Abasic Sites Induce Triplet-repeat Expansion during DNA Replication in Vitro*

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The occurrence of triplet-repeat expansion (TRE) during transmission of genetic information is involved in many neurological and neuromuscular diseases including fragile X syndrome and myotonic dystrophy. DNA slippage during replicative synthesis appears to cause TRE. The causes of DNA slippage, however, remain mostly unknown. We investigated the effects of abasic sites on the occurrence of TRE during DNA replication in vitro using Escherichia coli Klenow polymerase I as the model polymerase. Here we show that a single abasic site analog, synthesized in the triplet-repeat tract at the 5′ end of the template strand, induced dramatic TRE during DNA synthesis. The amount of TRE induced decreased when the abasic site was moved to the middle of the repeat tract, consistent with effectively decreasing the length of the repeat tract. Placing the abasic site in the primer did not induce TRE. TRE was sequence-dependent. The damage-induced increase in growing strand TRE depended on the sequence of the growing strand repeat as ATT > ATT > CAG > CTG. The expansions required replication from a primer complementary to the repeat tract. The expanded tracts were sequenced and contained multiple additions of the original repeat. The results imply that DNA damage can play a significant role in generating TRE in vivo.

The occurrence of triplet-repeat expansion (TRE) during transmission of genetic information is involved in many diseases including fragile X syndrome (1–3), the most common form of mental retardation; myotonic dystrophy, a neuromuscular disorder; and several neurodegenerative disorders (1, 2). DNA slippage (4) during replicative synthesis is believed to be a major contributor to TRE in vivo (5–8), because elimination of recombination in yeast (9) and in Escherichia coli (10) does not influence TRE, whereas elimination of mismatch repair does (10, 11). Pausing or blockage of DNA replication has been proposed to promote slippage by giving the DNA replication complex more time to dissociate and form misaligned DNA intermediates (12, 13). Hairpins (14–18), bulges (19, 20), tetraphosphates (18, 21), and possibly “slipped” structures (22, 23) have been proposed to promote DNA slippage. The structures were proposed to act either as intermediates, by their formation within the repeat or by blocking or pausing replication toward the end of the repeat (12, 24) or a combination of both (13, 17, 24). Replication pause sites are hot spots for nucleotide misincorporation (25). If pausing also induces slippage, the occurrence within the repeat tract of DNA damage that blocks DNA replication could profoundly effect TRE.

Studies of replication of DNA template-primers in vitro with DNA polymerase alone are important for their potential ability to uncover mutation mechanisms. Replication of triplet-repeat tracts in vitro shows TRE (26–29). Here, we report the effects of tract length, tract sequence, and DNA damage on TRE during DNA replication in vitro. The abasic site analog tetrahydrofuran (THF) was used as the model DNA damage lesion. Abasic sites and THF block DNA replication (reviewed in Refs. 30 and 31), and the abasic site is one of the most common lesions that arise in cellular DNA (32). THF was synthesized in the triplet-repeat tracts separately in the template and in the primer strands. Replication using E. coli Klenow pol I as the model polymerase and primed from within the repeat tract demonstrated that THF in the template strand greatly enhanced TRE during DNA synthesis.

EXPERIMENTAL PROCEDURES

Materials—Oligodeoxyribonucleotides (oligonucleotides) were synthesized by the Lineberger DNA synthesis facility using an Applied Biosystems automated synthesizer. Oligonucleotides were synthesized as templates for DNA replication. The templates contained ACTGT-GTCTGTGCU (U?) at the 5′ end and GCGACCTGTACCCU (U?) at the 3′ end, surrounding the triplet repeats (ATT)10, (ATT)20, (AAT)10, (AAT)20, (CTG)10, (CTG)20, (CAG)10, and (CAG)20, as indicated in figure legends. Templates containing 10-repeat tracts were also synthesized to contain the abasic site analog THF (phosphoramide purchased from Glen Research) in place of the last base at the 5′ end of the repeat tract. Primers were synthesized to be complementary to either U? or to the repeat tract. The latter were three repeats in length. To study the effect of THF placement, THF was also placed at the beginning of the fourth repeat of (ATT)10 in place of A, U, (ATT)?, (TTA)?, (ATT)U, where F is THF. THF was also synthesized in place of the middle residue of the primer annealed to the template. All the templates were synthesized to contain a three-carbon spacer (Glen Research) at their 3′ terminus that can not be removed or extended by DNA polymerase (not shown).

