CONSTRUCTION OF RECOMBINANT BACTERIOPHAGE

FOR THE STIMULATION OF STRUCTURAL GENE EXPRESSION

by

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'Tis all a Chequer-board of Nights and Days
Where Destiny with Men for Pieces plays:
Hither and thither moves, and mates and slays,
And one by one back in the Closet lays.

Edward Fitzgerald
The Rubáiyát of Omar Khayyám.

FOREWORD

The experiments described in this thesis and the composition of the text are my own work. The ideas behind the experiments emerged from discussion with my supervisor, Professor K. Murray, and other members of this Department.

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ABSTRACT

The bacteriophage maturation process includes a series of complex interactions between molecules of protein and DNA. Some of the essential protein components are present at very low levels in phage-infected cells. Systems have been developed to amplify the expression of these proteins in order to facilitate investigations of protein functions.

Hybrid bacteriophage derived from phage λ and P2 have been constructed *in vitro* using the R. *Eco* RI restriction endonuclease. The λ/P2 hybrid phage have contributed essential information for the identification of restriction enzyme recognition sites in P2 DNA and have provided a link between genetic and physical maps for phage P2. The expression of P2 structural genes in the hybrid phage has been studied and *in vivo* manipulation has demonstrated that transcription of P2 DNA can occur from phage λ promoters.

A λ duplication phage has been formed after a series of *in vitro* recombination reactions. The duplicated DNA includes the λ *cos* site and this creates the potential for 2 distinct linear monomeric phage DNA species. The patterns of protein synthesis from the duplication phage have been examined.

ABBREVIATIONS AND CONVENTIONS

Restriction endonucleases have been identified according to the system proposed by Smith and Nathans (1973). The prefix 'endo R' has been abbreviated to R. throughout this text.

Nudeotide sequences have been written in the 5' - 3' direction and phosphate groups have been omitted. Only deoxyribonucleotides are mentioned in the text and the prefix 'd' has been omitted.

OD_{260} nm represents the absorbance of light at 260 nm wavelength for a 1 cm light path.

pA, pB refer to the protein products from genes A and B.

The lengths of DNA molecules have been expressed in kilobases (kb).

The sizes of protein molecules have been expressed in daltons.

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CHAPTER 1 Introduction

The experimental work described in this thesis concerns the interactions between structural proteins and DNA during phage particle assembly. The temperate coliphage λ and P2 are the systems studied but comparisons are drawn with other bacteriophage. Definitive reviews of the genetic properties of λ and P2 are available (Hershey, 1971; Bertani and Bertani, 1971) and only a selection of properties is included here to illustrate similarities and differences between the 2 phage.

1.1 Temperate Bacteriophage

Temperate bacteriophage are characterised by their ability to follow lytic or lysogenic pathways after infection of a sensitive cell. The phage particle adsorbs to the cell surface and DNA is injected in a linear form. Immediately the DNA circularizes and bacterial ligase acts to seal single bond interruptions in the circle. Replication of the circular molecule begins and the division point between lysis and lysogeny occurs after several rounds of replication have been completed. DNA molecules circularize through short single strand projections of complementary sequence that are located at the 5' ends of the molecule. These projections have been termed cohesive ends (review, Yarmolinsky, 1971) and are a feature of some temperate bacteriophage; they appear during phage maturation as DNA is converted from a replication intermediate to a mature linear form that possesses cohesive ends. There is a specific

enzyme, designated *ter* (Mousset and Thomas, 1969) that recognises a particular nudeotide sequence in double strand DNA, the *cos* site, and produces a staggered break leading to single strand projections with complementary sequence and defined length.

1.2 Phage Genetic Structure

The λ genetic map (Fig 1.1) shows clustering of related genes; structural genes are located at the left of the genome and immunity and DNA replication genes are located at the right (Parkinson, 1968; Campbell, 1969; Davidson and Szybalski, 1971). The linear P2 genome is organised in a similar manner (Fig. 1.2) (Lindahl, 1969, 1974) but there is one main difference as the P2 lysis gene (gene K) is located between the head and tail genes whereas the λ lysis genes (genes S and R) are located at the extreme right of the genetic map.

DNA extracted from λ phage particles is a linear duplex molecule containing 49;000 base pairs (49 kb, Philippsen, P.and Davis, R. W. unpublished results). The functions essential for normal phage growth are confined to about 60% of this length but there is an element of physical size attached to phage maturation as DNA molecules lying outside the range 75 - 105% wild-type λ length are packaged very inefficiently (Henderson and Weil, 1977, a,b). P2 DNA is a linear duplex molecule containing 33,018 base pairs (Chattoraj, 1977); a combination of deletions will remove up to 17.7% of the P2 genome and this phage is still viable but it is likely that the remaining DNA is essential for normal phage development.

P2 was initially distinguished from λ on the basis of 3 general features a) very low recombination frequencies for P2, b) non-

λ GENETIC MAP

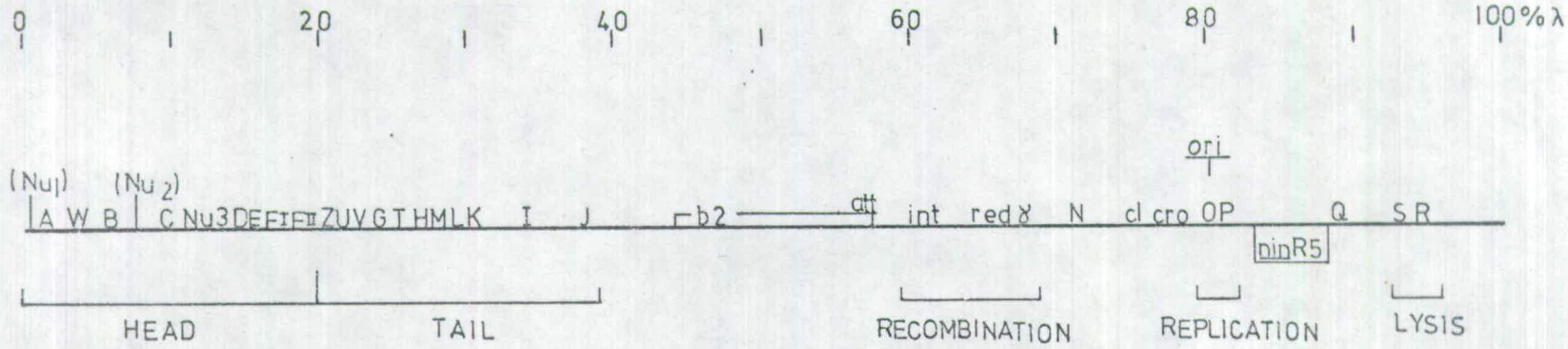


Fig. 1.1 (opposite)

Phage λ Genetic Map.

Genes A, W, B, C, Nu_3, D, E, F I and F II are involved with head assembly. Gene E codes for the major head protein.

Genes Z, U, V, G, T, H, M, L, K, I and J are involved with tail assembly. Gene V codes for the major tail protein and gene J determines the host range.

The b 2 region extends from 45.3% - 57.4% from the left end of the phage genome. This region can be deleted without removing any essential phage function.

att is the phage attachment site. The phage integrates into the bacterial chromosome at this site.

Genes int, red and ɣ are the phage recombination genes.

N is a positive regulator gene required during the phage lytic cycle.

c I is the phage repressor gene.

cro is a regulator gene for N-dependent transcription.

Genes O and P are required for phage DNA replication. The origin of replication is located within gene O and replication proceeds bi-directionally from this point.

nin R 5 is a deletion extending 83.8 - 89.2% from the left end of the phage genome.

Q is a positive activator gene required for transcription of the structural genes.

Genes S and R are responsible for bacterial cell lysis.

inducibility of a P2 prophage and c) the ability of P2 to occupy a number of host chromosomal sites when integrating to form a lysogen. An analysis of 42 distinct temperate bacteriophage isolated from nature revealed that 16 were serologically related to P2 and 12 to λ, showing that P2 and λ may be taken as typical members of 2 major groups of naturally occurring enterobacterial phage (Bertani and Bertani, 1971).

The factors involved with P2 recombination are not clearly understood. P2 apparently does not contain genes that would be analogous to phage λ recombination genes and the majority of recombination in P2 occurs across the attachment site and requires the int protein. The frequency of int-mediated recombination in P2 is approximately comparable with other temperate bacteriophage; P2 is distinct in recombination because of a significant reduction via alternative mechanisms. Recombination in P2 may be increased by ultra violet irradiation and results from this system suggest that the P2 genetic map may be circular (Bertani, 1975). The circular map can be justified as P2 DNA exists as a closed circular molecule during infection (Lindqvist, 1971) and recombination between circular molecules could occur by an exchange mechanism. In order to explain the pattern of normal int-mediated recombination, Bertani has suggested that a second recombination event occurs to the right of the attachment site and that this could be linked with DNA replication as the P2 origin of replication has been identified in this region of the chromosome.

P2 GENETIC MAP

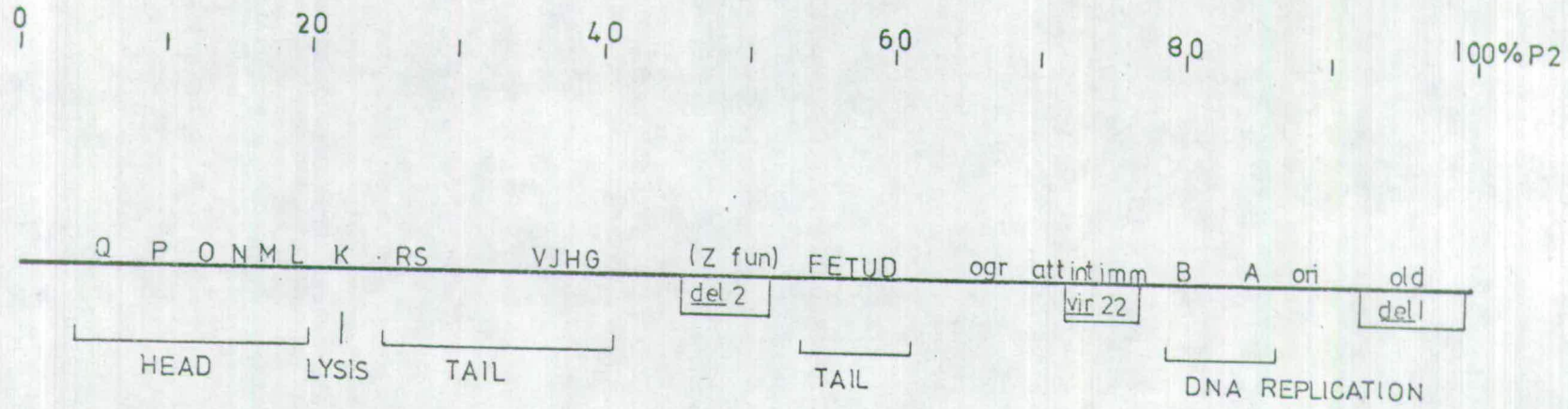


Fig. 1.2 (opposite)

Phage P2 Genetic Map.

Genes Q, P, O, N, M, and L are involved with P2 head synthesis. The major head protein is a modified form of the N gene product.

Gene K is the phage lysis gene.

Genes R, S, V, J, H, G, F, E, T, U, D are involved with P2 tail assembly. The major tail proteins are derived from gene F.

Gene Z is involved with the establishment of lysogeny (Bertani, 1976).

The fun gene causes increased sensitivity to FUDR with P2 lysogenic strains.

The ogr gene is involved with P2 late gene expression.

att is the phage attachment site. The phage integrates into the bacterial chromosome at this site. The majority of recombination in phage P2 occurs across the attachment site and requires the int protein.

imm is the phage immunity region and codes for the repressor protein.

Genes B and A are required for P2 DNA replication and late gene expression. The origin of replication, ori, is located 89% from the left end of the phage genome and replication proceeds rightwards from this point.

The old gene is involved with the phenomemon of λ-P2 interference. There is evidence for genetic linkage between the old gene and genes Q and P suggesting that the P2 genetic map may be circular.

P2 is able to form lysogens at high efficiency and can occupy several different chromosomal sites (Bertani and Six, 1958: Kelly, 1963; review Calendar *et al.*, 1977a). In *E. coli* C there is strong preference for one particular chromosomal site but there are several alternative sites; in *E. coli* K, 2 chromosomal sites are occupied at about the same frequency and one of these *E. coli* K sites is identical to a secondary site in *E. coli* C.

The non-inducible character of P2 lysogens has been explained by a model involving physical disruption of the *int* operon during prophage integration (Bertani, 1970). A P2 prophage is not excised from the host chromosome, even in a de-repressed lysogen, unless *int* function is provided by a superinfecting *int*$^+$ phage. When P2 repressor is active a superinfecting *int*$^+$ phage can integrate efficiently at a free attachment site and these observations suggest that P2 *int* expression is normally constitutive. Double lysogens formed by phage λ usually involve a tandem arrangement of 2 prophages (Calef *et al.*, 1965) whereas 2 distinct chromosomal sites would be occupied in a P2 double lysogen. Stable P2 tandem double lysogens only appear if one of the phage carries a mutation in the *int* gene (Bertani, 1971). A P2 tandem double lysogen would reconstitute an intact *int* gene and the structure is only stable if the mutant *int* gene is in a central position.

The orientation of prophage genes at different chromosomal sites has been shown to vary (Calendar and Lindahl 1969), but this does

not appear to affect the expression of P2 genes, fun and old, that are active in the lysogenic state. Strains of E. coli C that contain a P2 prophage show increased sensitivity to the base analogue 5-fluorodeoxyuridine (FUDR) (Bertani, 1964). Non lysogens have a bacteriostatic response to FUDR whereas there is a bacteriocidal effect for P2 lysogenic cells. A phage mutant (P2 fun fluorouracil non-converting) has been isolated that does not impart FUDR sensitivity to lysogenic cells. Bacteria lysogenic for P2 fun mutants show transient sensitivity to FUDR when superinfected with wild-type P2 confirming that the fun gene is expressed in the presence of P2 repressor (Bertani and Levy, 1964). The link between FUDR sensitivity and P2 fun gene expression is not understood. P2 phage carrying the del 2 deletion show the fun phenotype (Bertani, 1975).

Sironi (1969) isolated strains of E. coli C that could not be lysogenised by phage P2 (lyd strains lysogenisation defective). The lyd mutants have been shown by Sironi to be defective in recombination and the failure to form lysogens is due to cell killing. P2 mutants (P2 old, over lysogenisation defective) have been detected that are able to form lysogens normally on lyd strains. The P2 old mutation has been shown to be involved with λ-P2 interference (Lindahl, 1970): wild-type λ does not grow on a P2 lysogen, although adsorption and injection proceed normally, the block to wild-type λ growth is removed when the P2 prophage carries the old mutation. Phage λ red$^-$ gam$^-$ double mutants (λ Spi$^-$ mutants, sensitivity to P2 interference) grow normally on P2 lysogens (Zissler et al., 1971: Sironi et al., 1971). A double lysogen formed with wild-type

P2 and a P2 *old* mutant blocks λ growth suggesting an active role for the *old* gene in preventing λ multiplication. The phenomenon of λ-P2 interference has been the subject of a very careful biochemical and genetic analysis (Brégégère, 1974, 1976, 1978) and the results suggest that the *old* gene product acts at an early stage in the initiation of λ DNA replication. The exact mechanism of the interference still has to be determined. P2 phage carrying the *del* 1 deletion show similar properties to the P2 *old* mutants (Bertani, 1975).

1.3 DNA Replication

An examination of intracellular forms of λ DNA has revealed a rapidly sedimenting DNA species appearing late in the infectious cycle (Smith and Skalka, 1966). The rapidly-sedimenting form (later termed concatemeric DNA) seemed to consist of linear molecules 2-8 times the length of monomeric λ DNA and pulse-chase experiments suggested that concatemers are replication intermediates. A rolling circle model has been proposed to explain the derivation of concatemeric DNA from a replicating circle (Gilbert and Dressler, 1968; Eisen et al., 1968). The formation of concatemers is not affected by blocks to phage and bacterial recombination systems (Skalka, 1971) supporting the idea of concatemers being normal replication intermediates. Evidence from the electron microscope has confirmed that the closed-circular early replicating forms of λ DNA (θ forms, Schnös and Inman, 1970) are replaced by rolling circles (σ forms) at later times in the infectious cycle (Takahashi, 1974, 1975; Bastia et al., 1975; review, Skalka, 1977).

The linear concatemer is the normal substrate for λ packaging in vivo. (The term 'packaging' will appear frequently and it is used in the context of phage proteins and DNA interacting to form viable phage particles.) The development of λ in vitro packaging systems (Kaiser and Masuda, 1973: Hohn and Hohn, 1974) has shown that linear concatemeric and monomeric DNA can be efficiently converted into phage particles (Hohn, 1975). Linear monomeric DNA is not a substrate for packaging in vivo as DNA is not present in this form. Covalently

closed monomeric circles are not a substrate for packaging either *in vivo* or *in vitro*.

Normal λ DNA replication is disrupted if the phage has a *gam*$^-$ mutation (Zissler *et al.*, 1971; Enquist and Skalka, 1973). It appears that the *gam* gene product inactivates the bacterial *rec* BC nuclease to protect a transition state between closed circle and rolling circle replication. The rate of DNA synthesis from rolling circle replication is about three times greater than the rate of degradation by the *rec* BC exonuclease (Greenstein and Skalka, 1975). The *rec* BC nuclease has no action on closed circular DNA and, in the absence of *gam* gene product, replication produces only monomeric circles. Doubly mutant phage *red*$^-$ *gam*$^-$ do not grow on *rec* A$^-$ hosts but do grow on *rec* A$^-$B$^-$ hosts. These observations have been reconciled by the explanation that when *gam* protein is missing a recombination function (either phage or bacterial) converts some monomeric circles into multimeric circles and then monomers may be packaged from a multimeric circle. When all recombination is blocked, *gam*$^-$ phage replication is limited to monomeric circles and no phage particles are produced; growth on a *rec* A$^-$B$^-$ host is possible because the *rec* BC nuclease is non-functional and concatemeric DNA formation is not affected.

There is one instance where packaging of monomeric circular DNA has been demonstrated. A λ duplication phage with 2 copies of the *cos* site (Emmons, 1974) can be packaged by superinfecting φ 80 phage. The experiment is performed in a λ lysogen so that the λ duplication

phage is repressed and is present as covalently closed monomeric circles. There is no replication of the duplication phage and phage recovered after packaging have all lost the duplication demonstrating that both cos sites have been cut. There is no packaging in the control experiment with a normal λ phage in the φ 80-infected λ lysogen (Feiss and Margulies, 1973).

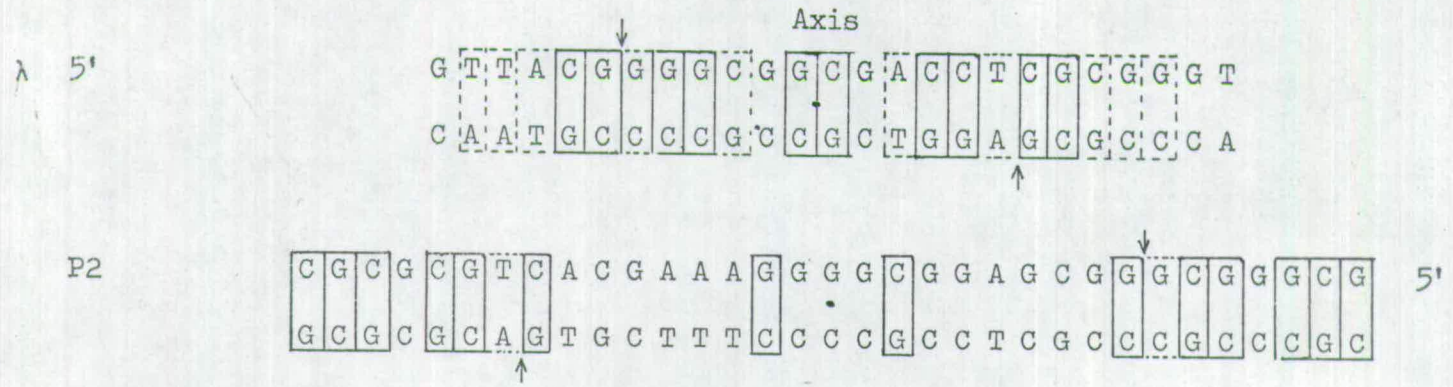
P2 DNA has been shown to exist in closed and open circular monomeric forms during the infectious cycle (Geisselsoder, 1976; Chattoraj, 1978); both forms apparently can be matured to give phage particles (Lindqvist, 1971). Observations of replicating P2 DNA molecules showed a modified rolling circle structure where the length of double strand DNA leading away from the circle does not exceed monomer P2 in length (Schnös and Inman, 1971). A model has been proposed in which the cis-acting P2 A protein promotes a site-specific recombination event to cleave the tail of the rolling circle and produce monomer circles (Pruss et al., 1975). The P2 in vitro packaging system (Pruss et al., 1974) has shown that both linear and monomeric circular DNA can be packaged in vitro. The efficiency is approximately 3 times higher for circular DNA when compared with linear DNA; linear concatemeric P2 DNA, prepared in vitro using DNA ligase, is packaged at about 20% the efficiency of linear monomeric DNA (Pruss et al., 1975).

1.4 Polarity in DNA Packaging

There is now considerable cumulative evidence demonstrating polarity for λ DNA packaging. The left cohesive end (adjacent to gene A) enters the head first (Emmons, 1974; Syvanen, 1975; Sternberg and Weisberg, 1975); the right cohesive end (adjacent to gene R) is positioned close to the phage tail (Padmanabhan et al., 1972; Saigo and Uchida, 1974; Chattoraj and Inman, 1974). (See Davidson and Szybalski, 1971 for a review of the evidence linking genetic and physical maps). The experiments of Chattoraj and Inman (1974) also revealed that the left cohesive end of P2 DNA is attached to P2 tails. This indicates that there is polarity for P2 packaging but further confirmatory evidence still has to be provided.

Fig. 1.3

Phage λ and P2 Cohesive End Sequences.



Sequences taken from Weigel et al., 1973 and Murray, K. et al., 1977.

Nucleotides enclosed in boxes are placed symmetrically with respect to a central axis of rotation, dashed lines indicate nucleotides that are symmetrical in purine or pyrimidine arrangements. The arrows show the locations of breaks introduced by ter enzymes.

1.5 Phage Cohesive Ends

The nucleotide sequence of λ cohesive ends has been determined (Wu and Taylor, 1971). The sequence has been confirmed for λ and an identical cohesive end sequence has been found for other lambdoid phage - φ 80, 82, 21 and 424 (Murray and Murray, 1973). The λ-type cohesive ends are 12 base pairs long and the sequence demonstrates hyphenated rotational symmetry about a central axis. An investigation of the sequence at the 3' ends of λ DNA showed that the hyphenated rotational symmetry extends through the cohesive ends into double strand DNA (Weigel et al., 1973). From the axis of symmetry, 5 of the first 8 base pairs show rotational symmetry and a further 5 of the first 11 base pairs show symmetrical purine-pyrimidine orientation. The single strand breaks produced by the ter enzyme are symmetrically placed with respect to the central axis (Fig. 1.3). A derivative of φ 80 (φ D 326, Rock et al., 1974) provides the only known deviation from the common lambdoid sequence; there are 2 changes in positions that do not affect the rotationally symmetrical distribution of bases and the altered sequence is still recognised by λ ter enzyme (Murray et al., 1975).

The chemical method for DNA sequencing (Maxam and Gilbert, 1977) has recently been applied to λ cohesive ends (Nichols and Donelson, 1978). These workers are able to identify 61 nucleotides from the left cohesive end and 117 nucleotides from the right end. Models proposed to explain the polarity of DNA packaging have invoked a recognition site close to the left cohesive end and the nucleotide sequence information revealed a perfect inverted repeat sequence,

10 nucleotides long, extending between positions 34 and 43 from the left 5' end. It is tempting to identify this inverted repeat as a site for interaction between DNA and protein but proof of this will only come from binding studies with DNA and purified protein. Another perfect inverted repeat sequence has been detected between positions 49 and 63 from the right 5' end. The only similarity between the inverted repeats is that both sequences are very A - T rich (80% AT); the 10 base inverted repeat close to the left cohesive end is immediately adjacent to a further 7 consecutive A - T pairs. The evidence for a recognition site close to the left cohesive end has been highlighted by in vitro packaging experiments (Hohn, 1975). The phage λ and ϕ 21 have identical cohesive end sequences yet linear ϕ 21 DNA is packaged very inefficiently by protein extracts from λ infected cells. There is no homology detectable at the left end of heteroduplexes formed between λ and ϕ 21 and so the low packaging efficiency probably reflects a change in the protein binding site.

The inverted repeat at the right end of λ DNA could be responsible for correct DNA-tail alignment. Micrococcal nuclease treatment of filled heads without attached tails removes 4 nucleotides from the right cohesive end of λ DNA but this does not reduce the efficiency of DNA injection (Padmanabhan et al., 1972). Cross-linking experiments between λ DNA and phage tails suggest that there are 130 ∓ 60 base pairs of DNA projecting from the head into the tail (Thomas, 1974). When these 2 results are considered together it appears that, after head-tail attachment, the right end of λ DNA

penetrates down the tail to a limited extent. The inverted repeat could be involved in this process and is perhaps interacting with the tail protein pZ - a protein that is essential for normal DNA injection (Thomas et al., 1978).

The cohesive ends of P2 are 19 bases long and the nucleotide sequence shows a marked absence of rotationally symmetrical bases (Fig. 1.3); in P2 there are 2 symmetrical arrangements in the 19 bases (Murray and Murray, 1973). Sequence determination at the 3' ends of the DNA does reveal hyphenated rotational symmetry (Murray, K., et al., 1977) and this is still consistent with symmetrically placed bases being involved with ter enzyme recognition. It has been suggested that if the P2 single strand projections contained symmetrically placed bases then stable hairpin loops could form and this would disrupt circularization of DNA molecules after injection.

1.6 Transcription

The λ repressor protein controls gene expression by binding to the DNA at 2 operator sites, O_L and O_R which are located on either side of the cI gene (Fig. 1.4) (review, Pirrotta, 1976). When repressor protein is absent, host RNA polymerase molecules initiate transcription at P_L and P_R. This immediate early transcription is independent of phage functions and begins as soon as repressor protein is released from the DNA. The transcription is terminated at sites to the left of gene N, t_L, and to the right of gene cro, t_{R1}; a small amount of transcription read through at t_{R1} is terminated at t_{R2}.

The next stage in transcription, the delayed early phase, requires λ N protein. RNA polymerase molecules are modified by N protein and this allows transcription to proceed beyond the termination sites t_L, t_{R1} and t_{R2} (Franklin, 1974; review, Adhya and Gottesman, 1978). The leftward, N-dependent, transcription leads to the formation of the recombination proteins red and ϒ. The rightward, N-dependent transcription leads to the formation of O and P proteins which are required for DNA replication and Q protein which is the activator for late gene transcription.

The gene cro is included in the immediate early rightward transcript and cro protein accumulates to depress transcription from P_L and P_R after about 10 minutes (Franklin, 1971 b; Echols et al., 1973).

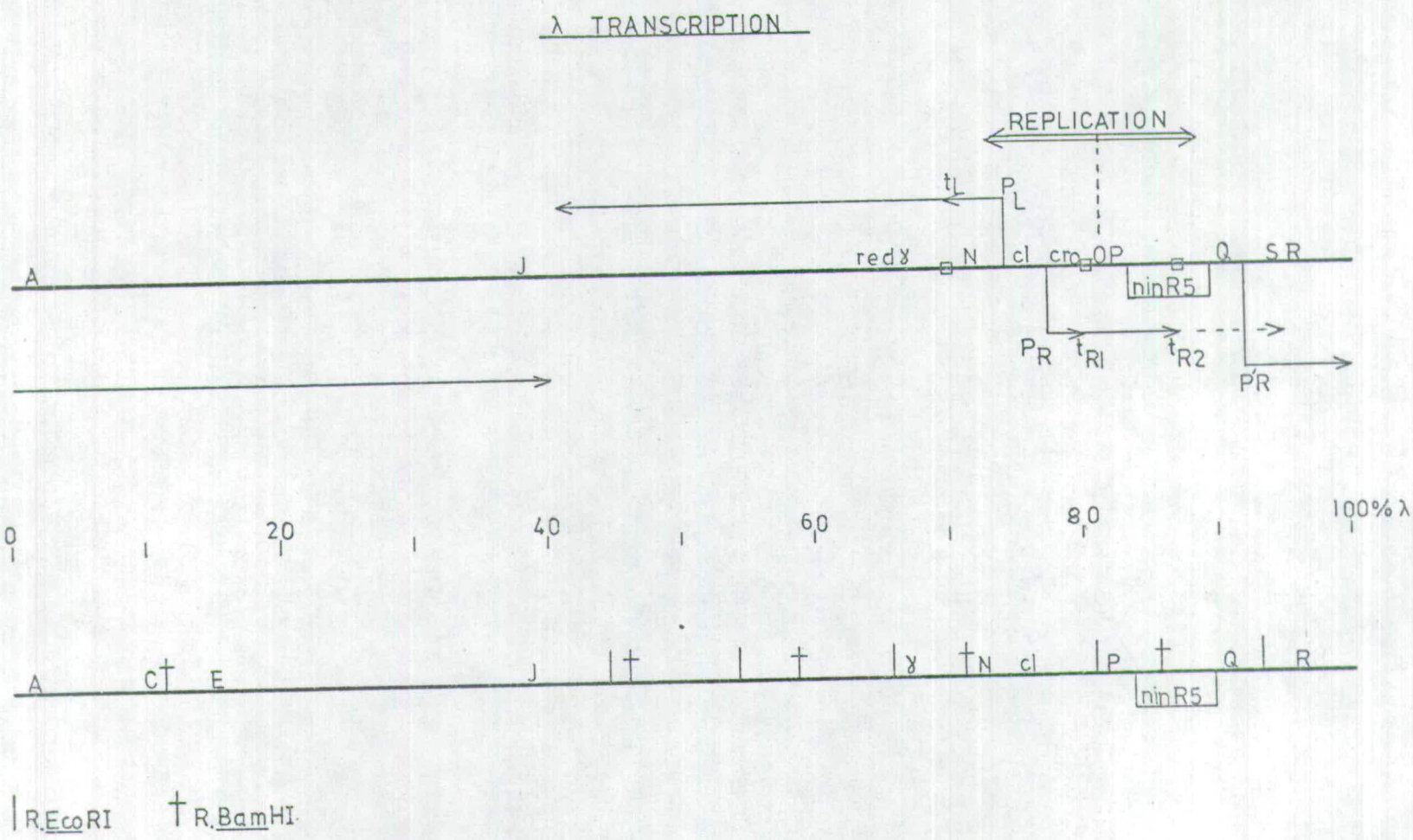

λ TRANSCRIPTION
REPLICATION
tL
PL
A
J
redγ
N
cI
cro
OP
Q
S R
ninR5
PR
tR1
tR2
P'R
0
20
40
60
80
100% λ
A
C
E
J
γ
N
cI
P
Q
R
ninR5
R.EcoRI
R.BamHI

Fig. 1.4 (opposite)

Phage λ Transcription.

Transcription initiates at P_L and P_R but is then terminated at t_L and t_{R1}, a small amount of read-through at t_{R1} is terminated at t_{R2}. In the presence of N protein, transcription can proceed beyond these termination sites. When Q protein has been synthesised, transcription of the late genes commences from P'R. The cro gene product acts to diminish N-dependent transcription after 5-10 minutes.

The termination site t_{R2} is removed by the nin R5 deletion.

The lower part of the diagram shows the relative positions of R. Eco RI and R. Bam HI recognition sites in λ DNA.

Transcription of the late genes initiates at P'R and is dependent on the presence of Q protein. The late genes, S - J, appear to be contained within a single transcript (Chowdhury and Guha, 1973; Ray and Pearson, 1974).

There is very little information available about early transcription in phage P2. The repressor protein binds at a site to the left of gene B to block transcription. Duplication of this region of the P2 genome causes constitutive expression of genes B and A (Chatteraj and Inman, 1974b; Bertani and Bertani, 1974). The B and A proteins are essential for DNA replication and late gene expression. The A protein shows cis specificity as A gene mutants cannot be complemented by any other P2 phage (Lindahl, 1970). Transcription of P2 late genes is apparently dependent on an interaction between P2A and ogr proteins (Sunshine and Sauer, 1975) but no details have been resolved.

The P2 structural genes have been grouped into 4 distinct units on the basis of polarity studies (Lindahl, 1971; Sunshine et al.,1971) and RNA - DNA hybridisation (Lindqvist and Bøvre, 1972). The genes P and Q are transcribed in the opposite direction and from the other DNA strand when compared with the remainder of the P2 structural genes (Fig. 1.5).

P2 TRANSCRIPTION

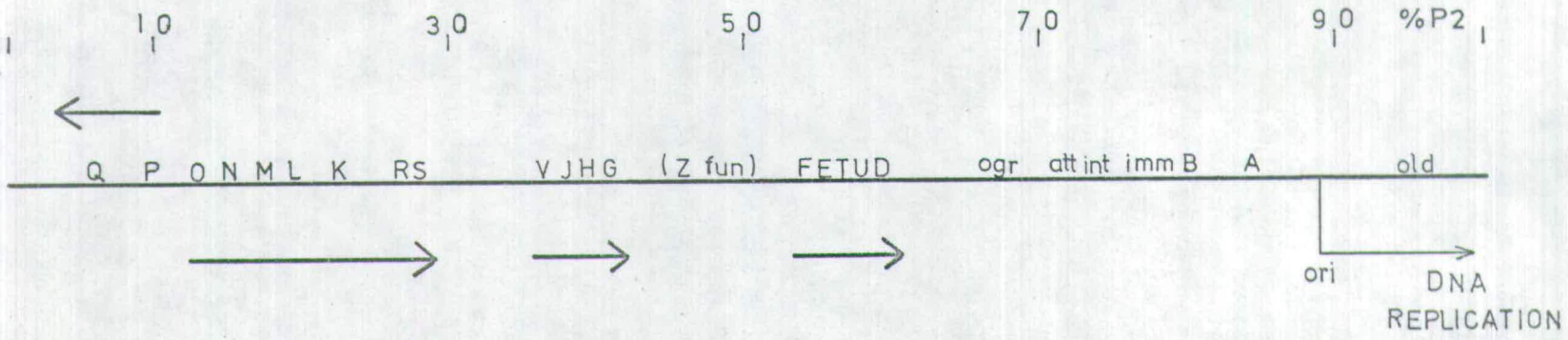


Fig.1.5(opposite)

Phage P2 Transcription.

The P2 structural genes are transcribed in 4 distinct units as shown by the arrows in the diagram. Genes P and Q are transcribed from the opposite strand to the remainder of the structural genes. There is an indication that the early genes, B and A, are transcribed from left to right. The genes fun and old are active in P2-lysogenic cells but nothing is known about transcription or regulation of these genes.

1.7 a Phage Head Assembly - λ

The head assembly process for λ requires the products of at least 9 phage genes - A, W, B, C, Nu_3, D, E, F I and F II; gene Nu_1, to the left of gene A, and gene Nu_2 between genes B and C are mentioned in some accounts but the published information is barely sufficient to confirm their existence (see Becker *et al.*, 1977 a, for a summary of the *in vitro* complementation properties of Nu_1). There is also a requirement for the host *gro* E gene product (Georgopoulos *et al.*, 1973; Sternberg, 1973 a, b; Georgopoulos and Hohn, 1978; Hendrix and Tsui, 1978) and a definite indication that a mutation in another bacterial gene significantly reduces the plating efficiencies of deletion phage (Henderson and Weil, 1977b).

The A gene product has been identified as the *ter* enzyme by Wang and Kaiser (1973). Unfortunately these experiments, involving the *in vitro* conversion of covalently closed monomeric circles to linear DNA cannot be repeated but the identification of pA as *ter* does appear to be correct. Linear concatemeric DNA accumulates in A^- infections but this does not absolutely confirm pA as *ter* because DNA processing is intimately linked to head assembly and DNA remains in concatemeric form if the correct head precursor structure is absent. There is however, cumulative evidence for pA being the *ter* enzyme. The A protein has been partially purified by assaying for activity with *in vitro* complementation reactions (Becker and Gold, 1975).

The proteins pB, pC and pE have been identified by SDS - polyacrylamide gel electrophoresis (Murialdo and Siminovitch 1972 a) and they are all structural components of the phage head. The protein pE is the major head protein while pB and pC are minor head proteins that are involved with cleavage reactions during head assembly (Hendrix and Casjens, 1974).

The protein pNu_3 has been assigned the role of a scaffolding protein as it is required for prehead assembly (Hohn et al., 1975). An additional role appears to be the protection of pB and pC from aberrant cleavage (Ray and Murialdo, 1975).

The protein pD is involved with stabilisation of the head structure and there are equal numbers of molecules of pE and pD in the phage head. (Hohn and Hohn, 1973; Hohn et al., 1975). Deletion phage with less than 82% wild-type λ DNA length can be packaged normally in the absence of pD (Sternberg et al., 1977; Sternberg and Weisberg 1977b).

The proteins pW and pF II are involved with head-tail attachment; pW appears to act before pF II (Casjens et al., 1972; Casjens, 1974). pF II is known to be a structural component of the completed phage particle and there are 5 - 6 molecules of the protein located at the head-tail joint. It is not known whether pW is incorporated into the phage head.

The protein pF I is thought to be involved with head enlargement

and there may be some role in the actual cleavage of DNA at cos sites (Boklage et al., 1973). The protein is present in large amounts in λ -infected cells but there has been no demonstration of the inclusion of pF I in phage particles.

Experiments and models relating to the λ head assembly process have appeared frequently in the literature. A comprehensive review was published recently (Hohn and Katsura, 1977) but a summary of current knowledge will be presented here. The λ in vitro packaging systems (Kaiser and Masuda, 1973; Hohn and Hohn, 1974; Becker and Gold, 1975; Sternberg et al., 1977; Hohn and Murray, 1977) have allowed the assembly process to be analysed in great detail as intermediates can be assayed for biological activity (Becker et al., 1977 a, b).

Precursor head structures (termed by various workers petit λ , preheads or proheads) have been observed in the electron microscope. These structures are 20% smaller in linear dimensions than phage heads and do not contain DNA. The precursor structures accumulate when DNA replication is inhibited or reduced. The head proteins pB, pC, pNu_3 and pE and the host protein gro E are known to be essential for the formation of biologically active proheads (Hohn et al., 1975). Phage mutants in gene E produce no head-related structures as pE is the major head protein. Phage mutants in gene C produce biologically inactive prohead-type structures. The phenotypes of mutations in genes B, Nu_3 and gro E are similar - inactive proheads and aberrant structures. Functional proheads

may be synthesised *in vitro* by mixing extracts of λ *E*⁻ and *Nu*$_3$⁻ infected cells; no other combination of extracts from mutants in genes B, C, *Nu*$_3$, E and *gro* E gives a similar reaction (Murialdo and Becker, 1977). The λ *Nu*$_3$⁻ extract is donating pE in monomeric or small oligomeric forms to the λ *E*⁻/λ *Nu*$_3$⁻ mixture and this suggests that an interaction between pE and p*Nu*$_3$ leads to the appearance of prohead structures. The minor component proteins pB and pC are incorporated at this stage into the prohead structure which consists of a pE shell enclosing a p*Nu*$_3$ core. The protein pC becomes fused to a minor fraction of pE and subsequent proteolytic cleavage generates the products X1 and X2. The core protein p*Nu*$_3$ is cut and removed in a process that requires pC and p *gro* E but the mechanism of this reaction remains to be clarified. The protein pB is cut to give pB* either during or after pC processing and the removal of p*Nu*$_3$. The pB/pB* present in the prohead may provide the recognition point for DNA entry (Murialdo and Ray, 1975). The pC may hold the p*Nu*$_3$ core centrally in the prohead so molecules of pC would be spread over the surface perhaps being finally located at the corners of the icosahedral head (Zachary *et al*., 1976).

The prohead is now available to interact with DNA but Becker *et al*. (1977 a, b) have been able to demonstrate that a specific complex forms between concatemeric DNA and pA before there is any complexing between DNA and prohead. The formation of the DNA/pA complex is dependent upon ATP, spermidine and unidentified host protein(s) - this host protein component could be provided by an extract from uninfected cells. The prohead interacts with the DNA/pA complex

and there appears to be some destabilisation at this stage as determined by loss of biological activity that can be recovered. However, the proheads / DNA/pA complex can be converted to viable phage without a further addition of proheads or pA. Temperature shift experiments with ts A protein have shown that pA molecules in the complex must remain functional for at least part of this reaction. An analysis of complex formation at different component concentrations suggests a stoichiometry of 1: 1: > 2 for proheads, DNA and pA respectively.

When pA is absent there is no productive interaction between concatemeric DNA and proheads. Mutations in 2 other phage genes, Nu_1 (Murialdo and Siminovitch 1972 b) and F I (Boklage et al., 1973) result in a similar phenotype; the roles of these proteins have not been clearly identified.

The conversion of the complex between prohead, DNA and pA into viable phage requires pD, pW, pF II and phage tails. However, before further protein interactions the prohead increases in size (Lickfield et al., 1976) and DNA enters the enlarged structure. There is no current indication as to how these processes occur.

The λ phage particle has an icosahedral head, radius 30 nm and a flexible tube tail 150 nm long, with a single tail fibre (review, Hohn and Katsura, 1977). The tail formation involves the products of at least 11 genes and, like the λ head assembly process, requires sequential interaction between protein components. An initiator

complex is formed and then the major tail protein, pV, polymerises to enclose the initiator complex. No polymerisation of pV occurs in the absence of the initiator and there is a specific protein, pU, that regulates the length of the tail and is necessary for correct attachment to the phage head.

1.7 b Phage Head Assembly - P2

The P2 head structure is formed by the products of 6 known genes - genes L, M, N, O, P and Q (Lindahl, 1973; Goldstein et al., 1974). The major head protein pN* constitutes about 90% of the protein in the head; synthesis occurs via a precursor form (pN) which is then cleaved (to pN*) during head assembly (Lengyel et al., 1973). The cleavage of pN is dependent on the simultaneous cleavage of pO, no form of pO has been detected in P2 head structures. Mutations in the other P2 head genes show normal processing of pN and pO indicating that the proteolytic activity is contained within pN, pO or both or that a bacterial enzyme is involved. Some of the minor protein components of the phage head appear to be different cleavage products derived from pN.

