Formation of DNA triple helices inhibits DNA unwinding by the SV40 large T-antigen helicase

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ABSTRACT

Previous studies have indicated that $d(TC)_n.d(GA)_n$ microsatellites may serve as arrest signals for mammalian DNA replication through the ability of such sequences to form DNA triple helices and thereby inhibit replication enzymes. To further test this hypothesis, we examined the ability of $d(TC)_i.d(GA)_i.d(TC)_i$ triplexes to inhibit DNA unwinding *in vitro* by a model eukaryotic DNA helicase, the SV40 large T-antigen. DNA substrates that were able to form triplexes, and non-triplex-forming control substrates, were tested. We found that the presence of DNA triplexes, as assayed by endonuclease S1 and osmium tetroxide footprinting, significantly inhibited DNA unwinding by T-antigen. Strong inhibition was observed not only at acidic pH values, in which the triplexes were most stable, but also at physiological pH values in the range 6.9–7.2. Little or no inhibition was detected at pH 8.7. Based on these results, and on previous studies of DNA polymerases, we suggest that DNA triplexes may form *in vivo* and cause replication arrest through a dual inhibition of duplex unwinding by DNA helicases and of nascent strand synthesis by DNA polymerases. DNA triplexes also have the potential to inhibit recombination and repair processes in which helicases and polymerases are involved.

INTRODUCTION

Eukaryotic genomes contain multiple repeats of short tracts of bases defined as microsatellites, or 'simple sequence' DNA. One group of microsatellites consists of purine repeats on one strand and pyrimidine repeats on the other strand (1,2). Of this group, $d(TC)_n.d(GA)_n$ sequences in particular are highly dispersed in eukaryotic genomes (3–8).

Studies performed in mammalian cells have indicated that $d(TC)_n.d(GA)_n$ microsatellites may cause pauses or arrests of DNA replication and amplification (9–14). A possible mechanism for such replication arrests has been suggested by *in vitro* primer extension assays performed with purified DNA polymerases and single-stranded DNA templates containing either $d(TC)_n$ or $d(GA)_n$ repeats. In these studies, DNA synthesis was found to be arrested in the middle of either type of repeat (15). Based on

other studies which indicated that $d(TC)_n.d(GA)_n$ duplexes may bind $d(TC)_i$ sequences and form $d(TC)_i.d(GA)_i.d(TC)_i$ triplexes (16–23), it has been proposed that in the primer extension reactions arrests of DNA synthesis in templates including $d(TC)_n$ repeats were caused by formation of $d(TC)_i.d(GA)_i.d(TC)_i$ triplexes between partially replicated $d(TC)_i.d(GA)_i$ duplexes and the remaining single-stranded $d(TC)_i$ sequences (15). Subsequently, data supporting this mechanism have been obtained (24). Similar experiments (15,24) have indicated that arrests of DNA synthesis in templates including $d(GA)_n$ repeats were caused by formation of triple-stranded DNA helices of the type $d(GA)_i.d(GA)_i.d(TC)_i$ which may be generated by interactions between $d(TC)_i.d(GA)_i$ sequences and single-stranded $d(GA)_i$ repeats (25–28). In line with these observations, DNA triplexes were also found to cause arrests of DNA replication during *in vitro* strand displacement assays carried out with double-stranded DNA templates and bacteriophage T7 DNA polymerase (29).

Although inhibition of DNA polymerases by triplex formation could account for the arrest of DNA replication observed at $d(TC)_n.d(GA)_n$ microsatellites in live cells, it was possible that inhibition of other components of the replication machinery might also play a role in the replication arrest. Hence, we have undertaken to determine whether helicases, which unwind DNA duplexes in front of replication forks (30–33), are also inhibited by formation of DNA triplexes at $d(TC)_n.d(GA)_n$ tracts. To this end, we have constructed appropriate substrates which could form DNA triplexes and compared the rates of unwinding of these substrates by helicases *vis à vis* the rates of unwinding of 'control' substrates which were not expected to form DNA triplexes.

In this report, we present the first study of this type carried out with the Simian Virus 40 (SV40) large tumor antigen (T-ag) helicase, an enzyme involved in both the initiation and the elongation steps of DNA replication (34–37). These data indicate that formation of $d(TC)_i.d(GA)_i.d(TC)_i$ DNA helices can inhibit the large T-ag helicase activity and suggest that triplex DNA has the potential to regulate the elongation stage of eukaryotic DNA replication.

