LYSOGENY OF LAMBDOID PHAGES STUDIED
WITH GENETIC FUSIONS MADE IN VITRO

by

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Restriction endonucleases that cleave double stranded DNA molecules to produce discrete DNA fragments with mutually cohesive ends, provide a simple method for the production "in vitro" of genetic rearrangements fusing genes normally unlinked.

This thesis describes the construction of two such genetic fusions and their use in the study of the two processes that govern lysogeny, namely, repression and integration.

In one case the trp genes of a λ trp transducing phage have been fused into the immunity region of the hybrid phage λ imm h3u, by deleting "in vitro" the DNA between two targets for the restriction enzyme R. EcoRI, one of which lies within the cI gene of phage h3u. This imm-trp fusion was used to study the control of repressor synthesis during the establishment and maintenance of lysogeny in the hybrid phage λ imm h3u.

The constitutive rate of expression of the cI gene is between 2 and 5% of the maximally stimulated rate. The products of the cII and cIII genes enhance expression of cI on infection of a sensitive host. The requirement for the cII product is more stringent than that for the cIII protein. The phage h3u repressor present in a h3u-immune cell stimulates the rate of cI expression from a superinfecting homoimmune phage about fifteen-fold. This result strongly suggests that repressor stimulates its own synthesis by a direct effect on transcription of the cI gene.

The second case involved the insertion of a fragment of DNA into the plasmid RP1. The fragment inserted carries the phage attachment region and was used to study the requirements for the
SUMMARY (Contd)

Integration of the phage $\lambda b^2$ into it. Integration of phage $\lambda b^2$ into the P.P' site inserted in the plasmid RPl shows a strict requirement for int protein and a partial requirement for xis. This finding is discussed in terms of the hypotheses put forward to explain the mechanism of the integration-excision reaction of bacteriophage $\lambda$.

Further applications of these plasmid insertions strains for the study of transfer of specific functions from E. coli into other bacterial genera are discussed.
a) The infection process

As a temperate phage, when bacteriophage lambda (λ) infects Escherichia coli, it can follow either of two developmental pathways: (a) the lytic pathway, which culminates in production of about one hundred phage particles and lysis of the cell; or (b) the lysogenic pathway, in which the infected cell survives, the lytic capacity of the virus is turned off, and the viral DNA is integrated into the host chromosome replicating as part of it. These cells have now acquired immunity to superinfection by λ and, either spontaneously (about 10^-5 cells/generation) or under appropriate conditions (releasing repression), they can produce a full burst of phage particles. Such bacteria are called lysogens, since they can give rise to cell lysis. The integrated phage is called a prophage.

In both the lytic and the lysogenic pathways, expression of λ genes takes place in an orderly sequence.

As defined above, the lytic, and lysogenic responses are mutually exclusive, and yet the phage proceeds initially along a common pathway before it is committed to one or the other. Later the two pathways diverge and a choice is made for lysis or lysogeny.

b) The lytic cycle

Immediately after entering the cell, λ DNA forms a circular molecule (Young and Sinsheimer, 1964). Circularisation occurs by covalent joining of the single-stranded, complementary ends, 12
nucleotides long, of the λ strands ("cohesive ends", m and m')
(Yarmolinsky, 1971).

Host RNA-polymerase begins transcribing genes N and cro, to the
left and right of the ρI gene (Fig. 1) The N message is transcribed
from the 1 strand and the cro message from the R strand (Taylor et al.,
1967).

The promoters and operators controlling this transcription are
defined PN and OQ for rightward and PL and O LI for leftward transcription. They map on opposite sides of ρI, within the immunity region.
The promoter regions PL and O LI are defined by sex and x mutants
(Roberts, 1969) and the operators O LI and O R by v2 and v1 v3 mutations
respectively (Hopkins and Ptashne, 1971; Sly et al., 1971).

In the absence of N protein or in the presence of chloramphenicol,
the leftward transcription terminates to the left of gene N and the
rightward to the right of the cro gene (Kumar et al., 1969),
corresponding to the two discrete m-RNAs 123 and 73 transcribed
in vitro in the presence of rho factor (Roberts, 1969) (see Fig. 1).
This stage of m RNA synthesis, carried out solely by the host RNA
polymerase, is called the "immediate early" stage in the sequential
pattern of expression of λ genes that follows infection of a cell
or induction of a lysogen. Sites on the λ DNA responsible for this
termination of transcription have been called L (site near N) and
QL (near cro) (Fig. 1).

In the presence of the N protein, transcription initiated at
PL proceeds leftwards through ρIII - int (even into the h2 region)
(Kumar et al., 1969) and from PL rightwards through ρII, Q, P and
gene θ (Heinemann and Spiegelman, 1970). These transcripts constitute
Legend to Fig.1

Detailed map of the immunity region of λ and adjacent genes and sites

The immunity region of λ is defined physically as the region of the DNA which differs between the genomes of λ and the hybrid phage λimm434 (Kaiser & Jacob, 1957). The genome of λimm434 is composed entirely of λ DNA except for a substitution of phage 434 DNA over a short region extending from 73.6 to 79.1%, measured from the left end of the λ + molecule. This region includes the following genes and sites: \( L, O, \text{rev (in the case of λ), } cI, \text{prm, } R, O \) and \( \text{cro} \) (Westmoreland et al., 1969).

The immunity region of λimm434 is 2% shorter in length and does not contain a \( \text{rex} \) gene.

Another hybrid phage λimm21 carries DNA different from both λ and λimm434 extending from the left of \( N \) till the right of \( \text{cII} \).

Shown as well in the figure are the immediate early transcripts and the transcripts of the \( \text{cI} \) gene.
the "delayed early" stage of phage expression (Fig. 2).

From recent genetic and biochemical experiments (Portier et al., 1972; Friedman et al., 1973; Adhya et al., 1974; Franklin, 1975), the following scheme for N-action has emerged. N protein interacts with transcription initiated at $\Omega_L$ or $\Omega_H$, at a site outside the immunity region, very close to the respective promoter (recognition sites), allowing transcription to be extended through the termination signals $t_{54}^L$, $t_{54}^H$ and $t_{54}^R$ (sites of action) (see Fig. 2). The effect of the presence of N in vivo on early transcription appears identical to that of the absence of rho in vitro, giving support to the antitermination hypothesis postulated by Roberts (1970). It is still unknown, however, whether N protein interacts with rho, with RNA polymerase or with the DNA.

We see therefore, that synthesis of N protein provides for the delayed early stage, characterised by maximal synthesis of proteins concerned with replication, recombination and regulation.

At this point, the lysogenic and lytic pathways diverge, and a choice is made to follow one or the other.

Lytic development into the "late stage" is activated by Q protein. The Q gene-product provides for efficient synthesis of RNA, complementary to the S strand, from the S to R and A to J regions (Dambly et al., 1968) which are covalently linked by joining via the cohesive ends after infection (Yarmolinsky, 1971).

As opposed to the N-dependent early functions, the bulk of late gene expression is independent of transcription initiation at the early promoter $\Omega_H$ (Thomas, 1970). An essential site located between genes Q and S (Herskowitz and Signer, 1970) is required for
Legend to Fig. 2

Positive controls on λ transcription

Relative genetic location of markers is accurate, but distances are not drawn to scale. Structural genes are placed between the two DNA strands, whereas genetic sites (promoters, operators and terminators) are indicated above or below the duplex.

- Represents immediate early transcripts and their extension in the presence of N protein.

- Indicates late RNA synthesis, dependent on Q protein.

- - - - - - - - Transcripts of the cI gene from the two promoters pre and prm.

Regions marked imm434 and imm31 represent areas of non-homology between these two hybrids and λ⁺.
Fig. 2
protein-dependent expression of late genes. Recent studies of in vivo transcription of \( \lambda \) DNA (Roberts, 1975) identify this essential site as the late \( \lambda \) promoter, \( \beta_\lambda^L \), and strongly suggest that \( \lambda \) protein acts by preventing termination of a short \( 63 \) RNA initiated at this late promoter.

In the absence of \( \lambda \)-protein, late functions are expressed to a level of 10-20% of the normal quantities (Dambly and Couturier, 1971), depending on active \( \lambda \) product and \( \beta_\lambda^L \) being accessible by the absence of repressor (or the presence of a mutationally acquired, repressor-insensitive promoter, i.e. \( \beta_{17}^L \) or \( \beta_{L}^C \)). This minor pathway of late expression is thus regulated in exactly the same way as expression of gene \( \lambda \). It consists of an extension, beyond the \( 63 \) RNA terminator site (i.e. \( t_{63}^L \)), of the transcriptional wave normally initiated in the immunity region (Fig. 2).

The \( \lambda \) late genes (\( S, R, \) and \( A \) through \( J \)) are probably transcribed into a single messenger RNA (Gariglio and Green, 1973) and, in order to explain the dramatic differences in the amounts of translation of adjacent transcripts, some post-transcriptional control must be invoked (Murialdo and Siminovitch, 1972).

We can summarize the positive controls of the \( \lambda \) and \( \lambda \) products of \( \lambda \) in the following manner.

Immediately after infection, the circular \( \lambda \) DNA molecule is transcribed by the host RNA polymerase from promoters \( \beta_\lambda^L \) and \( \beta_\lambda^R \) terminating to the left and right of genes \( \lambda \) and \( cro \) respectively (i.e sites \( t_{63}^L \) and \( t_{63}^R \)). The \( \lambda \)-protein will interact with the transcripts initiated at both \( \beta_\lambda^L \) and \( \beta_\lambda^R \), at a site near the transcription initiation site, allowing them to pass through the termination sites.
t_L and t_R2 (rho dependent) and t_R2. This early transcription permits expression of genes required for recombination (red, int), repression (cIII, cII, cro), DNA replication (Q and P) and the positive control element Q. Gene Q protein will permit late transcripts initiated at E_R to pass through the termination site t_R and synthesise late gene m-RNA.

Successful lytic growth requires not only activators N and Q. A gene cro, involved in negative control and its implications in the life cycle of λ, will be considered in the next section.

In summary, we can see that the sequential pattern of expression exhibited by λ during its lytic cycle leads to a maximal release of viral particles. N protein stimulates transcription of genes Q and P (DNA replication) and Q (activator of late genes). Q protein stimulates late gene expression (genes for cell lysis S, R and particle morphogenesis, A through J).

Active DNA replication will amplify late gene expression by increasing the number of gene copies. Cro product ultimately shuts off further synthesis of proteins no longer needed for lytic growth (genes cIII-int and cII-0-P-Q).

The lytic cycle takes approximately 60 minutes and results in the liberation of about 100 new phage particles and lysis of the host.
b-1) The role of cro in lytic growth.

Exonuclease, the product of the early gene $\alpha$, is one of the few genes for which there is available a biochemical assay (Radding, 1964). After induction of a lysogen or following infection, exonuclease synthesis proceeds for several minutes and then stops.

Mutants unable to turn-off exonuclease formation define a gene, cro, (tcf, fed and $\Delta 1$ are probably only alternative denominations) responsible for lowering early transcription initiated at $\alpha_{L}$ (Davison et al., 1971). Complementation studies performed by Pero (1971) had located the gene and its left site of action inside the immunity region (Fig. 1). This site of action seems to be defined by the mutation $\psi 2$ described above as defining the operator $\sigma_{R}$, based upon the observation that $\psi 2$ mutants are deficient in cro-mediated repression of exonuclease synthesis (Sly et al., 1971).

A new site of action for cro gene product (there is not yet evidence that cro codes for a protein) at $\sigma_{R}$ has been postulated. Evidence for this postulate was gained from rather indirect assays on $\lambda$ gene expression (Hampachemova et al., 1973; Echols et al., 1973). The apparent paradox about this site of action for cro-product, is that cro acting at $\sigma_{R}$ and decreasing expression of the $\alpha_{II} - 0 - P - Q$ region would have this same effect on cro-product itself, as the cro gene belongs to this transcriptional unit initiated at $\sigma_{R}$.

In the light of the described effect of cro, turning off early transcription, how does the effect of cro-product relate to the requirement for this product for successful growth of $\lambda$ phage?

The answer to this question has not gone beyond the level of
hypothesis, the form of which varies according to the presence or absence of an active $cI$ - protein. We will describe in section $c - 3$ the interactions between repressor and cro - product.

In summary, the postulated hypothesis to explain the growth defect of $\lambda$ cro - phage can be stated as follows:

cro - product is needed to decrease expression of early transcripts from $\rho_L$ (Kumar et al., 1970, Court and Campbell, 1972) and perhaps as well from $\rho_R$ (Hampecharova et al., 1973). Full level of expression of some of the functions contained in the early $\rho_L$ transcript, namely $cIII$, a hypothetical gene $kI1$ (apparently located between $y$ and $cIII$) (Herskowitz, 1973) and by analogy the $cII$ protein from the $\rho_{II}$ promoter (Fig. 2) are responsible for the inability of a $\lambda$ cro - phage to grow.

The dichotomy originates according to whether the phage carries a functional $cI$ gene or not. We will see in section $c - 3$, that synthesis of repressor (the protein product of the $cI$ gene), itself controlled by the $cII$, $cIII$ and cro products, allows the phage to establish repression midway in the infective cycle, and potentiates lysogeny.

In the presence of a functional $cI$ gene, overproduction by the cro - phage of the $cI$ - activators, $cIII$ and $cII$, leads to excessive and/or premature repression of every infective particles, which in turn will cause the lack of any lytic growth among the phage population and therefore failure to make a plaque on a bacterial lawn.

When an active $cI$ protein is not synthesized, the lack of growth of the cro - phage is explained by the deleterious effect on
the phage and/or the host, of the hyper-production of some $\lambda$ protein in the $\mathbb{N}$ - int operon, perhaps the so-called $\text{Kil}$ function or some other still unknown gene product in this region which is lethal for the virus or the host.

In summary, ocr is needed to decrease early transcription from $\mathcal{D}_L$ and $\mathcal{D}_R$ to achieve: (a) turn-off of repressor synthesis (b) prevent overproduction of $\lambda$ proteins which inhibit $\lambda$-growth.

We will describe next the basic phenomena involved in the lysogenic response.

c) The lysogenic cycle

We defined in section a the lysogenic pathway as the one leading to cell survival, immunity to superinfection and stable association between phage and bacterial DNA. Survival of the host would necessarily imply repressing late lytic functions; stable association of phage and bacterial genomes requires a covalent insertion of the phage DNA into the bacterial DNA (Gottesman and Weisberg, 1971). Successful repression and integration would therefore allow a phage to establish lysogeny.

We will consider as well how lysogeny is maintained, once being achieved.

c-l) General description of the repressor, operators and promoters

The protein product of the gene $\text{O}^L$ defines the repressor (Jacob and Monod, 1961). Repressor bound to operator $\mathcal{O}_L$ blocks leftward transcription of $\mathbb{N}$ and repressor bound to $\mathcal{O}_R$ blocks rightward transcription of ocr (Ptashne, 1971); thus repressor blocks formation
of the only two messages synthesized in the absence of N protein.

The sites of action of repressor, the operators, are defined by virulent mutants able to grow on \(\lambda\) lysogens. Classical virulent mutants of \(\lambda\) carry three mutations (v2, v1, v3) flanking the cI gene, which allow expression of genes N (v2 on the left of cI) and cro, o and P (v1, v3 on the right of cI) (Fig. 2). The hybrid phage \(\lambda\) imm, which has the DNA corresponding to the cI gene and the wild type allelic sites of v2, v1 and v3 replaced by analogous material from phage h3R (Kaiser and Jacob, 1957), is also able to grow in the presence of the cI product present in a \(\lambda\) lysogen.

Purification of the cI product, studies of its interaction in vitro with DNA (Chadwick et al., 1970) and recent nucleotide sequence studies of the operator regions (Maniatis et al., 1974; Waltz and Pirrotta, 1975; Melli et al., 1975) allowed the following picture for the repressor interaction with the operator sites (Fig. 2).

The \(\lambda\) repressor monomer (27,500 daltons M.W) associates into dimers or tetramers as a function of its concentration. The oligomers bind at two separate operators (o_L and o_R), each one of them consisting of multiple, non-identical repressor-binding sites, spanning about 100 base pairs. The sites are arranged in order of decreasing affinity, with the site of tightest binding located adjacent to the controlled operon. Operator mutations have been located in the terminal sites in o_L and o_R (v2, v101 and v3, v326) or in the weaker sites (v1, v2 both in o_R), implying that in vivo, both terminal and subterminal sites are important for operator function.
Legend to Fig. 3

Schematic representation of the λ operators and adjacent genes

The presumed repressor binding sites are indicated by brackets and the positions of some operator and promoter mutations are represented by arrows.

Broken lines represent AT-rich regions between repressor binding sites and the dots indicate unknown sequences in the O 3 region.

The wavy arrows show the direction of transcription of the repressor controlled genes N and cro. Note that the leftward operator is reversed from its usual orientation in the λ map, to emphasize the similarities between the blocks of nucleotides set-off by brackets.

Shown in the figure as well, are the cutting sites for endonuclease Hind II and the DNA regions protected by RNA polymerase and low concentrations of repressor (primary repressor binding sites).

Data extracted from Maniatis et al (1975) and Waltz & Pirrotta (1975).
When increasing amounts of repressor are added to operator DNA in vitro, repressor (dimer or tetramer) firstly fills the terminal sites (about 35 base pairs, S₁ and S₁') and subsequently the secondary ones (15 b.p., S₂ - S₆ and S₂' - S₆') are occupied (probably by addition of a monomer) in the opposite direction to that of transcription of the controlled operon. Three blocks of 17 nucleotides, with an axis of two-fold rotational symmetry drawn through the 9th base, are present in o_L and o_R. These sequences are strikingly similar in both operators and certain pairs of bases on them are always preserved at the same position. These conserved base-pairs are altered in nearly all of the mutant operators that decrease in vitro the affinity of the repressor for the operator sites.

Separating each of these sequences postulated as the repressor-binding sites are variable intervals of high AT-content.

Mutations impairing the function of the leftward promoter P_L (sex 1 and sex 3) and some of those that affect the rightward promoter P_R (sex 3 but not sex 3) are located in the Hind II sites in o_L and o_R (Allet and Solem, 1971), confirming early genetic evidence that in λ, operators and promoters interpenetrate (Ordal and Kaiser, 1973). Recent sequence information on the polymerase-protected fragment on P_R shows that it shares 25 nucleotide pairs with the 35 nucleotide primary repressor-binding site of the o_R operator (Waltz and Pirrotta, 1975). Only the transcription initiation sequence (17 base pairs) is unique to the polymerase site. As the fragment protected by polymerase, in accordance with the molecular size of the enzyme, is not longer than 40 base pairs, it follows that the
sites defined by the mutations sex 1, 3 and X3 define the sites of recognition and entry into the DNA, rather than the initiation site (Fig. 3).

From this information obtained from sequence studies, one can conclude that a mechanism of repression in λ is that repressor covers the promoter and prevents binding of RNA polymerase. The existence of multiple repressor-binding sites, and the finding that polymerase (Saucier and Wang, 1972) as opposed to repressor (Maniatis and Ptashne, 1973), unwinds DNA, allows for a graded control of expression from both P_r and P_L. Finally the existence of different interdigitating symmetries in the controlling regions on the side of the qI gene, could be correlated with the fact that several regulatory proteins, repressor, RNA polymerase and cro-product, interact with these regions of the DNA molecule.

We will refer to the above described nucleotide sequences, flanking the qI gene, in the following sections when describing the mechanisms of action of qI and cro products in controlling repression in an immune lysogen.

c-2) Establishment of repression

Bacteriophage λ forms turbid plaques when infecting E. coli. The turbidity seen in the centre of the areas of lysis is due to the growth of surviving lysogenic cells (Jacob and Wollman, 1954). Mutants that form clear plaques (Kaiser, 1957) and therefore defective in lysogenization, can be ascribed to four complementation groups on the basis of mapping (see Fig. 1), complementation tests and their effect on the frequency of lysogenization. These four
groups are called cI\textsuperscript{−}, cII\textsuperscript{−}, cIII\textsuperscript{−}, which define genes coding for proteins, and a minor group, cE\textsuperscript{−}, which defines an essential region in the DNA between genes cro and cII. Mixed infection of a sensitive cell with wild-type \(\lambda\) and any clear mutant in cI, cII or cIII yields cells lysogenic for both phages; thus the wild-type alleles are dominant over the mutant alleles. Furthermore, from cells infected with pairs of these mutants one may isolate double or single lysogens carrying the cII\textsuperscript{−} or cIII\textsuperscript{−} mutants only, but not cI\textsuperscript{−} mutants only.

These results suggest that the products of all three genes are needed to establish lysogeny, but that only cI product is required for its maintenance. cII\textsuperscript{−} and cIII\textsuperscript{−} mutants lysogenize rarely (frequency approximately \(10^{-4}\) and \(10^{-2}\)), but the lysogens, once formed, are indistinguishable from wild type lysogens. Further support for their role in establishing lysogeny came from the finding that genes outside the immunity region do not function in immune lysogens (Taylor et al., 1967). cY clear mutants complement cII\textsuperscript{−} and cIII\textsuperscript{−} mutants for lysogenization, but do not complement phage bearing amber mutations in the cI gene. On single infection they lysogenize rarely, at a frequency comparable with cII\textsuperscript{−} \((10^{-4})\), and the lysogens formed are perfectly stable (Kaiser, 1957). The mapping and properties of the cY mutants just described, together with the fact that the cII and cIII products are not immunity-specific, led Eisen et al. (1963) to propose that upon infection, repressor synthesis is initiated in the Y region (Fig. 1).

Direct measurements of the kinetics of cI protein production in infected cells has made use of the property of the repressor to bind homologous DNA (Echols and Green, 1971, Reichardt and Kaiser, 1971)
or used a radio-immunological assay (Reichardt and Kaiser, 1971; Reichardt, 1975a).

In this thesis I describe a novel method of following cl expression, consisting in fusing the trp genes of a λ trp transducing phage into the cl gene of the λ imm 434 hybrid phage.

