FEBS Letters 355 (1994) 11–14

FEBS 14752

pH-independent inhibition of restriction endonuclease cleavage via triple helix formation by oligonucleotides containing 8-oxo-2′-deoxyadenosine

Qing Wang, Satoru Tsukahara, Hidefumi Yamakawa, Kazuyuki Takai, Hiroshi Takaku*

Department of Industrial Chemistry, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275, Japan

Received 21 September 1994; revised version received 5 October 1994

Abstract The ability of homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine to form stable, triple helical structures with the sequence containing the recognition site for the class II-S restriction enzyme, *Ksp*632-I, was studied as a function of pH. The 8-oxo-2′-deoxyadenosine-substituted oligomers were shown to inhibit enzymatic cleavage and to bind within the physiological pH range in a pH-independent fashion without compromising specificity.

Key words: Triplex formation; Base modification; T_m value; Conformation; Native polyacrylamide gel electrophoresis; Inhibition of restriction enzyme

1. Introduction

Triple-helical nucleic acid structures can be formed from synthetic polymers as the result of internal disproportionation of polypurine arrays in duplexes. These structures may involve the binding of the third strand to the major groove of the DNA duplex. Binding results in the formation of hydrogen-bonded TAT and C^+GC triplets, in which pyrimidines in the third strand are Hoogsteen-bound to the purines of the DNA duplex [1–3]. The demonstration that homopyrimidine oligodeoxynucleotides can bind specifically to homopurine–homopyrimidine tracts of duplex DNA via intermolecular triplex formation has revealed new possibilities for the inhibition of enzymatic cleavage [4–7]. One limitation of this recognition motif is the strong pH dependence of triple helix formation. This pH dependence is due to the requirement for the protonation of the N3 of the cytidine in the third strand, in order to form the C^+GC triplet. Thus, triplex formation by homopyrimidines requires conditions considerably more acidic than the intracellular pH range of 7.1–7.6 [1]. Recent studies have shown that the requirements for base protonation can be eliminated by using bases or base analogues that are capable of interacting with the guanine of GC base pairs [8–11]. More recently, the triple helix-forming abilities of N^6-methyl-8-oxo-2′-deoxyadenosine [12,13] and 8-oxo-2′-deoxyadenosine (dA^{OH}) [14,15] as protonated deoxycytidine analogues were shown to be pH-independent within the physiological range. The C8 oxidation of deoxyadenosine therefore modifies the hydrogen-bonding characteristics of this base only when it adopts the *syn* conformation [16,17].

To examine the possibility of inhibiting sequence-specific DNA binding proteins by the oligonucleotide analogues containing dA^{OH} instead of deoxycytidine, we have tested the ability of homopyrimidine oligonucleotides containing dA^{OH} to inhibit 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 [18]. The *Ksp*632-I enzyme recognizes a 6-base pair homopurine–homopyrimidine sequence. We have synthesized 17-mer homopyrimidine oligonucleotides containing dA^{OH}, which are desgned to bind to the major groove according to Hoogsten base pairing (Fig. 1). We found that these oligonucleotides selectively inhibit the cleavage of SV40 DNA by the restriction enzyme *Ksp*632-I under physiological salt conditions.

2. Materials and methods

2.1. Oligonucleotide synthesis

The oligonucleotides were synthesized by means of the phosphoramidite method using an Applied Biosystems Model 392 DNA/RNA synthesizer on the 1 μM scale, and with controlled pore glass supports. Phosphoramidite units were prepared from the reaction of 5′-dimethoxytrityl N-protected-2′-deoxyribonucleosides or 5′-dimethoxytrityl N-protected-8-methoxy-2′-deoxyadenosine [19] with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite as a condensing unit. In the case of oligomers containing 8-methoxy-2′-deoxyadenosine, the support was first treated with thiophenol/triethylamine/dioxane (1:1:2, v/v/v) under N_2 gas for 5 days at 37°C. The support was treated with concentrated ammonia for 15 h at 55°C. The deprotected oligomers were purified by reverse-phase HPLC.

The nucleoside composition was determined after snake venom phosphodiesterase/bacterial alkaline phosphatase hydrolyses.

2.2. Thermal denaturation profiles

Thermal transitions were recorded at 260 nm using a Shimadzu UV-2200A spectrometer. The insulated cell compartment was warmed from 5°C to 90°C, with increments of 1°C and equilibration for 1 min after attaining each temperature, using the 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 6.5, 7.0, and 7.8), 1 mM EDTA, 10 mM $MgCl_2$, 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.

