Proc. Natl. Acad. Sci. USA
Vol. 76, No. 7, pp. 3445–3449, July 1979
Genetics

Occurrence of crossed strand-exchange forms in yeast DNA during meiosis

(Holliday structure/electron microscopy/2-μm DNA/*Saccharomyces cerevisiae*)

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Communicated by Herschel L. Roman, May 4, 1979

ABSTRACT **The crossed strand-exchange form (Holliday structure, half chiasma) has been predicted as an intermediate in the genetic recombination of eukaryotes. We report here the detection of this form in the yeast plasmid, 2-μm DNA, isolated during meiosis. Physical mapping has previously suggested that two forms of 2-μm DNA arise because of recombination between inverted repeat regions. After appropriate digestion with restriction endonuclease, a crossed strand-exchange form intermediate in this recombination would yield an X-shaped form resistant to loss by branch migration because of nonhomology in sequences flanking the region of homology. We first generated this X-shaped form artificially by reannealing melted restriction fragments of 2-μm DNA. This enabled us to develop a procedure for the physical separation of the X-shaped form by agarose gel electrophoresis. We then used this electrophoretic procedure to isolate a naturally occurring form of identical structure from the 2-μm DNA of meiotic cells. Electron microscopy demonstrated that the exchange junction had the expected configuration of strands and indicated that the junction occurred within the region of homology.**

It was postulated a number of years ago that genetic recombination in eukaryotes involves an intermediate that contains a junction at which single DNA strands from each duplex exchange pairing partners (1). Although the relative importance of this symmetric intermediate in the generation of nonreciprocal recombination remains unknown (2), the crossed strand-exchange form (also known as a Holliday structure or half chiasma) (3) serves as a useful model for explaining many aspects of reciprocal recombination.

The DNA of prokaryotes has been demonstrated to contain crossed strand-exchange forms which are recognized as pairs of DNA molecules joined together with a characteristic open center at their junction (4, 5). It has not previously been determined whether such forms also occur in the DNA of eukaryotic cells. We have therefore devised a sensitive method for their detection in a yeast plasmid and have used this method to assay their occurrence in meiotic cells.

The system

Most strains of *Saccharomyces cerevisiae* contain 50–100 copies per cell of a closed circular DNA plasmid that is double-stranded and has a contour length of 2 μm (consisting of 6 kilobases) (6). Every molecule of this species, termed 2-μm DNA (7, 8), contains two nontandem inverted repeats—each about 600 base pairs long—separated by interstitial regions of about 2700 and 2200 base pairs (7–10). The interstitial regions are arranged in either of two orientations (Fig. 1, forms A and A′), resulting in two populations of 2-μm DNA within a clone of cells (7, 8, 10). It has been proposed (7, 8, 10) that the two molecular types of 2-μm DNA are derived from one another by intramolecular reciprocal recombination between the two inverted repeats. If a crossed strand-exchange form is an intermediate in the proposed intramolecular recombination, then we expect the figure-8-shaped forms diagrammed as B in Fig. 1 to be present (perhaps only briefly) during recombination. The diagram shows two figure-8-shaped forms which could interconvert by the isomerization mechanism (2, 11) modified from the proposal by Sigal and Alberts (3), assuming that this event could occur within a circular molecule (12). An additional pair of figure-8-shaped isomers may be formed if the initial strand-exchange occurs between the alternative two single strands of the inverted repeat. Digestion of any of these forms with *Eco*RI, which cleaves each 2-μm DNA monomer at the two sites indicated, would produce an X-shaped form of the type diagrammed as C in Fig. 1.

The central portion of the X-shaped form consists of the symmetrically arranged sequences of the inverted repeat region. Within this region the form is bilaterally symmetrical, with two pairs of homologous arms, as in the "χ forms" that Potter and Dressler (5) detected after cleavage of recombining ColE1 plasmids. The site of the crossed strand-exchange is expected to undergo branch migration throughout the region of homology. Branch migration within typical crossed strand-exchange forms, such as those of the ColE1 plasmids, therefore continues to the ends of the homologous arms; the junction may then migrate off the ends of the arms, resulting in the separation of the crossed strand-exchange form into two separate duplex molecules (5, 13). In the proposed intermediate of 2-μm DNA, migration would be limited by the lack of homology in sequences flanking the inverted repeat (Fig. 1, abcd), thereby preventing loss of the junction.

