Effects of temperature, Mg\(^{2+}\) concentration and mismatches on triplet-repeat expansion during DNA replication in vitro

Tara Lyons-Darden and Michael D. Topal*

Lineberger Comprehensive Cancer Center and Department of Pathology, University of North Carolina Medical School, Chapel Hill, NC 27599-7295, USA

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ABSTRACT

The human genome contains many simple tandem repeats that are widely dispersed and highly polymorphic. At least one group of simple tandem repeats, the DNA trinucleotide repeats, can dramatically expand in size during transmission from one generation to the next to cause disease by a process known as dynamic mutation. We investigated the ability of trinucleotide repeats AAT and CAG to expand in size during DNA replication using a minimal in vitro system composed of the repeat tract, with and without unique flanking sequences, and DNA polymerase. Varying Mg\(^{2+}\) concentration and temperature gave dramatic expansions of repeat size during DNA replication in vitro. Expansions of up to 1000-fold were observed. Mismatches partially stabilized the repeat tracts against expansion. Expansions were only detected when the primer was complementary to the repeat tract rather than the flanking sequence. The results imply that cellular environment and whether the growing strand contains a nick or gap are important factors for the expansion process in vivo.

INTRODUCTION

Trinucleotide repeats in the human genome can become unstable and expand in size upon transmission. Expansions of CAG and AAG repeats give rise to neuromuscular and neurological disorders, including myotonic dystrophy (reviewed in 1,2), Huntington disease (1,2) and Freidreich’s ataxia (3). Expansions of CCG repeats are associated with the folate-sensitive group of chromosome fragile sites (1,2). One of these, fragile X (FRAXA), is responsible for the most common familial form of mental retardation (2). Expansions of both repeats can be remarkably unstable, frequently lengthening upon transmission from one generation to the next (4). Fragile site FRA16B results from an expansion of an AT-rich 33 base pair (bp) minisatellite repeat (5). This means that the process of dynamic mutation may be relevant to repeats of various composition and length.

Triplet-repeat expansion (TRE) appears to be a byproduct of DNA replication rather than DNA recombination because the expansions occur mainly towards the 3’ end of the repeat tract and are in linkage disequilibrium with flanking markers (6). In addition, mutations that affect mismatch repair but not recombination have dramatic effects on the stability of repeat tracts (7,8). DNA slippage, first used to explain the occurrence of frameshift mutations (9), has been proposed as a major contributor to DNA triplet-repeat instability in vivo (10–12). Model systems for studying the role of DNA slippage in trinucleotide repeat expansions include DNA replication in vitro (13,14), in Escherichia coli (10,12,15) and in yeast (16,17). The latter biological systems demonstrate polarity of expansion and therefore imply that DNA slippage has a bias for DNA lagging-strand replication, which is more error prone than leading-strand replication (10,15). DNA slippage is not well characterized. It is unclear what factors induce DNA sequences to slip during DNA replication. Sliding back and forth, possible when an Okazaki fragment occurs entirely within a triplet repeat, is an attractive mechanism proposed for expansion of triplet repeats by slippage (11). Hairpins (18–20), bulges (21,22), tetraplexes (23) and possibly ‘slipped’ structures (24,25) have been proposed to promote DNA slippage. They are proposed to work either as intermediates by their formation within the repeat or by blocking or pausing replication towards the end of the repeat (26,27), or a combination of both (23,28,29).

Replication of DNA template-primer in vitro with DNA polymerase alone represents a minimal system that enables observation of the spectrum of mutations occurring spontaneously and induced by the environment (30). Studies of TRE during DNA replication in vitro are important for their potential ability to uncover expansion mechanisms. Schlötterer and Tautz (13) studied expansion of a variety of complementary oligomers by bacterial DNA polymerases. Most trinucleotide repeats underwent limited expansion, including CTG and AGG which are associated with human inherited diseases (1–3). They were unable to detect expansion of GCC, which was later shown by others to expand similarly with one strand expanding preferentially over the other (14). The chromosomal fragile sites and some of the neurological and neuromuscular diseases such as myotonic dystrophy are characterized by larger TREs than generated in

