INVESTIGATION OF THE DOMAIN STRUCTURE OF THE *ESCHERICHIA COLI* DNA GYRASE A PROTEIN.

by

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For Judith.

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RICHARD JONATHAN REECE. INVESTIGATION OF THE DOMAIN STRUCTURE OF THE *ESCHERICHIA COLI* DNA GYRASE A PROTEIN.

Abstract.

Treatment of *Escherichia coli* DNA gyrase A protein with trypsin generates two large, stable fragments of molecular masses 64 kDa and 33 kDa which are derived respectively from the N- and C-terminus of GyrA. The trypsin-cleaved A protein (A'), can support DNA supercoiling, relaxation and other reactions of gyrase. The isolated 64 kDa fragment will also catalyse DNA supercoiling but the 33 kDa fragment shows no enzymic activity.

An amber mutation, introduced into *gyrA* near the point which corresponds to the tryptic cleavage site, yields GyrA(1-573) which shares the same properties as the 64 kDa tryptic fragment. Using genetic engineering, large numbers of 3'-*gyrA* deletion mutants have been produced; those encoding a protein smaller than 58 kDa (GyrA(1-523)) did not obviously over-produce truncated GyrA. GyrA(1-523) shows similar enzymic properties to GyrA(1-573) but cannot perform DNA supercoiling. Deletion of fifty C-terminal residues from GyrA(1-573) has the effect of disrupting part of the protein essential for supercoiling. I propose that the N-terminal 64 kDa represents the DNA breakage/reunion domain of the A protein, while the 33 kDa fragment contributes to gyrase-DNA complex stability.

Certain N-terminal deletion mutants of the GyrA protein were also constructed. Removal of the N-terminal 6 amino acids had no effect on the properties compared to GyrA. Removal of the N-terminal 69 amino acids yields a protein with no supercoiling or cleavage ability. The start of the N-terminal breakage-reunion domain is probably located within this 63 amino acid region.

The domains of GyrA were investigated by microcalorimetry. GyrA yields two unfolding transitions. GyrA(1-573) and GyrA(1-523) both yield a single unfolding transition corresponding to one of the GyrA transitions. Therefore GyrA contains two structural domains that can be assigned to the functional moieties described above. GyrA(1-573) has been crystallized, and four crystal forms identified. A diffraction pattern to 7 Å has been obtained.

Abbreviations.

$A_{595\ nm}$	Absorbance at 595 nm.
Amp	Ampicillin.
AMPS	Ammonium persulphate.
ADP	Adenosine 5'-diphosphate.
ADPNP	5'-adenylyl-β,γ-imidodiphosphate
ATP	Adenosine 5'-triphosphate.
ATPase	Adenosine 5'-triphosphatase.
BSA	Bovine serum albumin.
bp	Base pair.
CFX	Ciprofloxacin.
DNA	Deoxyribonucleic acid.
DNAse	Deoxyribonuclese.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetra-acetic acid.
FPLC	Fast protein liquid chromatography.
GyrA	DNA gyrase A protein.
GyrB	DNA gyrase B protein
IEF	Isoelectric focusing.
IPTG	Isopropyl-β-D-thiogalactopyranoside.
kb	Kilo-base pairs.
kDa	KiloDaltons.
MOPS	3-[N-Morpholino]propanesulphonic acid.
MPD	2-Methyl-2,4-pentanediol.
Mr	Molecular weight.
PAGE	Polyacrylamide gel electrophoresis.
PMSF	Phenyl methyl sulphonylfluoride.
PEG	Polyethyleneglycol.
RNA	Ribonucleic acid.
RNAase	Ribonuclease.
SDS	Sodium dodecyl sulphate.
SSC	Standard saline citrate.
TEMED	N,N,N',N'- tetramethylethylenediamine.
TRIS	Tris (hydroxymethyl) amino methane.
UV	Ultraviolet.

Summary.

Treatment of *Escherichia coli* DNA gyrase A protein with trypsin generates two large, stable fragments of molecular masses 64 kDa and 33 kDa which are derived respectively from the N- and C-terminus of GyrA. The trypsin-cleaved A protein (A'), can support DNA supercoiling, relaxation and other reactions of gyrase. The isolated 64 kDa fragment will also catalyse DNA supercoiling but the 33 kDa fragment shows no enzymic activity.

An amber mutation, introduced into *gyrA* near the point which corresponds to the tryptic cleavage site, yields GyrA(1-573) which shares the same properties as the 64 kDa tryptic fragment. Using genetic engineering, large numbers of 3'-*gyrA* deletion mutants have been produced; those encoding a protein smaller than 58 kDa (GyrA(1-523)) did not obviously over-produce truncated GyrA. GyrA(1-523) shows similar enzymic properties to GyrA(1-573) but cannot perform DNA supercoiling. Deletion of fifty C-terminal residues from GyrA(1-573) has the effect of disrupting part of the protein essential for supercoiling. I propose that the N-terminal 64 kDa represents the DNA breakage/reunion domain of the A protein, while the 33 kDa fragment contributes to gyrase-DNA complex stability.

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Chapter 1

Introduction.

1.1 DNA-Protein Interactions.

The interactions between proteins and nucleic acids are of fundamental importance in molecular biology. The functions of both DNA and RNA *in vivo* are controlled by their interactions with proteins. These interactions can be highly nucleotide sequence specific, such as the interaction between a restriction enzyme and its recognition site on a particular DNA molecule, or less specific in nature, for example the proteins involved in the elongation steps of DNA replication and transcription.

The manner in which regulatory proteins recognise specific DNA sequences has to be very precise. It has been calculated that some regulatory proteins are able to arrive at their base-specific DNA sites at a faster rate than can be explained by simple three-dimensional diffusion. The specific DNA target site may only occur once within the entire genome, and is consequently at a very low concentration (an *in vivo* concentration for a 17 bp sequence in the *Escherichia coli* genome of ~2 x 10^{-9} M). Also, the specific target site will be amongst many non-target sites (~10^7 per genome) some of which will share similarities to the target sequence (Berg *et al.*, 1981). One example of a specific DNA-binding protein is the *E. coli lac* repressor which controls the expression of the lactose degradation enzymes (Miller and Reznikoff, 1978). The repressor has been calculated to have an association rate constant (k_a) for binding to its specific DNA site of ~10^{10} $M^{-1}s^{-1}$. If the molecule were to find its specific site by simple diffusion then it has been estimated that k_a should not exceed ~10^7-10^8 $M^{-1}s^{-1}$, that is the calculated value is 100-1000-fold smaller than the experimentally observed value (Berg *et al.*, 1982). To explain the speed and accuracy with which transcription regulation occurs it has been postulated that the *lac* repressor binds to its specific 17 bp target site within the 10^6-10^7 bp of the genome in a two-step mechanism (Berg *et al.*, 1981; Berg *et al.*, 1982; Berg and von Hippel, 1988). Firstly, a repressor-DNA complex is formed at a non-specific site. The bound repressor then 'slides' along the DNA in a one-dimensional diffusion process until the target site is located. It has also been postulated that the repressor protein could also be transferred from one DNA

domain to another as a non-specific complex to further aid the formation of the specific DNA-protein complex (Berg *et al.*, 1982). In this way, rapid control of gene expression within the genome can be accomplished.

The ability of a protein to bind specifically to a DNA sequence is thought to be stabilised by a number of relatively weak chemical interactions, such as hydrogen bonds, van der Waals attractions and ionic and hydrophobic interactions. Individually these interactions can be weak, e.g. van der Waals attractions have energies of 1-2 kcal/mol and hydrogen bond energies are in the order of 3-7 kcal/mol (von Hippel, 1979). However, many different amino acid side-chains of the protein are able to interact with charged groups in the DNA, and the sum of these interactions can lead to the formation of a stable structure. Specific interactions between the protein and the DNA must occur at the base level. Non-specific interactions between the protein and the phosphate backbone of the DNA may be used to stabilise the more specific interactions. Base-specific contacts between the protein and the DNA occur with the bases exposed in the major groove of the DNA.

Some of the most extensively studied specific interactions between proteins and DNA are those between the λ repressor and its operator sites in λ DNA (reviewed by Ptashne, 1986). The λ repressor and the λ cro protein bind a 17 bp site within the operator, and play a role in the control of lysogeny during the phage life cycle. Crystallographic studies of such proteins has led to the idea of a helix-turn-helix DNA recognition motif within the protein structure (Pabo and Lewis, 1982). This motif consists of two relatively short stretches of α-helix (7-9 amino acid residues long) connected to each other by a β-turn of 4 residues. One of the α-helicies (the 'recognition helix') interacts with the bases in the major groove of the DNA, whilst the other α-helix of the motif appears to interact relatively non-specifically with the phosphate backbone (Sauer *et al.*, 1982). More recently, very high resolution crystallography of the protein-DNA complex (Jordan and Pabo, 1988) has allowed the

exact bonding interactions between the DNA and the protein to be elucidated. The protein and the DNA are very closely associated during this interaction, presumably this being essential for the recognition of a particular DNA site over all other possible sites. The complex is stabilised by an extensive network of hydrogen bonds and ionic interactions between the protein and the sugar-phosphate backbone. Several side chains form hydrogen bonds with sites in the major groove, and hydrophobic contacts also contribute to the specificity of binding. A number of other proteins are also known to interact with DNA via this helix-turn-helix motif, for example the repressor of phage 434 and P22 (Sauer *et al.*, 1982), and the catabolite gene activator protein (CAP) (Pabo and Sauer, 1984). A diagrammatic representation of the helix-turn-helix motif of the λ repressor is shown in Fig. 1.1.

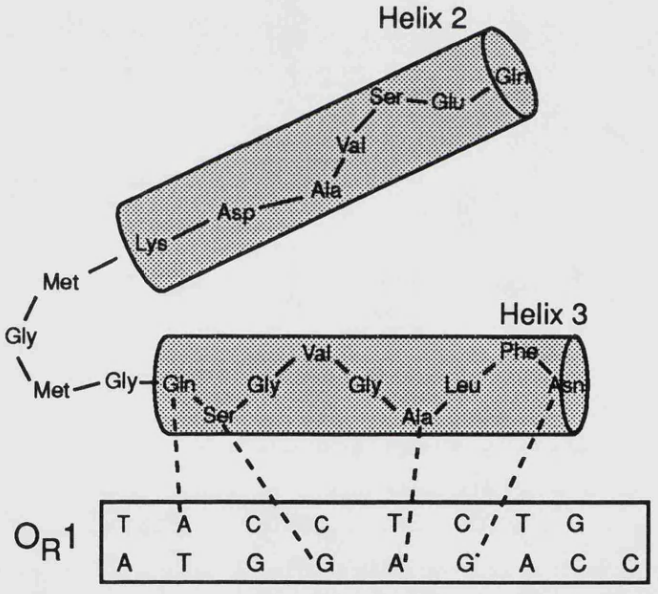


Fig. 1.1. The helix-turn-helix motif of the λ repressor, and some of the interactions it forms with its operator DNA (based on the data of Jordan and Pabo, 1988).

Helix-turn-helix structures are not apparent in a great number of DNA-binding proteins, and indeed a number of other DNA-binding motifs within proteins have been suggested, for example 'zinc fingers' (Klug and Rhodes, 1987) and 'leucine zippers' (Landschulz *et al.*, 1988; O'Shea *et al.*, 1989). In the case of zinc fingers, an atom of zinc is thought to be able to interact with certain amino acids to produce a finger-like

extension that is capable of interacting specifically with DNA. The zinc atom is co-ordinated tetrahedrally at the base of the finger, by four cysteine residues or by two pairs of cysteines and histidines. The eukaryotic transcription factor TFIIIA contains nine zinc-finger repeating units in its primary amino acid sequence (Klug and Rhodes, 1987). The 'tip' of the finger was originally thought to extend into the major groove of the DNA and make contacts there. However, nuclear magnetic resonance spectroscopy has indicated that, in solution, the zinc atom is actually utilised to position an adjacent α-helix and two β-sheets that are arranged in a hairpin structure (Lee, M.S. *et al.*, 1989). It is therefore suggested that the finger orientates the α-helix so that it is able to lie in the major groove of the DNA and make base specific contacts there. The β-sheet is thought to lie further away from the DNA helical axis and contacts the DNA backbone (Berg, 1988; Evans and Hollenberg, 1988).

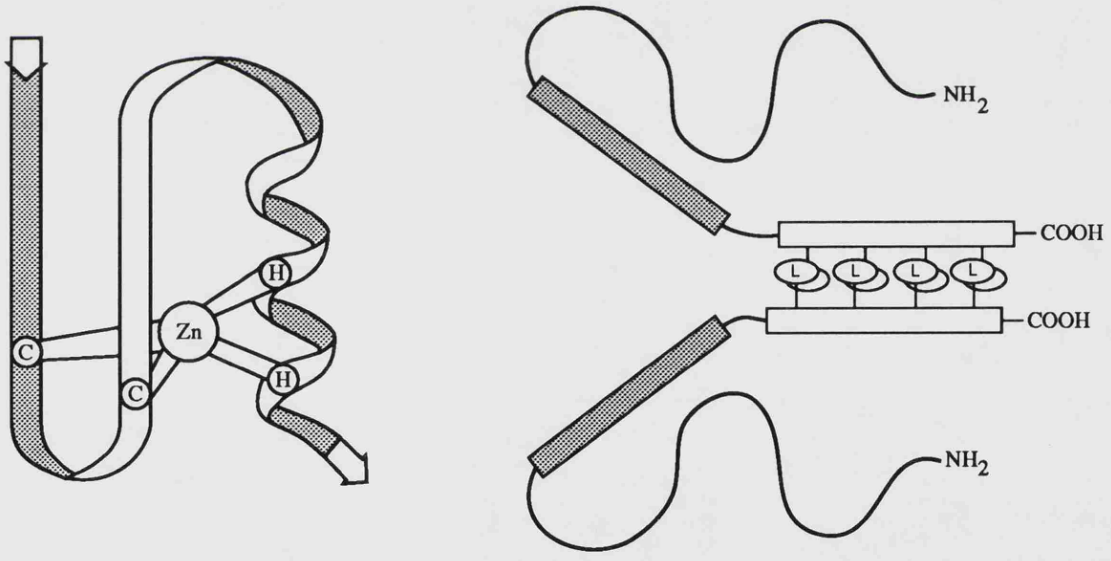


Fig. 1.2. The proposed structures for the zinc finger (left) and leucine zipper (right) (based on Johnson and McKnight, 1989).

The 'leucine zipper' is a theoretical secondary structure protein motif that is thought to act by allowing the peptide chain to obtain the correct quaternary structure to interact with DNA (Landschulz *et al.*, 1988). A number of dimeric proteins, including C/EBP, an enhancer binding protein, GCN4, the eukaryotic transcription activator,

and Myc, the cellular transforming protein, share sequence homology in two regions where leucine occurs at every seventh residue of the primary sequence. It has been suggested that if these residues form an α-helix then the leucines would all locate to the same side of the helix (O'Shea *et al.*, 1989). Two leucine-containing helicies could theoretically come together and interact, via the hydrophobic side chains to form a stable secondary structure (Johnson and McKnight, 1989). Originally it was speculated the the leucine residues could linearly interdigitate as shown in Fig. 1.2, but recent nuclear magnetic resonance data of a synthetic 33 amino acid peptide, taken from part of the GCN4 sequence, has indicated that the leucine zipper actually takes the form of a coiled coil (Oas *et al.*, 1990). The association of the two leucine-rich region is thought to bring two juxtaposed basic regions into such an orientation that they are capable of interacting with DNA (the shaded regions in Fig. 1.2). It has been speculated, because of the invariant distance of these regions from the zipper as observed in a number of such proteins, that these basic regions interact with the DNA via some sort of scissors-grip method (Vinson *et al.*, 1989). The two basic regions of the dimeric protein could interact with different faces of the DNA helix so that a number of specific contacts could be made.

Thus it can be noted that a number of specific DNA-protein interactions are mediated by definable secondary structural motifs in the protein. However, such motifs are not apparent in many DNA-binding proteins, and by necessity, not all proteins that interact directly with DNA are absolutely base sequence specific. Many must be able to interact with DNA of virtually any sequence so that, for example, correct replication and transcription can occur. Structural information about these proteins, and their associations with DNA, are not as extensive as those described above. Both the Klenow fragment of DNA polymerase I and the nuclease DNAse I have been subjected to X-ray crystallographic analysis (Joyce and Steitz, 1987; Suck *et al.*, 1988) The Klenow fragment of DNA polymerase I (a 68 kDa fragment of the polymerase originally produced by removing the 35 kDa N-terminal domain by limited

proteolysis) consists of two functionally and structurally distinct domains. The function of the domains is to provide the polymerase activity and the 3'-5' exonuclease activity of the intact DNA polymerase. The crystal structure of the polymerase domain shows it contains a positively charged cleft (20-24 Å wide and 25-35 Å deep) that could accommodate double-stranded DNA. When DNA is bound in the cleft, the protein is thought to wrap around the DNA to enclose it (Joyce and Steitz, 1987). By necessity, DNA polymerase must be able to interact with virtually any DNA sequence, so the interactions between the DNA and protein are likely to be less specific than those described above for repressor proteins. Another example of a DNA-binding protein which is capable of interacting with many DNA sequences is the type II topoisomerase DNA gyrase. Before considering this enzyme in detail, I will discuss aspects of DNA topology and the structure and mechanism of other topoisomerase enzymes.

1.2 DNA Supercoiling.

Virtually all the naturally occurring duplex DNA isolated from prokaryotes is found to be in a negatively supercoiled form; that is, the DNA is underwound and consequently in a higher energetic state than if it were fully relaxed. This negative supercoiling was first identified as a property of the topology of closed-circular DNA isolated from the animal virus, polyoma (Vinograd *et al.*, 1965).

In the well-known 'Watson-Crick' structure of DNA, the two antiparallel strands are coiled around each other (Watson and Crick, 1953). A direct consequence of this intertwining is that if a double-stranded DNA molecule is in the form of a ring with no discontinuity in the backbone bonds of either strand, the complementary single-stranded rings are linked. The topological state of covalently closed-circular DNA can be be described by a simple equation (Wang, 1980):

$$Lk = Tw + Wr.$$

The linking number (Lk) is the number of times that the two strands of a DNA duplex circle are interwound, and is invariant as long as the circle remains intact. Lk can be segregated into two components, twist (Tw) and writhe (Wr). The twist being the number of Watson-Crick turns of the helix in the circle, and writhe is a measure of the contortion of the helix in space. If the linking number of a relaxed DNA becomes altered then the resulting strain on the molecule can be relieved by a change in the writhe of the circle so that supercoiling occurs. Changes in linking number (ΔLk) can be expressed in terms of changes in twist (ΔTw) and changes in writhe (ΔWr). For plasmid DNA molecules, however, it is thought the main effect of moderate changes in Lk is to alter the writhe of the DNA, and so have an effect on the overall supercoiling of the molecule (Shore and Baldwin, 1983; Horowitz and Wang, 1984).

If nicked-circular DNA, which is under no torsional constraint, is closed by a DNA ligase the resulting DNA molecules have linking numbers approximately equal to the number of duplex turns in the nicked DNA. In this state, a closed DNA molecule is said to be relaxed. In fact, under such conditions, a Gaussian distribution of DNA topoisomers is formed whose linking numbers differ by one. These can be visualised as a ladder of bands on an agarose gel (Depew and Wang, 1975). A nicked DNA is free to change the number of duplex turns and so can remain in the relaxed state even under conditions affecting the geometry of the DNA helix, such as in the presence of intercalating agents like ethidium bromide. However, one way in which closed-circular DNA can respond to such conditions is by altering its tertiary structure; consequently the DNA becomes supercoiled and no longer relaxed (Depew and Wang, 1975; Pulleybank *et al.*, 1975). In the absence of such external agents, however, the only way a closed-circular DNA molecule can permanently change its overall topology is for there to be breakage and resealing of the phosphodiester backbone. This process results in a change in the linking number of that DNA species.

For large DNA circles, under physiological conditions, the linking number of the most abundant relaxed topoisomer, Lk^o, approximates to Tw^o. Under these conditions Tw^o is equal to the number of base pairs of the DNA divided by 10.5, the average number of base pairs per helical repeat (Shore and Baldwin, 1983; Horowitz and Wang, 1984):

$$Tw^o = N/10.5.$$

Naturally occurring closed-circular DNA molecules have linking numbers, Lk, that deviate from their respective Lk^o values. The difference between these values, $\Delta Lk = Lk - Lk^o$, is the linking difference of a DNA molecule (Crick *et al.*, 1979). Values of ΔLk may be either positive or negative depending on whether a DNA molecule is overwound or underwound with respect to its relaxed state. If the linking number of a given molecule exceeds Lk^o, ΔLk is positive and the DNA is said to be positively supercoiled. Similarly, a negative value of ΔLk defines negatively supercoiled DNA. In order to compare the topological properties of DNA molecules of different sizes the superhelical density, $\Delta Lk/Lk^o$ (sometimes called the specific linking difference) is usually quoted and normalises the linking differences with respect to the length of DNA circle in question.

The topological state of circular DNA molecules can be altered by a class of enzymes called DNA topoisomerases. These enzymes are able to catalyse changes in the tertiary structure of DNA both *in vitro* and *in vivo* (for reviews see Cozzarelli, 1980a,b; Gellert, 1981; Liu, 1983; Vosberg, 1985; Wang, 1985; 1987a,b; Maxwell and Gellert, 1986).

1.3 DNA Topoisomerases.

DNA topoisomerases are capable of carrying out a number of apparently diverse reactions *in vitro*, such as the supercoiling and relaxation of DNA, catenation and

decatenation of DNA circles (Kreuzer and Cozzarelli, 1980; Krasnow and Cozzarelli, 1982), and the intra-molecular knotting and unknotting of DNA molecules (Mizuuchi *et al.*, 1980; Liu *et al.*, 1980). Several models for the mechanism of action of such enzymes have been proposed (for example Liu and Wang, 1978a; Brown and Cozzarelli, 1979; Forterre, 1980; Gellert *et al.*, 1980; Wang, 1982; Dean and Cozzarelli, 1985), but all the reactions mentioned above can be explained by a transient break of the DNA backbone, followed by the passage of a section of DNA through the gap, and finally the break is resealed (Mizuuchi *et al.*, 1980). This mechanism and the problems associated with it will be discussed in more detail later.

There are two basic types of topoisomerase (termed type I and type II enzymes) that can be identified by an operational distinction. Type I topoisomerases act by the transient cleavage of a single-strand of DNA, while type II enzymes catalyse reactions involving transient double-stranded cleavage (Gellert, 1981). As a consequence of the extent of DNA strand cleavage, type I topoisomerases change the linking number of DNA in steps of one whilst the type II enzymes alter the linking number in steps of two (Fuller, 1978; Brown and Cozzarelli, 1979; Mizuuchi *et al.*, 1980). Hence type I and type II topoisomerases can be distinguished by incubation of the enzyme with a purified single DNA topoisomer to determine the incremental change in linking number (Liu *et al.*, 1980; Brown and Cozzarelli, 1981).

Topoisomerases have been isolated from viral, prokaryotic and eukaryotic sources. The first activity of DNA topoisomerases to be described was the relaxation of supercoiled closed-circular DNA by prokaryotic topoisomerase I (Wang, 1971). The only topoisomerase so far shown to be able to introduce negative supercoils into DNA is prokaryotic topoisomerase II; also called DNA gyrase (Gellert *et al.*, 1976a). A 'reverse gyrase' that introduces positive superhelical turns into DNA in the presence of ATP has been isolated from the thermophilic bacterium *Sulfolobus* and other archaebacteria (Kikuchi and Asai, 1984; Mirambeau *et al.*, 1984).

The supertwisting of DNA and the effect of topoisomerase action have been implicated to have functions in DNA replication, transcription and recombination (Wang, 1974; Nash, 1981; Liu and Wang, 1987). Supercoiling reduces the overall three-dimensional size of closed-circular DNA, so aiding its packaging into the cell. Negatively supercoiled DNA is partially unwound, and may thus allow RNA polymerase to bind more easily to the DNA to further unwind the helix and allow enhanced transcription of certain genes (Smith, 1981); although other reports show that transcription of certain genes may be suppressed by increased negative supercoiling of the DNA template (e.g. Menzel and Gellert, 1983). A series of recent reports suggest that prokaryotic DNA topoisomerases are involved in the buffering of supercoils during RNA polymerase-mediated transcription of the DNA template (Liu and Wang, 1987; Wu *et al.*, 1988; Giaever and Wang, 1988). Some of the eukaryotic topoisomerases are thought to be the intracellular targets for certain anti-cancer drugs (McVie, 1988; Hsiang *et al.*, 1988). The role of topoisomerases in these different processes will be discussed in more detail after a description of the enzymes themselves.

1.3.1 Type I Topoisomerases.

1.3.1.1 Prokaryotic Type I Topoisomerases.

The most widely studied prokaryotic type I topoisomerase is that isolated from *Escherichia coli* (originally termed the ω protein), first discovered by Wang (1971). Since then, similar enzymes have been discovered in several other bacterial species e.g. *Micrococcus luteus* (Kung and Wang, 1977) and *Agrobacterium tumefaciens* (LeBon *et al.*, 1978). The structural gene for the *E. coli* enzyme is located at 28 minutes on the standard K-12 chromosome map (Bachmann and Low, 1980) and is designated *top*A. The *top*A gene has been cloned (Wang and Becherer, 1983) and sequenced and found to encode a protein of 865 amino acids of molecular weight

97,413 (Tse-Dinh and Wang, 1986). This compares favourably to the denatured molecular weight of the enzyme estimated by SDS-polyacrylamide gel electrophoresis of 100,000 (Depew *et al.*, 1978). The native enzyme is a single subunit protein that requires magnesium ions for its activity as a DNA relaxing enzyme, but there is no ATP dependency. Highly negatively supercoiled DNA is a much better substrate for topoisomerase I than a more relaxed DNA (Kung and Wang, 1977). The *topA* gene appears to be essential. Some deletion mutants of *topA* are still viable, but these have other mutations in the DNA gyrase genes that are thought to compensate for the loss of topoisomerase I (see section 1.3.2.1).

The expression of the topoisomerase I gene requires the DNA template be in a supercoiled state and that the higher the level of supercoiling the greater the level of topoisomerase I expression (Tse-Dinh, 1985). It has therefore been suggested that the actions of topoisomerase I and DNA gyrase (see section 1.3.2.1) may have antagonistic effects to control the overall level of supercoiling within the prokaryotic cell (Menzel and Gellert, 1987).

Atomic absorption spectra analysis has indicated that *E. coli* topoisomerase I is a zinc-containing protein (Tse-Dinh and Beran-Steed, 1988). The enzyme contains at least three zinc atoms that could possible be coordinated by cysteine residues into 'zinc-fingers' that are capable of interacting with a DNA molecule, although the protein does not appear the have a specific DNA recognition sequence unlike, for example, the eukaryotic transcription factor TFIIIA (Klug and Rhodes, 1987). Limited proteolytic digestion of *E. coli* topoisomerase I with either trypsin or papain generates a C-terminal fragment, of molecular mass 14 kDa, that is relatively resistant to further digestion (Beran-Steed and Tse-Dinh, 1989). This fragment has been found to bind to DNA-agarose with an equal efficiency as the intact protein. Although the fragment is not essential for topoisomerisation activity (Zumstein and Wang, 1986; Beran-Steed and Tse-Dinh, 1989), mutants that lack this C-terminal fragment have less of an

affinity for DNA. Thus the topoisomerase reaction by the truncated protein is more sensitive to higher salt levels. The C-terminal portion of the protein could represent a DNA-protein stability function.

In order that topoisomerase I can perform the relaxation of supercoiled DNA, the DNA backbone must be broken so that a topological change can occur. The sites of cleavage on a particular DNA by topoisomerase I have been found to be non-random, although there seems to be no absolute sequence dependence, and proceeds via a phosphotyrosine linkage between the protein and the 5' end of the nick site (Liu and Wang, 1979; Tse *et al.*, 1980). If alkali is added to the protein-DNA complexes, fragmentation of the DNA chain occurs (Depew *et al.*, 1978). These complexes are susceptible to digestion by *E. coli* exonuclease I which act in a 3' to 5' direction. Also if single-stranded linear DNA is double labelled with ^{3}H internally and with ^{32}P at the 5' end then, on cleavage, the enzyme is only linked to ^{3}H-labelled material, indicating a bond to the 5' side at the nick. This 5' association is found with single-stranded and superhelical DNA, but not with relaxed DNA (Liu and Wang, 1979). It can also be assumed that there is a degree of non-covalent interaction between the protein and the 3' end of the nick site, so that the nick is completely held by the enzyme (Dean and Cozzarelli, 1985). If this were not the case then uncontrollable relaxation of DNA would occur rather than the step-wise relaxation reaction that is observed experimentally.

Topoisomerase I relaxes intact double-stranded supercoiled DNA by reducing the linking number in steps of one, and has therefore been postulated to proceed via a single-strand cleavage mechanism (Brown and Cozzarelli, 1979). The relaxation of supercoiled DNA by prokaryotic topoisomerase I generally does not go to completion. The lower the degree of superhelicity of the DNA template, the slower is the rate of reaction; and the relaxation is inhibited by low levels of single-stranded DNA. In fact topoisomerase I will form stable complexes with single-stranded DNA (Depew *et al.*,

1978). Topoisomerase I will not normally relax positively supercoiled DNA. However, this reaction has been observed following the construction of a positively supercoiled heteroduplex DNA molecule in which one strand contains a small deletion and hence has a single-stranded region within its structure. Such a plasmid is a substrate for relaxation by topoisomerase I, and topoisomerase I has been shown by nuclease footprinting to specifically bind to the single-stranded region (Kirkegaard and Wang, 1985). This result supports the idea that the specificity of topoisomerase I for negatively supercoiled DNA in its relaxation reaction derives from the requirement of a short single-stranded DNA segment in the active enzyme-substrate complex. When reacting with double-stranded DNA, denaturation of the topoisomerase binding site is driven by the preferential binding of topoisomerase I to single-stranded DNA. This is facilitated if the helix is destabilised by negative supercoils or a nick (Dean and Cozzarelli, 1985). Positive supercoils would not energetically favour the formation of short sections of single-stranded DNA (Wang, 1971).

Other reactions of prokaryotic topoisomerase I include the ability to catenate and decatenate single-stranded DNA circles, as well as double-stranded DNA circles provided at least one of the circles contains a single-stranded nick (Tse and Wang, 1980), and unknot a nicked, knotted DNA molecule. For these reactions topoisomerase I is thought to bind to the nick and introduces a single-stranded cut in the complementary DNA strand a few base pairs from the nick (Dean and Cozzarelli, 1985). The enzyme can also link (or intertwine) covalently closed single-stranded DNA rings containing complementary base sequences to form a duplex circle (Kirkegaard and Wang, 1978).

Two possible mechanisms have been proposed to explain the action of topoisomerase I. Firstly, the nick site of the DNA is allowed to swivel, rotating the duplex about its long axis. In this model the intact strand would be a stationary pivot for the rotational movement of the nicked strand (Wang, 1971). This can readily explain the relaxation

action of the enzyme, but it is difficult to envisage how other reactions, such as catenation and unknotting, can occur. A second, and more likely mechanism, involves DNA strand passage (Tse and Wang, 1980; Brown and Cozzarelli, 1981). In the catenation and unknotting reactions of double-stranded DNA the only requirement is that at least one of the duplex circles contains a nick. So, it is thought that the enzyme creates a cleavage site opposite this pre-existing nick and thus produces a double-stranded break through which another DNA duplex could be moved. In this way the catenation and unknotting reactions could proceed as well as relaxation (Tse *et al.*, 1980; Brown and Cozzarelli, 1981). With such a mechanism the section of single- or double-stranded DNA opposite the nick would pass through the single-stranded gate held by the enzyme. This mechanism also implies that the DNA molecule must pass through at least part of the protein structure if the gap is held by the enzyme throughout the reaction. This accounts for the step-wise action of the enzyme when it reduces the linking number of supercoiled DNA in steps of one (Dean and Cozzarelli, 1985).

In *top*A deletion mutants a weak type I topoisomerase activity can still be noted (Dean *et al.*, 1982; Srivenugopal *et al.*, 1984). A protein termed topoisomerase III (Mr 74,000) is thought to be responsible for the ATP-independent relaxation of supercoiled DNA observed. The *in vivo* function of topoisomerase III is not clear, although *in vitro* it does appear to be able to perform the decatenation reaction more efficiently than other topoisomerases (DiGate and Marians, 1988). Topoisomerase III has been calculated to perform the decatenation reaction 70-times faster than DNA gyrase and 10-times faster than topoisomerase I. The gene encoding topoisomerase III has been cloned and sequenced (DiGate and Marians, 1989). The gene, encoding a 73.2 kDa polypeptide, has been mapped to 38.7 min on the standard *E. coli* chromosome and designated *topB*. The gene product is found to share significant homology in its central region, although not at the N- or C-termini, to both topoisomerase I (DiGate and Marians, 1989) and to the yeast *top3* gene product (a

putative eukaryotic decatenase enzyme; Wallis *et al.*, 1989). It has been suggested that topoisomerase III is primarily involved in the decatenation of daughter circles at the end of a round of replication, and its initially observed relaxation activity may be of secondary importance to this decatenase activity. However, because the gene is non-essential (deletion mutants are still viable) the role of topoisomerase III *in vivo* remains in doubt.

Reverse gyrase is an ATP-dependent topoisomerase first isolated from the archaebacterium *Sulfolobus* that is able to increase the linking number of DNA and so introduce positive superhelical turns (Kikuchi and Asai, 1984; Mirambeau *et al.*, 1984). Reverse gyrase can also relax negatively supercoiled DNA. Originally the enzyme was described as a type II topoisomerase, but further experimentation has indicated that it belongs to the class I topoisomerases (Nakasu and Kikuchi, 1985). The protein has been purified to near homogeneity and consists of a single polypeptide of molecular mass 120 kDa (Nakasu and Kikuchi, 1985). The ATPase activity of reverse gyrase is stimulated in the presence of DNA. Single-stranded DNA is a much more potent activator of the ATPase than double-stranded DNA, but single-stranded DNA is an inhibitor of the topoisomerase activity (Shibata *et al.*, 1987).

Treatment of archaebacterium with inhibitors of DNA gyrase (covered in greater detail in section 1.3.2.1) results in all plasmids contained within such cells becoming positively supercoiled (Sioud *et al.*, 1988). This suggests that the DNA superhelical density in such bacteria is controlled by a balance between antagonistic DNA topoisomerase activities.

The function of reverse gyrase in archaebacterium is not entirely clear (Forterre *et al.*, 1986). Generally, positively supercoiled DNA is not found naturally, and can only be produced artificially. It is possible that because *Sulfolobus* grown at 75°C, reverse gyrase is required to prevent denaturation of the genome by positively supercoiling

DNA to tighten the pitch of the helix (Kikuchi and Asai, 1984). The presence of reverse gyrase in other archaebacteria that grow at lower temperatures cannot be readily explained.

1.3.1.2 Eukaryotic Type I Topoisomerases.

Eukaryotic type I topoisomerases have been identified in a wide range of lower and higher eukaryotic cells, for example mouse embryo cells (Champoux and Delbecco, 1972); *Drosophila melanogaster* eggs (Basse and Wang, 1974); HeLa cells (Liu and Miller, 1981); avian erythrocytes (Trask and Muller, 1983); yeast (Goto *et al.*, 1984). These proteins are found to be associated with the nuclear chromatin, and have a denatured molecular weight in the range of 70,000-135,000. The enzymes differ in several aspects from the prokaryotic type I topoisomerases, but eukaryotic type I topoisomerases isolated from different sources appear to have no gross mechanistic differences.

Eukaryotic type I enzymes have no ATP dependency, are active in the absence of magnesium, although at a slower rate than in its presence (Liu and Miller, 1981), and have the ability to relax both negatively and positively supercoiled DNA (Tang, 1978). Unlike the prokaryotic enzymes, they are able to completely relax supercoiled DNA *in vitro* without needing to resort to very high enzyme concentrations. The relaxation reaction is somewhat processive in nature. Eukaryotic enzymes are also able to promote a number of other reactions, such as the intertwining of complementary single-stranded DNA circles to form a duplex product (Lau *et al.*, 1981), the decatenation of single-stranded DNA circles and the unknotting of nicked, knotted DNA molecules. It appears that, unlike their prokaryotic counterparts, the eukaryotic type I topoisomerases interact covalently with the 3' end of the DNA nick site (Prell and Vosberg, 1980).

Avian topoisomerase I protects about 25 bp of the DNA helix from digestion by micrococcal nuclease (Trask and Muller, 1983). However, a simple sequence specificity for the binding and nicking of DNA cannot be readily noted, although the following possible putative cleavage sequence has been suggested (parentheses indicating a choice of nucleotide):

5' A(A/G)ACTTAGA(A/G)AAA(A/T)(A/T)(A/T) 3'
↑

Cleavage is thought to occur between the T and A in positions 6 and 7 (Bonven *et al.*, 1985). This sequence is similar, although not identical, to that described by other groups (Edwards *et al.*, 1982; Been *et al.*, 1984). The differences in cleavage specificity observed could be due to the effect of calcium ions in the media. Bonven *et al.* (1985) reported that omission of calcium from these reactions largely relieves the observed cleavage specificity, perhaps bringing into question the significance of the above topoisomerase recognition sequence.

The human gene encodes a 765 amino acid protein that shares 42% homology with prokaryotic topoisomerase I (D'Arpa *et al.*, 1988). The gene has been cloned and sequenced in two separate clones (D'Arpa *et al.*, 1988). The carboxy-terminal fragment can be expressed as a fusion protein that still retains the ability to relax DNA.

Topoisomerase activities have also been found associated with mitochondria. Rat liver mitochondria topoisomerase I is distinct from the nuclear enzyme in that it is sensitive to ethidium bromide and to the non-intercalating trypanocidal drug Berenil (Fairfield *et al.*, 1979). The enzyme, of native molecular mass 40 kDa, acts as a type I enzyme and has the ability to relax both negatively and positively supercoiled DNA in the absence of ATP (Fairfield *et al.*, 1985). Topoisomerase I and II, both found associated with purified yeast mitochondria are indistinguishable from the

topoisomerases of yeast nuclei (Goto *et al.*, 1984). The yeast enzymes are not inhibited by ethidium bromide or Berenil.

The mechanism of reaction of eukaryotic type I enzymes is less well understood than that of the prokaryotic enzymes, and although a strand passage mechanism is possible, the swivel hypothesis cannot be ruled out when trying to account for the differences in reactions between the two (Vosberg, 1985). The subtle difference in the relaxation properties could indicate a mechanistic difference between prokaryotic and eukaryotic type I topoisomerases. The complete and processive relaxation of DNA by the eukaryotic enzyme is in contrast to the prokaryotic case where relaxation is distributive and occurs less well at lower superhelical densities. For the eukaryotic enzyme, a strand-passage mechanism is likely to be employed to perform the decatenation and unknotting activities, but a swivel mechanism cannot be ruled out for the relaxation reaction.

In summary, although a general mode of action of topoisomerases would be attractive, it is not clear whether a unique mechanism can account for the reactions of the two kinds of type I topoisomerases. Some experimentally observed reactions may be best explained by a strand-passage route, whilst others may utilise a swivel-type mechanism. It is most likely that both types of enzymes proceed via a strand-passage mechanism, but that this mechanism may be slightly different in each case. It is possible that prokaryotic topoisomerase I relaxes DNA in multiple-hit events, whilst relaxation by eukaryotic topoisomerase I could involve a single-hit event. Such differences could explain the step-wise relaxation, and the inability to completely relax DNA, as observed with the prokaryotic enzyme, and the much more complete relaxation observed with eukaryotic topoisomerase I. Although there are a number of mechanistic and physical differences between prokaryotic and eukaryotic topoisomerase I, it has been found the yeast topoisomerase I will complement a conditional lethal mutant of topoisomerase I in *E. coli* (Bjornski and Wang, 1987). So

it appears that the two enzymes are capable of performing the same function in a prokaryotic environment.

1.3.2 Type II Topoisomerases.

1.3.2.1 DNA Gyrase.

The bacterial enzyme DNA gyrase is a type II topoisomerase that is able to reduce the linking number of (i.e. introduce negative supercoils into) relaxed closed-circular DNA in the presence of ATP. The enzyme has been discovered in a number of organisms including *Escherichia coli* (Gellert *et al.*, 1976a), *Micrococcus luteus* (Liu and Wang, 1978a), *Bacillus subtilis* (Sugino and Bott, 1980), and *Citrobacter freundii* (Aoyama *et al.*, 1988). The supercoiling reaction is dependent on a divalent cation, such as magnesium, and is stimulated several-fold in the presence of spermidine. The enzyme is also capable of relaxing supercoiled DNA in the absence of ATP (Gellert *et al.*, 1977; Sugino *et al.*, 1977), as well as catenating and decatenating two duplex DNA circles (Kreuzer and Cozzarelli, 1980; Krasnow and Cozzarelli, 1982; Marians, 1987) and unknotting a topologically knotted single DNA duplex (Mizuuchi *et al.*, 1980).

DNA gyrase is composed of two different subunits, A and B, which are the gene products of the *gyr*A (formerly *nal*A) and *gyr*B (formerly *cou*) genes, that map to 48 minutes and 82 minutes respectively on the standard *E. coli* K-12 chromosome map (Bachmann and Low, 1980). Temperature-sensitive or drug-resistant mutants of both the gyrase subunits (to be discussed in detail later) have been identified and found to map to the loci indicated here (Kreuzer and Cozzarelli, 1979; Orr and Staudenbauer, 1981). The two subunits can be purified to near homogeneity (Higgins *et al.*, 1978), and both the genes have been cloned into plasmids that allow their over-production (Mizuuchi *et al.*, 1984; Horowitz and Wang, 1987; Hallett *et al.*, 1990). The *M.*

luteus DNA gyrase appears to resemble the *E. coli* enzyme very closely (Liu and Wang, 1978a).

The number of copies of gyrase per *E. coli* cell is not exactly known. From the amounts that can be isolated from wild-type cells about 500 molecules per cell have been estimated for the A subunit , and the number for the B subunit may be lower by a factor of ten (Higgins *et al.*, 1978). The accuracy of such measurements, however, has to be questioned since much material may be lost during the purification procedures. The closed-circular DNA isolated from a typical *E. coli* cell has a specific linking difference of about -0.06. This is considerably less than the maximum level of supercoiling achievable by gyrase which can give specific linking differences of -0.11 for the plasmid pBR322 (Westerhoff *et al.*, 1988; Bates and Maxwell, 1989). It is likely, therefore, that the level of supercoiling observed *in vivo* is some sort of dynamic balance between the relaxation carried out by topoisomerase I and the supercoiling by gyrase (Menzel and Gellert, 1983). The synthesis of gyrase is itself controlled by the level of DNA supercoiling within the cell. Agents that block DNA gyrase activity, and thus decrease the level of intracellular supercoiling, can increase the *in vivo* rates of synthesis of the A and B subunits up to ten-fold to compensate (Menzel and Gellert, 1983). A systematic deletion analysis of the gyrase promoters indicates that a DNA sequence some 20 bp long, that includes the -10 consensus region, the transcription start point, and the first few bases of the gene, is responsible for the property of induction by DNA relaxation (Menzel and Gellert, 1987).

Both the *gyr*A and *gyr*B genes have been sequenced (Swanberg and Wang, 1987; Yamagishi *et al.*, 1986; Adachi *et al.*, 1987) and have been found to encode proteins of 874 (Mr 97,000) and 804 (Mr 90,000) amino acids respectively. The molecular mass values are in close agreement with those predicted from SDS-polyacrylamide gels (Mizuuchi *et al.*, 1978). The A protein seems to have its major role in the breakage and reunion of DNA by gyrase (Sugino *et al.*, 1977), whilst the B protein

has an ATPase activity associated with it (Sugino *et al.*, 1978; Mizuuchi *et al.*, 1978). From the results of protein cross-linking experiments the active enzyme is thought to be a tetramer of A_2B_2 (Klevan and Wang, 1980). The tetramer structure has subsequently been confirmed by accurate molecular weight measurements by small angle neutron scattering (Krueger *et al.*, 1990), a measured molecular mass of 353 kDa for gyrase means that the A_2B_2 structure (calculated to be 374 kDa) is very likely. In general, both subunits are required for all the reactions of gyrase, although the A subunit does appear to be able to bind to DNA as a dimer in the absence of the B subunit (Klevan and Tse, 1983; Moore *et al.*, 1983; Kirchhausen *et al.*, 1985). The B subunit of gyrase is also found to have a weak ATPase activity associated with it. The ATPase activity of GyrB is only fully stimulated in the presence of GyrA and DNA (Maxwell and Gellert, 1984).

Gyrase alters the linking number of DNA in steps of two per cycle of reaction for both supercoiling and relaxation (Mizuuchi *et al.*, 1980). This strongly suggests that passage of double-stranded DNA occurs through a double-stranded break in the sugar-phosphate backbone. Therefore the altering of DNA topoisomers by gyrase must involve double-stranded cleavage of the DNA with the free DNA ends being constrained by the protein whilst the reaction occurs.

One molecule of gyrase can produce a linking difference of about 100/min at 30°C (Higgins *et al.*, 1978). The enzyme can act catalytically in a processive manner; i.e. on a agarose gel, fully supercoiled products begin to appear whilst the majority of the relaxed starting material is still present (Gellert *et al.*, 1976a; Maxwell and Gellert, 1986). So perhaps the enzyme attaches to a single DNA molecule and carries out several catalytic cycles of the supercoiling reaction before dissociating. Under conditions of high salt concentrations, the supercoiling reaction appears to be much more distributive, with the ladder of topoisomers moving gradually down the gel to the supercoiled position. The relaxation of negatively supercoiled DNA by gyrase is

much more distributive than the supercoiling reaction (Gellert *et al.*, 1977; Higgins *et al.*, 1978).

Nuclease protection studies (Liu and Wang, 1978b; Fisher *et al.*, 1981; Morrison and Cozzarelli, 1981; Kirkegaard and Wang, 1981) indicate that about 140 bp of DNA are resistant to staphylococcal nuclease digestion when gyrase is bound. Digestion of the gyrase-DNA complex with DNAse I leads to about 120 bp being protected from DNAse action and to the generation of a series of DNA fragments whose length differ by approximately 10 bp (Liu and Wang, 1978b; Rau *et al.*, 1987). Binding gyrase to singly-nicked circular DNA, followed by sealing of the DNA with DNA ligase and deproteinisation leads to the introduction of positive superhelical turns into the DNA (Liu and Wang, 1978b). These two pieces of evidence strongly suggest that the DNA is wrapped around the outside of the enzyme with a positive superhelical sense. The wrapping of the DNA in this way probably facilitates the directionality of strand passage during the introduction of negative supercoils during the enzyme's normal function. Other studies on the binding of gyrase to DNA using DNAse I footprinting (Fisher *et al.*, 1981; Kirkegaard and Wang, 1981) have suggested that approximately 100-155 bp are protected by the gyrase tetramer with a central region of about 50 bp being the most strongly protected (Morrison and Cozzarelli, 1981). Nitrocellulose filter binding has shown that at 23°C the gyrase-DNA complex has a dissociation constant of about 10^{-10} M and a half-time of decay of about 60 hours (Higgins and Cozzarelli, 1982).

High resolution structural studies of the gyrase complex have not been performed, but a number of relatively low resolution experiments have given some indication as to the size and shape of the active complex. The complexes between gyrase and DNA have been observed by electron microscopy (Moore *et al.*, 1983; Lother *et al.*, 1984; Kirchhausen *et al.*, 1985; Rau *et al.*, 1987; Maxwell *et al.*, 1989). The gyrase tetramer is seen as an almost spherical structure of about 150-210 Å in diameter. At

higher resolution (Kirchhausen *et al.*, 1985) these particles have been interpreted as having a heart-shaped appearance with the A subunits forming the upper lobes of the heart, but a degree of caution must be used in the interpretation of this work. Electron microscopy of the gyrase complex is at the lower limit of resolution of the instruments and micrographs of gyrase appeared quite 'smudged' with no distinct structure. The heart-shaped structure only becomes apparent after multiple image processing. Electron microscopy has also indicated that gyrase associated with DNA can be found at the intersection of two duplex strands or apparently 'holding' out a loop of DNA. Some gyrase molecules have also been observed associated with more that one DNA molecule (Moore *et al.*, 1983). So, it is possible that the gyrase tetramer contains multiple DNA-binding sites. Transient electric dichroism measurements of gyrase complexed to a 172 bp piece of DNA, known to contain a preferred gyrase binding site, indicate that a single turn of DNA is wrapped around the enzyme with the entry and exit points located close together (Rau *et al.*, 1987). Addition of a non-hydrolysable ATP analogue (ADPNP) to the gyrase-DNA complex results in a structural change consistent with the DNA tails now also being wrapped around the protein.

Small angle neutron scattering (Krueger *et al.*, 1990) has indicated that the gyrase tetramer is a oblate particle approximately 175 Å wide and 52 Å thick that could appear heart-shaped when viewed from certain angles. The size and shape of the particle does not appear to change significantly on DNA binding, so it has been suggested that the DNA may lie in a groove or cavity within the protein. The radius of gyration of a compact globular protein of molecular mass 400 kDa can be calculated to be 43 Å, but the measured radius of gyration for the DNA gyrase tetramer is between 64-67 Å (Krueger *et al.*, 1990). This therefore also suggests that gyrase is not a solid structure and indeed contains cavities, grooves or indentations on its surface of the order of 15 Å in width. A model in which the gyrase contains cavities within its structure could suggest a route for the translocated DNA to pass through the centre of the protein

during the supercoiling reaction if some kind of protein gate is formed around one of these cavities.

ATP is required for all the reactions of gyrase except for the relaxation of negatively (but not positively) supercoiled DNA (Gellert, 1981); hence gyrase is actually able to bind to DNA, and carry out cleavage and some strand passage events in the absence of ATP (Higgins and Cozzarelli, 1982). The hydrolysis of ATP to ADP and inorganic phosphate by gyrase is greatly stimulated by linear, nicked-circular and relaxed closed-circular double-stranded DNA (Mizuuchi *et al.*, 1978), but is less well stimulated by highly negative supercoiled DNA (Sugino and Cozzarelli, 1980). The number of superhelical turns introduced into an initially relaxed circular DNA has been calculated to be approximately equal to the number of ATP molecules hydrolysed by gyrase (Sugino and Cozzarelli, 1980). Therefore it can be suggested that two ATP molecules are hydrolysed per cycle of reaction of gyrase, or that the hydrolysis of one ATP molecule is needed to introduce one superhelical turn into DNA by gyrase. This would seem to be appropriate since there are two B subunits in the active enzyme. The energy required to introduce superhelical turns into a closed-circular DNA is dependent on the superhelical density difference between the substrate and the product. It has been calculated that the free energy required to introduce the final two superhelical turns into pBR322 (maximum superhelical density achievable by gyrase is -0.11, and equivalent to 46 or 47 superhelical turns) is 114 kJ/mol (Bates and Maxwell, 1989). This is similar to the free energy that can be derived from the hydrolysis of two ATP molecules of -120 kJ/mol.

The gyrase B subunit has been found to have a very low level of intrinsic ATPase activity on its own (Maxwell and Gellert, 1984), which is appreciably stimulated when both the A subunit and double-stranded DNA are present. This stimulation is dependent on the length of the DNA used, but generally independent of DNA sequence. DNA molecules of less than about 70 bp in length can only stimulate the

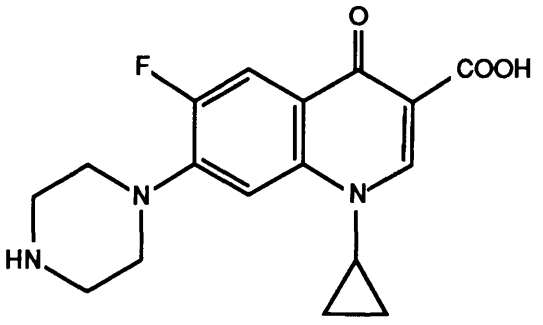
ATPase at very high concentration. It has been proposed that DNA must bind to two sites on the gyrase tetramer before the ATPase can be stimulated and supercoiling occur (Maxwell and Gellert 1984; Chen *et al.*, 1986). So, two short DNA molecules must bind to the gyrase before the ATPase can be stimulated and hence short DNA pieces only activate the ATPase at high concentration. With longer stretches of DNA, however, a single DNA molecule can bind to both the required sites to activate the ATPase at much lower DNA concentrations (Maxwell and Gellert, 1984; Maxwell *et al.*, 1986).

In the presence of the non-hydrolysable ATP analogue ADPNP (5'-adenylyl-β,γ-imidodiphosphate) limited DNA supercoiling can occur, stoichiometric with the amount of enzyme added. This suggests that the binding of ADPNP alone to gyrase is sufficient to allow a single cycle of supercoiling, but the hydrolysis of ATP appears to be required to return the enzyme to its starting state for another round of supercoiling (Sugino *et al.*, 1978).

Purification of the B protein of gyrase from certain bacterial strains results in the isolation a protein of molecular mass of approximately 50 kDa (Brown *et al.*, 1979; Gellert *et al.*, 1979). This protein complements with the A subunit to generate a complex, topoisomerase II', that cannot supercoil DNA, but has the ability to relax both negative and positive supercoils in the absence of nucleotides. The proteolytic digestion patterns of this protein suggested that it is a fragment of the B protein (Gellert *et al.*, 1979). When the *gyr*B gene was sequenced it became apparent that this fragment was the C-terminal half of the intact B protein that could have been produced by *in vivo* proteolysis (Adachi *et al.*, 1987). It appears that this fragment has lost either its ATPase activity or the energy transduction process to allow supercoiling activity of the DNA held by the A subunits. Hence it can be inferred that the ATPase activity of the B protein is located, at least partially, in the N-terminal of the B protein, whilst the C-terminal is required for interaction with the A protein.

DNA gyrase is inhibited by two classes of antibiotics; the quinolones (Gellert *et al.*, 1977; Sugino *et al.*, 1977; Drlica and Franco, 1988) and the coumarins (Gellert *et al.*, 1976b). The A and B subunits are the likely target proteins for the quinolones and the coumarins, respectively (Gellert *et al.*, 1978).

The structure of ciprofloxacin, a potent fluoroquinolone, is shown below:



In the presence of quinolone drugs (e.g. norfloxacin or ciprofloxacin), and subsequent addition of a protein denaturant, such as sodium dodecyl sulphate (SDS), gyrase induces double-stranded cleavage of duplex DNA with a 4 bp stagger at the cut site (Sugino *et al.*, 1977; Gellert *et al.*, 1977; Snyder and Drlica, 1979). The quinolones appear to uncouple the breakage and reunion reactions of gyrase. The cleavage site is approximately in the centre of the region protected from nuclease action (Morrison *et al.*, 1980). Gyrase seems to cut at preferred sites on a particular DNA molecule, but little sequence homology is immediately obvious from these preferred sites (Morrison and Cozzarelli, 1979; Lockshon and Morris, 1985). In the plasmid pBR322 there is a single major cleavage site at nucleotide position 990, but a total of 74 quinolone-induced cleavage sites have been mapped in this plasmid using high gyrase and drug levels (O'Connor and Malamy, 1985). Studies on the *in vitro* cleavage of chromosomal DNA indicate that the *E. coli* genome has approximately 45-50 major cleavage sites, so there are quinolone-induced gyrase cleavage sites about every 100

kb (Snyder and Drlica, 1979; Bejar and Bouche, 1984). On average this corresponds to about one cleavage event per topological domain within the chromosome. A large number of weaker sites also exist. A small, 10 kb region of the chromosome has been examined and found to contain 24 cleavage sites (Franco and Drlica, 1988). This means that the entire *E. coli* chromosome could have in excess of 10,000 cleavage sites, of which less than 0.5% have been identified as major cleavage sites. A possible explanation, assuming that the cleavage sites represent a major fraction of the places where gyrase interacts with DNA, is that a small number of strong interaction sites are used by gyrase to maintain superhelical tension in the chromosome as a whole and the weaker, dispersed sites allow gyrase to provide local swivelling needed for transcription and replication (Drlica and Coughlin, 1989). The role of gyrase during transcription will be discussed more fully in section 1.6.

The specificity of gyrase cleavage of DNA can be altered by the addition of ATP to the reaction mixtures, although the significance of this is not fully understood (Fisher *et al.*, 1981). Mutations at the major cleavage site of gyrase in the plasmid pBR322 can abolish, or reduce the cleavage activity at that site (Fisher *et al.*, 1986). The site has been extensively studied and it has been noted that 34 bp encompassing the cleavage site is not a substrate for the cleavage reaction, but if this sequence is extended 90 bp in either direction from the cleavage site then quinolone-induced cleavage at the site will occur. This phenomenon, however, is likely to be due to the length of DNA that is required by gyrase to perform a cleavage reaction, since removal of either, but not both, the flanking sequences results in a gyrase cleavable sequence. If calcium ions are used in a cleavage experiment, instead of magnesium, then they can also uncouple the breakage-reunion activities of gyrase and cleavage of the DNA can be observed (L.M. Fisher, M.H. O'Dea and M. Gellert, personal communication).

If uniformly radio-labelled DNA is used in a cleavage experiment, then, after protein denaturation and nuclease action, the protein can be shown to be labelled. Paper

chromatography of the acid-treated labelled protein shows that there is a covalent attachment between it and the cleaved DNA via phosphotyrosine linkages (Tse *et al.*, 1980; Sugino *et al.*, 1980). The labelled protein in such an experiment is the A subunit of gyrase. The 5' ends of the cleaved DNA are blocked to labelling by T4 polynucleotide kinase whilst the 3' hydroxyl ends of the cut site remain free and able to prime a DNA polymerase reaction (Morrison and Cozzarelli, 1979). The actual point of attachment between the protein and the 5' end of the DNA cleavage site has been found by a series of proteolytic digestions and sequencing of the labelled peptides to be tyrosine residue number 122 of the A subunit of gyrase (Horowitz and Wang, 1987).

The mechanism of action of cell-killing by the quinolones is not well understood. Although the intracellular target of the quinolones is thought to be DNA gyrase, *in vitro* studies have implicated single-stranded DNA as the preferential binding site of the quinolones (Shen and Pernet, 1985). These studies compared the binding of [^{3}H]-norfloxacin to DNA and DNA gyrase using membrane ultrafiltration and equilibrium dialysis. The results indicated little detectable norfloxacin binding to purified DNA gyrase, however the drug was reported to bind preferentially to single- rather than double-stranded DNA. These studies have been followed by further investigations into quinolone binding (Shen *et al.*, 1989a,b). The somewhat conflicting results obtained suggest that the quinolones still bind to DNA rather than to gyrase, but that they interact with the gyrase-DNA complex rather than with DNA on its own. It has therefore been postulated that the binding of gyrase to DNA induces the formation of some single-stranded character into the DNA which is then capable of interacting with the drug. Subsequently, Shen *et al.* (1989c) proposed a mechanism of inhibition of DNA gyrase by quinolones. The model suggests that gyrase binds to relaxed double-stranded DNA and cleaves both strands with a four base stagger. As the cleaved DNA strands are separated, to allow the subsequent strand passage event, the single-stranded DNA generated constitutes the quinolone binding site. The model proposes

that four drug molecules interact with each other through stacking of their planar rings, and tail-to-tail hydrophobic interactions between the drug molecules already hydrogen-bonded to opposing DNA strands. This model is, however, theoretical and only backed up with scant experimental data.

The ATPase activity of gyrase is inhibited by the coumarin drugs (e.g. novobiocin, coumermycin, and chlorobiocin), but not by the quinolones (Mizuuchi *et al.*, 1978). The coumarins do not inhibit the DNA breakage-reunion reaction of gyrase, and therefore do not inhibit the relaxation reaction, but the drugs will inhibit DNA supercoiling (Gellert *et al.*, 1976b). The coumarins appear to interfere with the binding of ATP to the B subunit of the enzyme since they interact competitively with ATP in the ATPase and supercoiling assays (Sugino *et al.*, 1978). The coumarins, however, appear to bear little structural similarity to ATP.

A number of mechanistic models have been proposed for the supercoiling reaction of DNA gyrase (reviewed in Maxwell and Gellert, 1986). Any model must account for the following features of gyrase reactions:

1. A DNA segment of about 120 bp must wrap around the enzyme in a single turn with a positive superhelical sense.
2. The cleavage of DNA in both strands occurs and there is covalent attachment of the 5'-phosphoryl termini to the A protein.
3. The linking number of DNA is altered in steps of two.
4. The ability to supercoil, relax, unknot, catenate and decatenate closed-circular duplex DNA.

The above requirements are fulfilled by a reaction that involves a double-stranded DNA segment being passed through a transient double-stranded break which is then resealed (Wang, 1982). There are, however, two major problems with this

mechanism. Firstly, since both DNA strands are broken the cleaved complex must be stabilised by the protein. If the DNA ends escaped then complete relaxation of the DNA would occur. However, whilst the enzyme holds the complex together, it must also allow the translocated DNA chain to pass through at least part of the protein structure. Secondly, it is not entirely clear whether the translocated DNA segment is contained within the region wrapped around the enzyme, or comes from a distant part of the chain. Some models suggest that the translocated DNA must be within the wrapped region in order to maintain directionality of strand transfer to introduce supercoils of the appropriate sign (Mizuuchi *et al.*, 1980), whilst others do not see this as a necessity (Brown and Cozzarelli, 1979). Gyrase is able to supercoil DNA circles as small as 174 bp (Bates and Maxwell, 1989) so this tends to suggest that the translocated segment of DNA could be located, at least in some cases, very close to the cleavage site. The exact mechanism of action of DNA gyrase is not as yet fully understood and further experimentation is required to elucidate such a difficult enzymological problem.

