Role of hnRNP A1 in NF-κB Transcriptional Activation.

David Colin Hay

School of Biomedical Sciences

University of St. Andrews

A thesis submitted for the degree of Doctor of Philosophy

November 1999

DECLARATION

I, David Colin Hay, hereby certify that this thesis, which is approximately 60,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for higher degree.

Date 16/03/2000 Signature of candidate

I was admitted as research student in October 1996 and as a candidate for the degree of Doctor of Philosophy in October 1996; the higher study for which this is a record was carried out in the Faculty of Sciences of the University of St Andrews between 1996 and 1999.

Date 16/03/2000 Signature of candidate..

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date..... Signature of supervisor.....

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 supplied to any *bona fide* library or research worker.

Date 16/03/2000 Signature of candidate

CONTENTS

LIST OF ABBREVIATIONS

3AT	3 Amino-Triazole
AMP	Adenosine monophosphate
ARD	Ankyrin repeat domain
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
c-IAP1	Cellular inhibitor of apoptosis
CAS1	Cellular apoptosis susceptibility protein
cDNA	Complementary DNA
CRM1	Chromosome region maintenance 1
D-MEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Eschericia coli*
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
ECL	Enhanced chemiluminesence
EDTA	Ethylenediaminetetracetic acid
eIF	Eukaryotic initiation factor
FCS	Foetal calf serum
GST	Glutathione S-transferase
GTF	General transcription factor
HAC	Histone acetylase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1
HPLC	High performance liquid chromatography
IBB	Importin β binding domain
ICER	Inducible cAMP early repressor
Ig	Immunoglobulin
Ig	Immunoglobulin
IKAP	IKK complex associated protein
IκB	Inhibitor kappa B

IKK1 (α)	IκB kinase 1 (α)
IKK2 (β)	IκB kinase 2 (β)
IL-1	Interleukin 1
iNAF	Inducible negatively acting factor
IPTG	Isopropyl-b-D-thiogalactopyranoside
ITF	Inducible transcription factor
KCl	Potassium chloride
KDa	Kilo Dalton
LB	Luria broth
LPS	Lipopolisaccharide
LTR	Long terminal repeat sequence
MAPKKK	Mitogen activated protein kinase kinase kinase
MW	Molecular weight
NEMO	NF-kappa B essential modulator
NES	Nuclear export sequence
NF-κB	Nuclear factor kappa B
NIK	NF-κB interacting kinase
NLS	Nuclear localisation signal
NP-40	Nonidet P-40
NPC	Nuclear pore complex
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PVDF	Polyvinyllidene difluoride
Ran-BP1	Ran binding protein 1
Ran-BP2	Ran binding protein 2
Ran-GAP	Ran-GTPase activating protein 1
RHD	Rel homology domain
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
RTF	Regulatory transcription factor
SCF	Skp1-Cdc53/ Cul1-F-box protein E3 complex
SDS	Sodium dodecyl sulphate
SMRT	Silencing mediator of retinoid and thyroid receptors
SUMO-1	Small ubiquitin modifier
TAF	TATA box associated factor
TBP	TATA binding protein
TFII	RNA polymerase II transcription factors
TNFα	Tumor necrosis factor alpha

TRAF	TNF receptor associated factor
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
U.V.	Ultraviolet
WT	Wild type

Abbreviations for amino acids

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	try	W
Tyrosine	tyr	Y
Valine	val	V

Genetic Code

TTT	phe	F	TCT	ser	S	TAT	tyr	Y	TGT	cys	C
TTC	phe	F	TCC	ser	S	TAC	tyr	Y	TGC	cys	C
TTA	leu	L	TCA	ser	S	TAA	OCH	Z	TGA	OPA	Z
TTG	leu	L	TCG	ser	S	TAG	AMB	Z	TGG	trp	W
CTT	leu	L	CCT	pro	P	CAT	his	H	CGT	arg	R
CTC	leu	L	CCC	pro	P	CAC	his	H	CGC	arg	R
CTA	leu	L	CCA	pro	P	CAA	gln	Q	CGA	arg	R
CTG	leu	L	CCG	pro	P	CAG	gln	Q	CGG	arg	R
ATT	ile	I	ACT	thr	T	AAT	asn	N	AGT	ser	S
ATC	ile	I	ACC	thr	T	AAC	asn	N	AGC	ser	S
ATA	ile	I	ACA	thr	T	AAA	lys	K	AGA	arg	R
ATG	met	M	ACG	thr	T	AAG	lys	K	AGG	arg	R
GTT	val	V	GCT	ala	A	GAT	asp	D	GGT	gly	G
GTC	val	V	GCC	ala	A	GAC	asp	D	GGC	gly	G
GTA	val	V	GCA	ala	A	GAA	glu	E	GGA	gly	G
GTG	val	V	GCG	ala	A	GAG	glu	E	GGG	gly	G

Acknowledgements

I would like to thank my supervisor Ron Hay for his patience and guidance through out my PhD project.

Also a big shout goes out to my family and friends, in and out of the laboratory, who have helped me during the last three years.

This work was supported by a grant from the BBSRC and benefited from collaboration under the European Communities Concerted Action (Project Rocio II).

ABSTRACT

The correct temporal and spatial expression of genetic material governs the phenotypic differences that characterise the multitude of cells observed in higher eukaryotic organisms. Gene expression is controlled at many levels. For most genes the primary control point is the regulation of transcriptional initiation. NF-κB is a ubiquitously expressed transcription factor that is retained cytoplasmically in an inactive form by the inhibitor protein IκBα. Following cell stimulation, by a variety of different inducers including UV irradiation, cytokines and bacterial or viral products, IκBα is degraded. Active NF-κB then translocates to the nucleus where it activates transcription in a protein synthesis independent manner. To identify proteins involved in the activity of the transcription factor NF-κB, proteins bound to the IκBα inhibitor were isolated by immuno affinity chromatography. The data clearly demonstrated the association of IκBα with both forms of hnRNP A1 *in vivo*. *In vitro* experiments with bacterially produced recombinant proteins indicated that the interaction between hnRNPA1 and IκBα was direct and was not mediated by a bridging protein present in the human cell extracts. Gel electrophoresis DNA binding experiments indicated that the ability of IκBα to inhibit the DNA binding activity of NF-κB p65 was not influenced by hnRNP A1. Sites of interaction between hnRNP A1 and IκBα were determined by deletion analysis and revealed that the acidic carboxy terminus of IκBα (residues 265-303) was the minimal region required for binding to hnRNP A1. A single RNA binding domain linked to part of the RGG box (residues 95-207) was the minimal region of hnRNP A1 required for interaction with IκBα. Immunofluoresence experiments demonstrated that hnRNPA1, in response to actinomycin D treatment, partially delocalises from the nucleus to the cytoplasm. Under these conditions IκBα wt also delocalised from the nucleus to the cytoplasm although the carboxy terminally truncated form of IκBα was not delocalised in response to actinomycin D treatment . Functional consequences of the interaction between IκBα and hnRNPA1 were investigated *in vivo* using a mouse erythroleukaemia cell line (CB3) which lacks endogenous hnRNPA1 (Ben-David et al., 1992). Cells

lacking hnRNP A1 are defective in NF-κB dependent transcriptional activation but the defect in these cells is restored by ectopic expression of hnRNP A1. Thus in addition to regulating mRNA processing and transport, hnRNP A1 may also contribute to the control of NF-κB dependent transcription.

1. INTRODUCTION

1.1 : Transcriptional Control of Gene Expression

The correct temporal and spatial expression of genetic material governs the phenotypic differences that characterise the multitude of cells observed in higher eukaryotic organisms. Gene expression is controlled at many levels. For most genes the primary control point is the regulation of transcriptional initiation. Following transcription the nascent mRNA transcript is processed extensively before its translation in the cytoplasm. Nuclear events such as 5' 7-methylguanosine capping, 3' poly(A) tail addition, RNA binding protein addition and the mRNA nucleotide sequence itself serve to modulate the half life of individual mRNA molecules. Pre-mRNA processing is also an important process in mRNA maturation and is carried out by the family of heterogeneous nuclear ribonucleoproteins (hnRNPs) which have also been implicated in mRNA transport out of the nucleus. Following nuclear translocation, mRNA translation initiation is also tightly controlled, generally by modification of the eukaryotic initiation factors (eIFs), several of which are phosphoproteins, but also through the structural features of the transcript itself. After mRNA decoding, post-translational events such as protein stability and modification also play an important role in the regulation of gene product.

As mentioned the primary control point of gene expression is the regulation RNA polymerase directed gene expression. RNA polymerases are large proteins in complexes of 8-14 subunits with a molecular mass of 500 kDa or more. In eukaryotic cells transcription is carried out by one of three RNA polymerases each with different specificities. The genes that encode 45S rRNA, the precursor of 18S, 5.8S and 28S ribosomal RNA, are transcribed by RNA polymerase I which accounts for approximately 50-70% of the total cellular RNA polymerase activity and is located in the nucleolus. Termination of elongation with RNA polymerase I occurs at a discrete 18 base pairs (bps) recognition sequence found between 100 and 4000 bps downstream of

the mature 3' end (Kerppola and Kane, 1991). RNA polymerase II is responsible for differential gene expression and is completely dependent on auxiliary transcription factors (TFs) to allow transcriptional initiation. The basal TFs required for initiation by RNA polymerase II have been separated into biochemically defined fractions referred to as TFIIA, B, D, E, F and H which congregate at the proximal promoter in a highly regulated and defined order (Buratowski et al., 1989; Conaway and Conaway, 1993; Weinmann, 1992) forming a complex of molecular mass greater than 2500 kDa (Zawel and Reinberg, 1995). The mechanism by which RNA polymerase II transcription is terminated is less clear, although it is not termination of elongation, but cleavage of the primary transcript at the poly (A) tail which is the significant event in determination of the 3' end. This post-transcriptional regulation is responsible for maturation of nascent RNA to messenger RNA and involves nuclear RNA processing and mRNA transport which affect mRNA stability and thus translation of message. RNA polymerase III synthesises tRNA, 5S RNA and small nuclear RNA and termination of RNA polymerase III directed transcription occurs at the second, third or fourth uridine in a run of four surrounded by GC pairs (Platt, 1986). Both RNA polymerase II and III are localised in the nucleoplasm and account for approximately 20-40% and 10% of the total RNA polymerase activity respectively.

The DNA sequences responsible for determining the exact start site and the level of mRNA synthesis are generically referred to as promoters. Promoters are recognised by RNA Polymerase and contain all the information necessary for accurate transcriptional initiation. The core promoter elements are the TATA boxes and initiator elements which determine and reside immediately 5' to and overlap with, mRNA start points. In most RNA Polymerase II promoters, an AT rich sequence, known as the TATA box, (consensus TATA$^{T}/_{A}$ A$^{T}/_{A}$ A) is the critical determinant for the multistep assembly of the basal transcription factors. However, there are many RNA Polymerase II genes that do not contain discernible TATA boxes, yet they appear to be transcribed by the same basal machinery. The pre-initiation complex on TATA-less promoters is typically specified by initiator elements that overlap the precise transcription start sites

(Weis and Reinberg, 1992). The functional consensus for initiator elements is rather loose (Py-Py-A(+1)-N-$^{T}/_{A}$-Py-Py) (Javahery et al., 1994). TATA-binding protein associated factors (TAFs) are required for basal transcription from TATA-less promoters (Chiang et al., 1993) and further, TATA-specific DNA binding activity of TBP is dispensable for transcription initiation from TATA less promoters (Verrijzer et al., 1994). Several TATA less promoters have been identified and appear to rely on DNA elements such as the ETS motif (GGCTTCCTGTCT) (Nye et al., 1992; Wasylyk et al., 1993) or the pyrimidine-rich initiator motif (CTCANTCT) (Smale and Baltimore, 1989; Smale et al., 1990) to initiate transcription (Kaufmann et al., 1996).

The presence of a core promoter sequence and assembly of the basal transcription apparatus is all that is required to initiate transcription of a gene. However, the levels of transcription that are achieved at such a promoter are minimal, and therefore upstream factors are required to modify transcriptional rates either by enhancing or repressing (Goodbourn, 1990). Enhancer elements are stretches of DNA upstream of the promoter which are specifically recognised by activator proteins that stimulate transcription. Enhancer elements are distinguished from promoters as they act in a position and orientation independent fashion. Many enhancers and their corresponding proteins have been identified and it is now clear that several are common to the majority of promoters. For example, the CCAAT box, named after its 9 bp consensus of 5' GGCCAATCT 3', is an enhancer generally located around 75bp 5' of the transcription initiation site. The GC box element is a 6 bp consensus sequence (5' GGGCGG 3'), which is present in the majority of eukaryotic promoters. It is generally located within the first 100 bp upstream of the transcription initiation site. The κB site is a degenerate decamer 5' GGGRN/NYYCC 3' (where R is any purine, N is any nucleotide and Y is any pyrimidine) which contains two half sites which reflect the specificity of the various NF-κB members to bind each half site. Elements such as CCAAT and GC boxes and the κB site are obviously paramount in increasing transcriptional rates to significant levels. However a large number of other motifs, or the 'response elements' such as the glucocorticoid response element (GRE) (Evans, 1988; Yamamoto, 1985) have also been

identified which respond to external stimuli and alter rates of gene expression. These motifs are usually located further upstream than the κB sites, GC and CCAAT boxes, but in general they exert their effects on transcriptional rates in the same manner. Thus promoter and enhancer units function in unison to regulate the gene expression observed in the cell.

Eukaryotic transcriptional machinery functions in a chromatin environment. The fundamental repeating unit of metazoan chromatin is the nucleosome which is composed of an octamer of four highly folded proteins, H2A, H2B, H3 and H4, and 147 base pairs of DNA wrapped around the octamer. A nucleofilament is then formed by linking nucleosome cores by short stretches of DNA bound in part by the 'linker' histones H1 and H5. Nucleosome core structure has been well characterised by X-Ray crystallographic studies, most recently to a resolution of 2.8A (Luger et al., 1997). In contrast to transcription in prokaryotes, strong promoter activity is essentially inactive in eukaryotes, thus the ground state for transcriptional activity in eukaryotic cells is generally restrictive. Chromatin maintains the transcriptionally repressive ground state by preventing the binding of TBP to the TATA element *in vitro* and the does not allow the association of TBP with the vast majority of yeast core promoters *in vivo* (in the absence of functional activator).

Following chromatin remodelling into an active state the basal transcription machinery assembles on core promoter elements. Commencing with the template commitment of a particular component of the TFIID complex referred as the TATA box binding protein (TBP). In eukaryotes the assembly of a functional class II pre-initiation complex involves the binding of the characterised GTFs (TFIIB, E, F, H), RNA polymerase II and TAFs to the core promoter region in an ordered fashion (Buratowski, 1994; Conaway and Conaway, 1993; Weinmann, 1992; Zawel and Reinberg, 1993). This may involve multiple, sequential steps that have been characterised by *in vitro* binding experiments (Zhou et al., 1992) or the recruitment of a pre-assembled holo-RNA polymerase that may contain a subset of GTFs (Goodrich and Tjian, 1994). In addition, promoters contain recognition sites for regulatory transcription factors. Thus

there are 2 general families of transcription factors, the general transcription factors (GTFs) and the regulatory transcription factors (RTFs). The former are constitutively expressed, bind with RNA polymerase to a specific DNA site where transcription commences and bridge to RTFs. The RTFs are generally sequence specific DNA binding proteins and influence transcription levels by either enhancing or antagonising the assembly or activity of the basal transcription machinery. It should be noted that assembly of the GTFs and the initiation of transcription differs with each RNA polymerase and is likely to change with different promoters (Roeder, 1996).

The mechanism by which transcriptional enhancers facilitate transcription from their target promoters *in vivo* remains unclear. Two models have been proposed how enhancers control transcription, the gradient and binary models (Walters et al., 1996). In the gradient model, activators control the level of transcription from each promoter template whilst the binary model predicts that activators increase the probability that a promoter will be active rather than controlling the level of transcription from each activated promoter. The assembly of enhancer complexes such as CTF/NF1 on CCAAT box (Jones et al., 1985; Santoro et al., 1988), Sp1 on the GC box element (Gidoni et al., 1984) and NF-κB on the κB site can also be referred to as the enhancesome (Gidoni et al., 1984). Formation of the enhancesome is facilitated by protein-protein interactions between the DNA bound factors. Two features of enhancesome assembly are important for transcriptional regulation, the first is the combination of multiple RTFs generates diverse patterns of regulation and secondly the highly co-operative manner in which RTFs bind DNA ensures the specificity of transcriptional control (Hochschild and Ptashne, 1986). RTFs also cooperate in activation of promoters. Transactivation by the short form of the STAT 3 protein and c-Jun, each binding to adjacent but different response elements, substantially increases the rate of transcription of rat alpha 2 macroglobulin promoter (Schaefer et al., 1995). A further aspect of RTF activation is their affinity for their DNA sequences, 80% Fos:Jun dimers dissociate from the Jun 1 site of the c-jun promoter within 1 minute, whereas Jun:ATF2 complexes remain attached for at least 5 minutes and thus maintain increased rates of transcription (Herr et

al., 1994). Cell stimulation activates second messenger systems whose end kinases, for example Mitogen Activated Protein Kinase, Jun N-Terminal Kinase/Stress Activated Protein Kinase, Protein Kinase A translocate to the nucleus and phosphorylate constitutively expressed GTFs such as CREB, ATF, SRF/TCF proteins already bound to DNA. Additionally cytoplasmic constitutively expressed GTFs are phosphorylated in response to cell stimulation. STAT 91 is an example of a phosphoprotein which directly translocates to the nucleus and binds to DNA or proteins already on the DNA (Edwards, 1994; Karin, 1995; Karin, 1994). This endows the enhancesome with increased DNA binding affinity, facilitates association with other co-induced or pre-existing RTFs and activates the GTF machinery to initiate synthesis of inducible mRNA.

As a consequence of the capacity of enhancers to function over several kilobases to regulate gene expression, it is critical that mechanisms exist which prevent inappropriate activation of neighbouring transcriptional units. If an enhancer can activate expression of a given gene from several kilobases then why does it not activate the expression of closely linked genes ? Insulators function over long distances to block interactions between cis-elements and inappropriate promoters (Kellum and Schedl, 1991). Scs and scs', which flank the *Drosophila* hsp70 locus, are perhaps the best characterised and most understood authentic insulators (Kellum and Schedl, 1991) which are believed to block distal enhancers via DNA bending, mimicking the structural changes mediated by TFIID binding to the promoter (Dunaway and Droge, 1989; Oelgeschlager et al., 1996). Alternatively, the mechanism of insulation may involve chromatin looping to separate genes into distinct nuclear compartments (Corces, 1995). Insulators appear to possess some regulatory specificity, for example the Drosophila gypsy insulator selectively blocks the activators, but not silencers, contained within the composite ventral repression element from the zerknullt (zcn) promoter region (Cai and Levine, 1997; Jiang et al., 1993; Jiang et al., 1992).

Although chromatin is often viewed as a general inhibitor of protein access to DNA, the degree to which nucleosomes inhibit DNA binding proteins from interacting with their cognate sites is highly variable (Workman and Kingston, 1998). Therefore

nucleosomes are not strictly repressors of transcription, but prevent the binding of TBP to the TATA box *in vitro* and the vast majority of yeast core promoters *in vivo*, in the absence of a functional activator (Kuras and Struhl, 1999; Li et al., 1999). Although nucleosomes prevent TBP association with DNA, they only have a very modest inhibitory effect on the ability of a variety of activator proteins to bind their target sites. Thus chromatin maintains the restrictive ground state by blocking the association of the basic RNA polymerase II machinery with the core promoter, while permitting many activators to bind their target sites. There are two mechanisms by which eukaryotic activators could enhance the association of the RNA polymerase II machinery with promoters. First, activators could directly recruit components of the RNA polymerase II machinery to the chromatin, which is analogous to the situation in prokaryotes system (Ptashne and Gann, 1997), although there are many more potential targets for the eukaryotic RNA polymerase II machinery. Secondly in a eukaryotic specific mechanism, activators could directly increase recruitment of the transcription machinery by altering chromatin structure. Thus local remodelling of chromatin is a key step in the transcriptional activation of genes as dynamic changes in nucleosomal packaging of DNA allow GTF contact with the DNA template. Several mechanisms have been identified which contribute to chromatin remodelling. Histone/DNA interaction can be modified by two opposing processes, histone acetylation or histone deacetylation, carried out by the histone acetyl transferase (HATs) or the histone deacetylases (HDACs) respectively. Proteins also exist to physically dissociate the histones from DNA in an ATP dependent manner. All of these processes act simultaneously and in concert to regulate access to the DNA template. The best understood mechanism of chromatin structure regulation is by histone acetylation (Bartsch et al., 1996; Grunstein, 1997; Kuo et al., 1998; Vettese-Dadey et al., 1996; Walia et al., 1998). It is now clear that eukaryotic enhancers also function, at least in part, by recruiting chromatin modifying activities to promoters. Gcn5 histone acetylase specifically acetylates histones in the vicinity of an activated promoter and enhancers can interact directly with histone acetylase or nucleosome remodelling complexes and stimulate transcription on

chromatin, but not on purified DNA templates *in vitro* (Kuo et al., 1998). A second mechanism for alteration of chromatin that is less well understood than acetylation of histones involves an ATP-dependent enzymatic activity that directly acts on nucleosomal structure. Based on analogous protein complexes in yeast and Drosophila, known as SWI/SNF (Burns and Peterson, 1997; Cote et al., 1998; Logie and Peterson, 1997; Ryan et al., 1998; Schnitzler et al., 1998), and RSC (Cairns et al., 1996; Lorch et al., 1998), it has been proposed that these complexes act as ATP dependent motors that track along the DNA strands and pull them away from the histone octamer cores (Burns and Peterson, 1997; Cote et al., 1998; Logie and Peterson, 1997; Ryan et al., 1998; Schnitzler et al., 1998), NURF (Ito et al., 1997; Mizuguchi et al., 1997). During this shift of histone-DNA contact points, the DNA would presumably become accessible to the transcriptional machinery. However there is conflicting evidence to suggest that these complexes serve to maintain chromatin in a repressive configuration, perhaps through dissociating other chromatin associated proteins from the DNA, such as the TBP (Auble et al., 1997). Reinforcing this concept of chromatin remodelling interplay is the finding that 2 members of the human p300-associated coactivator complex (Auble et al., 1997; Dallas et al., 1998) also posses a predicted ATPase activity requisite of the SWI/SNF type of engine.

Removal of acetyl groups by HDACs increases histone electrostatic attraction for DNA, causing histones to bind more tightly to DNA, creating a transcriptionally repressed chromatin architecture (Grunstein, 1997; Luger et al., 1997; Walia et al., 1998). N-CoR or Silencing Mediator of Retinoid and Thyroid Receptors (SMRT) (Yap et al., 1996) are examples of secondary repressor complexes. Both proteins bind to many sequence specific DNA-binding transcriptional repressor proteins such as Sin 3, which acts an intermediary protein and serves as a bridge to HDAC1 (Alland et al., 1997; Heinzel et al., 1997; Laherty et al., 1997). HDAC1 in turn restructures chromatin into a repressive configuration. N-CoR, Sin3 and HDAC1 function in many repressive pathways, including transcriptional silencing by the MAD/MAX members of the MYC family (Alland et al., 1997; Ayer et al., 1995; Harper et al., 1996; Laherty et al., 1997),

ETO (Gelmetti et al., 1998; Lutterbach et al., 1998), and other members of the steroid hormone receptor superfamily. DNA methylation has long been an accepted mechanism of transcriptional suppression (Razin and Cedar, 1991). A possible contributor to transcriptional-silencing effect of DNA-methylation may be the MeCP family of proteins, which specifically bind to regions of methylated DNA between adjacent nucleosomes (Nan et al., 1998). The MeCP2 protein directly binds Sin3 (Nan et al., 1998), which recruits HDAC1. Thus, MeCP2 binding leads to a change in the state of histone acetylation and in the chromatin structure of methylated DNA. Whether this mechanism directly mediates the transcriptional silencing effects of DNA methylation or whether it serves a synergistic or supporting role is not yet clear.

Changes in cellular transcription are induced by extracellular signalling molecules, such as growth factors and cytokines. Following ligand and receptor interaction these molecules induce a complex program of transcriptional events. The individual intracellular pathways activated, from the cell membrane, regulate transcription factor activity. The intracellular pathway activated can be traced, however the transcriptional response of the cell to different stimuli is more difficult to monitor. Following extracellular receptor stimulation, transcription factors can translocate to the nucleus, bind DNA, and interact with the basal transcription machinery to activate or repress transcription. The subcellular location in which transcription factors are activated is different. MAPK mediated phosphorylation of the TCF proteins (Elk-1 or SAP-1) provides a good example of nuclear activation (Treisman, 1994), JAK/STAT pathways are an example of cell membrane activation (Ihle et al., 1994) and NF-κB provides a classic example of cytoplasmic activation (Beg and Baldwin, 1993) . Although the activation of a single intracellular signalling pathway is sufficient for transcription factor activation, enhancesome assembly and transactivation are a result of multiple cell signalling pathways, thus introducing the concept of multiple transcription factor activation interfacing on one promoter to yield optimal transactivation combined with specificity.

As mentioned transcriptional activation is a key element in the activation of gene expression, promoter induction and expression has to be tightly controlled if specificity is to be maintained. Thus the initial transcriptional response of cells to stimulation is usually transient due to the inducible negatively acting factors (iNAFs). The iNAFs act either to reduce transcription factor activity directly or indirectly. IκBα which sequesters active NF-κB (Cheng et al., 1994; Chiao et al., 1994; LeBail et al., 1993) or ICER (inducible cAMP early repressor) which competes with CREB for target sites (Molina et al., 1993) are examples of directly acting iNAF whereas dual-specificity phosphatases that inactivate MAPK following pheromone or stress induction (Alessi et al., 1993; Doi et al., 1994; Marshall, 1995) are an example of indirect negative regulators of the signalling pathway in yeast and mammals. In reality the activity of all promoters requires the simultaneous presence of multiple transcription factors in equilibrium with the direct or indirect negative regulatory systems. Although the mechanistic basis of this interplay is still largely unknown, it is clearly the major determinant of transcription specificity in response to extracellular signals.

In summary, the transcriptional control of gene expression relies on tight regulation of transcription factors and the RNA pre-initiation complexes. Cell activation can lead to induction of a multitude of activating and repressing activities which have to be co-ordinated in order that gene expression is highly specific and 'timely'. All these requirements points to a hierarchical response of transcription factors to extracellular stimuli. The initial response facilitates the binding of the first transcription factor(s) to their DNA elements. This allows the recruitment of chromatin remodelling complexes to the nucleosome, whether they are activating or repressing. Following this the TATA boxes and initiator elements become available or inaccessible to pre-initiation complex assembly on the promoter depending on regulatory activity present upstream on the DNA.

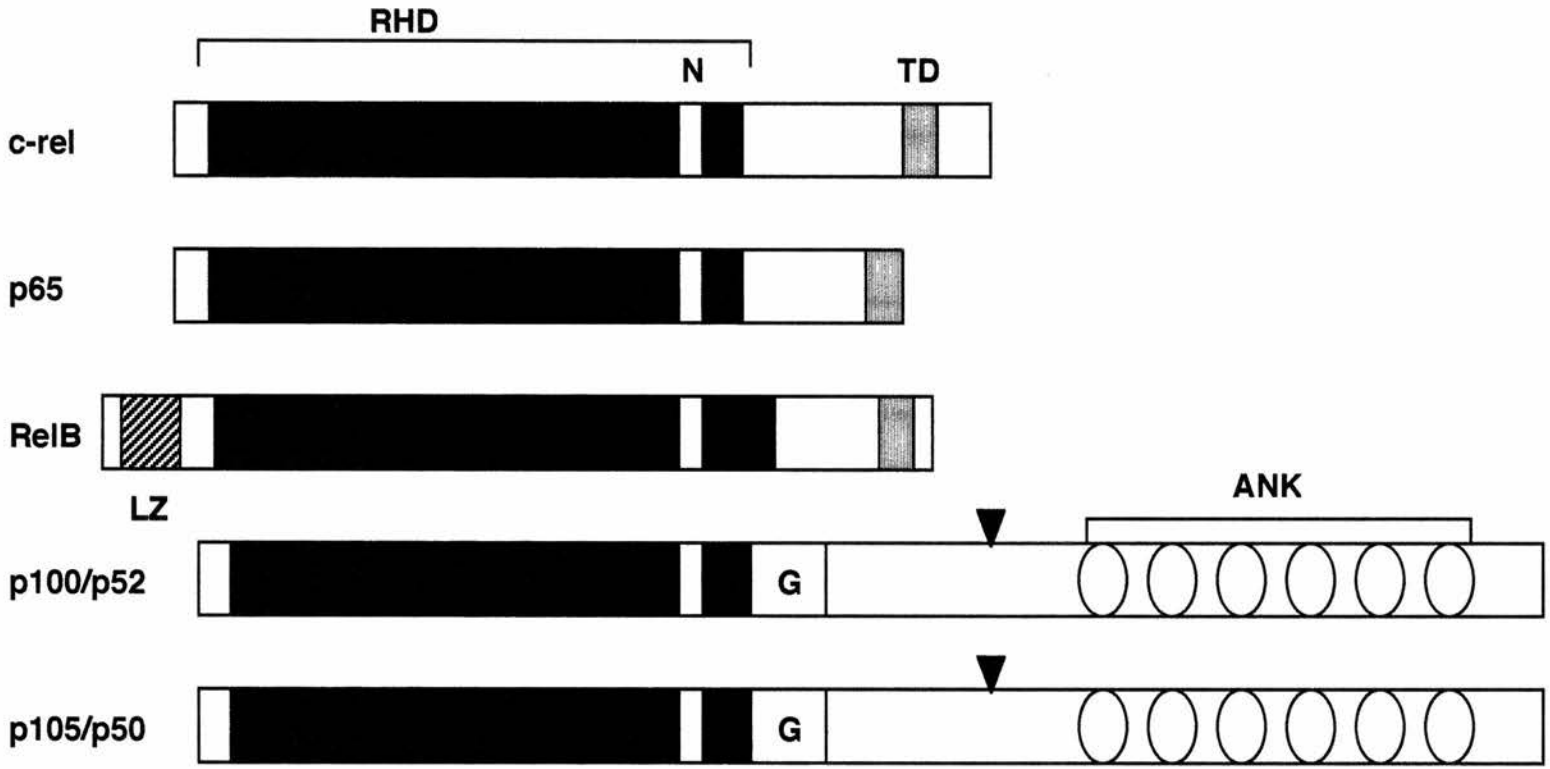
1.2 : Rel/NF-κB Family of Transcription Factors

NF-κB is a transcription factor that is inactive in most cells. Following cell stimulation NF-κB activates transcription in a protein synthesis independent manner. NF-κB was first identified as a B-cell nuclear factor and given its name on the basis of its ability to bind an intronic enhancer of the immunoglobulin κB light chain gene (Sen and Baltimore, 1986). The NF-κB family of transcription factors are critical in immune and inflammatory processes regulating the expression of more than 60 pro-inflammatory genes involved in controlling cell adhesion, immune stimulation, apoptosis, chemoattraction, differentiation, extracellular matrix degradation and redox metabolism. NF-κB is ubiquitously expressed and is activated by a variety of different inducers, including UV irradiation, cytokines and bacterial or viral products. NF-κB rapid induction and regulation, the complexity of its subunits and its involvement in the initiation and progression of several disease states such as autoimmune arthritis, glomerulonephritis, asthma, inflammatory bowel disease, septic shock, lung fibrosis, carcinogenesis and AIDS (Baldwin, 1996; Finco and Baldwin, 1995) have made this transcription factor a subject of intense study.

Presently, five mammalian NF-κB family members have been identified and cloned. these include NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB and c-Rel (Fig 1). NF-κB is sequestered in the cytoplasm of the majority of unstimulated cells as a consequence of interaction with the IκB family of inhibitor molecules. However, in response to extracellular signalling the most abundant activated form of NF-κB is a heterodimer composed of a p50 or p52 subunit and a p65 subunit which bind to a common DNA motif. Other dimeric forms exist, such as p50/p50, p52/p52, RelA/RelA homodimers, RelA/c-Rel heterodimers and p50/c-Rel heterodimers have also been detected in some cell types under certain conditions (Ganchi et al., 1993; Hansen et al., 1994; Kang et al., 1992; Parry and Mackman, 1994; Thompson et al., 1995). One exception is Rel B which only forms heterodimers with p50 or p52 (Ryseck et al., 1992; Ryseck et al., 1995). Although dimers bind a common κB binding motif, it has been shown that different dimers are able to recognise slightly different κB motifs, for

Figure 1. Members of the mammalian Rel/NF-κB family of proteins.

The arrows point to the endoproteolytic cleavage sites of p52/p100 and p50/p105. RHD, Rel Homology Domain; N, Nuclear Localisation Sequence; TD, Transactivation domain; LZ, Leucine Zipper Domain RelB; G, Glycine Rich Region and ANK, Ankyrin Repeats.


RHD
N
TD
c-rel
p65
RelB
LZ
ANK
p100/p52
G
p105/p50
G

example p50/65 binds the sequence 5'-GGGRN-NYYCC-3', with high affinity, whereas the RelA/c-Rel heterodimer prefers 5'-HGGARNYYCC-3' (where H is A,C or T; R is a purine; and Y is a pyrimidine)(Kunsch et al., 1992; Parry and Mackman, 1994).

A characteristic feature of NF-κB is that all of the family members share a highly conserved Rel Homology Domain (RHD). This domain is composed of approximately 300 amino acids responsible for NF-κB's nuclear translocation, protein dimerisation, DNA binding and interactions with the IκB inhibitor. As not all of the Rel/NF-κB proteins are transcriptionally active its members can be divided into two groups based on their transactivation capabilities. The carboxy-terminal regions of RelA, RelB and c-Rel contain a transactivating domain, important for NF-κB mediated gene transactivation (Blair et al., 1994; Ryseck et al., 1992; Schmitz et al., 1995; Schmitz et al., 1994). These domains contain abundant serine, acidic and hydrophobic amino acids which if mutated reduce the transactivation capacity. However NF-κB1 and 2, generated from precursor proteins, do not possess a carboxy terminal transactivation domains and thus do not generally act as transcriptional activators. In fact they have been shown to repress κB dependent transcription *in vivo* (Brown et al., 1994; Kang et al., 1992; Lernbecher et al., 1993; Plaksin et al., 1993). The mechanism by which p50/p52 homodimers inhibit transcription is not completely understood, but it is probable that they recruit chromatin repression complexes such as the histone deacetylases (W. Sands, Personal Communication).

Crystal structures with DNA have been solved for p50, p52, p65 homodimers and p65/p50 heterodimer (Chen et al., 1998; Chen et al., 1998; Cramer et al., 1997; Ghosh et al., 1995; Muller et al., 1995). The crystal structures containing the RHDs of three Rel/NF-κB family polypeptides complexed with various DNA targets obtained indicate that the RHD has a tripartite organisation (Chen et al., 1998; Chen et al., 1998; Cramer et al., 1997; Ghosh et al., 1995; Muller et al., 1995). The amino-terminal 180 amino acids fold into an Ig like domain and a short, 10 amino acid, flexible linker connects this amino-terminal domain to the second Ig like domain which is

approximately 100 amino acids in length. DNA recognition is primarily mediated by the amino terminal domain. The amino-terminal Ig fold (Bork et al., 1994) acts as a scaffold for the DNA contacting flexible loops which are also employed by p53 (Cho et al., 1994), STAT-1 (Chen et al., 1998) and NFATC1 (Zhou et al., 1998). All dimerisation contacts are mediated through the carboxy terminal Ig fold, referred to as the dimerisation domain. Fourteen residues from each subunit participate in the dimer interface, which is dominated by Van der Waals interactions. The Ig fold also contains the 13 amino acid Nuclear Localisation Sequence (NLS) required for nuclear translocation.

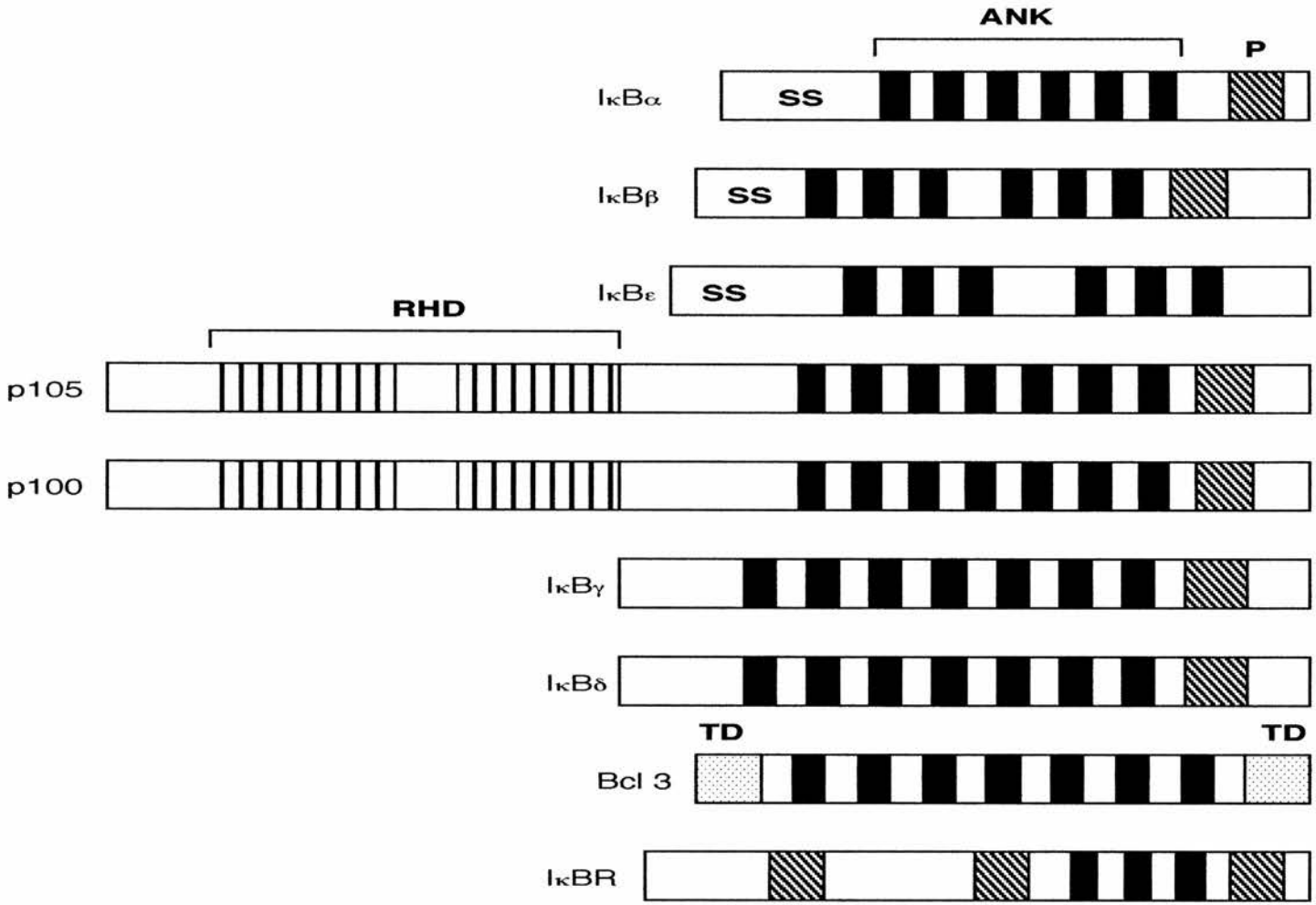
Crystallographic data shows that p50 interacts with DNA over a complete helical turn. Each p50 subunit contacts DNA through 5 flexible loops which connect the β strands. Two of these loops are in the amino-terminal domain, two in the carboxy terminal domain and one is the flexible linker connecting the domains. The most important NF-κB-DNA interaction is between the amino-terminal of the L1 loop that connects A and B β sheets, and the bases of the major groove of target DNA. This region of the AB loop is termed the recognition loop because of its interaction with DNA determines the sequence specificity of p50. In addition partial proteolysis (Hay and Nicholson, 1993; Matthews et al., 1995) and chemical modification (Bell et al., 1996) analyses suggest the carboxy-terminus of the L1 loops could also contact DNA. Lysine 80 residue, at the carboxy terminus of AB loop interacts with DNA through an additional lysine-phosphate backbone ionic bond, which makes a significant contribution to the binding energy thus stabilising the protein/DNA complex (Michalopoulos and Hay, 1999). This residue is conserved in all NF-κB/Rel/Dorsal molecules.

1.2.1 : Inhibitor Protein of NF-κB (IκB)

There are seven distinct mammalian IκB proteins identified so far, IκBα, IκBβ, IκBδ, IκBγ, IκBε, IκBR and bcl-3 (Fig 2) and these IκB molecules bear significant sequence and function homology with the cactus protein in Drosophila (IκBR is the

Figure 2. Members of the mammalian IκB family of proteins.

The black boxes represent the ankyrin repeat (ANK) motifs of each protein; the pair of serine residues which are phosphorylated in response to signalling and are necessary for the inducible degradation of IκBα, IκBβ and IκBε are represented by SS. Also shown are the PEST (P) domains (diagonal shading), the Rel homology domains (RHD) of p105 and p100 (vertical shading) and the transactivation domains (TD) of Bcl 3 (dotted boxes).


ANK
P
IκBα
SS
IκBβ
SS
IκBε
SS
RHD
p105
p100
IκBγ
IκBδ
TD
TD
Bcl 3
IκBR

most similar), which can inhibit the Rel-like protein Dorsal (Geisler et al., 1992; Kidd, 1992). All IκB family members contain multiple copies of the structural motif known as the ankyrin repeat, which are important for protein-protein interactions. The different IκB molecules show specificity for binding and inhibiting various Rel/NF-κB complexes for example IκBα and IκBβ interact with heterodimers of p50 or p52 complexed with RelA or c-Rel, as well as homodimers and heterodimers of RelA and c-Rel. In contrast, IκBε appears to be complexed almost exclusively with dimers that contain only RelA and/or c-Rel proteins. The hypothesis that different subsets of Rel/NF-κB responsive genes are controlled by different IκB molecules is supported by the observation that a subset of NF-κB responsive genes are transcriptionally active in cells derived from IκBα deficient mice (Beg et al., 1995).

IκBα is the most extensively studied human IκBα and is organised into three domains (Jaffray et al., 1995), firstly an unstructured regulatory amino-terminal domain involved in IκBα degradation and thus NF-κB induction. This region possesses the critical serine residues at position 32 and 36 which are phosphorylated by the IκBα kinase (IKK) complex. Site directed mutagenesis of these residues was found to block phosphorylation of IκBα in response to activating signals thereby preventing subsequent degradation of the protein (Brown et al., 1995; Roff et al., 1996; Traenckner et al., 1995). Following phosphorylation IκBα is ubiquitinated on lys 21 and 22 which act as the molecular signal for protein degradation via the proteosome 26S complex (Baldi et al., 1996; Rodriguez et al., 1996; Scherer et al., 1995). Specific inhibition of the proteosome activity prevents NF-κB activation and results in the accumulation of ubiquitinated forms of IκBα, thus demonstrating that IκBα is targeted for degradation by a phosphorylation dependent ubiquitin process (Alkalay et al., 1995; Chen et al., 1995; Roff et al., 1996). Secondly a central region consisting of 6 ankyrin repeat domains (ARDs) involved in protein-protein interactions with the NF-κB subunits and nuclear translocation. The second ankyrin repeat of IκBα contains the nuclear localisation sequence (NLS) responsible for IκBα nuclear translocation following resynthesis (Sachdev et al., 1998; Turpin et al., 1999). Thirdly a carboxy terminal region

containing a highly acidic domain rich in pro, glu, ser, thr known as the PEST domain. The PEST domain is a CKII phosphorylation site (Barroga et al., 1995) and modification of this domain has been implicated in regulating the stability of IκBα (Ernst et al., 1995; Lin et al., 1996; Schwarz et al., 1996). The carboxy terminal also contains the nuclear export sequence (NES). This leucine rich NES is recognised by the nuclear protein CRM1 (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997), which transports the NF-κB/IκBα trimeric complexes back to the cytoplasm.

