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Properties of Triple Helix Formation with Oligodeoxyribonucleotides Containing 8-Oxo-2′-deoxyadenosine and 2′-Modified Nucleoside Derivatives

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Abstract—The ability of homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine (dAOH), 2′-methoxyuridine (Um), 2′-fluorouridine (Uf), 2′-methoxycytidine (Cm), and 2′-fluorocytidine (Cf) to form stable, triple-helical structures with sequences containing the recognition site for the class II-S restriction enzyme, Ksp632-I, was studied as a function of pH. The 8-oxo-2′-deoxyadenosine substituted oligomers were shown to bind within the physiological pH range in a pH-independent fashion, without a compromise in specificity. In particular, the substitutions of three deoxycytidine residues with 8-oxo-2′-deoxyadenosine showed higher endonuclease inhibition than the substitution of either one or two deoxycytidine residues with 8-oxo-2′-deoxyadenosine. In contrast, the oligonucleotides containing 2′-modified nucleosides (Uf ,Um, Uf-Cf, Um-Cm, dAOH-Uf, and dAOH-Um) bind in a pH-dependent manner to the target duplex. Copyright © 1996 Elsevier Science Ltd

Introduction

Pyrimidine oligonucleotides bind specifically to purine sequences in double-stranded DNA to form local triple-helical structures.[1] 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).[2–7] However, protonation at the N-3 of C is required in order to form two hydrogen bonds with the G of the target strand. Owing to this requirement, triplex formation by homo-pyrimidines that contain multiple C residues is sensitive to pH and is usually restricted to pH values of 7 or below.

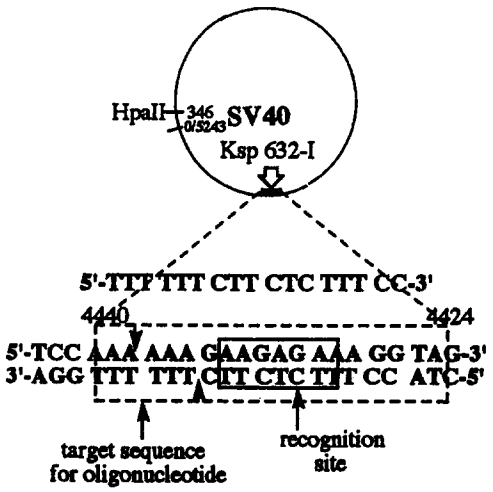
Recent studies have shown that the requirement for base protonation can be eliminated by using bases or base analogues that are capable of interacting with the guanines of GC base pairs.[8–11] More recently, the triple helix forming abilities of N^6-methyl-8-oxo-2′-deoxyadenosine,[12,13] 8-oxo-2′-deoxyadenosine,[14,15] and pseudoisocytidine[16] as protonated deoxycytidine analogues were shown to be pH-independent within the physiological range. Furthermore, Shimizu et al.[17] have shown that the triplexes formed with oligo-(2′-*O*-methylribonucleotides) or (2′-fluororibonucleotides) were thermally more 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.

In this paper, we report the possibility of inhibiting sequence-specific DNA binding proteins by oligonucleotide analogues containing 8-oxo-2′-deoxyadenosine (dAOH) and/or 2′-modified nucleosides (Uf, Um, Cf, and Cm) instead of deoxycytidine and deoxythymidine. We have tested the ability of homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine or 2′-modified nucleosides 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.[18,19] The *Ksp* 632-I enzyme recognizes a 6 base pair homopurine-homopyrimidine sequence. We have synthesized 17 mer homopyrimidine oligonucleotides, containing 8-oxo-2′-deoxyadenosine and/or 2′-modified nucleosides, which are designed to bind to the major groove according to Hoogsteen base pairing (Fig. 1). We found that the 8-oxo-2′-deoxyadenosine substituted oligonucleotides selectively inhibit the cleavage of SV40 DNA by the restriction enzyme *Ksp* 632-I, as compared with the 2′-modified uridine and cytidine substituted oligonucleotides, under physiological salt conditions.

