PURIFICATION AND CHARACTERISATION OF DNA BINDING PROTEINS WHICH EXHIBIT CONFORMATIONAL SPECIFICITY TO NON "B" DNA STRUCTURES.

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This thesis is submitted for the degree of Master of Science.

DECLARATION.

I, Ian R. Leith , hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been acccepted in partial or complete fulfilment of any other degree or professional qualification.

signed date 17/4/92

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ACKNOWLEDGEMENTS.

I would like to thank my supervisor, Professor W.C. Russell, for his guidance throughout the course of this work, and for his encouragement and advice during the production of my thesis. I am also indebted to Dr R.T.Hay for his enthusiasm and willingness to give helpful discussion and to Mr W.Blyth for photographic services.

Dedication.

To Jean.

PUBLICATION RESULTING FROM THIS WORK.

Leith I.R. Hay R.T. Russell W.C. 1988 Nucleic Acids Research 16 : 8277-8289

A further publication is in preparation.

Abbreviations.

A_{280}.....absorbance at 280nm.
A.....adenine or adenosine.
Å.....angstrong.
AAF.....acetylamminofluorin.
APT.....amminophenylthioether.
araC.....arbinosylcytosine.
ATP.....adenosine 5' triphosphate.
BBA.....bromoacetaldehyde.
BDE.....butandiol diglycidylether.
bp.....base pair.
BSA.....bovine serum albumin.
C.....cytosine or cytidine.
CAA.....chloroacetaldehyd.
CAT.....chloramphenicol acetyltransferase.
CD.....circular dichroism.
Ci.....curi.
CNBr.....cyanogenbromide.
CPG.....controlled pore glass.
cpm.....counts per minute.
DAPI.....4'6 diamidino-2-phenylindole.
DEPC.....diethylpyrocarbonate.
DES.....diethylsulphate.
d( ).....deoxyribose nucleotide.
DMS.....dimethyl sulphate.
DMTO.....dimethyloxytrityl.
DNA.....deoxyribonucleic acid.
DNase.....deoyribonuclease.
DTT.....dithiothreitol.
EDTA.....ethylenediaminetetraacetic acid.
EM.....electron micrscoscopy.
FPLC.....fast protein (peptide & polynucleotide) liquid chromatography.
g.....gravity.
G.....guanine or guanosine.
HEPES.....N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
IgG.....immunoglobulin G.

k.....thousand.
kb.....kilobases.
M.....molar.
mRNA.....messenger ribonucleic acid.
NFkB.....Nuclear Factor kappa B.
NF I.....Nuclear Factor I.
NMR.....nuclear magnetic resonance.
NOE.....nuclear overhauser effect.
oligo.....oligonucleotide.
p.....phosphate.
PAGE.....polyacrylamide gel electrophoresis.
PBS.....phosphate buffered saline.
PMSF.....phenylmethylsulfonyl fluoride.
RNA.....ribonucleic acid.
SDS.....sodium dodecyl sulfate.
SV.....simian virus.
T.....thymine or thymidine.
TBE.....Tris/borate electrophoresis buffer.
TCA.....trichloracetic acid.
TE.....Tris/EDTA buffer.
TEMED.....N,N,N',N' tetramethylethylenediamine.
TK.....thymidine kinase.
Tris.....tris(hydroxymethyl)aminomethane.
UV.....ultraviolet.

CONTENTS.

Results.

Abstract.

Considerable evidence now supports the concept that DNA is a dynamic molecule in which there is an equilibrium between different conformations. The equilibrium between righthanded "B" DNA and lefthanded "Z" DNA being one component. An equilibrium is strongly influenced by nucleotide sequence, ionic strength, negative superhelicity and protein environment.

The hypothesis that proteins which bind DNA are important factors in determining stabilisation and distribution of conformation is the basis of this study. Proteins are found to exist within cells which recognise and specifically bind to irregular DNA structures which have been induced by metabolic processes. The sequence of nucleotides determines the position and extent of these DNA structural changes and therefore is regarded as a form of conformational information in DNA in contrast to the more familiar coding sequence information.

A simple, fast and efficient method of detecting protein which binds to altered DNA structure (specifically "Z" DNA) has been established. Using this detection assay system proteins have been identified and isolated by ion exchange and DNA affinity chromatography from HeLa cell extracts that preferentially bind to non "B" DNA structures (particularly "Z" DNA). The purified protein fractions contain polypeptides with apparent molecular weights of 85, 68, 60 and 55k, with the 85 and 60k polypeptides being the major component. DNA binding affinity is stimulated by an altered DNA conformation. Competition studies demonstrate that these proteins do not bind single stranded DNA or RNA while the presence of excess carrier DNA eliminates any "B" DNA binding. In addition the recent proposal that "Z" DNA binding proteins are in fact phospholipid binding proteins has been shown to be incorrect.

Using this assay system the eukaryotic transcription factor NFkB is shown to have conformational specificity in addition to its DNA binding sequence recognition. The eukaryotic topoisomerase type I enzyme has also been shown to posses conformational specificity. This provides evidence that DNA binding proteins do

not all completely rely on specific recognition of nucleotide sequence with many interacting in a nonspecific but sequence dependent manner. These proteins read sequence information translated into structural microheterogeneity. It is important to note that many binding proteins will require a specific DNA sequence as well as conformational alterations to successfully make contact with the necessary helix components.

Introduction.

Background.

Over the past 25 years it has become increasingly clear that the DNA double helix has considerable conformational flexibility far beyond the original righthanded structure proposed by Watson and Crick (1953). In a fully hydrated low ionic strength medium, DNA is thought to exist predominately in what is termed "B" form (Langridge et al 1960). However controversy over DNAs ability to form structures other than the canonical Watson-Crick helix has raged for many years. In the past many long regarded the DNA duplex as having a very regular relatively stiff helical secondary structure, independent of nucleotide sequence and suggested that when tighter twists were required the helices would have to be kinked or broken (Wilkins 1956, Crick 1974, Crick & Klug 1975). However others held the view that DNA could assume different allimorphic forms allowing a greater affinity for specific DNA binding proteins but still held a relatively rigid molecule with the majority being in a "B" like conformation with only special sequences in an alternative conformation (Klug et al 1979). A similar theory was proposed by Arnott (1976) where DNA has one predominant conformation with many different short lived local conformations occurring along the molecule. In such an environment a sequence of nucleotides within the binding site of a protein might be induced to have a non "B" structure that would provide a unique tight binding site. Local changes in DNA secondary structure might also have an effect on more distant sites along the molecule. More recent studies have confirmed the existence of many different DNA conformations which may be induced by various environments depending on the base sequence.

Physical Evidence.

The early structural work on DNA involved X-ray diffraction studies on DNA fibres, the first pattern of an orientated DNA was obtained in the 1930s (Olby 1974) but the resolution was poor. Improved versions of the pattern were

not achieved until 1953 (Wilkins et al 1953). Interpretation of these patterns led to information which was used to propose a molecular model for DNA (Watson & Crick 1953, Crick & Watson 1954) known as the "B" allomorph. The "A" allomorph of the DNA double helix was also resolved in 1953 (Franklin & Gosling 1953) but neglected until the 1960s. The early DNA fibre diffraction studies however, led only to limited information on the general organisation of the sugar phosphate chains and the number of bases per helix turn. The technique provided only small amounts of data, due to the molecules being disordered to varying degrees, making interpretations difficult and atoms not being solved. Information on the averaged DNA conformation is all that could be achieved, local variations in the DNA induced by a particular nucleotide sequence could not be detected. The intimate structure of double helical DNA was only revealed on devising a method which could crystallise DNA fragments of fixed sequences and solve their three dimensional structure at atomic resolution. This development coincided with advances in organic synthetic nucleotide chemistry during the mid 1970s that could produce oligonucleotides of defined sequence in quantities sufficient for crystallisation. Single crystals diffract X-rays at or near atomic resolution (~1Å), solution of these crystal structures providing a wealth of detail on the geometry of individual components in contrast to the results of DNA fibre X-ray diffraction analysis which do not solve an unknown structure. The first visualisation of a double helix at atomic resolution was with dinucleoside monophosphates showing two righthanded base paired RNA fragments (Rosenberg et al 1973; Day et al 1973). In later studies it became evident that the structure of DNA clearly depends on its nucleotide sequence and has the ability to form unusual structures other than the canonical Watson and Crick double helix. (Viswamitra et al 1978, Dickerson & Drew 1981, Wang et al 1979 /82, McCall et al 1985, Nelson et al 1987).

Fibre X-ray studies proved useful after definition of the DNA conformation by single crystal analysis and it is important to note that the initial evidence suggesting

different DNA sequences could exist in different conformations and have varying properties were X-ray diffraction studies on DNA polymers (Marvin et al 1961, Davies & Baldwin 1963, Arnott et al 1974, Mitsui et al 1970).

Diffraction patterns of "B" DNA have been found in a variety of biological DNA sources, but the nature of X-ray diffraction from noncrystalline sources is such that it will only pick up highly repetitive elements of structure. Elements which are not repetitive will not register in the pattern. Thus although there is no doubt "B" DNA is present in a sample the technique will fail to see other conformations that are not in a regular periodic pattern.

DNA Secondary Structural Allomorphs.

Since the original structural hypothesis of DNA (Watson & Crick 1953) many different polymorphisms have been identified. Given the large number of degrees of freedom in the polynucleotide backbone with five rotatable single bonds and alternative sugar puckers and also the rotational freedom of the purine bases around the glycosidic bonds, it is not surprising alternative structures from the classic "B" duplex exist. Variation in "B" DNA involves the closing and opening of the minor groove of the duplex and changing the rotations by as much as 9° (Dickerson 1983). RNA double helices have yet to be found in the "B" form but RNA/RNA, RNA/DNA and DNA/DNA duplexes are all found in a variety of "A" forms. The morphological changes involved in this variability are more in the width of the major groove.

Table 1. summarises some of the various helix types known to exist within particular sequences and environments. These helices have distinctly different shapes that are due to the specific orientation and positioning of the bases with respect to the helix axis.

Table 1 DNA Helix Structure

Helix type	bp/turn	Rotation/bp	Vertical rise/bp	~Diameter
A	11.0	32.7°	2.56Å	23Å
B	10.0	36.0°	3.38Å	19Å
C	9.3	38.6°	3.32Å	19Å
D	8.0	45.0°	3.04Å	-
E	7.5	48.0°	3.25Å	-
Z	12	-30.0°	3.71Å	18Å

(Leslie et al 1980, Drew et al 1980)

Considerable variation is observed within these nominal helical structures. The range of conformational flexibility is most apparent when you consider that "A" DNA is 25% fatter than "Z" DNA, that 10bp of "Z" DNA is nearly 1.5 times longer than of "A" DNA and whilst 10bp in "Z" DNA is -0.83 of a helical turn it is 1.33 helical turns in "E" DNA. The lefthanded "Z" helix is quite unique in structure contrasting all the other helices, which are righthanded, as well as being the least twisted, thinnest conformation. Its existence is surprising to many biologists.

DNA with "B" like secondary structures may be subtly wrinkled or ruffled on their surfaces in ways which indicate the nature of the internal base sequence. "B" DNAs with alternating GC or AT sequences have structures which superficially resemble the general sequence but incorporate purine/pyrimidine nucleotides with altered conformation at C3'- O3' positions and consequently much changed phosphate group orientations (Arnott et al 1983). DNA in chromosomes may therefore have surface features signalling the presence of special internal base sequences.

Consideration of how these various helical forms are favoured by particular sequences and environments has been to the fore in studies of the physical chemistry of DNA for many years. On accepting their existence it is possible to

envisage that a living system may recognise and utilise this conformational flexibility in a biological role. Since the discovery of "Z" DNA a central and controversial question still remains : to what extent does "Z" DNA exist in vivo and what biological function does it have?

Lefthanded ("Z") DNA.

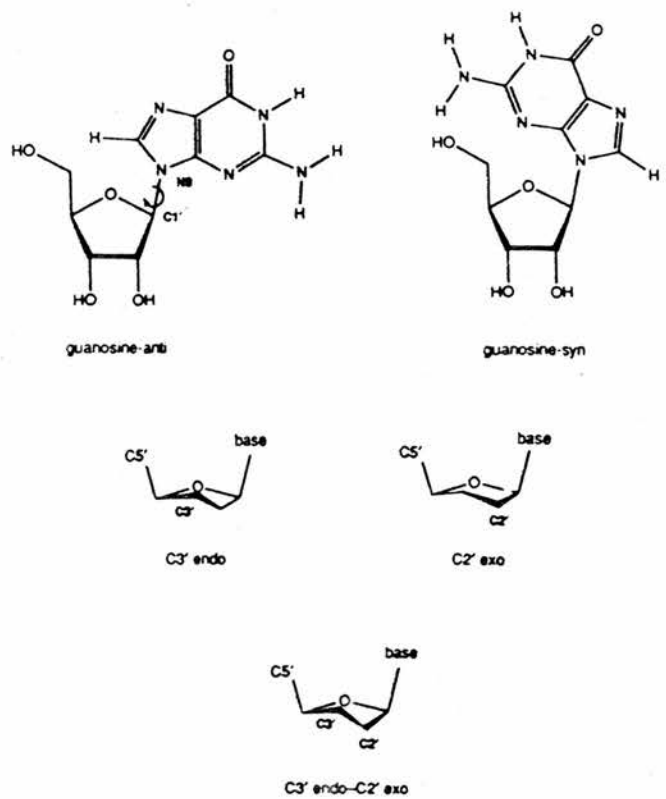
The possible existence of a novel form of double stranded helical DNA was suggested by the observation that the Circular Dichroism (C.D.) spectrum of the polymer Poly (dG-dC).Poly (dG-dC) undergoes an inversion when the salt concentration in solution is raised to 4M NaCl (Pohl and Jovin 1972). This was confirmed when the structure of a left-handed double helix was solved using a crystalline hexanucleoside pentaphosphate fragment with the sequence d(CpGpCpGpCpG) (Wang et al 1979). The crystal diffracted to 0.9Å and its solution produced a large amount of detail on the DNA molecule as well as the organisation of the many associated water molecules and ions. Raman spectroscopy proved that the spectra and therefore structure of the crystallised hexamer and the polymer Poly (dG-dC).Poly (dG-dC) in high salt were the same (Thamann et al 1981); while in low salt both produce a different spectrum (Pohl et al 1973) [existing in "B" form at low salt]. Subsequent studies using X-ray crystallography and Nuclear Magnetic Resonance (NMR) confirmed that a left-handed DNA helix existed (Drew et al 1980, Crawford et al 1981, Wang et al 1981, Fujii et al 1982, Patel et al 1982). Mitsui et al 1970 also suggested a novel DNA helical structure with the polymer Poly (dI-dC).Poly (dI-dC) showing unusual C.D. and fibre X-ray diffraction properties. However later vacuum ultra violet Circular Dichroism studies pointed to this being a right-handed conformation (Sutherland & Griffin 1983). In solution there exists an equilibrium in which both left-handed and right-handed DNA are represented. The equilibrium constant is determined by many factors including base sequence and ionic strength. The crystals of the left-handed DNA fragment studied by Wang et al 1979 were formed

in a solution that had a low concentration of cations so that the majority species in the solution was "B" form. However as these crystals grew the equilibrium shifted until all of the material was left-handed inside the crystal lattice. It is possible that spermine, present in the solution, may have facilitated this conversion (Russell et al 1983) although later studies (Wang et al 1981) indicate that it is not required to generate "Z" form in crystals, crystallisation alone driving the equilibrium towards the "Z" form with favourable sequences.

The Molecular Structure of the Lefthanded Helix.

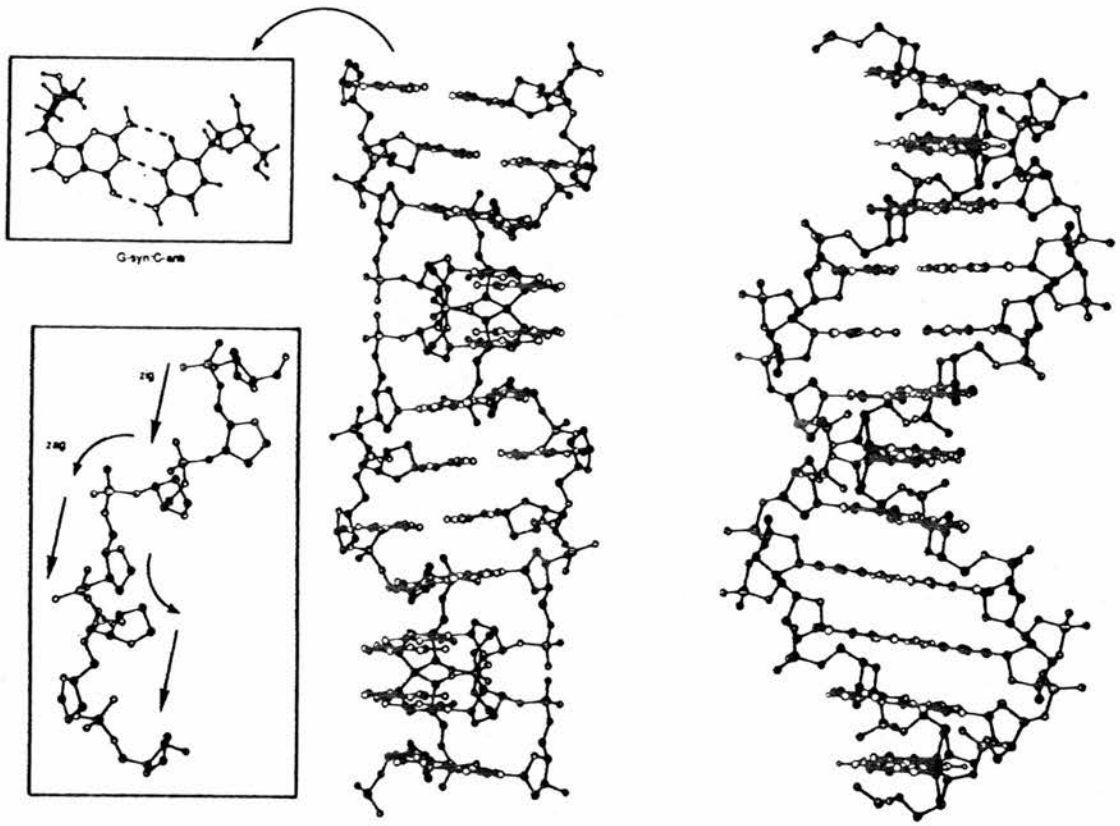
The left-handed DNA helical structure is similar to right-handed "B" form in that it is an antiparallel double helical fragment with Watson-Crick base pairing between the bases. However in "B" form DNA there are 10.5 base pairs per helical turn with a diameter of 20Å and a helical pitch of 34Å; in comparison to the left-handed helix which has 12 base pairs per turn, a diameter of 18Å and a helical pitch of 44.6Å resulting in a slimmer more elongated helix. In contrast to "B" DNA with a shallow minor groove and a larger major groove, the left-handed helix has only one deep groove, analogous to the "B" form minor groove, which extends to the axis of the double helix. A difference in the position of the helical axis accounts for the major change in the organisation of the molecule. The helical axis passes through the centre of the base pairs in "B" form DNA producing a molecule with two grooves with little difference in depth; whereas the helical axis in left-handed DNA falls outside the base pairs producing a molecule in which there is only one deep groove. The concave major groove of "B" DNA is transformed into the convex outer surface of the left-handed helix. In right-handed "B" DNA there is a linear ribose phosphate backbone where as the left-handed helix follows a zig zag course, hence it was named "Z" DNA. The asymmetric repeating unit of the nonlinear "Z" DNA backbone consists of a dinucleotide instead of a mononucleotide found in "B" DNA. All the nucleotides in "B" DNA have anti conformation and adopt a C2' endo pucker of the deoxyribose ring, conversion to

the left-handed helical duplex is accompanied by two conformational changes relative to the "B" form. There is a 180° rotation about the glycosidic bond at every alternate residue from the anti to the syn conformation with the pucker of the sugar ring changing to C3' endo. It is this conformational change which results in the zig zag organisation of the phosphate backbone. This was the first structure found to systematically use this conformation only the anti conformation is found in all other DNA structures.



Nucleotide stereochemistry. Depending on the rotation about the bond between C1' of the sugar and either N1 for pyrimidines or N9 for purines, a nucleotide can be described as either having an anti or syn conformation. Because of steric constraints, nucleotides are generally found in the anti configeration, with their Watson-Crick hydrogen bond donor acceptors swung outward away from the plane of the sugar ring. However guanosine is sometimes found in a syn conformation. In this form the bulk of the purine ring is positioned directly over the plane of the sugar. The ring can adopt different stereochemistries, these are labeled according to which group is bent out of the plane of the ring and in which direction. If a portion of the ring is bent upward toward the base this is known as endo, while if it is bent downward away from the base this is known as exo. In the figure plain lines represent bonds that are within the plane of the sugar, while bold lines indicate that the bond is bent out of the plane. Hence C3' endo - C2' exo describes a furanose ring in which the 2' and 3' carbons have been twisted in opposite directions and the bond connecting them crosses the plane of the ring.

"Z" DNA. "B" DNA.



Nucleic acid secondary structures. The structural consequence of the ability of nucleotides to form Watson-Crick base pairs is nucleic acid double helices. The figure illustrates the very different structures of the "B" and "Z" forms of the double stranded DNA helix.

These changes produced significant alterations in the relation between successive base pairs along the helix. The stacking of base pairs in "Z" form DNA is quite different in the sequence d(CpG) and in d(GpC), while in "B" DNA the stacking is similar. The base pairs in "B" DNA stack directly upon each other in the centre of the molecule. In the sequence d(CpG) in the "Z" DNA conformation the base pairs are sheared relative to each other so that the cytosine residue on opposite strands stack one over the other in the centre of the molecule, however the guanine residues now no longer stack on bases but stack upon the O1' oxygen atom of the sugar residue of the base below. In "Z" DNA the angle of twist between successive base pairs along the helix depends on the sequence, not so in "B" DNA which has a steady twist of 36° between successive base pairs. For the sequence

d(CpG) in "Z" DNA there is only a small rotation of ~15° between successive base pairs, while the sequence d(GpC) has a much larger rotation of ~45°. The shearing of the base pairs stiffens the left-hand duplex relative to the right-handed. Furthermore there is a considerable change in the relationship of the bases to the sugar phosphate backbone in comparing the "B" and "Z" form molecules. The imidazole ring of guanine is found predominantly on the periphery of the "Z" DNA molecule with exposure of the guanine N7 and N8. These atoms are both shielded in "B" DNA with the hydrogen attached to guanine C8 in van der Waals contact with the sugar phosphate chain on the outside of the molecule. This difference in accessibility explains some of the many differences in the chemical reactivity of the two molecules. In "B" DNA the base pair is situated in the centre of the molecule surrounded symmetrically by the sugar phosphate chains. In "Z" DNA the base pair is away from the centre so that the guanine imidazole ring is near the periphery.

It has been known for some time that both purines and pyrimidines can rotate about their glycosyl bonds forming the anti and syn conformations, observed in a variety of crystallographic and solution studies. One of the earliest deoxynucleoside complexes studied was deoxycytidine hydrogen bonded to deoxyguanosine in which the deoxyguanosine residues were in syn conformation (Haschemeyer & Sobell 1964). An early theoretical study of the stability of purines and pyrimidines in syn and anti conformations suggests that although purines can form the syn conformation without energy loss, there is a small amount of steric hindrance with pyrimidines (Haschemeyer & Rich 1967). Later studies (Son et al 1972, Davies 1978 and Neumann 1979) experimentally confirmed that purine residues form syn conformation relatively easily but it is less common to find pyrimidines in syn conformation. However it is possible to detect syn conformations of pyrimidines in nucleotide solutions. This data suggests that "Z" DNA formation would be favoured in sequences with alternations of purine and pyrimidine residues. Indeed the "Z" conformation is most favoured by alternations

in guanine and cytosine residues, in the $d(CG)_3$ crystal (Wang et al 1979); the deoxycytosines all have the anti conformation and the deoxyguanosines have syn conformation. Deviations from strict alternations of purines and pyrimidines have been found in "Z" DNA (Nordheim et al 1982a). This study suggesting the energy lost with one pyrimidine in the syn conformation is out weighed by the energy gain in forming a longer "Z" DNA segment. [Although there is some loss of energy due to close van der Waals crowding when pyrimidines are in syn conformation the loss is not very large]. More interestingly, crystallised sequences with greater than one base deviations have been shown to form lefthanded DNA (Wang et al 1985). If under certain favourable conditions nonalternating nucleotide sequences assume the "Z" conformation the surface features are different at these sites compared to alternating purine/pyrimidine sequences but the backbone is similar.

Sequence and Environment Requirments For Lefthanded DNA.

It is still not clear which DNA sequences will support the lefthanded "Z" DNA conformation and which environment is necessary to induce "Z" formation for a given base sequence. Since the initial discovery with the polymer Poly(dG-dC).Poly(dG-dC) in 4M aqueous salt (Pohl & Jovin 1972) several investigations with alternations in purine/pyrimidine sequences in solution have followed (Jovin et al 1983). However with the exception of $d(CG)_n$ where $n > 1$ [a dimer will not support the lefthanded form] either the studied sequence usually has to contain one or more modified bases before "Z" form is achieved in solution (Behe & Felsenfeld 1981, Wang et al 1985) or the conditions used to induce "Z" DNA are far more severe than that of 4M aqueous salt. Poly(dA-dC).Poly(dG-dT) requires 4M sodium perchlorate and 19% ethanol or methylation of the cytosine bases in order to form "Z" DNA (Jovin et al 1983, McIntosh et al 1983); only partial conversion is observed in high salt (Vorlickova et al 1982a, Zimmer et al 1982). Poly(dA-dT).Poly(dA-dT) has not been found to convert to "Z" form in high salt

(Vorlickova et al 1980/82b) but by adding nickel ions at high sodium concentrations an inversion of the circular dichroic spectrum, similar to that of high salt Poly(dG-dC).Poly(dG-dC), is detected (Bourtayre et al 1987, Ridoux et al 1988). A Raman spectra correlating with a lefthanded structure is also observed with the polymer Poly d(A-C).Poly d(G-T) under the same conditions (Taboury & Taillandier 1985, Ridoux et al 1987).

Results have shown the tendency of sequential dinucleotides in DNA sequences to form "Z" conformations in solution to be in the order $m^5CG>CG>TG=CA>TA$ (Wang et al 1984). The longer the $(GC)_n$ sequence the more stable the "Z" form becomes; Wang et al 1987 propose that sequential CG base pairs tend to stabilise the "Z" form, sequential TG and CA pairs are neutral in stability, where as AT sequences disrupt the stability of "Z" DNA in aqueous solutions and crystals (Pohl 1984, Cavailles et al 1985). When A and T bases are incorporated into oligomers the effect on the "Z" conformation is dependent on position [whether interrupting a CG run] (Chen 1988, Quadrifoglio et al 1981) and also whether it exists in solution or as a crystal where constraints are greater (Wang et al 1987).

The oligomer $d(CG_3TATACG_3)$ in solution with 4M $NaClO_4$ has been reported not exhibit "Z" conformation (Patel et al 1985), however others have shown "Z" DNA formation, but only in the CG segments (Ikuta & Wang 1989). Majumder et al 1989 observes that the polymer $d(ACGTACGT)_{8-12}$ will not form the "Z" conformation even in the strong stabilising conditions required for the transition of Poly d(AC).Poly d(GT) (Taboury & Taillander 1985). The GTA sequence being less favourable in "Z" formation than GTG or TGC and GCA sequences (Mishra et al 1988) indicating that exact base-base interaction decides the relative potential for "Z" formation within a given sequence.

The constraints of crystal formation are such that, sequences which would not be expected to form "Z" conformation, based on data from solution studies, do so. The oligomer d(CACGTG), which does not change from its righthanded structure in solution, forms "Z" DNA when high precipitant concentrations and long crystal

growth periods are used (Coll et al 1988). Similarly the oligomer d(CGCATGCG) forms a "Z" conformation in crystals but "B" in a 6M salt solution (Benevides et al 1984, Wang et al 1987).

On resolving the left-handed alternating purine/pyrimidine helical crystal structure with sequences containing AT inserts (Fujii et al 1982/85, Wang et al 1984/85, Benevides et al 1984,) the geometry of the TA base pairs appeared to be similar to that of the GC base pairs with the adenine residues in the syn conformation. A significant difference however is that the water molecules in the deep helical groove of the "Z" DNA are disordered near the TA base pairs in contrast to the high level of order found in the solvent of DNA segments containing CG pairs. This ordering is largely due to the presence of an amino group on the 2' position where hydrogen bonds to a water molecule and helps to organise others in the groove. This amino group is absent in TA pairs which undoubtedly leads to solvent disordering and contributes to the observation that TA pairs form "Z" DNA less readily than CG pairs (Wang et al 1984, Coll et al 1988). The polymer d(CGCGCGTGCA) can undergo a right to lefthanded helical transition although requiring higher salt concentrations than $d(GC)_n$ sequences [5.3M NaCl] (Schorschinsky & Behe 1986). Although less disruptive than AT sequences on "Z" DNA stability (Wang et al 1987), GT and CA sequences are not as effective as CG in "Z" DNA formation, it therefore follows that any interruption of consecutive CG sequences reduces the stability and ease of formation.

On using oligomers care must be taken not to produce hairpin formation (Chen 1988) which may reduce "Z" stability.

The negative driving force of negative supercoiling appears to be a more effective way of promoting "Z" formation than altering the environment or chemical modification of the DNA bases. The sequence d(CA/GT) does not form "Z" DNA easily in linear molecules but frequently does when in supercoiled circular DNA (Nordheim & Rich 1983b, Haniford & Pulleyblank 1983, Johnston & Rich 1985). Sequences of alternating AT base pairs are not easily induced to the "Z"

conformation as observed with linear DNA, but orientations of A-T bases (not necessarily alternating purine/pyrimidine) are found in lefthanded form when flanked with short runs of $d(G\text{-}C)_{3\text{-}5}$ in supercoiled plasmids (McLean et al 1986). However studies indicate that "Z" DNA does not form in stretches of d(A-T) longer than a few base pairs (Ellison et al 1986). Others have shown that runs of A-T adopt cruciforms under the influence of negative supercoiling (Greaves et al 1985, Haniford & Pulleyblank 1985). Supercoiling also forces ACGT tracts to adopt a cruciform structure (Nayloc et al 1988) implying that not all alternating sequences will be induced into the "Z" form even under the strong influence of supercoiling. This sequence could not be forced to form a lefthanded conformation in linear DNA either (Majumder et al 1989).

Negative supercoiling, like crystal formation, appears to have such a stabilising effect on "Z" DNA that alternating purine/pyrimidine sequences are not a necessary requirement (Kilpatrick et al 1984, Singleton et al 1983, Wang et al 1984/5, Ellison et al 1985, Johnston & Rich 1985, McLean et al 1986). This is demonstrated in vivo by the observation that in the supercoiled SV40 genome some sequences identified as being in "Z" form do not contain major segments of alternating purine/pyrimidine bases (Nordheim et al 1987).

Under stabilising conditions flanking $d(GC)_n$ sequences can induce neighbouring DNA sequences less likely to form "Z" DNA to do so.

Flexability within the "Z" DNA allomorph.

Crystals of "B" DNA do not show regularity in the molecule (Wing et al 1980), similarly all the crystals of "Z" DNA show a variety of slightly different conformations . In the case of "Z" DNA at least 3 major conformations have been identified (Wang et al 1979,81; Drew et al 1980; Crawford et al 1980; Drew & Dickerson 1981; Fujii et al 1982; Wang et al 1983, Harder & Johnson 1990). The variants designated Z_I (or Z), Z_{II} and Z_{III} differ with respect to sugar and sugar phosphate torsional angles, width of the concave groove and distance of base pairs

from the helical axis. Changes in the backbone structure of "Z" DNA are not reflected in base pair interactions to the same degree that is observed in "B" DNA due to the way the bases are linked in these conformations (Drew and Dickerson 1981). Thus many "Z" DNA variants probably exist which involve changes in backbone structure. Conformational variations appear to occur in all structural forms of DNA (Shakked et al 1989) and it is likely that slight modifications in structure commonly take place along the molecule. Local changes in the environment due to ions or proteins produce these effects which are often sequence dependent. It is important to note that the conversion from right handed to left-handed "Z" DNA is not brought about by a simple twisting of the double helix in the opposite direction. Instead it involves a series of complex internal rearrangements in which there is a change in the positioning of the bases relative to the backbone.

RNA and the "Z" conformation.

The RNA polymer Poly r(G-C) exists in the "A" conformation in low salt concentrations and "Z" form in high salt (Hall et al 1984). The "A" to "Z" transition of RNA is much less favourable than the corresponding "B" to "Z" transition in DNA. An integral part of the DNA backbone is sugar moiety. Substitution of the deoxyribose ring with ribose introduces a 2' hydroxyl group which significantly increases the rigidity of the sugar conformation holding it in the 3' endo conformation. The RNA double helix is less flexible for this reason. There is no steric reason to prevent Poly (rG-rC).Poly (rG-rC) forming "Z" RNA (Wang et al 1979), however it has proven difficult to convert this polymer from its usual "A" form to the lefthanded form (Klump & Jovin 1987). This resistance may be related to rigidity of the ribose ring in staying in the C3' endo conformation. In the "Z" form the pyrimidine residue has a C2' endo conformation; thus a change would require additional energy.

It has been shown that RNA has a dominant effect over DNA in helical conformation in RNA/DNA hybrids keeping them in the "A" form (Wang et al 1982).

"B" to "Z" DNA Junctions.

It is now recognised that contiguous right and lefthanded conformations can exist within the same DNA molecule. The interface between the two conformations being specified as a "B" to "Z" junction; relatively little is known about the structure of junctions. Early studies on plasmid DNA interpreted these junctions to be conformationally flexible, with different structures depending on sequence (O'Connor et al 1986), existing in nonalternating purine/pyrimidine segments, possibly partially single stranded, and may be containing unpaired bases. These regions were also described as being short 10 or 11 bases in length (Singleton et al 1982/83, Stirdivant et al 1982, Kang & Wells 1985, Johnston & Rich 1985, Kilpatrick et al 1983/84, McLean et al 1987, Hua et al 1989). Later results suggested junction length to be less than 8 base pairs (Azorin et al 1984), however spectroscopic studies on synthetic double stranded DNA oligomers indicate that the junction only spans 4 to 6 base pairs (Shready 1988) and recently suggest 3 base pairs or less with possible independence of base sequence (Dia et al 1989, Shready & Winkle 1989). Chemical modification by various modifying agents shows that several base pairs with broken or distorted watson Crick hydrogen bonds exist at "B"-"Z" junctions (Kang & Wells 1985, Johnston & Rich 1985, Johnston 1988, Nejedly et al 1988). The location and structure of "B"-"Z" junctions has been shown to depend upon superhelical density in plasmids (Johnston & Rich 1985, Rio & Leng 1986)

The fact that such short junctions can exist in DNA means that very short alternating forms of "B" and "Z" DNA could occur in native DNA if the environment was such as to stabilise these changes. It is important to be aware

such junctions may undergo deformations such as bending or twisting that could play a role in protein/nucleic acid interactions.

The phosphate-phosphate distance across the DNA helix is larger for dGpC than dCpG. The dGpC distance of 15Å is close to that found in "B" DNA (17.5Å). This prompted Wang et al 1979 to propose that a "B" to "Z" junction would form at this point and not at dCpG; therefore "Z" DNA in the middle of a "B" DNA segment would contain an even number of nucleotides.

A "Z"-"Z" junction is postulated to exist at the boundary between two regions of "Z" DNA that are out of phase with each other in respect to the alternation between syn and anti conformations of their bases (Johnston & Rich 1985).

Base order in purine/pyrimidine alternating sequences and its effect on "Z" DNA formation.

