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The SV40 large T-antigen helicase can unwind four stranded DNA structures linked by G-quartets

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ABSTRACT

We describe a novel activity of the SV40 large T-ag helicase, the unwinding of four stranded DNA structures linked by stacked G-quartets, namely stacked groups of four guanine bases bound by Hoogsteen hydrogen bonds. The structures unwound by the helicase were of two types: (i) quadruplexes comprising four parallel strands that were generated by annealing oligonucleotides including clustered G residues in a buffer containing Na^+ ions. Each parallel quadruplex consisted of four oligonucleotide molecules. (ii) Complexes comprising two parallel and two antiparallel strands that were generated by annealing the above oligonucleotides in a buffer containing K^+ ions. Each antiparallel complex consisted of two folded oligonucleotide molecules. Unwinding of these unusual DNA structures by the T-ag was monitored by gel electrophoresis. The unwinding process required ATP and at least one single stranded 3′-tail extending beyond the four stranded region. These data indicated that the T-ag first binds the 3′-tail and moves in a 3′→5′ direction, using energy provided by ATP hydrolysis; then it unwinds the four stranded DNA into single strands. This helicase activity may affect processes such as recombination and telomere extension, in which four stranded DNA could play a role.

INTRODUCTION

Short DNA sequence repeats, in which one strand consists of clusters of 3–8 guanine residues interspersed with other short sequences, are found in eukaryotic genomes at various chromosomal sites, including telomeres (1,2), immunoglobulin switch regions (3) and promoters (4,5). It has been shown that single stranded DNA sequences containing such repeats can form unusual DNA structures stabilized by interactions between the guanine bases (6–8). Of these structures, four stranded DNA complexes have been studied most extensively. In such complexes, groups of four guanine bases, one from each strand, are bound by Hoogsteen hydrogen bonds, thereby forming stacked G-quartets that hold the chains together (9–17) (for a review see ref. 18).

Four stranded DNA structures are formed *in vitro* in buffers containing physiological concentrations of Na^+ and K^+ ions at neutral pH. Therefore, it is conceivable that the same structures may occur in living cells and affect essential cellular processes, such as recombination and extension of telomeric sequences by the enzyme telomerase (19,20). In recombination, formation of four stranded DNA may account for initial chromosome pairing, as previously suggested (10,11). At telomeres, formation of four stranded DNA structures could inhibit the telomerase, as shown by *in vitro* assays of the enzyme (21). In both cases, the cells would be expected to possess a mechanism for resolving the four stranded DNA into single strands. This task could be accomplished by enzymes which specifically recognize the unusual four stranded DNA and cause separation of the strands. However, recent studies have indicated that helicases, which normally unwind DNA duplexes, can also unwind DNA triple helices (22,23). These results have led us to examine the possibility that helicases might be capable of unwinding four stranded DNA.

Here we report that the SV40 large tumour antigen (T-Ag), a *bona fide* helicase (24–26), can unwind four stranded DNA structures. The biochemical and biological implications of this finding are further discussed below.

MATERIALS AND METHODS

Preparation of parallel DNA quadruplexes

Oligonucleotides (purchased from Biotechnology General, Israel) were purified by polyacrylamide gel electrophoresis and were 5′-end labeled with ^{32}P, using the T4 polynucleotide kinase (27). One labeled oligonucleotide, or a mixture of two oligonucleotides, were dissolved in 10 µl of a buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA (TE buffer) at a concentration of 50 nmol/ml. The solution was heated 5 min at 95°C and fast cooled to 4°C. At this stage, NaCl was added to a final concentration of 0.50 M and the solution was annealed 40–72 h at 60°C, unless a different temperature was specified. The annealing was performed in an 1.5 ml Eppendorf tube without covering the solution with paraffin oil. This procedure was found to give a high yield of four stranded structures. The mixture was then diluted 5-fold into TE buffer and stored at –20°C until further use. Formation of DNA quadruplexes was monitored by polyacrylamide gel electrophoresis, as described in the section on helicase assays.

Preparation of four stranded DNA structures consisting of two folded chains

In this procedure, ^{32}P-labeled oligonucleotides were first heated 5 min at 95°C and fast cooled to 4°C. Then, the oligonucleotides were annealed, as described in the previous section, except that the annealing solution contained 0.30 M Kcl, instead of 0.50 M NaCl.

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Helicase assays

Helicase assays were carried out in 20 µl of a solution containing 10 mM Tris–HCl pH 8.0, 10 mM NaCl, or 10 mM KCl, 10 mM $MgCl_2$, 0.50 mM 1,4-dithiothreitol (DTT), 50 µg/µl bovine serum albumin, 2 mM ATP, 0.10 mM EDTA, 10% glycerol, 1.0 pmol/ml DNA substrate and 60 pmol/ml hexameric recombinant SV40 large T-ag prepared in insect cells (28). The reactions were performed at 37°C for 45 min, unless different reaction times were specified, and were terminated, as described (28). The mixtures were electrophoresed in 12% polyacrylamide gels in a buffer containing 45 mM Trizma base, 1.2 mM Na_2EDTA, 45 mM boric acid. The gels were dried on DE81 paper (Whatman) and autoradiographed. The electrophoresis was carried out either at 23°C, or at 4°C, as specified in the figure legends.

