Nucleic Acids Research, 1993, Vol. 21, No. 9 2131–2138

Repression of bacteriophage promoters by DNA and RNA oligonucleotides

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Received January 21, 1993; Revised and Accepted March 29, 1993

ABSTRACT

We are interested in creating artificial gene repressors based on duplex DNA recognition by nucleic acids rather than polypeptides. An *in vitro* model system involving repression of bacteriophage T7 RNA polymerase initiation has been employed to demonstrate that certain DNA oligonucleotides can repress transcription by site-specific triple-helix formation at two kinds of homopurine operator sequences [Maher, L. J., III, (1992) *Biochemistry* 31, 7587–7594]. Recognition in the purine motif is based on antiparallel oligonucleotide binding (G·G·C and T·A·T triplets). Recognition in the pyrimidine motif is based on parallel oligonucleotide binding (C^+·G·C and T·A·T base triplets). Using this system, we report that the concentration-dependence of repression by DNA oligonucleotides provides triple-helix inhibition constant (K_i) estimates of approximately 2×10^{-7} M for both purine motif and pyrimidine motif DNA complexes. RNA oligonucleotides are shown to repress promoters overlapping pyrimidine motif operators ($K_i = 6 \times 10^{-7}$ M), but not purine motif operators. Although competent to hybridize to complementary single strands, RNA oligonucleotides fail to bind the purine motif operator. Partial substitution of deoxyribose residues tends to rescue repressor activity by RNA oligonucleotides in the purine motif. These results suggest prospects for, and constraints on, natural and artificial RNA-based repressors.

INTRODUCTION

Control of transcription initiation is of fundamental importance in the regulation of gene expression. Negative transcriptional regulation often results from the binding of one or more repressor proteins to *cis*-acting promoter elements in a manner that reduces the rate of initiation by RNA polymerase. We are interested in exploring the design of site-specific transcriptional repressors based on nucleic acids (DNA or RNA) that can recognize regulatory sequences in duplex DNA. Such artificial repressors are of interest both for their therapeutic potential (1–3), and as models for exploring possible roles of ribonucleoproteins in transcriptional regulation (4–6).

One recent approach to repressor design involves oligonucleotide-directed triple-helix formation, wherein a pyrimidine (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 strategy permits sequence-specific DNA recognition (7–11). Probable hydrogen-bonding patterns and strand orientations for the two triple-helix motifs are shown in Fig. 1. Stabilization of pyrimidine motif triple-helical complexes by cytosine protonation requires slightly acidic pH, and is favored by substitution of 5-methylcytosine (12, 13). This requirement does not pertain to the purine motif. Triple-helical complexes are thermodynamically stable near physiological conditions with half-lives of several hours (14–16). Moreover, such complexes can inhibit DNA binding proteins (17–19), and can repress eukaryotic promoters *in vitro* (9, 20). Evidence has been presented to suggest that such complexes can form and alter gene expression after exposure of intact cells to oligonucleotides (21–23). Thus, oligonucleotide-directed triple-helix formation represents a possible strategy for designing artificial, sequence-specific regulators of DNA function.

With this goal in mind, we recently described a cell-free model system wherein oligodeoxyribonucleotide regulation was conferred upon a bacteriophage T7 RNA polymerase promoter by the introduction of an overlapping homopurine operator that could be recognized by oligonucleotide-directed DNA triple-helix formation (24). A schematic illustration of this model system is shown in Fig. 2A. After incubation of the duplex DNA template with 0.5 μM DNA oligonucleotide, recognition of operator sequences in either of the two triple-helix motifs caused inhibition of T7 RNA polymerase transcription initiation in a manner that was both promoter- and oligonucleotide-specific. Inhibition due to triple-helices of the pyrimidine motif was found to require slightly acidic pH, as expected. Inhibition by purine motif triple-helices did not require acidic pH, and was observed under optimum T7 RNA polymerase transcription conditions. Repression by triple-helix formation occurred rapidly after addition of purine motif repressor oligonucleotides, even when

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polymerase had been given prior access to the promoter. Footprinting analyses showed that repression results from occlusion of T7 RNA polymerase from the promoter by the triple-helix.

We now use this model T7 RNA polymerase repression system to address two questions. First, what is the concentration-dependence of promoter repression by DNA oligonucleotides? Second, can RNA oligonucleotides act as transcriptional repressors? Answers to these questions will be required in order to explore novel strategies for artificial gene control within living cells, wherein designed transcripts from a regulatory gene bind and repress a target promoter in *trans*.

MATERIALS AND METHODS

Materials

Radiochemicals ([α-^{32}P]CTP, [α-^{32}P]dATP, [α-^{32}P]dGTP) were purchased from Amersham. *E.coli* DNA polymerase I Klenow fragment, T4 DNA ligase, polynucleotide kinase, restriction endonucleases, and T7 RNA polymerase were purchased from New England Biolabs. Ribo- and deoxyribonucleoside triphosphates and glycogen were purchased from Boehringer Mannheim Biochemicals. RNase inhibitor was purchased from Stratagene. Monomers for RNA synthesis (base-protected *tert*-butyl dimethylsilyl β-cyanoethyl phosphoramidites) were purchased from MilliGen/Biosearch. Solid supports for RNA synthesis and deoxyuridine phosphoramidite were purchased from Glen Research. Tetrabutylammonium fluoride (1M in tetrahydrofuran) was purchased from Aldrich.

