Dinucleotide Repeat Expansion Catalyzed by Bacteriophage T4 DNA Polymerase in Vitro*

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DNA replication normally occurs with high fidelity, but certain "slippery" regions of DNA with tracts of mono-, di-, and trinucleotide repeats are frequently mutation hot spots. We have developed an in vitro assay to study the mechanism of dinucleotide repeat expansion. The primer-template resembles a base excision repair substrate with a single nucleotide gap centered opposite a tract of nine CA repeats; nonrepeat sequences flank the dinucleotide repeats. DNA polymerases are expected to repair the gap, but further extension is possible if the DNA polymerase can displace the downstream oligonucleotide. We report here that the wild type bacteriophage T4 DNA polymerase carries out gap and strand displacement replication and also catalyzes a dinucleotide expansion reaction. Repeat expansion was not detected for an exonuclease-deficient T4 DNA polymerase or for Escherichia coli DNA polymerase I. The dinucleotide repeat expansion reaction catalyzed by wild type T4 DNA polymerase required a downstream oligonucleotide to "stall" replication and 3' → 5' exonuclease activity to remove the 3'-nonrepeat sequence adjacent to the repeat tract in the template strand. These results suggest that dinucleotide repeat expansion may be stimulated in vivo during DNA repair or during processing of Okazaki fragments.

DNA polymerases replicate DNA with high fidelity except for certain DNA sequences, the so-called "slippery" DNAs, which are tracts of simple repeat sequences (reviewed in Ref. 1). The lengths of tracts of mono-, di-, and trinucleotide repeats are unstable, which can easily be detected as repeat-length polymorphisms in microsatellite sequences or as mutation hot spots, such as the classical frameshift hot spots identified by Benzer in the bacteriophage T4 rII genes (2), which are tracts of six A nucleotides (3). Hypermutability in repeat tracts can have serious consequences for human health as observed for an inherited mutation in the human APC gene, which converts the wild type sequence AAATAAAA to the A8 mononucleotide tract (4). The A8 sequence was found to create a small hypermutable region for gene inactivating mutations that predispose carriers to colorectal cancer (4). Streisinger et al. (5) suggested that frameshifts are produced in repeat sequences by a transient separation of the primer and template strands and then misalignment during reannealing to generate an intermediate in which one or more repeats is unpaired. This "slippage" intermediate is usually repaired by postreplication mismatch repair as revealed by the dramatic increase in repeat instability when this repair pathway is inactivated (6–8).

In vitro assays have been used to measure DNA polymerase-catalyzed "reiterative replication" of repeat sequences (for examples, see Refs. 9–14). The number of repeats can be amplified several hundred-fold by a variety of DNA polymerases, but most of the synthetic DNA substrates used are composed exclusively of repeat sequences. One objective of our studies was to develop an improved in vitro assay utilizing a DNA substrate that more closely resembles genomic DNA in which the repeat tract is flanked by nonrepeat sequences. A second objective was to use the more natural DNA substrate to probe the mechanism of DNA polymerase slippage. Both objectives were achieved with a DNA substrate in which a single nucleotide gap was positioned opposite the center of the dinucleotide repeat sequence (AC)9. The AC dinucleotide repeat was chosen because poly(GT10–30) is the most common simple repeat in many eukaryotic genomes (15). Nonrepeat sequences were positioned on both sides of the (AC)9 dinucleotide tract, as found in genomic DNA. The nonrepeat sequences also allowed precise annealing of the template and primer strands. A single nucleotide-gapped substrate is produced in vivo during base excision repair. A similar substrate is also produced when DNA polymerases have nearly completed repair synthesis of the larger gaps formed by nucleotide excision repair and postreplication mismatch repair and during lagging strand replication when the replicating DNA polymerase reaches the RNA primer of the next Okazaki fragment.

We examined replication of the single nucleotide-gapped DNA substrate by wild type and exonuclease-deficient T4 DNA polymerases and by Escherichia coli DNA polymerase I. Dinucleotide expansion was detected only for the wild type T4 DNA polymerase. While many previous in vitro assays detected repeat expansion by exonuclease-deficient DNA polymerases, our studies demonstrate that not only does the wild type T4 DNA polymerase, which has a potent 3' → 5' exonuclease activity, catalyze repeat expansion, but that the exonuclease activity stimulates the expansion reaction. We have proposed a model for repeat expansion based on our new findings.