Footprinting assays

^{32}P-labeled oligonucleotides were annealed in TE buffer containing 0.50 M NaCl, or 0.30 mM KCl, as described above. Other samples of the same oligonucleotides were dissolved in 0.10 M NaOH, heated 5 min at 95°C and fast cooled to 4°C. These samples were neutralized by addition of HCl and Tris–HCl pH 8.0 to final concentrations of 0.10 M. Methylation by dimethyl sulfate (DMS) was carried out 20 min at 22°C in a solution containing 10 mM Tris–HCl pH 8.0, 10 mM NaCl, or 10 mM KCl, 10 mM $MgCl_2$, 0.50 mM 1,4-dithiothreitol (DTT), 2 mM ATP, 100 pmol/ml DNA and 0.10% DMS. The reaction was terminated, as described (23), and the samples were electrophoresed in a 12% polyacrylamide gel. Bands containing the single stranded oligonucleotides and the two types of four stranded complexes were excised and incubated 16 h at 37°C in TE buffer containing 0.10 M NaCl to elute the radioactively labeled DNA molecules. The DNA was next precipitated in ethanol, dried, dissolved in 1.0 M piperidine and heated 30 min at 90°C. The samples were electrophoresed in a 12% Long Ranger sequencing gel, as previously described (23).

RESULTS

Formation of parallel DNA quadruplexes by association of oligonucleotides containing clusters of G residues

It has been previously found that oligonucleotides containing clusters of G residues, which are annealed in buffers containing Na^+ ions, form DNA complexes consisting of four parallel strands (10,17). Figure 1a shows examples of such quadruplexes which are generated by self-association of identical molecules (X_4, Y_4 and Z_4), or by association of two different molecules (XY_3). The single strands in these quadruplexes are held together by two groups of four and five stacked G-quartets, which are indicated in these drawings by squares. A detailed drawing of a G-quartet is illustrated in Figure 1c; it consists of four guanine bases—one per DNA chain—which are linked by Hoogsteen hydrogen bonds.

Table 1 presents six oligonucleotides containing G clusters (oligonucleotides A–F), which were employed for the present study. As noted in the caption to the table, these oligonucleotides correspond to sequences found in genomic DNAs. Figure 2 shows an experiment based on a previously reported approach (14), which confirmed that in the presence of Na^+ ions oligonucleotides containing G clusters form quadruplexes. The experiment was

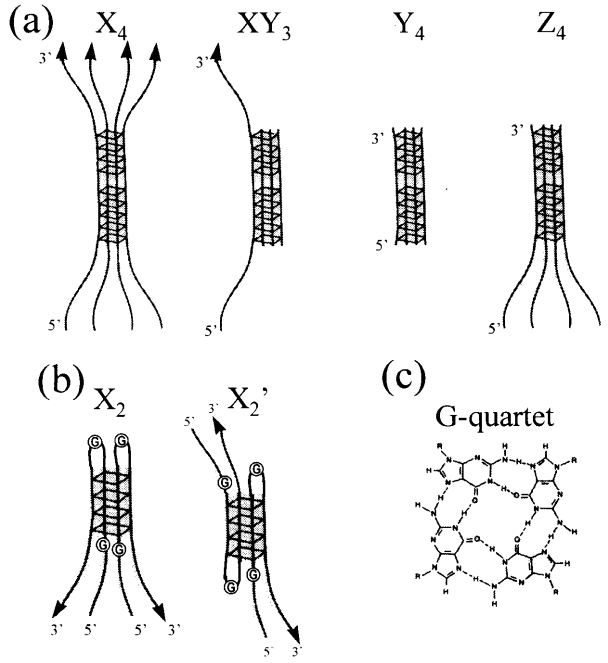

Figure 1. Illustrations of four stranded DNA structures. (**a**) Parallel quadruplexes. A detailed explanation of these drawings is presented in the Results. The squares denote G-quartets. (**b**) Four stranded DNA complexes formed by association of two identical folded chains. The complexes illustrated here are two of several possible isomers (16,18). The indicated G residues correspond to G17 and G23 in oligonucleotide D, which are underlined in Table 1 and are also indicated in the footprint shown in Figure 3b. (**c**) G-quartet.

carried out with oligonucleotides A and B, that differ in their lengths. The two oligonucleotides, which contain sequences derived from an immunoglobulin switch region, were radioactively labeled with ^{32}P and annealed in a buffer containing Na^+ ions. The annealed molecules were electrophoresed in a polyacrylamide gel that was subsequently autoradiographed. Lane a shows that self-annealing of oligonucleotide A generated a high molecular weight complex designated A_4 and some complexes having slower mobilities. Lane g shows a sample of the annealed oligonucleotide A molecules that was heated 5 min at 95°C to dissociate the A_4 complexes into single stranded A molecules. Lanes e and f show corresponding gel profiles of self-annealed and heated oligonucleotide B respectively. It can be seen that a high molecular weight complex designated B_4 was generated by self-annealing oligonucleotide B. In the assays shown in lanes b, c and d, oligonucleotides A and B were mixed at molar ratios of 4:1, 1:1 and 1:4 respectively, and were annealed under the same conditions. It can be seen that three additional complexes designated AB_3, A_2B_2 and A_3B were generated in these mixtures. The number of these complexes, their intermediate mobilities relative to those of the complexes A_4 and B_4 and their relative abundance are consistent with the notion that these complexes are DNA quadruplexes consisting of both oligonucleotides at the ratios indicated by the subscripts. This conclusion also implies that the complexes A_4 and B_4 are quadruplexes consisting of four molecules of oligonucleotides A and B respectively. The larger complexes seen in lane a could represent aggregates of more than four oligonucleotide molecules (18).

