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Title of Thesis SITE DIRECTED MUTAGENESIS

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I have designed and developed a novel method of site directed mutagenesis. Mutations are generated by introducing a single-stranded chemically modified restriction fragment into a supercoiled vector via a RecA protein/DNA intermediate. The system has been tested on the ampicillin gene of pBR322.

A new efficient method of preparing large quantities of small single-stranded restriction fragments is described. RecA protein has been purified from a new overproducing strain in this study.

The experiments described in this thesis show that D-loops can be generated with restriction fragments as small as 36 bases; chemically modified restriction fragments can be incorporated in the presence of cobalt ions; the displaced strand can be cut away with S1 nuclease without loss of the fragment. Finally, the fragment can be covalently joined to the vector.

Analysis of the final covalently closed product shows that the fragment is correctly located in the plasmid. A novel system to select for the mutant plasmid in vitro, based on the S1 nuclease sensitivity of supercoiled pBR322, has been tested.

SITE DIRECTED MUTAGENESIS

BY

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1983



D E C L A R A T I O N

I declare that this thesis
was composed by myself and that
the work described is entirely my own

signed

D. Rupert Holms.

This thesis is dedicated to my parents.

C O N T E N T S

	<u>PAGE</u>
<u>ABSTRACT</u>	1
<u>ABBREVIATIONS</u>	2
 <u>CHAPTER 1 : GENERAL INTRODUCTION</u>	 3
1.1 Introduction	3
1.2 Mutagenesis at Unique Restriction Sites	4
1.3 Mutagenesis between Unique Restriction Sites	8
1.4 Mutagenesis between any Restriction Sites	11
1.5 Mutagenesis at any Site	15
1.6 Discussion	20
1.7 Mutagenesis by Site Directed Heterologous Recombination	21
 <u>CHAPTER 2 : MATERIALS AND GENERAL METHODS</u>	 23
2.1 Materials	23
2.2 General Methods	23
 <u>CHAPTER 3 : FRAGMENT PREPARATION</u>	 33
3.1 Introduction	33
3.2 Preparation of Small Double-Stranded Restriction Fragments	34
3.3 Recovery of DNA Fragments from Gels	35
3.4 Strand Separation of Small DNA Fragments	36
3.5 Experimental Procedures	37
 <u>CHAPTER 4 : PREPARATION OF RecA PROTEIN</u>	 43
4.1 Introduction	43
4.2 RecA Protein Preparation from N1462-pRG3	44
4.3 Construction of a Stable Overproducing RecA Protein Strain	45
4.4 Purification of RecA Protein from DM936-pRG3	46
4.5 Experimental Procedures	48

	<u>PAGE</u>
<u>CHAPTER 5 : COMPLEX FORMATION PART I</u>	52
5.1 Introduction	52
5.2 Formation of Fragment-Vector Complexes	54
5.3 Characterisation of Complex F	55
5.4 Experimental Procedures	57
 <u>CHAPTER 6 : COMPLEX FORMATION PART II</u>	 60
6.1 Introduction	60
6.2 Production of Complex S in CMP Buffer	61
6.3 Production of Complex S in M Buffer	62
6.4 Discussion	63
6.5 Experimental Procedures	63
 <u>CHAPTER 7 : MODIFICATION OF COMPLEX S</u>	 67
7.1 Introduction	67
7.2 Deproteination of Complex S	68
7.3 Conversion of Complex S to CCRC Plasmid	69
7.4 Location of the Labelled Fragment in the CCRC Plasmid	70
7.5 Selective Digestion of Supercoiled pBR322 with S1 Nuclease	71
7.6 Discussion	72
7.7 Experimental Procedures	74
 <u>CHAPTER 8 : EXPERIMENTAL PROPOSALS AND GENERAL DISCUSSION</u>	 78
8.1 Introduction	78
8.2 Experimental Proposals	78
8.3 Discussion	81
 <u>ACKNOWLEDGEMENTS</u>	 83
<u>REFERENCES</u>	84

A B S T R A C T

I have designed and developed a novel method of site directed mutagenesis. Mutations are generated by introducing a single-stranded chemically modified restriction fragment into a supercoiled vector via a RecA protein/DNA intermediate. The system has been tested on the ampicillin gene of pER322.

A new efficient method of preparing large quantities of small single-stranded restriction fragments is described. RecA protein has been purified from a new overproducing strain in this study.

The experiments described in this thesis show that D-loops can be generated with restriction fragments as small as 36 bases; chemically modified restriction fragments can be incorporated in the presence of cobalt ions; the displaced strand can be cut away with S1 nuclease without loss of the fragment. Finally, the fragment can be covalently joined to the vector.

Analysis of the final covalently closed product shows that the fragment is correctly located in the plasmid. A novel system to select for the mutant plasmid in vitro, based on the S1 nuclease sensitivity of supercoiled pER322, has been tested.

ABBREVIATIONS

B.R.L.	Bethesda Research Laboratories
bp	base pairs
Bis	N,N'-methylenebisacrylamide
CCRC plasmid	Covalently closed relaxed circular plasmid
Complex F	Fast migrating labelled plasmid
Complex S	Slow " " "
DTT	Dithiothreitol
DE-52	Whatman DEAE-cellulose
Klenow Fragment	Large Fragment of E.coli DNA, polymerase 1
ml	millilitre
NaAc	Sodium Acetate
SDS	Sodium dodecylsulphate
SSB	Single-stranded DNA binding protein
SSDNA	Single-stranded DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
TBE	Tris. Borate. EDTA electrophoresis buffer
U.V.	Ultra Violet

SITE DIRECTED MUTAGENESIS

CHAPTER 1 : GENERAL INTRODUCTION

1.1 INTRODUCTION

The classical genetic approach to the study of protein function has been to isolate a characteristic mutant phenotype by selection and screening from a population of randomly mutagenised organisms. The mutations were then characterised by complementation or recombination analysis followed by protein isolation and sequencing. The method has been frequently applied to bacteria because their short generation times allows rapid analysis of the mutant clones.

The problem with this approach is that the experimenter has no control over where or how a gene will be modified. Mutations in a given gene are also rare. This means the screening procedure has to be simple so that thousands of clones can be tested. This may not be possible for a mutant protein with only a modified activity. To make screening easier, most studies concentrate on mutations that inactivate the protein either directly or by making it heat sensitive.

The rapid advances in site directed mutagenesis over the last five years have provided protein chemists with a powerful tool. It is now possible to modify either specific regions or specific bases in a given gene. Experiments can now be designed to assess the importance of an individual aminoacid in the structure, activity or stability of a protein.

Site directed mutagenesis has grown out of existing recombinant DNA technology. Earlier procedures relied on restriction sites to define the target for mutagenesis in a cloned gene. The emphasis has now shifted to synthetic DNA as the site specific mutagen. The problem of introducing defined changes, at a specific location, in high yield, has been tackled in a number of ways. The methods fall into four main categories in which the mutagenesis is specifically targeted, either at unique restriction sites, or between unique restriction sites, or between any pair of restriction sites, or at

any choice of site. Site specific deletions, insertions and point mutations have all been made. To the protein chemist, it is the point mutations that yield the most information as the changes are the most subtle.

A point mutation involves introducing a single base change into a DNA sequence. When a purine is replaced by a different purine or a pyrimidine by a different pyrimidine this is called a transition mutation. When a purine is replaced by pyrimidine or vice versa this is called a transversion mutation. Transitions have been generated by chemical mutagens such as nitrous acid and sodium bisulphite modifying the native DNA. Transition mutations have also been produced by introducing the base analogue N4-hydroxycytosine. Both transition and transversion mutations have been produced by forcing DNA polymerase to make errors. They have also been produced by introducing mismatched synthetic oligonucleotides. Only oligonucleotides define both the site and type of modification.

In the following sections site directed mutagenesis experiments will be reviewed in order of increasing flexibility and accuracy in producing and siting mutations in a cloned gene. The order is also roughly chronological.

1.2 MUTAGENESIS AT UNIQUE RESTRICTION SITES

A number of techniques have been developed to modify a gene at or around a unique restriction site. This has been achieved by cutting the site with the restriction enzyme then modifying and rejoining the ends. Alternatively a nick can be generated which on conversion to a gap produces a region of single stranded DNA which can be modified in a variety of ways.

1.2.1 DELETIONS AND INSERTIONS

Deletions have been generated at unique restriction sites in SV40 (Legerski et al 1978). First the circular molecule was linearised at a unique restriction site. Nucleotides were then removed from both strands of the termini by limited digestion with

either a combination of S1 nuclease and exonuclease III (Heffron et al 1977) or endonuclease BAL-31 (Legerski et al 1978). The linear molecules were then used to transfect cells. In vivo, the linear molecules cyclised to reform the gene with a deletion. A similar approach has been used to introduce deletions in the rRNA gene of E.coli (Gourse et al 1982). Linear molecules were generated by restriction enzyme digestion of one of four unique restriction sites in the 16S and 23S rRNA genes cloned into the plasmid pKK3535. The ends were digested with BAL-31 and religated with T4 DNA ligase to form circles. The circles were then treated with the same restriction enzyme. Molecules with a deleted restriction site were unaffected. Molecules that retained the site were converted to linears. E.coli was transformed with the DNA mixture and deletion mutants were isolated. The second restriction step reduced the wild type back ground as the linears formed could only transform at 0.1% of the frequency of the mutant circles.

Deletions have been generated from DNA gapped at a unique restriction site. pBR322 was digested in a solution containing ethidium bromide with BamH1 to generate nicks at the BamH1 site (Nilsson and Magnusson 1982). Nicks were then converted to 5-10 base pair gaps using the 5'-3' exonuclease activity of E.coli P1. The gaps were then ligated with high concentrations of T4 ligase displacing the uncut strand. E.coli cells were then transformed with these molecules and 30% of the plasmids were recovered with site specific deletions that inactivated the tetracyclin gene.

Insertions have been produced by limited digestion of the circular genome of bacteriophage P1 with Hinf1 (Boeke 1981). A variety of linear molecules was produced from linearisation at one of the multiple Hinf1 sites. The 3 base 5' extensions generated by the enzyme were filled in with DNA polymerase 1 and deoxynucleotide triphosphates (dNTPs). On recyclicalisation with ligase an insertion of 3 base pairs was formed, duplicating the Hinf1 sequence. The reading frame of the protein sequence was maintained as a whole codon had been introduced. A parallel experiment was carried out at the Hpa11 site with its 2 base extension. By filling in the cohesive ends and introducing a tetranucleotide during cyclisation a 2 codon insertion was formed.

1.2.2 POINT MUTATIONS

Point mutations have been produced at restriction sites by chemical modification of single stranded DNA, by incorporation of base analogues, by gap filling with inaccurate DNA polymerase and by replacing bases with synthetic linker molecules.

1.2.2.1 CHEMICAL MODIFICATION

Sodium bisulphite de aminates cytosine to uracil by catalysing nucleophilic substitution of the amino group. The reaction on single stranded DNA is a thousand times faster than on double stranded DNA (Shipiro et al 1973). On complementary strand synthesis the uracil pairs with adenine to produce a GC to AT transition mutation. This property has been used to produce mutations in single stranded DNA generated at restriction sites in two different ways.

Point mutations have been introduced in the cloned nematode t-RNA^{PRO} gene by cutting the plasmid with restriction enzyme Sma1 to blunt ended linears (Ciampi et al 1982). Single stranded ends were produced by limited 3' to 5' digestion with the Klenow fragment of DNA polymerase which lacks 5' to 3' exonuclease activity. The cytosines in the single stranded DNA were modified to uracils with bisulphite. Klenow fragment and dNTPs were then used to reform the blunt ends by complementary strand synthesis. The synthesis step introduced adenines opposite the uracils. The molecules were then ligated and used to transform bacteria.

An alternative approach has been used to generate point mutations at the Hpa11 site in SV40 (Shortle and Nathans 1978, Shortle et al 1979). A nick was introduced at the Hpa11 site by digestion with the enzyme in an ethidium bromide solution. The nick was extended to form a single stranded gap with micrococcus linteus Polymerase 1. The cytosines in the gap were then modified by sodium bisulphite. Second strand synthesis was performed using the polymerase and dNTPs to produce a fully duplex molecule.

1.2.2.2 NUCLEOTIDE ANALOGUES

N4-hydroxy dCTP has two tautomeric forms: an imino form (T like) that can pair with A and an amino form (C like) that can pair with G. This analogue can be incorporated either in gap filling or nick translation reactions. G to A transitions are generated when N4-hydroxy dCTP pairs with G on invitro synthesis and changes to a T like tautomer before replication in vivo. A to G transitions are generated when the analogue pairs with A then changes to a C like tautomeric form before replication in vivo (Flavell et al 1974).

A plasmid containing rabbit beta globin cDNA has been modified at the unique EcoR1 site in the globin gene (Muller et al 1978). The EcoR1 site was nicked with the restriction enzyme in the presence of ethidium bromide. Limited nick translation was initiated at this site using dATP, dCTP and N4-hydroxy dCTP. Cells were transformed with the modified plasmids and G to A transition mutants were isolated with the lesions around the EcoR1 site.

1.2.2.3 BASE MISINCORPORATION

Occasionally DNA polymerases incorporate an incorrect nucleotide during complementary strand synthesis thus producing a point mutation (Kunkel and Loeb 1979, 1980). This is normally removed by the 3' - 5' "proof reading" exonuclease of bacterial DNA polymerases (Brutlag, and Kornberg 1972). The correction process has been avoided in two ways to produce both transition and transversion mutations at a restriction site.

The method has been applied to four unique restriction sites in pBR322 (Shortle et al 1982). A nick was generated at the restriction site by digestion with the restriction enzyme in the presence of ethidium bromide. This was converted to a gap with the 5' - 3' exonuclease activity of micrococcus luteus Polymerase 1. Misincorporated bases were introduced in two ways. The gap was filled by the polymerase with only 3 of the 4 nucleoside triphosphates in a solution containing manganese ions. These conditions induce a high error rate (Kunkel and Loeb 1979). Proof-reading was blocked by rapidly closing the nicks with high concentrations of T4 ligase.

In the second method alpha thiophosphate nucleoside triphosphates were used. Once incorporated they are resistant to the 3' - 5' editing by bacterial DNA polymerases. Either dATP-alpha-S or dTTP-alpha-S was joined to the 3' hydroxy primer in the gap with the Klenow fragment. The gap was filled with dNTPs. After ligation and transformation between 12-40% of the plasmids isolated from the clones had transition and transversion mutations. Small deletions were also generated which may have resulted from the ability of T4 ligase to seal small gaps.

1.2.2.4 LINKER INCORPORATION

Specific base changes can be produced at a restriction site by cutting with the enzyme to produce linear vector with single stranded extensions which can be removed with an exonuclease. The molecule is then recyclised with a synthetic DNA linker molecule with the new sequence to replace the lost base pairs.

A specific base change has been produced at an HgiA1 site in the betalactamase gene of pBR322 by introducing a linker. The procedure was complicated by a number of cloning and mapping steps that were necessary to ensure that the correct mutant was selected (Sigal et al 1982).

1.3 MUTAGENESIS BETWEEN UNIQUE RESTRICTION SITES

These methods involve cutting a circular vector at two unique restriction sites to produce a shortened linear vector and a fragment. Deletions are generated by recircularising the shortened linear vector. Point mutations are generated by treating the fragment with a mutagen followed by ligation of the fragment back into its original location. Alternatively, a gapped heteroduplex can be formed by annealing single strands of the shortened linear vector with single strands of the full length vector. The single stranded gap produced becomes a target for bisulphite mutagenesis.

1.3.1 DELETIONS

Deletions have been generated in a fragment of phage T7 containing the late promoter cloned into plasmid pBR322 (Panayotatos and Truong 1981). Deletions were produced by cutting the plasmid to a linear molecule at the unique BamH1 site. The molecule was then cut at a near by unique Sal1 site. This divided the molecule into a shortened vector and a fragment. The vector was recircularised, after filling in the ends, to produce a circular molecule with a deletion between the BamH1 and Sal1 sites. A family of deletions extending away from the BamH1 site were produced by digesting the ends of the BamH1 opened vector with BAL31 nuclease. Repeating the Sal1 digestion, polymerase and ligation steps produced circular molecules with deletions extending in one direction from the Sal1 site.

1.3.2 POINT MUTATIONS

Mutagenesis of isolated fragments allows the use of a variety of mutagens to produce point mutations. Mutations have been produced with either bisulphite, nitrous acid or U.V. light.

Nitrous acid oxidatively deaminates both single stranded and double stranded DNA (Kotaka and Baldwin 1964, Shapiro and Yamaguchi 1972). Cytosine is converted to uracil which then pairs with adenine. Adenine is converted to hypoxanthine which then pairs with cytosine. Guanine is converted to xanthine which does not form stable base pairs. Nitrous acid will also cross link guanines to guanines and guanines to adenines (Singer and Kusmievsk 1982). All possible transition mutations can be generated by nitrous acid.

U.V. light induces free radical reactions which modify and cross link bases (Livneh 1983). In vivo, U.V. pretreated cells modify this altered DNA to transition, transversion and deletion mutations.

1.3.2.1 MODIFICATION OF ISOLATED FRAGMENT

Transition mutations were induced with nitrous acid in a restriction fragment containing 4% of the adenovirus5 genome (Solnick 1981). The fragment was generated by cutting the linear DNA at a unique Xba1 site. The Xba1 fragment was mutagenised with nitrous acid and religated to the shortened linear vector. 20% of the transformants had transition mutations in the region defined by the fragment.

CG to TA and GC to AT transition mutations were induced in a 109 base pair fragment containing the lac promoter/operator element (Weiher and Schaller 1982). The fragment was generated by cutting at unique EcoR1 and Hind111 sites in a pBR322 derivative plasmid. The fragment was then cloned in one orientation into Fd109 and in the other orientation into Fd109-2 (in which the EcoR1 and Hind111 sites were reversed). After transformation the single stranded viral plus strands were isolated and treated with sodium bisulphite. The minus strand was then synthesised in vitro to produce stable AU base pairs. The double stranded fragment was then cut out and recloned back into the Hind111-EcoR1 sites of the original plasmid and used to transform cells. 90% of clones carried multiple mutations in the Hind111-EcoR1 region.

Point mutations and deletions have been generated in the tetracyclin gene of pXf3 (Livneh 1983). pXf3 was cut at its unique BamH1 and Sph1 sites in the tetracyclin gene to produce a 185 base pair fragment. The fragment was U.V. irradiated then recloned back into its original location. SOS induced cells were transformed with the plasmid and 4% of the transformants contained mutations. Sequence analysis showed transition, transversion and point mutations had all been produced.

1.3.2.2 MODIFICATION OF GAPPED CIRCULAR HETERODUPLEXES

Point mutations have been targeted to a 275 base pair region of the tetracyclin gene of plasmid pKB280 (Giza et al 1981). The plasmid was cut at the unique Sal1 and BamH1 sites to generate a 275 base pair fragment and a shortened linear vector. The shortened linear

vector was denatured and the strands separated on an agarose gel. Another sample of pKB280 was cut at a unique Hpa1 site on the opposite side of the circle to the Sal1 site, to produce a full length linear vector. The full length linear vector was denatured and the strands were separated. The minus strand of the Sal1-BamH1 linear molecule was annealed to the plus strand of the Hpa1 linear vector. The result was a circular molecule with a nick in the plus strand at the Hpa1 site and a gap in the minus strand between the Sal1 and BamH1 sites. This gapped circular heteroduplex was treated with sodium bisulphite which only modified the cytosines in the gap. The gap was then closed and stable AU base pairs were created by in vitro complementary strand synthesis. More than 90% of the transformants were mutant.

An improved version of this experiment used single stranded phage vector M13 (Everett and Chambon 1982). The shortened vector linear was produced as before but it was denatured and annealed directly to the viral plus strand circle to generate the double stranded gapped circle. This method avoided the agarose gel steps.

1.4 MUTAGENESIS BETWEEN ANY RESTRICTION SITES

All these methods involve isolating a single stranded fragment to define the region to be mutated. Deletion mutations can be targeted between any two restriction sites. One method involves opening a double stranded vector at a D-loop produced by introducing the isolated fragment. The other involves opening a circular single stranded vector at restriction sites within a region of duplex produced by the annealed fragment. In both cases, the ends of the linear molecules are ligated to form a circular vector with a deletion.

Point mutations can be produced in four different ways. They can be generated in the previously constructed deletions. Point mutations can be introduced into the single stranded fragment which is annealed to the single stranded region of a gapped circular heteroduplex. The single stranded fragment can be used to prime complementary strand synthesis on a single stranded vector under

conditions that generate base misincorporation. Finally, a D-loop generated by the fragment can be converted to a single stranded gap then mutagenised with bisulphite.

1.4.1 DELETIONS

Deletions have been generated in the tetracycline gene of pBR322 (Green and Tibbets 1980). A 650 base pair EcoR1-Sal1 fragment was denatured and the strands separated in an agarose gel. The slow-migrating single-stranded fragment was extracted from the gel. It was then hydrolysed into pieces with an average length of 250 base pairs. The single stranded fragments were incubated with supercoiled pBR322 under conditions in which the fragments base pair with their complementary sequence in the plasmid. The other strand of the plasmid was displaced to form a single stranded D-loop. Treatment with high concentrations of S1 nuclease resulted in cleavage of the plasmid at the D-loop followed by limited digestion of the termini. The linears were ligated to form circles and used to transform bacteria. 5-20% of the clones were deletion mutants. The deletion varied between 6-19 base pairs on the region defined by the original 650 base fragment.

Deletions have been generated in phiX174 by annealing a restriction fragment to single stranded circles of the viral DNA (Humayun & Chambers 1979). The partial duplex circles were then treated with the multicutting restriction enzyme HincII. The enzyme cut out a small fragment between the two HincII sites in the duplex region. Sites in the single stranded region were insensitive to the enzyme. The linear vectors were ligated and used to transfect cells. 2% of the resulting phage carried the deletion.

1.4.2 POINT MUTATIONS

1.4.2.1 POINT MUTATIONS DEFINED BY A DELETION

Point mutations have been generated in a 21bp region of SV40 large T antigen (Kalderon et al 1982). Plasmid pPVU-0 was constructed by cloning the region coding for the large T antigen between the BamH1 and Pvu11 sites of pBR328. Plasmid pVU-1 was constructed by cloning a 21 bp deletion mutant from the same region. pPVU-0 was cut to linear molecules at the Pvu11 site. Deletion mutant pPVU-1 was cut to linear molecules at the BamH1 site. The two types of linear molecule were mixed, denatured and left to reanneal. The result was a circular molecule with a nick at the BamH1 site on one strand and a nick at the Pvu11 site on the other strand. At the site of the deletion, the wild-type strand from pPVU-0 formed a 21 base pair single stranded loop. The heteroduplex was treated with sodium bisulphite to convert the cytosines in the single stranded loop to uracils. The molecules were then used to transform a uracil-DNA-glycosylase deficient strain (*ung*⁻). 30% of the transformants carried transition mutations and all were limited to the 21 base pair region. A similar procedure has been used to produce point mutations in the tetracyclin gene of pBR322 (Peden and Nathans 1982).

The uracil-DNA-glycosylase-deficient strain was required as any uracils that appear in a DNA strand are excised *in vivo* by this enzyme (Duncan et al 1978, Lehman et al 1978). This was not a problem in previous protocols using bisulphite as the *in vitro* complementary strand synthesis step stabilised the mutation by introducing an adenine opposite the uracils. *In vivo*, after excision of the uracil, repair synthesis used the mutant adenine as a template.

1.4.2.2 ISOLATED FRAGMENT MUTAGENESIS

Single point transition mutations have been generated in the fibroin gene promoter cloned into f1 phage vector (Hirose et al 1982). Double stranded replicative form phage (RF-f1) was cut with two unique restriction enzymes to produce double stranded shortened

linear molecules and a 428 base pair fragment. The vector linear molecules were purified, mixed with single stranded circular fl, denatured and left to reanneal. The result was a circular duplex with a 428 base single stranded gap exposing the fibroin gene promoter. The target for mutagenesis was an 88 base Sau3A-Taq1 sequence in the gapped region. More RF-fl DNA was digested with Sau3A and Taq1 then electrophoresed. The double stranded fragment from this region was extracted from the gel. It was then denatured and strand separated on a second gel. One of the single stranded fragments was recovered and treated with nitrous acid. The modified fragment was then annealed to the single stranded gap producing U.G and HX.T base pairs. The remaining gaps were filled by in vitro complementary strand synthesis and the duplex used to transform ung⁻ cells. The uracil-DNA-glycosylase deficient strain was used to stop the uracils produced by the nitrous acid being replaced by cytosine. 21% of the clones carried transition mutations. Sequence analysis showed they were all in the 88 base target.

1.4.2.3 BASE MININCORPORATION

The 3' hydroxyl group of a restriction fragment has been used as a primer to direct inaccurate complementary strand synthesis through the am18 region of phiX174 (Zakour and Loeb 1982). The double stranded Hpa1(2) fragment was denatured and annealed to the single stranded circular phiX174 to produce a duplex region with the 3' end of the fragment 7 bases from the am18 locus. The primer was subjected to limited extension with Pol1 and with only two dNTPs, to bring the 3' end 2 bases from the am18 locus. Mutations were generated by extending the duplex through the am18 locus with three dNTPs and low fidelity avian myeloblastosis virus (AMV) DNA polymerase. Finally, the rest of the complementary strand was synthesised using four dNTPs and Pol1. The DNA was then treated with S1 nuclease to destroy the remaining single stranded and gapped circles. 33% of the clones isolated after transformation were am18 revertants. Sequence analysis showed they were the result of both transition and transversion mutations.

1.4.2.4 D-LOOP DIRECTED BISULPHITE MUTAGENESIS

Transition mutations have been generated in the beta-lactamase gene of pBR322 (Shortle et al 1980). A double stranded 128 bp Pvu1-Pst1 fragment was treated with Exonuclease 111 to produce single stranded fragments with an average length of 60 bases. The fragments were mixed with supercoiled pBR322 in the presence of RecA protein to produce D-loops in the Pvu1-Pst1 region. The RecA protein accelerated D-loop formation by generating D-loop /RecA protein complexes. The displaced strand of the D-loop was nicked with S1 nuclease which resulted in loss of the fragment. This treatment produced relaxed circles with nicks in the region of the target area. The nicks were converted to gaps with DNA polymerase. The single stranded gaps were treated with bisulphite then filled in with the polymerase and dNTPs. The DNA was used to transform E.coli. 7.5% of the transformants were ampicillin sensitive mutants. 4% of the mutations mapped outside the target region due to non-specific nicking.

1.5 MUTAGENESIS AT ANY SITE

Complete flexibility in siting mutations has come with the advent of rapid DNA sequencing and chemical synthesis of oligonucleotides. If the sequence of the target for mutagenesis is known then a short oligonucleotide can be produced with a complementary sequence to the region. It can then be used as a primer for complementary strand synthesis on a single stranded circle of vector DNA. Deletions, insertions and point mutations can be specifically generated at any base by introducing the mutations into the synthetic primer. The mismatched primer then directs the complementary strand synthesis to produce a vector with a mutation in one strand. Replication in vivo results in populations of mutant and wild-type vectors.

1.5.1 DELETIONS

The intron in the yeast tyrosyl transfer RNA (tRNA^{tyr}) suppressor gene cloned into plasmid pBR322 has been deleted by oligonucleotide mutagenesis (Wallace et al 1980). A 21 unit oligonucleotide was synthesised containing the flanking sequences of the intron and a 14 base deletion. Single stranded circular vector DNA was

produced by nicking the plasmid at the EcoR1 site and digesting the nicked strand with Exonuclease 111. The oligonucleotide was annealed to the complementary flanking sequences in the vector. At the site of the deletion a single stranded loop of vector DNA containing the intron was formed. The 3' end of the oligonucleotide was used to prime complementary strand synthesis with the Klenow fragment of E.coli Pol1 and dNTPs. The Klenow fragment lacks the 5'-3' exonuclease activity of Pol1 which would normally digest away the primer from the 5' end. The nicked duplex formed was sealed with T4 ligase and used to transform cells.