It is interesting that of the four selfcomplementary tetranucleotides d(CGCG), d(GCGC), d(CCGG), d(GGCC) only the first supports the "Z" conformation in high salt solutions (Thomas & Peticolas 1984) while others indicate a much easier "B" to "Z" transition for d(CG) than for d(GC) in longer sequences (Chen 1988, Quadrifoglio et al 1981/84). This is compounded by the demonstration that the octomer $d(CG)_4$ can completely transform into "Z" conformation in saturated NaCl solution whereas the decamer $d(GC)_5$ cannot (Wang et al 1987) suggesting that long GC alternating sequences are more difficult to enter into the "Z" conformation if they begin with guanine at the 5' end and that terminal GC sequences in short oligomers appear strongly inhibitory. It is important to note that AC 5' terminal sequences are also slightly inhibitory, although not being as destructive as GC, still indicating that beginning a sequence with a purine inhibits "Z formation [especially guanine] (Chen 1988).Oligomers which begin TG [5'] and end CA do not form "Z" DNA as easily as a sequence of similar length probably because they are not as stable in the left form as CG (Wang et al 1987) and reduce the number of consecutive CG bases. It is now popular belief that only short oligomers beginning

with a pyrimidine base can be converted to "Z" form in high salt solutions, however the sequence d(ACG_3T) has been shown to form "Z" DNA on the addition of 20% ethanol to the salt solution (Wang et al 1987).

Detection of "Z" form DNA.

(I) Physical-Chemical Methods.

Most DNA molecules of biological interest, such as promoters or origins in replication are difficult if not impossible to crystallise. Thus methods for studying DNA in solution are required. These methods however are generally qualitative and imprecise. In addition they are also often not capable of determining the three dimensional structure of DNA by themselves, relying on some known structure determined by X-ray crystallography.

Since "Z" DNA was first detected in solution with Poly (dG-dC).Poly (dG-dC) and it is accepted that the polymer readily forms "Z" DNA, most physical-chemical methods originally use this polymer to determine the change in properties involved in the "B"-"Z" DNA transition.

Ultra Violet Absorbance:- Pohl and Jovin 1972 reported that in a high salt solution Poly (dG-dC).Poly (dG-dC) shows a decrease in absorbance at A_{260} and an increase at A_{295} compared to the low salt spectrum. Although this effect is small it has become a useful index for "Z" DNA formation especially in polymeric solutions (Pohl et al 1972). UV analysis of the changes in the ratio of A_{295}/A_{260} (Soslau et al 1986) demonstrates that "B" DNA falls in the range 0.1 to 0.19 whereas "Z" DNA is between 0.28 to 0.38 with the aggregated "Z" form at 0.46-0.65. Similar results were reported by Castleman et al (1984).

Circular Dichroism:- This method monitors the effect of structure on the electronic transitions in the nucleotide bases. The near inversion of the (UV) Circular Dichroism spectra on raising the salt concentration in a solution of Poly (dG-dC).Poly (dG-dC) was demonstrated by Pohl 1971, Pohl and Jovin 1972 and remains one of the simplest methods of detecting "Z" DNA formation in polymers.

In low salt solutions there is a positive band at 280nm which is converted to a more intense negative band with a minimum at 290nm in 4M NaCl. There is also an inversion of a negative band at 253nm to a positive band at 265nm in high salt concentrations. However it is important to be aware that some chemical modifications introduce chiral centres on reaction with Poly (dG-dC).Poly (dG-dC) which yield an inversion of the UV C.D. spectrum without formation of "Z" DNA (Tomasz et al 1983). Synthetic polymers with alterations of purine/pyrimidines other than GC also show dichroic changes in high salt which suggested different conformations although they are not as obvious (Vorlickova et al 1980/82, Zimmer et al 1982).

The inversion of the C.D. spectrum also occurs in the Vacuum ultraviolet region between 180 and 230nm (Sutherland et al 1981). "B" DNA has a large positive peak at 187nm while "Z" DNA has a large negative peak at 194nm and a positive band at below 186nm. The magnitude of differences between "B" and "Z" DNA below 200nm are approximately ten times greater than those observed between 230 and 300nm. This sensitive region produced data which suggested that "V" form DNA is a 1:1 mixture of right and left handed DNA (Brahms et al 1982) [see later]. This method was also used to solve the question of the handedness of Poly (dI-dC).Poly (dI-dC) (Mitsui et al 1970, Sutherland & Griffin 1983). The Vacuum UV C.D. spectrum may be a more reliable index for the handedness of a helix as the absorption bands responsible for the spectrum arise largely from the backbone. When ethanol was used instead of NaCl as a dehydrating solvent to induce "Z" DNA in Poly (dG-dC).Poly (dG-dC) a change in the UV C.D. spectrum was observed when the ethanol concentration was increased from 50 to 80% (Pohl 1976). Initially this was described as change from the "Z" form to the righthanded "A" form; but Vacuum UV C.D. measurement proved it to be the "Z_I" lefthanded (Brahms et al 1982). A recent study acquired data from many DNA sequences providing a large amount of information on DNA conformations (Fairall et al 1989).

Raman Spectroscopy:- Raman spectroscopy is now well established as a powerful method of determining the physical state of biological molecules. A detailed Laser Raman Spectra can be obtained from a relatively small sample and involves the measurement of different types of vibrations from components of the DNA molecule. These vibrations being sensitive to conformational changes of the bases and DNA backbone, bond stretching, bending and other molecular changes. The spectra are independent of whether the molecule is fixed in a crystal lattice or free in solution and can identify the molecule in different physical states within a particular sample. There is an advantage in analysing spectra from crystals as the molecules are completely in a known conformation and the solvent environment of the vibrating groups is also known. Thus the extent to which the vibrations are perturbed or modified can be assessed. The Raman spectra of Poly (dG-dC).Poly (dG-dC) in 4M and 0.1M NaCl solutions, similar to those of Circular Dichroism, have significant differences (Pohl et al 1973). The spectra with the high salt solution is also identical to that produced by the crystal (Thaman et al 1981). Unlike Circular Dichroism the Raman spectra produce data which is quantitative and can therefore measure the fraction of the molecule in a particular conformation. On using this ability Wartell et al 1982 showed that regions of alternating GC residues in a DNA fragment at 4.5M NaCl were all in "Z" conformation. This was different to studies by Circular Dichroism which suggested that only some of the dG-dC inserts converted to "Z" DNA (Klysik et al 1981). Obtaining and interpreting Raman spectra of DNA and RNA oligonuleotide crystals for which high resolution X-ray crystal structures were available (Benevides et al 1984/86/88), facilitated the assignment of individual Raman bands in "A", "B" and "Z" DNA to specific backbone geometries and nucleoside conformers (Thomas 1986, Thomas & Wang 1988). These results were applied to the diagnosis of conformation in aqueous DNA and viruses (Thomas et al 1986/88).

A recent study shows that by using the novel technique of confocal Raman microspectrometry the spectra of a single intact cell, a chromosome, or a polytene

chromosome band and interband can be obtained providing information about DNA structure and protein/DNA ratios (Puppels et al 1990).

Nuclear Magnetic Resonance (NMR) Studies:- NMR is also used as a powerful method for studying the conformation of molecules in solution. As with CD and Raman spectroscopy the ^{31}P NMR spectra of Poly (dG-dC).Poly (dG-dC) is different in high and low salt solutions (Patel et al 1982). Instead of one resonance peak, found in "B" DNA, a split was observed in high salt implying a difference in the phosphate conformation. [Probably originating from the differences between the phosphodiester linkages in dCpG and dGpC sequences]. There has also been several proton ^{1}H NMR studies of "Z" DNA polymers (Patel et al 1982). These include studies that employ the Nuclear Overhauser Effect (NOE) [the change in the intensity of a given nuclear spin on saturation of a nearby dipolar coupled spin]. The data from NOE gives a measurement of distance between atoms and spectra can be used to determine the distribution of syn and anti nucleotides. When guanosine is in the syn conformation the C8 proton is close to the C1' proton of the deoxyribose. However when the guanine is in the anti conformation these two protons are further apart. In studies on Poly (dG-dC).Poly (dG-dC) a strong NOE effect was found between the deoxyguanosine H8 and H1' protons in 4M NaCl but not at 0.1M (Patel et al 1982). These transient NOE measurements demonstrated a syn glycosyl torsion angle at guanosine residues in contrast to the anti conformations found in cytidine. Two dimensional NMR allowed NOE studies to be carried out in a continuous fashion. This technique can determine all interactions within the molecule in a single study. Spatial relations of all the atoms in a particular conformation can be mapped. A study by Feigon et al 1983 gave a graphic demonstration of the equilibrium between "B" and "Z" DNA conformations. At 0.1M NaCl the hexamer (m^{5}dC-dG) produced a spectrum of largely "B" conformation with only 2-4% "Z" conformation; however on the addition of methanol to 40% the "Z" conformation was predominant. This technique is useful for studying the close interactions between nucleic acids and

other molecules, such as protein, and could be used to monitor the effects of a variety of ligands and other interacting substances on the equilibrium between "B" and "Z" DNA.

As most of the physical-chemical methods do not detect non "B" DNA conformations if they represent less than 5% of the total DNA, it would be useful to have a specific probe to test for the presence of "Z" helix as a small fraction of total nucleic acid structure. In addition interest is often not in the three dimensional structure of DNA per se but in its response to various tortional or flexural stresses imposed by the binding of a protein or other environmental factors such as supercoiling. Antibodies provide a solution to studies in both these conditions.

(II) Indirect Methods.

Antibodies:- Antibodies provide a good model system for examining specific DNA/protein interactions because large quantities of well characterised proteins are available. Antibodies also represent a very useful tool in local DNA structure research due to their high specificity, sensitivity and application in biological media. Antibodies to double stranded native DNA (Stollar 1975), RNA (Stollar et al 1978), RNA-DNA hybrids (Rudkin & Stollar 1977) and triple helical polynucleotides (Stollar & Raso 1974) can serve as specific agents sensitive to changes in helical shape (Lacour et al 1973). Some recognise antigenic sites comprising of the pentose-phosphate backbones of adjacent strands over a span of two or three base pairs (Leng et al 1978). Lafer et al 1981 describe the production and characterisation of antibodies which are specific for "Z" helical DNA. The chemically stabilised (brominated) high salt "Z" form of Poly(dG-dC).Poly(dG-dC) is found to be highly immunogenic and the resulting sera specific for the "Z" conformation. This is in contrast to "B" DNA, which is not very immunogenic and for which antibodies are not easily produced. It is interesting to note that the conformation of the alternating copolymer in physiological conditions appears to differ from native DNA based upon studies using sera of MRL/lpr mice which bind native DNA but not the polymer. Antibodies that react with "Z" DNA are

found in the sera of mice with an autoimmune disease similar to human systemic lupus erythematosus where anti-"Z" immunoglobulin in patient sera is also found (Lafer et al 1983). These are spontaneously occurring antibodies for which the natural antigen is unknown. Any organism probably becomes tolerant to "B" DNA during the early stages of embryological development and it is likely that "Z" DNA is not normally seen by the cells of the immune system during embryogenisis. When DNA is released from cells which have broken down, nuclease cleavage probably converts any "Z" DNA to "B" DNA by releasing torsional stress. A recent study has shown that immunogenic DNA is serum nuclease resistent allowing immunogenic responses (Braun & Lee 1988). Anti-"Z" DNA antibodies provide the first demonstration that a region of DNA could be stabilised by protein binding under physiological conditions (Lafer et al 1985).

Monoclonal antibodies can be produced against "Z" DNA using the same stabilised polymer (Moller et al 1982). These react with different parts of the surface of "Z" DNA. Some antibodies bind to the base pairs on the surface of the molecule (Nahon et al 1988) providing sequence specificity while others have a preference for the sugars and negatively charged phosphate groups showing conformational specificity. Some monoclonal antibody preparations display nucleotide sequence specificity in addition to their structural specificity in binding "Z" DNA (Nordheim et al 1986, Zarling et al 1984b) whereas others may not recognise "Z" DNA in sequences other than that of the immunogen. However bivalent anti-"Z" IgG have also been raised that recognise all known "Z" DNA conformational polymorphisms within sequences in synthetic polymers [Poly(dG-dC).Poly(dG-dC), Poly(dA-dC).Poly(dG-dT), Poly(dA-dT).Poly(dA-dT)] and other various mixed alternating purine/pyrimidine sequences (Zarling et al 1984a/84b). One of the significant observations with polyclonal (Lafer et al 1981) and some of the monoclonal antibodies is that they bind "Z" DNA even in the presence of high salt concentrations; particularly useful as varying the salt concentration can control the degree of "Z" DNA in a solution. The observation

that anti-"Z" DNA antibodies can shift the "B" to "Z" equilibrium to the "Z" form independent of salt (Jovin et al 1983, Zarling et al 1984b, Lafer et al 1985b/6, Runkel & Nordheim 1986) emphisises the fact that caution must be shown when using antibodies to demonstrate the existence of "Z" conformation in biological systems. The choice of "Z" DNA modification to stabilise the conformation before use as an antigen can influence both the immunogenicity and specificity of the antibodies produced. Chemical bromination appears to provide the best results; however antibody preparations should be screened for their specificity before carrying out analysis, as on modification these substituted atoms can be recognised by a number of antibodies. Indeed some monoclones can differentiate between different types of DNA modifications such as C_5 methylation of cytosines (Moller et al 1982).

Antibody studies show the chemically stabilised RNA polymer Poly (rG-rC).Poly (rG-rC) to contain a 1:1 mixture of "A" and "Z" form DNA under physiological conditions (Hardin et al 1987) [In contrast to Hall et al 1984]. Rabbit polyclonal antibodies against "Z" DNA specifically recognise the "Z" form of RNA (Zarling et al 1984a/b), indicating the presence of a "Z" DNA like structure element in "Z" RNA. Further studies suggested that the "Z" forms of RNA and DNA share a common phosphodiester backbone determinant (Hardin et al 1987). Rabbit polyclonal antibodies elicited against the "Z" form of the chemically modified RNA polymer also specifically recognise both modified and unmodified "Z" DNA (Hardin et al 1988). The binding sites of these anti-"Z" RNA antibodies span 6 to 14 base pairs and recognise features of the phosphodiester backbone, bases and the ribose 2' OH groups.

Antibodies have been used to specifically recognise "Z" DNA tracts in a variety of synthetic polynucleotides, negatively supercoiled plasmids and also in many viral, bacterial, plant, insect, protozoan and animal genomes. They are useful as structural and mechanistic probes in many other studies such as that of homologous recombination catalysed by Ustalago rec 1 protein (Kmiec &

Holloman 1984) or as kinetic probes in studying the "B"-"Z" transition in plasmid DNA (Peck et al 1986).

It is however important to realise that "Z" DNA may not be the only non "B" conformation these antibodies recognise.

Sedimentation:- Sedimentation studies have been most useful in identifying "Z" DNA formation in plasmids. Formation of "Z" DNA changes the supercoiling which directly influences its sedimentation rate. Using inserted regions of alternating GC residues sedimentation changes have been shown to be due to these entire segments forming "Z" DNA (Peck et al 1982). Velocity sedimentation has also been used to purify multimer populations of supercoiled circular DNA-IgG complexes (Hagen et al 1985). Differences in the aggregation state of "Z" DNA formed by increasing concentrations of $Co(NH_3)_6^{3+}$ ions can also be detected by changing S values (Soslau et al 1986).

Tritium Exchange:- This method involves the measurement of the exchange of solvent protons with those involved in base pair hydrogen bonding. Studies reveal that the exchange half time for protons on the cytosine amino group in "Z" form DNA is much longer than in "B" DNA (Ramstein and Leng 1980). This slower exchange rate is due to the "Z" DNA helix being far stiffer than the "B" form and therefore does not open as easily, thus hydrogen bonding protons are released more slowly.

Electron Microscopy (EM):- Direct EM analysis has shown that "Z" DNA formation is frequently associated with extensive self association (Castleman & Erlanger 1983). This self association takes place in an ordered fashion giving rise to toroidal or rope like structures. A variety of entangled and branched polymer filiments are seen that depend on the size of the aggregates (Revet et al 1983). Structures are observed which appeared to be four chain hairpins aggregated together.

EM analysis can give information on "Z" form DNA segments within plasmid DNA (Nordheim et al 1982a, Dicapua et al 1983, Castleman et al 1988) and other

genomes (Hagen et al 1985) by visualising the position of anti-"Z" antibodies. DNA loops can also be visualised between two antibody binding sites within supercoiled plasmids, bacteriophage and circular viral DNAs (Castleman et al 1988, Revet et al 1984, Hagen et al 1985).

The technique of **indirect immuno-electron microscopy** is where the antibody to "Z" DNA is added to fixed cytological chromosome preparations followed by a second antibody that binds the initial anti-"Z" immunoglobin. The second antibody contains a fluorescent chromophore [or is ferritin labelled] which visualises the position of "Z" DNA on the chromosome on illumination at the excitatory wavelength (Nordheim et al 1981, Arndt-Jovin et al 1983, Hill & Stollar 1983, Robert-Nicoud et al 1984).

Gel Electrophoresis:- Supercoiled minicircles differing by units of one in their topological linking number can be separated from each other by acrylamide gel electrophoresis on the basis of changes in DNA structure (Peck et al 1982). One-dimensional electrophoresis of plasmids on relaxation of supercoils allows correlation with right to lefthanded transition (Klysik et al 1981, Singleton et al 1982, Peck et al 1982, Stirdivant et al 1982) but data on individual topoisomers is difficult to quantitate. Two-dimensional gel electrophoresis separates positively and negatively supercoiled topoisomers and also permits quantitation (Lee et al 1981, Peck & Wang 1983, Connor et al 1986, Wang et al 1983, Haniford & Pulleyblank 1983). However this method still requires that a considerable fraction of the DNA molecules carry an alternative structure and will not detect a minority species. Topological isomers which form complexes with conformation specific proteins can be separated from protein free topoisomers by gel electrophoresis (Nordheim & Meese 1988). Changes in electrophoretic migration can also be attributed to the formation of various multimers of supercoiled circular plasmids or viral genomes by anti- "Z" DNA IgG (Hagen et al 1985).

Nitrocellulose Filtration:- "Z" form DNA induced by high salt conditions binds nitrocellulose on filtration where as "B" form [low salt] DNA remains in the

filtrate. Similarly brominated Poly (dG-dC).Poly (dG-dC) has a high affinity to nitrocellulose filters (Moller et al 1984). In 1M NaCl there is virtually no binding of "B" DNA, but a substantial binding of "Z" DNA occurs even after low levels of bromination. 2-(N-Acetoxyacetylamino)fluorene, known to bind to the C8 position in guanine stabilising "Z" DNA (Sage & Leng 1980, Santella et al 1981), also binds to nitrocellulose.

Antibody binding to DNA induces its retention on filtration. This can be used to detect DNA containing non "B" conformations and quantitatively estimate superhelical densities or other environments in which "B" to "Z" conversion occurs (Nordheim et al 1982). Deproteination followed by gel electrophoresis allows analysis of the bound DNA (Nordheim et al 1983). On chemical fixation of the antibody these antigenic sequences can be accurately mapped by restriction endonuclease cleavage (Nordheim et al 1986/7). This technique led to the first identification of a natural sequence that formed "Z" DNA within plasmids in bacteria (Nordheim et al 1982). Similarly analysis of nucleotide sequence requirements for the "B"-"Z" transition can also be studied using antibody binding assays (Nordheim and Rich 1983a, Dicapua et al 1983).

DNA Modifying Enzymes and Nucleases:- The prokaryotic Hha I methylase adds a methyl group to the CG sequence in $d(CG)_4$ in "B" DNA but not "Z" DNA (Vardimon & Rich 1984, Zacharias et al 1984) whereas murine and human methylases show no preference (Bestor & Ingram 1983, Pfeifer et al 1983). In supercoiled plasmids (dG-dC)n tracts in "Z" form are not methylated or cleaved by the restriction endonuclease Hha I but cleavage does occur when supercoiling is insufficient to induce a "B" to "Z" transition (Zaharias et al 1984). Studies using the linear synthetic polymer Poly (dG-dC).Poly (dG-dC) demonstrate that the rate of reaction of the restriction endonucleases Hha I and Cfo I are reduced with cobalt hexamine chloride induced "Z" DNA, but is dependent on aggregation and how the "Z" DNA is formed (Soslau et al 1986). When "Z" form DNA is induced by low concentrations of the Cobalt complex [50μM] both

endonucleases remain active but in the presence of higher concentrations [100μM], which increases aggregation, the endonucleases are inactive. The MHha I DNA methylase is found to react at equal rates with "B", "Z" and "Z" aggregates formed with low Cobalt concentrations, but at a greatly reduced rate with high cobalt concentration "Z" aggregates. These results are significantly different to those observed with "Z" form (dG-dC)n tracts in circular DNA and suggest that "Z" forms induced by supercoiling are similar in structure to aggregated "Z" DNAs with respect to restriction enzyme recognition.

The in vivo existence of lefthanded DNA witin plasmids in E.coli which elicits a biological response was shown utilising the fact that "Z" inhibits methylation in prokaryotes (Jaworski et al 1987). The in vivo assay was also based on the in the in vitro observation that an Eco RI recognition site was not methylated when near to or in a "Z" DNA helix. A plasmid encoding for a temperature sensitive Eco RI methylase was cotransfected with plasmids containing GC sequences with Eco RI restriction sites in the centre or at the end of potential "Z" forming blocks. Inhibition of methylation was observed with the plasmids containing long GC tracts.

Many other restriction endonucleases do not cleave sequences in "Z" form but will when in the "B" conformation (Azorin et al 1983). Poly (dG-dC).Poly (dG-dC) in both "B" and "Z" form is insensitive to nuclease S1 (Moller et al 1984). However nuclease S1 has been shown to recognise and cleave at the junction between right and left-handed DNA as does another bacterial endonuclease Bal 31 (Singleton et al 1982/83/84, Kilpatrick et al 1983/84, McLean et al 1986). The use of S1 nuclease, which preferentially cleaves single stranded regions of DNA, provided the first evidence of altered structure in supercoiled DNA (Lilley 1980, Singleton et al 1982, Hentschel 1982). The activity of Micrococcal nuclease which completely digests "B" form DNA is reduced by increasing the "Z" form of the polymer. This decreased activity is overcome by a higher concentration of enzyme (Singleton et al 1982); a similar effect is observed with Poly (dG-m^5dC).Poly (dG-m^5C)

requiring 20 fold more micrococcal nuclease to cleave "Z" form than "B" (Behe et al 1981). In both cases it is unclear whether the "Z" form is cleaved or whether it is a small amount of "B" form still in the equilibrium. The endonuclease DNase I and exonucleases Bal 31 and exonuclease III cleave "B" form but not "Z" form polymer. In these cases increased concentrations of enzyme do not have any effect. Restriction endonucleases are very sensitive to the DNA conformation of their recognition sequences and also those flanking them. Thus they can detect changes in the conformation and conformational flexibility of the DNA duplex. These changes are often not revealed by chemical modification (Singleton et al 1983, Azorin et al 1984). Digestion of DNA containing an insert in "Z" conformation indicated that with certain restriction endonucleases enzyme inhibition decreases as the distance between "Z" DNA inserts and enzyme recognition sequences increase (Lesnik et al 1991). Other enzyme activities are also effected by the proximity of "Z" DNA (Lesnik et al 1991). It is therefore concluded that alterations in DNA conformation occur over a large distance from DNA in "Z" conformation and influences the efficiency of enzyme DNA interactions. The nucleotide composition of the flanking sequences have an effect on structure (Wolfes et al 1985).

Light-Scattering:- Laser light scattering provides an excellent tool to study the static and dynamic properties of biological macromolecules in solution (Bloomfield 1981). Ionic effects on chain flexibility and hydrodynamic properties of DNA can be studied by this technique, showing an increase in flexibility of natural DNA at high ionic strength. Studies on Poly (dG-dC).Poly (dG-dC) in "B" or "Z" form revealed that the "Z" DNA molecule is stiffer and has greater length in solution. (Thomas & Bloomfield 1983).

Chemical detection:-Chemical probes can be used as a sensitive method in detecting alterations in DNA conformation. These reagents react with particular sites of the DNA bases and modified DNA bases may occur at unusual structural features. To reveal the sites of modification an adaptation of the Maxam-Gilbert sequencing protocol is used. A convenient DNA restriction fragment is created,

radioactively labelled at one end. The backbone is then cleaved at each modified site with piperidine and the resulting fragments resolved on a sequencing gel (Maxam & Gilbert 1980). The most common reagents are :

Diethylpyrocarbonate (DEPC) which has enhanced reactivity with purines particularly adenines in the syn conformation [ie in "Z" DNA] compared to those in the anti conformation [ie all other DNA forms including "B"] (Herr 1985). [DEPC also modifies primarily adenines in single strand DNA.]

Dimethylsulphate (DMS) or Diethylsulphate (DES) also react with purines (primarily guanines) in syn conformation but with a less pronounced effect (Johnston & Rich 1985). Whereas Hydroxylamine is highly reactive with pyrimidine residues, primariy cytosines, near putative "B"-"Z" junctions (single stranded DNA regions). Similarly Osmium tetraoxide reacts especially strongly and primarily with thymines at the same position (Johnston & Rich 1985). Both chemicals are specific base reagents in "B" DNA (Rubin & Schmid 1980, Friedman & Brown 1978, Burton & Riley 1966) but are hypereactive with other DNA conformations. Less specific probes used are chloroacetaldehyde, bromoacetaldehyde and potassium permanganate reacting with pyrimidine residues in single strand DNA (Kohhwi & Kohwi-Shigimatsu 1988, Lyamichev et al 1989). Bromoacetaldehyde (BAA) and chloroacetaldehyde (CAA) are used for the location of denatured regions in DNA, they react with adenine and cytosine residues when the bases are not paired (Lilley 1983, McLean et al 1987) detecting regions with a sigle stranded nature. Both chemicals also react with adenine residues in syn conformation but not cytosine residues in anti conformation within "Z" DNA. The modified residues are sensitive to piperidine cleavage, which allows mapping at the single nucleotide level (Vogt et al 1988). Potassium permanganate reacts preferentially with thymine, 5-methylcytosine and to a lesser extent with purine residues in double strand DNA. Cytosine remains unmodified. Permanganate oxidation is therefore a positive discriminator between methylated and unmethylated cytosine. Potassium permanganate selectively reacts with "B"-

"Z" or "Z" junctions in supercoiled plasmids within E. coli cells in vivo (Jaing et al 1991). Restriction enzyme recognition sites located at these junctions are not cleaved by the endonuclease after permanganate modification. This method provides one of the few assays able to detect and quantitate "Z" DNA in vivo. Hydrazine also has a lower activity with methylated cytosines. This is useful as methylation of cytosine is known to stabilise "Z" DNA (Behe and Felsenfeld 1981).

Such methods have been used to study natural DNA sequences for altered structure [eg Diethylpyrocarbonate probing of the SV40 genome (Jovin et al 1983)]. DMS treatment is not very sensitive because it requires a considerable fraction of the DNA molecules to be in the alternative structure. However all the other treatments give a result even if only a small propotion of the DNA has adopted the alternative structure.

Stabilisation of Lefthanded DNA.

All DNA sequences have an equilibrium between right and left-handed DNA with the position of the equilibrium being determined by nucleotide sequence and local environment. Using the knowledge that alternations of purine and pyrimidines favour "Z" DNA conformation, especially GC sequences, Poly (dG-dC).Poly (dG-dC) has been widely used in studying the factors influencing the "B" to "Z" equilibrium. In the equilibrium "B" DNA is usually the lower energy state with "Z" DNA only becoming the lower energy state when another stabilising factor is involved. The instability of "Z" DNA relative to "B" DNA is partly associated with the fact that the phosphate groups on opposite strands of the DNA become closer together in the "Z" conformation. The closest distance of the opposite phosphate groups in "Z" DNA being 7.7Å compared to 11.7Å in "B" DNA (Wang et al 1981). As the instability of "Z" DNA is largely electrostatic the initial observations with left-handed DNA were found in solutions with high salt concentrations which reduced the phosphate-phosphate repulsion (Pohl & Jovin 1972). On solution of

the structure of "Z" DNA a large number of chemical modifications and different environmental conditions were discovered which stabilise "Z" DNA or lower its energy allowing the equilibium to move in its favour.

Covalent Modifications.

chemical modifications :- Bromination of Poly (dG-dC).Poly (dG-dC), stabilised in "Z" conformation with 4M NaCl, occurs largely at the C8 position of guanine (38%) and to a lesser extent on the C5 position of cytosine (18%) (Moller et al 1984, Nordheim et al 1981). This reaction stabilises the molecule in "Z" form even after the salt is reduced to physiological concentrations by dialysis (Moller et al 1984). Covalent modification of only the guanine residues in Poly (dG-dC).Poly (dG-dC) also leads to stabilisation of "Z" DNA. The C8 bromination of guanine indicates that the purine residue is confined to the syn conformation, as this position is not sterically accessable in "B" DNA (Tavale & Sobell 1970, Uesugi et al 1982). The presence of a bulky substituent at this position prevents the assumption of the anti conformation.

Considerable stabilisation of "Z" DNA is also achieved by halogenation of cytosine at the C5 position alone. A fully brominated polymer at this position exists in "Z" conformation independent of salt concentration (Malfoy et al 1982, Jovin et al 1983). Stablisation of "Z" DNA by the substitution of an iodine atom at the same position is also reported (Jovin et al 1983).

The substitution of sulphur atoms for oxygen atoms in the phosphate group has been shown to influence the "B" to "Z" equilibrium, the thio phosphate being effective in "Z" DNA stabilisation depending on its position (Jovin et al 1983).

A number of reactions may also occur at the N7 position of guanine residues (see carcinogens). Platinum complexes such as Chloro(diethylenetriamine) Platinum (II) chloride stabilise "Z" form DNA by the contribution of the positively charged cation at the N7 position which may contribute to stabilisation by electrostatic means similar to high salt concentrated solutions. (Malfoy et al 1981, Ushay et al 1982). When Poly (dG-dC).Poly (dG-dC) is fully methylated at N7 it is stable in

"Z" conformation in physiological salt conditions (Moller et al 1981). Methylation at N7 will also introduce a positive charge.

Cytosine Methylation in CG Sequences.

Methylation of the cytosine C5 position in CG sequences is one of the most common modifications of eukaryotic DNA. Although CG sequences are not abundant in eukaryotic DNA (Bird 1980) they do exist, evidence suggests that methylation of CG residues is associated with gene inactivation and subsequent removal of the methyl group is associated with gene activation (Doerfler 1983). The conformation of the CG dinucleotide in "Z" form DNA is different to the CG sequence in "B" DNA and this may be important in the modification of gene expression by methylation (Wang et al 1979). On comparison of the "B" to "Z" equilibrium in Poly (dG-dC).Poly (dG-dC) and Poly (dG-m^5dC).Poly (dG-m^5dC) in a solution with 50mM NaCl, the midpoint of the "B" to "Z" transition using Mg^{2+} ions is reduced by three orders with the methylated polymer (Behe and Felsenfeld 1981). Similar results can be obtained with Na^+ and K^+ ions; with the polyamines spermine and spermidine being even more efficient, the transitional midpoint is only 2μM using spermine [see stabilisation by ions]. Methylation of the cytosine residues stabilises the polymer in "Z" conformation under physiological salt concentrations and is also effective in stabilising "Z" DNA inserts of alternating CG sequences in plasmids reducing the number of negative supercoils required to stabilise the "Z" form (Klysik et al 1983).

The structure of a methylated hexamer $(m^5C\text{-}dG)_3$ has been solved (Fujii et al 1982) at 1.3Å resolution. The methyl groups are found in pairs on the surface of the molecule filling a slight hydrophobic depression formed by the imidazole group of guanine from the next base pair and the C1' and C2' hydrogen atoms of the sugar. This depression is filled by water molecules in the unmethylated polymer. The methyl group forms a small hydrophobic patch on the surface of the molecule that stabilises it by excluding water from the hydrophobic pocket. The position of the methyl group in "Z" DNA is in contrast to its position in "B" DNA where it

projects into the major groove of the double helix and is surrounded by water molecules. The enviromental difference of the methyl group in "Z" and "B" DNA is a major factor in its strong stabilisation of "Z" DNA. In low salt solutions the methylated oligomer is largely in "B" form with only 2-4% in "Z" form, but addition of salt, methanol or $NaClO_4$ produces predominant "Z" form.

Carcinogens.

Most chemical carcinogens bind covalently to DNA. This covalent binding distorts the double helix and may be important in tumourigenic processes.

Covalent binding of acetylamminofluorine (AAF) residues to the DNA occurs at the C8 position of guanidines causing local distortions and effecting the conformation of certain DNA sequences (Spodheim-Maurizot et al 1979, Harvan et al 1977). Binding of AAF forces the guanidine residue into the syn conformation. If binding occurs in a DNA sequence which does not favour "Z" DNA the guanidine remains in the syn conformation with the fluorene ring inserted into the double helix and the guanosine residue on the outside of the helix. This causes a distortion of the double helix where the cytosine residues are no longer paired with the modified guanine residues. However if conditions and sequence favour a conformational change to "Z" form DNA the modified guanine residues are inside the helix paired with cytosine residues and the fluorene ring is outside the helix. Covalent binding of AAF residues to GC rich sequences has been shown to favour the "Z" DNA conformation (Sage & Leng 1980). The binding of the carcinogen N-acetoxyaminofluorene to the guanidine C8 position stabilises the "Z form (Sage & Leng 1980 /81, Hanau et al 1984) suggesting the presence of a bulky substituent at this position prevents the assumption of the anti conformation.

It has recently been argued that the reaction of carcinogenic amines (including AAF) with guanine residues does not occur in classical "B" DNA but with a transient conformational states, different amines stabilising different intermeadiate transient states (Daune et al 1985, Marrot et al 1987).

The carcinogen aflotoxin on reacting with the N7 of guanine in DNA prevents the "B" to "Z" conversion of the polymer on addition of salt by steric hinderance (Nordheim et al 1983) [Indicating that this position is accessible in "B" form DNA].

Chemical modification of DNA may cause important effects on cellular processes. The absence of base pairing may inhibit DNA replication (Sage & Leng 1980).

Other Chemical Modifications.

An anticancer drug arabinosylcytosine, effective in the treatment of human acute myelogenous leukemia is incorporated in DNA but not RNA (Kufe & Spriggs 1985). The insertion of araC into GC alternating sequences facilitates the "B" to "Z" conversion by providing a strong intramolecular O2'-N2' hydrogen bond, stabilising guanine in the syn conformation. The O2' hydroxyl groups of the araC residues are buried in the deep groove of the "Z" DNA helix and not accessible to the outer solvent region. AraC "Z" DNA therefore has the same surface as native unmodified "Z" DNA. All other chemical modifications that facilitate a "B" to "Z" transition introduce perturbations on the surface of the "Z" helix. Thus it may be useful to use this modification in protein binding studies (Teng et al 1989).

Other anticancer nucleotides also effect the "B" to "Z" transition (Coll et al 1989, Wang 1987).

Intercalating Agents.

Intercalators (Pohl et al 1972, Mirau & Kearns 1983), such as ethidium bromide, on addition to Poly (dG-dC).Poly (dG-dC) in "Z" form convert the polymer back to intercalated "B" DNA (Di Capua et al 1983, Jovin et al 1983, Hagen et al 1985). "B" form DNA has a more flexable backbone and is able to form an intercalated complex with calaters such as ethidium, whereas "Z" DNA is a more rigid molecule and may not be able to accomadate an intercalating agent between its base pairs (Thomas & Bloomfield 1983). The powerful ability of the calating agent to induce a "Z" to "B" DNA conversion is shown by the observation that bromination does not lock the polymer in "Z" form on the addition of ethidium bromide (Moller

et al 1984) insertion of the intercalating agent modifying the equilibrium providing calated "B" form DNA. In closed circular DNA the concentration of ethidium bromide required for reversal of the "B"-"Z" transition depends on superhelical density (Thomae et al 1983, Di Capua et al 1983). Ethidium bromide also inhibits the transition of "B" to "Z" DNA (Mirau & Kearns 1983).