Recently the crystal structure of the NF-κB/IκBα trimeric complex was solved by two groups (Huxford et al., 1998; Jacobs and Harrison, 1998). The structural details presented support a model by which both allosteric and direct modes of NF-κB DNA binding inhibition are performed by IκBα. The ARDs of IκBα forms a slightly bent cylinder with 5 loops, protruding from the packed arrangement of stacked α helices, which contain the residues that specifically recognise NF-κB. The contact area of the ARD with NF-κB is large, with the most significant contribution from the dimerised Ig like domain of the p50/p65 RHD and repeats 3-5 of IκBα. Both reports are in agreement as to how IκBα prevents the NF-κB dimer from binding to DNA. The sixth ankyrin repeat interacts with the DNA binding domain of p65 and profoundly changes its conformation. In a complex with the IκBα inhibitor, the N-terminal Ig like domain of the p65 RHD, flexibly linked to the C-terminal Ig like domain, is rotated by 180 degrees thereby critical residues for contacting DNA are occluded. Structural data from the two studies also demonstrate that interactions between the p50/p65 heterodimer and IκBα appear extensive, engaging all six ankryin repeats and at least 3 out of 4 Ig like domains of the p50/p65 dimer. Ankyrin repeats 1 and 2 serve to inhibit nuclear translocation by binding a long stretch containing the p65 NLS. Repeats 3 to 5 bind tightly over a large surface to the C-T Ig like domains of both p50 and p65 RHDs, however it is not clear whether this particular interaction directly interferes with the DNA binding activity of p65. The higher affinity of IκBα for p65 over p50 can be explained by two additional contact areas with p65 that fully engage ankyrin repeats 1,2 and 6 of IκBα, in addition to

repeats 3,4 and 5, which also bind p50. The amino-terminal regulatory domain of IκBα most likely protrudes from the NF-κB/IκBα complex, where it would be readily accessible to the IKK complex that phoshorylates serine residues 32 and 36, as well as enzymes that subsequently polyubiquitinates lysines 21 and 22.

Both crystal studies used heterotrimeric crystals that were obtained with the carboxy terminal Ig like domain of the p50 RHD, the entire RHD of p65 and a truncated IκBα protein, which largely consisted of its ARD and lacked most of the amino and carboxy terminal domains. The proteins used in this study provide us with useful information about the structure of a NF-κB/IκBα complex. However as the proteins are truncated one should interpret the data with care. The packing of the proteins into crystals and the overall trimeric complex structure may not directly resemble the trimeric complex observed physiologically. In addition, both amino and carboxy terminal regions are required for signal induced degradation (Brown et al., 1997; Kroll et al., 1997; Rodriguez et al., 1995) and nuclear export (Arenzana-Seisdedos et al., 1997; Fischer et al., 1995; Fritz and Green, 1996; Wen et al., 1995) of IκBα. It is therefore critical that the structure for IκBα is resolved for the full length protein, and its structural conformation adopted, in order to gain further insight into IκBα regulation of NF-κB.

1.2.2 : Signal Transduction Pathways Leading to NF-κB Activation

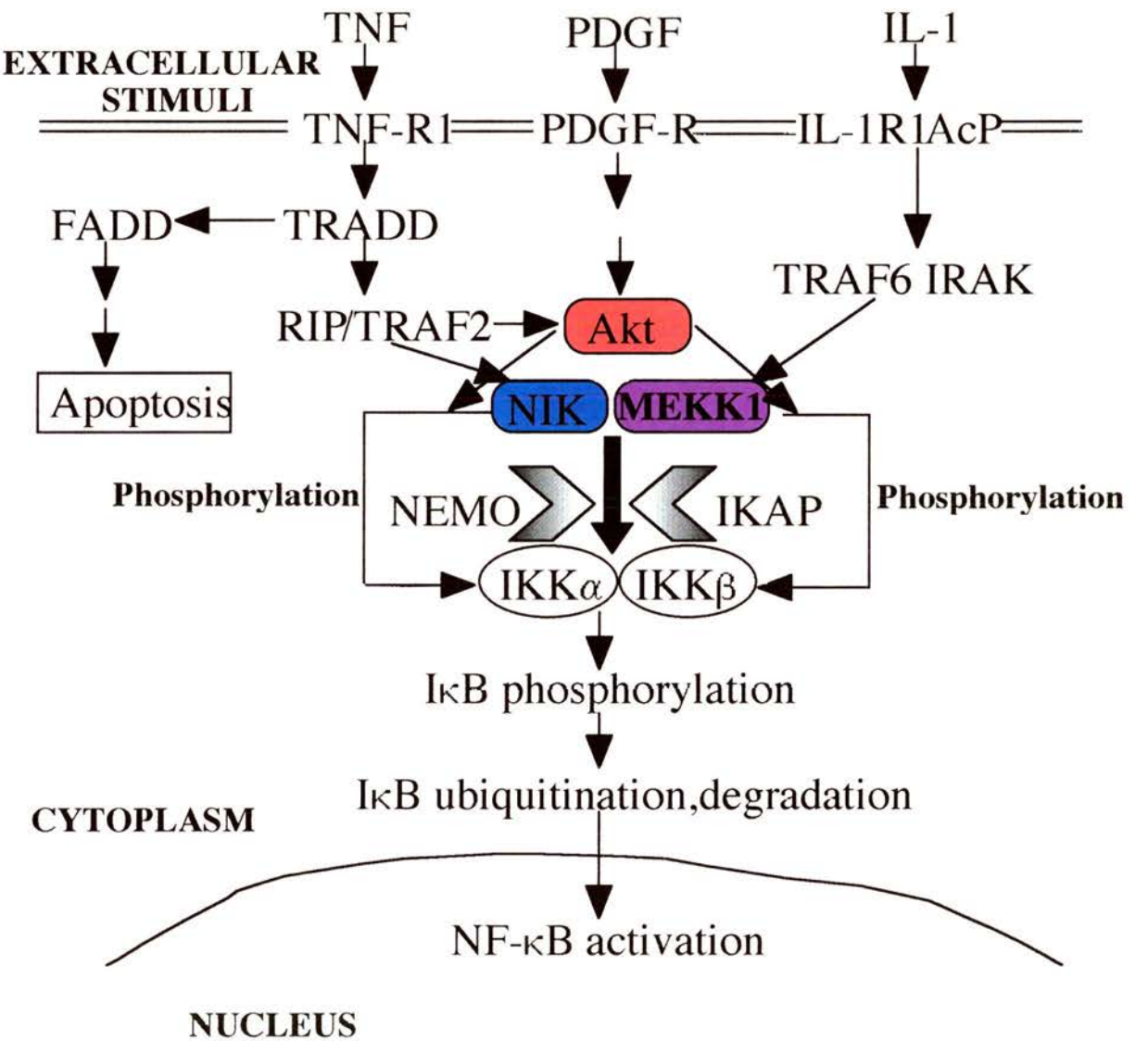
As mentioned NF-κB activation is mediated by a wide variety of stimuli. One of the best characterised activators of NF-κB transcriptional activity is TNFα. This pro-inflammatory cytokine, is produced by macrophages and monocytes (Hanazawa et al., 1993), and responses are elicited by the TNF Receptor (TNF-R). The TNF-R is composed of two distinct cell surface receptors, termed TNF-R1 (p55) and R2 (p75) (Smith et al., 1994). Although most of the biological activities of TNF appear to be transduced by TNF-R1, many can also be mediated by TNF-R2 (Tartaglia et al., 1993). Several protein kinases are activated rapidly in response to TNF, including ceramide kinase (Wiegmann et al., 1994), TNF-R1 associated ser/thr kinase (VanArsdale and Ware, 1994), as well as the molecularly identified Raf-1 (Belka et al., 1995), Jun N-T

kinases (JNKs) (Minden et al., 1994), p38/Mpk2 (Raingeaud et al., 1995) and the IKK complex (Didonato et al., 1996; Verma et al., 1995). TNF receptor associated factors (TRAFs) have emerged as the signal transducing units which are utilised by the TNF-Receptor superfamily and Interleukin 1 Receptor 1(IL-1R1)(Hsu et al., 1997; Rothe et al., 1995; Rothe et al., 1994; Sato et al., 1995; Song and Donner, 1995). TRAFs 2 and 6 are involved with the TNF receptor family and the IL-1R1 respectively, and activate the appropriate downstream protein kinases which continue the signal transduction process (Fig 3).

NF-κB Interacting Kinase (NIK) was first identified as a TRAF 2 interacting protein and has structural homology to the MAPKKK family (Malinin et al., 1997). NIK preferentially phosphorylates IKKα on serine 176 in the activation loop, leading to activation of IKKα activity (Nakano et al., 1998). A second MAPKKK, MEKK-1, has also been shown to co-purify with IKK activity (Mercurio et al., 1997). MEKK-1 preferentially phosphorylates the corresponding serines in the activation loop of IKKβ, leading to activation IKK activity (Nakano et al., 1998; Nemoto et al., 1998; Yin et al., 1998). NIK and MEKK-1 are activated by discrete stimuli which provides potential mechanisms for differential activation of the IKK members. Alternatively there is also potential for a mechanism for synergistic activation of the IKKs and consequently a more potent activation of the NF-κB pathway. Another protein kinase, Akt/Protein Kinase B, has recently been identified as an IKK activator and thus activates NF-κB. Akt has been shown to be activated in response to platelet derived growth factor (PDGF) and causes activation of NF-κB. This pathway depends on Akt phosphorylation of IKKβ in human and mouse fibroblast cells, however in their system TNFα does not activate Akt (Romashkova and Makarov, 1999). In contrast work done by Ozes et al, working with epithelial cells, demonstrates that TNFα activates Akt phosphorylation of IKKα, which induces NF-κB activation (Ozes et al., 1999). Although these results point to Akt involvement as an important modulator of NF-κB activation. One must interpret the results with care, between the two studies, as it appears that the activation of Akt and its cellular target are cell specific. Therefore it is not possible to construct a generic

Figure 3. Schematic representation of components of the NF-κB signal transduction pathway leading from TNF, IL-1 and PDGF receptors.

Signals emanating from the TNF, IL-1 and PDGF receptors activate members of the MEKK related family, including NIK and MEKK1. These proteins are involved in activation of IKKα and IKKβ components of the signalsome. These kinases phosphorylate members of the IκB family at specific serines within their amino termini, leading to site specific ubiquitination and degradation by the 26S proteosome. The released NF-κB is then free to activate expression of its target genes. NF-κB autoregulation (not shown) is facilitated by IκBα resynthesis as a consequence of NF-κB activation.


TNF
PDGF
IL-1
EXTRACELLULAR STIMULI
TNF-R1
PDGF-R
IL-1R1AcP
FADD
TRADD
TRAF6 IRAK
RIP/TRAF2
Akt
Apoptosis
NIK
MEKK1
Phosphorylation
NEMO
IKAP
Phosphorylation
IKKα
IKKβ
IκB phosphorylation
IκB ubiquitination,degradation
CYTOPLASM
NF-κB activation
NUCLEUS

model based on the observed phenomena to produce a hard and fast cellular mechanism of activation. TPL 2 kinase is activated in response to TNF and functions in the NF-κB activation pathway. Early studies on the signalling of TPL2 have shown that it transduces signals that are activated by the MAPK and SAPK pathways (Blank et al., 1991; Lin and Ghosh, 1996; Watanabe et al., 1997). The TPL-2 kinase has been shown to increase the ubiquitin mediated proteolysis of p105 to generate the p50 molecule (Belich et al., 1999).

The kinases which are directly responsible for phosphorylation of IκBα, IκBβ, IκBε are the IKKs which are components of a high molecular weight complex (900kDa) termed the IKK signalsome. The two catalytic subunits contained in this complex, termed IKKα and IKKβ, have been cloned and demonstrate cytokine inducible activity including rapid induction in response to known inducers of NF-κB (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Yaron et al., 1997) and are related members of a family of intracellular signal transduction enzymes, composed of an N-T ser/thr kinase domain, a central leucine zipper domain and a C-T helix-loop-helix domain. IKKα and IKKβ can form homodimers and heterodimers (or tetramers) *in vitro*, and purified recombinant forms of each can directly phosphorylate IκBα and IκBβ at the proper sites (Lee et al., 1998; Zandi et al., 1998). In addition, the IKK complex contains at least one regulatory subunit, IKKγ/NEMO (NF-κB essential factor) which is required for NF-κB activation (Rothwarf et al., 1998; Yamaoka et al., 1998). NEMO is a 419 amino acid long glutamine rich protein that lacks a known catalytic domain, but contains several coiled coil protein interaction motifs, including a leucine zipper next to its carboxy terminus (Rothwarf et al., 1998; Yamaoka et al., 1998). It has also been shown that the IKK complex can be bound by a carboxy terminal truncated version of NEMO, but once expressed it prevents IKK activation by a number of stimuli (Rothwarf et al., 1998). These results demonstrate the requirement of NEMO for IKK activation but also suggest a specific function whereby NEMO connects the IKK complex to upstream activators. Highly purified recombinant NEMO by itself can form upto tetramers, but it appears to bind IKKα-IKKβ as a dimer (Rothwarf et al.,

1998). It has also been demonstrated that in addition to IKKα, IKKβ , NIK, and NF-κB/IκBα, this complex also contains a 150 kDa protein named IKAP (IKK complex associate protein) (Cohen et al., 1998). IKAP is suggested to function as a scaffold protein due to its ability to assemble IKKα, IKKβ, NIK and NF-κB/IκBα however it remains to be determined whether IKAP is required for IKK activation at all, and whether it is involved in responses to all NF-κB activating stimuli or to only a subset of them. It should be noted however that it is not clear whether the complex found by Cohen et al, was purified to homogeneity and whether the reported polypeptides are its only components, for example they did not examine whether IKKγ was part of this complex.

The likely sites of activating phoshorylation on the IKKs have been identified as two serine residues within the kinase activation loop (Mercurio et al., 1997). Cell stimulation with TNF enhances the phosphorylation of all the IKK subunits (Delhase et al., 1999). However the bulk increase in IKK phosphorylation occurs with considerably slower kinetics than the increase in kinase activity and it is not yet clear to what extent phosphorylation of the activation sites at the T loop is due to the action of an upstream kinase and to what extent it is due to autophosphorylation. It can be concluded that at least two events are required for full IKK activity. One is the interaction of the HLH motif with the kinase domain and the other is the phosphorylation of specific sites in the T-loop of IKKα and IKKβ. In addition a preference for TNFα induced activation of IKKα and IKKβ heterodimers relative to IKKβ homodimers suggests that either IKKα or IKKα-specific associated proteins are required for the full activation of the IKK complex (Mercurio et al., 1999). To further elucidate the biological functions of IKKα and IKKβ mouse mutants lacking either IKKα and IKKβ were constructed and demonstrated that IKKα is not required for activation of IκBα in response to pro-inflammatory stimuli, whereas IKKβ was essential for this response (Hu et al., 1999; Takeda et al., 1999). Thus the IKK signalsome, by virtue of the virtue of the functional diversity of IKKα and IKKβ and their respective associated proteins, provides the

potential to integrate the diverse array of signalling pathways known to activate NF-κB in different cell types.

With a few rare exceptions, all stimuli which induce NF-κB activation target the IκBα molecule to degradation through a phosphorylation regulated process which directs the inhibitors to degradation via the Ub proteosome pathway (Baeuerle and Baltimore, 1996; Whiteside et al., 1997). Following kinase activation, IκBα is phosphorylated on serine 32 and 36 (Brockman et al., 1995; Brown et al., 1995; DiDonato et al., 1996; Roff et al., 1996; Traenckner et al., 1995), by the described IKK complex, and subsequently ubiquitinated on lys 21 and 22 (Baldi et al., 1996; Rodriguez et al., 1996; Scherer et al., 1995) which targets the molecule for degradation by the proteosome 26S complex. Selective protein degradation in eukaryotic cells is mainly carried out by the Ubiquitin (Ub) system and plays important roles in many cellular functions including cell cycle and cell division control, signal transduction, the nuclear transport process, receptor control by endocytosis, modulation of the immune system and inflammatory response, modulation of cell membrane receptors, ion channels and the secretory pathway, DNA repair and control of transcription.

The conjugation of Ub to proteins is a multiple enzymatic process. Ub is activated by ubiquitin-activating enzyme (E1) (Adams et al., 1992; Wilkinson et al., 1980) and in the presence of ATP is attached to a specific cys residue of the same enzyme (Haas et al., 1982) by a thioester bond. Ubiquitin is then transferred to a cys residue of the second enzyme, Ub conjugating enzyme E2 (Pickart and Rose, 1985). Whilst in many cases Ub can be directly transferred from the E2 and covalently linked to the substrate protein, Ub conjugation of some proteins requires the additional activity of a Ub ligase E3 (Ciechanover et al., 1982; Hershko, 1991; Reiss et al., 1989). The Ub moiety is covalently linked via an isopeptide bond to the amino group of a lysine residue in the target protein. Recently the E3 responsible for ubiquitination of IκBα was identified, the archetype of which was first identified in budding yeast, and contains Skp1, Cdc53/Cul1 and a specificity conferring F-Box containing protein (SCF complex) (Bai et al., 1996; Feldman et al., 1997; Patton et al., 1998; Skowyra et al.,

1997). β-transducin repeat containing protein (βTRCP) is a specific component of the IκBα ubiquitin ligase complex (Hatakeyama et al., 1999; Spencer et al., 1999; Winston et al., 1999; Yaron et al., 1998) (Vuillard et al., 1999) and SCF βTRCP complexes recruits IκBα into the SCF-Ub ligase complex, via the F-Box/WD40 repeat protein. The importance of this F-Box/WD40 domain has been demonstrated by deletion mutants, which act in a dominant negative manner to inhibit the degradation of IκBα (Spencer et al., 1999; Yaron et al., 1998).

The bound ubiquitin is in turn polyubiquitinated forming a branch of multiubiquitin chains and the tagged substrate is degraded by the proteosome complex with the release of free and reusable ubiquitin (Deshaies, 1995; Jentsch and Schlenker, 1995) (Ciechanover, 1996; Coux et al., 1996). Small ubiquitin related modifier 1 (SUMO-1) has been identified as a protein that is highly similar to ubiquitin (Boddy et al., 1996; Kamitani et al., 1997; Mahajan et al., 1997; Matunis et al., 1996; Shen et al., 1996). However, post translational protein modification by SUMO-1 has a role in other pathways rather than protein degradation, for example, SUMO modification of Ran GAP1 is required for its association with Ran BP2/Nmp 358, a component of the Nuclear Pore Complex (NPC), and the stable complex is required for the import of proteins into the nucleus (Saitoh et al., 1997) (Mahajan et al., 1998; Matunis et al., 1996). Conjugation of SUMO-1 to nuclear dot associated proteins PML and Sp100 appear to regulate the subnuclear partitioning of these proteins (Muller et al., 1998; Sternsdorf et al., 1997) and SUMO 1 modification of IκBα renders the protein resistant to ubquitination and thus resistant to signal induced degradation, therefore maintaining the cytoplasmic localisation of the transcription factor NF-κB (Desterro et al., 1998). From these examples SUMO-1 seems to regulate protein stability and intracellular localisation of certain proteins.

Following IκBα degradation stimulus dependent post translational modifications of NF-κB complexes has been reported to enhance or repress transcriptional activation of NF-κB. Signals that induce the phosphorylation of IκBα have also been shown to induce phosphorylation of NF-κB subunits (Mellits et al., 1993; Naumann and

Scheidereit, 1994). TNF induced transactivation of the p65 subunit has been shown to be potentiated following carboxy terminal phosphorylation of serine 529 which lies in the transactivation domain 1 (Wang and Baldwin, 1998). Transcriptional activity of NF-κB has also been shown to be stimulated upon phosphorylation of p65 on serine 276 by protein kinase A (Zhong et al., 1997). Redox state dependency of NF-κB has also been reported. One study demonstrated that a high level of oxygen radicals generated from hydrogen peroxide, promoted NF-κB activity (Schmidt et al., 1996). Paradoxically NF-κB DNA binding can be inhibited by oxidative modification of a reduced thiol group at position 62 in the p50 subunit (Mahon and O'Neill, 1995; Matthews et al., 1992; Matthews et al., 1993). Redox modification has also been shown in whole cell extracts suggesting this is important cellular mechanism of NF-κB inhibition (Brennan and O'Neill, 1998). It has been proposed that the oxidative inhibition of NF-κB may be a cytoplasmic event and nuclear translocation of the dimer allows the reversible modification and thus restores transcriptional activity (Galter et al., 1994).

Following activation NF-κB translocates to the nucleus, where it induces the transcription of several genes (Baeuerle and Baltimore, 1996; Baldwin, 1996; Verma et al., 1995) including that of its inhibitor, IκBα. Newly synthesised IκBα then accumulates in the cytoplasm but also in the nucleus, by virtue of the NLS found in the second ankyrin repeat (Sachdev et al., 1998; Turpin et al., 1999), and terminates NF-κB dependent transcription. This is accomplished by inhibition of the NF-κB/DNA interaction and export of NF-κB back to the cytoplasm (Arenzana-Seisdedos et al., 1995). The latter function is conferred by the leucine rich NES present in the carboxy terminus (Arenzana-Seisdedos et al., 1997).

1.2.3 : Apoptosis/Cell Survival

NF-κB transcriptional activity plays a pivotal role in the regulation of cell viability and cell senescence. Apoptosis is a major form of cell death, characterised by a series of distinct morphological and biochemical alterations (Arends and Wyllie, 1991). Apoptotic cell death occurs in two phases, first a commitment to cell death followed by

an execution phase characterised by dramatic stereotypic morphological changes in cell structure (Takahashi and Earnshaw, 1996). The death of an individual cell is an integral and continuing part of normal physiology (Jacobson et al., 1997) as inappropriate timing of cell death has been implicated in many human diseases including neurodegenerative diseases such as Alzheimer's disease and Huntingtons disease, ischaemic damage, autoimmune disorders and several forms of cancer (Nicholson, 1996; Thompson, 1995).

TNF engagement of TNF-R1 leads to its Death Domain (DD) recruitment of other DD containing proteins. These proteins include TRADD (TNF Receptor Associated Death Domain protein) (Hsu et al., 1996; Hsu et al., 1995), MORT1 (mediator of receptor induced toxicity) (Boldin et al., 1995), a ser/thr kinase RIP (receptor interacting protein) (Hsu et al., 1996; Stanger et al., 1995) and interaction of caspase 8 with FADD initiates the caspase cascade (Boldin et al., 1996; Muzio et al., 1996). On the other hand occupancy of TNF-R2 leads to the recruitment of TRAFs 1 and 2 (Rothe et al., 1994) and cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and 2) (Rothe et al., 1995). TRAF 2 association with TNF-R1 and 2 controls signal transduction pathways (Hsu et al., 1996; Rothe et al., 1995) of two transcription factors, AP-1 (Brenner et al., 1989) and NF-κB (Osborn et al., 1989). These transcription factors mediate induction of other cytokines and immunoregulatory genes as well as metalloproteinases.

NF-κB autoregulates the levels of mRNAs for TRAF1 and 2 and c-IAP 1 and 2 (Chu et al., 1997; Wang et al., 1998) and disruption of this pathway enhances the cytolytic effects of TNF (Enari et al., 1998; Hengartner and Horvitz, 1994; Kluck et al., 1997; Korsmeyer, 1995; Reed, 1997). Thus NF-κB activates a group of gene products that function co-operatively at the earliest checkpoint to suppress TNFα mediated apoptosis and that function more distally to suppress genotoxic agent mediated apoptosis. None of these transcription units are required for TNF induced killing, because the Death Response occurs in the absence of either protein synthesis (Wallach, 1986; Wallach, 1984) or NF-κB signalling(Hsu et al., 1996).

1.2.4 : Disease States

The inappropriate activation or expression of NF-κB is evident in several human cancers, including breast cancer (Nakshatri et al., 1997; Sovak et al., 1997) non-small cell lung carcinomas (Mukhopadhyay et al., 1995), Thyroid cancer (Gilmore et al., 1996), T or B cell lymphocyte leukaemia (Bargou et al., 1996) and several virally induced tumours (Berger et al., 1997; Blumberg, 1997; Miwa et al., 1997). NF-κB activity has been well characterised in Hodgkins lymphomas. Hodgkins lymphomas are composed of malignant Reed Sternberg and Hodgkins cells in a reactive background of lymphocytes, plasma cells, histiocytes and eosinophils. A common feature of Reed Sternberg and Hodgkins cells is that they display high levels of constitutively active nuclear NF-κB (Bargou et al., 1996; Gruss et al., 1994). In three Hodgkins cell lines (KM-H2, L428 and HDLM-2) NF-κB activity examined was not initiated by ligand/receptor interaction. Rather it is a direct consequence of defective IκBα expression in each cell line even though IκBα mRNA levels appear to be normal (Cabannes et al., 1999; Wood et al., 1998). IκBα is absent from KM-H2 cells, L428 cells possessed a carboxy terminal truncated version of IκBα and HDLM-2 produced a more slowly migrating form of IκBα which could associate with NF-κB, but increasing the levels of this failed to inhibit NF-κB DNA binding activity. In all three cell lines NF-κB could be could be inhibited using recombinant IκBα *in vitro* and plasmid expressed wild type IκBα *in vivo* (Wood et al., 1998)

Thus a role for NF-κB in the malignant transformation of cells is highly possible. Nevertheless, it is unclear whether over-activation or excessive expression of NF-κB in these transformed cells is linked directly to the transformation or whether NF-κB only provides an accessory signal for the transformation. NF-κB also plays a key role in controlling the expression of multiple inflammatory and immune genes involved in toxic shock, acute phase responses, radiation damage, asthma, rheumatoid arthritis, atherosclerosis, cancer, AIDS. Additional studies are clearly required to unveil the molecular basis for the activation and function of NF-κB in these disease states.

1.2.5 : Perspectives

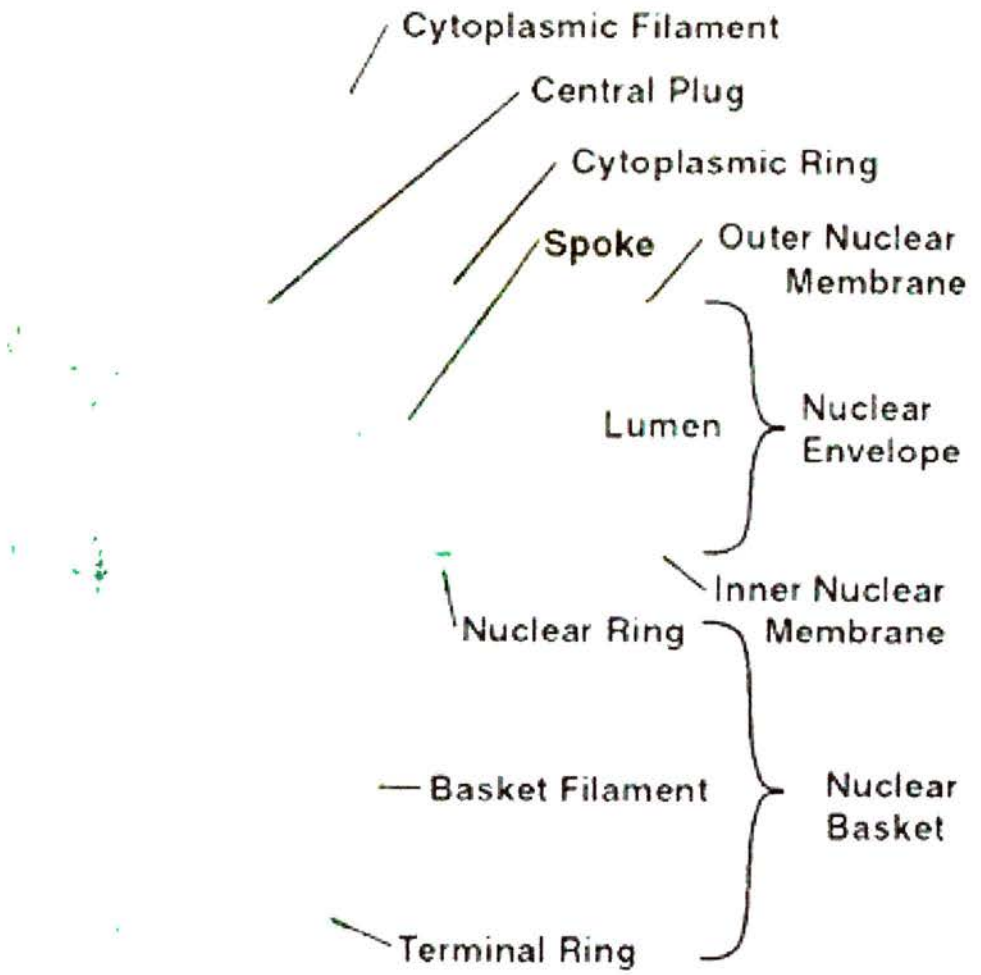
Due to the vast number of disease states induced by aberrant NF-κB activation, it makes this factor a central and favourable target for therapeutic intervention of diseases (Baldwin, 1996; Finco and Baldwin, 1995). However most of the biological and biochemical inhibitors of NF-κB presently available act by blocking the signalling pathways that lead to NF-κB activation or by compromising the binding activity of NF-κB to target DNA. NF-κB transcriptional activation can be modified by treatment with antioxidants (Schulzeosthoff et al., 1995), proteosome and calpain inhibitors (Beauparlant and Hiscott, 1996; Chen et al., 1997), glucocorticoids (Auphan et al., 1995; Scheinman et al., 1995; Unlap and Jope, 1997) NO (Matthews et al., 1996) and antisense oligonucleotide based interventions (Bielinska et al., 1990; Neurath et al., 1996; Roshak et al., 1996). Unfortunately either low selectivity exhibited by these molecules or in the case of antisense technology a wide variety of unexpected sequence independent effects have come to light both of which compromise rational drug design and single gene elimination.

1.3 : Nuclear and Cytoplasmic Transport

Nuclear and cytoplasmic shuttling is important in regulating many cellular functions including transcription factor activity, mRNA processing and translation. Greater than one million macromolecules per minute are actively transported between the nucleus and the cytoplasm of a growing mammalian cell. This bi-directional traffic is routed through the nuclear pore complex (NPC) which forms the aqueous channel through which all nucleocytoplasmic transport is thought to occur (See Figure 4). NPC are huge structures in vertebrates weighing125 MDa (Reichelt et al., 1990), and are composed of between 50 and 100 distinct polypeptides (Rout and Blobel, 1993) called nucleoporins. Molecules of upto approximately 9nm in diameter, corresponding to a globular protein of approximately 60 kDa, can in principle enter or leave the nucleus by diffusion through the NPC, although in practice very few proteins and no known RNAs

Figure 4. Overall NPC Architecture

The membrane spanning part of the NPC consists of an 8 fold symmetrical arrangement of spoke structures. These form a ring in which sits the central plug. On the cytoplasmic and nuclear surfaces of the ring of spokes form the cytoplasmic and nuclear rings. The cytoplasmic ring carries 8 cytoplasmic filaments that extend at least 30-50nm into the cytosol. The nuclear ring supports 8 thinner fibres of roughly 100nm that are joined by a terminal ring, forming a structure called the nuclear basket.. Adapted from Ohno et al., 1998.


Cytoplasmic Filament
Central Plug
Cytoplasmic Ring
Spoke
Outer Nuclear Membrane
Lumen
Nuclear Envelope
Inner Nuclear Membrane
Nuclear Ring
Basket Filament
Nuclear Basket
Terminal Ring

do so. The functional pore size for active transport is somewhat greater than 25 nm (Feldherr et al., 1984) and this difference between the size for diffusion and active transport channels means that active transport must be accompanied by large conformational changes in the NPC.

The membrane spanning part of the NPC consists of mainly of an 8 fold symmetrical arrangement of spoke structures. These form a ring in which sits the central plug, or transporter, through which active transport occurs (Akey and Goldfarb, 1989; Feldherr et al., 1984). The spokes penetrate into the lumen of the nuclear envelope and are therefore presumed to have transmembrane components and to be essential for anchoring the NPC in the membrane. On both the cytoplasmic and nuclear surfaces of the ring of spokes are annular structures that are of similar but not identical size and shape. These are called the cytoplasmic and nuclear rings. The primary docking sites for an import complex were identified by electron microscopic examination of the docking of NLS substrates that had been conjugated to colloidal gold (Feldherr et al., 1984). The docking sites were found on fibres that extend from the NPC into the cytoplasm.

1.3.1 : Nuclear Import

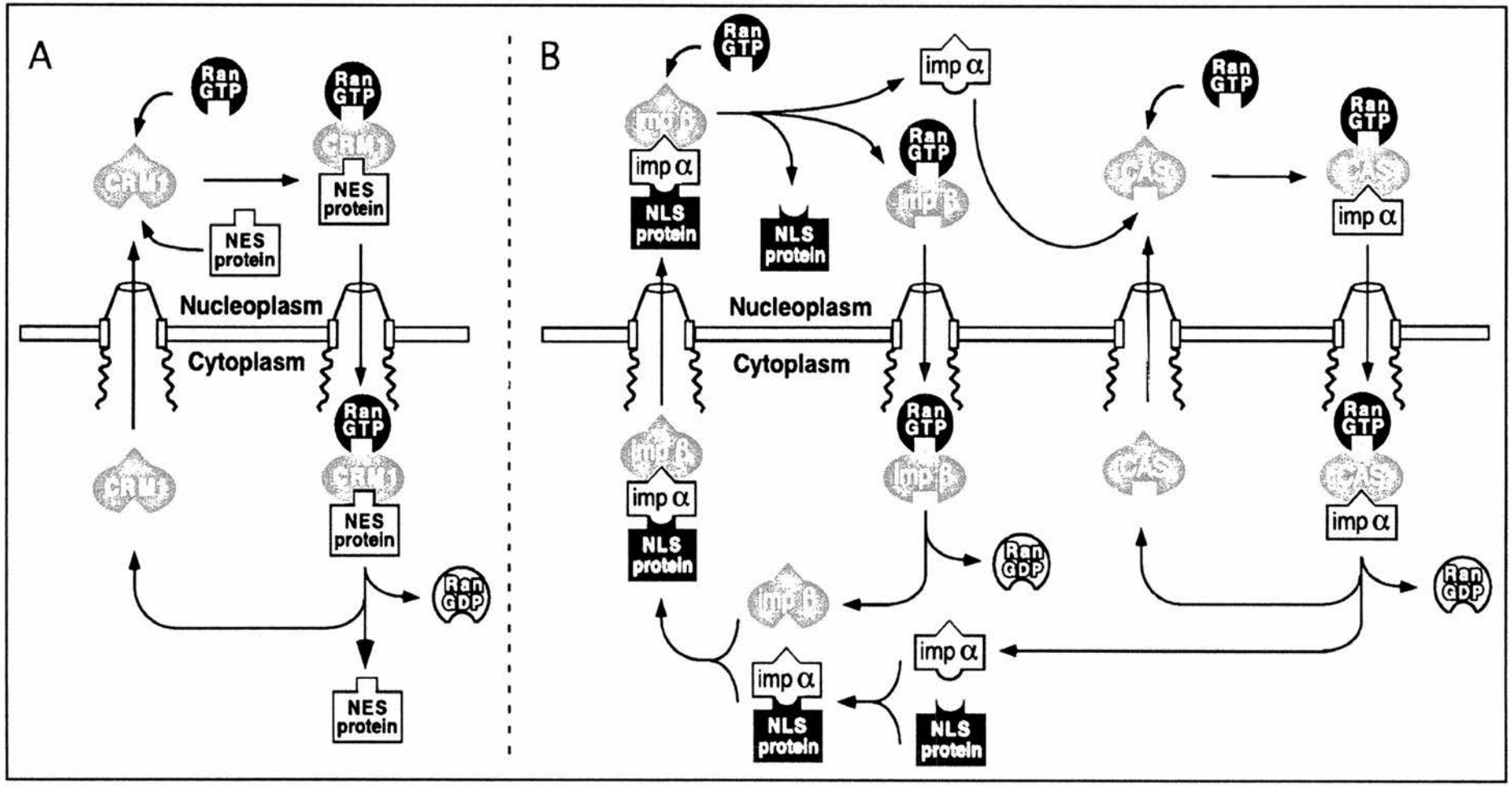
In many cell types nuclear proteins have to reaccumulate in the nucleus after each mitotic division unlike signal sequences involved in targeting proteins into either the endoplasmic reticulum or mitochondria which are generally removed during transit. This means that the nuclear targeting signals must be part of the mature nuclear protein rather than being removed on use (Bonner, 1975; De Robertis et al., 1978). The classical NLSs are those of the SV40 large T antigen and nucleoplasmin (Kalderon ct al., 1984; Robbins et al., 1991) which are short and contain several critical basic amino acids (Kalderon et al., 1984). Classical NLSs are bound by the protein import machinery, importin α and β, which recruits the transport substrate to the NPC (See Figure 5 (B)).

The two importin subunits α/β have specialised functions in docking. Importin α binds the substrate protein through recognition of the NLS, whereas importin β interacts with the NPC (Gorlich et al., 1995; Moore and Blobel, 1992; Moroianu et al., 1995).

Figure 5. Two examples of receptor mediated transport of soluble factors between the cytoplasm and the nucleus.

(A) In the nucleus, an NES containing protein binds to CRM1 co-operatively with RanGTP and is subsequently translocated through the NPC. In the cytoplasm the complex dissociates when Ran hydrolyses GTP under the influence of RanGAP and RanBP1 or 2. Empty CRM1 then re-enters the nucleus.

(B) In the cytoplasm, an NLS protein binds to importin β via the adapter importin α and is translocated to the nucleus. There the complex dissociates on RanGTP binding to importin β. CAS is the export receptor for importin α and is analogous to CRM1 in (A). Both CAS and importin β-RanGTP in the empty state must be recycled. Adapted from Ohno et al., 1998.


A
Ran GTP
CRM1
NES protein
Nucleoplasm
Cytoplasm
Ran GDP
B
imp α
imp β
NLS protein
CAS

Although this has been shown formally for the importin-NLS protein complex, all import receptors are likely capable of docking at the NPC with their substrate. NPC translocation of the importin-NLS protein complex requires two additional soluble proteins, the Ran GTPase activating protein 1 (RanGAP1) (Koepp and Silver, 1996) nuclear transport factor 2 (NTF2) (Moore and Blobel, 1994; Paschal and Gerace, 1995) and Ran (Corbett et al., 1995; Melchior et al., 1993; Moore and Blobel, 1993; Palacios et al., 1996; Richards et al., 1997; Schlenstedt et al., 1995)

The classical nuclear import system is not the only import pathway operating in the cell. The receptors involved in the transport of hnRNP and ribosomal proteins characterised to date are distantly related to importin β (Fornerod et al., 1997; Gorlich et al., 1997; Ohno et al., 1998) but do not follow the importin paradigm of division of function between two subunits. Rather, they are either known or thought to bind their substrates directly rather than via an α-like adapter. Transportin is the import receptor for hnRNP A1 (Fridell et al., 1997; Pollard et al., 1996) which directly binds the M9 import/export signal domain in hnRNP A1. The M9 domain is a region rich in glycine and aromatic amino acids rather than being basic in character. The human hnRNP K protein has 2 import signals. One is a bipartite basic NLS and the second an unrelated element called the KNS (Michael et al., 1997). KNS mediated *in vitro* import is not inhibited by saturating amounts of either the IBB domain (which blocks NLS protein import) or the M9 domain and thus is not dependent on either importin β or transportin (Michael et al., 1997) but some other distinct nucleocytoplasmic transport pathway.

1.3.2 : Nuclear Export

Many substrates for protein import are individual proteins that carry a nuclear import signal and thus, are relatively small and simple. This is probably not the case for export substrates, many of which are RNP particles that can be both large and complex in composition. Ribosomal subunits are one example while mRNPs are another (Franke and Scheer, 1974). Nuclear export is less well characterised than nuclear import, although the best characterised nuclear export proteins are the chromosome region

maintenance 1 protein (CRM1) (See Figure 5 (A)) and the cellular apoptosis susceptibility protein 1 (CAS1). Both export proteins are distantly related to importin β particularly in their amino terminal Ran binding region(Fornerod et al., 1997; Gorlich et al., 1997). CRM1 facilitates the nuclear export of leucine rich NES containing proteins such as Rev, PKI and IκBα (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997) where as the CAS1 binds importin α in the nucleus and recycles it to the cytoplasm following its import (Kutay et al., 1997).

1.3.3 : Ran

Ran is a critical component of almost all known nucleocytoplasmic transport pathways (Corbett et al., 1995; Melchior et al., 1993; Moore and Blobel, 1993; Palacios et al., 1996; Richards et al., 1997; Schlenstedt et al., 1995) and disruption of Ran function reveals pleiotropic phenotypes (Avis and Clarke, 1996). There is also good, but not definite, evidence that GTP hydrolysis by Ran supplies the energy for protein import (Melchior et al., 1993). However for export pathways at least some forms of nuclear export require nuclear Ran GTP, but may not depend on hydrolysis of the Ran bound GTP (Izaurralde et al., 1995; Richards et al., 1997). Unfortunately translocation assays currently available absolutely require the presence of an intact nucleus, making it difficult to pinpoint where the energy might be stored or what precise event accompanies hydrolysis of Ran associated GTP (Ohno et al., 1998).

Ran is extremely abundant and at steady state is mainly nuclear, although it is believed to move between the nucleus and the cytoplasm. Like many other regulatory GTPases, Ran has a low intrinsic activity and has to interact with a GTPase activating protein RanGAP1. However cytoplasmic interaction of RanGAP1 with the RanGTP bound protein also requires another protein, RanBP2, which increases the rate of RanGAP1 induced GTP hydrolysis to maximal level (Bischoff et al., 1995). Following cytoplasmic hydrolysis, Ran is transported across the nuclear membrane and the RanGDP is converted to RanGTP by virtue of the guanine nucleotide exchange factor

RCC1 (Izaurralde et al., 1997). Although other Ran-binding proteins exist, these are the major components involved in modulation of Ran function.

Thus the critical aspect of Ran's function relies on the fact that RCC1 is nuclear and stably bound to chromatin (Sweet and Gerace, 1996) while both RanBP1 and RanGAP1 are found either on the cytoplasmic side of the NPC or in the cytosol (Gorlich, 1997; Koepp and Silver, 1996) predicting that RanGTP concentration will be high in the nucleus and low in the cytoplasm providing part of the driving force behind nuclear and cytoplasmic transport. In fact treatments that collapses this RanGTP : RanGDP ratio or decreasing the nuclear RanGTP concentration, block nucleocytoplasmic transport (Corbett et al., 1995; Gorlich et al., 1996; Izaurralde et al., 1997; Melchior et al., 1993; Moore and Blobel, 1993; Palacios et al., 1996; Richards et al., 1997; Schlenstedt et al., 1995). Thus the differential RanGTP concentrations in the nucleus and the cytoplasm are likely to be a fundamental cause of directionality in receptor-mediated nucleocytoplasmic transport events. However this is unlikely to be the only determinant of transport asymmetry; other contributors to directionality are likely to include those of the structurally asymmetric NPC.

1.3.4 : Molecular Mechanisms of Nucleocytoplasmic Transport

The basic import machinery consists of importin α complexed with the NLS containing protein, importin β (RanGDP bound form) and NTF2. The model of nuclear import commences with the cargo-NLS recognition by the importin α subunit which then binds the importin β subunit (this strengthens the affinity for importin α and the NLS). Following assembly the importin β facilitates the importin complex docking at the cytoplasmic fibres of the NPC (Akey and Goldfarb, 1989; Feldherr et al., 1984), from there the complex is transferred the centre of the cytoplasmic face of the central plug (Akey and Goldfarb, 1989; Feldherr et al., 1984; Pante and Aebi, 1996). Alternatively a single productive step involving filament bending to present the transport complex bound at the tip of a cytoplasmic filament to the central plug may occur (Pante and Aebi, 1996). Once in the nucleus the β importin subunit is modified to a RanGTP bound state,

by RCC1. This decreases the affinity of importin β for importin α and the NLS-cargo thus the importin complex dissociates depositing the NLS containing protein in the nucleus and importin α and β are recycled back to the cytoplasm.

As mentioned, importin α although an import protein, also functions as a substrate for nuclear export. In this model of nuclear export, importin α binds the export adapter CAS1 in a RanGTP dependent manner (Kutay et al., 1997). Following this the exportin complex docks with the nuclear basket like structure at the NPC and then translocates the NPC to the cytoplasm. The cytoplasmic exportin complex is then a substrate for the RanGAP1/RanBP1 proteins (Floer et al., 1997; Gorlich, 1997; Kutay et al., 1997; Lounsbury and Macara, 1997) which facilitate CAS1 RanGTP hydrolysis. This causes disassembly of the exportin complex and importin α is deposited in the cytoplasm while CAS1 is recycled back to the nucleus.

In summary cytoplasmic import complexes are stable only in the absence of receptor bound RanGTP and they dissociate in its presence. In contrast, export complexes form in the nucleus in the presence of RanGTP and their dissociation in the cytoplasm involves the hydrolysis by the receptor bound Ran under the influence of the RanBP1 and RanGAP1 proteins that are either cytosolic or associated with the cytoplasmic face of the NPC (Floer et al., 1997; Gorlich, 1997; Kutay et al., 1997; Lounsbury and Macara, 1997).

1.4 AIMS / OBJECTIVES

To identify proteins involved in the activity of the transcription factor NF-κB.

To fully characterise this interaction *in vivo* and *in vitro*.

To find or postulate the physiological relevance of those interactions *in vivo*.

2. MATERIALS & METHODS

2.1: Antibodies

2.1.1: Antibodies used in experiments

IκBα (C-21, Santa Cruz) is a rabbit polyclonal antibody raised against a peptide corresponding to the carboxyl terminus of IκBα of human origin and used in immunofluoresence at 1:200 dilution. 4B10 monoclonal antibody to hnRNP A1 obtained from G. Dreyfuss, (University of Pennsylvania, Pennsylvania, USA) was used in immunoprecipitations, immunofluoresence at 1:200 dilution and western blotting at 1:1000 dilution. SV5 Pk tag monoclonal antibody (Hanke et al., 1992) was obtained from R.E. Randall, University of St. Andrews and was used as a control immunoprecipitation antibody. 10B monoclonal antibody which recognises IκBα (Jaffray et al., 1995) was obtained from Ellis Jaffray and was used in western blotting at a 1:100 dilution. The anti-myc monoclonal antibody was obtained from C. Dargemont, Institut Curie, Paris was used in immunofluoresence to detect the control protein localisation at 1:200 dilution.

