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Genetics

Evidence for inclusion of regions of nonhomology in heteroduplex products of bacteriophage λ recombination

(*red*, *recA*/replication-restricted crosses/negative interference/branch migration/asymmetric strand transfer)

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ABSTRACT **Total intracellular DNA was isolated from replication-restricted bacteriophage λ crosses in which the infecting parents were heteroallelic for wild-type and deletion mutant alleles. This DNA was examined for the presence of heteroduplex DNA molecules that contained wild-type sequences in one strand and deletion-mutant sequences in the other. Molecules hybrid for a 689-nucleotide deletion in the immunity region of λ were detected at significant levels only in crosses in which both the *red* recombination system of λ and the *rec* recombination system of *Escherichia coli* were active. Molecules hybrid for a 1300-nucleotide deletion in the central portion of the λ genome were detected at significant levels in DNA isolated from both *red*$^+$ and *red*$^-$ crosses in which *recA* function was present.**

Genetic experiments with a variety of organisms have suggested the possibility that regions of significant sequence nonhomology can be included in the heteroduplex products of recombination. Transformation of pneumococcus with DNA from deletion mutants results in introduction of the deletions with high efficiency (1). Pneumococcal transformation has been shown to proceed via a mechanism that involves single-strand insertion with the formation of heteroduplex DNA (2). Phage that are heterozygous for deletion mutations are present among the progeny of crosses of the nonpermuted rhizobiophage 16-3 (3). In yeast, substantial deletion mutations participate in meiotic gene conversion (4, 5). It has been suggested that gene conversion products result from the action of mismatch repair systems on heterozygous hybrid DNA molecules (6). If this is the case, it would appear that deletion mutations are also included in regions of heteroduplex DNA formed during yeast meiosis.

In normal bacteriophage λ crosses, in which either the *red* recombination pathway of λ or the *recA–recF* recombination pathway of *Escherichia coli* is active, inclusion of insertion or deletion mutations as central markers in multifactor crosses only modestly affects the yield of recombinants, and a high incidence of clustered multiple exchanges (localized negative interference) is observed (7, 8). One explanation for the phenomenon of localized negative interference is that apparent multiple crossovers result from the repair of mismatches present in the products of a single molecular exchange event that included some of the markers in question in heteroduplex DNA (9). This explanation provides a basis for suggesting that both λ and *E. coli* recombination systems are capable of including nonhomologies in regions of heteroduplex DNA. Localized negative interference is also observed in bacteriophage T4 crosses in which the central marker in a three-factor cross is a deletion mutation (10, 11).

To obtain more direct evidence for the formation of nonhomology-containing heteroduplex structures during recombination, we have applied a technique that allows physical detection of such DNA molecules (12) to an examination of total intracellular DNA isolated from replication-restricted bacteriophage λ crosses. Molecules hybrid for a 1300-nucleotide deletion in the *lac* region of λ*plac5* were detected at significant levels in DNA isolated from both *red*$^+$ and *red*$^-$ crosses when *recA* function was present. Heteroduplex molecules that contained both the wild-type and mutant alleles of a 689-nucleotide deletion in the immunity region of λ were detected only in crosses in which both *red* and *recA* functions were present.

MATERIALS AND METHODS

Bacterial and Phage Strains. Hosts for replication-restricted crosses were either FA77 (*dnaB*ts *sup*$^+$) (13) or FZ14, a *recA*$^-$ derivative of FA77 (14). Phage strains λMJL356 (*cIΔ-HindIII Pam80 Sam7 plac5ZamNG200*), λMJL370 (*cI857 Pam80 Sam7 plac5ZΔW4680*), λMJL381 (*cI857 red3 Pam80 Sam7 plac5ZamNG200*), and λMJL382 (*cIΔHindIII red3 Pam80 Sam7 plac5ZΔW4680*) were used. A physical map of λ*plac5* is shown in Fig. 1.