Transcription templates

Construction of plasmids pA1 and pA2 has been previously described (24). Briefly, synthetic oligonucleotide duplexes were inserted between proximal *Hin*dIII and *Pst*I sites located 397 bp from the existing T7 RNA polymerase promoter of plasmid pA0. These insertions each carry a 19-bp T7 RNA polymerase promoter (class III consensus sequence) and overlapping homopurine operator sequence (see Figure 2A). The inserts are oriented so that transcription of plasmids linearized with *Bam*HI yields T7 RNA polymerase run-off transcripts of 140 nt and 320 nt.

Oligonucleotides

Oligodeoxyribonucleotides were prepared, purified, and quantitated as previously described (14). Oligoribonucleotides and oligomers containing both ribose and deoxyribose sugars were synthesized at 1 μmole scale by phosphoramidite chemistry on an Applied Biosystems 380B synthesizer, with cycle modifications as suggested by the instrument manufacturer. Oligomers were removed from the solid support and partially deprotected by 16 h treatment with concentrated ammonia:ethanol (3:1) at 55°C. The dried residue was treated for 48 h with a 0.5 mL solution of 1 M tetrabutylammonium fluoride in tetrahydrofuran. After addition of 0.5 mL of 2 M triethylammonium acetate (pH 7.0) and 1 mL water, the oligomers were desalted by chromatography over P6 gel (BioRad). Fractions containing the oligomer were dried. The oligomers were then purified by electrophoresis through a 20% acrylamide gel containing 7 M urea, followed by ultraviolet shadowing and band excision. After elution overnight into 0.3 M ammonium acetate, oligonucleotides were desalted using NENsorb™ cartridges, as directed by the manufacturer (Dupont). RNA concentrations were calculated using the following molar extinction coefficients at 260 nm ($M^{-1}\cdot cm^{-1}$): 15 400 (A), 7 300 (C), 11 700 (G), 9 900 (U).

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 (0.05 mM), [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 mM), 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 ten-fold concentrate at 22°C. Transcription of a control template by T7 RNA polymerase is reduced 2 to 3-fold under the pH 6.8 reaction conditions relative to the pH 8.0 reaction conditions. 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 promoter concentration in these experiments was ca. 4 nM. 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 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. The value of P, the proportion of total incorporated label corresponding to the test transcript, was calculated for each lane using the following definition:

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

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 follows:

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

where subscripts t, bg, and c refer to the test lane, a background signal at the 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. Inhibition constants for triple-helical complexes were estimated using a two-state model:

$$\text{operator} + \text{oligomer} <=> \text{triple-helix}$$

by fitting binding data to the isotherm:

$$\theta = [O]/(K_i + [O])$$

where θ is the fraction of operator bound into a triple-helix, O is free oligonucleotide concentration, and K_i is the equilibrium inhibition constant for the triple-helical complex. Fitting was performed using KaleidaGraph™ (Abelbeck Software). This analysis is based on a model wherein promoter repression is taken

to be a direct measure of oligonucleotide binding ($K_i = K_d$). It remains possible that repressing oligomers can be displaced from the operator at a low rate by RNA polymerase competition at the promoter, in which case $K_i > K_d$.

RESULTS

Experimental design

The experimental design for this study is shown in Figure 2A (24). When linearized by *Bam*HI, plasmids pA1 and pA2 direct the synthesis of run-off transcripts from two T7 RNA polymerase promoters. A 320 nt transcript is produced from a constitutive T7 promoter. A 140 nt transcript is produced from a second T7 promoter that can be regulated by oligonucleotide binding to an overlapping homopurine operator sequence. Triple-helix formation at the operator has previously been shown to repress transcription initiation by occlusion of polymerase from the promoter (24).

The sequences of DNA, RNA, and mixed DNA/RNA oligonucleotides studied as repressors are indicated in Fig. 2B. The intended operator specificity of each oligonucleotide is listed. Specific oligomers were designed to bind duplex DNA operators by forming continuous base triplets in the purine motif (pA1 operator) or in the pyrimidine motif (pA2 operator). Nonspecific oligomers were designed to have a similar base composition but

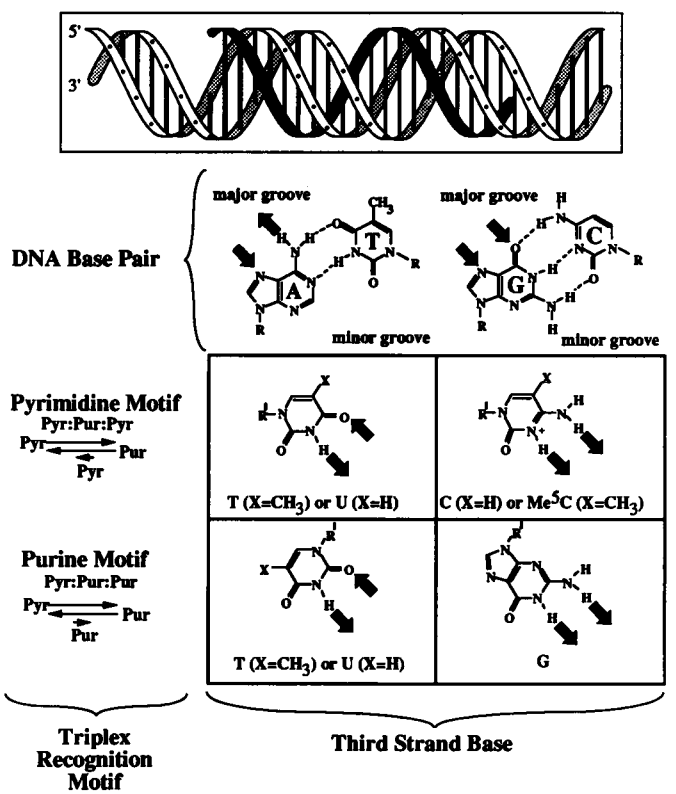
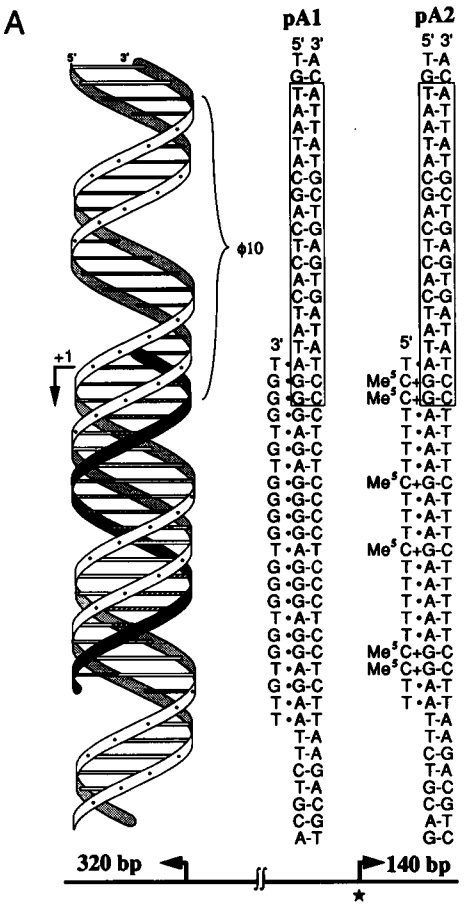


