Inhibition of Restriction Endonuclease Cleavage by Triple Helix Formation with RNA and 2′-*O*-Methyl RNA Oligonucleotides Containing 8-Oxo-adenosine in Place of Cytidine†

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ABSTRACT: The ability of homopyrimidine oligoribonucleotides (RNA) and oligo-2′-*O*-methyl-ribonucleotides (2′-*O*-methyl RNA) containing 8-oxo-adenosine (AOH) and 8-oxo-2′-*O*-methyl (AmOH) adenosine to form stable, triple-helical structures with sequences containing the recognition site for the class II-S restriction enzyme, *Ksp*632-I, was studied as a function of pH. The AOH- and AmOH-substituted RNA and 2′-*O*-methyl RNA oligonucleotides were shown to bind within the physiological pH range in a pH-independent fashion, without a compromise in specificity. The substitutions of three cytidine residues with AOH showed higher endonuclease inhibition than the substitution of either one or two cytidine residues with AOH. In particular, the 2′-*O*-methyl RNA oligonucleotide with only one cytidine substituted with AmOH showed higher endonuclease inhibition than the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides and the RNA oligonucleotides containing either one or two AOH moieties. Furthermore, the AmOH-substituted 2′-*O*-methyl RNA oligonucleotides were stable (53%) after an incubation in 10% fetal bovine serum for 8 h, whereas the RNA oligonucleotides were completely degraded. Increased resistance to nucleases is observed with the introduction of 2′-*O*-methylnucleosides. This stabilization should help us to design much more efficient third strand homopyrimidine oligomer and antisense nucleic acid-based antiviral therapies, which could be used as tools in cellular biology.

Homopyrimidine−homopurine sequences have been mapped to several sites in the regulatory regions of eukaryotic genes and are hypersensitive to single-strand-specific nucleases, such as S1 (*1*−*3*). These sequences are expected to exhibit unusual DNA structures, as they are known to undergo a transition to an underwound state in plasmids, under conditions of moderately acidic pH and negative supercoiling. Studies of these systems appear to support a model consisting of a triple-strand (pyr-pur-pyr) plus a single-stranded structure called H-DNA (*4*−*8*). Pyrimidine oligonucleotides bind in the major groove of the DNA, parallel to the purine Watson−Crick strand, through the formation of specific Hoogsteen hydrogen bonds to the purine Watson−Crick bases. Specificity is derived from thymine (T) recognition of adenine-thymine (AT) base pairs (T-AT triplet), and protonated cytosine (C+) recognition of guanine-cytosine (GC) base pairs (C+GC triplex) (*9*−*14*). However, protonation at the N-3 of C is required in order to form two hydrogen bonds with the G of the target strand. Due to this requirement, triplex formation by homopyrimidines that contain multiple C residues is sensitive to pH and is usually restricted to pH values of 7 or below.

Recently, the triple helix forming abilities of N^6-methyl-8-oxo-2′-deoxyadenosine (*15*, *16*), 8-oxo-2′-deoxyadenosine (*17*, *18*), and pseudoisocytidine (*19*) as protonated deoxycytidine analogues were shown to be pH-independent within the physiological range. Furthermore, Shimizu et al. (*20*) have shown that the triplexes formed with oligo-(2′-methoxylribonucleotides) or (2′-fluororibonucleotides) were more thermally stable than those formed by DNA oligonucleotides. The 2′-modified RNAs may bind more effectively and tightly to the DNA duplex than a third DNA strand, if the 2′-modified RNAs have the C3′-*endo* conformation and the 2′-substituted groups enhance the rigidity of the triple-helix structure. However, the oligo-2′-*O*-methoxylribonucleotides bind in a pH-dependent manner to the target duplex.

In this paper, we wish to report the possibility of inhibiting sequence-specific DNA binding proteins by RNA and 2′-*O*-methyl RNA oligonucletides containing 8-oxo-2′-adenosine (AOH) and/or 2′-*O*-methyladenosine (AmOH) instead of cytidine. We have tested the ability of homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH to inhibit the sequence-specific cleavage of simian virus 40 (SV40) DNA, at neutral and basic pH values, by the class II-S restriction endonuclease, *Ksp*632-I (*21*, *22*). The *Ksp*632-I enzyme recognizes a 6 base pair homopurine−homopyrimidine sequence. We have synthe-

