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Renaturation of complementary single-stranded DNA circles: Complete rewinding facilitated by the DNA untwisting enzyme

(superhelical simian virus 40 DNA/DNA relaxation/DNA recombination/heteroduplex DNA/nicking-closing enzyme)

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ABSTRACT Renaturation of two complementary single-stranded circles should be limited by topological constraints against the rewinding of the DNA helix. If a mixture of complementary single-stranded rings is annealed and then treated with the DNA untwisting enzyme, the DNA circles completely renature as judged by (*i*) the presence of interlocked rings that sediment at 53 S in alkali, (*ii*) the buoyant density of the renatured DNA in CsCl gradients containing ethidium bromide, and (*iii*) the resistance of the product to the single-strand-specific S1 nuclease. Therefore, the DNA untwisting enzyme is able to provide a transient single-strand break that is sufficient to allow the two strands to completely rewind. The possibility that the untwisting enzyme might facilitate the initiation of the process of genetic recombination is discussed.

The two strands of a covalently closed circular DNA molecule are topologically linked. Thus, if the DNA is denatured (e.g., with alkali), the two strands remain intertwined even after the helix-to-coil transition is complete (1, 2). The extent of the linkage is defined as the topological winding number (α) and is equal to the sum of the helical winding number (β) and the number of superhelical turns (τ) in the native form of the DNA ($\alpha = \beta + \tau$) (3). The two strands will physically separate after denaturation only if at least one of the strands is broken.

The renaturation of two single-stranded circles that are complementary poses the reverse topological problem. As duplex DNA is formed, the two strands wind around each other, forming the usual DNA helix. However, because in this case the topological linkage is zero, the formation of each helix turn requires that the unrenatured regions compensate by winding up one turn (not necessarily in a regular helical configuration) in the opposite direction. Alternatively, the newly formed duplex region could compensate for the formation of helix turns by becoming negatively superhelical ($\tau = -\beta$). In either case, eventually the loss of free energy (as a decrease in configurational entropy) that accompanies the renaturation of two circles will not be offset by the gain in free energy from base-pair formation and the process will cease. At this point the process will only proceed to completion if one of the strands is at least momentarily broken. In this paper, evidence is presented that the DNA untwisting enzyme (4) can provide the transient single-strand break required to facilitate the complete renaturation of two complementary single-stranded circles.

MATERIALS AND METHODS

General. The sources of most of the reagents and materials have been given elsewhere (4, 5). Hypoxanthine was obtained from Aldrich Chemical. Aminopterin was purchased from Nutritional Biochemicals and 5-bromodeoxyuridine from P-L Biochemicals. The procedure for the purification of the DNA untwisting enzyme from rat liver has been described (6). S1 nuclease was obtained from Miles Laboratories. Alkaline sucrose gradient sedimentation was carried out as previously described (7).

Simian Virus 40 DNA. The procedure for the preparation of ^{3}H-labeled DNA of simian virus 40 (SV40) has been described (5, 7). The specific activity of the DNA was 6.5×10^{3} cpm/μg. SV40 DNA containing 5-bromouracil (BrUra) in place of thymine was prepared as follows. African green monkey BSC-1 cells were infected with SV40 virus as previously described (5). At 48 hr after infection the culture medium was replaced with medium containing 20 μM 5-bromodeoxyuridine, 1 μM thymidine, 3 μM aminopterin, 0.1 mM hypoxanthine, and [^{3}H]thymidine at 2 μCi/ml ($\sim$20 Ci/mmol). The cells were extracted by the method of Hirt (8) on day 7. The BrUra-substituted DNA was purified by the procedure previously described for thymine-containing SV40 DNA (5) and had a specific activity of 1.3×10^{4} cpm/μg. The BrUra substitution was estimated to be approximately 80% from the observed buoyant density increment relative to fully substituted BrUra-DNA (9).

Isolation of Single-Stranded Circles and Linears. The procedure for the introduction of an average of one single-strand break per SV40 DNA molecule using controlled digestion by pancreatic DNase has been described previously (5). The DNA molecules containing single-strand breaks were separated from the unbroken molecules by CsCl/ethidium bromide centrifugation. After removal of the dye by extraction with isopropanol, the samples were dialyzed and layered onto a 5–20% sucrose gradient containing 0.25 M NaOH, 0.75 M NaCl, and 1 mM EDTA. The gradients were centrifuged for 16 hr at 30,000 rpm and 20° in a Beckman SW 41 rotor. The fractions containing the single-stranded circles ($\sim$18S) were pooled and dialyzed against 10 mM Tris·HCl (pH 7.5)/1 mM EDTA at 5°. Similarly, the unit-length linear strands ($\sim$16S) were obtained from the same gradient.