Figure 1. DNA recognition by triple-helix formation. Purine bases in homopurine DNA sequences can be recognized by oligonucleotide-directed triple-helix formation, forming site-specific complexes (diagram above chart). Specificity arises from the formation of specific base triplets involving the indicated hydrogen bond donor and acceptor groups (filled arrows). Chart depicts strand orientations (left) and third strand base interactions that were assumed in designing in the triple-helical complexes studied in this report. R, deoxyribose; R', ribose or deoxyribose.



B

Code	Sequence (5'-3')	Intended Specificity
D1	TCCTTTTCTTTCTTTTTCCTT	specific pA2
D2	TTTTTTCCTTCTTTCCTTCT	nonspecific pA2
D3	TTGTGGTGGGTGGGGTGTGGGT	specific pA1
D4	TGTGGTGGGTGGTGGTGGTGTG	nonspecific pA1
D5	AAGAGGAGGGAGGGGAGAGGGA	specific pA1
D6	ACCCACACCCCACCCACCACAA	complement of R3
D7	UUGUGGUGGGUGGGGUGUGGGU	specific pA1
R1	UCCUUUUCUUUCUUUUUCCUU	specific pA2
R2	UUUUUUCCUUCUUUCCUUCU	nonspecific pA2
R3	UUGUGGUGGGUGGGGUGUGGGU	specific pA1
R4	UGGGUGUGGGGUGGGGUGGUGUU	nonspecific pA1
RD3	TTGTGGTGGGTGGGGTGTGGGT	specific pA1
DR3	UUGUGGUGGGUGGGGUGUGGGT	specific pA1

Figure 2. Bacteriophage T7 promoter/operator constructs and repressor oligonucleotides. (A) Two copies of the ϕ10 promoter from bacteriophage T7 are present on each linearized plasmid (arrows in schematic below). One of the ϕ10 promoters (boxes in enlargement above, star in schematic below) overlaps a homopurine operator sequence that can bind oligonucleotide ligands by triple-helix formation (dark strand in ribbon diagram). For example, the 22-bp, G-rich operator of plasmid pA1 binds a G-rich oligodeoxyribonucleotide to form a pH-insensitive triple-helix (purine motif). The 21-bp, A-rich homopurine operator of pA2 binds a pyrimidine oligodeoxyribonucleotide under mildly acidic conditions. When compared to transcription from the linked constitutive ϕ10 promoter, transcription from the modified promoter reflects operator occupancy by oligonucleotide repressor. (B) Oligonucleotides tested in this study. Code designations: D, DNA; R, RNA; DR or RD, oligomers composed of purine (first letter) and pyrimidine (second letter) residues of the indicated sugar. Single underlining indicates 5-methylcytosine. Double underlining indicates ribose residues. The intended specificities of the oligomers are indicated at right.

