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In Vitro Genetic Recombination of Bacteriophage λ

(phage λ DNA/*in vitro* head assembly/site specific recombination)

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ABSTRACT **DNA of bacteriophage λ recombines in a cell-free extract prepared from an induced *Escherichia coli* lysogen of bacteriophage λ. The assay for recombination *in vitro* takes advantage of the ability of such an extract to package λ DNA and to assemble complete phage particles. For example, when λ DNA that has been extracted from phage with the immunity of 434 is added to an extract, infectious λ *imm* 434 particles are produced. The precursor DNA molecule in this packaging reaction is a multichromosomal polymer; circular monomers, for example, are not packaged.**

Nevertheless, when 434 circular DNA monomers are added to an extract, some phage that contain the *imm* 434 marker are produced. In this case, the circular DNA had recombined with λ DNA in the extract and thereby had become part of a polymeric structure, which by the normal packaging process could give rise to infectious particles with the *imm* 434 marker. Genetic recombination is demonstrated when *imm* 434 circular monomer DNA carries amber mutations in genes *A* and *B*; then most of the 434 plaque formers produced *in vitro* are A^+B^+, the genotype of the endogenous λ DNA. Genetic crossing-over occurs through a region that contains the prophage attachment site, suggesting that recombination is carried out by the λ Int functions. The 434 recombinant plaque formers are particles physically identical to wild-type 434 particles, as judged by their buoyant density in a CsCl equilibrium gradient.

Bacteriophage λ can undergo genetic recombination by three known pathways. The bacterial host specifies the Rec pathway. The phage codes for the Red pathway, which mediates general recombination (1, 2), and the Int pathway, which combines DNA at the prophage attachment site, and is responsible for integration of prophage during lysogenization (3).

The biochemical steps involved in recombination are only beginning to be understood. An exonuclease has been identified with the λ *red α* gene (4, 5), and another exonuclease has been associated with the host *recBC* genes (6, 7). Biochemical elucidation of genetic recombination has been hampered by the paucity of recombination-related activities that can be measured *in vitro*. Recombination between different DNA molecules that relies entirely on the functions provided by a genetically defined pathway has not been accomplished. A major difficulty with a direct attempt of this type is the lack of a simple assay for recombinant DNA.

In the present communication, I would like to describe a cell-free system that can carry out recombination between genetically distinct λ DNA molecules. The recombinant DNA so formed is then packaged into phage particles by *in vitro* viral head assembly. Recently Kaiser and Masuda showed that λ DNA added to a cell-free extract of an induced λ lysogen would be packaged into infective phage particles (8). Using that procedure, I then sought and found conditions in which recombination could proceed in the extract.

MATERIALS AND METHODS

Bacterial Strains and Phage. The heat-inducible strain W3101 (λ *CIts857 Sam7*), W3101 (λ *b506 CIts857 Sam7*) (11), or W3101 (λ *Jam27 CIts857 Sam7*) was used for preparation of the extract. *Y* Mel (λ), W3101 (λ *Aam32*), or W3101 (λ *Jam27*) was used as indicator bacteria to titer the various heteroimmune phage. λ *Aam32 Bam1 imm434 Cts56* (referred to here as A^-B^-434) was the source of *imm* 434 DNA.

Preparation of DNA. Linear *imm*434 DNA is extracted from a high-titer stock of λ *Aam32 Bam1 Cts56 imm*434 with phenol. Monomer circles of this DNA are prepared according to the procedure of Wang (9) as follows: The strain Hfr 64 *endo* 1^- is infected at a multiplicity of 20 with λ *Aam32 Bam1 imm434 Cts56* in the presence of chloramphenicol. After 1 hr at 37°, the infected cells are harvested and the super-coiled λ DNA is purified.

Preparation of Cell-Free Extract. The procedure given by Kaiser and Masuda (8) is used with the following exceptions. The heat-inducible lysogen is heated at 42–43° for 10 min to induce the prophage. After 40 min at 37°, the cells are harvested. In some experiments after the cells had lysed, cell debris and host DNA are pelleted by centrifuging at 30,000 × *g* for 5 min. The extremely gelatinous pellet occupies about half of the volume. The supernatant fraction is withdrawn and used as such.