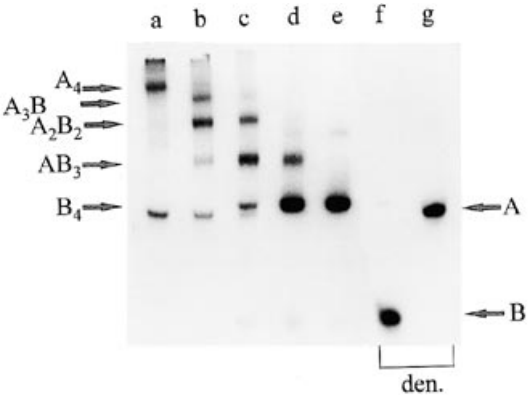

Figure 2. Formation of parallel DNA quadruplexes. Oligonucleotides A and B, whose sequences are shown in Table 1, were radioactively labeled with ^{32}P. Either or both oligonucleotides were annealed at the concentrations indicated below in a buffer containing Na^+ ions, as described in Materials and Methods. The annealed samples were then electrophoresed at 23°C and autoradiographed. Lane a, 50 nmol/ml oligonucleotide A; lane b, 40 nmol/ml oligonucleotide A and 10 nmol/ml oligonucleotide B; lane c, 25 nmol/ml oligonucleotide A and 25 nmol/ml oligonucleotide B; lane d, 10 nmol/ml oligonucleotide A and 40 nmol/ml oligonucleotide B. Lane e, 50 nmol/ml oligonucleotide B. In lanes f and g, which are marked den., the annealed oligonucleotides B and A respectively were heated 5 min at 95°C and fast cooled to 4°C, before being electrophoresed.

Table 1. Oligonucleotides[a]

A	33mer[b]	5′-GGTAC**GGGGG**AGCT**GGGG**TAGAT**GGG**AATGTTA-3′
B	15mer[c]	5′-C**GGGGG**AGCT**GGGG**T-3′
C	33mer[d]	5′-TAGAT**GGG**AATGTTAGGTAC**GGGGG**AGCT**GGGG**-3′
D	44mer[e]	5′-CAGGCTGAGCAGGTAC**G**GGGGAGCT**GGGG**TAGATTGGAATGTAG-3′
E	25mer[f]	5′-GGAGGT**GGG**T**GGGG**CGAGCAGAGTT-3′
F	24mer[g]	5′-TT**GGGG**TT**GGGG**CTACGCGATCAT-3′

[a]These oligonucleotides contain G clusters indicated by bold letters.
[b]Oligonucleotide A contains a sequence derived from the mouse immunoglobulin switch region (3).
[c]Oligonucleotide B represents a part of oligonucleotide A.
[d]In oligonucleotide C the 3′-terminal 15 nt sequence of oligonucleotide A was swapped to the 5′-end.
[e]Oligonucleotide D also contains a sequence derived from the immunoglobulin switch region. The underlined G residues no. 17 and no. 23 are indicated in Figure 3.
[f]Oligonucleotide E contains a human sequence flanking a chromosome truncation site associated with α-thalassaemia, to which a telomeric sequence has been added in the cells. It can also serve as a primer for the human telomerase *in vitro* (45).
[g]Oligonucleotide F contains a *Tetrahymena* telomeric repeat sequence to which a bacterial plasmid pBR322 sequence has been attached at the 3′-terminus. It can serve as a primer for the *Tetrahymena* telomerase *in vitro* (46).

Formation of quadruplexes was expected to protect the guanine bases which form G-quartets against methylation by dimethyl sulfate (DMS); for as shown in Figure 1c, the N7 atom of each of these bases, the target for the methylation, forms a Hoogsteen hydrogen bond with the NH2 group of another guanine in the quartet. Hence, DMS footprinting was used for a further analysis of these structures. Figure 3a shows footprinting assays of complexes generated from oligonucleotide D, which includes clusters of four and five G residues, like oligonucleotides A and B (Table 1). Lane a displays the pattern of methylation of the single stranded oligonucleotide D. Lane b shows the pattern of methylation

