Weak Strand Displacement Activity Enables Human DNA Polymerase β to Expand CAG/CTG Triplet Repeats at Strand Breaks*

Michael J. Hartenstein, Myron F. Goodman‡, and John Petruska

From the Department of Biological Sciences, Hedco Molecular Biology Laboratories, University of Southern California, Los Angeles, California 90089-1340

Using synthetic DNA constructs in vitro, we find that human DNA polymerase β effectively catalyzes CAG/CTG triplet repeat expansions by slippage initiated at nicks or 1-base gaps within short (14 triplet) repeat tracts in DNA duplexes under physiological conditions. In the same constructs, *Escherichia coli* DNA polymerase I Klenow Fragment exo− is much less effective in expanding repeats, because its much stronger strand displacement activity inhibits slippage by enabling rapid extension through two downstream repeats into flanking non-repeat sequence. Polymerase β expansions of CAG/CTG repeats, observed over a 32-min period at rates of ~1 triplet added per min, reveal significant effects of break type (nick versus gap), strand composition (CTG versus CAG), and dNTP substrate concentrations, on repeat expansions at strand breaks. At physiological substrate concentrations (1–10 µM of each dNTP), polymerase β expands triplet repeats with the help of weak strand displacement limited to the two downstream triplet repeats in our constructs. Such weak strand displacement activity in DNA repair at strand breaks may enable short tracts of repeats to be converted into longer, increasingly mutable ones associated with neurological diseases.

Lengthy expansions of triplet repeats in human DNA result in several neurological diseases (1). Triplet of bases repeated in tandem can form a variety of slipped structures (2), including single-strand hairpin loops observed in vitro (3–5) and in vivo (6). The slippery nature of tandemly repeated triplets may contribute to repeat expansions catalyzed by eukaryotic enzymes in DNA replication (7, 8), repair (9), or recombination (10). The degree to which a given process or enzyme contributes to expansion mutations in humans is not entirely clear but likely depends on the repeat type involved. Most studies to date have concentrated on the role that hairpin folding may play in slippage-expansion with DNA polymerase during DNA replication in regions of triplet repeats has been examined previously in connection with polymerase pausing and primer/template misalignment during DNA replication (11). Cells express pol β independently of the cell cycle (12) but show tissue specific differences in pol β expression, with particularly high levels of expression occurring in testis (13). During BER, following glycosylase and AP endonuclease activity, human pol β carries out DNA repair synthesis by filling single nucleotide gaps (short patch BER) or by strand displacement to create flaps of several nucleotides (long patch BER) (14). As observed in vitro under physiological salt conditions, pol β itself has weak strand displacement DNA synthesis activity (15, 16) that is stimulated in a cooperative fashion by poly(ADP-ribose) polymerase-1 (PARP-1) and flap endonuclease-1 (FEN-1), with the latter being absolutely required for this stimulation (17).

DNA synthesis by pol β in regions of triplet repeats has been examined previously in connection with polymerase pausing and primer/template misalignment during DNA replication (18–21). We have developed a 2-strand system to analyze repeat expansion in a DNA repair context at simple breaks (nicks or 1-base gaps) within double-stranded repeat tracts. Much like a recent study of dinucleotide repeat slippage and expansion by T4 DNA polymerase (22), we wanted to examine polymerase-catalyzed repeat expansions by slippage at breaks within a repeat tract surrounded by non-repeating sequence.

Here we present our results using human pol β to catalyze slippage-expansion at nicks and 1-base gaps within a DNA duplex of CAG/CTG repeats. For comparison we also present data obtained with a prokaryotic repair polymerase without proofreading, an exo− derivative of *E. coli* DNA polymerase I Klenow Fragment (KFexo−), which has a much stronger strand displacement activity than pol β.

### EXPERIMENTAL PROCEDURES

DNA Synthesis—DNA strands were synthesized by an Applied Biosystems 392 DNA/RNA synthesizer, using β-cyanoethyl phosphoramidites. Chemical phosphorylation reagent I (Glen Research) was used for non-radioactive phosphorylation of strand 5′-OH groups. DNA strand preparations were gel-purified (23) using 20 mM formamide as denaturant and dialyzed against chilled Milli-Q deionized water prior to lyophilizing. Lyophilized samples were dissolved in low ionic strength TE buffer (10 mM Tris, pH 7.0, 1 mM Na2EDTA), and dialyzed further against the same buffer to remove contaminants. After determining DNA strand concentrations using a Varian Cary 300 spectrophotometer, the solutions were stored frozen at −20 °C.

DNA Sequences Used for Nick and Gap Constructs—Two 72-mer

* This work was supported by National Institutes of Health-National Institute of Aging Program Grant AG17179. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biological Sciences, University of Southern California, SHS Room 172, University Park, Los Angeles, CA 90089-1340. Tel.: 213-740-5190; Fax: 213-740-8631; E-mail: mgoodman@mizar.usc.edu.

‡‡ The abbreviations used are: pol β, DNA polymerase β; BER, base excision repair; FEN-1, flap endonuclease-1; PARP-1, poly(ADP-ribose) polymerase-1; KFexo−, Klenow fragment polymerase 3′-exonuclease mutant (D355A, E357A); Ha, self complementary, hairpin sequence a; Hb self complementary hairpin sequence b.

