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# Unwinding of the third strand of a DNA triple helix, a novel activity of the SV40 large T-antigen helicase

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## ABSTRACT

**We present experiments indicating that the SV40 large T-antigen (T-ag) helicase is capable of unwinding the third strand of DNA triple helices. Intermolecular $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes were generated by annealing, at pH 5.5, a linearized double-stranded plasmid containing a $d(TC)_{27}\cdot d(GA)_{27}$ tract with a $^{32}$P-labeled oligonucleotide consisting of a $d(TC)_{20}$ tract flanked by a sequence of 15 nt at the 3′-end. The triplexes remained stable at pH 7.2, as determined by agarose gel electrophoresis and dimethyl sulfate footprinting. Incubation with the T-ag helicase caused unwinding of the $d(TC)_{20}$ tract and consequent release of the oligonucleotide, while the plasmid molecules remained double-stranded. ATP was required for this reaction and could not be replaced by the non-hydrolyzable ATP analog AMP-PNP. T-ag did not unwind similar triplexes formed with oligonucleotides containing a $d(TC)_{20}$ tract and a 5′ flanking sequence or no flanking sequence. These data indicate that unwinding of DNA triplexes by the T-ag helicase must be preceded by binding of the helicase to a single-stranded 3′ flanking sequence, then the enzyme migrates in a 3′→5′ direction, using energy provided by ATP hydrolysis, and causes release of the third strand. Unwinding of DNA triplexes by helicases may be required for processes such as DNA replication, transcription, recombination and repair.**

## INTRODUCTION

Polypurine·polypyrimidine sequences, which are highly dispersed in eukaryotic genomes (1–8), may undergo transitions into unusual structures containing DNA triple helices (9–18; for recent reviews see 19,20). Formation of these structures could be inhibitory to vital processes which require DNA strand separation, such as DNA replication and transcription. To overcome the inhibition eukaryotic cells might produce enzymes that specifically recognize and unwind DNA triplexes. Alternatively, helicases which unwind DNA duplexes (21–26) might also be capable of unwinding the third strand in DNA triplexes.

The latter possibility was suggested by studies recently carried out on interactions of the SV40 large T-antigen (T-ag) helicase with $d(TC)_i\cdot d(GA)_i\cdot d(TC)_i$ triplexes. We have found that unwinding of DNA substrates containing such triplexes by the large T-ag was inhibited relative to unwinding of similar 'control' substrates which could not form triplexes (27). Notwithstanding this observation, it became apparent that the T-ag helicase may unwind triplexes, because inhibition was not complete, particularly at neutral pH. However, in the substrates used for these earlier studies the third strand of the triplexes was covalently linked to one of the strands in the duplexes. Hence, in those experiments unwinding of the third strand was not assayed independently of unwinding of the duplexes. For this reason we were unable to rule out the possibility that unwinding of the third strand occurred spontaneously, but was only seen when the duplexes were unwound by the helicase. Others have reported that the bacteriophage T4-encoded dda helicase unwound a different type of DNA triplex (28). In those studies too, unwinding of the third strand of the triplex has not been assayed independently of duplex unwinding (28).

To address the question of whether helicases are capable of unwinding the third strand in DNA triple helices we have prepared substrates containing intermolecular $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes with a radioactively labeled third strand. Here we report studies carried out with these substrates in which the SV40 large T-ag was found to possess such a triplex unwinding activity.

## MATERIALS AND METHODS

### Oligonucleotides

The following three oligonucleotides were purchased from Biotechnology General (Israel) and were used for preparation of helicase substrates: (i) $d[(TC)_{20}TGACGCTCCGTACGA]$, designated 3′-tailed oligonucleotide; (ii) $d[AGCATGCCTCGCAGT(TC)_{20}]$, designated 5′-tailed oligonucleotide; (iii) $d(TC)_{20}$.

