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Template-directed pausing in *in vitro* DNA synthesis by DNA polymerase α from *Drosophila melanogaster* embryos

(DNA replication/template signals/termination)

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ABSTRACT **The activity of *Drosophila melanogaster* DNA polymerase α on DNA-primed single-stranded DNA templates has been examined. The DNA templates contain a 1471-nucleotide sequence from the heavy-strand origin region of mouse mtDNA inserted into the single-stranded bacteriophage vector M13G*ori*1. Preferred sites for pausing of *in vitro* DNA synthesis have been mapped within the cloned mtDNA insert and in the G4 cDNA strand origin which is contained within the vector DNA. Analysis of nascent DNA strands from replicative intermediates has revealed that pause sites are discrete and lie both at the positions of predicted stable dyads and in regions lacking the potential for formation of such structures. The pattern of kinetic pause sites observed for *Escherichia coli* DNA polymerase III holoenzyme is qualitatively similar to that found for DNA polymerase α. A subset of the observed kinetic pause signals are recognized by *E. coli* DNA polymerase I under similar conditions.**

We have examined the influence of template sequence and structure on polymerization by *Drosophila melanogaster* DNA polymerase α by constructing single-stranded templates for *in vitro* DNA synthesis which contain DNA sequences of functional importance *in vivo*. The heavy-strand (H-strand) origin region of mouse mtDNA was inserted into the duplex replicative form of the single-stranded bacteriophage vector M13G*ori*1. The 3′ ends of the five major displacement-loop (D-loop) strands of mouse mtDNA have been mapped to clusters of three to five nucleotides (nt) on the light-strand (L-strand) template in the D-loop region (1). Based on the identification of four repeated sequences which lie 24–63 nt upstream from the termination sites of D-loop strands, Doda *et al.* (1) postulated that the arrest of D-loop strand elongation is a template sequence-associated event. The vector M13G*ori*1 contains the bacteriophage G4 cDNA strand origin ($G4_{ori_c}$) (2) which has the potential to form two stable hairpin structures.

Sites of interruption of elongation on single-stranded DNA by *Escherichia coli* DNA polymerase II (3), vaccinia DNA polymerase (4), and T4 DNA polymerase (5) have been correlated with the positions of predicted stable hairpin structures in the DNA template. Furthermore, the affinity of KB cell polymerase α for primed single-stranded homo- and heteropolymers was shown to be markedly affected by the base composition of the primer template and by added unprimed inhibitor DNAs (6).

We show here that kinetic pause sites for DNA polymerase α from *D. melanogaster* embryos map within several nt of the *in vivo* termination points for mtDNA D-loop strands, within the hairpin structures in the $G4_{ori_c}$, and at other sites lacking the potential for formation of stable hairpins. These results are consistent with the hypothesis that properties of template secondary structure or primary sequence cause arrest of DNA synthesis by DNA polymerase α.

MATERIALS AND METHODS

Enzymes and Nucleic Acids. DNA polymerase α from *D. melanogaster* embryos (fraction VI, 6×10^4 units/mg) (7), *E. coli* DNA polymerase III holoenzyme (fraction V, 1×10^5 units/mg) (8), *E. coli* DNA polymerase I [2.5×10^3 units/mg (on activated DNA)] (9), and *E. coli* single-strand binding protein (SSB) (fraction IV, 4×10^4 units/mg) (10) were kindly provided by B. Sauer, P. Burgers, S. Scherer, and J. Kaguni, respectively. M13-type single-stranded DNAs were prepared as described (11). Primer-templates were prepared by hybridizing purified restriction fragments (at twice the molar concentration of single-stranded circles) to M13H*ori*8 and M13H*ori*3 single-stranded circles (15 μg/ml) for 5 hr at 37°C in hybridization buffer (0.6 M NaCl/0.2 M Tris·HCl, pH 7.5/0.02 M EDTA/50% formamide) after denaturation of the restriction fragments.

