

Efficient Double-Strand Break-Stimulated Recombination Promoted by the General Recombination Systems of Phages λ and P22

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ABSTRACT

To examine bacteriophage recombination *in vivo*, independent of such other processes as replication and packaging, substituted λ phages bearing restriction site polymorphisms were employed in a direct physical assay. Bacteria were infected with two phage variants; DNA was extracted from the infected cells and cut with a restriction endonuclease. The production of a unique recombinant fragment was measured by Southern blotting and hybridization with a substitution sequence-specific probe. High frequency recombination was observed under the following conditions: the substituted λ phages infected a wild-type host cell bearing a λ repressor-expressing plasmid designed to shut down phage transcription and inhibit phage DNA replication as well. The same plasmid expressed the λ *red* and *gam* genes. In addition, the host cell bore a second plasmid which expressed the *EcoRI* restriction-modification system. Both phage chromosomes possessed a single *EcoRI* site in the middle of the marked substitution sequence; however, as the site was modified in one of the parent phages, only the other partner was cut. Recombination was found to be dependent upon (1) *red*, (2) *recA*, (3) inactivation of the host *recBCD* function, either by Gam protein or by mutation and (4) double-strand breaks. The homologous recombination system of phage P22 could substitute for that of λ .

ONE impediment to the study of general recombination is that the process typically occurs at low frequency at unpredictable locations throughout the recombining chromosomes. Especially for biochemical experiments, it would be advantageous if a general recombination system could be made to operate at high efficiency, at a specified location and time, on nonreplicating DNA. Studies by STAHL and co-workers (STAHL, KOBAYASHI and STAHL 1985; THALER, STAHL and STAHL 1987a,b) suggest that the Red general recombination system of bacteriophage λ might be capable of functioning in this way. By analyzing recombinant progeny phage, produced in small numbers under conditions of severely limited DNA replication, these investigators concluded that the Red system promotes recombination events at high efficiency at the sites of double-strand breaks made by the phage DNA packaging apparatus, or by restriction endonucleases. To account for the high level of Red-mediated recombination occurring throughout the λ chromosome that is observed when phage replication takes place, these authors speculated that the phage replication apparatus might generate double-strand ends that initiate exchanges.

Indications of the activity of the Red system in promoting double-strand break repair have come from a number of additional studies. TAKAHASHI and KOBAYASHI (1990) showed that Red could promote the repair, *in vivo*, of plasmid DNA cut at restriction

sites *in vitro*, if the cut plasmid were provided with an uncut homologue in the transformed cell. EDDY and GOLD (1992) showed that the Red (and RecE) systems could promote an event much like the "homing" of mobile group I introns, in which an artificially inserted DNA segment would transfer from one chromosome to a cut—and previously noninsertion-containing—homolog. CLYMAN and BELFORT (1992) showed that Red, as well as the recombination system of bacteriophage T4, could promote the homing of the T4 *td* intron.

As a step toward biochemical study, we describe methods for direct characterization of a Red-mediated recombination event *in vivo*. The procedures combine the use of a restriction endonuclease to generate a double-strand break in one of the nonreplicating parent chromosomes (THALER, STAHL and STAHL 1987a,b), with recombination functions supplied by plasmids (POTEETE and FENTON 1984) and a direct physical assay of recombinants among intracellular DNA molecules (POTEETE, FENTON and MURPHY 1988). Results from the application of these methods to the λ Red system generally support the model advanced by STAHL, KOBAYASHI and STAHL (1985), under conditions in which the yield of recombinants (DNA molecules, rather than completed virions) is much higher. Extension of the methods to the P22 homologous recombination system reveals that its activity, too, is stimulated by double-strand breaks.

MATERIALS AND METHODS

Bacteria: *Escherichia coli* AB1157 (*argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 ml-1 xyl-5 thi-1 rpsL31 tsx-33 supE44*) and the otherwise isogenic strains JC10287 (Δ (*srlR-recA*)304), JC5519 (*recB21 recC22*), JC5547 (*recA13 recB21 recC22*), JC15329 (Δ (*srlR-recA*)306::Tn10 *recB21 recC22 sbcB15*), JC11451 (*sbcB15*), JC12190 (*recB21 recC22 sbcB15 recJ153*), JC9239 (*recF143*), JC8111 (*recB21 recC22 sbcB15 recF143*), JC11830 (*recB21 recC22 sbcB15*), and JC12123 (*recJ284::Tn10*) were obtained from A. J. CLARK. A derivative of AB1157, KM353, in which the allele *recD1901::Tn10* was installed by transduction, with subsequent selection for a tetracycline-sensitive variant, has been described, as has KM354, a tetracycline-sensitive derivative of JC12123 (MURPHY 1991).

Plasmid-bearing host bacteria for the experiments described below were not stored. Rather, freshly transformed clones were obtained and purified for each set of experiments. It was found that this procedure resulted in more efficient retention of pMB4 (data not shown).

Plasmids: Plasmid pMB4, encoding the *EcoRI* restriction and modification system, has been described (BETLACH *et al.* 1976; NEWMAN *et al.* 1981), as have pTP297, pTP302 and pTP304 (POTEETE, FENTON and MURPHY 1988). Plasmid pTP657, used for generating RNA hybridization probes, was constructed by insertion of an 1875-bp *EcoRI*-*BamHI* fragment containing P22 gene 9 sequences, between the *EcoRI* and *BamHI* sites of pGEM[®]-4Z (Promega).

Plasmids pTP178, pTP184, pTP223, pTP224 and pTP232, in which various λ and P22 recombination genes are fused to *P_{lacUV5}* and inserted into the *lacI*-expressing, tetracycline resistance-conferring vector pMC7 (a derivative of pMB9), have been described (FENTON and POTEETE 1984; POTEETE and FENTON 1984). Plasmid pTP535 was constructed by inverting the recombination gene-containing *EcoRI* fragment of pTP178, thus giving it the same orientation as the corresponding segments in the other plasmids (data not shown). The λ *cl* gene was inserted into each of these recombination function-expressing plasmids (except pTP178), by ligating a 2300 bp *DraI* fragment from λ *rex5a* into their filled-in *NdeI* sites, generating plasmids pTP692, pTP666, pTP699, pTP645, and pTP667, respectively. The control plasmid pTP648 was made from pTP645 by deletion of *P_{lacUV5}* and *red* genes with *EcoRI*.

