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# On the mechanism of genetic recombination: The maturation of recombination intermediates

(plasmid DNA/chi form/Holliday recombination intermediate/DNA multimers/*recA* gene/intramolecular recombination)

HUNTINGTON POTTER AND DAVID DRESSLER

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

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**ABSTRACT** **DNA molecules of the plasmid ColE1 are normally recovered from wild-type cells as a set of monomer- and multimer-size rings. The data of this paper show that the multimer-size species are a product of genetic recombination. Multimer rings do not arise after transfection of purified monomers into bacterial host cells lacking a functional *recA* recombination system. Analogously, purified dimers, trimers, and tetramers, transfected into *recA*$^-$ cells, can replicate, but are constrained to remain in those conformations. Only upon transfection into *rec*$^+$ cells can they regenerate the full spectrum of monomer- and multimer-size species.**

**In this paper we trace the flow of genetic information from the monomer to the multimer state and back again under the guidance of the *recA* recombination system.**

**The formation of multimer-size DNA rings is discussed as a natural consequence of the maturation of a Holliday recombination intermediate formed between two monomer plasmid genomes.**

Controlled genetic matings in eukaryotes have led to a wealth of information about the process of genetic recombination. From this information Holliday in 1964 suggested a molecular mechanism for recombination (1–3). The cornerstone of this mechanism is a central recombination intermediate that can be formed and matured to account for four genetic findings about recombinant eukaryotic chromosomes. These are: (*i*) All of the chromosomes that enter a eukaryotic meiosis and participate in recombination are recovered. That is, the recombination process is carried out with a net conservation of genetic information (4). (*ii*) The exchange of genetic information between homologous chromosomes (as observed over distances longer than a few thousand nucleotides) is almost always precisely reciprocal (4). (*iii*) An area of heterozygous DNA is frequently formed in the immediate region of the recombination event (5–10). (*iv*) The recombining chromosomes are apparently broken apart so that there is an equal chance that the genes on either side of the crossover position (the region marked by potential heterozygosity) will either be left in their parental linkage or emerge in a recombinant linkage (5, 6).

The Holliday recombination intermediate, which can account for these findings, is shown in Fig. 1. The intermediate is most easily thought of as arising from the pairing of two homologous DNA molecules that become nicked at roughly equivalent places and undergo a reciprocal strand invasion (Fig. 1 *A–C*). This sequence of events, or a related sequence (11), is expected to result in the formation of a stable recombination intermediate that, for convenience, can be represented in either of two planar configurations (Fig. 1 *F* and *G*). The subsequent maturation of the Holliday intermediate to yield recombinant chromosomes is shown in Fig. 1 *H* and *I*.

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While the Holliday model was evolved from a consideration of the genetic composition of recombinant chromosomes in eukaryotes, more recent evidence indicates that this mechanism may also be applicable to recombination in prokaryotes. Improved procedures for isolating nucleic acids and the introduction of electron microscopy (which allows the study of individual DNA molecules) have provided support for the Holliday intermediate in several prokaryotic systems; basically these relate to the recombination system of *Escherichia coli* as studied through its viruses S13, $\phi$X174, and $\lambda$ (12–15), and through the DNA of one of its plasmids, ColE1 (16, 17). In some of these instances it has been possible to directly visualize the recombination intermediate proposed by Holliday.

