ORGANIZATION AND FUNCTION OF THE PHAGE LAMBDA CHROMOSOME

Thesis by
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ABSTRACT

The process of prophage integration by phage λ and the function and structure of the chromosomal elements required for λ integration have been studied with the use of λ deletion mutants. Since $\underline{att}^{\varphi}$, the substrate of the integration enzymes, is not essential for λ growth, and since $\underline{att}^{\varphi}$ resides in a portion of the λ chromosome which is not necessary for vegetative growth, viable λ deletion mutants were isolated and examined to dissect the structure of $\underline{att}^{\varphi}$.

Deletion mutants were selected from wild type populations by treating the phage under conditions where phage are inactivated at a rate dependent on the DNA content of the particles. A number of deletion mutants were obtained in this way, and many of these mutants proved to have defects in integration. These defects were defined by analyzing the properties of Int-promoted recombination in these att mutants.

The types of mutants found and their properties indicated that $\underline{att}^{\varphi}$ has three components: a cross-over point which is bordered on either side by recognition elements whose sequence is specifically required for normal integration. The interactions of the recognition elements in Int-promoted recombination between att mutants was examined and proved to be quite complex. In general, however, it appears that the λ integration system can function with a diverse array of mutant att sites.

The structure of $\underline{att}^{\varphi}$ was examined by comparing the genetic properties of various att mutants with their location in the λ chromosome. To map these mutants, the techniques of heteroduplex DNA formation and electron microscopy were employed. It was found that integration cross-overs occur at only one point in $\underline{att}^{\varphi}$ and that the

recognition sequences that direct the integration enzymes to their site of action are quite small, less than 2000 nucleotides each. Furthermore, no base pair homology was detected between $\underline{att}^{\varphi}$ and its bacterial analog, $\underline{att}^{B}$. This result clearly demonstrates that λ integration can occur between chromosomes which have little, if any, homology. In this respect, λ integration is unique as a system of recombination since most forms of generalized recombination require extensive base pair homology.

An additional study on the genetic and physical distances in the left arm of the λ genome was described. Here, a large number of conditional lethal nonsense mutants were isolated and mapped, and a genetic map of the entire left arm, comprising a total of 18 genes, was constructed. Four of these genes were discovered in this study. A series of λ$\underline{dg}$ transducing phages was mapped by heteroduplex electron microscopy and the relationship between physical and genetic distances in the left arm was determined. The results indicate that recombination frequency in the left arm is an accurate reflection of physical distances, and moreover, there do not appear to be any undiscovered genes in this segment of the genome.

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GENERAL INTRODUCTION

Bacteriophage lambda (λ) has been extensively employed as a model system for the study of many biological processes because of its simplicity and the ease with which genetic and biochemical analyses can be carried out. The genetic map of the λ chromosome (Figure 1) demonstrates one very striking aspect of the simplicity of this organism: the functional organization of the genome is such that genes with similar functions are clustered together in the chromosome (1). In the left arm, genes A through F are concerned with DNA maturation (1, 2) and with formation of the phage head (3-6); genes Z-J control the assembly and structure of the phage tail (3, 4, 6). Thus all of the structural proteins of the phage particle are made and assembled by the genes in the left arm of the chromosome. The right arm of the λ genome is concerned with early events which take place soon after infection. The CIII-CII region controls the course of infection by regulating the expression of all other lambda genes (7-11). The O and P genes are necessary for λ DNA replication (12), and the Q-R region controls the expression of the A-J segment and the process of cell lysis (1, 13-15).

The genes in the left and right arms of the λ chromosome are essential for vegetative growth (4, 5, 7, 15-20), however the central portion of the λ DNA does not contain any essential genes (19, 21-23). Rather, this segment of the chromosome governs two recombinational processes: prophage integration (21, 22, 24-30); and generalized recombination (22, 31-33). Although neither of these functions is essential for growth, as we will discuss below, prophage integration is an essential step in the lysogenic pathway leading to stable lysogeny. As a temperate phage, λ can choose between the

Figure 1. A physical gene map of λ.

The distances on this map are meant to reflect the actual relationships between genes in the λ DNA molecule. The data used in constructing the map are from Simon, et al. (34) for the CI-R region, from Parkinson & Davis (35) for the J-CI region, and from Parkinson & Davis (36) for the A-J segment. Abbreviations are:

att$^{\varphi}$ = λ prophage insertion site

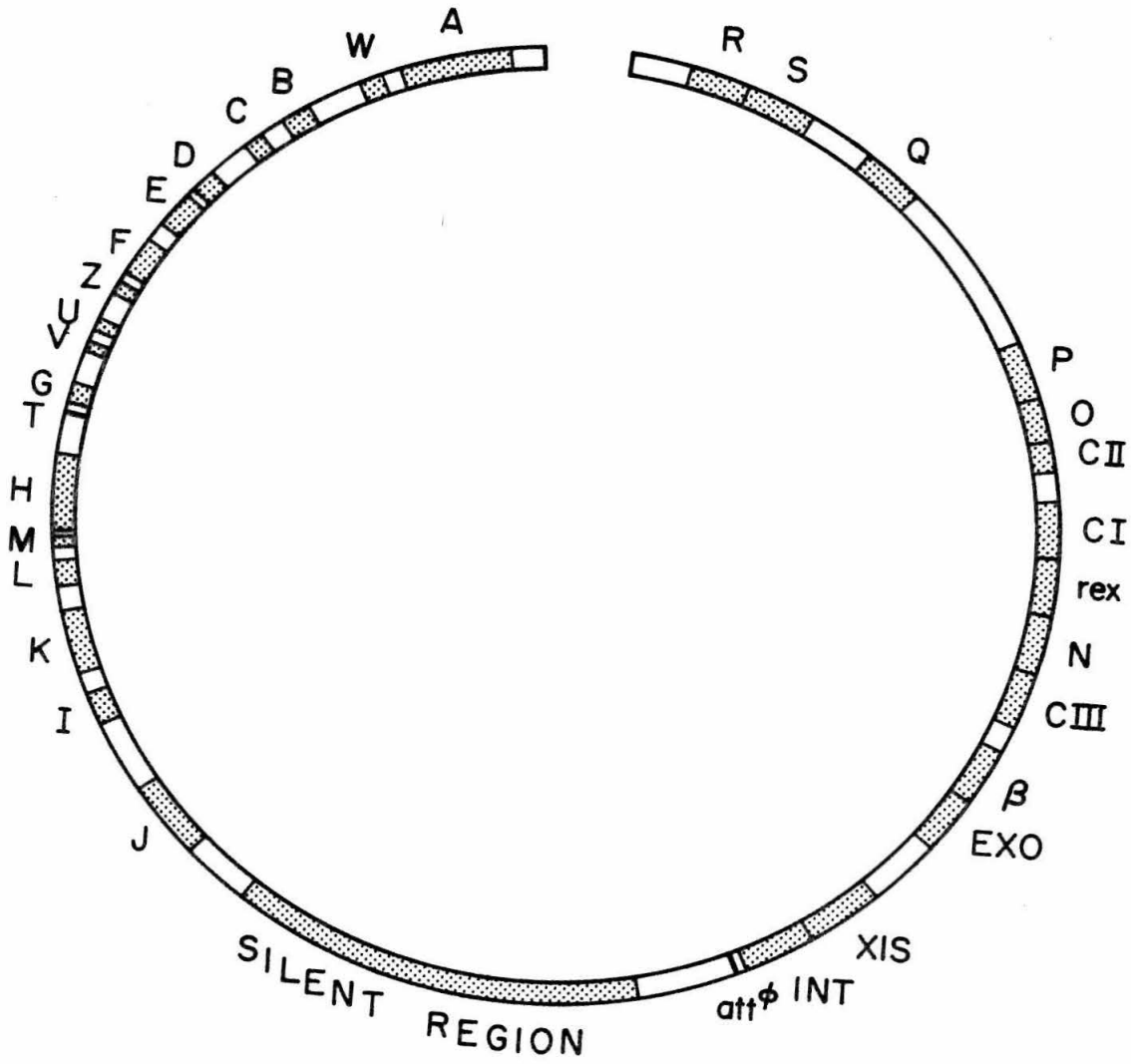
INT = integration gene

XIS = excision gene

EXO = λ exonuclease gene; needed for recombination

β = λ β protein; recombination gene

rex = λ gene responsible for excluding T4rII mutants.


A
W
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SILENT REGION
att φ INT
XIS
EXO
β
CIII
N
rex
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lytic pathway which results in cell death, and the lysogenic pathway which results in a stable association of the phage and bacterial DNA with no cell death. Thus infection by λ does not always lead to cell death as it does for virulent phage strains such as T4. Since lysogeny functions are not essential for lytic growth, the study of lysogeny presents several problems which will become apparent in Part I of this thesis.

Many of our current notions concerning processes such as morphogenesis, recombination, and development have originated with studies on λ. The work described in this thesis concerning two aspects of chromosome structure and function in λ should also provide some insight into a number of more general phenomena. This thesis is divided into two parts, with all results presented in the form of manuscripts, one of which has appeared in the literature, the rest of which have been submitted for publication. Part I deals with the mechanism of prophage integration and the special chromosomal structures involved in integrative recombination. Part II concerns a study of the genetics and physiology of mutants in the left arm of the λ chromosome, and the relationship between genetic and physical distances measured in this region of the λ DNA.

In part I, a novel method will be described for studying the process of prophage integration and the chromosomal structures required for integration. In this work a method for isolating λ deletion mutants will be described. These deletion mutants are used to analyze the genetic and physical properties of the λ integration apparatus. In particular, the technique of electron microscope mapping of heteroduplex DNA molecules has been exploited in several new ways. In part II, this technique was again used to determine the physical distances between genes in the left arm of the λ chromosome.

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PART I

PHYSICAL AND GENETIC STRUCTURE OF THE LAMBDA INTEGRATION APPARATUS

INTRODUCTION

After infecting a sensitive bacterium, temperate phage λ chooses whether to multiply vegetatively, thereby killing the host cell (lytic response) or instead assume a stable symbiotic relationship with the bacterial host (lysogenic response). The choice between the lytic and lysogenic pathways is made on the basis of the physiological condition of the host (1, 2) and probably assumes the form of a "race" between the early lytic functions and the initial events of lysogenization. The two pathways are mutually epistatic such that the initiation of lytic growth will prevent the expression of essential lysogeny genes (3), and likewise, the first steps in the lysogenic pathway turn off all events associated with the expression of lytic functions (4).

The establishment of lysogeny by phage λ takes place in two sequences of events. The first step is the repression of all lytic functions. This is the stage at which the fate of the cell is determined, for if repression cannot occur, the phage is forced to follow the lytic pathway, eventually destroying the host bacterium. Repression is ultimately achieved by the repressor, the product of the CI gene (5, 6). The repressor acts on two operator sites at each end of the CI gene to prevent both directly and indirectly the transcription of all other portions of the λ genome (7-9). Recent evidence, however, suggests that the CII and CIII genes of λ may also play a role in repressing lytic functions as a prelude to CI repression (10). Thus CI, CII and CIII mutants of λ are unable to establish lysogeny for the simple reason that repression of lytic functions cannot take place (3).

The repressed λ chromosome is called a prophage (11) and cells carrying a λ prophage acquire several new properties not found in non-lysogenic cells. Since the CI gene is the only functioning prophage gene (12, 13), the prophage continually produces repressor molecules which provide the cell with an immunity to superinfecting λ phages. When repression is lifted either spontaneously or by various inducing treatments, the prophage enters the lytic pathway and begins to make more progeny particles. Thus a λ prophage confers superinfection immunity on a cell, however the cell is continually faced with the prospect of undergoing spontaneous or induced lysis.

After repression, the next step in lysogenization involves the formation of a stable association between the prophage DNA and the replication machinery of the host. Although repression must occur shortly after infection, this final step in the lysogenic pathway may not take place for several cell generations (14). However, under normal conditions, the establishment of repression invariably leads to stable lysogeny in most of the infected cells (14). Since the replication functions of the phage are repressed, it is essential that the prophage become associated with the replication machinery of the host in order for the prophage to be duplicated and distributed to both daughter cells at each division. When this mechanism of association breaks down, the prophage cannot replicate and is transmitted unilinearly at each cell division, a condition known as abortive lysogeny (15). Cells which lose their prophage in this manner soon become sensitive again to superinfection because they no longer contain λ repressor (16).

Campbell (17) has proposed a simple, yet elegant model that accounts for the manner in which prophage and bacterial DNA are

replicated in synchrony in stably lysogenic cells. Campbell's model is outlined in Figure 1. The basic premise of this model is that the prophage DNA actually becomes inserted into the continuity of the bacterial chromosome by means of a reciprocal recombination event. After infection and subsequent repression, the phage genome was thought to circularize and a region of the phage DNA paired and recombined with a similar region in the bacterial chromosome. This leads to the insertion of the small circular λ DNA into the larger host chromosome. Prophage excision could occur by a reversal of the integration process after induction. Since the inception of this model, a vast amount of evidence has been obtained which clearly demonstrates the validity of the Campbell model in all major respects. This evidence will be summarized only briefly here.

The DNA of mature λ particles is a single linear molecule (18, 19). The Campbell model predicts that one of the initial stages during lysogenization must be the formation of circular λ chromosomes from these initially linear molecules. Circular forms of λ DNA have been demonstrated by electron microscopy (20) and by sedimentation velocity analyses following infection and induction (21-23). It is now clear that λ DNA circularizes through complementary single-stranded regions at the ends of the linear DNA molecule. These "sticky" or cohesive ends are 20 nucleotides long (24) and in vivo they can rapidly anneal to form hydrogen-bonded circles which are then covalently closed by the host's polynucleotide ligase (25).

The λ prophage, according to Campbell's model, is linearly inserted into the bacterial chromosome by recombination. Therefore the prophage DNA should be linear and physically bound to the bacterial chromosome; in addition, the prophage genetic map should be linear, but might be a cyclic permutation of the vegetative map

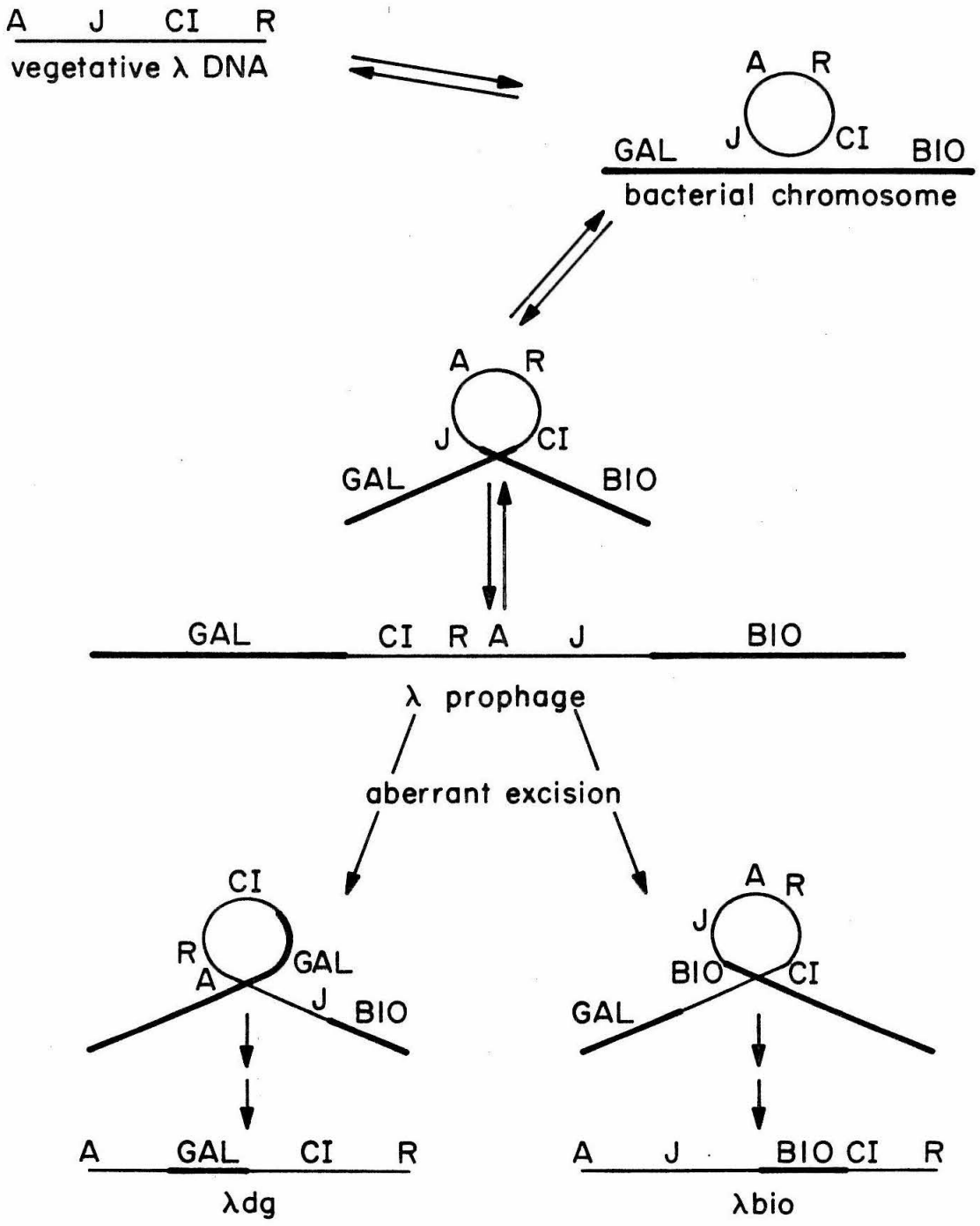
Figure 1. The Campbell model of prophage insertion and transducing phage formation.

After infection, the phage DNA circularizes, pairs with the bacterial chromosome, and recombines with the host chromosome. Excision usually occurs by a reversal of this process using the prophage ends for recombination, however, on rare occasions excision may occur by aberrant recombination acts between a portion of the host genome and a region in the phage chromosome, producing transducing particles with bacterial substitutions for some of the phage genes. Abbreviations are:

A, J, CI, R = phage genes included to demonstrate the permuted order of prophage genes compared to the vegetative order.

GAL and BIO = galactose and biotin operons of E. coli.

λdg and λbio = galactose- and biotin-transducing variants of λ, produced by aberrant excision.


A J CI R
vegetative λ DNA
A R
J CI
GAL BIO
bacterial chromosome
A R
J CI
GAL BIO
GAL CI R A J BIO
λ prophage
aberrant excision
CI
R
A
GAL
J BIO
A GAL CI R
λdg
A R
J
BIO CI
GAL
A J BIO CI R
λbio

order because of the circular intermediate between the vegetative and prophage states. Both genetic and physical experiments support the notion of linear insertion predicted by Campbell. For example, λ prophage behaves like any other bacterial marker in conjugation and transduction experiments (26, 27). The prophage maps between the galactose and biotin operons of E. coli and has a linear gene order which is a cyclic permutation of the vegetative genetic map. The prophage gene order is consistent with the integrative recombination event taking place always between the J and CI genes in the vegetative chromosome. Two lines of genetic evidence suggest that the λ prophage is physically inserted into the host chromosome. The linkage between gal and bio is decreased in the presence of an integrated λ prophage (25), and secondly, bacterial deletions can be obtained which extend into the prophage from either the gal or bio side removing prophage genes in the order predicted from the prophage map (28).

The most conclusive evidence for insertion is furnished by physical studies. Several workers have used density labels to follow the λ chromosome during integration and during excision (29-31). These experiments not only demonstrate that prophage DNA is covalently bound to bacterial DNA (29-31), but also that the recombination act leading to insertion and excision must occur in many, probably all, of the cells by breakage and reunion rather than copy-choice (30, 31). A second type of experiment involves the isolation of an entire lysogenic chromosome. Since it is not feasible to isolate the entire E. coli chromosome, F' episomes containing the gal-bio region from the host chromosome have been used to show that insertion has occurred. It is found that integration of λ prophage increases the size of the F' factor by an amount equivalent to the

size of one, and sometimes two, λ chromosomes (32).

One final line of evidence consistent with Campbell's model should be mentioned. Lambda is able to form specialized transducing phages which carry in place of some phage genes either the galactose or biotin genes from the host (33, 34). Campbell proposed that such variants were formed by aberrant excision after induction and are a consequence of linear insertion as shown in Figure 1. The available evidence is consistent with this interpretation. For example, λdg and λbio phages can only be formed after prophage induction which indicates that some feature peculiar to the prophage state, presumably insertion, is essential for transducing phage production. Furthermore, λ can only transduce genes which are adjacent to the prophage, which also supports Campbell's model of their formation. Finally, genetic (35-38) and physical (39, 40) studies of λdg phages and λbio phages confirm the structures shown in Figure 1 based on Campbell's model. Thus, the notion of linear insertion as the mechanism of stable lysogeny in λ appears well established.

How does prophage insertion come about? The fact that λ can establish stable lysogeny with normal frequency in recombination-deficient bacterial strains suggests that λ integration is carried out by some recombination system other than the Rec system which is responsible for all bacterial recombination (41, 42). At least one essential component of this integrative recombination system must be under phage control for the following reasons. When a λ phage superinfects a λ lysogen (homoimmune superinfection), it cannot integrate even though the resident prophage affords a large region of homology for recombination (43). Double lysogeny can be established by simultaneous infection with two different λ phages or

after heteroimmune superinfection (43, 44) which indicates that the hindrance to insertion in homoimmune superinfection is functional rather than structural. Since in homoimmune superinfections the infecting genome is immediately repressed, this indicates that a phage gene must be expressed to establish stable lysogeny.

One phage-specific integration factor has been identified by mutation and is designated the Int function (45). Mutants defective in the Int gene cannot integrate or, once inserted, cannot excise upon induction (45-47). Since int$^-$ mutants can be complemented for insertion and excision in trans, the int$^-$ defect must be recessive and the int$^+$ substance must be diffusable. Thus, although the function of the Int product is not known, it is clear that this product is essential for integration and excision. The simplest interpretation is that the Int gene makes an "integrase" which is necessary for insertional recombination.

A second phage-specific factor involved in the λ integration system has been recently identified. This gene is called Xis because xis$^-$ mutants are unable to excise from the bacterial chromosome after induction, even though they can insert normally (48). The xis$^-$ defect is also recessive which again suggests that the Xis gene like the Int may make an enzyme needed for excisive recombination.

With the discovery of λ mutants deficient in generalized recombination (49, 50), it was possible to demonstrate that integrative recombination occurs between two phage genomes as well as between the prophage and the bacterial chromosome (51, 52). This recombination occurs in the absence of all generalized recombination, but is strictly dependent on the Int function. Because Int-promoted recombination between two phage chromosomes only occurs at one site between the J and CI genes, it is believed that this type of recombination

process is identical to the prophage insertion event.

The locus at which Int-promoted recombination takes place is called the attachment (att) site. The phage chromosome contains att^{φ}, whereas the bacterial chromosome is thought to contain a similar site (att^{B}) located between gal and bio. Integration, therefore, is actually Int-promoted recombination between att^{φ} and att^{B}. This att combination does not require the Xis factor, however, Int-promoted recombination between the prophage ends does require Xis function (53).

The attachment site appears to play a purely structural role in the integration system since a number of λ mutants with defective att sites cannot be complemented for insertion in trans (13, 54-56). The known att mutants are deletions or substitutions with rather gross structural changes; since no att-defective point mutations have ever been reported, this suggests that att^{φ} has a structural role whose function cannot be destroyed by single base substitutions. The work described in this section of the thesis deals with studies into the fine structure of the attachment region in the λ chromosome. The rationale and general approach for these studies is outlined below.

Assume that att^{φ} and att^{B} are the substrates for the enzymatic component(s) of the λ integration system. We would like to know how these structures are recognized by the integration enzymes and what effect changes in the att site have on integration. Also we would like to know whether integrative recombination requires homology of the recombining chromosomes as generalized recombination does. To examine these aspects of λ integration, the following features of the att site were studied:

1) the genetic structure of att$^{\varphi}$
2) the role of att$^{\varphi}$ components in integration
3) the physical size and location of att$^{\varphi}$ and its components
4) the extent of base sequence homology between att$^{\varphi}$ and att^{B}.

In order to study att$^{\varphi}$, mutants with major structural defects are required. Since integration is not an essential function, viable deletion and substitution mutants of λ were isolated and examined for possible att defects. The methods used to isolate such mutants are described in the first manuscript below.

The integration defect of presumed att mutants was investigated mainly with the technique of Int-promoted recombination. Since the frequency of Int-promoted recombination between two phage genomes has been found to depend on the att sites of those phages (57), the att defects in these new mutants can be defined in terms of their effects on Int-promoted recombination. This type of test also allows a distinction to be made between mutants with structural integration defects and those with only functional defects. The genetic properties of att-defective mutants are described in the second manuscript.

Having established the phenotype and genetic properties of att-defective deletions and substitutions, I could apply the principles of deletion mapping to determine which portions of att$^{\varphi}$ are involved in any particular phenotype. It is necessary, however, to know the precise location of the deletion or substitution in each of these mutants in order to do this. To map the mutants, a technique developed by Davis & Davidson (58) was employed for visualizing the location of a deletion or substitution in heteroduplex DNA molecules by electron microscopy. With this technique an accurate physical map of the lesions in various att mutants could be constructed,

and with this physical map, we could locate very precisely the important att components missing in each of these mutants. To investigate the problem of homology between att^{φ} and att^{B}, a simple variation of the basic method described above allowed us to accurately measure the position and extent of very small regions of homology between two DNA molecules. This work was done in collaboration with Ron Davis of the Chemistry Division, and without his expertise and guidance, this most important aspect of the att^{φ} investigation would not have been possible. This work is described in the last manuscript of Part I.

Deletion Mutants of Phage Lambda

I. Isolation and Initial Characterization

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The inactivation rate of lambda phage at high temperature or low ionic strength is largely dependent on the DNA content of the lambda head. Deletion mutants of lambda have been selected, therefore, by isolating heat resistant or chelating agent resistant mutants of λ wild type. Because λ wild type stocks contain about .01% phenotypically resistant phage, it was necessary to select deletion mutants by recycling procedures which appear to select against certain types of deletions. Deletions have been isolated from UV-induced and vegetatively grown λ stocks and the two preparations are different in several respects. Based on the results of preliminary characterizations, all of the deletion mutants fall into three major classes with respect to their integration behavior on different host strains. Furthermore, none of the nearly 200 deletion mutants studied is defective in the ability to promote recombination in recombinationless host bacteria.

1. Introduction

Unlike the DNA of many bacteriophages, the chromosome of temperate phage lambda (λ) has specific fixed ends so that deletions of a portion of the λ chromosome result in particles which contain less DNA than wild type. Lambda deletion mutants can, therefore, be identified by virtue of their decreased buoyant density in CsCl equilibrium density gradients (Weigle, Meselson & Paigen, 1959); however, it is difficult to take advantage of this property of λ deletions to isolate new deletion mutants (see Kellenberger, Zichichi & Weigle, 1961a). We have developed methods for obtaining deletion mutants of λ based on the findings of Hertel, Marchi & Müller (1962) who demonstrated that heat resistance in the virulent phage T5 is accompanied by a decrease in buoyant density of the resistant particles. Heat resistant T5 phage are now known to be deletion mutants (Rubenstein, 1968), and we have found that λ deletion mutants can also be obtained by isolating heat-resistant λ phage.

Viable deletion mutants of lambda, having lost only non-essential segments of DNA, provide a way to study the organization and function of those regions of the λ chromosome which are not required for vegetative growth. Since Franklin (1967) has demonstrated that the entire central third of the λ chromosome is not essential for vegetative growth, we would expect most of our deletion mutants to lie within this region of the genome. Two very important, although dispensable, processes are controlled by the middle portion of the λ chromosome:

generalized recombination (Echols & Gingery, 1968; Signer & Weil, 1968) and prophage integration (Kellenberger et al., 1961a; Zissler, 1967).

In this paper we will describe two similar methods for selecting deletion mutants of phage lambda and the initial characterization of such mutants. In the accompanying papers (Parkinson, 1970; Parkinson & Davis, 1970) we have further studied these deletion mutants to analyze the genetic and physical properties of the lambda integration apparatus in the center of the λ chromosome. By studying λ mutants with structural defects, substitutions as well as deletions, we have been able to detect and characterize several structural elements in the λ genome which are involved in prophage integration.

2. Materials and Methods

(a) Phage strains

The λ mutants used in this work are listed in Table 1. Multiple mutant strains were constructed by crossing the appropriate parental strains together and then testing the desired recombinant by backcrossing to confirm the genotype. Stocks were made by the confluent lysis method or by UV or temperature induction of a lysogenic strain.

(b) Bacterial strains

The bacterial strains used in this study are described in Table 2. E. coli strain C600 was used for all phage assays.

TABLE 1

Phage strains

a) Derivatives of λ^{++} (λ PaPa)

Strain	Phenotype	Source and/or reference
CIt1	CIts	Lieb (1964)
CI509	CIsus	E. T. Young
int6	intA	E. Signer; Gottesman & Yarmolinsky (1968)
red3	redABC	E. Signer; Signer & Weil (1968)

b) Deletion mutants of λ

Strain	Density	% DNA change	Source and/or reference
λsd1	1.520	+8	E. Calef; Pica & Calef (1968)
λb1	1.512	+2	J. Weigle; Weigle et al. (1959)
λi^{434}	1.504	-3	J. Weigle; Kaiser & Jacob (1957)
$\lambda b5(\lambda i^{21})$	1.501	-5.5	J. Weigle; Kellenberger et al. (1961b)
λb2	1.491	-13	J. Weigle; Kellenberger et al. (1961a)
λb2b5c	1.484	-18.5	J. Weigle; Kellenberger et al. (1961b)

c) Special strains for recombination tests

red3 i^{434} sus R444

bioll i^{434} sus R5 (from M. Shulman)

TABLE 2

Bacterial strains

Strain	Relevant genotype[1]	Source and/or reference
C600	*su*$^+$ *rec*$^+$	Appleyard (1954)
594	*su*$^-$ *rec*$^+$	Weigle (1966)
CR63	*su*$^+$ λ^r,λh^s	Appleyard *et al.* (1956)
QR48	*su*$^+$ *recA*	E. Signer; Signer & Weil (1968)
QR14	*su*$^+$ *rec*$^+$ λcryh	E. Signer; Signer & Weil (1968)
QR15	*su*$^+$ *recA* λcryh	E. Signer; Signer & Weil (1968)
AB2480	*uvrA* *recA*	P. Howard-Flanders; Howard-Flanders & Theriot (1966)
SM0005	C600 (λ^{++})	
RS0744	594 (R444 CIts1)	
RS0746	594 (R5)	

[1]Abbreviations: *Su*$^+$ (*su*$^-$) = ability (inability) to support the growth of λ *sus* mutants.

rec$^+$ (*recA*) = ability (inability) to promote generalized recombination.

uvrA = UV-sensitive; unable to dark repair UV-damaged λ.

λcryh = cryptic prophage (no immunity) carrying extended host range marker (h).

λ^r,λh^s = resistant to λh^+, sensitive to λh.

(c) Media

TMG dilution buffer, K medium and BBL top and bottom agar for plates have been described previously (Parkinson, 1968). Tryptone broth (1% tryptone, 0.5% NaCl) was often used in place of K medium for growing bacteria. EMB glucose plates contained per liter of distilled H_2O: 27.5 gm EMB agar base (Difco) and 1 gm yeast extract. After autoclaving, sterile glucose solution was added to 0.05% final concentration and plates were poured rather thin ($\sim$ 25 ml./plate).

(d) Chemicals

Optical grade cesium chloride (Harshaw Chemical Co.) was used for CsCl density gradients, otherwise all chemicals used were standard reagent grade.

(e) Procedures described elsewhere

We have used a number of techniques which have been described by others. Except where noted, no modifications were made in these procedures as they are described in the literature.

(i) Plating bacteria, crossing bacteria and crossing procedure (Parkinson, 1968).

(ii) Red marker rescue spot tests (Signer & Weil, 1968). We used either QR14 + CR63 (1:2) or QR15 + CR63 (2:1).

(iii) Int spot test (Gottesman & Yarmolinsky, 1968).

(iv) Multiplicity reactivation was done with strain AB2480 as

described by Huskey (1969).

(f) Terminology

The abbreviations used in this paper are:

rec$^-$ = recombinationless bacterial mutants

red$^-$ = recombination deficient λ mutants

int$^-$ = integration negative λ mutants

λ cry = cryptic prophage containing only the λ genes from P through J on the phage chromosome

λ del$^+$ = λ phage which carries no deletions, that is, contains the wild type amount of DNA

3. Results

(a) Isolation of lambda deletion mutants

Two similar techniques have been developed for isolating deletion mutants of lambda. The method used initially--selection for heat resistance--has recently been replaced by a more sensitive method, selection for resistance to chelating agents. Although in all major respects both methods are virtually identical, we will describe in parallel the properties of both methods to point out several important quantitative differences in the two techniques as well as to emphasize the similarities in the selection procedures.

(i) Inactivation of λ^{++} by heat or by chelating agents.

Figure 1 shows the effect of high temperature and of chelating agents

Fig. 1. Inactivation patterns of λ^{++}.

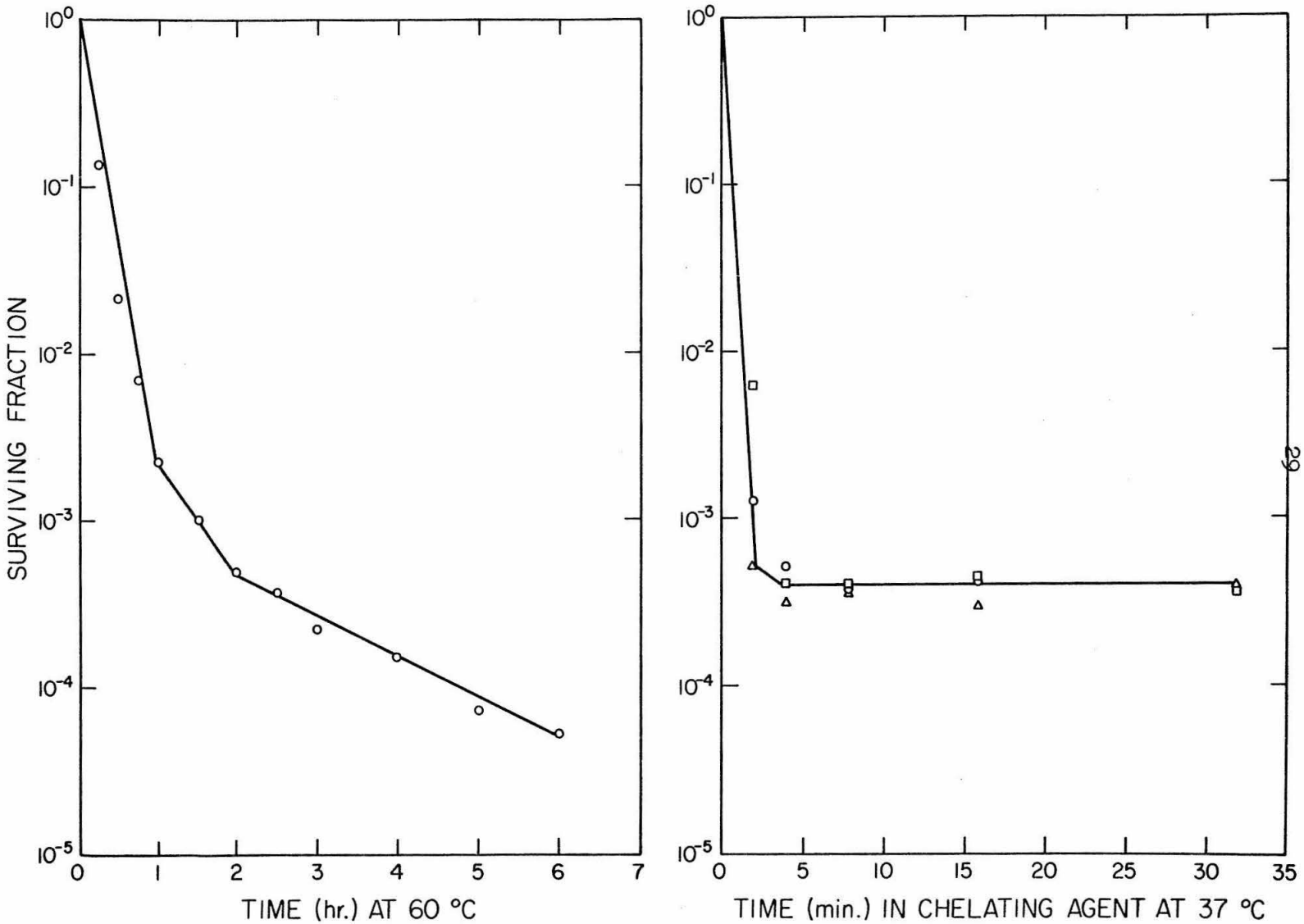
a) Heat inactivation. λ^{++} were diluted 50- to 100-fold into 10^{-1} M Tris, pH 7.4, at 60°C with 10^{-2} M $MgSO_4$. The reaction was stopped at various times by diluting samples into TMG buffer at room temperature.

b) Chelating agent inactivation. λCI509 $\underline{del}^{+}$ were diluted 50- to 100-fold into 10^{-2} M chelating agent, pH 8.5, at 37°C. The reaction was stopped at various times by diluting samples into TMG buffer at room temperature.

o——o = $Na_4P_2O_7$

□——□ = Na EDTA

Δ——Δ = Na Citrate


SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0 1 2 3 4 5 6 7
TIME (hr.) AT 60 °C
0 5 10 15 20 25 30 35
TIME (min.) IN CHELATING AGENT AT 37 °C

on the viability of λ^{++}. In both treatments a major portion of the λ^{++} population is rapidly inactivated, revealing a much more resistant minor component. Hereafter the conditions described in the legend of Figure 1 will be referred to as standard conditions. Moreover, we will only discuss pyrophosphate (PP) inactivation of λ phage as an example of chelating agent inactivation. It is evident from the results shown in Figure 1b that the ability to inactivate λ^{++} is not a unique property of any one chelating agent.