A possible scheme for the supercoiling reaction of relaxed closed-circular DNA by gyrase based on current experimental evidence is shown in Fig. 1.3. Gyrase is represented as a ovoid structure (as suggested by Krueger *et al.* (1990)) that is able to bind to DNA and wrap 120 bp around itself with a positive superhelical sense (Rau *et al.*, 1987; Liu and Wang, 1978b). This necessitates the formation of a negative writhe elsewhere in the molecule to relieve the strain of the positive writhe. Gyrase then promotes the formation of a double-stranded break in the wrapped DNA segment. The 5' ends of each of the break sites are covalently attached to the A subunits of gyrase via phosphotyrosine residues (Horowitz and Wang, 1987). This stabilises the break site and will not allow the DNA to untwist to relieve the strain of the negative writhe. The next stage in the process is that strand passage occurs through the break site, and also presumably through at least part of the protein structure if the gyrase remains covalently attached to the DNA. For this reason, models that predict channels

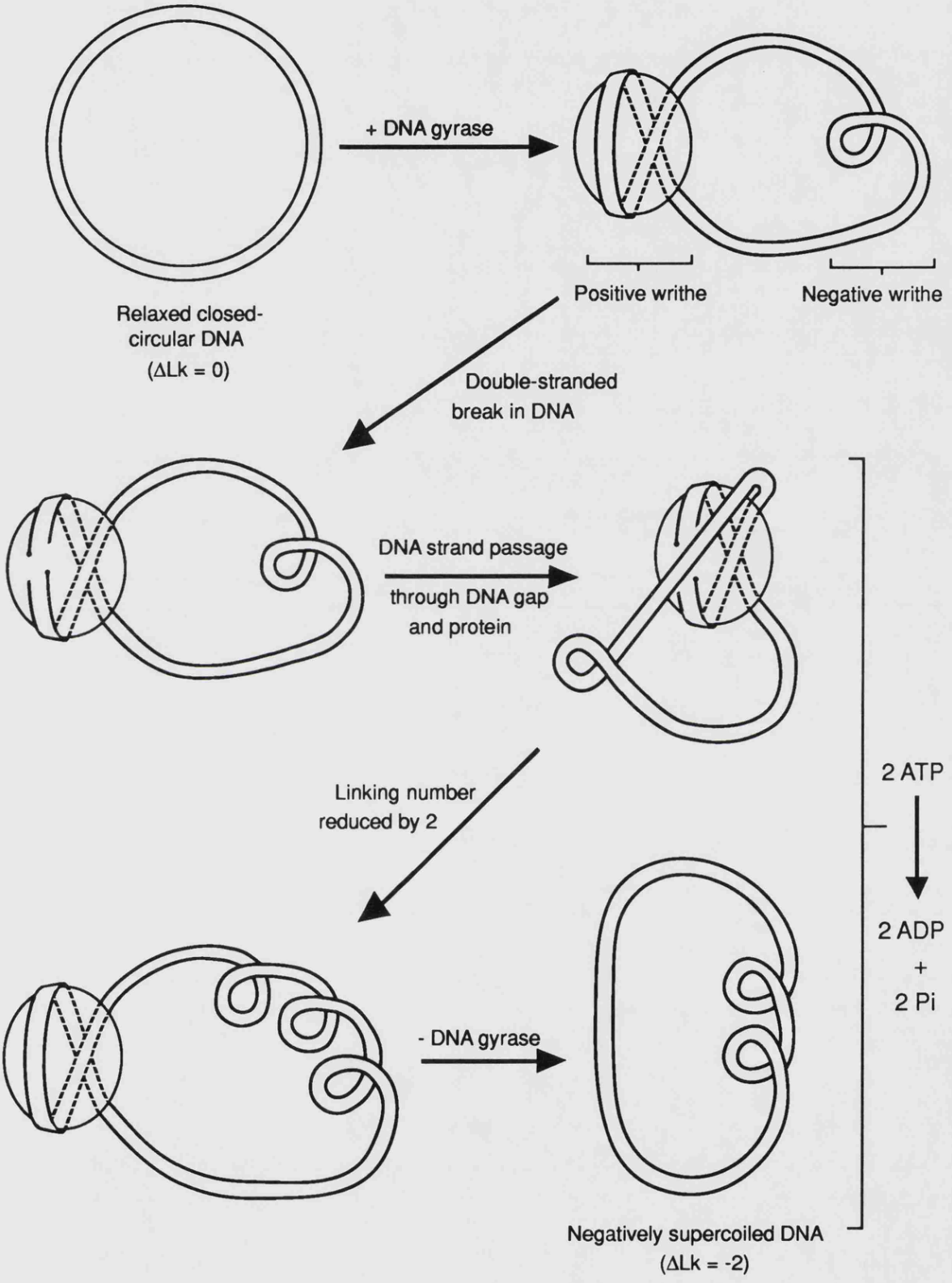


Fig. 1.3. A possible mechanism for the supercoiling of a closed-circular DNA by DNA gyrase.

or cavities within the protein structure are attractive in explaining how this occurs. It is not known if the passing strand is located close to the break site (or part of the wrapped DNA segment) or whether it is more remote. At least in some cases, the passing strand must be near to the wrapped DNA segment since gyrase will supercoil DNA circle as small as 174 bp (Bates and Maxwell, 1989). The passing of a DNA duplex through the double-stranded break means that the linking number of that DNA is reduced by 2 (indicated in the diagram as three negative writhes in the DNA and one positive writhe that occurs due to wrapping around gyrase). Two possibilities could then occur in the mechanism. Firstly, the break in the DNA could then be resealed and the gyrase dissociates from the DNA as indicated. This would result in distributive supercoiling since the gyrase would have to bind again to the DNA in order to carry out further reactions. Secondly, the break may or may not be resealed and the gyrase goes on to perform other cycles of reaction. This would result in the processive supercoiling reaction which is noted for gyrase (Maxwell and Gellert, 1986). If the break is not resealed then gyrase will remain covalently attached to the DNA and another DNA strand passage event could occur to further reduce the linking number of the molecule. If the break is resealed after each cycle of reaction then it must be broken again before further strand passage can occur. At some point in the reaction two ATP molecules are hydrolysed. However, it is not known how the ATP energy is utilised, or for exactly which part of the reaction it is required, and the energetics of the strand passage event are not well understood.

1.3.2.2 Eukaryotic type II topoisomerases.

Eukaryotic type II topoisomerases have been identified from a number of sources; these include yeast (Goto and Wang, 1982; Goto *et al.*, 1984), *Drosophila* embryos (Hsieh and Brutlag, 1980; Shelton *et al.*, 1983), HeLa cells (Miller *et al.*, 1981), rat liver (Duguet *et al.*, 1983) and calf thymus (Darby and Vosberg, 1985). All the above contain ATP-dependent enzymes that catalyse the relaxation of negative and positive supercoils, knotting, unknotting, catenation, and decatenation of DNA circles (Hsieh,

1983). A supercoiling activity has been reported in Xenopus oocyte fractions (Ryoji and Worcel, 1984; Glikin *et al.*, 1984), but such claims have not been substantiated by further evidence.

The enzymes isolated from different sources appear to have similar properties. The topoisomerase exists in its native form as a homodimer with a subunit molecular mass of about 160-175 kDa (Giaever *et al.*, 1986). For the *Drosophila* enzyme, the relaxation reaction is processive under conditions of maximum activity (Osheroff *et al.*, 1983). This can be contrasted to the relaxation reaction performed by prokaryotic DNA gyrase that is distributive in nature (Higgins *et al.*, 1978). The binding of ATP to the eukaryotic topoisomerase is required for the strand passage event and the hydrolysis of ATP for enzyme turnover. The ATPase activity of the enzyme is stimulated in the presence of negatively supercoiled DNA, and, by measurement of the amount of ATP hydrolysed per supercoil relaxed, the removal of one superhelical turn seems to be accompanied the hydrolysis of four ATP molecules (Osheroff *et al.*, 1983). This result, however, could be misleading since there may be some 'uncoupled' ATP hydrolysis during the processive reaction where the free energy liberated is not converted to relaxation of DNA. It is also found that fully relaxed DNA (i.e. the product) will stimulate the ATP turnover of the enzyme. This again could participate to give an anomalously high ATPase activity per supercoil relaxed. Some of the antibiotics that inhibit DNA gyrase (e.g. novobiocin) also inhibit eukaryotic topoisomerase II, although much higher drug levels are needed to inhibit the eukaryotic enzyme. The *Drosophila* enzyme has been cloned and sequenced and found to encode a protein of molecular mass 170 kDa (Nolan *et al.*, 1986).

The *top*2 gene of yeast encodes topoisomerase II (Goto and Wang, 1984) and has been cloned and sequenced (Giaever *et al.*, 1986). The protein consists of 1429 amino acids with the active tyrosine occurring at position 783 (Lynn *et al.*, 1986). It appears the the amino and carboxyl terminal halves of the single-subunit yeast enzyme

share homologies with the gyrase B and A subunit respectively. Alignment of the yeast topoisomerase II amino acid sequence to those of the subunits of gyrase yields an overall sequence homology of 22% identical residues and an additional 14% similar amino acids at corresponding positions (Lynn *et al.*, 1986). Thus it is likely that the N-terminal portion of the yeast enzyme is the ATPase whilst the C-terminal constitutes the DNA breakage and reunion activity. This could suggest a common mechanism between the eukaryotic and prokaryotic enzymes as well as a probable evolutionary relationship.

A number of anti-tumour drugs have been found to be inhibitors of eukaryotic topoisomerase II. A great deal of attention has been focussed recently on the effects of the weak intercalating drug 4'-(9-acridinylamino)methansulphon-m-anisidise (m-AMSA) and the non-intercalating epipidophyllotoxins VP16 and VM26 (Sioud *et al.*, 1987; McVie, 1988; Hsiang *et al.*, 1988). m-AMSA is a potent anti-tumour agent characterised by the ability to induce DNA strand breaks in mammalian cells with proteins tightly bound to the ends of the broken DNA (Zwelling *et al.*, 1981). m-AMSA stimulates the formation of DNA-protein complexes when used in conjunction with eukaryotic topoisomerase II (Nelson *et al.*, 1984). Single- and double-strand breaks are observed with topoisomerase II monomers covalently linked to the 5' ends of the breaks. The mechanism of action of these drugs is possibly the stabilisation of the 'cleavable-complex' formed between topoisomerase II and DNA. The 'cleavable-complex' is one involving the enzyme and DNA that can show cleavage on the addition of drug.

DNA cleavage by eukaryotic topoisomerase II occurs in a similar manner to DNA gyrase with four-base 5'-protruding ends. The 5' phosphoryl end is covalently linked to the enzyme, whilst the 3' hydroxyl at the cleavage site remains free. Again cleavage is non-random and sequence homologies have been suggested (Sander and Hsieh, 1983; Liu *et al.*, 1983; Fosse *et al.*, 1988; Spitzner and Muller, 1988), but no unified

consensus has emerged; e.g. the following sequence has been proposed for vertebrate topoisomerase II by sequencing a number of the drug-induced cleavage sites (Spitzner and Muller, 1988). The cleavage site being indicated by the arrow:

5' RNTNNCNNGYNG(G/T)TNYNY 3'.
↑

Whilst the sequence indicated below has been proposed from a similar study carried out with the *Drosophila* enzyme (Sander and Hsieh, 1985):

5' GTNATAYATTNATNNG 3'
↑

The differences in the above sequences may indeed represent species variations in the topoisomerase enzymes, or perhaps DNA sequence is not the overall controlling factor that determines cleavage specificity. More emphasis should perhaps be placed on other factors such as the inherent 'bendability' of a particular DNA sequence. The DNAse I footprint of *Drosophila* topoisomerase II to a synthetic preferred cleavage site has been measured (Lee, M.P. *et al.*, 1989). There are considerable differences between the footprint obtained here and that of the prokaryotic DNA gyrase. Most importantly, it appears that DNA is not wrapped around the enzyme since only some 25 bp in both strands of the duplex are protected from nuclease action (compared to approximately 120 bp for gyrase; see section 1.3.2.1). The site of DNA cleavage by the enzyme occurs in the centre of the nuclease-protected region. It has been suggested that the absence of DNA wrapping could probably be the mechanistic basis for the lack of DNA supercoiling action by eukaryotic topoisomerase II. A study of the major topoisomerase-induced cleavage sites on chromosomal DNA indicates they are generally found close to the 5' and 3' boundaries of genes (Udvardy *et al.*, 1985). In contrast there are few cleavage sites within genes. The full significance of this result is not understood, but perhaps the topoisomerase plays a role in the alteration of chromatin structure during the regulation of gene expression. The cleavage of DNA

by eukaryotic topoisomerase II, like that by DNA gyrase, can also be induced by calcium ions (Osheroff and Zechiedrich, 1987).

1.4 Viral Topoisomerases.

A number of viral topoisomerases have been described (e.g. Bauer *et al.*, 1977; Liu *et al.*, 1979). The most extensively studied of the virally encoded topoisomerases is that of the bacteriophage T4 (Liu *et al.*, 1979; 1980; Kreuzer and Alberts, 1984). The enzyme is multisubunit, coded for by the T4 genes 39, 52, and 60 with subunit molecular masses of 58 kDa, 51 kDa, and 16 kDa respectively (Liu *et al.*, 1979; Huang, 1986a,b). The enzyme catalyses the relaxation of positively and negatively supercoiled DNA, catenation/decatenation, and the knotting/unknotting of circular DNA molecules. Linking number changes occur in steps of two and so the mechanism involves strand passage through double-stranded breaks in the DNA. One or two ATP molecules are hydrolysed during each round of catalytic relaxation (Liu *et al.*, 1979).

The small subunit (16 kDa) is thought to play a role in holding the two larger subunits within the native enzyme complex (Seasholtz and Greenberg, 1983). DNA cleavage by T4 topoisomerase II can be stimulated by the anti-tumour drug m-AMSA, in a similar manner to eukaryotic type II topoisomerases. Under these conditions the 51 kDa protein is found to be covalently linked to the 5' ends of the broken DNA via phosphotyrosine linkages (Rowe *et al.*, 1984). Sequencing of the T4 52 gene (Huang, 1986a) indicates that the encoded protein shares significant amino acid sequence homology (32% identical residues) with the N-terminal half of the A subunit of bacterial DNA gyrase. There also seems to be significant homology in the region of the active tyrosine of the T4 enzyme (amino acid 116) and the gyrase active site (Huang, 1986a). The T4 39 gene product on the other hand, shares sequence homology (35% identical residues) with the N-terminal of the gyrase B subunit

(Huang, 1986b). So it would appear the 58 kDa protein acts as the ATPase whilst the 51 kDa protein is responsible for DNA breakage and reunion.

T4 topoisomerase II is essential for normal T4 replication and is thought to promote the initiation of replication of DNA. It has been speculated that this enzyme might induce some local supercoiling by forming a loop within the region of the replication origin (Liu *et al.*, 1979; 1980; Kreuzer, 1984).

A topoisomerase activity has also been identified from the poxvirus vaccinia (Bauer *et al.*, 1977). The enzyme appears to exist as a monomer of molecular mass 32-35 kDa. It is a type I enzyme that is inhibited by the coumarin class of gyrase inhibitors (Foglesong and Bauer, 1984). ATP is not required for the activity of this enzyme, however some stimulation of activity can be noted on addition of ATP. In a manner similar to the eukaryotic type I topoisomerase, vaccinia virus topoisomerase introduces single-stranded breaks into the DNA, and is covalently bound to the 3' end of the broken strand (Shaffer and Traktman, 1987).

1.5 Recombination Topoisomerases.

Two topoisomerase-like proteins have been described which have a defined function in site-specific recombination events, the λ Int protein and the resolvase of the γδ and Tn3 transposons. These enzymes share similar chemistry to the topoisomerases in that they are able break and rejoin phosphodiester bonds, but the control of overall DNA topology is not their main function.

The phage λ during its lysogenic pathway is able to integrate into the *E. coli* chromosome at a specific site. The recombination process is mediated by the λ *int* gene product and a host-encoded integration factor (Nash, 1981). The Int protein has a molecular mass of 44 kDa and its reaction involves the breakage of two double-stranded DNA molecules, those of the phage DNA and the *E. coli* chromosome, and

their rejoining by reciprocal crossover (Nash and Pollock, 1983). Since no energy is required for this rejoining, a topoisomerase-like mechanism would seem likely with transient covalent DNA-protein complexes (Craig and Nash, 1983). The linkages are similar to the eukaryotic type I topoisomerases, with covalent attachment of protein to the 3' phosphate at the cleavage site, despite being of prokaryotic origin. The Int protein is able to relax supercoiled DNA, although at a slow rate in comparison to other topoisomerases. The relaxation changes the linking number of the DNA in steps of one (Nash *et al.*, 1980).

γδ-resolvase is a small protein (21 kDa) which also acts as a type I topoisomerase (Krasnow and Cozzarelli, 1983). The protein is needed to allow the resolution of the γδ and Tn3 transposons. Transposition is essentially a two-step process with, firstly, the formation of a fused cointegrate from two separate replicons and, subsequently, resolution of this composite structure. In these cases recombination requires the presence of two DNA *res* sites in direct repeat. Resolvase action leads to the formation of catenated rings each carrying one res site. It is thought that DNA gyrase will complete the resolution of the cointegrates via decatenation (Krasnow and Cozzarelli, 1983).

1.6 Function of Topoisomerases.

Topoisomerases carry out a variety of reactions *in vitro*, and a number of these can be correlated to the *in vivo* situation. This section attempts to summarize the *in vivo* reactions of topoisomerases and give some indication of the function of these enzymes, both in prokaryotic and eukaryotic cells.

DNA gyrase is essential for DNA replication *in vivo*. The antibiotics that inhibit gyrase are also inhibitors of DNA replication. Also temperature-sensitive mutants of the *gyr*A and *gyr*B genes in *E. coli* block DNA replication at the non-permissive temperature (Kreuzer and Cozzarelli, 1979; Orr and Staudenbauer, 1981). It appears

that these mutants operate by a different mechanism to stop replication. The *gyr*B mutation only inhibits the initiation of replication, but not chain elongation (Orr and Staudenbauer, 1981), whereas the *gyr*A mutant leads to a rapid arrest of chain elongation (Kreuzer and Cozzarelli, 1979). It is likely therefore that the gyrase tetramer has a dual function in DNA replication and is required for both initiation and elongation, although the exact role of the protein in these processes is somewhat unclear. It has been suggested that gyrase acts in elongation at a site close to replication forks (Drlica *et al.*, 1980). Gyrase may also be required to complete DNA replication by segregating daughter chromosomes by decatenation. The nucleoids from *gyr*B mutants of *E. coli* are obtained as doublets if isolated from cells grown under non-permissive conditions. These doublets can be resolved by gyrase (Steck and Drlica, 1984).

The action of quinolone drugs on gyrase can lead to fragmentation of the DNA both *in vitro* and *in vivo*. Approximately 45-50 major specific gyrase cleavage sites have been estimated for *E. coli* (Snyder and Drlica, 1979). This is roughly equal to the number of supercoiled loops found in the *E. coli* chromosome. This may just be coincidence or gyrase binding may be involved in controlling the overall tertiary structure of the chromosome by separating loops of different superhelical density within the chromosome. Supporting evidence for this observation has also been noted by electron microscopy of gyrase-DNA complexes (Moore *et al.*, 1983). Often the gyrase is located at the intersection of loops of DNA, again suggesting a structural role for the protein. A structural role has also been suggested for eukaryotic type II topoisomerases (Earnshaw *et al.*, 1985).

Topoisomerases are also involved in the regulation of transcription of a number of prokaryotic genes. Negatively supercoiled DNA is found to have enhanced transcriptional ability over relaxed, nicked or linear DNA (Smith, 1981). The drugs that inhibit DNA gyrase activity can affect the level of gene expression (Sanzey,

1979). However some genes are inhibited by negative supercoiling. Indeed the gyrase genes themselves appear to be homeostatically controlled by the level of DNA supercoiling (Menzel and Gellert, 1983). Recently evidence has been growing about a role for topoisomerases in the transcriptional process itself. Transcription of a right-handed double helix requires the relative rotation of RNA polymerase around the DNA (Liu and Wang, 1987). It is possible that the advancing, rotating polymerase generates positive supercoils in the DNA template ahead of it and negative supercoils behind it. The transcription of a plasmid containing two genes in opposite orientations (e.g. the *tet* and *bla* genes of pBR322) means these two effects will reinforce each other, and inhibition of gyrase results in the formation of a highly positively supercoiled plasmid (Lockshon and Morris, 1983; Wu *et al.*, 1988). If one of the genes is deleted from the plasmid (e.g. removal of *tet* from pBR322) then gyrase inhibitors do not result in the production of a highly positively supercoiled plasmid (Pruss and Drlica, 1989). Topoisomerase I can also be inhibited in a similar experiment and in this case a highly negatively supercoiled plasmid is formed. Thus, it is probable that topoisomerase I removes negative supercoils and gyrase preferentially removes positive supercoils during normal transcription reactions (Wang and Giaever, 1988). This positive supercoiling can also occur in eukaryotic yeast cells that are deficient in their own topoisomerases, but are expressing *E. coli* topoisomerase I to specifically relax negative supercoils (Giaever and Wang, 1988). Thus both prokaryotic topoisomerases seem to play some role in controlling the topology of the DNA template during transcription. *In vitro* studies to quantify the extent of transcription-generated supercoiling have suggested that transcription is one of the principal factors affecting intracellular DNA supercoiling (Tsao *et al.*, 1989).

DNA gyrase is required for the site-specific integration of phage λ into the *E. coli* chromosome (Gellert *et al.*, 1976a). The function of gyrase is to supercoil the DNA molecule that carries the phage attachment site *att*P. Supercoiling also appears to be essential for other recombination reactions.

In *E. coli* deletion of the *top*A gene (encoding topoisomerase I) destroys the viability of the cells. However, a second mutation can occur to compensate for the loss of this enzyme and allow the cells to grow (Pruss *et al.*, 1982). These secondary mutants have been mapped to the gyrase genes; *gyr*A and *gyr*B. This suggests that excessive supercoiling is lethal to the cell, and that the expression of the topoisomerase genes must be under tight control. The gyrase genes themselves are under homoeostatic regulatory control. Increased supercoiling of the DNA template down-regulates the expression of the gyrase genes and a decrease in the superhelix density of the template leads to expression of the genes (Menzel and Gellert, 1983; 1987).

Eukaryotic topoisomerases appear to be involved in the replication of DNA, although their role is somewhat less clear than their prokaryotic counterparts. Deletion of the *top*1 gene of yeast appears to have no effect on the growth of the cells (Uemura and Yanagida, 1984), but the loss of this enzyme could be compensated for by the action of topoisomerase II. It appears that topoisomerase I may have a role in the control and maintenance of the chromatin structure throughout the cell cycle, whereas topoisomerase II is involved in chromosome segregation at the end of replication (Uemura and Yanagida, 1984; Uemura *et al.*, 1987). Immunofluoresence studies of topoisomerase II from chicken suggest that the enzyme is located as part of the mitotic chromosome scaffold (Earnshaw *et al.*, 1985; Earnshaw and Heck, 1985). It is possible therefore that this enzyme is also a structural protein of interphase nuclei and mitotic chromosomes. The eukaryotic topoisomerases may also be involved in other steps of the replication process, for example chain elongation, but as yet there is insufficient knowledge of the action of these enzymes. Little is known about the action of eukaryotic topoisomerases in the transcriptional process or their presumed role in DNA excision repair mechanisms (Downes and Johnson, 1988).

Eukaryotic topoisomerases are involved in the assembly of chromatin (Sekiguchi and Kmiec, 1988). The coiling of a topologically constrained DNA molecule is accompanied by the occurrence of conformational strain. Thus positive supercoiling is generated in compensation for negative left-handed supercoiling of DNA around the histone cores. Eukaryotic topoisomerase I can relax positive supercoils and could therefore play a role in the assembly of chromatin (Nelson *et al.*, 1979).

1.7 Aims of this project.

From all the evidence outlined above it appears that topoisomerases play a role in many vital cellular processes in both prokaryotes and in eukaryotes. Bacterial DNA gyrase provides a model system for the study of both DNA-protein interactions and the interactions of antibiotics with the protein-DNA complex. There are, however, a number of areas where our knowledge of the system is limited. No topoisomerase has so far been crystallized, and consequently no high resolution three-dimensional structure for any topoisomerase is available. Hence we have little idea of structure-function relationships within these proteins. The amino acid sequences of a number of topoisomerases appear to be evolutionary related (Huang, 1986a,b), so perhaps the existence of a common structure is not unreasonable. This structure could be segregated into a distinct domain system within the proteins. Another area of uncertainty about topoisomerase mechanism is how the hydrolysis of ATP at one site can be linked to the passage of a DNA strand at another. This also raises the question of the strand passage event itself and the exact manner in which a DNA molecule is able to pass through at least part of the protein structure if current models of topoisomerase action are correct.

There has been a number of reported cases where the domain structure of a DNA-binding protein has been partially elucidated by generating proteolytic fragments of the intact molecule (e.g. Pabo *et al.*, 1979; Abdel-Meguid *et al.*, 1984; Bear *et al.*, 1985; Jen-Jacobson *et al.*, 1986). A structural and/or functional domain should be a

relatively compact structure that, by allowing limited access, is resistant to proteolytic cleavage, whereas domains are connected by regions of protein that are more susceptible to attack. Thus it should be possible, under the correct conditions, to separate the domains of a particular protein in order to study them on their own and assess any activity that they may possess. With the λ repressor, papain digestion yields two stable fragments (Pabo *et al.*, 1979). The N-terminal fragment was found to bind to DNA and could be relatively easily crystallized. It was then possible to deduce the three-dimensional interactions that must exist between the protein and the DNA (Jordan and Pabo, 1988).

So, the initial aims of this study were to investigate any possible domain structure within the DNA gyrase subunits by generating proteolytic fragments, and attempting to study any activity they may possess both in isolation and in combination with the other subunit. Information should also be gained about the mechanistic aspects of gyrase action by the way in which proteolytic fragments may react in comparison to the native protein. It may be possible, through this study, to obtain suitable fragments of the gyrase proteins that are still active and are amenable to crystallization, to hopefully yield some detailed structural information about the active site and the general conformation of the protein.

Chapter 2

Materials and Methods.

2.1 Bacterial strains.

All the *E. coli* strains used in this study are derivatives of *E.coli* K12 unless otherwise stated.

Strain.	Genotype.	Source.
DH5	F', *end*A1, *hsd*R17, (r^-_k m^+_k), *sup*E44, *thi*$^{-1}$, λ^-, *rec*A1, *gyr*A96, *rel*A1.	W.J. Brammar.
E103S	*lon*$^-$	C.D. Thomas.
HB101	F$^-$, *hsd*S20 (r^-_B m^-_B), *rec*A13, *ara*14, *pro*A2, *lac*Y1,*gal*K2, *rsp*L20 (Sm^r), *xyl*5, *mtl*1, *sup*E44.	R.T. Simpson.
HB2154	Δ(*lac-pro*AB), *ara*14, *thi*$^{-1}$, F'(*pro*AB, *lacI*^{q}, *lac*ZΔM15), *mut*L::Tn10.	D.B. Wigley
JM109	Δ(*lac-pro*AB), *rec*A1, *end*A1, *thi*$^{-1}$, *sup*E44, *hsd*R17, *rel*A1, *gyr*A96, F'(*tra*D36, *pro*AB$^+$, *lacI*^{q}, *lac*ZΔM15).	W.J. Brammar.
KNK453	F$^-$, *pol*A1, *thy*A, *uvr*A, *phx*, *nal*A43 (ts 42°C).	A.S. Lynch.
RW1053	*rec*A Δ(*gal att* λ *bio*).	M. Gellert.
TG2	Δ(*lac-pro*AB), *sup*E, *thi*, *hsd*D5, *rec*A$^-$, F'(*tra*D36, *pro*AB, *lacI*^{q}, *lac*ZΔM15)	D.B. Wigley.

Bacterial stains were maintained in two types of stocks; as agar stab cultures in vials at room temperature, and at -70°C in LB Broth (see section 2.2) containing 15% glycerol.

2.2 Bacterial growth media.

All media for the growth of bacteria were prepared and sterilised according to standard microbiological techniques. For plate media 15 g/litre of agar (Oxoid code L11) were added to each of the below recipes.

'M9' Minimal medium:

K_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g
Water to 990 ml.	

After autoclave sterilisation of the above, the following filter-sterile solutions were added:

1 M $MgSO_4$	2.0 ml
20% Glucose	10.0 ml
1 M $CaCl_2$	0.1 ml
1 M thiamine-HCl	1.0 ml

Luria-Bertani (LB) medium:

Bacto-tryptone (Difco)	10.0 g
Bacto-yeast extract (Difco)	5.0 g
NaCl	10.0 g
Water to 1 L.	

2xYT medium:

Bacto-tryptone (Difco)	10.0 g
Bacto-yeast extract (Difco)	10.0 g
NaCl	5.0 g
Water to 1 L.	

H-TOP Agar:

Bacto-tryptone (Difco)	10.0 g
NaCl	8.0 g
Agar (Oxoid)	8.0 g
Water to 1 L.	

SOB Broth:

Bacto-tryptone (Difco)	20.0 g
Bacto-yeast extract (Difco)	5.0 g

NaCl	0.5 g
KCl	0.2 g
Water to 940 ml.	

After autoclave sterilisation of the above, the following filter-sterile solutions were added:

0.2 M $MgCl_2$	50 ml
1 M $MgSO_4$	10 ml

SOC Broth:

As SOB Broth except that 20 ml of filter-sterile 1 M glucose are added along with the $MgCl_2$ and $MgSO_4$ solutions.

Super Broth medium:

This medium was used for the growth of cultures for the preparation of plasmids in large amounts.

Bacto-tryptone (Difco)	12.0 g
Bacto-yeast extract (Difco)	24.0 g
Glycerol (50%)	8.0 ml
Water to 900 ml.	

After autoclaving the above, 100 ml of autoclaved 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 were added.

Antibiotics in growth media:

ANTIBIOTIC	[STOCK]	[IN MEDIA]
Ampicillin (sodium salt)	50 mg/ml	50-100 µg/ml media.
Tetracycline (hydrochloride)	15 mg/ml	15 µg/ml media.
Chloramphenicol	80 mg/ml in ethanol	250 µg/ml media.

2.3 Transformation of plasmids into *E. coli* cells.

Competent *E. coli* cells were prepared by a variation on the method of Hanahan (1983). A single colony was transferred to 100 ml SOB Broth and incubated until an optical density at 595 nm ($A_{595\ nm}$) of 0.5 was obtained. The cells were then pelleted by centrifugation and resuspended in 33 ml of 10 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM CH_3COOK (pH 5.8), 10 mM $CaCl_2$, 15% glycerol. The cells were then left on ice for 60 min, after which they were re-pelleted and resuspended in 8 ml of 10 mM MOPS (pH 7.0), 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% glycerol prior to freezing in 200 µl aliquots and storage at -70°C.

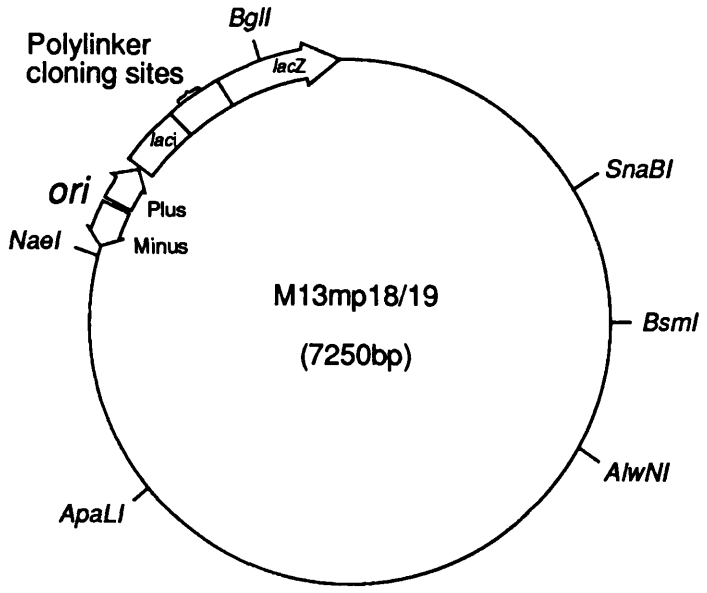
A 200 µl aliquot of competent cells was thawed and allowed to stand on ice for 30 min. DNA (in a volume of 20 µl of H_2O) was mixed with the competent cells and allowed to stand on ice for 60 min. The cells were incubated at 42°C for 90 seconds prior to adding 0.8 ml SOC Broth. The mixture was then incubated at 37°C for 1 hr, after which 200 µl were plated out on a suitable media. The plates were incubated overnight and the numbers of colonies counted the following day. The transformation efficiency of the competent cells was calculated using 50 ng of supercoiled pBR322. Efficiencies of 10^5-10^7 transformants/µg of DNA were generally observed.

2.4 DNA Plasmids used during this work.

M13mp18/19 (7250 bp). The double-stranded form (or replicative form; RF) of M13 bacteriophage. The mp series have been constructed to contain a number of unique restriction sites (Yanisch-Perron *et al.*, 1985). The mp18 and mp19 vectors differ only in the orientation of the multiple cloning site. See Fig. 2.1.

p5S172-25 (8.7 kb). Contains 25 head-to-tail repeats of a 172 bp DNA fragment, a preferred gyrase binding site within the sea urchin 5S rRNA gene (Simpson *et al.*, 1985; Rau *et al.*, 1987), cloned into the *AvaI* site of pAT153 by the method of Hartley and Gregori (1981). For the sequence of the 172 bp fragment see section 2.6.

Fig. 2.1. Restricion map of M13mp18/19.



Polylinker:

M13 mp18

5'....gattacgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcactggc.....3'

EcoRI *SacI* *KpnI* *SmaI* *XmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

M13 mp19

5'....gattacgCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGaattcactgg.....3'

HindIII *SphI* *PstI* *SalI* *AccI* *HincII* *XbaI* *BamHI* *SmaI* *XmaI* *KpnI* *SacI* *EcoRI*

p5S207-18 (8.1 kb). Contains 18 head-to-tail repeats of a 207 bp DNA fragment cloned into the *AvaI* site to pAT153 (Simpson *et al.*, 1985).

pAG111 (7.2 kb). A derivative of pTTQ18 that contains the *gyrB* gene linked directly to the *tac* promoter (Hallett *et al.*, 1990).

pBR322 (4363 bp). A multi-purpose general cloning vector with a number of unique restriction sites in the ampicillin-resistance and tetracycline-resistance determinants. See Fig. 2.2.

pDH24 (6.1 kb). A derivative of pSLS447 (Swanberg and Wang, 1987) constructed by D.S. Horowitz to enable the IPTG-inducible over-production of the GyrA protein in *E. coli lacI*^{q} hosts (Horowitz and Wang, 1987).

pMK47 (20 kb). Heat inducible GyrB over-producing plasmid (Mizuuchi *et al.*, 1984). The *gyrB* gene is placed downstream of a λP_L promoter.

pMK90 (23 kb). Heat inducible GyrA over-producing plasmid (Mizuuchi *et al.*, 1984). The gyrA gene is placed downstream of a λP_L promoter.

pPH2 (9.1 kb). A construct that contains the *gyrA* gene cloned into pTTQ18. The *gyrA* gene is approximately 1.1 kb downstream from the *tac* promoter, and has a *XhoI* restriction site silently mutated into the sequence 14 bp from the 5' end. pPH2 was an intermediate in the construction of pPH3 (Hallett *et al.*, 1990). See Fig. 2.3.

pPH3 (8.0 kb). A derivative of pTTQ18 that contains the *gyrA* gene linked directly to the *tac* promoter (Hallett *et al.*, 1990). See Fig. 2.3.

pRH43-117 (4.3 kb). A construct produced by Abremski and Hoess (1985) that contains the kanamycin resistance gene and two *loxP* sites, one of which has been mutated by the Bal31 nuclease to delete 8 bp at the 5' end of the inverted repeat. See Fig. 2.4. Recombination between the wild-type and mutant *loxP* sites, mediated by the cre recombinase, results in catenated products being formed.

pTTQ18 (4563 bp). A multi-purpose cloning vector constructed by M.R.J. Stark (1987) that contains an ampicillin-resistance determinant, a *lacI*^{q} allele, and the M13mp18 multiple cloning site directly down-stream from the *tac* promoter. See Fig. 2.5.

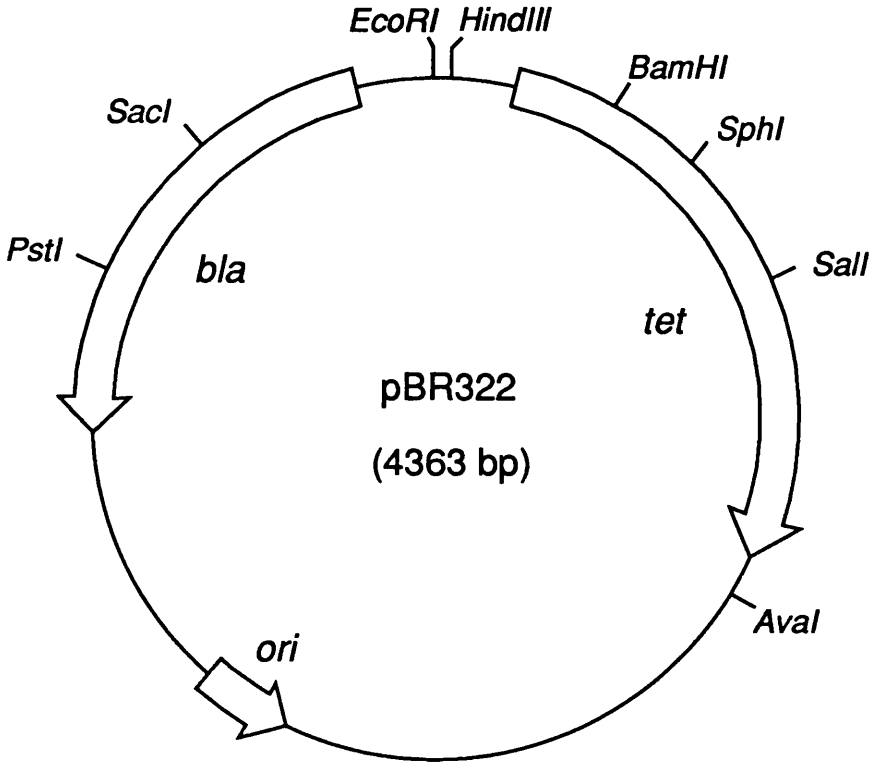


Fig. 2.2 Restriction map of pBR322.

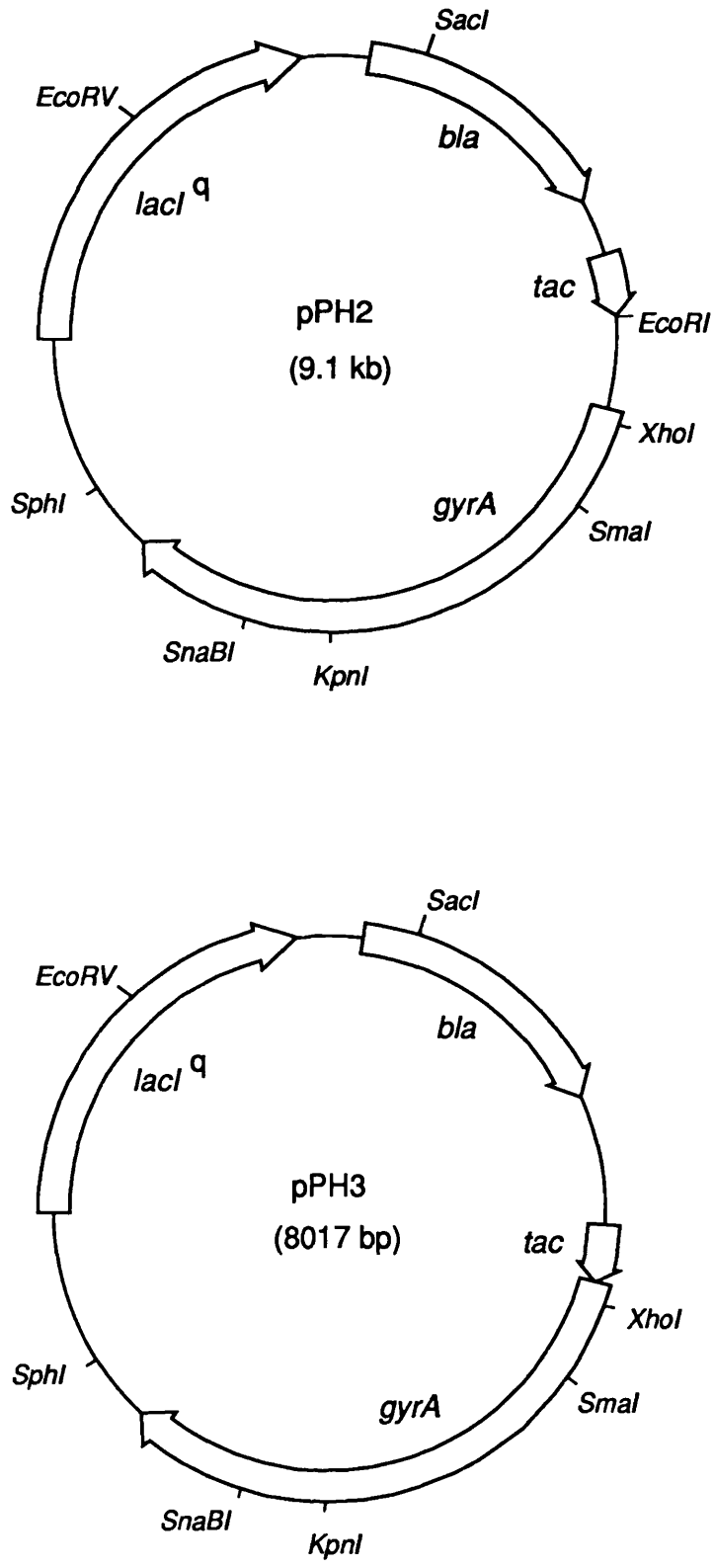
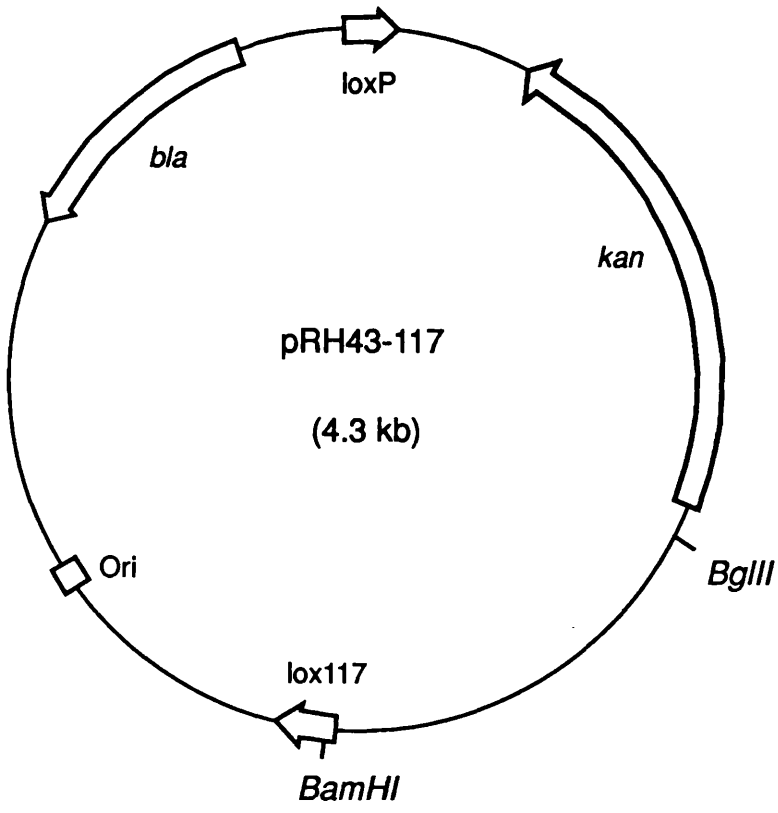
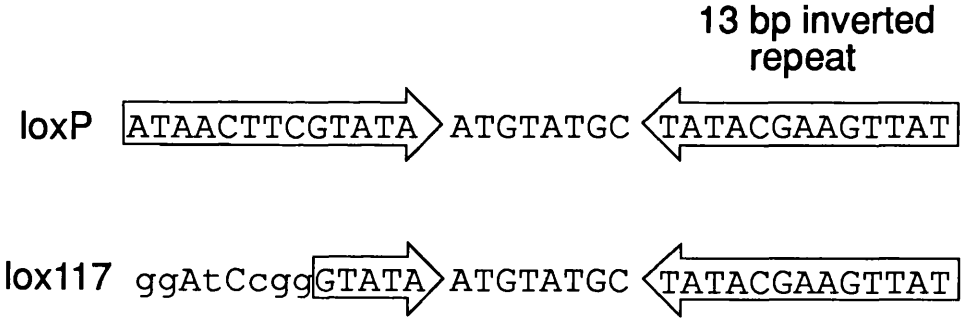


Fig. 2.3. Restriction maps of the plasmids pPH2 (top) and pPH3 (bottom). pPH2 contains the *gyrA* gene with a silent *XhoI* site mutated 14 bp 3' to the start of the coding sequence. pPH2 is an intermediate in the construction of pPH3, a GyrA over-producing plasmid (Hallett *et al.*, 1990).





lox117 has 8 bp deleted from the original loxP site.

Fig. 2.4. A restriction map of plasmid pRH43-117 and the sequence of the two *loxP* sites.

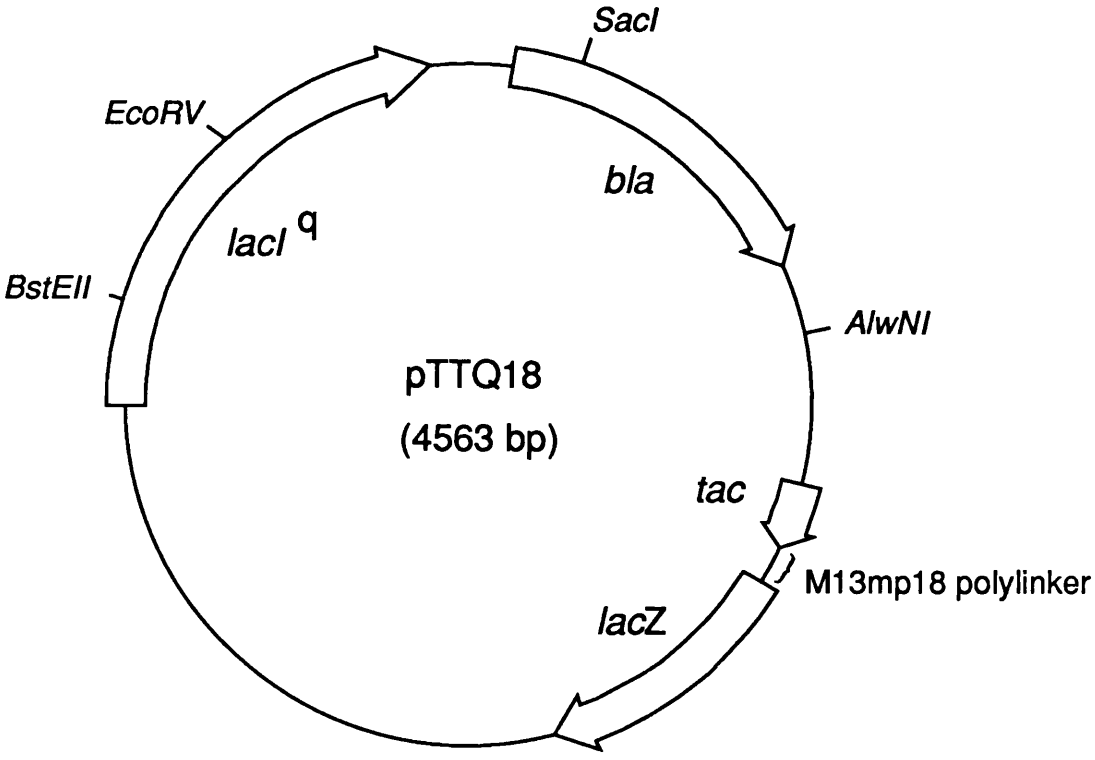


Fig. 2.5. Restriction map of pTTQ18.

2.5 Gel electrophoresis of DNA.

DNA can be visualised after electrophoresis through agarose and polyacrylamide gels. Agarose gels are convenient for the separation of DNA fragments ranging in size from a few hundred base pairs to over 20 kb, whilst polyacrylamide is used for the separation of smaller fragments. The gels themselves are a complex network of polymeric molecules through which negatively charged DNA migrates under the influence of an electric field. The DNA can subsequently visualised by fluorescence under UV light after staining with ethidium bromide.

Agarose (Miles), at a concentration of 0.8-1.0% (w/v) depending on the size of the DNA, was melted in TAE (40 mM Tris acetate, 2 mM EDTA) and poured into a suitable former. The solidified gel was then transferred to a horizontal gel electrophoresis tank and submerged in TAE buffer. One-fifth volume of Loading Buffer (40% sucrose, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mg/ml bromophenol blue) was added to the DNA samples before loading on the gel. Samples were electrophoresed at 75-150 mA until the bromophenol blue dye had migrated through approximately 75% of the length of the gel. The gel was then removed from the tank and stained for 10 min in a solution of ethidium bromide (2 μg/ml) in TAE buffer. The stained gel was destained in TAE prior to visualisation of the stained DNA using a UV transilluminator. Gels were photographed using Kodak T_{MAX} 100 (5" x 4") professional film.

For the visualisation and separation of DNA topoisomers from each other and from any nicked DNA that may be present, DNA samples were electrophoresed in the presence of chloroquine. Chloroquine is an intercalating agent that can be used to untwist closed-circular DNA, and consequently will have the effect making topoisomers run apparently more positively supercoiled in comparison to the positions which they run on TAE gels. Such gels were used to distinguish relaxed topoisomers from the nicked species. Agarose gels were made up in TPE (50 mM Tris phosphate,

1 mM EDTA (pH 7.2)) containing 1-10 μg/ml chloroquine. The DNA was loaded as described above and the gel run at not greater than 100 mA in TPE containing chloroquine to the same level as present in the gel. After electrophoresis the gel was stained and destained as described above.

Linear DNA fragments were electrophoresed through TAE agarose gels containing 1 μg/ml ethidium bromide, and run in TAE. The DNA could be observed at any stage during the electrophoresis by viewing on a UV transilluminator without the need for staining. If a particular DNA fragment was to be isolated from other DNA then the sample was electrophoresed through a 1% low-melting point agarose gel (NuSieve GTG agarose) containing 1 μg/ml ethidium bromide. DNA was visualised on a UV transilluminator and the appropriate DNA band excised. 100 μl of TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) was added to the gel slice and the mixture was then incubated at 65°C for 30 min and the DNA isolated by phenol extraction (Perbal, 1988). An equal volume of phenol (equilibrated with 1 M Tris-HCl (pH 7.5)) was added and the solution vortexed for 5 min. After centrifugation the upper aqueous layer was removed and the DNA was precipitated with three volumes of ethanol, washed in 70% ethanol, dried and then resuspended in an appropriate volume of TE.

Polyacrylamide gels were used for the study and isolation of DNA fragments under about 300-400 bp in size. The gels (6-20% polyacrylamide (19:1 acrylamide:bis) depending on the size of the DNA fragments) were made up in TBE (0.1 M Tris-borate, 2 mM EDTA) and were polymerised by the inclusion of 0.1% (w/v) AMPS and 0.1% (v/v) TEMED. Polyacrylamide gels were run vertically and the sample, in a 1/5 volume of Loading Buffer as above, was applied to the top of the gel. When the bromophenol blue marker reached the bottom, the gel was removed and stained and the DNA visualised in the same manner as an agarose gel. If fragment isolation was required, the polyacrylamide gel slice containing the DNA fragment of interest was cut out using a scalpel blade, chopped into small pieces, and transferred to a 1.5 ml

microcentrifuge tube. An equal volume of Crush Buffer (0.5 M CH_3COONH_4, 0.1% SDS, 10 mM $(CH_3COO)_2Mg$, 1 mM EDTA) was added, and incubated at 37°C in a shaking incubator overnight. The following day the buffer was pipetted into a fresh microcentrifuge tube, a further aliquot of Crush Buffer added to the gel fragments, and returned to a 37°C shaking incubator for 60 min. The pooled Crush Buffers were passed through a plug of sterile glass wool in a 1 ml syringe by spinning in a bench-top centrifuge at 5000 *g* for 2 min. The DNA was then recovered by ethanol precipitation, resuspended in a minimum volume of TE and the yield estimated by either electrophoresis of a sample on a polyacrylamide gel, or by optical density measurements.

2.6 Preparation and purification of DNA.

Plasmids were prepared from *E. coli* cells by a variety of different protocols. The method chosen was dependent on the amount and purity of the DNA required for subsequent *in vitro* manipulation. In general three sizes of bacterial cultures were employed: 1 ml ('mini-prep'), 5 ml ('midi-prep'), and 400 ml ('maxi-prep'). Plasmid DNA was extracted from the cells by the alkaline lysis method of Birnboim and Doly (1979), using the culture sizes and conditions recommended by Maniatis *et al.* (1982), except that large scale cultures ('maxi-preps') were grown in Super Broth medium and the DNA was subjected to two cycles of isopycnic centrifugation in caesium chloride and ethidium bromide gradients before use.

For a 5 ml overnight bacterial culture the following steps were performed to isolate plasmid DNA. The bacteria were pelleted by centrifugation and the supernatant removed by suction. The pellet was resuspended in 100 µl of ice-cold 25 mM Tris-HCl (pH 8.0), 50 mM sucrose, 10 mM EDTA, 0.1% (w/v) lysozyme, and incubated at room temperature for 5 min. The cells were then lysed by the addition of 200 µl of 0.2 M NaOH, 1% (w/v) SDS which was mixed by inversion, and incubated on ice for 5 min prior to the addition of 150 µl of ice-cold 3 M CH_3COONa (pH 4.8).

Incubation was continued on ice for a further 5 min. During this neutralisation step the chromosomal DNA denatures to form an insoluble precipitate together with the majority of the proteins and the high molecular weight RNA, whilst the covalently closed-circular plasmid DNA remains in solution. The plasmid DNA is removed as the supernatant after centrifugation at 10,000 *g* (12,000 rpm in an Eppendorf microfuge) for 5 min. Contaminating protein was removed by phenol extraction prior to ethanol precipitation of the DNA. The DNA pellet was resuspended in 20 µl of TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) containing 25 µg/ml Ribonuclease A (Sigma). These solutions were incubated for 30 min at 37°C before 200 µl of sterile water and 30 µl of 3 M CH_3COONa were added and the DNA was again extracted with equal volume of phenol/chloroform (25:24:1 phenol:chloroform:iso-amyl alcohol). The upper aqueous phase was removed and the DNA precipitated with three volumes of ethanol. Samples were incubated on dry-ice for 10 min before the DNA was pelleted by centrifugation at 10,000 *g* for 30 min. DNA pellets were subsequently washed with 70% ethanol before re-spinning at 10,000 *g* for 10 min. Pellets were then lyophilised and resuspended in TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). The DNA samples were analysed by agarose gel electrophoresis. Purified DNA was stored at 4°C.

Impurities were removed from DNA samples, e.g. after a restriction enzyme digest, in the following matter. The DNA solution was extracted with an equal volume of phenol, the upper aqueous phase was collected and extracted with phenol/chloroform and then with chloroform (24:1 chloroform:iso-amyl alcohol). A final extraction with an excess volume of ether was then carried out before the DNA was precipitated with three volumes of ethanol in the presence of 0.3 M CH_3COONH_4. The DNA was then collected by centrifugation, washed in 70% ethanol and dried. The DNA could then be dissolved in and appropriate volume of a suitable buffer.

Relaxed pBR322 DNA was prepared using chicken erythrocyte DNA topoisomerase I as described by Bates and Maxwell (1989). Supercoiled pBR322 (0.5 mg) was incubated in 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.25 mM EDTA, 5% (w/v) glycerol, 100 μg/ml BSA in a total volume of 0.5 ml. 3 μl topoisomerase I (~10^3 U/μl) were then added and the mixture incubated for 1 hr at 37°C. The extent of relaxation was assessed by agarose gel electrophoresis and the DNA purified on a caesium chloride/ethidium bromide gradient. Bacteriophage λ DNA (BRL) was extracted with an equal volume of phenol and chloroform and precipitated with ethanol prior to use.

Nicked pBR322 was prepared using DNAse I in the presence of ethidium bromide (Greenfield *et al.*, 1975). Due to topological constraints, supercoiled DNA can only untwist to a limited degree on ethidium binding and consequently has a limit on the amount of ethidium that can bind. Nicked DNA, on the other hand, has much more capacity to intercalate ethidium since there is much less of a constraint to untwisting. As the supercoiled starting material becomes nicked by the action of DNAse I, much more ethidium is able to intercalate into the DNA. The untwisting of DNA by ethidium bromide is thought to alter the conformation of the molecule to such an extent that the molecule is no longer a substrate for DNAse I. Consequently it is possible to produce a species containing a single nick in each DNA molecule. The following conditions were used: 200 μl of 1 mg/ml supercoiled pBR322, 1.8 ml 10 mg/ml ethidium bromide, 1.8 ml DNAse I buffer (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$) and 0.2 ml of 0.05 U/μl DNAse I (Boehringer). This mix was incubated at 20°C for 60 min. The reaction was stopped by chloroform extraction and the nicked DNA species was purified by banding on a CsCl/ethidium bromide gradient as described above.

A 172 bp DNA fragment, described as a preferred gyrase binding site in Sea Urchin 5S rRNA genes (Simpson *et al.*, 1985) was prepared from the plasmid p5S172-25.

The plasmid (2 mg) was digested with *HhaI* to liberate Fragment 1 (25 head-to-tail copies of the 172 bp DNA) which was purified from the smaller plasmid fragments by gel filtration through a 500 ml Sepharose 2B column (Pharmacia) equilibrated in TNE (10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA). Fragment 1 was then digested with *BstNI* to produce the 172 bp fragment. DNA fragments were 3'-labelled by incubation with [α-^{32}P]-dATP (3000 Ci/mmol; Amersham, UK) and Klenow fragment of DNA polymerase I (Boehringer) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT, 50 μg/ml BSA for 1 hr at 25°C. The labelled DNA was isolated by centrifugation through Sephadex G50, equilibrated in TE at 130 *g* for 2 min, followed by phenol extraction and ethanol precipitation. Alternatively the DNA was 5'-labelled using polynucleotide kinase (Boehringer) with [γ-^{32}P]-ATP (3000 Ci/mmol; Amersham, UK) as described by Maniatis *et al.* (1982). If the DNA was required to be uniquely labelled then one of the ends was removed from the main molecule by the action of the restriction enzyme *AatII* or *XmnI*, depending on which end was required to remain labelled. The sequence of the 172 bp fragments is shown below. The gyrase binding site, as determined by DNAse I footprinting studies, is indicated by the broken line (Rau *et al.*, 1987). The positions of various restriction enzymes are also indicated.

```
BstNI              AatII
AGGGATTTATAAGCCGATGACGTCATAACATCCCTGACCCTTTAAATAGCTTAACTTT
 CCCTAAATATTCGGCTACTGCAGTATTGTAGGGACTGGGAAATTTATCGAATTGAAA

                      Gyrase binding site
CATCAAGCAAGAGCCTACGACCATACCATGCTGAATATACCGGTTCTCGTCCGATCAC
GTAGTTCGTTCTCGGATGCTGGTATGGTACGACTTATATGGCCAAGAGCAGGCTAGTG

                                    AvaI              XmnI  BstNI
CGAAGTCAAGCAGCATAGGGCTCGAATTCCTCGGGGAATTCCAACGAATAACTTCC
GCTTCAGTTCGTCGTATCCCGAGCTTAAGGAGCCCCTTAAGGTTGCTTATTGAAGGT
```

A catenated DNA species, generated by reaction of the cre recombinase on plasmid pRH43-117 (Abremski and Hoess, 1985), was produced under the following

conditions: 100 μg of pRH43-117 was incubated with 60 μg of Cre protein (purified from cells containing plasmid pRK13, which contains the *cre* gene cloned under the control of the heat-inducible λP_L promoter, as described by Abremski and Hoess, (1984)) in 50 mM Tris-HCl (pH 7.5), 33 mM NaCl, 10 mM spermidine, 0.01 mg/ml BSA and in a total volume of 5 ml. The reaction mixture was incubated at 37°C for 1 hr, followed by addition of SDS to 0.2% and proteinase K to 0.1 mg/ml. The mixture was then incubated for another hour at 37°C. The DNA was isolated after phenol extraction and ethanol precipitation. The proportion of catenated material was assessed by agarose gel electrophoresis. Plasmid pRH43-117 contains a mutated *loxP* site that is still able to interact with the cre recombinase, but the catenated recombination products are unable to be resolved (Abremski and Hoess, 1985).

2.7 Isolation of M13 replicative-form DNA.

M13 is a filamentous, male-specific, *E. coli* bacteriophage. The replicative cycle of M13, following infection of the host cell, is biphasic. The infective single-stranded phage genome is copied to a duplex replicative-form (RF) which is amplified by bi-directional DNA replication to a level of 50-100 copies per cell. This is followed by a switch to rolling-circle synthesis which produces multiple concatemeric single-stranded copies of the viral genome which are cleaved and packaged in coat protein prior to export from the cell (Messing and Vieira, 1982). Replication of the phage DNA does not induce host lysis, instead the infected cells continue to grow at a reduced rate and extrude single-stranded progeny into the growth media.

The M13 vector series contain a portion of the *lac* operon of *E. coli* including the N-terminal 145 amino acids of the *lacZ* β-galactosidase gene. When such an M13 vector is inserted into a host that lacks a chromosomal β-galactosidase gene but carries a copy of the gene lacking amino acids 11-41 on the F episome, the two inactive polypeptides can reconstitute an active β-galactosidase enzyme. The active enzyme is IPTG inducible and acts on the chromogenic substrate X-Gal to produce the blue indoyl

derivative. Thus plaques on a lawn of male *E. coli* may be blue, indicating wild-type M13, or white, indicating the insertion of foreign DNA into the lacZ gene preventing successful intragenic complimentation.

M13 containing cultures were plated out onto 'M9' minimal plates as follows: The culture was mixed with 3 ml H-TOP agar warmed to 42°C and 200 µl of an overnight culture of TG2 cells grown in M9 media. The mixture was poured onto a pre-warmed M9 plate and allowed to set. The plate was then incubated at 37°C overnight. M13 turbid plaques could be noted in the lawn of TG2 cells.

A single M13 plaque was removed from a fresh transformation plate and transferred to 5 ml of sterile LB-media. 10 µl of an overnight culture of TG2 cells were added and the sample incubated at 37°C, with constant agitation, for 8 hr. The sample was transferred to four 1.5 ml tubes; the cells pelleted by centrifugation at 10,000 *g* for 5 min, and 4 ml of supernatant (containing phage particles) collected. The presence of phage particles was verified by precipitation of 0.5 ml of the supernatant with 25% polyethylene glycol (PEG) 6000, 2.5 M NaCl for 15 min at room temperature. If, after centrifugation at 10,000 *g* for 15 min, a pellet could be observed then this was used as an indication that phage particles were present in the supernatant. If sequencing of the DNA was required then the PEG pellet was resuspended in TE before precipitation with ethanol (see section 2.9 for the sequencing protocol).

To isolate the replicative form (RF) of M13 DNA, one litre of TG2 cells (2 x 500 ml LB-media in baffled flasks) were grown at 37°C to an optical density $A_{595\ nm}$ of 0.5 in an orbital shaker. 2 ml of the previously collected phage solution was added per flask and incubation continued for 90 min. Chloramphenicol was then added to a final concentration of 250 µg/ml and the flasks returned to the incubator for 8-10 hr to amplify the RF. The cells were then harvested and RF DNA isolated and purified by alkaline SDS lysis and CsCl-ethidium bromide gradient centrifugation as described

(section 2.6). The yield varied between 0.5 and 0.9 mg of purified RF DNA per litre of cells.

2.8 DNA modifying enzymes.

Restriction enzymes were purchased from New England Biolabs, Boehringer, Gibco BRL, Northumbrian Biotechnology, Pharmacia, and Sigma and were used according to the manufactures instructions. The DNA fragmentation pattern was observed by electrophoresis of a sample of the digest (approximately 300 ng of DNA) through a 1% agarose gel.