Reaction Conditions for Untwisting Enzyme. The standard reaction conditions were 10 mM Tris·HCl (pH 7.5)/1 mM EDTA/0.17 M NaCl. The enzyme was diluted in 20 mM KPO_4 (pH 7.4)/1 mM EDTA/0.5 mM dithiothreitol/10% (vol/vol) glycerol/bovine serum albumin at 100 μg/ml. Enzyme activity is given in units as previously defined for the fluorometric assay (6).

S1 Nuclease Reactions. Each reaction mixture (0.20 ml) contained, in addition to the DNA to be tested, 30 mM sodium acetate (pH 4.6), 30 mM NaCl, 1 mM $ZnSO_4$, boiled salmon sperm DNA at 30 μg/ml, and 100 units (Miles) of S1 nuclease. The reaction was terminated by the addition of 50 μl of salmon sperm DNA at 2.5 mg/ml and precipitation with an equal

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Abbreviations: SV40, simian virus 40; BrUra, 5-bromouracil.

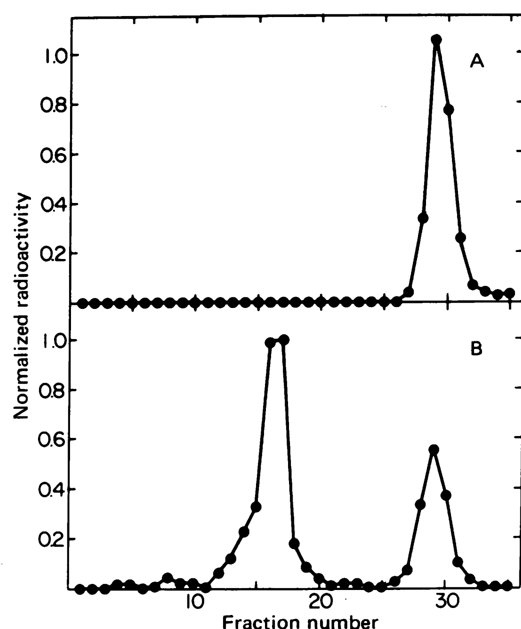

FIG. 1. Detection of interlocked circular strands by sedimentation in alkaline sucrose. Single-stranded circles from SV40 DNA (9.2 μg/ml) were annealed for 15 min at 60° in 10 mM Tris·HCl (pH 7.5)/1 mM EDTA/0.20 M NaCl. One aliquot (*A*) was sedimented directly in an alkaline sucrose gradient while a second aliquot (*B*) was treated with 0.2 units of the DNA untwisting enzyme (final volume 50 μl) for 10 min at 37° under the standard reaction conditions (see *Materials and Methods*). The sedimentation was carried out in a Beckman SW 56 rotor for 60 min at 55,000 rpm and 20°. Sedimentation is from right to left. The peak fraction in *A* contained 418 cpm; the peak fraction in *B* contained 116 cpm.

volume of 10% trichloroacetic acid. The precipitate was removed by centrifugation for 15 min at 8000 × *g* and the acid-soluble radioactivity in the supernatant was determined by counting in a toluene-based scintillation fluid containing Triton X-100.

RESULTS AND DISCUSSION

Formation of interlocked circular strands

Single-stranded circles of SV40 DNA were isolated by alkaline sedimentation of SV40 DNA that had been nicked by pancreatic DNase. Because this nuclease shows no specificity with regard to the strand it breaks, the resulting population of single-strand circles should contain approximately equal proportions of the two complementary strands. When this mixture is subjected to DNA renaturation conditions (0.20 M NaCl, 60°), one expects that pairs of complementary strands will partially renature. However, the two strands cannot become topologically interwound and thus should sediment in alkali as isolated rings (∼18S). The results given in Fig. 1*A* show that this is indeed the case. However, when the annealed mixture was treated with the DNA untwisting enzyme prior to alkaline sucrose gradient analysis (Fig. 1*B*), then a substantial proportion of the circles sedimented near the position of denatured closed circular SV40 DNA (∼53S) (1, 2). We have previously shown that the DNA untwisting enzyme will not seal single-strand breaks generated by pancreatic DNase (4). Thus, the fast sedimenting DNA could not have arisen by ligation of linear strands annealed to single-stranded circles.

