MOLECULAR AND CELLULAR BIOLOGY, Sept. 1994, p. 6097–6106
0270-7306/94/$04.00+0

Vol. 14, No. 9

A Role for RNA Synthesis in Homologous Pairing Events

HIDEHITO KOTANI AND ERIC B. KMIEC*

Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received 30 March 1994/Returned for modification 17 May 1994/Accepted 1 June 1994

The relationship between RNA synthesis and homologous pairing in vitro, catalyzed by RecA protein, was examined by using an established strand transfer assay system. When a short DNA duplex is mixed with single-stranded circles, RecA protein promotes the transfer of the minus strand of the duplex onto the complementary region of the plus-strand circle, with the displacement of the plus strand of the duplex. However, if minus-strand RNA is synthesized from the duplex pairing partner, joint molecules containing the RNA transcript, the plus strand of the DNA duplex, and the plus-strand circle are also observed to form. This reaction, which is dependent on RNA polymerase, sequence homology, and RecA protein, produces a joint molecule that can be dissolved by treatment with RNase H but not RNase A. Under these reaction conditions, product molecules form even when the length of shared homology between duplex and circle is reduced to 15 bp.

Among the most important phases of the recombination process is the homologous alignment of DNA molecules. The formation of this structure is a requirement for progression through the exchange of genetic material based on sequence homology. Once alignment has occurred, heteroduplexed molecules are created and subsequent resolution completes the recombination cycle. Much of the information about homologous pairing has been gathered from studies on the action of the *Escherichia coli* RecA protein (see reviews, see references 3 and 21). This remarkable 37,842-Da protein promotes the homologous pairing of a wide variety of DNA substrates, including duplex linear DNA and single-stranded circles. This duo has been used extensively in the in vitro characterization of homologous recombination, and the results of these experiments contributed to the identification of certain stages within the genetic exchange process.

One of the reaction parameters surrounding the duplex by circle pairing reaction is the length of homology shared by the two molecules. In recombination assays in which RecA protein acted as the recombinase, the lower limit of complementarity was originally defined to lie between 30 and 151 bp (6). The lower range was established because 30 bp of homology was insufficient to facilitate pairing even when reaction conditions that approached V_{max} were used. Hence, only stable reaction intermediates are formed when the length of homology is this low. Recently, Hsieh et al. (8) reported that RecA protein could catalyze the formation of low levels of joint molecules when there was 38 bp of homology between pairing partners. This same group extended this analysis by using a nonhydrolyzable ATP analog, 5′-[γ-thio]ATP, in pairing reactions (9). These workers found that stable joint molecules were formed when there is as little as 26 bases of homology between the DNA substrates. Furthermore, synaptic complexes which require the presence of RecA protein for stability were formed when 8 or 15 bases of shared homology was present.

We have been examining the process of homologous pairing on chromatin templates by using the duplex DNA–single-stranded circle pairing reaction. Our data indicate that a positioned nucleosome inhibits strand transfer by reducing the accessibility of RecA for the homologous regions within the duplex (14, 15). Subsequently, we demonstrated that transcription through the chromatin template destabilizes the nucleosome, facilitating RecA-promoted joint molecule formation. During the course of our preliminary studies on DNA templates devoid of nucleosomes, we detected the RNA transcript itself in the products of the strand transfer reaction. In some of these reactions, the positive strand of the duplex molecule was transferred in conjunction with the RNA transcript, forming a complex that consisted of plus-strand linear duplex DNA, minus-strand RNA, and plus-strand DNA circle. The creation of this complex was found to be dependent on RecA protein, single-stranded circles, minus-strand RNA, and DNA homology.

In this report, we focus on the length of DNA homology required to facilitate the (co)transfer of the positive-strand of the naked DNA duplex onto the single-stranded circle, RNA-mediated complex formation under reaction conditions that include ATP. In agreement with Gonda and Radding (5, 6) and Hsieh et al. (8), we find that a lower limit of DNA homology exists for the minus-strand transfer onto the plus-strand circle by the action of RecA protein. This barrier approaches 72 bases of homology under our reaction conditions. Plus-strand transfer which is dependent on RNA synthesis, however, occurs when the region of homology is as low as 15 bp.

MATERIALS AND METHODS

DNA templates and proteins. The 224-bp switch region ($S_{\gamma 1}$) of the γ constant region gene was a kind gift from Y. Chen (Temple University). This fragment was cloned into the expression vector pSP72 (Promega Biotec) via the *Pst-Bam*HI compatible sites. Such an arrangement places the fragment 49 bases away from a T7 RNA polymerase promoter. A construct containing this fragment and the T7 promoter was isolated by a *Pst*I-*Hpa*I double digestion, producing a 308-bp duplex DNA fragment. Radiolabeling of either the negative or positive strand was accomplished by the action of T4 polynucleotide kinase (U.S. Biochemicals). The 5′ end of the *Pst*I (recessed) or *Hpa*I (blunt) end was radiolabeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. To accomplish the labeling of only one strand, the plasmid was cleaved first with either *Pst*I or *Hpa*I, radiolabeled, and then recut with the other restriction enzyme.

* Corresponding author. Mailing address: Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, 233 S. 10th St., Philadelphia, PA 19107.

Because of the lower labeling efficiency expected from these two types of termini, an excess of T4 polynucleotide kinase (10 U/pmol of termini) was used. M13 mp18 phage DNA containing the 239 bases of homology was created by inserting the *Pst*I-*Bam*HI fragment with M13 mp18 replicative form I DNA at the compatible sites. M13 phage DNA, containing the $S_{\gamma 1}$ fragment described above, was purified and harbors 239 bases of homology with the duplex pairing partner. Single-stranded phage DNA was purified as described by Cunningham et al. (3).

For experiments addressing the homology requirements, the same parent clone as described above was used, with the following modifications. An *Apa*LI and *Pst*I digest of the parent clone produces a duplex containing 530 bp of nonhomologous sequence, 3′ relative to the T7 promoter element, and 239 bases of homologous sequence. Reduced lengths of homology were created at the 5′ end of this construct by digestion with *Dde*I (149 bp), *Bst*EII (72 bp), or *Bam*HI (21 bp). (The numbers in parentheses indicate the amount of homology to the circle remaining in each duplex.) For the duplex containing 15 bp of homology, a *Sac*I-*Nde*I fragment from plasmid pSPT19 (Boehringer Mannheim) was cloned into plasmid pSPT18. The duplex template was then extracted by *Pst*I-*Nde*I digestion, producing a molecule with 15 bp of homology at the 5′ end and 270 bases of nonhomology. This is the only *Pst*I site in this plasmid, and the 5′ recessed terminus was radiolabeled prior to digestion with *Nde*I. M13 mp18 phage DNA was used as the single-stranded pairing partner only for this reaction.

RecA protein and single-stranded-DNA-binding protein (SSB) were purchased from U.S. Biochemicals. T7 RNA polymerase was purchased from Boehringer Mannheim, and all nucleoside triphosphates (NTPs) were purchased from Pharmacia. Plasmid DNA was purified by organic extraction and was passed through two CsCl gradients prior to restriction enzyme digestion. RNase H and RNase A were purchased from Boehringer Mannheim and used according to the manufacturer's suggestions.

