Hybridization of a Complementary Ribooligonucleotide to the Transcription Start Site of the *lac*UV-5–*Escherichia coli* RNA Polymerase Open Complex. Potential for Gene-Specific Inactivation Reagents†

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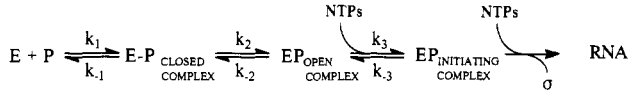
ABSTRACT: An ribooligonucleotide, UGGAA, complementary to the template strand of the *lac*UV-5 promoter can hybridize to the transcription "bubble" of the open complex formed by *Escherichia coli* RNA polymerase. Its site-specific binding, measured by gel retardation, enzyme inhibition, and chemical nuclease footprinting, is dependent on catalysis by RNA polymerase and the sequence of the hybridizing ribooligonucleotide. When UGGAA is linked to the chemical nuclease 1,10-phenanthroline copper, site-specific scission of the template strand of the transcriptionally active gene is observed. The formation of single-stranded DNA at transcription start sites by RNA polymerases provides a target for antigene strategies.

The synthesis of messenger RNA by DNA-dependent *Escherichia coli* RNA polymerase holoenzyme ($\alpha_2\beta\beta'\omega\sigma$) is a multistep enzymatic reaction proceeding through a series of distinct kinetic intermediates (eq 1) including a closed



complex representing the initial binding of the enzyme to the promoter, an open complex requiring Mg^{2+} for the formation of melted DNA, an initially transcribing complex capable of synthesizing the first few phosphodiester bonds, and finally a highly processive elongation complex resulting in full length transcript. The σ subunit serves an initiating role and dissociates from the enzyme after 10–11 nucleotides have been incorporated (Buc & McClure, 1985; Carpousis & Gralla, 1985; Krummel & Chamberlin, 1989, 1992; Kuwabara & Sigman, 1987; McClure, 1985; Shanblatt & Revzin, 1984).

The steady-state intermediates formed at the initiation of transcription have been studied by a variety of chemical approaches. For example, structural changes associated with the formation of the open complex composed of the *lac*UV-5 promoter and RNA polymerase have been examined with dimethyl sulfate, potassium permanganate, and the cuprous complex of 1,10-phenanthroline and its derivatives (Kirkegaard et al., 1983; Carpousis & Gralla, 1985; Kuwabara & Sigman, 1987; Spassky & Sigman, 1985; Thederahn et al., 1990). Dimethyl sulfate and potassium permanganate react with bases and modify single-stranded regions of both the template and nontemplate strands of the transcription bubble formed at the start of RNA synthesis. In contrast, the 1,10-phenanthroline–copper complex, $(OP)_2Cu^+$, which oxidizes the deoxyribose moiety, reacts exclusively with the template strand of this promoter. This redox-active complex generates strong cleavage sites at the the start of transcription with prokaryotic promoters (Spassky & Sigman, 1985; Frantz & O'Halloran, 1990) and the adenovirus late promoter (Buratowski et al., 1991). The specificity of this chemical nuclease for the start sites of RNA synthesis suggests that the "transcription bubble" formed in the open complex provides a specific and high-affinity binding site for the tetrahedral coordination complex: $(OP)_2Cu^+$. This hypothesis has been confirmed by the demonstration that the redox inactive and exchange stable 2:1 2,9-dimethyl-1,10-phenanthroline–cuprous complex [$(Me_2\text{-}OP)_2Cu^+$] blocks the template-specific scission reaction by the redox active complex and is an effective inhibitor of transcription (Mazumder et al., 1993).

Given the accessibility of the template strand to the tetrahedral cuprous complexes of 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline, the template strand should also be accessible for hybridization by a ribooligonucleotide complementary to the region immediately upstream of the start of transcription. Such hybridization would suggest a new approach for the development of gene-specific inactivation reagents in which recognition depends on the catalytic activity of RNA polymerase and the sequence of the hybridizing ribooligonucleotide. This paradigm has been tested in the present communication by synthesizing a ribooligonucleotide of sequence UGGAA which is complementary to the region from −3 to +2 of the template strand of the *lac*UV-5 promoter (Figure 1). Its specific interaction with the open complex has been demonstrated by gel retardation, chemical footprinting/protection, transcriptional inhibition, and the site-specific scission of the transcription initiation site by UGGAA covalently modified with 1,10-phenanthroline-copper.

MATERIALS AND METHODS

Promoter Fragment. A 203 bp *Eco*RI fragment containing the *lac*UV-5 promoter is overexpressed on a pUC-derived high copy number plasmid, restricted, and purified by 8% PAGE. The fragment is then phosphatased and 5′ labeled by T4 polynucleotide kinase (Gibco-BRL) in the presence of [γ-^{32}P]-ATP. *Pvu*II digestion of the 203 bp fragment yields a 186 bp fragment uniquely labeled on the template strand at the 5′ end.

