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SPECIFIC AND NONSPECIFIC INHIBITION OF TRANSCRIPTION BY DNA, PNA, AND PHOSPHOROTHIOATE PROMOTER ANALOG DUPLEXES

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Abstract: DNA duplexes analogous to the promoters for SP6 or T7 RNA polymerase inhibit transcription with exquisite selectivity. By contrast, phosphorothioate oligomers inhibit nonselectively, and peptide nucleic acid (PNA) duplexes and PNA:DNA heteroduplexes do not inhibit at all. The absence of recognition of proteins by PNAs may prove to be a substantial advantage for their use as anti-sense agents and nucleic acid probes.

Introduction

SP6 and T7 bacteriophage RNA polymerases (RNAP) are single subunit enzymes, and their simplicity relative to multi-subunit transcription complexes makes them attractive systems for the investigation of mechanisms for promoter recognition and transcription.[1] SP6 and T7 RNAP promoter sequences share 60% identity (Fig. 1a), but in spite of this similarity SP6 and T7 RNAP maintain stringent specificity for their respective promoter sequences. This strict discrimination suggests promoter-based oligonucleotide inhibitors as an approach to selective inhibition of transcription.

One of the limiting factors in the use of DNA oligonucleotides in vivo is their susceptibility to nuclease degradation. In addition to the use of phosphodiester (PO) oligonucleotides, we have examined inhibition of RNAP by phosphorothioate (PS) oligonucleotides,[2] where a nonbridging oxygen is replaced with sulfur, and peptide nucleic acids (PNAs) that contain linkages composed of *N*-(2-aminoethyl) glycine units rather than a deoxyribose phosphate backbone (Fig. 1b).[3]

We find that electronic and steric properties found in phosphodiester linkages of both strands of a promoter sequence are necessary for recognition by RNAP. Duplex PO oligonucleotides analogous in sequence to promoter DNA are selective inhibitors of RNAP, while PS oligomers inhibit transcription nonsequence selectively. Neither PNA:PNA nor PNA:DNA heteroduplexes inhibit transcription. These results suggest that for in vivo applications, PNAs will bind with high affinity to complementary nucleic acid targets and with minimal affinity to proteins likely to bind polyanions.

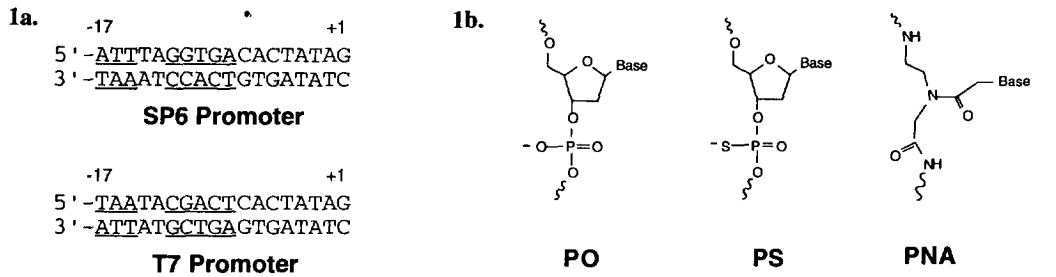

Figure 1. (a) Sequences of SP6 and T7 RNAP promoters. Underlined bases are residues that differ between T7 and SP6 promoter analog duplexes. (b) Phosphodiester (PO), phosphorothioate (PS), and PNA linkages.

Results and Discussion

Inhibition of transcription by phosphodiester oligonucleotides. We examined the inhibitory effects of DNA duplexes analogous in sequence to the promoter regions for either SP6 or T7 RNAP. We observe that these duplexes compete for binding to plasmid-borne promoter template in a concentration dependent manner. Addition of duplex DNA or a DNA hairpin containing the two strands of the promoter linked by a four base cytidine linker completely inhibited transcription by SP6 RNAP, with an IC_{50} of 20 nM (Table 1). Inhibition was selective, as SP6 RNAP was not inhibited by addition of the T7 promoter analog duplex. Similarly, addition of the T7 promoter analog duplex fully inhibited transcription by T7 RNAP, while no inhibition of T7 RNAP was observed upon addition of the SP6 promoter analog duplex. Single stranded oligonucleotides did not significantly inhibit transcription at concentrations up to 1 μM.

Table 1. IC_{50} values for inhibition of SP6 and T7 RNAP.