Construction and Isolation of M13H*ori*8 and M13H*ori*3. The mouse mtDNA heavy-strand (H-strand) origin is contained within a 1471-base-pair (bp) *Hin*cII/*Bal* I DNA fragment extending from position 15,188 to position 364 on the mouse mtDNA sequence (12). The vector M13G*ori*1 (13) contains a single *Pvu* II cleavage site within the cloned G4 sequence. M13G*ori*1 replicative form was cleaved with *Pvu* II and ligated to the blunt-ended *Hin*cII/*Bal* I mtDNA fragment, and the resultant chimeric DNAs were cloned. Two isolates, M13H*ori*8 and M13H*ori*3, containing the mouse mtDNA H-strand origin fragment inserted in opposite orientations in M13G*ori*1, were utilized. M13H*ori*8 contains the L strand of the mtDNA fragment in the viral (+) strand of M13G*ori*1.

DNA Synthesis and Analysis of Reaction Products by Gel Electrophoresis. Incubation mixtures (25 μl) for DNA polymerase α reactions contained 20 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 4 mM dithiothreitol, bovine serum albumin at 200 μg/ml, 100 μM dCTP, dGTP, and dATP, 50 μM [α-^{32}P]dTTP ($1.5–2.0 \times 10^4$ cpm/pmol), 230 pmol of single-stranded DNA, and 10 units of DNA polymerase α. DNA polymerase III holoenzyme or DNA polymerase I reaction mixtures contained 10 mM $MgCl_2$, 1.6 mM spermidine·HCl, and 0.8 mM ATP in ad-

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Abbreviations: H strand, heavy strand; D loop, displacement loop; nt, nucleotide(s); L strand, light strand; $G4_{ori_c}$, G4 cDNA strand origin; bp, base pair(s); SSB, single-strand binding protein.

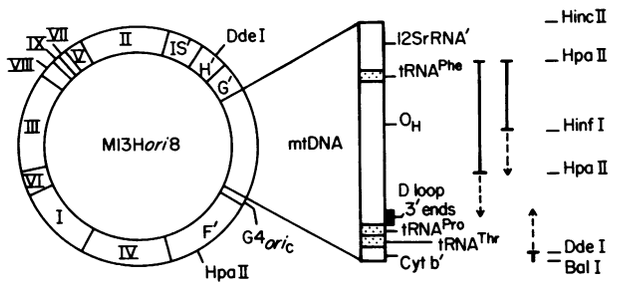

FIG. 1. Genetic map of the M13 chimeric phage genome M13H*ori*8, containing the mouse mtDNA origin of H-strand replication (O_H), in which both the 5′ and 3′ ends of D-loop strands map, and the adjacent $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Thr}$, and cytochrome *b* genes. M13 genes are indicated by Roman numerals (14), and G4 genes are indicated by capital letters (15). Primed letters indicate incomplete genes. The restriction fragments used to prime M13H*ori*8 and M13*ori*3 single-stranded DNA are indicated, and primer elongation proceeds in the direction of the dash-line arrows. Restriction fragments used to prime M13H*ori*8 single-stranded DNA contain the region of the 5′ ends of D-loop strands but do not extend into the region in which the 3′ ends map; the 692-nt *Hpa* II primer and the 434-nt *Hpa* II/*Hin*fI primer have the same 5′ end but differ by 258 nt at the 3′ end. M13H*ori*3 DNA was primed by a 717-nt *Dde* I fragment that has its 5′ end at the *Dde* I site within G4 DNA and its 3′ end at the *Dde* I site within the mtDNA insert.

dition to the above components, and 10 units of polymerase III holoenzyme or 0.2 unit of polymerase I, respectively. Reactions were carried out at 30°C. Products to be analyzed by polyacrylamide gel electrophoresis were made 1% in $NaDodSO_4$ and 20 mM in EDTA, heated for 10 min at 60°C, ethanol precipitated, digested with proteinase K at 1 mg/ml for 90 min at 37°C, and again ethanol precipitated. Unrestricted reaction products were denatured and electrophoresed in 4% polyacrylamide slab gels (13 × 30 × 0.15 cm) containing 7 M urea in 90 mM Tris borate (pH 8.3) and 2.5 mM EDTA.

