MAPPING OF INTEGRATION AND EXCISION CROSSOVERS IN SUPERINFECTION DOUBLE LYSOGENS FOR PHAGE LAMBDA IN *ESCHERICHIA COLI*[1]

E. CALEF

International Laboratory of Genetics and Biophysics, Naples, Italy

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IN a previous paper it was shown that one prophage apparently acts as a homology region for a superinfecting phage of the same kind (CALEF, MARCHELLI, GUERRINI 1965). A more rigorous test for the same possibility can be obtained by correlating the frequency of integration of superinfecting phage at any given region with the map length of that region. The work presented here consists primarily in a genetic mapping of the prophages in a double lysogen. The mapping was meant to verify that the process of integration of the second prophage into a segment of the first, under conditions which inhibit its function (homoimmunity), occurs at a frequency related to the length of the segment involved.

Concomitantly we were able to verify a number of features of the virus-host relationships such as linear insertion and order of markers.

A series of 19 independent double lysogens of independent origin have been analyzed genetically and are reported in this paper. The analysis has shown a positive correlation between map length and integration at any given segment. Some of the double lysogens, the structure of which had been deduced by P1 transduction mapping, were further analyzed by examining their monolysogenic segregants. The data obtainable by this method made it possible to: (1) map the prophage (confirming the known order of prophage genes), (2) have some insight into the modalities of haploidization, and (3) measure the relative length of those portions of the prophage map which lie outside the two extreme markers.

MATERIALS AND METHODS

Methods, procedure and material used were described by CALEF *et al.* (1965). A few changes are listed below.

Transduction: The selective medium for *bio*+ transductants was the Difco Biotin assay medium. This medium was chosen because it gave 50 to 100-fold more *bio*+ transductants than a selective bio minimal medium.

Phage: Two phages were used for the preparation of double lysogens; one is λ*cl, sus*54 and the other λ*sus*24. The marker *cl* is a clear mutant of the complementation group CIII; *sus* signifies suppressor sensitive. Lambda *sus*9 complementation group G was also used. The *sus* mutant is a gift of A. CAMPBELL (1961). Location and complementation group of the mutants can be seen in Figure 2.

Bacterial strain: Strain C600 (APPLEYARD 1954) was used to prepare the double lysogenic

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strains. We prepared two series of strains called A and B according to the order of lysogenization. Strains A received first *cI sus54* and then *sus24*; vice versa with strains B. In order to prevent extensive heterogeneity in the population, since a double lysogen can presumably undergo internal recombination, subculturing of these strains was kept to a minimum.

Double lysogens which showed lysis on the pm^- (nonpermissive) strain U279 were chosen. After one purification, a culture was grown to prepare the P1 lysate and to test heterozygosity of the three loci involved (*cI, sus54* and *sus24*).

Recipient strains in transduction experiments were strain U306 (CALEF *et al.* 1965) and the *gal⁻ bio⁻* strain (galactose negative biotin requiring) described by ROTHMAN (1965) as W602, which we have called U335.

Procedure: Classification of prophage type in transductants and in haploid segregants was made by successive replica platings of purified clones on the master plate to the permissive C600 to allow scoring of the marker *cI.* Since repeated replica platings were required, the lysogenic bacteria were arranged in regular rows of small streaks and were first replica-plated onto the C600 tester plate. About 8 hours later the C600 plate became the matrix of all successive replica platings on the nonpermissive strain U279 for scoring of the *sus* markers. The plates with strain U279 were prepared by brushing regularly 7 to 8 stripes of a suspension of the appropriate *sus* mutant. On this kind of plate the replica-plated bacteria will lyse the nonpermissive background only when they are lysogenic for sus^+ recombinants; on the other hand, replicas of lysogens containing *sus* mutants will lyse only when intersecting the λ*sus* stripes of the complementing type. In our case, we routinely used the two mutants involved in the preparation of the double lysogen and λ*sus9* to detect the double mutants. Unlike bacterial master plates which are usable only once, this secondary master plate, from which practically only phage is transferred, can be used up to five or six times and allows change of the velvet or filter paper after every successive replica.