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA Polymerases—Purifications of the bacteriophage T4 wild type and the exonuclease-deficient D112A/E114A-DNA polymerases have been described (16, 17). E. coli DNA polymerase I and Klenow fragment were purchased from Amersham Pharmacia Biotech.

DNA Substrates—The DNA substrates used in this study are described in Fig. 1. The oligonucleotides were synthesized using standard procedures by the DNA synthesis facility in the Department of Biological Sciences at the University of Alberta. Two types of hydrolysistresistant oligonucleotides were prepared. A 3'-phosphate was intro-

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 Mechanism of Dinucleotide Repeat Expansion

DNA substrate

| Sequence |
|----------|
| 3’ CCGCCACACACACACACATGGGGTG |
| 5’ pGCCGGTGGTGGTGG |
| 3’ CCGCCACACACACACACACATGGGGTTG |
| 5’ 30pGCCGGTGGTGGTGG GTGGTGGTACCCACC |
| 3’ CCGCCACACACACACACACATGGGGTTG |
| 5’ 30pGCCGGTGGTGGTGG GTGGTGGTACCCACC |
| 3’ CCGCCACACACACACACACATGGGGTTG |
| 5’ 30pGCCGGTGGTGGTGG GTGGTGGTACCCACC |
| 3’ CCGCCACACACACACACATGGGGTTG |
| 5’ 30pGCCGGTGGTGGTGG GTGGTGGTACCCACC |
| 3’ CCGCCACACACACACACATGGGGTTG |
| 5’ 30pGCCGGTGGTGGTGG GTGGTGGTACCCACC |

FIG. 1. DNA substrates constructed to detect dinucleotide expansion. Nine CA repeats are present in the template strand and are underlined; the CA repeats are flanked by nonrepeat sequences. What we define as the primer strand in this report is labeled with 32P at the 5’-end, except for in Fig. 1h. The GT repeat sequences in the primer strand and in the downstream oligonucleotide are underlined. s indicates the location of the nonbridging phosphorothioate, Sp isomer. p indicates a phosphate group at the indicated 3’-position. In the sketches of the DNA substrates, repeat sequences are illustrated as open rectangles, and the nonrepeat sequences are illustrated as solid lines. An asterisk indicates the position of the 32P label.

duced by using a phosphate-derivatized CPG (Glen Research, Sterling, VA). A 3’-phosphate prevents both DNA polymerase nucleotide incorporation and 3’→5’ exonuclease activities. Hydrolysis activity was prevented specifically at the 3’-end of the primer strand by a nonbridging phosphorothioate (Sp isomer). The phosphorothioate modification inhibits 3’→5’ exonuclease activity, but the phosphorothioate-modified primer still supports the nucleotide incorporation reaction. Phosphorothioate oligonucleotides were prepared using a sulferizing reagent (Glen Research). Two diastereomers, Rp and Sp, are produced in about equal amounts, but the Rp-phosphorothioate oligonucleotide is degraded by the 3’→5’ exonuclease activity of T4 DNA polymerase while leaving the exonuclease-resistant Sp-oligonucleotide (18, 19). All oligonucleotides were purified by gel electrophoresis. The appropriate bands were cut from the gels, and the DNA was eluted from the gel slices and further purified by chromatography through Sep-Pak cartridges (Waters). The primer or template strands were labeled at the 5’-end by T4 polynucleotide kinase and [γ-32P]ATP.

The template and primer strands were annealed in buffer containing 50 mM Tris-HCl, pH 8.0, and 25 mM NaCl. The 32P-labeled oligonucleotide was present at 50 nM, and unlabeled oligonucleotides were present in 2-fold excess at 100 nM. The solution was heated at 80 °C for 5 min and slow cooled to room temperature (90 min).

Methods

Reaction Conditions—Reaction mixtures (50 μl) contained 5 mM DNA substrate, 50 mM DNA polymerase, 67.5 mM Tris-HCl, pH 7.5, 25 mM NaCl, 100 μM dNTPs, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, and 0.5 mM EDTA. The mixtures were preincubated at 37 °C for 5 min, and then reactions were started by the addition of MgCl2 (final concentration, 8 mM). Reactions were stopped at various times by removing 5-μl samples and mixing with an equal volume of gel loading buffer, which contained formamide and gel buffer. Denaturing gel electrophoresis in 15% polyacrylamide plus 8M urea was used to separate the reaction products. The 32P-labeled products were visualized by using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Chemical Sequencing—32P-Labeled reaction products were cut from preparative polyacrylamide gels and purified as described above. The Maxam and Gilbert sequencing procedure was followed (20).

