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A Recombination Function Essential to the Growth of Bacteriophage P22

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Mutants of the *Salmonella* phage P22 have been isolated which cannot grow after infection of recombination-deficient (*rec*$^-$) hosts. These mutants (designated *erf*$^-$ for essential recombination function) are defective in a phage-specified recombination system, as shown by two- and three-factor crosses carried out in *rec*$^+$ and *rec*$^-$ hosts. Four *erf*$^-$ mutants examined in detail fall into one complementation group; *erf*$^+$ is dominant to *erf*$^-$. The *erf* gene is located in the "early" region of the phage genetic map.

The phenotype of *erf*$^-$ mutants after infection of *rec*$^-$ hosts includes failure to grow, to lysogenize, and to sustain phage DNA synthesis beyond about one round of replication. Late phage functions appear to be expressed normally by *erf*$^-$ mutants.

Circularization of the infecting circularly permuted, terminally repetitious phage DNA seems to be the essential step for which recombination is required. This idea is supported by the observation that an *erf*$^-$ phage can grow normally in *rec*$^-$ cytoplasm after induction of a lysogen; in this case the DNA is presumably circularized by the prophage excision mechanism.

We conclude that recombination is essential to growth because the linear infecting DNA must be circularized by recombination. The inability to sustain DNA synthesis in the absence of recombination suggests that circularization is topologically essential to successful replication of the phage genome.

1. Introduction

Each stage in the life cycle of the temperate *Salmonella* phage P22 is associated with a characteristic form of the phage DNA. The DNA found in mature phage particles is a single linear duplex (mol. wt 26 to 27 million) which is circularly permuted and terminally repetitious in its nucleotide sequence (Rhoades, MacHattie & Thomas, 1968). During the lytic propagation of the phage, continuous linear duplex phage DNA molecules two to five times the length of the mature form are observed (Botstein, 1968; Botstein & Levine, 1968*a*). These appear to be intermediates, and as such can account for the propagation of circular permutation and terminal repetition in the mature form of the DNA (Botstein & Levine, 1968*b*; Streisinger, Edgar & Denhardt, 1964; Séchaud *et al.*, 1965; Streisinger, Emrich & Stahl, 1967). During infections leading to lysogeny, closed circular duplex molecules of phage DNA are observed (Rhoades & Thomas, 1968). Apparently these are intermediates in the process of prophage integration as predicted by the Campbell model (Campbell, 1962).

One simple way to account for the metamorphosis of the mature DNA form into the circular or oversize form is to envision recombination events inside the terminal repetition. Recombination of a molecule with itself can produce a circular form. Recombination between two identical daughter molecules can produce a dimer. An alternative possibility is that oversize molecules are produced by some sort of rolling circle mechanism (Gilbert & Dressler, 1968). In this case a circular form must at some point be formed, presumably again by a recombination event. DNA replication would, in this case, be dependent upon recombination.

These considerations lead to the expectation that phage P22 cannot grow or lysogenize in the absence of recombination. Since phage P22 grows, recombines and lysogenizes in a recombination-deficient (*rec*$^-$)† host (Wing, Levine & Smith, 1968; Wing, 1968) the phage must direct the synthesis of a recombination system. A phage which has lost, by mutation, this recombination system would be expected to be unable to grow on a *rec*$^-$ host, but might well be able to grow on a *rec*$^+$ host.

We have isolated and characterized mutants, called *erf*$^-$, which have this phenotype. We report some of the properties of these mutants, supporting the idea that recombination is required for the metamorphosis of the mature phage DNA into circular or oversize molecules. The behavior of these mutants also indicates that DNA replication is specifically and characteristically affected by the defect in recombination, in a way which strongly suggests that circularization of infecting phage DNA is the critical step which requires recombination.

2. Materials and Methods

(a) *Bacterial strains*

All strains are derivatives of *Salmonella typhimurium* LT2. Table 1 summarizes the genotypes of strains used. DB25 is a thymine-requiring strain made from DB21 by selection with trimethoprim (a gift of Burroughs Wellcome & Co.) followed by selection for growth on 5 μg thymine/ml. DB31A is a fast-growing derivative of the *proA15 rec*$^-$ strain described by Wing *et al.* (1968). DB47 is a *rec*$^-$ prototroph made by mating DB43 (HfrB2 *rec*$^-$*metA Sm*^{r}; made by J. Wing) with DB25 for 15 min and selecting *thy*$^+$*rec*$^-$*Sm*^{s} progeny. It is thus nearly isogenic with DB21. DB74 was made by transduction of DB53 simultaneously to *cys*$^+$*his*$^+$ with *int4* (L4 of Smith & Levine, 1967) phage grown on DB38. It was tested for *su*$^+$ using *am* phage mutants. DB77 was made by selecting a *thy*$^-$ derivative of DB74, followed by a 15-min mating with DB43 and selection of *thy*$^+$*rec*$^-$*Sm*^{s}*su*$^+$. The suppressor phenotype was checked by using *am* phage mutants and the *rec*$^-$ phenotype by ultraviolet sensitivity as well as by inability to be transduced to streptomycin resistance.

(b) *Phage strains*

All phages are derivatives of phage P22 maintained by Dr M. Levine. The amber mutants were isolated by Mr Russell Chan in this laboratory: *am* N10 is in gene *1*, and a defect in this gene causes formation of empty heads; *am* N8 is in gene *5*, which appears to be the structural gene for the major head protein (Botstein & Levine, unpublished results). The *ts* mutant strains come from the collection of M. Levine. *ts* 21.1 and *ts* 6.1 are located in different early genes and were mapped first by Dr M. Gough. The *int* mutants were the gift of Dr H. O. Smith and are the L mutants of Smith & Levine (1967). The clear mutant alleles derive from the strains of Levine & Curtiss (1961). c_1 is c_1^7, c_2 is c_2^5, the temperature-inducible strain *ts* c_2^{29} is described by Levine & Smith (1964).

† Abbreviations used: General: *am*, amber nonsense mutation; *ts*, temperature-sensitive mutation. Phage markers: *int*, prophage integration; *sie*, superinfection exclusion; *erf*, essential recombination function; *c*, clear plaque. Bacterial markers: *cys*, *his*, *leu*, *met*, *pur*, *thy*, requirement for cysteine, histidine, leucine, methionine, purines, thymine; *su*, presence (*su*$^+$) or absence (*su*$^-$) of a nonsense (*am*) suppressor; *Sm*, resistance (*Sm*^{r}) or sensitivity (*Sm*^{s}) to streptomycin.

TABLE 1

Bacterial strains

	rec	Amber suppressor	Markers	Synonyms	Source
DB21	+	—	prototroph	strain 18	Botstein (1968)
DB25	+	—	*thy* 2	strain 18T⁻2	This paper
DB31A	−(W)†	—	*pro*A15*Sm*^{R}	—	J. Wing
DB28	+	*su*⁺527-1	*his*C527	—	Whitfield, Martin & Ames (1966)
DB30	+	—	*his*C527	—	Whitfield, Martin & Ames (1966)
DB38	+	*su*⁺19	*pur*H190	—	J. Gots
DB47	−(W)†	—	prototroph	—	This paper
DB53	+	—	*cys*A1348*his*C527	TR248	J. Roth
DB74	+	*su*⁺19	*cys*A1348*his*C527	—	This paper
DB77	−(W)†	*su*⁺19	*cys*A1348*his*C527	—	This paper
SB847	−(T)†	—	*his del* 22	—	P. Hartman
AE5073	−(E)†	—	*his del* 64	—	A. Eisenstark

† Abbreviations used: W, the *rec*⁻ allele described by Wing *et al.* (1968) and used throughout this work; T, the *rec* 101 allele of Takebe; E, the *rec* 13 allele of Eisenstark.

Strains carrying combinations of markers were made as needed, in all cases by recombination among existing strains. All stocks derive from single plaques after purification by single-plaque isolation at least twice.

(c) *Media*

M9CAA is described by Smith & Levine (1964), LB broth is described by Levine (1957), buffered saline by Botstein (1968). Agar plates are green indicator plates (Bresch, 1953; Levine & Curtiss, 1961) or λ plates (Signer & Weil, 1968); in either case, soft nutrient agar (Levine, 1957) was used. Dilutions are made in growth medium, buffered saline, or in DF (0·85% NaCl + 0·1% Difco nutrient broth).

(d) *Preparation and assay of phage stocks*

Assay of phage is described by Levine (1957). High-titer stocks were grown by suspending single plaques in 250-ml. Erlenmeyer flasks containing 30 ml. LB broth and 0·5 ml. of overnight culture of the appropriate permissive host (DB74 in most cases). After incubation at 25°C for 12 to 16 hr, chloroform was added, and after 10 min the usually turbid suspension was centrifuged at low speed to remove cells and debris. The supernatant fraction was centrifuged at 37,000 *g* for 70 min. The phage were resuspended in 2 ml. of buffered saline and stored over chloroform in the cold. The final titer was usually about 10^{12} phage/ml.