Several plasmids were constructed as intermediates in the construction of RFLP phages. Plasmid pTP354 contains, in order: phage λ sequences from the *EcoRI* site at λ coordinate 21,226 to the *HindIII* site at 23,130, a 1300-bp segment of pTP302 bounded by *HindIII* and *BamHI* sites (including phage λ sequences from the *XhoI* site at 33,498 to the *BamHI* site at 34,499), and the origin-containing segment of pBR322 bounded by *PvuII* and *EcoRI* sites (with the *PvuII* site converted to a *BamHI* site by ligation of the *PvuII* end to a filled-in *BamHI* end). Plasmid pTP356 was constructed by insertion of a *KpnI* linker (5'-GGGTACCC-3') into the filled-in *HindIII* site of pTP354. Plasmid pTP367 was constructed by insertion of a *SalI* linker (5'-GGTCCGACC-3') into the filled-in *XhoI* site of pTP356. Plasmid pTP368 was constructed by ligation of the large fragment generated by digestion of pTP367 with *KpnI* and *SalI*, the 1875-bp *KpnI*-*EcoRI* fragment containing phage P22 gene 9 sequences from pTP304, and the 3339-bp *SalI*-*EcoRI* fragment containing *lac* sequences from pTP297. Plasmid pTP378 was constructed by insertion of a *BglII* linker (5'-CAGATCTG-3') into the *KpnI* site of pTP368 (with the *KpnI* ends made flush by incubation with T4 DNA polymerase and dNTP's); pTP379 was similarly constructed by insertion of an *XbaI*

linker (5'-CTCTAGAG-3') into the *KpnI* site of pTP368. Plasmids pTP381 and pTP382 were constructed by insertion of *XbaI* and *BglII* linkers into the filled-in *SalI* sites of pTP378 and pTP379, respectively.

Phage: In λ strains bearing restriction fragment length polymorphism (RFLP) substitutions, λ sequences including genes *int*, *xis*, *red* and *gam* are replaced with sequences from the tail gene of phage P22 and the *lac* operon of *E. coli*. Methods for constructing and propagating them were as described previously (POTEETE, FENTON and MURPHY 1988), except that most of the phage stocks used in this study were grown in *E. coli* 594 (*sup*[°]). Stocks of *EcoRI*-modified phages were grown in strain 594 bearing plasmid pMB4. λ *sR1*[°] Δ *b527 sR3*[°] *sR4*[°] *Psus80 sR5*[°] (MMS1723) was obtained from M. M. STAHL. A *P*⁺ revertant was selected by plating on *E. coli* 594, and crossed with plasmids pTP381 and pTP382 to introduce the 5400-bp RFLP substitutions. Recombinants, in which the deletion Δ *b527* is replaced with the overlapping RFLP deletion-substitution, were selected on the basis of their formation of blue plaques on a *lac*⁻ indicator strain in the presence of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (Sigma). The genotypes of the resulting phages are *sR1*[°] *RFLP381 sR3*[°] *sR4*[°] *sR5*[°] and *sR1*[°] *RFLP382 sR3*[°] *sR4*[°] *sR5*[°], but they are referred to as λ RFLP381 and λ RFLP382 below. λ RFLP382 *imm21 cl-ts* was constructed by crossing λ *imm21 cl-ts* with pTP382. It was used to test for expression of λ *red* genes in plasmid-bearing cells. (In most of the *E. coli* strains used in these studies, expression of the *red* genes allows phages bearing the RFLP substitution to form larger plaques.)

Crosses: Cultures of host bacteria were grown overnight at 37° in LB broth supplemented with 0.2% maltose and antibiotics (tetracycline at 10 μ g/ml, ampicillin at 100 μ g/ml). Overnight cultures were diluted 100-fold into fresh medium and grown with aeration by swirling at 37° to a density of approximately 1×10^8 /ml. Isopropyl thiogalactoside (IPTG) was added to a final concentration of 1 mM and cultures were aerated at 37° for an additional 30 min, then placed on ice. Phage were added to 10^9 /ml each, typically resulting in a multiplicity of 5 each; MgSO₄ was added along with the phage, to a final concentration of 1 mM. Following a 15–30-min period of adsorption, 10- μ l portions were removed for determination of unadsorbed phage titers. In all cases reported, adsorption was >85%. Cultures were then diluted 5-fold with prewarmed medium containing antibiotics and IPTG and aerated at 37°. Immediately before the addition of phage, host bacteria were tested for retention of pMB4. Small portions of the cultures were streaked on LB-tetracycline plates. Following overnight incubation at 37°, 30 colonies from each culture were tested for growth on LB-ampicillin agar. Only experiments in which pMB4 was retained in a minimum of 28/30 were analyzed further; in practice, retention was generally 100%, except in *recA* strains.

Preparation and analysis of intracellular DNA: The extraction procedure employed was a modified version of that of SILBERSTEIN *et al.* (1990). At various times following addition of phage, 10-ml portions of culture were chilled by pipeting into tubes on ice, and infected cells were collected by centrifugation at 8000 rpm in a Sorvall SS-34 rotor at 4°. Cell pellets were frozen by immersion in a dry ice-ethanol bath, then thawed and resuspended in 0.5 ml 50 mM Tris-HCl, pH 8.0, 10 mM Na₂EDTA, 100 mM NaCl, 0.2% SDS. Hen egg white lysozyme (Sigma) was added, from a freshly prepared stock at 10 mg/ml in 50 mM Tris-HCl, pH 8.0, to a concentration of 100 μ g/ml, and the preparations were incubated for 10 min at room temperature. DNA was extracted twice with equal volumes of

phenol:chloroform:isoamyl alcohol (24:24:1), and once with diethyl ether, then precipitated with ethanol in the presence of 1.3 M ammonium acetate and 45 μg tRNA. Dried pellets were redissolved with 100 μl 10 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA. λ DNA concentrations were roughly quantitated by electrophoresis in a 0.7% agarose gel, followed by staining with ethidium bromide. In most experiments, the amounts of λ DNA extracted from infected cells were compared with amounts extracted from the same mixture of infecting phages, in the absence of bacteria, processed in parallel. Typical yields of intracellular DNA were in the range of 25–50% of the input. Occasionally, yields of phage DNA were lower, especially in samples taken at early time points (less than 30 min). Other than this, there was no apparent systematic variation of yield with time.

Samples containing approximately 20 ng of phage DNA (quantitated as described above) were digested overnight at 37° with *Bgl*II endonuclease (Boehringer Mannheim) and 100 μg/ml RNase A (Sigma). On occasion, DNA preparations were refractory to complete digestion with *Bgl*II. In these cases, satisfactory results were obtained by dialyzing 50-μl portions of the DNA preparations for 30 min on a cellulose acetate-cellulose nitrate filter (VSWP, Millipore) floating on 10 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA. Following electrophoresis through 0.7% agarose, the DNA was fragmented and transferred onto a membrane by overnight capillary transfer as described previously (POTEETE, FENTON and MURPHY 1988), except that Hybond N nylon filters (Amersham) were employed. A Stratagene UV cross-linker was used to immobilize the DNA on the filters. Filters were incubated for 45 min at 50° in a prehybridization mixture (volume 1 ml/10cm²) consisting of 50% (v/v) formamide, 6 × SSC, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM EDTA, 100 μg/ml yeast RNA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin. ³²P-Labeled RNA probe was added, and incubated with the filter overnight at 50°. Probe was synthesized *in vitro* by using T7 RNA polymerase on *Eco*RI-linearized pTP657, according to the instructions of the supplier of pGEM[®]-4Z (Promega). Sequences represented in the probe are RFLP substitution sequences to the left of the *Eco*RI site indicated in Figure 1. Filters were washed at room temperature for 0.5 hr in 1 liter 0.1 × SSC, 0.1% SDS, and then at 68° for 1 hr in 9 liters of the same solution. Filters were exposed to film at -80° with Dupont Lightning Plus fluorescence enhancing screens, for periods of time determined empirically to give the optimum visibility of any recombinant bands present.

