Nucleic Acids Research, 1993, Vol. 21, No. 17 4055–4058

Relief of triple-helix-mediated promoter inhibition by elongating RNA polymerases

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Received May 11, 1993; Revised and Accepted July 21, 1993

ABSTRACT

We have characterized triple-helix-mediated inhibition of an artificial bacteriophage promoter with respect to relief of inhibition by incoming RNA polymerases that initiate upstream or downstream from the operator sequence. Whereas oligonucleotide-directed triple-helix formation inhibits the test promoter, promoter activity is restored when the triple-helical complexes are disrupted by transcription of either strand of the homopurine operator sequence. The degree of relief from inhibition is related to the frequency of operator transcription. These observations demonstrate that this artificial repressor–operator complex is subject to antagonism by *cis* elements (other promoters) acting at a distance. Such antagonism might also arise between certain natural transcriptional control regions. Our results suggest that the efficiency of artificial repressors based on triple-helix formation may be limited by transcriptional activity in the gene control region.

INTRODUCTION

Repressor proteins inhibit the initiation of RNA polymerase by occluding polymerase binding to promoter DNA, or by preventing the transition between abortive cycling and elongation (1). Besides their effects on transcription initiation, certain repressor–operator complexes can act as transcriptional terminators. The extent to which different protein–DNA obstacles block transcription depends on the nature of the obstacle, its position relative to the promoter, and the RNA polymerase in question. For example, binding of the *Lac* repressor protein to its operator sequence blocks elongation and terminates transcription *in vivo* both for *E.coli* RNA polymerase and eukaryotic RNA polymerase II (2–4). In contrast, the more weakly bound *Trp* repressor does not block transcription by *E.coli* RNA polymerase if positioned beyond 16 bp from the transcription initiation site (5). Furthermore, elongation of RNA polymerases from bacteriophages T3 and T7 is not blocked by the presence of a *Lac* repressor-operator complex, unless the upstream border of the complex is positioned within approximately 20 bp of the initiation site (6). Phage polymerase elongation across obstacles such as TFIIIA (7) and GAL4 (8) has also been reported. Even the presence of a tight-binding *Eco*RI mutant protein causes only partial termination of transcription by T7 and SP6 RNA polymerases *in vitro* (9). These observations suggest that regulated promoters may be differentially susceptible to derepression by transcription across the operator sequence(s).

We have explored the potential impact of this type of derepression on an artificial repressor–operator interaction wherein a nucleic acid ligand binds to an operator sequence in duplex DNA by triple-helix formation, thereby blocking access to a promoter. A simple model of this type has been created and tested *in vitro* (10). In this model system, DNA or RNA oligonucleotides inhibit a T7 RNA polymerase promoter by binding to an overlapping homopurine operator sequence (10, 11). Inhibition arises through the steric occlusion of polymerase by the triple-helical complex, and occurs after addition of operator-specific oligonucleotide, even if polymerase has prior access to the DNA template. However, inhibition appears to act only at the level of transcription initiation; triple-helical complexes positioned far downstream from the promoter do not detectably block T7 RNA polymerase elongation.

To further characterize this artificial repressor–operator system, we determined if transcription across an inhibitory triple-helix restores the activity of the T7 promoter. We report that the activity of such a promoter is restored when elongating phage polymerases (initiating either upstream or downstream from the regulated T7 promoter) transcribe across triple-helices at the operator sequence.

MATERIALS AND METHODS

Materials

[α-^{32}P]CTP was purchased from Amersham. Restriction endonucleases, T7 and SP6 RNA polymerases and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs. Ribonucleoside triphosphates and glycogen were purchased from Boehringer Mannheim Biochemicals. RNase inhibitor was purchased from Stratagene.

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Transcription templates and oligonucleotides

The construction of plasmid pA1 has been previously described (10). Transcription templates were prepared by digestion of plasmid pA1 DNA with the indicated restriction endonucleases. When necessary, any resulting 3′ extensions were removed by treatment of the templates with the Klenow fragment of DNA polymerase I in the absence of deoxyribonucleoside triphosphates. Oligodeoxyribonucleotides were prepared, purified, and quantitated as previously described (10).