Recombination Assay. To 100 μl of the supernatant fraction is added 0.04–0.4 μg of DNA (either circles or linear prepara-

TABLE 1. *DNA substrate for in vitro packaging*

λ *imm* 434 DNA preparation	*imm* 434 plaques/0.1 μg of DNA
Annealed mixture with circular and linear aggregates*	14,000
Linear monomer†	50
Covalently-closed monomer circle‡	3

The DNA preparation is incubated with the extract for 1 hr at 37° before the addition of DNase I.

* A solution of A^-B^- 434 DNA at 40 μg/ml, which had been extracted from phage with phenol, was allowed to anneal by incubating at 47° for 2 hr then at 42° for 2 hr in 0.13 M KCl, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0 (15).

† The same DNA preparation used in (*) was heated at 70° for 10 min and cooled quickly on ice immediately before it was added to the extract.

‡ A^-B^-434 circular DNA prepared as described in *Methods*.

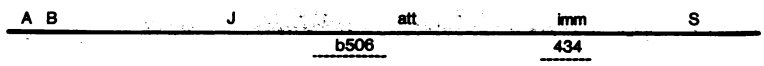

FIG. 1. Genetic map of bacteriophage λ, which shows the location of markers used in this study. *Broken lines* below the map give the extent of the deletion and deletion-substitution.

tions). The mixture is incubated at 23° for 60 min, then at 37° for 60 min at which time 200 μl of tryptone broth containing DNase I at 15 μg/ml is added. When the exogenous DNA is from A^-B^-434, the total number of 434 particles produced in the extract is determined by plating on Y mel (λ) while the number of 434 am^+ particles is determined by plating on the su^- strain, W3101 (λ *Aam*32).

RESULTS

Packaging Requires Polymeric DNA. When DNA that has been extracted from purified phage λ *imm*434 particles is added to the extract described above and allowed to incubate for 1 hr at 37°, infectious phage are produced that show the immunity of 434 (8). The DNA extracted from phage particles consists of double-stranded DNA molecules with identical cohesive ends. Such a DNA preparation consists of a mixture of linear monomers and monomer circles and polymeric structures held together through the cohesive ends. The data presented in Table 1 show that DNA that is packaged arises from the polymeric structures in the mixture. DNA with annealed cohesive ends has the highest activity; linear monomers only 0.5% as much; and circular monomer has the lowest activity. Therefore, the substrate for packaging in an active DNA preparation must be a dimer or a higher aggregate. This shows that the substrate for DNA packaging in the *in vitro* system is the same as in the bacterial cell. It has been shown with λ that monomer circles in infected cells will not package; however, if they either recombine with λ DNA or are replicated to concatamers, packaging of the DNA will occur (12–14).

DNA Circles Recombine with Endogenous λ DNA In Vitro. To assay for recombination *in vitro*, advantage is taken of the fact that cyclic monomers do not package directly into infectious phage particles. The rationale is as follows: If a monomer circle can recombine with another DNA molecule and thereby become part of a polymeric structure, it should become a substrate for packaging. To demonstrate recombination, 434 DNA with amber mutations in genes *A* and *B* (Fig. 1) is added to an extract of an induced λ lysogen. The extracts contain endogenous λ DNA that is A^+B^+ in the form of circles and linear polymers (10). If the circular exogenous A^-B^-434 DNA recombines with the endogenous λ DNA and is converted into a packageable substrate, some 434 am^+ particles should be formed.

TABLE 2. *Circular 434 DNA recombines with endogenous λ DNA*

λ *imm* 434 DNA preparation	*imm* 434 plaques/0.1 μg of DNA (time at 23°)			
	For 0 min		For 60 min	
	No.	% A^+B^+	No.	% A^+B^+
A^-B^- 434 linear	29,000	0.1	3,100	2.6*
A^-B^- 434 circular	44	60	185	77†

* The percent sus^+ was determined from the ratio of titer on W3101 (λA^-)/Y mel (λ).

† Plaques that appeared on Y mel (λ) were carried through one single plaque isolation on Y mel and tested against their ability to plaque on Y mel (λ) and W3101 (λA^-).