T4 DNA ligase (BRL) was used for the ligation of both blunt- and sticky-ended DNA fragments. The following conditions were used: 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) PEG 8000. DNA fragments were incubated at a molar ratio of insert:vector of 3:1, and in a total volume of 20 µl. One unit of ligase was added, and the mixture was either allowed to stand at room temperature for 2 hr (cohesive or 'sticky' end ligation), or placed at 15°C overnight (flush or 'blunt' end ligation). *E. coli* DNA ligase (NEB) was also used in cases where ATP had to be omitted from the reaction mixture. The ligation in this case was performed under the following conditions: 30 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 26 µM NAD, 50 µg/ml BSA. The total reaction volume was 30 µl, and 2 units of *E. coli* ligase were added per µg of DNA.

The large Klenow fragment of DNA polymerase I (BRL) can be used for 'filling-in' staggered DNA ends as well as in 'nick-translation' (Maniatis *et al.*, 1982). In either case the enzyme was used under the following conditions: 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DTT, 50 µg/ml BSA and an appropriate mixture of some or all the dNTPs at 0.1 mM. Reactions were incubated at room temperature for 30 min. The DNA was subsequently extracted with phenol and precipitated with ethanol.

T4 polynucleotide kinase (PNK; BRL) was used to add terminal phosphates to DNA. This includes the phosphorylation of single-stranded oligonucleotides and 5' end-labelling of DNA fragments. Conditions for 'blunt-' or 'sticky-ended' phosphate addition were different. In both cases the overall reaction conditions were 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 50 pmol ATP, 5% glycerol in a total volume of 50 µl. However, for blunt-ended DNA the Tris was buffered to pH 9.2, to assist fraying of the DNA ends, whereas pH 7.6 was used for sticky-ended DNA. Dried 'blunt-ended' DNA pellets were resuspended in 40 µl of 20 mM Tris-HCl (pH 9.5), 1 mM spermidine, 0.1 mM EDTA and heated to 70°C and chilled rapidly on ice before using PNK. 50 pmol of DNA were incubated with 20 Units of PNK for 1 hr at 37°C. The DNA was then extracted with phenol and precipitated with ethanol. Unincorporated ATP could be removed from the DNA by centrifugation through a 1 ml Sephadex G-50 column equilibrated in TE.

Calf intestinal phosphatase (CIP; Boehringer) was used to remove terminal phosphate residues from DNA fragments, generally prior to a ligation so that, for instance, a linearised vector could not religate back on itself without the presence of phosphorylated insert. The reaction conditions used were 50 mM Tris-HCl (pH 8.8), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine. In a total reaction volume of 20 µl, 0.1 Units of CIP were added per µg of DNA. The mixture was incubated at 37°C for 15 min, and then at 56°C for 15 min after which a second aliquot of CIP (0.1 U/µg) was added and the two incubations repeated. 20 µl H_2O, 5 µl 10 x TNE (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA) and 2.5 µl 10% SDS were then added and the mixture incubated at 70°C for 15 min to inactivate the CIP. The DNA was then phenol extracted and precipitated with ethanol (see section 2.6).

Bal31 nuclease, originally isolated from *Alteromonas espejiana* Bal 31, was used as an exonuclease to degrade DNA from both the 3' and 5' ends at a specific rate. The enzyme also has a highly specific single-strand endodeoxyribonuclease activity (Perbal,

1988). Bal31 reactions (240 µl) contained the following: 9 nmol linear DNA, 20 mM Tris-HCl (pH 8.1), 12 mM $MgCl_2$, 12 mM $CaCl_2$, 60 mM NaCl, 1 mM EDTA, 0.5U Bal31 nuclease (Boehringer). Incubations were carried out at 30°C and samples removed at specified times and the DNA extracted with phenol and precipitated with ethanol.

Mung bean nuclease is a single-stranded specific DNA endonuclease isolated from mung bean sprouts. The enzyme (obtained from Pharmacia, FPLC pure) was utilised to blunt over-hanging DNA ends after restriction endonuclease digestion. Mung bean nuclease was stored and diluted in 10 mM CH_3COONa (pH 5.0), 100 mM $ZnCl_2$, 1 mM cysteine, 50% (w/v) glycerol, 0.001% triton X-100. Reactions (50 µl) contained the following: 50 mM CH_3COONa, 30 mM NaCl, 1 mM $ZnCl_2$, 0.5 Units of nuclease per µg of DNA (8 kb in size). Reactions were incubated at 30°C for 30 min and terminated by phenol extraction of the DNA.

2.9 DNA sequencing.

Sequencing of DNA was mainly carried out using the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977) using the enzyme Sequenase version 2.0 (United States Biochemical Corporation). Sequenase is an *in vitro* genetically modified form of T7 DNA polymerase that completely lacks a 3'-5' exonuclease activity (Tabor and Richardson, 1987). Sequenase was used according to the manufactures instructions with DNA being prepared using the procedures recommended by Kraft *et al.* (1988). In this way M13 single-stranded DNA and double-stranded plasmid DNA could be sequenced. $\alpha[^{35}S]$-dATP (650 Ci/mmol; Amersham, UK) was used for all sequencing reactions. Sequencing reactions were run out on 6% polyacrylamide-urea gels, which were subsequently subjected to autoradiographic analysis (Maniatis *et al.*, 1982) using Fuji RX-100 X-ray film. Maxam and Gilbert chemical sequencing of $[^{32}P]$ 3'- or 5' end-labelled DNA fragments was carried out as described by Maniatis *et al.* (1982).

2.10 Oligonucleotide-directed mutagenesis.

Appropriate DNA fragments were cloned into M13 and the single-stranded form was isolated by PEG precipitation of the culture supernatant as described in section 2.7. An oligonucleotide (17-20 mer) was made on an Applied Biosystems 380B oligonucleotide synthesiser utilising phosphoramidite chemistry with controlled-pore glass supports, following standard Applied Biosystems protocols. The oligonucleotide was phosphorylated using T4 polynucleotide kinase (New England Biolabs) and 20 pmol was annealed to the single-stranded M13 DNA (2 μg) by warming to 80°C and allowing to cool slowly. Chain extension was performed under the following conditions for 12 hr at 4°C: 10 mM Tris-HCl (pH 8.1), 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM DTT, 0.5 mM dNTPs, 2.5 U/μl T4 ligase (Boehringer), 3 U/μl Klenow fragment of DNA polymerase I (Boehringer) in a total reaction volume of 20 μl. The reaction was stopped by the addition of 0.2 ml of 10 mM EDTA, 10 mM Tris-HCl (pH 8.1); 100 μl of the mix used to transform competent HB2154 ($mutL^-$) cells. M13 plaques were selected as described in section 2.7.

Colony hybridization was performed using a variation of the method described by Grunstein and Wallis (1979). 50 plaques were replica plated onto 2xYT plates and grown up overnight at 37°C. A nitrocellulose filter (Sartorious) was placed on one of the plates and left for 1 min at room temperature. The filter was removed and placed on Whatman 3MM paper, which had been soaked in 0.5 M NaOH, 1.5 M NaCl, for 5 min. The filter was then neutralised on 3MM paper which had been soaked in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, for 3 min before being allowed to dry and baked at 80°C for 1 hr.

The probe oligonucleotide was labelled with [γ^{32}P]-ATP (Amersham; 3000 Ci/mmol, 10 μCi/μl) in the following reaction for 30 min at 37°C: 10 μl [γ^{32}P]-ATP, 1.5 μl oligonucleotide (10 pmol/μl), 3 μl 10 x kinase buffer (0.5 M Tris-HCl (pH 8.1), 0.1

M $MgCl_2$), 1 µl 100 mM DTT, 25 µl H_2O, 0.5 µl polynucleotide kinase (0.5 U/µl). The reaction was then heated to 70°C for 10 min, and subsequently diluted to 4 ml with 1.2 ml 20x SSC (SSC is 0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7.2H_2O$ (pH 7.0)), 0.4 ml 100 x Denhardts solution (20 g/L BSA, 20 g/L Ficoll, 20 g/L polyvinyl pyrolidone) and 2.4 ml water. The filter was pre-washed in 100 ml 6x SSC (a 3 in 10 dilution of 20x SSC) and the colonies scrubbed off with a tissue. The filter was then placed in a tank (at 75°C) containing 100 ml prehybridisation solution (6x SSC, 10x Denhardts solution, 0.2% SDS) for 5 min. After washing the filter with 100 ml 6x SSC, which had been warmed to 65°C, the prehybridisation solution was replaced with the labelled probe for 10 min at 65°C. The tank was then allowed to cool to room temperature, with gentle agitation to allow even hybridization of the probe.

The melting temperature (Tm) of the oligonucleotide and the wild-type sequence was calculated according to the following formula (Wallace *et al.*, 1979)

$$Tm\ (°C) = 2(A+T) + 4(G+C).$$

The filter was then washed with successive 100 ml aliquots of 6x SSC pre-incubated at a temperature mid-way between the two melting temperatures. The principle behind this technique is that the labelled probe will preferentially remain annealed to the mutated sequence (Tm above the incubation temperature), but not to the wild-type sequence (Tm below the incubation temperature). The washes were monitored and continued until approximately half the counts had been removed from the filter. The filter was then sealed in a plastic bag and subjected to autoradiography. Any colonies that appeared darker than others (i.e. more counts detected than with other colonies) were picked onto minimal plates and covered with 3 ml H-TOP agar containing 200 µl of a fresh TG2 overnight culture, and incubated at 37°C overnight. M13 RF DNA was then prepared as described in section 2.7, and the presence, or absence, of the mutation confirmed by restriction enzyme digestion and DNA sequencing.

2.11 Preparation of DNA gyrase.

The A and B subunits of *E. coli* DNA gyrase were prepared as described by Hallett *et al.* (1990), based on the procedures of Mizuuchi *et al.* (1984), except that the cells were disrupted using a French press. The gyrase A and B subunits, and fragments of the A protein were all prepared in a similar fashion:

A 4 ml culture of *E. coli* cells (generally strain JM109) containing a plasmid encoding the protein of interest was grown up overnight at 37°C and then used to inoculate 1 L of 2xYT media, containing 100 μg/ml ampicillin, in a baffled flask. The culture was grown at 37°C until $A_{595\ nm}$ of 0.5 was obtained (approximately 3-4 hr). IPTG was then added to a final concentration of 50 μM and growth continued for a further 4 hr. The cells were then harvested by centrifugation at 13,500 *g* in a Beckman JA-10 rotor. The cell pellets were resuspended in a minimum volume (1 ml per g of cell pellet) of 50 mM Tris-HCl (pH 7.5), 10% sucrose and then frozen in liquid nitrogen and stored at -70°C.

An aliquot of cells were thawed the following were added; DTT to 2 mM, EDTA to 20 mM, and KCl to 100 mM. The cells were then disrupted using a French press at a pressure of 8,000-12,000 psi, or alternatively the sample was sonicated for 5 min (30 sec on and 30 sec off over a period of 10 min). Cell debris was removed by centrifugation at 130,000 *g* in a Beckman Ti50 rotor at 3°C for 1 hr. The supernatant was then diluted to a protein concentration of 20 mg/ml and 0.31 g of powdered solid ammonium sulphate were added per g of liquid. This mixture was then stirred on ice for 15 min. The ammonium sulphate pellet was collected by centrifugation at 10,000 *g* for 15 min at 3°C. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 5 mM DTT. The protein sample was then frozen in liquid nitrogen and stored at -70°C.

The protein sample was then dialysed for 4 hr against TGED (50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.2 mM EDTA, 5 mM DTT), before loading onto a Heparin-Sepharose column (Pharmacia) that was equilibrated in TGED + 25 mM NaCl. Protein was eluted from the column with a linear gradient of 25 mM to 0.4 M NaCl in TGED. Peak protein fractions were pooled and loaded directly onto an FPLC Mono Q 5/5 column (Pharmacia) equilibrated in TED (50 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5 mM DTT) in 5 mg aliquots. Protein was eluted from the column with a linear gradient of 0-0.6 M NaCl in TED. Peak fractions were pooled and dialysed into Enzyme Buffer (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM DTT, 1 mM EDTA, 10% (w/v) glycerol) overnight, frozen in liquid nitrogen and stored at -70°C. At this stage the proteins were estimated to be >95% pure as adjudged by SDS-polyacrylamide gel electrophoresis.

If further protein purification was required then ammonium sulphate was added to the pooled sample to a final concentration of 1.0 M. The sample was then loaded onto an FPLC Phenyl Sepharose 5/5 column (Pharmacia) in 5 mg aliquots. The column was equilibrated in TED + 1 M ammonium sulphate, and protein was eluted with a linear gradient of 1.0-0 M ammonium sulphate in TED. The final stage of the purification process involved the use of two FPLC Superose 12 columns run in series with each other. The columns were equilibrated in TED + 100 mM NaCl. Protein, no more than 5 mg per run, was applied to the columns in a small volume (250 µl). Peak fractions were pooled, dialysed into Enzyme Buffer overnight, frozen in liquid nitrogen and stored at -70°C.

The purified proteins were extensively dialysed against Enzyme Buffer prior to use. Protein concentrations were estimated by the method of Bradford (1976), using BSA as a standard. An appropriate volume of the protein solution was diluted to 100 µl with Enzyme Buffer and 1 ml of Bradford reagent was added (prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (Serva) in 50 ml 95% ethanol; to this was added

100 ml 85% phosphoric acid and made up to a final volume of 1 L with distilled water. The resulting solution was filtered through Whatman No 1 filter paper before use). The optical density at 595 nm was determined after 10 min at room temperature. The concentrations of the gyrase A and B proteins, and the proteolytic fragments of the A protein are expressed here as molarities of dimer.

2.12 SDS-polyacrylamide gel electrophoresis.

Proteins were analysed on 12.5% discontinuous polyacrylamide gels containing 0.1% SDS with a 4% polyacrylamide stacking gel, which were run using a Mini Protean II gel system (BioRad) according to the methods described by Hames (1981). The stacking gel (pH 6.8) was layered over the separating gel (pH 8.8) and were made up according to the following table:

	12% Separating gel	**4% Stacking gel**
30% Acrylamide		
(37.5:1 Acrylamide: Bis)	4.0 ml	1.3 ml
1 M Tris-HCl (pH 8.8).	3.75 ml	-
1 M Tris-HCl (pH 6.8).	-	1.25 ml
10% SDS.	0.1 ml	0.1 ml
10% Ammonium persulphate.	0.1 ml	0.1 ml
Water.	2.1 ml	7.35 ml
TEMED	10 µl	10 µl

Protein samples were prepared by adding an equal volume of sample application buffer (SAB is 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue) and placing in a boiling water bath for 4 min. Gels were run at 200V for 45 min; stained in 30% (v/v) methanol, 0.01% (w/v) Coomassie blue R (Sigma), 12% (w/v) trichloroacetic acid, 10% (w/v) sulfosalacylic acid (Sigma) for 30 min; and destained in 7.5% acetic acid, 5% ethanol. Alternatively,

gels were stained with silver using the following protocol: Gels were washed for 30 min in 250 ml 40% (v/v) methanol, 5% (v/v) glacial acetic acid, and then washed in three 250 ml changes of water for 15 min each. The gel was then transferred to 125 ml of 5 mM $AgNO_3$, 0.13% (w/v) NaOH, 0.025% (v/v) aqueous ammonia and agitated for 15 min before another 125 ml aliquot was added. The gel was then washed in three changes of water (for 15 min each) before the protein bands were developed using 250 ml aliquots of $5x10^{-3}$% citric acid, 0.02% (v/v) formaldehyde. After staining the gel was fixed in 40% (v/v) methanol, 5% (v/v) glacial acetic acid. Protein gels were photographed using Kodak T_{MAX} 100 (4" x 5") professional film.

2.13 Enzyme assays.

DNA supercoiling assays were carried out essentially as described by Mizuuchi *et al.* (1984). The reaction volumes were reduced from 70 µl to 30 µl and contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 0.36 mg/ml BSA, 9 µg/ml tRNA, 6.5% glycerol, 5 mM DTT, 1.4 mM ATP, 10 µg/ml relaxed pBR322 DNA and appropriate amounts of the gyrase A and B proteins. One unit of supercoiling activity is defined here as the amount of enzyme required to convert one half of the relaxed DNA to the supercoiled form in 1 hr at 25°C adjudged by electrophoresis in 1% agarose gels (see section 2.5). To assay DNA relaxation, conditions were the same as in supercoiling assays except 10 µg/ml supercoiled pBR322 replaced relaxed DNA, and ATP and spermidine were omitted. Decatenation assays were performed under the same conditions as supercoiling assays, but using the recombination-generated DNA catenanes of plasmid pRH43-117 as a substrate. As appropriate, 5'-adenylyl-β,γ-imidodiphosphate (ADPNP; Sigma) was included in place of ATP. The conversion of catenated DNA to monomeric plasmid was monitored by agarose gel electrophoresis as described above. Quinolone-induced cleavage of DNA by gyrase was carried out under the conditions described for supercoiling except that ciprofloxacin (CFX; gift of Bayer, W. Germany) was included at 3 µg/ml and ATP was omitted. Incubations were for 1 hr at 25°C and were terminated by the addition of

3 µl of 2% SDS and 3 µl of 1 mg/ml proteinase K (Sigma). Samples were further incubated for 30 min at 37°C then subjected to agarose gel electrophoresis as described above. For calcium-directed cleavage, CFX was omitted and 2 mM $CaCl_2$ replaced 4 mM $MgCl_2$.

2.14 Proteolytic cleavage of the gyrase proteins.

Samples of the gyrase proteins (approximately 1.0 mg/ml) were incubated with an appropriate protease (trypsin, chymotrypsin, and papain were all obtained from Sigma) in 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 5 mM DTT, 2 mM $MgCl_2$, 10% (w/v) glycerol for various times at 25°C. The protease was inactivated by adding an equal volume of SAB (see section 2.12) and placing in a boiling water bath for 4 min, and the samples were analysed by SDS-polyacrylamide gel electrophoresis. After staining and destaining, the gels were scanned using an LKB Ultrascan densitometer to determine the proportions of intact and cleaved protein. For larger scale preparations using trypsin, the protease was removed from the gyrase proteins by passing the reaction mixture through a 0.2 ml trypsin-inhibitor agarose column (Sigma) equilibrated in Enzyme Buffer. Fractions containing tryptic fragments were pooled and dialysed against Enzyme Buffer.

In order to determine the N-terminal sequence of the proteolytic fragments, a 50 µg sample of the protease-treated protein was subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were electroblotted onto polyvinylidene difluoride membranes (Matsudaira, 1987) using a Transphor semi-dry electroblotting apparatus (LKB). The sequence of the N-terminal amino acids of the peptides were determined using an Applied Biosystems 470A automated protein sequencer. This machine carries out automated cycles of the Edman degradation of the protein and analyses the products after each cycle.

Proteolytic fragments were isolated from SDS-polyacrylamide gels by the method of Hager and Burgess (1980). Protease-treated protein (20 μg per track) was applied to a 12.5% polyacrylamide SDS gel. After electrophoresis, the gel was stained for 5 min in 0.25 M KCl, 1 mM DTT and destained for 10 min in 1 mM DTT. Protein-containing gel slices were excised and 1 ml of 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.15 M NaCl, 5 mM DTT was added. The gel was then crushed and left for 2 hr. The resulting soluble protein was precipitated with four volumes of acetone and the pellet resuspended in 20 μl Enzyme Buffer containing 6 M guanidine hydrochloride. The sample was left at room temperature for 20 min and was then diluted to 1 ml with Enzyme Buffer and left at 4°C overnight to allow renaturation to occur. Samples were then dialysed extensively against Enzyme Buffer prior to use.

In order to investigate the effects of denaturing and refolding on proteins that had been gel purified, some of the fragments of the GyrA protein that were produced genetically were also completely denatured. The purified protein (at ~1 mg/ml) was precipitated with either 4 volumes of acetone, or ammonium sulphate to a final concentration of 50%. The protein was collected by centrifugation (10 min at 10,000 *g* in a microfuge) and resuspended in 20 μl of Enzyme Buffer containing 6 M guanidine hydrochloride. The sample was left at room temperature for 20 min and was then diluted to 1 ml with Enzyme Buffer and left at 4°C overnight to allow renaturation to occur. Samples were then dialysed extensively against Enzyme Buffer prior to use. Alternatively a sample of the protein was dialysed extensively (for at least 18 hr) against Enzyme Buffer containing 6 M guanidine hydrochloride, before extensive dialysis back into Enzyme Buffer. The supercoiling and cleavage activities of the proteins before and after this treatment were measured.

2.15 DNAse I footprinting.

Specific DNA-protein interactions can be revealed by the action of nucleases. Some DNA-binding proteins will, under certain conditions, bind to specific sequences of

DNA and protect them from nuclease digestion. Footprinting of the gyrase-DNA complex was carried out essentially as described by Galas and Schmitz (1978). Freshly labelled 172 bp DNA (prepared as described in section 2.6) was incubated, in the absence or presence of gyrase, under standard supercoiling assay conditions for 30 min at 25°C (section 2.13), except that ATP and pBR322 DNA were omitted. DNAse I (Boehringer; 23 U/µl) was then added to a final concentration of 0.002 U/µl. Incubation was continued for a further 10 min at 25°C. Reactions were terminated by the addition of 30 µl of 0.6 M CH_3COONH_4, 10 mM EGTA, 50 mg/ml sonicated λ DNA, 0.4% SDS. After ethanol precipitation and at least four 70% ethanol washes, the DNA pellet was resuspended in 4 µl formamide dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated to 80°C for 3 min and then loaded onto an 8% polyacrylamide-urea sequencing gel (19:1 acrylamide:bis). After running, the gel was washed in 10% CH_3COOH for 15 min to remove the urea. The gel was then dried under partial vacuum, and subjected to autoradiography using Fuji RX-100 X-ray film.

2.16 Nitrocellulose filter binding.

Protein-DNA complexes can be detected by the way in which such complexes adhere to nitrocellulose filters. Radio-labelled DNA will usually pass through such filters, but when protein is bound to the DNA the complex becomes trapped. Protein-DNA complexes can be detected by subjecting the filters to scintillation counting. Nitrocellulose filter binding has been used to quantify the gyrase-DNA complex (Higgins and Cozzarelli, 1982; Maxwell and Gellert, 1984).

Filter binding was performed using a 25 mm Millipore glass filtration apparatus and 25 mm diameter, 0.45 µm pore nitrocellulose filters (Schleicher and Schuell). Prior to use, filters were soaked for at least 1 hr in binding buffer (35 mM Tris-HCl (pH 7.5), 24 mM KCl, 6 mM $MgCl_2$, 6.5% (w/v) glycerol, 5 mM DTT). Reaction mixtures (30 µl) were as described for the supercoiling assay (section 2.13), except that labelled

172 bp DNA (either 5' or 3' radiolabelled, prepared as described in section 2.6) replaced pBR322, and ATP was omitted. Reaction mixtures were incubated for 1 hr at 25°C, and then 0.2 ml of binding buffer (at 25°C) was added to the sample and mixed. A 0.2 ml aliquot was immediately filtered at a flow rate of 1 ml/min through a filter previously washed in 0.5 ml binding buffer; the filter was subsequently washed twice with 0.5 ml of binding buffer. Filters were dried under heat lamps and the amount of DNA retained on each was determined by scintillation counting. This procedure was found to yield low levels (< 2%) of non-specific retention of DNA.

2.17 Detection of wrapping of DNA around gyrase.

When gyrase binds to a DNA molecule it has been shown that the DNA is wrapped around the outside of the enzyme with a positive superhelical sense (Liu and Wang, 1978b). In order to compare the wrapping DNA around of proteolytic fragments of gyrase to that observed with the intact molecule, the protein was allowed to bind to a nicked-circular DNA species and then the DNA was ligated and the protein removed to detect any changes in the topology of the resulting closed-circular plasmid.

0.8 µg of purified nicked-circular pBR322 (see section 2.6) was incubated with gyrase under the following conditions in a final volume of 30 µl: 50 mM Tris-HCl (pH 7.5), 55 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, 5% (w/v) glycerol, 18 µg/ml tRNA at 25°C for 15 min. Then 2 µl of 3 U/µl *E. coli* DNA ligase (BRL) and 1 µl of 1 mM NAD were added. Incubations were continued for 20 min at 25°C, before the DNA was extracted with chloroform and subjected to agarose gel electrophoresis. The extent of DNA wrapping was estimated by counting the change in linking number observed and expressed as the number of turns introduced per gyrase tetramer present.

2.18 Gel retardation assays.

The binding of protein to specific DNA sequences can be followed using gel electrophoresis (Garner and Revzin, 1986). Often a DNA-protein complex is stable

when electrophoresed through a polyacrylamide gel and the complex has a different mobility to that of the free DNA. The binding of gyrase to the 172 bp DNA fragment was performed essentially as described by Maxwell and Gellert (1984). Gyrase-DNA complexes were electrophoresed through 5% polyacrylamide gels (acrylamide:bis; 29:1) in 90 mM Tris-borate (pH 7.0), 10 mM $MgCl_2$. The gels were stained in 2 μg/ml ethidium bromide and viewed on a UV transilluminator.

2.19 ATPase assays.

The ATPase activity of DNA gyrase is thought to reside in the B subunit, but full ATPase activity is only observed in the presence of the A subunit and DNA (Maxwell and Gellert, 1984). Crude [γ-^{32}P]-ATP (specific activity >7000 Ci/mmol) was purchased form ICN and repurified by chromatography on a 0.5 ml Whatman DEAE 23-cellulose column (Chaykin *et al.*, 1965) and stored at -20°C. ATPase reaction mixtures (30 μl) contained the standard supercoiling conditions (see section 2.13), and also 10 mM potassium phosphate, [γ-^{32}P]-ATP (20 nCi) and 0.1 μM [^{3}H] glycine (40 nCi). Reactions were initiated by the addition of the B protein and incubated for 2 hr at 25°C. Reactions were terminated by the addition of 0.2 ml of ice-cold water and 0.8 ml of an ice-cold 20% (v/v) suspension of activated charcoal (HCl-washed Norit A, Sigma) in water and left on ice with occasional shaking for 10 min. Tubes were then spun in a microfuge for 10 min. A 0.5 ml sample of the supernatant was taken and the amount of ^{32}P-labelled phosphate determined by scintillation counting. [^{3}H]-glycine, present in all samples, was also counted and was used to normalise data and to compensate for pipetting errors. Results are expressed as the ratio of ^{32}P/^{3}H counts and were converted to per cent of ATP hydrolysed by comparison with the ^{32}P/^{3}H ratio in the total reaction, and are corrected any for novobiocin-insensitive and gyrase alone ATPase.

The phosphate release from the ATPase activity of the gyrase complex was also estimated using a malachite green assay (based on that described by Itaya and Ui

(1966)). ATPase reaction mixtures (30 µl) consisted of the standard supercoiling conditions (see section 2.13). The reactions were incubated for 6 hr and 1/2, 1/4, 1/8 and 1/16 dilutions of each sample were made in a microtitre plate using 30 µl of water for each dilution. 125 µl of malachite green reagent (1 volume of 4.2% ammonium molybdate/5 M HCl plus 3 volumes of 0.05% malachite green solution, incubated for 30 min and filtered) were added to each sample. The mixture was incubated for 30 min at 2°C before 25 µl of 0.5% Tween 20 (polyoxyethylene sorbitan monolaurate) were added and the samples incubated for a further 15 min at room temperature. The optical density at 640 nm for each sample was measured and compared to a standard curve prepared using known concentrations of sodium phosphate. Data were corrected for any novobiocin-insensitive phosphate release from the samples, but in most cases this was negligible.

2.20 Isoelectric focusing and western immunoblotting.

Samples of the gyrase A protein and various fragments (5 µg) were subjected to isoelectric focusing (IEF) by the method of O'Farrell (1975), using the conditions described by Duncan and Hershey (1985). Ampholines (pH range 3.5-10) were purchased from BDH. The rod gels were made up containing 6 M urea, 6% polyacrylamide, 3% nonidet, and 20% ampholines. The running buffers consisted of 50 mM NaOH (top) and 25 mM phosphoric acid (bottom). Samples were denatured by adding an equal volume of 9 M urea, mixing and leaving to stand for 15 min. Samples were then loaded onto the top of the rods and electrophoresed overnight as described (Duncan and Hershey, 1985). After running, the gels were either stained directly for protein (section 2.12) or equilibrated in 10% glycerol, 10 mM Tris-HCl (pH 7.5), 2% SDS, 5% DTT, 0.001% bromophenol blue, prior to SDS-slab gel electrophoresis. The IEF rods could be placed directly onto the top of a 1.5 mm wide SDS-PAGE gel, and electrophoresed as described in section 2.12. The markers used for IEF (obtained from Sigma) were lactate dehydrogenase (pI 8.55, 8.4, 8.3), β-lactoglobin (5.1), carbonic anhydrase (6.6) and soybean trypsin inhibitor (4.6).

Proteins were transferred from SDS-PAGE gels onto nitrocellulose membranes (Hybond-C, Amersham) using a Transphor semi-dry electroblotting apparatus (LKB) using the conditions suggested by Harlow and Lane (1988). The membranes were stained for protein using ponceau S (0.2% ponceau S (Sigma) in 3% trichloroacetic acid) for 5 min. The blot was photographed and the stain removed with a small volume of PBS (phosphate-buffered saline; 10 mM phosphate buffer (pH 7.3), 0.15 M NaCl), after which the membrane was dried and stored flat. Non-specific antibody binding sites on the blot were blocked by incubating the membrane sealed in a bag for 2 hr at 37°C in 5 ml of PBS containing 3% (w/v) nonfat dry milk (Marvel), 0.1% (v/v) Tween 20. The primary monoclonal antibody was then added at a dilution of 1/100 - 1/1000. The membrane was incubated in primary antibody at room temperature for 16 hr with gentle agitation.

The membrane was then transferred to a suitably sized tray and washed in 3 changes of PBS for 5 min each. The blot was then sealed in a fresh bag containing 2 ml of 3% nonfat dry milk, 0.1% Tween 20 in PBS containing a 1/1000 dilution of horseradish peroxidase conjugated secondary antibody. Incubation was continued for 1 hr at room temperature. The blot was then removed from the bag and washed in 3 changes of TBS (10 mM Tris-HCl (pH 7.3), 0.15 M NaCl). Detection of the bound secondary antibody was carried out in the following manner: 8 mg of 4-chloro-1-naphthol (Sigma) were dissolved in 2 ml methanol and added to 8 ml TBS. Half of this mixture was used to wash the blot for 10 min. To the remaining half were added 1.7 µl 30% H_2O_2. This was then washed over the blot until colour developed (generally 5-10 min). The H_2O_2 was then washed off with TBS and the dried blot stored flat wrapped in tin foil.

Primary antibodies used in this study are shown below. The purity of each of the antibodies was estimated by SDS-PAGE (A. Maxwell, personal communication).

	Name	Antibody Type	[Protein] (mg/ml)	Estimated purity (%)
Anti-*E. coli* GyrA protein;				
Mouse monoclonals:	4D3	IgG	2	20
	4F6	IgG	1	5
	6D9	IgM	4	50
	7F3	IgG	3	5
	7F11	IgG	1	5
	10C6	IgG	1	5

The secondary antibody used was goat-anti mouse IgG (H+L) horseradish peroxidase conjugate (obtained from BioRad).

2.21 Scanning microcalorimetry.

Scanning microcalorimetry is a technique that can be used to investigate the domain structure of protein samples. When a protein is heated up its tertiary structure will unfold. Energy is required to disrupt the non-covalent interactions that hold the protein in its native conformation. A microcalorimeter can be used to detect this energy (heat) absorption and consequently the point at which the protein unfolds. Therefore if a protein contains two, or more, domains and these unfold at different temperatures, then a calorimetric profile can be obtained that maps the different unfolding temperatures.

Samples of protein (2 ml of 1 mg/ml) were dialysed into 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA overnight at 4°C with several changes of dialysis buffer. A sample of the dialysis buffer was retained to use as a blank reference for the microcalorimeter. 1.5 ml of the dialysed protein sample was placed in the scanning microcalorimeter (MicroCal MC-2) and heated from 15°C to 100°C at a rate of 90° per hour. The difference in temperature between the protein sample and the buffer blank

was used to plot the unfolding profile of the protein. Transitions were separated into their component parts using the deconvolution software provided by MicroCal. The software package does not assume that there is a certain number of transitions, but uses as many peaks as are necessary to attempt to best-fit the experimentally obtained data

2.22 Crystallization of fragments of the GyrA protein.

Protein crystals can be grown by a variety of different methods (reviewed by Eisenberg and Hill, 1989). However the general principles involved in each of the techniques are essentially the same. The strategy used to induce crystallisation of macromolecules is to bring the system very slowly toward a state of minimum solubility and thus achieve a limited degree of supersaturation. At the same time, the component variables must be initially set to ensure that the macromolecules will have an opportunity to take advantage of the greatest number of favourable interactions with its neighbours. During this process the protein will tend to come out of solution either as an disorganised precipitate or as a highly ordered crystal. The conditions under which crystal growth is promoted appears to be different for nearly every protein (McPherson, 1982), and consequently many different conditions must be tried in order to get optimum crystal growth. A fundamental tenet of crystallization appears to be absolute homogeneity of the macromolecule. It is therefore necessary to ensure that the protein sample is purified as much as possible so only a single species is present in solution and specific interactions are not hindered by other species.

Samples of fragments of the DNA gyrase A protein (~10 mg/ml; concentrated in an Amicon ultrafiltration device using an Amicon diaflow PM30 membrane) were laid down for crystal growth using the hanging drop method (Eisenberg and Hill, 1989). This technique allows a drop of protein solution and a reservoir to equilibrate by vapour diffusion. The concentration of the precipitant and protein increase in the drop and crystallisation can occur. This method has the advantage that relatively small

quantities of protein are required (5 mg is sufficient for about 100 drops, each under different conditions) and different conditions can be surveyed rapidly. The precipitant solution (ammonium sulphate, polyethylene glycol, MPD etc) at various pH values (termed the mother liquor) was placed in the bottom of a 1.7 x 1.6 cm 24 well tissue culture plate (Linbro, Flow laboratories). Protein solution (10 μl) was mixed with an equal volume of the mother liquor and placed on a plastic cover-slip (Bel-Art) and inverted over the well. A air-tight seal between the well and the cover-slip was achieved using high vacuum grease. The culture plates were then left at various temperatures in a place where they would not be disturbed. Occasionally the plates would be observed using a binocular microscope (Wild M3C; Herrbrugg, Switzerland) and the presence of any precipitate or crystals noted. Photographs were taken using an Olympus OM-1 camera with Ilford PanF film.

Chapter 3

Proteolytic cleavage of the DNA gyrase A protein.

3.1 Introduction.

A structural and/or functional domain of a protein can be defined as a relatively compact structure that, by allowing limited access, is resistant to proteolytic cleavage; whereas domains may be connected by regions of protein that are more susceptible to attack. Thus it is theoretically possible, under the appropriate conditions, to separate the native domains of a particular protein in order to study them on their own and assess any activity that they may possess. This protease digestion approach has been used successfully to probe the domain structure in a variety of proteins, e.g. the muscle protein myosin, some immunoglobulins, and a number of DNA-binding proteins.

Treatment of myosin (540 kDa) with trypsin leads to the formation of two distinct fragments; termed light and heavy meromyosin. Light meromyosin, like intact myosin has the ability to spontaneously form filaments, but it lacks an ATPase activity and is unable to interact with actin unlike the intact molecule. Heavy meromyosin, on the other hand, is unable to form filaments, but is capable of interacting with actin and has an ATPase activity associated with it. Heavy meromyosin can be further fragmented by the action of the papain protease into two separate S1 ATPase sites (Alberts *et al.*, 1983).

Immunoglobulin G (molecular mass 150 kDa) can be split into three approximately equally sized 50 kDa fragments by the action of papain.

$$IgG \xrightarrow{\text{Papain}} 2\,F_{ab} + F_c$$

It has been found that the two F_{ab} fragments still retain the ability to bind antigen whilst the F_c fragment is thought to be involved in the trigger of the antigen-antibody complement cascade reaction that lead to the lysis of a foreign cell within the body.

Hence low resolution structural and functional information about these very large molecules has been obtained by fragmentation of the protein and subsequent testing the activities of each fragment.

There have been a number of reported cases where the domain structure of a DNA-binding protein has been partially elucidated by generating proteolytic fragments; for example the λ repressor (Pabo *et al.*, 1979), γδ resolvase (Abdel-Meguid *et al.*, 1984), *E. coli* transcription termination factor rho (Bear *et al.*, 1985), and the *EcoR*I restriction endonuclease (Jen-Jacobson *et al.*, 1986). With the λ repressor (26 kDa), papain digestion yields two fragments that are relatively stable to further digestion (Pabo *et al.*, 1979). The N-terminal fragment (10 kDa) was found to bind specifically to DNA, and was more amenable to crystallography than the intact protein. It was then possible to deduce the three-dimensional structure of the protein to very high resolution and to elucidate the precise interactions that must exist between the protein and the DNA (Jordan and Pabo, 1988). The C-terminal fragment of the λ repressor (11 kDa) is able to oligomerise, and thought to allow the dimerisation that occurs with the intact molecule. A connecting region, of some 40 amino acids, between the two domains is the protease sensitive part of the molecule and, beside being a connector, is not thought to serve any other functional role within the protein (Pabo *et al.*, 1979; 1982).

3.2 Results.

Proteolytic cleavage of the gyrase proteins.

(a) Trypsin.

Trypsin is a serine protease that cleaves polypeptide chains on the carboxyl sides of lysine and arginine residues. Incubation of the DNA gyrase A protein with trypsin initially results in the production of two fragments, with approximate molecular masses 64 kDa and 33 kDa, that are relatively stable to further digestion (Fig. 3.1).

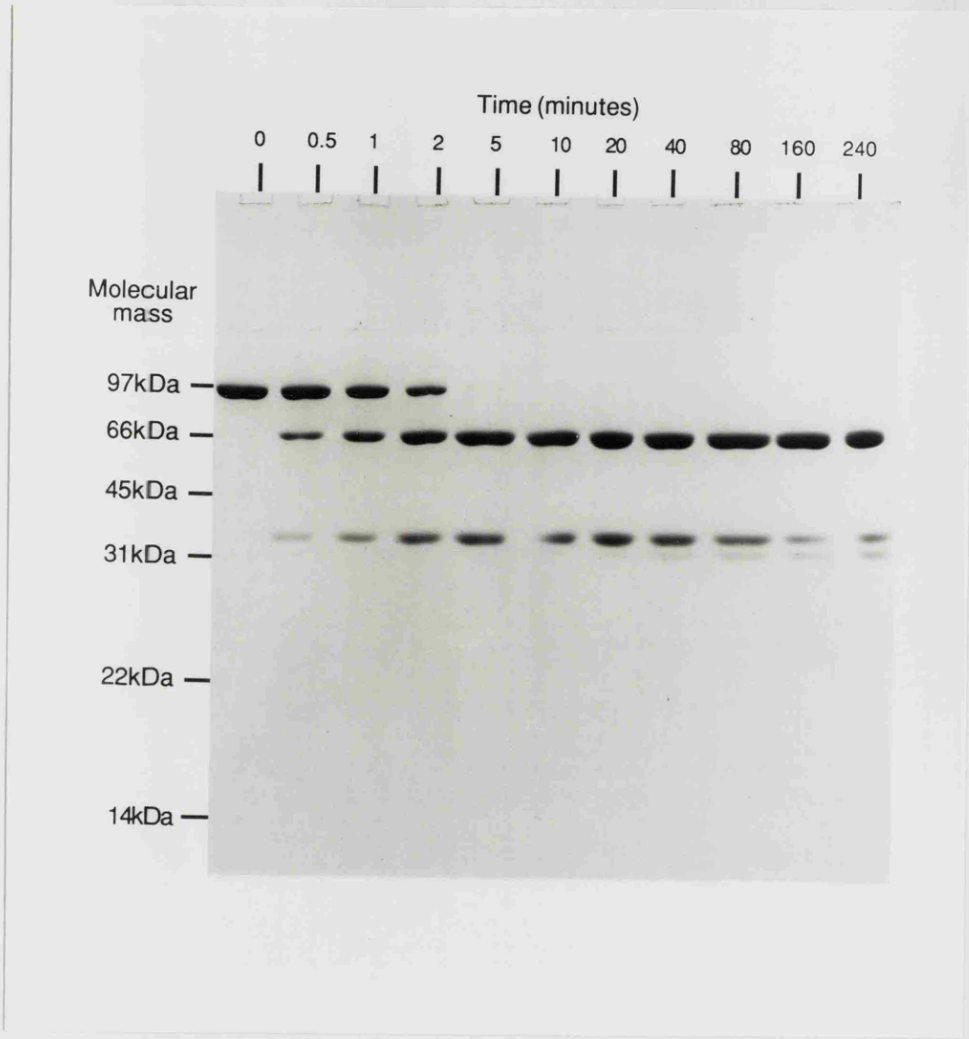


Fig. 3.1. Trypsin cleavage of the gyrase A protein. The DNA gyrase A protein (1.8 mg/ml) was incubated with trypsin (0.4 μg/ml) at 25°C. Samples (10 μl) were taken at the times indicated and the reaction stopped by boiling in 1% SDS for 3 minutes. Samples were analysed by SDS-polyacrylamide gel electrophoresis. The mobilities of protein standards are indicated.

The protein fragments were found to be resistant to further digestion with trypsin under the conditions of Fig. 3.1 for up to 1 hour, but prolonged incubation or 10-fold higher trypsin concentrations leads to further digestion of the 33 kDa fragment (Fig. 3.2). The 33 kDa fragment was degraded into a variety of products, including a metastable fragment of a molecular mass approximately 25 kDa that was itself degraded upon prolonged incubation with the protease. The 64 kDa fragment appears to be more stable to trypsin digestion than the 33 kDa fragment. Only at the later time points does a band at 45 kDa appear that is likely to be a degradation product of the 64 kDa fragment (Fig. 3.2). The presence of the gyrase B protein, or both the B protein and a 172 bp DNA fragment, did not alter the digestion pattern, although proteolysis was apparently less efficient under these conditions (data not shown).

The 64 kDa and 33 kDa tryptic fragments of the A protein were readily formed in the apparent absence of other contaminating fragments (Fig. 3.1). It was therefore desirable to map these fragments to the intact GyrA sequence so that any activity associated with the fragments could to assigned to particular region of the GyrA protein. The results of the determination of the ten N-terminal amino acid residues of the 64 kDa and 33 kDa tryptic fragments of the GyrA protein that were isolated from SDS-polyacrylamide gels and blotted onto PVDF membranes are shown below:

```
      1    5       10        15          565    570    575    580       871
GyrA: MSDLAREITPVNIEEELKS..GKSAARIKEEDFIDRLLVA..APEEE

            EITPVNIEEE...          IK?EDFID?L...

             64 kDa                  33 kDa
```

Previously, N-terminal sequencing of the GyrA protein has indicated that the mature protein has lost the N-terminal methionine (Menzel and Gellert; quoted in Swanberg and Wang, 1987), but in the convention used here sequence numbering will begin at the methionine residue. From the above sequences it can be noted that there are two trypsin cleavage sites, and that the 64 kDa protein begins at residue 7 (Glu) of the A

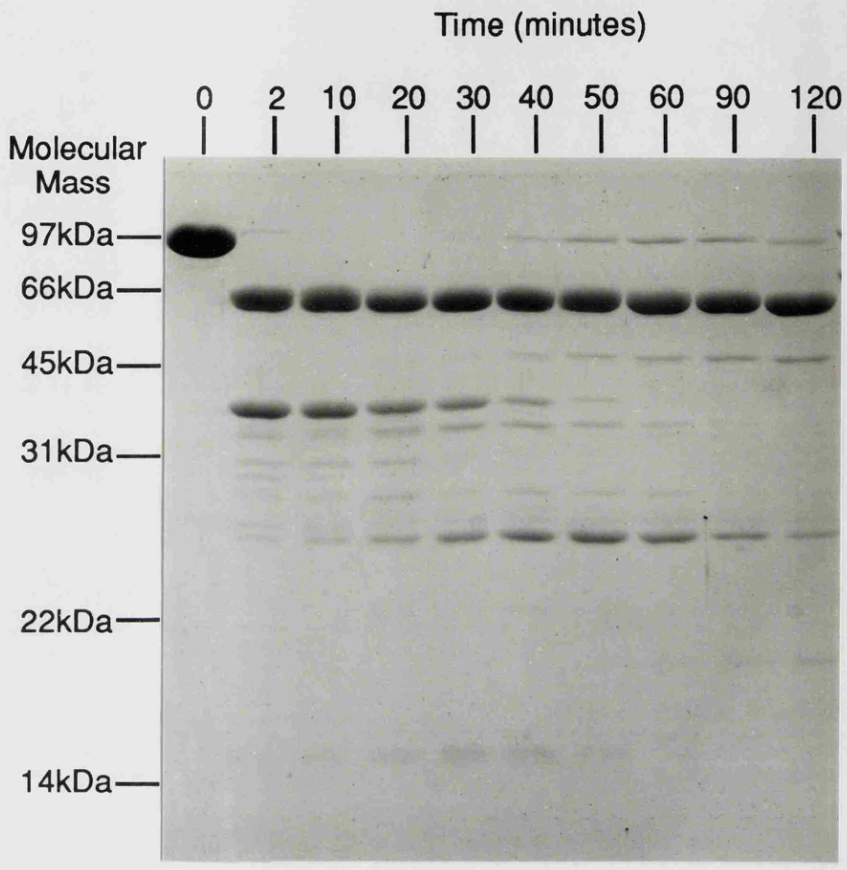


Fig. 3.2. Trypsin cleavage of the gyrase A protein at a higher trypsin concentration. The DNA gyrase A protein (1.8 mg/ml) was incubated with trypsin (4 μg/ml) at 25°C. Samples (10 μl) were taken at the times indicated and the reaction stopped by boiling in 1% SDS for 3 minutes. Samples were analysed by SDS-polyacrylamide gel electrophoresis. The mobilities of protein standards are indicated.

protein and the 33 kDa protein begins at residue 572 (Ile). Therefore, although there are a couple of ambiguities in the sequence (the automated amino acid analyser being unable to positively assign a residue), it is clear that the tryptic cleavages occur on the carboxyl side of residues 6 (Arg) and 571 (Arg). It is possible that further tryptic cleavages have occurred at the C-terminal ends of the 64 kDa and 33 kDa fragments. However, the fact that the molecular masses of these two fragments add up to the molecular mass of the gyrase A protein suggests that such peptides would be quite small. In addition, cleavage at the last Arg residue from the C-terminal end of the gyrase A protein would liberate an approximately 4 kDa fragment which would have been apparent on SDS-polyacrylamide gels, either directly or as a size shift of the 33 kDa fragment. An attempt was also made to sequence the C-termini amino acids of the 64 kDa and 33 kDa tryptic fragments of the GyrA protein using carboxypeptidase Y on the gel purified material. However, due to a number to technical difficulties, including the inability to obtain sufficient materials from the gel purification protocol, no sequence data was obtained.

(b) Other proteases.

Chymotrypsin is a serine protease that is able to cleave polypeptide chains on the carboxyl side of aromatic amino acids residues. Incubation of chymotrypsin with the A protein of gyrase yield the pattern observed in Fig. 3.3A. Chymotrypsin initially cleaves the A protein into a number of widely differing sized fragments; including fragments of sizes 64 kDa and 33 kDa which tend to predominate over the other fragments. These two major fragments may be similar to those obtained by the action of trypsin on the GyrA protein. The 64 kDa fragment appears to be relatively stable to further digestion by chymotrypsin. However, in the later time points there is evidence for the fragment appearing as a doublet on the SDS-polyacrylamide gel, suggesting that degradation is occurring. The smaller 33 kDa chymotryptic fragment is degraded into a number of smaller fragments, including two of approximate molecular weights 31 kDa and 29 kDa. The action of chymotrypsin on the B protein of gyrase generates

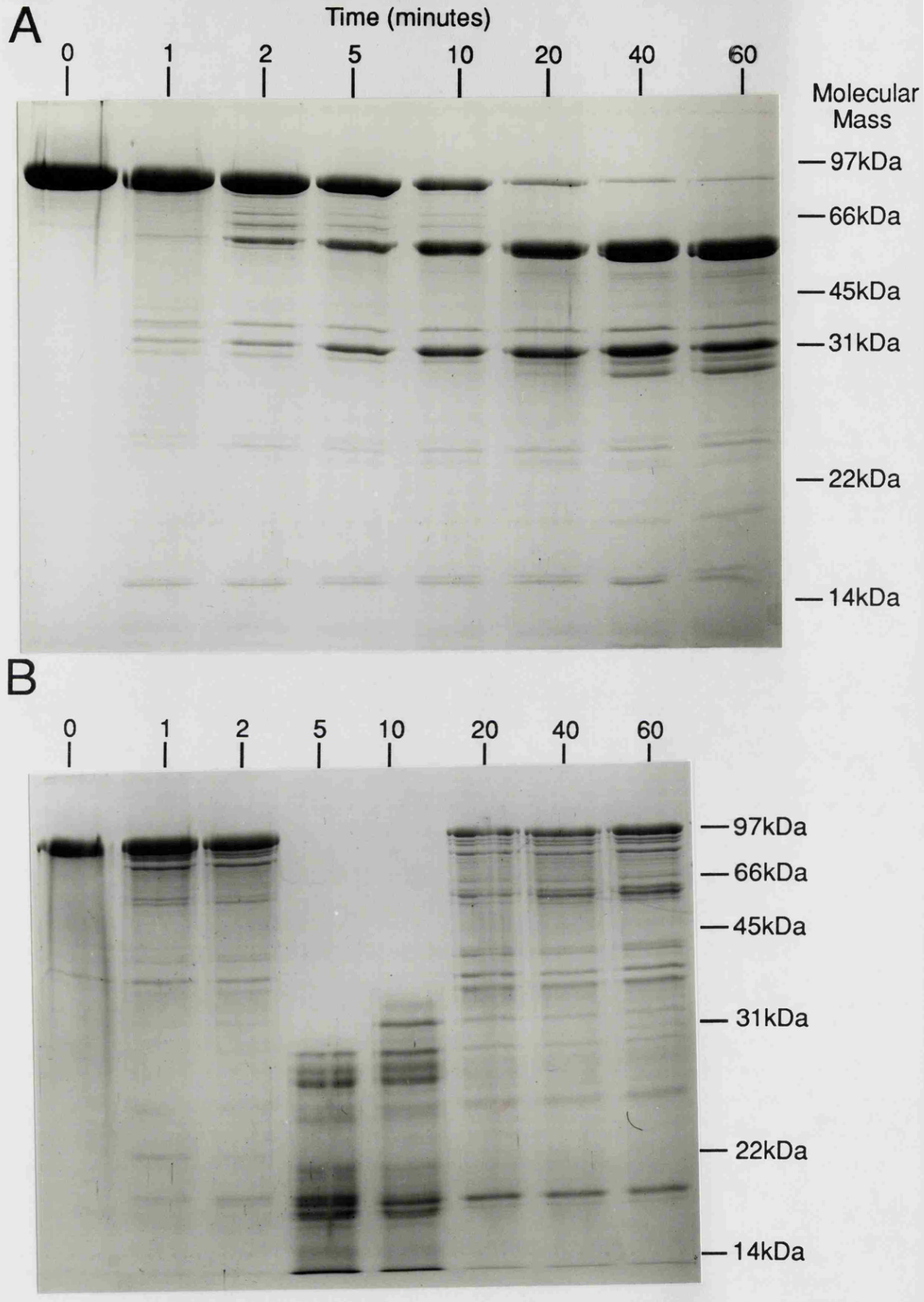


Fig. 3.3. Chymotrypsin and papain digestion of the gyrase A protein. The gyrase A protein (1.8 mg/ml) was digested with chymotrypsin (30 µg/ml; A) or papain (20 µg/ml; B) at 25°C, in a total reaction volume of 90 µl. Samples (10 µl) were removed at the times indicated and analysis performed on SDS-polyacrylamide gel electrophoresis.

many proteolytic products which tend to be degraded further, and again an A_2B_2 complex and that in association with DNA yielded no unique fragments, and again the overall digestion rate was slower with these complexes (data not shown). The fragments of chymotryptic digestion of the GyrA protein were greater in number than those of the trypsin digestion and the overall digestion pattern was more complex. It was therefore decided not to pursue these fragments further by carrying out amino acid sequence analysis or functional studies.

Papain is a sulphydryl protease that is relatively non-specific and will cleave most peptide bonds to various degrees (Arnon, 1970). Papain was found to cleave the A protein in many places (Fig. 3.3B) to yield a number of proteolytic fragments that are themselves further degraded into smaller fragments. None of the fragments appear to be very stable toward further papain digestion. It can also be noted that, although the reaction was stopped by boiling the samples in 1% SDS, this treatment does not appear to have completely halted the reaction, and in some cases further proteolytic degradation must be occurring to give the patterns observed in Fig. 3.3B. The ability of papain to survive such treatment is surprising, but it has been previously noted that the enzyme is still at least partially active in up to 8M urea and at high temperatures (Arnon, 1970). Incubation of the B protein of gyrase with papain yielded similar results in that a number of fragments were formed that were subsequently further degraded (data not shown). Papain was also used to digest an A_2B_2 gyrase complex and a gyrase complex that was associated with DNA. In both cases no new fragments were obtained, but the relative rates of degradation was observed to proceed at a somewhat reduced by comparison to those of the individual subunits.

Separation of 64 kDa and 33 kDa tryptic fragments of GyrA.

Using an appropriate trypsin digestion time (e.g. 10 min in Fig. 3.1), the 64 kDa and 33 kDa fragments can be prepared with minimal contamination by either intact A protein and by other degradation products. For larger scale preparation of the tryptic

fragments, the reaction mixture was passed through a trypsin inhibitor-agarose column. After this treatment no further digestion of the fragments could be noted (data not shown). The preparations of trypsin-treated A protein used in the experiments described below typically contained <1% contaminating intact A as judged by densitometry of the polyacrylamide gels. Although the two tryptic fragments were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 3.1), little success in separation has been achieved using column chromatography. This has included ion-exchange methods (DEAE Sepharose, FPLC Mono Q), hydrophobic interaction chromatography (valine Sepharose, leucine agarose), hydroxyapatite, HPLC reverse phase, and gel filtration (Sephadex, Sephacryl and FPLC Superose). Under the conditions tested none of the ion-exchange columns were able to separate the fragments to any extent, indicating that perhaps the two fragments remain tightly bound to each other even after cleavage by trypsin. Limited separation of the two fragments has been achieved by gel filtration, but only when 1% SDS, 8 M urea or 6 M guanidine hydrochloride were included in the elution buffers (Fig. 3.4). However the separation obtained was never complete; there was always some contamination of one of the fragments with the other, and problems were encountered in processing of the samples containing such high levels of urea or guanidine. Hence these methods proved impractical for the isolation of the two fragments. The 64 kDa and 33 kDa tryptic fragments of GyrA can, however, be successfully recovered from polyacrylamide gels following gel electrophoresis in the presence of SDS (Hager and Burgess, 1980). Control experiments have shown that, although material recovery is quite low at 10-20%, the intact A protein isolated from an SDS gel recovers approximately 80% of its supercoiling activity on refolding (data not shown).

In the experiments described below, studies were carried out principally using the trypsin-treated A protein containing both the 64 kDa and 33 kDa fragments (A') but, where feasible, experiments were also conducted using the purified 64 kDa and 33 kDa fragments isolated from SDS-polyacrylamide gels.

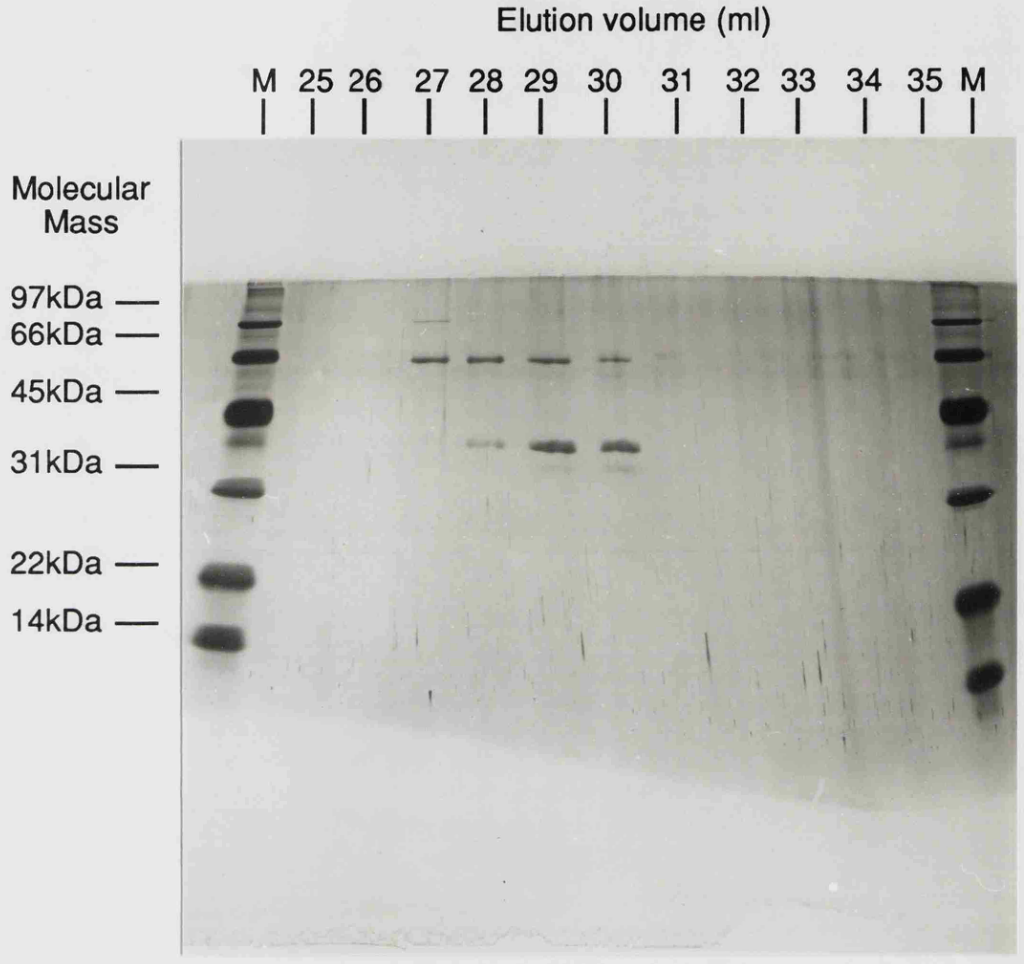


Fig. 3.4. Attempted separation of the tryptic fragments of the DNA gyrase A protein by gel filtration in 8M urea. The A' protein (0.5 ml of a 1.3 mg/ml solution) was passed down a 50 ml Sephacryl S-200 column equilibrated in buffer containing 6 M guanidine hydrochloride. Fractions from the column were analysed by SDS-polyacrylamide gel electrophoresis. The gel was stained with silver.

DNA Supercoiling.

In the presence of ATP, DNA gyrase catalyses the introduction of negative supercoils into closed-circular DNA. Under optimal conditions the supercoiling reaction is processive as evidenced by the appearance of supercoiled products while relaxed substrate still remains (Morrison *et al.*, 1980). Fig. 3.5 shows the supercoiling reaction of the trypsin-treated A protein (in combination with the B protein) compared with intact gyrase. Several observations can be made from these data. Firstly, the A' protein (consisting of the unseparated 64 kDa and 33 kDa tryptic fragments) will support DNA supercoiling in the presence of the B protein. Secondly, the supercoiling activity of the A' protein is approximately ten-fold less than that of the intact protein; the rate of supercoiling is greater than that which could be attributed to the low level of intact A protein which contaminates the preparation of A'. Approximate specific activities for the two proteins are calculated to be A= $1x10^6$ U/mg, A'= $1x10^5$ U/mg. Thirdly, whereas the supercoiling reaction of the A protein is largely processive, the reaction of A' is largely distributive as evidenced by the appearance of bands of intermediate superhelical density in Fig. 3.5. The fully supercoiled product is not apparent until many bands of intermediate superhelical density have been formed. The processive nature of the supercoiling reaction by the intact A protein is indicated by the appearance of a fully supercoiled product in the track of the lowest concentration of A (0.1 nM). Under other reaction conditions (100 mM KCl, 2 mM $MgCl_2$ and with the omission of spermidine) the supercoiling reaction of intact gyrase is largely distributive (A. Maxwell, personal communication). Experiments conducted with the A' protein show that the supercoiling reaction under these conditions is still also distributive but the rate of supercoiling is reduced by a factor of about 100 compared with the intact protein (data not shown). It appears therefore that the trypsin-treated A' protein can form a complex with the B protein which is able to support DNA supercoiling, but the reaction is slower than that of the intact protein and is distributive rather than processive.

