Inhibition of T7 RNA Polymerase Initiation by Triple-Helical DNA Complexes: A Model for Artificial Gene Repression†

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Received February 19, 1992; Revised Manuscript Received May 27, 1992

ABSTRACT: An experimental approach is presented for the creation of an artificial and functional repressor/operator interaction that does not involve polypeptides. This in vitro approach confers oligonucleotide regulation upon a bacteriophage T7 RNA polymerase promoter by introducing an overlapping homopurine operator that can be recognized by oligonucleotide-directed DNA triple-helix formation. Recognition of optimized operator sequences in either of two triple-helix motifs is shown to efficiently inhibit T7 RNA polymerase transcription initiation in both a promoter- and oligonucleotide-specific manner. Inhibition due to triple helices of the pyrimidine motif is pH-dependent, as expected. Inhibition by purine motif triple helices is not pH-dependent and occurs efficiently under optimum T7 RNA polymerase transcription conditions. Repression by triple-helix formation can be observed rapidly after addition of purine motif repressor oligonucleotides, even when polymerase has been given prior access to the promoter. The mechanism of repression is shown to be occlusion of polymerase from the promoter rather than trapping of the polymerase in unproductive preinitiation or initiation complexes. In contrast to their inhibition of T7 RNA polymerase initiation, the triple-helical complexes studied here do not detectably inhibit transcription elongation.

Promoter-specific control of transcription initiation is fundamental to cellular growth and differentiation. In eukaryotes, positive and negative influences on the rate of promoter utilization by RNA polymerase II arise from the complex interplay of multiple polypeptide transcription factors and repressors, the DNA double helix, other chromatin elements, and various small molecules (Lewin, 1990; Saltzman & Weinmann, 1989; Mitchell & Tjian, 1989; Levine & Manley, 1989).

The regulatory interactions responsible for controlling transcription initiation in prokaryotic operons provide models for understanding eukaryotic gene regulation. In particular, the mechanistic basis for transcriptional repression in prokaryotes has been well studied (Lewin, 1990; Collodo-vides et al., 1991). In some cases, steric exclusion of RNA polymerase from a promoter by the presence of a bound repressor may play a fundamental role in this process. Steric factors take the form of incompatible protein–protein interactions or higher-order nucleoprotein conformations that exclude RNA polymerase (Adhya, 1989). In other cases, RNA polymerase is able to bind to the repressed promoter but cannot engage in productive elongation (Lee & Goldfarb, 1991).

Despite the intricacy of transcriptional regulation, the design of artificial repressors is an attractive, albeit challenging, problem in molecular biology and chemistry. Such designs might eventually lead to rational approaches for gene-targeted chemotherapeutics (Kosturko et al., 1979; Nielsen, 1991; Maher et al., 1991; Hélène, 1991; Durland et al., 1991b).

One chemical approach to repressor design involves oligonucleotide-directed DNA triple-helix formation, wherein a pyrimidine-rich (pyrimidine motif; T·A-T and C+G-C triplets) or a purine-rich (purine motif; T·A-T and G·G-C triplets) oligonucleotide binds to a homopurine sequence in the major groove of double-helical DNA. This method allows highly sequence-specific DNA recognition (Moser & Dervan, 1987; Praseuth et al., 1988; Cooney et al., 1988; Dervan, 1990; Mergny et al., 1991). Base triplets and strand orientations for the two triple-helix motifs are shown in Figure 1A. Stabilization of pyrimidine motif triple-helical complexes by cytosine protonation generally requires slightly acidic pH. This requirement does not pertain to the purine motif. Triple-helical complexes are thermodynamically stable near physiological conditions with half-lives of several hours (Maher et al., 1990; Plum et al., 1990; Durland et al., 1991a). Moreover, such complexes can inhibit DNA binding proteins (Maher et al., 1989; François et al., 1989; Hanvey et al., 1989), and can repress eukaryotic promoters in vitro (Cooney et al., 1988; Maher et al., 1992). Evidence has been presented to suggest that such complexes can form and alter gene expression after exposure of intact cells to oligonucleotides (Birg et al., 1990; Postel et al., 1991; Orson et al., 1991). Thus, oligonucleotide-directed DNA triple-helix formation continues to present an interesting strategy for creating artificial, sequence-specific regulators of DNA function.

The current work involves creation of an artificial and functional repressor/operator interaction based on DNA triple-helix formation rather than polypeptide binding. The in vitro approach taken here confers oligonucleotide regulation upon a bacteriophage T7 RNA polymerase promoter by introducing an overlapping homopurine operator for oligonucleotide-directed DNA triple-helix formation. T7 RNA polymerase is a small, single-subunit enzyme of M_r ca. 100 000 (Chamberlin & Ryan, 1982). It is highly selective for its cognate promoter, and its transcription rate and efficiency in vitro and in vivo have been widely exploited (e.g., Studier et al., 1990). These characteristics make it a particularly useful tool for the model repression experiments reported here.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals. Radiochemicals ([α-^{32}P]CTP, [α-^{32}P]dATP, and [α-^{32}P]dGTP) were purchased from Am-

† Supported in part by Grant 5 P30 CA36727-08 from the National Cancer Institute.