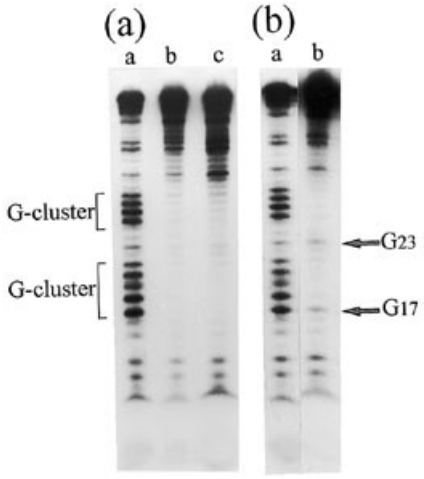

Figure 3. Footprinting of four stranded DNA structures. (**a**) Dimethyl sulfate footprinting of a four stranded DNA structure formed in the presence of Na^+ ions. Lane a, a footprint of single stranded oligonucleotide D that has been exposed to DMS; lane b, a footprint of oligonucleotide D that has been annealed in a buffer containing Na^+ ions and then exposed to DMS in a solution containing 10 mM NaCl; lane c: a footprint of oligonucleotide D that has been annealed in a buffer containing Na^+ ions and then was transferred into a solution containing 10 mM KCl and 1 mM NaCl and exposed to DMS. Details of the above procedures are described in Materials and Methods. (**b**) Dimethyl sulfate footprinting of a four stranded DNA structure formed in the presence of K^+ ions. Lane a, a footprint of single stranded oligonucleotide D; lane b, a footprint of oligonucleotide D that has been annealed in a buffer containing K^+ ions and exposed to DMS in a solution containing 10 mM KCl and 1 mM NaCl.

of D_4 quadruplexes generated by annealing oligonucleotide D molecules in a buffer containing Na^+ ions. It can be seen that the G residues in the four and five G clusters were protected against methylation. The complete protection of all the guanines in these G clusters indicates that the annealing generated D_4 quadruplexes consisting of parallel strands; for antiparallel arrangement should not yield full protection of all guanines in the five G cluster. Lane c presents the footprint of a sample of oligonucleotide D that was first annealed in a buffer containing Na^+ ions and was then transferred into a buffer containing K^+ ions. It can be seen that the pattern of methylation protection remained the same as in lane b. Similar footprints were also obtained in DMS methylation assays of A_4 quadruplexes (not shown).

Unwinding of parallel DNA quadruplexes by the SV40 large T-ag helicase

Figure 4A shows a polyacrylamide gel analysis of A_4 DNA quadruplexes and of complexes having slower electrophoretic mobilities, that were incubated at 37°C for various time periods in the presence of ATP and the SV40 large T-ag helicase. This incubation was performed in a buffer containing Na^+ ions. It can be seen that the T-ag helicase unwound the quadruplexes, as well as the slower migrating complexes, into monomeric oligonucleotide A molecules in a time dependent reaction. The reaction proceeded in a linear fashion up to 15 min and then it slowed down. In the absence of the T-ag, these complexes remained stable. It should be noted that in these assays, as in the other unwinding assays reported in this paper, the ratio T-ag/DNA substrates was 60:1. However, more recent studies have shown that the T-ag was

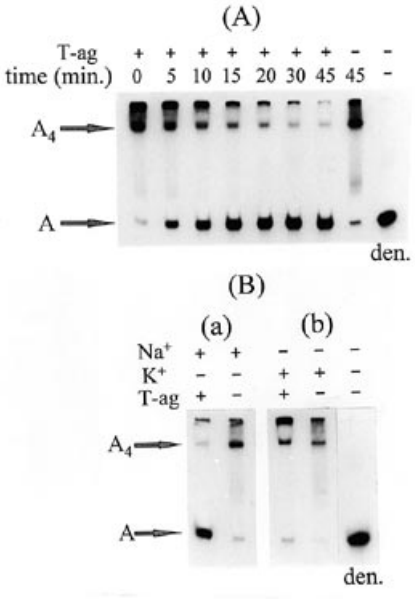

Figure 4. Unwinding of parallel DNA quadruplexes by the SV40 large T-ag helicase. (**A**) Time course of unwinding A_4 quadruplexes. Parallel DNA quadruplexes generated from oligonucleotide A (A_4 quadruplexes) were prepared and incubated at 37°C in the presence of the SV40 large T-ag for the indicated time periods, as described in Materials and Methods. The reaction mixtures were subsequently electrophoresed in a 12% polyacrylamide gel at 23°C and autoradiographed. This gel also displays a sample of the A_4 quadruplex that has been incubated 45 min at 37°C in the absence of the T-ag. den., A_4 quadruplexes were heated, as described in the legend to Figure 2. (**B**) Inhibition of quadruplex unwinding by replacement of Na^+ ions with K^+ ions. These reactions were carried out 45 min at 37°C, as described in (A), with buffers containing either 10 mM NaCl, or 10 mM KCl.