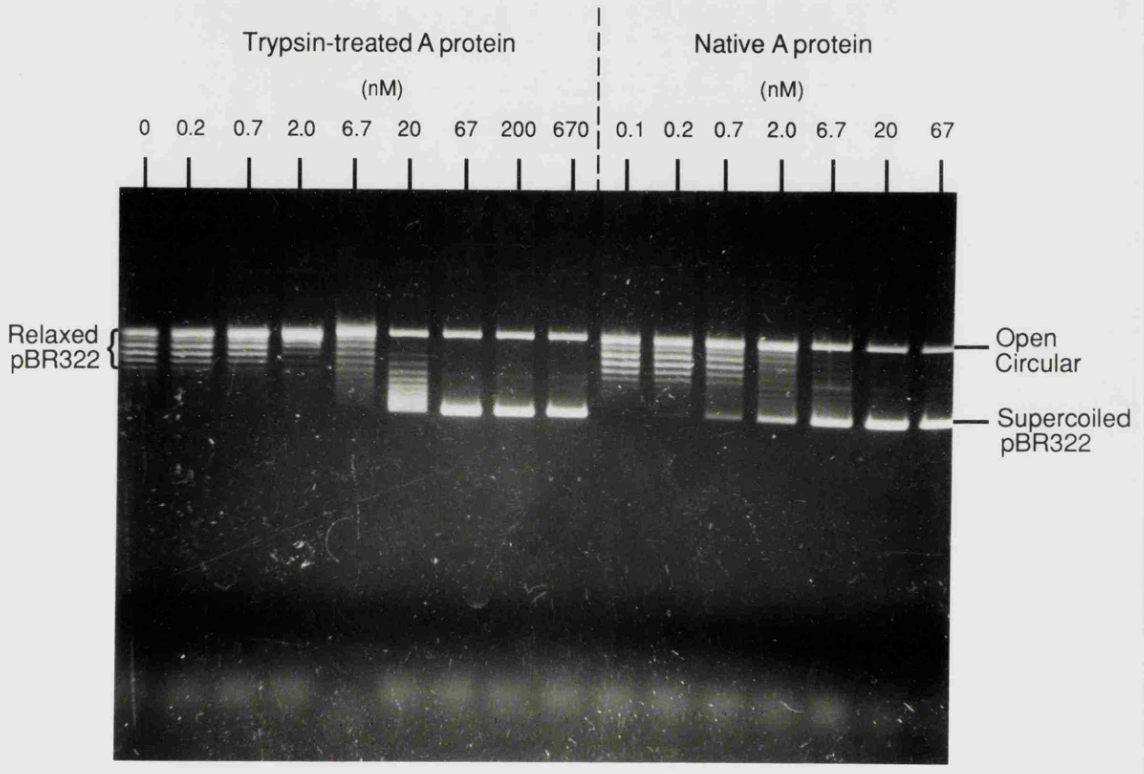
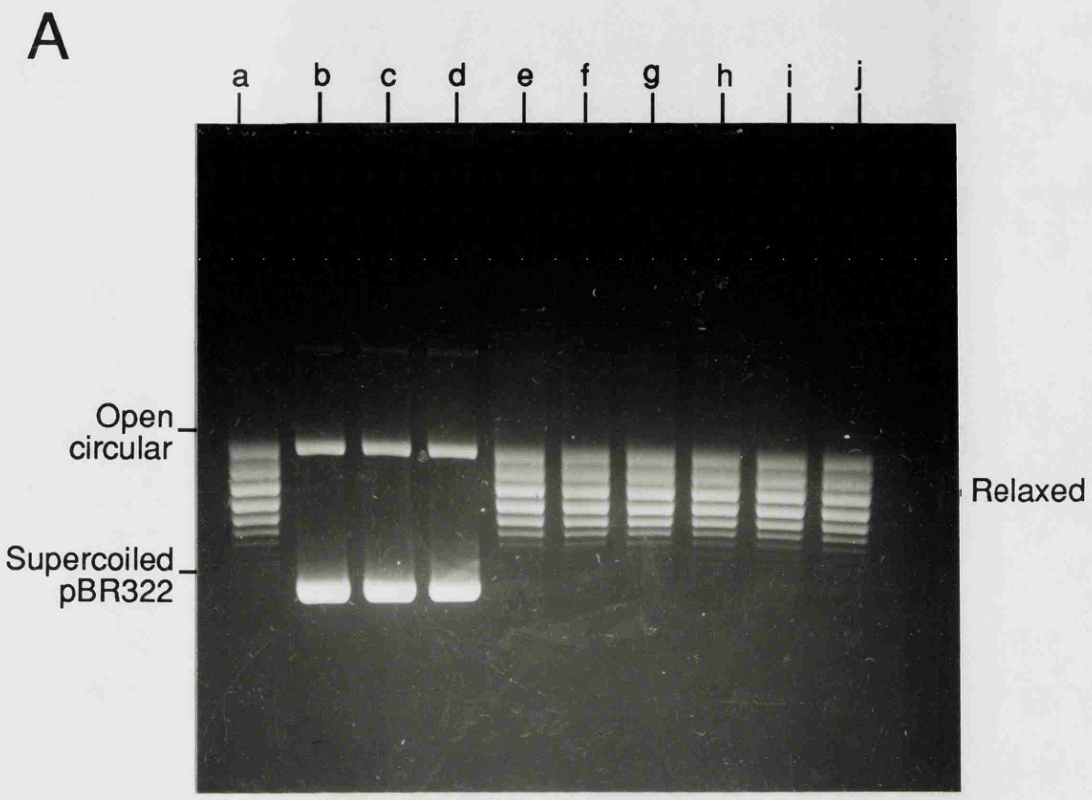


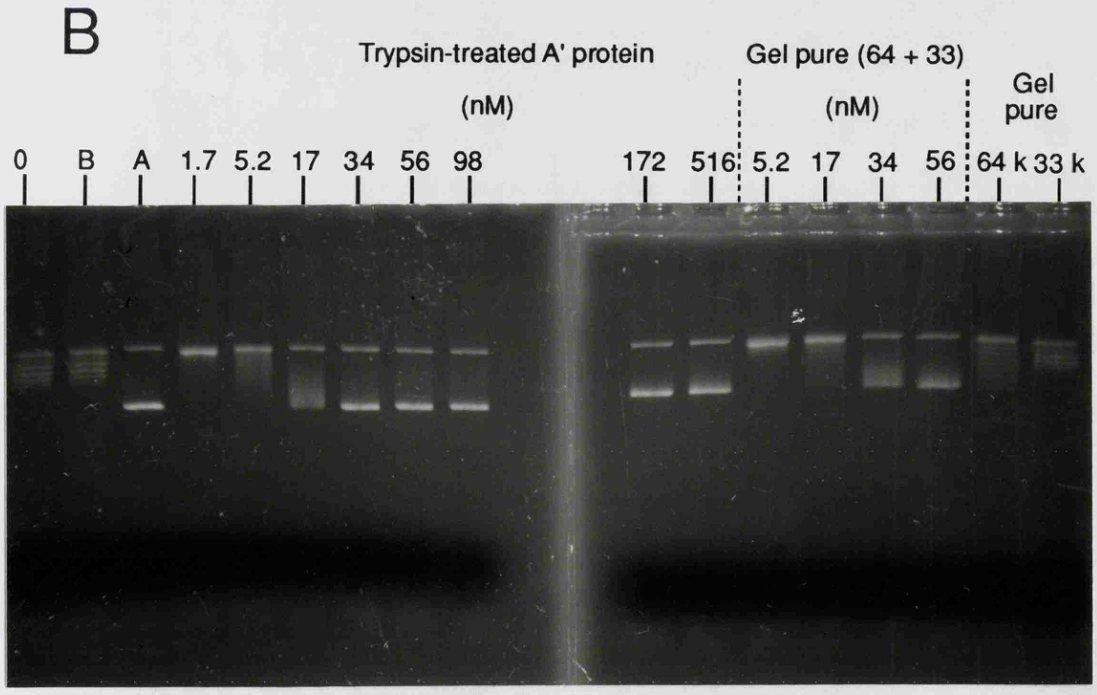
Fig. 3.5. DNA supercoiling by A and A'. Samples were incubated with excess gyrase B subunit (700 nM) and with the amount of A protein indicated, under the following conditions: 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 0.36 mg/ml bovine serum albumin, 9 µg/ml tRNA, 6.5% glycerol, 5 mM DTT, 1.4 mM ATP, 10 µg/ml relaxed pBR322 DNA in a total volume of 30 µl, for one hour at 25°C. A 10 µl aliquot was taken from each sample and electrophoresed through a 1% agarose gel run in TAE buffer.

The gel purified 64 kDa and 33 kDa fragments were also tested for their abilities to support DNA supercoiling in combination with the gyrase B protein. The 64 kDa fragment was found to support DNA supercoiling, again with an apparently distributive mechanism, whereas the 33 kDa fragment showed no supercoiling activity (Fig. 3.6A). Quantitation of the supercoiling ability of the 64 kDa fragment is uncertain because the amount of active material recovered from the SDS-polyacrylamide gel is unknown. If it is assumed, however, that 80% of the 64 kDa fragment is active after extraction from the polyacrylamide gel (by comparison with the level of activity which can be recovered for the intact A protein; see above) then a specific activity of $1x10^3$ U/mg can be calculated. Although DNA supercoiling by the complex involving the 64 kDa fragment is slow, control experiments indicate that the reaction is catalytic. For example, incubation of the 64 kDa fragment with the GyrB protein and relaxed DNA (at a level such that there is a molar excess of DNA over gyrase proteins) for 18 hr results in the introduction of more superhelical turns than can be explained simply by the amount of gyrase added (data not shown). Thus each enzyme molecule must be performing more than one cycle of reaction, albeit at a slow rate.

A number of experiments were also performed using the 64 kDa and 33 kDa gel purified tryptic fragments of the gyrase A protein that were added back together in equimolar amounts. The ability of such a reformed complex to perform the supercoiling of pBR322 in the presence of the gyrase B protein is shown in Fig. 3.6B. It is apparent that the supercoiling reaction of the reconstituted fragments, although not as efficient as the reaction for either the intact A protein or A', is considerably more efficient than the reaction for the 64 kDa protein alone. The 33 kDa fragment on its own has no enzymic activity (see above), but when added to the 64 kDa fragment, it is able to significantly improve the supercoiling ability of the 64 kDa fragment. This suggests that there is some sort of interaction between the two

Fig 3.6. DNA supercoiling by various fragments of the gyrase A protein. (on facing page) **(A)** Lane a, no protein addition. Lanes b, c, d, e, j each contained 30 nM B protein; lanes b and f, A protein (15 nM); lanes c and g, A' protein (15 nM); lanes d and h, gel purified 64 kDa (50 nM); lanes e and i, gel purified 33 kDa (50 nM). Samples were incubated under the conditions described in Fig. 3.5 for 9 hours at 25°C. **(B)** The supercoiling reaction of A', the 64 kDa and 33 kDa tryptic fragments (at 50 nM), and the fragments added back together (64 + 33), at the enzyme levels indicated. Conditions were as described for Fig. 3.5. Samples were incubated for 1 hr at 25°C. The products were electrophoresed through a 1% agarose gel. All samples, except for that labelled 0, contained the GyrB protein (at 30 nM). The sample labelled A contained purified GyrA protein (at 15nM).


A
a
b
c
d
e
f
g
h
i
j
Open circular
Supercoiled pBR322
Relaxed


B
Trypsin-treated A' protein
(nM)
Gel pure (64 + 33)
(nM)
Gel pure
0
B
A
1.7
5.2
17
34
56
98
172
516
5.2
17
34
56
64 k
33 k

polypeptides that have been gel purified, and also indicates a possible role for the 33 kDa fragment, in perhaps stabilising the DNA-protein complex during the supercoiling reaction. A specific activity for the reaction by the reconstituted A fragments can be calculated to be approximately $3x10^4$ U/mg. In a similar fashion to A' and the 64 kDa fragment on its own, the supercoiling reaction by the reconstituted fragments is distributive in nature.

DNA Relaxation.

In the absence of ATP, gyrase can relax negatively supercoiled DNA (Gellert *et al.*, 1977). During such a reaction it was found that the trypsin-treated A' protein in combination with the B protein could support relaxation (Fig. 3.7), but some differences could be noted between the intact and trypsin-treated proteins. Firstly, about five times more of the trypsin-treated enzyme was required to achieve the same extent of relaxation as the native DNA gyrase. Secondly, the end-point of the reaction appears to be different. With the native protein complex, after full relaxation has occurred the enzyme will form positively supercoiled products by wrapping the DNA around itself in a positively supercoiled turn (see section 1.5.1). This can be noted in Fig. 3.7 as an apparent shift back down the gel after full relaxation has occurs (see the 80 and 120 min time points for A_2B_2). Analysis of these products on a chloroquine-containing gel has shown that they are indeed positive supercoils (data not shown). Under the conditions used here, the trypsin-treated A' protein does not introduce positive supercoils after the relaxed state is achieved, or if positive turns are introduced there are fewer than is observed with the native protein complex.

In the experiment shown in Fig. 3.7 there is an eight-fold molar excess of gyrase over DNA, so when all the supercoiled DNA has been converted to the relaxed form, there is still likely to be associations between the protein and the DNA. Binding of gyrase to a relaxed DNA will result in a positive writhe being stabilised around the protein and the consequent formation of a negative writhe elsewhere in the molecule (see Fig.

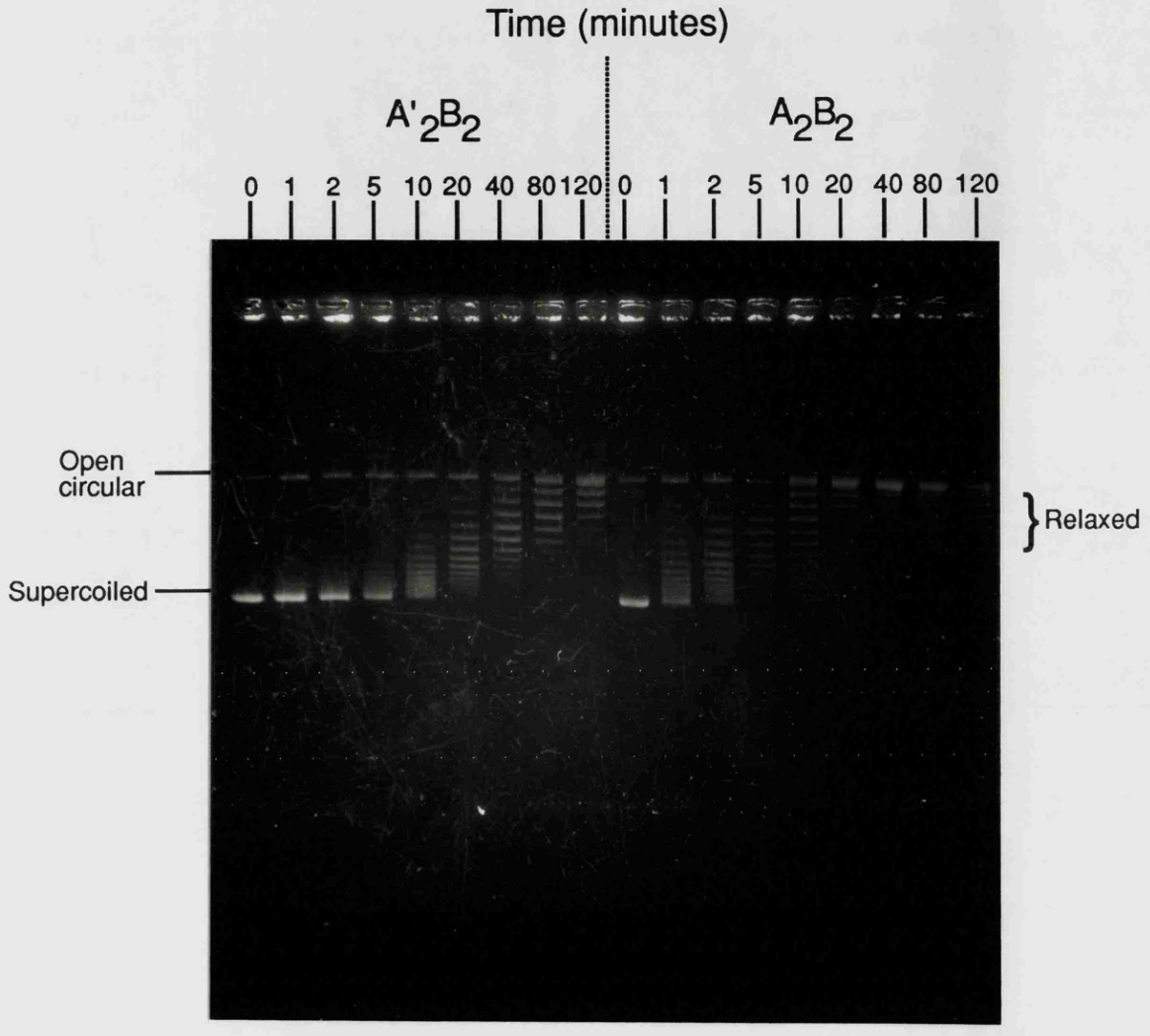


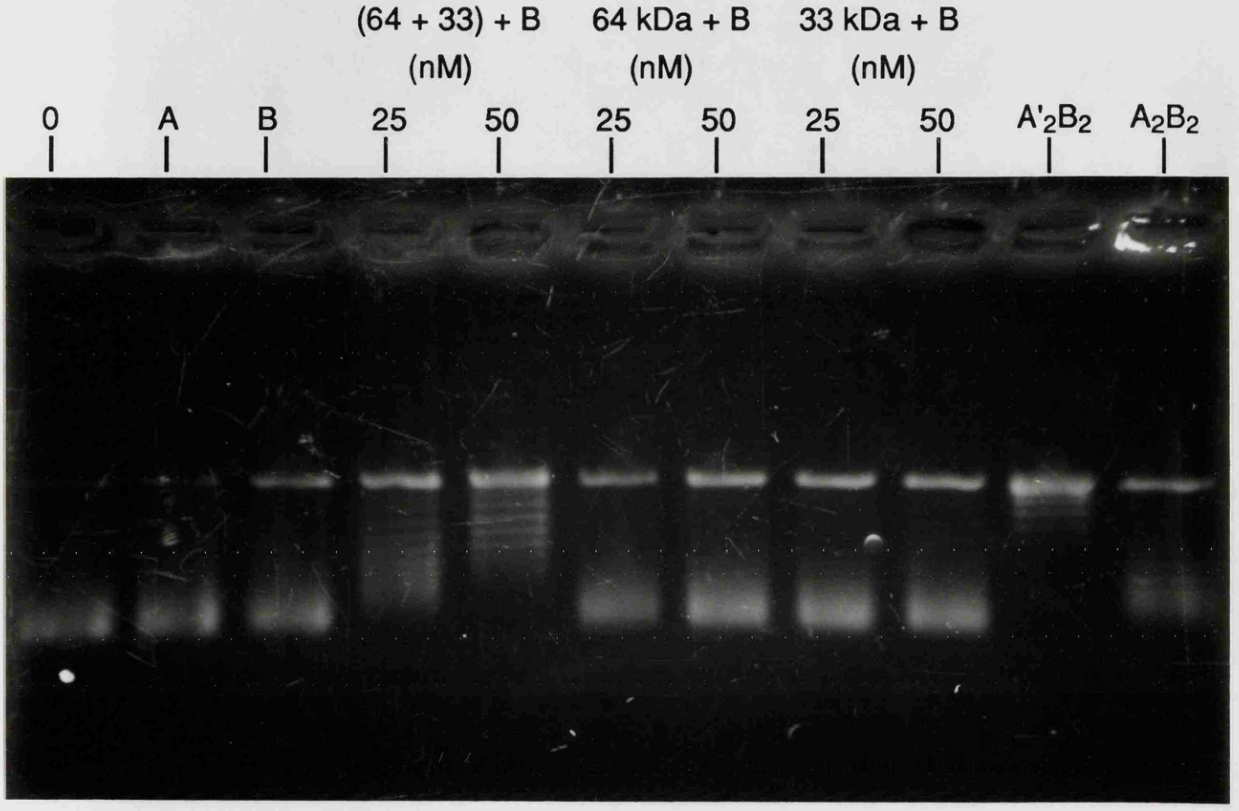
Fig. 3.7. Relaxation of negatively supercoiled pBR322. Samples of A_2B_2 or A'_2B_2 (both at 30 nM) were incubated at 25°C with supercoiled pBR322 (10 μg/ml) under the conditions described in Fig. 3.5, except that the ATP and spermidine were omitted and the reaction volume was increased to 100 μl. At the times indicated samples (10 μl) were removed and the reaction stopped. The samples were then subjected to electrophoresis through a 1% agarose gel.

1.3 for a diagrammatic representation of this process). The negative writhe could then be relaxed by other gyrase molecules, so that, on deproteinisation only positive writhes will remain. The lack of positive supercoil formation for the trypsin-treated protein complex (A'_2B_2) could indicate that this protein wraps DNA around itself less efficiently than the intact A_2B_2 complex.

The relaxation of negatively supercoiled pBR322 by the gel purified tryptic fragments of the gyrase A protein were also tested. No relaxation activity could be noted for either the 64 kDa or 33 kDa fragments when they were in combination with the gyrase B protein. However, using the fragments that have been added back together in equimolar amounts, relaxation activity could be noted (see Fig. 3.8). The efficiency of the relaxation reaction by the reconstituted fragments was estimated to be approximately equal to that of the A' protein, although exact quantification of this reaction is subjective since there is not an easily discernible end-point. It would be expected, from the supercoiling experiments, that the 64 kDa fragment on its own would perform the relaxation reaction since strand-passage must occur in both cases. The fact that none was detectable would therefore tend to suggest that relaxation by the 64 kDa fragment is too slow to be visualised by the methods used here. The limited recovery of material from an SDS-polyacrylamide gel also means that the protein concentration cannot be substantially increased to compensate for the slow reaction rate.

Decatenation.

In addition to intramolecular strand passage reactions (DNA supercoiling and relaxation) DNA gyrase can also carry out the intermolecular reaction of decatenation (Kreuzer and Cozzarelli, 1980). As a substrate for these reactions the catenated products of the interaction of the cre recombinase with mutant *lox*P sites in plasmid pRH42-117 were used (Abremski and Hoess, 1985). The trypsin-treated A protein, in the presence of the GyrB, was equally efficient in the decatenation reaction as intact



3.8. DNA Relaxation by GyrA fragments. Samples of the 64 kDa and 33 kDa gel purified GyrA tryptic fragments were incubated with supercoiled pBR322 for 1 hr at 25°C under the conditions described in Fig. 3.5, except that ATP and spermidine were omitted. The fragments were pre-mixed with an equimolar amount of the GyrB protein and incubated at 25°C for 30 min, and then added to the mixture at the concentrations indicated. The lanes labelled A and B contain either the GyrA or GyrB protein (at 30 nM) in the absence of the other. The lanes A'_2B_2 and A_2B_2 contain the trypsin-treated or native gyrase complex (at 85 nM).

DNA gyrase (Fig. 3.9). Consistent with previous observations (Kreuzer and Cozzarelli, 1980) it was found that the decatenation reaction is ATP-dependent. Fig. 3.9 also shows that the reaction cannot be supported by the non-hydrolysable ATP analog ADPNP, with either the intact or trypsin-treated A protein. It is not clear why ATP hydrolysis is required in the decatenation reaction, which in principle is not an energy-requiring process. Perhaps the directionality of strand-passage is important in determining the ATP-dependency of a process. It is possible to envisage that the supercoiling (ATP-requiring) and the relaxation (non ATP-requiring) reactions of gyrase are converse. Therefore DNA-strand passage may occur in one direction for one of these processes and in the opposite direction for the other. If this is the case, then the ATP-dependency of the decatenation reaction could be explained in terms of vectoral DNA-strand passage; that is, during decatenation strand-passage may occur in the same direction as that which occurs during supercoiling. It is also possible that decatenation is not strictly ATP-dependent, but that the reaction is much faster in the presence of ATP.

The decatenation reaction could not be detected with either the 64 kDa or 33 kDa gel purified gyrase A fragments on their own, but the reconstituted material showed the presence of decatenated products to approximately the same extent as the intact A or A' proteins (data not shown). Again, from the results of the supercoiling assays, it would be anticipated that the 64 kDa fragment, but not the 33 kDa fragment, would perform the decatenation reaction. Since this is not the case, then it must again be presumed that the slow reaction and lack of material have contributed to the absence of any decatenated products being formed.

DNA Cleavage.

An essential step in the DNA supercoiling pathway is the cleavage of DNA in both strands. Quinolone drugs (e.g. oxolinic acid, ciprofloxacin) are thought to inhibit the supercoiling of gyrase by interrupting the cleavage and rejoining of the DNA strands

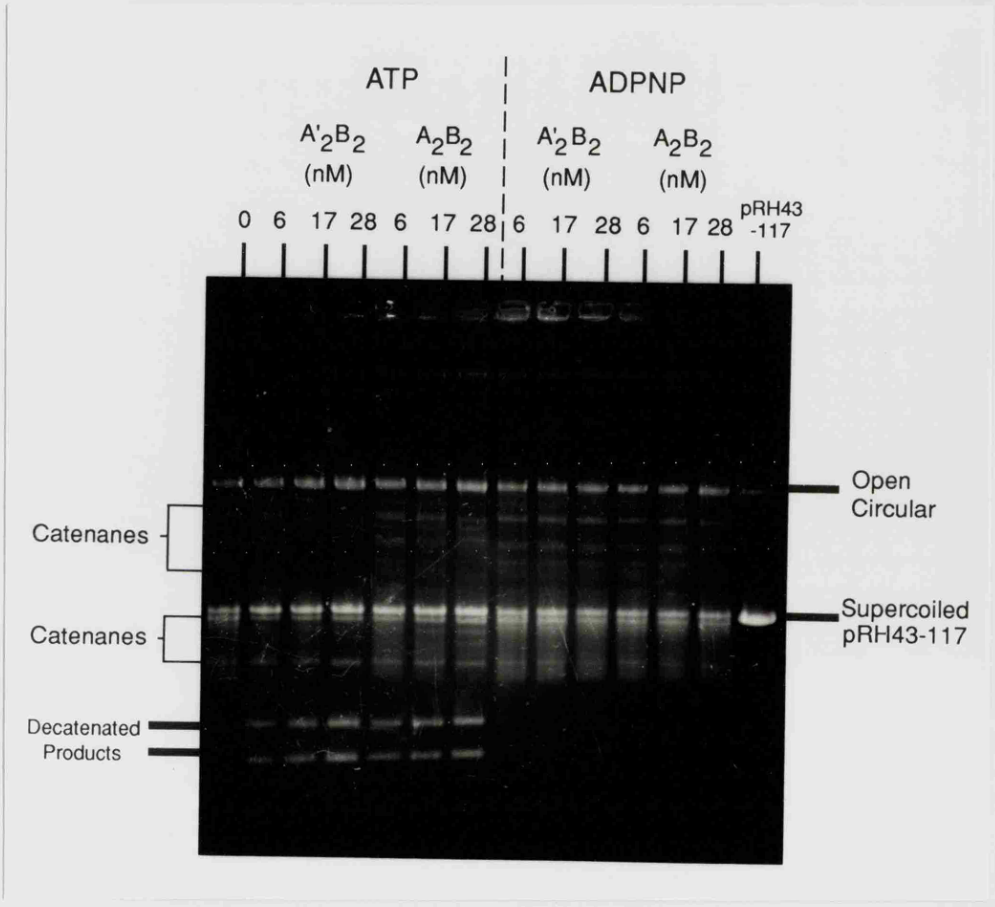


Fig. 3.9. Decatenation activity of A and A'. The catenated products of cre-mediated recombination of plasmid pRH43-117 (20 μg/ml) were incubated with the amount of native (A_2B_2) or trypsin-treated (A'_2B_2) gyrase indicated, either in the presence of 1.4 mM ATP or 1.4 mM ADPNP under the conditions described in Fig. 3.5. After 60 minutes at 25°C the products were electrophoresed through a 1% agarose gel.

(Gellert *et al.*, 1977). The addition of SDS to reactions containing DNA gyrase, DNA and a quinolone drug, and subsequent digestion of the protein, leads to the appearance of DNA cleaved in both strands. It is likely that the sites of cleavage represent the sites at which DNA breakage and reunion occur during the supercoiling reaction. It has also been found that the substitution of $CaCl_2$ for $MgCl_2$ in the gyrase reaction leads to double-stranded DNA breaks at the same sites as those revealed by quinolone drugs (L.M. Fisher, M.H. O'Dea, and M. Gellert, unpublished results). Quinolone-induced DNA cleavage of supercoiled plasmid pBR322 by gyrase leads to linearisation of the DNA under appropriate conditions (Fig. 3.10). The trypsin-treated complex (A'_2B_2) appears to cleave DNA with approximately the same efficiency as the intact complex. Ca^{2+}-directed cleavage also occurs to about the same extent with both complexes (Fig. 3.10). Experiments using the purified 64 kDa and 33 kDa fragments demonstrated that, in the presence of the B protein, only the 64 kDa fragment supported quinolone-induced DNA cleavage (Fig. 3.11). The efficiency of the cleavage reaction with the 64 kDa fragment is approximately equal to that of the intact A protein. Addition of the 64 kDa and 33 kDa fragments back together resulted in a complex that promotes the quinolone-directed cleavage of DNA with the same efficiency as both the 64 kDa fragment on its own and the intact A protein (data not shown).

The trypsin-treated complex will cleave a linear 172 bp DNA fragment with about the same efficiency as intact gyrase in the presence of the quinolone drug CFX (Fig 3.12). The major cleavage products were the same in both cases (of approximate sizes 105 and 71 bp), indicating that major cleavage-site specificity is unaltered. However, there were some differences in the minor cleavage products between the intact and trypsin-treated complexes. The A_2B_2 complex only showed one further, and relatively minor, cleavage site (generating fragments of sizes 95 and 82 bp), whilst the A'_2B_2 complex gave rise to a somewhat more elaborate cleavage pattern (118 and 59 bp & 98 and 80 bp).

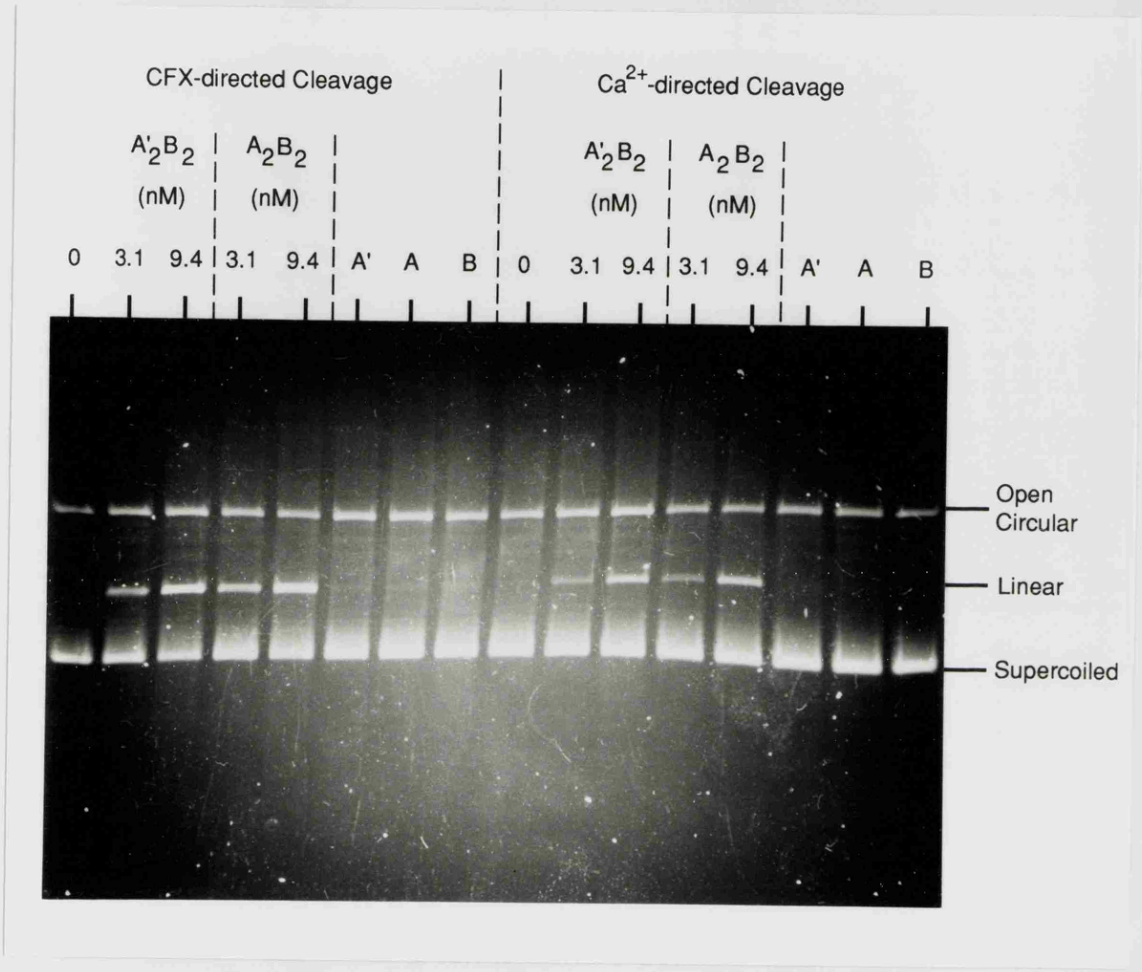


Fig. 3.10. DNA cleavage of A and A'. The supercoiled form of plasmid pBR322 (10 μg/ml) was incubated with native (A_2B_2) or trypsin-treated (A'_2B_2) gyrase at the concentrations indicated in the presence of either 3 μg/ml CFX or 2 mM $CaCl_2$ under the experimental conditions described in Fig. 3.5. The samples were then treated with SDS and proteinase K as described in section 2.13, and the products electrophoresed through a 1% agarose gel. A', A, and B indicate the individual subunits (at 100 nM) incubated with the DNA in the absence of the other subunit.

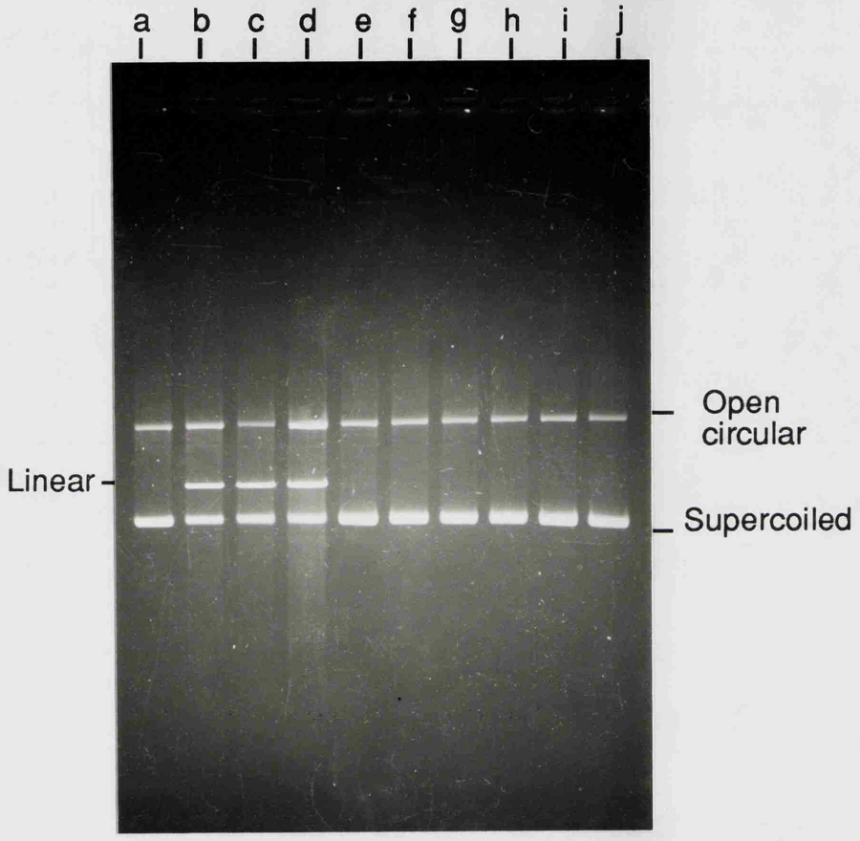


Fig. 3.11 Cleavage of supercoiled pBR322 by fragments of the A protein. The plasmid DNA (10 μg/ml) was incubated with CFX (3 μg/ml) under the experimental conditions described in Fig.3.5. DNA cleavage was observed by treating the samples as described in section 2.13. Lane a, no protein addition; lane b, 9 nM A_2B_2; lane c, 9 nM A'_2B_2; lane d, 20 nM 64 kDa fragment and 50 nM B; lane e, 20 nM 33 kDa fragment and 50 nM B; lane f, 100 nM A; lane g, 100 nM A'; lane h, 20 nM 64 kDa fragment; lane i, 20 nM 33 kDa fragment; lane j, 50 nM B.

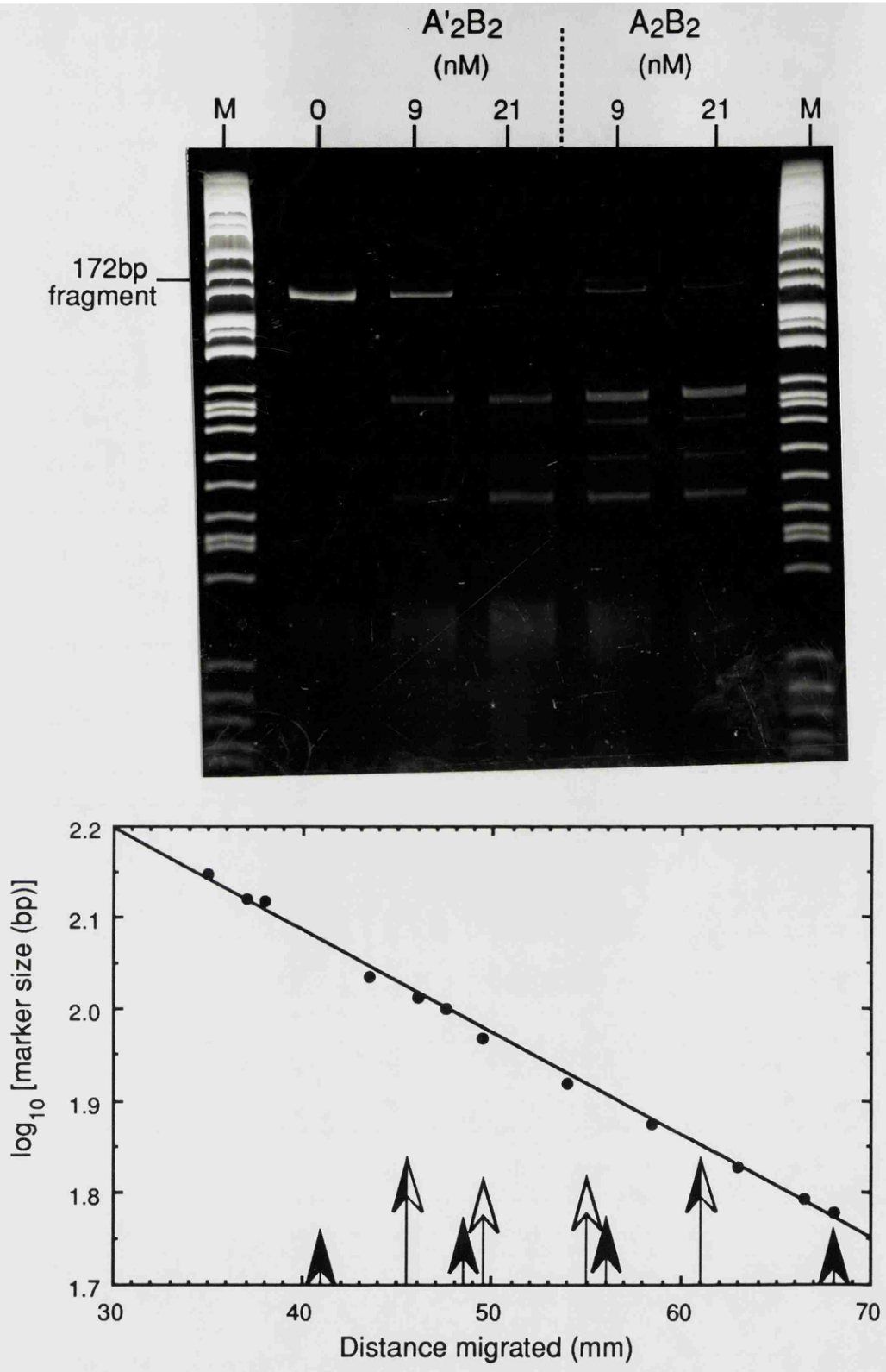


Fig. 3.12. Cleavage specificity of A and A' on a 172 bp DNA fragment. The CFX-directed cleavage reaction of A'_2B_2 and A_2B_2 on a 172 bp DNA fragment. The 172 bp DNA fragment (6 nM) was incubated with the amounts of gyrase indicated. After incubation the samples were electrophoresed through a 5% polyacrylamide gel. M indicates pBR322/*HhaI* size markers. Below is shown a graph of marker size against distance migrated and the positions of the A'_2B_2 fragments (filled arrows) and A_2B_2 fragments (open arrows). The mixed arrows represent the occurrence of the same DNA fragments with both types of complex, and the length of each arrow gives an approximate indication of the cleavage efficiency.

The specificity of quinolone-directed cleavage of DNA by gyrase has been shown to alter in the presence of ATP (Sugino *et al.*, 1978). The specificity of cleavage has been tested using λ DNA with the native and trypsin-treated gyrase complexes. The major cleavage products for A_2B_2 and A'_2B_2 are essentially the same (data not shown) and ATP alters the cleavage pattern similarly in both cases to yield a new cleavage pattern that was apparently identical for both types of complexes.

ATPase Activity.

The ATPase activity of DNA gyrase is associated with the B subunit (Mizuuchi *et al.*, 1978; Staudenbauer and Orr, 1981). The B protein alone possesses a very low level of intrinsic ATPase activity, but the presence of both the A protein and DNA greatly stimulates ATP hydrolysis (Maxwell and Gellert, 1984). Fig. 3.13 shows the DNA-dependent ATPase activity of gyrase as a function of the concentration of λ DNA, and the DNA-dependent ATPase activity of gyrase as a function of the gyrase concentration at a fixed DNA level. The data with the intact complex (A_2B_2) are similar to those reported previously (Maxwell and Gellert, 1984). The DNA-dependent ATPase activity of the complex formed between the trypsin-treated A' protein and the B protein was found to be reduced compared with that of the intact protein (Fig. 3.13). For a given DNA concentration, the difference between the ATPase activity of A_2B_2 and A'_2B_2 is approximately a factor of 4.

DNA Binding.

DNA gyrase forms an organised complex with DNA in which about 120 bp of DNA are wrapped around the protein in a single turn with a positive superhelical sense (Liu and Wang, 1978b; Rau *et al.*, 1987). The formation of such a complex can be detected by a number of techniques, including gel retardation, nitrocellulose filter binding and DNAse I footprinting (Maxwell and Gellert, 1986). In addition,

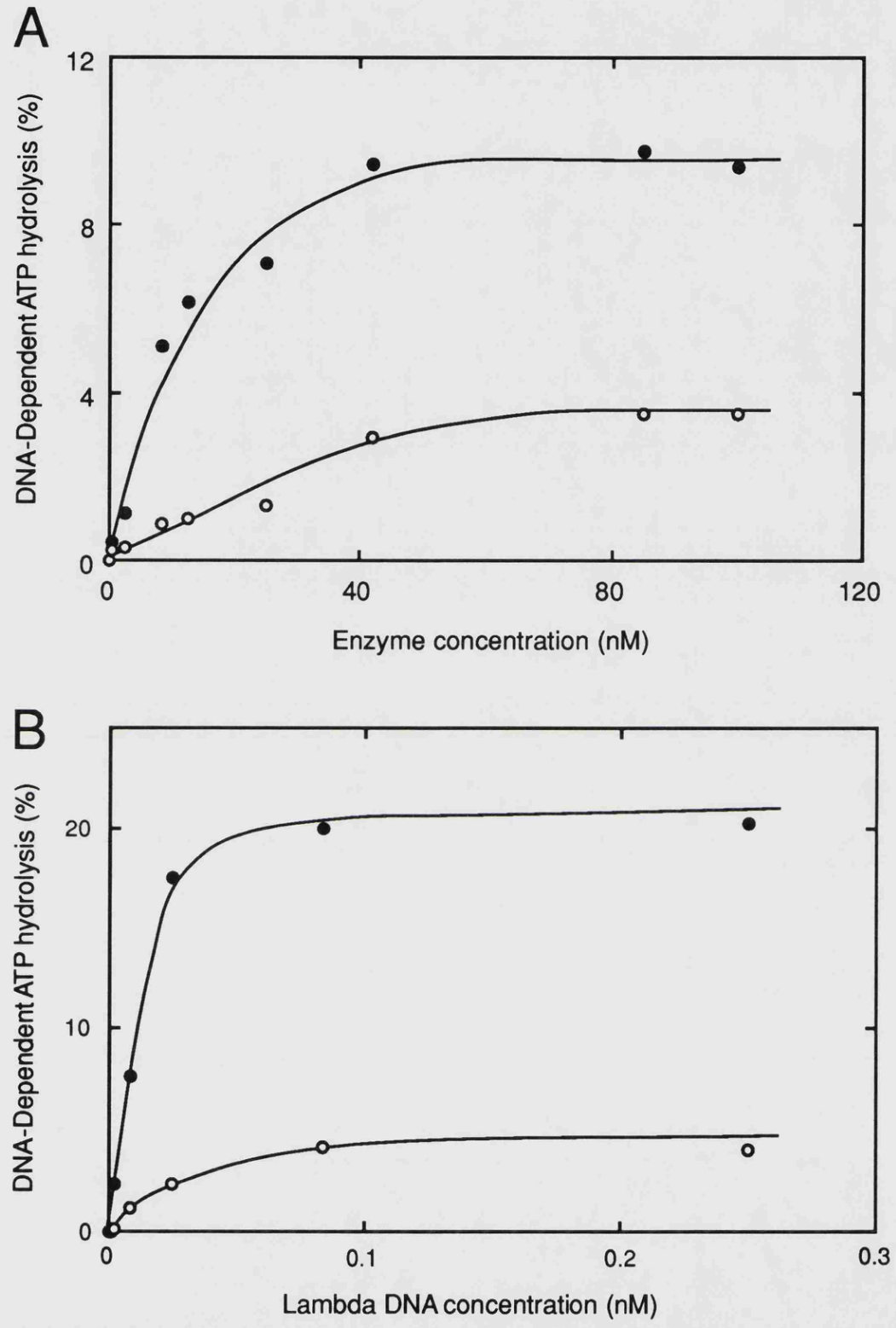


Fig. 3.13. ATP hydrolysis by A and A'. The native DNA gyrase A_2B_2 complex (•) or the trypsin-treated A'_2B_2 (o) were incubated with ATP (0.1 mM) and either 0.04 nM λ DNA and the gyrase levels indicated (A) or at a fixed gyrase level of 25 nM and the λ DNA at the concentrations indicated (B) for 2 hours at 25°C. The DNA-dependent hydrolysis of ATP was determined as previously described (Maxwell and Gellert, 1984) and is expressed as a percentage of the total ATP and have been corrected any for novobiocin insensitive and gyrase alone ATPase activities.

complexes between the A protein alone and DNA have been observed by electron microscopy (Moore *et al.*, 1983).

Complexes between gyrase and short DNA fragments have been found to be stable during electrophoresis on 5% polyacrylamide gels in the presence of Mg^{2+}; the complex forms a discrete band which can be resolved from free DNA (Maxwell and Gellert, 1984). The results of such a gel retardation experiment are shown in Fig. 3.14. DNA-protein complexes are observed with a linear 172 bp DNA fragment both for native gyrase and gyrase containing the trypsin-treated A protein. The mobility of these complex bands is similar (suggesting the the trypsin-treated complex is an A'_2B_2 tetramer), but at a given protein concentration there is less complex formed with the trypsin-treated than with the intact A protein. In addition, the complex band involving the trypsin-treated A protein is more diffuse than that with intact A (Fig. 3.14). These results suggest reduced stability of the complex formed by the trypsin-treated A protein with DNA. Fig. 3.14 shows that both the native A protein alone and the trypsin-treated A' protein alone form a complex with DNA that is detectable by the gel retardation assay. The mobilities of these complexes with the native and trypsin-treated A protein are apparently different. The observations of the binding of the A protein to DNA supports previous results obtained by electron microscopy (Moore *et al.*, 1983; Lother *et al.*, 1984; Kirchhausen *et al.*, 1985).

It has previously been shown that complexes between gyrase and DNA may be retained on nitrocellulose filters (Peebles *et al.*, 1978; Morrison *et al.*, 1980; Kirkegaard *et al.*, 1981; Higgins and Cozzarelli, 1982; Maxwell and Gellert, 1984). Using a 172 bp DNA fragment it has been shown that gyrase forms a bimolecular complex with this DNA molecule (Maxwell and Gellert, 1984). In a similar experiment (Fig. 3.15) it has been found that the trypsin-treated A protein, in combination with the B protein, will also form a filter-stable complex with the 172 bp fragment. It is clear that the complex involving the trypsin-treated protein is less stable

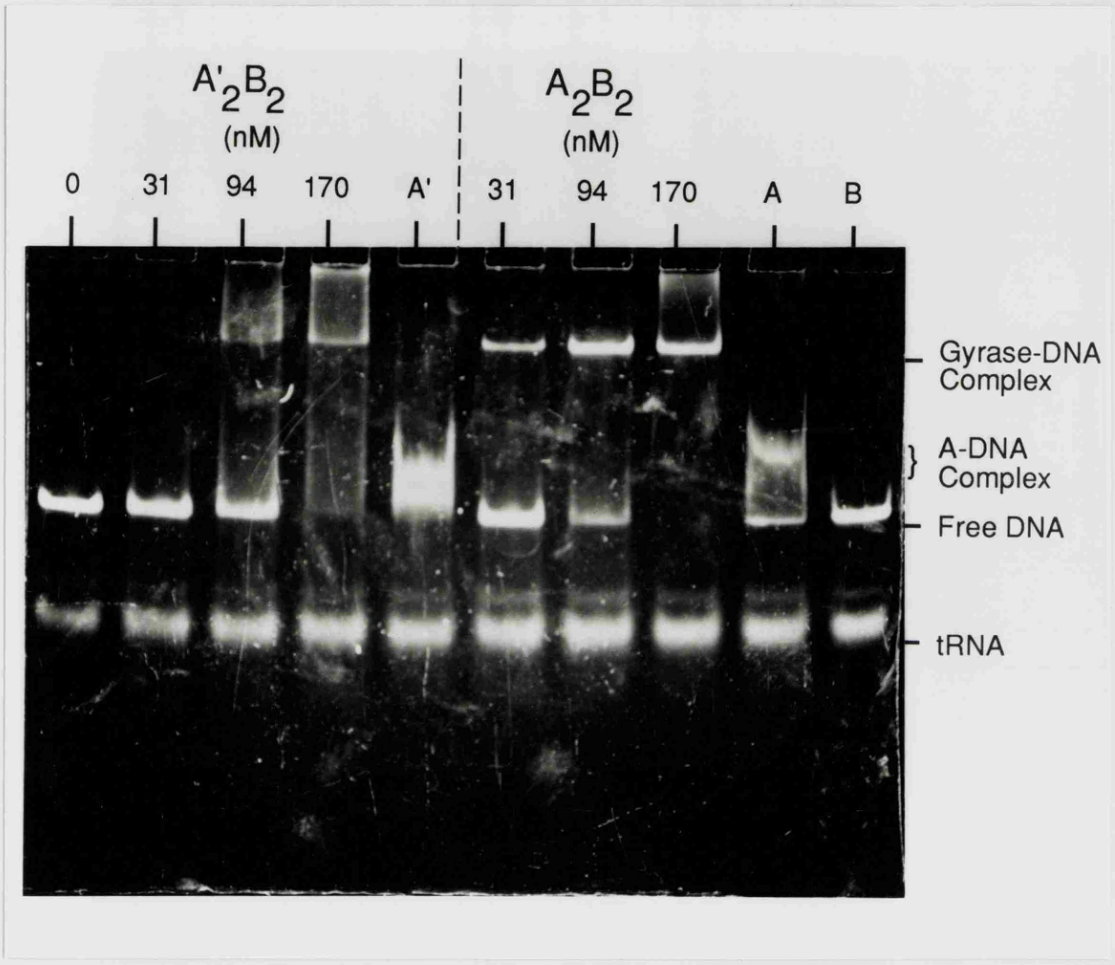


Fig. 3.14. Band-shift assay using A and A'. Native (A_2B_2) or trypsin-treated (A'_2B_2) gyrase at the concentrations indicated were incubated with a 172 bp DNA fragment (90 nM), for 60 min at 25°C, under the conditions described for DNA supercoiling in Fig. 3.5, except that ATP was omitted. The samples were then applied to a 5% polyacrylamide gel containing 5 mM $MgCl_2$. A', A, and B indicate samples containing one subunit (100 nM) in the absence of the other. Positions of protein-DNA complexes are indicated and were verified by protein staining. tRNA is a component of the reaction mixture.

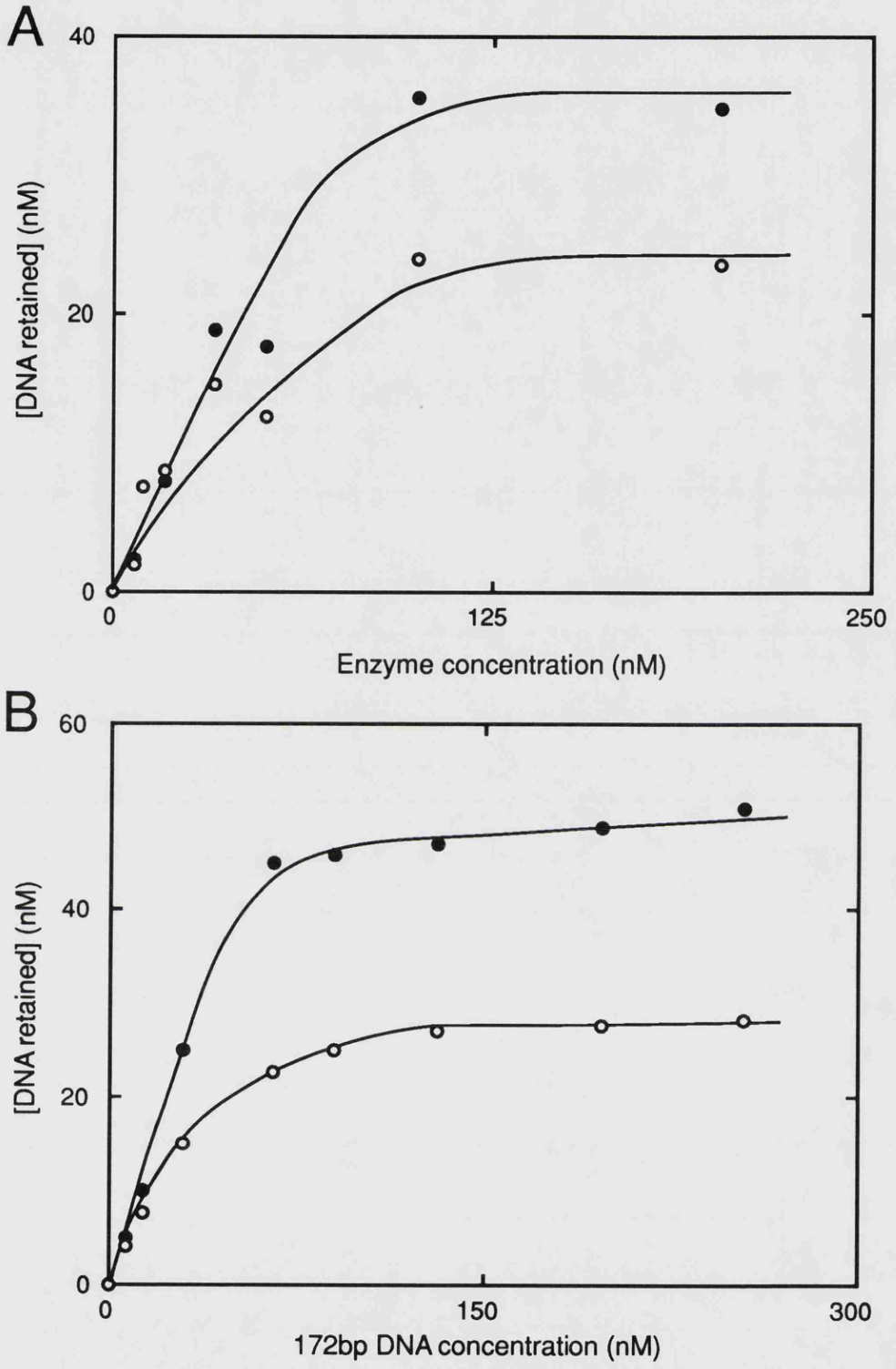


Fig. 3.15. Nitrocellulose filter binding. Native A_2B_2 gyrase (•) or the trypsin-treated A'_2B_2 derivative (o) were incubated with either 87 nM 3'-labelled 172 bp DNA fragment and the gyrase levels indicated (A) or 100 nM gyrase and the indicated 172 bp DNA concentration (B) of for 60 minutes at 25°C. The amount of complex formed between the DNA and the protein was estimated by the retention of label on nitrocellulose filters as described (Maxwell and Gellert, 1984). Data have not been corrected for efficiency of retention.

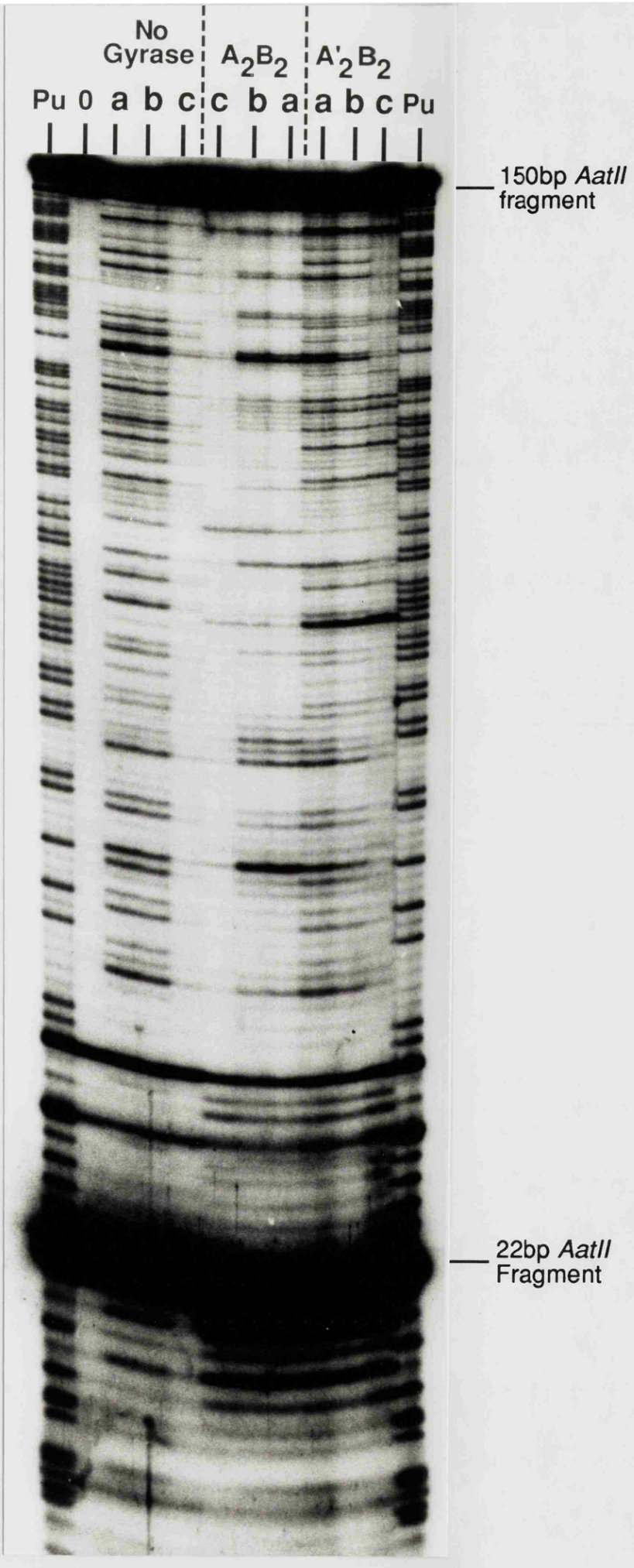
under these conditions compared with that involving intact A protein. The amount of DNA retained on the filter with the trypsin-treated complex is approximately 70% that obtained with the intact protein.

Details concerning the nature of the gyrase-DNA complex have been revealed by the analysis of the products of DNAse I digestion (Fisher *et al.*, 1981; Kirkegaard and Wang, 1981; Morrison and Cozzarelli, 1981; Rau *et al.*, 1987). The results from such studies suggest that gyrase protects a region of 100-155 bp of DNA from digestion by DNAse I. Within this region, sites of enhanced sensitivity to DNAse I spaced 10-11 bp apart can be distinguished and have been interpreted in terms of the wrapping of the DNA around the outside of the protein. Fig. 3.16 compares the DNAse I footprint of gyrase containing the intact A protein (A_2B_2) or the trypsin-treated A protein (A'_2B_2) complexed with a 172 bp DNA fragment. The complex involving the trypsin-treated A protein shows some similarities to that involving the intact A protein (e.g. periodic bands of enhanced sensitivity to digestion) but also some differences. These include a less extensive region of protection and a different pattern of enhanced and protected sites (Fig. 3.17). The A_2B_2 footprint covers approximately 119 bp of the DNA, whilst the A'_2B_2 footprint covers only 83 bp. Again this result supports the notion of an altered DNA-protein complex in the presence of the trypsin-treated A protein.

DNA wrapping.

When the gyrase complex binds to a relaxed DNA a single positive superhelical turn of DNA is wrapped around the enzyme (Liu and Wang, 1978b). This process can be observed by binding gyrase to a nicked-circular DNA species and sealing the nick with *E. coli* DNA ligase. In this case, when the gyrase is removed the sealed DNA will be positively supercoiled, by approximately one turn per gyrase tetramer (Liu and Wang, 1978b). The results of such an experiment comparing the wrapping abilities of the intact A protein complex to that with the trypsin-treated A' complex are shown in Fig. 3.18. In the presence of spermidine (Fig. 3.18A) there is a clear difference in the

Fig. 3.16. DNAse I footprinting. (On facing page) An autoradiograph of complexes of the 172 bp DNA fragment ^{32}P-labelled at the 5' ends, cut with *Aat*II, and subjected to DNAse I digestion. Samples containing DNA (10 μg/ml), and where indicated native (A_2B_2) or trypsin-treated (A'_2B_2) DNA gyrase (150 nM), were incubated with the following amounts of DNAse I: Lane 0, no DNAse I; lane a, 23 mU; lane b, 7 mU; lane c, 2 mU. Lanes labelled Pu were samples of the 172 bp fragment subjected to the Maxam-Gilbert sequencing reaction specific for purines (Maniatis *et al.*, 1982).


