Transcription-driven site-specific DNA recombination *in vitro*

(supercoiling/topological coupling/RNA polymerase)

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ABSTRACT   Transcription of a topologically relaxed, circular DNA triggers recombination between two directly repeated *res* sites by γδ resolvase *in vitro*. This activation of recombination depends on the *res* site-to-site distance and the orientation of sites with respect to the direction of RNA polymerase tracking. In addition to functioning as a site-specific recombinase, γδ resolvase acts as a site-specific topoisomerase and increases the topological linking number of templates during transcription. The data suggest that the link between transcription and recombination could be negative DNA supercoiling that transiently builds up on a relatively short DNA segment in the wake of an advancing RNA polymerase. Surprisingly, transcription-driven recombination is not inhibited by the presence of large amounts of eukaryotic topoisomerase type I, indicating that site-specific recombination can override relaxation by diffusible topoisomerases. This *in vitro* system might therefore serve as a model for some transcription-directed recombination events observed *in vivo*.

There is growing evidence from both *in vivo* and *in vitro* studies that proteins tracking along the DNA double helix can induce positive supercoils ahead of, and negative supercoils behind, the protein complex (1–7). This induction is a consequence of the helical structure of double-stranded DNA and can occur whenever an advancing protein complex is prevented from rotating around the helix axis. Such a protein "anchoring" is possible, for example, during transcription when the frictional drag of a growing RNA chain or an association of a transcript with cellular membranes prevents the attached polymerase from rotating (8, 9). In this situation, the transcribed template becomes topologically overwound in front of the protein complex and underwound behind it while the double helix axis rotates about itself (reviewed in refs. 10 and 11). However, the extent of transcription-induced DNA supercoiling and the local concentration of supercoils remain elusive.

Transcription processes can activate homologous as well as site-specific DNA recombination *in vivo* (12–16). The molecular mechanism(s) of activation, however, is obscure. One possibility is that transcription transiently alters the chromatin structure so that a recombinase gains access to its substrate. Another possibility is that topological consequences of transcription—e.g., transcription-induced DNA supercoiling—are directly involved in recombination processes (17).

I was interested in examining the latter possibility in an *in vitro* model system. For this purpose, the resolution system of bacterial transposon γδ/Tn3 is ideal because (*a*) it has been reconstituted in a simple buffer system with purified recombinase and DNA substrates containing two recombination (*res*) sites as direct repeats and (*b*) it requires negative DNA supercoiling for fast and efficient recombination (reviewed in ref. 18).

Two roles for negative supercoiling in resolution by γδ/Tn3 resolvase have been established. (*i*) Site synapsis between two resolvase-bound *res* sites is favored on a supercoiled substrate because a synaptic complex traps three negative plectonemic supercoils (19, 20). In addition, the probability that two *res* sites will be juxtaposed on a circular substrate appears to be significantly enhanced by supercoiling (21–23). (*ii*) During strand exchange, negative supercoiling may act as a driving force after strand cleavage to rotate DNA-linked resolvase protomers within a synaptic complex (24, 25). In a reaction that requires site synapsis but is uncoupled from recombination, γδ resolvase can also act as a site-specific DNA topoisomerase type I and relieve negative superhelical stress (26).

To test whether recombination by γδ resolvase is activated by localized, transcription-induced negative supercoiling, I combined the γδ system with transcription by phage T7 encoded RNA polymerase (T7 RNAP). With topologically relaxed, circular templates that lack specific stop sites for T7 RNAP and topoisomerases as probes, it was demonstrated that transcription generates positive and negative supercoils within a few minutes (4, 27). Furthermore, template supercoiling is absolutely dependent on both ongoing transcription and the generation of sizable nascent RNA chains.

The results of the present study demonstrate an example of a functional coupling through DNA topology between two basic biological processes—transcription and site-specific DNA recombination.

