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Formation of DNA triplexes accounts for arrests of DNA synthesis at $d(TC)_n$ and $d(GA)_n$ tracts

(Hoogsteen hydrogen bonds/triple helices/polypurines and polypyrimidines/DNA replication/gene amplification)

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ABSTRACT **To study the mechanism of arrest of DNA synthesis at $d(TC)_n$ and $d(GA)_n$ sequences, single-stranded DNA molecules including $d(TC)_{27}$ or $d(TC)_{31}$ tracts or a $d(GA)_{27}$ tract were used as templates for *in vitro* assays of complementary DNA synthesis performed by extension of a primer with the Klenow polymerase or the Taq polymerase (*Thermus aquaticus* DNA polymerase). Electrophoresis of the products revealed that arrests occurred around the middle of these tracts. The arrests in the $d(TC)_n$ sequences were eliminated when dATP or dGTP was replaced with the analogue 7-deaza dATP or 7-deaza dGTP, respectively, or when the templates were preincubated with the *Escherichia coli* single-strand binding protein (SSB). Preincubation of the template including a $d(GA)_{27}$ tract with SSB has also eliminated the arrests at this sequence. Furthermore, arrests did not occur at $d[G(7\text{-deaza A})]_{27}$ or $d[(7\text{-deaza G})A]_{27}$ tracts when molecules including such tracts were used as templates. These results are compatible with the notion that the arrests were caused by formation of $d(TC)_i$·$d(GA)_i$·$d(TC)_i$ and $d(GA)_i$·$d(GA)_i$·$d(TC)_i$ triplexes, in which the bases in the uncopied portions of the $d(TC)_n$ tracts, or of the $d(GA)_{27}$ tract, and the purine bases in the newly synthesized $d(TC)_i$·$d(GA)_i$ duplexes were bound by hydrogen bonds. In the assays performed with the Taq polymerase, the pH dependence (in the range of 6.0–9.0) and the temperature dependence of the arrests were determined. As the pH was lowered, the arrests in the $d(TC)_{27}$ tract were enhanced, in line with the expected properties of $d(TC)_i$·$d(GA)_i$·$d(TC)_i$ triplexes. The arrests in the $d(GA)_{27}$ tract were enhanced by an increase in the pH. At pH 7.2 the arrests in the $d(GA)_{27}$ tract persisted up to 80°C, whereas the arrests in the $d(TC)_{27}$ tract were eliminated at 50°C; these results presumably reflect the relative stabilities of the two triplexes mentioned above at this physiological pH value and could be biologically significant.**

Previous studies performed in living cells have indicated that $d(TC)_n$·$d(GA)_n$ sequences, which are highly dispersed in mammalian genomes (1–6), may be pause or arrest sites for DNA replication and amplification. First, in a polyomavirus-transformed rat cell line, wherein multiple rounds of replication of the chromosomally associated viral DNA could be induced by exposing the cells to carcinogens (7–9), the multifork replication was found to proceed into the flanking cell DNA and to be arrested at, or next to, a $d(TC)_{27}$·$d(GA)_{27}$ tract (9, 10). Subsequently, a 210-base-pair (bp) segment including this tract has been cloned into simian virus 40 and found to cause a pause in replication of the viral DNA in monkey cells infected with the mutant virus (11). More recently, single-stranded DNA molecules, including either $d(TC)_n$ or $d(GA)_n$ sequences of various lengths, were used as templates for *in vitro* synthesis of complementary DNA chains with bacterial or mammalian DNA polymerases. In these assays, DNA synthesis was arrested around the middle of $d(TC)_n$ and $d(GA)_n$ tracts of $n \geq 16$ repeats (12).

Following earlier studies, in which formation of triple helices consisting of oligopurines and oligopyrimidines has been demonstrated (13, 14), it was found that $d(TC)_n$·$d(GA)_n$ tracts cloned in double-stranded supercoiled DNA circles may undergo a transition into structures including $d(TC)_i$·$d(GA)_i$·$d(TC)_i$ triplexes (15–19). Triple helices may also be generated by association of $d(TC)_n$·$d(GA)_n$ duplexes with $d(TC)_n$ oligonucleotides (20–22). Recently, it was shown that a poly(dG)·poly(dC) sequence cloned into supercoiled DNA circles may undergo transitions into structures including either poly(dC)·poly(dG)·poly(dC) or poly(dG)·poly(dG)·poly(dC) triplexes (23). In view of these data, it has been proposed that the arrests of DNA synthesis observed in $d(TC)_n$ and $d(GA)_n$ tracts were caused by binding of the uncopied portions of these tracts to the newly synthesized $d(GA)_i$·$d(TC)_i$ duplexes, such that DNA triplexes were generated (12). Here we present experiments that lend support to this hypothesis.

