STUDIES OF PLASMID ENCODED RESTRICTION AND MODIFICATION SYSTEMS

by

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"The real cycle you're working on is

a cycle called 'yourself'".

Robert M. Pirsig

Zen and the art of motorcycle maintenance

FOREWORD

The work reported in this thesis was carried out part-time during my employment as a technician in the Department of Molecular Biology of the University of Edinburgh. The composition of this thesis and the work it describes are my own except where stated otherwise.

Stephen G.Hughes.

My other publications relevant to the work here presented are:

Hughes, S.G. and Brown, P.R. (1973);
Thompson, R., Hughes, S.G. and Broda, P. (1974);
Hughes, S.G. and Hattman, S. (1975);
Murray, K., Hughes, S.G., Brown, J. and Bruce, S.A. (1976);
Hughes,S.G. A Map of the Cleavage Sites for Endonuclease AvaI in the Chromosome of Bacteriophage Lambda (1976(7)), Biochem.J. accepted for publication.

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SUMMARY

Within the general context of the distribution, role, and origin, of restriction and modification systems among plasmids, two plasmid encoded systems have been studied.

The EcoRII (hspII) system

The sensitivity of the DNA of bacteriophage λ to endo R. EcoRII *in vitro* was found to depend on the presence or absence of methylation introduced by the cytosine specific DNA methylase of E.coliK. Subsequently, a revised minimum estimate for the number of cleavage sites for endo R. EcoRII and a map of these sites close to the ends of the λchromosome were made.

The EcoR124 (hspI) system

The restriction and modification system carried by the plasmid R124 was shown to be different from the EcoRI system carried by plasmid RY5 (subsequently NTP13) with which it had previously been assumed to be identical. Endo R. EcoR124 was isolated and found to be dependent on ATP and s-adenosylmethionine (SAM) (it is a class I restriction endonuclease).

A derivative of R124 was isolated, by chance, which has acquired a restriction and modification system of novel specificity. The derivative, R124/3, also has new cleavage sites for endo R. EcoRI and endo R. SalI in its DNA. It is proposed that the new system, EcoR124/3 arose by recombination between the determinants of EcoK and EcoR124.

ABBREVIATIONS and CONVENTIONS

The convention of Demerec et al 1966 for description of genotypes has been adhered to.

Nucleotide sequences have been written in the 5' —— 3' direction and the phosphates omitted.

Since only deoxynucleotides have been referred, to the prefix d has been omitted. In describing the specificity of modification of a phage the generally accepted notation has been followed, thus λ.K is phage λ modified against the EcoK system and λ.K.RII is modified against the EcoK and the EcoRII systems.

The acronymic system for naming restriction and modification enzymes and systems devised by Smith and Nathans (1973) has been followed. The prefix endo R. for restriction endonucleases has been reduced to R. or omitted completely wherever it was thought that this would not cause confusion, as has become the general habit in the literature.

The notation for cleavage sites for restriction endonucleases suggested by Arber and Linn (1969) has been used, srI and shindIII represent cleavage sites for EcoRI and HindIII respectively.

E^{260} represents extinction of light of 260 nm wavelength in a 1 cm pathlength cell.

SAM represents s-adenosylmethionine.

TEC represents triethylamine carbonate.

The sizes of protein molecules and DNA fragments have been expresed in Daltons (d) or megadaltons (Md) (10^6 Daltons).

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CHAPTER 1

Introduction

1.1 A general introduction to restriction and modification

Certain species of bacteria exhibit the ability to selectively degrade foreign DNA with which they are infected, be it from phage, plasmid or from another bacterial genome. DNA is recognised as foreign by the lack of a chemical modification pattern specific to the infected host, which is manifest as a methylation of adenine or cytosine within specific short nucleotide sequences. Unmodified DNA is cut by a special class of endonucleases which recognise these sequences and is thereafter further degraded by non specific nucleases.

This biochemical phenomenon is known as host controlled restriction and modification as it was first observed as a restriction of the plaque forming ability of certain phages upon challenge of a new host (Bertani and Weigle 1953). Phages which succeed in forming plaques on the new host subsequently plate with normal efficiencies on that host, their DNA having acquired the appropriate modification pattern.

Because of the many interesting and useful properties of the enzymes involved in restriction (restriction endonucleases) and modification (modification methylases) this field has been heavily reviewed. Outstanding reviews are: Arber and Linn (1969) which offers a very thorough and far sighted account of the developing field; Arber (1971) an extensive review of restriction and modification as it relates to phage\ ; Arber (1974) an updated survey; and Nathans and Smith (1975) a detailed review of the properties of the enzymes involved in restriction and modification. Additional reviews are by Meselson et al (1972) and Boyer (1971). The most recent review (Roberts 1976) gives a full account of the distribution, properties and applications of the restriction nucleases.

In the face of such intensive reviewing no attempt has been made in this introduction to provide an additional review. Instead the object has been to provide a factual background to the specific areas of the field covered in the experimental section of this thesis, and to draw attention to some areas of apparent conflict

between the published results and ideas of different workers, which bear on, and in some cases initiated the work in this thesis.

1.2 The distribution of restriction and modification systems

The ability to restrict the plating efficiency of one or more phages has been demonstrated in a number of bacteria, notably among species of Pseudomonas, the Enterobacteria and Haemophilus (for review see Boyer 1971). The phenomenon has proved of practical value in that the specificity of the host-modified phage interaction is part of the basis of phage typing systems, for instance that used for Salmonellae (Anderson & Williams 1956).

For historical and practical reasons the systems of Escherichia coli and Haemophilus influenzae strains are the best studied. Both sets of organisms contain a number of restriction and modification systems coded both by the chromosome and by plasmids.

Recent work on the characterization and isolation of class II restriction enzymes (Roberts 1976) suggests that restriction and modification may be of greater ubiquity than is indicated by the number of systems so far detected by the restriction of phage plating ability. Indeed, it is now felt by many that restriction and modification is an almost universal property of prokaryotes.

1.3 Restriction endonucleases

(i) General

Restriction endonucleases are characterized by the ability to introduce a limited number of cuts into a given double stranded DNA molecule. None of them produce significant amounts of acid soluble DNA when pure. Upon this, the specificity of their interaction with unmodified DNA, and the limited number of methyl groups necessary for modification (Gough and Lederberg 1966), was based the concept of specific "sites" on a DNA substrate , recognised by the restriction endonuclease, and protected by the action of the modification methylase (Arber and Linn 1969). This model has proved substantially correct, although different restriction endonucleases interact differently with these sites.

A number of restriction endonucleases have been isolated and compared in vitro with respect to their individual substrate recognition

	Class I Restriction Endonucleases	Class II Restriction Endonucleases
Co-factor requirements	Mg^{++} SAM ATP	Mg^{++}
Recognition	Genetically defined host specificity sites.	Specific unmodified targets containing rotationally symmetrical nucleotide sequences.
Cleavage	Random	As above.
Molecular Weight of purified enzyme	$\gtrsim$ 200,000	30 - 50,000
Additional properties associated with the purified enzyme.	methylation, ATPase	-
Archetypal systems mediated by Class I or Class II endonucleases.	Systems found in *E.coli* K (EcoK) *E.coli* B (EcoB) *Haemophilus influenza* (HindI)	The plasmid encoded systems EcoRI EcoRII The systems of *Haemophilus influenza* HindII HindIII

Table 1.1

and cleavage properties and their co-factor requirements. By these criteria they fall into two classes as shown in table II (Boyer 1974). The class I/class II distinction has been extended to the restriction and modification systems to which the enzymes belong.

(ii) Restriction by class I endonucleases

Sites which confer sensitivity to class I restriction systems have been defined genetically in phage and bacterial genomes (Smith et al 1972) (Murray et al 1973b) (Brammar et al 1974) as loci which can be mutated to give partial resistance to restriction in vivo, and which are necessary for the binding of purified restriction enzyme to DNA in vitro (Yuan and Meselson 1976) (Murray et al 1973a). Six sites have been demonstrated and localised in the chromosome of phage λ for the restriction system of E.coliK (Murray et al 1973b) and two in phage f1 for the E.coliB system (Arber and Kuhnlein 1967). The role of these so called host-specificity sites in the restriction reaction is not fully understood. However, it is clear that they are not sites of cleavage for the restriction endonuclease, as was shown by Horiuchi and Zinder (1972). They found that the products of digestion of the double stranded circular replicative form of f1 DNA with the purified restriction endonuclease of the E.coliB system, (endo R. EcoB abbreviated to EcoB) were full length linear molecules, which when melted and reannealed gave a high proportion of circular molecules, which, even if the original substrate had only one sensitive site, were once again a substrate for EcoB. Thus cleavage is remote from the genetically defined specificity site and is sufficiently random for the dissociated strands of the population of cut molecules to reassociate as circular or concatameric species.

Murray et al (1973a) in a study of the fragmentation of derivatives of phage λ containing one or two specificity sites for the E.coliK system in defined map positions failed to demonstrate cleavage at these sites. They concluded that cleavage is influenced by but remote from the genetically defined sites as would be expected if the enzyme bound to a specificity site and then moved a limited distance along the DNA before cutting. Bickle et al (1976) claim that cleavage occurs preferentially in "early melting" (presumably

A T rich) regions of the substrate. A model by Shulman (1974) proposes that a site of cleavage is determined by the collision site of endonuclease molecules moving along the substrate in opposite directions.

Dissection of the restriction reaction into a series of steps showed how the co-factors s-adenosylmethionine (SAM) and ATP participate in it. The restriction endonuclease first binds to SAM which acts as an allosteric effector, converting the enzyme to an activated species (Haddi et al 1975). Thus activated, the enzyme forms an unstable complex with the substrate DNA at a random point. This complex is stabilised by interaction with a host specificity site and by binding ATP (Yuan et al 1975). The status of the host specificity site, (whether it is unmethylated, half methylated or fully methylated) determines whether the restriction enzyme goes into a modifying on a restricting mode or dissociates from the DNA complex. In the restricting mode the enzyme then travels along the DNA substrate until a site of cleavage is determined. Cleavage ensues, first of one strand then of the other (Meselson and Yuan 1968). This is associated with, and followed by, a massive hydrolysis of ATP (Yuan et al 1972) (Horiuchi et al 1974) during which the enzyme remains bound to the substrate. The role of ATP in the cleavage reaction and thereafter is obscure.

The novel role of SAM as an allosteric effector rather than as a methyl donor may be a reflection of the relationship between restriction and modification. It has been demonstrated (Lark and Arber 1970) that methionine starvation (and as a consequence, SAM starvation) is lethal to cells containing class II restriction systems but not cells containing only class I systems, since the former cannot modify against their own restriction enzymes. For class I endonucleases to have evolved a SAM dependence, then, may reflect the need to inactivate these enzymes during periods of SAM depletion (Meselson et al 1972).

Attempts to add a 5' ^{32}P label to the ends of fragments generated by cleavage with class I enzymes, using polynucleotide kinase, (Murray 1973) have proved unsuccessful (Murray et al 1973a), in contrast to experience with class II systems (see below). It has

been concluded that either the 5' termini left by class I endonucleases are something other than a phosphate or hydroxyl group, or, the 5' terminal nucleotide is modified. Whatever the explanation it is clear that the mechanism of cleavage is unique among endonucleases and may reflect the observation that *in vitro* the class I restriction enzymes do not turn over but remain bound to the substrate after cleavage.

(iii) Class II restriction endonucleases

Class II restriction endonucleases have an apparently simpler mechanism than the class I enzymes (for review see Nathans and Smith 1975). In recognising and cutting DNA at short defined nucleotide sequences, they each reduce a given DNA molecule to a set of characteristic fragments (Sharpe *et al* 1973). Herein lies their popularity as tools for dissecting and analysing genomic structure, analogous to the use of trypsin in protein chemistry.

Nucleotide sequences at the ends of fragments generated by the cleavage of DNA molecules by a number of individual class II enzymes have been determined by a variety of methods (for review see Murray and Old (1974); Roberts (1976)) and the structures of the sites recognised by these enzymes deduced. The defined nucleotide sequences within the sites are from 4 to 8 nucleotide pairs long and all contain a two fold axis of symmetry through a central nucleotide pair or pair of nucleotide pairs. The recognition sequences of the class II restriction endonucleases studied or used in this thesis are shown as examples in table 1.2.

The class II restriction endonucleases fall into two subclasses: those which cut both DNA strands at the centre of symmetry of the recognition site to give a "straight break" e.g. the endonuclease from *Bacillus subtilis*X5 (BsuX) (Bron *et al* 1976); and those which cut one strand on either side of the centre of symmetry to produce "staggered breaks" with complementary single strand protrusions e.g. endonuclease III of *Haemophilus influenza* (Hind III). The latter class offer the possibility of rejoining restricted DNA fragments in new arrangements, which is the basis of the *in vitro* recombinational approach to genetic engineering by which heterologous DNA fragments may be inserted into specially constructed phage chromosomes or plasmids (Murray and Murray 1974) (Chang and Cohen 1974).

Table 1.2

Endonuclease R.	Nucleotide sequence at Recognition/cleavage site	Number of cleavage sites in the chromosome of phage λ	Relevant reference
EcoRI	N C-T-T-A*-A↓-G N 5' N G↓A-A-T-T-C N *	5	Allet et al. (1973) Hedgpeth et al. (1972)
EcoRII	N-G-G-A-C*-C↓ N 5' N↓C-C-T-G-G- N *	>20	Boyer et al. (1973) Bigger et al. (1973)
HindIII	T-T-C-G-A↓A* A↓A-G-C-T-T *	6	Old et al. (1975)
Sal I	?	2	
BamI	C-C-T-A-G↓G G↓G-A-T-C-C	4	Wilson & Young (1973)
AvaI	G-C-Py↓Pu-G-C C-G-Pu↓Py-C-G	8	Murray et al. (1976)
AvaII	C-C-A-G↓G G↓G-T-C-C	25	ibid Hughes (unpublished) 1976
HpaII	G-G-C↓C C↓C-G-G	50	Garfin & Goodman (1974)
Bsu x 5	C-C↓G-G G-G↓C-C	50	Bron & Murray (1975)

↓ show points of cleavage
* shows the base methylated by the corresponding modification methylase.

Class II restriction endonucleases can be readily identified and screened for by their ability to generate characteristic sets of fragments from a given DNA molecule, even in relatively crude isolates of microorganisms (see section 3.1). Roberts (1976) presents a list of some organisms from which such enzymes have been isolated, although it must be pointed out that not many of them have been shown to be involved in a classical restriction and modification system.

(iv) The restriction endonuclease of phage P1

This endonuclease (EcoP1) has been purified (Meselson and Yuan 1968) and extensively studied in vitro (Haberman 1974) (Morss 1976). It falls into neither class I or class II by the criteria of table 1.1. It requires ATP but does not have the high ATPase activity of class I enzymes. It cuts a given DNA molecule into defined fragments but does not cut at a single, symmetrical nucleotide sequence. It has a high (200,000) though somewhat lower molecular weight than the class I enzymes (300,000).

(v) The restriction endonuclease of Haemophilus parahaemolyticus

Another endonuclease with novel recognition and cleavage properties was isolated from Haemophilus parahaemolyticus (known as Hph). It makes a staggered break by cutting on either side of an A - T pair (AT) but the sequences surrounding these cleavage sites are otherwise random (Kleid et al 1976). A "common sequence" is found 8 to 9 nucleotides distant from the cleavage site. The common sequence contains no element of symmetry.

1.4. Modification Methylases

A number of modification methylases have been isolated and studied in vitro, with special reference to the identity of the sites on DNA with which they interact. Most extensively studied are the methylase of the E.coliB system (M.EcoB) (Lautenberger and Linn 1972), those of Haemophilus influenzae type d (M.HindII, M.HindIII) (Roy and Smith 1973), that of the phage P1 (M.EcoP1) (Brocks et al 1973) and those of the two class II plasmid encoded systems EcoRI and EcoRII (Boyer et al 1973) (Dugaiczyk et al 1974) (Yoshimori, 1971). With the exception of M.EcoRII these all methylate adenine residues to give 6-methyl aminopurine. M.EcoRII acts on cytosines

to give 5-methylcytosine (Hattman et al 1972). The methyl donor is always SAM. Again with the exception of M.EcoRII the number of methyl groups introduced by these enzymes is too small to be detected in in vivo modified DNA, against the background methylation by the cytosine and adenine specific methylases of the host cell. M.EcoRII introduces the same order of methyl groups as the host cytosine methylase (Hattman 1972). The nucleotide sequences of the sites methylated by these enzymes have been determined from DNA radioactively labelled in vitro using ^{14}C or ^{3}H methyl labelled SAM and the purified enzymes. For the class II systems it was found that the nucleotide sequences methylated were identical with those recognised by the corresponding restriction endonucleases. In an elegant experiment Dugaiczyk et al (1974) added a radioactive 5' terminal label to EcoRI digested fragments of DNA before ligating them back together, and using them as a substrate for in vitro modification with a different radioactive isotope in the methyl donor. The finding of both isotopes within the same oligonucleotides when this DNA was digested with endonucleases showed incontrovertibly that for EcoRI endonucleolytic cleavage and modification methylation occur at the same sites. Attempts to determine the nucleotide sequences methylated by class I modification enzymes and to demonstrate that these occur at the genetically defined host specificity sites have yielded less straightforward results (van Ormondt et al 1973) (Kuhnlein and Arber 1972). Short unique sequences containing the modifying methyl groups have not been found. However, it has been shown that radioactive label introduced by M.EcoB into phage f1 DNA lies only in those HaeIII fragments of this phage genome which carry the EcoB host specificity sites. This supports the proposition that for class I systems (besides class II systems) modification methylation occurs at or very close to the genetically defined host specificity sites.

The purified restriction endonucleases of the K, B, and P1 systems also have modifying activity (Vovis et al 1974) (Haberman et al 1972) in vitro. The class I restriction endonucleases and modification methylases are large multi subunit molecules and it is consistent with the mechanism of restriction presented in section 1.3(ii), that they should exist in vivo as a single complex molecule with both restricting and modifying activities. This is probably the

species isolated as the restriction endonuclease. The purified modification enzyme is probably a species which has shed the subunits involved in restriction. The analysis of the genetic determinants of restriction and modification (section 1.6) has given further insight into the subunit relationships between the endonucleases and methylases.

1.5 Plasmid controlled restriction and modification systems

(i) Plasmids in general

Plasmids are autonomously replicating extrachromosomal genetic elements and are widespread amongst bacteria and other prokaryotes. Many plasmids mediate their own transfer between microbial species or genera. Self transmisible plasmids are implicated in such phenomena as infectious multiple drug resistance (for review see Falkow 1975) which is so important to the chemotherapy of bacterial infections. Many are capable of the transfer of chromosomal genes between species and have been of great importance to bacterial genetic analysis. As a consequence, plasmids have been the subject of many reviews. Of these the comprehensive and up to date set of review papers in Microbiology 1974 (1975), and Falkow (1975) are strongly recommended.

The plasmids so far studied physically are small double stranded DNA molecules ranging in size from 1 to over 100 Md, and can be isolated as covalently closed circular molecules with superhelical turns (Roth and Helinski 1967). Use is made of the latter property in their separation from chromosomal DNA by isopycnic centrifugation in the presence of intercalating dyes.

Plasmids are of great diversity, but may be regarded as permutations of genes coding for a limited variety of functions which includes: resistance to antibiotics; bacteriocin production; degradative metabolic pathways; and host specific restriction and modification. In common they have a set of genes coding for their own replication (Willetts 1972) and the self transmissible plasmids have a set of genes coding for transfer functions (*tra* genes) (Achtman and Helmuth 1975). A functional classification of plasmids is presented as a Venn diagram, (Fig.1.1) which shows some of the sets of functions permuted among the known plasmids. An independent classification of plasmids is based on their interaction with the plasmid F and its transfer (Watanabe *et al* 1966a). Plasmids which

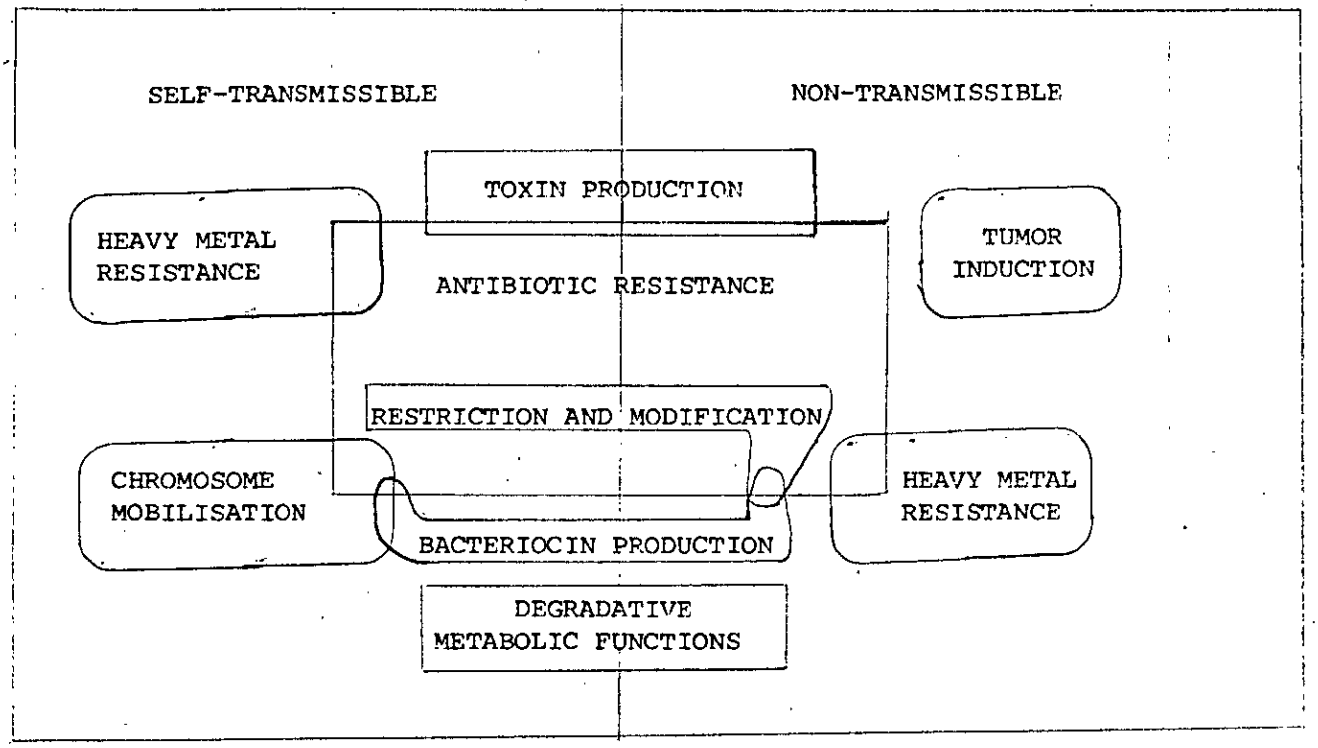


Fig.1.1 A Venn diagram of the distribution of functions among plasmids.

A comprehensive list of plasmid encoded functions is given by Novick et al (1976).

Other references are:

enterotoxin production, Smith & Halls (1968)
heavy metal resistance, Novick (1969)
Richmond & John (1964)
degradative metabolic functions, Rheinwald et al (1973)
tumor induction, Van Larebeke et al (1974)
bacteriocin production, Nomura (1967).

inhibit F transfer are called fi$^+$ and those which do not fi$^-$.

A further classification is based on the degree of relatedness of plasmids as indicated by their ability to co-exist as pairs in the same host (Datta 1975). This is called compatibility grouping. Plasmids of the same compatibility group are unable to coexist stably. So far, 25 compatibility groups have been identified (Novick et al 1976).

As a consequence of the several methods of classification there is as yet no systematic nomenclature, and plasmids are generally referred to by their isolation numbers.

The similarities and differences within and between functional and compatibility groups of plasmids have been examined by indirect comparisons of their nucleotide sequences. This has been done by DNA - DNA hybridization (Ingram 1973) and by electron microscopic analysis of DNA heteroduplexes (Davidson et al 1975). The latter studies have shown that many functionally related plasmids share considerable regions of nucleotide sequence homology, and in addition many contain specialised small segments of DNA some of which have been defined as insertion sequences (IS). In some cases these flank specific genetic determinants e.g. genes specifying antibiotic resistance. Determinants flanked in this way move readily from one plasmid to another independent of the host's recombination system (Heffron et al 1975) (Kopeco and Cohen 1975) and have been called transposons. They are grouped with insertion sequences under the heading translocation elements (Novick et al 1976). Insertion sequences appear to be involved in recombination with the host chromosome for the transfer of genes from one organism to another, and in the inter and intramolecular recombination which has produced the diversity of plasmids (for review see Kolata 1976 and Bukhari et al 1976) (see also Heffron et al 1975; and Cohen 1976).

In this thesis the term R plasmid will be used for any plasmid which carries an antibiotic resistance determinant regardless of the other functions it carries.

1.5(ii) The distribution of restriction and modification systems among plasmids

(a) E.coli Rplasmids

In a survey of 153 R plasmids Bannister (1969) found 10 groups

containing 61 plasmids in all, which conferred additional restriction properties on an E.coliK host. Those with the ability to restrict and modify a number of phages, in a superficially similar manner to the EcoK systems, fell into 2 groups. One group contained a number of plasmids which were fi$^-$ and which were subsequently all shown to be members of compatibility group N (Hedges 1972). The system carried by these plasmids was designated host specificity system II (hspII) and is identical with that described by Watanabe et al (1966b) and with the EcoRII system investigated by Yoshimori (1971). Not all N group plasmids carry hspII but there is a correlation between those which do and those which do not undergo thymineless elimination i.e. are not eliminated when a thy$^-$ host is starved for thymine (Birks and Pinney 1975). The hspII system when resident in E.coliK restricts K modified λ phage to give an efficiency of plating (e.o.p.) of 10^{-2}.

The other restriction and modification system discovered by Bannister (1969) was only found associated with one plasmid, R124. This is a self transmissible R plasmid and it is similar to the plasmid F (it is 65% homologous by DNA - DNA hybridization (H.Smith pers.comm.). It is the only member of compatibility group F IV (Datta and Hedges 1972). It is fi$^+$ and its transfer system is closely related to that of F, complementing mutants in all known transfer functions (Willetts pers.comm.). Resistance to tetracyclin is the only resistance determinant it carries and its restriction system, hspI, acts on phages T1, , Ø80 and P1. Phage λ is restricted to give an e.o.p. of 10^{-4}.