RESULTS

Replication of DNA Fragment-Primed M13H*ori*8 DNA by *D. melanogaster* DNA Polymerase α. The *Hpa* II and *Hpa* II/*Hin*fI fragment-primed M13H*ori*8 DNAs were used to examine synthesis by DNA polymerase α through the mtDNA D-loop template region and the $G4_{ori_c}$ (Fig. 1). The rate of replication was nearly equal for the two DNA-primed templates (Fig. 2 *A* and *B*) which contained a comparable level of primers as shown by assay with *E. coli* DNA polymerase III holoenzyme in the presence of saturating levels of *E. coli* SSB. Nascent DNA strands from replicative intermediates were analyzed by gel electrophoresis. Because the *Hpa* II and *Hpa* II/*Hin*fI primer fragments had the same 5′ end but differed by 259 nt at their 3′ ends, extended DNA primer strands were expected to be of identical length if points of termination on the DNA template were the same. Discrete nascent DNA strands were present after 5 min and 15 min of incubation of both primer templates with DNA polymerase α (Fig. 3 *Left*). The 15 numbered species correspond to DNA strands of 536–1402 nt, indicating extension of the primers by 103–969 nt. The number and lengths of products were independent of the 3′ end of the primer, suggesting that the discrete points of termination of the DNA strands were template specific. Two additional products observed with the *Hpa* II/*Hin*fI fragment-primed template (lanes

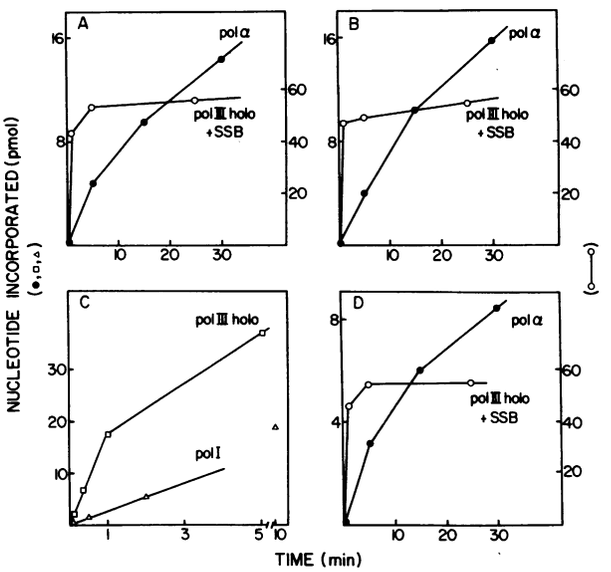

FIG. 2. Replication of DNA-primed M13H*ori*8 and M13H*ori*3 DNA by DNA polymerase α (pol α), *E. coli* polymerase III holoenzyme (pol III holo), and *E. coli* polymerase I (pol I). Reaction mixtures contained 0.6 μg of SSB where indicated. Each assay contained 230 pmol of M13H*ori*8 DNA (*A*, *B*, and *C*) or M13H*ori*3 DNA (*D*) primed by the *Hpa* II (*A*, *C*), *Hpa* II/*Hin*fI (*B*), or *Dde* I (*D*) fragments shown in Fig. 1.