MATERIALS AND METHODS

DNA Synthesis Catalyzed by the Klenow Polymerase. Single-stranded DNAs including $d(TC)_{27}$ or $d(TC)_{31}$ tracts or a $d(GA)_{27}$ tract were prepared from the phage M13 recombinants designated mpTC27, mpTC31, and mpGA27 (12) and were used as templates for DNA synthesis as described (12). Briefly, a single-stranded DNA template was annealed with the M13 17-mer sequencing primer (New England Biolabs). The primer was extended in a buffer containing 5 mM Tris·HCl (pH 8.0), 2.5 mM $MgCl_2$, 1.2 μM [^{32}P]dATP or [^{32}P]dCTP, and unlabeled deoxyribonucleotides (dNTPs) and dideoxyribonucleotides (ddNTPs) as previously specified (12). The polymerase/template ratio was 10 units/μg of DNA. The reaction mixtures were incubated 20 min at 33°C; then, each of the four dNTPs was added (150 μM) and the mixtures were further incubated 15 min at 33°C. The products were electrophoresed in sequencing gels and autoradiographed (12). In the reactions performed with the *Escherichia coli* single-strand binding protein (SSB; obtained from Z. Livneh, Weizmann Institute), the template–primer complexes were preincubated with SSB at 5.0 μg of SSB per 1.0 μg of DNA, before addition of the polymerase. These assays were performed at pH 7.5.

Synthesis of Single-Stranded DNA Containing 7-deaza A or 7-deaza G. DNA strands containing 7-deaza A were synthesized by a polymerase chain reaction using single-stranded mpTC27 phage DNA (at 2.5 μg/ml) as a template and the M13 17-mer (at 2.5 μg/ml) as a (single) primer. The reaction mixtures also contained 300 μM (each) 7-deaza dATP, dCTP, dGTP, and dTTP, 50 units of the Taq DNA polymerase (*Thermus aquaticus* DNA polymerase) per ml (New England

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Abbreviation: SSB, single-strand binding protein.
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Biolabs), 10 mM Tris·HCl (pH 8.3) (determined at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (wt/vol), 0.01% Tween 20, and 0.01% Nonidet P-40. Twenty-five cycles of amplification, including a polymerization step of 2 min at 65°C, were performed. Then, 50 units of fresh Taq polymerase per ml was added, and 25 additional cycles of the reaction were performed. DNA strands containing 7-deaza G, or the four normal bases, were similarly synthesized, except that the nucleotide precursors were dATP, dCTP, dTTP, and 7-deaza dGTP, or the four normal dNTPs, respectively. The amplified DNA was extracted with a mixture of phenol/chloroform/isoamyl alcohol (12) and purified by Sephadex G-50 spun column chromatography, followed by spin dialysis in Centricon 30 microconcentrators (Amicon) (24).

DNA Synthesis Catalyzed by the Taq Polymerase. Fifty micrograms of each single-stranded DNA template per milliliter was annealed 5 min at 68°C with 1.0 μg of a primer per ml in a buffer containing 100 mM Tris·HCl and 10 mM $MgCl_2$ at the pH values indicated in Figs. 4 and 5. The annealing mixtures were cooled 10 min at 25°C. DNA synthesis was performed in 7 μl of a buffer containing 50 mM Tris·HCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 60 μCi of [^{32}P]dATP (600 Ci/mmol; 1 Ci = 37 GBq), 25 μg of template–primer complex per ml, and 240 units of Taq DNA polymerase per ml. The pH, temperature, and duration of the reactions are specified in Figs. 4 and 5.

