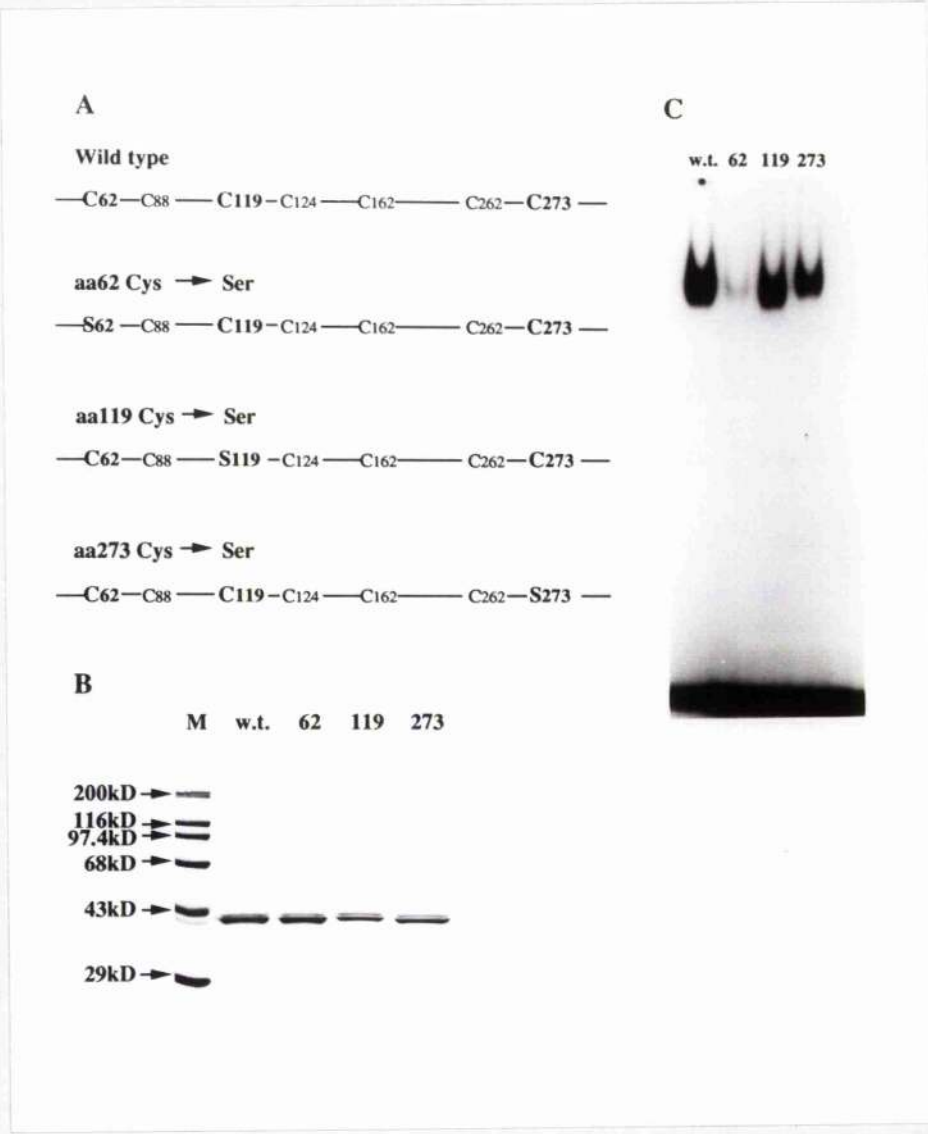
3.1 Initial characterisation of binding activity

Figure 3.1 Initial characterisation of κB motif DNA binding activity of the four NF-κB p50 aa35-381 protein variants.

A. Schematic of the positions of the conserved (bold type) and nonconserved cysteine residues, and the positions of the cysteine to serine mutations in the four NF-κB p50 aa35-381 protein species.

B. SDS-PAGE analysis of the four purified NF-κB p50 aa35-381 proteins, M indicates the protein molecular weight marker track, positions of the molecular weight standards are indicated.

C. Gel electrophoresis DNA binding assay with equal amounts of NF-κB p50 aa35-381protein in each track (1.9ng) carried out under standard conditions in the presence of spermidine with double stranded 16-mer κB motif oligonucleotide probe, upper bands represent the DNA-protein complex.



With purified homogeneous proteins now available, it was possible to begin meaningful *in vitro* characterisation of the DNA binding activity of the four NF-κB p50 aa35-381 species (Figure 3.1, Panel A). Initial trials demonstrated that the κB motif DNA binding activity of all four species was stimulated by treatment with the reducing agent DTT, and that addition of DTT to 25mM to the protein itself or to the total binding incubation mixture and incubating for 15 minutes on ice was sufficient to give full stimulation of κB motif DNA binding activity (data not shown).

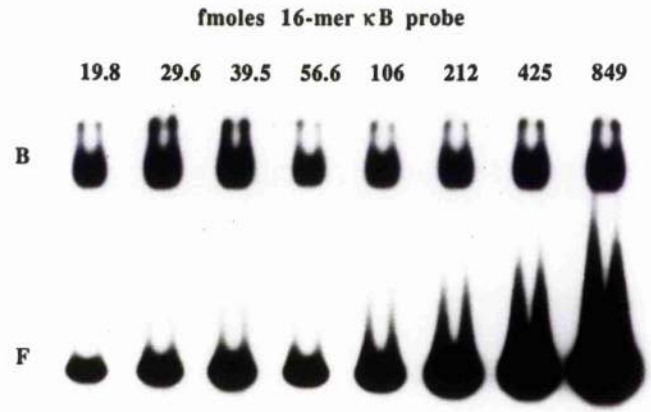
An initial gel electrophoresis DNA binding assay under the standard conditions (Materials and Methods) with the double stranded 16-mer κB motif oligonucleotide demonstrated that the four variant p50 proteins had significantly different DNA binding activities (Figure 3.1, Panel C) when the binding assays were performed with equal amounts of protein (1.9ng per track). Quantitation of the amounts of ^{32}P radioactivity in the DNA-protein complexes by liquid scintillation counting of dried gel slices yielded the following ratios of binding activity (normalised relative to the wild type protein) of :- wild type, 1.000; aa62 cysteine to serine mutant, 0.097; aa119 cysteine to serine mutant, 0.530; aa273 cysteine to serine mutant , 0.463. Thus the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein showed an interesting ten fold decrease in its binding affinity for the double stranded 16-mer κB motif oligonucleotide, with the two other cysteine to serine mutant proteins demonstrating intermediate binding affinities.

3.2 Determination of dissociation constants for interaction of the four NF-κB p50 aa35-381 proteins with DNA by Scatchard analysis

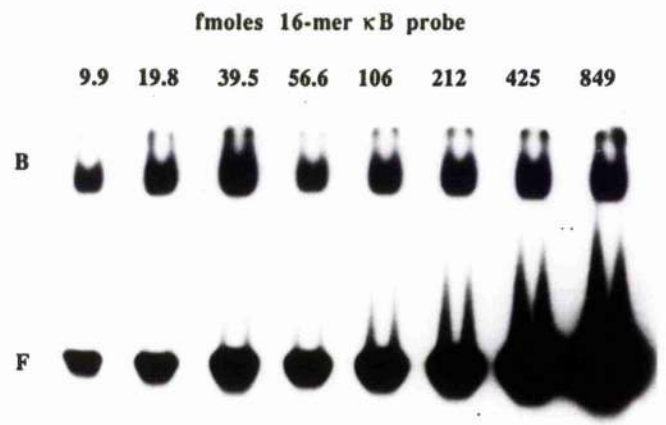
Figure 3.2 Gel electrophoresis DNA binding assays for dissociation constant analysis.

Gel electrophoresis DNA binding assays under standard conditions using large excesses of oligonucleotide probe in an attempt to saturate binding of the four NF-κB p50 aa35-381 protein species, all binding assays used 0.0029ug of p50 proteins. The bound and free 16-mer κB motif oligonucleotide probe bands are indicated by B and F respectively.

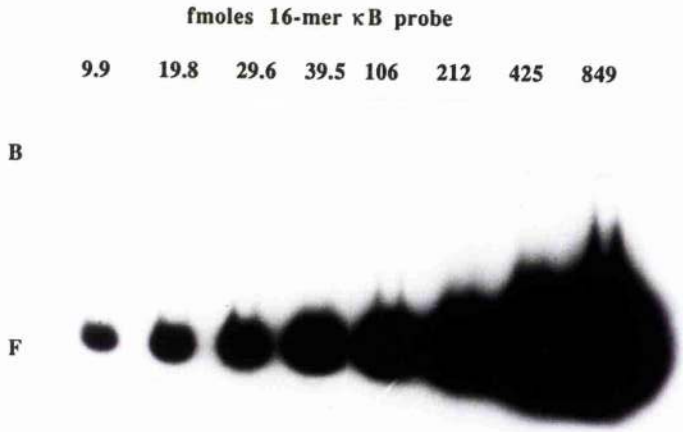
WT p50 aa35-381



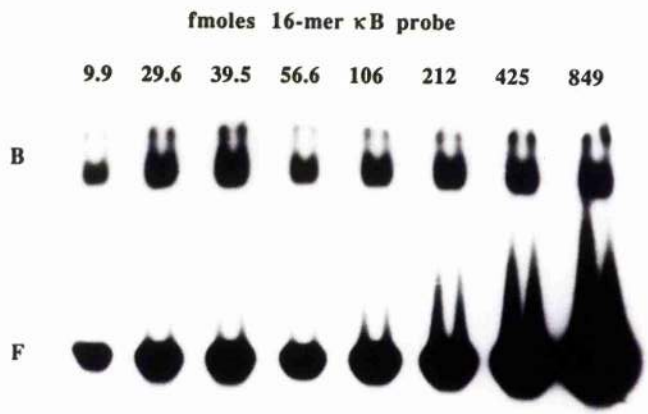
aa119 Cys to Ser



aa62 Cys to Ser



aa273 Cys to Ser

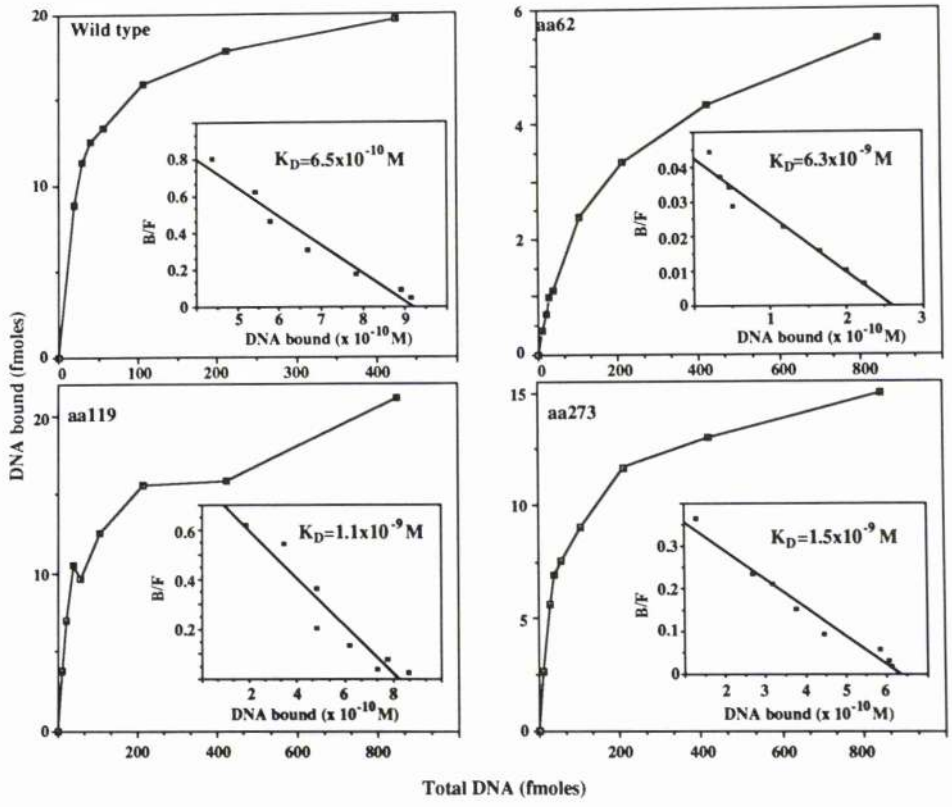


Although the previous experiment revealed the relative affinities of the four NF-κB p50 aa35-381 proteins for the κB motif, for the determination of absolute values for binding affinities, gel electrophoresis DNA binding assays were performed at 20°C in the presence of the standard 3.6mM spermidine on the four protein species and increasing amounts of double stranded 16-mer κB motif oligonucleotide (Figure 3.2). The dried polyacrylamide gel bands containing the DNA-protein complex and the free oligonucleotide probe were assayed for ^{32}P radioactivity by liquid scintillation counting and the concentrations of free oligonucleotide probe and bound oligonucleotide probe in the binding mixture calculated. From Scatchard plots of the ratio of concentrations of bound oligonucleotide probe/free oligonucleotide probe versus the concentration of bound oligonucleotide probe (Figure 3.3, small inserts) it was possible to derive estimates for dissociation constant (K_D) values for the various NF-κB p50 aa35-381 protein species with the double stranded 16-mer κB motif oligonucleotide under the standard DNA binding assay conditions (Figure 3.3).

The K_D values obtained for the various p50 protein derivatives are of the same general magnitude as values obtained for many other sequence-specific DNA binding proteins, although they are not as small as the K_D values obtained from some studies using native NF-κB proteins (Zabel et al., 1991; Urban and Baeuerle, 1990) - see Discussion section. The relative numerical values of the dissociation constants show good agreement (when expressed as association constants normalised to the wild type NF-κB p50 aa35-381 protein value) with the initial values measured in section 3.1 :- wild type, 1.000; aa62 cysteine to serine mutant,

Figure 3.3 Binding curves and Scatchard plot analysis of K_D values for the four NF-κB p50 aa35-381 proteins.

From a knowledge of the amount of 16-mer κB motif oligonucleotide probe in the bound and free bands, binding saturation curves and Scatchard plots were constructed for the four protein species. Straight lines were fitted to the Scatchard plot data by linear regression analysis and K_D values derived from the reciprocals of the negative gradients.



0.103; aa119 cysteine to serine mutant, 0.591; aa273 cysteine to serine mutant, 0.433.

3.3 Rate of dissociation of DNA-protein complexes at 0°C in the presence and absence of spermidine

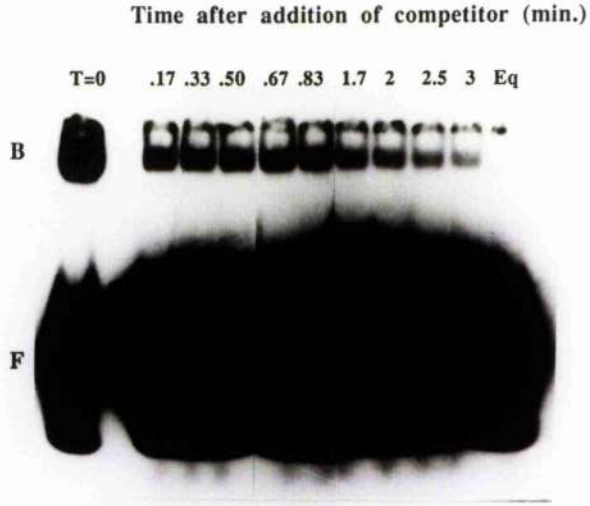
To explain these changes in dissociation constants at 20°C in the presence of spermidine some change must have occurred in either the rate at which the mutant proteins associate with DNA, or in the rate at which the DNA-protein complex dissociates, or in both of these factors. One early clue that some increase in the dissociation rate constant of the aa62 cysteine to serine mutant p50 protein might be at least partly responsible for its lower affinity for the κB motif came from observations that in gel electrophoresis DNA binding assays, a faint smear of radioactivity could often be seen running ahead of DNA-protein complexes involving the aa62 mutant protein, but not with complexes involving the other proteins (data not shown). Bearing in mind the known ability of the polyacrylamide gel matrix to stabilise protein-DNA interactions (Fried and Crothers, 1984), it seemed possible that the amino acid 62 cysteine to serine mutant NF-κB p50 aa35-381 protein - 16-mer κB motif oligonucleotide complex might have a larger dissociation rate constant than that of the DNA-protein complex involving the wild type p50 protein.

To answer this question, preformed radiolabelled 16-mer κB oligonucleotide-p50 protein complexes (with roughly equal DNA binding activity) in the presence of 3.6mM spermidine were challenged at 0°C with a 100-fold molar excess of unlabelled HIV-L κB motif oligonucleotide (previous attempts to

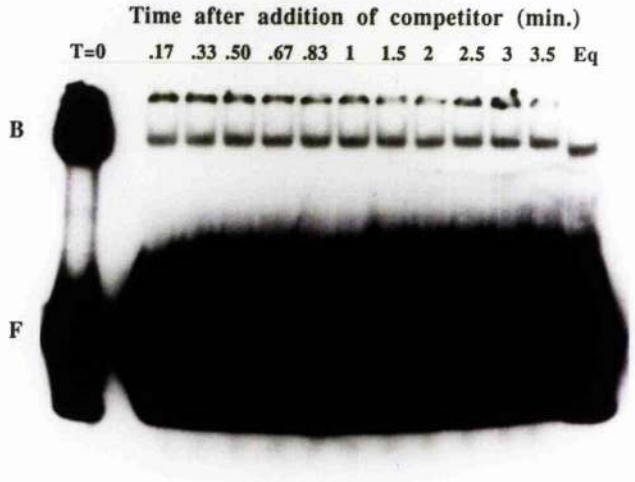
Figure 3.4 Gel electrophoresis DNA binding assays for determining dissociation rate constants at 0°C in the presence of spermidine.

Gel electrophoresis DNA binding assays (with roughly equal amounts of DNA binding activity for all four p50 protein species) were carried out at 0°C in the presence of 3.6mM spermidine. Preformed radiolabelled DNA-protein complexes were challenged with a 100-fold molar excess of unlabelled HIV-L κB motif oligonucleotide, samples were removed from the binding mixture at the times indicated and loaded immediately on a running non-denaturing gel. The T=0 tracks represent the amount of DNA binding activity before addition of unlabelled HIV-L competitor oligonucleotide. Eq indicates the amount of radiolabelled DNA-protein complex present at equilibrium - i.e. equivalent to the amount of radiolabelled DNA-protein complex seen when the probe and unlabelled HIV-L competitor oligonucleotide are added simultaneously. B and F represent the DNA-protein complex and free oligonucleotide probe respectively.

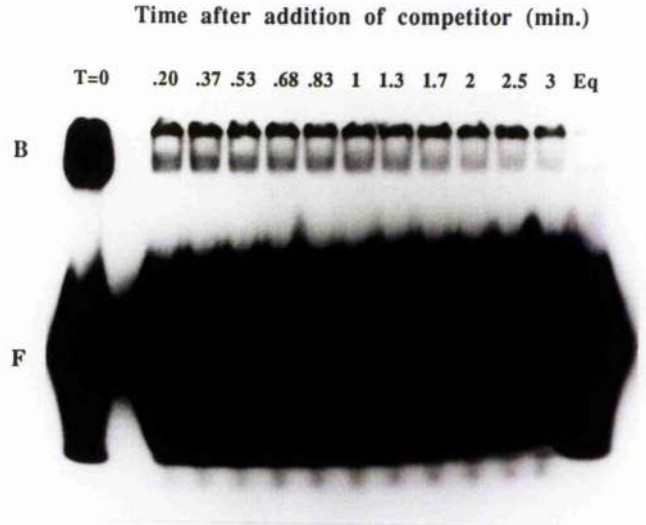
WT p50



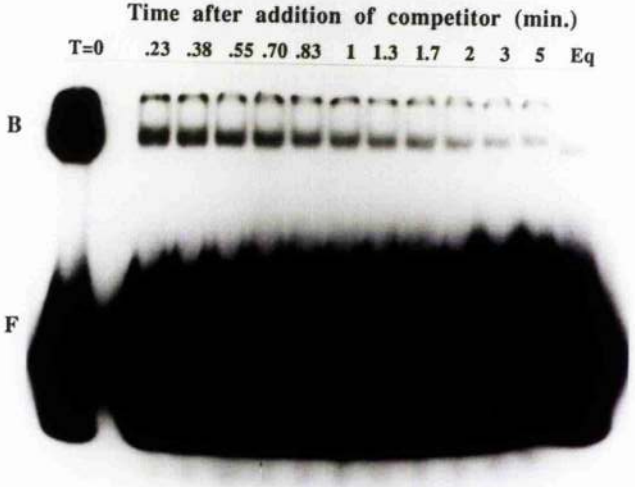
aa62



aa119



aa273



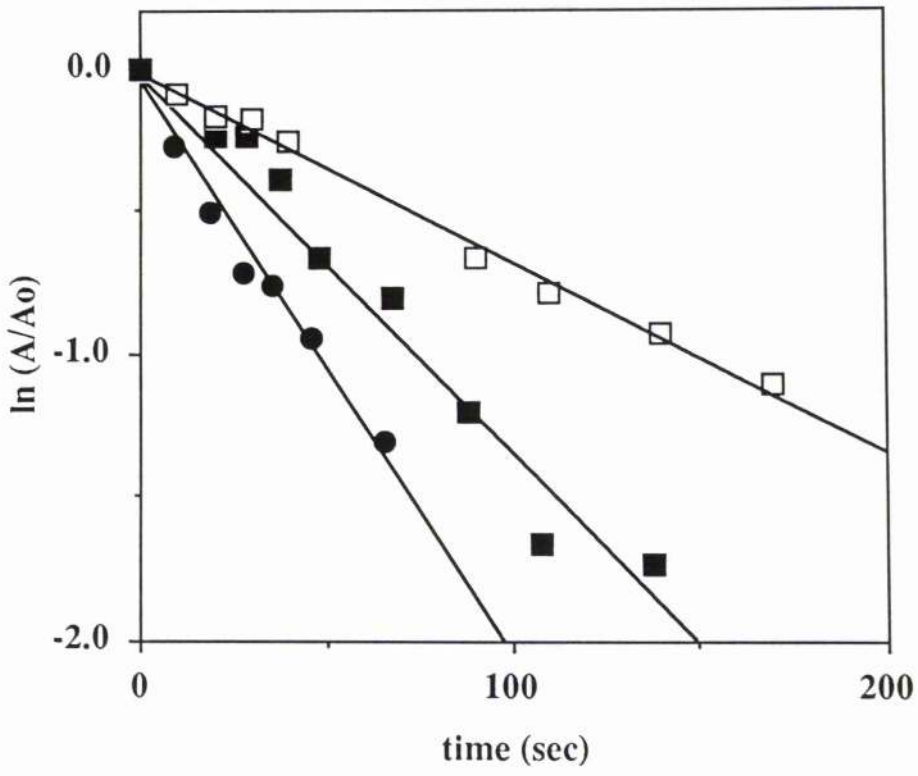
measure complex breakdown at 20°C had failed because dissociation was too rapid). Samples of the mixture were removed at specific timepoints and loaded immediately onto a running non-denaturing gel, while for longer timescale experiments, aliquots of the preformed complexes were removed and individually challenged with cold oligonucleotide to allow the simultaneous loading of all samples.

Because of the difficulty of estimating how long the radiolabelled DNA-protein complex takes to enter the gel and be effectively separated from free oligonucleotide, the effective time of all data points was taken relative to the time at which the first sample after unlabelled oligonucleotide challenge was loaded on the gel. The DNA binding assay unlabelled oligonucleotide challenge experiments carried out at 0°C in the presence of 3.6mM spermidine (Figure 3.4) showed that the initial suggestion that the aa62 cysteine to serine mutant p50 protein - 16-mer κB motif oligonucleotide complex was unstable seemed to be correct. It was immediately apparent that the DNA-protein complex involving the aa62 cysteine to serine mutant p50 protein had broken down sufficiently to give the equilibium level of radiolabelled DNA-protein complex by the time the sample from the 0.17 minute timepoint entered the gel. A further interesting observation was that radiolabelled DNA-protein complexes involving the other two mutant NF-κB p50 aa35-381 proteins seemed to be dissociating more rapidly than the complex involving the wild type protein.

After scintillation counting of the amounts of radioactivity in the various complexes, the data were plotted assuming that dissociation would be a first-order process. The plot of the integrated pseudo first-order rate equation, ln (A/Ao)

Figure 3.5 Integrated pseudo first-order rate equation plots for dissociation of wild type, aa119, and aa273 cysteine to serine p50 protein-DNA complexes in the presence of spermidine.

Integrated pseudo first-order rate equation plots for the dissociation of wild type, aa119, and aa273 cysteine to serine NF-κB p50 aa35-381 protein-16-mer κB motif oligonucleotide probe complexes in the presence of 3.6mM spermidine at 0°C. Open squares represent wild type p50 protein, solid squares represent aa119 cysteine to serine mutant p50 protein, solid circles represent aa273 cysteine to serine mutant p50 protein.



versus time, for dissociation of complexes involving the four p50 protein species in the presence of spermidine at 0°C (Figure 3.5) shows some interesting features. Firstly, the individual plots are linear - suggesting that for the wild type and aa119 and aa273 mutant p50 proteins dissociation is a first order process. The relative numerical values of the dissociation rate constants for the wild type, aa119, and aa273 NF-κB p50 aa35-381 proteins in the presence of spermidine (Table 3.1) measured at 0°C are in good agreement with their dissociation constants measured at 20°C in the presence of spermidine (Figure 3.3).

This carries the implication that if the dissociation constants remain broadly unchanged between 0°C and 20°C, then the association rate constants for these three proteins are likely to be very similar. However, the complex involving the aa62 cysteine to serine p50 mutant breaks down too rapidly to be measured by this gel electrophoresis method - leaving only the equilibrium level of binding. This observation is interesting in that if the association rate constant of the aa62 cysteine to serine mutant were the same as that of the other three proteins, then for an overall K_D value of approximately 6.3×10^{-9}M, a dissociation rate constant of approximately 0.07 s^{-1} would have been predicted - this should be within the detection limits of the technique (compare this with the value of approximately 0.35 s^{-1} obtained for the aa62 mutant in the absence of spermidine). Thus the implication is that in the presence of spermidine the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins posess similar association rate constants (with estimated values of approximately 10^7 $M^{-1}s^{-1}$ at 0°C), whereas the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein should have a significantly

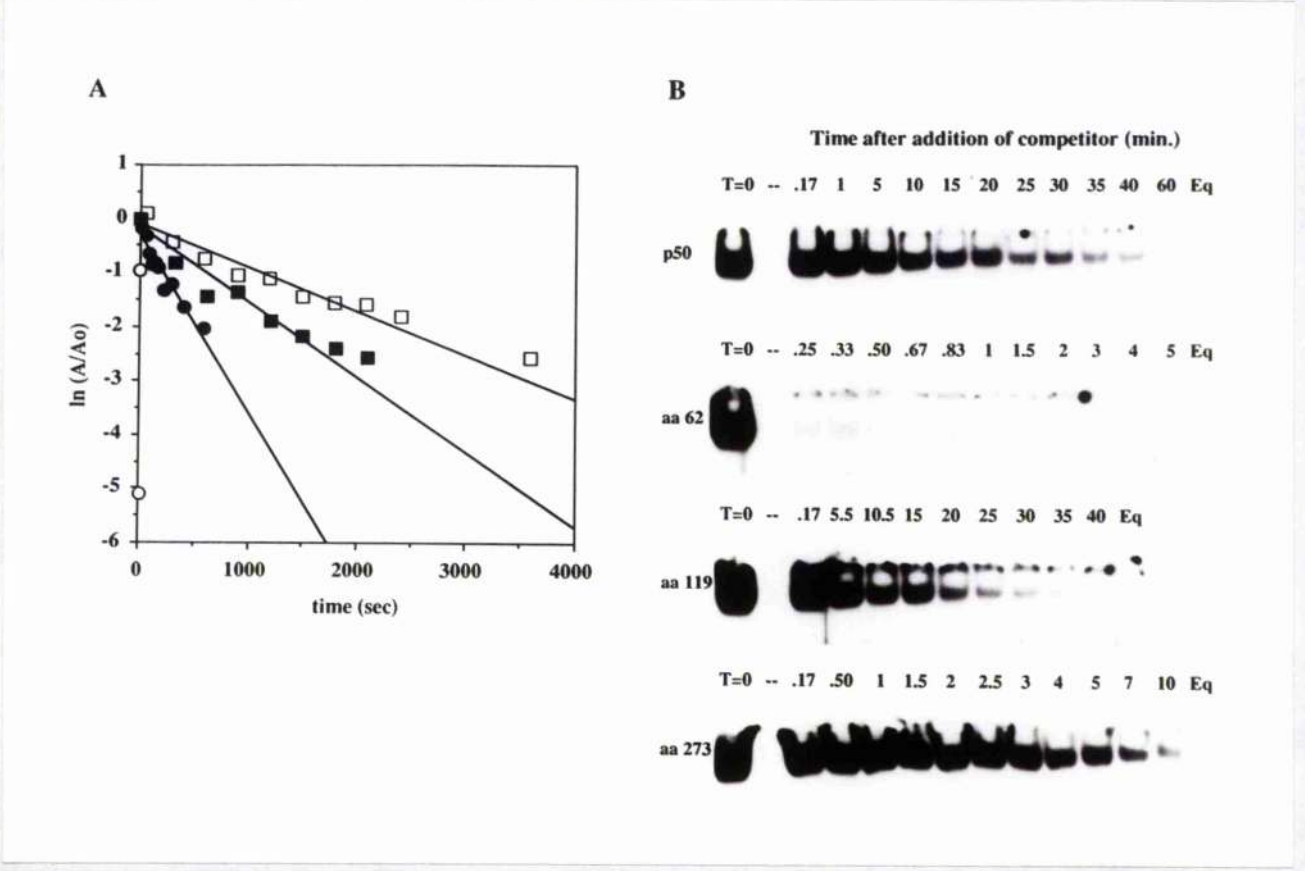
Table 3.1 Dissociation rate constants for the four NF-κB p50 aa35-381 protein-16-mer κB motif oligonucleotide complexes at 0°C in the presence and absence of 3.6mM spermidine.

	+ Spd.	- Spd.
W.T.	0.0070 s^{-1}	0.00071 s^{-1}
a.a.62	> 0.35 s^{-1}	0.35 s^{-1}
a.a.119	0.014 s^{-1}	0.0011 s^{-1}
a.a.273	0.019 s^{-1}	0.0033 s^{-1}

Figure 3.6 Gel electrophoresis DNA binding assays and integrated pseudo first-order rate equation plots for dissociation of all four p50 protein-DNA complexes in the absence of spermidine.

