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Stability of a CTG/CAG Trinucleotide Repeat in Yeast Is Dependent on Its Orientation in the Genome

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Trinucleotide repeat expansion is the causative mutation for a growing number of diseases including myotonic dystrophy, Huntington's disease, and fragile X syndrome. A $(CTG/CAG)_{130}$ tract cloned from a myotonic dystrophy patient was inserted in both orientations into the genome of *Saccharomyces cerevisiae*. This insertion was made either very close to the 5′ end or very close to the 3′ end of a *URA3* transcription unit. Regardless of its orientation, no evidence was found for triplet-mediated transcriptional repression of the nearby gene. However, the stability of the tract correlated with its orientation on the chromosome. In one orientation, the $(CTG/CAG)_{130}$ tract was very unstable and prone to deletions. In the other orientation, the tract was stable, with fewer deletions and two possible cases of expansion detected. Analysis of the direction of replication through the region showed that in the unstable orientation the CTG tract was on the lagging-strand template and that in the stable orientation the CAG tract was on the lagging-strand template. The orientation dependence of CTG/CAG tract instability seen in this yeast system supports models involving hairpin-mediated polymerase slippage previously proposed for trinucleotide repeat expansion.

Instability of repeated sequences in the human genome is an important disease-causing mechanism. One category, microsatellite instability, is caused by a dysfunctional mismatch repair system and is associated with colorectal cancer. Another category is expansion of trinucleotide repeats. Expanded arrays of trinucleotide repeats have been found at 11 human loci, and at 9 of these the expansions are associated with disease (for reviews, see references 2 and 44).

There are two general classes of trinucleotide repeat expansions. The first are relatively small expansions in which a CAG/CTG repeat within a gene coding region expands from a normal range of 5 to 35 copies to ~40 to 100 copies, resulting in an expanded polyglutamine tract in the protein product. This category of triplet expansion has been found in five neurodegenerative disorders, including Huntington's disease (2, 44). Recent evidence suggests that the dominant pathology of these diseases is due to an abnormally tight interaction between the protein with the expanded polyglutamine tract and other proteins in the brain (6, 27).

A second class of trinucleotide repeat disorders involves larger expansions, in some cases of as many as 2,000 or more repeats, in noncoding regions. In most cases, these large repeat expansions decrease production of protein from a nearby gene, either at the transcriptional or posttranscriptional level, with the decrease causing the disease phenotype. For example, a CGG expansion in the 5′ region of the *FMR1* gene, whose altered expression is responsible for fragile X mental retardation, results in methylation of a nearby CpG island and a decreased level of *FMR1* mRNA (17, 33). Expansion of a CTG/CAG tract located in the 3′ noncoding region of a serine-threonine protein kinase gene (*DMPK*) is responsible for myotonic dystrophy (DM), an autosomal dominant disorder that is the most common form of adult muscular dystrophy (46). In the case of DM, the location of the expanded CTG/CAG tract (CTG tract) in the 3′ noncoding region of the gene makes the mechanism of disease particularly intriguing. Several groups have reported a decreased level of *DMPK* mRNA transcripts and/or protein in muscle from DM patients (7, 13, 19). However, one study of mRNA levels found an apparent increase in mRNA of the affected gene (38), and two recent studies suggest that normal levels of mRNA are made but abnormally processed (45, 53). One mechanism that could explain decreased expression of the DM allele is if the expanded CTG repeats induce a heterochromatic or "silenced" chromatin structure that can spread to the nearby gene. Long tracts of CTG repeats have been shown to be preferential sites of nucleosome assembly (52, 53), and loss of DNase I cleavage at a hypersensitive site adjacent to the expanded alleles of three separate DM patients suggests that the chromatin structure of the region is altered (31).

The unstable triplet repeats associated with various genetic diseases have certain common features. First, like other repeated regions in the human genome, the number of repeats at a given locus is polymorphic, indicating the inherent instability of these sites. However, when the disease-associated trinucleotide repeats reach a critical threshold length, expansion becomes likely (see references 29 and 36 for reviews). Studies of DNA from affected families indicate that this threshold of instability occurs when an allele reaches a length of 34 to 37 uninterrupted repeats, which is the upper end of the normal size range for loci associated with triplet repeat expansion in the general population (11). Trinucleotide repeat tracts seem to show an even greater level of instability when they reach a size of 70 to 80 copies, since, at least for the DM and fragile X loci, very large expansions (as many as 1,000 to 2,000 copies) frequently occur when alleles of this size are transmitted (11, 36).

In the present study, we tested whether a CTG/CAG tract of approximately 130 repeats (390 bp) is able to silence a nearby gene. In the yeast *Saccharomyces cerevisiae*, silencing of genes occurs both at the silent mating type loci and at telomeres and in both cases is associated with an altered chromatin structure (25, 39). A tract of telomeric DNA, which consists of the inexactly repeated sequence $C_{1-3}A/TG_{1-3}$, can silence adjacent genes in a length-dependent manner (16, 24) when it is present

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either at the ends of chromosomes or at internal loci at various sites in the chromosome (42). If the CTG tract acts as a strong nucleosome-positioning element which can nucleate a region of silenced chromatin in vivo, this situation could possibly be mimicked in yeast cells which also contain nucleosomes and a silencing mechanism. However, we found that the 390-bp CTG tract was not able to silence a neighboring gene. In addition, we monitored the stability of the CTG tract in both orientations on the chromosome and over multiple generations. The stability of the tract was highly dependent on its orientation in the genome with respect to replication.

MATERIALS AND METHODS

Plasmid constructions. A 1.1-kb *Hin*dIII fragment containing the *URA3* gene from YEp24 was cloned into the *Hin*dIII site of pGEM$(CTG)_{130}$ (gift of J. Griffith) in both orientations. The resulting two plasmids, pCTG-URA and pCTG-URAr, were digested with *Pvu*II, and the 2.1-kb fragments containing the *URA3* gene and the $(CTG/CAG)_{130}$ tract [hereafter referred to as $(CTG)_{130}$] tract were isolated. Plasmid pTD27 (gift of T. Davis) was digested with *Eco*RV to remove most of the internal coding region of *LYS2* (3.7 kb). Each of the *Pvu*II fragments were cloned into the *Eco*RV sites of pTD27 in both orientations, creating a total of four plasmids: pCTG-URA-LYS, pCTG-URA-LYSr, pCTG-URAr-LYS, and pCTG-URAr-LYSr. The no tract control plasmid, pCF116, was made by ligating a 1.7-kb *Nru*I-*Bam*HI fragment from Yep24 containing the *URA3* gene into the *Hpa*I-*Bam*HI sites of pTD27, thereby replacing 1.2 kb of *LYS2* sequence with *URA3*.

