

A single base-pair change creates a Chi recombinational hotspot in bacteriophage λ

(A·T to T·A transversion/Rec-promoted recombination/deletion mapping/DNA sequencing)

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ABSTRACT χ^+ mutations, responsible for the Chi phenotype in phage λ , locally increase the rate of recombination promoted by the *Escherichia coli* recombination system (Rec). χ^+ mutations in the *cII* gene, one of a few sites in λ at which such mutations arise, were located genetically and physically with overlapping deletions. DNA sequence analysis of the deletion segment containing the χ^+C mutations showed that two independent χ^+C mutations arose by the same A·T to T·A transversion. Presumably, this change creates a nucleotide sequence recognized by a protein involved in a rate-limiting step of recombination.

Generalized recombination may involve exchange at any point along homologous chromosomes. However, several features of generalized recombination indicate that special sites influence the frequency of recombination in their neighborhood. (For reviews, see refs. 1 and 2.) Presumably, some part of the recombination machinery distinguishes these sites from other DNA sequences. In the chromosome of *Escherichia coli*, the existence of such sites, called Chi, has been demonstrated by Malone *et al.* (3). When Chi elements are introduced into bacteriophage λ , they locally stimulate generalized recombination catalyzed by the *E. coli* *recA recBC* system. In contrast, wild-type λ is apparently free of Chi elements, and its generalized recombination system (Red) is not influenced by them (4).

Mutations that locally stimulate recombination may arise in phage λ at a few widely separated loci (5, 6). Such mutations have been found at about 0.3, 0.6, 0.8, and 0.9 units on the λ genome (see Fig. 1). Each mutation stimulates recombination within approximately 0.2 units [10^4 base pairs (bp)] of its locus. Stimulation is maximal, about 10-fold, very near the locus and diminishes with distance (6). The properties of these mutations, called χ^+ , are indistinguishable from those of the *E. coli* Chi elements* (3).

Two possibilities for the similarity of the mutational λ χ^+ sites and the *E. coli* Chi elements suggest themselves. (i) At a limited number of places on the λ chromosome there are nucleotide sequences similar to the *E. coli* Chi elements that can become identical to them by mutation. (ii) The χ^+ mutations result from the insertion of *E. coli* Chi elements at a limited number of locations in λ . Therefore, we wished to determine whether the χ^+ mutations in λ are simple changes in the nucleotide sequence of λ or whether they are insertions of foreign DNA.

To distinguish between these possibilities, we have determined the DNA sequence change of χ^+ mutations arising in the *cII* gene of λ . We chose these mutations for analysis because deletions ending within this gene (7, 8) allowed us to locate the χ^+ mutations on both the genetic and the physical maps of λ .

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MATERIALS AND METHODS

Bacteriophage. λ J^{TS15} *int-4 red-3 gam-210 imm*⁴³⁴ χ^+C157 *susR5*, $\lambda\chi^+C151$ *susP902*, $\lambda\chi^+C152$ *susP902*, and $\lambda\chi^+C153$ *susP902* were obtained from Frank Stahl (University of Oregon). λ *imm*⁴³⁴ *cII60* was obtained from Ira Herskowitz (University of Oregon). λ *spi-380 nin-5* was obtained from Amos Oppenheim (Hebrew University, Jerusalem). Other phages are from our collection or were derived by crosses between them and the phages listed.

Bacteria. *E. coli* K-12 strain N3442 was obtained from Max Gottesman (National Institutes of Health). This strain does not suppress *sus* (amber) mutations and has a λ *imm*⁴³⁴ prophage with a deletion extending from gene *O* into the host *chlA* gene. Other *E. coli* strains are from our collection.