Of the remaining 8 groups of R plasmids which Bannister (1969) found were able to restrict but not modify one or more phage, it is possible that some restrict by other mechanisms than the breakdown of the infecting DNA. An example of this type of restriction is that directed against phage T7 by F, which acts by the blocking of phage specific transcription (Morrison and Malamy 1971). In a further survey of a series of freshly isolated R plasmids, Yoshimori (1971) identified the two restriction systems, originally designated RI and RII, which have come to be known as EcoRI and EcoRII. EcoRI was encoded by a single plasmid isolate (RY5) which was fi$^+$, and it restricted λ to

an e.o.p. of 10^{-4}. Thus EcoRI resembled hspI and by reviewers and researchers alike these two systems were assumed to be identical. The non identity of the two systems is the subject of part of this thesis. The original identity and subsequent lineage of the plasmid RY5 which codes for the EcoRI system has suffered much confusion. It is most likely that it was isolated as part of a plasmid aggregate carrying a number of antibiotic resistance markers and the fi^+ character. This segregated at various stages (Yoshimori 1971) and also the R plasmid RIdrd was added to the aggregate when it lost its self transmissibility, in order to mobilise it (Yoshimori et al 1972). This complex aggregate was analysed by Betlach et al (1975) and Smith et al (1975). Both groups showed that the EcoRI system is determined by a small (5.2 Md) non transmissible plasmid subsequently designated NTP13 which also carries the replicon and bacteriocin determinant of the plasmid colEI. It does not carry a drug resistance determinant. A derivative, NTP14, has been found which carries the ampicillin resistance determinant, owing presumably, to recombination between NTP13 and RI. It is unlikely that NTP13 was responsible for the fi^+ character of the original isolate. Also it is ironic that the EcoRI system is often referred to as being mediated by resistance transfer factor I (RTFI) when the plasmid responsible is neither self transmissible nor does it carry a resistance determinant.

(b) Other plasmid determined restriction and modification systems

The temperate bacteriophage P1 exists in the lysogenic state as a plasmid and confers on its host an additional restriction and modification system (Bertani 1953) which appears to be fundamentally different from the other systems studied so far (see sections 1.3(iv) and 1.6).

Plasmids mediating restriction and modification have been identified among strains of Salmonella typhimurium (Anderson et al 1968). They are of great importance in the phage typing systems used for the identification of Salmonellae (Anderson and Williams 1956) but none has so far been studied in sufficient detail to show whether the systems involved are related to those already described.

(c) The plasmid NI

NI (Watanabe et al 1966) carries a restriction system which is

different from that carried by N compatibility group plasmids in that it can restrict the non-glucosylated DNA of phage T4 DNA (Revel and Georgopoulos 1969). The EcoRII system of the N group plasmids does not act on the DNA of this phage whether glucosylated or not, presumably because it contains hydroxymethyl cytosine in place of cytosine (Kaplan and Nierlich 1974). In consequence, because of its name, NI has been a source of confusion in establishing the influence of methylation and hydroxymethylation of cytosine on restriction by EcoRII.

1.6. The genetics of restriction and modification

(a) General

Early investigators of the genetics of restriction and modification in E.coli found two classes of mutant which were phenotypically restrictionless. They occured at equal frequencies and at such a frequency as to suggest that both arose by single point mutations (Wood 1966) (Boyer 1964). The first class were deficient only in restriction (r^-) and the second in both restriction and modification (r^-m^-). They complemented one another in vivo (Boyer and Dussoix 1969) and in vitro, where the mixing of cell extracts restored restricting activity (Hadi and Yuan 1974). If the mutants were originally isolated in strains of different host specificities (E.coliK and B) the system reconstituted by complementation had the specificity of the r^- mutant. This suggested that the products of two cistrons are required for restriction. One of these is required also for modification and determines the specificity of the system. This supports the so called three gene model for restriction and modification, which emerged in 1969 (see Colson and Glover 1969) (Arber and Dussoix 1969). The model proposes that there are three determinants for restriction and modification: hsdr (host specificity determinant for restriction); hsds (specificity); and hsdm (modification). This model is consistent with the subunit structure of class I restriction endonucleases, which are an assembly of three different protein subunits in variable ratios (Eskin and Linn 1972), and the modification enzymes which contain two subunits, which are identical in size with two of those found in the endonuclease (Lautenberger and Linn 1972).

Further genetic analysis showed that the host specificity determinants of E.coliK and B not only complement but also recombine with one another (Glover and Colson 1969) and map in identical positions on their respective chromosomes (Glover and Colson 1969) (Hoekstra and de Haan 1965), that is, they are allelic. The E.coliA system and at least one system in Salmonella are allelic to the EcoK and B systems as demonstrated by map position.

Mutations in the proposed hsdm gene alone have not been isolated (Boyer and Dussoix 1969) nor have hsds mutants with altered specificity.

The determinants of the EcoP1 system are more complex. Two classes of modification deficient mutants were isolated (Rosner 1973), as mutants which gave clear plaques, since they were unable to modify their host and form lysogens. The two classes mapped at different sites on the P1 genome. It is predicted that more than three genes will be found to determine the EcoP1 system.

(b) The genetics of plasmid encoded restriction and modification systems

In their surveys of restriction and modification by plasmids, both Yoshimori (1971), and Bannister (1969) isolated phenotypically restrictionless mutants. Whereas Bannister's mutants of the hspI and hspII systems fell into the r^- and r^-m^- classes in approximately equal numbers, Yoshimori found only the r^- class. In the case of the hspI and EcoRI systems the disagreement could be explained if the two systems were different. This possibility appears to have been overlooked by reviewers of the field. In the case of the hspII and EcoRII systems which are identical the conflict is most likely due to the small number of r^- mutants on which Bannister made her observation. From a pool of 10 restriction deficient mutants she found 5 were r^- and 5 were r^-m^-. From this sample it is not possible to claim that the r^-m^- class necessarily arose at the same frequency as r^- and therefore this class may have resulted from deletions rather than single point mutations. Yoshimori's finding of only the r^- class for both the EcoRI and EcoRII systems suggested that they were fundamentally different from the class I systems previously studied, and correlated nicely with the subsequent finding that

the EcoRI and EcoRII systems differed from the class I systems in their endonucleolytic mechanisms.

CHAPTER 2

Materials and Methods

2.1 Materials

.1(i) Bacterial strains

A list of required strains and their relevant characters is given in table 2.1. Other strains were constructed by myself and are described in the experimental section.

.1(ii) Phage strains

The strain of λ wild type used was obtained from Dr.S Hattman of the University of Rochester N.Y.. It carries the thermo-inducible mutation C_I^{857} (Goldberg and Howe, 1969).

λ sk^o was obtained from Dr. N Murray. It is a hybrid of phages Ø80 and λ carrying the host range of Ø80 (structural genes) and the inmunity (control genes) of λ (Murray et al 1973). Also it contains no targets for the EcoK restriction system, and carries the thermoinducibility mutation C_I^{857}, and the lysis defective mutation Sam7 (suppressable by SuIII but not SuI).

λ clear (λc) was obtained from N.Murray. It will not lysogenise and will not propagate on a λ lysogen. λ virulent (λv) was obtained from N.Murray. It will not lysogenise but will grow on a λ lysogen.

.1(iii) Media

L broth Contained per litre: Difco tryptone 10g; Difco yeast extract 5g; NaCl 5g adjusted to pH 7.2 before autoclaving (Lennox 1955).

Porton Coli medium Contained per litre: tri-ammonium citrate 1.2g; yeast extract 1.5g; NaCl 2.4g; Na_2HPO_4 (anhydrous); NaH_2PO_4 $7H_2O$ 0.6g; $MgSO_4$ $7H_2O$ 1.0g; 2M K_2HPO_4 2.5ml; M $CaCl_2$ 1.0ml; M Ferric citrate 1.0ml; sterilised prior to the addition of the latter solutions and glycerol 14g.

LC Agar Contained L broth solidified with 1% Difco agar.

LB Agar Contained L broth solidified with 1.5% Difco agar.

BBL Agar Contained per litre: NaCl 5g; BBL trypticase peptone 10g; made solid with 1% Difco agar for bottom layer and 0.7% for top

Table 2.1

BACTERIAL STRAINS

Strain Designation	Relevant Characters	Source - Reference
E.coliK:		
803suIII	met$^-$ suIII$^+$ hsdr$_K^-$ hsdm$_K^-$	
1100	end1	Ditto, N Murray
1100 $T_{1,5r}$	end1: resistant to phages T_1 and T_5	Durwald and Hoffman-Burling (1968)
1100(λ sk^o)	end1, lysogenic for λsk^o	Ditto, N Murray
1100R245	end1 $T_{1,5r}$ containing the plasmid R245	Bigger et al (1973)
J53 R124	met, contains the plasmid R124	N Willets
WD9091	exonucleaseIII deficient; str^r;	Yajko and Weiss (1975) B Weiss
1100F$^+$(λ)mec$^-$	end1: lysogenic for C_I^{857} cytosine specific DNA methylase deficient	Hattman et al (1973)
1100F$^+$(λ)mec$^-$ N_3	ditto containing an hsdr$^-_{RII}$ hsdr$^+_{RII}$ derivative of the plasmid N_3	S Hattman
1100F$^+$(λ)	end1: lysogenic for C_I^{857}	"
5K	hsdrk$^-$ mk$^+$	S Glover
3736	str^r sensitive	S Mc Intyre
E.coliB251	λsensitive	S Meynard-Smith
E.coliC	wild type	N Murray

layer.

Low Phosphate Medium was prepared as required by mixing 100 volumes of solution A (KCl 1.5g/l; NaCl 5g/l; NH_4Cl 1g/l; Tris base 12.1g/l pH 7.4) with one volume of solution B (20% w/v bacto peptone dephosphated by precipitation at pH 9) made 0.4% in glucose and 1mM in Mg SO_4. (similar to the medium of Grosman 1967).

Pmedium was prepared by mixing: 25 ml of pstock (0.02M KPO_4 pH 7.0; 0.015M $(NH_4)_2SO_4$); 0.25ml 0.1M $MgSO_4$; 0.43ml 10^{-4}M $FeSO_4$; 0.125ml 20% glucose; and 0.1ml 20% acid hydrolysed casein.

Amino acid supplements were at a level of 50 ug/ml.

Minimal medium Contained per litre: 250ml of 4xM9 salts (Na_2HPO_4, 28g; KH_2PO_4, 12g; NaCl, 2g; NH_4Cl 4g); 8ml M $MgSO_4$; 10ml 20% glucose, and for the solid form for plates contained 3% agar.

.1(iv) Radiochemicals Carrier free P^{32} orthophosphate . H^3-thymidine at 16Ci/mmole . P^{32} labelled ATP at 15-20 Ci/mmole; were all obtained from the Radiochemical Centre Amersham, Bucks.

.1(v) Chemicals

ATP was purchased from Boehringer Corporation Ltd and was stored frozen in 0.1M solution at pH 8.

S-adenosyl methionine (SAM) was purchased from Sigma Chemical Corp. It was purified before use according to Meselson and Yuan (1968) and was stored at -20° in 4M acetic acid.

.1(vi) Enzymes

Bacterial alkaline phosphatase; Pancreatic deoxyribonuclease1 (ribonucleasefree); and venom phosphodiesterase were purchased from Worthington Biochemicals Corp. Freehold, New Jersey. They were stored at -20° dry or in aqueous solution. Polynucleotide kinase was prepared by Mr. A.Morrison by the method of Richardson (1965) from cells of E.coli infected with phage T_4amN122 (Hughes and Brown 1973).

Restriction Enzymes: EcoRI was prepared in this laboratory by different individuals and groups at various times, by an evolving method based on that of Yoshimori (1971) (see also Greene et al 1974). The enzyme was isolated from E.coliJK29 (strain 1100 $T_{1,5r}$

containing the small colicinogenic plasmid described in section (1.5) grown in Porton coli medium to a yield of 20-25g/1 wet weight.

EcoRII was prepared by the method of Yoshimori (1971) which is discussed further in section 3.1. Some batches of enzyme were obtained from other workers and some were prepared in collaboration with colleagues. Cells of 1100R245 grown in either Lbroth or Porton coli medium were used.

AvaI and AvaII were isolated from Anabaena variabilis as described by Murray et al (1976).

HindIII was obtained from Dr. H.Cook or Dr. H.Smith.

SalI was prepared by Karen Brown by a method devised by R.Roberts of Coldspring Harbor Laboratory (unpublished) from Streptomyces albus.

HpaII was a gift from A.Morrison.

1.1(vii) Antibiotics

Tetracyclin (tet) (Achromycin HCl) was purchased from Cyanamid, London, UK. and was used at a concentration of 50 ug/ml. A stock solution at 50 mg/ml was stoned at -20°.

Streptomycin sulphate was obtained from Glaxo Laboratories UK. It was used at 100 ug/ml for selection and was stored as a 100mg/ml solution at 4°. For nucleic acid precipitation, solutions were made up fresh at 5% w/v in E buffer.

.1(viii) Buffer solutions

E buffer used in gel electrophoresis was 0.04M tris base; 0.02M sodium acetate 2mM disodium EDTA, adjusted to pH 8.4 with acetic acid. It was made up and stored at 10x this concentration (Hayward and Smith 1972).

Extraction buffer contained 10mM tris HCl pH 7.4; $2x10^{-4}$M $MgSO_4$; 10^{-4}M disodiumEDTA; $2x10^{-3}$M 2-mercaptoethanol.

Phage buffer contained per litre: KH_2PO_4, 3g; Na_2HPO_4 (anhydrous), 7g; NaCl, 5g; 10ml 0.1M $MgSO_4$; 10ml 0.01M $CaCl_2$; 1ml 1% gelatin solution.

SSC - standard saline citrate contained per litre: 8.76g NaCl; 4.41g Na_3 citrate; adjusted to pH 7.0.

PE (phosphate extract) buffer contained: 10mM phosphate buffer pH7.0; mM EDTA; 7mM 2-mercaptoethanol.

TEC 30% triethylamine carbonate was prepared from distilled triethylamine by bubbling CO_2 through a mixture of equal volumes of triethylamine and water until the two phases became one. The solution was diluted to 30% by, addition of water and 3% extra triethylamine was added.

T4 ligase cocktail was prepared by mixing the following solutions: µl/ml

M tris HCl pH 7.5	660 "
0.4M EDTA pH 9.0	25 "
M $MgCl_2$	100 "
50mg/ml BSA	20 "
M dithiothreitol	100 "
0.1M ATP	10 "

2.2 Methods

.2(i) Bacterial Culture Methods

Small scale cultures (<21) were grown in conical flasks containing 1/10 of their volume of culture, and were aerated by shaking on an orbital, shaker (L H Engineering Co. Stoke Poges, UK., MK5) at 3.5 Hz. Growth was followed by measuring E^{650} (for broth grown cells an E^{650} of $1 \cong 10^9$ cells/ml) and cells were harvested by centrifugation at 10,000xg for 15 min at 4° an 8x50ml rotor in an MSE HS18 centrifuge.

Large scale cultures were grown in a 50l batch fermenter (Biotec FM50 Twin) with sparged aeration. The aeration rate (stirrer speed and air flow rate) was increased progressively as cultures grew so as to avoid oxygen limitation and retain the culture at maximum growth rate to the maximum possible cell density. Using LBroth yields of around, 2g/l were obtained and with Porton coli medium yeilds of up to 15g/l were obtained. A 1% volume inoculum was used. Cells were harvested in an Alfa Laval 102B continuous flow centrifuge and the cell pellet stored at -20°.

Methods

2.2(i) Cell Disruption

b. Sonication

Packed cells were resuspended in 2 volumes of buffer (this contains 2-mercaptoethanol which protects enzymes during sonication). The suspension was sonicated in a glass beaker, ($\frac{1}{2}$" step horn probe) at maximum power output (about 4 acoustic watts). Bursts of 2 minutes of sonication were given, in between which the sonicate was left to cool. In this way the temperature of the sonicate was maintained below 12°. Cell disruption was monitored by following the release of 280 nm absorbing material into solution. Samples (1ml) of sonicate were centrifuged (Quickfit micro centrifuge 2 min) and the supernatants diluted(1 in 200 in extract buffer and their E^{280} read and plotted against time of sonication. When the slope of the resultant protein release curve began to fall, sonication was stopped (see Fig 4.2)A.Branson S7 sonicator was used.

.2(ii) Preparation of Phage and Phage DNA

Cultures of E.coli lysogenic for λc_I^{857} were grown in L broth at 30° to a cell density of $4x10^8$ cells per ml. They were then incubated at 42° for 15 min to induce the phage and thereafter at 37° until lysis was complete (60-90min). Lysates were cleared of bacterial debris by centrifugation at 10,000xg in an MSE HS18 centrifuge for 15min.. Small volume lysates ($\leq$50ml) were made up to 41.5 w/w with caesium chloride directly, and centrifuged to equilibrium (48h at 75,000xg in an MSE SS65 centrifuge in a 3x23ml rotor). Large volume lysates were pelleted at 42,000xg in a 10x100 rotor over three hours and the pellets resuspended by gentle shaking in phage buffer (5ml) at 4° over night. The suspension was then centrifuged (MSE HS18 10,000xg for 15 min in a 8x50ml rotor) and the pellet re-extracted with a further 5ml. of phage buffer. The extracts were pooled and made up to 10 ug/ml with DNAseI and RNAseA and left to stand at room temperature for one hour. The pooled extracts were then cleared by centrifugation (MSE HS18 8x50ml rotor 12,000xg 30 min) and further treated as for small volume lysates.

Phage bands from equilibrium density gradient centrifugation

were detected visually and collected either by pasteur pipette from the top or by hypodermic syringe through the side of the centrifuge tube. Collected bands were pooled, diluted to 5ml with 41.5% CsCl and recentrifuged to equilibrium at 95,000 xg in a 3x5ml rotor for 24h at 4°. Phage bands were collected in the smallest possible volume and dialysed against DNA buffer at 4° to remove CsCl.

The phage preparation was then diluted (if necessary with DNA buffer until a slight Tindall effect was just visible. DNA was then extracted by the method of Kaiser and Hogness (1960) using freshly redistilled phenol equilibrated with 0.5M trisHCl pH 8 and three cycles of extraction. Residual phenol was removed by exhaustive dialysis against DNAbuffer. The concentration and relative purity of the resulting DNA preparation was established by measuring its E^{280} and E^{259}. Preparations routinely had E^{259} between 1 and 5 and E^{258}/E^{280} of 1.9 to 2. DNA preparations were stored at 0-4°.

(ii) Radioactive Phage DNA preparation

In the preparation of isotopically labelled phage DNA use was made of the S_7 mutation which is carried by λSK°. Since phage carrying this mutation do not lyse the host the phage may be concentrated by pelleting lysogenic bacteria two hours after induction at 42°: This avoids the problems of handling large volumes of radioactive lysates in fixed angle ultracentrifuge rotors. The cell pellet was resuspended in 5ml of phage buffer and the cells lysed by incubation with chloroform (0.1ml) for 1h at 0°. Bacterial debris was removed by centrifugation at 10,000xg at 4° for 15 min. The phage containing supernatant was thereafter purified exactly as for unlabelled DNA.

For the preparation of P^{32} labelled DNA lysogens were first grown to a cell density of $4x10^8$ cells/ml, induced at 42° for 15 min and the cells harvested by centrifugation (10,000xg 15 min). The cells were then resuspended in ½ the original culture volume of low phosphate medium and incubated at 37° for 2h following the addition of P^{32} orthophosphate (0.01 mCi/ml).

For the preparation of H^3 labelled DNA cells were grown up and induced normally, and H^3 thymidine (0.1 mCi/ml) was added prior to incubation at 37°.

Levels of incorporation were variable. P^{32} preparations generally contained 10^4-10^5 cpm/ug of DNA. Tritiated preparations were in the range of $2x10^3$-10^4 cpm/ug.

2.2(ii) Preparation of tritiated phage DNA by plate lysis

A sample of a late log phase culture of the appropriately modifying bacterium (3-5ml), and 1.5ml of a dilution of the required phage (10^5-10^6 pfu/ml) were added to 35ml of molten BBL top layer agar. 5mCi of ^3H thymidine were then added and the top layer spread on 15 BBL plates and allowed to set. The plates were then incubated at 37° for 8h by which time confluent lysis had been obtained. The phage were eluted from the plates by overlaying each with 2ml of phage buffer and leaving then to stand at 4° overnight. The phage buffer was then decanted and phage recovered as per a normal phage lysate.

.2(iii) The Titre of Phage and the Estimation of Efficiecies of Plating

Serial ten fold dilutions of phage lysates or preparations were made in phage buffer and 0.1ml of each dilution was added to 0.2ml of a fresh L.broth culture of a suitable plating host (1100 and derivatives for λ wild type and 803 suIII for λsk°). Each mixture was added to 2.5ml of molten LC top layer agar at 42° and poured onto the surface of a plate of BBL agar. The plate was incubated for 8-14h and the number of plaques formed at each dilution counted. The number of plaque forming units per ml (viable phage per ml-p.f.u./ml) = number of plaques per plate x the reciprocal of the dilution x 10 (to multiply the sample volume to 1ml). Efficiencies of plating were measured by titring the same set of serial dilutions of a phage on a restricting and non restricting host. When e.o.p.s were used to check the restriction efficiency and specificity of a host, controls of an appropriately modified phage and a known restricting host were run in parallel. When e.o.p.s were used to check the completeness or specificity of modification of a phage an additional control of an unmodified phage was run, and plating strains, if they contained plasmids were grown on selective media.

.2(iv) Preparation of Plasmid DNA

The polyethyleneglycol precipitation method of Humphries et al (1974) was followed. Their detailed protocol makes use of the method of Clewel and Helinski (1969) for cell lysis and the preparation of cleared lysates. Following dye-CsCl buoyant density equilibrium centrifugation, ethidium bromide was removed from the plasmid DNA either by extraction with iso-amyl alcohol (saturated with distilled H_2O) still in the presence of CsCl or by dialysis against the sodium form of Dowex 50-X8 ion exchange resin (see Thompson et al 1974). The former method gave a higher proportion of circular molecules in the final DNA preparation. Yields of DNA varied from 50 ug to 300 ug from a litre of cells and appeared to vary inversely with the severity of centrifugation required to obtain a firm pellet when clearing the lysate. Plasmid DNAs were evaluated and stored as for phage DNA.

.2(v) Plasmid Transfer

Over night cultures of the plasmid carrying donor and the intended recipient were diluted into LBroth and grown to a density of 2×10^8 cells/ml. Equal volumes of each were mixed and left to stand for 2h at 37°. The mixed culture was then diluted serially in phage buffer and plated on plates designed to select for the growth of a strain with the phenotypic properties of the recipient plus the plasmid, and to select against the donor. The mating mixture was also plated so as to allow growth of the donor alone and the recipient alone so as to determine the numbers of each at the time of plating and to check the effectiveness of the selection.

Colonies which appeared after incubation on selective/contra-selective plates were scored and the frequency of transfer per donor calculated. A few (5-10) of the selected colonies were picked and cloned twice on selective plates after which they were checked for retention of characters of the recipient, and characters of the plasmid other than those used in the selection. For the plasmid this generally involved checking the specificity of its restriction system.

.2(vi) Construction of λLysogens

Strains used for the construction of lysogens were 1100, 1100

$T_{1,5s}$ and B251. Lawns of these strains were spotted with a drop of a phage suspension containing 10^9 p.f.u. of the apropriate phage and incubated over-night at 30°. Cells from the centre of the spot were streaked on a plate which had been previously spread with 10^8 p.f.u. of λc and grown up at 30°. Surviving clones were purified by restreaking and then checked for lysogeny and the production of phage following induction at 42°.

.2(vii) Zone Electrophoresis on Agarose Gels

The use of agarose gels for the electrophoretic separation of DNA fragments was described by Hayward and Smith (1972). A method utilising slab gels of 1% agarose based on that of Hayward and Smith as modified by Sharp et al (1973) was devised in this laboratory and is described in detail by Thompson et al (1974). Gels were 38cmx17cmx4mm thick and were run in either the long or the short dimension. DNA was visualised on gels by ultraviolet fluorescence with ethidium bromide (Sharp et al 1973) or by autoradiography of the dried gel (when radioactively labelled DNA was used). Gels were dried onto Whatman 3MM paper under suction whilst heated over a boiling water bath, or directly in a hot air oven (70°) onto a glass plate. Gels were photographed by UV fluorescence in a ChromatoVue box (UV Products Inc. onto Ilford FP4 film, through a 4x red filter, and with an exposure of 5 min at f 4.5.

.2(viii) Zone Electrophoresis on Poly-acrylamide Gels

The procedure adopted for the setting up and running of poly-acrylamide slab gels was identical to that for agarose gels with the following exceptions:

The gelling mixture contained; 5% acrylamide 1% bis-acrylamide; 0.05% ammonium persulphate; 0.05% TEMED (N N N'N' tetra-methyl-ethylene-diamine); all w/v in Ebuffer.

The gel was 15cm longx8cm widex4mm thick and was run at a current of 10ma for 14h. Precoating of the glass plates was omitted.

Polyacrylamide gels were dried by suction onto 3MM paper and autoradiographed.

.2(ix) Assay of Restriction Endonucleases

a. Class I restriction enzymes were assayed by incubation with differentially labelled modified and unmodified DNAs in the presence of SAM and ATP. The reaction mixture contained per ml: 5 ul M $MgCl_2$; 0.5 ul 0.4M EDTA pH 9.3; 5 ul 0.1M 2-mercaptoethanol; 50 ul M tris-HCl pH 7.5; 940 ul distilled H_2O, to which was added 5 ul 15mM SAM in 4M acetic acid; 20 ul M tris base to neutralise; 10 ul 0.1M ATP. Each assay contained 100 ul of the above mixture plus 2-3 ug of H^3 unmodified DNA and 0.3-0.5 ug of P^{32} modified DNA. The volume of enzyme sample added varied from 1 ul to 20 ul depending on the concentration of the fraction being assayed. Incubation was at 37° for 5-60min. Each digest was analysed by sucrose density gradient centrifugation for the selective reduction of the sedimentation velocity (molecular weight) of the unmodified λDNA, as described by Meselson and Yuan (1968).

1300 ul digests were layered on top of 2.3ml gradients (6-20% sucrose in 10mM tris HCl pH 7.5, 0.02% sodium dodecyl sulphate mM EDTA) and centrifuged at 50000rpm in a 3x3ml swinging bucket rotor at 20° for 2h. Each gradient was collected as 4 drop fractions from the bottom of the tube onto 2.1 cm glass fibre filter disks which were dried and counted in butyl PPD scintillant in a BeckmanLS-230 liquid scintillation counter. Counts of tritium and P^{32} were plotted against fraction number.

This assay is sensitive to the presence of non-specific nucleases and thus cannot give a quantitative assay for partially pure fractions of enzyme during a preparation.

b. Class II restriction enzymes were assayed by incubation with unmodified DNA followed by analysis of the digest by agarose gel electrophoresis. In this way it was possible to establish the amount of a pure enzyme preparation required to completely digest a given amount (1 ug) of a particular substrate DNA. This figure was roughly applicable to other substrates. The assay cannot be regarded as quantitative for partially pure enzyme fractions.

Incubation conditions for the various class II restriction endonucleases were as follows: EcoRI, HindIII, SalI, - 10mM Tris-HCl pH 7.5, 8mM $MgCl_2$, 5mM 2-mercaptoethanol at 37° for 1h, in a

volume of 20-40 ul depending on the concentration of the DNA and enzyme samples used; EcoRII - 5mM $MgCl_2$, 25mM Tris-HCl pH 7.4 at 37° for 2h; AvaI, AvaII - 20mM Tris-HCl, 10mM $MgCl_2$, 37° 1h. Reactions were terminated by heating at 75° for 5 min or by the addition of an excess of EDTA to titrate out the Mg^{++} ion.