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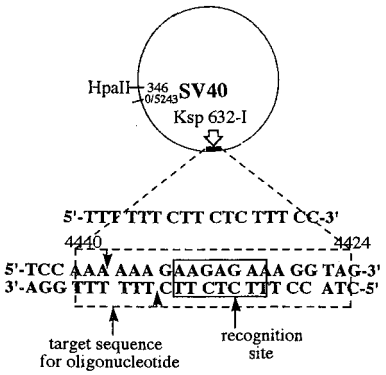

u-ODN 5'AAAAAAGAAGAGAAAGG3'

y-ODN 5'TTTTTTCTTCTCTTTCC3'

1	5'UUUUUUCUUCUCUUUCC3'
2	5'UUUUUUCUUAOHUCUUUCC3'
3	5'UUUUUUAOHUUCUAOHUUUCC3'
4	5'UUUUUUAOHUUAOHUAOHUUUCCC3'
5	5'UmUmUmUmUmUmCmUmUmCmUmCmUmUmUmCmCm3'
6	5' UmUmUmUmUmUmCmUmUmAmOHUmCmUmUmUmCmCm 3'
7	5' UmUmUmUmUmUmAmOHUmUmCmUmAmOHUmUmUmCmCm 3'
8	5' UmUmUmUmUmUmAmOHUmUmAmOHUmAmOHUmUmUmCmCm 3'

u-ODN:homopurine oligonucleotide; y-ODN:homopyrimidine oligonucleotide.

FIGURE 1: Schematic representation of SV40 DNA, showing the recognition site for the *Ksp*632-I enzyme and the target sequences for the homopyrimidine oligonucleotides containing 8-oxo-adenosine and 8-oxo-2′-*O*-methyladenosine, which are shown above the boxed target sequence. *Ksp*623-I and *Hpa*II were used for SV40 DNA linearization. Short arrows indicate the cleavage sites for the restriction endonucleases.

sized a 17 mer homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH, which are designed to bind to the major groove according to Hoogsteen base pairing (Figure 1). We found that the AmOH-substituted 2′-*O*-methyl RNA oligonucleotides selectively inhibit the cleavage of SV40 linear DNA by the restriction enzyme *Ksp*632-I, as compared with the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides and the RNA oligonucleotides containing either one or two AOH moieties, under physiological pH conditions. Furthermore, an increased resistance to nucleases was observed with the introduction of 2′-*O*-methylnucleosides.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The 5′-*O*-(4,4′-dimethoxytrityl)-8-hydroxyl-2′-*O*-(*tert*-butyldimethyl-silyl)- and 8-hydroxyl-2′-*O*-(methyl)-N^6-acetyladenosine-3′-*O*-[(*N*,*N*-diisopropyl-amino) (2-cyanoethyl)] phosphoramidites were synthesized by the procedures of Kim et al. (*23*). Oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA automatic synthesizer on a 1 μM scale with ribonucleotide phosphoramidite reagents; standard phosphoramidite chemistry was employed. The oligoribonucleotides were base-deprotected by an incubation in 3 mL of aqueous concentrated ammonia (28%)/ethanol (3:1) for 16 h at 55 °C in a screw-cap glass vial. After complete removal of the solvent by evaporation, the residue was taken up in 0.5 mL of 1.1 M tetrabutylammonium fluoride in THF and left at room temperature overnight. An OPC reverse-phase cartridge (PerSeptive Biosystems) was used for simple purification after desilylation of the RNA. The cartridge was first washed with 4% acetonitrile in 100 mM triethylammonium bicarbonate (pH 7.5) to remove the impurities. The RNA was then eluted with acetonitrile−methanol−water (3:3:4) and was evaporated in vacuo. The crude RNA was purified by chromatography on a YMC column with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0). After purification, the fragments were lyophilized to dryness.

Circular SV 40 DNA was linearized with the restriction enzyme *Hpa*II, which cleaves at position 346. The cleavage products were separated on 0.8% agarose gels. The products were eluted from the gel, and were recovered from the eluate by ethanol precipitation.

Thermal Denaturation Profiles. Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200 spectrometer. The insulated cell compartment was warmed from 5 to 90 °C, with increments of 1 °C and equilibration for 1 min after the attainment of each temperature, using a temperature controller, SPR-8 (Shimadzu). Samples were heated in masked 1 cm path length quartz cuvettes fitted with Teflon stoppers. Each thermal denaturation was performed in 33 mM Tris/acetate buffer (pH 6.0, 7.0, and 8.0), 1 mM EDTA, 10 mM $MgCl_2$, and 100 mM NaCl, containing 1 μM of each strand. The mixture of duplex and single strands was kept at 90 °C for 5 min, and then cooled to 5 °C. At temperatures below 20 °C, N_2 gas was continuously passed through the sample compartment to prevent the formation of condensate.