Phage Crosses. These were as described (7) except that five of each parental phage were used per cell. In crosses between *spi nin-5* phages and χ^+C *sus* phages, *Fec*⁺ *Sus*⁺ recombinants were selected on a *recA* *Su*⁻ strain (7) and scored as clear (χ^+C) or turbid (wild-type) plaque formers. In crosses between *imm*⁴³⁴ χ^+C157 and other χ^+C *susP902* phages, *imm*⁺ *Sus*⁺ recombinants were selected on strain N3442 and scored as in the preceding crosses.

Enzymes. Endonuclease *EcoRI* was a generous gift from Charles Woodbury (University of Oregon). *HindII*, *HindIII*, *Alu I*, *Mbo II*, *HinfI*, and *Pvu II* were purchased from New England BioLabs. *Taq I* was purchased from Bethesda Research Labs (Bethesda, MD). Polynucleotide kinase from phage T4 was a generous gift from Fred Ausubel and John Bedbrook (Harvard University).

Preparation of λ DNA. Phage were grown by infection of *E. coli* strain 594 with lysis-defective *susS7* derivatives of the desired phage. Five phage per cell were added to 0.5 liter of bacteria freshly grown at 37°C to 3.0×10^8 cells per ml in a broth of 10 g of Difco Tryptone and 5 g of NaCl per liter (pH 7.5). After 10 min for adsorption at 37°C, 0.5 liter of the above broth, supplemented with 5 g of Difco Yeast Extract per liter, 4 mM MgSO₄, 80 μ M FeCl₃, 160 μ M CaCl₂, and 20 μ g of thiamine per ml, was added. Growth was continued with vigorous shaking at 37°C for 3 hr. The cells were collected by centrifugation, resuspended in 10 ml of 10 mM Tris-HCl, pH 8.1/300 mM NaCl/5 mM Na₃citrate, and lysed by addition of 1 ml of CHCl₃. The viscosity was reduced by treatment for about 10 min at 37°C with about 0.5 μ g of pancreatic DNase per ml (Worthington) prepared in 1 mM MgSO₄ at about 50 μ g/ml. Cell debris was removed by centrifugation and MgSO₄ was added to a final concentration of 20 mM. Phage were further purified by centrifugation, first in a CsCl block gradient and then in a CsCl equilibrium gradient.

Abbreviation: bp, base pair(s).

* In accordance with the nomenclature proposed by Malone *et al.* (3), χ^+ designates the genotype of the active form of Chi sites in λ . Chi refers to the phenotype of the sites, whether in λ or in *E. coli*.

DNA was prepared from the purified phage by extraction with sodium dodecyl sulfate at 65°C (9, 10) and twice precipitated with ethanol. Final yields were 2–10 mg of DNA per liter of infected cells.

Preparation of "n" Fragment. (See ref. 11.) One milligram of DNA from λ 2 *cI857 susS7* (with or without a χ^+ C mutation) was digested for 15–30 hr at 37°C with 100 units each of *EcoRI* and *HindIII* endonucleases in 4.0 ml of 50 mM Tris-HCl, pH 7.6/100 mM NaCl/10 mM MgCl₂/100 μ g of bovine serum albumin per ml. After ethanol precipitation, the DNA was dissolved in 1.0 ml of 10 mM Tris-HCl, pH 8.1/1 mM EDTA/50 mM KCl and heated at 65°C for 4 min. EDTA (5 mM), sucrose (10%), xylene cyanol FF (60 μ g/ml), and bromphenol blue (60 μ g/ml) were added to the final concentrations indicated. The solution was layered on a 4% polyacrylamide gel, 0.6 \times 12 cm in cross section, in E buffer (12), and electrophoresed at 7 V/cm until bromphenol blue had migrated 10 cm. "n" fragment was located by staining with ethidium bromide and electroeluted (13) at 10 V/cm for 16 hr at 4°C into a dialysis bag in E buffer containing 0.1% sodium dodecyl sulfate to reduce sticking of the DNA to the dialysis tubing. The eluate was twice extracted with phenol/CHCl₃, 1:1, and the DNA was precipitated with ethanol. To remove non-nucleic acid contaminants eluted from the gel, we precipitated the DNA with cetyltrimethylammonium bromide as described by Sibatani (14). Traces of the detergent were removed by precipitating the DNA with ethanol three times from 0.20 M Tris-HCl, pH 7.5.

