An approach to gene-specific transcription inhibition using oligonucleotides complementary to the template strand of the open complex

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The single-stranded region of DNA within the open complex of transcriptionally active genes provides a unique target for the design of gene-specific transcription inhibitors. Using the *Escherichia coli lac* UV5 and *trp* EDCBA promoters as *in vitro* models of open complex formation, we have identified the sites inside these transcription bubbles that are accessible for hybridization by short, nuclease-resistant, nonextendible oligoribonucleotides (ORNs). Binding of ORNs inside the open complex was determined by linking the chemical nuclease bis(1,10-phenanthroline) cuprous chelate [$(OP)_2Cu^+$] to the ORN and demonstrating template-specific DNA scission. In addition, these experiments were supported by *in vitro* transcription inhibition. We find that the most effective inhibitors are 5 nt long and have sequences that are complementary to the DNA template strand in the region near the transcription start site. The ORNs bind to the DNA template strand, forming an antiparallel heteroduplex inside the open complex. In this system, RNA polymerase is essential not only to melt the duplex DNA but also to facilitate hybridization of the incoming ORN. This paradigm for gene-specific inactivation relies on the base complementarity of the ORN and the catalytic activity and sequence specificity of RNA polymerase for the site- and sequence-specific recognition and inhibition of transcriptionally active DNA.

The initiation of RNA synthesis in both prokaryotes and eukaryotes is catalyzed by RNA polymerase (RNAP) and requires the formation of an open complex (1–5). The open complex is a transient structure characterized by a region of unwound, single-stranded DNA (ssDNA) near the transcription start site. Work in this laboratory has demonstrated that bis(1,10-phenanthroline) cuprous chelate [$(OP)_2Cu^+$] and its derivatives have an unusual affinity for the open complex (6, 7). These chelates have been shown to inhibit transcription (8–11) and also have been used to footprint the transcription initiation complex by exploiting $(OP)_2Cu^+$-induced oxidative cleavage of the ssDNA template in this region (12–14). Melted DNA inside open complexes also has been detected by single-strand-specific DNA modification reagents like $KMnO_4$ and dimethyl sulfate (15, 16). However, none of these chemical agents have a recognition element capable of targeting a particular open complex within a transcriptionally active genome. They are not "gene-specific" (17).

Recently, substantial effort has been directed at developing compounds that can selectively inhibit a single gene within a genome (18, 19) as reagents of this type would have significant implications for the development of antibiotics (20) and antineoplastics (21). Current methods for gene-specific inhibition include antisense technology (22), triple-helix formation (23, 24), and the use of DNA-binding polyamides (25). None of these methods exploits the transiently formed ssDNA within the open complex. Specificity in these systems is based solely on the recognition of static features of the nucleic acid target. The accessibility of the open complex to reagents like $(OP)_2Cu^+$ led us to the notion that the ssDNA inside the transcription initiation "bubble" would be an ideal target for the design of compounds that can specifically recognize transcriptionally active DNA. Such open complex-targeted agents then might be used to inhibit transcription in a gene-specific manner if sufficient affinity and specificity were obtained. Oligonucleotides are a logical starting point for the design of open complex targeting agents as they harness the known specificity of Watson–Crick base pairing and can be easily modified (26, 27). Nuclease-resistant, 3′-deoxy oligoribonucleotides (ORNs), which are complementary to the accessible DNA template strand, bind to the open complex but are not substrates for elongation by RNAP (Fig. 1) (28, 29). Previously, we demonstrated that 3′-deoxy ORN pentamers can inhibit *in vitro* transcription only from their complementary promoter and can effectively target oxidative scission to the open complex when tethered to 1,10-phenanthroline cuprous chelate [$(OP)Cu^+$] (30). ORNs can be used to uniquely target an open complex; they are gene-specific.

We focus here on mapping the ORN-accessible regions within transcriptionally active open complexes. Our goals were to determine the constraints on ORN length and position within the open complex and to correlate the efficiency of ORN binding with transcription inhibition. We exploited the DNA scission chemistry of $(OP)Cu^+$-tethered ORNs (OP-ORNs) both by direct and indirect techniques to confirm the open complex hybridization of each ORN in two well-studied *in vitro* promoters, *Escherichia coli lac* UV5 and *trp* EDCBA.

Materials and Methods

Synthesis of *lac* UV5, *trp* EDCBA, and Artificial Open Complex Fragments. The *lac* UV5 promoter fragment extending from positions −144 to +67 was restricted from a pUC-derived plasmid and labeled on the 5′ terminus of the template strand as described (6). A *trp* EDCBA promoter fragment encompassing positions −81 to +47 was isolated as described (31) and used for transcription experiments. An artificial open complex was constructed from 80-mer template and nontemplate strand oligonucleotides (Genosys, The Woodlands, TX). The sequence of the artificial open complex is identical to the *lac* UV5 promoter in the region from −60 to +20 except for a 9-base mismatch on the nontemplate strand from positions −6 to + 3. The sequence of the nontemplate strand in the artificial open complex in the region −6 to +3 is 5′-CACACCTTA-3′.