EXPERIMENTAL

Transduction analysis of a double lysogen by selection of markers on both sides of the prophages: Although the linear insertion model has been repeatedly proved (CALEF and LICCIARDELLO 1960; ROTHMAN 1965; SMITH and LEVINE 1965), it was useful to check that a given lysogen contains the two prophages in the same linear structure, one phage closely linked to the *gal* gene and the other to the *bio* gene. This can be tested by making a P1 lysate using a given double lysogen and examining the type of prophage found in lysogenic transductants after selecting either for gal^+ or bio^+. Reciprocal types of prophages should be found linked to the two bacterial markers.

The data reported in Table 1 are from one experiment where a P1 lysate from a double lysogen was adsorbed to the recipient strain U335 according to the standard P1 transduction procedure. The infected cells were plated onto two different media to select either gal^+ or bio^+ transductants.

The prevalent class of prophage in one type of selection has a genotype reciprocal to the prevalent class in the other; hence the insertion of the two prophages in the same linear structure is confirmed.

Frequency of integration in relation to length of segments of prophage chromosome: In order to have a quantitative estimate of the relative number of integrations into segments of known length, we prepared 19 independent doubly lysogenic strains, as described in MATERIALS AND METHODS. Roughly 200 gal^+ transductants were collected and analyzed for the prophage carried. Altogether 19 independent donors were analyzed, 9 type A and 10 type B.

TABLE 1

Type of prophages found in Gal+ *or* Bio+ *transductants using as recipient strain U335* (gal⁻ bio⁻) *and as donor strain A15. The donor was prepared by superinfecting with* λ sus*24 the E. coli strain C600* (λ sus*54* cl)

Prophage genotype cl	*sus*54	*sus*24	Transductant Gal+	Bio+
+	+	+	36	12
—	—	—	1	8
+	+	—	7	**26**
—	—	+	**50**	4
+	—	—	0	0
—	+	+	9	0
—	+	—	2	2
+	—	+	13	0

The P1 lysate from double lysogen A15 was used to transduce to strain U305 *gal⁻ bio⁻* λs *leu⁻ thi⁻* the genes *gal+* and *bio+*. Approximately the same numbers of *gal+* and *bio+* transductants were found in the two selective media (180 and 140 respectively). Among the transductants, 118 and 52 respectively, proved to be lysogenic. After one purification by restreaking, the transductants were classified, with the results shown. The experiments reported in Table 2 made use of the same strain A15; however, an independent P1 lysate was used on that occasion.

Among the transductants, roughly 60% were nonlysogenic; among the lysogenic there were always some double lysogenic, the maximum incidence among the 19 lysates being 19%. The analysis of monolysogenic transductants is reported in Table 2. In each of the 19 cases there is a class which is largely prevalent over the others. The numbers of transductants in this prevalent class are given in boldface type. We took this class as representing the type of prophage nearest to *gal* and deduced from it the type of integration which occurred. Since the prophage has three markers, there are four possible integration regions (see Figure 1). Integration in *a* and *d* would result in parental type prophages while the others (*b* and *c*) would result in recombinant type prophages.

TABLE 2

Prophage types found in P1 gal+ *transductant from 19 independent doubly lysogenic donors on recipient U 306*