In our work we use plasmid DNA as an experimental system for studying recombination intermediates—both because the plasmid DNA molecules are small and can easily be isolated from the cell without breakage, and because the number of plasmid molecules can be selectively amplified to about 1000 per cell, potentially enhancing the opportunity for recombination by increasing the number of homologous DNA sequences. As discussed in a previous paper, we have been able to recover dimeric plasmid DNA molecules shaped like figure 8s, consistent with the interpretation of two circular genomes interacting at a point of DNA homology (see Fig. 2*A*, and also refs. 16 and 17). We confirmed this interpretation by linearizing the genomes of the plasmid with the restriction enzyme *Eco*RI: the figure-8 structures were converted to molecules with bilateral symmetry, shaped like the Greek letter chi ($\chi$) (an example is shown in Fig. 2*B*). Over 1000 such "$\chi$ forms" were photographed and analyzed. Each molecule contained two pairs of equal-length arms, indicating that two plasmid genomes were held together at a point of DNA homology. Moreover, in about 100 instances it proved possible to observe the polynucleotide strands in the region of the crossover. These strands could be seen connecting the two genomes, crossing over from one to the other (as in Fig. 2*C*).

The involvement of the $\chi$ forms in recombination was inferred from their absence in *recA*$^-$ cells.

We have interpreted our results as offering physical evidence in support of the recombination intermediate postulated by Holliday on genetic grounds: the experimentally observed $\chi$ forms correspond exactly to the two planar representations of the Holliday intermediate (compare Fig. 2 *B* and *C* with Fig. 1 *F* and *G*).

The physical evidence in support of the Holliday recombination pattern, such as shown in Fig. 2, deals specifically with the structure of the recombination intermediate. Two aspects of the recombination process that are less well understood concern the initiation events leading to the formation of the intermediate and the maturation steps by which the intermediate yields recombinant chromosomes. This paper is concerned with the maturation stage of the recombination process.

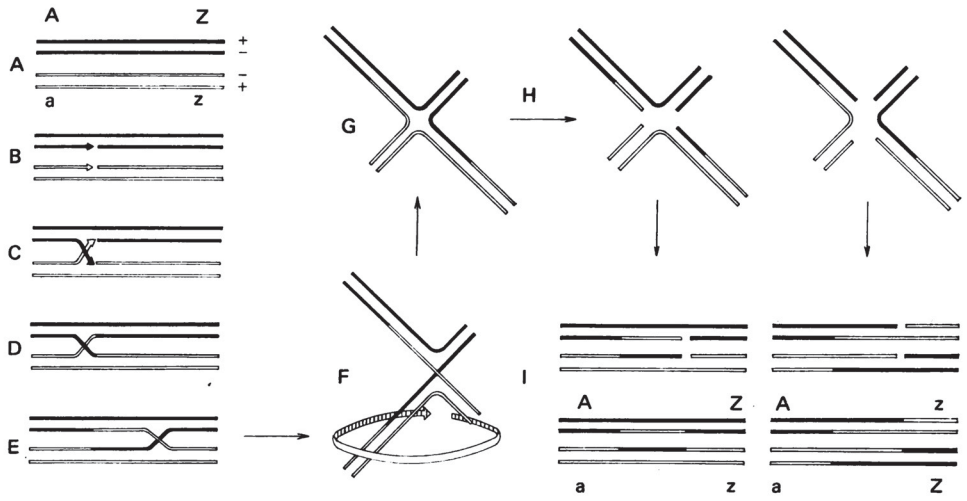


FIG. 1. Linear DNA molecules undergoing genetic recombination via a Holliday intermediate. The model is explained in the *text*.

## The maturation problem

One of the most interesting features of the Holliday intermediate arises because of its inherent symmetry. This symmetry leads to the expectation that the intermediate can be processed in either of two related ways to yield two different pairs of recombinant chromosomes. This dual maturation potential is most easily visualized if one considers the recombination intermediate in the planar representation shown in Fig. 1*G*. One of the maturation pathways cuts diagonally across the intermediate