RESULTS

Mechanism of Arrest of DNA Synthesis at d(TC)$_n$ Tracts. Fig. 1*a* displays a model that could account for the arrests observed at d(TC)$_n$ repeats. According to this scheme, a DNA chain complementary to a single-stranded DNA molecule including a d(TC)$_n$ tract is synthesized by extension of a primer. Once the d(TC)$_n$ tract is partially copied, the uncopied part folds back and binds to the d(GA)$_i$ strand in the major groove of the d(TC)$_i$·d(GA)$_i$ duplex. Thus, d(TC)$_i$·d(GA)$_i$·d(TC)$_i$ triplexes are generated, containing the C^+GC and TAT base triplets shown in Fig. 2*a*, in which the pyrimidines of the third strand are joined to the purines by Hoogsteen hydrogen bonds (see refs. 15–19). The most stable triplexes of this type should occur around the middle of the d(TC)$_n$ tract, and their formation should arrest DNA synthesis by causing dissociation of the polymerase or by preventing binding of previously dissociated polymerase molecules (12).

To test this model, the nucleotide analogue 7-deaza dATP or 7-deaza dGTP was used in assays of DNA synthesis instead of dATP or dGTP. In these compounds, the N-7 atoms of the bases A and G, which are indicated with arrows in Fig. 2*a*, are replaced with carbon atoms. These replacements eliminate one of the two Hoogsteen hydrogen bonds found in the TAT, or the C^+GC triplets. Therefore, the use of these analogues should prevent the formation of d(TC)$_i$·d(GA)$_i$·d(TC)$_i$ triplexes and eliminate the arrests.

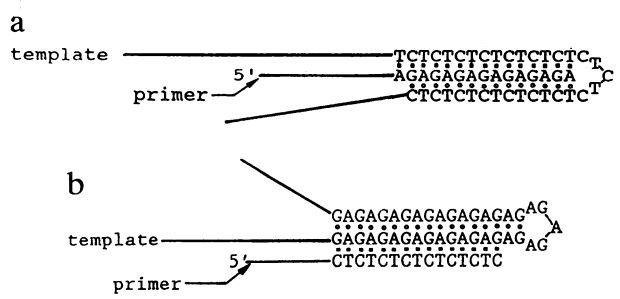

FIG. 1. Triple helix models for arrests of single-stranded DNA synthesis at d(TC)$_n$ and d(GA)$_n$ tracts. (*a*) Model for arrest of DNA synthesis at d(TC)$_n$ tracts (see details in the text). (*b*) Model for arrest of DNA synthesis at d(GA)$_n$ tracts. ■, Watson–Crick hydrogen bonds; ○, other hydrogen bonds.