Abbreviations: OP, 1,10-phenanthroline, *ortho*-phenanthroline; $(OP)_2Cu^+$, bis(1,10-phenanthroline) cuprous chelate; $(OP)Cu^+$, 1,10-phenanthroline cuprous chelate; OP-ORN, $(OP)Cu^+$-tethered oliguribonucleotide; RNAP, RNA polymerase; ORN, oligoribonucleotide; ssDNA, single-stranded DNA.

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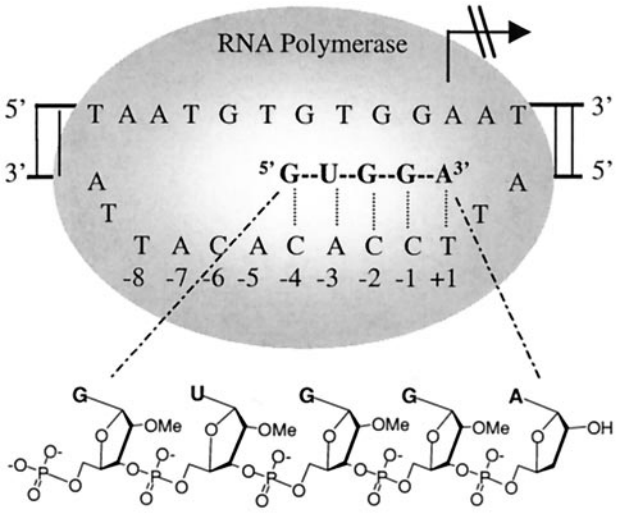

Fig. 1. Oligonucleotides lacking a 3′-hydroxyl group can be designed to target a specific open complex and inhibit *in vitro* transcription. The representation of RNAP is not meant to suggest that the enzyme is a single subunit or that it spans the promoter DNA over the sequences as drawn. By convention, the transcription start site is designated +1.

After gel purification of both oligonucleotides, the template strand was kinased with T4 polynucleoide kinase (Promega) and [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Pharmacia) according to the vendor's instructions. The labeled strand was combined with unlabeled nontemplate strand, heated at 92°C for 5 min, and then allowed to slowly cool to room temperature to anneal.

Synthesis of Oligonucleotide Inhibitors and 1,10-Phenanthroline (OP)-Linked Oligonucleotides. Modified ORNs were either synthesized by conventional automated synthesis using 3′-deoxyadenosine CPG columns and 2′-methoxyribose (2′-OMe) monomers (Glenn Research, Sterling, VA) or purchased from Genosys. The 5′ ends of all ORNs (except for 5′-GUGGA-3′-OP) were chemically phosphorylated (Glenn Research Chemical Phosphorylating Reagent II). Syntheses were performed trityl-on and ORNs were purified on a Glenn Research PolyPak cartridge. All ORNs were subjected to matrix-assisted laser desorption ionization-time of flight MS and visualized on polyacrylamide gel (20%) to confirm their sequence and purity. Treatment of phosphorylated ORNs (30 nmol in water) with 5-[β-alanylamido]-OP (32), in the presence of carbodiimide (33) and methylimidizole (pH 6.7) overnight at room temperature followed by polyacrylamide gel purification afforded OP-ORNs whose structures were confirmed by MS. Phenanthroline attachment to the 2′ position of 5′-GU(OP)GGA-3′ required the synthesis of 2′-aminouridine from cyclouridine as described by McGee *et al.* (34, 35). Carbodiimide coupling of the amine to 5-(propanoic acid)-OP afforded the 2′ amidophenanthroline uridine derivative. After conversion to the appropriate protected phosphoramidite, the monomer could be incorporated into 5′-GU(OP)GGA-3′ via standard oligonucleotide synthesis techniques (36).

Direct Targeted DNA Scission by OP-Linked Oligonucleotides. Open complex mixture is prepared from template-labeled *lac* UV5 promoter (3,000 cpm/μl) and 0.2 units/μl of *E. coli* RNAP (Amersham Pharmacia) in a transcription buffer containing 40 mM Tris (pH 7.9), 50 mM KCl, and 10 mM $MgCl_2$. Cleavage of the open complex by an OP-ORN (typically 15 μM) and analysis by gel electrophoresis has been described (26). DNase I footprinting was initiated by the addition of 0.05 units of enzyme (Promega) to 10 μl of open complex and allowed to proceed for 30 sec at 37°C before quenching. A Maxam-Gilbert G + A lane was generated from template-labeled promoter (37).