Table 2 shows that linear A^-B^-434 DNA gives rise to A^-B^- phage particles; only 0.1% are A^+B^+. Circular A^-B^- 434 DNA, however, generates A^+B^+ phage particles although the total number of *imm 434* phage is greatly reduced. Incubating the extract–DNA mixture at 23° before the 37° packaging incubation increased the yield of recombinants 4-fold. The 23° incubation also increases the yield of recombinants from linear DNA, although the total number of 434 plaques is reduced. From the circular DNA the absolute number of 434 DNA-containing phage increases after the 23° preincubation and the frequency of recombinants increases slightly. These results appear to justify the original idea; circular DNA may be converted into a packageable substrate after a recombination event *in vitro*.

In Vitro Recombination Is Site Specific. The generation of 434 am^+ phage from A^-B^-434 circular DNA shown in Table 2 indicates that crossing-over had occurred between *B* and *imm* (see Fig. 1). To test whether recombination might be specific to certain regions of the λ chromosome, additional crosses were conducted. As shown in Table 3, when the λ DNA in the extract contains an amber mutation in gene *S*, all of the 434 packaged were S^+. Therefore, the crossover event that integrates the cyclic monomer DNA molecule into some higher aggregate occurs between gene *B* and the immunity region but not between gene *S* and immunity (see Fig. 1). When the 434 DNA circles were added to an extract that contained

TABLE 3. *Genetic cross-over shows map preference*

Genotype of exogenous circular DNA	Genotype of endogenous DNA	*In vitro* packaged 434 phage	
		Genotype	Frequency
A^-B^- 434 S^+	λ S^-	am^+	77%
		A^-B^-	23%
		S^-	0/70
A^-B^- 434 S^+	J^- λ S^-	J^-	61%
		A^-B^-	31%
		am^+	7%
		S^-	0/92

When the extract was prepared from the induced J^- lysogen, 10 μl of extract prepared from W3101 (λ *Eam*4 *CIts*857 Sam7) was added after the addition of DNase I as a source of phage tails. In each case the DNase-resistant heteroimmune phage were detected by plating on Y mel (λ). After one single plaque isolation, individual plaques were tested for the ability to plate on Y mel, C600, or W3101. All phage plate on Y mel (contains *sup F*); the A^-, B^-, or J^- but not S^- will plate on C600 (contains *sup E*); and only am^+ will plate on W3101 (*sup*°). J^- 434 and A^-B^- 434 were distinguished by complementation on the indicator bacteria W3101 (λA^-) and W3101 (λJ^-). The 7% am^+ 434 phage produced in the second entry of the table were probably due to A^-B^- 434 or J^- 434 phage recombining with the prophage during the first plating on Y mel (λ).

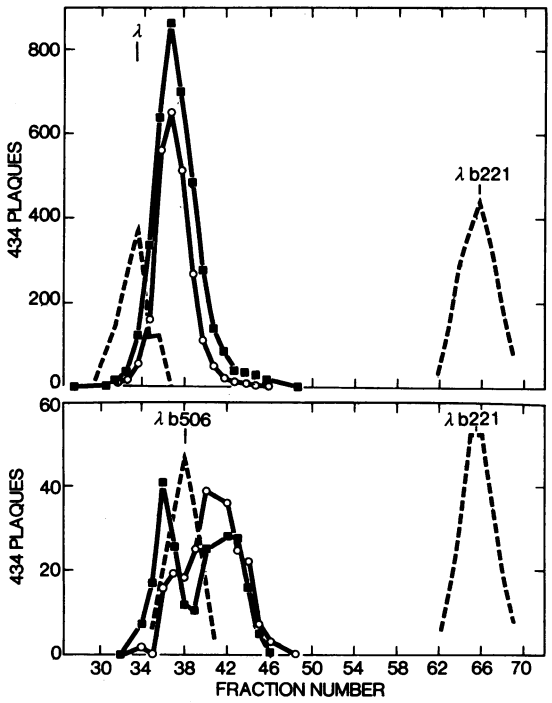

FIG. 2 (*top*). Density distribution of *in vitro* recombinants in a CsCl equilibrium gradient. Phage titers are given as a function of fraction number where the larger fraction number gives lower densities in the gradient; 0.07 ml of circular A^-B^- 434 DNA was added to 1.7 ml of extract containing λ *CI* 857 *Sam7* DNA. After the incubation period, the total volume was brought to 4.19 ml with buffer (20 mM Tris·HCl; 10 mM $MgCl_2$, pH 7.6), when 3.11 g of CsCl were added. The sample was centrifuged at 20,000 rpm for 48 hr at 5° in an SW39 rotor. The tube was pierced through the bottom and 5-drop fractions were collected into 0.1 ml each of T br. Total number of 434 phage in 0-05 ml of each fraction (■) or the number of am^+434 (○) in 0.05 ml are given against fraction number. (- - -) gives the distribution of phage λ found in the extract and the λ *b*221 added as a reference.