The increased rate of sedimentation in alkali could, however, be the consequence of (*i*) a change in the conformation of the single-stranded circles resulting in a lower frictional coefficient

Table 1. Formation of rapidly sedimenting circles in the presence of the DNA untwisting enzyme

DNA	Annealing	DNA untwisting enzyme	% 53S DNA
Single-stranded circles	+	−	<0.1
	−	+	3.4
	+	+	60
Single-stranded linears	−	−	<0.1
	−	+	<0.1
	+	+	<0.1

The annealing and enzyme reactions were carried out as described in the legend to Fig. 1. Sedimentation in alkali was used to quantitate the proportion of rapidly sedimenting DNA in each sample.

or (*ii*) the formation of a higher molecular weight species through the linkage of two or more circular strands. If a conformational change is responsible for the effect, then the data in Table 1 show that the strands must be circular, because no faster sedimenting species were observed when single-stranded linears were similarly treated. In fact, Liu *et al.* (10) have shown that treatment of single-stranded circles of phage fd DNA with *Escherichia coli* ω protein can result in the formation of a faster sedimenting species which appears to be a ring with a knot tied in it. From the data in Table 1, it can also be seen that the DNA strands must be subjected to renaturation conditions prior to treatment with the untwisting enzyme in order to form the fast sedimenting species. The small amount of fast sedimenting DNA formed after treating the circles directly with the untwisting enzyme is probably due to the occurrence of a limited amount of annealing during the handling and storage of the DNA. The requirement for annealing the strands prior to treatment with the enzyme is compatible with either of the hypotheses listed above.

In order to discriminate between these two possibilities, the reaction was carried out using a mixture of thymine-containing and BrUra-substituted circular strands. Both DNA samples were labeled with ^{3}H. The mixture was annealed and treated with DNA untwisting enzyme, and the products were separated by alkaline sedimentation as described above (see Fig. 1*B*). The two peaks from the alkaline sucrose gradient were subsequently analyzed by equilibrium centrifugation in CsCl. Fig. 2*A* shows the results for the slower sedimenting species. As expected, one only sees the heavy BrUra-substituted DNA, and the thymine-containing strands which band slightly denser than the native marker DNA. However, the fast sedimenting fractions from the alkaline sucrose gradient contained in addition a hybrid peak and only a small amount of fully heavy DNA (Fig. 2*B*). Therefore, most of the BrUra-substituted strands are associated with thymine-containing strands. The failure to see more fully heavy DNA is due to the fact that the ratio of thymine to BrUra-containing DNA in the original mixture was 2.2:1. This result, together with the fact that the association is stable in alkali where no base pairs can form, suggests that the fast sedimenting form is composed of two single-stranded circles that have become topologically interlocked. From the sedimentation velocity in alkaline sucrose it is difficult to discern whether the two strands were completely renatured or only partially rewound prior to the sedimentation (11) (see below).

The formation of a DNA species with a density midway between the densities of the fully heavy and the light DNA in the experiment described above shows that most sets of interlocked rings contained just two strands (or, less probably, four