Synthesis of UGGAA. The UGGAA pentaribonucleotide was synthesized by conventional automated synthesis from *tert*-butydimethyl-protected phosphoramidite precursor ribo-

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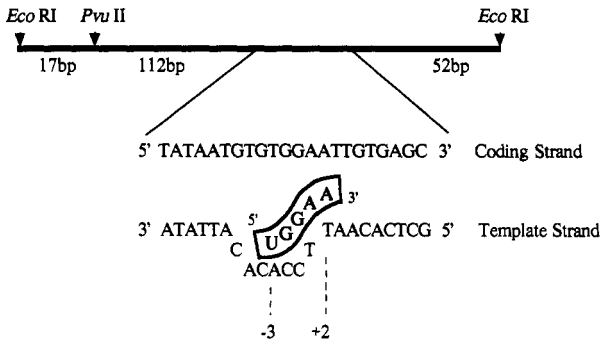
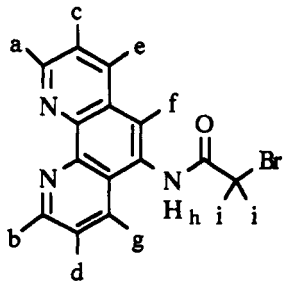


FIGURE 1: Schematic of UGGAA association with promoter–polymerase open complex. Shown above is the 203bp *Eco*RI fragment containing the *lac*UV-5 promoter while directly below is the bubble formed by RNA polymerase as it displaces the template strand. Boxed is the pentaribonucleotide UGGAA which is complementary to the template strand from positions −3 to +2. In later experiments, a *Pvu*II digest yields a 186 bp fragment uniquely labeled on the template strand.

nucleotides. The resulting RNA was then deprotected with TBAF (Aldrich), desalted over an HPLC-RPC4 column (Phenomenex), sized and purified over an HPLC-DEAE column (Nucleogen), and finally desalted for use over a disposable mini-C18 column. Five picomoles was kinased and analyzed by a 20% urea denaturing PAGE to confirm purity. In the following experiments, RNA concentrations were either monitored by UV absorbance (257 nm) or calculated from the specific radioactivity of the ATP.

Phenanthroline Derivatives and Synthesis of 5-(Bromoacetamido)-1,10-Phenanthroline. 2,9-Dimethyl-, 5-phenyl-, and 5-nitro-1,10-phenanthroline are commercially available from GFS Chemicals, Columbus, OH. 5-Nitro-1,10-phenanthroline is reduced in the presence of ammonium sulfide to give 5-amino-1,10-phenanthroline (Sigman et al., 1991). Synthesis of the 5-bromoacetamido derivative was carried out using a modified Schotten–Baumann procedure. 5-Amino-1,10-phenanthroline (0.5 g) is dissolved in 100 mL of $CHCl_3$. Twenty-five milliliters of 0.1 M NaOH is layered on top, and 1.1 equiv (0.3 mL) bromoacetyl bromide (Aldrich) is added slowly to the bottom organic layer with a Pasteur pipette. The reaction is stirred at room temperature for 2 h. The organic layer is then washed three times with 50 mM Na_2CO_3. The product is precipitated with 3 volumes of petroleum ether, and crystals are washed with 200 mL of cold petroleum ether, and then dried at 80 °C under house vacuum (yield: 50%). The product migrates as one spot on normal phase TLC silica plates (Kiesegel-Merck F_{60}) in 5% concentrated NH_4OH: 95% isopropanol (R_f, 0.44), reacts with 1-decanethiol, and gives a deep red color with ferrous ion characteristic of the 1,10-phenanthroline nucleus. NMR (Brüker 200-MHz NMR in DMSO-d_6) assignments:



H_a (doublet) δ = 9.04 ppm, J = 4.14 Hz; H_b (doublet) δ = 9.13 ppm, J = 4.29 Hz; H_c (quartet) δ = 7.75 ppm, J = 11.51 Hz; H_d (quartet) δ = 7.65 ppm, J = 12.46 Hz; H_e (doublet) δ = 8.50 ppm, J = 8.17 Hz; H_f (doublet) δ = 8.62 ppm, J = 2.66 Hz; H_g (doublet) δ = 8.18 ppm, J = 8.35 Hz; H_h (singlet) δ = 10.61 ppm, H_i (singlet) δ = 4.28 ppm. Elemental analysis (Galbraith Labs): % (expected) C:H:N:Br, 53:3.5:13.3:25; % (found) 52.57:3.28:12.89:24.69.

Synthesis of OP-NAc-SP-UGGAA. Deprotected, purified, desalted UGGAA (5 nmol) was kinased in volumes of 25–50 μL with a 5-fold molar excess of [γ-^{32}S]ATP (Boehringer-Mannheim) and [γ-^{35}S]ATP (Amersham) for 3 h at 37 °C with a specific radioactivity of 528 cpm/pmol. Twenty-five microliters of a 30 mM DMF solution of 5-(bromoacetamido)-1,10-phenanthroline, corresponding to a 3-fold molar excess over total thiol, was added followed by 1 μL of 10% triethylamine. Alkylation was complete after a 4 h incubation at 37 °C whereupon 2 μL of 100 mM DTT was added to quench the reaction. An equal volume of loading solution (0.1% bromphenol blue in formamide) was added and the reaction mixture purified by 21% (29:2 monomer to bis) nondenaturing PAGE at 900–1200 V constant in 1× TBE until the bromphenol blue had migrated 50%. Autoradiography reveals that the alkylated product runs 110% with respect to the dye while the free thiophosphorylated UGGAA runs 130% with respect to the dye. The alkylated product is eluted from the gel slice into 2 mL of water. In contrast to the starting material, the alkylated product is resistant to phosphatase. A final concentration of OP-NAc-SP-UGGAA is calculated from the specific radioactivity of the [γ-^{35}S]-ATP in the kinase mixture.