This paper is available on line at http://www.jbc.org

41379
DNA sequences, 5′-(CAG)2-Ha-(CTG)2-3′ and 5′-(CTG)2-Hb-(CAG)2-3′, referred to as CTG-Nick and CAG-Nick strands, respectively, were used to construct a double-stranded DNA molecule with a CTG/CAG repeat duplex having a nick between CTG repeats on one side (CTG Nick) and a nick between CAG repeats on the other side (CAG Nick), as illustrated (Fig. 1A, unslipped structure). The sequences Ha and Hb are 30-mer s chosen to form stable, intramolecular hairpin structures. Specifically, Ha and Hb were designed (CTG-Gap and CAG-Gap strands) to form stable, intrastrand hairpin structures (Fig. 1, A and B). The sequences Ha and Hb are 30-mer s chosen to form stable, intrastrand hairpin structures. Specifically, Ha and Hb were designed (CTG-Gap and CAG-Gap strands) to form stable, intrastrand hairpin structures (Fig. 1, A and B). The sequences Ha and Hb are 30-mer s chosen to form stable, intrastrand hairpin structures. Specifically, Ha and Hb were designed (CTG-Gap and CAG-Gap strands) to form stable, intrastrand hairpin structures (Fig. 1, A and B). The sequences Ha and Hb are 30-mer s chosen to form stable, intrastrand hairpin structures. Specifically, Ha and Hb were designed (CTG-Gap and CAG-Gap strands) to form stable, intrastrand hairpin structures (Fig. 1, A and B).

The CTG/CAG repeat duplexes between the two strands, before reducing temperature (at 0.5 °C/min) from 50 °C to 20 °C, and finally storing at 4 °C. The 2-fold excess of “cold” strand helped ensure that each “hot” strand was completely annealed in triplet-repeat duplex.

**Polymerase Reactions**—Annealed Nick and Gap DNA constructs, at 50 mM concentration of the radiolabeled strand, were equilibrated to 37 °C for at least 5 min in polymerase buffer (25 mM Tris-HCl pH 7.5, 6.7 mM MgCl2, 10 mM NaCl, 1.5 mM dithiothreitol, 0.2% glycerol) (26). Polymerase and dNTP substrate were equilibrated to 37 °C for at least 2 min in the same polymerase buffer in a separate microcentrifuge tube. Before combining reactionants, a 5-μl sample of DNA solution was removed (as a control) and added to 5 μl of stop solution (0.2 mg/ml proteinase K, 2% SDS, and 25 mM Na2 EDTA). To start extension reactions, radiolabeled DNA solution was micropipetted into 15 μl of polymerase-dNTP solution at which point the reaction time (t) began. The reactions at 37 °C were sampled in 5-μl aliquots taken at t = 0.25, 0.5, and 1 min, etc. (up to 32 min). Each sample was immediately added to an equal volume of stop solution and incubated at 50 °C for 1 h to allow sufficient polymerase digestion with proteinase K to obtain optimal DNA resolution by denaturing gel electrophoresis. Before loading samples on gel, 10 μl of denaturant (98% formamide, 2% bromphenol blue/xylene cyanol dye) was added to each 10-μl polymerase-digested reaction sample.

Each of the two polymers (pol β and KFexo-1) was assayed in the above manner at three enzyme concentrations (only one shown here) and three dNTP concentrations (all shown). In each case, reactions were sampled as a function of time (up to t = 32 min) and the extension products of the 32P-labeled strand were resolved as a series of bands by electrophoresis and phosphorimaging. The band patterns obtained as a function of time are shown (Fig. 2) for each enzyme at 5 μM concentration (0.05 and 0.5 μM not shown) and (Fig. 3) KFexo-1 at 0.6 μM concentration (0.06 and 0.8 μM not shown). Specific bands in a given panel are identified in terms of the number of bases added to the 3′-end of original 72-mer or 71-mer strands in Nick or Gap constructs, respectively.

**Electrophoresis and Phosphorimaging**—Extension products were separated into bands of increasing chain length by electrophoresis at constant power (55–65 watts) maintaining a temperature of 50 °C on a 30% polyacrylamide vertical stacking gel (40 cm × 3 mm) and CAG/CAG nick gel (40 cm × 3 mm) containing 20% formamide as denaturant, in TBE buffer (90 mM Tris borate, pH 8.3, 2 mM Na2 EDTA). Gels were dried on paper and scanned by a Molecular Dynamics Storm S800 Phosphorimager.

**RESULTS**

The double-stranded DNA molecules constructed to explore triplet repeat slippage and expansion with pol β have stable terminal hairpin structures flanking a less stable CAG/CTG triplet repeat duplex containing a strand break (nick or 1-base gap) on each side (Fig. 1, A and B). The hairpin structures effectively mimic a long stretch of surrounding, non-repetitive duplex DNA. If the polymerase bound to the strand break has enough strand displacement activity to extend the primer 3′-end into the non-repeating regions of terminal hairpins, the repetitive character of the primer will be destroyed and repeat expansion by slippage will be halted.

Our placement of strand breaks internally to the repeat/non-repeat boundary, at a distance of two CAG repeats on one strand and two CTG repeats on the other strand (Fig. 1, A and B), provides a weak (2-triplet) barrier against such termination of slippage by strand displacement into non-repeat sequence. As long as strand displacement is not strong enough to pass this barrier of two triplet repeats, the polymerase may expand triplet repeats by allowing the 3′-end to slip in the “positive” direction as indicated for +1 slippage, i.e. slippage by one strand to create a 3-base gap (Fig. 1, A and B). However, if strand displacement is strong enough to pass this barrier, the polymerase can only extend the 3′-primer end by continued strand displacement synthesis that creates the hairpin structure and uses the Ha or Hb sequence as template to create a blunt-end product of defined length (e.g. 42 bases added to the CTG-Nick or CAG-Nick strand, corresponding to 30 bases added on template Ha or Hb and 12 on 2 CAG + 2 CTG triplets). Since the 5′-end of one strand is 32P-labeled while the other is not, only extension products of the labeled strand are observed as
bands by denaturing gel electrophoresis and phosphorimaging.

