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On the mechanism of genetic recombination: Electron microscopic observation of recombination intermediates

(electron microscopy of DNA/chi form/Holliday model/plasmid DNA)

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ABSTRACT **This paper deals with the nature of recombination intermediates. Using the electron microscope to study the DNA of the plasmid colicin E1, we have observed more than 800 molecules that appear to represent intermediates in the process of recombination. Specifically, after isolating colicin DNA and linearizing it with the restriction enzyme *Eco*RI, we find crossed molecules with twice the normal colicin DNA content. These forms consist of two genome-length elements held together at a region of DNA homology. The molecules can be recovered from wild type and Rec B-C host cells but are not present among the colicin DNA forms isolated from recombination-deficient Rec A cells.**

We have termed the experimentally observed molecules "chi forms" and believe that they represent the recombination intermediate of the Holliday model.

The study of DNA metabolism falls into three general categories: replication, repair, and recombination. In contrast to our knowledge about replication and repair, relatively little is known about the nature of the intermediates or the enzymes involved in the process of recombination.

The purpose of this paper is to present evidence about the nature of recombination intermediates, using colicin DNA as an experimental system.

EXPERIMENTAL SYSTEM

The colicin DNA molecule is a small circular duplex ring that replicates autonomously in *Escherichia coli*, thereby maintaining itself as a plasmid (1). Its function is to code for a toxic protein that is secreted and, when absorbed by sensitive cells, destroys them.

Normally, the colicin DNA molecules (and derivative plasmids) are maintained at about 20 copies per cell. This number can be increased to about 1000 per cell by exposing the culture to chloramphenicol (2); this protein synthesis inhibitor prevents the replication of the bacterial chromosome but does not suppress colicin DNA replication. We have taken advantage of this phenomenon to fill the cell with homologous DNA molecules that might engage in recombination.

To search for recombination intermediates, we harvested the cells and prepared a lysate (see the legend to Fig. 1). The closed circular plasmid DNA molecules were then recovered from the cell lysate by equilibrium centrifugation in CsCl-ethidium bromide density gradients.

When the plasmid DNA molecules were treated lightly with DNase to remove superhelical twists and examined in the electron microscope, they were found to consist of a set of monomer-size DNA rings and related multimers. In a typical analysis of 1000 molecules, there were 655 simple monomer circles, 248 dimer-size circles, 81 larger multimer rings, and 16 linear molecules. A small percentage of these DNA rings appeared to be touching each other and thus are candidates for intermediates in recombination. Such "figure 8" structures have been observed in cells containing the DNA circles of viruses S13 (6, 7) and ϕX (8, 9). Fig. 1 shows an example of a colicin figure 8 molecule. To pursue these structures and to determine that they are not merely monomer circles interlocked like two links in a chain, but are genomes covalently connected at a point of DNA homology, we have opened the structures with the restriction enzyme *Eco*RI. This enzyme cleaves monomeric colicin DNA rings once, at a unique site, generating unit-size rods. The enzyme is expected to cleave recombinant figure 8 structures twice, generating molecules with bilateral symmetry shaped like the Greek letter chi. It is these chi-shaped molecules that are the subject of this paper.

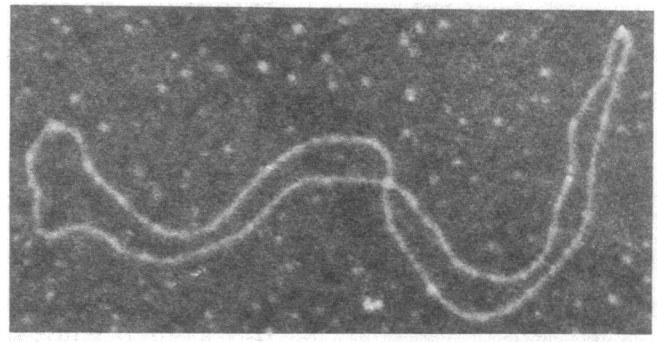

FIG. 1. A double-size colicin DNA molecule with the shape of a figure 8. Such molecules may represent two monomer circles in the crossover stage of recombination. Alternatively, they might simply consist of two monomer rings interlocked like links in a chain. Lastly, the structure could be a double-length DNA circle that accidentally crosses itself in the middle. This paper concerns such figure 8 colicin DNA molecules and their possible involvement in recombination.