2.1.2: Affinity purification of sheep antibodies

Primary antibodies to IκBα (wild type), p50 (residues 35-381) and p65 (residues 12-317) proteins were raised in sheep (Scottish Antibody Production Unit, Carluke) and were antigen affinity purified. NHS Hi-Trap™ affinity columns were used for the purification of the antisera from the crude sheep serum. The columns were washed with 10 volumes of coupling buffer (200mM $NaHCO_3$ and 500mM NaCl, pH 7.80). Following this 5mgs of the appropriate recombinant protein was recirculated over the column for 60 minutes and bound to the column. In order to deactivate any excess groups that have not coupled ligand and to wash out non-specifically bound ligand, the columns were washed in buffer A (500mM of NaCl and ethanolamine, pH 8.30) and buffer B (100mM sodium acetate and 500mM NaCl, pH 4.00). After column preparation a 1:10 dilution of sheep serum (diluted in PBS) was recirculated over the

column for 16 hours. Following loading, the column was washed extensively with 20mls of 10mM Tris, 500mM NaCl, pH7.50 and then eluted using 100mM glycine, pH 2.25. 500µl fractions were eluted into 50µl of Tris, pH 8.0 in order to neutralise the acidic eluate and maintain antibody activity. The eluted antibodies were supplemented with 1 mg/ml BSA, snap frozen in liquid nitrogen and stored at -70°C. Following characterisation antibodies were used in immunoprecipitation experiments and at 1:1000 dilution for western blotting.

2.2: Primers and expression vectors

2.2.1: Oligonucleotide Primers

Oligonucleotides were ordered from Oswel and the sequences are written 5' to 3' with restriction sites underlined.

FORWARD PRIMERS

hnRNP A1 1-320 : GTCGGATCCATGTCTAAGTCAGAGTCTCCT

hnRNP A1 196-320 : AGAGGATCCATGAGTGGTTCTGGAAACTTTGGT

hnRNP A1 65-320 : ATTATATGGATCCGTGGAGGAGGTGGATGCAGCT

hnRNP A1 75-320 : ATAGGATCCATGAGGCCACACAAGGTGGAT

hnRNP A1 85-320 : TAGGATCCATGGAACCAAAGAGAGCTGTCTCC

hnRNP A1 90-320 : CGGATCCATGGTCTCCAGAGAAGATTCT

hnRNP A1 95-320 : ATAGGATCCATGTCTCAAAGACCAGGTGCC

hnRNP A1 105-320 : GCGGGCGGATCCATGAAAAAGATATTTGTTGGTGGC

hnRNP A1 142-320 : AGTCGGATCCATGAGTGGCAAGAAAAGGGGCTTT

hnRNP A1 162-320 : GCGCCGCGGGATCCATGATTGTCATTCAGAAATAC CAT

hnRNP A1 182-320 : CGGGATCCATGTCAAAGCAAGAGATGGCTAGT

IκBα 1-317 : GTACTAGGATCCATGTTCCAGGCGGCCGAG

REVERSE PRIMERS

hnRNP A1 320-1 : GCCGCGAATTCTTAAAATCTTCTGCCACTGCC

hnRNP A1 267-1 :TATATTAAATTGAATTCGTTGTAATTCCCAAAATCATT

hnRNP A1 247-1	:CGCGGGAATTCAAATCCATTATAGCCATCCCC
hnRNP A1 227-1	:AGAATTCGCCACCACGACCACTGAAGTT
hnRNP A1 207-1	:CGAATTCTCCACGACCACCACCAAAGTT
hnRNP A1 196-1	:GGAATTCTCGACCTCTTTGGCTGGA
IκBα 296-1	:CGCGAATTCTGACGTGAACTCTGACTCTGT
IκBα 303-1	:CGAATTCTGACAGCTCGTCCTCTGTGAA
IκBα 313-1	:CGAATTCTGACTGGCCTCCAAACACACA

2.2.2: Expression vectors

pGEX-2T was obtained from Pharmacia. The transient mammalian expression vector pcDNA3 was purchased from Invitrogen Corporation. The luciferase reporter vectors 3EnhConALuc, ConALuc, AP1ConALuc were a kind gift from F.Arenzana-Seisdedos (Institut Pasteur). pV44ER.LexA and pACT/ pACT-cDNA were received from Colin Goding (Marie Curie Research Institute, Oxted, United Kingdom) and Stephen Elledge (Baylor College of Medicine, Houston, Texas), respectively, and both have been described previously (Durfee et al., 1993; Jayaraman et al., 1994). IκBα wt, deletions (1-256, 1-276, 1-280, 1-283 and 1-292) and β-Gal chimeras were a kind gift from Manuel Rodriguez (Rodriguez et al., 1995) (Kroll et al., 1997). All hnRNP A1 cDNAs and derivatives and IκBα truncations were amplified by PCR oligonucleotides containing Bam H1 and Eco R1 sites, digested with both enzymes and cloned into Bam H1 and Eco R1 cut pGEX 2T and pCDNA3. Vector maps of all constructs generated during this work are shown in Appendix I.

2.3: cDNA Cloning

2.3.1: Preparation of Electrocompetent Bacteria (DH5α)

E. coli DH5α (genotype: Φ80d*lac*ZDM15, *rec* A1, *end* A1, *gyr* A96, *thi*-1, *hsd* R17 (r_k-,m_k+), *sup E*44, *rel* A1, *deo*R, D(*lac*ZYA-*arg* F)U169) was used for routine DNA preparation and protein expression. Bacteria were grown in Luria-Bertani (LB) broth with antibiotics added when required. A 10 ml overnight culture grown in LB at

37°C was used to inoculate one litre of LB which was maintained in culture at 25°C until an OD_{600} of approximately 0.5. Cells were then chilled on ice for 30 minutes and centrifuged 15 minutes at 4000 rpm. Bacteria were resuspended in one litre of cold 1mM HEPES pH 7 and then centrifuged as described previously. Bacteria were resuspended in 500 ml of cold 1 mM HEPES pH 7 and centrifuged at 4000 rpm for 15 minutes. Bacteria were then resuspended in 20 ml of cold 10% Glycerol and centrifuged as described previously. Finally the bacteria were resuspended in a final volume of 2 to 3 ml of cold 10% glycerol and bacteria was frozen in dry ice and kept at -70 °C in small aliquots. All solutions were previously autoclaved and kept at 4 °C.

2.3.2: Transformation of electrocompetent bacteria

40 µl of electrocompetent bacteria were incubated with either plasmid DNA or ligation mixtures, on ice for at least 1 minute. 1ng of known plasmid DNA or no DNA were added to electrocompetent cells as positive and negative controls. After incubation the transformation mixture was transferred to a 2 mm electroporation cuvette (Flowgen), previously chilled on ice and the following electroporation conditions were used: V= 2500; C= 201; T= 5 msec. 1 ml of LB/20 mM glucose was immediately added to the transformed bacteria and the cells were incubated one hour at 37 °C before being plated on LB-Agar containing the relevant antibiotic.

2.3.3: DNA preparation

All the DNA preparations (minipreps, maxi-preps and gel extraction) used for cloning and transfections were prepared with Qiagen kits in accordance with manufacturers instructions. DNA restriction enzymes for cloning and characterisation of clones were obtained from New England Biolabs (NEB) and Promega. The Vent DNA polymerase used for PCR (polymerase chain reaction) was obtained from NEB. A Boeringher "Titan™ one tube RT-PCR System" was used for reverse transcription followed by PCR-amplification (RT-PCR). Quality and quantity of DNA was analysed by spectrophotometric readings at 260 nm and 280 nm and by electrophoresis in an agarose gel in the presence of ethidium bromide, followed by U.V.(Kozutsumi et al., 1989).

2.3.4: DNA sequencing

All constructions were used to transform *E. coli* DH5α to ampicillin resistance. Plasmid DNA of all constructs was isolated and inserts sequenced (ABI377) by Alex Houston of the University of St Andrews DNA sequencing facility.

2.4: Expression and purification of recombinant proteins

GST-hnRNP A1 wt and deletions, GST-IκBα wt and deletions, GST-IκBγ, GST-NFIII and GST constructions were expressed in *Escherichia coli* strain DH5α. Induction of expression, glutathione agarose affinity chromatography and thrombin cleavage of fusion proteins was as described (Jaffray et al., 1995).

2.5: Quantitation of protein

Protein concentrations were determined using BCA protein assay reagent (Pierce). Protein samples were mixed with the BCA reagent and left for 20 minutes at 37°C and the absorbance at 595 nm was measured on a spectrophotometer. Protein absorbencies were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbencies of a range of bovine serum albumin (BSA) concentrations.

2.6: SDS-PAGE and Western blot analysis

Protein samples were resuspended in disruption buffer (1X: 20 mM Tris/ HCl pH6.8, 2% SDS, 5% β-mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and denatured at 100°C for 5 minutes before loading on a 10% SDS-polyacrylamide gel. Bio-Rad mini gel equipment was used in accordance with the manufacturers instructions. Gibco protein molecular weight markers were used as standards to establish the apparent molecular weights of proteins resolved on SDS-polyacrylamide gels. Separated polypeptides were either stained with Coomassie Blue (0.2% Coomassie brilliant blue R250; 50% methanol; 10% acetic acid) for 30 minutes and then destained (20% methanol; 10% acetic acid) or transferred to a polyvinylidene difluoride membrane (Sigma) using a wet blotter (Biorad Systems). The membranes were blocked with PBS

containing 10% skimmed milk powder and 0.1% Tween 20 then incubated with monoclonal or polyclonal antibodies diluted in blocking buffer. Horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (Amersham) were used as secondary antibodies. Western blotting was performed using ECL detection system. After ECL detection and when necessary western blots were stripped as described (Roff et al., 1996).

2.7: Affinity purification of protein complexes containing IκBα

A frozen cell pellet from a 200 litre culture of B cells (Namalwa) was resuspended in lysis buffer (50mM Sodium Fluoride, 5mM Tetra-Sodium Pyrophoshate, 1mM Sodium Orthovanadate, 10mM β-glycerophosphate, 2mM EDTA, 0.05% NP40, 20mM sodium phosphate buffer pH 7.50) containing a cocktail of protease inhibitors (100μM Pefablock, 1μM TLCK, 50μg/ml Bestatin, 1μM Pepstatin and 1μM Leupeptin). Following cellular disruption by sonication the extract was clarified by centrifugation (20, 000 x g) for 30 minutes at 4°C. The supernatant was removed and recentrifuged (100,000 x g) for 60 minutes at 4°C to remove any particulate material. The supernatant was passed through a Protein A sepharose column, then through a pre-Immune column (pre-immune sheep IgG covalently crosslinked to Protein A sepharose), and finally through an anti-IκBα column (anti-IκBα covalently crosslinked to Protein A, this was used to purify IκBα complexes from the total protein extract). All protein A columns were prepared in accordance with the Antibodies Manual (Harlow and Lane, 1988). Subsequently anti-IκBα and anti-PI columns were washed extensively with 150mls lysis buffer and 50 mls 10mM triethylamine pH 8.0. The columns were eluted using 15mls 100mM acetic acid pH 2.25. The elutions from both columns were freeze dried overnight, the proteins TCA precipitated and resuspended in HPLC grade water, separated by 10% SDS PAGE and detected by Coomassie R450 staining.

2.8: In gel trypsin digestion

In gel trypsin digestion was carried out on the appropriate specific bands excised from the 10% SDS gel. A blank piece of gel and the 43kDa band (Actin) present in the specific and non-specific columns elutions were used as an internal control of protein sequencing. The gel was stained in 0.1% Coomassie Brilliant Blue R450/20% Methanol/0.5% Acetic acid and then destained in 30% Methanol until the bands were visible above a clear background. After excision, gel slices were washed twice in with 200mM ammonium bicarbonate containing 50% acetonitrile, pH 8.90 and freeze dried overnight. Gel slices were reswollen in 200mM ammonium bicarbonate, 1% Reduced Triton X-100 in 10% acetonitrile containing 10μg modified trypsin (PROMEGA) and incubated at 37°C for 20 hours. All supernatants were evaporated to near dryness, digested peptides fragments were reconstituted in 20ml of 0.1% TFA and separated by HPLC using a microbore HPLC System. The peaks present in the linear range of the trace were sequenced using the Edman Degradation Technique by Graeme Kemp and Paul Talbot University of St Andrews, protein sequencing unit.

2.9: *In vitro* transcription translation

In vitro transcription/translation was performed using 1-2 μg of plasmid DNAs and a TNT Coupled Wheat Germ Extract System (PROMEGA) according to the instructions provided by the manufacturer. 20 μCi of ^{35}S-methionine (Amersham) was used in the reactions to generate radiolabelled proteins. Proteins expressed were detected and standardised using a phospho-imager (Fuji Bas 1500).

2.10: Preparation of Namalwa, CB3 and HeLa cell fractions

Cell pellets of HeLa, CB3 and Namalwa cells was resuspended in lysis buffer (50mM Sodium Fluoride, 5mM Tetra-Sodium Pyrophoshate, 1mM Sodium Orthovanadate, 10mM β-glycerophosphate, 2mM EDTA, 20mM sodium phosphate buffer pH 7.5, 0.5% NP40) containing a cocktail of protease inhibitors (100μM Pefablock, 1μM TLCK, 50 μg/ml Bestatin, 1μM Pepstatin and 1μM Leupeptin).

Following cellular disruption by sonication the extract was clarified by centrifugation (20, 000 x g) for 30 minutes at 4°C. The supernatant was removed and recentrifuged (100,000 x g) for 60 minutes at 4°C to remove any residual particulate material before use in downstream applications.

2.11: Cell Culture and transfections

HeLa and CB3 cells were maintained in exponential growth in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum. 5-10 μg of plasmid DNAs were transfected by electroporation (Equibio). The media in which HeLa and CB3 cells were maintained was changed 1 hour pre-transfection (D-MEM/10% FCS). Following which the cells were trypsinised and counted and for each transfection. 5 X 10^6 cells were resuspended in 200μls of media supplemented with 15mM HEPES, pH 7.50. DNA to be transfected (10μg) was made up in 210mM NaCl, 30μg salmon sperm carrier and made up to a final volume of 50μl. The DNA and cells were mixed, added to the electroporation cuvettes (Equibio) and electroporated at 240V and 1200mFD. Following electroporation the cells were resuspended in 5mls of media containing 15mM HEPES, pH 7.50. Cells were centrifuged at 1,200 rpm for 2 minutes and the media changed back to normal culture conditions (D-MEM/10%FCS). The cells were left for 12 hours to express the transfected material before any downstream applications were carried out.

2.12: Luciferase and β-Galactosidase assays

2.12.1: Luciferase assays

CB3 cells were maintained in exponential growth in Dulbecco's-modified Eagle's medium, containing 10% fetal calf serum. 5-10μg of plasmid DNAs (an NF-κB dependent, 3EnhConALuc or AP1 dependent ConALuc or ConALuc) were co-transfected in triplicate with described plasmids for 12 h. The cells were harvested and washed 2 times in PBS. Cells were lysed using 25mM tris phosphate, 8mM $MgCl_2$, 1mM DTT, 1% Triton X-100 and 15% glycerol. Following cell extract protein

standardisation, luciferase activity was assayed in luciferase buffer (25mM luciferin, 1mM ATP, 1% BSA) made up in lysis buffer. Luciferase activity was measured using the MicroLumat (LB96P) plate reader and units of activity are given in relative light units (RLUs).

2.12.2 : β-Galactosidase assays

Following protein standardisation of the cell extracts, the Galacton Light Plus™ (Tropix) Chemilluminescent reporter assay was used to measure the relative light units (RLU) produced. Extracts were prepared and assayed in accordance with manufacturers instructions.

2.12.3 : Luciferase : β-Galactosidase Ratio

The luciferase : β-Galactosidase ratio was used to work out NF-κB units of activity, shown in the results section. The results presented is representative of 8 sets of data performed in triplicate. Data obtained was processed using the Kalieda Graph™ statistical analysis program and the standard error was calculated as a function of the standard deviation.

2.13: hnRNP A1/IκBα Immunoprecipitation

0.5μgs of anti-p65, anti-IκBα, PI (Sheep) and anti-hnRNP A1 and anti-SV5 tag were incubated with 10μl of protein A beads, on a rotating mixer, for 60 minutes at 4°C. Following this the beads were washed with 10 volumes of 3M NaCl, 50 mM sodium borate, pH 9.0, by centrifugation and aspiration. Subsequently the washed beads were resuspended in 10 volumes of 3M NaCl, 0.2M sodium borate, 45mM dimethylpimelimidate, pH 9.0, and incubated for 60 minutes at 4°C. The crosslinking reaction terminated by washing the beads twice in 10 volumes of 0.2M ethanolamine, pH 8.0 (Harlow and Lane, 1988). Following incubation of the crosslinked beads with cellular extracts the beads were washed 3 times with incubation buffer (100mM Potassium acetate, 1mM DTT, 20mM TRIS, 10mg/ml BSA and 0.05% NP40, pH 7.50) and 1 time in distilled water to remove excess BSA. Immobilised complexes were resuspended in SDS loading buffer (1X: 20 mM Tris/ HCl pH6.8, 2% SDS, 5% β-

mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and resolved by 10% SDS PAGE and western blotted for the appropriate protein.

2.14: Immunofluoresence Microscopy

HeLa cells grown on glass coverslips were cultured in the presence or absence of Actinomycin D (10 μg/ml) for 4 hours. Following this cells were fixed with 3% Parafromaldehyde/PBS for 10 minutes and fixation quenched by two 10 minute 0.1M Glycine/PBS incubations. Subsequently cells were permeabilised with a 10 minute 0.1% Triton X-100 (TX-100) incubation and cells were blocked (before antibody addition) using a 10 minute 0.2%BSA/PBS incubation. Immunostaining with the primary antibodies 4B10, 336 (anti-hnRNP A1 and anti-SV5 tag respectively), C21, (anti-IκBα) and anti myc were carried out at a concentration of 1:200. Immobilised antibody/antigen complexes were detected using the secondary antibodies (anti-mouse Texas Red and anti-rabbit FITC) at a concentration of 1:200. Following staining, cells were visualised and photographed using confocal and immunofluoresence microscopy. Pictures were taken by ordinary and confocal microscopy. The images obtained were processed by Adobe Photoshop 5.0 and annoted in Adobe Pagemaker 6.50.

2.15: *In vitro* Binding Studies

Glutathione beads containing 0.1μg of the respective fusion protein were blocked for 1 hour prior to use with PBS/BSA 10mg/ml. After blocking, the beads were washed once with the incubation buffer, resuspended in the buffer with the appropriate volume of the *in vitro* transcription/translation product and incubated for 1 hour at 4°C. The beads were washed 3 times with incubation buffer and 1 time in distilled water. Immobilised complexes were resuspended in SDS loading buffer, separated by 10% SDS PAGE. Following separation the gel was dried and interacting proteins were detected using a phosho-imager (Fuji Bas 1500).

2.16: Yeast II Hybrid Analysis

The yeast two hybrid system (Fields and Song, 1989) was used to test the interaction between hnRNP A1 and IκBα. The pV44ER.LexA expression vector was used to generate a fusion of the LexA DNA binding domain with the wt hnRNP A1. A second vector pACT was used to express a fusion of Gal4 activation domain with IκBα. Interacting species were identified in yeast Sacchromyces cerevisiae L40a reporter strain [His- Leu- Tryp- Ade- (LEXAop)4- HIS3; (LEXAop)8 - LacZ] and transformations were carried out using Clontech procedures. Following this co-transformants were grown on SD plates with differing levels of 3 Amino Triazole (0-30mM) and β-Galactosidase activity was measured qualitatively using the Clontech filter lift procedure. For quantitative analysis of β-Gal activity, single colonies were picked from -Trp, -Leu, -His plates and resuspended in 50mls of 100mM Potassium Phosphate buffer (containing 0.2% TX-100, 0.005% SDS and 7.5µl of chloroform). Yeast cells were lysed by vigorous vortexing for 10 seconds and their protein concentrations for each extract was estimated using BCA reagents (Pierce). Following protein standardisation the Galacton Light Plus™ (Tropix) Chemilluminescent reporter assay was used to measure the relative light units (RLU) produced by interacting proteins.

2.17: Electromobility Shift Assays (EMSAs)

1ng of NF-κB and varying concentration of the other proteins used were made up in 18µl of binding buffer (3.5mM Spermidine, 420µl GRABB salt [25mM HEPES, pH7.50, 1mM DTT, 1mM EDTA, 0.05% NP40, 10% glycerol, 100mM NaCl and 100mg/ml BSA], 6mM $MgCl_2$, 0.1% NP40, 1 mg/ml BSA, 25mM DTT) and incubated at 4°C for 30 minutes. Following this 2µl of the HIV-LTR probe was added to the tubes and left to incubate for a further 30 minutes at 4°C. After incubation 4µl of GRA dyes (25mM HEPES, pH 7.50, 1.6g glycerol, 10mM DTT and bromophenol blue) were added to the samples which were subsequently separated using a 6% non-denaturing PAGE, containing 0.5X TBE (45mM Tris, 45mM Borate, 1mM EDTA), for 2 hours at

200 Volts. Following separation the gel was dried and protein/DNA interactions were detected using a phosho-imager (Fuji Bas 1500).

2.18: Labelling of probe for use in EMSAs

10μCi of ^{32}P ATP, 2μl of 10X kinase buffer (New England Biolabs), 5 pmoles of the top and bottom strands of HIV-LTR DNA and 2μl of polynucleotide kinase (New England Biolabs) were incubated at 37°C for 30 minutes. Following incubation the labelling mixture was heated to 100°C for 2 minutes and allowed to cool to room temperature. The labelled material was separated from unlabelled probe and free isotope using 12% non-denaturing PAGE, containing 1X TBE (90mM TRIS, 90mM Borate, 2 mM EDTA), for 2 hours at 200 Volts. The gel was then exposed to film for 3 minutes. Following developing the correct band from the gel was excised and eluted overnight in 300μl of TEN (10mM Tris/HCl pH 8.00, 1mM EDTA, 100mM NaCl) at 4 °C.

3. RESULTS

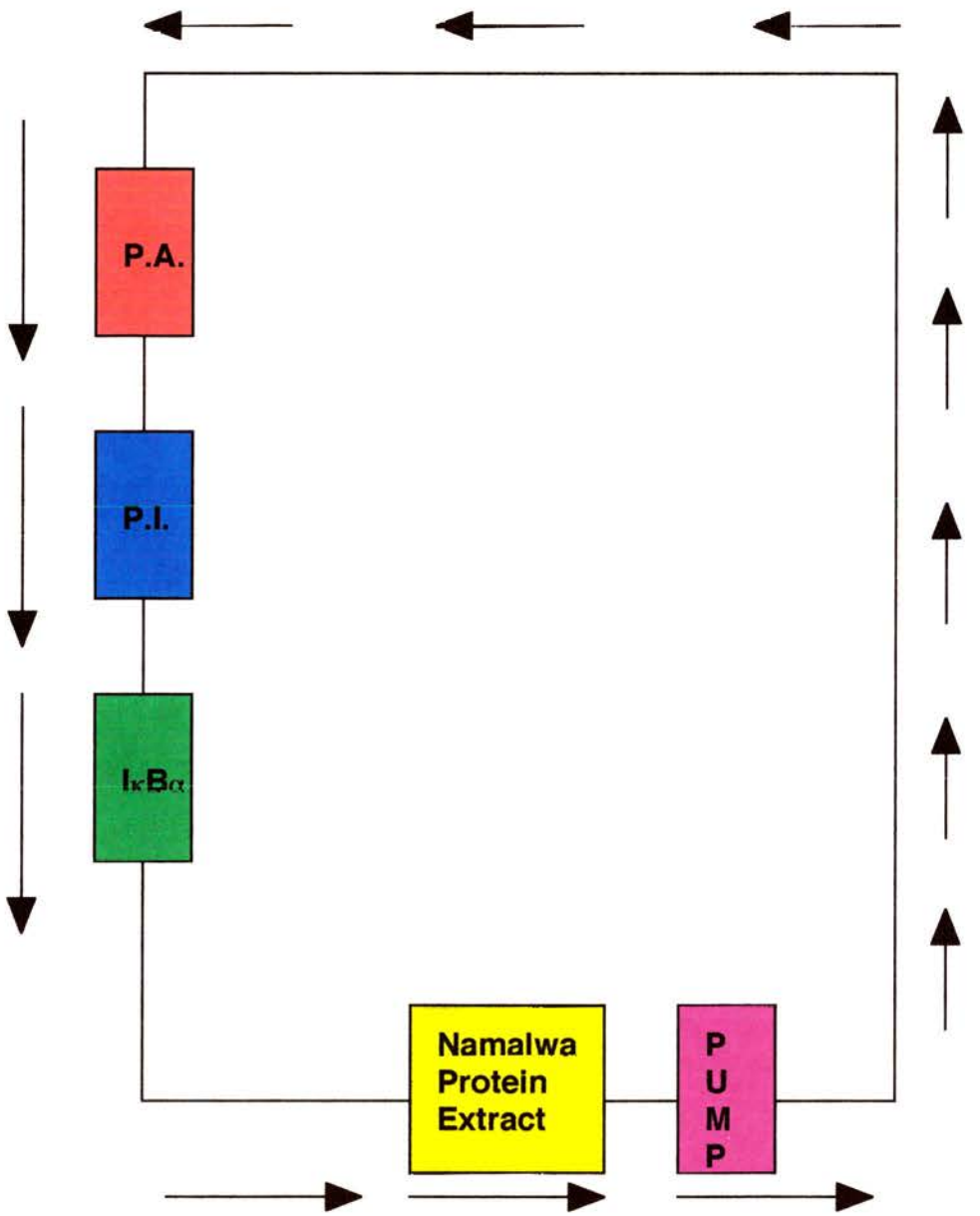
3.1 : hnRNPA1 interacts with IκBα

To identify proteins involved in the activity of the transcription factor NF-κB, proteins bound to the IκBα inhibitor were isolated by immuno affinity chromatography. An extract was prepared from a cell pellet collected from a 200 litre culture of Namalwa cells (B-cells) in a buffer containing a cocktail of protease and phosphatase inhibitors, used to preserve protein-protein complexes in the extract. We decided to use Namalwa cells as a source of protein as they contained constitutively active NF-κB. Also previous attempts using cells from a 100 litre culture of HeLa cells did provide enough material to successfully carry out protein sequence analysis on the isolated proteins.

The Namalwa extract was first passed over a column of protein A agarose column to remove any residual particulate matter found in the total protein extract. Following this the extract was passed through a Protein A column to which sheep preimmune IgG was linked. This column was used to remove any non-specific interacting proteins from the cell extract. The pre-immune IgG was purified from pre-immune sheep serum using protein A beads and following washing the beads were crosslinked before use. We crosslinked beads in order to prevent any antibody displacement when the proteins were eluted from the column and thus contaminate the downstream sequencing processes. The flow through from this column was then passed over a third column of protein A agarose to which anti-IκBα IgG was linked. Again the beads were crosslinked in order to prevent any antibody displacement. The anti-IκBα column was used to remove complexes containing IκBα from the protein extract (the affinity column set-up is shown in Fig. 6). The anti-IκBα used on the protein A column had been affinity purified from sheep serum, using IκBα recombinant protein conjugated to a Hi-Trap NHS activated column, and following washing the antisera eluted into aliquots and stored at -70°C. We used sheep polyclonal antisera, for affinity purification, as polyclonal antibodies recognise more epitopes than their corresponding monoclonals. This is of great advantage especially when purifying protein complexes from cell extracts, as

Figure 6. Affinity purification set-up used to purify IκBα complexes from the Namalwa cell extract.

The total protein extract prepared from the Namalwa cell pellet (shown in yellow) was recycled overnight through the affinity column set-up using a peristaltic pump (shown in crimson). The extract was first fed through a Protein A column (P.A. shown in red) to remove any residual particulate matter which could potentially block the column. Secondly through a Pre-Immune column (P.I. shown in blue) to remove any non-specific interactions from the cell extract and finally through the anti-IκBα column (shown in green) to remove IκBα and any complexed material. The arrows demonstrate direction of flow.


P.A.
P.I.
IκBα
Namalwa
Protein
Extract
P
U
M
P

protein epitopes *in vivo* may become occluded when protein-protein interactions are formed and ultimately this would reduce the efficacy of the affinity purification step. The Namalwa extract was left recycling through the affinity column set-up for 16 hours at 4°C. Following IκBα complex purification the Namalwa extract, pre and post-immunoaffinity purification, was separated by SDS PAGE (10%), and transferred to PVDF. Western blot analysis of the load and flow through fractions indicated that IκBα was present in the cell extract pre-affinity purification but was absent from the column flow through (Fig. 7A). As the affinity purification process was functioning efficiently both preimmune and anti-IκBα columns were separated and extensively washed before bound proteins were eluted from with acetic acid. Acetic acid was used to elute the proteins from the column as it was volatile and could easily be removed when the eluted samples were freeze dried overnight. Following freeze drying the fractions were resuspended in water and TCA precipitated, washed twice with ice cold acetone, resuspended in water and SDS PAGE sample buffer. Following SDS PAGE (10%), the eluted fractions were transferred onto PVDF membrane and western blotted for IκBα, p50 and p65 with sheep polyclonal antisera raised against the respective recombinant proteins. p50 and p65 polyclonal antisera used for western blotting was affinity purified in the same manner as for IκBα. Eluates indicated that IκBα along with NF-κB p50 and p65 were bound to and eluted from the anti-IκBα column, but were not present in the eluate from the pre-immune column (Fig. 7, B, C and D). These data indicated that immuno-affinity purification procedure was functioning efficiently.

After demonstrating that purification of IκBα was complete and specific, peak fractions were pooled separated by SDS PAGE (10%) and stained with Coomassie Brilliant Blue. In addition to NF-κB and IκBα proteins a prominent species migrating as a doublet of 34 and 38 KDa was identified (Fig. 8). Bands that differed, between the anti-IκBα and anti-Pre Immune IgG columns, and a blank piece of stained Coomassie SDS PAGE (10%) gel were excised from and washed twice with 50% acetonitrile in ammonium bicarbonate and freeze dried overnight. The following day the freeze dried products were resuspended in 50µl of ammonium bircabonate/1%Triton in 10%

Figure 7. Affinity purification of IκBα containing complexes from a total protein extract.

(A) Total protein extracts prepared from Namalwa cells was passed through an affinity column set-up. Anti-IκBα (α-IκBα) crosslinked to protein A beads was used to purify IκBα complexes from the extract. The anti-pre-immune column (α-PI) was used as a negative control. The columns were washed extensively, eluted and bound proteins were separated by SDS PAGE(10%). The presence of IκBα complexes were determined by western blot (WB α-IκBα) analysis (anti-IκBα was used at 1:1000 and detected using an anti-sheep HRP antibody at 1:5000). IκBα complexes were completely purified from the total protein extract if the load and flow through (shown as FT) lanes are compared. (B), (C), (D). The sheep anti-IκBα, anti-p65 and anti-p50 were used at 1:1000 to detect cellular IκBα, p65 and p50 respectively (WB α-IκBα, WB α-p65, WB α-p50). Immobilised antigen/antibody complexes were detected using an anti-sheep HRP antibody (1:5000) and the filter was exposed to Fuji film for 30 seconds. Figures B, C and D demonstrate that IκBα complexes were present in 4 of the fractions eluted from the affinity column set-up (shown as 1-4 in the diagram).

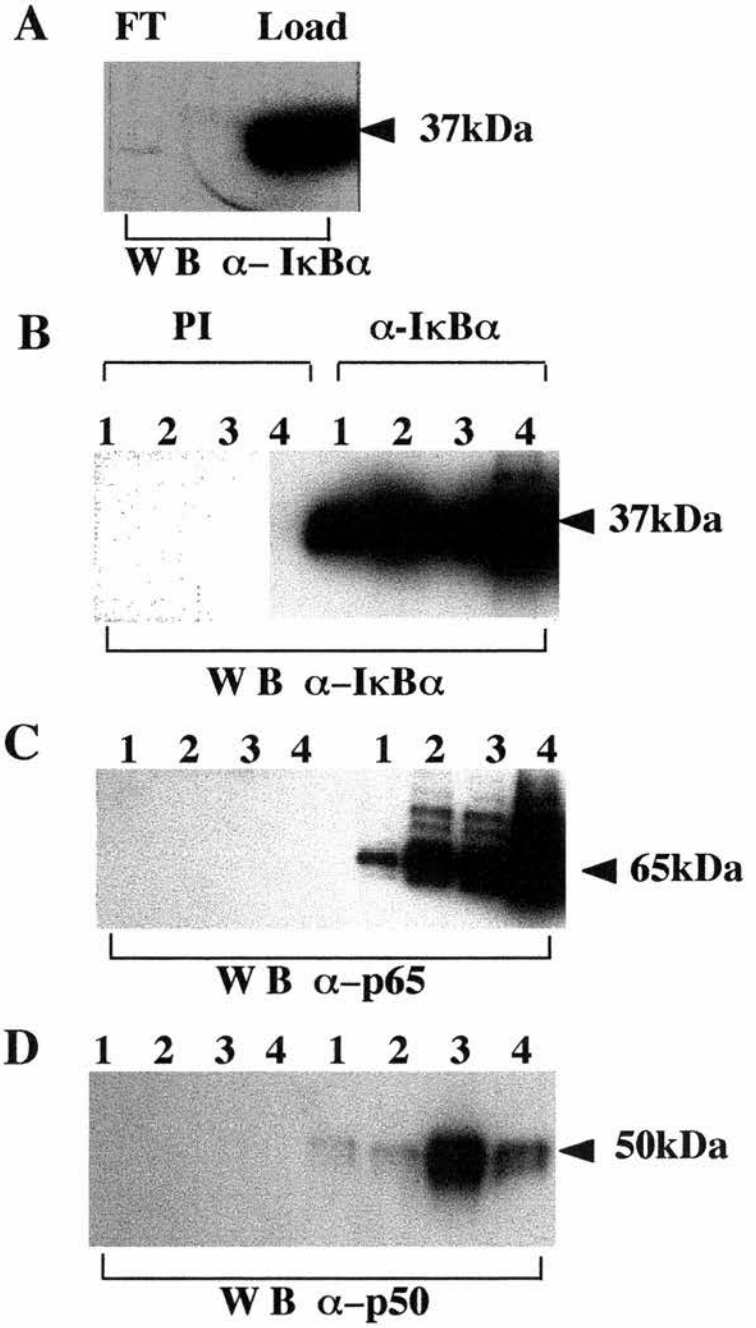
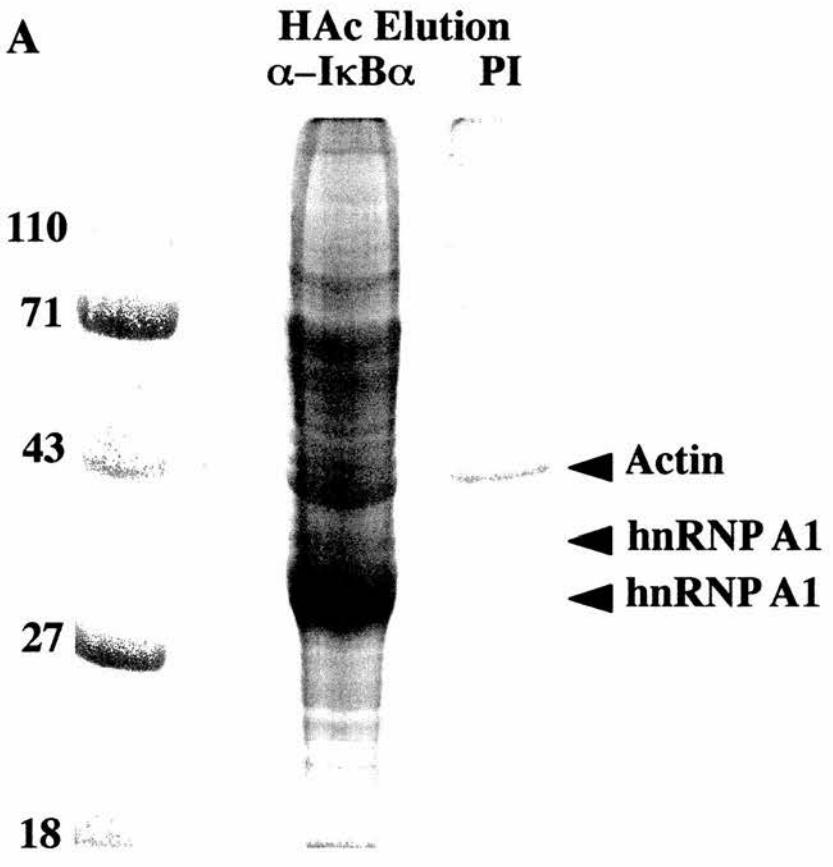

A
FT
Load
37kDa
W B α– IκBα
B
PI
α-IκBα
1 2 3 4 1 2 3 4
37kDa
W B α–IκBα
C
1 2 3 4 1 2 3 4
65kDa
W B α–p65
D
1 2 3 4 1 2 3 4
50kDa
W B α–p50

Figure 8. Characterisation of immuno-affinity purified IκBα interacting proteins.

The proteins were eluted from the anti-IκBα (α-IκBα) and the pre-immune column (α-PI) using acetic acid (HAc elution). The peak fractions were pooled and separated by SDS PAGE (10%). The bands that differed between the anti-IκBα and the pre-immune column were excised and treated for protein sequencing. Proteins identified by sequencing are shown at the right hand side of the gel. Protein weights were obtained by comparison with protein molecular weight markers shown on the left hand side of the gel.


A
HAc Elution
α–IκBα
PI
110
71
43
27
18
Actin
hnRNP A1
hnRNP A1

acetonitrile containing trypsin (modified porcine) and left for 20 hours at 37°C. After in gel digestion with trypsin the peptides were passed over a reverse phase column. In reverse phase non-polar or hydrophobic molecules bind preferentially to the stationary phase of the column and the polar molecules remain in the mobile phase. The stationary phase was then eluted from the column by reducing the polarity of the mobile phase and following this the eluted peptides were analysed by Edman degradation. BLAST sequence analysis of the peptides obtained at 34 KDa and 38 KDa indicated that both species were derived from the hnRNP A1 protein (Figure 9, Table 1).

To confirm the interaction observed in Namalwa cells, HeLa cell extracts were prepared using the same buffer containing the same cocktail of protease and phosphatase inhibitors. hnRNPA1 and IκBα complexes were immunoprecipitated using the sheep polyclonal antibodies to p65, IκBα or preimmune IgG (negative control) covalently crosslinked to protein A beads. Following extensive washing immunoprecipitated proteins were separated by SDS PAGE (10%), transferred to PVDF membrane and western blotted with monoclonal antibodies recognising hnRNPA1 or IκBα. Both forms of hnRNPA1 were immunoprecipiated by antibodies directed against IκBα or NF-κB p65, but not by preimmune IgG control (Fig. 10A) demonstrating again that the interaction was specific. As expected IκBα was immunoprecipiated by antibodies to both p65 and IκBα (Fig. 10A). The immunoprecipitation was carried out in the same manner using the hnRNP A1 monoclonal antibody covalently crosslinked to protein A beads. IκBα was also detected in these immunoprecipitates but was not detected when the irrelevant SV5 monoclonal antibody was used as the negative control (Fig. 10B) demonstrating the interaction was specific.

In summary immuno-affinity purification and immune precipitation procedures specifically purified IκBα containing complexes from the Namalwa and HeLa cell extracts. Using western blot and protein sequencing analyses we determined that NF-κB and the 2 forms of hnRNP A1 were present in these IκBα complexes. hnRNP A1 is a protein that shuttles between the nucleus and the cytoplasm and is implicated in export

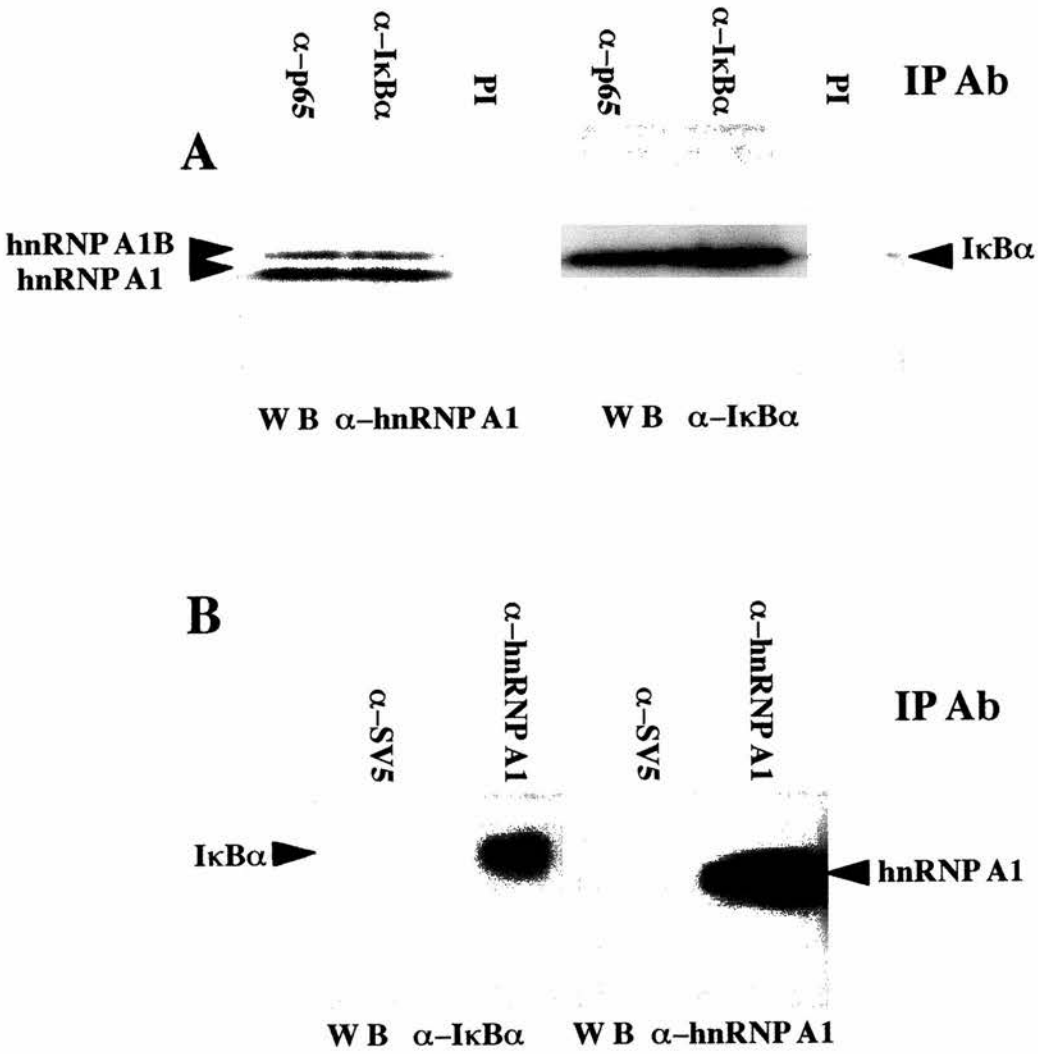
Figure 9. Protein sequencing of trypsin digested bands.

Bands excised from the SDS PAGE (10%) were trypsin digested and separated by HPLC purification. The fractions eluted from the reverse phase column were analysed for peptide content. The peptide sequences obtained are shown in Table 1. The molecular weight is denoted M.Wt, sequence refers to the peptide sequence obtained, identity refers to the protein which contains this sequence and location refers to the position of the peptide in the protein's primary structure.

M.Wt	Sequence	Identity	Location
34	EVIEIMTD	hnRNP A1	132-139
34	FAFVTFDDH	hnRNP A1	148-156
34	EDSQRPGA	hnRNP A1	93-100
38	FGFVTY	hnRNP A1	57-62
38	FAFVTFD	hnRNP A1	148-154

Figure 10. Immunoprecipitation of hnRNP A1 and IκBα complexes *in vivo*.

Total protein extracts prepared from HeLa cells were incubated with 0.5μgs of the immunoprecipitation antibody (IP Ab) : anti-p65 (α-p65), anti-IκBα (α-IκBα), anti-pre-immune (α-PI) (Figure A), anti-hnRNP A1 (α-hnRNP A1) and anti-SV5 (α-SV5) (Figure B). The beads were washed 3 times in incubation buffer, separated by SDS PAGE (10%) and detected by western blot analysis (denoted WB α-hnRNP A1 and WB α-IκBα). The 4B10 and 10B monoclonal antibodies were used to detect hnRNP A1 and IκBα respectively (1:1000). Immobilised antigen/antibody complexes were detected using an anti-mouse secondary antibody (1:5000) and the filter was exposed to Fuji film for 30 seconds.