No Gyrase
A_2B_2
A'_2B_2
Pu 0 a b c c b a a b c Pu
150bp *AatII* fragment
22bp *AatII* Fragment

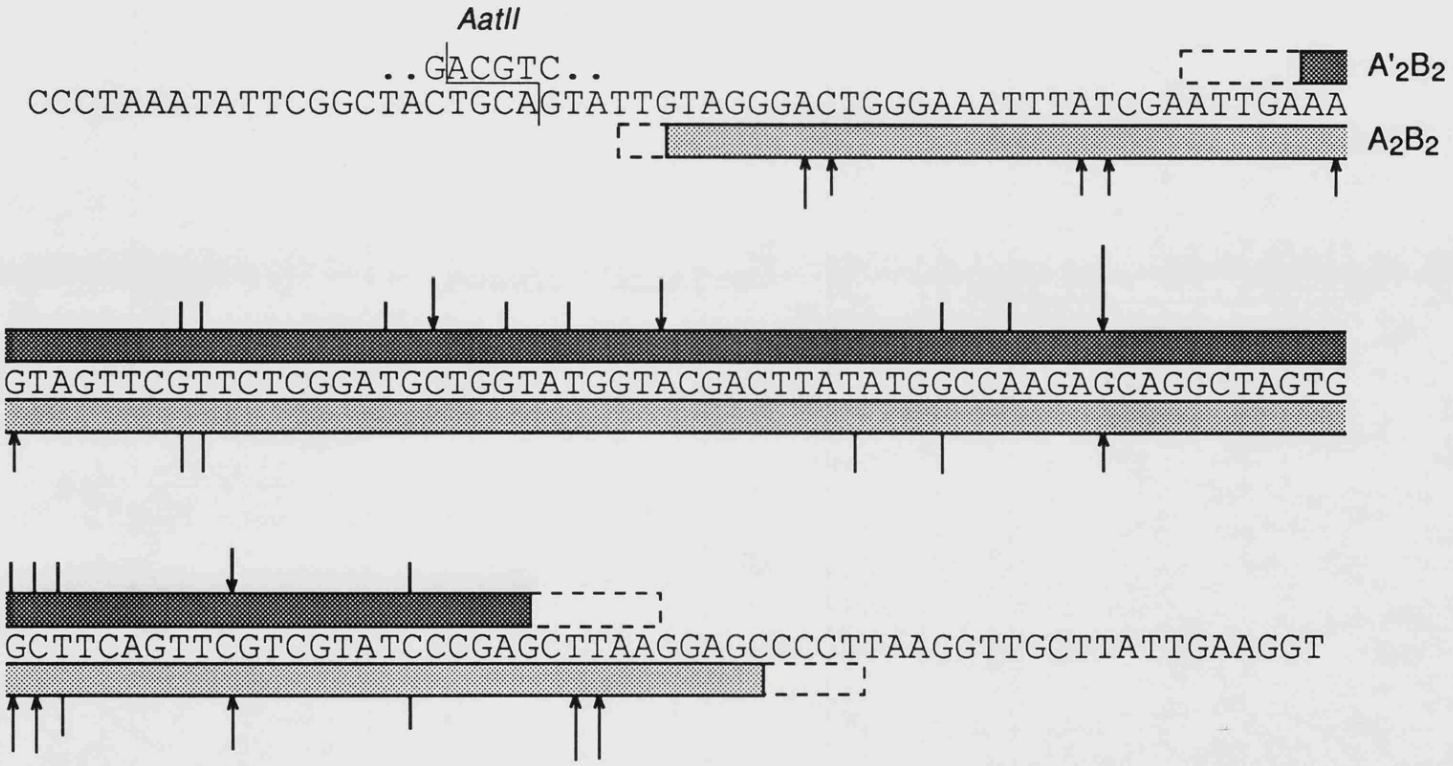


Fig. 3.17. Diagrammatic representation of the DNase I footprint of A_2B_2 and A'_2B_2 on a 172 bp DNA fragment. The protection data (taken from Fig. 3.16) is shown as a solid block for each of the protein complexes. The dotted lines indicate the boundaries of the footprint that are unclear due to either lack of resolution of the sequencing gel or a lack of DNase I cleavage bands to assess the exact nature of protection. The arrows indicate sequences of enchanced sensivity to DNase I in the presence of the protein, and the lines have been used to represent nucleotides that are incompletely protected by the protein (i.e. there is no difference the DNAse I pattern for DNA in the absence or presence of gyrase). In each case the length of the arrow has been used to represent the intensity of the band on the sequencing gel (Fig. 3.16). The total area of protection for A_2B_2 is 119 bp and for A'_2B_2 is 83 bp.

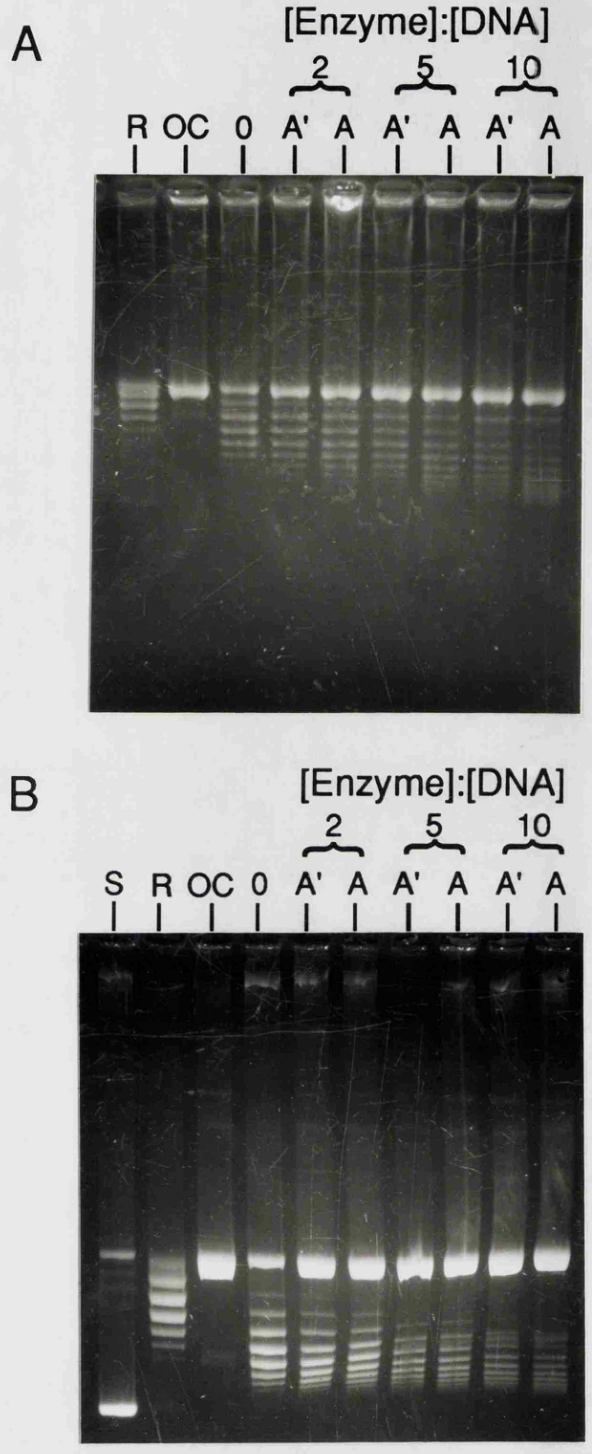


Fig. 3.18. Wrapping of DNA around gyrase in the presence and absence of spermidine. (A). A_2B_2 or A'_2B_2 (at 18, 45, or 90 nM) were incubated with open-circular pBR322 DNA (9 nM) for 30 min at 25°C, under the conditions described for Fig. 3.5, except that ATP was omitted. 2 µl of 2 U/µl *E. coli* DNA ligase and NAD to a final concentration of 26 µM were then added, and a further 30 min incubation at 25°C performed. Samples were then analysed on a 1% agarose gel run in TAE. **(B).** As in (A) except that spermidine was omitted from the reaction mixture. S, R and OC indicate supercoiled, relaxed and open circular pBR322 markers respectively.

amount of positively supercoiled product formed with A' when compared to the native A protein. Under the conditions of Fig. 3.18 the native complex appears to wrap 0.8 of a turn of DNA around itself, while the trypsin-treated complex wraps 0.6 of a turn. This difference corresponds to approximately 1/4 of a turn per gyrase tetramer. The values of the extent of wrapping are calculated from the number of gyrase tetramers present, and the number of positive turns inserted into a certain amount of DNA. However, in the absence of spermidine (Fig. 3.18B) this wrapping difference between A_2B_2 and A'_2B_2 seems to be virtually abolished and the extent of wrapping in both cases is almost identical (at approximately 0.7 turns per gyrase tetramer). An explanation for the results obtained in the absence of spermidine is not entirely obvious. However, less wrapping with the trypsin-treated protein does support the data obtained with the nuclease protection studies where less DNA was protected from DNAse I digestion with the trypsin-treated compared to the native protein.

3.3 Discussion.

Digestion of the DNA gyrase A protein with trypsin generates two stable proteolytic fragments of molecular masses 64 kDa and 33 kDa. No alteration in the digestion pattern can be observed if B protein and a 172 bp DNA fragment are included. The N-terminal amino acid sequence of these fragments has been determined, and it is found that the 64 kDa fragment comprises the N-terminal portion of the A protein from residue 7 onwards, while the 33 kDa fragment comprises the C-terminal portion beginning at residue 572. The stability of these fragments toward further proteolytic digestion suggests they may represent distinct domains of the gyrase A protein. However, failure to resolve the two fragments using standard chromatographic separation techniques suggests that either the domains are held together by strong non-covalent forces within the A protein, or that interactions stabilising the A_2 dimer prevent dissociation of the domains.

When combined with the B protein the trypsin-treated A protein was found to carry out all the known activities of DNA gyrase (Table 3.1), although with reduced efficiency in some cases. The 64 kDa fragment, when complexed with the B protein, was found to support both DNA supercoiling and cleavage. Although it was not feasible to test other activities of gyrase using the 64 kDa fragment, it can be assumed that this fragment will support DNA binding and ATP hydrolysis as these processes are intrinsic to the DNA supercoiling reaction. By contrast, the 33 kDa protein failed to show any measurable activity. It is therefore proposed that the DNA breakage and reunion activities of the trypsin-treated (A') protein can be ascribed to the 64 kDa (N-terminal) fragment alone. These data therefore support the idea that the amino acid residues involved in the breakage and reunion of DNA by gyrase are contained within the N-terminal portion of the A protein. It is possible that the reduced activity of the trypsin-treated A' protein could be attributed to the loss of the first six N-terminal amino acids. However, the results obtained with the 64 kDa and 33 kDa fragments which were gel purified and then added back together must dismiss this possibility. The gel purification process will exclude the small six amino acid fragment from the reconstituted preparations. The reconstituted fragments also tend to suggest that the enzymic activities of A' cannot be wholly explained by the 64 kDa fragment alone. It would appear likely that although the 64 kDa fragment is involved in the DNA breakage and reunion reactions, which can be performed in the absence of the 33 kDa fragment, the smaller fragment is required for stability of the protein-DNA complex. DNA supercoiling can be noted with the 64 kDa fragment alone, but the relaxation and decatenation reactions can only be visualised if both the 64 kDa and the 33 kDa fragments are present. Since the 64 kDa will perform the supercoiling there would appear to be no mechanistic reason why relaxation or decatenation should not occur. It must therefore be postulated that the 33 kDa fragment is required for the stability of the enzyme-DNA complex so the protein is able to perform the less efficient reactions of gyrase at a rate that is detectable by the methods used. It is possible that, in common with some other DNA-binding proteins; such as the λ repressor (Pabo *et al.*,

Reaction	Intact A (97 kDa)	A' (64+33 k)	Gel pure 64 kDa	Gel pure 33 kDa	Reformed Gel pure 64+33 kDa
Supercoiling	++++	+++	+	-	++
Relaxation	++++	+++	-	-	++
Decatenation	++++	++++	-	-	+++
Cleavage	++++	++++	++++	-	++++
ATPase	++++	+++			
DNA binding	++++	+++			

Table 3.1 Enzymic reactions of the proteins produced by trypsin cleavage of the DNA gyrase A protein. + indicates an approximate 10-fold change in activity compared to the intact protein. - indicates that no detectable activity could be observed, and a blank space shows that the activity has not been tested. All reactions were performed in the presence of the GyrB protein.

1979), 434 repressor (Aggarwal *et al.*, 1988), lexA repressor (Brent and Ptashne, 1985), and the GAL4 transcription activator (Keegan *et al.*, 1984); that the N-terminal of the gyrase A protein is involved in the interaction with DNA, whilst the C-terminal is required for the stabilisation of the complex, perhaps by mediating dimer interactions between the A proteins to allow DNA to wrap efficiently around the protein.

Fig. 3.19 shows a diagrammatic representation of the effect of the presence of the C-terminal 33 kDa of the gyrase A protein on the supercoiling ability by the N-terminal 64 kDa tryptic fragment. It is apparent that the 33 kDa fragment increases the efficiency of the supercoiling reaction, but that covalent attachment of the 64 kDa and 33 kDa fragments is required before processive supercoiling can be noted. Addition of the gel purified 33 kDa fragment results in a ten-fold increase in the supercoiling ability of the 64 kDa fragment. The activity of the reformed complex is only slightly less than that observed for the A' protein (whose fragments were not separated). The difference in activity between these two species could be explained as a result of some inactive protein being purified from the polyacrylamide gel, and experimental error in the determination of the point at which 50% supercoiling occurs (a measurement needed for all the specific activity calculations). The supercoiling reaction performed by the two separated fragment is still distributive in nature, and processive supercoiling can only be noted when the two fragments are covalently attached, as the intact GyrA protein.

Earlier evidence also supports the idea that the active site for DNA breakage and reunion by gyrase is contained within the N-terminal portion of the A protein. It has been shown that the tyrosine residue involved in the formation of the DNA-protein covalent bond is at position 122 of the A protein (Horowitz and Wang, 1987). The quinolone drugs are known to disrupt DNA breakage and reunion by gyrase (Sugino *et al.*, 1977). Quinolone-resistant mutations of the A protein have been found to map

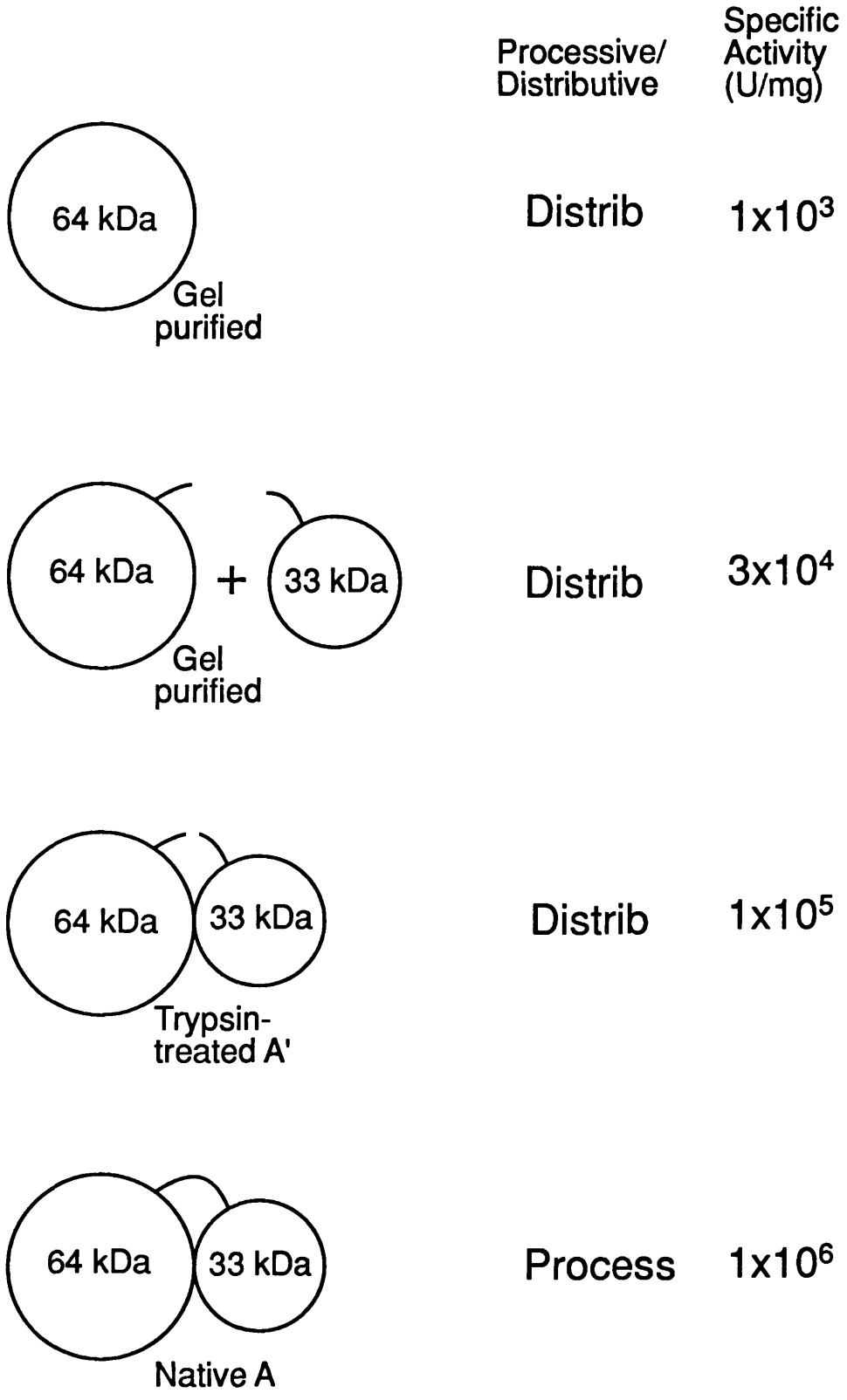


Fig. 3.19. Diagrammatic representation of the stabilising effect of the 33 kDa C-terminal fragment of the gyrase A protein on the supercoiling reaction by the N-terminal 64kDa fragment. The separate 64 kDa and 33 kDa fragments were purified from an SDS-polyacrylamide gel. All supercoiling reactions were performed in the presence of the GyrB protein.

within the N-terminal portion at amino acids Ala 67, Ser 83 and Gln 106 (Yoshida *et al.*, 1988). The subunit of T4 topoisomerase II known to be involved in DNA breakage and reunion (gene 52 protein) has been found to be homologous to the N-terminal portion of the gyrase A protein (Huang, 1986a). Taken together with the data described here, it is clear that the site of DNA breakage and reunion by gyrase lies within the N-terminal 571 amino acids of the A protein.

The principal differences between the gyrase complexes involving the trypsin-treated (A') rather than intact A protein are:

1. A DNA-protein complex with altered properties.
2. Reduced stimulation of the DNA-dependent ATPase reaction.
3. Reduced rate of the supercoiling and relaxation reactions.
4. Distributive rather than processive supercoiling.

A simple rationalisation of these observations is that the complex formed between the trypsin-treated A' protein, B and DNA is intrinsically less stable, and so is less capable of wrapping DNA around itself. As a consequence the DNA-dependent ATPase activity is less efficiently stimulated and during supercoiling, there is a higher probability of dissociation of the DNA-protein complex. The ability of the trypsin-treated complex to carry out the DNA cleavage reaction with the same efficiency as the intact complex, may reflect the fact that cleavage is essentially a stoichiometric reaction and does not require enzyme turnover. Similarly, the efficiency of the decatenation reaction of the trypsin-treated complex may be attributable to the fact that decatenation requires only one double strand-passage event to occur. Hence, the trypsin-treated complex appears to be able to perform 'single-hit' reactions with equal efficiency to the native complex, but is less able to carry out multiple turnovers. As indicated in Table. 3.1, the 64 kDa gel purified GyrA fragment reflects some of the properties of the A' protein. The 64 kDa fragment is able to support DNA supercoiling, and DNA

cleavage occurs with approximately equal efficiency to that observed with the A' protein. The 33 kDa gel purified fragment was unable to support any of these reactions. The supercoiling activity of the 64 kDa fragment could be increased by approximately a factor of ten by the addition of the 33 kDa fragment. Therefore, although the 64 kDa fragment clearly has the major role in DNA-protein interaction, the 33 kDa fragment appears to be involved in the stabilisation of the DNA-protein complex, for example during processive DNA supercoiling.

One major drawback in these results was the inability to satisfactorily resolve the 64 kDa tryptic fragment from the 33 kDa fragment under native conditions. This necessitated purification of the proteins from denaturing gels (with the attendant problems of potential alterations to the proteins) and the use of the unresolved fragments (A') in many experiments. One difficulty with the results using the A' protein is the unclear nature of the interaction between the two unresolved fragments in solution. This was, in some way, partially resolved by the use of the gel purified fragments that could be added back together to form a complex that was more stable than the isolated fragments and consequently able to carry out the more catalytic reactions of gyrase to an equal efficiency as the A' protein. The polyacrylamide gel purification process, however, also has the problem of low yields, thus reactions had to be carried out at relatively low enzyme levels.

One of the primary goals of this project is to gain some structural information about the gyrase proteins. The use of proteolytic fragments in, say, crystallization trials would be problematical. The likelihood of heterogeneous polypeptides must be high when all the other possible trypsin cleavage sites within the protein are considered. Also the method of separation of the 64 kDa and 33 kDa fragments must raise the possibility of alterations in the protein structure. Total protein denaturation, by boiling in 2% SDS followed by an attempt to recover and renature protein activity will inevitably lead to the formation of a subset of species that do not achieve correct

refolding (about 20% by calculation of the activity of the intact A protein). In order to achieve regular packing into a crystal the protein must be virtually homogeneous. It was therefore desirable to produce the 64 kDa fragment directly as a gene product so that large amounts of the material could be purified in the absence of any 33 kDa fragment and under native conditions.

Chapter 4

Probing the limits of the breakage-reunion domain of the DNA gyrase A protein.

4.1 Introduction.

The results obtained in Chapter 3 suggest that the N-terminal 64 kDa of the DNA gyrase A protein is responsible for the breakage-reunion activity of the enzyme and that the C-terminal 33 kDa, whilst not essential for the enzymic activity, is required for stability of the DNA-protein complex. The fragments obtained from a protease digestion, however, are unlikely to be sufficiently homogeneous, and are therefore of limited use, for high resolution structural studies. There are many other possible protease cleavage sites within the protein and other fragments are likely to arise, although presumably at a relatively low rate since they are not apparent on a polyacrylamide gel. For the growth of protein crystals, however, it is generally considered wise to have the protein sample as free as possible from any contaminations, especially modified forms of the same structure which may inhibit regular crystal lattice formation. The problems encountered with the separation of the 64 kDa and the 33 kDa fragments from each other must vitiate this protease route as a method of preparing large amounts of either fragment for crystallization trials or other structural studies. The possibility of permanently altering at least some of the protein molecules by isolation of the fragments by SDS-polyacrylamide gel electrophoresis must be high; only a maximum of 80% of the molecules being able to refold correctly to give an enzymic activity; but even the refolded forms may not be homogeneous. It was thus decided to attempt to over-produce the 64 kDa protein as a direct gene product.

The *gyrA* gene has been recently cloned into a high level expression vector that allows tight control of its expression (Hallett *et al.*, 1990). Using this plasmid it is possible to mutate the sequence at the appropriate point so that a stop codon is introduced at the end of the 64 kDa N-terminal fragment. Thus the 64 kDa protein will be produced directly upon induction of the plasmid-bearing bacteria. The tight regulation of the gene in this plasmid should mean that protein will only be produced in the presence of inducer, so if the gene product is lethal, bacteria bearing the mutant plasmid will still

be viable. The engineered protein can then be purified without any contamination with the 33 kDa fragment.

It is possible that the trypsin cleavage site in the GyrA protein (at amino acid residue 572) occurs within a region linking the two domains. The protease-susceptible site may represent a distinct and unique site, or be part of a larger linking region between the N-terminal 'breakage-reunion' domain and the C-terminal 'stability' domain. If there is a considerable linker between the two functional structures then this raises the possibility of further deletions of the *gyrA* gene in order to define the domain limits. Nuclease digestion of the gene and expression of the truncated proteins is likely to lead to a much better understanding of the extent of the breakage-reunion domain. Any smaller functional proteins, that perhaps represent a more compact domain, produced by this method could also be candidates for structural studies.

4.2 Results.

(a) Genetically produced 64 kDa protein.

The construction of a plasmid that over-produces the N-terminal 64 kDa of the GyrA protein was performed as shown in Fig. 4.1. A 749 bp *PstI-KpnI* fragment of pMK90 (Mizuuchi *et al.*, 1984) was purified from an agarose gel and ligated into a *PstI/KpnI* cut M13mp19 vector (see Fig. 2.1). The 749 bp fragment encompasses nucleotides 1206 to 1955 (encoding amino acids 403 to 652) of the *gyrA* sequence. The sequence of the *gyrA* gene at the start of the 33 kDa fragment is shown below.

```
        568                572                576
  ...LysSerAlaAlaArgIleLysGluGluAspPheIle...
5'...AAATCTGCCGCACGTATTAAAGAAGAAGACTTTATC...3'
                    ↑_____ 33 kDa fragment
```

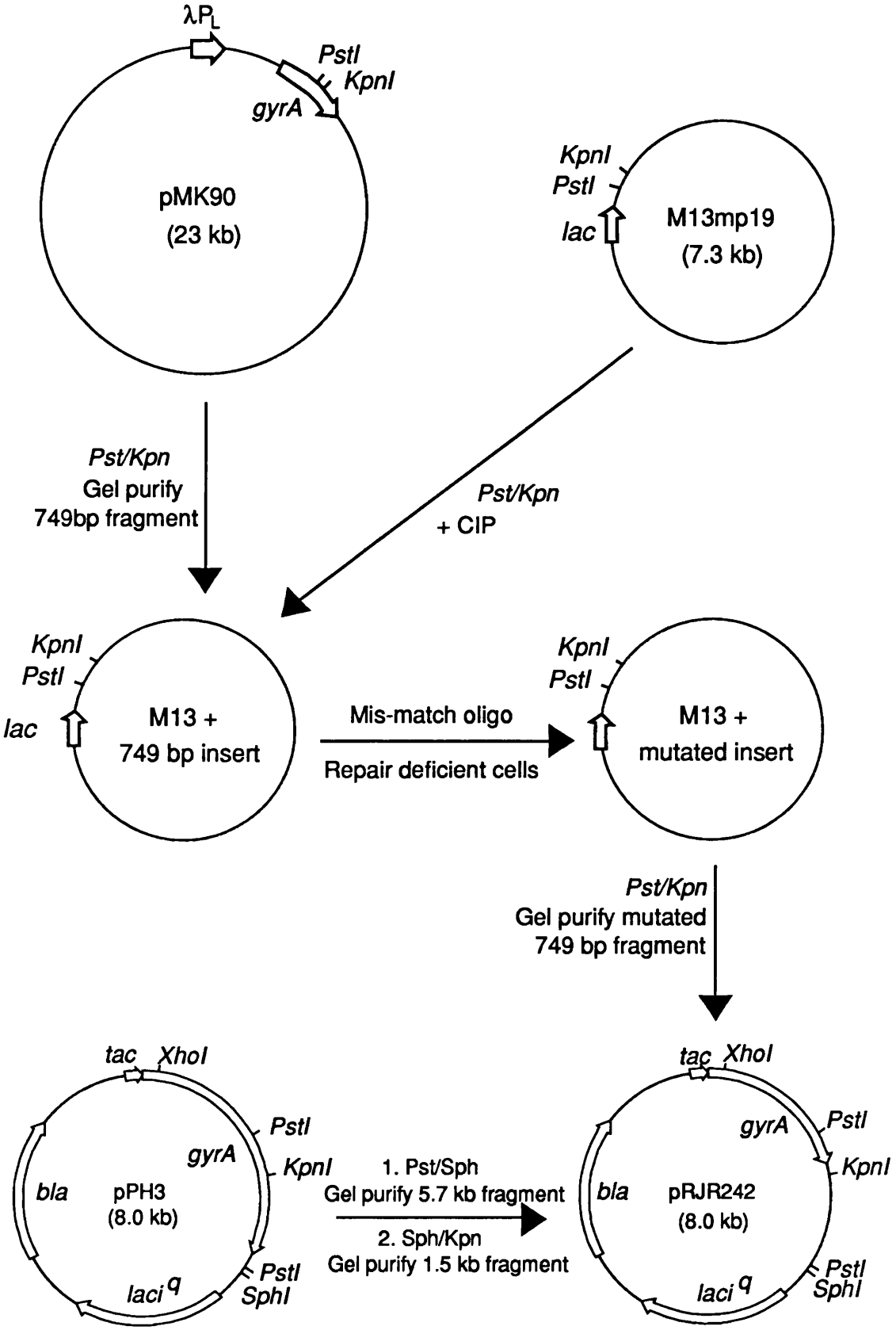


Fig. 4.1. Construction of pRJR242. A plasmid that over-produces the N-terminal 64 kDa of the DNA gyrase A protein (GyrA(1-573)).

To produce the 64 kDa fragment a stop codon is required to be inserted into this sequence. It was decided to perform this task by oligonucleotide-directed site-specific mutagenesis. Therefore a single-stranded oligonucleotide had to be designed such that a plasmid containing the oligonucleotide will have a stop codon in the appropriate place of the *gyrA* sequence. In order to maximise the probability of getting a mutant, and also having a sufficient melting temperature (Tm) difference to readily identify the oligonucleotide binding to the wild-type or mutant sequences during the screening of putative mutants, it was decided to attempt to change 2 bp in the native sequence. The closest place to the tryptic cleavage site where a two base change will lead to the insertion of a stop codon is directly after the start of the 33 kDa fragment. Mutation of AAA (encoding lysine 574) to TGA will result in a stop codon being placed into the sequence and in the correct reading frame. The following change was thus envisaged:

```
        568                 573
   ...LysSerAlaAlaArgIlesTOP
5'...AAATCTGCCGCACGTATTTGAGAAGAAGACTTTATC...3'
```

Therefore an oligonucleotide of the sequence indicate below was made:

```
3'  GCGTGCATAAACTCTTCTTCTG  5'
```

This single-stranded oligonucleotide was annealed to the single-stranded form of M13 which contained the 749 bp *PstI-KpnI gyrA* insert. After standard site-directed mutagenesis techniques (see section 2.10) resultant M13-containing bacterial colonies were probed with a [^{32}P]-labelled version of the above nucleotide. Fig. 4.2 shows an autoradiograph of 35 of the analysed colonies. The two darkest colonies were grown-up and large-scale M13 RF DNA preparations performed. The isolated DNA was then digested with *PstI* and *KpnI* and the resulting 749 bp fragment was gel purified. If mutation of the sequence had occurred then an *MseI* site would will be destroyed. That is, if the correct mutation occurs then the following change will take place: 5' GTATTAAAGA 3' will be converted to 5' GTATTTGAGA 3'. *MseI* cleaves the

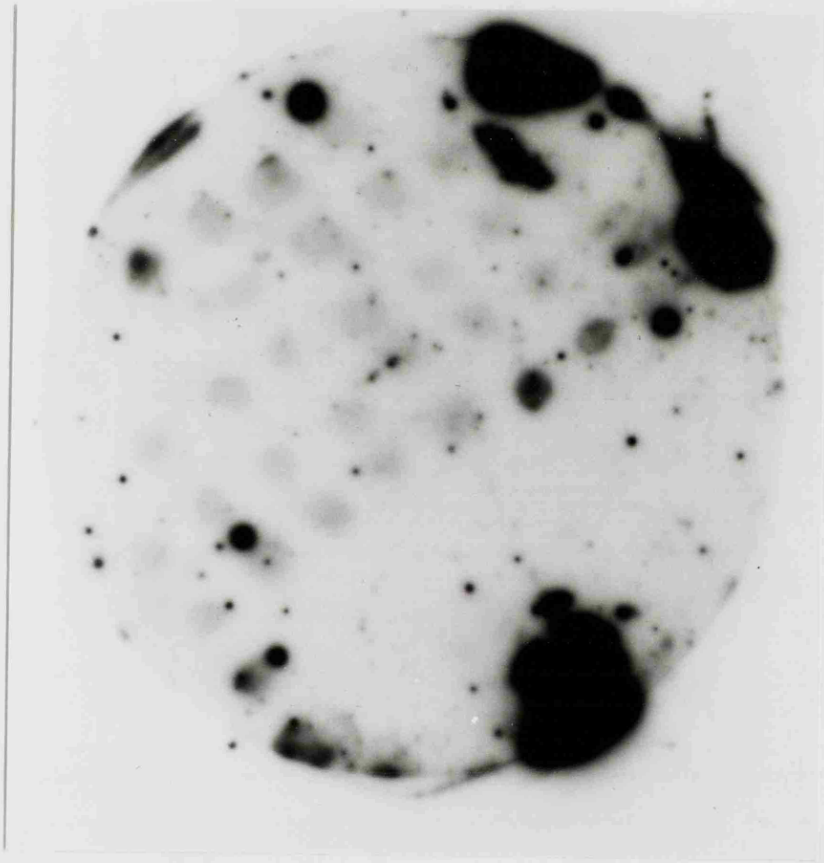


Fig. 4.2. Colony hybridisation. Nitrocellulose filter to which had been transferred the DNA from 35 bacterial colonies infected with M13mp19 containing the 749 bp *PstI-KpnI gyrA* fragment and subjected to oligonucleotide-directed site-specific mutagenesis as described in section 2.10. The DNA was then probed with [^{32}P]-labelled oligonucleotide. The two dark spots to the right of the centre of the filter were identified as putative mutants which could be further screened.

sequence 5' TTAA 3' and will thus cleave the native but not the mutant sequence. Cleavage of the native 749 bp *gyrA* fragment with *MseI* yields three fragments of sizes 438, 241 and 70 bp. These can be separated on a polyacrylamide gel. If the mutation has occurred at the correct site then fragments of sizes 438 and 311 bp will be generated. One of the putative positive gel purified 749 bp *PstI-KpnI* fragments retained all the fragments when digested with *MseI*, but one had lost the appropriate site (see Fig. 4.3). This clone was subjected to DNA sequencing using the universal sequencing primer in M13 to confirm that the correct mutation had actually occurred (see Fig. 4.4). The mutated sequence was indeed found to be correct. Approximately 350 bp can be read from this gel, including the *KpnI* site, the two base pair mutation and 50 bp beyond the mutation toward the *PstI* site at the 5' end of the fragment.

The mutated 749 bp fragment was then cloned back into a GyrA over-producing plasmid. Initially, this was pDH24 (Horowitz and Wang, 1987), and later into pPH3 (Hallett *et al.*, 1990). Digestion of the plasmid pDH24 with *KpnI* and *PstI* generated three fragments of approximate sizes 3.1 kb, 2.3 kb and 749 bp (arising from the *PstI* and *KpnI* sites in the *gyrA* gene and a *PstI* site in the *bla* gene). The two larger fragments were gel purified and ligated to the mutated 749 bp fragment produced above. Transformants were plated out onto ampicillin-containing media so that plasmids containing correctly reformed *bla* genes would be selected. The resultant plasmid (pRJR241) was found to over-produce a protein of approximate molecular mass 64 kDa, both in the presence and absence of IPTG inducer (see Fig. 4.5). This effect is also observed with GyrA production from pDH24 itself since the *gyrA* gene is somewhat downstream of the *tac* promoter and not tightly controlled.

Digestion of the plasmid pPH3 with *KpnI* and *PstI* generated three fragments of approximate sizes 5.7 kb, 1.5 kb and 749 bp which arise from the *KpnI* and *PstI* sites in the *gyrA* gene and a *PstI* site from the polylinker site of the original pTTQ18 vector (the *bla* gene of pTTQ has been silently mutated so that it no longer contains a *PstI* site

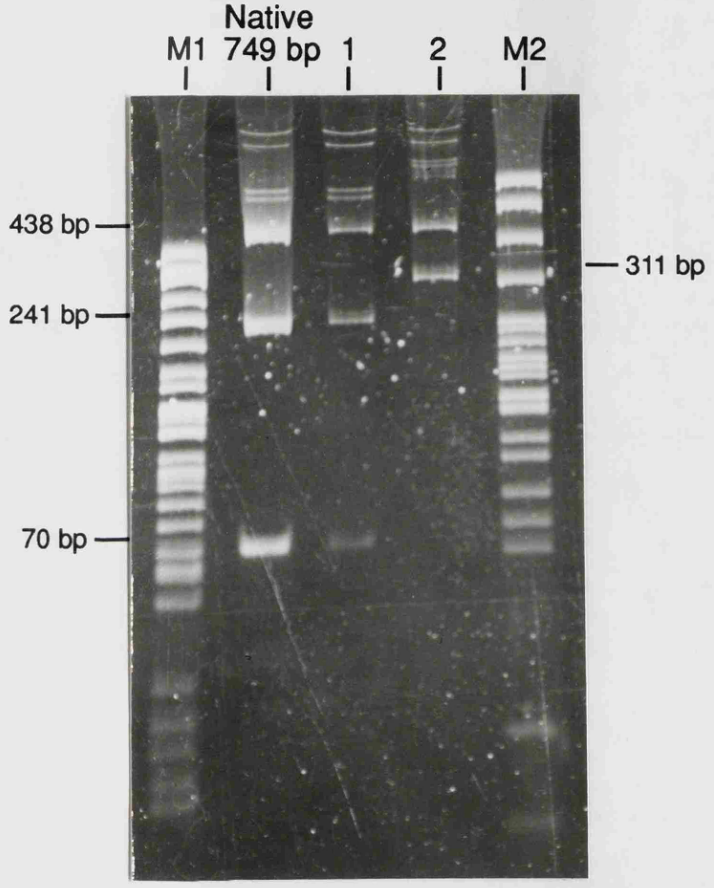


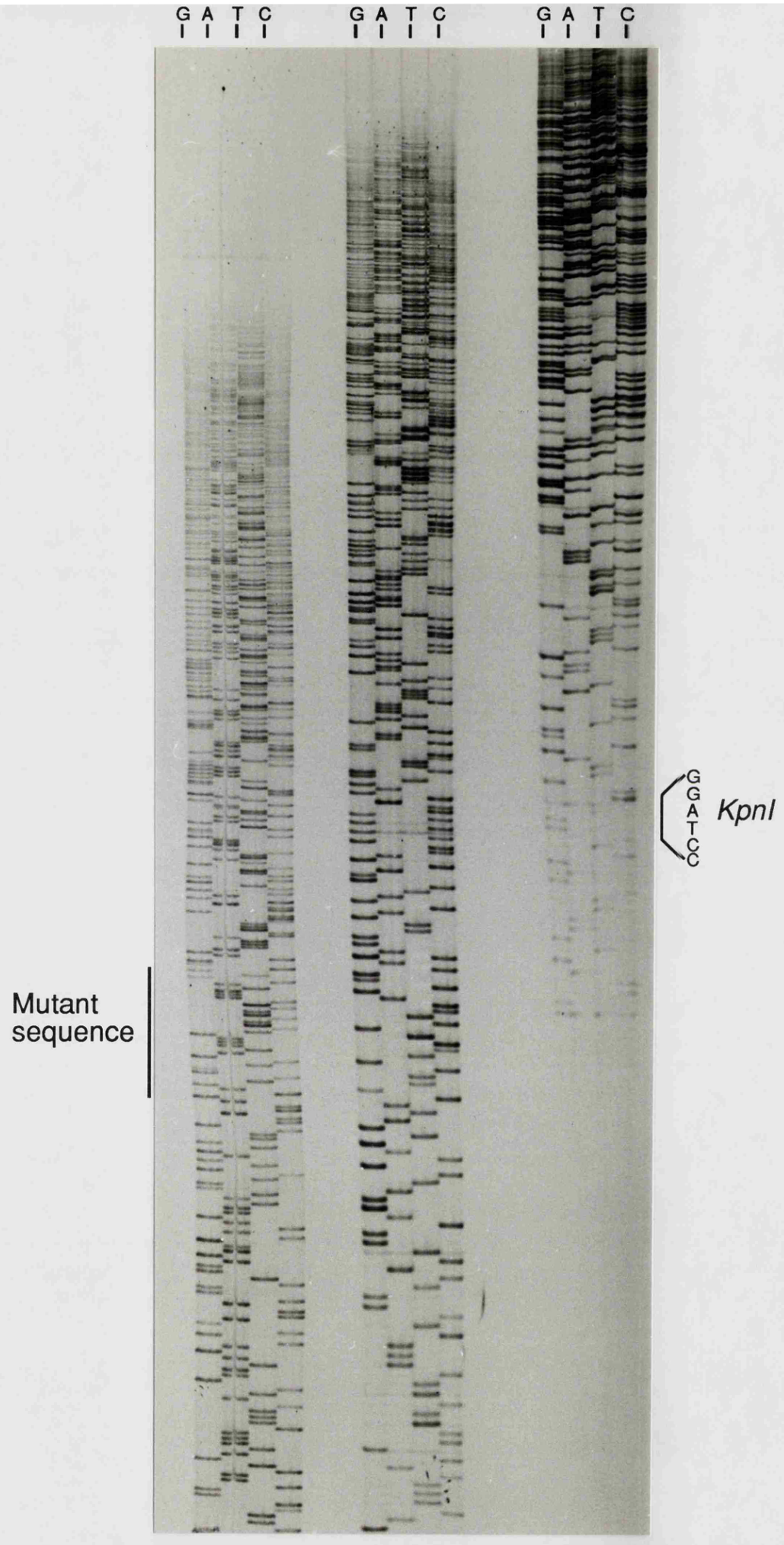
Fig. 4.3. *MseI* digests of two possible mutants. *MseI* restriction digestions of the native 749 bp *PstI-KpnI gyrA* fragment and that isolated from two putative mutants (1 and 2). Lanes M1 and M2 indicate pBR322/*HhaI* and pBR322/*MspI* markers respectively.

Fig. 4.4. Sequencing of the mutated 749 bp *PstI-KpnI gyrA* fragment in M13mp19 (facing page). The sequence was obtained using the universal M13 sequencing primer. The three sets of tracks represent loading onto the gel at different times so that more sequence could be read from the single gel. G, A, T, and C have been used to indicate the samples containing the appropriate dideoxynucleotide. The *KpnI* site is indicated and the mutant sequence (of the non-coding strand) indicated is shown below:

5' GCAGTCGGTCGATAAAGTCTTCTTCT**CA**AATACGTGCGGCAGA 3'

The equivalent *gyrA* sequence (Swanberg and Wang, 1987) is:

5' GCAGTCGGTCGATAAAGTCTTCTTCT**TT**AATACGTGCGGCAGA 3'


G A T C
G A T C
G A T C
Mutant sequence
G
G
A
T
C
C
KpnI

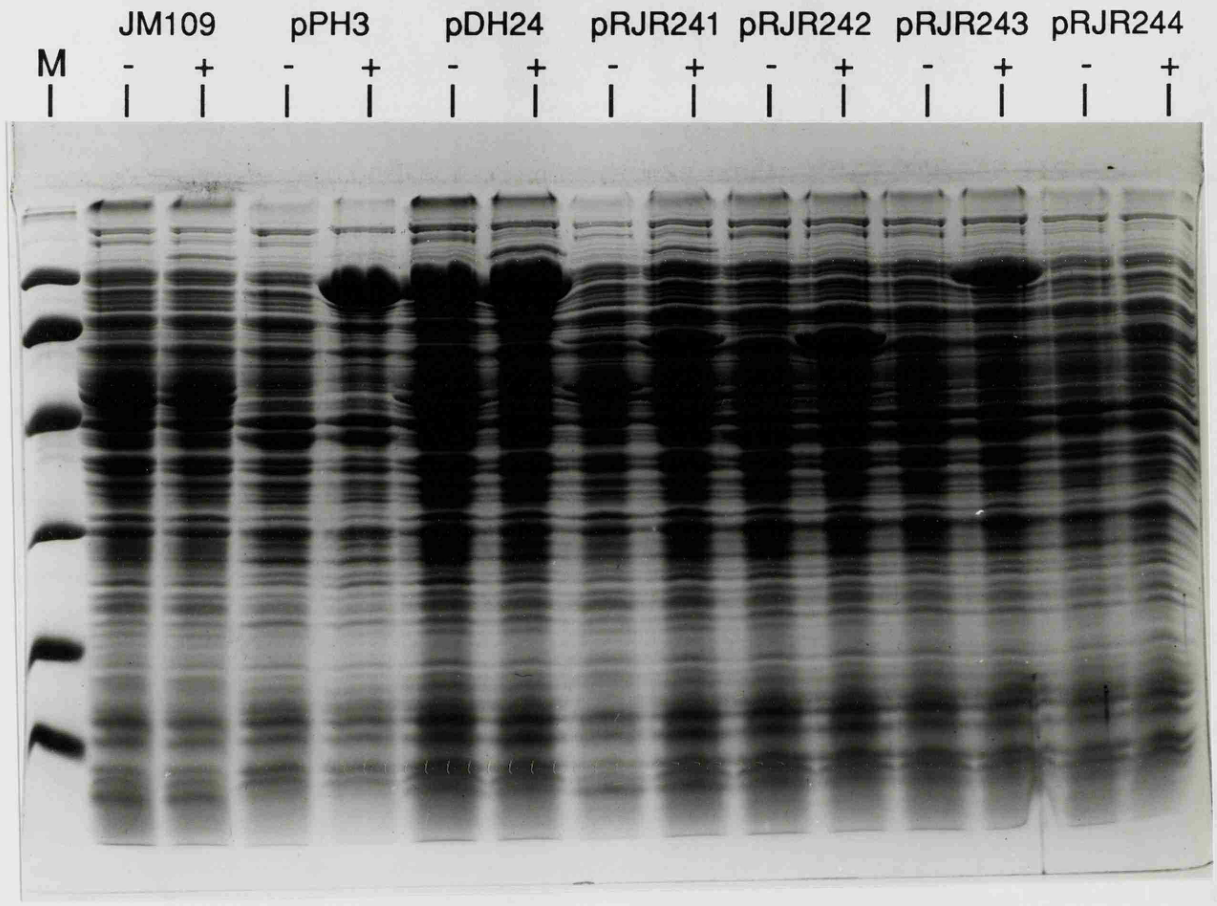


Fig. 4.5. Cell extracts of *E. coli* containing various plasmids grown in the absence or presence of the inducer IPTG. *E. coli* (all strain JM109) containing the plasmids indicated were grown in 5 ml of LB-broth. At an optical density ($A_{595\ nm}$) of 0.5, IPTG was added to a final concentration of 0.05 mM. Growth was then continued for a further 4 hr before cells were harvested, sonicated and soluble protein run out on a 12.5% polyacrylamide gel containing 0.1% SDS. The plasmids pDH24 and pPH3 over-produce the native 97 kDa GyrA protein. pRJR242 over-produces the 64 kDa GyrA(1-573) N-terminal fragment. pRJR243 over-produces GyrA(7-875) and pRJR244 over-produces GyrA(7-573).

(Stark, 1987)). Attempts to ligate the mutated 749 bp *PstI-KpnI* fragment to the gel purified 5.7 kb and 1.5 kb fragments proved unsuccessful. The transformants produced mainly consisted of the circularised 5.7 kb fragment that contained the intact *bla* gene (data not shown). Even dephosphorylation of the 5.7 kb fragment and using this in a ligating reaction did not have the effect of producing the correct sized plasmid. Therefore the following cloning strategy was devised (see Fig. 4.1). The plasmid pPH3 was digested with *PstI* and *SphI* and the 5.7 kb fragment gel purified. The plasmid was also separately digested with *KpnI* and *SphI* and the 1.5 kb fragment was gel purified. The two fragments were then mixed with the mutated 749 bp *PstI-KpnI* fragment and a ligation reaction performed. Consequently each of the three DNA fragments in the ligation reaction had different cohesive ends. The resulting plasmid (pRJR242) was identical in size to pPH3 and gave an expected restriction digestion map (data not shown).

(b) Purification and characterisation of GyrA(1-573).

Plasmid pRJR242 was used to over-produce the 64 kDa protein GyrA(1-573)*. Under optimal conditions, the protein was estimated, by densitometry, to constitute between 10 and 20% of the total soluble protein produced from plasmid-containing, IPTG-induced JM109 cells (see Fig. 4.5). This level of expression is not as great as that obtained for the intact GyrA protein from pPH3 which has been estimated at 40% of the soluble protein (Hallett *et al.*, 1990), but, owing to the size difference between GyrA and GyrA(1-573), the relative amounts of each protein observed by Coomassie staining are approximately equal. The promoter in pRJR242 is identical to that of pPH3 as judged by DNA sequencing of the first 90 nucleotides of the *gyrA* gene and approximately 100 bp of the 5' upstream region (data not shown).

---

* In the designation of the truncated GyrA proteins, the numbers refer to the amino acids encoded by the gene, and take no account of whether the N-terminal methionine residue is retained or not.

GyrA(1-573) was purified to virtual homogeneity using the following steps: ammonium sulphate precipitation, Heparin-Sepharose, FPLC Mono Q and FPLC Phenyl Superose chromatography (See Fig. 4.6). The purification process was followed at each stage by running peak protein fractions on SDS-polyacrylamide gels. After the final purification step the 64 kDa GyrA(1-573) was judged to be greater than 98% pure by scanning densitometry.

The ability of the truncated GyrA(1-573) protein to perform some of the reactions of DNA gyrase were measured. The supercoiling ability of the truncated protein was found to be very poor. Incubation of GyrA(1-573) with the gyrase B protein and relaxed pBR322 resulted in the introduction of negative supercoils at a very slow rate; the reaction being highly distributive in nature (see Fig. 4.7). A specific activity for supercoiling by the truncated protein can be calculated to be $8x10^2$ U/mg. This is quite low when compared to that of the intact GyrA protein ($1x10^6$ U/mg) and the trypsin-treated A' protein ($1x10^5$ U/mg), but is similar to that of the 64 kDa GyrA tryptic fragment that was purified from an SDS-polyacrylamide gel ($1x10^3$ U/mg; see chapter 3). It is apparent from Fig. 4.7 that on increasing the GyrA(1-573) protein level, the amount of supercoils introduced actually decreases. This could be due to one of several effects. For example, the reaction was carried out over a prolonged period of time (18 hr), so it is possible that during this time all the ATP in the reaction has been hydrolysed and that relaxation occurs preferentially. Incubation of the protein under conditions where relaxation by the intact GyrA protein is known not to occur (standard supercoiling conditions except for 6.5 mM spermidine and 1.8 mM $MgCl_2$; A.D. Bates and A. Maxwell, personal communication) no supercoiling activity of GyrA(1-573) could be noted (Fig. 4.7). It is possible that, since the rate of reaction of GyrA(1-573) is slow under optimal GyrA conditions, the reaction is simply too slow to see under these altered conditions. Another possible explanation for the apparent decrease in supercoiling could indicate that there may be some contamination

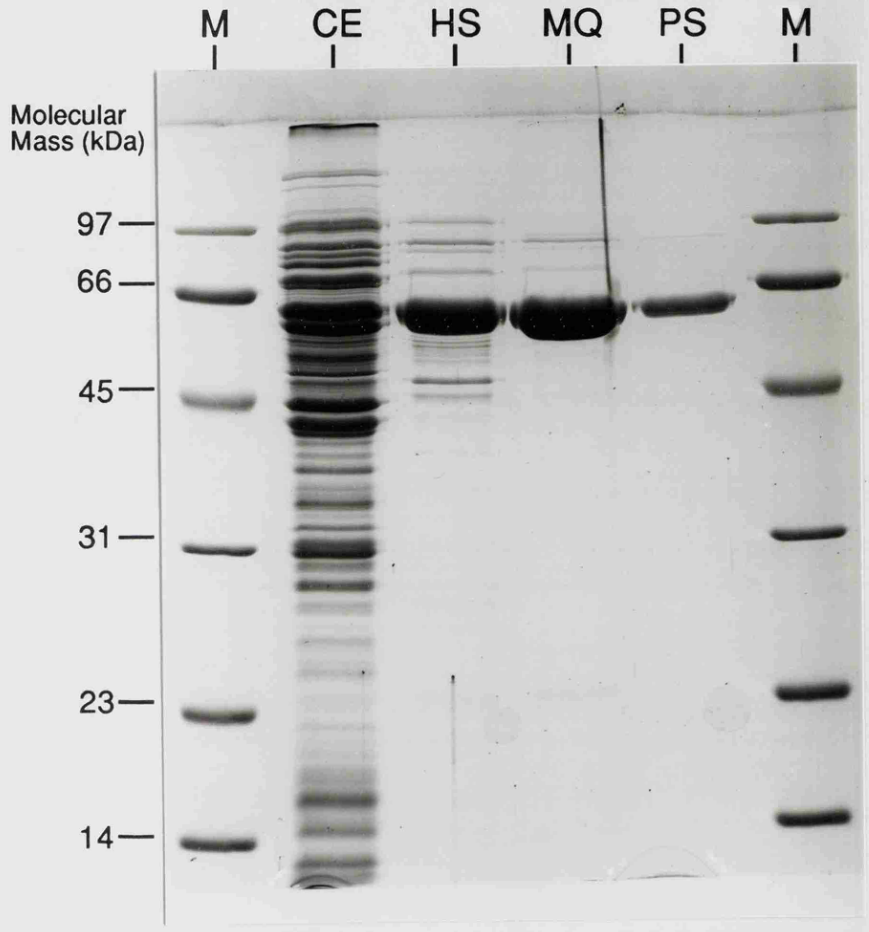


Fig. 4.6. Purification of GyrA(1-573). A 12.5% SDS-polyacrylamide gel showing the various purification stages used for the GyrA(1-573) protein. CE, HS, MQ and PS have been used to indicate the cell extract and the Heparin-Sepharose, FPLC Mono Q and the FPLC Phenyl Superose pools respectively. M indicates molecular mass markers whose sizes are also indicated.

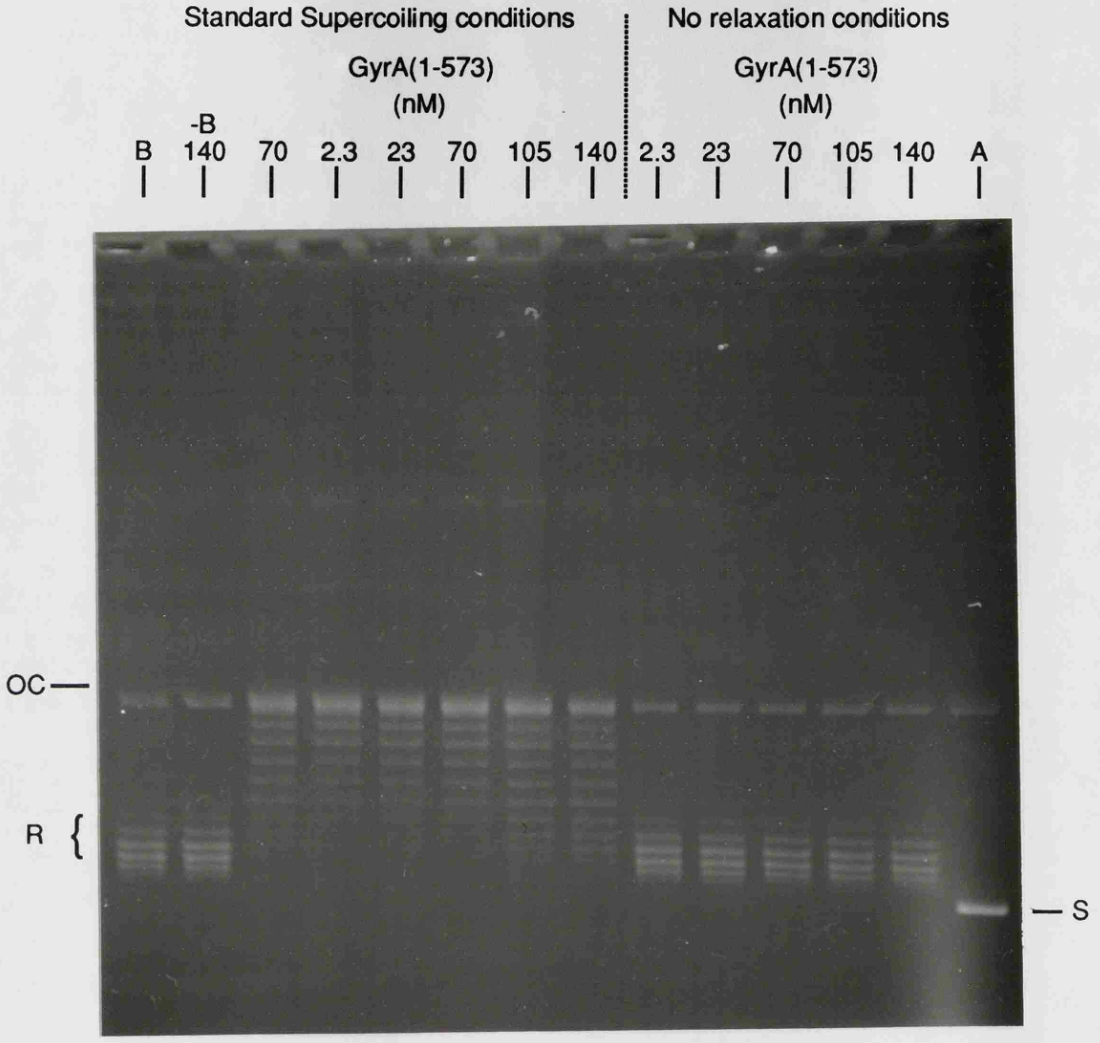


Fig. 4.7. The supercoiling reaction of GyrA(1-573). The GyrA(1-573) was incubated, at the concentrations indicated, under standard supercoiling reaction conditions, or no relaxation conditions. All samples contained the GyrB protein (at 90 nM), except for that marked -B, and the lane indicated A contained the GyrA protein (at 50 nM). Samples were incubated at 25°C for 18 hr and then 10 µl aliquots run on a 1% agarose gel (in TPE buffer) containing 1.5 µg/ml chloroquine. OC, R, and S have been used to indicate the positions of open-circular, relaxed, and supercoiled pBR322 DNA respectively.

in the GyrA(1-573) preparation that is inhibiting the supercoiling reaction at higher protein concentrations.

It is a possibility that the supercoiling reaction observed by the GyrA(1-573) protein is due to a low level of contamination of intact GyrA protein. However a number of lines of evidence tend to suggest that this is not the case. Firstly, the specific activity of the GyrA(1-573) protein is very similar to that of the gel purified 64 kDa tryptic GyrA fragment. Secondly, over-loading SDS-polyacrylamide gels with GyrA(1-573) does not lead to the appearance of a band at 97 kDa (the size of GyrA), and probing such gels with anti-GyrA antibodies does not reveal the appearance of a 97 kDa protein (data not shown). Thirdly, other C-terminal deletion mutants can be isolated that have no supercoiling activity (see below). Therefore, although very low level contamination cannot be ruled out, it would seem unlikely.

The 64 kDa tryptic fragment of the GyrA protein was isolated by excision from an SDS-polyacrylamide gel and the resultant protein renatured (see Chapter 3). It is feasible that the denaturation and renaturation process could have some effect on the activity of the protein. It was therefore decided to completely denature GyrA(1-573) and refold the protein to see if any effect on the activity of the protein could be observed. Samples of the truncated protein were precipitated with acetone and the pellet resuspended in buffer containing 6M guanidine hydrochloride. The protein was then allowed to renature by dilution with buffer. Samples of the denatured/refolded protein were then tested for their ability to perform the supercoiling reaction. It was found that the renatured protein had a supercoiling activity approximately equal that of the 'native' GyrA(1-573) protein (see Fig. 4.8). It can therefore be concluded that this denaturation and refolding process has little or no effect on the activity of the protein. Therefore the protein must be able to refold into the correct conformation to perform the supercoiling reaction after complete unfolding. It is also unlikely that the

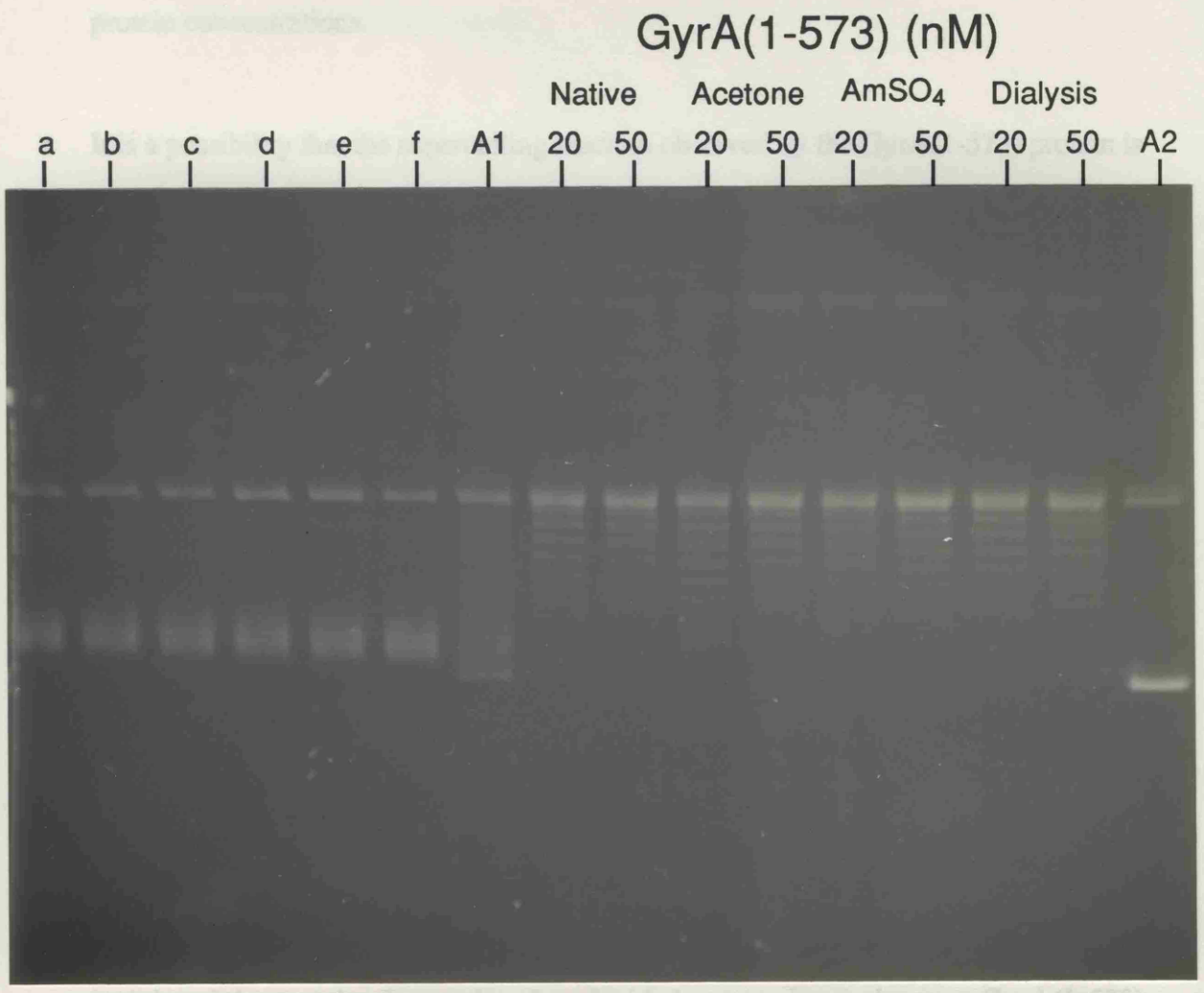


Fig. 4.8. Supercoiling of GyrA(1-573) that had been denatured and refolded. GyrA(1-573), at the concentrations indicated, was incubated with the GyrB protein and relaxed pBR322 DNA under standard supercoiling conditions at 25°C for 17 hr. 10 µl aliquots of each sample were run on a 1% agarose gel in TPE buffer containing 1.5 µg/ml chloroquine. The GyrA(1-573) protein was either in its native form, or had been precipitated with either acetone or ammonium sulphate, denatured and allowed to refold. Alternatively a sample of GyrA(1-573) had been dialysed into Enzyme buffer containing 6 M guanidine hydrochloride and then back into Enzyme Buffer. All samples, except for those labelled a, and c-f, contained GyrB protein (at 90 nM). Lanes c, d, e, and f contained native, acetone precipitated, ammonium sulphate precipitated and dialysed GyrA(1-573) respectively at 50 nM in the absence of GyrB. Lanes A1 and A2 contained native GyrA protein at 0.2 and 2 nM respectively.

supercoiling activity of the gel-purified 64 kDa GyrA tryptic fragment is attributable to the unfolding and refolding process.

The ability of GyrA(1-573) to relax negatively supercoiled pBR322 DNA was investigated (see Fig. 4.9). It was found that the truncated protein was able to relax the DNA, albeit at a very low rate when compared to the intact GyrA protein. Reaction mixtures had to be incubated for several hours before an appreciable change in the linking numbers of the DNA topoisomers could be noted (Fig. 4.9). Under the conditions used for these experiments, a relaxation end-point could not be noted. So it is not possible to say whether, if after relaxation had gone to completion, the DNA is wrapped about the enzyme with a positive superhelical sense (see chapter 3 for the effect of DNA wrapping with the A' protein). The denatured and refolded form of GyrA(1-573) also showed relaxation activity (Fig. 4.9).