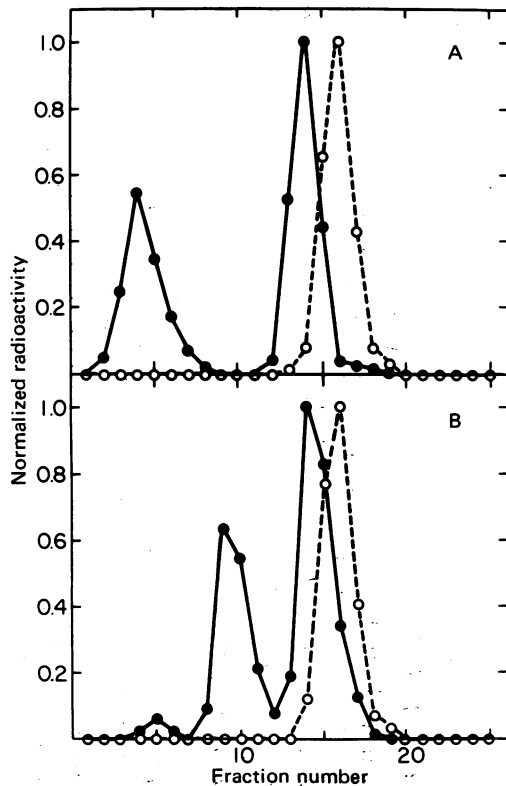

FIG. 2. Formation of hybrids after renaturation of a mixture of BrUra-labeled and light single-stranded circles. A mixture of ^{3}H-labeled thymine-containing single-stranded circles (2.4 μg/ml) and ^{3}H-labeled BrUra-labeled circles (1.1 μg/ml) was annealed for 60 min at 60° under the conditions described for Fig. 1. After treatment with 2.6 units of DNA untwisting enzyme (final volume 0.20 ml) under the standard reaction conditions, the products were sedimented in an alkaline sucrose gradient. (*A*) The pooled fractions from the slower sedimenting species were dialyzed against 10 mM Tris·HCl (pH 7.5)/1 mM EDTA and analyzed by equilibrium centrifugation in a CsCl gradient. Density increases from right to left. The peak fraction for the ^{3}H-labeled DNA (●) contained 109 cpm; the peak fraction for the ^{14}C-labeled marker native SV40 DNA (O) contained 267 cpm. (*B*) The pooled fractions from the rapidly sedimented species were similarly analyzed. The peak fraction for the ^{3}H-labeled DNA (●) contained 116 cpm; the peak fraction for the ^{14}C-labeled marker DNA (O) contained 252 cpm.

strands). Theoretically, higher forms are possible if more than two strands should combine during the annealing step, and one expects this event to become more probable as the DNA concentration is increased. That this may occasionally happen is suggested by the leading shoulder on the fast sedimenting peak in Fig. 1*B*. Consistent with this suggestion is the finding that this shoulder is observed when the annealing is carried out for 15 min at a DNA concentration of 9 μg/ml, but not when the DNA is annealed for the same length of time at 1 μg/ml (data not shown).

Fig. 3 shows a comparison of the kinetics of formation of interlocked circles with the kinetics of renaturation of single-stranded linears under the same conditions. The rate of formation of complexes that yield interlocked rings after treatment with the DNA untwisting enzyme is clearly slower than the rate of renaturation of linears. This may possibly be the result of formation of partially renatured circles that cannot be acted upon by the DNA untwisting enzyme. Presumably the substrate for the untwisting enzyme is a region of double-stranded DNA. However, the extent of duplex formation that occurs in the absence of added enzyme is unknown.

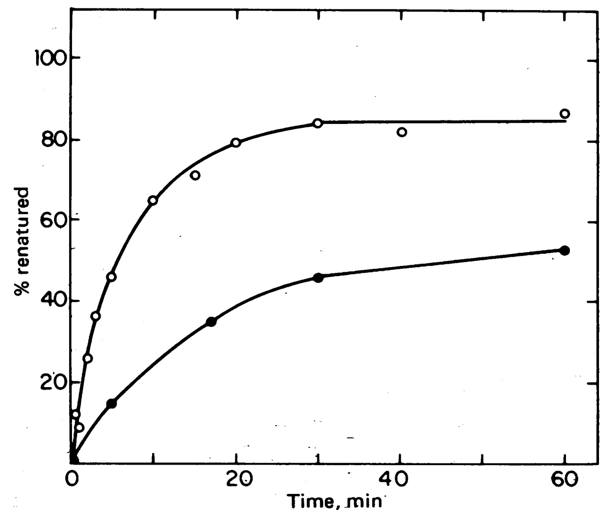

FIG. 3. Kinetics of renaturation of single-stranded circles versus single-stranded linears. The renaturation of linear strands (O) was measured by the resistance of the duplexes to S1 nuclease (see *Materials and Methods*). The renaturation of the circular strands (●) was measured as described for Fig. 1. In both cases the DNAs were annealed at a DNA concentration of 1 μg/ml in 10 mM Tris·HCl (pH 7.5)/1 mM EDTA/0.20 M NaCl at 60° for the indicated times.

In Fig. 3 the extent of formation of interlocked rings approaches 60%. In other experiments this value never exceeded 70%. This limit probably results from the presence of single-stranded linears in the circle preparation. The linears will renature with some of the circles and thereby reduce the number of circles that can participate with other circles in the annealing reaction. In one experiment, the circle preparation was estimated to have 18% linears by sedimentation in alkali. From this value one would predict a maximum of 67% conversion of the circles to interlocked rings. The observed value for this preparation was 67% which agrees with the predicted value.