(e) *Mutagenesis and isolation of mutants*

The mutagenesis procedure was adapted from Parkinson (1968). Strain DB28 or DB38 cells were infected in LB broth at 37°C with 1 phage/cell. 10 min after infection, 0·1 to 2 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/ml. (Aldrich Chemical Co.) was added. After lysis, chloroform was added and cell debris removed by centrifugation. A mutagenized stock typically contained 0·2% clear mutants. The phage were diluted and adsorbed to 10^7 exponential DB28 or DB38 (*rec*⁺*su*⁺) cells and layered on a λ-agar plate. As soon as the agar hardened, a second layer containing 2 to 5×10^7 DB31A or DB47 (*rec*⁻*su*⁻) cells was added. These plates were incubated for 20 hr at 25°C and plaques which looked faint or uniformly turbid were picked with toothpicks and stabbed onto plates seeded with DB28, DB31A or DB21 cells. *erf*⁻ Mutants (unable to grow on *rec*⁻ cells) and *amber* mutants (unable to grow on *su*⁻ cells) were distinguished in this way. Mutants were purified by streaking on the permissive host 2 or 3 times.

(f) *Standard cross*

The method is described by Gough & Levine (1968). In most cases this procedure was modified by using exponential phase cells grown in M9CAA medium and concentrated to about 6×10^8 cells/ml. by centrifugation and resuspension in buffered saline. Since the yield of phage in *erf*$^-$ × *erf*$^-$ crosses is very low, the dilutions after adsorption were only 1/1000. Plating was done immediately in order to minimize loss of titer due to the residual antiserum. In all crosses the unadsorbed phage before and after incubation with antiserum was measured, as well as the number of infective centers after treatment with antiserum. The input ratio of parental phage was measured directly by plating the mixture of phage under permissive conditions and counting the relative numbers of clear and turbid plaques. Sometimes the entire procedure was carried out using LB broth instead of M9CAA medium as the growth medium. In crosses in which morphological markers can be scored, the frequencies of recombination measured correspond well with the ones reported by Gough & Levine. In particular, in all mapping crosses these frequencies were checked after plating the cross under totally permissive conditions.

(g) *Test for immunity and integration*

Putative lysogens were streaked across a line of 10^8 c_2 phage on indicator plates. Interruption of growth or a dark green zone of lysis indicates sensitivity. In tests for lysogenization, surviving infected cells were plated together with antiphage serum (final value of K, 2 min^{-1}) and the colonies later tested for immunity.

(h) *DNA synthesis rate determination*

The method of Smith & Levine (1964) was used. These experiments were done at 30°C in order to improve resolution of the fluctuations in rate characteristic of c_2 mutants.

(i) *Transmission experiments*

Cells were prepared as described for the standard cross and infected at the desired multiplicity, treated with antiserum ($K = 5$) for 10 min, diluted in isothermal medium and plated on the permissive host.

(j) *Single-burst experiment*

The procedure is the same as for the transmission experiments, except that the cells were diluted to approximately 3 infective centers/ml. This dilution was distributed into 60 tubes (0·3 ml./tube) and incubated for 90 min at 37°C. Then 0·1 ml. of indicator bacteria was added directly to each tube. 10 min later, soft agar was added directly to each tube, and the mixture was plated.

(k) *Other methods*

Lysis was judged by observing the drop in turbidity in a Klett colorimeter (green filter). Assay for viable tailless heads and for base-plate parts was carried out according to Israel, Anderson & Levine (1967). Base-plate parts were purified from crude lysates by $(NH_4)_2SO_4$ precipitation followed by zone sedimentation in a sucrose density gradient. Transduction was assayed for two markers (*his*$^+$ and *leu*$^+$) by adsorbing phage to exponentially growing *his*$^-$ or *leu*$^-$ cells lysogenic for P22 *sie* 1 (Rao, 1968). This method makes possible the efficient assay of transducing particles in a lysate of a clear phage (J. Ebel, unpublished results).

3. Results

(a) *Isolation of* erf$^-$ *mutants*

Mutants unable to grow on *rec*$^-$ hosts were isolated after nitrosoguanidine mutagenesis using a double-layer recognition method. These mutants (called *erf*$^-$ mutants) not only fail to make a plaque on strains carrying the *rec*$^-$ allele described by Wing *et al.* (1968), but also fail to make a plaque on a *rec*$^-$ strain isolated by A. Eisenstark and on another strain isolated by S. Takebe. All of these *rec*$^-$ strains have a common

phenotype resembling the *rec*A phenotype of *Escherichia coli* (Wing *et al.*, 1968; Wing, 1968).

On *rec*$^+$ hosts, *erf*$^-$ mutants plate with near-normal efficiencies, although the plaque is typically reduced in size. On *rec*$^-$ hosts the efficiency of plating is about 10^{-5} and the plaques are of normal size. These plaques contain revertants which behave like wild type on *rec*$^-$ hosts with respect to burst size, efficiency of plating, lysogenization, and two-point recombination frequency. Thus it appears that the *erf*$^-$ phenotype can be the result of a single point mutation. The *erf*$^-$ alleles have been crossed into a great variety of wild-type P22 strains, without any noticeable change in phenotype.

(b) *Complementation behavior of* erf$^-$ *mutants*

The data in Table 2 show that the four *erf*$^-$ mutants form a single complementation group. Co-infection of *rec*$^-$*su*$^-$ cells with two different mutants results in no significant

TABLE 2

Complementation among erf$^-$ *mutants*

	None	*erf* 1	*erf* 2	*erf* 3	*erf* 4
erf 2 c_1	0·6	1·2	0·8	1·7	2·4
erf 3 c_1	1·1	1·3	0·9	0·6	2·1
am N10 c_1	<0·03	72	52	89	101
am N14 c_1	<0·01	81	77	81	110
c_1	166	121	107	95	142
None		1·6	0·6	1·3	2·2

Strain DB47 cells were infected at multiplicities of 7 to 10 of each phage per cell. Incubation was at 37°C for 90 min. Burst size equals the number of phage per infected cell and is corrected for dead cells found in *rec*$^-$ cultures (Wing *et al.*, 1968) and for unadsorbed phage. The average ratio of c_1/c^+ phage among the progeny varied from 0·5 to 3, indicating no significant selection in any of these experiments.

increase in burst size over infection with one mutant alone. However, co-infection of *erf*$^-$ mutants with *am* mutants in gene *12* (DNA synthesis) or gene *1* (head maturation) results in at least a 50-fold increase in burst size. Further, co-infection of *erf*$^-$ mutants with wild-type phage results in growth of both the *erf*$^-$ and the wild-type phage.

These results indicate that *erf*$^+$ is dominant over *erf*$^-$, and it is likely that the *erf* gene specifies the synthesis of a diffusible product.

(c) *Mapping of* erf$^-$ *mutants*

The four independent *erf*$^-$ mutants recombine with each other in *rec*$^+$ hosts. The frequency of recombination between them is smaller than the frequency of recombination between *erf*$^-$ mutants and the nearest known markers as shown in Table 3. The data in Table 3 also show that the *erf* gene lies in the early region of the P22 genetic map, near the *c* region.

The c_2 gene is known to lie to the right of both *ts* 6.1 and *ts* 21.1. Thus in the cross *ts* 6.1 $c_2 \times$ *erf* 1 (Table 3), the fact that most of the *ts*$^+$*erf*$^+$ recombinants are c_2 means that *erf* 1 lies to the right of *ts* 6.1. In the cross *ts* 21.1 $c_2 \times$ *erf* 1 few of the *ts*$^+$*erf*$^+$ progeny are c_2, indicating that *erf* 1 is to the left of *ts* 21.1. The order *ts* 6.1 —*erf* 1–*ts* 21.1–c_2 was confirmed in the five-factor crosses shown in Tables 4 and 5.

TABLE 3

Location of the erf$^-$ *loci*

Cross	Percentage recombination	Proportion of c^+ among recombinants	
erf 1 × *ts* 5.1 c_2	20	0·09	*ts* + *c*
erf 1 × *ts* 9.1 c_2	6·2	0·10	
erf 1 × *ts* 6.1 c_2	0·30	0·16	+ *erf* +
erf 1 × *ts* 21.1 c_2	0·60	0·86	+ *ts* *c* *erf* + +
erf 1 × *ts* 18.1 c_1	2·0	0·72	+ *c* *ts*
erf 1 × *ts* 12.1 c_1	3·0	0·62	*erf* + +
Deduce: *erf* 1 is between *ts* 6.1 and *ts* 21.1			
erf 2 × *erf* 1 c_2	0·08	0·17	− + *c* + − +
erf 3 × *erf* 1 c_2	0·01	0·86	
erf 4 × *erf* 1 c_2	0·16	0·88	
erf 1 × *erf* 2 c_2	0·07	0·88	+ − *c*
erf 3 × *erf* 2 c_2	0·07	0·90	− + +
erf 4 × *erf* 2 c_2	0·19	0·81	
Order deduced: (*erf* 4 − *erf* 3)† − *erf* 1 − *erf* 2 − c_2			

Standard cross conditions are described in Materials and Methods. The permissive host was DB21; non-permissive (*rec*$^-$) host was DB31A. The total adsorbed multiplicity of infection varied from 10 to 17 and the input ratio of parents never differed from equality by more than 50%. The ratio of *c* to c^+ was in each case measured in the total progeny: it never differed significantly from the input ratio. The proportion of c^+ among recombinants is corrected for the proportion in the total progeny. Each determination represents at least 100 plaques.

† These two markers are ordered with respect to each other solely on the basis of recombination frequency.