Yeast strain construction. Strain YPH500L was created by a one-step gene replacement by transformation of linearized pTD27 plasmid containing the wild-type *LYS2* gene into *S. cerevisiae* YPH500 (*ura3-52 ade2-101 trp1-Δ63 his-Δ200 leu2-Δ1 lys2-801^{am}*) (41) by the lithium acetate procedure (15, 40) and selection on plates lacking lysine. The *LYS2* gene was then replaced with each *5′lys2*-CTG-*URA3-3′lys2* construct or the control *5′lys2-URA3-3′lys2* construct by linearizing the four CTG plasmids or pCF116 (see above) with *Pvu*I and *Pvu*II and by transformation into strain YPH500L. Transformants were selected on plates containing 2.4 mg of α-aminoadipate (Sigma) per ml, which is toxic to cells expressing the *LYS2* gene (8). A total of 50 to 90 transformants of each orientation were restreaked directly to yeast extract-peptone-dextrose (YEPD) to create master plates; these cells are referred to as the master patch. Phenotypes were further characterized either by streaking to plates lacking lysine, lacking uracil, or containing 5-fluororotic acid (FOA; 1 g/liter; American Biorganics, Inc.) at the same time as creation of the master plate, or by replica plating cells from the master plate to plates lacking either lysine or uracil or containing FOA. It is estimated that cells underwent approximately 30 generations of growth after transformation to generate the master patch. Lys^- transformants were either Ura^+ FOA^S (10 to 30% of total transformants) or Ura^- FOA^r. Both types of transformants were analyzed by Southern blotting for the presence of the $(CTG)_{130}$ tract (see below). All of the Ura^+ FOA^s transformants checked by Southern analysis contained a CTG tract (37 analyzed), while none of the Ura^- FOA^r transformants contained a CTG tract (13 analyzed).

Southern blot analysis. A 5-ml yeast culture was grown until saturation (approximately 10 generations), and genomic DNA was prepared by a glass bead procedure (37). DNA was resuspended in 50 μl of 1× TE (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]), and 5 μl was digested with *Eco*RI and *Hin*dIII. Digested DNA was run on a 1.5% agarose gel alongside a 100-bp ladder and blotted to a nylon membrane under standard denaturing Southern blot conditions. Blots were hybridized to a 590-bp ^{32}P-labeled fragment containing a $(CTG)_{130}$ tract at 42°C overnight in super-Starks (50% formamide, 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's solution, 2% sodium dodecyl sulfate [SDS], 0.5% milk, 1 mg of herring sperm DNA per ml). The blots were washed twice for 10 min at 25°C in blot wash I (2× SSC, 0.1% SDS) and two to six times for 20 min at 65°C in blot wash II (0.1× SSC, 0.1% SDS) and were exposed to Kodak X-Omat-AR film. Increasing the number of blot II washes decreased hybridization to the background CTG-hybridizing sequences in the yeast genome; the optimal number of washes to decrease background without decreasing hybridization to the CTG tract integrated at the *lys2* locus varied from blot to blot.

*Eco*RI cuts 53 bp 5′ to the $(CTG)_{130}$ tract, and *Hin*dIII cuts 147 bp 3′ to the $(CTG)_{130}$ tract, yielding a fragment of 590 bp if the CTG tract is full length. Repeat sizes were estimated by subtracting 200 bp (the amount of nonrepeat sequence in the *Eco*RI-*Hin*dIII fragment) from the size of the CTG-hybridizing band and then dividing by 3.

PCR analysis. PCR primers hybridized to unique sequence 78 bp 5′ or 112 bp 3′ to the $(CTG)_{130}$ tract. T7 primer (5′-TAATACGACTCACTATAGGG-3′) and CTG-rev primer (5′-GTGGAGGATGGAACACGG-3′) were used to amplify the CTG tract directly from yeast cells by colony PCR. PCR mixtures contained 1 pmol each of the T7 and CTG-rev primers per μl, 0.2 mM deoxynucleoside triphosphate mix (0.05 mM each dATP, dGTP, dCTP, and dTTP), 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl (pH 8.3), 0.125 U of *Taq* polymerase (Boehringer Mannheim) per μl, and a small amount of yeast colony. The reaction mixtures were cycled 35 times at 94°C for 1 min, 57°C for 1 min, and 72°C for 3 min in a Perkin-Elmer thermal cycler. A portion of each PCR mixture was run on a 2.5% MetaPhor agarose gel (FMC) next to molecular weight marker VI (Boehringer Mannheim). DNA was visualized by ethidium bromide staining. The expected PCR product for a full-length $(CTG)_{130}$ tract is 580 bp (390-bp CTG repeat sequence plus 190-bp unique sequence).

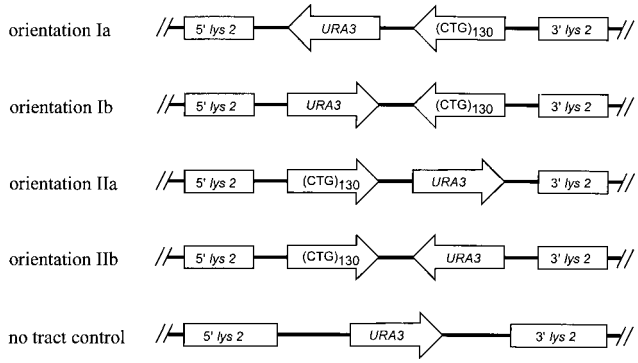

FIG. 1. Orientation of the $(CTG)_{130}$ tract and the *URA3* gene with respect to the *LYS2* gene on the right arm of chromosome II in the four yeast strains. The control strain (bottom) lacks a CTG tract and has 2.5 kb more of *lys2* coding sequence but is otherwise identical to the other strains. For *URA3*, directions of transcription are denoted by the arrows. For the $(CTG)_{130}$ tract, the arrows designate orientation in the genome: the centromere is to the left of *LYS2*, and the telomere is to the right.

2-D gel analysis. Genomic DNA for two dimensional (2-D) gel analysis was prepared from strain YPH500L by the method described by Wu and Gilbert (55). Approximately 3.7 μg of DNA was digested with *Eco*RI and *Pvu*II for 5 h at 37°C. DNA was precipitated and subjected to electrophoresis in a 0.4% SeaKem GTG agarose gel (FMC) at 10 mA (18 V) for 30 h. The gel was stained in 1 μg of ethidium bromide per ml, and the entire lane was cut out. The gel slice was washed for 20 min in 4 ml of restriction buffer without enzyme, the buffer was pipetted off, and 2 ml of restriction buffer plus 4,000 U of *Bam*HI (NEB) was added and incubated for 10 h at 37°C. The second dimension was subjected to electrophoresis in a 1.1% agarose gel containing 1 μg of ethidium bromide per ml for 4 h at 300 mA (~250 V) and Southern blotted as described above. A 700-bp fragment from the 5′ end of *LYS2* was used as the probe, and washes were as described above, except that blot wash II was repeated only twice, with the first wash II done at room temperature. 2-D gel analysis of strain IIa-2 was performed as described for strain YPH500L, except that enzymes *Xba*I and *Pvu*II were used in the first digest, and *Ava*I (which cuts ~950 bp off the 3′ end of the 3-kb *Xba*I-*Pvu*II fragment) was used before the second dimension.