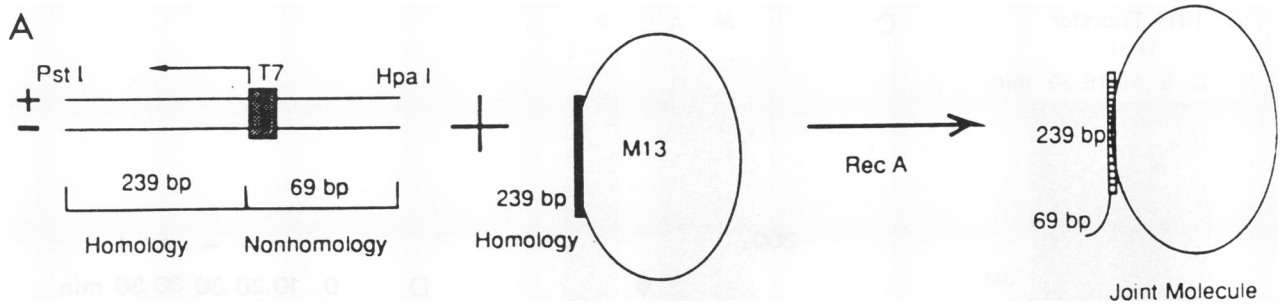
Strand transfer and transcription assays. Reaction mixtures (20 μl) containing 0.25 ng of ^{32}P-labeled duplex fragments, 12.5 ng of viral single-stranded circular DNA, 1 μg or indicated levels of RecA protein, and 0.1 μg of *E. coli* SSB in 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–3 mM ATP–1 mM dithiothreitol were incubated at 37°C for various times. We have found that the use of an ATP-regenerating system commonly found in RecA incubations interferes with the transcriptional activity of T7 RNA polymerase. To circumvent this problem we have used higher concentrations of ATP (3 mM). The reactions were terminated by the addition of sodium dodecyl sulfate (SDS; 0.1%), and the mixtures were incubated at 37°C for 60 min with proteinase K (10 mg/ml). The deproteinized samples were electrophoresed through 1% agarose for 4 h in a gel box (20 by 20 cm) at 80 V in Tris-glycine buffer without recirculation. Joint molecule formation was visualized by autoradiography of the dried gel.

Transcription reaction conditions were identical to those for strand transfer except that 0.5 mM GTP, UTP, and CTP were included in the reaction mixture at the start. ^{35}S-labeled RNA was generated by using 0.1 mM [^{35}S]UTP (1,327 Ci/nmol; Dupont) in place of unlabeled UTP. T7 polymerase (10 U) was incubated with the duplex template for 30 min prior to the start of the strand transfer reaction. In our hands, 10 U of SP6 or T7 polymerase generated approximately 30 transcripts per template per h, which is comparable to the rate measured by Losa and Brown (16). RecA protein, ATP (3 mM), and SSB were preincubated with the single-stranded circle for 5 min, and then the mixtures were combined. Runoff RNA was analyzed by polyacrylamide gel electrophoresis (8% gel containing 7 M urea) and quantitated by scintillating counting of the excised band. RNA length determination were carried out by the method of Wolffe and Drew (30), with the following modifications for joint molecules. The ^{35}S-labeled joint molecule was extracted from the agarose gel and isolated by filtration using a NIHON Millipore 0.45-μm-pore-size filter column. The RNA was precipitated by ethanol, denatured by urea, and electrophoresed on an 8% sequencing gel containing 7 M urea.

RESULTS

Experimental strategy. The strand transfer assay used in this study is similar to the one used by McCarthy et al. (19). These workers showed that RecA protein and a recombinase activity from *Drosophila melanogaster* catalyzed the transfer of one strand of a DNA duplex onto single-stranded circular DNA. The reaction was dependent on the presence of sequence complementarily between one of the duplex strands (negative polarity) and the circle. In terms of polarity, the minus strand is transferred onto the plus-strand circle, base pairing as a function of complementarity. We adapted this system for our purposes and created the construct diagramed in Fig. 1A. The 224-bp *Pst*I-*Bam*HI fragment from the immunoglobulin G1 switch region $S_{\gamma 1}$, cloned into pUC19 (kind gift of Y. Chen, Temple University), was isolated and subcloned into the expression vector pSP72 (Promega Biotec). The clone was then digested with *Pst*I and *Hpa*I to produce a duplex fragment containing a 308-bp-long T7 RNA polymerase promoter element. As the pairing partner, a single-stranded circle containing 239 bases of homology which includes the entire $S_{\gamma 1}$ region was used (Fig. 1A). Sixty-nine bases of heterology is located 3′ to the $S_{\gamma 1}$ region only in the duplex fragment. The addition of RecA protein to a reaction mixture containing both of these DNA substrates resulted in the transfer of the minus strand of the duplex onto the circle (Fig. 1B). In this reaction, only the minus strand was radiolabeled and the joint molecules migrate at the same position as the M13 phage DNA (data not shown). Although this construct contains heterologous DNA sequence at one terminus RecA protein was able to transfer the strand onto the circle. The amount of heterology (69 bases) falls below the 100- to 110-base barrier to full transfer identified by Bianchi and Radding (2) and Jwang and Radding (10). The positive strand of the DNA duplex does not comigrate with the reaction product, indicating that it is not transferred onto the single-stranded circle (Fig. 1C).

Transcription-mediated positive-strand transfer. When T7 RNA polymerase (10 U) is incubated with the duplex DNA substrate, RNA transcripts are produced at a rate of 30 transcripts per template per h. The RNA molecules are complementary to the positive strand of the duplex fragment and therefore designated minus. The fate of the minus-strand RNA was traced by including ^{35}S-labeled UTP in the reaction mixture. After the unlabeled duplex DNA template was transcribed by T7 RNA polymerase for 30 min, single-stranded circles, prebound by SSB and RecA protein, were added and incubation continued for various times. As seen in Fig. 2A, the ^{35}S-labeled RNA molecule comigrated with the M13 single-stranded circle. Because the sample was deproteinized, we believe that this structure represents a joint molecule containing RNA and DNA. A population of these joint molecules was extracted from the gel, incubated with either RNase H or RNase A, and loaded onto a 1% agarose gel. After electrophoresis, the sample treated with RNase A was observed to migrate at the same position, but RNase H treatment de-



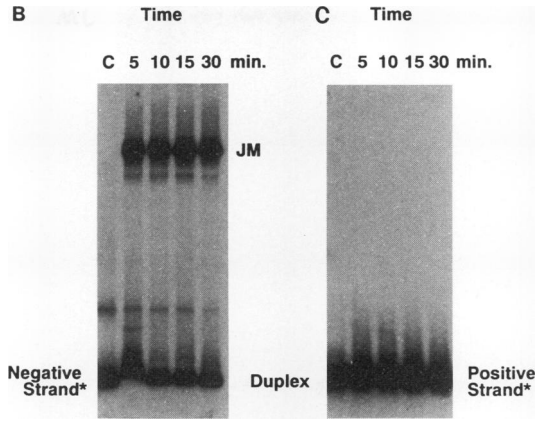


FIG. 1. Positive and negative DNA strand transfer promoted by RecA protein. (A) Diagram of reaction substrates and product. The duplex fragment (*Pst*I-*Hpa*I) is 308 bp in length and contains 239 bp of homology with a sequence within M13 phage DNA (single-stranded circles). The top strand is designated plus with regard to pairing polarity, and the bottom strand is designated minus. The T7 RNA polymerase transcribes 239 bases of the top strand (+) from the T7 promoter (shaded box), producing a minus-strand RNA molecule. RecA protein catalyzes the transfer of the minus strand of the DNA onto the plus-strand circle, producing a joint molecule containing 239 bp and 69 bases of unpaired DNA. (B) Reaction mixtures (20 μl) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3 mM ATP, 1 mM dithiothreitol, 0.1 μg of *E. coli* SSB, 12.5 ng of M13 phage DNA containing the 239-bp insert, and 0.25 ng of ^{32}P-labeled *Pst*I-*Hpa*I (labeled only on the minus strand) were incubated with 1.0 μg of RecA protein at 37°C. After the indicated times, SDS (0.1%) and proteinase K (10 mg/ml) were added, and each mixture was incubated at 37°C for 60 min. The samples were loaded onto a 1% agarose gel, electrophoresed, and dried for autoradiography. JM, joint molecule; Negative Strand; negative strand radiolabeled; C, control, no RecA protein added. (C) Reactions were identical to those in panel B except that the plus strand of the duplex fragment was uniquely radiolabeled.

stroyed the population of joint molecules (Fig. 2B). These results are consistent with the notion that the radiolabeled RNA is present and hybridized to the DNA within the joint molecule.