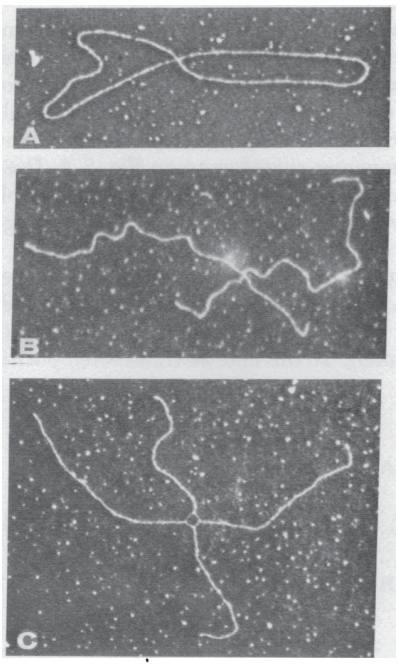


FIG. 2. Electron micrographs of plasmid DNA molecules in the process of recombination. (*A*) A figure-8 structure containing two monomer-size plasmid genomes. (*B*) A chi form: the plasmid DNA has been linearized with the restriction enzyme *Eco*RI (as in ref. 17); the two plasmid genomes are seen to be interacting at a region of DNA homology (compare with Fig. 1*F*). (*C*) An "open" $\chi$ form: during spreading for the electron microscope, the double helix has been disrupted in the region of the crossover, allowing the strand substructure to be seen (compare with Fig. 1*G*). Following purification from *recA*$^+$ cells, the plasmid DNA was examined in the electron microscope by a modification (18) of the Kleinschmidt and Zahn protein monolayer technique (19) as previously described (20).

along a north–south axis and leads to the formation of a pair of reciprocally recombinant chromosomes in which the genes on either side of the crossover emerge in a new linkage. This is a traditional single recombination event. But due to the symmetry of the recombination intermediate, a second maturation pathway is equally likely. In this case, diagonal nicking along an east–west axis leads to the separation of the two recombining chromosomes—but this time the genes on either side of the crossover are left in their original linkage. From a genetic point of view the recombination event would be silent, were it not for the possibility of forming heterozygous DNA in the immediate region of the crossover (as in Fig. 1 $D \rightarrow E$).

The maturation process, as discussed so far, relates to linear DNA molecules such as those found in eukaryotic chromosomes. The chromosomes of prokaryotes, viruses, and cellular organelles are, on the other hand, generally circular. It has long been recognized that this circularity poses a problem for the recombination process: a single recombination event between circular chromosomes is expected to produce a composite multimer structure. This sequence of events can be readily seen if one applies the processing steps we have considered to the maturation of the Holliday intermediate formed between two circles. As shown in Fig. 3, one of the two possible maturation pathways cuts the figure-8 intermediate apart into two independent monomer rings. But in these monomers, the outside markers are always left in their original linkage, and the only genetic recombination that arises does so because of the possible formation of heterozygous DNA in the immediate region of the crossover. On the other hand, if the alternative maturation nicks are introduced, the recombination intermediate is automatically converted into a dimer-size circle (Fig. 3*G*).

Thus monomer rings with a recombinant arrangement of the genes on either side of the crossover point are not directly produced by maturation of the recombination intermediate formed between two circles. It is the purpose of this paper to present evidence bearing on this problem.

## Multimers are formed in *recA*$^+$ cells

As discussed in the previous section, it is expected that circular dimers are a product of the maturation of two monomer circles engaged in the crossover stage of genetic recombination (see Fig. 3).

We have explicitly tested this prediction by purifying monomer-size plasmid rings and introducing them into *rec*$^+$ and *recA*$^-$ cells. The transplantation of the DNA rings into the