2.3. Gel electrophoresis

The duplex DNA was made by combining the designated amounts (200 μM) of oligonucleotides in 33 mM Tris-acetate (pH 7.0) buffer, 100 mM NaCl, 10 mM $MgCl_2$, keeping the mixture at 90°C for 10 min, and slowly cooling it to room temperature. The triplex DNA was made by the addition of an equimolar amount of the third strand to the duplex, followed by incubation at 4°C overnight. The concentration of

*Corresponding author. Fax: (81) (474) 71-8764.

SSDI 0014-5793(94)01139-7

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.

2.4. Inhibition of restriction endonuclease digestion at the Ksp*632-I site in SV40*

Enzymatic assays were performed in a buffer containing 33 mM Tris-acetate, 100 mM NaCl, 66 mM potassium acetate (pH 6.5, 7.0, and 7.8), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM spermine [20,21], and 1 μg/μl tRNA at 25°C. The pH of the incubation medium was changed as indicated in the text and legend of the figures. The concentration of SV40 linear DNA was usually 6 nM, and 20 units/μl restriction enzyme was used in each assay. After incubation, enzymatic reactions were stopped by the addition of EDTA (10 mM). Specimens were then analyzed by gel-electrophoresis on slab gels (0.8% agarose). Densitometric analysis of gels stained with ethidium bromide was performed on a Millipore Bio Image 60S.

Circular SV40 DNA was linearized with the restriction enzyme *Hpa*II, which cleaves at position 346. The SV40 linear DNA was separated by electrophoresis on an 0.8% agarose gel. The SV40 linear DNA band was eluted by crushing and soaking in 0.5 M ammonium acetate at 37°C for several hours, extracted with an equal volume of water-saturated phenol/chloroform (1:1), and precipitated with ethanol. The cleavage products were analyzed on 0.8% agarose gels stained with ethidium bromide.

3. Results and discussion

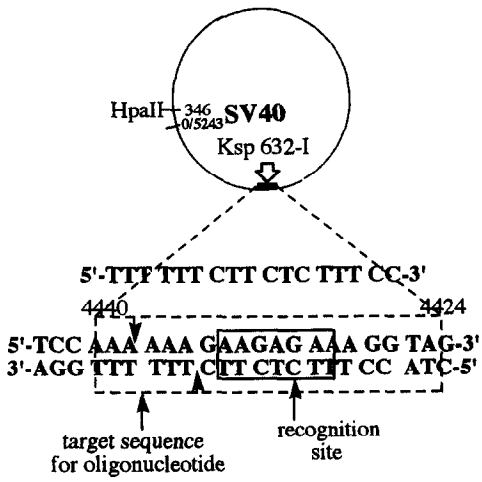
3.1. Triple-helix formation by homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine (dA^{OH})

In order to investigate whether an imperfect recognition site, containing more than a C/G-inversion, can form triplex structures, we synthesized the 17 nucleotide duplex, 5′AAAAAAAGAAGAGAAAGG3′/3′TTTTTTCTTCTCTTTCC5′ (SV40 target sequence) [22] and studied its interactions with the homopyrimidine oligonucleotides containing dA^{OH} (Fig. 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 oligonucleotides containing dA^{OH} bound to the 17-mer duplex. The influence of pH on the T_m was determined at pH 6.5, 7.0, and 7.8 for the 17-mer duplex/unmodified and modified homopyrimidine oligonucleotides. The unmodified (III) and modified (IV, V) homopyrimidine oligonucleotides bind in a pH-dependent manner to the target duplex. In contrast, the complete substitution of dA^{OH} for deoxycytidine base residues (VI) was pH-independent in the physiological range (pH 6.5–7.8). Thus, raising the pH should not favor the protonation of deoxycytidine, and thereby should decrease the stability of the triplex. Because dA^{OH} contains two hydrogen bond donors, at positions N7 and N6, and the pK of the hydrogen at position 7 is approximately 8.7 [16], its ability to participate in triplex formation should not be affected by an increase in pH, at least within the physiological pH range.

Table 1
Melting temperature, T_m (°C) of the 17-mer duplex and triplexes

pH	Duplex	Duplex–III	Duplex–IV	Duplex–V	Duplex–VI
6.5	60	43	41	36	33
7.0	60	30	32	29	31
7.8	60	20	25	24	31

Duplex, 5′AAAAAAGAAGAGAAAGG3′/3′TTTTTTCTTCTCTTTCC5′.