The specific colicin plasmid we study is PMB9 (obtained from Dr. Herbert Boyer), a 3.6 million dalton double-stranded DNA circle related to colicin E1. The DNA was prepared by a modification of the method of Clewell (2, 34). After purification (34), the DNA was examined in the electron microscope by the Davis, Simon, and Davidson modification (3) of the Kleinschmidt and Zahn protein monolayer technique (4) as previously described (5).

OBSERVATION OF CHI FORMS

Fig. 2 is an electron micrograph of one of more than 800 chi-shaped molecules found among colicin DNA after linearization with *Eco*RI. These dimeric forms have been observed amidst a simple background of about 80,000 unit-length monomer rods. In 25 different preparations, the frequency of chi forms ranged from 0.5 to 3%.

Could these crossed molecules have arisen from an accidental overlap between two monomer rods? This is unlikely for two reasons. First, the DNA was spread for the electron microscope at a low concentration so that accidental overlaps would be virtually nonexistent. Second, the crossed molecules always have a special symmetry. The point of contact between the unit-size

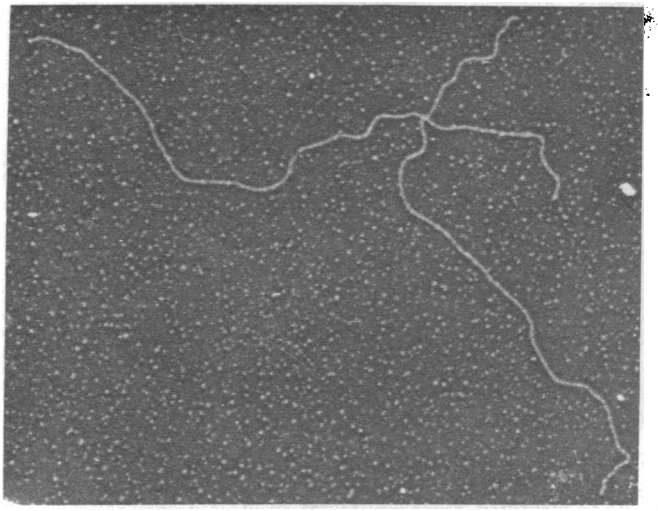

FIG. 2. An example of a chi form: two unit-size plasmid DNA molecules are held together at a region of homology. These molecules are observed after linearization of colicin DNA with the restriction enzyme *Eco*RI (see ref. 34 for specific conditions of the enzyme digestion).

After cutting by *Eco*RI, the chi forms are stable for days if maintained at 0°. A few minutes at 37°, however, is sufficient to allow almost all of them to terminalize (roll apart) into two separated monomer rods.

colicin genomes occurs so as to divide the structure into two pairs of equal-length arms (Figs. 2 and 3). Since the *Eco*RI cutting site defines a unique point on the colicin circle, the equality of the arms shows that the point of contact almost certainly occurs at a region of DNA homology. Thus, in these dimeric colicin DNA forms the two plasmid genomes appear to be held together at a region of homologous DNA.