MATERIALS AND METHODS

Materials

The SV40 large T-ag was purified from Sf9 cells infected with a recombinant baculovirus encoding the protein, as described (38).

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The cloning and the preparation of the recombinant M13 DNA including a d(TC)₂₇ tract was also described previously (15). Oligonucleotides were synthesized in an Applied Biosystems model 381A DNA synthesizer, or were purchased from Operon, Alameda, CA.

Preparation of helicase substrates

The substrates designated 'control [12]' and 'triplex [12]' were prepared by annealing a 34 base oligonucleotide at a concentration of 7.5×10^{-9} M with either of two complementary 78 base oligonucleotides at a concentration of 17.5×10^{-9} M. For the helicase assays, the 34 base oligonucleotide was 5' end-labeled with ^{32}P before the annealing, in a reaction catalysed by the T4 polynucleotide kinase (specific radioactivity: 2×10^8 to 5×10^8 d.p.m./ μg) (39). For the footprinting assays, the 78 base oligonucleotides were similarly labeled. The substrates designated 'triplex Hy-5' and 'triplex Hy-3' were prepared by annealing ^{32}P -labeled oligonucleotides at a concentration of 7.5×10^{-9} M with molecules of the recombinant M13 DNA mentioned above, which contains a d(TC)₂₇ tract, at a concentration of 17.5×10^{-9} M. The annealing was performed in a buffer solution containing 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM Na₂EDTA. The samples were heated 1.5 min at 90°C in a Dri-Block apparatus and were slowly cooled to 25°C. The slow cooling was accomplished by turning off the heating element of the Dri-Block apparatus. The cooling period lasted 150 min.

Helicase assays

These assays were performed in 20 μ l of a solution containing 34 mM Tris-acetate, 66 mM K-acetate, 10 mM NaCl, 11 mM $MgCl_2$, 0.10 mM EDTA, 0.50 mM dithiothreitol, 2 mM ATP, 0.40 mM spermine, 5% glycerol, 0.05 mg/ml bovine serum albumin (BSA) and $20\text{--}50 \times 10^{-12}$ M of DNA substrate. The pH of the solutions was adjusted to values between 6.1 and 8.7 by titration with acetic acid. T-ag was added to these solutions at the final concentrations stated in the legends to the figures. All assays were performed at 37°C. The reactions were stopped by addition of 5 μ l of a solution containing 15% glycerol, 3% sodium dodecyl sulfate, 20 mM Na_2EDTA , 8 mM Tris-HCl, 0.80 mM Bromophenol Blue, 1 mM Xylene Cyanol FF pH 8.0. In some of the assays, the stopping solution also contained 4.5 nM of unlabeled oligonucleotide of the same type as the radioactively labeled oligonucleotide in the substrate (see the legends to the figures). The mixtures were electrophoresed in 10% polyacrylamide gels which were later dried and autoradiographed, as described (40). The densities of bands in the autoradiograms were determined by microdensitometry, using a Cliniscan II Scanning Densitometer manufactured by Hellena Laboratories.

Footprinting assays

S1 endonuclease footprinting. S1 endonuclease digestions were carried out for 30 min at 37°C and pH 8.7, 6.9 or 6.1, in 5 µl of a solution containing the following components: 34 mM Tris-acetate, 66 mM K-acetate, 10 mM NaCl, 11 mM MgCl₂, 1.0 mM ZnCl₂, 0.10 mM EDTA, 0.50 mM dithiothreitol, 2 mM ATP, 0.40 mM spermine, 0.05 mg/ml BSA, 5% glycerol, 14 × 10⁻⁹ M of DNA substrate, and various concentrations of S1 endonuclease, as stated in the legend to Figure 3. The reactions were terminated