RESULTS

Primer Extension—In vitro replication of the AC repeat-containing template strand was first measured in a primer extension assay. The 32P-labeled primer was annealed to the template strand (Fig. 1a). T4 DNA polymerase extended the primer to produce full-length 30-mer product within the first 15 s of reaction (Fig. 2A). After 15 s of replication, the primer was extended by one nucleotide to fill in the gap (14-mer), but longer 15-mer, 16-mer, and 18-mer products as well as high amounts of full-length 30-mer product were also produced.
Synthesis of products longer than 14 nucleotides indicates that the T4 DNA polymerase can displace the downstream oligonucleotide. At later time points, the amounts of the shorter 14-, 15-, and 16-mer products decreased, while replication products longer than the full-length 30-mer were produced. By 1 min, and more clearly at 10 min, products were synthesized that appeared to be longer than 30 nucleotides by the addition of dinucleotide units, the gel bands indicated as 12, 14, 16, etc.

At 10 min, repeat expansion products represented about 13% of the replication products that were full-length or longer.

The band indicated at the +2-position (Fig. 2B) was excised from a preparative gel and sequenced by the Maxam and Gilbert chemical sequencing method (20); the sequencing gel is shown in Fig. 3. The primary sequence for the +2 product has 10 GT repeats instead of the nine expected from the nine AC repeats in the template strand. The 10 GT repeats are followed by the nonrepeat sequence (data not shown). Thus, wild type T4 DNA polymerase catalyzes a dinucleotide expansion reaction, which is followed by accurate replication of the nonrepeat template sequence.

The experiments were repeated with a similar DNA substrate but with 20 AC repeats instead of nine in the template strand. The same primer and downstream oligonucleotides were used, which produced a single-stranded gap of 23 nucleotides. Dinucleotide expansion by the wild type T4 DNA polymerase was detected with the longer DNA substrate to the same extent as observed with the shorter DNA substrate (data not shown).

FIG. 2. Primer extension and dinucleotide expansion by the wild type T4 DNA polymerase. Primer extension (A) and gap repair assays (B) were done with the DNA substrates described in Fig. 1, a and b, respectively. The reaction conditions are described in detail under "Experimental Procedures." The DNA replication products were separated by electrophoresis on a denaturing, 15% polyacrylamide gel. Samples were analyzed after 15 s, 30 s, 1 min, and 10 min of reaction. Lane C contains labeled primer and full-length DNAs. In B, the 14-mer product, which fills the gap, and longer 15-mer, 16-mer, 18-mer, and 20-mer products that were produced during displacement of the downstream oligonucleotide are indicated. Apparent dinucleotide expansion products are indicated as +2, +4, +6, etc.

FIG. 3. DNA sequence of the +2 dinucleotide expansion product. Products of chemical reactions to detect modified G, modified T, modified A+G, and modified C by the Maxam and Gilbert procedure (20) are in the indicated lanes. The DNA sequence is given by each band. There are 10 GT dinucleotide repeats followed by the nonrepeat ACCCCACC sequence. The bracket indicates bands due to a minor contaminant, which has an additional GT repeat.

Repeat Expansion by the Exonuclease-deficient D112A/31530
E114A-T4 DNA Polymerase—Experiments reported in Fig. 2 with the wild type T4 DNA polymerase were repeated with the exonuclease-deficient D112A/E114A-DNA polymerase. No dinucleotide repeat expansion was detected for the exonuclease-deficient T4 DNA polymerase with either the primer extension (Fig. 4A) or the single nucleotide-gapped DNA substrate (Fig. 4B). Displacement synthesis was more rapid by the exonuclease-deficient T4 DNA polymerase compared with the wild type enzyme, since little pausing to produce the gap-filled 14-mer product or other products shorter than full-length was observed (Fig. 4B, 15 s). A product apparently one nucleotide longer than the full-length product (+1) was detected at 10 min, which is probably due to the nontemplated extension of the primer strand that is observed for many exonuclease-deficient DNA polymerases at the ends of duplex DNA. Since dinucleotide expansion was not detected for the exonuclease-deficient T4 DNA polymerase, expansion by the wild type enzyme must require 3’→5’ exonuclease activity at some step in the expansion reaction.