Decatenation of catenated double-stranded DNA circles by DNA gyrase requires approximately 100-fold more enzyme to see the products than does the supercoiling reaction. GyrA(1-573) was tested for its ability to perform the decatenation reaction, but none could be noted even after prolonged incubation (8 hr; data not shown). It can therefore be assumed that either the enzyme lacks this activity or that the reaction is too slow to be followed using this assay. Since GyrA(1-573) is able to perform the ATP-dependent supercoiling reaction, it must be reasonable to assume that it will also carry out the decatenation reaction; strand-passage and ATP-dependence are common to both reactions. So it must be assumed that GyrA(1-573) will carry out decatenation of DNA, but that the assay used for this reaction is not sufficiently sensitive to detect any decatenated products.

GyrA(1-573) was tested for its ability to perform quinolone-directed cleavage of DNA (see Fig. 4.10). It was found that the truncated protein was able to perform the cleavage reaction with only slightly reduced efficiency when compared to the intact

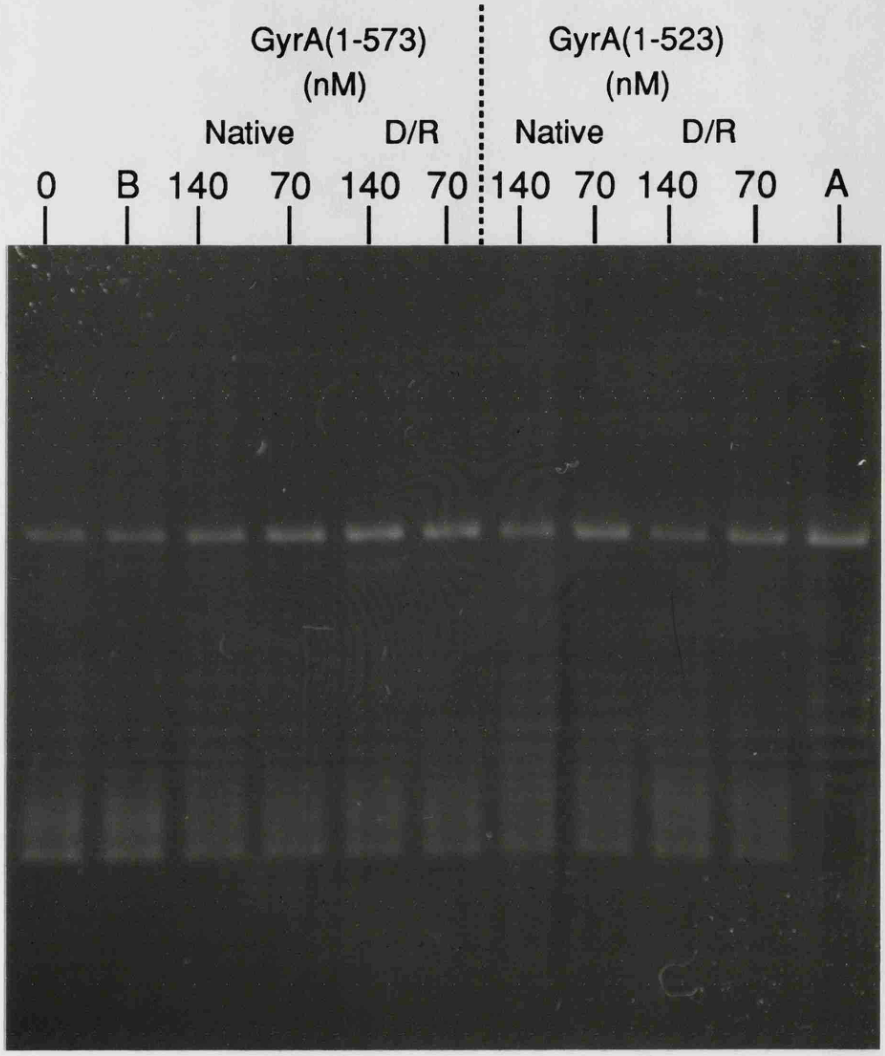


Fig. 4.9. Relaxation of supercoiled pBR322 by GyrA(1-573) and GyrA(1-523). GyrA(1-573), GyrA(1-523) and the acetone precipitated denatured and refolded (D/R) products, at the concentrations indicated, were incubated with the GyrB protein and supercoiled pBR322 DNA at 25°C for 8 hr. All samples, except for that labelled 0, contained GyrB protein (at 90 nM) and, where indicated, the GyrA protein (at 50 nM) was added. 10 µl aliquots of each sample were run on a 1% agarose gel containing 2.5 µg/ml chloroquine and run in TPE buffer.

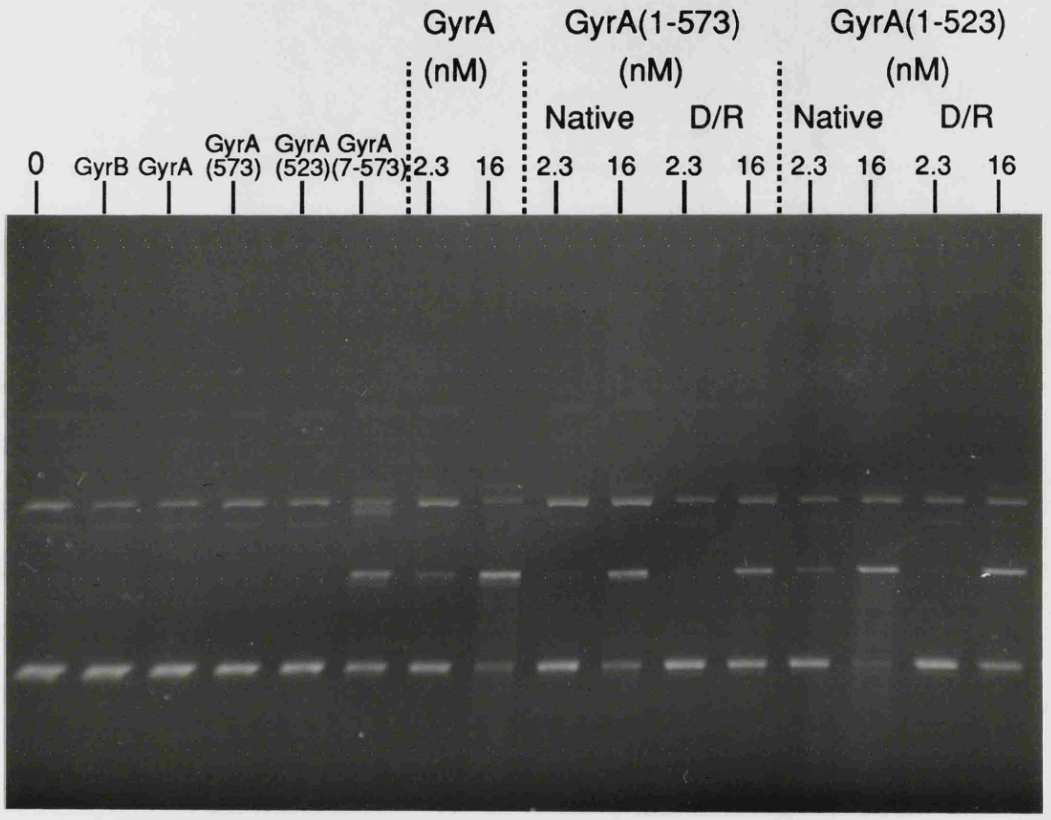


Fig. 4.10. CFX-directed cleavage of supercoiled pBR322 by GyrA(1-573) and GyrA(1-523). GyrA, GyrA(1-573) and GyrA(1-523), in the presence of an equimolar amount of the GyrB protein, were incubated at the concentrations shown with supercoiled pBR322 at 25°C for 1 hr. Samples were then treated with SDS and proteinase K as described (section 2.13) and then run out on a 0.8% TAE agarose gel. Lanes labelled GyrB, GyrA, GyrA(573) and GyrA(523) each contained samples of the appropriate protein (at 50 nM) in the absence of the other gyrase subunit. The lane GyrA(7-573) contains a crude extract from cells which over-produce that protein.

GyrA protein. Quantification of the efficiency of cleavage is not easy, but from the appearance of the linear DNA species and then its subsequent rate of degradation, it would appear that GyrA(1-573) is somewhat less efficient than intact GyrA (by less than a factor of two), but this difference is probably within the limits of experimental error. So, all the necessary amino acid residues are present in the first 573 amino acids of the gyrase A protein to allow efficient breakage of the DNA backbone.

(c) C-terminal deletion series of the GyrA protein.

In order to more accurately map the breakage-reunion domain of the DNA gyrase A protein it was decided to generate truncated *gyrA* genes that produce GyrA proteins of different sizes. The general principle behind this was to create fragments of the *gyrA* gene of differing lengths and clone them into a vector that will allow the production of the protein up to the end of an appropriate fragment. Therefore the first stage was to construct a vector that will allow the fragments to be cloned into it, and stop the translation of the produced mRNA transcript at a defined point.

The construction of plasmid pRJR133 is shown diagrammatically in Fig. 4.11. The plasmid pPH3 was cleaved with *KpnI* (which cuts the *gyrA* sequence some 300 bp downstream of the site that is mutated to yield GyrA(1-573); see above) and *SphI*. The resulting 6.5 kb fragment was gel purified from the 1.5 kb fragment that encoded the 3' end of the gene. Two (19 mer) oligonucleotides were made that had the following sequence:

```
      5'  TACGTAGATAGATAGCATG  3'
3'  CATGATGCATCTATCTATC  5'
```

The linker has *KpnI* and *SphI* overhanging ends so that ligation to the 6.5 kb *KpnI-SphI* fragment of pPH3 can occur. The resultant 6.5 kb plasmid (pRJR133) was sequenced directly as double-stranded DNA from the universal sequencing primer site

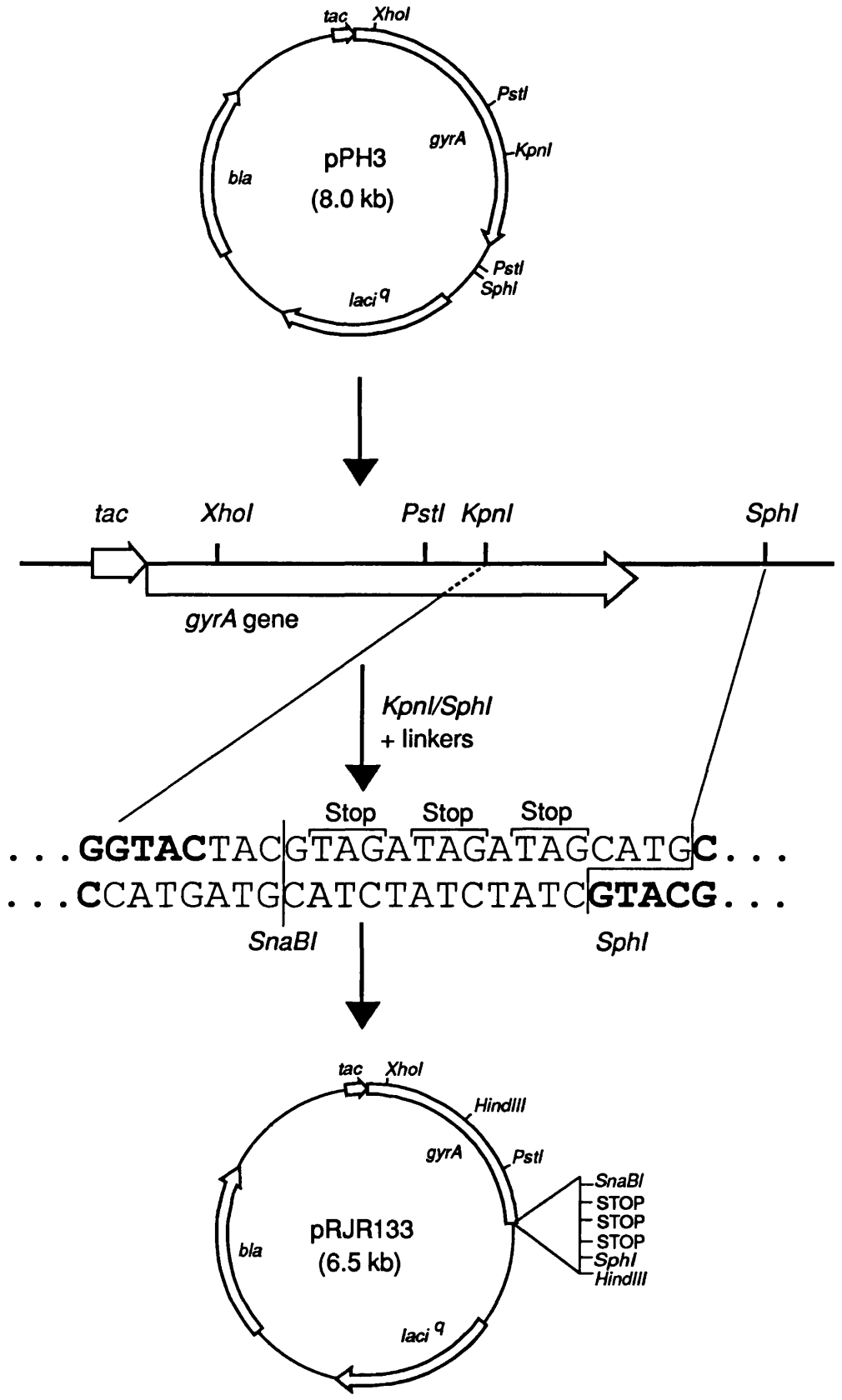


Fig. 4.11. Construction of the plasmid pRJR133. This plasmid encodes the first 73 kDa of the DNA gyrase A protein and three stop codons, each in a different reading frame, directly 3' of a *SnaBI* restriction site.

some 50 bp 3' to the *SphI* site (data not shown). A total of approximately 250 bp could be read as clear sequence and found to be consistent with a single copy of the linker being inserted. The linker also encodes a *SnaBI* restriction enzyme site (underlined in the above sequence). *SnaBI* is a blunt cutter that cleaves in the centre of its recognition sequence. Directly after the *SnaBI* site are three stop codons (TAG), each in a different reading frame. Therefore any blunt-ended *gyrA*-encoding DNA fragment can be ligated to the *SnaBI*-cut linker and, no matter what reading frame the fragment is in, a stop codon will be introduced into the transcript. In some cases, a few extra amino acids may be fused to the C-terminal end of the resulting protein depending on which stop codon is utilised, which itself is dependent on the reading frame of the nuclease-treated fragment. If the first stop codon is used then a single base (a G from the *SnaBI* site) will be added to the 3' end of the fragment. Therefore the following sequence will arise NNG TAG (N being the final nucleotides of the nuclease-treated fragment). This could result in a changed amino acid at the 3' end of the produced protein, however with the degeneracies in the genetic code it is possible that the correct sequence will still occur. If the second stop codon is utilised then the following sequence will arise NGT AGA TAG and will code for an unknown amino acid and then an arginine residue at the C-terminal end of the protein. If the reading frame of the nuclease-treated fragment is such that the third stop codon is used then the following 3' sequence will be found; GTA GAT AGA TAG. This would code for valine, asparagine, and arginine residues before the translation stop point is reached.

Fig. 4.12 diagrammatically shows the scheme for creating a large number of C-terminal deletion mutants of the *gyrA* gene. The plasmid pPH3 was linearised with *KpnI* and subsequently treated with the Bal31 nuclease for various times. The reaction was stopped and the DNA ends were blunted by the action of the single-strand specific mung bean nuclease. The blunted DNA was then cleaved with *XhoI* (which cuts 14 bp downstream from the 5' end of the *gyrA* gene) and the *XhoI*-blunt fragments were gel purified. These fragment appeared as a smear on an agarose gel

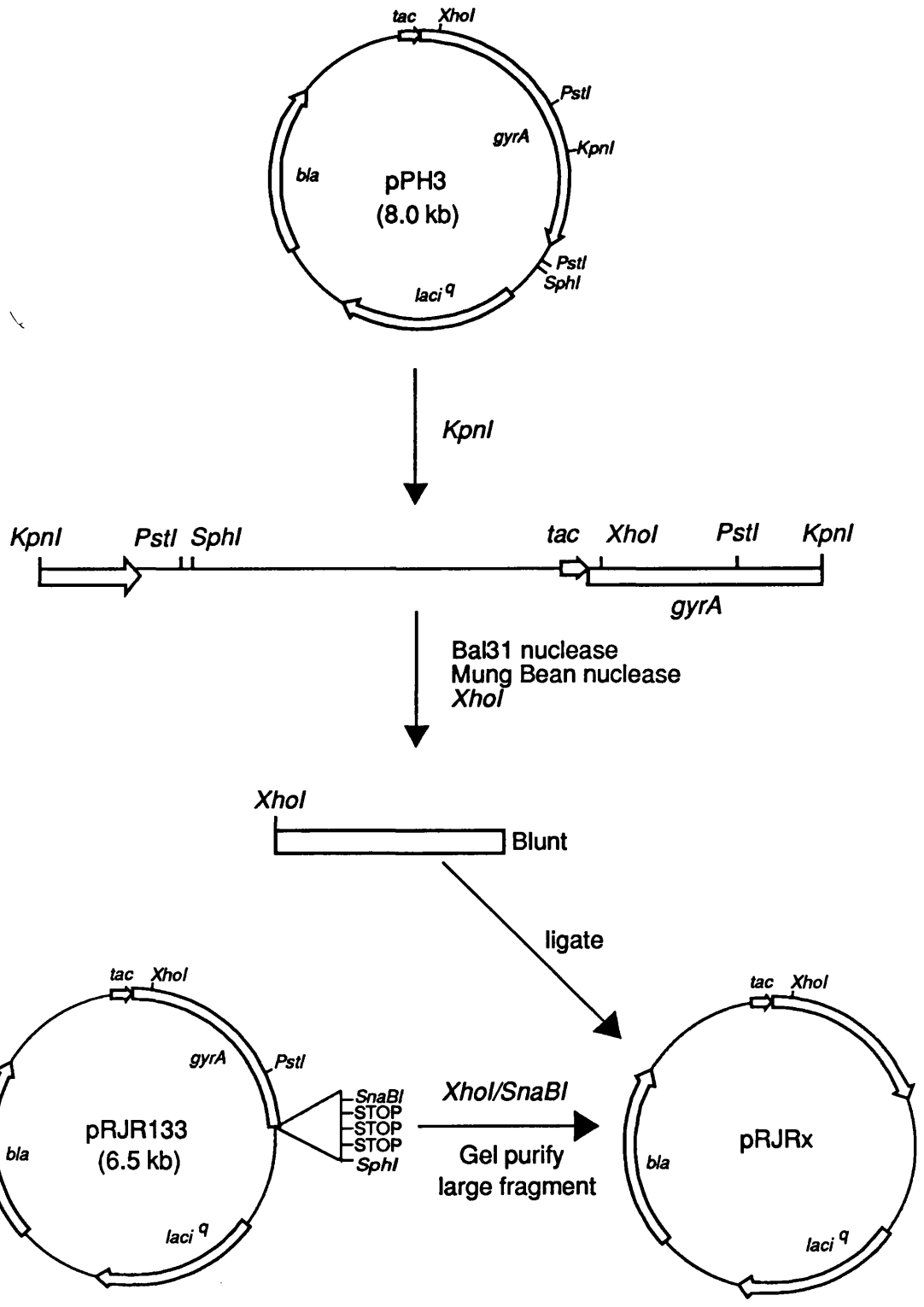


Fig. 4.12. Diagramatic representation of the way in which the Bal31-nuclease deletion series of the DNA gyrase A protein was constructed.

due to the variations in size of each of the time points (Fig. 4.13). Generally it was noted that the longer the incubation with the Bal31 nuclease the broader was the range of the fragments produced. The plasmid pRJR133 was cut with *XhoI* and *SnaBI* and the larger 4.6 kb fragment was gel purified. The 4.6 kb fragment was then ligated with the *XhoI*-blunt fragments and the resulting plasmids were transformed into competent JM109 *E. coli* cells. Over 5,000 colonies were produced.

The ability of the deletion series plasmids to over-produce protein was tested. A total of 256 of the colonies were picked into 5 ml of LB-Broth (containing ampicillin) and grown up at 37°C overnight in a shaking incubator. The following day, 5 µl of each culture was used to infect 1 ml of LB-Broth to which IPTG was added when an optical density ($A_{595\ nm}$) of 0.5 was obtained. After growth for a further four hours, the cells were collected by centrifugation and sonicated. A sample of the soluble protein was run on an SDS-polyacrylamide gel (see Fig. 4.14). Of the 256 colonies that were grown up only 24 obviously over-produced a soluble protein representing a truncated GyrA protein, and the majority of these were biased toward the shorter Bal31 nuclease incubation times. The plasmid DNA of all 24 over-producing colonies and 12 apparently 'non-producers' were isolated on a mini-scale and subjected to restriction enzyme analysis. Treatment of pRJR133 with *HindIII* yields two fragments of approximate sizes 5.3 kb and 1.3 kb. Deletion mutants produced by the above method show size reductions in the 1.3 kb fragment. The 24 of the 'producing' plasmids and 12 of the 'non-producing' plasmids were analysed by *HindIII* restriction digestion (see Fig. 4.15). In this way an idea of the size of the deletion could be estimated. 11 selected mutant plasmids (6 'producers' and 5 'non-producers') were cut with *SphI* and *SmaI* restriction enzymes and the smaller of the two fragments generated was gel purified. These fragments were then cloned into *SphI/SmaI* cut M13mp18 and subjected to DNA sequencing using the universal M13 primer. Table 4.1 summarises the data obtained for the deletion series of plasmids.

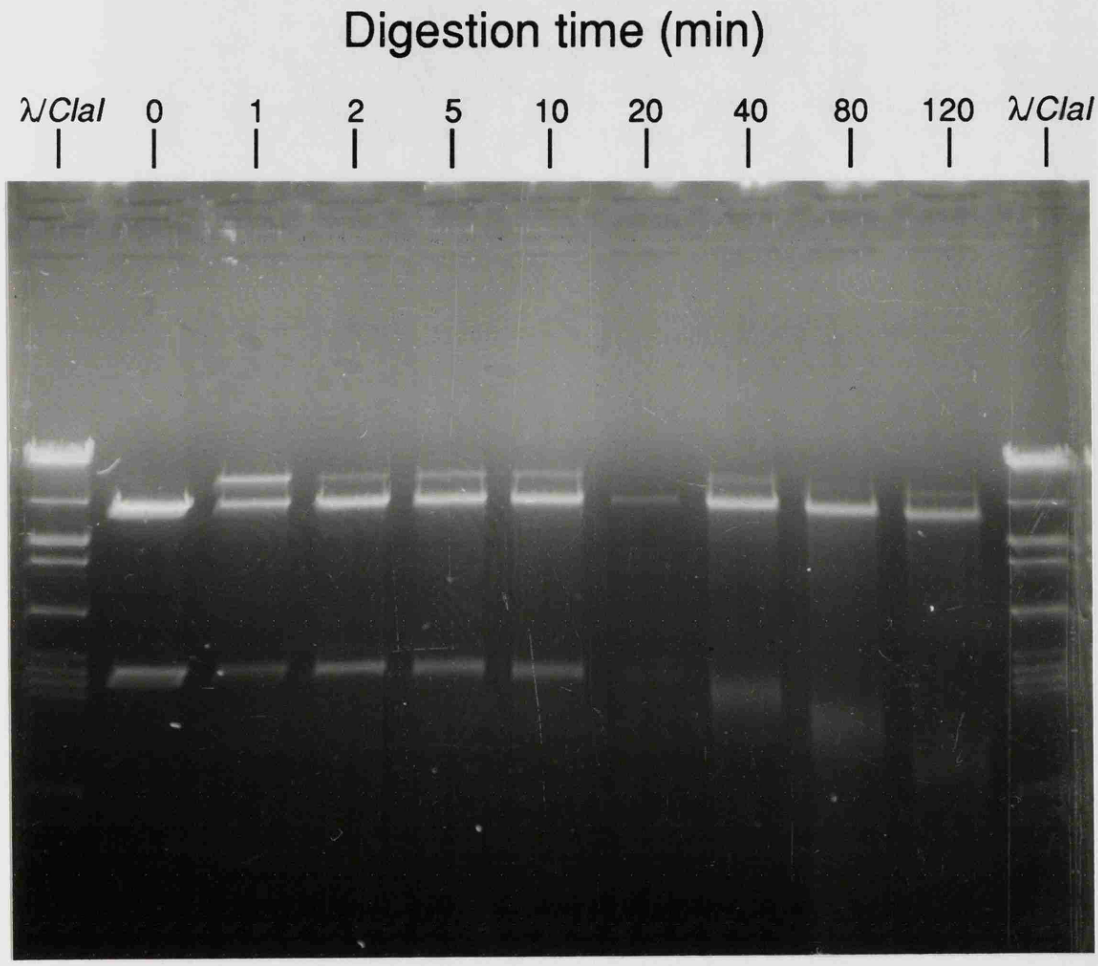


Fig. 4.13. Bal 31 nuclease digestion of linearised pPH3. *KpnI* linearised pPH3 was treated with the Bal 31 nuclease at 30°C under the conditions described in section 2.8. At the times indicated a sample was removed, extracted with phenol and precipitated with ethanol. The DNA pellets were resuspended in 5 µl TE and digested with *XhoI*. Samples were then run on a 1% agarose gel containing 1 µg/ml ethidium bromide.

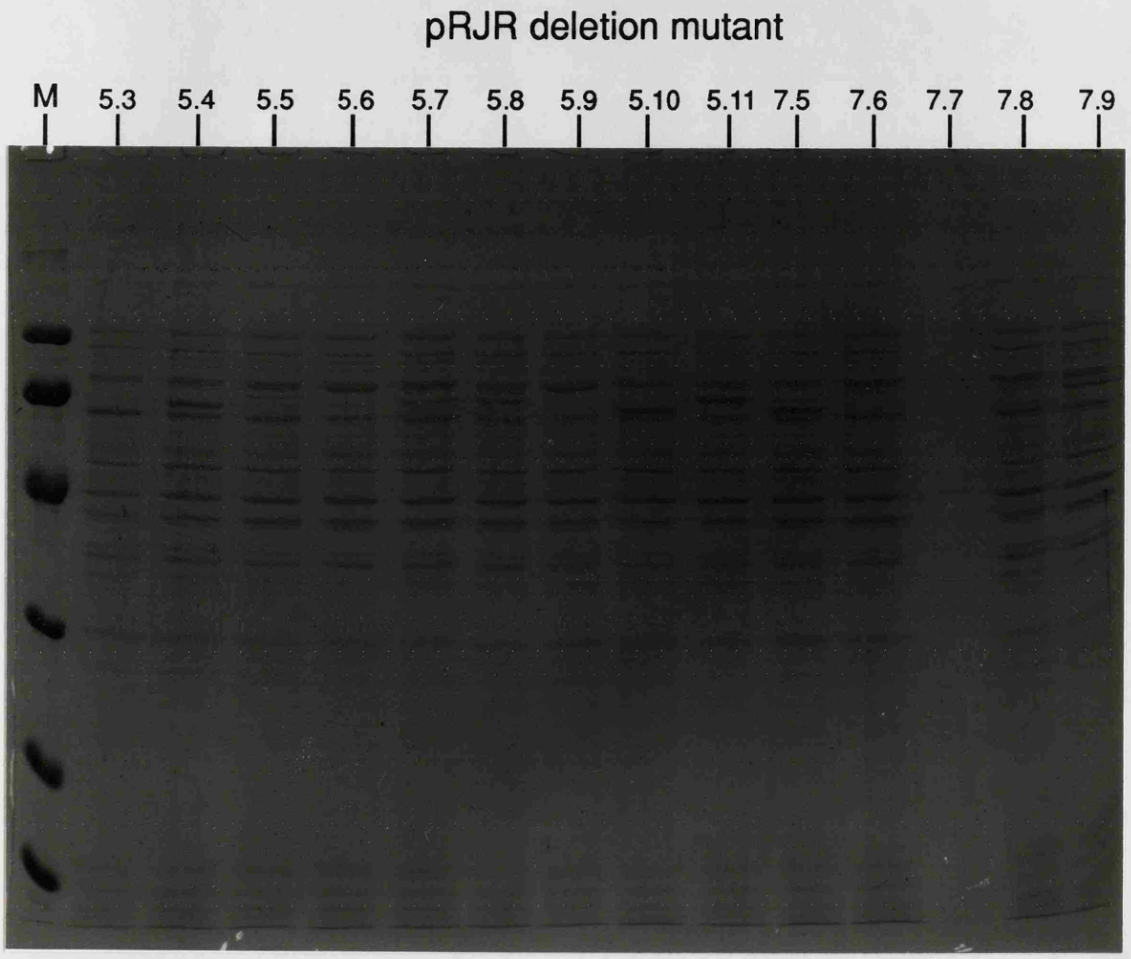


Fig. 4.14. Cell extracts from bacteria containing 3' deletions of the *gyrA* gene. Cultures (in 1 ml of LB-Broth) of JM109 cells containing a Bal31 nuclease-generated *gyrA* deletion plasmid were grown at 37°C until an optical density, $A_{595\ nm}$, of 0.5 was obtained. IPTG was then added to a final concentration of 0.05 mM and growth continued for a further 4 hr. The cells were then harvested, sonicated and a sample of the soluble protein visualised on a 12.5% SDS-polyacrylamide gel.

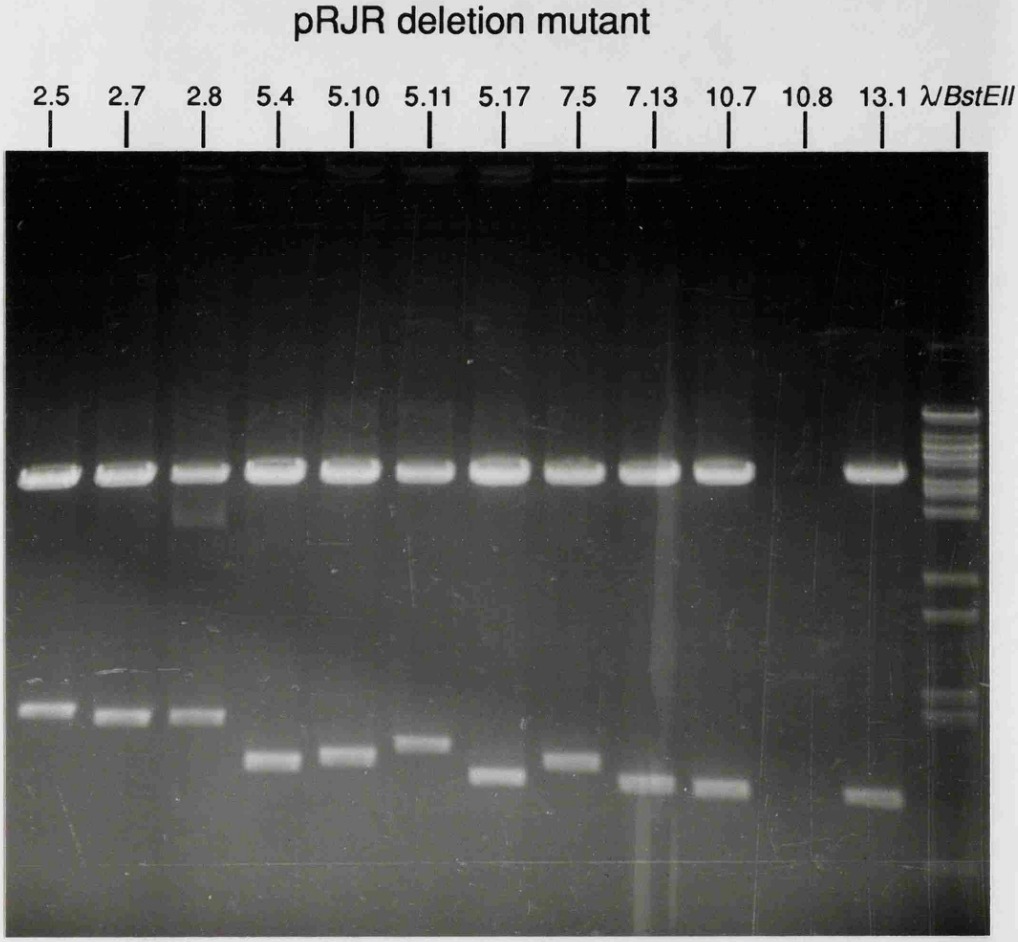


Fig. 4.15. *HindIII* restriction digests of *gyrA* 3' deletion mutant plasmids. A number of the Bal31 nuclease-generated *gyrA* deletion plasmids were digested with the restriction enzyme *HindIII* in order to estimate the size of the deletion made in each case. Digestion of pRJR133 (the construct into which the deletion mutants were cloned) with *HindIII* generates fragments of approximate sizes 5.3 kb and 1.3 kb. The 1.3 kb fragment encompasses the 3' end of the *gyrA* coding region in this construct. Therefore 3' deletions will be observed as reductions in the size of the 1.3 kb fragment. *λ/BstEII* is used to indicate the position of DNA size markers.

Plasmid pRJR	Protein est. size (kDa)	Approx gene size (bp)	Approx No of amino acids	Predicted Mr (kDa)	Sequenced gene size (bp)	Designation
2.5	70	1890	630	69.3		
2.7	67	1840	613	67.4		
2.8	67	1840	613	67.4		
5.4	63	1690	563	61.9		
5.10	64	1740	580	63.8	1712	GyrA(1-568+3)
5.11	66	1790	597	65.7	1772	GyrA(1-588+3)
5.17	60	1640	547	60.2		
7.5	63	1740	580	63.8		
7.13	60	1640	547	60.2		
7.21	60	1640	547	60.2		
7.22	-	1590	530	58.3	1628	GyrA(1-541+2)
7.23	59	1590	530	58.3		
7.24	64	1740	580	63.8		
7.29	-	1540	513	56.9		
10.7	60	1640	547	60.2	1610	GyrA(1-537)
10.8	60	1640	547	60.2		
10.17	-	1490	497	55.2	1547	GyrA(1-514+2)
10.18	58	1540	513	57.0	1568	GyrA(1-523)
10.19	-	1690	563	62.5		
13.1	60	1640	547	60.7	1601	GyrA(1-532+2)
13.8	62	1640	547	60.7		
13.15	-	1490	497	55.2		
13.20	58	1620	540	59.9		
13.25	60	1570	523	58.1		
13.27	-	1540	513	56.9	1493	GyrA(1-495+3)
13.28	68	1790	597	66.2		
13.29	60	1600	533	59.2	1577	GyrA(1-523+3)
16.4	-	1590	530	58.8		
16.13	63	1740	580	64.4		
16.16	-	1410	470	52.2	1385	GyrA(1-459+3)
16.23	66	1740	580	64.4		
19.1	-	1380	460	51.1	1358	GyrA(1-451+2)
19.13	-	1380	460	51.1		
19.22	60	1590	530	58.8		
30.5	-	1490	497	55.2		
30.7	-	1340	447	49.6		

Table 4.1. Sizes of the 3'-deletions of the *gyrA* gene and resultant proteins. The predicted protein molecular weights were calculated from the size of the *gyrA* gene estimated by restriction enzyme analysis.

The smallest of the *gyrA* genes that were able to obviously over-produce a truncated GyrA protein was derived from the plasmid pRJR10.18. The sequenced gene (1568 bp) should yield a protein of molecular mass 59 kDa, which was in close agreement with the molecular mass calculated from SDS-polyacrylamide gels. All of the sequenced plasmids whose *gyrA* genes were smaller than this did not yield over-produced protein. It may be that lower molecular mass proteins are unstable and are consequently degraded within the cell, or that the proteins are in an insoluble form (e.g. in inclusion bodies) that would not be picked up by this assay procedure. It is not the case, however, that there is a simple molecular mass cut-off below which obvious protein over-production will not occur. Some of the plasmids that should over-produce larger proteins are also not apparently over-produced. The starting plasmid for the cloning of truncated genes (pRJR133) should produce a 73 kDa protein (GyrA(1-654+1)). However there is no obvious over-production of such a protein in induced cell extracts that contain this plasmid.

The stop codon system that allows the cloning of *gyrA* fragments into the expression vector also means that some of the proteins may have a few extra amino acids attached to the the C-terminal end of the protein. Depending on the reading frame of the truncated fragment, a maximum of three extra amino acids may be added to the protein before a stop codon is reached. This does not, however, appear to have any effect on the stability of the produced proteins. For the sequenced plasmids there are examples of over-producers that have canonical sequence (10.7 and 10.18), two extra residues (13.1) and three extra residues (5.10, 5.11, and 13.29). Examples of the above can also be found for clones which are 'non-producers'. There would not appear to be a simple 'code' for the ability of over-production, e.g. there are examples of each of the different possible C-terminal ends in each class of protein. It is perhaps more likely that the secondary and/or tertiary structure of the protein will be more important in determining the stability of the protein. If the protein is truncated in the middle of an

α-helix or β-sheet region, for instance, then it is possible that the protein will not be able to fold correctly and will consequently be more susceptible to proteolysis.

The enzymic activities of some of the above proteins will be discussed later, after the construction of selected N-terminal deletion mutants of the GyrA protein have been described.

(d) N-terminal deletions of the GyrA protein.

To complement the C-terminal deletions of the DNA gyrase A protein detailed previously, a number of selected N-terminal deletions were also constructed. It was not considered practical to produce an N-terminal deletion series by the nuclease digestion method as described above. For the C-terminal deletion series it was possible, by inserting truncated DNA fragments directly before a series of stop codons, to ensure that all the genes were in the correct reading frame, with only the possibility of at most three foreign amino acids being placed at the C-terminal end of the truncated protein. It would also be possible to create a nuclease-digested series of DNA fragments that differed in their 5' ends. These could then be ligated to a translational start point to attempt to produce the 5' truncated protein. However, on average, two out every three constructs made will be in an incorrect reading frame. This would mean that the majority of the proteins could be made up of non-GyrA sequence. It is likely that the out-of-frame deletion mutants would not produce large proteins since the chances of encountering a stop codon must be high, and it is possible that these smaller proteins could confuse the results. It would be expected that N-terminal deletions of GyrA that extended beyond tyrosine 122 would produce an inactive protein. Site-directed mutagenesis of residue 122 to a serine or phenylalanine inactivates gyrase (A.J Wilkinson and J.C. Wang, personal communication), so presumably deletion of this residue would prove detrimental to enzymic activity. Hence, it would be expected that the breakage-reunion domain begins close to the N-terminus of the protein, and that the production of large deletion

mutants would not be fruitful. It was thus decided to make selected N-terminal GyrA mutants rather than a deletion series.

From the results obtained in Chapter 3, it has been shown that trypsin cleaves the GyrA protein at the C-terminal sides of the arginine residues at positions 6 and 572. A construct has already been described that over-produces GyrA(1-573) (see above). It was therefore desirable to produce the GyrA protein that lacks the first 6 amino acids to see if this has any effect on the functioning of the protein. A construct could then be engineered that over-produces a protein that represents the trypsin-generated N-terminal fragment (see chapter 3).

The construction of a plasmid that over-produces the GyrA protein lacking the first six amino acids is shown diagrammatically in Fig. 4.16. The plasmid pPH2 contains a complete *gyrA* gene with a silent mutation to create a *XhoI* site 14 bp downstream of the 5' end of the gene. The *gyrA* gene in pPH2 is some 1.1 kb 3' of the *tac* promoter. Part of the sequence of the pPH2 *gyrA* region is shown below. The *EcoRI* site is part of the pTTQ18 polylinker (Stark, 1987).

```
  1                                    ..6   7   8   9   10  11  12..
Met...                              ...ArgGluIleThrProValAsn...
ATGAATTC.........................CTCGAGAAATTACACCGGTCAAC...
TACTTAAC.........................GAGCTCTTTAATGTGGCCAGTTG...
   EcoRI            1.1 kb            XhoI
```

Cleavage of the plasmid pPH2 with both *EcoRI* and *XhoI* was performed. The restriction digest products were treated with mung bean nuclease to blunt the two overhanging ends and then the large 8 kb fragment was isolated by gel purification. The 8 kb fragment was then treated with DNA ligase to re-circularise the plasmid before the construct was transformed into competent JM109 *E. coli* cells. The sequence of the construct (pRJR243) was confirmed by DNA sequencing and is shown in part below:

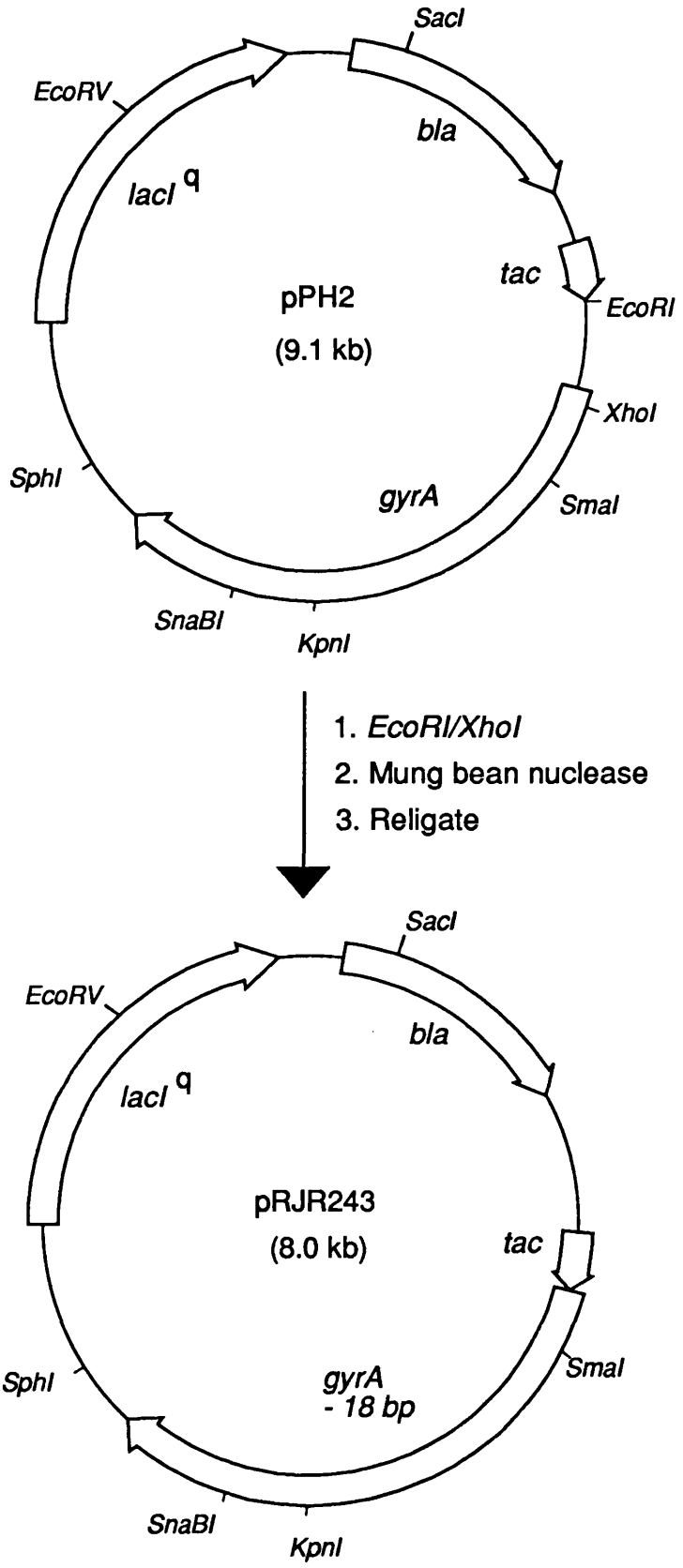


Fig. 4.16. Construction of the plasmid pRJR243 which codes for the entire GyrA protein except for the first six amino acids (GyrA(7-875)).

```
     1  2  3  4  5  6  7
    MetGluIleThrProValAsn...
5'..ATGGAAATTACACCGGTCAAC...3'
3'..TACCTTTAATGTGGCCAGTTG...5'
```

Thus pRJR243 over-produces a 96 kDa protein (see Fig. 4.5), designated as GyrA(7-875), that has a methionine (residue 1) linked to the rest of the coding sequence beginning at residue 7 (Glu). A second construct was also produced that utilises the the loss of the first six amino acids of the GyrA protein. The plasmid pRJR243 was digested with the restriction endonucleases *AatII* and *SphI* The smaller of the two fragments generated (2.5 kb) was gel purified and was ligated to the larger fragment of pRJR242 that had also been cut with *AatII* and *SphI* (5.5 kb). The resultant plasmid (pRJR244) over-produced the 64 kDa GyrA protein with the first 6 amino acids missing (see Fig. 4.5). This truncated protein was designated GyrA(7-573).

GyrA(7-875) and GyrA(7-573) were purified by Heparin-Sepharose and FPLC Mono Q chromatography. Samples of the partially purified protein were run on an SDS-polyacrylamide gel and blotted onto a PVDF membrane and subjected to automated protein sequencing (Matsudaira, 1987). The sequence of the six N-terminal amino acids in each case were determined. In both cases the following sequence was observed:

N-terminal: Met Glu Ile Thr Pro Val

Thus the N-terminal methionine residue of both the truncated proteins has been retained. This should be compared to the N-terminal sequence reported for the GyrA protein in which the N-terminal methionine is lost (Menzel and Gellert, quoted in Swanberg and Wang, 1987), and the sequences obtained for the GyrA(1-573) and GyrA(1-523) proteins that are shown below:

N-terminal: Ser Asp Leu

Both the C-terminal deletion fragments, in common with GyrA, lose the N-terminal methionine residue, whilst the N-terminal deletion mutants retain this residue. The enzymic reaction of the two proteins, GyrA(7-875) and GyrA(7-573), will be discussed later.

A second type of N-terminal deletion of the GyrA protein was also constructed that involved a larger deletion being made. Due to the lack of unique restriction sites 3' of the *XhoI* site in the *gyrA* gene a different approach to that adopted above had to be used. The next unique restriction sites 3' of the *XhoI* site in the *gyrA* gene is a *SacI* site 40 bp downstream (corresponding to approximately 13 amino acids) and an *AccI* site 293 bp downstream (approximately 98 amino acids). However neither of these sites were suitable for the production of a truncated protein since, if treated as above, the way in which they cleave the DNA would lead to an out-of-frame construct. It was therefore decided to use a non-unique site between these two and utilise an oligonucleotide linker to obtain the correct reading frame and control the size of the deletion that is made.

Plasmid pPH2 was digested with the *EcoRI* restriction endonuclease and treated with mung bean nuclease to remove over-hanging nucleotides (see Fig. 4.17). The linearised DNA was then digested with *BstXI* which cuts the DNA five times. The recognition sequence for *BstXI* is CCANNNNN/NTGG, and, as a consequence of the degenerate sequence, all the sites in pPH2 are found to be different. The sequences of the five *BstXI* sites are shown below (the numbering beginning at zero for the start of the *gyrA* gene in pPH2):

```
 229:    5' CCATCCCCATGG 3'
3232:    5' CCATTGTTGTGG 3'
```

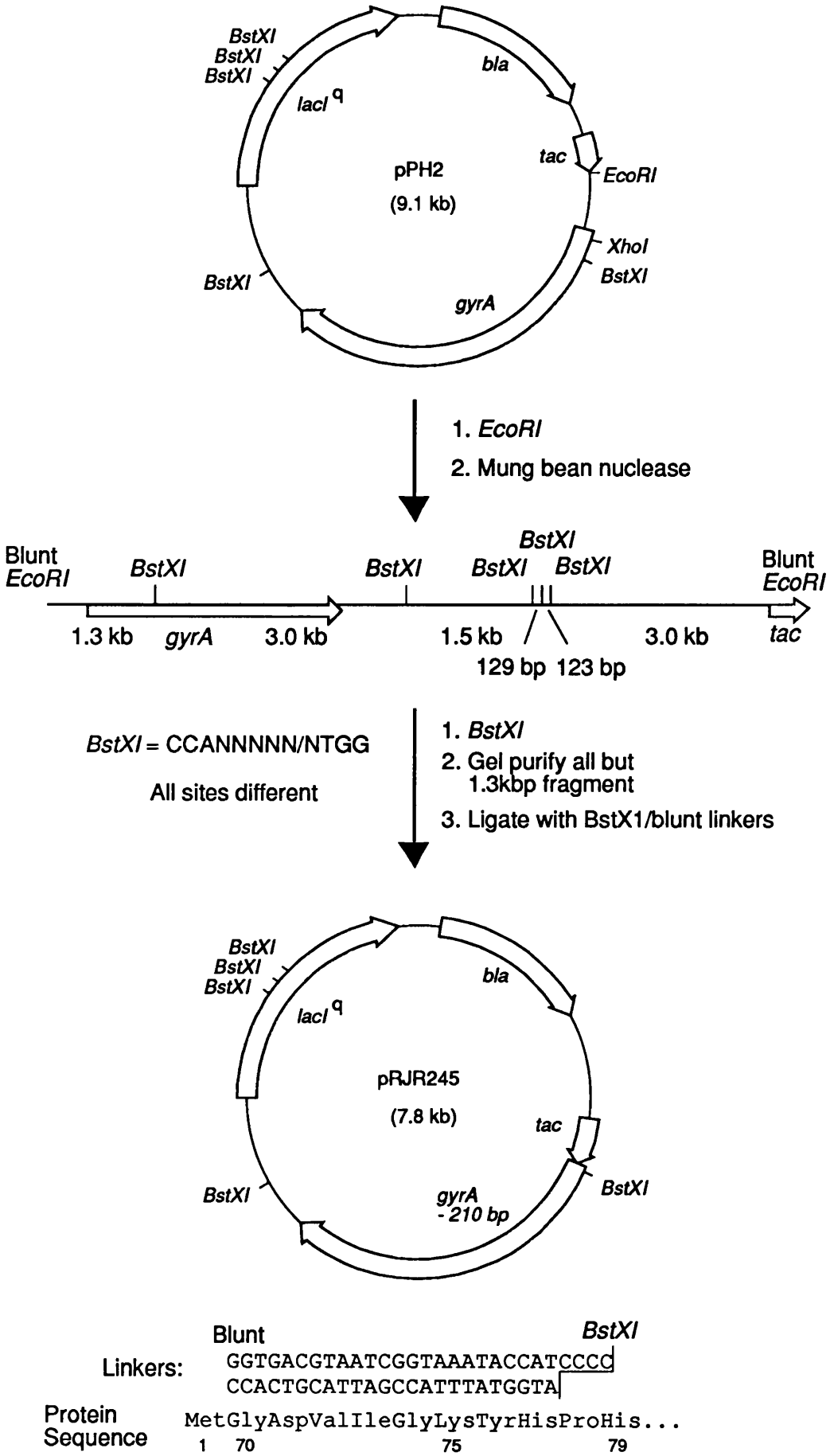


Fig. 4.17. Construction of pRJR245. A plasmid that over-produces a GyrA protein that has the first 69 amino acids missing (GyrA(70-875)).

```
4728:     5' CCAACCGCGTGG 3'
4857:     5' CCAGCGTGGTGG 3'
4980:     5' CCATGGCTGTGG 3'
```

Thus digestion of *EcoRI* linearised pPH2 with *BstXI* generates fragments of the following sizes; two of 3.0 kb, 1.5 kb, 1.3 kb, 129 bp and 123 bp. The fragments were run on a gel and all but the 1.3 kb fragment were purified. The 1.3 kb fragment represents the DNA between the *EcoRI* site directly 3' of the *tac* promoter and some 200 bp into the *gyrA* gene. The 1.3 kb fragment was replaced in a ligation mixture with a double-stranded oligonucleotide:

```
5' GGTGACGTAATCGGTAAATACCATCCCC 3'
3' CCACTGCATTAGCCATTTATGGTA     5'
```

This oligonucleotide pair will link the 3.0 kb blunted *EcoRI* fragment to the 3.0 kb *BstXI*-cut fragment of pPH2 (see Fig. 4.17). The resultant reformed plasmid (pRJR245) has a methionine linked directly to amino acid residue 70 of the GyrA sequence (a glycine residue). The DNA sequence of some 200 bp around this region in pRJR245 was confirmed by sequencing (data not shown). The produced protein, of molecular mass 89 kDa, is designated GyrA(70-875) and can be over-produced upon IPTG-induction of pRJR245 containing *E. coli* cells (data not shown).

GyrA(70-875) was partially purified by Heparin-Sepharose and FPLC Mono Q chromatography and a sample subjected to automated protein sequencing after blotting onto PVDF membranes after SDS-polyacrylamide gel electrophoresis. The sequence of the N-terminal six amino acids were determined and shown below:

N-terminal: Met Gly Asp Val Ile Gly

Therefore, once again, the N-terminal methionine residue is apparently retained by this protein. Enzymic reactions of this protein are discussed below.

(e) Enzymic activities of GyrA deletion mutants.

The enzymic reactions of a number of the deletions of the GyrA protein have been tested. Owing to the low supercoiling activity of the 64 kDa protein GyrA(1-573), and the consequent high levels of relatively pure enzyme needed to detect the reaction, it was decided to assay the C-terminal deletion mutants by their ability to perform quinolone-directed double-stranded cleavage of DNA. Six of the over-producing C-terminal deletion mutants (GyrA(1-588+3); (1-567+1); (1-537); (1-532+2); (1-523+3); and (1-523)) were grown up in 1L cultures and the IPTG-induced protein was partially purified by ammonium sulphate precipitation and FPLC Mono Q ion-exchange chromatography. After this treatment the protein was estimated to be approximately 50% pure, but was found to be free of nucleases at the enzyme levels used in a cleavage reaction (data not shown). Each of the truncated proteins was complexed with the gyrase B protein and incubated with supercoiled pBR322 DNA in the presence of the quinolone drug CFX. The cleaved products could be observed after denaturation of the DNA-protein complex. Fig. 4.18 shows the cleavage abilities of the six C-terminal deletion mutants. It is apparent that the smallest of the deletion proteins, GyrA(1-523), is able to perform the cleavage reaction equally as well as the largest of the deletion mutants tested, GyrA(1-588+3), and indeed as nearly well as the intact GyrA protein. The only anomaly in these results is GyrA(1-532+2) in which, although cleavage still occurs, the efficiency is somewhat diminished in comparison to the other proteins. A straightforward explanation for this result cannot be readily forwarded since proteins of sizes both larger and smaller than this are still able to efficiently perform the cleavage reaction. It is possible that GyrA(1-532+2) is completely inactive in its ability to cleave DNA, and that contamination by genome-encoded intact GyrA is the source of the weak cleavage activity observed with the

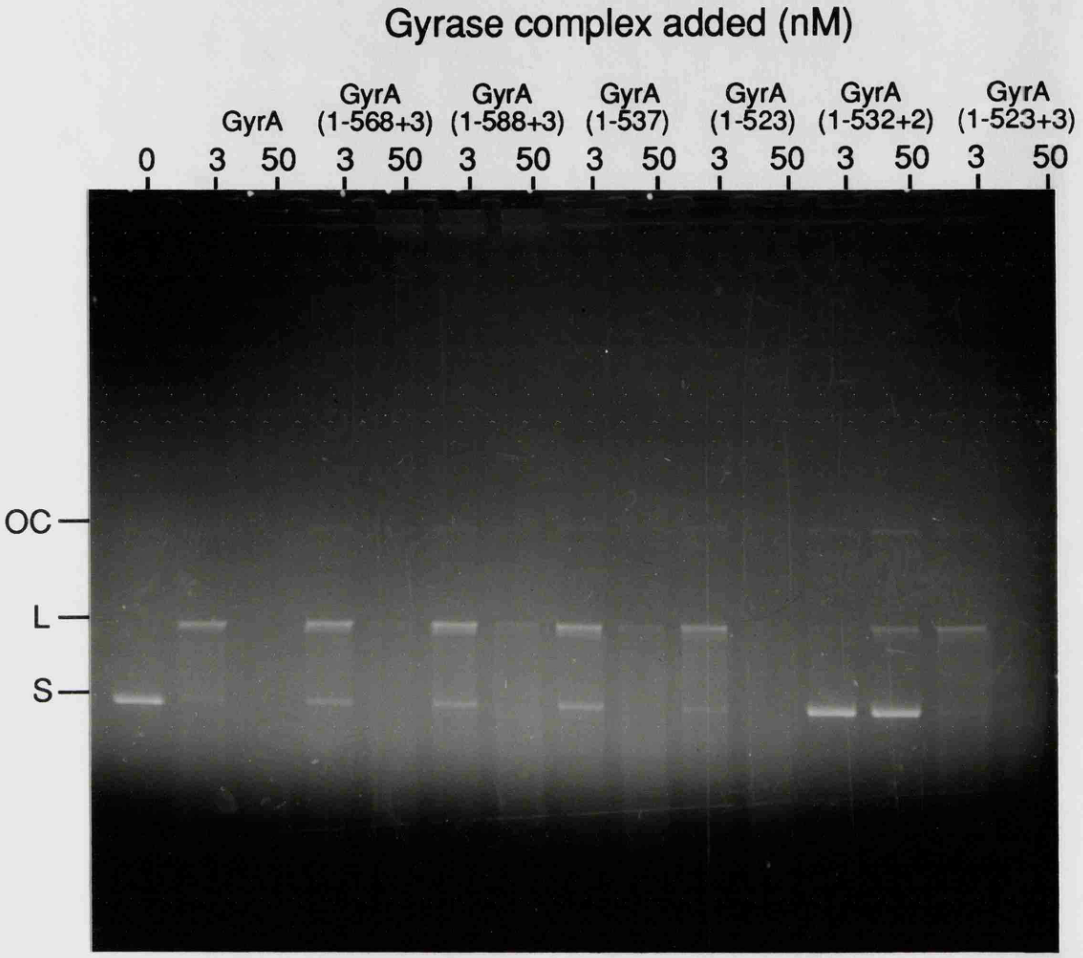


Fig. 4.18. CFX-directed DNA cleavage by C-terminal deletion mutants. Partially purified truncated GyrA proteins, at the concentrations indicated, were incubated with an equimolar amount of the GyrB protein and 10 μg/ml supercoiled pBR322 DNA in the presence of 3 μg/ml CFX for 1 hr at 25°C. After protein denaturation (as described in section 2.13) the samples were analysed on a 1% agarose gel run in TAE buffer.

fairly crude extracts used in these experiments. The extent of cleavage observed here is consistent with less than 1% contamination with the intact GyrA protein.

GyrA(1-523) appears to be the smallest protein to have all the breakage-reunion activities of the intact A protein, and has no foreign amino acids at the C-terminus, it was therefore decided to use this fragment to investigate the effects of the deletion on other gyrase reactions. GyrA(1-523) was purified to virtual homogeneity using Heparin-Sepharose, FPLC Mono Q, and FPLC Phenyl Superose chromatography (data not shown). The supercoiling activity of GyrA(1-523), in combination with the gyrase B protein, was tested. No detectable supercoiling by GyrA(1-523) could be noted, even using 10-times the enzyme level to detect supercoiling with GyrA(1-573) (data not shown). The effect of denaturation and renaturation of GyrA(1-523) was also tested. However, the refolded protein was also found to be inactive in terms of its supercoiling ability (data not shown). Therefore deletion of 50 amino acids from GyrA(1-573) to GyrA(1-523) has the effect of abolishing the weak supercoiling activity of the protein. Since quinolone-directed DNA cleavage is still known to occur efficiently with GyrA(1-523) there are several possibilities as to why the supercoiling activity has been nullified. For example, the strand-passage ability of the protein may have been affected in some way by the loss of the 50 amino acids so that, although cleavage occurs, DNA cannot be transferred through the gap in the sugar-phosphate backbone. Another possibility is that the energy transfer mechanism, by which the energy of ATP hydrolysis is used to drive the passage of a DNA double-helix through a gyrase-held gap, may have been interfered with. To examine this problem further some of the other reactions of gyrase were tested using GyrA(1-523).

The relaxation of supercoiled DNA by gyrase is an ATP-independent process. In the presence of the gyrase B protein, GyrA(1-523) was found to be able to support the relaxation of supercoiled pBR322 DNA (see Fig. 4.9). The reaction is slow but of a comparable rate to that found with GyrA(1-573). It was also found that the denatured

and refolded form of GyrA(1-523) will also perform the relaxation reaction equally as well as the 'native' truncated protein. Therefore it must be assumed that GyrA(1-523) is be able to carry out passive strand-passage involved in relaxation as well as being able to cleave DNA. The lack of active strand-passage, involved in DNA supercoiling, could suggest that the energy transfer process, by which the energy from the hydrolysis of ATP is converted into strand-passage, has been disrupted in GyrA(1-523). The strand-passage mechanism itself can still occur, as evidenced by the relaxation reaction, but ATP-dependent supercoiling does not occur.

The ability of GyrA(1-523) to decatenate catenated double-stranded DNA circles was investigated. However, in common with the larger GyrA(1-573) protein, no decatenation activity could be noted (data not shown). This is not perhaps surprising since if a supercoiling activity cannot be detected then it is unlikely that the ATP-dependent decatenation reaction, which requires more enzyme than the supercoiling reaction to be observed, will be detected.

The ability of the GyrA(1-573) and GyrA(1-523) proteins to stimulate the hydrolysis of ATP by the B subunit of gyrase was measured using the malachite green phosphate assay (see section 2.19). Samples of gyrase protein were incubated with 100 nM 172 bp DNA under standard supercoiling conditions for 6 hr at 25°C. The amount of phosphate in each sample was then assayed and corrected for any novobiocin-insensitive ATPase activity. The results of such an experiment are shown below:

Sample	Novobiocin-sensitive phosphate release (μM/hr)	GyrA-dependent phosphate release (μM/hr)
A_2B_2 (45 nM)	78.7	54.7
$GyrA(1\text{-}573)_2B_2$ (45 nM)	51.8	27.8
$GyrA(1\text{-}523)_2B_2$ (45 nM)	52.2	28.2
A (50 nM)	0.5	
GyrA(1-573) (45 nM)	0.8	
GyrA(1-523) (45 nM)	1.1	
B (50 nM)	24.0	

It can therefore be seen that the ability of GyrA(1-523) to stimulate the hydrolysis of ATP by the B protein is virtually identical to that of GyrA(1-573). It must therefore be postulated that the lack of supercoiling activity observed for GyrA(1-523) is not due to its inability to stimulate ATP hydrolysis. The ATPase activities of the two truncated proteins are approximately two-fold less than the values obtained for the intact GyrA protein. This is reasonably consistent with the level of ATPase observed for the trypsin-treated A' protein (see chapter 3). The ATPase activity observed for the A_2B_2 complex (at 45 nM) is approximately 80 μM/hr (22 nM/sec). This rate is consistent with the k_{cat} obtained by Maxwell and Gellert (1984).