Results and Discussion

In order to investigate whether imperfect recognition sites, containing more than a C–G inversion, can form triplex structures, we synthesized the 17 nucleotide duplex, 5′AAAAAAGAAGAGAAAGG3′/3′TTTTTT-CTTCTCTTTCC5′ (SV40 target sequence), and

studied its interactions with the homo-pyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine (dAOH) and/or 2′-modified nucleosides (Uf, Um, Cf, and Cm) (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 dAOH, Uf, Um, dAOH-Uf, Uf-Cf, dAOH-Uf, and Um-Cm, bound to the 17 mer duplex. The influence of the pH on the Tm was determined at pH 6.0, 7.0, and 7.8 for the 17 mer duplex/unmodified and modified homopyrimidine oligonucleotides. The unmodified, **1**, and modified (dAOH, Uf, Um, dAOH-Uf, Uf-Cf, dAOH-Um, and Um-Cm; **2**, **3**, **5**–**14**) homopyrimidine oligonucleotides bind in a pH-dependent manner to the target duplex. In particular, we expected the pH independent binding for the homopyrimidine oligonucleotide containing 2′-modified nucleosides (Um, Cm, Uf, and Cf), but the results showed the same pH dependence as the pyrimidine-rich OND (**1**). The mixed oligonucleotides containing 2′-modified nucleosides and dAOH destabilized the triplex by the incorporation of dAOH at pH 6.0–7.8. This triplex destabilization is influenced by the different conformations of the dAOH (*syn*) and the 2′-modified nucleosides (3′-*endo*). Furthermore, the substitution of only one or two deoxycytidine base residues with dAOH causes pH dependence. Thus, an increase in the pH should not favor the protonation of deoxycytidine, and thereby should decrease the stability of the triplex. In contrast, the oligonucleotide with the complete substitution of dAOH for the deoxycytidine base residues (**4**) exhibited pH-independent binding in the physiological range (pH 6.0–7.8). 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



	u-ODN	5'AAAAAAGAAGAGAAAGG3'
1	y-ODN	5'TTTTTTCTTCTCTTTCC3'
2	y-ODN-dAOH	5'TTTTTTCTTAOHTCTTTCC3'
3	y-ODN-2-dAOH	5'TTTTTTAOHTTCTAOHTTTCC3'
4	y-ODN-3-dAOH	5'TTTTTTAOHTTAOHTAOHTTTCC3'
5	y-ODN-3Uf	5'TTTTTTCUfUfCUfCTTTCC3'
6	y-ODN-3Uf-dAOH	5'TTTTTTCUfUfCUfAOHTTTCC3'
7	y-ODN-3Uf-2-dAOH	5'TTTTTTAOHUfUfCUfAOHTTTCC3'
8	y-ODN-3Uf-3-dAOH	5'TTTTTTAOHUfUfAOHUfAOHTTTCC3'
9	y-ODN-3Cf	5'TTTTTTCfUfUfCfUfCfTTTCC3'
10	y-ODN-3Um	5'TTTTTTCUmUmCUmCTTTCC3'
11	y-ODN-3Um-dAOH	5'TTTTTTCUmUmCUmAOHTTTCC3'
12	y-ODN-3Um-2-dAOH	5'TTTTTTAOHUmUmCUmAOHTTTCC3'
13	y-ODN-3Um-3-dAOH	5'TTTTTTAOHUmUmAOHUmAOHTTTCC3'
14	y-ODN-3Um-3Cm	5'TTTTTTCmUmUmCmUmCmTTTCC3'

u-ODN:homo-purine oligonucloetide; y-ODN:homo-pyrimidine oligonucleotide.

Figure 1. Schematic representation of SV40 DNA, showing the recognition site for the *Ksp* 632-I enzyme and the target sequence for the homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine and 2′-O-modified nucleosides, 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 endonuclease.

approximately 8.7, its ability to participate in triplex formation should not be affected by an increase in the pH range. 8-Oxo-2′-deoxyadenosine has been shown exist in the keto form, with the base in the *syn* conformation.[20,21] Recent NMR results suggest 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.[22] A similar hydrogen bond was recently proposed by Young et al.[12] for the interaction of N^6-methyl-8-oxo-2′-deoxyadenosine with GC base pairs in duplex DNA.