The basic kinetic result is shown in Fig. 8. Substantial production of cl protein does not begin until about 5 minutes after infection; there follows a period of rapid synthesis, followed by a shut-off. This kinetic pattern, and the levels of synthesis, are dependent upon the presence of the clII and clIII proteins (Schols and Green 1971; Reichardt and Kaiser 1971) and a functional clY site (Reichardt and Kaiser, 1971). The turn-off observed after 15-20 minutes is due to accumulation of cro gene product (Reichardt and Kaiser, 1971).

The stimulatory effect of the clII and clIII proteins on cl repressor synthesis is probably mediated by increasing transcription of the cl gene (Spiegelman et al., 1972). In addition to their effect in stimulating transcription of the cl gene, the clII and clIII might provide for an ancillary repression of lytic genes by retarding the synthesis of late proteins (Mcmacken et al., 1970). This repression activity is also eliminated by a mutation in clY−, suggesting that the complete role of the clII and clIII proteins in the establishment of repression may involve bifunctional regulation at a single site clY: positive regulation of the cl gene and negative regulation of lytic genes, exerted at the level of RNA transcription (Mcmacken et al., 1970; Spiegelman et al., 1972).

The fact that clY− mutants do not complement on coinfection with
cl−, might be interpreted by assuming that cY defines the promoter for cl transcription. This promoter has tentatively been called pre (promoter for establishment of repression) by Reichardt and Kaiser (1971). Location of pre in the γ region would imply that on infection of a sensitive host and under the stimuli of cII and cIII, transcription of cl would be accompanied by transcribing the anti-sense strand (the l-strand) of the cro gene. Spiegelman et al., (1972) have described the isolation of this anti-cro message and shown that its kinetics of synthesis parallels repressor protein synthesis or that of cl message. There was no proof, however, that the cl message was covalently linked with the l-strand cro RNA.

This observation that the x region is transcribed in both directions (Fig. 2), has prompted much speculation concerning whether transcription in one direction interferes with expression of genes transcribed from the other DNA strand. It has been invoked as a possible molecular mechanism by which the cII and cIII proteins could repress the expression of late lytic functions and therefore favour lysogeny (Echols 1972).

Another suggestion for the mechanism of action of cII and cIII products advocates a role in avoiding termination of a short transcript (ls) from the l-strand initiated in the genome region named ori (Roberts, 1975).

Clearly a number of possible molecular mechanisms exist, and in vitro biochemical analysis will be required to decide how cII/cIII regulation works.
c-3) Shut-off of repressor synthesis after infection

It was shown that repressor synthesis after infection of a sensitive host levels off approximately 15 minutes after infection (Fig. 5). Mutants in the cro gene fail to show turn off and continue to synthesize repressor for 10-15 minutes longer than cro + phage.

In section (b-1) we explained the role of the cro gene-product as an essential regulatory gene for lytic growth. We saw that cro product was responsible for lowering early transcription initiated at $\beta_L$ and probably rightward transcription as well. This postulated action of cro at $\beta_R$, besides that at $\beta_L$, has been deduced from studies of the growth pattern (Hampacherova et al., 1973) and ability to establish repression (Schols et al., 1973) of $\lambda$, on infection of a host constitutively expressing cro product. Those are defective lysogens of the type $(N^-, o^-, \theta^-)$ or $(N^-, v1 y3, \theta^-)$. The prophage is unable to replicate $(\theta^-)$ or to express any of its N-dependent genes and it has lost the cI function. It is capable however, of expressing the $\lambda$ region where cro is contained.

When $\lambda^+$ infects one of these host constitutively expressing cro, it makes small, clear plaques. The phage has an increased latent period, reduced burst size (Hampacherova et al., 1973) and repressor synthesis does not start (Schols et al., 1973). As $\lambda_{imm} h_{34}$ grows normally in this host, the effect seems to be immunity-specific.

To release $\lambda$ phage from this retarding effect ("channelling effect"), the phage has to have both operators, $\alpha_L$ and $\alpha_R$ mutated (virulent phage $v2, v1 y3$). This result suggests that cro exerts its
negative control not only over the genes to the left of the $\text{cI}$ gene but also over the genes to the right of it. The same conclusion was derived by Echols et al., (1973), studying the kinetics of repressor synthesis after infection by $\lambda^+$ of the cro - constitutive host.

The effect of cro on $D_R$, and possibly $D_R$, would explain how cro causes the turn-off of repressor synthesis seen at late times after infection of a sensitive host. Reducing expression from $D_L$ would diminish synthesis of $N$ - cIII, and from $D_R$ of cII. The instability of N as well as cII and cIII proteins (Greenblatt, 1973; Reichardt 1975a) would give rise to the observed turn-off of cI synthesis.

We have seen, however, that on infection of the cro - constitutive host, repressor synthesis does not start. This discrepancy in the time of appearance of the cro effect may simply be due to the different levels of cro present in a defective lysogen compared with the one reached by a phage on infection.

One problem that still remains unsolved is, why cro delays its action on infection until the onset of late expression? Synthesis of cro should start immediately after infection as a result of translation of the $N$ - independent $\text{cIII}$ message (Section b). The action of cro at $D_R$ will not help in building up a sufficient level of cro product before exerting its action.

Clearly there is not yet sufficient information to explain consistently the mechanism of action of cro product.

Is there any advantage for the phage in having evolved this complex and carefully timed regulatory circuit leading to establishment of repression? In Section c of this Introduction we defined the two
requirements for lysogeny as: repressing the lytic functions and stable integration of the phage DNA into the host chromosome.

We have just seen how repressor establishment depends upon the expression of the phage early functions, cIII and cII. One other early function, the int protein, would be coordinately expressed with cIII (Fig. 2). Int-protein catalyzes the integration of the phage DNA into the host chromosome (Zissler, 1963; Gingery and Echols, 1967) ensuring that stable lysogeny is achieved.

In comparing the two mutually exclusive cycles in λ, lytic and lysogenic, we realize that the phage proceeds into common stages of development (immediate and delayed early) before it is committed to one or the other. Both responses require transcription of coordinately regulated groups of genes, cro - cII - O - P - Q and N - cIII - int, in such a way that the cell at a particular moment has functions which catalyze either response (cI, cII, cIII v.s N, Q, cro). One additional feature of the cI protein, the ability to prevent the action of N and the replication proteins O and P (Thomas, 1966; Thomas and Bertani, 1964) on a repressed λ genome, explains how repression can take precedence over the lytic response. Likewise cro must take precedence over lysogenic processes (establishment of repression and perhaps integration). So, in a simplistic view, perhaps the balance between the cro and cI products may be the prime factor determining the choice in phage λ between the lytic or the lysogenic cycle.

c-h) Maintenance of repression

Once lysogeny has been established, its maintenance requires only
In trying to answer whether repressor was maintaining its own synthesis, several groups (Heinemann and Spiegelman, 1970; Kourilsky et al., 1970; Reichardt and Kaiser 1971; Hayes and Szymbalski 1973) have resolved to compare rates of synthesis of $\sigma I$ m-RNA or protein in a lysogen before and after thermal inactivation of the repressor. This can be studied in cells lysogenic for a temperature-sensitive prophage, $\sigma I$ 857, containing further mutations in gene $\lambda$ and in the $\lambda$ region to prevent killing of the host after thermal inactivation of the $\sigma I$ 857 protein (Eisen et al., 1968). $\lambda^{-}$ mutations are located in the $\sigma R$ - $\rho R$ region and severely reduce early $\rho$-strand transcription (Fig. 2).

The results of the experiments measuring the rate of $\sigma I$ - RNA have been contradictory. Two groups have reported a reduction in $\sigma I$ - RNA synthesis following the heat-pulse (Kourislsky et al., 1970; Heinemann and Spiegelman 1970), suggesting that active repressor was necessary for efficient $\sigma I$ transcription. This explanation was supported by direct measurements of the level of inactive repressor, using a radio-immunological assay (Reichardt and Kaiser 1971; Reichardt 1975b). In contrast, however, the DNA/RNA hybridisation measurements of Hayes and Szymbalski (1973) showed no difference in transcription of $\sigma I$ at the two temperatures.

When these strains were returned to the permissive temperature ($32^\circ C$) after prolonged growth at $42^\circ C$, they immediately began to regain immunity (Oppenheim and Slonim, 1971), suggesting that even after prolonged growth at a temperature ($42^\circ C$) that inactivates the $\sigma I$ 857 repressor, these cells still contain a considerable level of repressor molecules.
In Results section f-2 of this thesis I will present data which suggests that λ repressor has two separate activities; negative control of early RNA synthesis (repression) and positive control of gI expression (self-activation). In temperature-sensitive gI mutants only the repression activity is totally inactivated after the temperature-shift; the inactivated repressor retains its ability to stimulate its own synthesis.

Another line of evidence supporting the role of the repressor in stimulating its own synthesis derives from comparing the rate of repressor synthesis found after infection of a lysogen and a nonlysogen with a phage defective in synthesizing repressor by the establishment mode (i.e. gII-, gY-, or gIII- gII-). Production of gI protein under conditions of prior repression (on the lysogen) is much higher than on infection of the sensitive host (Eschs and Green, 1971; see results section f-1). This finding is readily interpretable in terms of a maintenance mode of gI gene-activation mediated by the gI protein itself. This interpretation is supported by the fact that synthesis of gI protein on a lysogen is markedly decreased by mutations that reduce the ability of gI protein to bind to its right binding site oR (Reichardt 1975b). This result is particularly consistent with the idea that the gI protein activates the maintenance mode of gI gene expression by interaction with a site in the right hand operator oR.

c-5) Cro gene product prevents the regaining of immunity

Derepression of a gI 857 lysogen carrying a prophage mutated in an essential gene (M-deficient or Q-deficient) results in killing of
the host even though no phage are produced (Eisen et al., 1966; Brachet et al., 1970). The phage genes responsible for killing have been identified by isolating mutants that survive prophage induction. In brief, the survivors obtained are defective in early 1-strand transcription (N<sup>-</sup>, sex<sup>-</sup>), causing reduced expression of an early 1-strand function (kil and possibly others. See Fig. 2) and are deficient in replication functions, i.e. are O<sup>-</sup>, F<sup>-</sup>, or x<sup>-</sup>. Although N<sup>-</sup> product is required for full level of Q and P expression, N<sup>-</sup> mutants produce a low level of Q and P proteins (Brachet et al., 1970). Basically two type of survivors are obtained, the one described in the previous section, N<sup>-</sup> cI 857 x<sup>-</sup> and N<sup>-</sup> cI 857 O<sup>-</sup> or P<sup>-</sup>. Both types are sensitive to λ infection at high temperature due to inactivation of the repressor, but differ in their response when returned to the permissive temperature.

Lysogens of the type N<sup>-</sup> cI 857 x<sup>-</sup> begin immediately to regain immunity after transfer to 32°C (Oppenheim and Slonim, 1971). The other type of defective lysogens, N<sup>-</sup> cI 857 Q<sup>-</sup> (or P<sup>-</sup>), do not regain immunity even after many generations at 32°C (Eisen et al., 1970; Neubauer and Calef 1970). Lysogenic carrying both types of defective prophages together, N<sup>-</sup> cI 857 O<sup>-</sup> and N<sup>-</sup> cI 857 x<sup>-</sup>, fail to recover immunity at 32°C. Since x<sup>-</sup> mutations block transcription of the regions x, y and genes cII through q a simple explanation is that the x<sup>+</sup> prophage produces something that blocks repressor synthesis. This explanation is supported by the isolation of derivatives of N<sup>-</sup> cI 857 O<sup>-</sup> which do recover immunity at 32°C after prolonged growth at 42°C (Calef et al., 1971). These prophages have acquired mutations in cro, which are recessive to cro<sup>+</sup> for recovery of immunity and are
located in the x region of the λ map (Fig. 2). The site of action of cro product to prevent the regaining of immunity has been located between the right end of cI and the cro gene itself (Castellazzi et al., 1972).

More recently it has been shown that cro can reduce repressor synthesis even in the presence of functional repressor (Sly et al., 1971; Reichardt 1975b). Therefore, cro product seems to block directly the promoter prm at a site which can not be protected by repressor binding to the right operator sites oR and oR2. One likely hypothesis is that both repressor and cro compete for the leftmost binding site, oR3 (Fig. 3), which is the location that is being assigned to the promoter prm (Mayer et al., 1975).

In summary, cro and cI are involved in a bistable switch. In the immune state, λ repressor is produced and cro product is not; repressor is blocking cro gene transcription (The mechanism of this action has been verified by in vitro experiments. See section c-1). In the anti-immune state (cro constitutive), cro product is synthesized and repressor is not. The mechanism for this action is not yet clear, but it might have evolved to ensure that repression in a prophage is not reestablished once lost, thereby facilitating prophage induction when adverse conditions emerge.

d) Prophage integration and excision

The second step of the lysogenic pathway, following repression, is the attachment of the repressed phage genome to the chromosome of the bacterial host (Lederberg and Lederberg, 1953).

These two steps, repression and attachment, were clearly shown
Fig. 4

Prophage integration and excision
and the origin of transducing phage
et al., 1968; Gottesman and Yarmolinsky, 1968a). The product of the λ int gene promotes the exchange between the E. coli and λ att sites, at the core sequence symbolized by the dot, to yield a prophage whose atts are reciprocal hybrids of bacterial and phage elements:

\[
P.P' + B.B' \overset{\text{int, xis}}{\Rightarrow} B.P' + P.B'
\]

The reciprocal event, excision, requires xis protein as well as int (Echols 1970). The need for both int and xis proteins to catalyze the excision reaction is the best demonstration that the four attachment sites P.P', B.B', B.P' and P.B' are not identical, and that unique elements of att lie on both sides of the cross-over locus, as was suggested by Signer (1968).

Although the nucleotide sequence of the attachment sites is not known, they are thought to have the following structure on the basis of recent genetic data (Shimada et al., 1972, 1973, and 1975). The bacterial att consists of two sequences, B and B', that flank a core sequence represented by the dot. The phage att contains two different sequences, P and P' that flank the same core.

The size of these elements is not known, although upper limits for the size of B and the common core have been estimated at 50 nucleotide pairs from electron microscopy studies (Parkinson, 1971). The cross-over point within the atts is not confined to a unique internucleotide point, but rather it occurs over a region along the common core sequence (Shulman and Gottesman, 1973).

A great deal of information towards the understanding of the integration-pathway derives from the study of the integration
characteristics of transducing phage lines derived from \( \lambda \) inserted at secondary sites. In bacteria lacking B.B' as a result of deletion, \( \lambda \ \text{int}^+ \) can still form lysogens at a reduced frequency (0.1% to 0.5% that of an att\(^+\) host), but still several orders of magnitude higher than that obtained with \( \lambda \ \text{int}^- \) (Shimada et al., 1972). The secondary sites at which the prophage integrates are not random, but seem to occur at loci where the nucleotide sequence resembles that of a normal attachment site, B.B'.

The sequences of these secondary sites are indicated as \( \Delta \cdot \Delta' \), where \( \Delta \) and \( \Delta' \) represent unknown sequences to the left and right of the core sequence, which is believed to be normal (Shimada et al., 1975).

The secondary prophage atts, \( \Delta \cdot \ P' \) and \( \ P \cdot \Delta' \), carried by transducing phage derived from these sites, are similar in structure to the atts of certain \( \lambda \) deletion mutants with specific parts of P.P' deleted (\( \lambda \ b2 \) and \( \lambda \ b517 \) are of the \( \Delta \cdot \ P' \) type and \( \lambda \ b522 \), \( \lambda \ b508 \) are \( \ P \cdot \Delta' \)).

As the formation of these deletion mutants requires int (Davis and Parkinson, 1971), it is suggested that they arise by a mechanism similar to \( \lambda \) insertion at secondary sites; Int-promoted recombination between P.P' and secondary \( \Delta \cdot \Delta' \) sites scattered along the phage chromosome, excises the DNA between the two att's:

\[
\begin{align*}
\Delta & \ P' \\
P & \ P' \\
\end{align*}
\]

These two classes of deletion mutants recombine very poorly with the
bacterial site B.B'. The $\lambda$ class (i.e. $\lambda_b^2$) recombines very efficiently with P.P'. We have made use of this efficient recombination to detect the clones carrying the att $\lambda$ fragment (P.P') inserted into the R-factor RPl. We have pointed out above that both integration and excision are extremely efficient processes. Since both genes int and xis are coordinately expressed from $P_L$, how is the decision to integrate or excise taken after infection?

One possible explanation rests on the finding that xis protein is relatively unstable compared with int (Weisberg and Gottesman, 1971). Following establishment of repression the decay in xis protein activity would shift the equilibrium $P.P' + B.B \rightleftharpoons B.P' + P.B'$ to the right, that is, toward the integrated state of the phage. Excision would be the favoured reaction immediately after the induction of a prophage because of rapid synthesis of the int and xis proteins.

Constitutive transcription of int from a promoter tentatively located in xis (Shimada and Campbell, 1974) will have the effect of directing the insertion-excision reaction towards insertion as well.
The discovery of sequence-specific endonucleases that cleave double-stranded DNA molecules to produce discrete DNA fragments with mutually cohesive single-stranded projections, has made the in vitro rearrangement of segments of DNA a facile procedure (Chang and Cohen, 1974; Morrow et al., 1974; Hershfield et al., 1974; Murray and Murray, 1974).

The R1 plasmid of E. coli specifies one such restriction enzyme, R. Eco R1 (Hedgpeth et al., 1972), which cleaves both strands of unmodified DNA at the specific DNA sequence possessing a 2-fold axis or rotational symmetry, written below:

\[
\begin{align*}
5' & \quad \text{N G A A T T G N} \\
3' & \quad \text{N C T T A A G N}
\end{align*}
\]

The fragments generated by purified endonuclease action on DNA in vitro can anneal together by the complementarity of the single-stranded ends and the breaks remaining in the reassociated molecules can be covalently closed by the action of polynucleotide ligase.

The DNA of bacteriophage \( \lambda \) has five targets for the endonuclease R. Eco R1 (Allet et al., 1973) and their position in the chromosome has been described (Murray and Murray, 1974).

By using deletions and isolation of restriction-resistant mutants, Murray and Murray (1974) have obtained phages carrying any combination of target sites.

From a \( \text{trp} \) transducing phage containing only two of these targets, I have (by deleting the DNA in between them) constructed a fusion of the \( \text{trp} \) genes into the \( \text{oI} \) gene of the \( \text{hI103} \) hybrid phage. Under conditions of tryptophan-mediated repression, the fused
trp genes can be expressed from the phage promoters that normally govern phage repressor synthesis. The activities of the readily assayed products of the trp genes can then be used to study the control of expression of the cI gene during the establishment and maintenance of lysogeny.

I have also isolated the DNA fragment between sites srl λ 2 and srl λ 3 and inserted it into the RI-target of the R-factor RPl. The recombinant plasmid carrying the phage attachment region P.P' has been used to study various aspects of the λ integration process.
a) Bacterial strains

Bacterial strains used in this thesis are listed below:

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<th>Description</th>
<th>Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>sup II, ton A, thr, leu, thi</td>
<td>Appleyard (1951)</td>
<td>N.M</td>
</tr>
<tr>
<td>W3350</td>
<td>sup o, gal -</td>
<td>Campbell (1961)</td>
<td>J.R.D</td>
</tr>
<tr>
<td>Wlh85</td>
<td>sup II</td>
<td>Franklin &amp; Dove (1969)</td>
<td>J.R.D</td>
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<td>CR63</td>
<td>sup I, λ-resistant</td>
<td>Appleyard et al., (1956)</td>
<td>N.M</td>
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<tr>
<td>W3101</td>
<td>sup o</td>
<td>Campbell (1961)</td>
<td>W.J.B</td>
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<td>594</td>
<td>sup o, P2-lysogenic</td>
<td>Henderson &amp; Weil (1975)</td>
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<tr>
<td>QR48</td>
<td>sup II, rec A</td>
<td>Weil &amp; Signer (1968)</td>
<td>N.M</td>
</tr>
<tr>
<td>W3110</td>
<td>sup o</td>
<td>Yanofsky &amp; Ito (1966)</td>
<td>W.J.B</td>
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<td>W3110 sup III trp A oc20</td>
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<td>W.J.B</td>
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<td>JG138</td>
<td>thy -, rha -, lac Rm, str R, sup o</td>
<td>Davison et al., (1974)</td>
<td>J.D.Gross</td>
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<td>ton B - trp AB deletion of JG138</td>
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<td>JD197</td>
<td>trp(AE)-deletion (λ ptrp BG2 N 7,53 ci 857)</td>
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<td>Bw</td>
<td>Partial prophage deletion derivative of JD197</td>
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<td>Bh</td>
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<td>Davison et al., (1974)</td>
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<td>Dx</td>
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<td>J.R.D</td>
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## Bacterial Strains (Contd)

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<tr>
<td>C600groP</td>
<td>gro P,sup II,ton A</td>
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<td>N.M</td>
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<td>KB8</td>
<td>pol A, end A,sup O</td>
<td>Murray et al., (1973a)</td>
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<td>W.J.B</td>
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a) **Bacterial strains** (Contd)

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<td>(1971)</td>
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<td>E. coli C sup^O</td>
<td>Bertani and Bertani</td>
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<td>C-85</td>
<td>E. coli C sup^O str^R</td>
<td>&quot;</td>
<td>W.J.B</td>
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The abbreviations used in the source of strains correspond to:

- Dr. W.J. Brammar (W.J.B), Dr. Noreen Murray (N.M) and
- Dr. J.R. Davison (J.R.D)
b) **Bacteriophage strains**

Bacteriophage strains used in this study are listed below. Recombinants prepared using these strains are described in chapters 3 and 4, and not included in the list.