Indirect Targeted DNA Scission by OP-5′-UGGAA-3′. A mixture of open complex is prepared from labeled *lac* UV5 as described above. Two microliters of ORN (30 μM final concentration) is added to 8 μl of mixture. The mixture is incubated at 37°C for 20 min then 1 μl of OP-5′-UGGAA-3′ (15 μM final concentration) is added and incubation is continued (5 min, 37°C). Targeted scission by OP-5′-UGGAA-3′ is initiated by the addition of $CuSO_4$ (15 μM final concentration) and ascorbic acid (2.5 mM final concentration). Scission is allowed to proceed for 25 min at 37°C. Samples are quenched and analyzed as described above.

***In Vitro* Transcription Inhibition of the *lac* UV5 and *trp* EDCBA Promoters.** The following experimental protocol was developed to provide reliable, quantitative inhibition data. A mixture of open complex was generated from unlabeled *lac* UV5 fragment (300 fmol/μl) and 0.2 unit/μl *E. coli* RNAP (Promega) in a buffer containing 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 mixture (10 μl) was added to lyophilized ORNs to yield the appropriate inhibitor concentration (25 μM). The mixture was preincubated (37°C, 20 min) then transcription was initiated by the addition of 1 μl of an rNTP mixture containing 0.5 mM each rNTP and 5.3 μCi/μl [α-^{32}P]UTP (3,000 Ci/mmol, Amersham Pharmacia). Transcription was allowed to proceed for 1 min at 37°C before quenching with 10 μl of formamide. Incubation times longer than 1 min tended to produce transcription artifacts (38). Samples were analyzed by gel electrophoresis.

Results

Direct Targeted Scission by OP-ORNs. To identify the length and position of accessible sites inside the open complex, ORNs of 5, 6, 7, 8, 10, and 11 nt were synthesized with sequences complementary to the template strand of either the *lac* UV5 or *trp* EDCBA promoter near the transcription initiation site (Fig. 2). One ORN, shown as a dashed line in Fig. 2, is complementary to the nontemplate (*Upper*) strand of *lac* UV5. ORNs are referenced to the transcription start site (+1) and written from 5′ to 3′ so that −3:+2 is a pentamer complementary to the DNA template positions −3, −2, −1, +1, and +2. All ORNs contain the following modifications: a 3′-deoxy-2′-hydroxyadenosine terminus, a 5′ phosphate, and 2′ methoxyribose moieties throughout (Fig. 1). These modifications create nonextendible (by RNAP), nuclease-resistant ORNs with functionality at the 5′ end for covalent phenanthroline attachment. To compare the hybridization affinities of the ORNs, we first used a direct targeted scission approach whereby each ORN was covalently linked to 5-[β-alanylamido]-OP and tested for its ability to direct oxidative damage to the DNA template within the open complex upon addition of copper and ascorbate. The location of the resulting DNA cleavage allowed the ORN's exact position to be identified (26, 39).

The results (Fig. 3) show intense cleavage of the DNA inside the open complex with OP-ORNs that are only 5 nt long (lanes 6–8). The cleavage patterns created by these OP-pentamers "walk" upstream as the 5′ termini of the ORNs move from positions −2:+3 to −3:+2 to −4:+1. This behavior is expected for a system that requires base pair-specific interactions. The requirement for RNAP to create a single-stranded hybridization site is clearly demonstrated by comparing the targeted scission created by OP-5′-GUGGA-3′ in the absence (lane 2) and presence (lane 8) of enzyme. Cutting is observed only in the latter case. The most striking result of this experiment is the dramatic drop-off in cleavage efficiency when OP-ORNs are

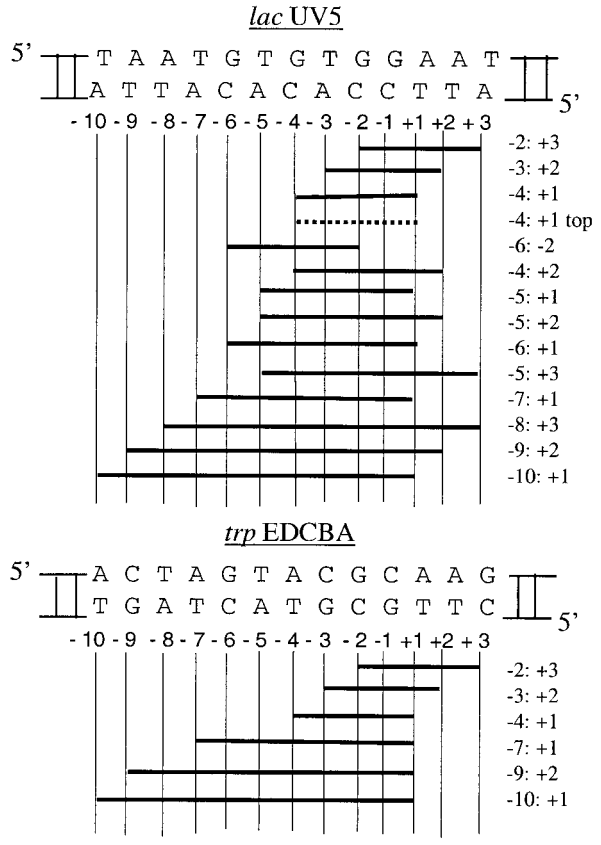

Fig. 2. Representation of ORNs tested for open complex hybridization. The black bars represent ORNs with sequences complementary to either the *lac* UV5 or *trp* EDCBA template strand. One pentamer is complementary to the nontemplate (*Upper*) strand from −4 to +1 and is shown as a dashed line.