.2(x) Ion Exchange Separation Methods

The general advice of Himmelhoch (1971) on the use of ion-exchange media in protein separation was followed.

The (Whatman) ion-exchange celluloses DEAE (DE52) and phosphocellulose (P11) were purchased through H.Reeve Angel & Co.Ltd., Islington London U.K.. DE52 was used without precycling but was pre-equilibrated with 10x the buffer concentration to be used in the ensuing separation, before being carefully equilibrated at the correct concentration. P11 was precycled according to Burgess (1969), and then equilibrated in the apropriate buffer. Great care was taken to ensure that complete equilibration had been attained.

.2(xi) Transformation/Transfection Procedure

The procedure of Jacob and Hobbs unpublished, see Bork et al 1976, which is a derivative of the method of Mandel and Higa (1970) for the uptake of DNA by calcium treated cells of E.coli was followed. E.coli (strain C or 803suIII) was grown up over-night in Pmedium then diluted into 25ml of fresh Pmedium and grown to a cell density $6x10^8$/ml. The culture was cooled in ice for 10 min and the cells then harvested and resuspended in 12.5ml cold 0.1M $CaCl_2$. The cell suspension was left in ice for 20 min. Then the cells were again harvested and resuspended in 2.5ml cold 0.1M $CaCl_2$. After 20 min at 0° these cells were competent and ready for use. Competent cells (0.2ml) were added to 0.1ml of SSC containing 0.01-0.1 ug of the relevant DNA in a 12mm bacteriological test tube. The mixture was incubated at 37° for thirty seconds and then stood in ice for 2h before plating. Transfections were serially diluted and 0.1ml samples plated in BHL top layer agar made mM with respect to Mg^{++} ion. Plasmid transformations were plated as described in section 4.5(iv).

With wild type λ DNA transfection frequencies of ~ 3000 transformants per ug were obtained.

.2(xii) P^{32} 5'Terminal Labelling

The method of Richardson (1966) as adopted in this laboratory (Murray 1973) was used.

For the 5'terminal labelling of whole λ molecules, freshly isolated DNA was used, the labelled DNA was separated from excess P^{32} ATP by extensive dialysis against M NaCl in DNAbuffer rather than by sucrose gradient centrifugation or gel filtration as described by Murray (1973). It was found that any residual γP^{32} ATP and Free Pi^{32} were well separated from labelled DNA in the gel electrophoresis experiments in which the DNA was used and did not constitute a problem. Labelled restricted fragments of DNA were separated from γP^{32} ATP by gel filtration as described in detail by Bigger et al (1974), followed by dialysis. Preparations of whole λ were labelled to a level of 100 - 1000 cpm/µg.

.2(xiii) Terminal Nucleotide Determination

Fractionated terminally labelled DNA samples were first digested with pancreatic DNAse and venom phosphodiesterase (the conditions used for this are given in detail with each experiment, and nucleotides recovered by adsorbtion to the points of ⌂ shaped pieces of AE81 ion-exchange paper which were washed in distilled water. (Sanger et al 1965). Adsorbed nucleotides were eluted with 30% TEC into capillaries and the eluate (200 ul) dried down under vacuum on a polythene sheet. The dried spots were taken up in distilled water and redried three times before being applied in a minimum volume to AE paper. Nucleotides were then separated by electrophoresis under the conditions specified by Sanger et al (1965). Mononucleotide markers were included and the blue dye marker was run a distance of 25cm. Following electrophoresis mononucleotide markers were located by UV fluorescence and marked with radioactive ink The electrophoretogamme was then radioautographed (for up to one month) and the labelled mononucleotides identified.

.2(xiv) Exonuclease assay

Uniformly labelled ^{32}P DNA (sufficient to give about 20,000cpm) was incubated with samples suspected of containing exonuclease activity under the same conditions as were used for the assay of the appropriate class II or class I restriction endonuclease. The digest was applied

to a strip (2cmx12cm) of polyethyleneimine thin layer chromatography medium (Whatman) which was developed in 2M HCl. The strip was then dried and the radioactivity moving with the solvent front (acid soluble) and remaining at the origin (acid insoluble) were determined by scintillation counting and compared with a control of undigested DNA. For a particular set of assays the results were expressed as the % age of the in put DNA made acid soluble in a given time.

CHAPTER 3

Experimental results and conclusions I:

Some properties of restriction endonuclease EcoRII

3.1 The purification of EcoRII

EcoRII was purified by the procedure devised by Yoshimori (1971) which is shown schematically in figure 3.1. In an initial purification fractions were assayed both by sucrose gradient analysis of mixedly digested, differentially labelled, modified and unmodified λDNA (as for class I enzymes) and by gel electrophoretic analysis of digested unmodified DNA. This showed (fig. 3.2) that as early as the streptomycin sulphate precipitation step of the purification the EcoRII containing fractions, when used to digest λDNA, gave a series of fragments which could be separated by gel electrophoresis, to give a characteristic band pattern. Subsequently therefore the latter assay was adopted for the routine purification of EcoRII.

Before being used in experiments EcoRII prepared as described above was tested, to ensure that it cut DNA at the same nucleotide sequences as the enzyme isolated by Bigger et al (1973) and Hedgpeth et al (1973). The purified enzyme was used to digest λDNA, and the resulting fragments were ^{32}P labelled at their 5'termini. Electrophoretic analysis of pancreatic DNAse and venom phosphodiesterase/pancreatic DNAse digests of the labelled DNA, (Fig 3.2a) showed that the predominant labelled nucleotide was pC, and that the small number of labelled oligonucleotides generated by pancreatic DNA'se were consistent with the 5'terminal sequences determined by Bigger et al (1973) Table 3.1. Unaccountable labelled oligonucleotides were not observed, which showed that the enzyme was EcoRII and in addition was free from other sequence specific or non-specific endonuclease, or significant exonuclease activity. Small amounts of the mononucleotides pG and pA were observed but could be accounted for as coming from the natural 5'ends of the λchromosome.

3.2 The sensitivity of λDNA to purified EcoRII

The results presented in this section have been published (Hughes and Hattman 1975). The extent of cleavage of λDNA in limit

Fig. 3.1

The Purification of EcoRII

1) Cells of 1100R245 resuspended in 2 volumes of PE buffer and disrupted by sonication.

2) Centrifugation 100,000xg, 1h, in MSE SS65

   supernatant — pellet discarded

3) Streptomycin sulphate precipitation (as in section 4.3)

   supernatant — pellet discarded

4) Ammonium sulphate fractionation

   0% to 50% cut

5) DEAE cellulose chromatography elution with a 0.2to 0.2M NaCl gradient. Fractions eluting between 0.14 and 0.17M pooled and dialysed against PE buffer + 30% glycerol.

6) Phosphocellulose chromatography , active fractioms eluted between 0.2 and 0.25M NaCl, pooled and dialysed as above.

7) Concentration on a small DEAE cellulose column eluted with 0.5M NaCl

8) Active fraction stored at -20° in 30% glycerol.

Legend to Fig. 3.2

Assays of EcoRII

Successive fractions off the DEAE-cellulose column which showed EcoRII activity in sucrose gradient assays were used to digest uniformly ^{32}P labelled λsk° DNA.

Each digest contained 2 µg of DNA, and 10 µl of a column fraction which contained enzyme, and was carried out under the conditions given in section 2.2(ix)b. The digests were analysed by electrophoresis on 1% agarose gel (tracks 2-9). The gel was dried and autoradiographed, and the autoradiograph photographed.

Track 1 shows a HindIII digest of λsk° DNA for comparison.

Track 10 shows a control of undigested λsk° DNA.

Track 11 shows a digest of 2 µg of λsk° DNA with 5 µl of a phosphocellulose fraction of EcoRII.

Track 12 shows a digest of 2 µg of unlabelled λ.k DNA with 10 ul of the 0 % to 50% ammonium sulphate fraction of an isolate of 1100R245. Here the gel was photographed by fluorescence with ethidium bromide. A characteristic EcoRII digestion pattern is visible even at this early stage in the purification.

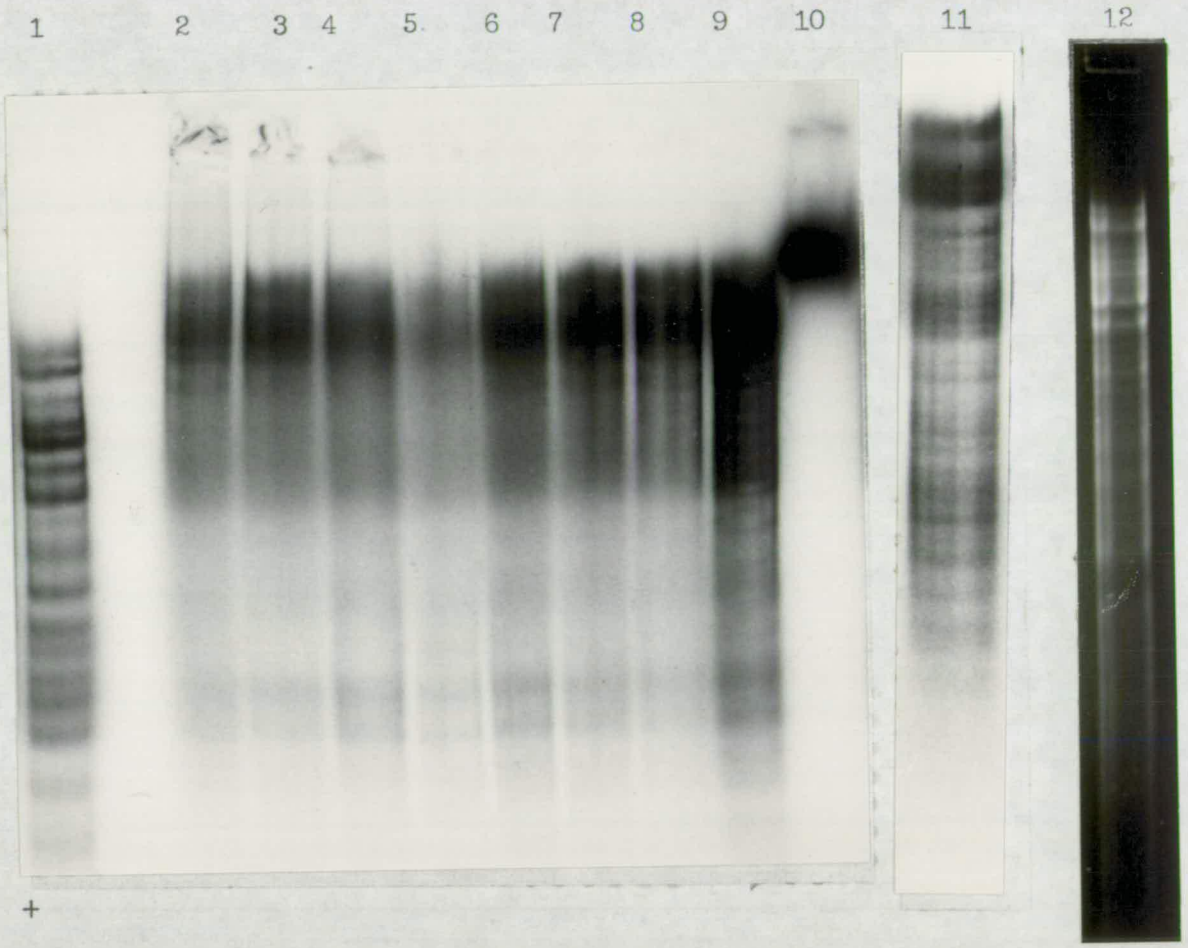


Fig.3.2 Assays of EcoRII by agarose gel electrophoresis , at various stages in its purification

Legend to Fig. 3.2a

Analysis of radioactive nucleotides generated from 5'terminally labelled EcoRII fragments of λ.k and λsk° DNA by digestion with pancreatic DNAse and vennom phosphodiesterase

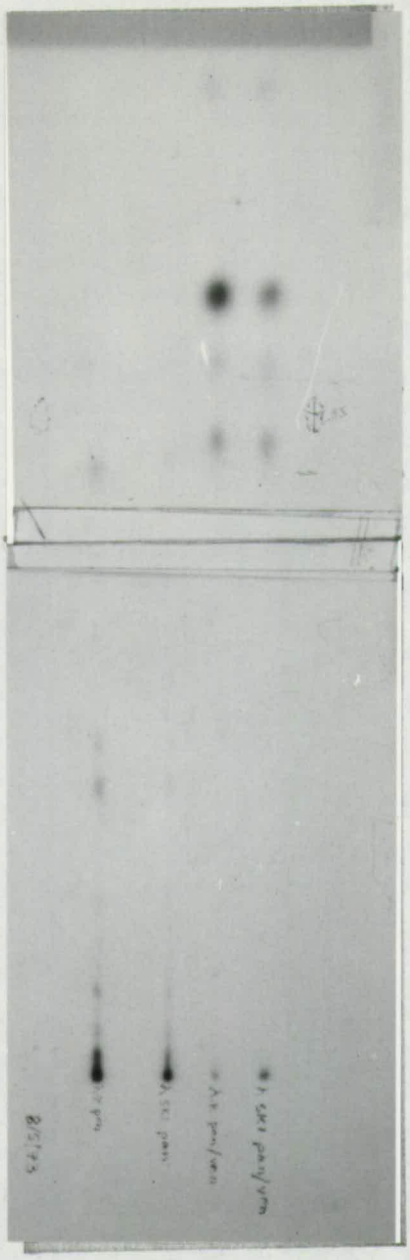
λ.k and λsk° DNAs were digested with EcoRII (concentrated phosphocellulose fraction), and the resulting fragments labelled at their 5' ends and purified as described by Bigger et al 1973. Samples of 0.25 ug of each DNA (about 2,000 cpm) were made up to 1mg/ml with pancreatic DNAse or vennom phosphodiesterase plus pancreatic DNAse and adjusted to 5mM with respect to $MgSO_4$ and 40mM with respect to trisHCl pH 7 prior to incubation at 37° for 2h. The digests were spotted onto AE 81 paper as follows:

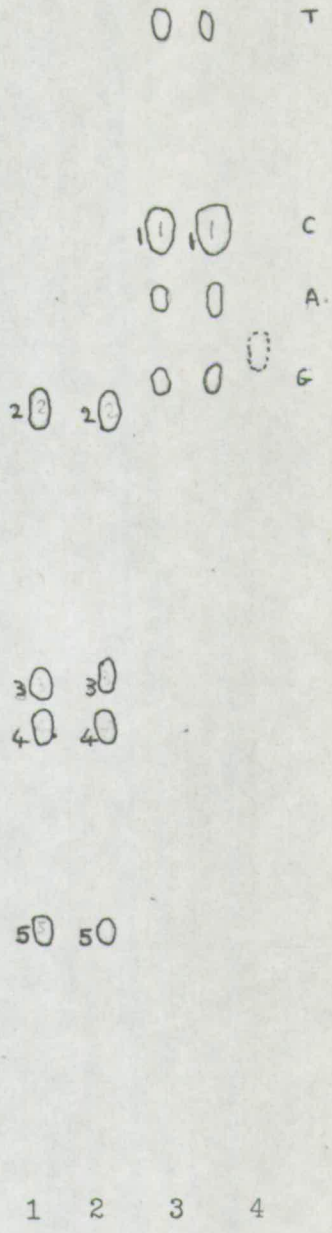
Track 1, λ.k with pancreatic DNAse,
2, λsk° with pancreatic DNAse,
3, λ.k with vennom phosphodiesterase + pancreatic DNAse,
4, λsk° with vennom phosphodiesterase + pancreatic DNAse

and electrophoresed at 2.5kV until the blue component of the xyanol XCFF marker had moved 25cm. The electrophoretogramme was dried, and autoradiographed for 2 weeks and the mobilities of each of the labelled nucleotides (numbered 1 through 5 on the tracing of the electrophoretogramme) compared with the blue marker, and tabulated below.

Table 3.1

nucleotide	mobility relative to blue (rf)	probable identity	rf according to Bigger et al 1973
1	1.17	PC	
2	0.901	PC-C	0.9
3	0.49	PC-C-T	0.5
4	0.43	PC-C-A	0.43
5	0.12	pCCAG/CCTG	0.13





1 2 3 4 1 2 3 4

Fig.3.2a Analysis of radioactive nucleotides generated from 5' ^{32}P labelled EcoRII fragments of λDNA and λsk° DNA , by digestion with pancreatic DNA'se and phosphodiesterase (from snake vennom.).

digests with EcoRII, was found to depend on the host upon which the phage was propagated, over and above the effect of EcoRII specific modification. A suggestion that this might be expected came from the work of Hattman et al (1973). They isolated mutants of E.coliK which as hosts to λ, produced phage which were 3 orders of magnitude more efficiently restricted by the EcoRII system than phage propagated on the wild type. These mutants were subsequently shown to be deficient in a cytosine specific DNA methylase and they were designated mec⁻, and the enzyme involved the mec methylase.

Comparison of the sensitivities of the DNAs of phages propagated on E.coli 1100 mec⁻ (λ.Kmec⁻) and the wild type (λ.K) to EcoRII (fig. 3.3), showed that at the same enzyme to DNA ratio λ.Kmec⁻ DNA is more extensively cut than λ.K DNA. Moreover, over the range of enzyme to DNA ratios used in the experiment, λ.K gave a consistent limit digest, whereas λ.Kmec⁻ DNA was more extensively degraded by increased treatment. In addition, the limit digest of λ.K DNA contained a larger number of fragments in the size range from 13 Md to 3.48 Md than could be accommodated in the λgenome, showing that the digest contained a large number of partially digested fragments. These results suggest that λ.K is partially protected against EcoRII, as a result of methylation at a random fraction of the .EcoRII recognition sequences, by the mec methylase.

The difference in sensitivity of λ.Kmec⁻ and λ.K to EcoRII is a property of the substrate DNA and not attributable to a previously unidentified endonuclease in the EcoRII preparation, since the DNA of λ.Kmec⁻. RII (propagated on a mec⁻ host harbouring the plasmid N3) was not degraded at all by EcoRII (fig. 3.3).

The observed difference in sensitivities could still be explained by the presence of a co-factor or inhibitor in either DNA preparation. However, the difference was observed with several DNA preparations, and the experiment in which uniformly ^{32}P labelled λ.B DNA and cold λ.K DNA were mixedly digested with EcoRII (fig. 3.4) argues against this possibility. E.coliB does not have a cytosine specific DNA methylase (Hattman 1972) and λ.B behaves in every way like λ.Kmec⁻ (see section 3.4). In a mixed digest, λ.B was more extensively degraded than λ.K (fig. 3.4).

Legend to Plate 3.3

Agarose gel electrophoresis of EcoRII digests of λ.k and λ mec⁻ DNAs

Tracks 1-9 were all run on the same gel.

Tracks 1, 2 and 3 show digests of mec⁻ DNA (2ug) and tracks 4, 5 and 6 show digests of λ.k DNA (2 µg). Treatment was as follows: tracks 1 and 4, 1 µl of EcoRII for 1h; tracks 2 and 5, 2 µl of EcoRII for 1h; tracks 3 and 6, 2 µl of EcoRII for 2.5h.

Track 7 contains untreated λ mec⁻ DNA.

Track 8 contains 2 µg of λ mec⁻ .RII (propagated on a mec⁻ strain harbouring an RII type R plasmid, and therefore RII modified) incubated for 2.5h with 2 µl of EcoRII.

Track 9 contains a digest of 2 µg λ mec⁻ digested with EcoRI which gives fragments of known molecular weight (Thomas and Davies 1975) which can be used to estimate the sizes of other fragments on the gel.

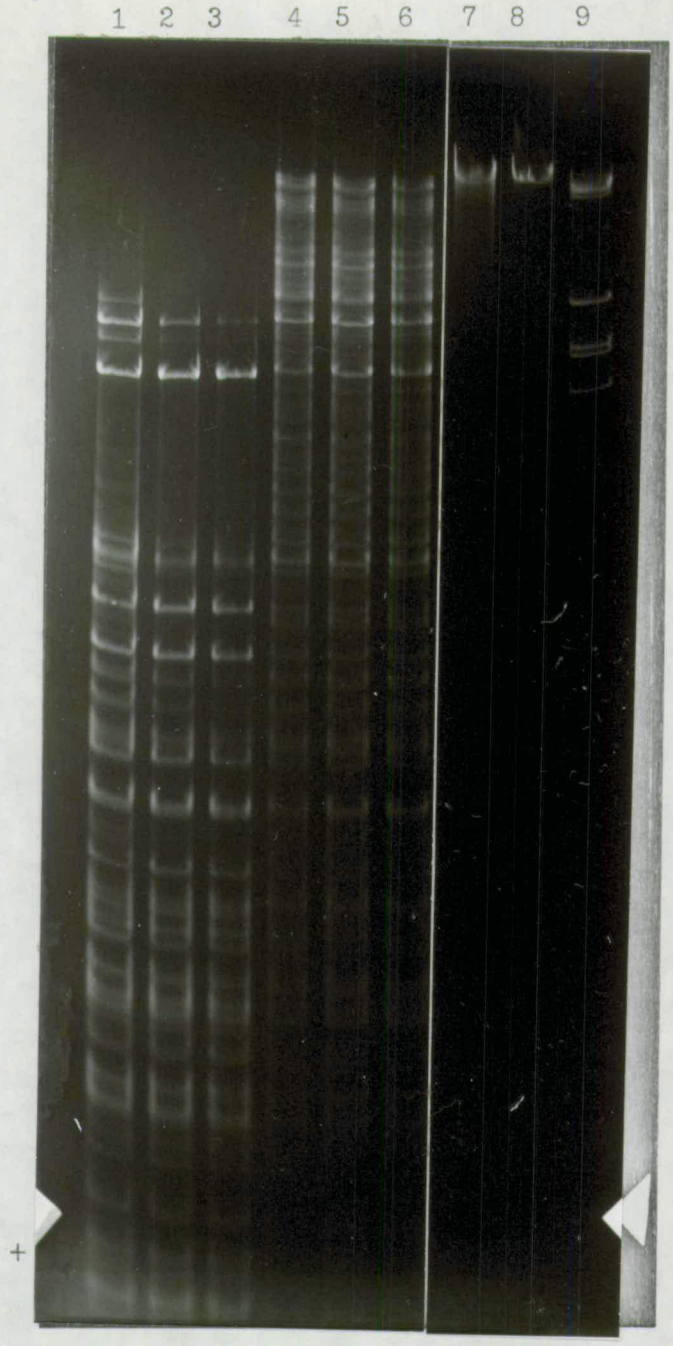


Fig.3.3 Agarose gel analysis of *Eco*RII digests of λ.K and λ*mec*⁻ DNAs

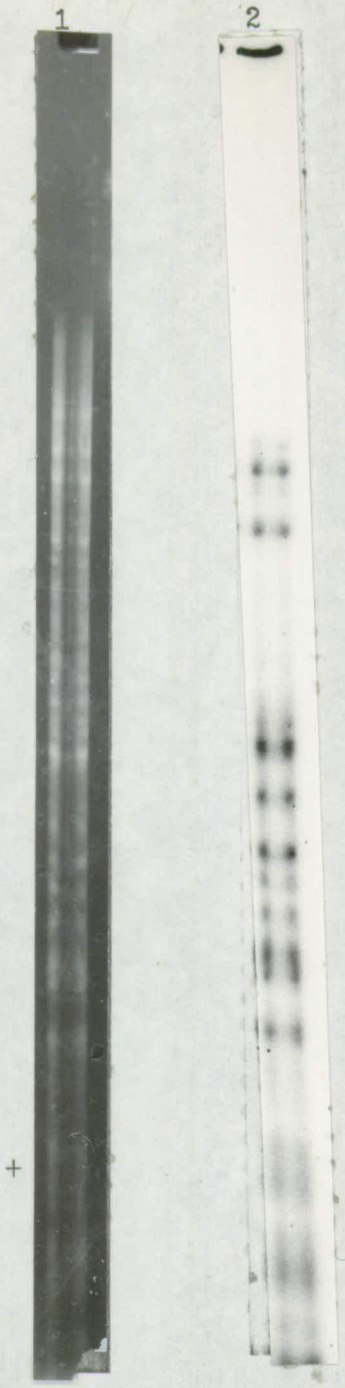


Fig.3.4 Agarose gel electrophoresis of a codigest of uniformly ^{32}P labelled λ.B DNA and non-radioactive λ.K DNA with EcoRII

5ug of λ.K DNA and 0.3ug of uniformly ^{32}P labelled λ.B DNA (30,000 cpm per ug) were mixedly digested with 20ul of EcoRII for 1h and the digest analysed on a 1% agarose gel. The gel was photographed by UV fluorescence (track 1) then dried and autoradiographed (track 2)

3.3 The digestion of *mec*$^+$ DNA by EcoRII

The number of methyl groups introduced per λ chromosome *in vivo* by the *mec* methylase is 60-85 (Hattman *et al* 1973) and by the EcoRII modification methylase 120-170 in the absence of *mec* methylation, and 60-80 in its presence. This suggests that about half the available sites for EcoRII specific methylation of which there are two per recognition site, (see table 1.2) are methylated by the *mec* enzyme. The large number of partially digested fragments in the EcoRII digest of λ DNA indicates that a random, rather than a specific fraction, of the EcoRII recognition sites are methylated. On this basis one might expect $\frac{1}{4}$ of the EcoRII recognition sites to be methylated at both sites in the recognition sequence, $\frac{1}{2}$ to be methylated at one site and the remaining $\frac{1}{4}$ unmethylated. Since a single methyl group in the recognition sequence is sufficient to prevent restriction (Roulland-Dussoix *et al* 1975) one would expect a total of $\frac{1}{4} \times 80 = 20$ sensitive targets per λ molecule. This figure corresponds well with the estimate of Bigger *et al* (1973) for the number of cuts put into the DNA of a *mec*$^+$ lambdoid phage.

In vitro the *mec* methylase will methylate 120-170 sites per molecule if allowed to go to completion, suggesting that it can methylate all the sites methylated by the EcoRII modification enzyme (May and Hattman 1975). It is likely that λ*mec*$^+$ owes its sensitivity to EcoRII to the inability of the *mec* methylase to saturate all the available sites, during the rapid DNA synthesis and packaging of the phage, in its lytic cycle. This assertion is supported by the demonstration (Fig. 4.11) that *mec*$^+$ plasmid DNA is totally insensitive to EcoRII despite being sensitive to all the other restriction enzymes tested, including. EcoRI, HindIII, AvaI, BamI, SalI and HpaII. Although the appropriate controls of mixed digestion were not performed, it is reasonable to conclude that plasmid DNA is fully methylated as a consequence of its slower replication.

The single stranded DNA phages fd and f1 are resistant to restriction by EcoRII *in vivo*, although the double stranded replicative form isolated from a *mec*$^-$ host is a substrate for the *mec* and EcoRII

methylases and for the EcoRII restriction endonuclease as well (Shlagman et al 1976) (Hattman et al 1973).

However, the single strand of DNA in the virion propagated on E.coliK is non-methylated, suggesting (a) that mec methylation does not act on single stranded DNA and (b) that following infection the phage DNA is methylated as rapidly as it becomes double stranded.

