Deoxynucleotide-containing oligoribonucleotide duplexes: stability and susceptibility to RNase V₁ and RNase H

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ABSTRACT

Oligoribonucleotide duplexes containing one to four 2'-deoxynucleotide residues were used as substrates for ribonuclease V₁ and RNase H. Either deoxyadenosine and/or deoxythymidine were incorporated into the duplex, 5'GGCCGGAUCCGCGC3'·5'GCGCGGAUCCGGCC3' by substitution of the appropriate deoxynucleoside triphosphate into a transcription reaction with T7 RNA polymerase. The melting temperature, T_m, of the duplex (1.8 μM in strands in 50 mM NaCl) containing only ribonucleotides was 79.9°C. Substitution of deoxyadenosine in both strands of the duplex lowered the T_m by 2.4°C. Substitution of deoxythymidine had no measurable effect on the T_m. Comparison of RNase V₁ digestion patterns of fully ribonucleotide and deoxy-substituted duplexes suggest that any distortion is localized to the site of the substitution. An oligoribonucleotide containing two deoxy residues directs specific cleavage of RNA by *E. coli* RNase H. Structural requirements for cleavage are proposed for RNase V₁ and RNase H.

INTRODUCTION

Double helical DNA generally forms a B-like helix in solution while RNA is assumed to form an A-form duplex. Fiber diffraction studies have shown that most DNA-RNA hybrid polymers form A-like duplexes (1). However, deoxynucleotides can retain B-form characteristics in hybrids (2,3). Fiber diffraction patterns obtained from poly(dA)·poly(rU) have been interpreted to be of heteronomous duplexes in which the DNA chain has C2' endo sugar puckers and the RNA strand has C3' endo sugar puckers (1). Hybrids which contain deoxyadenosine have been shown to be unstable in both polynucleotides (4) and oligonucleotides (5).

Sensitivity of DNA and RNA to nucleases depends upon both chemical and conformational differences. For example, the 2'-OH makes ribonucleotides susceptible to alkaline hydrolysis and to cleavage by ribonucleases like T₁ and U₂. Strand scission by these reagents occurs via a 2'–3' cyclic phosphate intermediate and products contain 5'-hydroxyl groups. This mechanism of hydrolysis is impossible for deoxynucleotide polymers. Certain ribonucleases, like RNase V₁ and RNase H, specifically cleave RNA, but have no intrinsic mechanistic requirement for the 2'-OH since hydrolysis leaves a 5' phosphate.

RNase V₁ from cobra venom is a widely used probe for double-stranded RNA (6). A model for RNase V₁ recognition was previously proposed based on digestion of tRNA^{Phe} (7). Cleavage was consistent with enzyme recognition of the minor groove of a stacked helical site. Lowman and Draper showed that the enzyme binds to DNA polymers with only slightly lower affinity as compared to RNA of the same sequence; however the minor amounts of DNA hydrolysis observed were dismissed as due to a DNase contaminant (8).

RNase H specifically degrades the ribonucleotide strand of an RNA·DNA hybrid (9). RNase H, like RNase V₁, leaves a 5' phosphate and so does not mechanistically require the 2' OH for cleavage. RNase H does not cleave DNA or double-stranded RNA. Discrimination between deoxy- and ribo-residues by RNase V₁ and RNase H could occur through direct contact with the 2' position of the sugar or by sensing a particular conformation either of the sugar or the helix.

In this study, 2'-deoxyribonucleotides were site-specifically incorporated within a 14 base oligoribonucleotide to allow study of duplexes containing covalently linked deoxy- and ribo-nucleotides. Synthesis of deoxy-containing oligoribonucleotides was accomplished using T7 RNA polymerase. The effect of substitution of one to four deoxy residues on the global structure of the duplexes was investigated using optically observed melting and circular dichroism. The local structure was probed using enzymatic digestion. Digestion of ribonucleotide- and deoxyribonucleotide-containing duplexes indicated the structural requirements for cleavage by RNase V₁ and *E. coli* RNase H.