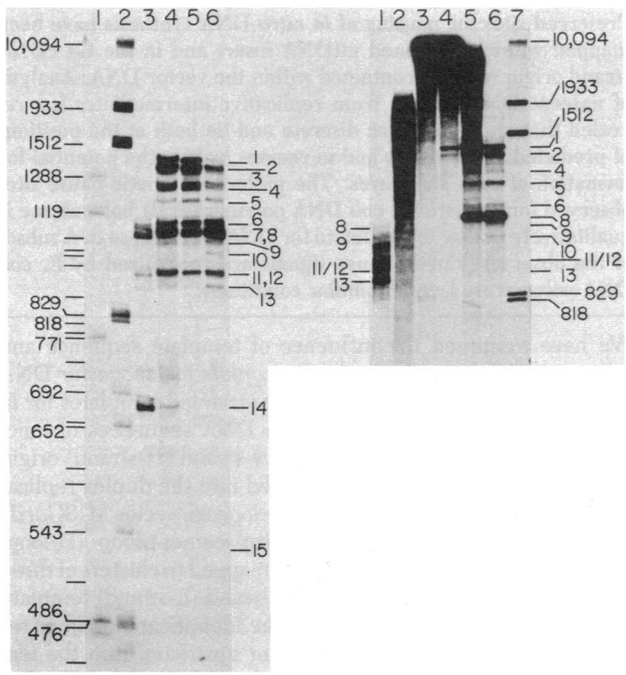

FIG. 3. Electrophoresis of *in vitro* products (17 μl) of DNA synthesis by DNA polymerases α, I, and III holoenzyme on 4% polyacrylamide/7 M urea gels. (*Left*) DNA polymerase α products resulting from 5 min and 15 min of DNA synthesis on *Hpa* II/*Hin*fI fragment-primed M13H*ori*8 DNA (lanes 3 and 4) and on *Hpa* II fragment-primed M13H*ori*8 DNA (lanes 5 and 6). The sizes of species 1–15 indicated on the right are 1402, 1340, 1240, 1222, 1170, 1080, 1040–1050, 1005, 970, 920, 883, 668, and 537 nt. Lanes 1 and 2 contain *Hin*fI and *Hpa* II restriction fragments of M13*bla*61 and M13H*ori*8 replicative form DNAs. The sizes of these fragments (in nt) are indicated on the left; also indicated are the positions of additional molecular weight markers electrophoresed in lanes not shown. (*Right*) DNA polymerase I and DNA polymerase III holoenzyme products resulting from DNA synthesis on *Hpa* II fragment-primed M13H*ori*8 DNA. Lanes 1–3: polymerase I reaction products after 30 sec, 2 min, and 10 min of DNA synthesis, respectively. The sizes of numbered species 8, 9, 11/12, and 13 indicated on the left are 1040, 1010, 912, and 887 nt. Lanes 4–6: polymerase III holoenzyme products after 1 min, 22 sec, and 8 sec of DNA synthesis, respectively. The sizes of numbered species 1–2, 4–6, and 8–13 indicated on the right are 1410, 1348, 1215, 1182, 1090, 1040, 1010, 971, 910, and 882 nt. Lanes 3 and 4 represent smaller aliquots (6.5 μl) of the reaction products. The 10,094-nt species is linear M13H*ori*8 single-stranded DNA.

3 and 4, numbers 14 and 15) mapped between the position of the 3′ end of the *Hpa* II/*Hin*fI primer and that of the *Hpa* II primer on the template.

To establish clearly that the elongated DNA strands resulted from differential extension of the two DNA primers to identical points on the DNA template, the reaction products were cleaved by *Hpa* II at the 3′ end of the *Hpa* II primer. Because the next *Hpa* II site in the template is 1512 nt from the *Hpa* II primer-end site (Fig. 1), and the largest synthetic DNA strand identified in Fig. 3 *Left* is 1402 nt (including the 692-nt primer itself), the lengths of the product strands observed should correspond to the lengths obtained from electrophoresis of the undigested reaction products after subtraction of the length of the *Hpa* II primer fragment. Furthermore, *Hpa* II cleavage of the reaction products should result in the generation of identical species for the two template primers. That this was the case was shown by electrophoresis of the *Hpa* II-digested products in a 6% polyacrylamide/7 M urea gel (autoradiograph not shown). Also consistent between these two analyses was the relative abundance of DNA strands at each position. The data suggest that various signals in the DNA template result in differential pausing in DNA synthesis. Because the DNA was not end labeled and initiation of synthesis was not synchronous, the efficacy of pause signals widely separated on the DNA template cannot be quantitated. However, it is evident that preferred pause sites exist among clusters of sites (Fig. 3 *Left*). Among the preferred pause sites are those that map in the $tRNA^{Pro}$ gene (corresponding to bands 6–8) and at the stems of two stable hairpin structures present in the $G4_{ori_c}$ (corresponding to bands 1 and 2).

To map the pause sites precisely within the mtDNA D-loop termination region of the template (those sites corresponding to DNA products 9–13 in Fig. 3 *Left*), and to determine the mode of termination of DNA strands at the pause sites (whether abrupt or gradual), *Hpa* II-cleaved denatured reaction products were subjected to electrophoretic sizing in parallel with the DNA sequence of a *Hpa* II/*Pst* I mouse mtDNA restriction fragment (Fig. 4). The *Hpa* II/*Pst* I DNA fragment was labeled at the same *Hpa* II site which was utilized for cleavage of the *in vitro* reaction products. The numbered species in Fig. 4 can be positioned to within 3 nt on the mtDNA template L strand, indicating abrupt termination of DNA synthesis. Three of the five