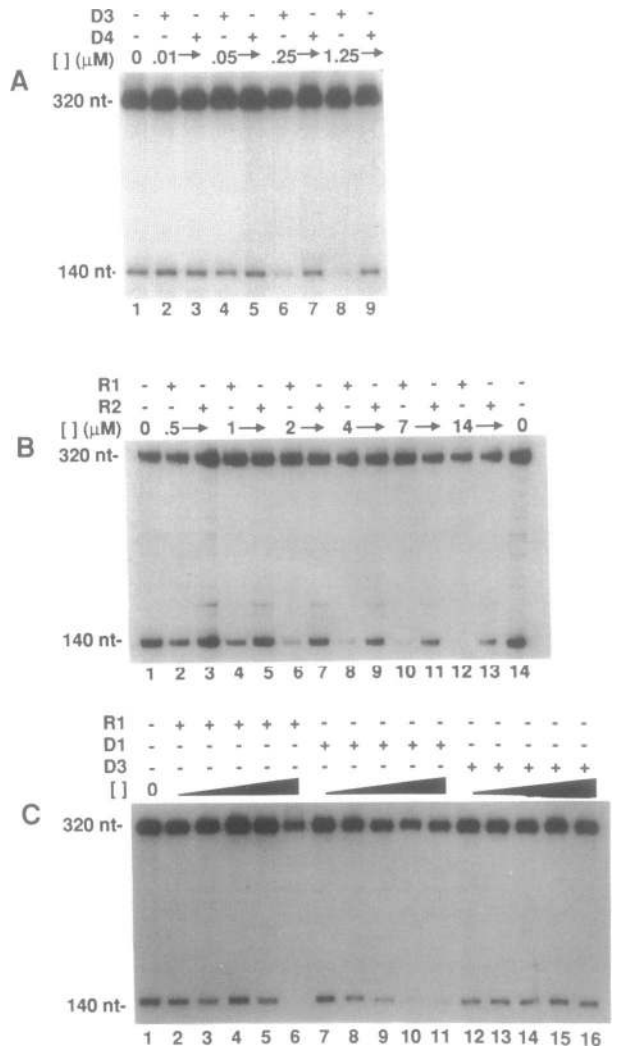


Figure 3. Examples of concentration-dependent transcriptional repression by oligonucleotides. Plasmid DNA was linearized by digestion with *Bam*HI, incubated with oligonucleotide at the indicated concentration for 90 min at 22°C, and transcribed by T7 RNA polymerase for 30 min at 37°C. Labeled transcripts were separated by electrophoresis and autoradiographed. Detailed reaction conditions are given in Experimental Procedures. Positions of internal control transcript (320 nt) and test transcript (140 nt) are indicated at left. A) Comparison of T7 transcription products after incubation of plasmid pA1 with oligomers D3 and D4 at pH 8.0. B) Comparison of T7 transcription products after incubation of plasmid pA2 with oligomers R1 and R2 at pH 6.8. C) Comparison of T7 transcription products after incubation of plasmid pA2 with oligomers R1, D1, and D3 at pH 6.8. Each oligomer was tested at concentrations of 0.01 μM (lanes 2, 7, 12), 0.05 μM (lanes 3, 8, 13), 0.25 μM (lanes 4, 9, 14), 1.25 μM (lanes 5, 10, 15), and 6.25 μM (lanes 6, 11, 16).

scrambled sequence relative to specific oligonucleotides. Oligonucleotides were assembled at various concentrations with linearized template DNA in a transcription buffer containing both Mg^{2+} and spermidine^{3+} or spermine^{4+}. Triple-helix formation was allowed to occur for 90 min at either pH 6.8 (pA2) or pH 8 (pA1). Templates were then transcribed *in vitro* by addition of T7 RNA polymerase.

The extent of repression was judged by transcript quantitation. A transcription index, *F*, was then computed (see Experimental Procedures). This index is useful for three reasons. First,

Table I. Representative transcription data

Plasmid[a]	Oligomer[b]	[Oligomer][c]	transcripts[d] 140 nt	transcripts[d] 320 nt	*F*[e]
pA1	none	0	2.21	7.76	1.00
	D3 (sp GT)	0.01	2.96	11.40	0.89
		0.05	2.15	9.64	0.79
		0.25	0.60	7.62	0.32
		1.25	0.24	7.65	0.15
	D4 (ns GT)	0.01	2.61	9.71	0.93
		0.05	2.68	11.70	0.80
		0.25	2.20	9.05	0.86
		1.25	1.52	6.23	0.89
pA2	none	0	1.28	3.18	1.00
	R1 (sp CU)	0.05	1.05	3.63	0.74
		0.25	0.23	1.92	0.37
		1.25	0.34	3.79	0.24
		6.25	0.07	1.26	0.08
	R2 (ns CU)	0.05	3.28	4.53	1.05
		0.25	2.26	3.46	1.02
		1.25	1.69	2.40	1.00
		6.25	1.05	2.87	0.78

[a]Transcription of plasmids pA1 and pA2 was performed at pH values of 8.0, and 6.8, respectively.
[b]Composition given in parentheses: sp, specific; ns, nonspecific; double underline indicates ribonucleotides.
[c]μM.
[d]Transcripts per 20 μL reaction (× 10^{11}).
[e]As defined in Materials and Methods, using P_t, P_{bg}, and P_c.

promoter-specific repression of the test promoter is reported by normalizing to transcription from an internal control promoter. This is important since nonspecific repression of the reference promoter (attributable to oligomer inhibition of T7 RNA polymerase; eg. see Table I) can sometimes be observed at high oligomer concentration. Except when such nonspecific inhibition becomes extreme, the *F* index is designed to remain a faithful measure of specific repression. Second, the index is not sensitive to intrinsic differences between levels of transcription from the test promoters (absolute transcription from the test promoter of pA1 appears somewhat weaker than from pA2, presumably due to the different sequence compositions of the transcribed operators). Third, the index is not sensitive to unavoidable imprecision in polymerase addition and sample loading prior to electrophoresis. Estimates of triple-helix inhibition constants were then obtained by fitting the repression data to a simple binding isotherm.

Examples of representative autoradiographs are shown in Fig. 3. An example of quantitative data from an experiment of this type is shown in Table I. Inhibition isotherms and deduced inhibition constants from multiple experiments are shown in Fig. 4 and Table II, respectively.