greater than 5 nt in length (lanes 10–18). The 6-mer complementary to positions −4 to + 2 (lane 9) binds tightly but the 6-mer complementary to positions −5 to + 1 has only weak affinity (lane 10). Longer ORNs have minimal or no affinity for the open complex.

There is also a strict position requirement for effective OP-ORN-targeted scission. Intense DNA cleavage is observed with pentameric OP-ORNs only when they are complementary to the DNA template strand at the transcription start site. The OP-pentamer complementary to positions −4:+1 shows strong targeted scission (Fig. 3, lane 8) whereas an OP-pentamer positioned upstream (−6:–2) shows absolutely no cleavage (Fig. 3, lane 13). This result is surprising given that the melted region has been shown to extend from approximately 12 bp upstream to 3 bp downstream from the transcriptional start site (15, 40). Strong targeted scission is observed only with pentameric ORNs whose sequence is complementary to the region from −4 to +3 along the promoter. A single or double mismatch in the ORN abolishes its affinity for the open complex (30).

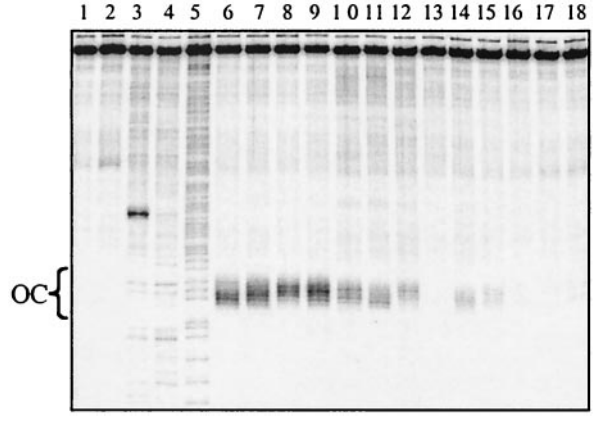

Fig. 3. Direct targeted scission of the *lac* UV5 open complex (labeled OC) by OP-ORNs. Lane 1: Template-labeled *lac* UV5 only; lane 2: OP(−4:+1) no RNAP; lane 3: DNase I + RNAP; lane 4: DNase I no RNAP; lane 5: G + A ladder; lane 6: OP(−2:+3) + RNAP; lane 7: OP(−3:+2) + RNAP; lane 8: OP(−4:+1) + RNAP; lane 9: OP(−4:+2) + RNAP; lane 10: OP(−5:+1) + RNAP; lane 11: OP(−5:+2) + RNAP; lane 12: OP(−6:+1) + RNAP; lane 13: OP(−6:+2) no RNAP; lane 14: OP(−5:+3) + RNAP; lane 15: OP(−7:+1) + RNAP; lane 16: OP(−8:+3) + RNAP; lane 17: OP(−9:+2) + RNAP; lane 18: OP(−10:+1) + RNAP.

Indirect Targeted Scission by OP-5′-UGGAA-3′. Tethering (OP)Cu^{+} to ORNs of various lengths and positions may force the chelate to adopt an unfavorable orientation, resulting in inefficient cleavage and thereby permitting the hybridization of longer ORNs to go undetected. To address this issue, an indirect targeted scission experiment was developed. This method relies on competitive hybridization to the melted region. First, an ORN of varying length is incubated with the open complex. Then, an OP-ORN with known hybridization and targeted scission ability is used to probe the open complex binding of the variable length ORN. If the DNA template is protected from cleavage then the ORN of interest does hybridize. If cleavage by the OP-ORN probe is observed, then no competitive hybridization occurred.

For these experiments OP-5′-UGGAA-3′, complementary to the *lac* UV5 promoter from −3 to +2 , was chosen to probe the binding affinity of several ORNs to the *lac* UV5 open complex. To determine what concentration of ORN is required to competitively quench OP-5′-UGGAA-3′-induced cleavage, a concentration-dependent experiment was run with 5′-UGGAA-3′ at 0, 1, 10, 30, and 50 μM against OP-5′-UGGAA-3′ at 15 μM (data not shown). Open complex scission was clearly attenuated with 10 μM 5′-UGGAA-3′, and total protection was observed with 30 μM 5′-UGGAA-3′.