An in vitro packaging system for P2 has contributed significantly to an understanding of the head assembly process (Pruss et al., 1974). Protein extracts from P2 Mam infected cells will package exogenous linear monomeric P2 DNA at high efficiency in vitro but this extract cannot convert monomeric closed circular DNA (the normal substrate for P2 packaging in vivo) into viable phage (Bowden and

Calendar, 1978). This result, coupled with pulse-chase DNA labelling experiments for different P2 head gene mutants (Pruss et al., 1975; Pruss and Calendar, 1978) and in vitro packaging experiments with defective extracts, suggests that gene M codes for the P2 ter enzyme. Bowden and Calendar (1978) have extensively purified P2 proteins M and P taking advantage of the in vitro packaging system to monitor the purifications and they have been able to demonstrate P2 ter cleavage of covalently closed monomeric circular DNA in vitro. The ter cleavage reaction involves purified M and P proteins, purified P2 empty heads, ATP, spermidine and closed circular P2 DNA; the cleavage is abolished if any of the components is omitted. The proteins M and P initially co-purify but they can be separated on a phosphocellulose column. Both M and P proteins have strong DNA binding properties and the final stage in each purification is elution from DNA cellulose columns.

The clear involvement of 2 P2 proteins in ter cleavage does not help explain the mechanism of ter action. The P protein has been shown to partition into a rapidly sedimenting fraction which suggests that either P is associated with the bacterial membrane or that it exists in a rapidly sedimenting aggregate. Extracts of P2 Pam infected cells do not package linear or closed circular DNA so P protein possibly has multiple functions. Both proteins M and P are present in large amounts in P2 infected cells but neither protein has been detected in the phage particle (Lengyel et al., 1973). This is in marked contrast to λ where the ter enzyme (pA) is only

present as a few molecules per cell.

The proteins L and Q have not been detected on polyacrylamide gels (Lengyel et al., 1973). Mutations in gene L do not affect phage maturation and L protein appears to act after DNA has entered the phage head. P2 heads that are lacking L protein show an increased sensitivity to DNase (Pruss and Calendar, 1978). The role of Q protein is unknown, P2 Qam extracts are defective for packaging with linear or closed circular DNA.

There is some evidence for a P2 head precursor structure (as determined by electron microscopy, Gibbs et al., 1973) but empty heads with normal dimensions are found early in the infectious cycle. The satellite phage P4 (Six and Klug, 1973) has provided an unusual approach to the study of P2 head assembly (Pruss et al., 1974, 1975; Goldstein et al., 1974). P4 requires the 6 known P2 head genes for the synthesis of the P4 head (45 nm diameter); this small head structure is never found during normal P2 infection (Gibbs et al., 1973). P4 appears to code for a protein that is directing the assembly of the smaller head structure (Barrett et al., 1976). However, P2 in vitro packaging reactions with P4 DNA (P4 DNA is approximately one third the size of P2 DNA) showed conclusively that trimeric P4 DNA is the optimum substrate (Pruss et al., 1974). A detailed explanation of the interactions between P2 and P4 is beyond the scope of this Introduction - a concise review has recently been published (Calendar et al., 1977 b). The important point here is

that the results with P2 and P4 suggest that DNA is packaged into preformed head structures rather than head proteins condensing round monomeric DNA.

The P2 phage particle has an icosahedral head, radius 31 nm and a contractile sheath tail 135 nm long (review, Goldstein et al., 1974; Lengyel et al., 1974). There are general similarities in the structures of the P2 and T4 contractile tails, P2 has 6 tail fibres but only a single tail spike instead of the 6 spikes found in T4. P2 tail formation requires the products of 11 known genes whereas tail formation in T4 involves 28 genes and 11 genes are necessary for the correct synthesis of the simple λ tail. The mechanism of P2 tail assembly has not been fully described but it seems that an initiator complex directs the polymerisation of the major tail proteins.

1.7 c Comparisons in Phage Particle Assembly

The T4 head requires the products of 18 genes and at least 11 proteins are detected in the phage particle, mutations in different T4 genes block head assembly at precursor stages (Laemmli and Favre, 1973). The major T4 head protein (p 23) can assemble to form polyheads in vitro and more recently, the in vitro assembly of T4 prehead-like structures has been demonstrated (van Driel, 1977; van Driel and Couture, 1978). These findings are analogous to the detection of λ polyheads in vitro (Wurtz et al., 1976) and the formation of λ proheads (Murialdo and Becker, 1977). The T4 protein p 23 is

cleaved during the conversion of preheads to head structures and a core protein (p 22) is eliminated from the prehead (Laemmli, 1970).

P 22, the temperate phage of S. typhimurium, does not appear to show protein cleavage during head assembly (Casjens and King, 1975). The P 22 head requires the products from 11 genes; a prehead is formed first and there is an increase in head size and loss of a core protein as the prehead interacts with DNA (Earnshaw, 1976).

Both T4 and P22 have circularly permuted DNA molecules and there are large terminal repeats. DNA packaging in these phage is thought to occur by a headful mechanism, the size of the P 22 terminal repeat has been shown to vary directly with the genome size (Tye et al., 1974 a). There is some nucleotide specificity as a site has been identified on concatemeric P 22 DNA for the initiation of packaging (Tye et al., 1974 b; Jackson et al., 1978).

Mechanisms by which DNA is taken into preformed head structures have not been clarified. An attractive idea is that the prehead represents a metastable state and that DNA is drawn into the structure during expansion. DNA condensation is thought to be assisted by the intracellular presence of polyamines but precise details are still unknown. The in vitro condensation of DNA by polyamines has recently been investigated (Gosule and Schellman, 1978; Chattoraj et al., 1978) and it is known that the omission of spermidine from in vitro packaging reactions results in a 10 fold reduction

in the numbers of phage particles that are recovered (Hohn and Hohn, 1974).

1.8 Construction of Recombinant Bacteriophage in vitro

The discovery and rapid exploitation of restriction endonucleases has greatly facilitated studies of DNA organisation and has created the potential for novel DNA sequence arrangements. The first type II restriction enzyme was characterised by Smith and Wilcox (1970) and since that time over 80 enzymes have been isolated from a wide range of organisms (reviews, Nathans and Smith, 1975; Roberts, 1976). The enzymes recognise specific nucleotide sequences in double strand DNA and cleave both strands of the duplex. The type II enzymes cleave at these specific sites and hence their importance for the rearrangement of DNA sequences. The type I enzymes, initially predicted from studies on phage restriction and modification (Luria and Human, 1952; Bertani and Weigle, 1953) do not cleave DNA molecules at specific sites (Meselson and Yuan, 1968; Arber, 1974) and will not be considered further here.

The recognition sites for type II enzymes are 4 - 6 base pairs long and possess two-fold symmetry. Some enzymes produce staggered breaks that result in single strand projection (5' or 3') whereas other enzymes cut both strands in the centre of the recognition sequence (Table 1.1). A characteristic of type II restriction endonucleases is that they cleave DNA to form 5' terminal phosphate groups and 3' terminal hydroxyl groups and the phosphodiester bond may be regenerated by the action of DNA ligase (Weiss and Richardson, 1967; Olivera and Lehman, 1967; Sgaramella et al., 1970; Sgaramella, 1972;

TABLE 1.1

Restriction Endonucleases.

Enzyme	Recognition Sequence	Number of Sites in DNA	Reference for Sequence Identification
R. *Eco* RI	5' G^{v}A A.T T C	5	Hedgpeth *et al.*, 1972
R. *Hind* III	5' A^{v}A G.C T T	6	Old *et al.*, 1975
R. *Bam* HI	5' G^{v}G A.T C C	5	Roberts *et al.*, 1977
R. *Sal* I	5' G^{v}T C.G A C	2	Arrand *et al.*, 1978
R. *Hpa* I	5' G T T.^{v}A A C	13	Garfin and Goodman, 1974
R. *Xho* I	5' C^{v}T C.G A G	1	Gingeras *et al.*, 1978

^{v} Denotes location of breaks in DNA strands.

Note that each sequence is symmetrical about a central axis.

Backman et al., 1976).

The first successful in vitro recombination experiment generated a hybrid between simian virus 40 (SV 40) and part of a λ gal transducing phage (Jackson et al., 1972). Reports then followed of the transfer of ribosomal genes from Xenopus laevis (Morrow et al., 1974) and the penicillin-resistance determinants of Staphylococcus aureus (Chang and Cohen, 1974) to bacterial plasmids. There has been a flood of publications since this early work and the diversity of experiments currently employing restriction enzymes is illustrated by a single text (Beers and Bassett, 1977).

The in vitro recombination experiments described in this work relied on phage λ vectors (Murray and Murray, 1974; Rambach and Tiollais, 1974; Thomas et al., 1974; Murray and Murray, 1975; Blattner et al., 1977). Detailed knowledge of λ genetics has allowed the phage genome to be manipulated in an attempt to increase protein synthesis from incorporated DNA segments (Murray, N. E., 1977).

The λ vectors are of two basic types - insertion vectors and replacement vectors. The insertion vector is deleted for inessential regions of the λ genome (deletions up to about 20% wild type λ length) and contains a single recognition site for a given restriction enzyme. The most convenient insertion vectors have the recognition site positioned within the phage repressor gene (e.g. λ 641, DNA is inserted at the single R. EcoRI target in imm^{434}).

Insertion of foreign DNA at this site interrupts the phage repressor gene causing a change in plaque morphology from turbid to clear. The recombinant phage has more DNA than the parental vector but the size increase (up to about 105% wild type λ) does not adversely affect phage viability.

The replacement vector contains 2 recognition sites for the particular restriction enzyme and DNA lying between the 2 sites in the vector is exchanged for foreign DNA (e.g. λ 596, an R EcoRI replacement vector that carries a fragment of E. coli DNA coding for the suppressor t-RNA gene, sup F). The identification of recombinants formed with replacement vectors relies on a genetic test to determine the presence or absence of the central fragment from the vector. The test for the suppressor fragment uses lactose MacConkey agar and a lac Z am bacterial host (ED 8538). Cells lysogenised by phage carrying a suppressor develop a red colouration from lactose metabolism, cells infected with phage that have replaced the suppressor fragment with foreign DNA are lysed and no distinctive colour develops (Murray N. E., et al., 1977). Critical points in the replacement vector system are that the central DNA fragment in the vector gives a recognisable phenotype in either orientation and that the vector has insufficient DNA for packaging when a central fragment is excluded.

1.9 Amplification of Protein Synthesis

Increased protein synthesis has been achieved with the lac repressor protein (Müller-Hill et al., 1968). Mutant repressor genes, selected by virtue of elevated levels of repressor protein, have been incorporated into phage chromosomes to raise the gene copy number. When cell lysis is blocked, the lac repressor comprises about 0.5% of the soluble protein in the cell (Müller-Hill et al., 1968).

Many aspects of this early experiment are important in current studies of gene expression. The in vitro recombination technique provides a mechanism for the formation of hybrids between specific DNA fragments and characterised vectors. There is good potential with phage λ vectors for gene expression in prokaryotic hosts because of detailed knowledge of the phage transcription system. Notable successes have been with E. coli DNA ligase (Cameron et al., 1974), E. coli DNA polymerase (Kelley et al., 1977) and T4 DNA ligase (Wilson, G. and Murray, N. E. in preparation).

There is a high initiation frequency for transcription from the λ leftward promoter, P_L and this promoter is often employed for transcription of foreign DNA in λ recombinants. Normally, λ P_L (and λ P_R) transcription is depressed by the phage cro gene product 5 - 10 minutes after phage infection (Franklin 1971 b). A mutation

in the cro gene prolongs P_L transcription throughout the infectious cycle and should maximise gene expression (Moir and Brammar, 1976).

If the direction of transcription of the foreign DNA is known and can be linked with restriction mapping information, then it should be possible to identify phage from in vitro recombination reactions that have the correct orientation for sense strand transcription from λ promoters. An extension of this approach employs 2 different restriction enzymes for the in vitro recombination reaction so that the orientation of the foreign DNA is predetermined. In many cases it is unlikely that the direction of transcription will be known but this information can be derived by separating the DNA strands of λ recombinant phage (Szybalski et al., 1971) and then hybridising each strand with labelled RNA (see for example Murray, K., 1977).

Protein synthesis in λ recombinants may be monitored by ^{35}S labelling after ultra violet irradiation to eliminate bacterial protein synthesis (Jaskunas et al., 1975). This complex mixture of labelled proteins may be analysed by high resolution SDS-polyacrylamide gradient gel electrophoresis or by iso-electric focussing (O'Farrell, 1975).

The following chapters describe in vitro recombinant phage that have been constructed to increase the synthesis of phage structural proteins. The expression of P2 genes in λ/P2 hybrids has been examined in both genetic and biochemical experiments. A λ

duplication phage has been formed *in vitro* and structural genes, including gene A, are now positioned for transcription from P_L.

CHAPTER 2 Materials and Methods

2.1 Bacterial Strains

Bacterial strains used in this work are described below. Some lysogens and phage resistant strains were constructed during the course of experiments.

Sources (N. E. M.) Dr N. E. Murray
(W. J. B.) Dr W. J. Brammar
(G. B.) Dr G. Bertani

E. coli K strains

Designation	Relevant Genotype	Source	Reference
C 600	*sup* E, *ton* A	NEM	Appleyard, (1954)
5 K	*hsd* R^- derivative of C 600	NEM	Hubacek and Glover, (1970)
5 K RI	5 K carrying the RI plasmids of Yoshimori	NEM	
803	*hsd* R^-, *hsd* M^-, *met* B, *sup* E	NEM	Wood, (1966)
QR 47	*sup* E	NEM	Weil and Signer, (1968)
N3098	*lig*^{ts} 7 *sup* F	NEM	
W 3350	*sup*^{o}	NEM	Campbell, (1961)
S 159	*uv* R^- *sup*^{o}	NEM	Buchwald *et al.*, (1970)

Designation	Relevant Genotype	Source	Reference
ED 8538	lac Z am, leu am, trp A 33	WJB	Borck et al.,(1976)
ED 8741	sup E hsd R^-, hsd M+ met^- BE9 trp R^-, Rec A56	WJB	
ED 8654	hsd R^-, hsd M^+ trp R derived from 803 sup F	WJB	Borck et al., (1976)

E. coli C Strains

Designation	Relevant Genotype	Source	Reference
C-1a	prototrophic E. coli C	NEM	Bertani and Bertani, (1970)
C-1055	F^+, auxotrophic, str-r	GB	Wiman et al., (1970)
C-1100	C-1055 lyd	GB	Sironi, (1969)
C-1757	F^-, auxotrophic, sup D, str-r	GB	Sunshine et al., (1971)
C-1966	sup D lyd	GB	Bertani (1975)

2.2 Phage Strains

Phage strains used in this work are listed below. λ/P2 recombinants are described in the text.

λ Strains

Number	Relevant Genotype	Reference
λ 1	$\underline{cI}^{ts}$ 857	
λ 143	$\underline{h}^{80}$, $\underline{att}$ λ, $\underline{imm}^{21}$	
λ 416	$\underline{b}$ 538, $\underline{cI}$ am, $\underline{S}$ am_7	
λ 570	$\underline{trp}$ 1A, $\underline{imm}^{21}$	
λ 578	$\underline{b}$ 508, $\underline{cI}$ am, $\underline{nin}$ R5	
λ 596	$\underline{sup}$ E, $\underline{\gamma}$ am, $\underline{cI}^{ts}_{857}$, $\underline{nin}$ R5	
λ 641	$\underline{sr}$ 1 λ 1^{o}, $\underline{sr}$ 1 λ $(2\text{-}3)^{o}$, $\underline{imm}^{434}$ $\underline{nin}$ R5, $\underline{sr}$ 1 λ $4,5^{o}$	Murray N.E. $\underline{et\ al.}$, (1977)
λ 671	$\underline{h}^{80}$, $\underline{att}$ λ, $\underline{cI}^{ts}$ At_2, $\underline{crol}$, $\underline{Q}$ am_{73}, $\underline{S}$ am_7	
λ 781	λ 596 γ^{+}	Murray N.E. $\underline{et\ al.}$, (1977)
λ 849	$\underline{h}^{80}$, $\underline{cI}^{ts}_{857}$, $\underline{cro}^{ts}$, $\underline{nin}$ R5, $\underline{Q}$ am_{73}, $\underline{S}$ am_7	

All λ strains were obtained from Dr. N. E. Murray and the phage numbers are Murray stock numbers.

P2 Phage Strains

Sources (NEM) Dr. N. E. Murray

(GB) Dr. G. Bertani

(JBE) Dr. J. B. Egan

Number	Relevant Genotype	Source	Reference
	P2 *lg cc*	NEM from GB	Bertani *et al.*, (1969)
	P2 vir_1	NEM from GB	Bertani, (1960)
	P2 vir_{22}	NEM from GB	Bertani, (1975)
635	P2 *P.* am_{137}, *del* 1	GB	Bertani, (1975)
737	P2 *A* am_{127}, *del* 1, (*lg*?)	GB	Bertani, (1975)
753	P2 Q^{ts}_{48}, *del* 1	GB	Bertani, (1975)
817	P2 P^{ts}_{15}, *vir* 1	GB	Lindahl, (1969)
1171	P2 *A* am_{127}, *B* am_{116} *del* 1, *del* 2	GB	
	P2 *M* am_{32} *vir* 1	JBE	Sunshine *et al.*, (1971)
	P2 *Q* am_{34} *vir* 1	JBE	Sunshine *et al.*, (1971)

The P2 phage numbers are Bertani stock numbers.

2.3 Chemicals and Enzymes

Acrylamide, N N^1 Methylenebis acrylamide (both specially pure for electrophoresis) and N N N^1 N^1- tetramethylenediamine (TEMED) were obtained from BDH Chemicals Ltd., Poole, England.

Lysozyme (Grade 1 egg-white enzyme) and pyruvate kinase (Type II from rabbit muscle) were obtained from Sigma Chemical Co., St. Louis, U.S.A.

T_4 polynucleotide ligase and electrophoresis grade agarose were purchased from Miles Laboratories, Ltd., Stoke Poges, Slough, Bucks.

Pancreatic deoxyribonuclease, pancreatic ribonuclease and snake venom phosphodiesterase were purchased from Worthington Biochemicals Corp., Freehold, N. J., U.S.A.

Bacterial alkaline phosphatase was obtained from The Boehringer Corporation (London) Ltd.

As far as possible A. R. grade chemicals were used Where appropriate, solutions were sterilized by autoclaving or by millipore filtration.

Restriction Enzymes

Some of the R. E co RI used for restriction analysis was prepared by myself using a method derived from that of Yoshimori (1971). At other times, samples of enzyme were provided by individuals in the laboratory.

R. Bam HI was prepared by R. Jefferson

R. Kpn I and R. Hpa I were kindly provided by Dr. B. Sain

All other enzymes were from stocks maintained by Professor K. Murray.

Radiochemicals

L - [^{35}S] - Methionine (about 1000 Ci/m mol) and γ - [^{32}P] - ATP (2000 - 3000 Ci/m mol) were obtained from the Radiochemical Centre, Amersham, Bucks.

2.4 Media and Solutions

L broth (Lennox, 1955)	Difco tryptone	10 g
	Yeast extract	5 g
	Na Cl	10 g
	+ H_2O to 1 litre	
	pH adjusted to 7.2 with NaOH	
L broth agar	L broth + 1.5% Difco agar	
BBL bottom agar	BBL trypticase	10 g
(Parkinson, 1968)	Difco agar	10 g
	Na Cl	5 g
	+ H_2O to 1 litre	
BBL top agar	As for BBL bottom agar but only 6.5 g agar	
L agarose	Bactotryptone	10 g
	Difco bacto yeast extract	5 g
	Na CL	10 g
	+ H_2O	
	pH adjusted 7.2 with NaOH + 15 g agarose and volume made up to 1 litre with H_2O	
BBL agarose top layer	BBL trypticase	2 g

	Na Cl	1 g
	+ H_2O	
	pH adjusted 7.2 with NaOH +	1.3 g
	agarose and volume made up to	
	200 ml with H_2O	
Phage Dilution Buffer	KH_2PO_4	3 g
	Na_2HPO_4	7 g
	NaCl	4 g
	0.1 M $MgSO_4$	10 ml
	0.01 M $CaCl_2$	10 ml
	1% Gelatin (w/v)	1 ml
	+ H_2O to 1 litre	
M9 salt mix (x 4)	Na_2HPO_4	28 g
	KH_2PO_4	12 g
	NaCl	2 g
	NH_4Cl	4 g
	+ H_2O to 1 litre	
M9 Maltose medium	M9 salt mix (x 4)	125 ml
	20% Maltose	10 ml
	0.1 M $CaCl_2$	0.5 ml
	1.0 M $MgSO_4$	0.5 ml
	+ H_2O to 1 litre	

A medium

(Bertani & Bertani, 1970)

Soln I	KH_2PO_4	15 g
	$(NH_4)_2SO_4$	3.6 g
	NaOH about	2.4 g
	+ H_2O to 1 litre, pH 6.8 with NaOH	
Soln II	Glucose 10% (w/v)	
Soln III	Casein Acid Hydrolysate	100 g
	NaCl	50 g
	+ H_2O to 1 litre	

A medium = 9 vols Soln I + 1 vol Soln II + 1 vol Soln III +
1.4 m$\underline{M}$ $MgSO_4$, 0.3 m$\underline{M}$ $CaCl_2$, 6×10^{-6} $\underline{M}$ $FeCl_3$
(Final concentrations)

Super T P G medium

(Lindqvist and Six, 1971)

(Pruss et al., 1974)

NaCl	0.5 g
KCl	8.0 g
NH_4Cl	1.1 g
Tris	12.1 g
KH_2PO_4	1.0 g
Na. pyruvate	0.8 g
$MgCl_2 . 6H_2O$ (20%)	1.0 ml
0.16 $\underline{M}$ Na_2SO_4	1.0 ml
pH to 7.4 with HCl	

+ H_2O to 1 litre then autoclave

then add	1.0 M $CaCl_2$	1.0 ml
	$FeCl_3 . 6H_2O$ (0.1 mg/ml)	1.0 ml
	Glucose (20%)	10 ml
	Casein amino acids (10%)	100 ml

each solution sterilized separately

then add	Amino acids	25 µg / ml
	Nucleic acid precursors	25 µg / ml
	Spermidine	0.1 mM
	Vitamin mix 1% v/v	

(The vitamin mix is described in Difco Supplementary Literature, 1968 p 469).

DNA Buffer	10 mM tris-HCl pH 8.0
	1 mM EDTA

R *Hind* III Restriction Buffer	10 mM tris-HCl pH 8.0
	10 mM $MgCl_2$
	10 mM β mercaptoethanol
	50 mM NaCl

Ligase Cocktail (x 10)	Stock solutions	µl/ml
	1.0 M tris-HCl pH 7.5	660
	0.4 M EDTA pH 9.0	25
	1.0 M $MgCl_2$	100

	1.0 M dithiothreitol	100
	0.1 M ATP	10
SSC (x 1)	NaCl	8.76 g
	Na_3 citrate	4.41 g
	+ H_2O to 1 litre	
Agarose gel loading buffer	Ficoll	10%
(final concentrations)	Bromophenol Blue	0.025%
	Xylene Cyanol FF	0.05%

SDS Polyacrylamide Gel Solutions

Stock Acrylamide	Acrylamide	30 g
	N N^1 Methylene bis	
	acrylamide	0.8 g
	+ H_2O to 100 ml	
	Filter and store in the dark.	
Lower tris (x 4)	Tris	18.17 g
	SDS (10% soln.)	4.0 ml
	+ H_2O, pH adjusted to 8.8 with	
	HCl then + H_2O to 100 ml.	
Upper tris (x 4)	Tris	6.06 g
	SDS (10% soln.)	4.0 ml
	+ H_2O, pH adjusted to 6.8 with	
	HCl then + H_2O to 100 ml.	

Sample Buffer	Glycerol	8.5 ml
	β mercaptoethanol	5.0 ml
	SDS (10% soln.)	30 ml
	Upper tris (x 4)	12.5 ml
	Bromophenol blue to 0.01%	
	+ H_2O to 100 ml	

Acrylamide Gradient Solutions	10%	20%
dist. H_2O	9.1 ml	2.0 ml
lower tris (x 4)	5.5 ml	5.5 ml
Acrylamide stock	7.33 ml	14.35 ml
Ammonium persulphate (10% soln.)	20 µl	16 µl
TEMED	10 µl	17 µl

Stacking gel	dist. H_2O	6.5 ml
	Upper tris (x 4)	2.5 ml
	Acrylamide stock	1.0 ml
	Ammonium persulphate (10%)	40 µl
	TEMED	15 µl

Electrophoresis Buffer	Tris	3 g
	Glycine	14.4 g
	SDS (10% soln.)	10 ml
	+ H_2O to 1 litre	

2.5 Methods

2.5 a Phage Titration

Phage were titered by mixing 0.1 ml of fresh plating cells with 0.1ml of the appropriate diluted phage solution. The mixture was left for 10 minutes at room temperature to allow phage adsorption to occur and then top agar (2.5ml at 45°C) was added. The solution was poured onto a dry plate and when the top agar had set the plate was incubated upside down for 8-12 hours.

Plating cells for λ were a 1 : 20 dilution from a fresh overnight culture grown with aeration at 37°C for 2 hours. The cells were then pelleted in a bench-top centrifuge and resuspended in half the initial volume of 10m$\underline{M}$ $MgSO_4$. Plating cells were stored at 4°C and were used over a period of a few days before being discarded. Phage λ was always titered on BBL plates and top agar was supplemented with 10m$\underline{M}$ $MgSO_4$.

Plating cells for P2 were prepared as indicated for λ except that the cells were resuspended in half the original volume of L broth containing 5m$\underline{M}$ $CaCl_2$. P2 phage were titered either on L plates containing 5m$\underline{M}$ $CaCl_2$ or on BBL plates; in this latter case the Ca^{2+} supplement was added to the top agar.

2.5 b Phage Spot Tests

A bacterial lawn was prepared by plating 0.1ml cells with 2.5 ml top agar (plating cells for λ or overnight culture cells supplemented with 10 mM $MgSO_4$, 5 mM $CaCl_2$ for λ and P2 spot tests). When the top agar had set, spots of diluted phage solutions (approximate volume 10 µl) were placed on the surface of the plate from sterile pipettes. After the spots had dried the plate was incubated upside down for 8-12 hours at 32 or 37°C.

2.5 c Phage Plate Lysates

Fresh, well separated plaques were picked with sterile toothpicks into 0.3 ml phage buffer containing 1 drop of chloroform. The mixture was left for about an hour and then 0.1 - 0.2 ml of the phage solution was mixed with 0.1 ml of fresh plating cells. After 10 minutes preadsorption, top agar was added and the mixture was plated on a moist L plate. Plates were incubated at 37°C for 6-8 hours and then overlaid with 3 ml L broth. The top agar was transferred in this broth to a small sterile bottle containing 1 drop of chloroform. After vigorous shaking, the agar was pelleted in a bench-top centrifuge and the supernatant carefully decanted. Titres for phage λ were generally in the range 1×10^{10} - 1×10^{11} p.f.u. /ml whereas the titres for P2 plate lysates were consistently 5 - 10 fold lower.

2.5 d Phage Crosses (λ only)

The E. coli strain QR 47 was used as the principal host for phage crosses. The strain is sup + and rec + and is sensitive to both h^{80} and h^{λ} phage. Fresh plating cells (0.1 ml) were infected at multiplicities of 5 for both the phage involved in the cross and the mixture was left at room temperature for 15 minutes to allow adsorption to occur. The volume was increased to 1.0 ml with L broth and the cells were pelleted in a bench-top centrifuge. Cells were resuspended in 1.0 ml prewarmed L broth and 0.1 ml of this solution was diluted into 10 ml prewarmed L broth. This diluted sample was shaken at 37°C for 90 minutes and then one drop of chloroform was added to lyse any remaining cells. The phage cross was titered on a totally permissive host to check for input of both parental phage and on selective hosts to look for recombinants.

2.5 e Lysogen Formation

Stationary phase overnight cultures (0.1 ml) were infected with phage at a multiplicity of 1 - 2 (approximately 10^9 p.f.u. /ml used) and adsorption was allowed to occur at room temperature for 15 minutes. The mixture was diluted with L broth (to 1.0 ml) and placed at 37°C for 1 hour (with λ c I_{857}, lysogens were selected at 32°C and the mixture was left for 2 - 3 hours). A loopful of the culture was then streaked for single colonies on a dried L plate that had been prespread with 10^8 clear phage of the same immunity as the desired lysogen. Single colonies appearing on the plate after overnight

incubation were picked with sterile toothpicks and streaked across dried tracks of indicator phage. The termination position of bacterial growth demonstrated which prophage if any, was present in the putative lysogen (e.g. a strain lysogenic for λ c I_{857} would grow through λ imm$^{\lambda}$ but would be lysed by λ imm^{434} and λ imm^{21}; a λ resistant strain could appear from the initial selection but this would be detected by growth through λ imm$^{\lambda}$, λ imm^{434} and λ imm^{21} in the cross-streak test). Cross-streaks that checked appropriately were re-purified through single colonies and re-tested before a single colony was picked and maintained as the lysogen stock.

2.5 f Phage Liquid Lysates

Large scale preparations of phage were made by infecting exponentially growing cultures of cells (Murray, et al., 1973; Bertani and Bertani, 1970). For phage λ, cells (C600 or ED8654) were grown in L broth supplemented with 10 mM $MgSO_4$ and when the culture reached OD_{650} nm 0.45, phage were added to give a multiplicity of infection of 1. Cells for P2 infection (C-1a or C-1757) were grown in A medium (section 2.4) and infected at OD_{650} nm 0.5 with phage at a multiplicity of 0.1 - 0.2. At the first signs of lysis in the P2 infected cultures (the appearance of clumps of bacterial debris) an excess of phosphate was added to remove calcium ions and thus prevent phage readsorption. When the OD_{650} nm reached a minimum (2 - 4 hours after infection) lysis was completed by the addition of chloroform (1.0 ml /litre) and 15 minutes later the lysate was clarified by centrifugation (H.S. 18, 6 x 250 ml Aluminium rotor 8k rpm, 10 minutes,

4°C, 10,000 g).

2.5 g Phage Concentration and DNA Extraction

Phage particles were concentrated from lysates either by pelleting in the ultracentrifuge or by polyethylene glycol (PEG) precipitation (Leberman, 1966). Pelleting was performed by centrifugation in a 10 x 100 ml Aluminium rotor (21 krpm, 3 hours, 4°C, 45,000 g) and phage were resuspended overnight in a small volume of buffer by gentle rotary shaking in the cold. Phage were precipitated by adding solid NaCl to 0.5 M and solid PEG 6000 to 10% (w/v). The mixture was swirled gently to dissolve the solids and then left to stand in the cold for at least one hour. The precipitate was collected by centrifugation (HS 18 6 x 250 ml Aluminium rotor 10 k rpm, 20 minutes, 4°C, 15,000 g) and resuspended overnight exactly as the ultracentrifuge phage pellet.

Concentrated phage solutions were treated with D.Nase and R.Nase (10 μg ml of each, digestion at room temperature for 1 hour) and then a drop of chloroform was added. Solutions were centrifuged (HS 18 8 x 50 ml Aluminium rotor 10 k rpm, 10 minutes, 4°C, 12,000 g) to remove any remaining bacterial debris. In the early part of this work, phage were purified through 2 cycles of caesium chloride equilibrium centrifugation. Solid caesium chloride (Cs.Cl) was added to concentrated phage solutions and the solid was allowed to dissolve by standing the mixture on ice (41.5% w/w CsCl for λ , 41.0% w/w for P2). Equilibrium centrifugation was performed in a 6 x 14 ml

Titanium swinging bucket rotor (33 k rpm, 36 hours, 4°C, 140,000 g) and phage bands were collected from above with a bent pasteur pipette. The recovered phage bands were mixed with an appropriate volume of pre-clarified CsCl solution (41.5% or 41.0% w/w, solution centrifuged HS 18 10 k rpm, 30 minutes, 4°C, 12,000 g), for the second equilibrium centrifugation using either 3 x 6 ml or 6 x 5 ml Titanium swinging bucket rotors (33 k rpm, 36 hours, 4°C, 90,000 g or 110,000 g respectively). Phage bands were collected through a hole in the bottom of the centrifuge tube.

More recently, the first equilibrium centrifugation has been replaced by a CsCl step gradient. This allowed a considerable saving in time and there was no apparent adverse effect on either the purity or quality of the final DNA. Step gradients were first demonstrated to me by Binie Klein and were made up on the following basis.

A stock saturated CsCl solution (at 20°C) was diluted with phage buffer in the proportions shown. Steps were formed in a 14 ml centrifuge tube by overlaying CsCl solutions sequentially from above.

0.5 ml	Sat. CsCl
0.5 ml	66% CsCl
1.0 ml	50% CsCl
1.5 ml	33% CsCl

About 9 ml of concentrated phage solution was layered on top of the CsCl in the centrifuge tube. The gradients were centrifuged in a 6 x 14 ml Titanium swinging bucket rotor (33 k rpm, 1¾ hours, 20°C, 140,000 g) and phage bands were collected through the side of the

centrifuge tube with a sterile syringe and needle. Phage preparations intended to provide DNA for restriction analysis were not taken beyond CsCl step gradients; when DNA was required for *in vitro* recombination experiments, phage were further purified by equilibrium centrifugation.

Phage bands were dialysed against DNA buffer to remove CsCl and DNA was released from phage particles by mixing with an equal volume of freshly distilled phenol (Kaiser and Hogness, 1960). (Phenol was distilled under nitrogen, collected in deaerated water and pre-equilibrated by shaking with an equal volume of 0.5 M tris-HCl pH 8.0.) The phage/phenol mixture was centrifuged briefly to separate the phases and the lower phenol layer was carefully removed with a drawn out pasteur pipette. The phenol extraction was repeated 2 - 4 times and then the aqueous phase was dialysed against several changes of DNA buffer to remove traces of residual phenol.

DNA concentration was determined by measurement of absorbance at 260 nm; readings at 235 nm and 280 nm were taken as an indication of the amount of contaminating protein. The ratio of absorbance at 260 and 280 nm was routinely in the range 1.7 - 2.0: 1 and zero readings at 320 nm confirmed that there was no interference from light scattering.

2.5 h DNA Preparation from Plate Lysates

The original method (Cameron *et al.*, 1977) was followed fairly

closely but minor modifications were adopted. Plate lysates were prepared by the standard method using L agarose plates and BBL agarose top layer (section 2.4). After the 37°C incubation, plates were overlaid with 4 ml 10 mM tris-HCl pH 7.5, 10 mM EDTA and left at 4°C overnight. If EDTA was replaced by 10 mM $MgSO_4$ viable phage could be recovered from the plate lysate. In this case only part of the supernatant was processed for DNA extraction while the remainder served as a high titre phage stock. The supernatant recovered from the plates was mixed with 0.25 M EDTA, 0.5 M Tris base, 2.5% SDS (0.8 ml this solution to 4.0 ml supernatant). Diethyl pyrocarbonate (10 µl) was added and the mixture was heated for 30 minutes at 65°C in open tubes in a fume cupboard. The solution was cooled on ice and 5 M potassium acetate was added to give a final concentration of 1.0 M. After 1 hour on ice the potassium dodecyl sulphate precipitate was pelleted (HS 18 8 x 50 ml Aluminium rotor 15 k rpm, 10 minutes, 4°C, 25,000 g) and the supernatant was carefully decanted. The supernatant was mixed with 2 volumes of ethanol and cooled to -70°C in a dry-ice/acetone bath for 10 minutes. Nucleic acids were pelleted (HS 18 8 x 50 ml Aluminium rotor 13 k rpm, 30 minutes, 0°C, 20,000 g) and the ethanol supernatant was discarded. After drying in a vacuum desiccator, the nucleic acid precipitate was redissolved in 0.2 - 0.3 ml 10 mM tris-HCl pH 7.5, 1 mM EDTA and 1.0 M tris-HCl pH 7.5 was added to give a final concentration of 0.1 M. The yield of DNA was variable, especially in terms of the amount of bacterial DNA that might be present, but generally 10 - 20 µl of redissolved solution was sufficient to

show phage DNA restriction bands after gel electrophoresis.

2.5 j Restriction of DNA

Samples of restriction enzymes were titered to determine optimum conditions with respect to digestion time and amount of enzyme required to give complete digestion of a fixed amount of DNA (generally 0.5 μg). Digests were analysed by agarose gel electrophoresis. Reactions were performed in sealed capillaries or snap-cap tubes. The R. Hind III restriction buffer (10 mM tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β mercaptoethanol, 50 mM NaCl) has been taken as standard and any deviation from this buffer is indicated in the text. The principal change in restriction buffer occurred with R. Eco RI digestions as these required 0.1 M NaCl to inhibit any RI* activity. Reactions were stopped by heating at 70°C for 10 minutes followed by rapid cooling on ice. This heat step dissociated phage λ cohesive ends but the longer P2 cohesive ends required heating at 75 - 80°C. Restriction reactions for gel electrophoresis were stopped at 70°C, mixed with loading buffer, concentrated in a vacuum desiccator, reheated at 70°C (75°C for P2) for 10 minutes, quick cooled on ice and then loaded onto the gel.

Restriction fragments that contain a phage cohesive end could be identified by mixing a slight excess of intact linear DNA (at 75°C) with the restriction digest (at 37°C) followed by rapid cooling on ice. When this mixture was run in parallel with a normal digest the 2 missing bands could be assigned as those containing cohesive

ends (Helling et al., 1974) (see Fig 5.2). The method was most convincing for terminal restriction fragments below 7 kb in size.

Restricted DNA required for in vitro recombination experiments was pre-checked by both transfection and gel electrophoresis. Transfection gave the cut back relative to unrestricted DNA and the gel showed if any of this cut back was due to incomplete/over digestion. (Unrestricted DNA $> 10^5$ pfu/μg, restricted DNA $< 10^3$ pfu/μg i.e. > 100 fold cut back).

2.5 k in vitro Ligation

Restricted DNA (5-20 μg/ml, generally not more than 1 μg per reaction) was mixed with 1/10th volume x 10 ligase cocktail (section 2.4) and then T4 DNA ligase was added. Ligation was initiated at 10°C for 3 - 6 hours and continued at 0°C for 2 - 4 days. Phage cohesive ends were ligated at 37°C with incubations of no more than 1 hour. Ligase activity was always checked in preliminary control experiments (e.g. restoration of plaque forming ability to restricted λ vector DNA in transfection or by the appearance of high molecular weight bands after agarose gel electrophoresis of restricted and ligated DNA). Ligation reactions were sampled by transfection at various times; a sample withdrawn at the end of the 10°C incubation period was a reliable indicator to the likely efficiency of the reaction.

2.5 1 Transfection

Competent cells were prepared by calcium starvation (Mandel and Higa, 1970) using a method modified from Lederberg and Cohen, (1974).

A fresh overnight stationary culture was diluted 1: 50 in L broth and grown with aeration at 37°C to OD_{650} nm 0.55 - 0.65. The culture was placed on ice for 20 minutes and then the cells were pelleted in a bench-top centrifuge. The cells were resuspended in half the initial volume of ice-cold 0.1 M $MgCl_2$ and re-pelleted immediately. The cells were then resuspended in one twentieth the initial volume of ice-cold 0.1 M $CaCl_2$ (i.e. a 50 ml culture produced 2.5 ml competent cells) and left on ice for at least 30 minutes before use.

DNA solutions were diluted to about 0.5 μg/ml in SSC: $CaCl_2$ (1 x SSC, 0.1 M $CaCl_2$ mixed in the ratio 3:4) and 0.1 ml portions of DNA solutions were mixed with 0.1 ml competent cells. The mixture was left on ice for 30 minutes, placed at 42°C for 2 minutes and then left on ice for a further 30 minutes. The mixture was plated on BBL plates and top agar was supplemented with 10 mM $MgSO_4$. Serial dilutions of the transfection mix were made just before plating.

Transfection efficiency varied in the range 10^5 - 10^6 pfu/μg depending on the DNA and host used. The normal transfection hosts were ED 8659 (*sup* E, *sup* F, *r* k^-, *mk*$^+$) and ED 8741 (*sup* E, *rk*$^-$, *mk*$^+$ *rec* A).

2.5 m Gel Electrophoresis (DNA)

Vertical agarose slab gels (20 or 40 cm long x 20 cm x 0.3 cm) were made routinely with an agarose concentration range 0.75% - 1.5% (w/v) in 40 mM tris-acetate pH 8.2, 1 mM EDTA (Sharp et al., 1973). Gels were made either between 2 glass plates or between a glass plate and the perspex face of an electrophoresis box apparatus. There was virtually no difference in the quality of results from the 2 types of gel but it was slightly more convenient to prepare gels in the box apparatus. Electrophoresis conditions were adjusted according to the separation required and marker dyes bromophenol blue and xylene cyanol FF (equivalent to approximately 0.5 kb and 5 kb respectively) were used as a visual indication of migration distances. Gels were stained after electrophoresis in ethidium bromide (1.0 μ g/ml solution in water), destained in water - both stages about 30 minutes - and then photographed under ultra violet light using Ilford FP 4 film (x 4 red filter) and Microphen developer. The background on gel negatives was reduced by transferring the gel to a sheet of black polythene prior to the photography. This also allowed the exposure time to be extended (normally 3 - 5 minutes going up to a maximum of 10 minutes) to facilitate the observation of very small quantities of DNA.

The sizes of restriction fragments have been estimated from calibration - curves based on R. E.co RI, R Hind III and R. E. co RI + R Hind III digests of λ c I_{857} DNA. The fragment sizes for these standard digests were determined by electron microscopy (Phillipsen, P. and

and Davis, R. W., unpublished results).

Polyacrylamide gels were made exactly as described by Maniatis et al., (1975) and served as a means of locating DNA fragments of less than 0.5 kb.

2.5 n Density Gradient Centrifugation

Neutral sucrose gradients were made from 6 - 20% (w/w) with a 50% sucrose shelf (sucrose dissolved in 10 mM tris-HCl pH 8.0, 0.1 M NaCl, sterile solutions prepared freshly for each experiment). Centrifugation conditions varied according to the experiment and details of individual gradients are given in the text. Gradients were collected dropwise through a hole in the bottom of the centrifuge tube. Labelled DNA (^{32}P) was detected by Cerenkov counting (Clausen, 1967). Unlabelled DNA was detected by electrophoresing samples of gradient fractions on agarose gels followed by ethidium bromide staining and photography under ultraviolet light.

2.5 o 5' Terminal Labelling of DNA and Ionophoresis

The experimental procedure was basically as reported by Murray (1973). The labelling reactions involved 2 stages catalysed by the enzymes alkaline phosphatase and polynucleotide kinase. The phosphatase removed 5' phosphate groups to leave 5' hydroxyl groups which could then accept a phospate group from ATP (i.e. γ -^{32}P-ATP) in a reaction catalysed by kinase. DNA for labelling was freshly

extracted from caesium banded phage; both the phosphatase and kinase enzymes recognise nicks so it was essential to have good DNA preparations.