Next, the formation and stability of 8-oxo-2′-deoxyadenosine or 2′-modified uridine-containing triplexes were confirmed by gel retardation assays. As Figure 2 shows, the single-, double-, and triple-stranded 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 all oligonucleotide analogues lacking the complete substitution of 8-oxo-2′-deoxy-adenosine for deoxycytidine. We could not detect any stable triple-helical structures with the 2′-modified nucleoside derivatives.

Table 1. Melting temperatures, T_m (°C), of the triplexes

Oligonucleotide number	pH 6.0	pH 7.0	pH 7.8
1	51.3	31.6	27.9
2	49.1	31.5	28.7
3	40.3	31.8	28.5
4	34.0	31.7	31.8
5	51.7	31.0	26.9
6	47.3	31.1	27.6
7	36.5	26.9	23.6
8	30.1	27.2	26.3
9	42.3	27.0	20.0
10	52.2	31.9	25.1
11	40.8	29.4	21.4
12	30.2	20.5	15.9
13	34.1	23.3	13.9
14	49.0	33.1	27.9

Duplex, 5′AAAAAAGAAGAGAAAGG3′/3′-TTTTTTCTTTCTCT-TTCC5′.

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 (Fig. 1). We have synthesized homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine and/or 2′-modified nucleosides (Uf, Um, Cf, and Cm), 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 reports, this oligonucleotide was shown to bind to the major groove of the duplex DNA, in the homopurine sequence.[22,23]

In order to test the ability of the 17 mer homopyrimidine oligonucleotide analogues containing dAOH, Uf, Um, dAOH-Uf, Uf-Cf, dAOH-Um, and Um-Cm to recognize selectively their *Ksp* 632-I site within the SV40 DNA, the experiments were carried out at 30 °C and at either pH 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 DNA. The oligonucleotides were added to the SV40 DNA before the addition of the restriction enzyme. Figure 3A (lane 3) shows the digestion of the SV40 DNA by *Ksp* 632-I in the presence of the unmodified oligomer (**1**) at a 10 μM concentration. Densitometric analysis of the gels indicated that the inhibition of restriction enzyme cleavage at a 10 μM oligomer concentration was 20%. When the pH of the cleavage reaction was increased to 7.8, no inhibition of the restriction enzyme was observed at a concentration of oligonucleotide (**1**) of 10 μM (lane 3, Fig. 3B). This was also supported by the gel retardation assay (lane 3, Fig. 2). Further evidence for the oligonucleotide binding to the 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 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.

In a subsequent experiment, we incubated the SV40 DNA with *Ksp* 632-I in the presence of the homopyrimidine oligonucleotides containing dAOH, Um, Uf,

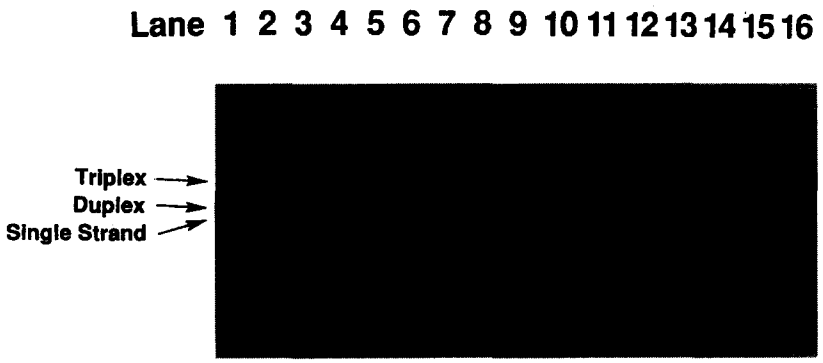


Figure 2. Methylene Blue stained, 15% non-denaturing polyacrylamide gel run in 33 mM Tris–acetate buffer (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$. Lane 1: ODNs; lane 2: duplex DNA; lanes 3–16: oligomers **1–14**.