These observations all support the contention that the mec methylase and the EcoRII modification methylase have the same sequence specificity. This is further supported by the observation that the pyrimidine tracts which contain 5 methyl cytosine are the same whether generated from mec^+ DNA or EcoRII modified DNA (May and Hattman 1975).

3.4 Analysis of the EcoRII digest of λ.Kmec⁻ DNA

The digests of λ.Kmec⁻ in fig. 3.3 show in a semi-quantitative manner that doubling the enzyme to DNA ratio results in additional cleavage but only a small amount, sufficient in fact to cause the disappearance of 4 or 5 of the minor partially digested fragments. Treatment with additional enzyme for a longer time removed some of the residual partially digested fragments but again not completely. This suggests that over the range of enzyme to DNA ratios used, the digest is nearing completion, and some fragments of DNA contain cleavage sites which are poor substrates for EcoRII. Both λ.B and λ.Kmec⁻ showed the same pattern of recalcitrant, partially digested fragments, so this observation cannot be explained in terms of a residual mec activity in E.coliK mec^- which can still methylate a subset of the EcoRII cleavage sites for which it normally has a slightly higher affinity.

The completeness of the digests of λ.Kmec⁻ shown in fig. 3.3 was established by analysing a digest of λ.Kmec⁻ DNA which had been previously labelled at its 5' ends with ^{32}P. Figure 3.5 shows that an EcoRII digest of λ.Kmec⁻ DNA which contains a few obviously partially digested fragments as seen in fig. 3.3 only contained one labelled fragment . This fragment split to give 2 smaller fragments if the DNA was heated to dissociate the cohesive ends of the λ chromosome, before gel analysis. This confirms that the digests shown in fig. 3.5 & fig. 3.3 are essentially complete, since otherwise

one would expect to observe a series of partially digested, labelled fragments. Such a series of labelled fragments was observed with 5' ^{32}P labelled λ.K DNA (fig. 3.5).

In summary, λ. mec⁻ and λ.B DNA contain targets for EcoRII which are poor substrates for this restriction enzyme and in limit digests give a series of 4-5 residual partially digested fragments, within an otherwise complete digest.

3.5 An estimate of the number of cleavage sites for EcoRII in the λ chromosome

A lower limit was set on the number of cleavage sites by determining the number of cleavage sites by determining the number of identifiable fragments in an EcoRII digest of λ.Kmec⁻ DNA. This analysis was complicated by the fact that many of the bands on an agarose gel electrophoretograph appear to contain more DNA than bands which move more slowly and obviously contain a larger piece of DNA i.e. they contain more than one fragment. To establish the number of fragments (stoichiometry) in each band the individual bands from a digest of uniformly ^{32}P labelled λ.B DNA (fig. 3.6) were cut out from a gel and their ^{32}P content measured (table 3.2). The experiment was carried out in parallel with an EcoRII digest which was heated to separate the cohesive ends of λ before gel analysis and a digest of λ DNA made with AvaII.

This endonuclease gives a similar fragment size distribution to EcoRII and like EcoRII it has a pentanucleotide recognition sequence. A plot was made of ^{32}P content versus electrophoretic mobility (fig. 3.7) for gel bands from both EcoRII and AvaII digests. A series of 3 parallel lines could be drawn connecting points on the plot corresponding to bands containing 1, 2 and 3 fragments (singlet doublet and triplet bands respectively). Lines drawn in this way for EcoRII bands, were parallel to those drawn for AvaII bands, showing that this was a valid procedure. Having established the stoichiometry of each band in this way (table 3.2), the sizes of the fragments contained in each of the bands which fell close to a unit stoichiometry were calculated using the formula:

$$\text{fragment size} \quad \frac{^{32}P \text{ content of band}}{\Sigma\, ^{32}P \text{ content of all bands}} \times \frac{\text{size of whole}}{\text{stoichiometry of band}},$$

Legend to Fig. 3.6

Analysis of an EcoRII digest of uniformly ^{32}P labelled λ.B DNA

Uniformly labelled λ.B DNA (1.5 ug per track at 20,000 cpm/ug) was digested with EcoRII (tracks A and B) and with AvaII (track C), and analysed by electrophoresis on a 1% agarose slab gel. The EcoRII digest run on track A was incubated at 75° for 10 min to separate the cohesive terminal fragments. The gel was dried and autoradiographed. The autoradiograph was used to locate the radioactive bands which were cut out, suspended in liquid scintillant and counted to a less than 5% error.

The results are plotted in Fig. 3.7 and shown in Table 3.2. Track D shows a digest of 5'^{32}P terminally labelled λ*mec*$^-$ DNA (see Fig.4.5) run in parallel.

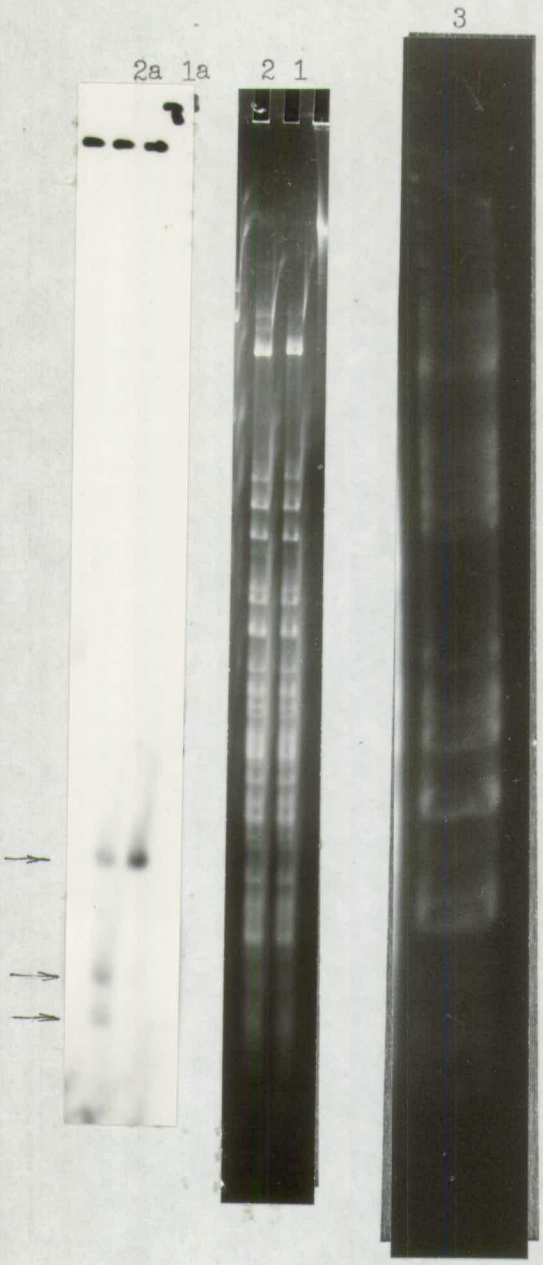


Fig.3.5 Agarose gel electrophoretic analysis of EcoRII digests of 5'^{32}P terminally labelled λmec$^-$ and λ.K DNAs

TABLE 3.2

EcoRII gel band	^{32}P content (cpm) Track A (deconcatenated)	Track B (undeconcatenated)	Stoichiometry	Fragment Size Md Track A	Track B
1	248	66	1	2.45	2.59
2		72	1		
3	981	1698	1		
4	74	301	1		
5	636	1084	1	1.59	1.66
6	468	931	1	1.16	1.43
7	45	129	1		
8	358	511	1	0.892	0.782
9	435	799	1	1.09	1.22
10	856	1444	2	1.067	1.105
11	478	781	1 +		
12	789	1086	2	0.984	0.831
13	598	1202	2	0.747	0.919
14	748	1051	2	0.933	0.805
15	1109	1684	3	0.922	0.859
16	550	1030	2	0.687	0.789
17	502	647	2	0.626	0.495
18	673	1273	2 +	Cohesive terminal fragment	
19	471	585	2	0.588	0.448
20	441	641	2	0.550	0.491
21	468	769	2	0.584	0.589
22	750	1157	3 +	Cohesive terminal fragment	
23	272	428	1 +	" " "	
24	584	937	4	0.364	0.358
25	174	378	1 +		

Sum of stoichiometries 38

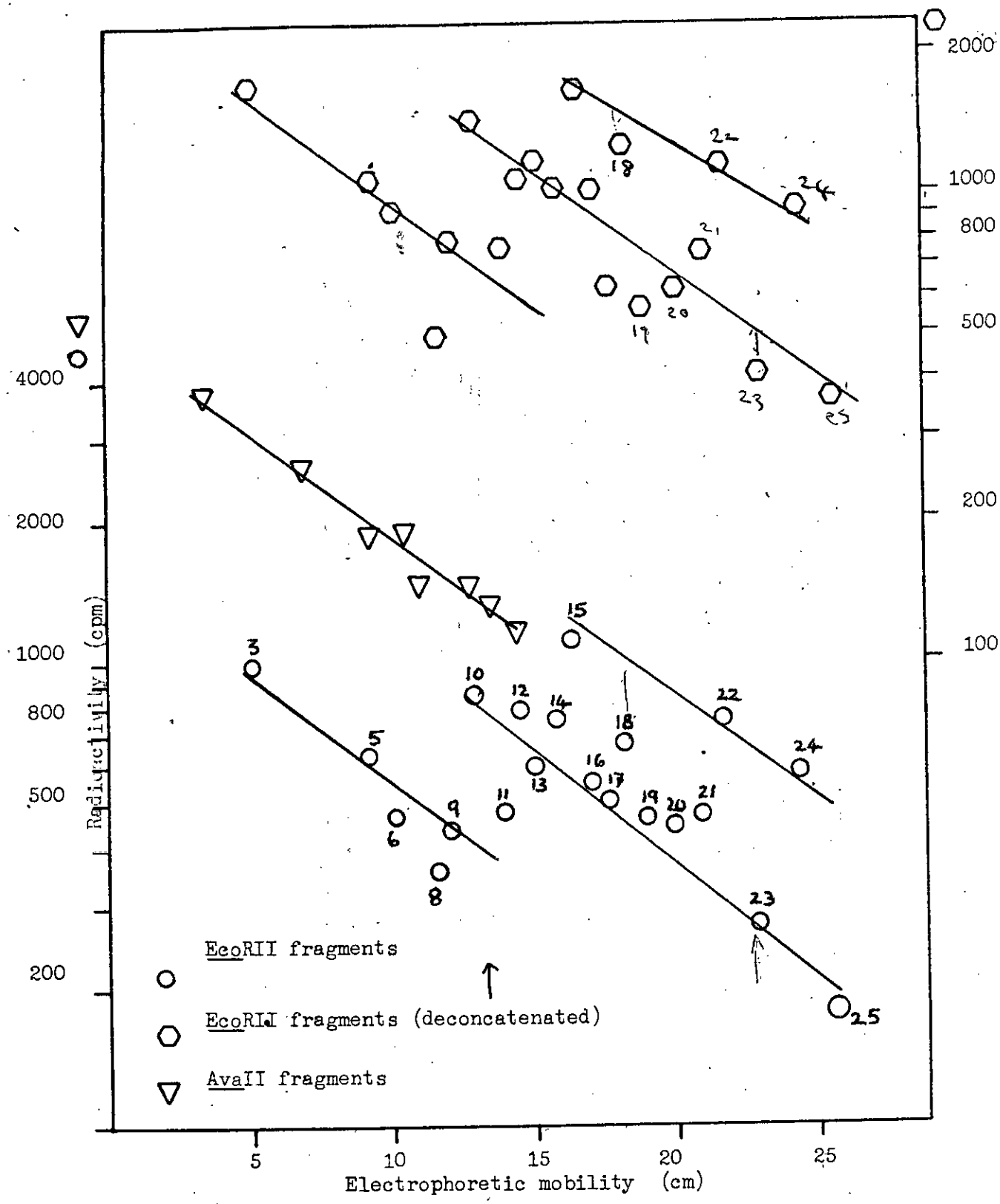


Fig.3.7 A plot of mobility versus ^{32}P content for fragments generated from uniformly labelled λ B DNA by EcoRII and AvaII.

and listed in table 3.2. The size of the whole λ genome was taken as 30.8 Md (Davidson and Szybalski 1971). The size of the fragment in band 3 (one of the set of singlet bands) determined by this method is 2.45 to 2.59 Md, which agrees reasonably with the size of this fragment determined from its electrophoretic mobility on an agarose gel, relative to EcoRI fragments of λ DNA. This supports the contention that band 3 is a singlet.

Attention was then turned to those bands of fractional stoichiometry i.e. those which fell between the lines on fig. 3.7. A few bands fell well below the line connecting single bands and it was concluded that they contain residual, partially digested fragments (bands 1, 2, 4, 7 and 8). However, band 8 has a stoichiometry of 0.75 - 0.6 (calculated by dividing the ^{32}P content of band 8 by the amount it would contain if it fell on the straight line joining singlet bands) and it was concluded that this band contains either a fragment with a highly resistant EcoRII target, a series of partially digested fragments or a fragment which for some reason of nucleotide composition has a true stoichiometry of one but a much reduced mobility. Because of the latter possibility, and its high stoichiometry relative to bands 1, 2, 4 and 7, band 8 was given the status of a singlet band. Other bands fell between the lines joining singlet and doublet bands, and between doublets and triplets. Some of these obviously contain partially digested fragments in addition to fully digested fragments, or result from inaccurate cutting out of closely spaced bands, but the fractional stoichiometries of bands 18, 22 and 23 can be explained in another way. The analysis in section 3.5 shows that bands 18, 22 and 23 contain the cohesive terminal fragments of the λ chromosome. Band 18 contains the two terminal fragments annealed together and, as would be expected, has a reduced ^{32}P content following heating to separate the cohesive ends (fig. 3.7).

Conversely bands 22 and 23 have an increased ^{32}P content after heating. The stoichiometries of bands falling between the lines on figure 3.7 were rounded down to that of the line above which they lay.

Adding together the stoichiometries of the 25 bands resolved on a 1% agarose gel gives a total of 38 fragments in the EcoRII digest

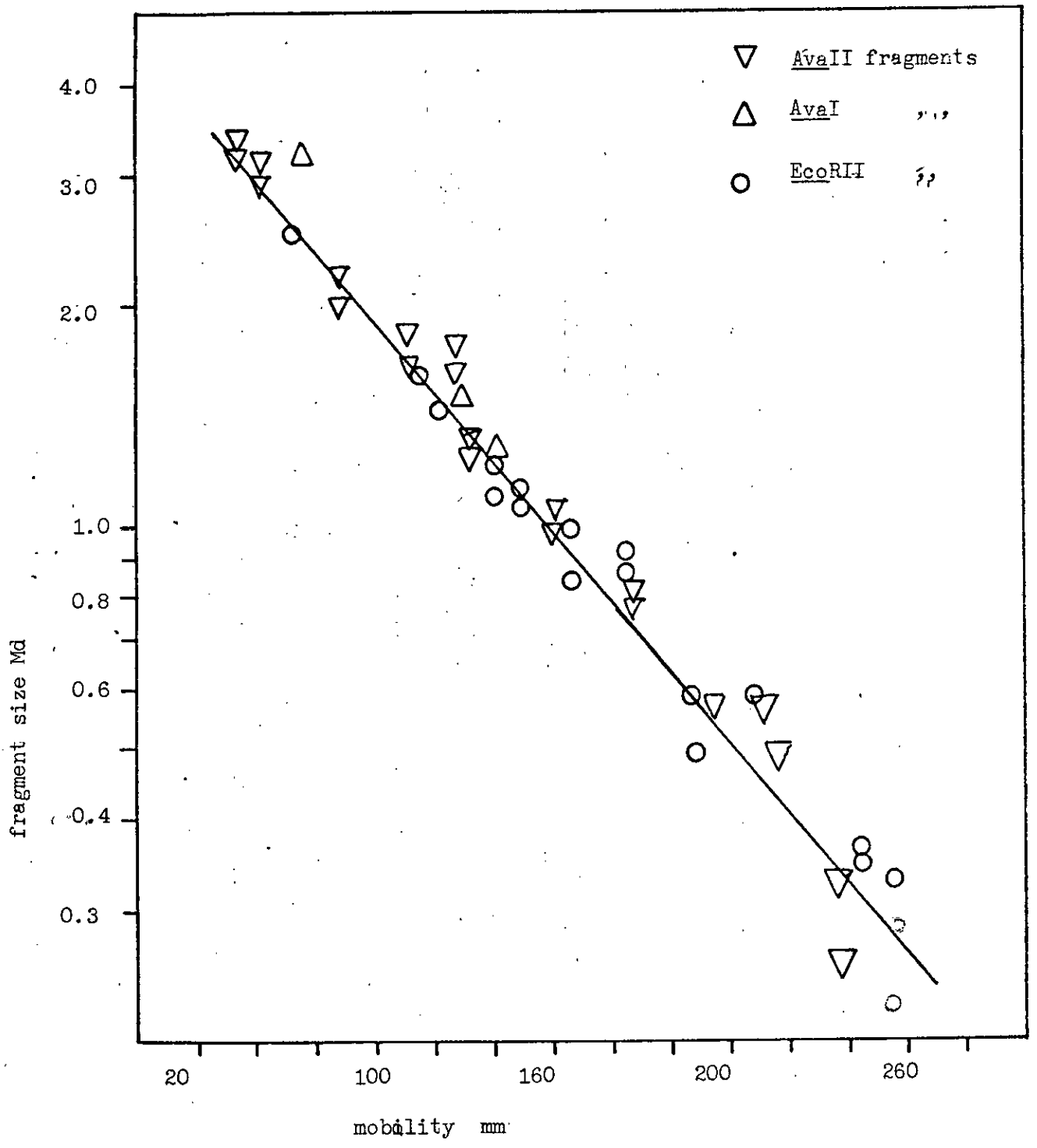


Fig. 3. Graph of fragment size versus mobility for AvaI , AvaII , and EcoRII fragments of λ DNA

of λ DNA. This predicts a total of 37 EcoRII cleavage sites in the genome. Acrylamide gel electrophoresis (on a 5% gel, fig. 3.9) resolves at least 11 bands of greater mobility than the smaller terminal fragment of λ DNA, instead of the 5 fragments resolved by agarose gel electrophoresis. The extra bands seen on the acrylamide gel probably represent fragments too small to be resolved on the agarose gel. Thus 6 fragments may be added to the 38 observed on the agarose gel, to give a total of 44 fragments, representing 43 EcoRII cleavage sites.

Although this figure must be regarded as a minimum estimate it is far short of the 80 sites for EcoRII specific modification made by Hattman et al (1973). On theoretical grounds one would expect a pentanucleotide sequence to occur once in 4^5 nucleotides. Because complementary pentanucleotide sequences are non-identical, the EcoRII recognition sequence should occur twice in 4^5 nucleotide pairs. So, if randomly distributed, the EcoRII recognition sequence should occur 80 times in the λ chromosome, which is in agreement with Hattman's figure.

This suggests that gel analysis has failed to detect about one half of the EcoRII fragments of the chromosome or that about one half of the EcoRII recognition sites are flanked by nucleotide sequences which make them completely resistant to EcoRII in vitro. A plot was made (fig. 3.8) of fragment size versus electrophoretic mobility for EcoRII fragments and AvaII fragments, to assist in the assignment of sizes to fragments of intermediate stoichiometry in particular the fragments containing the cohesive ends of λ . The mobility of AvaI fragments of known size (Hughes 1976) were normalised and added to the plot, where they fell close to the best line drawn through points representing EcoRII and AvaII fragments.

3.6 Identification of EcoRII fragments from the ends of the λ chromosome

a) Introduction. The ends of the λ DNA molecule have been extensively studied (for review see Yarmolinski 1971). The ends consist of single stranded 5' protrusions 12 nucleotides in length, which are complementary and consequently cohesive thus forming the basis for the circularisation of the linear form of λ DNA in vivo. The nucleotide

Legend to Fig. 3.9

Acrylamide gel electrophoretic analysis of an EcoRII digest of uniformly labelled ^{32}P λ.B DNA and 5'terminally ^{32}P labelled λ mec$^-$ DNA

Uniformly ^{32}P labelled λ.B DNA (1.5 µg) and 5'terminally ^{32}P labelled λ mec$^-$ DNA (2.5 ug) were each digested with EcoRII and half of each digest was analysed on a 1% agarose gel and the other half on a 5% acrylamide gel. The acrylamide gel was 125cm in length and was run for 10h at 10ma. Both gels were dried and autoradiographed. The terminally labelled fragments, shown by arrows on tracks 2 and 4 were used as reference markers in comparing the fragment patterns shown on the two gels.

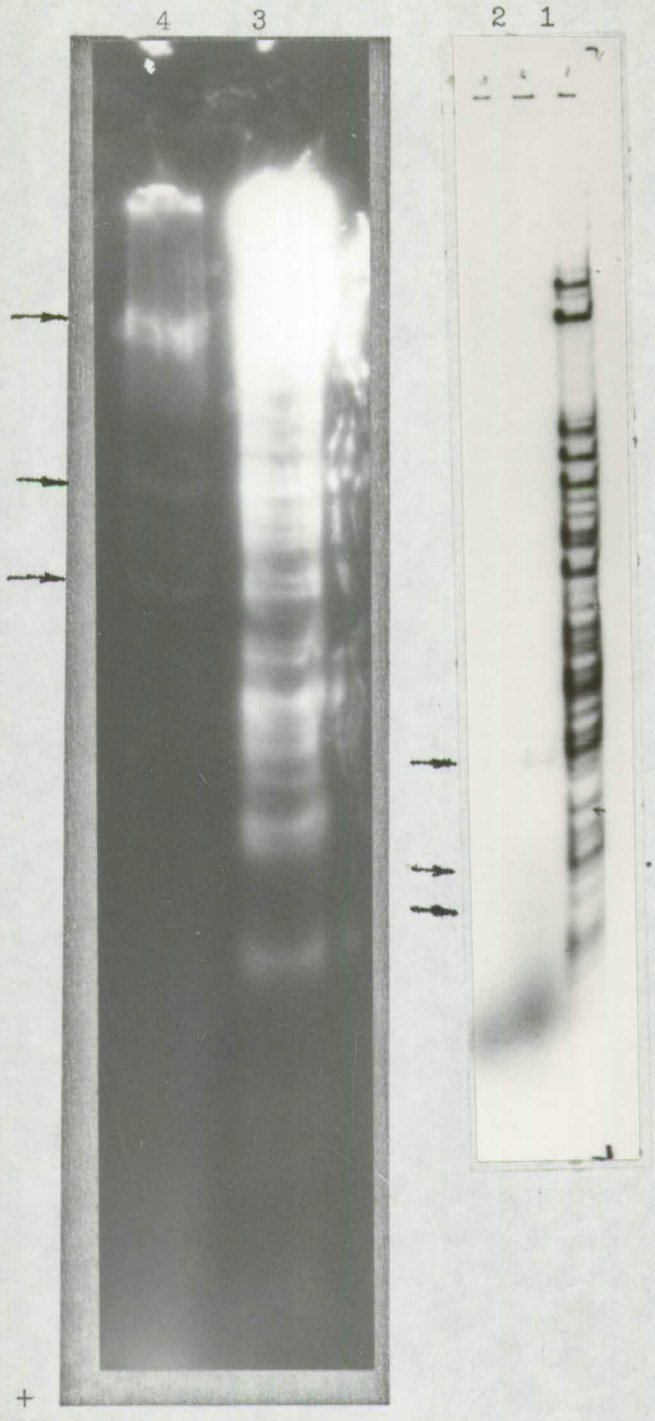


Fig.3.9 Acrylamide gel electrophoretic analysis of an EcoRII digest of ^{32}p uniformly labelled λ.B DNA

Legend to Fig. 3.10

The Separation of EcoRII fragments of 5' terminally labelled λ mec⁻ DNA by agarose gel electrophoresis

5' ^{32}P labelled λ mec⁻ DNA (3.75 ug at 130 cpm/ug) was digested with $\frac{1}{2}$ the amount of EcoRII required to give a complete digest, and the fragments separated by electrophoresis on a 1% agarose gel (track 1.). The gel was dried and autoradiographed (3 day exposure) and the labelled fragments identified and numbered 1 through 7.

A complete digest of uniformly ^{32}P labelled λ.B was run in parallel on the gel (track 2) for comparison.

This experiment is continued on Fig. 3.10.

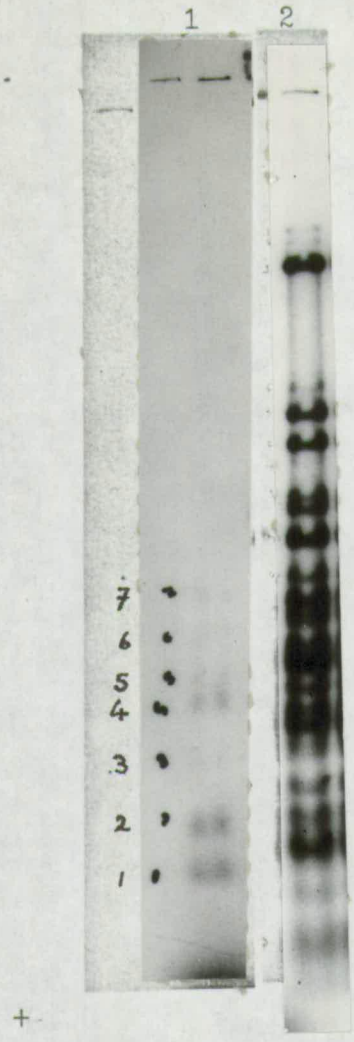


Fig3.10 The separation of EcoRII fragments of 5' terminally labelled λ mec⁻ DNA by agarose gel electrophoresis.

Legend to Fig. 3.11

Electrophoretic analysis on AE 81 paper at pH 3.5 of labelled nucleotides recovered from gel slices containing the EcoRII terminal fragments of λDNA

The gel bands containing the terminaly labelled fragments identified in fig. 3.10were cut out and each steeped for 12h in 200 ul of a solution of nucleases of the following composition:

10mg/ml solution of pancreatic DNAse	20 µl
10mg/ml solution of venom phosphodiesterase	20 µl
M Tris HCl pH 7.5	10 µl
M $MgCl_2$	2 µl
H_2O	150 µl

in order to release the labelled terminal nucleotide from each fragment in the gel. The supernatants were then counted for their radioactivity (table 3.2), and applied to AE 81 paper and electrophoresed at pH 3.5, and 2.4 kv until the blue marker had migrated about 25cm. Mononucleotide markers were run on the same paper. The electrophoretogramme was autoradiographed for 4 weeks. The identities of each of the labelled terminal nucleotides from bands 1 through 7 were determined from their mobilities relative to the mononucleotide markers and listed in table 3.3.

