Nishikawa, K., & Ooi, T. (1986) *Biochim. Biophys. Acta 871*, 45-54.
Ochi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakurai, T., Aihara, S., & Morita, Y. (1983) *J. Mol. Biol. 166*, 407-418.
Phillips, S. E. V., & Schoenborn, B. P. (1981) *Nature 292*, 81-82.
Presnell, S. R., & Cohen, F. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6592-6596.
Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990) *FEBS Lett. 262*, 20-24.
Qian, N., & Sejnowski, T. J. (1988) *J. Mol. Biol. 202*, 865-884.
Rao, S. T., & Rossman, M. G. (1973) *J. Mol. Biol. 76*, 241-256.
Rees, D. C., Deantonio, L., & Eisenberg, D. (1989) *Science 245*, 510-513.
Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111-152.
Richards, F. M., & Kundrot, C. E. (1988) *Proteins: Struct., Funct., Genet. 3*, 71-84.
Richardson, J. S., & Richardson, D. C. (1988) *Science 240*, 1648-1652.
Richmond, T. J., & Richards, F. M. (1978) *J. Mol. Biol. 119*, 537-555.
Roder, H., Eloeve, G. A., & Englander, S. W. (1988) *Nature 335*, 700-704.
Schiffer, M., & Edmundson, A. B. (1968) *Biophys. J. 8*, 29-39.
Schulz, G. E. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 1-21.
Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure*, Springer-Verlag, New York.
Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 296-299.
Sheridan, R. P., Dixon, J. S., Venkataghavan, R., Kuntz, I. D., & Scott, K. P. (1985) *Biopolymers 24*, 1995-2023.
Sikorski, A., & Skolnick, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2668-2672.
Sklenar, H., Etchebest, C., & Lavery, R. (1989) *Proteins: Struct., Funct., Genet. 6*, 46-60.
Skolnick, J., & Kolinski, A. (1989) *Annu. Rev. Phys. Chem. 40*, 207-235.
Skolnick, J., & Kolinski, A. (1990) *Science 250*, 1121-1126.
Smith, R. F., & Smith, T. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 118-122.
Steigemann, W., & Weber, E. (1979) *J. Mol. Biol. 127*, 309-338.
Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1983) *Acta Crystallogr., B 39*, 697-703.
Sternberg, M. J., & Gullick, W. J. (1990) *Protein Eng. 3*, 245-248.
Szebenyi, D. M. E., & Moffat, K. (1986) *J. Biol. Chem. 261*, 8761-8777.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694-699.
Weaver, L. H., & Matthews, B. W. (1987) *J. Mol. Biol. 193*, 189-199.
Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., & Weiner, P. (1984) *J. Am. Chem. Soc. 106*, 765-784.

Triple-Helix Formation Is Compatible with an Adjacent DNA–Protein Complex

Chiao-Chain Huang,[‡] Dat Nguyen,[‡] Ricardo Martinez,[§] and Cynthia A. Edwards*[,§]

Drug Discovery Systems Research Group and Molecular Virology Group, Genelabs Incorporated, 505 Penobscot Drive, Redwood City, California 94063

Received August 21, 1991; Revised Manuscript Received October 24, 1991

ABSTRACT: The effect of oligonucleotide-directed triple-helix formation on the binding of a protein to an immediately adjacent sequence has been examined. A double-stranded oligonucleotide was designed with a target site for the binding of a pyrimidine oligonucleotide located immediately adjacent to the recognition sequence for the herpes simplex virus type 1 (HSV-1) origin of replication binding protein, which is encoded by the UL9 gene of HSV-1. Since the optimal conditions for the binding of the UL9 protein and the pyrimidine oligonucleotide to the duplex DNA are markedly different, a pyrimidine oligonucleotide was designed to optimize binding affinity and specificity for the target duplex oligonucleotide. Consideration was given to length and sequence composition in an effort to maximize triple-strand formation under conditions amenable to the formation of the UL9–DNA complex. Using gel mobility shift assays, a trimolecular complex composed of duplex DNA bound to both a third oligonucleotide strand and the UL9 protein was detected, indicating that the UL9–DNA complex is compatible with the presence of a triple helix in the immediately adjacent sequences.