Gel Retardation Condition Used To Monitor the Binding of UGGAA to the Open Complex. UGGAA is labeled with excess chemical quantities of [γ-^{32}P]ATP (8800 cpm/pmol), purified by PAGE, and eluted in water. Aliquots of the stock solution of ^{32}P-UGGAA are withdrawn and evaporated to dryness. All samples are then resuspended in 1× transcription buffer: 40 mM Tris, pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM DTT, 100 μg/mL BSA, and 5% glycerol. The buffer also contained 30 fmol/μL unlabeled *lac*UV-5 promoter and 0.2 U RNA polymerase/μL, except where absence of polymerase or the promoter is indicated. Control lanes 1 and 2 (Figure 2) contained the labeled 203 bp *lac*UV-5 promoter. All samples were incubated for 20 min at 37 °C, heparin challenged (75 μg/mL final heparin) for 2 min at 37 °C, and loaded onto a 4% nondenaturing PAGE gel (29:1) run at 20 mA constant until the bromphenol blue had migrated 75% down the gel. The gel was dried on Whatman 3M paper, autoradiographed, and densitized. A K_d of 0.5 μM was calculated from a double-reciprocal plot of UGGAA concentration vs density R^2 = 0.986.

Footprinting the Binding of UGGAA to the Open Complex with 5-Phenyl-OP-Cu and DNase I. Appropriate amounts of UGGAA are dried down and resuspended in 10 μL of 1× transcription buffer containing 50 mM KCl, 40 mM Tris-HCl, pH 7.9, and 10 mM $MgCl_2$. Labeled *lac*UV-5 *Pvu*II restriction fragment (3000 cpm/μL) and 0.2 unit/μL RNA polymerase are included in the reaction mixture as indicated. Samples were incubated for 20 min at 37 °C. DNase footprinting: cutting proceeds for 1 min at 37 °C (0.83 mg/mL final DNase) and quenched with 1 μL of 0.5 M EDTA. For 5-phenyl-OP-Cu footprinting, final concentrations of 21 μM 5-phenyl-OP, 10 μM $CuSO_4$, and 5.8 mM mercaptopropionic acid are used. Cutting proceeds for 4 min at 37 °C before quenching with 3 μL of 40 mM 2,9-Me_2OP. All samples then receive 6 μL of a solution containing 1.5 M NaOAc, pH 5.2, and 1 mg/mL sonicated calf thymus DNA, followed by 150 μL of isopropanol. The samples are pelleted for 30 min at

14 000 rpm. The pellets are then washed with 70% ethanol, dried, resuspended in 25 μL of loading solution (deionized formamide, 0.1% bromphenol blue), heated to 90 °C for 5 min, and loaded onto a 10% urea denaturing PAGE. The gel is run at 55 W constant in 1× TBE until the bromphenol blue has just run off the gel.

Transcriptional Elongation of UGGAA. Elongation of UGGAA was assessed in the following manner. Negative controls received no UGGAA. UGGAA (10 pmol) was used for extension in each reaction. All lanes received 10 μL of a reaction mixture consisting of 30 fmol/μL unlabeled *lac*UV-5 promoter, 0.2 unit/μL RNA polymerase, 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl. After incubation for 20 min at 37 °C, 1 μl of [α-^{32}P]ATP or 1 μL of [α-^{32}P]UTP was added and the reaction mixture incubated for an additional 45 min at 37 °C. The reaction was arrested by addition of 2 μL of 0.5 M EDTA and 10 μL of formamide loading dye. Samples were then analyzed by 20% denaturing PAGE. Extension was observed only in the presence of UTP.

Inhibition of Nascent Strand Synthesis by UGGAA. Inhibition of *de novo* transcript synthesis was assayed with [γ-^{32}P]ATP as the sole source of label. Since background is high, a control lane of only [γ-^{32}P]ATP was run. The full length transcript control received no UGGAA. Increasing amounts of UGGAA were dried down and resuspended in 10 μL of a buffer consisting of 30 fmol/μL unlabeled *lac*UV-5 promoter, 0.2 unit/μL RNA polymerase, 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl to give the indicated concentrations. All samples were incubated for 20 min at 37 °C. For analysis of inhibition of full length transcript, samples received 1.1 μL of a nucleotide mixture of 500 μM ATP, UTP, CTP, GTP, and 5.3 μCi/μL [γ-^{32}P]ATP. Samples were transcribed 45 min at 37 °C, arrested with 2 μL of 0.5 M EDTA and 10 μL of formamide loading dye, and analyzed by 8% denaturing PAGE and autoradiography (Figure 4A). For analysis of inhibition of abortive transcripts, 1.1 μL of a partial nucleotide complement excluding CTP was used: 500 μM ATP, UTP, GTP, and 5.3 μCi/μL [δ-^{32}P]ATP. Reaction products were analyzed by 20% urea denaturing PAGE and the gel was dried onto Whatman 3M paper. The products were visualized by autoradiography, and, in the case of the abortive products, the bands representing abortive 7-mer and 8-mer transcripts were eluted from the paper into 500 μL of 1 M KOH and 6% H_2O_2 at 95 °C for 10 min, and counted in scintillation fluid. The counts from the [γ-^{32}P]ATP control were subtracted from all other samples as background. In the case of abortive transcript inhibition, the percent inhibition was plotted as a function of UGGAA concentration (Figure 4B).

