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Inhibition of T7 and T3 RNA polymerase directed transcription elongation *in vitro*

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ABSTRACT

A class of oligonucleotides which binds to naturally-occurring duplex DNA sites at physiologic pH to form triple helical structures was used as transcription attenuators in an *in vitro* transcription assay. Oligonucleotides were designed to form triple helices with a purine-rich, double-stranded target by binding in the major groove in an orientation anti-parallel to the most purine-rich strand of the target. A 45 base-pair purine-rich region located within the *gag* gene of Friend Murine Leukemia Virus (FMLV) was used as the duplex target. The target DNA was inserted by molecular cloning downstream of either the bacterial T7- or T3 promoter. The sequence-specific interaction of the triple helix-forming oligonucleotide (TFO) with the FMLV target was confirmed by DNAse I footprint analysis. The affinity of the TFO, as measured by the equilibrium dissociation constant of the TFO for the duplex, was determined by band shift analysis. When a TFO was allowed to form a triple helix with the target duplex in well-defined buffer conditions before the transcription reaction, truncated transcripts of a predicted size were observed. Attenuation of transcription was observed only when buffer conditions favorable to triple helix formation were used. In addition, oligonucleotides containing a high percentage of guanosine residues were able to inhibit mRNA production of the bacterial T7 polymerase by a mechanism independent of transcription attenuation. The ability of an oligonucleotide-directed triple helical structure to slow down, or even completely stop, RNA chain elongation may expand the utility of triple helix technology in the area of gene regulation.

INTRODUCTION

Synthetic oligonucleotides have been shown to bind to the major groove of duplex DNA at homopurine–homopyrimidine sequences in a sequence-dependent manner at physiologic pH, forming a local triple helix (1–3). One potential application of triple helix-forming oligonucleotides (TFOs) is the regulation of gene expression (4). For example, an oligonucleotide which formed a triple helix on a portion of the c-*myc* promoter was capable of inhibiting c-*myc* transcription *in vitro* (1,5). At present, TFOs, which are designed to bind in an anti-parallel or parallel orientation relative to the purine-rich strand of the target duplex, have been limited to DNA targets located within the promoter regulatory regions of genes (1,5–8). In theory, TFOs designed in this fashion inhibit gene expression by interfering with the initiation of transcription.

Restricting target selection to homopurine/homopyrimidine tracts within promoter regulatory regions of selected target genes greatly reduces the utility of the technology. Limitations in the target duplex to which triple helices could be formed may be overcome by easing the target constraints (homopurine/homopyrimidine restriction) or by increasing the available genetic sequences to which TFOs could be targeted. The latter approach would allow for the targeting of TFOs to greater stretches of DNA. In this scenario TFOs which bound to transcribed sections of the targeted genes could be used as attenuators of transcription.

Recently, Young *et al.* (9) described a TFO-mediated attenuation of transcription *in vitro*, using a duplex target located downstream of the adenovirus major late promoter. However, using an *in vitro* bacteriophage T7 promoter system, Maher (10) was able to detect TFO-mediated inhibition of the T7 RNA polymerase transcription initiation but not inhibition of transcription elongation (attenuation). In the present study we describe assay conditions needed to obtain *in vitro* attenuation of transcription using TFOs.

MATERIALS AND METHODS

Enzymes and biochemicals

Radiochemicals ([α-^{32}P]UTP, [α-^{32}P] dTTP, [α-^{35}S]dATP and [γ-^{32}P]dATP) were purchased from New England Nuclear. Restriction endonucleases, T4 DNA ligase, *Eschericia coli* (*E.coli*) DNA polymerase Klenow fragment, and T4 polynucleotide kinase were purchased from New England Biolabs. The bacterial T7 and T3 polymerases as well as the ribonucleoside triphosphates were purchased from Stratagene.

Oligonucleotide synthesis and modification

All oligodeoxynucleotides used in this study were synthesized on an Applied Biosystems Inc. (ABI) 380B or 3942 DNA synthesizer, using standard phosphoramidite methods at 0.2 or 1.0 μM scales. Protected nucleoside 3'-phosphoramidites and other reagents were obtained from Milligen with the exception of acetonitrile, which was obtained from Baxter. All oligonucleotides were synthesized with a 3'-Amino Modifier (Glen Research), which results in the covalent attachment of a propanolamine group to the 3'-hydroxyl group of the oligonucleotides. The oligonucleotides were desalted on an Amicon TCF cell containing an appropriate size membrane filter (11). The purity of the oligonucleotide was confirmed by analytical HPLC, electrophoresis of ^{32}P-labeled oligonucleotide on a 20% polyacrylamide gel containing 7 M urea, and by capillary electrophoresis analysis.

Recombinant plasmids

The plasmid pLRB215 containing the FMLV (clone 57) genome was kindly provided by S.Goff. The bacterial T7 polymerase vector (pT7-2) was purchased from United States Biochemical Corporation. The T3 polymerase vector pBluescript II KS$^+$(pBS KS$^+$) was purchased from Stratagene. To generate recombinant plasmids containing a portion of the FMLV *gag* gene downstream from either the bacterial T7 or T3 promoters, the HindIII/Eco R1 fragment of FMLV (1.97 kb) was inserted between the Hind III and Eco R1 sites of pT7-2 or pBS KS$^+$ (Figure 2C and 2D) using standard laboratory techniques (12). The orientation and integrity of the insert DNA was confirmed by DNA sequence analysis (12).