**Triplet Repeat Expansions Observed With DNA Pol β**

_A. Nick Construct_

At 1 and 10 μM dNTP concentration, pol β extends the 3’-end of the radiolabeled CTG-Nick strand to generate a band pattern with a periodicity of exactly three nucleotides, i.e., bases added in 3-base (triplet) steps (Fig. 2A, CTG Nick). The pattern builds in a progressive time-dependent manner, indicating CTG repeat expansion via slippage in steps of one triplet. With increasing reaction time at 10 μM dNTP, each band shows a characteristic rise in intensity to some maximum level, followed by a corresponding fall in intensity, as the molecules slip and are further extended by polymerase into bands of increasing number of bases added. Thus at 10 μM dNTP, bands ranging from 3 bases added (1 triplet) to 102 bases added (34...
FIG. 2. Comparison of extension products obtained with human DNA pol β acting on strands of CTG and CAG repeats in Nick and Gap constructs. Extension of the given radiolabeled strand (indicated in the upper left of each panel) by pol β at 37 °C was studied as a function of reaction time (min) and concentration (μM) of each dNTP (n = A, C, G, T). Extension products were resolved using 10% denaturing PAGE with 20 M formamide as the denaturant. Numbers and dashed lines aligned with specific bands indicate the number of bases added to original strand lengths of 72 bases for the Nick construct and 71 bases for the Gap construct. Although obtained in separate extension reactions, the pair wise extension reactions of like dNTP concentrations occur simultaneously with only the radiolabeled strand being visible. A, results obtained when radiolabeling the CTG Nick (upper left panel) or CAG Nick (upper right panel). At 10 μM for both CTG Nick and CAG Nick, the bands labeled at a triplet interval are added for reference and highlight the triplet periodicity of slippage expansion products. We also indicate the absolute extent of slippage expansion by labeling the band at 102 bases added (34 triplets). At 100 μM for both CTG Nick and CAG Nick, the band at 42 bases added is specifically labeled and corresponds to the predicted length of strands extended immediately into non-repeat sequence in the complete absence of slippage. B, results obtained when radiolabeling CTG-Gap (lower left panel) or CAG-Gap (lower right panel). We label the CTG Gap panel as in A. At both 10 and 100 μM for CAG Gap, the bands at 43, 40, and 37 bases added are specifically labeled and correspond to the predicted lengths of strands extended immediately into non-repeat sequence in the complete absence of slippage from both unslipped and negatively slipped states.
triplets) are formed as a result of triplet-repeat slippage and polymerase-catalyzed extension over a 32-min period (Fig. 2A).

After increasing dNTP concentration 10-fold, from 10 to 100 μM, we observe a dramatic change in the pol β extension pattern (Fig. 2A, CAG Nickel). The major product reaction is now a termination band at 42 bases added, which rises to a near maximum intensity within 1 min and fails to decrease in intensity with remaining reaction time. The accumulation of band intensity at this point (42 bases added) indicates that the major product is no longer extendable by pol β. Since this product corresponds to the predicted size dictated by the sequence of hairpin ends, we identify it as the blunt-ended product obtained by strand displacement through the 2-triplet barrier (into the non-repeat sequence) starting from the original unslipped state of CTG Nick.

Above this band, we also observe less intense ultimately static product bands with additional triplet repeats, corresponding to 42 + 3n bases added, where n = 1, 2, 3, etc. (Fig. 2A, CAG Nick, 100 μM). As before, the failure of these bands to decrease in intensity suggests that they also contain products that are blunt-ended and no longer extendable by pol β. These bands indicate to us that at this elevated dNTP concentration a substantial fraction of strands still undergo pol β catalyzed slippage-expansion a number of times before the downstream barrier of two triplet repeats is breached by strand displacement synthesis into non-repeat sequence. Determining how a given band below 42 bases added forms within the first 2 min of reaction (strand displacement or slippage expansion) is somewhat confounded by the fact that extension products from strand displacement into non-repeat sequence overlap in size with extension products formed by slippage expansion occurring prior to strand displacement into non-repeat sequence. However, once strand displacement into non-repeat sequence occurs, no further expansion by triplet-repeat slippage is possible. Therefore, molecules that have n triplets added by slippage end up in bands at 42 + 3n bases added, after reaching the blunt-ended state by the addition of 42 bases via strand displacement through the barrier.

As seen in Fig. 2A (CAG Nick), pol β extends the CAG-Nick strand in a similar fashion to the CTG-Nick strand, by catalyzing slippage-expansion at 1–10 μM dNTP concentrations and slippage-expansion largely terminated by strong strand displacement at 100 μM dNTP concentration. However, the slippage-expansion rate of the CTG-Nick strand, by the addition of CAG triplets with pol β, is not quite as high as the rate of the CTG-Nick strand by the addition of CTG triplets. Some differences in polymerase pausing on the two opposing strands are also evident.

Pol β Catalyzes Slippage-Expansion at Physiological Concentrations of dNTP—At 1–10 μM dNTP concentrations, our Nick construct (Fig. 2A), pol β catalyzes expansion by slippage with little or no strand displacement into non-repeat template. These concentrations roughly bracket published estimates of physiological dNTP concentrations in human, except for dTTP whose in vivo estimates are somewhat higher than 10 μM (29); human dNTP concentrations being on average as follows (in μM): n = A (2.4), G (2.7), C (4.5), T (17).

Pol β catalyzes terminal strand displacement into non-repeat sequence in our Nick construct only at much higher dNTP concentrations, e.g., 100 μM, well outside of the physiological range. Thus, pol β shows a much lower dNTP requirement for slippage-expansion than for terminal strand displacement, when acting on CTG Nick and CAG Nick (Fig. 2A).