RESULTS

A system for studying trinucleotide repeat arrays in yeast. To insert the triplet repeat tract into the yeast genome, plasmids were constructed with the $(CTG/CAG)_{130}$ tract either 371 bp 5′ (orientation a) or 287 bp 3′ (orientation b) to the *URA3* gene (Fig. 1). This $(CTG)_{130}$-*URA3* cassette was then cloned in both orientations into a plasmid containing the yeast *LYS2* gene, such that the cassette replaced the central portion of the *LYS2* gene. In orientation I, the CTG strand is in the direction opposite to that of *LYS2* transcription, and in orientation II, the CTG strand is in the same direction as that of *LYS2* transcription (Fig. 1). Transformation of these four plasmids into yeast resulted in integration of the $(CTG)_{130}$-*URA3* cassette at the *LYS2* locus on chromosome II in all four orientations (Ia, Ib, IIa, and IIb [Fig. 1]). In addition, a control strain was made with no $(CTG)_{130}$ tract, but only the *URA3* gene integrated into the *LYS2* locus (Fig. 1).

Transformants were plated on α-aminoadipate plates, which selects against Lys^+ cells. Since the α-aminoadipate plates contained uracil, no selection for expression of the integrated *URA3* gene was applied at this stage. The Lys^- phenotype was confirmed by the inability to grow on plates lacking lysine. Multiple independent transformants of each of the four constructs were selected for further analysis; we obtained 5, 30, 8,

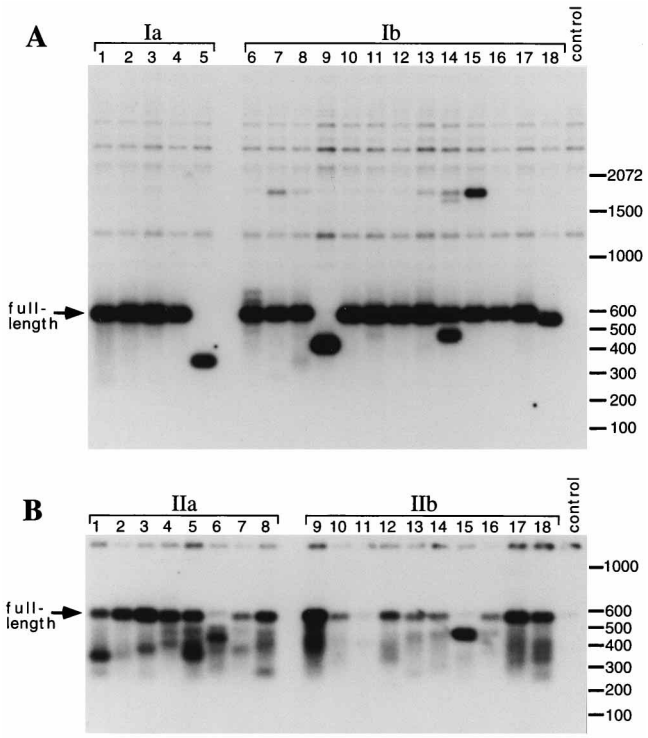

FIG. 2. CTG tract length for transformants from the master plate. DNA was prepared from cells on the master plate for each orientation, digested with *Eco*RI and *Hin*dIII, and analyzed by Southern blot hybridization to a CTG probe. (A) Transformants of orientations Ia and Ib. When the $(CTG)_{130}$ tract is full length, the expected band size is 590 bp, indicated by the arrow (390-bp repeat sequence plus 200-bp unique sequence). Higher-molecular-weight bands represent naturally occurring CTG-hybridizing sequences present in the yeast genome as seen by comparing the pattern of strains with the inserted CTG tract to the no-tract control strain (control). Darker CTG-hybridizing bands between 1.5 and 2 kb in lanes 7, 14, and 15 are not expansions but rather incomplete digestions, since they disappear upon reextraction and redigestion of the DNA (data not shown). Shortened CTG tracts are visible either as a discrete band (lanes 5, 9, 14, and 18) or as a smear underneath the full-length band. Sizes (in base pairs) were determined by the positions of a 100-bp molecular weight ladder run on the same gel. A size difference of about ±20 bp (~7 repeats) is resolved under these gel conditions. (B) Transformants in orientations IIa and IIb. Only the relevant portion of the gel is shown here and in subsequent gels.

and 20 transformants which contained the $(CTG)_{130}$ tract for orientations Ia, Ib, IIa, and IIb, respectively. The correct insertion of the repeat tract-*URA3* cassette was confirmed by Southern blot analysis for the subset of transformants used for further analysis (Fig. 2 and data not shown).

Proximity of a $(CTG/CAG)_{130}$ tract does not repress transcription of the *URA3* gene. The compound FOA kills cells expressing *URA3* (3). Thus, cells in which the *URA3* gene is transcriptionally repressed can be identified by their ability to grow on plates containing FOA. When *URA3* is placed near a telomere (16) or near an internal tract of $C_{1-3}A/TG_{1-3}$ DNA (42), a fraction of the cells are FOA resistant (FOA^r). However, because silencing both near a telomere (telomere position effect) and by an internal tract of telomeric DNA ($C_{1-3}A$-based silencing) is reversible, these FOA^r cells can also grow on plates lacking uracil. That is, cells that are FOA^r due to transcriptional silencing are also Ura^+. The ability to switch between a transcriptionally silent (FOA^r) and a transcriptionally active (Ura^+) state can be used to distinguish cells in which *URA3* is transcriptionally silenced from cells in which *URA3* is mutated. Growth on FOA is a very sensitive assay, since even a small subset of silenced cells can be detected (42).

We wished to test the hypothesis that a $(CTG)_{130}$ tract, by virtue of its property of being a strong nucleosome binding site in vitro (52, 53), would generate an altered chromatin structure that reduces transcription of the nearby *URA3* gene. To test this possibility, individual transformants were tested for their abilities to grow on medium lacking uracil (indicating expression of the *URA3* gene) or containing FOA (indicating no expression of the *URA3* gene) by simultaneously streaking cells from either the original transformant colony or a patch of cells grown from this colony (referred to as the master patch [see Materials and Methods]) to plates lacking uracil or containing FOA. None of the original CTG transformants had a Ura^+ FOA^r phenotype, indicating that the $(CTG)_{130}$ tract was not able to mediate transcriptional repression of *URA3*. In addition, the $(CTG)_{130}$ tract could be maintained for at least three successive restreaks (~75 generations) of selective growth on plates lacking uracil as monitored by Southern blotting (data not shown; Fig. 3A), suggesting that proximity of the tract to the *URA3* gene does not lead to any decrease in transcription which would confer a selective disadvantage to cells containing a full-length tract.