In vitro transcription

RNA polymerase transcription reactions (20 μL) contained linearized template DNA (100 ng; ca. 2 nM), ATP, GTP, and UTP (each at 0.5 mM), CTP (0.05 mM), [α-^{32}P]CTP (1 μCi; 400 Ci/mmol), dithiothreitol (10 mM), and RNase inhibitor (0.25 unit). Transcription reactions contained sodium chloride (10 mM), spermidine trihydrochloride (2 mM), magnesium chloride (10 mM), and Tris-hydrochloride (40 mM). The pH of the ten-fold buffer concentrate was 8.0 at 22°C. When indicated, oligonucleotide was added as a concentrated stock solution in water, and reactions were incubated at 37°C for 90 min prior to addition of T7 RNA polymerase. The total T7 and SP6 promoter concentrations in these experiments were 4 nM and 2 nM, respectively. Transcription was initiated by addition of T7 RNA polymerase (85 ng; 35 nM), and/or the indicated number of units of SP6 RNA polymerase, followed by 30-min incubation at 37°C.

Transcript analysis

Transcription reactions were terminated by addition of 180 μL of a solution containing glycogen (10 μg) and ammonium acetate (4 M). Labeled RNA transcripts were precipitated with ethanol and analyzed by electrophoresis on denaturing 5% polyacrylamide gels in 0.5× TBE buffer, followed by drying and autoradiography using Kodak XAR X-ray film. RNA transcripts were quantitated by scintillation counting of excised gel fragments. Specific inhibition of transcription from the test promoter is expressed in terms of a transcription index, F, allowing normalization to internal control transcripts (10). The resulting *F* values for transcription range from 0 (complete repression of the test promoter) to 1.0 (no repression of the test promoter).

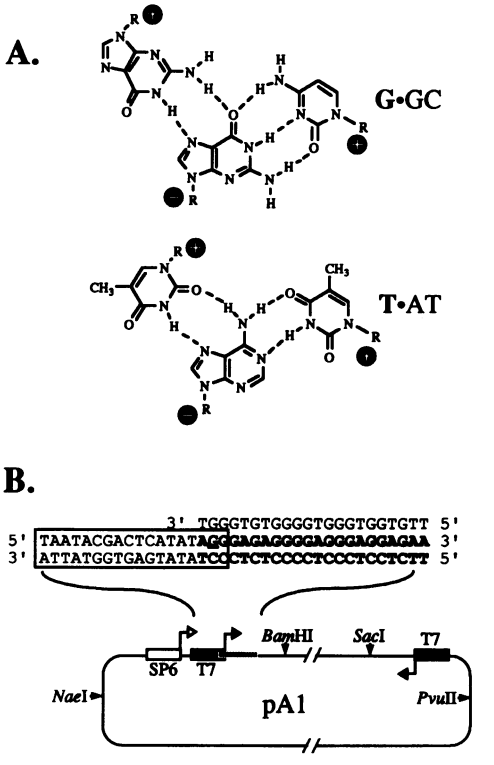

Figure 1. Experimental Design. **(A)** Base triplets involved in operator recognition by oligonucleotides. Filled circles indicate strand polarities. **(B)** Sequences of bacteriophage T7 promoter (19 bp class III consensus sequence, boxed); operator (22 bp homopurine sequence, bold letters); and repressor oligonucleotide (22 nt). This test promoter (filled box, arrow, and stippled line in lower schematic) was positioned 12 bp downstream from the transcription initiation site of an unregulated SP6 promoter (open box and arrow) that transcribes the same DNA strand. An unregulated T7 promoter (filled box and arrow) directing convergent transcription of the opposite DNA strand was positioned 460 bp downstream. Relevant restriction sites are indicated.