Antitumour agents daunomycin actinomycin D, mithramycin, adriamycin ,daunomycin, distamycin, netrospin, aureolic acid and bismethidiumspermine, have all been shown to facilitate the formation of "B" DNA on introduction to the polymer in "Z" form and inhibit the "B" to "Z" DNA transition by intercalation with DNA (Chaires 1984, Chen et al 1983, Zimmer et al 1983, Mirau & Kearns 1983, Shafer et al 1988). These drugs are from the intercalator classes of acridines, phenanthridiums, actinomycins and bisintercalators.

Ions.

Ions, especially cations strongly influence the equilibrium of "B" to "Z" DNA in solution (Pohl & Jovin 1972, Eichorn et al 1983, Zacharias et al 1983) and are likely to alter the ease of "Z" DNA formation in vivo. The predominant interaction being the cations clustering around the negatively charged phosphates and reducing the phosphate-phosphate repulsive forces. However if the transition occurs above molar salt concentration, electrostatic considerations are probably secondary to solvent effects. For instance the activity of the solvent water is lowered by increased NaCl concentrations which drives the DNA into the less solvated "Z" conformation. The monovalent cations, sodium, potasium and lithium have all been shown to influence the equilibrium with the midpoint for sodium "B" to "Z" conversion being 2.7M (Pohl & Jovin 1972). However lower concentrations of divalent ions are required with the mid point for magnesium being only 0.7M. Ions with higher valencies are even more effective such as the naturally occuring polyamines with charges of +3 [spermine] [putrescine] or +4 [spermidine] (Wang et al 1979, Behe & Felsenfeld 1981, Russell et al 1983, Chen et al 1984, Thomas & Messner 1988, Banville et al 1991, Ohishi et al 1991, Thomas et al 1991). In

addition to ionic interactions, hydrogen bonding and hydrophobic interactions lead to polyamines binding to specific sites on "Z" DNA (Basu et al 1988, Tomita et al 1989) and more tightly to "Z" than "B" form (Banville et al 1991). Recent X-ray crystallography studies (Williams et al 1990, Gessner et al 1989) show that spermine molecules are localised along the edge of the deep groove. As with rubidium (Thomas & Thomas 1989) the cobalt (III) hexamine complex is very efficient in stabilising "Z" DNA at micromolar concentrations (Behe & Felsenfeld 1981, Thomas & Bloomfield 1985, Thomas & Messner 1988, Peck et al 1982, Eichorn et al 1983). The cobalt hexamine cation is 5 orders of magnitude greater than the sodium ion and 4 orders greater than the magnesium in stabilising "Z" DNA (Behe and Felsenfeld 1981) although, as with polyamines (Chattoraj et al 1978, Gosule & Schellman 1976/78, Wison & Bloomfield 1979, Schellman & Parthasarathy 1984), aggregation can occur at high concentrations (Widom & Baldwin 1980, Shin & Eichhorn 1977, Schorschinsky & Behe 1986, Jordan et al 1972). It has been suggested that DNA condensation in aqueous solutions is caused by cations with a charge of 3+ or more which can be rapidly reversed by mono and divalent cations [Na^+, Mg^{2+}] (Widom and Baldwin 1980). DNA condensation in aqueous solution is proposed to occur when a critical fraction of the DNA phosphate charge is neutralised by cations (Wilson & Bloomfield 1979) also cation crosslinking and electrostatic bridging of adjacent helixes by trivalent or higher valence cations play a role (Widom and Baldwin 1980). However sequences which contain bases other than alternating GC prove more difficult to induce "B" to " Z" transitions by cations alone (Schorschinsky & Behe 1986). Binding of hydrated cations to bases and phosphate oxygens causes the "Z" variant Z_I to convert to Z_{II} at these sites (Wang et al 1981, Gessner et al 1985, Ho et al 1987, Harder & Johnson 1990).

Anions also influence the direction of the equilibrium; sodium perchlorate has a greater effect on the stabilisation of "Z" DNA than its cation alone (Pohl & Jovin

1972), while sodium acetate has been shown to produce an intermediate DNA form between the "B" and "Z" conformation (Zacharias et al 1983).

These studies suggest that ionic charges are also likely to influence the ease of "Z" DNA formation in vivo.

Solvents.

A variety of agents that change the dielectric constant of water have been found also to stabilise "Z" DNA. Studies report that ethanol, methanol, ethylene glycol and trifluoroethane can achieve stabilisation (Pohl 1976, Feigen et al 1983, Zimmer et al 1982, van de Sande et al 1982a&b, Zacharias et al 1982, Chen 1988). The mechanism for stabilisation is not clear; the lowering of the dielectric constant allows ionic interactions to have a greater effect resulting in a closer clustering of cations around the negatively charged phosphate groups reducing the phosphate-phosphate repusion. However other factors may play a role.

Temperature/polymer size.

The ability of sequences to adopt lefthanded conformation depends not only on sequence but also on temperature and length. In general the helical pitch of DNA increases with decreasing temperature (Depew & Wang 1975). Thus there is likely to be more torsional strain and hence more "Z" DNA formation at lower temperatures.

The "B" to "Z" transition of d(CG) sequences is influenced much more than in d(TG) sequences when in plasmid DNA. Analysis of thermodynamic factors indicates that d(TG) sequences of above 60bp in length within plasmid DNA at high temperatures [above $60^{o}c$] favour "Z" conformation more so than d(CG) sequences of a similar length (Connor et al 1986). The reverse being seen at lower temperatures. Other studies have postulated that at higher temperatures and long chain lengths d(CG) sequences in plasmids preferentially form cruciform structures (Frank-Kamenetskii & Vologodskii 1984) whereas d(TG) sequences do not.

In oligonucleotide studies "Z" formation appears to favour low temperatures under high NaCl concentrations (Chen 1988, Quadrifoglio et al 1981).

Widom and Baldwin 1980 report that cation-induced DNA condensation occurs more readily at high temperatures and restriction fragments as short as 400 base pairs form toroids by intramolecular condensation.

Negative Supercoiling.

The supercoiled state of DNA is associated with an unfavourable free energy relative to the relaxed state, consequently processes that reduce the number of superhelical turns are favoured. In negatively supercoiled circles these processes include base unpairing, strand separation, cruciform formation, unwinding of the double helix, protein binding as in nucleosome formation or "Z" DNA formation. The energy of supercoiling is used to stabilise a segment of "Z" DNA (Peck et al 1982, Singleton et al 1982) reducing the torsional strain. On the transition of a segment of righthanded helix to a lefthanded conformation approximately two negative supercoil turns are removed as a single turn of lefthanded "Z" DNA is created (Klysik et al 1981, Singleton et al 1982/83/84, Kilpatrick et al 1983/84, Stirdivant et al 1982, Peck et al 1982, McLean et al 1986). Any "Z" DNA present in living cells is likely to be induced at least partially by supercoiling.

Initial studies with plasmids containing inserts of alternating GC residues up to 42 bp in length (Nordheim et al 1982b, Stirdivant et al 1982) show that "B" to "Z" transitions can occur at natural negative superhelical densities (Peck et al 1982) and an inverse correlation between the superhelical energy required and the length of DNA segment stabilised in "Z" conformation exists. The torsional stress of negative supercoiling is a major force in stabilising "Z" DNA in 'natural' sequences. Lefthanded "Z" DNA regions can expand under high tortional stress to include nonalternating purine/pyrimidine sequences (Johnston & Rich 1985). In bacteria the negatively supercoiled pBR322 plasmid shows a naturally occurring sequence consisting of 14 base pairs of alternating purine/pyrimidine residues with one base pair out of alternation to form "Z" DNA conformation (Nordheim et al

1982a, Dicapua et al 1983). At high superhelical densities additional "Z" DNA segments are identified in pBR322 (Nordheim et al 1982a, Dicapua et al 1983). The negative superhelical density of plasmids in bacterial systems is generally in the range of -0.05 to -0.07 although higher levels have been reported. The highly supercoiled genome of the bacteriophage PM2 exists at a superhelical density of -0.12; and "B" to "Z" transitions are induced by this negative supercoiling (Kuhnlein et al 1980, Stockton et al 1983). The PM2 genome has been shown to contain eight different segments of alternating purine/pyrimidine sequences in the "Z" conformation (Miller et al 1983, Stockton et al 1983). Different "Z" DNA forming sequences also appear in the negative supercoiled bacteriophage ØX174 (Revet et al 1984). Small changes in salt concentrations are found to greatly influence the energy required for "B" to "Z" transitions induced by supercoiling (Singleton et al 1982, Peck et al 1982, Peck & Wang 1983, Nordheim & Rich 1983).

"Z" DNA conformation can also be observed in form "V" of plasmid pBR322 DNA (Lang et al 1982, Brahms et al 1982, Pohl et al 1982, Brahmachari et al 1987). "V" form DNA is prepared by annealing complementary single-stranded circles of DNA (Stettler et al 1979) creating a highly torsionally strained molecule with 80-90% of the residues in an ordered and base paired structure. If these plasmids reanneal to form righthanded helical segments it is topoilically constrained to also form a similar number of lefthanded helices (see "Z" DNA in recombination). These plasmids do not contain a high percentage of alternating puine/pyrimidine residues indicating that nonalternating base sequences can adopt "Z' conformation if suitably torsionally strained

"Z" DNA Binding Proteins and their Purification.

If "Z" DNA or other non "B" conformations are to have any biological significance then it seems reasonable to postulate that there will be specific protein/DNA interactions. These proteins will recognise the non "B" conformation and bind

preferentially to it, which may serve to stabilise the conformation. Two classes of proteins may be involved in this recognition, those that bind to "Z" DNA with sequence specificity and those that bind independent of sequence; inferring considerable functional differentiation. Proteins may have the property of binding both "B" and "Z" DNA and act to shift the equilibrium between these conformations. Thermodynamic reasoning proposes that high affinity binding of protein will shift the conformation equilibrium (Jovin et al 1983).

Addition of polyarginine but not polylysine to Poly(dG-dC).Poly(dG-dC) in the high salt form allows the retention of "Z" conformation on dialysis to low salt concentration (Klevan & Schumaker 1982). This suggests that "Z" DNA stablisation may involve specific interaction of the arginine side chain with the DNA helix as well as electrostatic forces. A later study (Russell et al 1983) established that as well as polyarginine, nucleosome core histones H_3, H_4, H_{2a} and H_{2b} and also protamine stabilise "Z" DNA and prevent its transition to "B" form. In contrast the linker histones H_1 and H_5 promote a "Z" to "B" transition. These linker histones bind more efficiently to left-handed Br-Poly(dG-dC).Poly(dG-dC) than the unmodified righthand form however a reduction in the characteristic "Z" DNA spectral features is observed upon binding (Mura and Stollar 1984).

Proteins from the nuclei of tissue culture cells derived from Drosophila Schneider have been isolated by "Z" DNA affinity chromatography (Nordheim et al 1982b). These proteins only bind "Z" form Br-Poly(dG-dC).Poly(dG-dC) and not natural "B" DNA sequences. Five major and several minor "Z" specific proteins are identified, many have a molecular weight over 70k, none being histones. These naturally occurring drosophila proteins also influence the "B" to "Z" equilibrium of Poly(dG-m^5dC).Poly(dG-m^5dC) in 0.1M NaCl and bind to negatively supercoiled plasmids containing "Z" form inserts $(GC)_n$ or $(CA/GT)_n$, but not to relaxed plasmids. Binding appears to be stronger with CA/GT segments which are known to occur in the drosophila genome suggesting sequence specificity. Nuclear

proteins from Drosophila melanogaster embryos bind specifically to the polymeric sequences Poly(dG-dT).Poly(dC-dA) and Poly(dG-dA).Poly(dC-dT) (Vashakidze et al 1988). Three proteins of molecular weights between 65-72k bound specifically to GT/CA sequences while various proteins in the region of 94-98k and 41-47k bound specifically to GA/CT sequences.

Proteins from wheat germ extracts can also be purified using "Z" DNA affinity columns (Lafer et al 1985). Three major proteins [90, 58 &40k] and two minor proteins [140 & 80k] have a 10^5 greater affinity for Poly (dG-dG).Poly (dG-dC) in the "Z" form than "B" form. Again sequence specificity is exhibited by a 2-3 fold increased affinity for $(dC\text{-}dA)_n(dT\text{-}dG)_n$ over $(dG\text{-}dC)_n(dG\text{-}dC)_n$ sequences in "Z" form within supercoiled plasmids.

A protein which preferentially binds "Z" form duplex DNA purifies from the cells of Deinococcus radiodurans (Kitayama et al 1985/88). This extremely radioresistant bacterium contains DNA which has a GC content of approximately 70%. This protein has an apparent molecular weight of 70k, binds both "Z" and "B" DNA but has a higher affinity for "Z" and is shown to stimulate RNA synthesis in vitro.

Azorin and Rich (1985) use affinity chromatography to isolate "Z" DNA binding proteins from purified SV40 microsomes. These proteins bind to left but not righthanded DNA, binding with greater affinity to "Z" DNA in supercoiled SV40 than in plasmids not containing SV40 sequences. This suggestion of sequence specificity is confirmed by mapping the binding to the Sph I sites in the SV40 control region. The binding activity is associated with a protein of 200k. Affinity for "Z" form $d(GC)_{12}$ in plasmid DNA is approximately 10^2 greater than for "B" form; higher affinities are reported for natural "Z" sequences in pBR322 and SV40.

Left-handed DNA affinity chromatography on E.coli cell extracts in the presence of excess "B" DNA purifies three proteins [50, 90 &100K]. On comparison to "B"

DNA binding the 50k protein has a 100 fold higher affinity to "Z" DNA, while the 90k has a 1000 fold and the 100k more than 1000 fold affinity.

The first isolation of "Z" binding proteins in mammalian tissue was from bull testis (Gut et al 1987). The proteins [31, 33 & 58k] have a high affinity to Poly (dG-dC).Poly (dG-dC) with the 58k protein binding with equivalent affinity to that of polyclonal anti-"Z" DNA serum. This study proposes that the proteins may have a role in recombination events.

Studies on the recombination protein rec 1 from the lower eukaryote Ustilago maydis observe a dramatic twenty fold preference for the "Z" conformation in DNA polymers and plasmids (Kmeic et al 1985, Kmeic & Holloman 1986, Zarling & Carlisle 1986).

Left-handed DNA binding studies involving the prokaryote rec A protein of E. coli, which mediates homologous recombination, indicates a two to seven fold preferential binding to "Z" DNA polymers (Blaho & Wells 1986).

A 74k protein, purified from human T-cell extracts, also catalyses homologous pairing reactions and DNA strand exchange (Moore & Fishel 1990), is shown to bind strongly to "Z" DNA (Fishel et al 1988).

It is important to note that conformation specific binding proteins are not restricted to lefthanded DNA structures; for instance cruciform structures are postulated to occur as a consequence of general and site specific recombination or in palandromic DNA sequences. Protein which binds specifically to cruciform DNA molecules has been purified from rat liver (Bianchi 1988) and human lymphoblasts (Elborough & West 1988).

The Existence and Role of "Z" DNA in vivo.

The anticipation that the formation of "Z" DNA in biological systems may have biological effects has fueled the search for the role of "Z" DNA in vivo.

All circular DNA isolated from natural sources exists in the negatively supercoiled underwound state. The discovery of DNA gyrase an ATP dependent enzyme that

catalyses the negative supercoiling of DNA in vitro suggested that intracellular DNA could exist in a metastable underwound state (Gellert et al 1976). Genetic evidence suggests that the degree of negative supercoiling in vivo is maintained by a dynamic balance between the actions of DNA gyrase (topoisomerase II) and DNA topoisomerase I (DiNardo et al 1982).

It is now accepted that supercoiled conformations are not restricted to covalently closed circular DNA molecules. Supercoiling with linear double stranded DNA genomes has also been reported; for instance in intracellular DNA in eukaryotic cells (Giaever & Wang 1988), bacteriophages and animal viruses (Sinden & Pettijohn 1981, Virrankoski-Castrodeza & Parish 1980, Wong & Hsu 1989). In each of these cases there is a requirement for either host or viral topoisomerases in their life cycles (Alonso et al 1981, Benson & Huang 1988, Constantinou et al 1986, Poddar & Bauer 1986). In mammalian cells, topoisomerase II has been implicated in formation of loop domains (Cockerill & Garrard 1986, Gasser & Laemmli 1986).

The topological tension of supercoiling in linear DNA is usually dissipated through free rotation of the teminal ends. However if the DNA is organised into loops with tight binding of proteins at the base, the loops will form topologically constrained psuedocircles. The presence of chromosomal DNA loops in both prokaryotic and eukaryotic cells has been suggested by a number of observations with each loop shown as an independent topological domain (Gross & Gerrard 1988, Jackson et al 1990, Wu & Liu 1991, Paulson & Laemmli 1977, Dickinson et al 1990). These observations indicate that DNA topology plays an important role in genomes which may be arranged in different topological forms through DNA-protein interactions within cells (Stonington & Pettijohn 1971, Worcel & Burgi 1972, Pettijohn & Hecht 1974). Protein-mediated DNA looping is not restricted to linear genomes as shown recently by in vivo studies using the lac repressor-operator system within plasmid DNA molecules containing two repressor binding sites (Wu & Liu 1991). Nuclear scaffold proteins organise yeast mating type genes into loops and some

bacterial regulatory proteins regulate genes from a distance by looping out the intervening DNA segment (Ptashne 1986). The possibility that looped DNA domains may regulate chromatin structure and gene expression in higher eukaryotes has been expressed (Weintraub 1985). Local topological stresses arising within these domains may be relieved temporarily by transient transitions between right and lefthanded DNA helices. Studies using anti-"Z" immunoglobulin agrees with the claim that local superhelical strain can be stabilised by polypeptides on creation of looped domains (Revet et al 1984).

Recent studies have suggested that in addition to DNA topoisomerases, RNA transcription can also significantly alter the supercoiling state of cellular DNA (Pruss & Drlica 1989, Brill & Sternglanz 1988). The transcription process generates positive supercoiling in the forward direction and negative supercoiling in the backward direction in respect to the RNA polymerase, (Liu & Wang 1987, Wu et al 1988, Wang & Giaever 1988, Tsao et al 1989, Koo et al 1990, Reece & Maxwell 1991, Wu & Liu 1991) and this would normally be dissipated by free rotation of the DNA causing topological tensions in constrained DNA loops. Chromosomal DNA loop attachment sites have been proposed to effectively restrict the rotational diffusion of supercoils generated by RNA transcription processes (Lui & Wang 1987, Wu & Liu 1991). The accumulation of torsional stress created by the elongating RNA polymerase has shown to be sufficient to induce and stabilise local "Z" DNA segments in circular plasmid DNA (Droge & Nordheim 1991) and can be relaxed by wheat germ topoisomerase I. Additional support for the model is found in a report showing that G/C alternating tracts can be detected in vivo 5' upstream from the tetracycline resistance (tet) gene of the plasmid pBR322 in E.coli but not downstream (Rahmouni & Wells 1989). These findings suggest that "Z" DNA formation may serve as a release mechanism for local accumulation of negative stress in vivo. DNA in a transient state can be highly negatively supercoiled (Baker et al 1987, Liu & Wang 1987) with the supercoiling generated by moving RNA polymerase similar to or higher than that experienced in

plasmids.The positive supercoils induced by the transcription process appear to displace nucleosome cores from chromatin in vitro (Benyajati & Worcel 1976) and perhaps in vivo (Pfaffle et el 1990, Pederson & Morse 1990, Clark & Felsenfeld 1991). This transient opening of nucleosomes associated with transcription may provide an additional source of unrestrained negative supercoiling sufficient to facilitate transient DNA conformational changes.

It is now understood that the overall extent of DNA supercoiling within a cell is much less relevent than the local transient degree of supercoiling which is altered by cellular enzymatic processes.

The polytene chromosome in Drosophila melanogaster was the first biological system to be studied in vivo and used antibodies specific for "Z" DNA (Nordheim et al 1981). The polytene chromosome is studied as it consists of 1000 to 2000 copies of the genome which allows amplification on detection of "Z" DNA in the multiple copies; also a variety of functional states can be seen at any one time since the chromosome is fully active in transcription and replication. These studies reproducibly yield strong antibody binding although localisation and strength of signal depend greatly on the fixation technique used in the preparation of the cytological specimen (Lemeunier et al 1982, Ardnt-Jovin et al 1983, Hill & Stoller 1983, Jovin et al 1983, Pardue et al 1983, Hill et al 1984, Robert-Nicoud et al 1984, Zarling et al 1984, Lancillotti et al 1985, Nordheim et al 1986). The classical method for the chromosome preparation employs the squashing of tissue in an acid fixative (Belling 1926) dissolving most cell structures, leaving the chromosomes fixed and ready for microscopic analysis. Some researchers found antibody binding prodominantly to interbands (Pardue et al 1983) while others largely to bands (Jovin et al 1983). The knowledge that solvents used in different fixation techniques affect to varying degrees parameters that influence DNA conformation, led to doubts about the existence of "Z" DNA in the native state. Solvents such as acetic acid or ethanol, in addition to denaturing DNA, remove proteins bound to DNA (eg histones, Dick & Jones 1968) releasing negatively

supercoiled, elastically strained DNA which may adopt "Z" DNA (Wittig et al 1989).

Reports of the absence of "Z" DNA in unfixed polytene chromosomes isolated by micromanipulation appeared to confirm these doubts (Hill & Stoller 1983, Robert-Nicoud et al 1984). These studies used a procedure avoiding exposure to acid fixatives maintaining the macromolecules close to the native state (Hill & Watt 1978) and found only background immunofluorescence. Whereas on exposure of the native chromosomes to acetic acid fixative, "Z" DNA immmunoradioactivity appears at the readily accessible interbands after 5 seconds and becomes more intense as penetration increases, activity being predominantly over the chromosome bands by 30 seconds. This observation was generally accepted as an explanation of the previously conflicting results although existence of "Z" DNA in vivo was not completely ruled out. Removal of the microdissected chromosome into a buffer environment different from the intact nucleus or the presence of active endogenous topoisomerases during chromosome isolation may have resulted in a loss of preexisting "Z" DNA. Indeed a more recent study (Lancillotti et al 1987) reported that in native conditions "Z" DNA forms are detected in specific sites on the transcriptionally active chromosomes of Drosophila Hydei. These results are obtained by procedures known not to induce the "B"-"Z" transformation and suggest that the cytological correlation observed between "Z" form DNA and transcription in regions of dispersed chromatin is restricted to specific genes. Various subdivisions of the chromosomes induced to high transcription rates showing different patterns of "Z" DNA reactivity. Strong anti-"Z" DNA antibody reactivity in transcriptionally active subregions induced by ecdyone (Lancillotti et al 1985) supports the observation that "Z" DNA segments in vivo are mainly localised in specific subdivisions of dispersed DNA involved in transcription. Lancillotti proposed that anti-"Z" DNA antibody binding was probably correlated with specific changes in the electrostatic enviroment of potential "Z" DNA segments during the transcriptional process. The more recent observation that the

transcription process generates positive supercoiling in the forward direction and negative supercoiling in the backward direction (Liu & Wang 1987, Wu et al 1988, Tsao et al 1989, Koo et al 1990, Wu & Liu 1991) confirms that immunoreactivity is likely to be related to transcription events with local accumulation of torsional stress being a more probable stabilising force for "Z" DNA. In a recent study "Z" DNA was found to be localised in limited areas inside the unfixed chromosomes of the primitive eukaryote dinoflagellate Prorocentrum micans. This organism shows a permenently well organised DNA structure with no histones and nucleosomal system that would modulate DNA supercoiling. Thus making it a very good model for studying altered DNA structures in vivo. The "Z" DNA was localised at the periphery or near to the segregation fork of dividing chromosomes indicating that it may be driven by torsional stress created by the DNA replication process (Soyer-Odile et al 1990). Similar experiments using immunoflourescence show the presence of "Z" DNA in the polytene chromosomes of Choronomus (Robert-Nicoud et al 1984, Jovin et al 1983), in chromosome material from the nuclei of four different plant species (Jones et al 1983); in the differentiated nuclear apparatus of the ciliated protozoan Stylonichia mytilus (Lipps et al 1983), in metaphase chromosomes of primates (Viegas-Pequinot et al 1983), in nuclei from gerbil tissue (Viegas-Pequinot et al 1982) and in the cell nuclei of several rat tissues (Morenegg et al 1983).

The question of to what extent DNA is negatively supercoiled in vivo, and its effect on the existence of "Z" DNA in vivo within the nucleus under physiological conditions was recently studied (Wittig et al 1989). Previous studies, described above, involved chromatin which was no longer in the physiological environment of the nucleus (Zarling et al 1984) and the relevence to in vivo situations is in doubt. The novel technique of encapsulating unfixed, metabolically active, permeabilized mammalian cell nuclei within agarose microbeads (Jackson & Cook 1985) in which the DNA was accessible to anti-"Z" DNA antibodies aberently solved these problems (Wittig et al 1989). The existence of "Z" conformation DNA

in nuclei was shown to be stabilised by torsional strain indicated by increased antibody binding on inhibition of topoisomerase I with camptothectin and addition of DNase I resulting in the complete loss of antibody binding (Wittig et al 1989). A dramatic increase in antibody binding shown to be associated with RNA transcription and DNA replication supports the finding that torsional stress in DNA is involved in the stabilisation of "Z" DNA (Wittig et al 1991). As previously described transcription and replication processes are known to generate supercoiling (Liu & Wang 1987) under the conditions used above transcription had a more profound effect than replication. However DNA synthesis only occurs during S phase and the number of cells in this phase was not known. The finding that high concentrations of anti-"Z" DNA antibodies induced "Z" DNA formation (Lafer et al 1985b/86) provides a reminder that caution must be taken in future studies using antibody to determine the existence of "Z" DNA in vivo and in interpretation of previously published experimental data. Wittig and coworkers have subsequently shown transcription dependent anti-"Z" DNA antibody binding localised within the c-myc gene.

Another early identification of "Z" DNA in a eukaryotic genome was in the negatively supercoiled circular minichromosome of the simian DNA tumour virus SV40 (Nordheim and Rich 1983b, Jovin et al 1983, Nordheim et al 1986). The sequences which form "Z" DNA map to three major regions, two are identical in sequence positioned within the 72bp repeat transcriptional enhancer element of the viral early promoter with the other just outside. Other studies suggest the existence of at least two sites of "Z" DNA (Zarling et al 1984a/b) and confirm "Z" DNA sites within the transcriptional enhancer (Hagen et al 1985). A more recent report (Nordheim et al 1987) identified previously undetected strong anti-"Z" antibody binding sites in the early and late promoter regions as well as those reported in the enhancer element. This study used higher superhelical densities and emphised the structural flexibility of regulatory DNA elements within the SV40 genome when under torsional stress. The extent of negative supercoiling of the

genome in vivo is not known although reports indicate that the transcriptionally active fraction of microchromosomes are under torsional strain. Enhancers are cis-acting components of eukaryotic promoters that stimulate transcription independently of position and orientation (Guarente 1988) and may represent entry sites for transcriptional factors into the chromatin such as RNA polymerase. The SV40 enhancer element is positioned within the viral control region which is believed to exist in a nucleosome-free chromatin-like structure in transcriptionally poised SV40 minichromosomes (Varshavsky et al 1978). DNase I hypersensitivity, usually associated with transcriptionally active chromatin (Smith et al 1983, McGee et al 1981), can be identified in this region and maps to the enhancer region. The cleavage locates to 25bp on either side of the "Z" segments which may be due to the presence of a DNA binding protein (Nordheim & Rich 1983, Azorin & Rich 1985).

At least two of the identified "Z" DNA segments are thought to be important in the physiological activity of the SV40 enhancer. SV40 mutants with altered sequence within and around the 72bp repeat enhancer region show a requirement for these "Z" segments (Fromm & Berg 1982, Banerji et al 1981) as does the spacing between them (Moreau et al 1981).

Computer searches of the SV40 DNA revealed one 9bp alternating purine/pyrimidine sequence, three 8bp and sixteen 7bp sequences (Readdy et al 1978, Fiers et al 1978). This proved an under representation of these sequences relative to a random distribution. The three "Z" DNA regions mapped to the 8bp sequences; the 9bp sequence contains the bases ATAT making it less likely to form the "Z" conformation and the 7bp segments appear not to create any detectable "Z" form.

A survey of other transcription enhancers reveals pairs of segments with potential for "Z" DNA formation in a number of enhancers with 50 to 80 base pairs between them (Nordheim & Rich 1983). This doubling may be of functional importance implying that the evolution of transcriptional activators as duplicated DNA

sequences (Weiher et al 1983) and suggests that "Z" DNA stabilised by specific binding proteins may have a significant role in the enhancer function. The presence of pairs of "Z" DNA segments invites the possibility that two binding proteins, subunits of a dimer, attach to the "Z" elements wrapping the DNA around them. Such a particle could effect local chromatin structure and provide a recognition site for RNA polymerase or other transcriptional factors. Nordheim and Rich (1983) speculate a mechanism whereby the removal of the "Z" DNA binding protein facilitates a local increase in negative superhelicity representing a form of transcriptional regulation (Smith 1981, Nordheim 1981). A 9bp "Z" DNA sequence essential for transcription is found in the 5' flanking region of the human metallothionein gene (Karin et al 1984) with two similar sequences located 43 and 73bp upstream. This "Z" segment is associated with transcriptional enhancer activity. A metallothionein apoprotein binding site is situated between these "Z" sequences and induction of the gene occurs when a heavy metal removes the protein. This mechanism may effect the "Z" binding protein activity in enhancer regulated transcription.

The relationship of "Z" DNA segments to transcriptional control may also be considered in relation to cellular oncogene activation by RNA retroviruses. Integration of enhancer-associated viral "Z" DNA sequences may occur independently of orientation both upstream and downstream of cellular oncogenes (Payne et al 1982). Oncogene activations may be governed by the length and potential for "Z" DNA formation within the regulatory sequences with mutational modifications altering the effect on transcriptional control.

"Z" DNA has been shown to be a negative effector of RNA polymerase III transcription (Hipskind et al 1983a&b). Thus removal of a 9bp DNA segment with "Z" forming potential from the 5' flanking sequence of the xenopus met-tRNA gene resulted in transcriptional activation. Aggregated "Z" form DNA is capable of acting as a template for RNA polymerase II although transcript production is slower than with "B" form templates (Durand et al 1983). On conversion of the

polymer Poly(dG-dC).Poly(dG-dC) to "Z" form, a decrease in transcriptional activity is also observed when used as a template with E. coli RNA polymerase (Butzow et al 1984). Insertion of a $d(GC)_{16}$ sequence downstream of a prokaryotic promoter and inducement of "Z" DNA by supercoiling, results in transcription terminating at the "B" to "Z" junction in vitro (Peck & Wang 1984). However the blockage of transcription was not observed when it was replaced by a $(dT\text{-}dG)_{21}.(dC\text{-}dA)_{21}$ insert (Peck & Wang 1985). This can be explained on the basis that the structure of the "B" to "Z" junctions formed with d(TG) segments is more ordered than those with d(CG) sequences (O'Connor et al 1986). Therefore the difference in junction structure from "B" form DNA is less of an obstacle with d(TG) than with d(CG) "Z" form segments. Insertion of d(GC) sequences into the lac Z gene of E. coli has also been shown to inhibit the expression of ß galactosidase in vivo (Horbach & Muller-Hill 1988). Recently $d(TG)_nd(CA)_n$ sequences upstream of the rat prolactin gene form "Z" DNA and inhibit gene expression (Naylor & Clark 1990). The basal level of transcription of this gene has been shown to be dependent on localised topoisomerase type II regulated changes in DNA torsional stress (White & Preston 1988). Thus supporting the model of transcription induced supercoiling facilitating conformational changes in DNA topology which inturn may have effects on metabolism if the torsional stress is not released by topoisomerase activity. It is thus possible that "Z" DNA formation in vivo may result in termination by the RNA polymerase thus acting as a regulatory system in highly supercoiled templates responsive to the supercoiled state. An interesting recent observation is removal of a transcriptional block caused by a lefthanded "Z" form segment or a cruciform structure in supercoiled DNA by HMG1 protein illustrating that the binding of protein to "Z" DNA or other altered DNA forms may be a regulatory form of control (Waga et al 1988/90).

Studies on the ability of DNA polymerase I [large fragment] from E.coli to recognise different DNA conformations indicate that "Z" form templates promote less activity (Ramesh et al 1986) suggesting the probable inability of the

polymerase to move along the conformationally rigid "Z" DNA molecule. Others observe that brominated Poly (dG-dC).Poly (dG-dC) in the "Z" form does not serve as a template for E. coli DNA polymerase I and avian myeloblastosis virus DNA polymerase (Brahmachari et al 1985).

A cluster of simple repeated sequences composed of $(GC)_5(AC)_{18}(AG)_{21}(G)_9(CAGA)_4$GAGGGAGAGAGGCAGAGA GGG$(AG)_{27}$ associated with the chinese hamster dihydrofolate reductase (dhfr) gene and located near the origin of replication (2kb 3' downstream) adopts multiple "Z" form and triplex DNA structures in recombinant plasmids (Bianchi et al 1990). The $(GC)_5(AC)_{18}$ tract forming "Z" structure and (AG) tracts intramolecular triplexes.When both structures are present in the same molecule it is described as a "Z" triplex DNA motif. DNA polymerase I and Taq polymerase are unable to efficiently extend sequencing primers annealed to M13 single stranded DNA templates through this motif. In vitro and in vivo studies indicated that DNA replicating was effected depending on the orientation of the sequence, with polymerases traversing the "Z" triplex motif more readily in the "Z" to triplex direction than triplex to "Z". Thus suggesting that "Z" DNA sequences may partially releive inhibition of the travelling replication fork by (AG) repeats. This suggestion leads to the proposal that this motif may be important in regulating the direction and rate of replication through the origin of the gene in vivo. The "Z" triplex region probably has the ability to adopt multiple non "B" form DNA structures in vivo, as DNA synthesis generates waves of superhelical tension that has effects over long distances (Skarstad et al 1990), approaching replication forks may induce transient alterations in DNA secondary structure thus slowing or pausing the replication fork (Weaver & DePamphilis 1984). It is also possible that this motif may effect the nucleosome distrobution or binding of other proteins which modulate gene expression in living cells. This unusual DNA structure have also been found in the mouse c-Ki-ras protooncogene promoter and several other genes (Prestov et al 1991). A recent report has indicated that these "Z"

DNA/intramolecular triplex conformations may enhance recombination (Weinreb et al 1990).

Although alternating GC repeating sequences are not common in biological systems, some evidence suggests that in E. coli there is a maximum length of d(GC) tolerated by the cells (Klysik et al 1982), blocks do occur (Bird 1980). The observation of the relative instability of d(GC) inserts cloned into plasmids (Klysik et al 1982) initiated a study demonstrating that left-handed DNA sequences in plasmids within E. coli are known to be hot spots for spontaneous deletions (Freund et al 1989). However the sequence $d(CA)_n(GT)_n$ is widely distributed in eukaryotic genomes (Hamada & Kakunaga 1982, Hamada et al 1982). [The human haploid genome has ~50,000 such regions of at least 50bp long]. Generally these sequences are found in the intervening sequences of genes although this may involve regulatory regions of particular genes (Miesfeld et al 1981, Saffer & Lerman 1983, Hamada et al 1982a&b, Kilpatrick et al 1984, Huijser et al 1987, Pardue et al 1987, Vasicek et al 1983, Naylor & Clark 1990, Tiesman & Rizzino 1990). In contrast Gross et al (1985) indicate that specific "Z" DNA conformations formed by $d(TG)_n(CA)_n$ sequences do not exist in isolated nuclei from exponentially growing mouse mastocytoma cells. Prokaryotic genomes also do not contain significant amounts (Hamada et al 1982, Gross & Gerrard 1986).