Band shift analysis

The affinity of oligonucleotides for the FMLV target duplex was determined using 'band shift' analysis as described by Durland *et al.* (2). Band shift experiments were conducted by incubating trace concentrations of end-labeled target duplex with increasing concentrations of TFO (10^{-10}M to 10^{-6} M) at 37°C overnight. The annealing buffer for these assays consisted of 20 mM Tris−HCl, pH 7.6, 10 mM $MgCl_2$ and 10% sucrose. In some experiments, 50 mM NaCl or 50 mM KCl was added to the annealing buffer. The midpoint in the titration of TFO at which the duplex target has shifted to a slower migrating species is the apparent equilibrium dissociation constant (K_D) for triplex formation (2).

DNAse I footprinting analysis

The Hind III to Stu I (172 base-pair) fragment of FMLV (Figure 2B) was excised from p275A, filled in at the Stu I site using the Klenow fragment of *E.coli* DNA polymerase and α-^{32}P dTTP. Footprinting was performed as described by Durland *et al.* (2). In these assays, target duplex was incubated with oligonucleotide for 4 hours at 37°C before the addition of DNAse I (0.125 units/ml) for 10 min at 37°C (2). The DNAse I cleavage pattern was analyzed by polyacrylamide gel electrophoresis.

In vitro transcription reactions

The recombinant plasmid DNAs p275A and pBSFMLV were cleaved to completion using Dde I, then treated with 50 μg/ml of proteinase K for 30 min at 37°C. The reaction mixtures were then extracted twice with an equal volume phenol:chloroform (24:1 chlololform:isoamyl alcohol), precipitated in ethanol, then resuspended in a buffer containing 10 mM Tris−HCl pH 7.6 and 0.1 mM EDTA. One μg of Dde I-digested plasmid DNA was annealed in 10 μl, with various concentrations of test oligonucleotides, for 16−20 hours at 37°C. The oligonucleotide component consisted of test oligonucleotide plus G101-50 so that the total amount of oligonucleotide in each reaction was 4×10^{-6}M. The annealing reaction was performed in 20 mM Tris−HCl, pH 7.6 and 10 mM $MgCl_2$. In several experiments the annealing buffer was modified by the addition of either 50 mM NaCl or 50 mM KCl. The annealed DNA (10 μl) was then incubated for 30 min at 37°C in a final volume of 20 μl consisting of 20 mM Tris−HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 mM GTP, ATP and CTP, 0.05 mM UTP, 1 μl of [α-^{32}P]UTP (800 Ci/mMol), and 2 units of either T7 or T3 polymerase. The reactions were stopped by cooling and precipitated by the addition of ethanol. The RNA transcripts were analyzed by electrophoresis on a 10% denaturing polyacrylamide gel containing 7M urea.

RESULTS

Experimental design

The modification of the 3' terminal hydroxyl group of an oligonucleotide with an amine group has been shown to enhance nuclease resistance of these molecules in culture and *in vivo* (13). For this reason, all oligonucleotides used in this study were synthesized so that a propanolamine group was attached at their 3' terminal hydroxyl group.

An oligonucleotide designed to form a triple helix (TFO) in an anti-parallel orientation (FMLV2ap) with the purine-rich target duplex (in the *gag* region of FMLV clone 57) is illustrated in Figure 1A. The purine-rich target spans nucleotides 931 to 975 of the FMLV genome (14). Oligonucleotides containing only guanosine and thymidine bases generally form triple helices with their target duplex when aligned in an anti-parallel orientation (2,3). Therefore, when the orientation of FMLV2ap was inverted, the resulting parallel-oriented molecule (FMLV2p) did not bind to the FMLV target and was used as sequence isomer control (Figure 1). The anti-parallel-oriented molecule (FMLV2ap), when bound to the viral genome, interacted with the non-coding strand such that the 5' terminus was oriented towards the incoming RNA polymerase. Other oligonucleotides used as controls in the various experiments (G101-50 and I100-11) are shown in Figure 1.

Binding affinity

The affinity of oligonucleotides for the FMLV target duplex was determined using band shift analysis as described by Durland *et al.* (2). In this experiment, ^{32}P-end-labeled target duplex was incubated with increasing concentrations of oligonucleotide at 37°C overnight before electrophoresis through a polyacrylamide gel. The midpoint in the titration of TFO at which the duplex target shifts to a slower migrating species is the apparent equilibrium dissociation constant (K_D) for triplex formation (Figures 1 and 3). The results of these assays demonstrated a tight interaction (10^{-9} M K_D) of FMLV2ap with the target duplex when no monovalent cation was present in the annealing buffer. However, when the assay was performed in the presence of 50 mM NaCl or KCl, the K_D was much larger. This observed change in the K_D in the presence of monovalent cations may be due to competition of the monovalent cation with the divalent Mg^{2+} ions which results in the induction of inter-

A

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        926                                                    978
5'-  ctaaacctcctcccctccttcttccccccttcagccccctctctcccacctgaa  -3' target pyrimidine strand
3'-  gatttggaggaggggaggaagaagggggaagtcgggggagagagggtggactt  -5' target purine strand

FMLV2ap  5'-   ggtggtggggtggttgttgggggttgttgggggtgtgtgggtggt -3'
FMLV2 p  3'-   ggtggtggggtggttgttgggggttgttgggggtgtgtgggtggt -5'


I100-11  5'-   gatccatgtcagtgacactgcgtagatccgatgatccagtcgatg -3'
G101-50  5'-   ggtgggtggtttgtgtggttggtgggtttt               -3'
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B

Oligonucleotide	K_D		
	No Salt	50 mM NaCl	50 mM KCl
FMLV2ap	2.5×10^{-9} M	7.6×10^{-8}M	7.1×10^{-6}M
FMLV2p	$>1 \times 10^{-6}$ M	$>1 \times 10^{-6}$M	$>1 \times 10^{-6}$M
I100-11	$>1 \times 10^{-6}$ M	$>1 \times 10^{-6}$M	$>1 \times 10^{-6}$M
G101-50	$>1 \times 10^{-6}$M	$>1 \times 10^{-6}$M	$>1 \times 10^{-6}$M