The mechanism of λ^{++} inactivation is not yet understood. A preparation of λ^{++} was purified by banding in CsCl and then examined in the electron microscope before and after heat inactivation. The samples were treated first with DNase (1 μg/ml.) and then negatively stained with phosphotungstic acid. The control sample contained 60% full-headed phage. The sample which was heat inactivated to 10^{-4} survival contained less than 0.1% full-headed phage particles. Evidently heat inactivation causes DNA to be released from the phage head. PP-inactivated particles also appear to have lost their DNA as shown by the following experiment. A λ^{++} preparation, purified by banding in CsCl, was analyzed by density in a Spinco Model E analytical ultracentrifuge before and after PP treatment. Before treatment, the UV-absorbing material (DNA) had a density of 1.5 g/cm^3 which corresponds to that of whole phage particles. After inactivation to 4×10^{-4} survival all of the DNA banded at 1.7 g/cm^3 which is the density of free λ DNA. Although DNA is clearly released from λ^{++} particles upon

heat or PP treatment, we do not know if the heads are being ruptured or if the DNA is escaping through the phage tail.

The biphasic inactivation patterns shown in Figure 1 are typical of all λ^{++} stocks. We will assume that the second component of these inactivation curves is due to the presence of heat or PP-resistant phage particles which we will designate λ^{*}. The frequency of λ^{*} phage, determined by back extrapolating the second component of the inactivation curves, is dependent on the inactivation conditions, but is independent of the origin of the λ^{++} stock which is used. Under standard conditions, this level of λ^{*} is quite reproducible for both heat and PP treatment. We have investigated the nature of λ^{*} particles and conclude that λ^{*} are genetically wild type but phenotypically resistant to heat or PP inactivation. The evidence for this conclusion is discussed below.

A preparation of λ^{*} particles was obtained by heat or PP inactivation of a λ^{++} stock to approximately 10^{-4} survival. The surviving phage were analyzed in preparative CsCl gradients and in both cases the surviving particles were heterogeneous in density. A narrow band of phage at wild type density was found and also a broad band of phage with a mean density of about 1.49 g/cm^3. We have not been able to test these phage for heat or PP sensitivity; however, the particles of wild type density could be contaminating λ^{++} phage and we concentrated our attention on the less dense particles. The progeny of the less dense phage are once again as sensitive as λ^{++} to heat or PP-inactivation. These progeny phage also have the same density as λ^{++}, which suggests

that the lighter density of the original λ^* particles was not a genetically determined property of the phage, but only phenotypic. Since resistance is correlated with density, it appears as though λ^* phage owe their resistance to a phenotypic modification of the particle which confers a lower density on the phage as well.

Since the amount of DNA in λ^* particles appears to be the same as in λ^{++}, the lower density of λ^* may be due to increased amounts of protein in these particles. This is probably the case since λ^* particles obtained by heat treatment have a faster sedimentation rate than λ^{++}, suggesting that the overall molecular weight of λ^* is greater than λ^{++}. By density and by sedimentation analysis, λ^* particles appear to be heterogeneous. The different frequencies of λ^* phage which are found with different inactivation treatments suggests that λ^* phage are inactivated by heat or PP until all of the λ^* particles which are sensitive to a particular set of inactivation conditions are destroyed. The more stringent the conditions, the more classes of λ^* will be inactivated and the lower the apparent frequency of resistant phage will be.

Since λ^* particles have a lower buoyant density than λ^{++}, if λ^* are present in λ^{++} stocks before inactivation, they could be removed from the λ^{++} stock by density purification. A λ^{++} stock was banded in CsCl and a very narrow fraction of phage of wild type density was examined for PP sensitivity. The density-purified λ^{++} did not have a biphasic inactivation pattern showing that λ^* particles do not arise during the inactivation treatment.

(ii) Influence of DNA content on inactivation rate. To show that heat or PP inactivation is a valid method for isolating λ deletion mutants, several standard deletion mutants of lambda have been examined. The results presented in Figure 2 show that each of these deletion mutants is less sensitive to heat or PP treatment than is λ^{++}. Figure 2 also demonstrates the greater sensitivity of PP inactivation in discriminating between phages with only small differences in DNA content. For example, λb5 is quite sensitive to heat inactivation, but is completely resistant to PP treatment. Even λi^{434} which is only missing about 3% of its DNA compared to λ^{++} is much more resistant than λ^{++} to PP inactivation.

Is the difference in sensitivity of λ^{++} and λ deletions due to differences in particle structural proteins? If the deletion mutants produce diffusible gene products which are responsible for increased resistance, a mixed infection of λ^{++} and λb2, for example, should produce resistant λ^{++} particles. Over 50 deletion mutants have been examined and never does heat or PP resistance of deletion mutants undergo phenotypic mixing with λ^{++}, which strongly suggests that the pattern of sensitivity to heat or PP is determined only by the size of the DNA in a λ particle and not by "resistant" structural proteins.

Five different λ deletion mutants, ranging from 5% to 23% less DNA than wild type, were studied to show that DNA content is directly related to PP or heat sensitivity. Figure 3 shows the PP inactivation patterns of these deletions at 45°C, a temperature at which all of the mutants are inactivated at measurable rates. The small initial

Fig. 2. Resistance of λ deletion mutants to heat and PP inactivation.

a) Heat inactivation, standard conditions.

o——o = λ^{++}

▽——▽ = λb5

◇——◇ = λb2

b) PP inactivation, standard conditions.

o——o = λCI509 <u>del</u>$^{+}$

□——□ = λi^{434}

▽——▽ = λb5

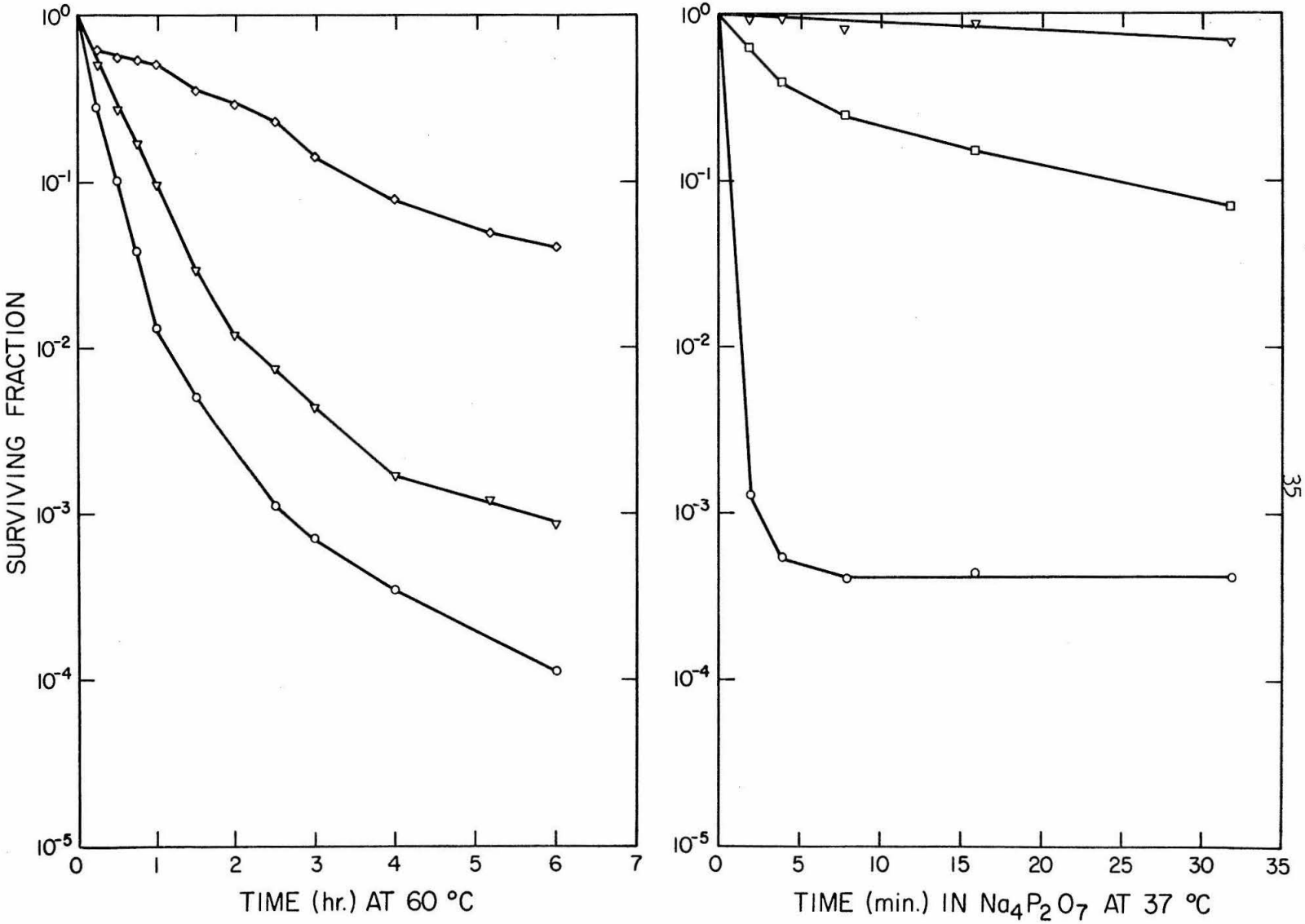
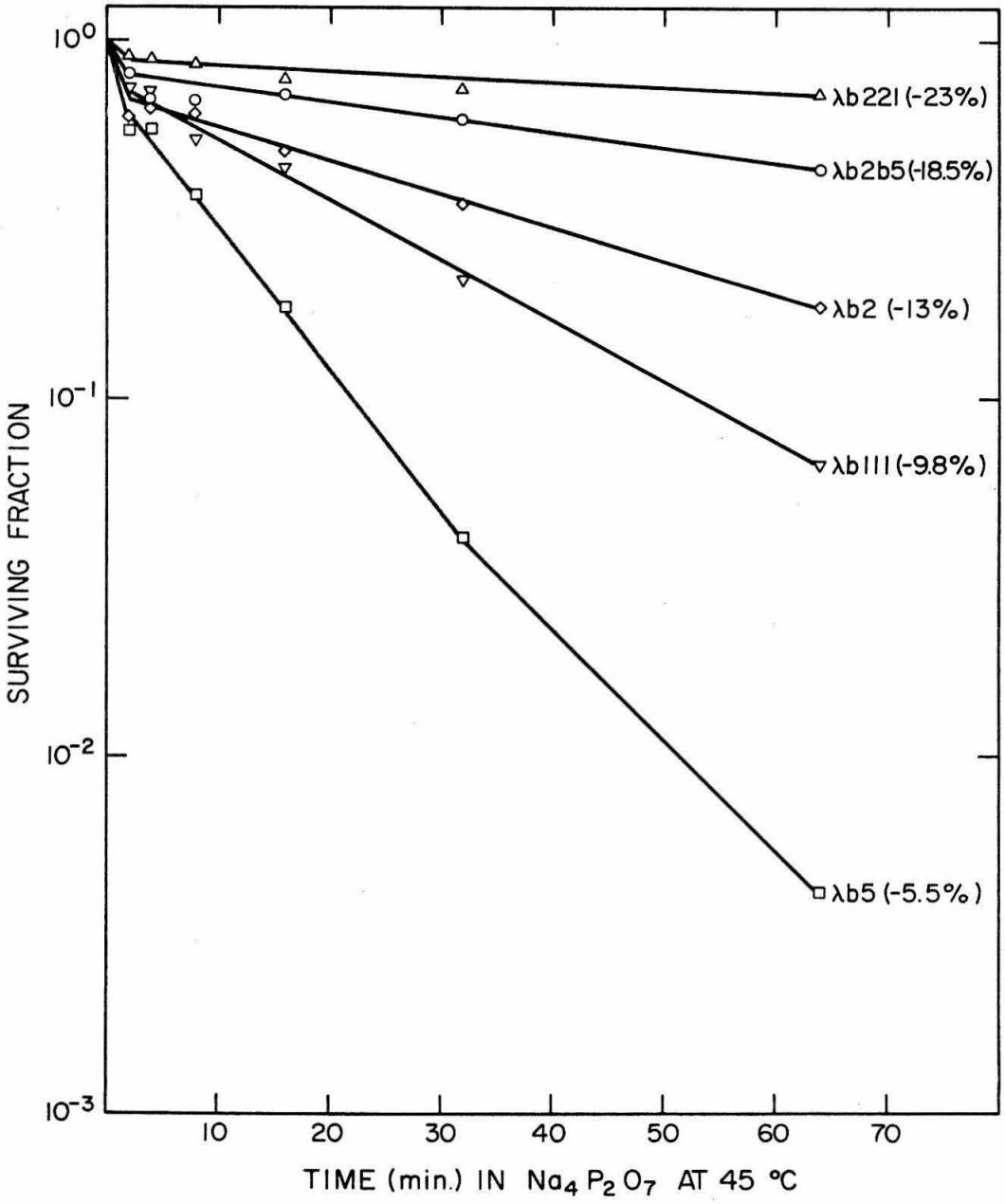

SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0 1 2 3 4 5 6 7
TIME (hr.) AT 60 °C
0 5 10 15 20 25 30 35
TIME (min.) IN $Na_4P_2O_7$ AT 37 °C

Fig. 3. Inactivation patterns of deletion mutants in PP at 45°C. Standard PP conditions were used except that the treatment was carried out at 45°C rather than 37°C.


SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10
20
30
40
50
60
70
TIME (min.) IN $Na_4P_2O_7$ AT 45 °C
λb221 (-23%)
λb2b5 (-18.5%)
λb2 (-13%)
λb111 (-9.8%)
λb5 (-5.5%)

inactivation, which appears to be very rapid as shown in Figure 3, is probably an effect of heat shock and has been disregarded. The inactivation rate of each deletion was calculated from the major linear component of each inactivation curve. As shown in Figure 4, the rate constant for each deletion is directly proportional to the DNA content of that deletion mutant. Analogous experiments have been done for heat inactivation and again we obtain a linear relationship between the log of the inactivation rate constant and percent deletion. This relationship has proven to be a useful method for rapidly estimating the DNA content of deletion mutants.

Since the DNA content of deletion mutants is related to the PP sensitivity of the mutants, we would expect that λ mutants with more DNA than λ^{++} will be even more sensitive than λ^{++} to PP inactivation. The experiment shown in Figure 5 confirms this prediction. Two mutants of lambda, λbl and λsdl, which have somewhat more DNA than λ^{++}, are rapidly inactivated at 20°C in pyrophosphate. At 20°C, however, λ^{++} is killed rather slowly, demonstrating that λbl and λsdl are more sensitive to PP treatment than λ^{++}. This difference is not due to protein differences between λ^{++} and the mutants, since no phenotypic mixing of density or PP-sensitivity is observed. These results emphasize the fact that there is nothing unique about the DNA content of λ^{++} particles with respect to PP inactivation. In fact, it is possible to discriminate between any two λ phages with different DNA contents by choosing the appropriate inactivation conditions.

Fig. 4. Relationship between DNA content and inactivation rate. PP inactivation rates were calculated from Fig. 3.

□ = λb5

▽ = λb111

◇ = λb2

o = λb2b5c

Δ = λb221

The DNA content of b111 and b221 was calculated from their buoyant density in CsCl as explained in Davis & Davidson (1968). The DNA content of b5, b2, b2b5c was calculated from electron microscope data of Davis & Davidson (1968) and Parkinson & Davis (1970).

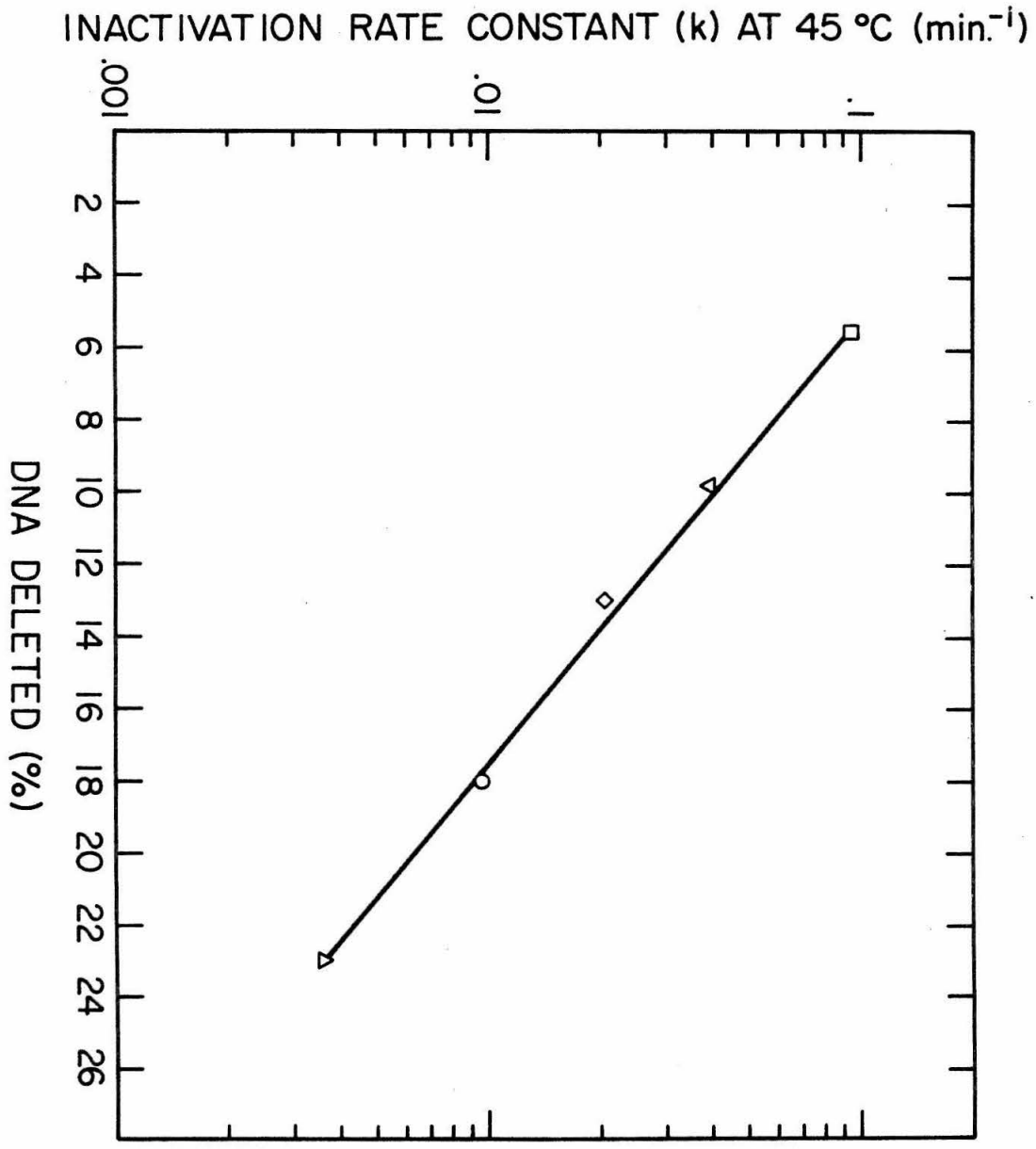
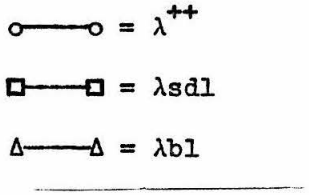
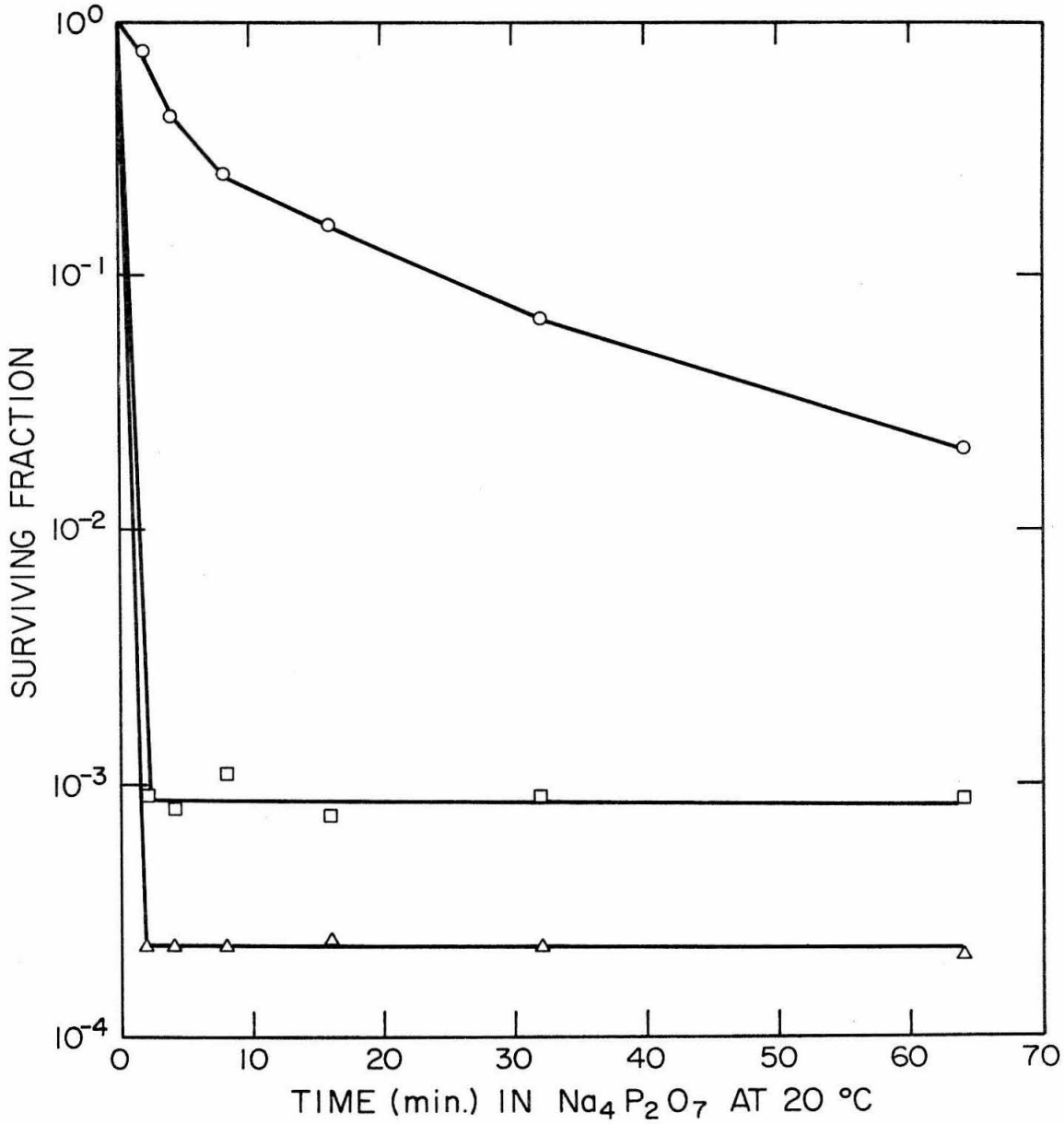

INACTIVATION RATE CONSTANT (k) AT 45 °C (min.$^{-1}$)
.001
.01
.1
DNA DELETED (%)
2
4
6
8
10
12
14
16
18
20
22
24
26

Fig. 5. Increased PP sensitivity of λ mutants with more DNA than λ^{++}. PP inactivation was carried out at standard conditions except that 20°C was the temperature rather than 37°C.




SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
10
20
30
40
50
60
70
TIME (min.) IN $Na_4P_2O_7$ AT 20 °C

(b) Isolation of λ deletions by heat or PP selection

A procedure for selecting λ deletion mutants was developed in the following way. The standard conditions which have already been described for heat and PP treatment were chosen on the basis of reconstruction experiments with λ^{++} and λ deletions. The purpose was to obtain conditions to optimize the viability of deletions and the inactivation of λ^{++}. Using these conditions, heat resistant or PP resistant mutants are then selected. Since λ^{++} stocks contain a relatively high frequency of λ^{*} compared to true resistant mutants, it is necessary to inactivate λ^{*} particles. This is done by simply regrowing the survivors of an inactivation treatment so that all of the λ^{*} phage return to a sensitive form. Each inactivation and regrowth constitutes one cycle of selection and produces an increase in the relative frequency of resistant mutants in the population as a whole. From reconstruction experiments, the enrichment factor for heat inactivation is about 300, that for PP treatment is 2000. Thus the initial frequency of resistant mutants in the population will dictate the number of selection cycles which are needed to produce nearly all resistant mutants. By using the enrichment factors, these initial frequencies can be estimated. Once a number of resistant mutants have been obtained they are tested to determine whether or not they are deletion mutants.

(i) Heat resistant mutants from UV-induced C600 (λ^{++}). A stock of λ^{++}, made by UV-induction of C600 (λ^{++}), was carried through two

cycles of heat selection as shown in Figure 6a. After the second cycle the population consisted mostly of heat resistant phage whose initial frequency in the λ^{++} stock is calculated to be about 1×10^{-4}. As shown in Figure 6b, these heat resistant mutants are less dense than λ^{++} and 200 of these density mutants were picked from the peak of lesser density for further study. The mutants obtained in this selection are numbered b101-b300 and are designated the "UV" class of mutants, 57 of which have been examined in detail.

(ii) PP-resistant mutants from vegetatively-grown λdel^{+}. A suppressible clear plaque mutant of λ^{++}, C1509, was grown on the su^{-} strain 594 to produce a vegetatively grown stock of λdel^{+}. Two cycles of PP selection were carried out, each time regrowing the surviving phage under vegetative conditions. The results of this selection are shown in Figure 7a from which it can be calculated that about 5×10^{-6} of the initial phage were PP-resistant mutants. Nearly all of the phage after two selection cycles are less dense than λ^{++} as shown in Fig. 7b. From the survivors of a third PP treatment, used to remove the few remaining wild type density phage, individual plaques were picked for further analysis. These mutants--the "VEG" class--are numbered b501-b642, and all 142 of these mutants have been at least partially characterized.

(iii) Resistant mutants are deletions. The UV and VEG mutants appear to be deletions for one or more of the following reasons:

1. The density of 60 UV mutants was measured and every mutant is less dense than λ^{++}.

Fig. 6. Selection of heat resistant λ mutants.

a) Heat inactivation of λ^{++} from C600(λ^{++}). C600(λ^{++}) was UV-induced and the phage were heat inactivated under standard conditions for 6 hours. The survivors were used to grow a new λ^{++} stock by the confluent lysis method and these phage were again heat inactivated. The progeny of the survivors of this second heat treatment were again inactivated by heating.

o——o = 1st inactivation

▽——▽ = 2nd inactivation

□——□ = 3rd inactivation

b) Density profile of heat resistant λ mutants. After the 2nd inactivation and regrowth, phage were examined in CsCl density gradients at 22,000 rev./min, 15°C for 18 to 24 hours in a Spinco SW 50 rotor.

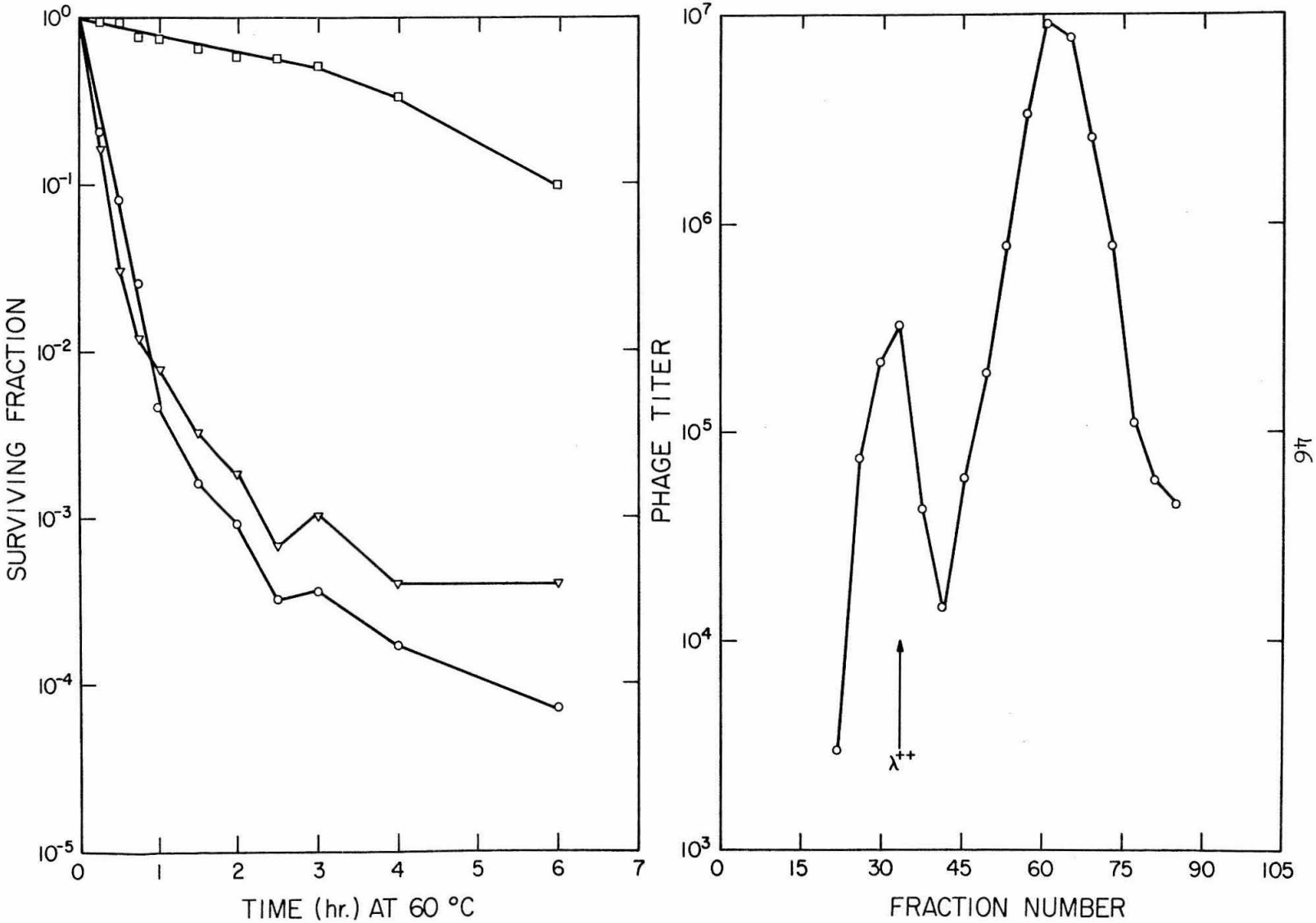

SURVIVING FRACTION
TIME (hr.) AT 60 °C
PHAGE TITER
FRACTION NUMBER
λ++
46

Fig. 7. Selection of PP-resistant λ mutants.

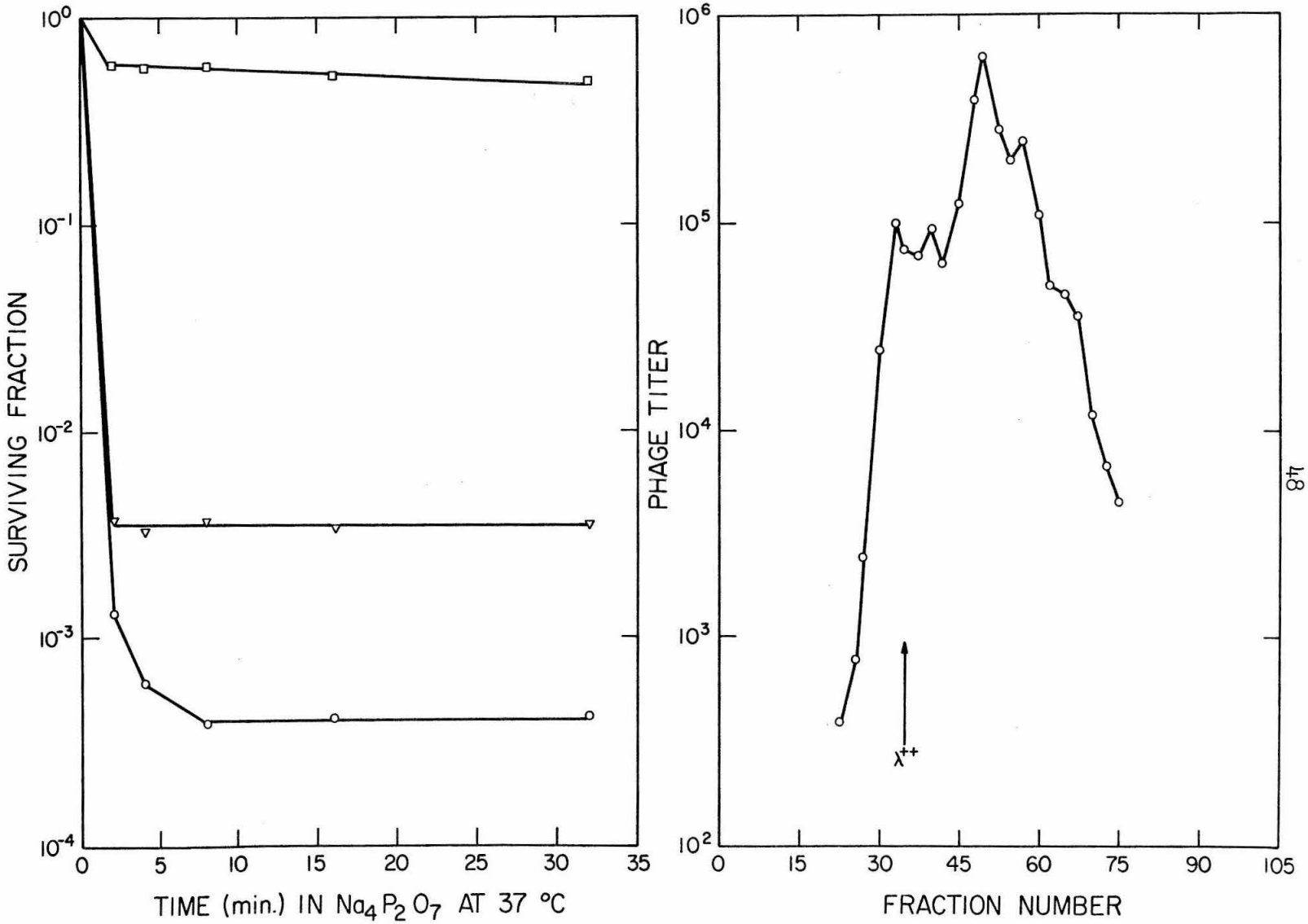
a) PP inactivation of λCI509 del^{+}. λCI509 del^{+}, grown by confluent lysis on strain 594, was PP inactivated for 4 minutes and the surviving phage were regrown by the confluent lysis method. This process was repeated and the phage were PP treated a third time.

o——o = 1st inactivation

▽——▽ = 2nd inactivation

□——□ = 3rd inactivation

b) Density profile of PP-resistant λ mutants. Survivors of the 2nd PP inactivation were regrown and examined in CsCl gradients as described in the legend of Fig. 6b.


SURVIVING FRACTION
TIME (min.) IN $Na_4P_2O_7$ AT 37 °C
PHAGE TITER
FRACTION NUMBER
χ^{++}
418

2. Of 12 UV mutants examined, all are more heat-resistant than λ^{++} and the rate of heat inactivation is directly related to the DNA content of these mutants calculated from their measured buoyant densitites according to the formula given by Davis & Davidson (1968).

3. All of the VEG mutants are more PP-resistant than λ^{++}; 4 UV mutants which were studied are also PP-resistant.

4. The heat resistance of the 12 UV mutants from part 2 above and the PP-resistance of the 40 VEG mutants and 4 UV mutants in part 3 above does not undergo phenotypic mixing with λ^{++}, suggesting that resistance is a property of the DNA of the particle rather than of the phage proteins in the particle.

5. The DNA of 3 UV mutants and of 21 VEG mutants has been measured in the electron microscope (Davis & Davidson, 1968; Parkinson & Davis, 1970). All of the mutant DNA's are shorter than λ^{++} DNA, indicating a deletion.

Taken together, all of this evidence indicates that the UV and VEG mutants are deletions. However, the size distribution of deletions in the two preparations is quite different. The DNA content of 60 UV deletions whose buoyant density has been measured ranges from 9% to 23% less DNA than wild type. The DNA contents of 21 VEG mutants studied with the electron microscope by Parkinson & Davis (1970) range from about 1.5% to 17% less DNA than λ^{++}. The difference in the UV and VEG preparations in this respect is most likely due to the different sensitivities of the two selection methods. It is possible, however,

that the initial size distribution of UV deletions was, in fact, different from the initial size distribution of the spontaneously-occurring VEG deletions.

(c) Properties of UV and VEG deletion mutants

As stated in the introduction, we expected most of the deletions to lie within the central portion of the λ chromosome, which controls the non-essential functions of prophage integration and generalized recombination. Below we will describe the preliminary characterization of UV and VEG deletion mutants with respect to integration and generalized recombination. At this time we only wish to show that many of the deletions do in fact alter the integration functions of the mutant phage, and that the deletions can be divided into 3 major classes with respect to their integration behavior. The implications of these results and a more detailed genetic analysis of these deletion mutants are presented in the accompanying paper by Parkinson (1970).