Targeted Scission of the Open Complex by OP-NAC-SP-UGGAA. Aliquots of OP-NAc-SP-UGGAA were dried down and resuspended in 10 μL of a 1× transcription mixture: 50 mM KCl, 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 3000 cpm/μL labeled promoter, 0.2 unit/μL RNA polymerase. OP-NAc-SP-UGGAA-directed scission was initiated with 2.5 mM sodium ascorbate, continued for 25 min at 37 °C, and quenched by 3 μL of 40 mM 2,9-Me_2OP. Samples were precipitated by the addition of 1.5 M NaOAc, pH 5.2, and 1 mg/mL sonicated calf thymus DNA, followed by 150 μL of 2-propanol. The samples were pelleted for 30 min at 14 000 rpm. The pellets were then washed with 70% ethanol, dried, resuspended in 25 μL of loading solution (deionized formamide, 0.1% bromphenol blue), heated to 90 °C for 5 min, and loaded onto a 10% urea denaturing PAGE run at 55 W constant in 1× TBE until the bromphenol blue has just run off the gel.

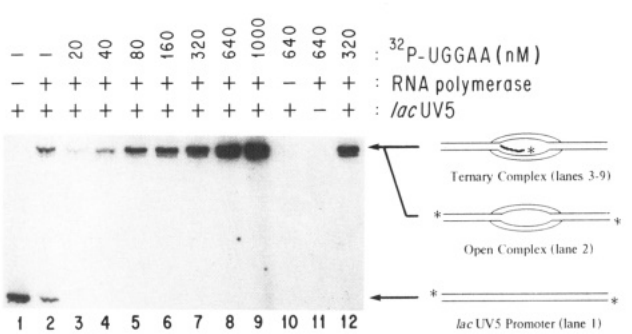


FIGURE 2: Stable ternary association of open complex with UGGAA in gel shift. (Lanes 1 and 2, positive controls) 5′-^{32}P-labeled *lac*UV-5 fragment and gel shifted open complex respectively. (Lanes 3–9, ternary complex, labeled pentamer) The promoter fragment is not labeled and increasing concentrations of 5′-^{32}P-UGGAA are used: 20, 40, 80, 160, 320, 640, and 1000 nM, respectively. (Lanes 10 and 11, negative controls) Each contains 640 nM UGGAA, but lane 10 contains only *lac*UV-5 promoter while lane 11 contains only RNA polymerase. Lane 12 contains 20 μM rifampicin and 320 nM 5′-^{32}P-UGGAA.

RESULTS

Demonstration of Hybridization Using Gel Retardation Analysis. Since the complex of *E. coli* RNA polymerase and the *lac*UV-5 promoter retains catalytic activity within the acrylamide matrix used in gel retardation assays (Kuwabara & Sigman, 1987; Shanblatt & Revzin, 1984; Straney & Crothers, 1985, 1987a,b), the hybridization of an RNA complementary to a sequence of the template strand upstream of the start of transcription was investigated using this method (Figure 2) (Crothers, 1987; Garner & Revzin, 1986). The hybridization of UGGAA to the open complex can be demonstrated with labeled UGGAA as the sole source of radioactivity. The open complex was formed in the presence of increasing concentrations of ^{32}P-UGGAA for 20 min at 37 °C. Before the samples were run into the gel, heparin was added to disrupt any nonspecific association of RNA polymerase with the promoter. A clear concentration dependence is observed in the shifted bands of lanes 3–9. No comparable band is apparent either with the *lac*UV-5 promoter fragment and ^{32}P-UGGAA in the *absence* of polymerase (lane 10) or with RNA polymerase and ^{32}P-UGGAA in the *absence* of the *lac*UV-5 promoter fragment (lane 11) even at a concentration of ^{32}P-UGGAA 300-fold greater than that necessary to observe a signal in the presence of RNA polymerase *and* the *lac*UV-5 promoter. Therefore this association involves a specific ternary interaction of the enzyme, the *lac*UV-5 promoter, and ^{32}P-UGGAA and not a binary association between RNA polymerase and the RNA or merely an R-loop with the promoter.

The dissociation constant (K_d) for the binding of ^{32}P-UGGAA to the open complex is 0.5 μM ± 0.2 μM, approximately 2 orders of magnitude lower than the K_m for the initiating nucleotide triphosphate, ATP. The affinity of ^{32}P-UGGAA for the open complex is not diminished by 20 μM rifampicin (Figure 2, lane 12), a concentration sufficient to completely inhibit transcription. This observation is consistent with the known affinity of rifampicin for RNA polymerase but not the promoter DNA (Jin & Gross, 1988; Kumar et al., 1992; McClure & Cech, 1978).