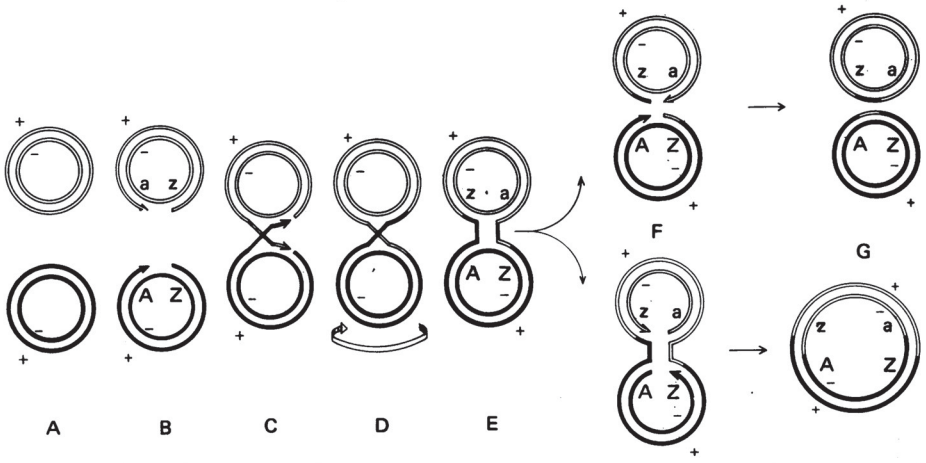


FIG. 3. Circular DNA molecules undergoing recombination via a Holliday intermediate. Because of the symmetry inherent in the recombination intermediate (*E*) there are two alternative maturation pathways. One leads to the formation of two monomer rings with the genes on either side of the crossover position retaining their original linkage. The other maturation pathway leads to the formation of a circular dimer.

desired experimental background is readily achieved by using recipient cells that have been made temporarily permeable to exogenous DNA by calcium treatment and a heat shock. Cells that receive a plasmid can be selected because they simultaneously gain a tetracycline resistance gene carried on the plasmid, and thus acquire the ability to form a colony on solid medium supplemented with tetracycline. Each colony that appears contains the entire lineage of plasmids resulting from the entrance of a single DNA molecule into a single recipient cell.

Several tetracycline-resistant colonies were picked from among the *rec*$^+$ and *recA*$^-$ recipients of purified plasmid DNA monomers. Each colony was further grown in the presence of tetracycline in logarithmic-phase culture for 70 generations. This was done to allow time for the cellular plasmid population to approach equilibrium. Plasmid DNA was then extracted from the *rec*$^+$ and *recA*$^-$ cells and analyzed for size heterogeneity using agarose gel electrophoresis.

As shown in Fig. 4, the monomers transfected into *recA*$^-$ cells were only able to replicate to form more monomers. In contrast, the transfection of monomers into *rec*$^+$ cells led to the development of a population containing both monomer *and* multimer plasmid genomes. This finding was further solidified by analysis using the electron microscope, which is of higher resolution. Table 1 shows the percentages of monomers, dimers, trimers, and tetramers found in *rec*$^+$ and *recA*$^-$ cells after transfection with purified monomers. In *recA*$^-$ cells 99% of the plasmids were monomers; only 1% were dimers. In *rec*$^+$ cells, after 70 generations, there were 60% monomers, 37% dimers, 1% trimers, and 2% tetramers.

The general conclusion is that in the absence of a functional *recA* system, multimeric plasmid DNA forms do not arise. Or, stated conversely, multimeric plasmid genomes appear to be the result of recombination.

## The action of the recombination system on multimers

As considered so far, the recombination system has produced a multimer-size plasmid instead of a pair of monomers that are recombinant for the genes on either side of the crossover. For the reciprocal recombination process to be completed, there must be a mechanism for the conversion of the larger circles back to the smaller ones. If not to achieve reciprocal recombination, such a mechanism would still be necessary to prevent multimer-size genomes from progressively accumulating and, as the result of recombination, dominating the cell population. The mechanism for regenerating monomers is not likely to be DNA replication because, as shown in Table 1, dimers, trimers, and tetramers transfected into *recA*$^-$ cells are locked into those configurations and can only replicate to form more dimers, trimers, and tetramers, respectively.

The experiments we will now discuss show that monomer genomes can in fact be obtained from a multimer-size plasmid. The key is to realize that the multimer circles that are produced as a result of the maturation of the first crossover need not be end products: they may themselves be substrates for the recombination system.