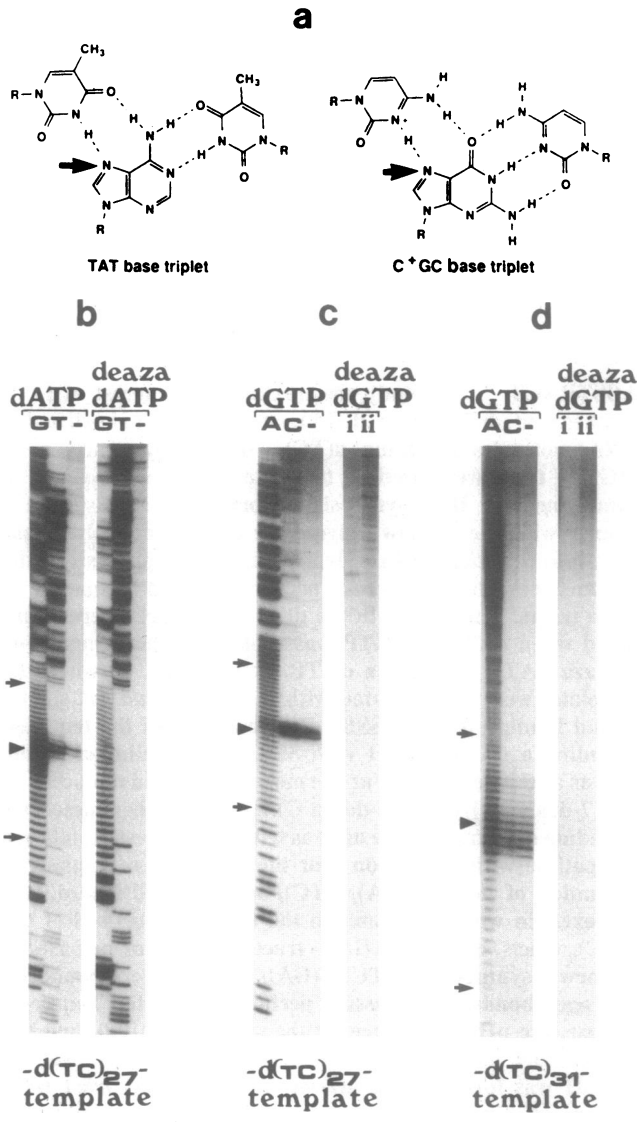

FIG. 2. Effect of the nucleotide precursor analogues 7-deaza dATP and 7-deaza dGTP on arrests of DNA synthesis at d(TC)$_n$ tracts. (*a*) Alternating TAT and C^+GC base triplets found in the triplex shown in Fig. 1*a*. The arrows indicate the N-7 atoms that are replaced with carbon atoms in 7-deaza adenine and in 7-deaza guanine. (*b*) Single-stranded DNA including a d(TC)$_{27}$ tract was used as a template for *in vitro* synthesis of complementary strands by extension of an M13 primer with the Klenow polymerase. In the lanes designated dATP, dATP and the three other dNTPs were used for DNA synthesis. In the lanes designated deaza dATP, dATP was replaced with 1.2 μM 7-deaza dATP. In the lanes designated G and T, the assays were performed in the presence of dideoxy GTP and dideoxy TTP, respectively. In the lanes designated (−), the assays were performed in the absence of any ddNTP. The products were electrophoresed in sequencing gels and autoradiographed as described (12). The arrows indicate the boundaries of the d(TC)$_{27}$ tract and the arrowhead points at the arrests. (*c*) The same as *b*, except that in the lanes designated deaza dGTP, dGTP was replaced with 7-deaza dGTP at a concentration of 1.0 μM (lane i) or 2.5 μM (lane ii). In the lanes designated A and C, the reactions were carried out in the presence of dideoxy ATP and dideoxy CTP, respectively. (*d*) The same as *c*, except that the template was single-stranded DNA including a d(TC)$_{31}$ tract (12).

Fig. 2 *b–d* displays sequencing gel patterns of the products obtained in these assays. In the experiments shown in Fig. 2*b*, the template was an M13 recombinant DNA molecule including a d(TC)$_{27}$ tract, the primer was a 17-mer complementary to M13 DNA, the replicating enzyme was the Klenow polymerase, and the precursors were dCTP, dGTP,

dTTP, and either dATP or 7-deaza dATP. Some reactions were performed in the presence of dideoxy GTP or dideoxy TTP. It can be seen that in the reactions performed with dATP, strong arrests, visualized as bands appearing across all lanes, occurred around the middle of the $d(TC)_{27}$ tract. Replacement of dATP with 7-deaza dATP caused elimination of the arrests in the $d(TC)_{27}$ tract but otherwise did not affect DNA synthesis. Similar results were obtained when dGTP was replaced with 7-deaza dGTP in reactions performed with the same template (Fig. 2*c*) or with a template including a $d(TC)_{31}$ tract (Fig. 2*d*). These data indicate that formation of Hoogsteen hydrogen bonds accounts for the arrests observed in the $d(TC)_n$ tracts and are, therefore, consistent with the model shown in Fig. 1*a*.

In a second test of this model, the effect of the *E. coli* SSB upon the arrests at the $d(TC)_{27}$ tract was examined. SSB binds strongly and cooperatively to single-stranded DNA molecules but does not inhibit replication of such molecules (25). SSB does not bind to double-stranded DNA (25). Therefore, in our assays, SSB that is bound to the template would be displaced from the copied portion of the $d(TC)_{27}$ tract but should remain associated with the uncopied portion. This association may prevent the interaction between these two portions and allow read-through by the polymerase. Fig. 3*a* shows that, indeed, arrests that occurred around the middle of the $d(TC)_{27}$ tract in the absence of SSB were eliminated in the presence of SSB.

Mechanism of Arrest of DNA Synthesis at $d(GA)_n$ Tracts. As mentioned above, DNA synthesis was also found to be arrested around the middle of $d(GA)_n$ tracts (12). The apparent similarity of the arrest profiles observed in $d(GA)_n$ and $d(TC)_n$ tracts suggested that similar interactions may account for the arrests at both sequences. To examine this hypothesis, assays of DNA synthesis analogous to those shown in Fig. 3*a* were performed with a single-stranded DNA template including a $d(GA)_{27}$ tract. These assays have shown (Fig. 3*b*) that arrests that occurred around the middle of the $d(GA)_{27}$ tract in the absence of SSB were eliminated by preincubation of the template with SSB. A plausible model that could account for these results is illustrated in Fig. 1*b*. This model assumes that during DNA synthesis the uncopied portion of the $d(GA)_{27}$ tract binds to the $d(GA)_i$ strand of the newly synthesized $d(GA)_i{\cdot}d(TC)_i$ duplex through formation of hydrogen bonds between the purine bases. Thus, $d(GA)_i{\cdot}d(GA)_i{\cdot}d(TC)_i$ triplexes are generated and cause arrests of DNA synthesis.