5-Phenyl-OP Copper Footprinting Demonstrates Pentamer Hybridization to Template Strand. Although the gel retardation analysis establishes that the pentamer binds to the open complex, it does not prove its sequence selectivity. Since (5-phenyl-OP)$_2$–Cu^+ footprinting can be used to visualize downstream displacement of the transcription bubble, the

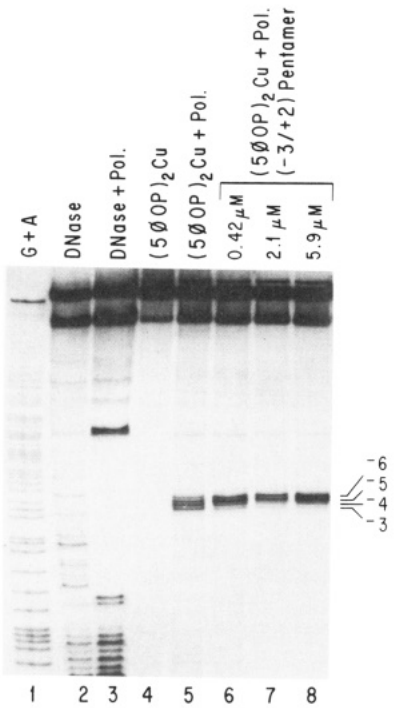


FIGURE 3: 5-Phenyl-1,10-phenanthroline–copper footprinting of UGGAA associated with the open complex. (Lane 1) Maxam–Gilbert G+A sequencing ladder. (Lanes 2 and 3) DNase footprint of promoter and open complex, respectively. (Lanes 4 and 5) 5ϕOP–Cu^+ footprint of promoter and open complex, respectively. (Lanes 6–8) 5ϕOP–Cu^+ footprint of UGGAA association with open complex with increasing concentrations of UGGAA, 0.42, 2.1, and 5.9 μM, respectively. 5ϕOP–Cu^+ is 10 μM in all cases.

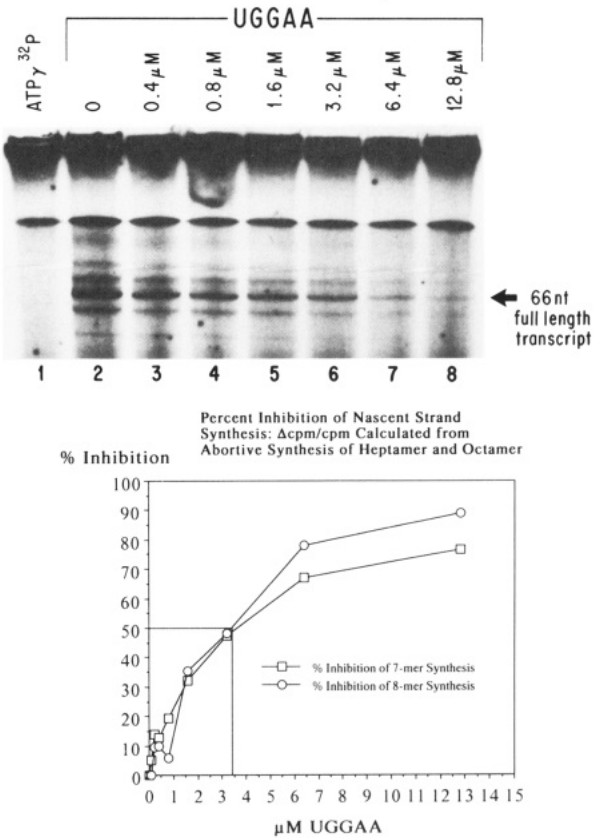


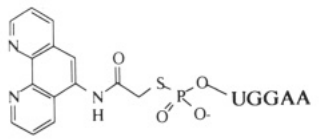
FIGURE 4: Unlabeled UGGAA inhibits nascent transcript synthesis. (Panel A, top) Autoradiogram of inhibition of *de novo* synthesis of full length transcript. (Lane 1) [γ-^{32}P]ATP. (Lane 2) Full length transcript, no UGGAA. (Lanes 3–8) Increasing concentrations of UGGAA: 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 μM. (Panel B, bottom) Inhibition of *de novo* abortive transcription was assayed in the following manner: Lane 1 received only [γ-^{32}P]ATP; lane 2 (full length transcript control) received no UGGAA; lanes 3–8 received appropriate amounts of UGGAA to give indicated concentrations. After 20 min at 37 °C, all samples received 1 μL of 500 μM ATP, UTP, CTP, GTP at 5.3 μCi/μL [γ-^{32}P]ATP and were incubated for 45 min at 37 °C, and the reaction products then excised from the gel and analyzed as described under Materials and Methods.