METHODS

Oligonucleotide Preparation

Oligoribonucleotides and 2'-deoxynucleotide-containing oligoribonucleotides were transcribed using synthetic DNA templates and T7 RNA polymerase (10). DNA was synthesized on an Applied Biosystems, Inc. Model 381A synthesizer and gel purified. T7 RNA polymerase was isolated from *E. coli* strain BL21, containing plasmid pAR1219, according to the procedure of Davanloo et al. (11). Transcription reactions contained 40 mM Tris (pH 8.1), 1 mM spermidine, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100, 80 mg/ml polyethylene glycol (relative molecular mass = 8000). DNA template strand concentrations were 0.4 mM. Ribonucleoside triphosphate concentrations were 2.5 mM; deoxyribonucleoside triphosphates were substituted at 5 mM concentrations. The MgCl₂ concentration was adjusted to maintain a Mg²⁺ to NTP ratio of 1.7 to 1. T7 RNA polymerase was used at 30 units/μl of reaction. Reactions were incubated at 37°C for 1 hour. Preparative 1 ml reactions were quenched by addition of EDTA to 50 mM followed by phenol extraction and ethanol precipitation. The full length product was isolated from a denaturing (7 M urea) 20% polyacrylamide gel by electro-elution and ethanol precipitated.

Figure 1. Autoradiographs of denaturing (7 M urea) 20% acrylamide gel-electrophoretic analysis of hydrolysis reactions. In each reaction the 5'-³²P end-labeled strand was 5'GGCCGGAUCCGCGC3' or the deoxy-containing analog. The sequence is written to the right of the final lane of each autoradiograph. Fully denatured oligonucleotides would migrate as indicated by the 3'-terminal C on the right of each autoradiograph. Lane headings denote whether the A and U residues in the oligonucleotides were deoxy or ribo. No hydrolysis is observed in the absence of enzyme or high pH (not shown).

- Alkaline hydrolysis reactions. No hydrolysis is observed after either dA or dT in the oligonucleotide in the second lane.
- RNase V₁ digestions. Lane headings indicate duplex present; upper strand is 5'-³²P end-labeled. Substitution of a deoxynucleotide results in considerably reduced cleavage at the phosphate immediately 3' of the substitution and no change in cleavage intensity elsewhere within the oligonucleotide.
- RNase H digestions of oligoribonucleotide and deoxy-containing oligoribonucleotides hybridized to DNA. Substitution of deoxynucleotides within the labeled strand oligonucleotide strand results in loss of cleavage at phosphodiester linkages 5' and 3' of the substitution.
- RNase H digestion of fully ribonucleotide duplex and oligoribonucleotide hybridized to dA- and dT-containing oligonucleotide. RNase H cleavage is directed to three specific sites by the deoxy-substitution.

Oligonucleotide Characterization

Partial enzymatic digestion and alkaline hydrolysis of 5'-³²P end-labeled transcripts insured that oligonucleotides were of correct length and sequence. Transcripts to be end-labeled were dephosphorylated using calf intestinal phosphatase (Boehringer) and then 5'-³²P end-labeled using [γ -³²P]-ATP and T4 polynucleotide kinase (U. S. Biochemicals). Each enzymatic digestion or alkaline hydrolysis reaction contained about 1 pmol 5'-³²P-labeled oligoribonucleotide and 3 μ g unfractionated yeast tRNA. Alkaline hydrolysis reactions were performed in 50 mM sodium carbonate (pH 9.2), 1mM EDTA at 90°C for 20 min. Ribonuclease digestions were done in 10 μ l of 25 mM sodium acetate (pH 5.2), 5 mM MgCl₂, 50 mM KCl at 37°C. Units of RNase per reaction and digestion times were as follows: RNase U₂ (Pharmacia), 10 units, 2 min; bovine pancreatic RNase (CalBiochem), 1 unit, 1 min; RNase T₁ (CalBiochem), 0.03 units, 1 min. Reactions were stopped by addition of 1 volume of 9 M urea in 50 mM Tris, 50 mM boric acid, 1 mM EDTA and freezing at -70°C. Reactions were analyzed using denaturing (7 M urea) 20% polyacrylamide gel electrophoresis and autoradiography. When a specific ribonucleoside triphosphate was omitted from a reaction mixture, a strong stop was observed at the first position where incorporation of that nucleotide should have occurred. Minor higher molecular weight bands were also observed, including a band which migrated at the position expected for the full-length product. Read through may have been due to cross-contamination of the nucleoside triphosphates, or to misincorporation. The transcription products of reactions using deoxynucleotides were resistant to alkaline hydrolysis and ribonucleases at sites of deoxynucleotide incorporation.