Gel Electrophoresis. The duplex DNA was made by combining the designated concentrations of oligonucleotides in 33 mM Tris-acetate (pH 7.0) buffer/100 mM NaCl/10 mM $MgCl_2$, incubating the mixture at 90 °C for 10 min, and slowly cooling it to room temperature. The triplex oligonucleotides were made by the addition of an equimolar amount of the third strand to the duplex, followed by an incubation at 4 °C overnight. The concentration of each strand was 1.23×10^{-3} μM in a total volume of 10 μL. Electrophoresis experiments were conducted using gels containing 15% polyacrylamide (acrylamide/bisacrylamide, 19:1) prepared in a Bio-Rad Protean II gel apparatus with $20 \times 22 \times 0.75$ cm glass plates. Tris/borate buffer (50 mM, pH 7.8)/5 mM $MgCl_2$ was used in the electrophoresis reservoirs. Electrophoresis was conducted at a constant temperature (4 °C) and 200 V for 16 h. The gel was stained using methylene blue.

Inhibition of Restriction Endonuclease Digestion at Ksp632-I Sites in SV40 Linear DNA. Enzymatic assays were performed in a buffer containing 33 mM Tris-acetate, 100 mM NaCl, 66 mM potassium acetate (pH 7.0, and 8.0), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM spermine, and 1 μg/μL tRNA at 30 °C (*24*). The pH of the incubation medium was changed as indicated in the text and the legend of the figure. The concentration of SV40 linear DNA was usually 6 nM, and 20 units/μL restriction enzyme was used in each assay. After the incubation, the enzymatic reactions were stopped by the addition of EDTA (10 mM). Specimens were then analyzed by electrophoresis on slab gels (0.8% agarose). Densitometric analysis of gels stained with ethidium bromide was performed on a Millipore Bio Image 60S.

Exonuclease Stability of Oligonucleotide Derivatives. The oligonucleotide was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The oligonucleotides (0.1 μM) were incubated with 100 μL of culture medium containing 10%

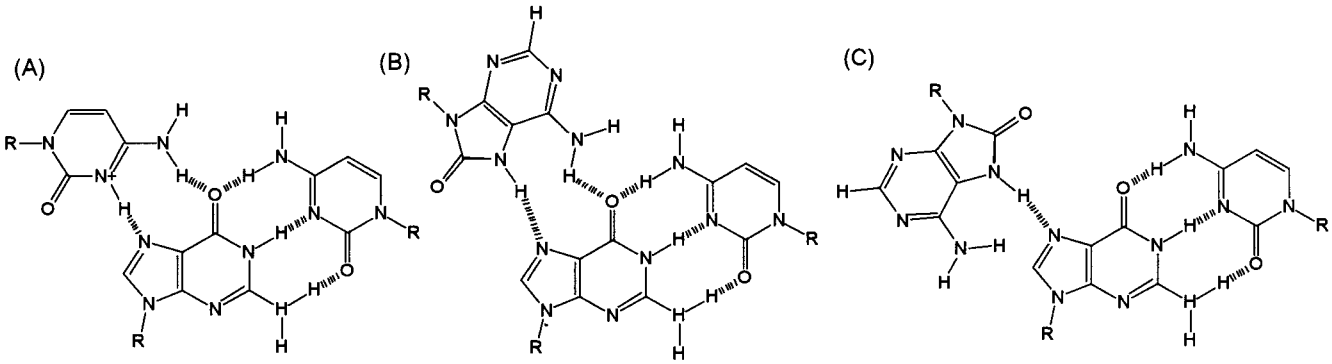

FIGURE 2: Possible hydrogen-bonding scheme for (A) the C^+·G·C triad, (B) the 8-oxo-A*(syn)*·G·C triad, and (C) the 8-oxo-A*(anti)*·G·C triad.

Table 1. Melting Temperatures, T_m (°C), of the Triplexes[a] and Inhibition of Restriction Endonuclease Activity by the Modified Oligonucleotides (1−8)

oligonucleotide number	T_m (°C)			IC_{50} (μM)[b]
	pH 6.0	pH 7.0	pH 8.0	pH 7.0
1	46	29	25	>10
2	35	32	26	10
3	30	31	24	8.9
4	28	28	27	2.5
5	52	30	28	>10.
6	34	34	34	1.8
7	37	36	36	0.51
8	40	39	38	0.08

[a] Duplex, 5′AAAAAAGAAGAGAAAGG3′/3′-TTTTTTCTTCTC-TTTCC5′. [b] The IC_{50} value is the concentration of the test compound that caused 50% inhibition of restriction enzyme cleavage.

fetal bovine serum for 24 h at 37 °C. Aliquots (5 μL) of the reaction were removed at the indicated times (15, 50, 60, 120, 240, 480, 960, and 1440 min), added to an equal volume of 80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue, and analyzed by electrophoresis on 20% polyacrylamide/7 M urea (29:1) gels. Autoradiograms were obtained by exposing the gels to Fuji (X-ray) film at −20 °C. The extent of cleavage was determined by comparing the radioactivity of the intact fragment to that of the cleaved fragments with a BioImage analyzer, BAS 2000 (Fuji Medical Systems, Tokyo, Japan).