Another test of this model would be to experimentally determine whether formation of hydrogen bonds between the purines accounts for the arrests observed at $d(GA)_n$ tracts. For this purpose, we synthesized single-stranded DNA molecules including either a $d(GA)_{27}$, a d[G(7-deaza A)]$_{27}$, or a d[(7-deaza G)A]$_{27}$ tract, and we used these molecules as templates for assays of complementary DNA synthesis. As illustrated in Fig. 4*a* (*i*), the single-stranded DNA molecule including the $d(TC)_{27}$ tract was used as a template for multiple cycles of synthesis of the molecules including these oligopu-

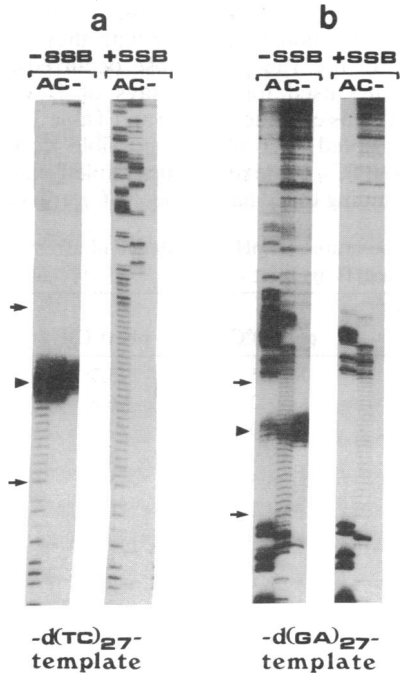

FIG. 3. Effect of the *E. coli* SSB on arrests of DNA synthesis at the $d(TC)_{27}$ and the $d(GA)_{27}$ tracts. (*a*) DNA synthesis was carried out with the Klenow polymerase in the absence of SSB (−SSB lanes) or in the presence of SSB (+SSB lanes) using the template including the $d(TC)_{27}$ tract. Other designations are defined in the legend to Fig. 2. (*b*) Same as *a*, except that the template was the single-stranded DNA molecule including a $d(GA)_{27}$ tract (12).