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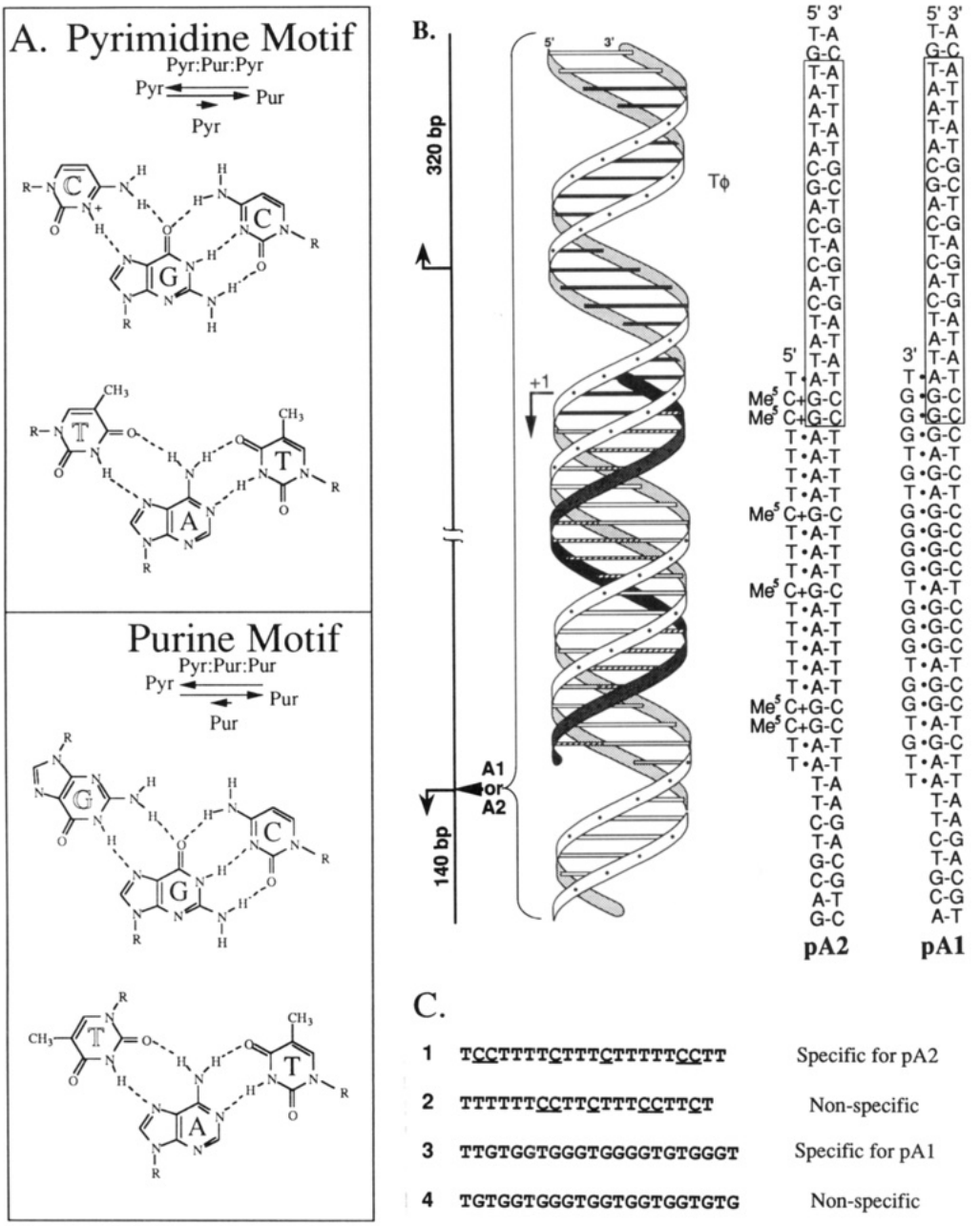

FIGURE 1: Schematic design of repressor/operator interaction. (A) Nucleic acid base triplets conferring triple-helix specificity. Strand orientations are shown above. The third strand (open letters) binds to the DNA duplex (filled letters) in the major groove. (B) When linearized by digestion with *Bam*HI as shown, plasmids pA1 and pA2 each contain two 19-bp bacteriophage T7 RNA polymerase promoters (arrows at left) that direct divergent transcripts. For each plasmid, one of the two promoters (upper boxes at right) overlaps a homopurine operator sequence that can bind an oligonucleotide ligand by triple-helix formation (dark strand in ribbon diagram). The 21-bp, A-rich homopurine operator of pA2 binds a pyrimidine oligonucleotide to form a pH-sensitive triple helix based on the pyrimidine motif. The 22-bp, G-rich homopurine operator of pA1 binds a G-rich oligonucleotide to form a pH-insensitive triple helix based on the purine motif. Relative strand orientations are indicated above each sequence at right. (C) Oligodeoxyribonucleotides tested as site-specific repressors of T7 RNA polymerase transcription initiation (underlining indicates 5-methylcytosine residues). Oligomer **1** binds specifically to the homopurine operator of pA2. Oligomer **3** binds specifically to the homopurine operator of pA1. Oligomers **2** and **4** are nonspecific controls with sequence compositions similar to oligomers **1** and **3**, respectively.

ersham. *Escherichia coli* DNA polymerase I Klenow fragment, T4 DNA ligase, polynucleotide kinase, restriction endonucleases, and T7 RNA polymerase were purchased from New England Biolabs. Unlabeled ribo- and deoxyribonucleoside triphosphates were purchased from Boehringer Mannheim Biochemicals. RQ1 RNase-free DNase I and RNase inhibitor were purchased from Promega and Stratagene, respectively. MPE[1] was the generous gift of P. B. Dervan (California Institute of Technology).