Thermodynamic Analysis

Following isolation, oligonucleotides were purified on Sep Pak C₁₈ cartridges (Millipore). Due to the method of transcription, all oligonucleotides terminate with 5' triphosphates. Buffer conditions for all optical experiments were 50 mM NaCl, 0.1 mM EDTA, 8 mM sodium phosphate (pH 7.0). Each melt sample was 50 μ M in nucleotides (1.8 μ M in total strand concentration). Samples were heated briefly at 95°C before each experiment to insure proper duplex formation. Helix to coil transitions were monitored at 260 nm using a Gilford 250 uv/vis spectrometer with a Gilford 2527 thermoprogrammer at a heating rate of 1°C per min. A 1 cm pathlength cuvette was used. Absorbance at 260 nm versus temperature data were converted to fraction single-stranded nucleic acid versus temperature and then smoothed.

Enzymatic Digestion Reactions

Duplexes were digested with RNase V₁ and RNase H. Approximately 1 pmol of 5'-³²P-labeled strand was combined with 10 pmol complementary strand, heated at 90°C for 1 min and allowed to cool slowly before addition of buffer and enzyme. RNase V₁ reactions in 20 μ l of 10 mM Tris (pH 8.1), 10 mM MgCl₂, 50 mM NaCl, contained 3 μ g unfractionated yeast tRNA and 0.1 units RNase V₁ (Pharmacia) and were incubated 3 min at 37°C. Reaction conditions for RNase H (Pharmacia) were 40 mM Tris (pH 8.1), 4 mM MgCl₂, 1 mM dithiothreitol, 30 μ g/ml BSA, 50% glycerol, 3 μ g unfractionated yeast tRNA in 15 μ l. When oligoribonucleotides were hybridized to DNA, 0.2 units of RNase H were added and reactions were incubated at 37°C for 10 min. Reactions containing oligoribonucleotides annealed to deoxy-substituted or fully ribo-oligonucleotides were incubated for 2 h at 37°C with 1 unit of RNase H. Ladders for band assignment were generated using 12 units of S₁ and 1 pmol 5'-³²P-labeled transcript in 20 μ l of 10 mM NaCl, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.3) for 2 min at 85°C.

Reactions were quenched by addition of 1 volume of 9 M urea and freezing at -70°C . Reactions were analyzed using denaturing (7 M urea) 20% polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Oligonucleotide Synthesis

Efficiency of 2'-deoxynucleotide incorporation into oligoribonucleotide transcripts by T7 RNA polymerase is dependent on template sequence and deoxynucleotide incorporated. A single deoxyadenosine (dA) was incorporated 70% as well as riboadenosine (rA) while dT or dU incorporation was about 85% that of rU. When dA and dT were both added to transcription reactions, the yield was about 25% compared to the fully ribonucleotide transcript. In separate transcription experiments on oligonucleotide sequences with fewer cytidines, there was a 50% loss in efficiency per deoxycytidine incorporated. Due to the number of guanosine residues in our templates and the requirement for guanosine residues in the +1 to +4 region of the transcript, dG incorporation was not investigated.

Substitution of manganese chloride for magnesium chloride in transcription reactions relaxed the specificity of T7 RNA polymerase for deoxyribonucleotides, as previously reported for *E. coli* DNA polymerase I incorporation of ribonucleotides (12). Although manganese allowed 90 to 100% incorporation of deoxynucleotides, overall yields were very low. For the transcript with only ribonucleotides, the yield was down by a factor of 30.