FIG. 3 (*bottom*). 0.04 ml of circular A^-B^- 434 DNA is added to 1.1 ml of extract derived from a lysogen whose prophage contains λ *b*506 *CI* 857 *Sam7*. Other steps are the same as in *legend* of Fig. 2.

polymeric λ DNA with the *Jam*27 allele, as in the experiment presented in the second line of Table 3, very few 434 am^+ particles were recovered; the majority were J^-. Thus, the crossover event frequently occurs between *J* and *imm* but rarely between *B* and *J*.

Because λ has a special system for recombination at the attachment site located between *J* and *imm* that is mediated by the λ *int* gene product, the results described above strongly suggest that *in vitro* recombination is occurring at the attachment site. If the generalized recombination pathways (the bacterial Rec or lambda Red) were responsible, crossover events between all of the markers tested would be evident; however, this is clearly not the case. In fact, when an extract prepared from *rec*A^- *E. coli* that has been infected with λ *red*$^-$ is used in preparation of the extract, an *in vitro* recombination activity is still present (data not shown).

Recombinant Phage Particles Contain a Single Chromosome of Normal Length. Recombinants are detected as plaques on *sup*° lambda lysogens. To show that the particles responsible for these plaques are, in fact, true haploid recombinants and not some type on aberrant particle containing two phage chromosomes that recombine in the indicator bacteria, the distribution of the recombinant particles in a CsCl sedimentation equilibrium gradient was measured as is shown in Fig. 2. Normal phage particles have a characteristic buoyant density that measures their DNA and protein content. Since the chromosome of *imm* 434 is 2% shorter than λ, 434 particles have a slightly lower density than λ particles. The distribution of λ *b*221 particles (whose DNA is 22% shorter than λ b^+) is also given in the figure; these were added as a density reference. Fig. 2 shows that the 434 particles produced *in vitro* have a slightly lower density than the λ particles, as expected for native 434 particles. Furthermore, the 434 am^+ particles (i.e., the recombinants made *in vitro*) show the same distribution through this region. This result indicates that the 434 am^+ recombinant is a phage particle containing a single 434 chromosome and a normal amount of protein.

This important point was tested in another way with an extract of cells lysogenic for λ *b*506. The *b*506 deletion is located just to the left of the attachment site on the λ chromosome (11). If *in vitro* recombination is *att* specific, all 434 am^+ recombinants should also be *b*506. Thus, when A^-B^- 434 circular DNA is recombined and packaged in an extract whose endogenous DNA is A^+B^+ *b*506, the 434 am^+ recombinant particles should have a lower density than either the A^-B^- 434 or λ *b*506 parents. This expected result is in fact obtained, as shown in Fig. 3. There are two peaks of 434 plaque-formers, one at a higher density than λ *b*506 and one at a lower density. The 434 am^+ recombinant particles occupy the peak of lower density; the region of the gradient where *b*506 434 particles are expected.

The high-density *imm* 434 peak contains nonrecombinant am^- particles. They may have arisen by the 434 DNA circles that recombine with one another or perhaps by some direct packaging mechanism that remains to be understood. The peak of 434 am^+ plaques in Fig. 3 has a shoulder toward higher density. These high-density 434 am^+ plaques probably do not represent recombinant particles made *in vitro;* more likely they are caused by recombination between λ *b*506 and A^-B^- 434 on the assay plate. The shoulder occurs where the higher density *imm* 434 peak (*solid squares*) overlaps the λ *b*506 peak, which rises to a height of 9×10^{11}/ml. Because the *imm* 434 particles are at a much lower concentration, plating at a dilution appropriate for the *imm* 434 results in mixed infection with A^-B^- 434 and λ *b*506. For example, in fractions 36 and 37 the multiplicity of λ *b*506 particles to indicator bacteria was 40 and 60. This means that in those fractions every A^-B^- 434 particle infects a cell that is also infected with many λ *b*506 particles; consequently the probability of recombination occurring under these conditions is high. Reconstruction experiments are consistent with this interpretation. The main peak of am^+ 434 particles (*open circles*) cannot be explained by recombination on the indicator bacteria; the titer of λ *b*506 is too low. This fact further supports the notion that the *in vitro* recombinants do indeed contain a single chromosome packaged in normal λ proteins. It also shows that genetic crossover occurs in the region between the *b*506 deletion and *imm*, which is consistent with an *att* specific recombination event.