The length of the RNA present in the heteroduplex product was determined next. The extracted ^{35}S-labeled RNA was treated with RNase A, denatured from the DNA, and electrophoresed on a sequencing gel. As seen in Fig. 2C, the majority of the RNA corresponds to a length of approximately 220 bases compared with ^{35}S-labeled RNA produced by the action T7 polymerase on the DNA template in the absence of strand transfer. The arrows in Fig. 2C indicate the position of the major transcript. Hence, we conclude that a stable RNA-DNA joint molecule is formed as a function of transcription and strand transfer. Furthermore, the RNA is approximately 220 bases in length, and thus over 80% of the full-length transcript is recombined and protected against digestion by RNase A. This result supports our assumption that the joint molecule contains the product of RNA synthesis from the transcribed duplex and not the single-stranded circle. In addition, when SP6 RNA polymerase was incubated with the duplex template and the single-stranded circle, no joint molecules containing RNA and DNA were produced; product formation was a function of the presence of duplex DNA and complementary RNA transcribed from the duplex.

The appearance of RNA in the joint molecule suggested that the positive strand of the duplex might cotransfer during a pairing reaction which involved RNA synthesis. As seen in Fig. 1C, we did not observe strand transfer of the positive strand in the absence of transcription. However, when the duplex template was transcribed by T7 RNA polymerase, transfer of the plus strand of the duplex was observed (Fig. 2D). In this experiment, the plus strand has been uniquely radiolabeled while the RNA transcripts are unlabeled, and thus radiolabeled joint molecule appear as a function of plus-strand DNA transfer. The importance of RNA in stabilizing this structure was revealed when the radiolabeled products disappeared after treatment with RNase H but not after incubation with RNase A. The formation of a joint molecule containing the plus strand of the duplex is dependent on the presence of RecA protein, RNA polymerase, single-stranded circles, and DNA homology (Fig. 2E).

RNA in the reaction product might arise from the annealing of free, radiolabeled transcripts onto the single-stranded circle by the action of RecA protein. In fact, Radding and colleagues (12, 13) have described this catalytic property of RecA protein. Although the sequence of the transcripts argues against this alternative pathway (see Discussion), we tested this possibility directly. ^{32}P-labeled RNA transcripts were produced by the action of T7 RNA polymerase and [α-^{32}P]GTP. The transcripts were isolated, quantitated, and mixed with the single-stranded circles under our standard reaction conditions. The amount of RNA synthesized in our transcription reaction is in the nanomolar range. Hence, a 10, 25, or 50 nM concentration of RNA transcripts was added to a reaction mixture that included RecA, ATP, and SSB prebound to the single-stranded DNA circle. The mixtures were incubated for various times, and the creation of radiolabeled products was determined by gel electrophoresis (Fig. 2F). No radiolabeled product migrating at the position of the circle is found at the RNA concentrations tested. It is likely that the secondary structure adopted by the transcript is refractory to the annealing reaction promoted by RecA.

We tested for this artifactual reaction in another way. The

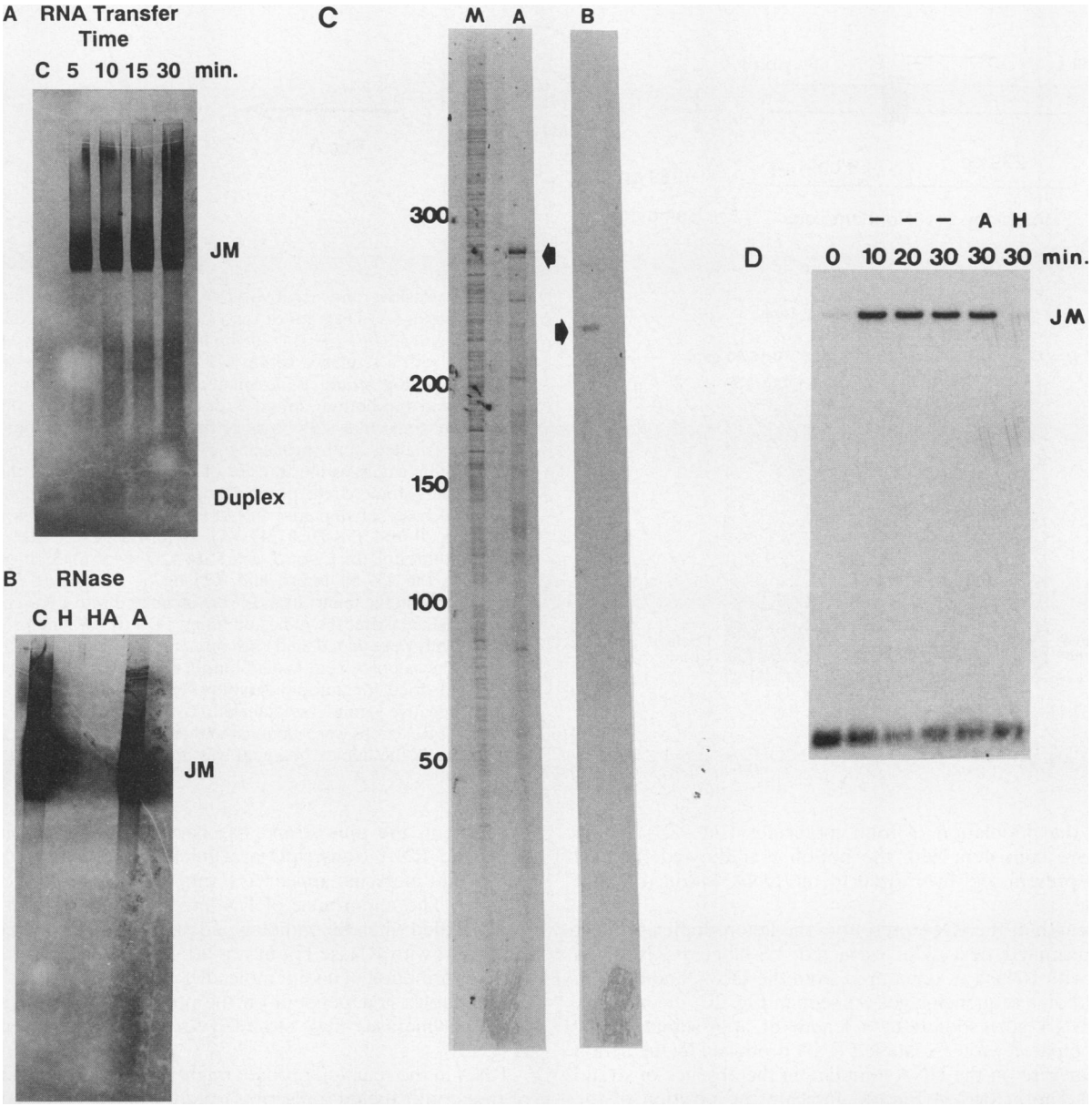
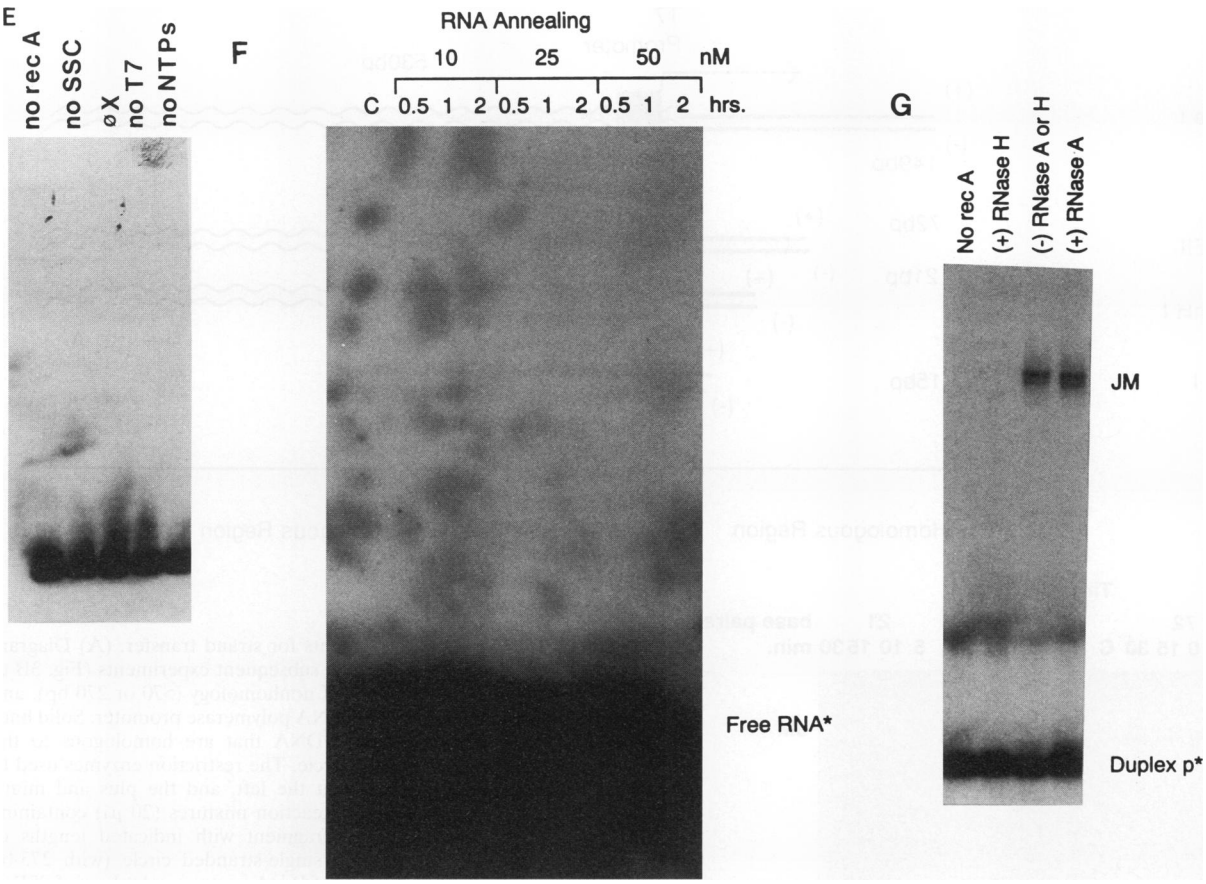