Isolation of Intracellular DNA. Replication-restricted crosses were performed as described (7). At 90 min after infection, Tris·HCl, pH 7.2, was added to a final concentration of 200 mM, followed by KCN to 50 mM and NaN_3 to 50 mM, and cultures were chilled by shaking in ice water for 3 min. Cells were centrifuged, washed in lysis buffer (200 mM Tris·HCl/50 mM KCN/50 mM NaN_3, pH 8.5), pelleted, resuspended in lysis buffer containing lysozyme at 0.5 mg/ml, and incubated for 10 min at 0°C. The cell suspension was made up to 0.2% (wt/vol) Sarkosyl, 0.1 mg/ml RNase I, 1 mg/ml proteinase K, and 0.1 M NaCl and incubated for 20 min at 20°C. Lysates were then extracted with phenol and precipitated with ethanol. Samples were stored in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0) at −20°C.

Restriction Enzyme and S1 Nuclease Digestion. All restriction enzymes were obtained from New England Biolabs and were used according to the supplier's recommendations. Restriction enzyme digestions were terminated prior to electrophoresis by adding a 1/5 vol of sample buffer [2.5% (wt/vol) Ficol/50 mM Na_3EDTA, pH 8.5/0.05% bromphenol blue] and were terminated prior to S1 nuclease treatment by extraction with phenol and precipitation with ethanol. S1 nuclease (Miles) digestions were performed in 30 mM sodium acetate, pH 4.5/0.3 mM $ZnCl_2$/0.3 M NaCl at room temperature and were terminated by addition of sample buffer.

Preparation of Radioactively Labeled DNA. Radioactive probes were prepared by nick-translation (19) of restriction fragments isolated from agarose gels (20). Plasmid pKB280 (21) was used as a source of the cI *Hin*dIII fragment. Plasmid pMC1403 (22) was used as a source of the *lacZ Cla* I/*Sac* I fragment.

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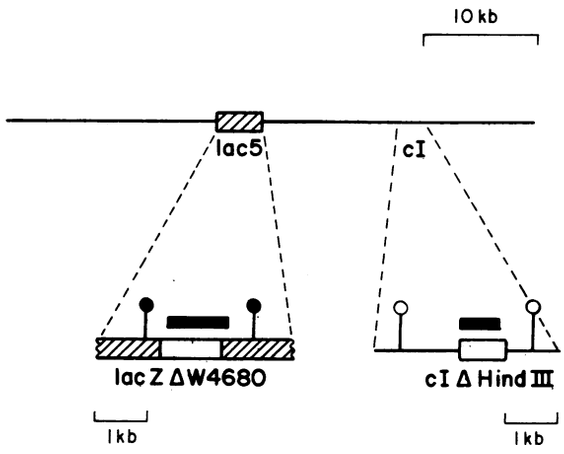

FIG. 1. Physical map of λ*plac5*. Locations and extent of the 0.69-kilobase (kb) deletion in *cI* (referred to in this work as *cIΔHindIII*) and of the 1.3-kb *lacZΔW4680* deletion are indicated by open rectangles. Sequences contained in the 0.59-kb *Hin*dIII/*Hin*dIII restriction fragment and the 1.1-kb *Sac* I/*Cla* I restriction fragment that were used to make radioactive probes are indicated by solid rectangles. Restriction enzyme recognition sites are indicated as follows: ●, *Bgl* I; ○, *Bgl* II. Positions of restriction sites and endpoints of *cIΔHindIII* were derived from DNA sequences (15–17). The endpoints of *lacZΔW4680* are from Malamy *et al.* (18) and R. Kurth (personal communication).

Detection and Quantitation of Nonhomology-Containing Heteroduplex DNA Molecules. Restriction-enzyme-digested DNA samples were subjected to electrophoresis, transferred to nitrocellulose, and hybridized with radioactive probe as previously described (12). Radioactive filters were exposed to film, and film density was determined as described (12). The relative amount of deletion-containing heteroduplex material present in each experimental sample was estimated by comparing the film density at the heteroduplex position in lanes containing experimental samples with the film density at the same position in lanes containing known amounts of artificial heteroduplex standards. To estimate the total amount of λ DNA loaded on each gel, a small aliquot of each DNA sample was displayed on agarose gels without restriction enzyme digestion, along with a series of dilutions of λ DNA of a known concentration. DNA in these gels was transferred to nitrocellulose by standard methods (15) and hybridized with radioactive λ DNA. Comparison of the total film density present in experimental lanes with the film density of lanes containing the λ DNA standards allowed estimation of the relative amount of λ DNA present in each experimental sample.