In order to determine the physical characteristics of the proposed recombinational intermediate, we generated this X-shaped form *in vitro* by the methods described below. Information about the physical properties of the artificial form then permitted us to investigate the natural occurrence of this crossed strand-exchange form.

Construction of an artificial crossed strand-exchange form

We constructed the artificial X-shaped form from circular 2-μm DNA consisting of the two types, diagrammed as A and A′ in Fig. 1, isolated from vegetatively growing cells. This DNA was cleaved with *Eco*RI (Fig. 1, steps 3 and 3′) to produce double-stranded linear fragments of four sizes, two from each monomer type (Fig. 1, D and D′). The entire population of linear molecules was then denatured to release eight types of single-stranded DNA (Fig. 1, E). Finally the mixture was reannealed, permitting not only the re-formation of the four original linear duplexes but also the novel formation of branched forms. The

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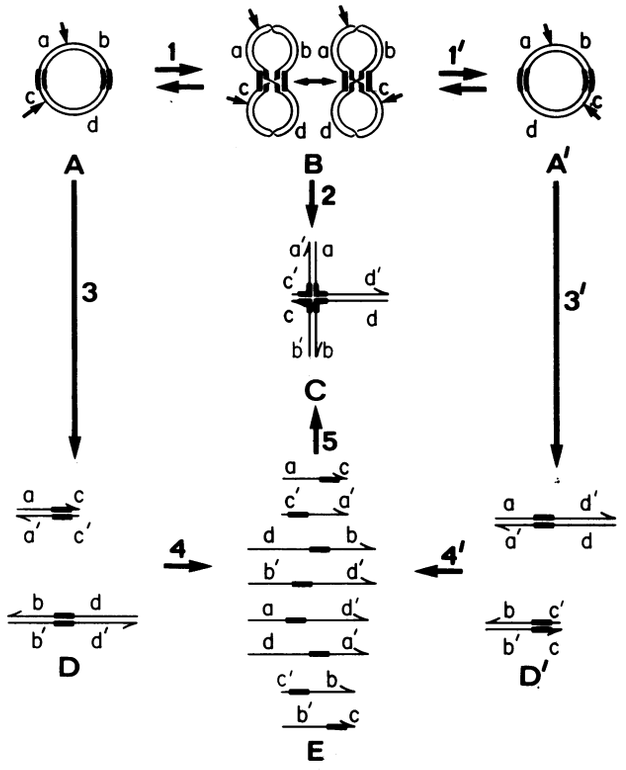

FIG. 1. Formation of native and artificial X-shaped forms. Each line represents a single strand of DNA. The small arrows designate *Eco*RI sites. The thick lines represent the inverted repeat regions, and the nonhomologous flanking sequences are indicated by lower-case letters. For further explanation, see the text.