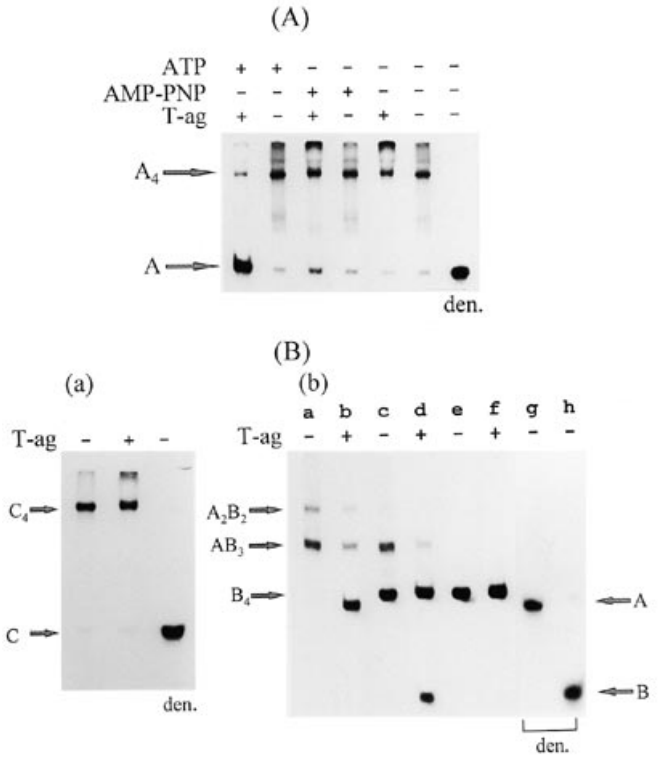

Figure 5. Dependence of the quadruplex unwinding reaction on the presence of ATP and a single stranded 3′-tail. (**A**) Dependence on ATP. The helicase reactions were carried out for 45 min. ATP, or AMP-PNP, were added at 2 mM, as indicated. Polyacrylamide gel electrophoresis was carried out at 23°C. den., A_4 quadruplexes were heated, as described in the legend to Figure 2. (**B**) Dependence on the presence of a 3′-tail. (a) SV40 large T-ag helicase unwinding assays of the parallel quadruplex C_4. The assays were carried out 45 min, as described in Materials and Methods. den., C_4 quadruplexes were heated, as described in the legend to Figure 2. (b) Lanes a and b, oligonucleotide A was radioactively labeled with ^{32}P and was mixed with unlabeled oligonucleotide B at a molar ratio of 4:1. Annealing, the helicase reaction and gel electrophoresis were carried out as described in Materials and Methods. Lanes c and d, same as lanes a and b, except that oligonucleotide B was radioactively labeled and oligonucleotide A was unlabeled. Lanes e and f, helicase assays carried out with ^{32}P-labeled oligonucleotide B alone. Lanes g and h, samples of oligonucleotides A and B respectively that were heated, as described in the legend to Figure 2. The various quadruplexes are denoted by the symbols used in Figure 2.

capable of efficiently unwinding quadruplexes even when the ratio T-ag/DNA substrate was 3:1 (Baran *et al.*, unpublished results).

Figure 4B shows an experiment in which A_4 DNA quadruplexes and complexes having slower mobilities were incubated 45 min at 37°C in buffers containing either Na^+ ions [panel (a)], or K^+ ions [panel (b)], with ATP and with, or without, the T-ag. It can be seen again that the T-ag efficiently unwound the parallel A_4 quadruplexes in the presence of Na^+ ions. However, replacement of the Na^+ ions with K^+ ions almost completely inhibited the unwinding. This result will be further addressed in the Discussion. It should be noted that all the experiments reported below on unwinding of parallel quadruplexes by the T-ag were performed in buffers containing Na^+ ions.

ATP and a 3′-tail are required for unwinding parallel quadruplexes by the SV40 large T-ag helicase

To find out whether unwinding of parallel quadruplexes was caused by T-ag helicase activity and not by a different mechanism, we sought to determine whether certain parameters of this reaction were compatible with a helicase mechanism. Helicases, including the SV40 large T-ag helicase, require ATP, or other hydrolysable nucleoside triphosphates, for unwinding duplex and triplex DNAs (23,24,29–33). Therefore, we tested the requirement for ATP as an energy source for unwinding quadruplexes. Figure 5A shows an experiment in which the A_4 quadruplexes were incubated with the SV40 large T-ag helicase in the absence, or the presence of ATP, or in the presence of the non-hydrolysable ATP analogue AMP-PNP. It can be seen that whereas unwinding of these complexes proceeded very efficiently in the presence of ATP, no monomer oligonucleotide was released from the complexes in the absence of ATP. Furthermore, in the presence of AMP-PNP, the helicase unwinding reaction was very inefficient. These data showed that ATP hydrolysis was required for unwinding the quadruplexes. Unless otherwise specified, all the helicase assays reported below were carried out in the presence of ATP.

Unwinding of duplex and triplex DNA structures by the T-ag helicase requires that these structures would be flanked by single stranded DNA on the 3′ side (23,24,26,33). Figure 5B presents experiments designed to determine whether there is a similar requirement for unwinding of parallel quadruplexes by the T-ag helicase. A C_4 quadruplex was generated from oligonucleotide C (Table 1). This complex contains four single stranded 5′-tails, but no single stranded 3′-tails, like the Z_4 complex illustrated in Figure 1a. As Figure 5B panel (a) shows, the quadruplex C_4 was not unwound by the T-ag helicase under the conditions in which the quadruplex A_4 was found to be unwound. Moreover,

complexes having slower electrophoretic mobilities than that of the C_4 quadruplex were generated in the presence of the T-ag. These complexes might consist of T-ag bound to single stranded 5′-tails present in these structures. We infer from these data that in the absence of single stranded 3′-tails, the T-ag is unable to unwind quadruplexes, or other slower migrating complexes.