Figure 1. Structure of the helicase substrates 'control [12]' and 'triplex [12]'. To generate the substrate designated 'control [12]', a 78 base oligonucleotide including a d(TC)₁₂ tract has been annealed to the indicated 34 base oligonucleotide including a d(GA)₁₂ sequence, which had been 5' end-labeled with ³²P. The complete sequence of the 78 base oligonucleotide was: 5'-TGCCATGACGACTGCTGACGATCCGTACGATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCATCTCCTGCCTATGTAATG-3'. The substrate designated 'triplex [12]' was formed by annealing the same 34 base oligonucleotide to a 78 base oligonucleotide including a d(TC)₂₇ tract. The sequence of nucleotides 65-78 in this 78 base oligonucleotide was the same as that of the corresponding segment of the 78 base oligonucleotide in the 'control [12]' substrate.

by addition of 14 μ l of a solution containing 35% formamide, 20 mM trizma base, 3.6 mM Na₂EDTA, 0.01% Bromophenol Blue, 0.01% Xylene Cyanol F,F pH 8.0. Samples of 4 μ l of these mixtures were electrophoresed in 12% polyacrylamide sequencing gels that were subsequently dried and autoradiographed, as described (15).

Osmium tetroxide footprinting. Osmium tetroxide modification was performed according to published procedures (41,42). The reactions were carried out for 15 min at 25°C and pH 8.7, 6.9 or 6.1, in 10 µl of a solution containing the following components: 34 mM Tris-acetate, 66 mM K-acetate, 10 mM NaCl, 11 mM MgCl₂, 0.10 mM EDTA, 0.50 mM dithiothreitol, 2 mM ATP, 0.40 mM spermine, 2% pyridine, 14×10^{-9} M of DNA substrate and various concentrations of osmium tetroxide, as stated in the legend to Figure 4. Subsequently, the DNA was ethanol precipitated and dried. Then, it was dissolved in 100 µl of 1 M piperidine and incubated at 90°C for 30 min. The piperidine was evaporated and the DNA was again dissolved in 19 µl of a solution containing 26% formamide, 15 mM Trizma base, 2.6 mM EDTA, 0.007% Bromophenol Blue, 0.07% Xylene Cyanol F,F pH 8.0. A sample of 4 µl of each mixture was electrophoresed in a 12% polyacrylamide sequencing gel that was subsequently dried and autoradiographed.

RESULTS

Helicase assays of a substrate capable of forming DNA triple helices and of a control substrate

Figure 1 illustrates the two helicase substrates used for the experiments described below. The molecule shown at the bottom, designated 'triplex [12]', consisted of a 34 base oligonucleotide including 12 d(GA) repeats, annealed with a 78 oligonucleotide including 27 d(TC) repeats. This molecule could form the triple helix shown in the drawing, as well as other triplexes generated by slippage of the d(TC)₂₇ repeats along the d(TC)₁₂.d(GA)₁₂ duplex. The molecule shown at the top, designated 'control [12]', consisted of the same 34 base oligonucleotide annealed with another 78 base oligonucleotide in which 15 d(TC) repeats (nucleotides 1–30) were replaced with a non-repeat sequence. This molecule lacked sequence elements that could generate

Figure 2. SV40 large T-ag helicase assays performed with the substrates 'control [12]' and 'triplex [12]'. (A) Unwinding of the 'control [12]' and the 'triplex [12]' substrates by the T-ag helicase. These reactions were carried out as described in Materials and Methods. The 34 base oligonucleotide in the substrate molecules was 5' end-labeled with ^{32}P . The concentration of the SV40 large T-ag helicase was 4.0 $\mu\text{g}/\text{ml}$. The products of the reactions were electrophoresed in 10% polyacrylamide gels that were subsequently dried and autoradiographed. Lanes d present samples of the 'control' substrate that were heated 5 min at 90°C in the absence of T-ag and quickly cooled to 4°C. The solution employed for stopping the reaction contained the unlabeled 34 base oligonucleotide at a concentration of 4.5×10^{-9} M. (B) Plots of the data shown in A. The data are presented as percent of oligonucleotide released from the substrate calculated as follows: $100 \times (\text{density of the band containing the released oligonucleotide} / \text{density of the band containing the oligonucleotide in the denatured sample})$.

DNA triplexes. Figure 1 also shows a schematic drawing of the SV40 large T-ag helicase which was expected to bind single-stranded DNA sequences in each of the two substrate molecules and to move in the direction 3'→5' into the duplex region, causing unwinding of the duplex (34–36,43,44).