One possibility for the lack of transcriptional repression of the *URA3* gene is that the $(CTG)_{130}$ tract is not long enough to nucleate a silenced state; however, silencing might occur in a subset of cells with expanded tracts. To test this possibility, a single colony (~10^7 cells; streaked from the master patch) of each orientation as well as the no-tract control was resuspended in water and plated on FOA. A small aliquot was simultaneously plated on yeast complete (YC) medium to determine the viable cell count. About 5×10^7 cells were scraped from the YC plate and again plated on FOA and YC medium, followed by yet another scraping-plating of ~3×10^8 cells. For all three platings, the frequency of FOA^r colonies was approximately the same as that of the no tract control, i.e., about 10^{-7}. None of the FOA^r colonies had a Ura^+ FOA^r phenotype indicative of silencing, and when they were analyzed by Southern blotting, all still contained either the full-length or a shortened CTG tract with no expansions. A few showed rearrangements that would be expected to compromise expression of the *URA3* gene. Some of the rearranged strains were able to grow poorly on both plates lacking uracil and FOA plates, indicating that our assay was sensitive enough to detect weak silencing of the *URA3* gene had it occurred. Thus, there was no evidence for transcriptional silencing of the *URA3* gene, including silencing in only a subset of cells or weak silencing. In addition, when colonies containing a subset of expanded tract (as detected by Southern blotting [see below]) were plated on FOA and YC medium, again no increase in the frequency of FOA-resistant cells compared to that of the no-tract control was detected.

Both telomere position effect (34) and silencing by an internal tract of telomeric DNA (42) are more efficient in a *ppr1*Δ strain, where *PPR1* is a transactivator of *URA3*, presumably because the *URA3* promoter is weakened. Therefore, we also tested two strains in each orientation for FOA resistance in a *ppr1*Δ background. However, even in a *ppr1*Δ strain, no evidence for CTG-mediated silencing was detected.

CTG/CAG tract stability is correlated with orientation in the genome. The lengths of the integrated CTG tracts were determined by two methods: Southern blot analysis of genomic DNA (see, for example, Fig. 2) and colony PCR (see, for example, Fig. 3B). Each method had advantages and disadvantages. Southern blot analysis was very sensitive and therefore useful for detecting repeat lengths that were present in only a

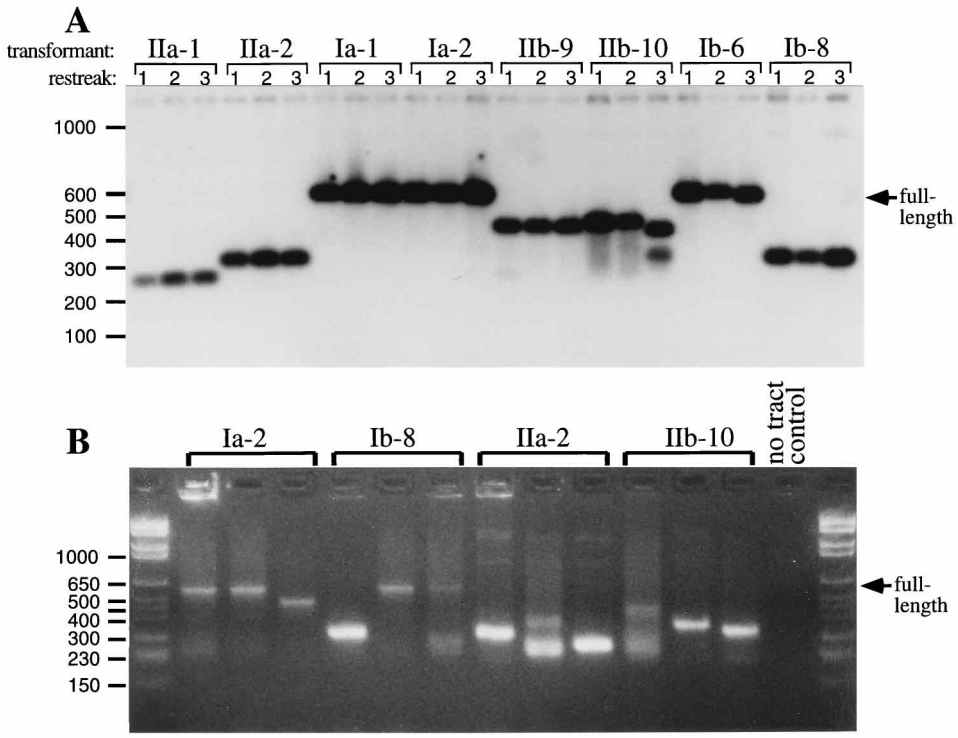

FIG. 3. CTG tract size stability over multiple generations. (A) Cells taken from the master plates were grown for approximately 25, 50, or 75 generations (restreaks 1, 2, and 3, respectively) before DNA was prepared and analyzed by Southern blotting as described for Fig. 2. Transformant numbers correspond to those used in Fig. 2. (B) PCR analysis of DNA from three independent colonies from the restreak 1 plate for one transformant in each orientation. Full-length $(CTG)_{130}$ tract produces a 580-bp band (390-bp repeat sequence plus 190-bp unique sequence), which is indicated by the arrow. The first colony of each set was also used to prepare DNA for Southern analysis (see corresponding restreak 1 colonies in panel A).

minority of cells. Expanded tracts would also be readily detected by this method. However, the shortest tracts (less than 30 to 40 bp) were not detected, since they did not hybridize to the CTG probe under the conditions used. Although PCR analysis was less reflective of the range of tract lengths that exist in vivo, it was useful for detecting small tract sizes and for analyzing large numbers of colonies. In addition, PCR analysis allowed direct analysis of DNA from cells in the colony without any additional growth, whereas approximately 10 generations of growth were required to prepare genomic DNA for Southern blot analysis. Comparison of CTG tract lengths from the same colony analyzed by both Southern blotting and PCR generally showed a similar distribution of tract lengths, although PCR tended to underestimate the amount of full-length tract, suggesting that smaller tracts were amplified more efficiently.

In addition to the inserted $(CTG)_{130}$ tract, other CTG-hybridizing bands which were presumably due to tracts of CTG or CTG-like repeats that occur naturally in yeast were detected by Southern analysis (Fig. 2, control lanes). Sequence analysis of the entire yeast genome revealed that the longest contiguous CTG tract has 11 repeats. However, there are several extended regions of interrupted CTG repeats, for example, a stretch of about 280 bp in *SNF5*.