Relative amounts of parental and recombinant bands were quantitated with the integrator of a Quick Scan R and D Electrophoresis/TLC densitometer (Helena Laboratories). The optical density of the film at a point slightly below the position of the 5400-bp recombinant band was taken as the background and subtracted from values measured in the zones of the various bands in the gel track. Recombination frequencies reported below represent the amount of 5400-bp recombinant band divided by the total of parental and recombinant bands, measured from the same film. The upper limit of the sensitivity of these determinations was assessed with measurements conducted on samples that contained no recombinants, as follows. DNA was extracted from singly infected cultures, mixed, and analyzed as described above. In several such tests, values of the "recombination frequency" (in the absence of recombination) thus measured ranged up to 0.3%. In the tables that follow, the cases in which no recombinant band was visible are thus reported as <0.3%.

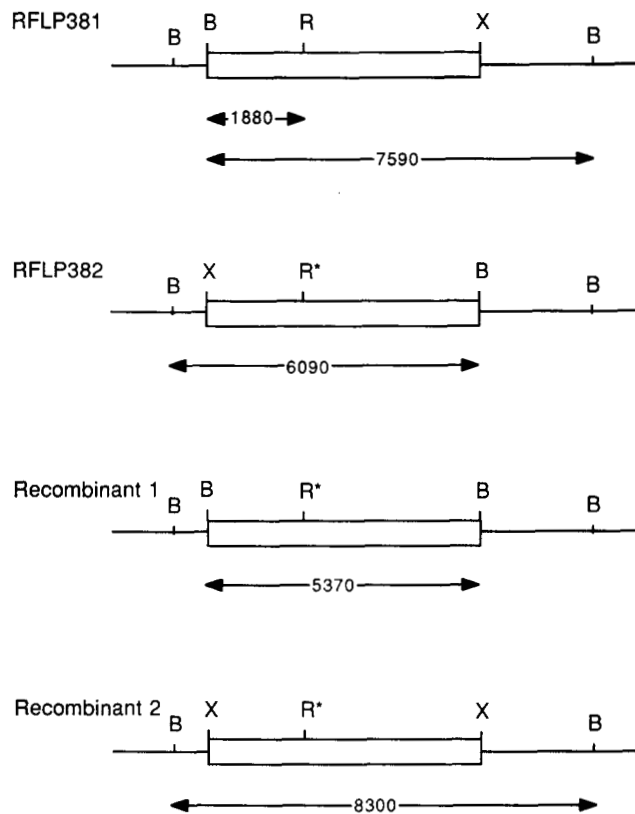


FIGURE 1.—Structures of parental and recombinant phages. Sequences in the RFLP substitution are indicated by boxes, λ sequences in the vicinity by thin lines. The RFLP substitution replaces λ sequences from the b2 region through gene *cIII* with sequences from the *lac* operon of *E. coli* and part of the tail gene of phage P22. B = *Bgl*II site, X = *Xba*I site, R = unmodified *Eco*RI site, R* = modified *Eco*RI site. Numbers and arrows under the maps indicate the sizes of the restriction fragments detected by hybridizing *Bgl*II-digested intracellular DNA with a probe that is specific for P22 tail gene sequences, to the left of the *Eco*RI site in the RFLP substitution.

RESULTS

Experimental system: The experimental system was designed for direct physical detection of general recombination events, without significant amplification of the recombinant DNA via replication. To this end, derivatives of phage λ were used to infect λ repressor-containing host cells, in which injection and circularization of the phage chromosome could occur, but in which transcription of phage genes was blocked. This blockade of transcription indirectly inhibited phage DNA replication. (For a review of λ replication, see FURTH and WICKNER 1983). Both λ and P22 recombination-promoting genes were expressed at high levels from plasmids. The technique of THALER, STAHL and STAHL (1987b) was employed to stimulate and focus recombination events: the phages bore single *Eco*RI restriction sites; in each cross, one parent's *Eco*RI site was modified, and the other's was unmodified. The host cells carried the *Eco*RI restriction-modification system on a plasmid, pMB4 (BETLACH *et al.* 1976).

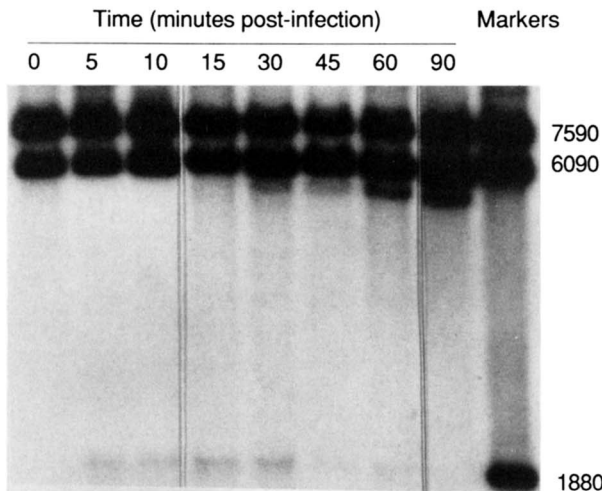


FIGURE 2.—Time course of recombination in the wild type host AB1157, with λ repressor, *red* and *gam* functions supplied by plasmid pTP666, *EcoRI* by plasmid pMB4. DNA was extracted from cells infected with λ RFLP381 m^- and λ RFLP382 m^+ , digested with *Bgl*III, electrophoresed in an agarose gel, transferred to a membrane and hybridized with a RFLP substitution-specific probe.

Recombination events were directed to a region in the substituted λ phages flanked by restriction site polymorphisms. This feature permitted detection of recombinants among DNA molecules extracted from the infected cells, by the use of restriction endonucleases, Southern blots, and hybridization with specific probes, as described previously (POTEETE, FENTON and MURPHY 1988). The structures of the parent and recombinant phages are shown in Figure 1. According to this scheme, the substituted λ chromosomes would enter the cell and circularize, then stop at that point in the phage life cycle. One phage's chromosome would be cut at its single *EcoRI* site. The double-stranded end thus produced would serve as a substrate for the proteins of the phage recombination system, which would use it to invade the unbroken duplex of its partner.

