Gene-Specific Transcription Inhibitors. Pentanucleotides Complementary to the Template Strand of Transcription Start Sites

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The single-stranded DNA template at the initiation site of RNA synthesis in transcriptionally competent open complexes formed with RNA polymerase provides a unique target for the design of gene-specific inhibitors.[1−5] Here, we show that the open complexes formed by *Eschericia coli* RNA polymerase (RNase) with the *lac* UV-5 and *trp* EDCBA promoters can be selectively inhibited *in vitro* by nuclease-resistant and nonextendible pentaribonucleotides complementary to sequences −3 to +2 of the template strand of each promoter. Promoter specific targeting of scission to the template strand by pentanucleotides linked to the chemical nuclease 1,10-phenanthrolinecopper (OP-Cu) confirms that hybridization to the template strand is responsible for the observed inhibition.[6]

The scission targeted by UGGAA linked to OP-Cu and its inhibition of *de novo* RNA synthesis of transcripts labeled at the 5′-end with P^{32}-γ-ATP have demonstrated that this pentaribonucleotide (5′-UGGAA-3′) complementary to sequence −3 to +2 of the *lac* UV-5 template strand, but not its deoxyoligonucleotide analog (5′-TGGAA), hybridizes to the open complex formed with *E. coli* RNA polymerase (Scheme 1).[6] Since the utility of complementary pentanucleotides which can arrest transcription of targeted genes in biochemical and pharmacological studies will depend on their hydrolytic stability and potency, in addition to their specificity, we have investigated the tolerance of hybridization sites created in the open complexes of *E. coli* promoters for structural variation within the pentamer by preparing RNase-resistant pentamers from 2′-*O*-methyl precursors containing 5-propynyluridines to enhance binding[7]

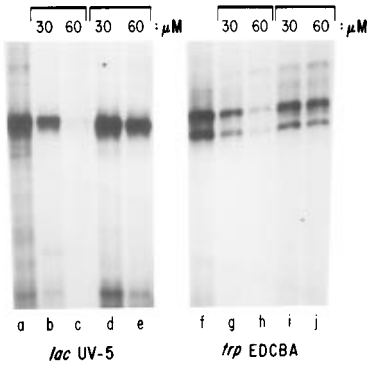
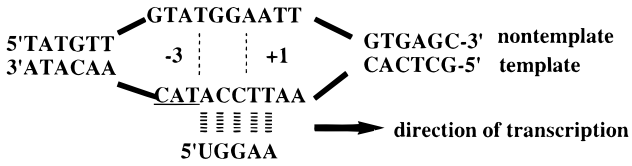
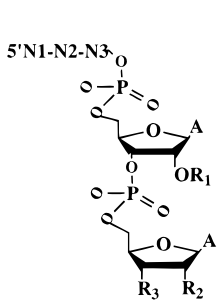


Figure 1. Selective inhibition of the *lac* UV-5 and *trp* EDCBA promoters by L-III and T-III oligonucleotides. Concentrations of inhibitory pentanucleotides are indicated. Transcription directed by *lac* UV-5, lanes a−e: lane a, control; lanes b, and c, L-III synthesized with propynyl U; lanes d, and e, T-III. Transcription directed by *trp* EDCBA, lanes f−j: lane f, control; lanes g, and h, T-III; lanes i,and j, L-III synthesized with propynyl U. Reactions were initiated by the addition of 100 μM each of ATP, CTP, GTP, and UTP with α-^{32}P-UTP to a solution of *lac* UV-5 or *trp* EDCBA and *E. coli* RNA polymerase which had been incubated for 20 min and then for 5 min with heparin (2 μg/mL). Conditions: pH 7.9, 80 mM Tris; 200 mM KCl; 6 mM $MgCl_2$, 0.1 mM DTT, and 200 μg/ml BSA; total volume 100 μL; T = 37 °C. Comparable inhibition is observed if the RNA polymerase is added last.