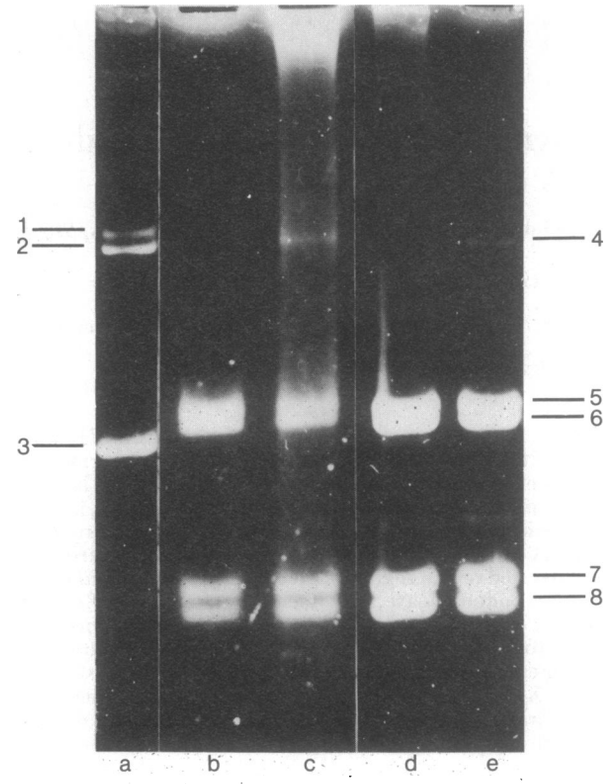

FIG. 2. Agarose gel electrophoresis of 2-μm DNA. Lanes: a, uncleaved, from vegetatively grown cells; b, vegetative DNA cleaved with *Eco*RI; c, vegetative DNA cleaved with *Eco*RI, denatured, and reannealed; d *Eco*RI-cleaved DNA 30 min after transfer to sporulation medium SPM; e, *Eco*RI-cleaved DNA 8 hr after transfer to SPM. Cells of strain 212-1 (14) were maintained in logarithmic growth on a shaker at 23°C in acetate presporulation medium PSP2 supplemented with adenine at 40 μg/ml (15) and generally labeled with [6-^{3}H] uracil (1 μCi/ml) for 12 hr to facilitate identification of fractions containing 2-μm DNA. Vegetative 2-μm DNA (lanes a, b, and c) was isolated when the culture reached a density of 1–1.5 $\times$ 10^7 cells/ml. For the induction of meiosis (lanes d and e), cells at a density of 2 $\times$ 10^7 cells/ml were transferred to a density of 1 $\times$ 10^7 cells/ml in sporulation medium SPM (15). For isolation of 2-μm DNA, cells were spheroplasted according to Peterson *et al.* (16) as modified by Byers *et al.* (17) except that 1 M sorbitol was used instead of 1 M NaCl in the spheroplasting medium. The DNA was extracted and 2-μm DNA was separated on CsCl/ethidium bromide gradients as described by Livingston and Klein (8). Denaturation and reannealing of *Eco*RI-cleaved (New England BioLabs) 2-μm DNA (lane c) was performed in 0.15 M NaCl/1 mM EDTA/0.1 M Tris·HCl, pH 7.5, by heating to 100°C for 10 min, quenching on ice for 3 min, and then incubating at 60°C for 30 min. The DNA was then concentrated by precipitation with 2 vol of ethanol at −20°C. All samples were treated with RNase A (Sigma) and then subjected to electrophoresis on vertical 0.7% agarose gels (12 $\times$ 12 $\times$ 0.6 cm; wells, 0.55 cm wide) at 15 V according to Greene *et al.* (18). Gels were stained with ethidium bromide and photographed. The visible bands demonstrate the following: lane a, uncleaved 2-μm DNA contains supercoiled circles (band 3) and relaxed, nicked circles (band 1 or 2) (7); lane b, *Eco*RI cleavage at two sites produces four bands because two isomers of 2-μm DNA are present; lane c, denaturation and reannealing of *Eco*RI-cleaved DNA produces the new band (band 4) identified as the artificial crossed-strand exchange form; lanes d and e, the naturally occurring crossed strand-exchange form is absent at 30 min after transfer to SPM (lane d) but is present (band 4) at 8 hr (lane e).

predicted X-shaped form (Fig. 1, C) would contain four of the denatured strands, which could have reannealed in any of several pathways. A representative pathway is as follows. The single-stranded fragments a′ c′ and cb′ could pair, at the c and c′ ends, to form an incomplete duplex with two single-stranded tails, a′ and b′. These tails could then pair with the single-stranded molecules ad′ and bd. Finally, the dd′ tails could pair to give the X-shaped form shown as C in Fig. 1. A second X-shaped form of identical configuration would be formed in a similar manner from the remaining four complementary single-stranded fragments.

The products of reannealing were analyzed by electrophoresis on agarose gels (Fig. 2). Whereas the products of digestion of 2-μm DNA by *Eco*RI consisted of four bands (Fig. 2, lane b), the reannealed sample contained the same four bands plus an additional band of lower mobility (Fig. 2, lane c). We eluted the fifth band and found by electron microscopy that it was composed of X-shaped forms of the expected type (Fig. 3*A*). Details of their structure will be discussed below.

Detection of naturally occurring crossed strand-exchange forms in meiotic cells

Our characterization of artificial X-shaped forms by agarose gel electrophoresis provided a sensitive method for detecting the natural occurrence of crossed strand-exchange forms in 2-μm DNA. If figure-8-shaped crossed strand-exchange forms (Fig. 1, B) were present in 2-μm DNA isolated from meiotic cells, cleavage with *Eco*RI (Fig. 1, step 2) would produce native X-shaped forms identical to the artificial forms (Fig. 1, C). These would migrate on agarose gels to their characteristic position, well separated from the linear products of digestion; they could then be electroeluted and examined by electron microscopy.