Oligonucleotide Characterization

Partial alkaline hydrolysis of 5'- ^{32}P -labeled 5'GGCCGGAUCCGCGC $^{3'}$ produces a ladder of bands of approximately equal intensity due to 2'-hydroxyl mediated cleavage after each nucleotide. Deoxynucleotides lack the 2'-hydroxyl necessary for cleavage through a 2'-3' cyclic phosphate intermediate and are not labile at high pH. When 5'- ^{32}P -labeled deoxynucleotide-containing transcript was partially hydrolyzed at high pH or by ribonucleases, no cleavage was observed at the phosphate immediately 3' of the deoxynucleotide residues (Figure 1a). Using bands in the same ladder as standards and subtracting background radioactivity, we estimate less than 5% cleavage at the site of expected deoxynucleotide incorporation; any cleavage was due to incorporation of ribonucleotide. The nucleoside triphosphates used in transcription reactions were not analyzed for cross contamination. Minor amounts of ribonucleotide incorporation may be due to incorporation of the correct ribonucleotide introduced as a contaminant or to misincorporation of an incorrect residue by T7 RNA polymerase.

Thermodynamic Analysis

The melting behavior of the fully oligoribonucleotide duplex, 5'GGCCGGAUCCGCGC $^{3'}$ ·5'GCGCGGAUCCGGCC $^{3'}$, was compared to those of duplexes with deoxynucleotide substitutions. The melting temperatures for all duplexes investigated are summarized in Table I. The derivatives of the fraction of single strand vs. temperature curves for the fully ribonucleotide duplex (I) and the duplex with dA substitutions on both strands (V) are shown in Figure 2. Completely analogous results were obtained for deoxynucleotide substitution within a second duplex sequence in which the UpA step was reversed to ApU: 5'GGCCGGUACCGCGC $^{3'}$ ·5'GCGCGGUACCGGCC $^{3'}$ (Table I). The duplexes studied are not melting in a two-state manner; stabilities will be discussed only in terms of T_m .

Circular Dichroism

Circular dichroism spectra (CD) of the oligoribonucleotide duplex (I) and the dA- and/or

Table I. Melting Temperatures of Oligonucleotide Duplexes.

Designation	Sequence	T _m (°C)
I	5'rGGCCGGAUCCGCGC3' 3'rCCGGCCUAGGCGCG5'	79.9
II	. . . rAdT dTrA . . .	79.4
III	. . . dArU rUrA . . .	78.5
IV	. . . rArU rUdA . . .	78.5
V	. . . dArU rUdA . . .	77.5
VI	. . . dAdT dTdA . . .	77.8
VII	5'rGGCCGGAUCCGCGC3' 3'rCCGGCCAUGGCGCG5'	79.7
VIII	. . . dTrA rAdT . . .	79.5
XI	. . . rUdA dArU . . .	77.7

Buffer conditions were 50mM NaCl, 0.1mM EDTA, 8mM sodium phosphate, pH 7.0. Concentrations were 50 μ M in nucleotides. The melting temperature (T_m) has an estimated precision of $\pm 0.5^\circ\text{C}$.

dT-substituted duplexes (II, V, VI) were recorded at room temperature under buffer conditions identical to those used in the melting studies. Over the range from 320nm to 200nm, the two spectra are identical and typical of double-stranded A-form RNA (results not shown). No conformational distortion of the helix due to the presence of the deoxy residues was observable by CD.

RNase V₁ Digestion Results

The oligoribonucleotide and the deoxy- substituted duplexes were digested with RNase V₁ and autoradiographs of the gel electrophoretic analysis of reactions are shown in Figure 1b. In each reaction only one of the strands, 5'GGCCGGAUCCGCGC3', was 5'-³²P-labeled. Similar results were obtained when the complementary strand was labeled. RNase V₁ cut the internal region of the duplex with varied intensity. Digestion left a dinucleotide at the 5' and a trinucleotide at the 3' end of the molecule intact. When dA and/or dT were substituted in each of the strands of the duplex cleavage was considerably reduced at the phosphate immediately 3' of the deoxynucleotides, but relative intensity of cleavage after ribonucleotide residues was unchanged. Cleavage was independent of the residues on the opposite strand, as shown by comparison of cleavage patterns of the 5'-³²P-labeled oligoribonucleotide strand hybridized to either a fully ribonucleotide or a deoxy-substituted strand. Deoxyuridine substitution resulted in cleavage patterns equivalent to those obtained for duplexes substituted with dT.