The earliest stage analyzed for tract length changes was represented by cells from the master patch, which had undergone approximately 30 generations of growth after transformation (~20 generations to form a colony from each transformed cell plus ~10 generations of growth on the master plate). Both PCR and Southern blot analysis of DNA from cells at this stage showed that the stability of the CTG tract differed for the various orientations. In orientations Ia and Ib, the majority of the transformants had full-length tracts of about 130 repeats (4 of 5 for orientation Ia and 12 of 14 for orientation Ib; Fig. 2A and data not shown). Although a faint smear of shorter-length tracts could be seen below the full-length band for many of the orientation I transformants, these shorter tracts represented a small minority of the total (Fig. 2A). The exception was transformant Ib-14, in which about 50% of the tract was shortened (Fig. 2A). In the four cases in which a large fraction of cells had a shortened tract, tract length appeared discrete, with about 53, 80, 100, and 123 repeats for Ia-5, Ib-9, Ib-14, and Ib-18, respectively (Fig. 2A). The discrete deletion products seen for transformants in orientation I could be due to an early $(CTG)_{130}$ tract deletion event during or after transformation, followed by relative stability of the CTG tract in progeny cells. For example, the transformant Ia-5 could have undergone a tract deletion event during transformation, during integration, or very early in colony formation, whereas transformant Ib-14, which had approximately 50% full-length and 50% shortened CTG tract, may have been full-length after integration but undergone a deletion event in one of the early cell divisions. For a transformant such as Ib-13, which had only a small amount of shortened tract, the deletion event probably occurred in a cell arising late in colony formation.

In contrast to orientations Ia and Ib, transformants of orientations IIa and IIb had a range of tract lengths from full-length to just a few repeats (Fig. 2B). PCR data indicated a minimum size of ~3 to 10 repeats (data not shown). There were no examples of a IIa or IIb transformant with an exclusively full-length repeat tract. Rather, in each transformant a

major fraction of the cells had shortened tracts. As seen for orientations Ia and Ib, discrete deletion products were sometimes present (see, for example, transformants IIa-1, IIa-3, and IIb-15). However, most transformants in orientations IIa and IIb showed a much darker smear of CTG-hybridizing fragments underneath the full-length band than orientation I cells, suggesting that a large proportion of cells were undergoing deletion events (see, for example, transformants IIa-5, IIa-8, IIb-9, and IIb-13). The number and range of deletion products seen in Fig. 2B indicate that deletion events occurred frequently during growth of orientation II transformants. PCR analysis directly from orientation II cells on the master plate showed approximately the range of tracts sizes seen by Southern analysis (data not shown). Therefore, deletion events occurred during the approximately 30 generations of growth between transformation and PCR analysis. The range of deletion products suggests that tract shortening was a constant event (rather than a process occurring during transformation or integration).

Occasionally, we saw larger CTG-hybridizing bands, suggesting that an expansion might have occurred in a subset of cells. All of the higher-molecular-weight CTG-hybridizing bands between 1.5 and 2 kb (Fig. 2a [Ib-7, Ib-14, and Ib-15]) were eliminated by reextraction and redigestion of the DNA, indicating that they were due to incomplete digestion rather than tract expansion. However, in two cases, both for orientation I transformants, a CTG-hybridizing band larger than that expected for the 390-bp tract was detected that persisted after reextraction and redigestion of the DNA, suggesting that they were in fact true expansions. The first example of a possible expansion was detected in DNA from transformant Ib-6 (Fig. 2A), with two species approximately 50 bp (17 repeats) and 130 bp (43 repeats) larger than the full-length 390-bp repeat. The second example was from DNA prepared from a restreak of transformant Ia-2, in which a subset of cells had a CTG-hybridizing band about 100 bp (33 repeats) larger than the full-length one (data not shown). In both cases, only a small percentage of cells had the possible expansion, and this band was lost upon further growth (i.e., see restreaks of Ib-6 in Fig. 3A), complicating attempts to verify the larger band as an expanded tract by PCR or restriction digests. That we detected so few expansions indicates that a CTG tract of 130 repeats was much more prone to deletion than to expansion in yeast. No cases of possible expansion were detected for tracts in orientation II (Fig. 2B and data not shown).

Orientation dependence of CTG/CAG tract stability is maintained over many generations. To investigate the orientation-dependent stability of the CTG repeat tract over longer time periods, two isolates of each orientation were restreaked from the master plate to either nonselective (YEPD) plates or plates lacking uracil for three successive restreaks. DNA was prepared from the colony used for restreaking.

For orientations Ia and Ib, the tract length could be maintained at full length over three restreaks on both YEPD (Fig. 3A [transformants Ia-1, Ia-2, and Ib-6; each restreak represents approximately 25 generations]) and medium lacking uracil (data not shown). However, tract shortening did occur in one of the four orientation I strains examined (Fig. 3A [Ib-8]). Once a shortened tract was detected, it appeared stable over the time period investigated. In a separate experiment, isolates Ia-2, Ia-3, and Ib-18 were restreaked five times (approximately 125 generations) with maintenance of either full-length tract (130 repeats for Ia-2 and Ia-3) or a slightly shorter tract (~123 repeats for Ib-18; data not shown).

To get a more quantitative idea of the variation in repeat sizes among the progeny of single transformants, tract lengths were measured after the first restreaking for 20 colonies from a single transformant of each orientation by PCR amplification (Table 1; see also Fig. 3B). For orientations Ia and Ib, 74 and 78%, respectively, of colonies had full-length tracts with no shortened tracts detectable by PCR, and the remaining 26 and 22%, respectively, had either only shortened tracts or a mixture of both full-length and shortened tracts (Table 1). For orientation Ia, the shortened tracts ranged from ~15 to 90 repeats, with an average of 61 repeats. For orientation Ib, the range was ~13 to 103 repeats, with an average of 40 repeats.

TABLE 1. PCR analysis of CTG tract lengths in transformants grown for one restreaking

Tract size	% Colonies in each category[a]:			
	Ia-2 (23)[b]	Ib-6 (23)[b]	IIa-2 (20)[b]	IIb-10 (20)[b]
Full length[c]	74 (17)	78 (18)	0 (0)	0 (0)
Shortened	17 (4)	4 (1)	100 (20)	95 (19)
Both[d]	9 (2)	17 (4)	0 (0)	5 (1)

[a] Numbers of colonies that fell into each length category are given in parentheses after percentage values. Percentages are rounded to the nearest whole number.
[b] Total numbers of colonies of each type examined are given in parentheses after orientation designations.
[c] Tract lengths of 130 ± ~7 repeats were counted as full length.
[d] Both full-length and shortened tracts present in DNA from a single colony.