<table>
<thead>
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<th>Phage</th>
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<td>(\lambda^+)</td>
<td>Hershey (1971)</td>
<td>W.J.B</td>
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<td>(\lambda_{eI} 90)</td>
<td>Kaiser (1957)</td>
<td>J.R.D</td>
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<td>(\lambda_{eII} 26)</td>
<td>Meselson (1961)</td>
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### Bacteriophage strains (Contd)

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c) **Growth media**

All media and solutions were sterilised by autoclaving at 15 lb/sq.in. for 15 minutes before use.

**L-broth (Lennox, 1955)**

- **Difco Bacto Tryptone**: 10g
- **Difco Bacto Yeast extract**: 5g
- **Na Cl**: 5g
- **Glucose**: 1g
- **Distilled water**: 1 litre

The solution was adjusted to pH 7.2 before autoclaving.

**L-agar**

Difco Bacto Agar to a concentration of 1% was added for bottom layer agar.

**Difco Tryptone Agar**

- **Difco Bacto Tryptone**: 10g
- **Na Cl**: 5g
- **Difco Bacto Agar**: 10g
- **Distilled water**: 1 litre

The pH was adjusted to 7.5 with NaOH.

**BBL Agar (Parkinson, 1968)**

- **Trypticase (Baltimore Biological Labs.)**: 10g
- **Na Cl**: 5g
- **Distilled water**: 1 litre

Difco Bacto Agar was added to concentration of 1% for plates and 0.65% for top layers.
c) **Growth media** (Contd)

**Spizizen minimal agar** - (Spizizen, 1958)

- Difco Bacto Agar 15g
- Distilled water 800ml

After autoclaving, the following additions were made.

- 5 x Spizizen salts 200ml
- 20% Glucose 10ml

**ACH agar**

Spizizen minimal agar was supplemented with Difco Bacto acid-hydrolysed casein (Vitamin free) to 0.05% w/v after autoclaving.

**Top layer for minimal and ACH agar plates**

- Difco Bacto agar 7g
- Distilled water 1 litre

**EMBO agar** (Gottesman and Yarmolinsky, 1968)

- Difco Bacto Tryptone 10g
- Difco Bacto Yeast Extract 1g
- Na Cl 5g
- Difco Bacto Agar 15g
- Distilled water 1 litre

The pH was adjusted to pH 6.5 and before autoclaving we added

- $K_2HPO_4$ 2g
- Eosin 0.1g
- Methylene Blue 0.065g
c) Growth media (Contd)

**Phage buffer**

- KH$_2$PO$_4$: 3g
- Na$_2$HPO$_4$: 7g
- NaCl: 5g
- MgSO$_4$, 0.1M: 10ml
- CaCl$_2$, 0.01M: 10ml
- Gelatin, 1% w/v: 1ml
- Distilled water: 1 litre

**5 X Spizizen salts**

- (NH$_4$)$_2$SO$_4$: 10g
- K$_2$HPO$_4$: 70g
- KH$_2$PO$_4$: 30g
- Tri-sodium citrate $\cdot 2$H$_2$O: 5g
- MgSO$_4$ $\cdot$ 7H$_2$O: 1g
- Distilled water: 1 litre

**50 X Vogel Bonner Salts** (Vogel and Bonner, 1956)

- MgSO$_4$ $\cdot$ 7H$_2$O: 10g
- Citric acid $\cdot$ 1H$_2$O: 100g
- K$_2$HPO$_4$: 500g
- Na(NH$_4$)$_2$HPO$_4$ $\cdot$ 1H$_2$O: 175g
- Distilled water: 670ml

2 ml of chloroform was added as preservative, and the solution was autoclaved before use.
37.

c) **Growth media (Contd)**

**Vogel Bonner nutrient medium** (Franklin, 1971b)

- 50X Vogel Bonner Salts 2ml
- 20% Glucose 1ml
- 20% Difco Bacto Casaminoacids 0.25ml
- 2 mg/ml Uridine 1ml
- 2 mg/ml 5-Fluorouracil 1ml
- Distilled Water 100ml

L-Tryptophan was added to a concentration of 2 μg/ml or 200 μg/ml as required.

**Medium A** (Gibson, 1968)

- 50X Vogel Bonner Salts 20ml
- Difco Bacto Yeast Extract 2g
- Difco Bacto Casaminoacids 2g
- L-Tryptophan 1mg
- Distilled water 1 litre

8 ml of sterile 20% glucose was added after autoclaving.

**Medium B** (Gibson, 1968)

- Na₂HPO₄ 12.8g
- KH₂PO₄ 1.36g
- Glucose 18g
- NH₄Cl 2.7g
- MgCl₂ 0.05M 2ml
- L-Tryptophan 2mg
- Distilled water 1 litre
MATERIALS AND METHODS

Methods

d) Plating bacteria

For assaying of phage, making phage stocks and estimation of restriction ratios, bacteria were grown in tryptone broth to mid-logarithmic phase. When the concentration of the culture was ca. $5 \times 10^8$ cells/ml., cells were harvested, washed and resuspended twice, at the same concentration, in $10^{-3}$ M MgSO$_4$.

e) Phage titration

Dilutions of phage stocks were carried out in phage buffer. Phage in a 0.1ml aliquot of suspension were absorbed to 0.1ml of plating cells for fifteen minutes at room temperature. 2.5ml molten BEL top layer agar were added, and the suspensions were immediately poured onto a BEL agar plate. After 12 hours or overnight incubation at the required temperature (usually 37°C), the plaques were analysed and counted.

If the plating strain was trp$^-$, tryptophan was added to a final concentration of 20 $\mu$g/ml throughout the plate.

f) Phage lysates

A well isolated single plaque was picked and transferred to 1ml of phage buffer containing a drop of chloroform, and agitated to release the phages into the buffer. 0.1ml of this suspension or of a 10-fold dilution of it was absorbed to 0.2ml fresh plating cells, mixed with 2.5ml BEL top agar and overlaid onto a fresh 5-agar or tryptone agar plate.
f) **Phage lysates (Contd)**

Once confluent lysis was observed (usually after 6-8 hours at 37°C), the top layer was harvested, combined with 3ml phage buffer and several drops of chloroform, and the mixture was vigorously shaken for 1 min. Cell debris, agar and chloroform were removed by centrifugation, the supernatant decanted, one drop of chloroform added and stored at 4°C.

In the case of phages having a very low burst size, which gave rise to very small plaques, phage stocks were made from dilutions of a previous one derived from a single plaque. An appropriate dilution of the stock (ca. 5x10^5 pfu/ml) was added to 0.2 ml of plating cells as described above.

To avoid recombination with the bacterial trp genes, stocks of λ trp phages were prepared on hosts of the same trp genotype or with those genes removed by deletion.

g) **Phage crosses**

Plating cells or mid-logarithmic cultures grown in L-broth harvested and resuspended in phage buffer, were infected with a mixture of two phages at a multiplicity of infection of 5 phages of each parental type per cell. After 15 min. adsorption at room temperature, unadsorbed phages were removed by centrifugation and the infected cells resuspended at a 100-fold dilution in L-broth.

The infected cultures were grown at 37°C with vigorous aeration for 2 hrs, and the cultures treated with chloroform. The supernatant containing the progeny phages was stored at 4°C.

Recombinant phages were selected on appropriate strains, as described in individual crosses.
g) Phage crosses (Contd)

To increase the recombination frequency between two very closely linked markers, crosses were done on a Thy⁻ host. Cells grown in minimal medium supplemented with thymine (50 μg/ml), to an OD of 1.0 (equivalent to \(5 \times 10^8 \text{cells/ml}\)), were washed and resuspended at the same concentration in phage buffer and left standing at 32°C for 30 min.

Infection and removal of unadsorbed phages were carried out as described above. The infected cells were suspended in minimal medium lacking thymine and agitated at 37°C for 1 hr, then thymine was added (50 μg/ml) and incubation at 37°C continued for an additional hour.

h) Lysogen construction

Cells were picked from the turbid area of lysis produced after o/n incubation, at the appropriate temperature, by a spot of a phage on top layer seeded with the required host, and streaked for single colonies in L-agar.

Individual colonies were tested for lysogeny by streaking a suspension of the colony against streaks of dilutions of the indicator phages, and the plates scored after o/n incubation.

Standard indicator phages were \(\lambda^{\text{cl-I}}\) and \(\lambda^{\text{vir}}\) to test for lysogens carrying \(\text{imm}\ \lambda\), and \(\lambda^{\text{immclhi}}\ \text{cl-I}^{-}\) and \(\lambda^{\text{vir}}\) for the ones carrying \(\text{imm hi}\).
1) **Measurement of the frequency of lysogenization** (Echols et al., 1973)

Fresh logarithmic cultures, at a density of $5 \times 10^8$ cells/ml., were harvested by centrifugation and resuspended in an equal volume of phage buffer. Cells were starved in this medium for 1 hr. at $37^\circ C$ and infected with phage at m.o.i $\leq 10$. After $15\text{'}$ at R.T to allow adsorption to occur, the infected cells were collected by centrifugation and the supernatant containing the unadsorbed phage removed. Pelleted cells were diluted into fresh, warm, L-broth and grown for 1 hr at $37^\circ C$. An aliquot of this suspension was plated on L-agar plates and incubated at $37^\circ C$ o/n to determine the frequency of survivors, the rest was plated on EMBO plates seeded with approximately $10^5$ clear phages of two different host ranges (i.e. $\lambda^R$ and $\lambda^S$), to eliminate resistant colonies ($\lambda^R$) growing on the plates.

Frequencies of lysogeny are normally referred as percentages of infected or surviving cells.

j) **Induction of a lysogen by ultraviolet light**

Lysogenic bacteria were grown to early exponential phase in L-broth ($OD_{650} = 0.5$), harvested in a benchtop centrifuge and resuspended in half the original volume of $10^{-2}$ M MgSO$_4$.

Aliquots of 2 ml were pipetted into an empty petri-dish and exposed to ultraviolet radiation for a dose of $400 \text{ ergs/mm}^2$, diluted 5 fold into fresh warm L-broth and grown, protected from light, for 2 hours at $37^\circ C$. Surviving cells were lysed by the addition of ca.2 drops of chloroform, shaken and cell debris removed by centrifugation.
k) Large-scale liquid lysate of phage

A fresh overnight culture was diluted 1:50 into 11. of L-broth supplemented with Mg SO₄ (final concentration of 10⁻³ M Mg SO₄ for 780, 10⁻² M for λ), using large flasks and vigorous shaking to provide good aeration (i.e. 100 ml of L-broth in a 1 litre flask). When the OD₆₅₀ reached 0.45 - 0.6, phages from a plate lysate were added to give a m.o.i of 1. The OD of the culture normally increased till 1.5-2 and then fell sharply when the cells lysed which usually took 2 to 3 hours. When the OD had reached a minimum 2ml. CH Cl₃ were added, the suspension was shaken for 10 minutes and cell debris removed by centrifugation. Titers between 2-6.10⁻¹⁰ phage/ml were usually obtained.

l) Complementation of clear plaque mutants

Mutants of λ in each one of the clear plaque complementation groups, αI (and αII), αII and αIII were used to test the presence of these genes in unknown phages.

A well dried BHI agar plate was seeded with 0.1 plating cells or 1 drop of a fresh o/n in top layer. Dilution of the phage to be tested, containing 10⁷, 10⁸ and 10⁹ phages/ml were applied as three drops near the side of the plate and allowed to run vertically down the surface of the tilted plate and to dry. At right angles were cross-streaked a suspension of lambda clear mutant containing the same concentrations of phages. The plates were allowed to dry and were incubated overnight. The response was scored as either turbid (tu) or clear (cl) according to whether bacteria were growing in the area of cross-streak. Turbidity signified that complementation between
1) **Complementation of clear plague mutants (Contd)**

The two clear phages had occurred.

Incubation was normally at 37°C except when dealing with its mutants, when either 32°C or 42°C was used.

m) **Conjugation**

Donor and recipient strains were grown to early exponential phase (ca. $2 \times 10^8$ cells/ml) in L-broth and equal volumes of them mixed in a large test tube. The mating mixture was incubated without shaking at either 32°C or 37°C, as appropriate for each case, for 1 hour. After vigorous agitation to disrupt the mating pairs, appropriate dilutions were plated to select the required transconjugants.

n) **Specialised transduction**

A suitable dilution of the phage suspension was adsorbed to 0.2 ml of the recipient strain in $10^{-3} \text{M MgSO}_4$ (plating cells), for 15' at room temperature. The mixture was overlayed onto selective plates.

**Trp** - transducing phages can be selected as "**trp**" - plaques", as described by Franklin (1971b). 0.1 ml of a **trp**" plating strain concentrated to $2 \times 10^9$ cells/ml was plated with 0.1 ml aliquots or, dilutions of an **LFT** (Low Frequency of Transduction) lysate in top layer agar on **ACH** agar plates.

Non-defective **trp** - transducing phages were identified after 36-72 hours incubation at 37°C as "**trp**" - plaques". A circle of lysis could be seen surrounded by a ring of bacterial growth, due to feeding of tryptophan from infected cells. If the **trp** phage is a
n) **Specialised transduction (Contd)**

turbid plaque-former it will produce heavy growth of \( \text{trp}^+ \) lysogens within the \( \text{trp}^+ \) plaque. Clear plaque formers are more difficult to score, and they are often made more easily visible by adding one drop of L-broth to the mixture before plating, to permit improved growth of the background lawn.

o) **Measurement of restriction ratios**

The titre of phages on the propagating, non-restricting host, divided by the titre on the restricting strain, is defined as the restriction ratio.

The RI restriction ratio \( (R_{RI}) \) was estimated as the titre of the phages on the propagating \( 0 \) or \( K \) host, divided by the titre on the \( 0/RI \) or \( K/RI \) host.

Restriction ratios were determined from plate counts of lysates at appropriate dilutions, except when large numbers of phages were handled when approximate values were estimated by spot tests. I will follow throughout this thesis the convention suggested by Arber and Linn (1969), to denote recognition sites for different restriction enzymes.

p) **Determination of phage sensitivity to pyrophosphate** (Parkinson and Huskey, 1971)

Phage lysates were diluted 50- to 100-fold into \( 10^{-2} \text{M P}_2 \text{O}_7 \) \( \text{Na}_4 \), pH 7.0, at \( 45^\circ \text{C} \). The reaction was stopped at various times by diluting samples into phage buffer at room temperature. The samples were plated on BBL-agar containing pyrophosphate added to the bottom layer of the plate, at the concentration previously determined as
p) **Determination of phage sensitivity to pyrophosphate** *(Contd)*
the one required to select for deletions in each particular phage. Concentrations of pyrophosphate, up to 20-30 mM did not interfere with the growth of the bacterial lawn.

q) **Phage buoyant density determinations** *(W. Szybalski in "Methods in Enzymology" vol. XII, part B, p.p. 330)*

Centrifuge tubes containing a 1.5% w/w CsCl solution in phage buffer, were loaded with approximately $5 \times 10^6$ phage, (in a volume ca. 20 ml), of the phage to be analysed, along with a similar number of the chosen density marker, i.e $\lambda^+$ (*ρ* = 1.505).

Centrifugation was for 1.5h at 25K rpm and 25°C in a 3 x 5 MBE rotor. Three-drop fractions were collected from a hole pierced in the bottom of the tube. The refractive index of alternate fractions was determined using an Abbe-31 refractometer and the critical angle values obtained converted into densities of CsCl solutions. The remaining fractions were used for titration of p.f.u by spot tests into selective hosts where one of the two phages contained in each tube was unable to plate (i.e $\lambda^R$, Gro N).

The titres and densities obtained were plotted against fraction number.

r) **Infection experiment** *(Franklin, 1971b; Moir 1975)*

Host bacteria ED 81h9 or ED 872l were grown with aeration in 100 ml L-broth from a 5 ml overnight inoculum grown in L-broth (thymine (100 $\mu$g/ml) was added for strain ED 81h9).

When an O.D. of 1.0 was reached, the culture was chilled on ice and the cells harvested by centrifugation for 10 minutes at 15K rpm at 4°C. The cells were washed and resuspended in ice-cold
Infection experiment (Contd)

$10^{-3} \text{M MgSO}_4$, (plus $20 \mu\text{g/ml 5-fluorouracil if thymine had been added previously}), to a final volume of 10 ml. The cells were infected with phage at the chosen multiplicity of infection. After 15 minutes of adsorption at $32^\circ\text{C}$, the infected cells were cooled on ice, centrifuged at 12 krpm for 10 minutes at $4^\circ\text{C}$ and resuspended in 100 ml. Vogel and Bonner nutrient medium, prewarmed to $37^\circ\text{C}$.

Unadsorbed phages in the supernatant were titred, to determine actual multiplicity of infection.

The infected cells were grown at $37^\circ\text{C}$. The time of resuspension of infected cells was taken as the zero time for measurement of enzyme synthesis after infection. At time intervals from 0-30 minutes, 20 ml aliquots were removed onto chilled tubes containing Chloramphenicol (200 $\mu\text{g/ml}$) and sodium azide ($10^{-2}\text{M}$), and used as samples for enzyme determination. The aliquots were centrifuged at $4^\circ\text{C}$, as before, washed in 0.1 M Tris-HCl, pH 7.8, and resuspended in 1 ml sonication buffer (0.05 M Tris-HCl, pH 7.8, $10^{-3}\text{M EDTA}$, $10^{-3}\text{M 2-mercaptoethanol}$).

Samples were sonicated for 3 x 6 second bursts on a MSE 100-watt ultrasonicator, then centrifuged for 10 minutes at 30,000 x g to remove cell debris. The supernatants were assayed for anthranilate synthetase activity and protein content.

Determination of anthranilate synthetase activity (Itô et al., 1969)

Anthranilate synthetase (A3ase) catalyzes the conversion of chorismic acid to anthranilic acid, both intermediates in the pathway of tryptophan biosynthesis. A mixture of substrates, prepared immediately before use, contained $2 \times 10^{-4}\text{M}$ chorismic acid
Determination of anthranilate synthetase activity (Contd)

(a 10-fold dilution of 1 mg dissolved in 4.4 ml. water), 4 x 10⁻² M MgSO₄, 10⁻² M L-glutamine, 10⁻² M potassium phosphate buffer, pH 7.4 and 10⁻³ M 2-mercaptoethanol. An aliquot of sample, normally 0.1 ml, was added to 1.5 ml of substrate mix prewarmed to 37°C in a quartz cuvette, mixed, and the reaction was allowed to proceed after transfer of the cuvette to a recording Locarte fluorimeter MkH. The fluorimeter was fitted with a water-heated cuvette holder to maintain the temperature of the contents of the cuvette at 37°C. Synthesis of anthranilate was followed as the increase in fluorescence of emission wavelength of 386 nm, using an excitation wavelength of 313 nm. The machine was calibrated with standard concentrations of anthranilic acid. The specific activity of anthranilate synthetase is expressed as units of activity per mg of protein. One unit of activity catalyzes the synthesis of 100 nmole of anthranilate in 20 minutes at 37°C.

The protein concentration in the sample was determined by the method of Lowry et al., (1951), using bovine serum albumin as the standard.

Purification of chorismic acid (Gibson, 1968, 1970; Moir 1975)

A culture of Aerobacter aerogenes, strain 62-1 was grown overnight in L-broth and diluted 10-fold into 10 x 200 volumes of medium A. The cells were incubated in this medium at 30°C for 6 hrs, when they reached an OD₆₅₀ 1.3.

The culture was then centrifuged at 4°C and the cells washed in half the original volume of 0.9% NaCl, centrifuged again and resuspended in the original volume of Medium B. The cells were
19.

**Purification of chorismic acid (Contd)**

Incubated in this accumulation medium for 12 hours at 30°C.

The cells were harvested at 4°C and the supernatant, containing the chorismic acid (a 10-fold dilution of it into 0.1 M NaOH had OD_{274} 1.1) was retained. 5 ml. of 10M NaOH were added per litre of supernatant, and the alkaline solution was passed through a column (2.7 x 7cm) of Permutit Deacidite FF (Cl-form), 200-400 mesh.

The resin was washed with 100ml deionised water, and the chorismate eluted in 1 M NH_{4}Cl at a flow rate of 1.6 ml/min.

8 ml fractions were collected, and after diluting them 200-fold in 0.1 M NaOH, their spectra around 274 nm was measured. The fractions containing the peaks of absorption were pooled and after acidification to pH 1.0 with conc. HCl, were extracted with an equal volume of anhydrous ether. This extraction was repeated twice, the three ether extracts combined and extracted once more with acidified distilled water.

The ether extracts were dried with anhydrous sodium sulphate, then bubbled with air to reduce the volume to ~ 20 ml. Petroleum ether (60-80 b.p) was added till the solution became just turbid and the walls of the flask scratched to facilitate crystallisation. The solution was left overnight at -20°C. The crystals were recovered, washed with cold 50:50 ether:petroleum ether mixture, redissolved in ether and recrystallised by the same procedure. Crystals were dried over conc. H_{2}SO_{4} and paraffin wax and stored dessicated at -20°C.

Pure dry crystals of chorismic acid, dissolved in water at 0.1 mg/ml have an OD_{274 umm} of 1.0.
Preparation of phage DNA

The phage in the supernatant of a large scale liquid lysate were pelleted at 40,000 x g for 3 hrs at 4°C. The pelleted phage were resuspended by gentle rotary shaking (60 rpm) overnight in 1/20 of the original volume of phage buffer. The resuspended pellets were spun at 20,000 x g for 10 min, the supernatant collected and the pellet reextracted with 5 ml of phage buffer, spun as before, and the supernatant added to the one from the first extraction. RNase and DNase were added to a final concentration of 10 μg/ml and the sample digested for 1 hr at room temperature. The phage were pelleted again at 40,000 x g for 3 hrs at 4°C, and the pellets handled in the same way as before.

Caesium chloride was added to 1.5% w/w and the suspension was clarified by centrifugation at 20,000 x g for one hour at 4°C. The suspension was decanted into clean tubes to remove pellicle and sediment and spun to equilibrium for 2h-4h0 hours at 100,000 x g, 4°C. The phage band was collected by puncturing the bottom of the tube, re-banded in pre-clarified 1.5% CsCl as before.