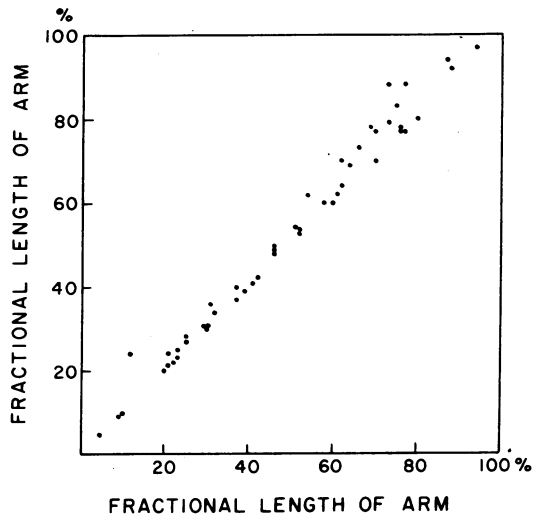

FIG. 3. An analysis of the lengths of the arms in 25 randomly chosen chi forms. The lengths of the four arms are measured, summed, and divided by two to obtain the unit genome length. The percentage lengths of the two shorter arms are then plotted, one as the abscissa and one as the ordinate. Similarly, the two longer arms are used to produce a single point for the curve. The fact that the points generate essentially a straight line of slope 1 establishes that the chi forms contain pairs of equal length arms. Furthermore, the finding that pairs of arms have different lengths indicates that the point of contact between the two genomes can occur at many locations, and perhaps randomly. All of the other chi forms have been similarly photographed, traced, measured, and analyzed, giving the same result.

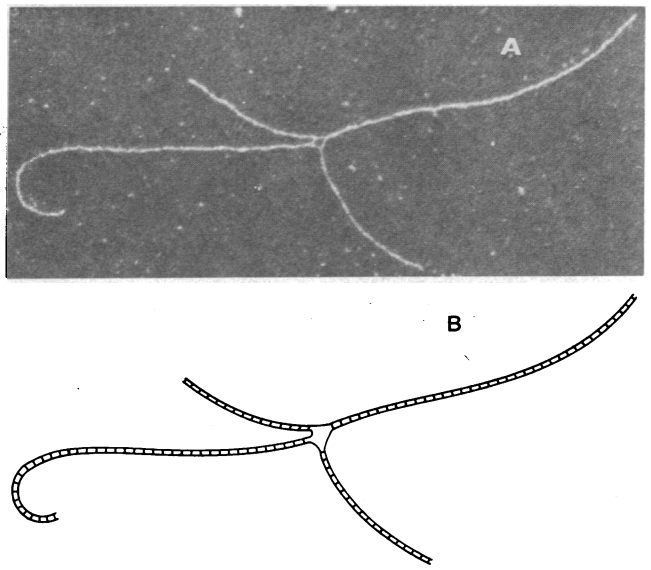

FIG. 4. (A) An example of one of 80 chi forms in which the covalent strand connections in the region of the crossover can be seen. Apparently the molecule has been strained during spreading for the electron microscope, and the single strands in the crossover have been pulled apart. The short arms of this molecule differ in length by 10%; the long arms differ in length by 8%. (B) A line diagram of the molecule, indicating the way in which the four arms are covalently interconnected in the region of the crossover.

The data in Fig. 3 provide evidence that the point of contact between the plasmid genomes can occur at numerous, and perhaps all, locations along the colicin DNA molecule—that is, at various distances from the *Eco*RI cutting site.

Not only can one see that the plasmid genomes are touching at a point of DNA homology, but often it is possible to observe the nature of the DNA strand substructure in the region of the crossover. Fig. 4A shows an example of one of 80 molecules that have become locally denatured in the region of the crossover during spreading for the electron microscope. The single strands connecting the four arms of the recombining molecules are visible, allowing the construction of the diagram in Fig. 4B.

Fig. 5 shows another example of a molecule that has become partially denatured during spreading for the electron microscope, in this case because the DNA was prepared in a high concentration of formamide. It is again possible to see the covalent connections in the region of the crossover. Furthermore, the homologous arms can be identified by their characteristic denaturation patterns, confirming the line diagram in Fig. 4B. Thus, Figs. 4 and 5 offer evidence that the chi-shaped molecules we see consist of two plasmid genomes covalently interacting at a region of DNA homology.