DNA Sequencing. Five to 10 μ g of "n" fragment was cleaved with an appropriate endonuclease and labeled with polynucleotide kinase by the exchange procedure (15). [γ -³²P]ATP was made by the method of Maxam and Gilbert (16). The labeled DNA was precipitated with ethanol, annealed at 65°C for 1 hr if it had been denatured before labeling, and cleaved with a second endonuclease. The fragments were separated on polyacrylamide gels, electroeluted, extracted with phenol/CHCl₃, and precipitated twice with ethanol. Removal of contaminants derived from the gel was not necessary at this stage. Chemical cleavage, separation on polyacrylamide gels, and autoradiography were done as described by Maxam and Gilbert (16).

RESULTS

Genetic Localization of Independent χ^+ Mutations at a Single Site within the *cII* Gene. One of the sites in the λ genome at which χ^+ mutations arise is in the *cII* gene (6). Because of the accessibility of this region to both genetic and biochemical analysis, we have begun the determination of the nucleotide sequence responsible for the Chi phenotype by studying four independent χ^+ mutations that arose spontaneously in *cII*. These mutants, called χ^+ C151, 152, 153, and 157, were isolated on the basis of their recombinational hotspot phenotype (6). Coincidentally, because the mutations inactivate the *cII* protein, they confer a clear-plaque phenotype. These two phenotypes are genetically inseparable (6). For convenience in scoring, the clear-plaque phenotype was used in the genetic experiments described here.