Transcription Templates. Plasmid pG$_5$E4T (Lin et al., 1988; Maher et al., 1992), herein termed pA0, contains the T7 RNA polymerase promoter from plasmid pGEM-3 (Promega) upstream of a recombinant RNA polymerase II promoter and adenovirus E4 gene sequences. Plasmids pA1 and pA2 were constructed by insertion into pA0 of oligonucleotide duplexes between proximal *Hin*dIII and *Pst*I sites located 397 bp from the existing T7 RNA polymerase promoter. Ths inserted sequence for pA1 (T7 promoter in

[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; MPE-Fe(II), methidiumpropyl-EDTA-Fe(II); nt, nucleotide(s); Tris-acetate, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol acetate; Tris-hydrochloride, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol chloride.

bold, homopurine operator underlined) is

AGCTG**TAATACGACTCACTATAGG**GAGAGGGGAGGGAGGAGAATTCTGCA
C**ATTATGCTGAGTGATATCC**CTCTCCCCTCCCTCCTCTTAAG

The inserted sequence for pA2 is

AGCTG**TAATACGACTCACTATAGG**AAAAGAAAGAAAAAGGAATTCTGCA
C**ATTATGCTGAGTGATATCC**TTTTCTTTCTTTTTCCTTAAG

These insertions each carry a 19-bp T7 RNA polymerase promoter (class III consensus sequence) and overlapping homopurine operator sequence (see Figure 1B). The inserts are oriented so that transcription of plasmids linearized with *Bam*HI yields T7 RNA polymerase runoff transcripts of 140 and 320 nt.

Oligonucleotides. Oligodeoxyribonucleotides were prepared, purified, and quantitated as previously described (Maher et al., 1990).

In Vitro Transcription. T7 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 (50 nM), [α-^{32}P]CTP (1 μCi; 400 Ci/mmol), dithiothreitol (10 mM), and RNase inhibitor (0.25 unit). Transcription reactions at pH 6.8 contained sodium chloride (70 mM), spermine tetrahydrochloride (0.4 mM), magnesium chloride (20 nM), Tris-acetate (25 mM), and bovine serum albumin (100 μg/mL). Transcription reactions at pH 8.0 contained sodium chloride (10 mM), spermidine trihydrochloride (2 mM), magnesium chloride (10 mM), and Tris-hydrochloride (40 mM). Buffer pH refers to the 10-fold concentrate at 22 °C. When indicated, oligonucleotide was added at 0.5 μM final concentration and reactions were incubated at 22 °C for 30 min prior to addition of T7 RNA polymerase. The total T7 promoter concentration in these experiments was ca. 4 nM, with the inhibiting oligonucleotide present in 250-fold excess. Transcription was initiated by addition of T7 RNA polymerase (85 ng; ca. 35 nM), followed by 30-min incubation at 37 °C.

Transcript Analysis. Transcription reactions were terminated by addition of 180 μL of a solution containing tRNA (1 μg) and ammonium acetate (4 M). After extraction with phenol–chloroform (1:1), labeled RNA transcripts were precipitated with ethanol and analyzed by electrophoresis on denaturing 5% polyacrylamide gels in 0.5× TBE buffer (Sambrook et al., 1989), followed by drying and autoradiography using Kodak XAR X-ray film. RNA transcripts were quantitated by scintillation counting of excised gel fragments. The value of *P*, the proportion of total incorporated label corresponding to the test transcript, was calculated for each lane using

$$P = [\mathrm{cpm}_{(140\mathrm{nt})}]/[\mathrm{cpm}_{(140\mathrm{nt})} + \mathrm{cpm}_{(320\mathrm{nt})}]$$

where cpm indicates radioactive counts per minute. Selective repression of the test transcript (140 nt) relative to the internal control transcript (320 nt) was then quantitated by calculation of an index, *F*, defined as

$$F = (P_t - P_{bg})/(P_c - P_{bg})$$

where subscripts t, bg, and c refer to the test lane, a lane representing pA0 (i.e., background signal at 140-nt position), and a lane representing the test promoter in the absence of added oligonucleotide, respectively. Thus, *F* values for transcription from pA0 (no 140-nt transcript) and for transcription from the test promoter in the absence of triple-helix formation are defined to be 0 and 1.0, respectively.

Footprinting. Plasmid pA1 was cleaved with *Bam*HI and *Kpn*I, and the resulting 2587-bp fragment was isolated. The fragment was uniquely labeled at the *Bam*HI site (140 bp from the homopurine operator sequence) by treatment with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dGTP, [α-^{32}P]dATP, dTTP, and dCTP.

DNase I footprinting reactions (25 μL) contained 70 ng of end-labeled restriction fragment (0.04 pmol; ca. 2 nM), sodium chloride (10 mM), magnesium chloride (10 mM), GTP (0.4 mM), Tris-hydrochloride, pH 8.0 (40 mM), and spermidine trihydrochloride (2 mM). Where indicated, oligonucleotide (0.2 μM final concentration) was incubated with the labeled DNA for 30 min. T7 RNA polymerase (340 ng; ca. 138 nM) was added to the labeled DNA, as indicated, and allowed to bind for 10 min at 37 °C prior to addition of DNase I. To each sample was added 2.5 μL of calcium chloride (100 mM) and 1.5 μL (0.033 unit) of DNase I. After 90 s at 22 °C, the reactions were terminated by addition of 75 μL of a stop solution (0.2 M sodium chloride, 0.03 M EDTA, 1% sarcosine, and 100 μg/mL tRNA). After extraction with phenol–chloroform (1:1), the labeled DNA was precipitated with ethanol.

