Joints formed by RecA protein from oligonucleotides and duplex DNA block initiation and elongation of transcription

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ABSTRACT

In the presence of the non-hydrolyzable analog of ATP, ATP γ S, RecA protein can polymerize on an oligodeoxy-ribonucleotide to form a stable oligonucleoprotein filament that can find its homologous sequence in double-stranded DNA. The homologous joint formed by the oligonucleotide and duplex DNA is stable only if RecA protein is not removed. Such a nucleoprotein joint, covering a part or all of the promoter region of T3 or T7 phage RNA polymerase, blocked transcription directed by those polymerases. The same kind of joint, located downstream of the RNA polymerase promoter, also inhibited elongation of transcription and caused accumulation of truncated transcripts. These observations suggest that RecA protein can be used to shut off transcription from any promoter of known sequence.

INTRODUCTION

The ability to interfere at will with transcription from any promoter might find a variety of applications in molecular biology and, eventually, in therapeutics. Recent observations have suggested a way in which transcription can be shut off experimentally. Polypurine or polypyrimidine oligodeoxyribonucleotides (ODN) can non-enzymatically form a sequence specific triple helix by hydrogen bonding to polypyrimidine or polypurine tracts within duplex DNA [1–5]. Cooney *et al.* and Postel *et al.* utilized such triplex formation to inhibit transcription from the *c-myc* promoter *in vitro* and *in vivo* [6, 7]. Young *et al.* and Duval-Valentin *et al.* observed inhibition of transcription elongation *in vitro* by a polypurine or polypyrimidine ODN that formed a triplex that was located downstream from a promoter [8, 9].

Previous observations have shown that RecA protein will form stable complexes either with ODNs or with single-stranded DNA in the presence of the non-hydrolyzable analog, ATP γ S, and that both such stable nucleoprotein filaments are active in homologous pairing [10–12]. More recent observations have implicated a triple-stranded intermediate in homologous recognition promoted by RecA protein [13–15]. Although the putative triplex intermediates made from some DNA substrates are surprisingly stable when RecA protein is removed, those made from a single-stranded ODN do not survive deproteinization unless the duplex DNA is superhelical (Jwang, B. and Golub, E., unpublished observations). However, the oligonucleoprotein joint formed by the ODN and

homologous duplex DNA encased in the RecA filament in the presence of ATP γ S is sufficiently stable to protect non-superhelical DNA against site-specific enzymatic methylation [16].

Although the nature of the triplex DNA intermediate and the conditions of its formation and stabilization by RecA protein are not yet well understood, the enzymatic reaction does not appear to require any specific sequence, but rather, can be targeted to any desired sequence such as a promoter. In this communication, we describe experiments which show that with specific ODNs, RecA protein can inhibit either the initiation or elongation of transcription by T3 or T7 phage RNA polymerase.

MATERIALS and METHODS

Plasmid and ODN

As a template for transcription we used plasmid pRK100, which is a derivative of pBS(+) (Stratagene) with a 215 bp *Hind*III fragment (with coordinates 1493–1708) of virus SV40 DNA, inserted into a multiple cloning site. The plasmid carries phage T3 and T7 RNA polymerase promoters. For most transcription experiments, plasmid pRK100 was digested by *Pvu*II. Full size transcripts made by T3 and T7 polymerases from this template consist of 394 and 487 nucleotides, respectively (see Fig. 1). For transcription experiment described in Fig. 5, plasmid pRK100 was digested by *Pvu*II and *Hind*III. Full size transcripts made by T7 polymerase from this template consist of 60 nucleotides.

The ODNs used in the study are listed in Table 1. All of the listed ODN sequences, except GM007, had complete homology to a region of the transcribed *Pvu*II fragment from plasmid pRK100. ODN GM007, which does not share homology to any part of the plasmid, served as the heterologous control.

Formation of oligonucleotide joints by RecA protein

ODN at 10 μ M was mixed with 5 μ M RecA protein in a solution containing 30mM Tris acetate, pH 7.2, 2.5 mM Mg acetate, and 1.2 mM ATP γ S. The mixture was incubated for 5 min at 37°C to allow RecA protein to coat the ODN. Then, 0.5–1.0 μ g (75–150 μ M) of plasmid pRK100, digested by *Pvu*II, was added and the concentration of Mg acetate was simultaneously increased to 5 or 12 mM. After 15–20 min incubation at 37°C, an aliquot of the mixture was used as a source of template for *in vitro* transcription. The conditions described above are adequate for oligonucleotide joint formation as judged by blockage of a