Sequence-specific recognition of double-stranded DNA by proteins is essential for the regulation of many cellular functions including replication, recombination, and transcription. Displacement of DNA-bound regulatory proteins from their recognition sites might provide a general strategy for the alteration of sequence-specific functions in eukaryotes. In principle, DNA–protein interactions could be disrupted by the presence of a DNA-binding molecule within or near the protein recognition sequence. Possible mechanisms of interference include steric hindrance or localized distortions in the physical

* To whom correspondence should be addressed.
‡ Molecular Virology Group.
§ Drug Discovery Systems Research Group.

0006-2960/92/0431-993$03.00/0

conformation of the DNA that disallow or hinder binding of the regulatory protein. This raises the possibility of regulating gene function by altering DNA conformations in vivo with sequence-specific DNA-binding molecules.

Oligonucleotides that bind to double-stranded DNA in a sequence-specific manner by triple-helix formation (Moser & Dervan, 1987) offer one approach to the experimental manipulation of sequence-specific protein binding. While the binding sites of regulatory proteins are the most desirable direct targets for triple-strand inhibition of expression, not all sequences are equally amenable to triple-strand formation. For this reason, we tested the possibility of using adjacent sequences as triple-strand target sites for the disruption of an adjacent DNA–protein complex. In a triple-helix complex, a pyrimidine oligonucleotide can be bound in the major groove parallel to the purine strand of the duplex DNA by Hoogsteen hydrogen bonds (Moser & Dervan, 1987). Both DNase I digestion studies on the oligonucleotide-directed triple helix (Cooney et al., 1988) and X-ray fiber diffraction studies on poly(A)·2poly(U) and poly(dA)·2poly(dT) triple helices (Arnott & Bond, 1973; Arnott & Selsing, 1974) suggest an A-form RNA-like conformation of the two Watson–Crick base-paired strands of the triple helix. Therefore, it appears that oligonucleotide-directed triple-helix formation can induce local conformational changes in the B-form duplex DNA.

The UL9 gene of herpes simplex virus type 1 (HSV-1) encodes a protein that binds specifically to the HSV-1 origins of DNA replication, ori_S and ori_L, and is required for viral replication (Elias & Lehman, 1988; Olivo et al., 1988; Koff & Tegtmeyer, 1988). We have expressed the DNA-binding domain of the UL9 protein (UL9-COOH) in *Escherichia coli* and have purified the recombinant protein to near homogeneity (Martinez et al., submitted for publication). Using purified UL9-COOH protein, we have designed experiments to test whether local conformational changes induced by pyrimidine oligonucleotide-directed triplex formation in the duplex oligonucleotides inhibit the sequence-specific binding of a protein at an immediately adjacent site. Our results indicate that there is no apparent inhibition of protein binding by the formation of a triple helix in the sequences immediately adjacent to the protein-binding site and suggest that sites targeted for the inhibition of regulatory protein binding by triplex formation must be targeted in a direct manner or with overlapping sequences to be effective.

Experimental Procedures

Oligonucleotide Synthesis and Preparation of ^{32}P-Labeled Duplex Oligonucleotides. Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer and purified using either Applied Biosystems purification cartridges or Rainin HPLC columns. The 5′ ends of the oligonucleotides were radiolabeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs) as per the manufacturer's instructions. After being annealed to a synthetic primer, the phosphorylated oligonucleotides were used as templates to synthesize the complementary strands, resulting in the formation of ^{32}P-labeled duplex oligonucleotides. Oligonucleotides were labeled to the same specific activity (approximately 10^8 dpm/μg), and equal numbers of counts were used for each experiment. Third-strand oligonucleotides were radiolabeled in a similar manner.

Expression and Purification of the UL9-COOH Protein. Details of the experimental procedures used to express and purify the recombinant UL9-COOH protein will be described elsewhere (Martinez et al., submitted for publication). Briefly, the gene segment of HSV-1 encoding approximately one-third of the carboxy terminus of UL9 was inserted into the pGEX-2T expression vector (Smith & Johnson, 1988). The DNA-binding domain of the UL9 protein is encoded in the carboxy-terminal one-third of the protein (Weir et al., 1989). The truncated UL9 gene was expressed as a fusion protein with the glutathione-S-transferase (GST) gene from *Schistosoma japonicum* under the control of a tac promoter. The GST fusion protein was purified from lysed cells under nondenaturing conditions using differential ammonium sulfate precipitation and affinity chromatography on glutathione–Sepharose 4B (Pharmacia). The fusion protein is greater than 95% pure. The carboxy-terminal UL9 protein (UL9-COOH) was released from the GST fusion protein using thrombin, which cleaves at the junction of the GST and UL9-COOH protein sequences. The resulting UL9-COOH preparation has retained stable binding activity when stored at −70 °C for several months.