*To whom correspondence should be addressed. Tel: +1 919 966 8208; Fax: +1 919 966 3015; Email: mdtopal@med.unc.edu
vitro. Here we investigate factors that might optimize the expansions observed by Schlötterer and Tautz (13). The effects were determined of temperature, Mg$^{2+}$ concentration and mismatched bases on the expansion of triplet repeats (AAT)$_n$ and (CAG)$_n$ during DNA replication in vitro. Characterization of the expansions showed double-stranded DNA products without nicks that are perfect expansions of the triplet-repeat sequences. The results demonstrate conditions necessary to generate dramatic expansions in the size of DNA triplet-repeats, and imply that cellular environment is important and that nicking of the growing strand may be an early step in the TRE pathway. The expansion of short repetitive DNA sequences to give longer DNAs is an early observation (31,32) that was used for preparation of DNAs for study of the genetic code. The studies reported here expand on these early studies and on later studies (13) of TRE in vitro.

MATERIALS AND METHODS

Materials

Escherichia coli DNA pol I and Klenow pol I were purchased from Promega. Taq DNA polymerase was from Boehringer Mannheim. Oligodeoxyribonucleotides (oligonucleotides) (AAT)$_3$, (ATT)$_5$, (CAG)$_3$, and (CTG)$_3$ were synthesized using an ABI synthesizer, so that annealing gave template primers for replication. Also synthesized were the oligonucleotides AATGATAAT and AATATATAAT; and CAGCCGACG, which when annealed with (ATT)$_5$ and (CTG)$_5$, respectively, give template primers containing single mismatches.

DNA replication assays

Equal concentrations of complementary oligonucleotides in distilled water were heated and mixed in reaction buffer lacking only DNA polymerase, then allowed to anneal for 30 min prior to the start of the reactions. Replication reactions, containing 50 mM Tris–HCl, pH 7.8, the concentration indicated of MgSO$_4$, 0.1 mM dithiothreitol, 5 µM of template primer complex and 0.1 mM of all four dNTPs were initiated by addition of either E.coli pol I, Klenow pol I or Taq DNA polymerase, as indicated. The reactions were incubated at the indicated temperature for 2 h. The reactions were stopped by additions of EDTA to 23 mM. Reaction products were resolved by electrophoresis on 2% agarose and stained with ethidium bromide. For denaturing conditions, NaOH was added to the finished reactions to a concentration of 50 mM and the gels contained 50 mM NaOH and 1 mM EDTA. The gel products were radiolabeled and visualized using a Storm PhosphorImager. Electron microscopy was performed as described elsewhere (33). All of the templates and all of the primers were tested individually for their ability to support expansion under the above reaction conditions. None of the oligonucleotides by themselves produced higher mobility bands in the presence of any of the DNA polymerases used in these studies.

RESULTS

Effects of Mg$^{2+}$ concentrations on DNA TRE

Lowering cation concentration reduces the stability of double-stranded DNA (34,35). We varied the concentration of Mg$^{2+}$ in the in vitro replication reactions to determine the effect this would have on TRE. Replication reactions with the triplet repeat (AAT)$_3$/ (ATT)$_5$, referred to below as AAT, used E.coli pol I and its large fragment, Klenow pol I, which cannot perform nick translation (36). The results uncovered a relatively narrow window through which higher mobility bands were observed by alkaline gel electrophoresis (Fig. 1). The denaturing properties of the gel eliminate the possibility that the differences in mobility were caused by changes in DNA secondary structure. The optimum expansion was observed at 0.2 mM Mg$^{2+}$ concentration. Similar results were found for the (CAG)$_3$/(CTG)$_5$, referred to below as CAG, using Taq DNA polymerase, except that 0.5 mM Mg$^{2+}$ was found to be the optimum concentration for expansion under the conditions used (not shown). The windows of Mg$^{2+}$ concentration were shifted up or down by a corresponding shift in dNTP concentration in the replication reaction (not shown).