Promoter Analog	IC_{50} T7 RNAP	IC_{50} SP6 RNAP
	DNA Oligomers	
SP6 PO duplex	ND[a]	20 nM
T7 PO duplex	15 nM	ND
SP6 PO single strand[b]	ND	ND
T7 PO single strand[b]	ND	ND
	PS Oligomers	
SP6 PS duplex	5 nM	10 nM
T7 PS duplex	5 nM	10 nM
T7 PS coding strand	5 nM	50 nM
Noncognate PS duplex	100 nM	100 nM
	PNA Oligomers	
SP6 PNA:DNA[c] heteroduplex	ND	ND
SP6 PNA:PNA duplex	ND	ND
T7 PNA:DNA[c] heteroduplex	ND	ND
T7 PNA:PNA duplex	ND	ND

[a]ND, No inhibition detected at oligomer concentrations ≥1 μM. [b]Both coding and noncoding strands were analyzed. [c]Both combinations of coding and noncoding PNA strands were evaluated.

Inhibition of transcription by phosphorothioate oligonucleotides. Duplex PS oligonucleotides were assayed for their ability to inhibit transcription by SP6 and T7 RNAP. Since PS:PS duplexes have lower melting temperatures they were synthesized as hairpin oligonucleotides to ensure stable formation of the duplex. Both the SP6 and the T7 PS promoter analog hairpins inhibit transcription by their cognate RNAP (Table 1). To examine the selectivity of inhibition of transcription by PS oligonucleotides, we analyzed the ability of the SP6 promoter PS hairpin to inhibit T7 RNAP and the T7 PS hairpin to inhibit SP6 RNAP. In contrast to the stringent selectivity observed for inhibition by DNA oligonucleotides, little selectivity was demonstrated by the PS oligonucleotides (Table 1). To further evaluate the specificity of inhibition we synthesized a scrambled version of the SP6 promoter containing the identical purine and pyrimidine content but in altered order. This PS oligonucleotide inhibited transcription by both polymerases with an IC_{50} of 100 nM. Also in contrast to DNA containing oligonucleotides, single-stranded PS oligonucleotides inhibited transcription by both T7 and SP6 RNAP. Inhibition was observed regardless of whether the single-stranded oligonucleotides were analogous in sequence to the promoter of the relevant polymerase.

The extent of nonsequence selective interactions of phosphorothioate oligonucleotides may appear surprising, as substitution of sulfur for oxygen seems conservative. However, sulfur is larger, its presence introduces a chiral center at the phosphate, and the geometry of the phosphorous center is altered.[2] It is likely, therefore, that a combination of steric and electronic effects contribute to the nonselective binding of PS oligonucleotides to proteins. Nonsequence-specific binding of PS oligonucleotides to proteins has also been reported for telomerase,[4] HIV reverse transcriptase,[5] and transcription factors,[6] and it may be a property of this class of oligomers.[7,8] One study suggests that sequence specificity can be regained by the use of a partially phosphorothioate-modified oligonucleotide.[6] In our study, however, a promoter analog duplex containing PS

linkages in the −6 to +1 region and phosphodiester linkages in the −17 to −7 region thought to be responsible for conferring specificity between T7 and SP6 polymerase,[9] was no more selective than a fully modified PS oligonucleotide.

Effect of peptide nucleic acid promoter analogs on transcription. PNAs bind to single-stranded DNA by Watson–Crick base pairing, and PNA-DNA heteroduplexes have been shown by NMR to adopt a B-like conformation[10] suggesting they might have the ability to bind proteins through interactions with the stacked bases and the remaining single phosphodiester backbone. To test whether SP6 or T7 RNAP can recognize PNA-containing duplexes we assayed inhibition of transcription by PNA homoduplexes or PNA:DNA heteroduplexes modeled after the SP6 or T7 promoter sequences. Neither PNA:DNA heteroduplexes with PNAs serving as either coding or noncoding strands nor PNA:PNA homoduplexes inhibited transcription by SP6 or T7 RNAP at concentrations up to 1.7 μM (Table 1). Apparently, interactions within the major or minor grooves or with the one remaining phosphodiester backbone of the heteroduplex are insufficient to yield observable association. The strikingly different affinities of PS, PO, and PNA oligonucleotides with proteins suggests that, while PNAs rapidly bind to their complementary DNA sequence, they have significantly less propensity to associate with proteins. This enhances their ability to target nucleic acids selectively. Advantages of this situation are evident for inhibition of telomerase through binding to its RNA template, where PS oligonucleotides inhibit nonsequence selectively, while PNA oligomers inhibit with high sequence selectivity.[4] In similar experiments we failed to observe interactions between TATA binding protein (TBP) and cognate PNA:PNA or PNA:DNA duplexes (G. Devora and D. Corey, unpublished results), even though the DNA:TBP cocrystal structure[11,12] shows that the complex is held together by protein contacts with the nucleotide bases, rather than the phosphodiester backbone.