Dinucleotide expansion was detected for the exonuclease-deficient T4 DNA polymerase, however, if only two of the four dNTPs, dGTP and dTTP, were supplied (Fig. 5B), but not for the wild type T4 DNA polymerase (Fig. 5A). Replication terminated for the wild type DNA polymerase once replication of the repeat region was completed (22-mer). For the exonuclease-deficient T4 DNA polymerase, a major product with an extra GT repeat (+2) was detected within the first minute of reaction. With longer reaction times, the amount of +2 product decreased, while the amount of +4 and longer products increased. Thus, elongation of misaligned primer-termini by the exonuclease-deficient T4 DNA polymerase produces relatively stable replication products. The absence of dinucleotide expansion products (longer than the 22-mer) for the wild type T4 DNA polymerase indicates that exonucleolytic proofreading efficiently corrects any expansion products. The presence or absence of the downstream oligonucleotide had no effect on reactions with dGTP and dTTP.

**Mechanism of Dinucleotide Repeat Expansion**

In order to determine at what step exonuclease activity is needed for the dinucleotide expansion catalyzed by the wild type T4 DNA polymerase, the three 3’-ends present in the gapped DNA substrate were modified. Besides the primer terminus, the downstream oligonucleotide and the template strand have 3’-ends that are potential substrates for the T4 DNA polymerase 3’→5’ exonuclease activity. A non-bridging phosphorothioate (Sp isomer) was placed at the 3’-
Mechanism of Dinucleotide Repeat Expansion

FIG. 6. The effect of DNA modifications on the dinucleotide expansion reaction. Reactions (10 min) catalyzed by the wild type T4 DNA polymerase with various DNA substrates are shown. The standard reaction conditions were used. Lane 1, dinucleotide expansion with a single nucleotide-gapped substrate like Fig. 1c, except that the downstream oligonucleotide was not phosphorylated at the 3’-end. Lanes 2 and 3, reactions with gapped DNA substrates, in which the template strands were phosphorylated on the 3’-ends (Fig. 1d and e, respectively). Lane 4, dinucleotide expansion with the gapped DNA substrate described in Fig. 1c, which has an Sp phosphorothioate at the primer-terminus and the downstream oligonucleotide is phosphorylated at the 3’-end. Lane 5, the template strand was synthesized without the nonrepeat sequence of the primer strand and the C residues in the repeat appear to be due to imprecise primer annealings in which there may be base pairing between the G residues in the nonrepeat sequence and with an Sp phosphorothioate at the 3’-end to prevent further exonuclease trimming (Fig. 1g). A strong dinucleotide expansion was observed (83% full-length) was observed (Fig. 6, lane 5). Also note that without the 3’-terminal nonrepeat sequence in the template strand, there was some imprecise annealing of the primer strand, which produced a few products shorter than the full-length 30-mer.

Removal of the nonrepeat sequence, however, still did not allow the exonuclease-deficient T4 DNA polymerase to carry out the reaction. Since the exonuclease-deficient T4 DNA polymerase may extend the phosphorothioate-modified DNA and resynthesize the nonrepeat sequence, the experiment was repeated with a DNA substrate in which the 3’-end of the template strand was protected with a 3’-phosphate, which prevents both degradation and extension (Fig. 1g). Products longer than full length were detected for the wild type (Fig. 7A) and 3’→5’ exonuclease-deficient DNA polymerases (Fig. 7B). The distinct expansion ladder was detected for the wild type T4 DNA polymerase but not for the exonuclease-deficient DNA polymerase; instead, products longer than the full-length template strand increased in length by apparent single nucleotide extensions, +1, +2, +3, etc. The single nucleotide extensions were probably produced by a combination of dinucleotide expansion followed by an untemplated primer extension, which is catalyzed by exonuclease-deficient DNA polymerases (Fig. 4). Products shorter than the full-length 30-mer were also observed (indicated by asterisks). These products appear to be due to imprecise primer annealings in which there may be base pairing between the G residues in the nonrepeat sequence of the primer strand and the C residues in the repeat tract in the template strand.