A. Integrated pseudo first-order rate equation plots for the dissociation of wild type, aa62, aa119, and aa273 cysteine to serine NF-κB p50 aa35-381 protein-16-mer κB motif oligonucleotide probe complexes in the absence of spermidine at 0°C. Open squares represent wild type p50 protein, solid squares represent aa119 cysteine to serine mutant p50 protein, solid circles represent aa273 cysteine to serine mutant p50 protein, and open circles represent the aa62 cysteine to serine mutant p50 protein.

B. Gel electrophoresis DNA binding assays (with roughly equal amounts of DNA binding activity for all four p50 protein species) were carried out at 0°C in the absence of spermidine. Preformed radiolabelled DNA-protein complex aliquots were individually challenged with a 100-fold molar excess of unlabelled HIV-L κB motif oligonucleotide at the appropriate time, then samples loaded simultaneously onto a running non-denaturing gel. The T=0 tracks represent the amount of DNA binding activity before addition of unlabelled HIV-L competitor oligonucleotide , Eq indicates the amount of radiolabelled DNA-protein complex present at equilibrium, B and F represent the DNA-protein complex and free oligonucleotide probe respectively.



larger association rate constant.

The plots determining dissociation rate constants in the absence of spermidine (Figure 3.6) at 0°C show additional interesting features - again the relative values of the dissociation rate constants for the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins correlate reasonably well with the K_D values determined in the presence of spermidine (Table 3.1). Although explicit K_D values have not been determined in the absence of spermidine, gel electrophoresis DNA binding assays differing only in the presence or absence of spermidine in the binding buffer have indicated that for the bacterially expressed NF-κB p50 aa35-381 constructs, the absence of spermidine has only a small effect on the overall binding affinities of the various proteins for the κB motif. The ratios of binding in the presence of spermidine to the absence of spermidine for the wild type, aa62, aa119, and aa273 cysteine to serine mutant NF-κB p50 aa35-381 proteins were 0.60, 2.21, 0.85, and 0.77 respectively.

However, the absolute values for the dissociation rate constants for the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins in the absence of spermidine were approximately 10-fold lower than in its presence. Also, in the absence of spermidine it was now possible to determine an approximate value for the dissociation rate constant for the aa62 cysteine to serine p50 mutant protein of approximately 0.35 s^{-1}, this suggests that the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein also has a significantly higher association rate constant than the other protein species in the absence of spermidine.

Chapter 4. Demonstration of a change in binding site specificity for the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

4.1 Competition of DNA binding activity by non-specific competitor DNA

Bearing in mind the above changes in the dissociation constants and kinetic behaviour of the cysteine to serine mutant p50 proteins towards the 16-mer κB motif oligonucleotide, it seemed possible that the DNA binding site specificity of the mutant p50 proteins might have altered. To test this possibility, competition studies were carried out for all four p50 protein species with sheared double stranded *E.coli* DNA in the presence of spermidine with ^{32}P radiolabelled HIV-L κB motif oligonucleotide as the probe. From an examination of gel electrophoresis DNA binding assays carried out with roughly equal amounts of binding activity for the four p50 protein species in the presence of increasing amounts of non-specific *E.coli* DNA competitor (Figures 4.1, 4.2) it is clear that the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein-HIV-L κB motif oligonucleotide complex is competed much more readily than the DNA-protein complexes involving the other three protein species. When the amounts of radiolabelled DNA-protein complex remaining in the above *E.coli* DNA competition experiments are plotted versus the amount of competitor DNA used (Figure 4.3), it seems clear that while the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins all show similar competition behaviour, the aa62 cysteine to serine p50 mutant is competed much more easily by the *E.coli* DNA . The fact that the aa119 and aa273 cysteine to serine mutant p50 proteins behave very similarly to the wild type protein suggests that even though their affinity for the κB motif is slightly reduced, their binding site specificity remains very similar, if not identical, to that of the wild type NF-κB p50 aa35-381 protein.

Figure 4.1 Gel electrophoresis DNA binding assays for E.coli DNA competition of wild type and aa62 cysteine to serine mutant p50 protein-HIV-L κB motif oligonucleotide complexes.

Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated amounts of competitor E.coli double stranded DNA before addition of the HIV-L κB motif oligonucleotide probe. B and F indicate the positions of the DNA-protein complex and free oligonucleotide bands respectively.

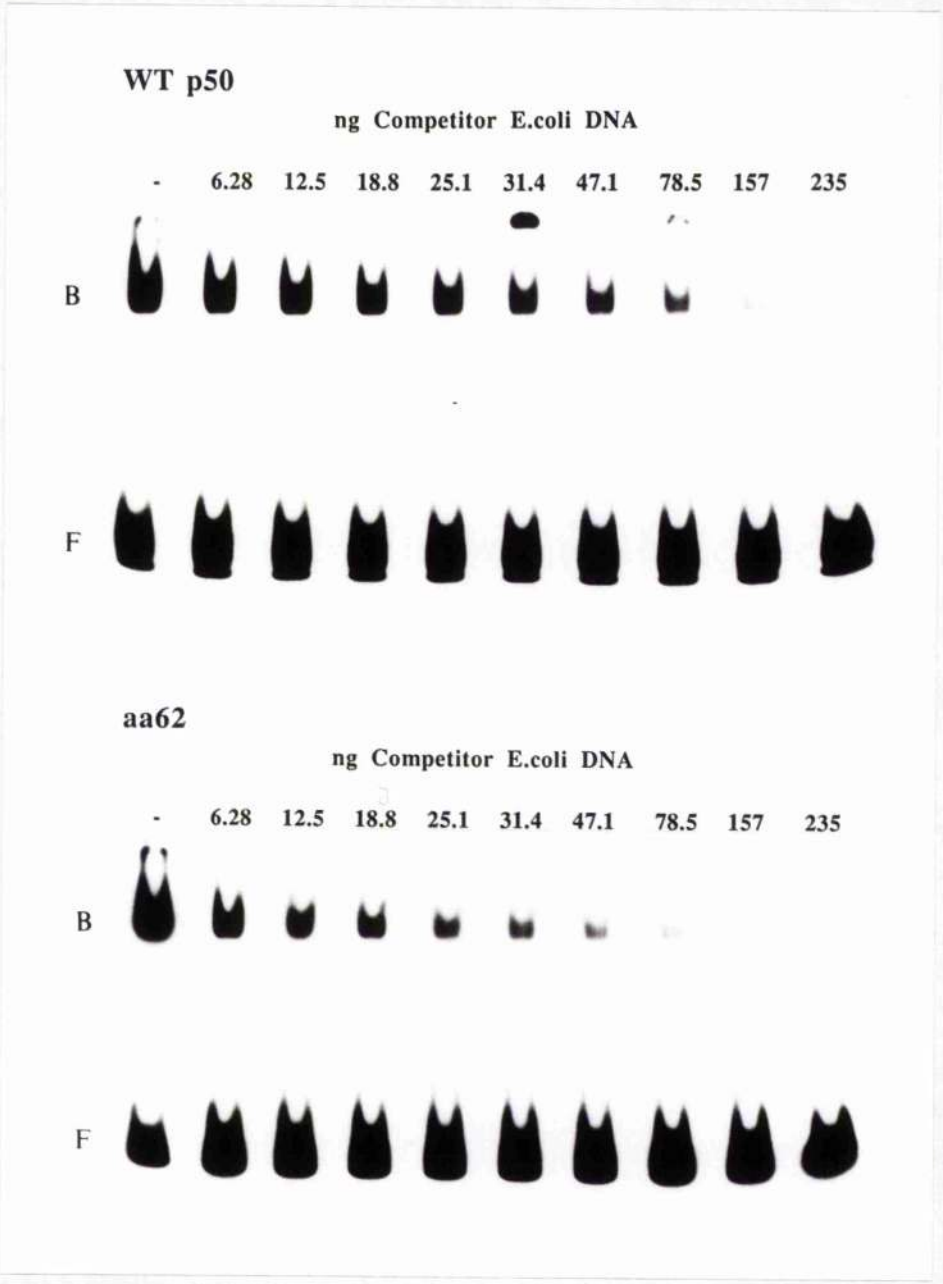


Figure 4.2 Gel electrophoresis DNA binding assays for E.coli DNA competition of aa119 and aa273 cysteine to serine mutant p50 protein-HIV-L κB motif oligonucleotide complexes.

Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated amounts of competitor E.coli double stranded DNA before addition of the HIV-L κB motif oligonucleotide probe. B and F indicate the positions of the DNA-protein complex and free oligonucleotide bands respectively.

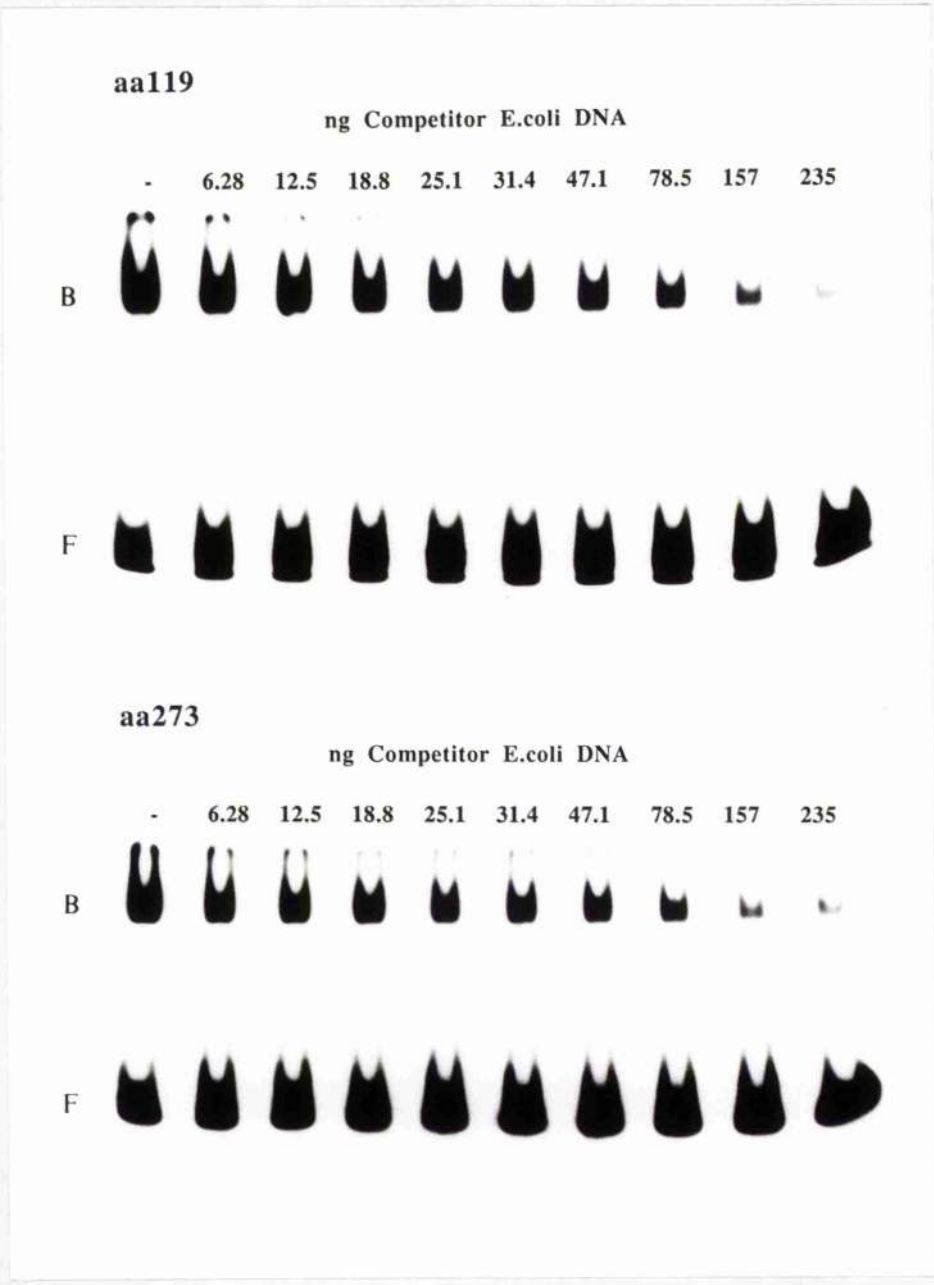
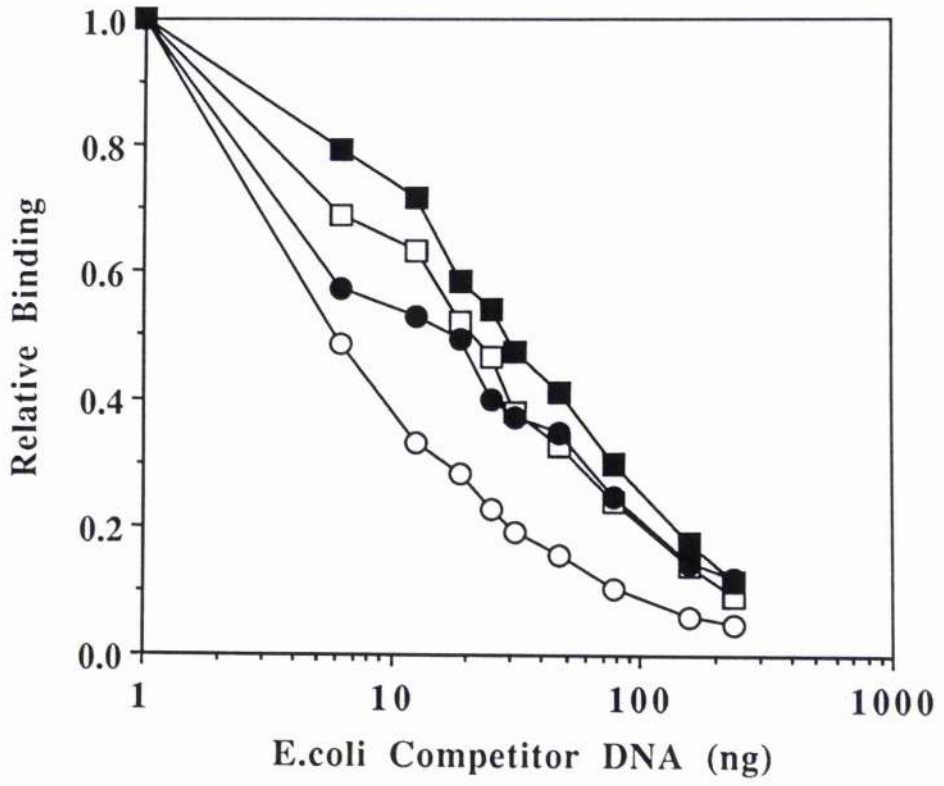


Figure 4.3 Non-specific E.coli DNA competition plot for the four NF-κB p50 aa35-381 protein species.

The relative amounts of HIV-L κB motif oligonucleotide DNA binding activity remaining under standard assay conditions in the presence of increasing amounts of non-specific E.coli double stranded DNA competitor are plotted for the four NF-κB p50 aa35-381 protein species. The DNA binding activities for the four p50 protein species in the absence of any competitor DNA were normalised, and this data point plotted equivalent to 1ng competitor DNA. Open squares represent wild type p50 protein, open circles represent the aa62 cysteine to serine mutant p50 protein, solid squares represent the aa119 cysteine to serine mutant p50 protein, and solid circles represent the aa273 cysteine to serine mutant p50 protein.



4.2 Change in DNA binding specificity of the aa62 cysteine to serine mutant p50 protein

Having some evidence from the *E.coli* DNA competition experiments that the DNA binding specificity of the aa62 cysteine to serine mutant p50 protein had altered, it was decided to test this directly, and to try to gain some insight about the nature of the new specificity. Thus both the wild type and aa62 cysteine to serine mutant p50 proteins in the presence of spermidine under the standard binding conditions were assayed for the ability of various oligonucleotide variants of the κB motif to compete for binding of the double stranded 16-mer κB motif oligonucleotide. The sequences of these competitor oligonucleotides are given in Table 4.1, with the κB-like motif indicated in bold.

Plots of the relative amounts of radioactive DNA-protein complex remaining versus the dilution factor - defined as (-(Molarity of probe oligo/(Molarity of probe oligo + Molarity of competitor oligo)) +1) should yield a straight line of gradient = -1 when the radiolabelled oligonucleotide probe is competed with identical unlabelled oligonucleotide. In contrast, when the competitor oligonucleotide represents a poorer binding site for p50, the data points will fall above the straight line - i.e. the molar excess of unlabelled competitor oligonucleotide needed to reduce the amount of radiolabelled DNA-protein complex to 50% will be greater than 1. Whereas for a better competitor κB motif, the data points will fall below the straight line - i.e. the molar excess of competitor oligonucleotide needed to reduce the amount of radiolabelled DNA-protein complex to 50% will be less than 1.

Competition specificity experiments were carried out in the presence of

spermidine for the human immunodeficiency virus type 1 enhancer HIV-L κB motif (HIV-L), the κB motif variant from the human ß-interferon gene regulatory element (IRE), a virtually symmetrical variant of the κB motif (EBP 'cons'), the κB motif variant from the mouse H-2K^b enhancer (H2TF1), a mutant κB site with a sequence of 8 continuous purine residues (SVUP), two mutants of the κB motif which show virtually no binding of native eukaryotic NF-κB (SV1-M1, SV1-M3), and a more subtle κB motif mutant which still shows some binding to native NF-κB (SV1-M2). The sequences of these oligonucleotides are given in Table 4.1.

Plots of the relative amounts of radiolabelled DNA-protein complex remaining versus the dilution factor of the competitor oligonucleotide are given for all eight competitor species (Figures 4.4 - 4.11) and the estimated relative molarities (compared with the molarity of the oligonucleotide probe) of competitor oligonucleotide needed to reduce the amount of radiolabelled DNA-protein complex by 50% are given in Table 4.1. From these analyses several interesting points emerge :- for example competition of the double stranded 16-mer κB motif oligonucleotide by the HIV-L oligonucleotide might be expected to yield a straight line plot since both κB sites in the HIV-1 LTR enhancer have the same sequence - however as can be seen from Figure 4.4 and Table 4.1 the HIV-L oligonucleotide is clearly a slightly poorer competitor. One significant difference between these two oligonucleotides may be the number of base pairs the κB motif is from the end of the double stranded region - three base pairs for the 16-mer κB motif oligonucleotide and two base pairs for the HIV-L oligonucleotide. Given observations from the *E.coli lac* repressor-operator system (Winter et al., 1981) that dissociation constants rose significantly as operator DNA fragments became

Figure 4.4 Gel electrophoresis DNA binding assays for HIV-L oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled HIV-L competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for HIV-L oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

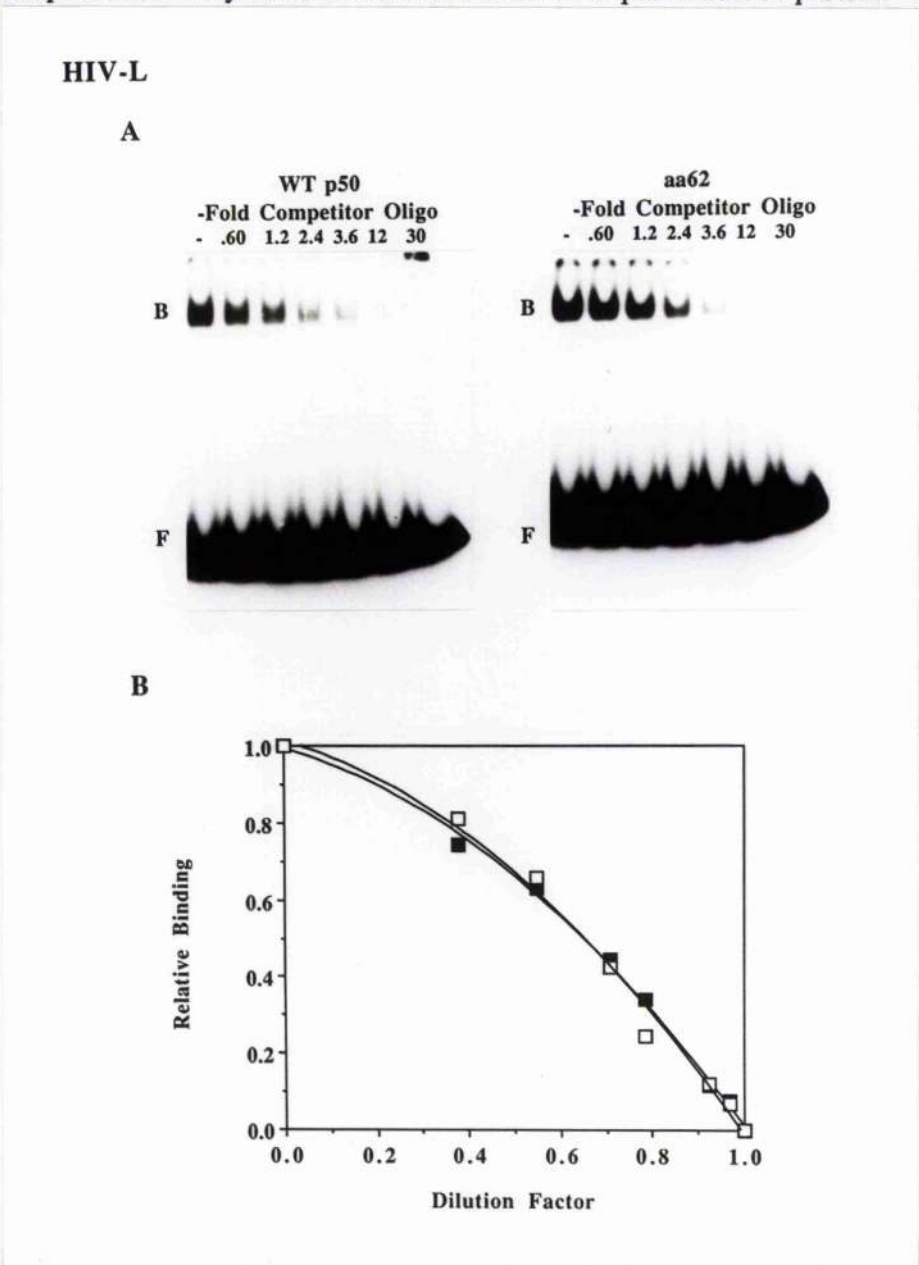


Figure 4.5 Gel electrophoresis DNA binding assays for IRE oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled IRE competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for IRE oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

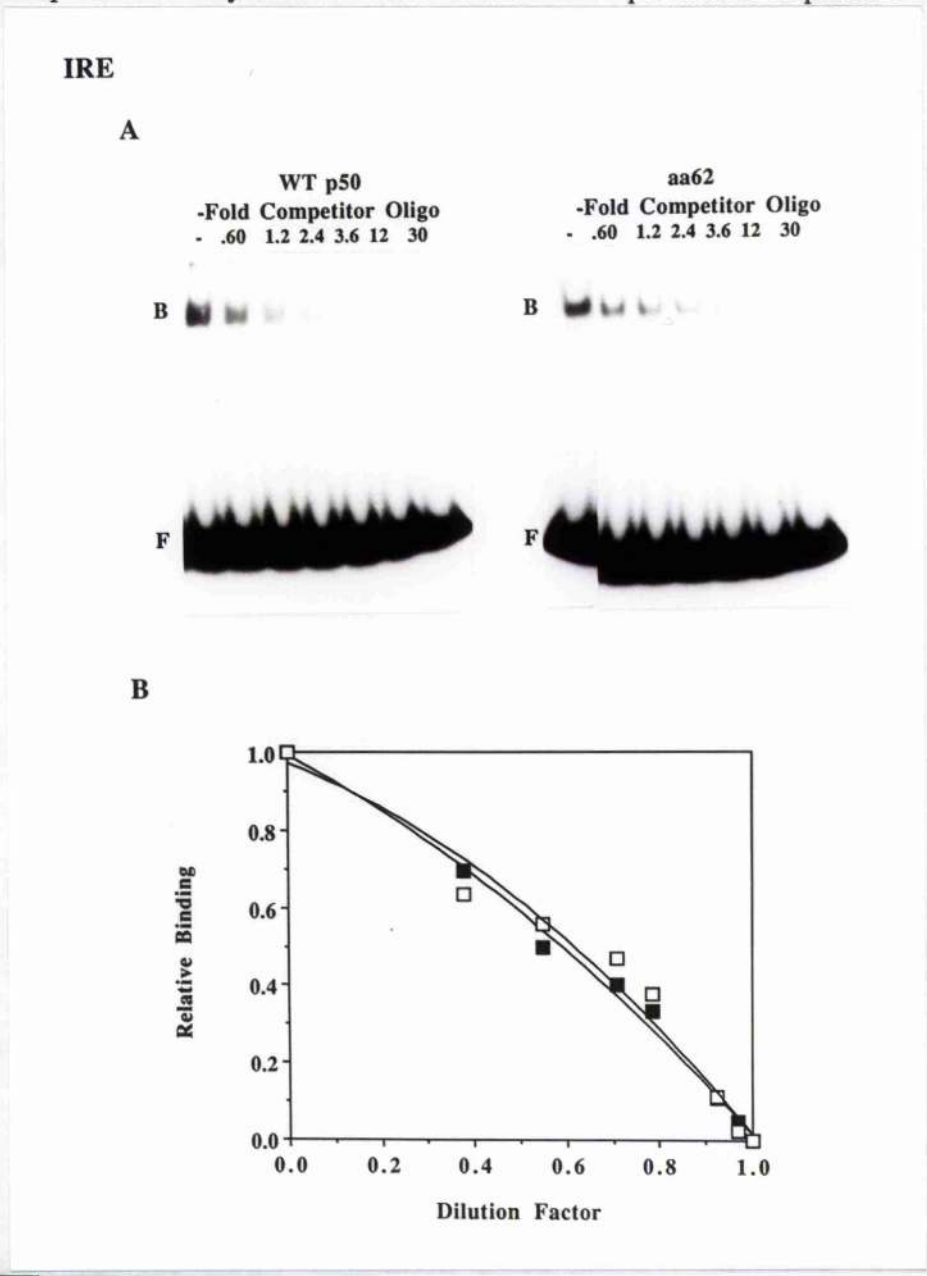
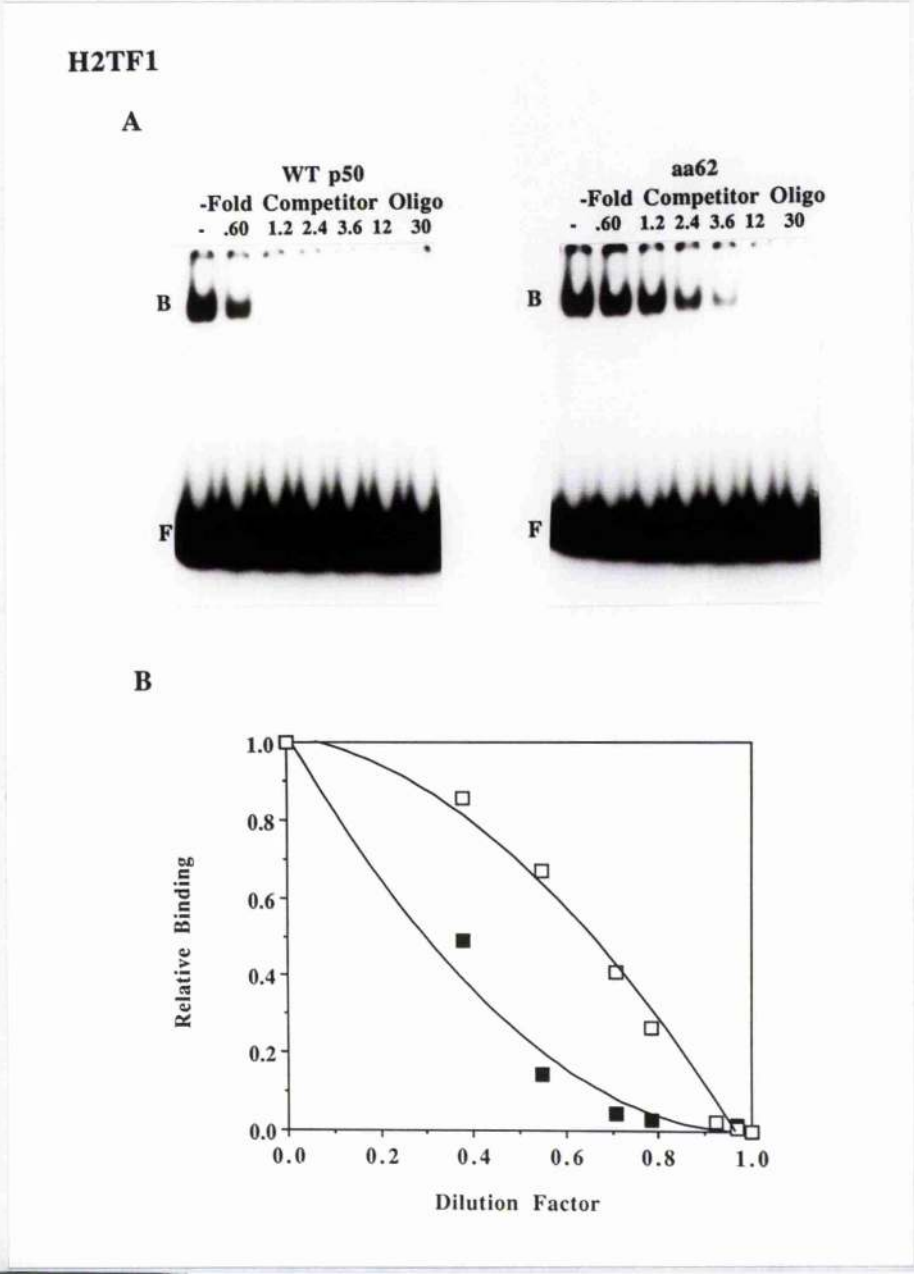


Figure 4.6 Gel electrophoresis DNA binding assays for H2TF1 oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled H2TF1 competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for H2TF1 oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.



shorter, it is likely that similar effects might occur if the κB motif is located near the end of the oligonucleotide. However, the above arguments should not prevent valid comparisons of the binding affinity of wild type and aa62 cysteine to serine mutant p50 proteins for each competitor oligonucleotide. As would be expected for the HIV-L oligonucleotide (Figure 4.4), the competition plots for the wild type and aa62 mutant p50 proteins coincide, giving identical relative molarities of competitor oligonucleotide for 50% binding (Table 4.1).

From the competition plots for the IRE type of κB motif oligonucleotide (Figure 4.5, Table 4.1), it can be seen that the two curves for wild type and aa62 cysteine to serine mutant proteins virtually coincide - suggesting no significant difference in binding specificity between the two proteins towards the IRE type of κB motif.