A
α–p65
α–IκBα
PI
α–p65
α–IκBα
PI
IP Ab
hnRNP A1B
hnRNP A1
IκBα
W B α–hnRNP A1
W B α–IκBα
B
α–SV5
α–hnRNP A1
α–SV5
α–hnRNP A1
IP Ab
IκBα
hnRNP A1
W B α–IκBα
W B α–hnRNP A1

of mRNA from the nucleus to the cytoplasm. hnRNP A1 exists in two forms. The 34 KDa hnRNP A1 species is more abundant whilst the 38 KDa species, termed A1B, is a differentially spliced form of hnRNP A1 containing an additional exon (Buvoli et al., 1990). Our results clearly demonstrate the association of IκBα with both forms of hnRNP A1. Although hnRNP A1 is found in IκBα complexes, we could not rule out the possibility that this interaction was indirect and could be mediated by a bridging protein found in both types of cell extracts. Therefore we decided to test the interaction *in vitro* in the absence of cell extracts using recombinant purified proteins.

3.2 : hnRNPA1 interacts directly with IκBα *in vitro*

We used recombinant proteins to determine that the interaction between hnRNPA1 and IκBα was direct and was not mediated by a bridging protein present in the human cell extracts. Interactions were studied using bacterially produced recombinant proteins. GST fusion proteins were purified from IPTG induced Escherichia coli (DH5α) lysate. The bacterial supernatant was incubated with glutathione agarose (1ml of a 50% (vol/vol) suspension) overnight at 4°C and the bound fusion protein was analysed by SDS PAGE (10%). Following SDS PAGE (10%) the concentrations of the fusions bound to the beads were calculated from the Coomassie stained gel and confirmed using the BCA (Pierce) protein estimation reagents. Subsequently the respective protein concentrations were standardised before use in the binding assays or recombinant protein preparation. Recombinant proteins prepared from the GST fusion proteins by a 2 hour thrombin digestion (2.5 units of Thrombin per 0.25μg of protein) at 25°C. 1mM PMSF was added to stop the reaction and the resulting solution was dialysed overnight (against PBS/0.5M NaCl/2mM DTT) to remove excess glutathione. Recombinant proteins were purified to homogeneity by removing GST and incompletely cut GST fusion proteins using a glutathione agarose column. The proteins were resolved by SDS PAGE (10%), following this the protein concentrations were calculated. hnRNP A1 cDNA was synthesised by reverse transcription and PCR using primers with a 5'Bam H1 and a 3' Eco R1 sites. Following digestion, hnRNP A1 cDNA was ligated into the pGex 2T vector and after amplification in DH5α's the DNA was tested by restriction digest and DNA sequencing to verify hnRNP A1 wt insert had been cloned. The vector containing hnRNP A1 was transformed into DH5α which were IPTG induced to express hnRNP A1 GST fusion protein. GST, GST-IκBγ and GST-NFIII pGEX 2T plasmids were obtained, expressed and purified as for hnRNP A1.

GST-hnRNPA1 was incubated with IκBα in the presence or absence NF-κB for 1 hour at 4°C. Following extensive washing in incubation buffer bound proteins were separated by SDS PAGE (10%), transferred to PVDF membrane and analysed by

western blotting using a monoclonal antibody to IκBα (10B). GST-hnRNPA1 bound IκBα irrespective of whether it was bound to p50 or p65 (Fig. 11A). As a positive control GST-p65 was shown to interact with IκBα under all conditions, while no interaction was demonstrable with either of the negative controls GST, GST-IκBγ or GST-NFIII (Fig. 11A). The recombinant purified proteins used in the assay (Fig. 11B) were IκBα wt, p50 (residues 35-381) and p65 (residues 12-317). The result obtained with the GST-IκBγ negative control was extremely important as it demonstrated that the association of hnRNP A1 was highly specific for IκBα, but not a structurally related IκB family member, IκBγ. The absence of cell extracts in this assay not only demonstrate that the interaction was direct, but also that no post-translational modifications of either proteins were required to mediate the interaction.

In summary the interaction between the IκBα and hnRNP A1 is direct and does not require a bridging protein *in vitro* and that post-translational modifications are not required for interaction. The association between the two proteins was also shown to be highly specific for IκBα when compared to another structurally related family member, IκBγ, which failed to interact with hnRNP A1.

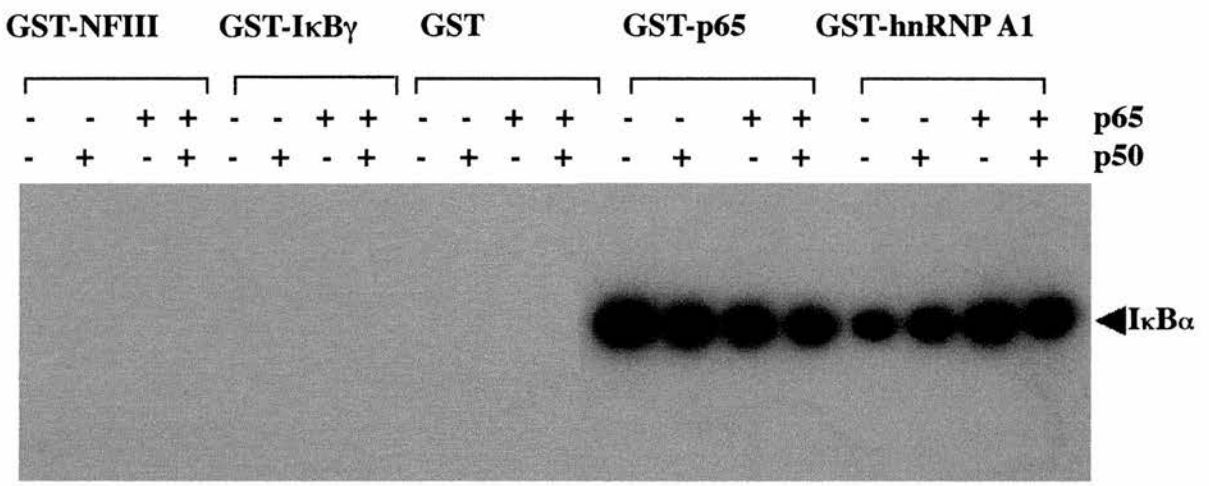
3.2.1 hnRNPA1 binds to the C-terminus of IκBα

Following the determination of direct association we decided to map the site of interaction between the 2 proteins *in vitro*. We employed deletion analysis in each protein to map the boundaries of the regions involved in the interaction. IκBα, the most extensively studied human IκB, is organised into three domains (Jaffray et al., 1995). Firstly an unstructured regulatory amino-terminal domain involved in IκBα degradation and thus NF-κB induction. Secondly a central region consisting of 6 ankyrin repeat domains (ARDs) involved in protein-protein interactions with the NF-κB subunits and nuclear translocation. The second ankyrin repeat of IκBα contains the nuclear localisation sequence (NLS) responsible for IκBα nuclear translocation following resynthesis (Sachdev et al., 1998; Turpin et al., 1999). Thirdly a carboxy terminal region which is essential in the maintenance IκBα protein stability and its signal induced

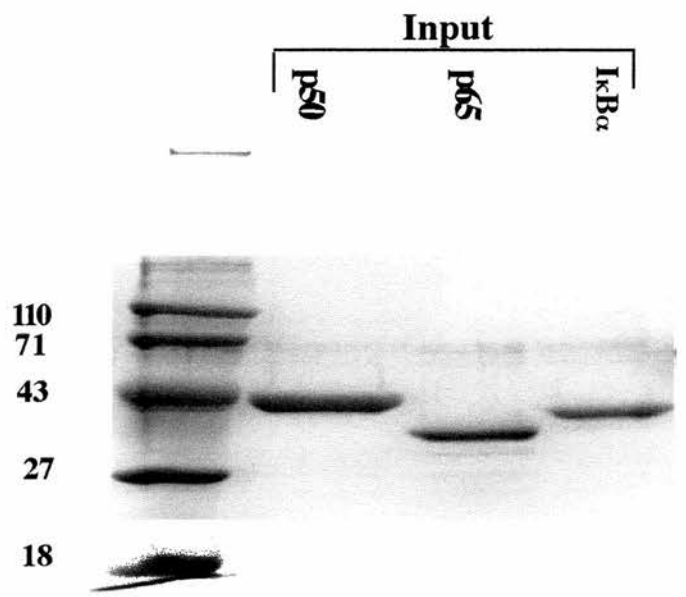
Figure 11. IκBα interacts with hnRNP A1 *In vitro*

(A) Recombinant IκBα (present in all lanes), p50 and p65 proteins (as indicated) were incubated with GST fusion proteins GST, GST-NFIII, GST-IκBγ, GST-p65 and GST-hnRNP A1 immobilised on glutathione agarose beads. The beads were washed 3 times with incubation buffer, bound materials resolved by SDS PAGE (10%) and western blotted. The 10B monoclonal antibody was used to detect IκBα (1:1000), immobilised antigen/antibody complexes were detected using an anti-mouse secondary antibody (1:5000) and the filter was exposed to Fuji film for 30 seconds.(B) The recombinant proteins used in this assay (Input) were separated by SDS PAGE (10%) and stained using Coomassie R450 and standardised before use. Protein weights were obtained by comparison with protein molecular weight markers shown on the left hand side of the gel.

A


GST-NFIII
GST-IκBγ
GST
GST-p65
GST-hnRNP A1
- - + + - - + + - - + + - - + + - - + + p65
- + - + - + - + - + - + - + - + - + - + p50
◀IκBα

B


Input
p50
p65
IκBα
110
71
43
27
18

degradation (Rodriguez et al., 1995; Whiteside et al., 1995). The carboxy terminal contains a highly acidic domain rich in pro, glu, ser, and thr, the so called PEST domain. The PEST domain is a target for CKII mediated phosphorylation (Barroga et al., 1995) and modification of this domain has been implicated in regulating the stability of IκBα (Ernst et al., 1995; Lin et al., 1996; Schwarz et al., 1996). The carboxy terminal also contains the nuclear export sequence (NES) required for the export of IκBα from the nucleus to the cytoplasm(Arenzana-Seisdedos et al., 1997).

A previously constructed series of amino and carboxy terminally deleted IκBα molecules were IPTG induced in Escherichia coli (DH5α) (Fig. 12A). As before the bacterial supernatant was prepared and incubated with glutathione agarose (1ml of a 50% (vol/vol) suspension) overnight at 4°C. The protein bound beads were washed with 50mls of Lysis buffer and resolved by SDS PAGE (10%). Following this the protein concentration of the beads were standardised before use in the assay. Subsequently the GST-IκBα fusions were tested for their ability to interact with ^{35}S labelled *in vitro* translated hnRNPA1 respectively.

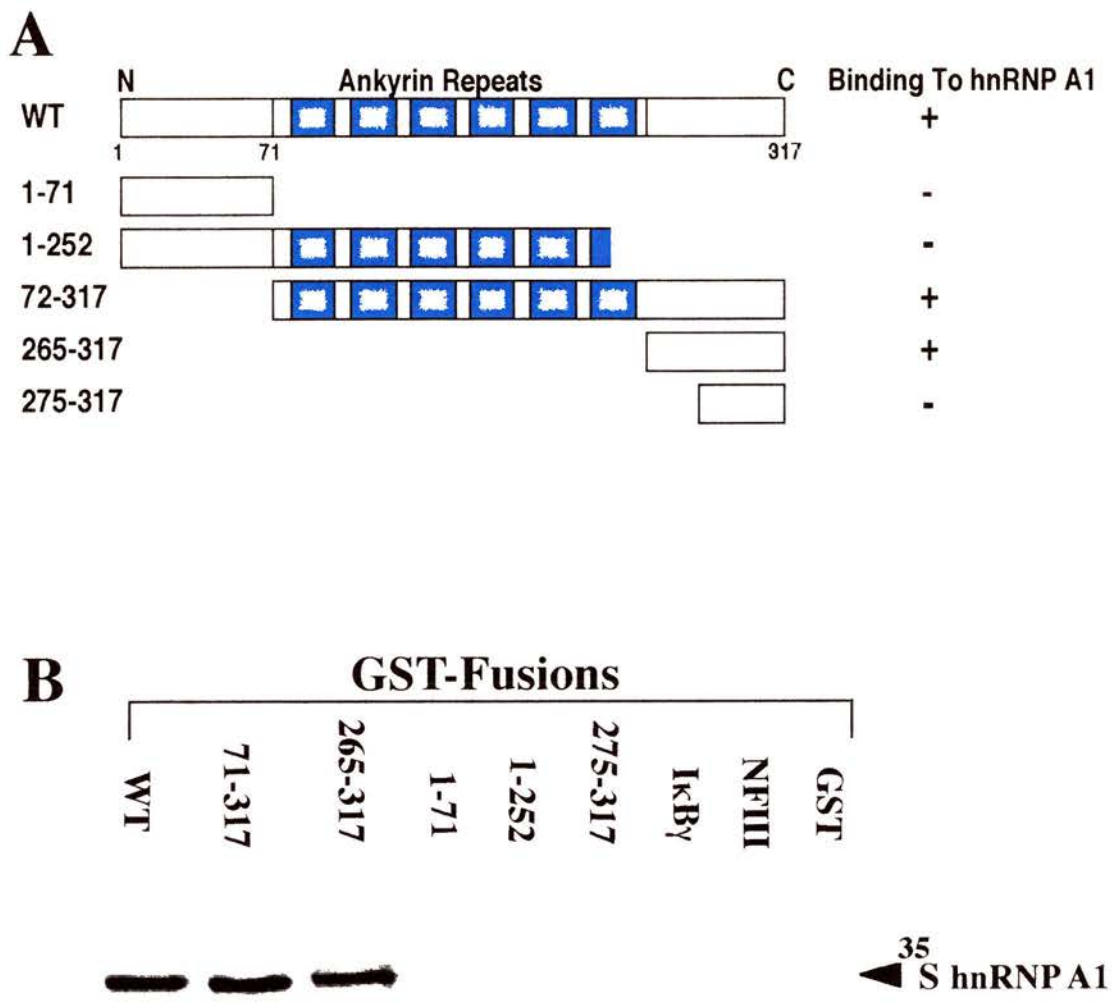
The cDNA for hnRNP A1 was cut from the pGex 2T vector and ligated into pCDNA 3 expression vector. Following amplification in DH5α's DNA was tested by restriction digest and DNA sequencing to verify the integrity of the hnRNP A1 wt insert. The pCDNA 3 vectors containing the hnRNP A1 fusion were *in vitro* transcription/translated using the PROMEGA TNT coupled system and standardised before use in the interaction assay. Equal molar amounts of each bacterially expressed fusion protein were incubated with the appropriate ^{35}S labelled protein for 1 hour at 4°C and following incubation were washed extensively in incubation buffer. Subsequently the fusion protein complexes were separated by SDS PAGE (10%), stained with Coomassie and the dried gel exposed to a phosphoimager screen overnight.

Fusion proteins which contained sequences from the carboxy terminus of IκBα were capable of binding with wild type affinity to hnRNP1 while GST fusions which lacked the full length carboxy-terminal region were unable to interact with hnRNPA1 (Fig. 12B). While the GST fusion containing IκBα residues 275-317 was unable to

Figure 12. Determination of IκBα binding region of hnRNP A1 *In vitro*.

(A) Schematic representation of full length and truncated forms of IκBα used for the interaction assays. The (+) or (-) denotes the ability to bind hnRNP A1.

(B) *In vitro* translated ^{35}S Methionine labelled hnRNP A1 (^{35}S hnRNP A1) was incubated with GST and GST-Fusions (GST-NFIII, GST-IκBγ, GST-IκBα wild type and GST-IκBα truncations) immobilised on glutathione agarose beads. The beads were washed 3 times with incubation buffer, bound materials were resolved by SDS PAGE (10%) and monitored using a phospho-imager.


A
N
Ankyrin Repeats
C
Binding To hnRNP A1
WT
1
71
317
+
1-71
-
1-252
-
72-317
+
265-317
+
275-317
-
B
GST-Fusions
WT
71-317
265-317
1-71
1-252
275-317
IκBγ
NFIII
GST
35
S hnRNP A1

bind hnRNP A1, the GST-IκBα fusion 265-317 could bind hnRNP A1. The data presented in Fig. 12 indicated that the first boundary to the interaction between hnRNP A1 and IκBα is between residues 265 and 275 in the carboxy terminus of IκBα.

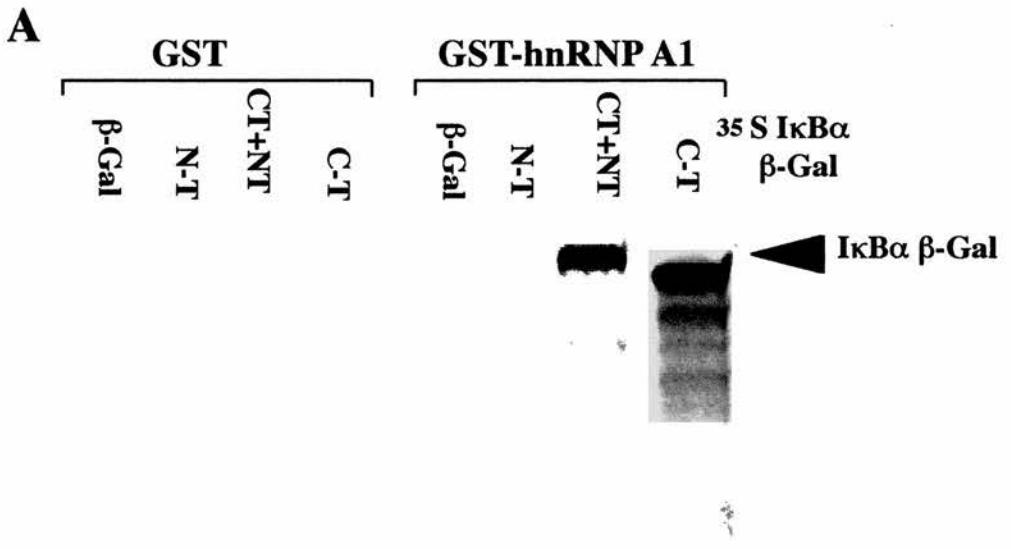
To confirm the role of the IκBα carboxy terminus in the interaction with hnRNPA1 a previously described series of β-Gal molecules linked to either the IκBα carboxy terminus, amino terminus or both the carboxy and amino termini were obtained (Rodriguez et al., 1996). These pCDNA 3 vectors containing the β-Gal fusions were *in vitro* transcription/translated using the PROMEGA TNT coupled system and standardised before use in the interaction assay. Equal amounts of each ^{35}S labelled *in vitro* translated protein were incubated and tested for interaction with either GST or GST-hnRNPA1 using the same method as above. While the construct containing only the IκBα amino terminal domain failed to interact with hnRNPA1, both constructs which contained the IκBα carboxy terminal region were bound by the GST-hnRNPA1 (Fig.13A).The ^{35}S labelled *in vitro* transcription/translation inputs were run on a gel and standardised before use in this assay (Fig. 13B).

Following confirmation of the carboxy terminal requirement it was necessary to delimit the carboxy terminal boundary of the IκBα region required for interaction with hnRNPA1. Another previously characterised series of IκBα molecules (constructs 1-256, 1-276, 1-280, 1-283 and 1-292) (Kroll et al., 1997) and a new series of IκBα molecules were prepared in which the carboxy terminus was progressively deleted (constructs 1-296, 1-303, 1-313). Primers with 5'Bam H1 and 3'Eco R1 sites were used in PCR reactions to generate the different IκBα deletion mutants (1-296, 1-303, 1-313). PCR products were ligated in to pCDNA3 and amplified in DH5α. Cloned cDNAs were checked by restriction digestion and DNA sequencing before use in the assay. These pCDNA3 vectors containing the various IκBα carboxy terminus deletions were *in vitro* transcription/translated using the PROMEGA TNT coupled system. Equal amounts of *in vitro* translated ^{35}S labelled IκBα molecules were tested for their ability to interact with either GST or GST-hnRNPA1 and treated as above (Fig. 14A and 14C).

Figure 13. The carboxy terminus of IκBα is required for interaction with hnRNP A1.

(A) *In vitro* translated ^{35}S Methionine labelled IκBα wild type and truncated forms fused to β-Galacatosidase (^{35}S IκBα β-Gal) were incubated with GST and GST hnRNP A1 wild type immobilised on glutathione agarose beads and the beads were washed and treated as in 12(B). C-T and N-T refers to carboxy terminus and amino terminus respectively.

(B) *In vitro* translated products used in this assay (Input) are shown in the bottom panel and were standardised before use.


A
GST
GST-hnRNP A1
β-Gal
N-T
CT+NT
C-T
β-Gal
N-T
CT+NT
C-T
35 S IκBα
β-Gal
IκBα β-Gal

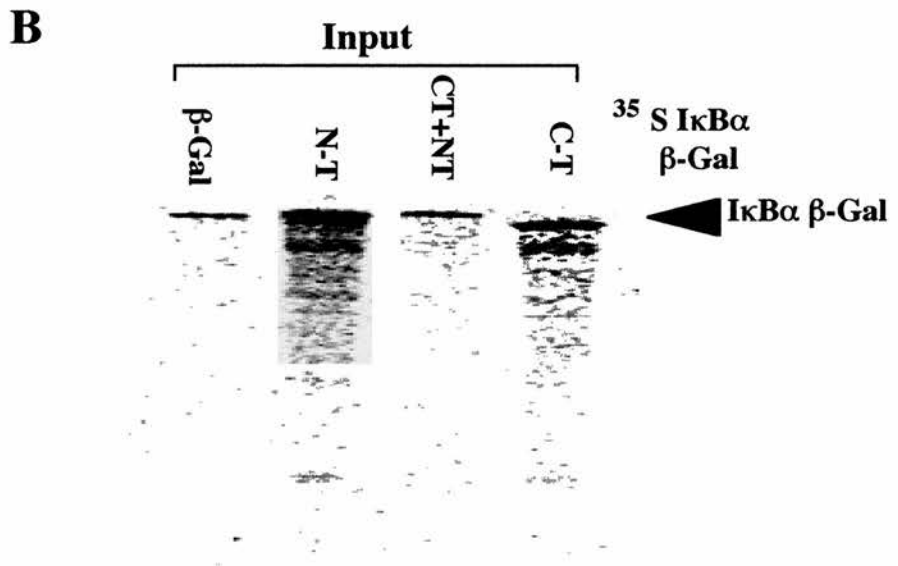
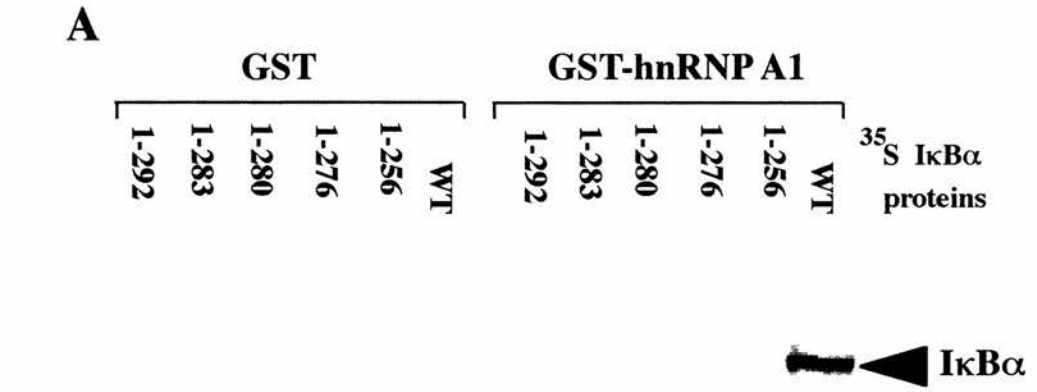
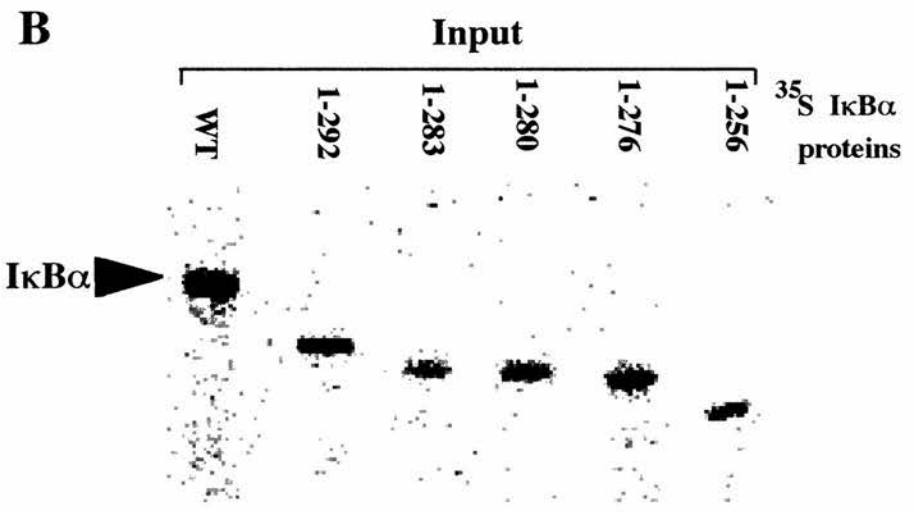

B
Input
β-Gal
N-T
CT+NT
C-T
35 S IκBα
β-Gal
IκBα β-Gal

Figure 14. IκBα carboxy terminal mapping of residues involved in interaction with hnRNP A1.

(A) *In vitro* translated ^{35}S Methionine labelled IκBα wild type and truncated forms (^{35}S IκBα proteins) were incubated with GST and GST-hnRNP A1 wild type immobilised on glutathione agarose beads. The beads were washed and treated as in 12(B).

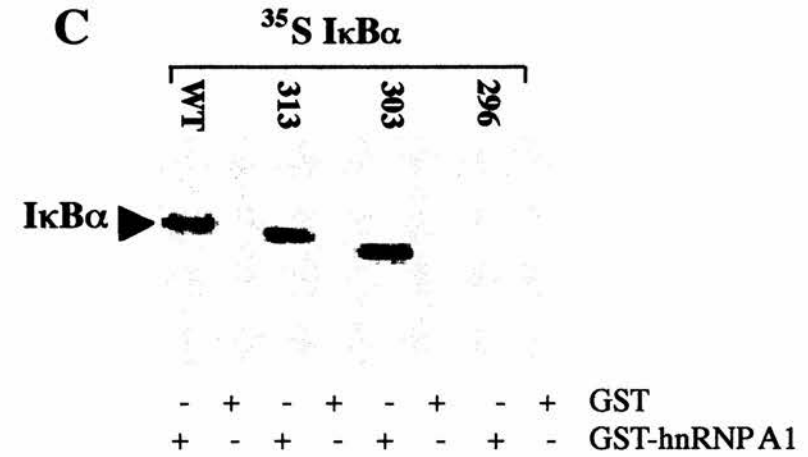
(B) *In vitro* translated products forms (^{35}S IκBα proteins) used in this assay (Input) are shown in the bottom panel and were standardised before use.

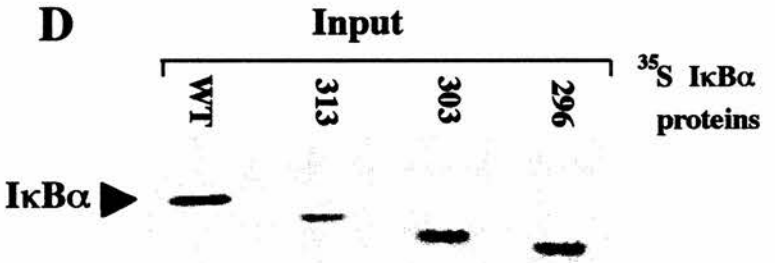

A
GST
GST-hnRNP A1
1-292
1-283
1-280
1-276
1-256
WT
35S IκBα proteins
IκBα


B
Input
WT
1-292
1-283
1-280
1-276
1-256
35S IκBα proteins
IκBα

(C) *In vitro* translated ^{35}S Methionine labelled IκBα wild type and truncated forms (^{35}S IκBα) were incubated with GST and GST-hnRNP A1 wild type immobilised on glutathione agarose beads. The beads were washed and treated as in 12(B).

(D) *In vitro* translated products (^{35}S IκBα proteins) used in this assay (Input) are shown in the bottom panel and were standardised before use.


C
35S IκBα
WT
313
303
296
IκBα
- + - + - + - + GST
+ - + - + - + - GST-hnRNP A1


D
Input
WT
313
303
296
35S IκBα proteins
IκBα

The ^{35}S labelled inputs were run on a gel and standardised before use in the assay (Fig. 14B and 14D). GST-hnRNP A1 could interact with the wild type IκBα and molecules containing residues 1-313 or 1-303, but were unable to interact with molecules containing residues 1-296 and more extensively deleted versions of IκBα. These data therefore define residues 296-303 as the carboxy terminal boundary of the region in IκBα required for interactions with hnRNP A1. Combined with the previous data these experiments indicate that residues located between 265-303 in IκBα are required for interaction with hnRNP A1 (Fig. 18).

3.2.2 An hnRNPA1 RNA binding domain is required for interaction with IκBα

To identify the boundaries of interaction in hnRNPA1 required for interaction with IκBα an analogous strategy to that employed with IκBα was adopted. hnRNPA1 is a multidomain protein that contains two RNA binding domains (RBDs) in the amino terminal half of the protein (Xu et al., 1997). The RBDs are an extremely ancient and common RNA binding motif within which the RNP-2 hexamer and RNP-1 octamer submotifs are highly conserved (Birney et al., 1993). The carboxy terminal half of the protein contains an RGG box which constitutes an RNA binding motif (Kiledjian and Dreyfuss, 1992) and its deletion results in complete loss of alternative splicing activity and a striking decrease in RNA binding and annealing (Mayeda et al., 1994). The carboxy terminal region also contains the M9 nuclear import/export sequence. Mutagenesis studies have indicated that the NES and NLS activities of M9 are either identical or overlapping as mutants which block NLS activity also abolish NES activity (Michael et al., 1995; Michael et al., 1995).

We used primers with 5' Bam H1 and 3' Eco R1 sites and PCR to synthesise the different hnRNP A1 molecules in which sequences from the amino and carboxy termini had been progressively deleted (Fig.15). Following this cDNAs were ligated in to pGEX 2T and amplified in DH5α. Inserts in plasmid were checked by restriction digestion and DNA sequencing before bacterial transformation. pGEX 2T vector and appropriate cDNAs were transformed into DH5α's. Following IPTG induction the GST-hnRNPA1 fusions were purified as before. The bacterial supernatant was prepared and incubated with glutathione agarose (1ml of a 50% (vol/vol) suspension) overnight at 4°C. The protein bound beads were washed with 50mls of Lysis buffer and a small fraction of the bound proteins resolved by SDS PAGE (10%) to enable equivalent amounts of each protein to be added to each assay. A Coomassie stained SDS PAGE (10%) gel in which most of the GST fusions used in the binding assays is shown in Fig. 16B. While GST-fusions of carboxy terminal deletions gave relatively homogeneous preparations of proteins, amino terminal deletions gave protein preparations which were much more heterogeneous. Equal molar amounts of each

Figure 15. Determination of hnRNP A1 binding region of IκBα *In vitro*

(A) Schematic representation of full length and truncated forms of hnRNP A1 used for the interaction assays. The (+) or (-) denotes the ability to bind IκBα. RBD 1 and 2 are denoted the RNA binding domains, a region rich in arginine and glycine is denoted the RGG box and the bifunctional nucleocytoplasmic transport signal is denoted M9.

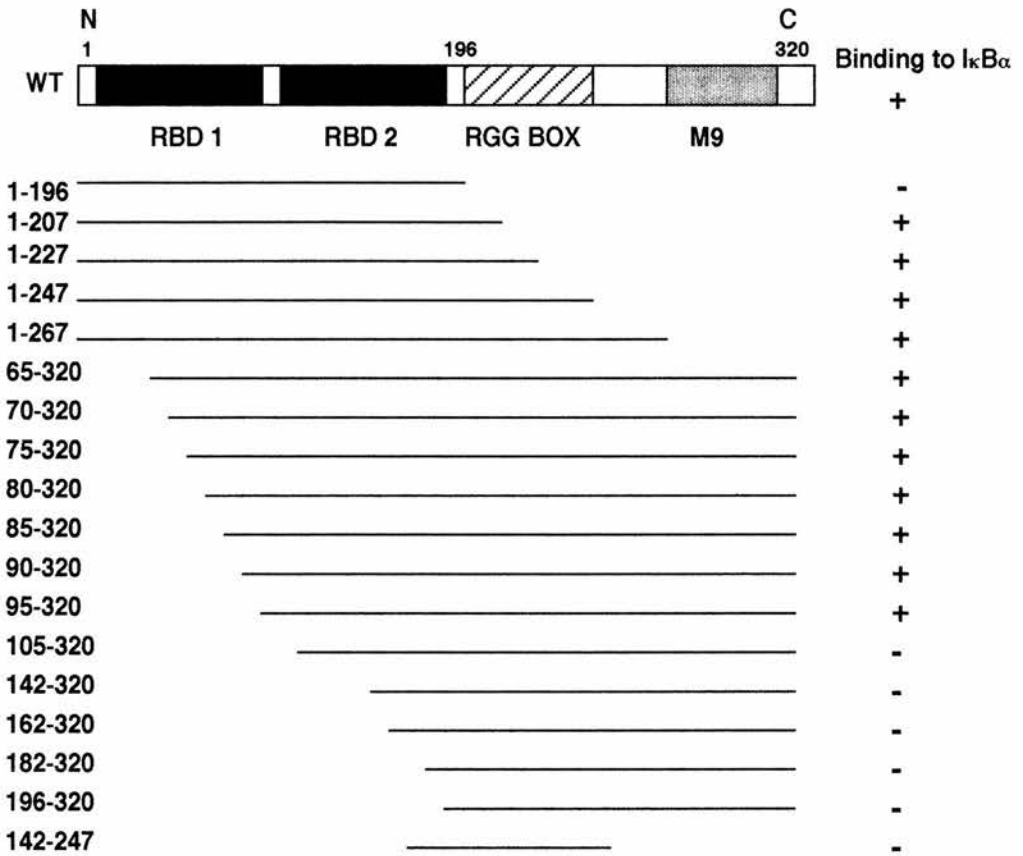
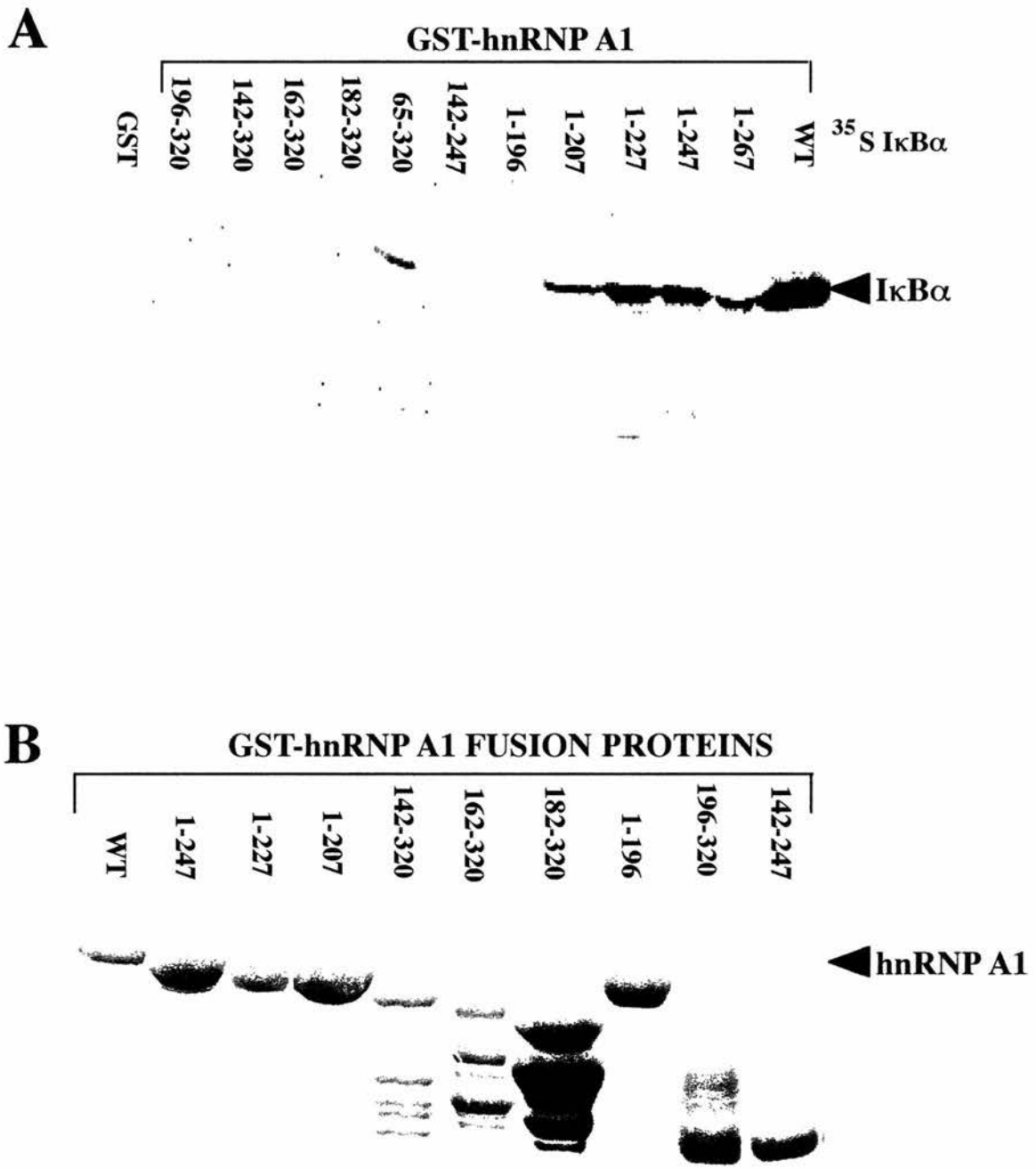

N
C
1
196
320
Binding to IκBα
WT
+
RBD 1
RBD 2
RGG BOX
M9
1-196
-
1-207
+
1-227
+
1-247
+
1-267
+
65-320
+
70-320
+
75-320
+
80-320
+
85-320
+
90-320
+
95-320
+
105-320
-
142-320
-
162-320
-
182-320
-
196-320
-
142-247
-

Figure 16. Determination of hnRNP A1 binding region of IκBα *In vitro*

(A) *In vitro* translated ^{35}S Methionine labelled IκBα (^{35}S IκBα) was incubated with GST or GST-hnRNP A1 wild type or GST-truncations immobilised on glutathione agarose beads. The beads were washed and treated as in 12(B).

(B) Bacterially expressed GST fusion products used in this assay are shown in this panel and were standardised before use.


A
GST-hnRNP A1
GST
196-320
142-320
162-320
182-320
65-320
142-247
1-196
1-207
1-227
1-247
1-267
WT
35S IκBα
IκBα
B
GST-hnRNP A1 FUSION PROTEINS
WT
1-247
1-227
1-207
142-320
162-320
182-320
1-196
196-320
142-247
hnRNP A1

bacterially expressed hnRNP A1 fusion protein were incubated with the appropriate ^{35}S labelled protein for 1 hour at 4°C and following incubation were washed extensively in incubation buffer. Bound proteins were separated by SDS PAGE (10%), stained with Coomassie and the gel exposed to a phosphoimager screen overnight. GST fusions containing hnRNPA1 sequences between 65-320 bound IκBα with wild type affinity whereas a fusion containing hnRNPA1 residues 142-320 was unable to bind IκBα (Fig. 16A) thus demonstrating the first boundary of interaction was between residues 65-142. Removal of sequences between residues 207 and the C-terminus (1-207) did not affect binding to IκBα whereas an hnRNPA1 molecule containing only residues 1-196 was unable to bind IκBα (Fig. 16A), thus determining the second boundary of interaction was between residues 196 and 207.

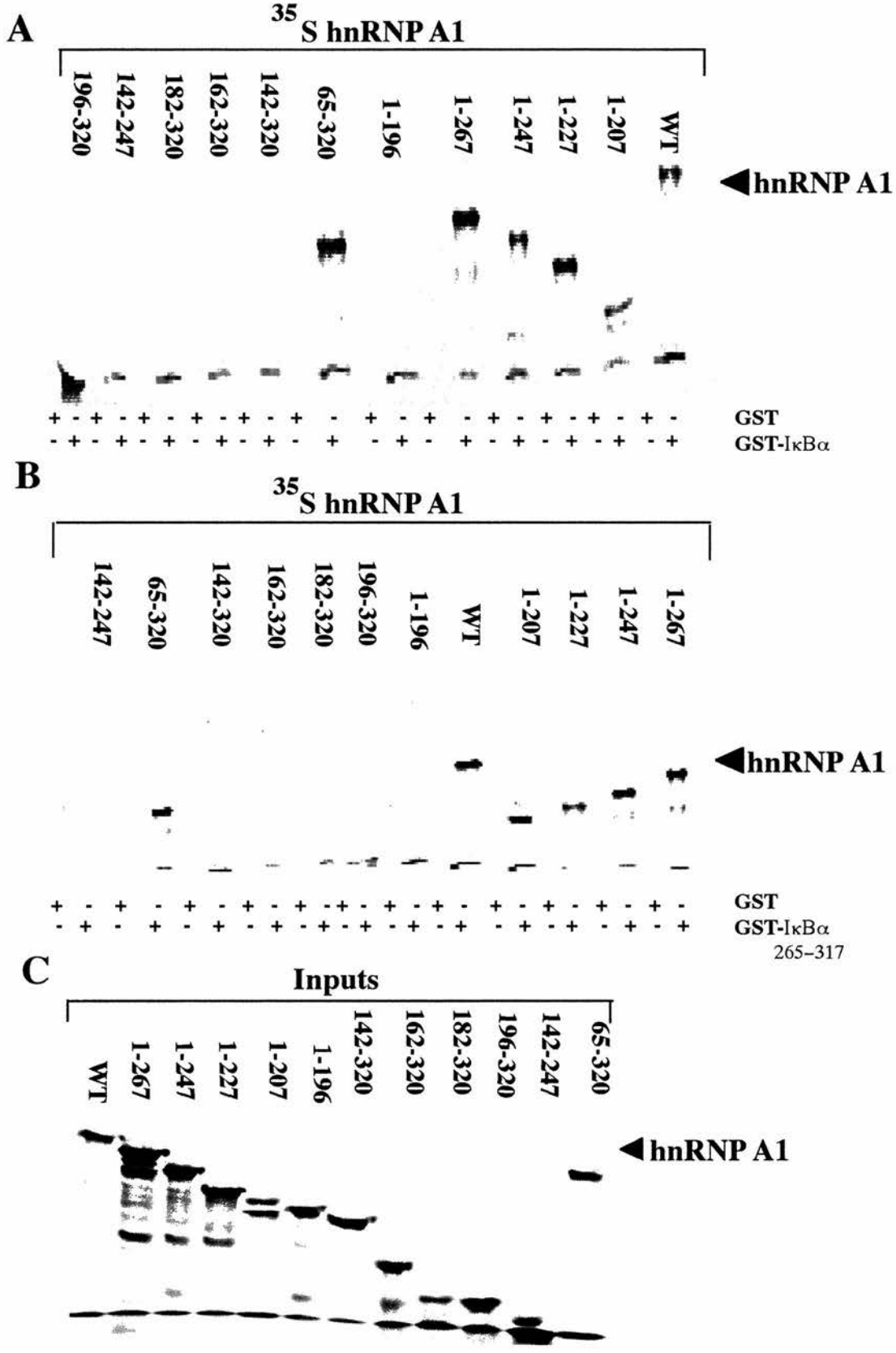
To confirm the previous result, we decided to test the interaction using ^{35}S labelled hnRNPA1 deletions and GST-IκBα$_{1-317}$ or GST-IκBα$_{265-317}$. cDNAs for the hnRNP A1 molecules were cut from the pGEX 2T vector and ligated into the pCDNA 3 vector. Following amplification in DH5α's, the cDNAs were tested by restriction digest and DNA sequencing. pCDNA3 vectors containing hnRNP A1 cDNAs were *in vitro* transcription/translated using the TNT coupled system, run on a gel (Fig. 17C) and standardised before use in the interaction assay. Equal molar amounts of each bacterially expressed fusion protein, GST-IκBα$_{1-317}$, GST-IκBα$_{265-317}$ or GST were incubated with the appropriate ^{35}S labelled hnRNPA1 deletion molecules for 1 hour at 4°C. Following extensive washing in incubation buffer, the protein-protein complexes were separated by 10% SDS PAGE, the gel dried and exposed to a phospho-imager screen over night. GST-IκBα$_{1-317}$ or GST-IκBα$_{265-317}$ interacted with hnRNP A1 with the same boundaries as observed above (Fig. 17A and B).

Following this result we prepared another set of deletions in hnRNP A1 to define the amino terminal boundary of the region required for interaction with IκBα. We used primers with 5' Bam H1 and 3' Eco R1 sites and PCR to synthesise the different hnRNP A1 molecules. The cDNA for the hnRNP A1 molecules synthesised were ligated into the pCDNA3 vector. Following amplification in DH5α's, the cDNAs were

Figure 17. Determination of hnRNP A1 binding region of IκBα *in vitro*.