I. 5′-AAAAAAGAAGAGAAAGG-3′
II. 3′-TTTTTTCTTCTCTTTCC-5′
III. 5′-TTTTTTCTTCTCTTTCC-3′
IV. 5′-TTTTTTCTTA^{OH}TCTTTCC-3′
V. 5′-TTTTTTA^{OH}TTCTA^{OH}TTT CC-3′
VI. 5′-TTTTTTA^{OH}TTA^{OH}TA^{OH}TTT CC-3′

Fig. 1. Schematic representation of SV40 DNA showing the recognition site for the *Ksp*632-I restriction enzyme and the target sequence for the homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine, which is shown above the boxed target sequence. The restriction enzymes *Ksp*623-I and *Hpa*II were used for the SV40 DNA linearization. Short arrows indicate the cleavage sites for the restriction endonuclease.

The formation and stability of dA^{OH}-containing triplexes were confirmed by gel-retardation assays. As Fig. 2 shows, the single-, double-, and triple-stranded species can be separated on a 15% non-denaturing polyacrylamide gel run at a low temperature. A band corresponding to the duplex (lane 2) and four bands corresponding to the triplexes were formed by the addition of III (lane 3), IV (lane 4), V (lane 5), and VI (lane 6), respectively. The relative stabilities of the triplexes found in the T_m analyses (Table 1) are consistent with the gel analysis, in which the amount of duplex (lane 2) reflects the instability of the particular triplex. It is worth noting that the band corresponding to a weaker mode of binding of the pyrimidine oligonucleotides to the imperfect CG sites was observed for all oligonucleotide analogues without the complete substitution of dA^{OH} for deoxycytidine.

3.2. Inhibition of restriction activity by a homopyrimidine oligonucleotide containing dA^{OH}

The restriction enzyme *Ksp*632-I recognizes the sequence 5′CTCTTC3′/3′GAGAAG5′ and cleaves the two strands asymmetrically outside the recognition sequence (Fig. 1). We have synthesized homopyrimidine oligonucleotides containing dA^{OH}, which are 17 bases in length and have a parallel orientation, as compared to the homopurine sequence of the 17 bp SV40 DNA sequence. In previous papers this oligonucleotide was shown to bind to the major groove of duplex DNA in the homopurine sequence [4,20,23].

In order to test the ability of the 17-mer homopyrimidine oligonucleotide analogues containing dA^{OH} to selectively recognize their *Ksp*632-I site within the SV40 linear DNA, the experiments were carried out at 25°C and either pH 6.5, 7.0 or 7.8, in the presence of 100 mM NaCl, 66 mM potassium acetate, 10 mM $MgCl_2$ and 1 mM spermine, conditions under which the enzyme makes a single double-stranded cut in the SV40 linear DNA. The oligonucleotides were added to the SV40 linear DNA before the addition of the restriction enzyme. Fig. 3A (lanes 3–5) shows the digestion of the SV40 linear DNA by *Ksp*632-I in the presence of the unmodified oligonucleotides (ODNs) at 1–10 μM. Densitometric analysis of the gels indicated that the inhibition of restriction enzyme cleavage at a 1 μM oligomer concentration was 100%. The above experiment was repeated at pH 7.0 using the unmodified ODNs. Under this condition, the unmodified ODNs afforded complete inhibition at 10 μM. Further evidence for the involvement of oligonucleotide binding to duplex DNA was provided using conditions that are known to either destabilize or stabilize triple-helical structures. Triple helix formation by a homopyrimidine oligonucleotide involves binding of thymidine and protonated deoxycytidine to AT and GC base pairs, respectively. An increase in the pH destabilizes the triple helix due to the loss of deoxycytidine protonation, which is required to form the CGC base triplet. When the pH of the cleavage reaction was increased to 7.8, no inhibition of the restriction enzyme was observed at 10 μM of oligonucleotide III (lane 3, Fig. 3B). This result was also supported by the gel-retardation assay (Fig. 2A). In a subsequent experiment, we incubated the linear SV40 DNA with *Ksp*632-I in the presence of the homopyrimidine oligonucleotides containing dA^{OH} (IV–VI). The analysis of the gels for the modified oligomers (IV–VI) indicated that they inhibited 100% (IV), 69% (V), and 29% (VI) at a 1 μM concentration of oligomers (lanes 8, 11, and 14, Fig. 3A). On the other hand, at the 1 μM concentration of modified oligomers (IV–VI) at pH 7.0, the corresponding inhibitions of the restriction enzyme were 18%, 48%, and 30%, respectively (lanes 8, 11, and 14, Fig. 3B). Furthermore, the above experiment was also repeated at pH 7.8 using modified oligomers (IV–VI). The inhibition of restriction enzyme cleavage at the 1 μM concentration of modified oligomers (IV–VI) was 17%, 25%, and 31%, respectively (lanes 8, 11, and 14, Fig. 3C). In particular, in the case of the substitution of three deoxycytidine residues with dA^{OH}, at 5 μM oligomer concentrations, the inhibition of the restriction enzyme was similar to that with a concentration of 10 μM (lanes 12 and 13, Fig. 3C). In contrast to the singly or doubly dA^{OH}-substituted oligonucleotides, oligonucleotide VI, containing dA^{OH} instead of three deoxycytidine residues, binds in a pH-independent manner to the target duplex. These findings suggest that, unlike the unmodified oligonucleotides, the homopyrimidine oligonucleotides containing dA^{OH} can specifically inhibit the DNA–protein interaction via triplex formation within the physiological pH range. Furthermore, the substitu-