TABLE 4

ts *21.1* m_3 c_2 h_{21} × erf 1

Selected markers ts^+ erf^+	*m* + *ts* *c* *h* + *erf* + + +	
Unselected markers	Number	Cross-over class
m + +	98	Single
+ + +	65	Double
m + *h*	13	Double
+ + *h*	5	Triple
m *c* *h*	13	Double
+ *c* *h*	5	Triple
m *c* +	8	Triple
+ *c* +	2	Quadruple
Order deduced: m_3 − *erf* 1 − *ts* 21.1 − c_2 − h_{21}		
Frequency of recombination: *erf* 1 − *ts* 21.1 = 0·6%		

The permissive host strain was DB21; non-permissive (*rec*$^-$) was DB31A. The multiplicity of infection was 8 of each parent. The frequency among the total progeny of the markers *m*, *c* and *h* was not significantly different from 0·5. The frequency of recombination between the *m*, *c* and *h* loci was measured among the total progeny and all values are similar to the values reported by Gough & Levine (1968).

TABLE 5

ts *6.1* m_3 c_2 h_{21} × erf 1

Selected markers ts^+ erf^+	*m* *ts* + *c* *h* / + + *erf* + +	
Unselected markers	Number	Cross-over class
+ *c* *h*	62	Single
m *c* *h*	30	Double
+ *c* +	17	Double
m *c* +	0	Triple
+ + +	20	Double
m + +	0	Triple
+ + *h*	1	Triple
m + *h*	0	Quadruple

Order deduced: m_3 — *ts* 6.1 — *erf* 1 — c_2 — h_{21}
Frequency of recombination: *ts* 6.1 — *erf* 1 = 0·30%

The multiplicity of infection was 6 of each parent. Permissive host was DB21; non-permissive (*rec*⁻) was DB31A. The controls described in Table 4 were done for this cross as well, and all marker and recombination frequencies were normal.

The segregation of the c_2 allele in the *erf*⁻ × *erf*⁻ crosses in Table 3 makes possible the ordering of the *erf* mutants. For example, the *erf*⁺ progeny of the cross *erf* 2 × *erf* 1 c_2 are primarily c_2; thus *erf* 2 must be nearer to c_2 and therefore to the right of *erf* 1. The reciprocal cross *erf* 1 × *erf* 2 yields, as expected, primarily c^+ among the *erf*⁺ progeny. In this way the order *erf* 4-*erf* 3-*erf* 1-*erf* 2-c_2 was deduced.

All of these results are summarized in Figure 1, which shows the map of the early region of phage P22, approximately to scale.

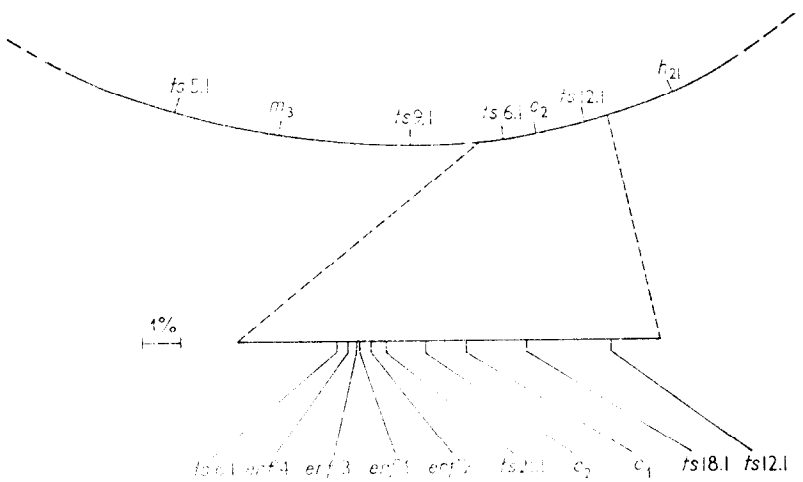

FIG. 1. Location of the *erf*⁻ mutants on the genetic map of phage P22. The Figure is approximately to scale. Mutants in genes *18*, *12*, *6* and *21* do not make phage DNA; gene *9* directs the synthesis of base-plate protein (Botstein & Levine, unpublished observations).

(d) *Phenotype of* erf$^-$ *mutants*

(i) *Growth of* erf$^-$ *mutants in* rec$^+$ *and* rec$^-$ *hosts*

In order to test the extent to which *erf*$^-$ mutants grow in *rec*$^-$ hosts, *rec*$^-$ strains were infected at low multiplicity with *erf*$^-$ phage and the infective centers plated with *rec*$^+$ indicator. A plaque indicates the capacity to produce at least one phage. Table 6 shows the results with several *erf*$^-$ mutants and their derivatives. In all cases, only about 5% of the infected *rec*$^-$ cells yielded any phage. Trivial explanations such as poor adsorption, infection of participation are made unlikely by the results of a rescue experiment also shown in Table 6.

TABLE 6

Phage strain	Helper	Percentage transmisssion
erf 1	None	3†
erf 1	*ts* 5.1 c_1	35†
erf$^+$	*ts* 5.1 c_1	33†
erf 2	None	6
erf 3	None	5
erf 4	None	7
erf 2 c_1	None	5
erf 2 c_2	None	5

DB47 (*rec*$^-$) cells were infected at a multiplicity of 0·1 to 0·2 phage/cell. Helper multiplicity was 10. After adsorption for 15 min at 25°C, antiphage serum ($K=3$) was added for an additional 10 min. The infective centers were then diluted and plated on DB21 (*rec*$^+$). Percentage transmission equals (infective centers observed/phage added) × 100.

† Plates were heated rapidly on a heating block and incubated at 40°C, which is non-permissive for *ts* 5.1. Temperature-shift experiments (Levine, unpublished observations) indicate that the *ts* 5.1 function is required late after infection.

In this experiment, *rec*$^-$ cells were infected not only with *erf*$^-$ phage at low multiplicity but also with many *erf*$^+$ but otherwise defective phage. The extent to which the minority *erf*$^-$ phage can contribute function and "rescue" the infective center depends on adsorption, injection and participation. The results in Table 6 show that *erf*$^-$ phage contribute function exactly as well as *erf*$^+$ phage in such an experiment.

(ii) *Ability of* erf$^-$ *mutants to recombine in* rec$^+$ *and* rec$^-$ *hosts*

The effect of the *erf* and *rec* alleles on recombination frequencies was measured in crosses between pairs of *ts* or *am* mutants. In any cross, both parents carried the same *erf*$^-$ mutation, or else were both *erf*$^+$. Representative data are shown in Table 7. An effect of the host *rec* allele alone on recombination is observed, but the reduction in frequency is at most twofold. Similarly, the *erf* allele alone has a slight effect on the recombination frequency. However, when the *erf*$^-$ phages are crossed in *rec*$^-$ hosts, the recombination frequency is reduced between 7- and 20-fold, depending on the *erf*$^-$ allele used. It must be remembered, however, that never more than 10% of the *rec*$^-$ cells infected with *erf*$^-$ phage yield any phage at all. In order to be sure that at least some infected *rec*$^-$ cells produce more than one progeny phage, a single-burst experiment, modified to simulate a cross, was carried out. The result (Fig. 2) shows

TABLE 7

Cross	*erf*	*rec*	Burst size	Percentage recombination
ts 12.1 × *ts* 18.1	+	+	180	2·6
25°C	+	−	125	1·2
	1	+	72	1·2
	1	−	26	0·14
am N9 × *am* N10 c_1	+	+	110	35
25°C	+	−	72	22
	3	+	24	20
	3	−	7	1·2
am N10 × *am* N8 c_1	+	+	131	15
39°C	+	−	78	12
	2	+	20	9·2
	2	−	6	2·2
	3	+	12	13
	3	−	8	0·7

Standard crosses were performed at the indicated temperatures. The total multiplicities of infection varied from 8 to 22 with parents within 50% of each other. Where possible, the c^+/c allele ratio was measured in the total progeny and this was always very close to the input. The same mixture of parental phage was used for both the *rec*$^+$ and *rec*$^-$ crosses. In the *ts* × *ts* cross, *rec*$^+$ was DB21; *rec*$^-$ was DB47. Percentage recombination equals (plaques at 40°C/plaques at 25°C) × 2 × 100; all platings were on DB21. In the *am* × *am* crosses, *rec*$^+$ was DB74 and *rec*$^-$ was DB77. Both carry the same *am* suppressor. Percentage recombination equals (plaques on DB53(*su*$^-$)/plaques on DB74(*su*$^+$)) × 2 × 100. All platings were at 37°C. Burst size equals total progeny phage/infective centers; both were plated on the *rec*$^+$ permissive host.

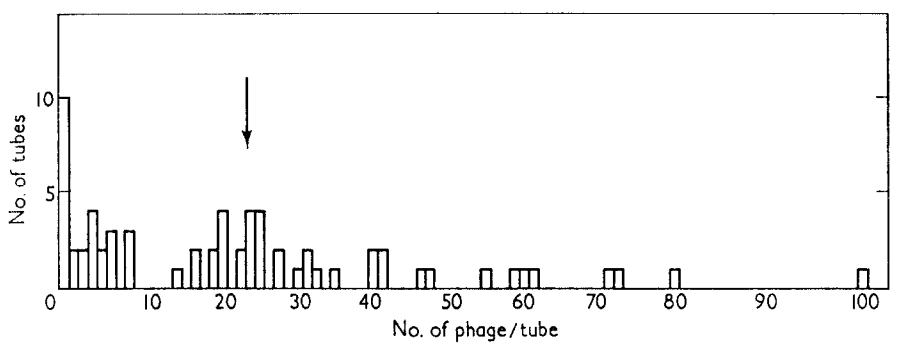

FIG. 2. A modified single-burst experiment, simulating a phage cross. Phage *erf* 2 c_1 were adsorbed to *rec*$^-$ (DB77) cells at a multiplicity of 12 phage/cell and diluted into single-burst tubes. The average burst per infective center was 14; the arrow shows the median at 21 phage per infective center.

that most of the progeny under these conditions derive from cells which yield about 20 phage per cell. Why a minority of infected cells should produce such a reasonable burst is still obscure, but the result makes it plausible that the reduction in recombination frequencies means that *erf*$^-$ mutants have indeed lost a function required for recombination in *rec*$^-$ hosts.