(A)(B) *In vitro* translated ^{35}S Methionine labelled hnRNP A1 wild type and truncated forms (^{35}S hnRNP A1) were incubated with GST or GST-IκBα wild type or with GST-IκBα carboxy terminus (GST-IκBα$_{265\text{-}317}$) immobilised on glutathione agarose beads. The beads were washed and treated as in 12(B).

(C)*In vitro* translated products used in this assay (Inputs) are shown in the bottom panel and were standardised before use.


A
35S hnRNP A1
196-320
142-247
182-320
162-320
142-320
65-320
1-196
1-267
1-247
1-227
1-207
WT
hnRNP A1
GST
GST-IκBα
B
35S hnRNP A1
142-247
65-320
142-320
162-320
182-320
196-320
1-196
WT
1-207
1-227
1-247
1-267
hnRNP A1
GST
GST-IκBα
265–317
C
Inputs
WT
1-267
1-247
1-227
1-207
1-196
142-320
162-320
182-320
196-320
142-247
65-320
hnRNP A1

tested by restriction digest and DNA sequencing. pCDNA3 vectors containing hnRNP A1 cDNAs were *in vitro* transcription/translated using the PROMEGA TNT coupled system, run on a gel and standardised before use in the interaction assay (Fig. 17E). The ^{35}S labelled hnRNPA1 deletion molecules were incubated with GST-IκBα for 1 hour at 4°C and processed as above. The results from this assay demonstrated (Fig. 17D) that a hnRNP A1 molecule containing residues 95-320 bound to IκBα, whereas the hnRNP A1 molecule containing residues 105-320 was unable to bind IκBα, thus demonstrating the minimal amino terminal boundary of hnRNP A1 required for interaction with IκBα.

The deletion analysis presented here has identified the minimal regions in hnRNP A1 that is required interactions with IκBα. The carboxy terminal boundary of this region is located between residues 196 and 207 while the amino terminal boundary is located between residues 95 and 105 (Fig. 18). Thus the region between residues 95 and 207 in hnRNP A1 contains the domains required for interaction with IκBα.

(E) *In vitro* translated ^{35}S Methionine labelled hnRNP A1 wild type and truncated forms (^{35}S hnRNP A1) were incubated with GST or GST-IκBα immobilised on glutathione agarose beads. The beads were washed and treated as in 12(B).

(F) *In vitro* translated products used in this assay (Inputs) are shown in the bottom panel and were standardised before use.

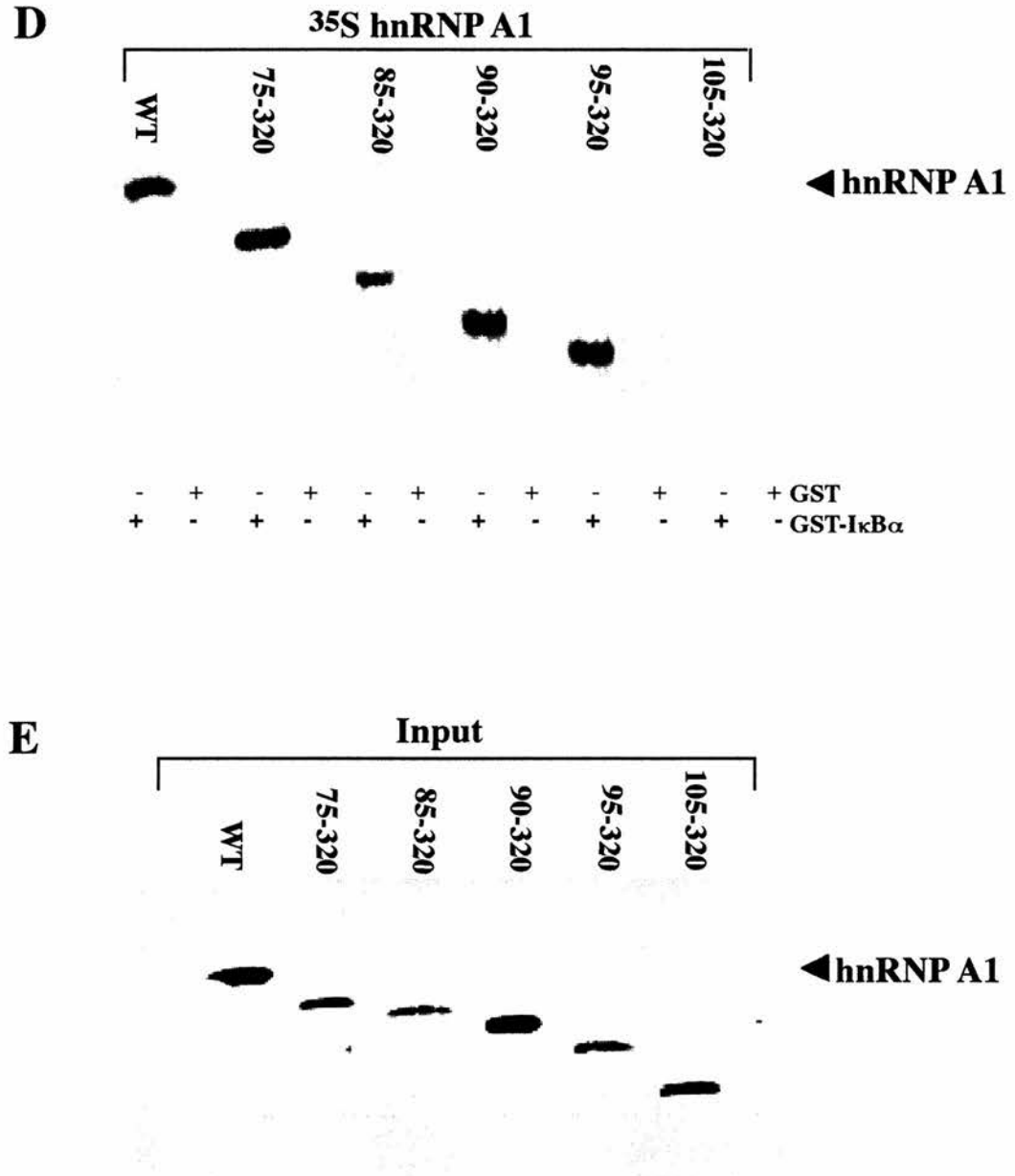
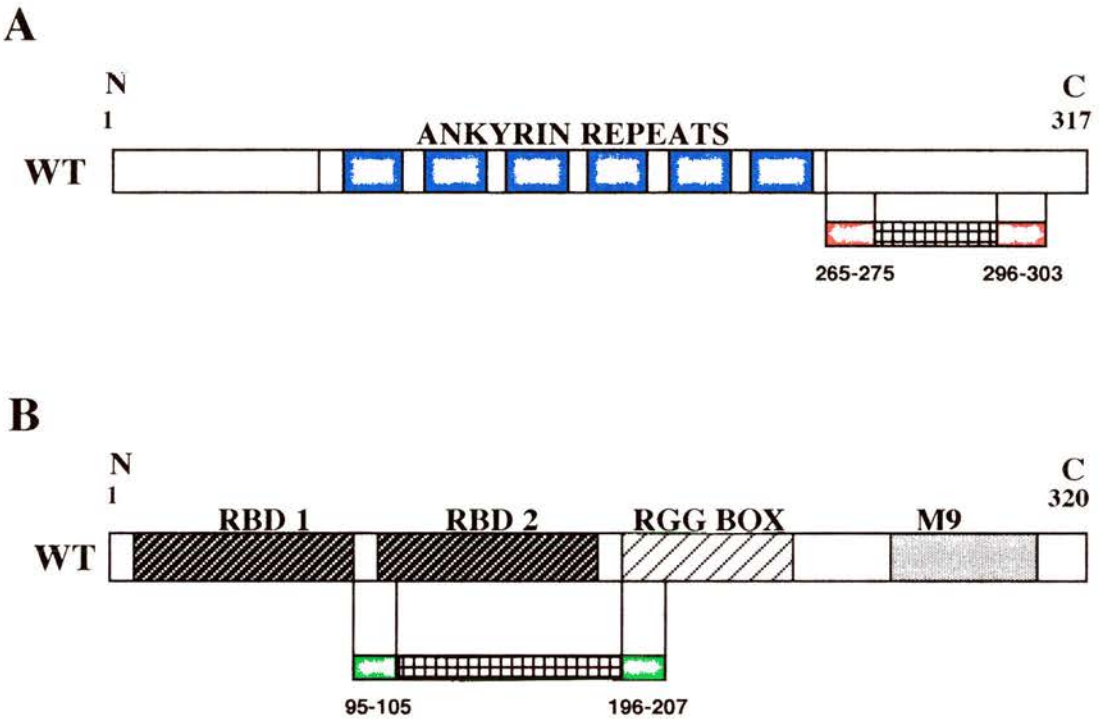

D
35S hnRNP A1
WT
75-320
85-320
90-320
95-320
105-320
hnRNP A1
- + - + - + - + - + - + GST
+ - + - + - + - + - + - GST-IκBα
E
Input
WT
75-320
85-320
90-320
95-320
105-320
hnRNP A1

Figure 18. The boundaries of hnRNP A1 and IκBα involved in interaction.

(A) The two boundaries to the interaction with hnRNP A1 are found within IκBα's carboxy terminus. The boundaries to the interaction are indicated by the red shading and the boxed lines indicates, in addition to the boundaries, the critical region of protein sequence required for interaction.

(B) The two boundaries to interaction with IκBα are found in hnRNP A1's amino-terminus and carboxy terminus. The boundaries to interaction are indicated by the green shading and the boxed lines indicates, in addition to the boundaries, the critical region of protein sequence required for interaction.


A
N
1
C
317
ANKYRIN REPEATS
WT
265-275
296-303
B
N
1
C
320
RBD 1
RBD 2
RGG BOX
M9
WT
95-105
196-207

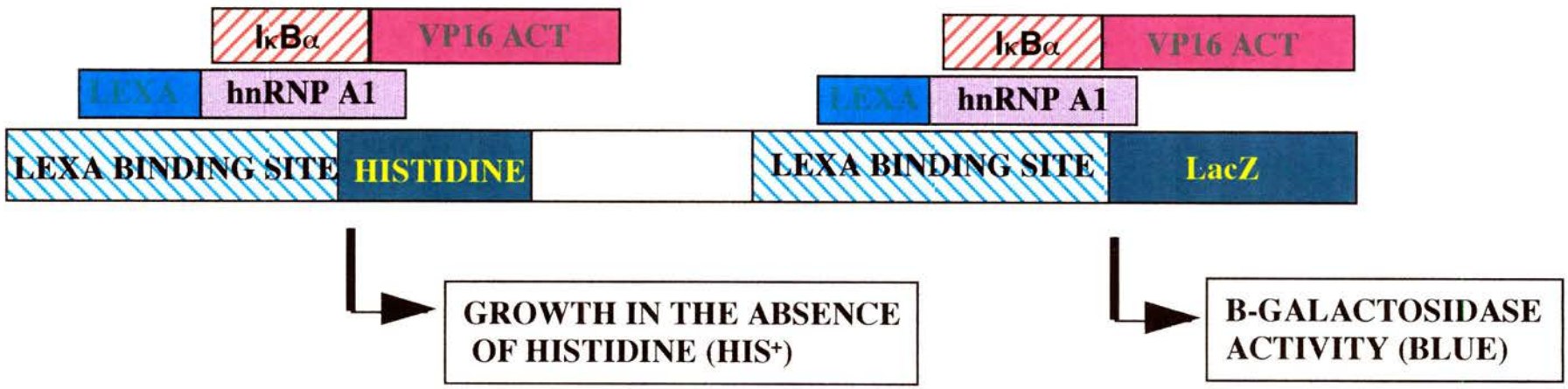
3.3 : IκBα interacts with hnRNP A1 *in vivo*

The yeast II hybrid system is a yeast-based genetic assay to detect specific protein-protein interactions *in vivo* by functional restoration of a eukaryotic transcriptional activator e.g. GAL 4. The yeast system is a sensitive method for detecting weak and transient interactions, which are found in large native complexes. Furthermore, the yeast II hybrid system has many useful features compared to *in vitro* methods. Most notably, since the assay is performed *in vivo*, the proteins involved are more likely to be in their native conformations. The system is based on the fact that many eukaryotic transcriptional activators consist of two physically discrete modular domains. One domain acts as the DNA binding domain while the other functions as the transcriptional activation domain. The proteins of interest are cloned into two vectors, one containing the DNA binding domain and the other containing the activation domain (Fig. 19). Both domains are required in close proximity to perform their normal transcriptional activation. This forms the basis of the yeast II hybrid system and the strength of interaction is measured via transcription of a β-Galactosidase and histidine reporter. To test the interaction between hnRNPA1 and IκBα *in vivo*, we examined the interaction in Sacchromyces cerevisiae strain L40a. The yeast L40a reporter strain was used to demonstrate the interaction between hnRNPA1 and IκBα as it possesses integrated histidine and β-galactosidase reporters.

hnRNP A1 wt was cut from the pCDNA 3 vector and ligated into the PV44ER.LexA vector. Following amplification in DH5α's the DNA was tested by restriction digest and by DNA sequencing. L40a were chemically co-transformed with pLexA-hnRNPA1 (DNA binding domain) and IκBα-VP16 (Transcriptional activation domain) expression constructs. An interaction between the two expressed proteins was detected as the yeast could grow on minimal medium containing 3-amino triazole but lacking histidine and could also activate the LexA dependent β-galactosidase reporter. Thus the activation and DNA binding domains were in close proximity and performed their normal transcriptional activation. Appropriate positive (pLexA-IκBαNT and pACT-Ubch9) and negative (pLexA-hnRNP A1wt and pACT-LysRSwt ; pLexA-hnRNP A1wt

Figure 19. hnRNP A1 interacts with IκBα *in vivo.*

To test the interaction between hnRNP A1 and IκBα *in vivo* the yeast II hybrid system was employed. The proteins were cloned into two vectors, hnRNP A1 with the LexA DNA binding domain (Lex A hnRNP A1) and IκBα with the VP16 activation domain (IκBα VP16 ACT). Both the activation domain and the DNA binding domain are required in close proximity to activate the β-Galactosidase and Histidine integrated reporters. Growth in the absence of Histidine and production of β-Galactosidase activity demonstrates promoter activity and thus activation of transcription in this system.


IκBα
VP16 ACT
LEXA
hnRNP A1
LEXA BINDING SITE
HISTIDINE
IκBα
VP16 ACT
LEXA
hnRNP A1
LEXA BINDING SITE
LacZ
GROWTH IN THE ABSENCE OF HISTIDINE (HIS+)
B-GALACTOSIDASE ACTIVITY (BLUE)

and pVP16 ; pLexA and IκBα-VP16) controls confirmed the specificity of this interaction. Qualitative data was obtained using the filter lift technique (Fig. 20A and B and summarised in Table 2). Following yeast cell lysis and protein standardisation the Galacton Light Plus (Tropix) Chemilluminescent reporter assay was used to measure quantitatively the relative light units (RLU) produced by interacting proteins (Fig. 20C).

The interaction in question between IκBα and hnRNP A1 grew upto a concentration of 7 mM 3 amino-triazole (3AT) and possessed β-gal activity, whereas the positive control, pLexA-IκBαNT and pACT-Ubch9, grew upto 30mM 3AT. This demonstrated that the interaction in question was not as strong as the positive control and was more likely to be of a transient nature *in vivo*. Nevertheless the proteins interacted *in vivo* and is likely to be of physiological significance.

Figure 20. IκBα interacts with hnRNP A1 in the yeast II hybrid system.

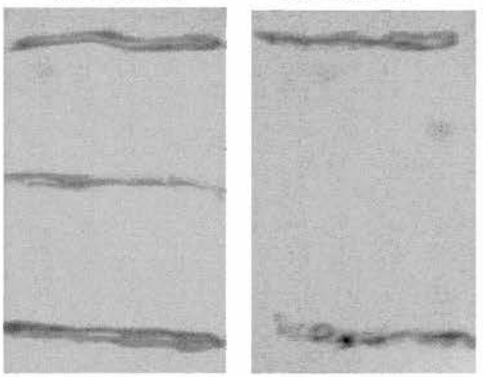
(A) Sacchromyces cerevisiae strain L40a was co-transfected with pLex-A hnRNP A1 wt and pVP16-IκBα wt (interaction in question); pLexA-hnRNP A1 wt and pACT-LysRS wt (negative control); pLexA-IκBα N-T and pACT-Ubch9 wt (positive control) and tested for β-Gal reporter activity.

(B) The same co-transformants were grown in the presence of 3 amino triazole (3AT) and tested for β-Gal activity.

(C) Following conformation of interaction using the qualitative data in (A) and (B), pLexA-hnRNP A1 wt and pVP16-IκBα wt co-transformants were grown in liquid culture and the strength of the interaction was measured quantitatively using the Galacton Light Plus Tropix kit ™.

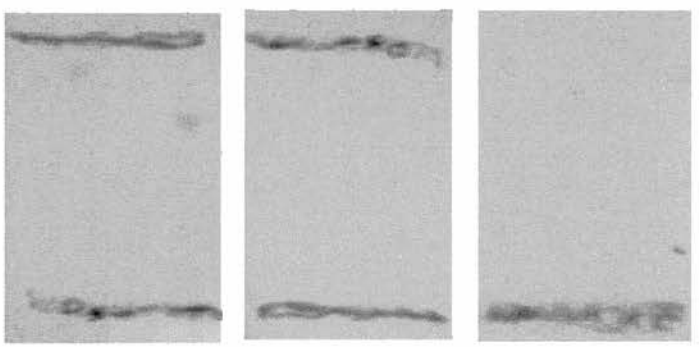
A

Transforming Plasmids	SD + His	SD - His	Colony Colour
pLexA-hnRNP A1 wt + pVP16-IκBα wt			BLUE
pLexA-hnRNP A1 wt + pACT-LysRS wt			WHITE
pLexA-IκBα 32/36A + pACT-Ubch9 wt			BLUE



B

Transforming Plasmids	-3AT	+ 7mM 3AT	+ 30mM 3AT
pLexA-hnRNP A1 wt + pVP16-IκBα wt			
pLexA-hnRNP A1 wt + pACT-LysRS wt			
pLexA-IκBα 32/36A + pACT-Ubch9 wt			



C

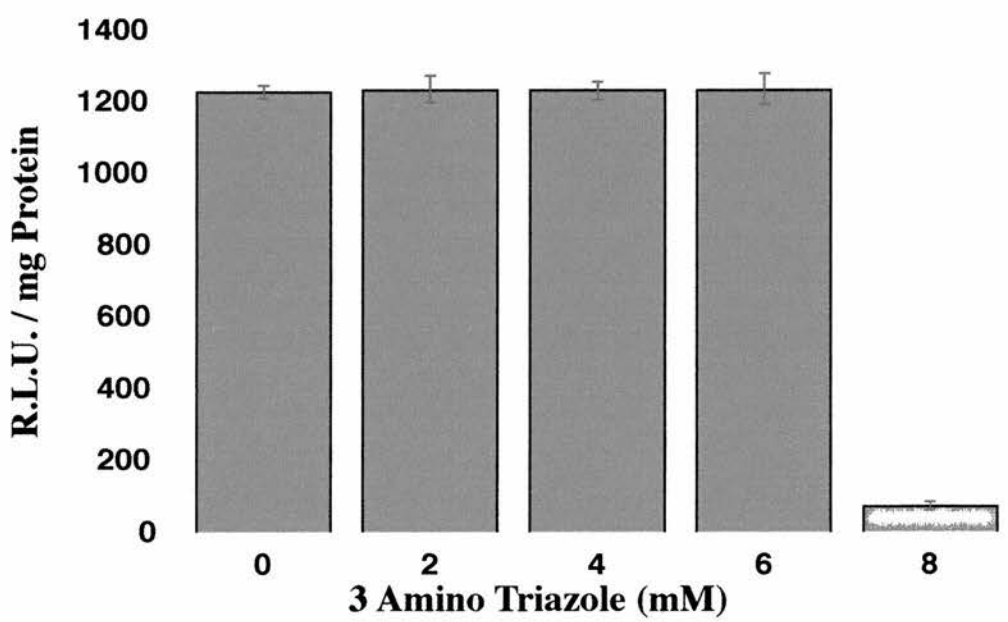


Table 2. hnRNP A1 Specifically Interacts with IκBα *in vivo*.

Sacchromyces cerevisiae strain L40a was co-transformed with hnRNPA1-LexA and IκBα-VP16; hnRNPA1-LexA and lystRNA Synthetase-ACT; IκBα N-Term-LexA and Ubch9-ACT (the interaction under investigation, the negative and positive controls respectively). Transformants were assayed for His prototrophy and β-Gal activity. Growth in the presence of 3-Amino Triazole (3AT)and the absence of Histidine and a high level of β-Gal activity are indications of an interaction between hybrid proteins.

Construct	Growth (+ His)	Growth (- His + 3AT)	Colony Colour
pLexA-hnRNP A1 pVP16.701 vector	+	-	WHITE
pLex A.44ER vector pVP16-IκBα wt	+	-	WHITE
pLexA-hnRNP A1 pVP16-IκBα wt	+	+	BLUE
pLexA-hnRNP A1 pACT-LysRS wt	+	-	WHITE
pLexA-IκBα N-T pACT-Ubch9	+	+	BLUE

3.4 : hnRNPA1 can influence the cellular localisation of IκBα

Shuttling of hnRNPA1 between the nucleus and the cytoplasm is known to depend on continuous transcription by RNA polymerase II (Pinol-Roma and Dreyfuss, 1991). hnRNP A1 is a predominantly nuclear protein during RNA Polymerase II transcription, however this protein is not confined to the nucleus. Rather it shuttles rapidly between the nucleus and cytoplasm. Inhibition of transcription by actinomycin D leads to the predominantly nuclear hnRNPA1, partially delocalising to the cytoplasm (Fig. 21A). Thus both hnRNPA1 (Michael et al., 1995; Michael et al., 1995; Pinol-Roma and Dreyfuss, 1992) and IκBα (Rodriguez et al., 1999) are known to rapidly shuttle between the nucleus and the cytoplasm. HeLa cells were transfected with pCDNA 3 containing the cDNAs for IκBα wt and IκBα$_{1-292}$, using electroporation, and grown on glass coverslips for 12 hours in D-MEM containing 10% FCS. Transfection by electroporation was chosen as it is a very efficient method by which the cells take up DNA and expression of the appropriate exogenous material can be assayed more quickly than other transfection methods (12 hours post transfection). Following cell fixation, permeabilisation and blocking, endogenous and exogenous expressed materials were monitored by indirect immunofluoresence. In this process of cell labelling the primary antibodies recognise the appropriate protein and the species specific secondary antibody, conjugated to either FITC or Texas Red, recognise the primary antibody and generate a fluorescent signal. Our control protein in this experiment was the NLS-Pk-tag construct. NLS-pk-tag contains the SV40 NLS region which is fused to the chicken pyruvate kinase (Pk) sequence tagged with c-myc and is transported to the nucleus using the classical import machinery. The NLS-pk-tag was used as an internal control of nuclear integrity, to demonstrate that IκBα and hnRNP A1 delocalisation was as a result of actinomycin D treatment and not a protein leaching process from damaged nuclei.

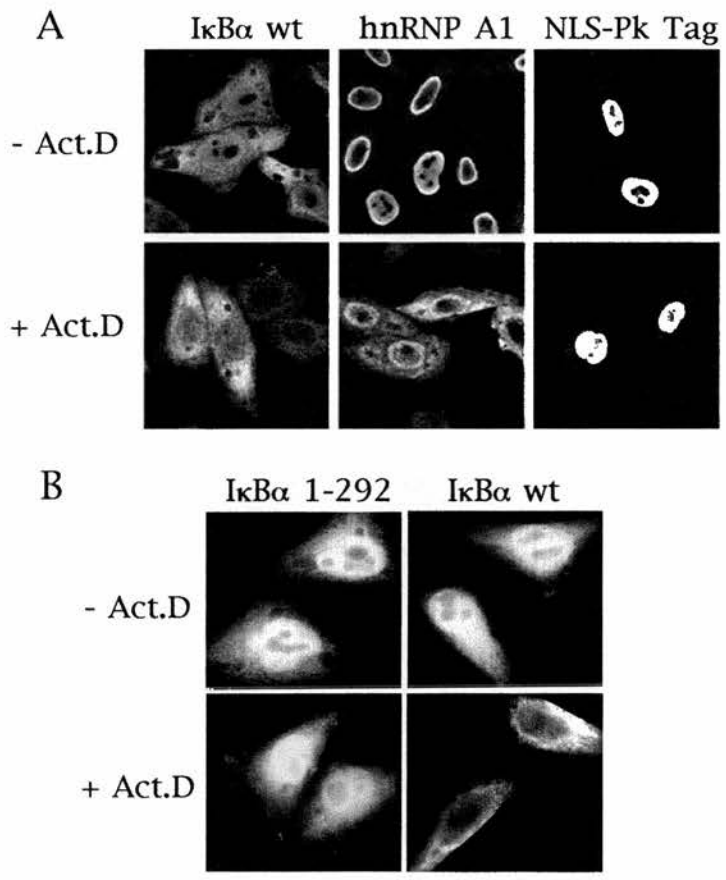
Exogenously expressed IκBα was present in both the nucleus and cytoplasm in untreated cells. However treatment of cells for 5 hours with actinomycin D resulted in IκBα delocalisation to the cytoplasm (Fig. 21A). The images shown in Fig. 17A were taken by confocal microscopy. To confirm the result that IκBα$_{1-292}$ does not interact

Figure 21. IκBα is cytoplasmically delocalised as a consequence of Actinomycin D treatment.

(A) Immunofluoresence carried out in HeLa cells monitored exogenous IκBα (IκBα wt) and endogenous hnRNP A1 in response to the presence/absence of Actinomycin D (10μg/ml). NLS-PK-tag and IκBα co-transformation was used as control of nuclear integrity.

(B) Exogenous IκBα wt and a carboxy terminal truncated form of IκBα ($IκBα_{1-292}$) were transfected into HeLa cells and incubated in the presence/absence of Actinomycin D as before.

Images in (A) and (B) were taken with confocal and fluorescence microscopy respectively.


A
IκBα wt
hnRNP A1
NLS-Pk Tag
- Act.D
+ Act.D
B
IκBα 1-292
IκBα wt
- Act.D
+ Act.D

with hnRNP A1, IκBα$_{1-292}$ was transfected in the HeLa cells. Following expression IκBα$_{1-292}$ had a similar localisation pattern to that of wild type IκBα, however IκBα$_{1-292}$ was not delocalised in the presence of actinomycin D (Fig. 21B). This demonstrated that there was no interaction between hnRNP A1 and IκBα$_{1-292}$. The images shown in Fig. 21B were taken by standard fluorescent microscopy.

The results clearly demonstrate that hnRNPA1, in response to actinomycin D treatment, partially delocalises to the cytoplasm. Delocalisation of endogenous hnRNP A1 also delocalised IκBα wt from the nucleus to the cytoplasm. However the carboxy terminally truncated form of IκBα which did not interact with hnRNP A1, IκBα$_{1-292}$, was not delocalised in response to actinomycin D treatment. Thus actinomycin D mediated IκBα delocalisation is only observed with IκBα molecules that are competent to interact with hnRNPA1.

3.5 : hnRNPA1 enhances NF-κB dependent transcriptional activation

To determine the functional consequences of the interaction between IκBα and hnRNPA1 we obtained a mouse erythroleukaemia cell line (CB3) which lacks endogenous hnRNPA1 (Ben-David et al., 1992). It is thus possible to introduce hnRNPA1 into these cells and evaluate its influence on NF-κB dependent transcriptional activation. CB3 cells were transfected by electroporation as this was found to be the only method which allowed these cells to efficiently take up exogenous DNA. CB3 cells were transfected with 1 of 4 hnRNP A1 constructs (95-320, 105-320, 1-196 and 1-207) ; 1 of 3 different luciferase reporters (3enh Con A Luc (NF-κB dependent), AP1 Con A Luc (AP1 dependent) and Con A Luc) and RSV β-Gal reporter either in the presence or absence of constructs expressing EBV latent membrane protein (LMP-1) (Fig. 22). EBV LMP-1 is a potent inducer of signal transduction pathways that lead to NF-κB activation (Sylla et al., 1998) and expression levels of this protein were adjusted to ensure that the NF-κB response was not saturated. 12 hours post transfection the cells were harvested, washed and lysed in luciferase or the β-Gal buffer. Subsequently the reporter activities and protein concentration of each extract were determined. The RLUs obtained for the luciferase assay were divided by the β-Gal RLUs to give the results in NF-κB activity units (Fig. 23A). In the absence of hnRNPA1, EBV LMP-1 expression results in an 13-fold increase in NF-κB dependent reporter activity. In the presence of hnRNPA1 and LMP-1 induced reporter activity was increased to 101-fold over that observed in the absence of hnRNPA1 and LMP-1 (Fig. 23A). hnRNP A1 mutants which did not interact with IκBα (105-320 and 1-196) failed to substantially increase LMP-1 activated NF-κB reporter activity whereas mutants which are capable of interacting with IκBα (95-320 and 1-207) still increased LMP-1 activated NF-κB reporter activity 69 and 107 fold respectively over that observed in the absence of hnRNP A1 and LMP-1 (Fig. 23A).

Neither LMP-1, nor hnRNP A1 had any influence on the activity of the Con A luciferase reporter (Fig. 23B) lacking NF-κB binding sites or on a LacZ reporter with an

Figure 22. The reporter constructs used in determination of hnRNP A1 effect on NF-κB dependent transcriptional activation.

The different plasmids used in the transfection experiments are shown above. The NF-κB binding sites are shown in Red ; the Con-A promoter shown in Blue ; the AP-1 binding sites shown in Green and the Luciferase, Lac Z (β-Galactosidase), hnRNP A1 and LMP-1 genes are shown in Yellow ; RSV promoter is shown as grey diagonal lines ; CMV promoter is shown in grey and as grey vertical lines.

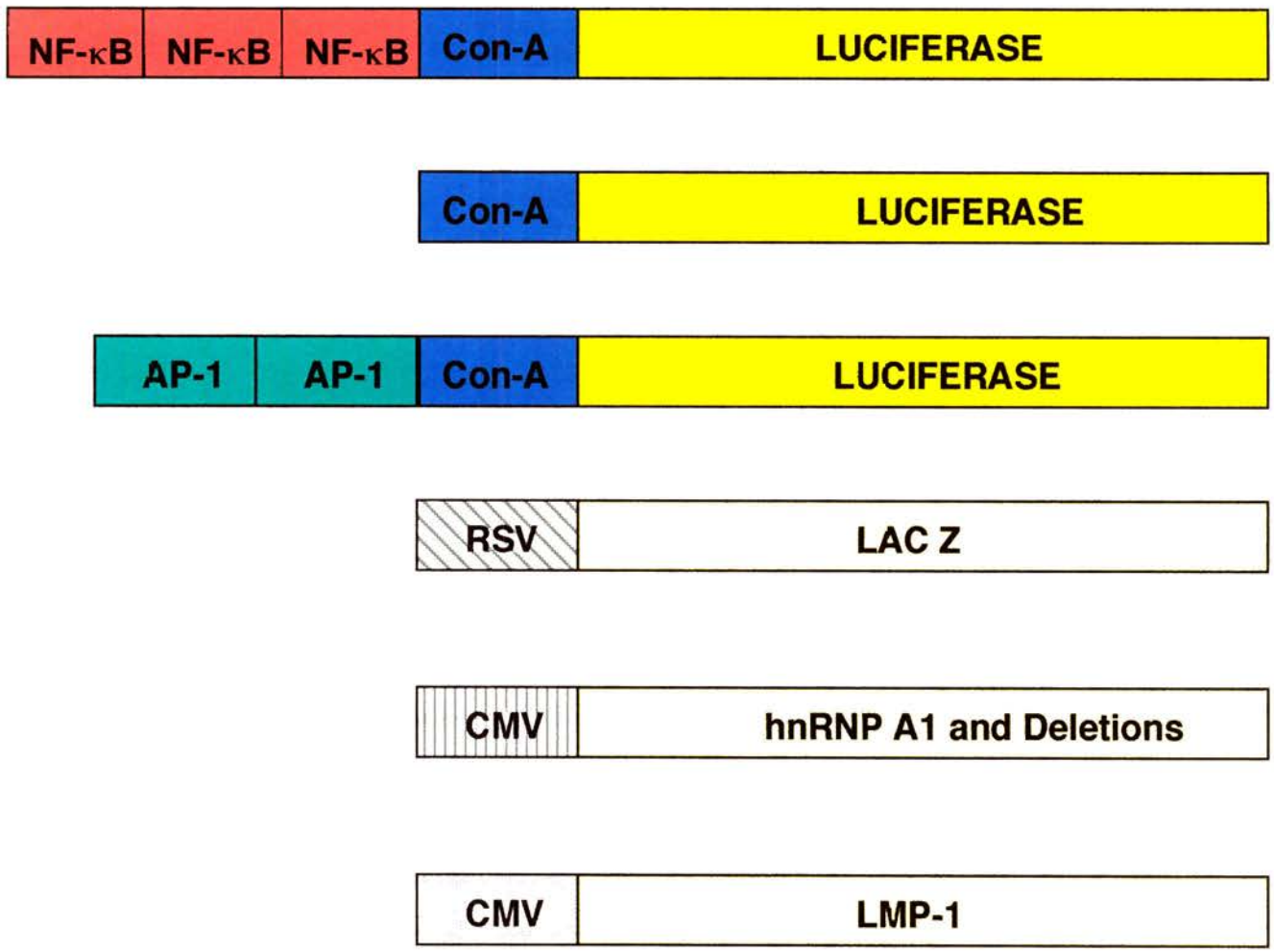
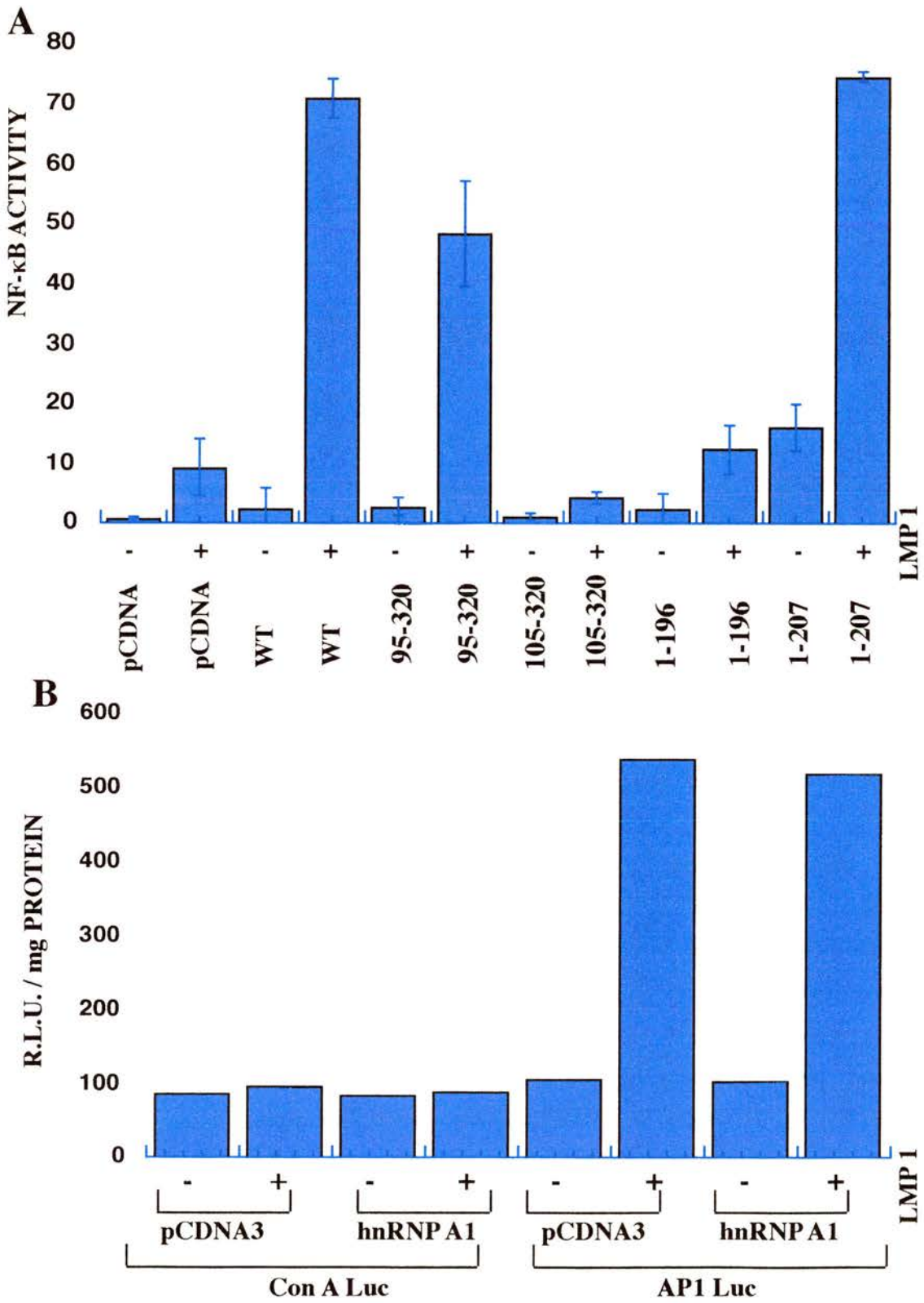

NF-κB
NF-κB
NF-κB
Con-A
LUCIFERASE
Con-A
LUCIFERASE
AP-1
AP-1
Con-A
LUCIFERASE
RSV
LAC Z
CMV
hnRNP A1 and Deletions
CMV
LMP-1

Figure 23. NF-κB activation by hnRNP A1.

(A) pCDNA empty vector, RSV β-Gal, Con-A Luc 3 enhancer reporter and pCDNA hnRNPA1 and deletions (wt, 95-320, 105-320, 1-196 and 1-207) encompassing the boundaries of interaction with IκBα, were transfected into a hnRNP A1 knock out cell line (CB3) in the presence or absence of LMP1 activation. The results above are representative of 8 experiments repeated in triplicate and the standard errors are calculated using 1 standard deviation. NF-κB activity was calculated by the Luc:βGal ratio.

(B) Con A Luc and AP1 Luc constructs were used as a negative control and to demonstrate hnRNP A1 induced activation was NF-κB specific respectively. The data shown in panel (B) was derived from 2 independent experiments (the units of activity are quoted in RLU per mg of protein) and thus could not be statistically processed to give error bars.


A
NF-κB ACTIVITY
80
70
60
50
40
30
20
10
0
- + - + - + - + - + - + - +
LMP 1
pCDNA
pCDNA
WT
WT
95-320
95-320
105-320
105-320
1-196
1-196
1-207
1-207
B
R.L.U. / mg PROTEIN
600
500
400
300
200
100
0
- + - + - + - +
LMP 1
pCDNA3
hnRNP A1
pCDNA3
hnRNP A1
Con A Luc
AP1 Luc

RSV promoter which was employed as an internal control. An AP1 dependent luciferase reporter was used to demonstrate that the transcriptional activation increase by hnRNP A1 was a NF-κB specific event. The AP1 construct was activated 5-fold by cotransfected LMP-1, however the AP1 promoter was not further activated by expression of hnRNPA1 thus demonstrating that the phenomena observed was specific for NF-κB transcription.

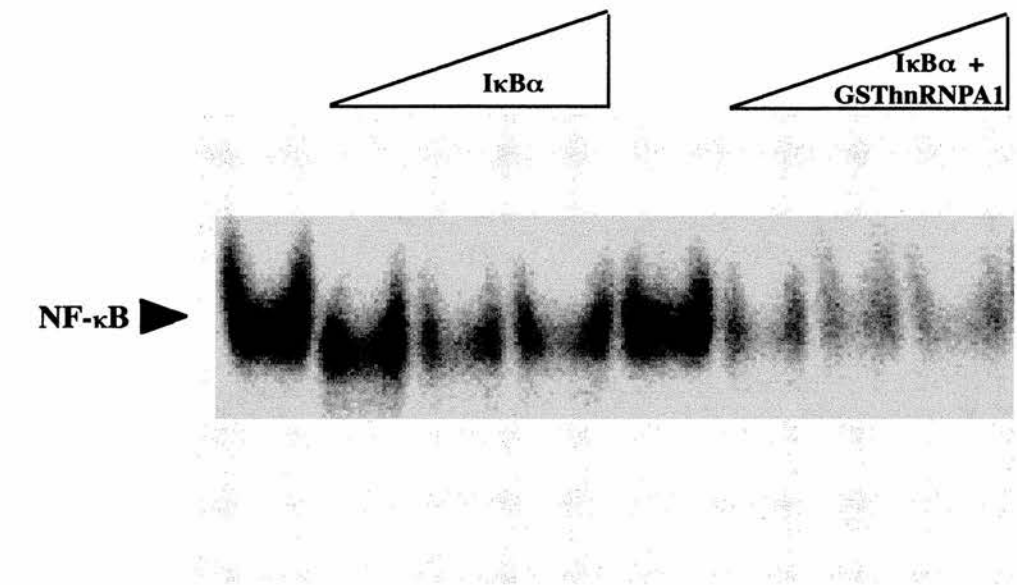
In summary the lack of hnRNPA1 expression in CB3 cells results in defective NF-κB dependent activation which can be rectified by expression of exogenous hnRNPA1. These experiments also indicate that the conclusions drawn from the *in vitro* deletion analysis were correct *in vivo*. Neither LMP-1, nor hnRNPA1 had any influence on the activity of a control luciferase reporter lacking NF-κB binding sites, or on a LacZ reporter with an RSV promoter or an AP1 dependent luciferase reporter. Thus demonstrating that the phenomena observed was specific for NF-κB dependent transcription.

3.5.1 IκBα inhibits NF-κB DNA binding in the presence or absence of hnRNP A1.

We used recombinant proteins and HIV-LTR γ^{32}P labelled probe in order to determine the functionality of the interaction between hnRNPA1 and IκBα. Interactions were studied using bacterially produced recombinant proteins prepared as before and the GST-hnRNP A1 fusion protein which was purified as before. Recombinant NF-κB was incubated in the presence or absence of IκBα or IκBα and hnRNP A1 for 30 minutes at 4°C. Subsequently 0.025 p moles of γ^{32}P labelled HIV-LTR probe was incubated with the proteins for a further 30 minutes at 4°C. Following incubation the complexes were separated by non-denaturing PAGE (6%), containing 0.5X TBE, for 2 hours at 200 Volts and the gel was dried before overnight exposure to a phoshoimager plate. As expected NF-κB bound the HIV-LTR probe with high affinity and NF-κB DNA binding activity could be reduced substantially with the addition of varying amounts of recombinant IκBα (Fig 24). Moreover the manner in which IκBα inhibited NF-κB

Figure 24. IκBα inhibits NF-κB DNA binding activity in the presence or absence of hnRNP A1.

NF-κB (1ng) was incubated with the HIV-LTR probe in the absence or presence of the IκBα inhibitor protein (1ng, 10ng and 100ng). The second region of the gel shows NF-κB (1ng) incubated in the presence/absence of IκBα and GST-hnRNP A1 (1ng, 10ng and 100ng).



p50	+	+	+	+	+	+	+	+
p65	+	+	+	+	+	+	+	+
HIV LTR	+	+	+	+	+	+	+	+

DNA binding was not affected by the addition of GST-hnRNP A1 (Fig 24). From this observation we can conclude, that *in vitro* , hnRNP A1 does not modulate the inhibitory manner of IκBα.

4. DISCUSSION

The experiments reported demonstrate that IκBα interacts specifically and directly with the pre-mRNA binding protein hnRNP A1. hnRNP A1 is a member of the hnRNP family, one of the most abundant families of eukaryotic nuclear proteins. There are about $7x10^7$ to $10x10^{10}$ molecules each of hnRNP A1 and C1 per cell nucleus (Kiledjian and Dreyfuss, 1992) and many other are similarly abundant. Although primarily nuclear, a subset of hnRNPs continuously and rapidly shuttle between the nucleus and cytoplasm in a RNA Pol II transcription dependent manner (e.g. A1, A2 and K) while others (e.g. C1, C2 and U) are restricted to the nucleus at all times (Pinol-Roma and Dreyfuss, 1992). Immunopurification and two-dimensional gel electrophoresis led to the discovery of > 20 hnRNP proteins, including the six core proteins, A1, A2, B1, B2, C1 and C2 (Beyer et al., 1977), that are components of hnRNP complexes in human cells (Pinol-Roma et al., 1988).