Genotype of prophage found in the transductant			Double lysogen used as donor																		
cl	*sus*54	*sus*24	A1	A3	A4	A5	A6	A7	A8	A11	A15	B2	B4	B5	B9	B11	B12	B16	B20	B27	B28
+	+	+	10	.	7	4	.	.	.	5	7	**28**	2	6	8	4	**67**	**42**	24	**14**	9
—	—	—	5	**28**	4	9	.	**37**	**17**	3	.	.	2	3	.	7	.	2	15	1	14
+	+	—	**52**	4	2	2	5	6	8	**32**	4	4	1	**33**	2	**25**	14	13	**45**	15	**31**
—	—	+	.	2	**47**	**21**	.	2	3	3	**32**	.	**14**	1	**24**	.	.	12	23	**37**	1
+	—	—	1	4	.	.	12	4	4	1	.	.	.	.	.	.	.	7	3	2	.
—	+	+	.	1	1	.	2	3	1	.	2	6	.	.	2	1	9	6	5	6	.
—	+	—	6	2	1	4	**32**	8	.	2	.	2	2	3	.	.	2	2	4	3	4
+	—	+	2	.	7	5	4	1	.	1	4	.	11	2	4	.	.	.	1	7	9
Integration crossover types deduced from prevalent class. Symbols according to Figure 1:			a	c	d	d	b	c	c	a	d	c	a	d	a	d	c	c	d	a	d

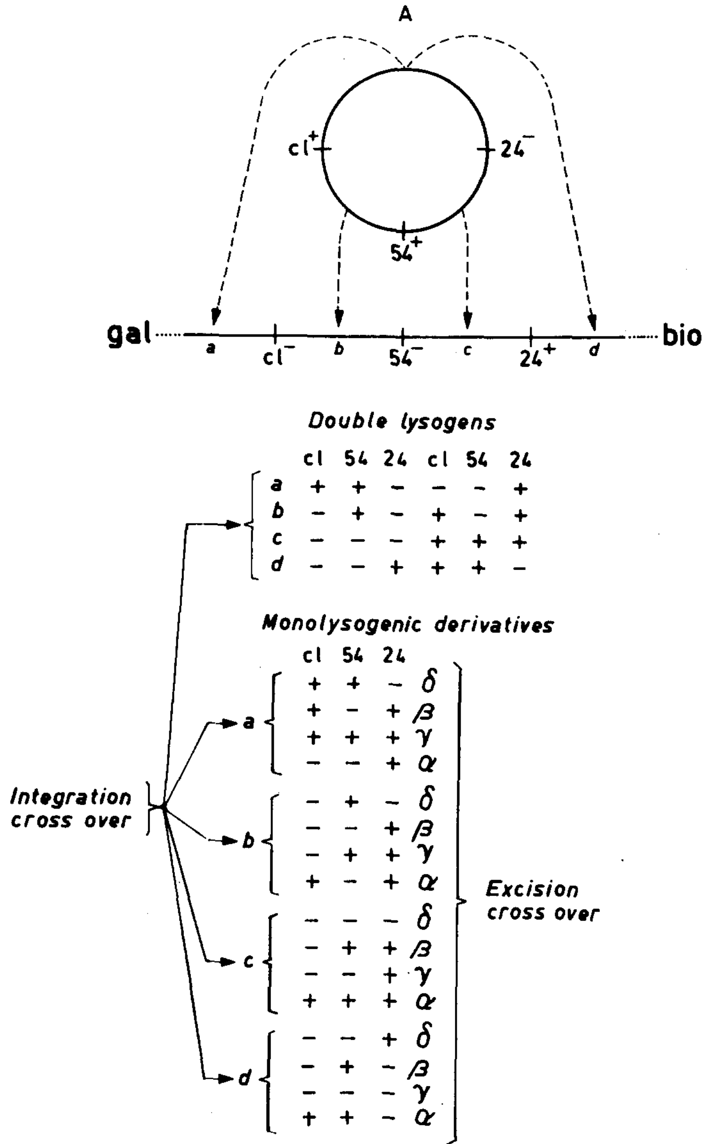

FIGURE 1.—Scheme of integrations occurring with superinfecting phage λ*sus*24 and *E. coli* strain C600 (λ*sus*54 *cI*). Under double lysogens there is the list of all possible types of integration. From these integration types all the monolysogenic derivatives occurring by single crossing over are listed.

The data of Table 2 can then be summarized with the following statement: Some 60% of the integration is outside of the two external markers in our map (*cI*, *sus*24). CIII is the first known gene on the *gal* side, the other is the third from the end on the *bio* side. The rest is distributed 1:6 among two segments whose relative lengths (measured in number of known genes content) are 5:11.