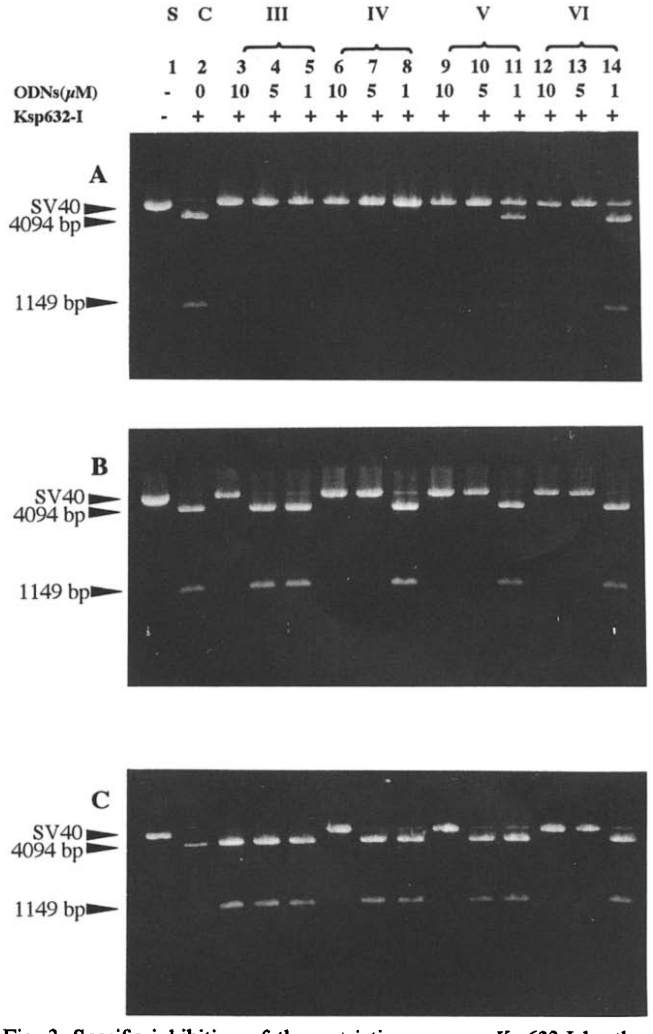


Fig. 3. Specific inhibition of the restriction enzyme *Ksp*632-I by the homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine. SV40 DNA was lineared at the *Hpa*II site (position 346). SV40 linear DNA (6 nM) was incubated at 25°C for 1 h with the restriction enzyme *Ksp*632-I (20 units/μl) and several concentrations (0, 1, 5, or 10 μM) of 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. The enzyme assay was carried out in the presence of the unmodified (III) and modified (IV, V and VI) oligonucleotides at pH 6.5 (A), 7.0 (B), and 7.8 (C). Lane 1, SV40 linear DNA incubated without the restriction enzyme and oligonucleotides. Lane 2, SV40 linear DNA incubated with the restriction enzyme.

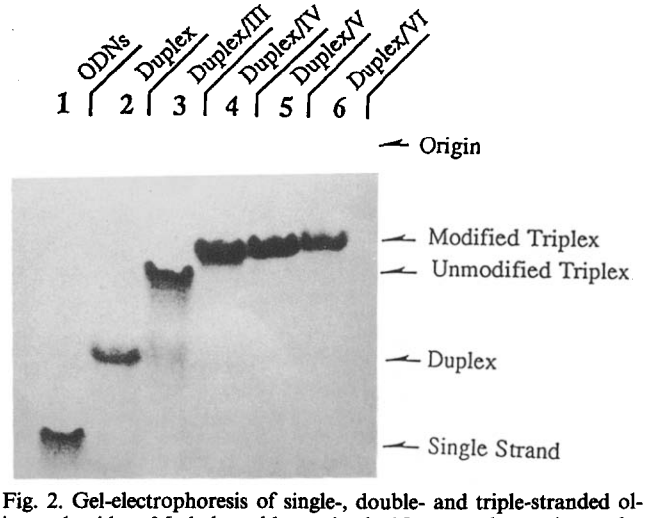


Fig. 2. Gel-electrophoresis of single-, double- and triple-stranded oligonucleotides. Methylene blue-stained, 15% non-denaturing polyacrylamide gel in 33 mM Tris-acetate buffer (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$.

tion of three deoxycytidine resides with dA^{OH} showed higher endonuclease inhibition than the substitution of only one or two deoxycytidine residues with dA^{OH}.