(i) General recombination in the deletion mutants. Signer & Weil (1968) and Echols & Gingery (1968) have isolated λ mutants which are unable to promote generalized recombination in a recombination-deficient host strain. These mutants, called red$^-$, define at least two genes, located adjacent to one another and near the center of the λ genome (Signer, Echols, Weil, Radding, Shulman, Moore & Manly, 1968). Since red$^-$ mutants are recessive, we can test for the presence of

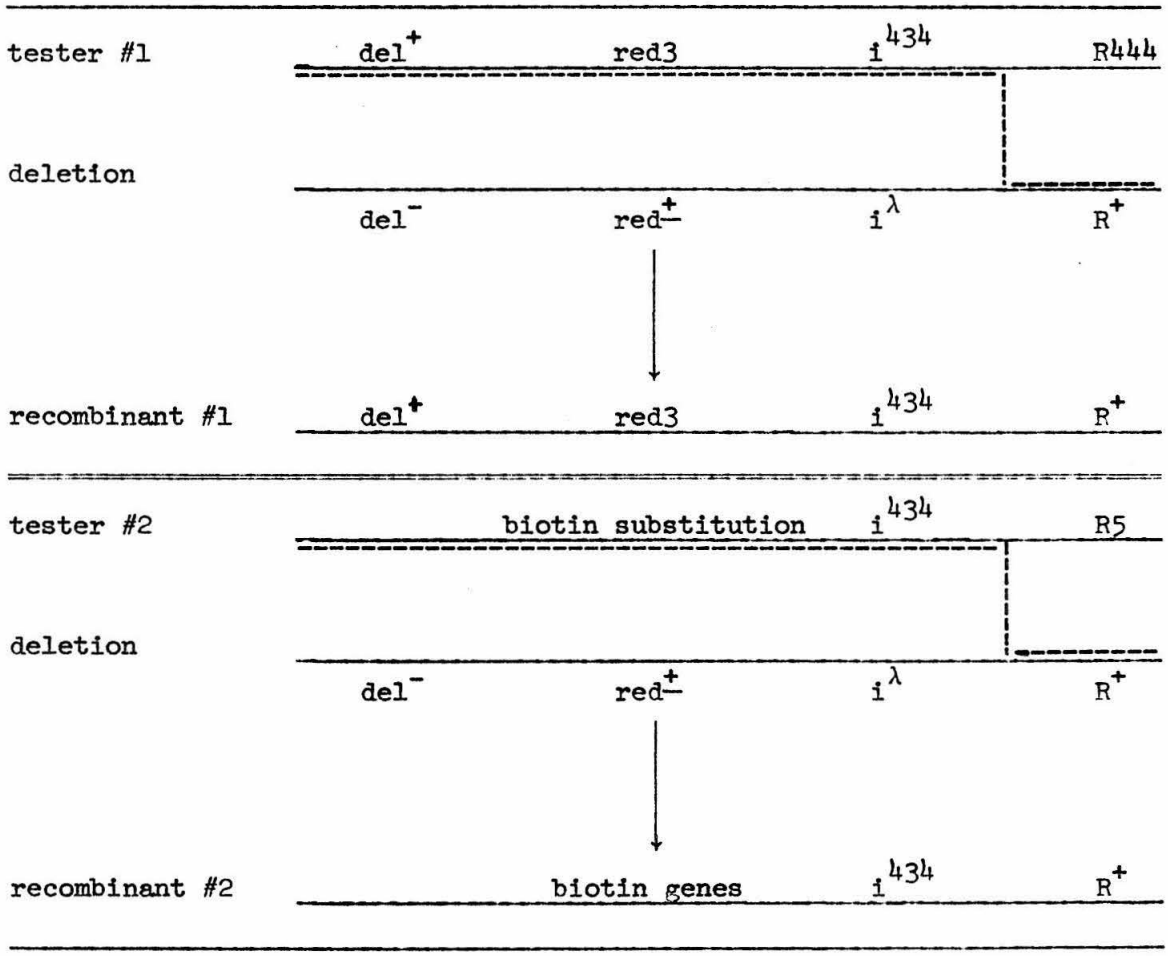
the Red genes in a deletion mutant by asking whether a deletion is able to furnish red$^+$ function in a cross with a red$^-$ tester phage. Table 3 shows the two types of tester phages used in these experiments and the types of recombinants that we looked for in each case. Both tester red$^-$ mutants were missing all of the known recombination genes so that if any one of these genes was defective in a deletion mutant, we could have detected it in this test. We tested 40 VEG mutants (b501-b540) and four UV mutants (b111, b130, b189, b221). None of these deletion mutants was defective in complementing the red$^-$ tester phage for generalized recombination; however, it is possible that some of these deletions were defective in other Red genes which are still functional in the tester phages. Two additional tests were done to examine this possibility.

Signer & Weil (1968) have described a marker rescue spot test for red$^-$ λ mutants. The test measures in a qualitative manner the ability of a phage to recombine with a defective prophage carried in a rec$^-$ host strain. We tested all 142 VEG deletions and 57 of the UV deletions in this way, but again all of the deletion mutants were able to promote recombination in the rec$^-$ host.

Huskey (1969) has devised yet another method for testing whether a phage strain is able to promote recombination. Huskey demonstrated that multiplicity reactivation--the formation of an active λ genome from a number of inactive ones--is dependent on the ability of the phage to promote its own recombination. An additional 27 UV deletions were examined with this test; however, all of these deletions gave

TABLE 3

Complementation test for recombination



Crosses were performed in QR48 (rec$^-$) at moi = 5 of each parent. Recombinant #1 was assayed on 594 (R444 i^λ); recombinant #2 was assayed on 594 (R5 i^λ). Complementation occurred if the frequency of recombination was greater than 0.1% which is the control frequency under these conditions using red$^-$ mutants. In all cases where complementation occurred, the frequency of recombinants was at least 3%.

substantial multiplicity reactivation and thus were red$^+$.

A total of 199 deletion mutants has been tested so far in one or more of these recombination assays without detecting any deletions which are red$^-$. We presume that few, if any, of the remaining 143 UV deletions which have not been tested, will prove to be red$^-$. It is clear that red$^-$ deletions are quite infrequent in both our UV and VEG preparations. However, it is important to emphasize the fact that we could only have detected Red genes which are phage-specific; that is, recombination functions which are not supplied by the rec$^-$ host strains which we used. If some phage Red genes are also present in the host, we would not be able to detect these phage functions.

(ii) Integration properties of the deletion mutants. We have tested 57 UV deletions and 142 VEG deletions for the ability to establish stable lysogeny in several different hosts. In order to establish stable lysogeny in a sensitive host strain, λ must have both a functional integration gene (Int) (Zissler, 1967) and a functional attachment site (att $^{\phi}$). In bacterial strains which carry a cryptic (defective) prophage or a heteroimmune lambda prophage, some mutants of λ which cannot integrate into the chromosome of sensitive bacteria, can integrate into the chromosome of these lysogenic strains (Fischer-Fantuzzi, 1967; Weisberg & Gottesman, 1969). We have tested the deletion mutants in both sensitive and lysogenic bacteria using a simple spot test described by Gottesman & Yarmolinsky (1968). This test is based on the fact that cells lysogenized with a non-integrating λ mutant will segregate sensitive cells with no prophage because the

phage genome is inherited unilinearly at each cell division (abortive lysogeny).

The results are presented in Table 4. All of the UV mutants and most of the VEG mutants are defective in establishing stable lysogeny in a sensitive host. A large number of these mutants, however, can establish stable lysogeny in the strain carrying a defective prophage. Thus we can define three classes of deletion mutants based on this test: class 1 which behave like λ^{++} in integration; class 2 mutants which have the integration pattern of λb2 or λdg; and class 3 deletions which behave like λ$\underline{int}^-$ mutants.

Since all of the deletion mutants are $\underline{red}^+$, the differences in the pattern of class 2 and 3 deletions cannot be due to the Red system. It is also likely that the ability of class 2 deletions to establish stable lysogeny in the cryptic prophage strain means that these deletions are $\underline{int}^+$. The properties of these deletions will be discussed in more detail in the accompanying paper by Parkinson (1970).

4. Discussion

(a) λ inactivation by heat and chelating agents

Heat and PP inactivation of λ^{++} appear to be nearly identical phenomena for several reasons. Both reactions are affected by the same parameters: temperature and ionic strength--divalent cations in particular. Furthermore, heat and PP treatments in some manner cause λ phage to lose DNA from their heads. There is, however, an obvious

TABLE 4

Integration patterns of λ deletion mutants

Class	Ability to integrate into: C600	QR14(λcry)	Integration pattern of:	Frequency UV	VEG
1	+	+	λ^{++}	0/57	43/142
2	0	+	$\lambda b2^{1}$;λdg^{2}	30/57	87/142
3	0	0	$\lambda \underline{int}^{-}$	27/57	12/142

Phage at ~ 10^{8}/ml. were spotted onto C600 and QR14 and incubated overnight. Bacteria from the turbid centers of the spots were transferred to EMB-glucose plates spread with ~ 10^{9} λ clear phage and incubated at 37°C. The tests were scored after 36-48 hours. In every series of tests, positive (λ^{++}) and negative ($\lambda \underline{int}^{-}$) controls were included.

[1]See Fischer-Fantuzzi (1967); we also confirmed this integration pattern for the λb2 mutant.

[2]See Guerrini (1969) and Weisberg & Gottesman (1969).

quantitative difference between heat and PP inactivation, in that PP inactivation is very sensitive and under standard conditions small deletion mutants are completely resistant to PP treatment. It would appear from our results that PP inactivation is a strictly DNA content dependent process. It may be that our conditions for heat inactivation bring about a slow inactivation which is independent of DNA content as well as the more rapid inactivation which is shown to be dependent on DNA content. In all major respects, however, PP and heat inactivations are probably identical in their mechanism of action.

Inactivation of phage T5 by heat has been described by Adams & Lark (1950) and more recently by Hertel, Marchi & Müller (1962). PP inactivation of T5 has also been described (Yamamoto, Fraser & Mahler, 1968). The λ and T5 systems with respect to heat or PP inactivation are remarkably similar and the mechanism of inactivation is probably the same for both phage. In both cases inactivation is dependent on the conditions of temperature and ionic strength and most importantly on the DNA content of the particles. Moreover, phenotypically resistant T5 phage occur in T5 stocks with a frequency similar to that for λ^{*} in stocks of λ^{++}. It is possible that heat or PP inactivation will prove to be useful methods with other phage as well.

The behavior of λ^{++} particles in low ionic strength conditions suggests a simple explanation for several puzzling results reported in the literature. Fraser (1966) has described the properties of "low density λ particles" which are produced by growing λ^{++} in broth with very low Mg^{++} and Ca^{++} concentrations. The burst size under these

conditions is very low and nearly all of the phage have a lower density than λ^{++}; however, their density returns to normal after one cycle of growth. Fraser suggested that the low density property of these phage was "intrinsic to the phage genome before maturation," however our results suggest an alternative explanation; that the low burst size of λ^{++} under Fraser's conditions is due to inactivation of λ^{++} particles after maturation and release from the cell. The growth conditions Fraser used are quite similar to PP inactivation and also Fraser found that λi^{434} produced higher burst sizes than λ^{++} under these conditions. As we have shown, λi^{434} is more resistant than λ^{++} to PP inactivation. Therefore, "low density phage" are probably equivalent to λ^{*} particles since they have similar density distributions, occur with similar frequencies, and are only phenotypically modified. It seems very unlikely to us that λ^{*} particles contain less DNA than wild type, as suggested for low density particles by Fraser, because of the fact that λ^{*} are not only less dense than λ^{++}, but also have a greater sedimentation rate than λ^{++}. The simplest explanation of these findings is that λ^{*} particles contain more protein than λ^{++}.

Hercules, Knacht & Zubay (1968) have reported that Ø80 dlac particles are produced in low yields after induction of a Ø80, Ø80 dlac double lysogen in a medium which contains little Mg^{++} or Ca^{++}. In a different medium with high ionic strength, Ø80 and Ø80 dlac particles were produced in equal quantities. It was suggested that the basis of this effect was differential DNA replication after induction which depended on the growth medium employed. Since the Ø80 dlac used by these

workers was shown to be more dense than Ø80 wild type, we believe that the differential yield of Ø80 and Ø80 dlac in low ionic strength medium is due to the inactivation of Ø80 dlac by a process analogous to PP inactivation of λ^{++}.

Both PP and heat inactivation of λ^{++} particles cause DNA to be released from the phage heads. In fact, several workers have reported the use of chelating agents to produce ghosted λ particles (Soller, Levine & Epstein, 1965; Villarejo, Hua & Evans, 1967). We are still not certain of the actual mechanism and site of action of inactivation treatments. On the one hand, increasing the repulsive forces of the DNA inside the phage head could cause the phage head to rupture, releasing the DNA. On the other hand, such forces within the head might trigger the injection process causing DNA to be extruded through the tail. It is difficult to imagine that either of these mechanisms would be as sensitive to changes in DNA content as is PP inactivation. Detailed studies of the inactivation of free λ heads and λ tails might serve to answer some of these questions.

(b) Properties of UV and VEG deletions

The final preparations of UV and VEG deletions which we have studied do not constitute a random sample of deletion mutants from the initial population before selection. Our selection methods necessarily introduce concomitant counter-selection pressures against certain classes of deletion mutants. For example, both heat and to a lesser degree, PP treatments cause small deletions to die at a faster rate

than large deletions. Thus the size distribution of deletion mutants in the UV and VEG preparations is skewed toward the larger deletions. This type of bias in our selection methods is not too serious, however, since we are only interested in using the deletion mutants to detect dispensable functions.

Another type of bias is exemplified by the absence of $\underline{red}^-$ deletion mutants in the UV and VEG preparations. Since $\underline{red}^-$ mutants have smaller burst sizes than $\underline{red}^+$ phage (see Signer & Weil, 1968), our recycling procedure would probably tend to deplete the population of $\underline{red}^-$ mutants. In fact, reconstruction experiments using mixtures of $\underline{red}^+$ and $\underline{red}^-$ phage prove that this is the case. We cannot be certain, therefore, that other deletion mutants with small burst sizes could be detected in this system.

Although the UV and VEG preparations are not random samples, nevertheless several lines of evidence suggest that UV and VEG deletions are basically different. First, the frequency of spontaneous (VEG) deletions is about 5×10^{-6} whereas the frequency of UV deletions is about 10^{-4}. Thus the UV preparation contains virtually no spontaneous (VEG) deletions. Since the UV deletions arose from an induced prophage, it is reasonable to suppose that most or all of the deletions are the result of aberrant excision of the prophage genome from the host chromosome, which has been suggested by Campbell (1962) as a mechanism of deletion formation. Secondly, the proportions of the three types of deletion mutants are quite dissimilar in the UV and VEG preparations. It seems to us unlikely that these differences can be

due merely to different size distributions of deletion mutants in the two preparations, but are rather caused by basic differences in the mechanism of formation of the deletion mutants. Further evidence for this notion is presented in the two companion papers by Parkinson (1970) and by Parkinson & Davis (1970).

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Deletion Mutants of Phage Lambda

II. Genetic properties of att-defective mutants

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Viable, integration-defective deletion mutants of phage λ were examined with the technique of Int-promoted recombination between phages. The types of mutants found and their properties are consistent with a three-component model of $\underline{att}^{\varphi}$. The results show that $\underline{att}^{\varphi}$ contains a unique locus for integration cross-overs which is bordered on each side by recognition sequences which are essential for normal integration. The role of these recognition sequences in integration was studied by analyzing the pattern of Int-promoted recombination between phage containing various types of mutant $\underline{att}$ sites. It was found that the integration system of λ can function with a variety of defective $\underline{att}$ sites, however, no simple pattern could be deduced from these results. Evidently, the λ integration system is considerably more complex than it was at first believed, and very unlike systems of generalized recombination.

1. INTRODUCTION

The establishment of stable lysogeny by temperate bacteriophage lambda takes place in two steps. After infection of a sensitive host, the phage lytic functions are first repressed. The lambda DNA then inserts itself into the host bacterial chromosome by a process known as prophage integration. Because integration appears to occur by reciprocal recombination, this system has been extensively studied as a model for recombination. Recent investigations of the lambda integration system indicate, however, that integration is a very special sort of recombination. The purpose of the present paper is to further examine these unique features of the lambda integration machinery.

The lambda integration system has been shown to have at least two essential components: an Int gene (Zissler, 1967) which seems to make an "integrase" needed for integration; and an attachment site ($\underline{att}^{\varphi}$) which appears to be the substrate for integrase action. Because $\underline{int}^{-}$ mutants of lambda can be complemented by $\underline{int}^{+}$ helper phage, the Int gene is thought to make a diffusible product. Mutants defective in $\underline{att}^{\varphi}$, however, cannot be complemented by a helper phage (Zichichi & Kellenberger, 1963; Campbell, 1965). Since $\underline{att}^{\varphi}$ appears to play a purely structural role in the integration system, in order to study $\underline{att}^{\varphi}$, it is necessary to employ mutants such as deletions or substitutions which contain gross structural defects.

In the first paper of this series (Parkinson & Huskey, 1970) we described the isolation of a number of lambda deletion mutants with integration defects. These deletions afford a means of exploring

the fine structure of att$^{\varphi}$. It will be shown that deletions and substitutions in the att region define three distinct elements of att$^{\varphi}$, and that modification of these elements results in a wide range of integration behaviors. In the accompanying paper by Parkinson & Davis (1970) we describe the use of deletion and substitution mutants to define the physical structure of these three att elements.

2. MATERIALS AND METHODS

(a) Phage strains

The λ mutants used in this work are listed in Table 1. Multiple mutant strains were constructed by crossing the appropriate parental strains, and then backcrossed to the parental strains to confirm the genotype. Selection methods for detecting various mutant phenotypes are described below. All stocks were made by the confluent lysis method.

(b) Bacterial strains

Table 2 lists the bacterial strains employed in this study. E. coli strain C600 was used for all phage assays.

(c) Media

TMG dilution buffer and BBL agar were described previously (Parkinson, 1968). Tryptone broth and EMB-glucose plates are also described elsewhere (Parkinson & Huskey, 1970).

TABLE 1

Phage Strains

(a) Derivatives of λ^{++} (λPaPa; Kaiser, 1957)

Strain	Phenotype*	Source and/or reference
CItl	CIts	Lieb (1964)
CI509	CIsus	E. T. Young
CI857	CIts	Sussman & Jacob (1966)
i^{434}	434 immunity	J. Weigle; Kaiser & Jacob (1957)
$i^{434}c$	CI^-	UV-mutagenesis of λi^{434}
L439 K424	susL susK	Parkinson (1968)
J418	susJ	Parkinson (1968)
bio69	$int^- red^-$	K. Manly; Manly et al. (1969)
bio7-20	$int^- red^+$	K. Manly; Kayajanian (1968)
int6	int^-	E. Signer; Gottesman & Yarmolinsky (1968)
red3	redABC	E. Signer; Signer & Weil (1968a)

(b) Deletion mutants of λ (see Parkinson & Huskey, 1970)

Mutant		DNA content (% missing compared to λ^{++})†
Class 1:	b503, b504	3.6
	b506	7.5
	b515	3.4
Class 2:	b2‡	12.0
	b130	10.6
	b189	17.5
	b511	8.9

TABLE 1 (continued)

Class 2 (continued)		
	b516	10.2
	b527	8.3
Class 3:	b221	22.3
	b508, b512, b517	3.9
	b522	7.1
	b538	16.3

*Abbreviations: sus = suppressor-sensitive nonsense mutants
ts = temperature-sensitive mutants
int⁻ = integration-negative mutants
red⁻ = recombination-deficient mutants

†DNA contents are taken from Parkinson & Davis (1970).

‡ See Kellenberger et al. (1961).

TABLE 2

Bacterial Strains

Strain	Relevant genotype*	Source and/or reference
C600	su^+ rec^+	Appleyard (1954)
594	su^- rec^+	Weigle (1966)
CR63	su^+ λ^r, λh^s	Appleyard et al. (1956)
QR48	su^+ recA	E. Signer; Signer & Weil (1968a)
QR14	su^+ rec^+ λcryh	E. Signer; Signer & Weil (1968a)
QR15	su^+ recA λcryh	E. Signer; Signer & Weil (1968a)
SA205	su^- att^L(rex-chlA)$^\Delta$	S. Adhya
SU740	su^- att^R(galT-att^L)$^\Delta$	S. Adhya
RMO705	594(L439 K424 red3 i^{434})	
RSO731	594(J418 CItl)	

*Abbreviations: su^+ (su^-) = ability (inability) to support the growth of λ sus mutants.

rec^+ (recA) = ability (inability) to promote recombination.

λcryh = cryptic (defective, non-immune) prophage carrying the extended host range marker (h).

λ^r, λh^s = resistant to λh^+, sensitive to λh.

att^L, att^R = left or right prophage end, respectively.

rex = T4RII exclusion locus in λ.

chlA = chlorate reductase gene.

galT = galactose gene.

(d) Methods

(i) Plating and crossing bacteria and crossing procedure are described by Parkinson (1968).

(ii) Marker rescue spot tests for red$^-$ phage are described by Signer & Weil (1968a).

(iii) Spot tests for int$^-$ phage were performed on the appropriate bacterial strains following the procedure of Gottesman & Yarmolinsky (1968).

(iv) Selection for deletion mutants in the progeny of a cross was done by pyrophosphate treatment of the phage at 37^{o}C or 45^{o}C as described by Parkinson & Huskey (1970).

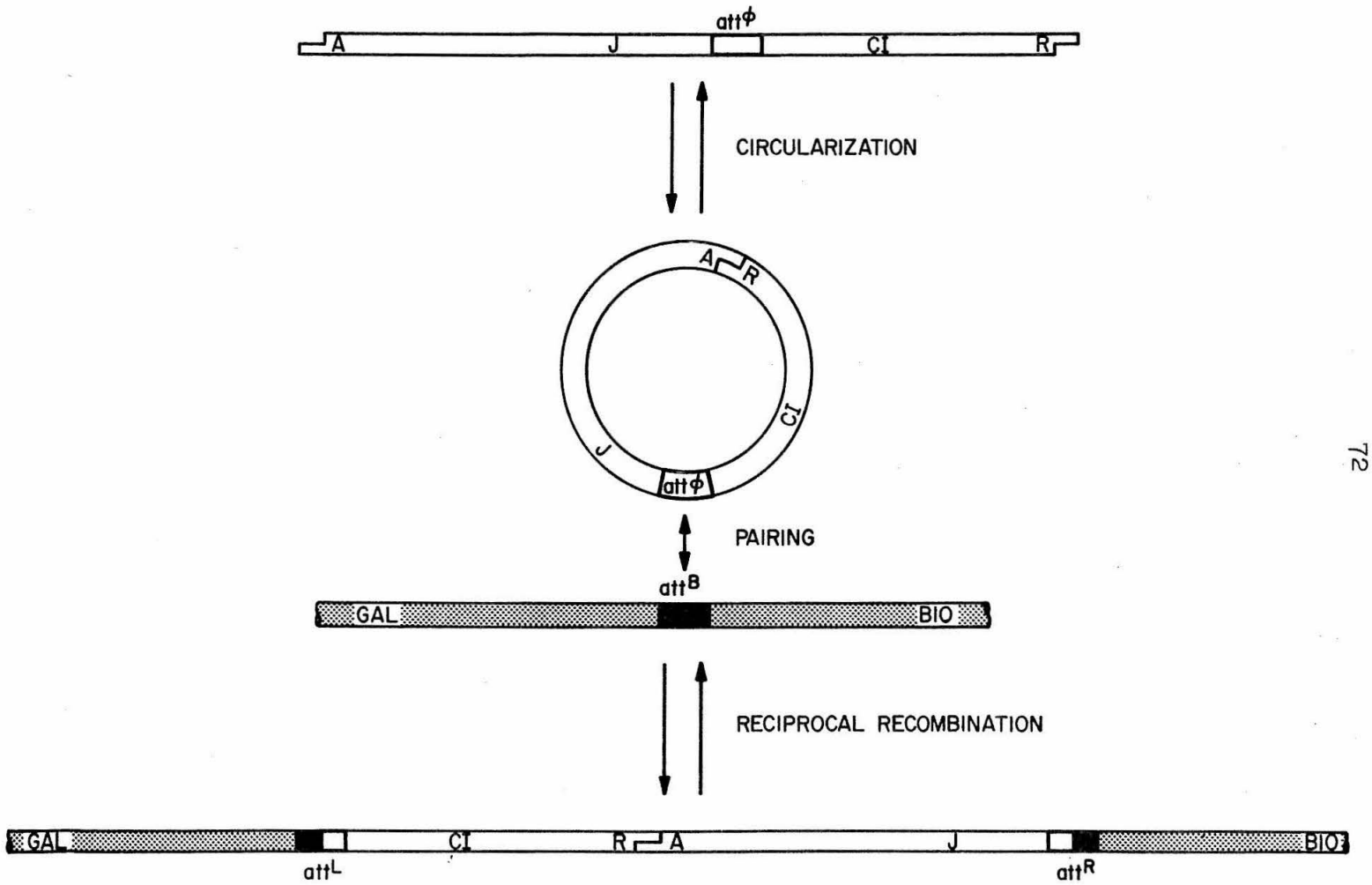
3. RESULTS

(a) A model of att$^{\varphi}$

The purpose of the present work is twofold: to define the various components of att$^{\varphi}$ with deletion and substitution mutants; and secondly, to examine the function of each att element by studying the interactions of various combinations of such elements in integrative recombination. At the outset it is useful to present a model of att$^{\varphi}$ as a convenient framework for discussing the types of att-defective deletions and substitutions that have been found and employed in this work.

The Campbell model (Campbell, 1962) of prophage insertion is depicted in Figure 1. In this model prophage integration involves a reciprocal recombination between the phage and bacterial

Fig. 1. The Campbell model of lysogeny. After infection, the phage chromosome circularizes, and pairs and recombines with the bacterial chromosome. Genes A, J, CI and R are included on the phage chromosome as reference points.


attφ
A
J
CI
R
CIRCULARIZATION
A
R
CI
J
attφ
PAIRING
attB
GAL
BIO
RECIPROCAL RECOMBINATION
GAL
CI
R
A
J
BIO
attL
attR

chromosomes at their respective attachment sites. $\underline{att}^{\varphi}$ in the phage chromosome recombines with $\underline{att}^{B}$ in the bacterial chromosome to form "hybrid" attachment sites at each end of the inserted phage DNA; $\underline{att}^{L}$ at the left end and $\underline{att}^{R}$ at the right end. The necessity of distinguishing between these four types of attachment sites comes from recent work which suggests that $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ are somehow different (Guerrini, 1969). For example, Weisberg & Gottesman (1969) have demonstrated that phage carrying $\underline{att}^{L}$ have different integration properties than phage carrying $\underline{att}^{\varphi}$. Thus $\underline{att}^{L}$ may be distinguished from $\underline{att}^{\varphi}$. Similarly, Manly, Signer & Radding (1969) have shown that $\underline{att}^{R}$ also differs from $\underline{att}^{\varphi}$. The simplest assumption which accounts for these findings is that $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ are not identical, and that crossing over takes place within these att sites during integration.

Because $\underline{att}^{L}$ has altered integration properties, it appears that a portion of $\underline{att}^{\varphi}$ essential for normal integration, has been replaced by bacterial DNA in $\underline{att}^{L}$. Likewise, another essential component of $\underline{att}^{\varphi}$ is missing in $\underline{att}^{R}$. Thus $\underline{att}^{\varphi}$ has at least two distinct components which I will call recognition elements. The presence or absence of these elements strongly affects the probability of integration. REL is the left-hand recognition element missing in $\underline{att}^{L}$; RER is the right-hand element missing in $\underline{att}^{R}$. By definition, the boundary between REL and RER in $\underline{att}^{\varphi}$ is the locus at which the cross-over event leading to integration actually takes place. Since this locus is still present in $\underline{att}^{L}$ and $\underline{att}^{R}$, its presence is not dependent on either REL or RER alone and therefore constitutes a third component of $\underline{att}^{\varphi}$ which I designate XOP for "cross-over point." In the following sections, the two $\underline{att}^{\varphi}$ components REL and RER are operationally defined as those segments of the attachment site which

are essential for normal integration. It is implicit in this definition that any lesion which involves either an entire recognition element or any portion of that element will produce a characteristic integration-defective phenotype.

To conveniently describe such mutant att sites, I will use a simple notation (adapted from Signer, 1968) which allows us to depict any integration event or attachment site in terms of the component att parts involved. For example, att^{φ} is designated P · P' where P refers to the origin of REL, P' refers to the origin of RER, and the dot corresponds to XOP. The extension of this notation to other forms of att sites is shown in Table 3. This type of attachment site notation will be frequently employed in later sections.

(b) Integration defects of λ deletion mutants

(i) Origin of λ deletion mutants. The deletion mutants used in this study and listed in Table 1 were selected from a large number of such mutants isolated by Parkinson & Huskey (1970). The deletions are of two major types depending on whether they arose spontaneously during vegetative growth (VEG deletions) or arose in a UV-induced stock of λ^{++} (UV deletions). An important difference between VEG and UV deletions is that the latter type has had the opportunity to incorporate prophage structures such as att^{L} or att^{R} during induction and excision from the host chromosome. Thus the integration defect of some UV deletions might be due to a small substitution carrying att^{L} or att^{R} rather than to a simple deletion of part of att^{φ}.

The population of UV and VEG deletions is composed of three classes of mutants based on the integration behavior of the deletions on different host strains (Parkinson & Huskey, 1970).

TABLE 3

att Site Notation

att	defect(1)	notation(2)
att^{φ}	none	P · P'
att^{B}	REL^{B} RER^{B}	B · B'
att^{L}	REL^{B}	B · P'
att^{R}	RER^{B}	P · B'
---	REL^{Δ}	X · P'
---	RER^{Δ}	P · X'

(1) The defect of an att site is defined relative to att^{φ}; Δ = deletion, B = bacterial substitution.

(2) The letters P, B, and X indicate the origin of the DNAin REL and RER: P = phage; B = bacterial; X = deletion.

These three classes are described in Table 4. Class 1 mutants are not integration-defective, whereas class 2 and 3 deletions do have integration defects. Class 2 and 3 deletions are different, however, in that class 2 mutants are able to establish stable lysogeny in a bacterial strain that contains the prophage ends, $\underline{att}^{L}$ and $\underline{att}^{R}$. A number of mutants from each of these three classes were selected at random for further study in order to define the att defects of these mutants in terms of the model of $\underline{att}^{\varphi}$ discussed above.

(ii) Integration-defective mutants predicted by the att model

A schematic representation of the λ attachment region is shown in Figure 2. In terms of this model, there are 13 possible kinds of integration-defective deletion mutants, listed in Figure 2, that we might expect to find. Of these 13 types, 10 are simple deletions of various att elements and 3 are bacterial substitutions which might be found among the UV deletions. Since substitution mutants of this sort probably arise by aberrant prophage excision these mutants should contain either $\underline{att}^{L}$ or $\underline{att}^{R}$, the prophage ends. The att defect in these strains will therefore be REL^{B} or RER^{B} and should begin at XOP. The class 1 deletions, which were found in the VEG population, must lie somewhere outside of the attachment region since they have no detectable integration defects. These deletion mutants will be discussed separately in a later section.

Two basic tests were used to classify the deletion mutants. These tests are outlined briefly below; whenever a variation of the basic tests was employed, this will be described at the appropriate time.

TABLE 4

Three Classes of Deletion Mutants

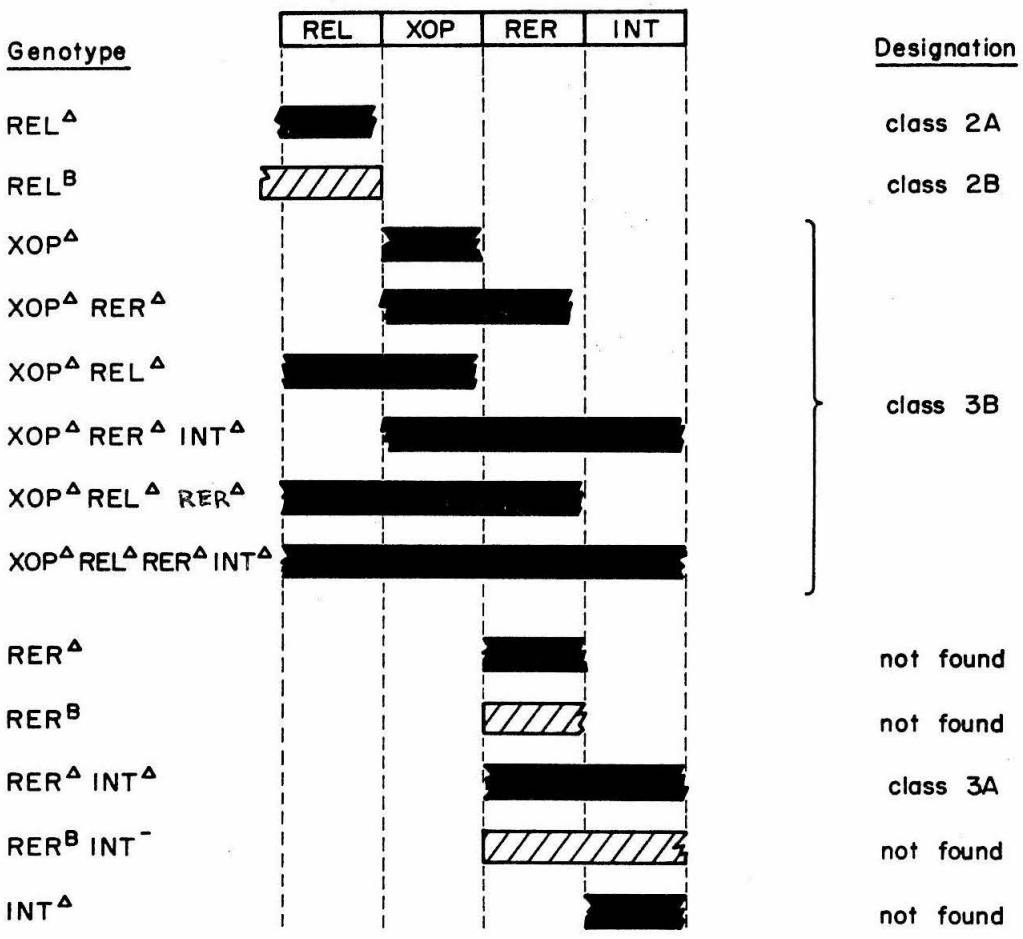
Deletion Mutant Class	Stable lysogeny of bacteria containing: $\underline{att}^{B(1)}$	$\underline{att}^{L}$ and/or $\underline{att}^{R(2)}$
1	+	+
2	0	+
3	0	0

These data are taken from Parkinson & Huskey (1970). Stable lysogeny was detected by spot tests as described by Gottesman & Yarmolinsky (1968).

(1) All sensitive bacterial strains such as C600 carry $\underline{att}^{B}$.

(2) Strains containing a heteroimmune prophage or a non-immune defective prophage carry $\underline{att}^{L}$ and $\underline{att}^{R}$.

Fig. 2. A model of the λ integration region and possible types of integration-defective deletion mutants. The mutant notation is described in Table 3. Deletions are indicated by solid areas, substitutions by stippled areas. On the right are listed the types of mutants that were actually found, and their designation which is used in this work.


Genotype
REL
XOP
RER
INT
Designation
REL^Δ
class 2A
REL^B
class 2B
XOP^Δ
XOP^Δ RER^Δ
XOP^Δ REL^Δ
XOP^Δ RER^Δ INT^Δ
XOP^Δ REL^Δ RER^Δ
XOP^Δ REL^Δ RER^Δ INT^Δ
class 3B
RER^Δ
not found
RER^B
not found
RER^Δ INT^Δ
class 3A
RER^B INT^-
not found
INT^Δ
not found

1. Integration tests

The deletion mutants can be tested for the ability to establish stable lysogeny in a number of different host strains. Integration, measured either qualitatively or quantitatively, can be tested on strains containing $\underline{att}^B$, $\underline{att}^L$, or $\underline{att}^R$. The procedure employed for detecting stably lysogenic cells is described by Gottesman & Yarmolinsky (1968).

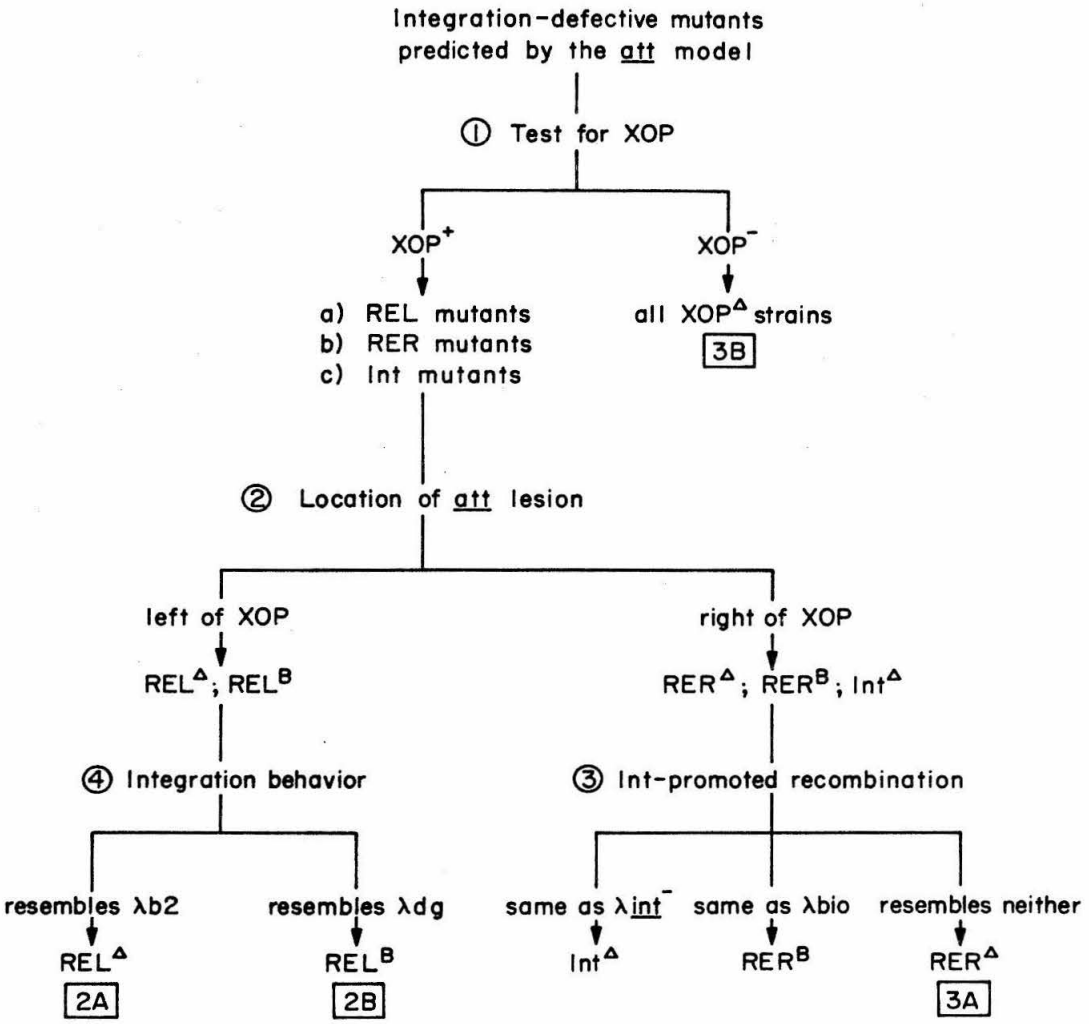
2. Int-promoted recombination

Weil & Signer (1968) and Echols, Gingery & Moore (1968) have demonstrated that the λ integration system is able to promote recombination between two phage chromosomes in vegetative crosses. Such Int-promoted recombination can occur in the absence of all generalized recombination promoted by the bacterial Rec system and the phage Red system. Because Int-promoted recombination only occurs at the attachment site and is dependent on a functional Int gene, we will assume that the mechanism of this recombination is the same as that for integration during lysogeny. Since the frequency of Int-promoted recombination depends on the attachment sites involved in the cross (Signer, Weil & Kimball, 1969), Int-promoted recombination can be used as a diagnostic test for analyzing the structure of the attachment site. In all of the tests described below, Int-promoted recombination has been measured under conditions where both the Rec and Red systems are inoperative so that all recombination that occurs must be Int-promoted.