In contrast, for the wild type p50 protein, the H2TF1 type of κB motif (Figure 4.6, Table 4.1) competes significantly better than the 16-mer κB motif, while for the aa62 mutant p50 protein, the H2TF1 type of κB motif is a slightly poorer competitor than the 16-mer κB motif oligonucleotide - this difference constitutes evidence of the aa62 mutant p50 protein having an altered DNA binding specificity. In the case of the EBP'cons' type of κB motif, for the wild type p50 protein this motif is a significantly better competitor than the 16-mer κB motif, and while the aa62 mutant p50 protein has a significantly lower affinity for this EBP'cons' sequence than the wild type p50 protein, this sequence is still a somewhat better competitor than the 16-mer κB motif oligonucleotide (Figure 4.7, Table 4.1). The SVUP κB motif variant containing eight sequential purine residues shows interesting competition behaviour (Figure 4.8, Table 4.1) -

Figure 4.7 Gel electrophoresis DNA binding assays for EBP'cons' oligonucleotide competition and binding site specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled EBP'cons' competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for EBP'cons' oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

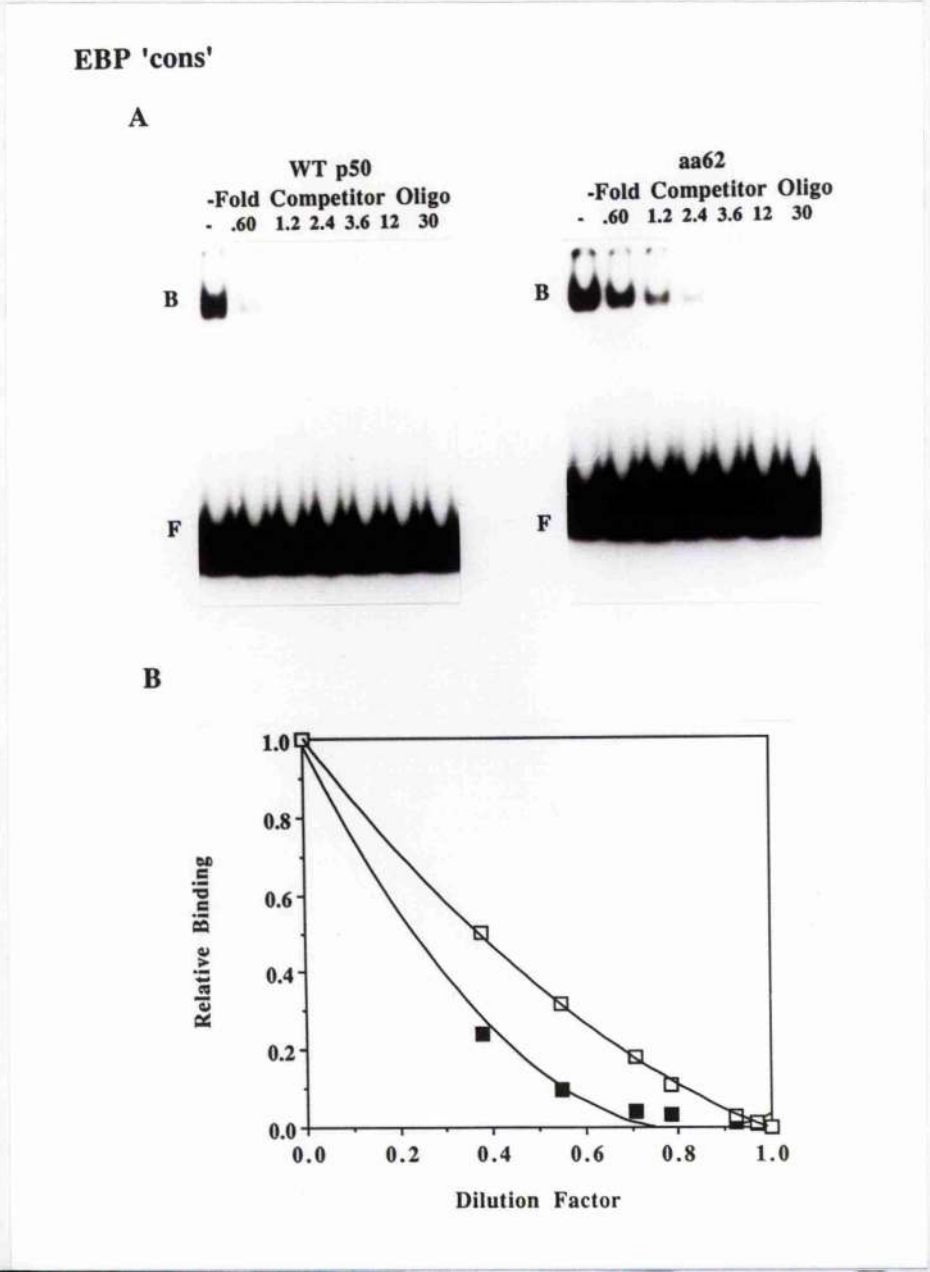
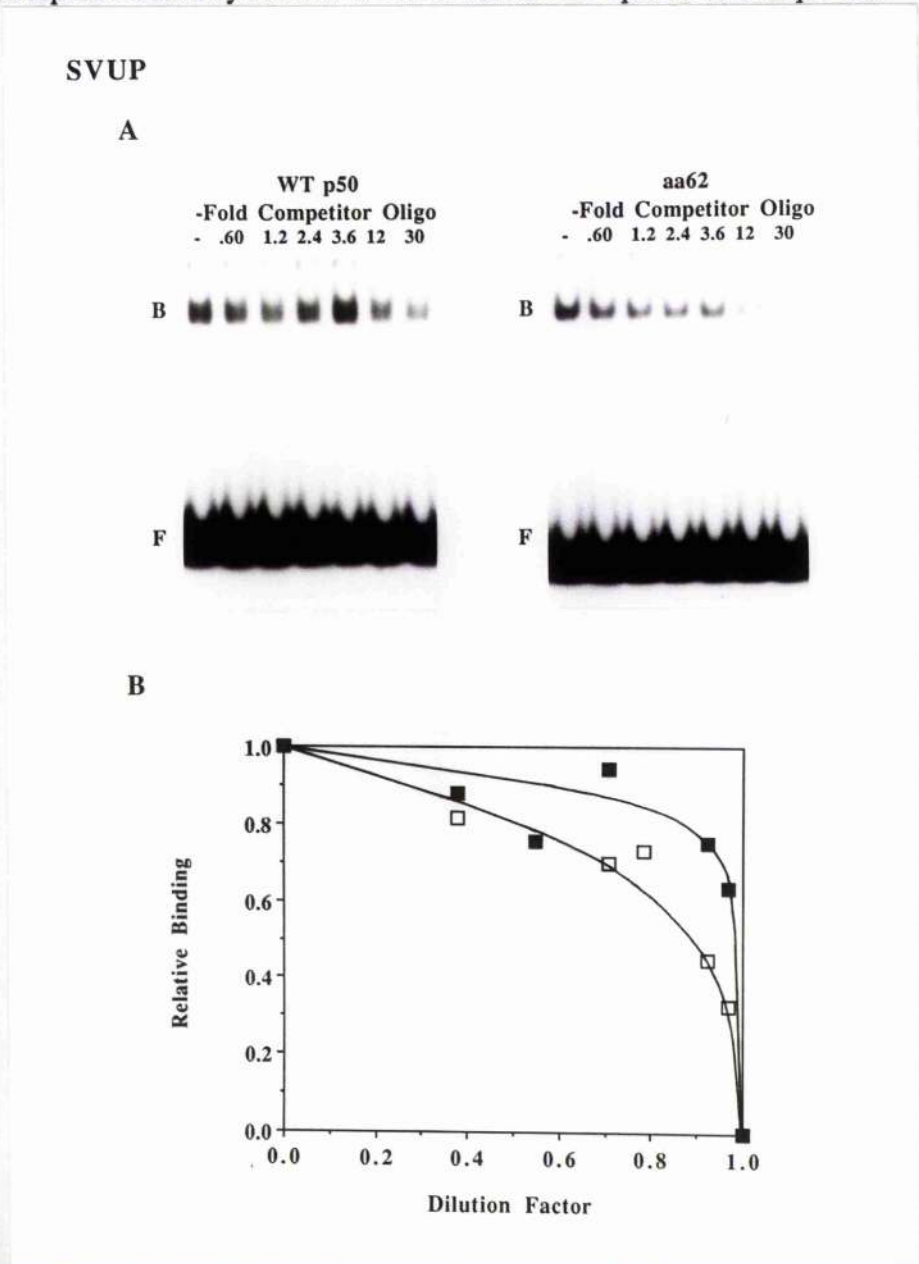


Figure 4.8 Gel electrophoresis DNA binding assays for SVUP oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled SVUP competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for SVUP oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.



although this sequence is a fairly poor competitor for both p50 protein species compared to the 16-mer κB motif oligonucleotide, it does seem to show a higher binding affinity towards the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

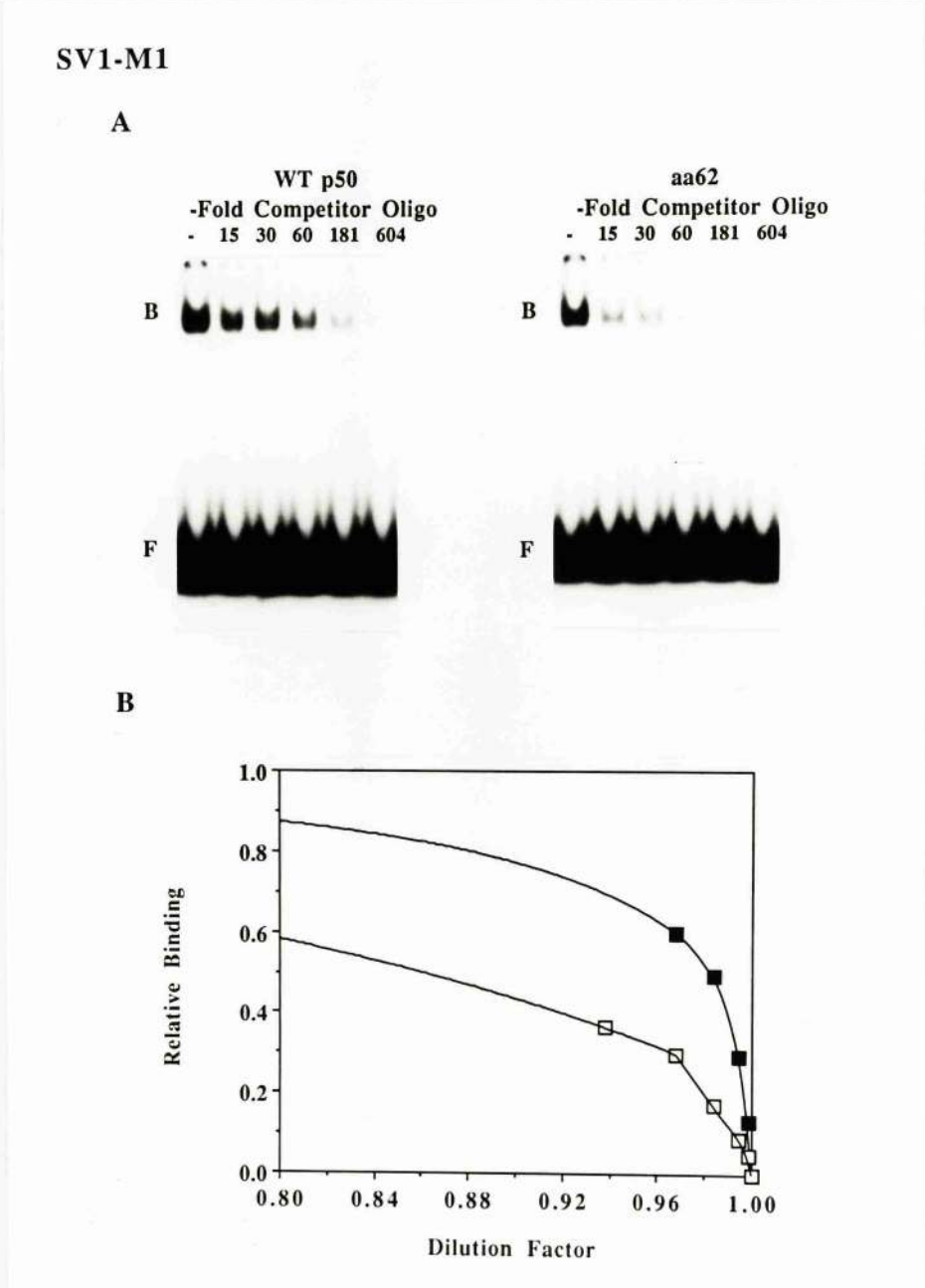
The three mutant κB motif oligonucleotides SV1-M1, SV1-M2, and SV1-M3 all show similar behaviour (Figures 4.9, 4.10, and 4.11, Table 4.1) - in that they are poor competitors for binding of both the wild type and aa62 cysteine to serine mutant p50 proteins, but again showing higher affinities towards the aa62 cysteine to serine mutant p50 protein.

The DNA binding assays in the above oligonucleotide competition experiments were all performed in the presence of the polyamine spermidine - which as demonstrated in Results, Chapter 3 causes an increase in the dissociation rate constants of the DNA-protein complexes (and which presumably also causes roughly corresponding changes in the association rate constants). One well-known effect of spermidine is to promote changes in the conformation of double stranded DNA which can be either profound such as the switch from the B-DNA to the Z-DNA conformation (Rich et al., 1984), or subtle - such as promoting or inhibiting transient breakdowns in base pairing (Plum and Bloomfield, 1990). To investigate if there was an effect of spermidine on some or all of the types of κB motif, data from the above competition studies performed in the presence of spermidine were correlated with new DNA binding assays performed in the absence of spermidine. The results of these comparisons suggested that there were no significant differences in competition behaviour by a range of oligonucleotides in the presence or absence of spermidine (data not shown).

oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled SV1-M1 competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

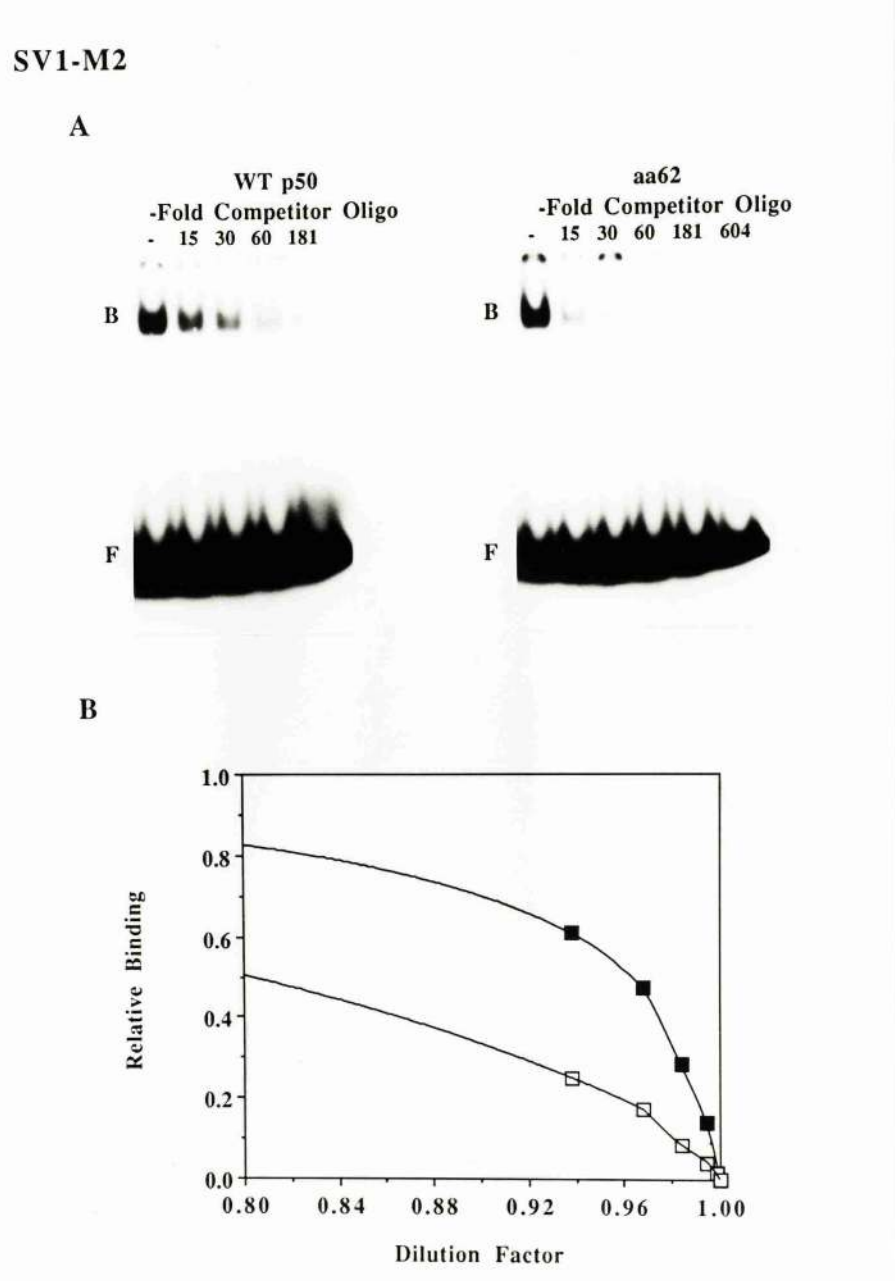
B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for SV1-M1 oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.



oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled SV1-M2 competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for SV1-M2 oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.



oligonucleotide competition and binding specificity analysis for wild type and aa62 mutant p50 proteins.

A. Gel electrophoresis DNA binding assays were carried out under standard conditions (see Materials and Methods) in the presence of spermidine with the addition of the indicated molar excess of unlabelled SV1-M3 competitor oligonucleotide before addition of the double stranded 16-mer κB motif oligonucleotide probe. Approximately equal amounts of wild type and aa62 mutant p50 protein DNA binding activity were used. B and F indicate the positions of the DNA-protein complex and free oligonucleotide probe bands respectively.

B. Plot of relative amount of radiolabelled DNA-protein complex remaining versus dilution factor for SV1-M3 oligonucleotide, solid squares represent wild type, and open squares represent aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein.

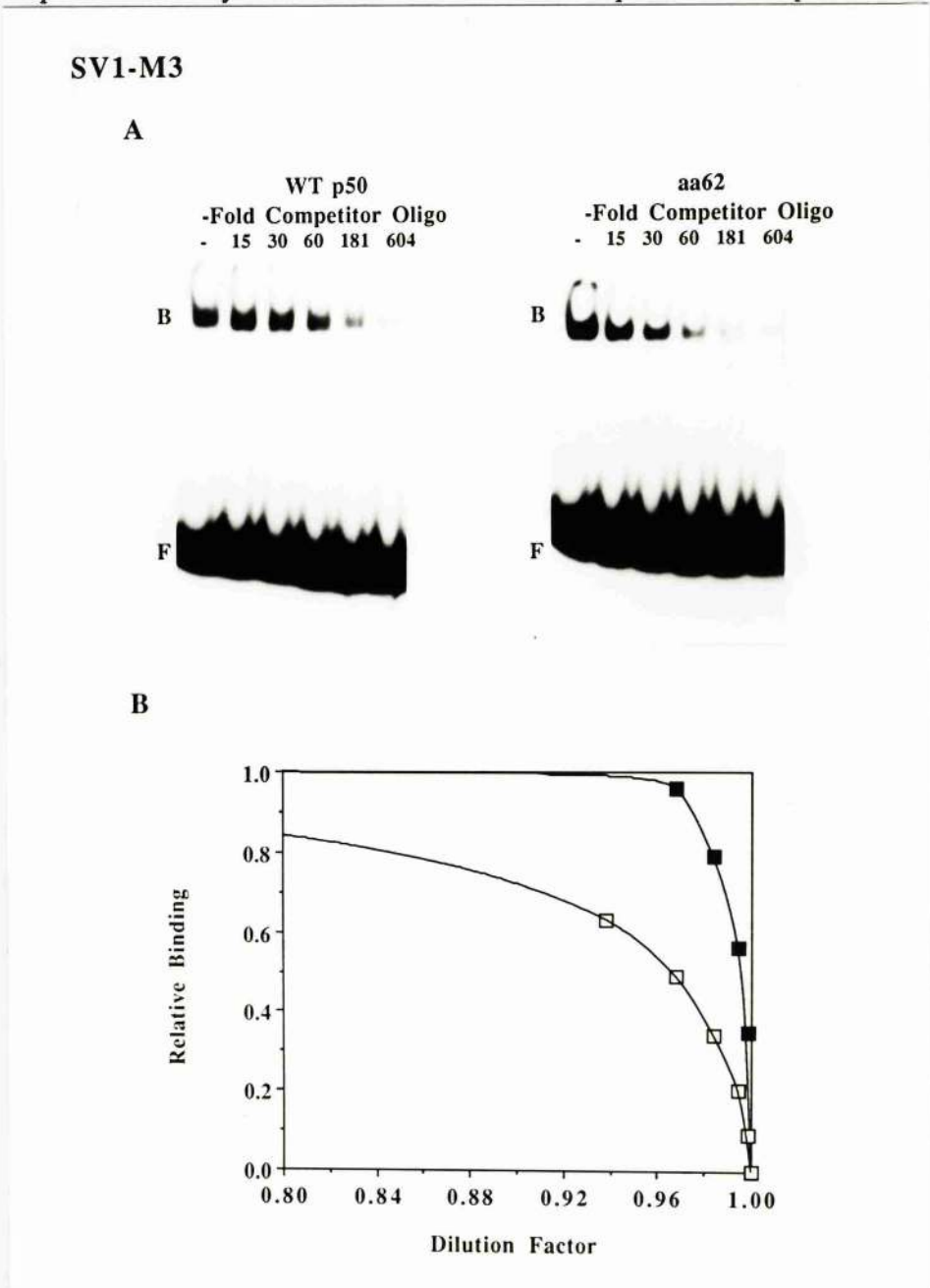


Table 4.1 Sequences of competitor oligonucleotides and relative molarities of competitor oligonucleotide needed to reduce the amount of radiolabelled DNA-protein complex by 50%.

Oligo	Nucleotide Sequence	Rel.Molarity 50% Binding	
		W.T.p50	aa62 p50
16bp WTκB	5' CTG **GGGACTTTCC** AGG 3' 3' GAC **CCCTGAAAGG** TCC 5'	-	-
HIV-L	5' GATCTA **GGGACTTTCC** GCG 3' 3' AT **CCCTGAAAGG** CGCCTAG 5'	1.81	1.81
IRE	5' GATCAAAGT **GGGAAATTCC** TCTG 3' 3' TTTCA **CCCTTTAAGG** AGACCTAG 5'	1.35	1.35
H2TF1	5' GATCT **GGGGATTCCCC** AG 3' 3' A **CCCCTAAGGGG** TCCTAG 5'	0.40	1.96
EBP'cons'	5' GATCATG **GGGAATTTCCC** CAG 3' 3' TAC **CCCTTAAAGGG** GTCCTAG 5'	0.30	0.58
SVUP	5' GATCTGAGGC **GGAAAGAACC** AGCTG 3' 3' ACTCCG **CCTTTCTTGG** TCGACCTAG 5'	39.0	9.64
SV1-M1	5' GATCTAGGGTGT **CCAAAGTCCC** G 3' 3' ATCCCACA **GGTTTCAGGG** CCTAG 5'	54.5	6.25
SV1-M2	5' GATCTAGGGTGT **GGAATGTCCC** G 3' 3' ATCCCACA **CCTTACAGGG** CCTAG 5'	25.3	4.00
SV1-M3	5' GATCTAGGGTGT **GGAAAGTGGC** CG 3' 3' ATCCCACA **CCTTTCACCG** GCCTAG 5'	199	27.6

Chapter 5. Direct demonstration of the presence of cysteine 62 in the DNA binding site of NF-κB p50 aa35-381, and regulation of DNA binding activity by reduction of a disulphide bond involving cysteine 62.

5.1 Protection of wild type NF-κB p50 aa35-381 DNA binding activity against iodoacetate inactivation by prior formation of a DNA-protein complex

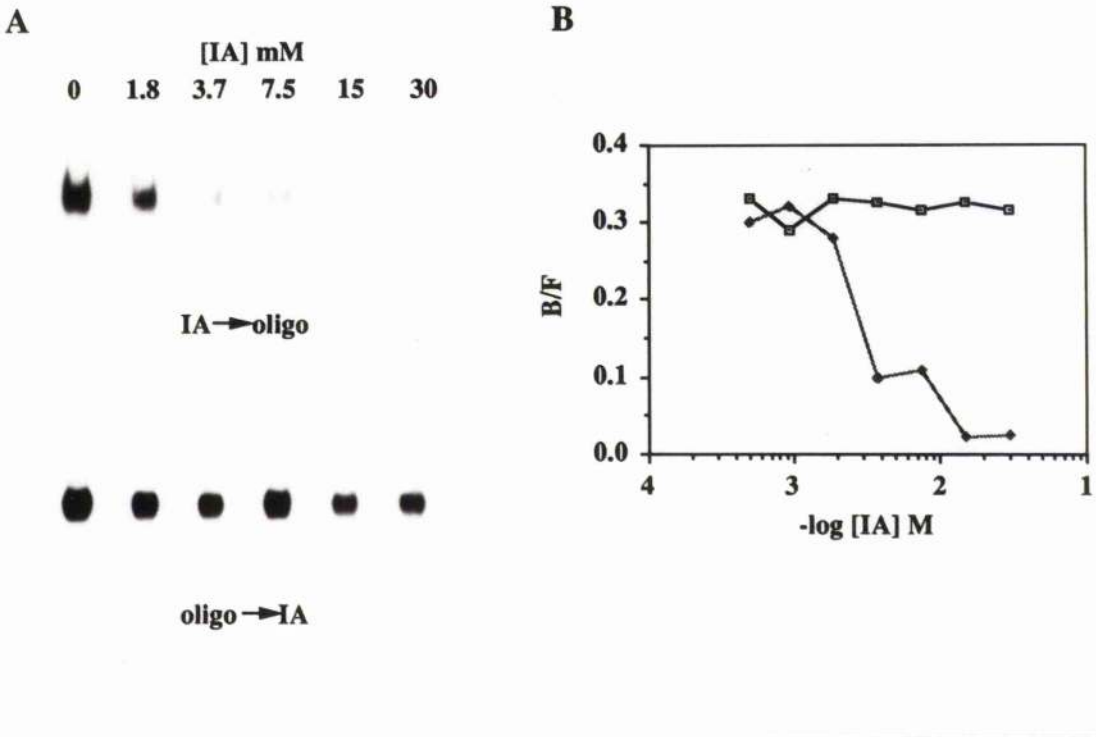
Following the demonstration in a study of redox regulation of the DNA-binding activity of the fos-jun heterodimeric transcription factor that incubation of the heterodimer with an oligonucleotide containing an AP-1 binding site prior to treatment with N-ethylmaleimide protected the DNA binding acivity from inactivation (Abate et al., 1990), a similar type of protection experiment was attempted with the wild type NF-κB p50 aa35-381 protein. Thus gel electrophoresis DNA binding assays were performed under standard conditions with fully reduced (by treatment with DTT to 25mM / incubating on ice 15 minutes before addition to the binding mixture) wild type p50 protein with increasing iodoacetate concentrations / incubation at 20°C for 15 minutes with addition of the iodoacetate to the indicated concentration both before (Figure 5.1, Panel A, upper section) and after (Figure 5.1, Panel A, lower section) addition of the radiolabelled 16-mer κB motif oligonucleotide probe. Dose-response curves for a similar experiment for iodoacetate inactivation by addition both before and after addition of the 16-mer κB motif oligonucleotide probe are shown in Figure 5.1, Panel B.

It is clear that when the wild type p50 protein was incubated with iodoacetate

Figure 5.1 Gel electrophoresis DNA binding assays for wild type p50 protein with addition of iodoacetate before and after addition of the double stranded 16-mer κB motif oligonucleotide.

A. DNA-protein complex bands from gel electrophoresis DNA binding assays carried out under staandard conditions (see Materials and Methods) in the presence of spermidine using wild type p50 protein with addition of iodoacetate to the indicated concentration and incubation at 20°C for 15 minutes before (upper panel) and after (lower panel) formation of the DNA-protein complex.

B. Dose-response curves for the amount of radiolabelled DNA-protein complex using wild type p50 protein with addition of iodoacetate to the indicated concentration before (solid diamonds) and after (open squares) formation of the DNA-protein complex.



prior to addition of the κB motif oligonucleotide, then a dose-dependent decrease in DNA binding activity was seen - however when the DNA-protein complex was allowed to form before the addition of the iodoacetate, then DNA binding activity was essentially unaltered even in the presence of 30mM iodoacetate. The observation that both iodoacetate and N-ethylmaleimide cause inhibition of p50 DNA binding activity strengthens the case for the involvement of cysteine residues in the redox modulation of p50 protein DNA binding activity, but more importantly (as in the case of the fos-jun heterodimer - Abate et al., 1990) protection against iodoacetate inactivation by preforming a DNA-p50 protein complex implies that these cysteine residues are intimately involved in binding to the κB motif.