Supercoiled 2-μm DNA was isolated from cells in pachytene of meiosis, when recombination is generally considered to occur. Pachytene was identified by the presence of synaptonemal complex (20) and by the time of commitment to recombination in *ade2* heteroalleles (21). When this 2-μm DNA was cleaved with *Eco*RI and subjected to agarose gel electrophoresis, we found five bands (Fig. 2, lane e), rather than the four present

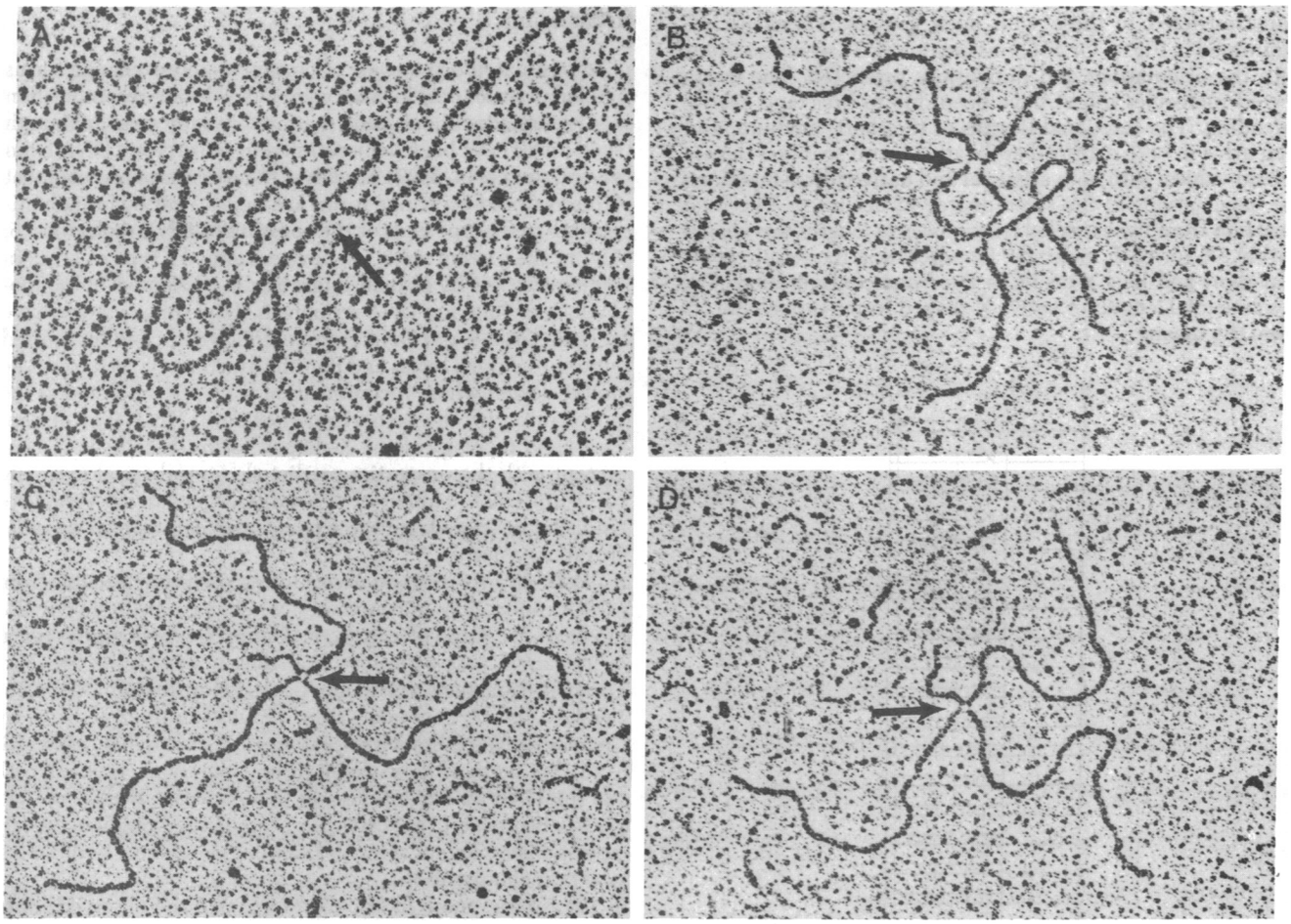

FIG. 3. Electron microscopy of crossed strand-exchange forms. (*A*) Artificial form. (*B–D*) Naturally occurring forms. Arrows indicate the open-centered junctions between DNA duplexes. DNA was electroeluted from the agarose gel by placing the gel fragment in dialysis tubing with a small volume of 100 mM Tris/10 mM EDTA, pH 8.0, and subjecting the sample to electrophoresis in 5 mM Tris/2.5 mM EDTA, pH 8.0, at 25 V for 12 hr or more. The DNA was then precipitated with ethanol and resuspended in 50 μl of 100 mM Tris/10 mM EDTA, pH 8.5. The DNA was spread for electron microscopic analysis by the method of Davis *et al.* (19) with a hyperphase of 50 mM Tris/5 mM EDTA, pH 8.5/40% formamide/cytochrome *c* at 0.1 mg/ml onto a hypophase of 5 mM Tris·HCl, pH 8.5/10% formamide. The artificial forms were spread from 30% formamide onto 15% formamide. Grids were stained with uranyl acetate and rotary shadowed with Pt/Pd, 80:20 (wt/wt). Contour lengths were measured with a Graf/Pen model GP3 digitizer connected to a Hewlett–Packard calculator. (×75,000.)

after similar analysis of 2-μm DNA from vegetative cells (Fig. 2, lane d). The additional band migrated to the same position as the artificial X-shaped form (Fig. 2, lane c). Electron microscopic analysis of the DNA electroeluted from this band confirmed that it consisted of branched molecules identical to the artificial forms (Fig. 3, *B–D*). From densitometry of the stained gel in combination with electron microscopic counts, we estimate that the X-shaped forms from meiotic cells are present at a frequency of approximately 1 per 1000 monomers of 2-μm DNA and that X-shaped forms are present at no more than 1/10th this frequency in vegetative cells.

Electron microscope analysis of artificial and native X-shaped forms