The transformants were screened for deletion mutations by end-labelling the oligonucleotide with ^{32}P and using it as a hybridisation probe. Lysed cells from the primary colonies containing the mutation gave a positive signal. 4% of the transformants carried the mutation.

This is a very powerful technique as it identifies the mutant at the level of the DNA sequence. This means that mutations can be introduced into a gene and identified in a system where the phenotype is not expressed.

1.5.2 POINT MUTATIONS

Specific transition and transversion mutations have been generated by using single base mismatched oligonucleotide primers. Occasionally, the primer anneals to the wrong site because a homologous sequence to the target exists somewhere else in the vector (Smith and Gillam 1981). This can be overcome by sequencing the gene and vector and using computer sequence search programmes to determine the minimum size of the oligonucleotide that is homologous to only one site. Early experiments had problems with low yields of mutants and a number of techniques have been developed to improve the mutant-to-wild-type ratio. The advances in colony hybridisation technology with small oligonucleotides has made this unnecessary as single base change mutants can be identified out of a background of thousands of wild type colonies.

The feasibility of generating point mutations with oligonucleotides was shown by reverting the lysis deficient am3 mutation of phiX174 (Razin et al 1978). Complementary strand synthesis on single stranded circles of phiX174 was primed with am11 base oligonucleotide with a mismatched cytosine in place of thymine. E.coli Pol1 was used to synthesise the second strand. It was found to digest the 5' end of the primer and eliminate the mismatch. A parallel experiment with a 12 base primer successfully reverted the am3 mutation using the Klenow fragment, which lacked the 5'-3' exonuclease activity, to synthesise the complementary strand (Hutchison et al 1978). The mutant yield was improved by treating the DNA with S1 nuclease after strand synthesis to eliminate the wild type single stranded circles. Including this step, 15% of the total progeny after transformation were am3 revertants.

Transversions have also been generated by a similar method in phiX174 (Gillam et al 1979).

1.5.3 METHODS TO IMPROVE MUTANT TO WILD TYPE RATIOS

The background of wild type transformants has been reduced by methods that involve in vitro or in vivo selection for the mutants. There are 3 main sources of wild types. First, contamination of the transforming DNA with highly infectious wild type single stranded phage. Second, repair of the gapped strand to wild type in vivo after incomplete complementary strand synthesis. Finally, mismatches can be converted to wild type by the mismatch repair machinery in vivo.

1.5.3.1 IN VITRO SELECTION FOR MUTANTS

The background of wild type phiX174 has been eliminated in an experiment to generate a single base deletion (Smith and Gillam 1981). A 10 base oligonucleotide carrying the one base deletion was used to direct complementary strand synthesis. Sequence analysis of the crude mixture of phiX174 DNA extracted after transforming the cells showed that only 5% was mutant. The levels of mutant phage were enhanced by adding the mutant primer to the crude mixture of single

stranded phage under conditions such that it hybridised only to the mutant phage. The oligonucleotide then specifically primed complementary strand synthesis on the mutant but not on the wild type phage. After S1 nuclease treatment to destroy the wild type single stranded circles the mutant covalently closed duplex phiX174 was used to transform cells. After 3 cycles, the extracted single stranded phage DNA was 100% mutant.

Mutant yields have also been improved by purifying the covalently closed duplex by either alkaline sucrose centrifugation (Zoller and Smith 1982) or electrophoresis in agarose/ethidium bromide gels (Laski et al 1982).

Mutant yields have been improved by cutting out the whole gene containing the primer mismatch from the newly synthesised partial duplex (Norris et al 1983). A high yield of a transversion mutant in the ompF signal peptide cloned into M13 has been achieved. A 17 base mismatch primer was used to generate the mutant. After the complementary strand synthesis step, there was a mixture of single stranded, gapped and fully duplex molecules. The ompF region was cut out of the duplex between unique EcoR1 and Sal1 sites and purified on an acrylamide gel. The fragment was then cloned between the EcoR1 and Sal1 sites of pBR322. On transformation 42% of the clones carried the transversion.

1.5.3.2 IN VIVO SELECTION FOR MUTANTS

In E.coli, DNA is often methylated at the N⁶ adenine position in GATC sequences by the dam methylase (Geier and Modrich 1979). When one strand of a heteroduplex is methylated and each strand has different genetic markers, on transformation, the majority of the markers recovered, are from the methylated strand (Radman et al 1980). Thus, mismatch repair mechanisms in vivo will use the methylated strand as the template to correct the mispairing. In phiX174 there are no GATC sites so the problem does not arise. Unfortunately M13 has three GATC sites and pBR322 has 18 GATC sites. When a mismatched duplex is formed by oligonucleotide primed synthesis, the wild type

strand from either M13 or pBR322 is methylated while the newly synthesised strand is not. On transformation, the mutant strand is recognised and selectively eliminated.

Mismatch repair can be harnessed to select for mutants by growing the vector as *dam*⁻ hosts to generate unmethylated DNA (Kramer et al 1982). The oligonucleotide primer is then annealed to the unmethylated single stranded circles along with a methylated single stranded linear molecule that covers most of the GATC sites. On transformation, the methylated mutant strand is selectively used as the replication template. An experiment using M13 as the vector has shown that a mutant yield greater than 50% can be achieved even when a gapped duplex is used to transform the cells.

IMPROVED SCREENING METHODS

It is now routine to use colony hybridisation with 12 to 20 base labelled mutant oligonucleotides to identify single base change mutants (Zoller and Smith 1982, Inouge et al 1982, Wallace et al 1981, Winter et al 1982, Montell et al 1982). The technique has been successfully used to select for point mutations as rare as one in a thousand wild types (Dalbadie-McFarland et al 1982). The technique can be inverted and used to identify which colonies are wild type by using a wild type probe (Matteucci and Heyneker 1983). This feature has been used to locate random transition and transversion mutations generated by an ambiguously synthesised mismatched oligonucleotide primer.

An interesting selection system has been developed to identify the mutant colonies by identifying the newly synthesised mutant strand (Traboni et al 1983). When a gene is cloned into M13 it usually generated a frame shift mutation that inactivates the betagalactosidase alpha peptide. White plaques are generated when these phage are grown on X-gal indicator plates. To identify the mutant strand a second primer containing a frame shift mutation that restores the betagalactosidase activity is added with the mismatched oligonucleotide. After the second strand synthesis and

transformation the plaques originating from the mutant strand metabolise the dye and appear blue on the X-gal plates.

1.6 DISCUSSION

There is no doubt that the use of oligonucleotides is the most versatile and accurate way of generating site specific mutations. A small drawback is that a successful mismatch primer experiment requires knowledge of the complete DNA sequence of the gene and vector. A bigger problem with oligonucleotides is that they are not cheap reagents to use. Directed chemical mutagenesis still provides an extremely useful way of modifying specific segments of a gene to determine which parts are functionally important.

Modification of protein coding sequences is best achieved using directed chemical mutagenesis techniques that are not limited to unique restriction sites as these are usually rare in a given gene. D-loop directed bisulphite mutagenesis (1.4.2.4) allows almost any site in a gene to be targeted because the fragment can be defined by any two restriction sites. Computer analysis of the restriction sites of known restriction enzymes in pBR322 shows that there is more than one restriction site in any fifty base pairs of sequence. Unfortunately there are three problems with this method. Bisulphite mutagenesis only allows half the bases in any sequence to be modified because it can only mutate GC base pairs. Second, there is a background of mutations outside the target. Finally, the yield of mutants is lower with small fragments. This makes precise targeting to a few residues in a protein sequence difficult with small DNA fragments.

Isolated fragment mutagenesis (see 1.3.2) allows the use of a variety of DNA modifying agents and thus any base in a given sequence can be modified. The problem is that returning the fragment to its correct location and generating the mutations efficiently requires convenient unique restriction sites near the area to be mutagenised.

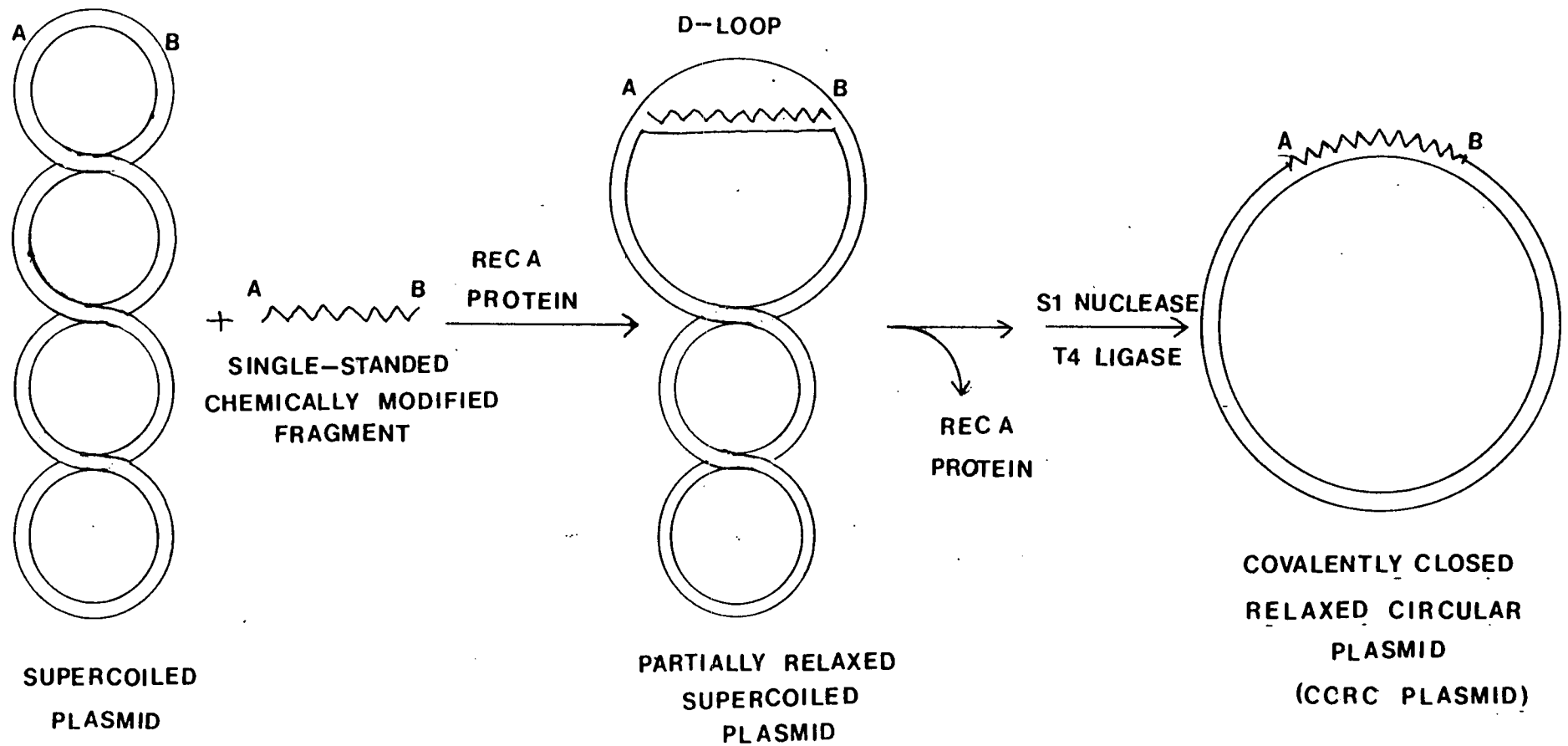
In this project I set out to combine these two approaches to create a procedure that was both flexible and accurate in mutagenising a target while being versatile and efficient at modifying the DNA. My method is based on recombination between mismatched DNA sequences. Site directed mutagenesis is achieved by in vitro heterologous recombination introducing a chemically modified single stranded fragment back into its original position in the gene via a D-loop/ RecA protein intermediate. (Fig. 1)

1.7 MUTAGENESIS BY SITE DIRECTED HETEROLOGOUS RECOMBINATION

To generate the relaxed covalently closed mutant heteroduplex (Fig. 1) is a four step process. First, vector DNA is digested with suitable restriction enzymes to produce a small double stranded fragment to define the target. The fragment is then denatured and the strands separated, purified and chemically modified. Second, the modified single stranded fragment is mixed with the supercoiled vector and incubated with RecA protein. The fragment forms a D-loop in the supercoiled DNA at the gene target in the presence of RecA protein. Third, the DNA/RecA protein complex is dissociated with detergent and the D-looped DNA purified free of RecA protein. Finally, the displaced strand of the D-loop is digested away with a mild treatment of single-strand-specific S1 nuclease. The cut ends are then ligated to the fragment to produce covalently closed circles.

The yield of covalently closed mutant circles is likely to be low because of the inefficiency of the first step. To combat this, I devised an in vitro selection system to eliminate the contaminating wild type supercoils. It has been found that pBR322 is sensitive to S1 nuclease when it is supercoiled. The enzyme cuts at specific palindromic sequences to convert the plasmid to blunt ended linear molecules. The sites are insensitive to S1 nuclease when the plasmid is relaxed. As blunt ended linear vector transforms cells poorly, treatment of the final mixture of supercoiled wild type and relaxed covalently closed mutant plasmid with high concentrations of S1 nuclease should improve the mutant recovery. This selection

FIG 1

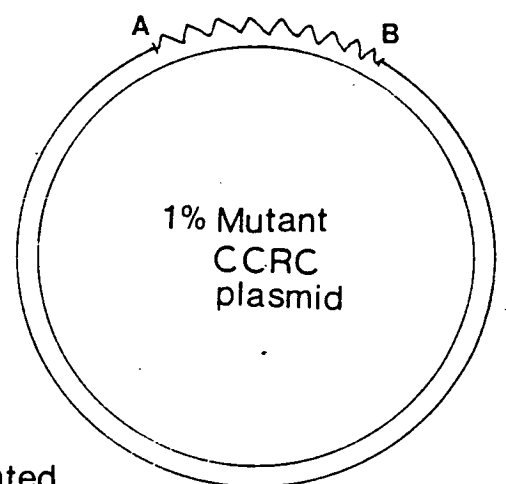
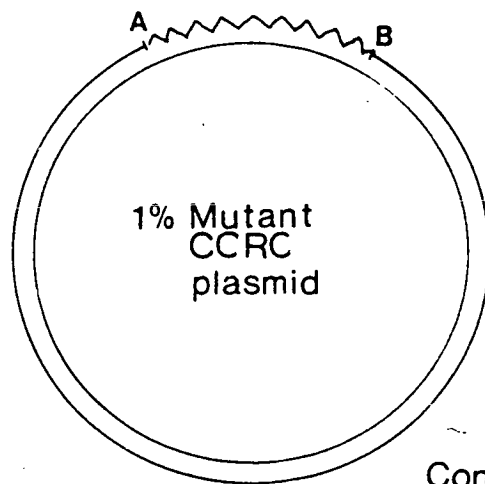
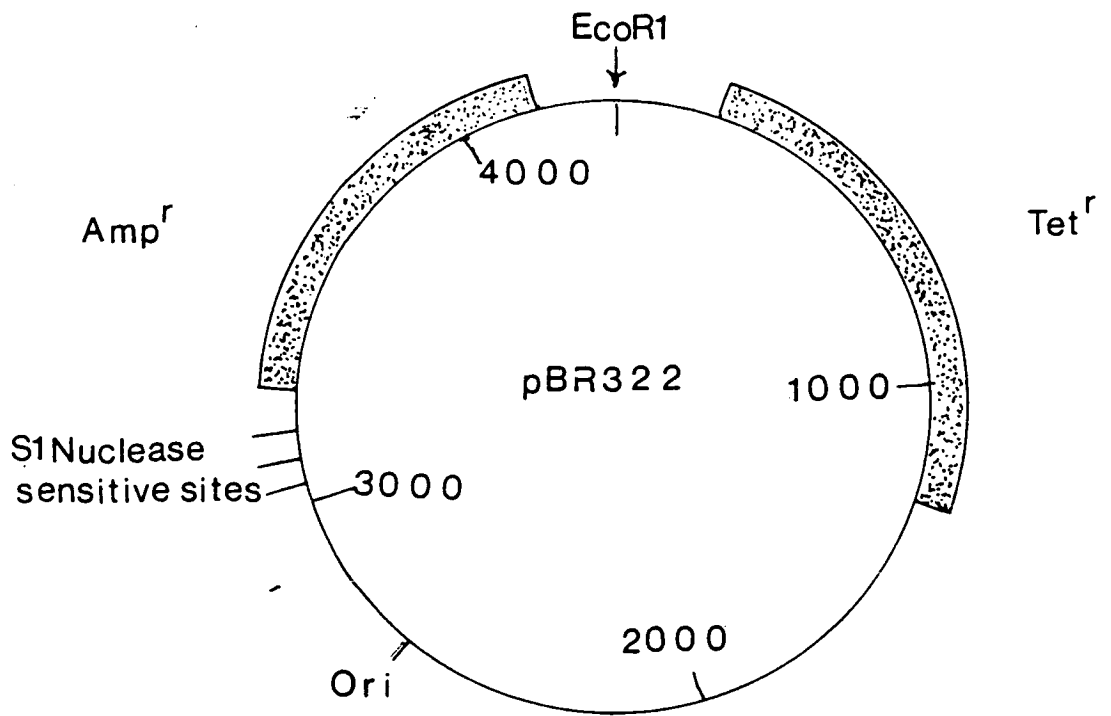


system is obviously limited to pBR322 derived plasmids that carry the S1 sites (Fig. 2).

The betalactamase gene of pBR322 was chosen as the model system for the following reasons. Changes in the drug resistant phenotype can be easily identified. The protein has been sequenced and simple activity assays are available. As the complete DNA sequence of the plasmid is known, any target in the protein sequence can be chosen by computer analysis of the restriction sites in the gene.

The first part of the project involved preparing the reagents. New gel systems had to be developed to produce large quantities of small single stranded fragments. A new RecA overproducing strain had to be constructed to isolate large quantities of pure RecA protein. Supercoiled plasmid DNA had to be prepared free of nicked and linear contaminants. The next stage involved investigating the conditions in which small mismatched fragments could be incorporated into the supercoiled plasmid. The uptake of radioactive fragments by the supercoils was followed by agarose gel electrophoresis and autoradiography. The same approach was used to monitor the conversion of D-loops to relaxed covalently closed circles. The location of the radioactive fragment was mapped with restriction enzyme digests and finally the in vitro selection system was tested.

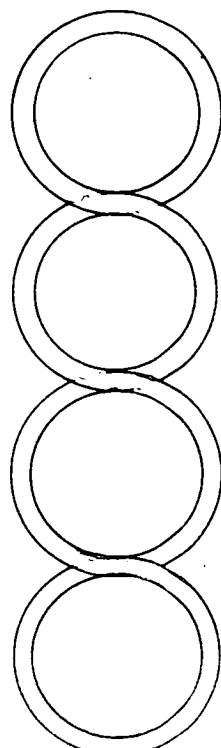
FIG 2



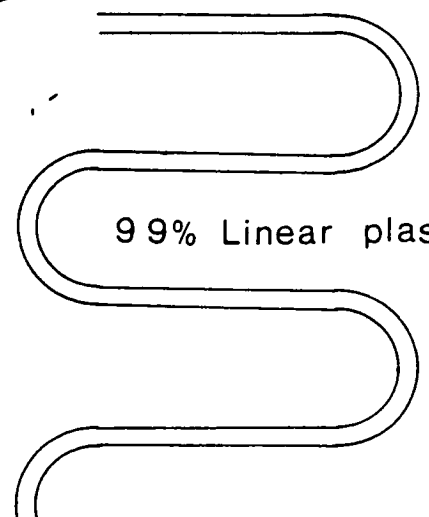
Concentrated

S1 Nuclease

99%
Wild-type
supercoiled
plasmid



99% Linear plasmid



CHAPTER 2 : MATERIALS & GENERAL METHODS

2.1 MATERIALS

RecA protein and restriction enzyme EcoR1 were purified in this study. S1 nuclease was purchased from Bethesda Research Laboratories then purified free of double stranded DNA nicking activity in this study. Restriction enzymes: Sau3A, Taq1 and HaeIII, and DNA modifying enzymes: T4 ligase and the klenow fragment of Ecoli Pol1 were also purchased from Bethesda Research Laboratories. Calf intestinal phosphatase was a gift from P Moulineau of this department. Polynucleotide kinase was purchased from P and L Biochemicals. Lysozyme was purchased from Sigma.

Calf thymus DNA, ethidium bromide, ficoll, sarkosyl, ATP dithiothreitol, TritonX-100 were purchased from Sigma. Reagents for acrylamide gels (acrylamide, N,N'-methylenbisacrylamide (bis), N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue, coomassie blue, ammonium persulphate) were purchased from BDH. Polymin-P was also purchased from BDH. dATP, dCTP, dTTP and dGTP were purchased from Boeringer Mannheim. Nitrocellulose membrane filters BA85 were purchased from Schleicher and Schull. Saran wrap was purchased from Dow Chemicals Corp. Filtration gels S-200, G-75 were purchased from Pharmacia, Cellulose ion exchanges DE-52 and P-11 were purchased from Whatman. Cellex 410 and Agarose were purchased from Biorad.

2.2 GENERAL METHODS

2.2.1 AGAROSE GELS

Uptake of radioactive fragments into supercoils were monitored on 1% w/v agarose gels. Conversion of D-loops to covalently closed relaxed circles was monitored on 1.2% w/v agarose gels. In vitro selection with S1 nuclease was monitored on 2% w/v gels.

Agarose gels were run in TAE : 40mM Tris, 20mM NaAc, 1mMEDTA adjusted to pH8.2 with HCl. DNA samples were loaded with an equal volume of 2 x loading buffer A : 40mM EDTA, 40% v/v Glycerol, 1% w/v Ficoll, 0.1% w/v sarkosyl, 0.1% w/v bromophenol blue, 20mM NaAc, 40mM Tris adjusted to pH8.2 with HCl. DNA/RecA protein samples were loaded with one fifth volume of 5X loading buffer S : 100mM EDTApH8.0, 2% sarkosyl, 0.05% Bromophenol blue, 25% glycerol.

Agarose gels were 18cm long, 12cm wide and 0.4cm thick. Gels were run between 1 and 8 V/cm until the bromophenol blue had run at least 10cm. DNA bands were located by staining the gel with a solution of 1 microgram per ml ethidium bromide at 20°C 5^{minutes} and viewing under short wave U.V. light.

2.2.2 ACRYLAMIDE GELS

End labelling of single stranded fragments was monitored on 8% Acrylamide 0.27% Bis 50% TBE gels. Restriction mapping of incorporated fragments was monitored on 5% Acrylamide 0.17% Bis TBE gels. Running buffer TBE : 1.080% w/v Tris, 0.550% w/v Boric acid, 0.093% w/v EDTA (finally pH8.1). Acrylamide gels were set by adding TEMED to 0.1% v/v, to a degassed solution of acrylamide, bis and 0.06% w/v ammonium persulphate in TBE(or 50% TBE). The gels were 20cm long, 15cm wide and 0.2cm thick. Gels were run between 2 and 20 V/cm. Gels were stained with 1 microgram per ml ethidium bromide at 20°C 30^{minutes}

2.2.3 SDS-ACRYLAMIDE GELS

The purification of RecA protein was analysed on gradient SDS-acrylamide gels. The lower gel was a 50ml gradient of 29% Acrylamide, 0.27% bis to 9% acrylamide, 0.27% bis in a solution of 0.09% w/v SDS 340mM Tris. HCl pH8.8. The gradient was set by adding TEMED to 0.06% v/v and ammonium persulphate to 0.012% w/v. The 10ml upper gel was 2.5% acrylamide, 0.6% bis in a solution of 0.1% w/v SDS, 125 mM Tris. HCl pH6.8. The upper gel was set by adding TEMED to 0.125% v/v and ammonium persulphate to 0.025% w/v.

The tank buffer was 0.1% w/v SDS, 1.44% w/v glycine, and 25mM Tris. Samples were loaded by adding an equal volume of 2 x loading buffer P: 62mM Tris, 2% w/v SDS, 10% w/v sucrose, 5% v/v 2 mercaptoethanol adjusted to pH7.0 with HCl. The gels were 20cm long, 15cm wide and 0.2cm thick. Gels were run between 7 and 15V/cm.

Protein bands were located by leaching out the SDS from the gel with 60% methanol 10% Acetic acid for 2 hours at 20% then staining with 0.1% coomassie blue in 50% methanol 10% acetic acid for 60 minutes at 20°C. Gels were destained for at least 20 hours in 5% methanol 7% acetic acid with chopped plastic sponges to soak up the stain.

2.2.4 AUTORADIOGRAPHY

End labelling of DNA fragments was monitored by autoradiography of wet acrylamide gels covered in saran wrap. Dupont cronex4 safety film was exposed at -70°C in a cassette with scintillation screens.

Agarose gels were dried before autoradiography. The gels were dried under saran wrap onto BA85 nitrocellulose filters at 80°C and under vacuum. The gel sandwich was taped to blotting paper to maintain its shape.

2.2.5 PLASMID PREPARATION

2.2.5.1 TRANSFORMATION

A single colony of RecA⁻HB101 (thr⁻leu⁻pro⁻recA⁻thi⁻hsdR⁻hsdM⁻SupE⁻), provided by D. Finnegan of this department, was grown overnight in 50ml L-broth at 37°C. The culture was diluted 1 in 100 into 50ml L broth then grown to mid log phase (35 klett) at 37°C. The culture was chilled at 0°C for 30 minutes then centrifuged for 10 minutes at 5000 r.p.m. (Sorvall 5534) at 2°C. The pellet was resuspended in 20ml cold (0°C) 0.1M CaCl₂ and incubated on ice for 20 minutes. The suspension was centrifuged for 10 minutes at 5000 r.p.m. (Sorval SS34) at 2°C. The pellet was resuspended in

0.5ml 0.1M CaCl_2 and stored on ice. The concentration of competent cells was 10^{10} per ml.

100 nanograms of a mixture of monomers and dimers of pBR322 (a gift from T. Brown of this department) were mixed with 0.1ml of competent cell suspension at 0°C . The mixture was then incubated at 0°C for 10 minutes, heat shocked at 37°C for 10 minutes then diluted with 1ml L-broth and incubated for 1 hour at 37°C . Cells were plated on L-broth agar containing 100 micrograms per ml ampicillin.

2.2.5.2. PLASMID MINI PREPARATION

Five ampicillin resistant colonies were picked and grown to stationary phase in 10ml L-broth, 100 micrograms per ml ampicillin. 0.75ml of culture was centrifuged for 1 minute. The cells were resuspended in lysis solution : 2mg/ml lysozyme, 25mM Tris. HCl pH8.0, 1mM EDTA and 50mM Glucose. The suspension was incubated for 30 minutes on ice. 0.2ml of 0.2M NaOH, 1% SDS was added and the suspension became viscous as the cells lysed during a 5 minute incubation on ice. 3M NaAc pH5.0 was then added and the solution was left for 1 hour at 0°C . The heavy white precipitate that formed was spun down for 5 minutes and DNA was precipitated out of the supernatant by adding 1ml Ethanol and chilling at -20°C for 30 minutes. The DNA was pelleted by centrifugation and the pellet was dissolved in 0.1ml 0.1M NaAc pH6.0. The DNA was reprecipitated by adding 0.2ml ethanol and chilling at -20°C for 10 minutes. The DNA was pelleted by centrifugation and dissolved in 20 microlitres of 10mM Tris. HCl pH8.0, 1mM EDTA. The plasmid content was analysed on a 1% TAE agarose gel. Four cultures contained monomer plasmid only (one contained plasmid dimer).