### Preparation of helicase substrates

A 2904 bp plasmid was constructed by insertion of a 210 bp rat cell DNA fragment containing a $d(GA)_{27}\cdot d(TC)_{27}$ tract into the *Kpn*I site of plasmid pUC18 (29). This plasmid, which we have designated pMA73, was digested with either the single cut restriction enzyme *Nde*I or the single cut restriction enzyme *Xba*I. A sample of 150 ng linearized double-stranded plasmid were annealed with 0.75 ng of either one of the three oligonucleotides described in the previous section, which had been 5′-end-labeled

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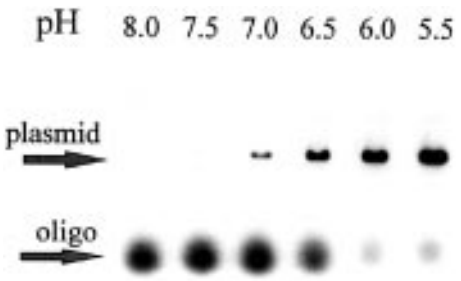


**Figure 1.** pH-dependent formation of intermolecular $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes. The 2904 bp pMA73 plasmid, which contains a $d(TC)_{27}\cdot d(GA)_{27}$ tract, was linearized by digestion with the single cut enzyme *Xba*I. The linearized plasmid was annealed with the oligonucleotide d[$(TC)_{20}$TGACGCTCCGTAC-GA] (3′-tailed oligonucleotide) which had been labeled with $^{32}P$ at the 5′-end. Agarose gel electrophoresis and autoradiography were performed as described in Materials and Methods.

with $^{32}P$ at a specific radioactivity of $2–5 \times 10^8$ c.p.m./μg (30). The molar ratio plasmid/oligonucleotide was 2:1. For some assays the linearized plasmid was 3′-end-labeled with $^{32}P$ as described below. The annealing was performed in 10 μl buffer containing 33 mM Tris–acetate, 66 mM potassium acetate, 100 mM NaCl, 10 mM $MgCl_2$ and 0.40 mM spermine. The pH of the buffer was 5.5 in all assays, except those shown in Figure 1, in which the pH values were as indicated. The samples were incubated at 56°C for 1 h and then slowly cooled to 25°C. Association between the oligonucleotide and the linearized plasmid duplex was determined by agarose gel electrophoresis, as described in the next section.

### Helicase assays

These assays were carried out at 37°C in 20 μl of a solution containing 36 mM Tris–acetate pH 7.2, 73 mM potassium acetate, 10 mM NaCl, 11 mM $MgCl_2$, 0.40 mM spermine, 0.50 mM 1,4-dithiothreitol (DTT), 0.05 mg/ml bovine serum albumin, 2 mM ATP, 5% glycerol, 0.037 ng/μl DNA substrate and 24 ng/μl SV40 large T-ag prepared as previously described (27). The reactions were terminated by addition of 5 μl of a solution containing 15% glycerol, 3% sodium dodecyl sulfate, 80 mM $Na_2$–EDTA, 8 mM Tris–HCl, pH 8.0, 0.80 mM bromophenol blue, 1 mM xylene cyanol and 3.0 nM unlabeled oligonucleotide. The mixtures were electrophoresed for 20 h at 4°C in a 1% agarose gel at 30 V. The running buffer consisted of 40 mM Tris–acetate, 5 mM sodium acetate, 1 mM $MgCl_2$, pH 5.5. The gels were dried and autoradiographed as described (27).