MATERIALS AND METHODS

Enzymes and DNA. γδ resolvase was purified as described (28). Purified calf thymus topoisomerase type I was a kind gift of A. Richter (University of Konstanz). T7 RNAP and restriction enzymes were purchased from New England Biolabs. RNase was supplied by Serva, and proteinase K was from Roth (Karlsruhe, Germany).

Templates were constructed from pTZ18R (Pharmacia), which contains a consensus promoter sequence for T7 RNAP upstream of a polylinker sequence. pTZres2 was constructed by inserting a 4.6-kb *Eco*RI–*Bgl* II fragment of pAO880 that contained two directly repeated *res* sites (29) into pTZ18R. A third *res* site was subsequently cloned into the unique *Eco*RI site to yield pT8res3. The orientation of *res* sites with respect to each other was determined by recombination assays. pT14res3 was constructed by cloning a 6.2-kb fragment of pAB3 (20) into pTZres2. pT10res2 was constructed by cloning *Bam*HI-linearized pAO880 into the single *Bam*HI site of pTZ18R. The orientation of the insert was determined by restriction analyses. All plasmid substrates were isolated by standard procedures from *Escherichia coli* strain DH1.

Reactions. Relaxation of templates was achieved by incubating 1 μg of supercoiled plasmid DNA with 10 units of calf thymus topoisomerase in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/150 mM NaCl/0.005% bovine serum albumin for 1 hr

Abbreviation: RNAP, RNA polymerase.

at 37°C. One unit is defined as the amount of enzyme required to relax 0.5 μg of DNA within 30 min under the above cited conditions. Reactions were stopped by NaDodSO₄ (0.5%, wt/vol), followed by phenol/chloroform extraction and ethanol precipitation. DNA was suspended in TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA). Mixtures of reference topoisomers for two-dimensional gel electrophoresis were generated as described (30).

Transcription and recombination were assayed in 20 mM Tris·HCl, pH 7.5/70 mM NaCl/10 mM MgCl₂/1 mM dithiothreitol/0.01% bovine serum albumin/2 mM spermidine/1 mM each NTP (Pharmacia) at 37°C for the times indicated. Reaction mixtures (30 μl) contained 5 nM template DNA, 70 nM T7 RNAP, and 800 nM γδ resolvase. Reactions were terminated by NaDodSO₄ (0.5%, wt/vol) and EDTA (25 mM), followed by RNase treatment (20 μg) at 37°C for 30 min, and proteinase K treatment (10 μg) at 55°C for 30 min. After phenol/chloroform extraction and ethanol precipitation, DNA was digested with either *Bgl* II or *Ava* I.

To measure transcription rates (see Fig. 3), RNA was labeled by adding 1 μCi (37 kBq) of [α-³²P]CTP (Amersham) without a reduction in the amount of unlabeled CTP. Reactions were terminated by the addition of 100 volumes of ice-cold 10% (wt/vol) trichloroacetic acid, and acid-insoluble ³²P was determined as described (6).

Gel Electrophoresis. DNA was displayed in 0.8% agarose gels which were run in TAE buffer (40 mM Tris·acetate, pH 8.1/2 mM EDTA). Two-dimensional gel electrophoresis was performed in 0.8% agarose gels with TBE buffer (90 mM Tris·borate, pH 8.3/2.5 mM EDTA) containing 2 μM chloroquine (Sigma) during electrophoresis in the second dimension. DNA was visualized by staining with ethidium bromide and photographed onto Polaroid 107 film.

DNA Quantification. DNA concentrations were determined in a DNA fluorometer (Hoefer Scientific Instruments, San Francisco) by using Hoechst dye 33258. DNA quantification from photographs was achieved with Scan Jet Plus (Hewlett–Packard) linked to a Macintosh IIcx (Apple). To ensure that the response of photographic band intensities was linearly proportional to the amount of recombination products, linearized pT8res3 was used as a standard. The recombination efficiency was evaluated from the total amount of DNA per assay, which was normalized to 100% recombination.