Pyrimidine Oligonucleotide-Directed Triple-Helix Formation. The DNA concentrations used are specified in the figure legends. In general, a pyrimidine oligonucleotide was added in vast excess (10 μM) to a ^{32}P-labeled duplex oligonucleotide (0.86 nM) in a 15-μL buffered solution containing 50 mM NaCl, 5 mM spermine, and 50 mM sodium acetate (pH 4.7), 100 mM Bis-Tris (pH 6.1) or 20 mM Hepes (pH 7.1). The reaction mixture was incubated at room temperature for the periods indicated in the figure legends. The formation of triple helix was typically monitored by polyacrylamide gel electrophoresis on 6–20% polyacrylamide gels.

Mobility Shift Assay of UL9-DNA Interactions. Unless indicated otherwise, 0.86 nM ^{32}P-labeled duplex oligonucleotides was used. UL9–DNA complexes were formed in 20-μL reaction volumes containing 100 mM Bis-Tris (pH 6.1) or 20 mM Hepes (pH 7.1), 50 mM NaCl, 5 mM spermine, 0.5 mM dithiothreitol (DTT), 1 mM EDTA, 120 ng of sheared herring sperm DNA, and 1% glycerol and then separated electrophoretically on 6% polyacrylamide gels in the presence of Tris–borate/EDTA buffer, pH 8.4 (Maniatis, 1982). The effect of adjacent triple-helix formation on the binding of UL9-COOH was analyzed in 20-μL sample volumes using the same binding buffers as described above. The gel running buffer contained 100 mM Bis-Tris (pH 6.1) or 20 mM Hepes (pH 7.1). Gels were dried, and the bands corresponding to the UL9–DNA complex were visualized by autoradiography.

Results

Analysis of the Recognition Sequences for the UL9-COOH Protein. The 18-nucleotide sequence, 5′-AGCGTTCGCACTTCGTCC-3′, located within one of the HSV-1 origins of replication (ori_S; Mocarski & Roizmann, 1982; Stow, 1982), is bound by UL9 as revealed by DNase I footprinting analysis (Elias et al., 1986; Koff & Tegtmeyer, 1988) and is required for the efficient replication of the virus (Hernandez et al., 1991). Furthermore, methylation interference studies define this sequence to contain at least eight essential contact residues, 5′-GTTCGCAC-3′ (Koff & Tegtmeyer, 1988). To further investigate the sequence requirements for UL9 recognition, two duplex oligonucleotides containing mutations in the outer sequences of the putative UL9 recognition sites were synthesized (see Figure 1) and tested in the mobility shift assay (Fried & Crothers, 1981; Garner & Revzin, 1981). Mutations in either the left or right side of the 11-nucleotide sequence 5′-CGTTCGCACTT-3′, within oligonucleotide HSVBox1, impaired the binding of UL9 protein (see Figure 1). The ΔL mutation did not bind detectable levels of UL9 at 5.4 μM UL9-COOH (in the presence or absence of nonspecific competitor DNA), a concentration

a) DELTA R: 5′-GGTCGTTCGCACTCCGC-3′

b) DELTA L: 5′-GGTCCTTCGCACTTCGC-3′

c) HSVBox 1: 5′-GGTCGTTCGCACTTCGC-3′

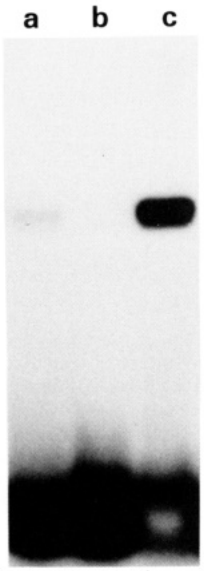

FIGURE 1: The effect of point mutations within the UL9 recognition sequence on UL9–DNA interactions. Shown are the top-strand sequences of the duplex oligonucleotides used in the UL9 binding assay. The locations of the mutations are underlined: lane a, ΔR; lane b, ΔL; lane c, HSVBox1.

```
                  <---triple helix--->__________
HU9-0   5′-GAGCCAAGAAAAGAAAGAAGAAAAACGTTCGCACTTCGTCCCAAT-3′
        3′-CTCGGTTCTTTTCTTTCTTCTTTTTGCAAGCGTGAAGCAGGGTTA-5′
                                      <---UL9--->

20-ts         5′-TTCTTTTCTTTCTTCTTTTT-3′

20-ct         3′-AAGAAAAGAAAGAAGAAAAA-5′

20-mix        5′-TTCTCTCTTTTTTCTCTTTT-3′

HSV                                5′-GGTCGTTCGCACTTCGC-3′
Box1                               3′-CCAGCAAGCGTGAAGCG-5′
```