RESULTS AND DISCUSSION

Triple-Helix Formation by the Modified Oligonucleotides. To investigate whether imperfect recognition sites, containing more than a C−G inversion, can form triplex structures, we synthesized the 17 nucleotide duplex, 5′AAAAAAGAA-GAGAAAGG3′/3′TTTTTTCTTCTCTTTCC5′ (SV40 target sequence), and studied its interactions with the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotide analogues containing AOH and/or AmOH instead of cytidine (Figure 1). The ability of the oligonucleotides to form triple helices was examined by melting temperature studies. Table 1 shows the results of the thermal melting of the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and AmOH (**1**−**8**) bound to the 17 mer duplex. The influence of the pH on the T_m was determined at pH 6.0, 7.0, and 8.0 for the 17 mer duplex/unmodified and modified homopyrimidine oligoribonucleotides (**1**−**8**). The unmodified, **1**, and modified (AOH; **2** and **3**) homopyrimidine oligoribonucleotides bind in a pH-dependent manner to the target duplex. Thus, an increase in the pH should not favor the protonation of cytidine and thereby should decrease the stability of the triplex. In contrast, the oligonucleotide with the complete substitution of AOH for the cytidine base residues (**4**) exhibited pH-independent binding in the physiological range (pH 6.0−8.0). On the other hand, the 2′-*O*-methyl RNA oligonucleotides with from one to three substitutions of AmOH showed pH-independent binding in the physiological range. Of particular interest was the 2′-*O*-methyl RNA oligonucleotide with only one substitution of AmOH, which was found to have pH-independent binding of the same order as that for the oligomer (**4**). In a previous study, we tested the pH-independent binding for homopyrimidine oligodeoxyribonucleotides containing 2′-modified nucleosides (Um, Cm, Uf, and Cf), but the results showed the same pH dependence as the pyrimidine-rich DNA oligomers (*25*). The mixed oligonucleotides containing 2′-modified nucleosides and 8-oxo-2′-deoxyadenosine (dAOH) destabilized the triplex by the incorporation of dAOH at pH 6.0−8.0. This triplex destabilization is influenced by the different conformation of the dAOH (*syn*) and the 2′-modified nucleosides (*3′-endo*). However, the 2′-*O*-methyl RNA oligonucleotides enhance the Hoogsteen bond stability by the incorporation of the AmOH at pH 6.0−8.0. These results suggest that the conformation of the 3′-*endo* sugar of the third strand pyrimidine and the *syn* conformation of the adenosine base favor the triple-helix structure and the increased pH independence of the binding of the triplex, due to the 2′-*O*-methyl and 8-hydroxyl groups (Figure 2). Since 8-oxo-2′-deoxyadenosine contains two hydrogen bond donors, at positions N-7 and N-6, and the p*K* of the hydrogen at position 7 is approximately 8.7, its ability to participate in triplex formation should not be affected by an increase in the pH. 8-Oxo-2′-deoxyadenosine has been shown to exist in the keto form, with the base in the *syn* conformation (*26*, *27*), and recent NMR results suggested that when incorporated in a DNA duplex, 8-oxo-2′-deoxyadenosine exists in the keto form, although in this case, the base appears to adopt an anti conformation in the duplex (*6*). The formation of a similar hydrogen bond was recently proposed by Young et al. (*15*) for the interaction of N^6-methyl-8-oxo-2′-deoxyadenosine with GC base pairs in duplex DNA.

Next, the formation and the stability of triplexes with RNA and 2′-*O*-methyl RNA oligonucleotide analogues containing 8-oxo-2′-adenosine (AOH) and/or 8-oxo-2′-*O*-methyladenosine (AmOH) were confirmed by gel retardation assays. As Figure 3 shows, the single-, double-, and triple-stranded

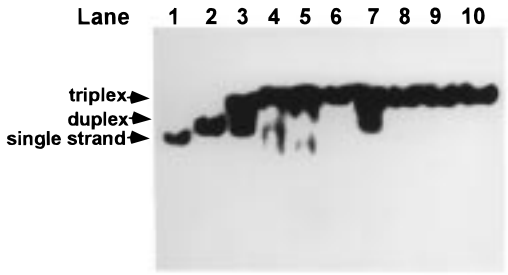

FIGURE 3: Methylene-blue stained, 15% nondenaturing polyacrylamide gel run in 33 mM Tris/acetate buffer (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$: lane 1, ODNs; lane 2, duplex DNA; lanes 3−10, oligomers **1**−**8**.