5.2 Iodoacetate sensitivity of wild type and mutant NF-κB p50 aa35-381 proteins

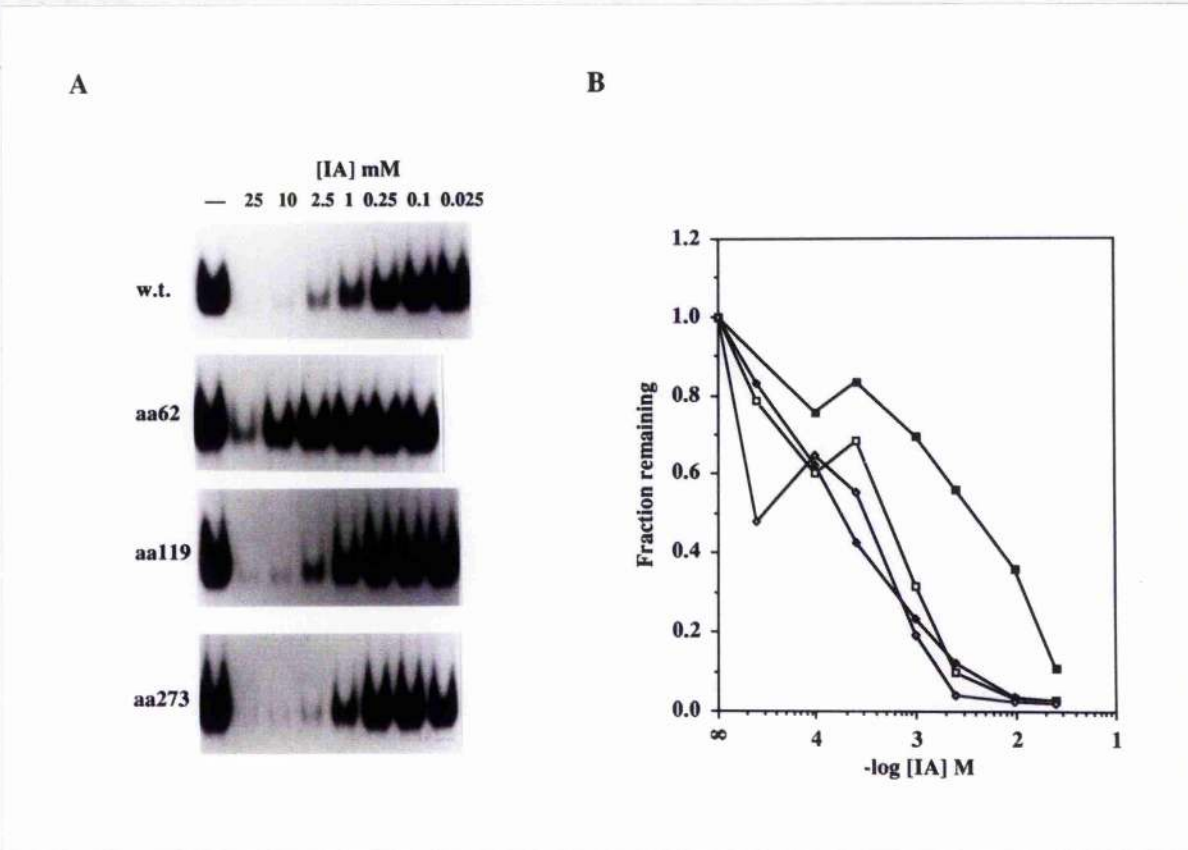
To identify the cysteine residue(s) located in the p50 DNA binding site, wild type and aa62, aa119, and aa273 cysteine to serine mutant p50 proteins were treated with increasing concentrations of iodoacetate before addition of the 16-mer κB motif oligonucleotide probe and the specific DNA binding activity remaining determined using the gel electrophoresis DNA binding assay. From the amounts of specific DNA-protein complex remaining (Figure 5.2, Panel A) over the range of iodoacetate concentrations studied, it was clear that the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins all showed similar titration behaviour, while the aa62 cysteine to serine p50 mutant was considerably less sensitive towards iodoacetate inactivation.

When the fractions of radioactive DNA-protein complex remaining (determined

Figure 5.2 Gel electrophoresis DNA binding assays for iodoacetate sensitivity of wild type and mutant p50 proteins.

A. DNA-protein complex bands from gel electrophoresis DNA binding assays carried out under standard conditions (see Materials and Methods) in the presence of spermidine with roughly equal amounts of κB specific DNA binding activity of all four p50 protein species, prestimulated with 25mM DTT / incubating 15 minutes on ice (wild type 2.1ng, aa62 5.7ng, aa119 2.9ng, aa273 4.2ng of p50 protein). Iodoacetate solution added to the indicated concentration, and the mixtures incubated at 20°C for 15 minutes, before addition of radiolabelled 16-mer κB motif oligonucleotide.

B. Dose-response curves for iodoacetate inactivation of the four p50 protein species, the amounts of radioactive DNA-protein complex in the absence of any iodoacetate treatment were normalised and the amounts of radioactive DNA-protein complex formed after iodoacetate treatment expressed relative to this. The symbols used for the various p50 protein species were :- solid diamond wild type p50, solid square aa62 cysteine to serine mutant p50, open square aa119 cysteine to serine mutant p50, open diamond aa273 cysteine to serine mutant p50 protein.



by scintillation counting) were plotted versus iodoacetate concentration (Figure 5.2, Panel B), the much lower iodoacetate sensitivity of the aa62 cysteine to serine p50 mutant was apparent. Thus, at an iodoacetate concentration of 2.5mM, the DNA-binding activity of the wild type, aa119, and aa273 mutant p50 proteins was reduced by approximately 90%, whereas the DNA binding activity of the aa62 cysteine to serine mutant was reduced by only 40%. The iodoacetate concentration needed to reduce DNA binding activity by 50% was 10-15 fold higher for the aa62 cysteine to serine mutant than for the other three NF-κB p50 subunit aa35-381 constructs.

These findings when coupled with the observations from previous chapters on the stimulation of κB-specific DNA binding activity by reducing agents, the weaker binding affinity of the aa62 cysteine to serine mutant for the κB motif, and the altered DNA binding specificity of the aa62 cysteine to serine mutant p50 protein all suggest that cysteine 62 is intimately associated with the DNA binding site, and is the site of redox regulation, of NF-κB p50 aa35-381.

5.3 ^{14}C Iodoacetate affinity labelling of cysteine 62 in the DNA binding site of wild type NF-κB p50 aa35-381

Although the above evidence was very suggestive of the intimate association of cysteine 62 with the DNA binding site of p50, more direct evidence was needed for a definitive assignment. The approach chosen was of differential labelling - a method first used to radiolabel the active site of an antibody (Koshland et al., 1959). The rationale behind this choice was that since preformation of the DNA-protein complex could protect the cysteine residue(s) in the DNA binding site of p50 against iodoacetate inactivation, reaction of the DNA-protein complex

with unlabelled iodoacetate should modify all the exposed cysteine thiol(ate) groups. Then, after removal of the excess unlabelled iodoacetate, the DNA-protein complex could be dissociated by increasing the NaCl concentration to 0.6M and the newly-exposed cysteine groups radiolabelled by reaction with ^{14}C iodoacetate. An outline of the ^{14}C iodoacetate differential labelling scheme used is shown in Figure 5.3 - this used HIV enhancer oligonucleotide-Sepharose resin to bind the wild type NF-κB p50 aa35-381 protein during the reaction with unlabelled iodoacetate, and allowed easy removal of the oligonucleotide binding site(s) from the reaction mixture.

The ^{14}C differential labelling (see Materials and Methods) of 100ug of wild type NF-κB p50 aa35-381 protein (Figure 5.4) illustrates some of the behaviour seen before with wild type p50 protein - thus comparing the amount of ≈38kD NF-κB p50 aa35-381 protein in the wash fractions (W1 and W2 - Figure 5.4, Panel A), it can be seen that there is no evidence of iodoacetate treatment of the preformed DNA-protein complex causing breakdown of the complex. From the autoradiogram (Figure 5.4, Panel B) of the SDS-PAGE gel, it is clear there is only one radiolabelled species (albeit as a doublet band) in the ^{14}C track. It seems likely that the higher molecular weight polypeptide bands present in all tracks in Panel A represent some contaminant proteins, possibly present in the SDS gel sample buffer since from other gels there was no evidence of their presence in the p50 protein used as the starting material, nor would they be expected to be present in the C30FT Centricon-30 flowthrough fraction when there was no evidence of the ≈38kD p50 protein in either the SDS-PAGE (Figure 5.4, Panel A) or gel electrophoresis DNA binding assays (Figure 5.4, Panel C).

Figure 5.3 Outline of ^{14}C iodoacetate labelling / substrate protection scheme for cysteine residues in the DNA binding site of wild type NF-κB p50 aa35-381 protein.

Given below is the outline of the DNA binding site substrate protection scheme used for 100ug of wild type p50 protein, the estimated recovery of radiolabelled wild type p50 for trypsin digestion was ≈75ug.

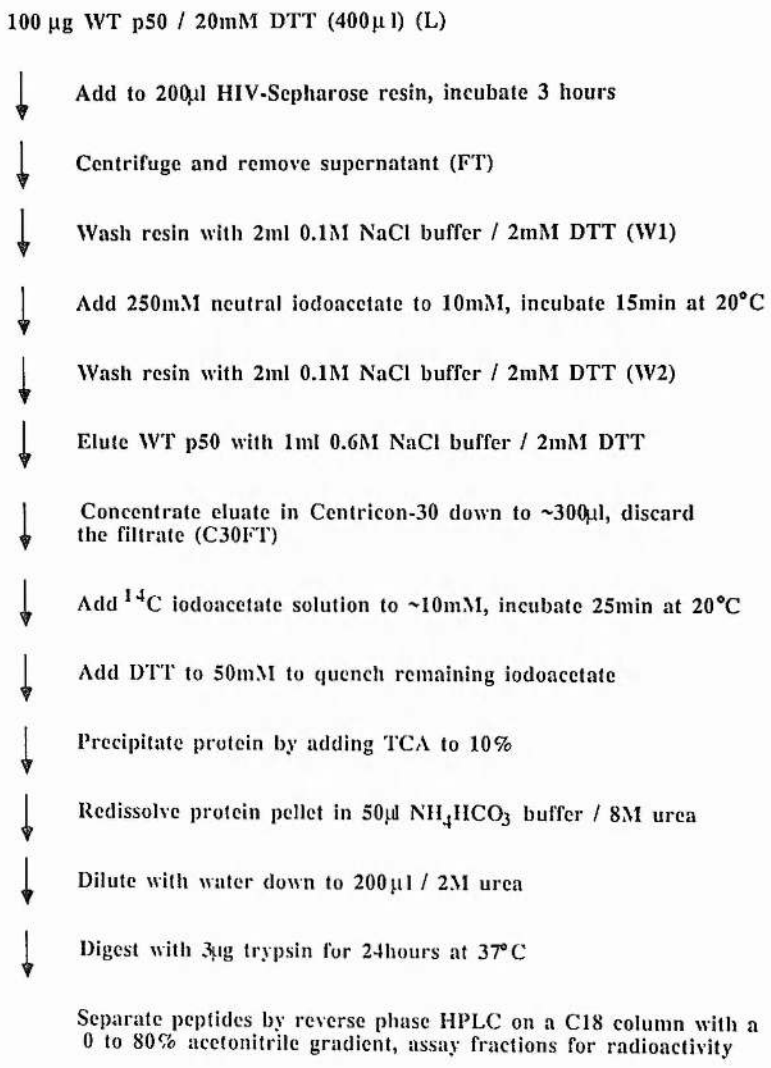
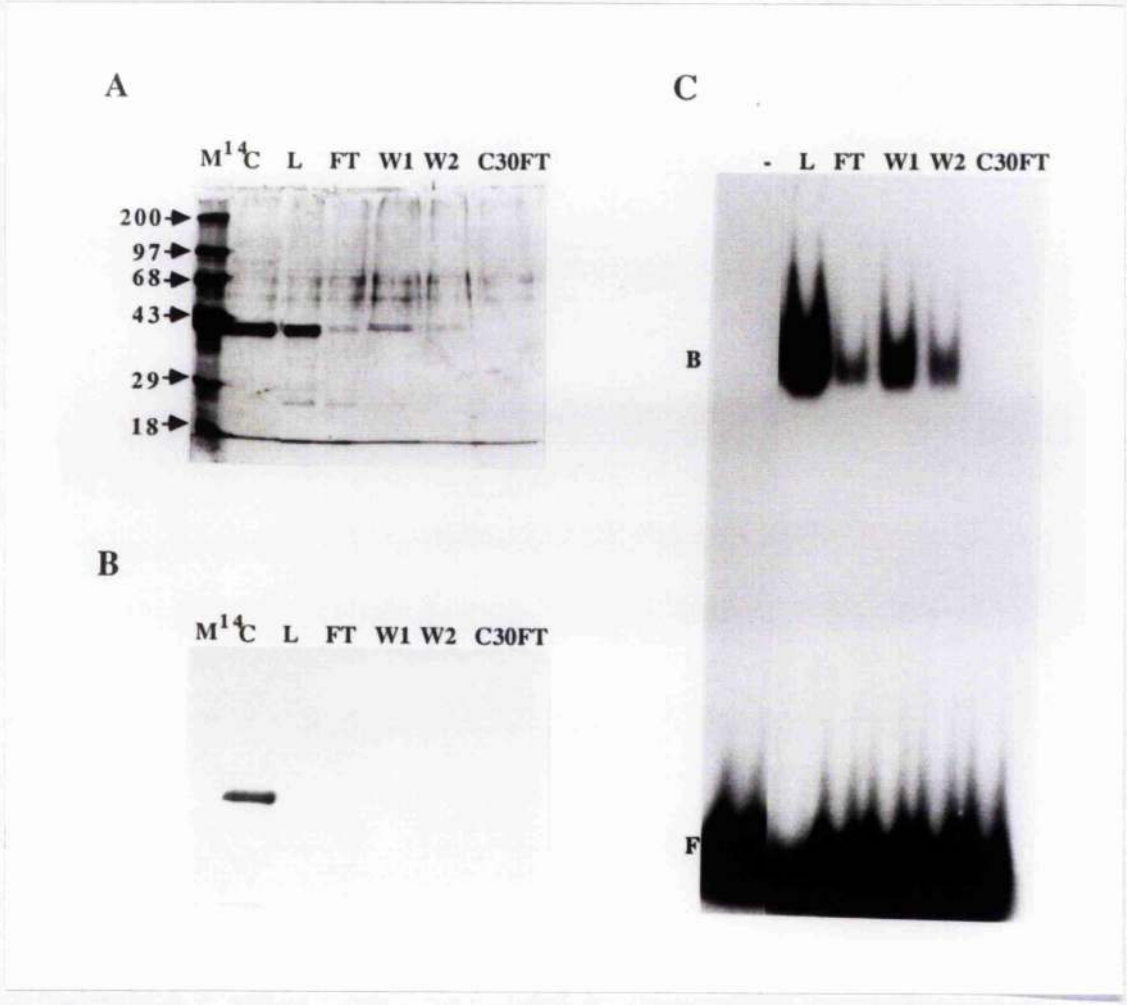


Figure 5.4 Analysis of fractions from the ^{14}C substrate protection labelling of cysteine residues in the DNA binding site of wild type NF-κB p50 aa35-381 protein.

A. SDS-PAGE analysis of ^{14}C substrate protection experiment (see Materials and Methods) fractions, visualised by Coomassie staining:- M, protein molecular weight standards; ^{14}C, final p50 protein eluate after ^{14}C labeling; L, initial load (100ug p50 protein, 400ul buffer) to HIV enhancer oligonucleotide-Sepharose affinity resin; FT, supernatant from loading p50 protein onto affinity resin; W1, first 0.1M NaCl wash of p50-loaded affinity resin; W2, second (after reaction of DNA-protein complex with unlabelled iodoacetate) 0.1M NaCl wash of p50-loaded affinity resin; C30FT, discarded Centricon-30 filtrate from concentration of eluted p50 protein prior to reaction with ^{14}C iodoacetate. The following volumes of protein fractions were used for the SDS-PAGE analysis ^{14}C - 8ul, L - 4ul, FT - 4ul, W1 - 20ul, W2 - 20ul, C30FT - 20ul. The molecular weights (in kD) of the marker proteins are indicated.

B. Direct autoradiogram of the above SDS-PAGE dried gel, abbreviations as in Panel A.

C. Gel electrophoresis DNA binding assays carried out under standard conditions (see Materials and Methods) in the presence of spermidine with ^{32}P radiolabelled HIV-L κB motif oligonucleotide. Abbreviations as used in Panel A, '-' indicates no protein addition, 'L' and 'FT' tracks used 1ul of protein solution, tracks 'W1', 'W2', and 'C30FT' used 5ul of protein solution. B and F indicate the positions of the DNA-protein complex and free oligonucleotide bands respectively.


A
M
14C
L
FT
W1
W2
C30FT
200
97
68
43
29
18
B
M
14C
L
FT
W1
W2
C30FT
C
-
L
FT
W1
W2
C30FT
B
F

The ^{14}C-labelled p50 protein, after quenching of the iodoacetate radiolabelling reaction with DTT (see Materials and Methods) was TCA precipitated - this had the advantage of removing unincorporated ^{14}C radioactivity without any need for dialysis - the p50 protein pellet was then redissolved in 50ul of 8M urea / ammonium bicarbonate buffer. The redissolved pellet was diluted with water to give a urea concentration of 2M, and then digested with 3ug of trypsin (i.e. a ≈25 : 1 substrate : trypsin ratio) at 37°C for 24 hours.

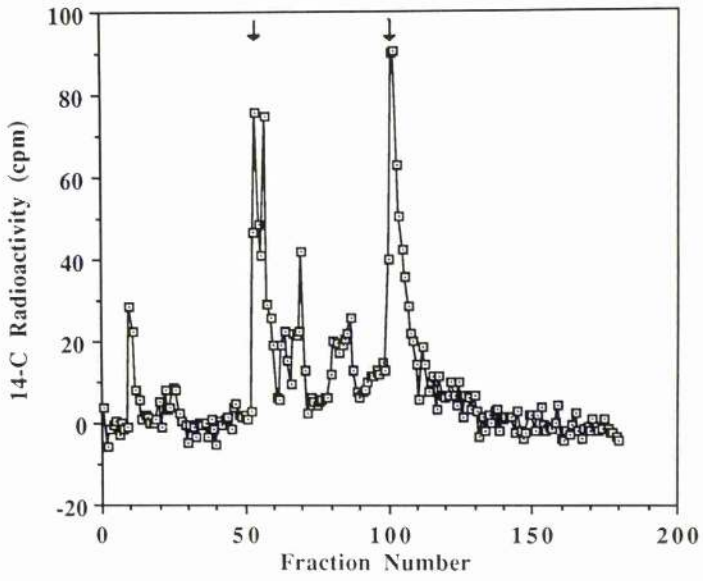
The trypsin-digested ^{14}C affinity labelled NF-κB p50 aa35-381 protein was then analysed by reverse phase HPLC chromatography using a Waters HPLC system with a Delta-Pak C18 column and a 0-80% acetonitrile gradient (see Materials and Methods). Peptides eluted from the reverse phase C18 column were collected over 180 x 0.25ml (30 second) fractions and 10ul aliquots of each fraction dotted onto (5mm x 5mm) squares of Whatman 3MM chromatography paper, after drying, the 3MM squares were immersed in 5ml of Ecoscint A and scintillation counted. The plot of ^{14}C radioactivity versus fraction number (Figure 5.5, Panel A) suggested a relatively simple pattern of radioactive peptides in the HPLC eluate. The two highest radioactivity eluate fractions - numbers 54 and 102 - were subjected to N-terminal amino acid sequencing (kindly performed by Dr.W.Kaszubska, Glaxo IMB, Geneva) this revealed (Figure 5.5, Panel B) that fraction number 54 contained three peptide species - the predominant one representing amino acids 60-69 of the NF-κB p50 aa35-381 protein (i.e. containing cysteine 62). The two minor peptides represented amino acids 158-163 (containing the non-conserved cysteine 162) and amino acids 195-197 of the NF-κB p50 aa35-381 protein. N-terminal amino acid sequencing of fraction 102

Figure 5.5 ^{14}C Radioactivity of eluate fractions from HPLC reverse phase chromatography of trypsin digested, DNA binding site labelled wild type NF-κB p50 aa35-381 protein, and amino acid sequence of peak fractions.

A. 10ul Aliquots of HPLC reverse phase chromatography eluate fractions from the 0-80% acetonitrile gradient spotted and dried on 3MM paper before liquid scintillation counting of ^{14}C radioactivity. The positions of the two peak radioactive fractions - numbers 54 and 102 - are indicated by arrows.

B. Amino acid sequence analysis of material in the two peak radioactive HPLC eluate fractions numbers 54 and 102 (N-terminal amino acid sequence analysis kindly performed by Dr.W.Kaszubska, Glaxo IMB, Geneva). Fraction 54 contained a major peptide species corresponding to amino acids 60 to 69 of NF-κB p50 aa35-381, and two minor species - one of these corresponding to amino acids 158-163 of the p50 protein, the other to amino acids 195-197 of the p50 protein. Fraction 102 contained material whose N-terminal amino acid sequence analysis indicated that it originated from the N-terminus of NF-κB p50 aa35-381.

A



B

HPLC Fraction 54 1) Y V - E G P S ? G G

2) M T E A - I

3) E L I

HPLC Fraction 102 S P N M A L P G A ? G ? Y L Q ? Q

yielded sequence corresponding to the N-terminus of the NF-κB p50 aa35-381 protein - suggesting that it might represent undigested material.

Surprisingly, no radioactivity could be detected in the sequencing cycle for cysteine 62 (nor for the cysteine 162), however this might have been due to a low level of radiolabelling efficiency in this experiment. The ^{14}C iodoacetate affinity labelling experiment has since been repeated with an estimated incorporation of 95% of the theoretical amount of ^{14}C, and has yielded definitive sequence data with only two significant radioactive peptide peaks - one radioactive peptide containing cysteine 62, and with radioactivity detectable only at the cysteine position, the other peptide radiolabelled at a non-cysteine residue (data not shown). It seems clear that cysteine 62 (and no other cysteine species) is intimately associated with the DNA binding site of the NF-κB p50 aa35-381 protein.

5.4 Demonstration that the wild type, aa119, and aa273 cysteine to serine mutant NF-κB p50 aa35-381 proteins can exist as intermolecular disulphide linked dimers

Bearing in mind the presence of cysteine 62 in the DNA binding site of the wild type (and presumably the aa119 and aa273 cysteine to serine mutants) NF-κB p50 aa35-381 protein, it seemed reasonable from symmetry arguments that the corresponding cysteine 62' from the other subunit would occupy a similar position. To try to gain some information if these two residues were close together or far apart in the DNA binding site, all four p50 protein species were fully reduced by treatment with DTT and then incubated with the reagent diazenedicarboxylic acid *bis* [N, N'-dimethylamide] (diamide) - a sulphydryl oxidising agent which induces the formation of disulphide bonds if cysteine

residues are appropriately positioned (Kosower et al., 1969).

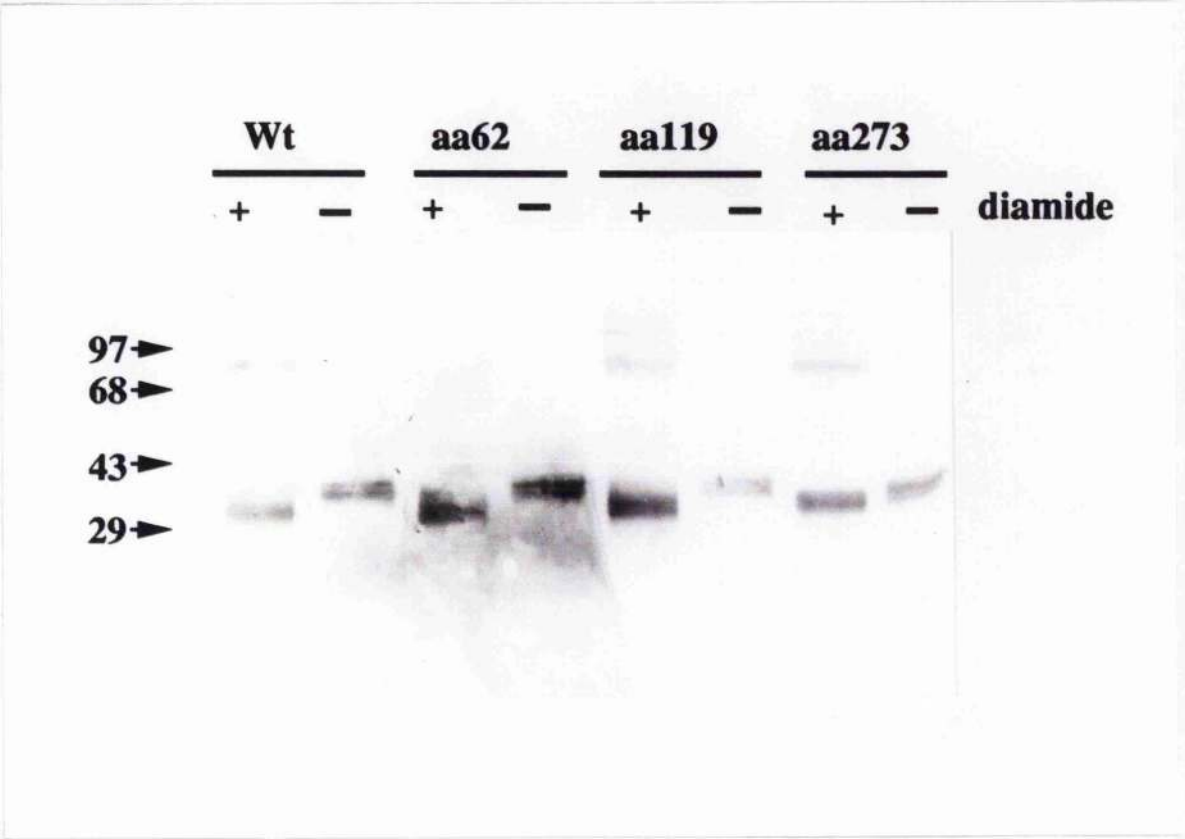
All four p50 protein species both with and without diamide treatment were then analysed by non-reducing SDS-PAGE and visualised by silver staining (Figure 5.6) - this revealed the presence of interchain disulphide links with the wild type, aa119, and aa273 cysteine to serine mutant p50 proteins, but no evidence of any interchain links with the aa62 cysteine to serine mutant NF-κB p50 aa35-381 protein. This finding indicated that the interchain disulphide bonds formed in the wild type protein must at least involve one amino acid 62 cysteine residue.

From symmetry arguments it seemed quite likely that cysteine 62 might form a disulphide bond with cysteine 62', however this experiment could not give a definitive answer to this question - in any case cysteine 62 does not form a disulphide bond with cysteine 119' or with cysteine 273' - since both the aa119 and aa273 cysteine to serine mutants can be interchain disulphide crosslinked by diamide treatment. However, as mentioned in Section 5.3, new affinity labelling results which demonstrate the incorporation of ^{14}C radioactivity into only the cysteine 62 residue lend weight to the idea of a cysteine 62-cysteine 62' interchain disulphide bond in the oxidised form of the wild type NF-κB p50 aa35-381 protein.

A further interesting observation from the diamide-treated tracks (Figure 5.6) is that the mobility of the monomer bands of all four p50 protein species is slightly faster after diamide treatment - which tends to suggest some intrasubunit disulphide bond formation. Since this behaviour is seen with all four NF-κB p50 aa35-381 species, the cysteines involved in this intrasubunit disulphide bond formation presumably belong to the non-conserved cysteine residues (at positions

Figure 5.6 Demonstration that the wild type, aa119, and aa273 cysteine to serine mutant NF-κB p50 aa35-381 proteins can exist as intermolecular disulphide linked dimers.

Equal amounts (≈1.04ug) of all four NF-κB p50 aa35-381 species fully reduced by treatment with DTT to 25mM and incubating on ice 15 minutes followed by either addition of (+) or no addition of (-) diamide to 33mM, incubation at 37°C for 1 hour, and quenching with excess iodoacetate (see Materials and Methods) were analysed by SDS-PAGE under non-reducing conditions, followed by silver staining of the gel. Positions of the molecular weight standard proteins (run under reducing conditions) are indicated.



88, 124, 162, and 262 of the NF-κB p50 subunit sequence (Kieran et al., 1990). The identification of which of these cysteines are involved in this intrasubunit crosslinking would require the individual mutagenesis of these non-conserved cysteine residues.

DISCUSSION.

This study has investigated the elements which a homodimer of the DNA binding and dimerisation region of the p50 polypeptide subunit of the transcription factor NF-κB uses to recognise and bind to the κB motif - a DNA regulatory element important in the transcriptional control of a wide range of cellular and viral promoters and enhancers. It has been shown that the DNA binding activity of native cellular κB-binding proteins and the bacterially expressed NF-κB p50 subunit amino acid 35-381 construct are regulated by their oxidation state, and that this regulation involves cysteine 62 forming a disulphide bond with a cysteine residue of the other subunit - probably with cysteine 62'. Further, the DNA binding specificity of the p50 aa35-381 amino acid 62 cysteine to serine mutant has been shown to have changed - the mutant protein has a 10-fold lower binding affinity for the κB motif - at least partly due to a much larger dissociation rate constant for this mutant protein compared to the wild type protein.

1. Characterisation and properties of native HeLa cell κB motif binding proteins

The initial aim of this work was the purification of the transcription factor NF-κB from HeLa cells to allow the eventual isolation of cDNA clones encoding this κB motif DNA binding activity, and the eventual overexpression of the factor to allow its use in studies of the initial transcriptional activation of the integrated HIV-1 provirus. Initial attempts to use HIV enhancer oligonucleotide-Sepharose affinity chromatography to isolate purified NF-κB were singularly unsuccessful - for example using four rounds of HIV enhancer oligonucleotide affinity

chromatography on HeLa cell nuclear extracts yielded a final eluate which appeared to contain at least 100 polypeptide species upon silver staining of SDS-PAGE gels (data not shown).

More sophisticated purification schemes (Results, Figure 1.2) involving multiple selection criteria yielded a much more satisfactory result - with two major polypeptide species of approximately 55 and 43kD molecular weight, and minor amounts of 140, 48, 36, and 34kD molecular weight polypeptides. However, since this final eluate material was still heterogeneous and contained only 1-2% of the initial κB-specific DNA binding activity, and with increasing evidence that the p50 and p65 subunits of NF-κB might belong to a family of related proteins, this purification route seemed unpromising as a source of material for amino acid sequence data, and was abandoned.

Unsuccessful attempts were also made during these studies to directly clone cDNAs encoding NF-κB subunits by screening a recombinant phage λgt11 cDNA expression library in *E.coli* with radiolabelled concatenated κB motif oligonucleotide. Although several putative positive phage clones were isolated, further characterisation suggested that the expressed fusion proteins also bound with high affinity to concatenated non-binding mutant variants of the κB motif - this was taken to indicate that the phages did not encode NF-κB subunits (data not shown).

Interesting reports of the presence of O-linked N-acetylglucosamine residues on nuclear proteins (Holt and Hart, 1986), of the presence of these residues to varying degrees on a variety of RNA polymerase II transcription factors (Jackson and Tjian, 1988), and of the successful use of wheat germ agglutinin-agarose

(WGA-agarose) lectin affinity chromatography as a first step in the purification of the transcription factor Sp1 (Jackson and Tjian, 1989) suggested the use of wheat germ agglutinin (this lectin shows highly specific binding towards N-acetylglucosamine and various sialic acid sugar species) -agarose affinity chromatography as a first step in the purification of NF-κB. This approach although unsuccessful, did yield some interesting insights - as detailed before (Results, Figure 1.4, Panel A), all κB-specific DNA binding proteins in HeLa cell nuclear extracts (see Materials and Methods) bound to the WGA-agarose affinity resin. However, these κB-specific DNA binding proteins could not be eluted from the resin under the conditions used for the purification of the Sp1 transcription factor (Jackson and Tjian, 1989) - nor could Sp1 DNA binding activity (used as a control). Increasing the N-acetylglucosamine concentration in the eluant to 0.7M (close to the solubility limit for N-acetylglucosamine) had no effect - neither κB-specific nor Sp1 DNA binding activity eluted from the WGA-agarose affinity resin.