Figure 1. (A) The oligonucleotide FMLV2ap was designed to form an anti-parallel triple helix with the purine-rich segment of the FMLV genome (nucleotides 931 to 975) located within the viral *gag* gene. Control oligonucleotides having different lengths and/or percentage of guanosine (I100-11 and G101-50) are also presented. (B) Band shift analyses were performed to obtain the apparent equilibrium dissociation constant (K_D) for each of these oligonucleotides. The K_Ds in the presence of the monovalent cations NaCl or KCl are also shown.

or intramolecular oligonucleotide associations, such as guanosine tetrads, mediated by hydrogen bonding between the guanosine residues (15). FMLV2p designed in the parallel orientation to the purine-rich strand of the duplex target did not bind to the double-stranded FMLV target and was used as an isomer control. I100-11 and G101-50 did not bind to the duplex target.

DNAse I footprint analysis

A more rigorous analysis of the interaction between the TFOs and their corresponding duplex target was performed by DNAse I footprint analysis. Specific binding of TFOs to the FMLV target can be assessed by DNAse I footprinting in a manner similar to the analysis of DNA–protein interactions. In this experiment, a 172-base-pair (bp), Hind III/Stu I, fragment of FMLV (Figure 2B) was radiolabeled, incubated with FMLV2ap or FMLV2p, then digested with DNAse I, as described in Materials and Methods. The results of this experiment demonstrate that FMLV2ap interacts with the FMLV duplex target at the predicted binding site (Figure 3). The control oligonucleotide, which was similar in length, base composition and terminal structures, did not protect the target sequence from DNAse I digestion (Figure 3). These data indicate that, within the limits of accuracy for DNAse I footprinting of triple helical nucleic acid structures, FMLV2ap bound to the duplex target as predicted.

In vitro transcription analysis

In order to assess the effects of the oligonucleotides on transcription, the 1973-bp Hind III to Eco R1 subfragment of the FMLV genome (Figure 2A) was inserted into the bacterial expression vectors pT7-2 and pBluescript. The resulting plasmids (Figure 2C and 2D) contained the TFO target sequence downstream from the bacterial T7 (p275A) or T3 (pBSFMLV) promoters. In plasmid p275A, the Hind III site is 10 bp downstream of the T7 mRNA start site, the beginning of the

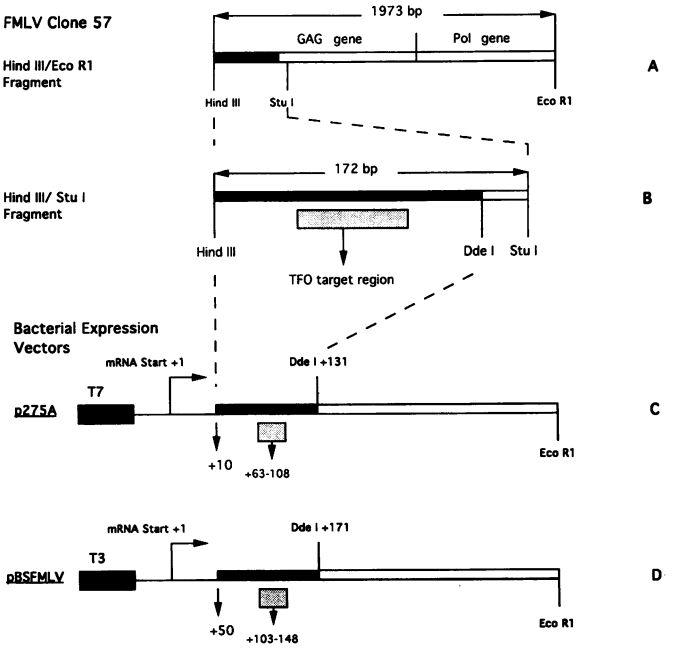

Figure 2. (A) The Hind III to Eco R1 subfragment (1973 base pairs) of FMLV (clone 57) genome is depicted. (B) A 172-bp (Hind III to Stu I) portion of the 1973-bp fragment is expanded. Within this fragment is the purine-rich target to which anti-parallel TFOs were directed. This fragment was used in the DNAse I footprinting experiment. (C) The entire Hind III/Eco R1 fragment was cloned into pT7-2, yielding p275A. In this recombinant, the Hind III site is 10 base pairs downstream of the T7 mRNA start site. The 5′ portion of the triple helix target region is 63 base pairs downstream of the mRNA start and the Dde I site is 131 base pairs downstream of the mRNA start site. (D) The Hind III/Eco R1 FMLV fragment was cloned into pBluescript, yielding pBSFMLV. In this recombinant, the Hind III site, duplex target and Dde I site are 50, 103 and 171 base pairs downstream from the mRNA start site, respectively.

purine-rich target is 63 bp 3′ to the mRNA start site, and the Dde I site is 131 bp 3′ to the mRNA start site (Figure 2C). When this plasmid is cleaved with Dde I and then used as a template in *in vitro* transcription reactions using the T7 polymerase, a 131-nucleotide (nt) run-off transcript is obtained. Plasmid pBSFMLV contains a Hind III site 50 bp downstream of the T3 mRNA start site, the target sequence is 103 bp 3′ to the mRNA start site, and the Dde I site is 171 bp 3′ to the mRNA start site (Figure 2D). *In vitro* transcription reactions using Dde I-linearized pBSFMLV ad T3 polymerase results in the generation of a 171-nt transcript.