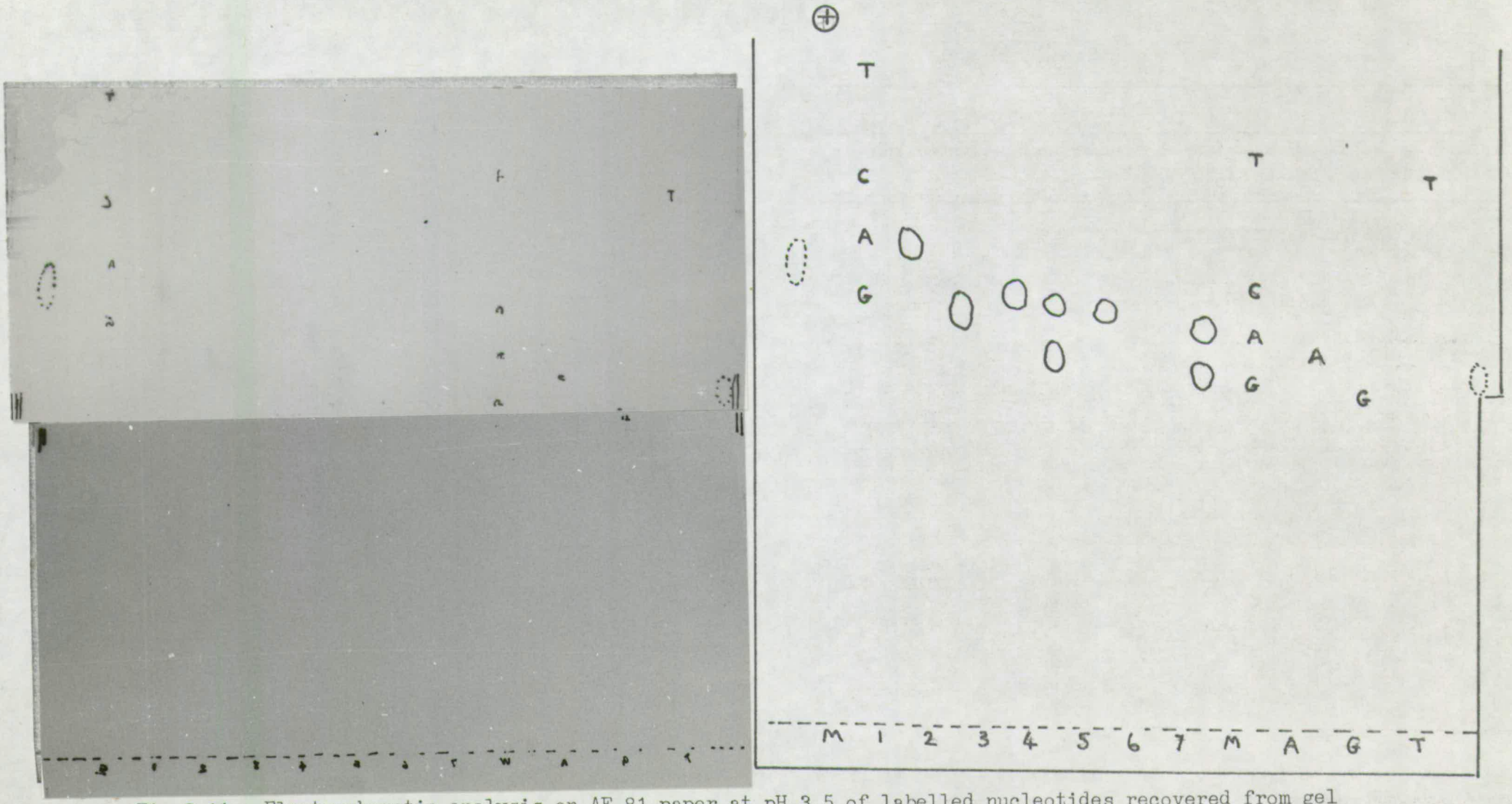


Fig.3.11 Electrophoretic analysis on AE 81 paper at pH 3.5 of labelled nucleotides recovered from gel slices containing the EcoRII terminal fragments of DNA.

TABLE 3.3

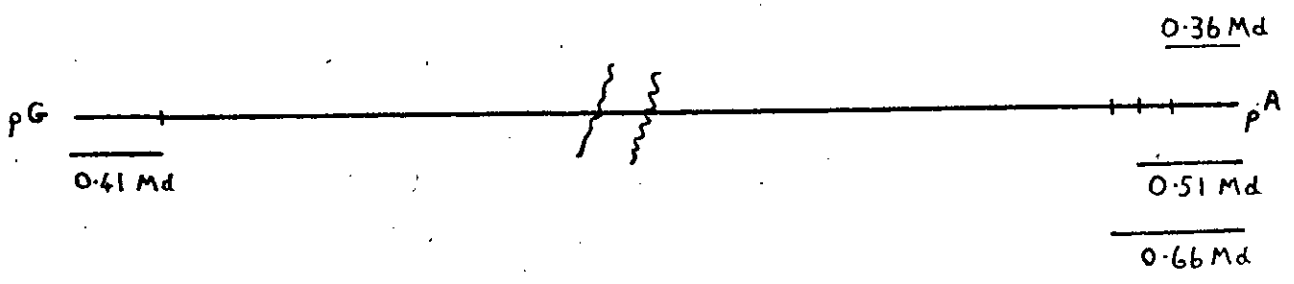
Labelled gel band number	c.p.m. in cut out band	Terminal nucleotide	Corresponding EcoRII band number	Size of Corresponding fragment in Md *
1	166	pA	22	0.365
2	138	pG	23	0.415
3	48	pA	20	0.51
4	89	pA + pG	18	0.63
5	46	pA	17	0.66
6	40	?	15	0.76
7	50	pA + pG	12	0.93

* scaled from Fig. 3.8.

sequences of the 5' protrusions have been determined (Wu and Taylor 1971) as have those of the adjacent double stranded regions (Weigel et al 1973) which together with them form the cos site of λ . With the map of drawn in the conventional way, with structural genes for head and tail proteins to the left and the genes for DNA replication and endolysin production to the right, (see Davidson and Szybalski 1971) the left hand 5' terminal nucleotide is pG and the right pA.

b) Experimental. The DNA of λ.Kmec⁻ labelled with ^{32}P at its 5' ends was digested with half the amount of EcoRII required to give the essentially complete digest shown in figure 3.6, so as to give some partially digested fragments. It was then analysed by agarose gel electrophoresis in parallel with a digest of uniformly ^{32}P labelled λ.B DNA (fig. 3.10). An autoradiograph of the dried gel was used to locate 7 bands contai..ing terminal label. These bands were cut out and the 5' terminal labelled nucleotide contained in each determined as described in fig. 3.11, and listed in table 3.3.

Labelled bands 1 and 2 correspond to EcoRII bands 22 and 21 respectively and as demonstrated in section 3.4 must be generated by cutting at targets adjacent to the termini of the λDNA molecule. Band 22 contains the right-hand terminus since it contains predominantly pA, and band 21 the left hand terminus as it contains pG. Labelled band 4 contains both pA and pG, which confirms the earlier observation that it contains the terminal fragments from bands 21 and 22 stuck together. Labelled bands 3 and 5 contain only pA and it was concluded that they were generated by cutting at the 2nd and 3rd EcoRII targets in from the right hand terminus respectively. Estimates of the sizes of these fragments from their electrophoretic mobility were made using the plot in figure 3.8. With this information it was possible to draw a map of the EcoRII cleavage sites close to the ends of the λ genome as shown below.



Band 7 (table 3.3) contains both pA and pG, but it is not possible to say whether this band contains separate or cohered fragments from the two ends of λ.

The analysis above, while it has given only limited information on the location of EcoRII cleavage sites offers, with suitable refinement and the use of terminally labelled DNA of higher specific activity a quick method of constructing restriction target maps for those restriction enzymes which have from 3 to 20 cleavage sites in a linear DNA molecule of interest.

CHAPTER 4

Experimental 2. Properties of the hspI (EcoR124) restriction and modification system in vivo and in vitro

4.1 Introduction

The hspI restriction and modification system was initially identified by Bannister (1969) associated with the F-like plasmid R124 and is hereafter referred to as the EcoR124 system. Subsequently this system was equated with the EcoRI system discovered by Yoshimori (1971) associated with the plasmid RY5. However, attempts to isolate the EcoRI restriction endonuclease following the now standard procedure (Greene et al 1974) from cells harbouring R124 (E.coli 1100R124) were unsuccessful. Thereafter, systematic fractionation and screening of extracts of the same cells for class II restriction endonucleases also proved negative (fig. 4.1). In this light the properties of the EcoR124 system in vivo and in vitro were examined.

4.2 Properties of the EcoR124 system in vivo

Many of the in vivo properties of this system were determined by Bannister (1969) and are mentioned in section 1.4(ii).

A direct comparison of the specificities of the EcoR124 and EcoRI systems had been omitted by other workers so this was undertaken. An initial set of results suggesting that the plasmids RY5 and R124 code for different restriction and modification systems was obtained with the plasmids in different host backgrounds. In order to rule out the possibility that the difference was augmented or determined by the host strain, the plasmids were each transferred to a common host lacking K specific restriction (E.coli5K). The transfers were carried out in collaboration with Dr N. Murray and are described in appendix I. Stocks of λv were propagated on 5K and each of the derivatives: 5KRY5; 5KR124; and in addition on 5KR245 (R245 codes for the EcoRII system). The efficiency of plating (e.o.p.) of each phage stock was then determined on 5K and each of the derivatives (table 4.1). The results clearly show that phage carrying EcoR124 modification is as efficiently restricted as unmodified phage by the EcoRI and EcoRII systems and vice versa for phage modified to EcoRI and EcoRII. This demonstrates that the

Legend to Fig. 4.1

An outline of the fractionation of E.coli 1100R124 for the detection of class II restriction endonucleases

1) Cells were grown harvested, disrupted and a crude isolate prepared as described in section 4.3(iii).

2) Streptomycin sulphate fractionation. The crude extract (10ml) was made up to 2% streptomycin sulphate by slow addition of 5% streptomycin sulphate solution. The pellet was recovered by centrifugation at 10,000xg for 20 min and redissolved by shaking over-night with 10ml of buffer.

3) Ammonium sulphate fractionation. Solid ammonium sulphate was added to the streptomycin sulphate supernatant (10ml) to bring it up to 30% saturation. The precipitate was recovered by centrifugation at 5,000xg for 10 min and redissolved in 10ml of buffer to give fraction 1. This process was repeated to produce cuts precipitating between 30 and 40% saturation (fraction 2), 40-50% (fraction 3), 50-70% (fraction 4) and a 70% + fraction (fraction 5).

This procedure was repeated with the redissolved streptomycin precipitate to produce fraction 6 (0-30%), fraction 7 (30-40%), fraction 8 (40-50%) and fraction 9 (50% +).

4) The crude extract was also subjected to ultracentrifugation (90,000xg for 90 min) and the supernantant the fractionated by ammonium sulphate precipitation, to produce fraction 10 (0-30% saturation), fraction 11 (30-40%), fraction 12 (40-50%), fraction 13 (50-70%) and fraction 14 (70% +)

5) Each of the fractions 1 through 14 were dialysed and then assayed for the presence of class II restriction endonucleases along with the unfractionated streptomycin supernatant (fraction 15), as described in section 2.2(ix)b. Uniformly labelled ^{32}P λsk^{o} DNA was used as a substrate and the dried 1% agarose gel autoradiographed. Assays of fractions 1 - 15 were run on corresponding tracks 1 - 15 and track 16 contains untreated DNA.

This figure should be compared with figure 3.2 in which an ammonium sulphate fraction from the purification of EcoRII was assayed by the same procedure.

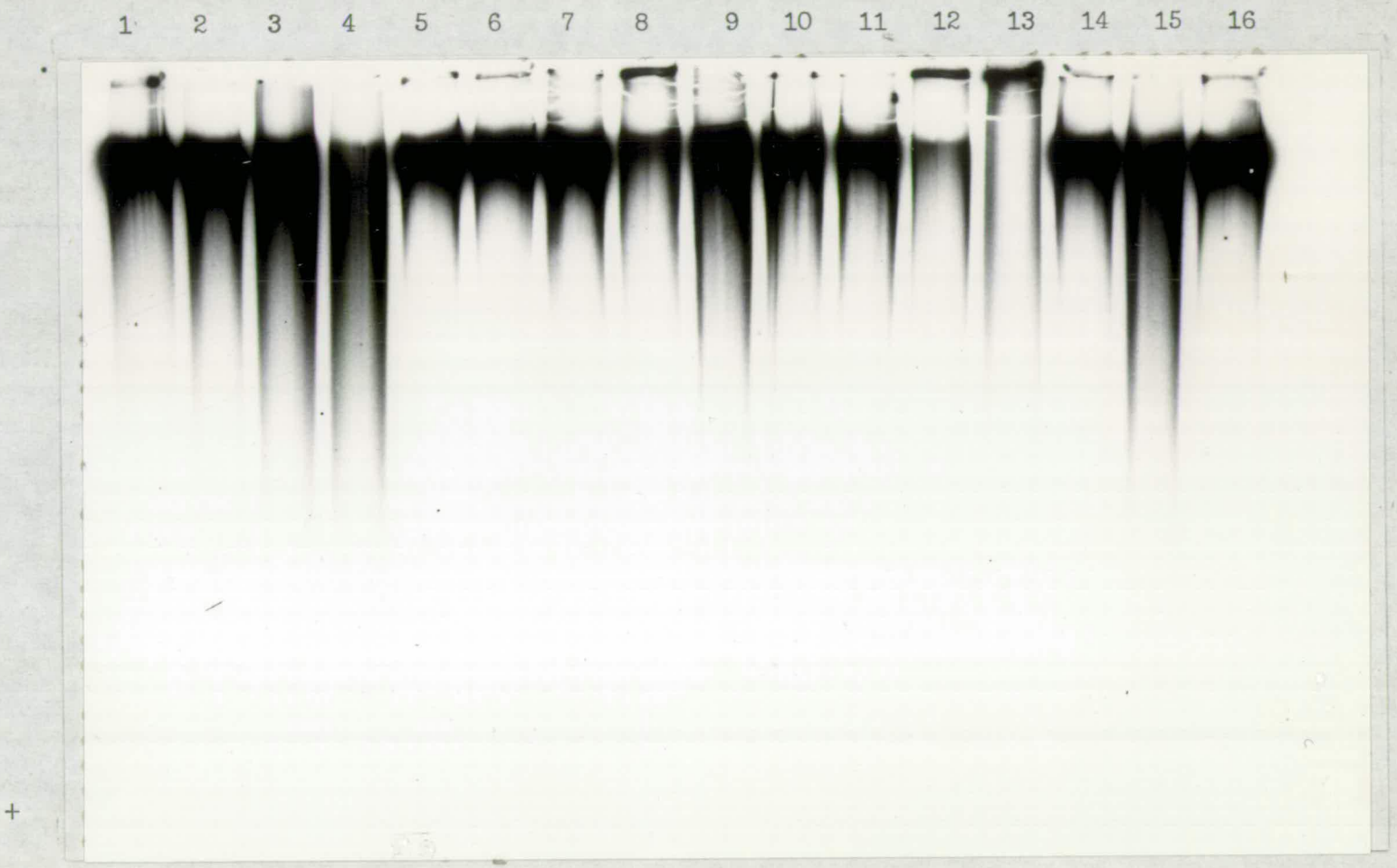


Fig. 4.1 Analysis of fractions of E.coli 1100R124 for class II restriition endonucleases

bacterium / phage	5KR124	5KR245	5KRY5	5K
λ.R124	1	0.6×10^{-2}	0.76×10^{-4}	1
λ.R245	1.5×10^{-4}	1	0.64×10^{-4}	1
λ.RY5	0.78×10^{-4}	$.6 \times 10^{-3}$	1	1
λ.K	1.16×10^{-4}	10^{-2}	3.2×10^{-4}	1

Table 4.1 The efficiencies of plating of phages modified against plasmid controlled restriction on 5K derivatives.

EcoR124 and EcoRI systems are different and mutually exclusive in both restriction and modification.

4.2(ii) The distribution of the EcoR124 system

The National Enteric Reference Laboratory's collection of plasmids includes two TP128 and TP129, which are both fi^+ and carry tet^r as their only resistance marker. TP129 is identical with R124. TP129 and TP128 are demonstrably different but highly related showing 65% homology by DNA-DNA hybridisation. For comparison TP128 shows 23% homology with F and 32% with plasmids from compatibility group FV (H Smith personal communication).

In view of the close relationship of the two plasmids it was of interest to see whether TP128 carried the EcoR124 restriction and modification system.

The two plasmids were first transferred to a λ sensitive streptomycin resistant host (3736) using streptomycin to select against the donor and tetracyclin to select for the plasmid. TP129 as expected showed the restriction and modification specificity of EcoR124 but TP128 no restriction or modification of phage λ . This observation added to the fact that the screening of over 200 plasmids by Bannister and Yoshimori only produced one, R124, with the EcoR124 system, emphasises the uniqueness of the plasmid R124 in this respect.

4.3. The isolation and characterisation of the restriction endonuclease of the EcoR124 system

4.3(i)

As a preliminary to the isolation of an endonuclease from cells harbouring R124 the plasmid was transferred to a number of E.coli strains. The conduct of the plasmid transfers, the properties of each resultant strain and its deployment in the isolation and assay procedure, are shown in table 4.2. The frequency of transfer obtained in 2h matings was about 1×10^{-4} per donor except in the case of transfer to the λsk^o lysogen where the frequency was lower by an order of magnitude.

4.3(ii) The assay procedure for restriction endonuclease activity

Since preliminary attempts to demonstrate the presence of a sequence specific endonuclease in cells harbouring R124 were unsuccessful, it was anticipated that if restriction by the R124 system was mediated by an endonuclease, it was likely to be of classI. Therefore an assay designed for the detection of class I or class II endonucleases was adopted. Differentially radioactively labelled modified and unmodified λsK^{o} DNAs were together incubated with samples of cell extract in the presence of SAM and ATP under the conditions specified in section 2.2(ix)a, and then analysed for the selective reduction of the molecular weight of the unmodified DNA, by sucrose gradient centrifugation. The orientation of the radioactive labels between modified and unmodified DNA (^{3}H in unmodified and ^{32}P in modified) was critical for the success of the assay. This feature is discussed further in section 4.3(iv). A typical set of results for the assay is shown in figure 4.3. The reduced rate of sedimentation of the ^{3}H unmodified DNA in frame shows that it has been selectively degraded by a nuclease in the fraction used in this assay which, since it did not degrade modified DNA, must be EcoR124.

4.3(iii) The isolation of EcoR124

a. Introduction. The procedure described below was established from a series of trial isolations in which the behaviour of EcoR124 activity in different purification steps was investigated. The enzyme was not purified to homogeneity.

b. Procedure. 1) Growth of cells

The chosen organism, E.coli 1100R124, was growth in L broth containing tetracyclin (50 ug/ml) to select for retention of the plasmid. The culture was harvested in late exponential phase at a cell density of $8x10^{8}$ to 10^{9} cells per ml. This cell density was found to be optimal for the isolation of other restriction endonucleases (R. Yuan personal communication). Yields of packed cells of 2g per 1 were obtained and the cells were stored frozen at -20^{o} until required.

b(2) Cell disruption - sonication

Stored cells (40g) were thawed and resuspended in 2 volumes of

TABLE 4.2

Donor	Recipient	Selection	Counter selection	Features of desired progeny tested	Deployment of strain
J53 R124	$1100T^{r}_{1,5}$	*tet*^{r}	growth on minimal medium + thiamine	*tet*^{r}, prototrophy Ø 80 resistance	1100R124 - isolation of *Eco*R124
J53 R124	803suIII	*tet*^{r}	growth on minimal medium + methionine	ability to plate S *am* 7, *tet*^{r}	803suIII R124 - the titre of modified phage carrying S *am* 7
J53 R124	1100 su^{-} (sk^{o} $C_{I}857$ S *am* 7)	*tet*^{r}	growth on minimal medium + thymine	growth at 32^{o} but not at 42^{o}, *tet*^{r}, R124 modified phage production at 42^{o}	1100 (sk^{o}) R124 - the production of R124 modified phage DNA

Construction of strains for the isolation of *Eco*R124

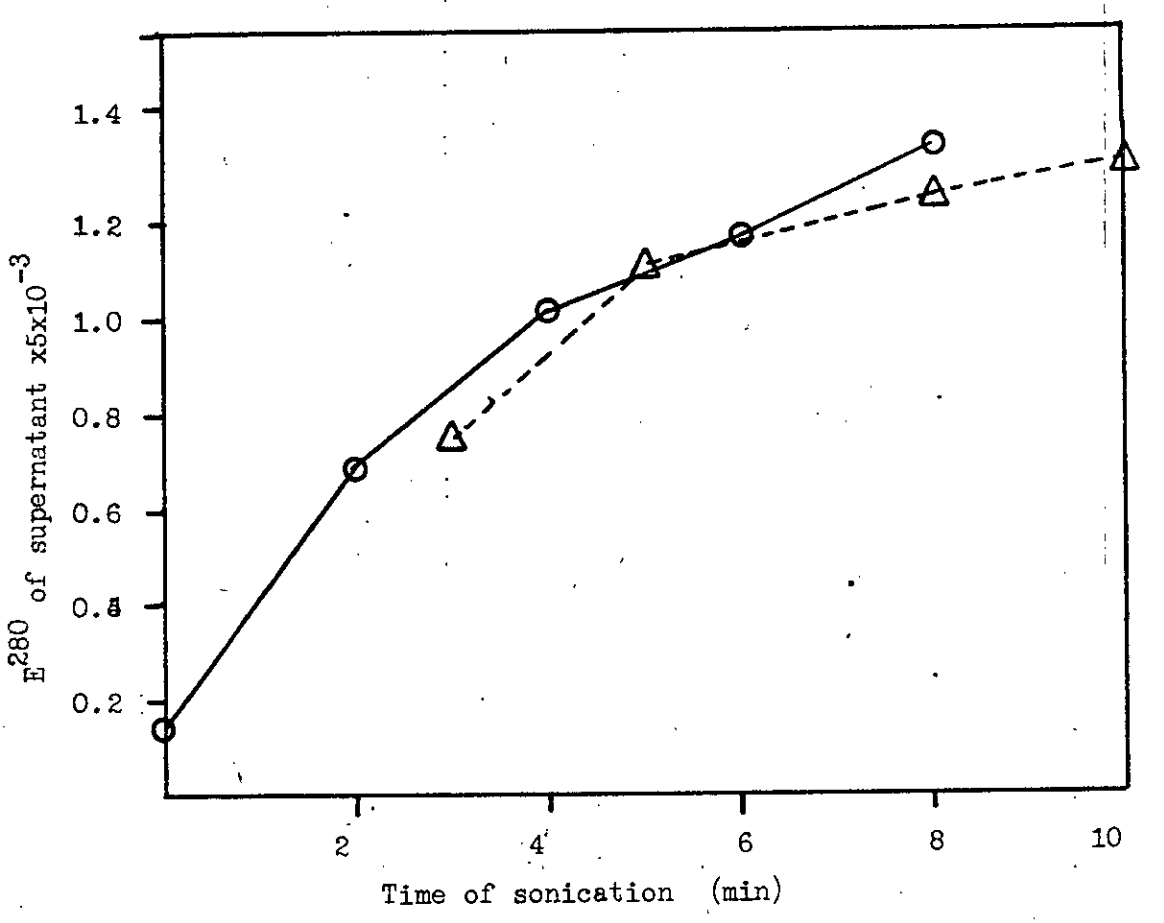


Fig. 4.2 Graph of the release of UV absorbing material into solution during sonication of a suspension of whole cells of 1100R124

tris extraction buffer. The suspension was sonicated as described in section 2.2(i)b and the kinetics of release of U.V. absorbing material into solution are shown in fig. 4.2. The bulk crude sonicate was centrifuged at 9,000xg for 15 min (MSE HS18 6x250 ml rotor) to separate cell debris which was discarded.

b(3) Streptomycin sulphate fractionation

A freshly prepared 5% solution of streptomycin sulphate in extraction buffer was added slowly to the crude supernatant with gentle stirring. A total of 0.4 volumes was added over a period of 1h. The mixture was then centrifuged (5,000xg for 20 min MSE HS18 8x50ml rotor) and the pellet discarded. The streptomycin concentration required for maximum precipitation of nucleic acid and protein without precipitation of EcoR124 was determined in a series of trial precipitations with increasing concentrations of streptomycin.

b(4) Ammonium sulphate fractionation

The supernatant from streptomycin precipitation was first made up to 35% saturation by addition of solid ammonium sulphate, slowly, with stirring over a period of 1h. The precipitate was recovered by centrifugation (5,000xg, 15 min in an MSE HS18 8x50ml rotor) and discarded. Further solid ammonium sulphate was added to the supernatant to make it up to 50% saturation and the precipitated protein was removed by centrifugation as above. The precipitate was redissolved in fresh extraction buffer ($\frac{1}{2}$ the volume of the streptomycin supernatant) and this fraction (30%-50% fraction) contained the majority of the EcoR124 activity. The fraction of protein precipitating between 50% and 70% saturation was found on occasion to contain some EcoR124 but always less than the 35%-50% fraction. The latter fraction was dialysed against fresh extraction buffer over night to remove residual ammonium sulphate.

b(5) Ion-exchange separation on DEAE-cellulose

This first ion-exchange fractionation was carried out by a batch method.