Although the purification by Jackson and Tjian (1989) of the Sp1 transcription factor showed a greater than 90% recovery of the intial Sp1 DNA binding activity applied to the WGA-agarose affinity column, other groups have demonstrated somewhat lower recoveries of glycosylated RNA polymerase II transcription factors from WGA-agarose affinity purification procedures. The purification from liver nuclear extracts of the liver-specific hepatocyte nuclear factor 1 (HNF1) transcription factor (Lichtsteiner and Schibler, 1989) involved heparin-agarose, WGA-agarose, and synthetic DNA oligonucleotide-Sepharose affinity chromatography stages, the recovery efficiencies over each of these stages

- 80%, 20-30%, and 80% respectively - indicated that a large fraction of the HNF1 applied to the WGA-agarose column could not be eluted with eluant buffer containing 0.3M N-acetylglucosamine.

Having some supporting evidence for the apparently irreversible binding of native, HeLa cell-derived RNA polymerase II transcription factors to WGA-agarose affinity resins from this study, it was interesting to note a report (Kearse and Hart, 1991) that lymphocyte activation in murine splenic T-cells and T-cell hybridomas resulted in rapid increases in the apparent levels of O-linked N-acetylglucosamine on many nuclear proteins, while in contrast the levels of O-linked N-acetylglucosamine on a distinct set of cytoplasmic proteins decreased rapidly after cell activation - with both types of changes reverting to control levels within a few hours. The appearance of a transient increase in the level of O-linked N-acetylglucosamine on most nuclear proteins of T-cell hybridoma cells when activated for sustained periods of up to 6 hours with a combination of the active phorbol ester PMA, and the calcium ionophore ionomycin was most pertinent to the present studies. A very similar type of transient activation was seen in the pattern of activation of κB DNA-binding activity in the pre-B-cell line 70Z/3 upon treatment with the active phorbol ester PMA for periods of up to 8 hours (Sen and Baltimore, 1986b).

Bearing in mind the observation that most HeLa cell line isolates do not possess constitutively active nuclear NF-κB DNA-binding activity - and the HeLa line used by Jackson and Tjian seems likely to have been one of these, whereas the HeLa cell line used in the present study does show constitutively active nuclear NF-κB DNA binding activity, it seems possible that the same cellular signalling

process responsible for the presence of constitutively active NF-κB in the HeLa cell nucleus is also responsible for significant upregulation of the number of O-linked N-acetylglucosamine residues carried by NF-κB (and presumably by other RNA polymerase II transcription factors such as Sp1). It seems likely that if different cell lines or normal tissue were used as the cell source in a WGA-agarose affinity purification scheme, that NF-κB (and other RNA polymerase II transcription factors) could be eluted from the WGA lectin affinity column with high yield under mild conditions.

The above type of purification scheme using cytoplasmic extracts from cell sources where NF-κB (and the other rel/dorsal protein family members) does not show constitutively active nuclear DNA binding activity would have the advantage that it could allow the recovery of the various IκB and related inhibitor proteins for study in their native forms. Similar arguments apply to other unrelated transcription factors also retained in the cytoplasm by interactions with inhibitor proteins - such as c-fos (Roux et al., 1990) - a transcription factor also known to be modified with N-acetylglucosamine residues in vivo (Jackson and Tjian, 1988), and the glucocorticoid receptor - complexed in the cytoplasm with heat shock proteins hsp 70 and hsp 90, and a 56 kD immunophilin protein (Ku Tai et al., 1992).

As regards the actual function of modification with N-acetylglucosamine residues, this remains unknown - for example suggestions that this modification might act as a nuclear targeting signal in an analogous manner to the mannose 6-phosphate lysosomal targeting signal are difficult to reconcile with the observation that after cell activation, the populations of O-linked

N-acetylglucosamine-modified proteins disappearing from the cytosol were largely distinct from those increasing in the nuclear fractions (Kearse and Hart, 1991). Other interesting possibilities derive from the observation that of the limited number of O-linked N-acetylglucosamine addition sites identified so far, a large proportion are similar to sites recognised by several serine/threonine protein kinases (Edelman et al., 1987) - with the possibility of some type of reciprocal relationship between glycosylation and phosphorylation (Kearse and Hart, 1991). Also possible is that the addition of O-linked N-acetylglucosamine residues might act to either block or target the protein for degradation by specific proteases - previous observations indicated that several proteins with intracellular half-lives of less than two hours contained one or more regions rich in proline, glutamic acid (and to a smaller extent aspartic acid), and serine or threonine - so-called 'PEST' domains (Rogers et al., 1986) - these motifs are similar to known sites of attachment of O-linked N-acetylglucosamine. Most interestingly, two of the proteins which were originally identified as rapidly degraded PEST proteins - c-fos and c-jun - have been shown to contain subsets modified by the addition of O-linked N-acetylglucosamine (Jackson and Tjian, 1988).

Bearing in mind that most O-linked N-acetylglucosamine-bearing proteins are present in small amounts, and those which have been identified seem to be transcription factors, components of transport systems, or involved in cytoskeletal interactions, coupled with the highly regulated nature of the removal/addition process, and the observation that this event seems to have an important role in the early stages of T-cell activation (Kearse and Hart, 1991), seems to indicate that an understanding of this process will be critical for analysis of the activation of

NF-κB DNA-binding activity and its translocation to the nucleus in response to cellular activation.

Since a common feature of DNA-protein complexes is the distortion - often bending - of the DNA recognised by the protein, it was decided to investigate if some such event occurred in the formation of the NF-κB-κB motif complex. Such bends at the protein recognition site on the DNA can be either intrinsic to the DNA as a result of some unusual nucleotide sequence, or the result of supercoiling of the DNA - further, protein binding can induce DNA bending - either as a curve on the surface of the protein, or by proteins bound at separate sites constraining a loop of DNA between them. The formation of such tightly-wrapped DNA-protein complexes is often a prerequisite for DNA-handling processes such as transcription, recombination, replication and topoisomerisation (Travers, 1990; Lilley, 1991; Echols, 1986).

Given observations from previous studies using highly purified κB-binding proteins in dimethylsulphate (DMS) methylation protection experiments with the HIV-1 LTR enhancer region that several bases within the promoter-proximal HIV-R κB motif of the HIV enhancer (-82A, -85C, -86A, and -87G), and -96A in the promoter-distal HIV-L κB motif, showed enhanced rates of reaction with DMS in the presence of κB-binding proteins (Clark et al., 1990), it seemed possible that some form of distortion was occurring at the κB motifs in the presence of the highly purified κB-binding proteins. Thus, making use of the p2xAT/HIV-R plasmid-derived circular permutation 600 bp probes in bending studies with crude HeLa cell nuclear extracts, the pattern of mobilities of circularly-permuted DNA-protein complexes did suggest the induction of a bend

centred on the κB motif (Results, Figure 1.5, Panel A). However, this protein preparation would contain all members of the NF-κB/c-rel family present in HeLa cell nuclear extracts - making it difficult to determine which member(s) of the family were responsible.

Similarly, although the 4xHIV enhancer oligonucleotide-Sepharose affinity purified κB-binding protein preparation was highly purified it had not been shown which of the members of the NF-κB/c-rel family were present. The pattern of mobilities obtained from this protein source was intriguing - with the four complexes with the Hind III, Sph 1, Avi II, and Eco RV-generated probes showing three sets of identical mobility bands, with the Bam HI-generated probe complex always showing a faster mobility (Results, Figure 1.5, Panel A). Whether these three species in each lane represent unique rel protein hetero- or homodimers, proteolysis products, or whether they represent differently posttranslationally modified forms is unknown. However, it does seem likely that the species giving rise to the fastest-migrating complexes can be isolated from the others by the HIV enhancer affinity / FPLC Mono-Q / *E.coli* DNA / HIV enhancer affinity procedure described in section 1.1 (Results, Figure 1.5, Panel B).

Although the circular permutation bending pattern data is difficult to interpret for the partially purified protein sources, the pattern of competition is most interesting - it is conceivable that the inability of a large molar excess of the HIV-R κB motif oligonucleotide to pre-compete binding of the HeLa cell Dignam nuclear extract may reflect a stabilising effect of a wrapping of the DNA surrounding the 10bp κB motif recognition site over the surface of the NF-κB/rel protein(s). Hence it might be possible to explain the ability of the undigested,

presumably supercoiled, p2xAT/HIV-R plasmid to compete at a much lower molar excess of κB sites. If the ability of the HIV-R oligonucleotide to compete for binding of the partially purified κB-binding proteins were due to the proteolytic removal of region(s) of the NF-κB/rel protein(s) which would otherwise stabilise wrapping of longer fragments of DNA, this might explain the unusual mobility pattern seen with the circular permutation studies. Although unexpected, this type of behaviour does not seem unique - studies of the binding of serum response factor (SRF) present in crude nuclear extracts to 152 bp probes carrying circularly permuted c-fos serum response element (SRE), and cardiac and skeletal actin gene promoter CArG box binding sites (Gustafson et al., 1989) showed that the DNA-protein complex involving the c-fos SRE was only poorly competed, if at all, by a 100-fold excess of unlabelled CArG box oligonucleotide. Further, when nuclear extracts were partially proteolysed by proteinase K treatment, the DNA-protein complex showed a much higher mobility, with complexes where the SRE binding site was located at the end of the fragment migrating more quickly than the other complexes which all showed a similar mobility.

Other studies of DNA bending induced by binding of purified NF-κB (Schreck et al., 1990) and crude nuclear extracts (Kuprash and Nedospasov, 1992) have indicated the induction of significant bending angles. Interestingly, the study by Schreck and coworkers (1990) found using purified NF-κB polypeptides, that a p50 subunit dimer induced a smaller angle of bending (75°) on binding to the immunoglobulin κB motif than did binding of the classical p50-p65 heterodimeric form of NF-κB (approximately 110°). Further, mapping of the bend centres

induced by the two species of protein complex demonstrated that the bend centre obtained with the p50 homodimer mapped to a position which corresponded to the centre of the κB motif, whereas the p50-p65 heterodimer induced a bend whose centre had been shifted towards the 3' end of the κB motif - significantly this location overlapped with previous determinations of a position at the 3' end of the κB motif where nicking of the DNA stimulated the binding of NF-κB to its recognition site (Schreck et al., 1990).

The study performed by Kuprash and Nedospasov (1992) using circularly permuted immunoglobulin κB site (and closely related κB site variant) probes with crude nuclear extracts demonstrated the presence of three distinct DNA-protein complex species - one, presumed to involve the p50 homodimer showing an induced bend of approximately 80°, another presumed to involve the p50-p65 heterodimer with an induced bend of approximately 102°, and a third complex species of unknown origin with an induced bend of approximately 131°.

The obvious difficulty with the above study (as with the bending patterns obtained with crude extracts and affinity purified κB proteins in the present work) is in identifying the protein species involved in any particular DNA-protein complex. In any case, it is clear that at least the p50 homodimer and p50-p65 heterodimer members of the NF-κB/rel/dorsal protein family are capable of inducing a severe bend in the DNA upon binding to the κB motif. It seems very likely that the analysis of the nucleoprotein complex assembled at the HIV-1 LTR will be essential for understanding the initial transcriptional activation event. However, considering one recent study of DNA bending by complexes of full-length and truncated variants of the fos and jun proteins (Kerppola and

Curran, 1991) which demonstrated large changes in both bend angle and direction depending on the protein constituents of the DNA binding dimer, this may prove a formidable problem with the NF-κB/rel protein family.

The identification of spermidine as a stimulatory factor for κB motif DNA binding activity in affinity purified protein preparations (Results, Figure 1.6, Panel A) was initially interpreted in terms of the promotion of a conformational change in the HIV-L oligonucleotide κB motif (Rich et al., 1984; Clark et al., 1990). It was thought that alteration of the conformation of the κB motif would allow a greater binding affinity for the NF-κB protein(s), and hence explain the very much greater amount of DNA-protein complex formed in the presence of 3mM spermidine. Subsequently, it was reported by other workers (Schreck et al., 1990) that the similar polyamine spermine (as well as spermidine) caused a similar massive increase in the amount of DNA-protein complex formed with purified p50-p65 heterodimeric NF-κB, much smaller increases were also found with addition of 5mM Ca^{2+}, 1mM Ba^{2+}, and 25uM $Co^{3+}(NH_3)_6$ to the binding mixture. This group also interpreted their results in terms of a promotion of a structural alteration of the cognate DNA binding site, rather than a direct interaction of the agents with the protein.

However, other observations began to suggest that the true reason for the massive stimulation in the amount of DNA-protein complex formed in the presence of spermidine might lie in the rates of formation and dissociation of the complex. It had been shown with affinity purified p50-p65 NF-κB that binding of κB motif oligonucleotide required 5-10 minutes to reach equilibrium in the absence of non-specific competitor DNA, while in the presence of non-specific

competitor DNA, equilibrium was only reached after approximately 60 minutes. In the same study, in experiments challenging pre-formed radiolabelled DNA-protein complexes with a 500-fold molar excess of unlabelled κB motif oligonucleotide, the half-life of the radiolabelled complex was approximately 45 minutes (Zabel and Baeuerle, 1990). While unlabelled κB motif oligonucleotide challenge experiments with preformed DNA-protein complexes using κB-binding proteins isolated from HeLa cells suggested that the amount of radiolabelled DNA-protein complex was essentially unchanged after 1 hour (L.Clark, unpublished observation).

The above observations all suggested that formation of complexes between native κB-binding proteins and κB motif oligonucleotides was relatively slow compared to other sequence-specific DNA-binding proteins, and that the dissociation of these complexes was also slow - however, all of these observations were made in the absence of spermidine (or spermine) in the binding mixture buffer. In the standard presence of 3.6mM spermidine in the gel electrophoresis DNA binding assay incubation mixture (except where specifically omitted) used in the present studies, it is clear that native κB-binding protein-κB motif oligonucleotide complexes must have greatly increased dissociation rate constants. From a consideration of Results, Figure 2.1, using various sources of native κB-binding proteins, comparison of tracks marked '-' and 'HIV-L' clearly shows that addition of 1ug of unlabelled HIV-L κB motif oligonucleotide after the formation of the radiolabelled DNA-protein complex competes away all of the radiolabelled complex in the ≈30 minutes before the samples are loaded on the mobility shift gel.

Thus, the dissociation rate constants for all of these DNA-protein complexes must be considerably higher in the presence of spermidine. This in turn prompted the realisation that the massive stimulation in the amount of DNA-protein complex formed in the presence of spermidine (and spermine) may not reflect some conformational change in the κB motif (although this need not be ruled out), but rather a failure to reach binding equilibrium within the reasonably short timescale of the DNA binding assay.

2. Characterisation of the DNA binding properties of bacterially expressed NF-κB p50 subunit aa35-381 proteins

The possible involvement of oxidation-reduction of cysteine residues in the modulation of the κB-specific DNA binding activity of proteins in the NF-κB/rel family was suggested by observations that both N-ethylmaleimide and iodoacetate inactivated DNA binding activity and that treatment with DTT stimulated κB-specific DNA binding activity (Results, Figure 2.1). The observation that formation of a DNA-protein complex prior to treatment with iodoacetate protected the DNA binding activity of the purified NF-κB p50 subunit (amino acids 35-381) further strengthened the case for some intimate involvement of cysteine residue(s) in the DNA binding site (Results, Figure 5.1).

During the course of the present study, a report appeared confirming that the DNA binding activity of native NF-κB, and of an *in vitro* translated amino acid 1-399 p50 protein was increased by treatment with the reducing agent 2-mercaptoethanol (Toledano and Leonard, 1991) - however, the above study attributed the stimulation effect solely to the p50 subunit. While the present study has not addressed the role of the NF-κB p65 subunit in the stimulation of

κB-specific DNA binding activity by reducing agents, it seems likely that it also contributes to this effect. The above considerations prompted a search of the primary amino acid sequence of the cloned NF-κB p50 subunit (Kieran et al., 1990; Ghosh et al., 1990). Three cysteine residues within the rel-homologous DNA binding and dimerisation region - at positions 62, 119, and 273 relative to the p50 sequence of Kieran et al. (1990) were identified which were absolutely conserved among all members of the NF-κB/rel/dorsal protein family (Results, Figure 2.2). The generation of cDNA encoding the wild type NF-κB p50 protein (amino acids 35-381), cDNAs encoding the amino acid 62, 119, and 273 cysteine to serine mutants (Results, Figures 2.3; 2.4) and expression of the encoded proteins in *E.coli* (Results, Figures 2.5; 2.6) using the pGEX-2T glutathione S-transferase expression vector proved essentially trouble-free, allowing the isolation of purified NF-κB p50 proteins in large quantities.

The dissociation constant values obtained from the Scatchard plot analyses of the κB motif oligonucleotide binding behaviour of the wild type and cysteine to serine mutant NF-κB p50 aa35-381 proteins (Results, Figure 3.3) are of the same general magnitude as values obtained for several other sequence-specific DNA binding proteins binding to their recognition sites on short oligonucleotides. For example a study of the 27 kD POU DNA-binding domain of the octamer binding protein oct-1 (NFIII) binding to its recognition site on an 18-mer double-stranded oligonucleotide yielded a K_D value of $7x10^{-11}$ M (Verrijzer et al., 1990), while a study of the DNA binding domain of NFI binding to a recognition site on a ≈100bp restriction fragment yielded a K_D value of $1.2x10^{-11}$ M (Cleat and Hay, 1989).

However, the dissociation constants obtained here are somewhat higher than those obtained by other workers using native NF-κB proteins: $2.7x10^{-12}$ M for affinity purified NF-κB (Zabel et al., 1991), $4x10^{-13}$ M for renatured, gel purified p50-p65 complex, and $9x10^{-13}$ M for the renatured, gel purified p50 homodimer (Urban and Baeuerle, 1990). Although the reason for this discrepancy is not clear, several interesting possibilities exist. It is very likely that native NF-κB protein purified from eukaryotic cells carries post-translational modifications which might influence its DNA binding behaviour. From the present study it seems likely that most if not all native NF-κB/rel protein isolates will be modified with O-linked N-acetylglucosamine residues - although it is unclear whether this would influence DNA binding behaviour. It also seems likely that native NF-κB/rel proteins will be phosphorylated to varying degrees - this could represent at least part of the reason for the vast majority of native HeLa cell κB motif DNA-binding activity applied to the FPLC Mono-Q (DEAE equivalent) column binding to the column, with only a small fraction of the κB motif DNA-binding activity eluting from the column before the application of the salt gradient (Results, Figure 1.3).

Other groups have reported *in vitro* binding studies with overexpressed NF-κB subunit proteins - one study using recombinant baculovirus expressed p50 and p65 proteins demonstrated that p65 homodimers bound to the immunoglobulin κB motif in a 16bp oligonucleotide with a lower affinity ($K_D = 32.2x10^{-12}$ M) than did the full length p50 homodimer ($K_D = 6.7x10^{-12}$ M), or the p50-p65 NF-κB heterodimer ($K_D = 5.7x10^{-12}$ M) (Fujita et al., 1992). Thus the dissociation

constant for the recombinant baculovirus expressed p50-p65 NF-κB heterodimer is in reasonable agreement with the value of $2.7x10^{-12}$ M for affinity purified native NF-κB (Zabel et al., 1991).

However, another study comparing bacterially expressed p50 protein derivatives with native purified NF-κB (Kretzschmar et al., 1992) suggested that bacterially expressed p50 bound the immunoglobulin κB site on a 37bp oligonucleotide with a much lower affinity than did the native NF-κB. The dissociation constant measured for purified native NF-κB of $1.3x10^{-12}$ M seems in reasonable agreement with the other studies quoted previously bearing in mind the larger oligonucleotide used in this study, however the dissociation constants obtained for a p50 amino acid 18-503 (55kD) homodimer ($K_D = 8.3x10^{-12}$ M) and for a p50 amino acid 18-443 (49kD - approximating to the size of the native p50 subunit) homodimer ($K_D = 2.6x10^{-11}$ M) are larger than expected. It is likely that the difference in K_D measurements for the baculovirus expressed ($6.7x10^{-12}$ M) and bacterially expressed ($2.6x10^{-11}$ M) p50 homodimers is an underestimate as the latter study used a significantly larger oligonucleotide in its DNA binding assays - larger DNA fragments causing an apparent decrease in dissociation constant values (Winter et al., 1981). In any case, it is clear that the bacterially expressed p50 protein binds the κB motif with a considerably lower affinity than does the baculovirus expressed p50 protein - it seems likely that some form of posttranslational modification is responsible for this effect.

Another interesting observation from the study by Kretzschmar et al.(1992) was the approximately 3-fold lower binding affinity of the p50 protein aa18-443

construct towards the κB motif compared with the p50 protein aa18-503 construct - if such a pattern were repeated for further deletions of the C-terminal p50 sequence it might explain the still higher dissociation constant value of $6.5x10^{-10}$ M seen with the wild type p50 protein aa35-381 construct in the present study. It is interesting to speculate on the reasons behind this lower binding affinity for the κB motif, it is possible that it may simply reflect some alteration in the relative orientation of those N-terminal regions of the p50 dimer implicated in the interaction with the κB motif. However a potentially more interesting possibility would be if each p50 monomer had a bipartite DNA-binding structure - it could be imagined that progressive C-terminal deletions of p50 might destroy the ability of the C-terminal component to bind DNA, leaving the N-terminal component functional but with a lower κB motif DNA binding affinity.

There may be hints that support such a model - the presence in the p50 amino acid sequence of a nonhomologous 30 amino acid 'insertion' (Kieran et al., 1990) in the DNA binding and dimerisation region with highly conserved regions on either side hints at the possibility of two domains. Since it has been demonstrated in this study that cysteine-62 in the extreme N-terminal region is involved in contacting DNA, the C-terminal 'domain' may in addition to its dimerisation function have a more direct role in contacting the DNA and increasing the binding affinity of the p50 homodimer. It has also been hinted that the ability of the wild type p50 aa35-381 protein to discriminate against mutant variants of the κB motif is impaired compared to both crude and highly purified preparations of native κB binding proteins. Such a bipartite DNA-binding structure model would have several precedents - for example, studies of the oct-1 (NFIII) POU domain

required for DNA binding have shown the requirement for both an oct-1 homeo domain and a POU-specific domain (Verrijzer et al., 1990) - where the isolated homeo domain was shown to bind 600 times less well to the canonical octamer sequence than did the intact POU domain. In any case, analysis of the DNA contacts made by full length and truncated derivatives of the NF-κB p50 subunit by hydroxyl radical footprinting, and dimethylsulphate methylation protection and interference would be worthwhile.

The variations in dissociation constants between the wild type and amino acid 119, and 273 cysteine to serine mutant p50 aa35-381 proteins in both the presence (Results, Figure 3.3) and absence of spermidine seem to be explicable in terms of altered dissociation rate constants (Results, Figure 3.4, Figure 3.5, Figure 3.6, Table 3.1). Hence the prediction that these three protein species should have similar association rate constants - if the dissociation constants remain broadly unchanged between 0°C and 20°C, the wild type, amino acid 119, and amino acid 273 cysteine to serine mutant p50 proteins should have association rate constants in the presence of spermidine in the region of $1x10^7$ $M^{-1}s^{-1}$ at 0°C. In contrast, the aa62 cysteine to serine mutant p50 aa35-381 protein in the presence of spermidine has a disproportionately larger dissociation rate constant, and a larger association rate constant compared to the three other species. One interesting possibility is that this might reflect a different mechanism of complex formation - some energetically favourable rearrangement of the DNA-protein complex may occur with the wild type protein after the initial association event - reducing the apparent dissociation rate constant, while the aa62 cysteine to serine mutant might not undergo this rearrangement and would hence show a higher dissociation rate

constant. With regard to this possibility, it would be interesting in the future to use the circular permutation DNA-bending analysis on the four different NF-κB p50 aa35-381 protein constructs.

Given the comparatively small effects on dissociation constant values of the absence of spermidine (Results, Chapter 3), but the approximately 10-fold lower dissociation rate constant values for the wild type, aa119, and aa273 proteins in the absence of spermidine (Results, Table 3.1), the association rate constants for these three NF-κB p50 aa35-381 proteins in the absence of spermidine would be expected to be in the order of 1-2x10^6 $M^{-1}s^{-1}$ at 0°C. Again, the aa62 cysteine to serine mutant p50 aa35-381 protein in the absence of spermidine shows a disproportionately large dissociation rate constant (approximately 500-fold larger than the 0.00071 s^{-1} value for the wild type p50 aa35-381 protein), and would be predicted to have a larger association rate constant (of approximately 2x10^7 $M^{-1}s^{-1}$ at 0°C) compared to the wild type, aa119, and aa273 cysteine to serine mutant p50 aa35-381 proteins.

Although other studies with purified native or recombinant NF-κB p50 and p65 subunits have not specifically addressed the question of dissociation rate constants of DNA-protein complexes, some observations on complex stability have been reported. Thus, as previously noted, the half-life of a preformed radiolabelled DNA-protein complex using affinity purified native p50-p65 NF-κB and a 34bp immunoglobulin κB motif oligonucleotide towards challenge by unlabelled oligonucleotide was approximately 45 minutes (Zabel and Baeuerle, 1990) in the absence of spermidine (or spermine). While in transcriptional activation studies using baculovirus expressed full length NF-κB p50 subunit protein, DNA-protein

complexes of p50 homodimer with H-2K gene and interferon-ß gene κB motif (5' -GGGATTCCCC-3' and 5'-GGGAAATTCC-3' respectively) ≈16bp oligonucleotides had half-lives of greater than 30 minutes - again in the absence of spermidine or spermine (Fujita et al., 1992). This compares reasonably closely with a half-life of approximately 16 minutes for challenge of the bacterially expressed wild type NF-κB p50 aa35-381 protein-16-mer κB motif oligonucleotide complex with unlabelled oligonucleotide in the absence of spermidine determined in the present study. As with dissociation constant determinations, it seems very likely that the size of the protein construct, the extent and nature of any posttranslational modifications, the size of the DNA fragment carrying the specific binding site, the presence of polyamines in the incubation buffer, and other factors will all influence dissociation rate constant measurements.

The dissociation rate constant values obtained from this study seem reasonable when compared with the range of values obtained in other eukaryotic systems - for example DNase I footprinting studies on purified native HeLa cell Sp1 transcription factor binding to its recognition site on a 450bp restriction fragment showed that Sp1 dissociated rapidly from its recognition site at 25°C with a half-life of less than 1 minute - i.e. with a dissociation rate constant greater than 0.011 s^{-1} (Schmidt et al., 1989). Whereas in the previously noted study of the DNA binding domain of native HeLa cell NFI protein (Cleat and Hay, 1989) binding to its recognition site on a ≈100bp restriction fragment, the dissociation rate constant for the DNA-protein complex was 3.2×10^{-4} s^{-1} at 20°C.

As indicated before, the influence of posttranslational modifications and the

presence of polyamines on dissociation and association rate constants is not restricted to NF-κB subunits - one interesting study of recombinant baculovirus expressed serum response factor (SRF) demonstrated that the recombinant SRF (phosphorylated at the major casein kinase II site) behaved identically to purified native HeLa cell SRF, and that dephosphorylation of the recombinant SRF, while not affecting its affinity for the *c-fos* serum response element (SRE), caused a large decrease in the rates of association with and dissociation from the *c-fos* SRE (Marais et al., 1992). Also observed in this study was that the rates of both formation and breakdown of phosphorylated and dephosphorylated SRF-*c-fos* SRE complexes were significantly increased by the presence of 3mM spermidine in the DNA binding assay buffer. The mechanism by which spermidine acted in the above study was unknown, but one proposal by the authors was that it might screen negative charges on the DNA backbone and in some way lower the activation energy requirement for complex formation and dissociation, it seems possible that a similar mechanism may apply in the case of the bacterially expressed NF-κB p50 aa35-381 proteins (and also presumably to native κB-binding proteins).

The above type of phosphorylation modulation of exchange rate kinetics is only one possible mechanism for regulating transcription factor activity, many other cases are known of phosphorylation altering such behaviour as the sequestration of transcription factors in the cytoplasm, positively or negatively altering the DNA binding activity of the factor, and affecting the interactions of the factors transactivation domains with other components of the transcriptional machinery (Reviewed in Hunter and Karin, 1992). In the activation of the NF-κB

transcription factor by phosphorylation, only one event is fairly well-characterised - that of the phosphorylation and inactivation of the various members of the IκB / ankyrin repeat protein inhibitor family (Baeuerle and Baltimore, 1988a; Baeuerle and Baltimore, 1988b; Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Haskill et al., 1991; Davis et al., 1991; Hatada et al., 1992). However, it is also possible that the NF-κB subunits themselves are phosphorylated under some or all cellular physiological conditions - a phosphorylation of NF-κB p50 might account for the significantly lower dissociation constant of baculovirus expressed p50 (Fujita et al., 1992) compared with that obtained with the bacterially expressed p50 (Kretzschmar et al., 1992). One plausible candidate for this modification is the potential serine phosphorylation site RRXS present (except in the cases of p49/p50B and RelB/I-Rel proteins) throughout the NF-κB/rel/dorsal family (Kieran et al., 1990; Nolan et al., 1991) located at amino acids 335 to 338 in the p50 sequence. Intriguingly, such sites which are potential substrates for casein kinase II phosphorylation have been identified in many proteins possessing a nuclear localisation signal. While the presence of a nuclear localisation signal determines the specificity of protein transport, the presence of such a potential casein kinase II phosphorylation site at a distance of ≈10-30 amino acid residues from a nuclear localisation signal has been identified as the factor determining the rate of nuclear transport (Rihs et al., 1991). This observation could thus provide an attractive explanation for the previous implication of casein kinase II phosphorylation in the transmission of growth signals to the nucleus - that it acted by controlling the rate of protein transport.

The competition experiment using non-specific *E.coli* DNA suggested that the DNA binding specificity of the aa62 cysteine to serine mutant p50 aa35-381 protein had indeed altered from that of the wild type and aa119 and aa273 cysteine to serine mutant p50 aa35-381 proteins (Results, Figure 4.3), and that the reason for the lower binding affinity of the aa119 and aa273 mutant p50 proteins towards the κB motif did not lie in an altered DNA binding specificity. To address this question a series of oligonucleotide competition experiments using variants of the immunoglobulin / HIV enhancer κB motif were carried out on the wild type and aa62 mutant p50 protein. The results obtained (Results, Figures 4.4 - 4.11, Table 4.1) demonstrate clearly that the aa62 cysteine to serine mutant p50 aa35-381 protein has an altered DNA binding specificity.