It is still not clear what conformation (dT-dG).(dC-dA) sequences hold in vivo. On cloning the sequence $(dT\text{-}dG)_{30}.(dC\text{-}dA)_{30}$ into simian virus 40 minichromosomes and comparing the linkage differences of topoisomers of viral DNA, with and without the insert, isolated from lytically infected cells, no significant change in superhelicity was associated with the presence of the insert concluding that the sequence was not stable in the "Z" conformation in vivo (Rodriguez-Campos et al 1986). It is however important to note that although potential "Z" forming sequences may largely be in "B" form in vivo transient formation may occur under certain conditions.

The sequence dCA-dGT has been shown to form lefthanded "Z" DNA in negatively supercoiled plasmids at physiological superhelical densities (Nordheim & Rich 1983, Haniford & Pulleyblank 1983). This confirms that negative supercoiling is a major force in forming "Z" DNA or other non "B" conformational changes in vivo. A $d(GT/CA)_n$ region of satellite DNA from Cebus is known to adopt "Z" DNA at highly supercoiled densities under topological constraint (Vogt et al 1988).

The distribution of $d(CA/GT)_n$ sequences in Drosophila chromosomes, evolutionary conservation (Pardue et al 1987) and the specificity of isolated proteins (Nordheim et al 1982b, Lafer et al 1985) for this sequence has been the basis of many studies. Evolutionary conservation of these sequences suggest a functional importance. Hybridisation experiments reveal ~2000 copies of these segments consisting of more than 50bp within the Drosophila chromosome (Hamada et al 1982a). Sites are distributed over most euchromatic regions but the X chromosome has a significantly higher density than the autosomes (Pardue et al 1984). Genes on the X chromosome of Drosophila are dosage compensated in males. [As males only have one X chromosome genes are expressed at a higher level than in females which have two X chromosomes (Huijser et al 1987)]. This compensation for different dosages of genes in the two sexes is proposed to be controlled by modification of transcriptional activity (Huijser et al 1987). An increase in transcription rate of X chromosome genes is the result of an interaction of a male specific factor and the increased amount of CA/GT sequences (Belote & Lucchesi 1980). This transcription enhancement must be due to sequences outside protein coding regions as genes from other genomic sites on translocation to the X chromosome also become susceptible to increased transcription (Pardue et al 1987). It is still not clear if "Z" DNA is involved in this process.

Libraries of Drosophila hydei nuclear DNA in recombinant plasmids have affinity with anti-"Z" DNA antibodies (Jimenez-Ruiz et al 1989). This affinity is observed at bacterial supercoiled density and locates to a short fragment containing a $d(GC)_3$

tract although there are also other regions of alternating purine/pyrimidine sequences within the fragment. This Drosophila DNA insert hybridises in situ with the 4-75C_{1-2} locus of the polytene chromosomes. Further investigation of this locus (Jimenez-Ruiz 1991) shows transcriptional activity is apparently developmentally regulated during the third instar. The generation of "Z" DNA in vivo is observed when transcriptional activity at the locus is high during late third instar. At the time of "Z" DNA formation an accumulation of nonhistone chromosomal protein (NHCP) is detected in the region. The "Z"-forming sequence is located between two transcriptional units whose transcription is regulated during third instar. The "B" to "Z" transition may be generated by the local level of DNA supercoiling during induction of transcription of the gene (Tsao et al 1989) the sequence being located upstream. The accumulation of the NHCP may have an additional influence on "Z" formation or be involved in stabilising the conformation. As the transcriptional unit upstream from the induced gene and the "Z" sequence is repressed at the developmental period in which "Z" DNA formation occurs, it may be that the left-handed conformation functions as a biological silencer of the upstream gene by inhibiting the movement of the RNA polymerase toward the polyadenylation site. [Acts as a transcriptional block when "Z" DNA is generated as a consequence of increased transcription of the 3' downstream gene (Peck & Wang 1985)]. Other reports have also shown d(CA/GT) sequences to be involved in the control of transcription rates (Shen & Rutter 1984, Hayes & Dixon 1985). All the early ecdysone-inducible loci of the polytene chromosomes in Drosophila hydei appear to have "Z" DNA epitopes.

The implication that "Z" DNA may have an effect on gene expression is supported by enhanced chloramphenicol acetyltransferase (CAT) gene transient expression in thymidine kinase deficient murine L cells by cotransfection of the CAT gene in plasmid vectors with polynucleotides that can form "Z" conformations (Banerjee & Grunberger 1986). Previous studies (Banerjee et al 1985) involving the cotransfection of alternating d(GC) polymers with the herpes simplex virus

thymidine kinase (TK) gene inhibited the level of gene transfection by reducing the number of transformed colonies. However it was presumed that these cotransfected polymers although not effecting DNA uptake, blocked the integration of the gene into the genomic DNA of the TK deficient L cells, but did not prevent transient expression of the gene in the unintegrated form. This assumption was shown to be the case (Poly (dG-dC).Poly (dG-dC) and its [m^5dC] methylated form by having a direct effect on transcription provide a large stimulation of gene expression (Banerjee & Grunberger 1986). Poly (dA-dC).Poly (dG-dT) which forms "Z" DNA less efficiently (Vorlickova et al 1980/82b) did not stimulate in this situation but has in other studies using $d(AT)_n d(GT)_n$ sequences in supercoiled plasmid DNA (Hamada et al 1984) which is known to stabilise the "Z" form (Nordheim & Rich 1983b, Haniford & Pulleybank 1983). Poly (dG).Poly (dC) and Poly (dA-dT).Poly (dA-dT) polymers which do not form "Z' confromation had little or no effect. The mechanism of enhancement is not clear, and it is possible that the cotranfected polymer on entering the cell becomes linked to the CAT vectors and by undergoing recombination (Miller & Temin 1983, Folger et al 1985, Blaho & Wells 1986, Kmeic et al 1985, Kmeic & Holloman 1986, Zarling & Carlisle 1986) affect gene expression. Another possibility is a repressor-like cellular factor which may bind "Z" DNA, specifically recognises the cotransfected polymers and increases transcription efficiency of the CAT gene due to competition for binding of the repressor.

Thus in conclusion, under physiological conditions short potential "Z" forming sequences of particular chromatin domains adopt the "Z" form through generation of negative supercoiling and that this conformation may negatively or positively affect DNA template function depending on their position within genes (Tsao et al 1989, Huijser et al 1987) and on the local genomic enviroment.

Effect of conformational alterations on transcription promoters has been previously observed by many other studies (Camilloni et al 1986), with supercoiling influencing the DNA topology and promoting transcription (Weintraub et al 1986).

The formation of "Z" DNA in vivo may effect DNA repair mechanisms. Some defects in DNA which are repaired in "B" form DNA are not repaired if the DNA is in the "Z" form (Lagravere et al 1984, Boiteux et al 1985).

Many studies have suggested that changes in methylation are associated with the control of gene expression (Bird 1984, Bird et al 1979). Doerfler (1983) suggests that methylation of CG sequences in the 5' flanking promoter region prevents transcription of the gene. However methylation of the CCGG sequences in the 5' flanking sequences of the chicken B-globin gene did not induce "Z" DNA formation in a supercoiled plasmid (Nickol & Felsenfeld 1983). Thus these studies could not conclude whether the effect of blocking transcription was due to "Z" DNA formation or even if "Z" DNA stabilisation by methylation occured in vivo.

Naturally occuring polyamines putrescine, spermine and spermidine are found essentially in all eukaryotes (Tabor & Tabor 1984, Pegg 1988) and are necessary for normal cell growth (Morris 1981, Sunkara et al 1987). A correlation between intracellular polyamine concentration and nucleic acid synthesis has been found in vivo and polyamines enhance both transcription and translation in vitro (Tabor & Tabor 1976, Sakai & Cohen 1976). It is not clear how polyamines are involved in these physiological effects but it is known that they readily bind to DNA forming a highly condensed toroidal structure at high concentrations (Chattoraj et al 1978, Gosule & Schellman 1976/78, Wison & Bloomfield 1979, Schellman & Parthasarathy 1984). Polyamines stabilise DNA against thermal denaturation and radiation damage (Thomas & Bloomfield 1981). Polyamines in the cell nucleus interact with DNA, nucleosomes and chromatin (Marton & Morris 1987, Morgan et al 1987 Sen & Crothers 1986, Smirnov et al 1988). They may also be involved in promoting carcinogenesis as there is ten times more polyamine in the cancerous organ compared to a normal one (Fujita et al 1976, Takami et al 1979). When genes which are activated in some types of cancers were identified it was suggested that lefthanded DNA plays an important role in the mechanism of carcinogenesis (Jean 1985). It is also known that polyamines modulate the

interaction of gene regulatory proteins with specific DNA sequences. Thus a twenty two fold increase in the binding of oestrogen receptor to Poly (dA-dC).Poly (dG-dT) is observed in the presence of polyamines which induce the "Z" form in DNA (Thomas and Kiang 1988). It is not unreasonable to suggest that induction of genes may be enhanced by effects on the DNA topology and its affinity with regulatory factors. Polyamines are capable of provoking "Z" DNA formation in small blocks of GC sequences embedded in righthanded "B" DNA (Thomas et al 1991).

DNA in eukayotic cells is packaged into nucleosomes organised in linear arrays which are thought to be further condensed through higher levels of supercoiling. Nucleosome positioning is thought to be intrinsically involved in the active modulation of gene expression. Nucleosome formation with "Z" DNA cannot be achieved using core histones alone (Nickol et al 1982) although formation of "Z" DNA nucleosomes is reported with an additional assembly protein (Miller et al 1982/83/85). Left-handed segments in supercoiled plasmids have a strong tendency for avoidance of nucleosome formation (Casasnovas & Azorin 1987) suggesting that a transition from the "B" to "Z" form in vivo might result in a significantly altered local placement of nucleosomes (Garner & Felsenfeld 1987). Examination of chromatin organisation with the relatively common $(dT\text{-}dG)_n.(dC\text{-}dA)_n$ sequence (Hamada et al 1982), known to undergo a "B"-"Z" transition under torsional stress (Haniford & Pulleyblank 1983), found it to be quantitatively packaged into typical nucleosome core particles (Gross et al 1985). This may indicate that "Z" form DNA is not stable in vivo for a long period of time. The formation of "Z" DNA is known to aggregate DNA therefore in conditions which stabilise this conformation (supercoiling) the formation may play a role in chromatin condensation.

Researchers suggested as early as 1967 a possible role for left-handed DNA in the formation of an early paranemic intermeadiate preceding the Holliday structure (Holliday 1964) in recombination (Meselson 1967). There has also been a good

correlation between a considerable number of hotspots for recombination and alternating purine/pyrimidine sequences potentially capable of assuming a "Z" conformation under negative stress (Slightom et al 1980, Murphy & Stringer 1986, Steinmetz et al 1986, Treco & Arnheim 1986, Weinreb et al 1988/90, Wahls et al 1990).

Studies on the recombination protein rec 1 from the fungus Ustilago maydis (Kmiec & Holloman 1982, Kmiec et al 1983) suggest that recombination may be facilitated by the interaction between "Z" DNA and rec1. It is not clear as to what extent the rec 1 protein plays in "Z" formation. The rec 1 catalyses formation of a paranemic joint and is accompanied by duplex unwinding with the generation of "Z" DNA (Kmiec & Holloman 1984). Two covalently closed supercoiled plasmids containing alternating $(dG\text{-}dC)_n(dG\text{-}dC)_n$ stretches in the "Z" conformation are paired and linked by the combined action of the rec 1 protein and topoisomerase I (Kmiec and Holloman 1986). This process produces a hemicatenated dimer in which the two circular DNAs are topologically intertwined at a region of "Z" DNA homology. Although involving potential "Z" DNA sequences confirmed by blocking the reaction with anti-"Z" DNA antibodies pairing is sequence dependent with d(GC).d(GC) unable to pair with d(GT).d(CA) sequences. These observations led to the hypothesis that recombination in eukaryotes may proceed following pairing at lefthanded sequences (Pohl 1967, Haniford & Pulleybank 1983b).

More recently DNA binding studies involving the rec A protein of E. coli, which is also involved in homologous recombination, indicate binding to linear "Z" DNA in preference to "B" DNA (Blaho & Wells 1986/7). There is a requirement for ATP and a preference for longer DNA sequences. The ATPase activity is stimulated by both the ""B" and "Z" conformations but the "Z" form is less sensitive to increasing pH than the "B" form. Kinetic studies show that "Z" DNA/rec A complexes are more stable than those with "B" DNA with a slower dissociation rate. In this study no conformational preference is detected in

supercoiled recombinant plasmids; but in other studies rec A independent recombination has been shown to be stimulated by the presence of potential "Z" forming Poly d(GT) d(CA) sequences in supercoiled plasmids (Murphy & Stringer 1986). Poly d(GT) sequences have been proposed to be recombinogenic elements in eukaryotic chromosomes (Skowronski et al 1984, Slightom et al 1980). An important feature of the prokaryotic rec A, in contrast to rec 1, is that it prefers to bind supercoiled compared to relaxed plasmid DNA irrespective of conformation. This appears to be an intrinsic difference between these two proteins which are considered to perform identical functions.

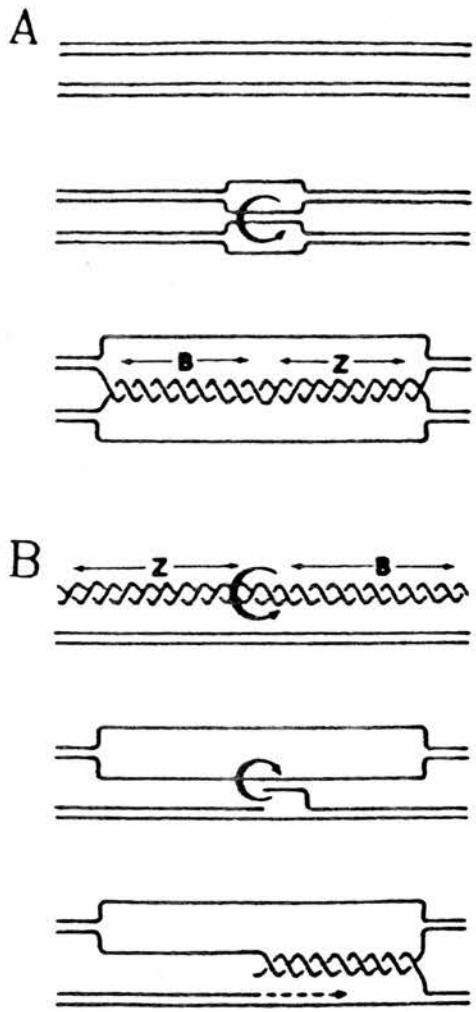
The capability of these enzymes to bind "Z" DNA with a higher affinity than for "B" DNA is consistent with left-handed DNA existing as a transient intermediate structure in recombination.

Identification of an enzymatic activity that catalyses ATP-dependent homologous pairing and strand exchange of duplex linear DNA and single stranded circular DNA was made in a nuclear extract of human T-lymphoblast cell cultures (Fishel et al 1988). Catalysis of homologous pairing reactions was later observed and studied (Moore & Fishel 1990).

Stabilisation of the paranemic intermediate heteroduplex has recently been considered (Holliday 1989) with the proposal that the lefthanded turns in "Z" DNA topologically balance righthanded turns as duplex formation proceeds. Subsequent relaxation of this paranemic joint by topoisomerase would then give a lower energy uniformly right-handed plectonemic heteroduplex. The production of "V" form DNA by reassociation of unlinked complimentary single stranded DNA circles (Brahms et al 1983) may provide a model for a paranemic intermediate structure early in recombination. Each right-handed turn being compensated by a left-handed turn, if this balancing is not evident then high superhelical densities will be reached. This may encourage "Z" DNA formation not only in purine/pyrimidine alternating sequences (M^{C}Lean et al 1986, Herr 1985) but also in sequences which would not normally be expected to form a "Z" conformation.

Left-handed DNA formation may also initiate pairing reactions by utilisation of the single stranded character of "B"-"Z" junctions which may act as a release for underwinding strain or providing points of recognition for proteins involved in recombination. There is an association of potential "Z" form alternating purine/pyrimidine tracts with chromosome breakage and reunion events responsible for translocations observed in human lymphoid tumours (Boehm et al 1989). These tracts on forming "Z" confornation may influence chromatin structure (Cereghini et al 1982) giving access to recombinase-mediated translocations. The formation of "Z" DNA provides an accessable signal-like structural element, without which recombinase mediated translocation can not take place. [The "B" to "Z" DNA transition may cause the untwisting of nearby negatively supercoiled regions, rendering chromatin more accessable.]

Interestingly the sequence $(dT\text{-}dG)_{30}$ can stimulate homologous recombination in human cells even when inserted into only one of the pairing partners (Wahls et al 1990) although is maximal when present in both. The substrate containing the "Z" motif preferentially acts as the recipient of genetic information during gene conversion events. The recombination model of Meselson and Radding (1975) accounts for preferential reception by "Z" DNA containing molecules during gene coversion events. Effects on recombination have been shown to be conferred by inserts outside the active region showing stimulation from a distance (Wahls et al 1990).



Models of how "Z" DNA may promote homologous recombination (Wahls et al 1990).

(A) Formation of "Z" DNA may increase the extent of paranemic joint formation during synapsis between two molecules by compensatory rotation of the participating strands. This would increase the length of heteroduplex DNA formed without altering the topological constraints or requiring free rotation of the participating molecules. Only the interaction of two strands is shown; however the other stands may also participate in paranemic joint formation.

(B) Unwound duplex at the junction between lefthanded and righthanded regions of the helix may facilitate interaction with free single strand ends. Since the formation of heteroduplex initiates adjacent to the "Z" DNA region, resolution of the resulting structure by the recombination model of Meselson and Radding (1975) can account for preferential reception of information by the "Z" DNA containing molecule during gene conversion events.

In conclusion eukaryotes may contain potential "Z" forming sequences which are widely scattered within the genome, providing regions where the release of negative torsional stress, arising during metabolic events, can occur. Alternatively DNA conformational changes may be actively involved in the elimination of nucleosomes or provide recognition or regulatory signals for molecules involved in gene activation or recombination. These processes may involve the binding of proteins to these altered DNA structures.

It is now generally accepted that higher energy DNA conformations can be a consequence of molecular pathways leading to functional transitions in chromatin. However a better knowledge of the kinetics of accumulation and decay of torsional stress in local domains of chromatin during metabolic processes is still required.

The existence of "Z" DNA in chromosomes has to be thought of as a dynamic rather than a static phenomenon with local accumulation of unrestrained torsional stress acting as the driving force for its formation. These transient non "B" DNA conformations are possibly stabilised by conformation specific DNA binding proteins in vivo.

Project Aims.

The case for a physiological role of non "B" DNA structures could be strengthened considerably if it were possible to show that proteins exist in nature which bind specifically to these structures. The in vivo existence of these atypical DNA helices and the knowledge that eukaryotic cells may be equipped with DNA binding proteins that selectively recognise distinct DNA conformations stabilising or further altering them may provide a basis for studies to elucidate a biological function for such proteins. A considerable number of putative "Z" DNA binding proteins from a variety of biological sources have been identified with suggestions that these proteins along with their DNA recognition sequences may participate in metabolic processes such as gene regulation, release of torsion stress, genetic recombination and chromatin organisation.

To establish a biological function these proteins will first have to be purified and their relative specificity to non "B" DNA structures characterised. The objectives of this project were firstly to establish a simple and efficient system for the detection of non "B" DNA bining proteins, specifiically "Z" DNA binding proteins. This assay system was then to be utilised in the detection of any such proteins in eukaryote cell extracts and in facilitating their isolation by following their binding activity during a purification procedure. A simple characterisation of the purified protein was then to be carried out with a view to eventually assigning a biological function in vivo.

MATERIALS and METHODS.

Chemicals.

Chemicals (Analar grade) were obtained from BDH Chemicals Ltd or May and Baker Ltd except where otherwise stated. Restriction endonucleases and DNA modifying enzymes were obtained from Northumbria Biologicals Ltd, New England Biolabs, or Boehringer Mannheim Ltd and used as specified by the manufacturer.

DNA Polymers.

The DNA alternating copolymer Poly (dG-dC).Poly (dG-dC), DNA duplex Poly (dG).Poly (dC) and single stranded polymer Poly (dT) [Pharmacia/P-L Biochemicals] were dissolved in sterile 25mM Tris HCl pH7.5, 100mM NaCl to give respective stock solutions at final concentrations of 0.5mg/ml, 4mg/ml and 1mg/ml.

DNA Concentration estimations.

The concentration of DNA was determined spectrophotometrically using a Perkin-Elmer lambda 5 UV/visible spectrophotometer. Concentrations were calculated assuming that 1 A_{260} unit is equivalent to 50µg/ml for double stranded DNA or 40µg/ml for single stranded DNA.

Preparation of dialysis tubing.

Dialysis tubing was immersed in 5% sodium carbonate, 1mM EDTA and boiled for 10 minutes, allowed to cool and rinsed in cold deionised water. After reboiling for a further 10 minutes in 1mM EDTA the cooled tubing was rinsed with cold deionised water and stored in 50% ethanol at 4°c.

Bacterial culture media.

Bacteria were grown in Luria Broth; 10g/L bacto-tryptone (Difco labs, Detroit, USA) and 5g/L yeast extract (Difco) in 10mM NaCl pH7.5 and sterilised by autoclaving. Agar plate cultures contained Luria broth supplemented with 1.5% agar (Difco). Culture media were supplemented with 100µg/ml ampicillin (Sigma) where selection was required.

Preparation of plasmid DNA.

(i) Preparation of competent cells.

Competent E. coli cells (K12 strain DH1) were prepared as described by Hanahan (1983). Colonies were picked off an agar plate containing a fresh streak of cells and resuspended in 6.5mls of 2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$. The culture was maintained at 37°c in an orbital shaker (275rpm) until a cell density of 4-7 x 10^7/ml (OD_{550} = 0.45-0.55) was reached (~2.5 hours). Cells were cooled on ice for 2 minutes, collected by centrifugation at 2,500rpm at 4°C for 10 minutes and resuspended in 2mls of ice cold, 10mM KOAc, 100mM KCl, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $Co(NH_3)_6Cl_3$; replaced on ice for 10 minutes then pelleted at 2,500 rpm at 4°c for a further 10 minutes. The pellet was resuspended in 0.5ml of the same buffer and DMSO added to 0.5%, mixed, and the suspension allowed to stand on ice for 5 minutes. A further 0.5% DMSO was added and incubation on ice continued for another 5 minutes. Competent cells were dispensed into 200µl aliquots, quick frozen in a dry ice/ethanol bath, and stored at -70°c until required.

(ii) Transformation of bacteria.

Competent cells were transformed by the method of Hanahan (1983). The cells were rapidly thawed in water at room temperature and placed on ice. Plasmid DNA (1-10ng in ~10µl of TE buffer pH7.5) was added to 200µl of the competent cells and incubated on ice for 30 minutes before heat shocking by immersion in a water bath at 42°c for 90 seconds. After 2 minutes on ice 800µl of 2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose was added and the mixture incubated at 37°c for 1 hour. Aliquots of 50-100µl were removed, plated onto Luria broth plates containing 100µg/ml ampicillin and incubated at 37°c overnight to establish colonies.

(iii) Preparation of plasmid DNA.

A colony of bacteria transformed by plasmid DNA was resuspended in 10ml of Luria broth containing 100µg/ml ampicillin and incubated overnight at 37°c, 2ml

this was then used to seed 2x500ml of the above broth in 2L baffled flasks at 37°c. The cells were grown overnight to stationary phase then harvested by centrifugation at 8,000 rpm for 10 minutes at 4°c (Beckman JA.20). The cell pellet was resuspended in 40ml of lysis solution (25mM Tris HCl pH8, 10mM EDTA, 50mM glucose, 1mg/ml lysosyme [added fresh]) and placed on ice for 5 mimutes. After which 80ml of 0.2N NaOH containing 1% SDS was added, and the lysed cells swirled on ice for a further 4 minutes before the addition of 60ml of ice cold 3M KOAc pH4.8. On further gentle mixing for 15 minutes on ice the mixture was clarified at 10,000 rpm for 10 minutes or filtered through a gauze mesh. Nucleic acid was precipitated from the supernatant by the addition of 100mls isopropanol and recovered by centrifugation at 10,000 rpm for 15 minutes after 1 hour at -20°c. The pellet was washed with 70% ethanol, dried and resuspended in 4mls of TE buffer pH7.5. Caesium chloride and ethidium bromide (sigma) were added to the DNA solution to final concentrations of 1.2g/ml and 500ug/ml respectively, mixed well and allowed to stand at room temperature for 10 minutes before clarifying by centrifugation 12,000g, for 3 minutes (MSE microfuge). The supernatant was loaded into 11x32mm Beckman polyallomer Quick seal tubes, heat sealed and the plasmid DNA centrifuged to equilibrium at 100,000rpm for 4 hours or 80,000rpm overnight at 20°c (Beckman TL100). Plasmid DNA bands were visualised under UV and removed by side puncture using hypdermic needles. Ethidium bromide was removed by several extractions with isobutanol saturated with a CsCl solution (1.2g/ml) [On shaking with an equal volume EtBr is left in the solvent phase].

Maxam and Gilbert (1980) DNA Sequencing of plasmid pAN 022.

Pan 022 plasmid DNA (10μg) was digested with EcoR1 restriction endonuclease (3 units/μg), extracted sequentially with phenol and chloroform then ethanol precipitated in a dry ice/ethanol bath. On centrifugation the pellet [of linearised plasmid DNA] was washed with 70% ethanol, dried, resuspended in 50mM Tris HCl pH8, 5mM $MgCl_2$, 1mM DTT and labelled with [$a^{32}P$] dATP using the

Klenow fragment of E.coli DNA polymerase (see later). The DNA was again phenol/chloroform extracted and ethanol precipitated before resuspension in 1x enzyme buffer and digested with HindIII restriction endonuclease (3 units/μg). This digest created two DNA fragments of 73 and 2635 base pairs each labelled at one end which were separated on a 6% polyacrylamide gel. The smaller 73bp fragment, which contained the 22bp alternating GC stretch, was excised from the gel and eluted overnight in 800μl elution buffer (500mM NH_4OAc, 1mM EDTA, 0.1% SDS, 10% methanol); the eluate filtered and the DNA in the supernatant ethanol precipitated, pelleted, washed (x2) in 70% ethanol before resuspension in 40μl of deionised water. Aliquots (10μl) of labelled DNA were placed into eppendorf tubes and treated as follows :

	Reaction Specicifity.			
DNA sample preparation.	G	G+A	T+C	C
DNA (μl)	10	10	10	10
DMS reaction buffer (μl)	200	-	-	-
Deionised water (μl)	-	-	10	5
5M NaCl (μl)	-	-	-	5
Base specific modification.				
DMS (μl)	1	-	-	-
Formic acid (μl)	-	25	-	-
Hydrazine (μl)	-	-	30	30
Times (minutes) at room temp	4	5	8	8
Reaction stop.				
DMS stop buffer (μl)	50	-	-	-
Hydrazine stop buffer (μl)	-	200	200	200
DNA precipitation				
Deionised water (μl)	200	200	200	200
3M sodium acetate (μl)	20	20	20	20
100% ethanal (μl)	500	500	500	500

On mixing tubes were immersion in a dry ice/ethanol bath for 20 minutes. The DNA was collected by centrifugation (5 minutes, 12,000g), rinsed with 70% ethanol twice and dried. The DNA was reprecipitated from deionised water and the dry pellet resuspended in 50µl of 1M piperidine, the tubes sealed and placed at 90°C for 35 minutes. On addition of 200µl of deionised water the DNA was lyophilised overnight (Howe Gyrovap); a further 20µl of deionised water was then added and lyophilised to dryness. The samples were resuspended into 10µl of formamide dyes, heated to 90°C for 3 minutes and placed immediately on ice before loading onto a prewarmed 20% sequencing gel. Gels were fixed in 10% acetic acid, baked dry onto the glass back plate and exposed to x-ray film (Hyperfilm MP Amersham) at -70°C with an intensifying screen.

Preparation of Brominated Poly (dG-dC).Poly (dG-dC).

Polymer (300µg) in 1ml of 50mM KOAc pH4.5, 3.5M NaCl was brominated by the addition of 5µl of freshly prepared bromine water (see below) and incubated for 5 minutes at room temperature. The reaction was cooled on ice and bromine removed by blowing nitrogen through the solution for a further 5 minutes before dialysis against 10mM Tris HCl pH 7.5. The presence of DNA in the "Z"-conformation was confirmed by U.V. absorption measuring the 295/260nM ratio. Bromination of polymer in "B" conformation was also carried out by lowering the NaCl concentration in the bromination buffer to 50mM.

Antibodies against the brominated Poly (dG-dC).Poly (dG-dC) were prepared by immunising rabbits using the schedule described by Lafer et al (1981) [carried out at the MRC NIMR Millhill, London].

Aqueous bromine reagent was prepared by adding bromine to an equal volume of deionised water and mixing thoroughly until the water became saturated with bromine at room temperature. Centrifugation (150g, 1 min, MSE microfuge) separated the bromine water from the insoluble lower layer of bromine.

Linkage of Brominated polymer to cellulose powder.

(i) Preparation of cellulose

Cellulose powder (Whatman CF-11) was washed successively in ten volumes of boiling ethanol (to remove pyridine), 0.1N NaOH (to remove organic acid), deionised water, 0.1N HCl and finally deionised water to pH 6 before lyophilisation.

(ii) Formation of Aminophenylthioether-cellulose.

Diazophenylthioether-cellulose forms complexes with DNA and protein molecules. Formation of this complex involves activating the cellulose with an oxirane group which then selectively couples with the strongly nucleophilic thiophenoxide anion of a primary amine thiophenol (Seed, 1982).

Five grams of dried cellulose, 13mls of 0.5N NaOH, 5mls of Bisoxirane [Butandiol diglycidylether, (BDE)] were mixed continuously overnight at room temperature. The BDE was removed by centrifugation (150g, 5 mins, IEC Centra-3R bench top centrifuge) and the cellulose incubated with 20mls of a solution containing 1% aminophenylthioether, 50% ethanol and 250mM NaOH for 4 hours with occasional mixing. After further centrifugation the cellulose was washed alternatively in 0.5N HCl and ethanol (x3) before drying at 4°C in darkness as APT-cellulose.

(iii) Activation of APT-cellulose (Diazotisation).

One gram of APT cellulose, 5mls of 1.2M HCl, 150mls of $NaNO_2$ (10mg/ml) were incubated at 4°C for 20 minutes [The resulting diazogroup causes a yellowing of the cellulose]. The cellulose was then washed (x2) with deionised water followed by sodium phosphate buffer pH 6 (x5) at 4°C.

(iv) Formation of a complex between Brominated Polymer and APT-cellulose.

One millilitre of Brominated Poly (dG-dC).Poly (dG-dC) (300µg) was incubated with 0.5g of activated APT-cellulose at 4°C overnight in darkness [Binding occurs by substitution of the Cl^- for NH_2 groups on the DNA bases]. After

centrifugation (150g, 5 mins, Centra-3R), U.V. absorption at 260nm of the supernatant determined the amount of DNA bound to the cellulose (~0.5mg DNA/g of cellulose). The DNA-cellulose was washed (x4) in sodium phosphate buffer pH6, to remove unbound DNA, before incubation at 37°C for 4 hours to inactivate unreacted diazo groups.

Linkage of Cyanogen Bromide-activated Sepharose 4B to Poly (dG-dC).Poly (dG-dC).

CNBr-activated sepharose (0.5g) was swollen in 2.5mls of 1mM HCl (pH2-3) followed by washing on a sintered glass filter funnel with 100mls of the same solution. The sepharose was then washed with 100mls of deionised water and 100mls of 10mM $NaPo_4$ buffered to pH 8 [The sepharose was never allowed to dry out on filtering]. Washed resin was resuspended in a minimal volume of 10mM $NaPo_4$ pH 8 buffer containing 1mM EDTA or 100uM $Co(NH_3)_6Cl_3$ [depending on the required DNA conformation] as a thick slurry and immeadiately mixed with Poly (dG-dC).Poly (dG-dC) polymer (350µg, 0.5µg/µl) which had been preincubated for 2 hours at 37°C in the same buffer [All subsequent incubations and washes contained either 1mM EDTA or 100uM $Co(NH_3)_6Cl_3$]. The DNA polymer was coupled to the resin overnight at room temperature by gentle rotation. Removal of unbound polymer and blocking of unreacted sites on the sepharose was achieved by washing with 25mls of 1M ethanolamine HCl pH 8 and gentle rotation of the slurry in ethanolamine for 4 hours at room temperature. The affinity resin was then washed sequentially with 25mls of 10mM $NaPo_4$ pH8, 0.1M NaCl; 25mls of 1M NaCl; 25mls of 10mM $NaPo_4$ pH8, 0.1mM NaCl and 25mls of 25mM Tris HCl pH7.5, 0.1M NaCl, 0.01% $NaNo_3$ before storage as a slurry at 4°C.

For DNA affinity chromatography resin was packed in 5ml disposable columns containing 70µm filter bed supports (Lab m., Burry, Lancs, England) and equilibrated in 25mM Tris HCl pH7.5, 10% glycerol, 1mM DTT, 0.1% NP40, 1mM PMSF and 1mM EDTA or 100µM $Co(NH_3)_6Cl_3$.

Immunoassay for Anti-"Z" DNA antibodies.

A stock suspension of DNA-cellulose [or sepharose] was made by resuspending 50mg of the Poly (dG-dC).Poly (dG-dC)/cellulose into 1ml of buffer A [25mM Tris HCl pH7.5, 100mM NaCl, 0.1mM EDTA, 0.5mM PMSF, 1% BSA]. Twenty-five µl of this suspension was then mixed with 25µl of a rabbit polyclonal antibody dilution against brominated "Z" form Poly (dG-dC).Poly (dG-dC) in buffer A and incubated at room temperature for 1 hour with occasional shaking. The cellulose was then sedimented (150g, 3 mins, Beckman bench microfuge) and washed (x3) with 200µl of buffer A. The amount of antibody retained on the cellulose was determined by adding 100µl ^{125}I labelled Protein A [Amersham International] 10^4 cpm (specific activity 40 mCi/mg) in buffer A; incubating for 30 minutes at room temperature and then washing by centrifugation. Retained radioactivity was measured by Cerenkov counting. In the competition experiments 25µl of an appropriate dilution of the rabbit antibody in buffer B [25mM Tris HCl pH 7.5, 100mM NaCl, 0.5mM PMSF, 1% BSA, 100µM $Co(NH_3)_6Cl_3$ or 1mM EDTA] was mixed with 25µl of a known concentration of Poly (dG-dC).Poly (dG-dC) which had been preincubated for 1 hour in buffer B [to ensure any conformational changes]. After a further incubation at room temperature for 1 hour the mixture was centrifuged (11,000g, 10 minutes, MSE microfuge) and the supernatant added to the DNA-cellulose suspension where the remaining antibody was assayed as described.

Preparation of double stranded synthetic oligodeoxynucleotides.

Olignucleotide synthesis.

Single stranded, synthetic oligonucleotides were synthesised on an Applied Biosystems Model 381A DNA Synthesiser using ß-cyanoethyl phosphoramidites. A solid phase sythesis is used in which the growing DNA chain remains covalently attached to an insoluble matrix, Controlled Pore Glass (CPG). CPG is easily derivitised with adenosine, guanosine, thymidine and cytosine phosphoramidites. An organic linker is attached to the CPG and the support is derivitised by covalent

attachment of the 3' hydroxyl of a nucleoside to the linker via an ester bond. Following derivitisation, all free amino groups are capped.

ß-cyanoethyl phosphoramidites are chemically modified nucleosides containing a diisopropylamine group on a 3' trivalent phosphorous moiety. A ß-cyanoethyl protecting group is present on the 3' phosphorous group of the nucleoside with a dimethyoxytrityl (DMTO) protecting group on the 5' hydroxyl.