Table 1. ODNs used in this study

Name	Number of Nucleotides	Sequence*	Comment
T3ASP ₂₀	20	<u>ACCCTCACTAAAGGGAACAA</u>	Carries 12ntd [†] sequence, homologous to part of T3 promoter
PBS-T3 ₄₂	42	<u>ATTACGCCAAGCTCGAAATTA</u> <u>ACCCTCACTAAAGGGAACAAA</u>	Carries 17ntd sequence, homologous to T3 promoter
PBS-T3 ₇₀	70	<u>ATTTACACACAGGAAACAGCTA</u> <u>TGACCATGATTACGCCAAGCT</u> <u>CGAAATTAACCCTCACTAAAG</u> <u>GGAACAA</u>	Carries 17ntd sequence, homologous to T3 promoter
PBS-T7 ₄₂	42	<u>GAGCTCGAATTCGCCCTATAG</u> <u>TGAGTCGTATTACAATTCAC</u>	Carries 17ntd sequence, homologous to T7 promoter
PBS-T7C ₄₂	42	<u>AGTGAATTGTAAATACGACTCA</u> <u>CTATAGGGCGAATTCGAGCTC</u>	Complementary to PBS-T7 ₄₂
PBS-T3UP ₄₂	42	<u>GAGCCGGAAGCATAAAGTGTA</u> <u>AAGCCTGGGGTGCCTAATGAG</u>	Homologous to upstream region of T3 promoter with coordinates -94 to -135 (relative to the transcriptional start signal)
GM007	48	<u>TGGATCACGAGGTCAGGAGAT</u> <u>CGAGACCATCCCGCTAAAAC</u> <u>GGTGAA</u>	No homology to any part of pRK100

* Sequences, homologous to T3 or T7 RNA polymerase promoters, are underlined.

[†] ntd = nucleotide

restriction site by an ODN homologous to the site (B. Jwang, personal communication). In experiments with PBS-T3₇₀, the concentration of the ODN was 67 μ M, and that of RecA protein was 8.3 μ M.

Transcription

Standard transcription mixtures contained 40mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM UTP, 12 μ M CTP, 1–2 μ Ci α -³²P-CTP (3,000 Ci/mmol), 1.2 U T3 or T7 phage RNA polymerase and 0.25–0.5 μ g DNA. The mixture was incubated for 5–60 min at 37°C. The reaction was stopped by adding 15 mM EDTA plus 0.3% SDS. Transcripts were analyzed by electrophoresis in a 1.5% agarose gel or a 6% or 8% denaturing polyacrylamide gel. Comparative estimations of the amounts of transcripts were carried out either from the gel by using a Phosphor-Imager (Molecular Dynamics), or by autoradiography by using a gel scanner, Visage 2000 (Millipore Corp.).

RecA protein was isolated as described [17]. T3 and T7 phage RNA polymerases and unlabeled ribonucleotides were purchased from Promega, α -³²P-CTP from New England Nuclear. ODNs were synthesized on a DNA Synthesizer, Model 394 (Applied Biosystems) and purified by separation on a polyacrylamide gel or by using NEN Sorb Prep Cartridges (New England Nuclear).

RESULTS

Inhibition of Initiation of Transcription by DNA Joints Made by RecA Protein

Transcription from the T3 and T7 promoters in the DNA fragment shown in Fig. 1 was assessed by gel electrophoresis as described in Materials and Methods. ODN PBS-T7₄₂ is homologous to the T7 promoter and flanking DNA (Table 1).

Figure 1. Map of the *PvuII* fragment of a pRK100 DNA that was used as a template for transcription from a T3 and a T7 promoter. Arrows 1 and 3 refer to the full size transcripts made by T7 and T3 RNA polymerases respectively; arrow 2 shows the expected length of a transcript initiated at the T7 promoter but interrupted by a RecA oligonucleoprotein joint formed with ODN PBS-T3₄₂ at the T3 promoter; arrow 4 refers to a transcript made by T7 RNA polymerase from pRK100 DNA digested by *HindIII*.