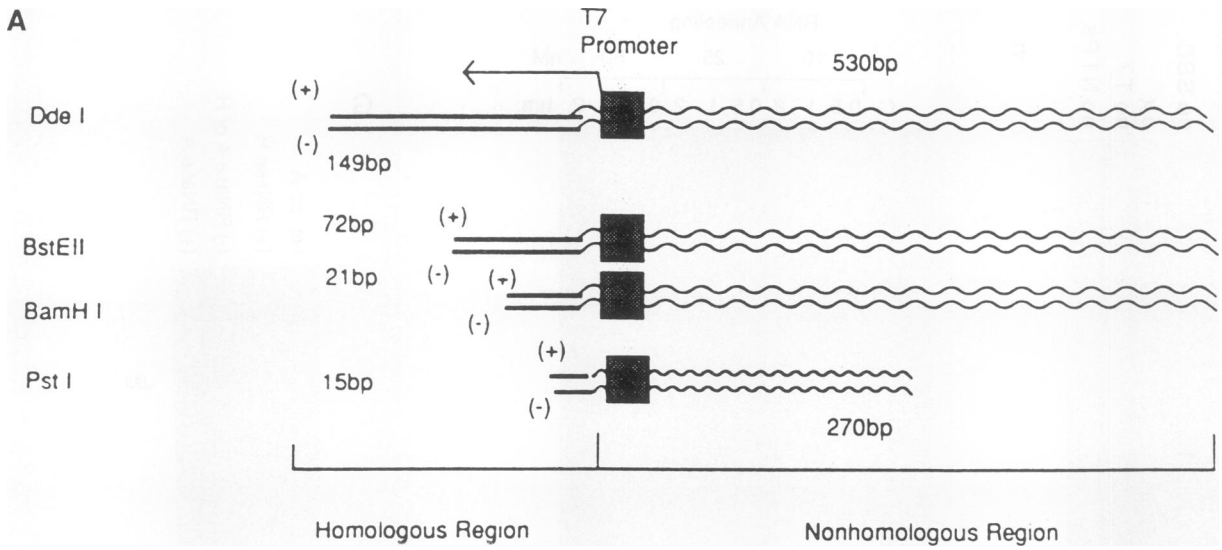
FIG. 2. RNA transcripts are present in the joint molecule. (A) Reaction mixtures were identical to those described in the legend to Fig. 1B except that neither DNA substrate was radiolabeled. Instead, 100 μM [^{35}S]UTP (1,327 Ci/nmol) 500 μM GTP, 500 μM CTP, and 10 U of T7 RNA polymerase were added to the reaction mixture for 30 min prior to the addition of SSB and RecA protein. In this and subsequent reactions, 3 mM ATP was included in the RecA, SSB, and single-stranded circle polymerization mixture. After the indicated times, the deproteinized samples were electrophoresed through 1% agarose and processed for autoradiography. JM, joint molecule; C, no RecA protein added. (B) A population of joint molecules assembled after 30 min of incubation were isolated from the gel, purified by organic extraction, and incubated for 6 h at 37°C with either RNase H (2 mg/ml), RNase A (2 mg/ml), or both. These reactions were terminated by addition of SDS (0.1%) and proteinase K (10 mg/ml), and the mixtures were incubated for 2 h at 37°C. The samples were then electrophoresed through 1% agarose and processed for autoradiography. C, no RNase added; H, RNase H added; HA, RNase H and RNase A added; A, RNase A added. (C) A 30-min reaction product (panel A) containing ^{35}S-labeled RNA was extracted from the gel, treated with RNase A (2 mg/ml) for 6 h at 37°C, and repurified by organic extraction and precipitation. The sample was electrophoresed on a denaturing sequencing gel as described in Materials and Methods. M, ^{32}P-labeled DNA markers; A, total ^{35}S-labeled RNA transcripts; B, ^{35}S-labeled RNA isolated from the joint molecule position of the 30-min time point. (D) Reaction mixtures (20 μl) containing 0.25 ng of ^{32}P-labeled duplex fragment (radiolabeled on the plus strand only), 500 μM GTP, UTP, ATP, and CTP, and 10 U of T7 RNA polymerase were incubated for 30 min at 37°C. RecA protein (1 μg) and SSB (0.1 μg) prebound to 12.5 ng of M13 single-stranded circular DNA were added, and the reaction continued for 10, 20, or 30 min. The deproteinized reaction mixtures were electrophoresed through 1% agarose and processed as described above. 0, no recA protein added; 10, RecA protein for 10 min; 20, RecA protein for 20 min; 30, RecA protein for 30 min; A 30, RecA protein for 30 min followed by the addition of RNase A (2 mg/ml), incubated at 37°C for 6 h; H 30, RecA protein for 30 min followed by the addition of RNase H (2 mg/ml), incubated at 37°C for 6 h. (E) Reaction mixtures were identical to those in panel C except that the indicated reaction component was left out or, in the case of ϕX, ϕX174 phage DNA was substituted for the M13 single-stranded circle (SSC) harboring the sequence homology. (F) Radiolabeled RNA transcripts were generated by incubating the unlabeled *Pst*I-*Hpa*I DNA fragment (0.25 ng) with 10 U of T7 RNA polymerase and 500 μM UTP, CTP, and ATP, respectively. [α-^{32}P]GTP (100 μM) was also included in the mixture, which was incubated for 60 min at 37°C. Transcripts were isolated by electroelution after agarose gel electrophoresis and quantitated by UV