The exonuclease trimming of the template strand suggested to us that DNA replication may also be taking place at the 3’-end of the template strand. This proposal was tested by using the same DNA substrate that supports dinucleotide expansion of the primer (Fig. 1c), except that the 5’-end of the template strand was labeled with 32P (Fig. 1h). Dinucleotide expansion in the template strand was detected only if the downstream oligonucleotide was present (Fig. 8), as observed for expansion in the primer strand (Fig. 2B). The apparent +2 template product was sequenced (Fig. 9). An extra dinucleotide repeat (AC) was present, and the repeat sequence was followed by the adjacent nonrepeat sequence.

Repeat Expansion by E. coli DNA Polymerase I—The experiments with the primer extension and gapped DNA substrates were repeated with E. coli DNA polymerase I and Klenow fragment. No dinucleotide expansion was detected (data not shown). Rapid displacement replication was observed for both bacterial DNA polymerases, as observed for the exonuclease-deficient T4 DNA polymerase (Fig. 4).

DISCUSSION

We have demonstrated that the wild type T4 DNA polymerase, which has a potent 3’→5’ exonuclease activity (16), can catalyze a dinucleotide expansion reaction with a single nucleotide-gapped DNA substrate with nine AC repeats in the template strand in the presence of all four dNTPs (Fig. 2B). Since the dinucleotide repeats are embedded between nonrepeat se-

terminal phosphodiester bond in the primer strand to protect the primer from exonuclease digestion, while still allowing the primer to be used for nucleotide incorporation. Dinucleotide expansion was detected with the phosphorothioate-modified primer (Fig. 6, lane 1) and when the downstream oligonucleotide was also modified with a 3’-phosphate, which prevents both exonuclease and nucleotide incorporation reactions (Fig. 1c; Fig. 6, lane 4). Protection of the 3’-ends of the primer and downstream oligonucleotide from exonuclease digestion increased the expansion reaction. About 21% of the full-length and longer replication products in Fig. 6, lane 1, were expansion products and 56% in Fig. 6, lane 4, but only 13% of the replication products were expansion products in the absence of any 3’-protection after a 10-min reaction (Fig. 2B).

Dinucleotide expansion was reduced to an almost undetectable level by phosphorylation of the 3’-end of the template strand (Fig. 1, d and e; Fig. 6, lanes 2 and 3, respectively). These results demonstrate that exonuclease trimming at the 3’-end of the template strand is required for production of high amounts of expansion products. The extent of exonuclease di-

gestion of the template strand required to support dinucleotide expansion was determined by placing nonbridging phospho-}

thioates at various positions at the 3’-end of the template strand. Dinucleotide expansion was restored only if the phosphorothioate was placed so that T4 DNA polymerase 3’→5’ exonuclease activity could remove the 3’-terminal nonrepeat sequence in the template strand. This point was demonstrated by constructing a template strand without the nonrepeat sequence and with an Sp phosphorothioate at the 3’-end to prevent further exonuclease trimming (Fig. 1f); a strong dinucleotide expansion reaction (83% full-length product compared with 17% full-length) was observed (Fig. 6, lane 5). Also note that without the 3’-terminal nonrepeat sequence in the template strand, there was some imprecise annealing of the primer strand, which produced a few products shorter than the full-length 30-mer.

Removal of the nonrepeat sequence, however, still did not allow the exonuclease-deficient T4 DNA polymerase to carry out the expansion reaction. Since the exonuclease-deficient T4 DNA polymerase may extend the phosphorothioate-modified DNA and resynthesize the nonrepeat sequence, the experiment was repeated with a DNA substrate in which the 3’-end of the template strand was protected with a 3’-phosphate, which prevents both degradation and extension (Fig. 1g). Products longer than full length were detected for the wild type (Fig. 7A) and 3’→5’ exonuclease-deficient DNA polymerases (Fig. 7B). The distinctive dinucleotide expansion ladder was detected for the wild type T4 DNA polymerase but not for the exonuclease-deficient DNA polymerase; instead, products longer than the full-length template strand increased in length by apparent single nucleotide extensions, +1, +2, +3, etc. The single nucleotide extensions were probably produced by a combination of dinucleotide expansion followed by an untemplated primer extension, which is catalyzed by exonuclease-deficient DNA polymerases (Fig. 4). Products shorter than the full-length 30-mer were also observed (indicated by asterisks). These products appear to be due to imprecise primer annealings in which there may be base pairing between the G residues in the nonrepeat sequence of the primer strand and the C residues in the repeat tract in the template strand.