Repression by DNA oligonucleotides

Studies of pyrimidine motif complexes involving plasmid pA2 are conveniently performed in the presence of Mg^{2+} and spermine^{4+} at pH 6.8 to stabilize oligonucleotide binding by facilitating protonation of cytosine residues in the oligonucleotide strand. Studies of purine motif complexes involving plasmid pA1 are performed in the presence of Mg^{2+} and spermidine^{3+} at pH 8 (no protonation is required). Incubation of template DNA with

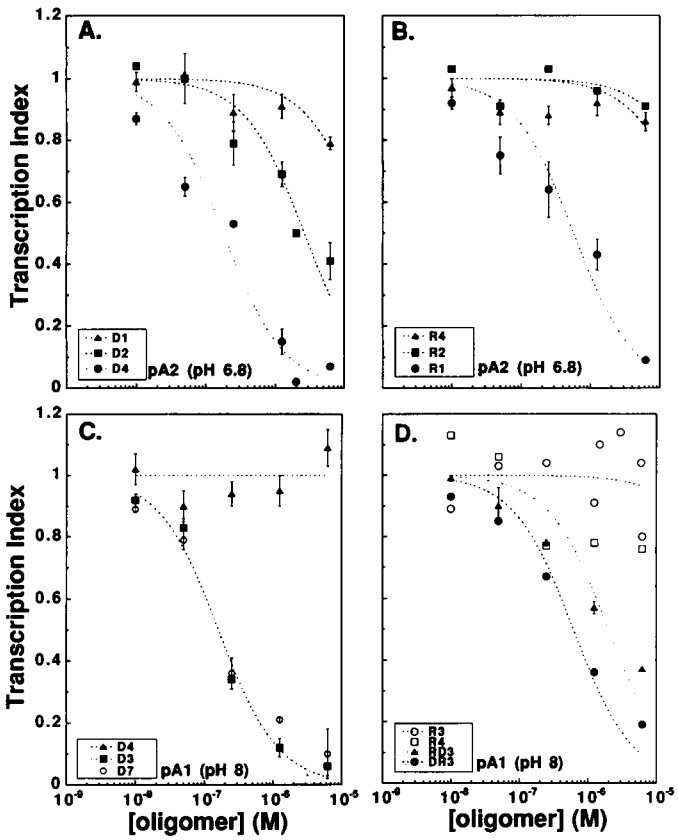


Figure 4. Repression isotherms for DNA and RNA oligonucleotides. Repression by specific and nonspecific DNA (panels A and C) and RNA (panels B and D) oligonucleotides was measured using plasmids pA2 (panels A and B) and pA1 (panels C and D). Repression by mixed DNA-RNA oligomers RD3 and DR3 is also shown in panel D. The pH of the transcription reactions is indicated in parentheses for each panel. Transcripts were quantitated by scintillation counting of gel slices. Transcription indices were calculated by normalizing repression of the experimental T7 promoter to the linked constitutive T7 promoter as described in Experimental Procedures. Curve fitting was performed as described in Experimental Procedures. Where indicated, error bars reflect the standard error of the mean for at least two experiments.

specific DNA oligonucleotides (0.5 μM) has previously been shown to result in repression of the test promoters in pA1 and pA2 under these conditions (24).

These binding interactions were further analyzed as a function of DNA oligonucleotide concentration. Examples of concentration-dependent repression of the pA1 test promoter by specific (D3) and nonspecific (D4) oligonucleotides in the purine motif are shown in Fig. 3A. Specific DNA oligonucleotide D3, but not oligonucleotide D4 (scrambled sequence) exhibits concentration-dependent repression of the test promoter (loss of 140 nt transcript relative to 320 nt transcript). The effect is observed over the concentration range 1×10^{-8} M to 1.25×10^{-6} M (Fig 3A, lanes 2–9). Similar experiments with DNA oligonucleotides D1–D4 were performed with both plasmids pA1 and pA2. Repression data of this type are plotted in panels A and C of Fig. 4, respectively. Inhibition constant estimates derived from curve-fitting are presented in Table II.

The data in panel A of Fig. 4 (summarized in Table II) show that at pH 6.8, specific DNA oligonucleotide D1 (K_i = 1.8×10^{-7} M) represses transcription from the test promoter of pA2 14-fold more effectively than nonspecific oligonucleotide D2 (scrambled sequence; K_i = 2.6×10^{-6} M). Nonspecific DNA oligonucleotide D4 (irrelevant sequence and composition) shows little repression at any concentration tested. These results suggest that the weak repressor activity of nonspecific oligonucleotide D2 might reflect the formation of one or more mismatched complexes at the pA2 operator. Such mismatched complexes presumably cannot occur for oligonucleotide D4.

The data in panel C of Fig. 4 (summarized in Table II) show that oligonucleotide D3 is a specific repressor of the test promoter of pA1 at pH 8 (K_i = 1.6×10^{-7} M), while oligonucleotide D4 has no detectable repressor activity ($K_i >> 10^{-5}$ M). Oligomer D7 (identical to D3 except for the substitution of deoxyuridine for deoxythymidine) was similar to oligomer D3 in its repression properties.

Table II. Inhibition constants from oligonucleotide repression data

Operator		Oligomer[a]	K_i (M)[b]
pA2	DNA	D4 (ns GT)	$>10^{-5}$[c]
		D2 (ns CT)	2.6×10^{-6}
		D1 (sp CT)	1.8×10^{-7}
	RNA	R4 (ns GU)	$>10^{-5}$
		R2 (ns CU)	$>10^{-5}$
		R1 (sp CU)	5.7×10^{-7}
pA1	DNA	D4 (ns GT)	$>10^{-5}$
		D3 (sp GT)	1.6×10^{-7}
		D7 (sp GU)	1.7×10^{-7}
	RNA	R3 (sp GU)	$>10^{-5}$
		R4 (ns GU)	$>10^{-5}$
	mixed DNA/RNA	RD3 (sp GT)	2.0×10^{-6}
		DR3 (sp GU)	6.2×10^{-7}

[a]Composition given in parentheses: ns, nonspecific; sp, specific; single underline indicates 5-methylcytosine; double underline indicates ribonucleotides.
[b]From least-squares analysis of repression isotherms.
[c]Highest tested oligomer concentration yields less than 50% repression.