Phage DNA (30 - 100 µg) was incubated with bacterial alkaline phosphatase (added at the rate of 20 µl/ml) in DNA buffer at 37°C and the reaction was terminated by phenol extraction. The solution was dialysed to remove phenol and the final dialysis buffer was normally 2mM tris-HCl pH 7.5. This allowed the DNA solution to be concentrated 10 fold in a vacuum desiccator for the kinase reaction. The kinase activity was critically dependent on the ATP concentration and in some case cold ATP was added to the reaction (Lillehaug and Kleppe, 1975). Reactions were performed in siliconised tubes that contained dried isotope (γ -^{32}P-ATP) and the final buffer was 20 mM tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β mercaptothanol. Kinase was purified from T4 infected cells by K. Murray and K. Brown (Richardson, 1965) and was used at 1 µl per 50 µl of solution. Reactions were incubated at 37°C for 1 hour and then stopped by the addition of excess EDTA followed by heating at 65°C for 5 minutes. DNA was separated from unincorporated nucleotide by sedimentation through neutral sucrose gradients (3 x 3 ml Aluminium swinging bucket rotor 50 k rpm, 2 hours, 20°C, 190,000 g). Gradients were fractionated by dripping through a hole in the bottom of the centrifuge tube and there was always a convincing separation between DNA and ATP. Fractions from the peak of radioactivity at the bottom of the gradient were pooled, dialysed to remove sucrose and then concentrated in a vacuum desiccator.

An alternative method for the separation of DNA and nucleotide was to dialyse the kinase reaction mixture against several changes of 0.3 M NaCl in DNA buffer. The salt was removed by dialysis against DNA buffer alone and then the solution was passed over a gel filtration column (Sephadex G-50 fine or Sephacryl S-200 superfine, Pharmacia Ltd.). Radioactivity eluting in the column void volume was pooled and concentrated.

Samples of labelled DNA were mixed with carrier DNA (1 - 5 µg/reaction) and were digested with pancreatic DNase (a non-specific endonuclease) at 37°C in a buffer containing 20 mM tris-HCl pH 7.5, 5 mM $MgCl_2$. Portions of pancreatic DNase digests were removed and digested with snake venom phosphodiesterase (a 3' → 5' exonuclease). The reaction products were analysed by ionophoresis on AE-cellulose (AE-81) paper at pH 3.5 or on DEAE-cellulose (DE-81) paper at pH 2.0 (Murray, 1970). In some cases a two dimensional separation was achieved by cutting out strips of paper after the first ionophoresis and sewing these onto a sheet of the other type of paper so that ionophoresis would then be at right angles to the initial direction. Nucleotides were detected by autoradiography and the identity of individual spots was determined from known mobility values and from expected shifts in mobility caused by the addition of different mononucleotides.

All autoradiography was performed with Kodak X-Omat H Film and Polycon developer (May and Baker Ltd., Dagenham, England). Developing times were carefully adjusted according to the temperature of the developer solution.

2.5 p P2 *in vitro* Packaging

Protein extracts from P2 vir_1 infected cells were prepared according to the published procedure (Pruss *et al.*, 1974). The standard assay for P2 *in vitro* packaging activity depended upon the addition of exogenous linear P2 vir_{22} DNA and the subsequent appearance of plaques on a lawn of a P2 lysogenic strain. P2 vir_1 has a weak virulent mutation, it does not form a lysogen and does not grow on a P2 lysogen; P2 vir_{22} has a strong virulent mutation - it does not form a lysogen but does grow normally on P2 lysogenic strains (Bertani and Bertani, 1971). The P2 lysogens (C-1a *ton* A /P2/ or C600 /P2/) eliminated any phage from endogenous (P2 vir_1) packaging and gave a direct measure of the exogenous (P2 vir_{22}) DNA packaging.

During the course of *in vitro* packaging experiments it was realised that an increase in the spermidine concentration for the packaging reaction (0.1 mM → 1.0 mM) gave roughly a 10 fold stimulation to the packaging efficiency. This effect has been noticed by other workers (Bowden and Calendar, 1978).

Packaging extracts were prepared by growing *E. coli* C-1a at 37°C to late log stage (OD_{650} nm 0.9 - 1.1, approximately 6 x 10^8 cells /ml) in Super TPG medium (section 2.4). The culture was then infected with P2 vir_1 phage at a multiplicity of 10 - 20 and allowed to continue growing at 37°C for 24 minutes. The culture was cooled quickly by pouring onto frozen growth medium and the cells were harvested by centrifugation (H.S. 18 8 x 50 ml Aluminium rotor,

10 k rpm, 10 minutes, 4°C, 12,000 g). The pellet was resuspended in lysozyme solution to give a 100 fold concentration (egg-white lysozyme, 2 mg / ml dissolved in 10 mM tris-HCl pH 8.0, 3 mM EDTA, 10 mM NaCl, 0.1 mM spermidine). The cells were lysed by freezing in liquid nitrogen and thawing briefly at 37°C (twice). After the second thaw, $MgCl_2$ (12 mM) and dithiothreitol (0.12 mM) were added and then the extract was sonicated (5 - 10 seconds with a Micro Tip probe at setting 5 on a Soniprobe Type 7530A sonicator, Dawe Instruments Ltd., London). The extract was then centrifuged to remove debris (H.S. 18 8 x 50 Aluminium rotor 5 k rpm, 15 minutes, 4°C, 3000 g); the supernatant was removed with a pasteur pipette and dispensed into sterile snap-cap tubes (50 µl of supernatant per tube). Tubes that were not required for immediate use were frozen in liquid nitrogen and stored at -70°C. The extracts appeared to be stable for storage periods of several months.

The packaging reaction was performed by incubating 50 µl of extract at 37°C for 1 hour with 5 mM phosphoenolpyruvate, 1 mM ATP, 1.3 µg pyruvate kinase, 1 mM spermidine and 0.6 - 0.9 µg P2 vir_{22} DNA. The reaction wasstopped by transferring to 0°C and diluting 10 fold with a pancreatic DNase solution (50 µg / ml DNase in 10 mM tris-HCl pH 7.2, 10mM $MgCl_2$, 1% ammonium acetate). The solution was then titered on bacterial hosts as a normal phage stock.

2.5 q ^{35}S Labelling of Proteins

Phage infection does not completely switch off bacterial protein

synthesis and this residual synthesis may obscure the identification of phage coded proteins. Bacterial protein synthesis may be effectively eliminated by ultraviolet irradiation; bacterial DNA is damaged and this seriously disrupts RNA and protein synthesis but the synthetic machinery survives largely undamaged and is able to interact with injected phage DNA. A pulse of radioactivity applied some minutes after the irradiation and infection of cells is incorporated almost exclusively into phage coded proteins.

The experimental procedure was adapted from an original paper (Jaskunas et al., 1975) by T. Linn (Linn et al. 1978) and I must acknowledge his advice when I practised the technique. E. coli strain 159 (Buchwald et al., 1970) was diluted 1: 50 into M9 minimal and maltose medium (section 2.4) from an L broth stationary phase culture and grown without shaking overnight at 32°C. The overnight culture was diluted to OD_{650} nm 0.1 with fresh M9 minimal + maltose and grown at 37°C with aeration to OD_{650}nm 0.5. The cells were pelleted in a bench top centrifuge and resuspended at 10^9 cells/ml in M9 maltose containing 20 mM $MgSO_4$. The cells (volume about 4 ml) were irradiated for 15 minutes at 9,000 ergs/mm^2 and then 50 µl portions were dispensed into sterile snap-cap tubes. The cells were infected with phage at multiplicity 10 and were placed on ice for 10 minutes to allow phage adsorption (phage titre was 10^{11} pfu/ml, therefore 5 x 10^7 bacteria per tube were infected with 5 x 10^8 phage i.e. 5 µl of phage stock solution). All phage preparations used for labelling experiments had been concentrated and purified on CsCl step gradients. After the phage adsorption period, the

cells were heated at 37°C for 3 minutes and then diluted with prewarmed buffer (200 µl buffer solution - M9 maltose containing 0.05 µg/ml L - methionine). In some experiments the radioisotope (10µ Ci L-^{35}S-methionine) was added with the prewarmed buffer, alternatively the isotope was added after an initial incubation at 37°C. Labelling was terminated by the addition of excess unlabelled L - methionine (50 µl 1 mg/ml solution). The tubes were transferred to ice, 10% (w/v) trichloracetic acid (250 µl) was added and, after thorough mixing, the tubes were left to stand on ice for 30 minutes. Protein precipitates were collected by centrifugation in a Quickfit Microcentrifuge (14,500 rpm, 6 minutes, 8°C, 14,000 g), rinsed with ice-cold acetone (1 ml) and redissolved in SDS sample buffer (50 µ l) (section 2.4). Protein solutions were heated at 100°C for 2 minutes before loading onto SDS polyacrylamide gels. Generally, 20-25 µ l of labelled protein was loaded on a 1 cm gel slot; the remainder of the protein solution appeared to be stable for 1 - 2 weeks when stored at -20°C.

2.5 r Polyacrylamide Gel Electrophoresis (Proteins)

Radioactively labelled proteins were analysed by sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (Laemmli, 1970) followed by autoradiography. Polyacrylamide gradient gels were selected for the analysis as both high and low molecular weight polypeptides could be resolved on a single gel.

The acrylamide solutions for gradient gels were made up according

to a recipe provided by T. Linn (section 2.4). The amounts of ammonium persulphate and N N N^1 N^1 Tetramethylenediamine (TEMED) were adjusted so that polymerisation did not occur for at least 1 hour and this allowed ample time for pouring the gel. The gel (20 cm x 20 cm x 0.15 cm) was formed between 2 glass plates and the acrylamide solution was pumped from a gradient maker (25 ml capacity for each chamber) to the top of the glass plates and allowed to run down slowly. The pump speed was set so that the gel (volume approximately 40 ml) was poured over about 20 minutes. The upper surface of the gel was overlaid with a small volume of iso-butanol and the gel was left for polymerisation to occur. The iso-butanol was then discarded and a stacking gel (3% or 5% acrylamide) was poured on top of the gradient (1 - 1.5 cms of stacking gel); a comb inserted into the acrylamide solution formed individual slots 1 cm long. When the stacking gel had set, the comb was carefully removed and the slots were washed thoroughly with electrophoresis buffer (0.025 M Tris, 0.19 M Glycine, 0.1% SDS). Protein samples were heated at 100°C for 2 minutes and were loaded under electrophoresis buffer from drawn out capillaries. All the gels had 1 or 2 slots with marker proteins (commercially available proteins dissolved in distilled water, approximately 10 µg of each protein loaded per slot) for molecular weight determination. Electrophoresis through the stacking gel was at 200 V, 12 mA (about 2 hours) then the voltage was increased to 350 V, 20 mA for electrophoresis through the separating gel (about 10 - 12 hours).

The gels were fixed (45 minutes, 37°C in 50% methanol, 10% acetic

acid), stained (90 minutes, 37°C 0.1% Coomassie Brilliant Blue - R 250 - in 45% methanol, 10% acetic acid) and destained (several hours, room temperature in 5% methanol, 7% acetic acid) until the marker protein bands were clearly visible. Gels were dried down under vacuum onto Whatman 3 MM filter paper, the marker proteins were outlined with radioactive ink (^{35}S) and the gels were set up for autoradiography. Exposure times were as indicated in the text but strongly labelled proteins could be readily detected after 24 - 36 hours.

2.5 s Electron Microscopy

DNA preparations were denatured in alkali and allowed to renature in 50% formamide at 27°C. Cytochrome C was added (final concentration 0.165 mg/ml) and spreading was then performed over a 15% Formamide hypophase, in a draught-free room. Parlodion coated grids were touched on the film near the origin of spreading and then were stained in uranyl acetate and washed in ethanol. The grids were rotary shadowed with platinum, coated with vaporised carbon and the parlodion, was removed with absolute ethanol.

The grids were examined in a Siemen Elmiskop 1A electron microscope. Photographic negatives were projected at 5 times magnification and traces of these projections were measured with a standard map measurer.

I am grateful to Pam Beattie and Dr. P. Highton for guidance in the

use of the electron microscope.

2.5 t Centrifugation

M.S.E. equipment was used for all large scale centrifugation (M.S.E. Scientific Ltd., Crawley, Sussex, England). Low speed spins were performed in a High Speed 18 (H.S. 18) centrifuge; all spins above 15k rpm were performed in preparative ultracentrifuges (S.S. 65 and S.S. 75).

A microcentrifuge (Jobling Laboratory Division, Stone, Staffs., England 14.5 k rpm, 14,000 g) was used for micro scale work. Tubes for this microcentrifuge (1.5 ml snap-cap tubes) were purchased from W. Sarstedt (U.K.) Ltd., Leicester.

CHAPTER 3 Identification of Restriction Enzyme Recognition Sites in P2 DNA and Molecular Cloning of P2 DNA

The *in vitro* recombination experiments described in this chapter had 2 basic objectives. It was hoped that the transfer of P2 DNA to λ vectors would enable the amplification of P2 structural gene products. The formation of λ/P2 recombinants and the simultaneous restriction mapping was intended to provide additional information to link P2 genetic and physical maps.

3.1 Preliminary Information on Restriction Enzyme Sites in P2 DNA

When this work was initiated there was very little information available on restriction enzyme recognition sites in P2 DNA. It appeared that there were 3 sites for the enzyme R. *Eco* RI in P2 DNA (Murray, K. *et al.*, 1977). This result was confirmed by an electron microscope examination of R. *Eco* RI digested P2 DNA (Chattoraj *et al.*, 1977). Length measurements for different restriction fragments were obtained and these fragments were placed in a unique order by extending the analysis to include characterised P2 deletion mutants. The arrangement of R. *Eco* RI sites was verified in this work using agarose gel electrophoresis to separate restriction digests of the deletion mutants.

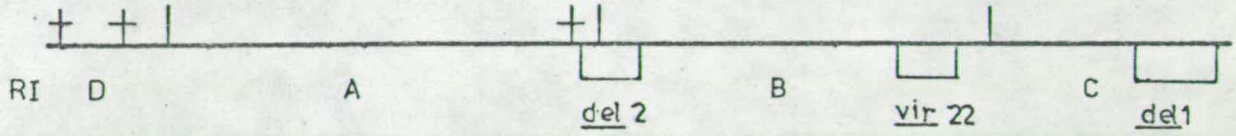
The R. *Eco* RI fragments were all of suitable size for cloning into phage λ vectors (after annealing the P2 cohesive ends, the 2 terminal

restriction fragments were considered as a single R. Eco RI fragment). Before any in vitro recombination was attempted, the restriction mapping was extended to permit easy recognition of the different P2 R. Eco RI restriction fragments. The recognition sites for R. Bam HI were determined from single digests and double digests with R. Eco RI (Fig. 3.1). The 79.9 - 10.5% RI fragment was cut twice by R. Bam HI and a characteristic 1.95 kb fragment was released. The 10.5 - 47.5% fragment was cut once asymmetrically by R. Bam HI and the 47.5 - 79.9% RI fragment was not cut by the enzyme. Therefore, R. Bam HI digests of λ/P2 recombinants would immediately reveal which P2 R. Eco RI fragment was present and, with knowledge of R. Bam HI recognition sites in the λ vectors, it would be possible to deduce the orientation for 2 of the 3 P2 R. Eco RI fragments.

The original interest in P2 DNA was centered on the left terminal R. Eco RI restriction fragment. There was an indication from pulse-chase experiments that P2 ter could be the Q gene product (Pruss et al., 1975) and gene Q had been placed near the left end of the linear genetic map (Lindahl, 1969). It was decided that the most convenient way to transfer the left P2 R. Eco RI terminal fragment to λ vectors was to allow P2 cohesive end reannealing after an R. Eco RI restriction digest and then incorporate this larger fragment containing both terminal restriction fragments and the P2 cos site into R. Eco RI vectors.

Fig. 3.1

Restriction Sites in P2 DNA



† R.Bam HI | R.EcoRI

R. Eco RI Fragments			R. Bam HI Fragments
(Chattoraj et al., 1977)			(Table 3.3)
A	37%	12.2 kb	18 kb
B	32.4%	10.7 kb	13.1 kb
C	20.1%	6.63 kb	1.95 kb
D	10.5%	3.46 kb	0.3 kb

The R.EcoRI + R.Bam HI double digest of P2 DNA produced new bands at about 11.5 kb, 1.35 kb and 0.76 kb (Fig 3.9). This information positioned the R. Bam HI sites as shown above.

The terminal R. Bam HI restriction fragments were initially identified from experiments with 5' terminally labelled DNA.

3.2 Molecular Cloning of P2 DNA

DNA from a P2 deletion mutant (P2 1171 A am_{127}, B am_{116}, del 1, del 2) was restricted with R. Eco RI (section 2.5 j) and the digest was loaded on top of a 5 - 20% (w/w) neutral sucrose gradient (section 2.5 n, centrifugation conditions: 3 x 6 ml Titanium swinging bucket rotor 40 k rpm, 4 hrs, 20°C, 130,000 g). No specific step for P2 cohesive end annealing was included as previous experiments had shown that about 50% of the cohesive ends would rejoin during the 37°C incubation with the restriction enzyme; the normal 75°C heat step to terminate restriction reactions and dissociate phage cohesive ends was omitted. The mutant phage P2 1171 contained 2 deletions which had some influence on the in vitro recombination reaction. The deletion del 2 covered the region 45.5 - 51.6% from the left end of the P2 genome (Bertani, 1975) and removed the central R. Eco RI recognition site. The R. Eco RI digest of P2 1171 DNA consisted of the 2 terminal fragments and a very large central fragment making the sucrose gradient separation very straightforward. The deletion del 1 covered the region 92 - 99% from the left end of the P2 genome (Bertani, 1975) and this reduction in the size of the right terminal fragment meant that the annealed cohesive end fragments could be accommodated more readily in λ insertion vectors.

The required fractions from the sucrose gradient were identified by gel electrophoresis (section 2.5 n), pooled and dialysed to remove sucrose. Ligation reactions were set up with the purified P2 DNA fragment and R Eco RI restricted λ 641 and λ 596 vectors (section

2.5 k). (λ 641 is an immunity insertion vector. λ 596 is a sup E replacement vector.) The numbers of plaques recovered by transfection (section 2.5 1) were rather low but 3 clear plaques, suggestive of recombinants, were recovered from the λ 641 reaction and 1 plaque from the λ 596 reaction was found to be sup^{o}. These 4 plaques were picked and taken through single plaque cycles of purification.

The phage from the λ 596 reaction gave a consistent mixture of small and large plaques. The vector, λ 596, was red^{-} γ am and it was expected that small plaques would be found after growth on a sup^{o} host. The large plaques had the correct immunity (i.e. $c\ I_{857}$) and stayed large whereas purified small plaques generated more large plaques when replated. The interpretation proposed was that the large plaque form had acquired a chi site from the bacterial chromosome and was now able to grow at much higher efficiency (Stahl, F. W. and Stahl, M. M., 1975). All subsequent analysis of the potential recombinant - λ/P2 (596) - was performed with a purified large plaque stock.

The potential recombinants with λ 641 were tested for phage immunity; the phage repressor gene had been inactivated by the insertion of foreign DNA but sensitivity to repressor was retained - the phage should fail to grow on strains lysogenic for λ imm^{434} and this was found to be the case. A consequence of DNA insertion into a single site vector was that the number of restriction sites for the 'cloning' enzyme increased from 1 to 2. With R. Eco RI this change

can be detected by determination of the phage restriction ratio (Murray and Murray, 1974). The restriction ratio is defined as the phage titre on a non-restricting strain divided by the phage titre on an iso-genic restricting strain (in this case, a strain carrying the RI plasmid). The ratios for the 3 potential recombinants were consistent with 2 R. Eco RI sites suggesting that the phage had inserted DNA.

Small scale liquid lysates (100 ml cultures) were prepared (section 2.5 f), phage were recovered by pelleting in the ultracentrifuge and purified by one round of equilibrium centrifugation. Sufficient DNA was recovered for restriction analysis with R. Eco RI and R. Bam HI and this confirmed that the 4 phage were genuine λ/P2 recombinants. The R. Bam HI restriction patterns for the λ/P2 (641) derivatives revealed a 1.95 kb fragment that was characteristic of the P2 left end of the genome but the 3 recombinants were not identical showing that both orientations of the P2 DNA had been isolated (Fig. 3.2). The single λ/P2 (596) phage fortunately had P2 genes P and Q placed adjacent to λP_L and, on the basis of the transcription map for P2 structural genes (Lindahl, 1971; Sunshine et al., 1971), sense-strand transcription of the P2 genes should occur from P_L.

Restriction maps for the 4 λ/P2 recombinants are shown in Fig. 3.3. The properties of these recombinants, as far as P2 DNA content is concerned, will be described in detail in Chapter 4 but marker rescue experiments at this stage provided absolute confirmation

Fig. 3.2 (opposite)

Restriction Analysis of λ /P2 Recombinant Phage.

1% agarose gel, all samples digested with R. Bam HI.

Track 1 λ 641 DNA

2 λ /P2 (641)a DNA

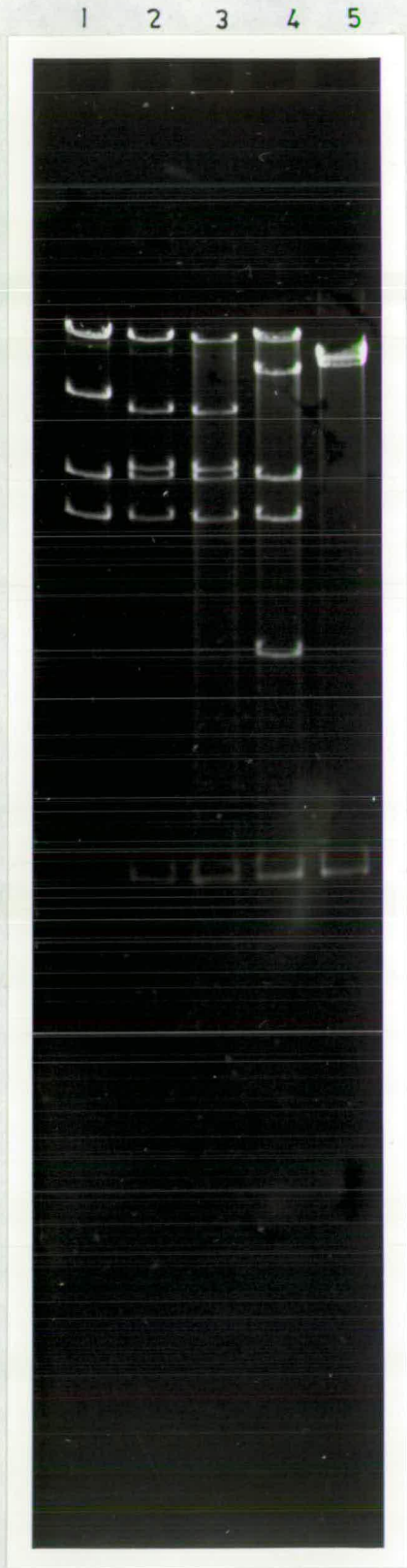
3 λ /P2 (641)b DNA

4 λ /P2 (641)c DNA

5 P2 1171 DNA

The 1.95 kb band in the P2 1171 track can be clearly seen in the λ /P2 recombinant DNA tracks. λ /P2 (641) a and b appear to be identical, λ /P2 (641)c has the P2 DNA inverted relative to λ sequences.

Bands are numbered to the right of the photograph and these numbers correlate with restriction maps (Fig. 3.3).


1 2 3 4 5

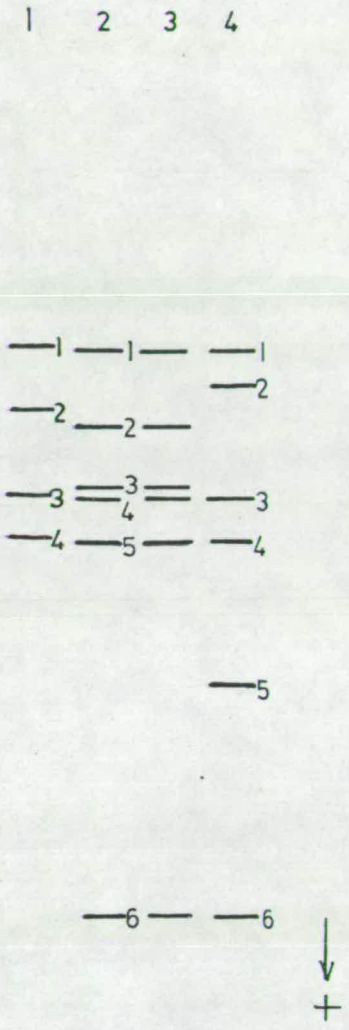
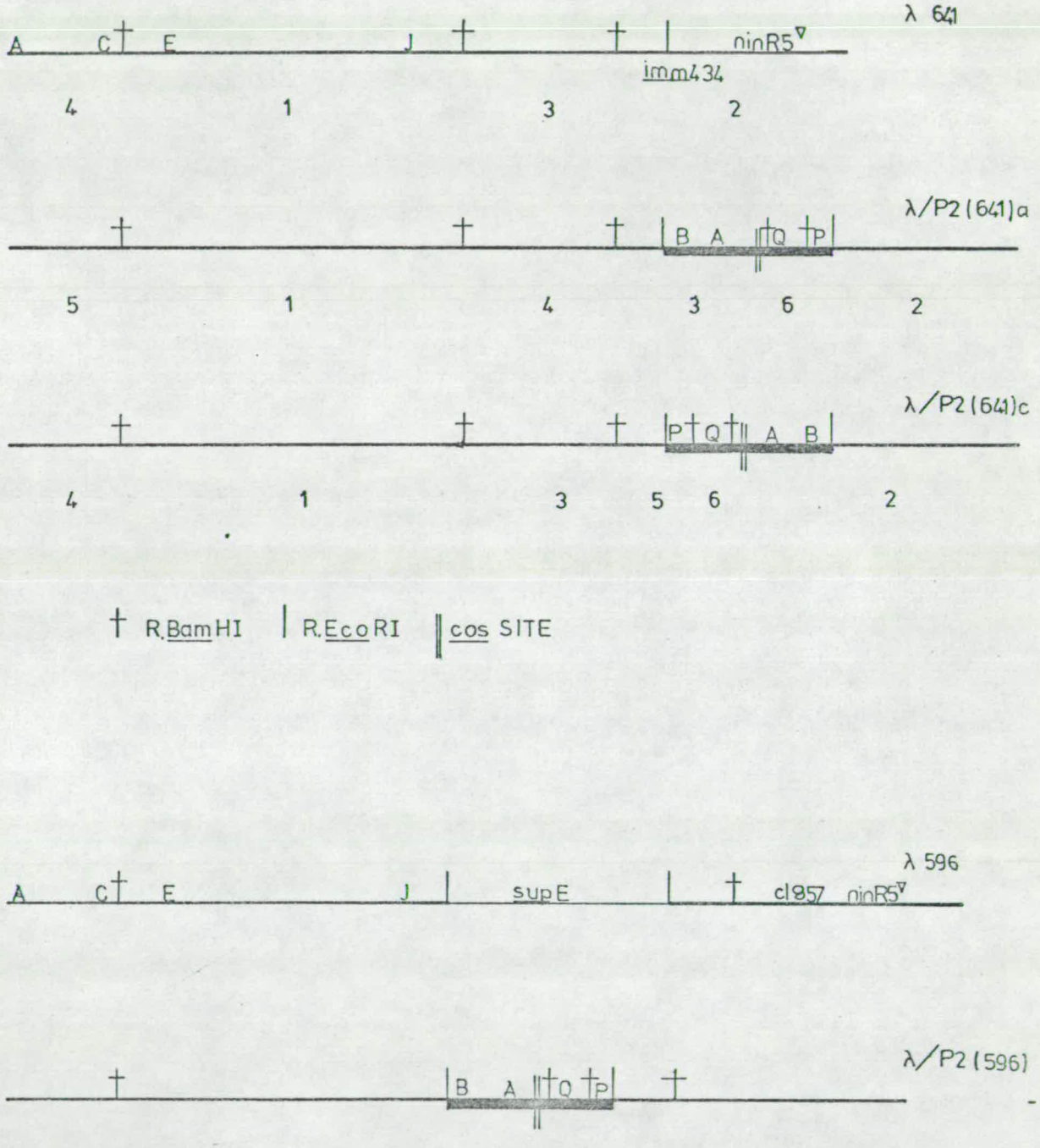

1 2 3 4
1
1
1
2
2
2
3
3
4
3
4
5
4
5
6
6
+

Fig. 3.3 (opposite)

Restriction Maps of λ/P2 Recombinant Phage.

The line diagrams show the positions of recognition sites for R. Eco RI and R. Bam HI. The numbers below the diagrams refer to the bands shown in Fig. 3.2.

The diagrams have been drawn approximately to scale.


λ 641
A
C
E
J
ninR5
imm434
4
1
3
2
λ/P2 (641)a
B
A
Q
P
5
1
4
3
6
2
λ/P2 (641)c
P
Q
A
B
4
1
3
5
6
2
R.BamHI
R.EcoRI
cos SITE
λ 596
A
C
E
J
supE
cI857
ninR5
λ/P2 (596)
B
A
Q
P

that these recombinants contained P2 DNA sequences.

Recent experiments from Calendar's group (Pruss and Calendar, 1978; Bowden and Calendar, 1978) have identified gene M as coding for the P2 ter enzyme and gene M was not located on the fragment of P2 DNA already transferred to λ vectors. More λ/P2 recombinants were constructed in an attempt to look at $\overset{P2\,M}{|}$gene expression in λ. Because there was some interest to determine whether each of the '3' P2 R. Eco RI fragments would generate viable recombinants, unfractionated R. Eco RI restriction digests of P2 DNA were ligated with restricted λ vectors. A number of small scale in vitro recombination reactions were set up with P2 vir_1 and P2 Q am_{34} vir_1 DNA and λ replacement vectors 596 and 781 - 781 is a Y^+ derivative of λ 596. The numbers of plaques recovered after transfection were again low but about 50% of the plaques tested were sup^o and potential recombinants. After plaque purification, plate stocks of the phage were prepared and these were used as a source of phage for L agarose plate lysates (section 2 - 5 h). DNA isolated from agarose plate lysates was digested with R. Bam HI and analysed by agarose gel electrophoresis (Fig. 3-4).

While the agarose plate lysate preparations were in progress a genetic test was devised as an alternative approach for screening the recombinants. A sup^o λ lysogen (C - la /λ cI_{857}/) was pre-infected with P2 M am_{32} and left at room temperature for adsorption to occur - 0.1 ml L broth overnight culture supplemented with 10 mM $MgSO_4$, 5 mM $CaCl_2$, infected with 2 - 5 x 10^6 pfu P2 mutant phage.

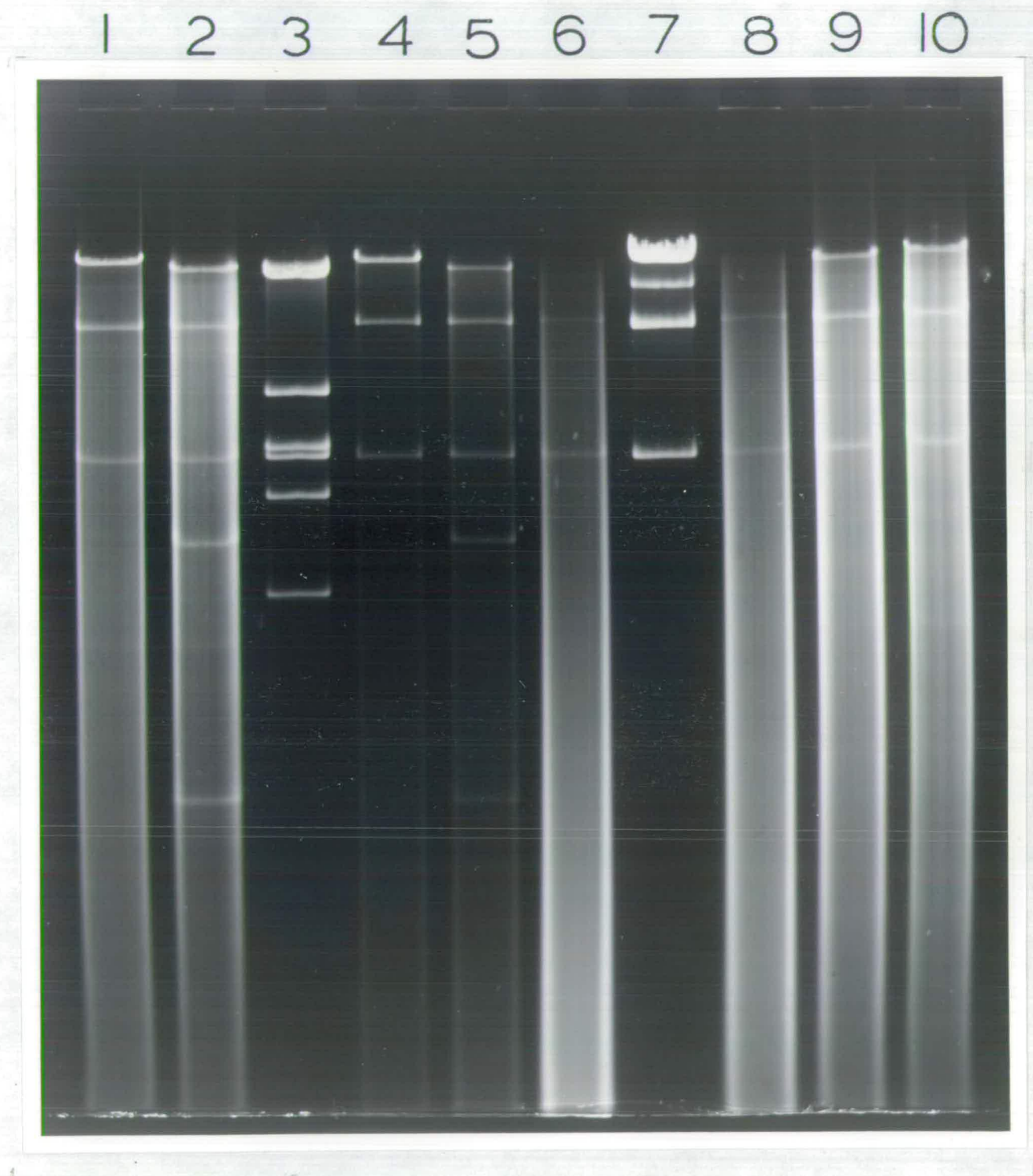

1 2 3 4 5 6 7 8 9 10
+

Fig. 3.4 (opposite)

Restriction Analysis to Identify λ/P2 Recombinants.

Track 1	λ/P2 (R1)	DNA + R. Bam HI
2	λ/P2 (R2)	DNA + R. Bam HI
3	λ cI_{857}	DNA + R. Eco RI
4	λ/P2 (R4)	DNA + R. Bam HI
5	λ/P2 (R5)	DNA + R. Bam HI
6	λ/P2 (R9)	DNA + R. Bam HI
7	λ 781	DNA + R. Bam HI
8	λ/P2 (R12)	DNA + R. Bam HI
9	λ/P2 (R13)	DNA + R. Bam HI
10	λ/P2 (12)	DNA + R. Bam HI

1% agarose gel.

Tracks 3 and 7 show DNA that had been extracted from CsCl purified phage, the other tracks are plate lysate DNA preparations.

The λ 781 vector (track 7) contains 2 recognition sites for R. Bam HI; the 2 fastest moving bands are the terminal fragments and the fainter, additional band is explained by annealing of the λ cohesive ends. λ/P2 (R2) and λ/P2 (R5) (tracks 2 and 5) contain 2 additional bands which show that these recombinants have the P2 RI C+D fragment in the same orientation as λ/P2 (596) (Fig. 3.3). The other λ/P2 recombinants have no additional R. Bam HI sites and so potentially contain RIB.

The mixture was then plated out and spots (about 10 µl) of λ/P2 recombinants were applied to the plate. It was essential to have high titre stocks of the λ/P2 phage so that about 10^8 pfu could be applied in the spot. A control plate with the λ lysogen alone was prepared in parallel to indicate the amount of 'killing' resulting from the high multiplicity of λ infection. There was always a convincing difference between a positive response indicating P2 phage growth after recombination between P2 Mam$_{32}$ and a λ/P2 phage carrying an intact P2 M gene and a negative response that showed inhibition to cell growth by killing. The λ/P2 (596) phage provided another control as this phage would produce a positive spot on a lawn of the λ lysogen that had been pre-infected with P2 Qam$_{34}$. The spot test gave no false positives and successfully identified all the P2 M^+ λ/P2 recombinants that were detected by examination of R. Bam HI restriction patterns.

The structure of 2 recombinants was not immediately apparent from the information provided by R. Bam HI restriction patterns and the M gene marker rescue test. One of the unusual recombinants - λ/P2 (14) - had an additional R. Bam HI fragment when compared with the λ vector but was negative in the spot test. An R. Eco RI digest of λ/P2 (14) showed only 1 cleavage site for this enzyme whereas both λ vector and the recombinants should have had 2 cleavage sites. λ/P2 (14) appeared to have undergone a deletion in vivo that had removed srl λ - 1 and P2 DNA up to and including the Mam$_{32}$ marker. λ/P2 (30), the other unusual recombinant, did not have an additional R. Bam HI site but contained a 6 kb fragment, flanked by 2 R. Eco RI

sites, that would allow rescue of the P2 Mam$_{32}$ marker. This phage also appeared to have been formed after a deletion *in vivo*; λ/P2 (30) will be considered again in Chapter 4.

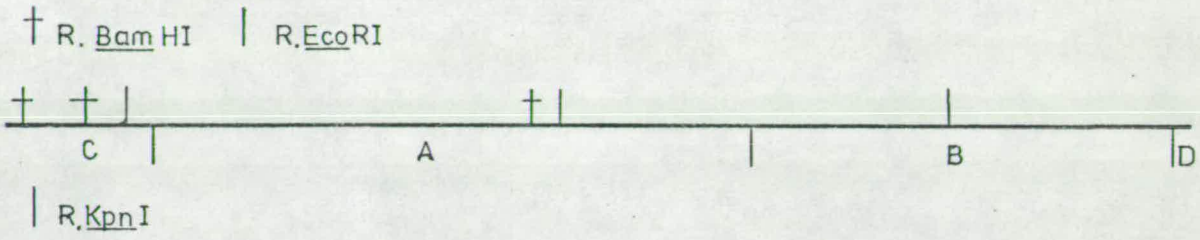
The *in vitro* recombination experiments resulted in the isolation of λ/P2 hybrid phage; the entire P2 genome was contained within 3 different λ/P2 recombinants. These recombinants were used to provide supplementary information for the P2 restriction mapping studies.

3.3 Restriction Mapping of P2 DNA

The locations of recognition sites in P2 DNA have been identified for the following restriction enzymes: R. Eco RI, R. Bam HI, R. Kpn I, R. Sal I, R. Hpa I and R. Rsp I (Yoshimori, 1971; Wilson and Young, 1975; Smith et al., 1976; Arrand et al., 1978; Sharp et al., 1973; unpublished information from M.R.E., Porton, Wiltshire). Information from double digests placed the recognition sites in a unique order (Fig. 3.10). The deduction of the arrangement of recognition sites will be described and illustrated for R. Kpn I (Figs 3.5 and 3.6); other restriction patterns will be represented principally by line diagrams although examples of some digests have been included.

The restriction analysis of P2 DNA relied on 4 different P2 DNA samples: P2 vir 1 the effective wild-type DNA, P2 753 del 1 ts Q_{48}, P2 1171 del 1, del 2, Aam_{127}, Bam_{116} and P2 vir_{22}. The deletions del 1 and del 2 were mentioned earlier (del 1 92.0 - 99.0%, del 2 45.5 - 51.6%); vir_{22} is a strong virulent mutation that corresponds to a complex chromosomal aberration involving a deletion of DNA 72.0 - 77.1% from the left end and a 0.5% insertion at the deletion site (Chattoraj and Inman, 1972; Bertani, G., 1975). It was assumed that the (point) mutations in P2 753 and P2 1171 had no influence on restriction patterns.

3.3 b Restriction Mapping: R. Kpn I



The P2 DNA samples were digested with R. Kpn I and analysed on a 1% agarose gel (section 2.5 m) (Fig. 3.5). The digests were not quite complete but there was no difficulty with the interpretations. (In an attempt to provide clear identification, major P2 vir_1 restriction bands have been designated by capital letters A - X in order of decreasing molecular weight and the restriction enzymes abbreviated to RI, Bam, Sal, Kpn, Hpa and Rsp; thus RI B, Bam D etc.). P2 vir_1 DNA apparently gave 3 major bands with R. Kpn I, DNA from the P2 deletion mutants also had 3 major bands suggesting that no site had been removed by a deletion. The band Kpn A was the same size for P2 vir_{22} and P2 753 but was smaller for P2 1171; the del 2 deletion therefore appeared to be contained within Kpn A. The band Kpn B was reduced in size by both the vir_{22} and del 1 deletions indicating that Kpn B originated from the right arm of the P2 genome. However, the size reduction in Kpn B was almost exactly the same for the vir_{22} and del 1 deletions and it was known that these deletions differed by 1.9% P2 length. There was the

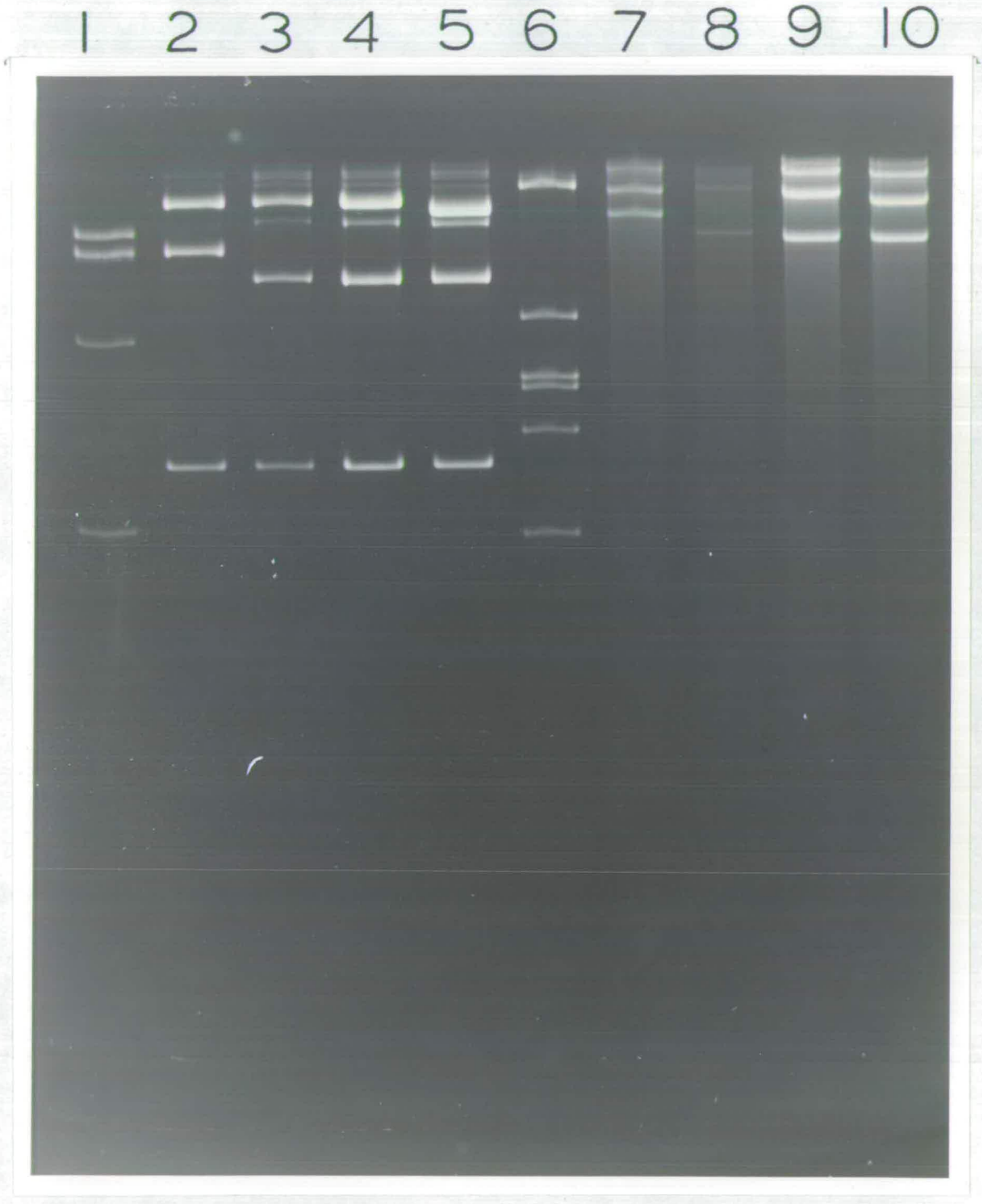

1 2 3 4 5 6 7 8 9 10

Fig. 3.5 (opposite)

R. Kpn I and R. Rsp I Restriction Analysis of P2 DNA.

Track 1	P2 vir_1 DNA + R. Eco RI		
2	P2 vir_1 DNA + R. Kpn I		
3	P2 vir_{22} DNA + R. Kpn I		
4	P2 753 DNA + R. Kpn I	(del 1)	
5	P2 1171 DNA + R. Kpn I	(del 1 and del 2)	
6	λ cI_{857} DNA + R. Eco RI		
7	P2 vir_1 DNA + R. Rsp I		
8	P2 vir_{22} DNA + R. Rsp I		
9	P2 753 DNA + R. Rsp I		
10	P2 1171 DNA + R. Rsp I		

1% agarose gel

The gel photograph shows the changes in band mobility caused by the different P2 deletions.