(iii) Classification of integration-defective deletion mutants

The screening procedure used to classify the deletion mutants is outlined in Figure 3. The steps numbered in Figure 3 are

Fig. 3. The screening procedure used to classify integration-defective deletion mutants. The individual steps in this screening are described in detail in the text. The mutant designations are listed in Table 3 and shown in Figure 2.


Integration-defective mutants predicted by the att model
① Test for XOP
XOP+
XOP−
a) REL mutants
b) RER mutants
c) Int mutants
all XOP^Δ strains
3B
② Location of att lesion
left of XOP
right of XOP
REL^Δ; REL^B
RER^Δ; RER^B; Int^Δ
④ Integration behavior
③ Int-promoted recombination
resembles λb2
resembles λdg
same as λint−
same as λbio
resembles neither
REL^Δ
REL^B
Int^Δ
RER^B
RER^Δ
2A
2B
3A

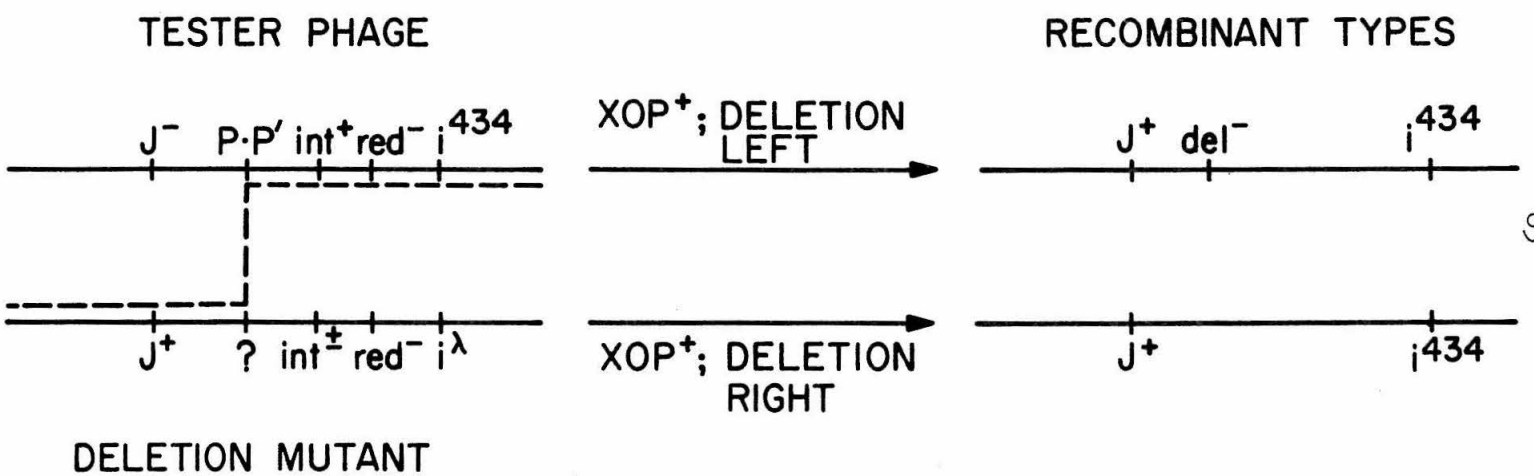
discussed in detail below.

1. Beginning with the class 2 and 3 deletions, which are known to be integration-defective, any mutants that did not contain a functional cross-over point were detected by the recombination test shown in Figure 4. In this test a red$^-$ derivative of each deletion mutant is first constructed and then crossed to a red$^-$ tester phage. Failure of a deletion mutant to undergo Int-promoted recombination is taken as evidence that the mutant is missing XOP. Since the tester phage supplies int$^+$ function, deletions that are int$^-$ should still be able to recombine if they are XOP$^+$.

Several of the UV and VEG deletions from class 3 failed to undergo Int-promoted recombination in this test. These mutants, designated class 3B, are presumably missing at least XOP. These mutants were not studied further since physical studies of these mutants (Parkinson & Davis, 1970) indicated that the entire att site is probably deleted in these phages.

2. The XOP$^+$ deletion mutants identified by the first test could have any of several integration defects as shown in Figure 3. Mutants with REL defects should map to the left of XOP, whereas deletion mutants with RER defects or an Int defect should lie to the right of XOP. Thus the next step was to locate the deletion in each mutant phage with respect to XOP. To do this, the recombinants from the cross shown in Figure 4 were analyzed for the presence of the deletion mutation. Since all recombination must take place at XOP, the selected recombinant class will carry the deletion if the deletion is located to the left of XOP. If the deletion is to the right of XOP, the recombinants will not carry the deletion.

Fig. 4. A recombination test for XOP and the position of a deletion relative to XOP. A $\underline{red}^-$ deletion mutant is crossed in QR48 to $J^- \underline{red}^- i^{434}$ and $J^+ i^{434}$ recombinants are measured on $594(J^- i^{\lambda})$. If the deletion mutant contains a functional att site, recombinants should be produced. If the recombinants contain the deletion this indicates that the deletion is located left of XOP. Otherwise the deletion must be to the right of XOP.


TESTER PHAGE
RECOMBINANT TYPES
J⁻ P·P′ int⁺red⁻ i⁴³⁴
XOP⁺; DELETION LEFT
J⁺ del⁻ i⁴³⁴
J⁺ ? int± red⁻ iλ
XOP⁺; DELETION RIGHT
J⁺ i⁴³⁴
DELETION MUTANT

On the basis of this test, all of the class 2 mutants mapped to the left of XOP, and the remaining deletions from class 3 mapped to the right of XOP. This finding indicates that class 2 deletions are REL-defective and the XOP^+ deletions from class 3 are either RER-defective or Int-defective.

3. The XOP^+ class 3 deletions therefore represent three possible att structures: $\underline{att}^{\varphi}$, RER^{Δ}, and RER^{B}. To distinguish between these three att's, class 3 deletions were crossed to a tester phage carrying $\underline{att}^{\varphi}$. The cross is diagrammed in Figure 5. For comparison, $\lambda\underline{int}^-$ and $\lambda\underline{bio}$ phages were also studied. If the integration defect of a class 3 mutant is merely functional, for example, an $\underline{int}^-$ deletion, that mutant should behave like wild type in Int-promoted recombination since the att site has not been altered. Structural att defects, however, could cause a significant change in Int-promoted recombination properties. The results (Table 5) indicate that the class 3 deletions that were tested do not resemble either $\underline{att}^{\varphi}$ ($\lambda\underline{int}^-$) or $\underline{att}^{R}$ ($\lambda\underline{bio}$) in their pattern of Int-promoted recombination. These mutants, designated class 3A, are probably RER^{Δ} as opposed to Int^{Δ} or RER^{B}. The failure to find these latter types of deletion mutants is not surprising in view of the small number of class 3 mutants that have been examined. Since $\lambda\underline{bio}$ phages carry $\underline{att}^{R}$, these transducing phages have been used to represent the RER^{B} type of att mutant.

The two 3A mutants that have been tested are both $\underline{int}^-$ as shown by their failure to complement an $\underline{int}^-$ phage for integration, and by failure to produce $\underline{int}^+$ recombinants when crossed to various $\underline{int}^-$ phages. Since all of the known $\lambda\underline{bio}$ transducing phages are also $\underline{int}^-$, this suggests that RER may be quite close to Int in the chromosome. In fact, it is not possible on the basis

Fig. 5. A cross used for quantitative measurement of Int-promoted recombination frequency between two att mutants. Crosses were performed in QR48 at 37°C. Both the i^{λ} (CI857) and the i^{434} (CI^{-}) phages cannot produce functional repressor at this temperature. $L^{+}K^{+}i^{\lambda}$ recombinants were assayed on 594 ($L^{-}K^{-}$ red3 i^{434}). Crosses of this type were always performed with both orientations of the selected markers in the parental phages.

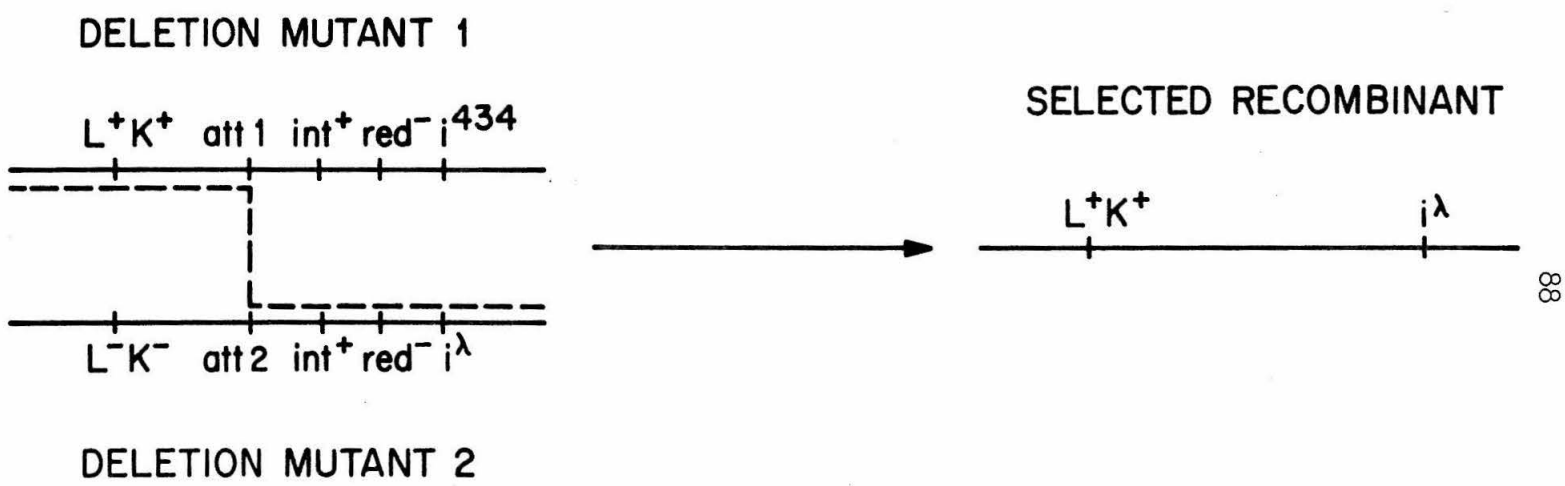

DELETION MUTANT 1
L+K+ att 1 int+ red− i434
L−K− att 2 int+ red− iλ
DELETION MUTANT 2
SELECTED RECOMBINANT
L+K+
iλ

TABLE 5

Int-promoted Recombination in XOP$^+$ Class 3 Deletions

	P · P'
Class 3 mutants	
b508	0.7
b522	1.4
Controls	
λbio69	16
λbio7-20	15
λ^{++}	9.7
λint$^-$	8.8
b221(3B)	< .01
b538(3B)	< .01

The numbers indicate the % recombination and are an average of at least 4 crosses and include both possible orientations of the selected markers in the two parents. The type of cross used is shown in Figure 5. The deviation in measured recombination frequency was always less than ±20% for crosses done on separate days.

of the present evidence to order Int and RER relative to XOP. It is likely, however, that RER will prove to lie next to XOP and to the left of Int, since RER is an essential component of $\underline{att}^{\varphi}$, which should be distinct from Int.

The high recombination frequency in Table 5 for crosses of $\lambda^{++} \times \lambda\underline{bio}$ is unexpected since others have found that this particular att combination is a poor one for Int-promoted recombination (Signer, Weil & Kimball, 1969). A plausible explanation for this discrepancy is presented in the Discussion section.

4. The final step in the deletion screening procedure involves the classification of the REL-defective class 2 mutants. These mutants could be either REL^{Δ} or REL^{B}. The REL^{B} class is expected to resemble $\lambda\underline{dg}$ phages which also carry REL^{B} ($\underline{att}^{L}$). The REL^{Δ} mutants may or may not behave like $\lambda\underline{dg}$. Guerrini (1969) has shown that $\lambda\underline{dg}$ phages integrate more frequently at $\underline{att}^{L}$ than at $\underline{att}^{R}$ in strains of bacteria that contain both of these att sites. Since all of the class 2 deletion mutants have been shown to establish stable lysogeny in such a host (Table 4), the att preference of these mutants was examined with strains containing only $\underline{att}^{L}$ or $\underline{att}^{R}$ (Table 6). The results indicate that, in fact, there are two types of class 2 deletions. Class 2A mutants integrate with greater efficiency into $\underline{att}^{R}$, whereas 2B mutants integrate more frequently into the $\underline{att}^{L}$ strain. Thus 2B deletions are presumed to carry REL^{B}. This is consistent with the fact that 2B mutants are only found among the UV deletion mutants.

As a check on whether or not 2B deletions carry $\underline{att}^{L}$, the Int-promoted recombination properties of the 2B mutants, λb130 and λb189, were further analyzed. Table 7 presents data comparing 2A and 2B deletions with respect to several properties ex-

TABLE 6

Integration Patterns of REL-defective Deletion Mutants

Class	Mutant	Fraction of surviving cells which are stably lysogenic ($\underline{att}^B$)	($\underline{att}^L$)	($\underline{att}^R$)
2A	b2	< .01	0.27	0.97
	b511	< .01	0.20	0.98
2B	b130	0.04	0.71	0.11
	b189	0.04	0.70	0.12
Controls				
	λ^{++}	0.90	0.47	0.24
	$\lambda\,\underline{int}^-$	< .01	< .01	< .01

C600 ($\underline{att}^B$), SA205 ($\underline{att}^L$) and SU740 ($\underline{att}^R$) were grown in tryptone and maltose to saturation and resuspended at 4×10^8/ml. in TMG buffer. Phage at a multiplicity of 5 were added to an equal volume of cells and adsorption was carried out for 15' at 37°. The cells were then diluted 2×10^4 into tryptone and glucose and anti-λ serum (k final = 0.1) and incubated for 5 hours at 37°. The growth tubes were plated for colonies and these colonies were tested on EMB-glucose agar (see Gottesman & Yarmolinsky, 1968) for stable lysogeny. At least 100 colonies were tested for each value shown. All phages contained the CItl marker (Lieb, 1964).

TABLE 7

Int-promoted Recombination in B · P' and X · P' Phages

	B · B'	X · B'	P · B'
P · P'	3.5/18	0.5/1.5	14/17
B · P'	0.5/2.2	0.4/0.7	60/24
X · P'	0.2/1.5	0.5/0.9	66/22

See the footnote of Table 5. These recombination values are averages of 2 or more determinations. The two values for each combination are from reciprocal crosses; the first value was obtained when the parent in the vertical column contained the L^-K^- markers; the second value was obtained when the other parent contained the L^-K^- marker. The mutants used were: B · P' (λb130, λb189); X · P' (λb2, λb511); B · B' (λb130bio69); X · B' (λb511bio69); P · B' (λbio69).

pected of B · P' phages. For example, a phage containing B · P' should recombine efficiently with a P · B' phage, since this att combination is similar to the excision event that occurs after prophage induction. Moreover, the two recombinants of such a cross should be P · P' and B · B'. The B · B' double mutant (att^B) should give high recombination with λ^{++} because this att combination resembles the actual integration event during lysogenization. Also B · B' should recombine poorly with both class 2A and 2B deletion mutants, since class 2 mutants cannot efficiently lysogenize bacteria containing att^B.

The data in Table 7 are consistent with the notion that 2B deletions contain att^L. However, these data also demonstrate an effect which has been repeatedly found in crosses of this sort: the frequency of reciprocal recombinants for a given pair of attachment sites may be grossly unequal. For example, λb130 × λbio69 gives a value of 23% when the b130 parent is L^-K^-, but a frequency of 63% when the bio69 parent is L^-K^-. These effects on recombination frequency do not, however, alter the interpretation of the results. Other instances of this phenomenon will be presented in a later section.

In summary, many of the integration-defective deletion and substitution mutants predicted by the att model discussed in (a) above have been found. These types are: XOP^Δ strains (class 3B); REL^Δ strains (class 2A); REL^B strains (class 2B); and RER^Δ strains (class 3A). In addition, non-integration-defective deletion mutants (class 1) have also been found. Two types of mutants not found were RER^B phages and phages containing lesions only in the Int gene. However, using int^- point mutants and λbio transducing phages, it was possible to complete the collection of integration-

defective mutants which were needed for the studies described below. The att mutants classified by this screening procedure and their properties are summarized in Table 8.

(c) Properties of Class 1 deletion mutants

Class 1 deletion mutants are those which have a wild type integration pattern. These mutants are of interest and are discussed here because I have been unable to find any properties other than the deletion itself which distinguish these phage from wild type. The following results have been obtained.

The frequency of stably lysogenic cells after infection by a Class 1 deletion is identical to a wild type control infection in att^B, att^L and att^R host strains. The efficiency of excision after induction of Class 1 lysogens appears normal based on two criteria: such induced lysogens produce a normal yield of particles; and the frequency of excision errors as judged by the number of λdg transducing particles is the same as λ^{++}. I have also checked a number of bacterial strains (su^-, rec^-, uvr^-) without finding one on which λ^{++} grows, but class 1 deletions do not.

(d) The function of REL and RER

The att mutants whose properties are described in Table 8 allow us to define three different forms of REL and three of RER. The portion of the attachment site which corresponds to REL or RER can be either of phage or bacterial origin or can be presumably non-specific DNA which has become joined to the att site by a deletion. Thus REL^{φ}, REL^B and REL^{Δ} can be thought of as three

TABLE 8

Properties of att mutants

Class of mutant	Genotype	att site Designation	integration preference	Int-promoted recombination	position of lesion relative to XOP	mutants studied
λ^{++}	wild type	P · P'	att^{B}	Yes	none	---
Class 1	del^{-} att^{φ}	P · P'	att^{B}	Yes	left	b503, b504, b506, b515
Class 2A	del^{-} REL^{Δ}	X · P'	att^{R}	Yes	left	b2, b511, b516, b527
Class 2B	del^{-} REL^{B}	B · P'	att^{L}	Yes	left	b130, b189
λdg phages	REL^{B}	B · P'	att^{L}	Yes	left	none
Class 3A	$del^{-}RER^{\Delta}int^{-}$	P · X'	----	Yes	right	b508, b512, b517, b522
λbio phages	RER^{B} int^{-}	P · B'	----	Yes	right	bio7-20, bio69, bio11
Class 3B	del^{-} att^{Δ} int^{-}	XX'	----	No	inclusive	b221, b538

A summary of the types of att-defective mutants that are known. Mutants b503, 504, 506, 508, 511, 512, 515, 516, 517, 522, 527, 538 are VEG deletions. Mutants b130, b189, b221 are UV deletions. The b2 deletion mutant has been described by Kellenberger, et al. (1961). Transducing phages bio11 and bio69 are described by Manly, et al. (1969). Transducing phage bio7-20 is described by Kayajanian (1968).

allelic forms of REL. Similarly, RER^{φ}, RER^{B} and RER^{Δ} are three allelic states of RER. Through Int-promoted recombination any form of REL can be combined with any form of RER to generate nine different att sites which are listed in Table 9. These att sites were examined by studying the patterns of Int-promoted recombination between att mutant phages. It was hoped that a pattern would emerge that would suggest a model for the function of REL and RER in integration. The type of cross employed in these studies has been shown in Figure 5. Since RER^{B} and RER^{Δ} phages are int^{-} as well, only results of crosses involving at least one phage with RER^{φ} will be presented because only RER^{φ} phages can supply the needed int^{+} function for these crosses.

The pattern of Int-promoted recombination between phage containing various att sites is shown in Table 10. In addition to these att combinations, crosses were done between phage, neither of which contained RER^{φ}, by using an int^{+} helper phage. All of these crosses yielded little or no recombination. Since proper controls for these helper-promoted crosses were not available, it is difficult to evaluate these results, however, it appears that RER^{φ} is probably necessary but not sufficient for effective Int-promoted recombination. It is evident from the results presented in Table 10 that Int-promoted recombination can occur between phage with many types of att defects, and that the resultant recombination frequencies vary over a wide range for different att combinations. Int-promoted recombination is therefore not restricted to phage with the naturally-occurring att forms for integration ($P \cdot P' \times B \cdot B'$) and for excision ($P \cdot B' \times B \cdot P'$).

A number of the att combinations shown in Table 10 are interesting in that reciprocal crosses yield significantly different

TABLE 9

Nine Different att Sites for Studying att Interactions

a) One-step att mutants

att	mutants used
P · P'	λ^{++}, b504, b506
X · P'	b2, b511
B · P'	b130, b189
P · X'	b508, b522
P · B'	bio7-20, bio69

b) Recombinant att mutants

att	mutants used
X · X'	b511b522
X · B'	b2bio69, b511bio69
B · B'	b130bio69, b189bio69
B · X'	b130b508

Recombinant phages were made by Int-promoted recombination between the appropriate parental strains so that the selected recombinants contained both deletions. The constitution of the X · X' and B · X' recombinants could not be confirmed by backcrosses; however, the properties of these phages indicate that they indeed have the indicated constitution.

TABLE 10

Patterns of Int-promoted
Recombination Between att Mutants

	P · P'	B · P'	X · P'
P · P'	9.7	28	25
B · P'	28	13	6.8
X · P'	25	6.8	2.2
P · B'	15	24/60	22/66
B · B'	3.5/18	1.4	0.8
X · B'	1.0	0.5	0.7
P · X'	1.0	18/7.2	7.2/0.8
B · X'	< .01	< .01	< .01
X · X'	< .01	< .01	< .01

See the footnote of Table 5. Combinations containing two recombination values are those in which recombination frequency exhibited a strong dependence on which recombinant was measured. The other values are averages of 2 or more sets of reciprocal crosses.

frequencies of recombination. The four most striking examples of this phenomenon are listed in Table 11. Three of these att combinations have the additional property of being essentially irreversible in recombination. That is, the att sites generated by such recombination are themselves unable to recombine with each other to generate the original att sites. All four of these att combinations have in common the property of generating one P · P' recombinant and one recombinant which contains neither REL^{φ} nor RER^{φ}. These combinations can all be considered excision-like events in which the relevant att configuration is P · -' × - · P'; that is, where REL^{φ} and RER^{φ} are each present but in different phages. The irreversibility of three of these combinations and the apparent inequality of reciprocal events for all four combinations may be related since it appears that the recombinant type which is found in excess generally contains an att site that cannot efficiently recombine with either of the parental att sites. With the exception of B · B', which recombines well with P · P', these att sites (X · B', B · X', X · X') are quite unreactive in all of the combinations which have been examined (see Table 10).

4. DISCUSSION

In the Results section a model of att^{φ} was discussed which states that att^{φ} is composed of three distinct elements: a cross-over point (XOP) at which integration occurs; and two recognition elements (REL and RER) on either side of XOP which are necessary for normal recombination. Many of the types of att mutants predicted by this model have, in fact, been found among a number of viable λ deletion mutants. It appears, therefore, as though this

TABLE 11

Examples of Irreversible and Non-reciprocal Int-promoted Recombination

B · P' × P · B' ⇌	P · P' × B · B'
18% / 3.5%	24% / 60%
B · P' × P · X' ⇌	P · P' × B · X'
< .01%/< .01%	7.2% / 18%
X · P' × P · X' ⇌	P · P' × X · X'
< .01%/< .01%	0.8% / 7.2%
X · P' × P · B' ⇌	P · P' × X · B'
1.5% / 0.5%	22% / 66%

See the footnote of Table 5. The recombination values given are those obtained when the recombinant containing the att site directly above that value was measured. For example, B · P' × P · B' gives 24% recombination if the P · P' recombinant is measured, and 60% recombination if the B · B' recombinant is measured.

model of $\underline{att}^{\varphi}$ is correct, at least in broad detail. In the accompanying paper (Parkinson & Davis, 1970) the physical properties of these att-defective deletion mutants are described. That work, in conjunction with the results reported here, permits us to locate and determine the size of the three att components discussed above.

The properties of these various aberrant integration mutants further strengthens the view that integrative recombination is uniquely distinct from generalized recombination. Unlike the latter, integrative recombination occurs only at one point, which is defined by the sequences in the flanking recognition elements. Furthermore, a certain degree of specific non-homology between att sites apparently enhances the efficiency of Int-promoted recombination between two att's. Thus in a cross between two phage genomes, the four recognition elements in the two att sites interact to produce characteristic levels of recombination which seem to be determined not only by the elements involved, but also by the orientation of those elements. An attempt has been made to reveal the underlying mechanism of integration by examining the interaction of att elements with the use of Int-promoted recombination. In adopting this approach, it was assumed that Int-promoted recombination between phage chromosomes operates in the same way as integration and excision of the λ prophage. Since Int-promoted recombination is both site-specific and Int-dependent (Weil & Signer, 1968; Echols, Gingery & Moore, 1968) and seems to be reciprocal as well (Signer & Weil, 1968b; Weil, 1969), there is sufficient reason to believe that integration and Int-promoted vegetative recombination are very similar processes.

The pattern of Int-promoted recombination between phages containing various att sites reveals no simple underlying basis that

we can detect (see Table 10). Some general features of this system can be described, however. For example, recombination can occur between phage containing a variety of mutant att sites, not merely the naturally-occurring ones for integration and excision. Secondly, efficient Int-promoted recombination seems to require that at least one of the recombining att sites contain RER^{φ}. The apparent complexity of the Int system is reflected in the wide range of recombination frequencies which can be obtained with different att combinations. For this reason, it is difficult to interpret the quantitative aspects of the results.

It seems likely that one factor contributing to the complexity of the present results is the existence of two discrete modes of Int-promoted recombination which could overlap for particular novel att combinations. Conceivably there are two kinds of recombination events each with its own special att requirements: one of these would resemble the normal integration mechanism; the other would resemble the mechanism of prophage excision. Echols (1969) has recently obtained evidence that a new gene called Xis (Guarneros & Echols, 1969) is essential for excessive recombination of P · B' × B · P'. Xis function is not required, however, for integrative recombination of P · P' × B · B'. Thus Int-promoted recombination between vegetative phage may occur in two ways, one of which is only Int-dependent (integration mode), and one which is both Int-dependent and Xis-dependent (excision mode). That both of these recombinational modes can operate on some att combinations is suggested by the finding that the frequency of Int-promoted recombination between some att sites (P · P' × P · P'; P · P' × B · P'; P · P' × P · B') decreases about 50% under $\underline{xis}^{-}$ conditions (Echols, 1969).

I have examined a number of simple models, based on the assumption that two modes of Int-promoted recombination exist, however, none of them can account for all of the qualitative features of the results. For example, the critical determinants for excision might be REL^{φ} and RER^{φ} if these elements are located in opposing att sites. Thus any att combination having the general form P · -' × - · P' could recombine by the excision mode. This type of analysis has the appealing feature of being able to account for the unexpectedly high recombination of many P · -' × - · P' combinations, some of which (Table 11) might be Xis-dependent. The analysis breaks down, however, when we try to define the integration mode in comparable terms of att element requirements. Is it possible that factors other than att structure are contributing to the apparent complexity of the results, confounding and obscuring any simple patterns that might exist? Some of these factors may be:

(1) Deletion mutants of REL or RER appear to be aberrant products of the Int system (Parkinson & Davis, 1970). Thus although it has been assumed that the DNA adjacent to XOP in REL^{Δ} and RER^{Δ} mutants has no sequence recognition value for the Int system, this may not be the case, especially in view of the Int-dependent origin of these deletion mutants.

(2) The phage mutants used to represent the various forms of att sites have very different DNA contents and a number of functional differences. It may not be valid to assume that att structure is the only important variable affecting Int-promoted recombination between these phages.

(3) The recombination patterns of att mutants could also be distorted by functional effects caused by differences in gene expression.

Two possible examples in this work can be cited. Int expression is under CI control (Signer, 1969) and it is possible that even under vegetative conditions, the presence of a functional CI gene in a cross might delay the onset of full Int expression and thereby decrease Int-promoted recombination frequency. This could account for the high recombination values obtained in this work with CI^- phages, and might also explain the very different efficiency of P · P' × P · B' under CI^- and CI^+ conditions (Signer, Weil & Kimball, 1969). A similar functional effect due to control of Xis expression could explain the different patterns of B · P' recombination during lysogenization and during vegetative growth. Upon integration, B · P' integrates more frequently at B · P' than at P · B'. In vegetative growth, Int-promoted recombination is much more efficient between B · P' and P · B' phages than between B · P' and B · P' phages. It is not unreasonable to suppose that Xis expression is repressed to a greater extent than Int expression during the establishment of lysogeny.

(4) Although Int-promoted recombination seems to be reciprocal (Signer & Weil, 1968b; Weil, 1969), several of the att combinations reported here do not produce equal numbers of the two recombinant types (see Table 11). The primary recombination event may be reciprocal in these cases, but one of the two recombinant types might replicate more slowly or have decreased viability relative to the other recombinant type. One way in which this could happen is by effectively withdrawing one recombinant type from the mating pool, allowing it to replicate unhindered by further mating events. This explanation is suggested by the fact that the favored recombinant type is generally the one which cannot recombine efficiently with either of the parental phages.

These studies appear to support the premise that integrative recombination in λ is in many respects quite unlike generalized recombination. Our previous notions about recombination do not seem to apply to this specific phenomenon, even considering the several factors which might obscure the analysis of the integration system. Clearly, however, the elucidation of this unique recombination event will require new methods and new insights.

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Deletion Mutants of Phage Lambda
III. Physical Structure of att$^{\varphi}$

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ABSTRACT

Deletion mutants and substitution mutants of phage λ have been used to examine the physical structure of the $\underline{att}^{\varphi}$ site in the λ chromosome which is essential for prophage integration. Integration-defective λ mutants were analyzed by constructing heteroduplex DNA molecules containing one wild type and one mutant strand and examining these heteroduplexes by electron microscopy. The results indicate that $\underline{att}^{\varphi}$ is less than 2500 base pairs in length and may be as small as 20 - 50 base pairs. Integration cross-overs between $\underline{att}^{\varphi}$ and its bacterial analog , $\underline{att}^{B}$, occur within a region which is less than 20 base pairs in length. Moreover, there is no detectable base sequence homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$. These results suggest that λ integration is a truly unique system of recombination.

1. INTRODUCTION

Bacteriophage lambda has evolved a highly specific recombination system for inserting its own DNA molecule into the continuity of the bacterial chromosome during lysogenization. The lambda integration machinery has at least two components, a functional one and a structural one. The functional part of this system is the Int gene (Zissler, 1967) whose product is essential for integration. It is not yet clear what role the Int product plays during integration, however, the Int product is assumed to be an integration enzyme or "integrase." The structural component of the integration system, called $\underline{att}^{\varphi}$, lies adjacent to the Int gene in the middle of the lambda genome. This phage attachment region is the site of action of the integrase and therefore must contain specific information necessary for recognition by the integration system.

The phage attachment site has at least three distinct components (Parkinson, 1970): a cross-over point (XOP) which is the site at which integrative recombination takes place, and a recognition element on each side of XOP. These elements are designated REL (recognition element left) and RER (recognition element right). While they influence the efficiency of integrative recombination, these elements are probably not uniquely essential for such recombination. In this paper we describe studies of the physical structure of $\underline{att}^{\varphi}$. In particular, we examined the following aspects of the physical organization of the attachment site:

(1) The size and location of XOP.

(2) The size and location of REL and RER.

(3) The extent of base sequence homology between $\underline{att}^{\varphi}$ and its bacterial analog $\underline{att}^{B}$.

In order to study $\underline{att}^{\varphi}$ which seems to have a purely structural role, it is necessary to employ mutants such as deletions or substitutions which contain major structural changes of the att region. The isolation and genetic properties of such att-defective mutants are described in the first two papers of this series (Parkinson & Huskey, 1970; Parkinson, 1970). For the work described in this paper we have employed the technique of electron microscopy of heteroduplex DNA molecules (Davis & Davidson, 1968; Westmoreland, Szybalski & Ris, 1969) to examine the physical properties of att-defective mutants. By comparing the genetic properties of these mutants with their physical size and location in the lambda chromosome, we have obtained a detailed picture of $\underline{att}^{\varphi}$ and of its role in the integration process.

2. MATERIALS AND METHODS

(a) Phage strains

The phage mutants used in this work are listed in Table 1. All of these phages are derived from λ^{++} (λPaPa) of Kaiser (1957). The deletion mutants in this table are described by Parkinson & Huskey (1970) and by Parkinson (1970).

(b) Bacterial strains

The bacterial strains employed in this study are described in Table 2.

TABLE 1

Phage Strains

a) Miscellaneous strains

Strain	Relevant Properties	Source and/or reference
λ^{++}	wild type	Kaiser (1957)
λint29	int⁻ sus$_{21}$	E. Signer
λb5	imm^{21}	J. Weigle; Kellenberger, et al. (1961b)

b) Deletion mutants (Parkinson and Huskey, 1970; Parkinson, 1970)

Class of mutant	Relevant Properties(1)	Mutant isolates employed
1	int^{+} att$^{\varphi}$	b501, b502, b504, b506, b509, b510, b515, b519, b520, b536
2A	int^{+} REL$^{\Delta}$	b2(2), b511, b516, b527, b540
2B	int^{+} REL^{B}	b130, b189
3A	Int$^{\Delta}$ RER$^{\Delta}$	b508, b512, b514, b517, b522
3B	Int$^{\Delta}$ att$^{\Delta}$	b221, b531, b538

c) Transducing phages

λdg isolate	Relevant Properties	Source and/or reference
P71	(A-J)$^{\Delta}$ att^{L}	isolated in this laboratory
P73	(C-J)$^{\Delta}$ att^{L}	"
P74	(G-J)$^{\Delta}$ att^{L}	"
P72	(H-J)$^{\Delta}$ att^{L}	"
λbio isolate		
bio16A	Int$^{\Delta}$ att^{R}	K. Manly; Manly, et al. (1969)
bio7-20	(Int-Xis)$^{\Delta}$ att^{R}	K. Manly; Kayajanian (1968)
bio69	(Int-Exo)$^{\Delta}$ att^{R}	K. Manly; Manly, et al. (1969)
bio11	(Int-β)$^{\Delta}$ att^{R}	"
bio10	(Int-CIII)$^{\Delta}$ att^{R}	"

TABLE 1 (Cont'd)

(1) Abbreviations:

$\underline{att}^{\varphi}$	=	wild type phage attachment site
$\underline{att}^{L}$	=	left prophage attachment site
$\underline{att}^{R}$	=	right prophage attachment site
REL^{Δ}	=	deletion of left recognition element in $\underline{att}^{\varphi}$
RER^{Δ}	=	deletion of right recognition element in $\underline{att}^{\varphi}$
REL^{B}	=	substitution of bacterial DNA for left recognition element in $\underline{att}^{\varphi}$
$\underline{att}^{\Delta}$	=	deletion of entire $\underline{att}^{\varphi}$ region
Int^{Δ}	=	deletion of Int gene

(2) Kellenberger, et al. (1961a)

TABLE 2

Bacterial Strains

Strain	Relevant genotype[1]	Source and/or reference
W3110	$\underline{su}^-$ prototroph	J. Weigle
C600	$\underline{su}^+ \underline{att}^B$	Appleyard (1954)
QR93	$\underline{su}^- \underline{att}^\Delta \underline{rec}^+$	E. Signer
QR94	$\underline{su}^- \underline{att}^\Delta$ recA	E. Signer
SA205	$\underline{su}^- \underline{att}^L$	S. Adhya
SU740	$\underline{su}^- \underline{att}^R$	S. Adhya

(1) Abbreviations: $\underline{su}^+$ ($\underline{su}^-$) = ability (inability) to support the growth of λ sus mutants

$\underline{att}^\Delta$ = deletion of the entire $\underline{att}^B$ region, which is the att site for λ

$\underline{rec}^+$ (recA) = ability (inability) to promote generalized recombination

$\underline{att}^L$, $\underline{att}^R$ = left and right prophage attachment sites

(c) Media

All media and buffers for phage work have been described previously (Parkinson, 1968; Parkinson & Huskey, 1970).

(d) Preparation of λ phage for DNA studies

W3110 was grown with aeration to $\sim 1 \times 10^8$/ml. at 37°C in tryptone broth supplemented with 10^{-2} M $MgSO_4$ and 0.2% glucose. Phage were then added at a multiplicity of 0.05 - 0.5 and aeration was continued for 5-6 hours at which time $CHCl_3$ was added to promote complete lysis. Phage yields of 5×10^{10} to over 10^{11}/ml. are routinely obtained with this procedure. Bacterial debris is removed by low speed centrifugation and the phage pelleted by centrifugation in the Spinco 21 rotor for 2 hours at 18,000 rev./min. The pellets are resuspended in 1/100 the initial volume with TMG buffer and the phage are then added to a saturated CsCl solution, adjusting the final density to about 1.49 g/cm^3. Centrifugation was for 20 to 24 hours at 30,000 rev./min. in an SW50 or SW50.6 rotor. The band of purified phage is collected by piercing the tube just below the band and collecting drops.