spectrophotometry as described by Kirkpatrick et al. (13). The indicated amounts of ^{32}P-labeled RNA were incubated with 1 μg of RecA protein, 3 mM ATP, 0.1 μg of SSB, and 12.5 ng of the single-stranded circles. Reaction mixtures were terminated and processed as described for panel A. Free RNA*, electrophoretic position of purified ^{32}P-labeled RNA transcripts. (G) Reaction mixtures were identical to those described for panel D. Prior to the addition of the RecA mixture, one set of reaction mixtures was incubated for 15 min with either RNase A or RNase H at 37°C, and then both sets were incubated with the RecA mixture for 60 min. The reactions were terminated by the addition of SDS and deproteinized by treatment with proteinase K. Products were visualized after agarose gel electrophoresis and autoradiography.

duplex template, containing the T7 RNA polymerase promoter, was radiolabeled only on the positive strand. The reaction conditions were identical to those used for Fig. 2D, wherein transcription of the positive strand is initiated by T7 RNA polymerase 30 min prior to the addition of the RecA filament. The only modification made was that RNase A or RNase H was added after the 30-min period and before the RecA reaction was begun. The pairing reaction was terminated after 60 min of incubation, and as seen in Fig. 2G, similar levels of joint molecules are formed in the absence and presence of RNase A. The 15-min treatment time with RNase A is sufficient to digest any free RNA molecules (data not shown). When RNase H was used in place of RNase A, no detectable joint molecules form. We suggest that the appearance of the positive strand in the joint molecule does not occur through an artifactual annealing reaction with free RNA transcripts.

Comparison between homology length requirements of negative- and positive-strand transfer. One of the parameters surrounding the strand transfer reaction catalyzed by RecA protein is the length of complementarity that exists between the pairing partners. Although a firm number has not been established, it is believed that between 38 and 151 bases of homology must be present in order for stable heteroduplexes to form (5, 6, 8). As described above, however, RecA protein can form synaptic complexes when there is as little as 8 bases of homology between pairing partners (8, 9). However, these products form only in the presence of a nonhydrolyzable ATP analog. In the present experimental design, only ATP is used. We wanted to compare the homology requirements for negative- versus positive-strand transfer. To carry out this experiment, the duplex molecules illustrated in Fig. 3A were constructed. Within the 239-bp homologous region, a number of unique restriction sites exist, and thus molecules harboring various lengths of complementarity can be created. To maintain an experimentally feasible length, the duplex molecules also contained 530 bp of nonhomology. In the first series of experiments only the minus strand was labeled, and in general agreement with Gonda and Radding (5) and Hsieh et al. (8), homologous pairing gradually disappears when the length of homology is reduced from 149 to 21 bp (Fig. 3B). No pairing is observed with 21 bp of DNA homology. Recently, we found that even 50 bases of homology is not sufficient to support the formation of stable joint molecules under our reaction conditions (data not shown). Hence, we believe that the homology requirement for DNA transfer of the minus strand of the duplex in this assay system lies between 50 and 72 bp.



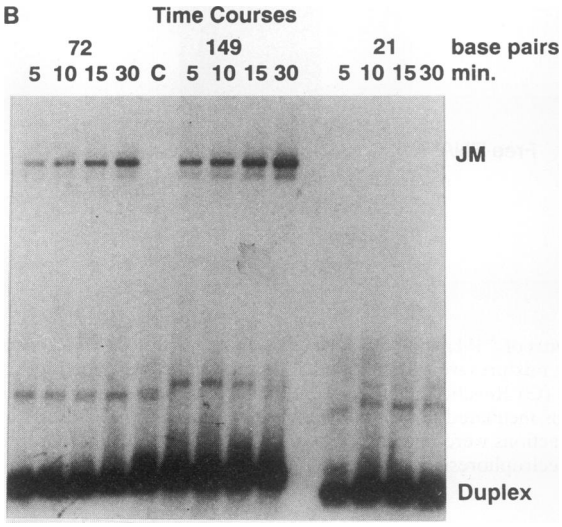


FIG. 3. Homology requirements for strand transfer. (A) Diagram of the DNA duplexes used in the subsequent experiments (Fig. 3B to 5). Wavy lines indicate regions of nonhomology (570 or 270 bp), and the black box represents the T7 RNA polymerase promoter. Solid lines indicate the various lengths of DNA that are homologous to the 273-base sequence in the M13 circle. The restriction enzymes used to create these lengths are listed at the left, and the plus and minus strands are also indicated. (B) Reaction mixtures (20 μl) containing 0.25 ng of ^{32}P-labeled duplex fragment with indicated lengths of homology and 12.5 ng of M13 single-stranded circle (with 273-bp insert) were incubated with 1 μg of RecA protein and 0.1 μg of SSB at 37°C. At the indicated times, SDS (0.1%) and proteinase K (10 mg/ml) were added, and incubation continued for 60 min at 37°C. Samples were electrophoresed through 1% agarose and then subjected to autoradiography. JM, migration position of joint molecule; Duplex, ^{32}P-labeled duplex DNA.

Since we observed an effect of RNA synthesis on the transfer of the positive strand of the duplex substrate, we attempted to identify the minimal homology requirement for positive-strand pairing. The experiment was carried out in duplicate; both sets were radiolabeled only on the positive strand of the duplex molecule having 21 or 15 bp of homology. In the absence of transcription, the plus strand of the duplex was not transferred onto the circle, as no product molecules are observed to form [Fig. 4A and B, (−)]. When the positive strands of the 21- and 15-base-homology templates were transcribed by T7 polymerase, joint molecules were observed to form after the addition of RecA protein [Fig. 4A and B, (+)]. With templates containing 72 or 149 bases of homology, no difference in the levels of negative-strand transfer was observed as a function of transcription (data not shown).