Figure 5B panel (b) presents additional data supporting this conclusion. In the assays shown in lanes a–d, mixtures of oligonucleotide A and oligonucleotide B at a molar ratio of 1:4 were annealed as described, and were subsequently incubated in the absence or in the presence of T-ag. For the assays shown in lanes a and b only oligonucleotide A was radioactively labeled with ^{32}P. Two mixed quadruplexes were observed in lane a: (i) AB_3, which contains one 3′-tail and one 5′-tail, and corresponds to XY_3 in Figure 1a. (ii) A_2B_2, which contains two single stranded 3′-tails and two single stranded 5′-tails. As shown in lane b, both quadruplexes were unwound by the T-ag. For the corresponding assays shown in lanes c and d, only oligonucleotide B was radioactively labeled with ^{32}P. Lane c displays the mixed quadruplex AB_3, as well as the quadruplex B_4 which corresponds to Y_4 in Figure 1a. A comparison of lanes c and d shows that whereas the AB_3 molecules were unwound by the T-antigen, the B_4 molecules found in the same reaction mixture were not unwound by the enzyme. For the assays shown in lanes e and f, ^{32}P-labeled oligonucleotide B was self-annealed, such that B_4 quadruplexes were generated. These assays confirmed that the T-ag did not unwind the B_4 quadruplexes. Based on these data, we conclude that at least one single stranded 3′-tail must be attached to parallel quadruplexes, for these structures to be unwound by the SV40 large T-ag helicase.

The SV40 large T-ag can unwind parallel quadruplexes generated from various types of oligonucleotides containing G clusters. Figure 6 shows T-ag unwinding assays of parallel quadruplexes generated from oligonucleotides D, E and F, which contain telomeric sequences and sequences derived from immunoglobulin switch regions (Table 1). It can be seen that the T-ag efficiently unwound the quadruplexes D_4, E_4 and F_4. Similar assays have revealed that the T-ag also unwound parallel quadruplexes generated by self-annealing oligonucleotides containing mammalian telomeric repeat sequences (not shown).

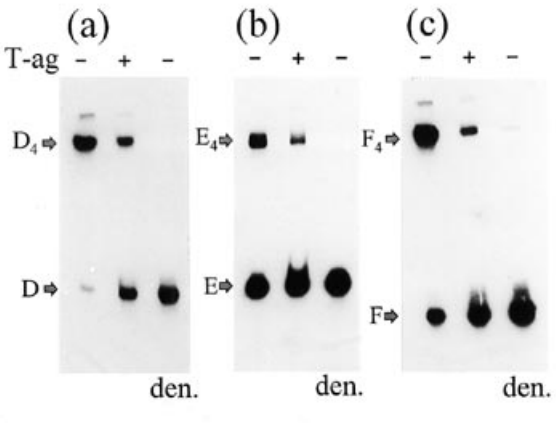

Figure 6. Unwinding of the parallel quadruplexes D_4, E_4 and F_4 by the SV40 large T-ag helicase. D_4 and F_4 quadruplexes were prepared by annealing the oligonucleotides D and F, as described in Materials and Methods. E_4 quadruplexes were prepared by annealing the oligonucleotide E 72 h at 50°C. The unwinding assays were performed, as described in the legend to Figure 5. Polyacrylamide gel electrophoresis was carried out at 4°C. The monomeric oligonucleotides and the corresponding quadruplexes are indicated. den., samples of the D_4, E_4 and F_4 quadruplexes that were heated, as described in the legend to Figure 2.

Formation of antiparallel four stranded DNA structures generated by association of two folded oligonucleotide molecules

It has been found that, in addition to parallel quadruplexes, oligonucleotides containing G clusters can also form four stranded DNA structures consisting of two parallel and two antiparallel strands. Structures of this type are generated when the oligonucleotides are annealed in buffers containing K^+ ions, instead of Na^+ ions (11,12,14–16). Figure 1b shows two examples of such complexes designated X_2 and X_2'. Like the parallel quadruplexes shown in Figure 1a, X_2 and X_2' complexes are also held together through formation of G-quartets. It should be noted that X_2 and X_2' represent just two of a larger number of fold-back antiparallel isomers (16,18) (see Discussion).

Figure 7 shows that self-annealing of the oligonucleotides A, D and F in a buffer containing K^+ ions generated complexes designated A_2, D_2 and F_2. Figure 3b presents a DMS footprint of the D_2 complex. In this footprint, eight G residues in the two clusters of Gs were fully protected against DMS methylation. The fifth G residue in the five G cluster, designated as G17 and underlined in Table 1, was not fully protected. In addition, the G residue designated as G23 and also underlined in Table 1, was unprotected. This pattern of protection differs from that of the parallel quadruplex generated by self-annealing oligonucleotide D in a buffer containing Na^+ ions, which is shown in Figure 3a; in that footprint, all nine G residues in the two clusters, as well as the residue No. G23 were protected. The pattern of protection shown in Figure 3b is consistent with the notion that pairs of oligonucleotide D molecules formed complexes such as those illustrated in Figure 1b, in which the encircled Gs correspond to the residues G17 and G23 described above. The notion that D_2 complexes, as well as the complexes A_2 and F_2, consisted of just two oligonucleotide molecules was supported by the observation that these complexes had faster electrophoretic mobilities than the corresponding parallel quadruplexes D_4, A_4 and F_4 (compare Figs 6 and 7). However, these experiments could not establish which of the various possible antiparallel four stranded isomers were obtained in the present studies.