formation of a heteroduplex upon hybridization of UGGAA to the template strand within the open complex should be detectable as well. The binding of UGGAA to the open complex was established using the chemical nuclease (5-phenyl-OP)$_2$–Cu^+, which cleaves the template strand of the promoter just upstream of the transcriptional start site with comparable intensity at positions –6 to –3 (Thederahn et al., 1990). The addition of UGGAA to the open complex at 37 °C protects positions –3 and –4 and causes a slight enhancement of scission at positions –5 and –6 with increasing concentrations (Figure 3). Comparable protection has been observed using 5′-^{32}P-UGGAA indicating that a terminal phosphate does not play a central role in the binding. However, protection is *not* observed with the corresponding *oligodeoxyribonucleotide* TGGAA at 100-fold higher concentrations. The stability of UGGAA binding at 37 °C and the fact that TGGAA failed to protect against the (5-phenyl-OP)$_2$–Cu^+ scission suggest that the RNA–DNA heteroduplex is preferentially stabilized in this steady-state intermediate and that the free energy associated with Watson–Crick base pairing is not the only factor contributing to the stability. Earlier studies have demonstrated deoxyoligonucleotides terminated with a 3′ ribonucleotide can serve as primers of transcription from the bacteriophage T7-A_2 promoter catalyzed by *E. coli* RNA polymerase but their efficiency as primers was not compared with ribooligonucleotides of identical sequence (Grachev et al., 1984).

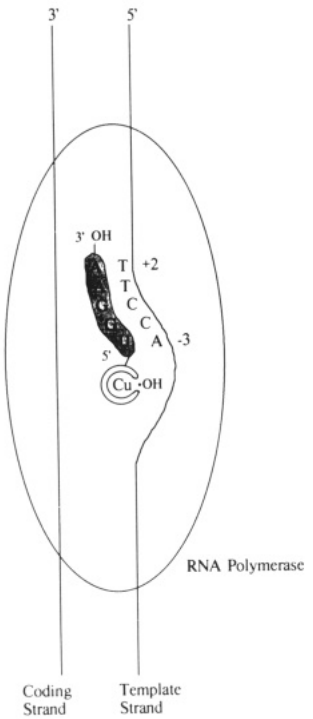
The footprinting experiments with (5-phenyl-OP)$_2$–Cu^+ further suggest that the pentamer does not randomly associate with the bubble or "slide" within the single-stranded region but that it is anchored specifically at the –3 position. Moreover, despite the observation that the region –2 to +2 is resistant to the chemical footprinting reagents, the protection of position –3 by the pentamer indicates that the entire region from +2 to –6 is solvent accessible.

UGGAA as a Primer and Inhibitor of Transcription. The fidelity of hybridization has been further demonstrated by the ability of labeled and unlabeled UGGAA to serve both as primers as well as effective inhibitors of transcription initiation. Ruetsch and Dennis (1987) previously demonstrated that tetraribonucleotides complementary to the template strand at positions –1 to +3 can prime the synthesis of short RNAs as long as their sequence does not extend beyond the third phosphodiester bond (Ruetsch & Dennis, 1987). To test if UGGAA hybridized to its complementary sequence at the transcriptional start site, it was used as a primer in a reaction mixture containing only [α-^{32}P]UTP, the nucleotide corresponding to positions +3 and +4. UGGAA was extended by exactly two bases. More importantly, *no* extension was observed in the presence of [α-^{32}P]ATP, the normal initiating nucleotide. This implies that UGGAA is correctly positioned at the +2 position and does not "slide" back to –1 to be extended in the presence of ATP.

Since UGGAA is extendable, it should also be a good inhibitor of [γ-^{32}P]ATP initiated transcription (Figure 4A,B). Since adenosine is the 5′ nucleotide of the RNA synthesized from *lac*UV-5, only *de novo* synthesized products will be terminally labeled with ^{32}P while those primed by UGGAA



OP-NAc-SP-UGGAA



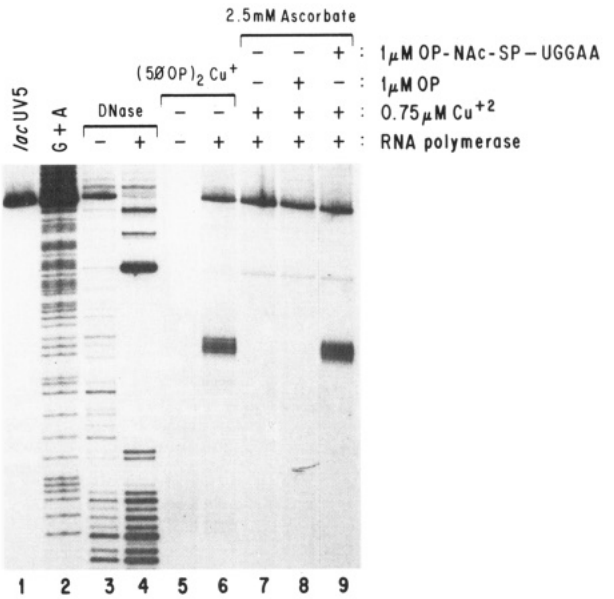


FIGURE 5: UGGAA directed oxidative scission of the open complex. (A, top left) OP-NAc-SP-UGGAA. (B, bottom left) Schematic of oligo-directed scission. (C, right) Autoradiograph. (Lane 1) 186 bp *lac*UV-5 promoter fragment labeled on template strand. (Lane 2) Maxam–Gilbert G+A sequencing ladder. (Lanes 3 and 4) DNase footprint of promoter and open complex, respectively. (Lanes 5 and 6) 5ϕOP–Cu^{+}(10 μM) footprint of promoter and open complex, respectively. (Lane 7) (negative copper control) 0.75 μM $CuSO_4$; (lane 8) (negative OP-Cu control) 0.75 μM $CuSO_4$, 1 μM OP (1,10-phenanthroline). (Lane 9) 0.75 μM $CuSO_4$, 1 μM OP-NAc-SP-UGGAA. Scission in lanes 7–9 was initiated with 2.5 mM sodium ascorbate, continued for 25 min at 37 °C, and then quenched by 3 μL of 40 mM 2,9-dimethyl-1,10-phenanthroline. The scission sites in lanes 6 and 9 are at positions –6 to –4 relative to the start of transcription.