2.2.5.3 LARGE SCALE PREPARATION OF SUPERCOILED PLASMID

1ml of a culture of HB101 transformed with plasmid monomer was used to inoculate 3 litres of L-broth, 50 micrograms/ml ampicillin. After 15 hours growth at 37°C the stationary cultures were pelleted in 500ml lots at 5000 r.p.m. for 15 minutes at 4°C in a GS3 sorval rotor. The cells were resuspended in 36ml cold 25% sucrose, 50mM Tris. HCl pH8.1, 40mM EDTA (buffer A). 6.0ml 10mg/ml lysozyme in buffer A and 6.0ml 0.5M EDTA were added and the mixture swirled gently for 30 minutes on ice. 78ml of 0.1% TritonX-100 62.5mM EDTA, 50mM Tris. HCl pH8.1 was added to lyse the cells. After swirling gently for 30 minutes at 0°C, 1ml of 10% TritonX-100 was added to complete the lysis.

The gelatinous mass was transferred to 50ml sorval tubes and centrifuged at 18,000 r.p.m. for 30 minutes at 4°C in an SS34 rotor. The supernatant was separated and 0.95g CsCl and 0.1ml 10mg/ml ethidium bromide were added per ml of supernatant. The solution was loaded into heat sealed tubes and centrifuged in a 50Ti rotor in a Sorval OTD-50B ultracentrifuge at 38,000 r.p.m. for 60 hours at 18°C.

The lower plasmid band was recovered by side puncture of the plastic tubes with a coarse syringe needle. The ethidium bromide was removed by 5 extractions with an equal volume of propan-2-ol. The CsCl was removed by a 3 hour dialysis against 5 litres of 170mM NaCl, 10mM Tris. HCl pH8.0, 1mM EDTA, followed by a gel filtration on a 200 ml G-75 column in 10mM Tris. HCl pH8.0 1mM EDTA(TE).

DNA was detected in the column fractions by mixing 5 microlitres of each 10ml fraction with 5 microlitres of 1 microgram/ml ethidium bromide on a plastic petridish. The droplets were then viewed under short wave U.V light. Orange fluorescence indicated the presence of DNA.

DNA-containing fractions from the column were pooled and incubated at 37°C for 30 minutes with 10 microgram/ml heat treated RNAaseA. The solution was then extracted with an equal volume of distilled phenol equilibrated in TE and applied to the top of a 200ml G-75 column in 1mM Tris. HC1 pH8.0 1mM EDTA. The DNA fractions from the void were pooled, the DNA concentration was determined by U.V. absorbance at 260nm ($A_{1\text{cm}}^{1\%} 260 \text{ } 200$) then stored in 100 microgram lots at -20°C. A 3 litre culture usually produced 2mg pBR322.

In previous preparation methods, most of the nicking of the plasmid DNA occurred during dialysis steps to remove the CsCl. The first gel filtration column avoided the long dialysis steps and reduced the level of nicked circular plasmid in the final DNA solution. tRNA was removed by the RNAaseA treatment and the second gel filtration step. Analysis of the plasmid DNA on a 1% agarose TAE gel showed that only 5% of the plasmid was nicked. The level of nicked circular plasmid could be further reduced by acid phenol extraction.

2.2.5.4. ACID PHENOL EXTRACTION

Nicked circular and linear plasmid can be selectively removed from a DNA solution by acid phenol extraction (Zasloff 1978).

10 micrograms of monomeric pBR322 in 100 microlitres 1mM Tris. HC1 pH8.0, 1mM EDTA (5% nicked circles and free of tRNA) was cooled to 4°C. 5 microlitres of 1.5M NaCl and 5 microlitres of 1M NaAc pH4.0 (5.72% v/v glacial acetic acid, 0.62% w/v NaOH) were added and the solution was mixed for 30 seconds with an equal volume of redistilled phenol equilibrated with 50mM NaAc pH4.0 at 4°C. The phases were separated by centrifugation for 2 minutes at 4°C (some DNA precipitated at the interface). The upper phase was removed and extracted with 500 microlitres ether. The solution was loaded onto a 5ml G-75 column in 1mM Tris. pH8.0, 1mM EDTA. The DNA containing fractions were pooled and the concentration determined by U.V. absorbance.

Between 10% and 50% of the plasmid was left after the acid phenol extraction. Analysis of the DNA on a 1% agarose gel showed that it was about 99% supercoiled. The supercoiled plasmid was stored at -20°C in 1 microgram lots.

2.2.6 PREPARATION OF RESTRICTION ENZYME EcoR1

Restriction enzyme EcoR1 was purified from strain JK29 provided by P. Ford of this department using an unpublished method by P. Ford.

140g frozen JK29 cells were suspended in 200ml E buffer : 10mM K₂PO₄ pH7.0, 7mM 2-mercaptoethanol, 1mM EDTA, with added protease inhibitors, 2mM Benzamide, 0.005% w/v trypsin inhibitor, 0.005% w/v ovatrpsin inhibitor. The 300ml cell suspension was sonicated in ten, one minute bursts at 0°C. The lysed cells were centrifuged at 20,000 r.p.m. for 20 minutes at 4°C in a sorval SS34 rotor. The supernatant was separated and 30ml 10% polyethyleneimine was added and stirred for 30 minutes at 0°C to precipitate the DNA. The DNA was pelleted by spinning at 15,000 r.p.m. for 20 minutes at 4°C in an SS34 rotor. The supernatantI was separated and contaminating proteins were precipitated by adding 20g ammonium sulphate per 100ml of supernatantI and stirring for 30 minutes. The precipitated protein was pelleted by spinning at 15,000 r.p.m. for 20 minutes at 4°C in an SS34 rotor. The supernatantII was separated. EcoR1 was precipitated by adding an additional 12g ammonium sulphate per 100ml of supernatantII and stirring for 30 minutes. Precipitated proteins containing EcoR1 were pelleted by spinning at 15,000 r.p.m. at 4°C in an SS34 rotor. The protein pellet was dissolved in a minimum volume of ENG buffer: E buffer plus 0.2M NaCl, 10% glycerol, 0.2% NP40. Protease inhibitor PMSF was added to 20 micrograms/ml.

This solution (15ml) was applied to a 400ml sephacryl S-200 column equilibrated in ENG buffer. The column was run in this buffer at 30ml per hour and 10ml fractions were collected. Protein was detected by U.V. absorbance at 280nm.

EcoR1 activity was detected by site specific digestion of DNA from phage lambda. 0.5 microlitres of each fraction was incubated at 37°C for 30 minutes with 5 microlitres of 60 micrograms/ml lambda DNA in R1 buffer : 100mM Tris. HCl pH7.5, 50mM NaCl, 5mM MgCl₂. The DNA was analysed on an 0.8% TAE agarose gel. EcoR1 activity was detected in fractions 15-29.

These fractions were pooled and loaded onto a 30ml phospho-cellulose p11 column equilibrated in ENG buffer. The column was washed with 100ml ENG buffer, then a 400ml salt gradient of 0.2M to 0.8M NaCl (ENG buffer to ENG buffer plus 0.6M NaCl). 10ml fractions were collected and every second fraction was assayed for EcoR1 activity. EcoR1 was eluted between 0.6M to 0.8M NaCl (fractions 36-50).

These fractions were pooled and loaded onto a 3ml HAP column equilibrated in ENG buffer. The column was washed with a 200ml gradient of 0.01M to 0.81M K₂PO₄ pH7.4 (ENG buffer to ENG buffer plus 0.8M K₂PO₄ pH7.4). 5ml fractions were collected and assayed for EcoR1 activity. EcoR1 was eluted between 0.45M and 0.8M K₂PO₄ (fractions 22-40).

These fractions were pooled, an equal volume of ENG buffer was added and the solution was applied to a 4ml P11 column equilibrated in ENG buffer. The column was washed with ENG buffer plus 0.6M NaCl (0.8M NaCl) and 0.7ml fractions were collected. Fractions 7-11 contained EcoR1 activity. 1.3ml of glycerol was added to each fraction. The EcoR1 was stored at -20°C.

One microlitre of peak fraction 7 was diluted ten fold by adding 9 microlitres of ENG buffer. This solution was then serially diluted by factors of two. One microlitre of each of these solutions was incubated at 37°C for 30' with one microgram of lambda DNA in 50 microlitres of R1 buffer. The extent of digestion was assayed on an 0.8% agarose gel. Complete digestion was obtained with one

microlitre of ten fold diluted fraction 7. This was equivalent to 20 units per microlitre (1 unit = hydrolysis of 1 microgram lambda DNA in one hour at 37°C). Peak fraction 7 contained 40,000 units of EcoR1.

2.2.7 S1 NUCLEASE PURIFICATION

B.R.L. S1 nuclease was repurified by DE-52 chromatography at pH4.6 (Vogt 1980).

30,000 units of B.R.L. S1 nuclease, contaminated with double stranded DNA nicking activity, in 100 microlitres of 20mM Tris. HCl pH7.5, 50mM NaCl 0.1mM ZnCl₂ 50% v/v glycerol, was added to 0.9ml of S buffer: 30mM NaAc pH4.6 0.1mM ZnSO₄ 50mM NaCl, 5% glycerol. The diluted S1 nuclease at pH4.6 was applied to a 0.1ml DE-52 column equilibrated in S buffer. The column was washed with 0.4ml S buffer, 0.4ml S buffer plus 50mM NaCl, 0.3ml S buffer plus 200mM NaCl and 0.1ml S buffer plus 450mM NaCl. 0.05ml fractions were collected and assayed for S1 nuclease and double stranded nicking activity.

S1 activity was identified by clean cutting of supercoiled pBR322 to linear plasmid. S1 nuclease contaminated with nicking activity was detected by the appearance of a heterogenous mixture of linear molecules from multiple cutting of the plasmid. 10 nanograms of supercoiled pBR322 in one microlitre of 30mM NaAc pH4.6, 1mM ZnSO₄, 50mM NaCl was incubated at 37°C for 10 minutes with one microlitre of each fraction. The digestion was analysed on a 1% TAE agarose gel. The nicking activity did not stick to the column and appeared in the S buffer wash. S1 nuclease was eluted in the S buffer plus 200mM NaCl fractions.

S1-nuclease containing fractions were pooled (0.5ml) and diluted with 2ml 30mM NaAc pH4.6 0.1mM ZnSO₄ 5% glycerol. This solution (2.5ml) was applied to an 0.05ml DE-52 column equilibrated in S buffer. The column was washed with 0.2ml S buffer and the S1 nuclease was eluted with 0.05ml S buffer plus 450mM NaCl.

The supercoil digestion assay showed that a residual nicking activity did not stick to this column. An equal volume of glycerol was added to the concentrated S1 nuclease fraction. The pure S1 nuclease in 100 μ l 15mM NaAc pH4.6, 250mM NaCl, 0.05mM ZnSO₄, 52% glycerol was stored at -20°C.

CHAPTER 3 : FRAGMENT PREPARATION

3.1 INTRODUCTION

Four class A beta-lactamases from different species of bacteria have been sequenced and have been shown to have related aminoacid sequences with a number of conserved residues (Ambler 1980). Assuming these evolutionary stable aminoacids are structurally or mechanistically important, targeting mutagenesis at any of these should result in a detectable change in phenotype. The Korn-Seq computer programme was used to search for suitable restriction sites that would generate small restriction fragments containing code for conserved aminoacids in the R-TEM beta-lactamase gene pBR322 (Korn et al 1977, Queen and Korn 1980).

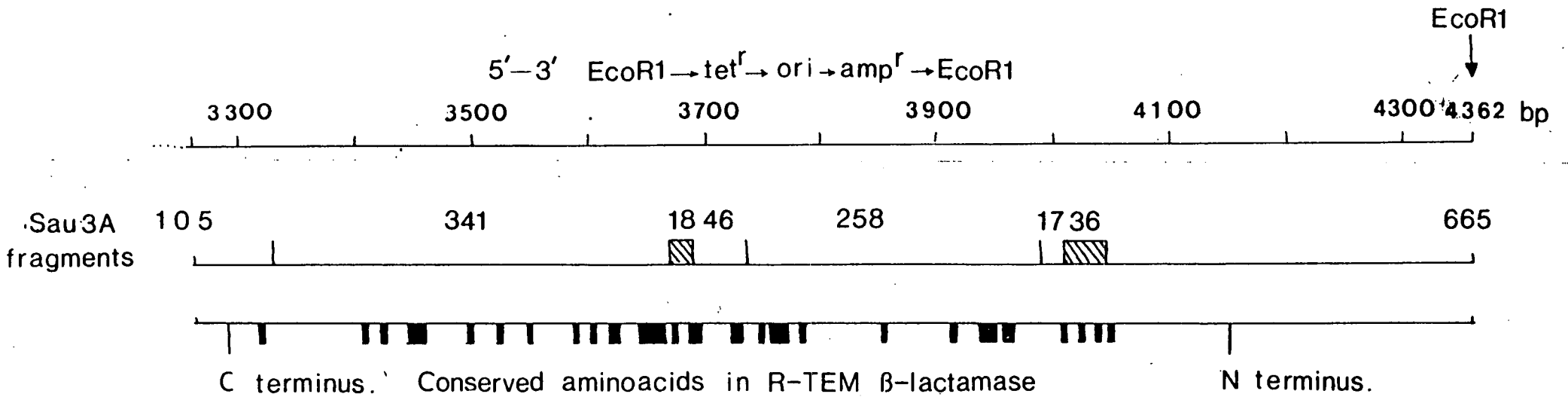
Sau3A breaks the beta-lactamase gene into eight pieces (fig.3). The 18 and 36 base pair fragments were chosen to define targets for mutagenesis as they are the smallest pieces that contain code for conserved aminoacids.

To prepare sufficient quantities of small fragments only 0.5% of the size of the plasmid, large quantities of DNA had to be digested and purified. Accurate measurement of DNA concentrations is limited to about 0.5 micrograms per ml with most spectrophotometers. This made it necessary to isolate about one microgram of each single stranded fragment. For the 18 base pair fragment this implied starting with a Sau3A digest of one milligram of pBR322 (to allow for purification losses). Accurate knowledge of the single stranded fragment concentration was essential as efficient production of D-loops is dependent on the stoichiometry of RecA protein and single stranded DNA (See Chapter 5).

The large quantity of DNA caused problems in resolving the double stranded fragments in a reasonable volume of acrylamide gel. To overcome this, I developed a gel system with good resolving power that tolerated very high DNA concentrations. To recover the

FIG 3

Location of Sau3A sites and conserved aminoacids in R-TEM gene of pBR322



DNA from the gel, I developed a quick and efficient extraction system. The quantity of DNA also made strand separation of the small fragments difficult in a small gel volume as single stranded fragments in high concentrations reassociate rapidly. To overcome this, I introduced a modification to standard strand separation protocols which allows relatively large concentrations of small fragment strands to be separated.

The single stranded fragments were isolated from the gel and their concentration was determined by U.V. absorbance. Batches were "end-labelled" with ^{32}P and used in the D-looping experiments. Some of the labelled fragments were then chemically modified with nitrous acid.

3.2 PREPARATION OF SMALL DOUBLE-STRANDED RESTRICTION FRAGMENTS

A normal loading quantity of a restriction digest is about one microgram per 0.15cm^2 of slot surface. To resolve a milligram of DNA such a gel would have to have a surface of 150cm^2 which is equivalent to a gel 0.15cm thick and 10 meters wide. The solution was to develop a gel system that was sufficiently porous at one end not to overload but to have the resolving power of a sequencing gel at the other end. High concentrations of acrylamide with high ratios of bis cross-linking give sequencing gels single base resolving power (Maxam and Gilbert 1980). Conversely, low acrylamide concentrations with lower ratios of bis cross-linking have less of a tendency to overload (personal observation). By trying various gradient gel systems using high concentrations of urea to smooth out running anomalies (Maniatis et al 1975), I developed a recipe for a gel that could be loaded with 500 micrograms of digested DNA per 0.15cm^2 of slot surface and completely resolve fragments between 15-100 base pairs.

The pBR322 DNA was digested with Sau3A and loaded onto the gradient gel (see 3.5, experimental procedures). To improve band resolution fragments smaller than 20 base pairs were run off the bottom of the gel into anion exchanger DE-52. The gel was stained

with ethidium bromide and U.V. illuminated (Fig. 4). The very small fragments from the DE-52 were run down a second identical gel for a shorter time (Fig. 5). DNA bands were cut out and stored in the gel at 4°C. An important property of this gel system is that the low molecular weight fragments are concentrated in a small volume of gel. This is essential as ethidium bromide staining of fragments less than 20 base pairs is very inefficient in 6M urea (personal observation).

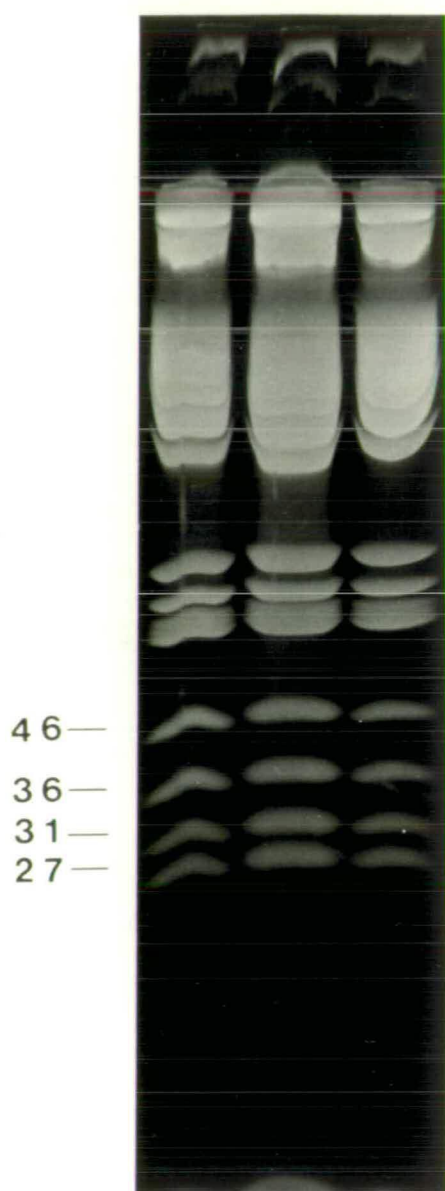
3.3 RECOVERY OF DNA FRAGMENTS FROM GELS

The next stage was to extract the DNA from the gel slab. Extraction methods based on electroelution (Davies 1981) and passive diffusion (Maxam and Gilbert 1980) were found to be unreliable with small fragments. I have designed a reliable method in which the DNA is electroeluted out of the gel onto DE-52. The apparatus is illustrated in Fig. 6.

The DE-52 was packed in column A under gravity then assembled with the chamber B containing the gel slab and the tubes connecting the two buffer tanks. The higher level of buffer in tank C kept the buffer syphoning through the pipe towards the positive electrode. As the flow of buffer and the migration of the DNA in the electric field were in the same direction the fragments were rapidly transferred from the gel slab to the DE-52. The apparatus was then dismantled and the column washed with a mild salt solution to remove any contaminants. The DNA was then eluted with a concentrated salt solution, extracted with n-butanol to remove ethidium bromide and ethanol precipitated. This is an improvement on HAP chromatography which requires a dialysis step to remove the phosphate ions before precipitation. Almost complete recovery of DNA between 17 and 5000 base pairs from acrylamide and agarose gels has been achieved with this method. It works equally well for single stranded DNA.

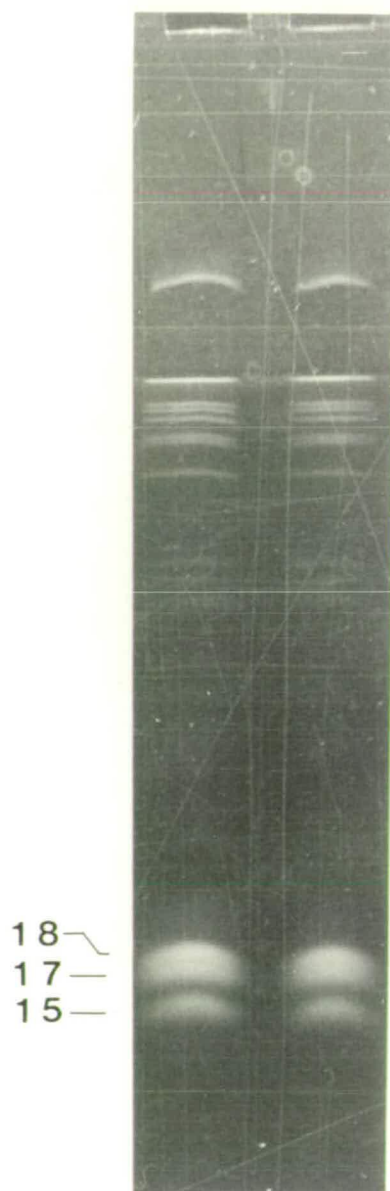
PURIFICATION OF DOUBLE-STRANDED
RESTRICTION FRAGMENTS

FIG 4



See 3.5.1

FIG 5

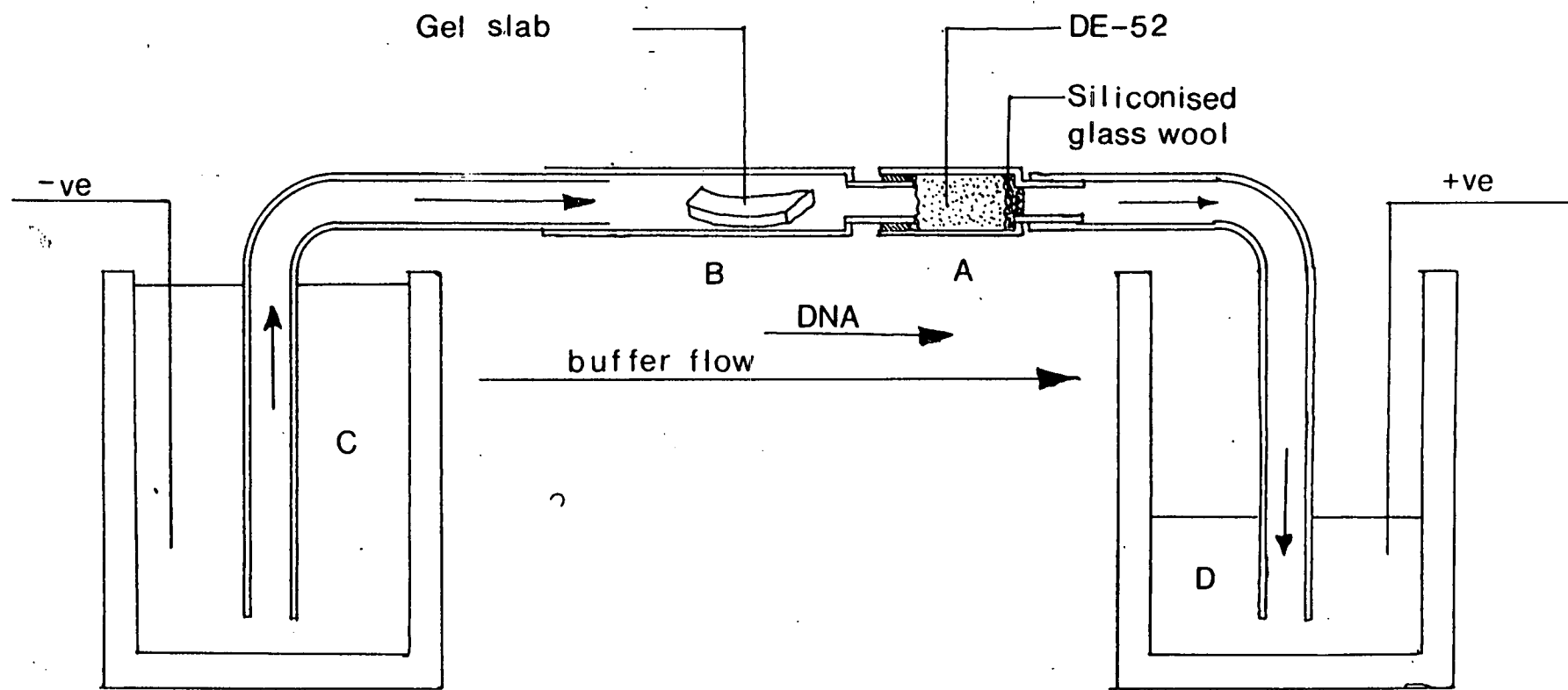


See 3.5.1

FIG 6

DE-52 ELECTROELUTION APPARATUS

(See 3.3 & 3.5.2)



3.4 STRAND SEPARATION OF SMALL DNA FRAGMENTS

The quantity of DNA created a problem in the strand separation step. Small fragments rapidly denature in 30% DMSO at 90°C (Maxam and Gilbert 1980) or in 0.2M NaOH (Szalay 1977) but will also rapidly reassociate as they concentrate at the interface of an acrylamide gel. The concentration effect is greater the denser the gel (personal observation). Unfortunately reducing the gel density reduces the band resolution as small fragments diffuse more easily. The problem can be eliminated by lowering the DNA concentration (fragment molarity) at the interface but this makes detection of the bands difficult without radio-labelling.

I reasoned that if the viscosity of the denaturing solution could be increased to a point where the rate of the fragments entering the gel equalled that of them arriving at the interface then the concentration effect would be eliminated. By adding large amounts of ficoll, a high molecular weight polysaccharide to the sodium hydroxide denaturing solution before loading the gel there was a dramatic increase in the production of independently migrating single stranded fragments.

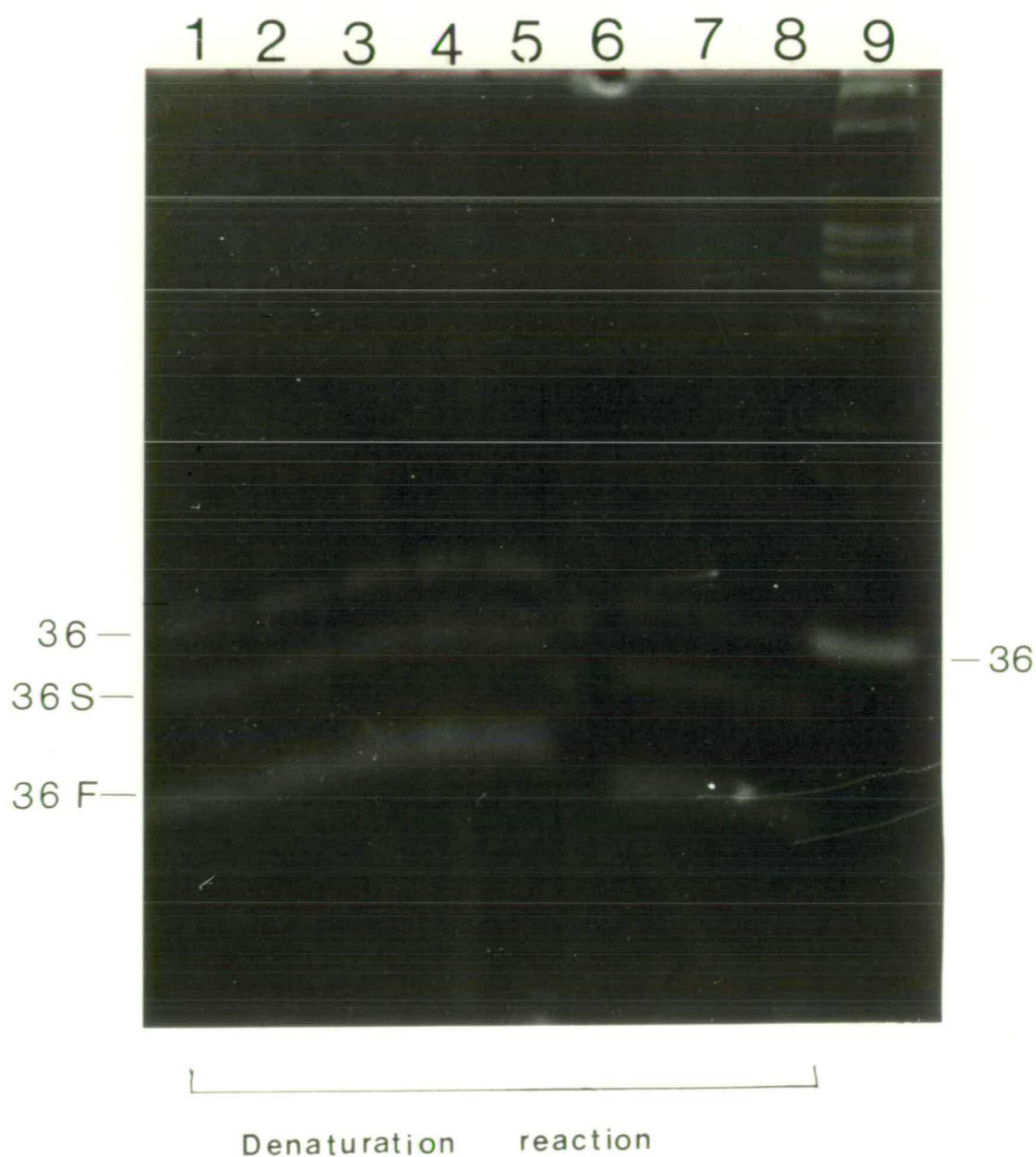
The 36 base pair fragment was denatured in sodium hydroxide, prevented from reannealing by adding ficoll and loaded onto a non-denaturing slab gel. The bands were detected by ethidium bromide staining and U.V. illumination (Fig. 7).