Figure 2. Fraction single strand vs temperature curves for duplexes I and V in 50mM NaCl, 0.1mM EDTA, 6mM Na₂HPO₄, 2mM NaH₂PO₄, pH 7.0. Nucleotide concentrations were 50 μ M. Data were collected at 260nm and smoothed. Substitution of two deoxyadenosines within the duplex results in a 2.4°C decrease in T_m .

RNase H Digestion Results

The effect of substitution of deoxy residues within the RNA strand of an RNA-DNA hybrid on RNase H cleavage was investigated by partial digestion with RNase H of the 5'-³²P-labeled oligoribonucleotide, 5'GGCCGGAUCCGCGC^{3'}, or 5'-³²P-labeled deoxy-containing analog while annealed to a complementary DNA strand. (This DNA strand was also used as the template for synthesis of the oligonucleotides and so extended 17 residues in a 3' overhang. The single-stranded region corresponds to the T7 RNA polymerase promoter region (10).) Digestion leaves dinucleotides intact at the 5' and 3' ends (Figure 1c). Substitution of deoxynucleotides within the 5'-³²P-labeled strand led to loss of cleavage at phosphodiester linkages both 5' and 3' of the deoxynucleotide (Figure 1c). Relative intensities of cutting elsewhere within the molecule were unchanged.

When hybridized to a complementary oligoribonucleotide containing both dA and dT, the 5'-³²P-labeled fully ribonucleotide strand was cleaved immediately 3' of the rU involved in the rU-dA base-pair and after two consecutive ribonucleotides in the 3' direction as indicated by arrows: 5'GGCCGGAU^vC^vC^vGCGC^{3'}. A single deoxy substitution, of dA, dT or dU, did not allow cleavage of the 5'-³²P-labeled ribonucleotide strand by RNase H.

DISCUSSION

We find that T7 RNA polymerase incorporates 2'-deoxynucleotides into oligoribonucleotides with greater than 50% efficiency per deoxynucleotide incorporated as compared to the yield of the fully ribonucleotide product. Previously, Axelrod & Kramer (13) showed that T7 RNA polymerase accepts 3'-deoxyribonucleotides, which act as chain terminators, with 25–60% reduced activity as compared to the corresponding ribonucleotides. While T7 RNA polymerase is not highly selective for the sugar portion of nucleotides, little is known about its base-specific fidelity under in vitro conditions. It has been suggested that a significant fraction of the RNA product synthesized using T7 RNA polymerase and synthetic

DNA templates is not the correct sequence (14). We estimate that less than 5% incorporation of ribonucleotide has occurred at each deoxy-position in the transcripts used in this study. The ribonucleotide at these sites may be the correct base as a result of impurity in nucleoside triphosphates or may be due to polymerase misincorporation. In either case, base-specific discrimination suffers little, if at all, when deoxynucleotides are substituted in transcription reactions.

Instability of DNA-RNA hybrids containing deoxyadenosine was previously reported (4,5,15). Substitution of a single deoxyadenosine destabilized the oligoribonucleotide duplex, $5'\text{GGCCGGAUCCGCGC}^{3'}$ · $5'\text{GCGCGGAUCCGGCC}^{3'}$, while deoxythymidine had no effect (Figure 2 and Table I). The effect of dA substitution was additive and independent of whether or not the base pair formed was a DNA-RNA hybrid; two deoxyadenosines on opposite strands paired with either rU or dT lowered the melting temperature by an average of 2.2°C. Destabilization occurs irrespective of nearest neighbors as shown by similar trends in thermodynamic behavior of the duplex $5'\text{GGCCGGAUCCGCGC}^{3'}$ · $5'\text{GCGCGGAUCCGGCC}^{3'}$ and deoxy-substituted analogs. Apparently, the specific combination of deoxy sugar and the adenine base are not readily incorporated into the A-form RNA helix. Any helix perturbation due to the deoxy substitution was not observable in the circular dichroism spectra. Although RNase V₁ cleavage was very weak after substituted deoxy residues, relative intensities at other sites on the strand, including nearest neighbors, remained unchanged despite the presence of the deoxynucleotide within the binding site of the enzyme. This suggests that the local RNA helical structure is not greatly disrupted by the presence of one or more deoxynucleotides.