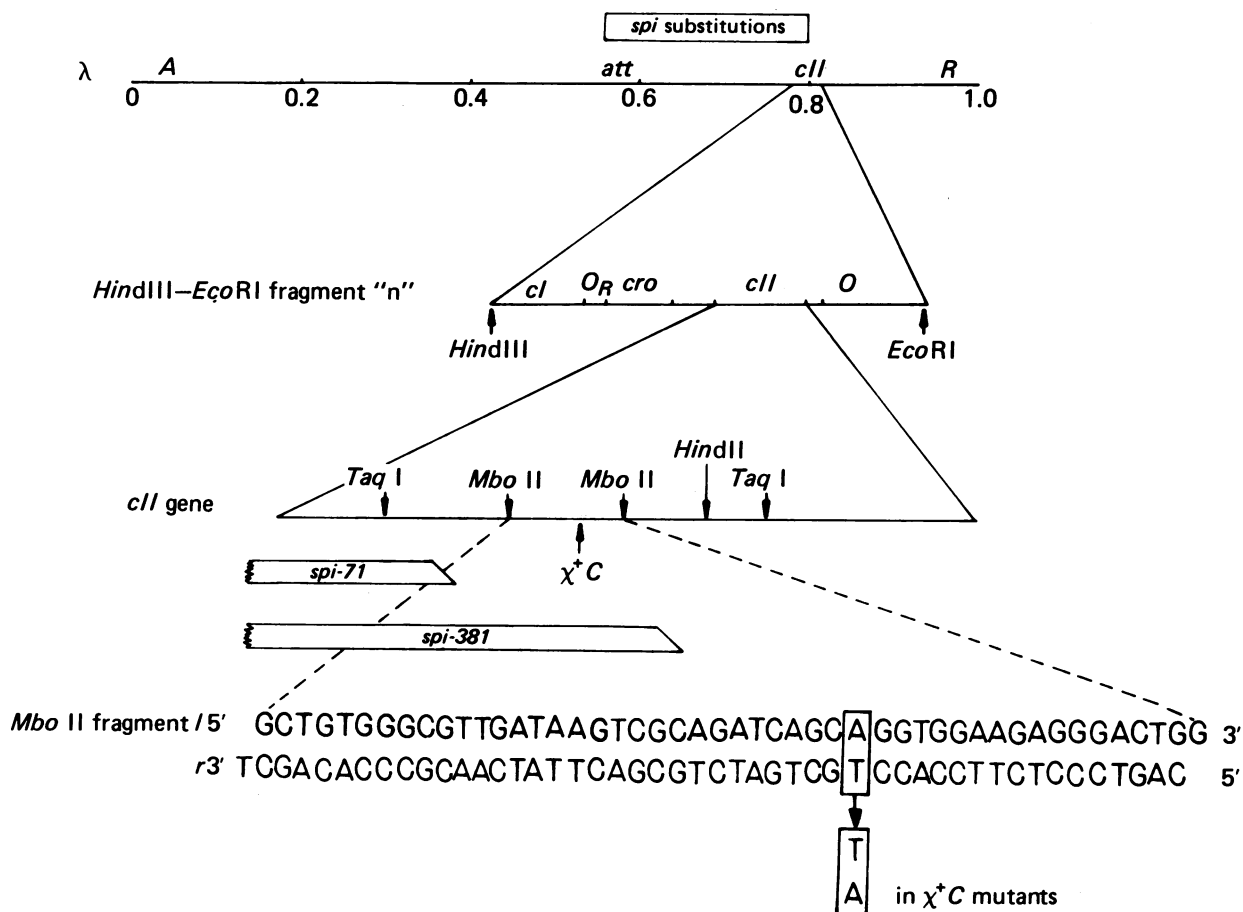


FIG. 1. Physical and genetic map of phage λ showing location and nucleotide alteration of χ^+ C. On each line, distances are drawn to scale. Top line shows the genes *A*, *cII*, and *R*, and the attachment site *att* at the indicated fractional distance from the left end of the λ chromosome (17). Second line shows a fragment of λ DNA, called "n" by Murray and Murray (11), which was prepared for sequence analysis of χ^+ C. Third line shows the location of endonuclease cleavage sites relevant to this paper (18). Bottom line shows the sequence of part of the *cII* gene (18) and the bp change in χ^+ C151 and χ^+ C157, as reported in this paper.

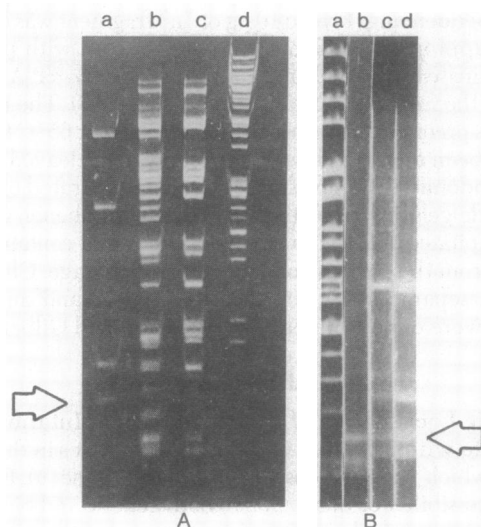


FIG. 2. Endonuclease cleavage sites in DNA from *spi* substitution phages. (A) *Taq* I cleavage of wild-type "n" fragment (lane a) and of the *Eco*RI fragment containing the junction between phage and host DNA in *spi-71* (lane b). Because the *spi-71* junction fragment was not well separated in this preparation from another fragment of similar length, the *Taq* I digestion pattern in lane b contains extra bands. The *Taq* I bands relevant to our analysis were identified as those not present in a digest (lane c) of a pure preparation of the other fragment, which was conveniently prepared from *spi-156* (7). A *Hind*II and *Hind*III digest of λ b2 *cI857* DNA in lane d provided size standards (20). Arrow, the 166-bp-long fragment resulting from cleavage between the two *Taq* I sites shown in Fig. 1. Products of *Taq* I digestion were subjected to electrophoresis in an 8% polyacrylamide gel. Migration was from top to bottom. (B) *Hind*II cleavage of wild-type "n" fragment (lane a) and of the *Eco*RI fragment containing the junction between phage and host DNA in *spi-71* (lane c) and in *spi-380* (lane d). A *Hind*II and *Hind*III digest of wild-type DNA in lane a provided size standards (20). Arrow, the 650-bp-long fragment extending from the *Hind*II cleavage site in the *cII* gene to the *Eco*RI site in the *O* gene, as shown in Fig. 1. Products of *Hind*II digestion were subjected to electrophoresis in 1.4% agarose gels. Migration was from top to bottom.