Double-stranded DNA gives a much stronger fluorescence than denatured DNA so the extent of denaturation was measured by comparison to a double-stranded DNA standard of known concentration. The 36 base single-stranded DNA was used in subsequent experiments as the 18 base denatured bands gave such a weak fluorescence signal they were impossible to locate.

The DNA was recovered from the gel by the electroelution procedure and ethanol precipitated. The 36 base DNA pellets were dissolved in distilled water and the absorbance of the single-stranded

PREPARATION OF SINGLE-STRANDED
RESTRICTION FRAGMENTS

FIG 7



See 3.5.3

DNA at 260nm was determined ($A_{1\text{cm } 260}^{1\%} 300$). 2 micrograms of each single-stranded 36 base pair fragment was recovered, an overall yield of 50%.

3.5 EXPERIMENTAL PROCEDURES

3.5.1 GRADIENT GEL SEPARATION OF RESTRICTION FRAGMENTS

One milligram of pBR322 was ethanol precipitated and the dry pellets combined and dissolved in 0.06ml of Sau3A buffer : 50mM NaCl, 6mM Tris. HCl pH7.5, 6mM MgCl_2 . 50 units Sau3A was added and the reaction was incubated for 16 hours at 37°C. 0.06ml 2 x loading buffer A was added and the DNA was loaded into three 1cm wells in the gradient gel.

The gel was a 50ml gradient of 15% w/v acrylamide, 0.5% Bis, 6M urea to 25% w/v acrylamide, 1.25% bis, 6.3M urea with a 10ml 3% w/v acrylamide 0.1% w/v bis 6M urea stacking gel. The gel was set and run in TBE buffer (see General Methods). The gel was set by adding ammonium persulphate to 0.008% w/v and TEMED to 0.2v/v. The stacking gel was set by adding ammonium persulphate to 0.04% w/v and TEMED to 0.4% v/v. The gel was 20cm long, 15m wide and 0.2cm thick. It was run for 90 hours at 3V/cm. A dialysis membrane was clipped over the bottom of the gel containing 5ml swollen DE-52 to collect fragments between 8 and 18 base pairs that ran off the end of the gel.

The gel was stained for 30 minutes in one microgram per ml ethidium bromide. The bands were located by illumination with short wave U.V. then cut out of the gel.

The DE-52 from the dialysis bag was packed in a 5ml 5cm column and washed with 10ml TBE plus 100mM NaCl. DNA was eluted by washing the column with 10ml TBE plus 1.5M NaCl. The volume of the DNA solution was reduced to 2ml with four extractions with n-butanol (twice the aqueous volume). This step also removed any remaining ethidium bromide. The DNA was then precipitated by adding a one

tenth volume of 0.1M $MgCl_2$ and 3 volumes of ethanol and freezing the solution in liquid nitrogen. The precipitate was pelleted by spinning in a microfuge for 10 minutes at 4°C. The pellet was washed with 70% ethanol then dried under vacuum. The pellet was dissolved in 0.02ml TBE. 0.02ml 2 x loading buffer A was added and the sample was loaded into a 1cm slot on a second identical gradient gel. The gel was run for 19 hours at 7.5 V/cm. The gel was stained and the 15, 17 and 18 base pair bands were located and cut out. Lower molecular weight fragments did not fluoresce.

3.5.2 ELECTROELUTION OF DNA FRAGMENTS ONTO DE-52

DNA was extracted from the gel slabs by electroelution onto DE-52. The apparatus was constructed out of modified plastic 1ml syringes (see Fig. 6). The complete apparatus was siliconised with B.D.H. Repellcote to prevent it binding DNA.

0.1ml DE-52 equilibrated in TBE was packed into column A and the gel slab was placed in chamber B. The apparatus was assembled vertically to avoid air bubbles then placed between the clean 800ml buffer tanks of TBE buffer (tank C had a 3cm head of buffer). The system was run for 2 hours at 200V (1mA) with the negative electrode in tank C. Column A was then detached and washed with 0.5ml TBE plus 0.1M NaCl. The DNA was eluted with 0.5ml TBE plus 1.5M NaCl, 0.05ml fractions being collected. The DNA content of the fractions was monitored by mixing 3 microlitres of the fraction with three microlitres of one microgram per ml ethidium bromide on a petri dish and viewing under short wave U.V.

The DNA fractions were pooled and extracted with 3 volumes of n-butanol. This both reduced the volume of the DNA solution and removed the ethidium bromide. The DNA was then precipitated as in 3.5.1.

3.5.3 STRAND SEPARATION OF THE 36 bp Sau3A FRAGMENT

The DNA pellet from the extracted 36 base pair band of the Sau3A digest (approximately 8 micrograms) was dissolved in 0.09ml distilled water. 10 microlitres of the solution was mixed with 5 microlitres of 2 x loading buffer A and loaded on the gel as the double-stranded standard. The remaining 0.08ml was mixed with 0.024ml of 3M NaOH, 0.01M EDTA and incubated for 10 minutes at 20°C. The solution was then cooled on ice to 0°C. 0.12ml of cool 20% w/v ficoll, 0.33% w/v bromophenol blue was added and the solution was loaded immediately onto a cooled 8% acrylamide, 0.27% Bis 50% TBE gel. 0.03mls of the solution was loaded in eight 0.5x1.0cm slots on the 0.5cm thick gel (20cm long, 15cm wide). The upper tank was cooled with a polythene bag containing crushed ice. The gel was run for 13 hours at 4.5V/cm. The gel was stained with one microgram 1ml ethidium bromide and viewed under short wave U.V. (Fig. 7).

The single-stranded bands were located running in front of the double-stranded DNA standard. The standard migrated just behind the bromophenol blue. More than 95% of the fragment DNA was single-stranded. The fast (36F) and slow (36S) migrating single-stranded bands were cut out and extracted from the 5ml of gel slab by a scaled up electroelution onto DE-52 (3.5.2). The strand separation gel also purified the 36 base fragments from residual contaminating higher molecular weight fragments (see Fig. 7, Track 9).

The single-stranded 36F and 36S DNA pellets were separately dissolved in 2ml of distilled water and their U.V. absorbance at 260nm was determined ($A_{260}^{1\% \text{ 1cm}}$ 300). 2 micrograms of 36F and 2 micrograms of 36S were recovered, an overall yield of 50%. The one microgram per ml DNA solutions were stored at -20°C.

3.5.4 END LABELLING OF 36bp DNA FRAGMENTS

36F and 36S were end labelled with ^{32}P in 0.2 microgram lots.

The 5' phosphates were removed from 0.2 micrograms of 36F (16.8×10^{-12} moles of ends in 0.2ml distilled water) by adding 0.02ml of 10 x CIP buffer: 50mM NaCl, 60mM Tris. pH7.5, 60mM MgCl_2 and incubating at 37°C for 30 minutes with 1 microlitre of calf intestinal phosphatase solution. The reaction was stopped by adding 0.02ml 0.2M EDTA pH8.0 and incubating at 65°C for 10 minutes. The DNA was precipitated by adding 0.25ml of 0.1M MgCl_2 , 0.025ml 3M NaAc, 1ml ethanol and freezing in liquid N_2 . The precipitate was pelleted by spinning for 10 minutes. The pellet was washed with 70% v/v ethanol and dried under vacuum.

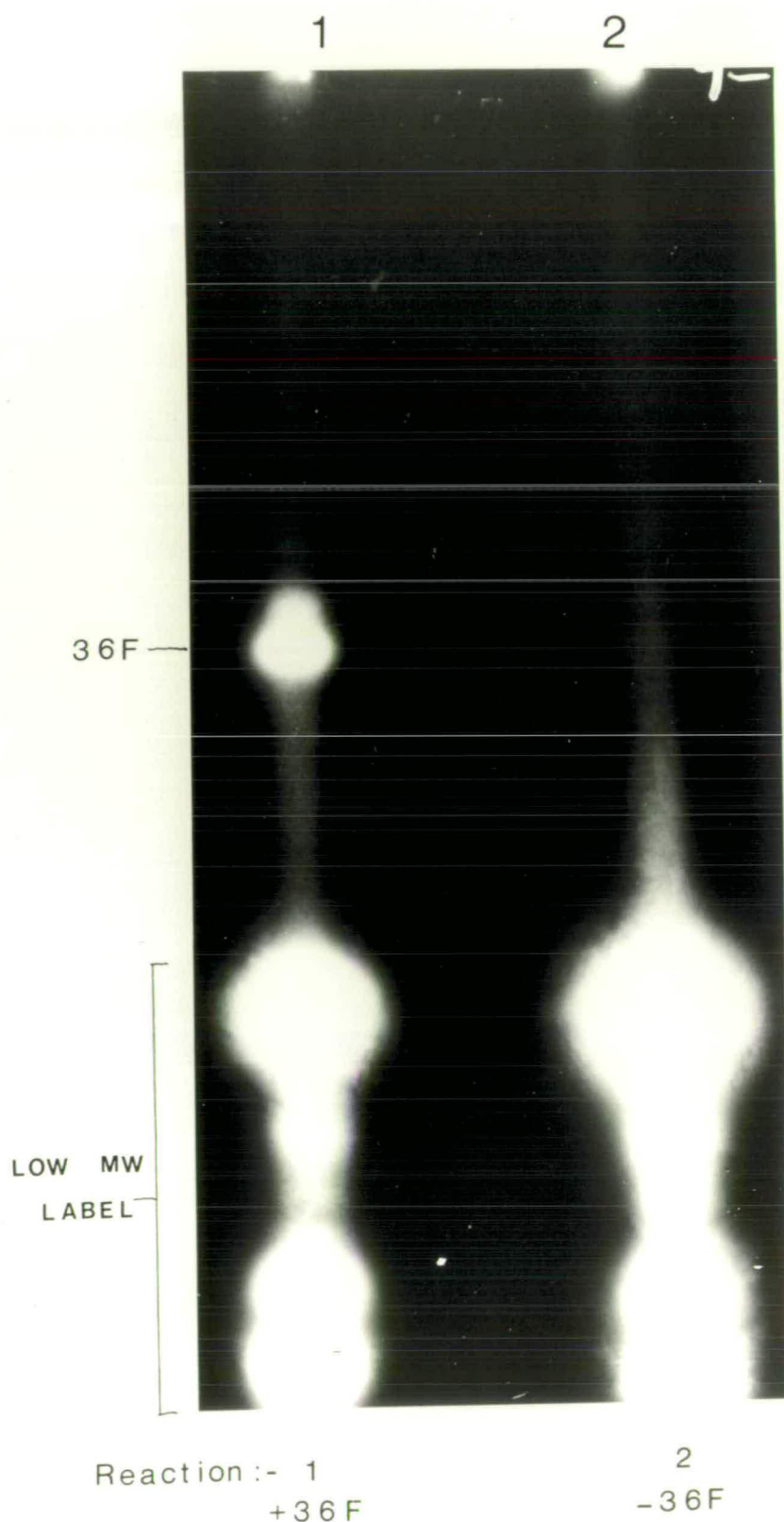
100 micro Curies of ^{32}P gamma labelled ATP (3000 Curries per m mole) was dried down in a 1.5ml eppendorf tube. The residual ammonium acetate was removed by adding 0.02ml dH_2O then drying under vacuum. This step was repeated. The label was then dissolved in 0.02ml water and added to the precipitated dephosphorylated 36F pellet and the mixture was dried under vacuum.

The mixture of DNA and label was dissolved in 0.015ml dH_2O and mixed with 4 microlitres of 5 x kinase buffer: 100mM Tris. pH7.5, 50mM MgCl_2 5mM 2-mercapto ethanol. One microlitre of P L Polynucleotide kinase was added and the reaction was incubated for 2 hours at 37°C . 0.025ml of water was added and the reaction was terminated by vortexing with 0.05ml distilled phenol equilibrated in 10mM Tris. pH7.5. The phenol was removed by extracting with 0.2ml diethyl ether.

The labelled DNA was then precipitated by adding 6 microlitres of 3M NaAc, 6 microlitres of 0.1M MgCl_2 and 0.15ml ethanol. The solution was frozen in liquid nitrogen, spun for 10 minutes and the supernatant removed. The labelled DNA pellet was resuspended in 0.05ml and 0.3M NaAc pH5.0 to dissolve the unincorporated label, 0.125ml ethanol was added and the mixture was frozen in liquid nitrogen and spun. The pellet was resuspended and the step repeated. Finally the

RADIOLABELLING OF THE 36F FRAGMENT

FIG 8



See 3.5.4

pellet was washed with 0.5ml cold 70% ethanol and dried under vacuum. The radioactive DNA was dissolved in 0.2ml 1mM Tris. HC1 pH7.5 1mM EDTA.

0.5 microlitres were mixed with 2 microlitres 2 x loading buffer A and heated at 100°C for 5 minutes. The sample was loaded onto an 8% acrylamide 0.27% bis 50% TBE gel (0.15cm thick) and run at 16V/cm until the bromophenol blue had migrated halfway. The gel was wrapped in Saran Wrap and the radioactive bands were located by autoradiography. The autoradiograph showed the radioactive 36F fragment was contaminated with low molecular weight radioactive breakdown products of ATP (Fig. 8). Track 1 is the sample of the labelling reaction, the slowest band is the labelled 36F DNA (running just in front of the bromophenol blue). Track 2 is a sample of a labelling reaction in which the DNA fragment was added after the kinase incubation had been terminated with phenol. The presence of only low molecular weight bands in control track 2 confirms the identity of the slow band as that of the labelled fragment.

Repeated ethanol precipitations at low pH failed to remove all these contaminants. The radioactive contaminants also remained bound to the single-stranded fragment during G-10 chromatography in 0.2M NaOH and eluted with the DNA in the void volume of the column. I decided not to purify the fragment free of these contaminants by electrophoresis because the variable efficiency of recovering the low concentrations of DNA from the gel introduced errors in the estimation of fragment concentration.

3.5.5 CHEMICAL MODIFICATION OF LABELLED 36F AND 36S

0.08 micrograms of ^{32}P 36F and ^{32}P 36S single-stranded fragments were chemically modified with nitrous acid (Hirose et al 1982).

0.08 micrograms of single-stranded fragment in 0.08ml 1mM Tris. pH8.0, 1mM EDTA were mixed with 0.08ml 0.5M NaAc pH4.3, 2M NaNO_2 (freshly prepared).

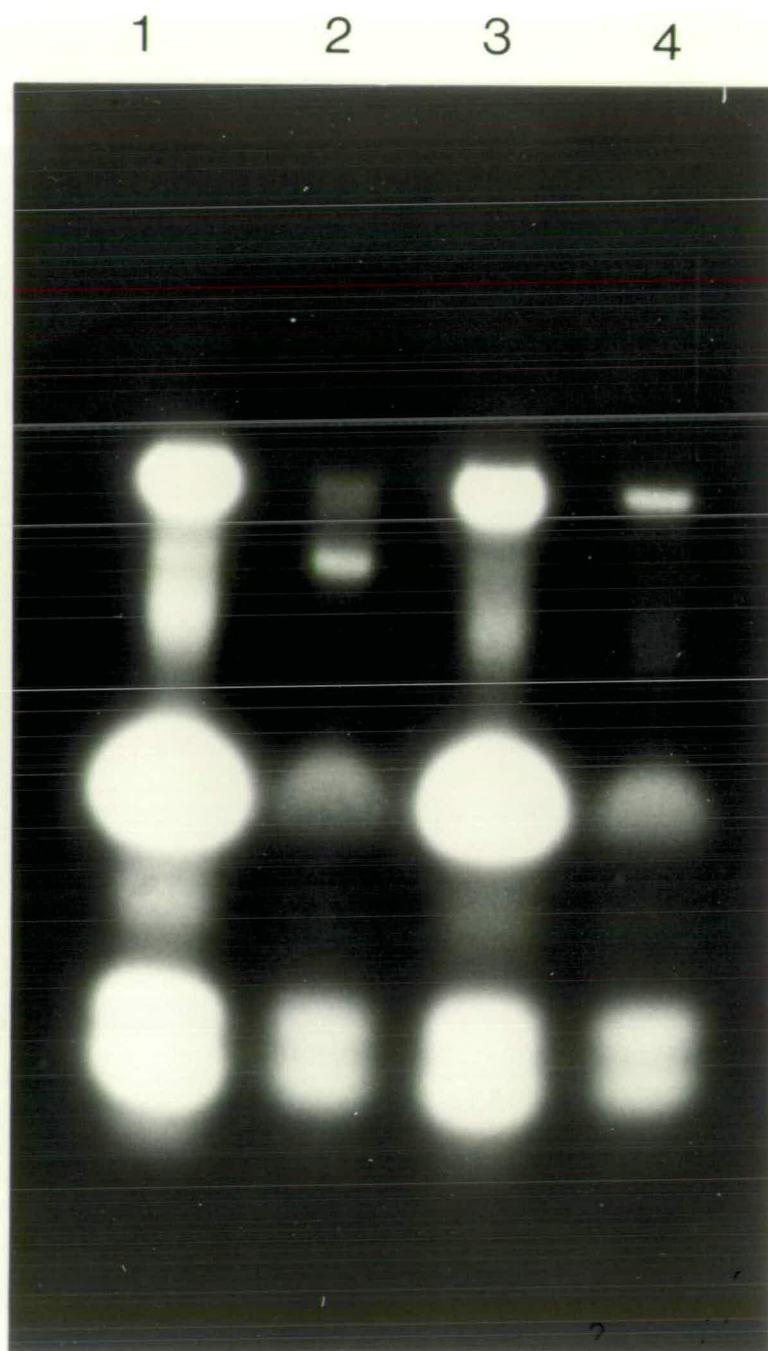
After 15 hours incubation at 20°C in the dark the reaction was terminated by ethanol precipitation. 0.03ml 0.1M MgCl₂, 0.03ml 3M NaAc, 0.08ml 1M Tris.HCl pH8.6 was added to the reaction and vortexed. 1ml ethanol was added and the solution was frozen in liquid nitrogen and spun for 10' in a microfuge. The pellet was washed with 0.5ml 70% Ethanol and dried under vacuum.

The pellet was redissolved in 0.08ml dH₂O 0.5 microlitres of 36F, nitrous acid modified 36F, 36S and nitrous acid modified 36S (Fig. 9, tracks 1, 2, 3 and 4 respectively) were run on an 8% acrylamide 0.27% bis 50% TBE gel (see 3.5.4).

A problem that was frequently encountered was that the chemically modified DNA bound irreversibly to the tube during ethanol precipitation so that only 10% could be redissolved (Fig. 9, tracks 1 and 3 versus tracks 2 and 4). The unmodified 36F and 36S have the same mobility on this gel (tracks 1 and 3). This may be due to the conformation the fragment adopts in 2 x loading buffer A. Chemical modification of 36F changes the mobility of the fragment while there is no effect with modified 36S. This could be explained by the distribution of bases susceptible to nitrous acid on the two strands. The sequence of the fragment (Sutcliffe 1979) indicates that one strand has 14 susceptible bases while the other has 22 susceptible bases.

CHEMICAL MODIFICATION OF LABELLED
FRAGMENTS

FIG 9



36F

36F*

36S

36S*

*=Chemically modified

See 3.5.5

CHAPTER 4 : PREPARATION OF RecA PROTEIN

4.1 INTRODUCTION

RecA protein (MW40,000) has been isolated from a number of *E.coli* strains that constitutively overproduce the protein from the RecA gene cloned into a multicopy plasmid (Weinstock et al 1979, Kuramitsu et al 1981, Cox et al 1981, Emmerson et al 1979, Hickson et al 1981, Cotterill et al 1982). Continuous expression was achieved by the *lexA* (*spr*) mutation that inactivates *lexA* protein, the repressor of the RecA gene.

The production of RecA is part of the SOS response to DNA damage and inhibition of DNA replication (Little and Mount 1982). The SOS response also involves enhanced capacity for DNA repair, mutagenesis and filamentation. The inducing signal (possibly gapped or fragmented DNA) activates low levels of constitutive RecA protein to become a protease that site specifically cleaves *lexA* protein. The effect is to depress the transcription of about ten genes including RecA resulting in several thousand monomers of RecA protein per cell (Weinstock et al 1979). The gene dose effect of RecA cloned into a multicopy plasmid pushes the expression levels up to 20%-30% of the total cell protein (Cotterill et al 1982).

RecA protein was first purified in this study using strain N1462 freshly transformed with plasmid pRG3 carrying the RecA gene, a generous gift of D. Bramhill of Department of Biochemistry, University of Newcastle-upon-Tyne. The protein was purified by phosphocellulose P11 and DE-52 chromatography to apparent purity by SDS-acrylamide gel electrophoresis (Kuramitsu et al 1981).

Two problems were encountered in purifying RecA protein: The ion exchange chromatography failed to eliminate a DNA nicking activity and overproduction of RecA protein from N1462-pRG3 was very unstable. To prepare RecA protein free of DNA nicking activity another purification route was adopted. The protein was selectively precipitated by a modification of the protocol used by Cotterill

et al 1982. The active RecA protein was then purified further by affinity chromatography (Cox et al 1981). To obtain reproducible high expression levels of RecA protein, I constructed a new overproducing strain by transforming the *lexA* temperature sensitive strain DM936 with pRG3.

4.2 RecA PROTEIN PREPARATION FROM N1462-pRG3

N1462, on *E.coli* strain with an (*spr*) inactivated *lexA* gene and a deleted RecA gene was transformed with pRG3, a multicopy plasmid carrying an ampicillin resistance gene and the RecA gene. Initial attempts failed to detect the overproduction of an MW40,000 protein in the cell lysates of a freshly transformed culture. RecA protein overproduction was identified on SDS-acrylamide gels by reducing the time between transformation and cell lysis to 48 hours. The MW40,000 band represented 10% of the soluble protein on the gel.

Starting from a nalidixic acid induced culture the protein was purified by PolyminP precipitation, ammonium sulphate fractionation, phosphocellulose P11 chromatography and finally DE-52 chromatography (Kuramitsu et al 1981). At the end of these steps the protein ran as a single band of 40,000 molecular weight on an SDS-acrylamide gel. Unfortunately the preparation possessed a nicking activity that converted supercoiled pBR322 to nicked circular plasmid. The level of nicking was lower when the protein was assayed in the presence of ATP. An explanation could be that RecA protein was binding to the double-stranded DNA in the presence of ATP and protecting it from degradation. (Fig. 10)

The transformation of N1462 with pRG3 was repeated to make more RecA protein. This time no overproduction of RecA protein was detected after 48 hours cell growth. To test the viability of the RecA gene cloned in pRG3 in this culture, the plasmid DNA was isolated by CsCl/ethidium bromide centrifugation and used to transform a fresh culture of N1462 to ampicillin resistance. A parallel culture of N1462 was transformed with the original pRG3 DNA. After only 15 hours growth the cultures were lysed and the lysates

analysed on a SDS-acrylamide gel. This time RecA protein overproduction was detected in the culture transformed by the original pRG3 DNA but not in the culture transformed with the freshly prepared pRG3. This indicated that the cloned RecA gene in plasmid pRG3 was stable for less than 48 hours at 37°C in N1462.

4.3 CONSTRUCTION OF A STABLE OVERPRODUCING RecA PROTEIN STRAIN

RecA protein was continuously expressed from the RecA gene in pRG3 in strain N1462 because *lexA*, the repressor of the RecA gene was inactivated by the *spr* mutation. The metabolic stress produced by this continuous production would strongly favour any modification to the cell that blocked the RecA synthesis. The RecA protein could have been responsible for DNA rearrangements that inhibited its own synthesis. For these reasons, I tried to construct an inducible RecA overproducing strain that could be propagated with the RecA gene repressed.

LexA protein is the repressor of the RecA gene (Brent and Ptashne 1981). The *lexA3* mutation makes *lexA* protein insensitive to cleavage by activated RecA protein and hence permanently represses the RecA gene (Mount et al 1972; Little and Harper 1979). The introduction of a temperature sensitive (*ts1*) mutation in the *lexA* gene makes the *lexA3* protein heat sensitive (Mount et al 1973). By raising the temperature to 42°C the *lexA3 ts1* protein is inactivated and RecA protein is expressed. Thus, to propagate the strain, cells are transformed and grown at 30°C. To isolate the protein the temperature is raised to 42°C inactivating the repressor allowing transcription of the RecA gene. N. Sullivan of this department generously provided DM936 a RecA⁻ *E. coli* strain with the *lexA3* and *ts1* mutations (Mount et al 1975).

DM936 was transformed with pRG3 to give ampicillin resistant RH1. After 20 hours growth at 30°C the culture was split and half was incubated at 42°C. Both cultures had overproduced RecA protein to about 90% of the total cell protein by SDS-acrylamide electrophoresis. RH1 still overproduced RecA protein after 100

hours growth at 30°C and 6 months storage at 4°C. The unexpected expression at 30°C could have been due to the multiple copies of the RecA operator litrating out the active *lexA3* repressor (McEntee 1977). The stability of the strain may have been the result of growing the cells at 30°C.

RH1 was grown to mid log at 30°C then incubated at 42°C with nalidixic acid. Cells lysates were analysed on an overloaded SDS-acrylamide gel after partial (fig.11a) and complete destaining (Fig.11b). RecA protein was overproduced to similar levels at 30°C and 42°C (Tracks 3 and 4). An MW50,000 band in the cells grown at 30°C (Track 3) disappeared when the temperature was raised to 42°C. This may have been a dimer of *lexA3* *tsl* protein. The addition of nalidixic acid doubled the amount of RecA protein expressed (Track 5). Expression in cells grown at 42°C with nalidixic acid resulted in at least 90% of the total soluble protein being RecA protein (Track 5, Fig. 11b).

Nalidixic acid is thought to induce RecA protein synthesis by inhibiting DNA gyrase which is necessary for DNA replication (Cozzarelli 1980). Replication intermediates are then broken down by RecBC DNAase to produce DNA fragments that activate RecA protein to cleave *lexA* protein, depressing RecA transcription (Little and Mount 1982). In RH1, *lexA* protein is protease insensitive and at 42°C is already inactive. Therefore the nalidixic acid induction must be via another route.