FIGURE 2: The nucleotide sequences of the single-strand oligonucleotides and the duplex oligonucleotide used in this work. The target site for the binding of the 20-ts oligonucleotide is underlined, and the recognition sequence of the UL9-COOH protein is indicated.

sufficiently high to bind 99.99% of the wild-type ori$_S$ sequence (data not shown). These calculations are based on an estimated binding affinity constant for the UL9–ori$_S$ interaction of 2×10^9 M^{-1} (Edwards, unpublished results). Our results demonstrate that the 11-bp recognition sequence is necessary and sufficient for maximum binding to the UL9-COOH protein. This 11-nucleotide sequence is also contained within an origin of DNA replication of varicella zoster virus, another member of the human herpesviruses (Stow & Davison, 1986). These data were used in the design of the oligonucleotide described below.

Pyrimidine Oligonucleotide-Directed Triple-Helix Formation. A duplex oligonucleotide, HU9-0, in which the target site for oligonucleotide binding is located immediately adjacent to the 11-bp recognition sequence of the UL9 protein (Figure 2) was designed to study the effect of oligonucleotide-directed triple-helix formation on the UL9 binding. In addition to sequence composition and length, the binding affinity and specificity of a pyrimidine oligonucleotide for a duplex DNA has been shown to be sensitive to pH, organic cosolvent, cations, and temperature (Moser & Dervan, 1987). Table I illustrates the effect of increasing pH on the binding of pyrimidine oligonucleotides of various lengths at room temperature to a duplex oligonucleotide. The results suggest the importance of acidic pH and length in the formation of triple helix. Since the optimal DNA-binding conditions for the UL9 protein (data not shown) are not compatible with the optimal conditions for triple-helix formation (e.g., low pH and organic cosolvent) (see above; Moser & Dervan, 1987), a pyrimidine oligonucleotide, 20-ts, was designed to optimize binding affinity and specificity. Consideration was given to length and sequence composition in an effort to maximize triple-strand formation (see Figure 2) under conditions amenable to UL9–DNA complex formation.

Table I: Effect of Increasing pH on Triple-Helix Formation

length of the pyrimidine oligonucleotide (in bases)	pH	triple-helix formation[a]
20[b]	4.7	+
	6.1	+
	7.1	+
17[c]	4.7	+
	6.1	+
	7.1	–
11[c]	4.7	+
	6.1	–
	7.1	nd[d]
7[c]	4.7	–
	6.1	–
	7.1	nd[d]

[a] Triple-helix formation was monitored by a gel mobility shift assay as described in the text. [b] The 20-ts oligonucleotide described in Figure 2 was used here. [c] The 17-base oligonucleotide is 5′-TCTGTTCTGTCTTCTCC-3′. The 7- and 11-base oligonucleotides are nested deletions from the 5′ end of the 17-base sequence, 5′-CTGTCTTCTCC-3′ and 5′-CTTCTCC-3′, respectively. [d] nd, not determined.

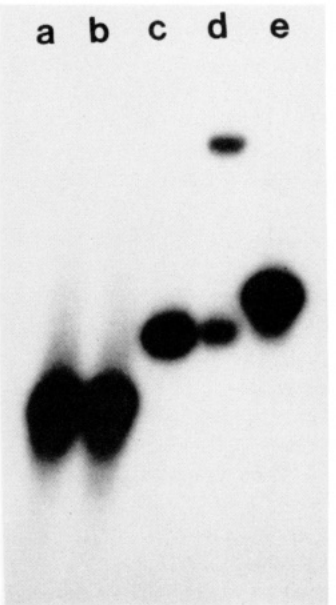

FIGURE 3: Analysis of triple-helix formation at pH 7.1 by the gel mobility shift assay: lane a, ^{32}P-labeled 20-ct oligonucleotide (2.8 nM); lane b, ^{32}P-labeled 20-ct (2.8 nM) mixed with the HU9-0 duplex oligonucleotide (140 nM); lane c, ^{32}P-labeled 20-ts oligonucleotide (2.8 nM); lane d, ^{32}P-labeled 20-ts (2.8 nM) complexed with the HU9-0 duplex oligonucleotide (140 nM); lane e, ^{32}P-labeled 20-ts (2.8 nM) preincubated with its complementary oligonucleotide, 20-ct (70 nM), and then mixed with the HU9-0 duplex oligonucleotide (140 nM).