MPE footprinting reactions (10 μL) contained 70 ng of end-labeled restriction fragment (0.04 pmol), sodium chloride (10 mM), magnesium chloride (6 mM), Tris-hydrochloride, pH 8.0 (10 mM), and dithiothreitol (4 mM). Where indicated, oligonucleotide (0.2 μM final concentration) was incubated with the labeled DNA for 30 min. T7 RNA polymerase (136 ng; ca. 138 nM) was added to the labeled DNA, as indicated, and allowed to bind for 5 min at 37 °C prior to addition of MPE-Fe(II). GTP (0.4 mM) was added in some cases to allow formation of initiation complexes. MPE-Fe(II) was prepared by incubating equal volumes of MPE solution (1 mM) and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (2 mM) for 5 min followed by dilution to a final concentration of 50 μM MPE. MPE-Fe(II) (1 μL) was added to each binding reaction and allowed to react for 3.5 min at ambient temperature. Reactions were terminated by addition of 90 μL of a solution containing sodium acetate (0.1 M), EDTA (0.016 M) and glycogen (20 μg), followed by precipitation with ethanol.

Samples were subjected to electrophoresis in a denaturing 5% polyacrylamide sequencing gel. The gel was dried and analyzed by autoradiography. A purine sequence ladder was generated by performing an A+G chemical cleavage reaction on the labeled DNA fragment (Maxam & Gilbert, 1980).

RESULTS

Experimental Design. The experimental design for this study is summarized in Figure 1. Plasmids pA1 and pA2 each contain an unmodified bacteriophage T7 RNA polymerase promoter that serves as an unregulated internal control in transcription experiments. In addition to this internal control promoter, each plasmid also contains a second T7 RNA polymerase promoter that overlaps a homopurine operator sequence for regulation by triple-helix formation. In plasmid pA1, the 22-bp homopurine operator was designed to be G-rich, a requirement for recognition by the antiparallel binding of a G-rich oligomer in the purine motif (Beal & Dervan, 1991; Durland et al., 1991a). In plasmid pA2, the 21-bp homopurine operator was designed to be A-rich, permitting recognition by the parallel binding of a T-rich, homopyrimidine oligomer in the pyrimidine motif (Moser & Dervan, 1987). When the plasmid is linearized by treatment with *Bam*HI and transcribed by T7 RNA polymerase in vitro, distinct runoff transcripts of 320 and 140 nt are produced by

the internal control promoter and test promoter, respectively. The ability of triple-helical DNA complexes at the homopurine operators of these plasmids to specifically repress transcription initiation from the test promoters was judged by transcript quantitation. An index was computed to normalize the test transcript signal to the internal control transcript signal (see Experimental Procedures). Alternative plasmid linearization sites allow investigation of the effects of triple-helical DNA complexes on transcription elongation by T7 RNA polymerase (see below).

Four oligodeoxyribonucleotides were studied as repressors (Figure 1B). Oligomers **1** and **2** were tested for binding to the homopurine operator of pA2. Oligomer **1** is designed to be specific for this operator, while nonspecific control oligomer **2** has a similar nucleotide composition but in scrambled sequence. Oligomers **1** and **2** contain 5-methylcytosine substitutions, shown to stabilize triple-helix formation in the pyrimidine motif (Povsic & Dervan, 1989; Maher et al., 1991). Oligomers **3** and **4** were tested for binding to the homopurine operator of pA1. Oligomer **3** is designed to be specific for this operator, while nonspecific control oligomer **4** has a similar nucleotide composition but in scrambled sequence.

T7 Promoter Repression. Plasmids pA1 and pA2 were linearized and incubated with or without 0.5 μM oligonucleotide under conditions that support triple-helix formation. T7 RNA polymerase was then added and transcription was allowed to proceed for 30 min. The result of such an experiment is shown in lanes 1–14 of Figure 2A. The 320-nt transcript is the sole product of pA0 (lanes 1 and 8), whereas transcription of pA2 and pA1 in the absence of oligonucleotides yields both 320- and 140-nt products at pH 6.8 (lanes 2 and 5, respectively). Similar results were obtained at pH 8.0 (lanes 9 and 12).

At pH 6.8, triple-helix formation at the homopurine operators of pA1 and pA2 selectively reduced transcription from the test promoter by 99.7% and 92%, respectively (lanes 3 and 6). Oligomers of similar nucleotide composition but scrambled sequence did not cause inhibition (lanes 4 and 7).

At pH 8.0 (under conditions considered optimal for T7 RNA polymerase activity), transcription from the test promoter of pA1 (purine motif) in the presence of specific oligomer **3** was again inhibited by 95% (lane 10). As at pH 6.8, nonspecific oligomer **4** had no effect on transcription from the test promoter (lane 11). In contrast to results obtained at pH 6.8, transcription of the test promoter of pA2 was not inhibited by specific or nonspecific oligomers at pH 8.0 (lanes 12–14).

The results of several experiments of this type are summarized in Figure 2B. The *F* index for plasmids with no test promoter is defined as 0, and for no oligomer added, defined as 1.0. As indicated by the bar graph, transcription from the test promoter of pA1 (operator recognized in the purine motif) is inhibited in an oligomer-dependent, pH-independent manner. This result demonstrates that the pH-independent purine motif can be employed to regulate T7 initiation under standard in vitro transcription conditions. Transcription from the test promoter of pA2 (operator recognized in the pyrimidine motif) is inhibited in an oligomer- and pH-dependent manner. This result is consistent with the typical protonation requirements of pyrimidine motif triple helices (Moser & Dervan, 1987; Maher et al., 1990). Mechanistic aspects of the latter type of repression by purine motif triple helices were further characterized as described below.