RESULTS

We have previously described a method that selectively and sensitively detects nonhomology-containing heteroduplex molecules (12). Upon electrophoresis in agarose, deletion-containing heteroduplex DNA fragments migrate to an intermediate position, between the positions of the mutant and wild-type homoduplex fragments. DNA is then transferred in the native state to nitrocellulose filters, using a modification of the method of Southern (23) in which the initial denaturing soak is omitted. DNA fragments immobilized on nitrocellulose filters are then hybridized with radioactive probe that contains sequences absent from the deletion mutation. Because of the partial single-stranded character of deletion-containing heteroduplex DNA molecules, they are selectively bound to nitrocellulose in the native state, and, once bound, selectively hybridize to radioactive probe (12).

This technique was used to examine total intracellular DNA isolated from λ crosses between phage containing wild-type and deletion mutant alleles. Since loss of heteroduplex structures would be expected as a consequence of DNA replication, crosses were performed under conditions in which DNA synthesis was restricted by mutations in both the host bacteria (*dnaB*ts at 42°C) and in the phage (*Pam80*). Crosses were performed in both *recA*$^+$ and *recA*$^-$ bacterial hosts, using both *red*$^+$ and *red*$^-$ parental phage. Parental phage were heteroallelic for the mutant and wild-type alleles of both *lacZΔW4680* and *cIΔHindIII*, thus allowing examination of the same DNA sample for the presence of molecules that contained either deletion mutation in heteroduplex.

In a typical experiment, the host bacteria were divided into four cultures just prior to infection. The first culture was infected with both parents at an equal multiplicity (about 10 phage of each parent per cell). The second and third cultures were each infected with a single parent at a multiplicity of 20 phage per cell. Equal volumes of these two cultures were mixed just prior to lysis. DNA isolated from this mixture of single-parent infected cells served as a control for formation of deletion-containing heteroduplex molecules during the lysis and DNA isolation procedure. The fourth culture was not infected with phage and was processed along with the other three cultures. DNA from this culture was used as carrier DNA for position markers and quantitation standards.

Detection of *lacZamNG200/lacZΔW4680* Heteroduplex Molecules. To detect hybrid molecules containing both the mutant and wild-type alleles of *lacZΔW4680*, DNA isolated from mixedly infected cultures and from mixtures of singly infected control cultures was digested with *Bgl* I, resolved on an agarose gel, and transferred to nitrocellulose without prior denaturation. The nitrocellulose filter was hybridized with radioactive probe prepared from the *lacZ Cla* I/*Sac* I fragment. An autoradiogram of this filter is shown in Fig. 2.

A clear band is visible at the position expected for the *lacZamNG200/lacZΔW4680* heteroduplex in DNA derived from both *recA*$^+$ *red*$^+$ and *recA*$^+$ *red*$^-$ mixed infections. This band is present at an intensity that corresponds to about one *lacZamNG200/lacZΔW4680* heteroduplex structure per 200 λ genomes. A much weaker signal (visible on the original autoradiogram) is present at the same position in the corresponding control samples, and it is also present in experimental and control DNA isolated from *recA*$^-$ *red*$^+$ infections. No signal is detectable at this position in DNA isolated from *recA*$^-$ *red*$^-$ infections. Lane background is prominent in DNA isolated from *recA*$^+$ infections and is less evident in DNA isolated from *recA*$^-$ infections. In addition, there is evidence of hybridization of probe, at variable levels, at the *lacZamNG200* homoduplex position.

Detection of *cI857/cIΔHindIII* Heteroduplex Molecules. To detect hybrid molecules containing both the mutant and wild-type alleles of *cIΔHindIII*, DNA isolated in the same experiments was digested with *Bgl* II, resolved on an agarose gel, and transferred to nitrocellulose without prior denaturation. The filter was then hybridized with a radioactive probe prepared from the *cI Hin*dIII fragment. Autoradiograms of these filters are shown in Fig. 3.