The joint made with PBS-T7₄₂ strongly inhibited transcription from that promoter (Fig. 2, compare lanes 1 and 5). The inhibition of transcription was observed only in the presence of functional RecA protein (compare lanes 4 and 5) and it required the presence of ATP γ S (compare lanes 2 and 5), which is necessary for the action of RecA protein but which itself did not inhibit transcription (see lanes 1, 3 and 4). ODN GM007, which shared no homology with template DNA, did not influence transcription (compare lanes 1 and 7), nor did RecA protein alone (lane 3). ODN PBS-T7C₄₂ whose sequence is complementary to PBS-T7₄₂ (Table 1) blocked transcription to the same degree at PBS-T7₄₂ (compare lanes 5 and 6). Thus, inhibition of transcription showed no strand-specificity. A densitometer tracing

Figure 2. Inhibition of transcription from a T7 promoter by a homologous RecA oligonucleoprotein joint. Joints were formed prior to the initiation of transcription, which was carried out for 60 min. Transcription was analyzed by electrophoresis in a 1.5% agarose gel, and visualized by autoradiography as described in Materials and Methods. In lanes 1 and 4 RecA protein was omitted and replaced by an equal volume of R buffer (50 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.3 mM EDTA, 10% glycerol), which is used for dilution of RecA protein). The arrow shows the position of a band whose mobility is consistent with that expected for a full length T7 transcript (487 nucleotides, see Fig. 1) as judged by comparison with a '1 kb' ladder of fragments (BRL) on a 6% denaturing polyacrylamide gel (see Fig. 4).

Figure 3. Inhibition of transcription from a T3 promoter. Transcription, directed by T3 RNA polymerase, was carried out for 30 (panel A) or 60 min (panel B) and its product were analyzed on 1.5% agarose or 6% denaturing polyacrylamide gel, respectively. In lane 1 (panel A), RecA protein was omitted and replaced by an equal volume of R buffer. Lane 1 (panel B): 32 P end-labeled pBR322 digested by *MspI* (New England Biolabs), which was used as a marker. The numbers identify the lengths of the fragments.

of the autoradiogram shown in Fig. 2 indicated that the RecA oligonucleoprotein joint inhibited transcription by more than 98%.

Transcription from a T3 promoter was affected to a similar extent by homologous RecA joints formed from ODN PBS-T3₄₂ (Fig. 3A, lanes 1 and 2). Note that ODN GM007, which shares no homology with template DNA, and ODN PBS-T3UP₄₂, which is homologous to a site located upstream of T3 promoter (see Table 1), did not influence transcription (Fig. 3A, compare lanes 1, 3 and 4). The 70mer ODN, PBS-T3₇₀, also caused practically complete inhibition (>99%) of T3 RNA polymerase directed transcription (Fig. 3B, compare lanes 2 and 3); 20mer ODN, T3ASP₂₀, which covers only part of T3 promoter (12

Figure 4. Inhibition of the elongation of transcription initiated at a T7 promoter. Transcription, directed by T7 RNA polymerase, was carried out for 15 min and its products were analyzed by electrophoresis in a 6% denaturing polyacrylamide gel. Lane 1: 32 P end-labeled 1 kb DNA ladder, which was used as marker. The numbers identify the lengths of the fragments. Lane 2: no ODN, and RecA protein was replaced by R buffer. Lane 3: 10 μ M 42mer ODN PBS-T3₄₂ and 5 μ M RecA protein.

Fig. 5. Retention of RNA polymerase molecules by RecA joints. Transcription, directed by T7 RNA polymerase, was carried out for 15 min and its products were analyzed by electrophoresis in an 8% denaturing polyacrylamide gel. In lanes 4 and 5, the second template (pRK100, digested by *PvuII* and *HindIII*) was added 4 min after transcription from first template (pRK100 digested by *PvuII*) started. Both templates were added in equal amounts: 250ng. Lanes 1 and 8: 32 P labeled pBR322 digested by *MspI* used as marker.

nucleotides out of 17 implicated in the T3 promoter), also inhibited transcription but to some lesser degree (approximately 93% inhibition) (Fig. 3B, compare lanes 2 and 4).

Inhibition of Elongation of Transcription

Figure 4 shows the results of an experiment in which the DNA joint was located 276–317 nucleotides downstream from the T7 promoter. The RecA joint interrupted the propagation of transcription by T7 RNA polymerase and caused accumulation of a smaller transcript than the complete one, which was 487 nucleotides (see Fig. 1). The truncated transcript formed a single band whose migration was consistent with the expected size, 275 nucleotides, if transcription were arrested at the beginning of the joint located in the T3 promoter region. According to quantitative estimations made by using a gel scanner, and calculations that took into account the difference in size of the uniformly labeled full and truncated transcripts, joint formation decreased the number of transcribed molecules approximately 4-fold (see Discussion) and caused the synthesis of around 3 times more truncated transcripts than full-sized ones.