Competition between Polymerase and Oligonucleotide Repressor. Inhibition of DNA binding proteins by triple-helical DNA complexes has generally been assayed after the

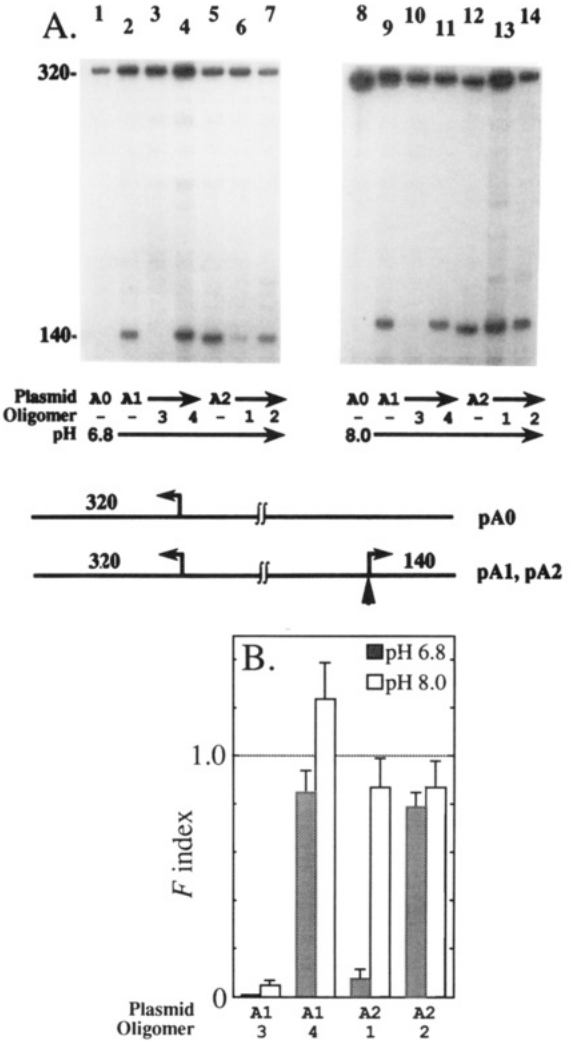

FIGURE 2: Promoter-specific repression of T7 RNA polymerase transcription initiation by triple-helical complexes. (A) The indicated oligonucleotides and template DNA (linearized as shown below) were incubated at pH 6.8 (lanes 1–7) or pH 8.0 (lanes 8–14), transcribed by T7 RNA polymerase, and analyzed as described in Experimental Procedures. (B) Data derived from at least three experiments. The *F* index was calculated as described in Experimental Procedures. Error bars indicate the standard error of the mean.

prebinding of oligonucleotides to homopurine targets (Maher et al., 1989, 1992; Hanvey et al., 1989; François et al., 1989). Depending on oligonucleotide concentration, prebinding reactions of 0.5–24 h are usually performed to overcome the relatively slow kinetics of triple-helix formation, particularly when competition with a high-affinity DNA binding protein is involved (Maher et al., 1990). It was therefore of interest to determine if an oligonucleotide ligand could repress transcription from the pA1 test promoter even if added *after* incubation of the promoter with T7 RNA polymerase and ribonucleoside triphosphates. The results of such an experiment are shown in Figure 3. The experimental protocol is shown in panel A. Transcription of the pA1 template was initiated by incubation at 37 °C with T7 RNA polymerase and unlabeled ribonucleoside triphosphates for 5 min. Oligonucleotide was then added at 1 μM final concentration and allowed to compete with polymerase for template binding for 0–40 min. After this varied competition period, 30-min labeling reactions were initiated by addition of [α-^{32}P]CTP, followed by transcript isolation and analysis. Lanes 1–3 of Figure 3B show results obtained after oligonucleotide prebinding. As in the experiment shown in Figure 2, complete

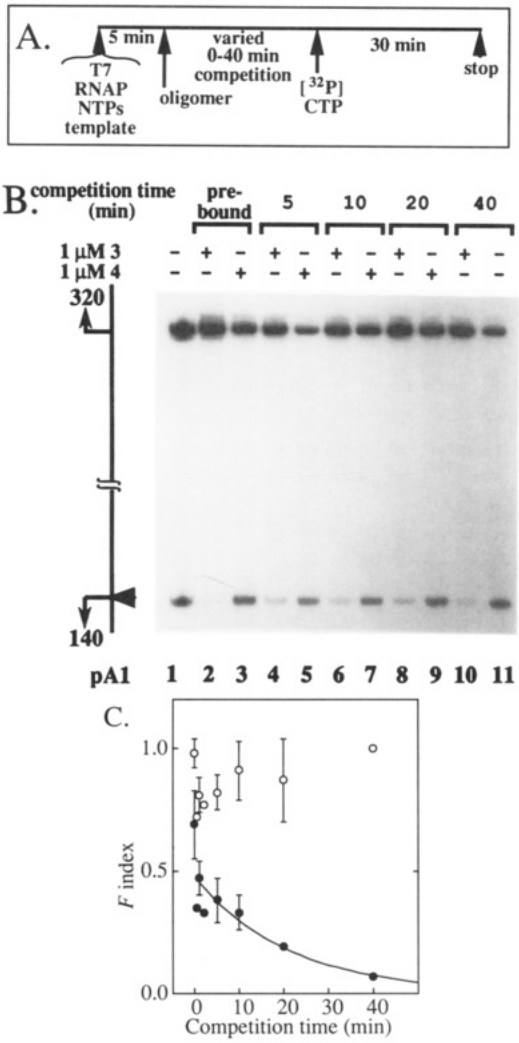

FIGURE 3: Competition between polymerase and oligonucleotide repressor. (A) Schematic illustration of repression protocol. T7 RNA polymerase was allowed to transcribe from promoters on the linearized pA1 template for 5 min at pH 8.0. Specific or nonspecific oligomer was then added to give 1 μM final concentration. After varied (0–40 min) competition times, [^{32}P]CTP was added for a further 30 min to label new transcripts. T7 RNAP indicates T7 RNA polymerase. (B) Electrophoretic analysis of transcripts labeled for 30 min after exposure of template to T7 RNA polymerase and oligomer for the indicated times. (C) Pooled data from multiple experiments. The *F* index was calculated as described in Experimental Procedures. Error bars indicate the standard error of the mean. (●) Oligomer **3**; (○) oligomer **4**.