4.1 hnRNP A1 Function

The proteins of an hnRNP complex are involved in diverse aspects of pre-mRNA metabolism. There is also considerable evidence that suggests a role for some hnRNPs in the export of mRNA from the nucleus to the cytoplasm (Michael et al., 1995; Michael et al., 1995; Pinol-Roma and Dreyfuss, 1992; Visa et al., 1996). However the precise mechanism by which mRNAs are exported to the cytoplasm is poorly defined (Izaurralde and Mattaj, 1995; Nakielny et al., 1997). In addition to its presumed role in pre-mRNA packaging and transport, hnRNP A1 has other activities of biological importance. Both *in vitro* and *in vivo* studies demonstrate A1 has the potential to influence 5'splice site selection in pre-mRNAs that contain multiple 5'splice sites

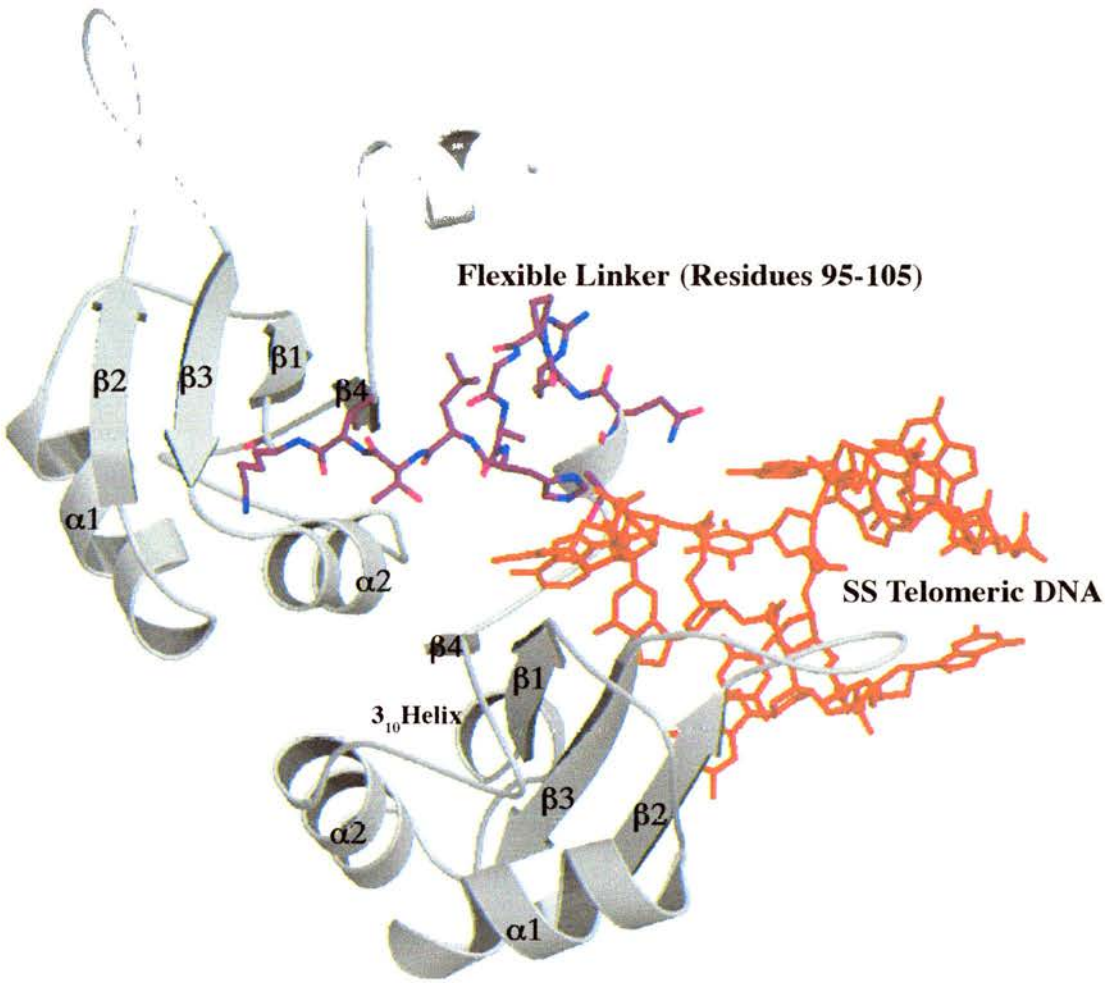
(Caceres et al., 1994; Mayeda and Krainer, 1992; Yang et al., 1994) and promotes the renaturation of complementary single-stranded nucleic acids (Munroe and Dong, 1992; Pontius and Berg, 1992). hnRNP A1 has been shown to interact with the HCMV IE2 protein (Wang et al., 1997), although as yet, no function has been attributed to the interaction. hnRNP A1 has also been shown to cooperate with Rev, which is an HIV-1 encoded RNA binding protein, and another 50kDa protein in the export of unspliced mRNA from the nucleus (Najera et al., 1999). It should be noted that Rev, like IκBα, contains both a leucine rich nuclear export signal and a nuclear localisation signal, which together allow the proteins to shuttle between the makers and the cytoplasm

4.2 hnRNP A1 Structure

The hnRNP A1 protein consists of a single polypeptide chain of 320 amino acids with a molecular weight of 34 kDa. An amino terminal proteolytic fragment spanning the first 196 amino acids, is also known as unwinding protein 1 (UP1) (Herrick and Alberts, 1976; Williams et al., 1985) and possesses two RNA recognition motifs (RRMs) separated by a short linker. The atomic structure of UP1 (residues 8-190), including the flexible linker, has been solved (Ding et al., 1999) and shows that the folding of both RRMs closely resembles that of previously determined individual RRM structures. The independently folded RRMs are held rigidly by two Arg-Asp salt bridges, and are positioned with the carboxy terminal α helices of each RRM adjacent and parallel to each other, such that the four stranded β sheets form an extended RNA binding surface (Ding et al., 1999; Shamoo et al., 1997; Xu et al., 1997). Each RRM of hnRNP A1 is folded into a compact globular domain which consists of six strongly conserved elements, β1 α1 β2 β3 α2 β4 (Figures 25 and 27). The four β strands form a single anti-parallel β-sheet and the 2 α helices pack tightly on one side of it. There are two conserved solvent exposed phenylalanine residues found at the centre of the β-sheet in each RRM, which have been shown to contact RNA directly (Mayeda et al., 1994; Merrill et al., 1988). The inter-RRM flexible linker is located on the surface between the 2 β-sheets, and its position suggests that it is also capable of contacting bound RNA

Figure 25. The crystal structure of UP-1 as determined by Ding et al., 1999.

The secondary protein structure of the amino terminus of hnRNP A1. The protein structure is drawn in a conventional manner. The flexible linker (residues 95-105) that joins the two RNA recognition motifs (RRMs) is shown in a ball and stick configuration. UP-1 in this structure binds single stranded telomeric DNA (SS Telomeric DNA) using RRM1.


Flexible Linker (Residues 95-105)
β2
β3
β1
β4
α1
α2
SS Telomeric DNA
β4
β1
3₁₀Helix
β3
β2
α2
α1

directly and may allow a 'hinge' like motion to bring RNAs together, or to allow more than one RRMs to bind simultaneously to a single high affinity RNA binding site (Ding et al., 1999; Shamoo et al., 1997; Xu et al., 1997). Also a short 3_{10} helix immediately amino terminal to the first β strand of the first RRM (Figure 25) occupies a spatial position corresponding to regions involved in critical RNA contacts in other RRM containing proteins. The protein structure of hnRNP A1 RRM 1 and 2 provides insight into the architecture of multi-RRM proteins suggesting that an anti-parallel arrangement of RNA binding platforms can function to condense RNA (Ding et al., 1999; Shamoo et al., 1997; Xu et al., 1997). The carboxy terminus of hnRNP A1 is rich in glycines (45%) and the high glycine content and proteinase sensitivity suggest that the carboxy terminal domain is largely unstructured or at least highly flexible. Unfortunately the carboxy terminal structure of hnRNP A1 has not been solved. This region of hnRNP A1 includes several Arg-Gly-Gly (RGG) tri-peptides repeats that also constitute a RNA binding motif (RGG Box)(Kiledjian and Dreyfuss, 1992). In addition the carboxy terminus of the protein contains a 38 amino acid domain, termed M9. M9 was initially identified as the hnRNPA1 nuclear localisation sequence (Siomi and Dreyfuss, 1995; Weighardt et al., 1995) and following this observation M9 was also shown to activate nuclear export (Michael et al., 1995). Mutageneis studies so far have been unable to separate the NLS from the NES activities of M9, suggesting that some of the factors required for hnRNP A1 nuclear import may also function in its nuclear export (Michael et al., 1995). The carboxy terminus is also known to be involved protein-protein interactions (Cartegni et al., 1996; Nadler et al., 1991) and an RNA strand annealing activity (Pontius and Berg, 1992). The amino and carboxy termini of hnRNP A1 therefore function to bind RNA and recruit distant RNA elements onto a single protein/RNA complex. Following complex formation the hnRNPs function in concert to correctly orientate RNA before alternative splicing takes place. The mature mRNA transcript is then transported to the cytoplasm, presumably in association with hnRNP A1, where it is deposited and translated.

4.3 hnRNP A1 Interaction with RNA

The arrangement of structural domains RRM1-RRM2-Gly rich, is characteristic of all hnRNPA/B proteins (Dreyfuss et al., 1993). The deletion of the glycine rich domain results in complete loss of alternative splicing activity and a striking decrease in RNA binding and annealing activities (Mayeda et al., 1994). To determine whether each of the RRMs contributes equally to these properties, each RRM was altered by mutation of two Phe residues in β3 (Mayeda et al., 1994). The replacement of these residues in either RRM resulted in the complete loss of alternative splicing, demonstrating that both RRMs are essential for splicing activity. In contrast, the RNA binding and annealing activity of hnRNP A1 decreased only slightly upon substitution of the Phe residues in either RRM, and only upon simultaneous mutation of both RRMs were these activities strongly affected. One explanation for the different effects of substitutions within individual RRMs on splicing as compared with RNA binding or annealing is that splicing requires the maintenance of precise and stringent interactions with both RRMs of hnRNP A1. Thus the 2 RRMs function in a co-operative or co-ordinated manner with the glycine rich carboxy terminus to achieve functional RNA binding, annealing and alternative splicing activities.

4.4 hnRNP A1 promotes alternative splicing

Most mammalian pre-mRNAs contain multiple introns, that allow different mRNA molecules to be generated from the same pre-mRNA by alternative splicing and this process plays an important role in the tissue specific regulation of gene expression (McKeown, 1992; Smith et al., 1989). With the exception of the 20mer winner oligo containing 2 copies of the high affinity binding site : consensus UAGGGA/U (Burd and Dreyfuss, 1994), hnRNP A1 appears to have a relatively narrow range of affinities for naturally occurring RNA oligo sequences examined and this range can be accounted for almost entirely in terms of base content rather than sequence specificity (Bai et al., 1999). As mentioned above both the amino and carboxy terminal domains are required for the alternative-splicing activity of hnRNP A1 (Mayeda et al., 1994). The regulation

of alternative splicing usually depends on choosing candidate splice sites, either alternative 5' or alternative 3'. Previous work has shown that several proteins of a highly conserved family of general splicing factors (SR proteins) are widely distributed among eukaryotes and promote the use of the proximal 5' sites that can splice to a fixed 3' site (Krainer et al., 1990). Conversely hnRNP A1 influences the choice between alternative 5' splices in the opposing manner, promoting splicing at the distal site (Mayeda and Krainer, 1992) (Caceres et al., 1994; Yang et al., 1994).

4.5 Nucleocytoplasmic shuttling of hnRNP A1

Following alternative splicing the mature mRNA has to be exported to the cytoplasm where it translated by the protein synthesis machinery. The transport of macromolecules between the nucleus and the cytoplasm is a bi-directional process. The possibility that hnRNP proteins might be directly involved in the nucleocytoplasmic trafficking of mRNA molecules was suggested by the observation that several hnRNPs (A1,A2,D,E,I,K) shuttle continuously and rapidly between the nucleus and the cytoplasm and are associated with Poly (A)+ mRNA in both compartments (Michael et al., 1995; Pinol-Roma and Dreyfuss, 1992). It has been estimated that at least 120,000 molecules of hnRNP A1 are exported to the cytoplasm per minute but then rapidly reimported such that the steady state localisation of hnRNP A1 is nuclear (Michael et al., 1995). Interestingly an hnRNP A1-like protein in insect cells (*Chironomus tentans*) has also been shown by immunoelectron microscopy to be associated with a specific mRNA in transit to the cytoplasm through the NPC (Visa et al., 1996). Taken together, these results suggest that hnRNP A1 and other shuttling hnRNPs such as A2,D,E,I,K could play a significant role in mRNA transport. The nucleocytoplasmic transport of hnRNP A1 has been studied in detail, and the signals that mediate its shuttling have been identified (Michael et al., 1995; Siomi and Dreyfuss, 1995; Weighardt et al., 1995). Nuclear import (Siomi and Dreyfuss, 1995) and export (Michael et al., 1995) of hnRNP A1 is mediated by a carboxy terminal 38 amino acid domain, termed M9. Other hnRNPs such as A2 and B1 bear sequences with striking similarities to M9 (Siomi and

Dreyfuss, 1995). Mutagenesis studies indicate that the NES and NLS activities of M9 are either identical or overlapping as mutants which block NLS activity also abolish NES activity (Michael et al., 1995). M9 bears no sequence similarity to 'classical' NLSs of the SV40 large T or nucleoplasmin bipartite basic types (Dingwall and Laskey, 1991) and thus does not use the classical importin mediated import pathway but requires a 90 kDa protein, termed transportin, as its nuclear import mediator (Nakielny et al., 1996; Pollard et al., 1996).

4.6 Post translational modification of hnRNP A1

Reversible phosphorylation is utilised by the cell to regulate a variety of cellular processes such as protein degradation and activation. Previous studies have demonstrated that hnRNPs may be regulated by phosphorylation. hnRNP C1 and C2 proteins are phosphorylated *in vivo* by caesin kinase II and dephosphorylation is required for binding of hnRNP C proteins to interact with nucleic acids. (Holcomb and Friedman, 1984; Mayrand et al., 1993). The same phenomena has been demonstrated for hnRNP A1. hnRNP A1 has also been found as a phosphoprotein in HeLa cells and phosphorylation *in vitro* by PKA was observed in a peptide sequence that contains serine 197 and 199(Cobianchi et al., 1993). CKII has also been reported to phosphorylate A1, *in vitro*, at serine 199 although other sites may also be phosphorylated (Idriss et al., 1994). hnRNP A1 is also phosphorylated *in vitro* at Serines 95, 192 and 199 by PKC and dephosphorylated by phosphatase 2A (Idriss et al., 1994). The phosphorylation of hnRNP A1 inhibits its interstrand annealing activity but this can be regenerated by treatment of the hnRNP A1 phosphoprotein with phosphatase 2A but not phosphatase 1 (Idriss et al., 1994). The *in vitro* phosphorylation of hnRNP A1 by the afore mentioned kinases (CKII, PKA and PKC) thus offers a potential mechanism for regulation of the multiple activities of the hnRNP A1 *in vivo*. hnRNP A1 is also post-translationally methylated by an unidentified methyl transferase activity. The 4 sites of hnRNP A1 arginine methylation have been located at residues 193, 205, 217 and 224 in the glycine rich, COOH terminal one-third of HeLa hnRNP

A1 protein (Kim et al., 1997; Williams et al., 1985). All four sites fall within a span of sequence between residues 190-233, which contains the RGG sequences interspersed with phenylalanine residues, and have been postulated to represent an RNA binding motif (Kiledjian and Dreyfuss, 1992). Arginines 205 and 224 have been methylated *in vitro* by a nuclear protein methyltransferase (histone methylase I) using recombinant A1 as substrate (Kim et al., 1997). This suggests A1 may be an *in vivo* substrate for this enzyme. An intriguing suggestion (Najbauer et al., 1993) is that methylation might help to lock proteins like A1 into a non-specific binding mode by preventing the formation of arginine-dependent H-bonds of the type that have been proposed to account for specific interactions. The carboxy terminal A1 domain that extends from 196 to 320 contributes to all or most of the cooperativity of hnRNP A1 binding (Kumar et al., 1990) and it is possible that arginine methylation might effect binding by modifying the co-operative protein:protein interactions that occur between adjacent hnRNP A1 molecules bound to a nucleic acid lattice. Thus this finding that all the sites of A1 methylation occur within its RGG domain (Kim et al., 1997) lends strong support to previous data suggesting that methylation may modulate the nucleic acid binding properties of A1. This evidence is supported by experiments done in yeast where the hnRNP shuttling hnRNP proteins Npl3p and Hrp1p which take part in mRNA processing and export, are methylated by the Hmt1p methylase. In the absence of methylation the shuttling proteins are localised to the nucleus and cannot be shuttle to the cytoplasm (Shen et al., 1998).

As mentioned serine 199 abrogates the ability of hnRNP A1 to facilitate interstrand annealing (Cobianchi et al., 1993) and is very close to 2 methylation sites (194 and 205) (Kim et al., 1997). Since phosphorylation of hnRNP A1 abrogates the ability to facilitate *in vitro* strand annealing this provides further support for the notion that this region plays an important role in modulating interaction of hnRNP A1 with nucleic acids. In this respect it would be of interest to determine the importance of these 2 post-translational modifications on each other and whether the effects on hnRNP A1 are additive.

4.7 IκBα/NF-κB Complex

IκBα is organised into 3 domains with an unstructured amino terminus (residues 1-72) which acts as a signal response domain and a central ankyrin repeat domain (ARD) (residues 73-242). The carboxy terminal domain (243-317) contains a highly acidic region (residues 276-317) which is joined to the ARD via a flexible linker (residues 243-275).

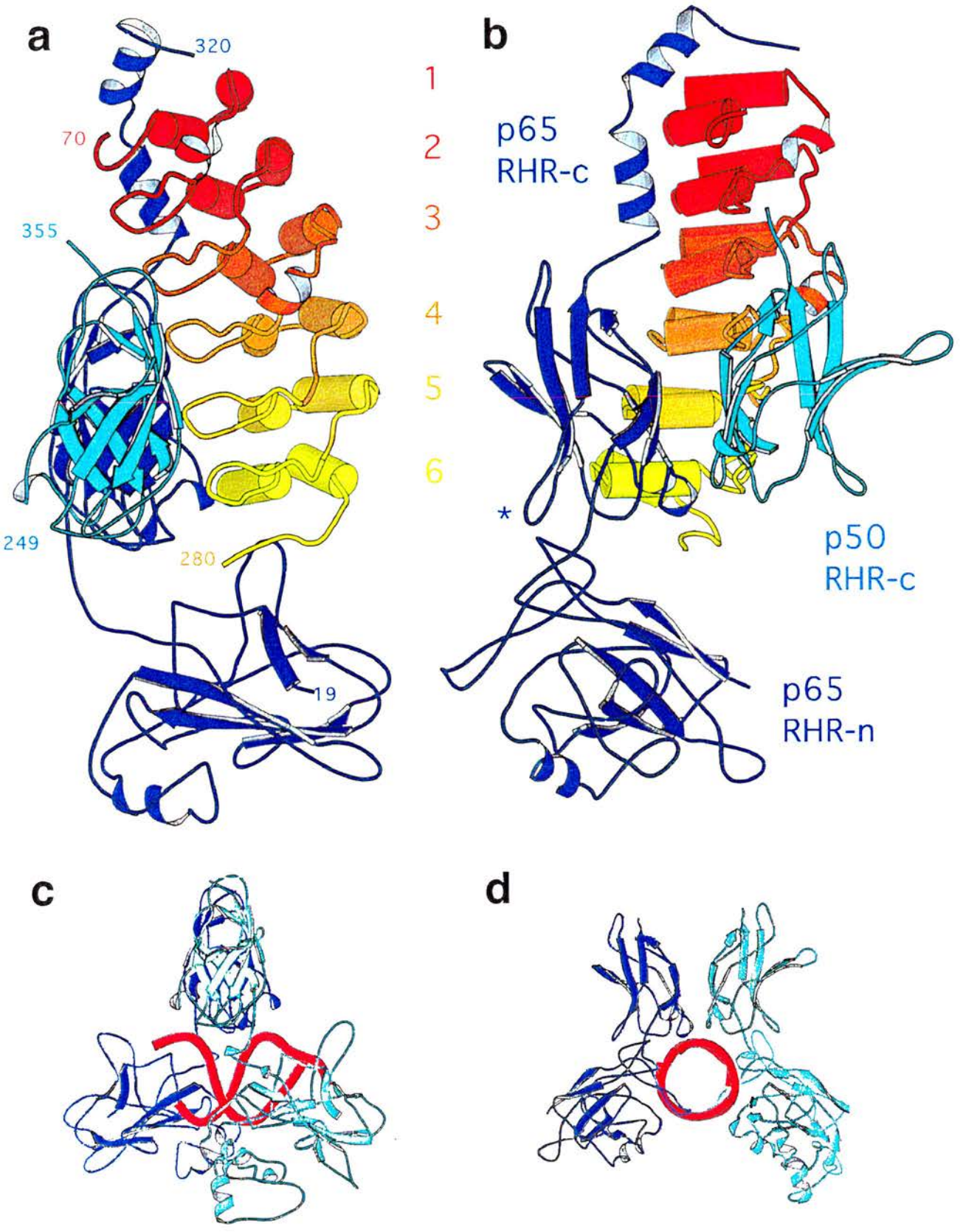
The crystal structures of an IκBα/NF-κB complex demonstrates that the interactions between IκBα and the p50/65 heterodimer appear extensive (Huxford et al., 1998; Jacobs and Harrison, 1998)(Figure 26), engaging all six ankyrin repeats (Huxford et al., 1998; Jacobs and Harrison, 1998). Each ankyrin repeat, consists of a β loop and 2 anti-parallel α helices, stacks on the previous one at a small angle causing the stack of repeats to face toward the inner helix. The concave side of the ARD faces the NF-κB dimer and masks p65's NLS extension. The masking of p50's NLS was absent from the crystal data as a truncated form of the protein lacking the NLS was used. However it has been shown that residues 44-52, not present in either model are necessary for cytoplasmic retention of p50 homodimer (Latimer et al., 1998). The amino terminal of IκBα is flexible, therefore it could easily fold back to mask the NLS or prevent directly or indirectly with the nuclear import machinery. The amino and carboxy termini of IκBα, although not present in the crystal structures, appear to localise consistently with their known or likely functions. That is amino and carboxy termini are expected to be largely unstructured and accessible to both post translational modification and the nuclear import and export machinery. The accessibility exhibited in the carboxy terminal region of IκBα means that it can be recognised and bound by hnRNP A1.

4.8 IκBα carboxy terminus

The carboxy terminus has been an area of intense study and it has been demonstrated that within the carboxy terminal domain of IκBα there are 2 important regions. The nuclear export sequence (NES) found between residues : 265-277 and the PEST region from residues 278-317. The leucine rich region of sequence, 265:

Figure. 26 The structure of the NF-κB/IκBα complex, with DNA bound NF-κB as a reference.

(a) A ribbon drawing of the ankyrin domain of IκBα bound to NF-κB. The RHR-c and -n domains of p65 are shown in dark blue, the RHR-c domain of p50 in light blue, and the ankyrin domains shaded from red at the N-terminus to yellow at the C-terminus. The RHR-n domain of p50 is not present in this structure. Each ankyrin repeat is numbered in its corresponding colour, and the α helices of IκBα are represented as cylinders. Residue numbers of the termini of the model are shown in the left panel. (b) View of a NF-κB/IκBα complex rotated 90° from the view in panel (a). The asterisk indicates the p65 phosphorylation site, Ser 276. (c,d) NF-κB bound to DNA (Chen et al., 1998). These view correspond to the orientations of NF-κB in panels (a) and (b) respectively and use the same colours. The DNA is shown in red as a ribbon through backbone phosphate positions. These figures were taken from Jacobs et al 1998.


a
320
70
355
249
280
19
1
2
3
4
5
6
b
p65
RHR-c
*
p50
RHR-c
p65
RHR-n
c
d

IQQQLGQLTLQNL :275, functions as an autonomous NES and confers IκBα with this capacity (Arenzana-Seisdedos et al., 1997). The leucine residues critical for nuclear export activity of IκBα were identified by mutagenesis. Leucine to alanine changes at positions 272, 274 and 277 inhibited the ability of IκBα to be exported from the nucleus to the cytoplasm (Arenzana-Seisdedos et al., 1997). In addition this triple mutant was shown to be the most defective for export as compared to dual mutations 269 and 272 ; 272 and 274 ; 274 and 277 (Arenzana-Seisdedos et al., 1997). It should be noted that in addition to the carboxy terminal NES there also appears to be an amino terminal region from residues 45-54 which could be a canonical leucine rich NES sequence. Homologues of the NES are found in HIV-1 Rev, a protein that promotes nuclear export of unspliced/partially spliced viral mRNAs (Fridell et al., 1996), and PKI (the inhibitory subunit of PKA) (Wen et al., 1995) which returns the catalytically active PKA subunit back to the cytoplasm. Thus the NES constitutes a transferable transport signal that is necessary and sufficient to mediate rapid and active export from the nucleus to the cytoplasm. The nuclear protein CRM-1 has been identified as the receptor for the leucine rich NES, required nuclear export (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). CRM-1 forms a complex with IκBα which is facilitated by the presence of Ran in its GTP bound form. Following nuclear translocation it has been proposed that the cytoplasmic disassociation of the export complex is mediated by Ran bound GTP hydrolysis mediated by RanGAP and Ran BP1 (Fornerod et al., 1997).

Deletion of residues 266-317 from the carboxy terminus of IκBα renders the protein resistant to signal induced degradation (Rodriguez et al., 1995). Within this region is the PEST domain which is rich in proline, glutamic acid, serine and threonine residues and constitutes a large part of IκBα carboxy terminus. Residues 288-317, which represents most of the PEST region, have been shown to be dispensable for signal induced degradation of IκBα (Beauparlant et al., 1996) where as further deletion down to residue 279 renders IκBα unresponsive to TNF and LPS mediated degradation (Beauparlant et al., 1996; Whiteside et al., 1995). The stretch of amino acids in the

carboxy terminus required for IκBα signal induced degradation are found between residues 279-287 and has the sequence MEPESEDEE (Barroga et al., 1995; Ernst et al., 1995; Lin et al., 1996). The half life of wild type IκBα is 2 hours, however removal of the carboxy terminal region between 269 and 317 decreases the protein half life to 15 minutes (Beauparlant et al., 1996). This phenomena was not observed for the 288-317 deletion mutant, which had a normal 2 hour half life indicating the importance of the 269-287 region in regulation of IκBα stability. Within the region lies an important CKII phosphorylation site located between residues 283-286, the SEDE motif. Mutation of Glutamate 284 and 286 or Aspartate 285 in the SEDE motif of IκBα inhibits the ability of IκBα to dissociate of NF-κB from DNA (Ernst et al., 1995) (Wright, PhD Thesis 1996). Furthermore mutation of the polar residues in and surrounding the SEDE motif at positions serine 283 , threonine 291 and 299 also decreases the intrinsic stability of IκBα (Lin et al., 1996). CKII phosphorylation of serine 283 demonstrated that a wt SEDE region renders IκBα a slightly stronger inhibitor of NF-κB DNA binding (McElhinny et al., 1996). This phosphorylation aids, but is not absolutely necessary for IκBα inhibition of NF-κB, furthermore it has been shown that decreases in the number of negative charges in the carboxy terminus can reduce the binding activity of IκBα for NF-κB (Ernst et al., 1995). This is not the case in another IκB family member. IκBβ interacts very weakly with NF-κB, however when its two CKII motifs are phosphorylated it binds NF-κB more tightly (Thompson et al., 1995). The importance of the IκBβ PEST region phosphorylation in inhibition of NF-κB DNA binding may be due to IκBα and IκBβ carboxy terminal difference in charge. IκBα contains 14 negatively charged amino acids whereas IκBβ contains 9 negative amino acids. However following CKII phosphorylation of IκBβ there is a greater net negative charge, 13, which aids inhibition of NF-κB DNA binding.

In IκBα the acidic carboxy terminal region appears to represent a distinct domain (Jaffray et al., 1995) that is required for inhibition of Rel A DNA binding but is dispensable for simple interaction with Rel A (Ernst et al., 1995). The carboxy terminal domain of IκBα is in close vicinity to the flexibly linked DNA binding Ig-like domains

of the dimer, where it could potentially interfere with NF-κB DNA binding (Huxford et al., 1998; Jacobs and Harrison, 1998). Although the acidic residues in IκBγ which contact the DNA binding loops of p50 have not been identified, deletion of the acidic sequences in p105 converts it from a non-DNA binding form into a form capable of binding DNA (Blank et al., 1991). Thus a model for interaction of IκB molecules with DNA bound Rel proteins would involve initial recognition of the Rel protein in the region of the NLS to form a ternary complex with DNA, containing IκB, Rel and DNA. Thus displacement of NF-κB bound DNA maybe mediated by a highly specific interaction between the polycation carboxy terminus of IκBα and IκBγ competing against DNA for interaction with p65 and p50 respectively (Bell et al., 1996). This mechanism of action is remarkably similar to that of *Bacillus subtillis* σ factor, which contains a highly acidic region thought to be directly responsible for the displacement of RNA from RNA polymerase (Lopez de Saro et al., 1995). In conclusion the carboxy terminus of IκBα plays an important role in IκBα protein stabilisation, regulation of NF-κB DNA binding and the nuclear export of the NF-κB/IκBα trimeric complex.

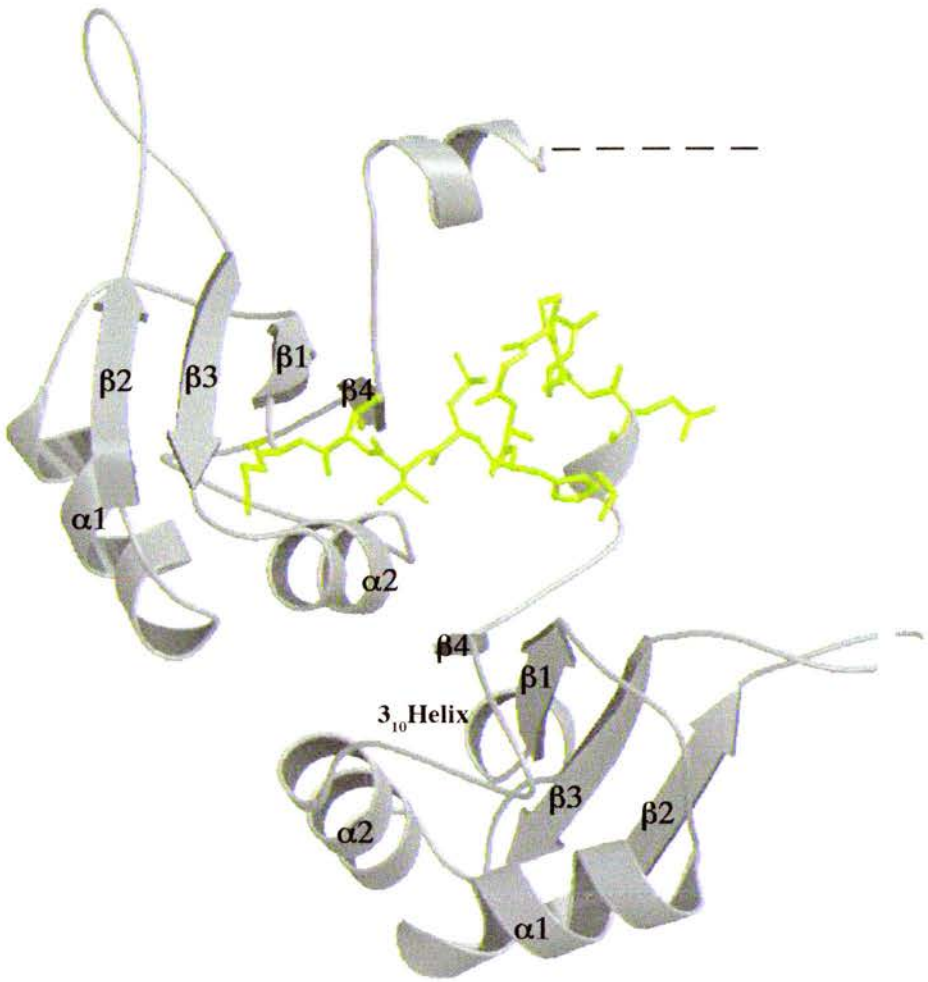
4.9 hnRNP A1 interaction with IκBα

The *in vitro* data presented demonstrates that hnRNP A1 interacts with a carboxy terminal region of IκBα and in addition experiments with recombinant protein demonstrate that post translational modifications of neither hnRNP A1 nor IκBα are required for interaction *in vitro*. As hnRNP A1 and IκBα are both post translationally modified *in vivo*, we cannot rule out the possibility that post translational modifications modulate the interaction between the proteins *in vivo*.

The region of hnRNP A1 required for the interaction extends from residue 95-207 and contains a single RRM. However the RRM alone is not sufficient for interaction with IκBα. Also required is part of the flexible linker between the RRMs (residues 95-105) and part of the RGG box (residues 196-207) (Figure 27). The flexible linker between the RRM domains has the sequence 95: SQRPGAHLTVK :105 and contains two potential phosphorylation sites of which serine 95 has been shown to

Figure 27. The crystal structure of UP-1 as determined by Ding et al., 1999.

The protein secondary structure of each RRM is drawn in a conventional manner and is joined by the flexible linker (shown in yellow). The flexible linker found at residues 95-105 is the first boundary to interaction with IκBα and the black dotted line demonstrates where the second boundary, residues 196-207, to interaction with IκBα maybe positioned.


β2
β3
β1
β4
α1
α2
β4
β1
3₁₀Helix
β3
β2
α2
α1

be phosphorylated by PKC *in vitro* (Idriss et al., 1994). Removal of the sequence 197: RSGSGNFGGGR :207 from hnRNP A1 abolishes the interaction with IκBα and again contains two potential phosphorylation sites which are modified by PKC *in vitro* (Idriss et al., 1994). The region of IκBα required for interaction extends from residues 265-303 and contains the NES and part of the PEST region. The NES (residues 265-277) sequence required for interaction stretches from 265: IQQQLGQLTLQ :275 which contains 1 phosphorylation site at threonine 273. Also required is part of the PEST region (residues 296-303). Removal of the sequence 296: TEFTEDEL :303 from IκBα abolishes the interaction with hnRNP A1. This region also contains potential phosphorylation sites at threonine 296 and 299. Both the flexible linker region (residues 95-105) which is found at the surface of the molecule between the 2 β sheets (Ding et al., 1999) and part of the flexible RGG box (Xu et al., 1997) are accessible for interaction with IκBα. The 296-303 region in IκBα is also an exposed region of structure, as demonstrated by proteolysis studies. IκBα upto residue 275 is protected from proteolysis by its interaction with p65 and thus is not a flexible region in the NF-κB/IκBα trimeric complex context. However the region after residue 275 is not protected suggesting flexibility (Jaffray et al., 1995). Structural data also suggests that IκBα interacts with p65 in this region and that amino acids 280-291 which are carboxy terminal to this region are devoid of secondary structure and give a serpentine structure, suggesting flexibility (Huxford et al., 1998). The structural data of the regions required for interaction between hnRNP A1 and IκBα are summarised in Figure 28

We also demonstrated *in vitro* that another IκB family member, IκBγ, was incapable of interaction with hnRNP A1 even though it possesses ARDs and an acidic carboxy terminus like IκBα. Following this observation we compared the sequences of four other IκBα molecules and other family members IκBβ, IκBε, cactus and IκBγ. We used the 'CLUSTAL' program to align the primary protein sequences (Figure 29). This was done in order to determine if the carboxy terminal region in human IκBα was conserved between IκBα in other species and in the different members of the IκB family. From the alignment it can been seen that residues in IκBα (between 265-303)

Figure 28. The required regions for interaction between IκBα and hnRNP A1 are accessible for protein-protein interaction.

(a) The ribbon drawing of the ankyrin domain of IκBα bound to NF-κB (details as in Figure 26).

(b) View of a NF-κB/IκBα complex rotated 90° from the view in panel (a) (details as in Figure 26).

(c) The protein secondary structure of each hnRNP A1 RRM is drawn in a conventional manner (details as in Figure 27).

The structures presented in this figure demonstrate that the regions required for interaction between the two proteins appear to be largely accessible for interaction in 3 dimensional space. The flexible linker of hnRNP A1 (shown in green) and the uncrystalized carboxy terminus of hnRNP A1 (shown as a black dotted line) interacts with the carboxy terminus of IκBα (shown as broken red box).

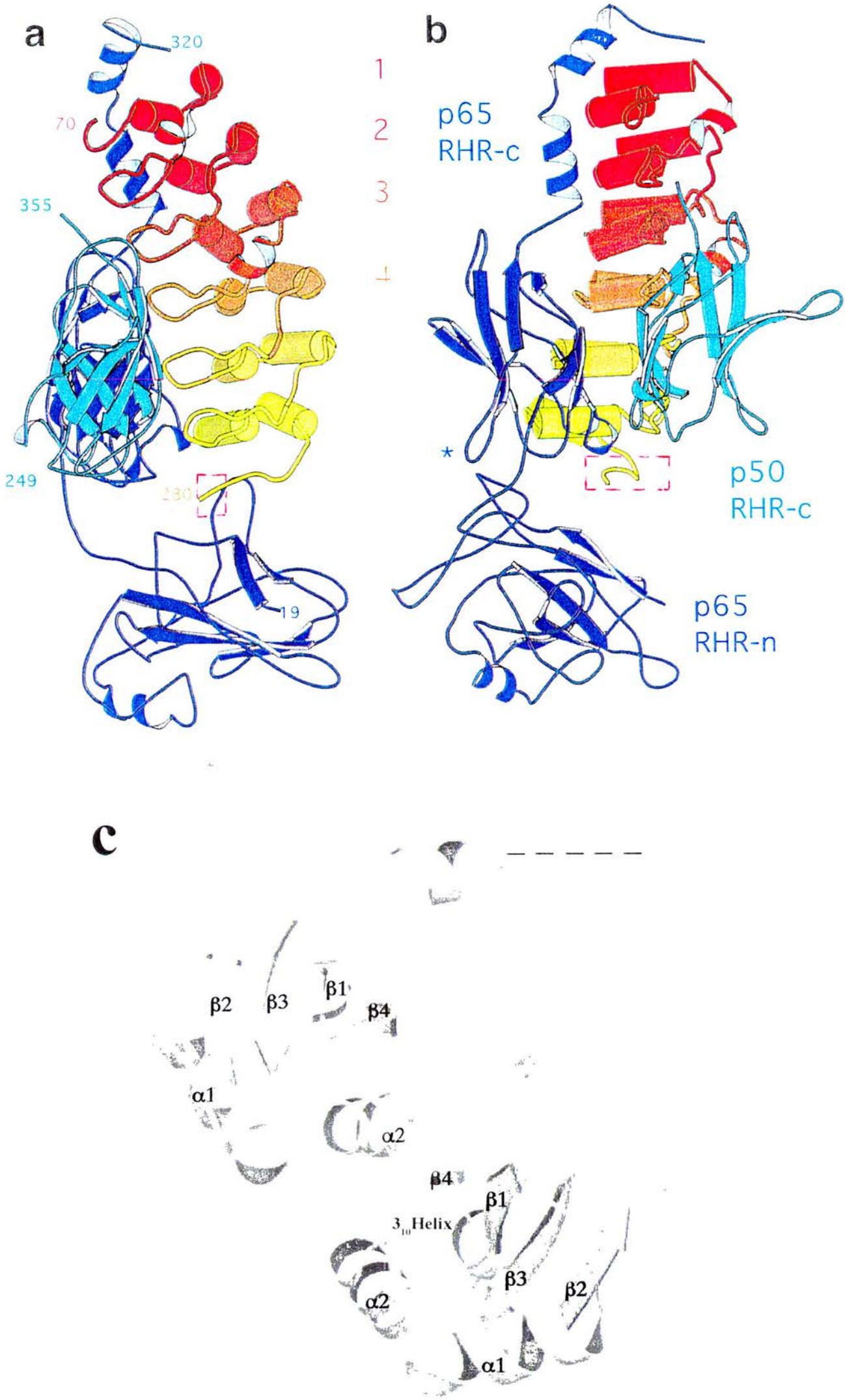
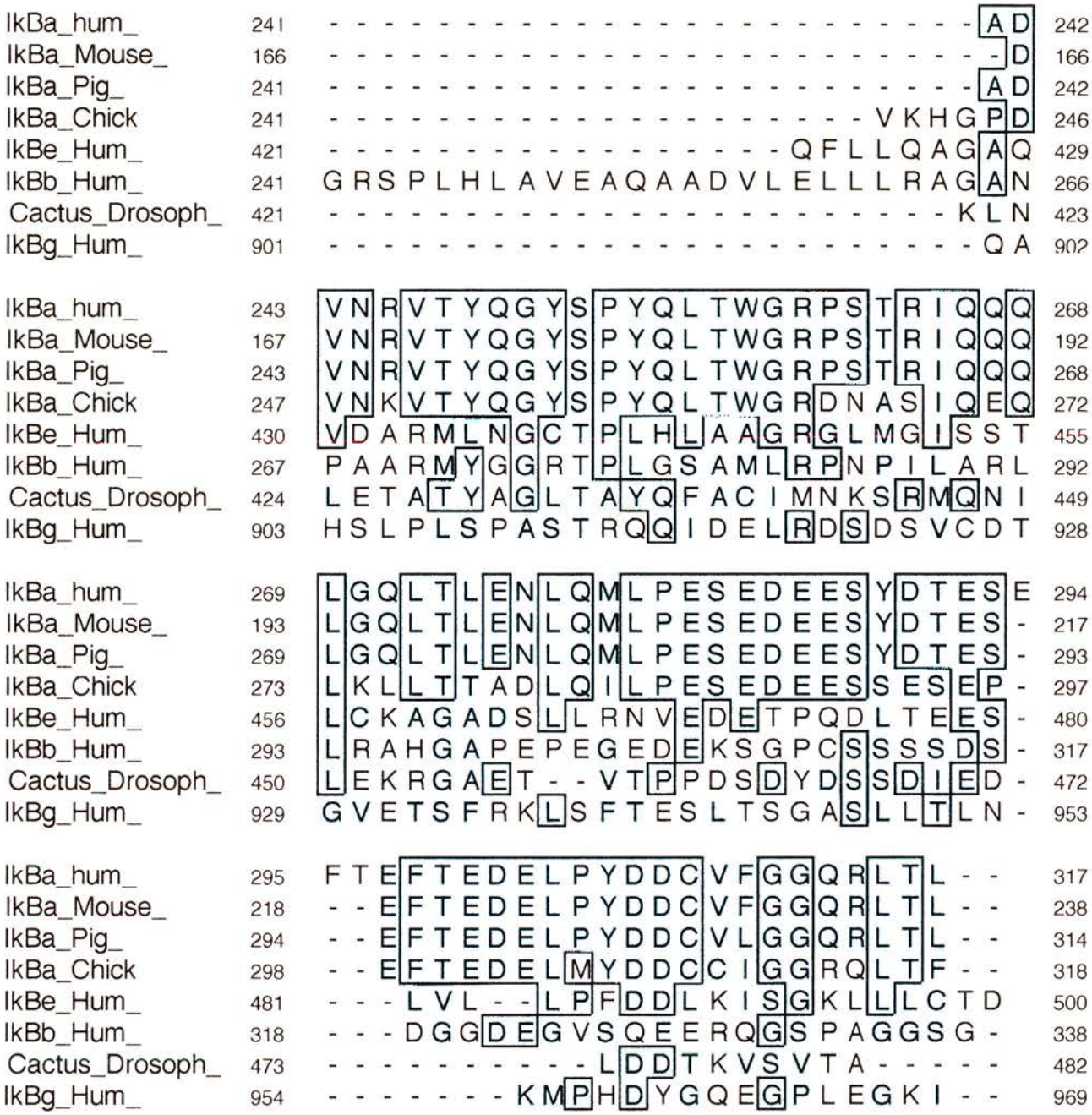

a
320
70
355
249
230
19
1
2
3
4
b
p65
RHR-c
p50
RHR-c
p65
RHR-n
c
β2
β3
β1
β4
α1
α2
β4
β1
3 10 Helix
β3
β2
α2
α1

Figure 29. Carboxy terminal alignments of IκBα (from four different species), IκBβ, IκBε, IκBγ and Cactus.

The primary protein structure of the carboxy terminal regions of each protein (IκBα (from four different species), IκBβ, IκBε, IκBγ and Cactus) were aligned using the CLUSTAL alignment program. Regions of homology are shown in blue and regions of identity are shown in boxed blue.