Deletion mapping was used to localize the χ^+C mutations within the *cII* gene. For this purpose, *spi* derivatives of λ , containing substitutions of bacterial DNA for phage DNA, were used as the deletion parents in crosses with χ^+ mutants (7, 8). As shown in Fig. 1, the particular *spi* phages used are deleted for λ DNA from *att* rightward to various points within the *cII* gene. Crosses between *spi-71* and each of the four χ^+C mutants showed significant recovery of wild-type (*CII*⁺) phage. Among the progeny selected to have a crossover in the *cII* to *P* interval, 2% formed turbid (*CII*⁺) plaques. In contrast, crosses between *spi-380* and the χ^+C phages produced no (<0.01%) turbid recombinants. Thus, these four χ^+C mutations map between the right end points of *spi-71* and *spi-380*, an interval of less than 160 bp (see below).

The proximity of the χ^+C mutations to each other was determined by crossing them with each other and with *cII60*, a mutation that is in the same deletion interval (8). Crosses involving χ^+C157 and χ^+C151 or χ^+C157 and χ^+C153 gave one turbid plaque per 40,000 progeny selected to have a crossover in the *imm*⁴³⁴ to *P* interval, an interval of less than 2500 bp (17). Similarly, a cross between χ^+C157 and χ^+C152 yielded two turbid plaques per 42,000 selected recombinants. (These rare turbid plaques may have arisen by recombination with the *cII*⁺ prophage in the bacteria on which the recombinants were selected.) In contrast, the frequency of turbid recombinants was approximately 100-fold higher (0.4–0.8%) when any of the χ^+C

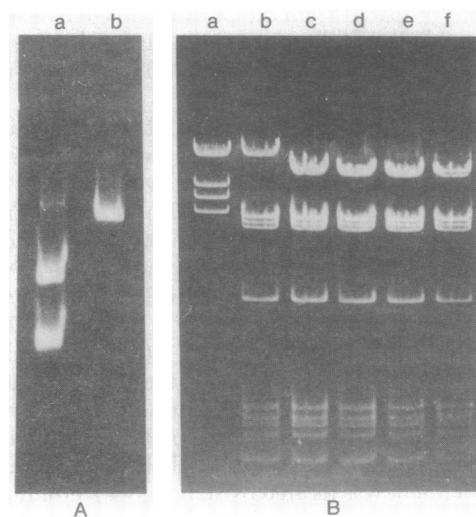


FIG. 3. Presence of an extra *Pvu* II endonuclease cleavage site in χ^+C DNA. (A) *Pvu* II cleavage of "n" fragment from χ^+C157 (lane a) or from wild-type DNA (lane b). Products of *Pvu* II digestion were subjected to electrophoresis in a 4% polyacrylamide gel. Migration was from top to bottom. (B) *Pvu* II cleavage of λ b2 *cI857 susS7* derivatives of wild-type (lane b), χ^+C157 (lane c), χ^+C153 (lane d), χ^+C152 (lane e), and χ^+C151 (lane f). Products of *Pvu* II digestion were subjected to electrophoresis in a 1.2% agarose gel. Migration was from top to bottom. An *Eco*RI digest of λ b2 *cI857 susS7* DNA in lane a provided size standards (22). The smallest fragment produced by *Eco*RI is not seen here because it remained annealed to the largest fragment.

mutants was crossed with a phage carrying the *cII60* mutation, which is separated from χ^+C by less than 160 bp. We conclude, therefore, that the four χ^+C mutations we have examined are very close to each other and, in fact, may be at the same site.