Gel mobility shift assays were used to monitor the formation of oligonucleotide-directed triplex, taking advantage of the reduction of DNA charges that are likely to accompany triplex formation (Cooney et al., 1988). It has been suggested that if a triple-stranded oligonucleotide has the overall dimensions

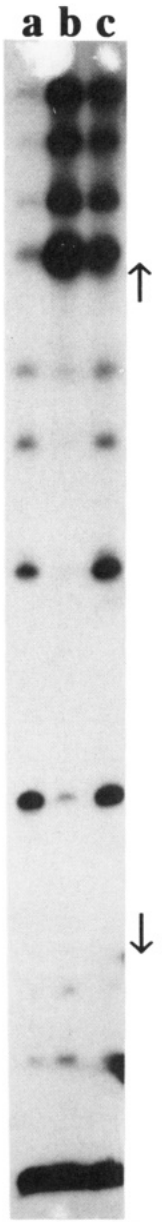

FIGURE 4: Results of purine-strand modification by dimethyl sulfate (DMS). The 5′ end of the ^{32}P-labeled purine strand of the HU9-0 duplex oligonucleotide is located at the bottom of the gel (not shown). The region that forms the triple helix is indicated by the arrows: lane a, ^{32}P-labeled HU9-0 duplex oligonucleotide (100 nM); lane b, ^{32}P-labeled HU9-0 duplex oligonucleotide (100 nM) incubated with 20-ts (9 μM); lane c, ^{32}P-labeled HU9-0 duplex oligonucleotide (100 nM) incubated with the 20-mix oligonucleotide (9 μM). The methylation reaction was performed in a buffered solution containing 100 mM Bis-Tris, 50 mM NaCl, and 5 mM spermine, pH 6.3.

similar to those of a duplex oligonucleotide but a significantly reduced phosphate charge, it will migrate more slowly than the duplex oligonucleotide through a gel matrix (Cooney et al., 1988). The result of a mobility shift assay on a 20% polyacrylamide gel is shown in Figure 3. When the ^{32}P-labeled pyrimidine oligonucleotide 20-ts (see Figure 2) was mixed with the duplex oligonucleotide HU9-0, a slowly migrating band appeared, presumably the triple-strand complex, as illustrated in lane d of Figure 3. The generation of this distinct species is sequence-specific as demonstrated by the failure of the complementary oligonucleotide of 20-ts, 20-ct, to produce the novel band (lane b of Figure 3). Furthermore, competition for the 20-ts oligonucleotide by preincubation with the complementary strand, 20-ct, prevented the formation of the triple-strand complex (lane e in Figure 3).

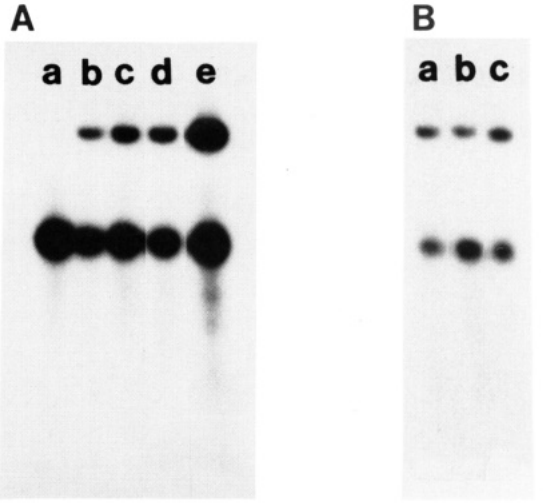

FIGURE 5: Kinetic analysis of triple-helix formation. (A) The rate of triplex formation was monitored by incubating ^{32}P-labeled 20-ts (3.4 nM, lane a) with unlabeled HU9-0 duplex oligonucleotide (125 nM) for 1 min, 5 min, 60 min, or 48 h (lanes b, c, d, and e, respectively). (B) The kinetic stability of the triple helix was measured by a competition assay in which the products of the 48-h binding reaction were challenged with a 55-fold molar excess of unlabeled 20-ts: lane a, no competitor added; lane b, 45 min with unlabeled 20-ts; lane c, 48 h with unlabeled 20-ts.