IkBa_hum_ 241 - A D 242
IkBa_Mouse_ 166 - D 166
IkBa_Pig_ 241 - A D 242
IkBa_Chick 241 - V K H G P D 246
IkBe_Hum_ 421 - Q F L L Q A G A Q 429
IkBb_Hum_ 241 G R S P L H L A V E A Q A A D V L E L L L R A G A N 266
Cactus_Drosoph_ 421 - K L N 423
IkBg_Hum_ 901 - Q A 902
IkBa_hum_ 243 V N R V T Y Q G Y S P Y Q L T W G R P S T R I Q Q Q 268
IkBa_Mouse_ 167 V N R V T Y Q G Y S P Y Q L T W G R P S T R I Q Q Q 192
IkBa_Pig_ 243 V N R V T Y Q G Y S P Y Q L T W G R P S T R I Q Q Q 268
IkBa_Chick 247 V N K V T Y Q G Y S P Y Q L T W G R D N A S I Q E Q 272
IkBe_Hum_ 430 V D A R M L N G C T P L H L A A G R G L M G I S S T 455
IkBb_Hum_ 267 P A A R M Y G G R T P L G S A M L R P N P I L A R L 292
Cactus_Drosoph_ 424 L E T A T Y A G L T A Y Q F A C I M N K S R M Q N I 449
IkBg_Hum_ 903 H S L P L S P A S T R Q Q I D E L R D S D S V C D T 928
IkBa_hum_ 269 L G Q L T L E N L Q M L P E S E D E E S Y D T E S E 294
IkBa_Mouse_ 193 L G Q L T L E N L Q M L P E S E D E E S Y D T E S - 217
IkBa_Pig_ 269 L G Q L T L E N L Q M L P E S E D E E S Y D T E S - 293
IkBa_Chick 273 L K L L T T A D L Q I L P E S E D E E S S E S E P - 297
IkBe_Hum_ 456 L C K A G A D S L L R N V E D E T P Q D L T E E S - 480
IkBb_Hum_ 293 L R A H G A P E P E G E D E K S G P C S S S S D S - 317
Cactus_Drosoph_ 450 L E K R G A E T - - V T P P D S D Y D S S D I E D - 472
IkBg_Hum_ 929 G V E T S F R K L S F T E S L T S G A S L L T L N - 953
IkBa_hum_ 295 F T E F T E D E L P Y D D C V F G G Q R L T L - - 317
IkBa_Mouse_ 218 - - E F T E D E L P Y D D C V F G G Q R L T L - - 238
IkBa_Pig_ 294 - - E F T E D E L P Y D D C V L G G Q R L T L - - 314
IkBa_Chick 298 - - E F T E D E L M Y D D C C I G G R Q L T F - - 318
IkBe_Hum_ 481 - - - L V L - - L P F D D L K I S G K L L L C T D 500
IkBb_Hum_ 318 - - - D G G D E G V S Q E E R Q G S P A G G S G - 338
Cactus_Drosoph_ 473 - - - - - - - - - - - L D D T K V S V T A - - - - - 482
IkBg_Hum_ 954 - - - - - - - K M P H D Y G Q E G P L E G K I - - 969

required for interaction with hnRNP A1 are highly conserved between all the four species and thus may constitute a binding site for hnRNP A1. The conservation within this region is much less when other members of the IκB family are observed and this may explain why we did not observe an interaction between hnRNP A1 and IκBγ in the *in vitro* assays. In summary the carboxy terminus of IκBα is highly conserved between the four different species and maybe recognised by hnRNP A1. Also the interaction between hnRNP A1 and IκBα is highly specific if compared to another ARD and acidic carboxy terminal protein of the same family, IκBγ, which is incapable of interaction.

4.10 Physiological consequences of the interactions between hnRNP A1 and IκBα.

Although the interactions between hnRNP A1 and IκBα clearly has functional consequences for NF-κB activity *in vivo*, the mechanism by which signal induced activation of NF-κB dependent transcription is potentiated has yet to be established. However it is known that 2 viral proteins, HIV-1 Rev and HCMV IE2 also interact with hnRNP A1 and have some characteristics in common with IκBα. A comparison of the properties of the three hnRNP A1 interacting proteins may therefore prove instructive.

4.11 Direct Interaction of hnRNP A1 with IκBα.

hnRNP A1 acts in synergy with the HIV-1 Rev protein to ensure stabilisation and transport of a pool of unspliced and partially spliced mRNAs. Splicing of HIV-1 mRNA must be inefficient to provide a pool of unspliced messages which encode viral proteins and genomes for new virions. hnRNPs of the A and B group are transactivating factors required for the function of the tat exon 2 exonic splicing silencer (ESS). A high affinity hnRNP A1 binding sequence can substitute functionally for the ESS in tat exon 2 and this mediates repression of splicing. Moreover the HIV-1 p17gag instability elements also carry a high affinity binding site for hnRNP A1 and a 50 kDa protein which permits the formation of a complex (Najera et al., 1999). hnRNP A1 can cooperate with Rev to promote the export of unspliced RNA from the nucleus (Najera et

al., 1999) by creating a pool of unspliced mRNA in the nucleus that is separated from the splicing machinery and can be recognised by Rev. Moreover the RNA complex exported from the nucleus contains hnRNP A1 and Rev (Najera et al., 1999).

The interaction between hnRNP A1 and IκBα takes place over the second RRM of hnRNP A1. Each RRM has been shown in Drosophila to possess slightly differing affinities for mRNA (Zu et al., 1998). Thus the interaction observed between IκBα and hnRNP A1 could block the second RRM from interaction with mRNA and thus give hnRNP A1 a slightly different affinity for newly synthesised mRNA. This may have very important implications in certain types of mRNA processing and nuclear export. Preferential binding to sub classes of mRNA in the nucleus could allow limited processing and efficient export to the cytoplasm (as for Rev) or could allow mRNA multiple processing and transport to the cytoplasm. If we consider p65 and IκBα mRNAs as examples, rapid processing and cytoplasmic synthesis of both proteins could be achieved in this manner with activating or repressive effects on transcription respectively. The hnRNP A1 knockout cell line we used was shown to be dysfunctional in NF-κB activation (Fig 23). However when the cell line was transfected with exogenous hnRNP A1 wild type or deletions which interacted with IκBα (1-207 and 95-320) the level of transcription substantially increased in comparison to non-transfected cells or cells transfected with hnRNP A1 non-interacting deletions which were unable to interact with IκBα. The interaction presented in this context would be responsible for suppressing/promoting splicing of the appropriate mRNA transcript and would be responsible for its nuclear export. In this manner hnRNP A1 nuclear function would be a chaperone/promoter of nuclear splicing and also act as a nuclear exporter of the mRNA particle.

4.12 IκBα **Nuclear Activity**

hnRNP A1 has also been shown to interact with the HCMV immediate early protein, IE2, which is a transactivator and repressor of transcription. HCMV genes are synthesised immediately following infection and can be expressed without any previous

viral protein synthesis. The best characterised IE proteins are the 72 KDa IE1 protein and the 86 KDa IE2 protein polypeptides. IE2 utilises various viral and cellular promoters and appears to function by binding to a specific target sequence in the promoter. Support for this notion was first provided by the demonstration the IE2 can negatively regulate its own transcription (Ghazal et al., 1991; Lillie et al., 1986) by binding at a cis acting repression site near the start of transcription (Chang et al., 1989; Kadonaga et al., 1988; Macias and Stinski, 1993). IE2 can also form complexes with transcription factors such as CREB, c-jun, SP1 (Hagemeier et al., 1994; Jupp et al., 1993; Sommer et al., 1994) the tumor supressor products RB, p53 (Hagemeier et al., 1994; Speir et al., 1994) and the basal transcription machinery proteins TBP and TFIIB (Hagemeier et al., 1994; Jupp et al., 1993; Sommer et al., 1994) which augments transcription from the appropriate promoters. Although the biological function of interaction between hnRNP A1 and IE2 interaction remains unresolved, the chaperone/escorting function demonstrated for hnRNP A1 and Rev could be applied with respect to the IE2 protein. In this model IE2 function as a transactivator or a repressor could be facilitated by virtue of interaction with hnRNP A1. hnRNP A1 would be responsible for escorting the IE2 protein to the correct position on the DNA and thus enable the protein to carry out its nuclear function whether activating or repressing transcription.

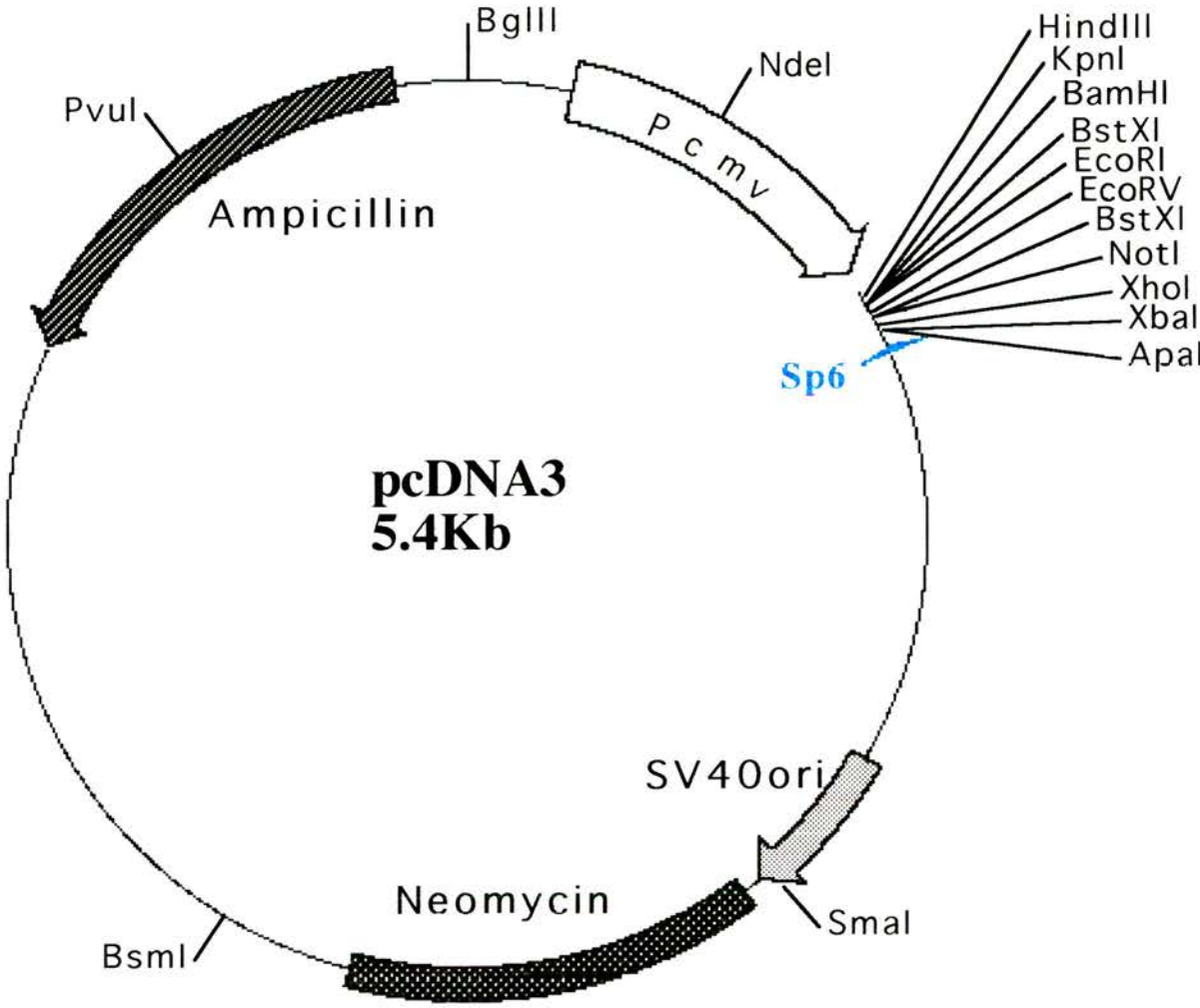
Although IκBα is a transcriptional regulator which can repress NF-κB dependent transcription it has also been shown to be capable of activating transcription in the yeast II hybrid system when fused to the Lex A DNA binding domain (Morin and Gilmore, 1992). In this sense IκBα functions similarly to the IE2 protein, which can act as a transcriptional activator and repressor, and thus may be regulated in a similar fashion to what we have postulated for IE2. IκBα in this context could also be escorted to the DNA by hnRNP A1 to carry out its function as a transcriptional regulator, whether activating or repressing transcription. The hnRNP A1 knockout cell line we used was shown to be dysfunctional in NF-κB activation. However when the cell line was transfected with exogenous hnRNP A1 wild type the level of NF-κB dependent

transcription was substantially increased, thus suggesting an important role for hnRNP A1 in the regulation of NF-κB activity.

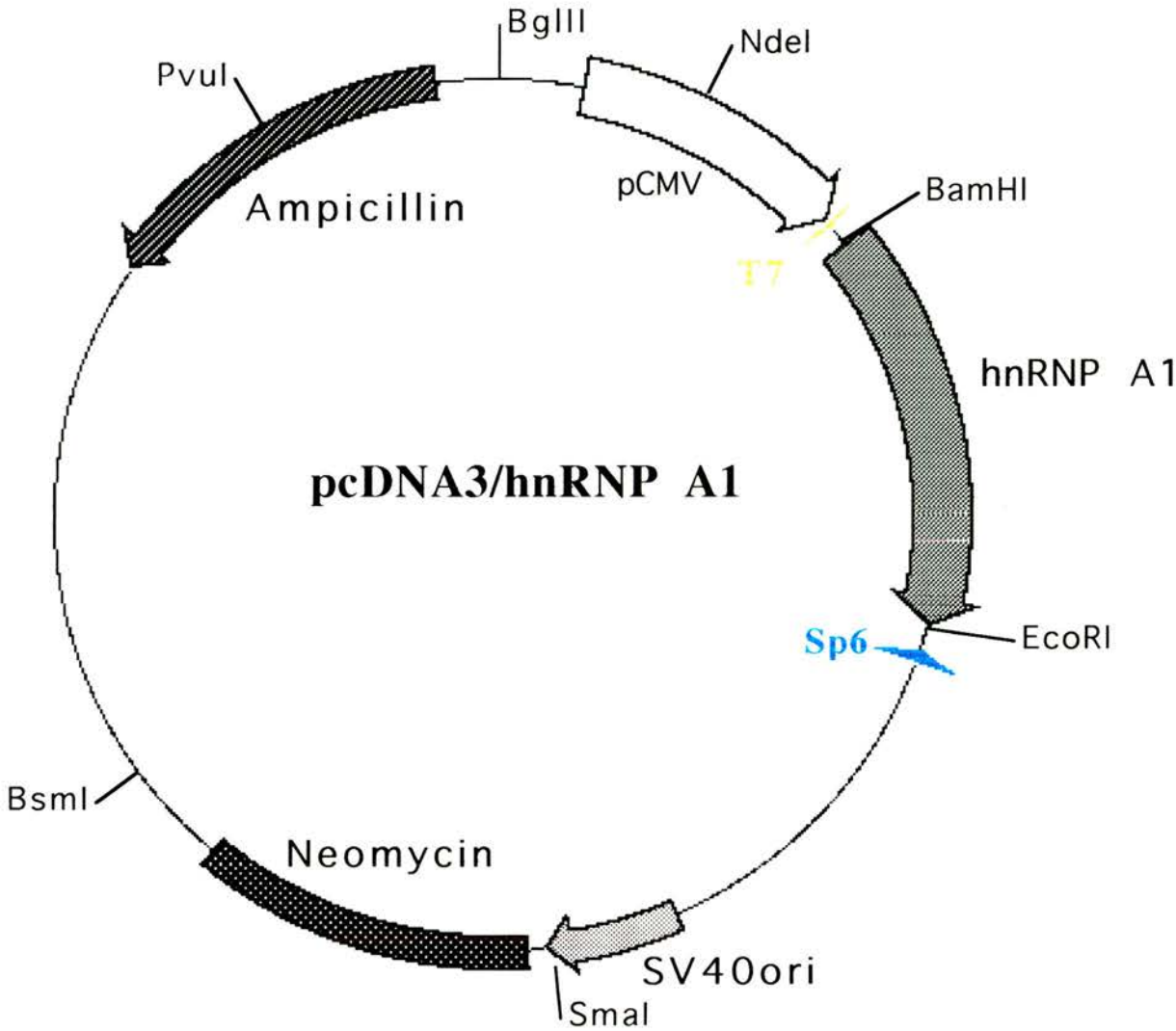
4.13 Nuclear Export of IκBα and NF-κB Complexes

In addition to the effect of hnRNP A1 on NF-κB dependent transcription we also demonstrate that hnRNP A1 associates with IκBα/NF-κB complexes *in vivo* (Figure 10) and that IκBα is exported from the nucleus in a complex with hnRNP A1 following actinomycin D treatment (Figure 21). Although the precise function of this interaction remains unresolved comparison with the hnRNP A1 and HIV-1 Rev interaction suggests that hnRNP A1 could be performing a chaperone/escort function which delivers IκBα to template bound NF-κB where IκBα fulfils its negative regulatory role. Following removal of NF-κB complexes from the DNA we also postulate that hnRNP A1 facilitates the export of IκBα/NF-κB through the M9 export pathway as well as the leucine rich nuclear export pathway. The use of 2 export pathways would allow efficient IκBα/NF-κB export to the cytoplasm and thus allow reactivation of NF-κB. This is supported by the observation that cells lacking hnRNP A1 exhibit dysfunctional NF-κB activation. In these cells the dysfunctional activation of NF-κB could be due to inefficient nuclear export of the complexes. Indeed nuclear IκBα appears to be resistant to signal induced phosphorylation and degradation, and this results in nuclear accumulation of transcriptionally inactive IκBα/NF-κB complexes (Rodriguez et al., 1999). Thus NF-κB would be retained in the nucleus for a greater time in a non-DNA binding form, also IκBα would be protected in the nucleus from degradation and thus transcription observed from the luciferase reporter would be reduced.

5. APPENDIX



Comments for pcDNA3 (5446 nucleotides)
Supplier: Invitrogen
Acession number: IG1046
CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
NeoR ORF: bases 2151-2932
SV40 poly A: bases 3120-3250
pUC19 backbone: bases 3272-5446
AmpR ORF: bases 4450-5310



CLONING OF hnRNP A1 WT AND DELETIONS

FORWARD PRIMERS

hnRNP A1 1-320 : GTCGGATCCATGTCTAAGTCAGAGTCTCCT
hnRNP A1 196-320 : AGAGGATCCATGAGTGGTTCTGGAAACTTTGGT
hnRNP A1 65-320 : ATTATATGGATCCGTGGAGGAGGTGGATGCAGCT
hnRNP A1 75-320 : ATAGGATCCATGAGGCCACACAAGGTGGAT
hnRNP A1 85-320 : TAGGATCCATGGAACCAAAGAGAGCTGTCTCC
hnRNP A1 90-320 : CGGATCCATGGTCTCCAGAGAAGATTCT
hnRNP A1 95-320 : ATAGGATCCATGTCTCAAAGACCAGGTGCC
hnRNP A1 105 -320 : GCGGGCGGATCCATGAAAAAGATATTTGTTGGTGGC
hnRNP A1 142-320 : AGTCGGATCCATGAGTGGCAAGAAAAGGGGCTTT
hnRNP A1 162-320 : GCGCCGCGGGATCCATGATTGTCATTCAGAAATACCAT
hnRNP A1 182-320 : CGGGATCCATGTCAAAGCAAGAGATGGCTAGT

REVERSE PRIMERS

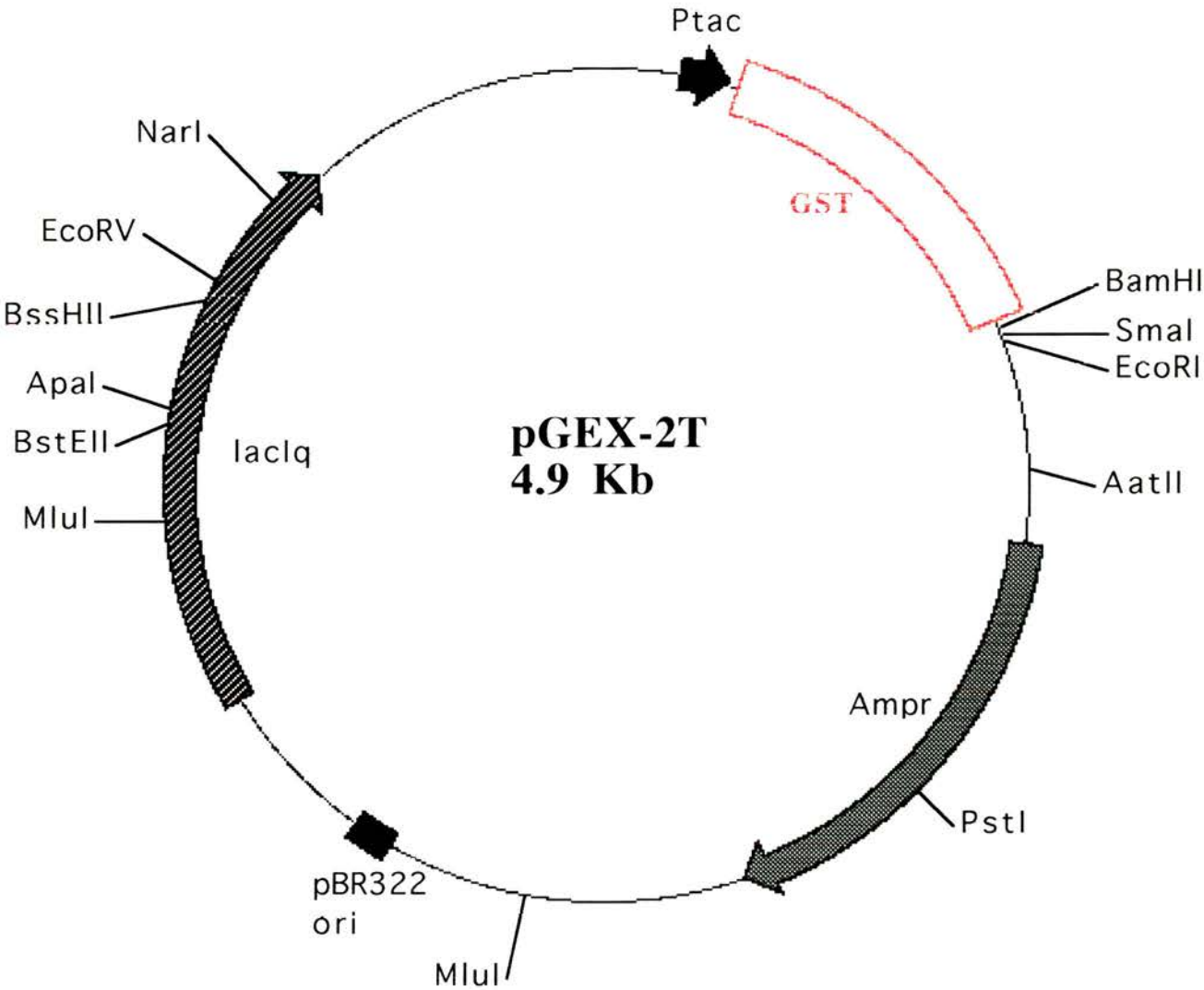
hnRNP A1 320-1 :GCCGCGAATTCTTAAAATCTTCTGCCACTGCC
hnRNP A1 267-1 :TATATTAAATTGAATTCGTTGTAATTCCCAAAATCATT
hnRNP A1 247-1 :CGCGGGAATTCAAATCCATTATAGCCATCCCC
hnRNP A1 227-1 :AGAATTCGCCACCACGACCACTGAAGTT
hnRNP A1 207-1 :CGAATTCTCCACGACCACCACCAAAGTT
hnRNP A1 196-1 :GGAATTCTCGACCTCTTTGGCTGGA

hnRNP A1:

ORGANSIM : HOMO SAPIENS
CODING REGION : 962 BP
GB ACCESSION NUMBER : X79536

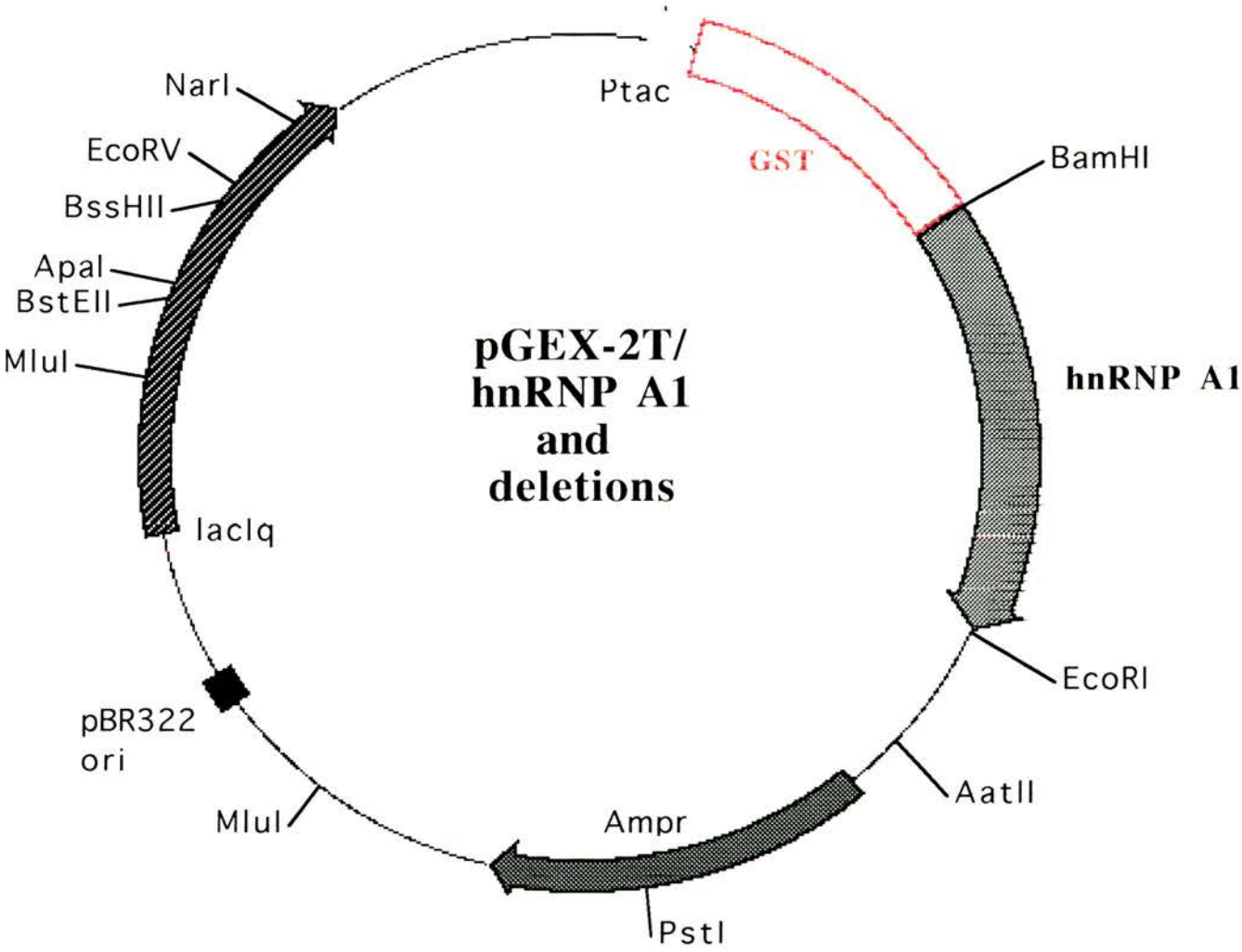
ANTIBODIES:

4B10 MONOCLONAL ANTIBODY



Comments for pGEX-2T (4948 nucleotides)
Supplier: Pharmacia
Acession number: U13850
tac promotor-10: bases205-211
tac promotor-35: bases183-188
lac operator: bases217-237
Start codon for GST: base 258
Ampr ORF: bases 1356-2214
laclq ORF: bases 3297-4377
Site of replication initiation: base 2974
Multiple Cloning Site: bases 930-945

For inducible, high level expression of genes as fusions with glutathione S-transferase (GST) gene from *Schistosoma japonicum*, which forms an affinity tail on the protein products of genes inserted into the multiple cloning site (MCS). Expression is under the control of a tac promoter which enables inducible, high-level production of fusion proteins.



CLONING OF hnRNP A1 WT AND DELETIONS

FORWARD PRIMERS

hnRNP A1 1-320 : GTCGGATCCATGTCTAAGTCAGAGTCTCCT
hnRNP A1 196-320 : AGAGGATCCATGAGTGGTTCTGGAAACTTTGGT
hnRNP A1 65-320 : ATTATATGGATCCGTGGAGGAGGTGGATGCAGCT
hnRNP A1 75-320 : ATAGGATCCATGAGGCCACACAAGGTGGAT
hnRNP A1 85-320 : TAGGATCCATGGAACCAAAGAGAGCTGTCTCC
hnRNP A1 90-320 : CGGATCCATGGTCTCCAGAGAAGATTCT
hnRNP A1 95-320 : ATAGGATCCATGTCTCAAAGACCAGGTGCC
hnRNP A1 105 -320 : GCGGGCGGATCCATGAAAAAGATATTTGTTGGTGGC
hnRNP A1 142-320 : AGTCGGATCCATGAGTGGCAAGAAAAGGGGCTTT
hnRNP A1 162-320 : GCGCCGCGGGATCCATGATTGTCATTCAGAAATACCAT
hnRNP A1 182-320 : CGGGATCCATGTCAAAGCAAGAGATGGCTAGT

REVERSE PRIMERS

hnRNP A1 320-1 :GCCGCGAATTCTTAAAATCTTCTGCCACTGCC
hnRNP A1 267-1 :TATATTAAATTGAATTCGTTGTAATTCCCAAAATCATT
hnRNP A1 247-1 :CGCGGGAATTCAAATCCATTATAGCCATCCCC
hnRNP A1 227-1 :AGAATTCGCCACCACGACCACTGAAGTT
hnRNP A1 207-1 :CGAATTCTCCACGACCACCACCAAAGTT
hnRNP A1 196-1 :GGAATTCTCGACCTCTTTGGCTGGA

hnRNP A1:

ORGANSIM : HOMO SAPIENS
CODING REGION : 962 BP
GB ACCESSION NUMBER : X79536

ANTIBODIES:

4B10 MONOCLONAL ANTIBODY

6. REFERENCES

Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C., and Venter, J. C. (1992). Sequence identification of 2,375 human brain genes. Nature *355*, 632-4.

Akey, C. W., and Goldfarb, D. S. (1989). Protein import through the nuclear pore complex is a multistep process. J Cell Biol *109*, 971-82.

Alessi, D. R., Smythe, C., and Keyse, S. M. (1993). The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in Xenopus oocyte extracts. Oncogene *8*, 2015-20.

Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A., and Ben-Neriah, Y. (1995). Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. Proceedings of National Academy of Science USA *92*, 10599-10603.

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature *387*, 49-55.

Arends, M. J., and Wyllie, A. H. (1991). Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol *32*, 223-54.

Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1995). Inducible nuclear expression of newly synthesized IκBα

negatively regulates DNA-binding and transcriptional activities of NF-κB. Molecular And Cellular Biology *15*, 2689-2696.

Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J. L., and Dargemont, C. (1997). Nuclear localization of IκBα promotes active transport of NF-kB from the nucleus to the cytoplasm. Journal of Cell Science *110*, 369-378.

Auble, D. T., Wang, D., Post, K. W., and Hahn, S. (1997). Molecular analysis of the SNF2/SWI2 protein family member MOT1, an ATP- driven enzyme that dissociates TATA-binding protein from DNA. Mol Cell Biol *17*, 4842-51.

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., and Karin, M. (1995). Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. Science *270*, 286-290.

Avis, J. M., and Clarke, P. R. (1996). Ran, a GTPase involved in nuclear processes: its regulators and effectors. J Cell Sci *109*, 2423-7.

Ayer, D. E., Lawrence, Q. A., and Eisenman, R. N. (1995). Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell *80*, 767-76.

Baeuerle, P. A., and Baltimore, D. (1996). NF-κB: ten years after. Cell *87*, 13-20.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). Skp1 Connects Cell-Cycle Regulators to the Ubiquitin Proteolysis Machinery Through a Novel Motif, the F-Box. Cell *86*, 263-274.

Bai, Y., Lee, D., Yu, T., and Chasin, L. A. (1999). Control of 3' splice site choice *in vivo* by ASF/SF2 and hnRNP A1. Nucleic Acids Res *27*, 1126-34.

Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996). Critical Role For Lysine-21 and Lysine-22 Signal-Induced, Ubiquitin- Mediated Proteolysis Of IκBα. Journal Of Biological Chemistry *271*, 376-379.

Baldwin, A. S. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annual Review of Immunology *14*, 649-683.

Bargou, R. C., Leng, C., Krappmann, D., Emmerich, F., Mapara, M. Y., Bommert, K., Royer, H.-D., Scheidereit, C., and Dorken, B. (1996). High-level nuclear NF-κB and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. Blood *87*, 4340-4347.

Barroga, C. F., Stevenson, J. K., Schwarz, E. M., and Verma, I. M. (1995). Constitutive phosphorylation of IκBα by casein kinase II. Proceedings Of The National Academy Of Sciences Of The United States Of America *92*, 7637-7641.

Bartsch, J., Truss, M., Bode, J., and Beato, M. (1996). Moderate increase in histone acetylation activates the mouse mammary tumor virus promoter and remodels its nucleosome structure. Proc Natl Acad Sci U S A *93*, 10741-6.

Beauparlant, P., and Hiscott, J. (1996). Biological and biochemical inhibitors of the NF-kappa B/Rel proteins and cytokine synthesis. Cytokine Growth Factor Rev *7*, 175-90.

Beauparlant, P., Lin, R. T., and Hiscott, J. (1996). The role of the c-terminal domain of IκBα in protein- degradation and stabilization. Journal Of Biological Chemistry *271*, 10690-10696.

Beg, A. A., and Baldwin, A. S. (1993). The IκB proteins - multifunctional regulators of rel/NF-kB transcription factors. Genes & Development *7*, 2064-2070.

Beg, A. A., Sha, W. C., Bronson, R. T., and Baltimore, D. (1995). Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα-deficient mice. Genes and Development *9*, 2735-2746.

Belich, M. P., Salmeron, A., Johnston, L. H., and Ley, S. C. (1999). TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105. Nature *397*, 363-8.

Belka, C., Wiegmann, K., Adam, D., Holland, R., Neuloh, M., Herrmann, F., Kronke, M., and Brach, M. A. (1995). Tumor necrosis factor (TNF)-alpha activates c-raf-1 kinase via the p55 TNF receptor engaging neutral sphingomyelinase. Embo J *14*, 1156-65.

Bell, S., Matthews, J. R., Jaffray, E., and Hay, R. T. (1996). IκB gamma inhibits DNA binding of NF-kappaB p50 homodimers by interacting with residues that contact DNA. Mol Cell Biol *16*, 6477-85.

Ben-David, Y., Bani, M. R., Chabot, B., De Koven, A., and Bernstein, A. (1992). Retroviral insertions downstream of the heterogeneous nuclear ribonucleoprotein A1 gene in erythroleukemia cells: evidence that A1 is not essential for cell growth. Mol Cell Biol *12*, 4449-55.

Berger, C., Brousset, P., McQuain, C., and Knecht, H. (1997). Deletion variants within the NF-kappaB activation domain of the LMP1 oncogene in acquired immunodeficiency syndrome-related large cell lymphomas, in prelymphomas and atypical lymphoproliferations. Leuk Lymphoma *26*, 239-50.

Beyer, A. L., Christensen, M. E., Walker, B. W., and LeStourgeon, W. M. (1977). Identification and characterization of the packaging proteins of core 40S hnRNP particles. Cell *11*, 127-38.

Bielinska, A., Shivdasani, R. A., Zhang, L. Q., and Nabel, G. J. (1990). Regulation of gene-expression with double-stranded phosphorothioate oligonucleotides. Science *250*, 997-1000.

Birney, E., Kumar, S., and Krainer, A. R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res *21*, 5803-16.

Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP dissociation by Ran- GTP binding protein RanBP1. Embo J *14*, 705-15.

Blair, W. S., Bogerd, H. P., Madore, S. J., and Cullen, B. R. (1994). Mutational analysis of the transcription activation domain of RelA - identification of a highly synergistic minimal acidic activation module. Molecular And Cellular Biology *14*, 7226-7234.

Blank, V., Kourilsky, P., and Israel, 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 Journal *10*, 4159-4167.

Blumberg, B. S. (1997). The current state of the prevention of HBV infection, the carrier state and hepatocellular carcinoma. Res Virol *148*, 91-4.

Boddy, M. N., Howe, K., Etkin, L. D., Solomon, E., and Freemont, P. S. (1996). Pic-1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukemia. Oncogene *13*, 971-982.

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell *85*, 803-15.

Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J Biol Chem *270*, 7795-8.

Bonner, W. M. (1975). Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J Cell Biol *64*, 431-7.

Bork, P., Holm, L., and Sander, C. (1994). The immunoglobulin fold. Structural classification, sequence patterns and common core. J Mol Biol *242*, 309-20.

Brennan, P., and O'Neill, L. A. (1998). Inhibition of NF-κB by direct modification in whole cells--mechanism of action of nordihydroguaiaritic acid, curcumin and thiol modifiers. Biochem Pharmacol *55*, 965-73.

Brenner, D. A., O'Hara, M., Angel, P., Chojkier, M., and Karin, M. (1989). Prolonged activation of jun and collagenase genes by tumour necrosis factor-alpha. Nature *337*, 661-3.

Brockman, J. A., Scherer, D. C., Mckinsey, T. A., Hall, S. M., Qi, X. X., Lee, W. Y., and Ballard, D. W. (1995). Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. Molecular And Cellular Biology *15*, 2809-2818.

Brown, A. M., Linhoff, M. W., Stein, B., Wright, K. L., Baldwin, A. S., Basta, P. V., and Ting, J. P. Y. (1994). Function of NF-κB/Rel binding sites in the major histocompatibility complex class II invariant chain promoter is dependent on cell-specific binding of different NF-κB/Rel subunits. Molecular And Cellular Biology *14*, 2926-2935.

Brown, K., Franzoso, G., Baldi, L., Carlson, L., Mills, L., Lin, Y.-C., Gerstberger, S., and Siebenlist, U. (1997). The signal response of IκBα is regulated by transferable N- and C-terminal domains. Molecular and Cellular Biology *17*, 3021-3027.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of IκBα proteolysis by site-specific, signal-induced phosphorylation. Science *267*, 1485-1488.

Buratowski, S. (1994). The basics of basal transcription by RNA polymerase II. Cell *77*, 1-3.

Buratowski, S., Hahn, S., Guarente, L., and Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA Polymerase II. Cell *56*, 549-561.

Burd, C. G., and Dreyfuss, G. (1994). RNA binding specificity of hnRNP A1: significance of hnRNP A1 high- affinity binding sites in pre-mRNA splicing. Embo J *13*, 1197-204.

Burns, L. G., and Peterson, C. L. (1997). The yeast SWI-SNF complex facilitates binding of a transcriptional activator to nucleosomal sites *in vivo*. Mol Cell Biol *17*, 4811-9.

Buvoli, M., Cobianchi, F., Bestagno, M. G., Mangiarotti, A., Bassi, M. T., Biamonti, G., and Riva, S. (1990). Alternative splicing in the human gene for the core protein A1 generates another hnRNP protein. Embo J *9*, 1229-35.

Cabannes, E., Khan, G., Aillet, F., Jarrett, R. F., and Hay, R. T. (1999). Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IκBα. Oncogene *in press*.

Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994). Regulation of alternative splicing *in vivo* by overexpression of antagonistic splicing factors. Science *265*, 1706-9.

Cai, H. N., and Levine, M. (1997). The gypsy insulator can function as a promoter-specific silencer in the Drosophila embryo. Embo J *16*, 1732-41.

Cairns, B. R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B., and Kornberg, R. D. (1996). RSC, an essential, abundant chromatin-remodeling complex. Cell *87*, 1249-60.

Cartegni, L., Maconi, M., Morandi, E., Cobianchi, F., Riva, S., and Biamonti, G. (1996). hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins. J Mol Biol *259*, 337-48.

Chang, C. P., Malone, C. L., and Stinski, M. F. (1989). A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. J Virol *63*, 281-90.

Chen, F., Lu, Y. J., Kuhn, D. C., Maki, M., Shi, X. L., Sun, S. C., and Demers, L. M. (1997). Calpain contributes to silica-induced IκBα degradation and NF-κB activation. Archives Of Biochemistry and Biophysics *342*, 383-388.

Chen, F. E., Huang, D. B., Chen, Y. Q., and Ghosh, G. (1998). Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. Nature *391*, 410-3.

Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J. E., Jr., and Kuriyan, J. (1998). Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell *93*, 827-39.

Chen, Y. Q., Ghosh, S., and Ghosh, G. (1998). A novel DNA recognition mode by the NF-κB p65 homodimer. Nat Struct Biol *5*, 67-73.

Chen, Z. J., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes & Development *9*, 1586-1597.

Cheng, Q., Cant, C. A., Moll, T., Hoferwarbinek, R., Wagner, E., Birnstiel, M. L., Bach, F. H., and De Martin, R. (1994). NF-κB subunit-specific regulation of the IκBα promoter. Journal Of Biological Chemistry *269*, 13551-13557.

Chiang, C. M., Ge, H., Wang, Z., Hoffmann, A., and Roeder, R. G. (1993). Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerases II and III. Embo J *12*, 2749-62.

Chiao, P. J., Miyamoto, S., and Verma, I. M. (1994). Autoregulation of IκBα activity. Proceedings Of The National Academy Of Sciences Of The United States Of America *91*, 28-32.

Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-55.

Chu, Z.-L., McKinsey, T. A., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. (1997). Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proceedings of National Academy of Sciences *94*, 10057-10062.

Ciechanover, A. (1996). Ubiquitin-Mediated Proteolysis and Male-Sterility. Nature Medicine *2*, 1188-1190.

Ciechanover, A., Elias, S., Heller, H., and Hershko, A. (1982). "Covalent Affinity" Purification of Ubiquitin-activating Enzyme. The Journal of Biological Chemistry *257*, 2537-2542.

Cobianchi, F., Calvio, C., Stoppini, M., Buvoli, M., and Riva, S. (1993). Phosphorylation of human hnRNP protein A1 abrogates *in vitro* strand annealing activity. Nucleic Acids Res *21*, 949-55.

Cohen, L., Henzel, W. J., and Baeuerle, P. A. (1998). IKAP is a scaffold protein of the IkappaB kinase complex. Nature *395*, 292-6.

Conaway, R. C., and Conaway, J. W. (1993). General initiation factors for RNA polymerase II. Annu Rev Biochem *62*, 161-90.

Corbett, A. H., Koepp, D. M., Schlenstedt, G., Lee, M. S., Hopper, A. K., and Silver, P. A. (1995). Rna1p, a Ran/TC4 GTPase activating protein, is required for nuclear import. J Cell Biol *130*, 1017-26.

Corces, V. G. (1995). Chromatin insulators. Keeping enhancers under control [news; comment]. Nature *376*, 462-3.

Cote, J., Peterson, C. L., and Workman, J. L. (1998). Perturbation of nucleosome core structure by the SWI/SNF complex persists after its detachment, enhancing subsequent transcription factor binding. Proc Natl Acad Sci U S A *95*, 4947-52.

Coux, O., Tanaka, K., and Goldberg, A. L. (1996). Structure and Functions Of the 20s and 26s Proteasomes. Annual Review Of Biochemistry *65*, 801-847.

Cramer, P., Larson, C. J., Verdine, G. L., and Muller, C. W. (1997). Structure of the human NF-κB p52 homodimer-DNA complex at 2.1 A resolution. EMBO Journal *16*, 7078-7090.

Dallas, P. B., Cheney, I. W., Liao, D. W., Bowrin, V., Byam, W., Pacchione, S., Kobayashi, R., Yaciuk, P., and Moran, E. (1998). p300/CREB binding protein-related protein p270 is a component of mammalian SWI/SNF complexes. Mol Cell Biol *18*, 3596-603.

De Robertis, E. M., Longthorne, R. F., and Gurdon, J. B. (1978). Intracellular migration of nuclear proteins in Xenopus oocytes. Nature *272*, 254-6.

Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999). Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science *284*, 309-13.

Deshaies, R. J. (1995). Make It or Break It - the Role Of Ubiquitin-Dependent Proteolysis In Cellular-Regulation. Trends In Cell Biology *5*, 428-434.

Desterro, J. M. P., Rodriguez, M. S., and Hay, R. T. (1998). SUMO-1 modification of IkBa inhibits NF-kB activation. Molecular Cell *2*, 233-239.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996). Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Molecular and Cellular Biology *16*, 1295-1304.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997). A cytokine-responsive I kappa B kinase that activates the transcription factor NF-kappa B. Nature *388*, 548-554.

Ding, J., Hayashi, M. K., Zhang, Y., Manche, L., Krainer, A. R., and Xu, R. M. (1999). Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA. Genes Dev *13*, 1102-15.

Dingwall, C., and Laskey, R. A. (1991). Nuclear targeting sequences--a consensus? Trends Biochem Sci *16*, 478-81.

Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K., and Matsumoto, K. (1994). MSG5, a novel protein phosphatase promotes adaptation to pheromone response in S. cerevisiae. Embo J *13*, 61-70.

Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., and Burd, C. G. (1993). hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem *62*, 289-321.

Dunaway, M., and Droge, P. (1989). Transactivation of the Xenopus rRNA gene promoter by its enhancer. Nature *341*, 657-9.

Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev *7*, 555-69.

Edwards, D. R. (1994). Cell signalling and the control of gene transcription. Trends Pharmacol Sci *15*, 239-44.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43-50.

Ernst, M. K., Dunn, L. L., and Rice, N. R. (1995). The PEST-like sequence of IκBα is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. Molecular And Cellular Biology *15*, 872-882.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-95.

Feldherr, C. M., Kallenbach, E., and Schultz, N. (1984). Movement of a karyophilic protein through the nuclear pores of oocytes. J Cell Biol *99*, 2216-22.