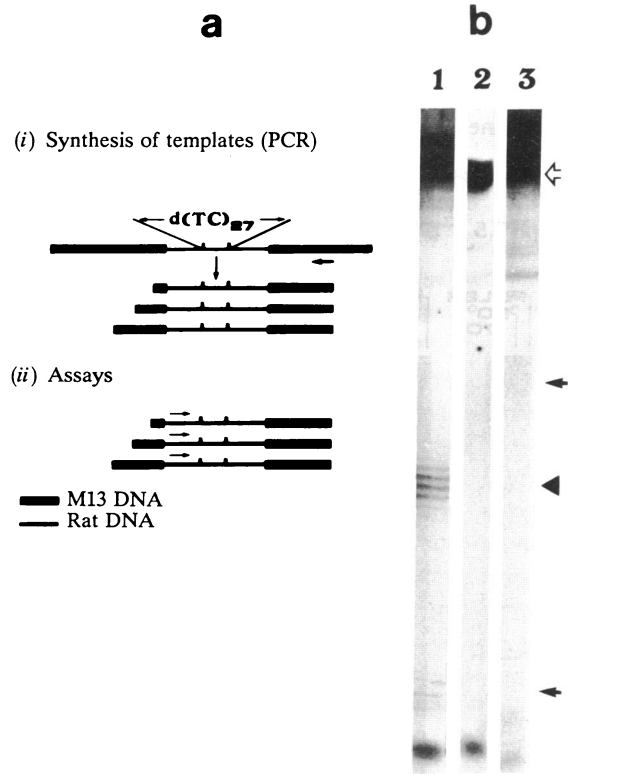

FIG. 4. Absence of arrests at d[G(7-deaza A)]$_{27}$ and d[(7-deaza G)A]$_{27}$ tracts. (*a*) Schematic illustration of the experiment. (*i*) The DNA template including a $d(TC)_{27}$ tract was used for synthesis of single-stranded DNA molecules including either a $d(GA)_{27}$, a d[G(7-deaza A)]$_{27}$, or a d[(7-deaza G)A]$_{27}$ tract. PCR, polymerase chain reaction. The short horizontal arrow indicates the M13 primer used for the synthesis. (*ii*) The single-stranded DNA molecules that were synthesized as illustrated in *i* were employed as templates for DNA synthesis assays performed with the Taq polymerase. The primer, 5′-AAATATATGTGACCAA-3′, employed for these assays was complementary to a rat DNA sequence mapping 220 nucleotides downstream of the M13 17-mer used for synthesizing the template molecules. This primer is indicated by short arrows. (*b*) The single-stranded DNA templates including a $d(GA)_{27}$ tract (lane 1), a d[G(7-deaza A)]$_{27}$ tract (lane 2), or a d[(7-deaza G)A]$_{27}$ tract (lane 3) were annealed with the primer shown in *a* (*ii*). DNA synthesis was performed for 10 min at 65°C and pH 9.0 (determined at 20°C). The small solid arrows indicate the boundaries of the oligopurine tracts, the arrowhead indicates the position of the arrests, and the open arrow indicates the fragment generated when the polymerase reached the end of each template—i.e., the 5′ terminus of the M13 17-mer primer.

rine tracts. This synthesis was performed with the Taq polymerase under conditions in which the enzyme was not arrested in the $d(TC)_{27}$ tract (see the next section). The Taq polymerase was also used for the assays of complementary DNA synthesis, which are illustrated schematically in Fig. 4*a* (*ii*). The results of these assays, which were performed under conditions that favored arrests in $d(GA)_n$ tracts, are displayed in Fig. 4*b*. It can be seen that arrests occurred in the $d(GA)_{27}$ tract but did not occur in the $d[G(7\text{-deaza A})]_{27}$ or the $d[(7\text{-deaza G})A]_{27}$ tract. These results support the notion that formation of hydrogen bonds (in which the N-7 atoms participate) between purine residues in the $d(GA)_{27}$ tract accounts for the arrests of DNA synthesis at this sequence repeat (see *Discussion*).

pH and Temperature Dependence of Arrests at $d(TC)_n$ and $d(GA)_n$ Tracts. We reasoned that the temperature and pH dependence of the arrests of DNA synthesis at $d(TC)_n$ and $d(GA)_n$ tracts should provide information on the stability of the structures that cause the arrests, and used the Taq polymerase to perform such studies. Fig. 5*a* shows the results of assays of the single-stranded DNA template including the $d(TC)_{27}$ tract that were performed at pH values of 6.5 or 7.2 (determined at 20°C). The lanes designated "pulse" show the products of reactions that were carried out 45 sec at 60°C. In the other lanes, a similar pulse was followed by incubation