The behavior of the experimental system is illustrated in Figures 2 and 3, and quantitated in Figure 4. In the experiment shown in Figure 2, the host was wild type, and the λ recombination functions *red* α (exonuclease), *red* β , and *gam* were supplied by a plasmid. Cutting by the *EcoRI* endonuclease is shown by the appearance of a 1880-bp fragment, and, in theory, by a relative depletion of a 7590-bp DNA fragment from the unmodified parent. In practice, the 1880-bp fragment produced by *in vivo* digestion with *EcoRI* endonuclease and *in vitro* digestion with *Bgl*III never amounted to more than one percent of the total DNA hybridizable with the probe, and relative depletion of the 7590-bp fragment from the unmodified parent was partial (Figure 2 and other experiments not shown).

Results of control experiments indicated that the evidently low steady-state level of *EcoRI*-digested

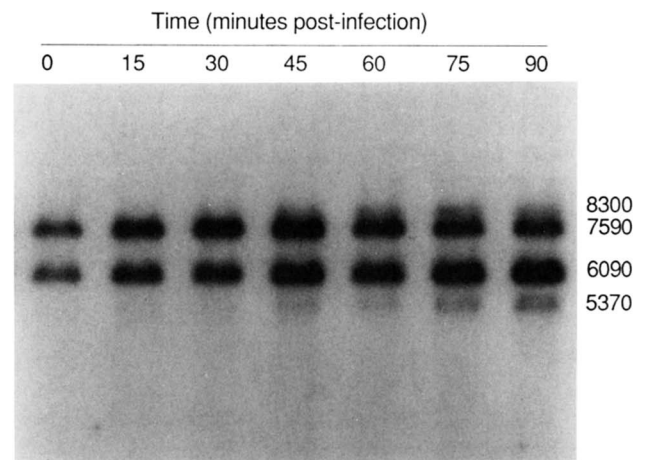


FIGURE 3.—Time course of recombination between λ RFLP381 m^- and λ RFLP382 m^+ in the *recB recC* host JC5519, with *EcoRI* supplied by plasmid pMB4, and λ repressor and *red* (no *gam*) supplied by plasmid pTP645.

phage DNA was not due to lack of *EcoRI* activity. As described under MATERIALS AND METHODS, retention of the *EcoRI*-bearing plasmid pMB4 was tested and found to be nearly complete in all experiments. Moreover, cultures used in some of the experiments were tested directly for their ability to restrict plaque formation by unmodified heteroimmune λ . In the case of the *recA recB recC* strain bearing pMB4 and a plasmid expressing *cI*, *red* and *gam*, the efficiency of plating of unmodified phage (bearing multiple *EcoRI* sites) was 0.23; in all other strains it was less than 0.04.

Recombinant bands were measurable in most experiments by 15 min postinfection (Figures 4 and 5), though they are not highly visible in the photograph of an autoradiogram shown in Figure 2 until 30 min postinfection. Recombinant bands continued to increase in amount for at least an hour (Figures 4 and 5); the two reciprocal recombinants were produced in approximately equal amounts, approaching 20% of the total in some experiments. Phage DNA replication could not be accurately quantitated in these experiments, but was apparently held to low levels at most. The efficiency of extraction of DNA varied from one sample to another, but there was little tendency of samples extracted from cells at later time points to contain more phage DNA than those at earlier time points. In contrast, because cellular replication was unimpeded in the infected cells, recovered bacterial DNA increased in amount during the time course. In otherwise similar infections of non repressor-containing cells, phage DNA was greatly amplified (data not shown). The plasmid-encoded repressor apparently inhibited replication more strongly than lysogen-encoded repressor (presumably due to its presence at higher levels in the plasmid-bearing cells); some replication of λ DNA was evident in similar experiments involving the infection of λ lysogens (data not shown).

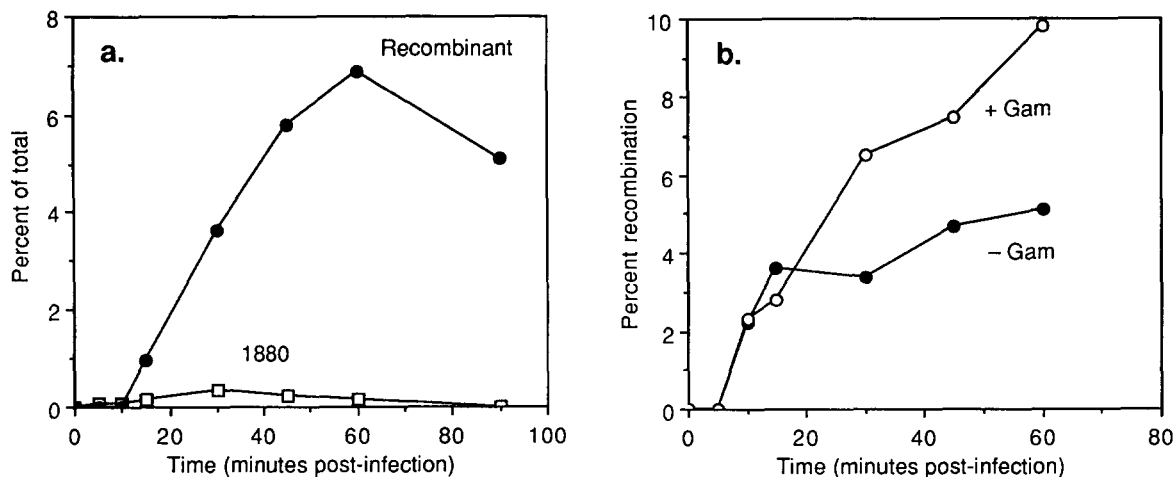


FIGURE 4.—Time courses of recombination between λ RFLP381 m^- and λ RFLP382 m^+ : densitometric measurements of autoradiographs as in Figures 2 and 3. (a) Wild-type host (AB1157), with λ repressor, *red* and *gam* functions supplied by plasmid pTP666, *EcoRI* by plasmid pMB4. Percentages of total hybridized radioactivity in the form of the 5370-bp recombinant band (filled circles) and the 1880-bp parental fragment produced by intracellular digestion by the *EcoRI* endonuclease (open squares) are indicated. (b) *recB recC* host (JC5519), with *EcoRI* supplied by plasmid pMB4, and either λ repressor, *red* and *gam* functions supplied by plasmid pTP666 (open circles), or λ repressor and *red* (no *gam*) supplied by plasmid pTP645 (filled circles). Percentages of total hybridized radioactivity in the form of the 5370-bp recombinant band are indicated.