The length of nonhomology in the two duplex substrates harboring 15 and 21 bases of homology is extensive. Thus, we suspected that the population of joint molecules formed as a function of transcription might actually contain both strands of the duplex; because of the length of DNA heterology, it was unlikely that the negative strand would be displaced during the transfer reaction. Our suspicion was confirmed by the experimental results depicted in Fig. 5A. Here, the minus strand of the duplex was radiolabeled while the positive strand was left unlabeled. When the positive strand was transcribed, joint molecules were found to contain the negative strand. No transfer of the negative strand of the 21-base-homology duplex was observed in the absence of transcription (Fig. 3B). Next, we wondered whether the transcript itself could again be found in the joint molecule. For this experiment, the DNA templates were unlabeled while [^{35}S]UTP was included in the reaction mixture. In addition, RNase A (2 mg/ml) was added to the mixture to remove unbound RNA. The results (Fig. 5B) reveal the presence of hybridized RNA within joint molecules formed with 21 and 15 bp of homology. Subsequent treatment with RNase H (2 mg/ml) after joint molecule formation led to the disappearance of the ^{35}S-labeled RNA molecule (Fig. 5B, right panel). Hence, these results indicate that the RNA is complexed with DNA because joint molecules are dissolved after treatment with RNase H but not RNase A. Taken together, the data suggest that RNA-mediated strand transfer can occur at levels of homology lower than those required for DNA strand transfer in the absence of RNA synthesis.

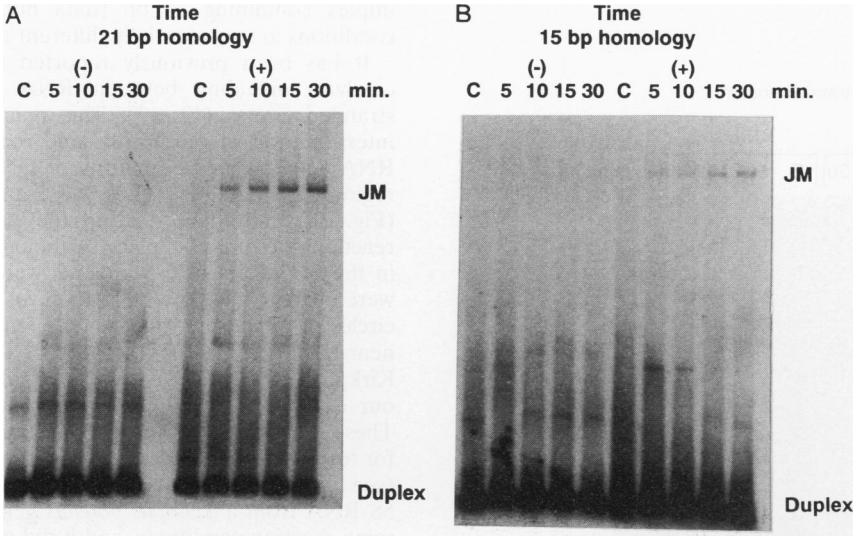


FIG. 4. RNA synthesis reduces the minimal homology requirements for joint molecule formation. (A) Duplicate reaction mixtures (20 μl) containing 0.25 ng ^{32}P-labeled 21-bp-homology duplex fragment DNA (with the radioactive tag on the plus strand), 500 μM GTP, UTP, ATP, and CTP, and 10 U of T7 RNA polymerase were incubated at 37°C. To one set, 10 U of T7 RNA polymerase was added [(+)], while the other set received no T7 polymerase [(−)]. After 30 min, 0.1 μg of SSB and 1 μg of RecA protein, prebound to 12.5 ng of M13 single-stranded circle for 5 min, were added, and aliquots (4 μl) were removed at the indicated times at 37°C. The reactions were terminated by the addition of 0.1% SDS and deproteinized by addition of 10 mg of proteinase K per ml. Samples were electrophoresed through 1% agarose and processed for autoradiography. C, reaction mixtures stopped at 0 time; JM, joint molecule; Duplex, position of ^{32}P-labeled plus-strand DNA fragment. (B) Reactions were identical to those on panel A except that the fragment containing 15 bases of homology was used in place of the 21-base-homology fragment.

DISCUSSION

The data presented in this report couple the processes of RNA synthesis and homologous recombination. We used a simple assay system in which a minus strand of a short DNA duplex is transferred onto a single-stranded circle harboring sequence complementarity by the action of RecA protein. The constructs were designed so that distal joints (20) were formed because these types of joints are considered the most stable compared with proximal or medial pairing partners. We have recently begun a comparative analysis of proximal, medial, and distal joint molecule formation under similar reaction conditions, and our preliminary findings suggest that RNA obviates this discrimination.

The experiments here were designed to analyze the influence of transcription on DNA strand transfer promoted by RecA protein, and three fundamental observations can now be reported. First, the RNA transcript is incorporated into a recombined, joint molecule. Second, the transfer of the plus strand of the duplex pairing partner onto a recipient single-stranded circle can occur even though both molecules are positive in polarity. The formation of this structure, however, is dependent on the synthesis of RNA that is complementarity (minus) to the circle. Third, strand transfer is observed even when the level of sequence homology between the two DNA molecules falls below that required for RecA-mediated DNA transfer. These data suggest homologous pairing of short stretches of homology may be influenced by the synthesis of complementary RNA molecules. These molecules are likely to remain bound to the transcription template because a correlation exists between the number of RNA molecules and the number of plus-strand DNA molecules found in the heteroduplex or triplex product. Such data suggest that the RNA-DNA hybrid is transferred as a complex onto the single-stranded circle, although we cannot rule out the possibility of an ordered RNA strand-DNA strand transfer mechanism. Preliminary gel shift experiments reveal that the RecA protein interacts with a stable RNA-DNA duplex, providing some support for the foregoing explanation. Since the RNA transfer reaction occurs in the presence of RNase A, we believe that the mechanism does not involve regions of single-stranded (free) RNA.

The complex formed in some of our reactions could conceivably be a triplex consisting of DNA-RNA-DNA hybrids. Alternatively, the RNA may bridge both DNA molecules at different sequences, creating a heterogeneous structure of intertwined strands. In a preliminary analysis of thermal stability, the joint molecule formed under these conditions has a melting temperature exceeding that of RNA-DNA hybrids or DNA-DNA hybrids. The entire duplex pairing template (including the minus strand) is, in all likelihood, present in the joint molecule because the region of nonhomology is so extensive that displacing the entire strand as a function of positive-strand transfer seems unlikely (Fig. 5A). Jwang and Radding (10) have demonstrated that 110 bases of nonhomologous DNA located at the termini of a duplex pairing partner can block the full displacement of the positive strand. Independent of structural possibilities, what is clear is that 21 or 15 bp of homology is not sufficient to support the transfer of the negative strand onto the circle in the absence of transcription. Camerini-Otero and colleagues (8, 9) reported that RecA protein could catalyze strand transfer of the negative strand of the duplex when only 38 bp of homology exists between the pairing substrates. Furthermore, synaptic complexes were observed to form between molecules sharing 8 bases of sequence homology when the RecA-promoted reaction mixtures contained a nonhydrolyzable ATP analog (9). We have also measured negative-strand transfer in more detail as a function of decreasing homology and have detected no transfer with a