Cm, and Cf (**2–13**). Gel analysis of the modified oligomers (**2–14**) indicated that they inhibited cleavage by 35% (**2**), 49% (**3**), 52% (**4**), 10% (**5**), 11% (**6**), 15% (**7**), 0% (**8**), 0% (**9**), 8% (**10**), 18% (**11**), 0% (**12**), 0% (**13**), and 20% (**14**) at a 10 μM concentration (lanes 4–16, Fig. 3A). Furthermore, the above experiment was also repeated at pH 7.8, using modified oligomers (**2–13**). The percentages of inhibition of restriction enzyme cleavage at the 10 μM concentration of modified oligomers (**2–13**) were 2, 5, 49, 6, 5, 0, 10, 0, 5, 0, 0, 0, 0, and 0%, respectively (lanes 3–16, Fig. 3B). These results suggest that the oligonucleotide analogues lacking two or three substitutions of 8-oxo-2′-deoxyadenosine provided only weak cleavage protection. In particular, in the case of the substitution of three cytidine residues with 8-oxo-2′-deoxyadenosine, the inhibition of the restriction enzyme was quite high. In contrast to the oligonucleotides with either one or two 8-oxy-2′-deoxyadenosine substitutions, the oligonucleotide (**4**) containing 8-oxo-2′-deoxyadenosine, instead of three cytidine residues, binds in a pH independent manner to the target duplex. However, very weak cleavage protection was observed with the 17 mer homopyrimidine oligonucleotide analogues containing Uf, Um, dAOH-Uf, Uf-Cf, dAOH-Um, and Um-Cm (**2**, **5–14**). These findings suggest that, unlike the unmodified oligonucleotides, the homopyrimidine oligonucleotides containing dAOH can specifically inhibit the DNA–protein interaction via triplex formation, within the physiological pH range. The substitution of three deoxycytidine resides with 8-oxo-2′-deoxyadenosine showed higher endonuclease inhibition than the substitution of either one or two deoxycytidine residues with 8-oxo-2′-deoxyadenosine. In contrast, the oligonucleotides containing 2′-modified nucleosides did not interfere with the endonuclease activity in the physiological pH range.

In conclusion, the recognition of a DNA sequence by a restriction endonuclease can be specifically inhibited by a homopyrimidine oligonucleotide analogue containing 8-oxo-2′-deoxyadenosine under physiological (pH 6.0–7.8) conditions, which allow triple helix formation. However, the homopyrimidine oligonucleotide