Physical Localization of χ^+C within the *cII* Gene. Since the entire sequence of the wild-type *cII* gene has been determined (18, 19), localization of the deletion end points bracketing χ^+C should permit identification of a DNA fragment containing this locus. Direct nucleotide sequence information about the *spi-71* and *spi-380* end points is not available. Therefore, we have located the right ends of these deletions relative to known endonuclease cleavage sites within the *cII* gene.

For physical analysis of χ^+C , we prepared a DNA fragment containing the *cII* gene, called "n" by Murray and Murray (11). This fragment contains about 1580 bp between a *Hind*III site in the *cI* gene and an *Eco*RI site in the *O* gene, as shown in Fig. 1. Fragments of *spi-71* and *spi-380* DNA containing the junction between host and phage DNA and extending rightward into the *O* gene were similarly isolated after *Eco*RI digestion. These fragments were cleaved with either *Taq* I or *Hind*II, and the resulting fragments were compared with those produced by cleavage of wild-type "n" fragment.

As shown in Fig. 2A, *spi-71* removes the leftmost *Taq* I site in the *cII* gene. Cleavage at this site is required to produce the 166-bp-long fragment that is present in *Taq* I digests of wild-type "n" fragment DNA. This 166-bp-long fragment is absent from comparable digests of the *spi-71* junction fragment. In contrast, the next rightward *Taq* I site appears to be intact in *spi-71* DNA, since a fragment 196 bp long located just to the right of the 166 bp-long fragment is present in *Taq* I digests of both wild-type and *spi-71* DNA. Thus, the right end point of *spi-71* must lie between these two *Taq* I sites (see Fig. 1).

Similarly, the right end point of *spi-380* was located to the left of the *Hind*II cleavage site in the *cII* gene. As shown in Fig. 2B, a fragment about 640 bp long is produced when either