The X-shaped forms observed by electron microscopy displayed a range of configurations consistent with the model diagrammed in Fig. 1. The four arms differed from one another in length because of the fixed lengths of the four nonhomologous sequences (Fig. 1, abcd) that flank the inverted repeat region. In addition, the arm lengths were variable because of variation in the position of the junction as it underwent branch migration within the homologous inverted repeat sequences. This generated a family of configurations which the X-shaped forms assumed under branch migration.

Both artificial and native X-shaped forms often appeared in the electron microscope to be "open-centered" at the junction because of localized separation (denaturation) of strands. This appearance, which has been described previously for the recombinational intermediates of prokaryotes (4, 5), demonstrates unequivocally that the convergence of arms represents a crossed strand-exchange form with covalent continuity of all four strands, rather than one duplex molecule lying on top of another. Moreover, we gain the useful information that opposite arms (in *trans*) in an open-centered junction are homologous within the inverted repeat region, whereas adjacent arms (in *cis*) are nonhomologous. The pattern of flanking sequences in forms with open centers is therefore predicted by the model 2-μm DNA structure (Fig. 1). The short arm will nearly always be opposite the long arm because the shortest and longest flanking regions are always attached to homologous central regions. This relationship is valid for all states of branch migration within the homologous inverted repeat region except when the short arm is at its shortest and the three other arms are of nearly equal length.

We measured 41 artificial X-shaped forms, 9 of which had open centers. Most of these (27 of those with closed centers and all with open centers) were consistent with the predicted form. Measurements of native X-shaped forms from meiotic cells

were restricted to those with open centers. These are diagrammed in Fig. 4. Of 40 forms measured, 37 were consistent with the orientations and dimensions predicted. The unexpected molecules might have been due to breakage during spreading. The fractional lengths of the short, medium, and long arms in both artificial and native forms spanned the expected values, with both artificial and native forms matching the expectations to the same degree of accuracy. The total variation from the expected fractional arm lengths was always within 5% for each molecule, incorporating the requirement that short and long arms be in the *trans* configuration. The absolute total lengths of native X-shaped forms varied only 5% from the mean.