(e) Heteroduplex formation

To a small test tube were added: 50 µl. of 0.2 M EDTA at pH 8.0; 0.350 ml. H_2O (minus the volume of phage to be added); 5×10^{10} particles of phage A (approximately 2.5 µg of DNA in 10 µl. of solution) and 5×10^{10} particles of phage B. The phage DNA was extracted and denatured by the addition of 50 µl. of 1.0 N NaOH. After standing for 10 minutes at room temperature to allow complete

strand separation, the solution was neutralized by the addition of 50 µl. of 1.8 M Tris-HCl, 0.2 M Tris-base (pH ~7). Renaturation was carried out by adding 0.5 ml. of formamide (Mallinckrodt, 99%) to give a final volume of 1 ml. at pH 8.5.

Renaturation was carried out at room temperature for 1 to 2 hours, after which time the pH of the solution was 7.5 to 8.0. Further renaturation was prevented by dialysis against 10^{-2} M Tris, 10^{-3} M EDTA, pH 8 at 4°C. This preparation is then stored at 4°C; however, after several days of storage a significant fraction of the renatured molecules become cyclized. Immediately before use, decyclization is achieved by heating the heteroduplex preparation to 50°C for 5 minutes followed by an ice water quench.

(f) Electron microscopy

Grids were prepared by the basic protein film technique (Kleinschmidt & Zahn, 1959) and stained with uranyl acetate or shadowed with platinum-palladium. Two modifications of this technique were used.

(i) Aqueous technique

This method is essentially that described by Davis & Davidson (1968) and in more detail by Davis, Simon & Davidson (1969). 50 µl. of a solution containing about 0.5 µg/ml. of the heteroduplex DNA preparation and 0.1 mg/ml. of cytochrome c in 0.5 M ammonium acetate, pH 7, were spread onto a clean glass slide. This solution (hyperphase) was then allowed to flow down the slide onto a clean surface of 0.25 M ammonium acetate (hypophase). A parlodion-coated grid was touched to the film about 5 mm. from the glass slide

before all of the hyperphase had run off the slide. Grids were stained with uranyl acetate as described previously (Davis & Davidson, 1968).

(ii) Formamide technique

Immediately before use the hyperphase solution is prepared containing 10 µl. of the renatured DNA preparation (0.05 µg. DNA); 10 µl. of 1 mg/ml. cytochrome c in 1 M ammonium acetate and 0.1 M Tris, pH 7.9; 40 µl. H_2O; 40 µl. formamide. The hypophase, also freshly prepared to eliminate pH changes which can occur in formamide solutions upon standing, contained 10^{-2} M ammonium acetate, 10^{-3} M Tris, pH 7.9, and 10% formamide or else 10^{-2} M Tris, pH 7.9, and 10% formamide. 50 µl. of the hyperphase were spread onto the hypophase as described in part (i) above. The film was picked up with parlodian-coated grids after one minute and the grids were stained in uranyl acetate and shadowed with platinum-palladium.

(iii) Microscopy

A Phillips EM300 electron microscope using a 50 µ objective aperture and 60 KV accelerating voltage was employed. Negatives (~3000X) were enlarged 20X or 50X on a Nikon shadowgraph and traced onto paper. Lengths were measured on these tracings with a map measurer. Occasionally the dark field mode of operation of the Phillips EM300 electron microscope was used.

3. RESULTS

(a) Heteroduplex mapping of deletions and substitutions

(i) Electron microscopy of heteroduplex DNA molecules

Accurate physical maps of deletions and substitutions in the λ chromosome have been described by Davis & Davidson (1968) and by Westmoreland, Szybalski & Ris (1969). The basic principle of this mapping method involves constructing heteroduplex DNA molecules, one strand of which contains a deletion or substitution relative to the other strand. As described in the Methods section, these heteroduplexes are formed by mixing two DNA preparations, alkaline denaturing to allow strand dissociation, and subsequently neutralizing in formamide to give gentle renaturation. The resulting DNA preparation will contain not only the two original homoduplex DNA's, but also a large proportion of heteroduplex molecules containing one strand from each of the two original molecules. If, for example, heteroduplexes were prepared with wild type DNA and DNA from a deletion mutant, the heteroduplex would contain a single-stranded loop of DNA in the wild type strand corresponding to the size and position of the DNA missing in the deletion strand. Below we describe briefly two methods for visualizing such loops.

Heteroduplex molecules mounted by the aqueous technique (see Methods section) permit accurate measurements of duplex contour lengths; however, the loops of single-stranded DNA collapse into "bushes" (MacHattie et al., 1967; Davis & Davidson, 1968). Apparently in high ionic strength and basic protein the more flexible single-stranded DNA collapses because of nonspecific base interactions. Thus in this technique the lengths of single-stranded DNA

cannot be measured even though the positions of single-stranded regions can be accurately determined because they form characteristic bush-like structures.

The formamide mounting technique (Westmoreland et al., 1969) described in the Methods section allows us to directly measure the length of single-stranded DNA in a heteroduplex molecule. Formamide is a DNA denaturing agent which under certain conditions (30 - 70% v/v, ionic strength = 0.1, 25°C) does not affect either duplex DNA or protein but does prevent single-stranded DNA from collapsing by melting out all nonspecific base interactions. In the electron microscope duplex DNA mounted with the formamide method appears essentially the same as if mounted by the aqueous method. However, single-stranded DNA appears as a flexible, non-tangled filament whose contour length can be accurately measured.

Although in principle the formamide technique will yield more information, we generally used the aqueous technique for mapping deletions and substitutions. The major reasons for choosing the aqueous technique are its reliability and simplicity and the relatively short time involved in preparing and scanning grids made in this manner. In addition, the aqueous technique gives considerably fewer molecules ($\sim 10\%$) which must be discarded because of overlaps and tangles than does the formamide method. Furthermore, metal shadowing is not only unnecessary but actually obscures the single-strand bushes in heteroduplexes prepared by the aqueous mounting procedure. The formamide technique was employed whenever it was necessary to examine the length or nature of the single-stranded DNA loops in heteroduplex molecules.

(ii) Mapping principles

Figure 1 illustrates the basic principles for mapping the location and size of deletions by heteroduplex microscopy. Substitutions are also mapped in the same manner. We desire to know the fractional lengths of the two deletion endpoints from the left end of the wild type λ chromosome, the left end being defined genetically by gene A, the right end by gene R. By hybridizing the deletion mutant with λb5 DNA which contains a substitution in the right arm, we are able to mark the right arm of the heteroduplex physically and thus relate the physical map to the standard genetic map. The b5 substitution in the reference DNA also provides an internal calibration for each heteroduplex molecule so that length fluctuations between molecules, even those in the same grid, do not influence the accuracy of the measurements. Furthermore, by using an internal standard in each heteroduplex molecule, it is not necessary to include standard reference DNA molecules for length calibrations. Because of this calibration technique, we have abandoned the convention of expressing DNA lengths in microns and instead refer to sizes and locations in terms of the fractional length of λ^{++} DNA. The values we determine are x_1 and x_2, the fractional positions of the left and right deletion or substitution endpoints measured from the left end of the λ DNA molecule.

In this mapping procedure we must first know very accurately the size and position of the b5 marker substitution. Methods for calibrating this substitution are discussed in the next section. With the b5 marker calibrated, the mapping procedure outlined in Fig. 1 is quite straightforward. We measure the three separate duplex segments (A, B and C) shown in the heteroduplex molecule in Fig. 1. The length C from the right end of the molecule to the beginning of

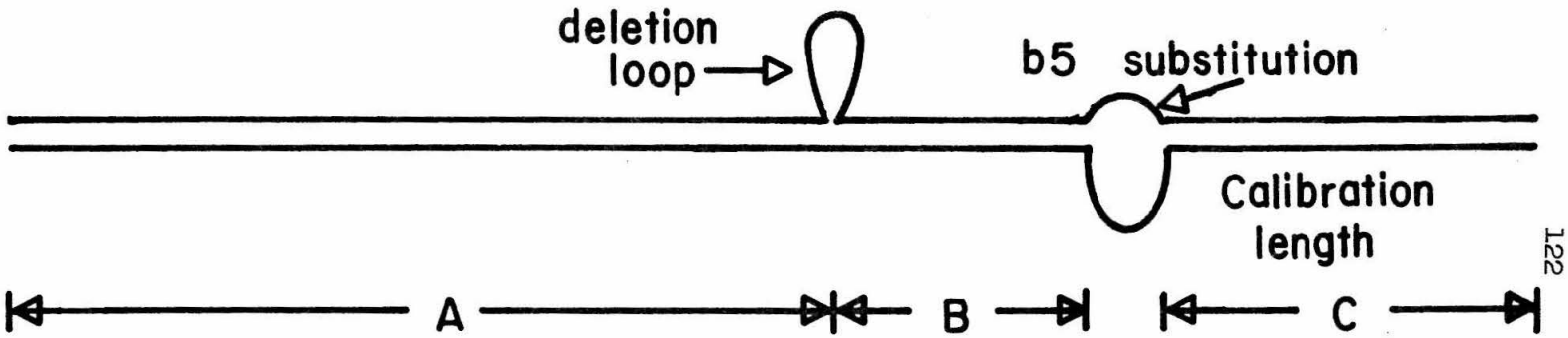
FIGURE LEGENDS

Fig. 1. A schematic heteroduplex molecule illustrating the technique of physical mapping with the aid of an internal calibration standard. A, B and C are the measured duplex DNA lengths from the left end of the molecule to the deletion bush (A); from the deletion bush to the b5 marker substitution (B); and from the b5 substitution to the right end of the molecule (C). The fractional lengths of A and B are obtained by using length C as a standard distance, which for the b5 substitution was determined to be equivalent to 0.206 λ units. Thus,

a = fractional length of A = A/C (.206)

b = fractional length of B = B/C (.206)

The fractional position of the left deletion endpoint (x_1) is simply equal to a. The fractional position of the right deletion endpoint (x_2) is equal to 0.711, the left endpoint of the b5 substitution, minus the fractional length b. The amount of DNA deleted is therefore $x_2 - x_1$.


deletion
loop
b5 substitution
Calibration
length
A
B
C

the b5 substitution provides the necessary calibration factor in terms of λ^{++} DNA for each molecule. The lengths A and B are converted to fractional lengths (a and b) by taking the ratio of A to C and of B to C and multiplying by the previously determined fractional length of C (designated as c). The left endpoint of the deletion (x_1) will equal a, the fractional length of A, which is the distance from the left end of the molecule to the beginning of the deletion. The right deletion endpoint (x_2) is equal to the fractional position of the beginning of the b5 substitution minus the fractional length b from the right end of the deletion to the b5 substitution. The size of the deletion is merely $x_2 - x_1$.

(iii) Calibration of reference markers

Several reference markers have been used in this work. We will describe here the methods used to calibrate the b5 marker which was most often employed. Other markers were either similarly calibrated or calibrated against b5.

The b5 substitution involves a deletion of the λ immunity region and a substitution of a different immunity region. The amount of λ^{++} DNA missing in this mutant was determined by measuring the single-stranded lengths in heteroduplexes of $\lambda b5/\lambda^{++}$ mounted with the formamide technique. It has been found that the length ratio of single- to double-stranded DNA is not always constant; therefore ØX174 DNA which is circular and single-stranded was included on these grids as a means of calibrating single-strand lengths. This DNA is a useful calibration standard because it is circular and the possibility of measuring single-strand fragments does not exist. The size of ØX DNA relative to λ^{++} DNA was determined by comparing the lengths of λ^{++} DNA to those of ØX174 RFII which is circular and

also double-stranded, both DNA's being mounted on the same specimen grid. It was found that the length ratio of double-stranded ØX174 DNA to that of duplex λ^{++} DNA was 0.112. Therefore the length ratio of single-stranded ØX DNA to a single strand of λ^{++} DNA should also be 0.112. In this way the b5 substitution proved to be missing 0.083 of the λ^{++} DNA which was replaced by 0.037 λ units of DNA with the immunity of phage 21. The net deletion, therefore, must be 0.083 - 0.037 = 0.046. This value is the same as that obtained by comparing the duplex lengths of b2b5 DNA with b2 DNA. Measured against λ^{++}, the b2 deletion is missing 0.121 and the b2b5 double deletion is missing 0.166 of the wild type DNA. Thus the net amount of DNA missing in the b5 deletion is 0.166 - 0.121 = 0.045, which compares quite well with the value above.

The position of the b5 substitution was determined by measuring 60 heteroduplexes of $\lambda b5/\lambda^{++}$ mounted by the aqueous method. The length from the right end of the heteroduplexes to the beginning of the b5 substitution was measured relative to the total length of the heteroduplex. We know that the total length must be 1.000 - 0.083 so that the fractional position of the right end of the b5 substitution can be computed by simply correcting for the size of the b5 substitution. This position proves to be 0.794 ($= x_2^{b5}$) and x_1^{b5} must be 0.711 (0.794 - 0.083).

The calibration values used in this work are 0.083 which is the fractional length of λ^{++} DNA missing in the b5 substitution and 0.206 which is the fractional distance from the right end of the λ molecule to the right end of the b5 substitution. The values obtained by Westmoreland, Szybalski & Ris (1969) of 0.090 and 0.202, respectively, although quite similar to ours, were not used because we were unable to obtain consistent mapping data with these values.

(b) Location and size of XOP

(i) Use of transducing λ phages to locate XOP

Prophage integration, according to the Campbell model (Campbell, 1962), takes place through a reciprocal recombination of the phage and bacterial chromosomes at their respective attachment sites. The act of integration thus forms two hybrid attachment sites, $\underline{att}^{L}$ and $\underline{att}^{R}$, which are located at the left and right end, respectively, of the inserted prophage DNA as shown in Figure 2. The locus at which $\underline{att}^{\varphi}$ is divided during integration behaves genetically as though it were a point and therefore is designated as the cross-over point (XOP) (Parkinson, 1970). It is quite possible, however, that $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ contain homologous regions at XOP so that integration cross-overs might really occur anywhere within this segment of homology. We will explore the question of homology in a later section; however, we can show by the experiments below that $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ probably contain only one cross-over point each.

As may be seen from Figure 2, the cross-over region in $\underline{att}^{\varphi}$ must lie at the junction between phage and bacterial DNA in the two hybrid attachment sites $\underline{att}^{L}$ and $\underline{att}^{R}$. Thus XOP can be mapped by examining heteroduplexes between λ^{++} and $\underline{att}^{L}$- or $\underline{att}^{R}$-containing phages. In order to recover $\underline{att}^{L}$ and $\underline{att}^{R}$ from the bacterial chromosome we have employed λ$\underline{dg}$ and λ$\underline{bio}$ transducing phages and several deletion mutants whose origins are diagrammed in Figure 3. The Campbell model specifies that transducing lambda mutants are produced by aberrant excision events in which the two excision break-points are thought to occur at random. If this is the case, then λ$\underline{dg}$ phages should contain $\underline{att}^{L}$ and λ$\underline{bio}$ phages should contain $\underline{att}^{R}$.

Fig. 2. The Campbell model of lysogeny. After entering the cell, the phage DNA circularizes by means of base pairing at the cohesive ends, pairs with the bacterial chromosome, and undergoes reciprocal recombination to insert the phage DNA into the bacterial chromosome. This recombination occurs between the att regions of the phage and bacterium which are different, to form the hybrid att sites located at the ends of the prophage.

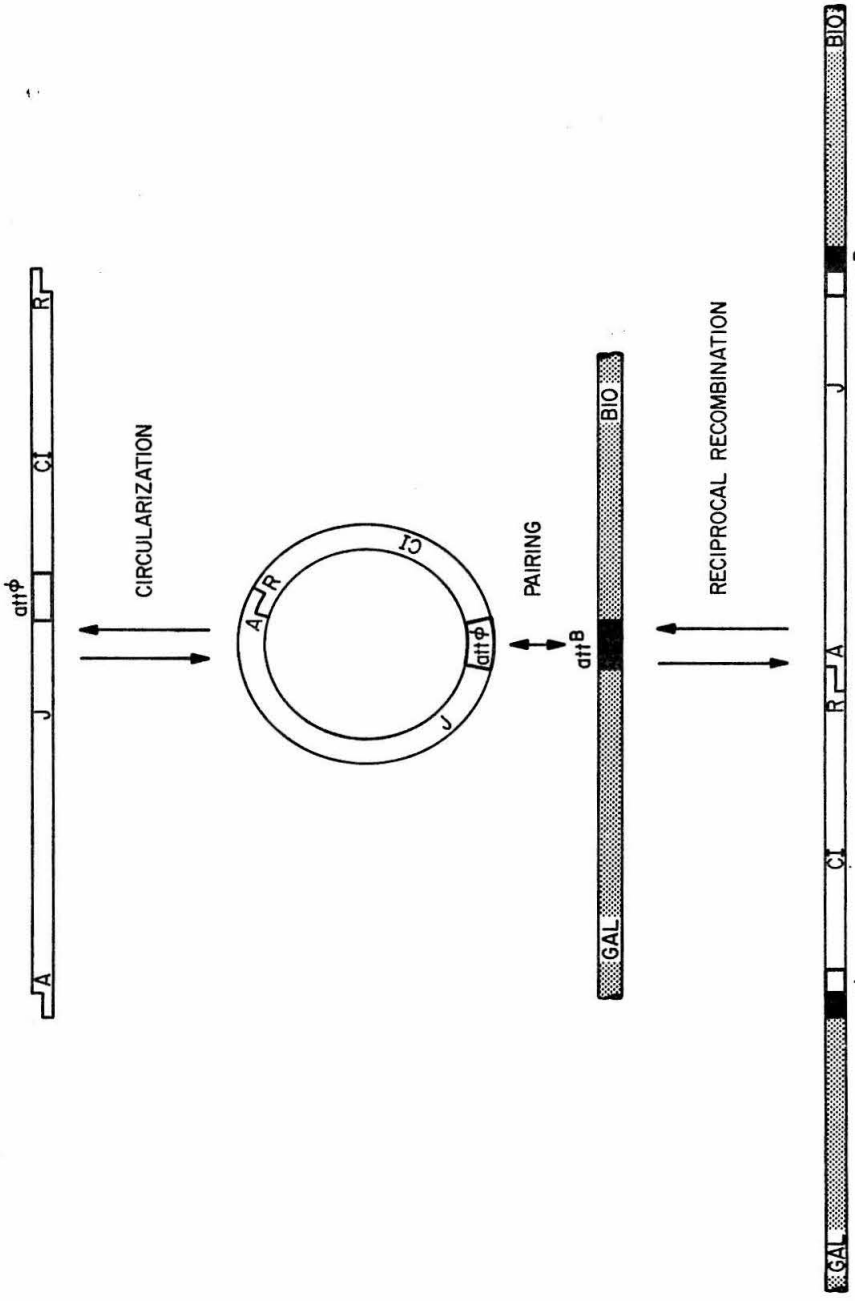
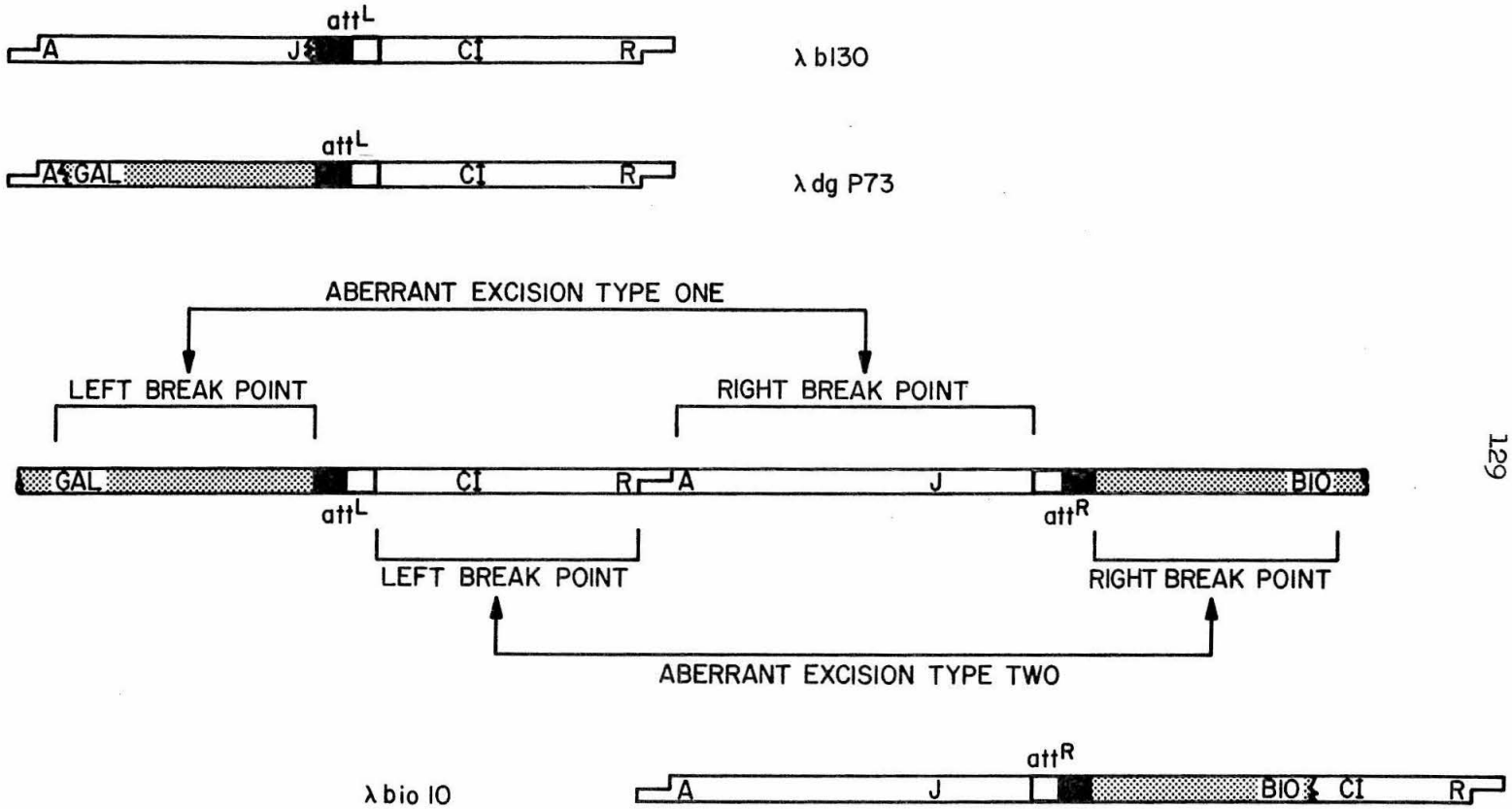

attΦ
A
J
Cl
R
CIRCULARIZATION
A
R
Cl
J
attΦ
PAIRING
attB
GAL
BIO
RECIPROCAL RECOMBINATION
GAL
attL
Cl
R
A
J
attR
BIO

Fig. 3. Formation of λ phages containing $\underline{att}^{L}$ or $\underline{att}^{R}$ by aberrant prophage excision. After induction, prophage excision normally occurs by recombination of $\underline{att}^{L}$ and $\underline{att}^{R}$ to release the phage genome. Campbell (1962) proposed that rare aberrant excisions can occur in which a portion of the prophage chromosome recombines with a portion of the bacterial chromosome to produce phages containing bacterial DNA. Type one excisions are here defined as those which incorporate $\underline{att}^{L}$ DNA into the excised phage genone. Two examples of such phages are λ<u>dg</u> transducing mutants and deletion mutants, such as λb130, which contain $\underline{att}^{L}$, but which are not defective because no essential phage genes were lost during excision. Type two excisions produce $\underline{att}^{R}$-containing phages, exemplified by λ<u>bio</u> transducing mutants.


att^L
A
J
CI
R
λ b130
att^L
A
GAL
CI
R
λ dg P73
ABERRANT EXCISION TYPE ONE
LEFT BREAK POINT
RIGHT BREAK POINT
GAL
CI
R
A
J
BIO
att^L
att^R
LEFT BREAK POINT
RIGHT BREAK POINT
ABERRANT EXCISION TYPE TWO
λ bio 10
att^R
A
J
BIO
CI
R

Heteroduplex molecules of λdg/λb5 and λdg/λ^{++} are shown in Plate I. The large region of nonhomology in these molecules is due to the replacement of a region of phage DNA by a region of DNA containing the galactose genes from the bacterial chromosome. As expected, this region of nonhomology is in the left half of the chromosome. The right terminus of the λdg substitution should therefore correspond to the first sequence of bacterial DNA in att^L which is not homologous to the corresponding phage DNA in att^{φ}. Thus the left-hand limit of the position of XOP is defined by the right end point of λdg substitutions. In a similar manner, the left terminus of λbio substitutions should correspond to the right-hand limit of the position of XOP.

The results given in Table 3 and summarized in Figure 4 indicate that λdg and λbio substitutions have a common end point located at 0.574 fractional length from the left end of the lambda genome. The deletion mutants, λb130 and λb189, which are shown below to contain att^L also begin at 0.574. This end point is presumably equivalent to XOP. Since the error in these measurements corresponds to a length of no more than about 100 nucleotides, it appears most likely that XOP is in fact a fixed point or at most a region 100 nucleotides in length. The fact that λdg and λbio substitutions have a common end point at 0.574 indicates that the bacterial DNA in att^L and att^R is not homologous to more than 100 base pairs of the DNA in att^{φ}. Therefore, any homology between att^{φ} and att^B at XOP must be quite small. We will return to this problem again in a later section.

Although the data of Table 3 show that there is probably only one position in att^{φ} at which integration can take place, it remains to be demonstrated that att^B also has only one XOP. If, for example,

PLATE CAPTIONS

Plate I λdg/λ heteroduplexes

a) Heteroduplex of λdgP73/λb5 mounted by the aqueous method. The arrows mark the ends of the dg bush and of the b5 marker bush. The integration cross-over point (XOP) is defined by the right terminus of the dg substitution.

b) Heteroduplex of λdgP74/λ^{++} mounted by the formamide method. The right end of the molecule is at the right of the picture and the arrows mark the endpoints of the dg substitution. This micrograph demonstrates that dg bushes such as the one in (a) above are composed of two non-complementary single strands, one containing λ^{++} DNA, the other containing bacterial DNA.

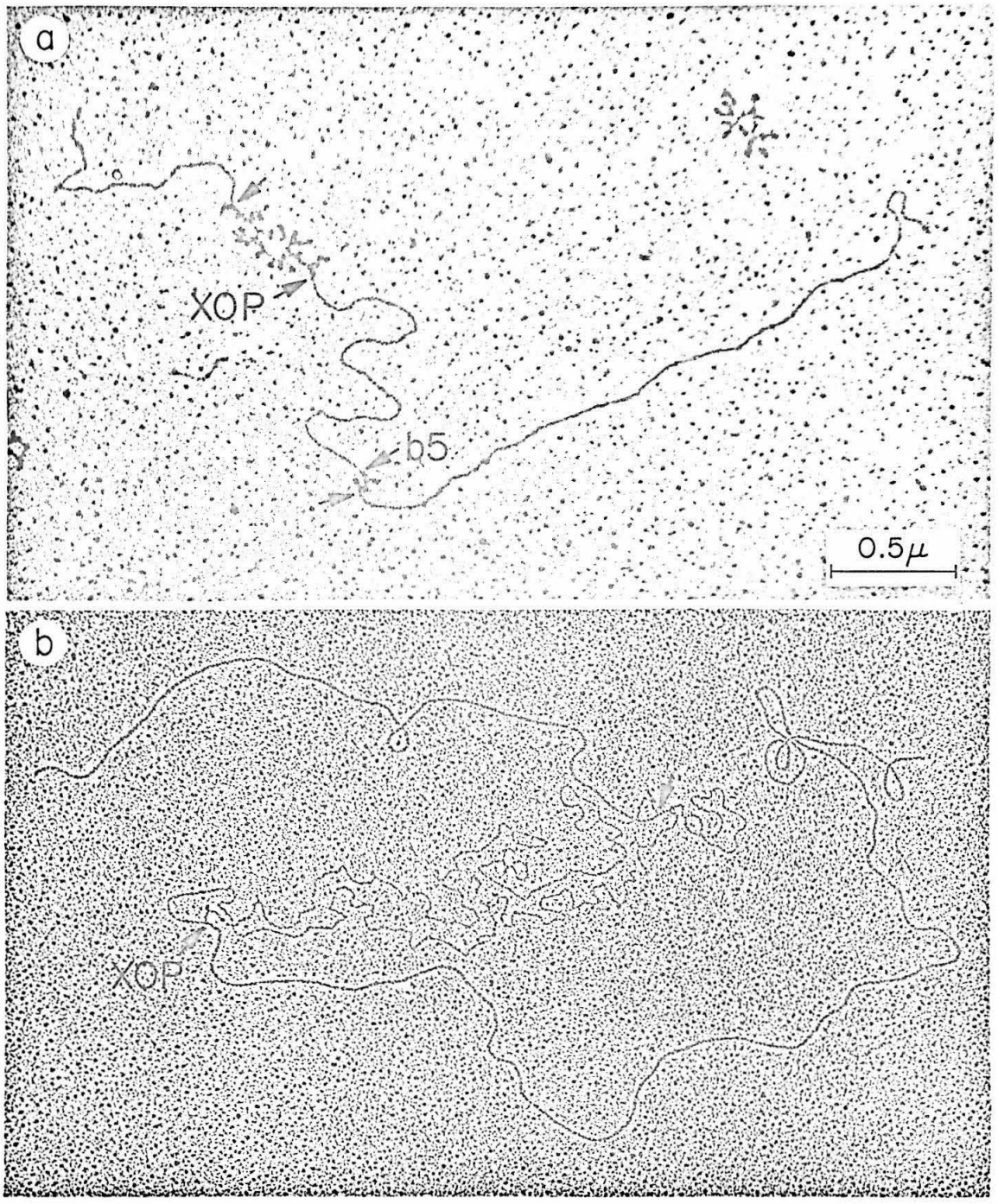

a
XOP
b5
0.5μ
b
XOP

TABLE 3

Substitution Endpoints in $\underline{att}^{L}$ and $\underline{att}^{R}$ Phages

$\underline{att}^{L}$ phages	marker strand	number of molecules measured	Physical Position[1] x_1	error	x_2	error	% λ DNA deleted
λ dg P72	b5	14	.225	±.004	.571	±.003	34.6
λ dg P74	b5	16	.193	±.003	.576	±.002	38.3
λ dg P74[2]	λ^{++}	22	.191	±.001	----	-----	38.5
λ dg P73	b5	18	.094	±.002	.575	±.002	48.1
λ dg P71	b5	16	.030	±.001	.576	±.002	54.6
λ b130[3]	b5	17	.423	±.006	.572	±.002	14.9
λ b189[3]	b5	20	.397	±.005	.572	±.002	17.5
SUMMARY		101	Variable		.574	±.001	

$\underline{att}^{R}$ phages	marker strand	number of molecules measured	Physical Position[1] x_1	error	x_2	error	% λ DNA deleted
λbio16A	b536	18	.574	±.002	.585	±.004	1.1
λbio7-20	b536	20	.573	±.002	.625	±.004	5.2
λbio69	b536	24	.573	±.002	.663	±.003	9.1
λbio11	b536	17	.574	±.002	.677	±.004	10.3
λbio10	b536	20	.574	±.002	.709	±.003	13.5
SUMMARY		99	.574	±.001	Variable		

TABLE 3 (Cont'd)

(1) The physical positions of the left (x_1) and right (x_2) substitution endpoints were calculated by the procedure described in the legend of Figure 1. The error values indicate the interval which contains the mean of the population from which this sample was drawn at the 95% confidence level. The error was calculated from the equation:

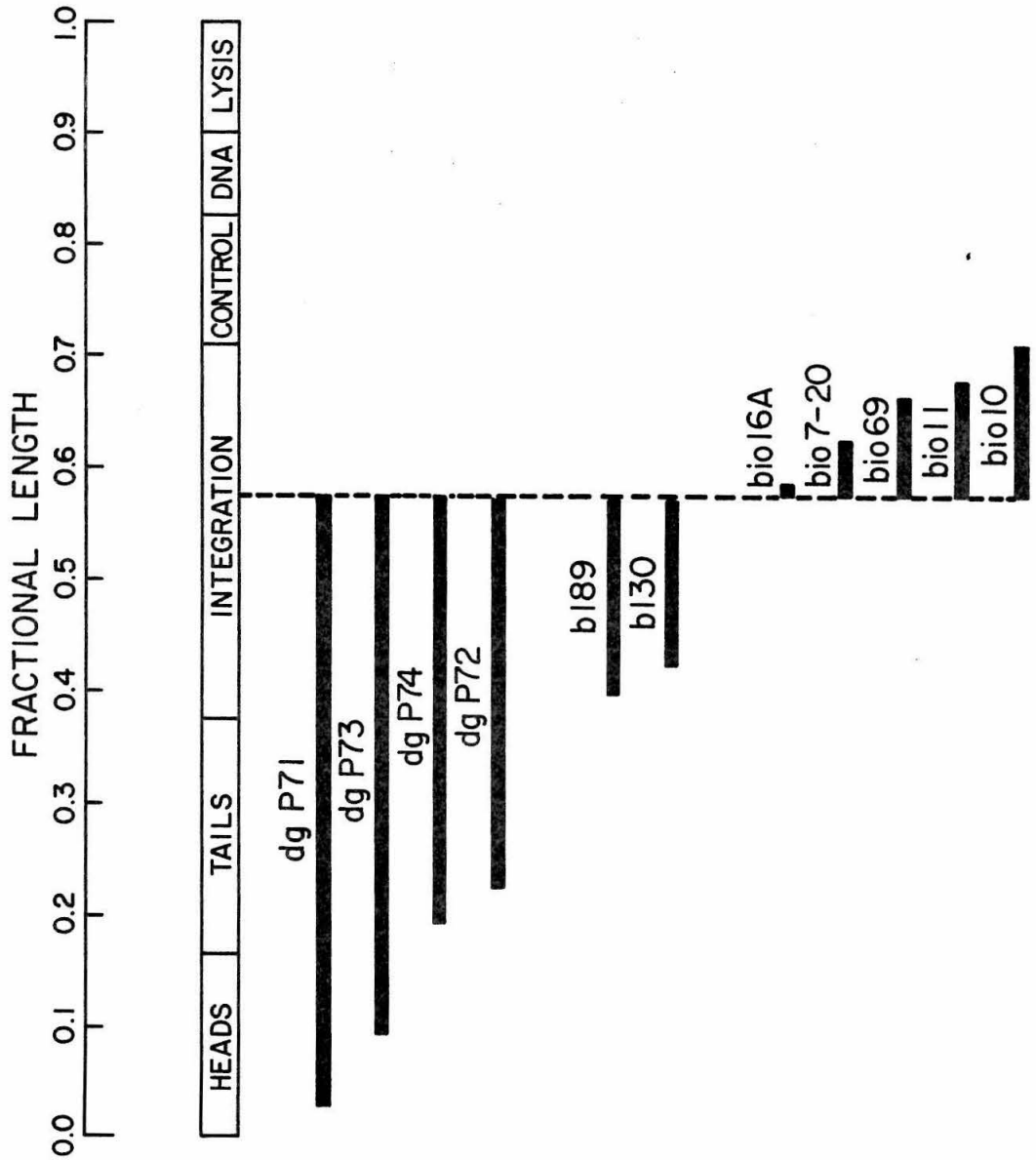
$$\text{error} = 2L_x \sqrt{1/n\,(1/L_x + 1/L_c)}$$

where L_x is the average length of the duplex DNA segment measured for locating a substitution endpoint; L_c is the average length of the calibrating DNA; and n is the number of measurements. This error analysis is described in more detail by Davis, et al. (1969).

(2) The left endpoint of this mutant was calculated by assuming that the substitution began at 0.574 and using the length from 0.574 to the right end as a calibration distance. This provides a check on the accuracy of the method used for the other heteroduplexes.

(3) The deletion mutants λb130 and λb189 appear to contain $\underline{att}^L$ by genetic evidence (Parkinson, 1970). In a later section, λb130 is shown to contain $\underline{att}^L$ by physical evidence as well.

Fig. 4. Substitution endpoints of phages containing $\underline{att}^{L}$ or $\underline{att}^{R}$. The physical locations of the substitutions in a number of phage mutants produced by aberrant excision are shown. The mapping data were taken from Table 3. All of these mutants have a common endpoint at 0.574 in the λ chromosome which is presumed to be the location of XOP. The model which describes the formation of such phage (Figure 3) predicts that one endpoint of these substitutions should not be fixed, and, in fact, this proves to be the case as shown.


FRACTIONAL LENGTH
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
HEADS
TAILS
INTEGRATION
CONTROL
DNA
LYSIS
dg P71
dg P73
dg P74
dg P72
b189
b130
bio16A
bio7-20
bio69
bio11
bio10

$\underline{att}^{B}$ contained two cross-over points separated by more than about 50 nucleotide pairs, we could detect this with the following experiment. A pair of λ$\underline{dg}$ or λ$\underline{bio}$ phages in which different bacterial cross-over points were used for prophage integration would form heteroduplexes containing a region of nonhomology at 0.574 due to the presence of different sequences of bacterial DNA adjacent to the phage DNA in $\underline{att}^{L}$ and $\underline{att}^{R}$. A number of λ$\underline{dg}$/λ$\underline{dg}$ and λ$\underline{bio}$/λ$\underline{bio}$ heteroduplex DNA's have been constructed between independently isolated λ$\underline{dg}$ and λ$\underline{bio}$ phages; however, no evidence has been found which suggests that $\underline{att}^{B}$ contains more than one XOP. Some typical examples of such heteroduplex molecules are shown in Plate II.

(ii) Use of λ deletion mutants to locate XOP

Parkinson (1970) has described several types of deletion mutants of lambda which can be used to locate XOP independently of the transducing phage method. For example, genetic mapping shows that class 2A deletions lie to the left of XOP in $\underline{att}^{\varphi}$, whereas class 3A deletions lie to the right of XOP. Therefore XOP must lie between the right end of 2A deletions and the left end of 3A deletions. When these deletions were mapped by the heteroduplex method (see Plate III), the data in Table 4 were obtained. Both the right termini of all 2A deletions and the left termini of 3A deletions map at 0.574 which is the same position found from the mapping of λ$\underline{dg}$ and λ$\underline{bio}$ substitutions. Thus XOP again is found to be located at 0.574 in the lambda chromosome.