The 1880-bp fragment produced by *EcoRI* digestion *in vivo* and *BglII* digestion *in vitro* appeared in samples taken five minutes after infection, and persisted, at a low level, during the entire period in which recombinants were produced. It is shown in Figure 2, and quantitated in Figure 4a.

Requirements for high frequency recombination:

The effects of changes and omissions on the efficiency of recombination were tested in an experiment summarized in Table 1. Switching the genotypes of the modified and unmodified phages had little effect, except that the 1880-bp fragment was replaced by a 2580 bp fragment (see the diagram in Figure 1). Omitting *red*, the *EcoRI* restriction system, or *gam* made recombinants undetectable.

The requirement for *gam* suggested that inactivation of RecBCD might have a role in the recombination reaction. If so, the need for Gam might be bypassed in a *recB recC* mutant host. This was observed to be the case, as shown in Figures 3 and 4(b), and Table 1. As in the wild type host, the observed recombination was dependent upon *red* and *EcoRI* and was not significantly affected by switching the modified and unmodified phages. The observed recombination frequency in the *recB recC* host was reproducibly lower than in wild type, however, suggesting that Gam has a second role in this process, in addition to inactivating RecBCD function. This notion was strengthened by the experiment illustrated in Figure 4b; it was found that Gam stimulated recombinant formation in a *recB recC* host.

The observed frequency of *red* and *gam*-promoted recombination in the wild-type host could be doubled by infecting the wild-type host first with the unmodi-

fied phage, then, after ten minutes at 37°, infecting with the modified phage, as shown in Figure 5a. The opposite result was found in the *recB recC* host in the absence of Gam (Figure 5b). Sequential infection, in this case, produced fewer recombinants than simultaneous infection. When Gam was supplied in the *recB recC* host, however, the wild-type pattern of recombination was observed (Figure 5c). As in the previous experiments, there was relatively little variation in the yields of recombinants from cells infected simultaneously with both parent phages: slightly more from wild type than from the *recBC* mutant, with the yield from the latter increased slightly when *gam* function was supplied by a plasmid. These effects associated with switching between simultaneous and sequential infection suggest that the hypothetical (non-RecBCD) second cellular target of Gam might destroy the recombinogenic potential of double-strand ends if pairing with a homologue does not occur quickly after their generation.

P22 and host recombination functions: The homologous recombination system of bacteriophage P22 consists of genes *arf* (accessory recombination function; POTEETE, FENTON and SEMERJIAN 1991), *erf* (essential recombination function; BOTSTEIN and MATZ 1970), *abc1* and *abc2* (anti-RecBCD; MURPHY, FENTON and POTEETE 1987). It could substitute for its λ counterpart, as shown by the experiment summarized in Table 2. A plasmid expressing these four P22 genes stimulated recombination that was, as in the case of λ functions, dependent upon double-strand breaks and upon the RecBCD-modifying proteins of the phage (*Abc1* and *Abc2* of P22).

The question of the involvement of host recombi-

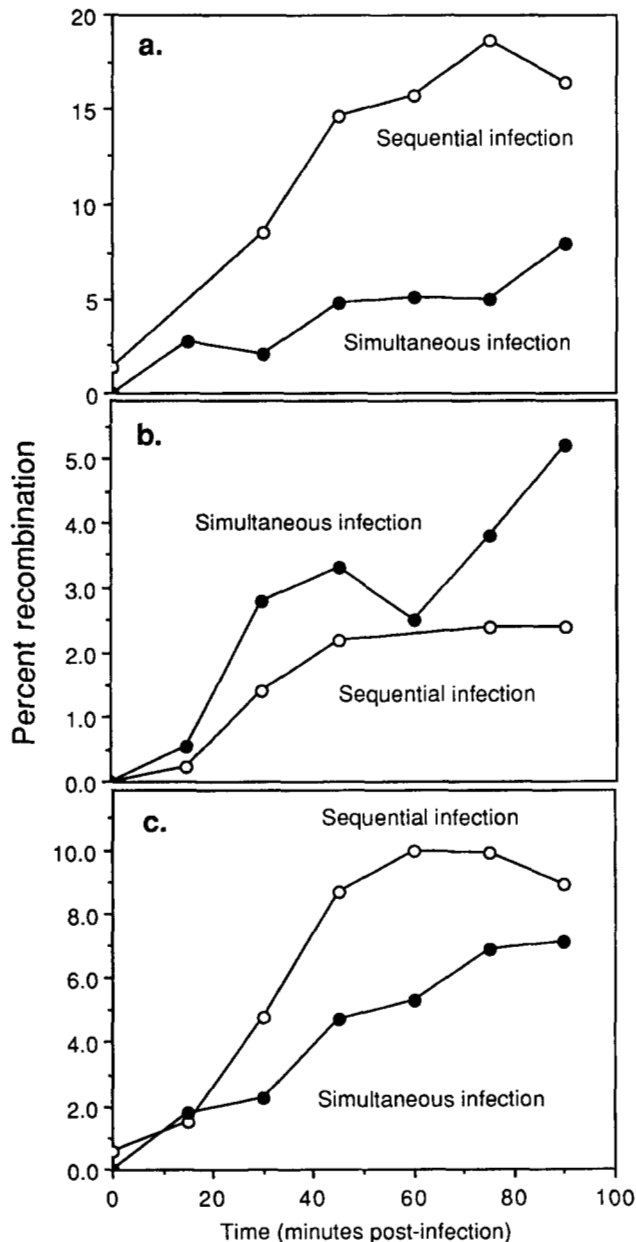


FIGURE 5.—Recombination following simultaneous and sequential infection with λ RFLP381 m^- and λ RFLP382 m^+ . All hosts expressed the *EcoRI* restriction-modification system from plasmid pMB4. (a) The wild-type host (AB1157), with repressor, *red* and *gam* supplied by plasmid pTP666, was infected simultaneously with modified and unmodified phages (filled circles); or first with unmodified phage, followed, after 10 min at 37°, by modified phage (empty circles). (b) The *recB recC* host (JC5519), with repressor and *red* supplied by plasmid pTP645, was infected simultaneously (filled circles) or sequentially (open circles), as in (a). (c) The *recB recC* host (JC5519), with repressor, *red* and *gam* supplied by plasmid pTP666, was infected simultaneously (filled circles) or sequentially (open circles), as in (a).

nation functions in *red*-mediated recombination in this system was investigated in the experiment summarized in Table 3. Recombination was not observed in a *recA* strain expressing *red* and *gam*, regardless of whether the strain was additionally mutant in *recB* and

recC as well. In contrast, recombination was seen not to depend upon *recB* or *recC*, as observed previously, or upon *recD*, *recF* or *recJ*.