RESULTS

Experimental design

Previous experiments have shown that placement of a G-rich homopurine operator sequence in an overlapping configuration relative to a T7 RNA polymerase promoter allowed specific inhibition of the promoter by oligonucleotide binding (10). Oligonucleotide recognition of one such operator derives from the formation of base triplets in a pattern termed the 'purine motif' (Fig. 1A). To determine how oligonucleotide inhibition of the test promoter was affected by transcription elongation across the operator, potentially disruptive bacteriophage RNA polymerases were allowed to initiate transcription upstream or downstream from the operator (Fig. 1B). An SP6 promoter directs transcription initiating 29 bp upstream from the initiation site of the test T7 promoter, using the same DNA template strand. An additional T7 polymerase promoter lies 460 bp downstream from the test promoter, directing convergent transcripts utilizing the opposite DNA strand as template. By selection of appropriate restriction endonuclease(s) for template cleavage, phage promoters could be selectively coupled or uncoupled. Two kinds of experiments were performed. First, activity of the test promoter was measured during coupled transcription of opposite DNA strands by T7 RNA polymerase initiating from convergent promoters. Second, activity of the test T7 promoter was measured during coupled transcription of the same strand initiating from an upstream SP6 promoter.

Relief of promoter inhibition by convergent elongation

We wished to determine if transcription of the purine strand of the operator would restore the activity of the test T7 promoter. Restriction endonucleases were used to cleave plasmid pA1 in three different ways. The resulting templates and T7 RNA polymerase transcripts are shown schematically below Fig. 2. Template cleavage by *Bam*HI uncouples the convergent T7 promoters resulting in a 140 nt transcript initiating from the test promoter (subject to oligonucleotide inhibition), and a constitutive 320 nt transcript. As has been previously described (10), initiation of the 140 nt transcript is inhibited by oligonucleotide binding (compare lanes 1 and 2 of Fig 2.). As expected, promoter inhibition by oligonucleotide binding is still observed after

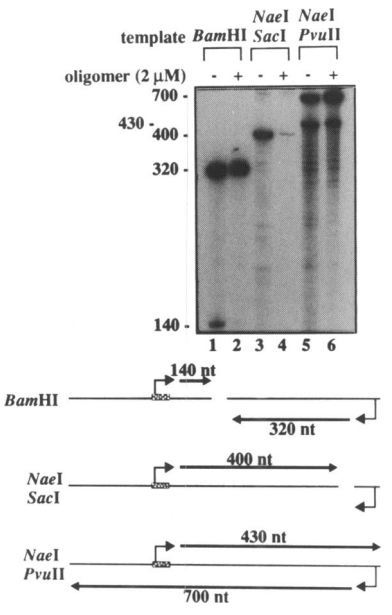

Figure 2. Relief of inhibition by convergent elongation. Transcription templates (see lower schematic) were prepared by digesting plasmid pA1 with the indicated restriction endonuclease(s). Thin lines with filled arrows indicate T7 transcription initiation sites. Stippled lines indicate homopurine operators. Bold arrows indicate RNA transcripts. Addition of T7 RNA polymerase resulted in transcripts shown in lanes 1, 3, and 5. Template incubation with inhibitory oligonucleotide (2 μM) prior to addition of T7 RNA polymerase produced the transcripts shown in lanes 2, 4, and 6.

template cleavage by *Nae*I and *Sac*I, which uncouple the promoters such that only the 400 nt transcript initiating from the test promoter can be visualized (lanes 3 and 4 of Fig. 2).

In contrast to the promoter-specific inhibition observed for uncoupled promoters, oligonucleotide inhibition of the test promoter cannot be detected when the T7 promoters remain coupled. This effect can be observed in the case of templates treated with *Nae*I and *Pvu*II (compare levels of 430 nt transcripts in lanes 5 and 6 of Fig. 2). Thus, transcription elongation across the homopurine strand of the operator substantially restores the activity of the test promoter.

In addition to the restoration of promoter activity by incoming polymerases initiating downstream, evidence for premature termination of transcription can be seen in the form of ladders of shorter RNA products below the 430 nt transcript in lanes 5 and 6 of Fig 2. Such premature termination products may reflect transcriptional interference between the promoters (5).