### Footprinting assays

The pMA73 plasmid containing the $d(GA)_{27}\cdot d(TC)_{27}$ tract was first prepared as described (31) and was further purified by CsCl–ethidium bromide equilibrium centrifugation (30). The plasmid was digested with the single cut restriction enzymes *Xba*I and *Hin*dIII, whose recognition sites are 24 bp apart. The cleaved DNA was 3′-end-labeled with $^{32}P$ (specific radioactivity $4 \times 10^6$ c.p.m./μg) by filling the 5′ staggered *Xba*I cuts using Klenow polymerase, such that the labeled end was located 104 bases beyond the $d(GA)_{27}$ tract. Molecules containing the other labeled strand consisted of 24 bp and did not interfere with the footprinting assays. An aliquot of 5.7 ng 3′-tailed oligonucleotide was annealed with 250 ng radioactively labeled duplex plasmid DNA, constituting a 2-fold molar excess of the oligonucleotide. The annealed molecules and plasmid molecules that had not been annealed with the oligonucleotide were exposed to dimethyl sulfate (DMS) in a 100 μl solution containing 36 mM Tris–acetate, 73 mM potassium acetate, pH 7.2, 10 mM NaCl, 11 mM $MgCl_2$, 0.40 mM spermine, 0.50 mM DTT, 2.0 mM ATP and 2.5 ng/μl DNA. DMS concentrations were either 0, 0.08 or 0.16%. The samples were incubated for 15 min at 22°C and the reactions were then terminated by addition of β-mercaptoethanol to a final concentration of 0.60 M. The samples were ethanol precipitated, dissolved and cleaved in 1 M piperidine at 90°C and electrophoresed in a 6% Long Ranger sequencing gel (AT Biochemical), as described (32).

## RESULTS

### Preparation of a helicase substrate containing intermolecular $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes

Previous studies have shown that $d(TC)_i\cdot d(GA)_i\cdot d(TC)_i$ triplexes could be generated by annealing linear double-stranded DNA molecules containing $d(TC)_n\cdot d(GA)_n$ tracts with oligonucleotides containing $d(TC)_i$ sequences (33,34). We used this approach to prepare a DNA substrate that was suitable for our helicase assays. A long (2904 bp) linearized double-stranded plasmid, designated pMA73, which contained a $d(TC)_{27}\cdot d(GA)_{27}$ tract was annealed with a short (55 nt) $^{32}P$-labeled oligonucleotide designated 3′-tailed oligonucleotide. This oligonucleotide consisted of a $d(TC)_{20}$ sequence flanked by a sequence of 15 nt at the 3′-end. The molar ratio plasmid:oligonucleotide was 2:1. Figure 1 shows an experiment in which annealing of these two molecules was carried out at several pH values under the conditions specified in Materials and Methods. Then the annealed molecules were electrophoresed in a 1% agarose gel at 4°C and pH 5.5 and the gel was autoradiographed. It can be seen that electrophoresis separated the unbound oligonucleotide from the complex formed between the oligonucleotide and the linear duplex plasmid, which co-electrophoresed with the plasmid molecules (see Fig. 4 below). No binding of the oligonucleotide to the linear plasmid occurred at pH 8.0 and 7.5. Some binding occurred at pH 7.0. The binding increased as the pH was lowered to 6.5 and reached a maximal value between pH 6.0 and 5.5.

The pH dependence of the association between the oligonucleotide and the duplex was a strong indication that this association was due to formation of $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes, in which the $d(TC)_{20}$ repeats in the oligonucleotide were bound by Hoogsteen hydrogen bonds to $d(TC)_{20}\cdot d(GA)_{20}$ repeats in the double-stranded plasmid molecule (35); for one of the two Hoogsteen bonds between C residues in the third strand and G residues in the duplex could only be generated if those C residues were protonated (9–13,33,34). The possibility that association between these two molecules was due to strand displacement appears unlikely in view of the large difference in the lengths of the two molecules. Also, strand displacement would not be expected to exhibit the observed pH dependence. Furthermore, oligonucleotides of comparable lengths which were homologous to other regions of the plasmid did not associate with the plasmid molecules (not shown).