RESULTS

Activation of Site-Specific Recombination by Transcription. Four templates were utilized in this study (Fig. 1). Each contained a promoter consensus sequence for T7 RNAP and either two or three *res* sites as direct repeats but in different positions and orientations with respect to the T7 promoter. Reaction conditions were established that allowed both efficient transcription and site-specific recombination. A control reaction in the absence of transcription with negatively supercoiled pT8res3 showed that >60% of the DNA was recombined within 30 min (Fig. 2A, lane M). Furthermore, all three possible combinations of *res* site pairing resulted in recombination. The preference for strand exchange between sites 1 and 3 is best explained by two recombination events on the same substrate, since the dimeric negatively supercoiled catenanes that result from recombination between either *res* 1 and 2 or *res* 2 and 3 still have two *res* sites located on one of the two interlocked rings in cis. These catenanes are substrates for a second recombination event.

To test for an activation of recombination by transcription, relaxed pT8res3 was incubated with resolvase in the presence or absence of T7 RNAP. While recombination was not detectable when transcription was prohibited (Fig. 2A, lane 1), recombination products accumulated within 7 min in the
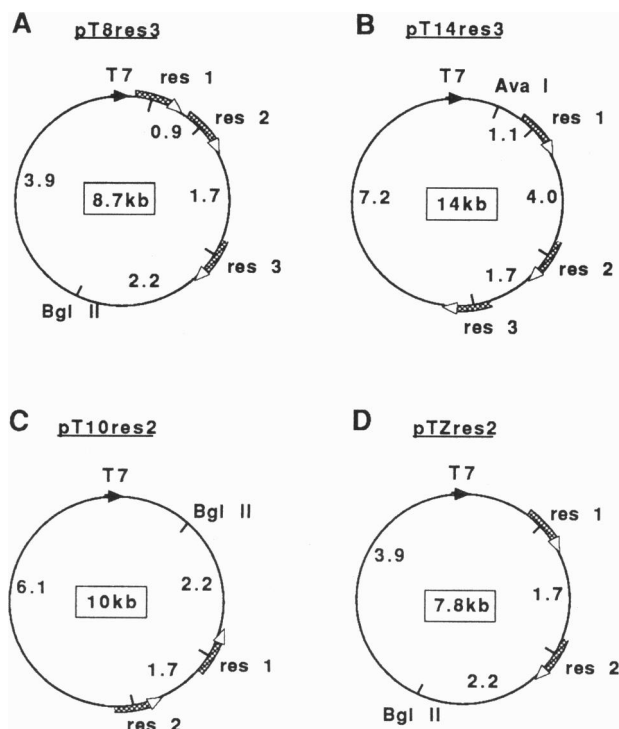

FIG. 1. Substrates for transcription-induced recombination. Each substrate contains a promoter consensus sequence for bacteriophage T7 RNAP (black arrowhead) and either two or three recombination sites for γδ resolvase as direct repeats (arrows with open heads, denoted by *res*). The numbering of *res* sites starts at the promoter and follows the direction of transcription. The numbers between the landmarks on the circular templates indicate the distances in kilobase pairs.

presence of T7 RNAP (lanes 2–6). Compared with the control reaction, transcription-activated recombination was precluded between sites 1 and 3.

In an attempt to increase the yields of recombination products, the recombinase was added at different times before or after transcriptional initiation (Fig. 2B). Recombination was repressed if resolvase was prebound (lane 2), or if T7 RNAP was added for 5 or 15 min before resolvase (lanes 4 and 5, respectively). Cleaved intermediates, the result of abortive recombination events (24), showed the same dependency on the sequence of addition of T7 RNAP and resolvase. Hence, transcription-induced recombination appears most effective when the recombinase is added simultaneously with T7 RNAP to the template (lane 3). I found that under these conditions, resolvase typically converted about 5% of substrate DNA into recombination products.