The phage were dialysed against 10⁻² M Tris-HCl, 10⁻³ M EDTA, pH 8.0 for at least an hour to remove the CsCl. The phage DNA was extracted four times, with equal volumes of phenol, freshly distilled and pre-equilibrated with 0.5 M Tris, pH 8.0. The phenol was removed by exhaustive dialysis against four changes of 10⁻² M Tris, 10⁻³ M EDTA, during at least 2hrs. Phage DNA prepared in this way should give a ratio of OD₂₆₀nm between 1.8-2.
v) **Isolation of plasmid DNA** (Thompson et al., 1974)

Cells were grown in 1 or 21 of L-broth to $5 \times 10^8$ cells/ml, harvested by centrifugation and resuspended in 48 ml. of cold 25% sucrose, 0.05 M tris-HCl, pH 8.0. Lysozyme (6.9 ml of freshly prepared solution at 10 mg/ml in 0.25 M tris-HCl, pH 8.0) was added; the mixture was shaken for 30 secs. at 37°C and then placed on ice. After 5 min, 26 ml of 0.25 M EDTA, pH 8.0 were added and after a further 5 min the cells were lysed by adding 54 ml of a solution containing 2% Triton x-100, 0.05 M tris-HCl, pH 8.0, and 0.0625 M EDTA. A period of 20 min was allowed for lysis, and then the crude lysate was cleared at 17 Krpm in MSE 8 x 50 rotor for 15 min at 4°C. (Large plasmids may need shorter spins to avoid pelleting).

The supernatant (non viscous upper layer only), usually ca. 130 ml, was divided into equal volumes and gently layered over 3 ml. saturated CsCl cushions (61.7% W/W) in three Spinco SW 25.2 tubes. Tubes were completely filled by layering distilled water over the cleared lysates.

The DNA was pelleted into the CsCl cushion by centrifugation at 23 Krpm at 10°C in a Spinco SW 25.2 rotor for 16-20 hrs. The lowest 8 ml of each tube were collected and these fractions pooled.

Ethidium bromide was added to a final concentration of 0.5 mg/ml from a stock solution at 10 mg/ml. The density was increased to 1.55 g/cm³ (48.4% W/W CsCl) by adding CsCl. The DNA was banded at 13 Krpm, 15°C in a Spinco SW 50.1 rotor for 24 hrs (Hadlof et al., 1967). The lower, red band, comprising closed circular plasmid DNA, was collected by aspiration (19 gauge needle, 1 ml disposable syringe). The plasmid bands were pooled and recentrifuged in a 5 ml tube for 24 hrs. The plasmid band was collected and the ethidium bromide
**v) Isolation of plasmid DNA (Contd)**

removed by dialysis against 15-20 g. Dowex Na\(^+\) (50 W-X 8, 20-50 US mesh), in 150 ml buffer 0.8 M NaCl, 0.05 M tris-HCl, 0.01 M EDTA, pH 8.0 (Sharp et al., 1972). Resin was prewashed with 2 M HCl, distilled water, 1 M NaOH to convert it to the Na\(^+\) form.

The DNA solution was finally dialysed against 0.01 M tris-HCl, 0.001 M EDTA, pH 7.2, for 2 hr with four changes of the buffer.

**w) Restriction of DNA with Eco RI and Hind III endonucleases**

(Murray and Murray, 1974; Allet and Sollem, 1974; Allet et al., 1973)

DNA was restricted in a mixture of tris-HCl pH 7.5, MgCl\(_2\) and
B-mercaptoethanol, all to a final concentration of 10\(^{-2}\)M. For Eco RI, NaCl to a concentration of 0.1 M was added to inhibit Eco RI activity. In the case of endo R. HindIII, the final concentration of NaCl was 0.05 M.

The mixture was incubated for 1 hr at 37\(^\circ\)C and the reaction was stopped by adding excess EDTA (e.g. to 10\(^{-2}\)M) or heating for 10 min at 70\(^\circ\)C depending upon whether the restricted DNA was going to be used for fragment separation on gels or for subsequent ligation.

**x) Agarose gel electrophoresis** (Brammar et al., 1974)

Samples of restricted DNA were diluted with an equal volume of gel buffer containing 50% glycerol and 0.005% bromophenol blue and added to the wells of a 1% Agarose slab gel (20 cm x 10 cm x 0.3 cm) containing 0.1 \(\mu\)g of ethidium bromide/ml (Sharp et al., 1973). Electrophoresis was continued for approximately 16 hrs at 40 mA, using a continuous buffer system of 40 mM Tris-acetate, 20 mM-sodium acetate and 1mM-EDTA, pH 8.2 (Hayward and Smith, 1972).
x) **Agarose gel electrophoresis (Contd)**

The gel was photographed under U.V light (Chromato-Vue viewing box, U.V products Inc., San Gabriel, Calif.) on Ilford FP4 film with an uX red filter (exposure about 5 min.at f8), and the film was developed for 9 min at 20°C in Microphen developer.

y) **In vitro recombination of DNA fragments using Th-ligase**

(Murray and Murray, 1974)

After stopping the restriction of DNA by a heat pulse, the DNA was cooled, water was added to bring the final DNA concentration to 10-30 μg/ml (Additional Na Cl should be added to maintain it 0.1 M in the mixture), and the mixture was incubated at 0°C for 2h hrs, then at 37°C for 10 min. To this mixture, ligase cocktail was added in 10th. the final volume, from the following stock solution, 0.66 M Tris, pH 7.5; 0.01 M EDTA, pH 9.0; 0.1 M Mg Cl₂; 1 mg/ml BSA; 0.1 M dithiothreitol; 0.01 M ATP, then ligase was added and the mixture was incubated at 10°C for 5 hrs, then 2-10 days at 0°C. Samples were assayed for activity by transfection at periodical intervals.

z) **Transfections** (Modified from Mandel and Higa (1970))

Cells from a fresh culture in L-broth were diluted 100-fold into P-medium and grown overnight at 37°C. This culture was diluted 20-fold into fresh P-medium and grown to an OD₆₅₀ 0.6.

The culture was chilled on ice for 10 minutes, then the cells were harvested by centrifugation in the cold and resuspended in half the original volume of cold 0.1 M Ca Cl₂ and maintained on ice for 20 min. They were then harvested as before and resuspended in one
2) **Transfections (Contd)**

tenth of the original volume of 0.1 M CaCl₂. The cells were kept now on ice for at least 15 min before being used for transfection.

0.1 ml of DNA diluted at a concentration of 3µg/ml in 1 x SSC (0.15 M NaCl; 0.015 M tri-sodium citrate, pH 7.0), were added to 0.2 ml of competent cells. The mixture was heat shocked for 30 secs. at 37°C, then left on ice for about 90 min., with occasional shaking. Samples of the mixture were plated on EHL plates, using EHL top layer with MgSO₄ added to a final concentration of 10⁻³M. If dilutions of the mixture were plated, additional competent cells were added to obtain more vigorous growth of the bacterial lawn.

α) **Separation of DNA fragments by sucrose gradients**

The DNA which had been restricted and heat-pulsed was cooled in ice and diluted into 10⁻² M Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 M NaCl to a concentration of 0.1 mg/ml. The sample in a volume of 0.5 ml was carefully loaded onto a 5-20% continuous sucrose gradient dissolved in 10⁻² M Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 M NaCl, in a tube of an MSE SW 6 x 14 rotor. Centrifugation was at 35 K rpm for 15 hrs at 20°C. The fractions were extracted by pumping a more concentrated sucrose solution (usually 30%) into the bottom of the tube and the OD of the fractions (usually 0.3-0.5 ml fractions) measured in an Isco 222 Ultraviolet Analyser, at 254nm connected to a recorder.

Fractions corresponding to the peaks of a absorption were pooled and the DNA precipitated by adding 2 volumes of absolute ethanol, and stored at -20°C for 2hrs. The DNA was pelleted by centrifugation at 12 K rpm, 4°C for 30' and the pellet resuspended
Separation of DNA fragments by sucrose gradients (Contd)

in $10^{-2}$M Tris-HCl, pH 8.0, 1 M EDTA, 0.2 M NaCl and precipitated again with ethanol as before. The final pellet was resuspended in an appropriate volume of buffer, $10^{-2}$M Tris, 1 M EDTA, pH 8.0 and dialysed against several changes (3) of the same buffer.
CONSTRUCTION AND CHARACTERISATION OF THE imm-lrp FUSION

a) Isolation of the appropriate starting strain RPO

The parent strain RPO, was conceived as a phage having only two targets for the restriction endonuclease R. Ecc RI, sri \( \lambda \) and sri i h3h (see Fig. 5).

The fusion that we were intending to isolate, phage REL2, would involve the deletion of the intervening DNA between those two targets. This region of the \( \lambda \) genome carries only non-essential functions except for the \( \Pi \) gene, which is essential for the full expression of almost all other \( \lambda \) genes (Thomas, 1966). It was therefore necessary to introduce the deletion \( \text{min} R \_\Pi \) (Court and Sato, 1969), that bypasses the \( \Pi \) requirement.

As we were intending to study the role of the different gene products involved in repressor control, it was desirable to start with a phage deficient in some of the genes that participate in repressor regulation (Reichardt and Kaiser, 1971; Echols and Green, 1971), namely \( \text{cII} \) and \( \text{cIII} \). The \( \text{cIII} \) gene maps in the region between sri \( \lambda \) and sri i h3h (Fig. 5) and it was going to be deleted, therefore it was only needed to cross a \( \text{cII} \) mutant allele into the starting strain RPO. No attempt was made to introduce a mutant\( \text{cro} \) gene, another known regulatory gene affecting repressor synthesis (Eisen, et al., 1970). The reason was that \( \text{cro} \) product is needed for phage growth in the absence of active repressor (Herskowitz, 1971) via a still unknown mechanism of action.
a) Isolation of the appropriate starting strain RPO (Contd)

The parental phage RPO was constructed in the following steps.

A \( \lambda \) trp phage, retaining sri \( \lambda \) 3 (Brammar et al., 1974) was crossed with \( \lambda \) pbiolo as illustrated in the diagram below:

\[
\begin{array}{c}
\text{h}^{-80} \text{att}80 \text{trp}^{13} \text{red}^{+} \text{imm} \\
\hline \\
\text{h}^{\lambda} \text{att} \text{pbio}10 \text{imm} h34 \text{cl168 Pam80} \\
\end{array}
\]

Recombinants selected on WL185 (\( \lambda / \lambda \)) as clear plaques arose at a frequency of 0.013%.

The left arm of this recombinant was substituted by that of a phage devoid of targets for R. Eco RI. This phage had a small deletion removing sri \( \lambda \) 1 (N.E. Murray, personal communication) and sri \( \lambda \) 2 was eliminated by replacement with \( \delta 80 \) DNA:

\[
\begin{array}{c}
\text{h}^{-80} \text{trp}^{13} \text{red}^{+} \text{imm} h34 \text{cl2 Pam} \\
\hline \\
\text{h}^{\lambda} \text{trp BG-2} \text{cl1 857} \text{nin-5} \\
\end{array}
\]

The selection was carried on QR 48 tonA, and the frequency of recombination was 0.05%. The recombinants were checked for their trp-content and the presence of the cl2 68 and Pam 80 alleles.

The above recombinant was back-crossed to the Eco RI restriction resistant parent, as follows:

\[
\begin{array}{c}
\text{h}^{\lambda} \text{trp}^{13} \text{imm} h34 \text{cl2 Pam} \\
\hline \\
\text{h}^{\lambda} \text{trp BG-2} \text{cl1 857} \text{nin-5} \\
\end{array}
\]
Legend to Fig. 5

Characteristics of the physical maps of phages RPO and RP12

(a) Map of wild type λ showing the position of the recognition sites for the EcoRI restriction system (▼), the HindIII restriction system (●) and several genes and sites in the λ genome. The scale 0-100% represents the length of λ+ DNA.

(b) RPO, the parental λtrp imm434 hybrid phage, was generated by conventional genetic crosses as described in the text.

(c) and (d) RP12 derives from RPO by deleting in vitro the DNA between sites srIλ3 and srI434, fusing the trpD and E genes to the immunity region.
a) **Isolation of the appropriate starting strain HPO** (Contd)

The selection was carried on W3101 sup^0 rec A for clear phage at 32°C. The recombinants (freq. 0.3%) tested after purification had acquired imm 1,3l, the nin 5 deletion, were F^+ and contained only trp D and E genes from their trp 13 parent. The RI restriction coefficient of this recombinant was 50, indicative of the presence of two targets, srl 3 and srl 1,3l. This was further confirmed by gel electrophoresis analysis of the phage DNA with endonuclease R Eco RI, as will be described in section d of this chapter. The fact that the RI restriction coefficient was only about 50 might be explained by assuming that the RI target contained in srl 1,3l is not as effectively cut as some others in the λ genome (see Murray and Murray, 1974, for comparison of Rc with other targets).

b) **Deletion "in vitro" of the DNA between srl 3 and λ srl 1,3l**

The DNA of the parental phage, HPO, was digested with endonuclease as described in Materials and Methods. The generated fragments were incubated at 10°C for 2 hr in the reaction mixture for T1 polynucleotide ligase, but in the absence of that enzyme, in order to facilitate preferentially the joining of the fragments via the phage cohesive ends and therefore delete the fragment between srl 3 and srl 1,3l.

This mixture was used to transfect the host B03 OEM, obtaining a yield of 1.2 plagues per µg of DNA. Under the same conditions, untreated HPO DNA yielded 5.10^4 plagues per µg of DNA. As B03 OEM is deficient in K-type restriction and modification (hsdR^K, hsdM^K), the plagues obtained from RI-restricted HPO DNA, were replica tooth-picked into strain EK, to K-modify their DNAs.
b) Deletion "in vitro" of the DNA between srl$\lambda$3 and srl$\lambda$h37

Phage from each of the 1h2 plaques obtained, were tested for the phenotype expected of a phage that had lost the DNA between srl$\lambda$3 and srl$\lambda$h37.

Table 1 summarizes the phenotypic differences between the starting strain RPO and a chosen isolate from the transfection, RP 12. Sixteen out of 1h2 isolates appeared to behave identically to isolate RP 12.

c) Comparative physical analysis of the genome of phages RPO and RP 12

The rates of inactivation of lambda phage particles by heat shock or chelating agents (i.e. EDTA, $P_2$O$_7$Na$_4$) is a function of the length of their genomes (Parkinson and Huskey, 1971). The inactivation rate constant ($K$, min$^{-1}$) decreases linearly as the DNA complement of the phage becomes shorter. This property has been used by the above authors to select for $\lambda$ phage deletions.

The kinetics of inactivation of wild type $\lambda$ (genome length 100%), phage RPO and RP 12 by 10 mM sodium pyrophosphate, pH 7.0, at 45°C are presented in Fig.6.

The results obtained allowed the conclusion that the DNA content of RP 12 is considerably less than that of its parent RPO which itself has a shorter genome length than $\lambda$ wild type. By comparison of the rates of inactivation with those of well characterised deletions (i.e. $\lambda$ b 111 91%, b 221 78%, in Parkinson and Huskey, 1971), estimates of 93.5% for RPO and 83% for RP 12 were obtained.
Table 1
Comparative phenotypes of phages RPO and RP 12

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RPO</th>
<th>RP 12</th>
<th>Functions missing in RP 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fec phenotype</td>
<td>+</td>
<td>-</td>
<td>red, gam</td>
</tr>
<tr>
<td>Spi phenotype</td>
<td>+</td>
<td>-</td>
<td>red, gam</td>
</tr>
<tr>
<td>R^ £0 Target for R. ECO 31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cIII - complementation</td>
<td>+</td>
<td>-</td>
<td>cIII</td>
</tr>
<tr>
<td>cI - complementation</td>
<td>+</td>
<td>-</td>
<td>cI</td>
</tr>
</tbody>
</table>

Legend to Table 1
Tests for the presence of the structural genes responsible for the Fec and Spi phenotypes were carried out by spotting appropriate dilutions of phage RPO and RP 12 onto lawns of rec A^- and (P2) lysogenic bacteria, respectively.

The presence of genes cIII and cI was assessed by complementation tests against \(\lambda_{\text{am}}\) \(\lambda_{\text{cIII am 611}}\). Phage suspensions were cross-streaked on a lawn of W3350 on BBL plates and incubated overnight at 37°C. The area of cross-streak was examined for the turbid response characteristic of successful complementation. The loss of a recognition site for R. ECO RI was monitored by the decrease in the restriction coefficient (R. RI) on titration of the phages or appropriately restricting and non-restricting hosts (Murray and Brammar, 1973).
Pyrophosphate treatment of phage $\text{RP}0(\Delta), \text{RP}12(\bullet)$ and $\lambda^+(O)$. The phage were diluted 100 fold into 10mM $P_2O_7Na_4$, pH7.0, at 45°C. The reaction was stopped at various times by diluting the sample into phage buffer at room temperature. Survival is calculated as $\frac{\text{final phage titer}}{\text{phage titer before treatment}}$. 
c) Comparative physical analysis of the genome of phages RPO and RP 12 (Contd)

This result was confirmed by measuring the buoyant densities of both phages in 1M NaCl. The results are presented in Fig. 7.

From the values of the buoyant densities the lengths of the genomes were determined by applying the equation of Bellet et al., (1971),

\[ f = 8.6L \Delta \theta (1-2.14 \Delta \theta) \]

where \( f \) is the length of the deletion in \( \lambda \) units \[ f = \left( \frac{L \lambda - L}{L} \right) \] and \( \Delta \theta \) is the difference in buoyant density of the phage from \( \lambda^+ \left[ \Delta \theta = (\theta \lambda - \theta) \right] \) in 1M NaCl at 25°C.

We obtained values of 93.6% and 83.4% for RPO and RP 12, respectively.

d) Gel electrophoresis of restriction fragments

In Plate I and in the diagram annexed Fig. 8, are represented the products of treating with restriction enzymes R. Eco RI and R. Hind III, the DNAs of the following phages: \( \lambda^+K \), \( \lambda_b 538 \) imm 43h, RPO and RP 12.

The R. Eco RI digestion of RPO DNA produces three fragments of sizes 66.6%, 17% and 10.4% of the \( \lambda \) genome. Digestion of RP 12 DNA with the same enzyme reveals the absence of the 10.4% fragment as the only discernable difference between the two phage DNAs. This fragment must represent the DNA between sri 3, located at 65.6% from the left end of the \( \lambda \) genome (Murray and Murray, 1971; Thomas and Davis, 1975) and sri 1 43h, which locates the latter site at 76% from the left end of the physical map of \( \lambda \).
Legend to Fig. 7

CsCl density gradient analysis of phage X (O), RPO (A), and RP12 (D). Greater density is to the left.

The left. Bp12 (O) greater density is to the right. y (O), Bp12 (A) and of phage y (O), Bp12 (A) and C5L density gradient analysis.

Legend to Fig. 7.
d) Gel elec trophoresis of restriction fragments (Contd)

This result was confirmed by analysis of the digestion products obtained with endonuclease R. Hind III and from double digests with both R. Eco RI and R. Hind III. The positions of the targets for R. Hind III on the λ genome were taken from Murray and Murray (1975) and within the trp operon of E. coli from Hopkins (1975).

RP 1 DNA digested with R. Hind III gives four fragments 55.3, 21.5, 9 and 8.2 % of λ respectively.

RP 12 DNA gives instead three fragments: 55.3, 19.3 and 9 %.

The DNA loss in RP 12 compared with RP 1 amounts to 10.1 %, the same value obtained from the analysis of the RII products.

The lack on the RP 12 digest with R. Hind III of the two fragments 21.5 % and 8.2 % from RP 1, and their replacement by a unique fragment 19.3 %, can only be interpreted by assuming that shd III lies to the left of sri 1, and that the former target has been lost in the construction of RP 12.

Double digests of RP 1 and RP 12 DNA by R. Eco RI and R. Hind III agree consistently with these conclusions and allow the location of shd III to the left of and very close (probably less than 0.5 % of λ) to sri 1.

e) Kinetics of repressor synthesis

Transcription initiated at an upstream promoter was known, in two previously studied operon fusions (Reznikoff et al., 1969; Franklin, 1971), to continue unabated past an operator under conditions that would normally activate repression. It was therefore expected that phage RP 12 would express its trp E and D genes, in the
Kinetics of repressor synthesis (Contd)

Presence of repressing concentrations of L-tryptophan, by transcription initiated at the upstream immunity promoters, pre and prm (Fig. 1), promoters for establishment and maintenance of repression (Reichardt and Kaiser, 1971; Echols and Green, 1971).

We sought to study expression from these two promoters by following the synthesis of anthranilate synthetase, the product of the trp E and D genes, during infection of a sensitive or immune host strain carrying deletions of the trp E and D genes.

e-1) Expression from pre

Repressor synthesis initiated at the promoter for establishment of lysogeny was studied by following the synthesis of anthranilate synthetase after infection of the sensitive host ED 81k9 with phage RP 12, in the presence of 200 μg/ml of L-tryptophan. Replication of the phage was stopped by the absence of Thymine on the medium. Infection of the above host with phage RP 12 results (Fig. 9) in a very low and constant rate of AS ase (anthranilate synthetase) formation. Part of this residual level of expression comes from the repressed trp promoter and the weak, constitutive promoter in xis (Shimada and Campbell, 1971) and can be subtracted from parallel infections with the parental phage RFO (insert to Fig. 9).

Transcription of the λ cI – rex region after infection of a sensitive host requires the active products of genes cII and cIII (Reichardt and Kaiser, 1971; Echols and Green, 1971; Spiegelman et al., 1972; Astrachan and Miller, 1972).

In order to study the specific effects of these controlling
Legend to Fig. 9

Rate of synthesis of Anthranilate synthetase from promoter pre

Sensitive bacteria ED8149 was used as host for infection under conditions of repression of autonomous trp functions.

(0).—The sensitive bacteria ED8149 were infected with RP12 and no helper. Replication was prevented by thymine-starvation.