If two homologous areas within a dimer DNA ring can initiate an *intramolecular* recombination event, a new figure-8 intermediate will be created, at a new crossover position (see Fig. 5). This intermediate will be identical in strand substructure to the figure 8 that is formed by reciprocal strand invasion between two monomer rings (compare Fig. 5 *A–D* with Fig. 3 *A–D*). The maturation of this figure 8 should, 50% of the time, yield two monomer rings with recombinant arrangements of genetic markers (Fig. 5*H*).

Thus, we sought to demonstrate that, when dimeric circles are placed in *rec*$^+$ cells, the circles are able to undergo an intramolecular recombination event leading back to the formation of monomers. To this end we transfected purified dimers

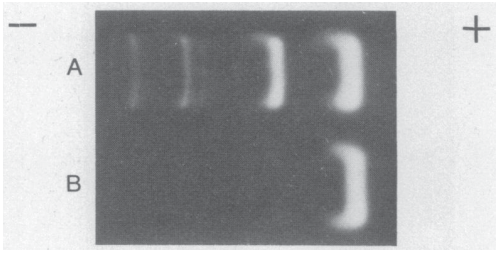


FIG. 4. (*A*) Both monomer- and multimer-size plasmid DNA rings are recovered after transfection of monomers into *rec*$^+$ cells. (*B*) Monomers transfected into *recA*$^-$ cells are able to replicate, but cannot recombine to form multimeric structures. To obtain this result, plasmid DNA was recovered from individual *rec*$^+$ and *recA*$^-$ clones and analyzed for size heterogeneity by agarose gel electrophoresis. The gel contained 0.75% agarose in 40 mM Tris·HCl/20 mM NaOAc/1 mM EDTA, pH 7.6; after electrophoresis, the DNA was visualized by staining with ethidium bromide (0.5 μg/ml for 60 min). The major bands, from right to left, are supercoiled plasmid monomers, dimers, trimers, and tetramers. The faint bands result from a low percentage of nonsuperhelical rings.

Table 1. Fate of purified monomers, dimers, trimers, and tetramers transfected into *rec*$^+$ and *recA*$^-$ cells

| Transfection | | Resulting plasmid population | | | | | |
|---|---|---|---|---|---|---|---|
| DNA | Recipient | Monomers | Dimers | Trimers | Tetramers | Higher multimers | Total |
| Monomers | *rec*$^+$ | 595 | 367 | 13 | 24 | 1 | 1000 |
| | *recA*$^-$ | 994 | 6 | 0 | 0 | 0 | 1000 |
| Dimers | *rec*$^+$ | 43 | 693 | 8 | 241 | 15 | 1000 |
| | *recA*$^-$ | 1 | 989 | 0 | 9 | 1 | 1000 |
| Trimers | *rec*$^+$ | 167 | 136 | 675 | 5 | 17 | 1000 |
| | *recA*$^-$ | 1 | 0 | 984 | 0 | 15 | 1000 |
| Tetramers | *rec*$^+$ | 68 | 432 | 62 | 407 | 31 | 1000 |
| | *recA*$^-$ | 2 | 10 | 2 | 967 | 19 | 1000 |

Monomer and multimer plasmid DNA rings were purified by repeated sucrose velocity gradient centrifugation, passaged through *recA*$^-$ cells, and then used to transfect, in parallel, *rec*$^+$ and *recA*$^-$ cells (*E. coli* strains 294 and 152 from M. Meselson). Individual cells that received a plasmid were selected immediately after transfection by virtue of their conversion to a tetracycline-resistant state. From each transfection 2–6 recipient cells were recovered and grown into cultures, from which superhelical and nonsuperhelical plasmid DNA was purified (17, 21). The numbers of monomer and multimer plasmid species were then determined using the electron microscope. Plasmid sizes were easy to distinguish by inspection; these estimates were confirmed by selective photography and molecule measurement. The experiment has been done four times.

into *rec*$^+$ cells, selected individual tetracycline-resistant recipients, grew these cells through 70 generations, and then isolated their plasmid DNA.