Owing to the relatively small sample, the correlation between gene content and integration of any given segment seems to support the assumption that any part of the lysogenic chromosome which contains λ homologous genetic material can play an equivalent role in the insertion process. These data, however, can be considerably augmented by examining the details of the process which is the inverse of incorporation, namely haploidization of the double lysogen.

Frequency of excision in relation to length of segment of prophage chromosome: Figure 1 diagrams the integration of a superinfecting phage into the chromosome and tabulates both the types of double lysogens and the monolysogens which in turn can be derived from them. The four double lysogens are named after the type of integration crossing over which generates them as *a*, *b*, *c* and *d*. It can be seen that for any of the four types of double lysogens only four types of monolysogenic derivatives are possible by a single crossing over. This imposes a very stringent control to the conclusion previously obtained from the mapping by transduction. In other words, we could take four double lysogens classified as different types of integration crossovers by transduction and analyze the types of haploidization which originate from them. If the previous classification were correct and if the haploidization occurred mainly by a single crossing over, there should be only four major classes of haploids (Figure 1). Double lysogens A1, A6, A7 and A4, which according to Table 2 are classified respectively as *a*, *b*, *c*, *d* integration types, were grown in broth, and plated in tryptone in order to have some 150 colonies per plate. Afterwards, the plates were replica-plated onto other plates seeded with C600. This plating revealed that the lysed areas corresponding to roughly 2.3% of the colonies lost the characteristic mottled appearance and appeared either clear or turbid. Nearly all of these colonies were monolysogens as shown by further tests which demonstrated that they produced only one type of phage. Table 3 gives the final results of such an experiment by classifying all the monolysogens collected. Each of the double lysogens yields chiefly the types predicted by the transduction classification.

Having found a good agreement between integration type and pattern of hap-

TABLE 3

Types of prophage found in monolysogenic segregants from four different double lysogens

Prophage genotype			Double lysogenic strain of origin			
cI	*sus*54	*sus*24	A1	A6	A7	A4
+	+	+	**70**	2	**57**	2
—	—	—	1	7	**37**	**66**
+	+	—	**53**	. .	2	**13**
—	—	+	**39**	**29**	**36**	**64**
+	—	—	7	. .	4	. .
—	+	+	. .	**78**	**66**	9
—	+	—	. .	**89**	4	**36**
+	—	+	**18**	**11**	6	. .
			188	216	206	190

loidization, one can ask whether the four types of monolysogens derivable from each double lysogen occur with a frequency compatible with the length of the segment available for their formation. To answer this question we have classified the data of Table 3 for excision crossing overs, designating each region with a Greek letter to distinguish it from integration crossing over regions:

α	:	β	:	γ	:	$\delta\alpha'$	:	β'	:	γ'	:	δ'
	cI		*sus*54		*sus*24		*cI*		*sus*54		*sus*24	

Once classified (see Table 4), the frequencies from different double lysogens are pooled and the percent of crossing over is computed. Such pooled frequencies represent an estimate of the map lengths of the segments indicated by the crossing over symbols.

The relative percentage of recombination in each region is shown in Figure 2. In the figure all the known genes are shown to allow a comparison of percent of excision crossing over and known number of genes in a region. Also shown is the percent recombination obtained by AMATI and MESELSON (1965) over closely comparable segments in vegetative crosses.

It can be seen that by our measurements, the segments CIII-*sus* R and *sus* R—*sus* K closely resemble in relative length the analogous segments as measured by AMATI and MESELSON. Our data also allow estimation of the two portions of the prophage map lying outside the two extreme markers. These two portions contain region *b*2 of the phage and its bacterial counterpart. If all the chromosome missing in a *b*2 mutant is used in pairing (since the bacterial counterpart must have the same length), we can compute for this portion 40% of the total prophage map. In our map the two segments which include two genes beside *b*2 comprise 55% of the map.