Unwinding of antiparallel four stranded DNA structures by the SV40 large T-ag helicase

Figure 7 shows that the antiparallel four stranded complexes A_2, D_2 and F_2 were efficiently unwound by the T-ag helicase. It should be noted that these experiments and the subsequent experiments on unwinding of antiparallel complexes were all performed in a buffer containing K^+ ions. Figure 8A shows that ATP hydrolysis was required for unwinding the A_2 complexes. Figure 8B shows that the antiparallel complexes C_2 and B_2, which do not contain 3′-tails, were not unwound by the T-ag helicase under conditions in which the complexes A_2, D_2 and F_2 were unwound by the enzyme. Thus, ATP and a 3′-tail were required for unwinding the antiparallel, as well as the parallel four stranded structures.

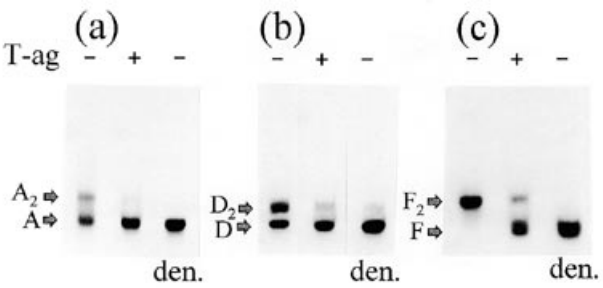

Figure 7. SV40 large T-ag helicase assays of antiparallel four stranded DNA structures formed in the presence of K^+ ions by association of two folded oligonucleotide molecules. The four stranded DNA structures designated A_2 and D_2, which consist of two folded molecules of the oligonucleotides A and D respectively, were prepared by annealing these molecules in a buffer containing K^+ ions. F_2 structures were prepared by annealing oligonucleotide F 40 h at 45°C in the same buffer. Helicase assays of these complexes were also performed in a buffer containing K^+ ions, as described in Materials and Methods. Polyacrylamide gel electrophoresis was carried out at 4°C. den., A_2, D_2 and F_2 structures that were heated, as described in the legend to Figure 2.

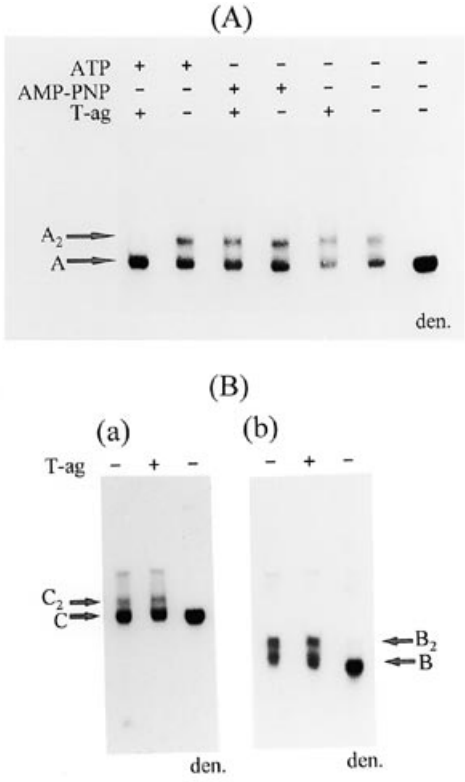

Figure 8. Unwinding of antiparallel four stranded DNA structures by the SV40 large T-ag helicase requires ATP and a single stranded 3′-tail. (**A**) Requirement for ATP. The helicase reactions were carried out for 45 min, as described in the legend to Figure 7. ATP, or AMP-PNP, were added at 2 mM, as indicated. Polyacrylamide gel electrophoresis was carried out at 4°C. den., A_2 structures were heated, as described in the legend to Figure 2. (**B**) Dependence of the unwinding reaction on 3′-tails. The antiparallel four stranded DNA structures C_2 and B_2, which consist of two folded molecules of the oligonucleotides C and B respectively, were prepared by annealing these molecules in a buffer containing K^+ ions, as described in Materials and Methods. (a) The unwinding assays of the antiparallel quadruplex C_2 were carried out, as described in the legend to Figure 7. den., C_2 complexes were heated, as described in the legend to Figure 2. (b) The unwinding assays of the antiparallel quadruplex B_2 were also carried out as described in the legend to Figure 7. den., B_2 complexes were heated, as described in the legend to Figure 2.

DISCUSSION

The experiments reported in this article have shown that the SV40 large T-ag helicase can unwind parallel DNA quadruplexes and four stranded complexes containing two parallel and two antiparallel strands. The unwinding process requires ATP hydrolysis and at least one single stranded 3′-tail. Based on these data, we suggest that the unwinding proceeds as follows: first, the T-ag binds a single stranded 3′-tail. Next, the enzyme migrates in a 3′→5′ direction along the single stranded DNA, using the energy provided by ATP hydrolysis, until it encounters the G-quartets; then, it causes disruption of these quartets.