repression of the test promoter was observed under these conditions. In lanes 4–11, oligomer was added after T7 RNA polymerase. Lane 4 shows that substantial repression is observed when the 30-min labeling reaction is initiated as little as 5 min after oligomer addition. If oligonucleotide and T7 RNA polymerase are allowed to compete for up to 40 min prior to the labeling reaction, transcription from the test promoter is almost completely repressed (lanes 6, 8, and 10). Nonspecific oligomer **4** does not repress transcription in this assay (lanes 5, 7, 9 and 11). Pooled data from multiple experiments of this type are illustrated graphically in Figure 3C. A control experiment demonstrated that the apparent rate of triple-helix formation was not increased by the presence of initiating polymerase (data not shown). Thus, promoter-specific repression by triple-helix formation occurs in the presence of T7 RNA polymerase and does not require oligonucleotide prebinding to the operator.

Mechanism of Repression. The observation that triple-helical complexes at the homopurine operators of pA1 and pA2 can repress T7 RNA polymerase initiation from the corresponding test promoters raises a mechanistic question. Repression could result, in principle, from either (i) exclusion of the polymerase from the promoter, (ii) trapping of the polymerase in an unproductive preinitiation complex, or (iii) trapping of the polymerase in an unproductive initiation complex. Footprinting procedures were employed to distinguish between these mechanisms. The results of such an experiment with pA1 are shown in Figure 4A. Binding of oligomer **3** to the homopurine operator at the test promoter confers DNase I resistance to the entire operator and an additional 3 bp of the T7 promoter (compare lanes 2 and 3). Oligomer **4** does not cause a DNase I footprint (lane 4). In the presence of GTP, T7 RNA polymerase creates a DNase I footprint that covers the entire 19-bp promoter as well as 8–9 bp downstream of the initiation site (lane 5). Binding of oligomer **3** prior to incubation with T7 RNA polymerase completely abolishes the polymerase footprint (lane 6), while nonspecific oligomer **4** had no effect on polymerase binding (lane 7). These results indicate that oligonucleotide-directed triple-helix formation at the homopurine operator prevents formation of initiation complexes by T7 RNA polymerase. However, DNase I does not sensitively detect T7 RNA polymerase preinitiation complexes formed in the absence of GTP. Therefore, MPE-Fe(II) footprinting was employed to discern whether the triple-helical complexes acted by blocking formation of a preinitiation complex (Gunderson et al., 1987). A typical result is shown in Figure 4B. In the absence of GTP, formation of a preinitiation complex by T7 RNA polymerase can readily be detected by MPE-Fe(II) between -16 and -4 relative to the site of initiation (lane 11). Upon formation of an initiation complex, the MPE-Fe(II) footprint extends downstream to +8 (lane 12). In the presence of specific oligomer **3**, the MPE-Fe(II) footprint is centered on the homopurine operator (lane 13). When both T7 RNA polymerase and oligomer **3** are present, no polymerase footprint can be detected in either the absence (lane 14) or presence (lane 15) of GTP. As in Figure 4A, nonspecific oligomer **4** does not affect polymerase binding (data not shown), and previous results (Figures 2 and 3) confirm that none of the molecular assemblies at the test promoter of Figure 4 has any effects on the internal control promoter. Thus, it appears that triple-helix formation at the homopurine operator of pA1 represses transcription initiation by blocking polymerase binding to the promoter rather than by trapping bound polymerase in unproductive preinitiation or initiation complexes.

Effects on Transcription Elongation. The preceding experiments show that triple-helical complexes spanning approximately two turns of the DNA helix and impinging on a T7 promoter sequence can block transcription initiation by T7 RNA polymerase. What are the effects of such complexes on an elongating polymerase? This question was addressed by linearizing plasmids pA1 and pA2 as shown in Figure 5. This approach allows RNA polymerase molecules initiating at the internal control promoter to encounter triple-helical complexes that are repressing the test promoter 397 bp downstream. Lanes 1 and 2 of Figure 5 provide molecular weight standards for the expected runoff transcripts (670 nt) or the truncated transcripts (397 nt) expected to arise if transcription terminates at the triple-helical complex (wherein the third strand is hydrogen-bonded to the template strand). As shown in lanes 3 and 4, no 397-nt transcripts are observed upon transcription through the triple-helical complexes. Thus, neither purine motif nor pyrimidine motif complexes cause detectable termination of T7 RNA polymerase elongation