The recombinant plasmids were linearized with Dde I, incubated at room temperature overnight with or without oligonucleotide, then used as template for *in vitro* transcription reactions. The bacterial T7 (p275A) or T3 (pBSFMLV) polymerases were used in these assays. Radio-labeled [(α-^{32}P)UTP], full-length or truncated mRNA transcripts were analyzed by polyacrylamide gel electrophoresis, then quantitated by cutting out the specific transcript band and measuring the radioactivity in a scintillation counter.

In initial transcription experiments it was observed that a non-sequence-dependent inhibition of T7 RNA polymerase occurred when oligonucleotides containing a high percentage of guanosine residues were pre-incubated with p275A. For example, I100-11, a random sequence oligonucleotide which contains only 25% guanosine, had a stimulatory effect on the production of full-length (131 nt) T7 transcripts (Figure 4 lane 2, Table 1). However, G101-50 (53% guanosine) consistently demonstrated minor inhibition of full-length T7 transcripts (Figure 4A lanes 3 and 10, Table 1). In dose response assays, the level of G101-50 inhibition leveled off when concentrations in the range of 10^{-6}M were used (data not shown). Therefore, to separate non-sequence-dependent inhibitory events from TFO-mediated inhibition, we used G101-50 as a carrier oligonucleotide in all transcription reactions. When I100-11 was pre-incubated with p275A in the presence of G101-50 (to a final reaction concentration of 4×10^{-6}M oligonucleotide), the stimulatory effect of I100-11 was still observed in a dose-dependent fashion (Figure 4 and Table 1). However, when FMLV2p (64% guanosine) was pre-incubated with p275A in the presence of G101-50, an additional dose-dependent inhibition of T7 transcription was observed (Figure 4A and Table 1). In assays designed to monitor the effects of I100-11, G101-50 and FMLV2p on T7-directed transcription, no detectable accumulation of truncated transcripts (63 to 108 nt) was observed. This result, obtained in multiple experiments, implies that transcription inhibition by FMLV2p occurred via a mechanism independent of attenuation.

To investigate TFO-mediated transcription attenuation, Dde I-digested p275A was pre-incubated with FMLV2ap (2×10^{-6}