Some enzymic activities of the N-terminal deletion mutants of the GyrA protein were measured. The supercoiling reaction was performed by a cell extract containing GyrA(7-875) with approximately equal efficiency of that containing the intact GyrA protein (see Fig. 4.19). Under these conditions used here the specific activity for the supercoiling reactions can be calculated to be 6.7×10^5 U/mg for GyrA and 4.5×10^5 U/mg for GyrA(7-875). The small difference in these two values could be explained by the reduced level of expression of the truncated protein, when compared to the intact gyrase A protein, in the extracts used for this experiment (see Fig. 4.5). The supercoiling activity of the GyrA(70-875) was also investigated and found to be completely absent (data not shown). Therefore it seems that deletion of six amino acids from the N-terminal of GyrA has virtually no effect on the supercoiling activity of the protein. Since the supercoiling activity of the protein is unaltered then it can also be assumed that the protein will also carry out all the other reactions of the intact GyrA protein. However, deletion of the first 69 amino acids of the GyrA protein has the effect of producing a protein that is unable to support the supercoiling reaction. The ability of GyrA(70-875) to stimulate ATP-hydrolysis by the GyrB subunit was also measured. Samples of gyrase protein were incubated with 100 nM 172 bp DNA under standard supercoiling conditions for 5 hr at 25°C. The amount of phosphate in

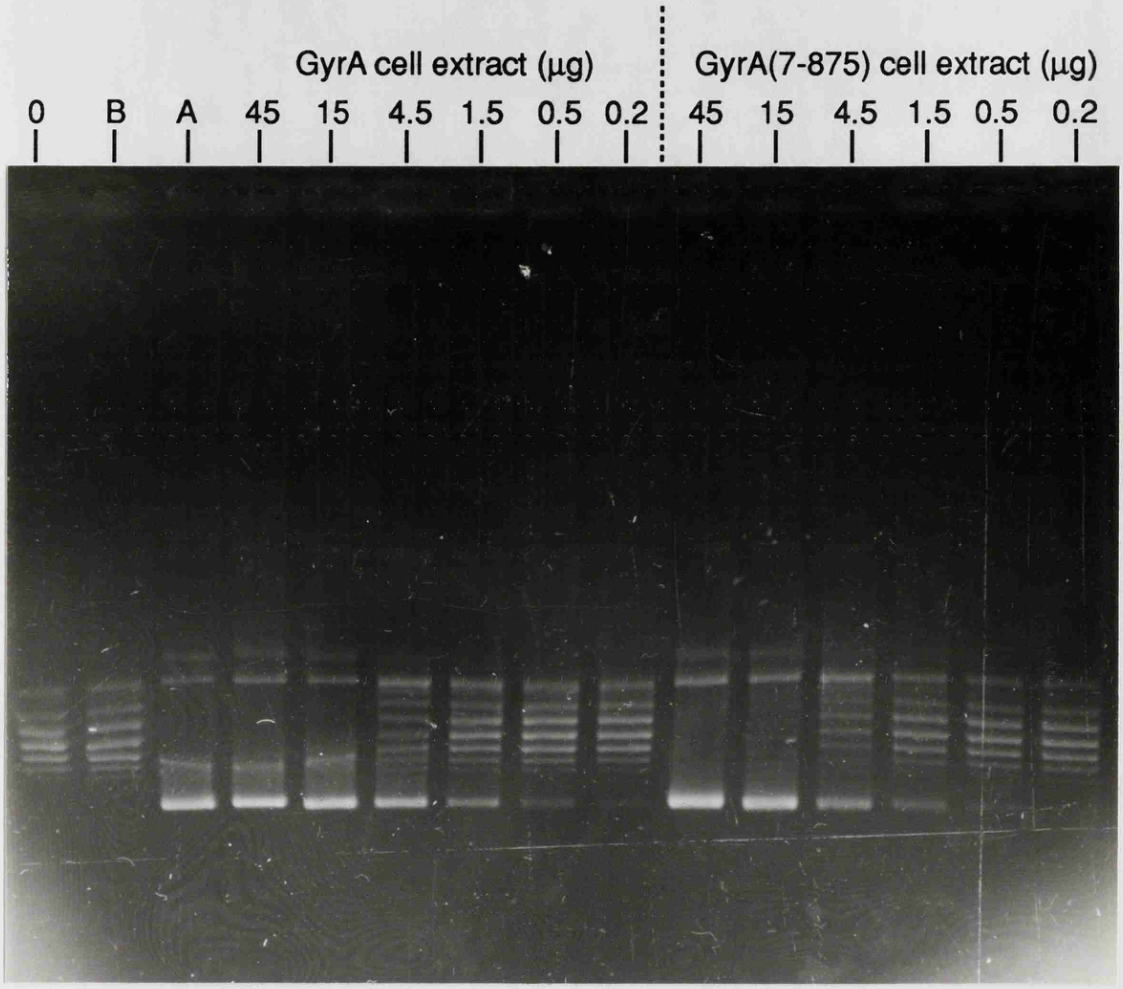


Fig. 4.19. Supercoiling abilities of cell extracts of pPH3- and pRJR243-containing cells. GyrA or GyrA(7-875), at the approximate concentrations shown, were incubated with the GyrB protein and relaxed pBR322 DNA under the standard supercoiling conditions for 1 hr at 25°C. Samples were analysed on a 1% agarose gel run in TAE buffer. All samples, except for that labelled 0, contained GyrB (at 50 nM); the lane labelled A contained highly purified GyrA (at 90 nM).

each sample was then assayed and corrected for any novobiocin-insensitive ATPase activity. The results are shown below in tabulated form:

Sample		Novobiocin-sensitive phosphate release (μM/hr)	GyrA-dependent phosphate release (μM/hr)
A_2B_2	(45 nM)	98	62
GyrA(70-875)$_2B_2$	(45 nM)	96	60
GyrA	(45 nM)	0.9	
GyrA(70-875)	(45 nM)	1.5	
GyrB	(45 nM)	34	

Thus it can be seen that GyrA(70-875) is able to stimulate the ATPase activity at a level comparable with that of the intact protein. It can therefore be assumed that GyrA(70-875) is able to interact with both the GyrB protein and DNA. This shows that the protein is correctly folded and that the lack of supercoiling activity noted with this protein can be attributed to the loss of the N-terminal 69 amino acids only.

The quinolone-directed cleavage of DNA by the N-terminal deletion mutants of the GyrA protein were also studied. It was found, as expected, that GyrA(7-875) and GyrA(7-573) have the ability to cleave DNA with approximately the same efficiency as their counterparts that retained their N-terminal ends. However GyrA(70-875) does not show any trace of cleavage activity (Fig. 4.20). Thus the first six amino acids of the GyrA protein are not essential for the reactions of the intact GyrA protein, but deletion of the first 69 amino acids leads to the production of a protein with no enzymic activity.

Table 4.2 summarises the enzymic reactions of a number of the deletion mutants of the GyrA protein. Included in this table are the trypsin-treated A' protein and the gel purified 64 kDa and 33 kDa fragments, both alone and in combination with each other (see Chapter 3), the cloned 64 kDa protein, the smallest of the C-terminal over-

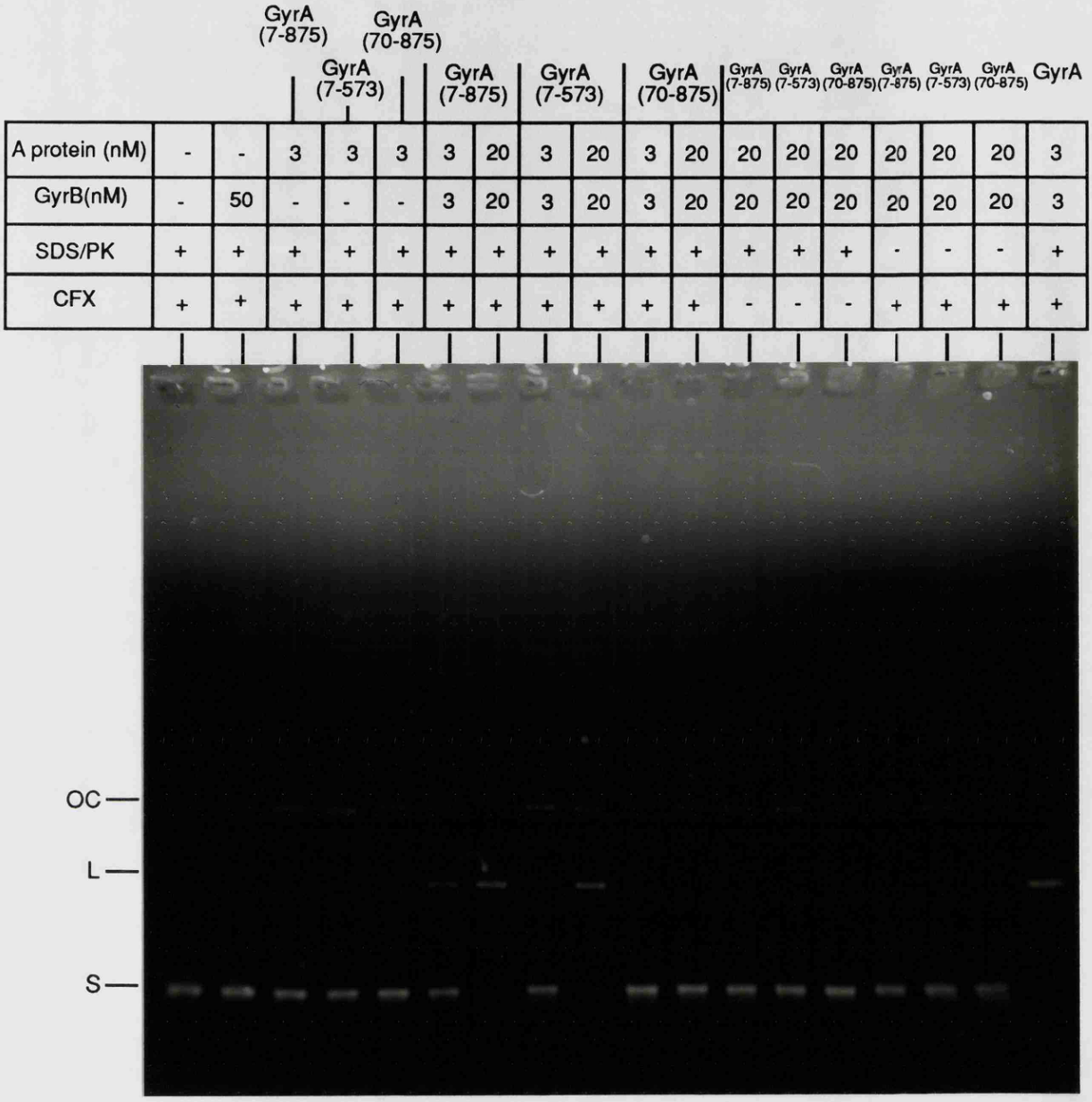


Fig. 4.20. CFX-directed DNA cleavage by GyrA, GyrA(7-875), GyrA(7-573) and GyrA(70-875). Gyrase proteins, at the concentrations shown, were incubated with supercoiled pBR322 at 25°C for 60 min. All samples, except those indicated, contained 3 μg/ml CFX. Also indicated are samples that were not treated with SDS or proteinase K before running on a 1% agarose gel.

Reaction	Intact A (97 kDa)	A' (64+33 k)	Gel pure 64 kDa	Gel pure 33 kDa	Reformed Gel pure 64+33kDa	GyrA (1-573) (64 kDa)	GyrA (1-523) (58 kDa)	GyrA (7-875) (96 kDa)	GyrA (7-573) (63 kDa)	GyrA (70-875) (89 kDa)
Supercoiling	++++	+++	+	-	++	+	-	++++		-
Relaxation	++++	+++	-	-	++	+	+			
Decatenation	++++	++++	-	-	+++	-	-			
Cleavage	++++	++++	++++	-	++++	++++	++++	++++	++++	-
ATPase	++++	+++				+++	+++			++++
DNA binding	++++	+++								

Table 4.2 Enzymic reactions of the proteins produced by trypsin cleavage of the DNA gyrase A protein and of those produced by the pRJR24 series of plasmids. + indicates an approximate ten-fold change in activity compared to the intact protein. - indicates that no detectable activity could be observed, and a blank space shows that the activity has not been tested. All reactions were performed in the presence of the GyrB protein.

producing deletion mutants (GyrA(1-523)) and the various N-terminal deletion mutant derivatives.

4.3 Discussion.

From the results obtained in Chapter 3 it was apparent that the protease trypsin cleaves the 97 kDa DNA gyrase A protein into fragments of sizes 64 kDa and 33 kDa. The 64 kDa fragment has been proposed to represent the breakage-reunion domain whilst the 33 kDa fragment seems to play a role in the stability of the protein-DNA complex. By mutation of the *gyrA* sequence, the 64 kDa fragment (GyrA(1-573)) has been produced as a direct gene product, and has been found to share very similar properties to the gel purified 64 kDa tryptic GyrA fragment (see Table 4.2). GyrA(1-573) has the ability to supercoil, relax and cleave double-stranded DNA, although the efficiencies of the supercoiling and relaxation reactions are greatly reduced when compared to the intact GyrA protein. Although the 64 kDa fragment has the ability to interact with DNA on its own, it is likely the process is aided greatly by the presence of the 33 kDa C-terminal fragment of the GyrA protein. The results obtained with the cloned 64 kDa fragment also dismiss the possibility of the first six N-terminal amino acids playing a role in the reactions of the GyrA protein. These six residues are cleaved from the 64 kDa fragment by trypsin (see Chapter 3) and will not be present in the gel purified preparations. However, the cloned and over-produced fragment behaves in a very similar manner to the trypsin-generated fragment even though it retains the first six residues.

Further C-terminal deletions of the GyrA protein have been made. The ability of 256 such plasmids, encoding shortened versions of the *gyrA* gene, to over-produce their respective truncated protein was investigated. Only 24 were noted to obviously over-produce a truncated GyrA protein upon induction. It is possible that many more of the constructs do actually produce a truncated GyrA protein; but this may be either in quantities too small to be detected by the assay used, or the protein may be insoluble

due to the exposure of, say, hydrophobic regions that should be in the interior of the native protein. Of the obviously over-produced proteins, the molecular masses ranged from 70 kDa down to 59 kDa. From the construction of the starting plasmid (pRJR133; Fig. 4.11) the maximum molecular mass of a protein that could be made by this method is 73 kDa (GyrA(1-654+1)). However no obvious over-production could be noted from this plasmid. Indeed, the majority of the plasmids containing shortened *gyrA* genes did not over-produce truncated protein. From restriction enzyme and DNA sequencing analysis, the majority of the constructs would appear to be correct, in that there has been no gross changes in the DNA sequence. All the plasmids investigated, both 'producers' and 'non-producers', give an expected restriction digest pattern and the sequences obtained were consistent with a *XhoI*-blunt *gyrA* fragment being inserted into the stop-codon linker system of plasmid pRJR133 (see Fig. 4.12).

It would therefore appear that rather than the DNA construct being the problem for the lack of expression of certain truncated genes, it is the produced protein itself that is unstable. All constructs that were supposed to over-produce a protein less than a molecular mass of 59 kDa were all found to be 'non-producers'. Many of the larger constructs also were apparent 'non-producers' even though the DNA was apparently of correct sequence. This is shown diagrammatically in Fig. 4.21. There is not a simple rationale for the over-production of protein from the C-terminal deletion series of GyrA proteins. For example, GyrA(1-532+2) is over-produced whereas GyrA(1-541+2) is not. Both of these proteins should have the following sequence at their C-termini -Asp Arg, but only the smaller is actually produced. It is therefore possible that the termination point lies within some secondary or tertiary structure within the protein that will lead to the exposure of, say, hydrophobic residues to solvent. This may make the protein insoluble, or yield it more susceptible to the action of proteases.

Fig. 4.21. The sizes and quinolone-directed DNA cleavage ability of some of the proteins made by deletion of the *gyrA* gene. The * indicates that although cleavage occurs, the reaction is inefficient. The sizes of the proteins have been calculated from the DNA sequence.

The smallest of the C-terminal deletions that can be efficiently over-produced as soluble protein is GyrA(1-523). This protein is still able to efficiently cleave DNA in the presence of quinolone drugs, but has apparently lost the ability to supercoil DNA. Strand-passage can still occur with GyrA(1-523) since relaxation of supercoiled DNA can be observed. Relaxation is a not an energy requiring process. However, efficient DNA cleavage appears not to be the only factor important in the relaxation process. The efficiency of relaxation is greatly reduced for the proteins that lack the 33 kDa C-terminal fragment of the GyrA protein.

It is possible that deletion of the 50 amino acids between GyrA(1-573) and GyrA(1-523) has in some way affected the ATPase activity of the gyrase complex. However, the ATPase activity of GyrB is stimulated equally well in the presence of GyrA(1-523) and GyrA(1-573). It is possible that the energy transfer process between the ATP-hydrolysis site, on the gyrase B protein, and the actual site of DNA strand-transfer, presumably situated in the N-terminal 64 kDa of the GyrA protein, has been disrupted by deletion to GyrA(1-523). It can be envisaged that there must be some sort of interconnecting network between the A and B proteins so that when DNA binds to the gyrase complex the ATPase activity of GyrB is stimulated and supercoiling occurs. It is possible that deletion of the C-terminal 50 amino acids from GyrA(1-573) has the effect of disrupting this network so that the energy of ATP hydrolysis cannot be transferred into a strand-passage event.

Deletion of the first 6 amino acids of the GyrA protein appears to have no effect on the activity of the protein. GyrA(7-875) has the ability to supercoil DNA with approximately the same efficiency as the native GyrA protein. The only other activity of this protein that has been tested is its ability to cleave double-stranded DNA in the presence of quinolone drugs. Again GyrA(7-875) has an activity comparable to that of the intact protein. It can be assumed that this protein also has the ability to relax supercoiled DNA and decatenate catenated DNA species. GyrA(7-573) appears to be

identical in its reactions to GyrA(1-573) in terms of the efficiency of the cleavage reaction.

Deletion of the first 69 amino acids of the GyrA protein leads to the production of a protein that is unable to either supercoil or even cleave DNA, but is still capable of interacting with the GyrB protein and DNA (judged by the ability to stimulate the ATPase activity of GyrB). It has previously been reported that a change of GCC (encoding Ala 67) to TCC (encoding Ser) leads to the formation of a quinolone-resistant protein (Yoshida *et al.*, 1988). Deletion beyond this residue (as in GyrA(70-875)) has the effect of disrupting quinolone-directed DNA cleavage, and due to the proximity of the residue to the active tyrosine (122) it is perhaps not surprising that other activities are also abolished.

All of the N-terminal deletion mutants produced retained their N-terminal methionine residue as judged by amino acid sequencing. It has previously been reported that the intact GyrA protein loses this residue (Menzel and Gellert, quoted in Swanberg and Wang, 1987). However, this would not appear to affect the enzymic reactions of these proteins since GyrA(7-875) is just as an efficient supercoiling enzyme as GyrA.

In order to gain some structural information about the gyrase proteins it is desirable that they should be as small as possible, so that if crystals were obtained then the analysis of the data obtained would be considerably easier. However, it would also be advantageous to have an active protein so that any structure obtained could be interpreted as that of the native protein. The work described in this chapter throws up two obvious candidates for structural investigation. The 64 kDa GyrA(1-573) and the smaller 59 kDa GyrA(1-523). GyrA(1-573) is still active in terms of supercoiling ability, whereas the smaller GyrA(1-523) appears to have no supercoiling activity but is still able to cleave DNA efficiently. Several groups have attempted to crystallize the gyrase protein with little success. Perhaps there will be a greater chance of obtaining

suitable crystals if a single, active domain of the protein is used. The C-terminal deletion mutants described here probably represent shortened versions of the breakage-reunion domain of gyrase. They are still able to interact with the quinolone drugs and DNA, although GyrA(1-523) has lost its ability to perform the supercoiling reaction. Thus any structural information that can be obtained from these fragments would probably be representative of the native GyrA protein.

Chapter 5

Structural studies on fragments of the DNA gyrase A protein.

5.1 Introduction.

From the work presented in chapters 3 and 4, I have suggested that the GyrA protein is composed of two domains. The N-terminal 64 kDa breakage-reunion domain and the C-terminal 33 kDa 'stability' domain. This has also recently been suggested by Dimri and Das (1990) by more indirect methods. They have cloned and sequenced the *gyrA* gene from *Klebsiella pneumoniae* and compared the sequence obtained to that of the *E. coli* and *B. subtilis* genes. The *K. pneumoniae* GyrA protein has approximately 90% identity to that of *E. coli* and 50% to *B. subtilis*, but in both cases the strongest regions of homology are located in the N-terminal region of each protein. The C-terminal of GyrA shows a wider cross-species variability. It has therefore been suggested that each of the GyrA proteins consists of a conserved N-terminal domain, that is involved in DNA contact, and a variable C-terminal domain, that is not essential for the supercoiling activity of the proteins. So some structural information about the N-terminal domain of gyrase would seem to be the most important.

One possible method for elucidating the domain structure of a protein is to subject it to calorimetric analysis (Creighton, 1984). As the temperature of a protein in solution rises it will begin to unfold and denature. At a sufficiently high temperature the non-covalent interactions, that hold a protein into a secondary and tertiary structural motifs, will breakdown and the protein will take up a random-coil structure. If a protein consists of two domains then it is possible that they will unfold at different temperatures, depending on the relative thermal stability of each domain. The unfolding of a protein domain requires energy which can be detected in a scanning microcalorimeter as a difference in temperature between a buffer blank and a protein-containing sample. Such an approach has been used to successfully elucidate the domain structure of the λ repressor protein (Pabo *et al.*, 1979). A number of lines of evidence, discussed in chapters 3 and 4, strongly suggest the existence of two domains within the GyrA protein, so it would seem reasonable to test this hypothesis using calorimetry. Comparison of the specific heat capacity at constant pressure (Cp)

of the intact GyrA protein at different temperatures to those obtained for the fragments should yield some information about the domain structure of the protein and the relative stability of any domains present.

Although much information has been gained about the mechanism of action of DNA gyrase (reviewed in Maxwell and Gellert, 1986), to date there is relatively little structural information available. The structural studies so far performed have been at relatively low resolution. Active DNA gyrase is a tetramer of A_2B_2 (Klevan and Wang, 1980; Krueger *et al.*, 1990) and the gyrase complex, both alone and in the presence of DNA, has been studied by electron microscopy (e.g. Kirchhausen *et al.*, 1985), transient electric dichroism (Rau *et al.*, 1987) and small angle neutron scattering (Krueger *et al.*, 1990). Direct observation of the complex by electron microscopy has indicated that gyrase is a nearly spherical or heart-shaped particle of approximate diameter 150-210 Å (these values have been corrected for platinum decoration). The gyrase A protein is thought to form the upper lobes of the heart structure. However, the results obtained by Kirchhausen *et al.* (1985) require a great deal of interpretation from the original micrographs before any recognisable structure can be noted. This, together with the problems of sample disruption during preparation for such microscopy, must bring into question the validity of the results obtained.

Transient electric dichroism of gyrase-DNA complexes have indicated that about 120 bp of DNA is wrapped around the enzyme in a single turn with the entry and exit points located close together (Rau *et al.*, 1987). This is consistent with the results observed by electron microscopy of gyrase-DNA complexes. The dichroism data suggest that the gyrase-DNA particle is almost spherical, approximately 84 Å in radius. The size of the particle and the slow relaxation time observed when the electric current was removed suggest that the gyrase tetramer is not a compact globular structure. Using this technique on a compact, globular protein of molecular mass 400

kDa should yield a sphere of radius 66 Å. It has therefore been suggested that the complex may contain cavities or channels within its structure. There are a number of drawbacks in the used of the electric dichroism technique. For example, only the DNA in the DNA-protein complex can be observed. Another possible drawback is that the fitting of the data is model dependent so that a unique solution is not necessarily obtained.

Small angle neutron scattering of the gyrase tetramer suggests that the gyrase particle is oblate in shape of approximate dimensions 175 Å wide by 52 Å thick. The calculated radius of gyration of the particle (at 64-67 Å) is considerably greater than that which would be expected for a 400 kDa compact globular protein (estimated to be 43 Å). This has been interpreted as the gyrase molecule having channels or cavities up to 15 Å wide. The radius of gyration of the particle does not alter greatly on binding DNA, so it is possible that the DNA is somewhat embedded into the protein. The cavities suggest a route for DNA strand passage through the protein, which must occur if the DNA is to traverse an enzyme-bridged gap in the DNA. For example, it is possible to envisage a model where there is a channel between the A and B proteins that could open to allow the transfer of a DNA strand through the complex, whilst the A proteins are bridging a gap in the DNA. A hinge mechanism could therefore be acting in the gyrase tetramer. Such a mechanism could be separated into the following steps: DNA binds to the protein and is cleaved, a strand from elsewhere in the DNA is the passed through the gap, a conformational change then occurs in the protein to allow the DNA to pass through a channel in the protein. However, such a model is somewhat speculative and high resolution structural studies would go a long way to elucidate the actual method of strand-passage. Small angle neutron scattering data has a number of drawbacks; again the data is model-dependent, and the data obtained is spherically averaged since the particles take up all possible orientations in solution.

To date, there have been no reports of any crystal structures or other high resolution structural data, such as nuclear magnetic resonance data, for the gyrase proteins. A high resolution structure of gyrase could lead to important advances in several areas. Only with advent of such data can rational drug design be attempted to find greater potency antibacterials that are capable of interacting with gyrase. Of particular importance to this work are the quinolone drugs whose intracellular target is thought to be the gyrase A protein; although recently it has been suggested that the actual drug target may be the DNA in the gyrase-DNA complex (Shen *et al.*, 1989c). All quinolone-resistant GyrA proteins have been mapped to the N-terminal 64 kDa of the protein (Yoshida *et al.*, 1988; P. Hallett and A. Maxwell, personal communication). High resolution structural data will also be of use in the determination of the precise mechanism of supercoiling by DNA gyrase, and should also facilitate the study of the more general topics of protein-DNA and protein-protein interactions.

A number of groups have previously attempted to crystallize the gyrase proteins with little or no success. It is possible that gyrase crystals have not been grown due to the nature of the proteins themselves. Gyrase may be, by its very nature, a flexible protein in order to allow the passage of a section of DNA through an enzyme-held DNA gap, and consequently through part of its structure. This flexibility could hinder the formation of a regular crystal lattice array. Since both the gyrase proteins are large, it is perhaps wise to attempt crystallographic studies on the smaller fragments whose possible results could be easier to interpret. The 64 kDa GyrA(1-573) and the 59 kDa GyrA(1-523) are obvious candidates for such study since both can be over-produced in large amounts and purified to virtual homogeneity (see chapter 4). GyrA(1-573) represents the tryptic cleavage fragment that could represent a natural boundary in the GyrA protein. GyrA(1-523) is the smallest GyrA fragment that I have been able to produce that is at least partially active. Attempts to crystallize these fragments of the GyrA protein may be more successful. Since these fragments are active, any structure obtained is likely to be relevant to the intact protein. The

fragments of GyrA may be somewhat less flexible proteins than the intact molecule and hence easier to crystallize. The reduction in size between the deletion fragments and intact GyrA should mean that any diffraction pattern obtained for the fragments should be somewhat easier to interpret.

5.2 Results.

(a) Scanning microcalorimetry.

In order to further probe the nature of the GyrA domain structure, samples of the gyrase proteins were subjected to scanning microcalorimetry (performed in collaboration with Dr. P.M. Cullis, Dept. of Chemistry, University of Leicester). Samples of the GyrA protein (0.89 mg/ml), GyrA(1-573) (0.97 mg/ml) and GyrA(1-523) (1.05 mg/ml) were dialysed against the same buffer overnight (see section 2.21). The samples were then placed in turn into the calorimeter with a sample of the dialysis buffer being used as a blank. The temperature was then raised from 15°C to 100°C at a rate of 1.5°C per min. The trace obtained for the specific heat capacity of each protein against temperature is shown in Fig. 5.1. There are two major transitions revealed by these data. The first occurs at about 60°C for all three proteins and the second at about 80°C for GyrA and 75°C for the two deletion derivatives. The second of these two transitions is thought to represent the precipitation of the protein, since after the run the protein sample was a thick white precipitate. The differences in the precipitation points between the intact protein and the N-terminal fragments could indicate that the truncated GyrA proteins are somewhat less stable than their full length counterpart. The transition curves obtained, and shown here, were repeatable with two different protein preparations.

It is proposed that the first transition shown in Fig. 5.1 represents the unfolding, or denaturation of the protein, and is somewhat different for the GyrA protein when compared to the two deletion derivatives. The unfolding transition for GyrA(1-573) and GyrA(1-523) seem to be identical in that a single, sharp, almost symmetrical

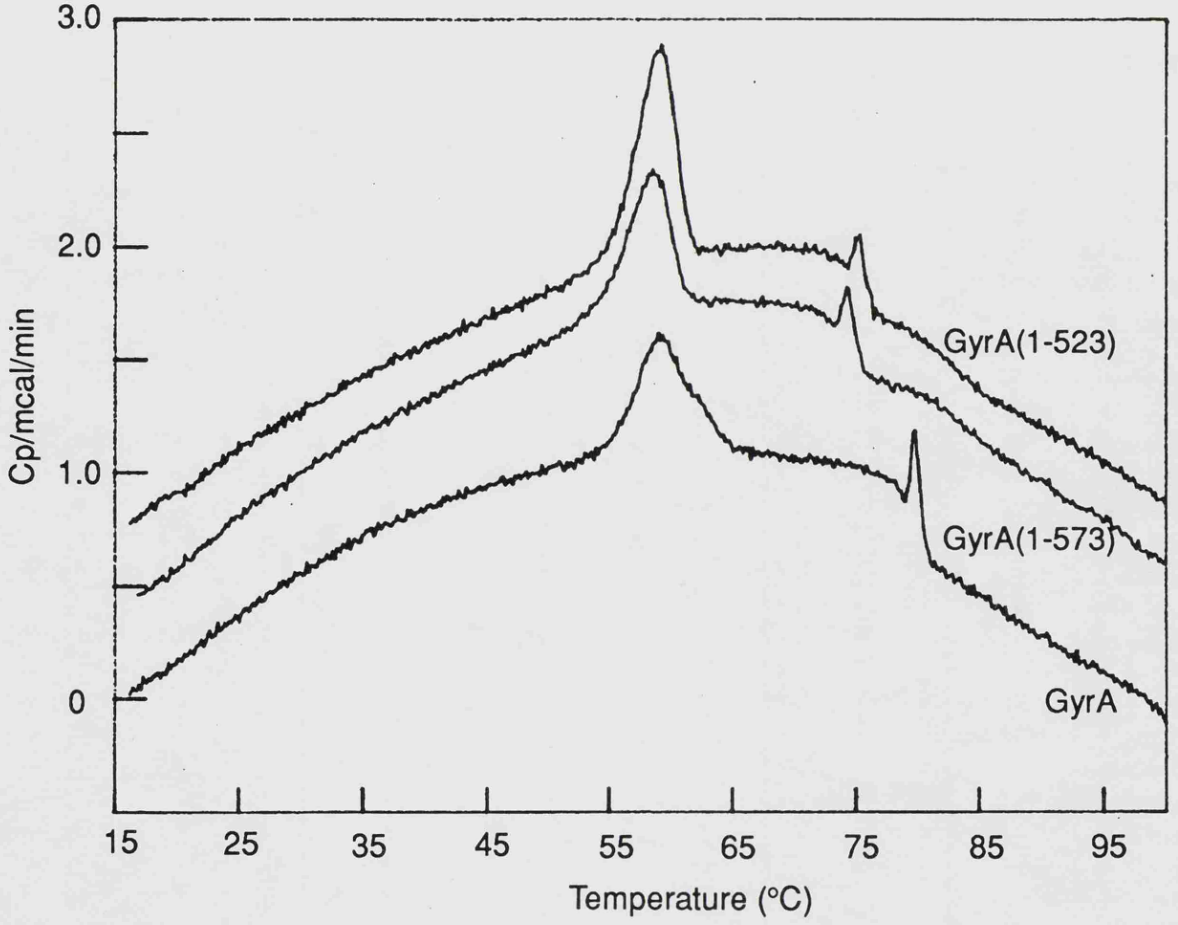


Fig. 5.1. The specific heat capacity of GyrA, GyrA(1-573) and GyrA(1-523) as measured as a function of temperature by scanning microcalorimetry.

transition occurs at approximately 58°C. The GyrA transition is, however, asymmetric being broader and has a 'bulge' on the high temperature side of the peak. The denaturation process was irreversible for all the proteins studied. If the protein samples were heated to 70°C so that the first transition occurred and then cooled back to 15°C before re-heating then no transition would recur until the precipitation temperature was attained (data not shown).

To account for the asymmetry of the GyrA unfolding transition, it can be assumed the the data has arisen from more than one separate transition. In this case, the experimentally obtained curve can be deconvoluted into two or more curves. This operation has been performed on both the GyrA protein (Fig. 5.2) and GyrA(1-573) (Fig. 5.3). Deconvolution was not performed with the GyrA(1-523) curve since this was essentially identical to that obtained for GyrA(1-573). Splitting the GyrA transition into two separate symmetrically-shaped curves yields information shown in Fig. 5.2. From these data, the combination of the two curves shown results in a line that is able to fit the experimentally observed data very closely. The combination of these two are shown on Fig. 5.2 as the smooth line close to the somewhat uneven data line. The GyrA transition can be best fitted to this two transition model. The two transition temperatures are 58.7°C and 59.6°C and have ΔH values (calculated by integrating the curves) of 168 $kcalmol^{-1}$ and 139 $kcalmol^{-1}$ respectively. This therefore supports the idea of two domains within the GyrA protein, with transition temperatures that are close to each other.

Attempts to deconvolute the GyrA(1-573) transition into two separate events are shown in Fig. 5.3. It can be seen that the best symmetrical fit can be achieved using a single transition and that a second takes the form of a straight horizontal line that plays little, or no part in the combined curve. The transition temperature for the single transition model is 58.1°C and the ΔH value can be calculate as 173 $kcalmol^{-1}$. These values are similar to those calculated for the first transition of the GyrA protein. The

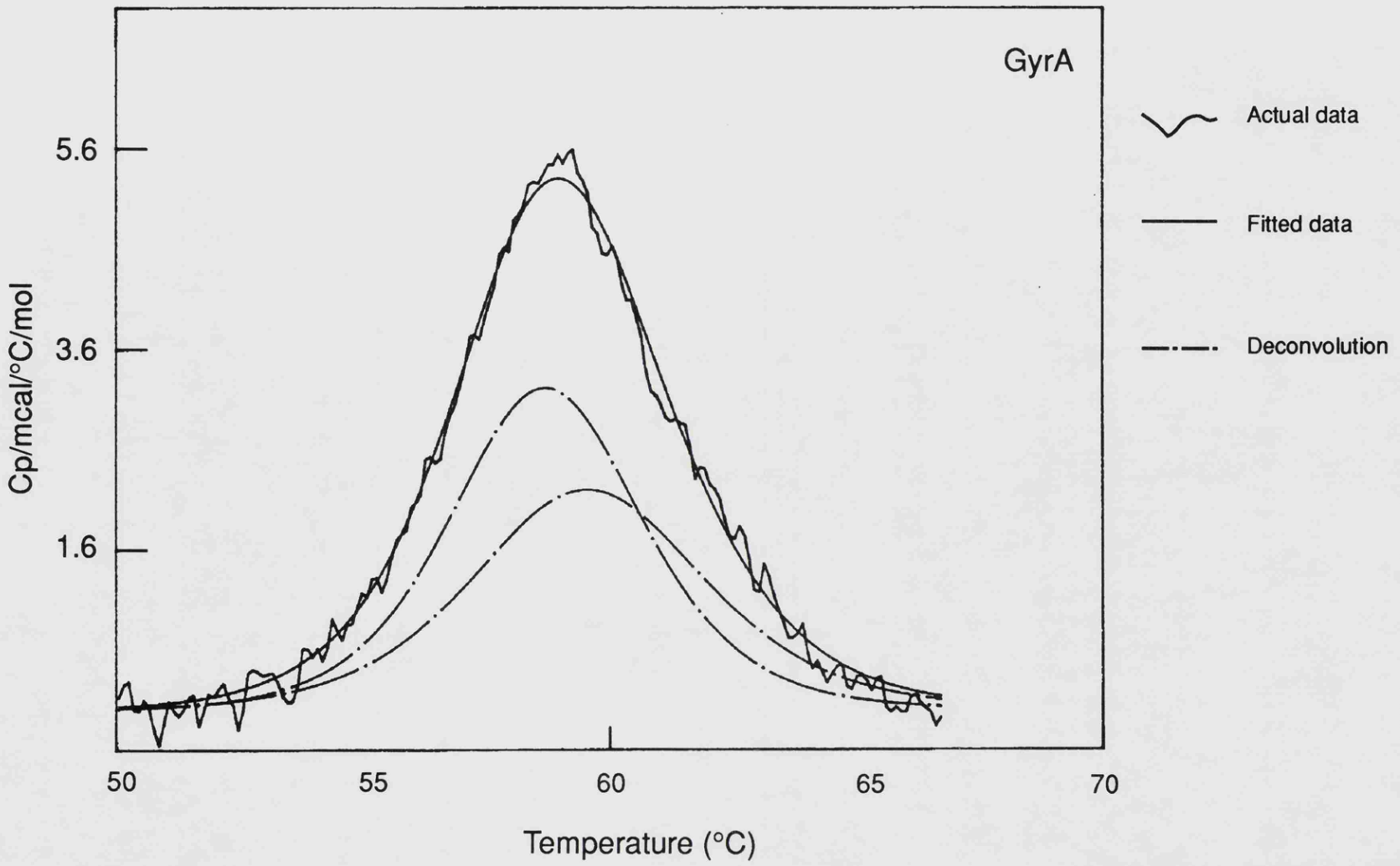


Fig. 5.2. Deconvolution of the GyrA temperature transition curve into two separate events.

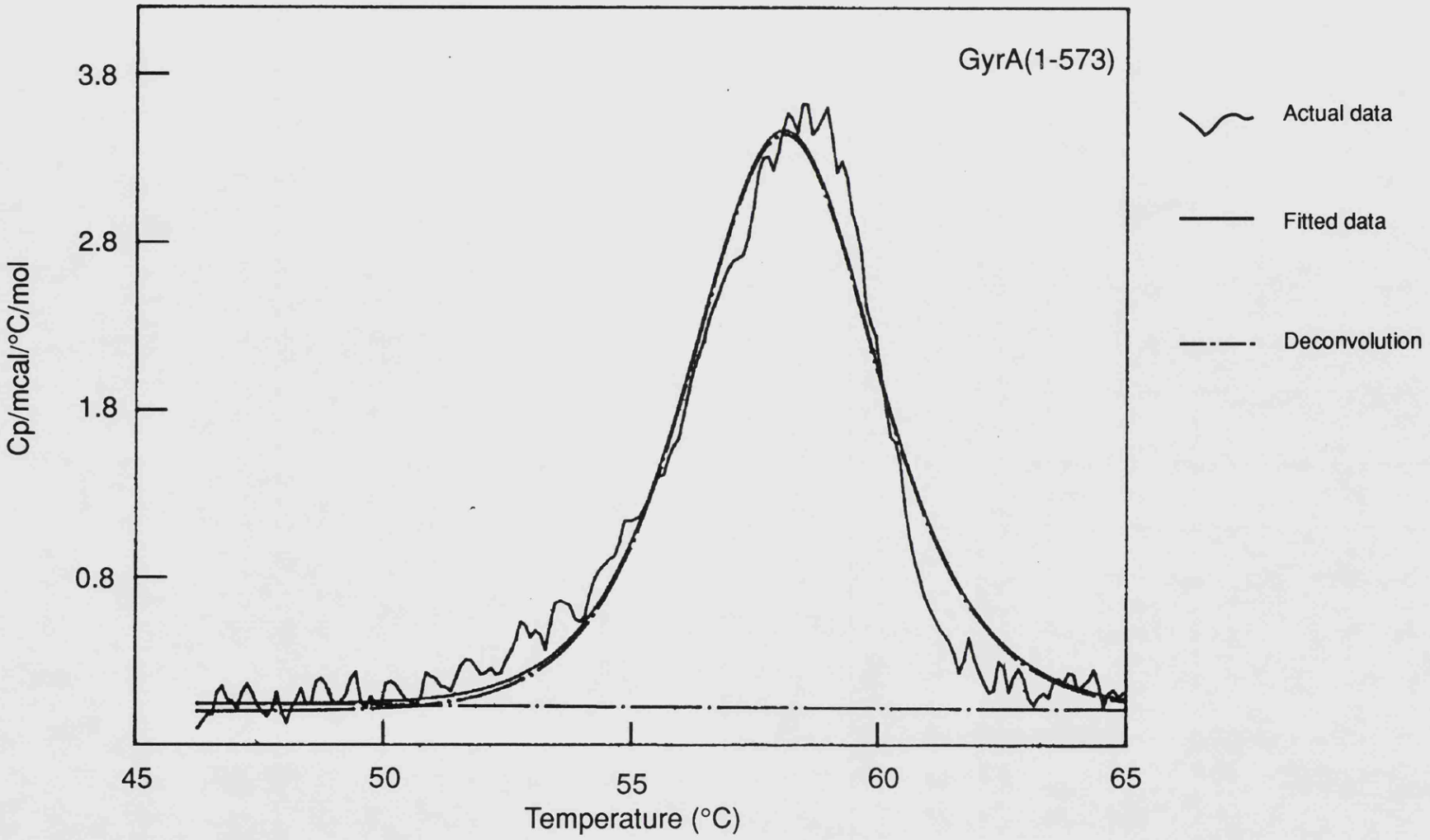


Fig. 5.3. Deconvolution of the GyrA(1-573) temperature transition curve into two separate events.

calculated data for the GyrA(1-573) do not fit the experimentally obtained data as well as the GyrA data (see Fig. 5.2), but is consistent with a single transition model. A summary of the Tm and ΔH values obtained for GyrA and GyrA(1-573) are shown below.

	Tm (°C)	ΔH ($kcalmol^{-1}$)
GyrA(1-573)	58.1	173
GyrA	58.7	168
	59.6	139

(b) Crystallization of GyrA fragments.

In order to obtain high resolution structural data of a protein it is necessary to grow crystals of the protein that can be subjected to X-ray analysis and the diffraction patterns interpreted. It is not possible to say under what conditions a protein will crystallize *a priori*, and many different conditions must be attempted so that correct parameters may be identified. However, it is generally considered vital to have pure protein preparations in order to achieve successful crystallization (McPherson, 1982).

(i) Protein purity.

One of the problems with attempts to crystallize of the truncated GyrA proteins has been identified as the purity of the protein preparations. Although the protein appears to be a single unique band on an SDS-polyacrylamide gel, overloading such gels, or staining with silver, leads to the appearance of a number of discrete bands beneath the major band. These are shown for the 64 kDa GyrA(1-573) in Fig. 5.4, but can also be noted for GyrA(1-523) and the intact GyrA protein itself (data not shown). These contaminating bands constitute less than 2% of the major protein as judged by densitometry. The minor bands in the GyrA(1-573) preparation (often four or five are visible) range in molecular mass from about 45 kDa up to just below 64 kDa, and

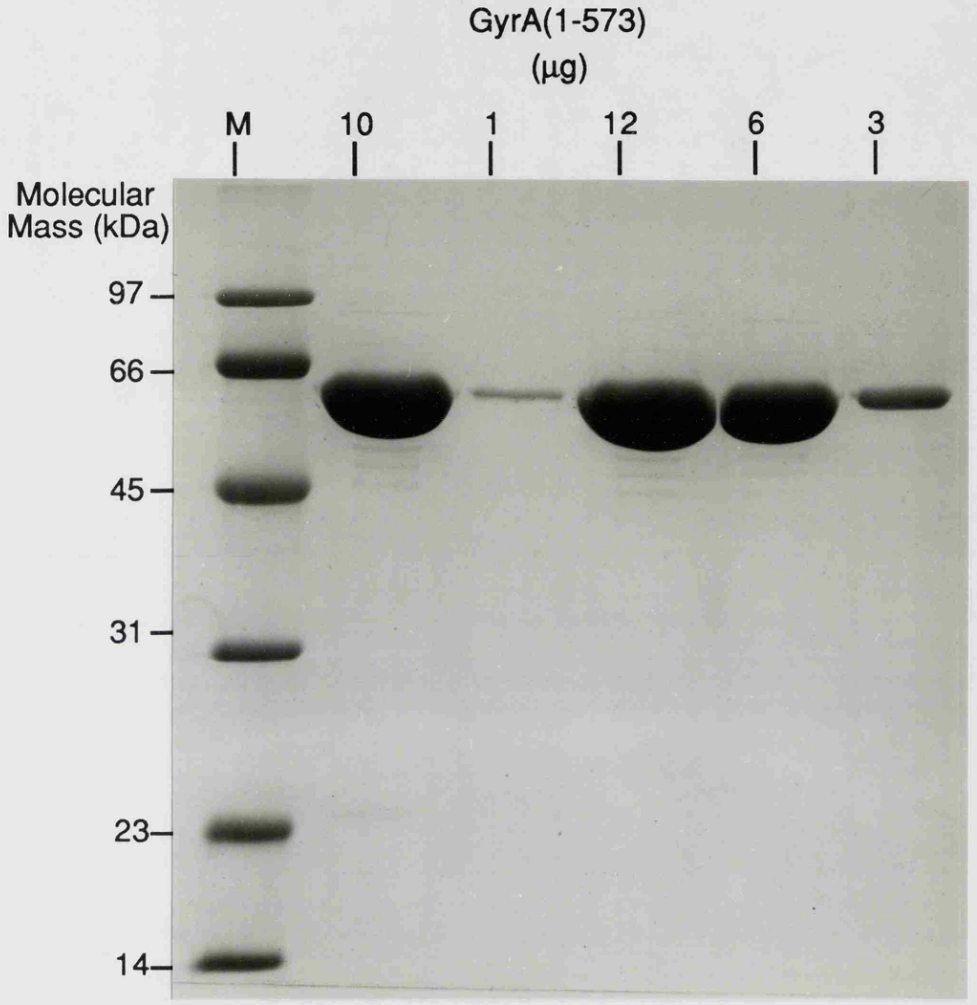


Fig. 5.4. An SDS-polyacrylamide gel of GyrA(1-573). GyrA(1-573), at the amounts indicated, were run out on an 12.5% polyacrylamide gel in the presence of 0.1% SDS. The lane M contained molecular mass markers, whose sizes are also indicated. The gel was stained with Coomassie blue.

there is always the possibility that there are other bands that are hidden by the GyrA(1-573) protein itself. These bands may represent different proteins that co-purify with the gyrase A proteins or may be digestion products of the gyrase proteins themselves, although the pattern is not altered by the inclusion of protease inhibitors, such as PMSF, in buffers (data not shown). It was therefore decided to separate the proteins in two-dimensions so that similar sized proteins should be well separated and then probe the bands obtained with anti-GyrA antibodies to see if the extra bands are indeed shortened versions of the GyrA protein.

Fig. 5.5 shows the results of isoelectric focusing gels of GyrA, GyrA(1-573) and GyrA(1-573) that had been denatured in guanidine and allowed to renature (see chapter 4). Although each of the preparations appears to be free of major contaminants on an SDS-polyacrylamide gel (e.g. Fig. 5.4) it is clear that there are a number of species present for each protein under these IEF conditions. The GyrA protein is composed of eight discrete bands spread over a pI range of 4.6-5.4. The band of highest intensity has an approximate pI of 5.3. There are no differences between the patterns obtained for GyrA(1-573) and the denatured and refolded version of the protein, but there are many discrete bands (at least ten) visible on the IEF rod for each. There are two major bands of nearly equal intensity at a pI value of approximately 5.2. There are, however, other discrete minor bands at pI values both above and below this, and a band much more 'smeary' in appearance at a pI value of 5.4. If such an IEF rod of GyrA(1-573) is subjected to SDS-PAGE in the second dimension then the pattern obtained in Fig. 5.6 can be noted. All of the protein has an molecular mass of approximately 64 kDa (as expected for GyrA(1-573)) except for the smeared band at pI 5.4 on the IEF rod which now appears to be mainly 64 kDa, but smears back toward the top of the gel. It is likely that this is artifactual and that all the protein is in fact of molecular mass 64 kDa. A gel such as that shown in Fig 5.6 was blotted onto a nitrocellulose membrane so that it could be probed with anti-GyrA antibodies to investigate the nature of the 64 kDa protein.

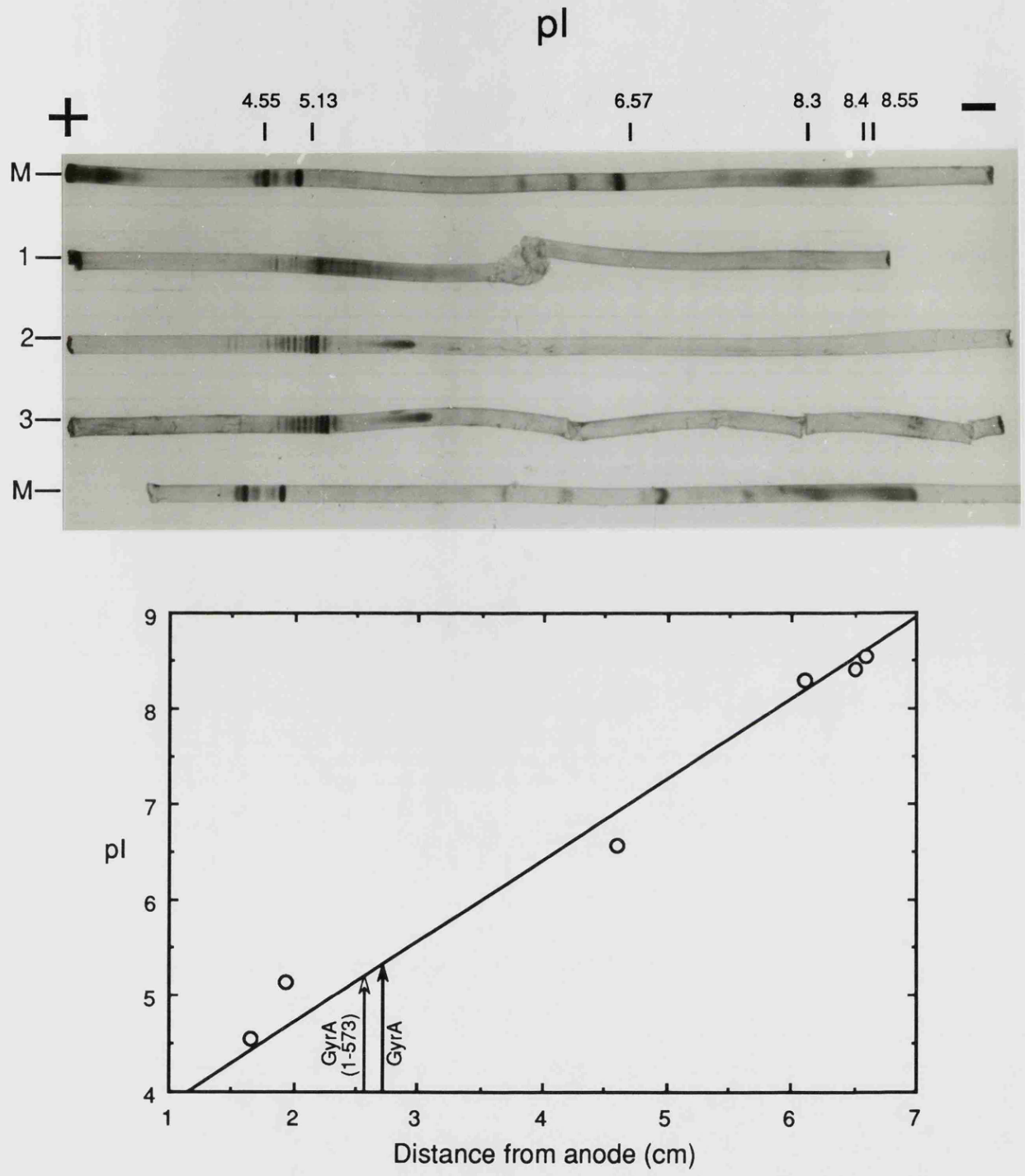


Fig. 5.5. Isoelectric focussing of GyrA and GyrA(1-573). 5 μg samples of the GyrA protein (1), the GyrA(1-573) protein (2), and the GyrA(1-573) protein that had been denatured and allowed to refold (3) were run on IEF rods (as described in section 2.20). The two samples labelled M contained protein whose pI value is known. The marker proteins (and pI values) are as follows; soybean trypsin inhibitor (4.55), β-lactoglobin (5.13), carbonic anhydrase (6.57), and lactate dehydrogenase (8.3, 8.4, and 8.55). Below is shown a graph of pI against distance from anode and the position of the major bands in GyrA (filled arrow) and GyrA(1-573) (open arrow).

Fig. 5.6. A two-dimensional gel of GyrA(1-573). A 5 μg sample of GyrA(1-573) was subjected to IEF as described for Fig. 5.5. The rod was then subjected to SDS-PAGE at 90° to the original gel. The + and - indicate the orientation of the IEF rod, and the position of molecular mass markers (used only for the SDS-PAGE) are indicated. The gel was stained with Coomassie blue.

There were six anti-GyrA monoclonal antibodies available in partially purified form (from A. Maxwell), but these were relatively uncharacterised and there was no indication as to which part of GyrA they will react. In order to further characterise the numerous bands obtained on the IEF gel it is necessary to have an antibody that will recognise the N-terminal 573 amino acids of the GyrA protein. Fig. 5.7 shows the probing of intact GyrA, the A' protein (containing both the N-terminal 64 kDa and the C-terminal 33 kDa tryptic fragments of GyrA; see chapter 3) and GyrA(1-573) with each of the six antibodies. The reactions of the antibodies are shown below in tabulated form:

Anti-GyrA antibody	Reaction with intact GyrA	Reaction with N-terminal of GyrA	Reaction with C-terminal of GyrA
4D3	Yes	Yes	No
4F6	Yes	Yes	No
6D9	Yes	Yes	No
7F3	Yes	No	No
7F11	Yes	Yes	No
10C6	Yes	Yes	No

There are a number of the anti-GyrA antibodies that specifically recognise the N-terminal 64 kDa fragment of GyrA (and also the cloned GyrA(1-573) protein) whilst none of the antibodies used only recognised GyrA and the C-terminal 33 kDa fragment. This could suggest the the N-terminal 64 kDa of the GyrA protein is somewhat more antigenic than the C-terminal portion of the protein. The antibody 7F3 recognised GyrA but failed to recognise either the N- or C-terminal tryptic fragments or GyrA(1-573). This could indicate that the 7F3 antibody binds to regions contained in both fragments that is disrupted upon their separation. The strength of the observed signal varied depending on which antibody was used. For example, 7F11 and 10C6 recognise GyrA, the 64 kDa tryptic fragment and GyrA(1-

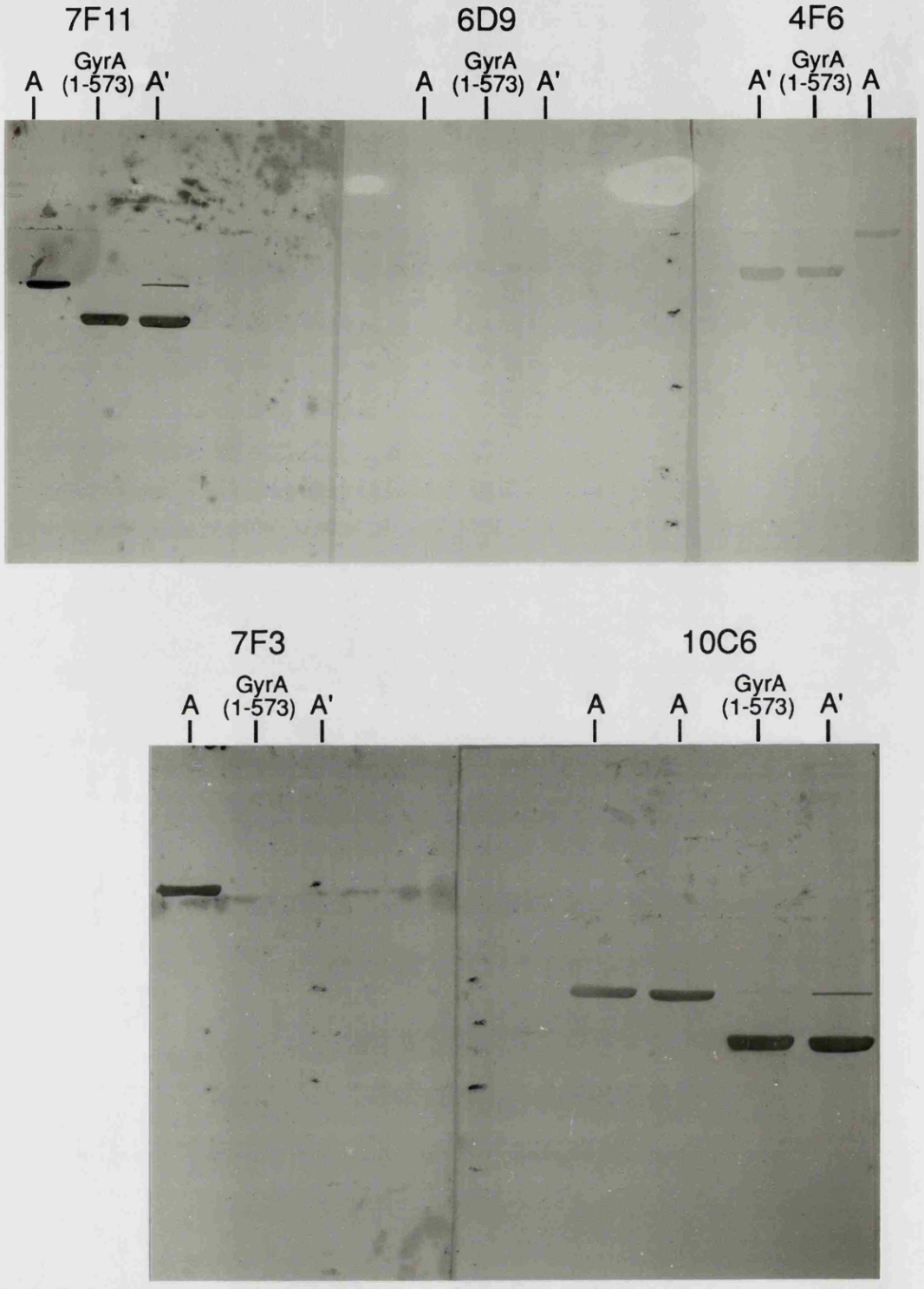


Fig. 5.7. Samples of GyrA, A' and GyrA(1-573) probed with anti-GyrA monoclonal antibodies. 2 μg samples of GyrA, the trypsin-treated A', and GyrA(1-573) were run on 12.5% SDS-PAGE and blotted to nitrocellulose membranes. The membranes were then probed with anti-GyrA antibodies as described in section 2.20. Antibody detection was performed using goat-anti mouse IgG (H+L) horseradish peroxidase conjugate.

573) with about the same efficiency, and the amount of bound antibody detected by the methods utilised here is considerably greater than that for some of the other antibodies. It was therefore decided to use these two antibodies to probe the multiple bands obtained by IEF to order to elucidate whether they are, in fact, all part of the same protein.

A nitrocellulose membrane, to which either GyrA or GyrA(1-573) had been transferred from a 2-D gel, was incubated with the 7F11 anti-GyrA antibody. After antibody detection (as described in section 2.20) the membranes shown in Fig. 5.8 were obtained. It can be seen that in both cases all the protein from the 2-dimensional IEF/SDS-polyacrylamide gel is detected by the antibody. This therefore indicates that the many species obtained by IEF of GyrA and GyrA(1-573) are all different forms of the same protein. That is, rather than being foreign protein contaminants, the multiple bands must be altered variants of GyrA and the truncated GyrA(1-573).

The initial rationale for running IEF gels of GyrA(1-573) was to establish the nature of the lower molecular mass contaminant bands that co-purified with the protein. It was not possible, however, to overload an IEF gel to a sufficient extent so that these bands are visible on the second dimension polyacrylamide gel, or to be distinguished from the many bands visible on the IEF gel itself. This therefore necessitated the probing of a nitrocellulose membrane to which proteins had been transferred from an over-loaded SDS-polyacrylamide gel. The results of incubation of such a membrane with the 10C6 anti-GyrA antibody are shown in Fig. 5.9. It is clear that the bands beneath the major protein band are indeed recognised by the anti-GyrA antibody, and this therefore suggests that they are degradation products of the main protein.

(ii) Crystallization trials.

GyrA(1-573) and GyrA(1-523) were purified to near homogeneity as described previously (see chapter 4). The protein preparations used for crystallization trials

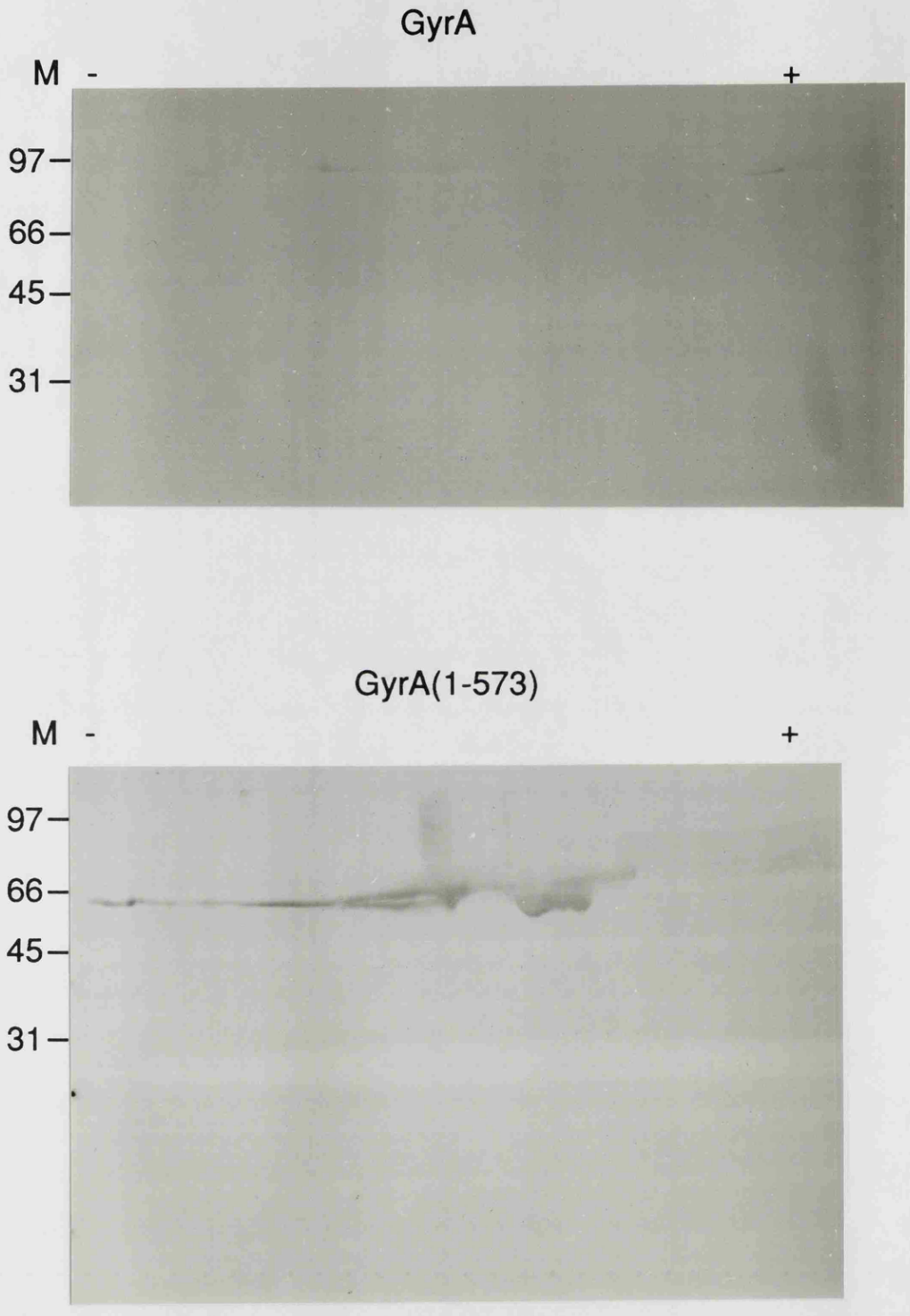


Fig. 5.8. Two-dimensional gels of GyrA and GyrA(1-573). 5 µg samples of either GyrA or GyrA(1-573) were subjected to IEF and then SDS-PAGE. The proteins were then blotted onto a nitrocellulose membrane and probed with the 7F11 anti-GyrA antibody, and detection performed as described in section 2.20. The positions of molecular mass markers are also indicated, and the + and - indicate the orientation of the IEF rod.