Figure 2A shows the results obtained in helicase unwinding assays of the 'triplex [12]' and the 'control [12]' substrates carried out at the pH values 8.7, 6.9 and 6.1. In these assays, the 34 base oligonucleotide in the substrates was 5' end-labeled with ^{32}P . Samples withdrawn 0, 5, 10 and 15 min after initiation of the reactions were mixed with a solution that inactivated the helicase. Other samples were heated, such that the substrate molecules had been denatured. The helicase treated and the denatured DNA samples were electrophoresed in 10% polyacrylamide gels that were subsequently dried and autoradiographed. The lanes designated 'd' contain samples of the denatured 'control [12]' substrate. Samples of the denatured 'triplex [12]' substrate gave similar profiles and are not shown here. In the autoradiograms shown in Figure 2 the upper bands contain the unwound substrates. The lower bands contain the 34 base oligonucleotide released by the helicase, or by the heating. It should be noted that the upper bands containing the 'triplex [12]' substrate molecules were somewhat retarded relative to the bands including the 'control [12]' substrate molecules. This retardation was due to interaction of the 'triplex [12]' substrate molecules with unlabeled 34 base oligonucleotide that was added in these assays to the stopping solution to minimize reannealing of the radioactively labeled oligonucleotide; such retardation did not occur in other assays in which the unlabeled oligonucleotide was omitted from the stopping solution (not shown).

Since the fraction of substrate molecules that were unwound by the helicase was rather low under the conditions used for these assays, the unwinding reaction could be most accurately monitored by measurements of the released oligonucleotide. Hence, microdensitometry was used to estimate the intensities of the bands containing the oligonucleotide released by the enzyme and of the bands containing the oligonucleotide released by denaturation (lanes d). Figure 2 shows plots of the ratios of these numbers versus time. It can be seen that in the helicase assays performed at pH 8.7, the extent of unwinding of the 'triplex [12]' molecules after 5 min was higher than that of the 'control [12]' molecules. The extents of unwinding of the two substrates were approximately the same after 10 min and remained the same after 15 min. In contrast, in the assays performed at pH 6.9 and 6.1 the unwinding of the 'triplex [12]' substrate was strongly inhibited at all time periods, compared to the unwinding of the 'control [12]' substrate. Since d(TC)_id(GA)_id(TC)_i triplexes are stabilized by protonation of the cytosines in the third strand (17–23), the pH dependence of these reactions is compatible with the notion that unwinding of the 'triplex [12]' substrate molecules by the SV40 large T-ag helicase was inhibited in these assays by formation of such triplexes.

Footprinting assays of the 'triplex [12]' and the 'control [12]' substrates

S1 endonuclease footprinting. In order to assess the extents of DNA triplex formation in the 'triplex [12]' and the 'control [12]' substrates, we have carried out S1 endonuclease footprinting assays of these molecules. For these assays the substrate molecules were end-labeled with ^{32}P in the 78 base strands

Figure 3. Endonuclease S1 footprinting assays of the substrates 'control [12]' and 'triplex [12]'. In these assays the 78 base oligonucleotides in the substrate molecules were 5' end-labeled with ^{32}P . Endonuclease S1 digestions were performed at pH values 8.7, 6.9 and 6.1, as described in Materials and Methods. The concentrations of the enzyme were as follows: pH 8.7: 'control [12]' substrate, lanes 1–7, or 'triplex [12]' substrate, lanes 8–14: 0, 0.10, 0.30, 0.90, 2.70, 8.10 and 24.2 U/ μl , respectively. pH 6.9: 'control [12]' substrate lanes 1–7, or 'triplex [12]' substrate, lanes 8–14: 0, 0.01, 0.03, 0.10, 0.30, 0.90 and 2.70 U/ μl , respectively. pH 6.1: 'control [12]' substrate, lanes 1–7, or 'triplex [12]' substrate, lanes 8–14: 0, 0.003, 0.01, 0.03, 0.10, 0.30 and 0.90 U/ μl , respectively. Electrophoresis was carried out in 12% polyacrylamide sequencing gels. The numbers designate bands containing fragments of the 78 base oligonucleotide that were cleaved at the indicated residues. The lengths of these fragments were determined by running oligonucleotide markers in the same gels.