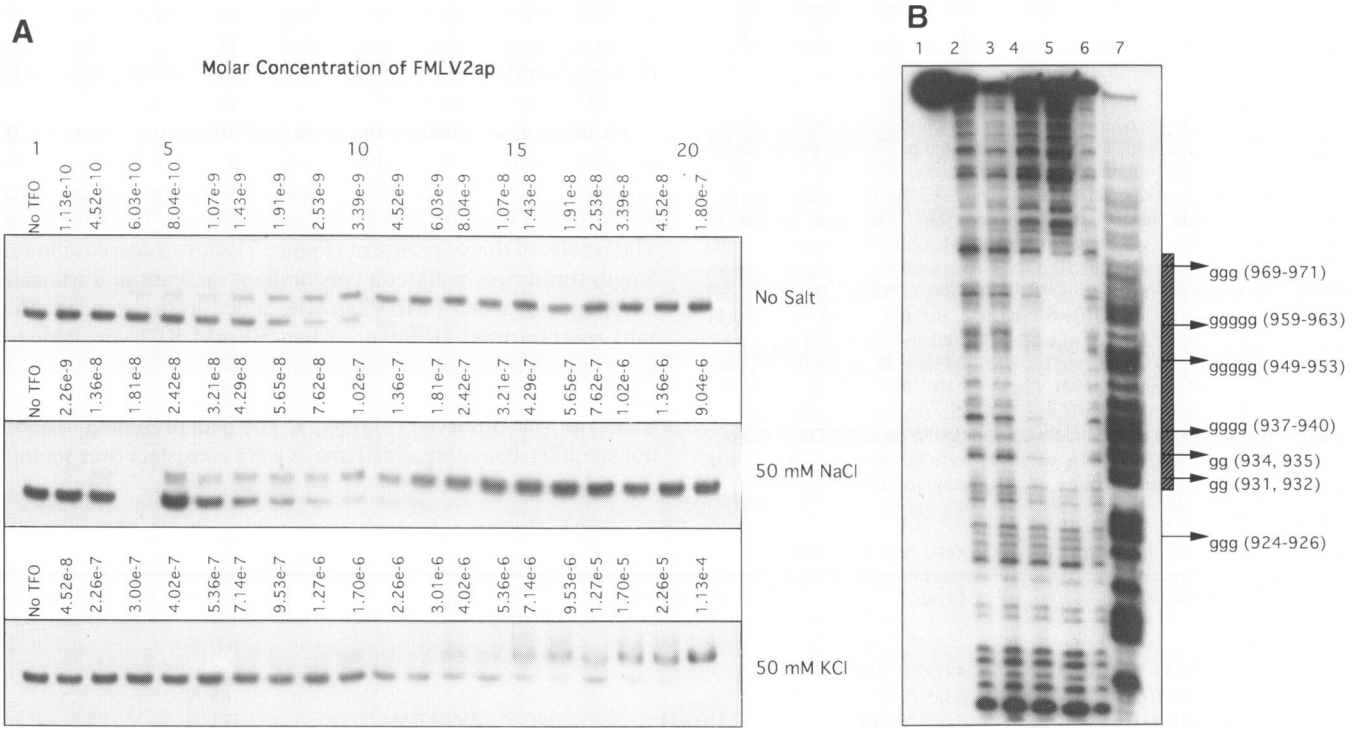

Figure 3. (A) Determination of the apparent dissociation equilibrium constant using band shift analysis. In the top panel (No Salt), TFOs were mixed with target duplex in the standard low salt conditions. The lanes (from left to right) contain no TFO, followed by increasing amounts of FMLV2ap from 1.13×10^{-10}M to 1.8×10^{-7}M. In the middle panel the standard annealing buffer was modified to include 50 mM NaCl. The lanes in the center panel contain no TFO, followed by amounts of FMLV2ap from 2.26×10^{-9}M to 9.0×10^{-6}M. In the bottom panel the standard annealing buffer was modified to include 50 mM KCl. Lanes in the bottom panel contain no TFO, and then increasing increments of FMLV2ap from 4.52×10^{-8}M to 1.13×10^{-4}M. In lane 4 of the middle panel, the sample was inadvertently left out of the lane during electrophoresis. **(B)** DNAse I footprint analysis of anti-parallel TFOs. The Hind III to Stu I, 172-bp fragment of FMLV was filled in at the Stu I site using Klenow polymerase and α-^{32}P-labeled dTTP. Footprinting was performed as described in Methods. Lane 1 contains the radio-labeled, 172-bp fragment without DNAse I treatment. In lane 2, the 172-bp fragment was digested with DNAse I, but not pre-incubated with an oligonucleotide. In lanes 3 to 5, the DNA fragment was pre-incubated with 5×10^{-9}M (lane 3), 5×10^{-8}M (lane 4) or 5×10^{-7}M (lane 5) FMLV2ap. In lane 6, the target duplex was pre-incubated with 5×10^{-7}M of the control oligonucleotide FMLV2p. Lane 7 shows the DMS reaction results. To the right of the figure the extent of the DNAse I protected region is marked (striped box) as well as the nucleotide position of selected guanosine bases determined in the DMS reaction.

to 2×10^{-9}M). In these assays, a dose-dependent decrease in the full-length (131 nt) transcript was observed, with a maximum decrease of approximately 80% when 2×10^{-6}M or 2×10^{-7}M FMLV2ap was used (Figures 5A and 5B). At the same time, an accumulation of truncated transcripts of a size corresponding to the position of the TFO binding site (63 – 108 nt) was observed (Figure 5A). The control oligonucleotide G101-50 alone (4×10^{-6}M) decreased the amount of full-length transcripts by approximately 20%, but no accumulation of truncated mRNA was observed (Figures 4 and 5A). The quantitation of the relative amounts of accumulated truncated transcripts is presented in Figure 6. It is interesting to note that the accumulated truncated transcripts did not account for the total decrease in full-length transcripts (Figure 6). The data from this experiment, when plotted as the ratio of TFO to target duplex, suggests that maximum attenuation was achieved with an effector to target ratio of approximately 1:1 (data not shown).

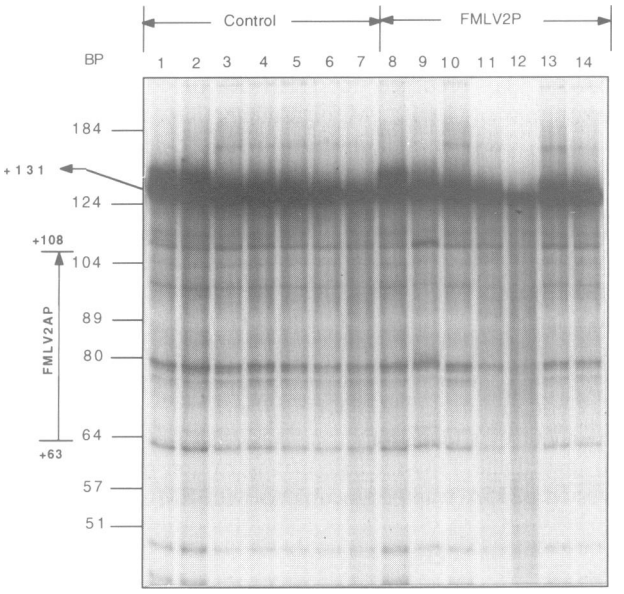

Figure 4. Effects of I100-11 and FMLV2p on transcription directed by the bacterial T7 promoter. In each reaction, 1 μg of Dde I-digested p275A was pre-incubated overnight with or without oligonucleotide. The plasmid DNA was then used as template in the transcription reaction. In lanes 1 and 8, p275A DNA was transcribed without oligonucleotide in the pre-incubation step. The total amount of oligonucleotide in all other reactions was 4×10^{-6}M. G101-50 was used to bring the oligonucleotide concentration up to this level when needed. The oligonucleotides used in these reactions included 4×10^{-6}M I100-11 (lane 2), 4×10^{-6}M FMLV2p (lane 9), 4×10^{-6}M G101-50 (lanes 3 and 10), 2×10^{-6}M I100-11 (lane 4), and 2×10^{-6}M FMLV2p (lane 11). Reactions depicted in lanes 5–7 contained I100-11 at 2×10^{-7}M (lane 5), 2×10^{-8}M (lane 6), or 2×10^{-9}M (lane 7), while lanes 12–14 contained FMLV2p at 2×10^{-7}M (lane 12), 2×10^{-8}M (lane 13) or 2×10^{-9}M FMLV2p (lane 14). The size of the transcripts, in nucleotides, was estimated using a radio-labeled double-stranded DNA marker. In the gel conditions used, the DNA standards were denatured so that the size estimates were based on the mobility of single-stranded DNA.

A dose-dependent decrease in full-length (171 nt) transcripts and a concomitant increase in truncated transcripts (103 to 148 nt) was observed in assays designed to monitor the effects of TFOs on T3 polymerase elongation (Figure 5A and 5B). Little to no decrease in full-length RNA transcript was observed when pBSFMLV DNA was pre-incubated with 4×10^{-6}M of the control oligonucleotide G101-50 (Figure 5).