will remain unlabeled and thus undetected. Incomplete and complete nucleotide triphosphate complements were used to observe inhibition of both abortive and full length transcription respectively. The I_{50} of UGGAA for transcription initiated by [γ-^{32}P]ATP was 3.4 μM.

Targeted Scission by OP-NAc-SP-UGGAA. In addition to providing a new direction in the design of gene-specific inactivation reagents, the targeted scission of the template strand by UGGAA covalently linked to 1,10-phenanthroline–copper would suggest a further test for the stringency of hybridization. UGGAA was covalently derivatized with the 1,10-phenanthroline moiety via alkylation by 5-bromoacetamido-1,10-phenanthroline of the 5′ thiophosphate which is introduced with polynucleotide kinase and [γ-^{35}S]ATP, yielding the chimera OP-NAc-SP-UGGAA.

The open complex was formed in the presence of 1 μM OP-NAc-SP-UGGAA. UGGAA-directed scission of the template strand was observed at positions –6, –5, –4, and –3 exclusively on the template strand (Figure 5). Neither free copper nor 1,10-phenanthroline–copper (1.33:1) at identical concentrations cleaved the template strand. The efficiency of scission approaches 33%. *In addition to unambiguously establishing the antiparallel hybridization of UGGAA to the template strand, this experiment demonstrates that RNA polymerase can potentiate the sequence-specific scission of a gene which is activated for transcription.*

DISCUSSION

Gel retardation, chemical nuclease footprinting, inhibition studies, and targeted scission have established the hybridization of UGGAA to the template strand of the *lac*UV-5 promoter. Gel retardation analysis demonstrated the specific binding of the ^{32}P-labeled pentamer to the open complex. Using the pentamer as the sole source of radioactivity, the ternary complex composed of RNA polymerase, *lac*UV-5 promoter DNA, and UGGAA exhibited the same rate of migration as the open complex itself in the polyacrylamide matrix. No binding of the pentamer was observed with enzyme alone or with the promoter fragment alone. This experiment proves that the retarded band represents a ternary complex and that the binding of RNA polymerase to the *lac*UV-5 promoter creates a specific binding site for the pentamer.

The demonstrated association of UGGAA with the *lac*UV-5 open complex complements the recent results of Daube and von Hippel, who demonstrated that RNA polymerase could elongate a synthetic dodecaribonucleotide which had been prehybridized to a double-stranded DNA fragment containing several mismatches engineered to simulate the single-stranded regions of DNA in the open complex (Daube & von Hippel, 1992). However, the association of UGGAA is distinct from that of Daube and von Hippel in that a transcriptionally competent open complex is formed on a B-DNA promoter to

which the pentamer then associates. In addition, this association is unusual since the association of a pentamer corresponding to regions +1 to +5, synthesized by RNA polymerase, is not isolable under gel-shift conditions (Krummel & Chamberlin, 1989). Our studies suggest a straightforward approach for probing the size of the transcription bubble and investigating structural and kinetic aspects of RNA polymerase–promoter association (Meares, 1991).

The gel retardation experiment by itself does not identify the binding site within the open complex. Since the chemical nuclease (5-phenyl-OP)$_2$Cu$^+$ specifically cleaves the single-stranded region upstream of the start of transcription, this coordination complex provides an ideal footprinting reagent for investigating the binding of the pentamer. UGGAA both protects and perturbs the sites cleaved by (5-phenyl-OP)$_2$Cu$^+$ in a concentration-dependent manner. Sequence positions −3 and −4 are protected from scission while the sites of scission at −5 and −6 are enhanced. The concentration range in which UGGAA influences the scission pattern is similar to that at which binding had been established by gel retardation analysis.

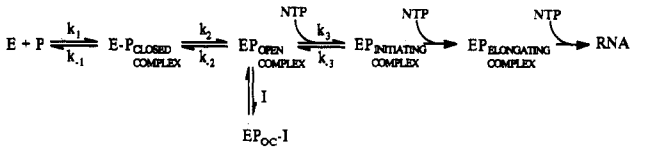
Since the protection of scission could occur by an indirect conformational change, two kinetic experiments were carried out in order to test the sequence specificity of the binding. In one experiment, UTP or ATP was used to extend the pentamer. If the UGGAA bound in a complementary fashion to the template strand and remained correctly positioned at the +2 position, only U, and not A, should have been added to the 3′ end of the pentamer since the first four nucleotides of *lac*UV-5 mRNA are AAUU (Figure 1). The fact that U and not A was added to the 3′ end of UGGAA strongly indicates that the pentamer is hybridizing to its complementary sequence on the template strand in the open complex.