As shown in Table 1, we observed that the dimers were processed by the *recA* recombination system to generate both larger and smaller plasmid DNA rings. After 70 generations, the percentages of monomers, dimers, trimers, and tetramers in *rec*$^+$ cells were 4%, 69%, 1%, and 24%. Similarly, purified trimers and tetramers transfected into *rec*$^+$ cells regenerated the full spectrum of monomer and multimer-size rings.

In sum, these results indicate that *recA*-mediated intramolecular recombination can occur in DNA circles, allowing the production of pairs of reciprocally recombinant genomes.

Our results may have relevance to a model recently proposed by Holloman *et al.* (22). They have made the interesting proposal that the initiation of *recA*-mediated recombination is an inherently asymmetric event. Specifically, one recombining DNA molecule is supercoiled and the other relaxed, so that the former (by giving up its superhelical twists and acquiring a region of local denaturation) may serve as the recipient for a strand invasion from the latter (22). Under such a model, a circular dimer would be expected to be unable to initiate in-

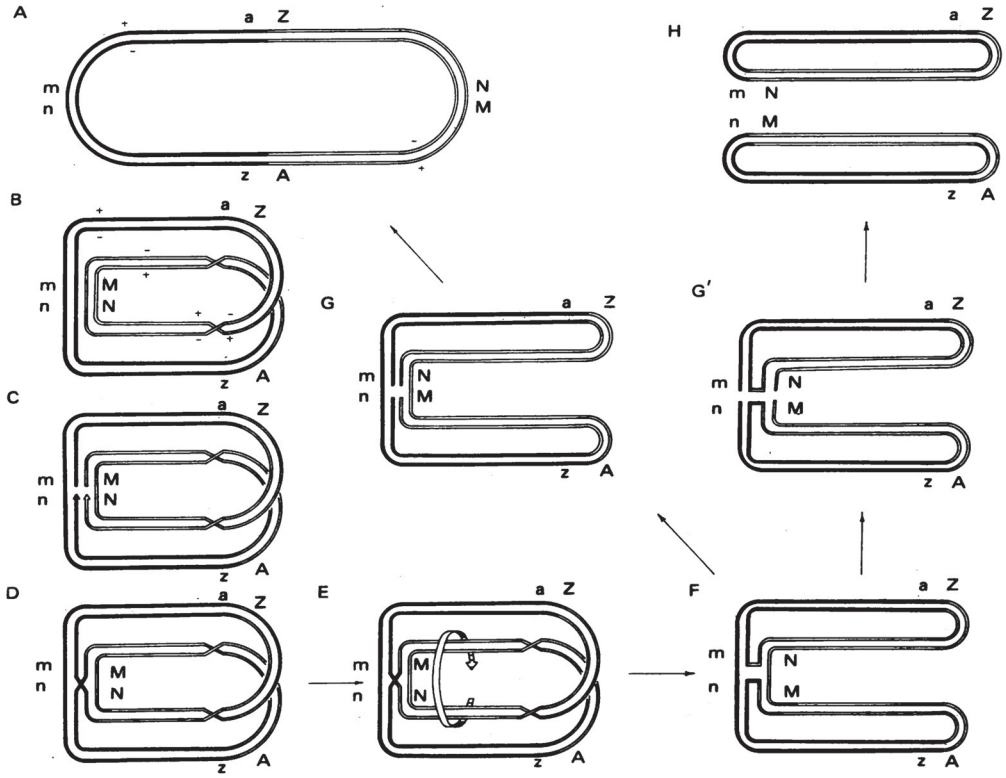


FIG. 5. Steps by which a circular multimer can undergo an intramolecular recombination event. The aligning of homologous DNA sequences is followed by the nicking and exchange of DNA strands of like polarity, leading to the formation of a figure-8 structure. The strand substructure in the crossover region is identical to that of the recombination intermediates shown in Figs. 1 and 3. The two alternatives for maturation of this recombination intermediate allow: (*i*) the re-formation of the multimer ($G \rightarrow A$), or (*ii*) the creation of a pair of reciprocally recombinant DNA rings ($G' \rightarrow H$).