In order to demonstrate that triple helix formation was necessary for the generation of the truncated transcripts, 50 mM KCl was added to the pre-incubation and *in vitro* transcription buffer. In the presence of KCl, the measured K_D of FMLV2ap for the target weakened 100-fold, from 2×10^{-9}M to 3×10^{-7}M (Figures 1 and 3). In these experiments, if FMLV2ap was allowed to pre-incubate with p275A DNA before the transcription reaction, then no decrease in full-length transcript or accumulation of the attenuated species was observed (Figure 7). The control oligonucleotide FMLV2p had the same effect on transcription as FMLV2ap under these assay conditions (Figure 7).

To determine whether the observed inhibitory effect of the oligonucleotides was dependent on interaction of the oligonucleotide with the DNA template, the oligonucleotides were added to the reaction mix at the same time as T7 polymerase. The results of this experiment (Figure 7) show a decrease in full-length transcripts without a concomitant increase in a truncated RNA species when FMLV2ap was added to the standard (low salt) reaction mix. However, when 50 mM KCl was added to the reaction mix, and oligonucleotides were added at the same time as the T7 polymerase, then no decrease in full-length transcript was observed (Figure 7). The data presented in all the transcription assays presented above were consistent over multiple repetitions.

Table 1. Quantitation of full length (131 nucleotide) run off transcript

Lane	Oligo # 1	Conc	Oligo #2	Conc	Percent 131 Base Transcript[a]
1	–	–	–	–	100 %
2	I100-11	4.0×10^{-6}M	–	–	132 %
3	G101-50	4.0×10^{-6}M	–	–	83 %
4	G101-50	2.0×10^{-6}M	I100-11	2×10^{-6}M	127 %
5	G101-50	3.8×10^{-6}M	I100-11	2×10^{-7}M	120 %
6	G101-50	4.0×10^{-6}M	I100-11	2×10^{-8}M	86 %
7	G101-50	4.0×10^{-6}M	I100-11	2×10^{-9}M	83 %
8	–	–	–	–	100 %
9	FMLV1p[b]	4.0×10^{-6}M	–	–	60 %
10	G101-50	4.0×10^{-6}M	–	–	83 %
11	G101-50	2.0×10^{-6}M	FMLV2p	2×10^{-6}M	53 %
12	G101-50	4.0×10^{-6}M	FMLV2p	2×10^{-7}M	27 %
13	G101-50	4.0×10^{-6}M	FMLV2p	2×10^{-8}M	97 %
14	G101-50	4.0×10^{-6}M	FMLV2p	2×10^{-9}M	79 %

[a]The values presented are the average of two or more experiments.
[b]FMLV1p is a shorter version of FMLV2p (26 nucleotides in length) and spans nucleotides 956 to 931 of the FMLV genome.