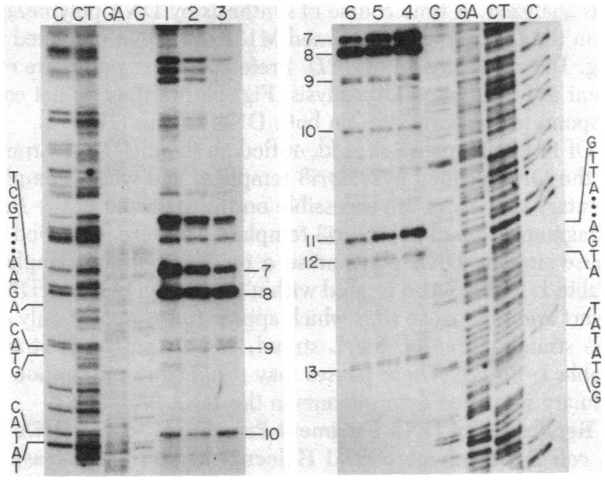

FIG. 4. Structural mapping of the 3′ ends of nascent DNA strands. *In vitro* reaction products (25 μl) were cleaved by *Hpa* II at the 3′ end of the *Hpa* II primer, denatured, and electrophoresed in a 6% polyacrylamide/7 M urea gel (16) in parallel with a DNA sequence ladder generated by chemical cleavage (16) of a mouse mtDNA restriction fragment labeled at the 5′ end at the same *Hpa* II site. Lanes 1–3 and 4–6: first and second loadings, respectively, of *Hpa* II-digested nascent DNA strands isolated after 5, 15, and 30 min of DNA synthesis. The leftward four lanes and the rightward four lanes are the DNA sequence ladders loaded in parallel with the *in vitro* synthesis products. The nascent DNA species that are mapped precisely in this analysis are given the same numbers as corresponding DNA species in the analysis presented in Fig. 3 *Left*.

pause points for *in vitro* DNA synthesis map within 0–3 nt of the *in vivo* termination points for the D-loop strands of mouse mtDNA (Fig. 5). The two additional *in vitro* pause points map downstream from the first repeated template sequence implicated in D-loop strand termination (1).

Reverse-Polarity Replication of DNA Fragment-Primed M13H*ori*3 DNA. To examine the possible correspondence between template-specific signals present on the mtDNA D-loop template strand and its complement, reverse-polarity DNA synthesis through the D-loop region, using the H strand of the cloned H-strand origin region of mouse mtDNA as template,

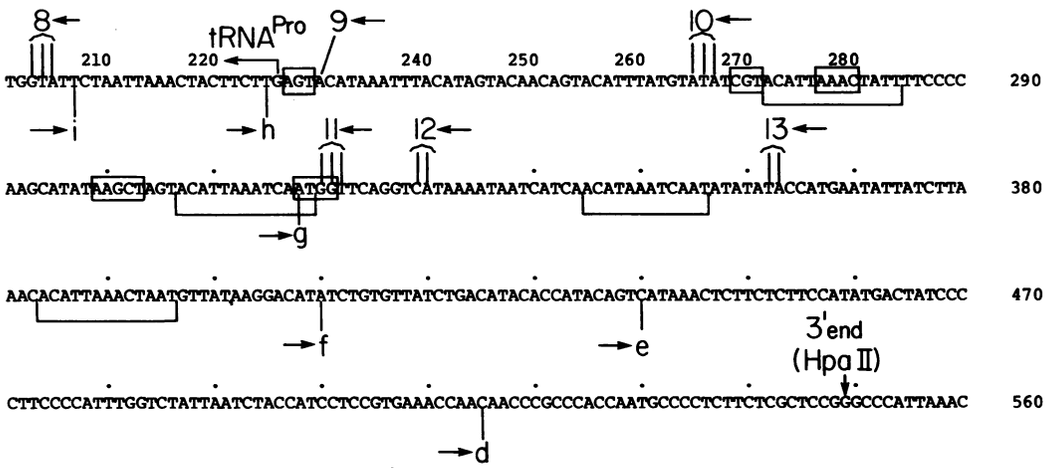

FIG. 5. Nucleotide assignment of the 3′ ends of *in vitro* DNA product strands in the region of the 3′ ends of mtDNA D-loop strands; 360 nt of the mouse mtDNA D-loop template region are shown (12). This L-strand sequence (which is present in M13H*ori*8) encodes $tRNA^{Pro}$ starting at nucleotide 226 in the coordinate defined in Table 1. The 3′-terminal nucleotide of the *Hpa* II primer is indicated at position 549, and that of the *Dde* I primer is at position 52 (not shown). The template sequences at the map positions of the 3′ ends of the five major D-loop strands from mouse mtDNA (1) are boxed; the D-loop strand termination-associated sequences (1) are indicated by brackets below the sequence. The 3′-end positions of DNA strands resulting from DNA synthesis (leftward in this figure) on *Hpa* II fragment-primed M13H*ori*8 DNA, and mapped to within 3 nt in the analysis shown in Fig. 4, are indicated by leftward arrows. Rightward arrows indicate map positions of the 3′ ends of DNA strands resulting from DNA synthesis (rightward in this figure) on the H-strand template in *Dde* I fragment-primed M13H*ori*3 DNA (identified in Fig. 6).