A strong band is present at the position expected for the *cI857/cIΔHindIII* heteroduplex only in DNA derived from the *recA*$^+$ *red*$^+$ mixed infection. This band is present at an intensity that corresponds to a frequency of about one *cI857/cIΔHindIII* heteroduplex structure per 1000 λ genomes. A much weaker band (visible on the original autoradiogram) is present at this position in the *recA*$^+$ *red*$^+$ control and in experimental and control DNA isolated from *recA*$^-$ *red*$^+$ infections. This faint band is not detectable in DNA isolated from cells that were infected with *red*$^-$ phage. Lane background is present in samples derived from *red*$^+$ infections and is not visible in DNA derived from *red*$^-$ infections. In addition, there is evidence of hybridization of probe, at variable levels, at the *cI857* homoduplex position.

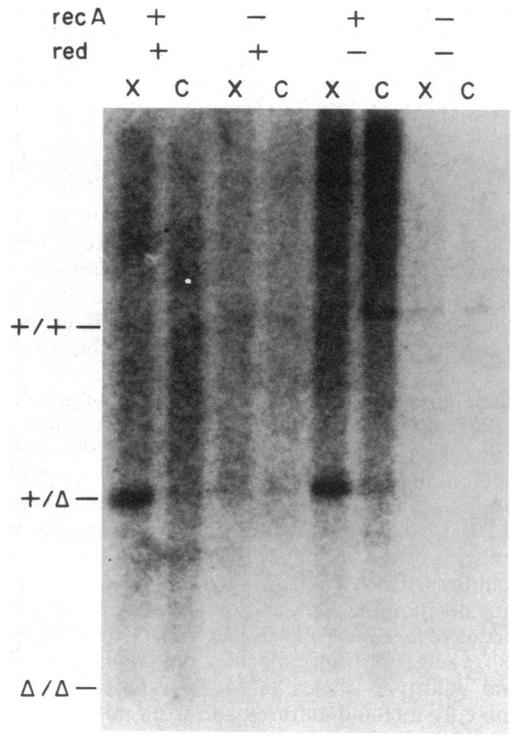

FIG. 2. Detection of *lacZamNG200/lacZΔW4680* heteroduplex molecules in DNA isolated from replication-restricted crosses. Crosses were performed in the *recA*$^+$ host FA77 or the *recA*$^-$ host FZ14 with *red*$^+$ or *red*$^-$ parental phage, as indicated. Lanes containing *Bgl* I digests of DNA isolated from mixedly-infected cultures and a mixture of single-parent infected cultures are indicated by the letters "X" or "C," respectively. Lines indicate the expected positions of the *lacZamNG200* homoduplex (+/+), the *lacZamNG-200/lacZΔW4680* heteroduplex (+/Δ), and the *lacZΔW4680* homoduplex (Δ/Δ) *Bgl* I fragments.

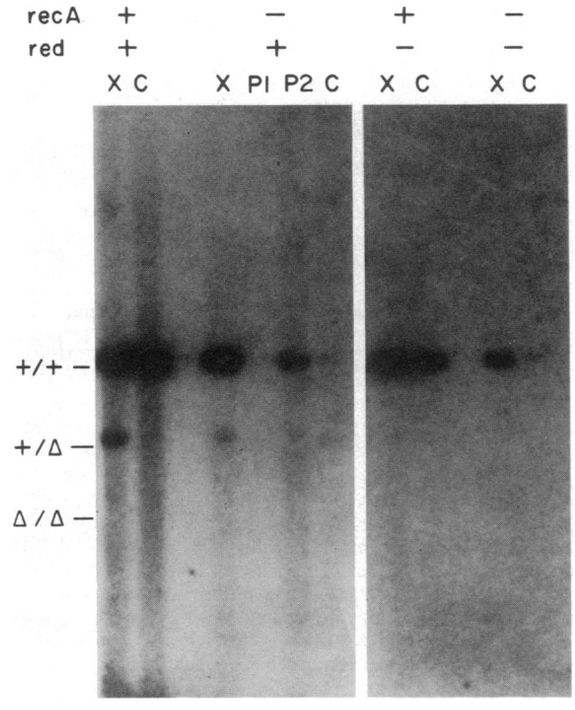

FIG. 3. Detection of *cI857/cIΔHindIII* heteroduplex molecules in DNA isolated from replication-restricted crosses. Crosses were performed in the *recA*$^+$ host FA77 or in the *recA*$^-$ host FZ14 with *red*$^+$ or *red*$^-$ parental phage, as indicated. Lanes containing *Bgl* II digests of DNA isolated from mixedly-infected cultures and a mixture of single-parent infected cultures are indicated by the letters "X" and "C," respectively. Lanes P1 and P2 contain *Bgl* II digests of DNA isolated from single-parent infected cultures infected with λ382 and λ381, respectively. Lines indicate the expected positions of the *cI857* homoduplex, the *cI857/cIΔHindIII* heteroduplex, and the *cIΔHindIII* homoduplex *Bgl* II fragments.