Free phage found in the supernatant of a double lysogen: One might ask which kind of phages are released by a double lysogen of known structure. Since the free phages spontaneously occurring in a lysogenic culture are believed to be present owing to removal of lysogenic immunity in individual cells, one does not

TABLE 4

Frequencies of excision crossover pooled according to the region of excision

Excision crossing-over type	Double lysogenic donors A1	A6	A7	A4		Percent
α	39	78	57	13	= 187	24.5
β	18	29	66	36	= 149	19.58 (4.5)
γ	70	11	36	66	= 183	24. (5.55)
δ	53	89	37	64	= 243	31.7
					762	
Integration crossover type deduced from transduction analysis:	a	b	c	d		

Frequency of excision crossovers pooled according to region of excision (data from Table 3). In brackets in the last column are values normalized for comparison to the data of AMATI and MESELSON (1965).

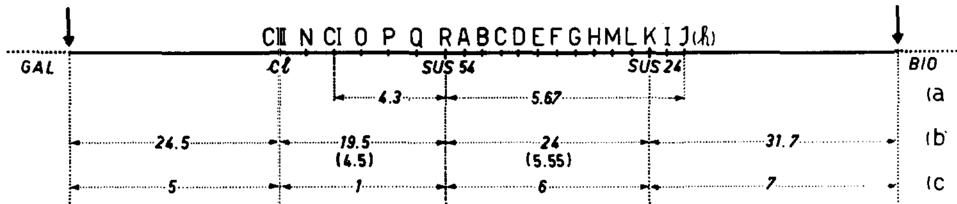

FIGURE 2.—Homology map of λ prophage. (a) Recombination values found by AMATI and MESELSON (1965) normalized. (b) Percent haploidization crossovers, values from Table 5; in brackets are values normalized for comparison with AMATI and MESELSON. (c) Integration crossovers, values from Table 2. Vertical arrows indicate termini of the λ homology in the integrated virus.

expect any correlation between genotype frequency of free phage and monolysogenic phage to be found in the same culture.

During the analysis of double lysogeny, A_4 along with the monolysogens the free phages present in the culture were analyzed. The data are given in Table 5, where frequency of haploid types and frequency of free phage types are compared. The distribution is significantly reciprocal in frequency. This feature, which is not expected on the current theory of prophage regulation, strongly suggests that loss of one prophage occurs by reciprocal crossing over.

DISCUSSION

By a study of formation of double lysogens and their disassembly, data were collected bearing on the mode of integration and excision while the phages are in repressed condition.

By selecting transductant at both sides of the prophages, we confirmed the accepted notion that both prophages in a double lysogen lie on the same linear structure. Indeed, selection of *gal*+ (left side) gave a prevalence of one type of prophage, while selection of *bio*+ (right side) gave prevalence of the reciprocal prophage type. This result also implies that the proposed method of transduction

TABLE 5

Frequency of genotypes of free phage and prophage of monolysogenic segregant in the culture of A1

	Genotypes			
cl	*sus*54	*sus*24	Prophage in the monolysogenic segregants	Free phage in the supernatant
+	+	+	70	7
—	—	—	1	97
+	+	—	53	18
—	—	+	39	31
+	—	—	7	2
—	+	+	0	8
—	+	—	0	31
+	—	+	18	5
			188	199

into sensitive cells is acceptable. In a previous paper (CALEF *et al.* 1965), the possibility that this method might be misleading as to the linkage relation between a bacterial marker and the prophages was discussed. The result obtained here would have been highly improbable if either the hypothesis underlying the method or the theory about the structure were not as postulated.

The main part of the work consisted of mapping several double lysogens. This mapping was obtained by P1 transduction and by haploidization. The first method is well known and has been described previously (JACOB 1955; ROTHMAN 1965; IKEDA and TOMIZAWA 1965). By this method one can evaluate the number of integration crossovers occurring at any given point. The method itself does not allow analysis of a large number of double lysogens and the figures obtained have a qualitative value rather than quantitative. In spite of this limitation, the comparison made between the number of integrations at any given segment and gene content of the segments appears to be a good support to the assumption that the superinfecting phage does not distinguish between standard attachment sites and other λ homologous parts.