was analyzed. A time course of synthesis by DNA polymerase α on the *Dde* I fragment-primed M13H*ori*3 DNA (depicted in Fig. 1) is presented in Fig. 2*D*. Preferred pause points are evident as in the previous analysis (Fig. 6), but they do not correspond to the same sites on both DNA strands (Table 1).

Of 11 kinetic pause sites identified on the mtDNA L strand in the DNA-primed M13H*ori*8 template, and whose complementary sequences are accessible on the H strand in the *Dde* I fragment-primed M13H*ori*3 template, six were identified as pause sites by DNA polymerase α on the H-strand template (Table 1; Fig. 5). Also located within this region of the mtDNA insert are nine pause sites which appear to be present only on one strand—three on the L strand, and six on the H strand (Table 1; Fig. 5). These pauses may result from recognition of primary sequence determinants in the DNA template.

Replication of DNA Fragment-Primed M13H*ori*8 DNA by *E. coli* DNA Polymerase III Holoenzyme and Polymerase I. Because DNA polymerase α pauses at numerous sites that cannot be correlated with stable hairpin structures, we determined whether or not pausing at these sites is specific to DNA polymerase α by comparing its activity to that of *E. coli* DNA polymerase III holoenzyme and *E. coli* DNA polymerase I. Several analogies have been drawn between the *D. melanogaster* DNA polymerase α and polymerase III holoenzyme (17): both are multisubunit enzymes capable of replicating long stretches of single-stranded DNA, and both contain a subunit that has DNA polymerase activity on activated DNA but requires the additional subunits to replicate long single-stranded DNAs. In contrast, polymerase I, which efficiently replicates long single-stranded DNAs, contains only a single subunit and is capable of displacing a DNA strand to allow synthesis through duplex regions.

Fig. 2*C* shows a time course of DNA synthesis by the two polymerases on the *Hpa* II fragment-primed M13H*ori*8 DNA. Because the rate of synthesis by polymerase III holoenzyme and polymerase I was greater than that by DNA polymerase α on the single-stranded DNA template, sampling times were adjusted so that products resulting from approximately equal extents of replication could be analyzed. Comparison of the polymerase III holoenzyme reaction product profile (Fig. 3 *Right*, lanes 4–6) with that of DNA polymerase α (Fig. 3 *Left*, lanes 5 and 6) reveals a qualitatively similar pattern. Ten of 13 nascent DNA species identified in the DNA polymerase α analysis also were present in the polymerase III holoenzyme analysis. Among the preferred sites for pausing by DNA polymerase α, those in the $G4_{ori_c}$ and one of the three in the $tRNA^{Pro}$ gene also were preferred sites for pausing by polymerase III holoenzyme (bands 1, 2, and 8 in Fig. 3). However, other preferred sites were not common between the two enzymes. In addition, most of the pause signals recognized by DNA polymerase α appear either to be recognized poorly by polymerase III holoenzyme or to result in a more brief interruption of DNA synthesis.

Most replicative intermediates produced in the polymerase I reaction contained DNA strands whose 3′ ends were positioned randomly on the DNA template (Fig. 3 *Right*). However, discrete DNA species were identifiable, with four of five corresponding to those obtained in the DNA polymerase α analysis. Unlike that of DNA polymerase α and polymerase III holoenzyme, the rate of DNA synthesis by polymerase I through the pause sites must be nearly equal to the rate between sites.