DNA synthesis begins on a column containing the required 3' support bound nucleoside and proceeds in a 3' to 5' direction. The first step involves the removal of the acid labile, dimethoxytrityl (DMTO) group by trichloroacetic acid (TCA) yielding a reactive 5' hydroxyl which reacts with the next phosphoramidite during the following coupling. During the coupling step, tetrazole and the next phosphoramidite mix on entering the reaction chamber generating a highly reactive species which rapidly reacts with the free 5' hydroxyl group of the support bound nucleotide. Tetrazole protonates the nitrogen of the diisopropyl group on the 3' phosphorous which converts the amine into a very good leaving group upon nucleophilic attack by the 5' hydroxyl group. Thus an internucleotide (3'-5') linkage is is formed through a trivalent phosphorous group. During the coupling stage a small percentage of oligonucleotides may fail to couple; these truncated sequences are chemically modified to prevent further participation in subsequent synthesis. Acetic anhydride and dimethylaminopyridine upon mixing form a powerful acetylating agent which terminates (caps) any unreacted chains following the coupling step. Immediately after capping, the labile trivalent phosphorous linkage formed on coupling is oxidised to the stable pentavalent phosphorous linkage of biologically active DNA using iodine as a mild oxident. DNA synthesis is continued by removal of the DMTO group at the 5' terminus of the oligomer and repitition of another cycle of base addition. When the required length and sequence of oligomer has been sequenced the chain is cleaved from the CPG support and the ß-cyanoethyl protecting groups on the phosphates removed by four fifteen minute washes with concentrated ammonium hydroxide. The base protecting groups are

removed by heating to 55°C in ammonium hydroxyde for 15 hours. Following cleavage and deprotection the ammonia is removed by evaporation under vacuum, the DNA ethanol precipitated and the dry DNA pellet resuspended in deionised water.

Hybridisation of Complementary Single Stranded Oligonucleotides.

Complementary, single stranded synthetic oligonucleotides were annealed by heating to 100°C in 10mM Tris pH7.5, 1mM EDTA, 0.1M NaCl followed by slow cooling to room temperature.

Fragmentation and labelling of Poly (dG-dC).Poly (dG-dC).

Poly (dG-dC).Poly (dG-dC) was diluted to 125µg/ml in 10mM Tris HCl pH7.5, denatured by heating to 100°C for 5 minutes and renatured by slow cooling to room temperature. Alternatively the polymer was digested with the restriction enzyme BssHII at 50°c for 2 hours (8 units/2.5µg DNA). Poly (dG-dC).Poly (dG-dC) either in the native state or as fragmented above was 3' end labelled using the Klenow fragment of E. coli DNA polymerase I [Northumbria Biologicals Ltd] (Klenow et al,1971).

DNA fragments were 3' end labelled by end repair (Drouin,1980). The above polymerase was used to fill in from a recessed 3' end using the corresponding 5' extention as a template for DNA synthesis. Polymer was incubated with 20µCi[a^{32}P]dCTP, 100µM unlabelled dGTP, 1µg gelatin, and 8 units of the Klenow polymerase in a 20µl reaction containing 50mM Tris HCl pH8, 5mM $MgCl_2$, 1mM DTT, at room temperature for 20 minutes. Unlabelled dCTP was added to a final concentration of 100µM and incubation at room temperature allowed to proceed for a further 15 minutes. The reaction was stopped by the addition of one fifth volume of 50 % glycerol, 100mM EDTA, bromophenol blue and xylene cyanol or by extraction with phenol/chloroform and precipitation with ethanol. Polymer was labelled to approximately 10^7 cpm/µg using this method.

Labelling of other DNA fragments.

On end labelling the DNA fragment BamHI$(GC)_{11}$BamHI excised from the plasmid pAN 022 or the double stranded oligonucleotide AccI$(GC)_{16}$AccI the DNA was incubated with the appropriate restriction enzyme (creating a 3' recess) and then labelled by end repair as above. The labelling reaction mixes contained 20μci [^{32}P]dATP and 100μm unlabelled dCTP, dGTP, dTTP proceeded by a further 20 minute incubation with 100μm unlabelled dATP.

Filter assay.

Labelled Poly (dG-dC).Poly (dG-dC) [~10ng, from resuspended ethanol precipitation] was incubated in buffer B [25mM Tris HCl pH7.5, 1% BSA, 100mM NaCl, 0.5mM PMSF, 100μM $Co(NH_3)_6Cl_3$] at room temperature for 1 hour [to facilitate a "B" to "Z" conformation transition] with a 100 fold excess of unlabelled carrier "B" DNA Poly (dG).Poly (dC) [1μg]. Aliquotes were mixed with equal volumes of rabbit antisera dilutions in buffer B for a further 1 hour at room temperature and filtered through 0.22μm nitrocellulose filters (Whatman GSWP) which had been prewashed in buffer B. Filters were washed with 5ml of buffer B , dried and counted [Packard liquid scintillation system, 300] with 5mls of scintillation fluid [Ecoscint A, National Diagnostics]. In some experiments DNA on damp filters was extracted by cutting the filter into small pieces and incubating for 1 hour at 37°c in 400μl of 25mM Tris HCl pH7.5, 1% SDS, 1mM EDTA, 100μg/ml Protease K (Boehringer). Eluted DNA in the supernatant was extracted sequentially with phenol and chloroform before precipitation with cold ethanol. The pellet was dried (Howe, Gyrovap) and dissolved in 50mM Tris HCl pH 8, 100mM EDTA, 50% glycerol, with bromophenol blue and xylene cyanol dyes and submitted to agarose or polyacrylamide gel electrophoresis. Labelled size markers were electrophoresed in parallel wells [these having been prepared from Adenovirus type 2 DNA digested with AccI or BamHI followed by end labelling]. Gels were fixed in 10% acetic acid (v/v aqueous) and autoradiographed for approximately 16 hours at -70°c using an intensifying screen.

Gel retardation assay.

Labelled, native (Pharmacia/P-L Biochemicals) or fragmented (P-L Biochemicals) Poly (dG-dC).Poly (dG-dC) was electrophoresed in a 6% polyacrylamide gel along with labelled size markers; DNA of 400-1000 bp was excised from the gel. The gel was crushed using a glass rod and DNA eluted at 37°c overnight in 800µl 500mM ammonium acetate, 1mM EDTA, 0.1% SDS, 10% methanol with constant shaking. Eluted DNA was filtered through glass wool, precipitated with cold ethanol and resuspended in 10mM Tris/HCl pH7.5 in a volume equivalent to 3-4 x 10^3 cpm/µl (by Cerenkov).

One µl of labelled DNA was mixed with 17µl of binding buffer [25mM Tris HCl pH7.5, 10% glycerol, 1mM DTT, 0.1% NP40, 50mM NaCl, 250µg/ml BSA, 1mM PMSF, 100µM $Co(NH_3)_6Cl_3$] and 1µl of carrier "B" conformation DNA [Poly(dG).Poly(dC) at 1mg/ml]. After incubation for 1 hour at 37°c 2µl of diluted rabbit antibody or 2µl of cell extract] was added and incubation continued for a further 20 minutes at room temperature; 4µl of 25mM Tris HCl pH7.5, 50% glycerol, 0.1% bromophenol blue was added prior to loading onto a 6-8% [55:1, acrylamide: bisacrylamide] polyacrylamide gel prepared in 40mM Tris/glycine pH8.5 containing 100µM Hexamine cobalt (III) chloride. In control gels 1mM EDTA replaceded the cobalt in the binding buffer, gel and running. Gels were fixed in 10% acetic acid (v/v aqueous) dried in vacuo and visualised by autoradiography. The labelled BamHI$(GC)_{11}$BamHI fragment from the plasmid PAN 022 and the oligonucleotides were also prepared and used in the assay as above except elution was from a 10% polyacrylamide gel.

Tissue Culture

HeLa S_3 spinner cells [WS line] were grown in suspension in Earle's minimal essential media [Gibco] containing 50 units/ml penicillin [Glaxo], 50µg/ml streptomycin [Evans medical Ltd], 2.2g/L sodium bicarbonate and supplemented with 7% new born calf serum [Sera-lab] at a density between 3 x 10^5 and 6 x 10^5 cells/ml by dilution every 3-4 days with fresh medium. Cultures were periodically

monitored for contamination by mycoplasmas using 4'-6-diamidino-2-phenylindole [DAPI] (Russell et al , 1975).

Fractionation of cell extracts.

Cytoplasmic and nuclear extracts were prepared as described by Challberg and Kelly 1979. Hela S_3 cells at a density of 6 x 10^5 cells/ml were pelleted by centrifugation at 2000rpm, 10 minutes (MSE 6L), washed in cold PBS and resuspended in cold hypotonic buffer [20mM HEPES/NaOH pH7.5, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT] and allowed to swell on ice for 15 minutes. The cells were then subject to 10/15 strokes of a tight fitting Dounce homogeniser (pestle B) and the disrupted lysate centrifuged at 2000g for 10 minutes. The pelleted nuclei were resuspended in 50mM HEPES pH7.5, 10% sucrose [2.5ml/l of original cell culture] and frozen in liquid nitrogen before storage at -70^{o}c.

Ammonium Sulphate Precipitation.

The cytoplasmic supernatant was clarified by centrifugation at 100,000g for 1 hour (8x50ml AR, Beckman L8) before fractionation. Solid ammonium sulphate was slowly added to cytoplasmic extract to the required saturation, maintained at 4 ^{o}c for 30 minutes while stirring and the precipitate collected by centrifugation (15,000g, 20 minutes, 8 x 50ml AR, Beckman JA20). The supernatant was further precipitated by adding solid ammonium sulphate and continued in this manner until 80% saturation was reached. In each case the precipitate was dissolved in buffer C [25mM Tris pH7.5, 10% glycerol, 1mM DTT, 0.1mM NP40, 1mM PMSF] containing 50mM NaCl and dialysed against the same buffer.

FPLC (Pharmacia) Analysis.

Crude cytoplasmic extract or ammonium sulphate precipitated extract (~30mg) was filtered through a 0.22μM nitrocellulose disc [or centrifuged at 100,000g) before loading onto a FPLC anion exchange column (Mono Q) further purification involved cation exchange chromatography (Mono S). Elution of bound protein to both columns was with a salt gradient from 50mM to 1M NaCl in the appropriate buffer, monitored by UV absorption at 280nm, and 0.6ml fractions were collected.

Anion exchange chromatography used Tris/HCl based Buffer C whereas cation exchange used Buffer D [50mM HEPES pH 7.5, 10% glycerol, 1mM DTT, 0.1% NP40, 1mM PMSF [glycerol concentration was reduced to 5% and NP40 to 0.01%] in later runs. Pooled fraction samples from the anion exchange column were dialysed into buffer D containing 50mM NaCl overnight before cation exchange chromatography. Subsequently pooled fractions from the Mono S column were dialysed back into buffer C containing 50mM NaCl before DNA affinity chromatography.

DNA Affinity chromatography.

Prior to loading onto a 0.75ml "Z" DNA/sepharose column equilibrated with 50mM NaCl in buffer C, 10µg of Poly (dG).Poly (dC) carrier "B" DNA was added to the sample and loading commenced at a rate of 1.5mls/hour. The column was then washed with 3 column volumes of low salt buffer C and bound protein eluted in buffer C containing 1M NaCl at the same rate. Ninety µl fractions were collected, alliquoted, and immeadiately frozen in liquid nitrogen before transfer to -70°c.

During a later purification fractions with positive "Z" DNA binding activity from the FPLC Mono S column were loaded onto a 1.5ml "B" DNA column equilibrated in buffer C containing 100mM NaCl. The flow through from this column was immediately loaded onto a 1.5ml "Z" DNA column equilibrated in the same buffer. The column was washed with three column volumes of equilibration buffer before the bound protein was eluted in buffer C containing 1M NaCl.

Protein Concentration Estimations.

The concentration of protein in cell extracts were estimated by the method of Bradford (1976). Protein samples (10µl) were mixed with 990ul of Bradfords reagent (100mg Coomassie brilliant blue G250, 100ml orthophosphoric acid and 50mls of ethanol in 1 litre of deionised water) and the absorbance at 595nm measured [Perkin-Elmer lambda 5].

Concentrations were calculated from a standard curve constructed using Bovine Serum Albumin (BSA).

Trichloroacetic acid (TCA) Precipitation of protein.

Protein was precipitated by the addition of a 1/10 volume of 100% trichloroacetic acid (TCA), mixing and incubation for 1 hour on ice. The precipitate was collected by centrifugation at 50,000rpm for 15 minutes at 4°C (Beckman TL100) and washed in ice cold acetone before being resuspended in SDS PAGE boiling mix.

Gel Electrophoresis.

(i) Agarose gel electrophoresis.

Flat bed slab gels containing 1% agarose (w/v) in 1 x TBE were run at 55 volts for 16 hours (260mm x 160mm) or 1hour (70mm x 100mm) submerged by 1 x TBE containing 1µg/ml ethidium bromide. Prior to loading the sample, a one fifth volume of dye mix containing 50% glycerol, 100mM EDTA , 0.1% bromophenol blue and 0.1% xylene cyanal FF was added. DNA was visualised under long wave UV light.

(ii) Native poly acrylamide gel electrophoresis.

Gels (175mm x 150mm) used in DNA analysis and purification were cast to the required acrylamide concentration (usually 6% or 10%) from a 29% acrylamide:1% N,N' Methylene-bisacrylamide stock solution and contained 1 x TBE buffer. Polymerisation was by the addition of 1/10 volume of 1% ammonium persulphate solution and a 1/800 volume of TEMED (Eastman Kodak Co., Rochester, New York, USA). Gels were poured between glass plates separated by 1mm teflon spacers and well comb, sealed with adhesive tape. Electrophoresis was at 200v for 2 hours at room temperature in 1 x TBE running buffer.

Native gels for gel retention assays were made to the required concentration (usually 6-8%) from a 44% acrylamide: 0.8% N,N' Methylene-bisacrylamide stock solution and contained 40mM Tris buffered to pH8.5 by glycine and also 100µM cobalt hexamminochloride (III). Polymerisation was as above. Electrophoresis was at 200v for 2 hours at 4°c in 40mM Tris bufferd to pH8.8 by

glycine containing 100μM $Co(NH_3)_6Cl_3$ running buffer. In control gels cobalt complex was replaced by EDTA.

(iii) SDS polyacrylamide gel electrophoesis.

(Laemmli,1970)

Resolving gels containing 10 to 16% polyacrylamide were prepared from a 50% acrylamide:0.235% N,N' Methylene-bisacrylamide stock solution and contained 0.375M Tris. HCl pH 8.3 and 0.1% SDS. Polymerisation was carried out as for native polyacrylamide gels. Gels were cast between glass plates (75mm x 90mm) using 0.75mm thick teflon spacers and sealed by adhesive tape. The resolving gels were poured to 1cm below the sample wells to allow a stacking gel to be included. On pouring the resolving gel an overlay of isobutanol [saturated with H_2o] was added to ensure an even surface and facilitate polymerisation. Stacking gels containing 5% acrylamide were prepared from a 50% acrylamide:1.3% N,N' Methylene-bis acrylamide stock solution and contained 0.105M Tris. HCl pH 6.8 and 0.1% SDS. Samples were denatured by boiling for 3 minutes in a solution containing 12.5% glycerol, 2.5% SDS, 62.5mM Tris. HCl pH 7, 0.875M ß-mercaptoethanol and 0.015% bromophenol blue. Electrophoresis was carried out on a minigel rig (Biorad) in 1x TGS buffer, at 40 volts for 20 minutes and then increased to 120 volts for 2 hours at room temperature until the dye front was 0.5cm from the bottom of the gel. Gels were fixed in 10% acetic acid, 40% methanol prior to stainning.

DNA Sequencing gel electrophoresis.

One of the glass plates (430mm x 230mm) was treated with surfacil siliconising agent (Pierce Chemical Company). While the other with silane A-174 (sigma) which covalently cross-links the acvrylamide to the surface of the glass plate. Gels containing 20% acrylamide were prepared from a 40% stock consisting of a 19: 1 ratio of acrylamide to bisacrylamide. Gels were made in 1 x TBE and contained 46% urea. Electrophoresis was carried out in 1 X TBE at 2,000 volts for 5/6 hours.

Protein Blotting and Probing with "Z" DNA.

Aliquots (25µl, 100µg) from resuspended ammonium sulphate precipitates of HeLa cell cytoplasmic proteins which contained "Z" DNA binding activity [60,50,40% by gel retention] were electrophoresed in triplicate on a 16% SDS polyacrylamide gel under denaturing conditions. Purified adenovirus type 2 and protein molecular weight markers (Gibco/BRL) were also electrophoresed in parallel. Polypeptides were renatured in situ by washing the gel in 4M urea (Silva et al 1987) for one hour at room temperature (x2 buffer changes) followed by a series of decreasing concentrations [ranging from 4M to 0M] prior to 'western' blotting on to nitrocellulose (Russell & Precious 1982). The nitrocellulose sheet was cut into strips containing individual gel tracks and one strip of each of the cytoplasmic precipitates stained with napthalene black along with the markers to determine transfer efficiency. The remaining two strips were washed with 5% Marvel in 10mM Tris/HCl pH 7.5, 50mM NaCl containing 1mM EDTA ("B") or 100µM $Co(NH_3)_6Cl_3$ ("Z") by shaking at room temperature for two hours before adding [^{32}P] labelled DNA probe, Poly (dG-dC).Poly (dG-dC), (1-2 x 10^5 cpm in 5mls of washing buffer) either in "Z" or "B" conformation. [The labelled DNA probes were preincubated for one hour at 37°C in the appropriate buffer to ensure the required conformation.] The probe buffer always contained ~100 fold excess unlabelled Poly (dG).Poly (dC) carrier DNA. Nitrocellulose strips were incubated with probe and gently agitated for three hours at room temperature, washed twice in the appropriate buffer for 30 minutes, dried and submitted to autoradiography.

Silver Stainning.

The detection of proteins after SDS PAGE was carried out using a Bio-Rad silver stain kit [derived from the method of Merril et al, 1982]. Gels were fixed for 30 minutes in 40% methanol, 10% acetic acid (v/v), followed by two further 15 minute washes in 10% ethanol, 5% acetic acid (v/v). The gels were then immersed in 200mls of freshly prepared oxidiser [contains potassium dichromate and nitric acid] for 5 minutes before washing twice in 400mls of deionised water for 5

minutes. Freshly prepared silver reagent (200mls) [contains silver nitrate] was then added and the gel incubated for 20 minutes before being rinsed with deionised water and developed in 200mls of freshly prepared developer at 25°C. On addition of the developer a brown smokey precipitate appeared this was poured off and the gel briefly rinsed in deionised water before adding fresh developer. When the gels were sufficiently developed 400mls of 5% acetic acid was added and the gels photographed or sealed in plastic bags. All incubations and washes were carried out in a pyrex glass baking dish placed on a rocker and gels handled carefully using vinyl gloves.

Renaturation of Polypeptides from Excised Bands after SDS Polyacrylamide Gel Electrophoresis.

This method was essentially as described by Hager and Burgess 1980 with modifications described by Baeuerle and Baltimore 1988/89.

Crude cell extract (300µg) was subject to SDS polyacrylamide gel electrophoresis on a 10% acrylamide gel, the gel was washed for 3x 10 minutes in 1mM DTT, gel pieces from different molecular weight regions [determined from neighbouring stained and prestained mw markers (Gibco/BRL, Sigma) were excised, ground and proteins eluted overnight at 4°C in 500µl of 50mM Tris/HCl pH7.5, 0.1% SDS, 0.02mg/ml BSA, 1mM DTT, 0.2mM EDTA, 0.1mM PMSF, 50mM NaCl, 2.5% glycerol (method 2 contained 100µg/ml BSA) by gentle agetation. On centrifugation (2 minutes, MSE microfuge), the supernatant was removed and recentrifuged for a further 10 minutes to remove small gel debris. Four volumes of cold acetone were added to the supernatant and the proteins allowed to precipitate for 2 hours at -20°C. The precipitate was collected by centrifugation (10 minutes, MSE microfuge), washed with 1ml of cold 80% methanol in 25mM Tris/HCl pH7.5, 50mM NaCl, 1mM DTT, 0.1mM PMSF and dried under vacuum (Howe Gyrovap). The dried pellet was either redissolved in 2.5µl of a saturated urea solution containing 25mM Tris/HCl pH7.5, 50mM NaCl, 1mM DTT, 0.1mM PMSF allowed to stand at room temperature for 20 minutes before dilution with

125µl (50 fold) of 25mM Tris/HCl pH7.5, 50mM NaCl, 1mM DTT, 0.1mM PMSF, 0.1% NP40, 5% glycerol. Renaturation was allowed approximately 18 hours at 4°C before "Z" DNA binding activity was detected by mobility shift assays (method 1). Alternatively the dried pellet was redissolved in 80-125µl of 6M guanidine hydrochloride in 25mM Tris/HCl pH7.5, 50mM NaCl, 1mM DTT, 0.1mM PMSF, 0.1% NP40, 5% glycerol incubated for 30 minutes at room temperature before overnight dialysis at 4°C against a litre of 25mM Tris/HCl pH7.5, 50mM NaCl, 1mM DTT, 0.1mM PMSF, 0.1% NP40, 5% glycerol. The dialysate (15µl) was assayed for "Z" DNA binding activity by gel retention. The assay conditions were adapted as follows - the standard gel retention assay incubation buffer was diluted 1: 2 with 25mM Tris HCl pH 7.5 before preincubation and only 200ng of carrier DNA was used.

Glycerol Gradients

Glycerol gradients were constructed by carefully layering the appopriate glycerol concentrations on top of each other starting with the highest at the bottom of the tube and finishing with the lowest at the top. The protein sample was then layered on top. Sedimentation was by centrifugation at 49,000rpm for 21 hours at 4°C (Beckman L8, SW50.1). Fractions (50µl) were collected from the bottom of the tube after piercing.

Buffers.

TE :-	10mM Tris pH7.5, 1mM EDTA.
TBE :-	89mM Tris, 89mM boric acid, 2mM EDTA.
TGS :-	40mM Tris, 5mM glycine, 0.1% SDS.
PBS A :-	137mM NaCl, 2.7mM KCl, 3.2mM Na_2PO_4, 1.5mM KH_2PO_4.
DMS reaction buffer :-	50mM sodium cacodylate pH8, 1mM EDTA.
DMS stop buffer :-	1.5M sodium acetate pH7, 10M ß-mercaptoethanol.
Formamide dyes :-	80% formamide, 10mM NaOH, 1mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol.

Hydraze stop buffer :- 0.3M sodium acetate pH7, 0.1mM EDTA

RESULTS.

A model system for exploring the the parameters important in the binding of proteins to DNA in "Z" conformation was provided by an immunoassay which utilised a rabbit antibody against the brominated "Z" form of Poly (dG-dC).Poly (dG-dC). This antibody was shown to react specifically with "Z" form polymer bound to cellulose [or sepharose] as determined by binding of iodinated Protein A (Amersham UK). [Protein A binds to the Fc fragment of immunoglobulin, thus a measurment of the radioactivity retained on the antigen/antibody complex gave a direct indication of bound antibody.] Figure 1a. shows the amount of radioactivity retained expressed in counts per minute as a function of serum dilution. Only nonspecific binding was observed with preimmune serum or when anti-"Z" serum was incubated with "B" form polymer bound to cellulose.

The efficacy of $Co(NH_3)_6^{3+}$ ions in promoting "Z" DNA formation of the polymer Poly (dG-dC).Poly (dG-dC) was demonstrated in an assay where binding of the antibody to "Z" DNA antigen was competed out by Poly (dG-dC).Poly (dG-dC) only in the presence of $Co(NH_3)_6^{3+}$ ions. Figure 1b. shows the effect of increasing amounts of "Z" form competitor on the radioactivity retained, expressed as counts per minute. Only background competition was observed when $Co(NH_3)_6^{3+}$ ions were replaced with 1mM EDTA. The ability of Poly (dG-dC).Poly (dG-dC) to assume the "Z" conformation in the presence of $Co(NH_3)_6^{3+}$ ions was also shown by a filtration assay using [^{32}P] end labelled polymer both in the native form and on renaturation after boiling. Retention of [^{32}P] radioactivity on the filter was significantly greater after incubation with anti-"Z" serum than with preimmune serum. Figure 1c. indicates that the quantity of labelled polymer retained on the filter was related to dilution of the antibody. Agarose gel electrophoretic analysis of the DNA retained on the filters demonstrated that antigenicity was evident over a wide range of molecular weights (figure 2). Native Poly (dG-dC).Poly (dG-dC) [P-L Biochemicals] was extremely heterogeneous in

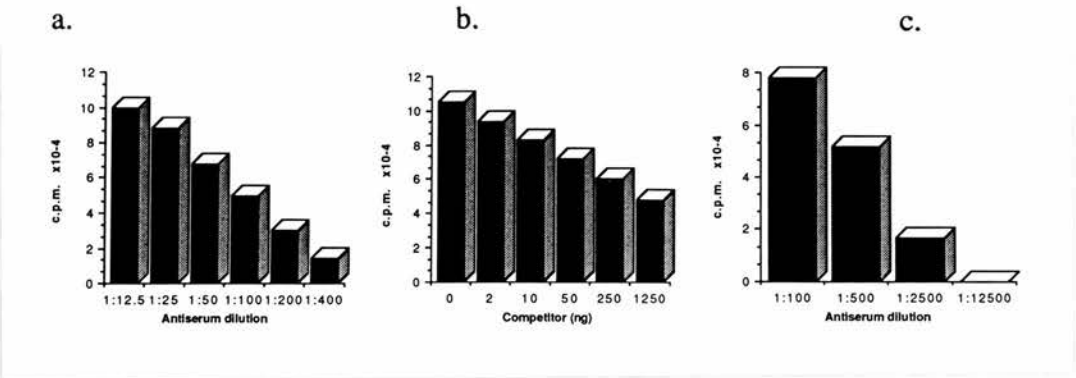


Figure 1. Immunoassays for "Z" DNA Antibodies.
(a) Incubation of antiserum dilutions with a suspension of cellulose/polymer complex was followed by extensive washing to remove all unbound antibody. A further incubation with ^{125}I labelled Protein A followed by more washing determined the amount of antibody bound to the antigen by cerenkov counting.
(b) Prior to incubation with the cellulose/"Z" form polymer antigen, a 1 in 25 dilution (1: 50 final) of the anti-"Z" DNA serum was incubated with increasing amounts of competitor Poly (dG-dC).Poly (dG-dC) in the presence of 100μM $Co(NH_3)_6Cl_3$. [The competitor polymer DNA was preincubated for 1 hour at 37°C in the presence of $Co(NH_3)_6^{3+}$ ions to ensure "Z" conformation].
(c) Filter retention assays with [^{32}P] labelled polymer were carried out before and after incubation with dilutions of "Z" DNA antiserum in the presence of 100μM $Co(NH_3)_6Cl_3$. Antibody binding was expressed as radioactive counts per minute [cpms] retained on the filter after filtration and subsequent washing. Nonspecific binding by the rabbit serum was compensated for by subtraction of the cpms retained with preimmune antiserum at the appropriate dilutions. [Preincubation of the polymer at 37°C for 1 hour in the presence of $Co(NH_3)_6^{3+}$ ions ensured "Z" conformation].

its fragment size, ranging from over 14,000 to less than 300 base pairs. Denaturation by boiling reduced the fragment length to below 1,000 base pairs (renaturation by 'snap' cooling on ice or slow cooling in a water bath to room temperature, did not appear to differ in the fragment size generated). Partial digestion of the native polymer with the restriction endonuclease BssH II [which recognises the sequence d(G^CGCGC)] produced similar low molecular weight fragments on analysis by agarose and polyacrylamide gel electrophoresis (figures 3a & 3b). Sonication also reduced the polymer to a more uniform fragment size but was found to create a large amount of single stranded DNA at fragment ends. This was evident when sonicated "end labelled" polymer was treated with S_1 nuclease (specific for single strand DNA) leading to a 90% loss of radioactivity from the polymer (data not shown).

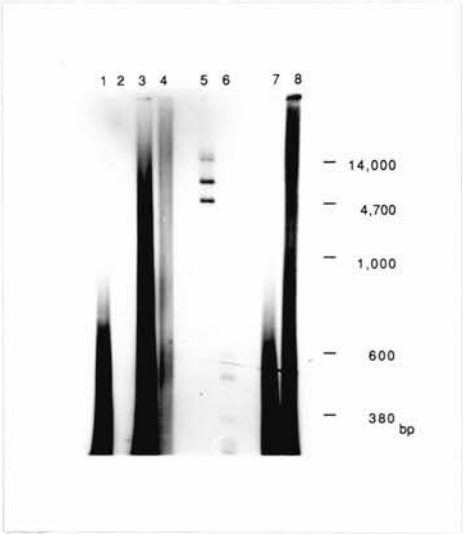
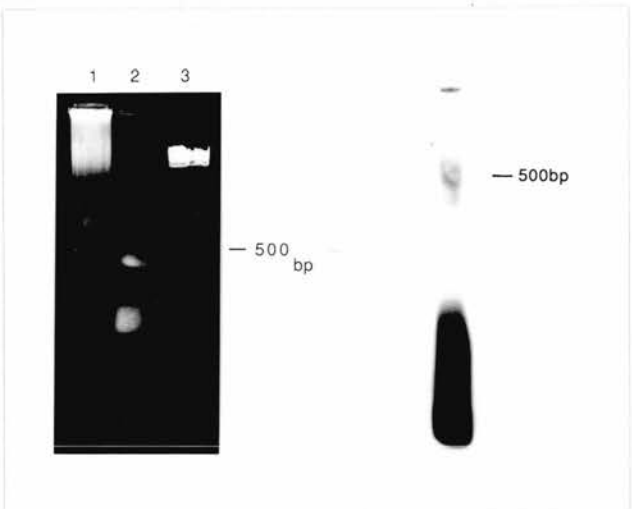


Figure 2. Autoradiogram of an agarose electropherogram showing the labelled material retained on filters after incubation with anti-"Z" DNA and preimmune rabbit serum.

Prior to electrophoresis on a 1% agarose gel, [^{32}P] labelled Poly (dG-dC). Poly (dG-dC) was boiled and renatured then incubated with a 1 in 25 dilution of anti-"Z" DNA serum (1: 50 final) (lane 1) and preimmune serum (lane 2) followed by filtration. Native labelled polymer was also incubated with anti-"Z" DNA serum (lane 3) and preimmune serum (lane 4) before filtering. After washing the antibody retained on the filters was degraded by protease, the DNA phenol/chloroform extracted and ethanol precipitated before loading onto gels. Control lane 7 [denatured] and lane 8 [native] show a profile of the total polymer DNA before incubation and filtration. The central lanes 5 and 6 contain [^{32}P] labelled Adenovirus type 2 DNA restriction fragment [Bam HI & Acc I] markers giving fragment lengths in base pairs as indicated.
The gels were dried under and subject to autoradiography.

a. b.

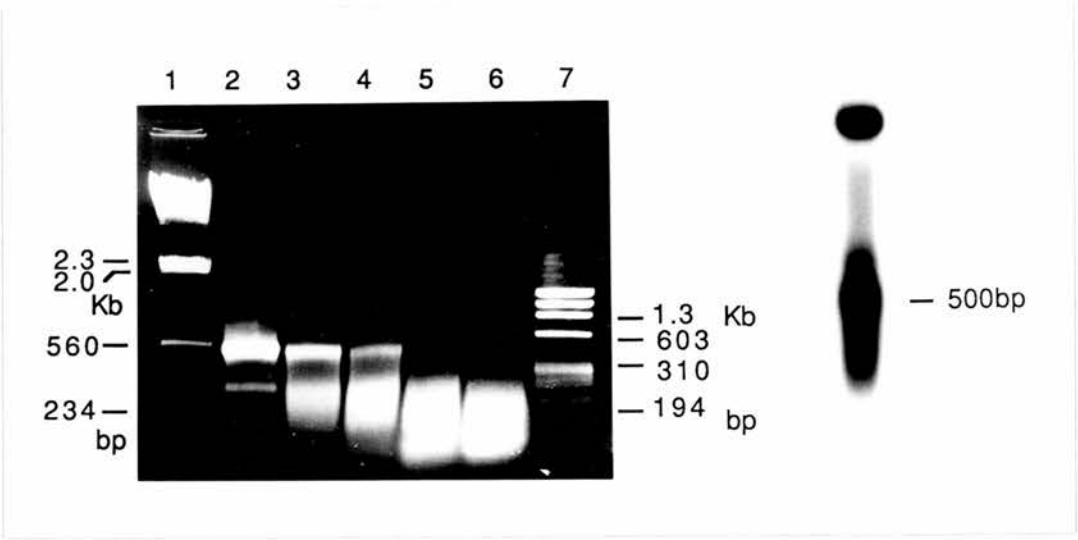


Figures 3a and 3b. Partial digestion of Poly (dG-dC).Poly (dG-dC).

(a) Native Poly (dG-dC).Poly (dG-dC) was incubated with 8 units of BssH II restriction enzyme/µg of polymer at 50^{0}C for 2 hours before analysis on a 1% agarose gel and staining with 1µg/ml ethidium bromide in 1 x TBE. Lane 1 contains 2.5µg of uncut polymer, lane 2 contains 2.5µg of the BssH II cut polymer and lane 3 Lambda DNA restriction enzyme fragment [Hind III] markers as indicated in base pairs.
(b) Partial BssII II digested polymer (0.5µg) was [^{32}P] end labelled and electrophoresed on a 6% polyacrylamide gel along with size marker as indicated in base pairs and subject to autoradiography after drying.

On purchasing a new batch of the alternating polymer Poly (dG-dC).Poly (dG-dC) from the recently merged Pharmacia/P-L Biochemicals, fragment size on electrophoresis was found to be far more homogeneous, with an average length of 450 base pairs (figure 4a).

Radioactively labelled fragments of polymer were utilised in developing a gel shift assay which would detect specific binding of protein. Labelled Poly (dG-dC).Poly (dG-dC) of a defined fragment length (~500bp) was purified by elution out of bands excised from 6% polyacrylamide gels (figure 4b). Labelling 0.5µg of polymer produced enough radioactive material to use as probe for numerous gel shift assays [see methods].The more uniform the fragment size the more labelled material could be retrieved from individual excised bands. The conditions used in the assays were similar to those developed for the detection of sequence specific DNA binding proteins previously described (Barrett, Clark and Hay 1987), but as amended in the methods section. In all experiments unlabelled Poly (dG).Poly (dC) was added in a large excess to eliminate binding to "B" form DNA. After incubation with antibody in the presence of $Co(NH_3)_6Cl_3$ the labelled probe was analysed by electrophoresis in non denaturing 6% polyacrylamide gels. Incubation of [^{32}P] labelled probe with the rabbit anti-"Z" serum in 100µM $Co(NH_3)_6Cl_3$ followed by electrophoresis revealed specific retention of the labelled DNA at the top of the gel. Incubation with preimmune rabbit serum showed no such retention and no retention was evident when the incubations and electrophoresis were carried out in the absence of $Co(NH_3)^{3+}$ ions (figure 5a). Specific retention of the labelled DNA probe was demonstratable out to a final dilution of 1 in 40,000 anti-"Z" serum [with different probe preparation] (figure 5b). Incorporation of $Co(NH_3)_6Cl_3$ into the gel and running buffer on electrophoresis, to ensure a non "B" 'probe' DNA conformation, resulted in labelled bands of slower mobility to that obtained with control gels in the absence of $Co(NH_3)_6^{3+}$ ions (cf figure16a &b). Competition experiments were carried out, with the minimum antiserum dilution

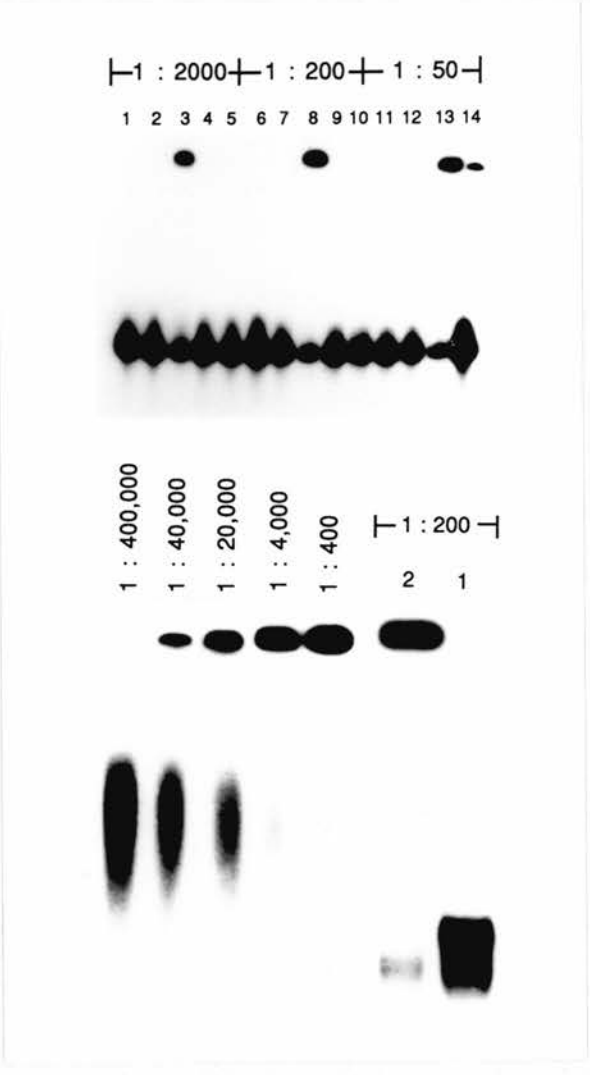


a. b.