For orientations IIa and IIb, the repeat tract always shortened upon restreaking (Fig. 3A [transformants IIa-1, IIa-2, IIb-9, and IIb-10] and data not shown). In most cases, a tract size of 20 to 80 repeats was dominant after one to two restreaks, and this size repeat tract was then fairly stable. PCR analysis of orientation IIa and IIb transformants showed that 100% of colonies that had gone through one restreaking had shortened tracts (Table 1). No full-length tracts were detectable in DNA from 20 colonies from transformant IIa-2, and only 1 of 20 colonies from transformant IIb-10 had any detectable full-length tract (Table 1). Shortened tract lengths ranged from ~10 to 83 for orientation IIa and ~17 to 80 for orientation IIb, with both having an average tract length of 53 repeats. The instability of the longer CTG tracts in orientation II upon restreaking indicates that the deletion event occurred during mitotic growth and, therefore, is not solely a transformation-associated event.

An example of a PCR gel showing CTG tract length for three first restreak colonies in a strain of each orientation is shown in Fig. 3B. As seen on Southern blots, CTG tracts were seen both as discrete bands and as a smear of heterogeneous products, depending on the colony examined. For comparison, the first colony of each set was also used to prepare genomic DNA that was examined by Southern blot analysis (Fig. 3A [see restreak 1 colonies for comparison]).

When cell populations were examined early (at ~30 generations), the deletions arising from transformants in orientation I appeared more discrete than those arising in orientation II transformants. However, further deletions that occurred during continued propagation of orientation II transformants also led to discrete shortened products in many cases. There was no one size of deletion product for all transformants, but a size of between 20 and 80 repeats appeared to be most common. One possibility is that the difference between discrete and heterogeneous deletions is due to the greater frequency of deletions occurring in orientation II colonies. Alternatively, the difference could reflect a difference in mechanism, with infrequent but large-scale tract deletions more typical in orientation I, and

small but frequent deletions occurring more commonly in orientation II.

In summary, analysis of a large number of $(CTG)_{130}$ tracts that had gone through approximately 55 to 105 generations of growth since transformation highlighted the difference in stability between tracts in orientation I and II. $(CTG)_{130}$ tracts in orientation I were quite stable, with about 75% of cells maintaining full-length tracts after 55 generations, and with maintenance of full-length tracts possible for at least 155 generations. In contrast, CTG tracts in orientation II were extremely unstable, with virtually no full-length tracts remaining by 55 generations of growth. However, for both orientations, tracts which had shortened to a size of 10 to 80 repeats could be stably maintained for at least 75 generations.

Direction of replication through the LYS2 locus. One factor that could be responsible for the difference in stability between repeats in orientations I and II is the direction of replication through the region (see Discussion). To determine the direction of replication through this portion of chromosome II, a modification of the 2-D gel technique was employed (12). 2-D gels separate linear DNA molecules from nonlinear molecules of equal mass, thus enabling the visualization of branched replication intermediates (5). In the modified 2-D gel method, an in-gel restriction digestion before the second dimension cuts off one end of the replication intermediates, resulting in a shift of the arc of Y-shaped replication intermediates (Fig. 4A). The direction of the shift is indicative of the direction of replication fork movement through the fragment.

Genomic DNA from strain YPH500L was first digested with enzymes that cut 5′ and 3′ to the *LYS2* gene, and the restriction fragments were separated by mass in the first dimension. The entire lane was then excised, digested in situ with a second restriction enzyme (*Bam*HI) which cuts off 1.1 kb from the 3′ end of the 4.5 kb *LYS2* fragment and subjected to second dimension electrophoresis. Southern hybridization with a 5′ *LYS2* fragment as the probe detected both full-length undigested replication intermediates (Fig. 4B [upper arc]) and *Bam*HI-digested intermediates which were shorter and thus ran faster (Fig. 4B [lower arc]).

The results show that the Y arc shifted to the right after digestion with *Bam*HI, indicating that the replication fork moves from left to right through the *LYS2* locus, which is the same direction as that of *LYS2* transcription. A similar result was obtained for a strain containing the integrated CTG tract-*URA3* cassette (strain IIa-2; data not shown), although forked replication intermediates appeared less abundant in this strain compared to YPH500L. As a consequence, the CTG strand of the trinucleotide repeat will be replicated by lagging-strand synthesis when the tract is in orientation I and by leading-strand synthesis when the tract is in orientation II.