Relief of promoter inhibition by collinear elongation

Experiments were designed to explore how transcription elongation across the homopyrimidine strand of the operator affects the activity of the test promoter. Template linearization by *Bam*HI digestion results in the transcription map shown schematically below Fig. 3A. Two T7 promoters are oriented divergently, with the test and constitutive promoters directing synthesis of 140 nt and 320 nt transcripts, respectively. In addition, a 168 nt transcript can initiate from an SP6 promoter just upstream of the test T7 promoter. The spacing of the SP6 initiation site (about three helical turns of DNA upstream of the operator) suggests that SP6 RNA polymerase will encounter the

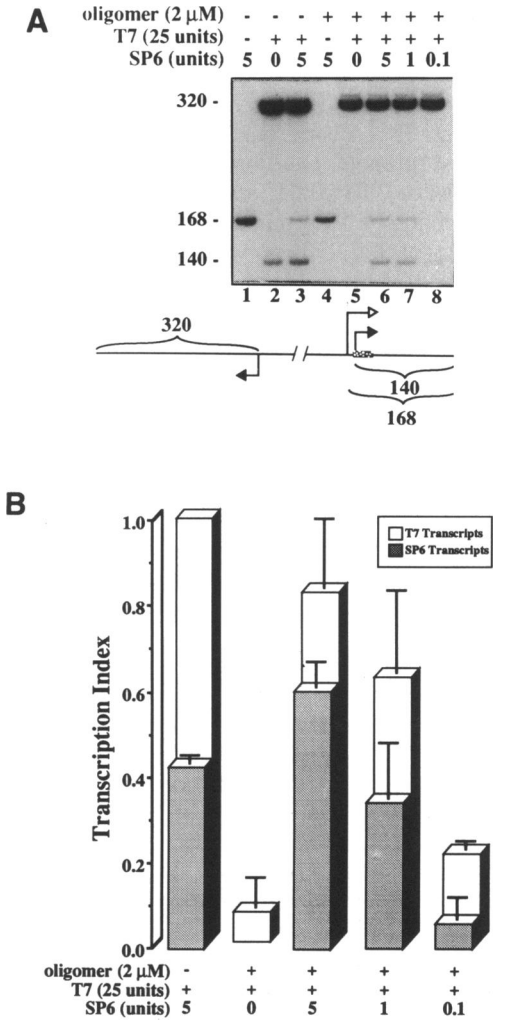

Figure 3. Relief of inhibition by collinear elongation. **(A)** Transcription template (see lower schematic) was prepared by digesting plasmid pA1 with *Bam*HI. Filled and open arrows indicate T7 and SP6 transcription initiation sites, respectively. Stippled line indicates homopurine operator. Lengths of T7 transcripts (320 nt and 140 nt) and SP6 transcript (168 nt) are indicated. **(B)** Quantitation of promoter activity. Transcription index is described in Materials and Methods. T7 and SP6 transcript levels are indicated by open and filled columns, respectively. Data reflect averages (+ standard error of the mean) from two experiments.

operator during elongation rather than abortive cycling. Transcription experiments were performed using this template and T7 RNA polymerase in the presence of different concentrations of SP6 RNA polymerase. The result of such an experiment is shown in Fig. 3A. Data based on the quantitation of two experiments of this type are shown in Fig. 3B.

Incubation of the template with SP6 RNA polymerase (5 units) results in a 168 nt transcript (Fig 3A, lane 1). Transcription by T7 RNA polymerase (25 units) produces 140 nt and 320 nt transcripts (lane 2). Template incubation with both polymerases produces all three transcripts (lane 3). Oligonucleotide binding at the operator does not inhibit transcription elongation from the upstream SP6 promoter (lane 4), but selectively reduces transcription initiating from the test promoter in the absence of SP6 RNA polymerase (lane 5). When both polymerases are added after oligonucleotide binding to the template, little promoter-specific inhibition is observed (compare lanes 3 and 6).

Relief of test promoter inhibition decreased with decreasing concentrations of SP6 RNA polymerase (Fig. 3A, lanes 6–8). SP6 RNA polymerase levels of 5, 1, and 0.1 units produced average SP6 transcript indices of 0.60, 0.34, and 0.06, respectively (Fig. 3B). A corresponding reduction in transcription from the T7 test promoter was observed under these conditions (average indices of 0.81, 0.61, and 0.20; Fig. 3B). Thus, the extent of restoration of T7 promoter activity appears to depend on the frequency of SP6 RNA polymerase elongation across the pyrimidine strand of the operator.