The results of an indirect targeted scission experiment are shown in Fig. 4 with full-strength cutting by OP-5′-UGGAA-3′ in lane 8. Neither the 11-mer (−9:+2) (lane 1) nor the 8-mer (−6:+2) (lane 2) are able to protect the open complex from targeted scission by OP-5′-UGGAA-3′. These results confirm that neither of these longer ORNs effectively hybridizes to the open complex. Protection of the DNA template is seen only with the 6-mer (−4:+2) and 5-mer (−3:+2), both of which overlap the transcription start site (lanes 4–5). Marginal protection is observed with the 7-mer (−5:+2) (lane 3). Specificity of an ORN for its complementary promoter is demonstrated by the inability of the *trp* EDCBA-specific ORN 5′-CGCAA-3′ to protect the melted region of the *lac* UV5 promoter from targeted scission by OP-5′-UGGAA-3′ (lane 7). Also, the ORN complementary to the nontemplate strand of the *lac* UV5 promoter does not prevent OP-5′-UGGAA-3′ from cleaving the DNA template strand (lane 7). Oligonucleotide length, position, and sequence requirements identified by indirect hybridization analysis are in agreement with the results of the direct targeted scission experiments.

***In Vitro* Transcription Inhibition.** To demonstrate that open complex-targeted ORNs can inhibit transcription and to confirm the length and position effects identified in the scission experiments, we compared the inhibitory properties of several ORNs in an *in vitro* transcription experiment with the *lac* UV5 promoter (Fig. 5*A*). Lane 1 contains no inhibitor and lanes 2–8 contain ORN pentamers that are complementary to various positions along

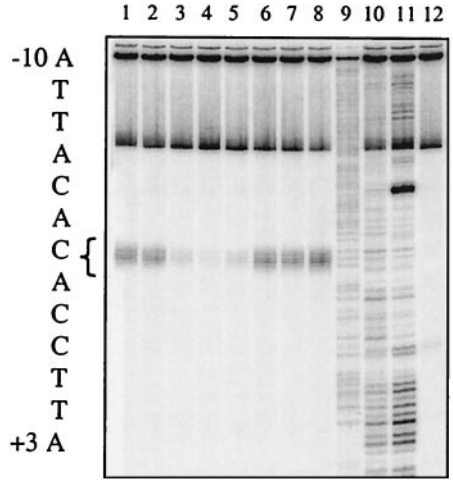

Fig. 4. Indirect targeted scission of the *lac* UV5 open complex (labeled OC). Lanes 1–8 contain 15 μM OP-5′-UGGAA-3′ in addition to the competing ORN. Lane 1: −9:+2; lane 2: −6:+2; lane 3: −5:+2; lane 4: −4:+2; lane 5: −3:+2; lane 6: *trp* EDCBA (−3:+2); lane 7: *lac* UV5 (−4:+1) top; lane 8: OP-5′-UGGAA-3′ only; lane 9: G + A ladder; lane 10: DNase I no RNAP; lane 11: DNase I + RNAP; lane 12: *lac* UV5 only.

the *lac* UV5 open complex. Transcription is strongly inhibited with pentamers −4:+1, −3:+2, and −2:+3 (lanes 5–7). ORNs positioned too far upstream (−12:−8) (lane 2) or downstream (−1:+4) (lane 8) do not inhibit transcription presumably because these sites are not accessible to the incoming ORN. This result is consistent with those obtained by direct and indirect targeted scission.

In addition to position, the length limitation that was observed in the DNA scission experiments is reproduced in the transcription inhibition studies. Transcription is almost completely abolished by the 5-mer (−3:+2) (Fig. 5*A*, lane 9), less so by the 8-mer (−6:+2) (Fig. 5*A*, lane 10), and not at all by the 11-mer (−9:+2) (Fig. 5*A*, lane 11). All of these ORNs contain the original 5-nt sequence (−3:+2). Gel retardation experiments (data not shown) demonstrate that RNAP remains bound to the promoter