Pause Patterns of DNA Pol β Suggest Low Processivity in Slippage-Expansion—At 10 μM dNTP, during slippage expansion at both CTG Nick and CAG Nick (Fig. 2A), pol β produces repetitive “pause” bands indicating low processivity, i.e., a tendency to fall off DNA after each insertion. The greatest pausing (most intense pause bands) appears after T insertion during addition of CTG repeats to the CTG-Nick strand (Fig. 2A, left), and after C and A insertions during addition of CAG repeats to the CAG-Nick strand (Fig. 2A, right). The observation of such pausing suggests slippage-expansion is not simply occurring by slippage and gap filling but also may involve non-processive strand displacement in the repeat region.

A simple slippage event in a triplet repeat sequence might be expected to create a 3-base gap as indicated for +1 slippage (Fig. 1A). Pol β has been shown to fill small gaps in non-repeating DNA in a highly processive manner in the presence of a 5′-phosphate group (30). If there were simply a processive filling of 3-base gaps created by slippage in our system, we would expect a single-band pause pattern occurring at insertion of G after adding 3 nucleotides with G being the last one as dictated by the sequence of the gap, for both CTG-Nick and CAG-Nick strands. The observation of pausing after other insertion events in such gap filling has led us to consider how slippage and gap-filling may be associated with non-processive strand displacement in the repeat region.

Extension Reactions of Gap Construct with Pol β—The gap construct (Fig. 1B) differs from the Nick construct (Fig. 1A) only by the removal of 3′-terminal G from each of the two strands. The CTG-Nick and CAG-Nick strands with G removed from their 3′-ends are referred to as CTG-Gap and CAG-Gap strands, respectively. In the resultant CTTG/CAG duplex formed between the two strands, we expect 1-base gaps as illustrated (Fig. 1B, unslipped structure).

Pol β extends the CTG-Gap strand (Fig. 2B) in much the same way it extends the CTG-Nick strand (Fig. 2A). As expected, we see a shift in the gel patterns by 1 base (consistent with the fill-in of the 1-base gap) (Fig. 2B). Otherwise, we observe pol β catalyzing almost the same slippage-expansion process at lower dNTP concentrations (1 and 10 μM) and only a slightly different terminal strand displacement reaction at higher dNTP concentration (Fig. 2B).

At 100 μM dNTP, in addition to pol β extending the unslipped structure via terminal strand displacement to form the 43 bases-added product (corresponding to the 42 bases-added product formed in the Nick construct), we see a strand displacement product at 40 bases added as well (Fig. 2B). The intense band at 40 bases added is consistent with extension of a 3′-primer end that has been slipped in the “negative” direction to create a 5′-flap of 2 bases, as illustrated for −1 slippage (Fig. 1B, top). We note that the 40 bases-added band appears even more intense than the 43 bases-added band, indicating that the negatively slipped state is energetically more favorable than the unslipped state.

DNA Pol β Shows More Strand Displacement Activity at the CAG Gap at All dNTP Concentrations—A marked difference between pol β extension of the CAG-Nick and CAG-Gap strands is observed in the 10 μM dNTP reaction (Fig. 2B). At 10 μM dNTP, pol β shows much more strand displacement activity at the 3′-end of the CAG Gap compared with CAG Nick and CTG Nick (Fig. 2A) and to CAG Gap (Fig. 2B). In the CAG Gap case (Fig. 2B, right), pol β extends 3′-primer ends by strand displacement to a blunt-end state not only from the unslipped and −1 slipped states, but also from the −2 slipped state to yield a termination band at 37 bases added (Fig. 2B, right). The 40 bases-added band appears most intense, with the 37 bases-added band being about half as intense, comparable to the intensity of the 43 bases-added band. The greater intensity of bands from negatively slipped primer ends again indicates that negatively slipped states are energetically more favorable than
the unslipped state in the gap context.

**Strong Strand Displacement by KFexo/11002 Prevents Slippage—Expansion**—In contrast to our results with pol β, extension of the Nick and Gap constructs by KFexo/11002 presents a much simpler result, extremely rapid strand displacement into non-repeat sequence with little or no expansion of triplet repeats (Fig. 3, A and B). Unlike pol β (Fig. 2, A and B), where triplet repeat expansions progress with time at dNTP concentrations up to 10

**Fig. 3.** Comparison of extension products obtained with *E. coli* DNA pol I, KFexo/ acting on strands of CTG and CAG repeats in Nick and Gap constructs. Extension of the given radiolabeled strand (indicated in the upper left of each panel) by KFexo/ at 37 °C was studied as a function of time (min) and of concentration indicated (μM) of each dNTP (α = A, C, G, T). Extension products were resolved using 10% denaturing PAGE with 20 M formamide as the denaturant. Numbers and dashed lines aligned with specific bands indicate the number of bases added to original strand lengths of 72 bases for the Nick construct and 71 bases for the Gap construct. Bands corresponding to the end products of immediate strand displacement synthesis from unslipped or negatively slipped primers are specifically identified. Results obtained when radiolabeling A, the CTG-Nick (upper left panel) or CAG-Nick (upper right panel) and B, CTG-Gap (lower left panel) or CAG-Gap (lower right panel). In panel A, poor well formation required that the 8 and 16 min samples be analyzed on a separate gel (data not shown). Although obtained in separate extension reactions, the pair wise extension reactions of like dNTP concentrations occur simultaneously with only the radiolabeled strand being visible.
DNA Polymerase β Expands Triplet Repeats

μM and are only inhibited by strand displacement into non-repeat sequence at high dNTP (100 μM), KFexo⁺ shows immediate strand displacement into non-repeat sequence at all dNTP concentrations used (Fig. 3, A and B, dNTP = 0.2, 2, and 20 μM). Even at 0.2 μM dNTP, a limiting substrate condition, the extension reaction with KFexo⁺ is almost entirely by strand displacement into non-repeat sequence, yielding some complete termination product within 0.5 min, e.g. 42 bases added (Fig. 3A, CTG Nick); 40 and 43 bases added (Fig. 3B, CTG Gap).