The low yields of recombination products and the inhibition of recombination by prebound resolvase could indicate that transcription is affected by DNA-bound resolvase. This is demonstrated in Fig. 3. While transcription was measurable for at least 20 min in the absence of resolvase, the rate of transcription dropped by a factor of ≈5 when T7 RNAP and resolvase were added simultaneously. Preincubation of templates with resolvase affected transcription even more. Thus, the small amounts of recombination products may, at least in part, be explained by transcriptional inhibition due to DNA-bound resolvase.

Previous studies revealed that transcription-induced supercoiling *in vitro* depends upon the generation of long nascent RNA chains. Furthermore, topoisomerases can remove superhelical stress built up before and behind an advancing RNA polymerase (3, 4). I anticipated that, if transcription-induced DNA supercoiling were indeed in-
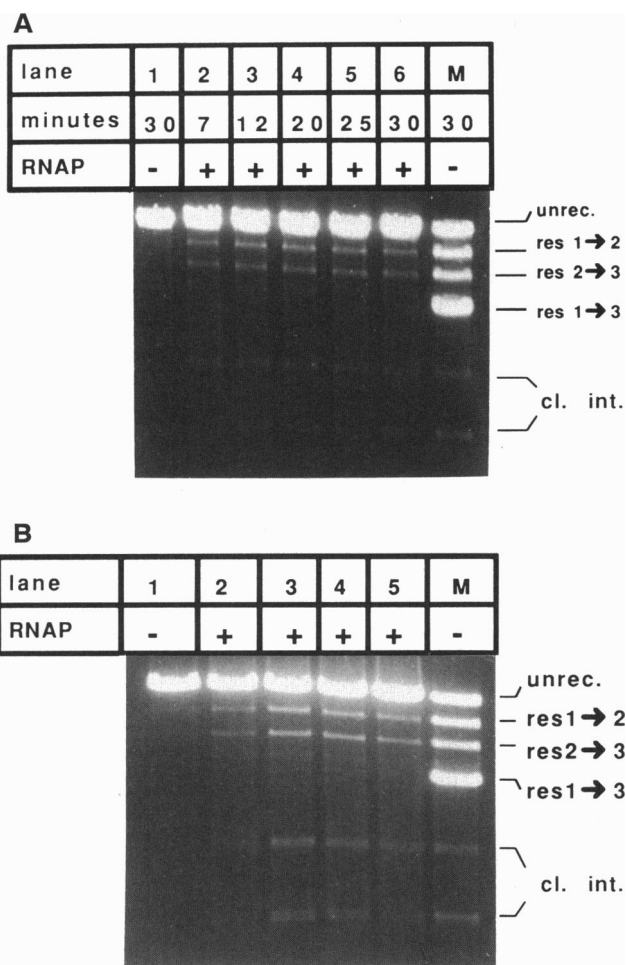
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A

B

FIG. 2. (*A*) Activation of recombination by transcription. T7 RNAP was added simultaneously with γδ resolvase to topologically relaxed pT8res3 and incubated at 37°C for the times indicated (lanes 2–6). Lane 1, T7 RNAP was omitted. Lane M, recombination of supercoiled pT8res3. Recombination was assayed after digestion with *Bgl* II restriction endonuclease. unrec., Unrecombined pT8res3; res 1 → 2 (etc.), recombination between res 1 and res 2 (etc.); cl. int., cleaved intermediates. (*B*) Sequence of resolvase addition. Resolvase was either preincubated with pT8res3 for 15 min at 37°C (lane 2), added simultaneously with T7 RNAP (lane 3), or added 5 and 15 min after transcription was initiated (lanes 4 and 5, respectively). In each case, recombination was assayed 15 min after addition of the second component. Lane 1, incubation without T7 RNAP. Lane M, recombination of supercoiled pT8res3.

volved in recombination, the presence of either RNase or eukaryotic topoisomerase type I would interfere with recombination. Recombination was completely suppressed when RNase was added (Fig. 4, lane 5). Surprisingly, the presence of even large amounts of topoisomerases did not interfere with, but instead stimulated, both recombination and DNA strand cleavage about 2-fold (compare lanes 2 and 4; data not shown).