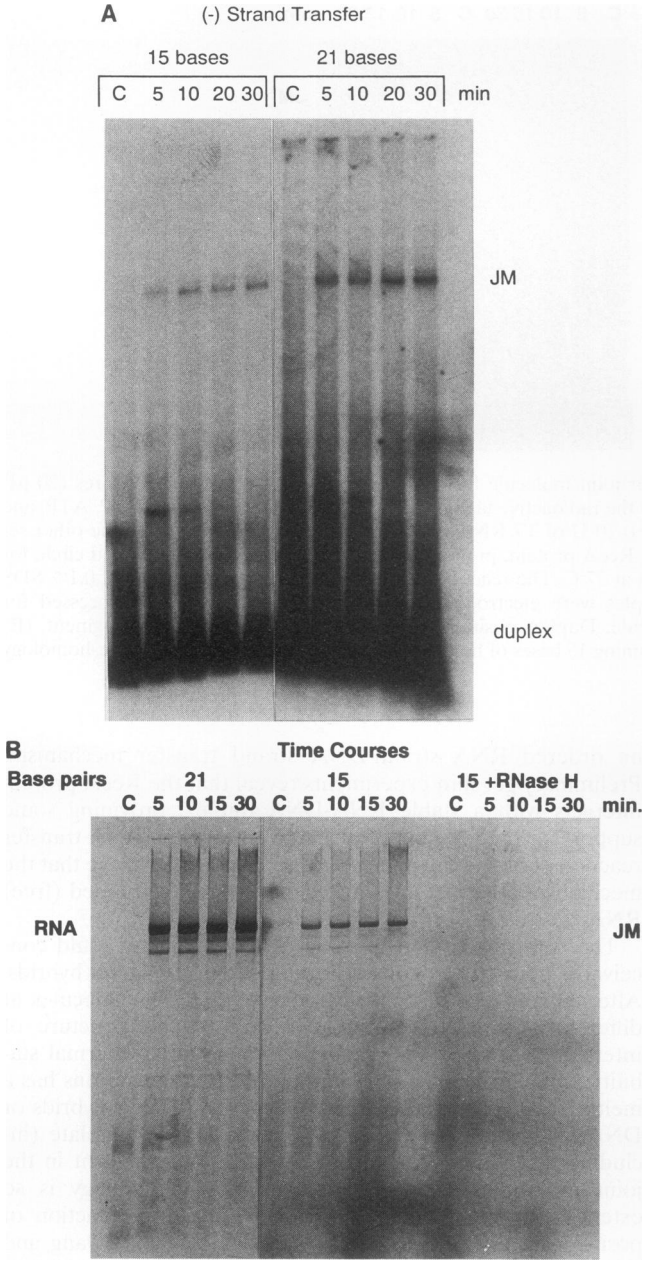


FIG. 5. RNA is found in the joint molecules harboring only 21 or 15 bases of DNA homology. (A) Reactions were identical to those described for Fig. 4A except that the minus (−) strand of the duplex DNA was radiolabeled and T7 RNA polymerase and NTPs were included in the reaction mixture. (B) The reaction mixture contained unlabeled DNA but included ^{35}S-labeled UTP, unlabeled CTP and GTP, and 10 U of T7 RNA polymerase. Prior to termination of the reaction, RNase A (all panels) and RNase H (right panel only) were added at 2 mg/ml for 1 h at 37°C. 21 and 15, lengths (in bases) of homologous sequence; 15 +RNase H, samples treated with RNase A and RNase H; RNA, position of labeled RNA transcript; JM, joint molecule position; C, no RecA protein added.

duplex containing 50 bp (data not shown). Our reaction conditions are substantially different and always include ATP.

It has been previously reported that RecA protein will catalyze annealing between RNA transcripts and single-stranded circles (12, 13). This reaction could obscure the interpretation of our results and reduce the possibility that RNA acts to facilitate pairing of DNA molecules with short regions of homology. Thus, we examined this issue directly (Fig. 2F) and found that in the present system, this side reaction does not take place. Although the reaction conditions in the two series of experiments were similar, the substrates were not. First, we used a lower level of single-stranded DNA circles in our assays. Second, the length of our transcripts is near the lower end of the range of RNA transcripts used by Kirkpatrick et al. (13). Most importantly, the transcript used in our experiments is part of the Sγ switch region transcript. These RNA transcripts as free molecules have a high potential for forming secondary structure under our reaction conditions (see reference 7 for a review). In addition, we have tested the 5S RNA from a *Xenopus borealis* gene transcribed under the same reaction conditions, and it did not participate in annealing reactions promoted by RecA (data not shown); rRNA transcripts can also adopt significant secondary structure. Kirkpatrick and Radding (12) proposed that rRNA and tRNA transcripts do not compete in a RecA-promoted annealing reaction because of the highly folded structure. Thus, we believe that the degree of RNA folding or the adoption of secondary structure dictates which class of RNA molecules can be annealed directly onto the single-stranded circle by the action of RecA protein. Furthermore, the association of the transcript with the positive (transcribed) strand is important for positive (DNA) strand transfer since treatment with RNase H eliminates joint molecule formation.

It is also possible that such joint molecules are formed by a transcript paired with the switch region on the circle and an unrelated region on the duplex template. This seems unlikely, as the transcript is created only from the homologous region of the duplex. If this were the case, one might predict that we would see a shift in duplex mobility in the absence of the circle. Such a structural alteration has not been observed. We are now in the process of analyzing the structure of the joint molecule, with particular emphasis on the notion of triplex formation.

What is the significance of RNA-mediated DNA transfer? The answer may lie in the second group of results presented in this paper. These results suggest that joint molecules are formed even when the homologous regions are reduced to 15 bp if the region of complementarity is transcribed. The significance of this observation may interface with the mechanisms that underlie events such as immunoglobulin switch region recombination.

A correlation between RNA synthesis and genetic recombination has been suggested by several in vivo studies (17, 31). Such relationships are most often associated with studies surrounding heavy-chain immunoglobulin switching (see references 7 and 23). Located 5′ to the immunoglobulin heavy-chain gene is a switch region used as a target site for induced rearrangement in B cells (26). Deletional DNA recombination is preceded by preswitch transcriptional activation, and it is believed that RNA synthesis may serve primarily to increase the accessibility of homologous DNA regions to the switch recombinase (13a, 17). Recent data from Griffin and colleagues (6a; see also reference 23) suggest that the RNA transcript itself may participate in the recombination event by hybridizing to two strands of DNA simultaneously.

Since many of these regions lack extensive amounts of DNA homology, below the requirement of accepted recombinase

activity, perhaps these types of recombination intermediates develop through a pathway involving RNA. In addition, a large number of studies have revealed recombination events occurring in prokaryotes and eukaryotes when the region of homology is less than 30 bp. Pont et al. (20) found that extrachromosomal elements purified from *D. melanogaster* embryos arose most likely from a recombination event involving 8- to 15-base genomic direct repeats. Some of these events are thought to occur via a homologous recombination pathway. Elegant work from Whoriskey et al. (29) showed that RecA protein mediates genetic duplications at short nucleotide repeats (<15 bp) when, by experimental design, the recombining substrates are transcriptionally active. These data indirectly implicate RNA synthesis as part of the recombination process that generates duplications and ultimately leads to gene amplification. In earlier studies, Albertini et al. (1) showed that spontaneous deletions in *E. coli* occur through short stretches of sequence homology. These reactions are 25 times more frequent in $RecA^+$ strains of *E. coli*, although the mechanism by which those deletions occur remain unclear. Our data suggest that such RecA-mediated events are more likely to happen if the recombining substrates are transcriptionally active. In a more recent but related study, Schofield et al. (24) demonstrated that inversions between short (12- to 23-bp) stretches of DNA in *E. coli* are reduced 1,000-fold in $RecA^-$, $RecB^-$, or $RecC^-$ strains.