(▲).—The same host infected with RP12 and cI90 as helper. The ratio of helper to test phage was 2:1. Replication was prevented by thymine-starvation.

(□).—Open squares represent the same experiment as above, but allowing replication of the phage by supplying thymine at 40 µg/ml.

Data corrected for the efficiency of adsorption of the phage.

Insert.—The expression of the trp genes from p1 of parental phage RPO

The strain ED8149(●) and the 434-immune derivative ED8721(x) were infected with phage RPO in the presence of 200 µg/ml L-tryptophan to repress transcription from the trp promoter. Replication of phage DNA was prevented by thymine-deprivation and by immunity respectively.

The specific activities of anthranilate synthetase are normalised to 1.0 phage genome per cell.
Expression from pre (Contd)

elements on pre activity, we co-infected ED 8149 bacteria with RP 12 and various helper phages.

Co-infection by RP 12 and λ cI 90 gives a pattern of enzyme synthesis that is characteristic of gene expression from pre (Reichardt and Kaiser, 1971; Echols and Green, 1971). Enzyme synthesis proceeds at a very low rate during the first five minutes after infection (Fig. 9), then becomes very rapid for the next 7-10 minutes, and later declines sharply. This pattern is basically maintained when varying the ratios of helper/test phage; however the optimal levels of synthesis were achieved for a ratio of \( \frac{2}{1} \) helper/test phage, indicating that the activators provided by the helper phage are in limiting amounts.

A similar pattern of synthesis is seen when the phage DNAs are allowed to replicate, though the yields of enzyme are increased substantially (Fig. 9). The functions provided by the helper phage leading to the trans stimulation of pre are the products of genes cII and cIII, as can be concluded by examining the effect of different helper phages in Table 2.

Comparing the stimulatory effect that λ cI 90 provides, (provision of cII and cIII), in column 2 with the one obtained on mixed infection with the double mutant λ cII 68 cIII 67 (column 5), we can conclude that only the products of genes cII and cIII have an effect in activating expression from promoter pre. This excludes any direct involvement of any other protein of λ in expression from pre (i.e. N protein). The separate effect of cII and cIII proteins can be assessed by using helper phages lacking either of them.
### Table 2

**Effect of cII and cIII on expression from preAnthranilate synthetase (rel. spec. act)**

<table>
<thead>
<tr>
<th>Phage</th>
<th>None</th>
<th>cI 90</th>
<th>cIII 611</th>
<th>imm 21</th>
<th>cII 68</th>
<th>cIII 67</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP12</td>
<td>10</td>
<td>100</td>
<td>46.5</td>
<td>24</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

**Legend to Table 2**

Results are expressed as the percent of the level of ASase accumulated in ED 8149 cells 15 minutes after being infected with RP12 and cI 90 as helper.

The multiplicities of infection were 2 for RP12 and 4 for the helper. Replication of the phage was prevented by thymine-starvation of the thy- host.
e-1) Expression from pre (Contd)

For instance, column h shows that the provision of the cIII-product alone, by λ imm 21, allows only 24% of the maximum activity.

The provision of the cII-product from a λ cIII- helper allows 96% of maximum helper activity (column 3), confirming the more stringent requirement of the cII protein to achieve normal levels of repressor after infection of sensitive bacteria (Reichardt and Kaiser, 1971).

e-2) cro-gene product turns off expression from pre

We saw in Fig.9, that expression from pre does not continue indefinitely at a high rate, but levels off at about 15 minutes after infection. Phage with a mutant cro gene fail to stop repressor synthesis at the normal time (Reichardt and Kaiser, 1971). The cro-product is known to decrease cIII and cII gene expression by acting at or near the promoters P_L and P_R (Fero, 1971; Hampacherova et al., 1973; Echols et al., 1973) that govern their expression.

It was therefore reasonable to hypothesize that the cII and cIII proteins would be unstable, as otherwise the action of cro-product would only stabilize, not reduce the rate of repressor synthesis. This hypothesis has been proved to be correct by Reichardt (1975a) for the cro product of λ imm l3h and in this thesis for the analogous one from λ+.

The experiment shown in Fig. 10, test this hypothesis. The host ED 8lhl9 was co-infected with phage RP 12 and with λ cI ts or λ cII ts cro 1 as a helper, at multiplicities of 2 and h respectively. Replication was stopped by Thymine starvation of the host, and the cells were incubated at 42°C so that the repressor protein of the
Comparison between a \textit{cro}^+ versus a \textit{cro}^- helper phage in promoting expression from \textit{pre}.

Conditions for infection are the same as in Fig. 9.

The ratio helper to test phage was 2:1.

(▲) Sensitive bacteria ED8149 infected with \textit{RP12} and \textit{λcIts} as helper.

(●) The same with helper \textit{λcIts cro}^-.

(The values represent average of two experiments)
e-3) Promoter pre is insensitive to catabolite repression (Contd)

by their phages \( \lambda \) and P22, suggested that some host-controlled
elements are important in bacteriophage development, affecting the
choice between the lytic or lysogenic cycle.

We have investigated directly whether the expression from pre
was affected under conditions causing catabolite repression in the
host. In this way we could dissociate from the complex lysogenic
response, any effect of c-AMP on the rate of repressor synthesis.

In Fig. 11, we see that the rate of synthesis of AS ase from
ED 8149 cells mixedly infected with RP 12 and helper \( \lambda gI 90 \)
(m.o.i 2 and 4) does not differ in cells grown with glucose or with
glycerol as carbon source. The samples taken were simultaneously
assayed for anthranilate synthetase and \( \beta \)-galactosidase.

Cells grown in glycerol show the anticipated difference in
capacity to produce \( \beta \)-galactosidase, proving that conditions of
catabolite repression were achieved (insert to Fig. 11).

We conclude from this experiment that c-AMP is probably not
a major physiological effector for regulation of the synthesis of
gI protein from promoter pre in lambdoid phages. It seems reasonable
that the effects on lysogency attributed to the cyclic AMP concentration
in the host are probably due to indirect effects of the deranged
gene activation system in the mutant strain (Jordan et al., 1973).

It should be remembered that the establishment of lysogeny
requires both repression and integration. For instance, mutants in
the cro gene showing increased expression of genes under the \( \beta_L \)
promoter, \( \beta \) through int (Court and Campbell, 1972), and lack of
turn off of gI expression initiated from pre (Reichardt and Kaiser,
Fig. 11

Effect of glucose on expression from pre

Bacteria ED8149 were starved in 10mM MgCl$_2$ and 40 $\mu$g/ml thymine for 60 minutes at 37°C before being infected with phage RP12 and $\lambda$CI90 at multiplicities of 2 and 4 respectively.

The infected cells were diluted into high tryptophan minimal medium (Brammar et al., 1974) with glucose (O) or glycerol (●) as carbon source. Samples were taken and assayed for anthranilate synthetase and $\beta$-galactosidase (Insert).

Cells grown in glycerol show the anticipated difference in capacity to produce $\beta$-galactosidase, proving that conditions of catabolite repression were achieved.

Key to insert: (o) cells grown in glucose.

(●) cells grown in glycerol.
e-3) Promoter pre is insensitive to catabolite repression (Contd)

1971) were "a priori" supposed to lysogenize every infected cell. They are known now to do so only very rarely (Galland et al., 1975). This example should be taken into account when measuring the effect of host mutations, impairing the levels of expression of early operons of \( \Lambda \) (Buchanan and Markowitz, 1973; Belfort and Wulff 1971, 1973; Rolfe et al., 1974), on lysogenization frequencies, as this process seems to require a delicate balance in timing and levels of expression of the repressor protein and other early functions coordinately expressed under \( \Lambda \) control.

f) Expression from prm

f-1) Kinetics of de ase on infection of a lysogenic host

In a lysogenic cell, the only functions that the prophage expresses are the ones encoded inside the immunity region (Fig. 1), namely the \( \sigma I \) gene (and \( \sigma R \) in the case of \( \Lambda \)).

The repressor acting at the two operators \( \sigma_L \) and \( \sigma_R \) flanking the immunity region, abolishes expression of any other phage function (Thomas, 1966; Ptashne, 1967; Chadwick et al., 1970). The same mechanism accounts for the immunity of the lysogen to superinfection by another phage with the same immunity.

Therefore, infection of a lysogenic cell by another phage with the same immunity should provide a way of determining how repressor synthesis is maintained in a lysogen.

We have seen in Results-section e-1 that infection of the sensitive host, trp- ED 81h9, by phage RP 12 in the presence of 200 \( \mu g/ml \) of L-tryptophan results in a very low rate of anthranilate synthetase synthesis.
Legend to Fig. 12

Rate of synthesis of Anthranilate synthetase from promoter prom

(●) Lysogenic bacteria ED8721 infected with RP12 alone. Replication in this case was prevented by the immunity of the λimm434 lysogenic host.

(O) The sensitive bacteria ED8149 infected with RP12 and no helper. Replication was prevented by thymine-starvation.

Activities in both cases are normalised to an effective m.o.i=1, to compensate for gene dosage effects.

The description of the Insert is the same as in Fig. 9.
Anthranilate synthetase (Specific activity)

Minutes after infection

0 5 10 15 20 25 30 35

0.01 0.02 0.03 0.04 0.05 0.06 0.07

0 5 10 15 20 25 30 35
Kinetics of AS ase on infection of a lysogenic host (Contd)

The rate of enzyme production is markedly increased when RP 12 infects an immune host, ED 8721, under similar conditions; anthranilat synthetase starts immediately after infection and proceeds at a linear rate for at least 25 minutes (Fig. 12).

This pattern of expression contrasts with that shown on infection by the parental phage, RPO, when trp gene expression from the phage promoter, $P_L$, is repressed in the immune host (insert to Fig. 12). It is clear from these results that the fusion phage RP 12 can express its trp genes from a phage promoter that is activated in the immune host.

This promoter should be located inside the immunity region, in the only operon that is expressed in a lysogen in the presence of active repressor. This promoter has been named $prm$ (Reichardt and Kaiser, 1971).

We can see in these experiments that the presence of the imm $H^3$ repressor stimulates the rate of expression from $prm$ of the superinfecting RP 12 about fifteen to twenty fold above the level obtained on infection of the sensitive host. We believe that the repressor present in the lysogenic host is responsible for this increase in $cI$ expression. The way in which repressor enhances expression from $prm$ could be a projection of a dual activity: a) Indirectly, by inhibiting cro-product from acting at $prm$ (Eisen et al., 1970) b) Directly by stimulating $cI$ transcription (Reichardt, 1975b).

Complementation between $\lambda cI$ ts and phage deficient in repression establishment

The complementation pattern obtained on mixed-infection of a
f-2) Complementation between $\lambda$ $\alpha$I ts and phage deficient in repression establishment (Contd)

Sensitive host with different pairs of clear mutants is presented in Table 3.

$\lambda$ $\alpha$I - shows a pattern basically similar to that of $\lambda$ $\alpha$I - except for the fact that the former can be complemented to lysogeny by temperature-sensitive $\alpha$I mutants at the non-permissive temperature (Column 2 of table 3) (Brachet and Thomas, 1969). Complementation was obtained as well when $\lambda$ $\alpha$I and other phages defective in repressor establishment ($\alpha$II - , $\alpha$III - , $\alpha$II$^*$$\alpha$III - ) singly infected a host Bh, containing a $\alpha$ro-deleted defective prophage, carrying the $\alpha$I 857 allele (Table 1, column 2).

The prophage genetic map of the defective lysogen Bh is shown in Fig. 13. This strain was isolated by Dr J. Davison (Davison et al., 1971) as a survivor at $h_2^0$C of a lysogen containing $\lambda$ $p$ $t$ $r$ $p$ BG-2 $N$ $\alpha$I 857 as a prophage. Some of the bacteria surviving the heat treatment at $h_2^0$C carry extensive deletions from the prophage late region into the $\alpha$ro gene, as shown by marker rescue experiments and inability to show constitutive expression of $\alpha$ro (anti-immune state) when the culture is returned to $32^0$C after being grown for several generations at $h_2^0$C.

The characteristic clear-plague morphology produced on infection of the standard host W 3350 by $\lambda$ $\alpha$I - contrasts with the turbid one obtained when the same phage infects the defective lysogen Bh at $h_2^0$C (See plates in annexed paper in the Appendix).

In Table 4 we present evidence that the effect is immunity specific, since there is no response with any $\lambda$ $\text{imm}$ $h_3$: clear.
## Table 3

**Complementation pattern of λ cY mutants**

<table>
<thead>
<tr>
<th></th>
<th>cl</th>
<th>cl ts</th>
<th>clI</th>
<th>cl II</th>
<th>cl III</th>
<th>cl Y 112</th>
<th>cl 857</th>
</tr>
</thead>
<tbody>
<tr>
<td>cl</td>
<td>cl</td>
<td>cl</td>
<td>tu</td>
<td>tu</td>
<td>cl</td>
<td>cl</td>
<td></td>
</tr>
<tr>
<td>clY</td>
<td>cl</td>
<td>tu</td>
<td>tu</td>
<td>tu</td>
<td>cl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend to Table 3**

Phage suspensions were cross-streaked on a lawn of W 3500 on ERL plates and incubated overnight at 37°C. The area of cross-streak was scored as either turbid (tu) or clear (cl). Clear mutants used were those described in section b of Materials and Methods. Experiments involving cl ts mutants were performed at 42°C.
<table>
<thead>
<tr>
<th>Genotype of infecting phage</th>
<th>W 3350 or Bh cI</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ</td>
<td>tu</td>
<td>tu</td>
</tr>
<tr>
<td>λ cII 90</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ cII 14</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ cII ts 857</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ cIII 68</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ cIII 611</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ cII 68 cIII 67</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ cIV h2</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ cIV 8h4</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ cIV 2001</td>
<td>cl</td>
<td>tu</td>
</tr>
<tr>
<td>λ imm h3h</td>
<td>tu</td>
<td>tu</td>
</tr>
<tr>
<td>λ imm h3h cI</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ imm h3h cII 61</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ imm h3h cIII 67</td>
<td>cl</td>
<td>cl</td>
</tr>
<tr>
<td>λ imm h3h cIV h2</td>
<td>cl</td>
<td>cl</td>
</tr>
</tbody>
</table>

**Legend to Table h**

Phage suspensions were plated to give single plaques and scored as clear (cl) or turbid (tu), after overnight incubation at 42°C. Recipient bacteria were grown overnight and subcultured at the same temperature.
Legend to Fig. 13

Genetic map of the right arm of bacteriophage λ and of the defective lysogen Bh

Strain Bh derives from a λtrp transducing phage in which the genes int-cIII of λ are replaced by tonB and the trp operon of E.coli, λtrpBG-2. The transducing phage was made to lysogenize the tonB-trp deletion strain JD197 and a second deletion isolated which removes all the λ genes except N, rex and cI (Davison et al., 1974).
(Contd) Complementation between λ cI ts and phage deficient in suppression establishment

This observation confirms at the same time the absence from the deleted-prophage strain Bh of both cII and cIII genes and therefore eliminates any interference in the described turbid-response observed with λ cY− being mediated via pre. (The heteroimmune λ imm h3l cIII−, cIII− or cY− should induce prophage complementation for cII and cIII if they were present, and therefore become turbids).

To prove that the trans acting product from the prophage Bh is in fact the repression-inactive cI 857 protein, we isolated mutants of Bh which have permanently lost immunity by acquiring an additional mutation in cI besides the cI 857 allele. The isolation of such mutants was possible since strain Bh expresses the trp operon from the leftward promoter Lr, which is subject to negative control by the λ repressor. Thus at 32°C, Bh is both immune (since it is cRO defective) and a trp auxotroph. Selection of revertants of Bh able to grow at 32°C without exogenous tryptophan allows the isolation of mutants of Bh carrying a cI defect in addition to the cI 857 allele already present (Davison et al., 1971). Twenty independent, spontaneously arising cI− defective mutants of Bh were isolated and tested for their ability to give turbid plaques when various clear-plaque mutants were plated on them (Table 4, column 1). All twenty isolates behaved identically in that they were unable to give a turbid plaque with homo-immune λ cY, λ cII and cIII mutants, showing that the inactive cI 857 repressor was responsible for the complementation observed in the parent strain Bh.

A similar result was obtained on mixed infection of the sensitive host W 3350 by λ cY and the double mutant λ cY cI 857 at 42°C.
71.

f-2) Complementation between \( \lambda \) cI ts and phage deficient in repression establishment (Contd)

(Table 3, column 5). This proves that on mixed infection, the positive complementation obtained between \( \lambda \) cY and \( \lambda \) cI ts depends on a full level of synthesis of the inactive cI ts protein from pre and that if this synthesis is limited by a mutation in the pre promoter (\( \lambda \) cI ts cY) complementation is not obtained.

The above results can be explained in two ways.

a) Cells infected with a \( \lambda \) cY- or \( \lambda \) cII cIII produce a low but detectable level of active repressor monomers (approximately 1-2% of wild type levels. Reichardt and Kaiser, 1971). If the inactive cI 857 monomers, produced at normal rates, can form active hybrid dimers with wild type repressor monomers, then the active dimer level on mixed infection by \( \lambda \) cI ts and \( \lambda \) cY-, or on infection by \( \lambda \) cY- or cII-cIII of the defective cI 857 lysogen B\(_h\), could be as high as four percent of the normal level, and might permit complementation to occur.

This hypothesis would be strengthened if active repressor, instead of being a dimer (Ptashne 1971), is a higher order oligomer.

However, this explanation is considered unlikely because this hypothetical twofold increase in the amount of active repressor, would probably not be sufficient to account for the intense turbid response obtained. Furthermore, mutants in gene cIII exhibit a clear phenotype, despite the fact that they produce 4-7 fold more repressor than cY mutants (Reichardt and Kaiser, 1971).

In the light of the evidence supporting the hypothesis that wild-type repressor stimulates cI transcription from pre (Reichardt 1975b; This Thesis, results section f-1), we will propose the following alternative hypothesis to explain the complementation observed between cI ts v.s cY.
b) The repression-inactive $\lambda_{cl857}$ protein synthesized at $42^\circ$C remains active in the positive control of $\lambda_{cl}$ transcription from $\text{prm}$ (Pastrana and Davison, 1974. See paper enclosed in the Appendix).

The temperature-sensitive repressor activates transcription of the wild-type $\lambda_{cl}$ gene, (present in both $\lambda_{clY}$ and $\lambda_{clIIcIII}$) from the maintenance promoter, $\text{prm}$, resulting in synthesis of wild-type repressor molecules.

This hypothesis attributes both the presence of $\lambda_{cl857}$ repressor in strain $\text{Eh}$ at $42^\circ$C and its ability to complement $\lambda_{clY}$ at $42^\circ$C, to the same mechanism. However as these results are strictly qualitative they do not demonstrate that the ability of the $\lambda_{cl}$ ts repressor for self-activation of $\lambda_{cl}$ transcription is the same at $42^\circ$C as at $32^\circ$C.
IN VITRO INSERTION OF THE ATTACHMENT SITE INTO THE PLASMID RP1

a) Choice of the plasmid vector RP1 and of a suitable phage donor of the att fragment -

RP1 is the prototype of resistance factors belonging to the incompatibility group P. Group R factors were originally identified in Pseudomonas aeruginosa (Datta et al., 1971), and since then they have been found in other genera, such as Proteus, and Providencia.

Several properties of plasmid RP1 made it a very attractive choice as a vector for the insertion of a DNA fragment via the Eco RI restriction endonuclease.

1st. The RP1 DNA molecule (M W approx. 3.6 x 10^6 daltons) was known to contain only one target for this restriction enzyme (Jacob and Grinter, 1975).

2nd. Plasmids of the P group are notable for their extremely broad host-range, which includes Pseudomonas, the Enterobacteriaceae, Proteus, Rhizobium, Azotobacter, Agrobacter, Chromobacterium, Neisseria, and Vibrio (Sykes and Richmond, 1970; Datta and Hedges, 1972; Olsen and Shipley, 1973; Chandler and Krishnapillai, 1974).

This property was specially advantageous for further studies of expression of genes from one determined source into another background following their insertion into the hybrid plasmid and subsequent transfer into another host.
a) Choice of the plasmid vector RP 1 and of a suitable phage donor of the att \( \lambda \) fragment (Contd)

3rd. The plasmid conferred to E. coli resistance to the antibiotics Ampicillin, Kanamycin and Tetracycline, providing a convenient set of markers for its selection.

The fragment we chose to insert into RP 1, was the \( \lambda \) DNA fragment between the Eco RI targets srl \( \lambda 2 \) and srl \( \lambda 3 \) (Fig. 1b). This fragment extends from positions 54.3 to 65.6% from the left end of the \( \lambda \) DNA molecule (Murray and Murray, 1971) and includes from left to right: Part of the inessential \( b2 \) region, the attachment site (P.P'), genes \textit{int} and \textit{xis} and part of \textit{red} \( \chi \) (Thomas et al., 1971).

This fragment has, besides its size that facilitates its isolation from other restriction fragments, the attraction of containing the att \( \lambda \) site (P.P') and therefore potentiates the study of new facets of the integration-excision reaction. (Gottessman and Weisberg, 1971).

b) Extraction and analysis of plasmid RP 1 DNA

Bacteria W 3350 containing RP 1 were grown in 2 l. of L-broth + Kanamycin (25 \( \mu \)g/ml), and the plasmid DNA was extracted as described in Materials and Methods. Yields of about 250 \( \mu \)g. DNA/2l. culture were obtained. This DNA was assayed for its ability to confer resistance to Ampicillin (50 \( \mu \)g/ml) Kanamycin (25 \( \mu \)g/ml) and Tetracycline (10 \( \mu \)g/ml) to sensitive E. coli strains, on transformation.

Competent cells of E. coli C were transformed with intact and RI restricted RP 1 DNA. The average efficiency of transformation,
b) **Extraction and analysis of plasmid RP 1 DNA (Contd)**

taken from several experiments was: 5,600 resistant colonies/μg of DNA, well in agreement with results obtained by other authors using similar systems (i.e. see Jacob and Hobbs 1974).