4.4 PURIFICATION OF RecA PROTEIN FROM DM936-pRG3

RecA protein was purified from the nalidixic acid induced culture at 42°C by a modification of the RecA polymerisation method (Cotterill et al 1982), followed by single-stranded DNA cellulose affinity chromatography (Cox et al 1981). RH1 was lysed, RecA protein and DNA were precipitated from the lysate with polymin P leaving behind most of the contaminating proteins in the supernatant (Fig.11a, Track 6). RecA protein was extracted from the pellet with a dilute ammonium sulphate solution then reprecipitated with high

concentrations of ammonium sulphate (Track 7). The pellet was redissolved in a minimum volume of phosphate buffer and dialysed for 3 days against a large volume of the same buffer. Most of the impurities stayed in solution while the RecA protein polymerised and precipitated as a dense white solid (Track 11). This step did not require magnesium ions to precipitate the RecA protein. The RecA precipitate was redissolved in a small volume of phosphate buffer containing 1M NaCl, the solution was applied to a G-75 gel filtration column and RecA oligomers were collected from the void volume. This step removes the low molecular weight factors that precipitate the protein. The RecA protein solution was then passed through a phosphocellulose P11 column to remove any remaining polymin P. At this point the RecA protein appeared pure (Track 12). Unfortunately the RecA protein preparation still extensively nicked supercoiled pBR322.

The final purification step was based on RecA protein's affinity for single-stranded DNA and its dissociation in the presence of ATP. The RecA protein was applied to a single-stranded DNA cellulose column (Alberts and Herrick 1971). Most of the RecA protein bound to the column. The column was washed with a low salt buffer (Track 13) then RecA protein was elution with 1mM ATP (Track 14). RecA protein was precipitated out of the ATP fractions with ammonium sulphate. The RecA protein free of ATP, was redissolved and the ammonium sulphate was dialysed away.

At the end of this step the RecA protein did not contain any detectable nicking activity on supercoiled pBR322. The advantage of single-stranded DNA cellulose chromatography is that it specifically purifies RecA protein, active in binding to, and ATP-dependent release from, single-stranded DNA.

My purest RecA protein preparations always showed a minor component, moving more rapidly in electrophoresis (Fig. 11). Since the distribution of material between these two bands varied from gel to gel rather than from preparation to preparation, I assume that the minor band is an artefact of electrophoresis or of sample preparation. A standard sample, provided by an experienced group, showed the same pattern (Fig. 11).

FIG 10

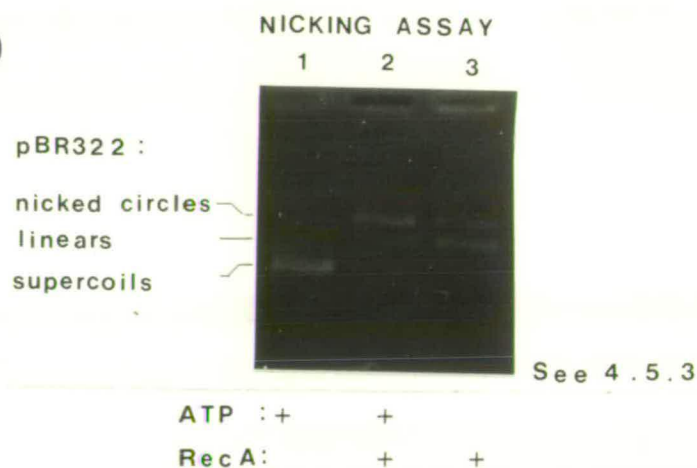


FIG 11a

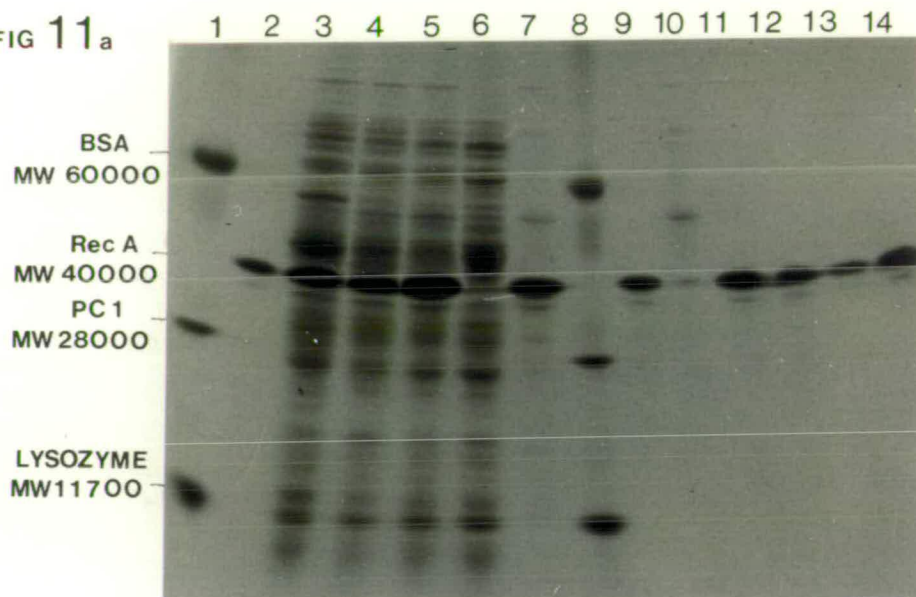


FIG 11b
(11a fully
destained)

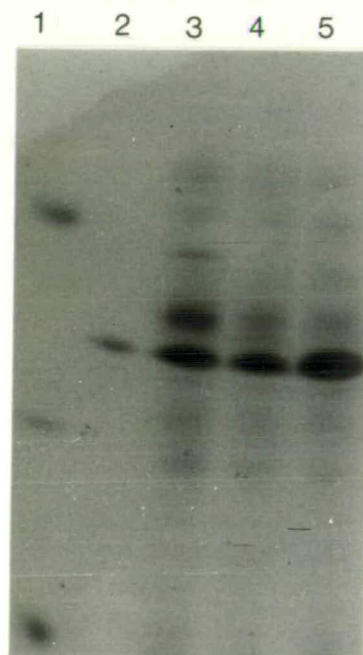


FIG 11a/11b

**PURIFICATION OF RECA
PROTEIN FROM RH1**

- 1 Protein standards
- 2 RecA standard
- 3 Lysate 30°C
- 4 Lysate 42°C
- 5 Lysate 42°C/nalidixic acid
- 6 Polymin-P supernatant
- 7 300 mM (NH₄)₂SO₄ extraction
- 8 Protein standards
- 9 RecA Standard
- 10 Supernatant from P-Buffer dialysis
- 11 Precipitate " " applied
- 12 SSDNA cellulose column: sample
- 13 " " " washings
- 14 " " " protein eluted with ATP buffer

See 4.5.5

4.5 EXPERIMENTAL PROCEDURES

4.5.1 TRANSFORMATION OF N1462 WITH pRG3

See 2.2.5.1 for details. N1462 was grown in L-broth plus 50 micrograms per ml streptomycin. Strain N1462 is described in Cotterill et al 1982.

4.5.2 PURIFICATION OF RecA PROTEIN FROM N1462-pRG3

RecA protein was purified from a 6 litre culture of N1462-pRG3 (10^8 cells/ml). Following a scaled down version of the method used by Kuramitsu, 1981, 6mg of RecA protein pure by SDS-acrylamide chromatography was recovered. The protein was contaminated with a DNA nicking activity.

4.5.3 DNA NICKING ACTIVITY ASSAY

One microlitre of the RecA protein solution was mixed with 50 nanograms of supercoiled pBR322 in 10 microlitres of M buffer: 20mM Tris.HCl pH8.0, 10mM MgCl₂, 1mM Dithio thrietol (DTT) and incubated for 10 minutes at 37°C. A parallel sample was incubated in M buffer plus 1mM ATP. The reaction was terminated by adding 10 microlitres 2 x loading buffer A and heating for 5 minutes at 60°C. The sample was then analysed by 1% agarose gel electrophoresis.

4.5.4 TRANSFORMATION OF DM936 WITH pRG3

DM936 is a lexA temperature sensitive, streptomycin resistant Ecoli K12 strain. DM936: Thr⁻, leu⁻, ProA⁻, RecA⁻ ArgE⁻, thi⁻, ara⁻, leu⁻, gal K⁻, xyl⁻, mtl, tsx33, lambda sup E44; lexA3, lex ts1, rps L31.

A 50ml culture of DM936 was grown to 10^8 cells/ml at 30°C in L-broth plus 50 micrograms per ml streptomycin. Competent DM936 cells were prepared as in 2.2.5.1. Competent DM936 was transformed with pRG3 DNA by adding 100 nanograms pRG3 DNA to 0.1ml cell suspension (10^{10} cells/ml) at 0°C, and incubating for 10 minutes. The culture was heat shocked at 30°C for 10 minutes then diluted with 1ml of

L-broth and incubated for 2 hours at 30°C. Transformants were selectively grown by adding 1 litre of L-broth plus 50 micrograms per ml streptomycin, 25 micrograms per ml ampicillin and incubating at 30°C. After 16 hours the culture reached 10⁸ cells/ml. The culture was stored at 4°C. The Dm936/pRG3 culture still overproduced RecA protein after 6 months storage at 4°C and repeated subculturing at 30°C (100 hours cell growth).

4.5.5 PURIFICATION OF RecA PROTEIN FROM DM936-pRG3

4.5.5.1 STAGE 1 PURIFICATION

Two 500ml cultures of DM936/pRG3 were grown to 10⁸ cells/ml in L-broth plus 50 micrograms per ml streptomycin 25 micrograms per ml ampicillin in shaken 2 litre flasks.

5ml of 4mg/ml nalidixic acid in 100mM NaOH was then added (final concentration 40 micrograms per ml) and incubated for 20 hours at 42°C.

The cells were harvested at 5000 r.p.m. for 5 minutes at 4°C in sorval GS3 rotor. The cells were resuspended in 5ml cold 50mM Tris.HCl pH7.5 25% w/v sucrose. 1ml 0.5M EDTA pH8.0 was added and mixed. 1ml 10mg/ml lysozyme in 0.25M Tris.HCl pH7.5 was added and the suspension was incubated for 10 minutes at 0°C. 8ml 1% w/v triton X-100, 50mM Tris.HCl pH7.5 2mM DTT was added and the suspension was left to lyse at 0°C for 30 minutes. The cell debris was pelleted by spinning at 15,000 r.p.m. for 30 minutes at 4°C in a sorval SS34 rotor.

The supernatant was recovered and PolymixP (10%v/v pH7.5) was added to a final concentration of 0.5% and mixing was continued until an even white precipitate appeared. The precipitate was pelleted by spinning at 15,000 r.p.m. for 10 minutes at 4°C in a sorval SS34 rotor.

The pellet was resuspended in 3ml (half the volume of the lysate) R buffer: 20mM Tris.HCl pH7.5, 10% glycerol, 1mM DTT, 0.1mM EDTA plus 300mM ammonium sulphate. The precipitate was pelleted at 10,000 r.p.m. for 15 minutes at 4°C and discarded.

The supernatant was recovered and RecA protein was precipitated with the addition of 0.4g ammonium sulphate per ml of supernatant and left for 60 minutes at 0°C. The precipitate was pelleted by spinning at 10,000 r.p.m. for 30 minutes at 4°C. The precipitate was dissolved in 2ml P buffer: 20mM K₂PO₄ pH6.8, 10% v/v glycerol, 1mM DTT, 0.1mM EDTA and dialysed for 60 hours against 2 litres of P buffer at 4°C.

The heavy white precipitate of RecA protein that appeared during dialysis was spun down at 10,000 r.p.m. for 30 minutes at 4°C and redissolved in 1ml P buffer plus 1M NaCl.

This solution was applied to a 20ml G-75 column equilibrated in P buffer plus 1M NaCl. The RecA protein eluted in the void volume. The protein containing fractions were applied to a 4ml phosphocellulose P11 column equilibrated in P buffer plus 1M NaCl. The column was washed with 20ml P buffer plus 1M NaCl. 2ml fractions were collected. The RecA protein fractions were pooled (4ml) and dialysed against P buffer. The protein concentration was determined using $A_{280}^{1\%}$ 5.17. 56mg RecA protein, pure by SDS-acrylamide electrophoresis had been recovered from the 1 litre culture of DM936-pRG3.

4.5.5.2 STAGE II PURIFICATION BY AFFINITY CHROMATOGRAPHY

DNA nicking activity was removed by single-stranded DNA cellulose chromatography.

Single-stranded DNA cellulose was prepared as described in Alberts and Herrick 1971 from cellex 410 cellulose and calf thymus DNA.

A 25ml column of SSDNA cellulose was washed with 50ml P buffer at 4°C. The dialysed RecA protein solution (4ml of 14mg/ml RecA protein) was loaded onto the column. The column was washed with 50ml P buffer plus 50mM NaCl, 20ml P buffer plus 50mM NaCl and 1mM ATP, 25ml P buffer plus 50mM NaCl. These buffers were autoclaved before use (ATP was added after the buffers had cooled). 5ml fractions were collected and the protein precipitated by adding 2 grams ammonium sulphate to each tube then incubating at 0°C for 15 hours. 90% of the precipitated protein was in the ATP fractions. 10% appeared in the fractions collected from the first wash of the column with P buffer plus 50mM NaCl. The precipitate from the ATP fractions was pelleted in a bench centrifuge, the supernatant was removed and the pellets were resuspended in steril R buffer plus 0.3g/ml ammonium sulphate. The precipitate was pelleted in a bench centrifuge. The supernatant was discarded and the pellets dissolved with 1ml R buffer and dialysed against 2 litres of R buffer.

The concentration of RecA protein was determined using $A_{280}^{1\%} = 5.17$. 18mg of pure RecA protein free of DNA nicking activity was recovered after affinity chromatography. An equal volume of glycerol was added to the RecA protein solution to give a final concentration of 4mg/ml. The solution was stored in 0.5ml aliquots at -20°C.



5.1 INTRODUCTION

In the presence of RecA protein and ATP, supercoiled DNA takes up homologous single stranded DNA fragments to form displacement loops (D-loops). The single stranded fragments base pair with one strand of the vector displacing the other strand as a single stranded loop (Shibata et al 1979a, Shibata et al 1979b, Cunningham et al 1979, McEntee et al 1979). The rate and extent of the reaction is enhanced by the presence of single stranded DNA binding protein (SSB), (McEntee 1980, Shibata 1980).

D-loops are transiently stable structures in the presence of RecA protein. Apart from D-loops formation RecA protein catalyses a branch migration reaction (Cox and Lehman 1981). Branch migration is a polar reaction in which the displaced strand of the D-loop exchanges with the 5' bound fragment followed by extension of the branch point in a 5'-3' direction with respect to the fragment. (DasGupta and Radding 1982a). The effect of this is to collapse the D-loop and release the fragment.

The rate and extent of branch migration is increased in the presence of SSB (Cox and Lehman 1982, West et al 1982). SSB increases the rate of reaction by stabilising RecA protein-DNA complexes (Cox et al 1983, Soltis and Lehman 1983). RecA protein drives branch migration by binding to double stranded DNA and unwinding the helix to produce positive superhelical turns (Ohtani et al 1982; Shibata et al 1982). As a branch migration is slower than D-loop formation, D-loops accumulate early in the reaction then slowly decay.

In the absence of RecA protein, D-loop formation can only occur with homologous fragments at a temperature sufficient to partly denature the supercoiled double stranded DNA (Beattie et al 1977). The fragment uptake results in the relaxation of negative supercoils, one superhelical turn being relaxed for every 10 base pairs of D-loop formed. As there is no net change in base pairing during homologous D-loop formation the reaction is driven by the net increase

in entropy from the supercoil relaxation. The kinetic barrier to D-loop formation is the activation energy of melting the double stranded DNA. This can be overcome either by raising the temperature or by the addition of RecA protein which couples ATP hydrolysis to this process, in the presence of single stranded DNA.

When approximately equal DNA concentrations of single stranded fragment and supercoiled vector are incubated together in the presence of RecA protein, the formation of D-loops depends on the concentration ratio between single stranded DNA and RecA protein (Shibata et al 1979b). The optimum yield of D-loops occurs when there is one molecule of RecA protein for every 5-10 bases of single stranded DNA. When the double stranded DNA is in excess, D-loop formation is favoured by concentrations of RecA protein greater than one molecule of RecA protein per 20 base pairs of double stranded DNA (Masukata et al 1983).

D-loop formation in the presence of RecA protein can occur with fragments that are not completely homologous to the supercoiled vector (DasGupta and Radding 1982b). D-loops were formed between supercoiled G4 and phiX174 single stranded DNA fragments (and vice versa) even though there was only 67% homology. In the absence of RecA protein D-loop formation with heterologous fragments did not occur. In the presence of RecA protein but in the absence of vector supercoiling, D-loop formation with heterologous fragments was again not observed. The formation of heterologous D-loops had an absolute requirement for a supercoiled vector and RecA protein.

In this study, the first experiments to demonstrate D-loop formation using the conditions described by Shortle et al 1980, were unsuccessful. Initially, D-loop formation was assayed by following the relaxation of the supercoils during fragment uptake. Unfortunately, the sensitivity of the assay was poor. After a long series of experiments, this assay was abandoned in favour of monitoring the uptake of radio labelled fragments by the plasmid.

To detect the formation of complexes between supercoiled pBR322 and either the homologous or the chemically modified 36 base Sau3A fragment, the fragment was end labelled with ^{32}P (see 3.5.4). The complexes were separated on an agarose gel and the radioactive bands located by autoradiography.

A single stranded fragment with an average length of 60 base pairs is the smallest homologous fragment that has been incorporated into a supercoiled vector (Shortle et al 1980). The main problem with producing smaller D-loops is fragment loss due to branch migration. Dissociation of D-loops has been blocked by the addition of cobalt ions to the magnesium containing reaction buffers (Resnick and Sussman 1982). Complexes between supercoiled pBR322 and the 36 base Sau3A fragment were first successfully produced in this study using this cobalt-magnesium buffer (CMP buffer).

In the presence of RecA protein, supercoiled pBR322 and homologous 36 base fragment, two high molecular weight radioactive complexes were detected. One had the expected properties of a D-loop while characterisation of the other, revealed it to be a complex of labelled fragment and single stranded circular pBR322.

A quantitative estimation of the amount of complex formed, was made difficult by radioactive ATP in the labelled fragment preparation.

DNA concentrations in the next two chapters will be expressed as follows : N = DNA concentration on moles of nucleotide per litre, P = DNA concentration in moles of polymer per litre.

5.2 FORMATION OF FRAGMENT-VECTOR COMPLEXES

Complexes were first detected at unexpectedly high RecA concentrations. A fixed concentration of acid-phenol-extracted supercoiled pBR322 (N = 13 micromolar, P = 1.5 nanomolar) and labelled 36F fragment (N = 0.26 micromolar, $P = 7.2 \text{ nanomolar}$) were incubated at 37°C in CMP buffer with increasing concentrations of RecA protein. Two radioactive bands were observed when the reaction was analysed

on an agarose gel (Fig 12). The faster band (complex F) was favoured by lower RecA protein concentrations, the slower band (complex S) was favoured by higher RecA protein concentrations. The optimum RecA protein concentration range was between 1 and 10 molecules of RecA protein per base of single stranded DNA.

If the D-loop reaction depended on the stoichiometry of single stranded DNA to RecA protein, the quantity of RecA protein was between 5 and 50 times more than expected. An explanation could be that the majority of the RecA protein preparation was inactive. This seems unlikely, because the affinity chromatography step in preparing the RecA protein specifically selected for protein active at least in binding to, and ATP-dependent release from, single-stranded DNA.

The two complexes could not be exactly matched to the position of the supercoiled DNA as the ethidium bromide staining of the DNA bands was poor. The sarkosyl detergent that was used for deproteinating the complexes interfered with the ethidium bromide staining and reduced the fluorescence by a factor of 5 (experiments not described here).

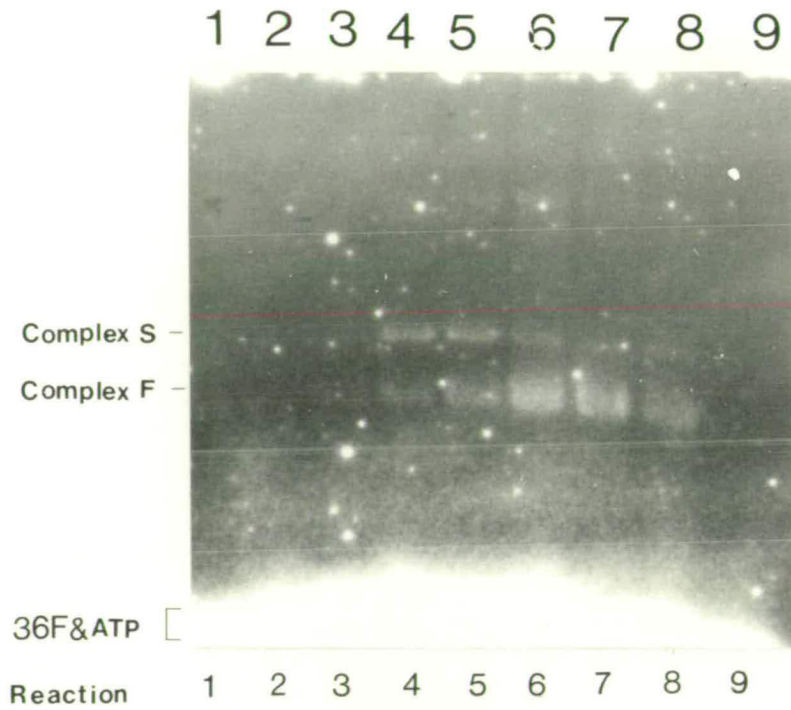
5.3 CHARACTERISATION OF COMPLEX F

Complex F was found to migrate in front of the supercoiled band when the reaction conditions were altered to facilitate detection of ethidium bromide stained DNA. The concentrations of fragment, plasmid, and RecA protein were increased ten-fold (maintaining the molar ratios). This allowed detection of the DNA bands by ethidium bromide staining. The reaction was incubated at 0°C in a modified cobalt-magnesium buffer (CM buffer). These conditions selected for the production of complex F (complex S was not formed).

Complex F was stable for at least 2 hours at 0°C in the presence of RecA protein (Fig. 13). The relative mobility of complex F compared to supercoils and bromophenol blue changed when the gel was run at 4°C rather than 25°C (Fig. 14). Complex F migrated faster at low temperatures.

("e" = ethidium bromide stained DNA)

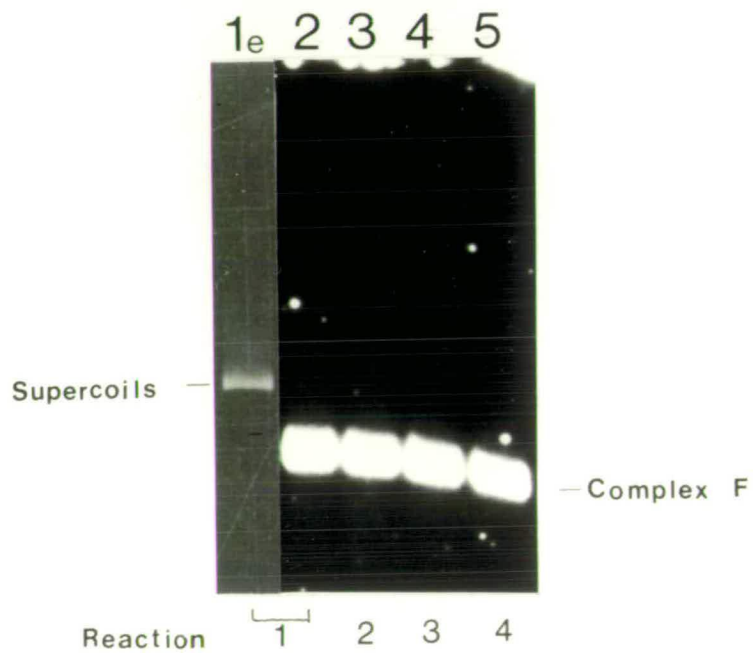
FIG12. GENERATION OF COMPLEX-F AND COMPLEX-S



See 5.4.1

FIG13

SELECTIVE GENERATION OF COMPLEX-F



See 5.4.2

Complex F could be degraded by S1 nuclease (Fig 15, Tracks 3-10). The effect of S1 nuclease digestion was to produce a smear of labelled DNA of higher molecular weight than the original single stranded fragment.

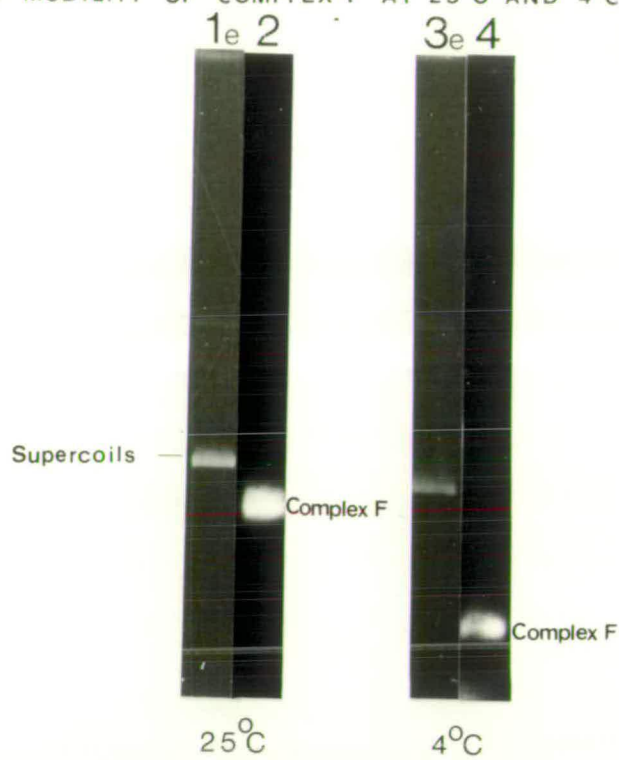
Complex F was insensitive to restriction enzyme digestion by EcoR1 and Ava1, even when the ethidium bromide stained plasmid was fully digested. It was sensitive to Sau3A (Fig. 16).

Complex F was recovered from the gel and the DNA was examined by electron microscopy. The only polymeric material found was single stranded DNA, 10 per cent of which was single stranded circles of similar size to relaxed pBR322 (Fig 17).

The insensitivity to restriction enzymes and the temperature dependent conformation change were consistent with complex F being a single stranded plasmid circle with an annealed labelled fragment. Sau3A sensitivity could be explained by the annealed fragment regenerating a Sau3A site. The low molecular weight labelled smear generated by S1 nuclease degradation was consistent with digestion of a labelled single stranded plasmid.

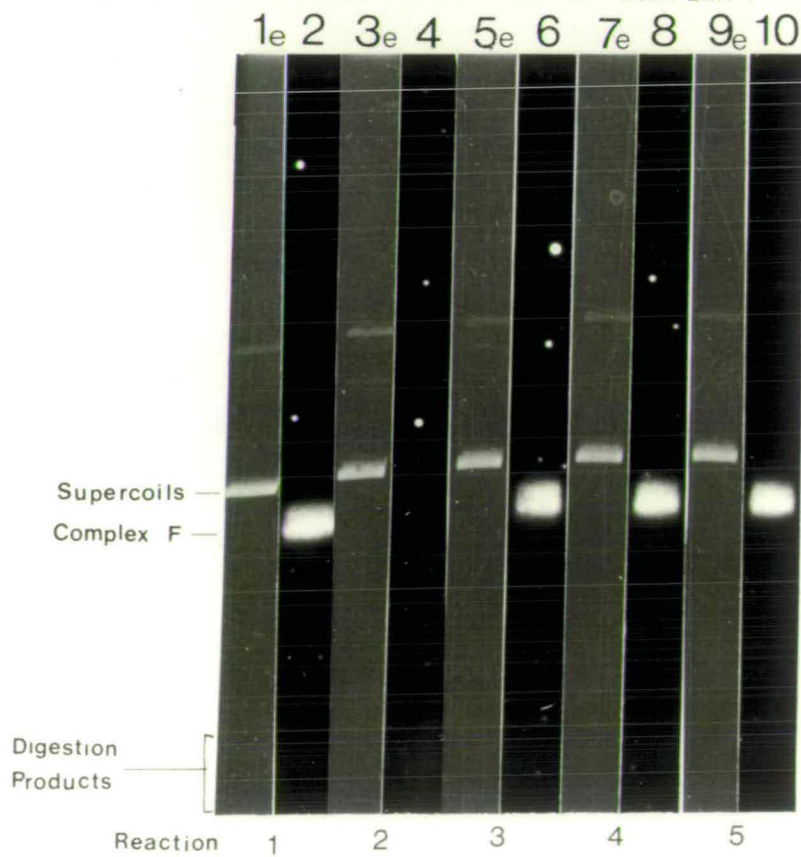
The next question was whether the single stranded plasmid had been generated by the RecA reaction. To test this, supercoiled plasmid DNA generated by acid-phenol extraction was pretreated with S1 nuclease before the RecA incubation. The S1 nuclease was removed by phenol extraction and the supercoils were incubated with the fragment and RecA protein. This time no complex F formed. This experiment revealed that the single stranded circles were present in the supercoil preparation and had not been generated by interaction with the RecA protein. The quantity of this contaminating single stranded DNA was small as it could not be detected by ethidium bromide staining of the plasmid.