Dimethyl sulfate (DMS) was also used to probe triplex formation (Voloshin et al., 1988). DMS modifies the N7 position of guanines leading to chain scissions after treatment with piperidine. This chemical will not react with the pyrimidine–purine–pyrimidine triplex because the N7 position of purines is protected by Hoogsteen base pairing (Moser & Dervan, 1987; Voloshin et al., 1988). The duplex oligonucleotide HU9-0 was labeled with ^{32}P on the top strand and incubated with the oligonucleotide 20-ts before the addition of DMS. As shown in lane b of Figure 4, the guanines located within the target site for pyrimidine oligonucleotide binding are less reactive with DMS than the guanines external to the third-strand target site indicating triplex formation under the experimental conditions. Another pyrimidine oligonucleotide, 20-mix, which has the same length and nearly identical base composition but different absolute sequence than the oligonucleotide 20-ts (see Figure 2), was used to test sequence specificity; 20-mix failed to protect the guanines located within the triple-helix region against methylation by DMS (lane c of Figure 4). These data suggest that the pyrimidine oligonucleotide 20-ts binds in a sequence-specific manner to the duplex oligonucleotide HU9-0 to form a triple helix.

Kinetic Analysis of Triple-Helix Formation at the Target Site. The rate of triplex formation was monitored by incubating duplex oligonucleotide HU9-0 (125 nM) with ^{32}P-labeled oligonucleotide 20-ts (3.4 nM) for various periods of time. As shown in Figure 5A, the products were assayed by gel electrophoresis. At pH 7.1, and room temperature, approximately 30% of 20-ts binds to HU9-0 after 1 min of incubation and about 50% of 20-ts binds to HU9-0 after 48 h of incubation. A competition assay was used to measure the kinetic stability of the triple helix under these conditions (Figure 5B). The products of the 48-h binding reaction were challenged with a 55-fold molar excess of unlabeled 20-ts. Comparison of lane a to lane c of Figure 5B indicates no substantial triplex dissociation for at least 48 h under these physical conditions (i.e., pH 7.1 and room temperature).

No Apparent Inhibition of UL9-COOH Binding by Adjacent Triplex Formation. UL9-COOH was added (8.6 nM) to a binding reaction mixture (at pH 6.1) in which the ^{32}P-labeled

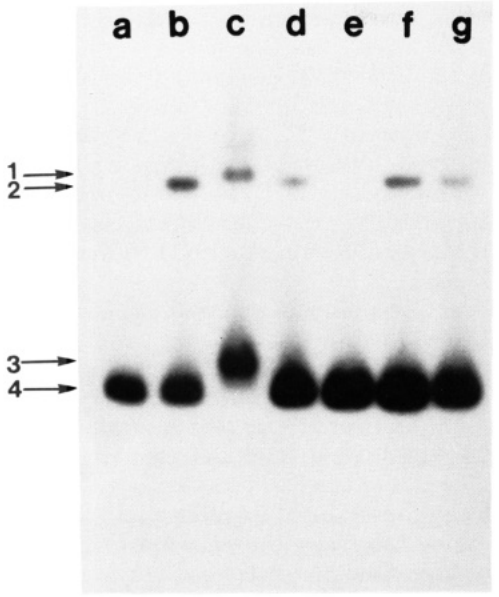

FIGURE 6: The effect of triple-helix formation on the binding of the UL9-COOH protein. All lanes contain ^{32}P-labeled, double-stranded HU9-0 as the probe: lane a, probe (0.86 nM) alone; lane b, UL9-COOH added (8.6 nM); lane c, probe preincubated overnight with an 11 600-fold molar excess of 20-ts, followed by incubation with UL9-COOH (8.6 nM) for 2 h; lane d, probe preincubated overnight with the nonspecific oligonucleotide, 20-mix (11 600-fold molar excess), followed by incubation with UL9-COOH; lane e, competition with HSVBox1, the double-stranded oligonucleotide 5′-GGTCGTTCGCACTTCGC-3′ (1.3 μM), which contains the recognition sequence for UL9-COOH; lane f, competition with the unrelated, double-stranded oligonucleotide 5′-GCTTCCA-GAGGGGACTTTCCGAGAGGCT-3′ (800 nM); lane g, probe preincubated overnight with an 11 600-fold molar excess of both 20-ts and the complementary strand, 20-ct, followed by the addition of UL9-COOH.