Figure 2 shows footprinting assays which more directly demonstrated that the association between the plasmid and the oligonucleo-

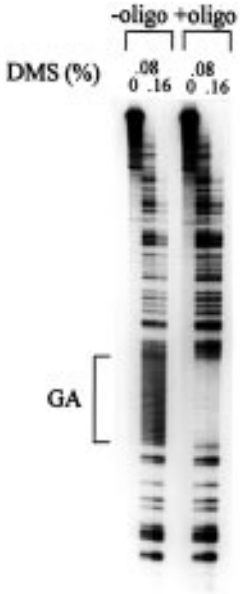


**Figure 2.** DMS footprinting of the complex between the 3′-tailed oligonucleotide and the linearized duplex plasmid pMA73. The footprinting assays were carried out as described in Materials and Methods. The letters GA denote the region including 25 G residues in the $d(TC)_{27}\cdot d(GA)_{27}$ tract that were protected from DMS modification by annealing with the 3′-tailed oligonucleotide.

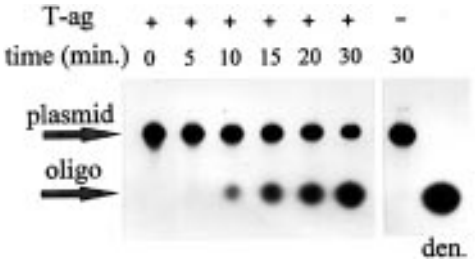


**Figure 3.** Time dependence of unwinding of the third strand in the $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes by the SV40 large T-ag helicase. The helicase substrate was prepared by annealing, at pH 5.5, the pMA73 plasmid, which had been linearized by cleavage with the enzyme *Xba*I, with the $^{32}$P-labeled 3′-tailed oligonucleotide, as shown in Figure 1. The large T-ag helicase reactions were performed at pH 7.2 for the indicated times and the products analyzed by agarose gel electrophoresis. In the lane marked den. the sample was heated for 5 min at 90°C.

tide molecules was due to formation of DNA triplexes. In this experiment the plasmid DNA was cut with a restriction enzyme near the $d(TC)_{27}\cdot d(GA)_{27}$ tract and the strand containing the $d(GA)_{27}$ sequence was end-labeled with $^{32}$P. Samples of this $^{32}$P-labeled plasmid DNA were annealed with the oligonucleotide at pH 5.5 at a molar ratio plasmid:oligonucleotide of 1:2. The mixtures were then brought to pH 7.2, the pH at which helicase assays were performed (see below) and exposed to two different concentrations of DMS. Other samples of the linearized plasmid duplex to which no oligonucleotide was added were similarly treated. Finally, these samples and samples that were not exposed to DMS were treated with piperidine and electrophoresed in a sequencing gel (36). It can be seen that binding of the oligonucleotide protected the N7 atoms of 25 out of 27 G residues in the $d(GA)_{27}$ repeats against methylation by DMS (36). This result indicates that the 25 G residues were bound to C residues of the $d(TC)_{20}$ repeats in the oligonucleotide by Hoogsteen hydrogen bonds. In view of the excess oligonucleotide in these samples the protection of 25, rather than 20, G residues may have been due to binding of two oligonucleotide molecules to one plasmid duplex. Alternatively, this result could have been due to slippage of the $d(TC)_{20}$ repeats along the $d(TC)_{27}\cdot d(GA)_{27}$ tract and a consequent statistical distribution of the $d(TC)_{20}$ repeats in the oligonucleotide over the $d(TC)_{27}\cdot d(GA)_{27}$ tract within the duplex DNA.