Eco RI-restricted RP 1 DNA gave 20 resistant colonies/μg of DNA. This effective cut-back on restriction (~250 fold) was taken as a proof that our plasmid DNA was a good substrate for restriction with endo R. Eco RI.

Gel electrophorosis analysis of both intact and RI-restricted DNA confirmed the result, showing (Plate 2): how RP 1 DNA migrates faster after Eco RI endonuclease treatment.

c) **Construction of a suitable phage donor of the attλ fragment**

The following features: 1, 2 and 3 were considered desirable for our starting strain.

1) From "a priori" considerations of the properties of a hybrid plasmid with the λ fragment between Eco RI sites sri λ 2 and sri λ 3 inserted on it, it was decided to introduce an int" (int am 29) mutant gene into the donor phage. The reason for this was that synthesis of int protein from either the phage promoter tentatively located in xis (Shimada and Campbell, 1974) or from some other upstream promoter on the plasmid RP 1 may catalyze the integration of the hybrid structure into the bacterial attachment site (Campbell 1962; Zissler, 1967), when it was not desired.

2) The isolation of the fragment concerned would be made easier from a phage containing only the two flanking targets, making the two other fragments produced on digestion with endo R. Eco RI sufficiently different in size (Fig. 1h).
Legend to Plate No. 2

Electrophoretic analysis of the following DNAs:

Track(1) \( \lambda^+ \) DNA, endoR.EcoRI.
Track(2) \( \lambda_{b519} \ sr1\lambda2 \ sr1\lambda3 \ cI857 \ ninR \) DNA, endoR.EcoRI.
Track(3) Plasmid RP1 DNA, endoR.EcoRI.
Track(4) Plasmid RP1 DNA, undigested.
Track(5) DNA from sucrose gradient, peak 1.
Track(6) DNA from sucrose gradient, peak 2.
c) Construction of a suitable phage donor of the \texttt{att} \lambda fragment (Contd)

3) Making the phage temperature sensitive for repression, by introducing the \texttt{cl 857} allele, would help in obtaining higher yields of phage from lysates. The starting phage strain was constructed from the two crosses illustrated below:

\[
\begin{array}{c}
\text{h} & \text{(b 519)} & \text{att} \lambda & \text{cl am} \\
\hline
\text{h 60} & \text{att} \lambda & \text{int 29} & \text{cl 857}
\end{array}
\]

The distribution of markers along lines representing phage genomes is not to scale. The broken line represents the recombinant genotype obtained. Deletion b 519 removes \texttt{srl} \lambda 1 site (Murray and Murray, 1974).

Recombinants of genotype h ^ b 519 cl 857 were identified as clear plaques when plating the progeny of the cross on C 600, a \texttt{phl} -resistant \texttt{sup} II strain, at 37°C. The frequency of recombination was 2%

Recombinants were purified again on C 600 at 37°C and then screened for possession of the \texttt{int am 29} marker by stabbing from the turbid plaques they produced on W 3350, 32°C, into EMB-0 plates (Section 1, Materials and Methods). Six out of 26 recombinants tested revealed themselves as integration-deficient, indicating that their genotype was: h ^ b 519 \texttt{int am 29} cl 857.

In order to eliminate \texttt{H} targets \texttt{srl} \lambda 4 and \texttt{srl} \lambda 5, the above recombinant was crossed against the \texttt{trp-immunity} fusion strain RP 12 (see Fig.5), as depicted below:
c) Construction of a suitable phage donor of the att \( \lambda \) fragment

\[
\begin{align*}
\text{h}^\lambda & \quad (b \ 519) \quad 2^+ & \quad \text{int}^- \ 3^+ & \quad \text{eI} 857 \\
\text{h}^\lambda & \quad (1 \ 5) & \quad \text{attSO trp} 13 & \quad \text{l}34 & \quad \text{l}0 \quad \text{(nin} 5; \ 5^c)
\end{align*}
\]

Recombinants were obtained on Gro K (\( \lambda ^\text{imm} \ l34 \)), that selects for phage with \( \lambda \) immunity having acquired the N-independent deletion \( \text{nin}^R_5 \) (Georgopoulos and Herskowitz, 1971). Recombinants were purified by plating again on Gro K and from several of them the RI restriction coefficient was measured. One isolate with a restriction coefficient of 30, indicative of the presence of two targets, was checked for its complete genotype being \( \text{h}^\lambda \ b \ 519 \ \text{sri} \ \lambda 2^+ \ \text{int} \ l\text{am} \ 29 \ \text{sri} \ \lambda 3^+ \ \text{eI} 857 \ \text{sri} \ \lambda 4^0 \ \text{nin} \ 5 \ \text{sri} \ \lambda 5^0 \) (abbreviated as \( \text{h}^\lambda \ 2^+ \ \text{int}^- \ 3^+ \)) and lysates made from it.

d) Isolation of the DNA fragment from \( \lambda \text{sri} 2 \) to \( \lambda \text{sri} 3 \)

Liquid lysates of the above recombinant were prepared and its DNA was extracted as described in section u of Methods. The DNA was digested with endo R. \( \text{EcoRI} \) (Fig. 1h and annexed Plate 3) and as expected three fragments were obtained, one of which, the fastest moving fragment, corresponds to the DNA between \( \text{sri} \ \lambda 2 \) and \( \lambda 3 \) as seen by comparison with the digestion products obtained with \( \lambda^+ \) DNA digested with endo R. \( \text{EcoRI} \) (Track No l4). The other two fragments correspond to the DNA between the left-end of the \( \lambda \) molecule and \( \text{sri} \ \lambda 2 \) (Slowest moving fragment: 16%) and from \( \text{sri} \ \lambda 3 \) to the right end of the molecule (Intermediate fragment: 29%). This pattern of digestion is entirely consistent with the one predicted
Legend to Plate No.3 and Fig. 14

Electrophoretic analysis of endo R.EcoRI digests of DNA of the following phage:

Tracks (1), (2) and (3): Increasing amounts (0.5, 1 and 2 μg) of λb519 srIλ2 srIλ3 cI857 ninR5 DNA, digested with endoR.EcoRI.

Track (4): 1 μg of λ+ DNA, endoR.EcoRI.

In the upper part of Fig. 14 are shown the genetic maps of phage λ(a) and λb519 2+ int- 3+(b), with the targets for endoR.EcoRI indicated by arrows. The lower part of the figure shows an idealized representation of Plate 3, where tracks No. 1, 2 and 3 in the plate correspond to diagram (b) and track No. 4 to diagram (a).
d) Isolation of the DNA fragment from \( \lambda \) srl 2 to \( \lambda \) srl 3 (Contd)

from the known position of the RI targets in the \( \lambda \) genome (Murray and Murray, 1974) and the extent of the deletions \( h 519 \) ( - 6.2\%) and \( \text{min } R_y \) ( - 5.4\%).

Preparative sucrose gradients was the method chosen for the separation of the fragment \( \text{srl } \lambda 2-3 \) from the other two. Fig. 15, represents a typical OD profile of a sucrose gradient fractionation of the Eco RI-digested DNA of the phage \( h \lambda 2^+ \text{ int}^-3^+ \).

The DNA from the fractions corresponding to the peaks of absorption (Peaks 1 and 2 in Fig. 15) was analysed on gels and the result is shown in Plate. 2.

It can be seen that the DNA corresponding to peak 1 from the sucrose gradient (Track 5) co-migrates with the 11.3\% fragment of \( \lambda \) (Track 1) and that the DNA corresponding to peak 2 (Track 6) does the same with the 29\% fragment that extends from \( \text{srl } \lambda 3 \) till the right end of the molecule of phage \( h \lambda 2^+ \text{ int}^-3^+ \) (Track 2).

The conditions chosen for the separation of the fragments by sedimentation in neutral sucrose gradients (described in Methods, section \( \alpha \) ) were such that the biggest fragment (h8\%) and the complete, undigested molecules were going to be pelleted, in favour of a better resolution between the smallest and intermediate fragments. We obtained up to 7\( \mu \)g of purified \( \text{srl } \lambda 2-3 \) fragment from 100\( \mu \)g of RI-restricted \( h \lambda 2^+ \text{ int}^-3^+ \) DNA, representing a yield of over 60\%.
Legend to Fig. 15

5-20% sucrose gradient showing the sedimentation of DNA fragments from phage λ b519. srIλ2 srIλ3 cI857 ninR5 after digestion with endonuclease EcoRI.

The absorbance $A_{254}$ profile shows two prominent peaks (1 and 2) corresponding to fragments srIλ-3 and from srIλ3 till the right end of the molecule.

Gradients were centrifuged for 15 hours at 35Krpm at 20°C in a 6x14 swing rotor of an MSE65 ultracentrifuge.

Sedimentation is from left to right.
e) Construction and isolation of hybrids RP 1 - attλ (see illustrative diagram in Fig. 16)

Four micrograms of RP 1 DNA were digested with Eco RI endonuclease and the success of the reaction was verified by following the decrease in its ability to confer antibiotic resistance (AmpR, KanR and TetR) to competent E. coli C by transformation. The restriction reaction was stopped by heating at 70°C for 10 min. (Section w, of Methods). Four micrograms (10 fold molecular excess) of fragment sri λ 2 to sri λ 3, previously heated at 37°C for 10 minutes to separate pre-annealed fragments, were added and the mixture was adapted to conditions for ligation.

When samples from the Th-ligase mixture were taken after two days and intervals thereafter and assayed on transformation of calcium-treated, competent E. coli C, up to 700 transformants/μg of DNA were obtained. This represents a recovery of about 35 fold over the transformation ability of HI-restricted RP 1, indicating that conditions for effective covalent closing of restricted circles were achieved.

The procedure followed to detect the plasmid RP 1 with the att (P.P') fragment inserted into it, was based upon the observation that phage λ b2, which has an altered att site (Davis and Parkinson, 1971), does not recombine efficiently with the bacterial site B.B'; but it does undergo efficient int-promoted recombination with certain other attachment sites such as P.P' and P.B' (Fischer-Fantuzzi, 1967; Weil and Signer, 1968; Echols et al., 1968). Therefore, a cell transformed with the hybrid insert: RP 1-sri λ 2 P.P' sri λ 3 would provide a site (P.P') for efficient integration of λ b2.#

* b2 does not entirely delete P.P'; it retains a segment of att corresponding to P'. This is usually represented by Δ P', where Δ stands for the nucleotide sequence moved close to the dot by the deletion.
Fig. 16

Construction and isolation of hybrids RP1-attλ

Plasmid RP1 DNA

\[ \text{Eco RI} \]

Phage λDNA

\[ 2^+ \text{RP}^* \]

\[ 3^+ \]

\[ \text{Eco RI} \]

Separation of fragment 2-3

Anneal & ligase

\[ \text{RP1-att} \lambda \]

Transformation of E.coli C to Amp Kan Tet

\[ \text{E.coli C/RPI-att} \lambda (\text{RP}) \]

Infection with \( \lambda_b2 \)

Stable lysogens
e) Construction and isolation of hybrids RP 1 - att λ (Contd)

Competent E. coli C cells were transformed with samples from the ligase mixture and the transformants were either plated directly or after infection with λ b2 red 3 (m.o.i = 3),** on L-plates containing the three antibiotics Amp., Kan. and Tet.

The b2 - infected resistant clones obtained (68 clones, representing a frequency of 240 clones/μg RP 1 DNA), were subsequently stabbed into EMB-0 plates, to screen for stable lysogens. One clone, designated as C-2, was found in this way. The genotype of this lysogen could be written as: E. coli C/RP 1( λ b2 red 3).

The remaining uninfect ed resistant clones (101 clones, representing a frequency of 700 clones/μg of DNA, were grown individually in L-broth and screened, basically in the same way, for their ability to give an increased frequency of stable lysogens on infection by b2 red 3. A culture of E. coli containing wild type RP 1 acted as a control. Three clones designated: C-31, C-39 and C-50 were obtained. The genotype of these non-lysogenic strains could be written as:

E. coli C/RP 1-srI λ 2-3 or E. coli C/RP 1-att λ for short.

The four plasmid inserts obtained (C-2, C-31, C-39 and C-50) represent a frequency of 3% inserts among the total number of plasmid molecules ligased. From this result, it was considered very likely that the insertion did not affect either the plasmid antibiotic resistance markers or its ability to replicate vegetatively.

** The presence of the red 3 allele in our phage stock, was entirely fortuitous and is irrelevant in our selection.
f-1) Lysogenization frequency by $\lambda_{b2}$ red 3 infecting C-31, C-39 and C-50 (Contd)

The finding that $\lambda_{b2}$ red 3 lysogenizes between 500-1000 fold more frequently the cultures C-31, C-39 and C-50, believed to carry the P.P' site inserted into their plasmid, should reflect the more efficient integration of the partially deleted attachment site of $\lambda_{b2}$ at the P.P' site, rather than at the chromosomal site B.B'.

This result qualitatively agrees with previous observations by other authors (Weil and Signer, 1968; Echols et al., 1968) and made the basis of our screening method.

As expected, $\lambda$ wild type, with a P.P' attachment site, integrates equally well at the chromosomal B.B' site carried by the control strain E. coli C/A'P 1 as in each one of the other three cultures: C-31, C-39 or C-50 (Column 2, Table 5).

f-2) Transfer experiments

Lysogenic derivatives of $\lambda_{b2}$ red 3 integrated into each one of the four plasmid inserts: C-2 C-31, C-39 and C-50 should confer simultaneously upon transfer into recipient cells of E. coli C Sm \( ^R \) (\( \lambda \) cI 857) \( \lambda \) \( ^R \), the antibiotic resistances contained in their plasmid plus the ability to survive at non-permissive temperatures (\( 42^\circ C \)), as a result of acquiring the wild type cI gene from $\lambda_{b2}$ red 3.

The recipient strain dies upon induction at \( 42^\circ C \) as a consequence of repressor inactivation and subsequent expression of lethal and lytic functions from its (\( \lambda \) cI 857) prophage. Making the strain resistant to h\( ^A \) phage, eliminates the possibility of being infected during the mating by spontaneously liberated $\lambda_{b2}$ red 3 phage from the donor.
<table>
<thead>
<tr>
<th>Genotype of donor</th>
<th>Clone</th>
<th>Genotype of recipient</th>
<th>Efficiency of transfer (of transconjugants/ml) x 10^7</th>
<th>% of transconjugants surviving at 12°C</th>
<th>% of recipients (5 x 10^7) x 10^7</th>
<th>Genotype of recipient</th>
<th>% of transconjugants surviving at 12°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli c p red I</td>
<td>C-2</td>
<td>E. coli c p red I</td>
<td>2.2 x 10^-3</td>
<td>0.00</td>
<td>100</td>
<td>E. coli c p red I</td>
<td>100</td>
</tr>
<tr>
<td>E. coli c p red I</td>
<td>C-2</td>
<td>E. coli c p red I</td>
<td>9.3 x 10^-3</td>
<td>0.00</td>
<td>100</td>
<td>E. coli c p red I</td>
<td>100</td>
</tr>
<tr>
<td>E. coli c p red I</td>
<td>C-2</td>
<td>E. coli c p red I</td>
<td>2.2 x 10^-3</td>
<td>0.00</td>
<td>100</td>
<td>E. coli c p red I</td>
<td>100</td>
</tr>
<tr>
<td>E. coli c p red I</td>
<td>C-2</td>
<td>E. coli c p red I</td>
<td>1.3 x 10^-3</td>
<td>0.00</td>
<td>100</td>
<td>E. coli c p red I</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 6: Simultaneous transfer of antibiotic resistance and ability to survive at 12°C.
83.

f-2) Transfer experiments (Contd)

Table 6, represents the results of this transfer experiment. The matings were done statically at 32°C for 1 hour, and the ratio of donor to recipient cells was approximately 1. The donor was counter-selected with Streptomycin (100 mcg/ml).

Survivors at 42°C were scored either by transferring the transconjugants to a 42°C incubator after being allowed to grow at 32°C for 2-3 hours or by replica-plating the transconjugants obtained overnight at 32°C into selective plates and incubation overnight at 42°C.

A strain carrying wild type RP 1 and having (λ b2 red 3) prophage inserted in the chromosome acted as a donor control in this experiment. This strain gives rise to 42°C survivors with a frequency at least 10⁻¹⁰ times smaller than each one of the four plasmid inserts (Table. 6). Some of these rare survivors could probably arise from recipient cells spontaneously cured of their prophages (We have not investigated further these survivors for their lack of immunity, to prove this hypothesis).

In another experiment we measured zygotic induction upon transfer from one of the four plasmid inserts, strain C-2, into either E. coli C Sm R or its lysogenic derivative E. coli C Sm R (λ+).

A sensitive E. coli C carrying wild type RP 1 and a lysogenic derivative of it with λ b2 red 3 integrated in the chromosome, acted as controls. The matings were done statically at 37°C for 30' and Streptomycin was used for counterselection.

The results are given in Table 7,
f-2) Transfer experiments (Contd)

The value obtained for the effect due to zygotic induction, 33, agrees well with the ones given by other authors (see Miller, 1972, pp 297), and is taken to represent the effect of killing the sensitive recipient cell, following transfer of the plasmid with λ b2 red 3 integrated in it.

Another point to emphasize from the above experiment is that the efficiency of transfer from strain C-2 into the lysogenic recipient, 8 x 10⁻³, agrees very well with the value obtained with the two control strains; sensitive (1.3 x 10⁻²) and chromosomal-lysogen (5 x 10⁻³).

During the 30' mating experiment, the strain C-2 transferred its plasmid with λ b2 red 3 inserted in it, as efficiently as each one of the two controls did with their wild type RP 1.

This result and the one presented in Table 6, suggest very strongly that each one of the isolates: C-2, C-31, C-39 and C-50, transfers both the antibiotic-resistance markers and the prophage λ b2 red 3 as part of the same transfer system. This system is likely to be the entity resulting from the integration of λ b2 red 3 at the P.P' site inserted "in vitro" into plasmid RP 1.

g) Electrophoretic analysis of the plasmid DNA from isolate C-31

Final proof for the hypothesis, derived from the preceding experiments, that each of the isolates: C-2, C-31, C-39 and C-50 carried the λ DNA fragment from σRI λ 2 to 3 inserted into RP 1, was sought by electrophoretic analysis of the plasmid DNA extracted from one of them; strain C-31.
g) **Electrophoretic analysis of the plasmid DNA from isolate C-31**

(Contd)

Digestion with endo R *Eco RI* of the plasmid DNA obtained from this strain should give rise to a fragment of size equal to 11.3% of \( \lambda \), representing the fragment from \( srI \lambda^2 \) (51.3%) to \( srI \lambda^3 \) (65.6%) inserted "in vitro". Comparison of tracks 2 and 3 in Plate I and Fig. 17 proves this assertion. The fastest moving band obtained after RI-digestion of phage \( \lambda b 5192^+int^-3^+ \) is seen to co-migrate with the smaller fragment produced from the plasmid DNA.

The identity of this fragment was further confirmed by double digestion with endonucleases *Eco RI* and *Hind III* of C-31 plasmid DNA. The RI fragment, \( srI \lambda^2 - 3 \), was known to contain a Hind III target, \( s_{hind} III \lambda^3 \) at 56.1% from the left end of molecule (Murray and Murray, 1975).

Therefore on digestion of C-31 plasmid DNA with RI and Hind III, the 11.3% fragment should give rise to two fragments: one 9.2% of \( \lambda \) in size and the other 2.1%.

In track No. 5 we can distinguish a band with a mobility corresponding to the 9.2% fragment. The other fragment, 2.1%, unfortunately, is difficult to see, probably due to incompleteness of the digestion.

From this electrophoretic analysis carried out on one of the isolates, C-31, taken at random we conclude that in fact each one of the four strains: C-2, C-31, C-39 and C-50 carries a hybrid plasmid structure made from inserting the \( \lambda \) fragment that extends between positions 51.3 to 65.6%, from the left end of the \( \lambda \) molecule, into the RI site characteristic of plasmid RP 1 DNA.
Legend to Plate 4 and Figure 17

Electrophoretic analysis of the following DNAs digested with restriction endonucleases. From left to right, the tracks correspond to:

Track (1) $\lambda^+$ DNA, endo R.EcoRI.

(2) $\lambda_{b519}$ srflA2 srflA3 ninR5 DNA, endo R.EcoRI.

(3) Plasmid-att$\lambda$ insert DNA from strain C-31, endo R.EcoRI.

(4) Plasmid-att$\lambda$ insert DNA from strain C-31, endo R.HindIII.

(5) Plasmid-att$\lambda$ insert DNA, digested with both RI and HindIII.

In Fig.17 the targets for RI and HindIII are indicated by arrows above and below the genetic maps. The lower part of Fig.17 represents schematically Plate 4.
g) Electrophoretic analysis of the plasmid DNA from isolate C-31

(Jacob and Grinter, 1975). This fragment contains, among other functions, the phage attachment site, P.P', providing an efficient site for integration of the attachment-deficient phage λb2 (Δ.P'). The study of the phage functions that catalyze this particular integration event will be described in the following section.

h) Requirements for the integration of phage λb2 red 3 at the P.P' site

The nature of the recombination event used by phage λb2 red 3 to integrate into the P.P' site contained in our plasmid insertion strains, has been analysed through measurements of lytic recombination between phage DNA molecules carrying the structural determinants characteristic of this type of recombination.