Scheme 1. Hybridization of UGGAA to *lac* UV-5 Open Complex Where Underlined Nucleotides Indicate Sites Cleaved by OP-Conjugate



and a nonextendible terminal 3′-deoxyribose to block the *in situ* generation of elongated products.[8] To establish gene-specific inhibition, we compared the kinetic effects of a UGGAA analog (L-III) and a CGCAA analog (T-III) on transcription from the *lac* UV-5 and *trp* EDCBA promoters[9] (Figure 1; see structures below). We chose these promoters because both are relatively strong and neither requires activator proteins for maximal activity.[10,11] L-III inhibited transcription more effectively from *lac* UV-5 than from *trp* EDCBA. Conversely, T-III, which is complementary to *trp* EDCBA, inhibited transcription from this promoter more effectively than that directed by *lac* UV-5. In each case, the oligonucleotide analogs inhibited their targeted promoters greater than 85% at 60 μM but the nontargeted promoter only 15−25%. This assay provides a challenging test of stringency because L-III and T-III are identical at three of five positions (−2, 1, and 2).



UGGAA

R_1= H; R_2=H; R_3=OH
L-I
R_1= CH_3; R_2=H; R_3=OH
L-II
R_1= CH_3; R_2=OH; R_3=H
L-III

CGCAA

R_1= CH_3; R_2=H; R_3=OH
T-II
R_1= CH_3; R_2=OH; R_3=H
T-III

Since nonspecific binding of oligonucleotides to RNA polymerase could account for a component of the observed inhibition

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(9) All pentanucleotides were purified either on a Perkin-Elmer oligonucleotide purification cartridge while still bearing the 5′-dimethyltrityl group or by 20% nondenaturing acrylamide gels. They were pure as judged by HPLC. Sodium and potassium counterions were carefully avoided during the purification. Characterization was achieved by matrix-assisted laser desorption ionization (MALDI). Samples were made approximately 100 μM and contained 1% triethylamine (TEA). Results were within the error limit of 0.2%. T-III: expected 1767.3; found 1768.3, 1764.1, and 1765.8 in three runs. L-III: expected 1762.3; found 1763.9, 1762.7, and 1761.5 in three separate runs. All nucleotides were 5′-thiophosphorylated using chemical phosphorylation and Beaucage reagents (Glen Research). Cleavage and deprotection were carried out overnight in NH_4OH at 55 °C.

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(typically 15−25%) of the noncognate promoters, we sought additional verification for the sequence-specific hybridization of the pentanucleotides to their complementary template strand sequences. The targeted scission of the template DNA within the open complex by pentanucleotides L-II and T-II covalently modified with the chemical nuclease OP-Cu provides a direct test for the sequence and site specificity of this interaction within the open complex.[6,12] The OP-linked pentanucleotides were prepared with 2′-*O*-methyl nucleotides, 2′-deoxy termini, and unmodified uridines.[6,13,14] Selective hybridization is demonstrated (Figure 2) by showing that OP-UGGAA cleaves the template strand of *lac* UV-5 (lane 14) but OP-CGCAA (lane 15) does not. In contrast, OP-CGCAA cleaves the template strand of *trp* EDCBA (lane 8) but not *lac* UV-5 (data not shown). The cleavage sites of OP-CGCAA correspond to those generated with the cuprous chelate of 3,4,7,8-tetramethyl-1,10-phenanthroline, a scission reagent with preference for the open complex of the *trp* EDCBA promoter. This chelate also cleaves the *lac* UV-5 open complex.[6,15] The cleavage of the open complex by the cuprous chelate of 5-phenyl-1,10-phenathroline confirms the formation of the open complex while DNA polymerase (DNase) I footprinting (Figure 2 lanes 3 and 4 and lanes 10 and 11) proves that RNA polymerase binds quantitatively to the *trp* EDCBA and *lac* UV-5 promoters, respectively. Targeted scission is observed at concentrations of UGGAA and CGCAA analogs 10-fold less than those needed to observe inhibition. These results are consistent with competitive binding between the pentanucleotides and the NTPs which were not present during targeted scission.