Fates of *EcoRI*-digested DNA *in vivo*: Blocking recombination in this system does not lead to accumulation of *EcoRI*-digested DNA in the infected cell, as shown in Figure 6. The first five lanes show the time course of infection of a *recA* host. As in the wild type host, the 1880-bp fragment resulting from *EcoRI* digestion *in vivo* and *BglII* digestion *in vitro* appears early in the infection, and is seen, in gradually decreasing amounts, thereafter; and there is a gradual depletion of the band corresponding to the unmodified parent. No recombinant bands appear. The last three lanes show 30-min time points from infections of wild-type cells bearing plasmids that supply all the necessary components but one: *EcoRI*, *red* and *gam*, respectively. In samples taken from the *EcoRI*-less infection, the unmodified parent band is not depleted, and no 1880-bp fragment appears. In the *red*-less infection, a small amount of the 1880-bp fragment, and a slight depletion of the unmodified parent band are seen, as in the *recA*-less infection. In the *gam*-less infection, depletion of the unmodified parent band is more pronounced, and no 1880-bp fragment is detectable; these latter observations presumably reflect the destructive activity of unmodified RecBCD nuclease.

DISCUSSION

The λ Red system, acting in concert with RecA, in the absence or near-absence of normal phage DNA replication, can promote recombination events at a high frequency, if one partner in the cross has a double-strand break. This finding is consistent with the mechanism of Red-mediated recombination proposed by STAHL, KOBAYASHI and STAHL (1985). Subsequent research by THALER, STAHL and STAHL (1987a,b) provided support for this mechanism through studies of the properties of recombinant progeny phage. The present study extends these earlier results by direct analysis of intracellular DNA substrates, possible intermediates, and products of recombination.

Intracellular digestion of the unmodified parent phage chromosome by the *EcoRI* endonuclease could be monitored by the production of the 1880-bp DNA fragment upon digestion with *BglII* *in vitro* (Figures 2 and 4). This fragment was present, in low amounts, in all cases in which recombinant formation could be detected. It was additionally detectable in cases in which recombinant formation was blocked by omission of *red* or *recA* function, but not when *gam* was omitted (thus leaving RecBCD nuclease active), or when *EcoRI* was omitted (Figure 6). We were initially surprised by the apparently sluggish activity of the *EcoRI* nuclease in cutting the unmodified parent

TABLE 1
Requirements for high frequency recombination in wild-type and *recBC* mutant *E. coli*

Host	Plasmids	Functions ^a	Phages ^b	Percent recombination ^c
Wild type (AB1157)	pTP666 + pMB4	<i>red</i> + <i>gam</i> + <i>EcoRI</i>	λRFLP381 m ⁺ λRFLP382 m ⁻	2.7
	pTP666 + pMB4	<i>red</i> + <i>gam</i> + <i>EcoRI</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	3.6
	pTP645 + pMB4	<i>red</i> + <i>EcoRI</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	<0.3
	pTP699 + pMB4	<i>gam</i> + <i>EcoRI</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	<0.3
	pTP666	<i>red</i> + <i>gam</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	<0.3
<i>recBC</i> (JC5519)	pTP645 + pMB4	<i>red</i> + <i>EcoRI</i>	λRFLP381 m ⁺ λRFLP382 m ⁻	1.5
	pTP645 + pMB4	<i>red</i> + <i>EcoRI</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	1.3
	pTP648 + pMB4	<i>EcoRI</i>	λRFLP381 m ⁻ λRFLP382 m ⁺	<0.3
	pTP648	None	λRFLP381 m ⁻ λRFLP382 m ⁺	<0.3

^a λ repressor was present in every case.

^b m⁺ = *EcoRI*-modified, m⁻ = *EcoRI*-unmodified.

^c 5370-bp recombinant band as percentage of total recombinant + parental bands at 30 min after infection.

TABLE 2

Double-strand break-stimulated recombination by λ and P22 systems

Plasmids	Functions ^a	Percent recombination ^b
pTP666 + pMB4	<i>red</i> + <i>gam</i> + <i>EcoRI</i>	3.0
pTP666	<i>red</i> + <i>gam</i>	<0.3
pTP645 + pMB4	<i>red</i> + <i>EcoRI</i>	<0.3
pTP648 + pMB4	<i>EcoRI</i>	<0.3
pTP667 + pMB4	<i>arf</i> + <i>erf</i> + <i>abc1</i> + <i>abc2</i> + <i>EcoRI</i>	1.1
pTP692 + pMB4	<i>arf</i> + <i>erf</i> + <i>EcoRI</i>	<0.3
pTP667	<i>arf</i> + <i>erf</i> + <i>abc1</i> + <i>abc2</i>	<0.3

Host was AB1157 bearing the indicated plasmids. Phages were λRFLP381 m⁻ and λRFLP382 m⁺.

^a The λ repressor was present in every case.

^b 5370-bp recombinant band as percentage of total recombinant + parental bands at 30 min after infection.

phage chromosomes *in vivo*. This observation contrasted with the efficiency of the same host cells in restricting plaque formation by unmodified λ, as described in RESULTS. On the other hand, a study by HEITMAN, ZINDER and MODEL (1989) has indicated that *E. coli* strains with normal ligase activity have a great capacity to repair *EcoRI* scissions in their chromosomes. We surmise that the low level of *EcoRI*-cut DNA observed in our experiments reflects a nearly steady state, with a substantial rate of turnover mediated by the processes of endonucleolytic digestion, recombination, ligation, and exonucleolytic destruction. The present study leaves unanswered the question of whether the *EcoRI*-ended 1880-bp fragment

TABLE 3

Dependence of high frequency recombination on host functions

Host	Percent recombination
Wild type	1.0
<i>recA</i>	<0.3
<i>recBC</i>	0.8
<i>recABC</i>	<0.3
<i>recD</i>	1.0
<i>recF</i>	1.1
<i>recJ</i>	0.9

Phages were λRFLP381 m⁻ and λRFLP382 m⁺. Host cells bore the *EcoRI* plasmid pMB4 and the λ repressor + *red* + *gam* plasmid pTP666. Recombination was measured as the amount of the 5370-bp recombinant band as a percentage of total recombinant + parental bands at 30 min after infection. Control crosses were carried out in each bacterial strain bearing pMB4 and the non-*red* or *gam*-expressing plasmid pTP648; in all such cases, recombinant bands were not detectable.

detectable *in vitro* is part of an actual recombination intermediate. The kinetics of its appearance in the cell, shortly before and during the period in which recombinants accumulate, suggest that it is. On the other hand, the 1880-bp fragment does not accumulate appreciably when recombination is blocked by omission of *recA* or *red*; it is possible that the actual intermediate has too short a lifetime for detection by these methods, and that the only *EcoRI* ends we observe are those that are destined for exonucleolytic degradation, for instance. Investigation of this and related questions, by the use of biochemical techniques *in vitro*, is needed.