Activation of Recombination Depends on the Distance Between Sites and on Their Orientation with Respect to Polymerase Tracking. One interpretation of the results obtained so far is that the distance between *res* sites might be important for an activation of recombination by transcription. While recombination between sites separated by 0.9 and 1.7 kb was equally efficient, recombination on pT8res3 was not detected between *res* sites 1 and 3, which are separated by 2.6 or 6.1 kb, respectively (see Fig. 1*A*). To further test the impact of the site-to-site distance on the reaction, transcription-induced recombination was assayed on pT14res3. This sub-
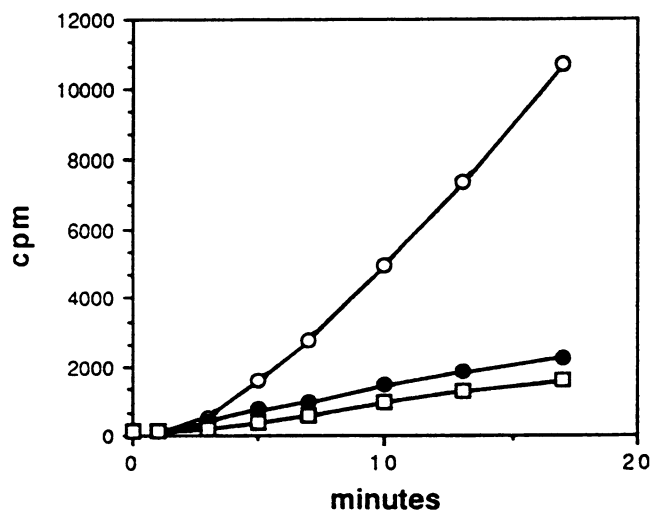

FIG. 3. Transcription rates in the presence and absence of resolvase. Transcripts were labeled by incorporation of [^{32}P]CMP, and acid-insoluble ^{32}P was determined at the times indicated. ○, Transcription in the absence of resolvase; ●, resolvase added simultaneously with T7 RNAP; □, resolvase prebound to pT8res3 for 15 min before the onset of transcription.

strate also contains three *res* sites as direct repeats, but now in a different spatial arrangement (Fig. 1*B*). Recombination required transcription and occurred between sites 2 and 3 (Fig. 5). Strand exchange between *res* 1 and *res* 2, however, was not observed, indicating that the distance between adjacent sites is important and reaches a critical value between 1.7 and 4.0 kb on pTZ14res3.

To investigate whether the orientation of *res* sites with respect to the direction of transcription might be important, I tested a third substrate, pT10res2, which contains two directly repeated *res* sites inversely oriented to the direction of transcription (Fig. 1*C*). In this case, neither recombination products nor cleaved intermediates were detectable by ethidium bromide staining (data not shown). In contrast, transcription-induced recombination on pTZres2, which contains two *res* sites separated by 1.7 kb and oriented in the same
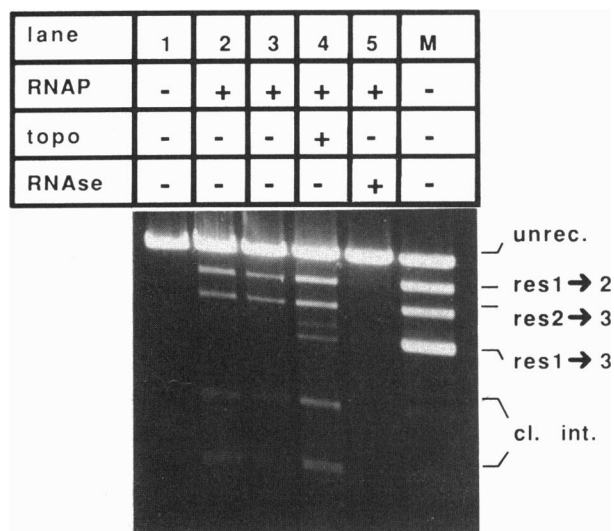