Figure 4. Osmium tetroxide footprinting assays of the substrates 'control [12]' and 'triplex [12]'. These assays were carried out with substrate molecules in which the 78 base oligonucleotides were 5' end-labeled with ^{32}P . The following osmium tetroxide concentrations were used at all three specified pH values: 'control [12]' substrate, lanes 2–7, or 'triplex [12]' substrate, lanes 9–14: 0, 4, 8, 12, 16 and 20 mM, respectively. Lanes 1 and 8 represent samples that were incubated for 15 min at 25°C in the absence of osmium tetroxide and were also not exposed to piperidine. The numbers designate the lengths of cleavage products of the 78 base oligonucleotide. Each of these cleavage products is smaller by one base than the number of the corresponding thymidine residue in Figure 1.

including the $\text{d}(\text{TC})_{27}$ repeats. The radioactively labeled molecules were partially digested with the single-strand-specific endonuclease S1 at pH 6.1, 6.9 or 8.7, the pH values employed for the helicase assays, using several enzyme/substrate ratios. The digests were electrophoresed in denaturing polyacrylamide gels and the gels were autoradiographed. Figure 3 shows the autoradiographs obtained in these assays. Inspection of the footprint of the 'control [12]' substrate obtained at pH 8.7 shows that it consists of three regions. One region (nucleotides 1–30), corresponding to a single-stranded region of the molecule (Fig. 1), contains S1-sensitive sites distributed throughout its length. The second region of the footprint, which begins at nucleotide 31,

and corresponds to the double-stranded portion of the molecule, was protected against endonuclease S1 cleavage. This region is followed by another S1-sensitive region. The boundary between the last two regions could not be accurately mapped, due to the rather poor resolution of the bands at the top of this 12% polyacrylamide gel and to some heterogeneity of the 5'-end of the 78 base oligonucleotide. A similar pattern of S1 cleavage is seen in the footprint of the 'control [12]' substrate generated at pH 6.9. However, at this pH value, the sites in the region extending between the nucleotides 24–30 were slightly more susceptible to cleavage by the enzyme than the other sites. This pattern was even more pronounced at pH 6.1.

In the 'triplex [12]' footprint produced at pH 6.1, both the region extending beyond nucleotide 30, and the sequence extending between nucleotides 2 and 22, were protected against endonuclease S1 cleavage. An S1 hypersensitive region found between these two protected regions extended in most of the 'triplex [12]' molecules between nucleotides 22 and 30. This pattern of cleavage is compatible with formation of the triplex shown in Figure 1; thus, the S1-hypersensitive region corresponds to the single-stranded loop, and the protected region extending between nucleotides 2 and 22 corresponds to the third strand of the triplex. It is not clear to us why the region including nucleotides 25–30 is also slightly more susceptible to S1 cleavage in the 'control [12]' substrate. However, at the highest enzyme/substrate ratios used in this experiment (lanes 7 and 14 in the pH 6.1 footprint), the strong bands at the nucleotides 22–30 are still observed in the footprint of the 'triplex [12]' substrate, whereas the corresponding bands in the 'control [12]' substrate are barely visible. The footprint of the 'triplex [12]' substrate obtained at pH 6.9 resembles the pH 6.1 footprint, except that the hypersensitive region is somewhat broader; this difference is indicative of a slightly greater heterogeneity of the triplexes generated at pH 6.9. The footprint of the 'triplex [12]' substrate obtained at pH 8.7 revealed a much broader distribution of the hypersensitive sites throughout the region extending between nucleotides 10 and 30. This result indicates that at this higher pH value only very short unstable triplexes, or no triplexes, have been generated, and is in line with the observation that at pH 8.7 unwinding of the 'triplex [12]' substrate by the T-ag helicase was not inhibited.