Effects of temperature and composition on DNA TRE

Increasing temperature melts DNA hydrogen bonds (34,35). Increasing the temperature of the replication reaction increased expansion of the AAT repeat by pol I, at all Mg$^{2+}$ concentrations used (Fig. 2A); less expansion was observed as the Mg$^{2+}$ concentration increased. The mobilities of the products on agarose gels were not altered by treatment with proteinase K, so protein binding was not responsible for the mobility shifts observed. Expansion was variable: in repeat experiments at 0.3 and 0.5 mM Mg$^{2+}$ and 47°C, the AAT expansion reaction products always reached sizes measured by gel electrophoresis of >600 bp and occasionally >20000 bp (Fig. 2A, lanes 4 and 7). The AAT expansion products at 47°C (Fig. 2A) extended up to and into the sample loading well for electrophoresis. Use of lower percentage gels and higher molecular weight markers demonstrated that the largest expansion products were >20 000 bp in size (not shown): an expansion of >1000-fold (a 15 bp template-primer expanded to 20 000 bp). Similar results were obtained using Klenow pol I (not shown) and by analysis of the products on alkaline gels (see below). The CAG triplet repeats, having higher GC base pair composition and thus more stable hydrogen bonding, required higher temperatures for expansion (see below). Expansion reactions increased in an approximately linear manner with respect to time for the first 20 min of the reaction (Fig. 2B). The increase with time indicated that, on average, 13 bp were added per minute during the linear part of the reaction under our conditions and incubation temperature (42°C). Adding unique sequences to both sides of the (ATT)$_5$ template strand gave similar results to those shown in Figure 2 only when the primer was complementary to the repeat tract. If the primer was made complementary to the 3′ unique sequence of the template, expansion was not detected.
Effects of mismatched base defects on TRE

The effects of mismatch repair defects (7,8) on simple-repeat instability led us to test the effect of a mismatch on the expansion of AAT and CAG repeats in vitro. Simple repetitive sequences are susceptible to the occurrence of mismatches during DNA replication because of the opportunity for DNA slippage (9,37). To test the effect of a mismatch on the expansion reaction, a single mismatch was introduced into both the AAT and CAG repeats (see Materials and Methods). For the AAT repeat, the G and T mismatches used inhibited expansion at lower temperatures (compare lanes 2 and 3 with lanes 5 and 6, and 8 and 9, respectively). At 47°C the mismatches gave a mixture of both greater and less expanded product compared with that without the mismatch (compare lane 4 with lanes 7 and 10). For CAG, the C mismatch inhibited expansion from 52 to 62°C. Only at 67°C did the mismatch show increased expansion during DNA replication.

Characterization of the expansion products

The expansion reaction products were characterized by denaturing gel electrophoresis, electron microscopy (EM), DNA sequencing and restriction endonuclease cleavage. It was possible that the long expansions observed on native gels were the result of filling in by polymerase of gaps caused by overlapping slipped intermediates. Since the replication reactions lacked DNA ligase, the reactions, if they involved overlapping intermediates, would give nicked DNA duplexes. We ran the expansion reactions in the presence of radiolabeled dNTPs and resolved the reaction products on both native and alkaline denaturing gels (Fig. 4). The presence of nicks would have been manifested as significantly smaller products on the denaturing gels. The results, however, show identical size products, arguing against overlapping intermediates. The results were the same, independent of whether pol I, Klenow pol I or Taq pol was used for primer extension (not shown).

For EM, expansion products from 37 and 47°C AAT expansion reactions both with and without a mismatch and containing 0.3 mM Mg^{2+} were studied. The products from the 37°C reaction were isolated from an agarose gel slice and complexed with E.coli

Figure 2. The effects of temperature and Mg^{2+} concentration on expansion of the triplet repeat (AAT)_3/(ATT)_5 during DNA replication in vitro. The reactions were resolved by gel electrophoresis on native 2% agarose gels and stained with ethidium bromide. (A) (AAT)_3/(ATT)_5 was incubated with pol I (0.1 U/µl) under the reaction conditions described in Materials and Methods, and at the Mg^{2+} concentrations and temperatures indicated. Lane 1 contains a base pair ladder with sizes as indicated. (B) (AAT)_3/(ATT)_5 was incubated with Klenow pol I, as described above, at 42°C and 0.3 mM Mg^{2+}, and the reactions stopped at the times indicated.