When given 2 μM dNTP or higher (20 μM), KFexo⁺ extends all strands almost exclusively in a terminal strand displacement mode, to yield prominent termination bands like those seen with pol β at much higher (100 μM) dNTP (Fig. 2, A and B). In the cases of CTG-Nick and CAG-Nick with KFexo⁺ (Fig. 3A), within 15 s of reaction, almost all the products are in the termination band at exactly 42 bases added and there is very little change after this time. When extending CTG-Gap (Fig. 3B, left), KFexo⁺ produces strong termination bands at 43 and 40 bases added (with the 40 bases-added band being most intense). When extending CAG-Gap (Fig. 3B, right), KFexo⁺ produces termination bands at 43, 40, and 37 bases added (again with 40 bases-added band being most intense and all bands reaching their maximum intensity within 0.5 min). The strand displacement by KFexo⁺ not only occurs much more quickly than with pol β but also at 10–100-fold lower enzyme and dNTP concentrations.

Although KFexo⁺ extends a majority of strands immediately by strand displacement into non-repeat sequence, some strands still undergo limited slippage-expansion as evidenced by bands forming in a triplet periodicity above the major strand displacement products. However, the faintness of these upper bands in KFexo⁺ reactions leaves little signal to assign to slippage-expansion products including fill-in of gapped molecules. Thus, it is evident that only a small percentage of molecules could initially be in slipped states with small gaps of 1 or 2 triplets and essentially none could have larger gaps.

Negatively Slipped 3′-Primer Ends Occur Specifically in the Gap Construct—In Figs. 2 and 3, only the Gap construct shows terminal strand displacement products smaller than expected from the unslipped state (42 or 43 bases added for Nick and Gap, respectively). We expect these shorter termination products (40 or 37 bases added, Fig. 2B) to form by extension of 3′-ends that have displaced one or two 5′-downstream repeat units as illustrated for −1 slippage (Fig. 1B). In the Gap construct, such displacement of downstream repeats by −1 and −2 slippage yields small 2 or 5 base flaps at the 5′-end. We term the downstream realignment of repeat units as “negative” slippage as opposed to “positive” slippage that occurs in the opposite direction away from 5′-sequence.

Polymerase Extensions of CTG Strands in the Absence of dATP and CAG Strands in the Absence of dTTP—In designing our dual hairpin molecules (Fig. 1, A and B), we specifically anticipated the possibility that slippage and extension of triplet repeats at a given 3′-primer end of one strand could affect the expansion rate at the 3′-primer end of the other strand. To address the issue we performed extension reactions in the absence of dATP (−dATP) when using radiolabeled CTG-Nick or CTG-Gap strands and in the absence of dTTP (−dTTP) when using radiolabeled CAG-Nick or CAG-Gap strands (Fig. 4). In the absence of the appropriate nucleotide, polymerase can only add triplet repeats to the radiolabeled strand and synthesis by strand displacement is limited to the triplet repeat region. While allowing strand displacement into the 2 downstream triplet repeats, strand displacement into non-repeat sequence is inhibited, because the missing nucleotide is required to correctly cross the repeat/non-repeat boundary in each case (Fig. 1, A and B). The results shown for pol β (Fig. 4A) were obtained with 10 μM concentrations of each of the three dNTPs indicated; those shown for KFexo⁺ (Fig. 4B), with 5 μM concentrations.

Further Evidence of Limited Displacement-assisted Repeat Expansion—By withholding one dNTP to limit strand displacement to the repeat region, we observe dramatic changes in the results with KFexo⁺ (Figs. 4B versus 3) but relatively small changes in the results with pol β (Figs. 4A versus 2), as expected. In stark contrast to the static patterns produced by strand displacement in the presence of all four dNTPs (Fig. 3), KFexo⁺ now produces a dynamic slippage expansion pattern that builds over time (Fig. 4B) much like the patterns produced by pol β at 10 μM dNTP concentration (Figs. 2 and 4A). The ability of KFexo⁺ to extend triplet repeats when strand displacement is limited to within the repeat tract lends credence to the idea that a limited strand displacement activity is capable of assisting slippage-expansion.

While expanding triplet repeats in the absence of one dNTP, KFexo⁺ shows a tendency to strand displace into non-repeat template sequence by misinsertion with some probability. Misinsertion at the first non-repeat template base is to be expected in the absence of the correct dNTP. Such misinsertion converts some fraction of each major triplet repeat expansion product into a static termination band longer by 1 nucleotide in each Nick and Gap case (Fig. 4B). The occurrence of prominent misinsertion only after major triplet pause bands indicates that KFexo⁺ catalyzes slippage-expansion by strand displacement up to the repeat/non-repeat boundary rather than by filling in gapped molecules. The observation provides further evidence that strand displacement is involved in the slippage-expansion process.

CTG Strands Slip and Expand Independently of Extension and Slippage Realignment of CAG 3′-Primer Ends—Extensions of CTG-Nick and CTG-Gap strands by pol β −dATP show a remarkable degree of consistency with each other and in particular with extension in the presence of all four dNTPs (Figs. 2 and 4A). Therefore, in the absence of a growing CAG-Nick or CAG-Gap strand, CTG-Nick and CTG-Gap strands are slipping and being extended by pol β independent of extension from the CAG strand. Additionally, the CTG-Gap strand slips and expands in the presence of pol β and 10 μM of all four dNTP, despite a pronounced strand displacement of the CAG-Gap strand into non-repeat sequence that prevents 3′-primer realignment (Fig. 2B). Therefore, CTG strands may slip and expand independently of slippage realignment at the CAG 3′-primer ends as well.