EFFECT OF THE *rec A* LOCUS

In 25 different preparations of colicin DNA from wild-type cells, the frequency of chi forms ranged from 0.5 to 3%. A similar percentage of chi forms was observed in material from a Rec B-C strain (MM 486 from Matthew Meselson). However, when we examined material obtained from three independent recombination-deficient (Rec A^-) strains (MM 152 from Matthew Meselson, HB 101 from Herbert Boyer, and RM 201J from Frank Stahl), few structures larger than monomers, and no chi forms, were found among 8000 molecules. The fact that the chi forms were absent in these recombination-deficient cells forms the basis for our belief that they represent intermediates in recombination.

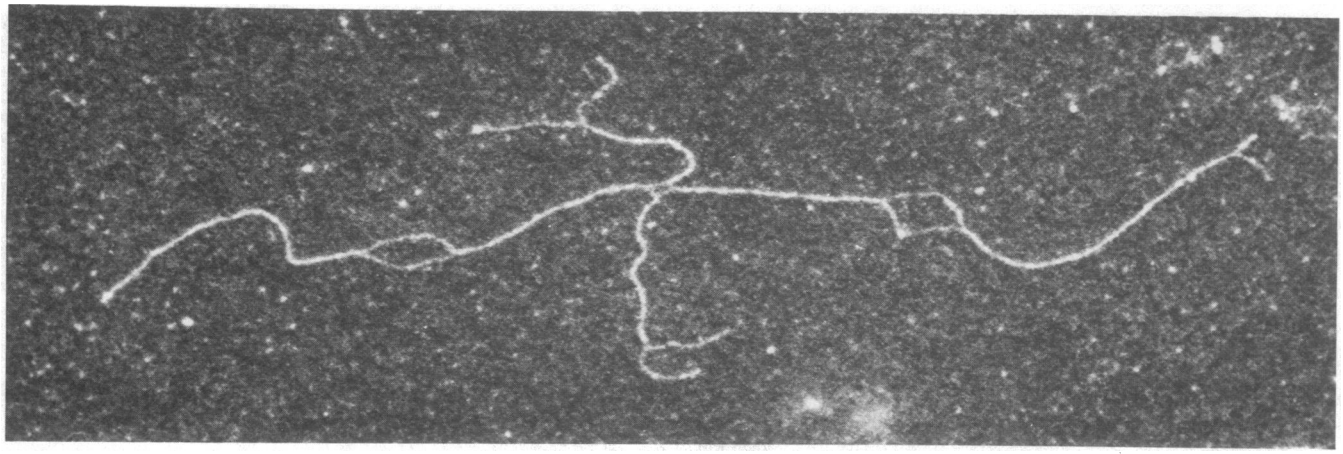

FIG. 5. A chi form that has been prepared for the electron microscope in the presence of a high concentration of formamide (5). Under these conditions the DNA double helix is stressed, and those regions particularly rich in A-T base pairs undergo a localized denaturation. This sequence-specific denaturation allows the homologous arms in the molecule to be identified. Furthermore, the covalent strand connections in the region of the crossover can be seen. In this, and the other 80 open molecules, the homologous arms are in a *trans* configuration. This geometry is expected, as will be discussed in connection with Fig. 6 (compare Figs. 4 and 5 with Fig. 6i).

INTERPRETATION

We would now like to consider our results in terms of current models for recombination (for a review, see refs. 10 and 11), with special emphasis on a set of models initiated by Holliday (12–14). This set of models has been advanced on the basis of genetic data. Our results offer support for the physical existence of the recombination intermediate proposed by Holliday.

The Holliday class of models was proposed in order to explain the behavior of eukaryotic chromosomes at meiosis. Recombination during meiosis is the most highly ordered form of gene reorganization known, and is associated with four general principles. These are: (*i*) the total conservation of genetic information (15); (*ii*) a potential for the reciprocal exchange of genes between homologous chromosomes (15); (*iii*) the frequent occurrence of a region of heterozygous DNA in the area of the recombination event (16–20); and (*iv*) a potential for maturation of the recombinant chromosomes so that on either side of the heterozygous region, the genes can either be left in their original linkage or reciprocally exchanged (16–17).