Table 1. Pauses in *in vitro* DNA synthesis by *D. melanogaster* polymerase α

Pause	Nascent DNA strand length, nt	Sequence position			Nascent DNA strand length, nt	Pause
1*†	710		$G4_{ori_c}$			
2*†	648		$G4_{ori_c}$			
3	548	1	Cyt *b*			
4	530	19	Cyt *b*			
5‡	478	71	Cyt *b*	73	21	o‡
			Cyt *b*	85	33	n§
			$tRNA^{Thr}$	105	53	m§
			$tRNA^{Thr}$	135	83	l*§
6*	388	161	$tRNA^{Pro}$	170	118	k*
7*†‡	356–358	191–193	$tRNA^{Pro}$	190	138	j†‡
8*†‡	344–346	203–205	$tRNA^{Pro}$	207	155	i†‡
9‡	319	230	$tRNA^{Pro}$	225	173	h‡
10§	282–284	265–267	D loop			
11*‡	227–229	320–322	D loop	318	266	g‡
12§	219, 220	329, 330	D loop			
13§	186, 187	362, 363	D loop			
			D loop	410	358	f§
			D loop	440	388	e*§
			D loop	515	463	d*§
14*‡	234	574	D loop	575	523	c‡
			D loop	661	609	b†
15†	103	705	D loop			
			D loop	885	833	a

The numbers at the left refer to pause sites corresponding to DNA species resulting from DNA synthesis on DNA-primed M13H*ori*8 DNA; the letters at the right refer to those resulting from synthesis on M13H*ori*3 DNA. "Nascent DNA strand length" refers to the distance from the 3′ end of the *Hpa* II primer (pauses 1–13), the *Hpa* II/*Hin*fI primer (pauses 14 and 15), or the *Dde* I primer (pauses a–o) to the 3′ end of the synthetic DNA strand. Sequence positions 1 and 1471 are defined as the *Bal* I and *Hin*cII cleavage sites bounding the mtDNA insert in the M13 chimeric DNAs. The sequence positions of the pauses listed are contained within the genetic elements indicated in the center of the table and whose map locations are shown in Fig. 1. Cyt *b*, cytochrome *b*.

* Preferred site.

† Maps at the site of a potentially stable dyad.

‡ Identified on complementary template strands.

§ Identified on one template strand only.

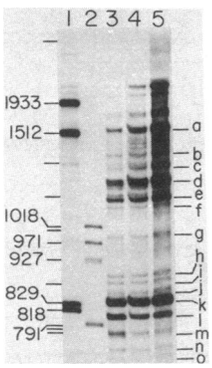

FIG. 6. Electrophoresis, on 4% polyacrylamide/7 M urea gels, of products of DNA synthesis by DNA polymerase α on *Dde* I fragment-primed M13H*ori*3 DNA. Lanes 3–5: products (15 μl) resulting from 5, 15, and 30 min of DNA synthesis, respectively. The sizes of the 15 species (a–o indicated on the right) are 1550, 1326, 1240, 1180, 1105, 1075, 983, 890, 872, 855, 835, 800, 770, 750, and 738 nt. Lanes 1 and 2: *Hpa* II and *Taq* I restriction fragments of M13H*ori*8 and M13 RF DNAs, respectively; molecular weights are indicated on the left; also indicated are the positions of additional molecular weight markers electrophoresed in lanes not shown.

DISCUSSION

DNA polymerase α from *D. melanogaster* embryos is capable of replicating DNA-primed single-stranded DNA by a mechanism in which the polymerase pauses at discrete sites on the

DNA template as DNA synthesis proceeds. Because the polymerase pauses at the same sites regardless of the position of the primer, the pause sites are template specific. The fact that the *E. coli* DNA polymerase III holoenzyme pauses at many of the same sites indicates that recognition of template-specific signals may be a general feature of DNA polymerases. DNA strands from replicative intermediates mapping at seven of the sites were positioned to within several nucleotides on the DNA template, indicating that recognition of the template-specific signals results in sharply attenuated synthetic products (Fig. 4).

Three potential types of pause signals are present among those we have analyzed. A computer and visual search for dyad symmetries and homologies was made of the DNA sequence containing all of the pause sites and of the sequences around each individual pause site. The pause sites in the $G4_{ori_c}$ (Table 1, sites 1 and 2) map at the stems of the two stable hairpins [ΔG, -16.1 and -20.7 kcal (1 kcal = 4.18 kJ)] (Fig. 7). Within the mtDNA sequence are two potential hairpin structures of lesser stability (ΔG, -13.0 and -10.1 kcal) which may account for pausing at sites 15 and b and 7, 8, j, and i (Table 1). In the case of the two hairpins in $G4_{ori_c}$ and at position 664–692 in the mtDNA insert sequence, the 3′ ends of nascent DNA strands map on the 5′ side (in the template DNA strand) of the dyad stem. Within the DNA sequence containing the least-stable hairpin structure are four size classes of nascent DNA strands. This sequence encodes the anticodon stem-loop of $tRNA^{Pro}$. Because of the clustering of pause sites within the $tRNA^{Pro}$ gene, one might invoke other template features as responsible for impeding DNA synthesis through this region.