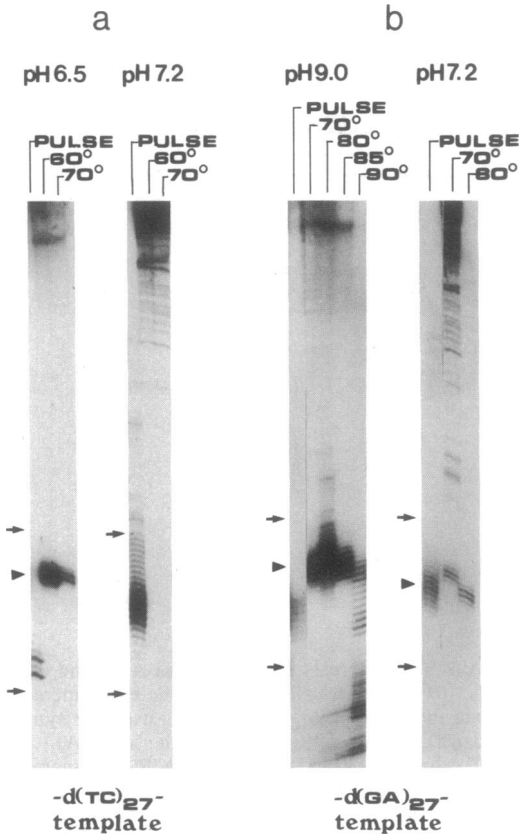

FIG. 5. pH and temperature dependence of arrests of the Taq polymerase at the $d(TC)_{27}$ and the $d(GA)_{27}$ tracts. (*a*) The DNA template including the $d(TC)_{27}$ tract, the M13 17-mer primer, and the Taq polymerase were used for assays of complementary DNA synthesis. The lanes designated pH 6.5 and pH 7.2 display the products of assays performed at pH 6.5 and pH 7.2 (determined at 20°C). The lanes designated "PULSE" and 60°C show the products of reactions that were carried out 45 sec and 16 min, respectively, at 60°C. The lanes designated 70°C show the products of reactions that were initiated at 60°C and after 45 sec were transferred to 70°C and allowed to proceed for 15 additional min. (*b*) The same as *a*, except that the pH values and temperatures were as specified, and the template was the single-stranded DNA molecule including the $d(GA)_{27}$ tract.

periods of 15 min at 60°C and 70°C. The extension of the primer during the pulse ensured that the primer did not dissociate from the template at temperatures of >60°C. These and additional data are summarized in Table 1. Table 1 also shows the pH values determined at the temperatures in which the reactions were performed, which were considerably lower than the values determined at 20°C. It can be seen that in the reactions performed at pH 6.5 (determined at 20°C) and at temperatures of 50°C, or 60°C, strong arrests occurred in the $d(TC)_{27}$ tract; much less prominent arrests occurred at 70°C. However, when the pH was ≥7.2, no arrests were detectable even at 50°C.

Fig. 5*b* and Table 2 show the pH and temperature dependence of arrests of the Taq polymerase at the $d(GA)_{27}$ tract. It can be seen that in reactions performed at pH 9.0 (determined at 20°C), strong arrests occurred at temperatures of 70°C, 80°C, and 85°C. At 90°C the reaction was inhibited such that the polymerase did not extend the DNA chains beyond the site attained during the 45-sec pulse. Arrests of lesser strengths were observed at a pH of 7.2 (determined at 20°C). Nevertheless, even when the temperature was raised to 80°C, the arrests were not completely eliminated at this pH value. At higher temperatures, the reactions were inhibited.

DISCUSSION

The hypothesis that formation of $d(TC)_f$·$d(GA)_f$·$d(TC)_i$ triplexes accounts for the arrests of DNA synthesis at $d(TC)_n$ tracts (Fig. 1*a*) is supported by the elimination of the arrests in the assays performed with the dNTP analogues (Fig. 2) or in the presence of SSB (Fig. 3*a*). This model is also supported by the observation that the arrests occurred around the middle of the $d(TC)_n$ tracts, for in this region the longest and the most stable triple helices should be generated. The pH dependence of the arrests in the $d(TC)_n$ tracts is also consistent with this model, for, as shown in Fig. 2*a*, formation of the G^+GC triplets in $d(TC)_f$·$d(GA)_f$·$d(TC)_i$ triplexes depends upon hemiprotonation of the cytosines. Hence, such triplexes should be destabilized by an increase in the pH and the arrests should be eliminated. In line with these predictions, strong arrests of the Taq polymerase occurred only at the lowest pH value used for the assays of DNA synthesis performed with this enzyme (Fig. 5*a* and Table 1; see ref. 12). It should be noted that of two possible conformers of $d(TC)_f$·$d(GA)_f$·$d(TC)_i$ triplexes in supercoiled plasmid DNA, molecules including the 3′ half of the $d(TC)_n$ tract as the third

Table 1. Temperature and pH dependence of arrests of the Taq polymerase at a $d(TC)_{27}$ tract

Temperature, °C	pH (20°C)*	pH (t°C)†	Arrest‡
50	6.5	5.9	++
	7.2	6.6	−
	8.0	7.5	−
	9.0	8.5	−
60	6.5	5.6	++
	7.2	6.3	−
	8.0	7.3	−
	9.0	8.3	−
70	6.5	5.4	+
	7.2	6.2	−
	8.0	7.2	−
	9.0	8.2	−
75	6.5	ND	RI

Assays of DNA synthesis were performed as shown in Fig. 5*a*.
*The pH of each solution was determined at 20°C.
†The pH of each solution was determined at the temperature at which the reaction was performed. ND, not determined.
‡++, Strong arrest; +, arrest of moderate strength; −, arrest not detectable. RI, the reaction was inhibited.