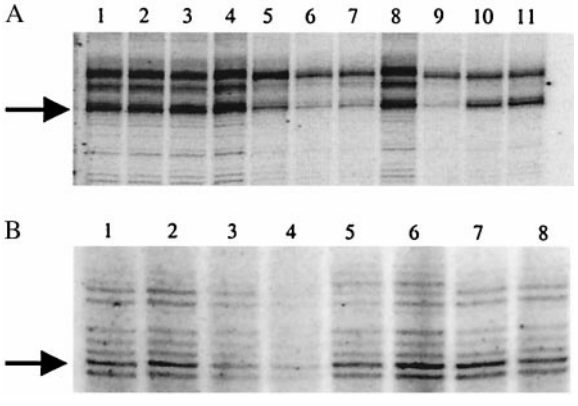

Fig. 5. (*A*) Inhibition of runoff transcription (*in vitro*) from the *lac* UV5 promoter fragment. Full-length transcript has been designated with an arrow. ORNs were tested at 25 μM. Lane 1: No inhibitor; lane 2: −12:−8; lane 3: −6:−2; lane 4: −5:−1; lane 5: −4:+1; lane 6: −3:+2; lane 7: −2:+3; lane 8: −1:+4; lane 9: −3:+2; lane 10: −6:+2; lane 11: −9:+2. (*B*) Inhibition of runoff transcription (*in vitro*) from the *trp* EDCBA promoter fragment. Full-length transcript has been designated with an arrow. ORNs were tested at 25 μM. Lane 1: No inhibitor; lane 2: −2:+3; lane 3: −3:+2; lane 4: −4:+1; lane 5: −7:+1; lane 6: −10:+1; lane 7: *lac* UV5 complement (−4:+1); lane 8: no inhibitor.

in the presence of these ORNs, which, in conjunction with the targeted scission data, confirms that the observed transcription inhibition is not the result of the ORN dislodging the enzyme from the promoter. Inhibition in this system is caused by the sequence-specific recognition of the open complex by the ORN.

ORN hybridization to the *trp* EDCBA open complex was investigated to assess the generality of open complex targeting. *In vitro* transcription inhibition data from the *trp* EDCBA promoter and its complementary ORNs yielded length and position constraints analogous to those observed with *lac* UV5 (Fig. 5*B*). The *trp* EDCBA pentamers 5′-CGCAA-3′ (−3:+2) and 5′-ACGCA-3′ (−4 + 1) inhibited transcription well (Fig. 5*B*, lanes 3 and 4). The octamer (−7:+1) (Fig. 5*B*, lane 5) was less effective, and the 11-mer (−10:+1) was completely ineffective (Fig. 5*B*, lane 6). Open complex specificity is demonstrated in Fig. 5*B*, lane 7 where the *lac* UV5 complement (−4:+1) is shown to be ineffective at inhibiting the *trp* EDCBA promoter.

Conformation of Antiparallel Heteroduplex Formation. Targeted scission by OP-ORNs also can be used to demonstrate that binding within the open complex corresponds to the formation of an antiparallel, Watson–Crick base-paired heteroduplex. Although the observed sequence specificity of ORN binding is suggestive of hybridization, direct analysis was needed to confirm the formation of an antiparallel heteroduplex. To this end, the cleavage patterns of three phenanthroline derivatives of the ORN 5′-GUGGA-3′ (*lac* UV5 complement, −4 to +1) were compared (Fig. 6*A*). If an antiparallel heteroduplex is formed, terminal phenanthroline modification at either the 5′ or 3′ end of this pentamer should create DNA cleavage patterns that bracket the ORNs site of hybridization. Likewise, internal phenanthroline attachment to this ORN via a 2′-aminouridine should direct cleavage toward sites that are intermediate to those seen with the end-modified ORNs. The conformation of the 2′-amino-OP uridine also should constrain the phenanthroline to the putative minor groove of the ORN:DNA heteroduplex thereby directing the reactive $(OP)Cu^{+}$ toward the oxidatively sensitive C-1′ hydrogen. This modification would be expected to cause an increase cutting intensity and/or specificity.

The observed patterns of scission by OP-5′-GUGGA-3′ and 5′-GUGGA-3′-OP are fully consistent with the formation of an antiparallel heteroduplex (Fig. 6*B*, lanes 1 and 3). Cleavage by OP-5′-GUGGA-3′ is localized to sequence positions −5 through −7 whereas the scission sites created by 5′-GUGGA-3′-OP are displaced downstream to positions −2 and −1. Interestingly, the intensity of cleavage by 5′-GUGGA-3′-OP is less than that observed with the 5′-linked derivative. Because $(OP)Cu^{+}$ does not generate a diffusible reactive intermediate, this decreased efficiency may reflect a reduced accessibility to the ribose C-1′ hydrogen for the 3′-linked $(OP)Cu^{+}$ (41, 42). Alternatively, the binding affinity of this ORN may be reduced because of the steric bulk of a 3′ modification. Additional evidence for the formation of an antiparallel heteroduplex comes from the scission pattern created by the internally tethered phenanthroline analog 5′-GU(OP)GGA-3′ (Fig. 6*B*, lane 2). Scission by 5′-GU(OP)GGA-3′ occurs at positions −5 and −4, which are directly between the sites generated by the 5′- and 3′-linked OP-ORNs. Helix formation between an OP-ORN and a target DNA strand in an antiparallel orientation is expected to cause a 2- to 3-base 3′ stagger of the DNA cut sites generated by the minor-groove-centered attack of $(OP)Cu^{+}$. The 3′ staggered scission patterns of these OP derivatives of 5′-GUGGA-3′ provide direct evidence for the formation of an antiparallel heteroduplex between the ORN and the ssDNA inside the open complex.