FIG. 4. Characterization of transcription-induced recombination. Recombination was assayed after incubation with T7 RNAP for 20 min in the presence of either topoisomerase type I (20 units, lane 4) or RNase (10 μg, lane 5). Lane 1, T7 RNAP was omitted. Lanes 2 and 3, incubation of resolvase with T7 RNAP for 20 and 10 min, respectively. Lane M, recombination of supercoiled pT8res3.

lane	1	2	3	M
RNAP	−	+	+	−

— unrec.
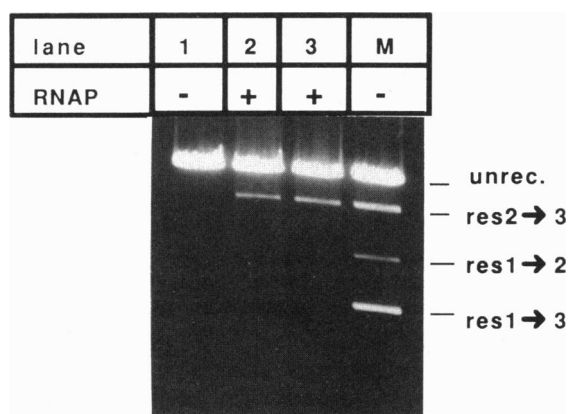
— res2 → 3

— res1 → 2

— res1 → 3

FIG. 5.   Distance dependency of transcription-induced recombination. Topologically relaxed pT14res3 was incubated with T7 RNAP and resolvase for 10 and 20 min (lanes 2 and 3, respectively). Lane 1, incubation for 20 min without T7 RNAP. Lane M, recombination on supercoiled pT14res3.

direction as transcription proceeds (Fig. 1*D*), was comparable to that observed with pT14res3 (data not shown). Hence, successful activation of recombination depends on both the distance between two adjacent sites and their orientation with respect to polymerase tracking. Furthermore, it is clear that the presence of a third *res* site on the transcribed template is not required for an activation of recombination.

Transcription-Induced Site-Specific Relaxation by Resolvase Yields Positively Supercoiled Templates. Resolvase can function as a site-specific type I topoisomerase and release negative superhelical stress of a substrate in a reaction uncoupled from recombination (26). I tested whether resolvase acts as a topoisomerase to alter the topological linking number of pT8res3 upon transcription. The template topology was not changed when transcription was prohibited (Fig. 6, lane 1) or when transcription occurred in the absence of resolvase (data not shown). In contrast, when resolvase was present during transcription, a ladder of topoisomers was generated (Fig. 6, lanes 2–4). The changes in template linking number upon transcription were not detectable with pT10res2 (lanes 5–8). The latter result concurs with the observation that transcription cannot activate recombination

when sites are oriented opposite to the direction of transcription.

Two-dimensional gel electrophoresis was employed to determine whether the topoisomers generated by resolvase during transcription are positively or negatively supercoiled. A sample analogous to that in Fig. 6, lane 4, was analyzed along with mixtures of *in vitro* generated reference topoisomers. Fig. 7 presents an example of such an analysis (*Upper*) and a description of the various DNA species (*Lower*). The two spots denoted by *a* consist of the nicked form of pT8res3, while the streak of spots denoted by *c* consists of negatively supercoiled topoisomers and that denoted by *b* is made up of moderately positively supercoiled DNA. This streak was extended when pT8res3 was transcribed in the presence of resolvase, revealing that positively supercoiled topoisomers were generated (arc to the right, denoted by ′*b*).