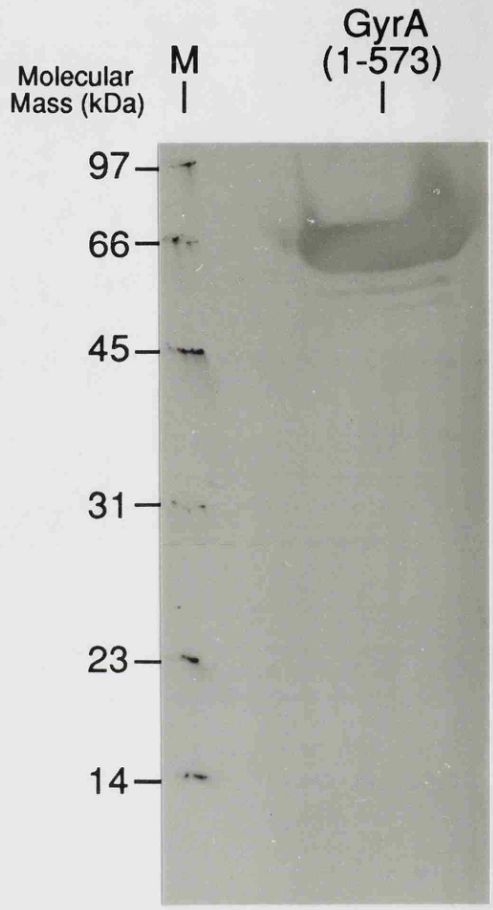


Fig. 5.9. SDS-PAGE of GyrA(1-573) probed with anti-GyrA antibody. A 15 μg sample of GyrA(1-573) was run on a 12.5% SDS-PAGE and the protein blotted onto a nitrocellulose membrane. The membrane was probed with 10C6 anti-GyrA antibody and detection performed as described in section 2.20. The positions of molecular mass markers are also indicated (marked with ball-point pen after staining with Ponceau S).

contained a low proportion of truncated protein contamination (see above). Protein samples were concentrated to 8-15 mg/ml in low salt buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT). Samples were then used in crystallization trials utilising the 'hanging-drop' method. A variety of different conditions were used in an attempt to get the proteins to form crystals. Three protein precipitants were used; ammonium sulphate, MPD, and polyethylene glycol (average molecular weights ranging from 600 to 20,000). The amount of precipitant in each sample, the pH of the solution and the temperature of incubation were varied. The results of all of the trials on GyrA(1-573) and GyrA(1-523) are shown in Fig. 5.10.

As represented by Fig. 5.10, under the majority of conditions it is possible to get the protein to precipitate, but, with a few notable exceptions, below the precipitation point there is no evidence for crystal formation. Several general conclusions can be drawn from these data. Firstly, the two protein preparations are very sensitive to precipitation by PEG, with concentrations down to 1%, in certain cases, being sufficient to bring the protein out of solution as a precipitate. Secondly, the effect of altering the temperature of the crystallization tray appeared to have some effect on the precipitation point of the protein in that increase temperature lead to lower precipitation points, but did not generally aid crystal formation. This effect could be most graphically illustrated for the trays left at room temperature. On warm days, or after a spell of hot weather, the precipitation point was lower than if the trays were observed on cold days. Thirdly, changes in the pH of the mother liquor and the protein drop seem to have effects on the solubility of both of the proteins used here. Generally, it was noted that raising the pH of the solution had the effect of lowering the concentration of, say, ammonium sulphate required to precipitate the protein.

There were five observed fates for the protein each of the sample drops:

(1) Nothing would apparently happen to the drop. No precipitate would form and the drop would remain clear.

Fig. 5.10. (shown on the next seven facing pages). Summarisation of all the crystallization trials of the 64 kDa GyrA(1-573) and the 59 kDa GyrA(1-523). Protein (10 μl), at the stated concentration, was mixed with an equal volume of the mother liquor and pipetted onto a plastic cover-slip. The cover-slip was then inverted over a well containing 2 ml of the mother liquor and a seal between the two made using high-vacuum grease. The plates (each having 24 such wells) were checked at regular intervals and the state of the droplet noted over many months. In the tables here; ppt indicates that an insoluble thick precipitate formed in the drop; grainy has been used to represent the presence of a semi-crystalline precipitate in the drop; oil indicates the formation of clumped oil-like droplets; and crystal is used to indicate the tentative presence of protein crystals in the drop. The results were scored after 10 months for the GyrA(1-573) samples and three months for the GyrA(1-523) samples.

GyrA(1-573) at 10 mg/ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

pH 6.8	1%	2%	4%	6%	8%	10%
PEG600	-	-	ppt	ppt	ppt	ppt
PEG1K	-	ppt	ppt	ppt	ppt	ppt
PEG4K	-	-	-	ppt	ppt	ppt
PEG6K	-	-	ppt	ppt	ppt	ppt

pH 6.8	1%	2%	4%	6%	8%	10%
PEG8K	ppt	ppt	ppt	ppt	ppt	ppt
PEG20K	ppt	ppt	ppt	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	ppt	ppt	ppt

pH 7.5	1%	2%	4%	6%	8%	10%
PEG600	-	-	ppt	ppt	ppt	ppt
PEG1K	-	ppt	-	-	ppt	ppt
PEG4K	-	-	ppt	ppt	ppt	ppt
PEG6K	-	-	-	-	ppt	ppt

pH 7.5	1%	2%	4%	6%	8%	10%
PEG8K	-	ppt	ppt	ppt	ppt	ppt
PEG20K	-	ppt	ppt	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	ppt	ppt	ppt

pH 8.5	1%	2%	4%	6%	8%	10%
PEG600	-	ppt	ppt	ppt	ppt	ppt
PEG1K	-	-	-	ppt	ppt	ppt
PEG4K	-	ppt	ppt	ppt	ppt	ppt
PEG6K	-	ppt	ppt	ppt	ppt	ppt

pH 8.5	1%	2%	4%	6%	8%	10%
PEG8K	-	ppt	ppt	ppt	ppt	ppt
PEG20K	ppt	ppt	ppt	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	ppt	ppt	ppt

GyrA(1-573) at 10 mg/ml in 100 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 6.8	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	ppt	ppt	ppt	ppt	ppt	ppt
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 7.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 8.0	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	ppt	-	**crystals**	**crystals**
	22%	23%	24%	25%	26%	27%
	ppt	ppt	ppt	ppt	ppt	ppt
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

GyrA(1-573) at 10 mg/ml in 100 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 8.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	grainy	ppt	ppt
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 9.0	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	grainy	**crystals**	-
	22%	23%	24%	25%	26%	27%
	crystals	ppt	ppt	ppt	ppt	ppt
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 9.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	ppt	ppt	ppt	ppt	ppt
	22%	23%	24%	25%	26%	27%
	ppt	ppt	ppt	ppt	ppt	ppt
	28%	29%	30%	31%	32%	33%
	ppt	ppt	ppt	ppt	ppt	ppt

GyrA(1-573) at 9 mg/ml in 20 mM Tris-HCl. RT.

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 7.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	crystals	**crystals**	**crystals**	ppt	ppt	ppt

GyrA(1-573) at 9 mg/ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. 4°C.

$(NH_4)_2SO_4$	10%	15%	20%	25%	30%	35%
pH 6.5	-	-	-	ppt	ppt	ppt
pH 7.5	-	-	-	-	ppt	ppt
pH 8.5	-	-	-	ppt	ppt	ppt
pH 9.5	-	-	-	ppt	-	ppt

GyrA(1-573) at 9 mg/ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

$(NH_4)_2SO_4$	10%	15%	20%	25%	30%	35%
pH 6.5	-	-	ppt	ppt	ppt	ppt
pH 7.5	-	-	-	-	ppt	ppt
pH 8.5	-	-	-	ppt	ppt	ppt
pH 9.5	-	-	-	ppt	ppt	ppt

GyrA(1-573) at 9 mg/ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. 30°C.

$(NH_4)_2SO_4$	10%	15%	20%	25%	30%	35%
pH 6.5	-	ppt	ppt	ppt	ppt	ppt
pH 7.5	-	-	ppt	ppt	-	ppt
pH 8.5	-	-	ppt	ppt	ppt	ppt
pH 9.5	-	ppt	ppt	ppt	ppt	ppt

GyrA(1-523) at 11 mg/ml in 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

pH 6.8	1%	2%	4%	6%	8%	10%
PEG600	ppt	-	ppt	ppt	ppt	ppt
PEG1K	-	ppt	ppt	ppt	ppt	ppt
PEG4K	-	ppt	ppt	ppt	ppt	ppt
PEG6K	-	ppt	ppt	ppt	ppt	ppt

pH 6.8	1%	2%	4%	6%	8%	10%
PEG8K	-	-	ppt	ppt	ppt	ppt
PEG20K	-	-	ppt	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	ppt	ppt	ppt

pH 7.5	1%	2%	4%	6%	8%	10%
PEG600	-	oil/ppt	ppt	ppt	ppt	ppt
PEG1K	-	oil/ppt	ppt	ppt	ppt	ppt
PEG4K	oil	oil	oil/ppt	ppt	ppt	ppt
PEG6K	oil	ppt	ppt	ppt	ppt	ppt

pH 7.5	1%	2%	4%	6%	8%	10%
PEG8K	-	-	-	ppt	ppt	ppt
PEG20K	-	-	grainy	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	-	ppt	ppt

pH 8.5	1%	2%	4%	6%	8%	10%
PEG600	-	-	-	ppt	ppt	ppt
PEG1K	-	-	-	-	ppt	ppt
PEG4K	-	-	ppt	ppt	ppt	ppt
PEG6K	grainy	-	ppt	ppt	ppt	ppt

pH 8.5	1%	2%	4%	6%	8%	10%
PEG8K	-	-	ppt	ppt	ppt	ppt
PEG20K	-	-	ppt	ppt	ppt	ppt
	5%	10%	15%	20%	25%	30%
MPD	-	-	-	-	grainy	grainy

GyrA(1-523) at 11 mg/ml in 100 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 6.8	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	-	-	-	-	-	-

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 7.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	-	-	-	-	-	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 8.0	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	
	28%	29%	30%	31%	32%	33%
	-	-	-	grainy	ppt	ppt

GyrA(1-523) at 11 mg/ml in 100 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT. RT.

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 8.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	-	-	-	-	-	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 9.0	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	ppt	-	grainy	ppt	ppt	ppt

$(NH_4)_2SO_4$	10%	11%	12%	13%	14%	15%
pH 9.5	-	-	-	-	-	-
	16%	17%	18%	19%	20%	21%
	-	-	-	-	-	-
	22%	23%	24%	25%	26%	27%
	-	-	-	-	-	-
	28%	29%	30%	31%	32%	33%
	-	-	grainy	grainy	ppt	ppt

(2) The protein would come out of solution in the form of a thick white, precipitate (see Fig. 5.11A). This is thought to represent denatured protein.

(3) The protein may still precipitate, but a rather granular, semi-crystalline structure is formed. This is shown in Fig. 5.11B.

(4) The GyrA(1-523) protein, at pH 7.5 and with some concentrations of PEG, formed an oil-droplet like structure (see Fig. 5.12). These are obviously interacting in some fashion due to their ability to clump together, but the structure is by no means ordered, and there no indication of crystal formation.

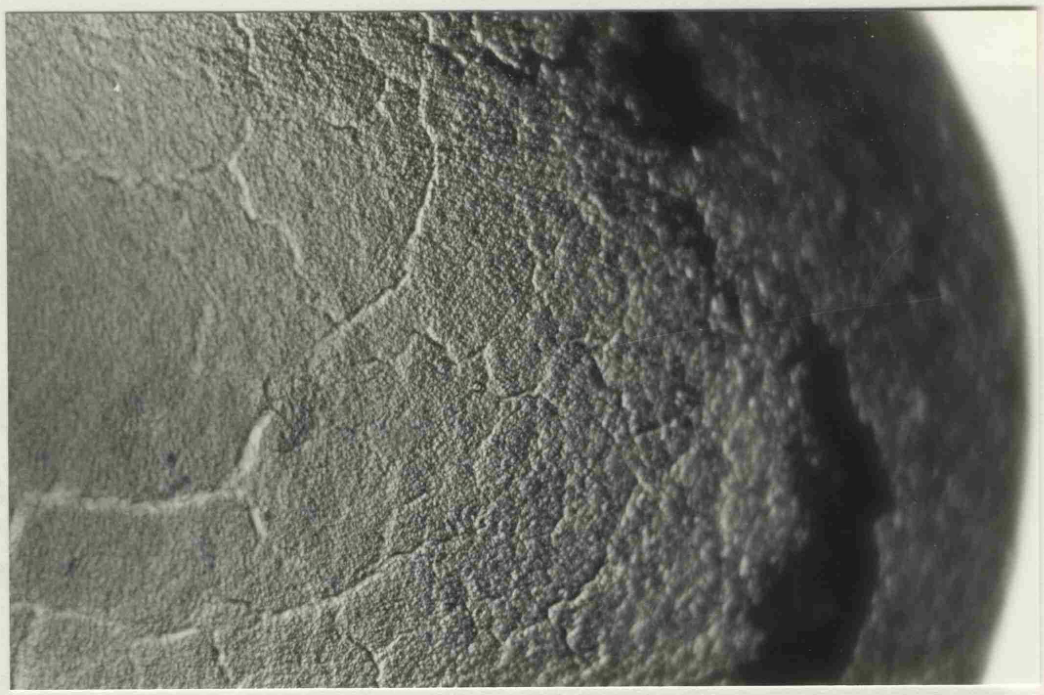
(5) Putative protein crystals could form.

Four different crystal types have been identified for the GyrA(1-573) protein:

(a). From a solution of 100 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 5 mM DTT, 22% ammonium sulphate, 'rugby-ball' shaped crystals could be noted after 2-3 months at room temperature (Fig. 5.13A). The approximate size of the crystal shown is 50 μm by 20 μm. These crystals were not very regular, with few distinct faces, and no diffraction pattern could be observed for this 'rugby-ball' type crystal when subjected to X-ray analysis at the synchrotron source at Hamburg, West Germany (D.B. Wigley, personal communication). A number of these crystals were grown, but none were any larger than the one shown.

(b). From a solution of 100 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 5 mM DTT, 20% ammonium sulphate, 'pyramid'-shaped crystals could be noted after 2-3 months at room temperature (Fig. 5.13B). The approximate size of these crystal forms were 20 μm by 20 μm. The 'pyramid' crystals had much more of a regular appearance, but their small size excluded them from X-ray analysis. Both the above crystal forms were grown at pH 9.0. At this high pH, intact gyrase is still active as a supercoiling enzyme; DNA gyrase having quite a broad pH profile (D.P. Weiner and A. Maxwell, personal communication), so it is reasonable to assume that if any structure can be

A



B

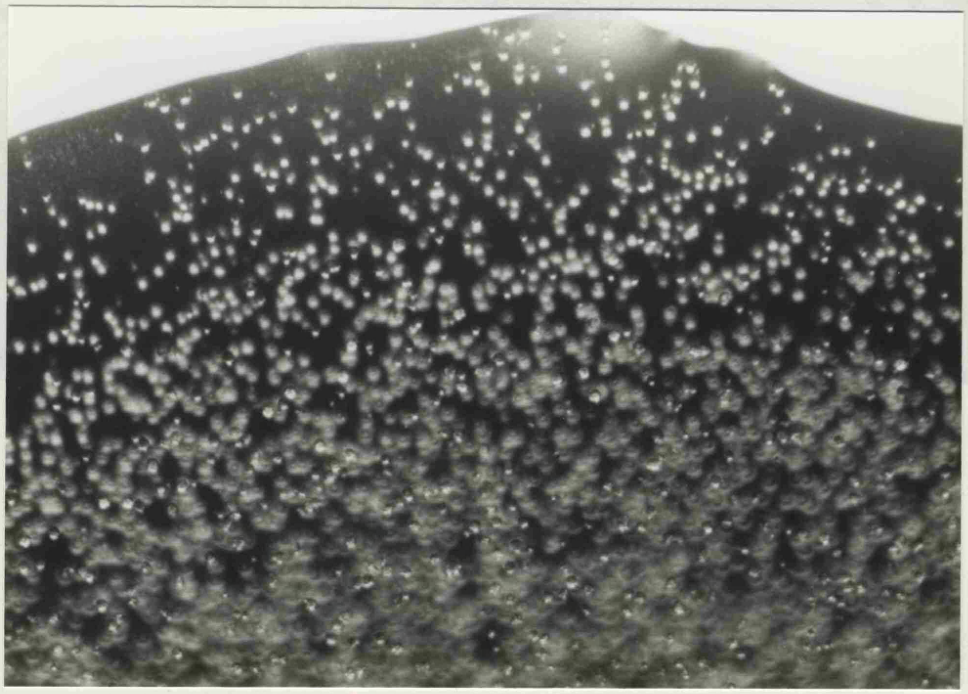


Fig. 5.11. Precipitates formed by GyrA(1-573) during crystallography trials. **(A).** A thick amorphous precipitate formed by GyrA(1-573), at 9 mg/ml in 20 mM Tris-HCl (pH 7.5) and 33% ammonium sulphate. **(B).** A granular precipitate of GyrA(1-573), at 10 mg/ml in 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM DTT and 25% ammonium sulphate. Both (A) and (B) were photographed at 40X magnification.

Fig. 5.12. Oil-droplets of GyrA(1-523). GyrA(1-523), at 11 mg/ml in 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 1% PEG 4000, formed an 'oil-drop' precipitate. The above photograph was taken at 100X magnification.

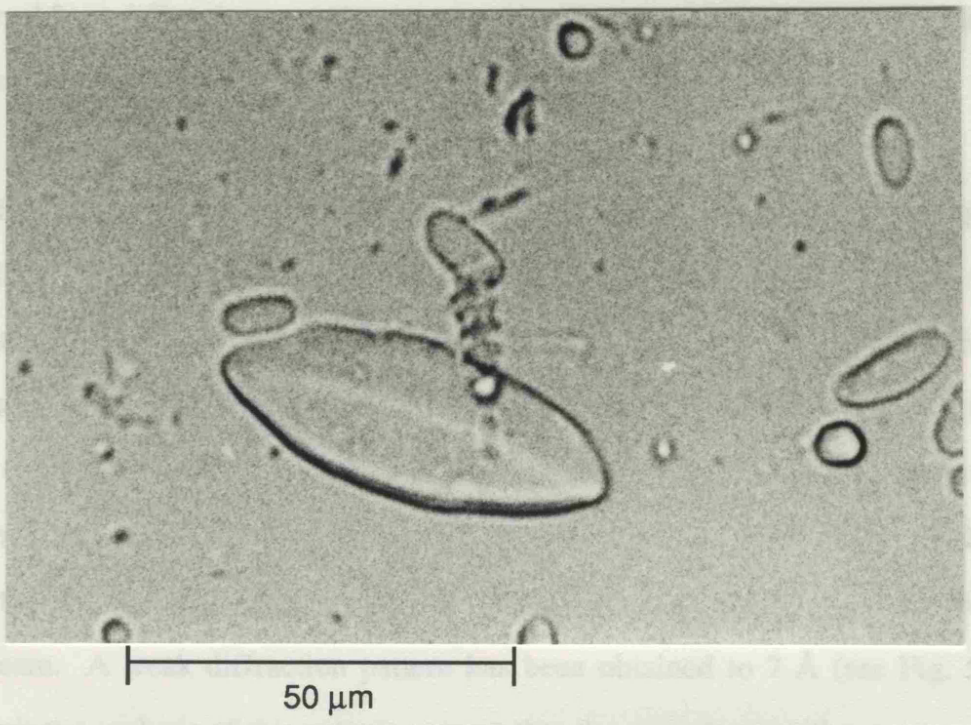


Fig. 5.13. Crystal forms of GyrA(1-573) grown at pH 9. (A). 'Rugby-ball' crystals grown from a solution of GyrA(1-573), at 10 mg/ml in 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM DTT and 22% ammonium sulphate. **(B).** 'Pyramid' crystals grown from a solution of GyrA(1-573), at 10 mg/ml in 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM DTT and 20% ammonium sulphate. In both cases the drops were incubated at room temperature and crystals could be noted after several months.

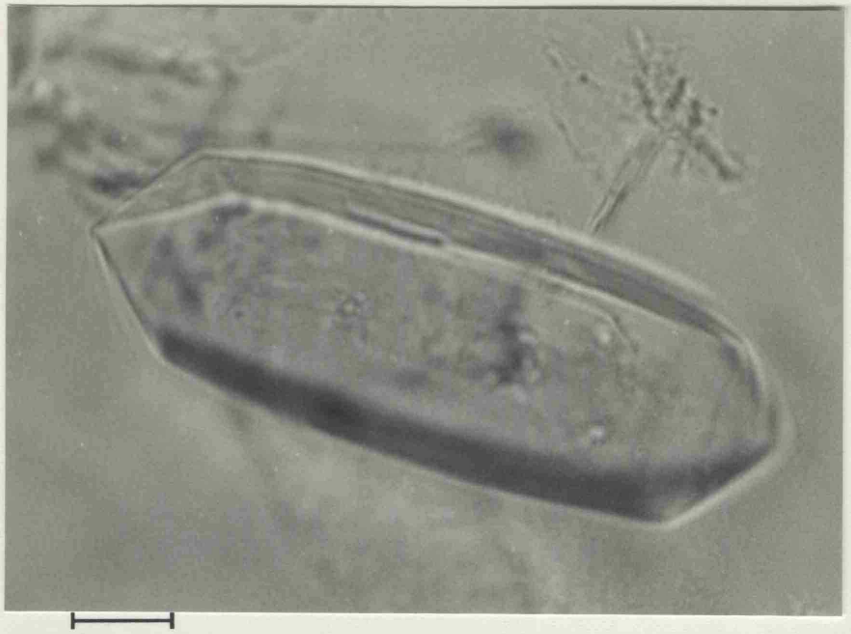
obtained from diffracting crystals grown at this pH then they are likely to represent an active protein structure.

(c). From a solution of 100 mM Tris-HCl (pH 7.5), 28-30% ammonium sulphate, 'needle' crystals could be noted after 8-9 months at room temperature (Fig. 5.14). The largest crystal obtained under these conditions measured 350 μm by 75 μm, but the majority were about 100 μm in length. A number of the crystals obtained under these conditions had the appearance of the 'rugby-balls' obtained at pH 9.0, but some of them had distinct sharp faces. The largest crystal obtained was damaged during transit to the X-ray source at Hamburg, so one of the smaller crystals was inserted into the beam. A weak diffraction pattern has been obtained to 7 Å (see Fig. 5.15). Preliminary analysis of this pattern suggest that the crystals are either tetragonal or orthorhombic with unit cell dimensions of 170 x 170 x 150 Å. It is therefore likely that there are four or eight molecules in the asymmetric unit, and that the protein could be arranged in a tetrameric structure. The lack of resolution of the diffraction pattern has been attributed to the small size of the crystals used rather than any disordered nature of the crystals themselves (D.B. Wigley, personal communication).

(d). From a solution of 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 20-21% ammonium sulphate, 'barrel' shaped crystals could be noted after 5-6 months at room temperature (Fig. 5.16). The approximate size of these crystal forms were 40 μm by 20 μm, and were regularly organised into hexagonally-shaped barrels. The size of the crystals, however, was considered too small for X-ray diffraction analysis.

No crystal forms of the GyrA(1-523) protein have thus far been obtained, but at pH 7.5 with low concentrations PEG600, 1000, 4000 and 6000, an 'oil droplet'-like cluster of material could be noted and is shown in Fig. 5.12. The exact nature of this interaction is not clear, but it is possible that the protein is forming some kind of

A



B

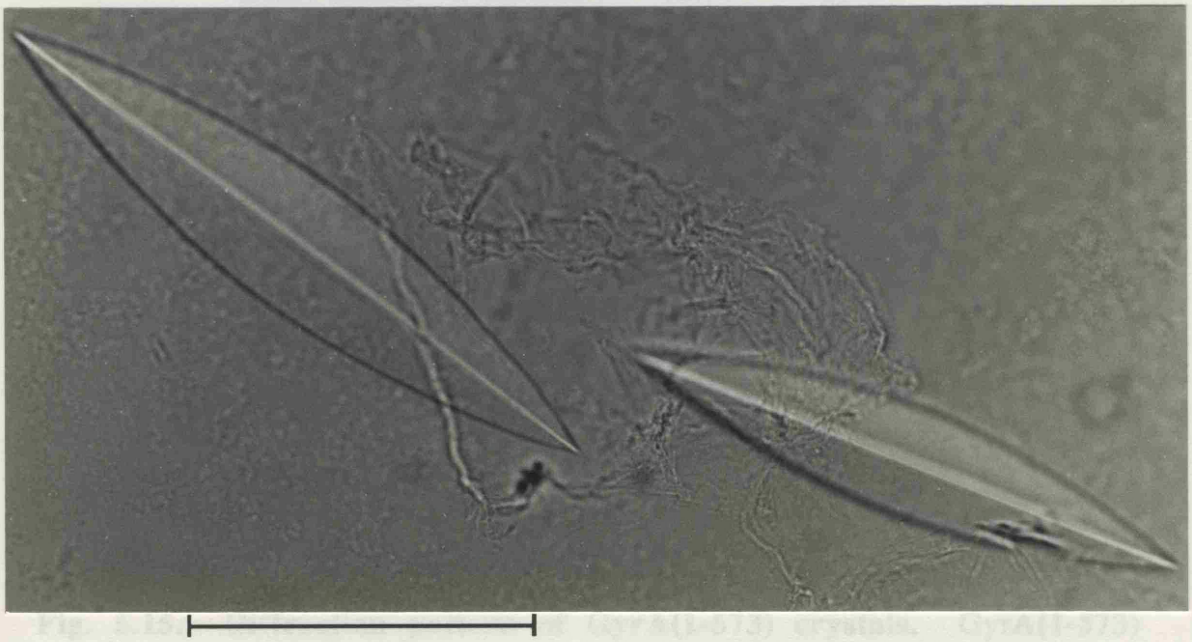


Fig. 5.14. Crystal forms of GyrA(1-573) grown at pH 7.5. The two 'needle' crystal types shown here (A and B) were grown from a solution of GyrA(1-573), at 9 mg/ml in 20 mM Tris-HCl (pH 7.5), and 28% and 30% ammonium sulphate respectively. The drops were incubated at room temperature and crystals could be noted after approximately eight months.

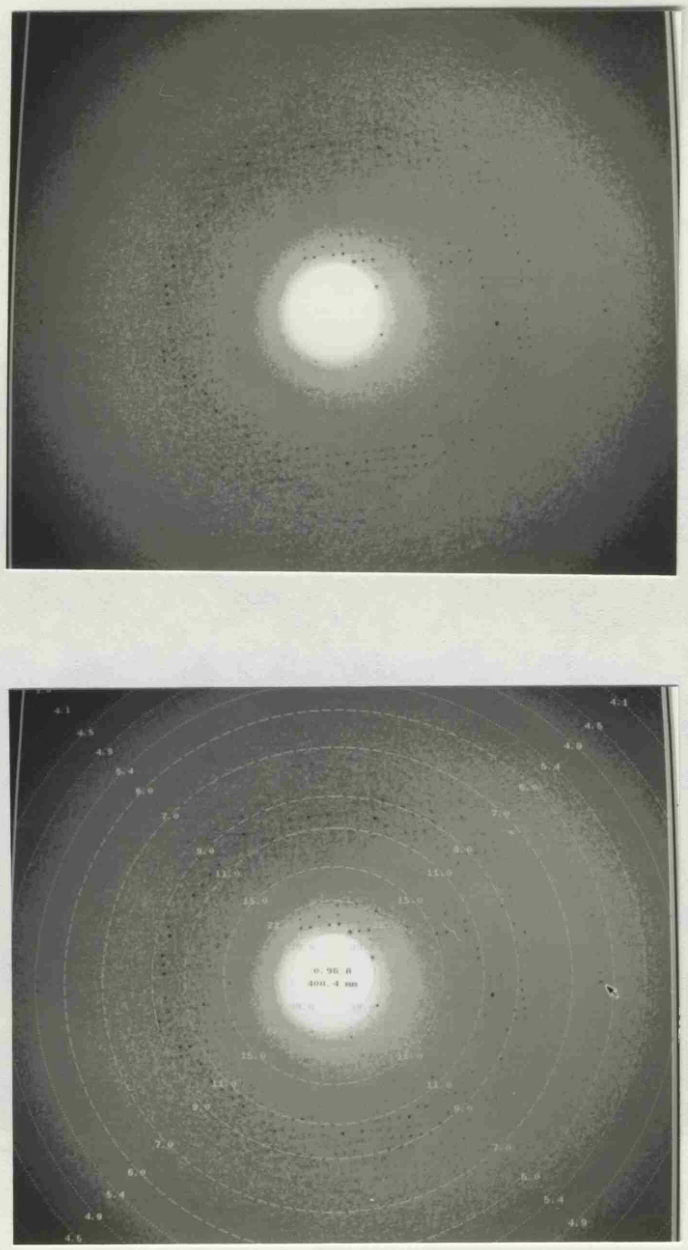


Fig. 5.15. Diffraction pattern of GyrA(1-573) crystals. GyrA(1-573) crystals (of the type shown in Fig. 5.14B) were subjected to X-ray analysis. Shown here is a 1° oscillation image, taken on port X-11 at DESY, Hamburg. X-rays of a wavelength of 0.96 Å were used. The pattern was recorded on an imaging plate, which could then be represented on a computer screen and photographed. The resolution of the pattern (in Å) is shown on the lower photograph; the arrow indicating the spot of highest resolution.

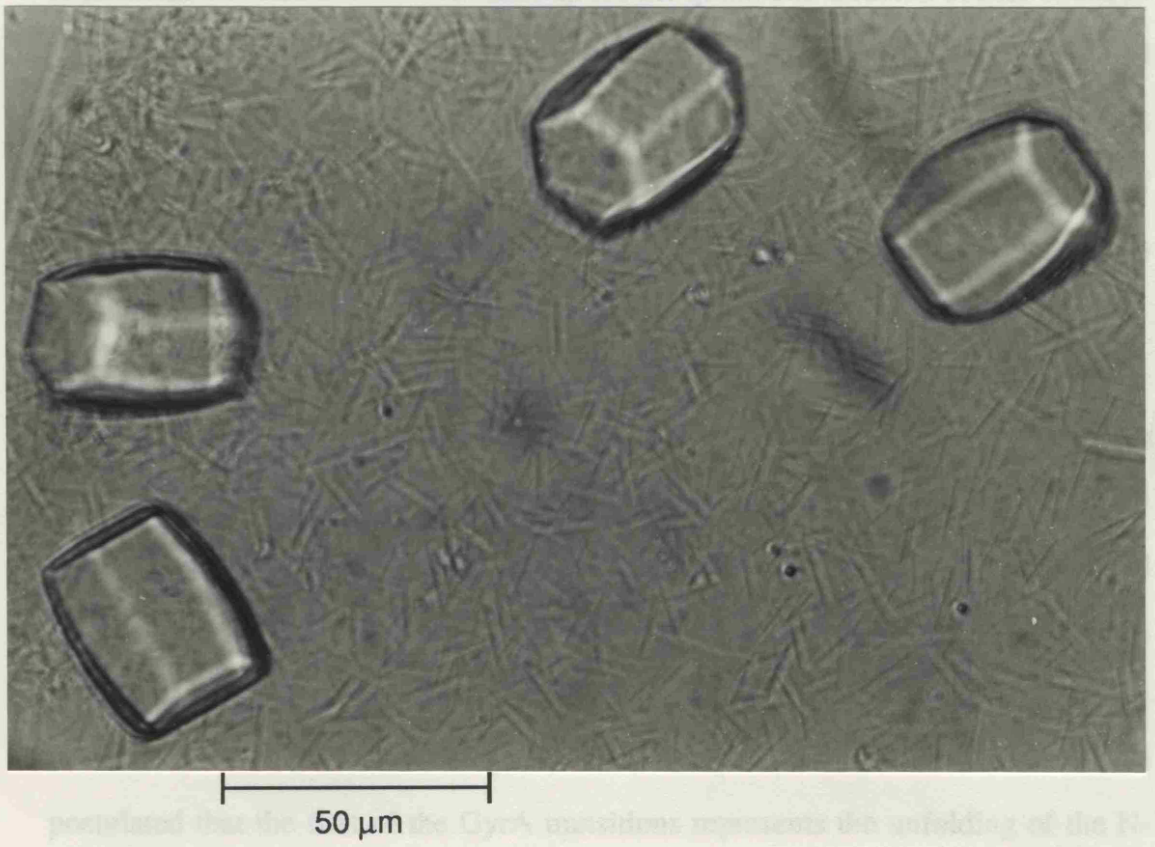


Fig. 5.16. Crystals of GyrA(1-573) grown at pH 8.0. The 'barrel-shaped' crystals were grown from a solution of GyrA(1-573), at 10 mg/ml in 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT and 21% ammonium sulphate. The drops were incubated at room temperature and crystals could be noted after several months.

ordered array, but the interaction is not sufficiently specific so that a regular crystal lattice is formed.

5.3 Discussion.

Previous chapters in this thesis have inferred the presence of distinct functional domains within the DNA gyrase A subunit. Enzymic evidence has suggested that the N-terminal 523 amino acids are essential for the quinolone-directed double-stranded cleavage of DNA. The C-terminal 33 kDa of the GyrA protein appears to be required for the stability of the enzyme-DNA complex, and is needed to allow efficient supercoiling to occur. It has been speculated that the GyrA protein is composed of two domains; an N-terminal breakage-reunion domain and a C-terminal domain involved in the stability of the gyrase complex.

Scanning microcalorimetry of GyrA yields a non-symmetrical unfolding transition at approximately 59°C. The experimental data obtained can be best fitted to a dual transition model where the transition temperatures are close together (58.7°C and 59.6°C). This therefore suggests the presence of two domains within the GyrA protein. The first of the transitions of GyrA corresponds almost exactly to the single transition obtained with either GyrA(1-573) or GyrA(1-523). It can therefore be postulated that the first of the GyrA transitions represents the unfolding of the N-terminal breakage-reunion domain, whilst the second transition shows the C-terminal 'stability' domain unfolding. One problem with these data is the proximity of the two GyrA transitions. Although the data obtained were reproducible over two separate runs, the significance of the bulge on the non-symmetrical transition curve must be questioned. Ideally, the two transitions should be separated by at least several degrees so that the two transitions can be seen clearly on the raw data. However, this is not the case and the only way to clearly visualise the two separate peaks is by deconvolution of the transition into its constituent parts. This process has been performed for both GyrA and GyrA(1-573). Whilst the intact GyrA protein can be

separated into the two peaks mentioned above, attempts to perform the same function on the truncated protein result in a single peak that can best fit the data. Any second peak takes the form of a horizontal line that plays no part in the combined curve. This information, taken together with the fact the the transition temperature and the calculated ΔH value for the GyrA(1-573) transition are similar to those obtained for the first GyrA transition must give credence to the idea of the two domain model of the GyrA protein. Therefore, these data, when combined with the enzymic data presented in this thesis (chapters 3 and 4) suggest the presence of two distinct domains within the DNA gyrase A protein. There would appear to be some structural basis for this separated domain model by the double unfolding transition obtained by calorimetry.

Previously, it has been found that the supercoiling activity of the gyrase A_2B_2 complex can be heat-inactivated at 46°C, but this inactivity has been attributed to the thermal instability of the gyrase B protein (Peebles *et al.*, 1978). At 46°C the gyrase A protein was found to be stable and retain full supercoiling activity for up to one hour. The microcalorimetry experiments suggest that the A protein will not be denatured until a temperature of about 59°C is reached. Once past this point, however, the protein cannot be renatured by lowering the temperature. If this is attempted, then no unfolding transition recurs when the temperature is once more raised. The data presented here also suggest that intact GyrA is less susceptible to heat precipitation than its truncated relatives, and this could indicate a reduced stability of the isolated N-terminal domain.

Other calorimetric experiments that could be performed include the addition of a quinolone drug to the protein to assess the effect on the transitions of the GyrA protein, or performing the calorimetric analysis in the presence of the GyrB protein to see if this has any effect on the individual transitions of the A protein or the fragments. It is possible that the addition of quinolone drugs to the GyrA protein will stabilise the

N-terminal domain so that the unfolding temperature would now occur at a higher temperature.

Calorimetry is a technique from which information about the domain organisation of a protein can be obtained. Crystallography should yield much higher resolution structural data, but the attempts to crystallize the N-terminal domain of the DNA gyrase A protein have proved only partially successful. Some crystals of GyrA(1-573) have been obtained from ammonium sulphate solutions at a range of pH values. However, by their overall shape, the majority of the crystals are not very well ordered and indeed only a weak diffraction pattern to 7 Å has thus far been obtained. There are two problems that have been identified with the attempts to grow protein crystals of truncated GyrA. The first of these concerns the purity of the protein sample, and the second to the time taken for crystals to appear. There are reported cases where crystals can be grown from crude protein preparations, e.g. ferritin (see McPherson, 1982), but it is generally considered vital to have a pure sample before attempting crystallography. Over-loaded SDS-polyacrylamide gels of GyrA and GyrA(1-573) show the presence of a number of bands that co-purify with the gyrase proteins and cannot be separated from it by conventional chromatographic techniques. When these proteins are run on an IEF gel, a complex pattern of bands emerges. Probing these bands with anti-GyrA antibodies has shown that they are indeed multiple forms of the gyrase proteins. Each of these bands must represent a species of the protein that differs in charge from the other bands, but an explanation as to how these species arise is not immediately forthcoming. It is possible that limited proteolysis could be occurring during the preparation of the protein, so that charged amino acid residues are removed and consequently many differently charged species are present.

Such a heterogeneous mixture of differing charged polypeptides must reduce the possibility of successful crystal growth. As a crystal grows protein molecules come out of solution and form a regular lattice on the pre-existing structure, built around a

nucleation centre. If a non-homogeneous species adds onto the lattice then it is likely that the regular, ordered structure will break down. This will either result in no, or poor crystal formation. It is possible that the crystals observed here are not more regularly shaped due to this phenomenon.

The crystals of GyrA(1-573) obtained took several months to grow. In the case of the pH 7.5 crystals, up to nine months elapsed before any crystals could be noted. This could be due to the length of time required for the formation of a nucleation centre for the crystallization process to begin, or be due to the equilibration time necessary to build up a sufficient amount of a single species that is susceptible to crystallization. The effect of incubating the protein at room temperature for this length of time is not known, but it must be assumed that the protein is somewhat less than fully active after such treatment. Therefore, it is possible that a denatured form of the protein that has undergone the crystallization process. From the data obtained, it is not possible to tell whether this is the case or not. If the lack of nucleation sites is the rate-limiting step, the one possibility for further experimentation would be to use the already obtained crystals for seeding other protein solutions. This should facilitate crystal growth at a more rapid rate, since a nucleation centre will already be present. There are a number of reported cases where crystals have been grown after several months, e.g. *E. coli* L-asparaginase and rice cytochrome C each take 3-4 months before crystal growth can be noted (reviewed by McPherson, 1982).

If sufficient crystals had been obtained then a number of experiments could be performed in order to elucidate the nature of the protein after it had been at room temperature for several months. Firstly, a crystal could be re-dissolved and run on a gel to ensure that the all the crystal types are actually composed of protein. Secondly, an activity assay, such as DNA supercoiling or cleavage could be performed in order to see if the protein has remained in a native and active conformation. Other possibilities for crystallization trials include growth in the presence of a quinolone

drug, which could have the effect of stabilising the crystal structure and allow a diffraction pattern to be obtained.

In summary, it appears that, although I have obtained some structural information about the GyrA protein and some crystals of fragments of the protein have been grown, there is still some way to go before the three-dimensional structure of the gyrase proteins can be elucidated.

Chapter 6

Discussion and future directions.

Detailed knowledge of the structure of DNA gyrase will have important consequences for our understanding of both the mechanism of action of the enzyme, and the way which it interacts with both DNA and certain antibacterial drugs. Precise details of the gyrase reaction mechanism remain unclear despite a large array of biochemical data. The advent of high resolution structural data for the enzyme will assist in the clarification of, for example, the way in which DNA strand passage occurs, the nature of the active site in gyrase, and the way in which the DNA and protein interact. The manner in which the gyrase proteins interact with certain antibacterial drugs may also be assessed, and novel drugs designed, once structural information is available.

Summary of the results obtained.

The results presented in chapters 3, 4, and 5 have essentially been driven by the underlying desire for structural information about the gyrase proteins and the way in which the structure is related to enzymic function. Initially, the domain structure of the proteins was investigated by proteolysis. Trypsin readily cleaves the GyrA protein at the C-terminal side of residues Arg 6 and Arg 571, yielding two large fragments of molecular masses 64 and 33 kDa, mapping to the N- and C-terminal portions of the protein respectively, which were comparatively easily formed in the absence of contamination. The trypsin cleavage site may represent a natural separation point in the GyrA structure. Chromatography under native conditions, however, was not useful in separating the fragments, but denaturing conditions were partially successful. The fragments appear to be tightly associated, suggestive of strong non-covalent interactions holding the fragments together in the native state. A number of enzymic experiments were performed with the purified individual fragments, but due to the technical difficulties in fragment separation, the enzymic properties of the unseparated fragments (the A' protein) were also investigated. A' will support all the reactions of the intact GyrA protein, although with reduced efficiency in some cases. The results suggest that a gyrase complex involving the A' protein is less able to wrap DNA around itself, and the DNA-protein complex formed is less stable than the native

A_2B_2-DNA complex. The purified 64 kDa tryptic fragment will support DNA supercoiling at a low rate; the rate being enhanced in the presence of the 33 kDa C-terminal GyrA tryptic fragment. The N-terminal fragment also supports other reactions of gyrase with equal efficiency as the native protein, such as quinolone-directed DNA cleavage. It is therefore suggested that the 64 kDa fragment represents a breakage-reunion domain and that the 33 kDa C-terminal fragment is required for the stability of the enzyme-DNA complex.

Site-directed mutagenesis of the *gyrA* gene has been used to introduce an amber mutation into the sequence near the point which corresponds to the tryptic cleavage site. The over-produced protein (GyrA(1-573)) has been purified, and shares common properties with the 64 kDa tryptic fragment. A large number of *gyrA* deletion mutants were produced in order to more accurately define the breakage-reunion domain. Any construct that encoded a protein smaller than 59 kDa (GyrA(1-523)) did not obviously over-produce a truncated GyrA protein. It is conceivable that amino acid 523 of the GyrA protein is close to the boundary of the breakage-reunion domain and deletions beyond this point may result in an unstable structure that is unable to fold correctly, and consequently degraded *in vivo*. The 59 kDa GyrA(1-523) protein shares some enzymic properties with GyrA(1-573) but is unable to perform the DNA supercoiling reaction. However, other reactions, such as relaxation, quinolone-directed DNA cleavage and ATPase activity, are similar for both proteins. Therefore, deletion of fifty amino acids from GyrA(1-573) has the effect of destroying part of the structure that is essential for DNA supercoiling, but is not required for relaxation or cleavage.

Two N-terminal deletion mutants of the GyrA protein were constructed. The first, in which the N-terminal six amino acids were removed, has identical properties to GyrA. Whilst the second, lacking the first 69 amino acids, has lost the ability to supercoil or

even cleave DNA. It is likely, therefore, the start of the N-terminal breakage-reunion domain is located within this region.

The results outlined above are indicative of two functional domains within the GyrA protein. The domain organisation within the GyrA protein has also been investigated using scanning microcalorimetry. Intact GyrA yields two unfolding transitions with similar, but distinct Tm values. GyrA(1-573) and GyrA(1-523) both yield a single unfolding transition that corresponds almost exactly to one of the GyrA transitions. Therefore GyrA contains two structural domains that can be assigned to the functional moieties described above.

GyrA(1-573) has been crystallized from ammonium sulphate solutions, and four seemingly distinct crystal forms have been identified. A weak diffraction pattern to 7 Å has been obtained from one of the crystal types. Preliminary analysis of the diffraction pattern suggests the cell dimensions are 170 x 170 x 150 Å, and that there are four or eight molecules in the asymmetric unit. The lack of higher resolution data is thought to be due to the small size of the crystals rather than any disordered nature.

Significance of results.

The results presented in this thesis have shown that the DNA gyrase A protein of *E. coli* is composed of two separate domains. The N-terminal 64 kDa breakage-reunion domain is functionally and structurally distinct from the C-terminal domain that seems to be required for the stability of the enzyme-DNA complex (see Fig. 6.1). It is also apparent that the two domains are tightly held together owing to the lack of success in their separation under native conditions. So, it is likely that the channels, thought to exist on the gyrase tetramer structure, are located between the A and B subunits of the protein rather than between the N- and C-terminal domains of the A protein.

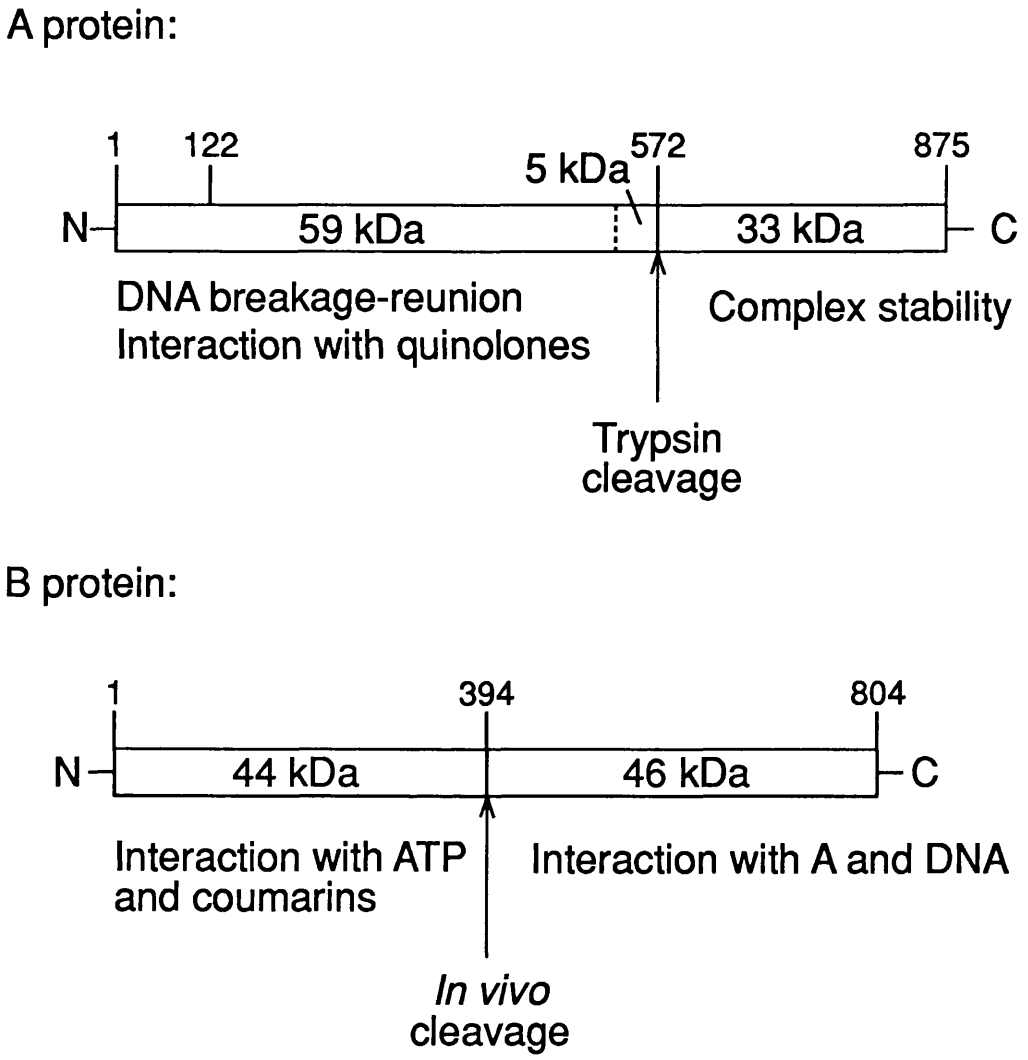


Fig. 6.1. The possible domain structure of the DNA gyrase proteins. Each of the proteins has been represented as a linear block with various amino acid positions indicated.

Both electric dichroism and small angle neutron scattering data have suggested that the gyrase particle is larger than expected for a globular protein of molecular mass 374 kDa. The discrepancy in size has been interpreted in terms of grooves or channels within the protein (Rau *et al.*, 1987; Krueger *et al.*, 1990). In order to account for all the observed reactions of gyrase by a single mechanism, DNA must be able to pass through at least part of the protein structure (reviewed by Maxwell and Gellert, 1986). It is likely that there is a channel between the gyrase subunits to allow strand-passage to occur. Gyrase is an oblate particle 175 Å by 52 Å, that is able to wrap 120 bp of DNA around itself. If the DNA is wrapped in a smooth circle then the diameter of the resulting ring will be approximately 130 Å (centre-to-centre; the DNA itself being 20 Å thick). Since the size of the gyrase particle does not appreciably alter on binding DNA, it has been suggested that DNA in embedded somewhat into the protein. From this data, it is conceivable that the DNA is held by the enzyme in a groove in the protein structure. The DNA cannot be completely enclosed by the protein since many studies have shown that the DNA remains somewhat susceptible to nuclease action (e.g. Klevan and Wang, 1980; Fisher *et al.*, 1981; Morrison and Cozzarelli, 1981; Kirkegaard and Wang, 1981). The DNA-holding groove is likely to be distinct from the channel through which strand-passage must occur. A possible structure of the gyrase-DNA complex that accounts for the above data is outlined in Fig. 6.2. The DNA is held in a groove in the protein, and channels exist between the subunits to allow strand passage to occur.

As shown in Fig. 6.2, the DNA is represented being wrapped around the protein in a symmetrical groove. This may not necessarily be the case, and the groove may occur on one side of the protein only, or be somewhat asymmetrical in nature. The channels on the gyrase molecule have been estimated to be of the order to 15 Å wide (Krueger *et al.*, 1990), but this size must increase to allow a section of double-stranded DNA to pass through. Also shown in Fig. 6.2 is the position of the proposed cleavage site on DNA. Gyrase cleaves DNA in both strands to yield phosphotyrosine linkages with

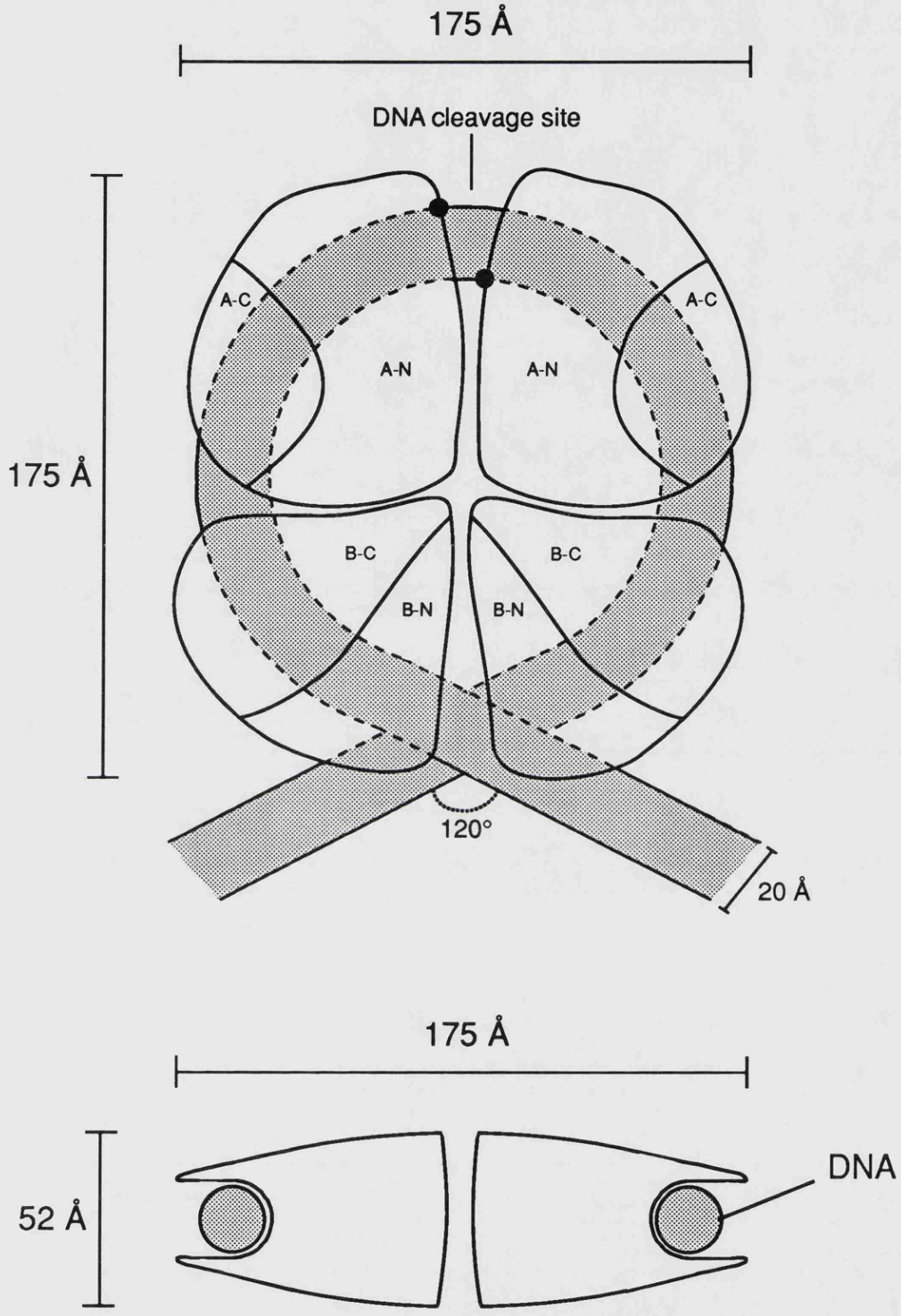


Fig. 6.2. A possible model for the structure and domain organisation for DNA gyrase. In the diagram, the DNA is shaded and the annotation A-N, A-C, B-N, and B-C used to represent the N- and C-terminus of the GyrA and GyrB proteins respectively. This model is based on the electric dichorism data, the small angle neutron scattering data, and the data presented in this thesis, and is approximately to scale. Shown are a plan (above) and a transverse section (below) of the particle.

amino acid 122 of GyrA, and a four-base stagger between each of the nick sites. For the sake of clarity, the cleavage site is shown in the figure as occurring on both sides of the DNA. However, since DNA is helical with 10.5 bp per turn (Shore and Baldwin, 1983; Horowitz and Wang, 1984), a difference a 4 bases will mean that nucleotides in different strands will be on approximately the same side of the helix, and separated by approximately 15 Å. Therefore there is no need for asymmetry of the complex, and the cleavage site could be at the mouth of the channel shown.

The results obtained in this thesis suggest a channel is not located within the GyrA protein. If a channel were present it could possibly represent the boundary between the two domains and is likely to have protease sensitive regions on its surface. However, if this were the case, cleavage of the peptide backbone should result in fragments that can be easily separated with few non-covalent interactions holding them together. The lack of success in the separation of the GyrA tryptic fragments therefore suggests the two domains are in close association with each other. The channels in the gyrase tetramer are more likely to be located between the subunits themselves, thereby providing a means for the DNA to be translocated through the protein. It is possible however, that the protease-sensitive site is located on or near a groove postulated to hold the DNA. If this were the case, then the two fragments could remain non-covalently bound to each other.

The proximity of the two GyrA unfolding transitions obtained by microcalorimetry could also be indicative of interactions between the two domains. It is possible that there is a degree to cooperativity in the unfolding of the domains. As temperature increases, one of the domains may begin to unfold (most likely the N-terminal breakage-reunion domain since it has a slightly lower Tm) and this may have consequences on the stability of the other domain which may also now unfold. The Tm values for the two domains, therefore, will appear to be very close to each other.

Perhaps one of the most important findings of the present study is the ability to crystallize fragments of the GyrA protein. To date, no crystal structure for a topoisomerase is available, and indeed a number of groups have attempted to obtain crystals of the gyrase proteins with little or no success. Thus the crystallization of the N-terminal breakage-reunion domain of GyrA must represent a significant step forward in the structural elucidation of gyrase. Obviously it would be more desirable to be able to study a crystal of the whole gyrase protein but, since this has proved unsuccessful, the active fragments produced here are likely to represent a starting point in obtaining a high resolution data set. The N-terminal breakage-reunion domain is likely to be important because of the interactions of the fragment with DNA and with the quinolone class of antibacterials.

Future work.

The work presented here is not complete and certainly leads to the possibility of further study. Below is listed a number of possible areas for further experiments which the current work has suggested:

(1). Much of the work involved with this thesis has concentrated on the enzymic and structural properties of the breakage-reunion domain of the GyrA protein. The 33 kDa C-terminal domain plays no role in the cleavage of DNA, and strand-passage occurs in the absence of this fragment. However, the C-terminal domain is required for efficient DNA supercoiling and appears to be involved in the stability of the DNA-protein complex. Using cloning methodology it should be possible to over-produce the 33 kDa fragments directly as a gene product. It will then be feasible to perform a number of experiments with this fragment; e.g. investigate whether it will interact with DNA, or the N-terminal GyrA fragment. It is likely that the 33 kDa C-terminal domain will be able to interact with the breakage-reunion domain since the reconstituted gel purified GyrA tryptic fragments are able to interact to form an active species. If the function of the C-terminal fragment is indeed involved in the stability

of the enzyme-DNA complex, then nitrocellulose filter binding or gel retardation assays could be utilised to study any such interaction.

The cloned and purified 33 kDa fragment could also be reconstituted with the cloned and purified 64 kDa GyrA(1-573) to more accurately investigate the effects of the smaller fragment on the enzymic activities of the breakage-reunion domain. If the 33 kDa fragment can be over-produced then this raises the possibility of crystallization trials on the C-terminal fragment. Structural information about the C-terminal fragment could yield important information about the way in which the two domains interact with each other and with DNA. The cloned C-terminal fragment could also be used in microcalorimetry experiments to investigate the dependency of the unfolding of each domain on the other.

(2). Purification of the gyrase B protein of gyrase (molecular mass 90 kDa) from certain *E. coli* strains results in the isolation of a 46 kDa fragment (Brown *et al.*, 1979; Gellert *et al.*, 1979). This protein binds to the GyrA subunit to generate a complex, termed topoisomerase II', that cannot supercoil DNA but has the ability to relax both negative and positive supercoils in the absence of nucleotides. The proteolytic digestion patterns of this protein suggested that it is a fragment of the B protein (Gellert *et al.*, 1979). Sequencing of *gyrB* has shown this fragment to be the C-terminal half of the intact B protein, which could have been produced by *in vivo* proteolysis (Adachi *et al.*, 1987). It appears this fragment has lost either its ATPase activity or the energy transduction process to allow supercoiling activity of the DNA held by the A subunits. Hence, it can be inferred that the ATPase activity of the B protein is located, at least partially, in the N-terminal part of the B protein, whilst the C-terminus is required for interaction with the A protein. It is therefore possible that the GyrB protein also contains two domains. These are shown diagrammatically in Fig. 6.1.

One of the problems associated with working on the GyrB protein has been the lack of a suitable vector that will direct the over-production of the protein in large amounts. However, a vector has recently been constructed in which the *gyrB* gene is linked directly 3' of the *tac* promoter (Hallett *et al.*, 1990). It is now possible to perform mutagenesis of *gyrB* and obtain large amounts of the mutated protein. Therefore, it should be possible to over-produce the putative GyrB domains directly as gene products so that the enzymic and physical properties of each can be studied. In a similar fashion to the study described here, structural information about the GyrB protein may also be elucidated. Recently, the N-terminal 44 kDa of the GyrB protein has been cloned and over-produced (A.P. Jackson and A. Maxwell, personal communication). The fragment is found to be have a novobiocin-sensitive ATPase activity associated with it.

(3). Perhaps the most most exciting aspect of the present study is the crystallography of the breakage-reunion domain of the GyrA protein. A number of crystal forms have been identified and a diffraction pattern obtained. However, much research remains to be performed before the three-dimensional structure of the domain can be solved. Larger crystals must be obtained, and heavy metal derivatives must be sought. Some of the major problems associated with the crystallography described here include the time taken for crystals to form, and the small size of the crystals themselves. Crystal seeding may overcome both of these problems. Using the crystals already obtained, it may be possible to find conditions where they will grow larger. Seeding can take one of two forms; micro- or macro-seeding (Thaller *et al.*, 1985). Micro-seeding involves the isolation of a single crystal and its disruption by vortexing. The crystal pieces are then placed into a solution of the protein under conditions close to those where the original crystal formed. The microscopic crystal fragments provide nucleation centres for fresh crystal growth. Macro-seeding involves the addition of a single small crystal to fresh mother liquor in the hope that the crystal will grow larger. These two techniques should provide larger crystals for analysis at higher resolution.

The GyrA(1-573) protein is still able to interact with quinolone drugs. It would therefore be interesting if a quinolone-resistant protein could be crystallized and the structure compared to that of the native protein. A number of quinolone resistant mutants have been identified within the N-terminal domain of the A protein (Yoshida *et al.*, 1988; P. Hallett and A. Maxwell, personal communication). It should therefore be possible to create a GyrA(1-573) protein that contains the quinolone-resistant mutation. If this could be crystallized then the basis for resistance might be identified. Other possibilities for crystallization include attempts to crystallize the N-terminal GyrA domain in the presence of quinolone drugs to see where they interact with the protein and the manner of this interaction.

The initial aims of this project were to investigate the domain structure of the *E. coli* DNA gyrase proteins and attempt to gain some structural and mechanistic information about the proteins. These have, in part, been realised using a variety of protein chemistry, genetic, and physical techniques. The results obtained may provide a starting point for the elucidation of the three-dimensional structure of the gyrase proteins.

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Appendix.

Some of the work presented in this thesis has already been published elsewhere:

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