As would be expected for competition of the 16-mer κB motif oligonucleotide by unlabelled HIV-L κB motif oligonucleotide, the competition behaviour of the wild type and aa62 cysteine to serine mutant p50 aa35-381 proteins are identical (Results, Figure 4.4). The reason for the relative molarity of unlabelled competitor needed to reduce the amount of radiolabelled complex by 50% being 1.81 rather than 1.00 (Results, Table 4.1) probably lies in the size of the regions flanking the κB sites - as discussed in the Results section. For competition of the 16-mer κB motif oligonucleotide by the unlabelled IRE type of κB motif, the competition behaviour of the wild type and aa62 cysteine to serine mutant p50 proteins are identical (Results, Figure 4.5), while the comparatively similar affinity of the wild type p50 construct homodimer for the IRE and HIV-L κB motifs (the relative molarities of unlabelled competitor for a 50% reduction in radiolabelled complex being 1.35 and 1.81 respectively - Results, Table 4.1) are

in good agreement with the results of κB motif binding affinity studies using baculovirus expressed p50 homodimers (Fujita et al., 1992).

In the case of the competition of the 16-mer κB motif oligonucleotide by unlabelled H2TF1 type κB motif (Results, Figure 4.6), interesting differences appear with regard to binding of the wild type and aa62 cysteine to serine mutant p50 proteins - the relative molarity of the H2TF1 competitor oligonucleotide needed to cause 50% reduction in the amount of radiolabelled complex is only 0.40 for the wild type p50 construct, but 1.96 for the aa62 cysteine to serine mutant p50 construct (Results, Table 4.1). Puzzlingly, the higher affinity of the p50 homodimer construct in this study for the H2TF1 type of κB motif is at variance with dissociation constant determinations for the baculovirus expressed p50 homodimer with immunoglobulin / HIV enhancer, interferon-ß, and H2 gene κB motif oligonucleotides (Fujita et al., 1992) - all these types of κB motif showed similar dissociation constants of approximately $6x10^{-12}$ M. However, other studies with both recombinant and purified native p50 subunit proteins have shown the binding affinity of the p50 homodimer to be significantly greater towards the H2TF1 type of κB motif compared to the immunoglobulin / HIV enhancer κB motif (Kieran et al., 1990; Baldwin and Sharp, 1988). In addition, this study demonstrates that the aa62 cysteine to serine p50 mutant has the same binding affinity for the HIV-L κB motif as for the H2TF1 type of κB motif.

Competition of the 16-mer κB motif oligonucleotide by unlabelled, highly symmetric EBP'cons' κB motif oligonucleotide (this like the H2TF1 κB motif having a 3' half-site which was a mirror image of the 5' half-site), there were again some differences in behaviour between the wild type and aa62 cysteine to

serine mutant p50 proteins (Results, Figure 4.7). The molar excess of EBP'cons' competitor oligonucleotide required to reduce the amount of radiolabelled DNA-protein complex by 50% (0.30 and 0.58 respectively for the wild type and aa62 cysteine to serine mutant p50 proteins - Results, Table 4.1) indicate that the EBP'cons' κB motif is a better competitor for the wild type p50 protein than is the 16-mer κB motif oligonucleotide. This finding is supported by other studies showing that full-length bacterially expressed p50 bound similar palindromic κB sites with an affinity at least 5-fold greater than for the immunoglobulin / HIV enhancer κB motif (Kretzschmar et al., 1992).

The other four species of mutant κB motif - SVUP, SV1-M1, SV1-M2, and SV1-M3 (Results, Figures 4.8, 4.9, 4.10, and 4.11, Table 4.1) all show similar patterns of behaviour - as noted before, they are poor competitors for binding of both the wild type and aa62 cysteine to serine mutant p50 aa35-381 proteins, but with higher affinities for the aa62 mutant p50 protein - implying that the aa62 mutant p50 protein has lost the ability to discriminate between specific and non-specific DNA binding sites.

Although the substitution of serine in place of a cysteine residue is often thought of as being among the most conservative amino acid substitutions possible, it should be noted that their side chain bond lengths and angles are significantly different - thus it is possible that the substitution by serine could disturb potential hydrogen bond contacts with the κB motif on the DNA, and hence reduce the stability of the DNA-protein complex. That such effects can be dramatic is illustrated by the sulphate binding protein - where the substitution of a serine residue which contributes hydrogen bonds for ligand binding by cysteine results in

the mutant protein sulphate binding affinity being over 1000-fold lower (He and Quiocho, 1991).

Many examples have been documented of relatively subtle amino acid substitutions affecting the sequences recognised and/or the binding affinities of sequence specific DNA binding proteins, most of the well-studied examples are prokaryotic repressor proteins, but some eukaryotic transcription factors have also been analysed. The 10-fold change in dissociation constant for the aa62 cysteine to serine mutant p50 protein is paralleled by examples such as the *E.coli* CRP protein (Ebright et al., 1984) - where it was shown that substitution of glutamic acid 181 by lysine, leucine or valine residues resulted in the mutant proteins preferentially recognising A.T base pairs (rather than the wild type G.C base pairs) at positions 7 and 16 of the symmetrical CRP recognition site. Thus, with the wild type CRP recognition site, the aa181 glutamate to lysine change resulted in approximately 5-fold lower levels of reporter enzyme activity.

Eukaryotic examples of such changes in DNA binding specificity include the yeast bZIP type transcriptional activator GCN4 - which normally binds the optimal site ATGACTCAT with much higher affinity than the mutant site TTGACTCAA, mutation of a single invariant asparagine in the basic region (Asn-235) to tryptophan resulted in a mutant protein which bound both sites with similar affinity (Tzamarias et al., 1992). Another recently characterised change in DNA binding specificity was that of the yeast TFIID general transcription factor, which normally binds to the consensus TATA element - TATAAA, genetic selection techniques allowed the identification of a mutant TFIID which allowed transcription from promoters containing the mutant TATA element - TGTAAA.

The mutant TFIID protein contained three substitutions within a 12 amino acid region - two of which were necessary and mainly responsible for the new specificity - isoleucine-194 to phenylalanine, and leucine-205 to valine (Strubin and Struhl, 1992). Both of the above mutants were isolated by genetic methods in yeast - such a technique could have great advantages for identifying mutant NF-κB species with novel binding specificities. Indeed, *Saccharomyces cerevisiae* has been shown to lack endogenous κB-binding transcriptional activator proteins and has been used for transfection studies to map transcriptional activation domains of p105 and IκBß (Morin and Gilmore, 1992). Intriguingly however, the fission yeast *Schizosaccharomyces pombe* has been shown to possess endogenous κB-binding transcriptional activation protein(s) (Toyama et al., 1992).

3. Identification of cysteine residues in the NF-κB p50 subunit DNA binding site

The initial impetus behind the study of the oxidation-reduction regulation of NF-κB p50 subunit DNA binding activity came from *in vitro* studies demonstrating increased AP-1-specific DNA binding activity of the fos-jun heterodimeric transcription factor after treatment with the reducing agent dithiothreitol (DTT) (Abate et al., 1990) and the mediation of this stimulation by the oxidation-reduction state of a single conserved cysteine residue in each subunit. An initial experiment with various sources of crude κB-binding proteins suggested that such a regulatory mechanism might apply to NF-κB DNA binding activity, since treatment of the gel electrophoresis DNA binding assay incubation mixture with additional DTT to 25mM and incubating 15 minutes on ice caused a significant, in some cases massive, increase in specific κB motif DNA binding

activity (Results, Figure 2.1). The possibility that this stimulation involved some intermediary protein rather than the direct action of DTT was lessened by the observation that extensively affinity purified κB binding proteins 'Aff.Pur.NF-κB' also showed the same stimulation by DTT treatment. The fact that N-ethylmaleimide pretreatment of all protein sources prior to DNA-protein complex formation inactivated κB motif DNA binding activity strengthened the case for the involvement of a cysteine residue(s) in this process.

Comparison of the predicted amino acid sequences of a variety of members of the NF-κB/rel/dorsal protein family in the region corresponding to the previously roughly defined DNA binding and dimerisation region of the NF-κB p50 subunit (Results, Figure 2.2) (Kieran et al., 1990; Ghosh et al., 1990) indicated that within the amino acid 35 to 381 region of p50, were three cysteine residues which were absolutely conserved among all family members - at p50 amino acid positions 62, 119, and 273. The bacterial expression of the wild type and amino acid 62, 119, and 273 cysteine to serine mutants of the p50 protein aa35-381 region (Results, Figure 3.1) yielded enough protein for extensive studies.

Measurements of dissociation constants for the four p50 construct species with a 16-mer κB motif oligonucleotide (Results, Figure 3.3) and dissociation rate constants in the presence and absence of the polyamine spermidine (Results, Figures 3.5, 3.6, Table 3.1) suggested a different DNA binding specificity for the aa62 cysteine to serine mutant p50 aa35-381 protein - and hence that cysteine 62 might be involved in hydrogen bonding contacts with the κB motif DNA. These suggestions were strengthened by DNA binding competition analysis of the wild type and aa62 cysteine to serine mutant p50 constructs with a range of κB motif

variants (Results, Figures 4.4 - 4.11, Table 4.1).

As noted before, during these studies a report of the *in vitro* modulation of NF-κB DNA binding activity appeared (Toledano and Leonard, 1991), indicating that for both crude sources of κB binding proteins and an *in vitro* translated p50 subunit aa1-399 construct, specific κB-binding activity was stimulated by treatment with 2-mercaptoethanol. It was also observed that in the activation of cytoplasmic, inhibitor protein-complexed, forms of κB binding activity from uninduced T-cell extracts, that there was a synergistic effect of 2-mercaptoethanol and the deoxycholate/NP40 detergent treatment previously shown to activate the DNA binding activity of IκB-shielded NF-κB (Baeuerle and Baltimore, 1988b). Thus raising at least the possibility that oxidised, non DNA-binding forms of κB-binding proteins (not necessarily complexed to ankyrin repeat proteins) could exist in the cytoplasm. Further, as seen in the case of the fos-jun heterodimer (Abate et al., 1990), the native κB binding proteins and the p50 protein aa1-399 construct were inactivated by N-ethylmaleimide, and also by the sulphydryl-oxidising reagent diamide (Kosower et al., 1969).

Bearing in mind the previously noted hints that cysteine 62 of the p50 protein aa35-381 construct is intimately involved in the DNA binding site, and that the incubation of the fos-jun heterodimer with an oligonucleotide containing an AP-1 binding site, prior to treatment with N-ethylmaleimide protected the protein against inactivation (Abate et al., 1990), a similar oligonucleotide protection experiment was carried out with the wild type p50 protein aa35-381 construct. This demonstrated (Results, Figure 5.1) that the DNA binding activity of the preformed DNA-protein complex was essentially unchanged even in the presence

of 30mM iodoacetate, whereas incubation of the protein with this concentration of iodoacetate prior to addition of the radiolabelled 16-mer κB motif oligonucleotide resulted in complete inactivation of DNA binding activity - firm evidence for the presence of a cysteine residue(s) in the p50 protein aa35-381 construct DNA binding site.

Although the above experiment could give no information as to which particular cysteine(s) was involved in intimate contacts with the κB motif DNA, the previous binding affinity and specificity studies and the results of the titration of the DNA binding activity of all four p50 construct species (Results, Figure 5.2) with iodoacetate all indicate that cysteine 62 is the residue involved in the redox modulation of the DNA binding activity of the NF-κB p50 protein aa35-381 construct. It should be noted that the difference in iodoacetate sensitivities between the aa62 mutant and the three other species may well be an underestimate as the presence of comparatively large amounts of the carrier protein bovine serum albumin, which is rich in cysteine residues, may act as a thiol buffer.

To confirm the presence of cysteine 62 in the DNA binding site of the NF-κB p50 subunit aa35-381 construct, the substrate protection of iodoacetate-reactive residues in the DNA binding site (Results, Figure 5.3) was performed with ^{14}C-iodoacetate. The N-terminal amino acid peptide sequence analysis of HPLC fraction number 54 revealed a predominant peptide species (Results, Figure 5.5, Panel B) with sequence corresponding to amino acids 60 to 69 of the NF-κB p50 sequence (Kieran et al., 1990). A repeat of this ^{14}C-iodoacetate affinity labelling experiment with the incorporation of 95% of the theoretical amount of ^{14}C recently demonstrated the identity of cysteine 62 as the only radiolabelled cysteine

- indicating that cysteine 62 is the only cysteine residue in the NF-κB p50 subunit aa35-381 construct DNA binding site.

A recent study of the v-rel oncoprotein identified the homologous cysteine 35 in the conserved motif RxxRxRxxC as being essential for v-rel protein-DNA contact and suggested that reduction of this conserved cysteine was necessary for optimal DNA binding (Kumar et al., 1992). Making use of an unusual combination of UV cross-linking of DNA-protein binding reactions and immunoprecipitation to quantitate binding suggested that mutation of all arginine residues in the RxxRxRxxC motif resulted in loss of DNA binding activity. It was also proposed on the basis of this binding assay that mutation of cysteine 35 to serine resulted in a slight increase in binding affinity for a bromodeoxyuridine-labelled palindromic κB motif, and that mutation of v-rel cysteine residues 92 and 216 (the homologues of p50 cysteine residues 119 and 273) to serine had no effect on DNA binding activity.

Whether these differences in the effects of cysteine to serine mutations are inherent between the v-rel oncoprotein and NF-κB p50, or possibly due to the truncated nature of the p50 subunit aa35-381 construct is unknown. However, the authors of the above study did identify one significant philosophical problem with the proposal that the cysteine 35 to serine mutation increased and deregulated DNA-binding activity - that unlike the case with the oncogenic v-jun cysteine to serine mutation which both escapes redox control and has increased DNA binding activity (Maki et al., 1987), no such oncogenic mutants are known for NF-κB/rel/dorsal family proteins - indeed v-rel itself has a cysteine residue rather than serine at this position. It is then necessary to propose that cysteine 35 is also

involved in some other function such as modulating the interaction of v-rel with other cellular proteins.

The demonstration of cysteine 62 as the only cysteine residue in the NF-κB p50 subunit aa35-381 construct DNA binding site suggested from symmetry arguments that its pair, cysteine 62', would occupy a symmetrically related position - prompting the question of whether they were close together or widely separated in the DNA binding site. The results of diamide treatment of the four NF-κB p50 aa35-381 constructs demonstrated that only the aa62 cysteine to serine mutant protein failed to be interchain disulphide crosslinked (Results, Figure 5.6). This result combined with the ^{14}C affinity labelling results showed that cysteine 62 and 62' are close enough in the DNA binding site to form a disulphide bond.

It is becoming clear that arrangements of cysteine sidechains that allow interchain and intrachain disulphide bonds to form in cytoplasmic and nuclear protein complexes may be quite common - examples include the previously noted fos-jun heterodimeric transcription factor (both the fos and jun proteins belong to the basic region - leucine zipper (bZIP) class of DNA binding proteins) which is converted from an active DNA-binding species to a non-binding species by formation of an interchain disulphide bond between the two conserved cysteine residues in their basic DNA binding motifs (Bannister et al., 1991), also the human glucocorticoid receptor - with evidence for the formation of inter- and intramolecular disulphide bonds and a close link between the reduction of intramolecular disulphide bond(s) and the ability of the receptor monomer to bind DNA (Silva and Cidlowski, 1989), and the human general transcription factor TFIIIC (Cromlish and Roeder, 1989). Other examples include other bZIP class

proteins such as the liver and fat cell specific transcriptional regulator CCAAT / enhancer binding protein (C/EBP) (Landschulz et al., 1989; Williams et al., 1991) and the Epstein-Barr virus BZLF1 transactivator protein (Bannister et al., 1991).

Intruigingly, the bZIP class protein examples mentioned above (fos-jun, C/EBP, and BZLF1) while all showing the ability to be interchain crosslinked *in vitro* (for C/EBP via a conserved cysteine at the C-terminus of the leucine zipper) by diamide treatment do not all show a significant change in DNA binding activity with changes in redox state. Thus, while the wild type C/EBP dimer did not show any difference in DNA binding activity for the reduced and disulphide crosslinked forms (Williams et al., 1989), the introduction of a serine to cysteine mutation at position 12 of the C/EBP basic motif (the equivalent of the conserved redox-sensitive cysteine in the core basic DNA binding motifs of the fos, jun , and BZLF1 DNA binding domains) results in the generation of a redox-sensitive protein which can only bind to its recognition site in the presence of DTT (Bannister et al., 1991).

This concentration on the bZIP class of transcriptional regulators reflects their potential special relevance for the NF-κB/rel/dorsal protein family - with the identification of a cDNA encoding the rel B protein (Ryseck et al., 1992) which posesses an N-terminal putative leucine zipper structure between amino acids 22 and 51. Homodimers of this rel B protein could not bind κB sites with high affinity, but heterodimers with the NF-κB p50 subunit could bind with high affinity to the κB motif and were transcriptionally active. Given the large number of bZIP type transcriptional regulator species within the cell, it seems likely that some types of functional heterodimers could be formed between a rel B monomer

and another bZIP species - with the generation of hybrid transcriptional regulators with new DNA binding specificities.

An interesting parallel between all of the NF-κB/rel/dorsal family and redox-sensitive bZIP proteins such as fos, jun, and BZLF1 is that the redox-sensitive cysteine is surrounded by a short but well conserved sequence of basic residues (different for the two groups of proteins). It has been known for some time that such arrangements of basic residues near a cysteine can increase the cysteines reactivity dramatically - the oxidation equilibrium constant can change over several orders of magnitude (Snyder et al., 1981). Thus it may be that one function of these basic regions is to make that particular cysteine thiol exquisitely sensitive towards changes in the intracellular redox environment - this is in addition to any other roles these basic regions may play in contacting the DNA of the recognition motif.

4. Implications for the control of gene expression

It is clear that the control of gene expression by trans-acting factors of the NF-κB/rel/dorsal protein family binding to κB-like motifs is a highly complex problem, and the control of the initial transcriptional activation of the integrated HIV-1 provirus significantly more so. However, this study has at least identified the redox control of DNA binding activity in the NF-κB/rel/dorsal family as one regulatory mechanism with the possibility of therapeutic intervention.

At this point it seems pertinent to explore the possible mechanisms which a cell might use to alter its internal redox environment - one seemingly ubiquitous activity is the thioredoxin family of dithiol reducing enzymes - originally identified as a hydrogen donor for ribonucleotide reductase (Holmgren, 1985).

The human equivalent of this activity is thought to be the oxido-reducing enzyme adult T-cell leukaemia derived factor (ADF) which has been isolated and cloned from both the Epstein-Barr Virus (EBV) infected 3B6 B-cell line and the Human T-cell Leukaemia Virus type I (HTLV-I) infected adult T-cell leukaemia line ATL2 (Tagaya et al., 1989). Most interestingly, while the mRNA for ADF cannot be detected by Northern blotting of resting lymphocytes, it was inducible by treating lymphocytes with either the mitogenic lectin phytohaemagglutinin (PHA) or the active phorbol ester, phorbol myristyl acetate (PMA) (Tagaya et al., 1989). It has also been demonstrated that high levels of ADF mRNA are present in EBV-LCL and HTLV-I positive ATL cells (Wakasugi, 1992). These conditions under which ADF mRNA is expressed are also those where transcription from the HIV-1 LTR occurs, and which show activation of the DNA binding activity of NF-κB.

To investigate the physiological importance of such oxido-reducing enzymes as ADF in the control of the DNA binding activity of redox-sensitive transcriptional regulators such as the NF-κB/rel/dorsal family we became involved in a collaboration with N.Wakasugi and J.-L.Virelizier. Cotransfection of Cos7 cells with an ADF/human thioredoxin expression vector and a luciferase reporter construct under the control of a wild type HIV-1 LTR gave a 5-fold increase in luciferase activity compared to the absence of the thioredoxin expression plasmid, while use of a luciferase construct with an HIV-1 LTR deleted for the κB sites gave no increase in luciferase activity in cotransfections with the thioredoxin expression plasmid. In addition *in vitro* DNA binding assays demonstrated that both ADF/human thioredoxin and *E.coli* thioredoxin could directly stimulate

κB-specific DNA binding activity of the purified wild type NF-κB p50 subunit aa35-381 protein construct, while the aa62 cysteine to serine mutant p50 aa35-381 protein DNA binding activity could not be stimulated by ADF/human thioredoxin (Matthews et al., 1992). Thus for NF-κB p50 subunit homodimers at least, there seems to be no requirement for an intermediary protein such as the Ref-1 nuclear protein involved in stimulating (via the reduction of the conserved cysteines in the basic DNA binding motifs) the DNA binding activity of the fos-jun heterodimer transcription factor (Xanthoudakis and Curran, 1992).

It seems possible that the same may not be true for the p65 subunit of NF-κB however, as studies on the overexpressed Ref-1 protein (Xanthoudakis et al., 1992) have shown that while p65 homodimer DNA binding activity was significantly stimulated by 10mM DTT, it could also be stimulated to a lesser degree by treatment with bacterially expressed Ref-1 protein. Transcription factor species such as fos-jun and myb were in contrast stimulated to a greater extent by the Ref-1 treatment than by DTT. The authors suggested that Ref-1 mediates its redox activity through a KCR motif around the redox-active cysteine which is absolutely conserved throughout all the fos-related and jun-related proteins identified to date, and noted that a similar motif, KICR, occurred in the p65 sequence at amino acids 195-198 (Nolan et al., 1991) - with the implication that this is the redox-sensitive cysteine of p65 - interestingly enough this cysteine is conserved among NF-κB p65; dorsal; mouse, human, and turkey c-rel (Nolan et al., 1991) and v-rel proteins (Kieran et al., 1990), the only exception being the NF-κB p50 subunit. However it is also true that around the p65 homologue of the completely conserved p50 cysteine 62 is the similar sequence RYKCEGR -

conserved throughout the rel family which might possibly act as a Ref-1 substrate, while the homologous p50 amino acid sequence RYVCEGP may be too distantly related for this. Thus the potential relevance of Ref-1 in acting as an intermediary in ADF/human thioredoxin-mediated reduction of, and stimulation of the DNA binding activity of, the NF-κB p50-p65 heterodimer, and p65 homodimers is as yet, unclear.

Up to this point the interior of the cell has been considered solely as a reducing environment where the presence of disulphide bonds might not be expected, however several suggestions have been made that even under normal metabolic conditions the existence of intracellular disulphide bonds is allowed. For example studies of 3-hydroxy-3-methylglutaryl-CoA reductase (Cappel and Gilbert, 1988) have suggested the regulation of enzyme activity by formation and reduction of disulphide bonds in response to normal changes in the thiol/disulphide oxidation state of the cellular glutathione redox buffer.

However, the cell must also face severe challenges such as oxidative stress and ultraviolet radiation, in both prokaryotes and eukaryotes such insults can lead to the so-called pro-oxidant state - characterised by the build up of reactive oxygen species of which the major forms are the superoxide anion O_2^-, the hydroperoxy radical $HO_2\cdot$, singlet oxygen $O_2{}^1*$, the hydroxyl radical $HO\cdot$, and hydrogen peroxide (Reviewed in Cerutti, 1985). Prooxidant states can vary depending on the target cell and the type of induction mechanism and cause chromosomal damage by indirect action, however such states can be prevented or suppressed by a system of cellular antioxidant defense enzymes and low molecular weight scavenger molecules.

The possible involvement of an interplay between the reactive oxygen species generated in such situations as the inflammatory response and the intracellular antioxidant enzyme/scavenger molecule system was demonstrated *in vivo* by studies showing that treatment of cell cultures with the active phorbol ester PMA or tumour necrosis factor α (TNF-α) resulted in the activation of κB DNA-binding activity, in lower levels of intracellular reduced glutathione, and the activation of transcription from the HIV-1 LTR. In contrast, treatment of cell cultures with N-acetyl-L-cysteine (NAC) prevented the (PMA or TNF-α-induced) fall in intracellular reduced glutathione levels, blocked the activation of κB DNA-binding species, and blocked the activation of transcription from the HIV-1 LTR (Staal et al., 1990; Roederer et al., 1990).

Further *in vivo* studies showed that the activation of NF-κB DNA binding activity and of transcription from the HIV-1 LTR in the Jurkat T-cell line could be induced by treating cells with micromolar concentrations of hydrogen peroxide (Schreck et al., 1991), and that this activation process could be blocked by preaddition of NAC. However, if NAC were added at the end of the hydrogen peroxide treatment no inhibition was observed, nor could hydrogen peroxide alone activate the DNA binding activity of purified NF-κB-IκB complex or the complex in cytoplasmic extracts - leading to the suggestion that NAC might be reacting with some metabolite of hydrogen peroxide. NAC also blocked the activation of NF-κB by agents such as cycloheximide, double stranded RNA, calcium ionophore, TNF-α, PMA, interleukin-1, bacterial lipopolysaccharide, and lectin - leading to the suggestion that these diverse pathways might all act via a common intermediate involving reactive oxygen species.

Whether such mechanisms also apply in the case of other inducible transcription factors such as the fos-jun heterodimer is unclear - both κB and AP-1 specific DNA binding activities are known to be increased in soluble extracts of UV-treated cells (Stein et al., 1989). At present it is also unclear what role reactive oxygen species play in the action of the HTLV-1 Tax protein, hepatitis virus B X protein, and C-terminally truncated forms of the hepatitis virus B middle and large surface antigen proteins which have been shown to transactivate several species of inducible transcription factor (including NF-κB) by a process which can be inhibited by antioxidants and which presumably involves reactive oxygen species (Meyer et al., 1992).

5. Future studies

The evidence presented in these studies suggests that the DNA binding activity of the NF-κB/rel/dorsal transcriptional modulator family may be modulated by the redox state of a conserved cysteine residue (aa62 in p50) in their DNA binding and dimerisation regions. Whether the redox mechanism regulating the DNA binding activity of the other family members involves the formation of an interchain disulphide bond as in the p50 protein, or whether it involves some other reversible modification such as the generation of a sulfenic acid is a question of immediate interest.

It will also be of great interest to determine which other amino acid side chains in the DNA binding site are involved in making hydrogen bonds with bases in the κB motif. One potentially useful approach would be the use of different specificity chemical reagents in substrate protection experiments to attempt to identify side chains other than cysteine. Another powerful approach to analysing the

DNA-protein interaction could lie in determining the new DNA binding specificity of the aa62 cysteine to serine mutant by a PCR amplification/random oligonucleotide binding site method.

Having identified the role of one conserved cysteine residue (aa62) in the p50 DNA binding and dimerisation region as a redox state modulator in the DNA binding site, an obvious question concerns the function of the other conserved cysteine residues (aa119 and aa273). One possibility is a role in protein dimerisation - it has been suggested that NF-κB may be a zinc-dependent protein, thus it could be imagined that the four cysteine residues (aa119, aa273, aa119', aa273') might tetrahedrally coordinate a zinc ion. However, it might have been expected that a cysteine to serine mutation at either of the 119 or 273 positions would then be very detrimental to dimer stability - in contrast to the modest effects of these mutations on DNA binding activity *in vitro*.

Further regulatory possibilities for the NF-κB/rel/dorsal family centre around the dramatic increase in on- and off-rates for p50 in the DNA-protein complex in the presence of spermidine - it seems likely that effects at least as dramatic are seen with native proteins. Given the potential phosphate-shielding activity of polyamines such as spermidine, and the tight cell-cycle control of spermidine synthesis, it seems possible that some regulatory interplay between free spermidine levels and protein phosphorylation may exist to coordinately regulate the DNA binding activity of a wide range of factors such as transcriptional modulator proteins, DNA replication proteins, and topoisomerases. *In vitro* studies with purified protein kinases and purified members of the NF-κB/rel/dorsal family would be most useful for understanding this potentially

important mechanism for regulating DNA binding activity.

REFERENCES.

Abate, C., Patel, L., Rauscher, F.J. III and Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. Science **249**, 1157-1161.

Allen, J.S., Coligan, J.E., Darin, F., McLane, M.F., Sodroski, J.G., Rosen, C.A., Haseltine, W.A., Lee, T.H. and Essex, M. (1985). Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. Science **228**, 1091-1094.

Anderson, M.G. and Clements, J.E. (1991). Comparison of the transcriptional activity of the long terminal repeats of simian immunodeficiency viruses SIVmac251 and SIVmac239 in T-cell lines and macrophage cell lines. J. Virol. **65**, 51-60.

Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T. and Karin, M. (1988). Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. Nature **332**, 166-170.

Arima, N., Molitor, J.A., Smith, M.R., Kim, J.H., Daitoku, Y. and Greene, W.C. (1991). Human T-cell leukemia virus type I Tax induces expression of the rel-related family of κB enhancer-binding proteins: evidence for a pretranslational component of regulation. J. Virol. **65**, 6892-6899.

Atchison, M.L. and Perry, R.P. (1987). The role of the κ enhancer and its binding factor NF-κB in the developmental regulation of κ gene transcription. Cell **48**, 121-128.

Bachelerie, F., Alcami, J., Arenzana-Seisdedos, F. and Virelizier, J.-L. (1991). HIV enhancer activity perpetuated by NF-κB induction on infection of monocytes. Nature **350**, 709-712.

Baeuerle, P.A. and Baltimore, D. (1988a). Activation of DNA binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**,

211-217.

Baeuerle, P.A. and Baltimore, D. (1988b). IκB: A specific inhibitor of the NF-κB transcription factor. Science **242**, 540-546.

Baeuerle, P.A. and Baltimore, D. (1989). A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3**, 1689-1698.

Baeuerle, P.A., Lenardo, M., Pierce, J.W. and Baltimore, D. (1988). Phorbol-ester-induced activation of the NF-κB transcription factor involves dissociation of an apparently cytoplasmic NF-κB/inhibitor complex. Cold Spring Harbor Symp. Quant.Biol. **53**, 789-798.

Baldwin, A.S.Jr., Azizkhan, J.C., Jensen, D.E., Beg, A.A. and Coodly, L.R. (1991). Induction of NF-κB DNA-binding activity during the G0-to-G1 transition in mouse fibroblasts. Mol. Cell. Biol. **11**, 4943-4951.

Baldwin, A.S.Jr. and Sharp, P.A. (1987). Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2K^b class I major histocompatibility gene. Mol. Cell. Biol. **7**, 305-313.

Baldwin, A.S.Jr. and Sharp, P.A. (1988). Two transcription factors, NF-κB and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter. Proc. Natl. Acad. Sci. USA **85**, 723-727.

Ballard, D.W., Böhnlein, E., Lowenthal, J.W., Wano, Y., Franza, B.R. and Greene, W.C. (1988). HTLV-I Tax induces cellular proteins that activate the κB element in the IL-2 receptor α gene. Science **241**, 1652-1655.

Ballard, D.W., Böhnlein, E., Hoffman, J.A., Bogerd, H.P., Dixon, E.P., Franza, B.R. and Greene, W.C. (1989). Activation of the interleukin-2 receptor α gene: regulatory role for DNA-protein interactions flanking the κB enhancer. New Biol. **1**, 83-92.

Ballard, D.W., Dixon, E.P., Peffer, N.J., Bogerd, H., Doerre, S., Stein, B. and Greene, W.C. (1992). The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. USA **89**, 1875-1879.