The background smear in the R. Rsp I digests indicates that a low level of non-specific nuclease activity is present in the restriction enzyme preparation.

The enzyme R. Rsp I was isolated from Rhodopseudomonas spheroides (unpublished information, M.R.E., Porton, Wilts.). The enzyme digests were performed in a buffer containing 6 mM tris-HCl pH 7.5, 6 mM $MgCl_2$, 10 mM DTT.

possibility that the del 1 deletion had affected an R Kpn I site close to the right cohesive end. Kpn C appeared to be the same size for all the P2 DNA samples.

One more fact had to be considered from these digests; a faint band could be detected above Kpn C which did not seem to be present in the other tracks. The faint band suggested that there was another major band, too small to be detected on this gel; either the faint band was a partial digestion product or it had been formed by some reannealing between Kpn terminal fragments which would have to be Kpn C from the left and a tiny fragment, Kpn D, from the right. The faint band above Kpn C was not detected in the other P2 DNA samples which appeared to be less well digested and so the partial digestion product seemed unlikely. P2 753 and P2 1171 differed from P2 vir_1 close to the right end of the DNA and could well have a different Kpn right terminal fragment. These preliminary R. Kpn I digests suggested the band order C, A, B, D but the alternative arrangements D, C, A, B and C, D, A, B could not formally be excluded.

Comparisons between double digests of P2 vir_1 DNA with R. Kpn I and R. Eco RI or R. Bam HI and the respective single digests provided additional information (Fig. 3.6). The digest of P2 vir_1 with R. Kpn I alone again showed the faint band above Kpn C but it was now possible to identify a low molecular weight band, Kpn D. Marker tracks provided mobility values for a calibration curve and it was possible to estimate the lengths of different P2 restriction fragments. The band Kpn C was equivalent to 4.3 kb and the faint band above was

Fig. 3.6 (opposite)

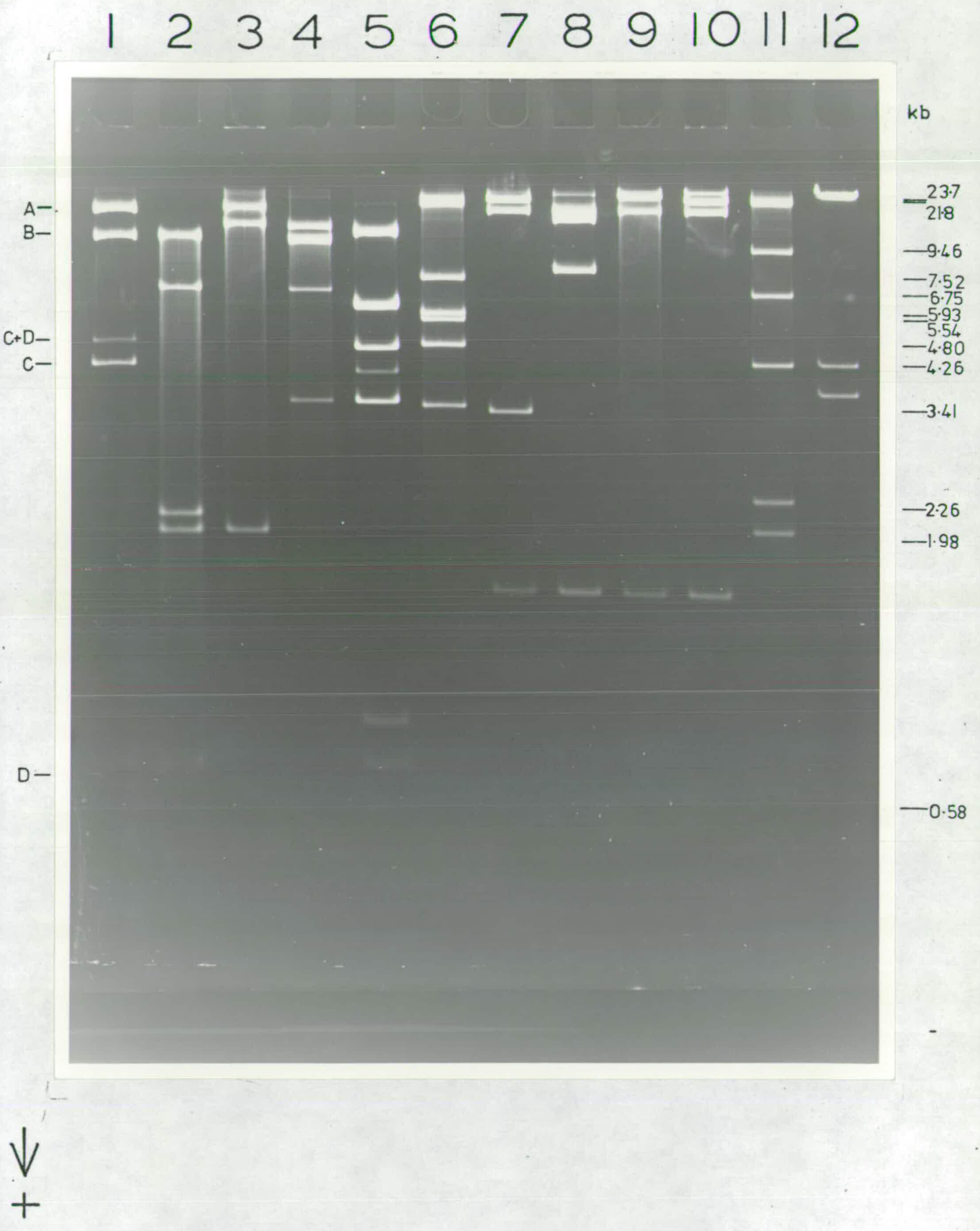
R. Kpn I Double Digests of P2 DNA.

Track 1 P2 vir_1 DNA + R. Kpn I
2 P2 vir_1 DNA + R. Kpn I + R. Bam HI
3 P2 vir_1 DNA + R. Bam HI
4 P2 vir_1 DNA + R. Eco RI
5 P2 vir_1 DNA + R. Eco RI + R. Kpn I
6 λ cI_{857} DNA + R. Eco RI
7 λ/P2 (29) DNA + R. Kpn I
8 λ/P2(30) DNA + R. Kpn I
9 λ/P2(596)DNA + R. Kpn I
10 λ cI_{857} DNA + R. Kpn I
11 λ cI_{857} DNA + R. Hind III
12 λ/P2(29) DNA + R. Hind III

1% agarose gel

The R. Kpn I double digests are discussed in the text. The R. Kpn I digest of λ/P2 (29) shows that P2 RIA contains a single R. Kpn I recognition site i.e. 1 extra band when compared with λ cI_{857} + R. Kpn I (track 10). The R. Kpn I digest of λ/P2 (30) (track 8) shows that λ/P2 (29) and (30) contain P2 DNA in opposite orientations (see Chapter 4).

The R. Hind III digest of λ/P2 (29) shows that shn λ 5 and shn λ 6 are the only recognition sites present. This band pattern was reproduced for λ/P2 recombinants carrying P2 RI B and P2 RI C + D.


1 2 3 4 5 6 7 8 9 10 11 12
kb
A
B
C+D
C
D
23·7
21·8
9·46
7·52
6·75
5·93
5·54
4·80
4·26
3·41
2·26
1·98
0·58
-
+

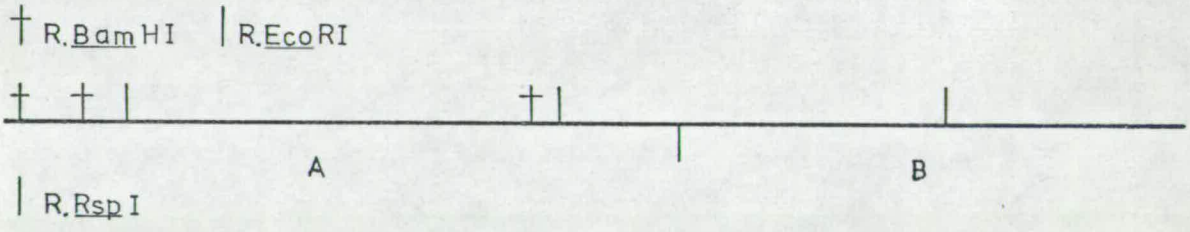
equivalent to 4.9 kb; Kpn D appeared to be slightly slower than the 0.58 kb λ c I_{857} Hind III G band (i.e. Kpn D approximately 0.6 kb) and this fitted with the faint band being the sum of Kpn C and Kpn D.

The bands RI D and Kpn D from single digests remained in the P2 vir_1 + R.Kpn I + R.Eco RI double digest (Fig. 3.6, tracks 1, 4 and 5). RI D, the left terminal fragment, was estimated from this gel to be 3.6 kb and there was now a faint band above RI D in the double digest at a position equivalent to 4.1 kb which could have arisen by P2 cohesive end annealing between RI D and Kpn D. There was a slight discrepancy (RI D 3.6 kb + Kpn D 0.6 kb = 4.2 kb and the length measured was 4.1 kb) but this could probably be attributed to small errors in the length measurements. In the P2 vir_1 + R.Kpn I + R.Eco RI double digest the largest band was slightly smaller than RI A indicating that there was an R.Kpn I site close to one end of the RI A fragment. The new band at about 0.7 kb must be the DNA lying between the R.Kpn I site in RI A and the R.Eco RI site at an end of RI A. The band RI B disappeared completely in the double digest and new bands at about 6 kb and 4.6 kb were formed. The 6 kb band was a doublet as RI C was cut by R. Kpn I to give a 6 kb fragment and Kpn D. The figures for this RI C change were very close but not exact: RI C 6.85 kb = a new band at 6 kb + Kpn D 0.6 kb.

The R.Kpn I site in RI B was placed asymmetrically but confirmation was still required for the relative positions of the 6 kb and 4.6 kb fragments. Bands from R.Kpn I and R.Bam HI single digests present

in the double digest were Kpn B, Kpn D, Bam C and Bam D (Fig. 3.6 tracks 1, 2 and 3); Bam D, the extremely small fragment from the left end of the genome cannot be seen on this gel. In the R. Bam HI + R.Kpn I double digest, the largest band was a doublet comprising Kpn B and a shortened form of Bam B; the new band at 2.15 kb was formed by cleavage at an R. Kpn I site within Bam B. The new band at 7 kb was derived from Bam A; the R. Kpn I sites in Bam A produced the 7 kb fragment, Kpn B and Kpn D. This 7 kb fragment removed the doubt about the positioning of the R. Kpn I site within RI B - the 6 kb RI/Kpn fragment must be closer to the left end of the P2 genome. The band fragments from the double digests provided verification of the R. Kpn I fragment order C, A, B, D.

3.3 c Restriction Mapping: R. Rsp I



The preliminary information for the R. Rsp I restriction map is shown in Figure 3.5 (tracks 7 - 10 inclusive). The fragment lengths suggested that the largest band in each digest was unrestricted DNA and this would mean that there was a single recognition site for R. Rsp I located in the right half of P2 DNA. The R. Rsp I digestion

TABLE 3.1

P2 *vir*$_1$ Restriction Fragment Lengths (First Estimates)

	R. *Eco* RI	R. *Bam* HI	R. *Sal* I	R. *Kpn* I	R. *Rsp* I	R. *Hpa* I	
A	13.5	20	22	20	20	6.1	A
B	11.5	14	13	11.8	13.5	5.8	B
C	6.8	1.95	1.8	4.2		5.2	C
D	3.6	0.3		0.6		3.35	D
						3.25	E
						3.1	F
						2.43	G
						1.8	H
						1.0	J
						0.6	K
						0.45	L

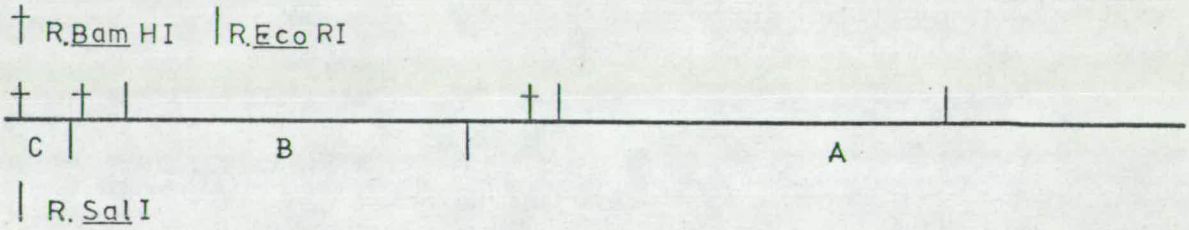
Lengths are expressed in kb.

No accuracy is attached to values above 7 kb or below 0.5 kb.

The figures represent averages from a number of experiments.

patterns of λ/P2 recombinants containing P2 fragments RI A, RI B and RI C + D could be explained with knowledge of the R. Rsp I recognition sites inλDNA (Murray, K., unpublished information) and were consistent with a single R. Rsp I site in P2 RI B. The λ/P2 RI C + D recombinant was particularly useful for mapping because the annealed P2 cohesive ends were now located in the centre of a λ DNA molecule and restriction enzyme recognition sites close to the P2 cohesive ends could easily be identified. Double digests with R. Rsp I plus R. Eco RI or R. Bam HI fixed the position of the R. Rsp I site within RI B (Fig. 3.7).

3.3 d Restriction Mapping: R. Sal I



Preliminary digests with R. Sal I suggested that there were2recognition sites for this enzyme in P2 vir_1 DNA; the restriction fragments were about 22 kb, about 13 kb and 1.8 kb. Terminally labelled DNA (section 2.5 o) identified the 22 kb and 1.8 kb fragments as belonging to the ends of linear P2 DNA and the deletions del 1, del 2 and vir_{22} were all located within the 22 kb fragment. The location of R. Sal I sites was deduced from double digests with R. Eco RI and confirmed by double digests with R. Bam HI (Fig. 3.7).

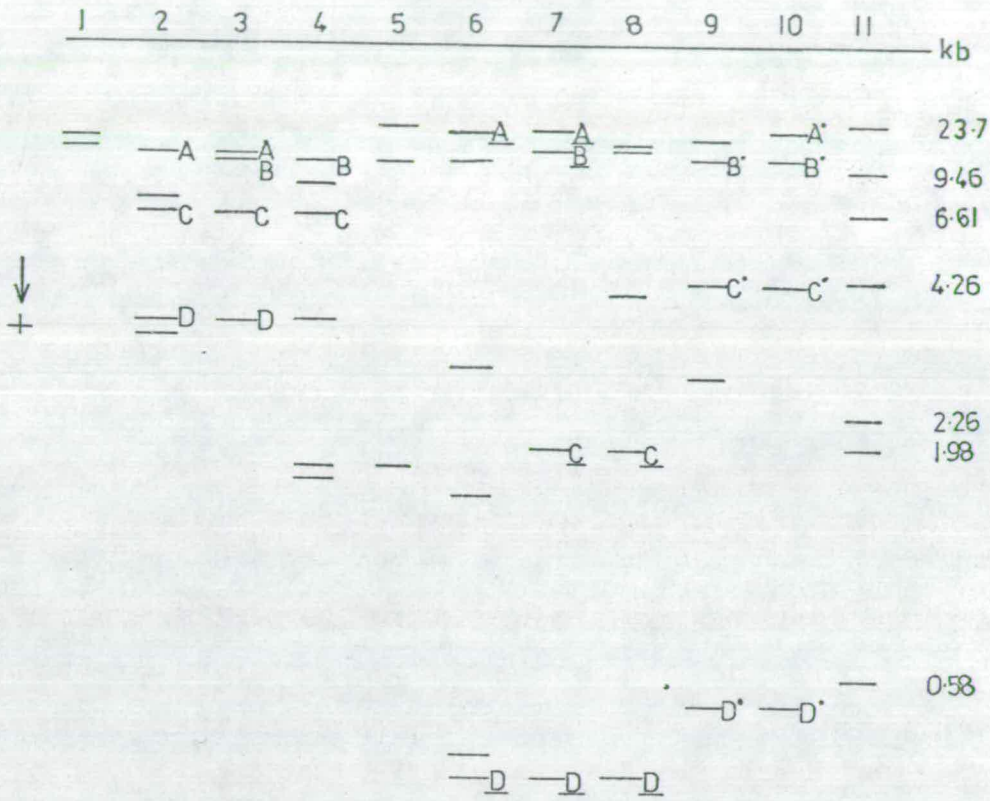

1 2 3 4 5 6 7 8 9 10 11
kb
A
B
C
D
A'
B'
C'
D'
23·7
9·46
6·61
4·26
2·26
1·98
0·58

Fig. 3.7 (opposite)

Diagrammatic Representation of a 1% Agarose Gel.

Track 1	P2 *vir*$_1$ DNA	+	R. *Rsp* I			
2		+	R. *Rsp* I	+	R. *Eco* RI	
3		+	R. *Eco* RI			
4		+	R. *Eco* RI	+	R. *Sal* I	
5		+	R. *Sal* I			
6		+	R. *Sal* I	+	R. *Bam* HI	
7		+	R. *Bam* HI			
8		+	R. *Bam* HI	+	R. *Rsp* I	
9		+	R. *Kpn* I	+	R. *Rsp* I	
10		+	R. *Kpn* I			
11	λ *cI*$_{857}$ DNA	+	R. *Hind* III			

RI bands denoted A ..., Bam bands A, Kpn bands A°.

A single R. *Rsp* I recognition site was located in the right part of the P2 genome (Fig. 3.5). The R. *Rsp* I + R. *Eco* RI double digest (track 2) showed that RI B (track 3) was cut by R. *Rsp* I to give new fragments at 7.3 kb and 3.2 kb. The R. *Rsp* I + R. *Bam* HI and R. *Bam* HI digests (tracks 7 and 8) showed that Bam A was cut by R. *Rsp* I to give new fragments at about 14 kb and 3.95 kb. From the relative positions of the R. *Eco* RI and R. *Bam* HI sites (Fig. 3.1) the R. *Rsp* I site must be 3.2 kb to the right of the central R. *Eco* RI site. This arrangement was confirmed by the appearance of a 2.7 kb fragment that was cut

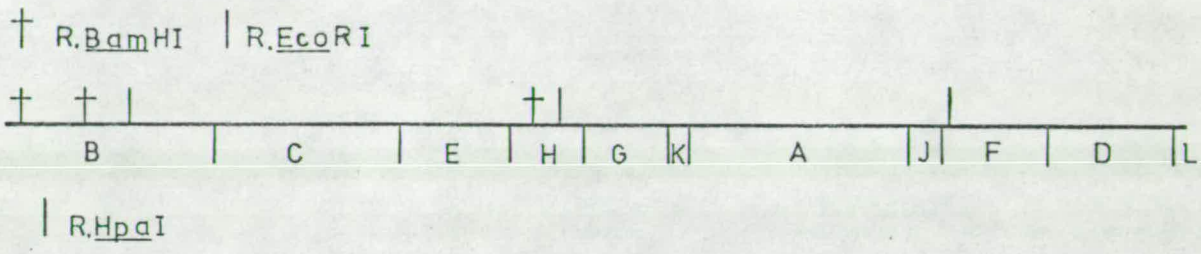
Fig. 3.7 (continued)

from Kpn A in the R. Rsp I + R. Kpn I double digest (tracks 9 and 10).

The R. Eco RI + R. Sal I digest showed that RI B and RI C remained in the double digest (tracks 3 and 4). RI A was cut to give new bands at about 9.5 kb and 3.6 kb. RI D was cut to give new bands at 1.8 kb and 1.75 kb, the 1.8 kb band was identified as an R. Sal I terminal fragment with terminally labelled DNA. The R. Bam HI + R. Sal I digest showed that Bam A remained in the double digest (tracks 6 and 7). A 2.9 kb fragment was cut from Bam B by R. Sal I and Bam C was cut to give a new band at 1.55 kb and a tiny fragment of about 0.4 kb. These digests gave the R. Sal I fragment order as C, B, A.

The R. Sal I site was measured to be 3.6 kb away from the central R. Eco RI site and 2.9 kb away from the central R. Bam HI site. This gave an independent estimate of 0.7 kb for the distance between the R. Eco RI and R. Bam HI sites which agreed with the figure of 0.76 kb obtained by direct measurement.

3.3 e Restriction Mapping: R. Hpa I



There were 10 recognition sites for the enzyme R. Hpa I in P2 vir_1 DNA. Each of the P2 deletions appeared to remove a single R.Hpa I recognition site as judged by the disappearance of one band and a change in one other band (Fig. 3.9 and also Fig. 5.2). An experiment with terminally labelled P2 vir_1 DNA showed that the del 1 deletion changed the right terminal fragment; a 0.5 kb fragment for P2 vir_1 increased to a 1.5 kb fragment when the del 1 deletion was present. The left terminal restriction fragment was a band at 5.8 kb. The assignment of cohesive end fragments was confirmed by demonstrating the specific removal of the end fragments after the addition of excess intact linear DNA (section 2.5 j) (Fig. 5.2).

The λ/P2 recombinants provided most of the additional information for completion of the restriction map. R. Hpa I digests of the vector λ596, P2 vir_1 and λ/P2 recombinants could be interpreted in a straightforward manner (Fig. 3.8). Bands in common between λ596 and λ/P2 recombinants originated from recognition sites in λ DNA, bands in λ596 not present in λ/P2 recombinants originated from the

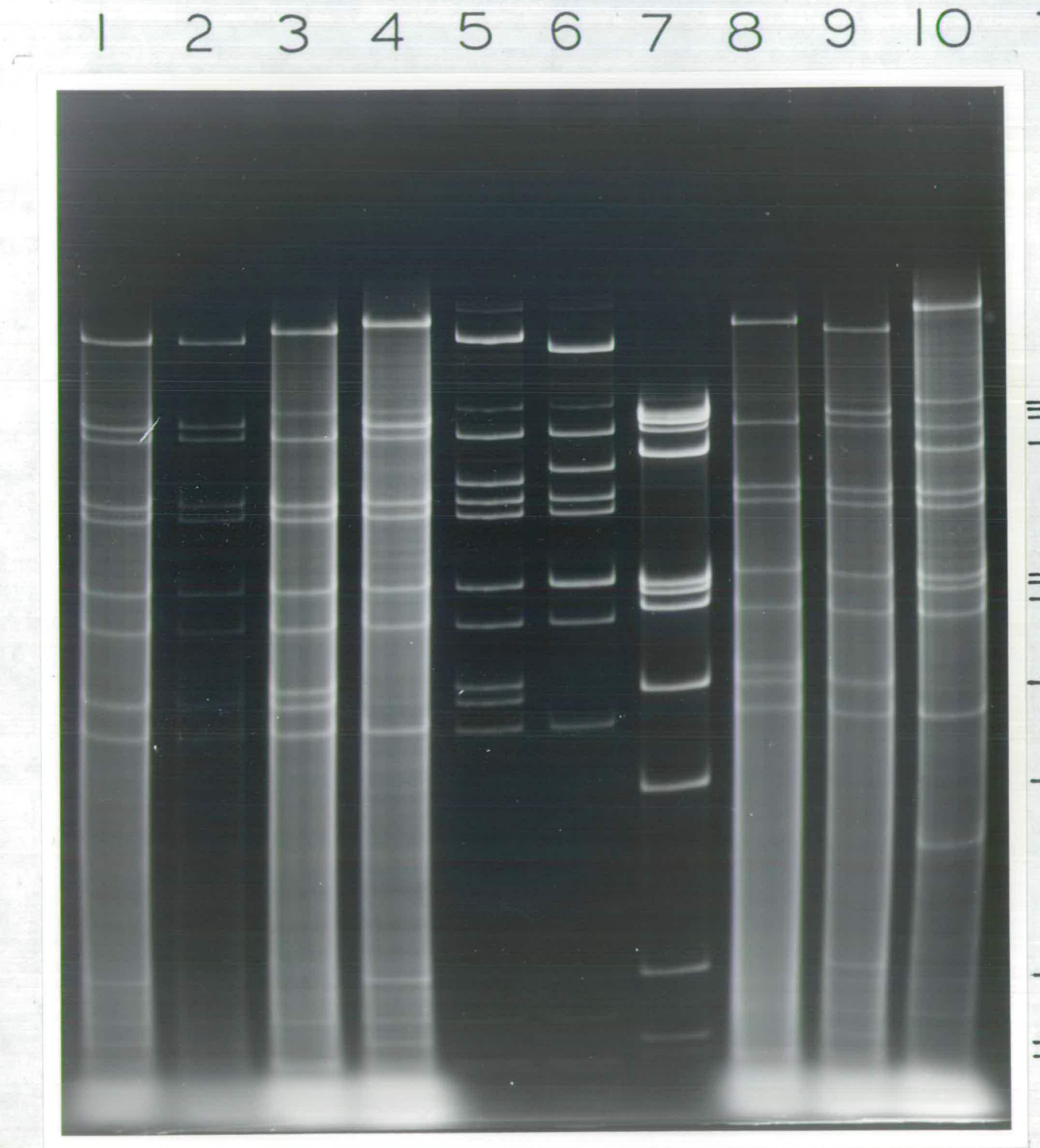

1 2 3 4 5 6 7 8 9 10 7

Fig. 3.8 (opposite)

R. Hpa I Restriction Analysis of λ/P2 Recombinants.

1% agarose gel, all samples digested with R. Hpa I.

Track 1	λ/P2 (R1)	P2 RI B
2	λ/P2 (R4)	P2 RI B (inv.)
3	λ/P2 (R13)	
4	λ/P2 (12)	P2 RI B (inv.)
5	λ781	vector
6	λ/P2 (596)	P2 RI C + D (inv.)
7	P2 vir_1	
8	λ/P2 (R11)	
9	λ/P2 (16)	P2 RI B (inv.)
10	λ/P2 (29)	P2 RI A (inv.)

P2 RI restriction fragments in the λ/P2 recombinants are listed above; the abbreviation (inv.) signifies that the restriction fragment is inverted with respect to the normal orientation in P2 DNA.

Tracks 5, 6 and 7 contained DNA that had been extracted from CsCl purified phage, all the other DNA samples were prepared from L-agarose plate lysates.

The R. Hpa I digests of λ/P2 recombinants showed that:

Fig. 3.8 (continued)

RI A contained Hpa C and Hpa E

and RI B contained Hpa A, Hpa G, Hpa J and Hpa K.

No P2 bands were cut from λ/P2 (596) - there was only 1 R. Hpa I recognition site in the P2 DNA, the site very close to the right cohesive end had been removed by the del 1 deletion.

Terminally labelled DNA had identified Hpa B as the left cohesive end and Hpa L as the right cohesive end. The del 1 deletion affected both Hpa L and Hpa D showing that these fragments were adjacent at the right cohesive end (Fig. 3.9). Bands Hpa A and Hpa J in RI B were changed by the vir_{22} deletion (Fig. 5.2) and this meant that the fragment order was A, J. The bands Hpa H and Hpa G were changed by the del 2 deletion but Hpa G appeared to be within RI B so this suggested the order H, G. Bands Hpa C and Hpa E were cut from RI A and clearly belonged to the left part of the P2 genome. The bands Hpa K and Hpa F were placed by inference. Hpa K was not affected by the deletions at the ends of RI B and so must be positioned in the central part. From size considerations, Hpa F and Hpa D probably comprised the majority of RI C.

The R. Hpa I fragment order could now be written

B (C, E), H, G, K, A, J, F, D, L.

Close comparison of the R. Hpa I digests of λ781 and λ/P2 (596) showed that there were 3 band changes in the recombinant. It had been anticipated that only 2 changes would occur after the addition

Fig. 3.8 (continued)

of P2 DNA that contained a single R. Hpa I site. The unexpected change occurred in the largest molecular weight band as this was reduced in size for λ/P2 (596). The only satisfactory explanation was that a new R. Hpa I recognition site had appeared in the λ DNA at a position close to the left end of the P2 DNA.

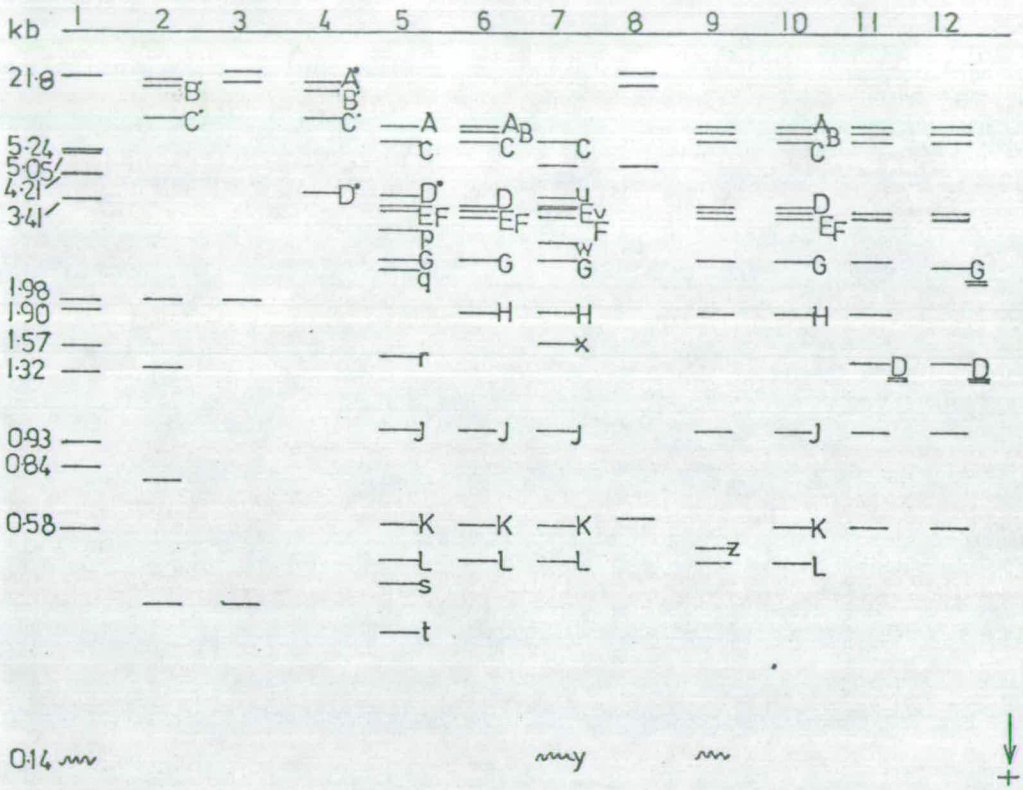

kb
1
2
3
4
5
6
7
8
9
10
11
12
21·8
5·24
5·05
4·21
3·41
1·98
1·90
1·57
1·32
0·93
0·84
0·58
0·14
A
B
C
D
E
F
G
H
J
K
L
P
Q
R
S
T
U
V
W
X
Y
Z

Fig. 3.9 (opposite)

Diagrammatic Representation of a 1.5% Agarose Gel.

Track 1	λ *cI*$_{857}$ DNA	+	R. *Eco* RI	+	R. *Hind* III
2	P2 *vir*$_1$ DNA	+	R. *Eco* RI	+	R. *Bam* HI
3		+	R. *Bam* HI		
4		+	R. *Eco* RI		
5		+	R. *Eco* RI	+	R. *Hpa* I
6		+	R. *Hpa* I		
7		+	R. *Hpa* I	+	R. *Kpn* I
8		+	R. *Kpn* I		
9		+	R. *Hpa* I	+	R. *Rsp* I
10		+	R. *Hpa* I		
11	P2 753 DNA	+	R. *Hpa* I		
12	P2 1171 DNA	+	R. *Hpa* I		

RI bands denoted A*..., Hpa bands A ...

New bands in double digests denoted p ...,

new bands in P2 deletion mutants D ... .

The R. *Eco* RI and R. *Bam* HI digests (tracks 2, 3 and 4) showed that a 0.76 kb fragment was cut from RI A by R. *Bam* HI. The band RI D was cut by R. *Bam* HI to produce Bam C, Bam D and a new band at 1.35 kb (Fig. 3.1).

The R. *Eco* RI and R. *Hpa* I digests (tracks 4, 5 and 6) showed that

Fig. 3.9 (continued)

RI D was present in the double digest, Hpa B was cut to give RI D and a new band, q, at 2.3 kb. Hpa F was cut to give new bands p and t at 2.85 kb and about 0.3 kb; this confirmed the location of Hpa F in the right part of the genome. Hpa H was cut to give new bands r and s at 1.47 kb and about 0.35 kb.

The R. Hpa I and R. Kpn I digests (tracks 6, 7 and 8) showed that Kpn C was present in the double digest, Hpa B was cut to give Kpn C and a new band x at 1.5 kb. Hpa A was cut to give new bands u and w at 3.5 kb and 2.65 kb. Hpa D was cut to give a new band v that comigrated with Hpa E and a very tiny fragment, y, at the base of the gel. The new bands in the R. Hpa I + R. Kpn I double digest were all consistent with the R. Hpa I fragment order B, (C, E), H, G, K, A, J, F, D, L.

The R. Rsp I + R. Hpa I digest showed that only band Hpa K changed from the R. Hpa I single digest (tracks 9 and 10). This located the R. Rsp I site within the Hpa K and confirmed the positioning of Hpa K in the centre of the RI B fragment.

The R. Hpa I digests of P2 vir_1, P2 753 (del 1) and P2 1171 (del 1, del 2) showed that the del 1 deletion removed Hpa L and Hpa D and produced a new band D. Digests of 5' terminally labelled DNA identified Hpa L and D as terminal fragments, the left terminal fragment was Hpa B. The del 2 deletion removed Hpa G and Hpa H and produced a new band G.

Fig. 3.9 (continued)

The ambiguity over the order of fragments Hpa C and Hpa E was resolved by digests of λ/P2 (14) and λ/P2 (30) (see page 73).

λ/P2 (14) was derived from the right part of RI A and contained Hpa E. λ/P2 (30) was derived from the left part of RI A and contained neither Hpa C or Hpa E. This meant that the fragment order was C, E.

TABLE 3.2

P2 *vir*$_1$ Restriction Fragment Lengths from Double Digests.

(1% agarose gel, Fig, 3.6 and Fig. 3.7)

R. *Rsp* I + R. *Eco* RI	R. *Rsp* I + R. *Bam* HI	R. *Rsp* I + R. *Kpn* I	R. *Eco* RI + R. *Sal* I	R. *Eco* RI + R. *Kpn* I	R. *Bam* HI + R. *Sal* I	R. *Bam* HI + R. *Kpn* I	R. *Hpa* I + R. *Eco* RI
12.2	14.2*	15*	13	12.1*	20	11.8 (x 2)**	6.15
7.35*	12.8	11	9.9*	6.0 (x 2)**	12.4*	7.0*	5.23
6.5	3.95*	4.16	6.85	4.6*	2.88*	2.14*	3.57
3.45	1.88	2.68*	3.62*	3.6	1.55*	2.0	3.35
3.23*	0.3	0.6	1.80	0.7*	0.4*	0.6	3.27
			1.75*	0.6	0.3	0.3	2.88*
							2.41
							2.28*
							1.42*
							+ others

Lengths are expressed in kb.

No accuracy is attached to values above 7 kb or below 0.5 kb.

New bands, not present in either single digest are marked by asterisks.

TABLE 3.2 (continued)

P2 vir_1 Restriction Fragment Lengths from Double Digests.

(1.5% agarose gel, Fig. 3.9)

R. Eco RI + R. Bam HI + others	R. Eco RI + R. Hpa I + others	R. Hpa I + R. Kpn I + others	R. Hpa I + R. Rsp I + others
1.95	p 2.85	w 2.64	
1.35	G 2.43	G 2.43	G 2.45
0.76	q 2.32	H 1.84	H 1.87
0.3	r 1.47	x 1.5	K 1.0
	J 1.0	J 1.0	z 0.55
	K 0.6	K 0.6	L 0.45
	L 0.45	L 0.45	~0.05
	s 0.35	y~0.1	
	t 0.30		

Lengths are expressed in kb.

the E. coli suppressor fragment in the vector. Any band in the λ/P2 samples not present in λ 596 was due to cleavage at recognition sites in P2 DNA. If these new band(s) lined up with a band(s) in the P2 vir_1 digest then there were 2 or more R. Hpa I sites in the λ/P2 RI fragment that were being cut to produce normal P2 R. Hpa I restriction fragments.

Verification of the order of R. Hpa I restriction sites was provided by a series of double digests (Fig. 3.9).

3.3 f Restriction Mapping: R. Hind III and R. Xho I

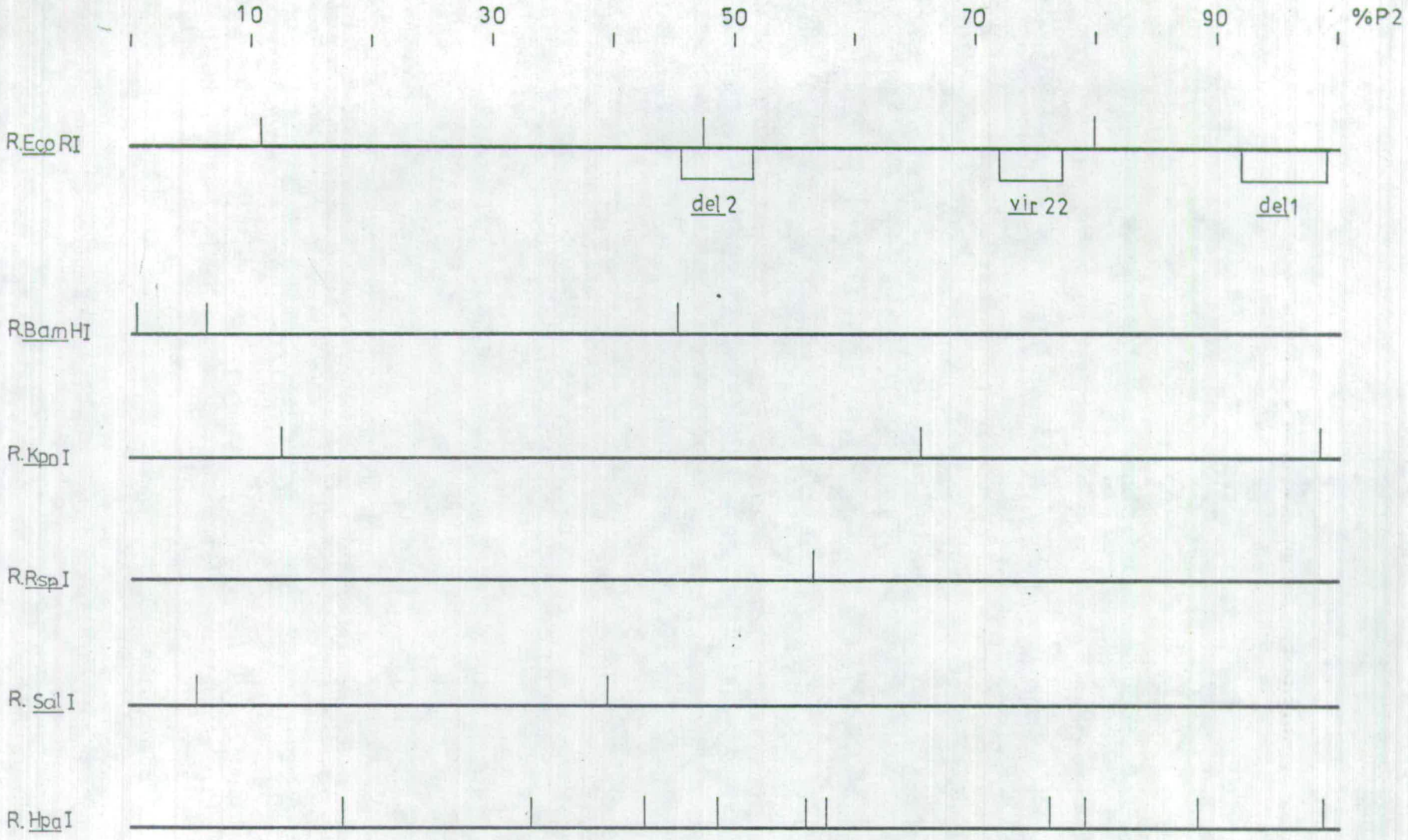
Repeated attempts to digest P2 vir_1 DNA with either R. Hind III or R. Xho I proved to be unsuccessful. The activity of enzyme preparations was always tested in parallel reactions with λ DNA. The R. Hind III digestion patterns of the 3 λ/P2 recombinants that spanned the P2 genome were virtually identical; the patterns could be explained by knowledge of the enzyme recognition sites in λ DNA and the only differences were caused by variation in the P2 DNA content of the λ/P2 recombinants. The same observations applied to R. Xho I digests of λ/P2 recombinants. This seems to be good evidence that the recognition sites for R. Hind III and R. Xho I are not present in P2 vir_1 DNA.

TABLE 3.3

Best Estimates of Restriction Fragment Lengths for P2 *vir*$_1$ DNA.