FIG14 MOBILITY OF COMPLEX-F AT 25°C AND 4°C



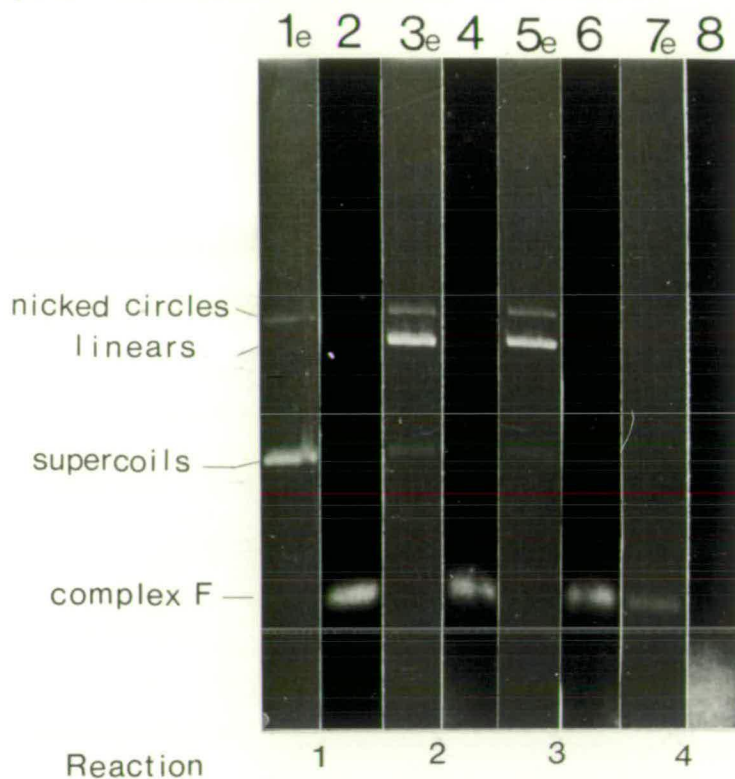
See 5.4.3

FIG15 S1 NUCLEASE DIGESTION OF COMPLEX-F



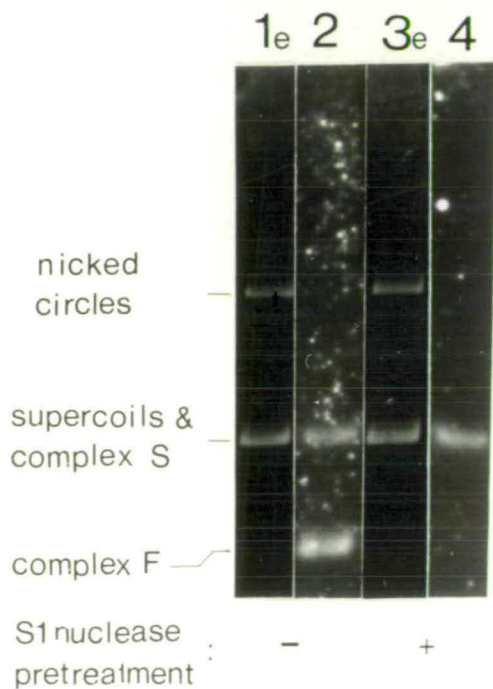
See 5.4.4

FIG16 RESTRICTION ANALYSIS OF COMPLEX F



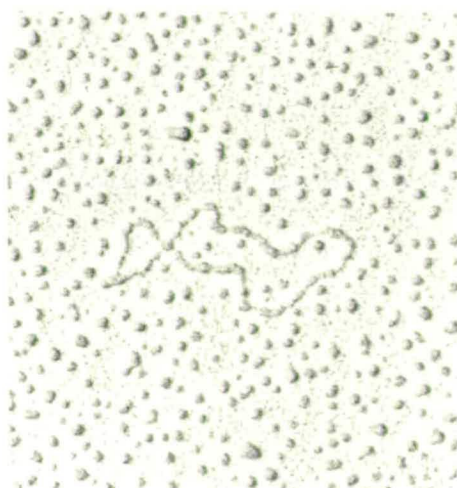
See 5.4.5

FIG18 S1 NUCLEASE
PRETREATMENT OF SUPERCOILS



See 5.4.7

FIG17 COMPLEX-F



See 5.4.6

5.4 EXPERIMENTAL PROCEDURES

5.4.1 GENERATION OF COMPLEX F AND COMPLEX S

Complex F and S were generated in 5 microlitres of CMP buffer : 30mM Tris, 10mM KH_2PO_4 , 20mM CoCl_2 , 10mM MgCl_2 , 2mM ATP adjusted to pH8.0 with HCl. Each reaction contained 4 micrograms per ml supercoiled pBR322 and 0.08 micrograms per ml 36F. RecA protein was added as follows to give final concentrations of:

Reaction;	1	2	3	4	5	6	7	8	9
RecA protein;-	480	240	120	60	30	15	7.5	3.5	micrograms per ml

The reactions were incubated at 37°C for 5 minutes then terminated with one microlitre of 5 x loading buffers (see 2.2.1). The reactions were analysed on an agarose gel (see 2.2.1 and 2.2.4). Fig 12.

5.4.2 SELECTIVE GENERATION OF COMPLEX F

Complex F was selectively generated in 2 microlitres of CM buffer: 30mM Tris. HCl pH8.0, 10mM CoCl_2 , 40mM MgCl_2 , 2mMATP. Each reaction contained 50 micrograms per ml supercoiled pBR322, 1 microgram per ml 36F and 2mg per ml RecA protein. The reactions were incubated at 0°C for the following times:

Reaction;	1	2	3	4
Time;	15	30	60	120 minutes

0.5 microlitres 5 x loading buffer S was added and the reactions were analysed on 1% agarose gels. Fig 13.

5.4.3 MOBILITY OF COMPLEX F AT 4°C. AND 25°C

A 4 microlitre reaction of 50 micrograms per ml pBR322, 1 microgram per ml 36F and 2mg per ml RecA protein in CM buffer was incubated for 15 minutes at 0°C. The reaction was terminated with 1 microlitre of 5 x loading buffer S. 2.5 microlitres were loaded onto a 1% agarose gel and run at 4°C. The other 2.5 microlitres were loaded onto a 1% agarose gel and run at 25°C. Fig. 14.

5.4.4 S1 NUCLEASE DIGESTION OF COMPLEX F

Complex F was generated in 20 microlitres of CM buffer containing 50 micrograms per ml supercoiled pBR322, 1 microgram per ml 36F and 2mg per ml RecA proteins. The reaction was incubated for 15 minutes at 0°C then terminated by the addition of 2.5 microlitres of 5% w/v sarkosyl. The reaction was extracted with 20 microlitres of phenol equilibrated in CM buffer, to remove the denatured RecA protein. The phases were separated by centrifugation. The supernatant was extracted twice with 0.1ml ether to remove this phenol. These steps generated a solution of protein-free complex F.

3 microlitres of 5M NaCl and 3 microlitres of 0.5M NaAc pH5.0 were added and the solution was made up to 30 microlitres with water. The solution was split into 5 fractions and S1 nuclease was added as follows:

Reaction;	1	2	3	4	5
S1 nuclease;	-	40	4	0.4	0.04

units added in one microlitre 0.5M NaCl, 50mM NaAc pH5.0.

The reactions were incubated at 0°C for 30 minutes then terminated by adding 3 microlitres of 2x loading buffer A. The reactions were immediately analysed on a 1% agarose gel.

5.4.5 RESTRICTION ANALYSIS OF COMPLEX F

Protein free complex F was produced as in 5.4.4. The solution was split into four fractions. Restriction enzymes were added as follows:

Reaction;	1	2	3	4
Enzyme	-	2 units EcoR1	2 units Aval	2 units Sau3A

The reactions were incubated at 20°C for 15 minutes then terminated with 5 microlitres of 2 x loading buffer A. The reactions were analysed on a 1% agarose gel run at 4°C. Fig. 16.

5.4.6 PREPARATION OF COMPLEX F FOR ELECTRON MICROSCOPY

Protein free complex F was produced as in 5.4.4 then mixed with 5 microlitres of 5 x loading buffer S. The solution was loaded onto a single 1cm x 0.15cm slot in a 1% agarose gel and electrophorised. The radioactive band was located by autoradiography of the wet gel wrapped in saran wrap. The band was cut out and the DNA was recovered by electroelution onto DE-52 and ethanol precipitated. The DNA pellet was dissolved in 10mM Tris.HCl pH8.0, 1mM EDTA. P. Beattie of this department mounted and photographed the sample under the electron microscope. Fig. 17.

5.4.7 S1 NUCLEASE PRETREATMENT OF SUPERCOILED pBR322

100 nanograms of supercoiled pBR322 (acid-phenol extracted) was incubated with 40 units of S1 nuclease in 15 microlitres of 0.5M NaCl, 50mM NaAc pH5.0, 10mM MgCl₂ at 0°C for 15 minutes. The reaction was adjusted to basic pH with a tenth volume of 1M Tris.HCl pH8.6 and extracted with 20 microlitres phenol. The phenol was removed by two 0.1ml ether extractions. 2 nanograms of 36F were added and the solution was ethanol precipitated. The DNA was redissolved in one microlitre of 2 x CM buffer and one microlitre of 4mg/ml RecA protein was added and the reaction incubated at 37°C for 5 minutes. The reaction was terminated with the addition of 0.5 microlitres 5 x loading buffer S and loaded onto a 1% agarose gel. A parallel reaction using untreated supercoiled DNA was also loaded onto the gel. Fig. 18.

6.1 INTRODUCTION

In the previous chapter, two labelled complexes (complex F and complex S) were identified from the analysis of the reaction between the labelled single stranded fragment (36F) and supercoiled pBR322 in the presence of RecA protein. Complex F was characterised as single stranded plasmid which had picked up the labelled fragment. The single stranded plasmid was present in low concentrations in the plasmid preparation.

In the experiments described in this chapter, complex S was selectively produced by incubating the mixture of supercoils and fragment in the presence of higher concentrations of RecA protein. The low levels of complex F formed in the experiments varied between batches of acid phenol extracted DNA. In CMP buffer and in the presence of RecA protein, the complex S concentration reached a peak, after 2 minutes and then decayed. Complex S also comigrated with the supercoil band. These two facts made complex S a good candidate for a D-looped plasmid.

Complex S could also be generated with chemically modified 36F in the presence of RecA protein in CMP buffer. The complex concentration reached a peak after 1 minute with chemically modified 36F.

Production of complex S was more extensive in M buffer, a buffer containing magnesium ions but no cobalt ions. The peak concentration of complex S occurred after 10 minutes incubation. A number of incubations in M buffer, containing chemically modified 36F, pBR322 and RecA, failed to generate complex S. Cobalt ions appeared to be necessary for heterologous D-loop formation with small fragments.

When supercoils of pBR322, that had not been acid phenol extracted, were used in the RecA reaction, no complex F was produced. This identified the acid phenol extraction step as the source of single stranded plasmid DNA.

Single stranded DNA binding protein (SSB) has been reported to increase the rate and extent of D-loop formation (see 5.1). When SSB was added to the reaction after preincubation with RecA protein, the yield of complex S was unexpectedly reduced.

6.2 PRODUCTION OF COMPLEX S IN CMP BUFFER

Formation of the complex S could be selectively enhanced by increasing the RecA protein concentration. In a reaction in CMP buffer containing acid-phenol extracted supercoiled pBR322 (N = 13 micromolar, P = 1.5 nanomolar) and labelled 36F (N = 0.26 micromolar, P = 7.2 nanomolar), only complex S was produced when the RecA protein concentration was increased to 24 molecules of RecA protein per base of single stranded DNA (6.25 micromolar). The concentration of complex S decreased after two minutes incubation at 37°C. Fig. 19.

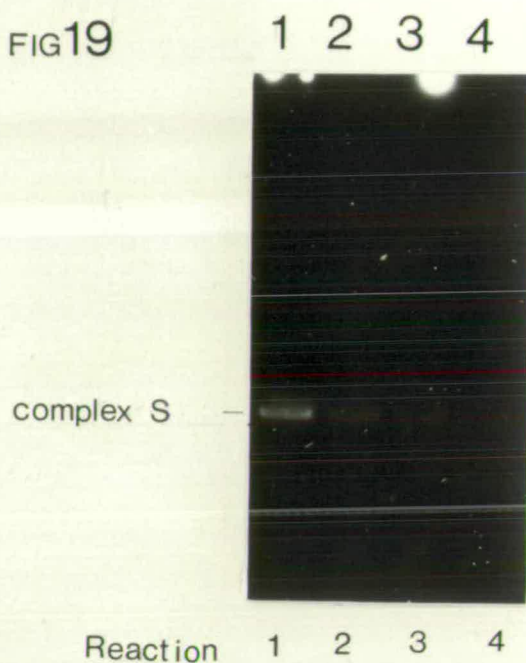
To facilitate the location of the supercoiled band, the DNA concentration was increased 12 fold while maintaining the above ratio of 36F to plasmid. RecA protein was added to a concentration of 2 molecules of RecA protein per base of single stranded DNA (6.25 micromolar). The reaction was incubated at 37°C in CMP buffer. Complex S was found to migrate at the same location as the supercoiled plasmid. The concentration of complex S increased for 2 minutes then decayed. Fig. 20.

The concentration of complex S formed in the reaction was proportional to the RecA protein concentration when the reaction was incubated for 2 minutes at 37°C. The maximum observed concentration of complex occurred at a RecA protein concentration of 50 micromolar. Fig. 21.

Complex S could also be generated with chemically modified 36F using the reaction conditions of the previous experiment. The reaction contained chemically modified 36F (N = 3.2 micromolar, P = 90 nanomolar) supercoiled pBR322 (N = 162 micromolar, P = 18.6 nanomolar) and RecA protein (50 micromolar). The reaction was incubated at 37°C in CMP buffer. This time complex S formed, but the concentration decreased steadily after one minute at 37°C. Fig. 22.

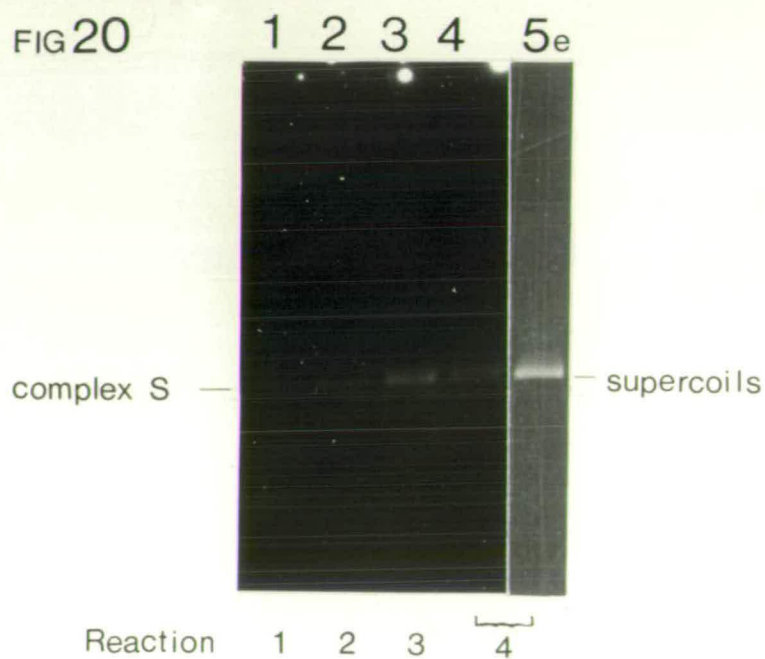
THE TIME COURSE OF COMPLEX-S FORMATION IN C.M.P BUFFER

FIG19



See 6.5.1.1

FIG20



See 6.5.1.2

FIG 21 DEPENDENCE OF
COMPLEX-S ON RECA
PROTEIN
CONCENTRATION

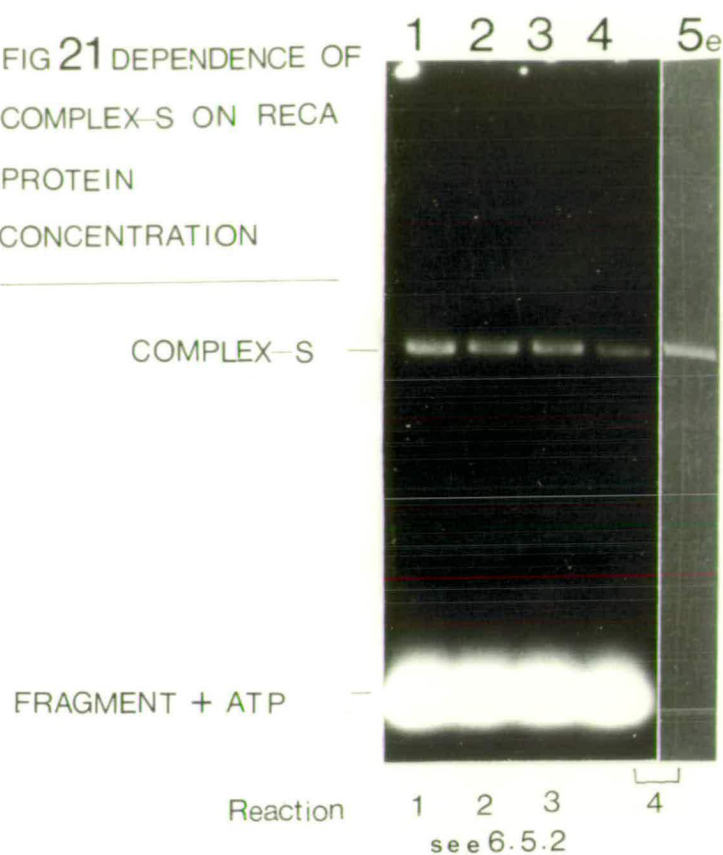
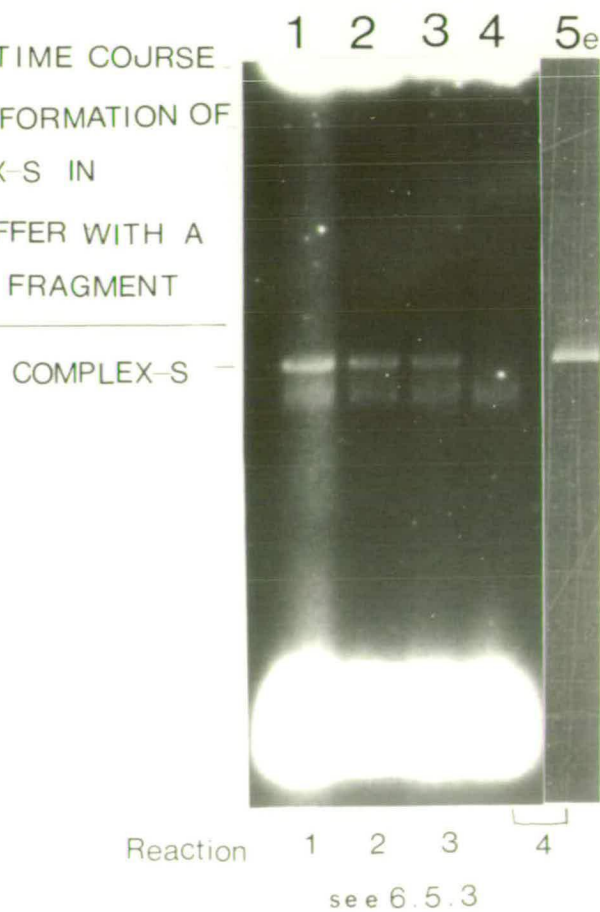


FIG 22 TIME COURSE
OF THE FORMATION OF
COMPLEX-S IN
CMP BUFFER WITH A
MUTANT FRAGMENT



6.3 PRODUCTION OF COMPLEX S IN M BUFFER

The life time of complex S could be increased by incubating the reaction in M buffer, a buffer that contained magnesium but not cobalt. The reaction contained ^{36}F ($N = 6.2$ micromolar, $P = 180$ nanomolar) supercoiled pBR322 ($N = 162$ micromolar, $P = 18.6$ nanomolar) and RecA protein (50 micromolar). The reaction was incubated at 37°C . The concentration of complex S increased for 10 minutes then decreased and finally disappeared after 30 minutes.^{Fig 23} The complex F concentration increased during the first 10 minutes of the reaction then remained stable for 60 minutes. Fig. 24.

When the reaction was incubated at 37°C for 10 minutes and the concentration of RecA protein was varied, the concentration of complex S varied in proportion. The maximum observed concentration of complex S occurred in the reaction containing 50 micromolar RecA protein. Fig. 25a.

Contaminating single stranded plasmid was responsible for the production of complex F. A parallel experiment was performed with pBR322 direct from the CsCl gradient purification to find out if the acid phenol extraction was the source of the single stranded DNA. This time no complex F was detected. Fig. 25b. Acid phenol extraction had been generating the contamination by denaturing the plasmid DNA. Twice the amount of complex S was produced (compared to the previous reaction), for a given concentration of RecA protein. This could be explained by the small fragment not having to compete with single stranded plasmid for the RecA protein.

SSB (single stranded DNA binding protein) increases the rate and extent of D-loop formation with a heterogenous mixture of homologous single stranded fragments (see 5.1). The optimum stoichiometry is one monomer SSB to 8 bases of single stranded DNA. To test whether SSB would enhance D-loop formation with the small ^{36}F fragment, increasing concentrations of SSB were added to the reaction after a brief preincubation with RecA protein. The reaction was incubated for 5 minutes at 37°C . SSB lowered the amount of complex S formed by a factor of 5. Fig. 26.

TIME COURSE OF COMPLEX-S FORMATION AT 37°C & 0°C

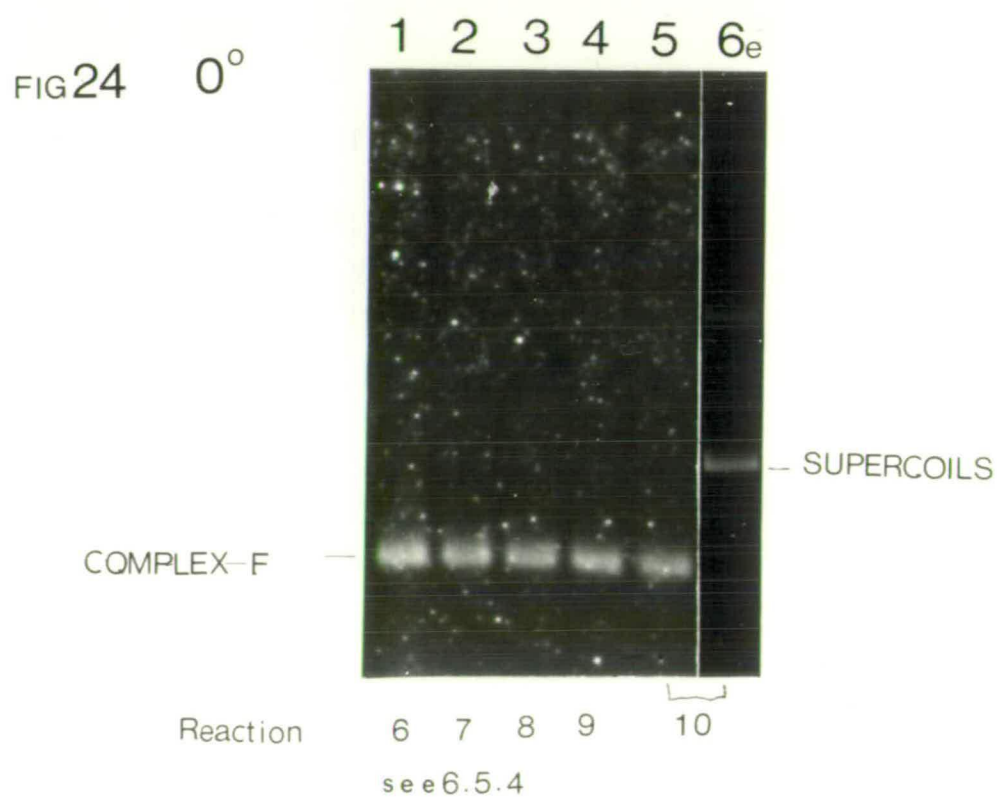
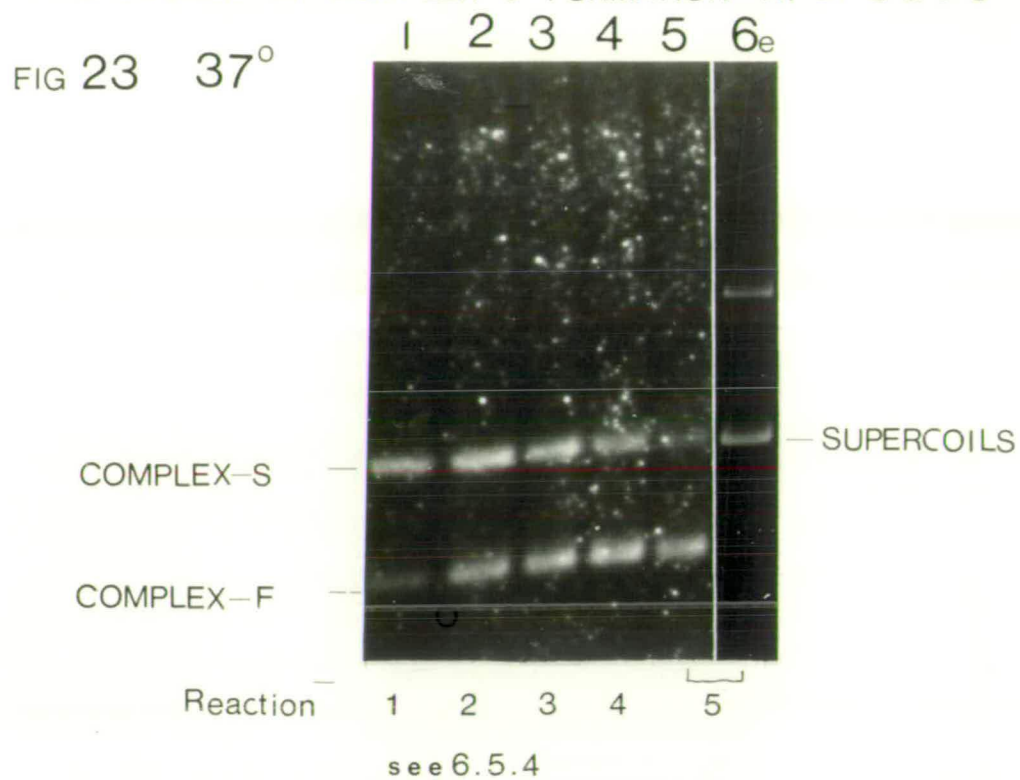
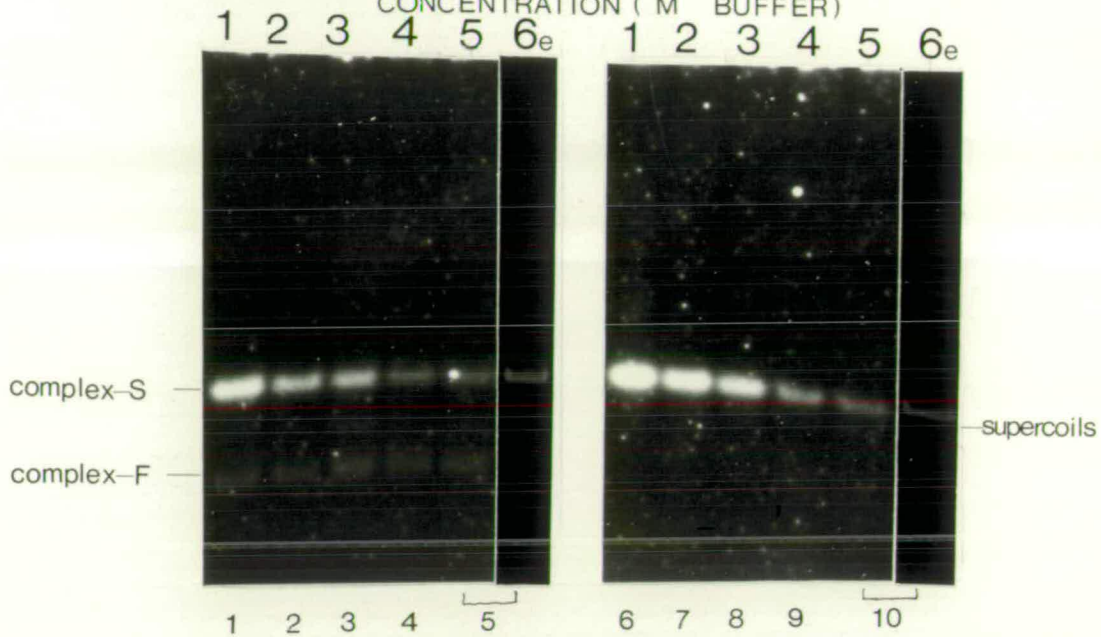
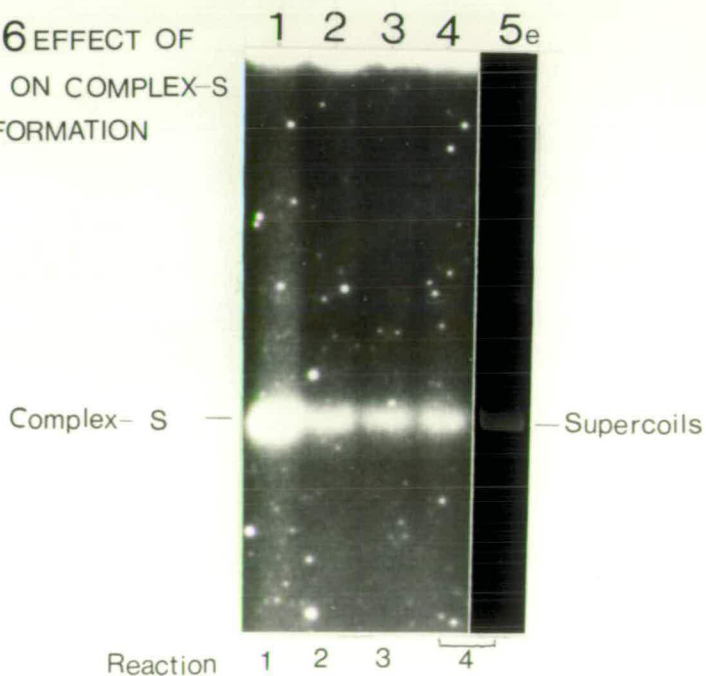