Ballard, D.W., Walker, W.H., Doerre, S., Sista, P., Molitor, J.A., Dixon, E.P., Peffer, N.J., Hannink, M. and Greene, W.C. (1990). The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**, 803-814.

Bannister, A.J., Cook, A. and Kouzarides, T. (1991). *In vitro* DNA binding activity of Fos/Jun and BZLF1 but not C/EBP is affected by redox changes. Oncogene **6**, 1243-1250.

Berkhout, B., Gatignol, A., Rabson, A.B. and Jeang, K.-T. (1990). TAR-independent activation of the HIV-1 LTR: evidence that *tat* requires specific regions of the promoter. Cell **62**, 757-767.

Berkhout, B. and Jeang, K.-T. (1992). Functional roles for the TATA promoter and enhancers in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **66**, 139-149.

Blanar, M.A. Burkly, L.C. and Flavell, R.A. (1989). NF-κB Binds within a region required for B-cell-specific expression of the major histocompatibility complex class II gene Ea^d. Mol. Cell. Biol. **9**, 844-846.

Blank, V., Kourilsky, P. and Israël, A. (1991). Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J. **10**, 4159-4167.

Böhnlein, E., Lowenthal, J.W., Siekevitz, M., Ballard, D.W., Franza, B.R. and Greene, W.C. (1988). The same inducible nuclear proteins regulates mitogen activation of both the interleukin-2 receptor-α gene and type 1 HIV. Cell **53**, 827-836.

Böhnlein, E., Siekevitz, M., Ballard, D.W., Lowenthal, J.W., Rimsky, L., Bogerd, H., Hoffman, J., Wano, Y., Franza, B.R. and Greene, W.C. (1989). Stimulation of the human immunodeficiency virus type 1 enhancer by the human T-cell leukemia virus type I *tax* gene product involves the action of inducible cellular proteins. J. Virol. **63**, 1578-1586.

Bomsztyk, K., Rooney, J.W., Iwasaki, T., Rachie, N.A., Dower, S.K. and Sibley, C.H. (1991). Evidence that interleukin-1 and phorbol esters activate NF-κB by different pathways: role of protein kinase C. Cell Reguln. **2**, 329-335.

Bours, V., Burd, P.R., Brown, K., Villalobos, J., Park, S., Ryseck, R.-P., Bravo, R., Kelly, K. and Siebenlist, U. (1992). A novel mitogen-inducible gene product related to p50/p105 NF-κB participates in transactivation through a κB site. Mol. Cell. Biol. **12**, 685-695.

Bours, V., Villalobos, J., Burd, P.R., Kelly, K. and Siebenlist, U. (1990). Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature **348**, 76-80.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Brasier, A.R., Ron, D., Tate, J.E. and Habener, J.F. (1990).A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1α induced, NF-κB mediated *trans*-activation of the angiotensinogen gene acute-phase response element. EMBO J. **9**, 3933-3944.

Briegel, K., Hentsch, B., Pfeuffer, I. and Serfling, E. (1991). One base pair change abolishes the T cell-restricted activity of a κB-like proto-enhancer element from the interleukin 2 promoter. Nucl. Acids. Res. **19**, 5929-5936.

Briskin, M., Damore, M., Law, R., Lee, G., Kincade, P.W., Sibley, C.H., Kuehl,

M. and Wall, R. (1990). Lipopolysaccharide-unresponsive mutant pre-B-cell lines blocked in NF-κB activation. Mol. Cell. Biol. **10**, 422-425.

Briskin, M., Kuwabara, M.D., Sigman, D.S. and Wall, R. (1988). Induction of κ transcription by interferon-γ without activation of NF-κB. Science **242**, 1036-1037.

Brownell, E., Mittereder, N. and Rice, N.R. (1989). A human *rel* proto-oncogene cDNA containing an *Alu* fragment as a potential coding exon. Oncogene **4**, 935-942.

Bull, P., Morley, K.L., Hoekstra, M.F., Hunter, T. and Verma, I.M. (1990). The mouse c-*rel* protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain. Mol. Cell. Biol. **10**, 5473-5485.

Bushman, F. and Craigie, R. (1991). Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. Proc. Natl. Acad. Sci. USA **88**, 1339-1343.

Calman, A.F., Busch, M.P., Vyas, G.N., McHugh, T.M., Stites, D.P. and Peterlin, B.M. (1988). Transcription and replication of human immunodeficiency virus-1 in B lymphocytes in vitro. AIDS **2**, 185-193.

Capobianco, A.J., Chang, D., Mosialos, G. and Gilmore, T.D. (1992). p105, the NF-κB p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. J. Virol. **66**, 3758-3767.

Cappel, R.E. and Gilbert, H.F. (1988). Thiol/disulphide exchange between 3-hydroxy-3-methylglutaryl-CoA reductase and glutathione. J. Biol. Chem. **263**, 12204-12212.

Carthew, R.W., Chodosh, L.A. and Sharp, P.A. (1985). An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**, 439-448.

Cerutti, P.A. (1985). Prooxidant states and tumor promotion. Science **227**, 375-381.

Chung, C.T., Niemela, S.L. and Miller, R.H. (1989). One-step preparation of competent *Escherichia coli* : Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86**, 2172-2175.

Clapham, P.R., Weber, L., Whitby, D., McIntosh, K., Dalgleish, A., Maddon, P., Deen, K., Sweet, R. and Weiss, R. (1989). Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. Nature **337**, 368-379.

Clark, L. (1989). Ph.D. Thesis, University of St. Andrews.

Clark, L., Pollock, R.M. and Hay, R.T. (1988). Identification and purification of EBP1: a HeLa cell protein that binds to a region overlapping the 'core' of the SV40 enhancer. Genes Dev. **2**, 991-1002.

Clark, L., Nicholson, J. and Hay, R.T. (1989). Enhancer Binding Protein (EBP1) makes base and backbone contacts over one complete turn of the DNA double helix. J. Mol. Biol. **206**, 615-626.

Clark, L., Matthews, J.R. and Hay, R.T. (1990). Interaction of enhancer-binding protein EBP1 (NF-κB) with the human immunodeficiency virus type 1 enhancer. J.Virol. **64**, 1335-1344.

Cleat, P.H. and Hay, R.T. (1989). Kinetic analysis of nuclear factor I and its DNA-binding domain with the adenovirus origin of replication. FEBS Letts. **258**, 51-54.

Collart, M.A., Baeuerle, P. and Vassalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. Mol. Cell. Biol. **10**, 1498-1506.

Cooney, A.J., Tsai, S.Y., O'Malley, B.W. and Tsai, M.-J. (1991). Chicken

ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. J.Virol. **65**, 2853-2860.

Crabtree, G.R. (1989). Contingent genetic regulatory events in T lymphocyte activation. Science **243**, 355-361.

Cromlish, J.A. and Roeder, R.G. (1989). Human transcription factor IIIC (TFIIIC). J. Biol. Chem. **264**, 18100-18109.

Cross, S.L., Halden, N.F., Lenardo, M.J. and Leonard, W.J. (1989). Functionally distinct NF-κB binding sites in the immunoglobulin κ and IL-2 receptor α chain genes. Science **244**, 466-469.

Cullen, B.R. and Greene, W.C. (1989). Regulatory pathways governing HIV-1 replication. Cell **58**, 423-426.

Dalgleish, A.G., Beverleym, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F. and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312**, 763-767.

Davis, J.N., Bargmann, W. and Bose, H.R.Jr. (1990). Identification of protein complexes containing the c-*rel* proto-oncogene product in avian hematopoietic cells. Oncogene **5**, 1109-1115.

Davis, N., Ghosh, S., Simmons, D.L., Tempst, P., Liou, H.-C., Baltimore, D. and Bose, H.R. Jr. (1991). Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253**, 1268-1271.

Demarchi, F., D'Agaro, P., Falaschi, A. and Giacca, M. (1992). Probing protein-DNA interactions at the long terminal repeat of human immunodeficiency virus type 1 by in vivo footprinting. J.Virol. **66**, 2514-2518.

Dewhurst, S., Embretson, J.E., Anderson, D.C., Mullins, J.I. and Fultz, P.N. (1990). Sequence analysis and acute pathogenicity of molecularly cloned

SIVSMM-PBj14. Nature **345**, 636-640.

Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. **11**, 1475-1489.

Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A.D., Singh, M. and Skinner, M.A. (1990). HIV-1 *tat* protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO J. **9**, 4145-4153.

Drouet, C., Shakhov, A.N. and Jongeneel, C.V. (1991). Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-α promoter in primary macrophages. J. Immunol. **147**, 1694-1700.

Durand, D.B., Shaw, J.-P., Bush, M.R., Replogle, R.E., Belagaje, R. and Crabtree, G.R. (1988). Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**, 1715-1724.

Duyao, M.P., Buckler, A.J. and Sonenschein, G.E. (1990). Interaction of an NF-κB-like factor with a site upstream of the c-*myc* promoter. Proc. Natl. Acad. Sci. USA **87**, 4727-4731.

Ebright, R.H., Cossart, P., Gicquel-Sanzey, B. and Beckwith, J. (1984). Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E.coli*. Nature **311**, 232-235.

Echols, H. (1986). Multiple DNA-protein interactions governing high-precision DNA transactions. Science **233**, 1050-1056.

Edbrooke, M.R., Burt, D.W., Cheshire, J.K. and Woo, P. (1989). Identification of *cis*-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor κB-like transcription factor. Mol.

Cell. Biol. **9**, 1908-1916.

Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987). Protein serine / threonine kinases. Annu. Rev. Biochem. **56**, 567-613.

Englund, G., Hoggan, M.D., Theodore, T.S. and Martin, M.A. (1991). A novel HIV-1 isolate containing alterations affecting the NF-κB element. Virology **181**, 150-157.

Ensoli, B., Lusso, P., Schachter, F., Josephs, S.F., Rappaport, J., Negro, F., Gallo, R.C. and Wong-Staal, F. (1989). Human herpes virus-6 increases HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. EMBO J. **8**, 3019-3027.

Faktor, O. and Shaul, Y. (1990). The identification of hepatitis B virus X gene responsive elements reveals functional similarity of X and HTLV-I tax. Oncogene **5**, 867-872.

Fan, C.-M. and Maniatis, T. (1991). Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature **354**, 395-398.

Farnet, C. and Haseltine, W. (1990). Integration of human immunodeficiency virus type 1 DNA in vitro. Proc. Natl. Acad. Sci. USA **87**, 4164-4168.

Felber, B.K., Paskalis, H., Kleinman-Ewing, C., Wong-Staal, F. and Pavlakis, G.N. (1985). The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. Science **229**, 675-679.

Feng, S. and Holland, E.C. (1988). HIV-1 *tat trans*-activation requires the loop sequence within TAR. Nature **334**, 165-167.

Folks, T.M., Clouse, K.A., Justement, J., Rabson, A., Duh, E., Kehrl, J.H. and Fauci, A.S. (1989). Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. USA **86**, 2365-2368.

Franza, B.R.Jr., Josephs, S.F., Gilman, M.Z., Ryan, W. and Clarkson, B. (1987). Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature **330**, 391-395.

Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K. and Siebenlist, U. (1992). The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature **359**, 339-342.

Fried, M.G. and Crothers, D.M. (1984). Kinetics and mechanism in the reaction of gene regulatory proteins with DNA. J. Mol. Biol. **172**, 263-282.

Fujii, M., Sassone-Corsi, P. and Verma, I.M. (1988). c-*fos* promoter trans-activation by the tax_1 protein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **85**, 8526-8530.

Fujita, T., Nolan, G.P., Ghosh, S. and Baltimore, D. (1992). Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. **6**, 775-787.

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. Cell **46**, 401-407.

Garcia, J.A., Harrich, D., Soultanakis, E., Wu, F., Mitsuyasu, R. and Gaynor, R.B. (1989). Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J. **8**, 765-778.

Garcia, J.A., Wu, F.K., Mitsuyasu, R. and Gaynor, R.B. (1987). Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J. **6**, 3761-3770.

Garson, K., Percival, H. and Kang, C.-Y. (1990). The N-terminal env-derived

amino acids of v-rel are required for full transforming activity. Virology **177**, 106-115.

Gelderblom, H., Hausmann, E.H., Ozel, M., Pauli, G. and Koch, M.A. (1987). Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **168**, 171-176.

Gélinas, C. and Temin, H.M. (1988). The v-*rel* oncogene encodes a cell-specific transcriptional activator of certain promoters. Oncogene **3**, 349-355.

Ghazal, P., Lubon, H. and Hennighausen, L. (1988). Multiple sequence-specific transcription factors modulate cytomegalovirus enhancer activity in vitro. Mol. Cell. Biol. **8**, 1809-1811.

Ghosh, S. and Baltimore, D. (1990). Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature **344**, 678-682.

Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P. and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF-κB : Homology to *rel* and *dorsal*.. Cell **62**, 1019-1029.

Gilmore, T.D. and Temin, H.M. (1986). Different localization of the product of the v-*rel* oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell **44**, 791-800.

Gimble, J.M., Duh, E., Ostrove, J.M., Gendelman, H.E., Max, E.E. and Rabson, A.B. (1988). Activation of the human immunodeficiency virus long terminal repeat by herpes simplex virus type 1 is associated with induction of a nuclear factor that binds to the NF-κB/core enhancer sequence. J. Virol. **62**, 4104-4112.

Goh, W.C., Sodroski, J., Rosen, C., Essex, M. and Haseltine, W.A. (1985). Subcellular localization of the product of the long open reading frame of human T-cell leukemia vius type I. Science **227**, 1227-1228.

Golub, E.I., Li, G. and Volsky, D.J. (1990). Differences in the basal activating of

the long terminal repeat determine different replicative capacities of two closely related human immunodeficiency virus type 1 isolates. J. Virol. **64**, 3654-3660.

Greene, W.C., Leonard, W.J., Wano, Y., Svetlik, P.B., Peffer, N.J., Sodroski, J.G., Rosen, C.A., Goh, W.C. and Haseltine, W.A. (1986). *Trans*-activator gene of HTLV-II induces IL-2 receptor and IL-2 cellular gene expression. Science **232**, 877-880.

Griffin, G.E., Leung, K., Folks, T.M., Kunkel, S. and Nabel, G.J. (1989). Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature **339**, 70-73.

Grosschedl, R. and Marx, M. (1988). Stable propagation of the active transcriptional state of an immunoglobulin μ gene requires continuous enhancer function. Cell **55**, 645-654.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Münsterberg, A., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature **346**, 245-250.

Gustafson, T.A., Taylor, A. and Kedes, L. (1989). DNA bending is induced by a transcription factor that interacts with the human c-fos and α-actin promoters. Proc. Natl. Acad. Sci. USA **86**, 2162-2166.

Hagman, J., Rudin, C.M., Haasch, D., Chaplin, D. and Storb, U. (1990). A novel enhancer in the immunoglobulin l locus is duplicated and functionally independent of NF-κB. Genes Dev. **4**, 978-992.

Hannink, M. and Temin, H.M. (1989). Transactivation of gene expression by nuclear and cytoplasmic *rel* proteins. Mol. Cell. Biol. **9**, 4323-4336.

Hannink, M. and Temin, H.M. (1990). Structure and autoregulation of the c-*rel* promoter. Oncogene **5**, 1843-1850.

Harrich, D., Garcia, J., Wu, F., Mitsuyasu, R., Gonzalez, J. and Gaynor, R. (1989). Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. J.Virol. **63**, 2585-2591.

Haseltine, W.A. (1991). Molecular biology of the human immunodeficiency virus type 1. FASEB J. **5**, 2349-2360.

Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A.S. Jr. (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65**, 1281-1289.

Hatada, E.N., Nieters, A., Wulczyn, F.G., Naumann, M., Meyer, R., Nucifora, G., McKeithan, T.W. and Scheidereit, C. (1992). The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. Proc. Natl. Acad. Sci. USA **89**, 2489-2493.

He, J.J. and Quiocho, F.A. (1991). A nonconservative serine to cysteine mutation in the sulfate-binding protein, a transport receptor. Science **251**, 1479-1481.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Karn, J., Lowea, D., Singh, M., and Skinner, M.A. (1990). HIV-1 regulator of virion expression (*rev*) protein binds to an RNA stem-loop structure located within the *rev*-responsive element region. Cell **60**, 685-693.

Henkel, T., Zabel, U., van Zee, K., Müller, J.M., Fanning, E. and Baeuerle, P.A. (1992). Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell **68**, 1121-1133.

Herzog, N.K. and Bose, H.R.Jr. (1986). Expression of the oncogene of avian reticuloendotheliosis virus in *Escherichia coli* and identification of the transforming protein in reticuloendotheliosis virus T-transformed cells. Proc.

Natl. Acad. Sci. USA **83**, 812-816.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**, 51-59.

Hohmann, H.-P., Remy, R., Pöschl, B. and van Loon, A.P.G.M. (1990). Tumor necrosis factors-α and -ß bind to the same two types of tumor necrosis factor receptors and maximally activate the transcription factor NF-κB at low receptor occupancy and within minutes after receptor binding. J. Biol. Chem. **265**, 15183-15188.

Hohmann, H.-P., Remy, R., Aigner, L., Brockhaus, M. and van Loon, A.P.G.M. (1992). Protein kinases negatively affect nuclear factor-κB activation by tumor necrosis factor-α at two different stages in promyelocytic HL60 cells. J. Biol. Chem. **267**, 2065-2072.

Holmgren, A. (1985). Thioredoxin. Annu. Rev. Biochem. **54**, 237-271.

Holt, G.D. and Hart, G.W. (1986). The subcellular distribution of terminal N-acetylglucosamine moieties. J. Biol. Chem. **261**, 8049-8057.

Hoyos, B., Ballard, D.W., Böhnlein, E., Siekevitz, M. and Greene, W.C. (1989). Kappa B-Specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. Science **244**, 457-460.

Hromas, R., Pauli, U., Marcuzzi, A., Lafrenz, D., Nick, H., Stein, J., Stein, G. and Van Ness B. (1988). Inducible DNA-protein interactions of the murine kappa immunoglobulin enhancer in intact cells: comparison with *in vitro* interactions. Nucl. Acids Res. **16**, 953-967.

Hunter, T. and Karin, M. (1992). The regulation of transcription by phosphorylation. Cell **70**, 375-387.

Inoue, J.-I., Kerr, L.D., Ransone, L.J., Bengal, E., Hunter, T. and Verma, I.M.

(1991). c-rel activates but v-rel suppresses transcription from κB sites. Proc. Natl. Acad. Sci. USA **88**, 3715-3719.

Inoue, J.-I., Kerr, L.D., Kakizuka, A. and Verma, I.M. (1992a). IκBγ, a 70kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68**, 1109-1120.

Inoue, J.-I., Kerr, L.D., Rashid, D., Davis, N., Bose, H.R.Jr. and Verma, I.M. (1992b). Direct association of pp40/IκBß with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89**, 4333-4337.

Inoue, J.-I., Seiki, M., Taniguchi, T., Tsuru, S. and Yoshida, M. (1986). Induction of interleukin 2 receptor gene expression by $p40^{x}$ encoded by human T-cell leukemia virus type 1. EMBO J. **5**, 2883-2888.

Ip, Y.T., Kraut, R., Levine, M. and Rushlow, C.A. (1991). The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila. Cell **64**, 439-446.

Isoda, K., Roth, S. and Nüsslein-Volhard, C. (1992). The functional domains of the *Drosophila* morphogen *dorsal*: evidence from the analysis of mutants. Genes Dev. **6**, 619-630.

Israël, A., Kimura, A., Kieran, M., Yano, O., Kanellopoulos, J., Le Bail, O. and Kourilsky, P. (1987). A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and ß2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**, 2653-2657.

Israël, A., Le Bail, O., Hatat, D., Piette, J., Kieran, M., Logeat, F., Wallach, D., Fellous, M. and Kourilsky, P. (1989a). TNF stimulates expression of mouse MHC class I genes by inducing an NF-κB-like enhancer binding activity which displaces constitutive factors. EMBO J. **8**, 3793-3800.

Israël, A., Yano, O., Logeat, F., Kieran, M. and Kourilsky, P. (1989b). Two purified factors bind to the same sequence in the enhancer of mouse MHC class I genes: one of them is a positive regulator induced upon differentiation of teratocarcinoma cells. Nucl. Acids Res. **17**, 5245-5257.

Jacks, T., Power, M.D., Masiarz, F.R., Luciw, P.A., Barr, P.J. and Varmus, H.E. (1988). Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression. Nature **331**, 280-283.

Jackson, S.P. and Tjian, R. (1988). O-Glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell **55**, 125-133.

Jackson, S.P. and Tjian, R. (1989). Purification and analysis of RNA polymerase II transcription factors by using wheat germ agglutinin affinity chromatography. Proc. Natl. Acad. Sci. USA **86**, 1781-1785.

Jain, J., Valge-Archer, V.E., Sinskey, A.J. and Rao, A. (1992). The AP-1 site at -150bp, but not the NF-κB site, is likely to represent the major target of protein kinsae C in the interleukin 2 promoter. J. Exp. Med. **175**, 853-862.

Jamieson, C., McCaffrey, P.G., Rao, A. and Sen, R. (1991). Physiologic activation of T cells via the T cell receptor induces NF-κB. J. Immunol. **147**, 416-420.

Jantzen, H-M., Admon, A., Bell, S.P. and Tjian, R. (1990). Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature **344**, 830-836.

Jeang, K.-T., Shank, P.R. and Kumar, A. (1988). Transcriptional activation of homologous viral long terminal repeats by the human immunodeficiency virus type 1 or the human T-cell leukemia virus type I tat proteins occurs in the absence of *de novo* protein synthesis. Proc. Natl. Acad. Sci. USA **85**, 8291-8295.

Jiang, J., Rushlow, C.A., Zhou, Q., Small, S. and Levine, M. (1992). Individual

dorsal morphogen binding sites mediate activation and repression in the *Drosophila* embryo. EMBO J. **11**, 3147-3154.

Jones, K.A. (1989). HIV trans-activation and transcription control mechanisms. New Biol. **1**, 127-135.

Jones, K.A., Kadonaga, J.T., Luciw, P.A., Tjian, R. (1986). Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science **232**, 755-759.

Jones, K.A., Luciw, P.A. and Duchange, N. (1988). Structural arrangements of transcription control domains within the 5' untranslated leader regions of the HIV-1 and HIV-2 promoters. Genes Dev. **2**, 1101-1114.

Kadonaga, J.T. and Tjian, R. (1986). Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**, 5889-5893.

Kang, S.-M., Tran, A.-C., Grilli, M. and Lenardo, M.J. (1992). NF-κB Subunit regulation in nontransformed $CD4^+$ T lymphocytes. Science **256**, 1452-1456.

Kanno, M., Fromental, C., Staub, A., Ruffenach, F., Davidson, I. and Chambon, P. (1989). The SV40 TC-II(κB) and the related $H\text{-}2K^B$ enhansons exhibit different cell type specific and inducible proto-enhancer activities, but the SV40 core sequence and the AP-2 binding site have no enhanson properties. EMBO J. **8**, 4205-4214.

Kao, K.R. and Hopwood, N.D. (1991). Expression of a mRNA related to c-*rel* and dorsal in early *Xenopus laevis* embryos. Proc. Natl. Acad. Sci. USA **88**, 2697-2701.

Kato, H., Horikoshi, M. and Roeder, R.G. (1991). Repression of HIV-1 transcription by a cellular protein. Science **251**, 1476-1479.

Kawakami, K., Scheidereit, C. and Roeder, R.G. (1988). Identification and

purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter *in vitro*. Proc. Natl. Acad. Sci. USA **85**, 4700-4704.

Kearse, K.P. and Hart, G.W. (1991). Lymphocyte activation induces rapid changes in nuclear and cytoplasmic proteins. Proc. Natl. Acad. Sci. USA **88**, 1701-1705.

Kerppola, T.K. and Curran, T. (1991). DNA bending by fos and jun: the flexible hinge model. Science **254**, 1210-1214.

Kerr, L.D., Inoue, J.-I., Davis, N., Link, E., Baeuerle, P.A., Bose, H.R.Jr. and Verma, I.M. (1991). The Rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBß and regulation by phosphorylation. Genes Dev. **5**, 1464-1476.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., LeBail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A. and Israël, A. (1990). The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62**, 1007-1018.

Kim, S., Ikeuchi, R., Byrn, R., Groopman, J. and Baltimore, D. (1989). Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**, 9544-9548.

Kiyokawa, T., Kawaguchi, T., Seiki, M. and Yoshida, M. (1985). Association of the pX gene product of human T-cell leukemia virus type-1 with nucleus. Virology **147**, 462-465.

Klein, S., Gerster, T., Picard, D., Radbruch, A. and Schaffner, W. (1985). Evidence for transient requirement of the IgH enhancer. Nucl. Acids Res. **13**, 8901-8912.

Klimkait, T., Strebel, K., Hoggan, M.D., Martin, M.A. and Orenstein, J.M.

(1990). The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. J. Virol. **64**, 621-629.

Kochel, T., Mushinski, J.F. and Rice, N.R. (1991). The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. Oncogene **6**, 615-626.

Koshland, M.E., Englberger, F. and Koshland, D.E. Jr. (1959). A general method for the labeling of the active site of antibodies and enzymes. Proc. Natl. Acad. Sci. USA **45**, 1470-1475.

Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969). Diamide, a new reagent for the intracellular oxidation of glutathione to the disulphide. Biochem. Biophys. Res. Comm. **37**, 593-596.

Kretzschmar, M., Meisterernst, M., Scheidereit, C., Li, G. and Roeder, R.G. (1992). Transcriptional regulation of the HIV-1 promoter by NF-κB in vitro. Genes Dev. **6**, 761-774.

Ku Tai, P.-K., Albers, M.W., Chang, H., Faber, L.E. and Schreiber, S.L. (1992). Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. Science **256**, 1315-1318.

Kumar, S., Rabson, A.B. and Gelinas, C. (1992). The RxxRxRxxC motif conserved in all rel/κB proteins is essential for the DNA-binding activity and redox regulation of the v-rel oncoprotein. Mol. Cell. Biol. **12**, 3094-3106.

Kuprash, D.V. and Nedospasov, S.A. (1992). Interaction of transcription factors of NF-κB/rel family with DNA results in its significant bending at the binding site. Doklady Akademii Nauk CCCP **322**, 421-426.

Lacoste, J., Cohen, L. and Hiscott, J. (1991). NF-κB activity in T cells stably expressing the Tax protein of human T cell lymphotropic virus type I. Virology **184**, 553-562.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature **227**, 680-685.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**, 1759-1764.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**, 1681-1688.

Laspia, M.F., Rice, A.P. and Matthews, M.B. (1989). HIV-1 *tat* protein increases transcriptional initiation and stabilizes elongation. Cell **59**, 283-292.

Law, R.E., Stimmel, J.B., Damore, M.A., Carter, C., Clarke, S. and Wall, R. (1992). Lipopolysaccharide-induced NF-κB activation in mouse 70Z/3 pre-B lymphocytes is inhibited by mevinolin and 5'-methylthioadenosine: roles of protein isoprenylation and carboxyl methylation reactions. Mol. Cell. Biol. **12**, 103-111.

Leblanc, J.-F., Cohen, L., Rodrigues, M. and Hiscott, J. (1990). Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. Mol. Cell. Biol. **10**, 3987-3993.

Leonard, J., Parrott, C., Buckler-White, A.J., Turner, W., Ross, E.K., Martin, M.A. and Rabson, A.B. (1989). The NF-κB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63**, 4919-4924.

Lenardo, M.J. and Baltimore, D. (1989). NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. Cell **58**, 227-229.

Lenardo, M.J., Kuang, A., Gifford, A. and Baltimore, D. (1988). NF-κB protein purification from bovine spleen: nucleotide stimulation and binding site

specificity. Proc. Natl. Acad. Sci. USA **85**, 8825-8829.

Lenardo, M., Pierce, J.W. and Baltimore, D. (1987). Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science **236**, 1573-1577.

Leung, K. and Nabel, G.J. (1988). HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature **333**, 776-778.

Libermann, T.A. and Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol. Cell. Biol. **10**, 2327-2334.

Lichtsteiner, S. and Schibler, U. (1989). A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. Cell **57**, 1179-1187.

Lilley, D.M.J. (1991). When the CAP fits bent DNA. Nature **354**, 359-360.

Lim, M.-Y., Davis, N., Zhang, J. and Bose, H.R.Jr. (1990). The v-*rel* oncogene product is complexed with cellular proteins including its proto-oncogene product and heat shock protein 70. Virology **175**, 149-160.

Lin, B.B., Cross, S.L., Halden, N.F., Roman, D.G., Toledano, M.B. and Leonard, W.J. (1990). Delineation of an enhancerlike positive regulatory element in the interleukin-2 receptor α-chain gene. Mol. Cell. Biol. **10**, 850-853.

Lindholm, P.F., Marriott, S.J., Gitlin, S.D, Bohan, C.A. and Brady, J.N. (1990). Induction of nuclear NF-κB DNA binding activity after exposure of lymphoid cells to soluble Tax_1 protein. New Biol. **2**, 1034-1043.

Lindholm, P.F., Reid, R.L. and Brady, J.N. (1992). Extracellular Tax_1 protein stimulates tumor necrosis factor-ß and immunoglobulin kappa light chain expression in lymphoid cells. J. Virol. **66**, 1294-1302.

Link, E., Kerr, L.D., Schreck, R., Zabel, U., Verma, I. and Baeuerle, P.A. (1992). Purified IκB-ß is inactivated upon dephosphorylation. J. Biol. Chem. **267**, 239-246.

Liou, H.-C., Nolan, G.P., Ghosh, S., Fujita, T. and Baltimore, D. (1992). The NF-κB p50 precursor, p105, contains an internal IκB-like inhibitor that preferentially inhibits p50. EMBO J. **11**, 3003-3009.

Liu, J., Chiles, T.C., Sen, R. and Rothstein, T.L. (1991). Inducible nuclear expression of NF-κB in primary B cells stimulated through the surface Ig receptor. J. Immunol. **146**, 1685-1691.

Lowenthal, J.W., Ballard, D.W., Böhnlein, E. and Greene, W.C. (1989). Tumor necrosis factor α induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. Proc Natl. Acad. Sci. USA **86**, 2331-2335.

Lu Y., Stenzel, M., Sodroski, J.G. and Haseltine, W.A. (1989). Effects of long terminal repeat mutations on human immunodeficiency virus type 1 replication. J.Virol. **63**, 4115-4119.

Lucito, R. and Schneider, R.J. (1992). Hepatitis B virus X protein activates transcription factor NF-κB without a requirement for protein kinase C. J. Virol. **66**, 983-991.

Maki, Y., Bos, T.J., Davis, C., Starbuck, M. and Vogt, P.K. (1987). Avian sarcoma virus 17 carries the jun oncogene. Proc. Natl. Acad. Sci. USA **84**, 2848-2852.