	R. *Eco* RI	R. *Bam* HI	R. *Sal* I	R. *Kpn* I	R. *Rsp* I	R. *Hpa* I	
A	12.1	18.0	20.9	17.4	19	6.1	A
B	10.5	13.1	10.5	10.3	14	5.8	B
C	6.8	1.95	1.8	4.2		5.2	C
D	3.6	0.3		0.6		3.35	D
						3.25	E
						3.1	F
						2.43	G
						1.80	H
						1.0	J
						0.6	K
						0.45	L

These figures have been derived from double digests and represent lengths in kb.



NO SITE FOR R.HindIII OR R.XhoI .

Fig. 3.10 (opposite)

Map of Restriction Enzyme Recognition Sites in P2 DNA.

The following points can be emphasised from the map. The R. Bam HI site lies outside the del 2 deletion.

At the right end of the map, the R. Kpn I and R. Hpa I sites are removed by the del 1 deletion. The R. Hpa I site is closer to the right cohesive end of the DNA than the R. Kpn I site.

The R. Rsp I site is located between the 2 closely spaced R. Hpa I sites.

The lengths of restriction fragments are given in Tables 3.2 and 3.3.

3.4 Conclusions

The final restriction map (Fig. 3.10) shows that recognition sites have been located throughout the P2 genome. The information presented here gives an unambiguous order for the various restriction sites. The best estimates for the size of restriction fragments have been calculated from double digests (Tables 3.1 - 3). The general distribution of mapped restriction sites allows specific fragmentation of the P2 genome and small segments could be incorporated into phage λ or relaxed plasmids for fine structure genetic analysis.

The positions of P2 restriction sites identified here are in good agreement with results from an independent investigation (Westöö, A., Ljungquist, E. and Bertani, G., unpublished results). These workers confirmed the location of sites for R. Eco RI, R. Bam HI, R. Sal I, R. Hpa I and R. Kpn I, the absence of a site for R. Hind III and they also mapped cleavage sites for the enzymes R. Bgl II, R. Sma I, R. Bal I and R. Ava I.

CHAPTER 4 Genetic Information Derived from λ/P2 Hybrid Bacteriophage

4.1 Introduction

The λ/P2 recombinants isolated from *in vitro* restriction and ligation experiments (Chapter 3) were studied with respect to their P2 DNA content. The formation of λ/P2 recombinants represented a unique purification of the different P2 R. *Eco* RI fragments and allowed each fragment to be examined in isolation. The information included here represents a preliminary investigation of some P2 properties and hopefully this will be extended by workers possessing greater familiarity with P2 genetics.

4.2 Marker Rescue Experiments

The λ/P2 recombinants containing RI B or RI C + D were initially characterised by marker rescue experiments involving λ/P2 lysogens and superinfecting P2 phage mutants. A double lysogen formed by λ/P2 (596) and λ imm^{434} helper allowed marker rescue for P2 Q am$_{34}$ and P2 P am$_{137}$. The helper phage was necessary for lysogen formation because the λ 596 vector has been deleted for the phage attachment site. An alternative method for marker rescue has already been mentioned (Chapter 3, p 72) and this was more convenient as it allowed rapid screening of recombinants without requiring the formation of large numbers of λ/P2 lysogens.

The λ/P2 recombinants that have been extensively studied are represented in Figure 4.1.

The marker rescue experiment confirmed the restriction mapping information by indicating which genes were contained within the restriction fragments but there was no indication about P2 gene expression in λ/P2 recombinants. Restriction mapping of λ/P2 (596) had shown that P2 genes P and Q were correctly positioned for sense strand transcription from P_L. A cro$^-$ version of λ/P2 (596) was constructed by phage crosses (section 2.5 d) to create the potential for extended λ P_L transcription.

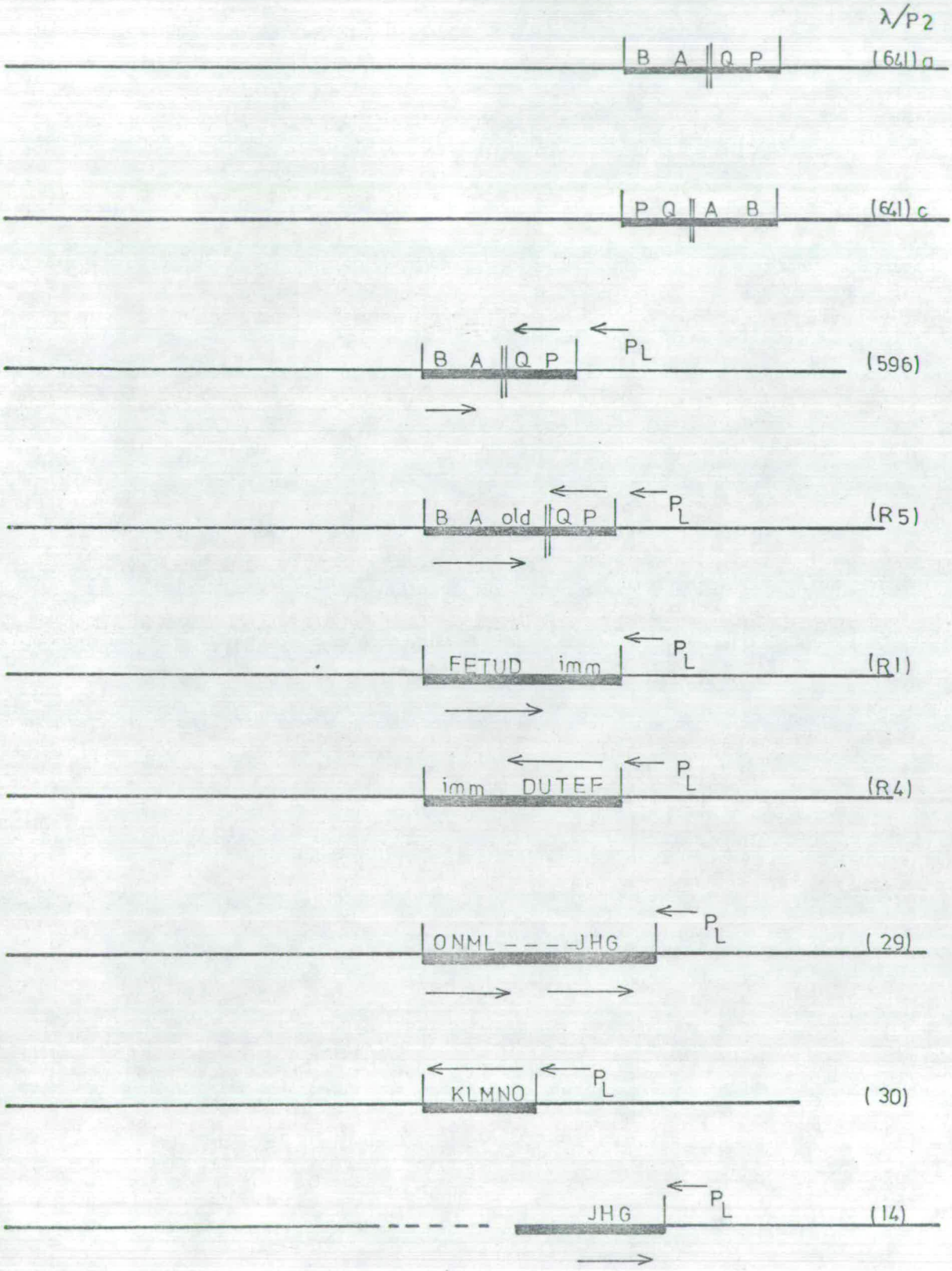

λ/P2
B A Q P
(641)a
P Q A B
(641)c
B A Q P
PL
(596)
B A old Q P
PL
(R5)
FETUD imm
PL
(R1)
imm DUTEF
PL
(R4)
ONML - - - JHG
PL
(29)
KLMNO
PL
(30)
JHG
PL
(14)
R.Eco RI
cos SITE

Figure 4.1 (opposite)

λ/P2 Recombinant Bacteriophage.

The diagram shows the P2 DNA content of the recombinants that have been studied in some detail. The known directions of P2 transcription are shown (indicated by arrows) and there is potential for increased synthesis of P2 proteins from recombinants that have aligned P2 and λ P_L transcription.

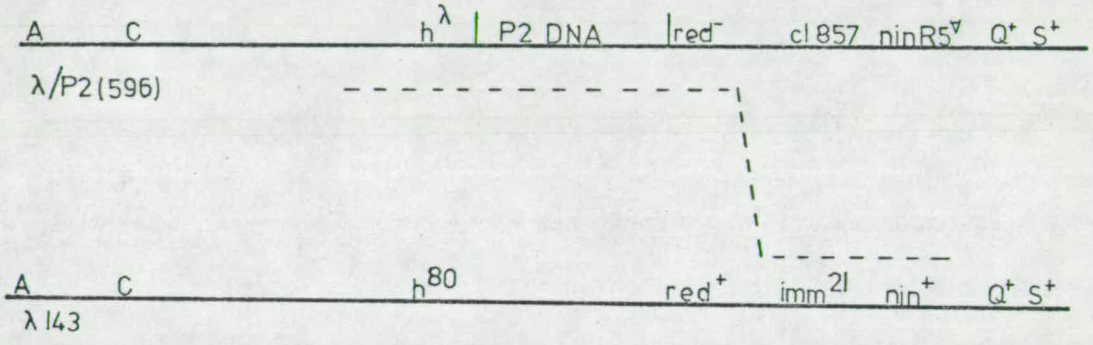
λ/P2 (641) and λ/P2 (596) are described in detail in Chapter 3.
λ/P2 (R5) contains RI C + D from P2 Q $\underline{am}_{34}$ $\underline{vir}_1$. In contrast to λ/P2 (596), λ/P2 (R5) is B^+, A^+, $\underline{old}^+$.
λ/P2 (30) is an *in vivo* deletion phage containing sequences from P2 RI A. The 6 kb P2 DNA fragment gives marker rescue for P2 M am, L am, and K am. P2 genes O and N are assumed to be present but this has not been confirmed by marker rescue. There is a single R. Hpa I site in the 6 kb fragment positioned 2.3 kb from an R. Eco RI site (see Fig. 3.10).
λ/P2 (14) is an *in vivo* deletion phage containing sequences from P2 RI A. The P2 DNA content has not been determined but there is an estimate of ⩾ 4.8 kb. There are 2 R. Hpa I recognition sites in the P2 DNA that are cut to produce Hpa E.

Recombinant λ/P2	λ vector	P2 RI Fragment	Number Isolated	Comments
(641) a	641	C + D	2	B am, A am, del 1
(641) c	641	C + D	1	Bam, Aam, del 1
(596)	596	C + D	1	Bam, Aam, del 1
(R5)	781	C + D	2	Q am
(R1)	596	B	2	$\underline{vir}_1$
(R4)	781	B	2	$\underline{vir}_1$
	596	B	10	$\underline{vir}_1$ orientation not determined
(29)	596	A	3	
(30)	596	(A)	1	Deletion removing P2 DNA
(14)	596	(A)	1	Deletion removing P2 and λ DNA.

4.3 Phage Crosses

The cro gene lies within the immunity region and it is almost impossible to separate immunity and cro by genetic recombination. Both λ/P2 (596) and the cro⁻ donor phage carried imm$^{\lambda}$ so the phage cross had to proceed through 2 stages.

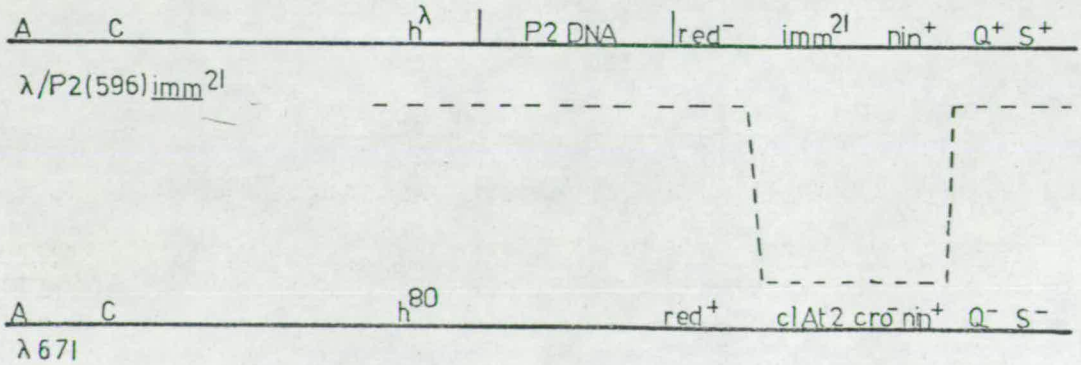
λ/P2 (596) x λ143 λ 143 h^{80} imm^{21}



Recombinants were selected directly on C 600 (λ) and then tested for the red⁻ character by failure to grow on a lig ts strain. Plaques were picked into phage buffer and $CHCl_3$, purified through single plaque cycles and retested. One of these purified plaques was maintained as the stock of λ/P2 (596) imm^{21}.

λ/P2 (596) imm^{21} x λ 671

λ671 h^{80}, red⁺, c I At_2, cro 1, Q am_{73}, S am_7



The cross was plated on a totally permissive host at 37°C and tiny plaques were screened for growth on Y mel ton A and C 600 (imm^{21}). Recombinants should grow on both hosts and were isolated at very low frequency. All the recombinants were of the form cro$^-$ Q$^+$ S$^+$ and one of these phage was plaque purified and maintained as the stock of λ/P2 (596) cro$^-$. This cro$^-$ phage would still permit marker rescue for P2 Q am$_{34}$ and P2 P am$_{137}$ verifying that the P2 DNA had been retained through the 2 phage crosses.

4.4 *in vivo* Complementation Experiments

An experiment, termed *in vivo* complementation, was devised to look for complementation between P2 mutants and λ/P2 recombinants. A *sup*^{o} *E. coli* strain (W 3350 *r* k^{-} *m* k^{+}) was doubly infected with λ/P2 (596) and P2 *Q* am_{34} - both phage were added at a multiplicity of 2 - 3 - and shaken at 37°C until lysis occurred. The phage solution was treated with $CHCl_3$, centrifuged to remove bacterial debris and then titered on *sup*$^{+}$ and *sup*^{o}, λ-resistant strains (C-1757 λ R and C-1a *ton* A λ R respectively). A parallel infection with the P2 mutant alone provided an indication of the 'background' from unadsorbed P2 phage that were still present.

Complementation was detected consistently between λ/P2 (596) and P2 *Q* am_{34} although there was some fluctuation between experiments (Table 4.1). The λ/P2 (596) *cro*$^{-}$ phage produced a significant increase in the complementation for P2 *Q* am_{34} (Table 4.1) confirming an involvement for λ P_L in the transcription of P2 DNA. Rather surprisingly, there did not seem to be any complementation with P2 *P* am_{137} (Table 4.1). This implied that P2 gene P was not intact in the λ/P2 (596) recombinant although the marker rescue experiment had shown that at least part of gene P was present.

An assumption made in the interpretation of the *in vivo* complementation - results was that complementation would be roughly comparable for Q and P proteins and the marginal increase (1-2 fold) for λ/P2 + P2 *P* am over P2 *P* am alone was not considered significant. Bowden

TABLE 4.1

in vivo Complementation Experiments.

	C-1757 λ R	Stimulation	C-la *ton* A λ R
λ/P2 (596) + P2 *Qam*	5.7×10^7 pfu/ml	3.4	
P2 *Qam*	1.5×10^7		
λ/P2 (596) + P2 *Qam*	2.9×10^8	5.6	
P2 *Qam*	5.2×10^7		
λ/P2 (596) + P2 *Qam*	8.4×10^8	21	6.1×10^4 pfu/ml
P2 *Qam*	4.0×10^7		1.9×10^3
λ/P2 (596) + P2 *Qam*	4.2×10^8	12.4	2.9×10^4
P2 *Qam*	3.4×10^7		2.2×10^4
λ/P2 (596) + P2 *Qam*	1.1×10^7	3.7	5.0×10^3
P2 *Qam*	3.0×10^6		2.0×10^3
λ/P2 (596) + P2 *Qam*	8.6×10^7	12.8	3.7×10^4
P2 *Qam*	6.8×10^6		2.8×10^4
λ c + P2 *Qam*	7.1×10^6	1	3.1×10^4

TABLE 4.1 (continued)

	C-1757 λ R	Stimulation	C-la *ton* A λ R
λ/P2 (596) + P2 *Q*am	1.9×10^8	11.9	
P2 *Q*am	1.6×10^7		
λ/P2 (596) + P2 *P*am	2.8×10^7	2.0	
P2 *P*am	1.4×10^7		
λ/P2 (596) + P2 *Q*am	5.0×10^7	6.5	
P2 *Q*am	7.7×10^6		
λ/P2 (596) *cro*$^-$ + P2 *Q*am	2.2×10^8	29.3	
λ/P2 (596) *cro*$^-$ + P2 *Q*am	7.1×10^8	124.6	
P2 *Q*am	5.7×10^6		
λ/P2 (596) *cro*$^-$ + P2 *P*am	4.3×10^7	1.6	
P2 *P*am	2.6×10^7		

C-1757 λ R, *sup*$^+$ strain, C-la *ton* A λ R, *sup*^{o} strain.

'Stimulation' is the titre for λ/P2 + P2 am divided by the titre for P2 am alone.

The figures have been grouped to show experiments from different days.

and Calendar (1978) have demonstrated an activity for P2 P protein *in vitro* and complementation has been demonstrated between P2 *P* am phage and a co-infecting P2 mutant defective in a different gene (Lindahl, 1974). These findings supported the validity of a complementation analysis for P2 P protein. One possibility that could not be discounted was that the intracellular concentration of P2 P protein in the complementation reaction was too low to support normal P2 growth - P is one of the proteins readily detected during P2 phage infection (Lengyel *et al.*, 1973).

The λ/P2 recombinants containing P2 RI A were also used for the *in vivo* complementation experiment in conjunction with P2 *M* am_{32}. The λ/P2 recombinants used were λ/P2 (29) containing the complete RI A fragment in the wrong orientation for λ P_L transcription and λ/P2 (30), the *in vivo* deletion phage containing DNA sequences from P2 RI A (Fig. 4.2). The results from 3 experiments were ambiguous, suggesting that complementation from the recombinants was either very low or non-existent. At this stage, the orientation of P2 DNA in λ/P2 (30) was unknown (the R. *Kpn* I restriction map for P2 had not been derived) and it was tentatively concluded that λ/P2 (29) and λ/P2 (30) contained P2 DNA in the same orientation. However, restriction digests with R. *Kpn* I established that λ/P2 (29) and λ/P2 (30) possessed different orientations of P2 DNA and that the P2 genes in λ/P2 (30) were correctly aligned for sense strand transcription from P_L. Further-marker rescue experiments with P2 *L* am_{302} and P2 *K* am_{12} showed that both of these mutations were covered by the P2 DNA in λ/P2 (30) and this suggested that P2 gene M would be intact in the recombinant.

1 2 3 4 5 6

→

↓
+

Figure 4.2 (opposite)

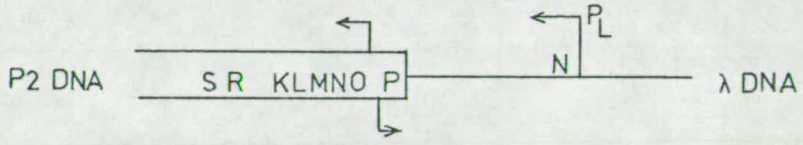
Restriction Analysis of λ/P2 (29) and λ/P2 (30).

1% agarose gel.

Track 1	λ/P2 (29)	+	R. Bam HI			
2	λ/P2 (30)	+	R. Bam HI			
3	λ/P2 (29)	+	R. Bam HI	+	R. Eco RI	
4	λ/P2 (30)	+	R. Bam HI	+	R. Eco RI	
5	λ/P2 (29)	+	R. Eco RI			
6	λ/P2 (30)	+	R. Eco RI			

In the R. Eco RI digests (tracks 5 and 6) the fastest moving bands are the P2 DNA fragments and this shows the difference in P2 DNA content for the 2 recombinants. The faint, high molecular weight bands are caused by λ cohesive end reannealing. The R. Bam HI digests (tracks 1 and 2) show that λ/P2 (29) has an additional R. Bam HI recognition site - this is the site at the left end of the P2 RI A fragment. In the double digests (tracks 3 and 4) a faint band from λ/P2 (29) can be detected near the bottom of the gel. This is the 0.7 kb fragment that is located between the R. Eco RI and R. Bam HI recognition sites in P2 RI A.

The P2 genes O, N, M, L form a single unit of transcription with a promoter located near gene O: the P2 genes P and Q are transcribed from the opposite DNA strand in the opposite direction, presumably from a promoter near gene P (Lindahl, 1971; Lindqvist and Bovre, 1972; Geisselsoder et al., 1973). If the R. Eco RI site has been correctly positioned within P2 gene P then the recombinant λ/P2 (30) would have the following arrangement of promoters



Transcription from P_L may be disrupted by transcription initiating at the P2 promoter for genes P and Q but it was anticipated that λ P_L transcription would predominate (Ward, 1978). It was possible that the short burst of P_L transcription from the λ/P2 (30) cro$^+$ phage produced insufficient P2 M protein for efficient complementation; M protein is found in large amounts in P2 infected cells (Lengyel et al., 1973). Initiation of transcription at the P2 promoter near gene O may be blocked because of the absence of P2 A protein; the P2 A protein is essential for the expression of all other P2 genes (Lindahl 1970; Lengyel and Calendar, 1974).

The analysis of λ/P2 (29) and λ/P2 (30) was curtailed in the light of a manuscript (Bowden and Calendar, 1978), sent by Dr. Richard Calendar, which described the purification of M protein from P2 infected cells.

4.5 Analysis of Protein Synthesis with λ/P2 Recombinant Phage

P2 Q protein was not identified in the original analysis of P2 structural proteins (Lengyel *et al.*, 1973) and this was explained by proposing that Q was a minor protein involved with head assembly. The results from *in vivo* complementation experiments suggested that there was significant synthesis of P2 Q protein with the λ/P2 (596) *cro*⁻ recombinant and this phage provided the basis of an attempt to identify P2 Q protein. The experimental procedures for ^{35}S labelling of proteins and SDS-polyacrylamide gel electrophoresis have already been described (sections 2.5 q and 2.5 r).

The simple predictions were that P2 Q protein would not be present in wild-type λ controls, there would be a (weak) Q protein band from λ/P2 (596) and there would be a strong Q band from λ/P2 (596) *cro*⁻. A third recombinant λ/P2 (R5) would only show the (weak) Q protein band in the presence of a suppressor. λ/P2 (R5) contains RI fragments C + D from P2 *Q* am_{34} *vir* $_1$ with P2 genes P and Q adjacent to λ P_L (Fig. 4.1). P2 *Q* am_{34} vir_1 does not contain the *del* 1 deletion but it was considered extremely unlikely that this difference would affect transcription of P2 genes P and Q from λ P_L in the λ/P2 recombinants.

Labelled proteins synthesised after λ/P2 phage infections are shown in Figure 4.3. A polypeptide of molecular weight about 36,000 appears to meet all the criteria for P2 Q protein. This molecular weight would mean that Q protein would comigrate with

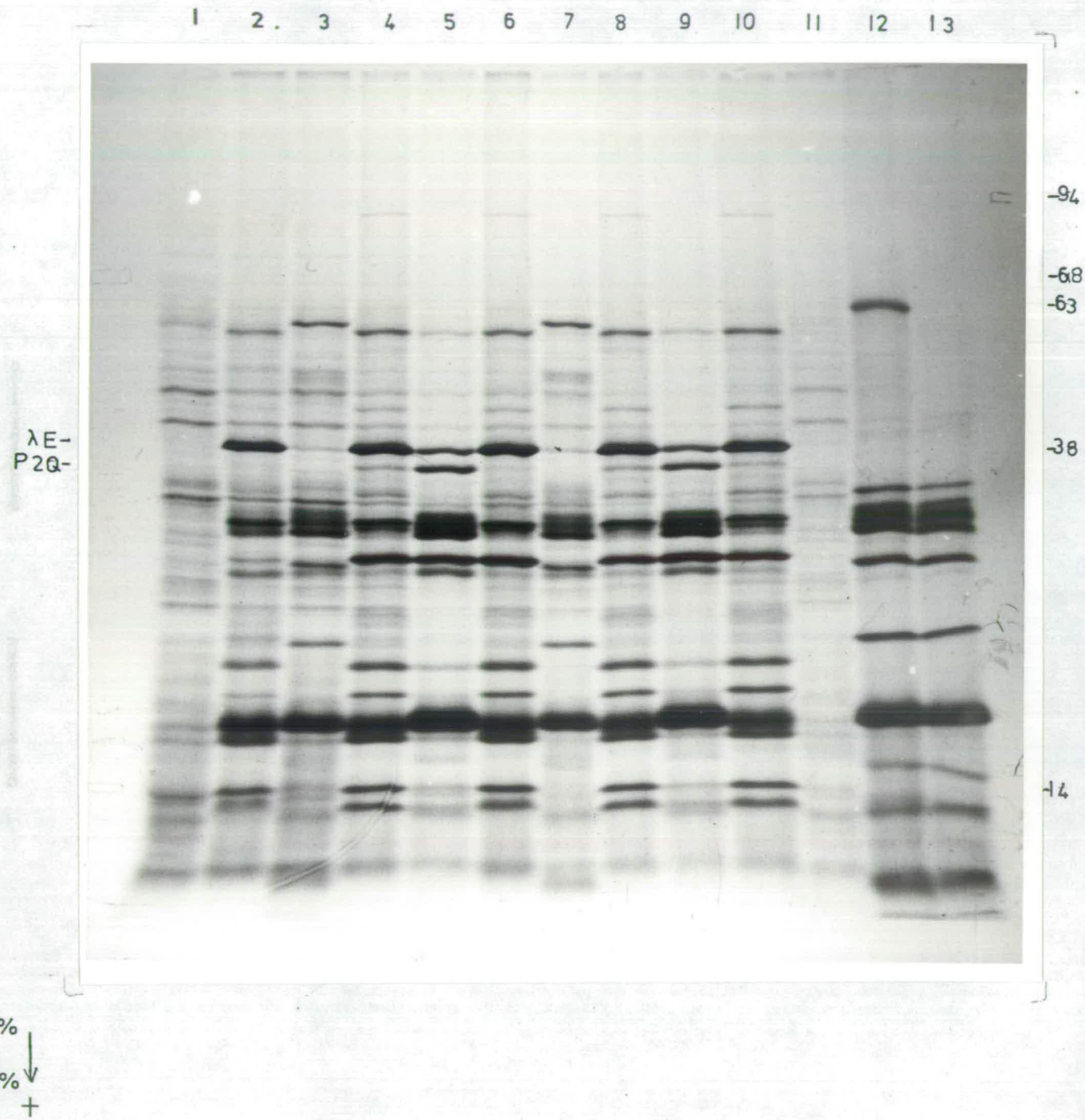

1 2. 3 4 5 6 7 8 9 10 11 12 13
λE-
P2Q-
-94
-68
-63
-38
-14
10%
20%
+

Figure 4.3 (opposite)

Analysis of Labelled Proteins from λ/P2 Phage Infected Cells. 14-20% SDS polyacrylamide gradient gel, approximate molecular weights (x 10^{-3}) are indicated at the right of the photograph.

Track	Bacterial Host	Infecting Phage
1	S 159	Uninfected control
2		λ dup 74
3		λ c I_{857} S am_7
4		λ/P2 (596)
5		λ/P2 (596) cro⁻
6		λ/P2 (R 5)
7	S 159 (λ imm^{434}) sup F	λ c I_{857} S am_7
8		λ/P2 (596)
9		λ/P2 (596) cro⁻
10		λ/P2 (R 5)
11		Uninfected control
12		λ 967
13		λ 968

The band provisionally identified as P2 Q protein is missing from the P2 Q am phage, λ/P2 (R5), (track 6) but is observed when this phage is grown with a suppressor (track 10).

There are significant differences in the levels of λ structural protein synthesis (compare λ pE in tracks 2-5), this point is considered in Chapter 6.

The phage λ dup 74 is described in Chapter 6.

The phage λ 967 and λ 968 are λ/T4 'hybrid immunity' phage (Murray, N.E., 1977). After infection of the S 159 (λ imm^{434}) sup F host, the phage are repressed for all λ rightward transcription. The protein of molecular weight 63,000 is T4 DNA ligase (N. E. Murray, personal communication).

The autoradiograph was exposed for 12 hours.

P2pN*, the major capsid protein and this would explain the previous failure to detect P2 Q on one dimensional gels. Unfortunately, the shortened polypeptide resulting from the P2 Q am mutation cannot be detected but, with a suppressor, the P2 Q am gene shows a new band that corresponds exactly with the Q band for λ/P2 (596) and λ/P2 (596) cro⁻. The λ/P2 (R5) cro⁻ phage is being constructed in an attempt to identify the P2 Q am polypeptide.

Absolute verification of the correct assignment for Q protein may not be straightforward. The role of Q protein in P2 head assembly is unknown; P2 Q am extracts are defective for all in vitro packaging and there is a preliminary indication that Q protein has no part in P2 ter cleavage (Bowden and Calendar 1978). If Q protein is incorporated into P2 phage heads, possibly to determine the point for tail attachment, then anti-sera raised against purified P2 phage particles could be used for immunoprecipitation with the labelled proteins from λ/P2 infections (Epp and Pearson, 1976; Simmons and Martin, 1978). Results from an in vivo complementation reaction performed in the presence of ^{35}S methionine were obscured by a high background of bacterial protein synthesis. The intention had been to recover P2 phage from the complementation reaction and determine whether or not the phage particles contained labelled protein. This approach is worthy of further consideration but anomalous results would be obtained if Q protein is present in P2 phage heads in a processed form.

The P2 in vitro packaging system may not be appropriate as an assay

for P2 Q protein. It is possible that extracts of P2 Q am infected cells could be complemented *in vitro* by Q protein from λ/P2 infected cells. Fractionation of the λ/P2 protein extract could eventually identify P2 Q protein. However, if Q protein is required at an early stage in P2 head assembly then head structures formed in the absence of Q may not be competent for packaging when Q protein is provided at a later time. Experiments are required to distinguish between these possibilities.

4.6 Lysogen Formation with λ/P2 Recombinant Phage

λ/P2 recombinants containing P2 RI B were initially identified by the absence of R. Bam HI recognition site in the P2 DNA i.e. λ vector and λ /P2 RI B recombinants gave very similar R. Bam HI restriction patterns (see Fig. 3.4). Restriction digests with R. Hpa I confirmed the presence of P2 RI B (see Fig. 3.8), and R. Kpn I digests revealed the orientation of the RI B fragment in λ/P2 recombinants.

The phage attachment site has been deleted in the vector, λ 596, and this results in an effective block to lysogen formation. However, λ 596 derivatives containing P2 RI B were able to lysogenise at high frequency. The fragment RI B includes the P2 attachment site and so the lysogens have probably formed via integration at this P2 site. Transduction experiments are required to determine the site of prophage integration and this information could confirm that lysogeny had occurred via the P2 attachment site at one of the normal P2 chromosomal sites.

The recombinants containing RI B were generated from P2 DNA carrying the vir_1 mutation. As a consequence, lysogens formed by these phage do not confer P2 immunity but it is anticipated that a λ/P2 recombinant containing RI B from wild type P2 DNA would confer both P2 and λ immunity. The lysogens were isolated at 32°C because of the λ temperature sensitive repressor mutation c I_{857} that was present

in the λ/P2 recombinants. At 32°C the lysogens were stable and expressed λ immunity. At 42°C the lysogens grew poorly and colonies appearing at this temperature were no longer λ immune when retested at 32°C. The mechanism of prophage excision in these high temperature survivors has not been investigated.

4.7 General Considerations of λ/P2 Hybrid Bacteriophage

Although only a small number of λ/P2 hybrid phage have been examined (Fig. 4.1) it is perhaps significant that one class of recombinant has not been isolated. The missing recombinant would contain P2 RI C + D with P2 gene A positioned for sense strand transcription from λP_L. The P2 A^+ DNA used for *in vitro* recombination reactions was *old*$^+$ and it is likely that synthesis of the *old* gene product would prevent the isolation of a viable λ/P2 recombinant. Further *in vitro* recombination reactions with P2 A^+ *del* 1 DNA would clarify this point.

It was thought that a recombinant carrying P2 RI A might be inviable if P2 K protein, the lysis protein, was produced at early times from λP_L. These doubts were diminished by the characterisation of λ/P2 (30) which contained at least part of gene K as judged by marker rescue with P2 *K* am_{12}. There is probably sufficient P2 DNA in λ/P2 (30) to include the whole of gene K and this can easily be tested by additional marker rescue experiments. If the K gene is intact in λ/P2 (30) then there is no obvious barrier to the formation of λ/P2 recombinants containing P2 RI A in either possible orientation.

There is no explanation for the deletions observed in λ/P2 (14) and λ/P2 (30). Normally DNA sequences are preserved during *in vitro* recombination experiments (see for example, Taniguchi *et al.*,

1978). Heteroduplexes between the λ/P2 deletion phage and P2 DNA would provide further information about the P2 DNA content of the recombinants. These deletion phage may be useful in contributing to the detailed alignment of P2 genetic and physical maps.

CHAPTER 5 P2 ter Enzyme and Cohesive End Sequences

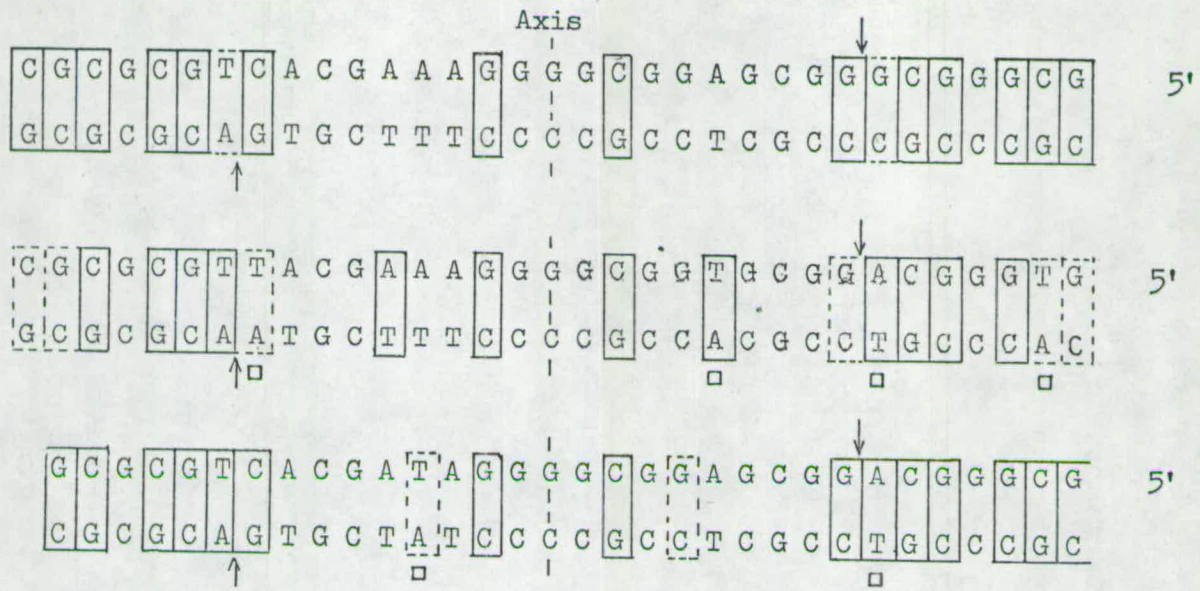
5.1 Introduction

The cohesive end sequences for the related phage P2, 186 and 299 contain minor variations (Fig. 5.1) (Murray and Murray, 1973: Murray, K., et al., 1977). An analysis of 13 independently isolated phage, all serologically related to P2, but exhibiting 7 different classes of immunity, revealed no deviation from the P2-type cohesive end sequence (Murray, K. and Bertani, G., unpublished results). This conservation of nucleotide sequence has confirmed the importance of cohesive end sequences during DNA maturation and phage particle assembly.

The sequence differences between phage P2 and 186 are confined to 2 positions out of the 19 that compose the single strand projections. The sequences are very G. C. rich and a strong cross reaction can be demonstrated between P2 and 186 cohesive ends (Wang et al., 1973; see also Fig. 5.2). Base pairing between P2 and 186 cohesive ends has been forced by selection for P2/186 hybrid phage formed after illegitimate recombination in vivo (Bradley et al., 1975: Younghusband et al., 1975). These hybrids (designated Hy 2 and Hy 5) contain P2 structural genes but have the immunity region and DNA replication genes from phage 186. The hybrids would have arisen by a single crossing over event between 2 phage DNA molecules and would initially have contained the P2 left cohesive end and the 186 right cohesive end. It was of interest to determine how the

Fig. 5.1

P2-type Phage Cohesive End Sequences (Murray, K. et al., 1977)



Nucleotides enclosed in boxes are placed symmetrically with respect to the central axis of rotation, dashed lines indicate nucleotides that are symmetrical in purine or pyrimidine arrangements. The arrows show the locations of breaks introduced by ter enzymes and changes from the P2 sequence are marked (▫).

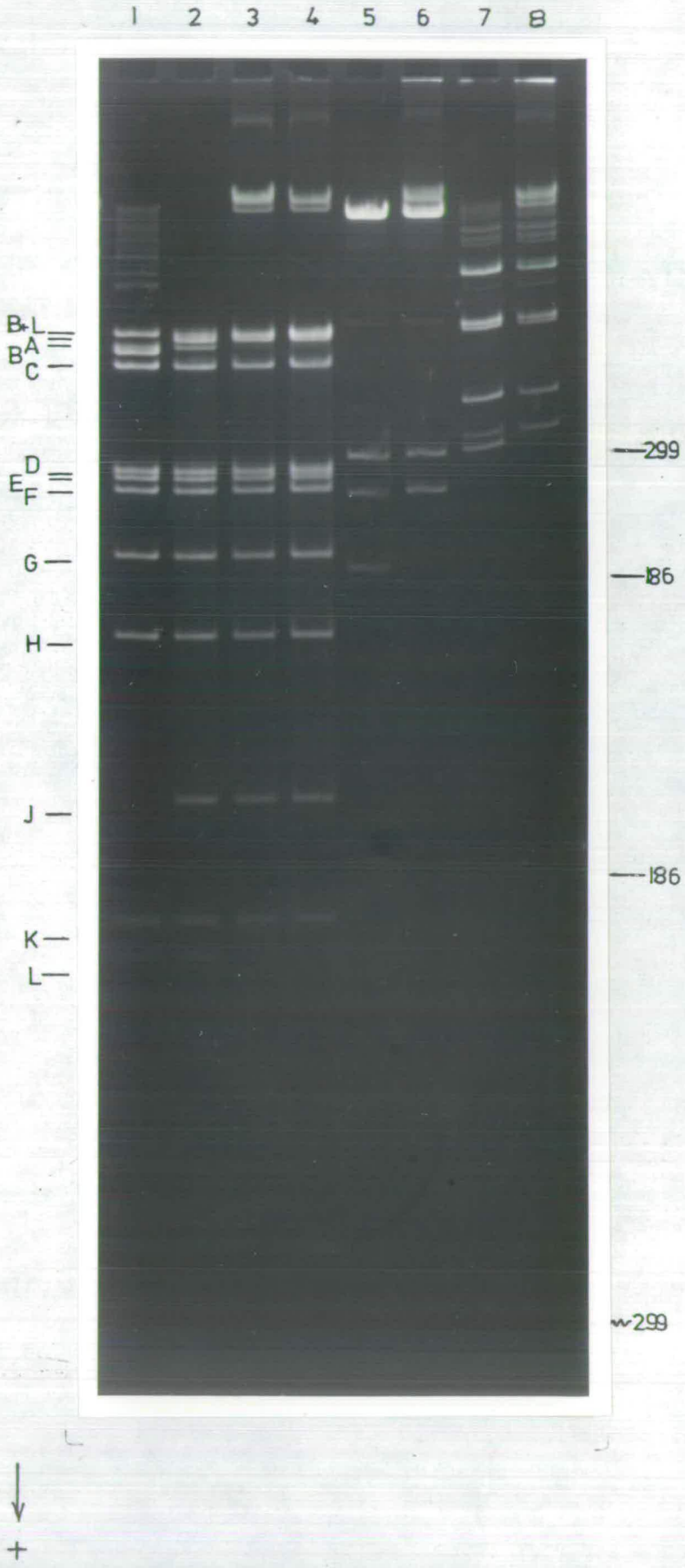

1 2 3 4 5 6 7 8
B+L
BA
BC
D
EF
G
H
J
K
L
299
186
186
299
+

Fig. 5.2 (opposite)

End to End Aggregates of P2, 186 and 299 Phage DNAs.

Intact linear phage DNA was added to some of the samples at the end of the restriction digests to 'remove' terminal restriction fragments (section 2.5 j).

Track 1	P2 $\underline{vir}_{22}$ DNA	+	R. Hpa I		
2	P2 $\underline{vir}_{1}$ DNA	+	R. Hpa I		
3	P2 $\underline{vir}_{1}$ DNA	+	R. Hpa I	+	186p DNA
4	P2 $\underline{vir}_{1}$ DNA	+	R. Hpa I	+	299 DNA
5	186 p DNA	+	R. Eco RI		
6	186 p DNA	+	R. Eco RI	+	299 DNA
7	299 DNA	+	R. Eco RI		
8	299 DNA	+	R. Eco RI	+	186 p DNA

1% agarose gel. The P2 $\underline{vir}_{1}$ R. Hpa I restriction fragments are identified by letters at the left of the photograph. The terminal R.EcoRI restriction fragments for 186 and 299 phage DNAs are indicated at the right of the photograph (Murray, K. et al., 1977).

Tracks 1 and 2 show that the bands Hpa A and Hpa J are changed by the $\underline{vir}_{22}$ deletion.

The P2 R. Hpa I terminal restriction fragments Hpa B and Hpa L interact specifically with both 186 and 299 DNA (tracks 2, 3 and 4).

The terminal restriction fragments from 186 and 299 DNA are specifically removed by the addition of intact DNA from the other phage (tracks 4 - 8).

These cross reactions between terminal restriction fragments and intact phage DNA provide a simple demonstration of the similarity in cohesive end sequence for the P2-type phage.

mispaired bases present in the hybrid cohesive sites had been repaired. The sequences had to be recognised by P2 ter enzyme to ensure viability of the hybrid phage.

5.2 Nucleotide Sequence Information

DNA was extracted from concentrated Hy 2 and Hy 5 phage kindly provided by Dr. J. B. Egan. Samples of the hybrid phage DNAs were digested with restriction enzymes to check that the band patterns were composites of the known band patterns for P2 and 186 DNAs. The hybrid DNAs were labelled at 5' termini by incubation with γ - ^{32}P - ATP and polynucleotide kinase (section 2.5 o). Labelled DNA was digested to oligonucleotides in reactions with pancreatic DNase and was further digested to mononucleotides by the addition of snake venom phosphodiesterase. The products of these reactions were analysed by ionophoresis.