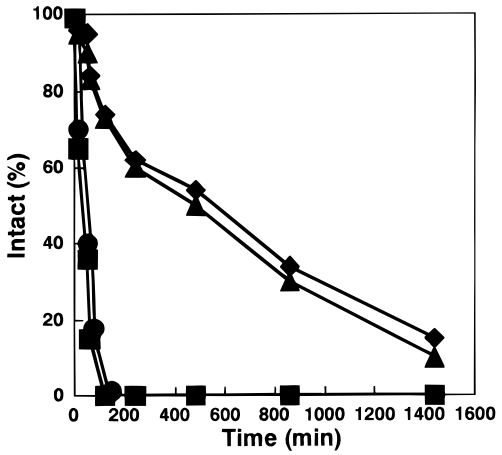

FIGURE 4: Stability of oligonucleotide derivatives (**1**, **4**, **5**, and **8**) in the presence of 10% calf serum. Oligonucleotides were incubated in the presence of 10% calf serum for the indicated times, as described in the Materials and Methods. Full-length ("intact") and digested oligonucleotides were resolved on 20% denaturing polyacrylamide gels, and the full-length oligonucleotide was quantitated at the indicated time points, as described in the Materials and Methods section. Oligonucleotides tested are indicated as follows: ■, oligo **1**; ●, oligo **4**; ▲, oligo **5**; and ◆, oligo **8**. The percentage of the oligonucleotide that remained intact was calculated by comparison with the full-length oligonucleotide levels in the samples.

species can be separated on a 12% polyacrylamide gel at pH 7.0. It is worth noting that a band, corresponding to a weaker binding mode of the pyrimidine oligonucleotides to the imperfect C−G sites, was observed for the oligoribonucleotide analogues lacking the complete substitution of 8-oxo-adenosine for cytidine. On the other hand, we could detect more stable triple-helical structures with the 2′-*O*-methyl RNA oligonucleotides with from one to three AmOH substitutions.

Exonuclease Resistance of Modified Oligonucleotides. The exonuclease resistance of the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and AmOH was examined in the medium used for anti-HIV assays, which contains 10% fetal bovine serum (FBS) (Figure 4). For comparison, the unmodified oligoribonucleotide (**1**) was chosen as a control. The 2′-*O*-methyl RNA oligonucleotide (**5**) and the 2′-*O*-methyl RNA oligonucleotide containing AmOH (**8**) were stable (50% and 53%) after 8 h of incubation, whereas the oligoribonucleotide (**1**) and the RNA oligonucleotide containing AOH (**4**) were completely degraded (100%) after 2 h. An increased resistance to nuclease degradation has been observed in oligo-2′-*O*-methylribonucleotide derivatives incubated with 3′-exonucleases (*28*−*31*). However, the results reported by Monia (*29*) clearly indicate that the nuclease resistance conferred on an adjacent P−O linkage by a 2′-*O*-methyl substituent still does not allow for potent antisense activity in cellular systems. On the other hand, the 2′-*O*-alkyl-RNA binds complementary sequences with high affinity, relative to analogous DNA or RNA oligonucleotides. The clinical applications of 2′-*O*-methoxyethyl RNA with a phosphorothioate backbone are being explored in phase I/II clinical trials directed against PCK-α in refractory solid tumors (*32*). This stabilization should help us to design much more efficient chemical recognition enzymes, which could be used as tools *in vitro* and *in vivo*.

Inhibition of Endonuclease Cleavage Activity by Modified Oligonucleotides. The restriction enzyme *Ksp*632-I recognizes the sequence 5′-CTCTTC-3′/3′-GAGAAG-5′ and cleaves the two strands asymmetrically outside the recognition sequence (Figure 1). We have synthesized homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH instead of cytidine, which are 17 bases in length and have a parallel orientation as compared to the homopurine sequence of the 17 bp SV40 DNA sequence. Previous reports showed that this oligonucleotide binds to the major groove of the duplex DNA in the homopurine sequence (*6*, *11*).