Figures 4a and 4b. Analysis of Poly(dG-dC).Poly(dG-dC) from Pharmacia/P-L Biochemicals.

(a) Analysis of native Poly(dG-dC).Poly(dG-dC) from the newly merged Pharmacia/P-L Biochemicals on a 1% agarose gel followed by ethidium bromide staining. Lambda DNA restriction fragment (Hind III) size markers (lane 1) and Øx174 restriction enzyme (Hae III) DNA markers (lane 7) are shown in base pairs. Poly(dG-dC).Poly(dG-dC) (2.5µg) is shown in lane 2 while lanes 3-6 contain polymer (2.5µg) incubated with BssH II restriction endonuclease (8 units) at increasing time intervals; 1hour (lane 3), 2 hours (lane 4), 4 hours (lane 5) and 6 hours (lane 6).

(b) Native Poly(dG-dC).Poly(dG-dC) [Pharmacia/P-L Biochemicals] was [^{32}P] end labelled and electrophoresed on a 6% polyacrylamide gel and subject to autoadiography.



Figures 5a and 5b. Detection of "Z" DNA binding activity using anti-"Z" DNA serum in a gel retention assay.

(a) Gel retention assays were carried out with [^{32}P] labelled Poly(dG-dC).Poly (dG-dC) as 'probe' DNA and rabbit polyclonal antibody raised against the brominated "Z" form of the polymer. Preimmune rabbit serum was used in control assays. Labelled probe (~1ng, 40 counts/second by Cerenkov) was preincubated in a reaction mix (18µl) containing 25mM Tris HCl pH 7.5, 10% glycerol, 1mM DTT, 0.1% NP40, 50mM NaCl, 250µg/ml BSA, 1mM PMSF, 100µM $Co(NH_3)_6Cl_3$ or 1mM EDTA and 1µg Poly (dG).Poly (dC) [carrier "B" DNA] for 1 hour at 37^{o}C prior to adding 2µl of diluted rabbit serum. Incubation was continued at room temperature for a further 20 minutes, gel dyes (4µl) were then added [25mM Tris HCl pH 7.5, 50% glycerol, 0.01% bromophenol blue] and the sample loaded onto a 6% polyacrylamide gel prepared in 40mM Tris/glycine pH 8.5 which was electrophoresed in the same buffer at pH 8.8.

Rabbit serum was added to the assays at a final dilution of 1: 2000 (lanes 1-4), 1: 200 (lanes 6-9) and 1: 50 (lanes 11-14).

Lanes 1,6 and 11 contain preimmune serum in EDTA whilst lanes 2, 7 and 12 contain the same serum in cobalt.

Lanes 4, 9 and 14 contain anti-"Z" serum in EDTA whereas lanes 3, 8 and 13 contain the same serum in cobalt.

Control lanes 5 and 10 do not contain serum and are in cobalt containing buffer.

Retention of the labelled DNA probe is only observed with the anti-"Z" DNA serum in assays containing $Co(NH_3)_6Cl_3$ to promote "Z" conformation (lanes 3, 8 and 13).

(b) **Detection of "Z" DNA versus anti-"Z" serum dilution.**

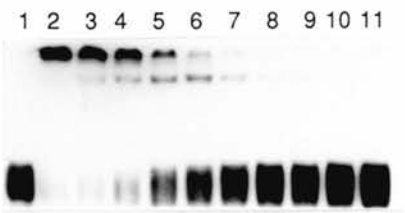
Gel retention assays were performed using serial dilutions of anti-"Z" DNA serum in cobalt conditions. Final anti-"Z" serum dilutions are as indicated; lane 1 contains control preimmune serum. Retention is observed out to a final dilution of 1: 40,000.

that provided maximal probe retention (therefore anti-serum not in excess of DNA antigen), to assess the effect of adding unlabelled polynucleotides as competitors for antibody binding. Figure 6a shows that competition was obtained with unlabelled probe, Poly(dG-dC).Poly(dG-dC), in ng amounts whereas no retention was achieved with the single stranded polymer Poly (dT) (figure 6b). Boiled and renatured salmon sperm DNA also competed for binding to the labelled probe, but 100 times more competitor was required compared to the unlabelled competitor 'probe' DNA (figure 6c). This possibly reflecting the presence of a small number of "Z" DNA like sequences in the salmon sperm DNA. In these competition experiments additional quantities of unlabelled Poly (dG).Poly (dC) carrier DNA were added to at least 1,000 fold excess with no effect on the results obtained.

Using this system as a model to detect "Z" DNA/protein interactions in the presence of $Co(NH_3)_6^{3+}$ ions, the parameters of the binding reactions were studied with respect to time and temperature of incubation and the conditions described in the materials and methods section were utilised in all subsequent experiments. In every gel shift assay the efficacy of the labelled probe was monitored by the ability of the anti-"Z" serum to retain the labelled DNA at the top of the gel, using preimmune rabbit serum as a negative control.

The minimum number of base pairs required to form "Z" DNA in an enviroment containing $Co(NH_3)_6^{3+}$ ions was monitored by gel shift assays using probes of decreasing fragment size. These probes were prepared from the partial digestion of Poly (dG-dC).Poly (dG-dC) [P-L Biochemicals] by the restriction enzyme BssH II (figures 3a & b). A plasmid containing a stretch of 22 alternating guanidine and cytidine bases flanked at either side by a Bam HI restriction site (pAN 022) was kindly gifted by Alfried Nordheim. Published data has shown that the 22 base purine/pyrimidine alternating sequence maintains a "Z" conformation within the supercoiled plasmid. In our studies the fragment was excised from the plasmid via the BamHI restriction endonuclease sites and the [^{32}P] labelled isolated fragment

a.

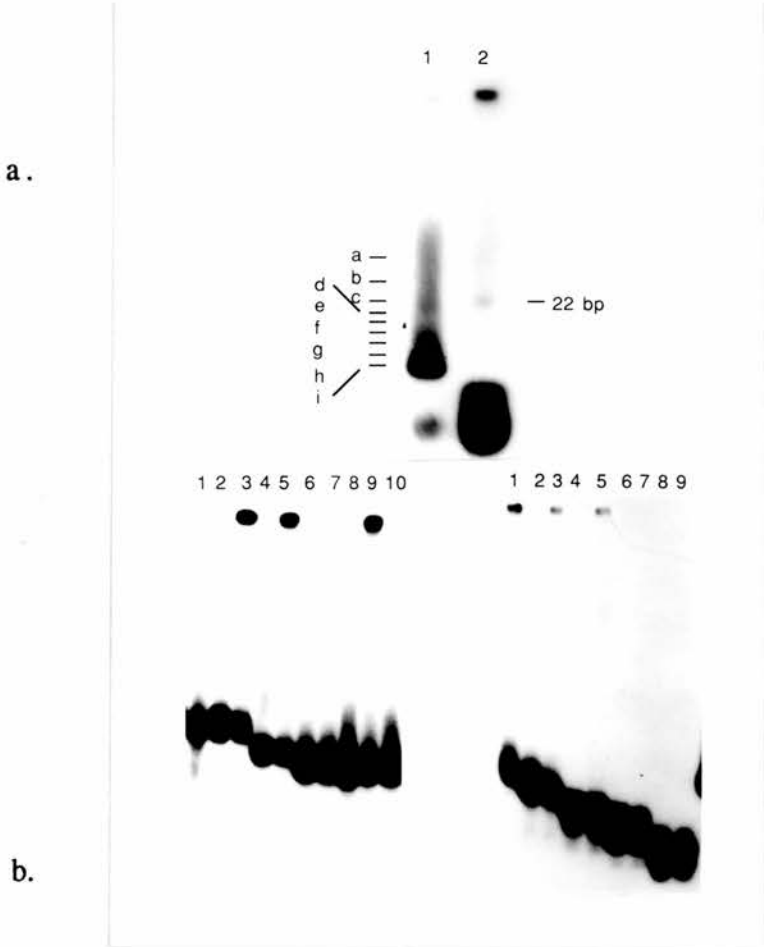


b.

c.

Figures 6a, b & c. Competition assays.
Gel retention assays were carried out as previously described (figure 5a) with anti-"Z" DNA polyclonal rabbit serum at a final dilution of 1: 500 in cobalt conditions. This dilution facilitated approximately 100% retardation of the particular preparation of labelled 'probe' DNA but did not result in a large excess of anti-"Z" antibodies in the assay. Thus an accurate indication of competition was achieved. Carrier DNA (1µg) was present in all the assays and incubation with preimmune rabbit serum gave no retention at this dilution (lane 1).
Unlabelled competitor DNA was included in the assay reaction mix before the one hour preincubation in order that "Z" conformation could occur in competitor DNA in addition to the 'probe' DNA.
(a) Unlabelled 'probe' [Poly(dG-dC).Poly(dG-dC)] DNA was added to the assays in increasing amounts; 0ng (lane 2), 0.1ng (lane 3), 0.25ng (lane 4) 0.5ng (lane 5), 1ng (lane 6), 2ng (lane 7), 4ng (lane 8), 5ng (lane 9), 10ng (lane 10) and 25ng (lane 11). Lane 1 contained control preimmune serum at a final dilution of 1: 500.
(b) Unlabelled single stranded homopolymer Poly (dT) was also added to the assays in inceasing quantities; 0ng (lane 1), 50ng (lane 2), 100ng (lane 3), 1000ng (lane 4).
(c) Increasing amounts of boiled salmon sperm DNA was added into assays in increasing amounts; 0ng (lane 1), 10ng (lane 2), 50ng (lane 3), 100ng (lane 4), 500ng (lane 5), 1000ng (lane 6), 2000ng (lane 7) and 3000ng (lane 8).

used in antibody binding experiments. In the presence of $Co(NH_3)_6^{3+}$ ions no retention was observed on either filters or in retention assay gels. When the antigenicity of the labelled 22 base pair fragment was compared to labelled fragments of similar size (generated by partial digestion of Poly (dG-dC).Poly (dG-dC) [P-L Biochemicals] by BssH II) using the gel shift assay several observations were made (figures 7a,b & c). The 22 base pair fragment originating from the plasmid was not antigenic but an excised fragment of Poly (dG-dC).Poly (dG-dC) which migrated with a similar mobility was antigenic and indeed faster migrating fragments of polymer were also antigenic (figure 7b & c). This observation was originally thought to possibly be due to the Bam HI restriction enzyme cleavage sites on either side of the $d(GC)_{11}$ plasmid fragment reducing the number of alternating d(GC) base pairs compared to a polymer fragment of similar size (pAN 022 fragment after BamHI cleavage $GATCC(GC)_{11}G$). However on analysis of these fragments by resolution on 20% polyacrylamide DNA sequencing gels along with size markers, it was evident that the polymer was running anomalously [the equivalent and faster migrating antigenic polymer fragments on the 6% gel were in fact still larger than the 22 base pair plasmid fragment]. By assaying sequentially faster migrating bands of polymer which had been sized on a sequencing gel antigenicity appeared to require more than 33 alternating GC bases (figures 7b & c) thus possibly explaining why no antibody binding was observed with the fragment originating from the plasmid. The plasmid fragment was sequenced using the method described by Maxim and Gilbert 1980 and a 22 base alternating GC stetch of DNA flanked by BamHI sites was confirmed (data not shown).



Figures 7a, b and c. Antigenicity of a BamHI$(GC)_{11}$BamHI restriction fragment and analysis of the number of alternating GC bases required for anti-"Z" conformation serum antigenicity in a 100μM $Co(NH_3)_6Cl_3$ enviroment.

(a) Poly(dG-dC).Poly(dG-dC) [P-L Biochemicals] digested with BssHII restriction endonuclease (lane 1) and the BamHI restriction digested plasmid pAN 022 (lane 2) were ^{32}P radioactively end labelled and electrophoresed on a 10% polyacrylamide gel. The excised DNA fragments which were used to measure antigenicity are indicated. Polymer fragment c. has the same electrophoretic mobility as the plasmid born BamHI$(GC)_{11}$BamHI restriction fragment. Free ^{32}P nucleotide can be seen at the bottom of the gel below the labelled DNA fragments.

(b & c) Autoradiograms showing antigenicity of the excised DNA fragments with anti-"Z" DNA serum at a final serum dilution of 1: 2000.

(b) Polymer fragment a. (lane 1), Polymer fragment a. with preimmune control serum (lane 2), Polymer fragment a. with anti-"Z" serum (lane 3).

Polymer fragment b. with preimmune control serum (lane 4), Polymer fragment b. with anti-"Z" serum (lane 5).

pAN 022 $(GC)_{11}$ fragment with preimmune serum (lane 6), pAN 022 $(GC)_{11}$ fragment with anti-"Z" serum (lane 7).

Polymer fragment c. with preimmune serum (lane 8), Polymer fragment c. with anti-"Z" serum (lane 9), Polymer fragment c. (lane 10).

(c) Polymer fragment d. with anti-"Z" serum (lane 1), Polymer fragment e. with preimmune serum (lane 2), Polymer fragment e.with anti-"Z" serum (lane 3).

Polymer fragment f.with preimmune serum (lane 4), Polymer fragment f. with anti-"Z" serum (lane 5).

Polymer fragment g.with preimmune serum (lane 6), Polymer fragment g. with anti-"Z" serum (lane 7).

Polymer fragment h.with preimmune serum (lane 8), Polymer fragment h. with anti-"Z" serum (lane 9).

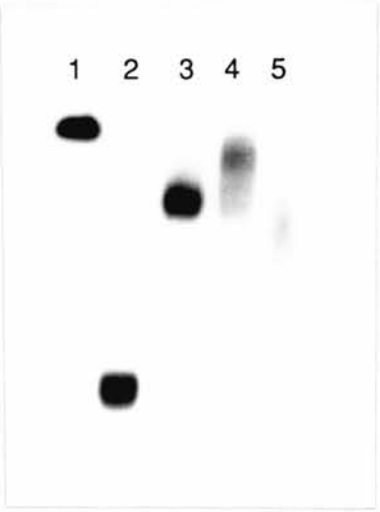
From information gained by migrating the excised polymer fragments on a 20% polyacrylamide DNA sequencing gel along with size markers it was evident that fragment e. contains 42bp, fragment f. 36bp and fragment g. 33bp. Thus suggesting that fragments of less than 36bp are not antigenic in the presence of 100μM $Co(NH_3)_6Cl_3$ in this assay system.

Detection of "Z" DNA [non "B" DNA] binding Proteins in Mammalian cells.

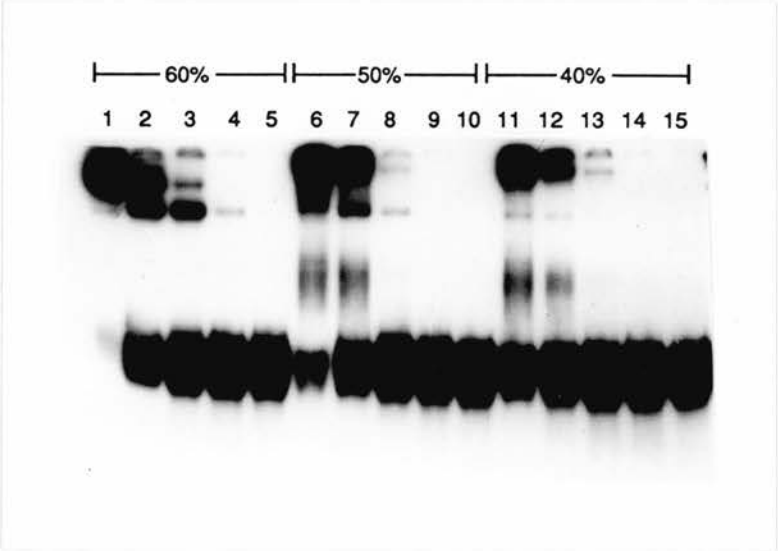
Initial studies carried out on extracts of HeLa S_3 spinner cells indicated that proteins binding to the "Z" DNA probe could be detected using the gel shift assay in both cytoplasmic and nuclear extracts (data not shown). Since it was assumed that there would be fewer DNA binding proteins in the cytoplasmic extracts [simplifying identification and purification] further fractionation of this extract was carried out. Sequential step-wise ammonium sulphate precipitations with centrifugation and dialysis of the redissolved precipitates provided the first crude separation. Specific "Z" DNA binding activity was evident in those fractions precipitated by 40, 50 and 60% ammonium sulphate (figure 8), no binding in gel shift assays was evident in buffer containing EDTA. [Similar results were obtained with cytoplasmic extracts from baby hamster kidney cells (BHK 21)]. Assaying the binding activity from serial dilutions of the dissolved precipitates produced numerous bands in gel shift assays which may correspond to different species of proteins [not all common to a particular precipitation] (figure 9).

Preliminary characterisation of these binding proteins was attempted by separation of polypeptides in the 60, 50 and 40% ammonium sulphate precipitates by SDS PAGE, renaturation in situ and electrophoretic transfer to nitrocellulose. Polypeptides were then probed with ^{32}P labelled Poly (dG-C).Poly (dG-C) either in the presence or absence of 100μM $Co(NH_3)_6Cl_3$ in buffer containing a large excess of unlabelled Poly(dG).Poly(dC). In $Co(NH_3)_6^{3+}$ ions, a polypeptide with an apparent molecular weight of 56,000 was detected by autoradiography as "Z" binding; however it was only present in the 40 and 50% precipitates (figure 10). The polypeptide was not detected in the absence of $Co(NH_3)_6^{3+}$ ions; nor was binding observed with histones electrophoresed and blotted in parallel.

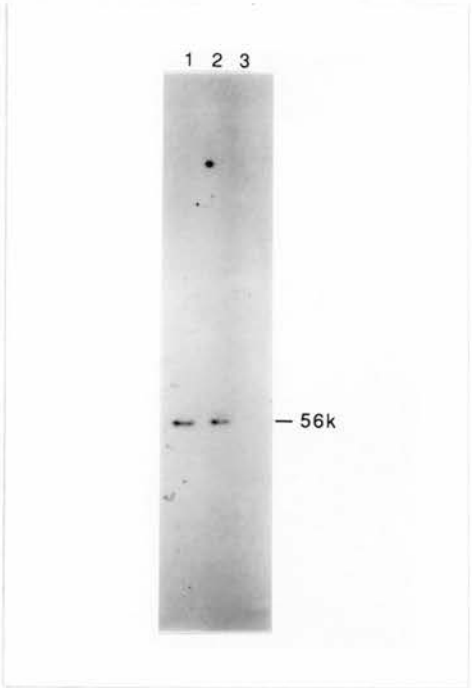
Fractionation of crude cytoplasmic extract by FPLC (Pharmacia) anion (Mono Q) exchange chromatography followed by gel shift analysis of the fractions indicated that "Z" binding activity was confined to fractions which composed a minor



Detection of "Z" DNA binding activity in mammalian cell extracts.
Figure 8.
Reactions were as described previously (figure 4a) containing ~1ng of radioactively labelled alternating GC polymer, with 1µg of unlabelled non alternating (G)(C) homopolymer in each assay (20µl). Ammonium sulphate precipitates of protein from HeLa cell cytosol were assayed for "Z" DNA binding activity by gel retention under standard cobalt conditions. An autoradiograph of the assay gel is shown with lanes containing :-
Anti-"Z" DNA serum at a final dilution 1: 1000 (lane1), preimmune serum at a final dilution 1: 1000 (lane 2), 2µl (42µg) 60% ammonium sulphate precipitate (lane 3), 2µl (30µg) 50% ammonium sulphate precipitate (lane 4) and 2µl (21µg) 40% ammonium sulphate precipitate (lane 5).



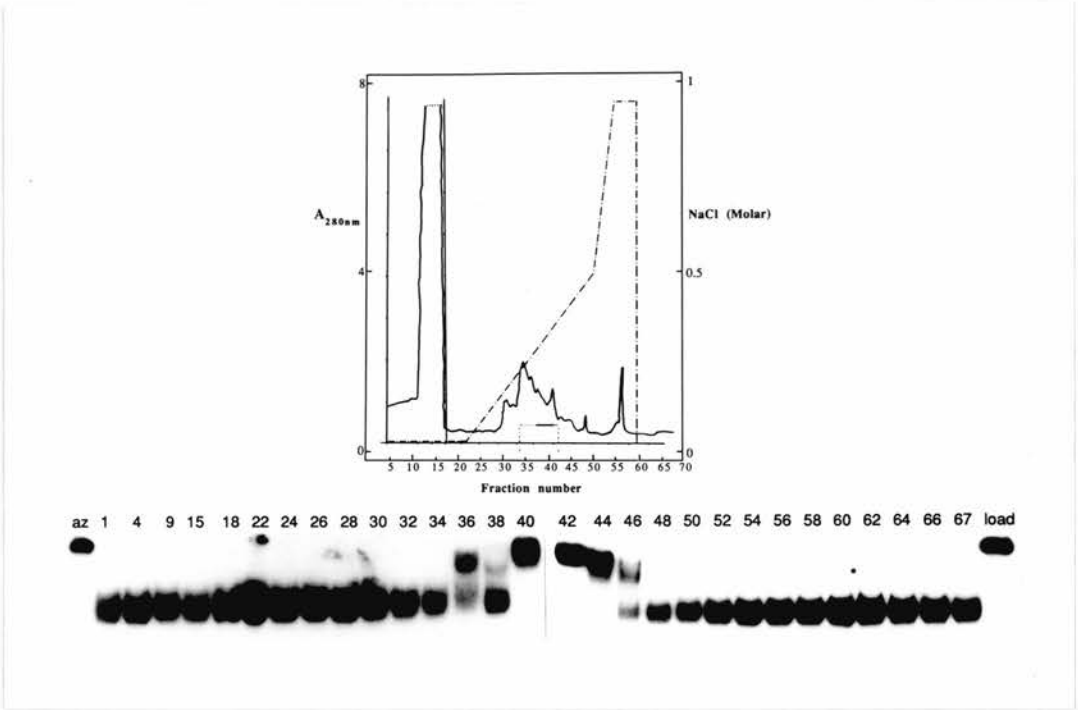
"Z" DNA binding activity in serial dilutions of ammonium sulphate precipitates from HeLa cell cytosol.
Figure 9.
As shown in figure 8, "Z" binding activity was evident in the 40, 50 and 60% ammonium sulphate precipitates. The protein concentration in each of the dialysed precipitates was determined (Bradford 1976) and serial dilutions of each made in dialysis buffer. Activity in each dilution was monitored by gel retention as previously described. The amount of protein added into assays was as follows:
Lanes 1-5, 60% ammonium sulphate precipitate; 2µg (lane 1), 1µg (lane 2), 0.5µg (lane 3), 0.25µg (lane 4), 0.125µg (lane 5).
Lanes 6-10, 50% ammonium sulphate precipitate; 3µg (lane 6), 1.5µg (lane 7), 0.75µg (lane 8), 0.375µg (lane 9), 0.187µg (lane 10).
Lanes 11-15, 40% ammonium sulphate precipitate; 2µg (lane 11), 1µg (lane 12), 0.5µg (lane 13), 0.25µg (lane 14), 0.125µg (lane 15).



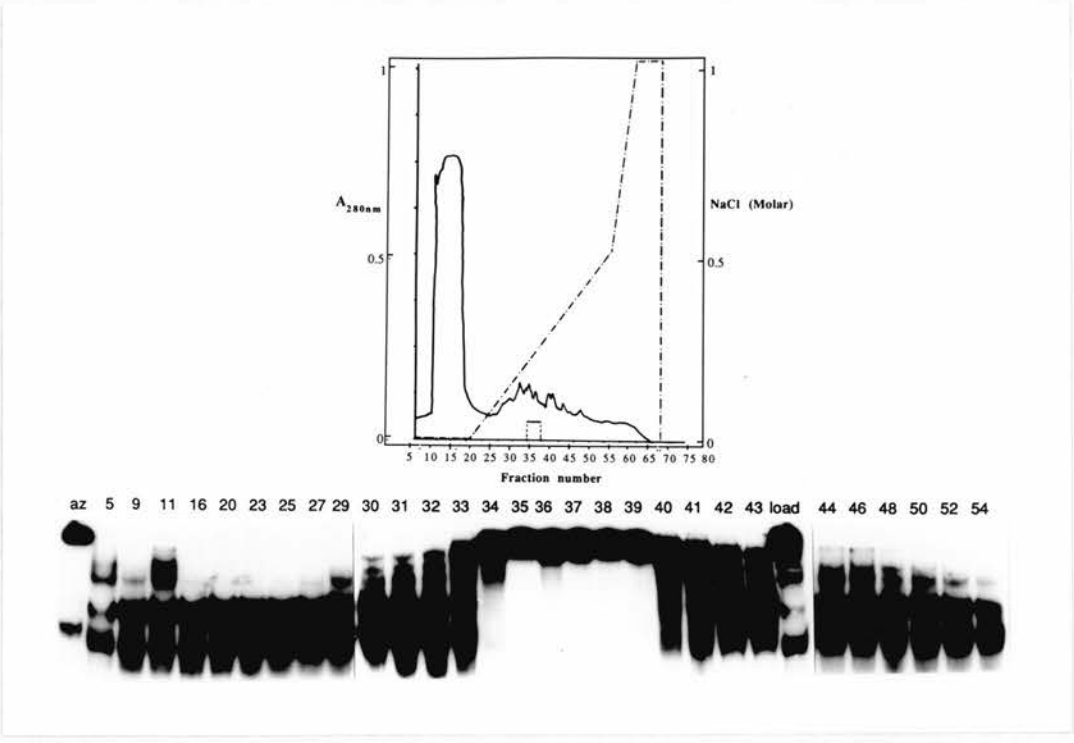
Identification of "Z" DNA binding polypeptides in ammonium sulphate precipitates from HeLa cell cytoplasm.
Figure 10.
Polypeptides contained in the the dialysed 40, 50, 60% ammonium sulphate precipitates (100µg) were separated by SDS PAGE analysis, renatured in situ, and transfered to nitrocellulose. "Z" binding activity was detected on probing with ^{32}P labelled Poly (dG-dC).Poly (dG-dC) in an excess of unlabelled nonalternating "B" form polymer in the presence of cobalt ions. No activity was observed in EDTA.
The figure shows an autoradiogram of the nitrocellulose after probing in cobalt conditions. Lane 1. contains 40% ammonium precipitate, lane 2. 50% precipitate and lane 3. 60% precipitate. The molecular weight of the labelled polypeptide band was derived by comparison with parallel stained blots containing adenovirus type 2 proteins as markers.

fraction of the total protein in the initial extract (data not shown).

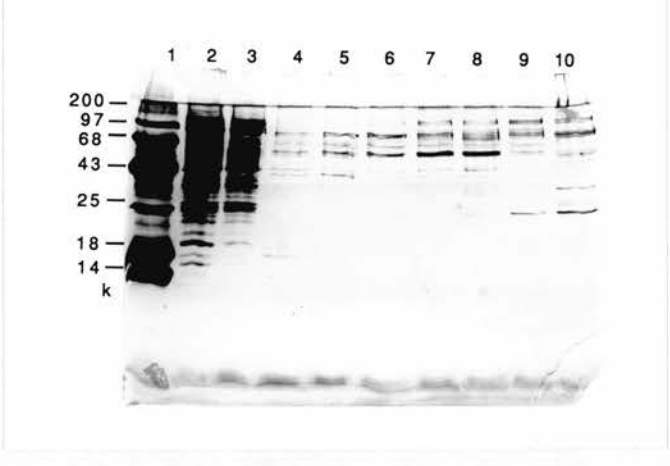
FPLC fractionation of the 60% ammonium sulphate precipitation (25mg) was carried out to isolate and identify proteins involved in binding to "Z" DNA conformations. Initial anion exchange (Mono Q) separation at pH 7.5 resulted in binding activity being detected by gel shift assays in fractions eluting between 0.25M and 0.4M NaCl (figure 11). While cation exchange (Mono S) chromatography of the pooled and dialysed active fractions at the same pH revealed binding activity in fractions eluting from the column between 0.2 and 0.25M NaCl (figure 12). This activity was contained in a minor component of the total protein. Figure 13 shows the analysis of the active fractions eluting from the Mono S column by SDS PAGE followed by silver staining (Biorad). Dialysis of the pooled fractions from the Mono S column was followed by further fractionation by DNA affinity chromatography.



Fractionation of the 60% ammonium sulphate precipitate from HeLa cell cytosol by FPLC anion exchange chromatography.
Figure 11.
The dialysed redissolved 60% ammonium sulphate precipitate from the HeLa cytoplasm was either filtered through a 0.22µm filter or centrifuged at 100,000g for 30 minutes to remove particulates before loading onto a Mono Q (Parmacia) anion exchange column. Protein fractions (0.6ml) eluting with salt were collected and assayed (2µl) for "Z" DNA binding proteins by gel retention. The figure indicates fractions 36 to 46 exhibit retention of ^{32}P labelled Poly (dG-dC).Poly (dG-dC). The protein OD_{280nm} and NaCl gradient profiles shown above suggest that "Z" DNA binding activity eluted from the column between 0.25 and 0.4M NaCl. No activity was evident in the protein which did not bind to the column.



Further fractionation of the HeLa cytosol 60% ammonium sulphate precipitate by FPLC cation exchange following previous anion exchange chromatography.
Figure 12.
Active "Z" DNA binding fractions eluted from the FPLC anion exchange column were pooled and dialysed overnight to lower the salt concentration to 50mM NaCl and to replace the Tris HCl pH7.5 buffer with HEPES at the same pH. On loading the Mono S (Pharmacia) cation exchange column fractions (0.6ml) were again collected and eluting protein assayed (2µl) for "Z" DNA binding activity in gel retention assays. The autoradiogram in the figure shows peak activity to be in fractions 34 to 39 with the profiles indicating elution between 0.2 and 0.25M NaCl.



SDS PAGE analysis of "Z" DNA binding protein after FPLC cation exchange chromatography.
Figure 13.
Aliquots of fractions eluted from the Mono S column which were defined as containing "Z" DNA binding activity by gel retention (figure 12) were electrophoresed along with a sample of the column load [load = pooled and dialysed active fractions eluted from the Mono Q column] on a 16% polyacrylamide gel. The separated polypeptides were visualised by silver staining (Biorad).
Gel lanes consisted of :- molecular weight protein markers (BRL) (lane 1), 10µl [~6µg] of the Mono S column load (lane 2), 5µl [~3µg] of the Mono S column load (lane 3), 15µl [~2µg] of the active "Z" DNA binding fractions eluted from the Mono S column (figure 12) (lanes 4 - 10); fraction 40 (lane 4), fraction 39 (lane 5), fraction 38 (lane 6), fraction 37 (lane 7), fraction 36 (lane 8), fraction 35 (lane 9), fraction 34 (lane 10).

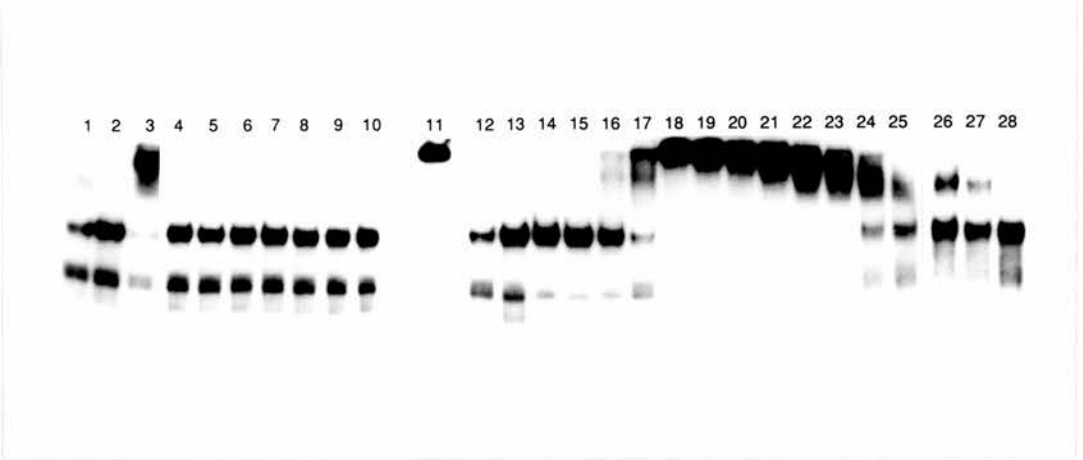
Affinity chromatography.

Carrier "B" form DNA (10μg) was added to the active protein (250μg) prior to loading onto a "Z" DNA/sepharose column in low salt (50mM). Bound protein eluting from the column in 1M NaCl was collected in small fractions (90μl) which were assayed by gel retention for specific "Z" DNA binding (figure 14a). The polypeptide profiles of an active fraction was then analysed by SDS PAGE after TCA precipitation and visualised by silver staining (Biorad) (figure 14b). Figure 14b shows, by comparing the column load (lanes 1 & 2) to the peak activity eluting from the affinity column (lane 4), a purification of two polypeptides with apparent molecular weights of 60k and 85k evidently binding with the same molar ratio with possibly a further weaker 68k protein band. A purification profile is shown below.

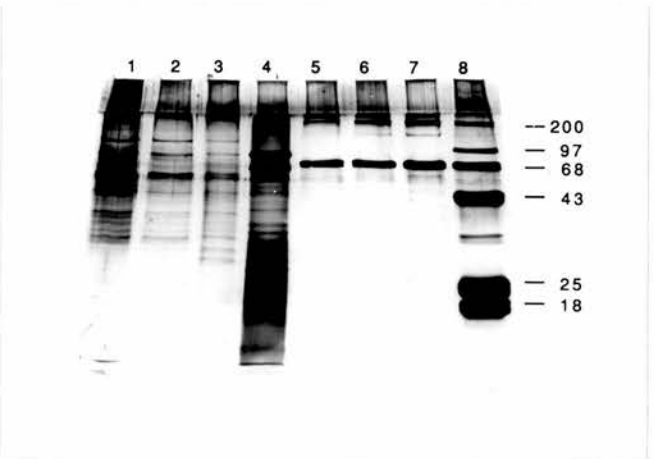
	Protein (mg)	volume (ml)	Units of activity	Yield (%)	Specific activity	fold purified
Mono Q Load	21	5	20,000	100	952	1
Mono S Load	3	6	15,000	75	5,000	5
"Z" DNA affinity load	0.36	3.6	9,000	45	25,000	26
"Z" DNA affinity eluate	0.005	0.54	810	25	162,000	170

1unit of activity retains 50% of the labelled 'probe' DNA in a gel retention assay.