Table 2. Temperature and pH dependence of arrests of the Taq polymerase at a $d(GA)_{27}$ tract

Temperature, °C	pH (20°C)*	pH (t°C)†	Arrest‡
60	7.2	6.3	+
	9.0	8.3	++
70	7.2	6.2	+
	9.0	8.2	++
80	7.2	6.1	+
	9.0	8.0	++
85	7.2	ND	RI
	9.0	ND	++
90	7.2	ND	RI
	9.0	ND	RI

Assays of DNA synthesis were performed as shown in Fig. 5*b*. *†‡, Same notations as in Table 1.

strand were found to be the major conformer (16–18). However, triplexes of the type proposed here to account for the arrests of DNA synthesis at $d(TC)_n$ tracts (Fig. 1*a*), in which the 5′ half of the $d(TC)_n$ tract is the third strand, have been also found in supercoiled plasmid DNAs (26).

The observation that the arrests occurring at the $d(GA)_{27}$ tract were eliminated by SSB and the experiments showing that DNA synthesis did not stop at $d[G(7\text{-deaza A})]_{27}$ and $d[(7\text{-deaza G(A)}]_{27}$ tracts have indicated that formation of hydrogen bonds between the purines in the uncopied and the copied portions of $d(GA)_n$ tracts accounted for the arrests at these oligopurine repeats. It has been reported that single-stranded oligopurine chains may associate in the absence of complementary oligopyrimidines (14, 27–29). However, we failed to detect interstrand associations between the single-stranded DNA molecules including the $d(GA)_{27}$ tract under the conditions of our assays (N.B., unpublished results). On the other hand, the $d(GA)_i$·$d(GA)_i$·$d(TC)_i$ triplex arrest model shown in Fig. 1*b* is consistent with the data discussed above and with the observation that the arrests occurred around the middle of the $d(GA)_n$ tracts. We have already pointed out that (poly dG)·(poly dG)·(polydC) triplexes have been previously found to form in supercoiled plasmid DNA (23). Recently, $d(GA)_i$·$d(GA)_i$·$d(TC)_i$ triplexes were also found to form in supercoiled plasmid DNAs in the presence of Zn^{2+} ions (30). In these triplexes, as in the triplexes proposed to account for the arrests of DNA synthesis at $d(GA)_n$ tracts [but unlike the (poly G)·(polyG)·(polyC) triplexes], the 5′ half of the $d(GA)_n$ tract was found to be the third strand. Formation of poly(A·A·U) triplexes has been also observed in solutions of poly(A) and poly(U) (31).

The observation that a $d(TC)_{27}$·$d(GA)_{27}$ tract apparently stops multifork "onion skin" replication in a polyomavirus-transformed rat cell line (10) has suggested that the highly dispersed $d(TC)_n$·$d(GA)_n$ repeats may stop similar gene amplification events in many replicons (32); this would be a useful function, since it should be advantageous to cells to impose limits on the extent of the amplified regions. $d(TC)_n$·$d(GA)_n$ tracts might also serve as pause or arrest signals for normal chromosomal DNA replication. However, the recent discovery of $d(TC)_n$·$d(GA)_n$ repeats in the initiation region of an amplified dihydrofolate reductase replicon (33) has indicated either that these sequences may not completely stop replication and amplification at all chromosomal sites or that arrests of replication occur at these sequences in just one orientation relative to the direction of replication. It is interesting to note, in this connection, our observation that at physiological pH values the arrests at the $d(GA)_{27}$ tract survived much higher temperatures than the arrests at the $d(TC)_{27}$ tract (Fig. 5 and Tables 1 and 2). Such a result would be expected if, under these conditions, $d(GA)_i$·$d(GA)_i$·$d(TC)_i$ triplexes were more stable than $d(TC)_i$·$d(GA)_i$·$d(TC)_i$ triplexes and suggests a possible mechanism for orientation-dependent arrests of replication at $d(TC)_n$·$d(GA)_n$ tracts; it is conceivable, for example, that the replication may be arrested only in the orientation placing the $d(GA)_n$ tract in the template strand for the retrograde chain of the replication fork. On the other hand, it is possible that orientation-dependent arrests occur in $d(TC)_n$·$d(GA)_n$ tracts due to binding of a specific asymmetric protein that blocks replication, as observed in bacteria (34–36). A $d(TC)_n$·$d(GA)_n$ binding protein found in rodents and primates (H.M., unpublished results) could perform this function.

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