The interpretation of DNA sequence information was critically dependent on the fidelity of the labelling reaction. The Hy 2 and Hy 5 double digests with pancreatic DNase and venom showed counts only in 5' pG. This result had 2 consequences a) the 5' terminal labelling had been very specific, in these reactions counts incorporated into 5' pC or 5' pT represented a measure of non-specific labelling, b) the mispairing that resulted from the 5'pA of 186 DNA aligning with a C in the P2 cohesive end had, in both cases, been repaired to give the G. C. base pair found in P2.

The distribution of oligonucleotides on DE 81 paper at pH 2.0 suggested that there was a difference between Hy 2 and Hy 5 (Fig. 5.3). These oligonucleotides were separated in a second dimension on AE paper at pH 3.5 (Fig. 5.4) and this confirmed and identified

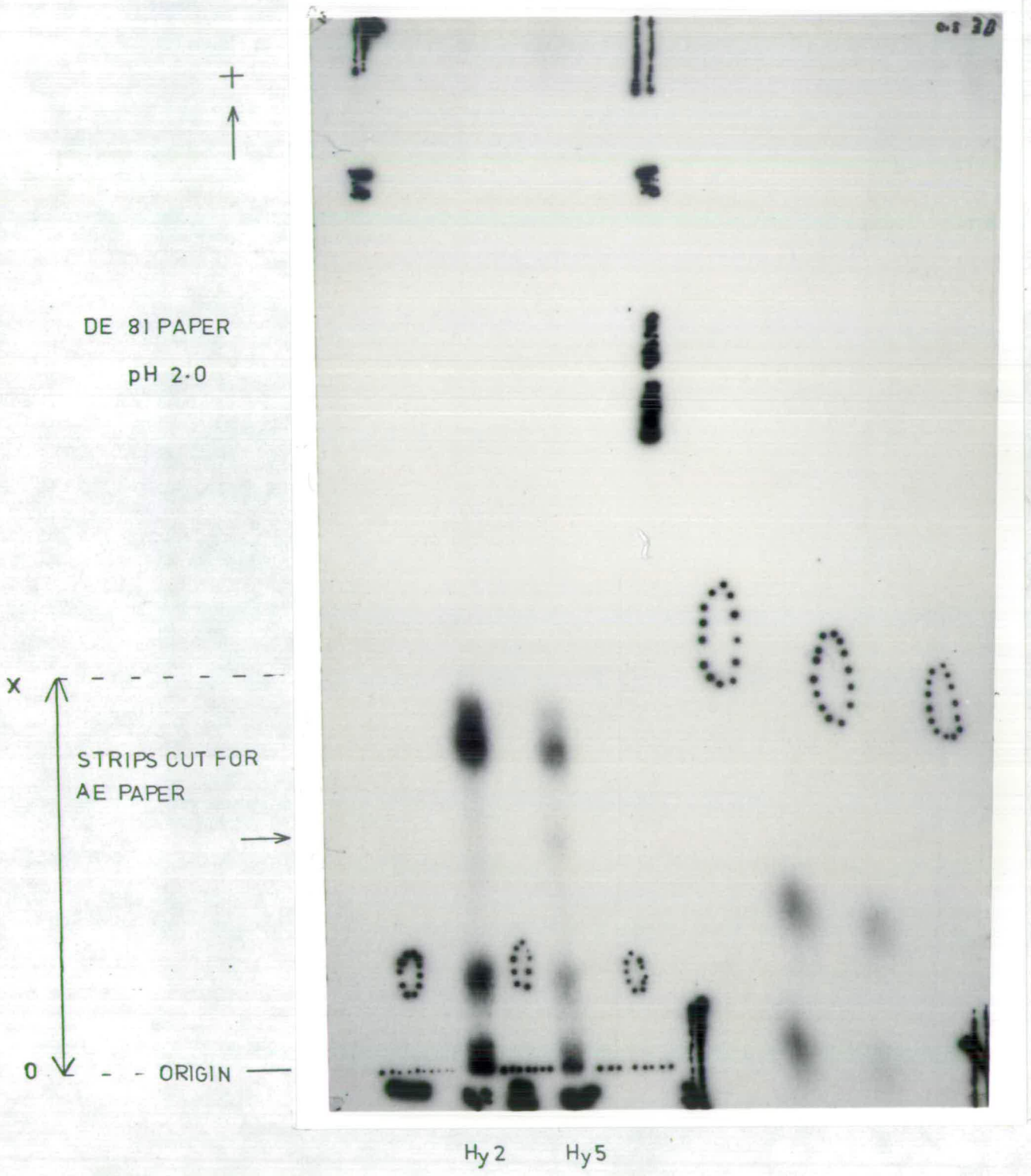

+
DE 81 PAPER
pH 2·0
X
STRIPS CUT FOR
AE PAPER
0
ORIGIN
Hy 2
Hy 5

Fig. 5.3 (opposite)

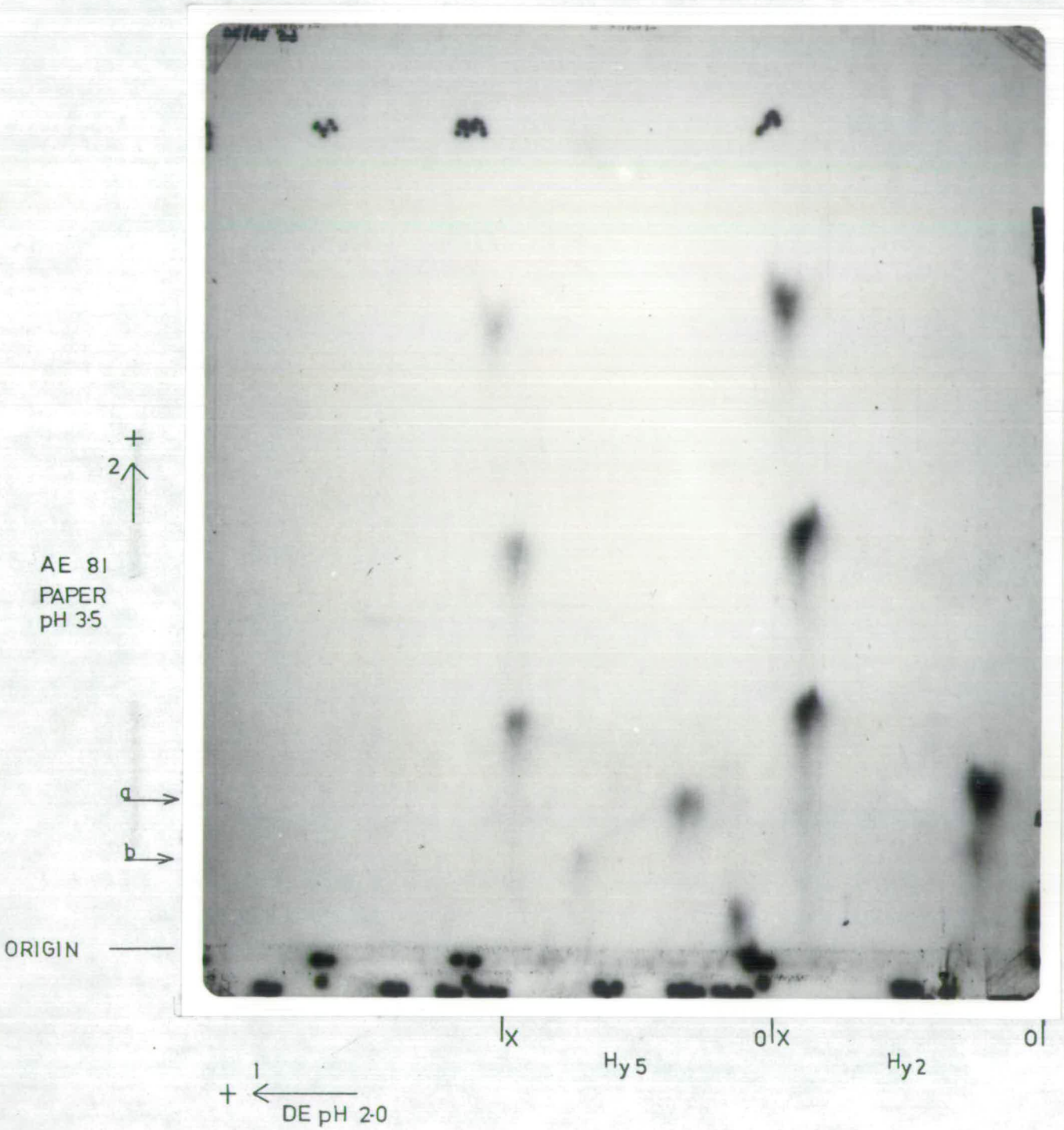
Autoradiograph of Labelled Nucleotides from Hy 2 and Hy 5 DNA.

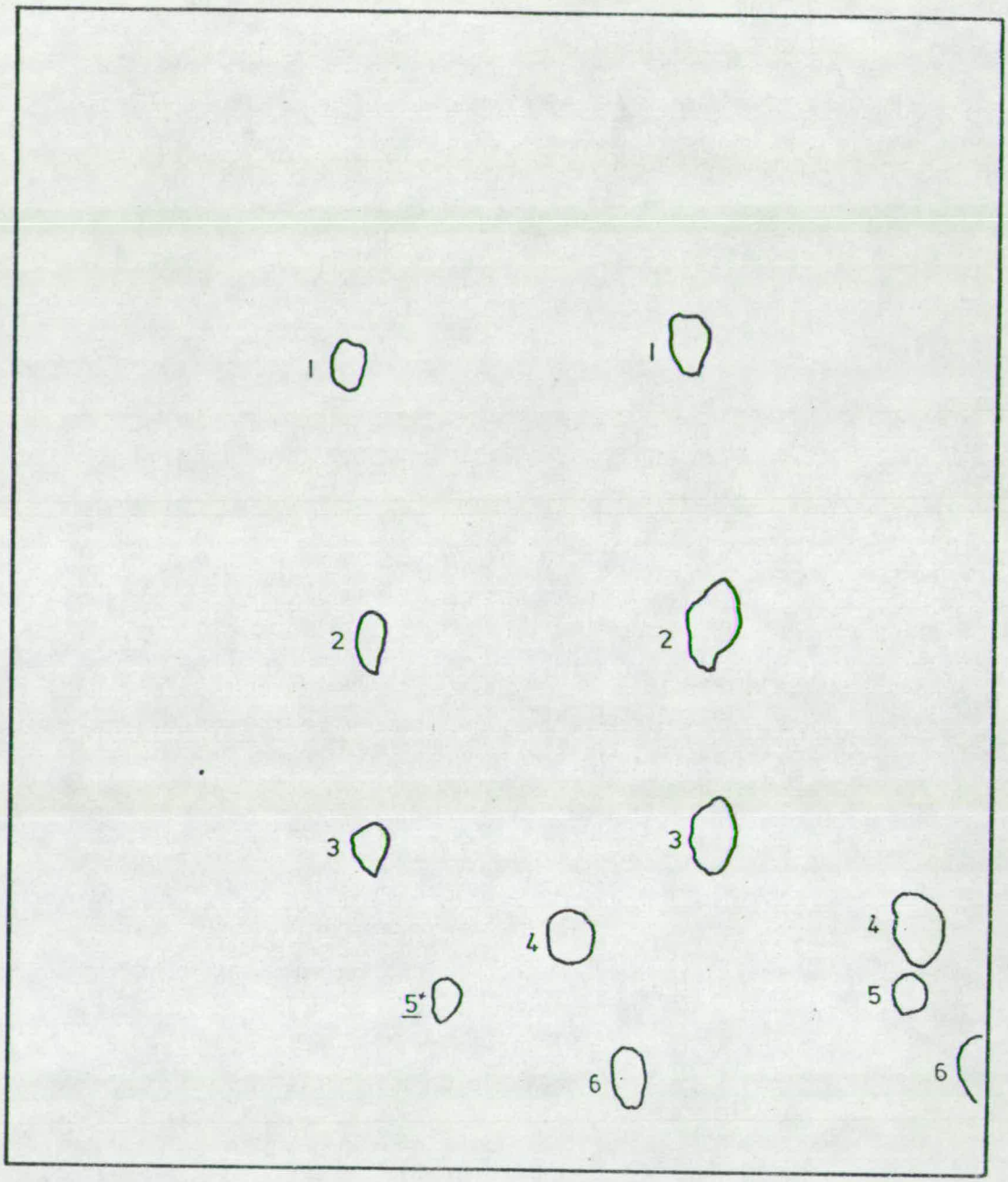
The nucleotides have been separated by ionophoresis on DE 81 paper at pH 2.0. This autoradiograph suggested that there was a difference between the patterns of nucleotides for the Hy 2 and Hy 5 samples. Strips of the DE paper were cut out for ionophoresis in a second dimension on AE 81 paper at pH 3.5 (see Fig. 5.4).

Fig. 5.4 (following pages)

Autoradiograph of a Two Dimensional Separation of Labelled Nucleotides from Hy 2 and Hy 5 DNA.

The labelled nucleotides have been identified on the basis of known mobility values (Murray and Murray, 1973) and the nucleotides are shown in a tracing of the autoradiograph.


+
2
AE 81
PAPER
pH 3·5
a
b
ORIGIN
lx
olx
ol
Hy 5
Hy 2
+
1
DE pH 2·0



+ 2 AE pH 3·5

IGIN

|X 0|X 0|

+ 1 DE pH 2·0

Hy 5

	5'p
1	GTG
2	GTGC
3	GGCG
4	GTGCT
5*	GGCGA
6	GTGCTT.

Hy 2

	5'p
1	GTG
2	GTGC
3	GGCG
4	GTGCT
5	GGCGT
6	GTGCTT.

the difference between Hy 2 and Hy 5. At the internal 'mispairing' position Hy 2 has the 186 T residue (i.e. 5' p GGCGT) whereas Hy 5 has the P2 A residue (i.e. 5' p GGCGA). Sequence analysis of another P2/186 hybrid (Hy 10, Egan, J. B., unpublished information) has given a preliminary indication that the 5' pG residue is present and that the sequence from the other 5' terminus is 5' p GGCGA.

The finding of 5' G in the 3 hybrids studied so far is beginning to imply that this G. C. base pair is important for correct P2 ter recognition. The sequence information shows that the internal base pair can change, at least between A. T and T. A, without affecting phage viability. The P2/186 hybrid formation would also have altered the rotational symmetry at the 3' ends of the DNA (Fig. 5.1). The number of base pairs involved in symmetrical arrangements remains the same (5 out of the first 7 bases from the 3' ends of the DNA) but 2 positional shifts occur in the transition from P2 to P2/186 hybrid. This minor change also does not appear to have affected P2 ter recognition. The significance of nucleotide sequence arrangements for ter enzyme recognition and cleavage will be considered in Chapter 7.

5.3 *in vitro* Packaging

The P2 *in vitro* packaging system (Pruss *et al.*, 1974) has provided an alternative approach for the analysis of P2 and 186 cohesive ends. Linear exogenous 186 p DNA (a gift from R. Saint) and linear exogenous P2 vir_{22} DNA were packaged at approximately comparable efficiencies (10^4 - 10^6 pfu/μg) by protein extracts from P2 infected cells (section 2.5 p). The products of a packaging reaction with 186 p DNA would have been P2 heads containing 186 p DNA attached to P2 tails, but after propagation on a bacterial lawn normal 186 p phage particles were formed. No attempt was made to verify that 186 p DNA was actually located inside P2 head structures as a simple genetic test provided evidence for authentic 186 p packaging. The phage P2 and 186 are heteroimmune (Bertani and Bertani, 1971) and phage 186 does not grow on *E. coli* C strains (Woods and Egan, 1974); the 186 p packaging reactions were plated on C 600 (P2) and plaques were picked into phage buffer and chloroform for spot tests on C 600 (P2) and C-1a *ton* A (P2). No phage was found from 186 p DNA packaging reactions that could grow on C-1a *ton* A (P2) and the packaging reaction did not generate plaques if exogenous DNA was omitted. These observations confirmed that 186 DNA was an acceptable substitute for P2 DNA during phage particle assembly.

Heteroduplexes formed between P2 and 186 DNAs contained about 25% sequence homology, 25% partial homology and 50% non-homology

(Younghusband and Inman, 1974). The homology is mainly confined to the structural gene region located at the left of the linear genetic and physical maps; there is no detectable homology at the right. This heteroduplex information is of some relevance to the *in vitro* packaging result. Both P2 and 186 DNAs are packaged asymmetrically *in vivo* as the left end of both DNAs is attached to the phage tail (Chattoraj and Inman, 1974). This must mean that the right cohesive end enters the head first and that, despite the non-homology observed in the electron microscope, the nucleotide sequence elements required for efficient interaction with P2 packaging proteins are present on 186 p DNA. The recognition site(s) may be limited to the cohesive ends and the hyphenated rotational symmetry arrangement at the 3' ends of DNA molecules or may extend beyond the known nucleotide sequence.

5.4 Information from Genetic Studies

The potential cross reaction between P2 and 186 *ter* enzymes could have been tested by a genetical approach but there are some difficulties and the method was not attempted. Phage P2 is normally propagated on *E. coli* C strains but phage 186 does not adsorb to C strains (Woods and Egan, 1974). The genetic map for P2 has been established for some time (Lindahl, 1974) and the P2 *ter* gene has now been identified (Bowden and Calendar, 1978). However, a linear genetic map for phage 186 has only just become available and the identification of gene products is not yet complete (Hocking, 1977). There is some indication that 186 *ter* can complement P2 *ter*$^-$ mutants (Calendar, R., unpublished information) and this is consistent with the general similarity in cohesive end sequence and DNA packaging for P2 and 186 DNA.

CHAPTER 6 Gene Duplication in Bacteriophage λ

6.1 Introduction

Tandem duplications of DNA sequences in the λ genome have been known for several years (Bellett *et al.*, 1971; Busse and Baldwin, 1972). Phage carrying duplications appeared *in vivo* following the propagation of λ deletion phage; the duplication phage were separated from the starting phage by virtue of an increased bouyant density resulting from the addition of DNA. The duplicated segment of DNA could appear in higher multimeric forms in the phage genome but an upper size limit was imposed by the phage packaging system. The identification of tandem duplications has relied heavily on electron microscope analysis of heteroduplexes formed between the duplication and the parental phage DNAs. These heteroduplexes contained a single strand loop of constant length but the position of the loop varied over the length of the duplicated segment.

Among a population of duplication phage Emmons (1974) identified a phage that had duplicated the *cos* site - the duplication extended from 94.9% to 4.9% wild type λ length. Heteroduplexes formed between the duplication and parental phage DNAs suggested that the duplication phage DNA contained a mixed population of linear DNA molecules. The duplication was usually located at the left end of linear monomeric molecules. (This observation contributed towards the evidence for asymmetrical λ packaging with the left end of linear DNA entering the head first.) Unlike most duplication

phage, the cos duplication was not stable during growth in the absence of generalised recombination. Triplications and parental phage were segregated at high frequency as a result of ter cutting at cos sites during packaging of concatemeric DNA.

Further experiments demonstrated that the appearance of tandem duplications was independent of known recombination systems (Emmons et al., 1975). Tandem duplications have been isolated to cover every region of the λ genome (the b 221 region, 40.6 - 62.9%, has not been duplicated as this region is deleted in order to accommodate additional DNA) and there was no apparent pattern to the end points of the duplications(Emmons and Thomas, 1975). On these criteria, tandem duplications were identified as being the products of illegitimate recombination (Franklin, 1971a).

Restriction endonucleases have been employed to rearrange DNA sequences in vitro and this application has been used to create duplications in bacteriophage genomes (Thomas et al., 1974; Moore et al., 1977). A curious observation has emerged from in vitro recombination experiments as it has been found that inversion of the λ DNA between 57% and 77% has no adverse effect on phage viability (Philippsen et al., 1978).

The in vitro duplications need not necessarily assume tandem arrangements and there is no indication of instability during phage growth. The objective of the experiments described in this Chapter was to construct in vitro a derivative of phage λ that carried a second

copy of gene A and the cohesive site in the middle of the genome. The orientation of the second gene A could be controlled through the use of 2 different restriction enzymes so that sense strand transcription would occur from λ P_L. In this way it was hoped to stimulate levels of λ A protein to facilitate purification and characterisation of the protein.

As these experiments were reaching a conclusion, my attention was drawn to some work from Nomura's group (Williams et al., 1977). In the course of molecular cloning experiments with λ transducing phage they had, by chance, isolated phage carrying a second copy of the cos site together with ribosomal protein genes. The analysis of these phage with the second cos site in the middle of the linear genetic map is entirely consistent with the information presented here. Phage analogous to those of Williams et al. (1977) have been isolated from molecular cloning experiments with λ d rif^d 18 (Newman et al., 1978) and I have some results from stocks of these phage kindly provided by A. Newman.

The λ cos site has also been inserted into bacterial plasmids in experiments with restriction endonucleases (Fukumaki et al., 1976; Collins, J. and Hohn, B., in preparation).

6.2 Construction of a λ Duplication Phage in vitro

The experimental design required the reconstruction of a phage genome in vitro. A stepwise procedure was adopted in an attempt to favour the formation of particular intermediates. The scale of reactions did not permit isolation and characterisation of intermediates so there was no verification that the scheme outlined (Fig. 6.1) was actually followed. However, it was considered extremely unlikely that restriction and ligation of a simple mixture of composite phage DNAs would permit the isolation of the desired recombinants.

DNA fragments were prepared from phage λ derivatives with specific lesions that had been introduced to remove various restriction sites (Murray and Murray, 1974). λ 416 has a single target for the restriction enzyme R. Eco RI located at 93% from the left end of the linear genetic map. (There is an amber mutation in gene S - S am_7, suppressed by sup F - of λ 416; the reason for including this mutation will be explained later in the text - page 113).

λ 570 has a single target for the restriction enzyme R. Bam HI at 11.4% from the left end of the linear genetic map. The restriction digests λ 416 + R. Eco RI and λ 570 + R. Bam HI were essentially similar in appearance, in each case there were 2 fragments one very large and one small. The small fragments were purified by sucrose density gradient centrifugation (section 2.5 n, 6 x 5 ml Titanium swinging bucket rotor 40 k rpm, 4 hours, 20°C, 155,000 g).

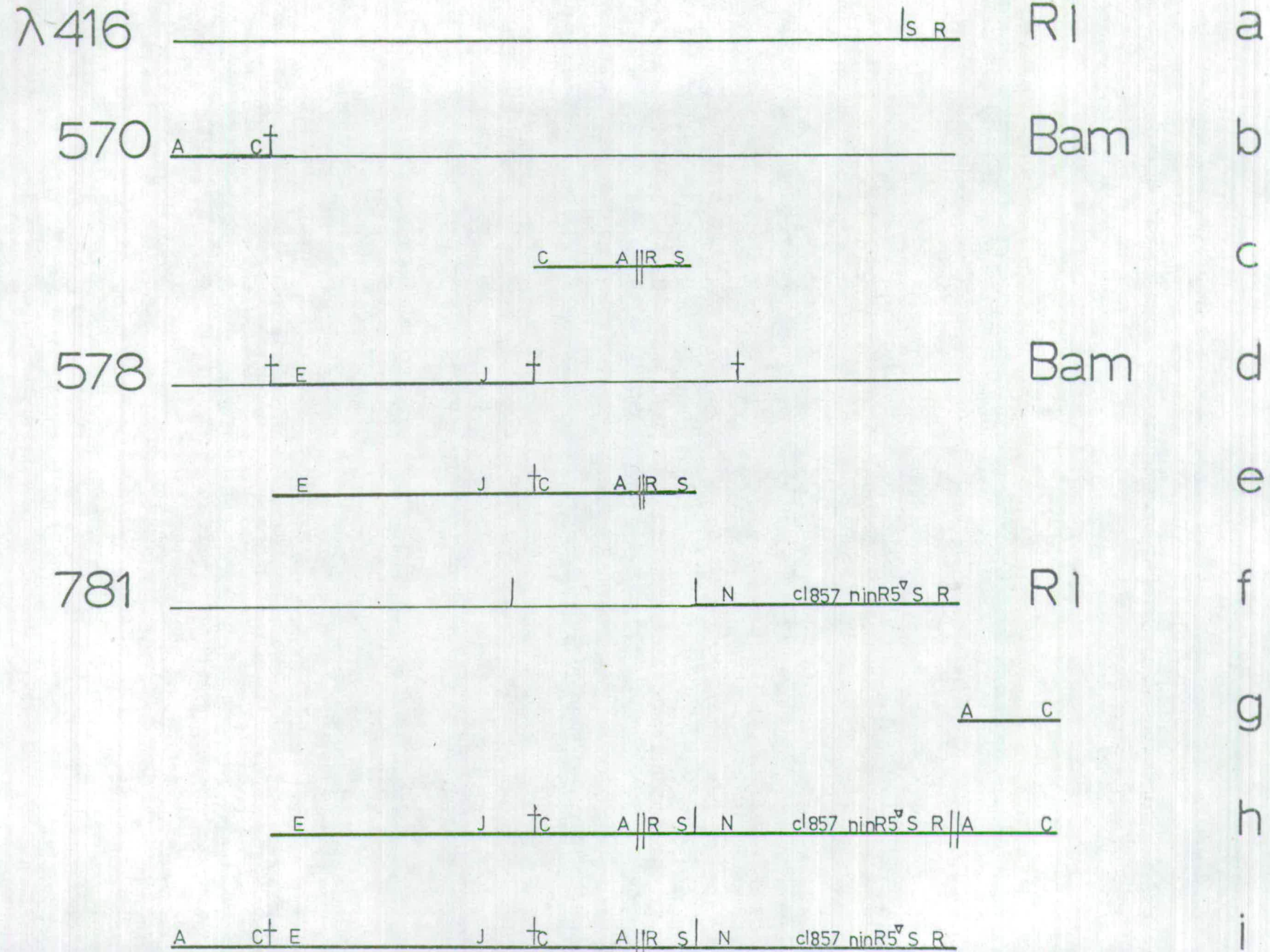

λ416
S R
R1
a
570
A
C
Bam
b
C
A
R S
c
578
E
J
Bam
d
E
J
C
A
R S
e
781
N
cI857 ninR5▽S R
R1
f
A
C
g
E
J
C
A
R S
N
cI857 ninR5▽S R
A
C
h
A
C
E
J
C
A
R S
N
cI857 ninR5▽S R
i

Fig. 6.1 (opposite)

in vitro Phage Reconstruction Scheme .

a λ 416 *b* 538, *c* Iam *S* am_7

Sucrose gradient to purify 7 % R. *Eco* RI fragment.

b λ 570 *trp* 1A, imm^{21}, *nin* R5

Sucrose gradient to purify 11.4% R. *Bam* HI fragment.

c Restriction fragments from λ 416 and λ 570 ligated at 37°C to join λ cohesive ends.

d λ 578 *b* 508, *c* I am, *nin* R5

Sucrose gradient to enrich for 11.4 - 46.0% R. *Bam* HI fragment.

e R. *Bam* HI fragment from λ 578 ligated to c.

f λ 781 *sup* E, *c* I_{857}, *nin* R5

Restricted with R. *Eco* RI.

g Excess R. *Bam* HI fragment from b mixed with f for hydrogen bonding at the λ cohesive ends.

h Ligation reaction between e and g.

i Conventional representation of the linear molecule after the final ligation.

λ 578 has recognition sites for the enzyme R. Bam HI at 11.4%, 46.0% and 71.0%; the largest restriction fragment (11.4 - 46.0%) was required for the in vitro reconstruction process and while the fragment could not be completely purified it was considerably enriched by sucrose density gradient centrifugation (conditions as for the λ 416 and λ 570 restriction digests). Attempts to remove the R. Bam HI site at 11.4% have so far been unsuccessful and λ 578 was the best compromise for R. Bam HI restriction fragments from the left arm of the λ genome.

Fractions from the sucrose gradients were analysed by agarose gel electrophoresis (section 2.5 n, see for example Fig. 6.2). The gradient fractions that contained the required restriction fragments were pooled, dialysed against 1/10 th DNA buffer and concentrated 5 - 10 fold in a vacuum desiccator.

The small terminal restriction fragments from λ 416 and λ 570 were joined together by ligation of the λ cohesive end sequences (Fig. 6.1 c, section 2.5 k). The restriction fragment from λ 578 was then ligated to the joined terminal restriction fragments (Fig. 6.1 e). The λ 578 fragment could be joined in either of 2 possible orientations but it was anticipated that the inverted orientation (with reference to the normal λ gene order) would not subsequently give rise to viable phage.

The reconstructed phage genome was missing the left 11.4% R. Bam HI fragment and also the entire right arm. An R. Eco RI replacement


1
20
26
32
7%
+

Fig. 6.2 (opposite)

Sucrose Gradient Separation of the R. Eco RI Digest of λ 416 DNA.

Gel analysis (1% agarose gel) of fractions recovered after sucrose gradient centrifugation. The numbers at the top of the photograph are gradient fraction numbers 1, the bottom of the gradient and 32, the top. The 7% restriction fragment (peak in Fraction 26) is well separated from high molecular weight material.

vector was used to provide the right arm; this DNA fragment was not purified as genetic tests could be used to identify recombinants. The replacement vector, λ 781, contains an *E. coli* DNA fragment that codes for a suppressor t RNA and a test with a *lac Z* am bacterial host (ED 8538) identifies phage that have lost the suppressor fragment.

A sample of the 11.4% R. *Bam* HI terminal fragment was mixed with an R. *Eco* RI digest of λ 781 to allow annealing of the λ cohesive ends. This mixture was then ligated with the products from the λ 416 + λ 570 + λ 578 restriction fragment ligation reactions (Fig. 6.1 h). Plaque forming units were recovered by transfection (section 2.5 l) and 249 plaques were obtained from one third of the final reaction.

Rather surprisingly, there were approximately equal numbers of clear and turbid plaques - the right arm donor λ 781 has the temperature sensitive repressor mutation *c* I_{857} and at 37°C should give exclusively clear plaques. There were no turbid plaques on control plates that had been set up with unrestricted vector DNA to check the competence of the transfection cells. Individual plaques were picked into phage buffer plus chloroform for spot tests:

Turbid plaques:	30 ex 30	*sup*°	*imm*^{21}
Clear plaques:	43 ex 45	*sup*$^+$	*imm*$^\lambda$
	2 ex 45	*sup*°	*imm*$^\lambda$

Tests on 30 tiny plaques (too small to allow initial differentiation

between clear and turbid plaque type) yielded 4 more phage of the sup^{o}, imm$^{\lambda}$ class.

The turbid plaques were taken to be λ 570 as judged from genetic spot tests (the turbid plaques were red $^{+}$, imm^{21}). The appearance of λ 570 could be explained from a defect in the fractionation of the sucrose gradient used to prepare the R. Bam HI 11.4% fragment from λ 570 DNA. When gradients were collected from the bottom there was sometimes a slight mixing back so that the densest fragment subsequently washed out in the other fractions. In this way a trace of the large R. Bam HI fragment could have been added to the ligation reactions, leading to the appearance of λ 570 plaques after transfection.

The 6 potential recombinants were plaque purified and single plaques were picked to prepare plate lysate stocks (section 2.5 c). The plate stocks provided sufficient phage for small scale liquid lysates (100 - 200 ml of culture). DNA was extracted from phage purified by equilibrium centrifugation and R. Eco RI digests were analysed by agarose gel electrophoresis (sections 2.5 j and 2.5 m).

While the DNA preparations were in progress some of the phage were tested for their restriction ratio (Murray and Murray, 1974). The ratio (titre of phage on an E. coli K strain divided by the titre on an iso-genic E. coli K strain carrying the RI plasmid) was indicative of the number of R. Eco RI sites located in a given phage genome. Restriction ratios and information from agarose gel

TABLE 6.1

R. Eco RI Recognition Sites in the Potential λ Duplication Phage.

Recombinant	Restriction Ratio	Probable number of R. Eco RI sites[a]	Number of bands after R. Eco RI digestion and gel electrophoresis[b]
66	100	2	3
74	100	2	6
79	20	1 or 2	2
80	-	-	2
91	130	2	3
96	-	-	2
λ781 control	70	2	3

a The correlation between restriction ratio and the number of R. Eco RI sites is defined by Murray and Murray (1974).

b For a linear DNA molecule, the number of bands is 1 greater than the number of restriction sites.

electrophoresis are displayed in Table 6.1.

It was anticipated that any phage with the desired arrangement of DNA fragments would have 2 R. Eco RI sites - srl λ - 3 used for the attachment of the right arm from λ 781 and srl λ - 1 that had been carried silently on the R. Bam HI fragment from λ 578. Recombinant 74, now designated λ dup 74, immediately appeared unusual as the restriction ratio suggested 2 R. Eco RI sites but 6 bands were observed after R. Eco RI digestion and gel electrophoresis of the purified phage DNA. A tentative interpretation was that λ dup 74 represented the end product of the in vitro construction; a phage with 2 copies of the cos site could give rise to linear DNA in 2 forms which would have different restriction patterns. The tandem duplications of the cos site (Emmons, 1974; Feiss and Campbell, 1974) were analysed by electron microscopy and not by gel electrophoresis however, the DNA structures observed would be consistent with unusual restriction patterns.

A trivial explanation was that the λ dup 74 DNA had been prepared from a mixed population of phage, each having 2 different R. Eco RI sites. The λ dup 74 phage stock was plated out for single plaques and 6 well separated plaques were purified through 2 single plaque cycles. Single plaques from each of the purified series were used for lysates on L-agarose plates (section 2.4). Phage DNA was recovered from the plates (section 2.5 h) and digested with R. Eco RI. The restriction patterns of the 6 purified plaques were found to be identical with each other and also with the λ dup 74 DNA prepared

from the liquid lysate (Fig. 6.3). This result eliminated the possibility of contaminating phage and gave the first indication of the stability of the duplication phage. The restriction patterns for λ dup 74 DNA with the enzymes R. Bam HI and R. Hind III fitted exactly with the proposed structure (Fig. 6.4).

During the in vitro construction process there were some doubts about the potential viability of a phage carrying additional copies of gene A and the cos site. If A protein was synthesised in large amounts then the protein might recognise and cleave cos sites at an early stage in the infectious cycle to prevent the appearance of plaque forming units. These doubts were dispelled by the isolation of the phage λ dup 74. A similar type of reasoning was behind the inclusion of the S am_7 mutation from λ 416; phage carrying this mutation are blocked in host cell lysis (Goldberg and Howe, 1969). There was concern that the early appearance of S protein, as a result of P_L transcription would cause premature cell lysis. The initial transfection and propagation were with sup^o hosts but in subsequent tests λ dup 74 appeared to grow as well in the presence or absence of sup F.

Fig. 6.3 (opposite)
Restriction Analysis of λ dup 74 Phage DNA.

1% agarose gel, all DNA samples digested with R. Eco RI

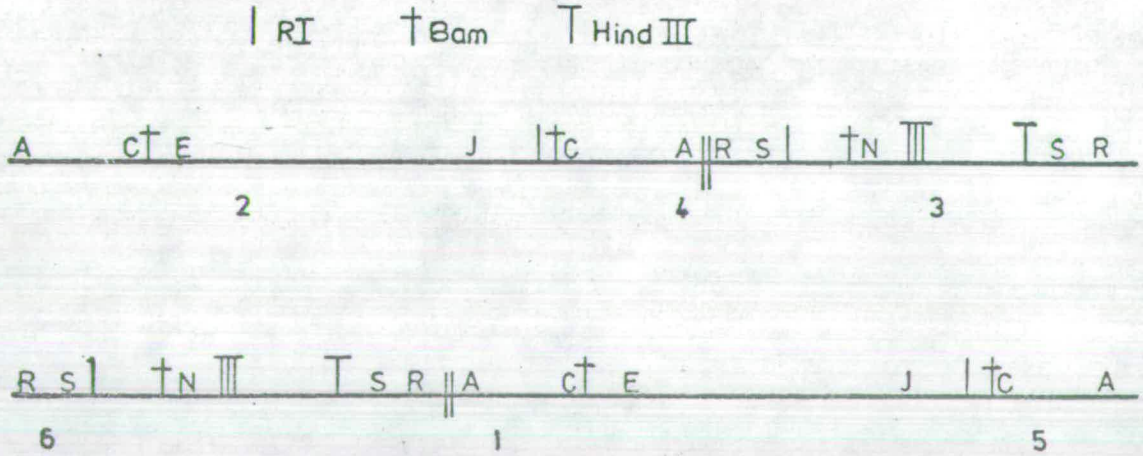
Track 1 λ c I_{857} S am_7 DNA
5 λ dup 74 DNA
9 λ c I_{857} S am_7 DNA

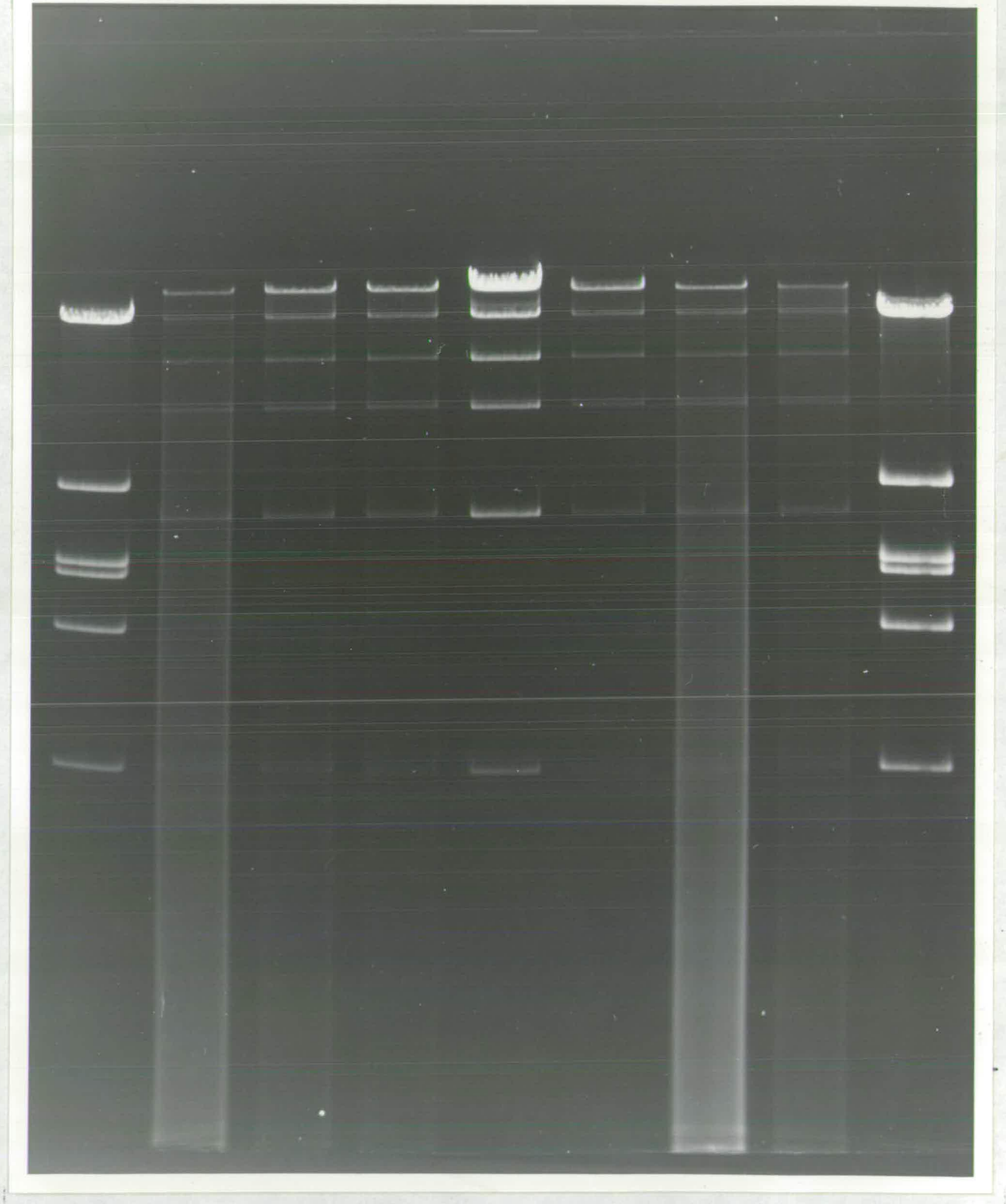
2-4 and 6-8 Purified single plaque isolates from λ dup 74 phage stock.

Tracks 2-4 and 6-8 show DNA samples prepared from L-agarose plate lysates. The backgrounds are variable because of differing amounts of bacterial DNA. The 6 bands present with the purified λ dup 74 DNA are also present for the re-purified single plaque isolates.

Fig. 6.4 (below)
Alternative Linear Forms for Phage λ dup 74.

The λ dup_{74} DNA can exist in 2 linear forms depending on which pair of cos sites is recognised during packaging of concatemeric DNA. R. Eco RI restriction fragments are numbered (1-6) and correspond to the 6 bands (in order of decreasing molecular weight) that are visible in the gel photograph opposite. Restriction targets for R. Bam HI and R. Hind III are included in the diagram.