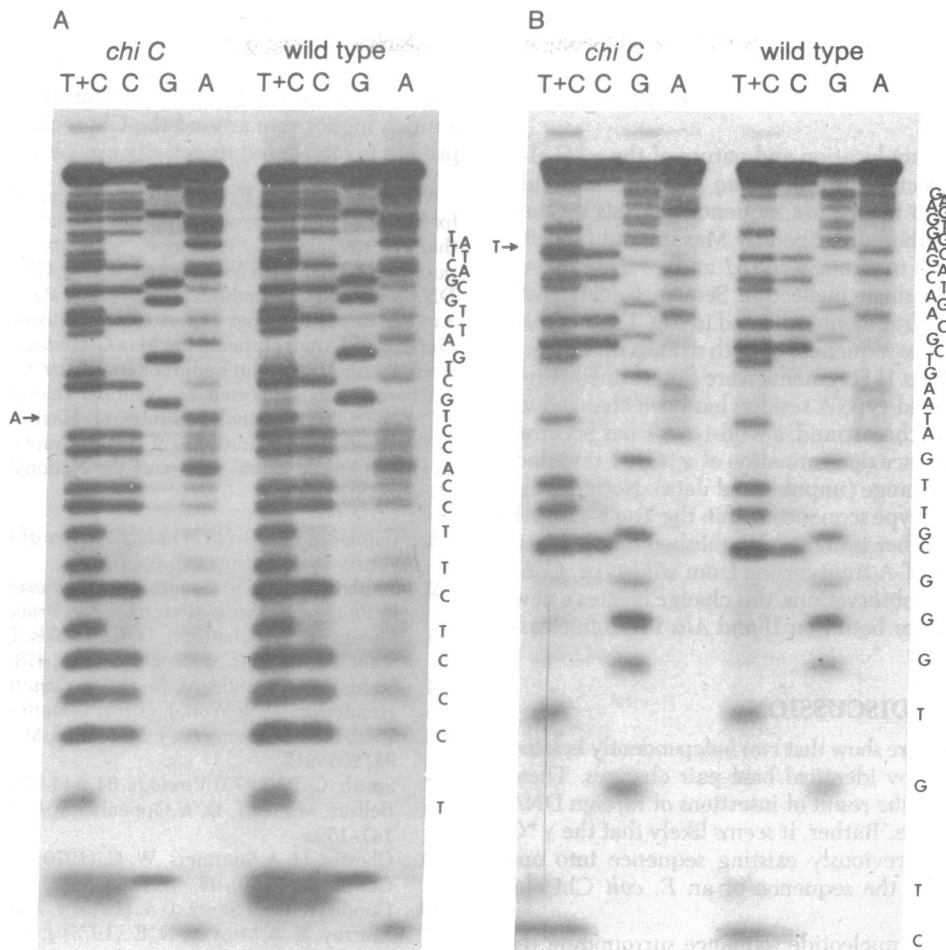


FIG. 4. DNA sequence analysis of the *Mbo* II fragment containing the χ^+C mutations. Fragment "n" from derivatives of λ with or without the χ^+C157 mutation was cleaved with *Mbo* II (see Fig. 1) and labeled with ^{32}P at the 5' ends. A fragment 48 bp long was isolated by electrophoresis in a 12% polyacrylamide gel, and the strands were separated (16). The isolated strands were subjected to chemical cleavage, and the products were separated by electrophoresis on a 20% polyacrylamide gel and autoradiographed (16). (A) *r* strand (see Fig. 1); (B) *l* strand. Arrows, positions of the base changes in χ^+C157 . A similar experiment with the *Mbo* II fragment from χ^+C151 showed the same base changes (unpublished data).

wild-type "n" fragment or the *spi-380* junction fragment is digested with *Hind*II. The 640-bp fragment results from cleavage at the *Hind*II site in *cII* and at the *Eco*RI site in the *O* gene (18, 21). Because χ^+C has been genetically mapped between the *spi-71* and *spi-380* deletion end points and because these end points lie between the leftmost *Taq* I site in *cII* and the *Hind*II site in *cII*, we conclude that χ^+C must be located within the 152-bp *Taq* I-*Hind*II interval.

Nucleotide Sequence Analysis of χ^+C . Sequence analysis of χ^+C was facilitated by our finding that the four χ^+C mutations genetically analyzed create recognition sites for endonuclease *Pvu* II. Fig. 3A shows that wild-type "n" fragment is not digested by *Pvu* II, whereas χ^+C157 "n" fragment is cleaved into two fragments the sum of whose lengths equals that of "n." The lengths of these fragments are consistent with location of the new *Pvu* II cleavage site between the *Taq* I and *Hind*II sites, the interval already shown above to contain χ^+C . Thus, it is likely that χ^+C157 creates the *Pvu* II recognition sequence d(C-A-G-C-T-G) (T. Gingeras and R. Roberts, personal communication), a sequence that is not present in wild-type "n" fragment.

Each of the three other χ^+C mutations also creates a new *Pvu* II cleavage site. This additional cleavage can be detected in digests of total λ DNA because of the limited number of frag-

ments produced. Fig. 3B shows that a large fragment in wild-type DNA is replaced by two smaller ones in *Pvu* II digests of χ^+C DNA. The sizes of these fragments are consistent with the location of a new *Pvu* II cleavage site within the *cII* gene.