Assays—The reaction replication contained 50 mM Tris-HCl (pH 7.8), either 0.3 mM or 0.5 mM MgSO4, as indicated in the figure legends, 0.1 mM dithiothreitol, 0.1 mM of all four dNTPs, 5 μM of the primer and template, and either 0.1 unit/μl or 0.2 unit/μl of Klenow pol I (Promega). For annealing, primer and template were mixed, incubated at 90 °C in 50 mM Tris-HCl and MgSO4, and allowed to cool to room temperature. The reaction was started by addition of dithiothreitol, dNTPs, and polymerase and was incubated at the indicated temperatures for 2 h. The reactions were stopped by addition of EDTA to 23 mM and by cooling to 4 °C. Reaction products were resolved by either 2% agarose native or alkaline gel electrophoresis. The gels were either stained with ethidium bromide or, if radiolabeled substrates were used, analyzed using a Storm PhosphorImager (Molecular Dynamics). All of the templates and primers were tested individually for their ability to support expansion under the above reaction conditions. Only primed templates supported TRE under our reaction conditions.

DNA Sequencing—To determine the sequences of the TREs, the expanded primers were extended to the end of the template 5′-terminal unique sequence (U?). For reactions involving abasic sites, the reaction products were melted and renatured twice. First, the reaction products were melted and the extended primers were renatured to templates identical to the reaction templates but lacking the abasic site. The new
annealed template-primers were extended with Klenow polymerase to the end of the template downstream 5' unique sequence (Uₜ). Second, the extended primer strand was melted and reannealed to complementarily sequencing primer for sequencing by automated methods.

Restriction Enzyme Digestion—Restriction enzymes BcaI (New England Biolabs), which recognizes and cleaves the sequence GCAGC(8/12), and AciI, BsgI, MboII, MsiI, NlaIII, and PstI (New England Biolabs), and ThaI (Life Technologies, Inc.), which recognize variants of the CTG repeat, were used according to the manufacturer's recommended conditions.

RESULTS

We analyzed the effect of THF on TRE during DNA replication in vitro. Template-primers were constructed to contain a repeat tract flanked by two different unique sequences (Uₜ and Uₜ in Fig. 1). The 3' ends of the templates were blocked by a three-carbon spacer (Glen Research), which blocks extension by polymerase (not shown). Primers were constructed to be complementary to either the triplet-repeat tract (floating primer) or the unique flanking sequence 3' to the repeat tract (fixed primer). The former mimics the sliding model (6, 7) for expansion by DNA slippage and the latter mimics models involving bulge and hairpin intermediates (14–18). Replication from a floating primer generates long TREs under conditions of low Mg²⁺ (29). The presence of unique sequences flanking the repeat tract, however, significantly reduces the amount of TRE. Control experiments, using end-labeled template and primer, demonstrated that templates and primers by themselves did not support DNA synthesis by Klenow polymerase (not shown). DNA synthesis and TRE were detectable only when annealed primer-template was used for DNA replication.

The model abasic site analog THF (Fig. 1A) was introduced at a single position at the 5' end of the 10-repeat tract just before the unique flanking sequence (Fig. 1B). Replication with both floating and fixed primers is portrayed in Fig. 1B, 1 and 2, respectively. The presence of THF at the end of the repeat tract gave significant TRE with the primer/template (AAT)₆:Uₜ(FTT)(ATT)₇Uₜ (Fig. 2), whereas there was little expansion in the absence of THF under these conditions. The conditions used previously to observe significant amounts of spontaneous TRE during DNA replication in vitro lacked unique sequences flanking the repeat template (26–29). The amount of THF-induced expansion that occurred at 42 °C increased with increased reaction time to approximately 1500 bases for primer AAT after 4 h (Fig. 2). Significant THF-induced expansion was also observed at 37 °C (Fig. 3, lane 3). Shifting the position of THF to the middle of the repeat tract (see “Experimental Procedures” for sequence) decreased the amount of expansion observed at 37 and 42 °C. The result is consistent with effectively shortening the length of the repeat tract in the template (Fig. 3, lanes 4 and 9). Placing THF in the middle of the primer did not enhance TRE (Fig. 3, lanes 5 and 10) and instead appeared to reduce the amount of fill-in replication product as measured by the intensity of the lowest molecular weight band (lanes 5 and 10).