Despite its wide use as a probe for double-stranded RNA, little is known about the binding and cleavage specificity of RNase V₁. Minor RNase V₁ cleavage after deoxynucleotides was too intense to be due to cleavage of misincorporated ribonucleotide (compare Figure 1a with Figure 1b) and must have been due to RNase V₁ cleavage of DNA. Since the binding constant for RNase V₁ binding to DNA is only 2-fold lower than that for binding to RNA of the same sequence (8), RNase V₁ interaction with a 2'-hydroxyl must be necessary for efficient cleavage although not for binding.

RNase V₁ digestion of oligoribonucleotide duplexes in this study and of hairpins in previous work (16) shows that cleavage leaves a dinucleotide at the 5' end of a duplex and trinucleotide at the 3' terminus intact. RNase V₁ apparently requires two nucleotides 5' and three nucleotides 3' of the cleavage site for optimal cleavage. Results are consistent with a model in which RNase V₁ binds to the phosphates and/or sugars five nucleotides on one strand of a helical region which may consist of typical double-stranded duplex, DNA-RNA hybrid duplex or single-stranded stacked bases. For cleavage to be efficient, the residue at the cut site must be ribose, but deoxynucleotides elsewhere in the binding site have no effect. Even in complementary duplexes cleavage intensity varies considerably, although no simple sequence specificity is apparent. Binding and cleavage may be influenced by groove width or conformational flexibility throughout the binding site.

E. coli RNase H has previously been used in conjunction with hybridized DNA probes to specifically cleave long RNA molecules (17). Shibara and coworkers (18) used chimeric probes containing three or more deoxynucleotides linked to 2'-O-methylribonucleotides. These chimeric probes offer the advantage of increased length, which allows disruption of RNA secondary structure and more stable hybridization, without increasing the number of cleavage sites as would occur with DNA probes of comparable size. Cleavage at a single site on the ribonucleotide strand occurred with probes containing four deoxy residues;

multiple cut sites are observed with probes containing three or five deoxynucleotides. We have found that RNase H cleaves an oligoribonucleotide when the complementary probe strand contains only two deoxynucleotides. When two deoxynucleotides are contained in the probe, the ribonucleotide strand was cleaved on the 3' side of the uridine involved in the rU·dA hybrid base pair and after the next two residues in the 3' direction as shown by the arrows: ^{5'}GGCCGGAU^{3'}C^vC^vGCGC^{3'}. For optimum binding the enzyme may need to contact four deoxy sugars; the two deoxy residues in our probe may allow RNase H to bind at three fairly equivalent positions. Digestion with RNase H leaves dinucleotides at both the 5' and 3' ends of the ribonucleotide strand of the DNA-RNA hybrid duplex. This suggests that a minimum of four residues on the ribonucleotide strand are contacted by the enzyme. The enzyme probably binds in the minor groove since it has no sequence specificity and since the 2' position of the sugar is located in the minor groove. RNase H does not bind to four base pairs, but rather binds to residues close together across the minor groove. The first residue contacted on the ribo strand is two nucleotides in the 3' direction from the first deoxynucleotide contact.

Oligoribonucleotides containing two to four deoxy residues used in conjunction with RNase H will allow directed cleavage of large RNA molecules. The probes will increase site-specificity of cleavage and minimize secondary structure problems often encountered with RNase H fragmentation using oligodeoxynucleotides. Probe sequences are limited by the method of synthesis; some sequence flexibility can be gained by incorporating deoxynucleotides outside the cleavage site since a single hybrid base pair is not recognized by RNase H. While chemically synthesized probes containing deoxynucleotides and either ribonucleotides or 2'-O-methylribonucleotides would allow more sequence variability and will ultimately be the method of choice, the techniques for enzymatic synthesis are more generally available.

Oligoribonucleotides containing one or more deoxy residues may be useful in structural studies of splice sites or ribonuclease cleavage sites since the substitutions, while presumably altering structure very little, would be resistant to 2' OH mediated cleavage. Deoxy residues within oligoribonucleotides can be used for NMR studies; the change at the 2' position would aid in assignment.

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