The data of Table 4 are quite surprising in that all 2A and 3A deletions seem to begin just at XOP. Apparently these deletions have not been produced by a random process and in fact appear to be related in some way to the integration system since they have a

Plate II λdgA/λdgB and λbioA/λbioB heteroduplexes

a) Heteroduplex of λbio16A/λbio69 mounted by the formamide method and shadowed with Pt-Pd. The non-homologous region in this molecule is due to different amounts of phage and bacterial DNA's in the two λbio's. There is no detectable non-homology at XOP.

b) Heteroduplex of λbio10/λbio16A mounted as in (a) above.

c) Dark field electron micrograph of a λbio16A/λbio69 heteroduplex stained with uranyl acetate and mounted by the formamide technique. Only the region around XOP (arrow) is shown.

d) Heteroduplex of λdgP72/λdgP73 mounted as in (a) above. The segment around XOP contains no detectable non-homologous regions. Note the proximity of the molecular ends; this molecule is probably a pulled-apart circle (see Table 8).

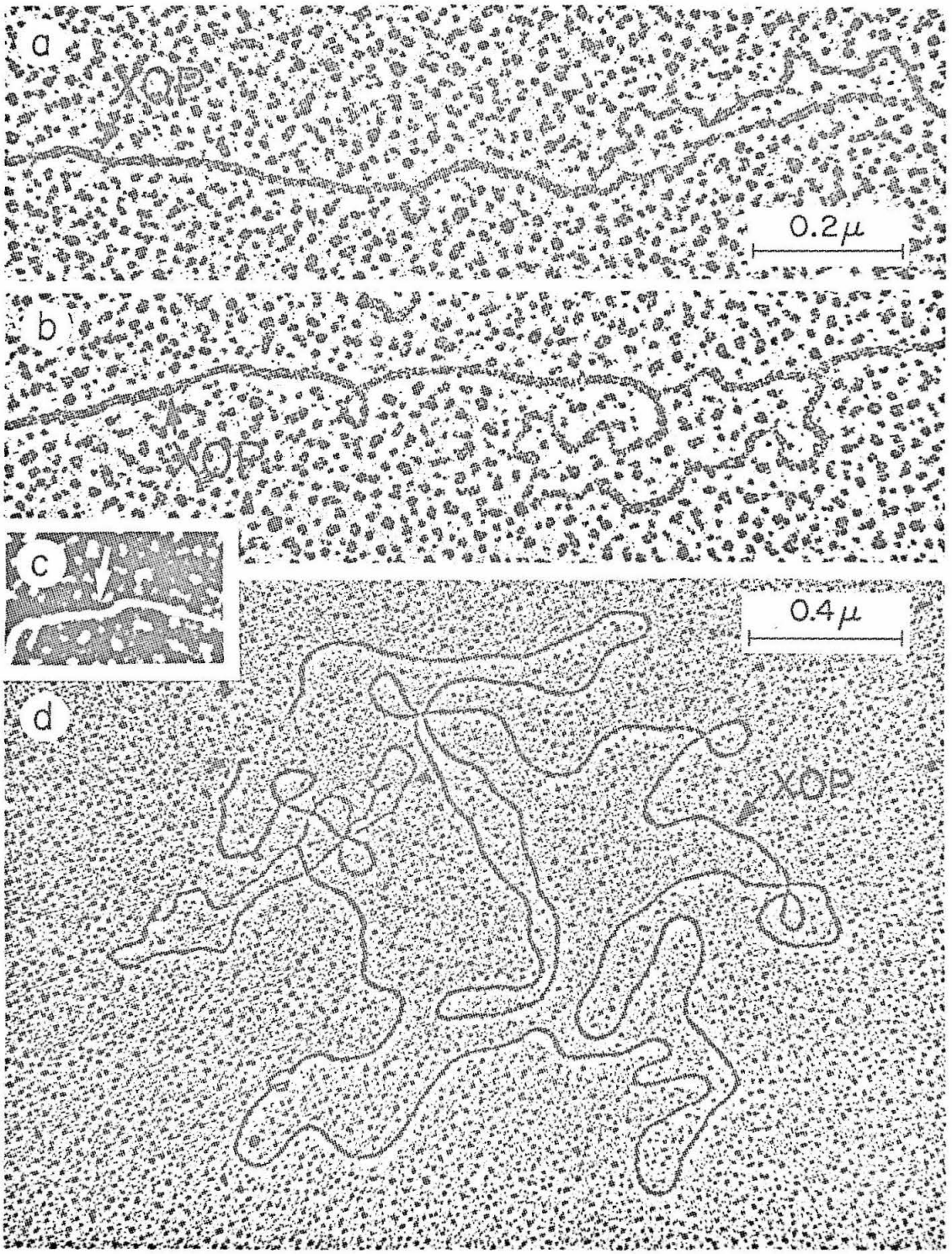

a
XOP
0.2μ
b
XOP
c
0.4μ
d
XOP

Plate III Physical mapping of deletion mutants

a) Heteroduplex of λb517/λb5 mounted by the aqueous technique. The arrows mark the position of the deletion bush and the ends of the b5 substitution bush used as a marker.

b) An enlarged view of a b5 substitution seen by the formamide mounting procedure. The arrows mark the ends of the substitution region.

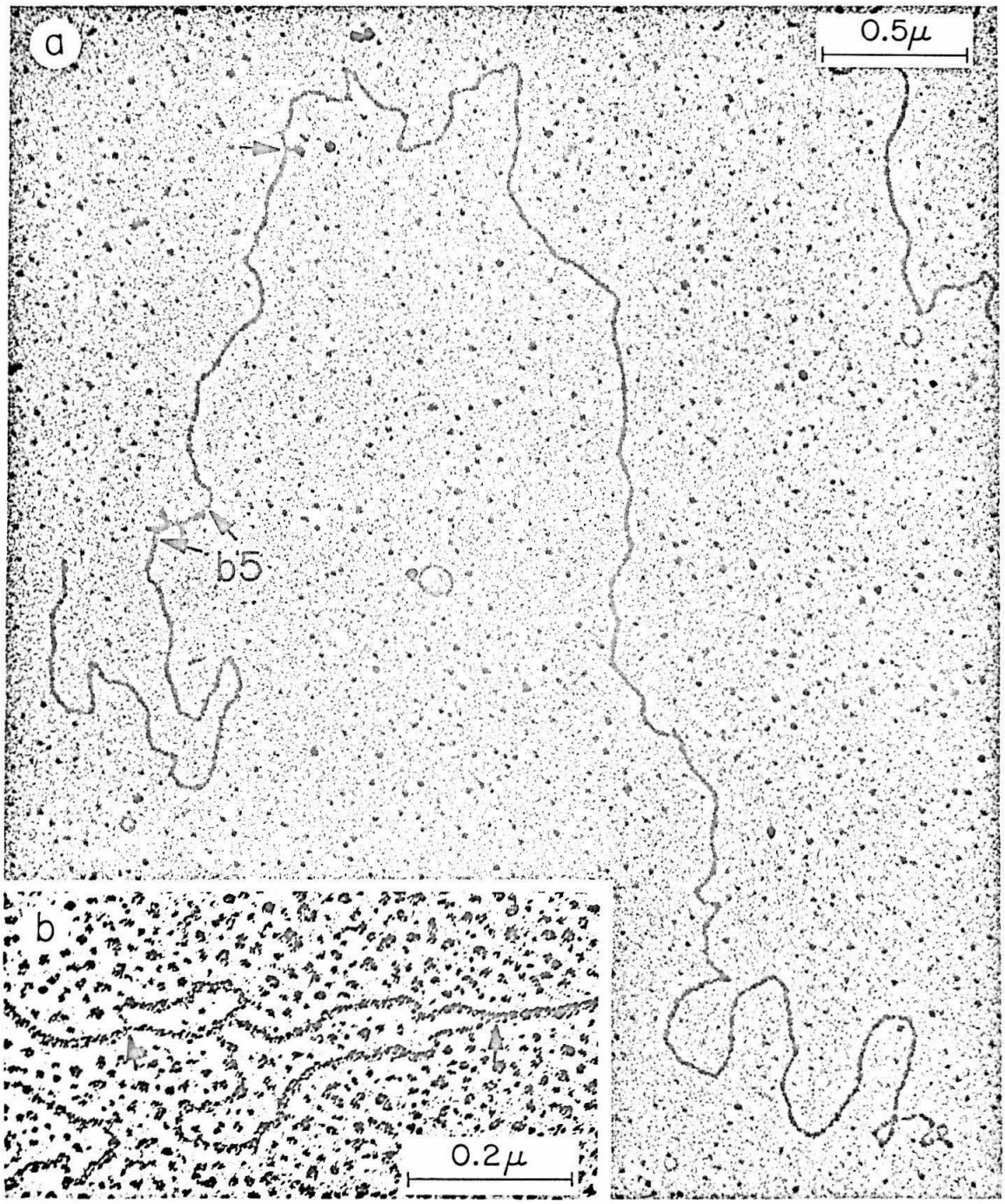

a
0.5μ
b5
b
0.2μ

TABLE 4

Deletion Endpoints of Class 2A and 3A Deletion Mutants

Class 2A mutants	marker strand	number of molecules measured	Physical Position x_1	error	x_2	error	% DNA deleted
b2(1)	λ^{++}	50	.453	±.001	.574	±.001	12.1
b2	b5	26	.454	±.005	.573	±.002	11.9
b511	b5	12	.486	±.007	.575	±.003	8.9
b516	b5	9	.473	±.008	.575	±.003	10.2
b527	b5	16	.491	±.006	.574	±.002	8.3
b540	b5	20	.451	±.005	.574	±.002	12.3
SUMMARY		83	Variable		.574	±.001	

Class 3A mutants	marker strand	number of molecules measured	Physical Position x_1	error	x_2	error	% DNA deleted
b508	b5	14	.584	±.008	.613	±.002	2.9
b512	b5	18	.570	±.007	.614	±.002	4.4
b514	b5	17	.574	±.007	.614	±.002	4.0
b517	b5	20	.574	±.007	.616	±.002	4.2
Average		69	.575	±.004	.614	±.001	3.9
b508	b536	20	.573	±.000	.610	±.005	3.7
b512	b536	15	.575	±.001	.614	±.006	3.9
Average		35	.574	±.001	.612	±.003	3.8
b522	b5	18	.572	±.007	.643	±.001	7.1
SUMMARY		122	.574	±.001	Variable		

See the footnote of Table 3. Mutants b508, 512, 514, 517 appear to be identical. These deletions are not independent isolates (Parkinson and Huskey, 1970).

(1) Data of Davis and Davidson (1968).

common end point at XOP which is presumed to be the substrate for integrase action. To test this notion spontaneous deletion mutants were isolated from int$^+$ and int$^-$ phages. Each deletion preparation was then tested for the presence of 2A deletion types by a simple spot test procedure to determine whether a functional Int gene was essential for 2A deletion formation. The data in Table 5 indicate that this is the case. In both int$^-$ preparations no class 2A deletion mutants were found, whereas the int$^+$ preparations each contained a large proportion of 2A deletion mutants. Since no spot tests are available to distinguish 3A mutants from 3B mutants, it is not possible to conclude that 3A deletions are also dependent on the Int product for their formation. This seems likely, however, since 3A deletions as well as 2A deletions begin at XOP.

Both transducing phages and deletion mutants locate XOP in att$^{\varphi}$ at 0.574 from the left end of the chromosome. A further check on this position is provided by class 3B deletion mutants which appear to lack XOP entirely (Parkinson, 1970). Mapping data for three of these deletions are presented in Table 6 and are consistent with the previous results. All of these 3B deletions are quite large and each spans the region of 0.574 where XOP is located.

(c) Properties of REL and RER

The existence of "recognition elements" on each side of XOP in att$^{\varphi}$ has been demonstrated with att-defective deletion and substitution mutants. Class 2A deletions, for example, appear to be REL deletions since they map to the left of XOP and yet have an integration defect (Parkinson, 1970). Similarly 3A deletions are missing RER, a region to the right of XOP which is essential for normal integration

TABLE 5

Dependence of 2A Deletion Formation on Int Function

phage	host strain	frequency of deletion mutants in original stock	number of deletions examined	fraction of deletions that were: class 1	class 2	class 3
int$^+$	QR93	6.0×10^{-7}	131	0.44	0.22	0.34
int29	QR93	2.8×10^{-7}	154	0.47	0.00	0.53
int$^+$	QR94	1.2×10^{-6}	156	0.47	0.24	0.29
int29	QR94	8.6×10^{-7}	156	0.86	0.00	0.14

Stocks of λ^{++} and λ int29 were grown on QR93 or QR94 by the confluent lysis method and then carried through two cycles of deletion selection with pyrophosphate inactivation as described by Parkinson and Huskey (1970). Individual plaques from the resultant deletion preparations were stabbed onto lawns of C600 and SA205, and after overnight incubation at 37°C, the turbid centers from these spots were transferred with sterile toothpicks to EMB-glucose plates spread with $\sim 10^9$ of a clear plaque mutant to test for stable lysogeny. Class 1 mutants form stable lysogens on both strains, class 3 deletions cannot stably lysogenize either strain, and class 2A mutants integrate in SA205 but not in C600 (Parkinson, 1970).

TABLE 6

Location of XOP by Class 3B Deletion Mutants

Class 3B mutants	marker strand	number of molecules measured	Physical Position x_1	error	x_2	error	% DNA deleted
b221[1]	λ^{++}	33	.406	±.003	.629	±.003	22.3
b531	b5	13	.432	±.006	.605	±.002	17.3
b538	b5	13	.436	±.006	.599	±.002	16.3

See the footnote of Table 3. Mutants b531 and b538 are probably identical.

(1) Data of Davis and Davidson (1968).

behavior. From the physical positions of these and other types of deletions and the principles of deletion mapping we can locate REL and RER in the lambda chromosome and make an estimate as to their size.

By definition REL must be within the deleted region which is common to all class 2A mutants. However, class 1 deletion mutants also map to the left of XOP but are not REL-defective. Therefore REL must also lie outside of the region defined by class 1 deletions. Mapping data for a total of five different class 1 deletion mutants are presented in Table 7. These deletions all lie between 0.404 and 0.538 in the chromosome so REL must not be anywhere between these positions. Similar data for class 2A deletions have been presented in Table 4 which shows that the region between 0.491 and XOP at 0.574 is deleted in all 2A mutants that were studied. Since REL cannot lie to the left of 0.538 because of the class 1 deletions, REL must be between 0.538 and 0.574 in the chromosome. This region is approximately 2000 nucleotide pairs in length.

The problem of locating RER is more difficult because we do not yet have a class of deletion mutants on the right of XOP which are not RER-defective. Nevertheless, RER must lie somewhere between XOP at 0.574 and 0.613 which is the common region missing in both of the 3A deletions described in Table 4. This region is also about 2000 base pairs long. In Figure 5 we have summarized the use of deletion mutants to map the various components of $\underline{att}^{\varphi}$. In the Discussion section we will return to the problem of estimating the position and size of REL and RER.

TABLE 7

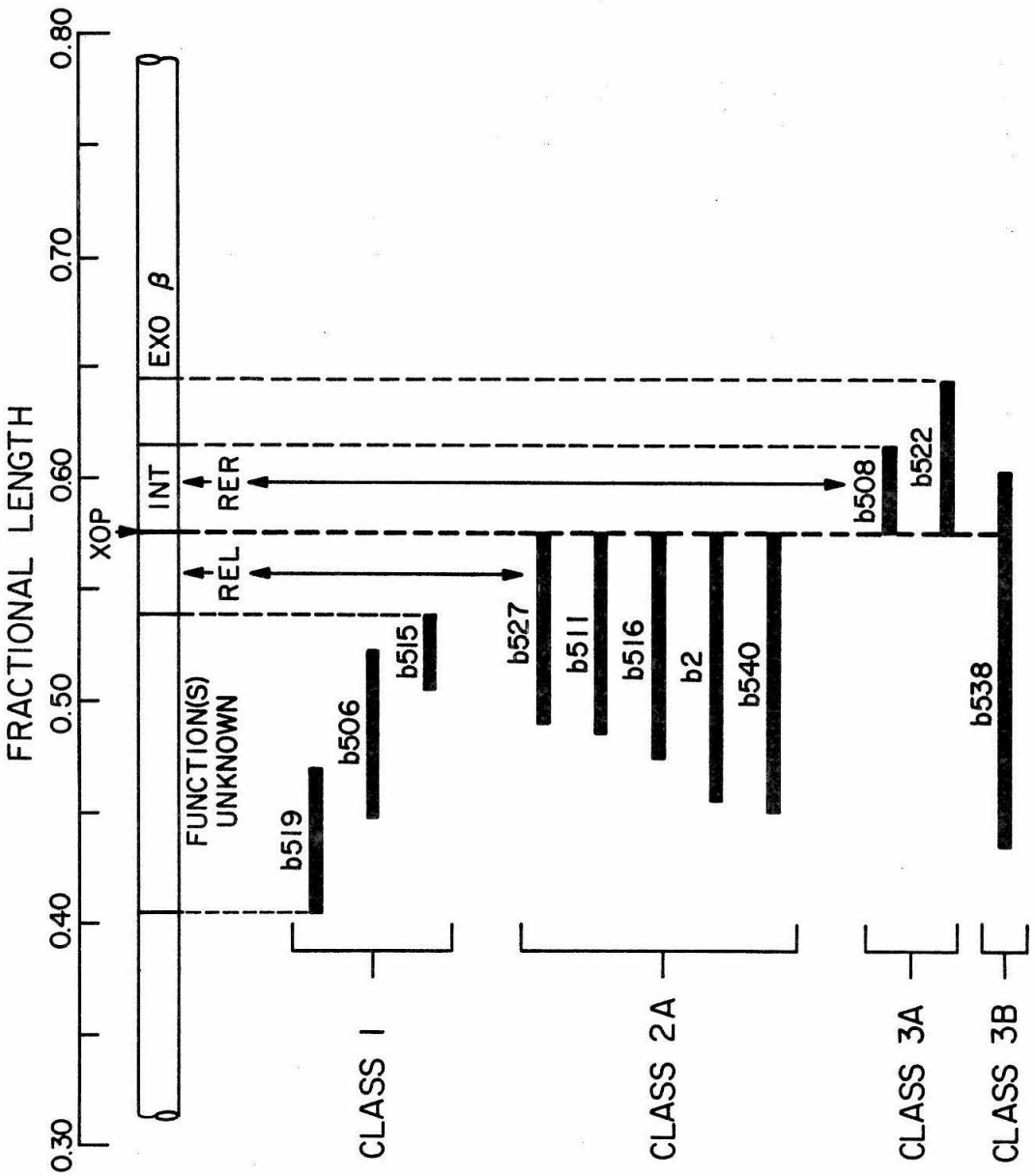
Physical Position of Class 1 Deletions

Class 1 mutants	marker strand	number of molecules measured	Physical Position x_1	error	x_2	error	% DNA deleted
b501	b5	12	.465	±.007	.499	±.004	3.4
b502	b5	12	.465	±.007	.503	±.004	3.8
b504	b5	13	.467	±.007	.505	±.004	3.8
b509	b5	11	.470	±.007	.499	±.004	2.9
Average		48	.467	±.004	.502	±.002	3.5
b506	b5	10	.447	±.008	.522	±.004	7.5
b510	b5	14	.399	±.006	.470	±.004	7.1
b519	b5	13	.407	±.006	.470	±.004	6.3
b520	b5	14	.405	±.006	.470	±.004	6.5
Average		41	.404	±.003	.470	±.002	6.6
b515	b5	11	.504	±.008	.538	±.003	3.4
b536	b5	20	.428	±.003	.468	±.003	4.0
b536(1)	λ^{++}	19	.426	±.003	.463	±.003	3.7

See the footnote of Table 3. Mutants b501, 502, 504, 509 appear to be identical as do b510, 519, 520. These mutants are not independent isolates (Parkinson and Huskey, 1970).

(1) These values were calculated by the procedure described by Davis and Davidson (1968), and provide a check on the heteroduplex calculations for the other mutants in this table.

Fig. 5. A deletion map of the center of the λ chromosome. This map, constructed from the data of Tables 4, 6 and 7, summarizes the use of deletion mutants to study the structure of att$^{\varphi}$ and the surrounding portions of the λ genome. The class 1 deletions which have no known defects (Parkinson, 1970) define a "silent region" between 0.403 and 0.538 in the chromosome. Class 2A deletions are REL-defective and thus REL must lie between XOP and 0.538. Class 3A deletions which are RER-defective, locate RER between XOP and 0.614. The Int gene must also lie in this interval since 3A mutants are int^{-}. Class 3B deletions such as b538 appear to be missing the entire att region.


FRACTIONAL LENGTH
0.30
0.40
0.50
0.60
0.70
0.80
EXO β
INT
XOP
RER
REL
FUNCTION(S) UNKNOWN
b519
b506
b515
b527
b511
b516
b2
b540
b508
b522
b538
CLASS 1
CLASS 2A
CLASS 3A
CLASS 3B

(d) A search for $\underline{att}^{\varphi}$ - $\underline{att}^{B}$ homology

(i) Class 2B deletions contain $\underline{att}^{L}$

Parkinson (1970) has demonstrated that class 2B deletion mutants behave genetically as though they carried $\underline{att}^{L}$. We examined two of these deletion mutants (λb130, λb189) to see whether this notion can be supported by physical evidence. The location of the non-homology between these mutants and λ wild type (Table 3) begins at XOP and extends to the left, which is consistent with both λ$\underline{dg}$ phages containing $\underline{att}^{L}$, and with simple deletions like the 2A mutants. To prove that λb130 and λb189 are not simple deletion mutants, heteroduplexes were constructed between these mutants and λ wild type and mounted for electron microscopy by the formamide procedure (Plate IVa, b). Plate IVa clearly shows that the non-homology in λb130/λ^{++} is due to a substitution in the λb130 DNA; however, the λb189/λ^{++} molecule in Plate IVb appears to contain a deletion loop only, with no detectable substitution in the λb189 strand.

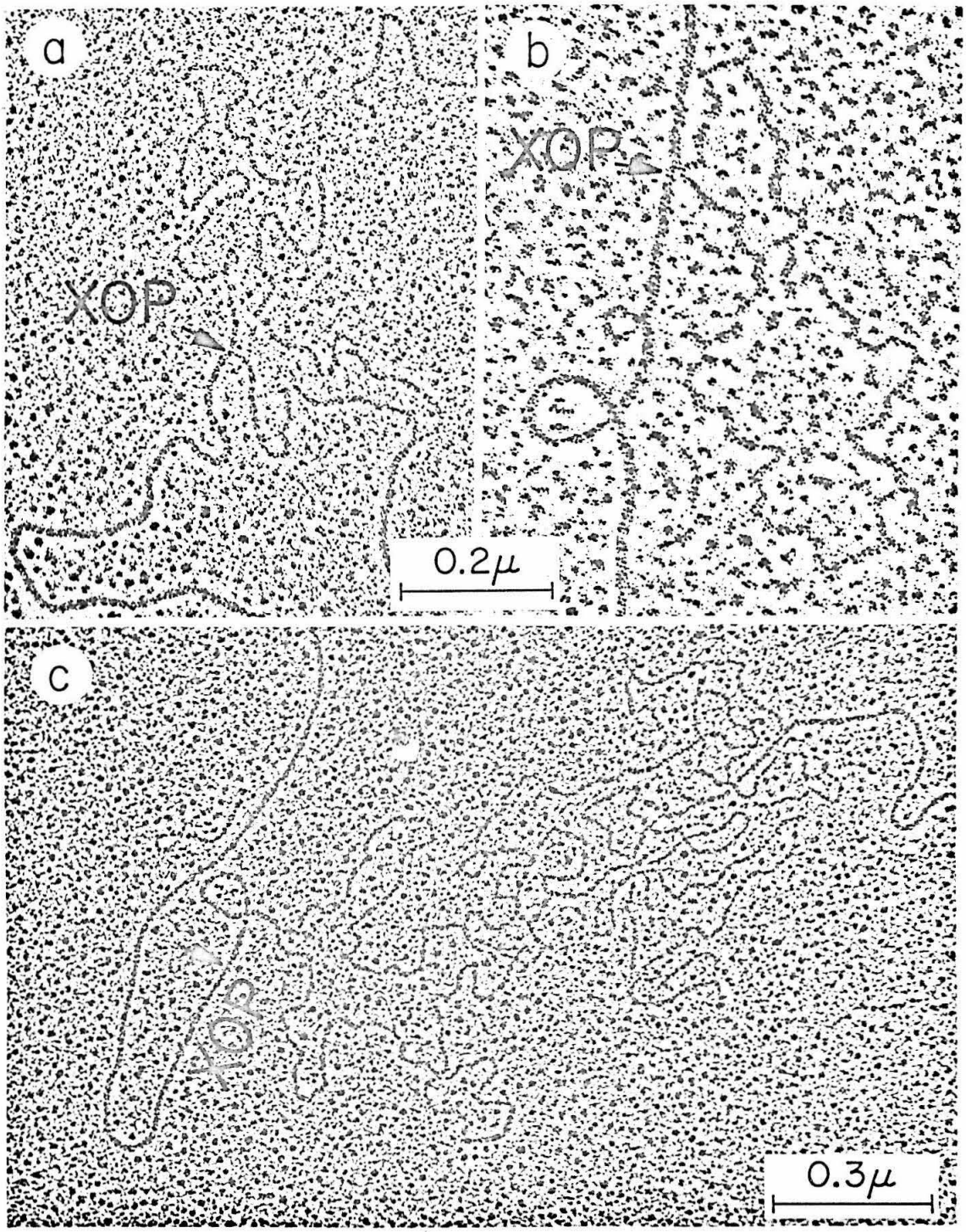
The size of the substitution in λb130 is 0.042 ± .001 λ unit. This DNA appears to be identical to the bacterial DNA at $\underline{att}^{L}$ in λ$\underline{dg}$ phages as indicated in Plate IVc which shows a λb130/λ$\underline{dg}$ heteroduplex molecule. Whereas the non-homology in λb130/λ^{++} molecules begins at XOP, the λb130/λ$\underline{dg}$ molecules contain a duplex segment of 0.042 λ unit in length adjacent and to the left of XOP. Thus all of the substituted region in λb130 is homologous to the corresponding DNA at $\underline{att}^{L}$ in the λ$\underline{dg}$ phages. Although we cannot detect any bacterial DNA in λb189, the similarity in integration properties of λb189 and λb130 (Parkinson, 1970) supports the supposition that this mutant probably contains a small amount of bacterial DNA.

Plate IV Heteroduplex molecules containing class 2B deletions

a) Heteroduplex of λb130/λ^{++} mounted by the formamide method. Only the region around XOP is shown to demonstrate that λb130 contains a small substitution rather than a simple deletion.

b) Heteroduplex of λb189/λ^{++} mounted by the formamide method. The non-homology at XOP appears to be a simple deletion.

c) Heteroduplex of λb130/λdg mounted with the formamide method. A portion of λb130 DNA to the left of XOP (arrow) is homologous to the corresponding DNA from λdg. The length of λb130 DNA in (a) above that is not homologous to λ^{++} DNA is completely homologous to λdg DNA.


a
XOP
b
XOP
0.2μ
c
XOP
0.3μ

(ii) $\underline{att}^{\varphi}$ and $\underline{att}^{B}$

It has been proposed that a portion of the phage and bacterial attachment sites have the same base sequence and that integration takes place by homologous crossing-over. We have already pointed out in section (b) above that there is little, if any, homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ in the vicinity of XOP. To test this notion more carefully and in a direct manner, we constructed a phage which contains $\underline{att}^{B}$ to search for homology in heteroduplexes of $\underline{att}^{B}/\underline{att}^{\varphi}$. The $\underline{att}^{B}$ phage was made by introducing into one chromosome the two halves of $\underline{att}^{B}$ from $\underline{att}^{L}$ and $\underline{att}^{R}$ by crossing λb130 ($\underline{att}^{L}$) by λ$\underline{bio}$69 ($\underline{att}^{R}$) and selecting the λb130$\underline{bio}$69 recombinant. The structure of this recombinant was checked by constructing the following heteroduplexes: λb130$\underline{bio}$69/λ^{++}; λb130$\underline{bio}$69/λ$\underline{bio}$16A; and λb130$\underline{bio}$69/λ$\underline{dg}$P72 (Plate Va, b, c). The structures of these heteroduplex molecules show that λb130$\underline{bio}$69 contains bacterial DNA on both sides of XOP and will be hereafter designated as $\underline{att}^{B}$. The genetic properties of this phage are consistent with those expected of $\underline{att}^{B}$ (Parkinson, 1970).

In a heteroduplex of λb130$\underline{bio}$69/λ^{++} we might be able to detect a small region of homology at XOP if, in fact, $\underline{att}^{B}$ and $\underline{att}^{\varphi}$ are partly homologous. However, a nucleation event is required in order to form a short duplex segment between regions of non-homology in a heteroduplex molecule. The possibility that such nucleation events cannot take place during our normal renaturation procedure was eliminated in all of the experiments described below by heating the renatured DNA in 0.1 M NaCl, 0.01 M EDTA, pH 7.3 for several hours at 45°C followed by slow cooling to 25°C.

Plate V Heteroduplex molecules containing $\underline{att}^{B}$ (λb130$\underline{bio}$69)

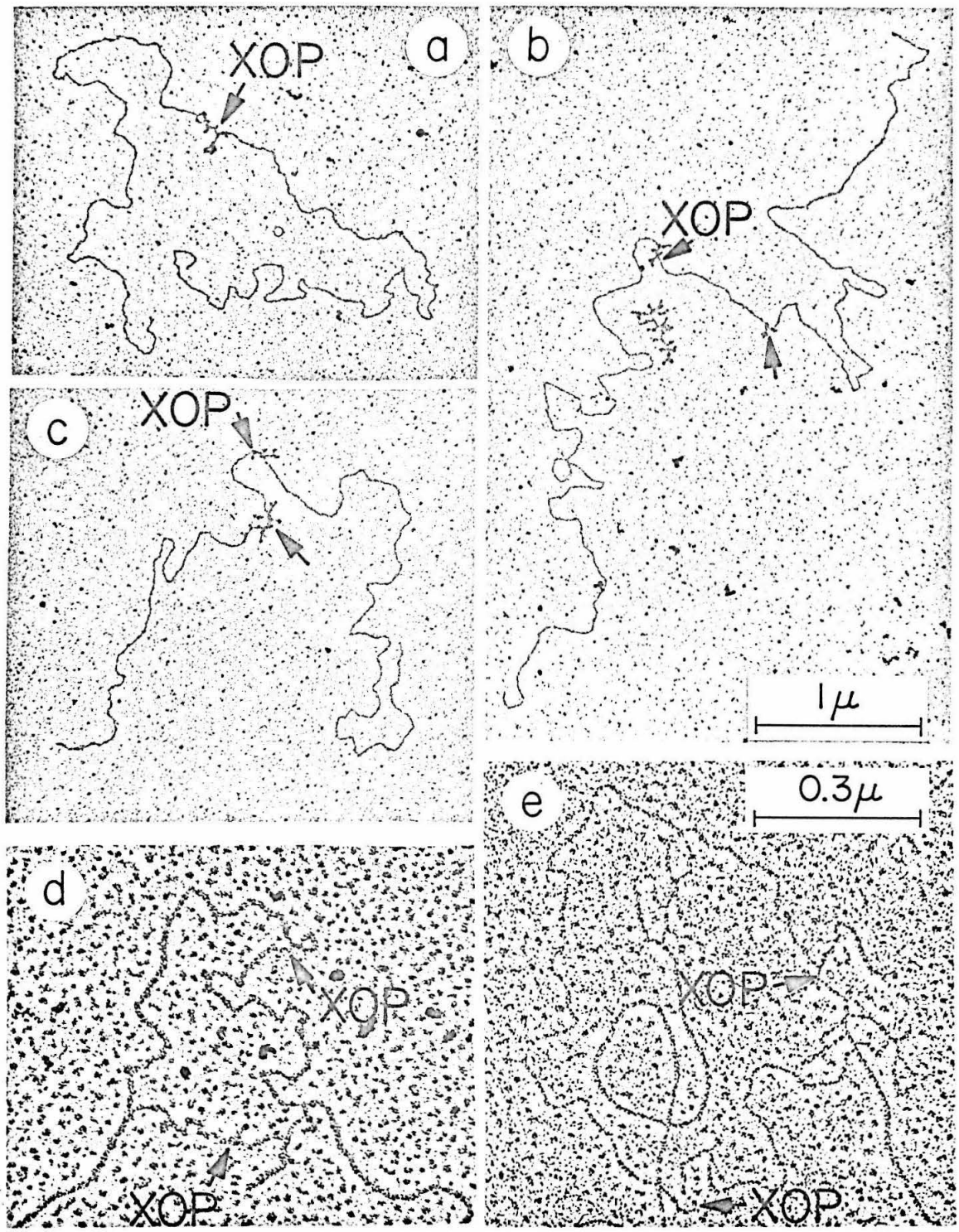
a) Heteroduplex of λb130$\underline{bio}$69 ($\underline{att}^{B}$)/λ^{++} ($\underline{att}^{\varphi}$) mounted by the aqueous method. The molecule contains one large bush at XOP with no detectable region of duplex DNA within the bush.

b) Heteroduplex of $\underline{att}^{B}/\underline{att}^{R}$ (λb130$\underline{bio}$69/$\lambda\underline{bio}$16A) mounted by the aqueous method. The bush adjacent to XOP corresponds to the λb130 deletion (Plate IVa). The other bush is due to the different substitutions in the two $\lambda\underline{bio}$'s (see Plate IIa). The region of homology between these two bushes demonstrates that λb130$\underline{bio}$69 contains $\underline{att}^{R}$ DNA.

c) Heteroduplex of $\underline{att}^{B}/\underline{att}^{L}$ (λb130$\underline{bio}$69/$\lambda\underline{dg}$P72) mounted with the aqueous technique. The region of non-homology adjacent to XOP is due to the $\lambda\underline{bio}$69 substitution; the other bush is from the different substitutions in λb130 and $\lambda\underline{dg}$ (see Plate IVc). The duplex DNA between XOP and the $\underline{dg}$ bush corresponds to the length of bacterial DNA in λb130 and proves that λb130$\underline{bio}$69 contains $\underline{att}^{L}$ DNA.

d) Enlargement of the non-homology region in a heteroduplex of λb130$\underline{bio}$69 ($\underline{att}^{B}$)/λb506 ($\underline{att}^{\varphi}$) mounted by the formamide procedure using condition (g) of Table 8. There is no detectable homology between $\underline{att}^{\varphi}$ (upper strand) and $\underline{att}^{B}$ (lower strand).

e) Same as (d) except mounted in formamide with condition (a) of Table 8. The different single-strand lengths in (d) and (e) are due to differences in formamide concentration and ionic strength.


a
XOP
b
XOP
1μ
c
XOP
d
XOP
XOP
e
0.3μ
XOP
XOP

In an attempt to detect homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$, heteroduplexes of λb130bio69/λ^{++} were prepared as described above and mounted for electron microscopy under a wide variety of formamide and salt concentrations (Table 8). These conditions cover the range from complete melting out of random base interactions to stability of such interactions resulting in the collapse of single-stranded DNA into a bush. Even with the most favorable conditions (Plate Va), we never detected a unique region of duplex DNA in the $\underline{att}^{B}/\underline{att}^{\varphi}$ heteroduplexes (see also Plate Vd and e).

(e) Physical detection of small regions of homology

A number of deletion mutants were selected from those described in Tables 4 and 7 and heteroduplexes were constructed between pairs of deletions which should not overlap. We then searched for a short duplex segment located between the two deletion loops in these heteroduplexes to determine how close together two deletions could be before we failed to detect a region of homology between the non-overlapping ends of the two deletions. Several molecules of this type are shown in Plate VI (d, e, f). Table 9 lists the heteroduplexes that were constructed and gives a comparison of the measured length of homology with the length expected from the previously determined positions of each deletion mutant. These values compare very well, which indicates that the presence of non-homologous segments on each side of a short homologous region does not seem to cause any denaturation of that duplex segment. The λb501/λb536 heteroduplex shown in Plate VI (d, e, f) contains only about 100 base pairs of homology between the deletions and yet is perfectly stable under all formamide conditions used. Thus we feel confident that regions of

TABLE 8

Stability of Base Pairing under Various Mounting Conditions

	Hyperphase concentration of:			Hypophase concentration of:			Presence of:		Relative stability of random base interaction
	Formamide	NH_4Ac	Tris	Formamide	NH_4Ac	Tris	stable circles	"pulled-apart" circles	
(a)	50%	0.1M	0.01M	10%	-----	0.01M	-	+	unstable
(b)	40%	0.1M	0.01M	10%	-----	0.01M	-	+	unstable
(c)	30%	0.1M	0.01M	10%	-----	0.01M	-	+	unstable
(d)	20%	0.1M	0.01M	10%	-----	0.01M	-	+	partially stable
(e)	10%	0.1M	0.01M	10%	-----	0.01M	-	+	stable
(f)	30%	0.1M	0.01M	10%	0.01M	0.001M	-	+	unstable
(g)	20%	0.1M	0.01M	10%	0.01M	0.001M	-	+	partially stable
(h)	25%	0.1M	0.01M	5%	-----	0.01M	-	+	partially stable
(i)	20%	0.1M	0.01M	5%	-----	0.01M	-	+	stable
(j)	50%	0.5M	0.01M	10%	0.1M	0.01M	+	-	partially stable
(k)	40%	0.5M	0.01M	10%	0.1M	0.01M	+	-	stable
(l)	0%	0.5M	-----	10%	-----	0.01M	+	-	stable
(m)	0%	0.5M	-----	0%	0.25M	-----	+	-	stable

Stability of base pairing in heteroduplexes of $\underline{att}^{\varphi}/\underline{att}^{B}$ was estimated by the appearance of the single-stranded regions. Self structure of this DNA appeared as short bushlike regions in the single strands. Under all of these conditions, cohesive ends of λ are stable, however, mechanical forces in the protein film during mounting often pull the joined ends apart. This type of mechanical denaturation should not be important in the stability of $\underline{att}^{\varphi}/\underline{att}^{B}$ molecules, since the region of possible homology should not be subjected to mechanical stress from the rest of the molecule.