DISCUSSION

We find simple strand breaks in the form of nicks or 1-base gaps within CTG/CAG repeat tracts are sufficient to cause reiterative repeat expansion in the presence of human DNA pol β at physiological dNTP concentrations. At 10 μM or less of each dNTP, the strand displacement activity of pol β is sufficiently low to prevent extension of 3′-primer ends through a downstream barrier of 2 repeat units and into surrounding non-repeat template. The inability of the weak strand displacement activity to extend the 3′-primer end beyond the repeat template into non-repeat template preserves the repeat motif of the primer and explains the continuation of slippage-expansion with time.

A recent study of T4 DNA polymerase and dinucleotide repeat stability highlighted the need for a barrier to replication into surrounding non-repeat sequence for microsatellite instability to occur (22). In that study, proofreading 3′-exonuclease activity and a downstream oligonucleotide enabled the 3′-
primer end to remain within the repeat tract sufficiently long to allow for primer misalignment and T4 polymerase extension to catalyze expansion. Here we observe how limited strand displacement activity in the complete absence of exonuclease activity may achieve similar results in causing microsatellite instability. Others have also observed the inverse relationship between strand displacement and slippage (31). Pausing by pol β at particular bands during slippage-expansion.
The present study reveals the ability of pol β to expand triplet repeats in the absence of any other proteins. Some repair proteins (FEN-1 and PARP-1) increase the strand displacement activity of pol β (17) while others (XRCC1 and Lig III) reduce its strand displacement activity in favor of gap filling and ligation (32). In both cases (increased strand displacement or gap filling with ligation), we expect a reduced amount of expansion in the experiment presented. Also, there is the natural comparison to be made between the ability of pol β and homolog polymerase λ (pol λ) to expand triplet repeats. Relative to pol β, pol λ shows less strand displacement activity and lower $K_{m}$ for dNTP (39).

In the presence of a 5′-exonuclease activity such as FEN-1, the rate of repeat expansion with pol β is expected to decrease for two reasons. First, removal of 5′-flaps prevents the kind of flap exchange that may contribute to slippage (Fig. 5). Second, such removal may enable primer 3′-ends to be extended more rapidly to the repeat/non-repeat boundary. In fact, disruption of FEN-1 activity in yeast causes significant expansion of triplet repeats (34). The presence of FEN-1 counteracting the pol β-catalyzed expansion observed here provides a simple way of explaining eukaryotic DNA repeat instability observed in the absence of FEN-1.

The dramatic difference between pol β and KFexo + extension of our dual hairpin molecules illustrates the impact of strand displacement on the slippage-expansion process. In light of the similar roles played by pol β in eukaryotes and pol I in prokaryotes, the large difference in strand displacement activity between these two polymerases may be one reason why triplet repeat expansions are more common in eukaryotic genomes than in prokaryotic genomes. Interestingly, expression of pol β complements a pol I-deficient bacteria strain in Okazaki fragment processing (35) and such a strain may provide an immediate way of ascertaining the effect of these differing strand displacement activities on the stability of triplet repeats in a bacterial genome. As might be expected, inactivation of the 5′-exonuclease activity in bacterial DNA pol I increases repeat expansion relative to wild type (36), consistent with our expectation that FEN-1 inactivation may allow eukaryotic pol β to expand triplet repeats by a flap exchange mechanism (Fig. 5).

In the presence of a 5′-flap, ligase activity is capable of creating ligation-mediated expansions even in the presence of significantly higher amounts of FEN-1 (9). With ligase winning the competition with FEN-1, a lingering competition remains between polymerase extension of 3′-ends and ligation of 5′-ends during slippage occurring in the presence of a flap. Regardless, we are likely observing the greatest amount of expansion to be expected from pol β with these additional protein activities only serving to stabilize repair synthesis at strand breaks in triplet repeats.

While recent studies suggest the involvement of simple gap formation in triplet repeat expansion despite any direct observation of such gaps (37), spontaneous gap formation requires more activation energy than displacement-assisted gap formation (Fig. 5). At the top of Fig. 5, starting from a nick in a CTG repeat strand, we illustrate the spontaneous formation of a 3-base gap by slippage without polymerase. Such slippage requires the melting of at least 4 base pairs to enable the 3′-G to move from its nick position (top, left) to its gap position (top, right). In the proposed displacement-assisted mode (left, top to bottom), polymerase extension by strand displacement extends the 3′-end while creating a 5′-flap. At low dNTP concentration (10 μM or less), strand displacement is slow enough to allow the 5′-flap to reanneal and displace a 3′-flap (unextendable). The 3′-flap promotes slippage by reducing the activation energy for slippage (i.e. reducing the number of base pairs that need to be

---

**Fig. 5. Model of polymerase displacement-assisted slippage and expansion of trinucleotide repeats by human pol β.** Weak, non-processive 5′-end displacement accompanied by 3′-end slippage and gap filling are shown contributing to polymerase-catalyzed slippage and expansion of triplet repeats at a nick. On the left (indicated by vertical arrows), polymerase-catalyzed 5′-end displacement occurs in steps of single nucleotide insertion creating 5′-flaps that rearrange to form 3′-flaps (shown in equilibrium). The 3′-flaps act as intermediates to promote 3′-slippage in steps of triplet repeats, enabling repeat expansion by gap-filling (indicated on the right by vertical arrows). In our construct (Fig. 1), the non-repeat sequence (Hb or Ha) placed next to the 5′-end repeat sequence (5′-CTGCTG or 5′-CAGCAG) inhibits 5′-slippage of the kind proposed in ligation-mediated expansions (7, 9), allowing us to observe polymerase-catalyzed repeat expansions by 3′-slippage accompanying 5′-displacement.