Replications of all four of the 10-repeat templates with and without THF were compared under identical reaction conditions. The order of primer repeat expansion induced by THF, located at the 5' end of the template repeat tract, was AAT > ATT > CAG > CTG (summarized in Fig. 4). A small fraction of longer replication products in the absence of THF is visible in Fig. 4, lanes 2 and 4. These products appear to be the product of processive replication, since only a small fraction of substrate is extended to long product. Alternatively, the THF results imply that a low level of oligonucleotide with contaminating damage within the repeat could be responsible for the small background of long products visible in Fig. 4.

Sequencing of the expanded ATT- and CAG-repeat primer DNA strands (see “Experimental Procedures”) showed that the expansion sequences mimicked the primer repeat (not shown). CAG expansion products, from reactions in which the primer and template strands were separately labeled, were analyzed on alkaline gels. The results demonstrated that only the primer strand (and only from floating primers) was expanded (Fig. 5). The labeled products were also digested with restriction enzymes and analyzed on denaturing gels (Fig. 5, lanes 3 and 6). Both strands of the expansion products were cleaved by BbvI (GCAGC8/12) but not by enzymes that recognize variants of the CAG repeat (AciI, BsgI, MboII, MsiI, NlaIII, PstI, and ThaI) (not shown). The presence of multiple bands in lane 4 (template
only) is probably due to residual secondary structure caused by hairpin formation by CTG repeats (14–18). No breakage at the abasic site was detectable above background. Strand breakage caused by the THF site would have given a band in Fig. 5 corresponding to a labeled 12- or 13-mer (Fig. 5, lane 4).

Using agarose gel electrophoresis, we analyzed the effects of temperature and tract length on replication of CTG template repeat tracts. Ten- and twenty-repeat tracts with and without THF were replicated. Only floating primers (i.e., primers complementary to the repeat tract) gave detectable levels of expansion. Increasing temperature increased TRE both with and without THF. The presence of THF in the template repeats enhanced expansion of triplet repeats over that without THF at all temperatures studied (Fig. 6). 20-repeat tracts reproducibly gave significantly more TRE than 10-repeat tracts. This was true at all temperatures studied whether or not THF was present. The difference between TRE with and without THF in 20-repeat tracts was not as dramatic as that for 10-repeat tracts. This was because of the large amount of "spontaneous" TRE for 20-repeat tracts. Similar increases in TRE with increasing repeat-tract length were found for all of the template-primers (not shown).

**DISCUSSION**

The effects of an abasic site in the triplet-repeat tract on the occurrence of TRE during DNA replication in vitro were determined. The model abasic site THF blocks DNA replication (33–36). Introduction of THF into a triplet-repeat tract was found to dramatically increase TRE during replicative synthesis in vitro. Low Mg\(^{2+}\) concentration was used to generate dramatic TRE (29) similar to the type II TREs observed in Fragile X syndrome and myotonic dystrophy (1–3). Although
The steady-state levels of abasic sites vary with the tissue

Abasic sites arise spontaneously through hydrolysis of DNA with a high enough frequency to have meaningful impact.

It has been suggested that DNA slippage should be enhanced by an idling polymerase at a pause site caused by a DNA structure or bound protein (12). The suggestion, if correct, leads to the hypothesis that DNA damage that blocks replication could have profound effects on TRE. To test the hypothesis in vitro, we introduced the abasic site analog THF into triple-repeat tracts and demonstrated that its presence dramatically increased the occurrence of expansion. This demonstration only has importance in vivo if abasic sites occur within repeat tracts with a high enough frequency to have meaningful impact. Abasic sites arise spontaneously through hydrolysis of DNA N-glycosyl bonds (37) and are estimated to occur at a rate of 10−23 to 10−25 per nucleotide per cell per day (38). The steady-state levels of abasic sites vary with the tissue sampled from a low of ~1 abasic site per 106 nucleotides to a high of 35 abasic sites per 106 nucleotides (38). Chemical exposures of cells in culture induced higher levels (38). Thus, given triplet-repeat tracts of 107 nucleotides and spontaneous steady-state levels of abasic sites, we would expect 0.0001–0.0035 abasic sites per given repeat tract. Assumption that the presence of an abasic site in the triplet-repeat tract results in a 10^3-fold (it could be significantly higher) increase in TRE per

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