Repression by RNA oligonucleotides

Using DNA oligonucleotide repression data as a starting point, we next compared T7 RNA polymerase repression by RNA oligonucleotides. Panels B and C of Fig. 3 show examples of data of this type. Panel B of Fig. 3 demonstrates that micromolar concentrations of specific RNA oligonucleotide R1, but not nonspecific RNA oligonucleotide R2, repress the test promoter of plasmid pA2 at pH 6.8. Panel C of Fig. 3 compares repression by specific RNA oligonucleotide R1, specific DNA oligonucleotide D1, and nonspecific DNA oligonucleotide D3. The data from RNA repression experiments of this type were quantitated and compiled in panels B and D of Fig 4. Inhibition constant estimates derived from curve fitting are presented in Table II.

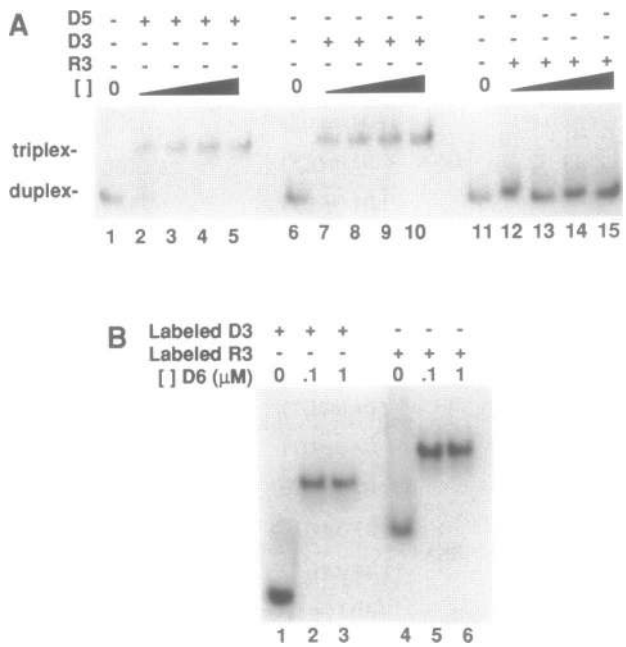


Figure 5. Analysis of triplex (panel A) and duplex (panel B) formation by DNA and RNA oligomers. **(A)** The following DNA duplex was labeled by end-filling with [^{32}P]-deoxyribonucleoside triphosphates and Klenow fragment of DNA polymerase (triplex target site underlined):

5′AGCTGTAATACGACTCACTATAGGGAGAGGGGAGGGAGGAGAATTCTGCA
3′CATTATGCTGAGTGATATCCCTCTCCCCTCCCTCCTCTTAAG

The labeled duplex DNA was incubated in transcription buffer for 2 h with 0 μM, 0.75 μM, 1.5 μM, 3 μM or 6 μM of unlabeled oligonucleotide D5 (lanes 1–5), D3 (lanes 6–10), or R3 (lanes 11–15). Duplex and triplex forms of the labeled probe were separated by electrophoresis in a 20% polyacrylamide gel using 1× TBE buffer supplemented with 20 mM $MgCl_2$. **(B)** Oligonucleotides D3 (lanes 1–3), and R3 (lanes 4–6) were radiolabeled by phosphorylation using polynucleotide kinase. Labeled oligomers were incubated in transcription buffer with 0 μM, 0.1 μM, or 1 μM unlabeled complementary oligomer D6 for 90 min at 22°C, followed by electrophoresis as in panel A.

The data in panel B of Fig. 4 confirm that repression of the test promoter of plasmid pA2 at pH 6.8 is observed only for specific RNA oligonucleotide R1. Nonspecific oligonucleotides R2 (scrambled sequence) and R4 (irrelevant sequence) showed no significant repression at the concentrations tested. As shown in Table II, the estimated K_i value for the complex of R1 at the test promoter of pA2 is 5.7×10^{-7}. This value suggests a binding interaction with RNA oligonucleotide R1 (which contains cytosine and uracil residues) that is about three-fold weaker than that involving DNA oligonucleotide D1 (which contains 5-methylcytosine and thymine residues; see Discussion).

In contrast to the promoter repression observed upon binding of RNA oligomers to the operator of plasmid pA2 in the pyrimidine motif, neither RNA oligonucleotide R3 (specific) nor R4 (nonspecific) significantly repressed the test promoter in plasmid pA1 in the purine motif (Fig. 4D). Two experiments were performed to further analyze this apparent difference.

Binding of R3 to duplex and single-stranded targets

We tested the hypothesis that RNA oligonucleotide R3 binds tightly to the target operator of plasmid pA1 in the purine motif but fails to repress the test promoter due to steric differences relative to the repressive complex involving DNA oligomer D3. An electrophoretic mobility shift assay was therefore performed to assess duplex operator binding by oligonucleotides (16). The results of this experiment are shown in Fig. 5A. We tested the binding of oligonucleotides D5 (specific for pA1 operator; G·G·C and A·A·T triplets), D3 (specific for pA1 operator; G·G·C and T·A·T triplets) and R3 (specific for pA1; G·G·C and U·A·T triplets) to a labeled duplex DNA target containing the homopurine operator from plasmid pA1. Labeled duplex probe was incubated in transcription buffer for 2 h with various concentrations of unlabeled oligonucleotide. Duplex and triplex forms of the labeled probe were separated by electrophoresis in a 20% polyacrylamide gel using 1× TBE buffer supplemented with 20 mM $MgCl_2$. Whereas DNA oligonucleotides D5 (lanes 1–5), and D3 (lanes 6–10) caused a concentration-dependent mobility shift indicating triple-helix formation, RNA oligonucleotide R3 showed no binding activity (lanes 11–15).