DISCUSSION

We have previously shown that whereas triple-helical DNA complexes can strongly inhibit transcription initiation by bacteriophage T7 RNA polymerase at a T7 promoter, such complexes are destabilized by elongating T7 or SP6 RNA polymerases (10). We now report that oligonucleotide inhibition of a T7 promoter is relieved when such elongating phage polymerases (initiating either upstream or downstream from the regulated T7 promoter) transcribe across DNA triple-helices at the operator sequence.

In our test system, relief of inhibition is caused by incoming T7 or SP6 RNA polymerases. Transcription elongation by these polymerases has been shown to proceed with high efficiency through DNA-bound obstacles including triple-helical complexes (10) and a variety of proteins (6–9). In contrast, eukaryotic RNA polymerase II has been reported to transiently pause at triple-helical complexes (12). The effect of such complexes on *E.coli* RNA polymerase remains to be studied. The sensitivity of triple-helical complexes to disruption by phage polymerase elongation is therefore likely to be an extreme case. Nonetheless, our studies show that although they can act as strong inhibitors of RNA polymerase initiation, triple-helical complexes may be sensitive to disruption by transcription elongation directed by upstream or downstream promoters.

The orientation of bacteriophage promoters in our studies allows us to conclude that transcription of either strand of the homopurine/homopyrimidine operator leads to relief of promoter inhibition by a triple-helix. This observation argues against the possibility that repressor oligonucleotides remain bound to the purine strand of the operator when the pyrimidine strand acts as the template.

Previous experiments indicate that oligonucleotide-directed DNA triple-helix formation is an intrinsically slow process. This is true for complexes based on T·AT and C+·GC triplets (pyrimidine motif) and for complexes based on G·GC and T·AT triplets such as the repressor-operator described here (13, 14). T7 RNA polymerase association with promoter DNA is likely to occur with a higher rate constant than oligonucleotide binding. Although repressor oligonucleotides can fully block transcription initiation from an isolated T7 promoter in the presence of T7 RNA polymerase (10), continual operator clearance by incoming polymerases produces a constant binding competition between T7 RNA polymerase and inhibitory oligonucleotide. Loss of triple-helix inhibition probably reflects the higher association rate of T7 RNA polymerase relative to oligonucleotides under these concentration conditions. Restoration of promoter activity by increasing concentrations of disruptive polymerase (Fig. 3) can be interpreted in light of these kinetic considerations. If transcription elongation across the operator is sufficiently rare, the oligonucleotide can rebind the operator, maintaining inhibition.

The intermolecular triple-helix under study in these experiments amounts to an artificial repressor–operator system derived totally from nucleic acid components. However, our observation that two linked promoters influence one another positively (derepression by disruption of a repressor–operator complex) could also apply to natural gene regulation. Such interactions might arise in either prokaryotes or eukaryotes when adjacent transcription units are not separated by a strong terminator. Elongating RNA polymerases might thereby disrupt regulatory proteins bound at downstream promoters. Like DNA replication, such a process could facilitate rapid gene activation or repression by making the downstream promoter constantly sensitive to changes in the pool of free transcriptional regulatory factors.

From the perspective of artificial repressor design, the results reported here establish an important constraint on artificial repressors derived from intermolecular triple-helix formation. In particular, the triple-helix repressor–operator interaction may be subject to antagonism by *cis* elements (other promoters) acting at a distance. Unless additional stabilizing factors exist (eg. triple-helix binding proteins or covalent modification chemistries), artificial gene regulation by triple-helical complexes may require that target sequences are located in control regions that are seldom transcribed. It is not trivial to meet this requirement in prokaryotic systems, and disruptive transcription may also represent a significant problem in eukaryotes. Therefore, the activities of artificial repressor–operator complexes such as those described here may best be investigated in genetic constructs that have been insulated by terminators in order to prevent disruptive transcription of the operator.

ACKNOWLEDGEMENTS

This work was supported, in part, by a Young Investigator's Award from Abbott Laboratories, and by Grant 5 P30 CA36727-08 from the National Cancer Institute.

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