S1 Nuclease Treatment of DNA Isolated from *recA*$^+$ *red*$^+$ Crosses. S1 nuclease preferentially degrades single-stranded DNA (24). To confirm that the band present at the *cI-857/cIΔHindIII* heteroduplex position was due to a species that was partially single stranded, DNA samples isolated from *recA*$^+$ *red*$^+$ infections were first digested with *Bgl* II and then treated with various amounts of S1 nuclease. The DNA samples were then examined for the presence of *cI-857/cIΔHindIII* heteroduplex molecules as described above. Treatment of samples containing about 0.5 μg of DNA with 5 units of S1 nuclease for 30 min at room temperature resulted in complete loss of material hybridizing to the *c*I *Hin*dIII probe in gels transferred to nitrocellulose without prior denaturation (Fig. 4*A*). To assess degradation of duplex DNA, a small aliquot of these same samples was displayed on an agarose gel, transferred to nitrocellulose *with* prior denaturation, and hybridized with a radioactive probe prepared from total λ DNA. The treatment with S1 nuclease did not measurably affect the duplex λ DNA present in these samples (Fig. 4*B*).

DISCUSSION

In these experiments, total intracellular DNA was isolated from replication-restricted λ crosses with infecting parents that were heteroallelic for the wild-type and deletion mutant alleles. A novel species, identified as a heteroduplex DNA molecule in which one strand contained wild-type sequences and one strand contained deletion mutant sequences, was detected. These observations provide supporting evidence for the earlier genetic inference that regions of significant sequence nonhomology can be included in heteroduplex DNA during recombination.

Identification of Deletion-Containing Heteroduplex DNA Molecules. On the basis of electrophoretic mobility in agarose, selective detection upon transfer to nitrocellulose without prior denaturation, and, in one case, sensitivity to S1 nuclease, the novel species detected in these experiments have been identified as deletion-containing heteroduplex DNA molecules. Is it possible that this species does not have this structure, but is rather some other nucleic acid species that contains a single-stranded region? Other partially single-stranded structures, such as RNA-containing D-loop structures and partially single-stranded joint molecules, have been observed among intracellular DNA molecules isolated from λ infections (25, 26). However, these structures exhibited considerable heterogeneity, both in their size and in their location on the λ genome, and therefore should not migrate as a discrete species in agarose. The presence of such structures, or other structures that contain single-stranded regions, may have contributed to the broadly distributed lane background that is visible in some of the lanes on the filters shown in this work. The small amounts of material present at the heteroduplex position in some of the control samples isolated from mixtures of single parent-infected cultures are presumably the result of the hybridization of such partially single-stranded molecules during the isolation procedure.

Two observations suggest that the majority of the deletion-containing heteroduplex DNA molecules detected are not the products of an *in vitro* annealing process, but are rather the result of an intracellular interaction between pa-