Experiments described above show that the homol-

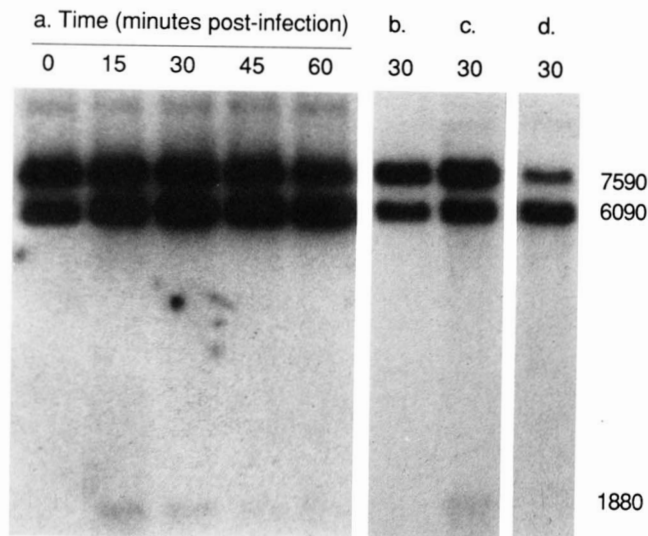


FIGURE 6.—Fates of the parental DNA in infections in which recombination is blocked. λ RFLP381 m^- and λ RFLP382 m^+ infected the indicated strains: (a) the *recA* host (JC10287), with λ repressor, *red* and *gam* functions supplied by plasmid pTP666, *EcoRI* by plasmid pMB4; (b) the wild-type host (AB1157), with λ repressor, *red* and *gam* functions supplied by plasmid pTP666 (*EcoRI* omitted); (c) the wild-type host, with λ repressor and *gam* functions supplied by plasmid pTP699 (*red* omitted), *EcoRI* by plasmid pMB4; (d) the wild-type host, with λ repressor and *red* functions supplied by plasmid pTP645 (*gam* omitted), *EcoRI* by plasmid pMB4.

ogous recombination system of phage P22, like that of λ , is stimulated by double-strand breaks. This finding is unsurprising in light of the functional interchangeability of the recombination systems of the two related phages (POTEETE and FENTON 1984). However, it is intriguing in light of the apparent structural unrelatedness of the various parts of the two recombination systems (PROTEETE, FENTON and SEMERJIAN 1991).

The RecE recombination pathway of *E. coli* resembles the Red pathway of phage λ in some respects (GILLEN, WILLIS and CLARK 1981). It depends upon activation of recombination genes in the cryptic prophage Rac. SYMINGTON, MORRISON and KOLODNER (1985) showed that *E. coli* strains in which the RecE pathway was active could circularize linear plasmid dimers by recombination following transformation. KOBAYASHI and TAKAHASHI (1988) found that such cells could carry out double-stranded gap repair on plasmid DNA introduced by transformation. NUSSBAUM, SHALIT and COHEN (1992) have described experiments, similar in concept to those reported here, demonstrating double-strand break-stimulated homologous recombination of plasmids by the *E. coli* RecE pathway. Their experiments differed, though, in that recombination was slower, and was accompanied by extensive DNA replication in the linear multimer synthesis mode (COHEN and CLARK 1986).

High frequency Red-mediated double-strand break-stimulated recombination depends upon inactivation

of RecBCD, either by mutation of *recB* or *recC* or by expression of phage-encoded RecBCD-modulating proteins. This observation is consistent with the idea that RecBCD is destructive to ends of double-stranded DNA lacking properly oriented Chi sites (STAHL *et al.* 1990). However, the Gam protein of λ evidently has a second role in the process, since supplying it stimulates recombination in a *recB recC* mutant infected sequentially with the two test phages. KULKARNI and STAHL (1989) have shown that the *gam* gene is essential for the growth of λ derivatives bearing long DNA palindromes in *sbcC*⁺, but not in *sbcC*⁻ hosts. Possibly, the product of *sbcC*, or some other function under its control, interferes mildly with recombination in the crosses described here, and Gam prevents this interference via an interaction with *sbcC*.

It is formally possible that the effects attributed to *gam* in these studies are wholly or partially due to some other, as yet unknown λ function expressed (in addition to *gam*) by plasmid pTP666 but not by pTP645. The former differs from the latter only in containing λ sequences between positions 32,966 and 33,248 (SANGLER *et al.* 1982). Computer-assisted inspection of this sequence does not reveal any likely protein-encoding reading frames other than those of *gam* and *red β* (not shown). However, as noted by FRIEDMAN and HAYS (1986), there are two feasible translation initiation sites in the *gam* open reading frame; both are contained in pTP666. MURPHY (1991) has shown that Gam protein has an amino acid sequence consistent with initiation at the second of these sites, but these data do not rule out the possibility that a second protein is encoded by the longer open reading frame.

An unexpected finding in these experiments was the apparent enhancement, in the wild type host, of the recombinogenic potential of the cuttable phage chromosome when it was injected before that of the uncuttable partner. This observation suggests that some recombination-promoting component, one that operates only on the double-strand end-bearing partner, is partially sequestered by the first (uncut) phage chromosomes injected into the cell.

The requirement for RecA in this system illustrates the complexity of its mechanistic relationship with Red β . On one hand, when λ replicates freely, it recombines efficiently in the absence of RecA (ECHOLS and GINGERY 1968; SIGNER and WEIL 1968). In addition, the single strand-pairing properties of Red β resemble those of RecA, although RecA can promote strand exchange, while Red β apparently cannot (KMIEC and HOLLOWAN 1981; MUNIYAPPA and RADDING 1986). Moreover, Red β can suppress the deficiency in plasmid recombination of a *recA* mutant, operating via the RecF pathway (BERGER and COHEN 1989). On the other hand, *recA* strains containing

Redβ are in other ways phenotypically RecA⁻: they are UV-sensitive, and incapable of forming stable recombinants following conjugation (our unpublished observations). When DNA replication is blocked, the λ recombination system is nearly inactive in the absence of RecA (STAHL, STAHL and MALONE 1978); overexpression of *red* and *gam* by a plasmid does not remedy this deficiency (A. R. POTEETE and F. W. STAHL, unpublished observations). It would appear that RecA has an activity, not shared with Redβ, that is essential specifically for recombination of nonreplicating DNA. CLYMAN and BELFORT (1992) reported a similar requirement for *recA* in *red*-mediated intron transfer; they speculated that the special role of RecA protein in the process might be to direct strand invasion.