## Unwinding of the intermolecular $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes by SV40 large T-ag helicase

Figure 3 shows an experiment in which substrate molecules containing $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes were prepared at pH 5.5, as shown in Figure 1. Then the pH was raised to 7.2 and the samples were incubated with the SV40 large T-ag at 37°C for various time periods in the presence of ATP (see below). Following incubation the samples were electrophoresed in an agarose gel. A sample that was similarly incubated for 30 min in the absence of large T-ag and a sample that was heated for 5 min at 90°C were also electrophoresed in the same gel. It can be seen that in this experiment: (i) all detectable oligonucleotide molecules formed a complex with the linearized plasmid molecules; (ii) no oligonucleotide molecules were released from the complex in the sample incubated for 30 min at 37°C in the absence of T-ag; (iii) a time-dependent release of oligonucleotide was observed in samples that were incubated with T-ag. Release was apparently due to unwinding of the $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes, which accounted for association between the two molecules. Microdensitometric analysis of these data (not shown) revealed that the extent of oligonucleotide released increased linearly during the period of the experiment, such that after 30 min 70% of the triplexes were unwound. It can also be seen that all oligonucleotide molecules were released by heating the complex to 90°C (lane marked den.).

## Unwinding of the $d(TC)_{20}\cdot d(GA)_{20}\cdot d(TC)_{20}$ triplexes by large T-ag helicase releases the third strand and leaves intact duplex molecules

Since the SV40 large T-ag helicase has been reported to unwind long duplex molecules (37), it was interesting to find out whether at the end of the triplex unwinding reaction the linearized double-stranded plasmid DNA molecules were also unwound. For this purpose DNA triplexes were generated by annealing unlabeled or $^{32}$P-labeled linearized plasmid molecules with unlabeled or $^{32}$P-labeled 3′-tailed oligonucleotide. These complexes and $^{32}$P-labeled plasmid molecules which had not been annealed with the oligonucleotide were incubated with or without large T-ag and were electrophoresed in an agarose gel, as described in the previous sections. Figure 4 shows the data obtained in these assays. Clearly, electrophoresis resolved denatured single-stranded plasmid molecules from the native double-stranded plasmid molecules (which co-electrophoresed with the complex formed between the plasmid and the oligonucleotide) and from the released oligonucleotide. Inspection of these data reveals that unwinding of the $d(TC)_{20}\cdot d\,(GA)_{20}\cdot d\,(TC)_{20}$ triplexes by large T-ag helicase resulted in release of the oligonucleotide, while the long linear duplex molecules remained double-stranded.

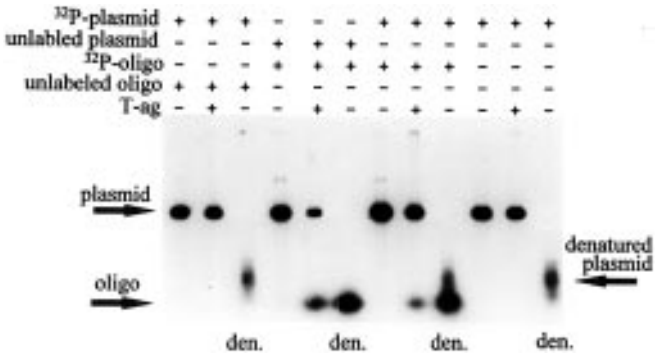


**Figure 4.** Triplex unwinding assays in which either or both the linear plasmid and the 3′-tailed oligonucleotide were $^{32}$P-labeled. The plasmid pMA73 was digested with the single cut enzyme *Xba*I and 3′-end labeled with $^{32}$P. The 3′-tailed oligonucleotide was 5′-end labeled with $^{32}$P. Helicase assays were carried out for 15 min as described in Materials and Methods. The unlabeled molecules were used at the same concentrations as the labeled molecules. In the lanes marked den. the samples were heated for 10 min at 100°C and fast cooled by insertion into ice before being electrophoresed.