In order to define the requirements of this reaction, we compared the frequencies of site-specific recombination in pairs of identical crosses done in the presence or absence of either int or xis products.
h) Requirements for the integration of phage $\lambda b2 \text{ red } 3$ at the $F_P'$ site (Contd)

A schematic representation of the crosses is presented below:

**xis - effect**

\[
\begin{array}{c}
\begin{array}{c}
\text{h}^\lambda(b2) \cdot \text{P}' \text{ red}^- \text{cI}^+ \\
\text{h}^\lambda \text{ susJ} \cdot \text{P}' \text{ xis}^- \text{cI857}
\end{array}
\end{array}
\]

\[
\begin{array}{c}
\begin{array}{c}
\text{h}^\lambda(b2) \cdot \text{xis}^- \text{cI} \\
\text{h}^\lambda \text{ susJ} \cdot \text{xis}^- \text{cI857}
\end{array}
\end{array}
\]

**int - effect**

\[
\begin{array}{c}
\begin{array}{c}
\text{h}^\lambda(b2) \cdot \text{red}^- \text{cI}^+ \\
\text{h}^{80} \text{ att}^\lambda \text{ int}^- \text{cI857}
\end{array}
\end{array}
\]

\[
\begin{array}{c}
\begin{array}{c}
\text{h}^\lambda(b2) \cdot \text{P}' \text{ int}^- \text{cI}^+ \\
\text{h}^{80} \text{ att}^\lambda \text{ int}^- \text{cI857}
\end{array}
\end{array}
\]

Crosses were carried out by growing lytically the two parent phage on strain W 3101 sup rec A'. Since both pairs of crosses were heterozygous for the b2 marker, most of the recombinants h cI 857 obtained should result from site specific recombination at the attachment site (Weil and Signer, 1968) and therefore no attempt was made to eliminate the contribution of the phage red system.

The complete genotype of the phage and the frequencies of recombination obtained in each pair of crosses are given in Table 8.
Table 8

<table>
<thead>
<tr>
<th>Cross</th>
<th>% Recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>h(^\text{b2}) red 3 x h(80) att (\lambda) int 29 cI 857</td>
<td>20%</td>
</tr>
<tr>
<td>h(^\text{b2}) int 29 x h(80) att (\lambda) int 29 cI 857</td>
<td>0.07%</td>
</tr>
<tr>
<td>h(^\text{b2}) red 3 x h(^\text{sus}) J xis 1 cI 857</td>
<td>1.1%</td>
</tr>
<tr>
<td>h(^\text{b2}) xis 1 x h(^\text{sus}) J xis 1 cI 857</td>
<td>1.1%</td>
</tr>
</tbody>
</table>

Legend to Table 8

Recombinants h\(^\lambda\) cI 857 in the first pair of crosses were scored on a strain ton A sup\(^0\), and h\(^\lambda\) sus cI 857 recombinants from the second pair on W 3101 sup\(^0\). Total progeny were scored by plating on W 3101 sup\(^0\) or 1485 at 37\(^\circ\)C. Details of cross procedures are given in Materials and Methods, section g.
h) Requirements for the integration of phage λ b2 red 3 at the P,P' site (Contd)

The results in Table 8, prove quite conclusively that active int and xis genes are essential for the efficient integration of λ b2 red 3 at the P,P' site.

The quantitative discrepancy between the values obtained for the effect due to int (approx. 300 fold) compared with xis (about 15 fold), could either reflect a more stringent requirement for int-protein or it may be due to the fact that whereas the int-mutation used was an amber the xis was a missense mutation. A more realistic estimate of the contribution of these two products to the integration of λ b2 at P,P' could be obtained by measuring frequencies of stable lysogenization after infection with each one of the mutants. Frequencies of integration derived from site specific recombination data, although qualitatively significant, are known to differ sometimes from measurements of stable lysogenization. However, a repeat of this experiment done by measuring lysogenization frequencies on infection of one of the plasmid insertion strains confirmed the result obtained from site specific recombination crosses (Table. 9).

We could represent the recombination λ b2 X P,F 1 - att λ, according to the nomenclature suggested for the different sites by Gerrini (1969) as follows:

\[ \Delta .P' + P,F' \overset{\text{integration}}{\rightarrow} \Delta .P' + P,F' \]

Examining this reaction, we realize that no change is produced in the structure of the participating sites as a result of integration or excision. Therefore, both reactions should have the same
<table>
<thead>
<tr>
<th>Phase</th>
<th>W3101 sup^0/RPl att(\lambda)</th>
<th>W1185 supII/RPl att(\lambda) int</th>
<th>xis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda^b2) red 3</td>
<td>70%</td>
<td>32%</td>
<td>&gt;70</td>
</tr>
<tr>
<td>(\lambda^b2) int am29</td>
<td>&lt;1%</td>
<td>12%</td>
<td>fold</td>
</tr>
<tr>
<td>(\lambda^b2) xis 1</td>
<td>10%</td>
<td>5%</td>
<td>7</td>
</tr>
</tbody>
</table>
h) Requirements for the integration of phage $\lambda$ b2 red 3 at the P.P' site (Contd)

requirements for int and xis products. In fact, the same requirement for int and xis has been found, for the integration of b2 at the other site P.B' for which it was known to have a high affinity (Shimada et al., 1975).

Had int been the only requirement for the above integration reaction, we would have expected the phage to be very unstable in its association with the plasmid site P.P'. Even if transient integration were achieved, the basal level of int expression from a repressed prophage genome (Kaiser and Hasuda, Shimada and Campbell 1974; a, b) should cause higher spontaneous liberation of phage from such a site, than from a $\lambda$ wild type chromosomal lysogen that requires both int and xis for prophage excision (Echols, 1970). In fact the liberation rates found were even smaller ($\sim 1.7 \times 10^3$ P.f.u/ml) than for a wild type lysogen ($\sim 2 \times 10^6$ P.f.u/ml), becoming only comparable after UV induction ($1.6 \times 10^6$ vs $2 \times 10^9$ P.f.u/ml).

This observation agrees with the earlier conclusion that excision of $\lambda$ b2 red 3 prophage from the plasmid site (P.P' $\Delta_{P'}$) requires both int and xis proteins.
CHAPTER 5

DISCUSSION

I will consider in turn the two processes governing the lysogenic response: repression and integration; concentrating the discussion mainly in those aspects of these processes which have been studied in this thesis in more detail. Finally I will discuss some further applications of the plasmid-att\(\lambda\) insertion strains.

1) Control of repressor synthesis

The level of expression of the\(c1\) gene from the immunity promoters,\(pre\) and\(prm\), was assayed by monitoring the synthesis of anthranilate synthetase during infection of sensitive or immune host strains, as described in chapter 3.

Infection of a sensitive host with phage\(RF12\), under conditions of\(trp\)-mediated repression, leads to a very low rate of expression of\(c1\) gene. After subtracting the background levels of anthranilate synthetase formation from the repressed\(trp\) promoter and the weak, constitutive promoter in\(xis\) (Shimada and Campbell, 197b) estimated from parallel infections with the parental phage\(RF0\), the rate of constitutive expression of\(c1\) appears to be between two and five percent of the fully stimulated rate from\(pre\). It is not possible to determine what proportions of this synthesis are initiated at\(pre\) and\(prm\).

Co-infection of this host by\(RF12\) and the helper phage\(\lambda\)\(cI90\) greatly stimulates repressor synthesis from\(pre\). The products responsible for this stimulation provided by the helper, are the
Control of repressor synthesis (Contd)

proteins coded by the genes gII and gIII. The requirement for the gII product (1-5 fold stimulation) is more stringent than that for gIII product (2 fold), as previously found by other authors (Schols and Green, 1971; Reichardt and Kaiser, 1971, Reichardt 1975a).

The N protein is not directly required for gI transcription from pre; instead it acts indirectly by allowing expression of the gII and gIII genes.

The rate of synthesis of repressor from pre levels-off at about fifteen minutes after infection due to the action of the oro gene-product of the helper, since a \( \lambda \)oro helper does not produce this turn-off. The helper phage's oro-product reduces expression from pre of the trp phage, RP12, by reducing the intracellular levels of the gII and gIII proteins provided by the helper. These proteins are unstable (Reichardt 1975a), so reduced synthesis eventually lowers their activities and when their rates of inactivation exceed their rates of synthesis, then repressor synthesis will be reduced.

Several host functions have been described as having an effect on the frequency of lysogenization. I have investigated the direct effect of c-AMP, reported to promote lysogeny (Grodzicker et al., 1972), in the rate of expression of repressor synthesis from promoter pre. Conditions that produced marked catabolite repression of \( \beta \)-galactosidase synthesis had no detectable effect on anthranilate synthetase formation from RP12. Since transcription from the establishment promoter, pre, does not seem to be sensitive to cyclic AMP concentrations, the way in which c-AMP promotes lysogeny might be via some indirect effect of the deranged catabolite
1) **Control of repressor synthesis** (Contd)

Gene activation system in the mutant strain. Other workers (Jordan et al., 1973) have reached the same conclusion by direct assay of lambda repressor. In summary, the pattern and rate of synthesis of cI from promoter pre in the hybrid lambdoid phage \( \lambda_{imm3} \) is basically similar to the one previously obtained for \( \lambda \) by other authors. This result was logically anticipated since \( \lambda^+ \) and the hybrid \( \lambda_{imm3} \) have in common the two genes responsible for activation, cII and cIII, as well as the site where they carry out their activation, cY (pre).

Another lambdoid phage, \( \lambda_{imm21} \), provides an active cIII-product capable of promoting repressor synthesis in phage \( \lambda_{imm3} \) or \( \lambda^+ \), in spite of having a different cY site and making a different cII protein (Kaiser and Jacob, 1957). As the requirement for cIII protein to activate pre is a minor one (2-fold stimulation), it might be argued that cIII promotes lysogeny by increasing the efficiency or stability of the cII protein, perhaps in a rather unspecific way. In contrast with cIII, cII may well be directly required to activate cI transcription from pre by interacting at or near the cY region of the phage DNA, preventing termination of short RNA molecules in a manner analogous to the N-protein (Roberts, 1975).

Infection of a \( h^L \)-immune host by RP12 results in a considerably higher rate (15-20 fold) of anthranilate synthetase formation than similar infection of a sensitive host. Since repressor, the only product from the \( imm h^L \) prophage, blocks expression of all genes of the superinfecting homo-immune phage, except cI itself, the repressor must be responsible for this
1) **Control of repressor synthesis** (Contd)

increase in cI expression. Since the cro gene-product can inhibit expression of the cI gene from prm (Eisen et al., 1970; Reichardt 1975b), part of the stimulatory activity attributed to the repressor may reflect an indirect effect by preventing the cro protein from acting at prm. However, the cro product is known to be an inefficient repressor of the transcriptions that it controls (Davison et al., 1971) and in fact its effect at prm has been estimated as a decrease of about 6 fold, which will not be sufficient to explain the total stimulatory effect due to the cI-product that I have found on infection of a lysogen. Recently, Reichardt (1975b) has shown that the lambda repressor has a stimulatory effect (5 fold) on prm expression either in the presence or in the absence of the cro gene product. The comparison between the constitutive rate of expression of phage RF12 in the sensitive host versus the cI-activated in the immune lysogen is rather elaborate, due to the lack of a direct estimate for the effect of cro acting at prm on infection of the sensitive host. Why did I not start with the parent phage RFQ mutated in the cro gene, so as to eliminate this interference? The reason stems from the lack of solid knowledge about the causes of the inability of cro phage to grow in the absence of repressor.

Two explanations have been proposed to account for the growth defect of cro mutants in the absence of repressor (Herskowitz, 1971).
1) **Control of repressor synthesis** (Contd)

1) **Growth interference.** Cro\(^-\) mutants overproduce some function(s) which inhibit phage and/or host growth; at least one lambda gene, \(k_{11}\), has been implicated in this effect.

2) **Transcriptional interference.** In the absence of cro product, transcription of the \(q_1\) gene from \(pre\) never stops causing interference with transcription of genes \(Q\), \(P\) and \(Q\).

   Bearing that in mind, I have attempted, earlier on in this thesis, to isolate in vivo a deletion fusing the \(trp\) genes of the transducing phage \(\lambda_{trpBC-2}itsaro-cII\) into the immunity region. The method involved pyrophosphate-treatment of the phage lysate and growth of the surviving particles in a host that expressed the \(N\)-gene constitutively (Davison et al., 1974). None of the numerous and extensive \(N\)-deletions obtained entered the immunity region, in spite of the fact that the phage \(\lambda_{ptrpBC-2}\) lacks the region of \(\lambda\) where \(cIII\) maps and where \(k_{11}\) has been tentatively located; in addition, this phage carried a mutant \(cII\) gene.

   Therefore, neither growth interference nor transcriptional interference could be invoked in this case to explain the failure to obtain a deletion extending from the \(N\)-gene into \(cIt\)s.

   Consequently, and in the light of this negative evidence, no attempt was made to make the starting phage \(R\)F\(O\) mutant in the cro gene, as it was suspected that a deletion such as the one that originated the immunity-\(trp\) fusion phage \(RFL2\) would be a lethal event in the presence of a defective cro gene, as the phage became deleted for the \(imm\_{3}\)-\(cI\) gene.
1) **Control of repressor synthesis** (Contd)

It is attractive to imagine that repressor binding at $o_2$ activates transcription from the $cI$ promoter, $prn$. Dottin et al., (1975) have shown that the addition of purified lambda repressor to a DNA-dependent protein-synthesizing system stimulates the synthesis of the $cI$ gene-product about four fold. Their data, however, together with those of Reichardt (1975b) are equally consistent with the stimulation by repressor of a post-transcriptional step in repressor-polypeptide synthesis. The fact that I find a similar stimulatory effect of the $imm^{-}$ repressor enhancing the synthesis of the unrelated enzyme anthranilate synthetase from the immunity/trp fusion operon of $RFL2$ makes a specific post-transcriptional step much less likely.

Comparison of the rates of repressor synthesis from promoters $pre$ and $prn$ in the case of $\lambda$ phage, revealed that $pre$ was about 7-8 times more efficient than $prn$ (Reichardt and Kaiser, 1971). The immunity-trp fusion phage, $RFL2$, expresses repressor from $prn$ at a rate not less than half the one from $pre$. Since, as discussed before, $pre$ is identical in $\lambda$ and the hybrid $\lambda$ $imm^{-}$; the difference must reflect a more efficient $prn$ promoter being carried by the $\lambda$ $imm^{-}$ hybrid parent strain $RPO$. 
ii) Integration and excision

From the functional and structural requirements, the integration and excision reaction of lambda DNA into and out of the host DNA, can be written as follows:

\[
P.P' + B.B' \xrightarrow{\text{int}} P.B' + B.P'
\]

Several proposals have been put forward to explain this asymmetric feature of the integration-excision reaction, namely, why the \textit{xis} protein is essential for excision but dispensable for integration? One of them, is an energy-level hypothesis (Dove, 1970) which in short, can be stated as follows:

a) The integrated state (the prophage) lies at a lower energy than the excised state.

b) The \textit{int} product acts as a simple catalyst for the reaction pathway.

c) The \textit{xis} product acts to provide energy to make the excision process possible.

The energy by which the prophage is more stable is postulated to arise from nucleotide stacking interactions within the sequences which bound the prophage.

A prediction derived from this model is that exchanges between two attachment sites not causing any change in the structure of the participating sites (conservative changes), cannot involve an energy change and therefore should be \textit{xis} independent (i.e. P.P' + B.P'; P.P' + P.B'; B.P' + B.P')

The integration of phage \( \lambda b^2 \text{red}3 \) into the P.P' site inserted in the plasmid \( \Phi F1 \), represented as:

\[
\Delta P' + P.P' \xleftrightarrow{\text{int}} \Delta P' + P.P'
\]
does not lead to the creation of new structures (conservative) and
11) **Integration and excision** (Contd)

therefore it should be only dependent on int product. However, I found that the effect of int, measured in site-specific recombination crosses, was about 300 fold and that of xis about 15 fold. In all other site specific crosses where both products are required, such as: B.P' + P.B'; B.P' + P.\( \Delta' \); \( \Delta.P' + P.\Delta' \) and \( \Delta.P' + P.B' \), the absence of xis protein from these crosses causes as big a drop in the recombination frequencies as that of int; however all these recombinational events generate new structures (generative) and therefore agree with the energy-level hypothesis.

In addition, all other known crosses of the conservative type: P.P' + P.P'; P.P' + P.B'; P.P' + P.B and B.P' + B.P' show no requirement for xis gene product.

So, I have studied the only recombinational exchange between att sites not leading to the creation of new structures which shows a requirement, albeit small, for xis protein. This finding would tend to suggest that the energy-level hypothesis postulated by Dove (1970) is not sufficient to explain the factors that govern the insertion and excision of \( \lambda \).

Some other predictions of the above hypothesis have been found by other authors not to be fulfilled (Shulman and Gottesman, 1973).

The stability of the prophage state when \( \lambda.b2 \) is integrated at the P.P' site of the plasmid is consistent with the findings from phage crosses. Had the integration of \( \lambda.b2 \) into the plasmid been dependent only on int-protein, then excision of the prophage from that structure, and subsequent liberation of phage particles,
would have been much more frequent, as a consequence of the expression of the \textit{int} gene from a repressed prophage genome (Kaiser and Masuda, 1970; Shimada and Campbell, 1971a, 1971b).

Examination of the specific exchanges showing a requirement for \textit{xis} protein, in the search for a model revealing the pattern of recognition by \textit{xis}, reveals that a DNA substrate with the \textit{B} \text{ configuration}, or \textit{F} \text{ + F}, or might be recognized by \textit{xis} protein, reacting with it and changing it to a form which could be recognized by \textit{int} enzyme. Alternatively the \textit{xis} protein might react with the \textit{int} enzyme to change its catalytic specificity.

With either of these hypothetical mechanisms, the "decision" by a phage as to whether to integrate or to excise would depend on the levels of both proteins after infection. Metabolic instability of one of the catalytic components versus the other (Weisberg and Gottesman, 1971) or continuation of the synthesis of one of them when the other has already ceased (Shimada and Campbell, 1971a), could explain why integration and excision of phage \textit{\lambda} can proceed so effectively under circumstances appropriate to each process, namely following infection after repression has been established, and after prophage induction. Clearly, however, the elucidation of this unique recombination event will require new methods and new insights.
iii) Further applications of the plasmid-att \( \lambda \) insertion strains

The \( \lambda \) DNA fragment inserted into the plasmid RPl extends from positions 54.3% to 65.6% from the left end of the \( \lambda \) molecule. It contains, part of the inessential \( b^2 \) region, the phage attachment site P.P', genes int and xis and part of red \( \alpha \). The int allele contained in the inserted fragment was the amber mutant \( \text{int} \text{am}29 \), easily suppressible by a permissive supII host.

Transcription of the inserted DNA fragment either from the phage constitutive promoter P'I located in xis (Shimada and Campbell, 1972) or from some other upstream promoter on the plasmid RPl would, after translation in the supII host, give rise to the formation of a functional int protein. Since int is the only requirement for the reaction: P.P' + B.B'; the suppression of the \( \text{int am}29 \) mutation in the supII host would cause some integration of RPl-att \( \lambda \) into the B.B' site of the E. coli chromosome (17' minutes) and from that location it should give rise to chromosomal transfer from that fixed origin. The polarity of transfer would depend upon the orientation of the inserted \( \lambda \) DNA fragment with respect to the origin of transfer of the plasmid RPl. In principle this method could be extended to integration of the plasmid RPl-att \( \lambda \) into bacterial secondary sites (Shimada et al., 1972) by using bacteria deleted for the chromosomal site B.B'.

Integration of \( \lambda \) transducing phage into the plasmid-inserts would provide regions of homology with the bacterial chromosome and therefore potentiate the generation of novel Hfrs by general recombination. The integration of transducing phage into the P.P' site however, will not give rise to the creation of new sites, and
iii) Further applications of the plasmid-attλ insertion strains (Contd)

therefore, integration of transducing phage like λgal or λbio into it, (an "int-only" event), may turn out to be easily reversed by the low level of int expression from either the integrated repressed prophage (case of λgal) or from the int am29 allele contained in the inserted DNA fragment, which would have to have been suppressed to help the integration of the λbio phage.

Derivatives that become stable will select for those mutants that have acquired an Int− phenotype. Transducing phage derived from secondary sites with an attachment site of the type Δ.P (i.e. λlyn, λgal K, λtrp E) will be ideal in this respect as their integration into P.P' should be, by analogy with λb2, both int and xis dependent and therefore after integration they should not be able to excise and will be as stable as λb2 is after integration into RP1-attλ.

The wide host-range of the P-group of plasmids affords a means of studying the functionality of E.coli or λ phage genes into any of the diverse bacterial genera into which RP1 can be transferred. Expression of genes from distant bacterial genera, like Bacillus licheniformis into E.coli has been observed in this laboratory (Bramm and Muir, unpublished observations). The restriction barrier following transfer of foreign DNAs might not be very difficult to override as proved by recent reports of in vivo intergeneric transfer and expression of several bacterial genes (Olsen and González, 1974; Cannon et al., 1976). If expression of the transferred foreign DNA is not obtained it could be further analysed to see whether transcription or translation
iii) Further applications of the plasmid-att \( \lambda \) insertion strains (Contd)

is the cause of the failure. Again the use of transducing phage particles as vehicles for the transferred \( \textit{E. coli} \) genes enables one to identify those RNA species coded by the transducing phage, from the complex mixture of the total extracted RNA, with relative ease.

A final point of interest to be made is that the insertion of the \( \lambda \) DNA fragment, \( \text{srI} \lambda^2-3 \), brings about a single target for the restriction endonuclease \( \text{HindIII} \) (\( s\text{ hindIII} \lambda^3 \), at 56.4% from \( \lambda \) left end) opening the possibility of employing the plasmid insertions \( \text{RMl-att} \lambda \) as a receptor system for DNA fragments obtained via restriction endonuclease \( \text{R. HindIII} \).
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The following abbreviations are used:

Advanc. Genet.
Ann. Inst. Pasteur
Biochem. J.
Biochem. Prep.
Cold Spring Harbor Symp. Quant. Biol
Compt. Rend.
Genet. Res.
J. Bacterial
J. Biol. Chem
J. Gen. Microbiol
J. Gen Virol
J. Mol. Biol.
J. Virol.
Methods Enzymol.
Mutation Res.