A freshly equilibrated slurry of DEAE cellulose (DE-52) was titrated into the 35-50% ammonium sulphate fraction slowly with stirring, until the addition of further slurry caused no further decrease in

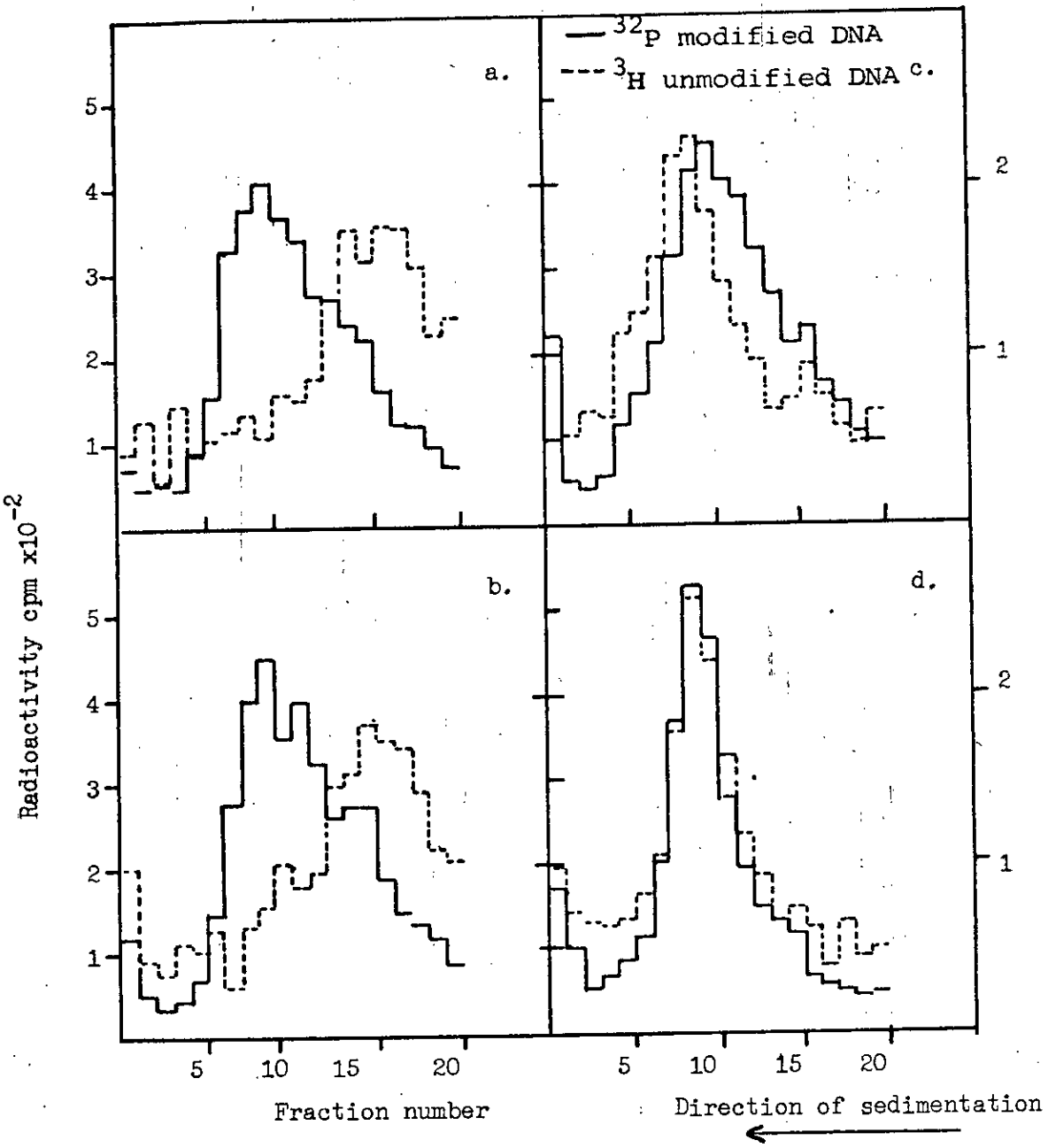


Fig. 4.3 Sucrose gradient analysis of assays of the active DEAE cellulose fraction of R.EcoR124 to establish co-factor requirements. Assays were carried out as per section 2.2(ix)a. using : a,10 l of active DEAE fraction +ATP +SAM ; b.,10 ul of active DEAE fraction +ATP -SAM ; c.,10 ul of active DEAE frction -ATP +SAM ; d., control

the E^{280} of the supernatant. Assays at this stage showed that no EcoR124 remained in the supernatant. The ion-exchange cellulose was recovered by filtration through a Buchner Funnel and washed with one volume of tris extraction buffer. The washed pellet was resuspended in one volume of tris extraction buffer containing 0.2M NaCl and gently stirred for one hour and allowed to settle. The supernatant was decanted and a further volume of this eluant was mixed with the ion-exchanger for 30 min, recovered by filtration and pooled with the previous supernatant. This stepwise elution procedure was repeated with eluant containing 0.3M NaCl, 0.4M NaCl and 0.6M NaCl. Assays showed that the majority of the EcoR124 activity eluted in the 0.3M NaCl fraction. The conductivity of this fraction after elution was found to be equivalent to 0.25M NaCl as a result of the dilution which is unavoidable in the batch elution method.

b(6) Characterisation of EcoR124

Assays of the active ammonium sulphate and DEAE-cellulose fractions in the absence of ATP or SAM (fig. 4.3) showed that these fractions of EcoR124 required ATP but not SAM for their activity.

The products of incubation of modified and unmodified λsk^{o} DNAs with the active DEAE-cellulose and ammonium sulphate fractions were also analysed by agarose gel electrophoresis (fig. 4.4). Compared with the untreated DNA modified DNA remained as a relatively intact band as did unmodified DNA in the absence of ATP. Unmodified DNA digested in the presence of ATP gave a smear of faster moving DNA suggestive of random cleavage and similar to the digests of λDNA with the class I restriction endonuclease EcoK (Murray et al 1973). Since it was possible that the observed streak was the product of the action of contaminating endonucleases or exonucleases on a discrete set of fragments produced by the action of EcoR124, the above analysis was repeated using EcoRI digests of unmodified λsk^{o}/DNA as a substrate.

The agarose gel electrophoretic analysis in figure 4.4 shows that the characteristic EcoRI banding pattern is conserved during incubation with the 35-50% ammonium sulphate fraction of EcoR124 in the presence or absence of ATP. This suggests that under the conditions of this assay the contaminating nucleases are not sufficient to obliterate a characteristic band pattern and that the observed

Legend to Fig. 4.4

The ammonium sulphate fraction of EcoR124 was used to digest modified and unmodified λsk° DNA as described in section 2.2(ix)a and the products were analysed by electrophoresis on a 1% agarose gel.

Track 1. λsk° DNA no enzyme
" 2. λsk° 124 DNA "
" 3. λsk° DNA + enzyme - ATP + SAM
" 4. λsk°.124 DNA " " "
" 5. λsk° DNA + enzyme + ATP + SAM
" 6. λsk°.124 DNA " " "
" 7. λsk° DNA + enzyme + ATP + SAM
" 8. " + enzyme - ATP + SAM
" 9. λsk° DNA predigested with EcoRI + enzyme + ATP + SAM
" 10. " " " + enzyme - ATP + SAM
" 11. λsk° DNA + enzyme + ATP + SAM
" 12. λsk° predigested with EcoRI no enzyme

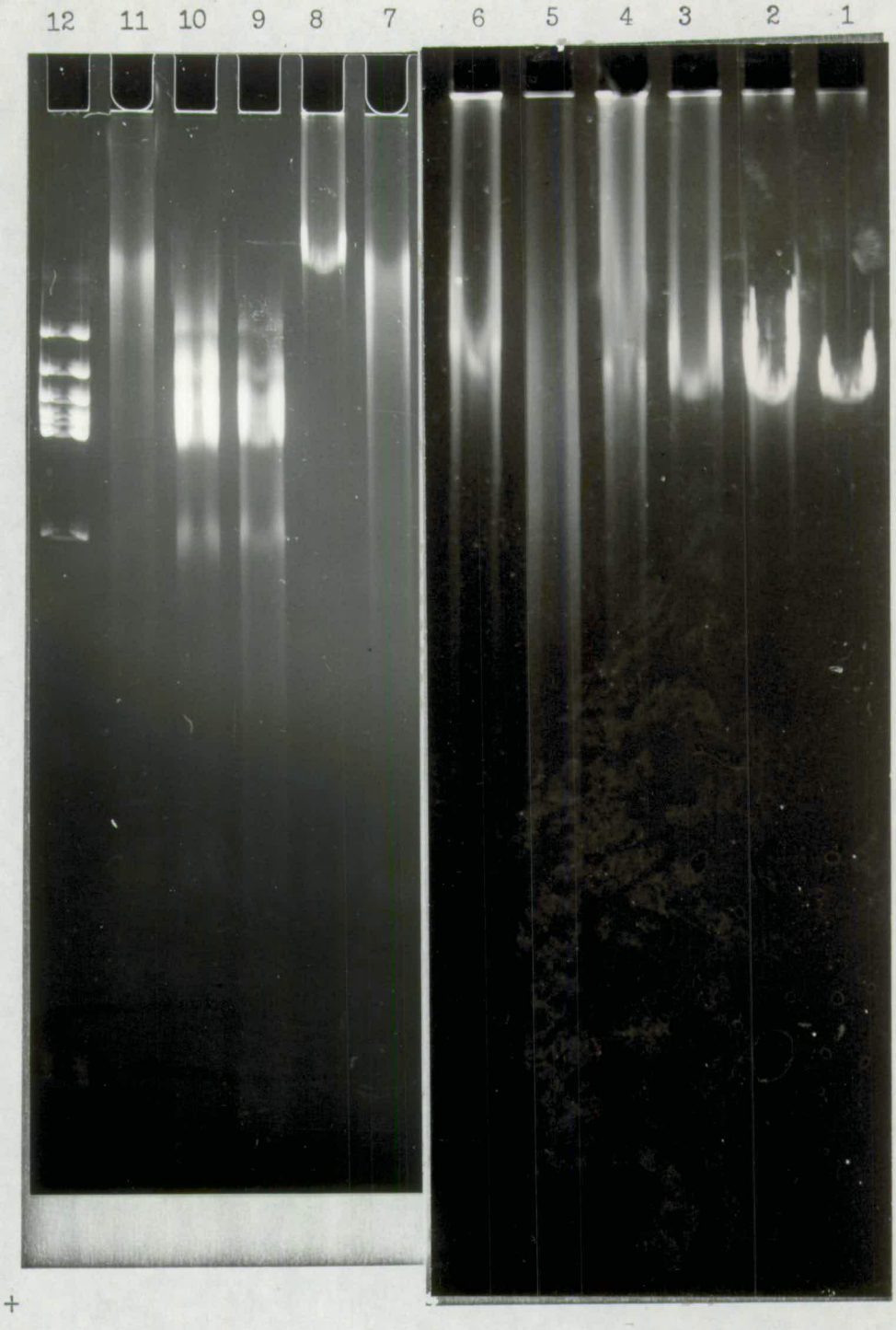


Fig.4.4 Agarose gel electrophoretic analysis of digests of modified and unmodified λsk^{o}DNA with EcoR124.

smear is the product of EcoR124. It also suggests that EcoRI fragments of λsk^{o} are a poor substrate for EcoR124.

4.3(iv) Further purification

Repeated attempts to further purify the EcoR124 activity by ion-exchange column chromatography were unsuccessful both with DEAE and phosphocellulose. A small amount of EcoR124 activity was recovered from one phosphocellulose column in which a step was accidentally produced in the elution gradient. Since this produced the most extensively purified fraction of EcoR124 ever obtained and subsequent characterisation revealed a new property of EcoR124, full details of the procedure followed, the elution profile, and assays of the active fractions are shown in figures 4.5, 4.6 and 4.7.

The assays of fractions 5, 6 and 7 (figs. 4.6 and 4.7) demonstrate the partial separation of a contaminating nuclease from the EcoR124 activity. In the absence of both ATP and SAM fractions 5 and 6 gave specific breakdown of the ^{32}P labelled modified DNA suggesting that they contained an additional nuclease activity distinct from EcoR124, with novel properties. The possible identity of this activity and its influence on the observed properties and isolation of EcoR124, are considered further in section 4.3(v). Under the same conditions fraction 7 showed EcoR124 activity in the presence of cofactors and very little breakdown of either DNA in their absence. In the presence of ATP and absence of SAM this fraction gave much reduced activity showing that in the absence of the additional nuclease, EcoR124 is at least stimulated by SAM and probably has a stringent requirement for it. Thus on the basis of cofactor requirement EcoR124 is a classical class I restriction endonuclease. This shows that both class I and class II restriction and modification systems exist on plasmids and is in accord with Bannister's observation (Bannister and Glover 1969) that the three determinants characteristic of class I systems are found coding for this system.

4.3(v) The nature of the contaminating nuclease

Unlike EcoR124 the contaminating nuclease with specificity for ^{32}P DNA was repeatedly recovered from phosphocellulose columns. It was a cause of confusion in early attempts to purify EcoR124 in which

Legend to Fig. 4.5

Ion-exchange chromatography on phosphocellulose

Freshly precycled phosphocellulose was set up in a glass column (7cm x 1.5cm diameter) and carefully equilibrated with tris extraction buffer. The dialysed active fraction from DE AE cellulose fractionation (50ml) was pumped slowly (10ml per h) through the column and the unbound fraction collected. The bound fraction was eluted with a 200ml gradient of from 0 to 0.5M NaCl in tris-extraction buffer. Fractions (10ml) were collected and assayed for EcoR124 activity which was found in fractions 5,6 and 7. The unbound fraction contained no EcoR124 activity.

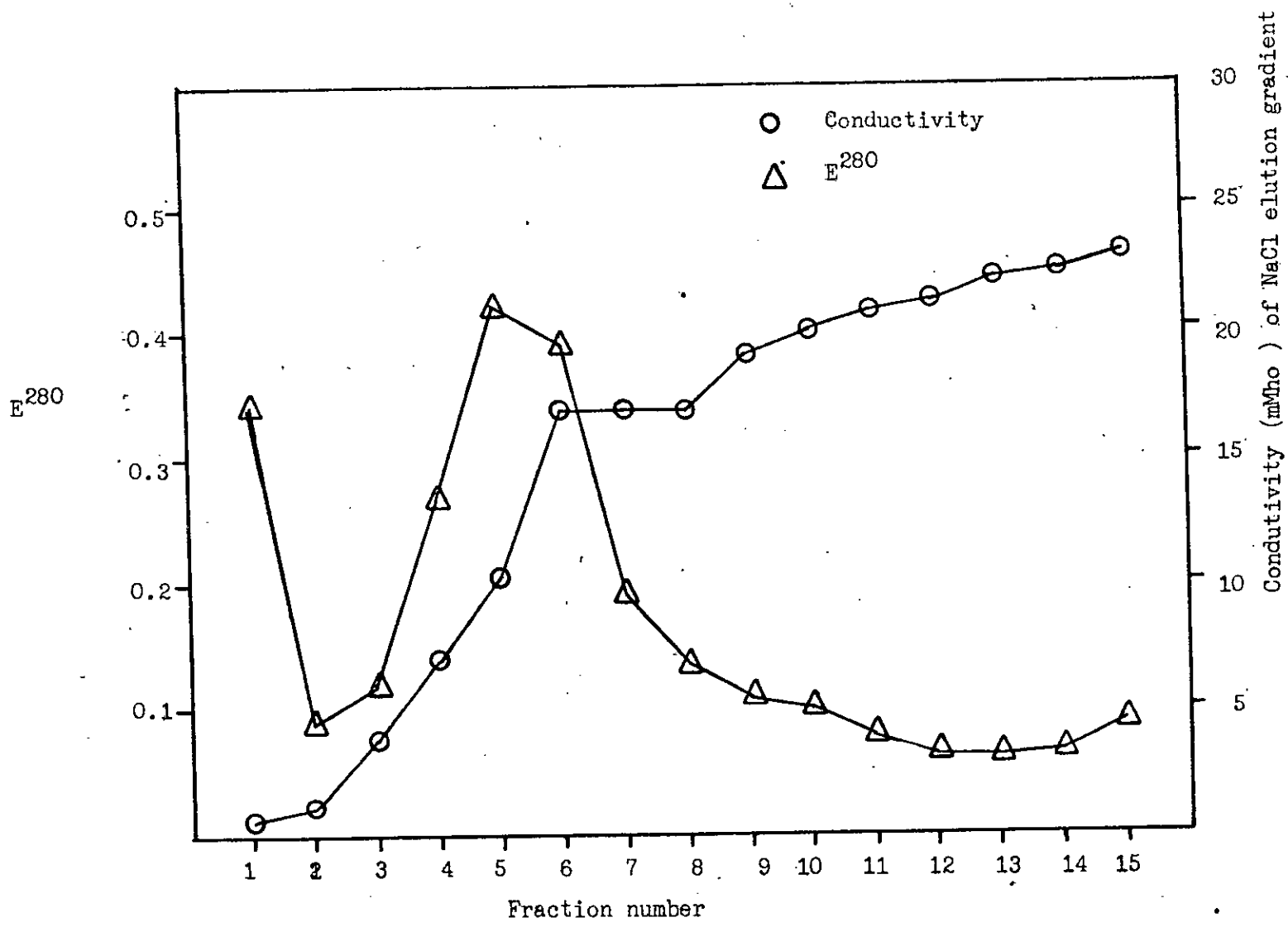


Fig. 4.5 Elution profile of phosphocellulose column used in the further purification of R.EcoR124

Legend to Fig. 4.6

Sucrose gradient analysis of assays of phosphocellulose fractions of EcoR124 contaminated with an additional nuclease

Assays carried out as per section 2.2(ix)a

a. Control
b. Fraction 5 + ATP + SAM
c. Fraction 5 - ATP - SAM
d. Fraction 6 + ATP + SAM
e. Fraction 6 - ATP - SAM

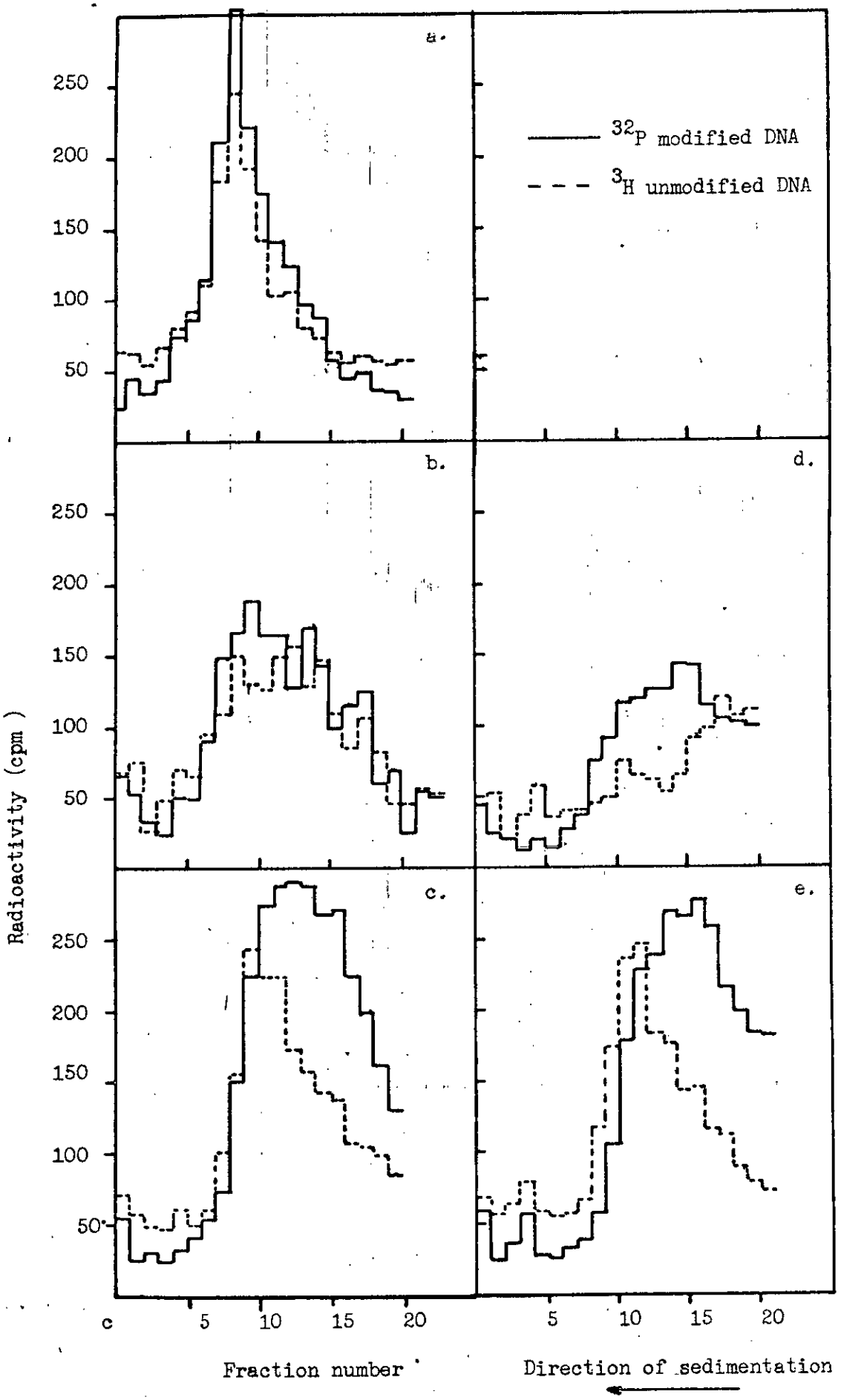

a.
b.
c.
d.
e.
250
200
150
100
50
^{32}P modified DNA
^{3}H unmodified DNA
Radioactivity (cpm)
c
5
10
15
20
Fraction number
Direction of sedimentation

Fig. 4.6

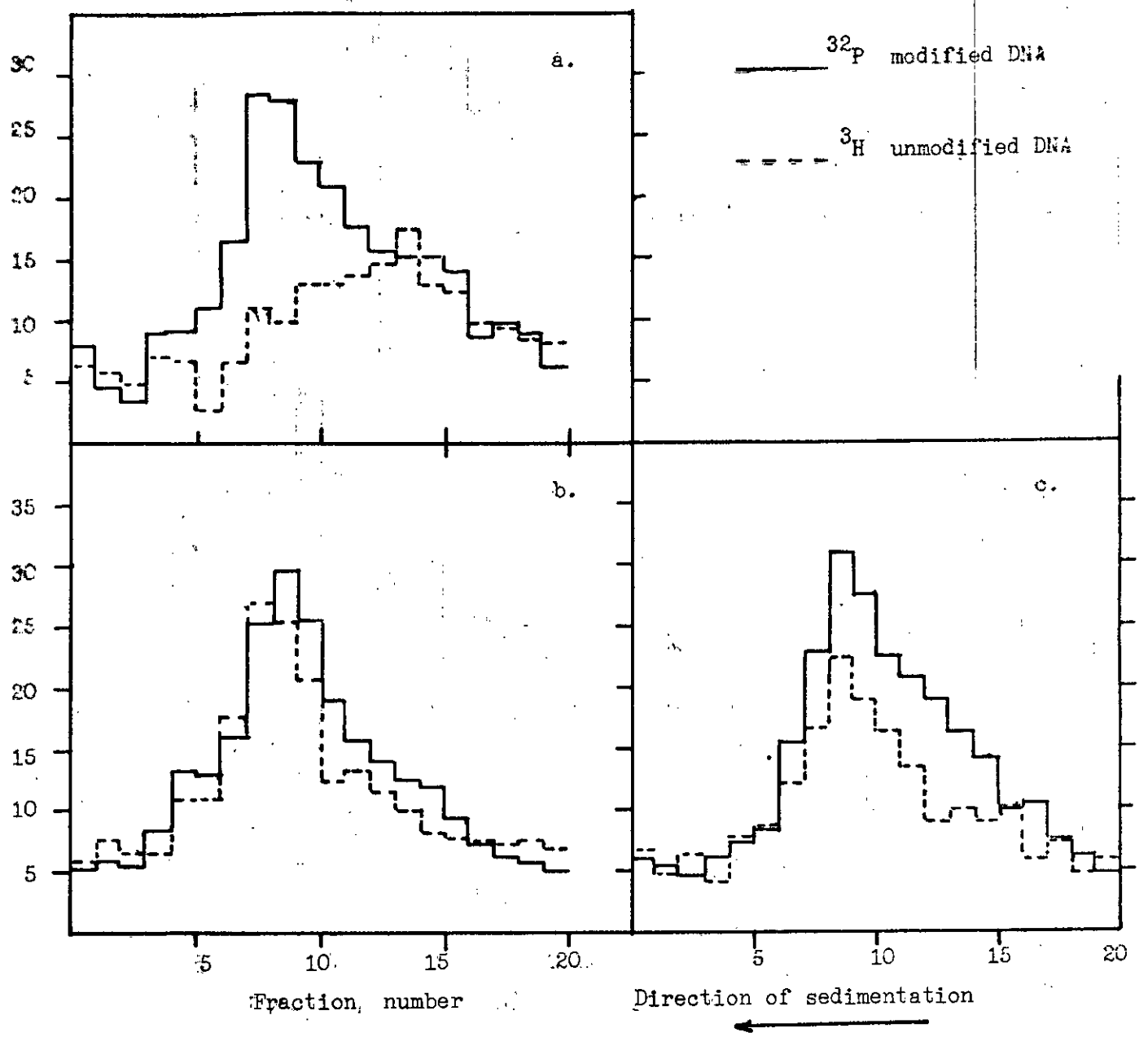


Fig. 4.7 Sucrose gradient analysis of assays of active phospho-cellulose fraction (fraction 7) of R.EcoR124 to test co-factor requirements.

The assays were carried out as per section 2.2(ix)a, using; a., 10 µl fraction7 +ATP + SAM b., 10 µl fraction 7 -ATP +SAM c., 10 µl fraction 7 +ATP -SAM .

the endonuclease assay utilised ^{32}P labelled unmodified DNA and ^{3}H labelled modified DNA. By this assay the EcoR124 activity and contaminating nuclease were indistinguishable and this led to the erroneous conclusion that EcoR124 was SAM and ATP independent. Consequently in the established assay the radioactive labels were reversed and the ^{32}P DNA was labelled to low specific activity.

It is not possible to say whether the activity with a preference for ^{32}P DNA was an endo or an exonuclease. Certainly acid solublising activity indicative of an exonuclease was demonstrated in standard exonuclease assays of all those and only those phosphocellulose fractions which contained the ^{32}P DNA prefering nuclease. It cannot be ruled out however, that the activity is a mixture of enzymes and that the specificity for ^{32}P resides in an endonuclease. In any event it is extremely likely that the contaminating nuclease exaggerates the breakdown of unmodified DNA initiated by EcoR124 and this may offer an explanation for a) the apparent SAM independence of EcoR124 prior to phosphocellulose chromatography, and b) the loss of EcoR124 activity during column chromatography. It is possible that prior to phosphocellulose chromatography there was sufficient SAM either bound to the enzyme or free in solution to give the enzyme a weak activity which was amplified by the contaminating nuclease. It may even be the case that EcoR124 by itself does not make double strand cuts in DNA but makes single strand scisions similar to the way in which ^{32}P decay damages DNA, and as a consequence requires the action of an additional nuclease in order to be detected by the assay. Thus it might be expected that separation of the two nucleases by column chromatography would lead to loss of the EcoR124 activity.

An observation which possibly relates to the above explanation was made by Kimball and Linn (1976). They found that the class I endonuclease EcoB releases about 75 nucleotides as acid soluble oligonucleotides per endonucleolytic cut. This implies that EcoB makes something other than a straight double stranded cut in DNA. In this case it is claimed that the acid solublising activity is a function of the EcoB itself and it is SAM and ATP dependent.

A possible candidate for the contaminating activity is exonuclease III. This enzyme will operate on double stranded DNA and

in addition will act as an endonuclease on depurinated DNA (Haddi et al 1973) (Yajko & Weiss 1975) in which guise it is called endonuclease II. It is possible that as well as recognisng and acting at strand scisions caused by depurination it can recognise and act at scisions caused by ^{32}P decay.

4.4 Is exonuclease III required for restriction by EcoR124 in vivo?

To test the possibility that exonuclease III is involved in restriction by EcoR124 the plasmid R124 was transferred to an exonuclease III deficient (xth) strain. The strain (BW9091) was obtained from Dr.B.Weiss and carried besides xth the chromosomal streptomycin resistance marker str^r. Recipients of R124 were selected on medium containing streptomycin and tetracyclin and were found at a frequency of 10^{-3} per donor. Four str^r tet^r colonies were purified and tested for EcoR124 specific restriction. Three of the four clones restricted λ.k with an e.o.p. of 10^{-4} and did not restrict λ.k.R124. This showed that exonuclease III is not required for the expression of normal restriction by R124.

The fourth str^r tet^r clone restricted both λ.k and λ.k.R124 with an e.o.p. of 10^{-3}. This isolate was at first thought to be a contaminant, but surprisingly further tests (section 4.5) showed it to carry a derivative of the plasmid R124 with a novel restriction and modification system. The isolate was designated BW9091 R124/3.