(iii) *Origin of the residual recombination between* erf⁻ *mutants in* rec⁻ *hosts*

Even in the case of crosses between *erf*⁻ mutants in a *rec*⁻ host, however, there is some residual recombination. This recombination could be the result of leakiness in the *rec*⁻ or *erf*⁻ mutations, or it could be the result of yet another recombination system which has been revealed by the removal of the much more active *rec* and *erf* systems. One such system might be the *int* system (by analogy to phage λ (Weil & Signer, 1968; Echols & Gingery 1968)). In the case of a phage with circularly permuted DNA such as P22, a site-specific recombination system can catalyze recombination between any two markers, regardless of their position with respect to the site of the recombination event. An example of how events limited to the attachment region of phage P22 might cause the formation of recombinants between sites which do not span the attachment region is shown in Figure 3. The example assumes the

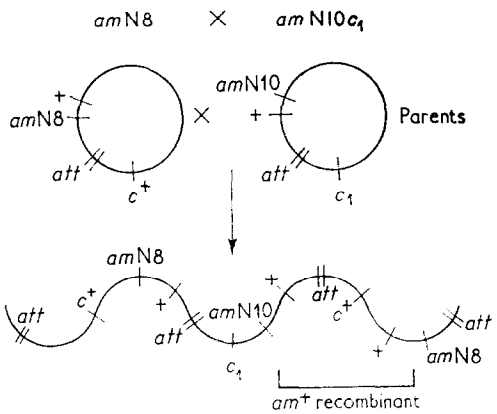

FIG. 3. Structure of recombinants in a three-factor cross where cross-overs occur only at a single site.

If the point marked *att* is the only point where cross-over events occur, then the *am*N10c_1 phage can be joined to the *am*N8 phage in only two ways, both of which are shown in the form of a single molecule of oversize intermediate DNA. Only one *am*⁺ structure can be cut out; all *am*⁺ recombinants should be c^+ in this case. The data for this cross are in Table 8. The circular maps are drawn roughly to scale.

formation of an oversize continuous piece of DNA as an intermediate in phage maturation (see Botstein & Levine, 1968*b*). The example shows, however, that site-specific recombination does have some unusual linkage properties; in the case shown all *am*⁺ progeny resulting from site-specific recombination at a site between c_1 and *am* 8 must be c^+. In the case of generalized recombination c_1 is linked to the *nearer am* marker; by site-specific recombination the c_1 marker is linked to the nearest *am* marker only if the site does not lie between.

Table 8 shows the results of crosses carried out according to the design of Figure 3. In each case the cross (*am*N8c_1 × *am*N10) and the reciprocal cross (*am*N8 × *am*N10c_1) were done, in the presence and absence of the *erf* system and/or the host *rec* system. As before, both parents always carried the same *erf*⁻ allele in *erf*⁻ crosses. In each cross the ratio of c^+ to c in the total progeny and among the *am*⁺ progeny was measured. In the last column of Table 8, the latter ratio is normalized to the same ratio from a cross carried out in a *rec*⁺ host; the value thus obtained is a measure of increase of

TABLE 8

Linkage in three-factor erf⁻ *crosses*

Forward cross: *am* N10c_1 × *am* N8
Reciprocal cross: *am* N10 × *am* N8c_1
Reciprocal cross values are in parentheses.

Cross *erf*	Cross *rec*	Burst size	Recombination frequency (%)	Ratio c^+/c total	Ratio c^+/c am^+	enrichment†
Crosses at 25°C						
+	+	120(80)	6·2(5·5)	0·92(1·1)	0·83(1·0)	0·90(1·1)
+	−	51(23)	7·5(6·2)	1·0 (1·0)	0·85(1·0)	0·85(1·0)
3	−	3(2)	0·6(0·3)	0·7 (1·9)	4·3 (0·33)	6·1 (5·8)
3	+	13(11)	7·6(4·4)	0·90(2·1)	0·93(2·1)	1·0 (1·0)
2	−	5(3)	2·2(2·6)	1·6 (1·6)	7·6 (0·34)	4·8 (4·7)
2	+	20(12)	9·2(6·4)	1·5 (1·5)	2·2 (1·1)	1·5 (1·3)
Crosses at 37°C						
+	+	131(100)	12 (15)	1·1 (1·5)	0·83(1·6)	0·75(1·1)
+	−	78(92)	11 (15)	0·83(1·1)	0·76(1·3)	0·86(1·2)
3	−	4(4)	0·74(1·1)	0·56(2·1)	2·2 (0·51)	3·9 (4·1)
3	+	12(4)	13 (11)	0·7 (2)	0·82(1·7)	1·2 (1·2)
2	−	6(4)	3·5(1·8)	1·0 (1·7)	19 (0·21)	19 (8)
2	+	32(22)	8·4(4·3)	1·5 (1·4)	1·1 (1·4)	1·4 (1·0)

The crosses were performed as described in Materials and Methods; the design is explained in the text. The *rec*⁺*su*⁺ host was DB74; the *rec*⁻*su*⁺ host was DB77. Infective centers were always measured by plating on DB74. Burst size is calculated as total progeny (scored on DB74) per infective center. am^+ Progeny were scored on DB53 (*rec*⁺*su*⁻). The total adsorbed multiplicity varied from 11 to 20; the parental c^+/c ratio (not shown) was always within 20% of the ratio among the progeny of the *rec*⁺ cross. The same mixture of parents was always used for both the *rec*⁺ and *rec*⁻ crosses. Determinations of ratio depend on at least 250 total plaques.

† This number equals (c^+/c in *rec*⁻ cross)/(c^+/c in *rec*⁺ cross) for the forward cross and (c/c^+ in *rec*⁻ cross)/(c/c^+ in *rec*⁺ cross) for the reciprocal cross. It is thus a measure of linkage between the *am*N10 locus and the c_1 locus (see Fig. 3).

linkage. In the case of the reciprocal cross, the reciprocal value is shown, so that the numbers may be compared directly.

The results show that linkage of the c_1 marker to the *am*N10 marker is increased 4- to 20-fold by removal of both the *erf* and *rec* systems. Removal of either the *rec* or *erf* system alone does not disturb linkage in this test. These results confirm that the *erf*⁻ mutation causes a failure to recombine normally, even among those phage (am^+) which must have recombined at least once.

Another, more tentative, conclusion from Table 8 is possible using the published map of Gough & Levine (1969). The *am*N10 mutation lies in gene *1* and maps within 2 map units of *ts* 1.1. The *am*N8 mutation lies in gene *5* and maps within 1 map unit of *ts* 11.1 (C. Lawrence & D. Botstein, unpublished results). This means that *am*N8 is about 28 map units from c_1 and *am*N10 is close to 46 map units from c_1. Nevertheless, Table 8 shows that *am*N10c_1 becomes linked to c_1 in an *erf*⁻*rec*⁻ cross. Thus the data suggest that there is a site-specific recombination system revealed by removal of the *erf* and *rec* systems and that the specific site lies between *am*N8 and c_1.

(iv) *Ability of* erf⁻ *mutants to lysogenize* rec⁺ *and* rec⁻ *hosts*

If recombination is required to circularize phage DNA and if circularization of some sort is required for lysogenization, then *erf* mutants should lysogenize *rec*⁺

hosts (where recombination is nearly normal) but not *rec*$^-$ (where recombination is decreased).

This prediction was tested by infecting *rec*$^-$ and *rec*$^+$ cells with *erf*$^-$ phages under conditions favoring lysogeny. Table 9 shows the result: *erf*$^-$ phage lysogenize *rec*$^+$ cells, albeit somewhat less well than *erf*$^+$ phage do; however, *erf*$^-$ phage do not detectably lysogenize *rec*$^-$ cells, whereas *erf*$^+$ phages lysogenize *rec*$^+$ cells quite normally.

Also shown in Table 9 are two complementation tests for lysogenization. The first shows that *erf*$^-$ phage can probably be integrated into a *rec*$^-$ host, since good complementation was obtained with a c_2 phage (missing repressor). This subject is dealt

TABLE 9

Lysogenization of erf$^-$ *phages*

Phage	Host	Survival (%)	Immune fraction	
			number	percentage
erf$^+$	*rec*$^+$	92	25/30	83
erf$^+$	*rec*$^-$	82	21/30	70
erf 2	*rec*$^+$	92	23/39	59
erf 2	*rec*$^-$	78	0/43	<3
erf 3	*rec*$^+$	85	34/54	63
erf 3	*rec*$^-$	81	0/61	<2
erf 2 + *erf*$^+$ c_2	*rec*$^-$	72	13/27	48
int_3	*rec*$^-$	86	0/35	<3
erf 2 + int_3	*rec*$^-$	79	15/33	46

DB21 (*rec*$^+$) or DB47 (*rec*$^-$) cells growing exponentially in LB broth at a density of 2×10^8 cells/ml were infected with 20 phage/cell. After 10 min, antiphage serum ($K = 4$) was added for 5 min. The cells were then diluted and plated for survival on plates containing antiphage serum (final $K = 2$). The colonies which grew up the next day were then tested for immunity.

with more extensively below. The second test shown is with an *int*$^-$ mutant (called L mutants in Smith & Levine, 1967; Smith, 1968) which is by itself unable to integrate but which grows normally. Although we have not determined the structure of these lysogens, the finding of lysogens in substantial numbers after this mixed infection means that the *int* gene is intact in *erf*$^-$ mutants.