Figure 3. The effects of temperature and a single mismatched base pair on the expansion of triplet repeats (AAT)_3/(ATT)_5 and (CAG)_3/(CTG)_5 during DNA replication in vitro. Reaction conditions and oligonucleotides used to generate template primers containing single mismatches are described in Materials and Methods. Mg^{2+} concentration for AAT repeats was 0.3 mM and pol I was used. CAG repeats were replicated by Taq polymerase under identical conditions, except Mg^{2+} concentration was 0.5 mM (optimized by titration). Identical molecular size markers are shown in lanes 1, 11 and 20.
EM visualization of reaction products from expansion of (AAT)3/(ATT)5 at 0.3 mM Mg2+ by E.coli pol I, as shown in the above figures. The DNA reaction products were incubated with E.coli single-stranded DNA binding protein, cross-linked with gluteraldehyde, and prepared for electron microscopy as described previously (31). The products varied in size and reached lengths $>$1600 bp (right panel). No single-stranded regions were detected other than at rare DNA ends (arrow, left panel). Bar, 100 bp.

Figure 5. EM visualization of reaction products from expansion of (AAT)3/(ATT)5 at 0.3 mM Mg2+ by E.coli pol I, as shown in the above figures. The DNA reaction products were incubated with E.coli single-stranded DNA binding protein, cross-linked with gluteraldehyde, and prepared for electron microscopy as described previously (31). The products varied in size and reached lengths $>$1600 bp (right panel). No single-stranded regions were detected other than at rare DNA ends (arrow, left panel). Bar, 100 bp.

DISCUSSION

The effects of varying temperature, varying Mg2+ and dNTP concentration, and introducing a mismatched base on the ability of AAT and CAG triplet repeats to expand during DNA replication in vitro were determined. Titrating Mg2+ concentration revealed a relatively narrow window of concentration, which depended on dNTP levels, through which expansion could be observed. The ability of the dNTP concentration to shift the window most likely results from chelation of Mg2+ by dNTPs (38). The lower boundary of the window is caused by the requirement of E.coli pol I activity for Mg2+; the upper boundary

single mismatches (Fig. 3) were isolated and sequenced. The results showed perfect (AAT)140 and (AAT)120 repeats. Evidence of the mismatches in the expanded DNA was not detected by sequencing, probably because the mismatches were repaired in vivo. (CAG)3 expansion was sequenced directly by attachment of a unique flanking sequence for binding the sequencing primer. Results of this sequencing reaction showed perfect repeat expansion from (CAG)3 to (CAG)42. Very long DNAs were not sequenced in this study because they are unstable in E.coli and difficult to sequence.

Digestion of product DNA with restriction enzymes was used to investigate the sequences of longer expansions of (CAG)3. The CAG repeat can be cleaved with the enzyme BbvI, which recognizes and cleaves the sequence GCAGC(8/12), where the numbers in parentheses indicate points of cleavage in the two strands after that number of bases. Expansion products from the (CAG)3/(CTG)5 replication reaction after digestion with BbvI gave the expected sized fragments demonstrating the expected sequence of the reaction products (Fig. 6). Seven other restriction enzymes (AcI, BglII, MboII, MmiI, NlaIII and PstI from NEB and Thal from Gibco-BRL) that recognize variants of the CTG repeat in vivo were determined. Titrating Mg2+ concentration revealed a relatively narrow window of concentration, which depended on dNTP levels, through which expansion could be observed. The ability of the dNTP concentration to shift the window most likely results from chelation of Mg2+ by dNTPs (38). The lower boundary of the window is caused by the requirement of E.coli pol I activity for Mg2+; the upper boundary
is most likely caused by the ability of Mg$^{2+}$ to stabilize DNA against localized denaturation needed for DNA slippage.