The Holliday model involves a single recombination intermediate that can be formed, processed, and matured to satisfy all of the above conditions. The model has evolved over 10 years, with contributions from several sources (12–14, 21–25).

Recombination, according to the prototype Holliday model, proceeds as shown in fig. 6. Two homologous double helices are aligned, and in each the positive strands (or alternatively the negative strands) are broken open in a given region. The free ends thus created breathe away from the complementary strands to which they had been hydrogen-bonded and become associated instead with the complementary strands in the homologous double helix (Fig. 6a–d). The result of this reciprocal strand invasion is to establish a tentative physical connection between the two DNA molecules. This linkage can be made stable through a process of DNA repair, which in this case can be as simple as the formation of two phosphodiester bonds by the enzyme ligase (Fig. 6e).

The recombination intermediate thus formed is the Holliday structure. Sigal and Alberts have shown, by building a space-filling model of DNA, that in this intermediate steric hindrance is minimal and virtually all of the bases can be paired (26).

Once formed, the intermediate need not be static. A continuing strand transfer by the two polynucleotide chains involved in the crossover can occur, moving the point of linkage between the two DNA molecules to the right or the left (Fig. 6f). It is this dynamic property of the Holliday structure that can account for the development of regions of heterozygous DNA during recombination (12–14). Meselson has calculated that the rate for this process, which he discusses in terms of the rotary diffusion of DNA double helices, is high enough to allow the rapid formation of hybrid regions under physiological conditions (27).

The maturation of the Holliday intermediate can occur in either of two reciprocal ways. This key property of the Holliday structure is most easily appreciated if one draws the intermediate in another planar form (14, 23, 26) (Fig. 6i). Then, cutting on an east-west (or a north-south) axis allows the release of recombinant DNA molecules in which, on either side of the potentially heterozygous region, the parental alleles are either conserved in their original linkage or reciprocally exchanged (Fig. 6l). Genetic maps would then arise from the 50% of the cases in which the flanking markers are exchanged.

When necessary, the recombination event is completed either by the repair of heterozygous regions, using enzymes that recognize base pair mismatches, or by the conversion of these regions to a homozygous state through subsequent rounds of DNA replication.

We believe that our results offer direct physical evidence in support of the recombination intermediate postulated by Holliday on genetic grounds. The chi forms (Figs. 2 and 6) correspond exactly to the two planar representations of the Holliday structure (Fig. 6g and i).

The structures we have observed do not have the geometry expected on the basis of alternative recombination models (10, 28–30). For instance, an interesting model proposed by Broker and Lehman, and supported by data in the phage T4 experimental system, postulates the interaction of two input genomes leading to the construction of a single recombinant chromosome, with the rest of the input DNA being ultimately lost as fragments. This model, the H-shaped intermediate it postulates, and the evidence supporting it, are discussed in refs. 28 and 29.

DISCUSSION

The chi forms are observed after *Eco*RI digestion of colicin DNA. Before this linearization, the structures have the form