(e) *Effect of the* erf$^-$ *mutation on phage DNA replication*

The phage function most likely to require recombination directly is the replication of the phage DNA. Fortunately, the P22 system lends itself to the examination of phage DNA synthesis, because phage infection results in a fairly complete cessation of host-specific DNA synthesis which, although temporary, lasts longer than a normal latent period (Smith & Levine, 1964; Botstein, 1968). Thus any DNA which is made soon after infection is almost exclusively phage-specific. Smith & Levine (1964) measured the rate of DNA synthesis after infection with various clear mutants of phage P22 by measuring the amount of [^{3}H]thymidine taken up in one-minute pulses administered at various times after infection. They found that c_1 and c_2 mutants have characteristic patterns of DNA synthesis. In this way the pattern of DNA synthesis following infection of *rec*$^+$ or *rec*$^-$ hosts with *erf*$^-$ clear mutants was compared to the pattern obtained with the corresponding *erf*$^+$ clear phage.

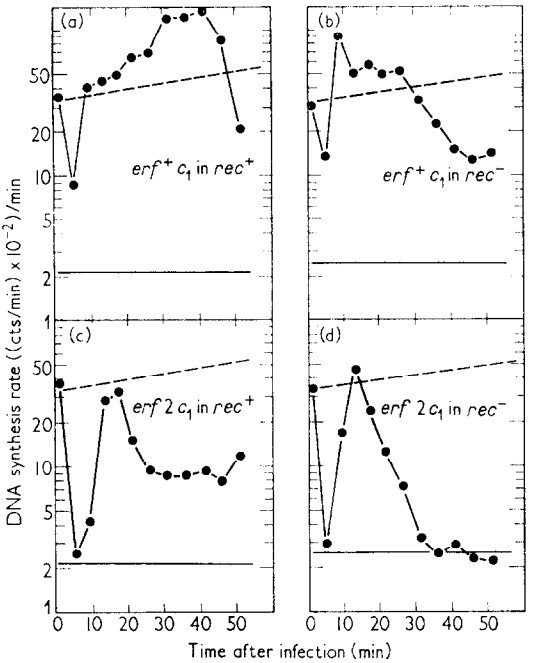

FIG. 4. Rate of incorporation of [^{3}H]thymidine into DNA after infection with various c_1 phages in *rec*$^+$ and *rec*$^-$ hosts.

Cells growing in M9CAA medium at 30°C were infected with 20 phage/cell. 1-min pulses of [^{3}H]thymidine were administered as described by Smith & Levine (1964). The *rec*$^+$ host was DB21 and the *rec*$^-$ host was DB47. (a) *erf*$^+$ c_1 in *rec*$^+$. (b) *erf*$^+$ c_1 in *rec*$^-$. (c) *erf* 2 c_1 in *rec*$^+$. (d) *erf* 2 c_1 in *rec*$^-$. The dotted lines show the rate of incorporation in uninfected control cultures; the solid horizontal line shows the background control level ([^{3}H]thymidine with no cells).

The rate of DNA synthesis at various times following infection with c_1 phage in a *rec*$^+$ host is shown in Figure 4(a). Immediately after infection, the rate drops as bacterial synthesis is shut off. Soon thereafter phage DNA synthesis begins and continues at a substantial rate until lysis. Infection of *rec*$^-$ cells with c_1 phage (Fig. 4(b)) produces a similar pattern although the rate of synthesis starts to drop off somewhat before lysis. After infection of *rec*$^-$ cells with *erf* 2 c_1 phage (Fig. 4(d)) the picture is quite different. After the cessation of host synthesis, the rate of phage DNA synthesis rises to a normal level, but shortly thereafter the rate falls to zero. In *rec*$^+$ cells, infection with *erf* 2 c_1 phage (Fig. 4(c)) results in an intermediate pattern; phage DNA synthesis begins normally, and although the rate falls shortly thereafter, it does not drop to zero, but stays at a significant level until lysis occurs. Figure 4 thus shows that the rate of DNA synthesis is affected by both the *rec* and *erf* alleles; but it is absolutely affected (i.e. the rate drops to zero) only when both the *rec* and *erf* systems are absent. In all cases, however, the same maximum rate is achieved immediately after the onset of phage DNA synthesis; it is as if the *rec* and *erf* alleles affect only the continued synthesis of DNA.

The situation is somewhat more clearly illustrated by the case of infection with c_2 phages. Figure 5 is a record of a set of experiments with c_2 and *erf* 2 c_2 phages. After infection of *rec*$^+$ (Fig. 5(a)) with c_2, phage DNA synthesis begins and then immediately ceases again, resuming a few minutes later and continuing thereafter until lysis. The

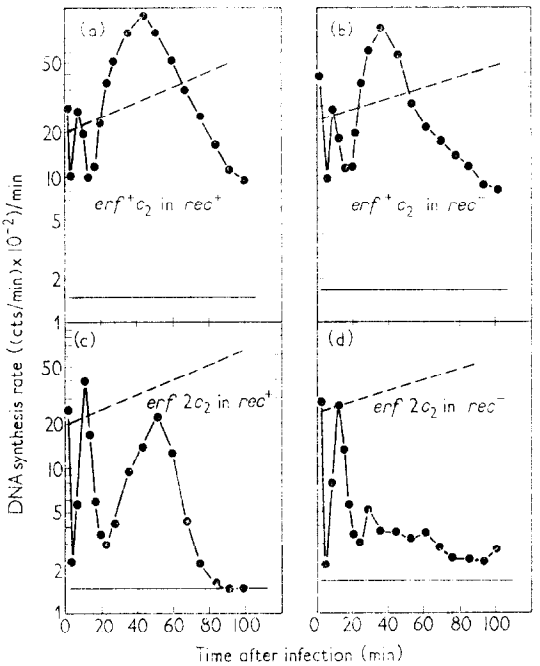

FIG. 5. Rate of incorporation of [^{3}H]thymidine into DNA after infection with various c_2 phages in *rec*$^+$ and *rec*$^-$ hosts.

The procedure is described in the legend to Fig. 4. (a) *erf*$^+c_2$ in *rec*$^+$. (b) *erf*$^+c_2$ in *rec*$^-$. (c) *erf* 2 c_2 in *rec*$^+$. (d) *erf* 2 c_2 in *rec*$^-$. The dotted lines show the rate of incorporation in uninfected control cultures. The solid horizontal line shows background control level ([^{3}H]thymidine with no cells).

pattern after c_2 infection of *rec*$^-$ is similar (Fig. 5(b)). Upon infection of *rec*$^+$ with *erf* 2 c_2 (Fig. 5(c)) the early peak in rate is absolutely normal; however, when synthesis resumes the second time, the maximum rate achieved is substantially lower than in the two preceding cases. In the case of infection of *rec*$^-$ with *erf* 2 c_2 (Fig. 5(d)), the early peak is again quite normal, but the second peak is absent, and DNA synthesis is never resumed at a significant rate. Here again the absolute defect is observed only in the *erf*$^-$*rec*$^-$ case; in all other cases, significant rates of synthesis are observed at the expected times. Most clear in Figure 5 is the observation that the early behavior of the DNA synthesis rate is normal both in duration and magnitude even in the *erf*$^-$*rec*$^-$ case.

Similar experiments carried out with *erf* 3 c_1 and *erf* 3 c_2 phage give the same results. From this we conclude that the onset of the synthesis of phage DNA does not require either the *erf* or *rec* function, but that continued DNA synthesis does require one or the other. This in turn implies that early DNA synthesis does not require recombination, but that extensive and continued DNA replication does.

(f) erf *and the control of late functions*

The synthesis of DNA has been implicated in the control of late phage functions in some phage systems. As is the case with some other phages, P22 mutants unable to replicate DNA at all do not turn on their late functions (D. Botstein & M. Levine, unpublished results). Likewise, cells infected with wild-type (c^+) P22 phage which are destined for lysogeny and which synthesize phage DNA only very shortly after

infection (Smith & Levine, 1964) do not synthesize late proteins (Cohen & Levine, 1966). *erf*$^-$ Mutants in *rec*$^-$ hosts also make DNA, but only for a short period soon after infection (like the c^+ case), so it is of interest whether late functions are expressed in this case. A preliminary indication that they are expressed was that *rec*$^-$ cells infected at high multiplicity with *erf*-clear phages lyse at the normal time. A more exact indication was that these lysates contain substantial amounts of base-plate protein (Israel *et al.*, 1967). However, the capacity of P22 to encapsulate bacterial DNA into phage heads (producing generalized transducing particles) makes possible the simultaneous assay of all functions required for production of assembled particles. *erf*$^-$ and *erf*$^+$ phage produce the same number of transducing particles per infected *rec*$^-$ cell, even though the phage titer in the *erf*$^-$ lysate is 50-fold reduced. Thus, with the exception of some of those required to replicate phage DNA, all phage proteins required for maturation appear to be present in reasonable amounts after infection of *rec*$^-$ with *erf*$^-$ phage.