Six pauses on the L strand of the mtDNA insert in M13H*ori*8 DNA are coincident with pause sites on the H-strand complement in M13H*ori*3 DNA (Table 1, 6 and k), map approximately 9 nt apart on the L and H strands. With the exception of the dyad structures presented in Fig. 7, no other potential hairpin of $\Delta G \leq -5.0$ kcal was found in the computer search conducted. No common sequences around the pause sites were identified, nor were any palindromic sequences found which might signal pausing on opposite strands. The fact that the pause sites map within several nucleotides of each other on the two DNA strands argues

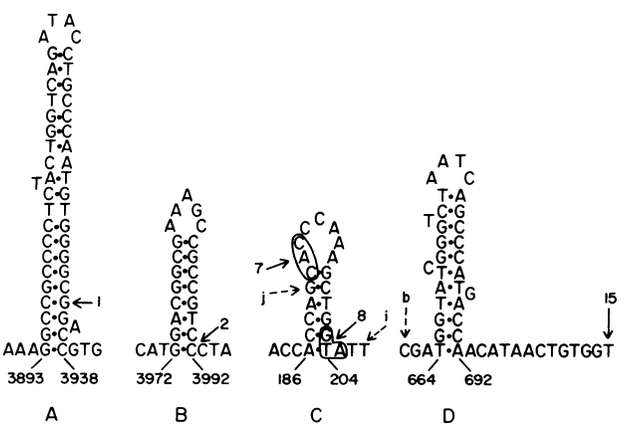

FIG. 7. Potential dyad structures correlated with pause sites in DNA synthesis. Structures A and B are contained within the $G4_{ori_c}$ (2) and are viral (+) strand sequence; sequence coordinates are taken from the G4 DNA sequence map (15). Structures C and D are contained within the mtDNA insert in M13G*ori*1 and are L-strand sequence (12); sequence coordinates are as indicated in Table 1. Numbered pauses (→) are those identified in Figs. 3 *Left* and 4 and Table 1 and result from DNA synthesis proceeding leftward on the M13H*ori*8 template strand shown. Lettered pauses (←) are those identified in Fig. 6 and Table 1 and result from DNA synthesis proceeding rightward on the M13H*ori*3 template strand, which is complementary to that shown.

that a second type of template structure is responsible for interruption of DNA synthesis at these points. All of the pause sites in the $tRNA^{Pro}$ gene, one in the cytochrome *b* gene, and two which map at the *in vivo* positions of the 3′ ends of D-loop strands are included in this group (Table 1).

Nine pause sites were located only on the L or the H strand of the mtDNA sequence (Table 1). These include two sites in the $tRNA^{Thr}$ gene, one in the cytochrome *b* gene, and six in the D-loop region. An intriguing possibility is that pausing in *in vitro* replication at these sites is due to the presence of primary sequence determinants in the DNA template. Three of five pause sites in the region of the *in vivo* map positions of the 3′ ends of D-loop strands are of this third type, and the two others are not. Thus, although there is good correlation between *in vivo* termination points for D-loop strands (1) and pause sites in *in vitro* DNA synthesis (Fig. 5), we cannot propose a singular mechanism by which template sequences might act as termination signals *in vivo* and *in vitro*.

The highly clustered nature of pause sites in the D-loop termination region, the coincidence of pause sites on complementary DNA strands, and the general absence of predicted stable hairpin structures in the DNA template indicate that a single mechanism involving destabilization of the replication apparatus at the position of a hairpin barrier is insufficient to explain the phenomena reported here. It is also clear that a common primary control sequence cannot be implicated in the pausing of *in vitro* DNA synthesis even when the exact termination sites of DNA strands have been identified. Further, although DNA polymerase α, polymerase III holoenzyme, and polymerase I either recognize the template signals to differing extents or replicate DNA through a pause site at different rates relative to the rate of replication between sites, many of the same pause sites are observed in analyses of replicative intermediates during *in vitro* synthesis by these enzymes.

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