We have shown that the recognition of a DNA sequence by a restriction endonuclease can be specifically inhibited by a homopyrimidine oligonucleotide analogue containing dA^{OH} under physiological pH conditions, which allow recognition of the target site by triple helix formation. Selective binding of homopyrimidine oligonucleotide analogues containing dA^{OH} might modulate transcription, either by interfering with the binding of regulatory protein factors or by preventing the elongation of RNA. DNA replication might be similarly inhibited. This stabilization should help us to design much more efficient transcription and replication inhibitors, which could be used as tools in cellular biology.

Acknowledgements: This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas No. 0562102 and No. 05265220 from the Ministry of Education, Science and Culture, Japan, and by a Research Grant from the Human Science Foundation.

References

[1] Moser, H.E. and Dervan, P.B. (1987) Science 238, 645–650.
[2] Strobel, S.A., Moser, H.E. and Dervan, P.B. (1988) J. Am. Chem. Soc. 110, 7972–7929.
[3] Hélène, C. and Téoule, J.J. (1990) Biochim. Biophys. Acta 1049, 99–125.
[4] François, J.C., Saison-Behmoaras, T., Thoug, N.T. and Hélène, C. (1989) Biochemistry 28, 9617–9619.
[5] Maher, L.J., Wold, B. and Dervan, P.B. (1989) Science 245, 725–730.
[6] Hanvey, J.C., Shimizu, M. and Wella, R.D. (1990) Nucleic Acids Res. 18, 157–161.
[7] Xodo, L.E., Alunni-Fabbroni, M., Manzini, G. and Quadrifoglio, F. (1993) Eur. J. Biochem. 212, 395–401.
[8] Lee, J.S., Woodsworth, M.L., Latimer, L.J.P. and Morgan, A.R. (1984) Nucleic Acids Res. 12, 6603–6614.
[9] Ono, A., Ts'o, P.O.P. and Kan, L.-S. (1991) J. Am. Chem. Soc. 113, 4032–4033.
[10] Koh, J.S. and Dervan, P.B. (1992) J. Am. Chem. Soc. 114, 1470–1478.
[11] Ono, A., Ts'o, P.O.P. and Kan, L.-S. (1991) J. Org. Chem. 57, 3225–3230.
[12] Young, S.L., Krawczyk, S.H., Matteucci, M.D. and Toole, J.J. (1991) Proc. Natl. Acad. Sci. USA 88, 10023–10026.
[13] Krawczyk, S.H., Milligan, J.F., Wadwani, S., Moulds, C., Froehler, B.C. and Matteucci, M.D. (1992) Proc. Natl. Acad. Sci. USA 89, 3761–3764.
[14] Miller, P.S., Bhan, P., Cushaman, C.D. and Trapane, T.L. (1992) Biochemistry 31, 6788–6793.
[15] Davison, E.C. and Johnsson, K. (1993) Nucleosides Nucleotides 12, 237–243.
[16] Cho, B.P. and Evans, F.E. (1991) Nucleic Acids Res. 19, 1041–1047.
[17] Leonard, G.A., Guy, A., Brown, T., Téoule, R. and Hunter, W.N. (1992) Biochemistry 31, 8415–8420.
[18] Bolton, B.J., Schmitz, G.G., Jarsch, M., Conner, M.J. and Kessler, C. (1988) Gene 66, 31–43.
[19] Holmes, R.E. and Robins, R.K. (1965) J. Am. Chem. Soc. 87, 1772–1776.
[20] 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.
[21] François, J.C., Saison-Behmoaras, T., Chassignol, M., Thuong, N.T. and Hélène, C. (1989) J. Biol. Chem. 264, 5891–5898.
[22] Reddy, V.B., Thimmappaya, B., Dhar, R., Subramanian, K.N., Zain, B.S., Pan, J., Ghosh, P.K., Celma, M.L. and Weissman, S.M. (1978) Science 200, 404–502.
[23] Praseuth, D., Perrouault, L., Le Doan, T., Chassignol, M., Thuong, N.T. and Hélène, C. (1988) Proc. Natl. Acad. Sci. USA 85, 1349–1353.