Several factors affect the strand displacement activity of pol β including enzyme concentration (30), salt concentration (16), auxiliary repair proteins (17, 32), and in our own experiments dNTP concentration. The high pol β concentration (5 μM) used in our experiments is sufficient for near maximum strand displacement activity since we obtain nearly identical results at 10-fold lower concentration (data not shown). However, complete strand displacement (to a blunt-ended state) by pol β is obtained only at very high dNTP (100 μM) suggesting a high $K_{m}$ for strand displacement. At lower dNTP concentrations of 1–10 μM, the strand displacement activity is clearly much weaker, allowing slippage to occur within the repeat sequence without extension into flanking non-repeat sequence. At the 100 mM NaCl concentration used here, the strand displacement activity of pol β is likely to be only slightly greater than the very low level observed in physiological, 150 mM saline (16).
melted for 3′-end slippage to occur.

Our model of displacement-assisted expansion (Fig. 5) raises interesting possibilities with regard to break placement and repeat length. Trinucleotide repeats show increasing probability of expansion with increasing lengths of pure repeat sequence. Increasing length of repeat tract not only increases the probability of strand breakage within the repeat tract, but also allows breaks to occur farther from non-repeat boundaries and therefore allows for greater amounts of strand displacement without inhibiting slippage realignment. Breaks within a repeat tract occurring farther from the non-repeat boundary would be expected to increase the probability of triplet repeat expansion involving strand displacement and would allow other polymerases with stronger strand displacement activity to catalyze repeat expansion. Thus, longer repeats might be expected to expand more easily than shorter repeats as observed in triplet repeat expansion diseases (38). While increasingly longer repeat tracts may allow polymerase with strong strand displacement to catalyze repeat expansion, polymerase with weak strand displacement of the type observed here with pol β may specifically allow expansion of initially shorter repeat tracts that would naturally have breaks closer to the non-repeat boundary. Therefore, weak strand displacement would provide an effective means of generating longer (more mutable) repeat tracts from shorter ones.

We also observe the importance of break type on repeat expansion. The CAG-Gap strand shows less repeat expansion than the CAG-Nick strand apparently because pronounced negative slippage at CAG Gap (Fig. 2B) places the 3′-end of the CAG-Gap strand closer to the non-repeat boundary. Negative slippage seen in similar amounts with pol β (Fig. 2B) and KFexo− (Fig. 3B), indicate that such slippage (creating 5′-flaps) occurs initially, in absence of polymerase. Our observation that pol β and KFexo− both show a majority of CTA-Gap and CAG-Gap strands adopting a negatively slipped state (Figs. 2 and 3), indicates that negative slippage can result in energetically more favorable conformations in the presence of a 1-base gap. In converting from unslipped to −1 slipped, the gap construct replaces each 1-base gap with a 2-base 5′-flap, with more degrees of freedom in the flap than in the gap. Since purines but not pyrimidines undergo anti-syn rotation, the greater negative slippage at CAG-Gap ends might be related to the greater number of degrees of freedom obtainable with an extra purine base (A) in CAG flaps versus an extra pyrimidine base (T) in CTG flaps.

In our Nick construct (Fig. 1A), the formation of a 3-base flap by −1 slippage (not shown) requires the loss of 3 base pairs as shown for +1 slippage to form a 3-base gap. However in our Gap construct, the formation of a 2-base flap by −1 slippage only requires the loss of 1 base pair relative to the unslipped structure. The resultant difference of several kcal/mol in activation energy explains the observation of negative slippage in the Gap construct but not in the Nick construct with both pol β and KFexo−.

In addition to break type, repeat sequence composition clearly affects the rate of repeat expansion at a break. Expansion occurs faster or more efficiently on the CSG strand than on the CAG strand, probably because single strands of CTG repeats form hairpin loops with more stable secondary structures (3, 24) facilitating larger amounts of slippage. Our previous work on slippage within hairpin loops of triplet repeats demonstrates the effect of sequence composition on slippage rate as well as on hairpin loop conformation (25). The greater stability of CAG repeat hairpins relative to CAG repeat hairpins causes slippage within hairpin loops to occur more slowly. However, in the present study, more stable CTG repeat hairpins appear to be enabling greater amounts of polymerase-catalyzed slippage expansion of the CTG strand within a CAG/CTG repeat duplex.

Our data for pol β (Figs. 2A and 4A) indicate that repeat expansions of the CTG strand occur independently of those on the CAG strand and appear to be dominant to expansion on the CAG strand, suggesting that CTG repeat expansions would occur at a break on the CTG strand without requiring a break on the CAG strand. Expansion at a single strand CTG break would lead to heteroduplex with more CTG than CAG repeat units. Such heteroduplex has been seen to be stably transcribed in yeast to fix a repeat expansion (39). Furthermore, our system allows for further exploration of the effect of base composition and sequence through analysis of other triplet sequences associated with neurological diseases, including the Friedrich’s ataxia GAA/TTG repeat not known or expected to form normal hairpin structures (40).

Notwithstanding all the interesting questions our experimental system is capable of addressing, we already see that pol β readily expands triplet repeats at strand breaks under physiological dNTP and salt concentrations. Our observations raise the possibility that pol β may be an important contributor to triplet repeat expansions in human neurological diseases. Further, our results combined with the observations of others suggests a testable model (Fig. 5) indicating how the weak strand displacement activity of this eukaryotic gap-filling polymerase enables triplet repeats to be expanded by slippage at strand breaks.