DISCUSSION

The main result of this study is that transcription can activate site-specific recombination *in vitro*. Two lines of evidence suggest that this activation is due to transcription-driven negative supercoiling. First, fast and efficient recombination by γδ resolvase requires negative supercoiling of the substrate. Consequently, topologically relaxed templates are not recombined when either T7 RNAP or rNTPs are omitted, indicating a requirement for ongoing transcription. Second, and more important, transcription of pT8res3 in the presence of resolvase yields positively supercoiled topoisomers, an observation which is best explained by selective relaxation of transcription-induced negative supercoils in a resolvase-catalyzed topoisomerase reaction that requires *res* site synapsis but is uncoupled from recombination (26).

The presence of a large amount of RNase prevents an activation of recombination by transcription. One possible explanation for this observation is that in order to induce template supercoiling, long nascent RNA chains are needed to "anchor" a polymerase during transcription (4, 8). However, because of the large amount of RNase, a direct inter-

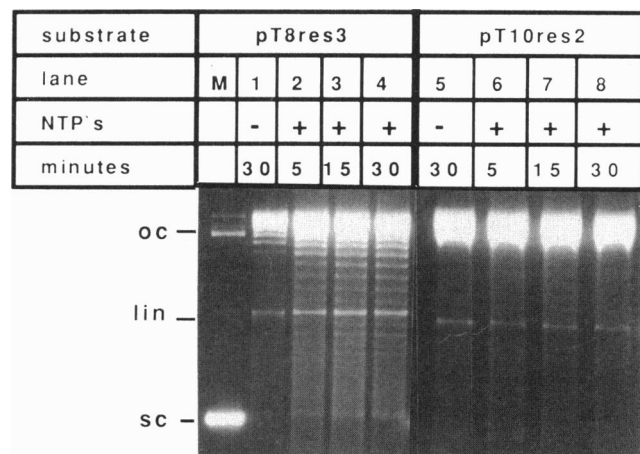| substrate		pT8res3				pT10res2			
lane	M	1	2	3	4	5	6	7	8
NTP′s		−	+	+	+	−	+	+	+
minutes		30	5	15	30	30	5	15	30

oc —

lin —

sc —

FIG. 6.   Transcription-induced topoisomerase activity of resolvase. Topologically relaxed pT8res3 (lanes 1–4) or pT10res2 (lanes 5–8) was incubated with both resolvase and T7 RNAP for the times indicated. Topoisomers were displayed by agarose gel electrophoresis. Samples analyzed in lanes 1 and 5 lacked rNTPs to prevent transcription. Lane M, supercoiled pT8res3. oc, Open circular; lin, linear; sc, supercoiled.

2nd →

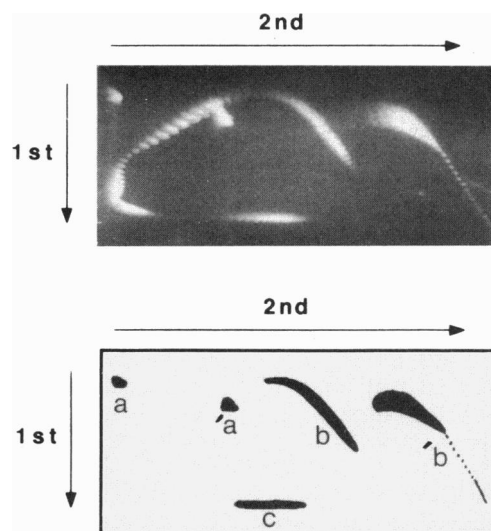1st ↓

2nd →

1st ↓

a   ′*a*   *b*   ′*b*

c

FIG. 7.   Two-dimensional analysis of transcription-generated topoisomers. A sample such as that analyzed in Fig. 6, lane 4, was resolved by two-dimensional gel electrophoresis (right arc) along with mixtures of *in vitro* generated reference topoisomers (left arc). The directions of DNA migration during the first and second dimensions are indicated. (*Upper*) Photograph of a gel after ethidium bromide staining. (*Lower*) Interpretation of the analysis, which is explained in detail in the text. DNA species present in the resolvase reaction are marked by primed letters (′*a* and ′*b*).