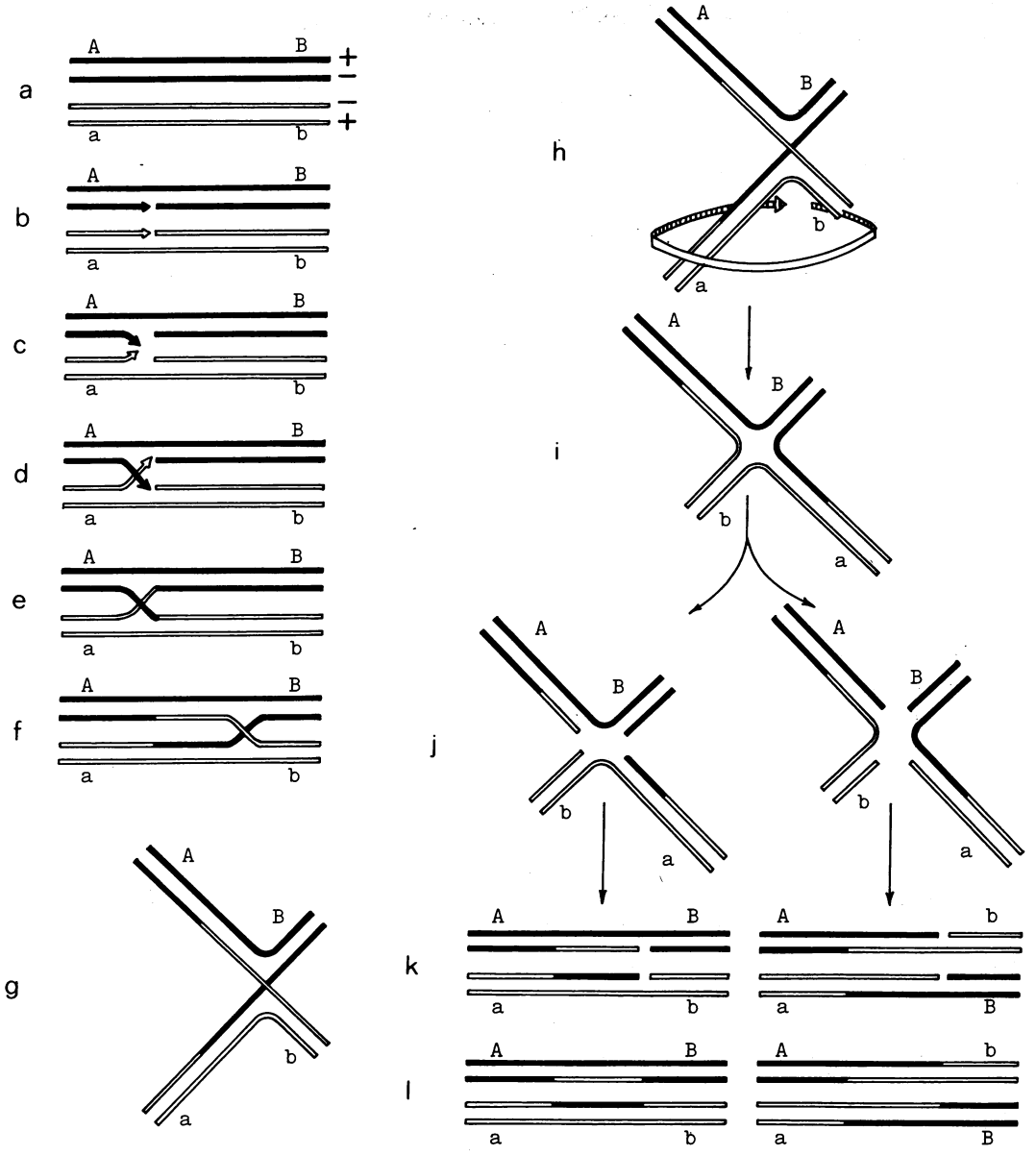

FIG. 6. The diagram shows the prototype Holliday model for genetic recombination (12–14); details of the model are explained in the *text*.

of a figure 8, which is a special type of dimeric DNA circle. Important evidence concerning dimer DNA circles and figure 8 forms and their involvement in recombination exists.