Conclusions

The composition of oligonucleotide backbone linkages not only affects the affinity[13] and rate of association,[14] but also the specificity of recognition. Promoter recognition appears to be finely balanced to yield effective sequence-specific discrimination, and relatively subtle ionic and steric modifications in the backbone can upset this recognition and abolish selectivity. Nonselective protein binding is a problem that has hindered the use of PS oligonucleotides. PNAs do not bind nonselectively, which may facilitate their use as selective antisense molecules.

Experimental

General. Radiolabeled α-^{32}P rCTP, SP6/T7 grade (800 Ci/mmole), was purchased from Amersham. SP6 and T7 RNAPs were purchased from Promega. In vitro synthesis of mRNA using template plasmid p629E10 was carried out using reagents provided in the Riboprobe™ systems kit from Promega. Synthesis of phosphorothioate modified oligonucleotides was performed using tetraethylthiuram disulfide (TETD, Applied Biosystems). PNA oligomers were synthesized as described[15,16] using monomer reagents supplied by PerSeptive Biosystems. PNA purity was evaluated by mass spectral analysis.

Transcription templates and oligonucleotides. Template DNA used in these studies was plasmid p629E10, a 4.07 Kb pGEM-4Z (Promega) derivative. p629E10 was obtained from Dr. Narayan Iyer and generated by introducing in frame a 1.3 Kb insert containing the gene p62 with a 9E10 epitope tag into the *BamH*I site of the polylinker of parent plasmid pGEM-4Z. Plasmid DNA was linearized with *Sca*I so that the potential transcript with SP6 RNAP is 3620 bases and the transcript with T7 polymerase is 2796 bases.

The SP6 promoter analog PO and PS duplex sequences used in the inhibition studies are listed in the 5′ to 3′ direction as follows: GGATTTAGGTGACACTATAGA and TCTATAGTGTCACCTAAATCC. The T7 promoter sequences were TCTAATACGACTCACTATAGG and CCTATAGTGAGTCGTATTAGA. The PO and PS hairpin sequences were complementary repeats linked by four cytidine bases. The SP6 promoter PNA sequences were NH_2-CCTATTTAGGTGACACATAGT-COOH and NH_2-CTATAGTGTCACCTAAATAGG-COOH. The T7 promoter PNA sequences were NH_2-TCTAATACGACTCACTATAGG-COOH and NH_2-CCTATAGTGAGTCGTATTAGA-COOH. The sequence of the PS hairpin based on a scrambled version of the SP6 promoter sequence was TGTCCTAAATATAGACCTCCCCAGGTCTATATTTAGGACA. The complementary oligonucleotide, PNA pairs, and hairpin oligonucleotides were annealed at 65 °C for 15 min and cooled gradually to room temperature over a period of 60 min to form duplexes. The formation of base-paired

structures by DNA and PS hairpin oligonucleotides was confirmed by determining their melting temperature profiles using a Hewlett Packard 8452 diode array spectrophotometer equipped with a Peltier temperature controller in a solution of 40 mM Tris-Cl pH 7.5, 6 mM $MgCl_2$, and 10 mM NaCl.

In vitro inhibition of transcription. The reactions were initiated by the addition of SP6 or T7 RNAP, 0.5 μL (20 U/μL) in the presence of varying concentrations of oligonucleotide (0 to 1700 nM). Assays were carried out at 37 °C for 60 min, and the reactions were stopped upon the addition of 1/5th volume of 0.5 M EDTA. Transcribed products were separated on a 4% polyacrylamide gel, and relative incorporation of radiolabel in each transcript was quantified using a Molecular Dynamics model 425F phosphorimager with ImageQuant software version 3.3. Background radioactivity was determined from mixtures where no polymerase was added and subtracted from all measurements.

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