In preliminary experiments, the parameters affecting the annealing reaction (e.g., ionic strength, temperature) were investigated. To the extent it was tested it was found that the optimum conditions fit well with those found for the renaturation of DNA in general (12, 13). Therefore, this aspect of the problem was not investigated further.

Characterization of product

In order to determine the extent to which the interlocked circles are renatured at the end of the enzyme treatment, the product was banded directly in a CsCl gradient containing ethidium bromide (Fig. 4). If the two strands have become only partially rewound at the end of the reaction, the molecules should be deficient in topological turns and thus be negatively superhelical (3). If the number of negative superhelical turns exceeds the number of turns present in native SV40 DNA (~24) (14), then the ^{3}H-labeled product should band between the ^{14}C-labeled closed circular marker DNA and the nicked form of the same DNA. If the DNA becomes completely rewound, then the product should band slightly denser than native SV40 DNA, at the position of relaxed closed circular DNA (15). As can be seen in Fig. 4, the latter result is obtained. In addition there is a small amount of material banding as a shoulder on the light side of the ^{3}H peak, which may represent molecules that had not become completely rewound.

The fractions indicated by the bar in Fig. 4 were pooled and dialyzed against 10 mM Tris·HCl (pH 7.5)/1 mM EDTA. Alkaline sucrose gradient analysis (not shown) confirmed that this

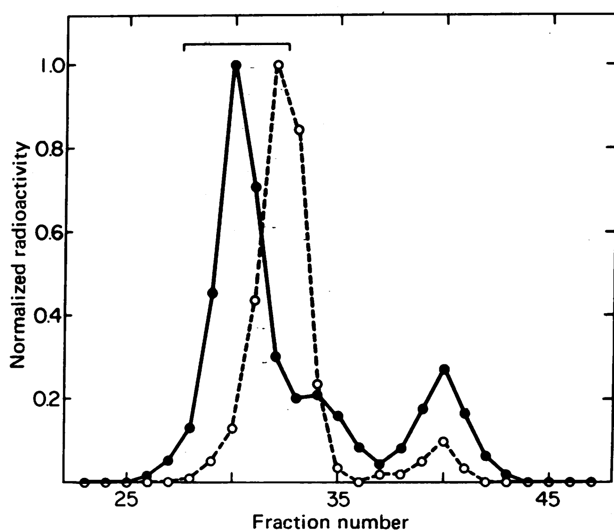

FIG. 4. CsCl/ethidium bromide gradient analysis of the interlocked circular strands. ^{3}H-Labeled single-stranded circles (9.2 μg/ml) were annealed and treated with DNA untwisting enzyme exactly as described for Fig. 1. The products were banded to equilibrium in a CsCl gradient (initial density 1.59 g/cm^3) containing ethidium bromide at 300 μg/ml. The sample was centrifuged for 48 hr at 35,000 rpm and 20° in the SW 56 rotor. An aliquot representing $^1/_{10}$ of each fraction was assayed for radioactivity. Density increases from right to left. The peak fraction for the ^{3}H-labeled DNA (●) contained 203 cpm; the peak fraction for the ^{14}C-labeled marker SV40 DNA (○) contained 115 cpm. The indicated fractions were pooled, the ethidium bromide was extracted with isopropanol, and the sample was dialyzed against 10 mM Tris·HCl (pH 7.5)/1 mM EDTA before further characterization (see *text*).

material represented the interlocked rings (~53S) detected by direct alkaline sedimentation of the products. In addition, the DNA in the pooled fractions was tested for its sensitivity to the single-strand-specific S1 nuclease. Under conditions in which single-stranded linears were digested to 95% acid solubility, none of the label in this DNA was rendered acid soluble by S1 nuclease. Thus, the product of the reaction has all the properties of a double-stranded, relaxed closed circular DNA molecule. This indicates that complete renaturation has occurred. Two single-strand circles thus become topologically linked under the driving force of DNA helix formation. The DNA untwisting enzyme provides the requisite swivel.

Significance

During the process of DNA replication the two parental strands must unwind, and it has been suggested that the DNA untwisting enzyme may provide the swivel that allows strand separation (4). In addition, each newly made daughter strand must eventually pair with and wind around another DNA strand. Because replication is semiconservative, this process no doubt involves the interaction of the new strand with the template strand that directed its synthesis. Nascent strands have free ends, and unless they are very long they may not encounter the topological constraint to winding exhibited by two single-strand circles. It seems unlikely therefore, that the reaction described here is important in this phase of DNA replication.