We have also observed that both χ^+C151 and *157* create a new *Alu* I cleavage site in "n" fragment. Digestion of wild-type "n" fragment with *Alu* I produces a fragment 730 bp long. This fragment extends from the *Eco*RI end of "n" fragment leftward to a point between the *Mbo* II sites shown in Fig. 1 (18). On the other hand, *Alu* I digestion of "n" fragment from two χ^+C mutants, χ^+C151 and χ^+C157 , yields two fragments in place of the 730-bp-long fragment. One of these is about 30 bp long and the other is about 700 bp long (unpublished data). These results are consistent with the creation, by the χ^+C mutation, of a new *Alu* I cleavage site within the *cII* gene. Since the *Alu* I recognition sequence, d(A-G-C-T) (23), is contained within the *Pvu* II recognition sequence, d(C-A-G-C-T-G), the simultaneous creation of both sites by a single mutational event is possible.

χ^+C does not change either the number or the sizes of the fragments produced from "n" fragment by the other endonucleases we have tested, *Hind*II, *Hinf*I, *Mbo* II, and *Taq* I. Thus, χ^+C does not appear to result from the insertion or deletion of a significant amount of DNA. Rather, our results are consistent

with the idea that χ^+C is the consequence of a single base change. However, within the *Taq* I-*Hind*II interval in which χ^+C is located there are three sequences at which a single base change could create both a *Pvu* II and an *Alu* I recognition sequence (18).

To determine the exact location and nature of the χ^+C alteration, we have subjected the entire *Taq* I-*Hind*II interval in two χ^+C mutants to nucleotide sequence analysis by the chemical cleavage method described by Maxam and Gilbert (16). We find that both mutants, χ^+C151 and χ^+C157 , have the same alteration of a single nucleotide. Sequence analysis of the 48-bp-long *Mbo* II fragment indicated in Fig. 1 shows this change for χ^+C157 . The sequences of both strands of the mutant and wild-type *Mbo* II fragments were determined (Fig. 4). In the *l* strand, a wild-type A residue has been changed to T in χ^+C157 , while in the *r* strand, a wild-type T has become A in the mutant. Sequence determination of χ^+C151 revealed the same nucleotide change (unpublished data). No other alteration from the wild-type sequence within the *Taq* I-*Hind*II interval was seen in either mutant (unpublished data). Thus, χ^+C is a single A-T to T-A transversion from wild-type. Consistent with our earlier observations, this change creates a new recognition sequence for both *Pvu* II and *Alu* I endonucleases.

DISCUSSION

The results presented here show that two independently isolated χ^+C mutations arose by identical base-pair changes. These mutations are thus not the result of insertions of foreign DNA containing Chi elements. Rather, it seems likely that the χ^+C mutations change a previously existing sequence into one similar or identical to the sequence of an *E. coli* Chi element.

What section of the nucleotide sequence surrounding the χ^+C mutation is responsible for the Chi phenotype? The sequence in Fig. 1 shows that χ^+C creates a 6-bp palindrome, the *Pvu* II recognition sequence d(C-A-G-C-T-G). This sequence cannot be the only requirement for Chi sites, however, since wild-type λ contains many *Pvu* II sites (see Fig. 3) yet is free of active Chi sites. Since no other obvious symmetrical sequence is created by the χ^+C mutation, Chi appears not to be a symmetrical sequence. Two properties of Chi support this conclusion: Chi stimulates recombination to a greater extent on one side of itself than on the other side (24), and Chi elements become inactive when they are inverted in the λ chromosome (unpublished data). In the sequence surrounding the χ^+C mutation, no striking features other than this 6-bp palindrome are apparent. We expect to deduce the structure of Chi by comparing the sequence around χ^+C with the sequence around other Chi elements and by isolating mutations near χ^+C that inactivate this site.

How does the nucleotide sequence created by the χ^+C mutations stimulate recombination in its neighborhood? We imagine that the Chi sequence is recognized by a protein operating at a rate-limiting step in the *recBC* pathway of *E. coli*.

This pathway acts at a low, roughly uniform rate on wild-type λ , which is devoid of Chi sites, and acts at an increased rate around introduced Chi sites (4). The protein that recognizes Chi may act at a low rate on DNA without this sequence but act at a much higher rate around the Chi sequence. The particular protein involved and its activity are yet to be identified.

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