Plate VI Detection of small regions of homology between two deletions

a), b), c) Heteroduplex of λb511/λb508 mounted by the aqueous (a) and (b) or the formamide (c) method. Any homology between these deletion mutants must correspond to the position and size of XOP. The molecules shown in (a) and (c) are typical of those found with these mounting conditions, showing that there is little or no homology between λb511 and λb508 at XOP. (b) shows an infrequently found type of molecule which shows two adjacent deletion bushes with no duplex DNA between the bushes.

d), e), f) Heteroduplex of λb501/λb536 mounted by the aqueous (d) and (e) or the formamide (f) method. A region of homology corresponding to about 100 base pairs can be detected between the deletions in these molecules. This length agrees quite well with the length expected from the position of each deletion mapped separately (see Table 9).

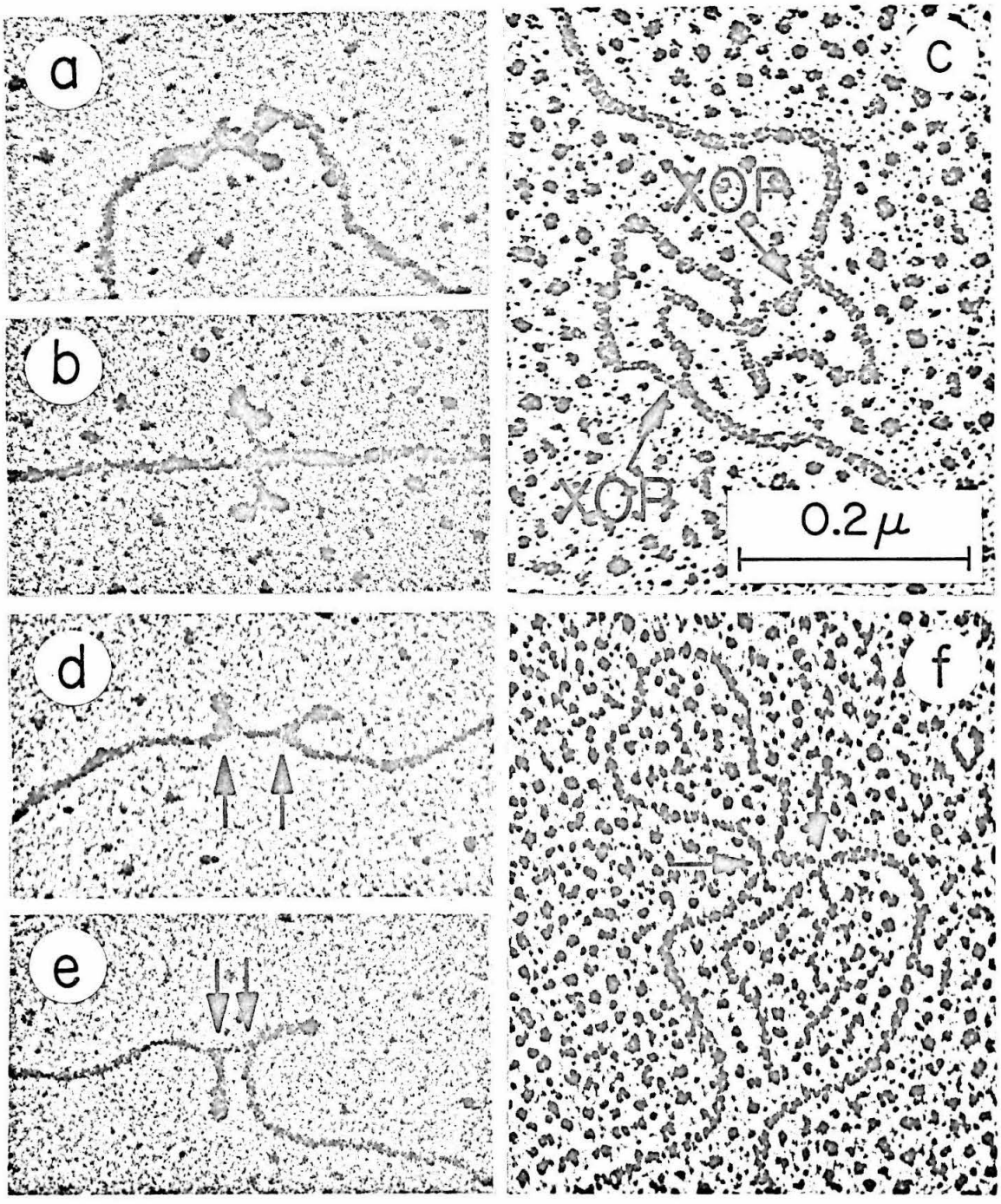

a
b
c
XOP
XOP
0.2 μ
d
e
f

TABLE 9

Two Methods for Determining the Distance Between the Endpoints of Two Non-overlapping Deletions

deletion mutants		Length of DNA between the ends of non-overlapping deletions	
		From data of Tables 4 and 7	From heteroduplexes of:
ΔA	ΔB	$x_2^A - x_1^B$	ΔA/ΔB
b536	b501	.002 ± .007	.0025 ± .0002
b536	b509	.007 ± .007	.0027 ± .0005
b536	b511	.023 ± .007	.027 ± .0005
b536	b516	.010 ± .008	.026 ± .001
b519	b511	.016 ± .010	.014 ± .0005

Heteroduplexes between two deletion mutants were examined with the aqueous and formamide techniques, and the length of duplex DNA between the deletion loops or bushes was measured to give the values in the last column. Errors were calculated as described in the footnote of Table 3. These data indicate that b511 and b516 are probably identical.

homology even smaller than 100 nucleotides in length could be detected with the milder formamide conditions of Table 8.

The closest pair of non-overlapping deletions to be examined were 2A/3A combinations. Although each of these deletions begins at XOP, 2A and 3A mutants must not overlap since they all contain XOP and are able to recombine by Int-promoted recombination to generate wild-type recombinants (Parkinson, 1970). However, the amount of DNA between the endpoints of 2A and 3A deletions is too small to be detected by heteroduplex methods which indicates that XOP is quite small.

Heteroduplexes of λb511/λb508 do not contain any duplex segments between the deletions even when mounted under very mild formamide (Plate VIc) or aqueous conditions (Plate VIa and b). Since aqueous conditions promote complete stability of random base interactions, we should expect to detect small regions of homology under these conditions. Occasionally we found molecules like the one shown in Plate VIb that had two closely spaced deletion bushes. If we assume that the length of duplex DNA between these deletion bushes represents the maximum possible homology between 2A and 3A deletions, this homology cannot be more than 20 base pairs. More likely, the structure shown in Plate VIb is caused by the random collapse of the non-homology region into a bush which resembles two separate deletion bushes and therefore there may be no homology at all between 2A and 3A deletions at XOP.

Another homology test that we attempted involved a λb189/λb130bio69 heteroduplex molecule. If $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ are actually identical at XOP, but the extent of this homology is too small to be detected with our methods, we could increase this homology with λb189 which must contain a short sequence to the left of XOP which

is identical to that in λb130. Again, however, no homology in the form of a duplex segment was detected under a variety of mounting conditions. This result suggests that the total length of XOP plus most or all of REL is still too short to be stable under these conditions.

4. DISCUSSION

(a) XOP and the Int system

(i) Size and location of XOP

We have obtained two independent determinations of the location of XOP, the point at which the lambda chromosome is broken during integration. Analysis of both transducing phages and deletion mutants locates XOP at 0.574 in the λ genome. This value is in excellent agreement with the results of several others based on transducing phage studies. Hradecna & Szybalski (1969) reported a value of 0.573 based on heteroduplex studies of λb2 and λbio phages. Yamagishi & Skalka (1969) obtained a value of 0.57 from analyses of segments of differing base composition in λ^{++} and λdg DNA.

The size of XOP has been estimated from studies on heteroduplexes between deletion mutants which begin at XOP and extend either to the left (Class 2A) or to the right (Class 3A). Because of the unique structure of deletion loops, which originate from a point, homology between 2A and 3A deletion mutants can be studied even in the presence of random base interactions. Two findings from these studies indicate that XOP is very small: molecules which appear to have two distinct deletion bushes are very uncommon in 2A/3A heteroduplexes; moreover, the points of origin of the two deletion bushes in

such molecules are separated by less than 20 base pairs. Since nearly all of the heteroduplex molecules do not contain two deletion bushes, the amount of homology, if any, between the XOP of 2A and 3A deletions is certainly less than 20 base pairs and perhaps less than 10 base pairs. It is difficult to estimate accurately the lower limit of detection in this case because denaturation of a small homology region could conceivably receive mechanical assistance from the forces on the rest of the molecule. This effect, however, may not be very large.

Thus XOP in $\underline{att}^{\varphi}$ is less than 20 base pairs in length which is consistent with the genetic and physical evidence that demonstrates that only one site exists in both $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ for integrative cross-overs to take place. If multiple cross-over points exist, they must all be within 20 base pairs of one another and would probably not have been detected in our experiments.

(ii) Int-produced deletions

The integration and excision of the lambda prophage occurs by reciprocal recombination which appears to involve some sort of breakage and reunion event (Hoffman & Rubenstein, 1968; Ptashne, 1965). Thus one of the steps during integrative recombination is likely to involve the introduction of single- or double-stranded breaks into the phage DNA at XOP. The existence of a large number of deletions beginning at XOP and dependent on the Int gene for their formation is consistent with the notion of Int-produced nicks in $\underline{att}^{\varphi}$ at XOP. Deletions of this sort might be produced in a number of ways as a result of errors in the Int system itself or as a result of mistakes subsequent to Int action on $\underline{att}^{\varphi}$. For example, the Int

system might promote occasional unequal crossing-over between the att site of one phage and a nonhomologous region in the chromosome of another phage. Or the Int system might introduce nicks at XOP which are subsequently "chewed back" by nucleases with occasional rejoining of the chewed ends.

The most likely models of Int-dependent deletion formation seem to begin with Int-produced nicks in the attachment site at XOP. Assuming that this is the case, we can develop arguments concerning Int action based on the properties of Int-produced deletion mutants. The most striking property of the 2A and 3A deletion mutants which are produced by the Int system is that both classes of mutants contain a functional cross-over point. This suggests that XOP may actually be the junction between the recognition elements REL and RER and that one recognition element in an attachment site is sufficient to define XOP. An alternative possibility is that XOP is actually a short region similar to the cohesive ends of λDNA and generated by two staggered nicks in opposite strands of the attachment site. If this were the case, it becomes necessary to account for the fact that XOP is never lost in Int-produced deletions. One could imagine, for example, that chewing by nucleases can only occur in one direction so that XOP is never chewed away during deletion formation.

It is impossible to choose between these two alternative models of XOP and Int action on the basis of present evidence. Perhaps, however, further studies of Int-produced deletions will provide a means of studying these questions.

(b) Structure of REL and RER

In addition to XOP, the λ att site contains two sequences of DNA, designated REL and RER, which border XOP and somehow influence the efficiency of the att site in Int-promoted recombination. Correlation of the REL-defective or RER-defective properties of deletion mutants with the physical location of these deletions in the λ chromosome allows us to set an upper limit on the size of these two recognition elements of att^{φ}. For example, REL cannot be more than 2000 nucleotides long, the limits being set on the one side by class 1 deletions which are not REL-defective, and on the other side by XOP which is present in all of the REL-defective mutants so far examined (class 2A and 2B deletion mutants).

Since all of the RER-defective mutants presently available (3A deletions, λbio transducing phages) are also int^{-}, we cannot as yet precisely localize RER and Int relative to XOP. However, λbio16A which is RER-defective, is only missing part of the Int gene (Manly, 1969), which suggests that RER is located between XOP and Int. Moreover, λbio16A is missing phage DNA from XOP to 0.585 in the λ chromosome. This indicates that RER is less than 500 nucleotides long.

It is quite possible that REL and RER are much smaller than the upper limits discussed above. A rather indirect line of reasoning leads us to believe that this may be the case. Our argument is based on the fact that λb189 behaves genetically as though it contained REL^{B}, and yet we cannot detect any bacterial DNA in this mutant. If, in fact, λb189 does contain the REL sequence from att^{B}, REL^{B} must be less than 20 base pairs in length. Since all of the genetic and physical evidence we now have indicates that att^{B} may be

structurally and functionally analogous to $\underline{att}^{\varphi}$, this implies that REL^{φ} is perhaps as small as REL^{B}, of the order of 20 nucleotides or less. Further analysis of the att site is necessary to demonstrate whether or not this conclusion is tenable.

(c) Homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$

It has been suggested that integrative recombination occurs through the creation of cohesive ends at the attachment site in phage and bacteria (Signer, 1968; Wu & Kaiser, 1968). This model requires only a very small amount of base sequence homology, 15 - 20 nucleotide pairs, between the two att sites. Our results, however, raise doubts as to whether there is even this much homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$. Operationally, all homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ must be within the region designated as the cross-over point. We have already shown that this region appears to be smaller than 20 base pairs long and could be much smaller.

A more direct approach to this problem involved the construction of $\underline{att}^{\varphi}/\underline{att}^{B}$ heteroduplexes. No homology between these two att's was ever seen even using a wide variety of mounting conditions for electron microscopy. To estimate the sensitivity of these methods, several controls were done. Most importantly, it was demonstrated that two deletions whose endpoints are only 100 base pairs apart form two loops with a short 100 nucleotide homology region between them. This length of homology was stable even under the most denaturing conditions employed in our experiments. This suggests we could have detected considerably less homology with our methods.

Another estimate of the sensitivity of these techniques in detecting homology is that λ cohesive ends which are 20 base pairs long (Wu & Kaiser, 1968) are stable under the mild mounting con-

ditions used. This situation, however, is not strictly comparable to the case in which a small homology region is bounded by large segments of non-homology. The reason for this is that the cohered ends of λ DNA could gain additional stability through base stacking interactions at the ends of the cohered region. This effect is probably small since loss of these stacking interactions does not significantly alter the stability of cohered ends (Wang & Davidson, 1968).

There is also associated with the cyclization of λ DNA an unfavorable entropy. The entropy for forming a short duplex region in the middle of two single-stranded segments is presumably more favorable than for cyclization. Therefore, although we cannot quantitatively evaluate the stability of small homology regions bounded by large portions of non-homology, it seems likely that a region of 20 base pairs would be stable. Moreover, since the denaturing conditions used to mount the DNA were barely sufficient to melt out random base interactions, we cannot detect any homology between $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ in excess of random base pairing which probably involves fewer than 10 base pairs.

Thus our results indicate that the mechanism of λ prophage integration must be a very special sort of recombination event. The enzymatic portion of this system seems to specifically recognize the short sequence of nucleotides comprising $\underline{att}^{\varphi}$ and $\underline{att}^{B}$ to bring about a reciprocal recombination between these two attachments sites. Although we do not yet know how this recombination is accomplished, it is evident that little or no base sequence homology is required for integration. Furthermore, integration cross-overs occur at only one point within the phage and bacterial $\underline{att}$ sites. In contrast, systems of generalized recombination are neither site-specific nor homology-independent. It appears, therefore, as though $\underline{att}^{\varphi}$ and the

enzymatic components of the λ integration apparatus constitute a highly specialized recombination system which may operate by quite different principles than do systems of generalized recombination.

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Discussion

The three known components of the λ integration system, Int (45-47), Xis (48) and att$^{\varphi}$ are all located in the center of the λ chromosome and are not essential for vegetative growth. In the past, λ mutants defective in integration were isolated by searching for a particular mutant defect among large numbers of mutagen-treated phage. A novel approach to the study of λ integration was made possible by the development of methods for selecting λ deletion mutants from a large wild type population. Since the entire central third of the genome is not essential for lytic growth (59, 60), most viable deletion mutants contain lesions in the center of the chromosome and many of these mutants prove to be defective in integration.

The method used in this study for isolating λ deletion mutants depends on the finding that the stability of λ phages in various environments is often related to the DNA content of the particles. At high temperature or low divalent cation concentration, the amount of DNA in the head of a λ particle becomes critical; the larger the DNA, the more likely it becomes that the head will rupture, releasing the DNA and inactivating the particle. Thus conditions such as pyrophosphate treatment inactivate λ wild type at a much greater rate than λ deletion mutants. By inactivating preparations of wild type phage in this manner, it was possible to detect and isolate the small fraction of deletion mutants in such populations. Upon subsequent examination many of these deletion mutants were found to be defective in integration. Thus it was possible to isolate integration-defective λ mutants without first assuming what the nature of the integration-defective phenotype should be.

The integration-defective deletion mutants used in this work provided the means with which to examine the phage attachment site ($\underline{att}^{\varphi}$) which plays a purely structural role in the integration process. These mutants were examined by their integration properties and by their behavior in Int-promoted vegetative recombination and proved to be of four major types. Some deletion mutants, designated class 3B, seemed to be missing the entire $\underline{att}^{\varphi}$ region and thus could not be used for dissecting the various components of $\underline{att}^{\varphi}$. However, the remaining three classes of deletion mutants behaved as though only part of $\underline{att}^{\varphi}$ was missing. If we define the point in $\underline{att}^{\varphi}$ at which integration cross-overs occur as XOP, then class 2A and 2B deletions were missing DNA to the left of XOP and class 3A mutants were missing DNA to the right of XOP. Since these three deletion types all displayed non-complementable integration defects, it was proposed that $\underline{att}^{\varphi}$ contains recognition sequences on each side of XOP which are necessary for normal integration behavior. These elements are designated REL and RER. It was found that class 2A mutants were deletions of REL and class 3A mutants were deletions of RER. The defect of class 2B deletions proved to be due to a substitution of bacterial DNA from $\underline{att}^{B}$, the bacterial $\underline{att}$ site, in place of the phage sequence for REL. The $\lambda\underline{bio}$ transducing phages appear to have an analogous substitution for RER.

The function of REL and RER was investigated by employing class 2A, 2B and 3A deletion mutants and $\lambda\underline{bio}$ phages to define two mutant forms of REL and two of RER. A number of mutant $\underline{att}$ sites were constructed by combining these mutant elements in various ways and the interactions between $\underline{att}$ elements was studied by analyzing patterns of Int-promoted recombination between these mutants. No clear pattern emerged, however. In general, we can conclude that the Int system is not totally specific for the naturally-occurring $\underline{att}$

forms $\underline{att}^{\varphi}$ and $\underline{att}^{B}$, but can tolerate a wide range of att lesions and still function. It also appears that there is a minimum amount of sequence specificity necessary for an att site to be recognized by the enzymatic components of the integration system, however, the orientation of att elements in two recombining chromosomes also has a great effect on the efficiency of Int-promoted recombination between those att's.

With a general notion about the role of REL and RER in Int-promoted recombination, we turned to a physical investigation of the properties of $\underline{att}^{\varphi}$ and its component elements. The chromosomal location of the deletion or substitution in the various types of att mutants was precisely determined by constructing heteroduplex DNA molecules containing one mutant strand and one wild type strand. These heteroduplexes were examined by electron microscopy and the physical position of each mutation was measured. We then compared the att defect of each mutant with its physical location to obtain an accurate picture of the size and location of each of the att components REL, XOP and RER. It was found that XOP is located 0.574 of the distance from the left end of the wild type λ chromosome. Consistent with the genetic evidence which indicates that XOP is a true point, we found that XOP was smaller than 20 base pairs in length and could, in fact, be merely the boundary between REL and RER. We also determined that the essential recognition sequences in REL and RER lie adjacent to XOP and are quite small; each is less than 2000 base pairs in length.

What does the structure of $\underline{att}^{\varphi}$ reveal concerning the mechanism of integration? Apparently integration cross-overs only take place at one precise point in the λ chromosome and short nucleotide sequences on each side of this point are responsible for directing the

integration enzymes to their site of action. It is unlikely, however, that integration requires base sequence homology between the two att's, att^{φ} and att^{B}. To test this notion, we constructed heteroduplexes between phages, one of which contained att^{φ} and one of which contained att^{B}. We were unable to detect any homology between these att sites. Thus we conclude that there is less than 20 base pairs of homology between att^{φ} and att^{B}. This result indicates that integration is a unique type of recombination since other systems of generalized recombination require extensive regions of homology for crossing over. It will be most interesting and instructive to learn how integration actually occurs; however, new approaches will be required to answer this question.

There are many other examples of integration-like behavior in both microorganisms and in higher organisms. The possibility that many of these integration systems have features in common with the λ system justifies an intensive exploration of λ integration as a model system. Some of these other integration systems (in order of decreasing specificity of insertion) are:

1) Phage P22 of Salmonella typhimurium and the lambdoid phages Ø80, 21, 82, and 434 all exhibit site-specific integration similar to that of λ. An integration gene analogous to Int has been identified in P22, which suggests that P22 may integrate in much the same manner as does λ (61).

2) Temperate coliphage P2 can use a wider variety of insertion sites in the coli chromosome, however it is clear that P2 prefers some sites over others, suggesting that these sites are not identical. Another distinctive feature of P2 lysogeny is that phages which have integrated at one site in the host genome, may have altered att site preferences after induction. This suggests that P2 may routinely

incorporate a small amount of host DNA during excision (62). Further study of the P2 system might reveal basic differences in the mechanism of P2 integration compared to λ integration.

3) Bacterial episomes such as F^+ factors exhibit rare integration which is promoted largely by the host Rec system. Since there are a limited number of chromosomal sites that accept F^+ factors, this type of integration may require partial homology between the recombining structures (63).

4) Mul, a temperate coli phage unrelated to λ or P2, apparently inserts into a large number of bacterial sites. This phage causes bacterial mutations by integrating into various genes. Although the number of available sites for integration must be quite large, we do not know how many sites can be used by Mul for integration, nor the mode of Mul integration (64).

In higher organisms, several possible examples of integration can be cited. The most interesting of these are the "controlling elements" found in Maize, which alter gene expression perhaps by inserting into or adjacent to the gene that is changed. The best-studied example is McClintock's work (65) on the modulator-dissociator elements which influence the expression of several genes in Maize. Both of these elements seem to be capable of popping on and off the chromosome, altering gene expression at the sites where they are localized on the genome. Another possible example of integration is that of paramutation in Maize which has been studied by Coe and Brink (66, 67) and their coworkers. The case for insertion is less clear for paramutation, however the heritable changes in the genes that are studied occur at the locus of that gene rather than elsewhere in the genome. These heritable modifications of gene

function can be transmitted through the cytoplasm, which is suggestive of bacterial episome behavior.

Several animal viruses which cause transformation of cells in tissue culture resemble temperate phages in that the viral genome can be carried by the cell for many generations and can be recovered by various (inducing?) treatments. This phenomenon is best documented for SV40 virus and to a lesser extent for polyoma virus (68). There is also evidence which indicates that the SV40 genome may in fact be inserted into the chromosomal DNA since it appears to become associated with large DNA-containing structures in transformed cell lines (69). If SV40 transformation proves to result from integration of the SV40 genome into the cellular DNA, it becomes even more imperative that we learn how λ can accomplish such integration.

Finally, the process of deletion formation in microorganisms may be still another example of integration-like behavior. It has been proposed that deletions are formed by the abberant excision of small circular DNA fragments from the chromosome by a process similar to prophage excision (17). Although the bacterial Rec system is not involved in deletion formation (70), partial homology may be a necessary requirement as suggested by the work of Demerec and his collaborators on the "ditto" deletions in the cysC region of the Salmonella genome (71). These deletions occur frequently and appear to have one common endpoint. The possibility that deletions are formed by abberant excision events is further cause for thoroughly understanding the mechanism of λ integration.

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PART II

GENETICS AND PHYSIOLOGY OF MUTANTS IN THE LEFT ARM OF THE LAMBDA CHROMOSOME

Introduction

Because bacteriophage λ is one of the most completely characterized organisms from the genetic standpoint, much attention has been devoted to the use of λ as a model system for development and gene regulation. The work described in this portion of the thesis was primarily intended to further extend our knowledge of lambda genetics. This study began at the urging of Dr. Robert Huskey and the late Dr. Jean Weigle, who introduced me to the fascinating world of lambdology. In the first manuscript below, I describe the isolation and characterization of a number of nonsense mutants in the left arm of the λ chromosome. Previously, this portion of the genome, which encodes phage structural proteins, had been rather poorly characterized. Several new genes were found and a genetic map of the left arm was constructed. When the technique of heteroduplex microscopy was developed, it seemed advisable to reinvestigate the left arm in terms of the physical locations of the genes. This was accomplished through the use of λ<u>dg</u> transducing phages as described in the second manuscript.

GENETICS OF THE LEFT ARM OF THE CHROMOSOME OF BACTERIOPHAGE LAMBDA[1]

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THE chromosome of temperate bacteriophage lambda consists of three functionally distinct regions of approximately equal size. The right arm contains the genes for regulation (EISEN *et al.* 1966), DNA synthesis (JOYNER *et al.* 1966) and cell lysis (HARRIS *et al.* 1967). The central portion of the λ DNA molecule is concerned with the processes of prophage integration (KELLENBERGER, ZICHICHI and WEIGLE 1961; ZISSLER 1967) and vegetative recombination (FRANKLIN 1967). Neither of these latter functions is essential for vegetative growth and the DNA in this region can be deleted with no loss of viability (FRANKLIN 1967; HUSKEY 1968). The left arm of the lambda chromosome is comprised of genes controlling DNA maturation (DOVE 1966; SALZMAN and WEISSBACH 1967) and genes which code for the structural components of the lambda phage particle (WEIGLE 1966).

Although many studies have been devoted to the right and central portions of the lambda chromosome, the little that we know about the left arm is mostly of a qualitative nature. CAMPBELL (1961) isolated suppressor-sensitive (*sus*) nonsense mutants of λ and defined 13 complementation classes in the left arm on the basis of complementation spot tests. He also ordered these 13 classes with respect to one another by deletion mapping with λdg transducing phage. The present study was prompted by this lack of quantitative genetic information concerning the left arm of the lambda chromosome.

Many of the *sus* mutants of CAMPBELL cannot be used to analyze the left arm in a quantitative manner. This is due to the fact that CAMPBELL's mutants give small burst sizes under permissive conditions, suggesting that the mutant phenotype is not fully suppressed in the permissive host. To obtain reproducible recombination values in order to establish the size and spatial arrangement of genes in the left arm of the λ genome, the burst size of *sus* mutants under permissive growth conditions should approach that of lambda wild type. Therefore, new conditions for performing crosses have been devised and new *sus* mutants have been isolated which appear to be fully suppressed in the permissive host. The complementation between genes in the left arm has been studied by measuring the total phage yield of non-permissive cells which have been infected with two different *sus* mutants. Evidence is presented that the left arm of the lambda

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chromosome contains at least 18 cistrons and that three groups of genes in this portion of the lambda genome are co-transcribed.

MATERIALS AND METHODS

Strains used: The bacterial strains used were: C600 (APPLEYARD 1954), 594 (WEIGLE 1966) and W3350 gal 1⁻ gal 2⁻ pm⁻ (CAMPBELL and BALBINDER 1958). C600 is permissive and 594 is restrictive for λ *sus* mutants.

All of the λ mutants isolated in this laboratory were derived from the lambda PaPa strain of KAISER (1957). A series of λ *sus* mutants obtained from DR. A. CAMPBELL was used to define genes A-J in preliminary complementation tests. Temperature-sensitive (*ts*) mutants of lambda were obtained from J. WEIGLE and A. HARRIS. In addition, *ts* mutants defective in cistrons V, H, L and I were isolated in this laboratory among *sus*+ revertants of *sus* mutants defective in those genes. All of the *ts* mutants could grow on C600 or 594 at 37°C but not at 42°C.

Media: Nutrient agar plates for phage and bacterial assays contained: Bacto-agar (Difco) 10 gm; BBL trypticase (Baltimore Biological Laboratories) 10 gm; NaCl 5 gm in one liter of distilled H_2O. BBL soft agar is of the same composition except that 6.5 gm of Bacto-agar is used per liter.

All bacteria were grown in K medium, which is M9 buffer (ADAMS 1959) containing 1.5% charcoal-filtered casamino acids (Difco), 0.005 M $MgSO_4$, and 0.05% NaCl. For adsorption of λ particles, bacteria were grown in K medium containing 0.2% maltose (KM); otherwise, K medium containing 0.2% glucose (KG) was used. For growth of C600, thiamine hydrochloride was added to a final concentration of 10 μg/ml.

Dilutions were done in TMG buffer which contains 0.01 M tris HCl, pH 7.4, 0.01 M $MgSO_4$, and 0.01% gelatin.

Plating bacteria: Phage were assayed with bacteria grown to stationary phase in KM, chilled, centrifuged, and resuspended in 2/3 volume of 0.01 M $MgSO_4$.

Crosses and liquid culture complementation: A saturated culture of C600 for crosses or 594 for liquid culture complementation tests was diluted 2000-fold into KM at 37°C and grown with aeration to a density of 5×10^7–1×10^8 cells per ml. The bacteria were chilled, centrifuged, and resuspended in cold TMG at 4×10^8/ml. Log phase bacteria prepared in this manner give much higher phage yields than do stationary phase bacteria. A mixture of the two parental phages at 4×10^9/ml is added to an equal volume of bacteria to give a concentration of bacteria at 2×10^8/ml and a multiplicity of infection of 10. Adsorption is carried out at 37°C for 15 minutes. Under these conditions, phage adsorption is better than 95% when bacteria have been grown in maltose. The complexes are then diluted 4×10^4 into KG at 37°C and incubated for 90 minutes. The use of antiserum to inactivate unadsorbed phage and chloroform to promote lysis did not alter either the background or the final yield and was omitted in most experiments.

Mutagenesis and isolation of sus *mutants:* Two different mutagenic treatments were used. In one, a stock of lambda wild type (λ++) was treated with hydroxylamine following the procedure of FREESE, BAUTZ-FREESE and BAUTZ (1961). In the other treatment, 594 carrying a temperature-inducible lambda prophage (λCItl, LIEB 1964) was grown to 2×10^8/ml in KG at 37°C. The prophage was induced by placing the cells at 43.5°C for 20 minutes. The induced cells were returned to 37°C and N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wisconsin) was added to a final concentration of 5 μg/ml. The culture was aerated until lysis and chloroformed.

sus mutants were selected by plating the treated phage stocks on C600 and overlaying with soft agar containing an equal amount of 594. Since the *sus* mutants grow only on C600, they make uniformly turbid plaques which can be easily distinguished from the plaques made by *sus*+ phage.

Isolation of λdg and λdg spot tests: A series of W3350 (λdg) lysogens was made from a lysate of UV-induced C600 (λ++) following the general procedures described by CAMPBELL (1958). To order mutant sites by deletion mapping with λdg's (CAMPBELL 1959) an agar plate was seeded

with 0.15 ml of W3350 (λdg) and 0.15 ml of 594, both of which had been grown to saturation in KM. The plates were spotted with a drop containing about 2×10^6 particles of the unknown mutant and allowed to dry, after which each plate was UV-irradiated with the dose used for optimal induction of liquid cultures. Each mutant was also spotted on 594 alone as a control. The plates are incubated overnight at 37°C.

The UV-irradiation serves two purposes in this test: the λdg prophage is induced and immunity to superinfection is destroyed; and secondly, recombination between the superinfecting *sus* mutants and λdg is increased several fold. Since *sus* mutants cannot grow on W3350 or 594, a positive test (an area of lysis on the plate) can only result if the *sus* mutation lies outside the deletion so that *sus*+ recombinants can be formed.

Complementation spot tests: Spot tests were done by seeding a plate with 594 and then spotting the plate with a drop containing approximately 2×10^6 particles of each of two different *sus* mutants. The plates are incubated overnight at 37°C. A positive test—an area of lysis—indicates that the two *sus* mutants are defective in different genes. Recombination between *sus* mutants defective in the same gene to produce *sus*+ phage will not give a positive test under these conditions.

RESULTS

Isolation and classification of new sus *mutants: sus* mutants were selected after growing the mutagen-treated stocks for several cycles on the permissive strain C600 to eliminate the possibility of mutational heterozygotes. Uniformly turbid plaques were transferred from the double layer plates with a sterile pin onto plates seeded with 594 and C600, respectively. Phage that grew on C600 but did not grow on 594 were purified from the C600 plate by a single plaque isolation. Approximately 0.3% of the phage that survived either mutagenic treatment were *sus* mutants, however no precautions were taken to insure that the mutants arose independently. The level of spontaneous *sus* mutants which would have been detected with this technique was found to be less than 0.01% in untreated control samples. A total of 310 *sus* mutants was isolated and stocks were prepared by the confluent lysis method (ADAMS 1959) for further studies.

A three-step screening procedure was employed to determine the complementation class and approximate location of each *sus* mutant in the left arm. The following procedure applies only to *sus* mutants in the left arm of the lambda chromosome.

(1) Each *sus* mutant was first spot tested against a series of λdg prophages whose deletion end-points had been previously established by tests with the reference *sus* mutants defective in genes A through J provided by DR. A. CAMPBELL. This allowed each mutant to be assigned to a group of two or three adjacent genes within the left arm.

(2) Complementation spot tests were done using six reference *sus* mutants defective in genes spanning the region indicated by the λdg test. This test was almost always adequate for making a definite cistron assignment for each new *sus* mutant.

(3) Many of the *sus* mutants were further tested by liquid culture complementation. In this test, non-permissive bacteria were infected with equal multiplicities of a reference *sus* mutant and an unknown *sus* mutant, and the total phage yield produced during one lytic cycle was measured. Two mutants defective

in the same gene typically produce fewer than 1/10 of a phage per infected cell. Two *sus* mutants which complement each other yield 100–250 phage per cell, indicating that the mutants are defective in different functions and therefore different genes. Some pairs of *sus* mutants, however, produce intermediate yields of 10–30 phage per cell. These combinations will be discussed in detail in the section on polarity.

Complementation classes in the left arm: A summary of the new *sus* mutants obtained in this study is presented in Table 1. No new *sus* mutants were obtained in cistron D or in cistron T, a new gene between G and H (MOUNT, *et al.* 1968). *sus* mutants defective in four additional new genes were isolated; these genes

TABLE 1

New sus *mutants in the left arm of the lambda chromosome*

Cistron	Number of isolates	Number of sites	Order of mutant sites*
A	4	4	—11†—914—420—(701—854)—
W	6	3	—403,748,805,822—859—812—
B	2	2	—915—427—
C	5	5	—737—884—434—407—818—
D	none	..	—15†—123†—
E	2	2	—815—723—
F	17	7	—785,826,865—471,478—730,893—421,889—
			—804,910—423,431,739,762,876—784—
Z	11	5	—718,735,740—405,712,834,916—744,758—
			—453—797—
U	6	5	—733,897—869—413—(824—858)—
V	11	4	—(438,466,475,817,852,877—750,760—736—
			—769,873)—
G	15	9	—447—703,917,919—764,799—759,872—
			—832—825—436,880—874,898—901—
T	none	..	—*ts*40‡—
H	10	5	—866,885—706—435—717,745—437,457,463,848—
M	3	3	—888—481—793—
L	16	7	—756,853,908—771—780,860—441,708,
			725,836—439—778—749,814,841,850—
K	13	10	—429—768,819—770,847—763—424—702—
			—755—(704—761,833)—892—
I	2	2	—811—838—
J	7§	5	—442—449—486—418,428,433—426—

* The cistrons are listed in the order in which they occur on the genetic map; each mutant site is separated by dashes, and sites enclosed in parentheses have not been ordered with respect to one another. Mutants numbered between 401 and 488 were induced with hydroxylamine; those numbered from 700–922 were induced with nitrosoguanidine. Two mutations were considered to be at the same site if no recombination could be detected between them after each parent had been UV-irradiated with a dose of two phage lethal hits, which increases recombination frequencies approximately 20-fold.

† These mutants were obtained from A. CAMPBELL and were used to construct a map of the left arm. They are shown in their correct order, but have not been included in the columns headed "Number of isolates" or "Number of sites."

‡ Cistron T has been defined by temperature-sensitive mutants (MOUNT, *et al.* 1968), and *ts*40 from A. HARRIS was used to establish the map position of the T cistron.

§ 30 mutants were isolated in the J cistron, but only 7 of these have been analyzed in detail.

are designated by the letters W, Z, U and V in accordance with the nomenclature proposed by CAMPBELL (1961). Gene U has been identified independently by MOUNT, *et al* (1968) using *ts* mutants.

The distribution of mutant isolates among the 78 sites shown in Table 1 closely approximates a Poisson distribution with a mean of 0.75, which indicates that more than one-half of the sites that could be defined by this type of *sus* mutation have already been found. Moreover, the mean number of sites per gene is 5 and none of the 16 genes defined by this class of *sus* mutants has fewer than two mutational sites. This suggests that few new genes would be found by isolating more *sus* mutants of this type.

In vitro *phenotypes of genes in the left arm:* The *in vitro* complementation test devised by WEIGLE (1966) can be used to determine one aspect of the defective phenotypes of *sus* mutations in the left arm of the lambda chromosome. WEIGLE has shown that *sus* mutants defective in genes A, B, D and E prevent the assembly of functional phage heads; *sus* mutants defective in genes G, H, M, L, K, I and J prevent the synthesis of functional phage tails. In the terminology of WEIGLE, genes A, B, D and E are "tail donors" and genes G, H, M, L, K, I and J are "head donors" because defective lysates of mutants in these genes produce normal tails and normal heads, respectively. At the time, cistron F could not be classified in this manner because the available mutants defective in the F cistron were very leaky.

An *in vitro* complementation analysis has been made of *sus* mutants defective in cistrons W, C, F, Z, U and V to complete the classification of the phage structural genes in this respect. Although the T cistron was not studied, it appears to control a step in phage tail synthesis (KEMP, HOWATSON and SIMINOVITCH 1968). The data summarized in Table 2 show that W, C and F mutants only make phage tails under restrictive conditions, and must, therefore, control the production of functional phage heads. Cistrons Z, U and V are seen to be head donor genes, indicating that these genes are involved in the formation of complete

TABLE 2

In vitro *complementation patterns of previously untested cistrons*

Mutant lysate	Phage made after mixing a mutant lysate with: Head donor (L_{es})	Tail donor (A_{11})	Self (Background)
W_{403}	250	<.001	<.001
C_{434}	410	<.001	<.001
F_{423}	200	<.01	<.01
Z_{405}	<.01	350	<.01
U_{413}	<.001	560	<.001
V_{438}	<.001	310	<.001

Defective lysates were made by growing 594 carrying a temperature-inducible *sus* prophage to 2×10^8 in KG at 37°C. The cells were induced at 43.5°C for 20 min and returned to 37°C until lysis occurred. *In vitro* complementation was measured by mixing equal volumes of a mutant lysate with a head or tail donor lysate (see WEIGLE 1966). The reaction was stopped after 3 hours incubation at room temperature and the total phage were assayed. The data give the number of phage made $\times 10^{-7}$.

phage tails. Thus all of the genes in the left arm control the assembly and structure of the mature lambda particle, which further strengthens the notion (see DOVE 1966) that the lambda chromosome is organized into groups of genes whose functions are related.