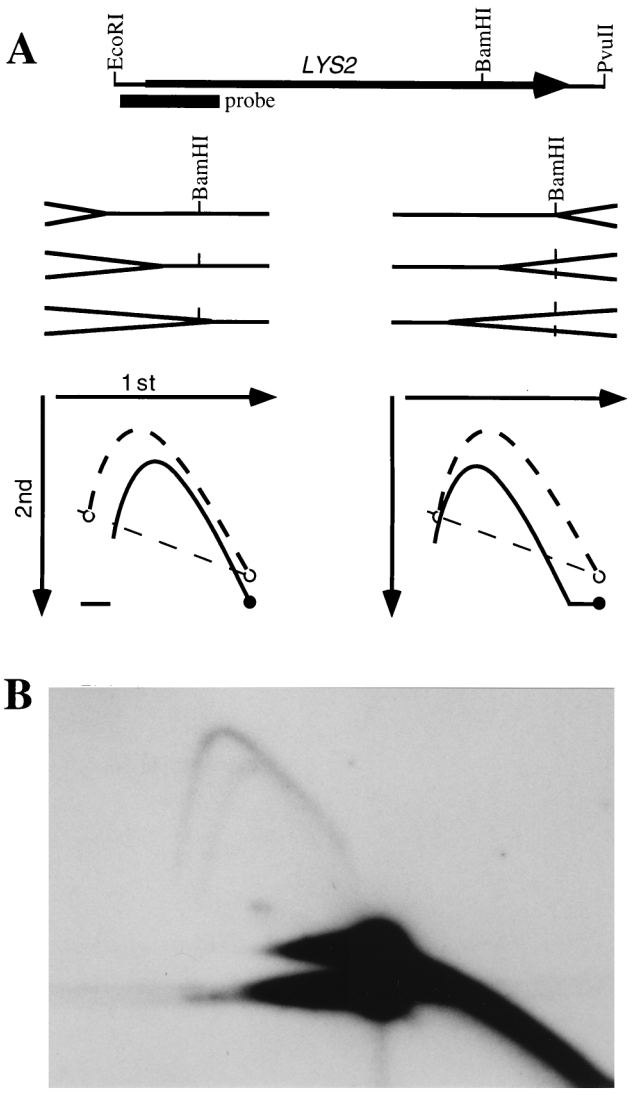

FIG. 4. Direction of replication through the *LYS2* locus. (A) Schematic of the modified 2-D gel system as adapted from Fangman and Brewer (12). Genomic DNA prepared from strain YPH500L was digested with *Eco*RI and *Pvu*II, which cut on either side of the *LYS2* gene to generate a 4.5-kb fragment, and was separated on a first-dimension gel. The gel slice was incubated with *Bam*HI, which cleaves 1.1 kb off the 3′ end of the *Eco*RI-*Pvu*II fragment, and the resulting cleaved replication intermediates were separated on the second-dimension gel. Because the in situ digestion was not complete, hybridization to a probe from the 5′ end of *LYS2* detected both the undigested replication intermediates (Y arc [dotted line]) and *Bam*HI-cleaved replication intermediates (smaller Y arc [solid line]). The smaller *Bam*HI-cleaved Y arc will be shifted either to the left or the right compared to the original Y arc of replication intermediates, depending on the direction of replication through the region. If replication forks move from left to right through the fragment, late replication intermediates will be cut off by the in-gel digestion, and the arc of Y-shaped intermediates will shift to the right. If replication forks move from right to left through the fragment, early replication intermediates will be cut off and the Y arc will shift to the left. (B) Results of the 2-D gel analysis. DNA was treated as described for panel A and prepared for Southern analysis. Since the replication intermediates shifted to the right after *Bam*HI cleavage, replication proceeds from left to right through the *Eco*RI-*Pvu*II fragment.

DISCUSSION

The mechanism by which the expanded CTG/CAG repeat at the 3′ end of the DMPK gene causes DM is still unclear. Several studies have shown decreased levels of *DMPK* mRNA in muscle tissue from affected patients, and both DNase I cleavage and electron microscopy studies of nucleosome binding to expanded CTG repeat sequences indicate that an altered chromatin structure may exist. By analogy to position effect variegation in *Drosophila* or telomere position effect and internal $C_{1-3}A$ silencing in yeast, the data are consistent with a model in which the expanded CTG tract induces an altered chromatin structure that inhibits mRNA transcription of neighboring genes. We tested this model in yeast but could find no evidence for triplet repeat-mediated silencing. There are several possible explanations for this result. For example, yeast might lack a critical *trans*-acting factor involved in the proposed silencing, such as a CTG or CAG binding protein (35, 47, 56). Alternatively, the expanded CTG tracts could act

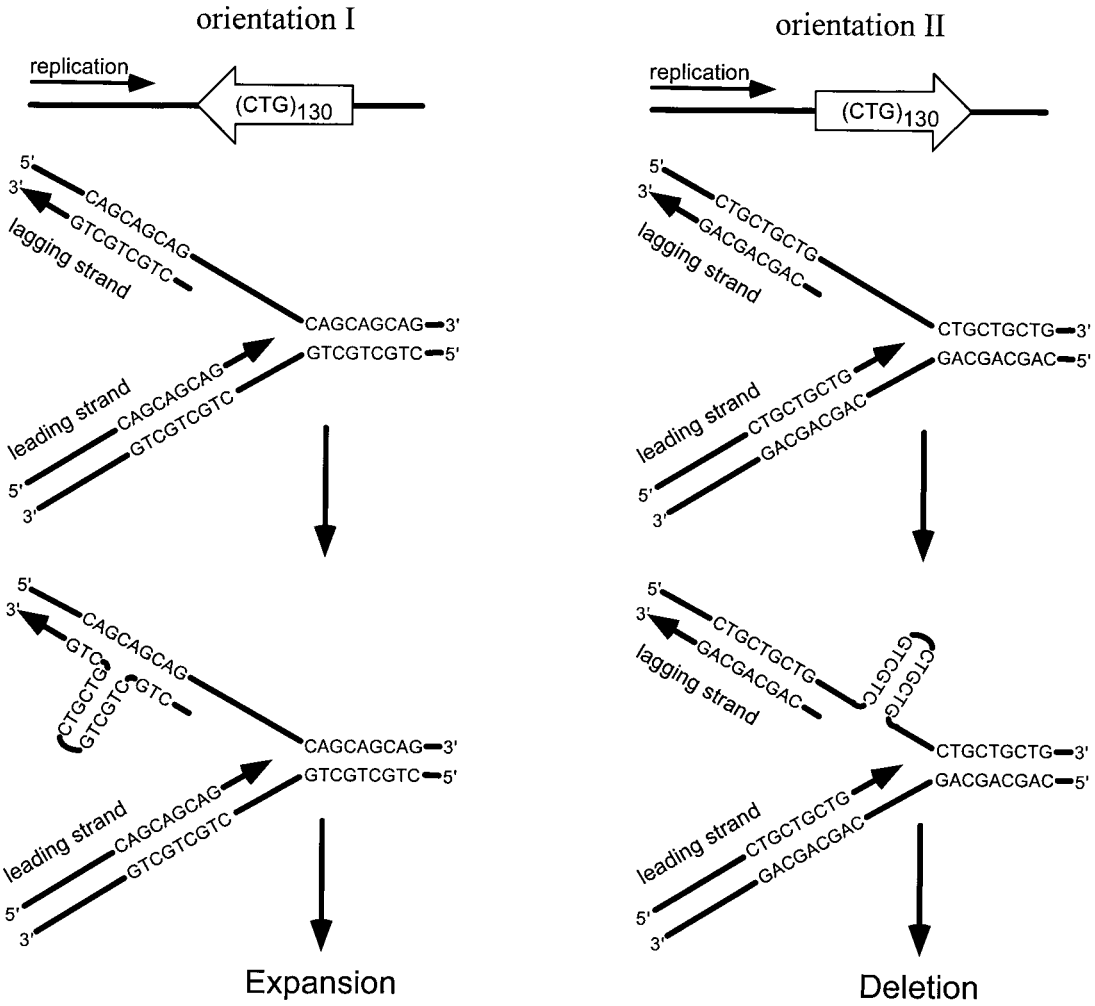

FIG. 5. Model to explain the orientation dependence of CTG tract stability at the yeast *LYS2* locus. The model is based on (i) the datum that CTG repeats form hairpins more stable than those of CAG repeats (30, 32, 57) and (ii) the assumption that hairpins are more likely to form on the lagging-strand template than the leading-strand template. In orientation II, the CTG repeat is on the lagging-strand template and is thus likely to form hairpins, leading to tract deletions. Hairpin formation could be favored when a portion of the lagging-strand template is single stranded during replication. In orientation I, the CAG repeat is on the lagging-strand template and is thus less likely to undergo hairpin formation leading to deletions (although this process would happen at a low frequency). Occasional expansions seen for orientation I could be explained by hairpin formation of CTG repeats on the Okazaki fragments.

through a completely different mechanism, such as interference with mRNA processing (45, 51) or DNA methylation (4).