Similar effects of inhibition of elongation of transcription were observed in experiments with T3 RNA polymerase. ODN PBS-T7₄₂ and PBS-T7C₄₂ which are complementary to each other, show the same effect on the inhibition of elongation (data not shown).

Retention of RNA polymerase molecules by RecA joints

In our experiments, the blockage of elongation by the RecA joints was manifested not only by the appearance of truncated RNA molecules but also by a considerable decrease in the total number of transcribed RNA molecules. It was suggested that molecules of RNA polymerase, which are arrested when they encounter the RecA joint, cannot be released from template DNA and are excluded from participation in further rounds of transcription. To substantiate that possibility we designed an experiment in which a second template that contains the same promoter but lacks the site for joint formation was added several minutes after transcription from a template with joints was started. In support of the above hypothesis, we found that the amount of RNA transcribed from the second template, was about 3-fold less when the first template had an arresting RecA nucleoprotein joint (Fig. 5, compare lanes 4 and 5).

DISCUSSION

Experiments carried out in this laboratory and others have shown that in the presence of RecA protein and ATP γ S an ODN can pair with covalently closed circular (CCC) dsDNA [12, 16, 18]. The DNA product, or joint molecule, formed under these conditions is stable and its formation can be monitored by the co-migration of a ³²P-labeled ODN with dsDNA upon electrophoresis in a neutral agarose gel. However, such co-migration was not observed in experiments with linear dsDNA even if linearization was executed after the ODN-CCC dsDNA triplex was formed (Jwang, B. and Golub, E., unpublished observations). These observations suggest that whatever the structure of the initial joint that RecA protein makes between an oligonucleotide and duplex DNA, deproteinization leads to the formation of a D-loop that is unstable except in superhelical DNA [19]. The concept that RecA protein mediates homologous recognition by initially forming triplex DNA [20] is supported, however, by observations of RecA reactions involving other substrates, which to various extents form three-stranded intermediates that survive deproteinization [13–15]. In the experiments reported here, the intermediates made by RecA

protein were not deproteinized, and the effects observed are almost certainly attributable to the oligonucleoprotein joint consisting of RecA protein and the oligonucleotide at a homologous site in duplex DNA.

The joints just described blocked transcription from a linear dsDNA template. An ODN, homologous to one strand of a promoter, inhibited the initiation of transcription and an ODN homologous to a site downstream from the promoter stopped the elongation of transcription. By contrast an ODN homologous to a site 94 base pairs upstream from the promoter had no effect, indicating that inhibition results from creation of a direct barrier, rather than long distance effects like unwinding. The effective blockage of transcription by the RecA joint formed in the presence of the non-hydrolyzable analog, ATP γ S, provides further evidence of the ability of such joints to bar access of other enzymes to DNA, as recently shown in the case of restriction/modification enzymes [16].

Under the experimental conditions of the present experiments, nucleoprotein joints formed in the region of a promoter, inhibited transcription almost completely. The degree of inhibition did not depend notably on the time that was allowed for transcription: we observed the same inhibition when transcription lasted for 5 min or 60 min (data not shown). Furthermore, we observed a similar high degree of inhibition of initiation of transcription when the triplex joint was formed and kept at 37°C for 4 h prior to transcription (data not shown).

In experiments on inhibition of elongation we observed that a majority (approximately 70%) of transcribed RNA molecules were truncated and the rest were full-sized transcripts. This contrasts with the more complete inhibition of initiation of transcription. A similar incomplete inhibition of elongation by homopurine-homopyrimidine triplexes was reported by Young, *et al.* which they attributed to 'dissociation of the triple helix complex during the course of transcription elongation.' These authors [8] found that truncated transcript could be elongated to full length transcripts upon prolonged incubation, especially if during the incubation an excess of a ribonucleoside triphosphate was added (so-called 'chasing'). In similar experiments on the blockage of transcription by RecA protein, the ratio of truncated to full-sized transcripts was however unaffected by chasing (data not shown).

The principal advantage of the RecA pairing reaction as a means to interfere with transcription, or the action of any DNA enzyme, is the ability to target an oligonucleotide to any known sequence. As an example of the potential usefulness of these observations, it may be possible to examine directly the role of *cis* regulatory elements in transcription by blocking them with RecA protein-coated ODNs *in vitro*. The usefulness of this reaction to study the effect of shutting off specific promoters *in vivo* is limited in the short run by the need to introduce into the cell both RecA protein and an oligonucleotide; but further investigation may conceivably overcome such problems and provide a powerful new tool for research and therapy.

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