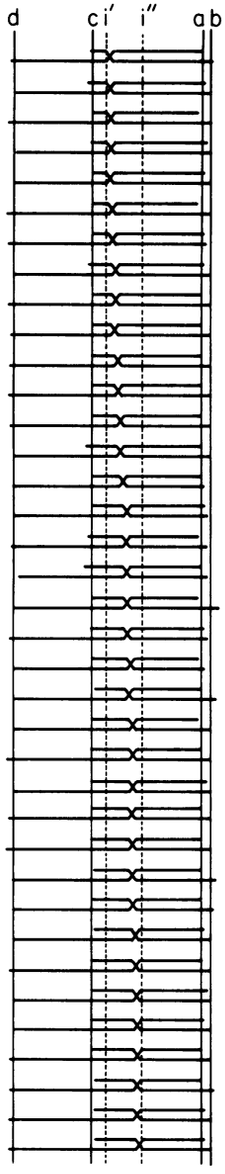

FIG. 4. Distribution of arm lengths of 37 crossed-strand exchange forms isolated from meiotic yeast 2-μm DNA after digestion with *Eco*RI. Open centers were seen in all forms selected for measurement. Vertical lines (a, b, c, and d, corresponding to the letters in Fig. 1) indicate the fractional arm lengths predicted by physical mapping (9). Arms c and d were in *trans* configuration; they are both diagrammed to the left in order that the homologous inverted repeats (flanked by vertical lines i′ and i″) could be aligned in parallel. The observed limits of branch migration are coincident with the predicted limits (i′ and i″). The original sample also included three forms (not shown) that did not have arms of the predicted lengths.

Discussion

The postulated occurrence of crossed strand-exchange forms (Holliday structures) in eukaryotic DNA during recombination has remained without confirmation for many years, although such forms have been demonstrated in prokaryotic DNA from several sources (4, 5, 22). Studies of the latter have revealed that crossed strand-exchange forms are rapidly lost by branch migration to the free ends of the constituent DNA molecules (5, 13). In the present study we have examined a type of DNA in which the loss by branch migration is prevented by nonhomology of flanking DNA sequences. These observations have revealed that 2-μm DNA from meiotic yeast contains crossed strand-exchange forms and that the junctions of such forms lie in the region where recombination has been predicted as the mechanism for the isomerization of 2-μm DNA. The dimensions and topology of the X-shaped forms described are consistent with the properties predicted from physical mapping of 2-μm DNA. Moreover, the naturally occurring forms are indistinguishable from the model forms constructed by renaturation *in vitro*.

Both the artificial and native forms are distinguishable from DNA replication intermediates, which have been shown (23) to have the shape of a theta, θ. These θ forms contain typical replication forks with three branches, in contrast to the four-branched junctions seen in the X-shaped forms. Forms with four arms could be generated from replication intermediates by reannealing and branch migration of nascent daughter strands (24), but many of these would differ from those observed, both in their overall dimensions and in the relative lengths of their arms. Moreover, replication intermediates would necessarily possess homologous arms and so would be subject to rapid loss by branch migration.

It appears more likely, therefore, that the observed forms represent intermediates in the intramolecular recombination event believed to cause isomerization in the 2-μm DNA population. Isomerization has, in fact, recently been demonstrated in a line of yeast cells transformed by cloned 2-μm DNA (25); although the transforming DNA consisted of only one type, cells eventually gained the second isomer. Such isomerization may occur by a simple recombination within a single plasmid as diagrammed in Fig. 1 (A-B-A′), but intermolecular recombination may also be involved. Guerineau *et al.* (10) have postulated that recombination between inverted repeats of two identical circles would generate a dimeric circle. We note that recombination within this dimer, between the second pair of inverted repeats, could produce the alternative isomeric monomers. This mode of isomerization again involves recombination with inverted repeats flanked by nonhomologous sequences and could thereby yield the observed X-shaped forms.

The abundance of the X-shaped forms in the meiotic, but not vegetative, cultures provides a further indication that these forms may be recombination intermediates. Although the relative rates of isomerization in vegetative and meiotic cells are unknown, it seems reasonable that intracellular conditions would be favorable for recombination of 2-μm DNA during meiosis, when chromosomal DNA recombines. An increased rate of recombination would probably be accompanied by an increased frequency of recombination intermediates. Alternatively, the abundance of X-shaped forms could result in part from a decrease in the rate of their resolution.

Although the location of 2-μm DNA within the cell is unknown (6, 26), this plasmid has come to serve as a useful model for chromosomal processes. Several of the nuclear genes that control chromosomal replication are also required for 2-μm

DNA replication (23, 27). Furthermore, the replication of 2-μm DNA during the beginning of S phase (28) suggests the occurrence of common control mechanisms. The observations reported in this paper suggest that there may also be functions common to the recombination of both chromosomal DNA and 2-μm DNA. Further analysis of the isomerization of 2-μm DNA may therefore provide insight into some of the functions essential to chromosomal recombination. We note, however, that functions required for chromosomal pairing may well be unnecessary for intramolecular recombination of the plasmid.

We thank Dr. A. H. Doermann and Dr. H. L. Roman for criticism of the manuscript. This research was supported by a research grant (GM 18541) from the National Institutes of Health. L.B. is a Predoctoral Fellow supported by the National Institutes of Health.

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