**REFERENCES**

1. Bowater, R. P., and Wells, R. D. (2001) Proc. Natl. Acad. Sci. USA 98, 5122–5127.
2. Celli, B., and Bacolla, A. (1998) in Genetic Instabilities and Hereditary Neurological Diseases (Wells, R. D., and Warren, S. T., eds.), pp. 561–583, Academic Press, San Diego.
3. Petruska, J., Arneheim, N., and Goodman, M. F. (1996) Nucleic Acids Res. 24, 1992–1998.
4. Mitas, M., Yu, A., Dill, J., Kemp, T. J., Chambers, E. J., and Haworth, I. S. (1995) Nucleic Acids Res. 23, 1050–1059.
5. Gacy, A. M., Goellner, G., Janusiec, N., Macura, S., and McMurray, C. T. (1995) Cell 81, 535–540.
6. Darfor, J. M., and Leach, D. R. (1995) Genes 11, 1411–1418.
7. Gordenin, D. A., Kunkel, T. A., and Resnick, M. A. (1997) Nat. Genet. 16, 116–118.
8. J. H., Okert, R., Burgers, P. M., Kunkel, T. A., Resnick, M. A., and Gordenin, D. A. (2001) Proc. Natl. Acad. Sci. USA 98, 5122–5127.
9. Henrickson, L. A., Veeraraghavan, J., Chafin, D. R., and Bambara, R. A. (2002) J. Biol. Chem. 277, 23834–23841.
10. Jankowski, C., Nasar, F., and Nag, D. K. (2000) Proc. Natl. Acad. Sci. USA 97, 2134–2139.
11. Sow, R. W., Horton, J. K., Kuhn, R., Gu, H., Singhai, R. K., Prasad, R., Rajevsky, K., and Wilson, S. H. (1996) Nature 379, 183–186.
12. Mitchell, J., Karwawa, E., Kinzea, T., and Wilson, S. (1985) Mutat. Res. 146, 295–300.
13. Intaras, G. W., McManah, A., Walter, R. B., McCurry, J. R., and Walter, C. A. (2001) Nucleic Acids Res. 29, 1366–1372.
14. Klungland, A., and Lindahl, T. (1997) EMBO J. 16, 3341–3348.
15. Wang, T. S.-F., and Kern, D. (1980) Biochemistry 19, 1782–1790.
16. Randall, H., Elliott, G. C., and Linn, S. (1988) J. Biol. Chem. 263, 12225–12234.
17. Prasad, R., Davenport, O. J., Kim, S. J., Kedra, P., Yang, X.-P., Vande Berg, B. J., and Wilson, S. H. (1991) J. Biol. Chem. 276, 23411–23417.
18. Kang, S., Ohshima, K., Shimizu, M., Amarihaeri, S., and Wells, R. D. (1995) J. Biol. Chem. 270, 27014–27021.
19. Oshika, K., and Wells, R. D. (1997) J. Biol. Chem. 272, 16788–16806.
20. Ji, J., Cleg, N. J., Petersen, K. R., Jackson, A. L., Laird, C. D., and Loeb, L. A. (1998) Nucleic Acids Res. 26, 2835–2840.
21. Kreisler, L. C., and Kunkel, T. A. (1999) Nucleic Acids Res. 27, 3481–3486.
22. Fidalgo da Silva, E., and Reha-Krantz, L. J. (2000) J. Biol. Chem. 275, 31532–31539.
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J., Smith, J. A., and Struhl, K. (eds.) (1987) Current Protocols in Molecular Biology, Vol. 1, pp. 2.12.1–2.12.7 John Wiley & Sons, Inc., New York.
24. Paravastu, J., Hartenstein, M. J., and Goodman, M. F. (1998) J. Biol. Chem. 273, 5204–5210.
25. Hartenstein, M. J., Goodman, M. F., and Petruska, J. (2000) J. Biol. Chem. 275, 18382–18390.
26. Abbotts, J., SensGupta, D., Zmudzka, B., Widen, S., Notario, V., and Wilson, S. (1988) Biochemistry 27, 901–909.
27. Derbyshire, V., Freemont, P., Sanderson, M., Beese, L., Friedman, J., Joyce, C., and Steitz, T. (1988) Science 240, 199–201.
28. Berger, S. L., and Kimmel, A. R. (1987) Methods Enzymol. 152, 100–101.
29. Traut, T. W. (1994) Mol. Cell. Biochem. 140, 1–22.
DNA Polymerase β Expands Triplet Repeats

30. Singhal, R. K., and Wilson, S. H. (1993) J. Biol. Chem. 268, 15906–15911
31. Canceill, D., Vigueria, E., and Ehrlich, S. D. (1999) J. Biol. Chem. 274, 27481–27490
32. Kubota, Y., Nash, R. A., Klangland, A., Schar, P., Barnes, D. E., and Lindahl, T. (1996) EMBO J. 15, 6662–6670
33. Garcia-Diaz, M., Bebenek, K., Sabariegos, R., Dominguez, O., Rodriguez, J., Kirchhoff, T., Garcia-Perdero, E., Picher, A. J., Juarez, R., Ruiz, J. P., Kunkel, T. A., and Blanco, L. (2002) J. Biol. Chem. 277, 13184–13191
34. Kaglin Schweitzer, J., and Livingston, D. M. (1998) Hum. Mol. Genet. 7, 69–74
35. Sweasy, J. B., and Loeb, L. A. (1992) J. Biol. Chem. 267, 1407–1410
36. Morel, P., Reverdy, C., Michel, B., Ehrlich, S. D., and Cassuto, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10003–10008
37. Kovtun, I. V., and McMurray, C. T. (2001) Nat. Genet. 27, 407–411
38. Richards, R., Holman, K., Friend, K., Kremer, E., Hillen, D., Staples, A., Brown, W., Goonewardena, P., Tarleton, J., Schwartz, C., and Sutherland, G. (1992) Nat. Genet. 1, 257–260
39. Moore, H., Greenwell, P. W., Liu, C.-P., Arnheim, N., and Petes, T. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1504–1509
40. Sakamoto, N., Chastain, P. D., Parniewski, P., Ohshima, K., Pandolfo, M., Griffith, J. D., and Wells, R. D. (1999) Mol. Cell 3, 465–475