Osmium tetroxide footprinting. Figure 4 shows a series of osmium tetroxide footprints of the 'control [12]' and the 'triplex [12]' substrates that were also generated under the conditions employed for the helicase assays. Osmium tetroxide, in the presence of pyridine, modifies primarily thymidine (T) residues in single-stranded DNA; subsequent treatment of the DNA with piperidine cleaves the DNA next to the modified residues (42). Inspection of the 'control [12]' footprints revealed that at all three pH values, the T residues in the segment extending between nucleotides 1 and 30 of these substrate molecules were highly susceptible to osmium tetroxide modification. However, some cleavage products were also observed at T residues mapping beyond nucleotide 30, within the expected double-stranded region. We believe that these cleavage products were generated from an excess of the 78 base oligonucleotide in the reaction mixtures; for these reactions were carried out under the conditions employed for the helicase assays, in which an excess of the 78 base oligonucleotide was needed to obtain complete annealing of the 34 base oligonucleotide.

In the osmium tetroxide footprints of the 'triplex [12]' substrate that were generated at pH 6.1 and 6.9, the T residues 23, 25 and 27 (which correspond to the band numbers 22, 24 and 26 in the footprints) were clearly hypersensitive to the modifying chemical reagent. These thymidines map in the region found to be S1-hypersensitive in the corresponding footprints shown in Figure 3. However, the T residue 29, which is included in the S1 hypersensitive region, is less sensitive to the osmium tetroxide modification. The reason for this apparent discrepancy is unclear. Unlike the footprints generated at pH 6.1 and 6.9, in the osmium tetroxide footprint of the 'triplex [12]' substrate generated at pH 8.7, the T residues 22, 24 and 26 were not hypersensitive to osmium tetroxide modification. At all three pH values the region

Figure 5. Structure of the helicase substrates 'control', 'triplex Hy-5' and 'triplex Hy-3'. To form the substrate designated 'control', the M13 DNA molecule containing a 190 base fragment of rat DNA including a d(TC)₂₇ tract (see Materials and Methods), has been annealed with the indicated 24 base oligonucleotide, which is complementary to a sequence within the rat DNA. The substrates designated 'triplex Hy-5' and 'triplex Hy-3' were generated by annealing molecules of the same recombinant M13 DNA with the indicated 26 base oligonucleotides. The arrows depict the direction of movement of the large T-ag helicase. Further details are presented in the text.

extending beyond the T-residue 27 in the 'triplex [12]' substrate (but not in the 'control [12]' substrate) was protected against the osmium tetroxide modification. Possibly, the triple-stranded structures provided better protection of the T residues beyond 27 than the double-stranded configuration. Also, in the footprints of the 'triplex [12]' substrate generated at pH 6.1 and 6.9, but not in the footprint of this substrate generated at pH 8.7, the T residues mapping between nucleotides 4 and 21 were also better protected against osmium tetroxide modification than the thymidines 23, 25 and 27. In conclusion, like the corresponding S1 footprints, the osmium tetroxide footprints show that at pH 6.1 and 6.9 the 'triplex [12]' substrate molecules formed rather stable triple helices. The absence of a hypersensitive region in the osmium tetroxide footprint of the 'triplex [12]' substrate obtained at pH 8.7, indicates that stable triplexes were not generated at this higher pH value.

Helicase assays of substrates capable of forming either of two triplex isomers

It has been shown that d(TC)_n.d(GA)_n duplexes can undergo transitions into two different d(TC)_i.d(GA)_j.d(TC)_k triplex isomers [(45), see below]. Hence, it was interesting to find out whether the SV40 large T-ag helicase is only inhibited by isomers having the polarity of the 'triplex [12]' substrate, or is also inhibited by isomers of the second type. Figure 5 illustrates three helicase substrates which were used to examine this question. To generate the substrate designated 'control', a recombinant M13 DNA molecule, including a d(TC)₂₇ tract, has been annealed with the indicated 24 base oligonucleotide. This substrate lacks sequence elements which may form DNA triplexes. In the substrate designated 'triplex Hy-5', an identical M13 DNA molecule has been annealed to a 26 base oligonucleotide consisting of eight d(GA) repeats and 10 nt complementary to the 10 nt sequence flanking the d(TC)₂₇ tract at its 3' end. As shown in the drawing, this substrate could form triplexes of the type Hy-5 (45), which have the same polarity as that of the 'triplex [12]' substrate. In the substrate designated 'triplex Hy-3', another