(g) *Role of circularization in phage growth*

Circularization of the phage DNA might be the step for which recombination is required and which is necessary for continued phage DNA replication. In this case, phage growth after induction of a lysogen might not be dependent on recombination,

TABLE 10

Strain		Survival (%)	Infective centers (%)† *rec*$^+$	*rec*$^-$	Burst size† No tails *rec*$^+$	With tails *rec*$^+$	With tails *rec*$^-$
DB47 (*erf* 2 *ts* c_2)	no. 1	16‡	100	<0·1	3·8	78	<0·02
	no. 2	14‡	130	<0·1	3·5	112	<0·02
	no. 3	13‡	80	<0·1	2·6	53	<0·02
	no. 4	31‡	110	<0·1	3·9	80	<0·03
	no. 5	30‡	70	<0·1	3·5	123	<0·01
DB47 (*ts* c_2)		3	120	122	5·2	221	220
DB47 infected with *erf* 2 *ts* c_2			4	<0·1	1·1	1·6	<0·003
DB47 infected with *ts* c_2			98	106	232	251	260

erf 2 *ts* c_2 lysogens of DB47 were made by isolating colonies which were immune at 25°C from the survivors in the center of a spot of 10^6 *erf* 2 *ts* c_2 plus 10^6 c_2 phage on a plate seeded with DB47 cells. Each colony was streaked 4 times; one colony which produced *erf*$^+$ phage at 40°C was discarded. The remainder were grown to exponential phase at 25°C in LB broth, plated for viable counts at 25°C, and then diluted into LB broth at 40°C. Survival at 40°C and infective center titer at 40°C were plated immediately. The number of free phage (chloroform-insensitive plaque-forming units) was also determined at this time. Less than 0·01 times the number of viable cells at 25°C were found in all cases. After 90 min of incubation at 40°C, the cultures were chloroformed and the phage titer was determined with and without previous addition of a 100-fold excess of purified base-plate protein ("tails").

Infections were performed at a multiplicity of 1 phage per cell. After adsorption for 10 min at 40°C, unadsorbed phage were measured and the cultures treated exactly as above.

† All values are calculated with respect to viable count at 25°C for inductions or to number of phage added for infections.

‡ At least 95% of these survivors are sensitive to phage.

since the excision of the prophage should presumably produce a circular form of the phage DNA (Campbell, 1962; Botstein & Levine, 1968*b*).

Several *rec*$^-$ lysogens containing temperature-inducible *erf*$^-$ prophage were constructed by complementation, as described in the legend to Table 10. These lysogens are immune to phage at 25°C, but at 40°C they die. The surviving cells are sensitive to phage, and are presumably cured of the prophage. The reason for this high curing rate is not known; however, mutants in several early phage genes share this property. The critical test is whether or not these lysogens produce phage at 40°C. Purified base-plate protein was added to all the lysates, since induction at high temperature in general produces tail-deficient particles (Israel *et al.*, 1967; Israel, 1967). As shown in Table 10, upon induction at 40°C, *erf* 2 *ts* c_2 lysogens of *rec*$^-$ cells all become infective centers and produce normal bursts of *erf* 2 *ts* c_2 phage upon induction. The same *erf* 2 *ts* c_2 phage cannot grow in the *rec*$^-$ host after infection.

4. Discussion

Previous work on the replication of phage P22 DNA (Botstein & Levine, 1968*b*) had suggested that physical recombination events have an essential role in the growth of this phage. The experiments reported here substantiate this view. We have isolated mutants (*erf*$^-$) which are apparently defective in a phage-specified recombination function; this defect renders these phage incapable of growth after infection of bacteria which are themselves unable to catalyze successful recombination events (Wing *et al.*, 1968).

The phenotype of the *erf*$^-$ mutants is entirely dependent on the host which they infect: the mutant phenotype is only expressed in *rec*$^-$ hosts. The ability of the host *rec*$^+$ allele to suppress all aspects of the *erf*$^-$ mutant phenotype is a major reason for believing that the *erf* gene specifies a component of the phage recombination system.

We can summarize the main features of the *erf*$^-$ mutant phenotype and our interpretation of each of them as follows:

(1) *erf*$^-$ Mutants do not grow after infection of *rec*$^-$ hosts. The wild-type allele is dominant over *erf*$^-$ in mixed infections. We conclude that the *erf*$^+$ gene directs the synthesis of a diffusible product the presence of which is essential to growth after infection of *rec*$^-$ bacteria.

(2) A small minority of *rec*$^-$ cells infected with *erf*$^-$ phage yield substantial bursts of progeny. The frequency of recombination among these progeny is substantially smaller than that found when either the host *rec*$^+$ allele or the phage *erf*$^+$ allele is present. In three-factor crosses between *erf*$^-$ phage carried out in *rec*$^-$ hosts, linkage of an unselected marker is increased from 50% (i.e. random assortment) to between 80 and 95% linkage. In this experiment the increase in linkage is observed among progeny which must have undergone at least one recombination event. Thus, by two criteria, phage recombination was shown to be reduced in *rec*$^-$ cells infected with *erf*$^-$ phage. If either the *rec*$^+$ or *erf*$^+$ allele is provided in any of these crosses, recombination is almost completely normal. We interpret these results as showing that the *erf* gene directs the synthesis of an essential part of a phage-specified recombination system.

(3) *erf*$^-$ Mutants cannot lysogenize *rec*$^-$ hosts, although they can lysogenize *rec*$^+$ bacteria at high frequency. Under conditions ordinarily favoring lysogeny, *rec*$^-$ cells infected with *erf*$^-$ mutants survive even though they are not lysogenized. In mixed infections of *rec*$^-$ cells, *erf*$^-$ phage can provide c^+ function to c_2 mutants

(defective in phage repressor; Levine & Smith, 1964); *erf*$^-$ mutants can also provide *int*$^+$ function to *int*$^-$ mutants (defective in prophage integration; Smith & Levine, 1967). Furthermore, *erf*$^-$ phage can become stable prophage if "helped" to integrate by co-infecting *erf*$^+c_2$ phage. Thus we conclude that *erf*$^+$ function is required to establish, but not to maintain, lysogeny.

(4) DNA replication is initiated after infection of *rec*$^-$ cells with *erf*$^-$ phage, but synthesis continues only for a very short time. By integrating the synthesis rate over time, we estimate that under these conditions *erf*$^-$ mutants replicate their DNA about once. Thus it appears that recombination events are essential for sustained synthesis of phage DNA after infection.

(5) Although *erf*$^-$ phage cannot grow after infection of *rec*$^-$ cells, induction of an *erf*$^-$ lysogen of a *rec*$^-$ host strain results in normal growth. This result, as explained below, can most easily be interpreted if the circularization of the infecting phage DNA is the step for which recombination is required. According to this interpretation, the prophage excision mechanism (cf. Campbell, 1962; Signer, 1968) circularizes the prophage DNA after induction of a lysogen. After infection, however, recombination events are required to circularize the permuted, terminally repetitious linear DNA molecules. Either the *rec* system of the host or the phage *erf* system can catalyze these required recombination events. Failure to circularize the infecting phage DNA in the absence of both the *erf* and *rec* systems would also explain the inability of *erf*$^-$ phage to lysogenize *rec*$^-$ bacteria.

(a) *Relation of recombination to phage growth*

There is an inherent difficulty in any attempt to demonstrate that a phage cannot grow because it cannot recombine. If the failure to grow were absolute, then no measurement of recombination frequency would be possible. Fortunately, a small minority of *rec*$^-$ cells infected with *erf*$^-$ phage produce reasonable bursts of progeny, among which we were able to measure recombination frequencies and linkage. The frequencies of recombination which we observed under *rec*$^-$*erf*$^-$ conditions were greatly reduced compared to the frequencies normally observed between the pairs of markers which we used. This need not have been the case. If recombination is essential to growth, one might reasonably expect recombinants to have a growth advantage. In such a case, the observed recombination frequencies need not be reduced, and might even be increased when the number of physical recombination events occurring in the infected cells is limited. For this reason, it is possible that the reduction in recombination frequency which we observed with the *erf*$^-$ mutants may considerably underestimate the actual defect in physical recombination.

Although the three-factor crosses unambiguously demonstrate recombination deficiency because of the increase in linkage observed between the c_1 marker and the *am*N10 locus, this measurement probably also seriously under-estimates the extent of the physical recombination deficiency. The c_1 site is normally not linked to the *am*N10 site. From this we know only that many cross-over events normally occur between c_1 and the *amber* alleles, but not how many. Therefore we cannot know how much reduction in recombination is required in order for these markers to become linked. Thus the linkage determination can only place a lower limit on the magnitude of the deficiency.

The point is underscored by the effect of the *erf*$^-$ mutation on the rate of DNA synthesis. Late in infection, the *erf*$^-$ mutation alone (in *rec*$^+$ cells) makes a difference

of a factor of 10 in the rate of phage DNA synthesis. Under these conditions, the observed difference in recombination frequency in a two-factor cross is at most a factor of 2, and the effect on linkage of c_1 to *am* N10 in a three-factor cross is nil. Thus we have good reason to believe that the *erf*$^-$ mutation has an effect on the growth of the phage much larger than that indicated by the recombination measurements.