To test the ability of the 17 mer homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH instead of cytidine to recognize selectively their *Ksp*632-I site within the SV40 DNA, we conducted the experiments under physiologically relevant pH conditions. SV40 DNA was digested with *Hpa*II (position 346) and was used as a substrate to assay the restriction enzyme cleavage activity of the homopyrimidine-oligoribonucleotide derivatives. Cleavage of linear SV40 DNA with *Ksp*632-I yields fragments of 1150 and 4090 base pairs in length. The experiments were carried out at 30 °C and at either pH 7.0 or 8.0, in the presence of 100 mM NaCl, 66 mM potassium acetate, 10 mM $MgCl_2$, and 1 mM spermine. The DNA fragments were visualized by ethidium bromide staining and were subjected to quantitative analysis, assuming that the fluorescent intensities were proportional to the lengths of the DNA fragments. The oligonucleotides were added to the SV40 linear DNA before the addition of the restriction enzyme. Figures 5A (lane 3) and 3C (lane 3) show the digestion of the SV40 DNA by *Ksp*632-I in the presence of the oligo- and oligo-2′-*O*-methyl- ribonucleotides (**1** and **5**) at a 10 μM concentration. Densitometric analysis of the gels indicated that the percentages of restriction enzyme cleavage inhibition at a 10 μM oligomer concentration were 35% and 10%, respectively. When the pH of the cleavage reaction was increased to 8.0, no inhibition of the restriction enzyme was observed at a 10 μM concentration of oligonucleotide (*1*) (Figure 5B, lane 3). In the case of **5**, very weak cleavage protection was observed (Figure 5C, lane 3). This was also supported by the gel retardation assay (Figure 3). Further evidence for the oligonucleotide binding to the duplex DNA was obtained using conditions that are known to either destabilize or stabilize triple-helical structures. Triple helix formation by a homopyrimidine oligonucleotide involves binding of uridine and protonated cytidine to A−T and G−C base pairs, respectively. An increase in the pH destabilizes the triple helix, due to the loss of cytidine protonation, which is required to form the C−G−C base triplet.

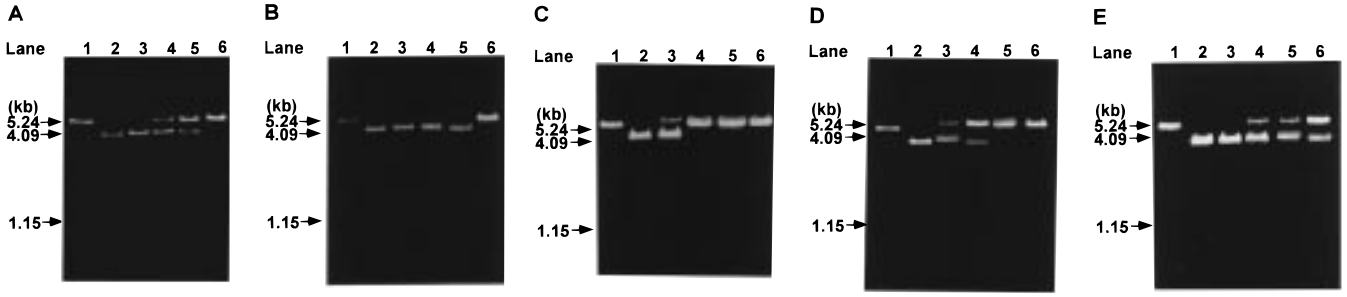

FIGURE 5: Specific inhibition of the restriction enzyme *Ksp*632-I by the homopyrimidine oligonucleotides containing 8-oxo-adenosine and 8-oxo-2′-*O*-methyladenosine. The enzymatic assay was performed by incubating SV40 linear DNA (6 nM) at 30 °C for 1 h with *Ksp*632-I (20 units/μL) and oligonucleotide in a buffer containing 33 mM Tris-acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM spermine, and 1 μg/μL tRNA. (A) The enzymatic assay was carried out in the presence of the unmodified (**1**) (lane 3) and modified (**2**−**4**) (lanes 4−6) oligonucleotides (10 μM) at pH 7.0. (B) The enzymatic assay was carried out in the presence of the unmodified (**1**) and modified (**2**−**4**) oligonucleotides (10 μM) at pH 8.0. (C) The enzymatic assay was carried out in the presence of the modified (**5**−**8**) oligonucleotides (10 μM) at pH 7.0. (D) The enzymatic assay was carried out in the presence of the modified (**5**−**8**) oligonucleotides (10 μM) at pH 8.0. (E) The enzymatic assay was carried out in the presence of the modified (**5**−**8**) oligonucleotides (0.1 μM) at pH 7.0. Lane 1: SV40 linear DNA incubated without restriction enzyme. Lane 2: SV40 linear DNA incubated with restriction enzyme.