In principle, the lack of operator binding by RNA oligonucleotide R3 could reflect either i) a fundamental inability of this RNA to participate in a purine motif triple-helix with the DNA duplex, or ii) an artifact of chemical synthesis wherein RNA oligonucleotide R3 was unable to participate in base-pairing interactions due to some defect such as incomplete removal of protecting groups. We tested the second possibility by evaluating whether RNA oligonucleotide R3 was fully competent to bind its complement by Watson–Crick hybridization. Because the functional groups required for base-pairing contacts with duplex DNA targets are the same as those required for binding to complementary single strands (Fig. 1), the observation of hybridization activity would argue against residual chemical modification. The results of such a hybridization experiment are shown in Fig. 5B. Oligonucleotides D3 (lanes 1–3), and R3 (lanes 4–6) were radiolabeled by phosphorylation using polynucleotide kinase. Tracer amounts of labeled oligomers were incubated in transcription buffer with 0 μM, 0.1 μM, or 1 μM unlabeled complementary DNA oligomer D6 for 90 min at 22°C, followed by electrophoresis. Electrophoretic mobility shifts are observed for both DNA oligomer D3 and RNA oligomer R3. The lower mobility of purine-rich RNA vs. DNA oligomers (compare lanes 1 and 4) was observed for several different sequences. The mobility shift data demonstrate that the oligonucleotide R3 preparation is homogeneous, and that it does not contain variants that are unable to bind to the complementary sequence. Thus, RNA oligonucleotide R3 is competent to participate in base-pairing interactions. Because a denaturation step was not employed prior to the hybridization reaction, this result also tends to argue against the possibility that oligomer R3 self-associates into unproductive intra- or intermolecular complexes. The observed failure of this oligonucleotide to bind the target operator or repress transcription from plasmid pA1 may therefore reflect a fundamental inability of RNA molecules to participate in triple-helix formation at duplex DNA operators in the purine motif. This issue was further explored by analysis of the repressor activity of oligonucleotides composed of mixtures of ribose and deoxyribose nucleotides.

Repression by mixed DNA/RNA oligonucleotides

Experiments were designed to determine whether repressor activity by RNA oligonucleotide R3 could be rescued by partial deoxyribose substitution. Sequences and compositions of chimeric oligonucleotides RD3 and DR3 are listed in Fig. 2B. Both oligonucleotides are analogs of RNA oligonucleotide R3. In oligonucleotide RD3, guanosine residues bear ribose sugars while

the remaining residues are deoxythymidines. In oligonucleotide DR3, guanosine residues bear deoxyribose sugars, while the remaining residues are uridines.

Concentration-dependent repression of the test promoter of plasmid pA1 was measured as described above. Interestingly, repressor activity was ameliorated by both kinds of deoxyribose substitution (Fig. 4D and Table II). Thus, repression of the test promoter by oligonucleotide DR3 ($K_i = 6.2\times10^{-7}$ M) was only about four-fold lower than repression by DNA oligonucleotide D3 ($K_i = 1.6\times10^{-7}$ M). Substitution of deoxythymidine residues for uridines within the sequence of oligonucleotide R3 also conferred a degree of repressor activity on oligonucleotide RD3 (Fig. 4D). As indicated in Table II, the resulting repressor activity of oligonucleotide RD3 ($K_i = 2\times10^{-6}$) is about 13-fold lower than that of DNA oligonucleotide D3. These results suggest that interrupted blocks of one or more ribose residues in the third strand are tolerated to a large extent in purine motif triple-helices at duplex DNA targets. However, a continuous RNA backbone in the third strand is apparently not compatible with triple-helix formation under these circumstances. The observed similarity in repression by oligomers D3 and D7 demonstrates that absence of a thymine methyl group is not responsible for loss of repressor activity by RNA oligomers designed to bind in the purine motif.

DISCUSSION

Analysis of promoter repression by DNA oligonucleotides

We used the concentration-dependence of T7 promoter repression by DNA oligonucleotides to estimate the apparent inhibition constants for triple-helical complexes in the pyrimidine motif [d(T)·d(A)·d(T) and d(^{5}MethylC$^+$)·d(G)·d(C) triplets] and in the purine motif [d(T)·d(A)·d(T) and d(G)·d(G)·d(C) triplets]. Using a two-state binding model, estimates of K_i for specific DNA repressor complexes on both plasmids pA1 and pA2 were approximately 2×10^{-7} M. These values are higher (implying weaker complexes) than previous dissociation constant estimates for similar complexes based on electrophoretic mobility shift assays (10^{-7} M to 10^{-9} M; 16) or restriction endonuclease inhibition (10^{-8} M; 14). Besides differing experimental conditions, two factors may be responsible for this difference. First, the slow association kinetics displayed by triple-helix formation reactions imply that very long reaction times are required for equilibration in the presence of low oligonucleotide concentrations (14, 25). The 90-min binding reactions in the current study may therefore underestimate repression in the lower portion of the binding curve. Second, it is possible that T7 RNA polymerase actively displaces triple-helical complexes from the operator at some low rate. The apparent K_i value based on repression might therefore be expected to be higher than estimates based on physical binding assays.