A genetic map of the left arm: To insure that the crossing procedure described in the METHODS section was indeed valid for genetic analyses of the new *sus* mutants, some of the factors which can influence the measurement of recombination frequencies were checked. The reversion frequencies and leakiness of the *sus* mutants used in a cross limit the accuracy with which one can measure sus^+ recombinants among the many *sus* progeny of that cross. The reversion frequencies of the new *sus* mutants range between 10^{-5} and 10^{-8}, and with the exception of several sites in the F cistron, none of the mutants are leaky—that is, the burst size of these *sus* mutants in single infection of a non-permissive host is very much less than one. Thus all of the new *sus* mutants are suitable for genetic mapping.

The generation and formation of wild-type recombinants in a cross does not seem to be favored under the crossing conditions used here. To show this, a series of crosses was performed between *sus* and sus^+ phage and the ratio of *sus* to sus^+ phage among the progeny was compared to that of the parental input. The allele ratio among the progeny was always the same as that of the parental input over a range of allele ratios from 1:10 to 10:1. Thus lambda wild type does not have a selective advantage in mixed infection with a *sus* mutant under the conditions employed.

The left arm of the lambda genome was mapped by crossing several mutants defective in a gene by mutants defective in the two nearest genes on either side of that gene. The position of mutant sites within each gene was established by three-factor crosses in which the CI gene (KAISER 1957) was employed as an unselected outside marker by using the wild type (CI^+) and temperature-inducible (CIt1) alleles of the CI gene. At 42°C phage carrying the CIt1 mutation form clear plaques but phage with the CI^+ allele make turbid plaques. The segregation of the outside marker among the sus^+ recombinants was followed by plating the progeny from a cross on 594 at 42°C and scoring clear and turbid plaque-formers. This method was reliable for ordering mutant sites which are separated by at least 0.1% recombination. Markers closer than this could not be reliably ordered with respect to the outside marker because of high negative interference (AMATI and MESELSON 1965).

In order to evaluate the effects of negative interference on the additivity of recombination distances, and to determine whether a mapping function (STAHL, EDGAR and STEINBERG 1964) could be constructed, the results of a representative sample of 2-factor crosses were analyzed as shown in Figure 1. The data indicate that recombination values are very nearly additive from 0.1% to about 3%. Apparently, negative interference does not appreciably affect the additivity of recombination distances derived from 2-factor crosses. Since a mapping function was unnecessary, the map of the left arm shown in Figure 2 was constructed by

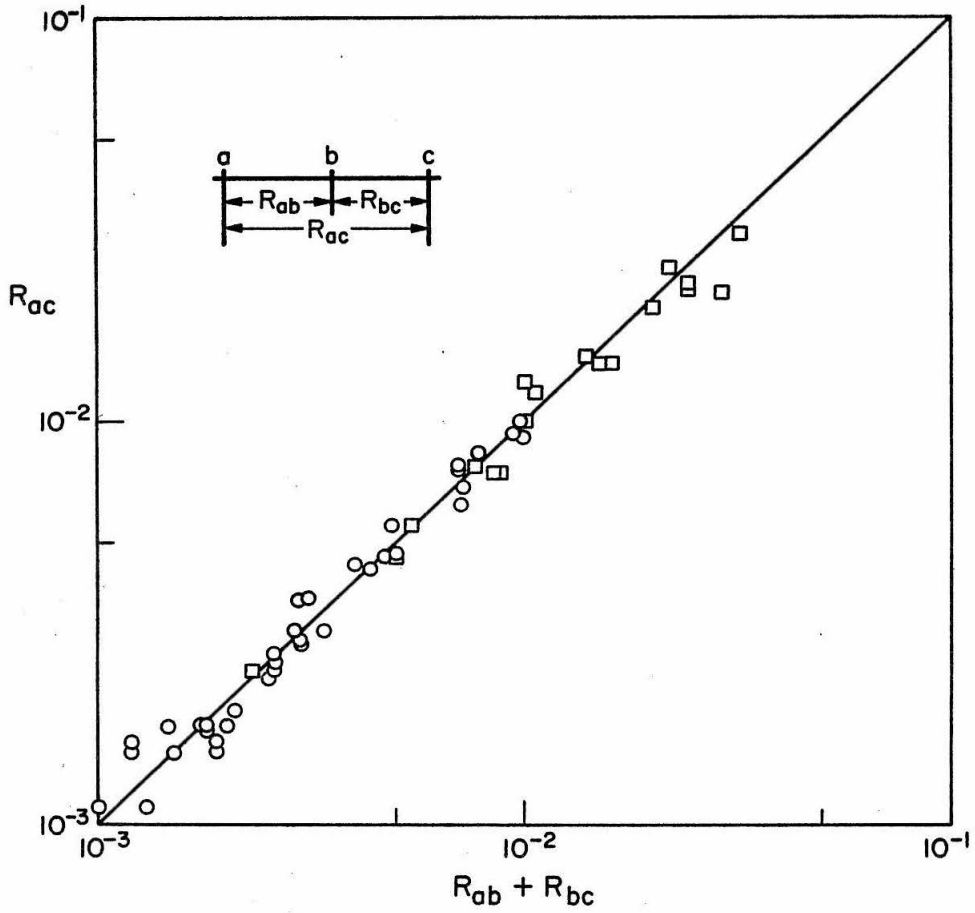

FIGURE 1.—The additivity of recombination distances in the left arm of the lambda chromosome. The frequency of recombination (R) is defined as 2 × plaques on 594/ plaques on C600 for the progeny of a cross. The frequency of recombination between sites *a* and *c* (R_{ac}) has been plotted on log-log paper as a function of the sum of the primary intervals R_{ab} and R_{bc}. The data are taken from a sample of two-factor crosses between *sus* mutants in the left arm. Burst sizes in these crosses usually ranged from 150–300 phage per cell, and no cross was accepted if the burst size was below 100 phage per cell. ○ = intragenic crosses; □ = intergenic crosses.

converting recombination frequencies directly into relative distances on the genetic map.

Polar effects between genes in the left arm: The left arm of the lambda chromosome is divided into two non-overlapping gene clusters: genes A-F which synthesize the mature phage head; and genes Z-J which synthesize the functional phage tail. We can further characterize this portion of the λ genome at the level of transcription by studying polar effects among the genes of this region. The evi-

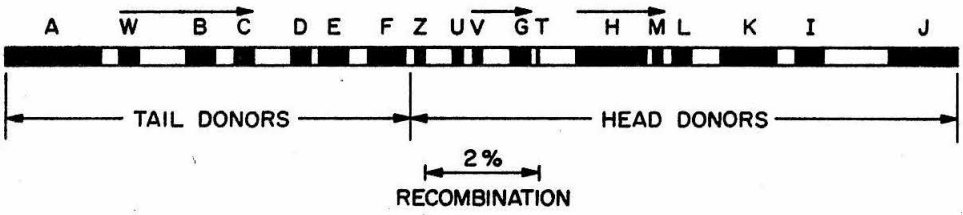

FIGURE 2.—A genetic map of the left arm of the lambda chromosome. The observed recombination frequencies between *sus* mutants in the left arm were converted to relative distances on the genetic map. The shaded areas represent the minimum estimate of the size of a gene based on recombination between the most distant *sus* mutants defective in that gene. Genes A-F are tail donors and genes Z-J are head donors as determined by *in vitro* complementation patterns (WEIGLE 1966). The extent and orientation of polarity in this region of the genome is indicated by the arrows above some of the cistrons.

dence, from work on bacterial operons, strongly suggests that polarity—the reduction of one gene's activity by a nonsense mutation in a neighboring gene—is due to the fact that some genes are transcribed and translated together as a unit (IMAMOTO and YANOFSKY 1967a, b). The inability of nonsense codons to specify an amino acid under non-permissive conditions somehow prevents the expression of any genes beyond the nonsense mutation when these genes are organized as a unit of transcription. The basis of polarity may be the postulated coupling of transcription and translation (STENT 1966) and it is clear that in bacterial operons, the orientation of polar effects is the same as the direction of transcription and translation.

STAHL *et al.* (1966) have demonstrated with phage T4 that the phage yield from non-permissive cells which have been mixedly infected with *sus* mutants defective in different genes can be used as a measure of polarity. If the two mutants are not polar, the phage yield should be identical whether the functioning wild-type alleles of these genes are on the same (*cis*) or on different (*trans*) chromosomes. A low yield in the *trans* configuration compared to the *cis* control can be indicative of polarity. *Cis-trans* tests such as this have been done with each pair of adjacent genes in the left arm and the results (Table 3) indicate that mutations in cistrons W-B, B-C, V-G and H-M may be polar since the complementation between mutants defective in these genes is reduced when the mutations are in the *trans* configuration.

The polarity of cistrons W-B, B-C, V-G and H-M has been confirmed by demonstrating that poor complementation between mutants defective in these genes is due to the presence of a *sus* (nonsense) mutation in only one of the genes of each pair. One way to show this is to replace the *sus* mutants with *ts* mutants and repeat the *trans* complementation tests to see whether the *cis-trans* position effect still occurs (see STAHL *et al.* 1966). The results of this test for gene pairs B-C, V-G and H-M are shown in Table 4. In each case one combination of *ts* × *sus* no longer exhibits poor complementation, whereas the opposite combination still complements poorly. The example, U-V, has been included to show that a gene

TABLE 3

Cis-trans tests of adjacent genes in the left arm

Cistrons tested	*Cis* test $\left(\frac{+\ +}{sus\ sus}\right)$ Percent of λ++ yield	*Trans* test $\left(\frac{+\ sus}{sus\ +}\right)$ Percent of λ++ yield	Percent of *cis* yield
A × W	76(2)	40(5)	53
W × B	53(4)	10(6)	*19*
B × C	45(4)	12(4)	*26*
C × D	.....	40(4)	..
D × E	.....	40(4)	..
E × F	76(2)	50(2)	66
F × Z	80(1)	50(2)	62
Z × V	100(2)	95(2)	95
U × V	62(1)	73(1)	117
V × G	82(1)	13(5)	*16*
H × M	54(4)	7(7)	*13*
M × L	85(2)	61(5)	72
L × K	64(1)	60(1)	94
K × I	50(1)	50(1)	100
I × J	52(2)	40(4)	77

594 was infected with 5 particles of each parent and the total phage yield was measured after 90 min incubation at 37°C. Under these conditions the yield of a λ++ control ranges from 150–250 phage per cell. The figures in parentheses refer to the number of times each test was performed. The data are averages of all the tests, but the values usually did not vary more than 15% for tests done on different days using different mutants in a gene.

The double mutants were constructed by crossing two *sus* mutants and spot testing the progeny against each parent. Those progeny which did not complement either parent were then crossed by each parent to verify the genetic constitution of the double mutant.

TABLE 4

A directional test to confirm the polarity of B-C, V-G and H-M

Test		Gene 1–Gene 2 B-C	V-G	H-M	U-V (Control)
$\frac{+\ +}{sus1\ sus2}$	(*cis* control)	100	80	50	65
$\frac{ts1\ +}{+\ ts2}$	(*ts trans* control)	70	45	50	55
$\frac{sus1\ +}{+\ sus2}$	(*sus trans* control)	25	10	10	50
$\frac{ts1\ +}{+\ sus2}$	(Experimental)	65	50	50	60
$\frac{+\ ts2}{sus1\ +}$	(Experimental)	25	10	10	50

594 was infected with 5 particles of each parent and the total phage yield was measured after 60 min incubation at 41.5°C. The data are expressed as per cent of a λ++ control yield, which is about 50 phage per cell at this temperature. Mixed infections of mutant and wild type were also done as controls. These always gave at least 60% of the wild-type yield for the mutants described in this table.

pair which exhibits no *cis-trans* differences in *sus* × *sus* tests also complements well in both *ts* × *sus* configurations. These data show that a *sus* mutant defective in cistron B, for example, prevents the full expression of cistron C which lies adjacent to B on the genetic map. However, a *sus* mutant defective in C does not affect the activity of cistron B. Thus the results show that poor complementation between *sus* mutants defective in genes B-C, V-G or H-M is caused by polarity. Each effect is unidirectional, which indicates that the direction of m-RNA synthesis most likely proceeds from B to C, from V to G, and from H to M.

The test described above could not be used with gene pair W-B because no *ts* mutant was available in the W cistron. A different test has been used to show that genes W and B are polarized. In this experiment, non-permissive bacteria were infected with *sus* mutants defective in cistrons W and B, and although the total multiplicity of infection was held constant, in separate tests different ratios of *sus* W to *sus* B phage were used to infect the cells. The burst size was then measured as a function of the number of *sus* W phage in the infected cells. STAHL *et al.* (1966) have shown that this test can be used to determine the direction of a polar effect. If, for example, *sus* mutants in W affected the expression of B, we would expect the burst size to increase with an increase in the number of *sus* W genomes in the infected cells, since more opportunities would exist for transcription and translation of the B cistron to occur. If W and B are not polarized, we should find that the burst size reaches a maximum when there is an equal number of *sus* W and *sus* B genomes in the infected cells. The results shown in Figure 3 demonstrate that a *sus* mutant defective in cistron W does decrease the expression of cistron B, since the burst size is directly dependent on the number of *sus* W mutants in the infected cell. The curves shown in Figure 4 demonstrate, as predicted, that non-polar genes complement most efficiently when the two parental inputs are equal in number.

The controls of $\lambda sus \times \lambda^{++}$ were also done to insure that input gene dosage or stoichiometric effects do not account for the differences in the results of Figures 3 and 4. The input ratio of *sus* to sus^+ phage was varied from 1:8 to 8:1 and the dosage dependence of each gene product was estimated from the dependence of burst size on the number of sus^+ genomes in the infected cells. None of the mutants tested exhibited gene dosage effects sufficient to account for the differences in the results presented in Figures 3 and 4.

Since W-B and B-C are polar, we would also expect W and C to be polar, and in the direction W to C. The results of Figure 3 confirm this notion, since the burst size in mixed infection by *sus* W and *sus* C phage is directly dependent on the number of *sus* W genomes in the infected cells. Thus, cistrons W-B-C constitute a polar triplet and are probably transcribed into messenger RNA as a unit.

Polarity studies on bacterial operons have shown that the position of a nonsense mutation within a gene determines the degree of polarity of that mutation. There exists a gradient of polarity throughout each gene of an operon such that mutations near the beginning of a gene cause much greater polar effects than do nonsense mutations near the end of that gene (NEWTON *et al.* 1965). Two *sus* mutants—*sus*403 and *sus*812—defective in gene W have been examined in com-

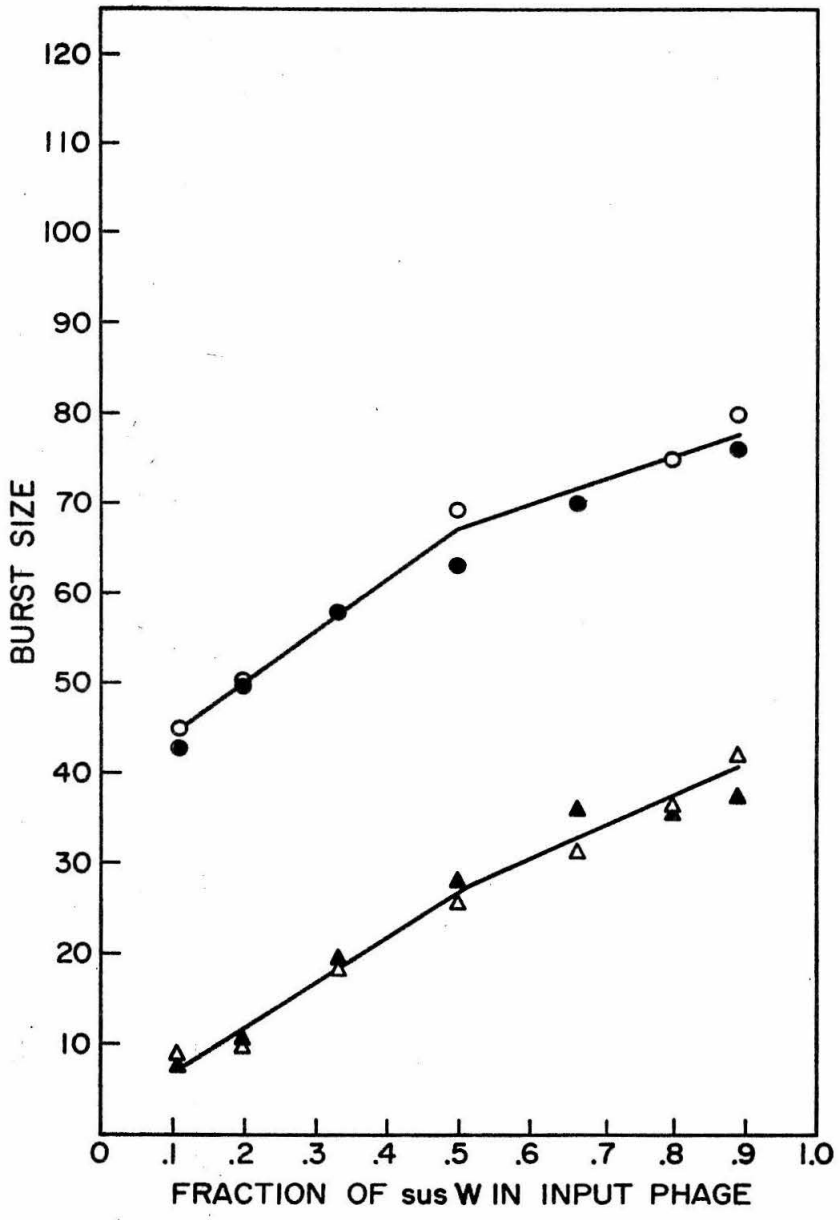

FIGURE 3.—The dependence of burst size on input ratio for polar gene pairs W-B and W-C. 594 was infected with two *sus* mutants at a total multiplicity of infection = 10. The input ratio of the two *sus* phages was varied from 1:8 to 8:1. After adsorption and anti-λ serum treatment to inactivate unadsorbed phage, the infected cells were plated on C600 before lysis to determine the number of cells yielding progeny phage. The total phage yield from these cells was measured after 90 min incubation at 37°C in KG medium. The burst size is defined as the ratio of total phage to cells which produced phage. △, ▲ $sus'_{W_{403}} \times sus_{B_{427}}$ ○, ● $sus_{W_{403}} \times sus_{C_{434}}$

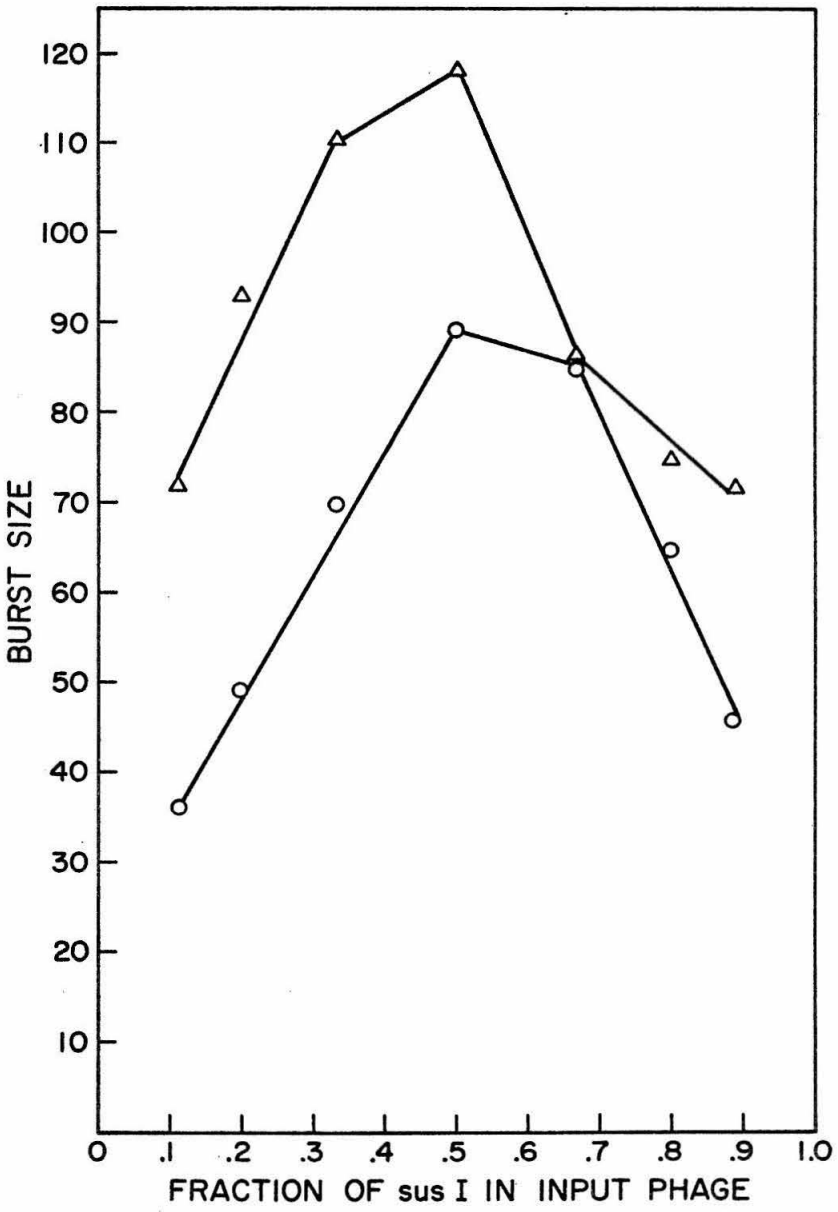

FIGURE 4.—The dependence of burst size on input ratio for non-polar gene pairs I-K and I-J. The procedure used was the same as that of Figure 3. ○ = $sus_{I_{811}} \times sus_{J_{418}}$ △ = $sus_{I_{811}} \times sus_{K_{424}}$

plementation tests with a *sus* mutant defective in cistron B to show that a gradient of polarity exists in cistron W. Five experiments were done with each *sus* mutant. *sus*403, which lies 0.25 recombination units to the left of *sus*812 on the genetic map, produces 7.8% (S.D. = 2.2%) of the wild-type yield in mixed infection with a *sus* B mutant. *sus*812 produces 13.1% (S.D. = 1.2%) of the wild-type yield in mixed infection with the same *sus* B mutant, indicating that a small gradient of polarity seems to exist within the W cistron. Since the burst size is a poor measure of any particular gene product, owing to the numerous interactions between phage precursors which give rise to an infective particle, this effect may be much more striking at the gene product level. NAKATA and STAHL (1967) have observed polarity gradients of a similar magnitude in phage T4.

DISCUSSION

It is quite probable that the 18 genes which have been defined by complementation between mutants in the left arm of the lambda chromosome account for nearly all of the genetic material in this region of the genome. If we assume that an average gene in this region contains 1,000 nucleotide pairs, and that this region is about ⅓ of the lambda chromosome or 20,000 nucleotide pairs long, we would expect about 20 genes in the left arm. Furthermore, there are no large gaps, as measured by recombination, between any of the known genes in the left arm of the lambda chromosome. Finally, the number of mutant sites per gene suggests that no more genes will be discovered with the type of *sus* mutants described in this paper.

Polar effects between *sus* mutants defective in adjacent genes such as those described here for phage λ have also been demonstrated in phage T4 by STAHL *et al.* (1966). In both phages a quantitative analysis is made difficult by gene dosage or precursor concentration effects, but it appears as though the basis for the *cis-trans* effects that have been observed in λ and T4 is in fact polarity of the same sort as that found in bacterial operons. Polar genes in both T4 (NAKATA and STAHL 1967) and lambda exhibit gradients of polarity. Moreover, in the lambda system the direction of transcription has recently been determined by examining the strand-specificity of messenger RNA made at early and late times during the vegetative cycle (TAYLOR, HRADECNA and SZYBALSKI 1967; COHEN and HURWITZ 1967). The left arm, which is only transcribed late in infection, is read in the direction from cistron A to cistron J. This is also the orientation of polar effects among three groups of genes in this portion of the lambda chromosome, and is consistent with the notion that the direction of transcription and the direction of polarity are the same.

In phage lambda the genes of the left arm are arranged into two non-overlapping clusters: one group of 7 genes concerned with DNA maturation and head formation; and another group of 11 genes which control phage tail assembly. The relationship, if any, between the clustering of functionally similar genes and the control of development during the final stages of the lambda growth cycle is not yet understood. It may be that the structural genes of the left arm are

expressed in a temporal sequence that parallels the order of those genes on the chromosome. The steps in the process of assembling mature lambda particles may also take place in the same sequence as the genes which control each step occur on the lambda chromosome.

Although previous polarity studies with phage T4 (STAHL *et al.* 1966) have failed to detect polar effects that involved more than two adjacent genes, the discovery in phage λ of polar genes W-B-C demonstrates that polarity need not be restricted to only two adjacent genes. The importance of joint transcription in phage morphogenesis is not known, but one possibility is that polarity provides a mechanism which insures that several critical gene products will be made at the same time, and in the correct relative amounts, during phage assembly. Phage lambda provides an excellent system with which to test this hypothesis.

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SUMMARY

The left arm of the chromosome of phage lambda was studied by using newly-isolated *sus* nonsense mutants. Four new genes were found which brings to 18 the number of known genes in this portion of the lambda chromosome. Recombination distances from two-factor crosses were found to be nearly additive in this region which allowed the construction of a genetic map of the left arm without the use of a mapping function. Physiological studies of these genes reveal two functional classes of genes in the left arm. Seven genes control phage head formation and another eleven genes are responsible for phage tail morphogenesis. It is clear from the mapping studies that the head genes are arranged into one cluster and the tail genes into another cluster on the chromosome. *Cis-trans* tests showed that three groups of genes in the left arm are polar, two polar groups contain two genes each and another polar group is composed of three genes. The orientation of the polarity effects is in all cases the same as the direction of transcription.

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A PHYSICAL MAP OF THE LEFT ARM OF THE LAMBDA CHROMOSOME

It is becoming increasingly important in biophysical and biochemical studies of bacteriophage to know the physical location of genes in the phage chromosome. A number of investigators have devised various methods for constructing physical gene maps in the phages T4 (Mosig, 1966, 1968; Goldberg, 1966) and λ (Kaiser, 1962; Jordan & Meselson, 1965; Hogness, Doerfler, Egan & Black, 1966). In general, these techniques involve the measurement of the length of a DNA fragment which is capable of bearing two genetic markers. However, the accuracy of these methods is limited either by the fragment size determination (Goldberg, 1966; Kaiser, 1962; Jordan & Meselson, 1965; Hogness, Doerfler, Egan & Black, 1966) or by the determination of the genetic content of the fragments (Mosig, 1966, 1968). We describe here a simpler, more direct technique for constructing accurate physical gene maps of the λ DNA molecule. Since this method is completely independent of recombination frequency or efficiency of marker rescue from the DNA fragment, the resolution is limited only by the number of available genetic markers and the size determination of the chromosome fragment.

As a source of uniform chromosome fragments, we employ λ*dg* phages which contain large substitutions in the left arm of the λ chromosome. Since these substitutions all have a common right terminus (Parkinson & Davis, 1970), λ*dg* phages constitute a series

of overlapping deletions of the left arm. The genetic contents of these left arm fragments are determined by marker rescue tests which do not depend on the efficiency of rescue since all of the left arm material is constant in any λ*dg* strain and thus a simple qualitative result is sufficient. The physical length of these fragments is then measured by electron microscopy of λ*dg*/λ^{++} heteroduplex DNA's (Parkinson & Davis, 1970; Davis, Simon & Davidson, 1970) and compared to the genetic content to construct a physical gene map.

The isolation and determination of the genetic content of λ*dg* phages are described elsewhere (Parkinson, 1968). The construction and measurement of heteroduplex DNA's are also described elsewhere (Parkinson & Davis, 1970; Davis, Simon & Davidson, 1970), but will be summarized briefly. Heteroduplexes are formed by mixing λ*dg* and λ^{++} phages, lysing in NaOH to release and denature the two DNA's, and finally renaturing in formamide at neutral pH. The duplex lengths in λ*dg*/λ^{++} heteroduplexes are measured by electron microscopy and the fractional length of the left arm fragment is calculated using an internal calibration standard (Parkinson & Davis, 1970).

The physical and genetic endpoints of 7 different λ*dg* strains are listed in Table 1. On the basis of these measurements, it is clear that the genetic and physical maps of the left arm are colinear. A direct comparison of the physical map and a genetic map

TABLE 1

Physical and genetic endpoints of 7 λdg phages

λdg	Marker	No. of molecules measured	χ_1	Error
A914-J	b5*	15	.030	± .001
C-J	b5*	17	.095	± .001
F730-J	i^{434}	10	.155	± .002
Z405-J	i^{434}	16	.168	± .0015
G-J	b5*	20	.193	± .0015
H706-J	b5*	12	.228	± .002
K-J	i^{434}	18	.284	± .002

χ_1, the length of phage DNA in the left arm of these λdg strains, was measured using the distance from the right end of the heteroduplex molecule to the beginning of the substitution at 0.574 (Parkinson & Davis, 1970) as a calibration length. The i^{434} or b5 substitution was used as a reference marker in the right arm. The error values are the limits within which the population mean should lie (Parkinson & Davis, 1970).

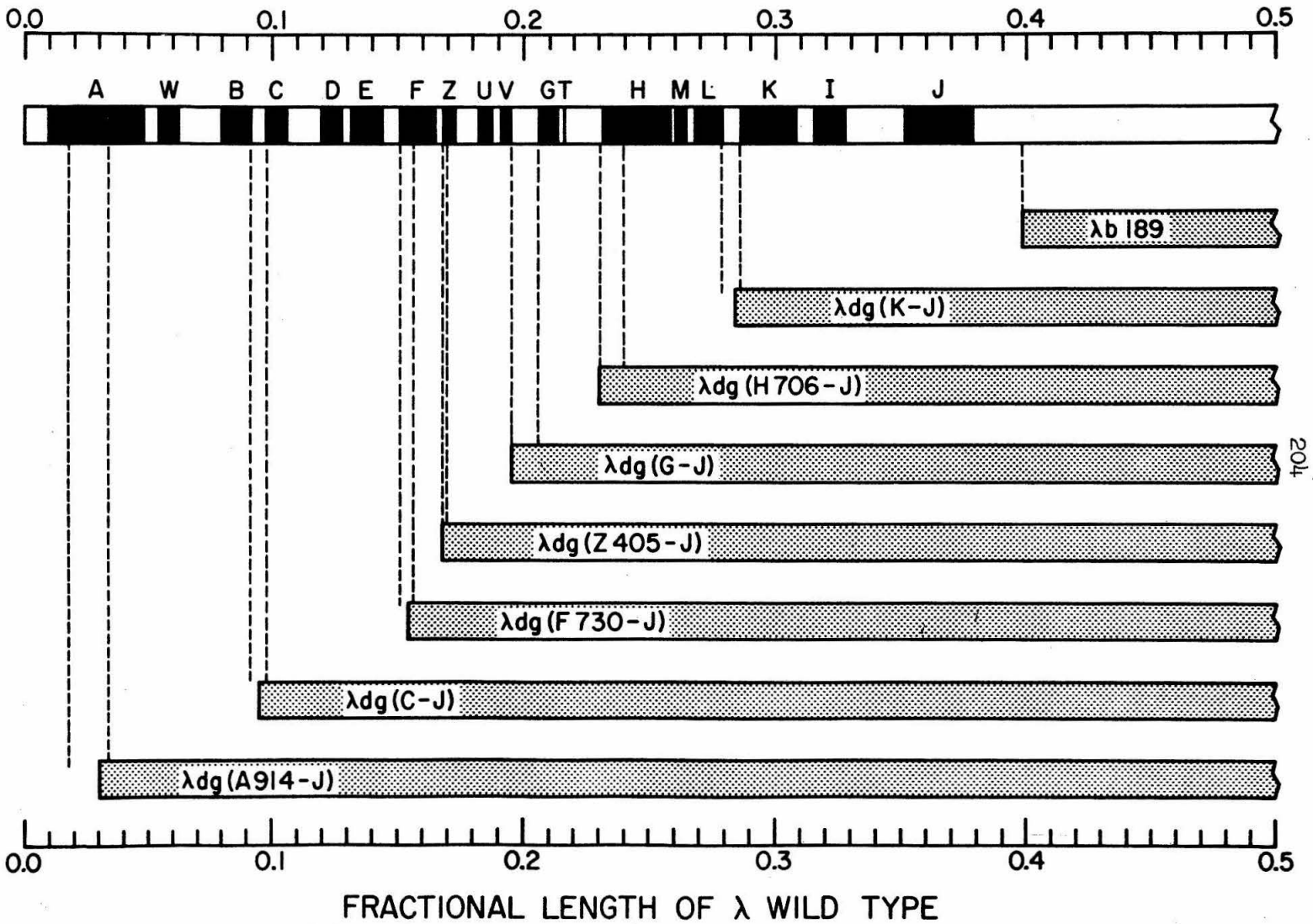
*These four λdg strains are also mapped in Parkinson & Davis (1970). The values have been recalculated using the entire right arm and central region as a calibration length as described above.

of the left arm is shown in Figure 1. The physical and genetic end-points of these 7 λdg strains can all be aligned. Thus, within the sensitivity of our measurements the frequency of recombination per unit distance is constant throughout this portion of the genome in contrast to the right arm which appears to have one region of very low recombination probability (Jordan & Meselson, 1965).

Several other features concerning the physical arrangement of genes in the left arm should be mentioned. The distance from the left end of the chromosome to the beginning of gene A is 1% of the total λ length. This segment of the genome may contain recognition sequences for the TER system which introduces the cohesive end cuts in λDNA (Gottesman & Yarmolinsky, 1968; Mousset & Thomas, 1968). Previously, this region was estimated to be about 4% in length (Jordan & Meselson, 1965). Secondly, it appears that the left arm contains few, if any, undiscovered genes. Assuming that an average gene is about 1000 nucleotides long (2% of λ length), the number of already identified genes is sufficient to fill the entire map of the left arm. Finally, the nature of the region between 0.38 and 0.4 in the genome is unclear. The right end of gene J based on these mapping data is at 0.378; however, no viable deletion mutants have ever been found to extend further to the left than 0.4. This suggests that J may extend more to the right or perhaps there is an essential gene between J and 0.4 which has not yet been identified.

In conclusion, the use of λ transducing phages to determine

FIG. 1. A comparison of genetic and physical distances in the left arm of the λ chromosome. The map at the top of this figure is the genetic map of the left arm described by Parkinson (1968). The genetic endpoints of the λ*dg* strains, indicated by dotted lines, were fitted to the physical endpoints by projecting the genetic map onto the physical map and then expanding and contracting the genetic map to obtain maximum correspondence of the genetic and the physical endpoints as determined by direct observation. In doing this, the error in physical lengths (see Table 1) was included so that all of the genetic endpoints fall within the error limits of the physical endpoints. The limits within which the A–J genes must lie are the end of the chromosome at 0.0 and the beginning of the silent region (Parkinson & Davis, 1970) whose left end is defined by the deletion mutant, λb189 (Parkinson & Davis, 1970). The position of λ genes in this map corresponds well with physical evidence of Simon *et al.* (1969); however Hradecna & Szybalski (1969) have reported that the A–E distance is only 0.076 λ units whereas we obtain a value of about 0.15. We suggest that the physical or genetic mapping of the λ*dg* strain used by these workers is incorrect.


0.0
0.1
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A W B C D E F Z U V GT H M L K I J
λb 189
λdg (K-J)
λdg (H 706-J)
λdg (G-J)
λdg (Z 405-J)
λdg (F 730-J)
λdg (C-J)
λdg (A 914-J)
0.0
0.1
0.2
0.3
0.4
0.5
FRACTIONAL LENGTH OF λ WILD TYPE

physical gene positions affords the most accurate method yet described for measuring distances between genes in the λ chromosome. This approach could also be employed for the right arm of the λ genome with the use of λdbio phages (Kayajanian, 1968). In conjunction with other similar mapping efforts (Parkinson & Davis, 1970; Simon, Davis & Davidson, 1969), it should be possible to construct a physical map of the entire λ chromosome which would be of great value in understanding the functional organization of the λ DNA molecule.

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Discussion

Based on the genetic evidence of polarized complementation, at least three groups of cistrons in the left arm of the λ chromosome appear to be cotranscribed. Since this work was completed, there have appeared several additional reports suggesting that the gene pair I-J is jointly transcribed as well (1, 2). It is possible that cotranscription of genes in the left arm is used by λ as a control mechanism for regulating the expression of these genes. Recent evidence, however, indicates that there may be only one initiation site for messenger transcription from the left arm (3). If this is true, it is not clear why all of the left arm genes do not exhibit polarized complementation. With the needed techniques now at hand, it is feasible to resolve this apparent paradox by examining the various species of messenger RNA made *in vivo* from the left arm. It is quite possible that a heretofore unsuspected mechanism of gene regulation is employed by λ to control the expression of genes in the left arm of the chromosome.

The studies reported here indicate that the recombination distances between mutations in the left arm appear to directly reflect the actual physical relationships between the mutations in the λ DNA. This work should be added to the small but growing list of studies which indicate that, with minor exceptions, recombination distances in DNA molecules correspond to physical distances (4-8).

The technique of using transducing phages to construct physical gene maps of λ could be easily extended to the right arm of the λ genome through the use of λ*bio* and λ*dbio* phages (9, 10). Indeed, the general methods we have used could be applied in other systems as well, for example, the F^+ episome, whose genetics are

only now being worked out. With minor modifications, the heteroduplex microscopy technique could be used with F^+ and a series of F' factors to study problems such as the location of insertion points in F^+ DNA, the distribution and size of essential DNA segments in F^+, the relationships between F^+ and the Col and RTF elements, and other problems. Combined with increased knowledge of F^+ genetics, heteroduplex electron microscopy should provide a valuable tool for attacking many of the problems which are difficult to answer by genetic techniques alone.

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