duplex oligonucleotide (0.86 nM) had been incubated overnight with or without the pyrimidine oligonucleotide (10 μM) to determine whether the pyrimidine oligonucleotide 20-ts bound to the duplex oligonucleotide HU9-0 could disrupt UL9-COOH binding. After a 2-h incubation, the products were analyzed by electrophoresis on an 8% polyacrylamide gel. The results of the experiment are shown in Figure 6. In the absence of oligonucleotide 20-ts, approximately 25% of the ^{32}P-labeled duplex oligonucleotides (arrow 4) binds to the UL9-COOH protein, as indicated by the appearance of a slowly migrating band (arrow 2) (see lane b, Figure 6). The formation of the DNA–protein complex is sequence-specific as demonstrated in a competition assay with competing duplex oligonucleotide containing the recognition sequence of the UL9 protein (i.e., HSVBox1 in Figure 1) (see lane e, Figure 6); an unrelated duplex oligonucleotide does not compete for UL9-COOH binding (lane f, Figure 6).

When the oligonucleotide 20-ts is present in the binding reaction mixture (at pH 6.1), the UL9-DNA complex (arrow 2) migrates higher in the gel. This "supershift" (arrow 1) is demonstrated by a comparison of lane c to lane b in Figure 6. A shift of the unbound duplex oligonucleotides is also observed, indicating the formation of a triple helix (arrow 3) (lane c, Figure 6). The formation of the supershift is sequence-specific, as demonstrated by the disappearance of the supershift when the oligonucleotide 20-mix (Figure 2), which cannot bind to the duplex oligonucleotide HU9-0 to form a triplex, was used to replace the oligonucleotide 20-ts in the UL9-binding reaction (lane d, Figure 6). Furthermore, when 20-ct, the complementary oligonucleotide of 20-ts, was preincubated with 20-ts in the overnight binding reaction mixture, the formations of the triple helix and of the supershift are blocked (lane g, Figure 6). These data demonstrate that the UL9-COOH protein binds to the DNA recognition site located immediately adjacent to a triple helix, resulting in a supershifted complex.

Competition assays were used to assess the kinetic stability of the UL9–triple helix complex by adding excess competing oligonucleotide HSVBox1 (Figure 2) after an initial 1-h binding reaction including the UL9-COOH protein and the triplex. Under the conditions used in these experiments, the half-life of the UL9–triplex complex is less than 30 s (data not shown). Within the limits of the measurement, this half-life is comparable to the half-life of the UL9–duplex oligonucleotide complex. The UL9–DNA complex is relatively unstable because the interaction is sensitive to the spermine and lower pH used for the formation of triplex DNA (data not shown). The effect of triplex formation, if any, on the stability of the UL9–DNA interaction is therefore difficult to assess.

Discussion

Micromolar concentrations of pyrimidine oligonucleotides have been shown to block recognition of double-stranded DNA by three different DNA-binding proteins at a homopurine target site (Maher et al., 1989). In that report, the target site for oligonucleotide binding was designed to overlap sequences recognized by the DNA-binding proteins. The oligonucleotides compete with site-specific DNA-binding proteins for the occupancy of an overlapping target site. However, the DNA–protein interactions were much less inhibited when the binding site for a third oligonucleotide strand was placed within a plasmid adjacent to the recognition sequence for *Eco*RI restriction/modification enzymes (Hanvey et al., 1990). In that report, since only the products of enzymatic reactions were examined, the formation of an intermediate composed of protein and triplex DNA and the potential role of conformational changes were not directly demonstrated. Here, we address the issue of the effect of local conformational changes in a duplex oligonucleotide on the binding of a protein by placing the target site for oligonucleotide binding immediately adjacent to the recognition site of the protein. Our data indicate that the local triple-helix formation does not prevent UL9–DNA interactions. This result is rather surprising in view of the deleterious effect on UL9-COOH binding (shown in Figure 1) of altering a single nucleotide pair at the 5′ end of the recognition sequence.