The exonuclease trimming of the template strand suggested to us that DNA replication may also be taking place at the 3’-end of the template strand. This proposal was tested by using the same DNA substrate that supports dinucleotide expansion of the primer (Fig. 1c), except that the 5’-end of the template strand was labeled with 32P (Fig. 1h). Dinucleotide expansion in the template strand was detected only if the downstream oligonucleotide was present (Fig. 8), as observed for expansion in the primer strand (Fig. 2B). The apparent +2 template product was sequenced (Fig. 9). An extra dinucleotide repeat (AC) was present, and the repeat sequence was followed by the adjacent nonrepeat sequence.

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DISCUSSION

We have demonstrated that the wild type T4 DNA polymerase, which has a potent 3’→5’ exonuclease activity (16), can catalyze a dinucleotide expansion reaction with a single nucleotide-gapped DNA substrate with nine AC repeats in the template strand in the presence of all four dNTPs (Fig. 2B). Since the dinucleotide repeats are embedded between nonrepeat se-
quences, this assay allows us to study dinucleotide expansion under conditions that may occur naturally in the cell during repair of gaps produced by various DNA repair activities and when lagging strand replication reaches the primer for the next Okazaki fragment. The expansion products have extra dinucleotide repeats that are followed by the adjacent nonrepeat sequence (Fig. 3). Thus, this assay detects both the strand misalignment phase of the expansion reaction and the switch to accurate replication of the nonrepeat template sequence. The critical step for repeat expansion was the requirement that DNA replication be hindered within the repeat tract. We combined results from in vitro dinucleotide expansion reactions with in vivo observations to formulate the following models for repeat expansion.

The first step in the repeat expansion reaction is DNA polymerase pausing and dissociation during replication of a repeat tract, which was forced in our assay due to the presence of a downstream oligonucleotide (Fig. 10A). Although our experiments were with the T4 DNA polymerase and not the highly processive T4 DNA polymerase holoenzyme, rapid dissociation for the holoenzyme is also observed when replication is barred under conditions that mimic an encounter with the 5′-end of an Okazaki fragment (21). Thus, results presented in this report are consistent with the proposal by others that frameshift mutagenesis is stimulated by DNA polymerase dissociation and reassociation (13, 22). We have extended these findings by demonstrating that DNA polymerase encounters with downstream DNA stimulate repeat expansion (Fig. 2, compare A and B). Furthermore, dramatic expansion may result if repeat expansion can take place on both strands (Figs. 6 and 8). These results suggest that DNA damage resulting in strand breaks on both strands within a repeat tract, as illustrated in Fig. 10A, has the potential, if acted on by DNA polymerases, to greatly expand the length of the repeat tract.

The second step is continued primer extension with displacement of the downstream strand. This step may involve several cycles of DNA polymerase dissociation and reassociation. Displacement of the 5′-end of the downstream strand creates a “flap” of unpaired single-stranded DNA (Fig. 10A). The 5′-flap structure may be in equilibrium with a structure in which the 3′-end of the primer strand is unpaired (3′-flap). The 3′-flap structure may spontaneously convert to a structure with a base-paired primer terminus but with one or more internal repeats not base-paired. Alternatively, the DNA polymerase may facilitate strand misalignment by binding the 3′-end of the single-stranded flap directly in the exonuclease active center (23) but then occasionally transferring the primer end to the polymerase active center in a misaligned configuration (24,
In either case, extension of the misaligned primer strand results in expansion of the repeat tract. The proposed role of a 5'-flap structure in repeat expansion is supported by the finding that deletion of the RAD27 gene in *Saccharomyces cerevisiae* increases repeat length instability, primarily to expand repeat tracts (26–28). The RAD27 gene encodes a flap endonuclease. If this activity is missing, then persistent flap structures may increase the opportunity to produce the misaligned DNA structures illustrated in Fig. 10A.