The advantage of directing these pentanucleotides to DNA as opposed to mRNA in this new, mechanism-based approach for the design of gene-specific transcription inhibitors is that recognition of only one or two target sites is sufficient to block gene expression.[16,17] Although triple helix forming oligonucleotides are also directed to DNA targets, only a restricted set of DNA sequences form stable triplexes, and such an approach cannot preferentially target actively transcribed DNA.[18] In addition, since deoxyoligonucleotides 15 nts long are required for high affinity binding and specificity, their transport across cell membranes is limited.[17]

Statistically, a pentaribonucleotide would be recognized each 1 kB within duplex B-form DNA[6] if it could spontaneously displace the DNA sequence of identical sequence and form a stable heteroduplex DNA under physiological conditions.

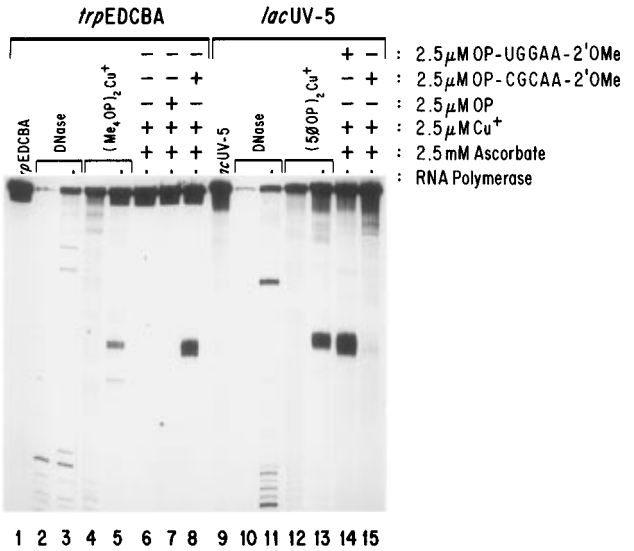

Figure 2. OP-rCGCAA-Directed, oxidative scission of the *trp* EDCBA open complex demonstrates gene-specific association with the *trp* EDCBA promoter and not the *lac* UV-5 promoter. Lane 1: *trp* EDCBA promoter fragment labeled on template strand. Lanes 2 and 3: DNase footprint of the promoter and open complex respectively. Lanes 4 and 5: 3,4,7,8-tetramethyl-1,10-phenanthrolinecopper footprint of the *trp* EDCBA promoter and open complex, respectively. Lane 6: copper control (2 μM), Lane 7: OP-Cu control (2.5−2 μM). Lane 8: site-specific scission directed by 2.5 μM OP-rCGCAA-(2′-OMe)$_{2-5}$ to *trp* EDCBA. Lane 9: *lac* UV-5 promoter labeled on the template strand. Lanes 10 and 11: DNase footprinting of the *lac* UV-5 promoter and open complex, respectively. Lanes 12 and 13: 5ϕOP-Cu footprinting of the *lac* UV-5 promoter and open complex respectively. Lane 14: (positive control) site-specific scission directed by 2.5 μM OP-rUGGAA-(2′-OMe)$_{2-5}$ to the *lac* UV-5 promoter. Lane 15: (negative control) site-specific scission directed by 2.5 μM OP-rCGCAA-(2′-OMe)$_{2-5}$ to the *lac* UV-5 promoter. Scission in lanes 6−8, 14, and 15 was initiated by addition of sodium ascorbate and continued for 25 min at 37 °C.

However, the formation of these stable heteroduplexes should not be expected since R-loops formed with RNAs less than 44 nts are unstable even under optimal conditions for the formation of these structures.[19] The key to the mechanism of transcription inhibition by the pentaribonucleotide is that RNA polymerase *creates* a single-stranded DNA at the start of transcription of the target gene. Despite a difference in nucleotides at only two sequence positions between CGCAA and UGGAA, we observed selective gene-specific inhibition and targeted scission of promoter DNA *in vitro*. Implications exist for the design of antiviral and antitumor drugs.

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