The parallel quadruplexes of the types shown in Figure 1a were generated from oligonucleotides including clustered G residues in a buffer containing Na^+ ions, and were unwound by the T-ag helicase in buffers containing Na^+ ions (Figs 4A, 5 and 6). However, as shown in Figure 4B, quadruplexes formed in a buffer containing Na^+ ions and then transferred into a buffer containing K^+ ions could not be efficiently unwound by the helicase. This result was not due to a major structural transition induced by the K^+ ions, since the DMS footprint of the quadruplexes was not altered by the ion replacement, as shown in Figure 3a. It could be caused by the selective stability imparted on these structures by K^+ ions binding to the O6 atoms in the cavities formed between pairs of stacked G-quartets (11,14).

The antiparallel four stranded structures (Fig. 1b), were generated by annealing the oligonucleotides containing the clustered G residues in a buffer containing K^+ ions. As Figures 7 and 8 show, these structures were efficiently unwound by the T-ag in a buffer containing K^+ ions. Apparently, these complexes were not stabilized by the K^+ ions to the same extent as the parallel quadruplexes. It should be noted that the isomer X_2' shown in Figure 1b was found by X ray analysis to occur in crystals formed in the presence of K^+ ions (15). However, a different isomer was found by NMR spectroscopy to occur in solution (16). As already pointed out in the Results, these isomers represent just two of a larger number of possible isomers. The footprints shown in Figure 3b could not reveal which isomer was generated by our annealing procedure. Thus, the question whether the T-ag can unwind all possible fold-back antiparallel four stranded isomers formed in the presence of K^+ ions, or just a subset of these isomers, remains open. It should also be noted that the intracellular concentration of K^+ ions is considerably higher than that of Na^+ ions (34). Hence, the unwinding of the structures generated in buffers containing K^+ ions might be physiologically more relevant.

In a recent study we have found that the SV40 large T-ag helicase was capable of unwinding the third polypyrimidine strand of polypyrimidine·polypurine·polypyrimidine DNA triple helices, which is linked to the polypurine strand by Hoogsteen hydrogen bonds (23). However, the data obtained in that study have not completely ruled out the possibility that the third strand and the polypyrimidine strand in the duplex were transiently exchanged, such that D-loop containing molecules were formed at a very low concentration and served as intermediates in the unwinding reaction. In this case, the helicase could have released the third strand by unwinding the duplex in these hypothetical D-loop intermediates. Unlike the triplexes, the four stranded DNA structures, in which the strands are solely linked by

Hoogsteen hydrogen bonds, could not possibly undergo a transition into normal duplex DNA. Hence, the data shown in the present article constitute a more conclusive demonstration that a helicase is capable of dissociating unusual DNA structures through unwinding DNA strands which are linked by hydrogen bonds other than those of the Watson–Crick type.

One way in which the T-ag helicase might unwind four stranded DNA structures is through a 'passive' creeping mechanism which is analogous to a mechanism previously proposed for unwinding DNA duplexes and triplexes by helicases (23,31,33). In this process, the Hoogsteen hydrogen bonds linking one of the G residues in the 3′-terminal quartet to the other Gs might be transiently opened. Then, the released G residue could be trapped by the helicase. The hydrogen bonds between the remaining Gs of the quartet could then be destabilized and spontaneously opened. Subsequently, the helicase might translocate, using the energy derived from ATP hydrolysis, and similarly disrupt the next G-quartet. Other 'active' mechanisms of unwinding four stranded DNA would require ATP hydrolysis not just for translocation along the single stranded DNA, but also for the unwinding process itself (31). Such mechanisms would also require binding of the enzyme to the four stranded DNA, for which there is no evidence. Clearly, more detailed studies are needed to unravel the mechanism by which the T-ag unwinds four stranded DNA.

Although biological roles for four stranded DNA structures have not been conclusively demonstrated, several studies appear compatible with functions suggested for these structures, such as regulation of telomere synthesis (20,21) and pairing of chromosomes in recombination (10,35). First, proteins which selectively bind four stranded DNA structures have been reported (36,37). Secondly, a subunit of a protein which specifically binds single strand extensions in telomeres was found to stimulate formation of four stranded DNA (38). Thirdly, the enzyme telomerase was found to be inhibited *in vitro* by formation of four stranded DNA structures (20,21). Fourthly, a yeast endonuclease that specifically cuts single stranded DNA next to four stranded structures has been reported (39). Furthermore, genetic evidence has been provided indicating that this endonuclease may affect telomere metabolism (40,41). As already pointed out, four stranded DNA structures are readily generated under physiological conditions. However, once formed, these structures are very stable and do not spontaneously dissociate (18). Nevertheless, such dissociation would presumably be required if four stranded DNA were formed in the course of telomere synthesis and during recombination processes. Our finding that the SV40 large T-ag helicase, a *bona fide* helicase, can unwind four stranded DNA structures, suggests that helicases may provide an enzymatic mechanism for dissociating these structures. It should be noted that helicases were found to be involved in both recombination (see, for example, ref. 42,43) and telomere synthesis (44), at stages which apparently require unwinding of duplex DNA, or DNA–RNA hybrids. Further studies would be needed to find out whether helicases are also involved in these processes through unwinding of four stranded DNA structures.

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