FIG 25_{a/b} DEPENDENCE OF COMPLEX-S ON RECA PROTEIN
CONCENTRATION (M BUFFER)



see 6.5.5

FIG 26 EFFECT OF
SSB ON COMPLEX-S
FORMATION



see 6.5.6

6.4 DISCUSSION

Complex S had two of the expected properties of a plasmid carrying a small D-loop. It was transiently stable and it comigrated with supercoils. Complex S was unusual in that its formation was favoured by very high relative concentrations of RecA protein to DNA. The amount of RecA protein that produced maximum complex S formation was sufficient to coat every base pair of the double stranded DNA in the reaction. In these experiments, the DNA concentration of single stranded DNA was one tenth of the double stranded DNA. An explanation for the unusual stoichiometry could be that the excess double stranded DNA was competing with the single stranded fragment for RecA protein.

The detrimental effect of SSB on complex S formation could be explained by accelerated branch migration speeding the decay of the complex.

To generate a D-loop containing a modified fragment, the cobalt containing CMP buffer had to be used. The effect of cobalt on heterologous complex formation requires further investigation. To test the subsequent biochemical steps of the mutagenesis procedure I used homologous D-loops generated in M buffer as these were stable for longer than the D-loops generated in CMP buffer.

6.5 EXPERIMENTAL PROCEDURES

6.5.1 TIME COURSE OF COMPLEX S FORMATION IN CMP BUFFER

EXPERIMENT 1

Complex S was generated in 5 microlitres of CMP buffer. Each reaction contained 4 micrograms per ml supercoiled pBR322, 0.08 micrograms per ml 36F and 250 micrograms per ml RecA protein. The reactions were incubated at 37°C for the following times:

Reaction;	1	2	3	4
Time;	2	5	10	15 minutes

1 microlitre of 5 x loading buffer S was added and the reactions were analysed on 1% agarose gels. Fig. 19.

EXPERIMENT 2

Complex S was generated in 2 microlitres of CMP buffer. Each reaction contained 50 micrograms per ml supercoiled pBR322, 1 microgram per ml 36F and 250 micrograms per ml RecA protein. The reactions were incubated at 37°C for the following times:

Reaction;	1	2	3	4
Time;	0.5	1	2	5 minutes

0.5 microlitres of 5 x loading buffer S was added and the reactions were analysed on a 1% agarose gel. Fig. 20.

6.5.2 RecA PROTEIN CONCENTRATION DEPENDENCE OF COMPLEX S IN CMP BUFFER

Complex S was generated in 2 microlitres CMP buffer. Each reaction contained 50 micrograms per ml supercoiled pBR322, 1 microgram per ml 36F and 250 micrograms per ml RecA protein. The reactions were incubated at 37°C for 2 minutes with the following RecA protein concentrations:

Reaction;	1	2	3	4
RecA protein;	2	1	0.5	0.25 mg/ml
concentration.				

0.5 microlitres of 5 x loading buffer S was added and the reactions were analysed on a 1% agarose gel. Fig. 21.

6.5.3 TIME COURSE OF COMPLEX S FORMATION IN CMP BUFFER WITH CHEMICALLY MODIFIED 36F

Complex S was generated in 2 microlitres CMP buffer - Each reaction contained 50 micrograms per ml supercoiled pBR322, 2mg/ml RecA protein and 1 microgram per ml chemically modified 36F (see 2.5.1). The reactions were incubated at 37°C for the following times:

Reaction;	1	2	3	4
Time;	1	2	3	4 minutes

0.5 microlitres of 5 x loading buffer S was added and the reactions were analysed on a 1% agarose gel. Fig. 22.

6.5.4 TIME COURSE OF COMPLEX S FORMATION AT 37°C AND 0°C IN M BUFFER

Complex S was generated in 2 microlitres M buffer: 20mM Tris. HCl pH8.0, 10mM MgCl₂, 1mMDTT, 1.3mMATP; containing 50 micrograms per ml supercoiled pBR322, 2 micrograms per ml 36F and 2mg/ml RecA protein:

Reactions 1-5 (incubated at 37°C) and Reactions 6-10

(incubated at 0°C) were incubated for the following times:

Reaction;	1	2	3	4	5	6	7	8	9	10
Time;	5	10	15	20	25	10	20	30	40	60
										minutes.

0.5 microlitres of 5 x loading buffer S was added, and the reactions were analysed on a 1% agarose gel. Fig 23/24.

6.5.5 RecA PROTEIN CONCENTRATION DEPENDENCE OF COMPLEX S IN M BUFFER

Complex S was generated in 2 microlitres M buffer containing 2 micrograms per ml 36F, and 50 micrograms per ml of either acid-phenol-extracted pBR322 (1-5) or pBR322 direct from CsCl gradient purification (6-10). The reactions were incubated for 10 minutes at 37°C with the following RecA concentrations:

Reaction;	1	2	3	4	5	6	7	8	9	10
RecA protein	2	1	0.5	0.25	0.125	2	1	0.5	0.25	0.125
Concentration										mg/ml

0.5 microlitres of 5 x loading buffer S was added and the reactions were analysed on a 1% agarose gel. Fig. 25a/25b.

6.5.6 EFFECT OF SSB ON COMPLEX S FORMATION

Complex S was generated in 4 microlitres M buffer containing 2 micrograms per ml 36F, 50 micrograms per ml supercoiled pBR322 (not acid-phenol-extracted), 2mg/ml RecA protein. The reactions were incubated for 30 seconds at 37°C then incubated with the following SSB concentrations:

Reaction;	1	2	3	4
SSB;	0	0.025	0.05	0.1 mg/ml SSB
Concentration.				

(SSB was heated at 95°C for 5 minutes to destroy contaminating nuclease activity). 1mg/ml SSB = 50 micromolar. One microlitre of 5 x loading buffer S was added and the reactions were analysed on a 1% agarose gel.

6.5.7 All reactions containing 2 micrograms per ml labelled ³²P fragment were adjusted to 10mM ATP before electrophoresis to reduce the background radiation on the gel from the contaminating labelled ATP.

7.1 INTRODUCTION

The next step in the formation of a recombinant molecule, was to remove the single stranded displacement loop in complex S with S1 nuclease and to ligate the ends of the fragment with the cut ends of the D-loop. In the presence of RecA protein, D-loops have been cut with S1 nuclease (Shortle et al 1980). Unfortunately, this resulted in strand exchange and the collapse of the D-loop to leave a nicked circular plasmid and a displaced fragment. D-loops formed in the absence of RecA protein, have been cut with S1 nuclease to generate a nicked circular vector without loss of the fragment (Radding et al 1977).

As soon as the displaced strand was broken, the supercoils relaxed and loss of the fragment became energetically favourable. The conditions for cutting the D-loops were a balance between under digestion, which generated an unstable relaxed intermediate which ejected the fragment, and over digestion, which cut the displaced strand back to generate single stranded gaps which on further digestion resulted in linearisation of the plasmid (Green and Tibbetts 1980).

The vector, with nicks at each end of the introduced fragment, was then converted to a covalently closed vector by ligation of the cut ends. One end of a 240 base homologous fragment in a double nicked vector, generated by S1 nuclease conversion of a D-loop, has been successfully ligated (Radding et al 1977). The failure to produce a fully covalently closed vector may have been the result of a single stranded gap at one end of the fragment.

In the present study complex S was deproteinated and treated with S1 nuclease. The double nicked vector carrying the fragment could only be produced over a narrow range of buffer conditions and enzyme concentrations. The covalently closed vector was finally produced in a concerted process involving the cutting of the D-loops and the ligation of the fragment in the same reaction.

The location of the fragment in the vector was identified by cutting the vector into fragments of defined size with restriction enzymes followed by autoradiography of the electrophoresed DNA.

As the ratio of complex S to unmodified supercoiled plasmid was low, an *in vitro* selection system developed. High concentrations of S1 nuclease were used to eliminate the supercoiled molecules.

Between the c-terminus of the beta-lactamase gene and the origin of replication of pBR322 are three S1-nuclease sensitive sites which can only be cut if the plasmid is supercoiled (Lilley 1980, Lilley 1981). The sites are small single stranded loops that appear transiently as flanking palindromic sequences form cruciform structures. Relaxed circles are insensitive to S1 as cruciform production is energetically unfavourable. When a mixture of supercoiled and covalently closed relaxed circular (CCRC) plasmids are treated with high concentrations of S1 nuclease only the supercoils are converted to blunt ended linear molecules. The blunt ended linear molecules transform cells very inefficiently.

7.2 DEPROTEINATION OF COMPLEX S

Before complex S could be treated with S1 nuclease, the RecA protein had to be removed. Complex F could be deproteinated by treatment with Sarkosyl (a non-ionic detergent) followed by phenol extraction. When sarkosyl treated complex S was phenol-extracted, the labelled fragment was lost. Complex S was successfully deproteinated by the addition of sarkosyl followed by an extraction with a mixture (1:1 v/v) of chloroform and phenol. The chloroform reduced the concentration of phenol in the aqueous phase. RecA protein precipitated at the interface when the phases were separated by centrifugation.

7.3 CONVERSION OF COMPLEX S TO CCRC PLASMID

Incubation of complex S with S1 nuclease in a low pH and high salt buffer containing zinc ions, converted the D-looped plasmid to nicked circles. Unfortunately, more than half the complexes lost the fragment. Conversion of complex S to nicked circular plasmid without fragment loss was achieved by manipulation of the buffer conditions. Optimum conversion occurred with incubations in a neutral pH, low salt buffer containing zinc ions. With these conditions, some complex S was converted to labelled linear molecules. The incubation had no effect on the supercoiled plasmid that had not picked up the fragment. Fig. 27.

Treatment of these nicked circles with T4 ligase failed to produce covalently closed relaxed circular (CCRC) plasmid. CCRC plasmid was assayed on agarose gels run in the electrophoresis buffer containing ethidium bromide. In the presence of ethidium bromide CCRC plasmid positively supercoiled and migrated in front of the negative supercoiled plasmid band (Shortle et al 1982).

The failure of ligase to form CCRC plasmid could have been due to the presence of short gaps at the ends of the fragment. In an attempt to overcome this problem, ligase was added to the S1 incubation in the hope that it would ligate the nicks before they were converted to gaps by the S1 incubation. Unfortunately, zinc the cofactor of the S1 nuclease reaction, inhibited the T4 ligase. The T4 ligase activity was assayed by monitoring the production of a spectrum of partially supercoiled plasmid from the combined action of T4 ligase and a contaminating nicking activity on supercoiled pBR322. Fig. 28.

The S1 incubation in the presence of T4 ligase was repeated in a buffer lacking zinc ions. There was only partial conversion of complex S to nicked circles. No CCRC plasmid was detected. The S1 nuclease concentration was increased to push the reaction to completion. T4 ligase and the klenow fragment of E.coli Pol1 and dNTPs were added to the same reaction to fill in and seal the

FIG 27 CONVERSION OF
COMPLEX-S TO NICKED
CIRCLES

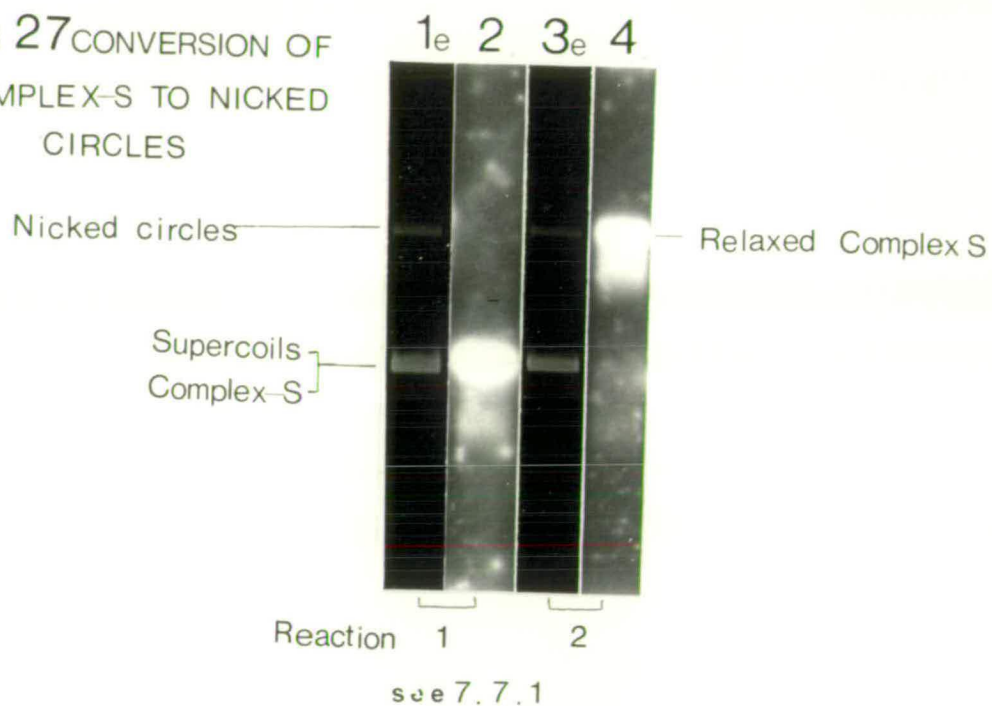
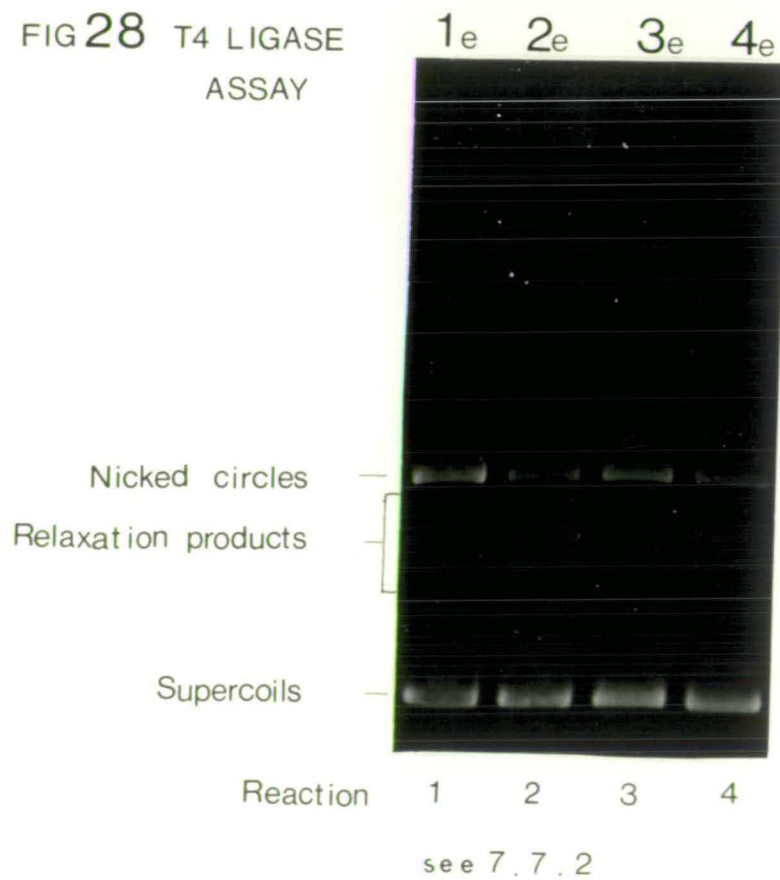


FIG 28 T4 LIGASE
ASSAY



hypothesised gaps. These conditions converted at least 80 percent of complex S to CCRC plasmid. Fig. 29.

In the absence of ethidium bromide in the gel, CCRC plasmid migrated just behind the nicked circles (Fig 29, Track 6). In the presence of ethidium bromide the CCRC plasmid migrated just in front of the supercoils (Track 8). The fragment was lost from complex S when it was electrophoresed in an ethidium bromide gel (Track 4). At the higher concentration of S1 nuclease, in the absence of T4 ligase and klenow fragment, the nicked circular intermediate containing the fragment was rapidly broken down (data not shown).

7.4 LOCATION OF THE LABELLED FRAGMENT IN THE CCRC PLASMID

The labelled CCRC plasmid was cut into fragments of defined size with restriction enzymes Hae111 and Taq1. The position of the labelled fragment in the CCRC plasmid was determined by identifying which restriction bands were radiolabelled.

When pBR322 is incubated with Hae111, a 587 basepair fragment that contains the sequence of the 36 base Sau3A fragment is generated.

A Taq1 site exists in the middle of the 36 base Sau3A sequence. Digestion with Taq1 splits the sequence between a 1444 and an adjacent 368 base pair fragment. The 368 base pair fragment contains the 5' end of the 36 base Sau3A sequence from the minus strand of the plasmid. The 1444 base pair fragment contains the 5' end of the 36 base Sau3A sequence from the plus strand of the plasmid. The plus strand sequence of pBR322 runs in a 5' to 3' direction from the unique EcoR1 site through the tet gene then through the amp gene (Sutcliffe 1978). As the original Sau3A fragment had a labelled 5' end, digestion of the CCRC plasmid with Taq1 not only locates the 36 base Sau3A fragment to a region of the plasmid defined by Taq1 sites but also to one strand of the vector. Fig. 30.

FIG 29

CONVERSION OF COMPLEX-S TO CCRC PLASMID

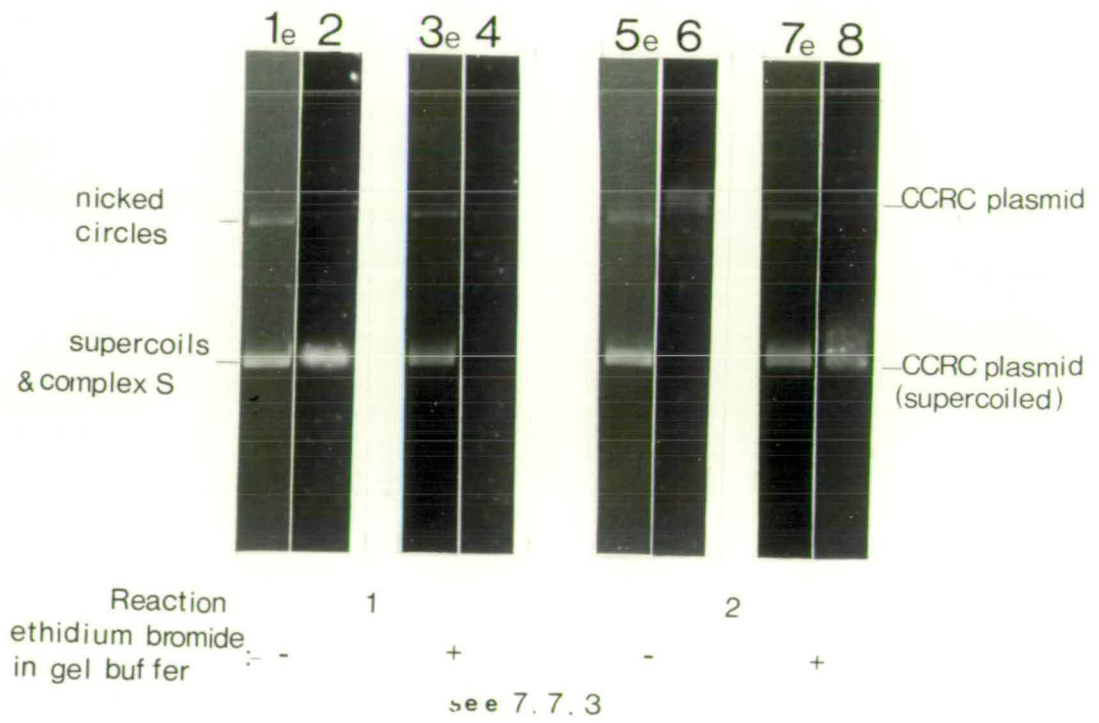
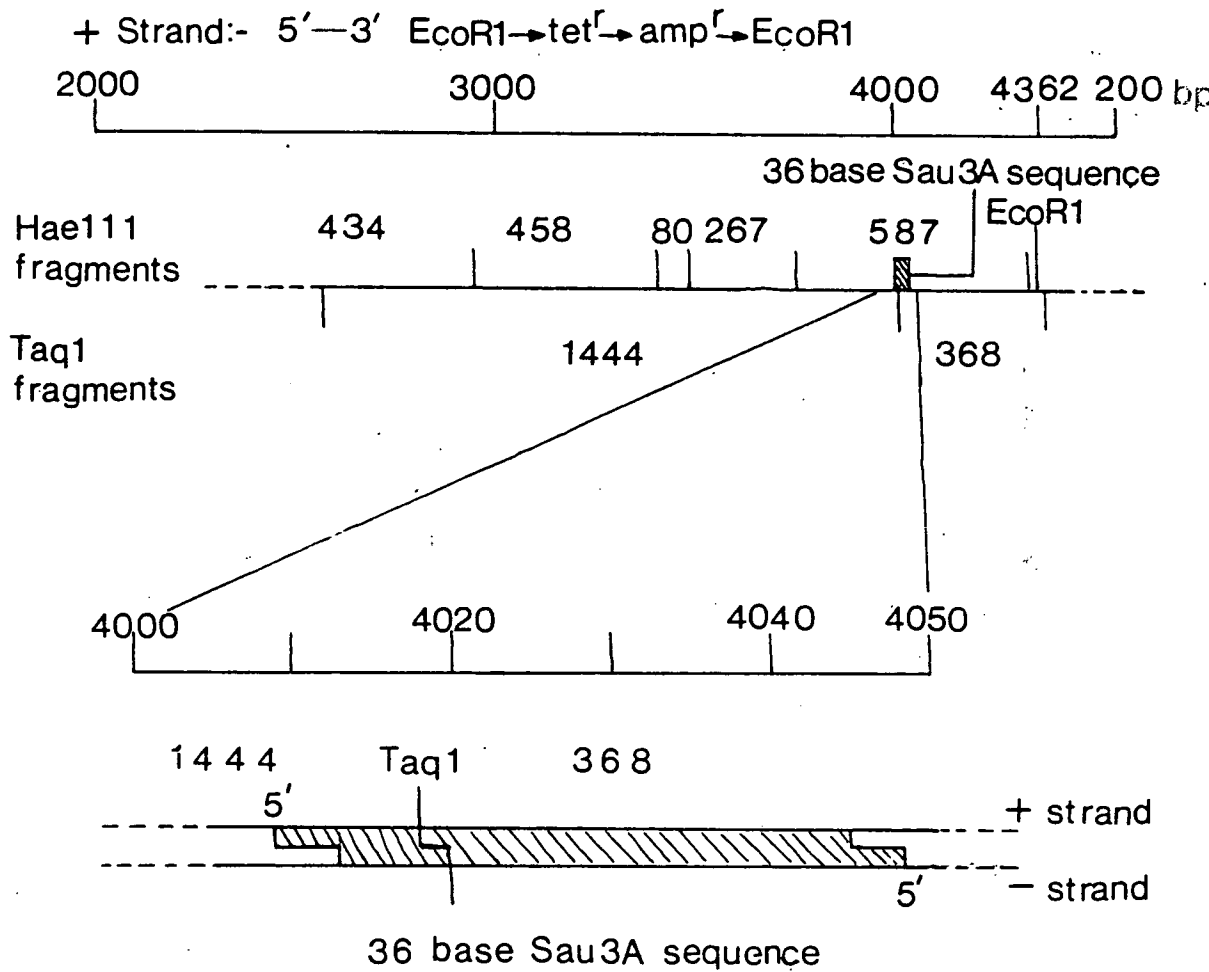


FIG30

Location of Taq1 and Hae111 sites in the Amp^r region
of pBR332



Before CCRC plasmid could be restriction-enzyme-mapped, the DNA had to be deproteinated. Gel filtration of the DNA on a small S-200 column in a high salt buffer turned out to be the only efficient deproteination method that did not result in substantial losses of CCRC plasmid.

Digestion of the mixture of labelled CCRC plasmid and supercoiled pBR322 with both Taq1 and Hae111 followed by gel electrophoresis, produced the expected restriction pattern of ethidium bromide stained DNA bands (Fig 31, Tracks 2, 4). In the Taq1 digest, the radiolabel appeared in the Taq1 368 base pair fragment indicating that the 36S Sau3A fragment had been introduced by RecA protein in the correct location, into the minus strand of pBR322, (Fig 31, Track 4).