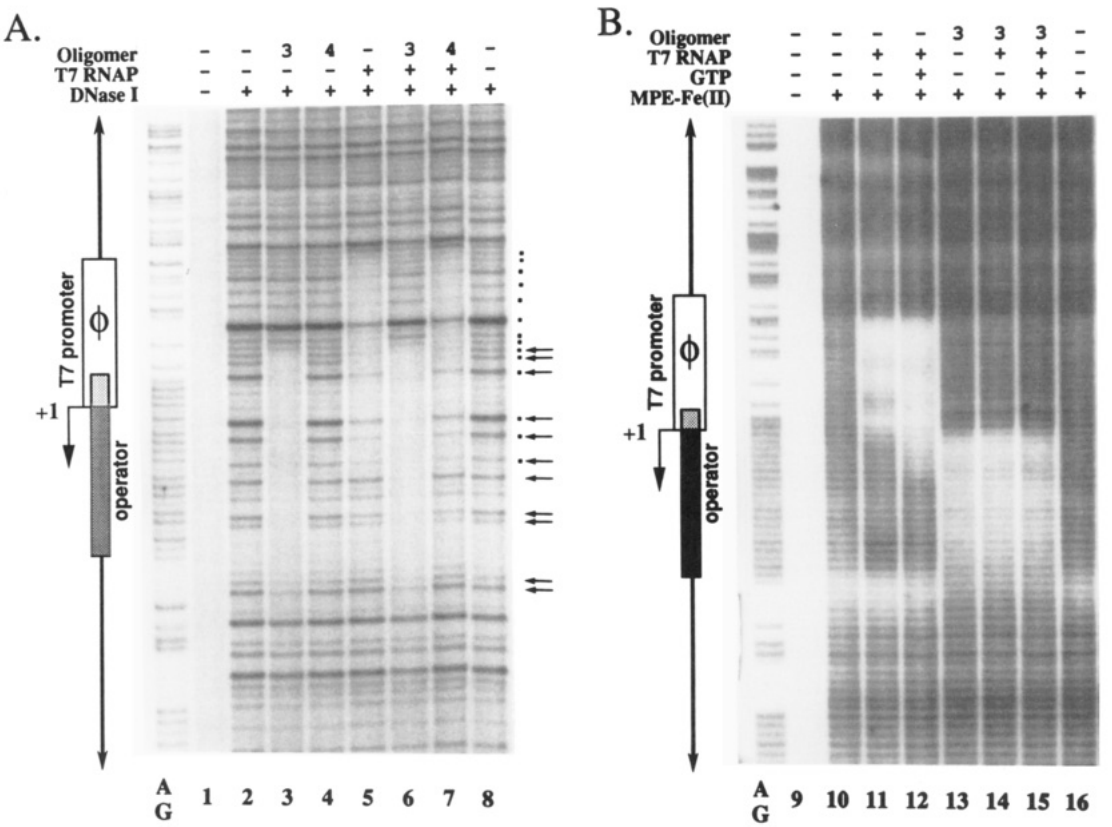

FIGURE 4: Exclusion of T7 RNA polymerase binding by triple-helix formation at the homopurine operator. End-labeled pA1 DNA was incubated in binding reactions with oligomer and/or T7 RNA polymerase, as indicated. Conditions for DNase I footprinting (panel A) and MPE-Fe(II) footprinting (panel B) are described in Experimental Procedures. The promoter sequence map is aligned at left of each panel (ϕ, T7 promoter; arrow, transcription initiation site; shaded bar, homopurine operator). A purine sequence ladder is displayed left of lanes 1 and 9. Symbols to the right of panel A indicate bands diagnostic for binding of oligomers (←) and T7 RNA polymerase (•). T7 RNAP indicates T7 RNA polymerase.

under conditions where they inhibit initiation almost quantitatively. This observation suggests that the elongating T7 RNA polymerase complex possesses a significant helix-destabilizing activity that is not observed during promoter recognition.

Although triple helices did not block transcription elongation, careful examination of the autoradiogram in Figure 5 reveals an increase in background radioactivity (transcripts shorter and longer than full length) in lanes 3 and 4. This phenomenon may be caused by a low level of transcription initiating from the test promoter. Although the test promoter is almost quantitatively repressed under these conditions (Figure 3), transcription complexes initiating from the internal control promoter presumably destabilize the triple helices as they elongate across the operator. This process may transiently reveal the test promoter and allow interference between the resulting convergent transcription units.

DISCUSSION

Inhibition of T7 RNA Polymerase Initiation. The experiments reported here describe a triple-helical DNA complex positioned to overlap part of a T7 RNA polymerase promoter. Guanosine-rich (pA1) and adenosine-rich (pA2) operators were designed to support stable triple helices in the purine and pyrimidine motifs, respectively. The data demonstrate that such complexes can substantially repress the T7 promoter in vitro. These results extend seminal observations concerning the repressive properties of polymeric triple helices first published by Morgan and Wells more than two decades ago (Morgan & Wells, 1968).

The T7 RNA polymerase repression described here is both promoter- and oligonucleotide-specific. Consistent with the apparent requirement for cytosine protonation, pyrimidine motif triple helices on plasmid pA2 repress transcription initiation under slightly acidic conditions (pH 6.8) but not at pH 8. That repression by pyrimidine motif triple helices requires slightly acidic conditions demonstrates the potential for T7 promoter regulation by [OH^-], in analogy with *lac* operon regulation by [allolactose]. In contrast, oligonucleotides bound to the homopurine operator of plasmid pA1 in the purine motif repress transcription at either pH and function at submicromolar concentrations under optimum T7 RNA polymerase transcription conditions.

In addition, purine motif complexes are shown to form and repress transcription even in the presence of active T7 RNA polymerase. The ability of an oligonucleotide to successfully compete with a preexisting DNA binding protein for occupation of mutually exclusive DNA sites has not previously been demonstrated. In most prior cases of direct competition between DNA binding proteins and triple helices, preformation of the latter complex has been required (Maher et al., 1989, 1992). It seems likely that the ability of oligomer **3** to effectively compete with T7 RNA polymerase for occupancy of the test promoter of pA1 in the current experiments reflects (i) the relative concentrations of the two ligands (ca. 30-fold molar excess oligomer relative to polymerase, (ii) the fairly weak equilibrium binding constant for the polymerase ($\leq 10^7$ M^{-1}; Gunderson et al., 1987) versus oligonucleotide ($\geq 10^8$ M^{-1}; Maher et al., 1990; Durland et al., 1991a), and (iii) the fact that the polymerase is mobile and vacates the promoter in a cyclical fashion as transcription proceeds. These observations suggest that inhibition of RNA polymerase II initiation in eukaryotes might similarly be achieved by oligonucleotide binding to operators overlapping the polymerase binding site,