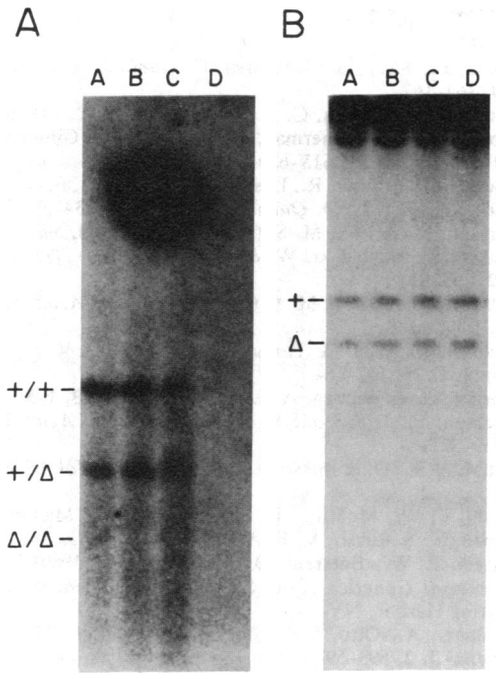

FIG. 4. S1 nuclease sensitivity of *cI857/cIΔHindIII* heteroduplex molecules. DNA isolated from a cross performed in the *recA*$^+$ host FA77 with *red*$^+$ parental phage was digested with *Bgl* II and subsequently digested with the indicated amounts of S1 nuclease. (*A*) Samples were displayed on a 1% agarose gel, transferred to nitrocellulose without prior denaturation, and hybridized with radioactive *c*I *Hin*dIII fragment. (*B*) Aliquots of the same samples were displayed on a 1% agarose gel and transferred to nitrocellulose *with* prior denaturation. The filter was then hybridized with radioactive λ DNA. Units of S1 nuclease per μg of total DNA were as follows: lanes a, 0; lanes b, 0.1; lanes c, 1; and lanes d, 10.

rental genomes. These heteroduplex molecules were detected in DNA isolated from certain mixed infections at much higher levels than in DNA isolated from the control mixtures of single-parent infected cultures. Therefore, it is unlikely that the strong signal seen at the heteroduplex position in lanes containing DNA isolated from mixedly infected cultures was due to a fortuitous association of parental DNA molecules that occurred after lysis. This conclusion is further strengthened by the observation that, while *lacZamNG-200/lacZΔW4680* heteroduplex molecules were detected at significant levels in samples containing DNA isolated from *red*$^-$ *recA*$^+$ mixed infections, *cI857/cIΔHindIII* heteroduplex molecules were not detected in samples from the same lysate.

In the autoradiograms described in this work, hybridization of radioactive probe was also observed at the position expected for homoduplex molecules. The extent of this hybridization is quite variable and can differ from sample to sample within the same filter. Since the signal present at the homoduplex position is eliminated by treatment with S1 nuclease prior to electrophoresis, it seems likely that this signal is due to the hybridization of the radioactive probe to small single-strand regions present in these otherwise duplex molecules.

Formation of Deletion-Containing Heteroduplex DNA Molecules. Recent studies of the *in vitro* activities of *recA* protein have indicated that the *recA* protein can promote the formation of joint molecules that include regions of substantial nonhomology in heteroduplex DNA (27). In the experiments reported here, deletion-containing heteroduplex molecules were detected at significant levels only in DNA isolated from crosses performed in the *recA*$^+$ bacteria, suggesting that the *recA* gene product may participate directly in the formation of such structures *in vivo* as well as *in vitro*. It is also possible, however, that *recA* was only indirectly responsible for the formation of the deletion-containing heteroduplex structures detected. For example, it is possible that products of genes induced during the SOS response [which is induced in *dnaB*ts cells at the nonpermissive temperature (28)] might have played a role. Such gene products would not be induced in *recA*$^-$ *dnaB*ts cells.

Stahl *et al.* have examined the distribution of crossovers along the genomes of the phage products of crosses in which replication was restricted and in which recombination was required for maturation of progeny (29). Formation of the vast majority of recombinant phage was found to be *recA* dependent, and the crossovers produced by the *red* system of λ were prominently clustered in the right-hand end of the λ genome. Crossovers mediated by *recA* in the absence of *red* function appeared to be distributed uniformly across the λ genome. In the experiments reported here, *cI857/cIΔHindIII* heteroduplex molecules were detected at significant levels only in DNA isolated from crosses in which both *red* and *recA* functions were present. In contrast, *lacZamNG-200/lacZΔW4680* heteroduplex molecules were more abundant, and they were detected in samples containing DNA isolated from either *red*$^+$ *recA*$^+$ or *red*$^-$ *recA*$^+$ crosses. Thus, while both *rec*- and *red*-encoded functions were necessary to form deletion-containing heteroduplex structures in *c*I (at the right-hand end of the λ genome), the *rec* recombination system was sufficient for efficient formation of such molecules in *lac*. These results provide further evidence to suggest that, in replication-restricted λ crosses, the *E. coli rec* and the λ *red* recombination systems play different roles in the formation of heteroduplex DNA in different parts of the λ*plac5* genome.