Increasing the temperature of the reaction from 37 to 42 and 47°C gave significantly higher expansion of AAT. Increasing temperature from 52 to 62 and 67°C using Taq DNA polymerase gave significantly higher expansion of CAG. Introduction of a mismatch into the triplet repeat stabilized the repeats against expansion at lower temperatures and reduced expansion to less than detectable levels under all conditions tested, when unique sequences flanked the triplet-repeat tract. While we were performing these experiments, a report was published suggesting that mismatches introduced into small triplet-repeat tracts of 15 bp can lead to expansion of the tract during DNA synthesis (39). In that report, however, up to six mismatches were necessary to increase expansion during replication of a repeat tract with unique flanking sequences. Stabilization of the repeat tract against expansion by a mismatch is consistent with findings that loss of interruptions from perfect repeat tracts predispose the Spinocerebellar ataxia type 1 (CAG)$_n$ and the Fragile X syndrome (CGG)$_n$ to expansion (40,41).

The expansion products from replication of the triplet repeats were characterized using native and denaturing gel electrophoresis, EM, DNA sequencing and restriction enzyme analysis. The results showed that the triplet repeats had expanded to give double-stranded DNA without detectable nicks or gaps resulting from overlapping slipped intermediates. This suggests that the small DNA template-primer may have gone through multiple rounds of slippage to reach the large expanded products observed possibly by the expansion scheme shown in Figure 7. In this scheme, factors that destabilize the DNA duplex ($\Delta$) overcome the kinetic barrier to misalignment, resulting in an equilibrium between fully duplex DNA and a low level of transient intermediates that can be extended by DNA polymerase. The DNA intermediates shown in the figure are of three types; slid (11), bulged (21,22) and hairpinned (18–20,23,42). Various combinations of these intermediates are possible and the pathways interconnect so which pathways predominate is unknown. ‘Slipped’ DNA, seen after denaturation and reannealing of expanded repeats (24,25,43), may play a role as transient intermediates. For simplicity, we assume that the n+1 cycle pathway is not influenced by the choice of the pathway used for cycle n, but this may not be true. For example, an intermediate slipped structure such as a bulge, still present in one strand from a prior round of slippage, may facilitate further slippage in that strand. Each cycle is initiated by the dissociation of polymerase to reassociate at a new intermediate.

The double-stranded nature of the reaction products when (AAT)$_n$/((ATT)$_n$ and (CAG)$_n$/((CTG)$_n$ were used without unique flanking sequences indicate that both strands are equally susceptible to expansion under our conditions. The equal participation of both strands suggests that because the probability of hairpin formation is different for the two strands (18,28), hairpin formation may not be a major contributor to expansion under our reaction conditions. Alternatively, hairpin formation may be rate limiting and unstable. For example, after hairpin formation on the CTTG strand of a CTTG/CAG repeat tract, replication would extend the foreshortened CTTG strand on the complementary CAG strand. The imperfect CTTG hairpin may come apart, thereby allowing the CAG strand to be extended by DNA polymerase on the longer CTTG strand. We speculate that expansion of a primer complementary to a repeat tract bounded by unique sequences proceeds by like mechanisms.

All of the expansions observed were independent of whether or not the template repeat tract was bounded by unique sequences. That was only true as long as the primer annealed within the repeat tract. No expansions were detectable when the primer was complementary to the unique flanking sequence. The requirement for a primer located entirely within the repeat tract implies that the ability to slide freely on the repeat tract is important for the expansion process. In vivo, the occurrence of a short primer with two free ends probably results either from an Okazaki fragment occurring completely within the repeat tract (11) or as the result of DNA damage and repair of the growing strand as it traverses the repeat tract. The latter could result in a nick or gap that frees the growing end for expansion.

In the experiments presented, the effects of increased temperature and decreased Mg$^{2+}$ concentration show the importance of environment on TRE. In addition, the results imply that the occurrence of a nick or gap in the growing strand as it traverses a repeat tract is important for TRE.

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REFERENCES