Several points in this model warrant further discussion. First, there was greater dinucleotide expansion activity with the *in vitro* assay than would be expected *in vivo*. Dinucleotide expansion may be limited *in vivo* if the optimal conditions for expansion are not met. For example, strand breakage in both strands within or near a repeat tract (Fig. 10A) is probably a rare event. Rapid DNA ligation or binding of other proteins to strand discontinuities would prevent DNA polymerase binding. The cell may also selectively utilize DNA polymerases for gap repair that are less prone to frameshift infidelity than T4 DNA polymerase, such as *E. coli* DNA polymerase I, which does not catalyze detectable repeat expansion in our *in vitro* reaction. The 5’→3’ exonuclease activity of *E. coli* DNA polymerase I would prevent formation of a 5’-flap and, thus, reduce opportunity for strand misalignment.

Another point to explain is the low level of dinucleotide expansion by the exonuclease-deficient T4 DNA polymerase in our *in vitro* assays (Figs. 4 and 7) but the high amount of repeat length instability detected for exonuclease-deficient DNA polymerases *in vivo*, such as for the exonuclease-deficient yeast DNA polymerase δ (29) and for the T4 D112A/E114A-DNA polymerase. There are two reasons why exonuclease deficiency reduces repeat expansion in our assay. First, there is little expansion detected if the nonrepeat sequence at the 3'-end of the template strand cannot be removed by exonuclease degradation (Fig. 6, lanes 2 and 3). Second, the short downstream oligonucleotide was displaced and dislodged too readily.

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**Fig. 9. DNA sequence of the template +2 dinucleotide expansion product.** Products of chemical reactions to detect modified G, modified T, modified A+G, and modified C by the Maxam and Gilbert procedure (20) are in the indicated lanes. The DNA sequence is given by each band. There are 10 AC dinucleotides that are flanked by the nonrepeat sequences.

**Fig. 10. Model for repeat expansion catalyzed by the T4 DNA polymerase.** Panel A, misalignment mutagenesis promoted by displacement replication. Panel B, misalignment mutagenesis promoted by DNA damage (X) in the template strand. The thicker lines represent repeat tracts. The black thick lines represent the primer strands and potential sites for strand misalignment.

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1. M. I. Hadjimarcou, R. Kokoska, T. D. Petes, and L. J. Reha-Krantz, manuscript in preparation.
2. L. J. Reha-Krantz, unpublished data.
The assay provides a starting point to further probe the mechanism that DNA replication is hindered within a repeat tract. The primary instigator of DNA polymerase-catalyzed expansion is DNA polymerase that functions in chromosome replication. Assays that stimulate dinucleotide expansion by a wild type DNA polymerase did not catalyze dinucleotide expansion when just dGTP and dTTP were supplied, the exonuclease-deficient DNA polymerase catalyzed a strong expansion reaction (Fig. 5B).

Exonuclease-deficient DNA polymerases may increase misalignment mutagenesis in vitro because of increased ability to displace downstream DNA. The exonuclease-deficient T4 DNA polymerase is more proficient at displacement replication than the wild type T4 DNA polymerase (compare Figs. 2 and 4, 15-s time points) because the elongating primer strand is not subject to proofreading by the mutant. A synergism between DNA polymerase proofreading and removal of RNA primers and 5'-flap structures has been observed in yeast; a haploid strain with an exonuclease-deficient DNA polymerase δ and no flap endonuclease is not viable (28). The lethal phenotype of the double mutant may be due to the higher production of 5'-flap structures due to increased strand displacement replication by the exonuclease-deficient DNA polymerase δ compared with the wild type enzyme, which could lead to persistent strand discontinuities and/or intolerable levels of misalignment mutagenesis (error catastrophe).

Exonuclease-deficient DNA polymerases may also be more prone to misalignment errors at sites of DNA damage than exonuclease-proficient DNA polymerases. DNA damage presents a severe block to replication (Fig. 10B). Exonuclease-deficient DNA polymerases may synthesize extra copies of the repeat as was observed in the reaction with dGTP and dTTP (Fig. 5B) and as illustrated in Fig. 10B. The wild type T4 DNA polymerase did not catalyze dinucleotide expansion when just dGTP and dTTP were supplied (Fig. 5A) because of correction by exonucleolytic proofreading; hence, less repeat expansion at DNA damage sites is expected for proofreading-proficient DNA polymerases.

In conclusion, we have identified conditions in an in vitro assay that stimulate dinucleotide expansion by a wild type DNA polymerase that functions in chromosome replication. The primary instigator of DNA polymerase-catalyzed expansion is that DNA replication is hindered within a repeat tract. The assay provides a starting point to further probe the mechanism(s) of DNA polymerase-catalyzed repeat expansion.

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