(b) *Formation of recombinants*

A necessary consequence of the permuted structure of the mature phage DNA is that two kinds of recombinant arrangements are possible between any two loci. If one imagines two loci which are close together on the circular genetic map, the two possible recombinant arrangements can be described as one in which the cross-over event has occurred in the short distance between the two loci, and one in which the crossover has occurred in the long distance between the two loci. This principle is illustrated in Figure 6, which shows how the two kinds of recombinant structures

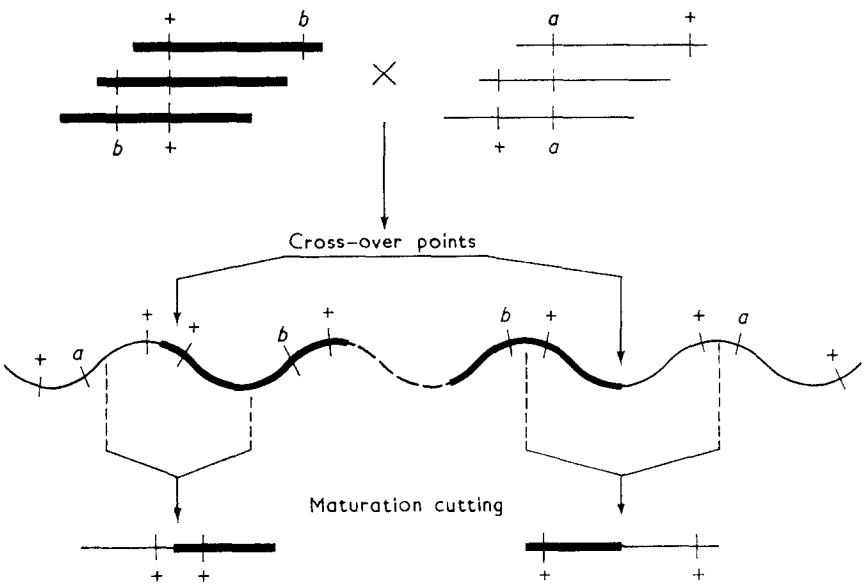

FIG. 6. Generation of two recombinant structures between two markers as a consequence of circular permutation of the mature phage DNA. Crossing-over is assumed to occur either before the formation of oversize intermediate DNA forms or between such forms. "Headfuls" of DNA are then cut out at random.

might arise from two genetically different populations of permuted DNA molecules. The Figure assumes an oversize DNA intermediate from which "headfuls" of mature phage DNA are cut, as suggested by Streisinger for phage T4 (Streisinger *et al.*, 1967). Evidence supporting the applicability of this maturation model to phage P22 has been reported previously (Botstein, 1968; Botstein & Levine, 1968*a*,*b*).

It is clear from Figure 6 that the generation of either kind of recombinant arrangement requires one physical cross-over event in the DNA and subsequent cutting of the recombined DNA by the maturation process in the right places so as not to destroy the recombinant. Thus the two kinds of recombinants do not differ in their origin, and both presumably require the same kinds of enzymological events. The crucial

point for the purposes of this discussion is the corollary that a cross-over event anywhere in the DNA can produce recombination between any two genetic loci regardless of their position with respect to the cross-over point. If cross-over events are equally probable at any point on the DNA, and the position of the maturation cutting is also random, then recombination is proportional to distance on the DNA. It is clear that in phages with permuted DNA the linearity of recombination with distance will be affected not only by the specificity of the enzymes which catalyze cross-over events, but also by the specificity of the maturation cutting system.

The possibility of two kinds of recombinant structures might also account in part for the very high frequencies of recombination per nucleotide observed with phage P22 and T4 relative to phage λ, which has DNA with unique ends, and in which only a single recombinant structure is possible.

(c) *Role of the prophage integration system in recombination and growth*

The three-factor crosses described above were performed in order to observe map shrinkage and thereby to show that *erf*$^-$ mutants are recombination-deficient. The increases in linkage which we found served this purpose. In addition, however, we observed that the unselected c_1 marker became linked, under *rec*$^-$*erf*$^-$ conditions, not to the *am* marker which is nearer on the map, but instead to the farther of the *am* markers. This result either means that the actual distances between c_1 and the *am* markers are very different from what was calculated from the map of Gough & Levine (1968), or else that there has been a relative enhancement of recombination in the region between c_1 and *am*N8, the nearer of the two *am* markers. The most attractive explanation would be to say that the prophage integration system is responsible for much of the residual recombination in *erf*$^-$*rec*$^-$ crosses. This is certainly what happens with phage λ (Signer & Weil, 1968; Echols & Gingery, 1968).

The explanation for the large bursts which arise from a small fraction of *rec*$^-$ cells infected with *erf*$^-$ phage is not known. One simple hypothesis is that these bursts represent cells infected with phage the DNA of which happens to be terminally repetitious at the prophage attachment site. The prophage integration mechanism might be active in circularizing these DNA molecules, thereby permitting growth.

Two observations support this hypothesis. One is that upon induction of a lysogen, *erf*$^-$ phage are produced in normal yield by *rec*$^-$ cells, presumably through the intervention of the prophage excision system. The other is the suggestion from the three-factor crosses that the residual recombination observed in *erf*$^-$ crosses performed in *rec*$^-$ cells might be site-specific in a region which includes the prophage attachment site.

The hypothesis is made somewhat unlikely, however, by the observations of Smith & Levine (1967), who showed that *int*$^+$ function is not essential, and may not be expressed, until very late after infection. More decisive against the hypothesis is our recent finding that *erf*$^-$*int*$^-$ double mutants grow almost as well as *erf*$^-$ mutants on *rec*$^-$ hosts. It is as yet not clear whether this means that the *erf*$^-$ alleles are all leaky enough to obscure the effect on growth of the removal of the *int* system or that the *int* system is itself not relevant in this context.

(d) *Recombination and phage DNA replication*

The experiments of Smith & Levine (1964) can be interpreted as dividing phage DNA synthesis into two stages defined by the action of the c_1 gene product. The "early" synthesis occurs before the c_1 gene product acts to stop synthesis and the

"late" synthesis begins shortly thereafter and continues until lysis. The experiments with *erf*$^-$ mutants clearly show that the early stage of phage DNA replication is unaffected by the capacity of an infected cell to catalyze phage recombination. It does not seem to matter whether a phage is *erf*$^+$ or *erf*$^-$, whether it is c_1 or c_2, or whether it is growing in *rec*$^+$ or *rec*$^-$ cells; in all cases, phage DNA replication begins in the same way. However, the late stage of DNA synthesis is affected by the capacity of the infected complex to catalyze successful recombination. If the phage is *erf*$^-$ growing in *rec*$^-$ cells, then the second stage of DNA synthesis is completely absent, whether the phage carries the c_1 or the c_2 mutation. Even in *rec*$^+$ cells, the extent of late-stage phage DNA synthesis is substantially reduced if the infecting phage are *erf*$^-$.

The amount of DNA synthesized by *erf*$^-$ phage in *rec*$^-$ cells can be estimated by integrating the observed rates of synthesis over time and comparing this to the total DNA synthesized in a normal infection. In agreement with similar calculations reported by Smith & Levine (1964), we estimate that early-stage DNA synthesis (i.e. DNA made before the action of the c_1 gene product) amounts to more than 0·3 and less than 2 phage equivalents per infecting phage particle. Thus *erf*$^-$ mutants in *rec*$^-$ cells appear to replicate their DNA about once. Preliminary experiments using density-labeled phage support this conclusion; the best estimate from these experiments is that about half the parental DNA replicates once under *erf*$^-$*rec*$^-$ conditions.

If these estimates are correct, then it would appear that in the absence of recombination only about one initiation of DNA replication is possible per infecting phage DNA molecule. This conclusion is similar to the one drawn by Mosig & Werner (1969) on the basis of their experiments on the replication of partial genomes of phage T4.

One simple hypothesis which would explain the limited synthesis of phage DNA in the absence of recombination is based on the assumption that there exists a genetically unique origin of replication and that replication proceeds in one direction only. In this case, replication in the absence of recombination should be able to proceed from the origin (which is at different points on different infecting phage DNA molecules because of the permutation of the DNA) to the end of the molecule and replication would then cease. Recombination would then be required in order to attach the unreplicated portion of the molecule to the end of the replicated portion so that replication could proceed further and eventually replicate all of the DNA. Our data are perfectly consistent with such a model, but much further work will be required in order to test it.

In the case of an integrated *int*$^-$ prophage which cannot excise (Smith, 1968), extensive replication of some (but not all) phage markers and some bacterial markers lying on one side of the prophage attachment site occurs. Presumably no circular form is possible after induction of these lysogens; abortive replication apparently proceeds in one direction from some point in the prophage very far into the bacterial genome. This situation is consistent with the *erf*$^-$ infection results. In the prophage case, replication continues into the DNA of the host; in the *erf*$^-$ case, replication ceases as the end of the molecule is reached. In the prophage case there are apparently many re-initiations of replication; in the *erf*$^-$ case there appear to be fewer. This might reflect a requirement for a large minimum distance between replication forks; a difference between the control of DNA replication after induction as opposed to infection; or a new control mechanism yet to be understood.