Repression by RNA oligonucleotides in the pyrimidine motif

Studies with repetitive polymeric sequences have demonstrated the ability of RNA strands to interact with duplex DNA in the pyrimidine motif under appropriate conditions (26–28). Oligo(2′-*O*-methyl)ribonucleotides (analogs of RNA oligomers) have also recently been reported to participate in triple-helical complexes in the pyrimidine motif (29). Sequence- (but not site) specific inhibition of RNA polymerase activity by RNA binding to duplex DNA was demonstrated in pioneering experiments by Morgan and Wells (26). These authors demonstrated that poly(U) inhibited transcription from either strand of poly[(dA)·(dT)] at pH 8, and that poly(U-C) inhibited transcription from either strand of poly[d(T-C)·d(G-A)] below pH 7. We now extend these results by showing that RNA oligonucleotides containing C and U residues can act as site-specific repressors of T7 RNA polymerase transcription by binding to a homopurine operator in the pyrimidine motif. Repression is both promoter- and oligonucleotide-specific, and occurs at pH 6.8 (37°C).

The apparent inhibition constant for triple-helical complexes involving RNA oligonucleotide R1 binding to the duplex DNA operator of plasmid pA2 is 5.7×10^{-7} M, only about three-fold higher than the K_i value for the binding of DNA oligonucleotide D1. This is notable because D1 contains 5-methylcytosine residues known to strongly stabilize triple-helices (12, 13), while R1 does not.

Our results are supported by the findings of Roberts and Crothers (39). After comparing the stabilities of triple-helical complexes of RNA and DNA strands (pH 5.5; no 5-methylcytosine substitution) these authors conclude that a Hoogsteen-bonded strand composed of RNA forms a particularly stable complex with duplex DNA in the pyrimidine motif.

Failure of RNA oligonucleotides to bind operators in the purine motif

In contrast to the results obtained with RNA-directed triple-helix formation in the pyrimidine motif, specific RNA oligonucleotide R3 did not bind or repress the homopurine operator of plasmid pA1 in the purine motif (Fig.4 and Table II). We report that formation of such purine motif complexes requires at least partial deoxyribose content in the backbone of the third strand. Comparison of the repression properties of oligomers D3 and D7 show that absence of binding activity for oligomer R3 cannot be attributed to the deletion of thymine methyl groups. Indeed, deoxyadenosine is also apparently interchangeable with deoxythymidine in purine motif DNA oligomers (40). The ability of RNA oligonucleotides to participate in duplex formation, but not triplex formation (Fig. 5) suggests that the purine motif cannot accommodate RNA recognition of duplex DNA.

This interpretation appears to contrast with the conclusions suggested by Letai et al., who noted the retention of labeled poly[(dG)·(dC)] by poly(G) affinity columns and concluded that specific (G)·d(G)·d(C) triplets were formed between the RNA third strand and duplex DNA (28). However, these authors studied homopolymeric targets rather than short, mixed sequence targets such as those described here. On the other hand, Morgan and Wells (26) reported that RNA poly(G-A) did not interact with DNA poly[d(T-C)·d(G-A)], in spite of the fact that triple-helices involving two purine strands and one pyrimidine strand had been shown for both DNA poly(dI·dI·dC) and RNA poly(G·G·C) (30, 31).

Results reported by Pei et al. are also significant (32). These authors selected high-affinity ligands for a homopurine sequence in duplex DNA using a combinatorial *in vitro* evolution approach at pH 5.5. The ligand sequences selected from a randomized pool of RNAs consisted exclusively of pyrimidine-rich oligomers that presumably recognized the target in the pyrimidine motif. Notably absent were any ligands with significant G content suggestive of a purine motif interaction. Although the particular experimental conditions and duplex target sequence might have selected against the formation of high-affinity complexes in the purine motif, these results tend to support our conclusion that RNA does not bind duplex DNA to form triplexes in the purine motif.

Implications for natural and artificial RNA-based repressors

Although individual base triplets have been detected in the folded structure of tRNA, 5S RNA, and the *Tetrahymena* self-splicing intron (33–35), and there is some evidence for the formation of *intra*molecular triple-helices in living bacterial cells (36), *inter*molecular triple-helix formation has not been observed in natural systems. It has long been suggested, however, that such interactions might permit gene regulation through the site-specific binding of ribonucleic acid or ribonucleoprotein to duplex DNA (37, 38). Some evidence for DNA recognition by ribonucleoproteins has been reported, although details remain to be elucidated (4–6).

We report evidence that places constraints on the kinds of RNA-directed triple-helices that might participate in site-specific interactions in natural systems. Repression by RNA-directed triple-helix formation is plausible in the pyrimidine motif, but requires acidic pH values (outside the presumed physiological range) for stabilization by cytosine protonation. Formation of such complexes under physiological pH conditions might therefore require alteration of the apparent pK_α of cytosine residues in the third strand of the triplex, either by extensive RNA tertiary structures or by the action of metals, small molecules, or proteins. Triple-helix formation and promoter repression by DNA oligonucleotides in the purine motif readily occurs at physiological pH (24). Initially, this result suggested that analogous interactions involving RNA third strands might be likely to occur in nature. However, we show here that a complex of the latter type does not spontaneously form. We propose that if recognition of duplex DNA by RNA occurs in natural systems, it is likely to require stabilization by accessory factors such as proteins.

DNA-directed triple-helix formation has been suggested as an approach to artificial gene repressors (1–3). Typical strategies involve the exogenous addition of DNA oligonucleotides or analogs with the goal of nuclear delivery. Although constrained by the limitations described above, RNA-directed triple-helix formation is an alternative strategy. Constructs encoding RNA repressors might be delivered to target cells by viral transduction. The resulting artificial regulatory genes would be designed to function by directing the synthesis of stable RNAs that, alone or in concert with accessory proteins, could bind and repress target promoters.

ACKNOWLEDGEMENTS

We acknowledge the excellent technical assistance of D.Eicher, C.Mountjoy, and W.Olivas. We thank W.Gmeiner, B.Gold, and M.Hollingsworth for comments on the manuscript. 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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