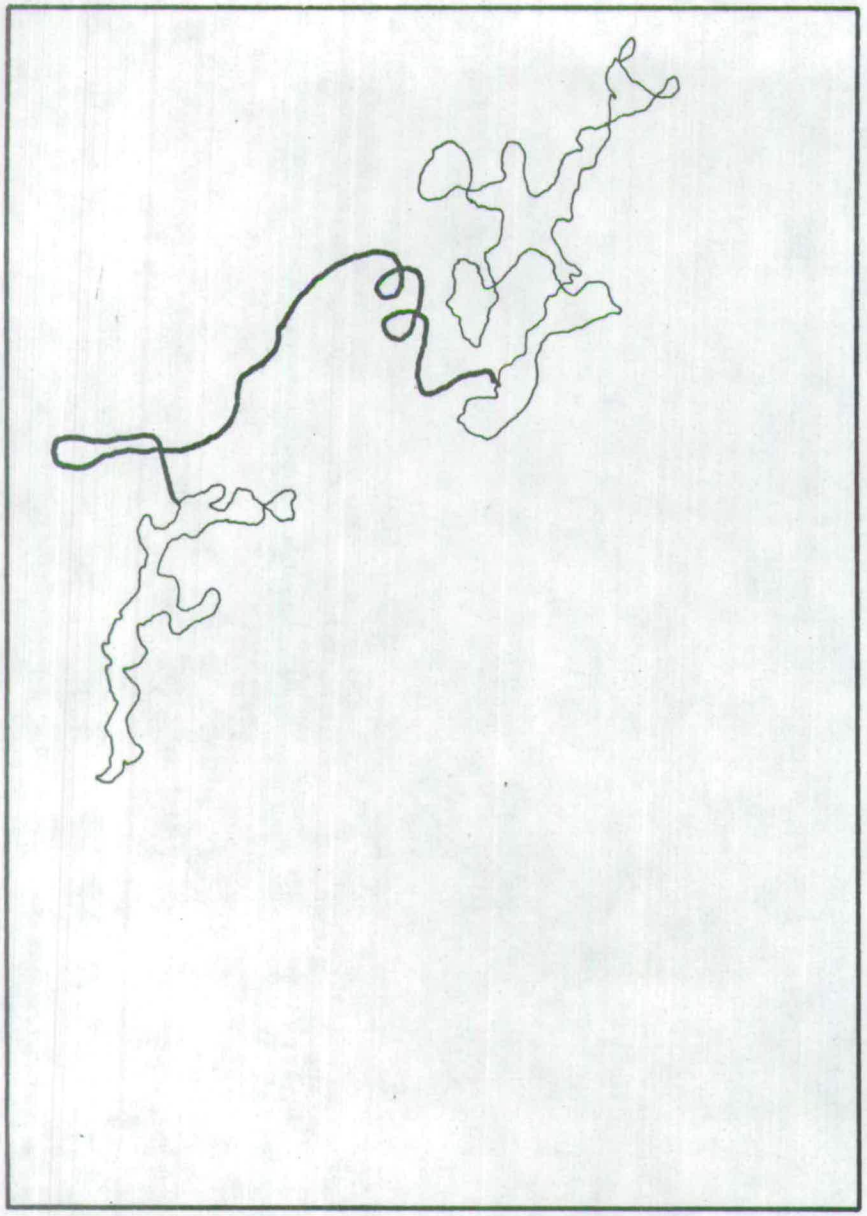



1
2
3
4
5
6
7
8
9
+

6.3 Electron Microscopy with λ dup 74 Phage DNA

The interpretation suggested for the phage λ dup 74 required that the duplicated genes were inverted with respect to their normal orientation in the λ genome. When DNA prepared from the duplication phage was denatured and allowed to renature, single strand structures should form and these should be visible in the electron microscope. As both ends of the linear λ DNA molecules have been duplicated the renatured species should have the appearance of a dumb-bell. The duplicated segments would generate double stranded DNA and the closed circles of the dumb-bell would be single strand DNA located between the duplications. The lengths of the component parts of the dumb-bell could be predicted fairly accurately from the known genotypes of phage involved with the duplication construction. The essential feature of the model for the electron microscopy was that the 2 single strand loops, formed as a result of intramolecular reassociation should be significantly different in length.

Double and single strand DNA length standards were mixed with renatured λ dup 74 DNA prior to spreading (section 2.5 s). (Samples of X-irradiated pSC101DNA and M 13 phage were kindly provided by Dr. J. Finnegan). Grids were scanned in the electron microscope and well-spread molecules adjacent to length standards were photographed (Fig. 6.5). This type of dumb-bell, from an intramolecular reaction (Fig. 6.6), was the predominant structure identified on the grids. However, dumb-bells could also arise from an intermolecular reaction between opposite strands of the 2 linear DNA types. These intermolecular dumb-bells could assume 2 forms depending on whether



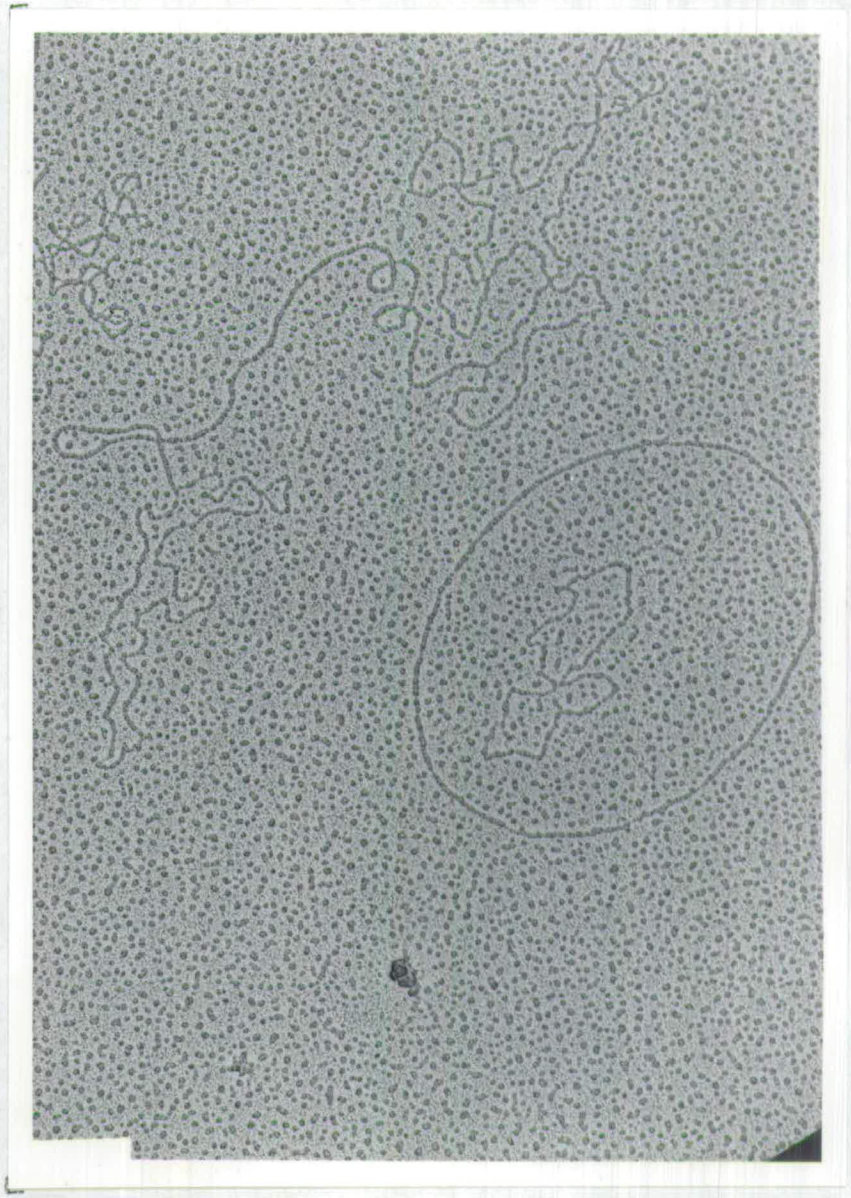


Fig. 6.5 (opposite)

Single Strand Structure formed by Intramolecular Reassociation of λ dup 74 DNA.

The structure consists of a length of double strand DNA that links 2 single strand loops; the loops are different in length. The molecule is traced below the photograph and the structure is represented schematically in Fig. 6.6.

Magnification of the electron micrograph x 27,000.

Structures in the top centre of the photograph are the length standards, the large oval is pSC 101 and it encloses a single strand molecule of M13 DNA .

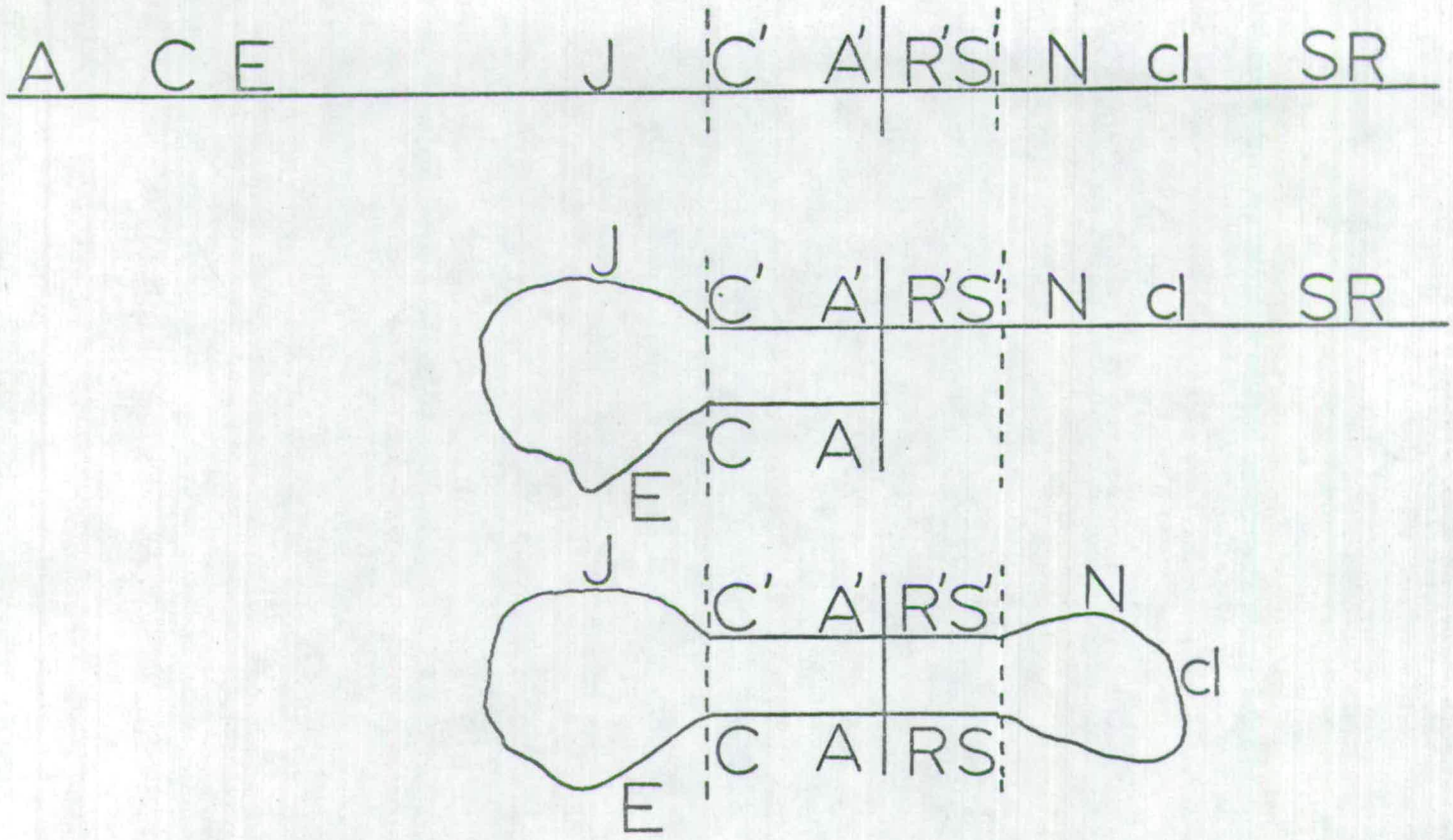

A C E
J
C' A' R'S'
N cl
SR
J
C' A' R'S'
N cl
SR
C A
E
J
C' A' R'S'
N
cl
C A RS
E
33%
18%
22%

Fig. 6.6 (opposite)

Representation of an Intramolecular Structure.

A single strand structure from λ dup 74 DNA. The structures should form rapidly so the intermediate with base pairing in the A - C segment is not expected to be stable and it is included for illustrative purposes only. The figures are the theoretical lengths of the component parts of the dumb-bell and are percentages of wild-type λ length.

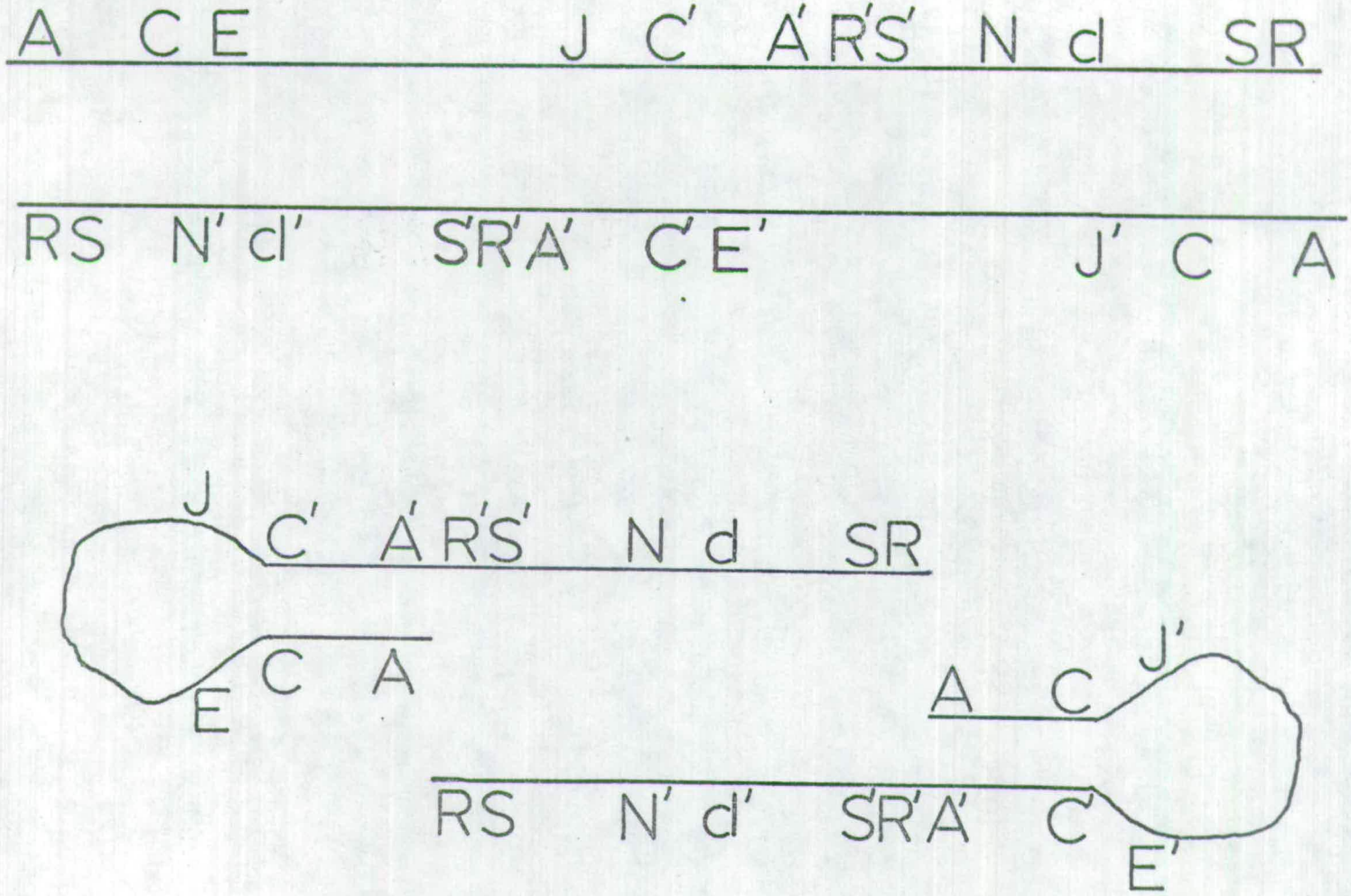
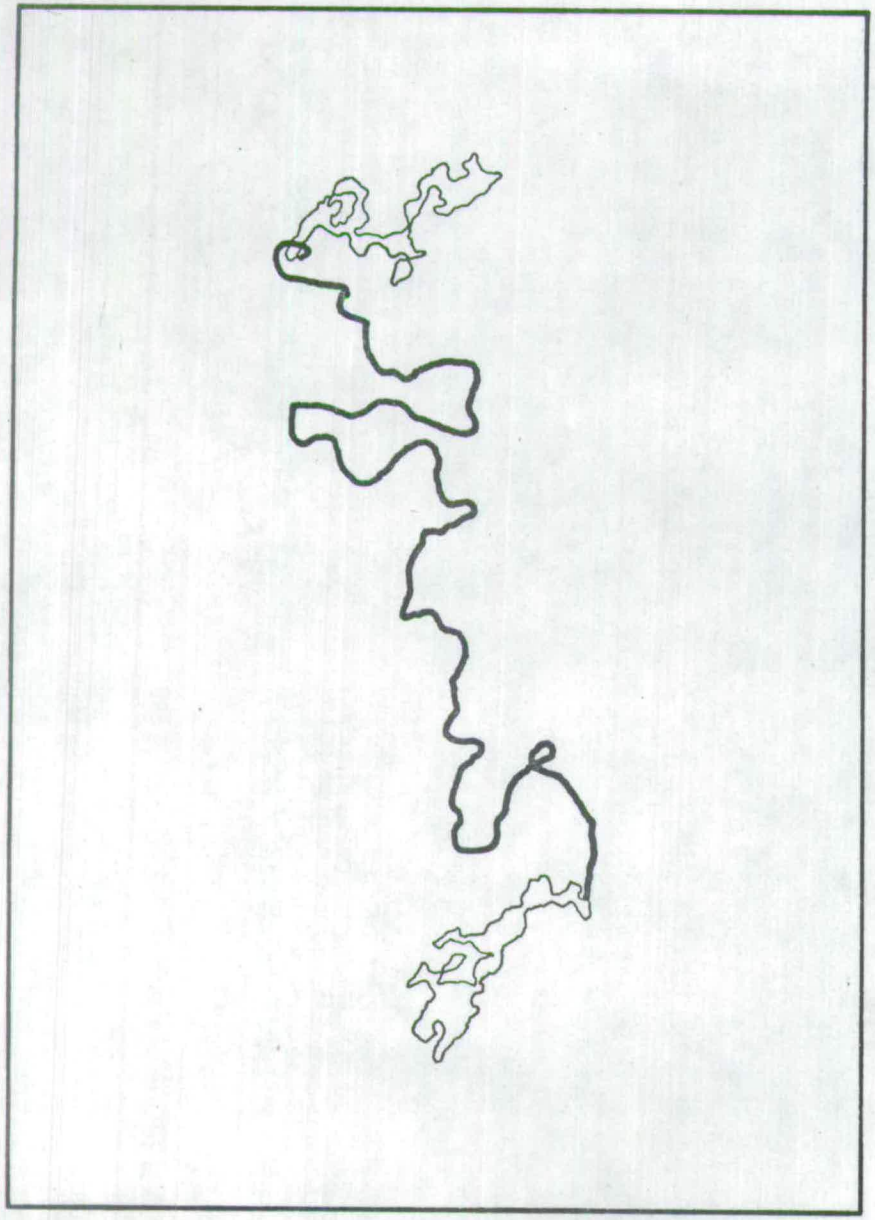

A C E J C' Á R'S' N d SR
RS N' d' S'R'A' C' E' J' C A
J C' Á R'S' N d SR
E C A
A C J'
RS N' d' S'R'A' C' E'
33%
58%
33%

Fig. 6.7 (opposite)

Representation of an Intermolecular Structure.

This structure is formed from 2 single strands of λ dup 74 DNA. At high DNA concentration, the transient intermediate with base pairing only in the A - C segment (or the S - R segment) may interact with a similar structure formed by the opposite strand of the other linear DNA type. The single strand loops are now the same size and are complementary. The figures are the theoretical lengths of the component parts of the dumb-bell and are percentages of wild-type λ length.



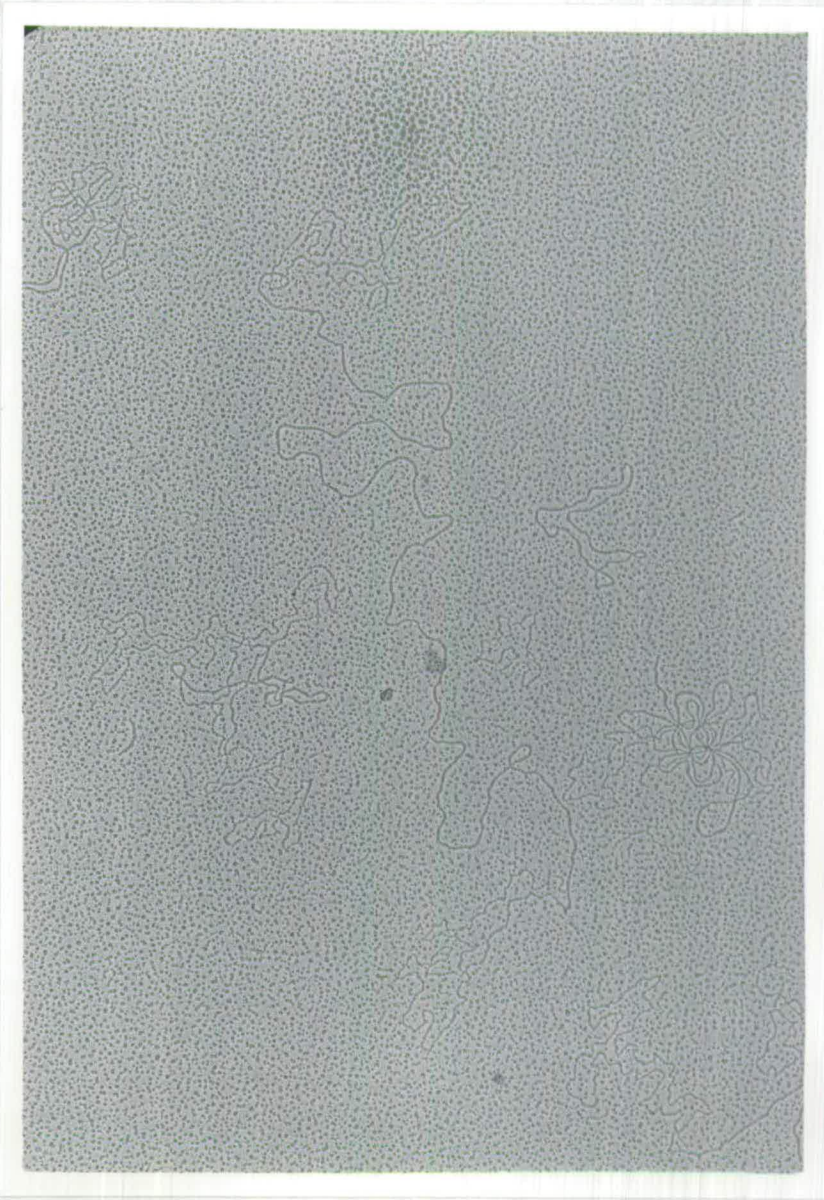


Fig. 6.8 (opposite)

Intermolecular Dumb-bell.

A structure formed from 2 single strands of λ dup 74 DNA. The structure is traced below the photograph.

Magnification of the electron micrograph x 13,000.

The photograph shows both length standards and a single strand loop from another dumb-bell can be clearly identified in the top right corner.

the initial base pairing occurred in the duplicated A - C region or in the duplicated S-R region. The A-C type has a double strand length corresponding to 58.2% wild type λ length, the single strand loops are identical and equivalent to 33.1% wild type λ length (Figs 6.7 and 6.8).

The S-R type has a double strand length of 71.3% wild type λ length and the single strand loops are both 22.5% wild type λ length. Only 1 each of the A-C and S-R type intermolecular structures were observed and this is in part explained by the fact that the single strand loops are now complementary and base pairing between loops would probably produce an unintelligible tangle of DNA.

The results from measurements of 10 intramolecular dumb-bells are shown in Table 6.2. The number of molecules measured is barely sufficient for statistical significance but there is fair agreement between predicted and observed lengths and the electron microscopy does confirm the DNA duplication in phage λ dup 74.

When DNA from recombinant 66 (Table 6.1) was denatured, renatured and examined in the electron microscope there was no indication of any structure comparable to the λ dup 74 dumb-bell. The R. Bgl II restriction patterns of recombinants 66, 79, 80, 91 and 96 were not consistent with a duplication of the R. Bgl II recognition site that is located very close to the left end of linear λ DNA. These recombinants, which were all quite different, were not studied any further.

TABLE 6.2

Length Measurements from Electron Microscopy of λ dup 74 DNA.

	pSC 101	Double Strand	Loop 1	Loop 2	M 13
Number of molecules measured (N)	8	10*	10*	10*	16
Mean lengths	11.14	10.93	12.34	19.43	7.48
Standard Deviation (SD)	0.92	0.31	0.61	1.17	0.55
Standard Error (se)	0.291	0.098	0.193	0.370	0.112

$$se = \frac{SD}{\sqrt{N}} \qquad (SD^2) = \left(s(x^2) - \frac{\overline{s(x)}^2}{N}\right)$$

$$\left(\frac{se}{mean}\right)^2 = \frac{(se\ (x))^2}{(\bar{x}^2)} \times \frac{(se\ (y))^2}{(\bar{y}^2)} + \frac{(se\ (x))^2}{(\bar{x}^2)} + \frac{(se\ (y))^2}{(\bar{y}^2)}$$

These equations were used to derive length relationships between molecules and standards.

* Measurements were from 10 intact dumb-bell structures.

Table 6.2 (continued)

Double Strand = 0.98 pSC101 $\bar{+}$ 0.03 pSC101

Loop 1 = 1.65 M13 $\bar{+}$ 0.036 M13

Loop 2 = 2.6 M13 $\bar{+}$ 0.062 M13

pSC101 9.66 kb, M13 6.2 kb

(D. J. Finnegan, personal communication)

	Theoretical Length	Measured Length
Double Strand	9.0 kb (18.35% λ)	9.47 kb $\bar{+}$ 0.29 kb (19.35% λ)
Loop 1	11.0 kb (22.45% λ)	10.23 kb $\bar{+}$ 0.22 kb (20.9% λ)
Loop 2	16.2 kb (33.1% λ)	16.1 kb $\bar{+}$ 0.38 kb (32.9% λ)

For the intermolecular structures:

	Double Strand DNA	Loops
A - C type	60.25% λ	35.7% λ
Predicted values	58.2% λ	33.1% λ
S - R type	75.5% λ	20.8% λ
Predicted values	71.25% λ	22.45% λ

6.4 Stability of Phage λ dup 74

DNA isolated from λ dup 74 phage particles exists in 2 alternative forms in a simpler but analogous arrangement to the DNA extracted from Herpes Simplex Virus (Wilkie and Cortini, 1976). It was of some interest to attempt a separation of the λ dup 74 DNA types to determine if both forms were infectious. The strategy behind the experiment was to 'destroy' the cohesive ends of a population of linear DNA molecules and then recover viable phage that were able to propagate using the cohesive site that had been protected in the centre of the genome.

DNA polymerase I was used to fill-in the cohesive ends (Strack and Kaiser, 1965) and attempts were made to form closed circles either *in vivo* after transfection with the linear treated DNA or *in vitro* with flush end ligation (Backman *et al.*, 1976) and then transfection to recover viable phage. An alternative approach relied on the single strand nuclease S1 to attack the 5' single strand projections at the cohesive ends. After a brief S1 treatment, the DNA was incubated with DNA polymerase I to repair ragged ends that might have arisen from S1 'nibbling' (Shenk *et al.*, 1975). This DNA was also used for flush end ligation *in vitro* and then transfected to recover viable phage.

In a number of experiments there were slightly more plaques recovered from the treated λ dup 74 DNA than from equivalently treated λ DNA control samples. Some of these plaques were purified and phage

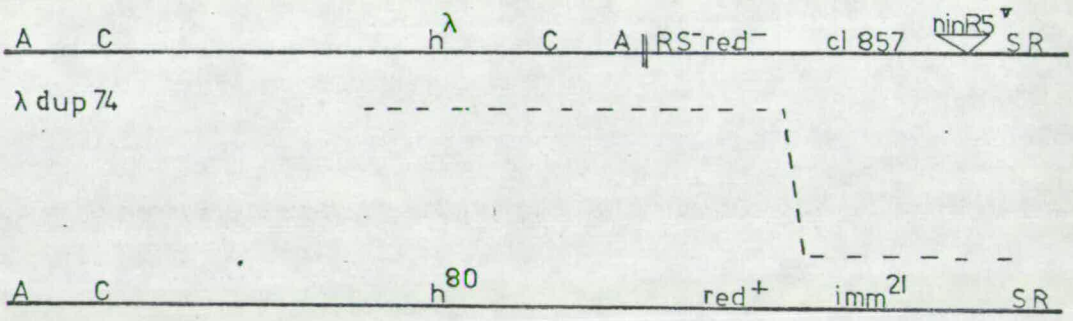
DNA was prepared from L-agarose plate lysates (section 2.5 h). The R. Eco RI restriction patterns of these single plaque isolates showed no deviation from the pattern of the original λ dup 74 DNA. Altogether, 62 independent plaques were examined for R. Eco RI restriction patterns and no change was detected. These phage were grown on either rec A^+ or rec A^- bacterial hosts and the complete absence of any change in restriction pattern is verification of the stability of phage λ dup 74.

The failure to separate the 2 DNA forms probably reflects inefficient in vitro flush end ligation. It seems likely that both DNA forms should be infectious but this does remain to be established. The λ dup 74 DNA could be an interesting substrate for in vitro site directed mutagenesis (Domingo et al., 1976) to determine which bases in the λ 5' single strand projections are involved with ter enzyme recognition and cleavage.

6.5 Phage Crosses

The duplicated genes in λ dup 74 were positioned for transcription from λ P_L. A *cro*$^-$ derivative of λ dup 74 was constructed by phage crosses (section 2.5 d) to study the effects of prolonged transcription from λ P_L. A two stage process was required for the isolation of the *cro*$^-$ phage.

λ dup 74 x λ 143 λ 143 *h*^{80}, *imm*^{21}



For simplicity, only 1 form of the λ dup 74 DNA is shown. The phage cross was plated out on Y mel *ton* A to select against the *h*^{80} of λ 143; recombinants should give turbid plaques. A direct selection on C 600 (*imm*$^{\lambda}$) was avoided because of possible interactions between recombinants and the prophage. Turbid plaques were picked and checked for failure to grow on a *lig* ts strain (i.e. still *red*$^-$) and one of these plaques was purified and kept as the stock of λ dup 74 *imm*^{21}.

As a precaution against abnormal recombination, phage DNA was

prepared from λ dup 74 imm^{21} for R. Eco RI restriction analysis. The restriction pattern differed from λ dup 74 DNA (Fig. 6.9 tracks 2 and 3, Fig. 6.10) because restriction sites had been introduced from λ 143 but the pattern was consistent with maintenance of the duplicated DNA.

λ dup 74 imm^{21} x λ 671

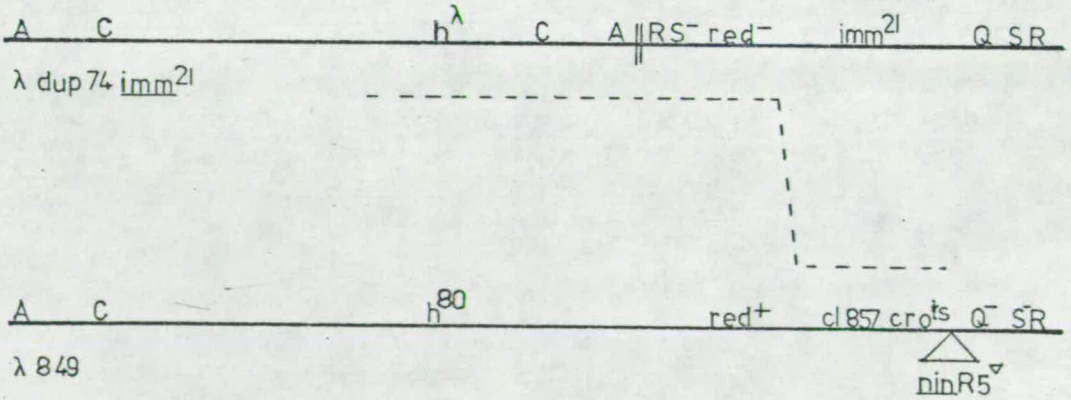
λ 671 h^{80}, att$^{\lambda}$ c I At2, cro 1, Q am$_{73}$, S am$_{7}$.

This phage cross was attempted several times but no duplication phage recombinants were isolated. The reason for this failure is not known.

A similar type of phage cross was attempted with a cro^{ts} mutant. At 30°C this phage grows normally but at elevated temperatures the cro protein is inactivated to leave P_L transcription undiminished.

λ dup 74 imm^{21} x λ 849

λ 849 h^{80}, c I$_{857}$, cro^{ts}, Q am$_{73}$, S am$_{7}$, nin R5.



The cross was plated on a permissive host, ED 8659, and plaques were screened on ED 8659 *ton* A and ED 8659 (*imm*^{21}) *ton* A. Phage that grew on both screening hosts were tested for failure to grow on a *lig* ts *sup* F strain (i.e. testing for *red*$^{-}$ phage). Two phage that checked appropriately were plaque purified and maintained as stocks: λ dup 74 *cro*^{ts} (2), required *sup* F for growth and λ dup 74 *cro*^{ts} (3), no suppressor requirement for phage growth.

Liquid lysates of these 2 *cro*^{ts} phage were prepared at 30°C (section 2.5 f) and phage DNA was extracted from phage particles that had been concentrated with PEG and purified in CsCl step gradients (section 2.5 g). Restriction analysis with R. *Eco* RI confirmed that the duplicated DNA segment was still present (Fig. 6.9 tracks 4, 5 and 6, Fig. 6.10). The phage λ dup 74 *cro*^{ts} (2) is Q^{+} *S* am_{7} and has the *nin* R5 deletion and must have been derived from multiple recombination events. The phage λ dup 74 *cro*^{ts} (3) is *nin*$^{+}$, Q^{+}, S^{+} and must result from a double crossing over that picked up *c* I^{857} and *cro*^{ts}.

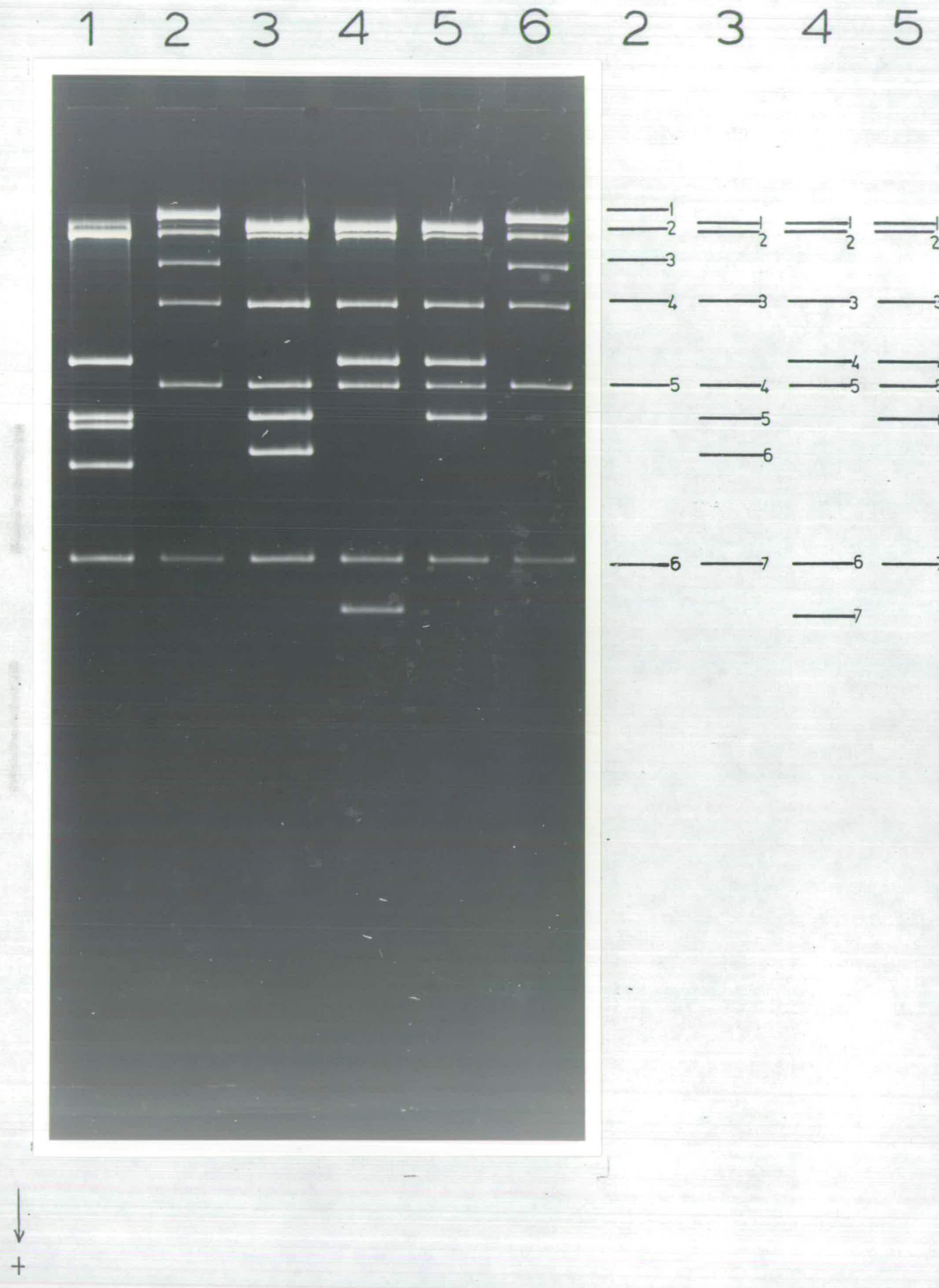

1 2 3 4 5 6 2 3 4 5
1
2
3
4
5
6
2
3
4
5
6
7
2
3
4
5
6
7
+

Fig. 6.9 (opposite)

Restriction Analysis of λ dup 74 Derivatives.

1% agarose gel, all DNA samples digested with R. Eco RI.

Track 1 λ c I_{857} S am_7 DNA
2 λ dup 74 DNA
3 λ dup 74 imm^{21} DNA
4 λ dup 74 cro^{ts} (2) DNA
5 λ dup 74 cro^{ts} (3) DNA
6 λ dup 74 DNA

Bands are numbered in a line diagram to the right of the photograph. The numbers refer to Fig. 6.4 (for track 2) and to Fig. 6.10 (for tracks 3, 4 and 5). All the digests show slight evidence of cohesive end reannealing - this is seen by the faint band between bands 1 and 2 in the λ dup 74 tracks and also by the apparent doublet for the heaviest molecular weight band in the λ cI_{857} S am_7 digest.

The differences between λ dup 74 (track 2) and λ dup 74 imm^{21} (track 3) are the loss of band 3 and the appearance of bands 5 and 6. The change is caused by the introduction of srl λ 4. λ dup 74 and λ dup 74 imm^{21} are effectively the same size, c I_{857} is replaced by imm^{21} (net loss 5%) and nin R5 is replaced by nin^{+} (net gain 5.8%).

The difference between λ dup 74 imm^{21} (track 3) and λ dup 74 cro^{ts} (3) (track 5) is a shift from band 6 to band 4. This represents the 5% gain for a change from imm^{21} to c I_{857} cro^{ts}.

λ dup 74 cro^{ts} (2) (track 4) shows an additional change as band 5 in λ dup 74 imm^{21} has been reduced in size by the nin R5 deletion to give band 7 in λ dup 74 cro^{ts} (2).

Fig. 6.10 (opposite)

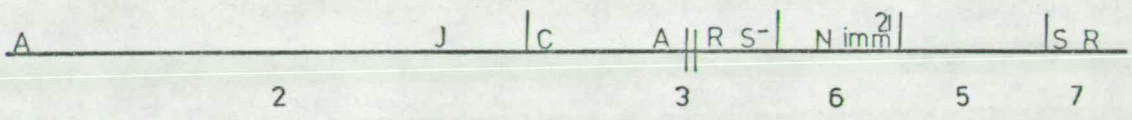
Line Restriction Maps.

Representations of the linear DNA forms for:

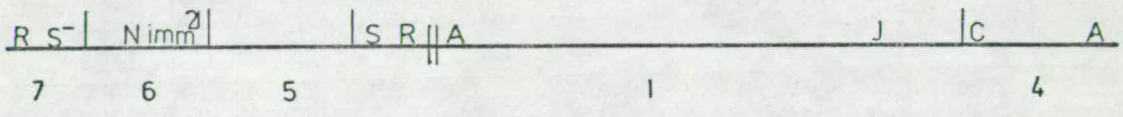
3. λ dup 74 imm^{21}
4. λ dup 74 cro^{ts} (2)
5. λ dup 74 cro^{ts} (3)

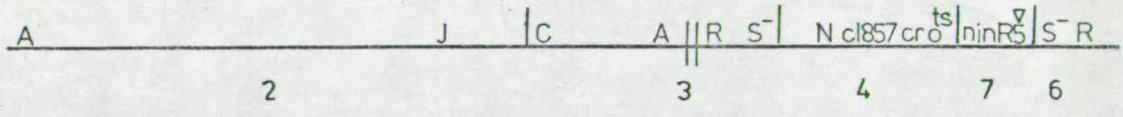
Single vertical lines represent R. Eco RI sites.
Double vertical lines represent covalently sealed phage cos sites.

The numbers (1-7) below the restriction maps refer to the R. Eco RI restriction fragments illustrated in Fig. 6.9. The gel photograph shows that some bands are present in greater than molar amounts and the occurrence of these bands can be explained from the restriction maps.

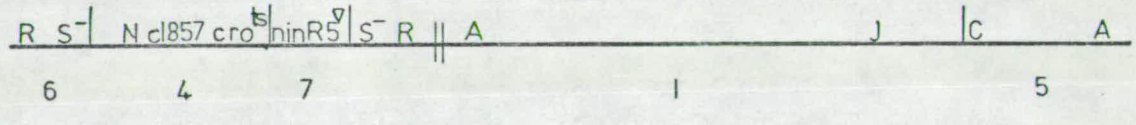


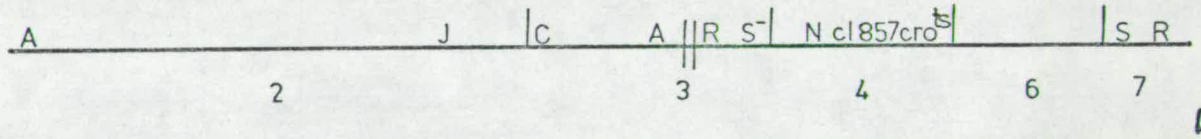
3



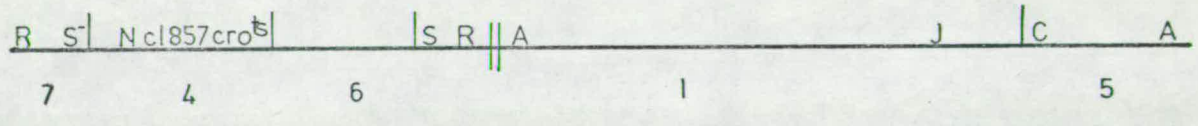


4





5



| R. EcoRI || cos SITE

6.6 Protein Synthesis with the λ Duplication Phage

Bacterial cells were irradiated with ultra violet light prior to phage infection and phage protein synthesis was followed by applying pulses of ^{35}S - L - methionine (Ptashne, 1967; section 2.5 q). Previous investigations, relying on ultra violet irradiation to depress host protein synthesis, have identified the λ structural gene products (Murialdo and Siminovitch, 1972 a; Ray and Murialdo, 1975; Ray and Pearson, 1976 and the references quoted in these papers). There had been indications from the early work (Murialdo and Siminovitch, 1972 a) that protein cleavage was involved with λ morphogenesis; results from pulse chase labelling experiments demonstrated the disappearance of high molecular weight polypeptides with the simultaneous appearance of lower molecular weight species. Ray and Pearson (1976) examined the patterns of protein synthesis from the λ tandem duplication phage isolated and characterised by Emmons and Thomas (1975) but unfortunately the cos site and gene A duplication was not included in these studies.