A further test for the stringency of hybridization is the ability of UGGAA to inhibit the *de novo* synthesis of mRNA. The pentamer was assayed as an inhibitor of full-length as well as abortive transcription (Figure 4, panels A and B, respectively). In both cases [γ-^{32}P]ATP was used as the sole source of radioactivity to visualize synthesis of unprimed transcripts. UGGAA inhibited the synthesis of transcript initiated by [γ-^{32}P]ATP. The I_{50}'s in both assays were comparable.

The conversion of UGGAA into a scission reagent by covalent derivatization with 5-bromoacetamido-1,10-phenanthroline to give the chimera OP-NAc-SP-UGGAA provided a final test for the hybridization of the pentamer to its target. Previous studies have demonstrated that 1,10-phenanthroline–copper linked to a carrier oligonucleotide accomplishes the sequence-specific cleavage of an RNA or DNA target (Chen & Sigman, 1986, 1988). Hybridization of OP-NAc-SP-UGGAA to the open complex followed by the addition of cupric ion and ascorbic acid resulted in the efficient scission of the template DNA strand just upstream of the predicted site of hybridization of the pentamer. This cleavage reaction establishes the antiparallel orientation of UGGAA with respect to the template strand.

The kinetic mechanism for the enzymatic synthesis of RNA catalyzed by both eucaryotic and procaryotic polymerases involves multiple intermediates. Since double-stranded DNA cannot serve as the template for RNA synthesis because the sequence information of the template is masked by the complementary noncoding strand, some of these steady-state enzyme intermediates must contain single-stranded template DNA to permit faithful transcription of the nucleotide sequence. Base-specific chemical modification reagents and the chemical nuclease activity of 1,10-phenanthroline–copper have provided experimental approaches for detecting these single-stranded regions (Kirkegaard et al., 1983; Sasse-Dwight & Gralla, 1989; Thederahn et al., 1990). However, the sensitivity of these chemical methods is a function of (a) the specificity of the underlying chemistry, (b) the concentration of kinetically competent RNA polymerase–promoter complexes, and (c) the steady-state levels of those enzyme intermediates containing single-stranded DNA. Obtaining positive evidence for enzyme-bound single-stranded DNA structures is therefore problematic. Although permanganate reacts with single stranded T's (Sasse-Dwight & Gralla, 1989; Wang et al., 1992), it is not clear if strained DNA structures formed at the active site of RNA polymerase might not also be hyperreactive to the reagent.

Reversible transcription inhibitors that are capable of recognizing and stably associating with single-stranded regions of DNA provide an alternative method to identifying these intermediates (e.g., eq 2). They will inhibit RNA synthesis



because they will cause the accumulation of dead-end (or slowly turning over) complexes. In the present experiments, we have demonstrated that the single-stranded region of the template strand in the open complex is accessible to hybridization by a complementary pentamer. The dependence of the efficiency of hybridization on the length of the ribooligonucleotides will be the subject of further investigations. Enhancement of the binding affinity by linking the hybridizing ribooligonucleotide to intercalating agents or general transcription inhibitors like the 2:1 2,9-dimethyl-1,10-phenanthroline–cuprous complex will also be explored. Although the *lac*UV-5 promoter provides a favorable case for demonstrating hybridization of a complementary ribooligonucleotide, the single-stranded DNA of other transcription units may be accessible to complementary oligonucleotides as suggested by the studies of Grachev et al. (1984) and Ruetsch and Dennis (1987).

The hybridization of UGGAA to the transcription start site of the open complex of *lac* UV-5 promoter and *E. coli* RNA polymerase is determined by three factors. The first is that the enzyme induces the formation of single-stranded DNA upstream of the start of transcription. The second is the sequence complementarity of the hybridizing ribooligonucleotide. The third is that RNA polymerase preferentially stabilizes the annealing of UGGAA relative to the deoxynucleotide analogs TGGAA.

The enzyme-mediated, high-affinity binding of a ribooligonucleotide as short as a pentamer to the open complex may be an important advantage for inhibiting gene expression in intact cells. Short oligonucleotides are more permeable and easier to synthesize. Their nuclease-resistant ribonucleotide analogs will also be more synthetically accessible.

In addition to suggesting an approach for developing reversible inhibitors of actively transcribing genes, our studies also indicate that the hybridizing ribooligonucleotide can direct the scission of single-stranded DNA at transcription start sites. Reactive functionalities other than 1,10-phenanthroline–copper might also be linked to hybridizing RNA or RNA analogs and may be more efficient in exploiting the vulnerability of activated genes. Efficient strategies for achieving gene-specific knockouts or mutagenesis might therefore be

envisioned. Alternative approaches for targeting specific genes have involved the formation of triple helices on DNA or heteroduplexes of mRNA and oligonucleotides (Cooney et al., 1988; Durland et al., 1991; Helene & Toulme, 1990; Orson et al., 1991; Maher et al., 1992) or a peptide–nucleic acid strand (Egholm et al., 1992). None of these strategies exploits the formation of single-stranded DNA at the active site of RNA polymerase. This feature should facilitate *in vivo* applications of these inhibitors. Moreover, an antigene strategy in which the DNA of an actively transcribing gene is targeted rather than multiple copies of the product mRNA may prove to be an effective approach in blocking gene expression.

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