An active fraction (showing retention of labeled "Z" probe) eluted from the "Z" DNA column was further purified by sedimentation through a 15 to 40% glycerol gradient, fractions (50μl) were collected from across the gradient and assayed for gel retention activity (figure 15a), the polypeptide content of the positive fractions were analysed by SDS PAGE after TCA precipitation (figure 15b). At first inspection of the silver stained gel separation of the polypeptides did not appear to be successful, with fractions which were negative in the binding assay containing the same polypeptide profile as those which were positive. As well as the bands described in figure 14b a band of similar intensity at a lower mobility was observed with an apparent molecular weight of 55k. However on closer inspection binding activity would appear to coincide with the weaker intensity 68k polypeptide.

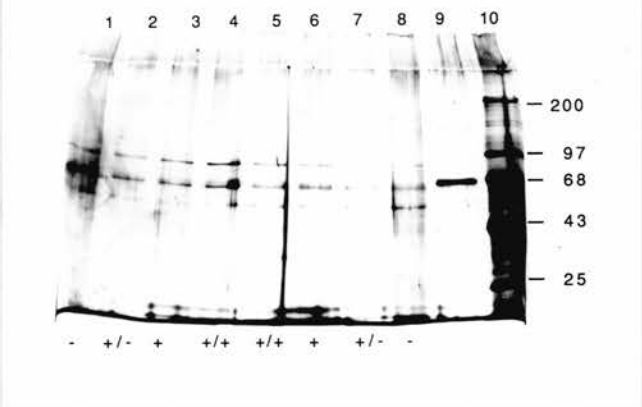


"Z" DNA/sepharose affinity separation in the presence of competitor "B" DNA following previous ion exchange chromatography.
Figure 14a.
Fractions eluting from the FPLC cation exchange column which contained "Z" DNA binding activity (figure 12) were pooled and dialysed into low salt buffer containing 100μM cobalt hexaminochloride. Excess carrier "B" form Poly (dG).Poly (dC) polymer DNA was then added before loading onto a "Z" DNA affinity column. Fractions containing bound protein eluting in high salt were collected and (2μl) assayed for "Z" DNA binding protein by gel retention. The figure shows autoradiographs of the assays with fraction numbers as follows :-
Low salt buffer column wash after loading (lane 1); column flow through whilst loading (lane 2); column load (lane 3); high salt buffer elution fractions 1-7 (lanes 4-10); anti-"Z" DNA serum at a final dilution of 1: 1000 (lane 11); high salt buffer elution fractions 33-49 (lanes 12-28)



SDS PAGE analysis of the peak activity fraction [bound in the presence of excess competitor "B" form DNA] eluting from the "Z" DNA affinty column with high salt buffer.
Figure 14b.
Protein in fraction 40 (figure 14a) containing the peak "Z" DNA binding activity eluting from "Z" affinity column was precipitated by TCA before loading onto a 16% polyacrylamide denaturing gel. Polypeptides were visualised by silver staining. The initial affinity column load and subsequent washes were also analysed in the gel. Apparent molecular weights and quantity of polypeptides were estimated by comparison with molecular weight markers (Gibco/BRL) and various concentrations of BSA electrophoresed in neighbouring lanes on the gel.
Gel lanes were as follows :- "Z" affinity column load (lane 1); low salt column wash (lane 2); column flow through whilst loading (lane 3); fraction 40 high salt elutant containing peak peak "Z" binding activity [figure 15] (lane 4); 0.5μg BSA (lane 5); 1μg BSA (lane 6); 2μg BSA (lane 7); protein molecular weight standards (lane 8).

Separation of proteins from a peak activity fraction [bound in the presence of excess competitor "B" form DNA] eluting from the "Z" DNA affinty column with high salt buffer by sedimentation through a glycerol gradient.
Figure 15a.
Protein in fraction 39 (figure 14a), containing "Z" DNA binding activity, eluting from "Z" affinity column was loaded onto a 15-40% glycerol gradient (5ml) and centrifuged for 21 hours at 4^0C. Fractions (50µl) collected from the gradient were assayed for specific "Z" DNA affinity by gel retention. In this instance because of the dilution factor, an attempt was made to increase the sensitivity of the assay. Five fold more labelled "Z" probe was included in each assay, carrier "B" form DNA was reduced ten fold (100ng) and BSA concentration in the assay increased two fold. The figure shows an autoradiogram of gradient fractions "Z" DNA binding activity by gel retention.
Gel lanes were as follows :- gradient fraction 51 (lane 1); 54 (lane 2); 57 (lane 3); 60 (lane 4); 63 (lane 5); 67 (lane 6); 71 (lane 7); 75 (lane 8); 79 (lane 9); 83 (lane 10); 87 (lane 11); 91 (lane 12); 95 (lane 13); 99 (lane 14); 103 (lane 15); anti-"Z" DNA serum at a final dilution of 1 : 1000 (lane 16).



SDS PAGE analysis of the "Z" DNA binding proteins eluted from the "Z" affinity column and separated by glycerol gradient sedimentation under non denaturing conditions.
Figure 15b.
Proteins in the gradient fractions which bound specifically to "Z" DNA in gel shift assays (figure 15a) were diluted with an equal volume of buffer C before precipitation with TCA. Analysis of the polypeptides by SDS PAGE in 10% polyacrylamide gels, along with those contained in neighbouring negative fractions was visualised by silver staining.
Gel lanes contain the following: glycerol gradient fraction 53 (lane 1); 58 (lane 2); 64 (lane 3); 68 (lane 4); 72 (lane 5); 76 (lane 6); 80 (lane 7); 86 (lane 8); 100ng BSA (lane 9); protein molecular weight markers (lane 10).
"Z" DNA binding by gel retention (figure 15a) is indicated below each gel lane.
- = no binding; +/- = weak binding; + = reasonable resonable and +/+ = strong "Z" DNA binding activity.

On a later purification of the HeLa cell cytoplasmic 60% ammonium sulphate precipitate, fractions containing "Z" DNA binding activity, which eluted from the FPLC Mono S cation exchange column, were pooled before concentration and desalting into affinity column low salt buffer by filtration (Centricon 30 microconcentrator, Amicon division, W.R.Grace & Co). Gel retention assays on the retained and filtered material demonstrated that no activity was lost in the filtrate (data not shown).

On passing the concentrated sample over a "B" DNA affinity column, proteins which did not bind in low salt (0.25M NaCl) were directly loaded onto a "Z" DNA affinity column, also equilibrated in low salt buffer. Protein which bound to the "Z" DNA matrix were then eluted with high salt buffer (1M) and "Z" DNA binding activity confirmed by gel retention.

Proteins which did bind to the "B" DNA affinity column in low salt (0.25M) were later eluted with high salt (1M) and fractions also assayed for any specific "Z" DNA binding activity.

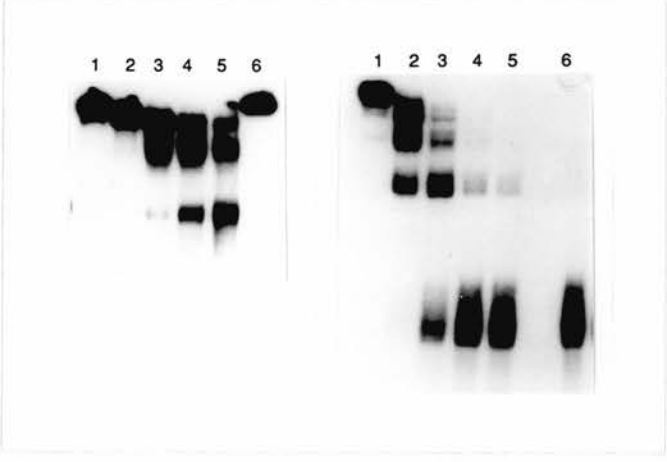
The elutions from the "B" and "Z" DNA affinity columns both contained "Z" DNA binding activity (by gel retention) and on analysis by SDS PAGE followed by silver staining appeared to have the same polypeptide content. These purified polypeptides had a similar apparent molecular weight profile as those previously purified by "Z" DNA affinity chromatography in the presence of excess "B" DNA (figure 14b, lane 4).

Competition assays.

The effect of the quantity of carrier Poly (dG).Poly (dC) polymer DNA included in the retention assay on protein binding was assessed in the presence of $Co(NH_3)_6^{3+}$ ions or EDTA (figure 16). At low concentrations binding of an active "Z" DNA binding protein fraction [eluted from the "Z" DNA affinity column in the presence of excess carrier "B" DNA (1.5µl fraction 42, figure 14a)] to the probe DNA was evident under both conditions, with a preference for greater binding in the presence of cobalt. The amount of carrier "B" DNA (1µg) used in the standard assays was shown to be satisfactory as a measure of specific binding of the purified protein to

cobalt induced probe DNA conformations. At this concentration (1µg, 50µg/ml) of carrier DNA, 75% of the labelled probe was retained in cobalt conditions whereas only <5% was retained in EDTA (figure 16). Thus this quantity of carrier DNA was retained in all further assays unless otherwise stated.

Gel retention assays with the active "Z" DNA binding purified protein fraction (1.5µl fraction 42, figure 14a) were utilised under standard conditions to observe the ability of unlabelled DNA fragments to compete for protein binding with the labelled Poly (dG-dC).Poly (dG-dC) probe DNA.



Cobalt. EDTA.

Effect of increasing quantities of carrier "B" DNA on protein binding
Figure 16.
Unlabelled carrier Poly (dG).Poly (dC) polymer DNA was introduced into retention assays containing labelled Poly (dG-dC).Poly (dC-dG) as DNA 'probe' and (1.5µl) an active "Z" DNA binding protein fraction (42, figure 14a) [eluted from the "Z" DNA affinity column in the presence of excess "B" DNA], in the following amounts 0ng (lane 1), 100ng (lane 2), 500ng (lane 3), 1000ng (lane 4) and 2000ng (lane 5). Control anti-"Z" DNA serum was added into an assay containing 800ng of carrier DNA at a final dilution of 1: 1000 (lane 6). Assays were carried out in both cobalt and EDTA conditions.
Autoradiographs after 16 are shown.

Using the data shown in figure 16, 750ng of carrier DNA was assessed as being sufficient to allow retention of the majority of 'probe' DNA without leaving an excess of unbound protein. Thus 750ng of carrier DNA was included in all the subsequent competition assays.

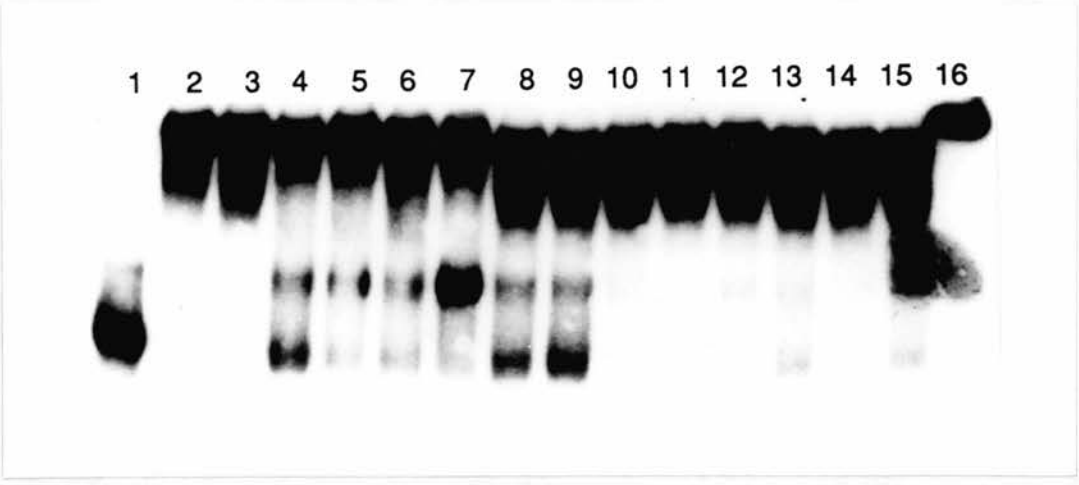
The ability of the labelled Poly (dG-dC).Poly (dG-dC) probe DNA to be competed for purified protein binding by unlabelled polymer probe in cobalt conditions was shown to require ~500ng of competitor to completely abolish labelled probe DNA

binding (figure 17). This is a large amount of competitor DNA compared to the quantity of 'probe' DNA (~1ng) in the assay. Studies with single stranded DNA competitors on protein binding in the same conditions indicated that although a 19 base synthetic oligonucleotide provided a very small amount of competition the single stranded DNA polymer Poly (dT) did not compete at all (figure 18a). Single stranded RNA purified from the Parainfluenza type 2 virus also did not compete for protein binding with the probe DNA (figure 18b).

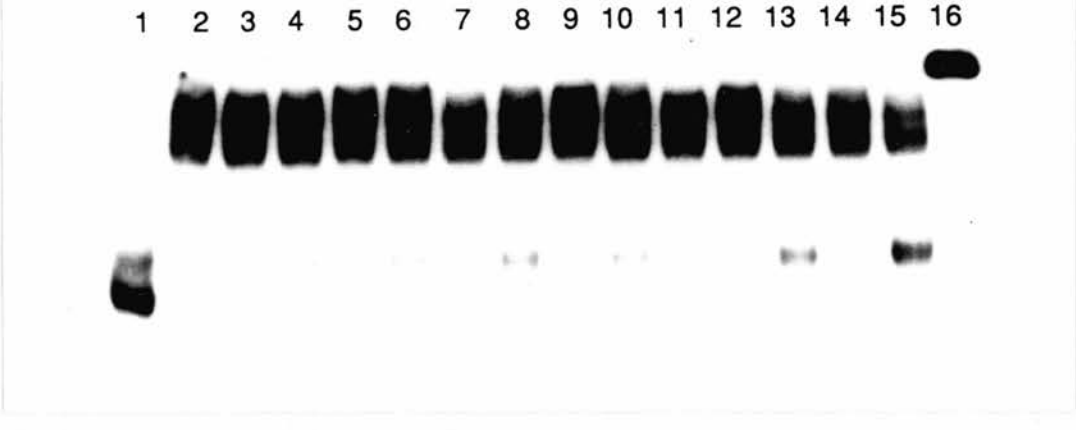
The claim of Krishna et al (1990) that "Z" DNA binding proteins are actually phospholipid binding proteins was also tested in competition assays (figures 19 & 20). Competition for 'probe' DNA binding to anti-"Z" DNA antibodies or the affinity purified HeLa cell protein fraction was carried out by including increasing amounts of the phospholipid cardiolipin into standard assays in both EDTA and cobalt conditions. The ability of the phospholipid binding protein glycophorin to bind to the polymer 'probe' DNA was also analysed in standard assay conditions (figure 19). A very slight competition of anti-"Z" DNA antibody binding was achieved with cardiolipin. An increase in retention with high concentrations of the phospholipid (100µg=5mg/ml) was shown to be due to nonspecific binding observed as retention of the "B" form probe DNA in EDTA conditions where no binding of antibody occurs. Cardiolipin had a marked effect on probe retention with the purified protein fraction, 25µg (1.25mg/ml) providing complete abolition of binding in both cobalt and EDTA conditions. Retention with higher concentrations again proved to be nonspecific, as shown by probe retention at the top of the gel (not characteristic of purified protein binding). No binding activity could be detected with the phospholipid binding protein glycophorin.

Competition assays.
Labelled Poly (dG-dC).Poly (dC-dG) DNA polymer probe was incubated with (1.5µl) an active "Z" DNA binding protein fraction (42), eluted from the "Z" DNA affinity column in the presence of excess carrier "B" DNA (750ng), under standard gel retention assay conditions. Competitor DNA was added into the assay at the preincubation stage (see Materials and Methods).

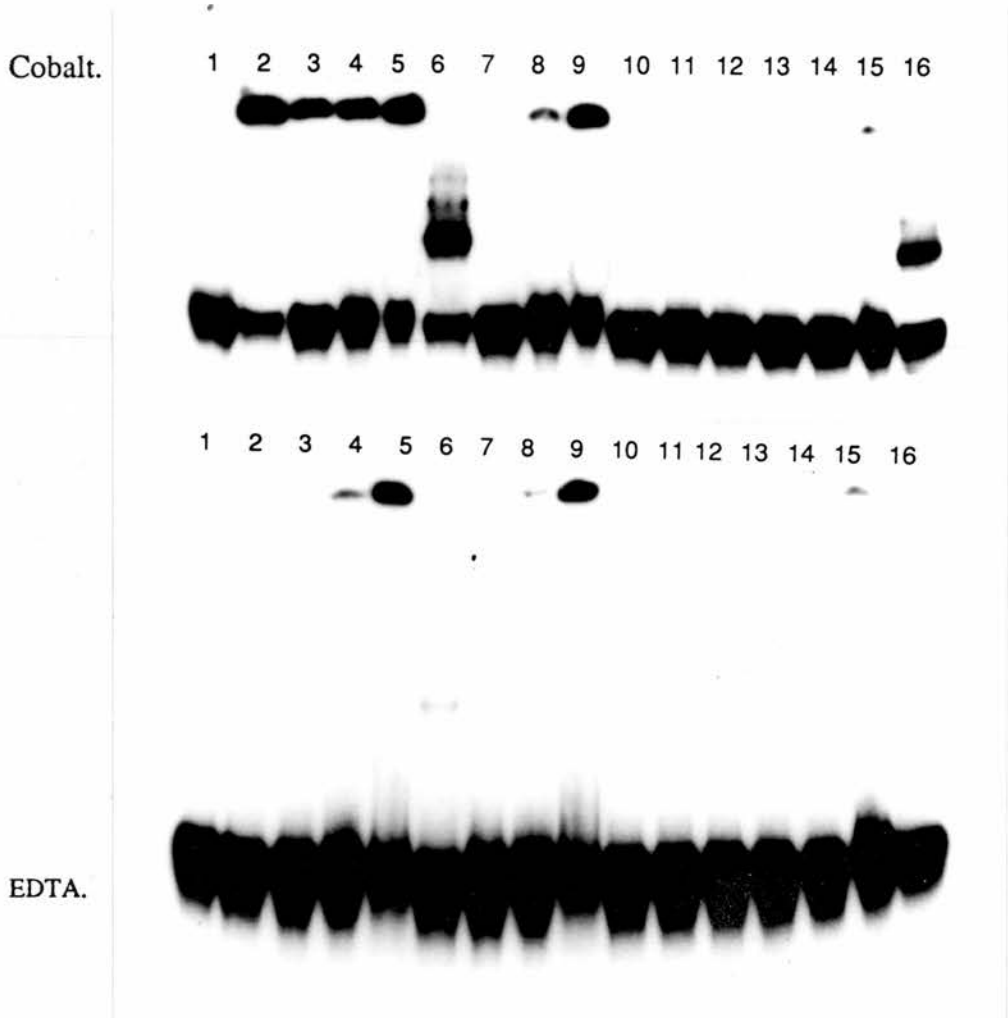
The effect of unlabelled competitor "Z" form Poly (dG-dC).Poly (dG-dC) on purified protein binding in the gel retention assay.
Figure 17.
Unlabelled Poly (dG-dC).Poly (dG-dC) 'probe' DNA was added into assays in the following quantities; 0ng (lane 1), 0.5ng (lane 2), 1ng (lane 3), 5ng (lane 4), 10ng (lane 5), 50ng (lane 6), 100ng (lane 7), 500ng (lane 8) and 1000ng (lane 10).



Effect of competitor single stranded DNA on the purified proteins binding to "Z" DNA.
Figure 18a.
Addition of a 19 base single stranded synthetic oligonucleotide [GATCTGGGGACTTTCCAGG] was made to assays in the following way 0ng (lane 2), 1ng (lane 3), 5ng (lane 4), 50ng (lane 5), 500ng (lane 6), 1000ng (lane 7), 2000ng (lane 8) and 3000ng (lane 9). Alternatively single stranded Poly (dT) poly DNA was added in the following amounts 0ng (lane 10), 50ng (lane 11), 100ng (lane 12), 500ng (lane 13), 1000ng (lane 14) and 2000ng (lane 15). Preimmune (lane 1) and anti-"Z" DNA (lane 16) rabbit serum were included in assays at a final dilution of 1:1000.

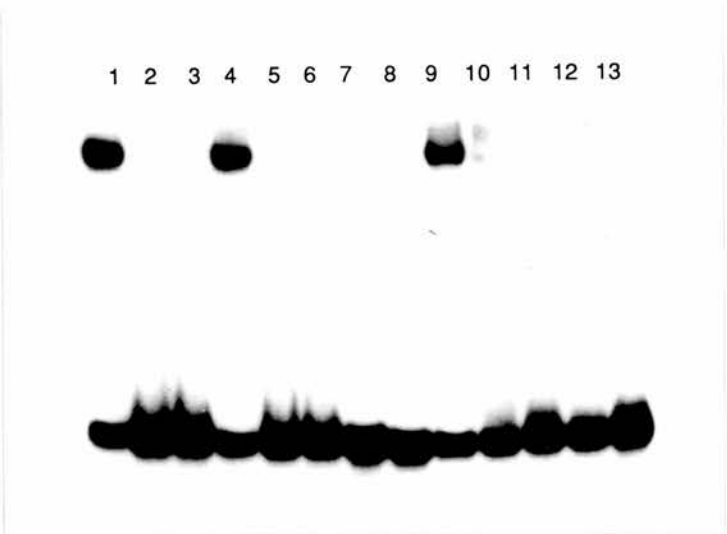


Effect of competitor single strand RNA on protein binding.
Figure 18b.
Introduction of Parainfluenza type 2 messenger RNA into retention assays was made in the following manner; 0ng (lane 2), 10ng (lane 3), 50ng (lane 4), 100ng (lane 5), 500ng (lane 6), 1000ng (lane 7) and 2000ng (lane 8). Total Parainfluenza RNA depleted in message was also included in assays : 0ng (lane 9), 10ng (lane 10), 50ng (lane 11), 100ng (lane 12), 500ng (lane 13), 1000ng (lane 14) and 2000ng (lane 15). Preimmune (lane 1) and anti-"Z" DNA (lane 16) rabbit serum at a final dilution of 1: 1000 were also included.



Competition of purified protein and anti-"Z" DNA antibody binding in gel retention assays by phospholipid.
[Do phospholipid binding proteins also bind "Z" DNA?]
Figures 19
Standard gel retention assays with labelled Poly (dG-dC).Poly (dG-dC) as 'probe' DNA were carried out in cobalt and EDTA conditions with anti-"Z" DNA rabbit serum, purified "Z" DNA binding protein or the phospholipid binding protein glycophorin. The phospholipid cardiolipin was added into the assays as a competitor for DNA binding.
Anti-"Z" DNA serum at a final dilution of 1: 1000 or affinity purified protein (1.5µl) were included in assays (lanes 2-5) and cardiolipin added as competitor in the following amounts 0µg (lane 2 & 6), 25µg (lane 3 & 7), 50µg (lane 4 & 8), 100µg (lane 5 & 9).
In other assays glycophorin was included in assays at varying amounts; 0µg (lane 10), 2.5µg (lane 11), 5µg (lane 12), 10µg (lane 13) and 20µg (lane 14). Glyophorin (5µg) was also competed with Cardiolipin (50µg) (lane 15).
Preimmune serum at a final diution of 1: 1000 (lane 1) and the purified protein fraction competed with BSA (25µg) (lane 16) were included as controls.

Competition assays on the affinity of the DNA binding domain of the transcription factor Nuclear Factor I (NF I) protein to its DNA recognition sequence with cardiolipin as phospholipid competitor were also carried out (figure 20). These studies were important in determining whether phospholipids would interfere with other protein/DNA interactions which involve sequence specificity . The binding domain of the NF I protein bound to a labelled DNA probe containing its recognition sequence in both cobalt and EDTA conditions. Cardiolipin at a concentration of 1.25mg/ml (25µg) completely abolished protein binding in both conditions. A titration of cardiolipin into binding assays at lower concentrations indicated that as little as 1µg (50µg/ml) vastly limited binding and 2.5µg (125µg/ml) inhibited all binding. Theses results suggest that phospholipids inhibit DNA/protein interactions irrespective of DNA secondary structure.



Competition of the transcription factor NF I binding to its specific DNA recognition sequence by phospholipid.
Figure 20.
Gel retention assays with the labelled NF I double stranded DNA binding sequence (GATCTTATTTTGGCTTGAAGCCAATATG) as probe DNA were carried out in EDTA and cobalt environments with the purified DNA binding domain of NF I protein (2µl) [Baculovirus expressed (Bosher et al 1991)]. The phospholipid cardiolipin was added to the assays as a competitor for DNA binding in the presence of excess carrier DNA (1µg, Poly (dG).Poly (dC)) in the following quantities 0µg (lanes 1,4 & 9), 1µg (lane 10), 2.5µg (lane 11), 5µg (lane 12), 7µg (lane 13), 25µg (lanes 2 & 5) and 50µg (lanes 3 & 6). Lanes 7 & 8 contain no protein.
Although assays were carried out in cobalt (lanes 1-3 & 7) and EDTA (4-6 & 8-13) conditions the polyacrylamide gel and electrophoresis buffer did not contain either.

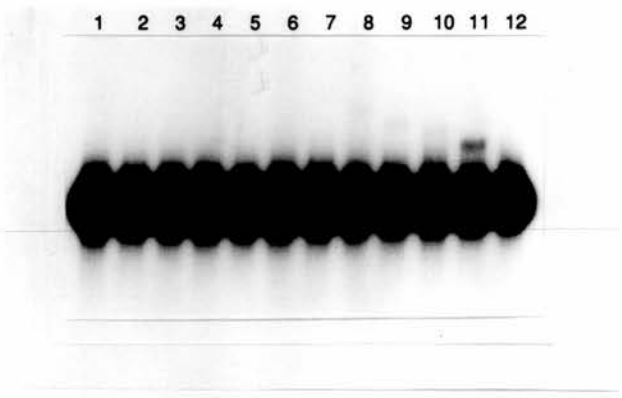
In an attempt to identify the number of DNA binding protein species in the crude cytoplasmic extracts after ammonium sulphate precipitation (in addition to the information obtained by ion exchange and affinity purification) and assign an apparent molecular weight to the activity. Polypeptides in the extracts were separated by electrophoresis under denaturing conditions, excised in gel slices with respect to their apparent molecular weight, passively eluted, concentrated and denatured before slow renaturation and the "Z" DNA binding activity assayed by gel retention.. This procedure could also indicate which of the polypeptides, affinity purified from the 60% ammonium sulphate precipitation, are involved in specific binding to non "B" DNA conformations and whether there is a requirement for more than one to retain activity. Two denaturation methods were applied with varying success. Polypeptides were denatured in the presence of 6M urea before a 50 fold dilution and renaturation allowed to slowly occur overnight at 4°C. Alternatively denaturation occurred in 6M guanidine hydrochloride followed by overnight dialysis at 4°C to facilitate renaturation of the protein.

Using the first method binding activity was detected by gel shift analysis with renatured polypeptides from the 50% ammonium sulphate precipitation (figure 21a). These polypeptides had an apparent molecular weight between 60 and 30k. The pattern of "Z" DNA activity in the gel shift assay was suggestive of a polypeptide of an individual polypeptide of between 60 and 50k being progressively cleaved by proteolysis down to a stable apparent molecular weight of between 35 and 30k. This method also resulted in activity in the 60% ammonium sulphate precipitate at at least two distinct apparent molecular weights. One between 72 and 66k with the other between 42 and 36k (figure 21b).

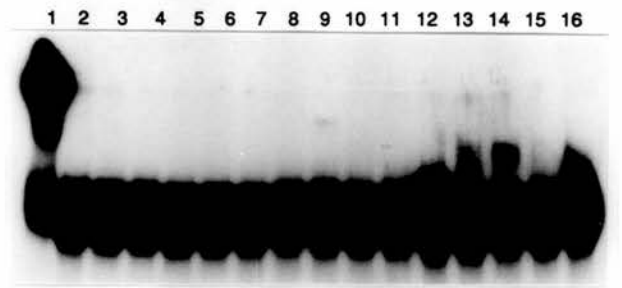
The second method found activity in most molecular weights within the 60% ammonium sulphate precipitate suggesting many different species of binding protein (figure 21c). The highest activity was detected between 84 and 58k although DNA retention was also observed at apparent molecular weights of between 116 and 97k, 97 and 84k, 58 and between 40 and 30k. Specific "Z"

DNA affinity was difficult to interpret as some binding also occured with 'probe' DNA in the "B" conformation.

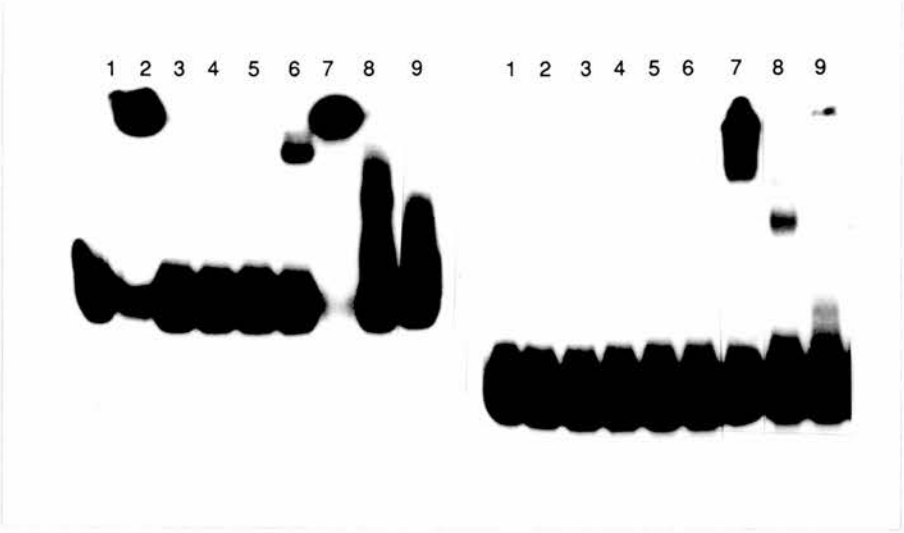
a.



b.



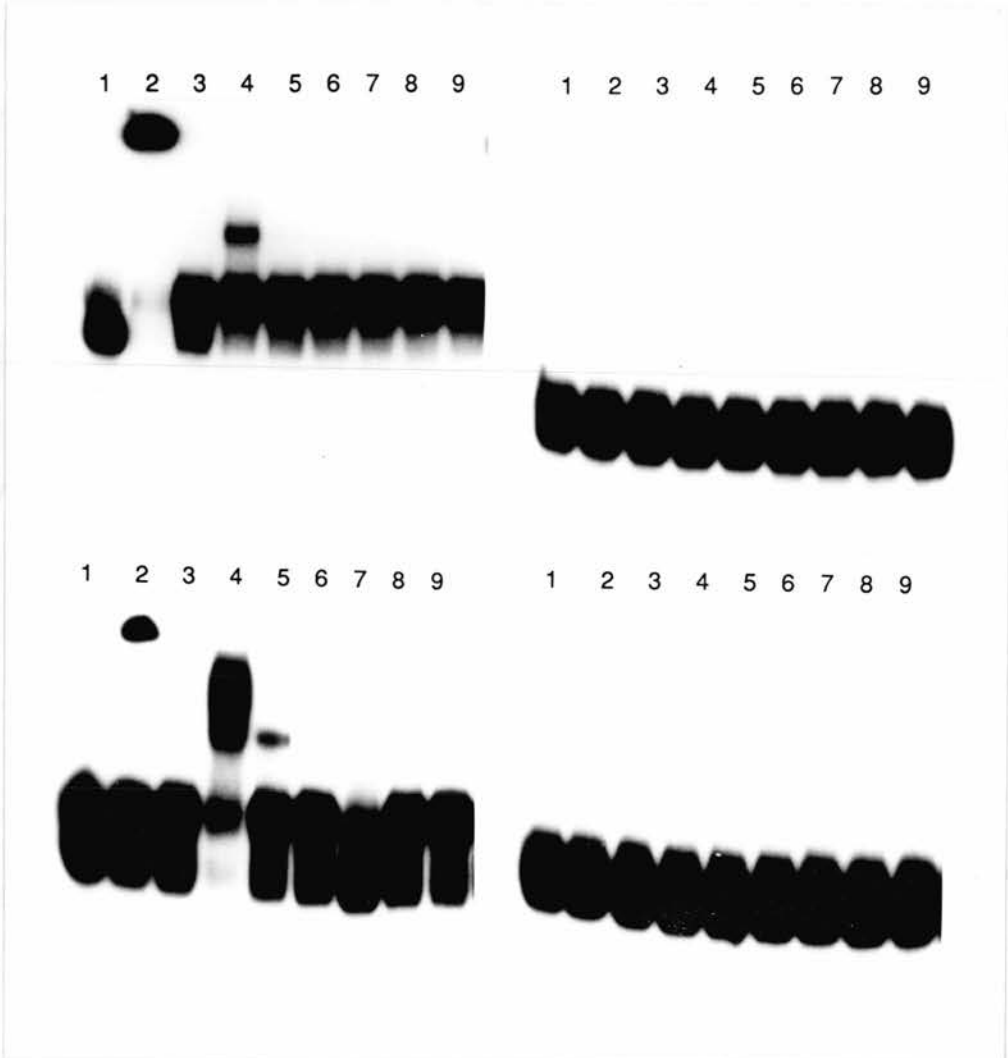
Renaturation of active "Z" DNA binding protein after fractionation by SDS PAGE [Determination of molecular weight].
Figure 21a and b.
(a) Protein from the 50% ammonium sulphate precipitation electrophoresed in a 10% polyacrylamide gel under denaturing conditions along with prestained molecular weight markers was excised from the gel in slices according to a particular molecular size range. Polypeptides were then eluted from the gel slices, concentrated by acetone precipitation and completely denatured by 6M urea before a 50 fold dilution and incubation overnight to allow a slow renaturation process (method 1); "Z" DNA binding activity was then assayed by gel retention (see Materials & Methods). The figure shows autoradiographs of the assays with renatured proteins (14µl) from the gel slices relating to molecular weights of
180-160k (lane 1), 160-120k (lane 2), 120-110k (lane 3), 110-100k (lane 4), 100-85k (lane 5), 85-70k (lane 6), 70-60k (lane 7), 60-50k (lane 8), 50-45k (lane 9), 45-35k (lane 10), 35-30k (lane 11) and below 30k (lane 12).
(b) Protein from the 50% ammonium sulphate precipitation of HeLa cytosol was also subject to method 1 as described above. The autoradiogram of the gel retention assays measuring "Z" DNA binding activity in the renatured proteins is shown, gel lanes relate to polypeptides in the apparent molecular weight ranges of
120-110k (lane 3), 110-100k (lane 4), 100-92k (lane 5), 92-84k (lane 6), 84-78k (lane 7), 78-72k (lane 8), 72-66k (lane 9), 66-60k (lane 10), 60-54k (lane 11), 54-48k (lane 12), 48-42k (lane 13), 42-36k (lane 14), 36-26k (lane 15), >26k (lane 16).
Control lanes were :- Anti-"Z" DNA serum at a dilution of 1 : 1000 (lane1), preimmune serum at a final dilution of 1 : 1000 (lane 2).



In the presence of $Co(NH_3)_6^{3+}$ ions. In the presence of EDTA.