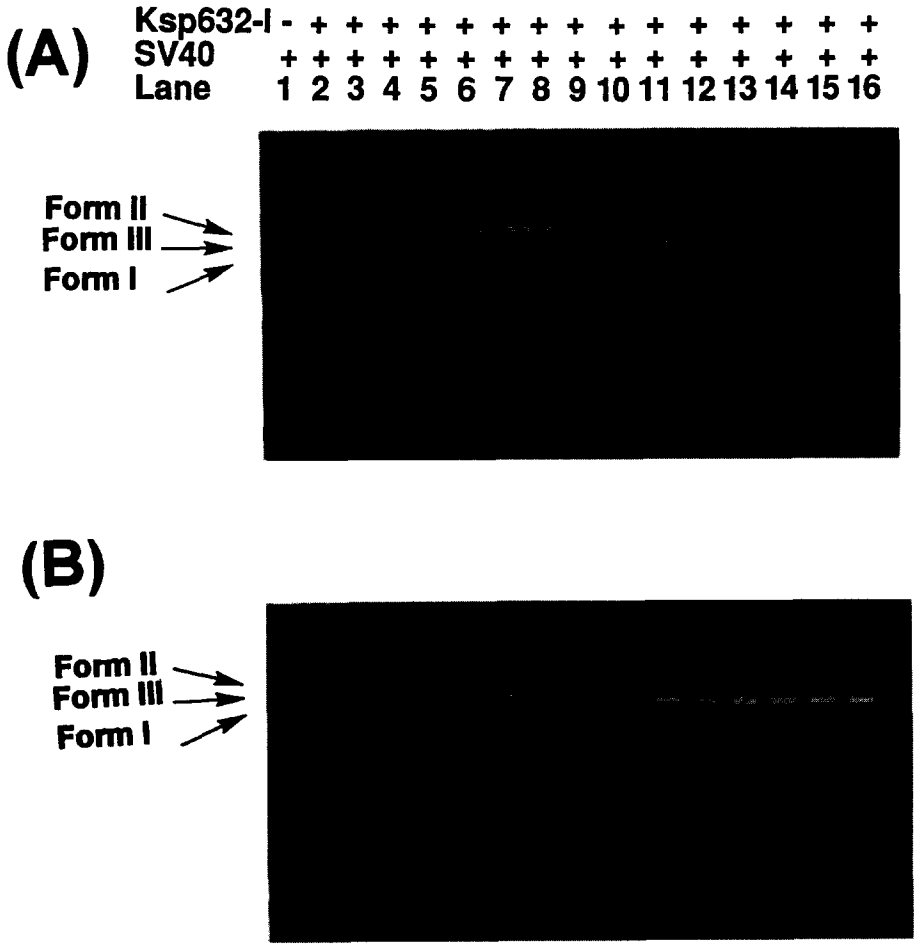


Figure 3. Specific inhibition of the restriction enzyme *Ksp* 632-I by the homopyrimidine oligonucleotides containing 8-oxo-2′-deoxyadenosine. The enzymatic assay was performed by incubating SV40 DNA (6 nM) at 30 °C for 1 h with *Ksp* 632-I (20 units/μL) and 10 μM of oligonucleotide in a buffer containing 33 mM Tris–acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM spermine, and 1 μg/μL tRNA. Form I: supercoiled DNA; form II: open circular DNA; form III: linear DNA. (A) The enzymatic assay was carried out in the presence of the unmodified (**1**) and modified (**2–14**) oligonucleotides at pH 7.0. Lane 1: SV40 DNA incubated without restriction enzyme and oligonucleotides. Lane 2: SV40 DNA incubated with restriction enzyme. (B) The enzymatic assay was carried out in the presence of the unmodified (**1**) and modified (**2–14**) oligonucleotides at pH 7.8. Lane 1: SV40 DNA incubated without restriction enzyme and oligonucleotides. Lane 2: SV40 DNA incubated with restriction enzyme. Lanes 3–16: oligomers **1–14**.

analogue containing 2′-modified nucleosides did not interfere with the endonuclease activity at the physiological pH value of 7.8. Specific binding of homopyrimidine oligonucleotide analogues containing 8-oxo-2′-deoxyadenosine 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. This stabilization should help us to design much more efficient transcription and replication inhibitors, which could be used for modulating gene expression, both in vitro and in vivo (chemotherapy).

Experimental

Oligonucleotide synthesis

The oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 392 DNA/RNA synthesizer on the 1 μM scale, and with controlled pore glass supports. 2′-Methoxyuridine and 2′-methoxycytidine were purchased from Yamasa Co. Phosphoramidite units were prepared from the reaction of 5′-dimethoxytrityl-*N*-protected-2′-deoxyribonucleosides, 8-methoxy-2′-deoxyadenosine,[24] 2′-methoxyuridine, 2′-methoxycytidine, 2′-fluorouridine,[25] and 2′-fluorocytidine,[25] with 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophos-phoramidite 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 compositions were determined after snake venom phosphodiesterase/bacterial alkaline phosphatase hydrolyses.

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 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.

Gel electrophoresis

The duplex DNA was made by combining 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 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 each strand was 1.23×10^{-3} μM in a total vol. 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 (30 °C) and 200 V for 16 h. The gel was stained with Methylene Blue.

Inhibition of restriction endonuclease digestion at Ksp 632-I sites in SV40 DNA

Enzymatic assays were performed in a buffer containing 33 mM Tris–acetate, 100 mM NaCl, 66 mM potassium acetate (pH 7.0, and 7.8), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM spermine, and 1 μg/μL tRNA at 30 °C.[26] The pH of the incubation medium was changed as indicated in the text and legend of the figure. The concentration of SV40 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 analysed by electrophoresis on slab gels (0.8% agarose). Densitometric analysis of gels stained with ethidium bromide was performed on a Millipore Bio Image 60S.

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