Requirement for RNAP. The activity of RNAP is central to the design of open complex recognition agents because it creates the

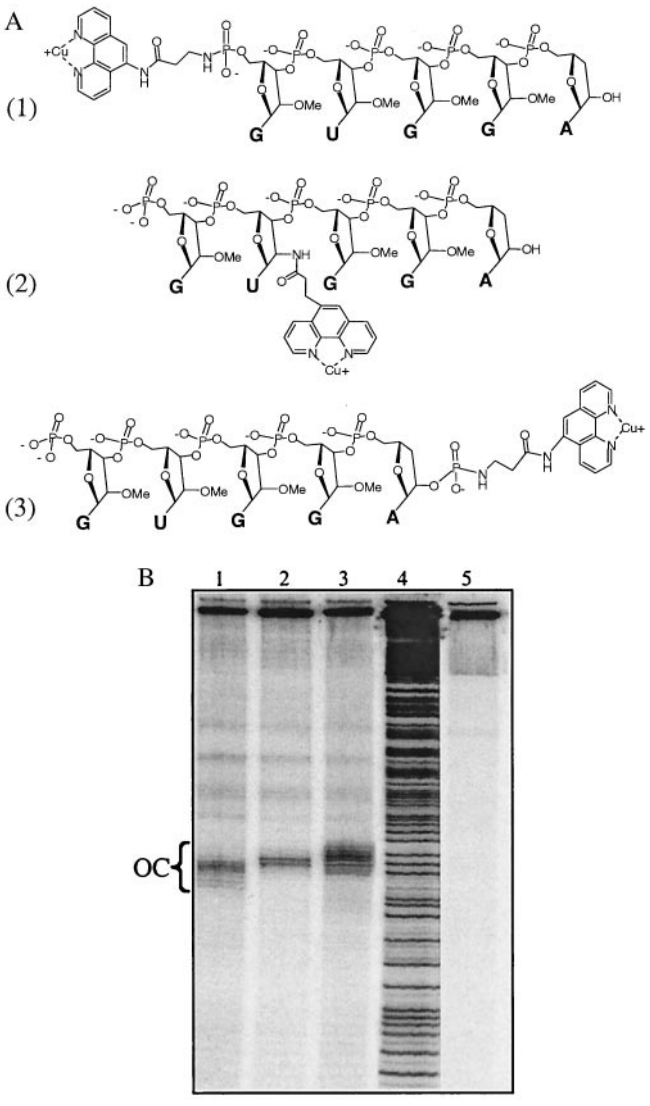

Fig. 6. (*A*) OP linked to either the 5′, 2′, or 3′ position of 5′-GUGGA-3′. 1) OP-5′-GUGGA-3′. 2) 5′-GU(OP)GGA-3′. 3) 5′-GUGGA-3′-OP. (*B*) Direct targeted scission of the *lac* UV5 open complex by 5′, 2′, and 3′ OP derivatives of 5′-GUGGA-3′. The open complex has been designated OC. Lane 1: *lac* UV5 scission by 30 μM 5′-GUGGA-3′-OP; lane 2: *lac* UV5 scission by 30 μM 5′-GU(OP)GGA-3′; lane 3: *lac* UV5 scission by 15 μM OP-5′-GUGGA-3′; lane 4: G + A ladder; lane 5: *lac* UV5 only.

single-stranded target site and contributes an additional 30–70 bp of sequence recognition. The specificity gained by the enzyme's DNA recognition elements allows us to achieve sequence selectivity with extremely short ORNs. To demonstrate that RNAP is intimately involved in the hybridization of short ORNs to the open complex, we created an artificial open complex containing a 9-nt mismatch from positions −6 to +3. Because of this mismatch, this artificial open complex does not require the catalytic activity of RNAP to create a melted region.

Gel mobility experiments demonstrated that RNAP retains its binding affinity to this artificial open complex (data not shown). Footprinting experiments (Fig. 7) reveal that DNase I does not hydrolyze the mismatched template in the region from −6 to +3 even in the absence of RNAP (Fig. 7, lanes 5 and 6). The open complex-specific cleavage agent 5-phenyl-OP-copper ion (12) cleaves the artificial bubble in the mismatched region only in the presence of RNAP (Fig. 7, lanes 3 and 4). Even more