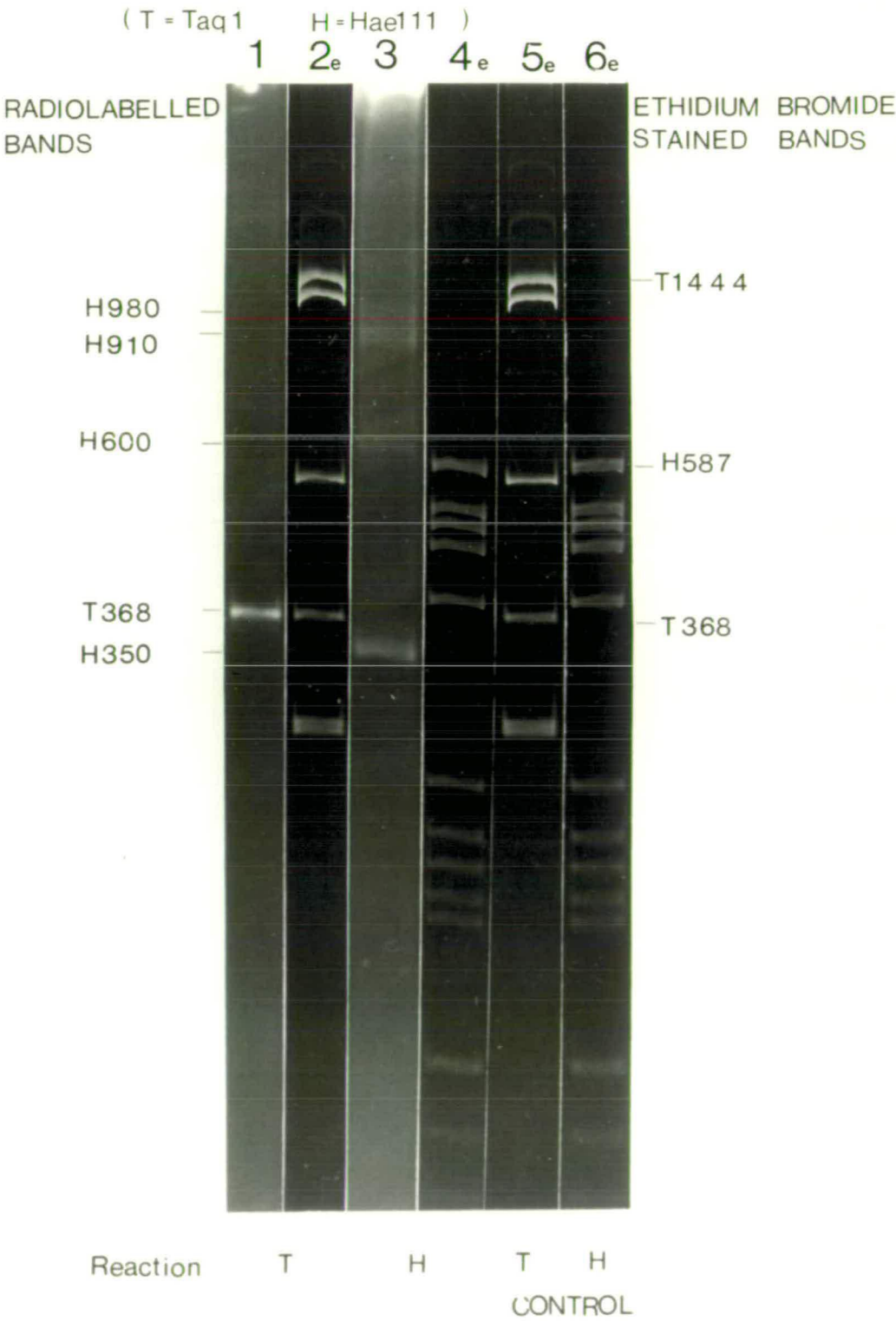
In the Hae111 digest, the radiolabel migrated as a strong 350 basepair band and weak bands at 600, 910 and 980. None of these bands corresponded to the position of a DNA band stained with ethidium bromide (Fig. 31, Track 3). (See 7.6).

7.5 SELECTIVE DIGESTION OF SUPERCOILED pBR322 WITH S1 NUCLEASE

To improve the final yield of mutant CCRC plasmid the unmodified supercoils had to be eliminated. The relative quantity of D-loops generated in the RecA reaction was very low compared to the total quantity of supercoils. This ratio was roughly calculated from the relative quantity of label incorporated into the plasmid band compared with the total amount of label on the autoradiograph. At best, complex S was not more than one per cent of the total plasmid DNA.

Pure S1 nuclease will cut a supercoiled pBR322 molecule at only one of the S1 sensitive sites (Fig 30). When pBR322 was incubated with S1 nuclease in the presence of zinc ions, one of the three sites was cut preferentially (Lilley 1980). When the pBR322 linearised with S1 nuclease was recut with EcoR1 at the unique EcoR1 site, predominantly two fragments were generated.

FIG31
 LOCATION OF THE FRAGMENT IN CCRC PLASMID BY
 RESTRICTION MAPPING



In the presence of cobalt ions, it was found in this study that there is no preference for which site S1 nuclease cuts. When the mixture of 3 types of linear pER322 molecules were recut with restriction enzyme EcoR1, six fragments were generated (Fig. 32).

Different batches of B.R.L. S1 nuclease were tested for purity using this assay. Most batches contained high levels of a contaminating double strand nicking activity which obscured the site specific cleavage by S1 nuclease. S1 nuclease was repurified by DE-52 ionexchange chromatography at low pH before it was used for selective digestion of supercoils.

The mixture of labelled CCRC and supercoiled plasmid were treated with S1 nuclease in a buffer containing zinc ions. Gel electrophoresis followed by ethidium bromide staining revealed that most of the plasmid DNA was converted to linear molecules (Fig 33 Track 5). When these linear molecules were recut with EcoR1, two fragments were produced (3060bp and 1300bp), indicating that S1 nuclease had cut at one site in the plasmid (Fig 33, Track 7).

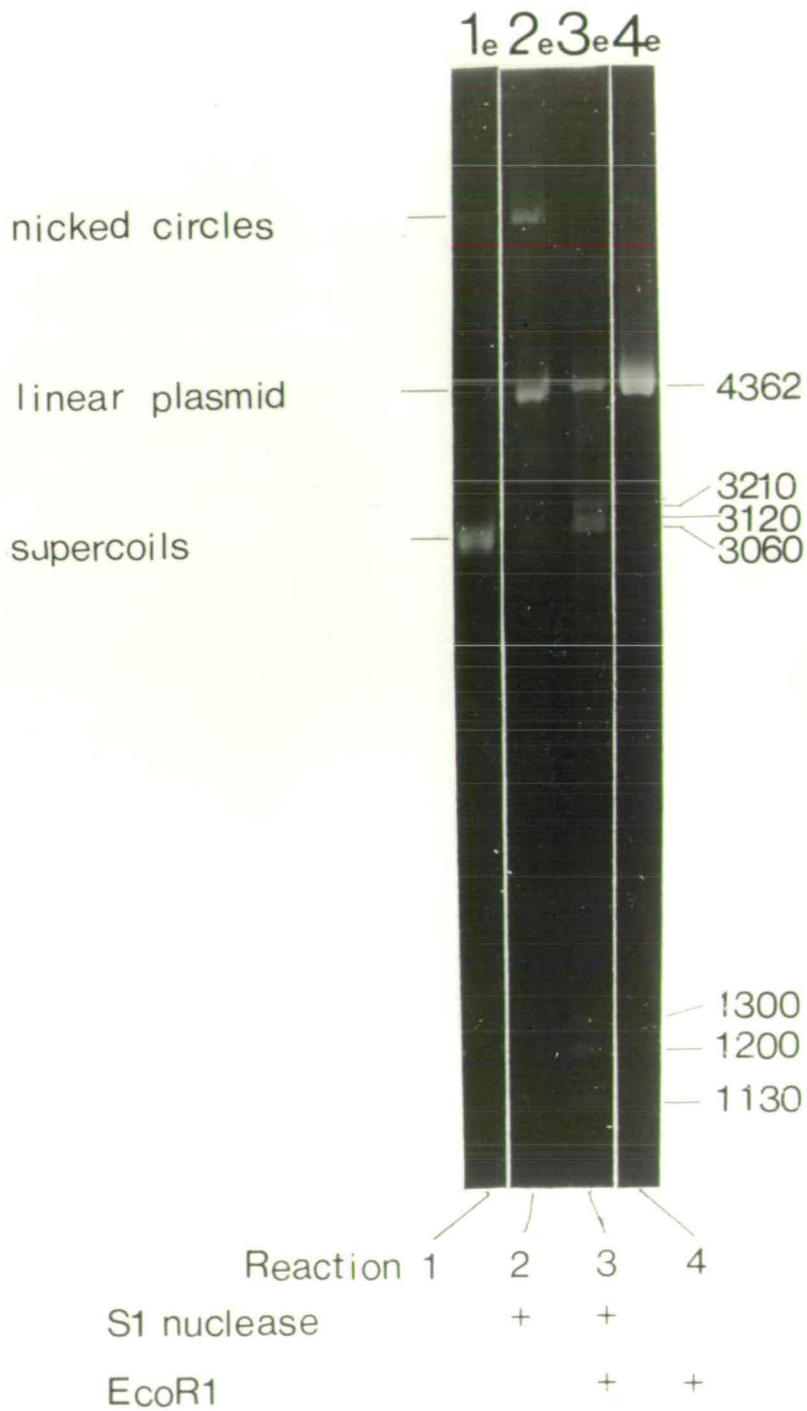
The effect on the labelled CCRC plasmid was complex. Digestion with S1 nuclease unexpectedly converted 10% of the labelled circles into linear molecules (Fig. 33, Track 6). When the S1 digested DNA was recut with EcoR1, three labelled bands were generated where only one was expected (Fig. 33, Track 8). Digestion of labelled CCRC plasmid with EcoR1 should have generated labelled linear plasmid. None of the labelled bands migrated with the ethidium stained linear plasmid DNA.

7.6 DISCUSSION

Treatment of the labelled CCRC plasmid with either Hae111, S1 nuclease or EcoR1 produced digestion products with unusual properties. The labelled CCRC plasmid was obviously not just a simple covalently closed relaxed circle of DNA.

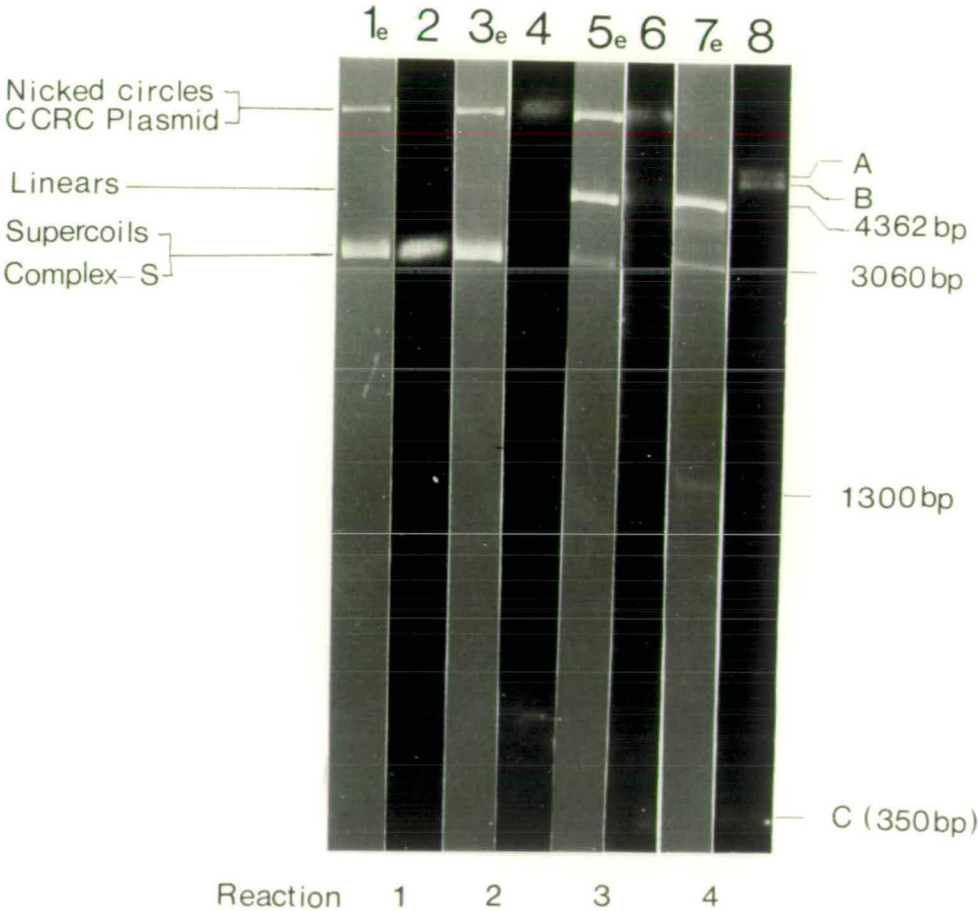
FIG 32

S1 NUCLEASE ASSAY



see 7.7.5

FIG 3 3
IN VITRO SELECTION FOR CCRC PLASMID WITH S1 NUCLEASE



see 7.7.6

A proposal that fits the experimental data involves postulating that the labelled fragment was located in the correct segment of the plasmid but the double helix was bent and partially denatured at either one or both ends of the incorporated fragment. T4 ligase is known to covalently close gapped DNA (Nilsson and Magnusson 1982). This process might generate permanent kinks in the double helix when the ends of the gapped strand were joined. Such kinks could have a long distance folding effect on a DNA molecule.

Taq1 digestion of the labelled kinked plasmid would not produce any fragments with very unusual secondary structure because, by cutting the Taq1 site in the middle of the 36 base Sau3A sequence, the long distance folding effect of the kink would be eliminated. Enzymes, such as Hae111 and EcoR1, which cut at sites far from the kink, would generate fragments with unusual secondary structure as the kink could transmit its effect over a long distance to produce a folded DNA molecule.

The S1 sensitivity of the labelled CCRC plasmid could be explained by a single stranded region in the kink. A small labelled fragment (approximately 350 bases) was generated after redigesting the S1 nuclease treated CCRC plasmid with EcoR1. This could be explained by S1 cutting the CCRC plasmid to a linear molecule at the site of the incorporated fragment. The 36 base Sau3A sequence is approximately 350 bases from the EcoR1 site.

No label was detected at the position of the 1300 base pair ethidium bromide stained DNA band generated by S1, followed by EcoR1 digestion. This confirmed that the palindromic sequences in the labelled CCRC plasmid were insensitive to S1 nuclease cleavage.

This proposal is still speculative. More experiments will have to be done before the identity of the labelled CCRC plasmid can be resolved. (See 8.2).

7.7 EXPERIMENTAL PROCEDURES

All the supercoiled pBR322 used in these experiments was acid phenol extracted, then S1 nuclease treated and phenol extracted as in 5.4.7.

7.7.1 CONVERSION OF COMPLEX S TO NICKED CIRCULAR PLASMID

Complex S was generated in 20 microlitres of M buffer containing 50 micrograms per ml supercoiled pBR322, 2 micrograms per ml 36S and 2mg/ml RecA protein. The reaction was incubated at 37°C for 10 minutes then terminated with the addition of 2.5 microlitres of 5% w/v sarkosyl. The reaction was partitioned with 20 microlitres of an equal mixture of chloroform and phenol equilibrated in M buffer. The upper aqueous layer was removed after centrifugation and partitioned twice with 0.1ml ether to yield protein-free complex S. (1µg pBR322).

Two microlitres of protein-free complex S was diluted to a 10 microlitre solution containing M buffer, 1mM ZnSO₄, 0.2 units per microlitre of S1 nuclease. The reaction was incubated at 16°C for 2 hours (Reaction 2). The reaction was then mixed with 3 microlitres loading buffer F: 10% ficoll, 0.2% w/v bromophenol blue, 30mM ATP and electrophoresed in a 1% agarose gel (Fig. 27, Tracks 3 and 4). A control sample of 2 microlitres protein-free complex S. Reaction 1 was diluted with 10 microlitres M buffer, mixed with 3 microlitres loading buffer F and electrophoresed on the same gel (Fig. 27, Tracks 1 and 2).

7.7.2 INHIBITION OF T4 LIGASE BY ZINC

0.02 units of B.R.L. T4 ligase was assayed in 5 microlitre solutions of M buffer containing 20 micrograms per ml supercoiled pBR322. In addition to the DNA solution, Reaction 1 contained 1mM EDTA, Reaction 2 contained 1mM EDTA and T4 ligase, Reaction 3 contained 1mM ZnSO₄ and T4 ligase, and Reaction 4 contained 1mM ZnSO₄ 2mM EDTA and T4 ligase. The reactions were incubated for 16 hours at 20°C then terminated with 2 microlitres 5 x loading buffer S and analysed on a 1% agarose gel.

The combination of a contaminating nicking activity and active T4 ligase generated a ladder of DNA bands on the gel from stepwise relaxation of the supercoils. In the presence of zinc, T4 ligase activity was abolished (Reaction 3). It was partially restored by the addition of excess EDTA (which chelates Zn^{2+} ions) Fig. 28.

7.7.3 CONVERSION OF COMPLEX S TO CCRC PLASMID

20 microlitres of protein-free complex S was generated as in 7.7.1. 7 microlitres was diluted to a 20 microlitre solution of M buffer plus 1mM EDTA, 0.5mM dNTPs, 0.04 units T4 ligase, 1 unit of Klenow fragment and 20 units S1 nuclease. The reaction was incubated for 2 hours at 16°C to produce CCRC plasmid (Reaction 2).

6 microlitres of loading buffer F (7.7.1) was added and 13 microlitres was loaded onto a 1.2% agarose gel in TAE buffer plus 0.5 micrograms per ml ethidium bromide. The other 13 microlitres was loaded onto an identical agarose gel and run in the absence of ethidium bromide.

Seven microlitres of protein-free complex S were diluted to 20 microlitres in M buffer, and mixed with 6 microlitres of loading buffer F (Reaction 1). 13 microlitres of this solution was loaded onto each gel. Fig. 29.

7.7.4 HAE111 AND TAQ1 DIGESTION OF CCRC PLASMID

20 microlitres of protein-free complex S (7.7.1) was converted to CCRC plasmid in 100 microlitres M buffer plus 1mM EDTA, 0.5mM dNTPs, 0.2 units T4 ligase, 50 units S1 nuclease and 5 units of klenow fragment.

5 microlitres of 5M NaCl was added and the volume of the solution was reduced to 25 microlitres by extraction with 0.2ml then 0.1ml butan-1-ol. The salt concentration was increased to 1M NaCl by this process. The proteins were separated from the DNA by applying the sample to an 0.4ml S-200 gel filtration column equilibrated in

1M NaCl, 10mM Tris.pH7.5 and 1mM EDTA. DNA fractions were monitored by mixing one microlitre of each fraction with a one microlitre droplet of one microgram per ml ethidium bromide and viewing the solution under U.V. light. Fluorescence due to DNA was detected in the fractions from the void volume of the column. The DNA fractions were combined and ethanol precipitated. The DNA pellet was dissolved in 10 microlitres water to produce a solution of protein-free CCRC plasmid.

3 microlitres of protein-free CCRC plasmid was diluted to a 5 microlitre solution containing BRL Taq1 buffer: 10mM Tris.HCl pH8.4, 6mM MgCl₂, 100mM NaCl, 6mM 2-mercaptoethanol; 1 unit of BRL Taq1 and incubated at 65°C for 4 hours (Reaction 1).

3 microlitres of protein-free CCRC plasmid was diluted to a 5 microlitre solution containing BRL Hae111 buffer: 50mM Tris.HCl pH7.5, 5mM MgCl₂, 0.5mM DTT; 1 unit of BRL Hae111 and incubated at 37°C for 4 hours. (Reaction 2).

Parallel Taq1 and Hae111 incubations were carried out with 0.2 micrograms supercoiled pBR322 (Reactions 3 and 4).

The reactions were terminated by the addition of 6 microlitres of 2 x loading buffer A. One microlitre of 0.1M ATP was added and the reactions were analysed on a 5% Acrylamide, 0.17% bis, TBE gel. The gel was stained with a solution of one microgram per ml ethidium bromide and the DNA bands were located with U.V. light. The gel was then dried down under saran wrap onto nitrocellulose for autoradiography. Fig. 31.

7.7.5 S1 NUCLEASE ASSAY

S1 nuclease specificity was assayed in a 60 microlitre solution containing 1mM CoCl₂, 30mM NaAc pH4.6, 450mM NaCl 0.2% SDS and 500 units S1 nuclease. The reaction was incubated at 0° for 3 hours then terminated by adding 30 microlitres of 4mM EDTA 1M

Tris.HCl pH8.6. The DNA was ethanol precipitated and the pellet was dissolved in 10 microlitres water. 5 microlitres was diluted to a solution of 6 microlitres EcoR1 buffer: 40mM Tris.pH8.0, 20mM MgCl₂, 2mM DTT. The solution was incubated with 1 unit EcoR1 for 30 minutes at 37°C then terminated by the addition of 2 microlitres of 2 x loading buffer A. The samples were analysed on a 2% agarose gel.

7.7.6 IN VITRO SELECTION FOR CCRC PLASMID WITH S1 NUCLEASE

The 10 microlitre solution of protein-free CCRC plasmid was produced as in 7.7.4. Two microlitres were diluted to a 10 microlitre solution containing 1mM ZnSO₄, 30mM NaAc pH4.6, 450mM NaCl, 0.2% SDS and 500 units repurified S1 nuclease. The reaction was incubated at 0°C for 3 hours then terminated by the addition of 5 microlitres of 4mM EDTA 1M Tris.HCl pH8.6. The DNA was ethanol precipitated and the pellet was dissolved in 20 microlitres water (Reaction 3). 10 microlitres was diluted to a solution of 12 microlitres EcoR1 buffer containing 1 unit EcoR1. The reaction was incubated for 30 minutes at 37°C then terminated by the addition of 3µl 2 x loading buffer A (Reaction 4). One microlitre of 0.1M ATP was added and the reaction was analysed on a 2% agarose gel.

10 microlitres of S1 treated CCRC plasmid (Reaction 3) was mixed with 3 microlitres 2 x loading buffer A and one microlitre of 0.1M ATP then loaded onto the same gel. Control samples of complex S (Reaction 1) and protein-free CCRC plasmid (Reaction 2) were also loaded onto the 2% agarose gel.

CHAPTER 8 : EXPERIMENTAL PROPOSALS AND GENERAL DISCUSSION

8.1 INTRODUCTION

The starting point for the work described in this thesis was the proposal outlined in 1.7 for a new technique for accurately generating site specific changes in a gene. The experiments described in chapter 6 show that D-loops can be generated with very small restriction fragments and that a certain degree of mismatch is tolerated. The experiments described in chapter 7 show that small D-loops can be cut away without the loss of the fragment and the fragment can then be covalently joined to the vector. These experiments also show that the fragment is incorporated at only one site in the plasmid and that the site of incorporation is in the correct region of the plasmid. Finally, it has been shown that contaminating supercoiled molecules can be selectively cut to linear molecules with S1 nuclease without the enzyme cutting the palindromic sequences in CCRC plasmid.

Two problems remain before mutant CCRC plasmid may be used to transform uracil-glycosylase-deficient bacteria. These are the nature of the labelled CCRC plasmid and the low D-loop yield. A routine application for mutants generated by this technique would be in saturating an area of a polypeptide chain with amino acid changes to determine whether it was essential for the activity, specificity or stability of a protein.

8.2 EXPERIMENTAL PROPOSALS

8.2.1 CHARACTERISATION OF THE LABELLED CCRC PLASMID

To learn more about the properties of the labelled CCRC plasmid three main types of experiment could be done. First, the labelled CCRC plasmid could be cut with various restriction enzymes and the products analysed on acrylamide gels in the presence and absence of denaturing concentrations of urea. Second, the restriction fragments could be analysed on gels containing a urea gradient perpendicular to the direction of electrophoresis. Finally, CCRC plasmid and its digestion products could be purified and analysed by electronmicroscopy.

Digestion of the labelled CCRC plasmid with restriction enzyme Sau3A followed by electrophoresis in a denaturing urea gel would reveal whether the two Sau3A sites at either end of the fragment had been modified. If these sites were insensitive to the enzyme, the label would be absent from the 36 base pair restriction band and a new labelled band equivalent to 718 base pairs would appear.

If secondary structure at the site of fragment incorporation, was responsible for the unusual mobility of the 587 base pair Hae111 fragment, then restriction analysis with other enzymes should produce a similar result. Labelled CCRC plasmid could be digested with Alu1 and Hpa11. Both these enzymes cut at sites distant from the 36 base Sau3A sequence to generate a fragment of similar size containing the sequence.

Digestion of the labelled CCRC plasmid with Mbo11 would produce a 78 base pair fragment that contains the 36 base Sau3A sequence. Comparison of the mobility of this fragment in the presence and absence of denaturing concentrations of urea would reveal whether the anomolous fragment mobility depends on secondary structure. If the anomolous mobility is due to gaps being closed by T4 ligase then one would expect the labelled strand to be shorter than 78 bases. This would be revealed on the denaturing gel.

The labelled CCRC plasmid could be digested with HgiA1. An HgiA1 site exists in the 36 base Sau3A sequence. The two flanking HgiA1 sites are 85 and 604 bases away. Labelled CCRC plasmids could be produced with the ^{32}P in both orientations by generating D-loops with either 36F or 36S. Both types of labelled CCRC plasmid could then be treated with HgiA1. In the absence of any structural anomaly, one would expect (from the result of the Taq1 digestion (7.7.4), the label from the 36S labelled CCRC plasmid to appear in the 604 base pair band while the label from the 36F labelled CCRC plasmid to appear in the 85 base pair band.

One route to generating CCRC plasmid would be to modify complex S in three separate steps (rather than the concerted process used in this study). First, the displaced strand in complex S would be removed by S1 nuclease. Second, the hypothesised gaps generated would be filled in with the klenow fragment and dNTPs. Finally, the nicked molecules would be closed with much lower concentrations of T4 ligase.

8.2.3 D-LOOP FORMATION

In this study attempts to increase the ratio of D-looped plasmid to non-D-looped plasmid stopped once a clearly detectable level of incorporation was achieved. The levels of incorporation might be enhanced by varying the SSB/RecA protein/DNA stoichiometry in further experiments.

At present, the ratio of D-loop plasmid to non-D-looped plasmid is too low to allow a reasonable yield of mutants even with the S1 nuclease selection system. If one per cent of the supercoiled plasmid preparation is nicked circular vector and at best, only one per cent of the supercoils carry D-loops, then even with the S1 selection system, the contamination of unmodified plasmid at the final stage will still be significant. Thus, the non-specifically nicked plasmids at the start of the process contributes significantly to the final wild type back-ground when small fragments are inefficiently introduced. If small fragments are incorporated inefficiently, the serious consequence for the method described by Shortle et al 1980 is that the high relative amount of nicked vector automatically results in a large percentage of the mutations falling outside the target (As it is the nicks that define the target in this method).

8.3 DISCUSSION

In the last few years a number of site directed mutagenesis techniques have been developed to a point where specific changes can be made in any part of a gene. Some techniques are better suited to generate relevant mutants that answer questions about the functionally

important parts of a protein. To relate functional changes in the protein to the structural effect of the mutation, the mutant protein has to be analysed by X-ray crystallography.

Techniques that involve the introduction of mismatched oligonucleotides to produce a single base change in the gene are the most precise, as individual amino acids in the protein can be changed. For structure-function studies of proteins this is not particularly important because the rate determining step in the overall experiment is the analysis of the mutant protein by X-ray crystallography. It is much simpler to saturate a small target in the protein with mutations. All the mutants could be rapidly characterised by DNA sequencing long before the structure of the first mutant protein had been determined. The technique described in this study could be used for such a task as it is potentially very accurate and could saturate the target with mutations in one experiment.

By this form of analysis, the general rules that govern the molecular architecture of proteins will be elucidated. With a theoretical knowledge of protein folding, new proteins could be created with novel specificities and activities.

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