The results from protein labelling with λ dup 74 have been difficult to interpret. A typical 10 - 20% gradient SDS - polyacrylamide gel (section 2.5 r) is shown in Fig. 6.11. There are several differences between the λ dup 74 samples and λ c I_{857} control samples but there is no simple correlation between the protein band changes and the known genetic composition of phage λ dup 74. The duplicated DNA segment in phage λ dup 74 should contain the following genes S am_7, R, A, W, B and C. Gene C may not be intact in the duplicated DNA

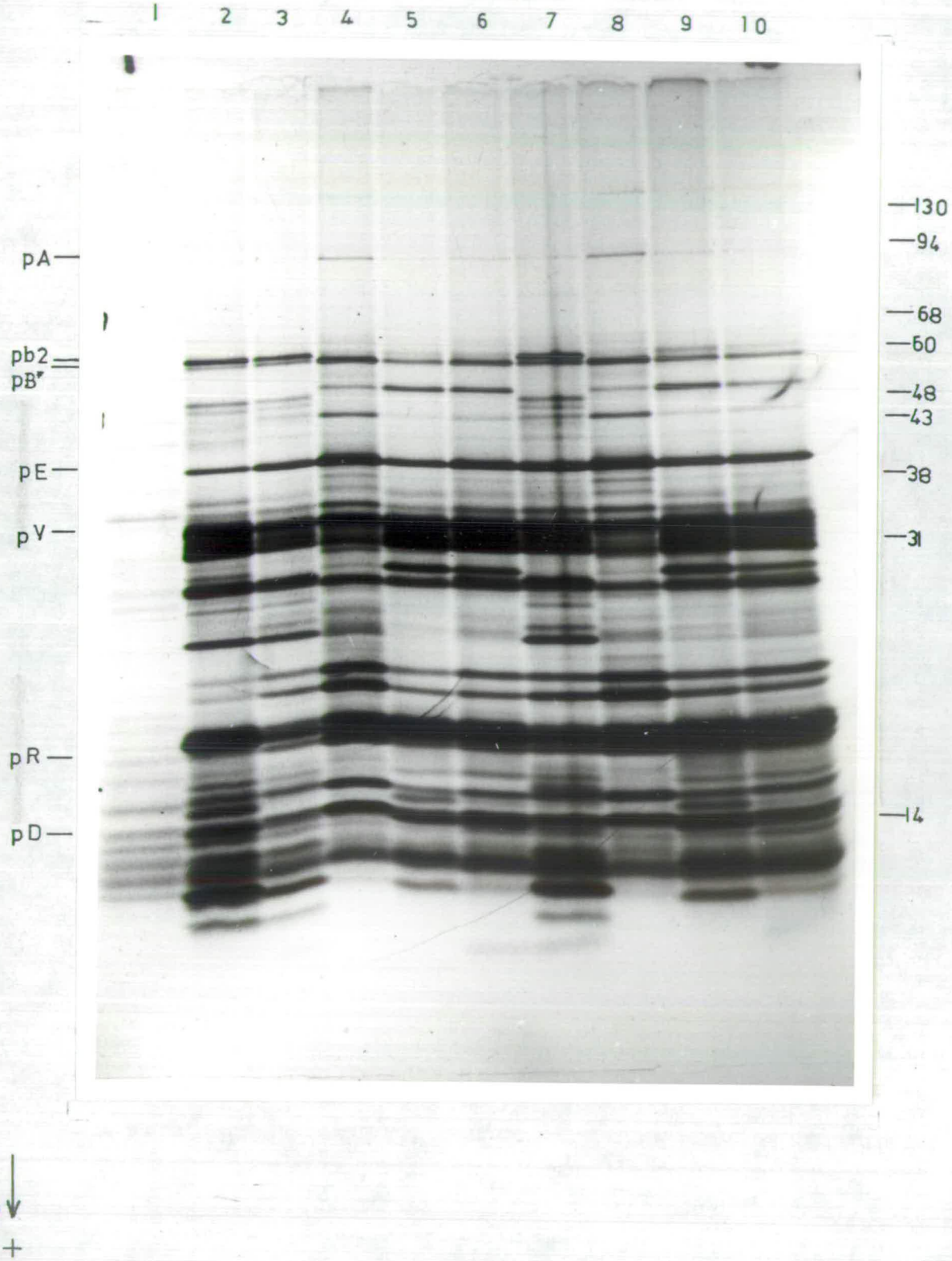

1
2
3
4
5
6
7
8
9
10
pA
pb2
pB*
pE
pV
pR
pD
130
94
68
60
48
43
38
31
14
10%
20%
+

Fig. 6.11 (opposite)

Analysis of Phage Proteins from λ dup 74.

10-20% SDS-polyacrylamide gradient gel

Track 1 Uninfected cells

2 λ^+

3 λ c I_{857} S am_7

4 λ dup 74

5 λ dup 74 cro^{ts} (2)

6 λ dup 74 cro^{ts} (3)

7 λ dup c I_{857} S am_7

8 λ dup 74

9 λ dup 74 cro^{ts} (2)

10 λ dup 74 cro^{ts} (3)

The ^{35}S labelling was performed at 41°C using S 159 as the bacterial host. Samples 1 - 6 were labelled for 5 minutes then chased with cold methionine for 3 minutes. Samples 7 - 10 were labelled for 12 minutes and then chased with cold methionine for 3 minutes.

The proteins pE, pU and pD were detected by staining the gel. Some of the proteins are identified at the left of the gel and approximate molecular weights (x 10^{-3}) are included at the right.

The autoradiograph was developed after 17 days.

because the precise genetic location of the 11.4% R. Bam HI recognition site has not been determined.

A polypeptide of molecular weight about 80,000 is prominent in the λ dup 74 samples (Fig. 6.11) and this is the right size for λ A protein (Murialdo and Siminovitch, 1972 a; Becker and Gold, 1975). There are 3 high molecular weight polypeptides normally synthesised after λ infection, pJ at 130,000 molecular weight, pH at 85,000 molecular weight and pA. The proteins pJ and pH are tail components and are synthesised at about the same time, late in the infectious cycle. The single band in the high molecular weight range can be provisionally identified as pA and the protein is formed at early times because of P_L transcription of the duplicated DNA.

The 60 - 55,000 molecular weight region shows a strongly labelled band in the λ c I_{857} S am_7 tracks that originates from the b 2 region (Hendrix, 1971; Murialdo and Siminovitch, 1972 a) and this band is absent for the λ dup 74 phage (Fig. 6.11). The band increase for λ dup 74 at about 55,000 molecular weight has been identified as pB*, the intact B protein should migrate slower than the b 2 protein in the λ c I_{857} S am_7 tracks. There are new bands at 48,000 and 43,000 molecular weight in the λ dup 74 samples and the relative amounts of these 2 bands differ for the λ dup 74 cro^+ and cro^{ts} phage (Fig. 6.11 tracks 4 - 6 and 8 - 10). The band at about 29,000 molecular weight is at the right position for pX 1 (a fusion product formed between pE and pC, Hendrix and Casjens, 1974); this band

is only present in the λ dup 74 cro^{ts} samples (Fig 6.11) but has been observed with λ dup 74 cro^{+} phage on other gels suggesting that it does represent a cleavage product.

The band changes in the lower molecular weight range have not been explained. Gene R codes for the phage endolysin and a polypeptide at about 18,000 molecular weight could be R protein (Hendrix, 1971). Gene W also produces a very small polypeptide of molecular weight 10 - 12,000 and again there is an increase in band intensity that could be pW. The duplicated gene S contains the am$_7$ amber mutation but the molecular weight of the shortened polypeptide, formed as a result of the amber mutation is unknown.

The identification of the various protein bands is only tentative and an extensive analysis involving different λ phage mutants and two-dimensional electrophoresis would be necessary to provide confirmation. There is no immediate distinction between specific proteolytic cleavage and protein degradation (both Hendrix and Murialdo have unpublished evidence that λ pE is partially degraded by boiling in SDS, quoted by Ray and Murialdo, 1975) but as the bands being considered are all discrete it is likely that they reflect faithful cleavage products. The normal interactions between protein components of the phage head may be disrupted by the presence of increased levels of certain proteins and this could be responsible for novel cleavage products.

6.6 b Synthesis of λ A Protein

The level of A protein synthesis from the λ dup 74 phage was not as high as had been hoped and the λ dup 74 cro^{ts} phage did not cause much stimulation to protein synthesis. Ray and Pearson (1974, 1975) have shown by DNA - RNA hybridisation that the amount of messenger RNA per unit length of DNA in the structural gene region is constant within experimental error. A survey of the inactivation of specific gene transcripts did not reveal any marked differences in messenger RNA stability but gene A was excluded from this survey. These results, together with the study of protein synthesis from tandem duplication phage, lead Ray and Pearson to conclude that regulation of structural protein synthesis is at the level of translation and involves m RNA conformation or ribosome binding. Differential protein stability is not considered as playing a major role in controlling the amounts of individual proteins.

The messenger RNA for gene A could be particularly susceptible to cleavage, or the RNA could assume a conformation that did not favour ribosome binding. Information on the properties of A gene m RNA could be obtained from RNA - DNA hybridisation experiments using restriction fragments of λ dup 74 DNA or different defective λ transducing phage that have replaced phage structural genes with bacterial DNA. Restriction fragments from λ dup 74 DNA could be used for *in vitro* transcription and translation (Yates *et al.*, 1977) as an independent approach to A protein synthesis.

The appearance of labelled proteins that are specific to λ dup 74 infected cells has demonstrated that the duplicated segment of DNA is being transcribed. There were some doubts expressed earlier (page 113) about the potential viability of the duplication phage with the cos site and gene A close to P_L. The single isolation of phage λ dup 74 may relate back to this point and it is possible that the phage λ dup 74 was only isolated because of base change(s) in the A gene coding sequence that prevent or reduce the synthesis of a functional protein (Sternberg, 1977). This could be tested by crossing λ dup 74 with a λ A am mutant to introduce the mutant gene into the left arm of λ dup 74. If duplication phage recombinants, isolated from this cross, can grow in the absence of a suppressor then presumably A protein is being synthesised from the gene adjacent to P_L. A similar cross with a λ C am mutant could establish whether the duplicated DNA contains an intact copy of gene C. There could be a problem with the identification of recombinants from these phage crosses as it might be necessary to perform back crosses in order to verify the presence of the amber mutations.

6.6 c Synthesis of λ Structural Proteins

The original investigations into λ protein synthesis relied on phage that differed by single mutations in the structural genes and missing bands on polyacrylamide gels have been equated with primary gene products (Murialdo and Siminovitch, 1972 a). Some of the λ derivatives described in this work include a mutation in the λ regulatory gene cro and this has a significant effect on the synthesis

of λ structural proteins (see for example Oppenheim et al., 1977). A λ cro$^-$ phage produces less E protein, and correspondingly less of all the other structural proteins, than the equivalent λ cro$^+$ phage (see Fig. 4.3). This observation is explained by a lower rate of DNA replication in the λ cro$^-$ phage so that the number of gene copies is reduced relative to the cro$^+$ phage (Folkmanis et al., 1977).

The phage λ c I_{857} S am_7 has been used as a control to show normal λ protein synthesis. There is a noticeable difference in the amount of λ E protein produced in the λ recombinant phage samples when compared with the λ control (Fig. 6.11 and Fig. 4.3). The λ control phage is nin$^+$ whereas most of the recombinants contain the nin R5 deletion which has removed the transcription termination site tR_2. The synthesis of late proteins begins at slightly earlier times after infection with phage containing the nin R5 deletion and this is the most probable explanation for the different levels of protein synthesis observed with the various λ phage.

6.7 Summary

The phage λ dup 74 allows an alternative approach to the study of λ late gene expression and regulation. The duplicated DNA in phage λ dup 74 has placed some late genes adjacent to the P_L promoter creating the potential for early synthesis of these gene products. Some of the λ structural proteins are synthesised in increased amounts by the λ dup 74 phage but this does not appear to affect phage viability. The duplication of the λ *cos* site provides some insight into the mechanism of λ DNA packaging and this is considered in Chapter 7.

CHAPTER 7 Discussion

7.1 Symmetry in Phage Cohesive End Sequences

Nucleotide sequence information has identified arrangements of symmetrically placed bases within, and immediately adjacent to phage cohesive ends (Wu and Taylor, 1971; Murray and Murray, 1973; Weigel et al., 1973; Murray, K. et al., 1977). The cohesive end sequence has been highly conserved in the lambdoid phage (Murray and Murray, 1973) and only a single variant (ϕ D 326, Rock et al., 1974) has been detected. The changes in sequence that have occurred do not disrupt the symmetrical arrangement of bases and the ter enzyme from phage ϕ D 326 also recognises the normal λ cos site (Murray et al., 1975).

The cohesive end sequences of the P2-type phage differ markedly from the lambdoid sequence and there are small differences in sequence between the members of the P2 class of phage (Murray and Murray, 1973; Murray, K., et al., 1977). The information presented in Chapter 5 is the first convincing demonstration that P2 ter enzyme can interact with an altered cohesive end sequence. Experiments with the satellite phage P4 have shown that phage P2 and 299 are better helpers than phage 186 (Six, E. W., unpublished results). The cohesive end sequences of P2 and P4 are very similar, as determined by an investigation of the thermal stability of end to end DNA aggregates (Wang et al., 1973), but the actual sequence for P4 is still unknown. The differences shown by the P2-type phage

regarding P4 growth may reflect changes other than minor alterations in cohesive end sequence. The identification of the P4 cohesive end sequence should provide further information about base changes that can be tolerated by phage P2, 186 and 299 *ter* enzymes.

7.1 b Theoretical Significance of Symmetrical Nucleotide Arrangements

The symmetrical sequences detected in phage cohesive ends have been examined by a theoretical approach (Brezinski, 1975). A sequence was considered to be exceptionally symmetrical from the probability of a random sequence of equal length having equal or greater symmetry. The calculated figures show that a 22 base pair sequence, with equivalent symmetry to the λ *cos* site, would occur only once in 44,000 base pairs and this does suggest that there is some function related to the symmetry at the λ *cos* site. The same argument may be applied to the symmetrically placed bases found at the 3' ends of P2, 186 and 299 DNA. The symmetry in these phage extends over 7 base pairs from the sites of *ter* enzyme cleavage and more nucleotide sequence information is necessary to locate the end points of the symmetrical arrangements.

A model proposed several years ago suggested that *ter* cleavage could involve 2 copies of the cohesive end sequence and a dimeric form of the *ter* enzyme (Wang and Brezinski, 1973). The 2 cohesive end sequences would be aligned in an anti-parallel sense by a hypothetical protein that would bind to individual *cos* sites

and then protein dimerisation would bring 2 *cos* sites together. The *ter* enzyme, probably 2 catalytic units, would then bind to the dimerised protein and cleave the DNA to form cohesive ends. The link that has been established between DNA maturation and packaging (Hohn, 1975; Bowden and Calendar, 1978) requires a more sophisticated explanation for *ter* cleavage but the notion of *ter* enzyme functioning as a dimer is still attractive. This idea has been supported by the finding that highly purified, concentrated λ A protein aggregates to form a dimeric molecule (Becker *et al.*, 1977 b).

The symmetrical base pairs in the P2-type cohesive end sequence are placed at the limits of a 33 base pair sequence. The putative recognition elements are therefore separated by a distance of about 112 Å but this distance could be spanned by a dimeric protein with spherical subunits of molecular weight about 75,000 (quoted in Murray, K., *et al.*, 1977). This is not immediately compatible with the identification of P2 M protein as the *ter* enzyme (Bowden and Calendar, 1978). Two P2 proteins, pM, molecular weight 28,000, and pP, molecular weight 64,000 (Lengyel *et al.*, 1973) have been shown to be required for P2 *ter* cleavage *in vitro*.

A model that would be consistent with these observations could be based on a dimeric form of P protein recognising the symmetrical arrangement of bases and binding to the P2 *cos* site. Molecules of M protein would then interact specifically with P protein molecules to cleave each DNA strand once at a predetermined site.

The P protein could also have a role in guiding the DNA correctly into the phage head. This suggested scheme would explain the initial co-purification of P2 M and P proteins and also the P2 *in vitro* packaging results which show that a P2 *P* am extract is defective for all packaging whereas a P2 *M* am extract is only defective for the packaging of closed circular DNA (Bowden and Calendar, 1978).

7.1 c Symmetry in Biologically Important Sequences

Nucleotide sequence information is now available for many sites of protein interaction. The *lac* operator contains a 35 base pair sequence in which 28 bases are arranged to show hyphenated rotational symmetry about a central axis and the *lac* repressor specifically protects this sequence from DNase digestion (Gilbert and Maxam, 1973; review Müller-Hill, 1975). More recent work has identified a 17 base pair core sequence that defines the extent of the DNA required for an interaction with the *lac* repressor protein (Bahl *et al.*, 1977).

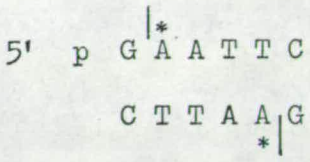
The sequence of the repressor binding site for the λ leftward operator, O_L, has the unusual property of containing 3 different axes which are the centres of hyphenated rotationally symmetrical arrangements (Maniatis *et al.*, 1974). Brezinski (1975) has commented that one of the symmetrical arrangements may not be statistically significant but the other two have sufficient symmetry to imply a biological function. It is attractive to suggest that repressor protein would recognise one symmetrical arrangement and RNA polymerase

would recognise another. There is considerable similarity between sequences for λ O_R and O_L and again 3 axes of two-fold symmetry have been detected (Pirrotta, 1975). Further sequence determination from O_R has included the promoter binding site P_R and 2 axes of symmetry have been detected with a hyphenated arrangement extending over 60 base pairs (Walz and Pirrotta, 1975).

The development of methods for rapid DNA sequencing (Sanger and Coulson, 1975; Maxam and Gilbert, 1977; Sanger et al., 1977) has facilitated the analysis of biologically important regions. Sequences from the λ origin of replication (Denniston-Thompson et al., 1977) and the SV 40 origin of replication (Subramanian et al., 1977) have again disclosed nucleotides in symmetrical arrangements. There has also been a suggestion that rotationally symmetrical bases may be implicated in RNA secondary structure (Schwarz et al., 1978).

The recognition sequences for the type II restriction and modification enzymes contain 4 - 6 nucleotides in a symmetrical arrangement about a central axis (see Table 1.1) (Nathans and Smith, 1975; Roberts, 1976). The 4 - 6 bases appear to define the extent of the sequence for the restriction or modification enzyme binding and this directly implicates the symmetrical arrangement as being responsible for the interaction.

The restriction endonuclease R. Eco RI recognises and cleaves the symmetrical sequence



(Hedgpeth et al., 1972), the modification enzyme interacts at this same sequence to methylate bases (*) (Dugaiczyk et al., 1974; Greene et al., 1974). The restriction enzyme has been purified to homogeneity and the physical and catalytic properties carefully investigated (Modrich and Zabel, 1976). The RI protein has a subunit molecular weight of 28,500 $\pm$ 500 and exists in solution as a mixture of dimeric and tetrameric forms; it is not clear whether both forms possess endonuclease activity. The enzyme cleaves double strand DNA at a recognition site in distinct stages by first introducing a single strand break and then cutting the other strand. The spatial arrangement of DNA and enzyme has not been resolved but it is reasonable to suggest that the dimeric or tetrameric enzyme binds so that the 2 DNA strands are cut by different catalytic units within the enzyme complex.

The occurrence of symmetrical nucleotide arrangements at phage repressor binding sites and restriction enzyme recognition sites implies that the symmetry in the phage cohesive end sequences has some functional significance. This is supported by the conservation of the symmetrical arrangements for all known lambdoid phage. A study of the mechanisms involved with ter enzyme recognition and cleavage can now proceed with purified protein components (Becker, A., unpublished results; Bowden and Calendar, 1978).

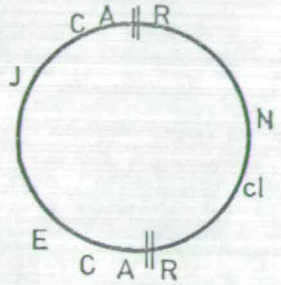
7.2 Phage λ DNA Packaging

The experimental results from the λ duplication phage λ dup 74 (Chapter 6) have some general implications for phage λ DNA packaging. DNA prepared from λ dup 74 phage particles consists of an apparently equal mixture of 2 linear species. It is anticipated that both linear forms would be equally infectious but this has not been verified. At no stage during propagation of the λ dup 74 phage has any tendency for the emergence of a predominant DNA form been noticed. The behaviour of λ dup 74 is not affected by changing to *rec* A$^-$ bacterial hosts; the phage is *red*$^-$ so the generalised recombination system is not responsible for the DNA forms. The interpretation of the mixed DNA types for λ dup 74 is based on DNA packaging from a linear concatemer that has the potential to generate 2 different monomeric units (Fig. 7.1). This situation is analogous to the packaging of the *in vivo cos* duplication phage (Fig. 7.2) (Emmons, 1974).

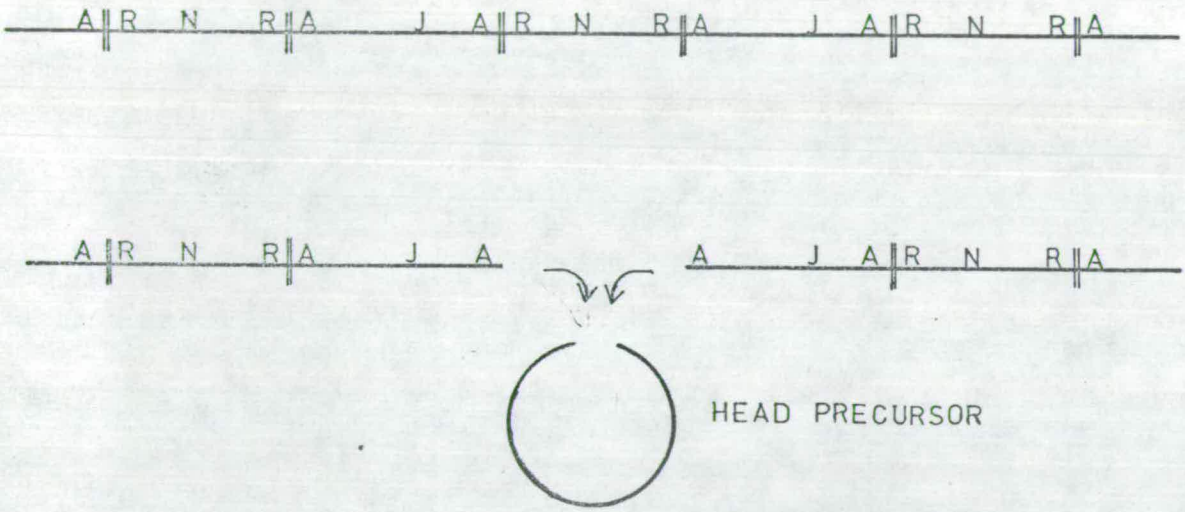
The equal representation of the 2 DNA forms for λ dup 74 indicates that DNA packaging initiates at random internal locations on linear concatemers. Electron microscope observations of λ rolling circle replication tentatively suggest that the leading free end of the rolling circle structure could come from the λ origin of replication (Takahashi, 1974, 1975). Problems with DNA strand breakage during sample preparation prevent a firmer conclusion being drawn from the electron microscope results. Under normal replication conditions, rolling circles pass from right to left i.e. λ gene N is closer to

λ dup 74

A


C A R
J
N
cI
E
C A R

B


A R N R A J A R N R A J A R N R A
A R N R A J A
A J A R N R A
HEAD PRECURSOR

C

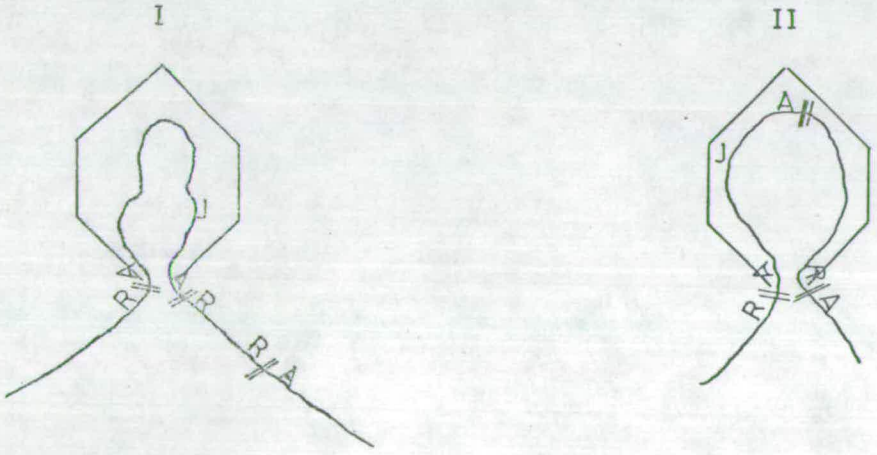

I
II
J
R A
R
R A
A
J
R A
R A

Fig. 7.1 (opposite)

DNA Packaging with Phage λ dup 74.

A A covalently closed monomeric circle of λ dup 74 DNA.

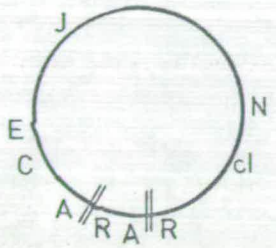
B A linear concatemer of λ dup 74 DNA that can generate 2 different linear monomeric DNA species depending upon which cos sites are recognised and cleaved during the packaging process. The 2 linear monomeric DNA species form identical covalently closed monomeric circular molecules. The packaging of the 2 linear DNA species must involve progress in different directions along the concatemer as the cos site adjacent to gene A enters the phage head first.

C When approximately 55% λ DNA monomer length has been packaged from a λ dup 74 DNA concatemer, 2 cos sites are in close proximity in a parallel arrangement (I). This arrangement does not appear to be cleaved efficiently by λ ter enzyme. When the remaining part of a monomer DNA length has entered the phage head, 2 cos sites are positioned in an anti-parallel arrangement (II) and ter cleavage occurs.

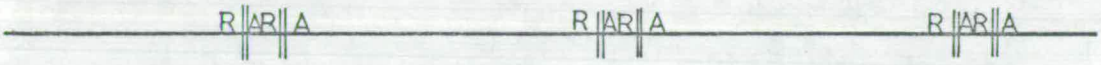
the free end of the replicating structure than λ gene A (Bastia *et al.*, 1975). There have been several independent approaches that have convincingly demonstrated a left to right polarity for λ DNA packaging (Emmons, 1974; Syvanen, 1975; Sternberg and Weisberg, 1975; Chattoraj and Inman, 1974; Saigo and Uchida, 1974). These results strongly suggest that packaging does not initiate at free ends of linear concatemers and, if there is a fixed starting point for rolling circle replication, packaging of DNA from the ends of concatemers could not account for the linear DNA species in phage λ dup 74.

The distribution of phage types found with the *in vivo cos* duplication phage is consistent with processive packaging along a concatemer (Emmons, 1974). Packaging initiates at a *cos* site, a monomer length of DNA is taken into the phage head and *ter* cleavage occurs. The newly generated cohesive end on the concatemer initiates the next packaging event. Emmons has calculated that 2 - 3 monomers are packaged sequentially from a linear concatemer and then packaging begins again at any *cos* site (Fig. 7.2). Estimates for the lengths of concatemeric λ DNA give a figure 2 - 8 times wild type λ length (Smith and Skalka, 1966). Packaging of the 2 types of monomer DNA found with λ dup 74 must involve progress in different directions along the concatemer (Fig. 7.1). This possibly indicates that there is only one packaging initiation event per concatemer because random multiple initiation with λ dup 74 concatemers could result in packaging complexes converging and this would probably reduce

A



B



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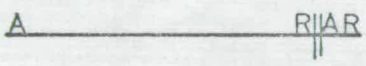

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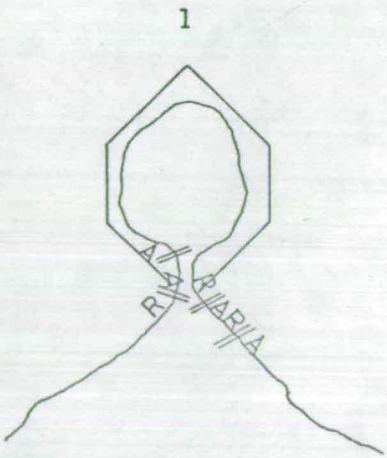


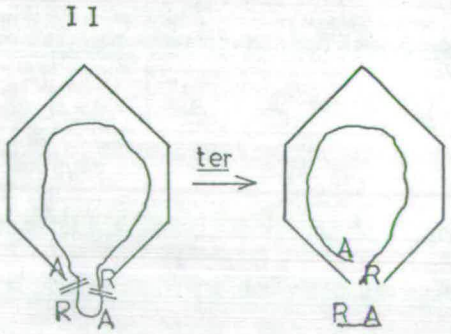


Fig. 7.2 (opposite)

in vivo λ *cos* Duplication Phage (Emmons, 1974).

A. Covalently closed monomeric circle of the *cos* duplication phage DNA showing the tandem arrangement of the duplicated DNA.

B. Linear concatemeric DNA from the *in vivo* cos duplication phage. DNA packaging initiates from *cos* sites adjacent to gene A and the linear concatemer has the potential to form 4 different linear monomeric DNA species as shown. The frequencies of occurrence for the different DNA species have been determined by a combination of density gradient centrifugation and electron microscopy.

C. The duplicated DNA segment is positioned mainly at the left end of linear DNA molecules. A monomer length of DNA enters a phage head and *ter* cleavage occurs at 2 *cos* sites in an anti-parallel arrangement (I). Covalently closed monomeric circles of *cos* duplication phage DNA can be packaged to give viable phage (Feiss and Margulies, 1973). The closed circle of DNA is taken into a phage head structure and λ *ter* enzyme cleaves the 2 cos sites that have become aligned in an anti-parallel sense (II). The phage recovered from this packaging reaction have all lost the duplicated segment of DNA.

the overall efficiency of packaging. An examination of DNA packaging intermediates in the electron microscope could provide further information on this point (Yamagishi and Okamoto, 1978).

Phage λ dup 74 DNA recovered from L-agarose plate lysates (Cameron et al., 1977) has been analysed by agarose gel electrophoresis for half-length DNA molecules that could have been formed by ter cleavage at 2 adjacent cos sites. The failure to detect half length molecules suggested that they do not arise at high frequency but this does not exclude occasional formation. With linear concatemers of λ dup 74 DNA, the appearance of half length molecules would require ter cleavage at 2 cos sites in parallel (Fig. 7.1). Recombinant phage have been isolated from molecular cloning experiments with λ d rif$^{d\ 18}$ that contain the λ cos site in both possible orientations (Newman et al., 1978). These phage appear to grow reasonably well but no specific search has been made for half length DNA molecules. These observations with the cos duplication phage suggest that a minimum length of DNA (probably greater than 50-60% wild-type λ length) enters the phage head before ter cleavage at cos sites can occur.

The packaging of half length DNA molcules has been demonstrated in vitro and, with an R. Bam HI digest of λ DNA, the left terminal 11.4% restriction fragment is specifically incorporated into phage head structures (Hohn, B., unpublished results). Phage heads containing a short fragment of DNA do not attach tails but the complex formed between the head and DNA is stable enough to allow isolation by

by CsCl density gradient centrifugation.

7.2 b λ Defective Particles and DNA Packaging

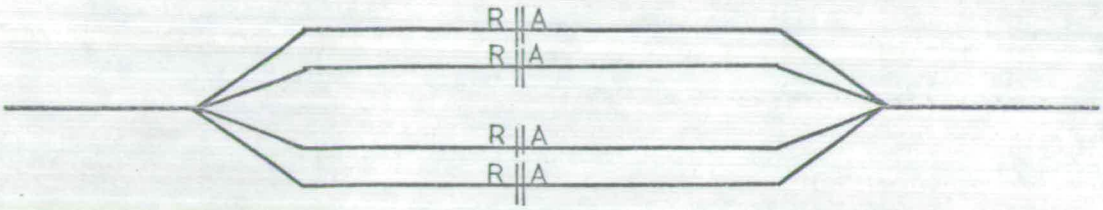
The defective phage particles, λ *doc*, (*doc*, defective one cohesive end) provide information on the specificity of λ packaging. The λ *doc* L and λ *doc* R particles (Fig. 7.3) appear after *ter* cutting and DNA packaging from an induced, excision defective λ prophage (Little and Gottesman, 1971; Sternberg and Weisberg, 1975, 1977 a, b). The particles contain a length of DNA that is part phage and part bacterial in origin and this DNA has only one phage cohesive end. The λ *doc* L and λ *doc* R particles differ significantly in their properties and this is directly related to the mechanisms of particle formation.

When the excision defective prophage is induced, phage replication begins and late gene products are synthesised. In the presence of prehead structures, *ter* cleavage at the prophage *cos* sites occurs and DNA located to the right of the *cos* site is packaged to give λ *doc* L particles. The yield of λ *doc* L particles is increased by DNase treatment *in vitro* to remove protruding ends of bacterial DNA from the phage heads so that tails can be attached. The λ *doc* L particles are non-infectious and this is probably due to a defect in DNA injection resulting from an incorrect alignment between the DNA and the tail (Thomas *et al.*, 1978).

The formation of λ *doc* R particles is not clearly understood.

λ doc PARTICLES

A



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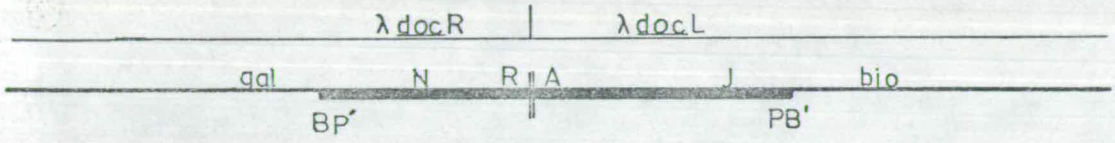

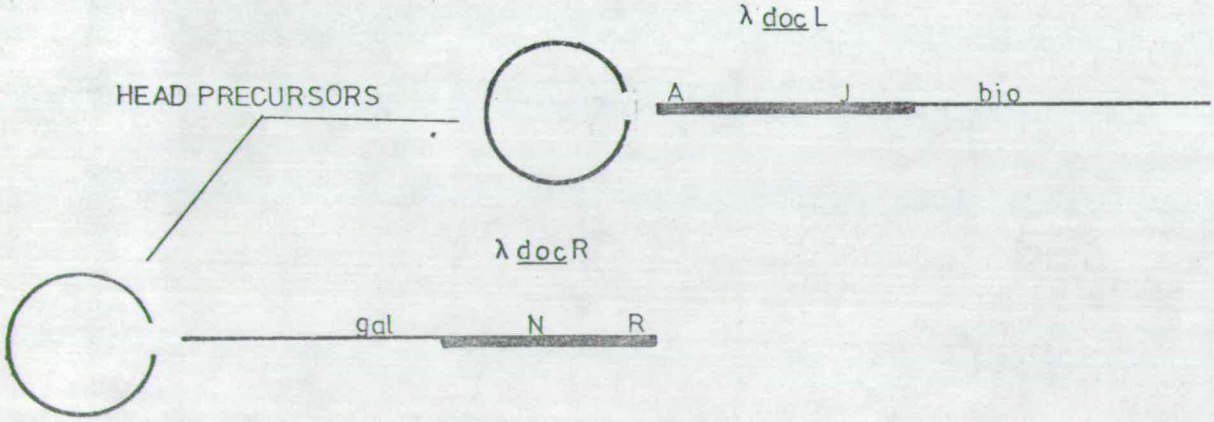

Fig. 7.3 (opposite)

λ Defective Particles.

A. After induction of an excision defective prophage, DNA replication begins from the origin of replication and a 'puff' is produced on the bacterial chromosome. The phage structural proteins are synthesised and, in the presence of head precursor structures, *ter* cleavage occurs at the prophage *cos* sites.

B. DNA to the right of the *cos* site is packaged to form λ *doc* L particles. The yield of these particles can be increased by DNase treatment *in vitro* to remove protruding ends of bacterial DNA so that phage tails can be attached. λ *doc* R particles probably arise by random cleavage of the bacterial DNA and then DNA extending from this break to the phage *cos* site is packaged.

Sternberg and Weisberg (1975) have calculated that there are 4 λ *doc* L and 0.01 - 0.02 λ *doc* R particles produced per induced cell.

Sternberg and Weisberg (1975) have calculated that there are 4 λ *doc* L particles formed per induced cell and that there are 0.01 - 0.02 λ *doc* R particles formed per cell. The λ right cohesive end is formed by *ter* cleavage of the prophage *cos* sites but with the left and right polarity for packaging this right cohesive end should be adjacent to the phage tail. A nuclease activity, λ *ter* or another enzyme, must generate random breaks in the bacterial DNA and, at very low frequency, the DNA between a break in the bacterial chromosome and the phage right cohesive end is packaged into a phage head. There is a considerable size variation for λ *doc* R particles as they range from 80 - 105% wild type λ length. All the λ *doc* R particles attach phage tails correctly and are infectious. The occurrence of λ *doc* R particles indicates that DNA lacking the λ left cohesive end sequences can occasionally be packaged into λ heads. After the initial cleavage of the prophage *cos* site λ pA may have no further role in the formation of λ *doc* R particles.

A study of missense mutations in the λ structural gene region has shown that the formation of λ *doc* L particles is affected by mutations in gene A (Sternberg and Weisberg, 1977 a). Missense mutations in other structural genes do not show this effect. No explanation has been found for this observation but it does suggest that pA has some role in identifying DNA for packaging.

7.2 c The ter Cleavage Reaction

There is cumulative evidence to indicate that ter cleavage at λ cos sites occurs at a late stage in the packaging process. DNA is taken up into the prohead structure and head enlargement occurs; with DNA molecules greater than 80% wild type λ length, D protein is necessary to stabilise the expanded head structure. If D protein is missing there is no cleavage at cos sites and the DNA rapidly dissociates from the head structures. DNA molecules less than 80% wild type λ length can be packaged normally in the absence of D protein showing that pD is not involved with the ter cleavage reaction (Sternberg et al., 1977).

The experiments that demonstrated specific packaging of λ left terminal restriction fragments also comfirmed that pD only binds to enlarged head structures. The left 11.4% R. Bam HI terminal fragment can be recovered from a head structure that has the same dimensions as λ proheads. However, the left terminal 44.5% R. Eco RI restriction fragment is associated with an enlarged head structure that contains pD (Hohn, B., unpublished results). This suggests that head enlargement occurs after a certain minimum length of DNA has entered the head and then the enlarged head interacts with pD. The process of enlargement may be responsible for completing DNA entry into the head.

The demonstration of processive packaging of monomeric units from a linear concatemer requires that the first packaging event differs

slightly from subsequent events. As DNA is taken into the phage head, the left end probably stays in the vicinity of the head opening so that when a monomer length is inside the head, ter cleavage can occur at 2 cos sites in close proximity. This is in general agreement with the model of 2 cos sites in an antiparallel arrangement being cut simultaneously by λ ter enzyme (Wang and Brezinski, 1973). The dimerisation or aggregation of pA molecules could be involved with the alignment of cos sites (Becker et al., 1977 b).

After ter cleavage, the newly generated left cohesive end interacts with a precursor head structure to begin packaging. The important difference in this second event is that a monomer of DNA can be packaged with ter cleavage at a single cos site. The molecules of A protein may still aggregate to cleave the single cos site. The fate of A protein molecules after ter cleavage is unknown. It seems likely that the A protein remains bound to the free left end of concatemeric DNA after ter cleavage to form part of the next packaging complex. The A protein molecules attached to the left end of the newly packaged DNA are not detected in phage particles, possibly these molecules are displaced from the DNA when a phage tail attaches to the head.

The A protein is necessary for packaging linear monomeric λ DNA in vitro confirming that the protein has more than a simple endonucleolytic function. An in vitro packaging system has been shown to be rapidly depleted for A protein suggesting that the protein may be released from DNA in an inactivated form (Hohn, 1975). The cos

duplication phage must contain an additional A protein 'binding site' and it is possible that molecules of A protein could be carried into the phage head during DNA packaging.

7.2 d Packaging of Closed Circular DNA

The failure to detect efficient packaging with monomeric closed circular λ DNA either *in vivo* (Enquist and Skalka, 1973) or *in vitro* (Hohn, 1975) has been explained by suggesting that the λ *ter* enzyme requires 2 copies of the cohesive site before cleavage can occur. The packaging of monomeric circular *cos* duplication phage DNA supported this idea because both *cos* sites were cleaved during the packaging reaction (Fig. 7.2) (Feiss and Margulies, 1973). However, the formation of λ *doc* L particles shows that *ter* cleavage and DNA packaging can occur either at a single *cos* site or at 2 *cos* sites that are arranged in parallel. An alternative explanation for the failure to detect efficient packaging of monomeric circular DNA has been proposed in terms of special structural features assumed by closed circular molecules (Sternberg and Weisberg, 1977 a).

During packaging of concatemeric DNA at least one end of the concatemer projects from the phage head before *ter* cleavage occurs. A closed circular monomer of DNA would be able to enter the phage head completely and may not adopt the appropriate configuration for *ter* cleavage. An examination of the closed circular monomeric DNA, found under conditions where late replication and recombination are blocked, has revealed that about one third of the molecules

have a site specific single strand nick (Reuben and Skalka, 1977). The nicks have been placed at either of 2 unique locations very close to the λ cos site but nucleotide sequencing is necessary to identify these locations precisely. These nicked circular molecules are possibly side products of abortive packaging reactions where a nick has been introduced at the cos site but ter cleavage has not proceeded to completion. It is not known whether λ A protein is involved with the formation of these nicks in closed circular DNA.

The packaging of covalently closed monomeric circular DNA molecules represents a major difference between the assembly processes for phage λ and P2. The basis of this difference is not understood but it is probably limited to the cohesive end sequences and the timing and mechanism of ter enzyme cleavage. In general, there is less information available about the P2 assembly process and the packaging of monomeric circular P2 DNA could be explained by requiring that P2 ter cleavage at a cos site occurs as an initial step in DNA packaging.

7.3 Conclusion

There are many aspects of the λ packaging system that remain unresolved. The introduction of *in vitro* packaging for the recovery of recombinant DNA molecules emphasises the importance of fully understanding the λ packaging process. The λ *cos* duplication phage provides an unusual substrate for λ DNA packaging and could yield further information on the interaction between λ *ter* enzyme and DNA. The λ/P2 recombinants containing the P2 *cos* site are potential substrates for P2 packaging *in vitro*. A complete picture of phage assembly may only emerge from reconstruction reactions involving purified and modified phage components. Some progress has already been made in this direction and it is hoped that the phage recombinants described in this thesis will assist investigations into the phage assembly process.

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