Malim, M.H., Tiley, L.S., McCarn, D.F., Rusche, J.R., Hauber, J. and Cullen, B.R. (1990). HIV-1 structural gene expression requires binding of the *rev* *trans*-activator to its RNA target sequence. Cell **60**, 675-683.

Marais, R.M., Hsuan, J.J., McGuigan, C., Wynne, J. and Treisman, R. (1992).

Casein kinase II phosphorylation increases the rate of serum response factor-binding site exchange. EMBO J. **11**, 97-105.

Marciniak, R.A., Garcia-Blanco, M.A. and Sharp, P.A. (1990). Identification and characterization of a HeLa nuclear protein that specifically binds to the *trans*-activation-responsive (TAR) element of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87**, 3624-3628.

Marriott, S.J., Lindholm, P.F., Brown, K.M., Gitlin, S.D., Duvall, J.F., Radonovich, M.F. and Brady, J.N. (1990). A 36-kilodalton cellular transcription factor mediates an indirect interaction of human T-cell leukemia/lymphoma virus type I TAX_1 with a responsive element in the viral long terminal repeat. Mol. Cell. Biol. **10**, 4192-4201.

Martin, M.E.D., Nicholas, J., Thomson, B.J., Newman, C. and Honess, R.W. (1991). Identification of a transactivating function mapping to the putative immediate-early locus of human herpesvirus 6. J. Virol. **65**, 5381-5390.

Matsuyama, T., Hamamoto, Y., Soma, G.-I., Mizuno, D., Yamamoto, N. and Kobayashi, N. (1989). Cytocidal effect of tumor necrosis factor on cells chronically infected with human immunodeficiency virus (HIV): enhancement of HIV replication. J. Virol. **63**, 2504-2509.

Matthews, J.R., Wakasugi, N., Virelizier, J.-L., Yodoi, J. and Hay, R.T. (1992). Thioredoxin regulates the DNA binding activity of NF-κB by reduction of a disulphide bond involving cysteine 62. Nucl. Acids Res. **20**, 3821-3830.

Mauxion, F., Jamieson, C., Yoshida, M., Arai, K.-I. and Sen, R. (1991). Comparison of constitutive and inducible transcriptional enhancement mediated by κB-related sequences: modulation of activity in B cells by human T-cell leukemia virus type I *tax* gene. Proc. Natl. Acad. Sci. USA **88**, 2141-2145.

Meyer, K.B. and Neuberger, M.S. (1989). The immunoglobulin κ locus contains a second, stronger B-cell-specific enhancer which is located downstream of the constant region. EMBO J. **8**, 1959-1964.

Meyer, K.B., Sharpe, M.J., Surani, M.A. and Neuberger, M.S. (1990). The importance of the 3'-enhancer region in immunoglobulin κ gene expression. Nucl. Acids Res. **18**, 5609-5615.

Meyer, M., Caselmann, W.H., Schlüter, V., Schreck, R., Hofschneider, P.H. and Baeuerle, P.A. (1992). Hepatitis B virus transactivator MHBst: activation of NF-κB, selective inhibition by antioxidants and integral membrane localization. EMBO J. **11**, 2991-3001.

Meyer, R., Hatada, E.N., Hohmann, H.-P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H.-W., Schlaeger, E.J., van Loon, A.P.G.M. and Scheidereit, C. (1991). Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **88**, 966-970.

Miller, R.H. and Robinson, W.S. (1986). Common evolutionary origin of hepatitis B virus and retroviruses. Proc. Natl. Acad. Sci. USA **83**, 2531-2535.

Miyatake, S., Seiki, M., Yoshida, M. and Arai, K.-I. (1988). T-cell activation signals and human T-cell leukemia virus type I-encoded $p40^{x}$ protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. Mol. Cell. Biol. **8**, 5581-5587.

Moore, B.E. and Bose, H.R.Jr. (1989). Expression of the c-*rel* and c-*myc* proto-oncogenes in avian tissues. Oncogene **4**, 845-852.

Morin, P.J. and Gilmore, T.D. (1992). The C terminus of the NF-κB p50 precursor and an IκB isoform contain transcriptional activation domains. Nucl.

Acids Res. **20**, 2453-2458.

Morrison, L.E., Kabrun, N., Mudri, S., Hayman, M.J. and Enrietto, P.J. (1989). Viral *rel* and cellular *rel* associate with cellular proteins in transformed and normal cells. Oncogene **4**, 677-683.

Muchardt, C., Seeler, J.-S., Nirula, A., Shurland, D.-L. and Gaynor, R.B. (1992). Regulation of human immunodeficiency virus enhancer function by PRDII-BF1 and c-*rel* gene products. J. Virol. **66**, 244-250.

Muesing, M.A., Smith, D.H. and Capon, D.J. (1987). Regulation of mRNA accumulation by a human immunodeficiency virus *trans*-activator protein. Cell **48**, 691-701.

Mukaida, N., Mahe, Y. and Matsushima, K. (1990). Cooperative interaction of nuclear factor-κB-and *cis*-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. J. Biol. Chem. **265**, 21128-21133.

Murre, C., McCaw, P. and Baltimore, D. (1989). A new DNA-binding and dimerization motif in immunoglobulin enhancer binding, daughterless, myoD, and myc proteins. Cell **56**, 777-783.

Nabel, G. and Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **326**, 711-713.

Nelson, J.A. and Groudine, M. (1986). Transcriptional regulation of the human cytomegalovirus major immediate-early gene is associated with induction of DNase I-hypersensitive sites. Mol. Cell. Biol. **6**, 452-461.

Nelson, K.J., Kelley, D.E. and Perry, R.P. (1985). Inducible transcription of the unrearranged κ constant region locus is a common feature of pre-B cells and does not require DNA or protein synthesis. Proc. Natl. Acad. Sci. USA **82**, 5305-5309.

Nerenberg, M., Hinrichs, S.H., Reynolds, R.K., Khoury, G. and Jay, G. (1987).

The *tat* gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science **237**, 1324-1329.

Nielsch, U., Zimmer, S.G. and Babiss, L.E. (1991). Changes in NF-κB and ISGF3 DNA binding activities are responsible for differences in MHC and ß-IFN gene expression in Ad5- versus Ad12-transformed cells. EMBO J. **10**, 4169-4175.

Nolan, G.P., Ghosh, S., Liou, H.-C., Tempst, P. and Baltimore, D. (1991). DNA Binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64**, 961-969.

Nonaka, M. and Huang, Z.-M. (1990). Interleukin-1-mediated enhancement of mouse factor B gene expression via NF-κB-like hepatoma nuclear factor. Mol. Cell. Biol. **10**, 6283-6289.

Novak, T.J., Chen, D. and Rothenberg, E. (1990). Interleukin-1 synergy with phosphoinositide pathway agonists for induction of interleukin-2 gene expression: molecular basis of costimulation. Mol. Cell. Biol. **10**, 6325-6334.

Numata, N., Ohtani, K., Niki, M., Nakamura, M. and Sugamura, K. (1991). Synergism between two distinct elements of the HTLV-I enhancer during activation by the trans-activator of HTLV-I. New Biol. **3**, 896-906.

Ogawa, K., Shibata, R., Kiyomasu, T., Higuchi, I., Kishida, Y., Ishimoto, A. and Adachi, A. (1989). Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. J. Virol. **63**, 4110-4114.

Orchard, K., Perkins, N., Chapman, C., Harris, J., Emery, V., Goodwin, G., Latchman, D. and Collins, M. (1990). A novel T-cell protein which recognizes a palindromic sequence in the negative regulatory element of the human immunodeficiency virus long terminal repeat. J.Virol. **64**, 3234-3239.

Osborn, L., Kunkel, S. and Nabel, G.J. (1989). Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation

of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**, 2336-2340.

Parrott, C., Seidner, T., Duh, E., Leonard, J., Theodore, T.S., Buckler-White, A., Martin, M.A. and Rabson, A.B. (1991). Variable role of the long terminal repeat Sp1-binding sites in human immunodeficiency virus replication in T lymphocytes. J. Virol. **65**, 1414-1419.

Paul, N.L., Lenardo, M.J., Novak, K.D., Sarr, T., Tang, W.-L. and Ruddle, N.H. (1990). Lymphotoxin activation by human T-cell leukemia virus type I-infected cell lines: role for NF-κB. J. Virol. **64**, 5412-5419.

Pessara, U. and Koch, N. (1990). Tumor necrosis factor α regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-κB-like factor to a promoter element. Mol. Cell. Biol. **10**, 4164-4154.

Pettersson, S., Cook, G.P., Brüggemann, M., Williams, G.T. and Neuberger, M.S. (1990). A second B cell-specific enhancer 3' of the immunoglobulin heavy-chain locus. Nature **344**, 165-168.

Phares, W. and Herr, W. (1991). Functional similarities between human immunodeficiency virus type 1 and simian virus 40 κB proto-enhancers. J. Virol. **65**, 2200-2210.

Pierce, J.W., Gifford, A.M. and Baltimore, D. (1991). Silencing of the expression of the immunoglobulin kappa gene in non-B cells. Mol. Cell. Biol. **11**, 1431-1437.

Pierce, J.W., Lenardo, M. and Baltimore, D. (1988). Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**, 1482-1486.

Plum, G.E. and Bloomfield, V.A. (1990). Effects of spermidine and hexaamminecobalt (III) on thymine imino proton exchange. Biochemistry **29**, 5934-5940.

Pomerantz, J.L., Mauxion, F., Yoshida, M., Greene, W.C. and Sen, R. (1989). A

second sequence element located 3' to the NF-κB binding site regulates IL-2 receptor-α gene induction. J. Immunol. **143**, 4275-4281.

Pomerantz, R.J., Feinberg, M.B., Trono, D. and Baltimore, D. (1990). Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. J. Exp. Med. **172**, 253-261.

Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984). Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**, 297-300.

Pozzatti, R., Vogel, J. and Jay, G. (1990). The human T-lymphotropic virus type 1 *tax* gene can cooperate with the *ras* oncogene to induce neoplastic trannsformation of cells. Mol. Cell. Biol. **10**, 413-417.

Radler-Pohl, A., Pfeuffer, I., Karin, M. and Serfling, E. (1990). A novel T-cell trans-activator that recognizes a phorbol ester-inducible element of the interleukin-2 promoter. New Biol. **2**, 566-573.

Randak, C., Brabletz, T., Hergenrother, M., Sobotta, I. and Serfling, E. (1990). Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. EMBO J. **9**, 2529-2536.

Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R.Jr, Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C. and Wong-Staal, F. (1985). Complete nucleotide sequence of the AIDS virus HTLV-III. Nature **313**, 277-284.

Rattner, A., Korner, M., Rosen, H., Baeuerle, P.A. and Citri, Y. (1991). Nuclear factor κB activates proenkephalin transcription in T lymphocytes. Mol. Cell. Biol. **11**, 1017-1022.

Raziuddin, Mikovits, J.A., Calvert, I., Ghosh, S., Kung, H.-F. and Ruscetti, F.W. (1991). Negative regulation of human immunodeficiency virus type 1 expression in monocytes: role of the 65-kDa plus 50-kDa NF-κB dimer. Proc. Natl. Acad. Sci. USA **88**, 9426-9430.

Rich, A., Nordheim, A. and Wang, A.H.-J. (1984). The chemistry and biology of left-handed Z-DNA. Annu. Rev. Biochem. **53**, 791-846.

Rihs, H.-P., Jans, D.A., Fan, H. and Peters, R. (1991). The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. EMBO J. **10**, 633-639.

Riviere, Y., Blank, V., Kourilsky, P. and Israël, A. (1991). Processing of the precursor of NF-κB by the HIV-1 protease during acute infection. Nature **350**, 625-626.

Roederer, M., Staal, F.J.T., Raju, P.A., Ela, S.W., Herzenberg, L.A. and Herzenberg, L.A. (1990). Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. Proc. Natl. Acad. Sci. USA **87**, 4884-4888.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234**, 364-368.

Roman, D.G., Toledano, M.B. and Leonard, W.J. (1990). Sp1 Represses IL-2 receptor α chain gene expression. New Biol. **2**, 642-647.

Ron, D., Brasier, A.R., Wright, K.A. and Habener, J.F. (1990). The permissive role of glucocorticoids on interleukin-1 stimulation of angiotensinogen gene transcription is mediated by an interaction between inducible enhancers. Mol. Cell. Biol. **10**, 4389-4395.

Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1985). Location of cis-acting regulatory sequences in the human T-cell lymphotropic virus type III

(HTLV-III/LAV) long terminal repeat. Cell **41**, 813-823.

Ross, E.K., Buckler-White, A.J., Rabson, A.B., Englund, G., and Martin, M.A. (1991). Contribution of NF-κB and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. J. Virol. **65**, 4350-4358.

Roulston, A., D'Addario, M., Boulerice, F., Caplan, S., Wainberg, M.A. and Hiscott, J. (1992). Induction of monocytic differentiation and NF-κB-like activities by human immunodeficiency virus 1 infection of myelomonoblastic cells. J. Exp. Med. **175**, 751-763.

Roux, P., Blanchard, J.-M., Fernandez, A., Lamb, N., Jeanteur, P. and Piechaczyk, M. (1990). Nuclear localization of c-Fos, but not v-Fos proteins, is controlled by extracellular signals. Cell **63**, 341-351.

Roy, S., Delling, U., Chen, C.-H., Rosen, C.A. and Sonenberg, N. (1990). A bulge structure in HIV-1 TAR RNA is required for tat binding and tat-mediated *trans*-activation. Genes Dev. **4**, 1365-1373.

Roy, S., Parkin, N.T., Rosen, C., Itovitch, J. and Sonenberg, N. (1990). Structural requirements for *trans* activation of human immunodeficiency virus type 1 long terminal repeat-directed gene expression by *tat*:: importance of base pairing, loop sequence, and bulges in the *tat*-responsive sequence. J.Virol. **64**, 1402-1406.

Ruben, S.M., Dillon, P.J., Schreck, R., Henkel. T., Chen, C.-H., Maher, M., Baeuerle, P.A. and Rosen, C.A. (1991). Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251**, 1490-1493.

Ruben, S.M., Klement, J.F., Coleman, T.A., Maher, M., Chen, C.-H. and Rosen, C.A. (1992b). I-Rel: a novel *rel*-related protein that inhibits NF-κB transcriptional activity. Genes Dev. **6**, 745-760.

Ruben, S.M., Narayanan, R., Klement, J.F., Chen, C.-H. and Rosen, C.A. (1992a).

Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. **12**, 444-454.

Ruben, S.M., Poteat, H., Tan, T.-H., Kawakami, K., Roeder, R.G., Haseltine, W.A. and Rosen, C.A. (1988). Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-I *tax* gene product. Science **241**, 89-92.

Rushlow, C.A., Han, K., Manley, J.L. and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in Drosophila. Cell **59**, 1165-1177.

Ryseck, R.-P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P. and Bravo, R. (1992). Rel B, a new rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12**, 674-684.

Sakaguchi, M., Zenzie-Gregory, B., Groopman, J.E., Smale, S.T. and Kim, S. (1991). Alternative pathway for induction of human immunodeficiency virus gene expression: involvement of the general transcription machinery. J. Virol. **65**, 5448-5456.

Sambucetti, L.C., Cherrington, J.M., Wilkinson, G.W.G. and Mocarski, E.S. (1989). NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8**, 4251-4258.

Schmid, R.M., Perkins, N.D., Duckett, C.S., Andrews, P.C. and Nabel, G.J. (1991). Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature **352**, 733-736.

Schmidt, A., Hennighausen, L. and Siebenlist, U. (1990). Inducible nuclear factor binding to the κB elements of the human immunodeficiency virus enhancer in T cells can be blocked by cyclosporin A in a signal-dependent manner. J. Virol. **64**, 4037-4041.

Schmidt, M.C., Zhou, Q. and Berk, A.J. (1989). Sp1 Activates transcription

without enhancing DNA-binding activity of the TATA box factor. Mol. Cell. Biol. **9**, 3299-3307.

Schmitz, M.L. and Baeuerle, P.A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10**, 3805-3817.

Schreck, R. and Baeuerle, P.A. (1990). NF-κB as inducible transcriptional activator of the Granulocyte-Macrophage Colony-Stimulating Factor gene. Mol. Cell. Biol. **10**, 1281-1286.

Schreck, R., Rieber, P. and Baeuerle, P.A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10**, 2247-2258.

Schreck, R., Zorbas, H., Winnacker, E.-L. and Baeuerle, P.A. (1990). The NF-κB transcription factor induces DNA bending which is modulated by its 65-kD subunit. Nucl. Acids Res. **18**, 6497-6502.

Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M. and Evans, R.M. (1990). Functional antagonism between oncoprotein c-*jun* and the glucocorticoid receptor. Cell **62**, 1217-1226.

Schultz, S.C., Shields, G.C. and Steitz, T.A. (1991). Crystal structure of a CAP-DNA complex: the DNA is bent by 90°. Science **253**, 1001-1007.

Seiki, M., Inoue, J.-I., Takeda, T. and Yoshida, M. (1986). Direct evidence that $p40^{x}$ of human T-cell leukemia virus type I is a *trans*-acting transcriptional activator. EMBO J. **5**, 561-565.

Selby, M.J. and Peterlin, B.M. (1990). *Trans*-activation by HIV-1 tat via a heterologous RNA binding protein. Cell **62**, 769-776.

Sen, R. and Baltimore, D. (1986a). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**, 705-716.

Sen, R. and Baltimore, D. (1986b). Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**, 921-928.

Shaw, J-P.,Utz, P.J., Durand, D.B., Toole, J.J., Emmel, E.A. and Crabtree, G.R. (1988). Identification of a putative regulator of early T-cell activation genes. Science **241**, 202-205.

Shibuya, H., Yoneyama, M. and Taniguchi, T. (1989). Transcriptional activation of homologous viral long terminal repeats by the human immunodeficiency virus type 1 or the human T-cell leukemia virus type I tat proteins occurs in the absence of *de novo* protein synthesis. Intern'l Immun. **1**, 143-149.

Shirakawa, F., Chedid, M., Suttles, J., Pollok, P.A. and Mizel, S.B. (1989). Interleukin 1 and cyclic AMP induce κ immunoglobulin light-chain expression via the activation of an NF-κB-like DNA binding protein. Mol. Cell. Biol. **9**, 959-964.

Shirakawa, F. and Mizel, S.B. (1989). In vitro activation and nuclear translocation of NF-κB catalysed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9**, 2424-2430.

Siddiqui, A., Gaynor, R., Srinivasan, A., Mapoles, J. and Farr, R.W. (1989). *trans*-activation of viral enhancers including long terminal repeat of the human immunodeficiency virus by the hepatitis B virus X protein. Virology **169**, 479-484.

Siekevitz, M., Feinberg, M.B., Holbrook, N., Wong-Staal, F., and Greene, W.C. (1987a). Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (*tat*) gene product of human T-cell leukemia virus, type I. Proc. Natl. Acad. Sci. USA **84**, 5389-5393.

Siekevitz, M., Josephs, S.F., Dukovich, M., Peffer, N., Wong-Staal, F. and

Greene, W.C. (1987b). Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-1. Science **238**, 1575-1578.

Silva, C.M. and Cidlowski, J.A. (1989). Direct evidence for intra- and intermolecular disulphide bond formation in the human glucocorticoid receptor. J. Biol. Chem. **264**, 6638-6647.

Sinensky, M. and Lutz, R.J. (1992). The prenylation of proteins. BioEssays **14**, 25-31.

Smale, S.T. and Baltimore, D. (1989). The 'initiator' as a transcription control element. Cell **57**, 103-113.

Smith, D.B. and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**, 31-40.

Snyder, G.H., Cennerazzo, M.J., Karalis, A.J. and Field, D. (1981). Electrostatic influence of local cysteine environments on disulphide exchange kinetics. Biochemistry **20**, 6509-6519.

Sodroski, J.G., Rosen, C.A. and Haseltine, W.A. (1984). *Trans*-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**, 381-385.

Southgate, C., Zapp, M.L. and Green, M.R. (1990). Activation of transcription by HIV-1 tat protein tethered to nascent RNA through another protein. Nature **345**, 640-642.

Staal, F.J.T., Roederer, M., Herzenberg, L.A. and Herzenberg, L.A. (1990). Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87**, 9943-9947.

Stein, B., Rahmsdorf, H.J., Steffen, A., Litfin, M. and Herrlich, P. (1989). UV-Induced DNA damage is an intermediate step in UV-induced expression of

human immunodeficiency virus type 1, collagenase, c-fos, and metallothionein. Mol. Cell. Biol. **9**, 5169-5181.

Steward, R. (1987). *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-*rel*. Science **238**, 692-694.

Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T. and Martin, M. (1987). The HIV'A'(sor) gene product is essential for virus infectivity. Nature **328**, 728-730.

Strubin, M. and Struhl, K. (1992). Yeast and human TFIID with altered DNA-binding specificity for TATA elements. Cell **68**, 721-730.

Suzan, M., Salaun, D., Neuvent, C., Spire, B., Hirsch, I., LeBouteiller, P., Querat, G. and Sire, J. (1991). Induction of NF-κB during monocyte differentiation by HIV type 1 infection. J. Immunol. **146**, 377-383.

Tabor, C.W. and Tabor, H. (1984). Polyamines. Annu. Rev. Biochem. **53**, 749-790.

Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K., Yokota, T., Wakasugi, H. and Yodoi, J. (1989). ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. EMBO J. **8**, 757-764.

Ten, R.M., Paya, C.V., Israël, N., LeBail, O., Mattei, M.-G., Virelizier, J.-L., Kourilsky, P. and Israël, A. (1992). The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J. **11**, 195-203.

Thevenin, C., Kim, S.-J., Rieckmann, P., Fujiki, H., Norcross, M.A., Sporn, M.B., Fauci, A.S. and Kehrl, J.H. (1990). Induction of nuclear factor-κB and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific

inhibitor of phosphatases 1 and 2A. The New Biol. **2**, 793-800.

Thompson, J.F. and Landy, A. (1988). Empirical estimation of protein-induced DNA bending angles: applications to λ site-specific recombination complexes. Nucleic Acids Res. **16**, 9687-9705.

Toledano, M.B. and Leonard, W.J. (1991). Modulation of transcription factor NF-κB binding activity by oxidation-reduction in vitro. Proc. Natl. Acad. Sci. USA **88**, 4328-4332.

Tong-Starksen, S.E., Luciw, P.A. and Peterlin, B.M. (1987). Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. Proc. Natl. Acad. Sci. USA **84**, 6845-6849.

Tong-Starksen, S.E., Luciw, P.A. and Peterlin, B.M. (1989). Signaling through T lymphocyte surface proteins, TCR/CD3 and CD28, activates the HIV-1 long terminal repeat. J. Immunol. **142**, 702-707.

Toyama, R., Bende, S.M. and Dhar, R. (1992). Transcriptional activity of the human immunodeficiency virus-1 LTR promoter in fission yeast *Schizosaccharomyces pombe*. Nucleic Acids Res. **20**, 2591-2596.

Travers, A.A. (1990). Why bend DNA ? Cell **60**, 177-180.

Twigg, A.J. and Sherratt, D. (1980). Trans-complementable copy-number mutants of plasmid ColE1. Nature **283**, 216-218.

Twu, J.-S. and Robinson, W.S. (1989). Hepatitis B virus X gene can transactivate heterologous viral sequences. Proc. Natl. Acad. Sci. USA **86**, 2046-2050.

Tzamarias, D., Pu, W.T. and Struhl, K. (1992). Mutations in the bZIP domain of yeast GCN4 that alter DNA-binding specificity. Proc. Natl. Acad. Sci. USA **89**, 2007-2011.

Urban, M.B. and Baeuerle, P.A. (1990). The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. Genes Dev. **4**,

1975-1984.

Urban, M.B., Schreck, R. and Baeuerle, P.A. (1991). NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10**, 1817-1825.

Vaishnav, Y.N. and Wong-Staal, F. (1991). The biochemistry of AIDS. Annu. Rev. Biochem. **60**, 577-630.

Veronese, P.D.M., Copeland, T.D., Oroszlan, S., Gallo, R.C. and Sarngadharan, M.G. (1988). Biochemical and immunological analysis of human immunodeficiency virus *gag* gene products p17 and p24. J. Virol. **62**, 795-801.

Verrijzer, C.P., Kal, A.J. and van der Vliet, P.C. (1990). The oct-1 homeo domain contacts only part of the octamer sequence and full oct-1 DNA-binding activity requires the POU-specific domain. Genes Dev. **4**, 1964-1974.

Van Lint, C., Burny, A. and Verdin, E. (1991). The intragenic enhancer of human immunodeficiency virus type 1 contains functional AP-1 binding sites. J. Virol. **65**, 7066-7072.

Visvanathan, K.V. and Goodbourn, S. (1989). Double-stranded RNA activates binding of NF-κB to an inducible element in the human ß-interferon promoter. EMBO J. **8**, 1129-1138.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985). Nucleotide sequence of the AIDS virus, LAV. Cell **40**, 9-17.

Wakasugi, N. (1992). Submitted for publication.

Wall, R., Briskin, M., Carter, C., Govan, H., Taylor, A. and Kincade, P. (1986). A labile inhibitor blocks immunoglobulin κ-light-chain-gene transcription in a pre-B leukemic cell line. Proc. Natl. Acad. Sci. USA **83**, 295-298.

Wano, Y., Feinberg, M., Hosking, J.B., Bogerd, H. and Greene, W.C. (1988). Stable expression of the *tax* gene of type I human T-cell leukemia virus in human T cells activates specific cellular genes involved in growth. Proc. Natl. Acad. Sci.

USA **85**, 9733-9737.

Waterman, M.L., Fischer, W.H. and and Jones, K.A. (1991). A thymus-specific member of the HMG protein family regulates the human T cell receptor Cα enhancer. Genes Dev. **5**, 656-669.

Waterman, M.L. and Jones, K.A. (1990). Purification of TCF-1α, a T-cell specific transcription factor that activates the T-cell receptor Cα gene enhancer in a context-dependent manner. The New Biologist **2**, 621-636.

Wen, L., Huang, J.K., Johnson, B.H. and Reeck, G.R. (1989). A human placental cDNA clone that encodes nonhistone chromosomal protein HMG-1. Nucleic Acids Res. **17**, 1197-1214.

Wightman, P.D. and Raetz, C.R.H. (1984). The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J. Biol. Chem. **259**, 10048-10052.

Wilhelmsen, K.C., Eggleton, K. and Temin, H.M. (1984). Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. J. Virol. **52**, 172-182.

Williams, J.L., Garcia, J., Harrich, D., Pearson, L., Wu, F. and Gaynor, R. (1990). Lymphoid specific gene expression of the adenovirus early region 3 promoter is mediated by NF-κB binding motifs. EMBO J. **9**, 4435-4442.

Williams, S.C., Cantwell, C.A. and Johnson, P.F. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev. **5**, 1553-1567.

Winter, R.B., Berg, O.G. and von Hippel, P.H. (1981). Diffusion-driven mechanisms of protein translocation on nucleic acids. 3. The *Escherichia coli lac* repressor-operator interaction: kinetic measurements and conclusions. Biochemistry **20**, 6961-6977.

Wirth, T. and Baltimore, D. (1988). Nuclear factor NF-κB can interact functionally with its cognate binding site to provide lymphoid-specific promoter function. EMBO J. **7**, 3109-3113.

Wong-Staal, F., Hahn, B., Manzari, V., Colombini, S., Franchini, G., Gelmann, E.P. and Gallo, R.C. (1983). A survey of human leukaemias for sequences of a human retrovirus. Nature **302**, 626-628.

Wu, F., Garcia, J., Sigman, D. and Gaynor, R. (1991). Tat regulates binding of the human immunodeficiency virus *trans*-activating region RNA loop-binding protein TRP-185. Genes Dev. **5**, 2128-2140.

Wu, H.-M. and Crothers, D.M. (1984). The locus of sequence-directed and protein-induced DNA bending. Nature **308**, 509-513.

Wulczyn, F.G., Naumann, M. and Scheidereit, C. (1992). Candidate proto-oncogene *bcl-3* encodes a subunit-specific inhibitor of transcription factor NF-κB. Nature **358**, 597-599.

Xanthoudakis, S. and Curran, T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. **11**, 653-665.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C.E. and Curran, T. (1992). Redox activation of fos-jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J. **11**, 3323-3335.

Xu, Y.-L., Adya, N., Siores, E., Gao, Q. and Giam, C.-Z. (1990). Cellular factors involved in transcription and *Tax*-mediated *trans*-activation directed by the TGACGT motifs in human T-cell leukemia virus type I promoter. J. Biol. Chem. **265**, 20285-20292.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains : nucleotide sequences of the M13mp18 and

pUC19 vectors. Gene **33**, 103-119.

Yano, O., Kanellopoulos, J., Kieran, M., LeBail, O., Israël, A. and Kourilsky, P. (1987). Purification of KBF1, a common factor binding to both H-2 and ß2-microglobulin enhancers. EMBO J. **6**, 3317-3324.

Yoshida, M. and Seiki, M. (1987). Recent advances in the molecular biology of HTLV-I : Trans-activation of viral and cellular genes. Annu. Rev. Immunol. **5**, 541-559.

Yoshimura, T., Fujisawa, J.-I. and Yoshida, M. (1990). Multiple cDNA clones encoding nuclear proteins that bind to the *tax*-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. EMBO J. **9**, 2537-2542.

Yu, D.-Y., Huang, Z.-M., Murakami, S., Takahashi, M. and Nonaka, M. (1989). Specific binding of a hepatoma nuclear factor to the NF-κB/H2TF1 recognition motif found in the C4 promoter, but not in the S1p promoter. J. Immunol. **143**, 2395-2400.

Zabel, U. and Baeuerle, P.A. (1990). Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61**, 255-265.

Zabel, U., Schreck, R. and Baeuerle, P.A. (1991). DNA Binding of purified transcription factor NF-κB. J. Biol. Chem. **266**, 252-260.

Zhang, Y., Lin, J.-X. and Vilcek, J. (1990). Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a κB-like sequence. Mol. Cell. Biol. **10**, 3818-3823.

Zhu, L. and Jones, P.P. (1990). Transcriptional control of the invariant chain gene involves promoter and enhancer elements common to and distinct from major histocompatibility complex class II genes. Mol. Cell. Biol. **10**, 3906-3916.

Zimmerman, K., Dobrovnik, M., Ballaun, C., Bevec, D., Hauber, J. and Böhnlein, E. (1991). *trans*-activation of the HIV-1 LTR by the HIV-1 Tat and HTLV-I Tax proteins is mediated by different *cis*-acting sequences. Virology **182**, 874-878.

Zorbas, H., Rogge, L., Meisterernst, M. and Winnacker, E.-L. (1989). Hydroxyl radical footprints reveal novel structural features around the NFI binding site in adenovirus DNA. Nucleic Acids Res. **17**, 7735-7748.