In our system, a 390-bp CTG/CAG repeat tract was markedly more unstable in one orientation than the other, regardless of whether the repeat was located 3′ or 5′ to the *URA3* transcription unit. When changes in tract length occurred, they were almost exclusively deletions, with only two possible expansions of alleles in orientations Ia and Ib (an increase of 17 to 43 repeats; Fig. 2A and data not shown). We started with a long repeat tract that clinically would be an expanded allele in the disease-causing range. One explanation for the bias toward deletions is that the tolerances of long repeat tracts are different in yeast and human cells. In addition, there might be *trans*-acting factors that control the expansion rate of triplet repeats that could be defective in patients with expanded alleles. If similar factors exist in yeast, they would presumably be wild type in the strains used here. Mutations in components of the mismatch repair system are known to cause instability in repetitive tracts, although large amplifications of trinucleotide repeats have not been observed in these backgrounds to date (21, 22, 43). Contractions of expanded trinucleotide repeats have been documented among parent-to-child transmissions, but expansions are 3- to over 100-fold more common, depending on the study (1, 26, 29). It is unclear whether these data reflect an inherent bias for expansions over deletions in humans, or whether the picture is skewed by the focus on DNA from families with disease. It is possible that either a component of the mismatch repair complex or another not yet identified repair system is required for repair of large loops or hairpins and that this correction is defective in patients with trinucleotide repeat disorders.

Several models have been suggested to explain the expansion of trinucleotide repeats (11, 18, 20, 23, 29, 36). The data are most consistent with a polymerase slippage model in which dissociation and subsequent misaligned reannealing of the newly synthesized strand in the repetitive tract results in addition (or deletion) of repeats. The polymerase slippage model can explain several features of trinucleotide repeat instability such as the stabilizing effect of variant repeats and the polarity of repeat addition to the 3′ ends of tracts (11, 18). Physical studies of different trinucleotide repeat sequences indicate that certain repeats, including CTG, CAG, and CGG, are able to

form hairpin structures in vitro (9, 14, 30, 57) and in vivo (10). Formation of a hairpin could overcome the thermodynamic barrier to dissociation of long stretches of repeat sequences, thereby allowing large expansions or deletions. It has been suggested that slippage occurs preferentially on the strand replicated by lagging-strand synthesis, perhaps because it is single stranded for a longer period of time and thus has more opportunity to engage in secondary structures (18, 29, 49). In *Escherichia coli*, a lagging strand with potential to form a hairpin if misalignment occurs has a mutation frequency 10- to 20-fold higher than that of a substrate without hairpin-forming potential (49). In addition, there is evidence that the *E. coli* lagging-strand polymerase is capable of elongating off a slipped intermediate at a rate 20-fold higher than that of the leading-strand polymerase, leading to a higher mutation frequency on the lagging strand (50).

The direction of replication through the yeast *LYS2* region can explain the orientation dependence of CTG/CAG tract stability seen in our experiments. In orientation II, the deletion-prone orientation, the CTG repeats are on the lagging-strand template, whereas the Okazaki fragments would contain the CAG repeat (Fig. 5). CTG DNA is predicted to form a more stable hairpin than that of CAG DNA (30, 32, 57). Since single-stranded DNA occurs naturally on the lagging-strand template, the CTG repeats could preferentially form hairpins, resulting in deletions (Fig. 5). In orientation I, the stable orientation, the CAG repeats, which are less likely to form hairpin structures, would be on the lagging-strand template, resulting in a more stable propagation of the trinucleotide repeat. Alternatively, because of the detection limit of the 2-D gel technique, we cannot rule out the possibility that replication forks are moving in the opposite direction in a small percentage of cells and that these forks are responsible for the occasional instability seen in orientation I. It has been suggested that expansions arise by slippage of Okazaki fragments (11, 18, 36). In orientation I, the CTG repeats would be on the Okazaki fragments, and thus this configuration would be more likely to allow expansions (Fig. 5). The two cases of putative expansions were both in this orientation. Unlike the lagging-strand template, the Okazaki fragments should not naturally have single-stranded regions, perhaps explaining why expansions are rare. Thus, our data are consistent with the model of polymerase slippage that was developed based on the behavior of trinucleotide repeats in human disease.

Other studies in yeast support a role for replication in repeat instability. Tran et al. (48) found that deletion events between short direct repeats of unique sequence in yeast are increased in strains with a temperature-sensitive polymerase δ, implicating a role for replication. Also, a paper submitted while our manuscript was in preparation found that the stability of a CTG tract of 80 to 90 repeats at the yeast *ARO2* locus is dependent on the absence or presence of a neighboring ARS element, supporting that direction of replication is an important factor in the stability of trinucleotide repeat tracts (28). Since the stability of the CTG tract with respect to replication direction is consistent in the two studies (reference 28 and this study), it appears to be a general phenomenon.

From the data presented here, we cannot rule out effects on the stability of the CTG tract due to transcription or recombination. Wierdl et al. (54) found that a $(GT)_{35}$ tract in yeast is destabilized six- to ninefold by very high levels of transcription through the tract and that an additional two- to threefold destabilization occurs in a mismatch repair-defective background. Unlike the instability of the $(CTG)_{130}$ tract, the alterations in the GT tract were limited to addition or deletion of one to two repeats, and there was no orientation dependence. Due to the placement of the *URA3* gene between the *LYS2* promoter and the tract, it is probable that transcription occurs through the CTG tract in orientation II but not in orientation I. One possibility is that this transcription or a transcription-coupled repair process is responsible for the greater instability of orientation II CTG tracts. However, any transcription levels from the *LYS2* promoter through the CTG tract would surely be low, since cells were maintained in complete medium for these experiments. Also, since in the study by Maurer et al. (28) transcription levels through a $(CTG)_{80}$ tract should be the same for both the stable and unstable orientations, one would have to invoke an orientation dependence of transcription-coupled repair. It is also possible that tract length changes can occur by recombination between sister chromatids rather than replication slippage, although the orientation dependence is less easily explained by a recombinational mechanism.

Our results also agree with experiments done with *E. coli* in which CTG/CAG repeats were also less stable when the CTG strand was the template strand for discontinuous synthesis (20). Taken together, the data suggest that similar deletion mechanisms operate in both prokaryotic and eukaryotic cells. A $(CTG)_{180}$ tract in orientation II in *E. coli* also had a higher frequency of deletions as the number of generations increased; however, shorter tracts of 20 to 40 repeats were stable (20). Thus, in agreement with clinical data, it appears that once a triplet tract reaches a size of about 30 repeats, in the range of normal allele sizes, it is no longer prone to replication-dependent slippage.

In summary, we have established that deletion of CTG/CAG trinucleotide repeats shows an orientation dependence in a region of a eukaryotic chromosome with a demonstrated direction of replication. The results agree with previously proposed models for generation of trinucleotide repeat expansions and deletions via polymerase slippage.

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