The formation of deletion-containing heteroduplex molecules in the *lac* region of λ*plac5* does not appear to be due to the elevated level of recombination conferred by the *chi* site present in the *lacZ* gene of λ*plac5* (30), since *lacZamNG-200/lacZΔW4680* heteroduplex molecules are produced in a *recA*-dependent *red*-independent manner in crosses using λ*plac5* derivatives that carry a mutation that destroys this *chi* site (data not shown).

Implications for Mechanisms of Recombination. Proposals describing the mechanism of recombination that include the formation of heteroduplex DNA as an intermediate step can, in general, be divided into two categories. In one class, typified by the Holliday model (31), heteroduplex DNA is formed by the branch migration of a symmetrical two-strand exchange structure. Both theoretical (32, 33) and experimental (34, 35) studies have suggested that branch migration could occur by thermal diffusion, requiring no substantial net input of energy. If this were the case, the presence of a region of substantial sequence nonhomology might constitute a barrier to branch migration, since inclusion of a nonhomology in heteroduplex DNA would involve formation of a considerable sequence of unpaired bases. Other models, such as those proposed by Meselson and Radding (36) and others (37, 38, 39), invoke enzymatic activities to produce regions of single-stranded DNA, which can then form heteroduplex DNA via asymmetric strand transfer. In such a model, part of the energy required for inclusion of regions of nonhomology in heteroduplex DNA would be provided by the initial formation of regions of single-stranded DNA.

Sodergren and Fox (40) provided evidence for the exclusion of nonhomologies from regions of heteroduplex DNA present in the genomes of phage emerging from replication-restricted λ crosses, and they suggested that this observation provided support for a role of branch migration of a Holliday structure in the formation of heteroduplex DNA. However, experiments that examined the effect of regions of nonhomo-

logy on recombination in normal replication-permitted λ crosses have provided indirect evidence that both λ and *E. coli* recombination systems are capable of including nonhomologies in regions of heteroduplex DNA (7, 8). The results of the experiments presented in this work indicate that heteroduplex DNA molecules that include deletion mutations are present in the total intracellular DNA isolated from replication-restricted crosses.

One way to reconcile these apparently contradictory observations is to suggest that the λ packaging system does not incorporate such heteroduplex DNA molecules in viable phage capsids. For example, it is possible that two different types of exchange events occur in replication-restricted crosses. The first type of event, an asymmetric strand transfer, would be capable of forming nonhomology-containing heteroduplex structures but would resolve to produce only monomer-length genomes, which, in the absence of subsequent DNA replication, would be inadequate substrates for λ encapsidation (41, 42). The second type of event, a symmetric strand exchange of the type suggested by Holliday (31), would be unable to include regions of nonhomology in heteroduplex DNA but would be capable of resolution to form a dimer-length genome suitable for encapsidation. Since Sodergren and Fox examined the recombinant phage that had matured from replication-restricted crosses, they would have detected only the products of exchange events of the second type.

Alternatively, it is possible that all of the exchange events that occur in replication-restricted λ crosses proceed via a mechanism that is capable of including nonhomologies in regions of heteroduplex DNA, but that such heteroduplex structures render the genome on which they reside unpackagable, or, if packaged, render the resulting phage inviable. Only exchange events that failed to include nonhomologies in regions of heteroduplex DNA would contribute to the yield of viable progeny.

Implicit in this discussion is the assumption that the deletion-containing heteroduplex DNA structures observed were present in DNA molecules that were products of recombination. This assumption is supported by the observation that these structures were detected only in crosses in which *recA* function was present. However, it remains to be demonstrated that deletion-containing heteroduplex structures are present in DNA molecules that would be expected to give rise to viable recombinants. Given this caveat, the experiments reported here provide evidence that at least some of the exchange events that occur in the course of λ recombination are capable of including regions of substantial nonhomology in heteroduplex DNA. This interpretation is most consistent with a mechanism of recombination in which heteroduplex DNA is formed by an active process, such as the asymmetric transfer of single-stranded DNA.

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