At least three explanations may account for the observed compatibility of triplex DNA with adjacent protein binding. First, the local conformational changes induced by triplex formation may dissipate and diminish at the triplex–duplex junction so that the recognition sequence of the UL9-COOH protein still assumes a B-form conformation suitable for protein binding. Computer modeling studies of contiguous DNA segments containing A-form and B-form DNA have suggested that the two different conformations can coexist with only one base pair and two internucleotide linkages between the two helical forms; a bend in the DNA at this junction is predicted (Selsing et al., 1979). When the 20-ts oligonucleotide binds to the HU9-0 duplex oligonucleotide, similar structural changes may occur without disrupting either the triple strand or the protein binding. Second, the DNA site recognition of the UL9 protein may be insensitive to the induced conformational changes transmitted from the target site of oligonucleotide binding. Third, although the pyrimidine oligonucleotide binds to the duplex oligonucleotide at the homopurine target site, some of the 3′ nucleotide residues may

remain unbound to the purine strand of the duplex oligonucleotide due to the "end-breathing" effect that has been well-documented in the literature for double-stranded oligonucleotides (Patel & Hilbers, 1975; Patel et al., 1982). If so, this 3′ end region would serve as a cushion to reduce the impact of the conformational changes on the UL9-binding site. Our preliminary data in a different model system suggest that at least the two most 3′ nucleotide residues of a similar pyrimidine oligonucleotide (in the absence of the UL9-COOH protein) were not paired with the purine strand of its target duplex oligonucleotide, as indicated by the observation that the corresponding purine bases were methylated by DMS (data not shown). Further studies are needed to distinguish these three possibilities.

In conclusion, our results suggest possible constraints on the design and use of oligonucleotides or their analogues in functioning as artificial gene-specific repressors in vivo as previously suggested (Maher et al., 1989). Since the affinity and specificity of binding for pyrimidine oligonucleotides are sensitive to sequence composition and length, pH, cation concentration, temperature, and solvent, consideration must be given in designing the oligonucleotides to maximize triple-strand formation under physiological conditions. More importantly, if the desired effect is to use oligonucleotides as therapeutics that function by interfering with DNA–protein interactions, special attention has to be given to the spatial relationship (e.g., overlapping vs nonoverlapping) between the target site for oligonucleotide binding and the recognition sequence of protein binding in the duplex DNA.

Acknowledgments

We thank Dr. Michael Lovett for critical review of the manuscript, Dr. Charles Cantor for helpful discussions, Rick Cuevas for assistance in the preparation of the manuscript, and John Fernandez for assistance in the preparation of figures.

References

Arnott, S., & Bond, P. J. (1973) *Nature, New Biol. 244*, 99–101.

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509–521.

Cooney, M., Czernuszewicz, G., Postel, E. H., & Hogan, M. E. (1988) *Science 241*, 456–459.

Elias, P., & Lehman, I. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2959–2963.

Elias, P., O'Donnel, M. E., Mocarski, E. S., & Lehman, I. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6322–6326.

Fried, M. G., & Crothers, D. M. (1981) *Nucleic Acids Res. 9*, 6505–6526.

Garner, M. M., & Revzin, A. (1981) *Nucleic Acids Res. 9*, 3047–3060.

Hanvey, J. C., Shimizu, M., & Wells, R. D. (1990) *Nucleic Acids Res. 18*, 157–161.

Hernandez, T. R., Dutch, R. E., Lehman, I. R., Gustafsson, C., & Elias, P. (1991) *J. Virol. 65*, 1649–1652.

Koff, A., & Tegtmeyer, P. (1988) *J. Virol. 62*, 4096–4103.

Maher, L. J., III, Wold, B., & Dervan, P. B. (1989) *Science 245*, 725–730.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, p 454, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mocarski, E. S., & Roizman, B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5626–5630.

Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.

Olivo, P. D., Nelson, N. J., & Challberg, M. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5414–5418.

Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2651–2656.

Patel, D. J., Kozlowski, S. A., Marky, L. A., Broka, C., Rice, J. A., Itakura, K., & Breslauer, K. J. (1982) *Biochemistry 21*, 428–436.

Selsing, E., Wells, R. D., Alden, C. J., & Arnott, S. (1979) *J. Biol. Chem. 254*, 5417–5422.

Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31–40.

Stow, N. D. (1982) *EMBO J. 1*, 863–867.

Stow, N. D., & Davison, A. J. (1986) *J. Gen. Virol. 67*, 1613–1623.

Voloshin, O. N., Mirkin, S. M., Lyamichev, V. I., Belotserkovskii, B. P., & Frank-Kamenetskii, M. D. (1988) *Nature 333*, 475–476.

Weir, H. M., Calder, J. M., & Stow, N. D. (1989) *Nucleic Acids Res. 17*, 1409–1425.