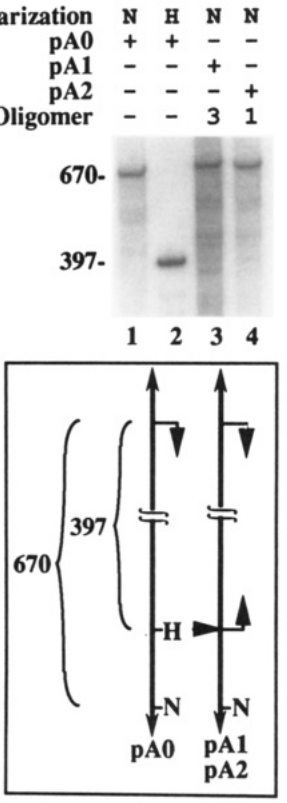

FIGURE 5: T7 RNA polymerase transcription elongation is not blocked by triple-helical complexes. Template DNA (see schematic diagram below) was linearized with the indicated restriction endonuclease, incubated with the indicated oligomer, and transcribed by T7 RNA polymerase as described in Experimental Procedures. The *Hin*dIII site (H) and *Nae*I site (N) of pA0 are located 397 and 670 bp downstream of the internal control T7 promoter, respectively. Thus, the *Nae*I site is 273 bp downstream of the triple-helix target site. The pH values of transcription buffers in lanes 3 and 4 were 8.0 and 6.8, respectively.

in addition to strategies targeting sites occupied by more stably bound activating proteins (Maher et al., 1989, 1992).

Mechanism of Repression. Footprinting experiments indicate that the mechanism of repression is exclusion of polymerase from the promoter rather than capture of the polymerase in unproductive preinitiation or initiation complexes. Thus, the artificial repressor appears to function in a manner historically ascribed to *lac* repressor, namely, steric hindrance (Majors, 1975). Interestingly, this mechanism appears not to be the actual basis for *lac* repressor function (Lee & Goldfarb, 1991) but may play a role in other prokaryotic and eukaryotic switches (Ptashne, 1986). Besides a direct steric effect, the triple-helical complex may prevent polymerase binding by causing a helix distortion over a short distance beyond the operator or by inducing local stiffening effects (Maher et al., 1992). In this regard, it is of interest to note that the DNase I footprint (but not the MPE footprint) due to triple-helix formation at the test promoter of pA1 (Figure 4) extends at least 4 bp further into the T7 promoter than that predicted for the operator sequence per se.

Failure of Triple Helices To Block Transcription Elongation. In marked contrast to their effects on transcription initiation, triple-helical complexes did not detectably block T7 RNA polymerase elongation when positioned downstream from the promoter. These results are consistent with previous observations that showed a lack of T7 RNA polymerase termination at sites of noncovalent oligonucleotide binding to either the template or nontemplate DNA strands (Maher, Povsic, Wold, and Dervan, unpublished observations). It is notable that the initiation and elongation phases of unrelated polymerization reactions such as protein biosynthesis have also been shown to be differentially sensitive to certain structural obstacles. Thus, scanning eukaryotic ribosomal subunits are inhibited by the binding of antisense oligonucleotides to mRNA sequences upstream of the initiation codon, while elongating ribosomes are not blocked by similar complexes (Maher & Dolnick, 1988).

It is interesting to compare the observed failure of triple-helical complexes to block T7 RNA polymerase elongation with results obtained using other RNA polymerases and molecular barriers. For example, whereas transcription elongation by *E. coli* RNA polymerase was completely blocked by the binding of noncatalytic *Eco*RI mutants, T7 RNA polymerase proceeded quite efficiently through such complexes (Pavco & Steege, 1991). Moreover, *lac* repressor has been shown to act as a barrier to elongation by *E. coli* RNA polymerase and eukaryotic RNA polymerase II, whereas the bound protein does not cause termination of T7 RNA polymerase elongation (Deuschle et al., 1986, 1990; Sellitti et al., 1987; Giordano et al., 1989; Dubendorff & Studier, 1991). In fact, T7 RNA polymerase has been shown to elongate through a variety of complexes, including mammalian RNA polymerase III, *Xenopus* 5S transcription complex, and nucleosomes (Pavco & Steege, 1991). It has recently been reported that certain (noncovalent) triple-helical DNA complexes transiently inhibit transcription elongation by human RNA polymerase II (Young et al., 1991b). These results suggest a possible hierarchical relationship between RNA polymerases in terms of their abilities to elongate through molecular barriers (T7 RNA polymerase > *E. coli* RNA polymerase ≥ human RNA polymerase II). The observed transcription rates for these enzymes also reflect this order (Pavco + Steege, 1991; Thummel, 1992).

Conclusions. The model system presented here shows that a functional repressor/operator interaction based only on nucleic acid components can be achieved in vitro. This observation suggests that similar nucleic acid interactions might be employed to create novel gene regulatory circuitry in vivo. This work also reinforces the interesting proposition that nucleic acids or ribonucleoproteins might play a natural role in certain sequence-specific DNA interactions that regulate transcription initiation (Miller & Sobell, 1966; Britten & Davidson, 1969; Minton, 1985; Davis et al., 1989; Beru et al., 1990; Young et al., 1991a).

ACKNOWLEDGMENT

I thank J. Harcia for suggesting this model system, J. Skoog for excellent technical assistance, P. Dervan for MPE, and B. Gold, M. Hollingsworth, and S. Rhode for comments on the manuscript.

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Registry No. RNA polymerase, 9014-24-8.