Figure 6. SV40 large T-ag helicase assays performed with the substrates 'control' and 'triplex Hy-5'. (A) Autoradiogram of a gel showing the unwinding of the 'control' and the 'triplex Hy-5' substrates catalysed by the T-ag helicase. In these assays the oligonucleotides were 5' end-labeled as described in Materials and Methods. The assays were carried out as described in the legend to Figure 2, except that the concentration of the large T-ag helicase was 24 μ g/ml. In these assays, no unlabeled oligonucleotide was added to the stopping solution. (B) Plots of the data shown in (A). Same presentation as in Figure 2.

identical recombinant M13 DNA molecule has been annealed with a 26 base oligonucleotide consisting of eight d(GA) repeats and 10 nt complementary to the 10 nt sequence flanking the d(TC)₂₇ tract at its 5' end. This substrate could generate triplex isomers of the type Hy-3 (*ibid.*). Figure 5 also shows the SV40 large T-ag helicase which, as already pointed out, initially binds single-stranded DNA and moves in the direction 3'→5'. Thus, in assays of the substrates 'triplex Hy-5' and 'triplex Hy-3', the helicase was expected to encounter the triplexes in two different orientations, as shown.

Figure 6A presents the results of T-ag helicase assays performed with the 'control' and the 'triplex Hy-5' substrates at the pH values 8.7, 7.2 and 6.3. In these assays, the oligonucleotides in the substrates were 5' end-labeled with ³²P. The upper bands contain the unwound substrates which barely moved into the gels. The lower bands contain the oligonucleotides released in the course of the helicase reactions, or by denaturation (lanes d). It should be noted that, unlike the helicase reactions performed with the 'control [12]' and the 'triplex [12]' substrates, no unlabeled oligonucleotide was added to the solution used for stopping these reactions, since the reactions were carried out under conditions in which no significant reannealing of the single strands was observed. Figure 6 shows plots of these data quantitated by microdensitometry. It can be seen that in the reactions performed at pH 8.7, after 10 min unwinding of the 'triplex Hy-5' substrate was inhibited by ~30% relative to the unwinding of the 'control' substrate. However, after 20 and 30 min the two substrates were unwound to approximately the same extent (~80%). In contrast, in the reactions performed at pH 7.2, there was a strong inhibition of unwinding of the 'triplex Hy-5' substrate after 10, 20 and 30 min. In the reactions performed at pH 6.3, the unwinding of the 'control' substrate was also inhibited. However, the unwinding of the 'triplex Hy-5' substrate was

inhibited to a much greater extent. Figure 7A and B shows similar assays performed with the 'control' and the 'triplex Hy-3' substrates at the same three pH values used for the experiment shown in Figure 6. These assays revealed a pH-dependent preferential inhibition of unwinding of the 'triplex Hy-3' substrate similar to that observed in the assays of the 'triplex Hy-5' substrate.

DISCUSSION

In this study we compared the rates of unwinding of two types of DNA substrates by the SV40 large T-ag helicase. Substrates of one type, the 'triplex forming' substrates, contained double-stranded d(TC)_n.d(GA)_n tracts linked to single-stranded d(TC)_n sequences and had the potential to form d(TC)_i.d(GA)_j.d(TC)_i triplexes (17–20). Substrates of the second type, the 'control' substrates, did not contain any d(TC)_n.d(GA)_n tracts, or did not contain d(TC)_n extensions, and were not expected to form DNA triplexes. We found that unwinding of the 'triplex forming' substrates by the helicase was strongly inhibited at ≤ pH 7.2, whereas unwinding of the 'control' substrates was not inhibited under the same conditions, or was inhibited to a lesser extent. In contrast, at pH 8.7 the two types of substrates were unwound by the helicase at approximately equal rates. Since formation of d(TC)_i.d(GA)_j.d(TC)_i triplexes is favored by protonation of the N3 of the cytosine in the third strand (17–23), the pH-dependence of the helicase inhibition supported the view that it was caused by formation of such triple helices. More definitive support for this inference was provided by the S1 endonuclease and the osmium tetroxide footprinting assays which directly demonstrated that DNA triple helices were formed in the 'triplex [12]' substrate, but not in the 'control [12]' substrate, under the conditions in which inhibition of the unwinding of this substrate by the helicase was

Figure 7. SV40 large T-ag helicase assays performed with the substrates 'control' and 'triplex Hy-3'. (A) Autoradiogram of a gel showing the unwinding of the 'control' and the 'triplex Hy-3' substrates catalysed by the T-ag helicase. The helicase assays were carried out as described in the legend to Figure 6. (B) Plots of the data shown in (A). Same presentation as in Figures 2 and 6.

observed. In preliminary studies we have found that unwinding of the same 'triplex forming' substrates by two other helicases, which had been purified from calf thymus, was completely inhibited under conditions in which the 'control' substrates were unwound (Peleg, Thömmes, Hübscher and Manor, unpublished results).