(e) *Recombination and lysogeny*

We have shown above that *erf*$^-$ phage will not lysogenize *rec*$^-$ cells although they will lysogenize *rec*$^+$ bacteria. The most likely reason for failure to lysogenize is that

recombination is essential to the circularization of the infecting permuted and terminally repetitious phage DNA. The possibility that for some reason a complete replication of the phage DNA is a prerequisite to integration is not excluded, and this might well account for the failure of DNA-negative mutants of phage P22 to lysogenize (Levine, 1967).

(f) *Expression of late phage functions in the absence of recombination*

erf$^-$ Mutants growing under non-permissive conditions (*rec*$^-$ host) exhibit a phenotype typical of "late" mutants: they cause lysis of the culture and produce considerable amounts of base-plate protein. This result is somewhat surprising in view of the fact that only very limited DNA replication occurs in these circumstances and of the position of the *erf* gene in the "early" region of the genetic map. However, this feature of the phenotype made possible experiments which show that during lytic infections *erf*$^-$ phage cause the production of normal numbers of generalized transducing particles per infected *rec*$^-$ cell. Since P22 transducing particles contain primarily host DNA made before phage infection (J. Ebel, unpublished experiments), this result implies that *erf*$^-$ mutants express all of the genes required for encapsulation of DNA into functional particles. Thus it is probable that only DNA synthesis is interfered with by failure to recombine, and that most of the late phage functions are expressed and are active.

(g) *Central role of circularization of the phage DNA in growth*

The observation that induction of a *rec*$^-$*erf*$^-$ lysogen results in normal growth of *erf*$^-$ phage in *rec*$^-$ cytoplasm suggests that circularization of the phage DNA is the step for which recombination is required after infection. The difference between infection (*erf*$^-$ phage cannot grow in *rec*$^-$) and induction (*erf*$^-$ phage grow in *rec*$^-$) is that after infection the phage DNA begins as a linear structure, whereas after induction the phage DNA is in a circular form, presumably because of the nature of the prophage excision event. Previous work indicates that a specific prophage integration and excision system exists for phage P22 (Smith & Levine, 1967; Smith, 1968) and recently a mutant which prevents excision but not integration has been isolated (H. O. Smith, personal communication).

The growth of *erf*$^-$ phage in *rec*$^-$ cells after induction shows that there is nothing wrong with *erf*$^-$ DNA *per se* which interferes with replication in *rec*$^-$ cells; *erf*$^-$ phage can direct the synthesis, in *rec*$^-$ cells, of all the proteins required for phage growth except for one or more of those required to circularize permuted linear infecting DNA.

The circularization idea provides a most attractive way of explaining the salient features of the *erf*$^-$ phenotype. Since the mature form of the phage DNA is terminally repetitious, circularization can be accomplished easily by a recombination event within the terminal repetition. Since the mature form of the DNA is circularly permuted, the enzyme system which can accomplish circularization must be able to recognize a large number of different base sequences. Such an enzyme system should also be able to catalyze recombination events between two different molecules at many different sites: i.e. it should be a general recombination system.

The inability of *erf*$^-$ phage to lysogenize *rec*$^-$ bacteria is easily explained as well, if we assume that circularization is not possible. It is very likely that the Campbell model for prophage insertion (Campbell, 1962) applies to phage P22 and that circular phage DNA is an intermediate in this process (Thomas, Kelly & Rhoades, 1968;

Botstein & Levine, 1968*b*). Failure of *erf*⁻ mutants to replicate their DNA extensively can also be explained (as outlined above) on the basis of circularization as the essential step requiring recombination. If we assume a unique origin and direction of replication, most infecting phage DNA molecules would be only partially replicated in the absence of circularization; a single origin cannot replicate all parts of the genome without recombination. There is, on the other hand, no reason inherent in this explanation why late functions should not be turned on, and why bacterial DNA should not be encapsulated to form transducing particles.

Several points should be emphasized in this connection. First, circularization does not necesssarily mean that closed circular DNA must appear as a long-lived intermediate form: circularization might be accomplished in one strand only and then only for a very short time. Previous work (Botstein & Levine, 1968*b*) indicates that long-lived circular intermediates may not exist at any time during the lytic growth phase of phage P22. Second, the requirement for circularization does not necessarily imply a rolling circle mechanism (Gilbert & Dressler, 1968; Eisen, Pereira da Silva & Jacob, 1968). Such a model is, however, attractive in view of the behavior of DNA replication under *erf*⁻*rec*⁻ conditions, which is exactly that predicted for phages T4 and λ by Gilbert & Dressler (1968).

The schematic outline of a model for DNA replication is shown in Figure 7. The model is based in part on the rolling circle idea, although we do not mean to imply all of the specific details of the rolling circle model. Upon infection of the host, DNA replication proceeds from a specific unique origin of replication in a single direction

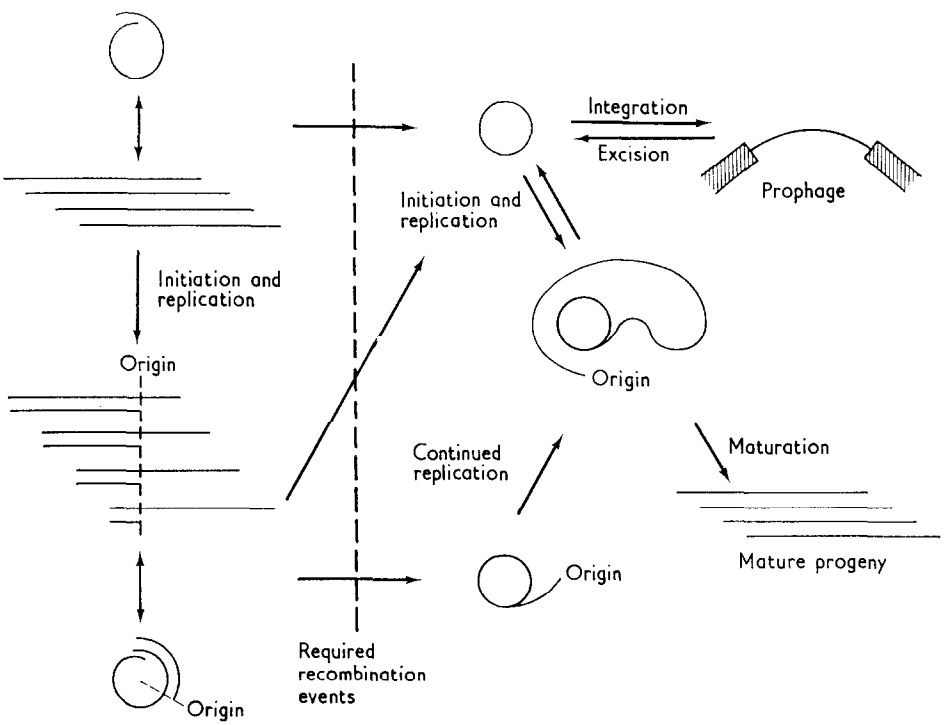

FIG. 7. A model for the topological transformations in phage DNA during the life cycle of phage P22. Single-headed arrows indicate a change in structure; double-headed arrows show topological equivalence and imply no change. Replication proceeds from the origin to the left.

until it reaches the end of the molecule. Since the linear parent molecules are circularly permuted, the sizes of the replicated piece will vary; on the average, one-half of the parental DNA will be replicated if initiation can occur only once per molecule. In order to proceed further, recombination must occur (crossing of the dotted vertical line in the Figure). In the case of an unreplicated molecule, since homology exists between the ends of the molecule, a single recombination event can circularize it. In the case of partially replicated molecules (shown with the homology lined up), one or more recombination events between the replicated and unreplicated portions of the molecules produce the rolling circle form, which can replicate many more times all regions of the chromosome, producing oversize continuous DNA molecules. These are cut up in the process of encapsulation of the DNA into a set of permuted and repetitious progeny molecules.

During infections leading to lysogeny, replicated or unreplicated molecules must be circularized in order to be integrated. This requires recombination events (crossing the dotted line).

After induction, the prophage excision mechanism produces a circular form on which DNA synthesis is initiated. The rolling circle form is thereby produced without the intervention of recombination events.

This kind of model may apply to all phages with permuted, terminally redundant DNA structure. In particular, there is some evidence that it may apply to phage T4. The recent work of Mosig & Werner (1969) suggests that partial genomes of phage T4 replicate from a unique origin to the end of the molecule; in this case circularization is impossible because there is no terminal repetition. Similarly, gene *32* mutants (which are apparently defective in recombination (Tomizawa, Anraku & Iwama, 1966)) appear to replicate only half their DNA upon infection of non-permissive hosts (Kozinski & Felgenhauer, 1967). These results can be made to fit our model if it is assumed that the *E. coli* recombination system is not active on T4 DNA (possibly because of the hydroxymethylcytosine) and that gene *32* is, like the *erf* gene, part of a recombination system essential for circularization of the infecting phage DNA.

The requirement for circularization as well as other features of the model may apply to phage λ as well. Mature phage λ DNA is not permuted, but has unique cohesive ends. The mature DNA can be circularized by a single enzymic step, ligation, of which the host alone is capable (Bode & Kaiser, 1965; Gellert, 1967). This would explain the observation that, unlike those of phage P22, recombination-deficient mutants of phage λ grow well in *rec*$^-$ *E. coli* (Weil & Signer, 1968; Echols & Gingery, 1968) even though the level of recombination is drastically reduced.

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Note added in proof: Yamagami & Yamamoto (1970) (*J. Mol. Biol.* **53**, 281) have recently published a preliminary report of their independent isolation of *ers*$^-$ mutants of phage P22.

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