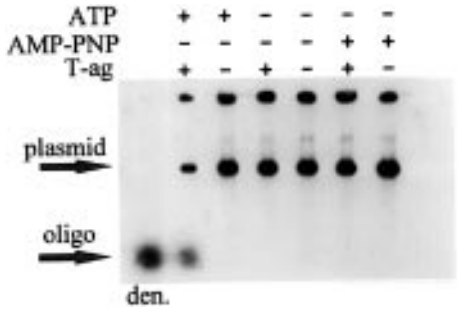


**Figure 5.** Dependence of the triplex unwinding reaction on ATP. The unwinding reactions were carried out for 30 min as described in the legend to Figure 3, except that the pMA73 plasmid was linearized by cleavage with the enzyme *Nde*I. ATP or AMP-PNP were added at 2 mM, as indicated. In the lane marked den. the sample was heated for 5 min at 90°C.

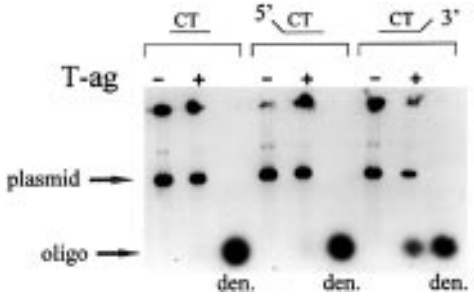


**Figure 6.** Dependence of the d(TC)$_{20}$·d(GA)$_{20}$·d(TC)$_{20}$ triplex unwinding reaction on the presence of a single-stranded 3′-tail. Three complexes containing d(TC)$_{20}$·d(GA)$_{20}$·d(TC)$_{20}$ triplexes were prepared by annealing the double-stranded plasmid pMA73, which had been linearized by cleavage with the enzyme *Nde*I, with the 3′-tailed oligonucleotide, the 5′-tailed oligonucleotide or the d(TC)$_{20}$ oligonucleotide (see Materials and Methods). Helicase unwinding assays were carried out with each of these three substrates for 30 min. The 3′- and the 5′-tails in the oligonucleotides are indicated at the top of the figure by diagonal lines.

## Requirement for ATP and a 3′-tail for unwinding of the third strand of the d(TC)$_{20}$·d(GA)$_{20}$·d(TC)$_{20}$ triplexes by T-ag helicase

To find out whether release of the oligonucleotide from the complex with the linearized plasmid was caused by T-ag helicase activity and not by a different mechanism we sought to determine whether certain parameters of the reaction were compatible with a helicase mechanism. Helicases, including the SV40 large T-ag, require ATP as an energy source for migration along single-stranded DNA and for unwinding duplex DNA (21,24–26,38). Therefore, we tested the requirement for ATP for release of the oligonucleotide from the complex. As Figure 5 shows, omission of ATP eliminated release of the oligonucleotide by the helicase. Inclusion of the non-hydrolysable ATP analog AMP-PNP in the reaction, instead of ATP, did not restore the activity. These results indicate that ATP hydrolysis is required for unwinding of the third strand of the triplex by T-ag helicase. It should also be noted that in this experiment association of the $^{32}$P-labeled oligonucleotide with the linearized plasmid also generated, in addition to complexes co-migrating with the plasmid molecules, slower migrating complexes. These species, which were also occasionally seen in other assays (see Fig. 6 below), might represent aggregates including two or more plasmid molecules bound to one oligonucleotide molecule.

Next we tested the requirement for single-stranded DNA for initial binding of the helicase to the substrate and the polarity of migration of the enzyme along the DNA (see 37,39). For this experiment two additional oligonucleotides were synthesized. One 55 base oligonucleotide, designated 5′-tailed oligonucleotide, contained d(TC)$_{20}$ repeats and a sequence of 15 nt flanking the 5′-end, instead of the 3′-end of the repeats. Another oligonucleotide consisted of just the d(TC)$_{20}$ repeats. The previous 3′-tailed oligonucleotide and the two new oligonucleotides were separately annealed with the linearized plasmid duplex. Each of these three complexes was incubated in the absence or presence of large T-ag under the conditions used for the assays shown in Figure 3 and the mixtures were analyzed by agarose gel electrophoresis. Figure 6 shows the data obtained in these assays. It can be seen that of the three complexes only the complex containing the 3′-tailed oligonucleotide was efficiently unwound by T-ag. These data are compatible with initial binding of large T-ag helicase to single-stranded DNA and with a 3′→5′ polarity of migration of the enzyme (see Discussion).