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ference with transcription or recombination via nonspecific DNA binding of the nuclease is not precluded.

While an activation of recombination by transcription is observable, it is also clear that this activation is very inefficient and occurs on only about 5% of templates. Several explanations could account for this low level of activation: (*i*) only a fraction of templates might be continuously transcribed in the presence of resolvase (Fig. 3); (*ii*) supercoiling by transcription might be inefficient because in this *in vitro* system, an advancing polymerase might not be perfectly anchored and prevented from rotating around the double-helix axis; (*iii*) the system employs circular templates, and the cancellation of positive and negative supercoils by diffusion might therefore become an important parameter in determining the local superhelical density.

If recombination is a consequence of localized negative superhelical stress, it is anticipated that the presence of topoisomerases during transcription would interfere with recombination. Surprisingly, even excess eukaryotic topoisomerase type I cannot suppress recombination. As discussed by Wang *et al.* (17), a scenario is possible in which a recombinase utilizes negative supercoiling before a diffusible topoisomerase is capable of releasing it. The data of the present study suggest that a site-specific recombinase can indeed have a kinetic advantage over a topoisomerase, which interacts primarily with DNA crossovers (31). This advantage may be due in part to the recombinase's presence at a *res* site at the time when a dynamic DNA tertiary structure recognizable by a topoisomerase is formed. Clearly, further experimentation is required to provide direct evidence for such a scenario.

The "twin-supercoiled-domain" model of transcription suggests that a gradient of positive supercoils in front of, and negative supercoils behind, a T7 RNAP is transiently built up on the template (8). The γδ system can be used to test this aspect of the model because both site synapsis and DNA strand transfer are promoted by negative supercoiling. The data indicate that an activation of recombination by transcription depends on the distance between *res* sites, reaching a critical value between 1.7 and 4.0 kb on pT14res3, and between 1.7 and 2.6 kb on pT8res3. One interpretation of this finding is that at some stage during transcription, sufficient negative superhelical stress to promote recombination is transiently present on a relatively short DNA segment behind an advancing polymerase. The actual length of a segment under superhelical stress, however, might depend on a variety of parameters, such as the size of a circular template, the rate of RNAP tracking, and the rate of supercoil diffusion.

Why is recombination/relaxation by resolvase activated only when *res* sites are oriented in the same direction as a T7 RNAP tracks along the template? In this case, it is likely that transcription-driven negative supercoiling fulfills two functional roles in γδ recombination: facilitating site synapsis and energetically driving strand exchange. If *res* sites are inversely oriented with respect to polymerase tracking, the results, in consideration with the current model of γδ recombination (18), suggest that transcription-driven negative supercoiling in the wake of a polymerase would act mainly on the accessory resolvase binding sites, which are apparently not directly involved in the strand exchange process. Thus, negative supercoiling would not be available as a driving force to promote strand exchange between the crossover regions of paired *res* sites. In addition, it is conceivable that because of the circular nature of templates, positive supercoiling in front of a polymerase might, in this case, interfere with DNA strand transfer.

Conclusion. Previous studies revealed that certain recombination processes observed *in vivo* correlate with transcription of the respective DNA segment. Elevated recombination rates between directly repeated sequences of the *GAL10* gene

in *Saccharomyces cerevisiae*, for example, correlate with gene expression (12). Additionally, site-specific immunoglobulin heavy-chain switching in higher eukaryotes appears to be induced by the developmentally regulated expression of unrearranged gene segments (13, 14). Finally, homologous recombination on yeast rDNA was shown to depend on transcription (15). In the latter case, an involvement of DNA topology was suggested by the results of Kim and Wang (16), who used yeast DNA topoisomerase double mutants. The present study indicates that topological consequences of transcription processes might indeed play an important functional role in recombination or perhaps other DNA transactions, such as the control of gene expression *in vivo* (32).

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