The thought that double-length DNA circles might be involved in recombination was first suggested by their discoverers, Rush and Warner (31). They found double-length ϕX duplex rings in virus-infected cells, and postulated that they could have arisen as the result of a recombination event between two monomer circles. It was suggested that the circular dimers would later be cut apart at a region of homology different from that used to form the dimer, thus yielding a pair of recombinant monomer rings. To obtain evidence for this hypothesis, Rush and Warner prepared dimeric circles from cells that had been infected with two kinds of mutant ϕX; they then showed that some of the dimers must be genetically heterozygous since, upon single infection into *E. coli* spheroplasts, they gave mixed bursts containing both types of phage. However, almost all of the dimeric circles produced only progeny of one genotype, indicating that they were genetically homozygous. It thus appeared that most dimeric circles were the result of DNA replication—for instance, the occasional traversing of a rolling circle template ring twice round before a progeny strand was matured (32). Nonetheless, the small percentage of heterozygous dimeric rings remained candidates for recombination intermediates.

Working from this base, Doniger, Thompson, Warner, and Tessman and their colleagues (6, 7) found that some of the dimers formed a subclass with a very special property: they crossed themselves exactly in the middle so as to have the configuration of a figure 8. Thus, these forms appeared to be composed of two interacting monomer circles. The figure 8 forms, though not dimeric circles in general, were absent in recombination-deficient strains. Benbow, Zuccarelli, and Sinsheimer (8, 9) reinforced and extended this result, showing that the figure 8 forms were sometimes composed of genetically different monomers; this was learned by co-infecting cells with

wild-type ϕX and with a second phage containing a small deletion. Among the figure 8 forms recovered were some that contained a monomer of each input size.

The advantage of the chi form over the figure 8 lies in the directness of its interpretation: it is clear that the touching of the two genomes occurs at a region of DNA homology and is not an accidental overlap or the result of two interlocked monomers.

During our study, Valenzuela and inman published a result similar to ours, using bacteriophage lambda as an experimental system (33). In their study, 26 lambda DNA molecules were photographed under conditions of partial DNA denaturation, and shown to be physically connected at homologous DNA positions. The structures observed are consistent with the Holliday intermediate. However, as noted (33), 18 of the 26 structures could equally well have arisen from a replicating intermediate (a Cairns form or a rolling circle) in which the parental strands had begun to reanneal, forcing the two daughter strands (which are of course complementary and capable of base pairing) to be extruded from the growing point, forming a fourth arm. The remaining eight molecules observed by Valenzuela and Inman were figure 8 forms; in some of these, single-strand connections were visible as in Figs. 4 and 5. These molecules thus are almost surely recombinants. The 800 chi forms analyzed in our study are all likely to be involved in recombination rather than to be the result of strand rearrangement within replicating intermediates (Cairns forms in the case of colicin DNA) because they possess two unit-size genomes—more DNA than could be derived from a partially replicated Cairns form. Furthermore, the colicin DNA forms, though not the lambda forms, have been correlated with the presence of a functional *rec A* gene.

SUMMARY

The experimental results presented in this paper deal with the nature of recombination intermediates, using colicin E1 DNA as an experimental system. The intermediates we have observed in the electron microscope provide physical evidence in support of the recombination intermediate postulated by Holliday on genetic grounds.

What is particularly encouraging is that our data, and the other data we have discussed, indicate that the class of models involving the Holliday structure, initially proposed to account for the highly ordered events that are involved in recombination between eukaryotic chromosomes at meiosis, also appears applicable to the potentially less demanding requirements of prokaryotic recombination. Thus, all of the experience and technological expertise available in prokaryotic systems can be brought to bear on the study of this recombination pattern.

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Note Added in Proof. Two recent publications present data relevant to the material described in this paper. Thompson, Camien, and Warner [(1976) *Proc. Natl. Acad. Sci. USA* **73**, 2299–2303] have detected X-shaped molecules in the bacteriophage G4 experimental system and found, as we do, that these structures roll apart in a matter of minutes. They have used this information to calculate that the rate of bridge migration is about 6000 base pairs/sec. In another publication, Bedbrook and Ausubel (submitted to *Cell*) have used polyacrylamide gel electrophoresis to show that multimer size plasmid forms do not arise in rec A$^-$ bacteria (see also ref. 34).

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