## DISCUSSION

The data presented in this paper indicate that SV40 large T-ag helicase is capable of unwinding the third strand of d(TC)$_i$·d(GA)$_i$·d(TC)$_i$ triplexes. The requirements for this activity of T-ag helicase are apparently similar to the previously established requirements for unwinding of double-stranded sequences within DNA molecules lacking an SV40 origin of DNA replication. To be efficiently unwound by T-ag double-stranded DNA in such molecules must be flanked by single-stranded DNA containing a 3′-end (37,39). Similarly, unwinding of the third strand of the d(TC)$_{20}$·d(GA)$_{20}$·d(TC)$_{20}$ triplexes occurred in the presence of a single-stranded 3′-tail attached to the third strand, but not in the presence of a 5′-tail or in the absence of a single-stranded tail. In addition, ATP was found to be

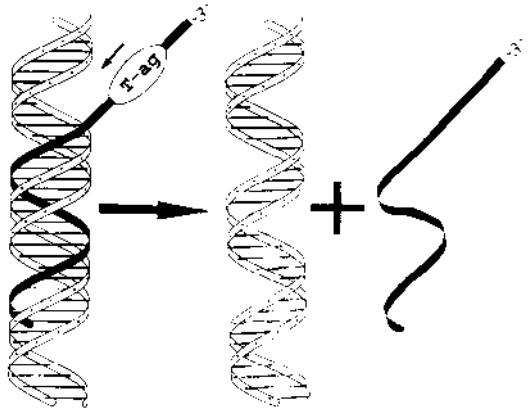


**Figure 7.** Schematic illustration of unwinding of the third strand in $d(TC)_i \cdot d(GA)_i \cdot d(TC)_i$ triplexes by the SV40 large T-ag helicase. For an explanation see Discussion.

required for unwinding of the third strand and could not be replaced by the non-hydrolysable ATP analog AMP-PNP. Furthermore, since AMP-PNP stimulates binding of T-ag to single-stranded DNA to a similar extent to ATP (40), it is clear that mere binding of T-ag to the 3′-tail of the third strand does not cause unwinding of this strand. Thus the triplex unwinding reaction is apparently coupled to ATP hydrolysis, like duplex unwinding by T-ag helicase (40). We have not yet carried out detailed studies of the efficiencies of triplex unwinding versus duplex unwinding by T-ag helicase. Nevertheless, a comparison of the present data with previous data on duplex unwinding (27) indicates that the concentrations of T-ag needed for unwinding a duplex substrate and the third strand in a triplex substrate are within the same order of magnitude.

Figure 7 presents a graphic illustration of the triplex unwinding reaction suggested by the data discussed above. Apparently T-ag first binds the 3′-tail of the oligonucleotide in the complex with the linear duplex molecules and then migrates in a 3′→5′ direction, using energy provided by ATP hydrolysis. As it encounters the triplex it causes unwinding of the third strand, such that at the end of the reaction the oligonucleotide is released and the duplex remains intact. Unwinding of the third strand by T-ag helicase indicates that the enzyme is not only capable of disrupting Watson–Crick hydrogen bonds, but may also cause disruption of Hoogsteen hydrogen bonds. This inference has gained further support from more recent experiments which indicated that T-ag helicase can also disrupt Hoogsteen hydrogen bonds in DNA quadruplexes which, unlike DNA triplexes, do not contain any Watson–Crick hydrogen bonds (Pukshansky, Baran and Manor, unpublished data).