Mapping by haploidization exploits the zygote-like nature of the double lysogens. Here, as in any genetic mapping, the haploidization gives the order of the genes and the distances between them, and also measures the amount of viral genetic material outside the two extreme markers. This last feature is especially significant in that it also measures the λ homologous region outside the two extreme markers. For this reason this type of mapping can be called "homology mapping".

Before analyzing the outcome of the experiments some aspects of the method of homology mapping will be considered, which might not be obvious. Two crosses are required to obtain the data used in this mapping. The first cross results in the integration of the second prophage; the second cross results in the excision leading to haploidization. The first cross can produce (given parents differing for three markers) four different sequences of these markers; two of these might be called "parental", two "recombinant". The "recombinant" sequences are those in which the integration occurs inside the markers of the first prophage, and in this case the genotype of the first prophage cannot be found in a continuous stretch along the sequence. During the second cross (excision) the four sequences will generate the haploid types of monolysogens which can be used in the analysis.

The experiments reported show that the segregation of phage from the double lysogens follows rules imposed by the type of integration. The types of prophage left integrated by the haploidization process are not random but belong almost exclusively to only four genotypic classes for each type of double lysogen. The pattern of haploidization sketched in Figure 1 is valid.

Let us now turn again to the question of the mode of integration of the second prophage. From the results of this paper we confirm the conclusion reached previously (CALEF *et al.* 1965) that integration of the second prophage into the first prophage is a common occurrence. We now have quantitative estimates of the frequency of recombination in given regions.

Our prophage was subdivided by the three markers into four regions, whose relative lengths can be estimated. Of these four regions *a* is without markers, *d* contains two known genes, and both *a* and *d* share the so-called *b*2 segment (KELLENBERGER, ZICHICHI and WEIGLE 1961). We estimated their combined lengths as approximately 40% of the prophage map. This value agrees nicely with the 50% value found.

The other two regions *b* and *c* cover respectively 4 and 5.25 recombination units (AMATI and MESELSON 1965). For these regions the relative frequencies of integration are respectively 1 and 6. We have respectively 19.58 and 24% of the total excision occurring in these regions. These figures conclusively indicate that the prophage chromosome is available for crossing over with homologous parts, and that its proneness to do so is uniform over all its length.

In this context, the question appears important whether integration in absence of immunity occurs by a special process affecting only the bacterial and phage homologous regions. The work of L. FISCHER-FANTUZZI (1966) and E. SIGNER (personal communication) suggests that a specific phage function is involved in lysogenization. Moreover, we know that hindrance in superinfection lysogenization is of an indirect or physiological nature (CAMPBELL and ZISSLER 1966; DAHL and CALEF 1966). Hence we can entertain the idea that the immunity is exerting its influence in lysogenization by blocking a special phage function needed for specific high efficiency integration or/and by preventing the accomplishment of some structural prerequisite to integration.

Finally, let us consider the observation regarding the frequency of free phage types in the supernatant. The distribution of phage types is definitely the reciprocal of the distribution of haploid segregants in the same culture. This fact strongly suggests that the crossing over leading to haploidization is of reciprocal type. In this fact there seems to be also another important implication; indeed it appears that in order to have a correlation such as the one found, it is necessary for the two distributions to originate from the same event, which in turn points out strongly that the decision to lyse follows rather than precedes excision.

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SUMMARY

Evidence is given that: (1) the two prophages in a double lysogen are linearly inserted in the bacterial chromosome; (2) integration in a lysogenic bacterium of a superinfecting phage at different segments of the prophage is in agreement with the length of the segments; (3) the frequency of haploidization crossing over at any given segment can be a measure of the prophage map and gives values useful for comparison with the values of segment lengths which were measured by other studies; (4) the loss of one prophage (excision) from double lysogens is analogous to the process of integration and occurs by reciprocal crossing over.

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