4.5 Characterisation of the novel restriction and modification system of BW9091 R124/3

(i) In vivo properties

To determine whether the novel restriction and modification system in BW9091 R124/3 was associated with a plasmid, tet^r was transferred from BW9091 R124/3 to strain 1100. Transfer of tet^r occurred at a frequency of about $3x10^{-4}$ per donor and of 4 colonies of 1100 tet^r tested all four restricted λ.k and λ.k.R124 with e.o.p.s of 10^{-3} to 10^{4}. This showed that the novel restriction system transferred with a plasmid marker and was therefore plasmid mediated. One of the clones,designated 1100 R124/3, was selected for further study.

phage / bacterium	λK.R124/3	λ.K	λK.R124
1100R124/3	1	3.79×10^{-4}	6.8×10^{-4}
1100	1	1	1
J53R124	3.73×10^{-4}	2.56×10^{-4}	1
1100	1	1	1
E.coli B251	$< 10^{-4}$	$< 10^{-3}$	$< 10^{-4}$
803 SuIII(P1)	$< 10^{-6}$	$< 10^{-4}$	$< 10^{-4}$

Table 4.3 Efficiencies of plating of phage modified against EcoR124 and EcoR124/3.

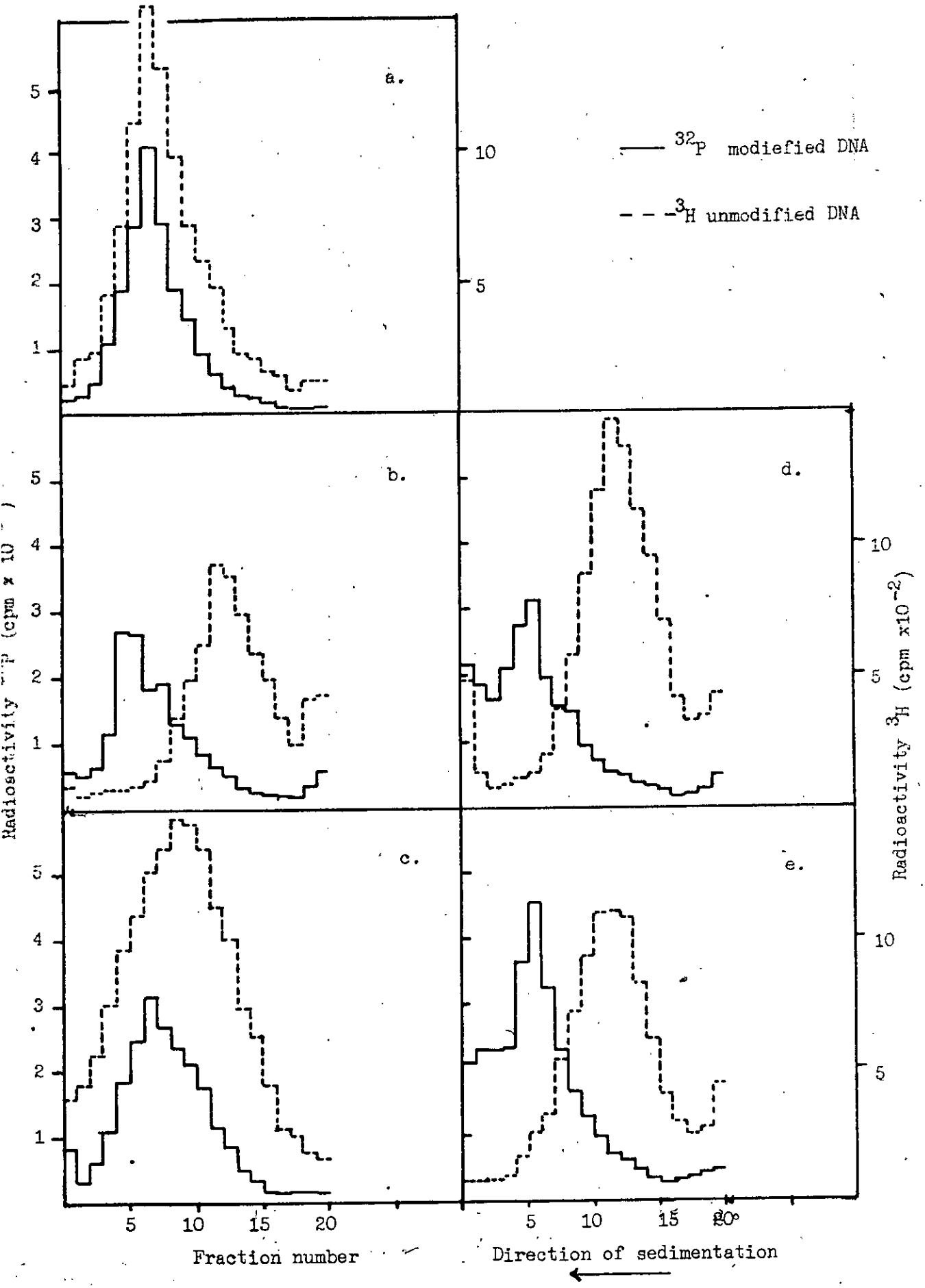

a.
b.
c.
d.
e.
^{32}P modiefied DNA
^{3}H unmodified DNA
Radioactivity ^{32}P (cpm x 10^{-2})
Radioactivity ^{3}H (cpm x10^{-2})
Fraction number
Direction of sedimentation

Fig. 4.8

Next, a stock of phage λv was propagated on 1100 R124/3 (λ.k.R124/3) and was used to examine the question of whether the novel system had a completely novel modification specificity or one which overlapped with R124 and other class I systems. The results of plating experiments with λ.k.R124/3 are shown in table 4.3.

These clearly show that the plasmid R124/3 specifies a completely novel restriction and modification system distinguishable from the EcoRI, EcoRII, B and k systems. This raises the question of how did this system arise?. Is it a derivative of the EcoR124 system which arose by mutation or recombination, or is the plasmid R124/3 a new isolate?. To answer these questions a preliminary isolation and characterization of the endonuclease coded by R124/3 was made and also the DNAs of the two plasmids R124 and R124/3 were compared by class II restriction enzyme digestion, which is a very useful technique for demonstrating the identity or near identity of plasmids (Thompson et al 1974).

4.5(ii) Preliminary isolation and characterisation of EcoR124/3

An identical procedure to that used for the isolation of EcoR124 (section 4.3) was followed.

In the construction of strains for the propagation and testing of appropriately modified λsk^{o} (for the preparation of ^{32}P labelled λsk^{o}.R124 DNA) strain BW 9091 R124/3 was used as a donor. As with R124 a reduced frequency of transfer to the lysogen 1100 (λsk^{o}) was observed (10^{-6} per donor).

The assay for EcoR124/3 made use of ^{32}P λsk^{o}.R124/3 and ^{3}H λ sk^{o} DNAs and both SAM and ATP were included in the assay mixture as in the assay for EcoR124. Digests were analysed by sucrose gradient centrifugation. EcoR124/3 activity was found in the 35 to 55% ammonium sulphate fraction just as was EcoR124. Further assays of this fraction (fig. 4.8) showed that at this stage EcoR124/3 activity required ATP but not SAM. Attempts to further purify this endonuclease activity by ion-exchange chromatography were unsuccessful and the isolation was not pursued further.

Thus from its properties in a crude isolate, EcoR124/3 appears very similar to EcoR124.

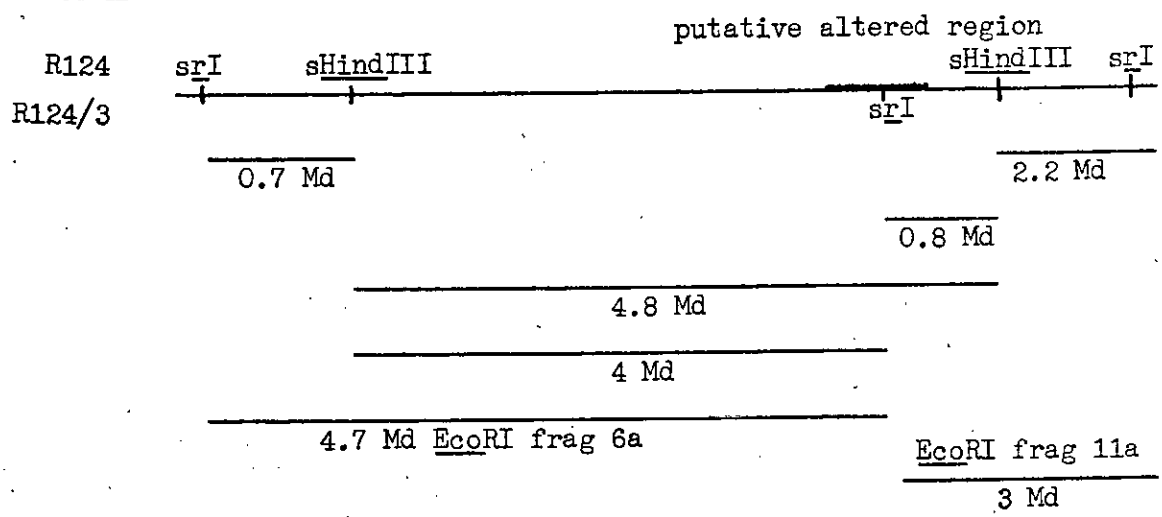
4.5(iii) Comparison of the DNAs of R124 and R124/3 by restriction enzyme digestion

The fragmentation patterns obtained by digesting the DNAs of R124 and R124/3 with a series of class II restriction endonucleases were compared by agarose gel electrophoresis. The patterns obtained for R124 and R124/3 are identical, except that those obtained with EcoRI and SalI show small differences, which are consistent with E124/3 containing an additional target for each of these enzymes.

In the case of EcoRI (fig. 4.9) cleavage at the additional site cuts fragment 3 (8 Md) to give new fragments 6b and 11a of 4.7 and 3 Md respectively. With SalI (fig. 4.10) fragment 5 (6.4 Md) is cut at the additional site to give fragments 7 and 11 of 4.9 and 1.65 Md respectively.

In view of the similarity of their fragment patterns it is concluded that R124/3 is a derivative of R124 and that a small region of R124 DNA has been altered so as to contain new SalI and EcoRI cleavage sites in R124/3.

Double digests with Hind III and EcoRI were used to further analyse the distribution of restriction enzyme targets around the altered region in R124/3 (fig. 4.13). These showed that EcoRI fragment 3 of R124 is cut by HindIII as is fragment 11 a of R124/3. Also a new fragment of 4.8 Md is present in the double digest of R124 and not in that of R124/3. Conversely a fragment of 4 Md is generated from R124/3 but not R124. It is concluded that these come from cleavage by HindIII of R124 fragment 3 and R124/3 fragment 6a respectively. These observations are consistent with the distribution of HindIII and EcoRI cleavage sites shown below.



Legend to Fig. 4.9

Comparison of R124 and R124/3 DNAs by digestion with EcoRI and HindIII followed by gel electrophoresis.

Tracks 1 and 4 contain undigested plasmid DNA
Tracks 2 and 5 contain plasmid DNA digested with EcoRI
Tracks 3 and 6 " " " " " HindIII

The fragmentation patterns of the two plasmids are shown schematically on the right of the figure. Bands which differ between R124 and R124/3 are marked with triangles. The sizes of these fragments were determined by comparing their mobilities with these of EcoRI fragments of λ DNA and are shown on the far right of the figure.

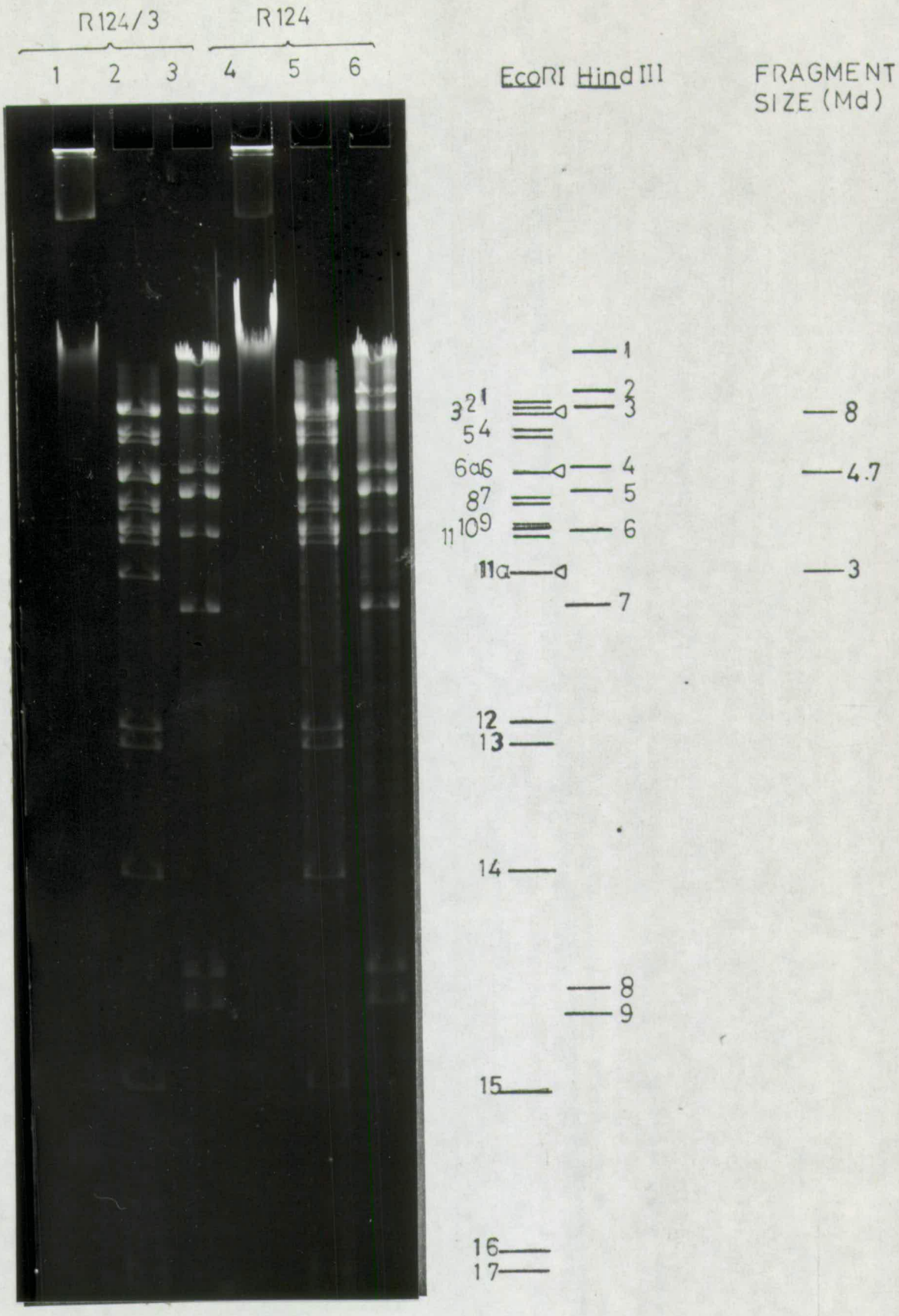


Fig. 4.9 A comparison of R124 and R124/3 DNAs by digestion with EcoRI and HindIII followed by agarose gel electrophresis.

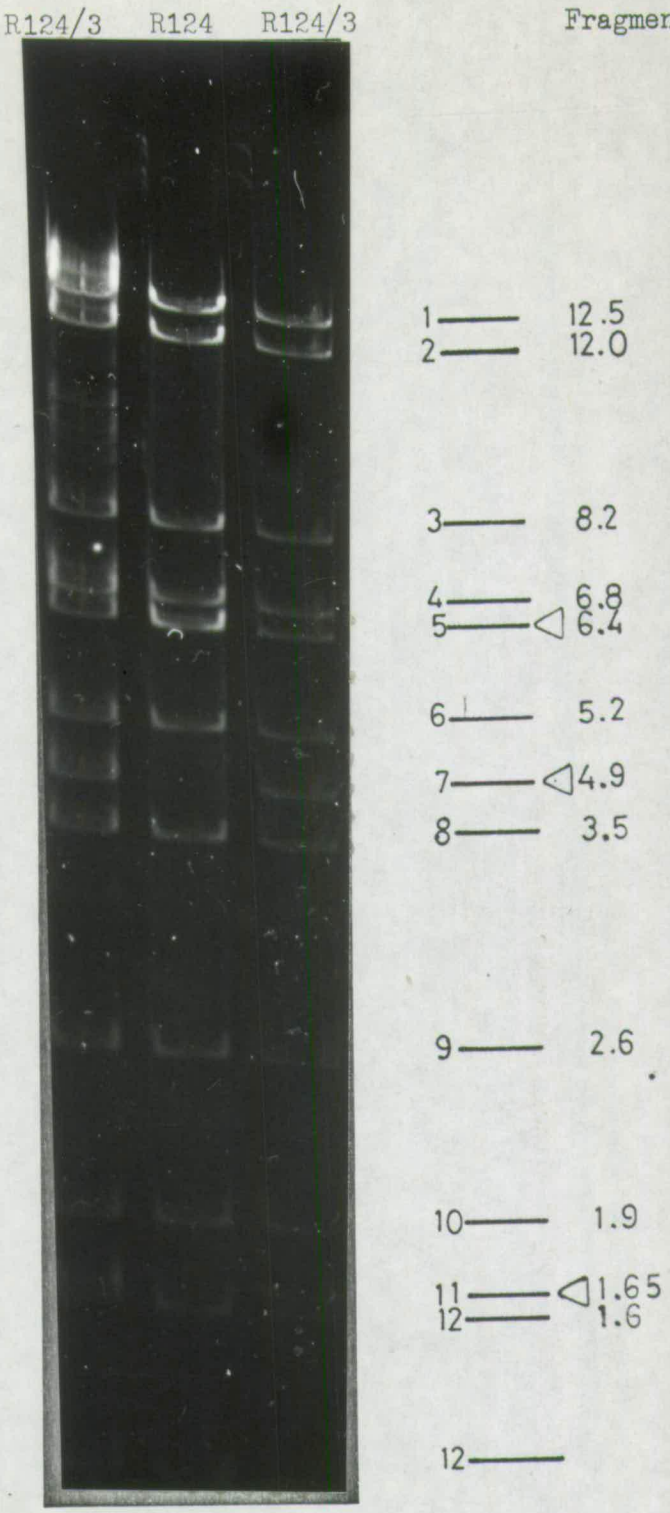


Fig. 4.10 Fragmentation of R124 and R124/3 DNA by SalI

Band 5 from R124 DNA contains 2 fragments of 6.4 Md . One of these is cut in R124/3 DNA to give new fragments of 4.9 and 1.65 Md

Legend to Fig. 4.11 (Continued)

Track 4 shows a digest of λ*mec*$^-$ DNA with *Eco*RII and tracks 5 and 6 show attempts to digest the DNA of R124 and R124/3 DNA with *Eco*RII under identical conditions.

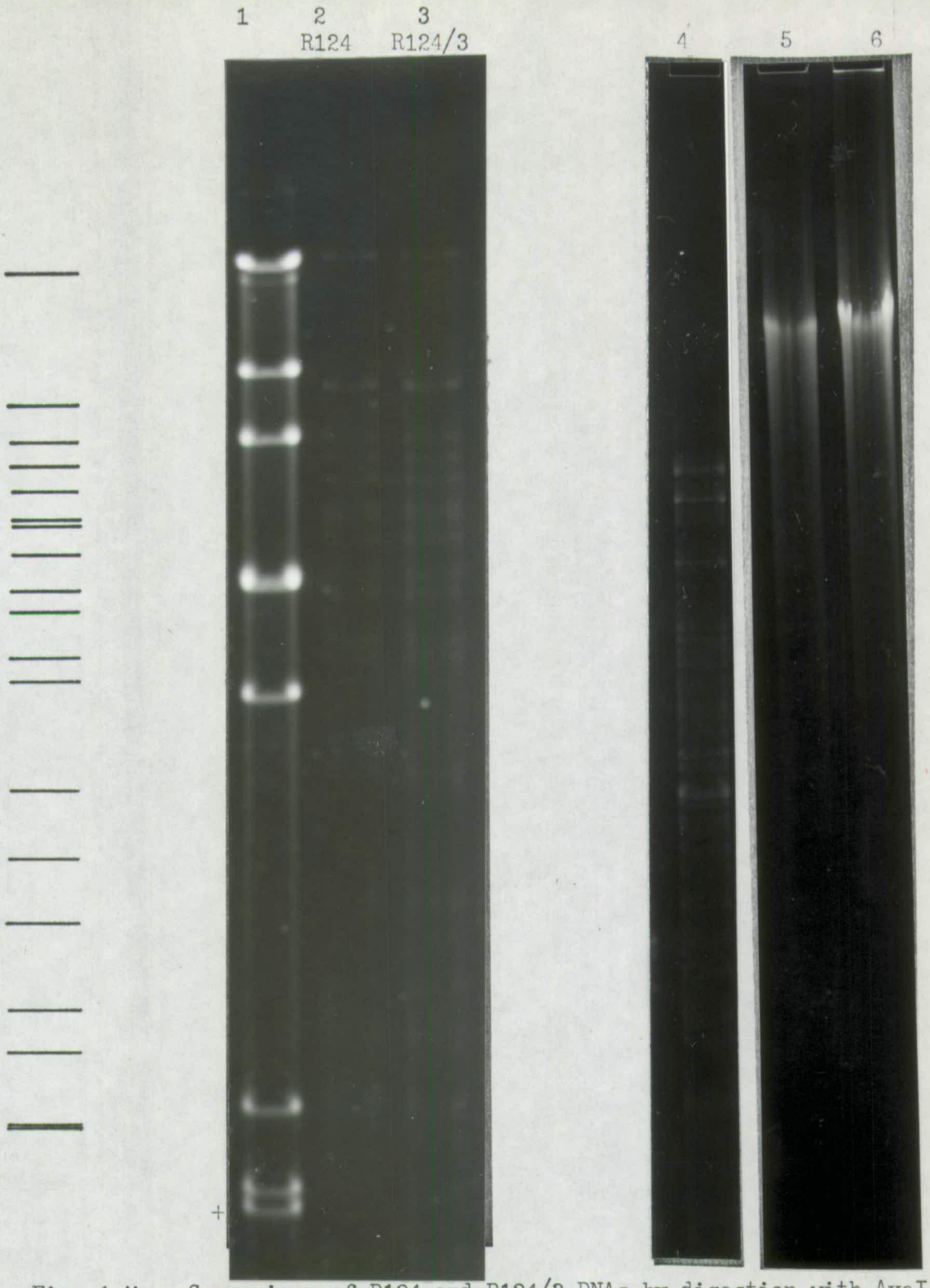


Fig. 4.11 Comparison of R124 and R124/3 DNAs by digestion with AvaI and EcoRII followed by agarose gel electrophoresis.

Tracks 1,2,3, show digests with AvaI of λ, R124, and R124/3 DNAs respectively. Since tracks 2 and 3 are rather feint on the print they are drawn schematically on the left. No differences were observed between tracks 2 and 3.

Fig.4.12 Comparison of BamI digests of R124 and R124/3 DNAs.

Legend to Fig. 4.13

Analysis of EcoRI/HindIII co-digests of R124 and R124/3 DNAs by agarose gel electrophoresis

Track 1. R124 DNA digested with EcoRI
Track 2. R124/3 DNA " " EcoRI
Track 3. R124 DNA digested with EcoRI + HindIII
Track 4. R124/3 DNA " " EcoRI + HindIII
Track 5. R124 DNA " " HindIII. The largest fragment was cut from this track prior to photography.
Track 4 shows incomplete digestion, consequently this digestion was repeated and run on track 7.
Track 6 shows an EcoRI digest of R124/3 DNA run on the same gel.

The result is shown schematically on the right of the figure. Bands which differ between R124 and R124/3 in the double digest are marked with a triangle.

Refer to Fig. 4.9 for the numbering of EcoRI fragments of R124.

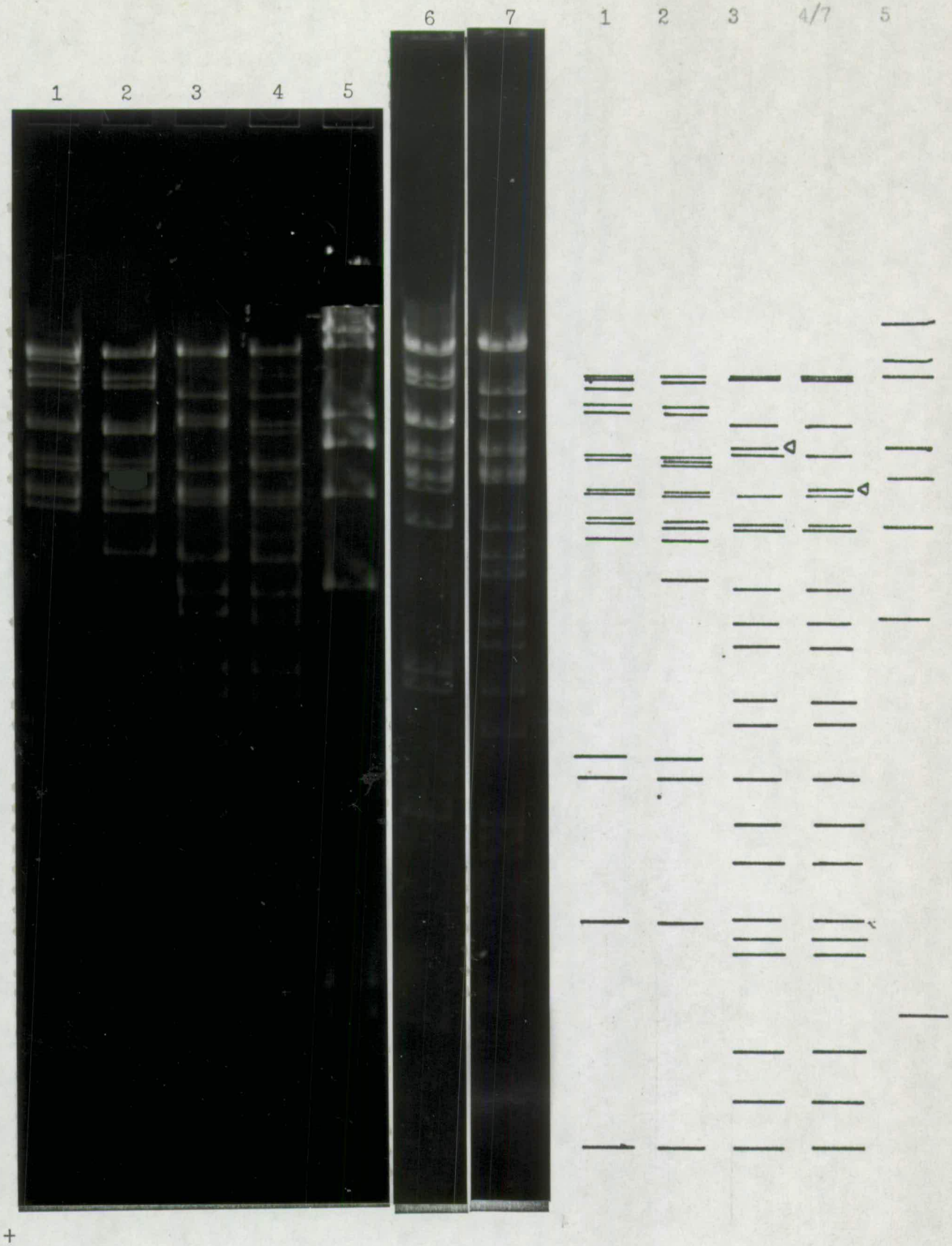


Fig. 4./3. Analysis of EcoRI/HindIII codigests of R124 and R124/3 DNAs.