RNA Polymerase and DNA Ligase Are Not Required for In Vitro Recombination. It seemed possible that the enzymes

TABLE 4. *Effect of rifampicin and nicotinamide mononucleotide on in vitro recombination*

	434 plaques/0.1 μg of DNA	(% am^+)
No additions	103	84
+ Rifampicin (10 μg/ml)	150	81
+ Nicotinamide mononucleotide (0.5 mM)	87	95

Rifampicin or nicotinamide mononucleotide is added to 50 μl of extract to give the indicated concentration. After 5 min on ice, 0.3 μg of A^-B^- 434 circular DNA is added. In a parallel set of assays (not shown), where covalently-closed 434 DNA polymers were tested for their ability to package into infectious particles, the nicotinamide mononucleotide caused a slight inhibition whereas the rifampicin stimulated the DNA packaging activity 4-fold.

RNA polymerase and polynucleotide ligase may be involved with the *in vitro* recombination. Therefore, inhibitors of these enzymes were tested for their effect on the recombination activity. High concentrations of nicotinamide mononucleotide, which inhibits the *E. coli* polynucleotide ligase (16), and rifampicin, a specific inhibitor of RNA polymerase (17), were used. As is shown in Table 4, neither of these ligands blocks the recombination activity. It was of interest to see if RNA polymerase was involved in this recombination because earlier reports based on studies *in vivo* have shown that transcription of the *att* region is required for integration and excision of prophage (18, 19). Apparently, the *in vitro* site specific recombination does not require transcription.

DISCUSSION

Recombination is understood to be a process that gives rise to normal chromosomes carrying genes derived from different parents. This implies two criteria by which to judge the adequacy of a claim that a cell-free system exhibits recombination. First, is the combination of genes contained in a single haploid phage structure? This question has been approached in some detail here. It is a well-established fact that the phage λ virion is haploid; it contains a single DNA molecule representing a single chromosome. If it can be shown that the putative recombinant particle is physically identical to the native phage particle, we will have established that the recombinant is haploid. This appears to be true. The recombinant 434 am^+ particles, as shown in the experiment in Figs. 2 and 3, have the bouyant density expected for the particular native 434 particles.

Second, we can say that the genes are indeed derived from different parents. The immunity region in the recombinant is derived from the purified DNA circle that is added to the extract, while the markers on the left arm, which become associated with the immunity marker, reflect the genotype of the endogenous λ DNA present in these extracts.

Granted that recombinants are produced in the extract, by what mechanism do they arise? The location of recombination implies that it results from the action of the attachment site specific recombination system. Crossing-over occurs between gene *B* and *imm* or between gene *J* and *imm*, but not between gene *B* and gene *J* nor between gene *S* and *imm*. Furthermore, when the endogenous λ DNA is deleted for *b*506, the am^+ 434 recombinants have the buoyant density of *b*506 *imm* 434, as demonstrated in the experiment of Fig. 3. This places the crossover site between the *b*506 deletion and *imm*, which contains *att*, the known target of attachment site specific recombination promoted by the λ *int* gene. Since no other examples of site specific recombination are known for lambda, we may presume recombination occurs by *int*. The possibility remains, however, that some unknown system, unique to our cell-free extracts, is responsible for the preferential recombination.

Thus far, the *E. coli* enzymes, polynucleotide ligase and RNA polymerase, do not appear to be required for the *in vitro* recombination. Preliminary observations indicate that nicotinamide mononucleotide, an inhibitor of DNA ligase (16), and rifampicin, an inhibitor of RNA polymerase (17), have no effect upon the formation of recombinants. This points to the possibility that if covalent resealing of the DNA is occurring at the synapse (a point not tested), then some factor, other than *E. coli* DNA ligase, is joining the DNA.

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