The process of DNA recombination involves recognition as well as physical interaction between two homologous duplex DNA molecules. Presumably the recognition must involve base pairing between two complementary strands of different duplexes at some stage in the process. Various models have been suggested to account for this interaction (refs. 16 and 17; for reviews see refs. 18 and 19). A feature common to these models is that the recombination process is initiated by the formation of one or more single-strand breaks. Beginning at the site of the break, one of the strands unpairs and subsequently interacts by base pairing with another protruding single strand or with a single-stranded region on the other DNA partner.

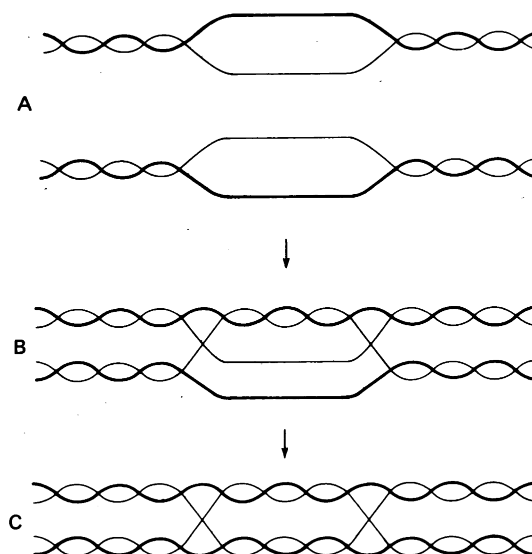

FIG. 5. A model for the initiation of recombination. (*A*) Strand separation at the same point on two DNA molecules. (*B*) Pairing and rewinding of two complementary strands facilitated by the DNA untwisting enzyme. (*C*) Pairing and rewinding of the two remaining complementary strands. See *text* for complete explanation.

The discovery that the renaturation of two single-stranded circles can be facilitated by the DNA untwisting enzyme suggests that recombination could be initiated by strand pairing followed by strand breakage rather than the usual break-and-then-pair scheme. This model is illustrated in Fig. 5. The initial event is the unpairing of the strands at the same region of two different DNA molecules (Fig. 5*A*). Strand separation could be facilitated by single-strand DNA binding proteins (20) or by the action of such proteins as the DNA unwinding enzymes (21–23) or the DNA gyrase (24). The separation of the strands of one DNA molecule is postulated to promote the "invasion" of a single-stranded segment from another molecule in the model proposed by Meselson and Radding (17). Moreover, Holloman and Radding (25) have recently shown that negatively superhelical DNA will recombine *in vivo* much more effectively than relaxed closed circles. In addition, superhelical, but not relaxed closed circular DNA will, under the right conditions, take up a single-stranded segment of DNA *in vitro* to form a D-loop structure (26).

The second step in the scheme presented in Fig. 5 is the pairing of two complementary strands from different DNA molecules. The winding of the helix that accompanies the pairing process would be facilitated by cycles of nicking and closing by the DNA untwisting enzyme to generate the structure shown in Fig. 5*B*. The two remaining unpaired strands shown in Fig. 5*B* are also complementary and could similarly wind up, forming a variant (27) of the Holliday (16) recombination intermediate (Fig. 5*C*). The resolution of this intermediate to yield recombinant structures and/or gene conversion has been previously described by others in the models cited above.

This model is subject to the objection that the initial unpairing of two DNA molecules at the same point would appear to be an improbable event. However, there is no evidence to exclude

the possibility that in some cases recombination proteins recognize certain structures or sequences in the DNA and that such regions are preferentially opened to initiate the process. In fact, in some systems evidence has been presented that "hot spots" for recombination do exist (28). Alternatively, the probability of intermolecular pairing may be increased if longer regions of single-stranded DNA exist intracellularly during DNA replication or perhaps due to the combined action of a DNA gyrase and a DNA unwinding enzyme.

Finally, it should be noted that the reaction described here, which is mediated by the untwisting enzyme, allows for the construction of an unusual kind of heteroduplex molecule for electron microscopic analysis. Normally, the region of single-stranded DNA in a deletion loop or substitution loop will not renature with a third homologous single-strand if the third strand is long relative to the length of the loop. This is because the winding of the helix requires that one end of the long strand find its way through the opening of the loop once for every 10 bases that pair. Treatment with the DNA untwisting enzyme should resolve such tangled structures to yield interpretable heteroduplexes involving three or possibly more strands.

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