In a subsequent experiment, we incubated the SV40 linear DNA with *Ksp*632-I in the presence of the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH instead of cytidine (**2**−**4** and **6**−**8**). Gel analysis of the modified oligomers (**2**−**4** and **6**−**8**) revealed cleavage inhibition by 52% (**2**), 68% (**3**), 94% (**4**), 100% (**6**), 100% (**7**), and 100% (**8**) at a 10 μM concentration (Figure 5, panels A (lanes 4−6) and C (lanes 4−6)). Furthermore, the above experiment was also repeated at pH 8.0, using the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH instead of cytidine (**2**−**4** and **6**−**8**). The percentages of inhibition of restriction enzyme cleavage at the 10 μM concentration of modified oligomers (**2**−**4** and **6**−**8**) were 0% (**2**), 11% (**3**), 95% (**4**), 86% (**6**), 100% (**7**), and 100% (**8**) (Figure 5, panels B (lanes 4−6) and D (lanes 4−6)).

To estimate the inhibitory effect of the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and/or AmOH instead of cytidine, we compared the 50% effective concentrations (IC_{50}) (Table 1). Figure 5E shows the digestion of SV40 DNA by *Ksp*632-I in the presence of the modified oligomers (**6**−**8**) at a 0.1 μM concentration. The modified oligomers (**6**−**8**) showed higher inhibition of the endonuclease activity (Table 1 and Figure 5E). This inhibition increased with the number of AmOH substitutions, as the oligomers with one, two, and three AmOH residues possessed IC_{50} values of 1.8, 0.51, and 0.08 μM, respectively. In contrast, the RNA oligonucleotide (**4**) with three AOH substitutions inhibited the endonuclease activity with an IC_{50} value of 2.5 μM, a substantially lower inhibitory effect than that observed for the 2′-*O*-methyl RNA oligonucleotide analogues (**8**). The RNA oligonucleotides (**2** and **3**) with one and two AOH substitutions showed less inhibition of the endonuclease activity than the 2′-*O*-methyl RNA oligonucleotides (**6** and **7**) with one and two AmOH substitutions. It is notable that this potent inhibition is achieved in a modified oligonucleotide (**8**) possessing a melting temperature of 39 °C, a higher T_m than those of the other modified oligonucleotides. These results suggest that the 2′-*O*-methyl RNA oligonucleotide analogues with from one to three AmOH substitutions provided very high cleavage protection in the physiological pH range. In particular, in the case of the substitution of only one cytidine residue with AmOH, the inhibition of the restriction enzyme was high. In contrast, the oligoribonucleotide analogues with one or two substitutions of AOH provided only weak cleavage protection. However, in the case of the substitution of three cytidine residues with AOH, inhibition of the restriction enzyme was observed. These findings suggest that the homopyrimidine RNA and 2′-*O*-methyl RNA oligonucleotides containing AOH and AmOH can specifically inhibit the DNA−protein interaction via triplex formation, within the physiological pH range. The substitution of one cytidine residue with AmOH showed higher endonuclease inhibition than the substitution of either one or two cytidine residues with AOH. Furthermore, the 2′-*O*-methyl RNA oligonucleotide analogues with from one to three AmOH substitutions provided very high cleavage protection in the physiological pH range. In particular, in the case of the substitution of only one cytidine residue with AmOH, the restriction enzyme inhibition was similar to that of the oligomer (**4**).

Conclusion. The recognition of a DNA sequence by a restriction endonuclease can be strongly inhibited under physiological pH conditions (pH 6.0−8.0) by homopyrimidine 2′-*O*-methyl RNA oligonucleotides containing AmOH, which bind with high affinity to duplex DNA. Specific binding of a homopyrimidine 2′-*O*-methyl RNA oligonucleotide containing AmOH might modulate transcription, either by interacting with the binding of protein factors or by preventing the elongation of RNA. DNA replication might be similarly inhibited. The oligo-2′-*O*-methylribonucleotide derivatives also display enhanced resistance to nuclease degradation by 3′-exonucleases. This stabilization should help us to design much more efficient transcription and replication inhibitors, which possess favorable pharmacological properties, thus facilitating their applications as antigene agents in animal testing and therapeutic development.