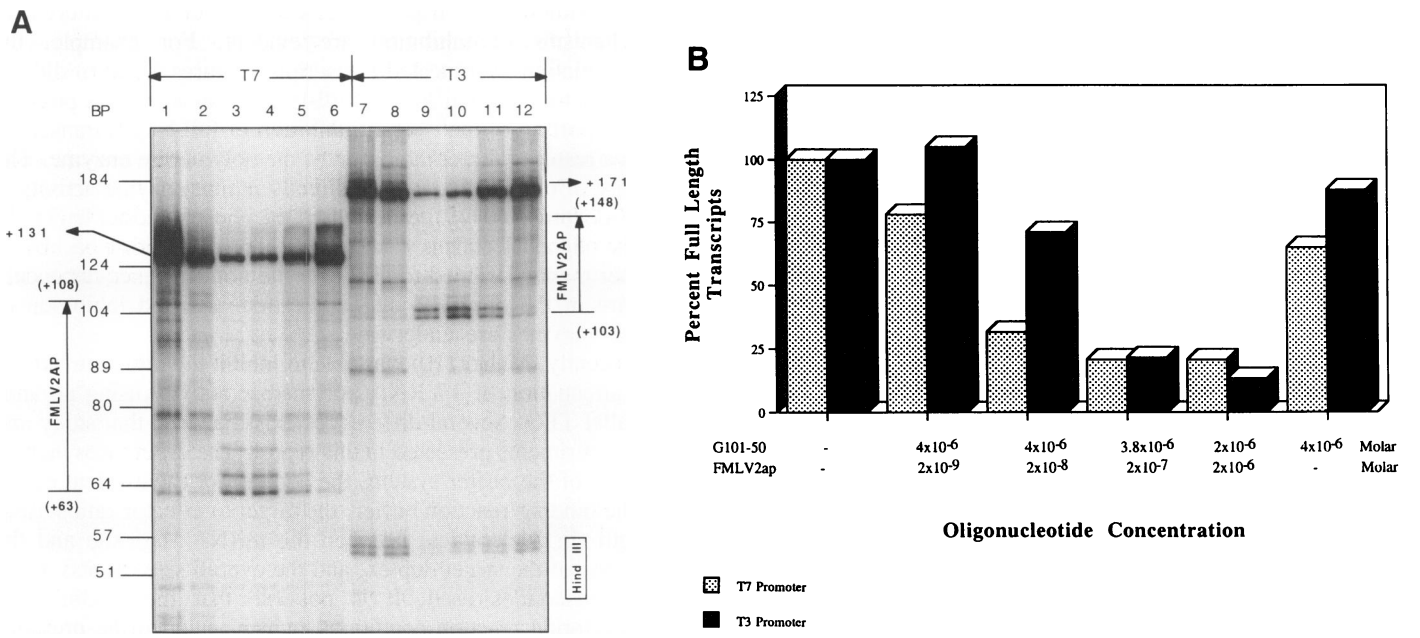

Figure 5. Attenuation of transcription by FMLV2ap. **(A)** Dde I digests of p275A (T7) or pBSFMLV (T3) were pre-incubated with G101-50 and various amounts of FMLV2ap for 16–20 hours before transcription reactions. Reactions depicted in lanes 1 and 7 contained only plasmid DNA, while reactions shown in lanes 2 and 8 contained plasmid DNA plus 4×10^{-6}M G101-50. The total amount of oligonucleotide in all other reactions was 4×10^{-6}M. G101-50 was used to bring the oligonucleotide concentration up to this level when needed. Lanes 3–6 and 9–12 contained 2×10^{-6}M, 2×10^{-7}M, 2×10^{-8}M, or 2×10^{-9}M FMLV2ap, respectively. **(B)** Quantitation of full-length RNA transcripts. Distinct bands corresponding to ^{32}P-labeled, full-length mRNA transcripts (131 or 171 nucleotides long) were removed from the gel and then quantitated by measuring the radioactivity in a scintillation counter. Oligonucleotides were pre-incubated with plasmid DNA as described in (A), using either p275A (T7 promoter) or pBSFMLV (T3 promoter). In column 1, no oligonucleotide was added to the transcription mix. Columns 2 through 5 contain the results obtained when decreasing amounts of FMLV2ap (2×10^{-6}M to 2×10^{-9}M) were used. Column 6 presents the results obtained when only G101-50 (4×10^{-6}M) was pre-incubated with template DNA. The molecular size standards are the same as described in the legend to Figure 4.

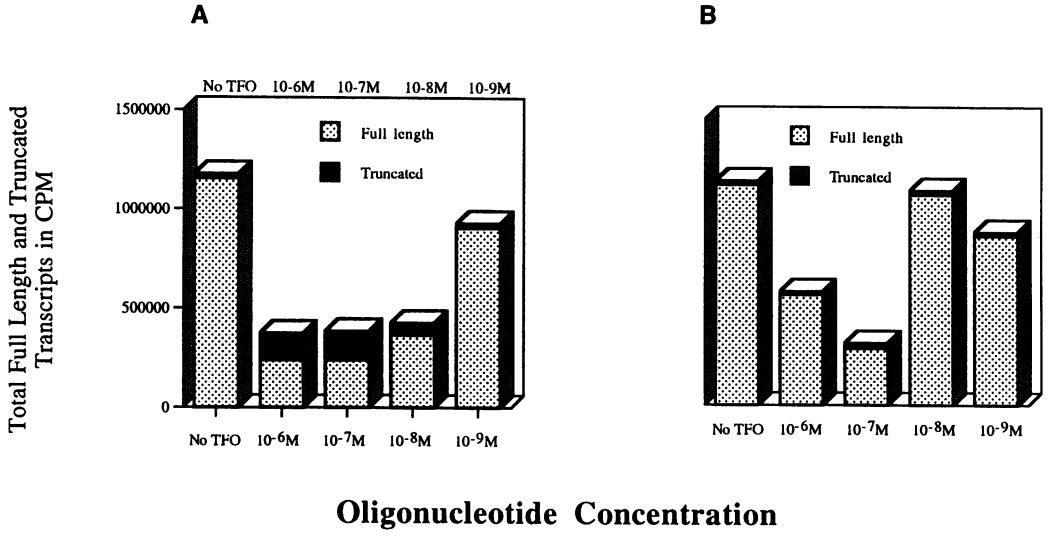

Figure 6. Quantitation of full-length and truncated transcripts. Quantitation of transcripts was performed by cutting out the distinct bands corresponding to ^{32}P-labeled full-length or truncated (63–108 nucleotides) RNA transcripts and then measuring the radioactivity in a scintillation counter. The effector to target ratios of 30:1, 3:1, 1:3 and 1:30 correspond to input FMLV2ap concentrations of 2×10^{-6}M, 2×10^{-7}M, 2×10^{-8}M and 2×10^{-9}M, respectively.

DISCUSSION

The data described in these experiments demonstrate that a triple helical DNA complex can, when positioned downstream from the either the bacteriophage T7 or T3 promoter, act as an attenuator of transcription in defined buffer conditions. Most experiments were conducted using p275A because of the well-documented ability of T7 polymerase to process through molecular blocks such as noncatalytic *Eco*R1 mutants and mammalian RNA polymerase III (16) or the *lac* repressor (17,18). These same factors act to block *E.coli* RNA polymerase and eukaryotic RNA polymerase II. The ability of the TFO to