Figure 21c.
Protein from the 60% ammonium sulphate precipitation of HeLa cell cytosol was electrophoresed under denaturing conditions along with prestained protein molecular weight markers. Polypeptides of a particular apparent molecular weight range were excised from the gel, passively eluted overnight, concentrated by acetone precipitation and denatured in 6M guanidine hydrochloride. Renaturation of the polypeptides was achieved by overnight dialysis at 4°c (method 2, see Materials & Methods). The DNA binding activity of the polypeptides was measured by gel retention under "B" and non "B" 'probe' DNA conformation conditions. The figure shows autoradiographs of the binding assays and gel lanes correspond to the following :-
No protein control (lane 1), anti-"Z" DNA serum at a final dilution of 1 : 1000 (lane 2), preimmune serum at a final dilution of 1 : 1000 (lane 3), a molecular weight range of between 180-116k (lane 4), 116-97k (lane 5), 97-84k (lane 6), 84-58k (lane 7), 58-40k (lane 8) and 40-30k (lane 9).

To ensure that DNA binding proteins in general, do not have an intrinsic preference for 'probe' DNA in the presence of $Co(NH_3)_6^{3+}$ ions, the cloned transcriptional activator proteins Nuclear Factor I (NF I) and NF-kB were assayed in both "B" and non "B" conditions (figure 22). NF I and NF-kB were purified after over expession in insect cells (Baculovirus expression vector system) and E.coli bacterial cells (pGEX 2T expression vector system) respectively and bind to specific DNA sequences within cellular DNA and viruses (Bosher et al 1991, Mathews et al unpublished data). NF I did not bind to the Poly(dG-dC).Poly(dG-dC) DNA 'probe' in either conformation confirming that cobalt does not intrinsically promote increased binding of DNA binding proteins. Interestingly however NF-kB possesed a marked affinity for DNA 'probe' only in the non "B" conformation.



In the presence of $Co(NH_3)_6^{3+}$ ions. In the presence of EDTA.

Binding of cellular transcription factors to 'probe' DNA "B" and non "B" conformations.

Figures 22a & b.

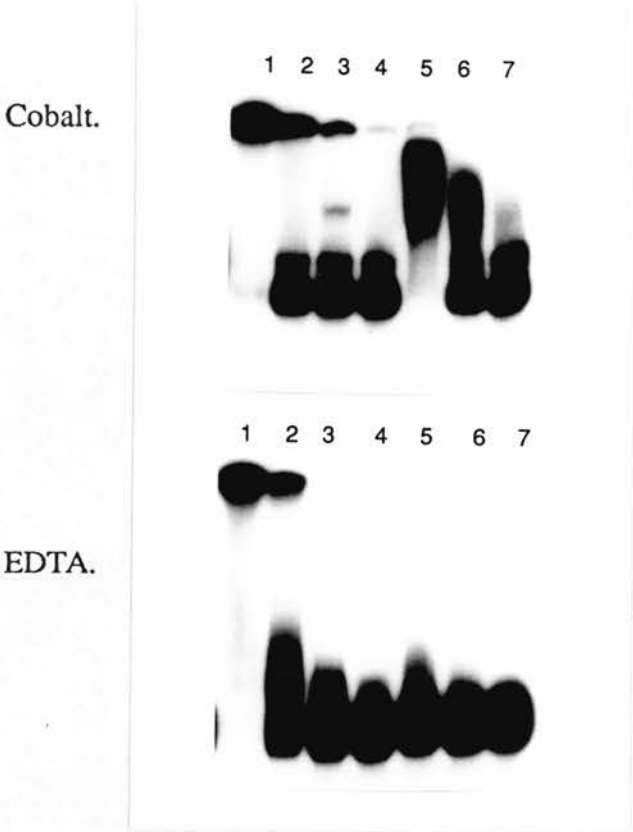
The cellular transcription factors NFkB and NF I were assayed for DNA binding under standard gel retention conditions for "B" and non "B" radiolabelled polymer 'probe' DNA (Poly (dG-dC).Poly (dG-dC) conformations.

The figures show autoradiographs of assay gels in both DNA 'probe' conformations, each lane consisting of the following :-

(a) No protein control (lane 1), anti-"Z" DNA serum at a final dilution of 1 : 1000 (lane 2), preimmune serum at a final dilution of 1 : 1000 (lane 3), 5ng of bacterially expressed, purified NFkB (lane 4), 0.5ng purified NFkB (lane 5), 0.05ng purified NFkB (lane 6); 5ng of baculovirus expressed, purified NF I (lane 7), 0.5ng purified NF I (lane 8) and 0.05ng purified NF I (lane 9).

(b)No protein control (lane 1), anti-"Z" DNA serum at a final dilution of 1 : 1000 (lane 2), preimmune serum at a final dilution of 1 : 1000 (lane 3), 100ng of purified NFkB (lane 4), 10ng purified NFkB (lane 5), 1ng purified NFkB (lane 6); 1000ng purified NF I (lane 7), 100ng purified NF I (lane 8) and 10ng purified NF I (lane 9).

As a change in the conformation of the DNA 'probe' occurs in the presence of $Co(NH_3)_6^{3+}$ ions, (not necessarily into the "Z" form) as indicated by anti-"Z" DNA antibody binding and an increased binding of the purified protein under these conditions it is therefore not unreasonable to propose that proteins involved in DNA structure recognition may bind probe DNA under the same conditions. To explore this question eukaryotic DNA topoisomerase I and prokaryotic DNA gyrase enzymes were assayed for DNA binding activity with labelled Poly (dG-dC).Poly (dG-dC) as DNA 'probe' in the presence of $Co(NH_3)_6^{3+}$ ions (figure 23). The two enzymes specifically bound to the 'probe' DNA, however after comparison of the enzyme binding affinities for 'probe' DNA in "B" form conformations it was evident that topoisomerase I specifically binds to DNA in a non "B" conformation whereas gyrase binds to both DNA conformations with only a slightly higher affinity for the non "B" DNA conformation.



DNA supercoiling enzymes show binding activity in gel retention assays.
Figure 23.
Using the gel retention assay system the binding potential of eukaryotic DNA Topoisomerase I [DNA supercoil relaxing enzyme, purified from calf thymus] and prokaryotic DNA gyrase [Topoisomerase II, DNA supercoiling enzyme, purified from Micrococcus luteus] (Gibco/BRL) were assayed under conditions in which the 'probe' DNA was in "B" and non "B" conformations.
The figures show autoradiograms of assay gelsinthe presence of $Co(NH_3)_6^{3+}$ ions (a) or EDTA (b) with lanes containing :-
8 units of gyrase (lane 1), 4 units of gyrase (lane 2), 2 units of gyrase (lane 3), 1 unit of gyrase (lane 4); 8 units of topoisomerase (lane 5), 4 units of topoisomerase (lane 6) and 2 units of topoisomerase (lane 7).
Topoisomerase I enzyme unit definition : one unit catalyses the conversion of 0.5µg superhelical øX174 RF DNA to a relaxed state in 30 minutes at 37°C.
Gyrase enzyme unit definition : one unit catalyses the conversion of 0.5µg relaxed pBR322 DNA to a supercoiled form in 30 minutes at 37°C.

DISCUSSION.

In this study, a method of detecting proteins which bind specifically to the lefthanded "Z" DNA conformation (but may also include other non"B" DNA conformations) is described. The assay system is utilised to purify HeLa cell cytoplasmic proteins which specifically bind these altered DNA conformations. Proteins with apparent molecular weights of approximately 85, 68, 60 and 55k were identified in an affinity purified fraction which actively bound the DNA 'probe' in a non "B" conformation. Proteins known to be involved in binding to altered DNA structures and the subsequent modification of them, such as topoisomerases, are also active in this system.

The double stranded DNA polymer Poly (dG-dC).Poly (dG-dC) was shown to have the ability to assume a lefthanded "Z" DNA conformation in the presence of 50 and 100μM cobalt hexaminochloride III ($Co(NH_3)_6Cl_3$) as shown by the binding of anti-"Z" DNA antibodies (figures 1 & 5). The homopolymer Poly (dG).Poly (dC) used as carrier DNA, clearly does not alter from a righthanded conformation in the presence of the polyvalent cobalt complex demonstrated by the absence of any competition for antibody binding even when carrier DNA is present in a large excess. The labelled alternating GC polymer 'probe' DNA in buffer containing EDTA in the absence of cobalt ions also remains in a "B" conformation as shown by the inability of anti-"Z" DNA antibodies to bind in the assay (figure 5a). Although retention assay polyacrylamide gels and gel running buffers usually contained either $Co(NH_3)_6^{3+}$ ions or EDTA, to ensure the desired conformation, results indicate that the requirement is only in the incubation buffers (figure 5a).

Anti-"Z" DNA rabbit polyclonal serum was prepared by immunising rabbits with Poly (dG-dC).Poly (dG-dC) chemically stabilised in the high salt concentration

(4M) conformation. However it is pertinent to note that although the antiserum was raised against an antigen regarded as being in the "Z" conformation it may not be specific to "Z" DNA, indeed it is possible that other non "Z", non "B" DNA forms are antigenic and present in the 'probe' DNA and may therefore be recognised. This also applies to DNA binding proteins in cell extracts. As a polyclonal serum is used in the assays it is important to realise that the serum may contain antibodies with different recognition properties. A portion may have sequence specificity binding to the base elements, while others will recognise the sugar phosphate backbone. It is important to note that if a small fraction of polymer 'probe' DNA were present in the "Z" conformation the binding of the antiserum may displace the "B" to "Z" equilibrium towards the lefthanded form (Malfoy & Leng 1981, Lafer et al 1985). This may also be the case with the interaction of cellular DNA binding proteins. However based on the assumption that in an equilibrium favouring the righthanded form (eg in EDTA) there may always be a small quantity of "Z" DNA present. The observation that no antibody binding is detected with Poly (dG-dC).Poly (dG-dC) in the righthanded form in this study suggests that antibody binding does not effect the "B" to "Z" transition equilibrium in this system. The lefthanded form of Poly (dG-dC).Poly (dG-dC) has been shown to accommodate one anti-"Z" DNA antibody molecule per 10 base pairs in the presence of excess antibody (Lafer et al 1985). Thus many antibodies will bind over the length of the 'probe' DNA which on average contains ~400 base pairs.

The competition assays with the unlabelled polynucleotides demonstrate that the antibodies do not bind to single strand DNA (figure 6b). Sensitivity of the assay is shown by the competition of antibody binding down to levels of unlabelled polymer equivalent to that of the labelled probe (~1ng)(figure 6a).

Competition achieved with the denatured salmon sperm DNA indicates that it may contain some potential non "B" DNA sequences (figure 6c). However the relatively large quantity of competitor salmon sperm DNA (0.5µg) required for partial competition of antibody binding suggests that the "Z" form is not abundant.

DNA FRAGMENT LENGTH AND ANTIGENICITY.

On ascertaining the fragment lengths of DNA 'probes' by electrophoresis on sequencing gels in the presence of DNA size markers. Antigenicity of Poly (dG-dC).Poly (dG-dC) fragments was shown to require more than 33 base pairs. This suggests that although smaller "Z" tracts are induced within supercoiled plasmid DNA, cobalt hexaminochloride III does not facilitate a "B" to "Z" DNA conformational transition in linear DNA fragments containing less than 36 base pairs. This explains why the $(GC)_{11}$ segment known to form "Z" DNA in a negatively supercoiled plasmid (Nordheim et al 1981) was not antigenic when excised from the supercoiled circular DNA via the Bam HI sites and incubated in the presence of cobalt. Higher concentrations of the cobalt ligand may provide a "B" to "Z" transition but also produces aggregation of the DNA preventing detection by gel shift analysis.

DETECTION AND ISOLATION OF DNA CONFORMATION SPECIFIC BINDING PROTEINS IN MAMALIAN CELLS.

Most sequence specific DNA binding proteins recognise and bind to their cognate DNA using structural domains that make sequence specific contacts with the DNA bases in the major groove (Schleif 1988). In contrast, the minor groove and phophate backbone are the likely primary recognition features for non sequence specific DNA binding proteins (Suck et al 1988, White et al 1989). However these two methods of DNA recognition are not mutually exclusive and are used in combination by many binding proteins (Freemont et al 1991).

Lefthanded "Z" DNA in vivo is likely to be stabilised by "Z" DNA binding proteins. Whether a protein can stabilise a given sequence in the "Z" conformation depends on how much energy is gained from its interaction with the DNA sequence and how much energy is required to maintain that particular base sequence in the lefthanded conformation. The free energy of binding is greater the

larger the number of base pairs involved in the protein DNA interaction and when energetic contributions come from both the phosphate backbone interactions as well as specific protein/DNA base contacts.

In this study analysis of cell extracts from a human epithelial cell line shows that although a DNA binding protein would be expected to reside in the nucleus, considerable binding activity to DNA in a non "B" conformation is present in cell cytoplasmic extracts (possibly due to nuclear leakage). This activity is demonstrated to be generated from the existence of different species of proteins which specifically bind non "B" DNA conformations. Purification of protein, which actively bound non "B" form DNA, from crude cell extracts revealed binding activity in a purified fraction containing two major polypeptides in equimolar amounts with apparent molecular weights of 85 and 60k and a minor component of 68k. A polypeptide with a lower apparent molecular weight of 55k, may also be involved.

These purified proteins bind to the Poly (dG-dC).Poly (dG-dC) 'probe' DNA in both its $Co(NH_3)_6^{3+}$ ion induced altered conformation and in "B" form, with a higher affinity for the non "B" structure. This suggests that the binding protein may have sequence specificity as well as conformational recognition. The protein obviously prefers to bind to the alternating purine/pyrimidine 'probe' DNA than to the large excess of nonalternating homopolymer carrier DNA. In this respect the use of Poly (dG).Poly(dC) as a carrier to eliminate nonspecific binding of protein to "B" DNA was not ideal. The homopolymer carrier DNA exists in a righthanded form which differs in structure from the canonical Watson and Crick (1953) DNA helix (McCall et al 1985), being more flexurally rigid than mixed sequence DNA (Rhodes 1979, Simpson & Kunzler 1979). However other DNA sequences would likely exhibit conformation alterations in the presence of the cobalt complex ions. It is therefore important to note that the carrier DNA will not obviate protein which has a sequence specificity for alternating GC sequences and therefore having a lower affinity for the nonalternating homopolymer.

The results suggest that these DNA binding proteins may also have a sequence dependent conformational specificity observed as a preference (higher binding affinity) for the 'probe' DNA in the presence of $Co(NH_3)_6^{3+}$ ions. Competition assays show that these proteins posses no affinity for single stranded DNA or RNA molecules.

The direct identification of "Z" DNA binding proteins with apparent molecular weights of 56k in the crude 40 and 50% ammonium sulphate precipitated extracts, after electrophoretic separation under denaturing conditions and renaturation in situ (on transfere to nitrocellulose), but not in the 60% precipitate points to significant differences between the polypeptide DNA binding activities present in native and denaturing conditions. The failure to detect any "Z" DNA binding activity in the 60% ammonium sulphate precipitated extract suggests that the polypeptides are not sufficiently renatured in the appropriate conformation to facilitate DNA binding or that more than one polypeptide subunit is required for binding.

Protein renaturation studies also detected a polypeptide with a molecular weight of between 60 and 50k in the 50% ammonium sulphate precipitated extract which was proteolytically cleaved down to a molecular weight of ~30k. DNA binding activity was also restored to the 60% ammonium sulphate precipitate on renaturation. It is not clear whether this was due the refolding of the appropriate conformation to allow active DNA binding or whether more than one polypeptide subunit was present in the excised gel fragment reassociating on renaturation.

Recent reports indicate that Poly (dG-dC).Poly (dG-dC) chemically stabilised in "Z" conformation by bromination is not a safe indicater of specific "Z" DNA binding by proteins. Three proteins were previously isolated from bull testis (Gut et al 1987), showing preferential binding to the brominated "Z" form polymer (Gut et al 1987, Christen et al 1990). On reassesment of their binding properties, no apparent descrimination between "Z" and "B" DNA conformations was detected in plasmids (Rohner et al 1990). These findings suggest that the proteins discriminate the brominated polymer on the basis of chemical properties different from the "Z" conformation, possibly an altered charge induced by electronegative bromine atoms. The same study casts doubt over the well documented ability of the rec A protein from E.coli to bind preferentially to the "Z" form of methylated Poly (dG-dC).Poly (dG-dC) (Blaho & Wells 1987). Failure to show any preferential binding to relaxed and supercoiled plasmids was again described. Other recent data alleges that the binding affinity of rec A to "Z" DNA represents a kinetic rather than an equilibrium effect (Kim et al 1989).

This is a clear demonstration that strong preferential binding to "Z" DNA is not sufficient as proof of the functional significance of the proteins interaction. Indeed it may prove prudent to assess to ability of the purified HeLa cell proteins to differentiate between "Z" and "B" DNA within plasmids.

Yolk proteins from nematodes, frogs and chicken eggs all have been unexpectedly reported as binding strongly and preferentially to lefthanded brominated Poly (dG-dC).Poly (dG-dC) (Krishna et al 1988). This interaction of yolk protein with "Z" DNA is strongly competed by phospholipids suggesting that "Z" DNA may interact with yolk protein phospholipid binding sites (Krishna et al 1988). A number of phospholipid binding proteins, such as bovine lung annexins and

human serum lipoproteins, have been demonstrated to also bind strongly and preferentially to "Z" DNA (Krishna et al 1990).

A number of "Z" DNA binding proteins have been isolated by "Z" DNA affinity chromatography using Poly (dG-dC).Poly (dG-dC) in a chemically stabilised "Z" form (Lafer et al 1988, Leith et al 1988). A recent study (Krishna et al 1990) using a similar method isolated a prominent "Z" DNA binding protein from E. coli cell extracts with an apparent molecular mass of 36k which was identified as an outer membrane porin protein and known to interact in vivo with phospholipids. "Z" DNA binding activity of this E.coli protein was strongly blocked by competition with acidic phopholipids such as cardiolipin (Krishna et al 1990) and led to the suggestion that many isolated "Z" DNA binding proteins are actually phospholipid binding proteins.

The results of this study were consistent with those of Krishna et al (1990) in that phospholipid did not inhibit the binding of anti-"Z" DNA antibodies to the polymer 'probe' DNA in the lefthanded conformation. The "Z" DNA binding activity in the affinity purified protein fraction was competed for binding by phospholipid, as similarly shown by Krishna et al with the putative "Z" DNA binding proteins purified from E.coli (Lafer et al 1988, Blaho & Wells 1987). However the transcription factor NF I is also competed by phospholipid from binding to its specific "B" DNA base recognition sequence present in the adenovirus genome (leegwater et al 1985). This suggests that the ability of phospholipid to compete for "Z" DNA binding proteins association with "Z" DNA is not because these proteins are actually phospholipid binding proteins, but probably due to the negative charge of the acidic phospholipids. This would agree with the observation of Krishna et al that phospholipid inhibits the binding of the lac repressor to its "B" form operator DNA sequence. These results however, urge caution in isolating "Z" DNA binding proteins from natural sources using physical properties to infer a "Z" binding function in vivo.

The NF-kB transcription factor originally found to be expressed in mature B lymphocytes (Sen & Baltimore 1986) can induce the expression of genes in many different cell types by binding to sequence motifs in promoter and enhancer elements (Baeuerle 1991). Activation of genes can be very rapid because NF-kB is present in the cytoplasm of unstimulated cells, requiring release from its inhibitory subunit IkB for activation and subsequent translocation into the nucleus (Baeuerle & Baltimore 1988). Activation of the transcription factor can occur on exposure to viruses, T cell mitogens, bacterial lipopolysacharide and cytokines.

NFkB exists as various multiprotein complexes. In the cytoplasm of unstimulated cells NF-kB exists in a non DNA binding heterotrimeric complex composed of protein subunits with apparent molecular weights of 50 (p50) and 65k (p65) along with a further subunit IkB. The prominent form of NFkB in the nuclei of activated cells is a DNA binding heterotetramer composed of two p50 and two p65 protein subunits. IkB prevents two p50-p65 heterodimers assembling into an active heterotetramer in the cytoplasm. Phosphorylation of IkB by protein kinases appears to be a requirement for its release which induces a translocation from the cytoplasm into the nucleus. The inhibitory subunit IkB is also translocated into the nucleus where the p65 subunit remains a receptor. NFkB may become inactivated on association with IkB even when bound to DNA with high affinity. The simplest DNA binding form of NFkB consists of the dimerised p50 subunit (Baeuerle and Baltimore 1989). As this homodimer was not thought to exist in vivo, doubt was held over its physiological relevance and it was suggested that it is an artifact (Shreck et al 1990). However recently a DNA binding factor KBF1, involved in the transcriptional control of class I genes of the major histocompatability complex, was found to be identical to the p50 DNA binding subunit of NFkB. KBF1 binds to an enhancer sequence similar to NFkB and both proteins display extensive amino acid sequence as well as functional homology with the v-rel oncogene and the Drosophila maternal morphogen dorsal (Kieran et al 1990).

The role of the p65 subunit of NFkB in the active nuclear form is to modulate the DNA binding specificity of the p50 subunit. In the absence of p65, the p50 dimer binds with high affinity to completely palindromic DNA binding sequences. The heterodimer binds with a 10-20 fold lower affinity to these sites but shows a two fold increase in affinity for the less symetric NFkB binding sequence GGGACTTTCC. It is unclear if p65 is required for transcriptional activation or how binding of NFkB to enhancer and promoter elements initiates the synthesis of mRNA. The binding of NFkB to its DNA motif has been shown to cause bending of the DNA molecule (Clark et al 1990, Schreck et al 1990, Mathews & Hay unpublished data). Bending of the DNA structure is known to be involved in the control of prokaryotic transcription (Bracco et al 1989, Collis et al 1989) and occurs either as an intrinsic property of DNA or induced after specific binding of protein to DNA sequences (Travers 1989/90).

Previous data has shown that a dramatic increase in the binding of the active NFkB heterotetramer to its DNA binding sequence GGGACTTTCC is observed in the presence of spermine (Clark et al 1990, Schreck et al 1990, Mathews & Hay unpublished data) with an optima at 0.5M spermine (Schreck et al 1990). It has been postulated that this stimulation may have been due to a direct interaction of spermine with the NFkB protein while others propose that the spermine may effect the stability of the active NFkB complex. In linear DNA electrostatic repulsion of phosphate groups in the DNA backbone poses as an energy barrier to bending. However in the presence of positively charged ions such as the polyvalent cation spermine (3^+), neutralisation of the negative charge reduces the energy requirement for bending and thus binding of NFkB is enhanced through an increase in the stability of the DNA/protein complex.

In addition to spermine, various metal ions are reported to promote and stabilise structural alterations of DNA such as bending of DNA (Laundon & Griffith 1987, Feuerstein et al 1990) and "Z" DNA (Thomas et al 1991). It is therefore possible that increased binding was a direct result of a structural alteration in the NFkB

DNA binding sequence. The results obtained in this study would appear to agree with this proposal with the NFkB p50 dimer binding to the DNA polymer Poly (dG-dC).Poly (dG-dC) only in the $Co(NH_3)_6^{3+}$ ion induced non "B" DNA conformation in preference to a large excess of polymer DNA, Poly (dG).Poly (dC), in the "B" form. The NFkB p50 dimer does not bind to the "B" form alternating GC polymer, this observation eliminates the possibility of any sequence specificity. Shreck et al (1990) reports the stimulation of the NFkB heterotetramer binding to its recognition sequence in the presence of metal ions at concentrations similar to those known to exhibit effects on DNA structure. Maximal effect was observed with 5mM Ca^{2+}, 1mM Ba^{2+} and 25μM $Co_{3+}(NH_3)_6$. In this study stimulation of binding was observed at 50 μM $Co_{3+}(NH_3)_6$. These findings in addition to studies with spermine clearly point to the interaction of NFkB with DNA being stimulated by modification of the cognate DNA structure. The NFkB transcription factor not only recognises a particular DNA sequence but an altered DNA conformation appears to influence the affinity for its binding motif. Recognition of this altered DNA structure is such that an affinity for a non "B" structure is observed independent of the DNA sequence. It is unlikely that the NFkB protein complex is recognising a bent DNA structure in this study as the polymer Poly (dG-dC).Poly (dG-dC) in 50μM $Co(NH_3)_6Cl_3$ is known to stabilise in "Z" conformation and is a relatively rigid structure. However it is possible that "Z" DNA is not the only non "B" conformation induced in the polymer by cobalt ions and other DNA structures (including bent and "Z" DNA) may have common recognition features influencing the binding of NFkB. The observation that the transciption factor NF I did not bind to the DNA 'probe' in the cobalt induced form (or in "B" form) provided evidence that protein binding was not due positive discrimination of the DNAs chemical properties. Competition analysis of the purified p50 homodimer binding to a radioactivly labelled double stranded oligonucleotide containing the NFkB DNA binding site with unlabelled Poly (dG-dC).Poly (dG-dC) polymer DNA (in the presence $Co(NH_3)_6^{3+}$ ions or EDTA)

demonstrated that the binding affinity to the recognition sequence motif could not be competed by DNA polymer in a non"B" conformation (data not shown). This suggests that although the protein specifically binds to an altered DNA structure binding affinity is also regulated by nucleotide sequence.

In vivo DNA binding proteins may superficially 'scan' the surface of DNA molecules recognising non"B" form conformations before becoming closely associated with the DNA making specific contacts with nucleotide bases and the phosphate backbone. This phenomena may serve as a primary recognition system for DNA binding proteins. Other studies have also led to the suggestion that transcription factors recognise and bind non"B" DNA forms of their sequence specific DNA binding site (Fairall et al 1989).

Topoisomerases are enzymes which change the topology of DNA and have been demonstrated to be essential for cell growth. DNA topoisomerase enzymes control and modify the topological state of DNA by transiently breaking and resealing the DNA helix. These enzymes are classified according to their mechanisms of action. Type I topoisomerases adjust DNA topology by breaking one of the helical strands and passing another through the transient break, characteristically changing the linking number in steps of one. Type II topoisomerase enzymes transiently break a pair of complementary strands and pass another double stranded segment through it, changing the linking number in steps of two (Brown & Cozzarelli 1979/81).

These enzymes also catalyse many interconversions between DNA topological isomers. Examples are catenation or decatenation of single and double stranded DNA rings and the removal or introduction of knots within these rings (Liu et al 1976, Tse & Wang 1980, Champoux 1977, Wang 1985).

Eukaryotic topoisomerase type I relaxes both positively and negatively supercoiled DNA (Wang 1985) with equal efficiency whereas the prokaryotic type I enzyme only relaxes negative supercoils (Kirkegaard & Wang 1985).The type II eukaryotic topoisomerase also relaxes positively and negatively supercoiled DNA but shows a requirement for the presence of ATP and Mg^{2+}. The eukayotic topoisomerase

enzymes do not introduce superhelical turns into relaxed DNA molecules which is in contrast to the bacterial type II enzyme, DNA gyrase which introduces negative superhelical turns into DNA rings as well as relaxing negative and positive supercoils. The prokaryotic gyrase enzyme forms a complex with DNA very differently to eukaryotic topoisomerase II enzymes. Gyrase coils DNA around itself in a right handed manner which leads to a binding preference for positively rather than negatively supercoiled regions. Coiling the DNA in a positive sense causes compensating negative supercoils to occur elsewhere in the DNA strand (Liu & Wang 1979). Synthesis of the gyrase enzyme is controlled by the level of DNA supercoiling within the cell, expression of gyrase genes are activated by a reduction in negative supercoiling (Menzel & Gellert 1983). A reverse gyrase that introduces positive superhelical turns into DNA has also been isolated from the thermophilic bacterium sulfolobus (Kikuchi & Asai 1984, Mirambeau et al 1984). Topoisomerasesare have been implicated in many important cellular processes (Wang 1985). The recently proposed model of Liu and Wang (1987) implies that two oppositely supercoiled domains may be introduced by transcription or other processes involving the tracking of a macromolecular assembly along DNA. The degree of local DNA supercoiling in vivo is determined by the speed of supercoil generation by the tracking process, how fast the oppositely supercoiled domains can neutralise each other through diffusion and how quickly the supercoils are removed by DNA topoisomerases. Type II topoisomerases are also involved in DNA replication, separating strands at the replication origin on initiation of replication and relaxing positive supertwists which are generated by unwinding of the template strands in front of the tracking polymerase during elongation. On termination of replication the topoisomerase type II enzyme assists segregation of daughter molecules by passing one helix through another (Sundin & Varsharvsky 1980). The topoisomerase of Saccharomyces cerevisiae is essential for effecting the separation of sister chromatids after DNA replication (DiNardo et al 1984). Studies on chromatin structure and assembly suggest the involvement of

topoisomerase II in chromosome condensation (To & Kmiec 1990) and as a structural protein in interphase (nuclear matrix/chromosome scaffolds), perhaps localised at the bases of radial loop domains in chromatin (Adachi et al 1991, Reece & Maxwell 1991, Howard et al 1990, Earnshaw & Heck 1985, Earnshaw et al 1985). A role in recombination has been ascribed but remains to be conclusively demonstrated (Wang et al 1990). Topoisomerase type II has also been shown to be cell cycle regulated (Heck et al 1988).

Type I (Liu & Miller 1981) and type II (Miller et al 1981) DNA topoisomerases have been purified to homogenicity from HeLa cell extracts. The native type I topoisomerase is a lysine rich monomeric protein with an apparent molecular weight of 100k. However as with other eukaryotic type I topoisomerases it is very sensitive to proteolysis with an additional species with apparent molecular weights of 67k and 57k observed (Liu 1983, Liu & Miller 1981). A later study demonstrated that on controlled proteolysis the 100k polypeptide converted initially to peptides of ~82 and 67k with the 57k peptide appearing on further proteolysis. Enzymatic DNA nicking activity was still evident in these proteolytic products. The eukaryotic type II topoisomerase exists as a homodimer of a polypeptide with a molecular weight of 172k. Proteolysis was also a problem on purification resulting in loss of activity. The bacterial DNA gyrase enzyme exists as a tetramer consisting of two subunits with apparent molecular weights of 97k encoded by the gyrase A gene and two subunits encoded by the gyrase B gene with apparent molecular weights of 90k. Subunit A contains the active site for DNA breakage and reunion while subunit B contains the ATP binding site.

Topoisomerases have specific DNA binding sequences with the consensus sequences of Drosophila topoisomerase type II (Sander & Hsieh 1985), chicken erythrocyte topoisomerase type II (Spitzner & Muller 1988) and topoisomerase type I (Shen & Shen 1990) recently published.

Earlier studies suggested that many topoisomerase cleavage sites correlate to regions of secondary structure in DNA (Kreuzer 1983). Eukaryotic topoisomerase

II has been shown to have a high affinity for purine/pyrimidine repeats compared to nonalternating sequences, with a considerable homology shown between the consensus binding sequence and alternating purine/pyrimidine sequences (Spitzner et al 1989). Enzyme activity appeared to be proportional to the length of the purine/purimidine repeats, with GT, AC and AT offering better cleavage subtrates than GC repeat sequences. A high binding activity towards a 54bp purine/pyrimidine repeat sequence in the human globin region that has been associated with recombination events in vivo (Semenza et al 1984, Kilpatrick et al 1984) is also observed with the topoisomerase type enzyme. These results imply that recognition of the DNA repeats involves more than sequence information alone, as alternating purine/pyrimidine DNA tracts have the potential for forming altered DNA secondary structures. Unexpected behaviour of topoisomerases towards unusual DNA sequences have already been observed. Strong topoismerase type II cleavage occurring with bent DNA sequences which moderately match the consensus sequence (Spitzner et al 1989). A recent report published after the completion of this thesis shows that Drosophila DNA topoisomerase II has a higher affinty for linear "Z" DNA than "B" DNA (Glikin et al 1991). The study also demonstrates a preference for alternating CG tracts in plasmids when supercoiling was sufficient to facilitate the "Z" conformation. The higher the topological stress within the plasmid the higher the binding affinity exhibited by the enzyme. DNA binding and cleavage sites are not thought to be synonymous and although a preference for binding to special DNA conformations is evident cutting may occur elsewhere on the molecule (Kirkegaard & Wang 1981). Cleavage may occur at a distant site by diffusion of the type II topoisomerase along the DNA (Osheroff 1986). Drosophila topoisomerase II also binds preferentially to bends, kinks and bubbles in DNA (Howard et al 1991).

Detailed studies of eukaryotic topoisomerase type I show its DNA binding site to encompass 18-25bp (Stevnsner et al 1989) with the cleavage site centrally located. The enzyme contacts the binding site in a highly symmetrical manner and is

functionally split into two regions. A region which lies almost entirely 5' to the cleavage site is essential for binding of the enzyme forming tight contacts with the DNA and stabilising the duplex (Svejstrup et al 1990). The region 3' of the cleavage site is also covered by the enzyme and until recently was regarded as nonessential.

A recent observation that the consensus DNA sequence is associated with a conserved structural motif in the essential region (Shen & Shen 1990, Caserta et al 1989) suggests that local aspects of helix geometry set the minimum recognition requirements for enzyme binding. In addition the topoisomerase type I/DNA interaction has been shown to be modulated by the helix conformation in the nonessential binding site region (Krogh et al 1991). Enzyme/DNA complex formation is markedly stimulated when intrinsically bent DNA is situated in the region 3' of the cleavage site. Enzyme contact points with the DNA are shown to be in the backbone. Topoisomerase type I as with the type II enzyme preferentially binds and cleaves supercoiled DNA (Muller 1985) with relaxation causing inactivation of the enzyme (Camilloni et al 1988).

Results in this study show that the eukaryotic topoisomerase type I enzyme binds preferentially to the GC alternating polymer 'probe' DNA only in the $Co(NH_3)_6^{3+}$ ion induced conformation. This is clearly a conformational specificity as binding was not detected when the polymer was in the "B" form demonstrating no sequence specificity for the alternating purine/pyrimidine 'probe' DNA over the homopolymer carrier DNA. This suggests that topoisomerases do indeed bind to altered DNA structures with higher affinity. However it is possible that the cobalt induced DNA form is more similar to supercoiled than linear DNA. It has been previously demonstrated that topoisomerase type I binds preferentially to supercoiled DNA over its relaxed form.

The prokaryotic DNA gyrase bound to the polymer 'probe' DNA in both forms showing a specificity for the purine/pyrimidine alternating 'probe' DNA over the carrier DNA. This may suggest the consensus binding sequence of DNA gyrase

has a higher homology to alternating purine/pyrimidine than nonalternating sequences similar to that of eukaryotic topoisomerase type II (Spitzner et al 1989). A slightly higher affinity was also shown for the probe in the presence of $Co(NH_3)_6^{3+}$ ions suggesting that there may also be a structural requirement for increased binding affinity.

Thus the mechanism by which topoisomerases recognise DNA targeting their enzymic action in vivo is probably not only due to sequence recognition but also to their affinity for altered DNA structure. Transitions in DNA conformation are clearly closely linked to the formation and modulation of topological units, for which DNA topoisomerases are primarily responsible. Thus a regulatory function for these enzymes in vivo may be based on the emergence of structural changes within DNA during metabolic processes. Whenever a conformational change occurs in DNA caused by its interaction with a protein, its unwinding related to transription or replication, environmental variations or torsion stress caused by the removal of a nucleosome DNA, topoisomerases will recognise the distortion targeting its enzymic action and returning it to its relaxed form.

It is interesting that the apparent molecular weights of the purified HeLa cell DNA topoisomerase type I enzyme and its proteolytic cleavage products are similar to those of the polypeptides contained in the "Z" DNA binding fraction eluting from the affinity column. To establish whether this enzyme may be related to the "Z" DNA binding activity purified in this study it would pertinent to assay fractions for topoisomerase type I activity over the purification procedure.

In summing up a biological function for proteins which bind to "Z" DNA has still to be confirmed. As it is know well established that non "B" DNA conformations transiently exist within cells with their occurrence and structural details dependent on the local DNA nucleotide sequence and superhelical density caused by cellular processes such as DNA replication and transcription. It is not suprising that protein may be purified from cells which bind to non"B" DNA including "Z" DNA.

However the characterisation of these proteins to develope an understanding of their cellular function remains a challenging topic for future investigations.

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