Feldman, R. M. R., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. Cell *91*, 221-230.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature *340*, 245-6.

Finco, T. S., and Baldwin, A. S. (1995). Mechanistic aspects of NF-kB regulation - the emerging role of phosphorylation and proteolysis. Immunity *3*, 263-272.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475-483.

Floer, M., Blobel, G., and Rexach, M. (1997). Disassembly of RanGTP-karyopherin beta complex, an intermediate in nuclear protein import. J Biol Chem *272*, 19538-46.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-60.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., Fransen, J., and Grosveld, G. (1997). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. EMBO Journal *16*, 807-816.

Franke, W. W., and Scheer, U. (1974). Pathways of nucleocytoplasmic translocation of ribonucleoproteins. Symp Soc Exp Biol *28*, 249-82.

Fridell, R. A., Bogerd, H. P., and Cullen, B. R. (1996). Nuclear export of late HIV-1 mRNAs occurs via a cellular protein export pathway. Proc Natl Acad Sci U S A *93*, 4421-4.

Fridell, R. A., Truant, R., Thorne, L., Benson, R. E., and Cullen, B. R. (1997). Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. J Cell Sci *110*, 1325-31.

Fritz, C. C., and Green, M. R. (1996). HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. Current Biology *6*, 848-854.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-11.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308-311.

Galter, D., Mihm, S., and Droge, W. (1994). Distinct effects of glutathione disulfide on the nuclear transcription factors kB and the activator protein-1. European Journal Of Biochemistry *221*, 639-648.

Ganchi, P. A., Sun, S. C., Greene, W. C., and Ballard, D. W. (1993). A novel NF-kB complex containing p65 homodimers - implications for transcriptional control at the level of subunit dimerization. Molecular And Cellular Biology *13*, 7826-7835.

Geisler, R., Bergmann, A., Hiromi, Y., and Nussleinvolhard, C. (1992). Cactus, a gene involved in dorsoventral pattern-formation of drosophila, is related to the i-kB gene family of vertebrates. Cell *71*, 613-621.

Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P. G., and Lazar, M. A. (1998). Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol *18*, 7185-91.

Ghazal, P., Young, J., Giulietti, E., DeMattei, C., Garcia, J., Gaynor, R., Stenberg, R. M., and Nelson, J. A. (1991). A discrete cis element in the human immunodeficiency virus long terminal repeat mediates synergistic trans activation by cytomegalovirus immediate-early proteins. J Virol *65*, 6735-42.

Ghosh, G., Vanduyne, G., Ghosh, S., and Sigler, P. B. (1995). Structure of NF-kB p50 homodimer bound to a κB site. Nature *373*, 303-310.

Gidoni, D., Dynan, W. S., and Tjian, R. (1984). Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature *312*, 409-13.

Gilmore, T. D., Koedood, M., Piffat, K. A., and White, D. W. (1996). Rel/NF-kappaB/IkappaB proteins and cancer. Oncogene *13*, 1367-78.

Goodbourn, S. (1990). Negative regulation of transcriptional initiation in eukaryotes. Biochim Biophys Acta *1032*, 53-77.

Goodrich, J. A., and Tjian, R. (1994). Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. Cell *77*, 145-56.

Gorlich, D. (1997). Nuclear protein import. Curr Opin Cell Biol *9*, 412-9.

Gorlich, D., Dabrowski, M., Bischoff, F. R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997). A novel class of RanGTP binding proteins. J Cell Biol *138*, 65-80.

Gorlich, D., Pante, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996). Identification of different roles for RanGDP and RanGTP in nuclear protein import. Embo J *15*, 5584-94.

Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. Nature *377*, 246-8.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature *389*, 349-52.

Gruss, H. J., Hirschstein, D., Wright, B., Ulrich, D., Caligiuri, M. A., Barcos, M., Strockbine, L., Armitage, R. J., and Dower, S. K. (1994). Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease. Blood *84*, 2305-14.

Haas, A. L., Warms, J. V. B., Hershko, A., and Rose, I. A. (1982). Ubiquitin-activating enzyme. Journal of Biological Chemistry *257*, 2543-2548.

Hagemeier, C., Caswell, R., Hayhurst, G., Sinclair, J., and Kouzarides, T. (1994). Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. Embo J *13*, 2897-903.

Hanazawa, S., Takeshita, A., Amano, S., Semba, T., Nirazuka, T., Katoh, H., and Kitano, S. (1993). Tumor necrosis factor-alpha induces expression of monocyte chemoattractant JE via fos and jun genes in clonal osteoblastic MC3T3- E1 cells. J Biol Chem *268*, 9526-32.

Hanke, T., Szawlowski, P., and Randall, R. E. (1992). Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. Journal of General Virology *73*, 653-660.

Hansen, S. K., Baeuerle, P. A., and Blasi, F. (1994). Purification, reconstitution, and I-κB association of the c-Rel p65 (RelA) complex, a strong activator of transcription. Molecular And Cellular Biology *14*, 2593-2603.

Harlow, E., and Lane, D. P. (1988). Antibodies: a laboratory manual: Cold Spring Harbor Laboratory).

Harper, S. E., Qiu, Y., and Sharp, P. A. (1996). Sin3 corepressor function in Myc-induced transcription and transformation. Proc Natl Acad Sci U S A *93*, 8536-40.

Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R. A., and Nakayama, K. (1999). Ubiquitin-dependent degradation of IkBa is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Procedings of the National Academy of Sciences of the U S A *96*, 3859-3863.

Hay, R. T., and Nicholson, J. (1993). DNA-binding alters the protease susceptibility of the p50 subunit of NF-kB. Nucleic Acids Research *21*, 4592-4598.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature *387*, 43-8.

Hengartner, M. O., and Horvitz, H. R. (1994). Programmed cell death in Caenorhabditis elegans. Curr Opin Genet Dev *4*, 581-6.

Herr, I., van Dam, H., and Angel, P. (1994). Binding of promoter-associated AP-1 is not altered during induction and subsequent repression of the c-jun promoter by TPA and UV irradiation. Carcinogenesis *15*, 1105-13.

Herrick, G., and Alberts, B. (1976). Purification and physical characterization of nucleic acid helix- unwinding proteins from calf thymus. J Biol Chem *251*, 2124-32.

Hershko, A. (1991). The ubiquitin pathway for protein degradation. Trends Biochem Sci *16*, 265-8.

Hochschild, A., and Ptashne, M. (1986). Cooperative binding of lambda repressors to sites separated by integral turns of the DNA helix. Cell *44*, 681-7.

Holcomb, E. R., and Friedman, D. L. (1984). Phosphorylation of the C-proteins of HeLa cell hnRNP particles. Involvement of a casein kinase II-type enzyme. J Biol Chem *259*, 31-40.

Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299-308.

Hsu, H., Solovyev, I., Colombero, A., Elliott, R., Kelley, M., and Boyle, W. J. (1997). ATAR, a novel tumor necrosis factor receptor family member, signals through TRAF2 and TRAF5. J Biol Chem *272*, 13471-4.

Hsu, H. L., Huang, J. N., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996). Tnf-dependent recruitment of the protein-kinase rip to the tnf receptor-1 signaling complex. Immunity *4*, 387-396.

Hsu, H. L., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495-504.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science *284*, 316-20.

Huxford, T., Huang, D. B., Malek, S., and Ghosh, G. (1998). The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. Cell *95*, 759-70.

Idriss, H., Kumar, A., Casas-Finet, J. R., Guo, H., Damuni, Z., and Wilson, S. H. (1994). Regulation of *in vitro* nucleic acid strand annealing activity of heterogeneous nuclear ribonucleoprotein protein A1 by reversible phosphorylation. Biochemistry *33*, 11382-90.

Ihle, J. N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K., Thierfelder, W. E., Kreider, B., and Silvennoinen, O. (1994). Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem Sci *19*, 222-7.

Ito, T., Bulger, M., Pazin, M. J., Kobayashi, R., and Kadonaga, J. T. (1997). ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell *90*, 145-55.

Izaurralde, E., Kutay, U., von Kobbe, C., Mattaj, I. W., and Gorlich, D. (1997). The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. Embo J *16*, 6535-47.

Izaurralde, E., Lewis, J., Gamberi, C., Jarmolowski, A., McGuigan, C., and Mattaj, I. W. (1995). A cap-binding protein complex mediating U snRNA export. Nature *376*, 709-12.

Izaurralde, E., and Mattaj, I. W. (1995). RNA export. Cell *81*, 153-9.

Jacobs, M. D., and Harrison, S. C. (1998). Structure of an IkB alpha / NF-kB complex. Cell *95*, 749-58.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-54.

Jaffray, E., Wood, K. M., and Hay, R. T. (1995). Domain organization of IκBα and sites of interaction with NF-kB p65. Molecular And Cellular Biology *15*, 2166-2172.

Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., and Smale, S. T. (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. Mol Cell Biol *14*, 116-27.

Jayaraman, P. S., Hirst, K., and Goding, C. R. (1994). The activation domain of a basic helix-loop-helix protein is masked by repressor interaction with domains distinct from that required for transcription regulation. Embo J *13*, 2192-9.

Jentsch, S., and Schlenker, S. (1995). Selective Protein-Degradation - a Journeys End Within the Proteasome. Cell *82*, 881-884.

Jiang, J., Cai, H., Zhou, Q., and Levine, M. (1993). Conversion of a dorsal-dependent silencer into an enhancer: evidence for dorsal corepressors. Embo J *12*, 3201-9.

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*, 3147-54.

Jones, K. A., Yamamoto, K. R., and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter *in vitro*. Cell *42*, 559-72.

Jupp, R., Hoffmann, S., Depto, A., Stenberg, R. M., Ghazal, P., and Nelson, J. A. (1993). Direct interaction of the human cytomegalovirus IE86 protein with the cis repression signal does not preclude TBP from binding to the TATA box. J Virol *67*, 5595-604.

Kadonaga, J. T., Courey, A. J., Ladika, J., and Tjian, R. (1988). Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science *242*, 1566-70.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499-509.

Kamitani, T., Nguyen, H. P., and Yeh, E. T. H. (1997). Preferential modification of nuclear proteins by a novel ubiquitin- like molecule. Journal Of Biological Chemistry *272*, 14001-14004.

Kang, S. M., Tran, A. C., Grilli, M., and Lenardo, M. J. (1992). NF-kB subunit regulation in nontransformed cd4+ lymphocytes-t. Science *256*, 1452-1456.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem *270*, 16483-6.

Karin, M. (1994). Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. Curr Opin Cell Biol *6*, 415-24.

Kaufmann, J., Verrijzer, C. P., Shao, J., and Smale, S. T. (1996). CIF, an essential cofactor for TFIID-dependent initiator function. Genes Dev *10*, 873-86.

Kellum, R., and Schedl, P. (1991). A position-effect assay for boundaries of higher order chromosomal domains. Cell *64*, 941-50.

Kerppola, T. K., and Kane, C. M. (1991). RNA polymerase: regulation of transcript elongation and termination. Faseb J *5*, 2833-42.

Kidd, S. (1992). Characterization of the Drosophila cactus locus and analysis of interactions between cactus and dorsal proteins. Cell *71*, 623-635.

Kiledjian, M., and Dreyfuss, G. (1992). Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. Embo J *11*, 2655-64.

Kim, S., Merrill, B. M., Rajpurohit, R., Kumar, A., Stone, K. L., Papov, V. V., Schneiders, J. M., Szer, W., Wilson, S. H., Paik, W. K., and Williams, K. R. (1997). Identification of N(G)-methylarginine residues in human heterogeneous RNP protein A1: Phe/Gly-Gly-Gly-Arg-Gly-Gly-Gly/Phe is a preferred recognition motif. Biochemistry *36*, 5185-92.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132-6.

Koepp, D. M., and Silver, P. A. (1996). A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? Cell *87*, 1-4.

Korsmeyer, S. J. (1995). Regulators of cell death. Trends Genet *11*, 101-5.

Kozutsumi, Y., Normington, K., Press, E., Slaughter, C., Sambrook, J., and Gething, M. J. (1989). Identification of immunoglobulin heavy chain binding protein as glucose-regulated protein 78 on the basis of amino acid sequence, immunological cross-reactivity, and functional activity. J Cell Sci Suppl *11*, 115-37.

Krainer, A. R., Conway, G. C., and Kozak, D. (1990). The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. Cell *62*, 35-42.

Kroll, M., Conconi, M., Desterro, M. J., Marin, A., Thomas, D., Friguet, B., Hay, R. T., Virelizier, J. L., Arenzana-Seisdedos, F., and Rodriguez, M. S. (1997). The carboxy-

terminus of IkBa determines susceptibility to degradation by the catalytic core of the proteasome. Oncogene *15*, 1841-1850.

Kroll, M., Conconi, M., Desterro, M. J. P., Marin, A., Thomas, D., Friguet, B., Hay, R. T., Virelizier, J. L., ArenzanaSeisdedos, F., and Rodriguez, M. S. (1997). The carboxy-terminus of I kappa B alpha determines susceptibility to degradation by the catalytic core of the proteasome. Oncogene *15*, 1841-1850.

Kumar, A., Casas-Finet, J. R., Luneau, C. J., Karpel, R. L., Merrill, B. M., Williams, K. R., and Wilson, S. H. (1990). Mammalian heterogeneous nuclear ribonucleoprotein A1. Nucleic acid binding properties of the COOH-terminal domain. J Biol Chem *265*, 17094-100.

Kunsch, C., Ruben, S. M., and Rosen, C. A. (1992). Selection of optimal kB/rel DNA-binding motifs - interaction of both subunits of NF-kB with DNA is required for transcriptional activation. Molecular And Cellular Biology *12*, 4412-4421.

Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., and Allis, C. D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes *in vivo*. Genes Dev *12*, 627-39.

Kuras, L., and Struhl, K. (1999). Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme. Nature *399*, 609-13.

Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, 1061-71.

Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997). Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell *89*, 349-56.

Latimer, M., Ernst, M. K., Dunn, L. L., Drutskaya, M., and Rice, N. R. (1998). The N-terminal domain of IkappaB alpha masks the nuclear localization signal(s) of p50 and c-Rel homodimers. Mol Cell Biol *18*, 2640-9.

LeBail, O., Schmidt-Ullrich, R., and Israel, A. (1993). Promoter analysis of the gene encoding the IκBα/mad3 inhibitor of NF-kB: positive regulation by members of the rel/NF-kB family. EMBO Journal *12*, 5043-5049.

Lee, F. S., Peters, R. T., Dang, L. C., and Maniatis, T. (1998). MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. Proc Natl Acad Sci U S A *95*, 9319-24.

Lernbecher, T., Muller, U., and Wirth, T. (1993). Distinct NF-kB rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature *365*, 767-770.

Li, X. Y., Virbasius, A., Zhu, X., and Green, M. R. (1999). Enhancement of TBP binding by activators and general transcription factors. Nature *399*, 605-9.

Lillie, J. W., Green, M., and Green, M. R. (1986). An adenovirus E1a protein region required for transformation and transcriptional repression. Cell *46*, 1043-51.

Lin, L., and Ghosh, S. (1996). A glycine-rich region in NF-kB p105 functions as a processing signal for the generation of the p50 subunit. Molecular and Cellular Biology *16*, 2248-2254.

Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996). Phosphorylation of IκBα in the C-terminal PEST domain by casein Kinase II affects intrinsic protein stability. Molecular and Cellular Biology *16*, 1401-1409.

Logie, C., and Peterson, C. L. (1997). Catalytic activity of the yeast SWI/SNF complex on reconstituted nucleosome arrays. Embo J *16*, 6772-82.

Lopez de Saro, F. J., Woody, A.-Y. M., and Helmann, J. D. (1995). Structural analysis of the Bacillus subtilis δ factor: a protein polyanion which displaces RNA from RNA polymerase. Journal of Molecular Biology *252*, 189-202.

Lorch, Y., Cairns, B. R., Zhang, M., and Kornberg, R. D. (1998). Activated RSC-nucleosome complex and persistently altered form of the nucleosome. Cell *94*, 29-34.

Lounsbury, K. M., and Macara, I. G. (1997). Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and karyopherin beta and reduces Ran GTPase-activating protein (RanGAP) inhibition by karyopherin beta. J Biol Chem *272*, 551-5.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-60.

Lutterbach, B., Westendorf, J. J., Linggi, B., Patten, A., Moniwa, M., Davie, J. R., Huynh, K. D., Bardwell, V. J., Lavinsky, R. M., Rosenfeld, M. G., Glass, C., Seto, E., and Hiebert, S. W. (1998). ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. Mol Cell Biol *18*, 7176-84.

Macias, M. P., and Stinski, M. F. (1993). An *in vitro* system for human cytomegalovirus immediate early 2 protein (IE2)-mediated site-dependent repression of

transcription and direct binding of IE2 to the major immediate early promoter. Proc Natl Acad Sci U S A *90*, 707-11.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. Cell *88*, 97-107.

Mahajan, R., Gerace, L., and Melchior, F. (1998). Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. Journal of Cell Biology *140*, 259-270.

Mahon, T. M., and O'Neill, L. A. J. (1995). Studies into the Effect of the tyrosine kinase inhibitor herbimycin A on NF-κB activation in T lymphocytes. Journal of Biological Chemistry *270*, 28557-28564.

Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997). MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature *385*, 540-4.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179-85.

Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R., and Hay, R. T. (1996). Inhibition of NF-kappaB DNA binding by nitric oxide. Nucleic Acids Res *24*, 2236-42.

Matthews, J. R., Nicholson, J., Jaffray, E., Kelly, S. M., Price, N. C., and Hay, R. T. (1995). Conformational-changes induced by DNA-binding of NF-κB. Nucleic Acids Research *23*, 3393-3402.

Matthews, J. R., Wakasugi, N., Virelizier, J. L., Yodoi, J., and Hay, R. T. (1992). Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. Nucleic Acids Res *20*, 3821-30.

Matthews, J. R., Watson, E., Buckley, S., and Hay, R. T. (1993). Interaction of the C-terminal region of p105 with the nuclear-localization signal of p50 is required for inhibition of NF-kB DNA-binding activity. Nucleic Acids Research *21*, 4516-4523.

Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. Journal of Cell Biology *135*, 1457-1470.

Mayeda, A., and Krainer, A. R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell *68*, 365-75.

Mayeda, A., Munroe, S. H., Caceres, J. F., and Krainer, A. R. (1994). Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. Embo J *13*, 5483-95.

Mayrand, S. H., Dwen, P., and Pederson, T. (1993). Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. Proc Natl Acad Sci U S A *90*, 7764-8.

McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996). Casein Kinase II Phosphorylates IκBα at S-283, S-289, S-293 and T-291 and is required for its degradation. Molecular and Cellular Biology *16*, 899-906.

McKeown, M. (1992). Alternative mRNA splicing. Annu Rev Cell Biol *8*, 133-55.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor [published erratum appears in J Cell Biol 1994 Jan;124(1-2):217]. J Cell Biol *123*, 1649-59.

Mellits, K. H., Hay, R. T., and Goodbourn, S. (1993). Proteolytic degradation of MAD3 (I kappa B alpha) and enhanced processing of the NF-kappa B precursor p105 are obligatory steps in the activation of NF-kappa B. Nucleic Acids Res *21*, 5059-66.

Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Motiwala, A., Zhu, H., Mann, M., and Manning, A. M. (1999). IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol Cell Biol *19*, 1526-38.

Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997). IKK-1 and IKK-2: Cytokine activated IkB kinases essential for NF-kB activation. Science *278*, 860-866.

Merrill, B. M., Stone, K. L., Cobianchi, F., Wilson, S. H., and Williams, K. R. (1988). Phenylalanines that are conserved among several RNA-binding proteins form part of a nucleic acid-binding pocket in the A1 heterogeneous nuclear ribonucleoprotein. J Biol Chem *263*, 3307-13.

Michael, W. M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal mediated, temperaturedependent nuclear protein export pathway. Cell *83*, 415-422.

Michael, W. M., Eder, P. S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. Embo J *16*, 3587-98.

Michael, W. M., Siomi, H., Choi, M., Pinol-Roma, S., Nakielny, S., Liu, Q., and Dreyfuss, G. (1995). Signal sequences that target nuclear import and nuclear export of pre- mRNA-binding proteins. Cold Spring Harb Symp Quant Biol *60*, 663-8.

Michalopoulos, I., and Hay, R. T. (1999). Role of the conserved lysine 80 in stabilisation of NF-kappaB p50 DNA binding. Nucleic Acids Res *27*, 503-9.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science *266*, 1719-23.

Miwa, M., Kushida, S., Maeda, N., Fang, J., Kawamura, T., Kameyama, T., and Uchida, K. (1997). Pathogenesis and prevention of HTLV-1-associated diseases. Leukemia *11 Suppl 3*, 65-6.

Mizuguchi, G., Tsukiyama, T., Wisniewski, J., and Wu, C. (1997). Role of nucleosome remodeling factor NURF in transcriptional activation of chromatin. Mol Cell *1*, 141-50.

Molina, C. A., Foulkes, N. S., Lalli, E., and Sassone-Corsi, P. (1993). Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell *75*, 875-86.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-3.

Moore, M. S., and Blobel, G. (1994). Purification of a Ran-interacting protein that is required for protein import into the nucleus. Proc Natl Acad Sci U S A *91*, 10212-6.

Moore, M. S., and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. Cell *69*, 939-50.

Morin, P. J., and Gilmore, T. D. (1992). The C terminus of the NF-κB p50 precursor and an IκB isoform contain transcription activation domains. Nucleic Acids Research *20*, 2453-2458.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995). Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci U S A *92*, 6532-6.

Mukhopadhyay, T., Roth, J. A., and Maxwell, S. A. (1995). Altered expression of the p50 subunit of the NF-kB transcription factor complex in non-small-cell lung-carcinoma. Oncogene *11*, 999-1003.

Muller, C. W., Rey, F. A., Sodeoka, M., Verdine, G. L., and Harrison, S. C. (1995). Structure of the NF-kB p50 homodimer bound to DNA. Nature *373*, 311-317.

Muller, S., Matunis, M. J., and Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. EMBO Journal *17*, 61-70.

Munroe, S. H., and Dong, X. F. (1992). Heterogeneous nuclear ribonucleoprotein A1 catalyzes RNA.RNA annealing. Proc Natl Acad Sci U S A *89*, 895-9.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell *85*, 817-27.

Nadler, S. G., Merrill, B. M., Roberts, W. J., Keating, K. M., Lisbin, M. J., Barnett, S. F., Wilson, S. H., and Williams, K. R. (1991). Interactions of the A1 heterogeneous nuclear ribonucleoprotein and its proteolytic derivative, UP1, with RNA and DNA: evidence for multiple RNA binding domains and salt-dependent binding mode transitions. Biochemistry *30*, 2968-76.

Najbauer, J., Johnson, B. A., Young, A. L., and Aswad, D. W. (1993). Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. J Biol Chem *268*, 10501-9.

Najera, I., Krieg, M., and Karn, J. (1999). Synergistic stimulation of HIV-1 rev-dependent export of unspliced mRNA to the cytoplasm by hnRNP A1. J Mol Biol *285*, 1951-64.

Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A *95*, 3537-42.

Nakielny, S., Fischer, U., Michael, W. M., and Dreyfuss, G. (1997). RNA transport. Annu Rev Neurosci *20*, 269-301.

Nakielny, S., Siomi, M. C., Siomi, H., Michael, W. M., Pollard, V., and Dreyfuss, G. (1996). Transportin: nuclear transport receptor of a novel nuclear protein import pathway. Exp Cell Res *229*, 261-6.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., and Sledge, G. W. (1997). Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Molecular and Cellular Biology *17*, 3629-3639.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386-9.

Naumann, M., and Scheidereit, C. (1994). Activation of NF-κB *in vivo* is regulated by multiple phosphorylations. Embo Journal *13*, 4597-4607.

Nemoto, S., DiDonato, J. A., and Lin, A. (1998). Coordinate regulation of IkappaB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-kappaB-inducing kinase. Mol Cell Biol *18*, 7336-43.

Neurath, M. F., Pettersson, S., zum Buschenfelde, K.-H. M., and Strober, W. (1996). Local administration of antiense phosphorothioate oligonucleotides to the p65 subunit of NF-κB abrogates established experimental colitis in mice. Nature Medicine *2*.

Nicholson, D. W. (1996). ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. Nat Biotechnol *14*, 297-301.

Nye, J. A., Petersen, J. M., Gunther, C. V., Jonsen, M. D., and Graves, B. J. (1992). Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev *6*, 975-90.

Oelgeschlager, T., Chiang, C. M., and Roeder, R. G. (1996). Topology and reorganization of a human TFIID-promoter complex. Nature *382*, 735-8.

Ohno, M., Fornerod, M., and Mattaj, I. W. (1998). Nucleocytoplasmic transport: the last 200 nanometers. Cell *92*, 327-36.

Osborn, L., Kunkel, S., and Nabel, G. J. (1989). Tumor necrosis factor-alpha and interleukin-1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kB. Proceedings Of The National Academy Of Sciences Of The United States Of America *86*, 2336-2340.

Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science *278*, 141-144.

Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science *278*, 141-4.

Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase [In Process Citation]. Nature *401*, 82-5.

Palacios, I., Weis, K., Klebe, C., Mattaj, I. W., and Dingwall, C. (1996). RAN/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus. J Cell Biol *133*, 485-94.

Pante, N., and Aebi, U. (1996). Sequential binding of import ligands to distinct nucleopore regions during their nuclear import. Science *273*, 1729-32.

Parry, G. C. N., and Mackman, N. (1994). A set of inducible genes expressed by activated human monocytic and endothelial-cells contain kB-like sites that specifically bind c-rel-p65 heterodimers. Journal Of Biological Chemistry *269*, 20823-20825.

Paschal, B. M., and Gerace, L. (1995). Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. J Cell Biol *129*, 925-37.

Patton, E. E., Willems, A. R., Sa, D., Kuras, L., Thomas, D., Craig, K. L., and Tyers, M. (1998). Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box proteincomplexes that regulate cell division and methionine biosynthesis in yeast [published erratum appears in Genes Dev 1998 Oct 1;12(19):3144]. Genes Dev *12*, 692-705.

Pickart, C. M., and Rose, I. A. (1985). Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl- terminal amides. J Biol Chem *260*, 7903-10.

Pinol-Roma, S., Choi, Y. D., Matunis, M. J., and Dreyfuss, G. (1988). Immunopurification of heterogeneous nuclear ribonucleoprotein particles reveals an assortment of RNA-binding proteins [published erratum appears in Genes Dev 1988 Apr;2(4):490]. Genes Dev *2*, 215-27.

Pinol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature *355*, 730-2.

Pinol-Roma, S., and Dreyfuss, G. (1991). Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. Science *253*, 312-4.

Plaksin, D., Baeuerle, P. A., and Eisenbach, L. (1993). Kbf1 (p50 NF-kB homodimer) acts as a repressor of h-2k(b) gene-expression in metastatic tumor-cells. Journal Of Experimental Medicine *177*, 1651-1662.

Platt, T. (1986). Transcription termination and the regulation of gene expression. Annu Rev Biochem *55*, 339-72.

Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F., and Dreyfuss, G. (1996). A novel receptor-mediated nuclear protein import pathway. Cell *86*, 985-94.

Pontius, B. W., and Berg, P. (1992). Rapid assembly and disassembly of complementary DNA strands through an equilibrium intermediate state mediated by A1 hnRNP protein. J Biol Chem *267*, 13815-8.

Ptashne, M., and Gann, A. (1997). Transcriptional activation by recruitment. Nature *386*, 569-77.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem *270*, 7420-6.

Razin, A., and Cedar, H. (1991). DNA methylation and gene expression. Microbiol Rev *55*, 451-8.

Reed, J. C. (1997). Double identity for proteins of the Bcl-2 family. Nature *387*, 773-6.

Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. D., and Rothe, M. (1997). Identification and characterization of an I kappa B kinase. Cell *90*, 373-383.

Reichelt, R., Holzenburg, A., Buhle, E. L., Jr., Jarnik, M., Engel, A., and Aebi, U. (1990). Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. J Cell Biol *110*, 883-94.

Reiss, Y., Heller, H., and Hershko, A. (1989). Binding sites of ubiquitin-protein ligase. Binding of ubiquitin-protein conjugates and of ubiquitin-carrier protein. J Biol Chem *264*, 10378-83.

Richards, S. A., Carey, K. L., and Macara, I. G. (1997). Requirement of guanosine triphosphate-bound ran for signal-mediated nuclear protein export. Science *276*, 1842-4.

Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell *64*, 615-23.

Rodriguez, M. S., Michalopoulos, I., Arenzana-Seisdedos, F., and Hay, R. T. (1995). Inducible degradation of I kappa B alpha *in vitro* and *in vivo* requires the acidic C-terminal domain of the protein. Mol Cell Biol *15*, 2413-9.

Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999). Nuclear retention of IκBα protects it from signal-induced degradation and inhibits NF-κB transcriptional activation. The Journal of Biological Chemistry *274*, 9108-9115.

Rodriguez, M. S., Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J. L., Hay, R. T., and Arenzana-Seisdedos, F. (1996). Identification of lysine residues

required for signal-induced ubiquitination and degradation of IkB-a *in vivo*. Oncogene *12*, 2425-2435.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem Sci *21*, 327-35.

Roff, M., Thomson, J., Rodriguez, M. S., Jacque, J.-M., Baleux, F., Arenzana-Seisdedos, F., and Hay, R. T. (1996). Role of IκBα ubiquitination in signal-induced activation of NF-κB *in vivo*. Journal of Biological Chemistry *271*, 7844-7850.

Romashkova, J. A., and Makarov, S. S. (1999). NF-kB is a target of AKT in anti-apoptotic PDGF signalling [In Process Citation]. Nature *401*, 86-90.

Roshak, A. K., Jackson, J. R., McGough, K., Chabot-Fletcher, M., Mochan, E., and Marshall, L. A. (1996). Manipulation of distinct NFκB proteins alters interleukin-1β-induced human rheumatoid synovial fibroblast prostaglandin E_2 formation. Journal of Biological Chemistry *271*, 31496-31501.

Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M., and Goeddel, D. V. (1995). The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell *83*, 1243-52.

Rothe, M., Sarma, V., Dixit, V. W., and Goeddel, D. V. (1995). TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. Science *269*, 1424-1427.

Rothe, M., Wong, S. C., Henzel, W. J., and Goeddel, D. V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kda tumor-necrosis-factor receptor. Cell *78*, 681-692.

Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998). IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature *395*, 297-300.

Rout, M. P., and Blobel, G. (1993). Isolation of the yeast nuclear pore complex. J Cell Biol *123*, 771-83.

Ryan, M. P., Jones, R., and Morse, R. H. (1998). SWI-SNF complex participation in transcriptional activation at a step subsequent to activator binding. Mol Cell Biol *18*, 1774-82.

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. Molecular And Cellular Biology *12*, 674-684.

Ryseck, R. P., Novotny, J., and Bravo, R. (1995). Characterization of elements determining the dimerization properties of RelB and p50. Molecular And Cellular Biology *15*, 3100-3109.

Sachdev, S., Hoffmann, A., and Hannink, M. (1998). Nuclear localization of IkBa is mediated by the second ankyrin repeat: the IkBa ankyrin repeats define a novel class of cis- acting nuclear import sequences. Molecular and Cellular Biology *18*, 2524-2534.

Saitoh, H., Pu, R., Cavenagh, M., and Dasso, M. (1997). RanBP2 associates with Ubc9p and a modified form of RanGAP1. Proceedings of the National Academy of Sciences of the United States of America *94*, 3736-3741.

Santoro, C., Mermod, N., Andrews, P. C., and Tjian, R. (1988). A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature *334*, 218-24.

Sato, T., Irie, S., and Reed, J. C. (1995). A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. FEBS Lett *358*, 113-8.

Schaefer, T. S., Sanders, L. K., and Nathans, D. (1995). Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. Proc Natl Acad Sci U S A *92*, 9097-101.

Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., and Baldwin, A. S. (1995). Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science *270*, 283-286.

Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995). Signal-induced degradation of IκBα requires site-specific ubiquitination. Proceedings of National Academy of Science USA *92*, 11259-11263.

Schlenstedt, G., Saavedra, C., Loeb, J. D., Cole, C. N., and Silver, P. A. (1995). The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)+ RNA in the cytoplasm. Proc Natl Acad Sci U S A *92*, 225-9.

Schmidt, K. N., Amstad, P., Cerutti, P., and Baeuerle, P. A. (1996). Identification of hydrogen peroxide as the relevant messenger in the activation pathway of transcription factor NF-kappaB. Adv Exp Med Biol *387*, 63-8.

Schmitz, M. L., Silva, M. A. D., and Baeuerle, P. A. (1995). Transactivation domain-2 (TA2) of p65 NF-κB: Similarlty to TA1 and phorbol ester-stimulated activity and phosphorylation in intact cells. Journal Of Biological Chemistry *270*, 15576-15584.

Schmitz, M. L., Silva, M. A. D. S., Altmann, H., Czisch, M., Holak, T. A., and Baeuerle, P. A. (1994). Structural and functional-analysis of the NF-κB p65 c-terminus - an acidic and modular transactivation domain with the potential to adopt an α-helical conformation. Journal Of Biological Chemistry *269*, 25613-25620.

Schnitzler, G., Sif, S., and Kingston, R. E. (1998). Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state. Cell *94*, 17-27.

Schulzeosthoff, K., Los, M., and Baeuerle, P. A. (1995). Redox signaling by transcription factors NF-kB and ap-1 in lymphocytes. Biochemical Pharmacology *50*, 735-741.

Schwarz, E. M., van Antwerp, D., and Verma, I. M. (1996). Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. Molecular and Cellular Biology *16*, 3554-3559.

Sen, R., and Baltimore, D. (1986). Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell *47*, 921-928.

Shamoo, Y., Krueger, U., Rice, L. M., Williams, K. R., and Steitz, T. A. (1997). Crystal structure of the two RNA binding domains of human hnRNP A1 at 1.75 A resolution. Nat Struct Biol *4*, 215-22.

Shen, E. C., Henry, M. F., Weiss, V. H., Valentini, S. R., Silver, P. A., and Lee, M. S. (1998). Arginine methylation facilitates the nuclear export of hnRNP proteins. Genes Dev *12*, 679-91.

Shen, Z., Pardington-Purtymun, P. E., Comeaux, J. C., Moyzis, R. K., and Chen, D. J. (1996). UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. Genomics *36*, 271-9.

Siomi, H., and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. J Cell Biol *129*, 551-60.

Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209-219.

Smale, S. T., and Baltimore, D. (1989). The "initiator" as a transcription control element. Cell *57*, 103-13.

Smale, S. T., Schmidt, M. C., Berk, A. J., and Baltimore, D. (1990). Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc Natl Acad Sci U S A *87*, 4509-13.

Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959-62.

Smith, C. W., Patton, J. G., and Nadal-Ginard, B. (1989). Alternative splicing in the control of gene expression. Annu Rev Genet *23*, 527-77.

Sommer, M. H., Scully, A. L., and Spector, D. H. (1994). Transactivation by the human cytomegalovirus IE2 86-kilodalton protein requires a domain that binds to both the TATA box-binding protein and the retinoblastoma protein. J Virol *68*, 6223-31.

Song, H. Y., and Donner, D. B. (1995). Association of a RING finger protein with the cytoplasmic domain of the human type-2 tumour necrosis factor receptor. Biochem J *309*, 825-9.

Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. (1997). Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J Clin Invest *100*, 2952-60.

Speir, E., Modali, R., Huang, E. S., Leon, M. B., Shawl, F., Finkel, T., and Epstein, S. E. (1994). Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science *265*, 391-4.

Spencer, E., Jiang, J., and Chen, Z. J. (1999). Signal-induced ubiquitination of IkBa by the F-box protein Slimb/b-TrCP. Genes and Development *13*, 284-294.

Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041-50.

Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995). RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell *81*, 513-23.

Sternsdorf, T., Jensen, K., and Will, H. (1997). Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. Journal of Cell Biology *139*, 1621-1634.

Sweet, D. J., and Gerace, L. (1996). A GTPase distinct from Ran is involved in nuclear protein import. J Cell Biol *133*, 971-83.

Sylla, B. S., Hung, S. C., Davidson, D. M., Hatzivassiliou, E., Malinin, N. L., Wallach, D., Gilmore, T. D., Kieff, E., and Mosialos, G. (1998). Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF-kappaB through a pathway that includes the NF-kappaB-inducing kinase and the IkappaB kinases IKKalpha and IKKbeta. Proc Natl Acad Sci U S A *95*, 10106-11.

Takahashi, A., and Earnshaw, W. C. (1996). ICE-related proteases in apoptosis. Curr Opin Genet Dev *6*, 50-5.

Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999). Limb and skin abnormalities in mice lacking IKKalpha. Science *284*, 313-6.

Tartaglia, L. A., Pennica, D., and Goeddel, D. V. (1993). Ligand passing - the 75-kda tumor-necrosis-factor (tnf) receptor recruits tnf for signaling by the 55-kda tnf receptor. Journal Of Biological Chemistry *268*, 18542-18548.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-62.

Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995). IκB-β regulates the persistent response in a biphasic activation of NF-κB. Cell *80*, 573-582.

Traenckner, E. B. M., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995). Phosphorylation of human IκBα on serine 32 and serine 36 controls IκBα proteolysis and NF-κB activation in response to diverse stimuli. EMBO Journal *14*, 2876-2883.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr Opin Genet Dev *4*, 96-101.

Turpin, P., Hay, R. T., and Dargemont, C. (1999). Characterisation of the IκBα nuclear import pathway. The Journal of Biological Chemistry *274*, 6804-6812.

Unlap, M. T., and Jope, R. S. (1997). Dexamethasone attenuates NF-kappa B DNA binding activity without inducing I kappa B levels in rat brain *in vivo*. Brain Res Mol Brain Res *45*, 83-9.

VanArsdale, T. L., and Ware, C. F. (1994). TNF receptor signal transduction. Ligand-dependent stimulation of a serine protein kinase activity associated with (CD120a) TNFR60. J Immunol *153*, 3043-50.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes and Development *9*, 2723-2735.

Verrijzer, C. P., Yokomori, K., Chen, J. L., and Tjian, R. (1994). Drosophila TAFII150: similarity to yeast gene TSM-1 and specific binding to core promoter DNA. Science *264*, 933-41.

Vettese-Dadey, M., Grant, P. A., Hebbes, T. R., Crane- Robinson, C., Allis, C. D., and Workman, J. L. (1996). Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA *in vitro*. Embo J *15*, 2508-18.

Visa, N., Alzhanova-Ericsson, A. T., Sun, X., Kiseleva, E., Bjorkroth, B., Wurtz, T., and Daneholt, B. (1996). A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. Cell *84*, 253-64.

Vuillard, L., Nicholson, J., and Hay, R. T. (1999). A complex containing b-TrCP recruits Cdc34 to catalyse ubiquitination of IkBa. FEBS Letters *455*, 311-314.

Walia, H., Chen, H. Y., Sun, J. M., Holth, L. T., and Davie, J. R. (1998). Histone acetylation is required to maintain the unfolded nucleosome structure associated with transcribing DNA. J Biol Chem *273*, 14516-22.

Wallach, D. (1986). Cytotoxins (tumour necrosis factor, lymphotoxin and others): molecular and functional characteristics and interactions with interferons. Interferon *7*, 89-124.

Wallach, D. (1984). Preparations of lymphotoxin induce resistance to their own cytotoxic effect. J Immunol *132*, 2464-9.

Walters, M. C., Magis, W., Fiering, S., Eidemiller, J., Scalzo, D., Groudine, M., and Martin, D. I. (1996). Transcriptional enhancers act in cis to suppress position-effect variegation. Genes Dev *10*, 185-95.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680-3.

Wang, D., and Baldwin, A. S., Jr. (1998). Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. J Biol Chem *273*, 29411-6.

Wang, Y. F., Chen, S. C., Wu, F. Y., and Wu, C. W. (1997). The interaction between human cytomegalovirus immediate-early gene 2 (IE2) protein and heterogeneous ribonucleoprotein A1. Biochem Biophys Res Commun *232*, 590-4.

Wasylyk, B., Hahn, S. L., and Giovane, A. (1993). The Ets family of transcription factors. Eur J Biochem *211*, 7-18.

Watanabe, N., Iwamura, T., Shinoda, T., and Fujita, T. (1997). Regulation of NFKB1 proteins by the candidate oncoprotein BCL-3: generation of NF-κB homodimers from the cytoplasmic pool of p50-p105 and nuclear translocation. EMBO Journal *16*, 3609-3620.

Weighardt, F., Biamonti, G., and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. J Cell Sci *108*, 545-55.

Weinmann, R. (1992). The basic RNA polymerase II transcriptional machinery. Gene Expr *2*, 81-91.

Weis, L., and Reinberg, D. (1992). Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. Faseb J *6*, 3300-9.

Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell *82*, 463-473.

Whiteside, S. T., Epinat, J.-C., Rice, N. R., and Israel, A. (1997). IκB epsilon, a novel member of the IκB family, controls RelA and cRel NF-κB activity. EMBO Journal *16*, 1413-1426.

Whiteside, S. T., Ernst, M. K., Lebail, O., Laurent-Winter, C., Rice, N., and Israel, A. (1995). N- and C-terminal sequences control degradation of MAD3/IκBα in response to inducers of NF-κB activity. Molecular And Cellular Biology *15*, 5339-5345.

Wiegmann, K., Schutze, S., Machleidt, T., Witte, D., and Kronke, M. (1994). Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell *78*, 1005-15.

Wilkinson, K. D., Urban, M. K., and Haas, A. L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. J Biol Chem *255*, 7529-32.

Williams, K. R., Stone, K. L., LoPresti, M. B., Merrill, B. M., and Planck, S. R. (1985). Amino acid sequence of the UP1 calf thymus helix-destabilizing protein and its homology to an analogous protein from mouse myeloma. Proc Natl Acad Sci U S A *82*, 5666-70.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCF β-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkBa and b-catenin and stimulates IkBa ubiquitination *in vitro*. Genes and Development *13*, 270-283.

Wood, K. M., Roff, M., and Hay, R. T. (1998). Defective IkappaBalpha in Hodgkin cell lines with constitutively active NF-kB. Oncogene *16*, 2131-9.

Workman, J. L., and Kingston, R. E. (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu Rev Biochem *67*, 545-79.

Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997). I kappa B kinase-beta: NF-kappa B activation and complex formation with I kappa B kinase-alpha and NIK. Science *278*, 866-869.

Xu, C. S., Fracella, F., RichterLandsberg, C., and Rensing, L. (1997). Stress response of lysosomal cysteine proteinases in rat C6 glioma cells. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology *117*, 169-178.

Xu, R. M., Jokhan, L., Cheng, X., Mayeda, A., and Krainer, A. R. (1997). Crystal structure of human UP1, the domain of hnRNP A1 that contains two RNA-recognition motifs. Structure *5*, 559-70.

Yamamoto, K. R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet *19*, 209-52.

Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998). Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kB activation. Cell *93*, 1231-40.

Yang, X., Bani, M. R., Lu, S. J., Rowan, S., Ben-David, Y., and Chabot, B. (1994). The A1 and A1B proteins of heterogeneous nuclear ribonucleoparticles modulate 5' splice site selection *in vivo*. Proc Natl Acad Sci U S A *91*, 6924-8.

Yap, N., Yu, C. L., and Cheng, S. Y. (1996). Modulation of the transcriptional activity of thyroid hormone receptors by the tumor suppressor p53. Proc Natl Acad Sci U S A *93*, 4273-7.

Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mercurio, F., Manning, A. M., Ciechanover, A., and BenNeriah, Y. (1997). Inhibition of NF-kappa B

cellular function via specific targeting of the I kappa B-ubiquitin ligase. Embo Journal *16*, 6486-6494.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998). Identification of the receptor component of the IkBa-ubiquitin ligase. Nature *396*, 590-594.

Yin, M. J., Christerson, L. B., Yamamoto, Y., Kwak, Y. T., Xu, S., Mercurio, F., Barbosa, M., Cobb, M. H., and Gaynor, R. B. (1998). HTLV-I Tax protein binds to MEKK1 to stimulate IkappaB kinase activity and NF-kappaB activation. Cell *93*, 875-84.

Zandi, E., Chen, Y., and Karin, M. (1998). Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science *281*, 1360-3.

Zawel, L., and Reinberg, D. (1995). Common themes in assembly and function of eukaryotic transcription complexes. Annu Rev Biochem *64*, 533-61.

Zawel, L., and Reinberg, D. (1993). Initiation of transcription by rna-polymerase-ii - a multistep process. Progress In Nucleic Acid Research And Molecular Biology *44*, 67-108.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB- associated PKAc subunit through a cyclic AMP-independent mechanism. Cell *89*, 413-24.

Zhou, P., Sun, L. J., Dotsch, V., Wagner, G., and Verdine, G. L. (1998). Solution structure of the core NFATC1/DNA complex. Cell *92*, 687-96.

Zhou, Q., Lieberman, P. M., Boyer, T. G., and Berk, A. J. (1992). Holo-TFIID supports transcriptional stimulation by diverse activators and from a TATA-less promoter. Genes Dev *6*, 1964-74.

Zu, K., Sikes, M. L., and Beyer, A. L. (1998). Separable roles *in vivo* for the two RNA binding domains of Drosophila A1-hnRNP homolog. Rna *4*, 1585-98.