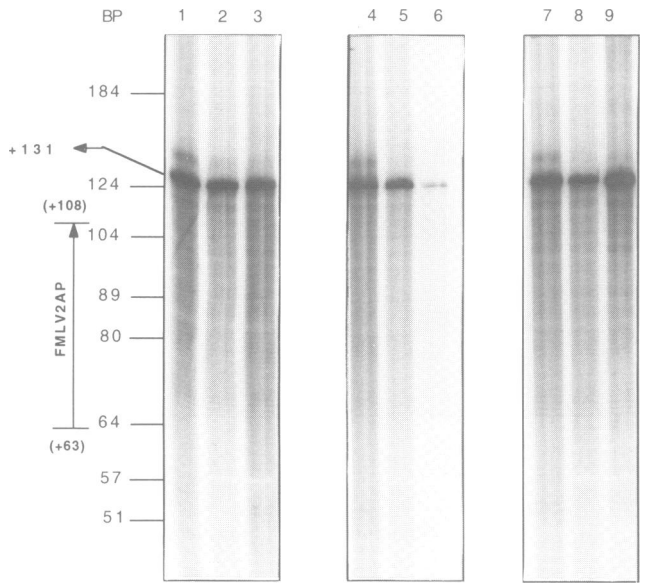

Figure 7. Effects of oligonucleotide on T7 polymerase transcription in the presence of KCl. Oligonucleotides were annealed with Dde I-digested p275A for 16–20 hours in annealing buffer before transcription reactions were performed. The annealing and transcription buffer was supplemented with 50 mM KCl (lanes 1–3). Oligonucleotides were added to the transcription reaction at the same time as T7 polymerase, and reactions were carried out in the low salt buffer (lanes 4–6). Oligonucleotides were added to the transcription reaction at the same time as T7 polymerase and the transcription buffer was supplemented with 50 mM KCl (lanes 7–9). Transcription reactions were performed either without TFO added (lanes 1, 4 and 7), in the presence of 4×10^{-6}M G101-50 (lanes 5 and 8), in the presence of 3.8×10^{-6}M G101-50 and 2×10^{-7}M FMLV2ap (lane 3, 6 and 9) or in the presence of 3.8×10^{-6}M G101-50 and 2×10^{-7}M FMLV2p (lane 2).

cause attenuation of this polymerase demonstrates a strong interaction with the target duplex. The recombinant vector containing the T3 promoter was used because the distance, in nucleotides, between the target duplex and the T3 promoter was over 100 bp, whereas the distance between the T7 promoter and the TFO target was only 60 bp. Observations of transcription attenuation using pBSFMLV as a template would help rule out the possibility that TFOs, when bound to a duplex target downstream of the mRNA start site, interfered with the initiation of transcription.

The triple helix-forming oligonucleotide, when pre-incubated with target duplex DNA at roughly 1:1 effector to target ratios (10^{-7}M oligonucleotide), significantly reduced full-length transcripts and caused the accumulation of an RNA product similar in size to that of the predicted truncated transcript. A shorter version of FMLV2ap (FMLV1ap which was 26 nt in length) was also able to inhibit transcription with an accumulation of a truncated transcript of the predicted size (data not shown). However, the observed attenuation effect was dependent on the buffer conditions used. When KCl was added to the binding and reaction mix, a decrease in the ability of the TFO to bind with its target duplex (Figure 1 and 3) and a loss in the ability to attenuate transcription were observed. This change in activity may be the result of guanosine tetrad formation in the presence of KCl (15). The formation of these stable structures would effectively reduce the amount of oligonucleotide available to form triple helical structures.

In addition to triple helix-induced effects, alternative mechanisms of inhibition are evident. For example, the accumulation of truncated transcripts (Figures 5 and 6) did not account for the total loss of full-length message. It is possible that a portion of the overall inhibition of full-length transcripts was a result of direct inhibition of the polymerase enzyme. The ability of oligonucleotides to directly inhibit enzyme activity is well-documented (19), especially if the phosphodiester backbone of the oligonucleotide is modified to a phosphorothioate backbone. Experiments designed to elucidate the non sequence-dependent nature of the observed oligonucleotide-mediated inhibition of these enzymes are underway.

Recently, Maher (10) was able to inhibit the initiation but not the attenuation of T7 RNA polymerase activity using an anti-parallel TFO. Several differences exist between that study and the experiments presented in this report. The differences include the pH of the buffer system, the addition of monovalent cation to the binding/reaction buffer, the target to effector ratios used, length (in nucleotides) between the mRNA start site and the position of the target duplex, and the overall size (in nts) of the oligonucleotides used. It is possible that the binding and transcription reaction conditions (which included the presence of monovalent cations) used in Maher's study (10) prevented the formation of a triple helix complex strong enough to attenuate RNA processing by the T7 promoter. The observations reported in this study, demonstrating that the presence of monovalent cations in the annealing/reaction buffer prevented attenuation of transcription, are consistent with Maher's (10) observations.

In another experimental system, Young *et al.* (9) used a parallel-binding oligonucleotide, which formed triple helical DNA at pH 7.2 in the presence of 50 mM KCl, to attenuate transcription in a eukaryotic system. Transcription attenuation was described as transitory in this system, in that the polymerase enzyme stalled in the region of a triple helix but could eventually 'read through' the blockage unless the TFO was covalently attached to the duplex (9). In our experiments, analysis of the transcription products reveals a banding pattern which is consistent with the position of the thymidine residues in the DNA template. Since UTP is the limiting nucleotide triphosphate in the reaction buffer, the position of these bands on the gel corresponds to where the polymerase has either stalled, due to lack of substrate, or has actually dissociated from the transcription complex, leaving a truncated transcript. The attenuation effect observed using FMLV2ap may have been enhanced by the fact that the enzyme is processing more slowly in the regions adjacent to the thymidine residues. It is not known whether the attenuated transcripts result from a stalled complex in the region of the triple helix or if they in fact represent dissociation of the enzyme from the transcription complex.

The *in vitro* experimental system presented in this report shows that oligonucleotides can form triple helices in a sequence-specific fashion and that these triple helical DNA structures alone are capable of disrupting RNA polymerase processing. At the present time, however, DNA sequence constrains the targeting of TFOs to only protein binding sites within promoter regulatory regions of selected target genes. The inability of the TFO to interact with target duplexes under more physiologic conditions greatly reduces the utility of the oligonucleotide-based triple helix technology. While we have addressed, in part, the ability to extend effective targets for TFO design, much work needs to be done to maximize the effectiveness of this class of transcription inhibitors. One potential solution to stabilizing the interaction of a TFO with its

target duplex *in vivo* would be to attach a cross-linking or intercalating agent to the TFO (9). In addition, advances in this technology may lead the way to the creation of TFOs capable of binding to a greater variety of target sequences, instead of only to purine-rich stretches of DNA under physiologic salt conditions. All of these advances may help expand the applications of TFO technology.

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