REFERENCES

1. Larsen, A., and Weintraub, H. (1982) *Cell 29*, 609−622.
2. Nickel, J. M., and Felsenfeld, G (1983) *Cell 35*, 467−477.
3. Elfin, S. C. R. (1984) *Nature 309*, 213−214.
4. Lee, J. S., Woodsworth, M. L., Latimer, L. P., and Morgan, A. R. (1984) *Nucleic Acids Res. 12*, 6603−6614.
5. Lyamichev, V. I., Miklin, S. M., Frank-Kamenetskii, M. D., and Cantor, C. R. (1988) *Nucleic Acids Res. 16*, 2165−2178.
6. Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J. L., Thuong, N. T., Lhomme, J., and Hélène, C. (1987) *Nucleic Acids Res. 15*, 7749−7760.

7. Hun, H., and Dahberg, J. E. (1988) *Science 241*, 1791−1798.
8. Johnston, B. H. (1988) *Science 241*, 1800−1804.
9. Moser, H. E., and Dervan, P. B. (1987) *Science 238*, 645−650.
10. Strobel, S. A., Moser, H. E., and Dervan, P. B. (1988) *J. Am. Chem. Soc. 110*, 7972−7929.
11. François, J. C., Saison-Behmoaras, T., Chassignol, M., Thuong, N. T., and Hélène, C. (1989) *J. Biol. Chem. 264*, 5891−5898.
12. Maher, L. J., Wold, B., and Dervan, P. B. (1989) *Science 245*, 725−730.
13. Hélène, C., and Téoule, J. J. (1990) *Biochem. Biophys. Acta 1049*, 99−125.
14. Hanvey, J. C., Shimizu, M., and Wella, R. D. (1990) *Nucleic Acids Res. 18*, 157−161.
15. Young, S. L., Krawczyk, S. H., Matteucci, M. D., and Toole, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10023−10026.
16. Krawczyk, S. H., Milligan, J. F., Wadwani, S., Moulds, C., Froehler, B. C., and Matteucci, M. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3761−3764.
17. Miller, P. S., Bhan, P., Cushaman, C. D., and Trapane, T. L. (1992) *Biochemistry 31*, 6788−6793.
18. Davison, E. C., and Johnsson, K. (1993) *Nucleosides Nucleotides 12*, 237−243.
19. Ono, A., Ts`o, P. O. P., and Kan, L.-S. (1991) *J. Am. Chem. Soc. 113*, 4032−4033.
20. Shimizu, M., Konishi, A., Shimada, Y., Inoue, H., and Ohtsuka, E. (1992) *FEBS Lett. 302*, 155−158.
21. Bolton, B. J., Schmitz, G. G., Jarsch, M., Conner, M. J., and Kessler, C. (1988) *Gene 66*, 31−43.
22. Wang, Q., Tsukahara, S., Yamakawa, H., Takaki, K., and Takaku, H. (1994) *FEBS Lett. 355*, 11−14.
23. Kim, S.-K, Yokoyama, S., Takaku H., and Moon. B.-J. (1998)-*Bioorg. Med. Chem. Lett. 8*, 939−944.
24. François, J. C., Saison-Behmoaras, T., Chassignol, M., Thuong, N. T., and Hélène, C. (1989) *J. Biol. Chem. 264*, 5891−5898.
25. Ishibashi, T., Yamakawa, H., Wang, Q., Tsukahara, S., Takai, K., Maruyama, T., and Takaku, H. (1996) *Bioorg. Med. Chem. 4*, 2029−2034.
26. Guschlbauer, W., Duplaa, A.-M., Guy, A., Téoule, R., and Fazakerley, G. V. (1991) *Nucleic Acids Res. 19*, 1753−1758.
27. Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J. L., Thuong, N. T., Lhomme, J., and Hélène, C. (1987) *Nucleic Acids Res. 15*, 7749−7760.
28. Fisher, T. L., Terhorst, T., Cao, X., and Wagner, R. W. (1993) *Nucleic Acids Res. 21*, 3857−3865.
29. Monia, B. P., Johnston, J. F., Sasmor, H., and Cummins, L. L. (1996) *J. Biol. Chem. 271*, 14533−14540.
30. Altmann, K.-H., Dean, N. M., Fabbro, D., Freier, S. M., Geiger, T., Häner, R., Hüsken, D., and Moser, H. E. (1996) *Chimia 50*, 168−176.
31. Altmann, K.-H., Fabbro, D., Dean, N. M., Geiger, T., Monia, B. P., Müller, M., and Nicklin, P. (1996) *Biochem. Soc. Trans. 24*, 636−637.
32. Dean, N. M., McKay, R., Miraglia, L., Howard, R., Cooper, S., Giddings, J., Nicklin, P., Meister, L., Ziel, R., Geiger, T., Müller, M., and Fabbro, D. (1996) *Cancer Res. 56*, 3499−3507.

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