tramolecular recombination because it would be either entirely supercoiled or entirely relaxed. Our results are at variance with this hypothesis, for we see the production of smaller circles when supercoiled multimers are placed in *rec*$^+$ cells. Perhaps a reconciliation is possible if the circular DNA molecules are able to possess independently superhelical regions. For instance, there could be a cellular system that maintains part of the DNA molecule in a superhelical state, while allowing other parts to remain relaxed. Independent superhelical regions have been observed in the *E. coli* chromosome (23), but their relevance to recombination has not been investigated.

## Interpretation

Overall, our evidence supports reciprocal recombination between DNA circles as a two-stage process leading first in a bimolecular reaction to a multimer-size structure. Then, a second, intramolecular, recombination event breaks the multimer apart into two smaller circles in which the original genes have been recombined into a new configuration. The data we have presented in this paper trace the flow of genetic information from the monomer to the multimer state and back again under the guidance of the *recA* recombination system.

There is a potentially important consequence of the finding that multimer-size DNA rings can be recombined into smaller circles, for this is essentially a deletion process. Accordingly, it would appear that the *recA* recombination system can work on tandem homologies whenever they occur in chromosomal DNA to catalyze the formation of a recombination intermediate (as in Fig. 5) and the excision of the DNA between the regions of tandem homology. How much difficulty this presents for the cells is unclear.

Because intramolecular recombination can occur (Table 1), one might be concerned that such control elements as promoters, which necessarily occur many times on a chromosome, would provide a source of repeated homologies inviting catastrophic deletions. What defense is there against such deletions? The simplest defense would result if the regions of homology were sufficiently small so that recombination events involving them would be rare. Also, subtleties in the design of such repeated structures as promoters may be able to prevent recombination. For instance, initial DNA sequencing studies indicate that promoters have been built so as to contain short recognition regions interrupted by nonhomologous nucleotide stretches. That is, although the RNA polymerase may sit on a 40- to 60-base-pair stretch of DNA, it makes contact with only a few key base pairs that actually constitute the promoter. The rest of the binding area appears to be nonhomologous material, perhaps designed to prevent recombination. Thus, the polymerase can recognize many promoters as being identical for the purpose of binding, but the promoters cannot recognize each other as being identical for the purpose of recombination.

The occurrence of whole genes repeated in tandem, such as ribosomal RNA cistrons, presents a more serious problem. Here design aspects minimizing the amount of DNA homology are not in evidence. Unless a mechanism exists for keeping some regions of the chromosome silent for the purpose of recombination, we would have to expect that intramolecular recombination involving these regions would result in a deletion that could prove lethal to the occasional cell in which it occurred.

## Two related studies

In a study similar to ours, but based on agarose gel analysis, Bedbrook and Ausubel (24) have also determined that multimeric plasmid genomes are a result of recombination. Where they overlap, our two sets of data reinforce each other.

Another study bearing on the recombinational origin of multimer DNA rings involves phage λ. λ genomes that contain mutations in genes *gamma* and *red* cannot synthesize concatemeric DNA characteristic of the late life cycle, nor can they engage in high efficiency phage-mediated recombination. Such phage, in attempting to grow, synthesize predominantly monomer-size rings. These rings cannot be packaged in coat protein, and the phage form small plaques. Lam *et al.* (10) have studied a class of second-site mutations that restore active phage production in this system. They have shown that these mutations, called χ mutations, enhance recombination 20- to 30-fold and have hypothesized that the restoration of normal phage growth results from an increase in the recombination-mediated production of packageable multimer DNA forms.

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