Advances in Genetics.
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Annual Reviews of Genetics.
Annual Reviews of Microbiology.
Biocmehanical and Biophysical Research Communications.
Biochemical Journal
Biochemical Preparations.
Comptes Rendues de l'Academie des Sciences (Paris).
Genetical Research.
Journal of Bacteriology.
Journal of Biological Chemistry.
Journal of General Microbiology.
Journal of Molecular Biology
Journal of Virology.
Molecular and General Genetics.
Mutation Research.
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Control of Transcription of the rex-cl Region of Bacteriophage λ

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Received February 10, 1974

Summary. A strain of E. coli, carrying a defective λ prophage in which the cro gene has been deleted and the cl gene carries the cl<sub>857</sub> allele, continues to synthesize both rex product and an inactive cl<sub>857</sub> repressor during growth at 42°C. The inactive repressor is able to act in trans to allow repressor synthesis by a super-infecting homo-immune λ<sub>y</sub> phage. This ability is lost when the cl gene of the defective prophage acquires an additional mutation to the cl<sub>845</sub> allele: the ability to synthesize rex product is often also lost. It is suggested that thermally inactivated cl<sub>857</sub> repressor is able to promote transcription of the rex and cl genes from the maintenance promoter, prm.

Introduction

The regulation of repressor synthesis in bacteriophage λ has been recently reviewed (Eisen and Ptashne, 1971; Echols, 1972; Davison, 1973). It is likely that there are two modes of repressor synthesis; the establishment mode and the maintenance mode, and that these use different promoters pre and prm respectively (Fig. 1). Repressor synthesis in the establishment mode requires active products to be made from genes cII and cIII and is prevented by the cis-acting mutation cy which is probably a defect in promoter pre (Reichardt and Kaiser, 1971; Echols and Green, 1971; Spiegelman et al., 1972).

In the maintenance mode it is clear that the transcription of the cl gene is subject to negative control by the product of the cro gene (Eisen et al., 1970; Calef et al., 1971, Castellazi et al., 1972). However there is disagreement about whether the λ repressor is involved in the positive control of cl transcription (Eisen et al., 1968). DNA/RNA hybridisation experiments indicated that the level of immunity specific RNA decreased immediately when the repressor of a λ<sub>cl<sub>857</sub</sub> cro-defective lysogen was thermally denatured (Heinemann and Spiegelman, 1970; Kourilsky et al., 1970). This suggested that an active repressor was necessary for cl gene transcription and this explanation was supported by direct measurement of the level of inactive repressor in a λ<sub>cl<sub>857</sub</sub> cro-defective lysogen at high temperature (Reichardt and Kaiser, 1972). Similar conclusions were reached by Echols and Green (1972) studying repressor levels after infection of a λ lysogen, and by Oppenheim and Slonim (1971), using a superinfection assay to determine repressor concentration. In contrast, however, the DNA/RNA hybridisation measurements of Hayes and Szybalski (1973) showed no difference in the transcription of the cl gene whether the thermosensitive repressor of a λ<sub>cl<sub>857</sub</sub> cro</sub>-defective lysogen was active or not.
Two mechanisms have been suggested whereby repressor may control its own transcription. According to the "flip-flop" model the promoter for rightward transcription (P_r) overlaps with the promoter for maintenance of repression (prm). Transcription proceeds preferentially rightwards through genes O and P (Fig. 1) unless prevented from doing so by repressor bound to operator OR in which case the cl gene is transcribed (Eisen et al., 1968). In contrast, repressor may play an active role in cl transcription (Echols, 1972). The hypothesis that cl transcription from prm requires repressor does not rule out the additional possibility that it is also transcribed constitutively at a lower rate (Echols, 1972).

This report attempts to rationalise the unexplained observation that while cy mutants fail to complement most cl mutants, they are able to complement temperature-sensitive mutants in the cl gene (at high temperature) (Brachet and Thomas, 1969). It is suggested that λ repressor has two separate activities; negative control of early RNA synthesis (repression) and positive control of cl transcription (self-activation), and that in temperature sensitive cl mutants, only the repression activity is inactivated at 42°C.

Materials and Methods

λ clear mutations used were: cl_90, cl_39, cl_98, cl_41, cl_611, cy_42 (Kaiser, 1957); cl_14, cl_611 (McMacken et al., 1970); cl_611, cy_44 (Sussman and Jacob, 1962); d_205 (Brachet and Thomas, 1969); cl_4A2, (Leib, 1966).

The non-permissive strain W3350 (Campbell and Baldwinder, 1958) was used to prepare lysates. The cryptic lysogen Bh has been described previously (Davison et al., 1974), and its genetic constitution is shown in Fig. 1.

Tests for rex function were performed by comparison of the plating efficiency of phages T4+ and T4rIIuvl87 (Dr. J. Drake, personal communication) Typically T4rII plated with an efficiency of 10^-4 on a Rex+ host, whereas T4+ was unaffected.
Results

Table 1, showing the complementation pattern obtained with \(\lambda cy\) mutants in establishing lysogeny or permitting survival of infected bacteria, is in perfect agreement with results reported by Brachet and Thomas (1969). \(\lambda cy\) mutants complement like \(cl\) mutants despite their location between \(cro\) and \(cl II\). This observation is in accordance with the hypothesis that \(cy\) mutants are defective in a promoter (pre) necessary for the establishment of repressor synthesis (Reichardt and Kaiser, 1971; Echols and Green, 1971). The fact that \(cy\) mutants are able to form stable lysogens at low frequency (Kaiser, 1957) suggests that they are not defective in the maintenance mode of repressor synthesis from \(pre\) (Fig. 1).

Column 2 of Table 1 shows the anomalous result that \(cy\) mutants can be complemented by temperature-sensitive \(cl\) mutants at the non-permissive temperature (Brachet and Thomas, 1969). Examination of the lysogens resulting from the complementation reveals them to be either single lysogens of the \(\lambda cy\) phage or double lysogens (\(\lambda cy\) \(\lambda cl IV\)), indicating that co-infection with a temperature sensitive \(cl\) mutant enables \(\lambda cy\) to establish lysogeny. The fact that the complementation is not seen with other \(cl\) mutants suggests that the inactive repressor itself plays a role in the complementation and that the “quality” of the inactive repressor is important. In agreement with this is the observation (column 5) that the double mutant \(\lambda cl IV cy 42\) fails to complement \(\lambda cy\), indicating that the inactive repressor in the above experiment is synthesized from \(pre\) and that if this is blocked by a \(cy\) mutation then it is not possible to make sufficient inactive repressor to complement the \(\lambda cy\) phage.

Two types of hypothesis may account for the complementation between \(\lambda cy\) and temperature-sensitive \(cl\) mutants. It is possible that the \(\lambda cy\) phage makes a small number of wild-type repressor subunits from the \(pre\) promoter and that these are able to interact with the temperature-sensitive subunits to form a mixed oligomer which may be temperature-insensitive. Since the active form of the lambda repressor is believed to be a dimer (Chadwick et al., 1970), such a mechanism would result in only a two-fold increase in the amount of active repressor. It is considered unlikely that this hypothetical twofold effect could account for the difference between the completely clear areas of lysis produced by the parent phages alone and the intense turbid area of the complementation cross streak. Furthermore, mutants in gene \(cl III\) exhibit a clear phenotype despite the fact that they produce 4–7 fold more repressor than \(cy\) mutants (Reichardt and Kaiser, 1971). We would suggest the alternative hypo-

| Phage suspensions were cross streaked on a lawn of W3350 on BBL plates (Parkinson and Huskey, 1971) and incubated overnight at 37°. The area of the cross streak was scored as either turbid (tu) or clear (cl). Clear mutants used were those described in Materials and Methods. Experiments involving \(cl IV\) mutants were performed at 42° C |
thesis that wild-type repressor stimulates $cl$ transcription from $prm$ and that this stimulatory activity is retained in the temperature-sensitive repressor protein at the non-permissive temperature. According to this hypothesis, temperature-sensitive repressor activates transcription of the wild-type $cl$ gene of the $\lambda cy$ phage from the maintenance promoter, $prm$, resulting in synthesis of wild-type repressor molecules.

**Complementation of $\lambda cy$ Mutants by Cryptic Prophage Deletion Bh**

The genetic constitution of the cryptic prophage deletion Bh is given in Fig. 1. Repressor synthesis in this strain is entirely from the maintenance promoter $prm$ since $cIII$, $cII$ and $pre$ are deleted. The gene $cro$ is similarly deleted so that the anti-immune state cannot be established, the cryptic lysogen being in the immune state at $32^\circ$C and in the sensitive state at $42^\circ$C due to the temperature-sensitive $cl_857$ mutation (Davison et al., 1974).

In the previous section it has been suggested that inactive repressor may activate $cl$ transcription at $42^\circ$C. According to this hypothesis, infection of strain Bh at $42^\circ$C by $\lambda cy$ would result in turbid plaques, due to the activation of wild-type repressor synthesis in the $\lambda cy$ phage by the inactive $cl_857$ repressor from the cryptic prophage. The results of this experiment are shown in Fig. 2. It can be seen that $\lambda cy$, which normally forms clear plaques, is able to form turbid plaques at $42^\circ$C when strain Bh is used as the host. This result is found even when the cryptic lysogen Bh has been grown continuously at $42^\circ$C for many generations. The phenomenon is analysed further in Table 2 where the plaque type of a variety of clear mutants is investigated using this strain. All $\lambda$ clear (immunity sensitive) mutants, with the exception of those defective in the $cl$ gene, are able to form turbid plaques on cryptic lysogen Bh. However, the effect is immunity specific, so that hetero-immune phages carrying clear mutations form clear plaques as usual. Identical results were obtained with three other, independently arising, $cro$ deletions (Davison, unpublished data) making unlikely the possibility that $cl$ transcription in Bh begins at some bacterial promoter rather than at $prm$. 

Fig. 2a and b. $\lambda cy_{42}$ was plated to give single plaques on: a) prophage deletion strain Bh. b) W3350 on BBL medium at $42^\circ$C. Bacterial strains had previously been grown at $42^\circ$C for several generations. When a $cl$ defective derivative of Bh was used as the host, the plaques resembled those on W3350.
Table 2. Plaque morphology of λ clear mutants on cryptic lysogen Bh and its cl derivatives

<table>
<thead>
<tr>
<th>Genotype of infecting phage</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W3350 or Bh</td>
</tr>
<tr>
<td>λ</td>
<td>tu</td>
</tr>
<tr>
<td>λcIₖ₆</td>
<td>tu</td>
</tr>
<tr>
<td>λcI₄₄</td>
<td>el</td>
</tr>
<tr>
<td>λcI₆₅₈₅₇</td>
<td>el</td>
</tr>
<tr>
<td>λcII₉₅</td>
<td>el</td>
</tr>
<tr>
<td>λcII₆₃₁₅₁</td>
<td>el</td>
</tr>
<tr>
<td>λcII₆₃₁I₃₇</td>
<td>el</td>
</tr>
<tr>
<td>λcy₄₂</td>
<td>el</td>
</tr>
<tr>
<td>λcy₆₄₄</td>
<td>el</td>
</tr>
<tr>
<td>λcy₂₀₀₁</td>
<td>el</td>
</tr>
<tr>
<td>λimm₄₃₄</td>
<td>tu</td>
</tr>
<tr>
<td>λimm₄₃₄cI</td>
<td>el</td>
</tr>
<tr>
<td>λimm₄₃₄cII₉₅</td>
<td>el</td>
</tr>
<tr>
<td>λimm₄₃₄cIII₆₅</td>
<td>el</td>
</tr>
<tr>
<td>λimm cII₄₂</td>
<td>el</td>
</tr>
</tbody>
</table>

Phage suspensions were plated to give single plaques and scored as clear (cl) or turbid (tu) after overnight incubation at 42°C. Recipient bacteria were grown overnight at 42°C and subcultured at the same temperature.

From these results it is obvious that the cryptic prophage deletion Bh makes a product able to act in trans to allow a clear cII, cIII or cy phage to form a turbid plaque. Furthermore, since heteroimmune clear mutants are unaffected, the trans acting product produced by strain Bh must either act within the immunity region of the incoming homo-immune phage or interact with a substance produced by that immunity region. These results suggest that the trans acting product is the inactive cI₆₅₇ repressor. To test this, mutants of strain Bh were isolated in which the cl gene was inactivated for repression even at 32°C. The isolation of such mutants was possible since the strain Bh expresses the trp operon from the leftward promoter of λ, and is subject to negative control by the λ repressor. Thus at 32°C Bh is both immune (since it is cro defective) and a trp auxotroph. Selection of revertants of Bh able to grow at 32°C without exogenous tryptophan allows the isolation of mutants of Bh carrying a cl defect in addition to the cI₆₅₇ allele already present (Davison et al., 1974). Twenty independent, spontaneously arising cl defective mutants of Bh were isolated and tested for their ability to give turbid plaques when various clear plaque mutants were plated on them (Table 2). All twenty isolates behave identically in that they are unable to give a turbid plaque with homo-immune cy, cII and cIII mutants, showing that the inactive cI₆₅₇ repressor is responsible for the complementation observed in the parent strain Bh.

In order to quantitate the frequency with which λcy phage lysogenises the cryptic deletion strain Bh, experiments were performed to measure the frequency of both survival and lysogenisation in strain Bh and one of its cl defective derivatives after infection by λ clear mutants. The results presented in Table 3 show
Table 3. Cell survival and lysogeny following infection of cryptic lysogen Bh by λ clear mutants

<table>
<thead>
<tr>
<th>Host</th>
<th>Genotype of infecting phage</th>
<th>% survival</th>
<th>% lysogeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bh</td>
<td>λ⁺</td>
<td>36</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>λimm^434</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>λclI₃₀</td>
<td>2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>λcy₁₃₂</td>
<td>50</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>λclI₃₆cIII₆₇</td>
<td>57</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>BhcI</td>
<td>λ⁺</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>λimm^434</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>λclI₃₀</td>
<td>2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>λcy₁₃₂</td>
<td>2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>λclI₃₆cIII₆₇</td>
<td>3</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

The experimental method derives from that of Echols et al. (1973). The cells were grown overnight at 42°C in L broth, diluted 100-fold in L broth supplemented with maltose (0.2%) and grown at 42°C to a density of 5 × 10⁸/ml. The cells were harvested by centrifugation resuspended in one tenth of the volume of phage buffer (0.01 M potassium phosphate buffer at pH 7.0, 0.01 M MgSO₄) and infected with the appropriate phage at MOI = 5. After 15 minutes at 42°C the infected cells were diluted to approximately 2 × 10⁷ cells/ml in supplemented L broth prewarmed at 42°C. The cells were shaken for 20 min at 42°C, diluted, plated on prewarmed L plates and incubated at 42°C to measure survivors. Survivors were screened for lysogeny using the EMBO plate technique (Gingery and Echols, 1967).

that Bh survives infection by λcy and λclI clIII some twenty times better than its cl defective derivative but that the frequency of stable lysogens is extremely low in both strains. Thus the turbid plaques of λcy on Bh must result from abortive lysogens similar to those of λ int (Gingery and Echols, 1967).

**Expression of rex Function**

The rex function of λ is identified by its ability to prevent the growth of rIII mutants of phage T4 in cells lysogenic for λ (Howard, 1967). It now seems clear that the cl and rex genes are distinct and specify separate polypeptides (Gussin et al., 1973; Astrachan and Miller, 1971), but that they form part of the same operon, subject to positive control by at least cII product (Astrachan and Miller, 1971) and negative control by cro product (Eisen et al., 1968; Hayes and Szybalski, 1973; Davison unpublished results). In view of the suggestion made above, that λ repressor activates transcription of the cl gene, and that this stimulatory activity remains in the temperature sensitive clI₃₆₇ repressor at 42°C, it was of interest to discover whether rex gene transcription is subject to the same control. According to this hypothesis the cryptic lysogen Bh grown at 42°C would be phenotypically Rex⁺ due to activation of rex-cl transcription by inactive clI₃₆₇ repressor. However, in the Bh cl derivatives in which the repressor has lost the ability to self-activate it would be expected that the rex gene would not be transcribed.

In accordance with the self-activation hypothesis it was found that the cryptic lysogen Bh is able to exclude T4rIII mutants even after prolonged growth
at 42°C. This result does not agree with a similar experiment by Eisen et al. (1968) who found that a cro-defective lysogen \((N_7 N_5 c^{f 57})\) lost the ability to exclude \(rII\) mutants following growth at 41°C. The reasons for the apparent contradiction are not understood but other cro defective lysogens of the same parentage as Bh (both cro deletions and x mutants) also express rex function at 42°C.

Investigation of the 20 Bh derivatives carrying additional cl mutations resulting in their being defective in self-activation (previous section), showed that the majority of these (13/20) had simultaneously lost the rex function. This result supports the idea that rex transcription in Bh at 42° is dependent on inactive \(c^{f 57}\) repressor. However, the presence of the unexpected class of cl mutant which is defective in self activation but not in rex function, allows alternative explanations which are discussed below.

**Discussion**

The experiments described in this report show complementation between cy mutants and temperature-sensitive cl mutants under two different conditions. When the two phages co-infect a non-lysogenic cell the mutant cl gene is transcribed in the establishment mode, since complementation is eliminated by the presence of a cy mutation cis to the temperature-sensitive mutant cl gene. Complementation also occurs when key or \(c^{f 57}\) cIII infect the cro-defective cryptic lysogen Bh, carrying the \(c^{f 57}\) allele. Under these conditions the temperature sensitive mutant cl gene can be transcribed only from prm, since pre is deleted and since, at least in the case of superinfection by \(c^{f 57}\) cIII, the functions necessary for establishment of repression are not provided. The two experimental conditions differ in that the rate of repressor synthesis from pre during infection is much greater than that from prm of a lysogen (Reichardt and Kaiser, 1971).

Complementation is not seen when the same cryptic cro-defective lysogen carries an additional cl mutation, showing that the complementation observed is a property of the cl\(^{f 57}\) product. The fact that inactive cl\(^{f 57}\) repressor is present in cryptic lysogen Bh grown at 42° suggests either that cl transcription is constitutive or that it is self-activated even by thermally-inactivated cl\(^{f 57}\) repressor. The complementation observed between the inactive repressor and the superinfecting key (or \(c^{f 57}\) cIII) phage can possibly be explained by an interaction between inactive cl\(^{f 57}\) subunits and wild type cl\(^+\) subunits produced by the key phage in limited amounts. However, as discussed above, \(a\) \(a\) \(p\) \(r\) \(i\) \(t\) \(r\) \(i\) \(v\) \(i\) \(o\) \(n\) \(t\) \(i\) \(o\) \( r\) \( e\) \( s\) \( c\) \( t\) \( o\) \( n\) arguments make this hypothesis seem unlikely. Instead it is suggested that a repressor protein is required for transcription of the cl gene and that the cl\(^{f 57}\) repressor, although inactive for repression at 42°C, remains active in the positive control of cl transcription. This unifying hypothesis thus attributes both the presence of cl\(^{f 57}\) repressor in strain Bh at 42°C and the ability of this cryptic lysogen to complement key at 42°, to the same mechanism. The hypothesis is incompatible with the “flip-flop” model, in which an active repressor is required to bind to the operator \(O_8\) and prevent rightward transcription in order to facilitate cl transcription (Eisen et al., 1968). It therefore remains an open question whether self-activation of cl transcription is mediated via the rightward operator. The “flip-flop” model has previously been criticised on other grounds (Oppenheim and Slonim, 1971).
The results obtained by the type of experiments used in this study are strictly qualitative and do not demonstrate that the ability of cl 857 repressor for self-activation of the cl transcription is the same at 42°C as that at 32°C. This reservation is important since, with one exception (Echols and Green, 1971), all workers on self-activation have used the cl857 mutation. If self-activation were less efficient at 42°C than at 32°C, our results would be compatible with reports of a reduction of cl transcription at 42°C (Heinemann and Spiegelman, 1970; Kourilsky et al., 1970; Reichardt and Kaiser, 1971; Oppenheim and Slonim, 1971). On the other hand, if cl857 repressor were equally efficient at self-activation at both temperatures, no effect would be found, giving the appearance of constitutive cl transcription (Hayes and Szybalski, 1973). One further point is relevant; since all experiments have used cl857 repressor and since it is possible that this may not be defective in self-activation at 42°C, there remains no compelling evidence that constitutive repressor synthesis can take place. This point could be best resolved by investigating the level of cl specific RNA in a defective λ lysogen carrying a cl mutant defective in self-activation.

The fact that rex function remains in strain Bh after growth for many generations at 42°C similarly suggests that rex transcription is either constitutive or activated by inactive cl857 repressor. The observation that the majority (13/20) of mutations in the cl gene result in loss of rex function strongly suggests the latter hypothesis. The finding that the remaining seven cl mutants remained rex+ may be explained by suggesting that these continue to self-activate rex-cl transcription, though to an extent too small to give a turbid plaque with λcy, but sufficient to give a rex+ phenotype. However, two trivial explanations must be considered. Firstly, it is possible that all thirteen rex-defective cl mutations are either deletions or polar mutations. This is somewhat unlikely since these types do not usually comprise the major class of spontaneously arising mutations. The polarity explanation is rendered less likely by the observation (Davison, unpublished data) that none of the twenty cl mutants were suppressed by the amber suppressor supD (suI). The second possibility is that rex product and repressor interact to produce the rex+ phenotype (Ravin and Polychina, 1968). This explanation seems very unlikely since amber mutants of the cl gene give normal expression of rex function (Astrachan and Miller, 1972).

The observation that although λcy forms turbid plaques on the cryptic lysogen Bh, it does not form stable lysogens, was unexpected. The explanation probably lies in the timing of repressor synthesis. During the infection of a non-lysogenic strain repressor synthesis is delayed, presumably due to the time taken to synthesize sufficient N product and cl II and cl III products (Reichardt and Kaiser, 1971; Echols and Green, 1971). However, in the cryptic lysogen Bh, according to our self-activation hypothesis, repressor synthesis would begin immediately, so that there may be insufficient time to accumulate the int product necessary for site-specific recombination leading to stable lysogeny.

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