Some of the unwinding reactions were carried out with the 'triplex Hy-5' and the 'triplex Hy-3' substrates that could form either of the triplex isomers Hy-5 or Hy-3, which had different orientations relative to the direction of movement of the large T-ag helicase (Fig. 5). The helicase was inhibited as it encountered the triplexes at either orientation. It is particularly interesting that strong inhibition of unwinding of these substrates and of the 'triplex [12]' substrate was observed at the physiological pH values of 7.2 and 6.9, respectively, as well as at acidic pH values. Furthermore, the footprinting assays of the 'triplex [12]' substrate molecules (Figs 3 and 4) have provided direct evidence for transitions of these molecules into stable triplexes at pH 6.9. It should be noted that other investigators have also reported the formation of $d(TC)_i \cdot d(GA)_j \cdot d(TC)_i$ triplexes at neutral pH, as well as acidic pH values [(20,46); Fresco, personal communication]. These data lend credence to the physiological significance of the helicase inhibition by such triplexes.

In both prokaryotes and eukaryotes helicases unwind the DNA strands at replication forks (30–33). Therefore, it appears plausible that arrest signals for DNA replication could operate by inhibition of helicases. Indeed, in bacteria, a specific DNA binding protein was found to arrest DNA replication in an orientation dependent mechanism by inhibition of helicases (47–49). Our present results raise the possibility that in live

mammalian cells inhibition of unwinding of $d(TC)_n \cdot d(GA)_n$ tracts by helicases due to their transitions into $d(TC)_i \cdot d(GA)_j \cdot d(TC)_i$ triplexes + single-stranded $d(GA)_j$ sequences (17,19,20) may cause replication arrests at such tracts. One possible objection to this model is that such transitions are unlikely to happen in double-stranded chromosomal DNA at physiological pH values; for unlike the substrates used in the present studies, these transitions require disruption of Watson–Crick hydrogen bonds and hence are energetically less favorable. However, we have shown that human cells synthesize a protein that selectively binds single-stranded $d(GA)_n$ tracts (40). Another protein that may also bind such sequences has been found by others (50). The binding of either of these proteins to the single-stranded $d(GA)_j$ tracts generated by the duplex–triplex transitions of $(TC)_n \cdot d(GA)_n$ sequences could stimulate these transitions at physiological pH values and thereby enhance the arrests (see ref. 51). A corollary of this hypothesis is that the arrests of DNA replication might be regulated by the $d(GA)_n$ -binding proteins. Moreover, since DNA triplexes were also found to inhibit DNA polymerases (15,24,29), it is possible that if, during DNA replication, the inhibition of unwinding of $d(TC)_n \cdot d(GA)_n$ sequences by the helicase is incomplete, then, in some cases, the polymerases engaged in lagging DNA strand synthesis may be subsequently inhibited by formation of DNA triplexes.

It should be noted that the substrates used for the helicase assays reported in this article could correspond to intermediates generated in the course of other processes of DNA metabolism such as recombination and repair which also require helicases (31–33). Hence, our data suggest that formation of $d(TC)_i \cdot d(GA)_j \cdot d(TC)_i$ triplexes may inhibit these processes by inhibition of the helicases. Since DNA polymerases are also involved in these processes, their activities may be similarly affected by formation of DNA triplexes. Finally, our studies of the interactions of DNA triplexes with these two groups of key enzymes raise the possibility that the activities of other enzymes involved in DNA and RNA metabolism are also affected by similar structural transitions. Therefore, we believe that interpretations of genetic and biochemical data regarding the interactions of such enzymes with their nucleic acid substrates, and predictions of the biological consequences of such interactions, should take into consideration not only the involvement of Watson–Crick hydrogen bonds in these reactions, but of other hydrogen bonds as well.

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