Although the data reported here did not provide information on details of triplex unwinding by T-ag helicase, it is interesting to consider possible mechanisms related to mechanisms suggested for duplex unwinding by helicases. The third strand in $d(TC)_i \cdot d(GA)_i \cdot d(TC)_i$ triplexes might be unwound by a passive 'creeping' mechanism (25,26). In this process the 3′-end of the third strand could be transiently released at the single strand–triple strand junction as a result of fraying of the end of the triplex. Then the released strand might be trapped by the approaching helicase. Next, the helicase could be translocated and trap the next segment of the frayed third strand and so on. This mechanism requires ATP hydrolysis only for translocation of the helicase and does not require the helicase to bind double-stranded or triple-stranded DNA. Furthermore, only one or a few nucleotides would be released by this mechanism at each step of unwinding.

In other previously proposed mechanisms for DNA duplex unwinding by helicases ATP binding and hydrolysis play a role not only in translocation of the enzymes along single-stranded DNA, but also in promoting conformational changes of helicase subunits bound to duplex DNA; such conformational changes presumably cause duplex unwinding (25). Analogous mechanisms of triplex unwinding by SV40 large T-ag helicase would require binding of T-ag to the triplex, for which there is no experimental evidence.

Even though the existence of both intramolecular and intermolecular DNA triple helices has been well documented *in vitro*, there is only scant evidence in support of DNA triplex formation in living cells (41–43). On the other hand, there is some evidence that sequences which could potentially form DNA triplexes *in vivo* may affect biological processes and that these effects might be caused by triplex formation. For example, we and others have found that $d(TC)_n \cdot d(GA)_n$ tracts may cause pausing and arrest of DNA replication and amplification in living cells (29,44–48). Moreover, studies performed *in vitro* have indicated that DNA polymerases are inhibited by formation of $d(TC)_i \cdot d(GA)_i \cdot d(TC)_i$ or $d(GA)_i \cdot d(GA)_i \cdot d(TC)_i$ or other related triplexes (49–51). These studies and the observation that SV40 large T-ag helicase and other cellular helicases are also inhibited by formation of $d(TC)_i \cdot d(GA)_i \cdot d(TC)_i$ triplexes (27; Peleg, Kopel, Thommes, Hübscher and Manor, unpublished results) have indicated that in living cells inhibition of polymerases and helicases by formation of triplexes could be the cause of the observed pausing and arrest. However, the prevalence of such sequences in eukaryotic genomes indicates that they cannot function as replication arrest signals under all circumstances, for they must themselves be replicated. Therefore, it appears reasonable that helicases may unwind the third strand of triplexes, as the present study shows. It is also possible that in living cells $d(TC)_n \cdot d(GA)_n$ tracts can become associated with proteins which they selectively bind (see for example 52,53). Such association might alter the frequency of duplex→triplex transitions and thereby affect the interactions of these sequences with helicases and polymerases. Thus replication arrests at these sequences may be regulated and only occur under appropriate circumstances.

It has also been reported that $d(TC)_n \cdot d(GA)_n$ and other polypurine·polypyrimidine tracts may facilitate recombination (54–56). Moreover, evidence has been presented that this characteristic too might be related to the ability of the polypurine·polypyrimidine tracts to form triplexes (56). Hence, triplex unwinding by helicases may also affect recombination events. It should be noted in this connection that the triplexes formed by polypurine·polypyrimidine tracts are distinguishable from triplexes which might be generated by enzyme catalyzed interactions between homologous sequences in the course of recombination events (57–60). In these triplexes the third strand is parallel to the strand containing the identical sequence (57–60), while in triplexes formed by polypurine·polypyrimidine tracts the corresponding strands are antiparallel (19,20).

Finally, viral origins of DNA replication, such as the polyomavirus and SV40 origins, and yeast origins contain polypurine·polypyrimidine tracts (61–64). These tracts might form DNA triplexes and helicases such as SV40 large T-ag helicase may be required to unwind these triplexes, as well as DNA duplexes, to allow initiation of replication.

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