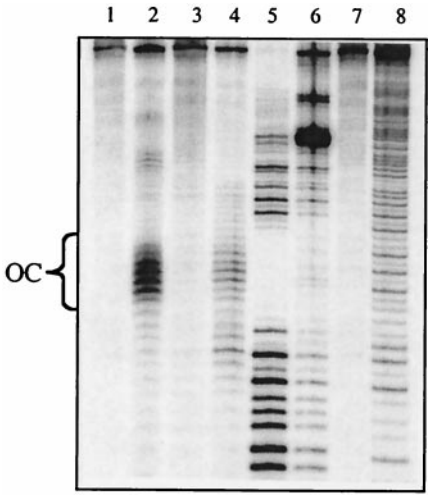

Fig. 7. Direct targeted scission of an artificial open complex with $(5\phi OP)_2Cu^+$ and OP-5′-UGGAA-3′. The open complex has been designated OC. Lane 1: Scission by 30 μM OP-5′-UGGAA-3′, no RNAP; lane 2: scission by 30 μM OP-5′-UGGAA-3′ + RNAP; lane 3: scission by 50 μM $(5\phi OP)_2Cu^+$ no RNAP; lane 4: scission by 50 μM $(5\phi OP)_2Cu^+$ + RNAP; lane 5: DNase I, no RNAP; lane 6: DNase I + RNAP; lane 7: labeled artificial bubble only; lane 8: G + A ladder of artificial bubble template strand.

striking is the observation that direct targeted scission of the artificial bubble by OP-5′-UGGAA-3′ occurs only in the presence of RNAP (Fig. 7, lanes 1 and 2). It is clear that the OP-ORN does not associate with the naked artificial open complex although a complementary, single-stranded hybridization site is available.

Discussion

The obligatory formation of the open complex during the initiation of both prokaryotic and eukaryotic transcription suggests that this essential intermediate is a viable target for the design of gene-specific inhibition agents. The hybridization properties of two transcriptionally competent open complexes have been probed as a function of the length, position, and orientation of inhibitory ORNs. Our experiments have been carried out in an *in vitro* model system to facilitate the reliable comparison between ORNs. However, this system required high concentrations of ORNs to be preincubated with the open complex before the addition of nucleotide triphosphates. Additional work under more physiologically relevant conditions is required. Nonetheless, our results demonstrate that the template strand within a transcriptionally competent open complex is available for hybridization by short, complementary ORNs. These experiments suggest an approach for the development of a novel class of antigene agents.

We have shown that RNAP enforces rigid length and position constraints on open complex-targeted ORNs. ORNs that are 5 nt long and are complementary to the DNA template strand between positions −4 and +3 were found to have the highest open complex binding affinity. ORNs that are 7 nt or longer and ORNs that are complementary to positions outside the region from −4 to +3 are not inhibitory, do not target cleavage to the open complex when linked to $(OP)Cu^+$, and do not protect the open complex from scission by an OP-ORN probe. Also, an ORN that is complementary to the nontemplate strand from −4 to +1 does not protect the template strand from OP-ORN-targeted cleavage. Hybridization of ORNs to the nontemplate strand seems unlikely as recent studies have indicated that *E. coli* RNAP interacts principally with the nontemplate strand, which therefore would seem to preclude ORN binding (43). The length

and position limitations described above have been observed in both the *lac* UV5 and the *trp* EDCBA open complexes.

The most efficient inhibitors are 5 nt long, a length that represents one-half turn of an A- or B-like helix. It is possible that the ssDNA inside the open complex remains in a helical conformation anticipating the formation of an A-type heteroduplex. Longer inhibitors may be precluded from forming an A-type helical heteroduplex by the presence of the transcription machinery. Although the exact nature of the putative helix cannot be determined at this time, direct evidence for the formation of an antiparallel heteroduplex structure is provided by comparing the DNA cleavage patterns generated by OP-5′-GUGGA-3′, 5′-GUGGA-3′-OP, and 5′-GU(OP)GGA-3′. As expected, the cutting pattern walks along the DNA template as the $(OP)Cu^{+}$ position varies. The exact nucleotide at which cleavage occurs is consistently 2–3 nt toward the 3′ terminus from the location of the OP-linked nucleotide. Such a 3′ stagger results from $(OP)Cu^{+}$ reaching across the minor groove of the newly formed heteroduplex and is diagnostic for a helical structure. The 2′-aminouridine used in this study provides a useful site of attachment for intercalating agents that should increase the oligonucleotides binding affinity without sacrificing specificity.

The central role of RNAP in defining this hybridization site has been investigated by examining the affinity of OP-5′-UGGAA-3′ for an artificial open complex. Our results demonstrate that binding by OP-5′-UGGAA-3′ to its ssDNA target occurs only in the presence of RNAP. Because the melting temperature of a ribonucleotide pentamer bound to naked ssDNA is low, no hybridization would be expected to occur at physiological temperatures without the stabilizing influence of RNAP. These data demonstrate the importance of the enzyme in maintaining the conformational integrity of the open complex. Recognition of the open complex by modified ORNs arises not only from their complementarity to the DNA template but also from the sequence recognition, strand separation, and stabilizing effects originating from the polymerase.

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