The 0.8 Md fragment which this map predicts should be present only in the double digest of R124/3 has not yet been observed. It is likely that it is masked by another fragment on the agarose gel.

These results imply that the region of R124 DNA which is altered in R124/3 lies on a HindIII fragment of 4.8 Md the most likely candidate for which is HindIII fragment 4 (see fig. 4.9).

Comparison of the cleavage patterns of R124 and F DNAs with EcoRI (fig. 4.14) showed several bands of the same mobility. These correspond to F bands 1,2 and 6 which come from the region of F responsible for transfer functions (tra) (Ohtsubo unpublished, see Skurray et al 1976). This is a region of strong homology among F-like plasmids as shown by DNA heteroduplex analysis (Sharp et al 1973; Davidson et al 1975). It might be expected that the plasmids F and R124 are identical in this region, since their transfer operons will complement for all tra functions, including traI which is otherwise plasmid specific. R124 and F DNAs are also similar in containing a large region of about 30 Md with no HindIII cleavage sites. It is likely that the large EcoRI fragments of R124 (fragments 1 and 2) which contain no HindIII cleavage sites comprise part of this large fragment. Since R124 fragments 1 and 2 are those with the same electrophoretic mobilities as F fragments 1 and 2, it will be interesting to see whether the tra genes of F lie on the large HindIII fragment, when the HindIII targets on F are mapped.

4.5(iv) The transforming ability of R124 and R124/3 DNA

Both plasmid DNAs were tested for the ability to transform competent cells of E.coliC simultaneously to tetracyclin resistance and to R124 or R124/3 specific restriction proficiency. The frequency of transformation obtained (10^{-7} per ug of DNA per competent cell) was in accord with those frequencies observed by other workers (Smith et al 1975) for similarly large plasmids. The frequency of transformation was determined from the least dilution of the transformed culture required to prevent recovery of tet^r colonies when grown overnight in L broth and plated on tetracyclin containing plates. All the tetracyclin resistant colonies recovered also carried the appropriate restriction system. This provides additional evidence that the novel restriction system of R124/3 has arisen from an

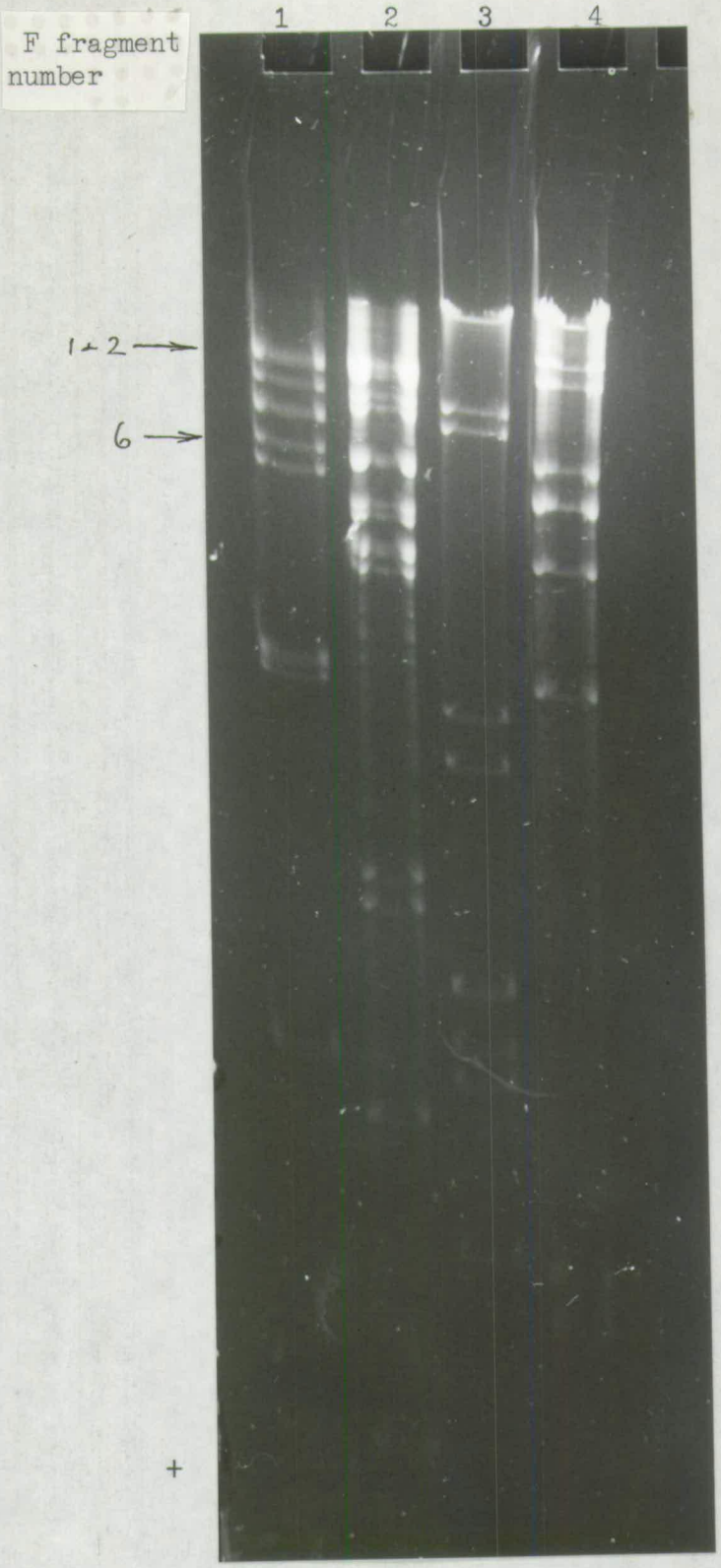


Fig. 4.14. Comparison of the fragmentation of F and R124 DNAs by EcoRI and HindIII by agarose gel electrophoresis.

Track 1, F DNA digested with EcoRI ; track 2, R124 DNA digested with EcoRI ; track 3, F DNA digested with HindIII ; track 4, R124 DNA digested with HindIII .

alteration of the plasmid R124.

4.5(v) Conclusions

From its fragmentation pattern with a series of class II restriction enzymes the plasmid R124/3 is clearly a derivative of plasmid R124 which in gaining new cleavage sites for EcoRI and SalI has become altered in the specificity of its restriction system. It will be argued that the latter event is a consequence of the former.

The DNAs of R124 and R124/3 were isolated on three separate occasions from separate clones and the patterns obtained with each set of isolates upon digestion with EcoRI were identical. This indicates that the altered distribution of EcoRI cleavage sites in R124/3 is a stable property of that plasmid and not a manifestation of some unstable rearrangement of the plasmid, such as the inversion of a segment of DNA lying between duplicated sequences. Consequently it is reasonable to assume that both the change in restriction specificity and the change in DNA structure of the plasmid represent rare events, and since the likelihood of them having occured simultaneously and independently is the product of their individual probabilities, it is most likely that they result from the same event. By a similar argument since the nucleotide sequences recognised and cut by EcoRI and SalI do not overlap (J Arrand unpublished) the acquisition of new cleavage sites for both these enzymes could not result from a single point mutation, as two independent mutations would be required. This all supports the proposition that EcoRI fragment 3 of R124 carries the hs determinants for the EcoR124 system and that a segment of DNA within this fragment has been altered by recombination with the host chromosome during plasmid transfer, to generate the hs determinants for EcoR124/3. Normally such a change in restriction specificity might be expected to be a lethal event since it would cause the bacterium to destroy its own DNA. However, plasmids must have some system for delaying the expression of restriction until a new host has had chance to become modified, as is seen during P1 infection (Arber and Dussoix 1962). This offers a possibility by which a novel plasmid encoded restriction and modification specificity which arises during transfer has a chance

to become established in the new host.

An obvious candidate for the host chromosomal gene with which the plasmid has recombined is the hsds gene of the EcoK system. Evidence that the EcoR124 and EcoK systems are evolutionarily related by both genetic and biochemical criteria has been presented in section 4.3(iv) and it is possible that recombination between the two hsds genes could produce a hybrid gene with a novel specificity . Such a phenomenon has recently been observed for recombinants formed by P1 transduction between the allelic restriction systems of Salmonella potsdam (sp) and an E.coli/Salmonella hybrid (sb) where a recombinant of novel specificity was observed (sq) (Bullas et al 1976). The recombinant system (sq) was found to be allelic to the sp and sb systems and also to complement mutants of the EcoK system. While no complementation has been observed between mutants of the EcoK and EcoR124 systems, this does not rule out the possibility that the determinants of the two systems, in diverging from a common ancestor, have retained sufficient regions of homology to undergo recombination.

The isolation of a λtransducing phage carrying the $hsds_k$ and $hsdm_k$ genes by Bonck et al (1976) offered a possible means of testing the above hypothesis, assuming that length of the piece of $hsds_k$ gene inserted in R124/3 is sufficiently long to be detectable by DNA heteroduplex analysis. The λhsd phage was made by inserting EcoRI fragments of E.coli DNA into a suitable λreceptor by the method of Murray and Murray (1974). Phage carrying and expressing hsds and hsdm were selected by serially passaging the pool of transducing phages on restricting and non restricting hosts alternately so as to enrich for phage with the ability to self modify. If phages carrying the hsd genes for EcoR124 and EcoR124/3 could be isolated in this way it should be possible by heteroduplex analysis and restriction enzyme analysis to determine the position and size of the DNA segment which is altered in R124/3. It should also be possible to show whether this segment of DNA has homology with the λhsd_k phage of Bonck et al (1976).

An attenpt to isolate λtransducing phages for the hsd genes of R124 and R124/3 using EcoRI is reported in Appendix 2. There are many possible explanations for the fact that despite the precautions

taken to ensure their isolation such phages were not isolated. None of these is sufficient reason for assuming that such a phage could not be isolated using a different receptor phage or possibly a small plasmid vector and a different restriction enzyme. This is the probable direction of future work.

CHAPTER 5

General Discussion

5.1 Introduction

The individual experimental results of this thesis have already been discussed in the relevant experimental sections. This general discussion will be confined to a consideration of the more fundamental conclusions and implications of the work, relative to the question of the occurrence, origin and role of restriction and modification systems in general, and their relationship to plasmids in particular.

5.2 The occurrence of restriction and modification systems among plasmids

The cause of the apparently haphazard distribution of restriction and modification systems among plasmids will remain obscure until evidence leading to a fuller understanding of their role *in vivo* is obtained. The only obvious correlation, that between the EcoRII system and those N group plasmids which do not undergo thymineless elimination, (Birks and Pinney 1974) still remains to be explained.

The contribution which this thesis has made to our knowledge in this area has been to introduce a new fact which will have to be accommodated in any explanatory models. This is, that both class I and class II restriction systems exist on plasmids. However, we still have only one isolate from the wild of a plasmid carrying a class I system.

5.3 The origin of plasmid encoded restriction and modification systems

The study of translocation elements and the discovery of plasmids with very wide host ranges, has underlined the fluidity of the distribution of genes among prokaryotes. This emphasises the futility of attempting to identify the source of a particular set of genes. However, if one can establish that genes of similar function also show structural homology, for instance by exhibiting their ability to recombine, or by demonstrating similarities in the amino acid sequences of their products, it is reasonable to claim that, at least segments of those genes have a common ancestral gene. The

work in this thesis suggests that such an evolutionary relationship exists between the determinants of the EcoK restriction and modification system and those of the EcoR124 system. It is likely that they recombined to produce the novel system EcoR124/3. Further support for this relationship comes from the observation (Bannister 1969) that the restriction of phage λ by the EcoR124 system is far more efficient when the plasmid is resident in E.coliC (e.o.p. 10^{-6}) than when it is in E.coliK (e.o.p. 10^{-4}) or E.coliB (e.o.p. 10^{-2}). E.coliC does not contain a class I restriction system. Although the important control experiment with R124 resident in an E.coliK or B derivative deleted for hsd was not performed, these observations suggest that the plasmid and host restriction enzymes interfere with one another, possibly by reassortment of enzyme subunits to give an inactive complex. This interpretation is consistent with the observation that mutants of the EcoK and EcoR124 systems do not complement in vivo. If, as this implies, the enzyme subunits of the EcoK and EcoR124 systems cross react abortively one must conclude that hsd_{R124} was largely conserved in the recombination event which generated EcoR124/3, and that the event was confined to a small region of the hsds gene This region is expected to be that which codes for the domain of the specificity subunit responsible for recognition of the host specificity site. A similar conclusion was reached by Bullas et al (1976) concerning their recombinant between the SP system of Salmonella and the allelic EcoB system.

The shared site specificity of the mec and EcoRII methylases suggests a possible evolutionary relationship between their determinants. However, no evidence of structural homology between the two methylases or their determinants has been demonstrated. Also, it must be noted that there are examples of restriction and modification systems, from widely diverged species, which have common nucleotide sequence recognition properties, for instance the systems of Bacillus subtilisX5 (BsuX5) and Haemophilus aegyptius (HaeIII) (Bron and Murray 1975). Until we have more information concerning homology between the determinants of these systems we must accept the possibility that the mec and EcoRII methylases and also the BsuX5 and HaeIII systems evolved independently and have converged on a common recognition sequence. Thus we await comparative

amino acid sequence analysis of the appropriate enzymes. An alternative approach which is inmediately available to the mec and EcoRII systems would be to isolate transducing phages carrying mec and hsd_{RII} and to compare them by heteroduplex analysis.

5.4 The role of plasmid encoded restriction and modification systems

Class I and class II restriction and modification systems may well have totally different roles in vivo. However, since the roles to be suggested for them are based on the superficial properties which the classes share, they will in general be treated together.

By degrading foreign DNA, restriction systems, besides providing an immediate barrier against virulent phages most probably defend members of a species or strain against the alteration of their genetic composition by recombination. As such they act as genetic isolating mechanisms. Genetic isolation has been proposed as prerequisite for the evolutionary divergence which leads to speciation (Dobzhanski 1951). Among higher eukaryotes, mechanisms of genetic isolation are immediately divisible into geographical and physiological (reproductive) isolation. The former has been proposed as an essential precursor of the latter, though the manner in which mechanisms of reproductive isolation might have arisen and been selected for is a central question in evolutionary theory. Examples of reproductive isolation include differential fertility timing and physical barriers to mating. Among prokaryotes the mechanism of exclusion by Teven phages (Pees and de Groote 1970 ; Russel and Huskey 1974) has been suggested as an example of a system which by limiting recombination between certain genes of T_2, T_4, T_6 and related phages, maintains their separate genetic identity i.e. it ensures their genetic isolation. To propose that restriction and modification act in this capacity among prokaryotes is to say for example that the restriction systems of E.coliK and E.coliB are responsible for stabilising the divergence which has taken place between these strains. Thus the acquisition of a new restriction specificity may be regarded as a critical step in prokaryotic evolution. An observation consistent with this proposition has been made by Hughes and Murray (unpublished work). Sequence specific endonucleases have been isolated and characterised from 2 closely related species of Anabaena, A.variabilis (AvaI and AvaII) (Murray et

al 1976), and A.subcylindrica (AsuI). It is assumed that these enzymes will subsequently be found to be part of a restriction and modification system. The nucleotide sequence recognised by AsuI is G-G-N-CC and that by AvaII G-G-($\frac{A}{T}$)-C-C. These sequences differ only in the limitations placed on the central nucleotide. This suggests strongly that one of these enzymes has evolved from the other by a subtle alteration in that part of the enzyme which recognises the central nucleotide, in the process of divergence between A.variabilis and A.catenula.

Plasmid controlled restriction and modification systems offer an immediate means by which an organism can erect a new genetic barrier. In addition the possibility of recombination between the determinants of the plasmid and host controlled systems offers a means of generating novel restriction and modification specificities.

Following the use of class II restriction endonucleases in in vitro recombination one is tempted to conclude that they have or have had such a role in vivo (see Dussoix et al 1975). Plausible models can be built to explain the way in which a plasmid borne system, upon transfer to a new host, might make the endonucleolytic cuts required for recombination between the host chromosome and genes mobilised or carried by the plasmid, before either have fully acquired the appropriate modification. Such a role is clearly more compatible with the known behaviour of plasmids than that of protection against recombination suggested above. With this in mind it is tempting to speculate that the class II restriction enzymes evolved from a set of recombination promoting enzymes which were important during early prokaryotic evolution for the assembly and permutation of genomes, and that at a critical stage they became associated with modification enzymes, together with which they stabilised the distribution of genes among species. This speculation offers a means of reconciling the opposing roles so far suggested for the plasmid controlled restriction and modification systems. One can think of restriction as a promoter of recombination during plasmid transfer and restriction and modification acting together to offer an additional fertility barrier during the stable association between host and plasmid. In this regard it will be interesting to learn whether either class of restriction endonucleases is related to the enzymes

involved in the recombination events implicated in the excision and insertion of translocation elements. The possibility of a relationship here is augmented by the observation that translocation elements themselves code for functions involved in their mobilisation (Heffron et al unpublished). Also, the presence of a particular translocation element on a plasmid prevents the transfer of another copy of that element to that plasmid although it does not prevent the transfer of that element to another plasmid (Robinson unpublished). This cis-specific phenomenon could be explained by a model in which all the sites on the plasmid at which the translocation element can insert are modified by an enzyme encoded on the resident element.

In assisting the process of speciation a plasmid may be seen as extending its host range and thus its ability to spread and survive. This offers a selective mechanism for the retention of restriction and modification systems on plasmids.

An association between the restriction endonucleases and the enzymes involved in recombination (recB recC exonuclease) was noted by Brammar et al (1974). In examining the effect of in vivo restriction on the expression of genes containing specificity sites for class I and class II systems they observed that restricted DNA is rapidly degraded by the recB recC exonuclease but that in its absence restricted DNA is stable. Those genes not actually cut by the restriction endonuclease may be expressed. Thus the restriction endonucleases help to make DNA a substrate for the degrading activity of recombination nucleases and by implication perhaps also for their recombinatory activity. The possibility of class I systems being involved in recombination was raised by Goodgal and Gromkova (1975). They found that non tranformable mutants of H.influenzae lacked the class I restriction system HindI, and that naturally occuring non-transforming strains also lacked this system. They also found that DNA restricted in vitro with HindI still had transforming activity but that linkage was reduced. This offers a possible refinement of the recombinatory role suggested for restriction. It is possible that restriction enzymes rather than abolishing and/or mediating recombination, exercise a control over the amount of genetic information which can be acquired in a recombination event. Thus they stimulate genetic exchange without allowing wholesale

acquisition of blocks of genes which might seriously disrupt the chromosome or abolish divergence. This refinement is clearly more applicable to class I systems than to class II.

Another possible role for plasmid controlled systems is the genetic isolation of the plasmid itself. In preventing superinfection by plasmids which might exclude or recombine with the resident plasmid restriction systems would take their place beside the mechanisms of incompatibility and superinfection immunity which are widespread among plasmids.

The roles suggested above are based on the gross properties of restriction and modification systems plus a few preliminary observations and must be regarded as highly speculative. Obviously there is a lot of work to be done in the area of recombination and population genetics to further analyse the *in vivo* role of restriction and modification.

5.5 The possible future scientific importance of restriction and modification

In addition to their technological importance in genetic engineering and genomic analysis, restriction and modification enzymes still have fundamental scientific importance in the area of nucleic acid protein interactions. It is crucial to our understanding in this area to learn more of the structures and mechanisms involved in the recognition of specific nucleotide sequences, especially for the class I restriction enzymes which interact with DNA in such a dramatic way. The recent construction of transducing phages carrying determinants for the EcoK and EcoRI systems (Bork *et al* 1976; Murray unpublished) should facilitate the isolation of large amounts of pure restriction and modification enzymes, which should in turn facilitate physical and chemical study of the structure and mechanism of these enzymes. At the same time, a genetic study of the fine structure of the *hsd*s genes of recombinant systems with altered specificities such as those described in this thesis and by Bullas *et al* 1976, should reveal the domains of the *hsd*s gene product involved in specific nucleotide sequence recognition. The genetic analysis will be best done with the *hsd* genes inserted in transducing phages. This will allow complementary electron

microscopic studies of heteroduplexes formed between parental and recombinant alleles to be undertaken.

In the field of restriction and modification many questions remain to interest those who look beyond the technological importance of class II restriction endonucleases.

Appendix 1

The transfer of plasmids RY, R124 and R245 into E.coli5K

Plasmids R124 and R245 were transferred directly to 5K from J53 using selection for tetracyclin resistance and prototrophy as contraselection (J53 is met$^-$). The transfer of RY5 was more complex. At the time it was not known that this plasmid was an aggregate. Information from Yoshimori (1971) indicated that the plasmid was non-self transmissable, carried streptomycin resistance, and resided in HB29, an end$^-$ gal$^-$ hsd_B derivative of E.coliK. Consequently it was reasoned that an additional plasmid would be required to mobilise RY5 from HB29 and for this purpose F' gal was chosen. The final recipient was a nalidixic acid resistant derivative of 5K (5K·nal^r).

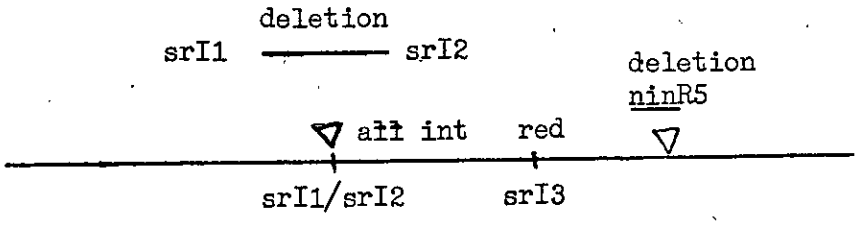
Exponentially growing cultures (at about $2x10^8$ cells/ml) of E.coli HB29 and ED2563 (F' gal donor, nal^s) were mixed (2ml of each) and left to stand for 30 mins. Then a culture of 5Knal^r (1ml at $2x10^8$ cells/ml) was added and the mixture left for 2 hrs before being plated onto medium containing nalidixic acid (40 ug/ml) and streptomycin 15mg/ml). Neither of the donor cultures nor recipient culture produced colonies on this medium.

Progeny from the mating selected on streptomycin and nalidixic acid were purified and tested for RY5 specific (EcoRI) restriction and modification. All the st^r nal^r clones tested proved positive in this respect and one of them was retained as 5K(RY).

Appendix II

The construction *in vitro* of transducing phages carrying segments of the plasmids R124 and R124/3

The procedures developed by Murray and Murray (1974) for the *in vitro* construction of λ transducing phages was followed. This utilises specially constructed λ derivatives containing only single targets for restriction endonucleases and from which inessential genes have been deleted, to make space for the insertion of exogenous DNA. The actual λ derivative chosen was number 574 (Murray *et al* 1976) which is a vector for EcoRI fragments. The phage is shown schematically below:



The insertion of a fragment of up to 9 Md between srI1 and srI3 is premitted since the phage may be reassembled without the fragment of λ DNA from between srI1/2 and srI3. Such recombinant phage having lost the *att* site, the *int* gene and part of the *red* gene are unable to lysogenise and are *red*⁻. Both these characters are readily tested and may be used in asessing the frequency of recombinant phage among progeny of an *in vitro* recombination experiment. The choice of the EcoRI *in vitro* recombination system was based on the observation that the EcoRI fragment of R124 likely to carry the *hs* determinants was between 8 and 9 Md in size and should be able to fit into the space available in phage 574. At that time it was erroneously thought that the *hs* determinants were located on the largest (30 Md) HindIII fragment of R124, which is too large to fit into a λ vector, and for this reason the HindIII recombination system (Murray and Murray 1975) was not tried.

Procedure

Stocks of phage λ574 DNA modified to EcoR124 and EcoR124/3 were produced. About 3 ug of each of these DNAs was restricted by incubation with EcoRI at 37° for 1h and then heated at 75° for 10 min. R124 DNA (3 ug) and R124/3 DNA (1 µg) were then restricted with

EcoRI, and tube gel electrophoresis of both phage and plasmid (only R124) DNA carried out to check restriction. Restricted λ574.R124 DNA (2 ug) was then added to 3 ug of restricted R124 DNA, and 1.25 ug of 574.R124/3 DNA was added to 1 µg of restricted R124/3 DNA. This gave volumes of 60 and 65 ul respectively, which were made up to 100 ul by addition of a solution of 0.1M NaCl 0.01M tris HCl pH 7.5. Ligase reaction cocktail (10 ul) was then added to each tube followed by 0.2 ul of T_4 ligase. The DNA mixtures were then incubated at 10^{o} for 4h and then at 0^{o} for 3 days. Then the ligated mixture was used to transfect a competent culture of E.coliC 5µl samples being used per 0.3 ml transfection reaction. Freshly grown E.coli5K cells* (0.2 ml of a borth culture) were added to each transfection reaction and the mixture plated on BBL agar in BBL top layer agar.

Simultaneous transfections were carried out with unrestricted 574 DNAs and with samples of restricted 574 DNAs removed prior to ligation as controls.

The numbers of phage plaques obtained from the various DNA samples are shown below

DNA	plaques per plate
574.R124 restricted	10
574.R124/3 restricted	56
574.R124+R124 ligated	146
574.R124/3+R124/3 ligated	119
574.R124 unrestricted	ca 10,000
574.R124/3 unrestricted	ca 10,000

These results show that the ligation reaction was successful in reconstituting viable phage genomes from the mixture of restricted DNAs.

Of the phage plaques recovered from each of the ligation reactions one hundred were separately picked into phage buffer containing chloroform. Each of these clones was then diluted 1 in 10 and spotted on lawns of E.coli5K and a polA⁻ strain of E.coli, to

* Foot note

The addition of 5K ensured that the progeny from each transfection centre were at least partly K modified.

test for the red⁻ character (red⁻ phages plate very poorly on polA hosts). Of the 100 clones tested, 39 from the ligation reaction with R124 DNA were red⁻ and from the reaction with R124/3 20 were red⁻. E.o.p. determinations on 1100R124 and 1100R124/ showed that none of the 100 clones was modified against EcoR124 or EcoR124/3. In total over 800 progeny from each ligation reaction were pooled.

The selection procedure for a λ phage carrying the EcoR124 or EcoR124/3 hs determinants

The rationale for the selection of a phage carrying host specificity determinants is that such a phage should be able to self modify. The passage of a pool of phages carrying segments of a plasmid or bacterial chromosome, alternately on a restricting and a non restricting host, should offer a strong enrichment for a phage capable of self modification. For the EcoR124 system, each cycle of passage should give a 10^4 fold enrichment for a self modifying phage. Two cycles of passage was all that was required to isolate a λ derivative carrying hsd_K (Borck et al 1976). After 3 cycles of passage the stocks of transducing phages for R124 and R124/3 showed no reduction in their sensitivity to R124 and R124/3 specific restriction. This indicated that the pools of phages recovered from the in vitro recombination of and R124 or R124/3 did not contain phages capable of self modification.

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