In *Saccharomyces cerevisiae*, transcription has been shown to stimulate recombination in several well-studied cases. First, Voelkel et al. (28) demonstrated that a DNA sequence (*HOT1*) of the rRNA gene cluster participates in recombination events at a higher frequency when the region around *HOT1* is transcriptionally active. Second, Thomas and Rothstein (27) showed that direct repeat recombination of the *GAL10* gene is heightened 15-fold when the gene is constitutively expressed. Both of these studies reinforce and extend the observations of Keil and Roeder (11), who found that fragment of DNA containing the RNA polymerase I promoter (*HOT1*) stimulated recombination of adjacent genes 25- to 100-fold. Although the results of these studies develop a correlation between transcription and elevated levels of recombination, none of them examine the role of RNA molecules in facilitating the reaction. Our studies suggest that the transcript which bears complementarity to at least one DNA strand of a pairing partner might serve to stabilize the crossover junctions, particularly within short stretches of sequence homology. Therefore, the half-life of the crossover is extended and the opportunity for genetic exchange is increased.

ACKNOWLEDGMENTS

We thank members of the Kmiec laboratory for discussion and critical readings of the manuscript. We are indebted to Linda Johnson for manuscript preparation.

This work was supported by a grant from the Tobacco Council of America (2821A).

REFERENCES

1. **Albertini, A., M. Hofer, M. Calos, and J. H. Miller.** 1982. On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:**319–328.
2. **Bianchi, M. E., and E. M. Radding.** 1983. Insertions, deletions and mismatches in heteroduplex DNA made by recA protein. Cell **35:**511–519.
3. **Cox, M. M., and I. R. Lehman.** 1987. Enzymes of general recombination. Annu. Rev. Biochem. **56:**229–262.
4. **Cunningham, R. P., T. Shibata, C. DasGupta, and C. M. Radding.** 1980. Homologous pairing in genetic recombination: recA protein makes joint molecules of gapped circular DNA and closed circular DNA. Cell **20:**223–245.
5. **Gonda, D. K., and C. M. Radding.** 1983. By searching processively RecA protein pairs DNA molecules that share a limited stretch of homology. Cell **34:**647–654.
6. **Gonda, D. K., and C. M. Radding.** 1986. The mechanism of the search for homology promoted by recA protein. Facilitated diffusion within nucleoprotein networks. J. Biol. Chem. **261:**13087–13096.

6a. **Griffin, J. A., et al.** Personal communication.

7. **Gritzmacher, C. A.** 1989. Molecular aspects of heavy-chain class switching. Crit. Rev. Immunol. **9:**173–200.
8. **Hsieh, P., C. S. Camerini-Otero, and R. D. Camerini-Otero.** 1990. Pairing of homologous DNA sequences by proteins: evidence for three-stranded DNA. Gen. Dev. **4:**1951–1963.
9. **Hsieh, P., C. S. Camerini-Otero, and R. D. Camerini-Otero.** 1992. The synapsis event in the homologous pairing of DNAs: RecA recognizes and pairs less than one helical repeat of DNA. Proc. Natl. Acad. Sci. USA **89:**6492–6496.
10. **Jwang, B., and C. M. Radding.** 1992. Torsional stress generated by RecA protein during DNA strand exchange separates strands of a heterologous insert. Proc. Natl. Acad. Sci. USA **89:**7596–7600.
11. **Keil, R. L., and G. S. Roeder.** 1984. *Cis*-acting recombination-stimulating activity in a fragment of the ribosomal DNA of *S. cerevisiae*. Cell **39:**377–386.
12. **Kirkpatrick, D. P., and C. M. Radding.** 1992. RecA protein promotes rapid RNA-DNA hybridization in heterogenous RNA mixtures. Nucleic Acids Res. **20:**4347–4353.
13. **Kirkpatrick, D. P., B. J. Rao, and C. M. Radding.** 1992. RNA-DNA hybridization promoted by *E. coli* RecA protein. Nucleic Acids Res. **20:**4339–4346.

13a. **Kotani, H., et al.** Submitted for publication.

14. **Kotani, H., and E. B. Kmiec.** 1994. Transcription activates RecA promoted homologous pairing of nucleosomal DNA. Mol. Cell. Biol. **14:**1949–1955.
15. **Kotani, H., and E. B. Kmiec.** Cruciforms facilitate *in vitro* homologous recombination on nucleosomal templates. Mol. Gen. Genet., in press.
16. **Losa, R., and D. D. Brown.** 1987. A bacteriophage RNA polymerase transcribes *in vitro* through a nucleosome core without displacing it. Cell **50:**801–808.
17. **Lutzker, S., and F. Alt.** 1988. Structure and expression of germ line immunoglobulin gamma 2b transcripts. Mol. Cell. Biol. **8:**1849–1852.
18. **Lutzker, S., P. Rothman, R. Pollock, R. Coffman, and F. W. Alt.** 1988. Mitogen- and IL-4-regulated expression of germ-line Ig gamma 2b transcripts: evidence for directed heavy chain class switching. Cell **53:**177–184.
19. **McCarthy, J. G., M. Sander, K. Lowenhaupt, and A. Rich.** 1988. Sensitive homologous recombination strand-transfer assay: partial purification of a Drosophila melanogaster enzyme and detection of sequence effects on the strand-transfer activity of RecA protein. Proc. Natl. Acad. Sci. USA **85:**5854–5858.
20. **Pont, G., F. Degroote, and G. Picard.** 1988. Illegitimate recombination in the histone multigenic family generates circular DNAs in Drosophila embryos. Nucleic Acids Res. **16:**8817–8833.
21. **Radding, C. M.** 1988. Homologous pairing and strand exchange promoted by *Escherichia coli* RecA protein. p. 193–230. *In* R. Kucherlapati and G. Smith (ed.), Genetic recombination. American Society for Microbiology, Washington, D.C.
22. **Rao, B. J., M. Dutreix, and C. M. Radding.** 1991. Stable three-stranded DNA made by RecA protein. Proc. Natl. Acad. Sci. USA **88:**2984–2988.
23. **Reaban, M. E., and J. A. Griffin.** 1990. Induction of RNA-stabilized DNA conformers by transcription of an immunoglobulin switch region. Nature (London) **348:**342–344.
24. **Schofield, M. A., R. Agbunag, and J. H. Miller.** 1992. DNA inversions between short inverted repeats in *Escherichia coli*. Genetics **132:**295–302.
25. **Shen, P., and H. V. Huang.** 1986. Homologous recombination in *Escherichia coli*: dependence on substrate length and homology. Genetics **112:**441–457.
26. **Starnvzer-Nordgren, J., and S. Sirlin.** 1986. Specificity of immu-

noglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. EMBO J. **5:**95–102.
27. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. Cell **56:**619–630.
28. **Voelkel, K., R. L. Keil, and G. S. Roeder.** 1987. Recombination-stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I. Cell **48:**1071–1079.
29. **Whoriskey, S. K., V.-H. Nghiem, P.-M. Leong, J.-M. Masson, and J. H. Miller.** 1987. Genetic rearrangements and gene amplification in *Escherichia coli*: DNA sequences at the junctures of amplified gene fusions. Genes Dev. **1:**227–237.
30. **Wolffe, A. P., and H. R. Drew.** 1989. Initiation of transcription on nucleosomal templates. Proc. Natl. Acad. Sci. USA **86:**9817–9821.
31. **Yancopoulos, G., R. DePinho, K. Zimmerman, S. Lutzker, N. Rosenberg, and F. Alt.** 1986. Secondary genomic rearrangement events in pre-B cells: V_HDJ_H replacement by a LINE-1 sequence and directed class switching. EMBO J. **5:**3259–3266.