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LITERATURE CITED

- BERGER, I., and A. COHEN, 1989 Suppression of RecA deficiency in plasmid recombination by bacteriophage λ β protein in RecBCD⁻ExoI⁻ *Escherichia coli* cells. *J. Bacteriol.* **171**: 3523–3529.
- BETLACH, M., V. HERSHFIELD, L. CHOW, W. BROWN, H. M. GOODMAN and H. W. BOYER, 1976 A restriction endonuclease analysis of the bacterial plasmid controlling the *EcoRI* restriction and modification of DNA. *Fed. Proc.* **35**: 2037–2043.
- BOTSTEIN, D., and M. J. MATZ, 1970 A recombination function essential to the growth of bacteriophage P22. *J. Mol. Biol.* **54**: 417–440.
- CLYMAN, J., and M. BELFORT, 1992 *Trans* and *cis* requirements for intron mobility in a prokaryotic system. *Genes Dev.* **6**: 1269–1279.
- COHEN, A., and A. J. CLARK, 1986 The synthesis of linear plasmid multimer in *E. coli* K-12. *J. Bacteriol.* **167**: 327–335.
- ECHOLS, H., and R. GINGERY, 1968 Mutants of bacteriophage λ defective in vegetative genetic recombination. *J. Mol. Biol.* **34**: 239–249.
- EDDY, S. R. and L. GOLD, 1992 Artificial mobile DNA element constructed from the *EcoRI* endonuclease gene. *Proc. Natl. Acad. Sci. USA* **89**: 1544–1547.
- FENTON, A. C., and A. R. POTEETE, 1984 Genetic analysis of the *erf* region of the bacteriophage P22 chromosome. *Virology* **134**: 148–160.
- FRIEDMAN, S. A., and J. B. HAYS, 1986 Selective inhibition of *Escherichia coli* RecBC activities by plasmid-encoded GamS function of phage lambda. *Gene* **43**: 255–263.
- FURTH, M. E., and S. H. WICKNER, 1983 Lambda DNA replication, pp. 145–174 in *Lambda II*, edited by R. W. HENDRIX, J. W. ROBERTS, F. W. STAHL and R. A. WEISBERG. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- GILLEN, J. R., D. K. WILLIS, and A. J. CLARK, 1981 Genetic analysis of the RecE pathway of genetic recombination in *Escherichia coli* K-12. *J. Bacteriol.* **145**: 521–532.
- HEITMAN, J., N. D. ZINDER, and P. MODEL, 1989 Repair of the *Escherichia coli* chromosome after *in vivo* scission by the *EcoRI* endonuclease. *Proc. Natl. Acad. Sci. USA* **86**: 2281–2285.
- KMIEC, E., and W. K. HOLLOWAN, 1981 β protein of bacteriophage λ promotes renaturation of DNA. *J. Biol. Chem.* **256**: 12636–12639.
- KOBAYASHI, I., and N. TAKAHASHI, 1988 Double-stranded gap repair of DNA by gene conversion in *Escherichia coli*. *Genetics* **119**: 751–757.
- KULKARNI, S. K., and F. W. STAHL, 1989 Interaction between the *sbC* gene of *Escherichia coli* and the *gam* gene of phage λ. *Genetics* **123**: 249–253.
- MUNIYAPPA, K., and C. M. RADDING, 1986 The homologous recombination system of phage λ: pairing activities of β protein. *J. Biol. Chem.* **261**: 7472–7478.
- MURPHY, K. C., 1991 λ Gam protein inhibits the helicase and χ-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. *J. Bacteriol.* **173**: 5808–5821.
- MURPHY, K. C., A. C. FENTON and A. R. POTEETE, 1987 Sequence of the bacteriophage P22 Anti-RecBCD (*abc*) genes and properties of P22 *abc* region deletion mutants. *Virology* **160**: 456–464.
- NEWMAN, A. K., R. A. RUBIN, S.-H. KIM and P. MODRICH, 1981 DNA sequences of structural genes for *EcoRI* DNA restriction and modification systems. *J. Biol. Chem.* **256**: 2131–2139.
- NUSSBAUM, A., M. SHALIT, and A. COHEN, 1992 Restriction-stimulated homologous recombination of plasmids by the RecE pathway of *Escherichia coli*. *Genetics* **130**: 37–49.
- POTEETE, A. R., and A. C. FENTON, 1984 λ *red*-dependent growth and recombination of phage P22. *Virology* **134**: 161–167.
- POTEETE, A. R., A. C. FENTON and K. C. MURPHY, 1988 Modulation of *Escherichia coli* RecBCD activity by the bacteriophage λ Gam and P22 *Abc* functions. *J. Bacteriol.* **170**: 2012–2021.
- POTEETE, A. R., A. C. FENTON and A. V. SEMERJIAN, 1991 Bacteriophage P22 accessory recombination function. *Virology* **182**: 316–323.
- SANGER, F., A. R. COULSON, G. F., HONG, D. F. HILL and G. B. PETERSEN, 1982 Nucleotide sequence of bacteriophage λ DNA. *J. Mol. Biol.* **162**: 729–773.
- SIGNER, E. R., and J. WEIL, 1968 Recombination in bacteriophage λ. I. Mutants deficient in general recombination. *J. Mol. Biol.* **34**: 261–271.
- SILBERSTEIN, Z., S. MAOR, I. BERGER and A. COHEN, 1990 Lambda Red-mediated synthesis of plasmid linear multimers in *Escherichia coli* K-12. *Mol. Gen. Genet.* **223**: 496–507.
- STAHL, F. W., I. KOBAYASHI and M. M. STAHL, 1985 In Phage λ, *cos* is a recombinator in the Red pathway. *J. Mol. Biol.* **181**: 199–209.
- STAHL, F. W., M. M. STAHL and R. E. MALONE, 1978 Red-mediated recombination of phage lambda in a *recA⁻ recB⁻* host. *Mol. Gen. Genet.* **159**: 207–211.
- STAHL, F. W., L. C. THOMASON, I. SIDDIQI and M. M. STAHL, 1990 Further tests of a recombination model in which χ removes the RecD subunit from the RecBCD enzyme of *Escherichia coli*. *Genetics* **126**: 519–533.
- SYMINGTON, L. S., P. MORRISON and R. KOLODNER, 1985 Intramolecular recombination of linear DNA catalyzed by the *Escherichia coli* RecE recombination system. *J. Mol. Biol.* **186**: 515–525.
- TAKAHASHI, N., and I. KOBAYASHI, 1990 Evidence for the double-strand break repair model of bacteriophage λ recombination. *Proc. Natl. Acad. Sci. USA* **87**: 2790–2794.
- THALER, D. S., M. M. STAHL and F. W. STAHL, 1987a Tests of the double-strand-break repair model for Red-mediated recombination of phage λ and plasmid λ-dv. *Genetics*, **116**: 501–511.
- THALER, D. S., M. M. STAHL and F. W. STAHL, 1987b Double-chain-cut sites are recombination hotspots in the Red pathway of phage λ. *J. Mol. Biol.* **195**: 75–87.