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Unstable Spinocerebellar Ataxia Type 10 (ATTCT)·(AGAAT) Repeats Are Associated with Aberrant Replication at the ATX10 Locus and Replication Origin-Dependent Expansion at an Ectopic Site in Human Cells[▽]

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Spinocerebellar ataxia type 10 (SCA10) is associated with expansion of $(ATTCT)_n$ repeats (where *n* is the number of repeats) within the ataxin 10 (ATX10/E46L) gene. The demonstration that $(ATTCT)_n$ tracts can act as DNA unwinding elements (DUEs) in vitro has suggested that aberrant replication origin activity occurs at expanded $(ATTCT)_n$ tracts and may lead to their instability. Here, we confirm these predictions. The wild-type ATX10 locus displays inefficient origin activity, but origin activity is elevated at the expanded ATX10 loci in patient-derived cells. To test whether $(ATTCT)_n$ tracts can potentiate origin activity, cell lines were constructed that contain ectopic copies of the c-*myc* replicator in which the essential DUE was replaced by ATX10 DUEs with $(ATTCT)_n$. ATX10 DUEs containing $(ATTCT)_{27}$ or $(ATTCT)_{48}$, but not $(ATTCT)_8$ or $(ATTCT)_{13}$, could substitute functionally for the c-*myc* DUE, but $(ATTCT)_{48}$ could not act as an autonomous replicator. Significantly, chimeric c-*myc* replicators containing ATX10 DUEs displayed length-dependent $(ATTCT)_n$ instability. By 250 population doublings, dramatic two- and fourfold length expansions were observed for $(ATTCT)_{27}$ and $(ATTCT)_{48}$ but not for $(ATTCT)_8$ or $(ATTCT)_{13}$. These results implicate replication origin activity as one molecular mechanism associated with the instability of $(ATTCT)_n$ tracts that are longer than normal length.

Spinocerebellar ataxia type 10 (SCA10; MIM 603516) is an autosomal dominant disease caused by large expansions of $(ATTCT)_n \cdot (AGAAT)_n$ [hereafter referred to as $(ATTCT)_n$] pentanucleotide repeats (where *n* is the number of repeats) in intron nine of the ataxin 10 (ATX10) gene (40). Recent work suggests that decreased production of the ATX10 protein or a gain-of-function mutation in the noncoding region of the ATX10 mRNA can contribute to the loss of cerebellar neurons (38, 47). SCA10 patients display progressive cerebellar dysfunction frequently evidenced by limb and gait ataxia, ocular movement abnormalities, and dysarthria (33). The pentanucleotide expansion in SCA10 is unstable during spermatogenesis as well as in somatic cells and is one of the largest expansions shown to cause human diseases (39). As in trinucleotide repeat diseases, the expansion of the microsatellite is often revealed to be much greater in affected children than in their parents (37). Intergenerational expansion is thought to be responsible for genetic anticipation, where phenotypic expression occurs at an earlier age and with more severity in successive generations (39). The repeat number, *n*, at the ATX10 site in normal individuals is in the range of 10 to 22, whereas the $(ATTCT)_n$ pentanucleotide repeat length of SCA10 patients can approach 4,500 (39, 40).

DNA unwinding elements (DUEs) are regions of easily unwound DNA frequently associated with replication origins in viruses, bacteria, and yeast, where they are proposed to facilitate unwinding of the template for replication. The helical stability of DUEs can be predicted under conditions of physiological temperature and superhelical stress (3, 8, 20), with an imperfect correlation existing between the thermodynamically calculated helical instability and plasmid autonomously replicating sequence (ARS) activity in the yeast *Saccharomyces cerevisiae* (19, 32, 56). AT-rich DUEs have also been reported at replication origins in the distantly related yeast *Schizosaccharomyces pombe* and in metazoans, where deletion of these sequences decreases replication efficiency (35, 42). While the *S. pombe* Orc4 contains a unique AT-hook motif which targets the origin recognition complex (ORC) to extended asymmetric AT-rich sequences (10, 26, 30), metazoan ORC binds with only modest preference to AT-rich DNA in vitro (48, 52). Thus, the function of AT-rich DUEs as protein binding sites, helically unstable regions, or both, is uncertain.

At the ATX10 locus a putative DUE region comprising a 124-bp AT-rich sequence and flanking $(ATTCT)_n$ repeats is predicted to have a low free-energy cost of unwinding (3, 20). In vitro, two-dimensional gel electrophoresis and atomic force microcopy detected local DNA unpairing in supercoiled plasmids containing the ATX10 $(ATTCT)_n$ repeats and the flanking AT-rich region (46). Moreover, supercoiled, but not relaxed, plasmids containing the $(ATTCT)_{23}$ DUE were semiconservatively replicated in HeLa cell extracts. Chemical probe analysis indicated the formation of single-stranded DNA within the pentanucleotide repeats dependent on repeat length and superhelical density (σ), with a threshold between 8 and 11 ATTCT repeats and σ values between -0.045 and -0.055; at a higher superhelical density,

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TABLE 1. Primers used in this work

Primer[a]	Forward primer sequence	Reverse primer sequence
Diagnostic PCR primers		
1	TGACTGGAGAGCGAGGCGAT	
2		GCAGTAGCGTGGGCATTT
3		TCAGAGCAGCCGATTGTCT
4	GCGTTAGCAATTTAACTGTG	
5		ATGATAAAGAAGACAGTCATAAG
6		GGAGGGAGAGAAAAGTTTAC
7		CCTAGTTCACTGACTCACAA
qPCR primers		
Hyg	TGCTCCGCATTGGTCTTGA	TGCGCCCAAGCTGCAT
pUV	TGCCTGACTGCGTTAGCAAT	CAAACCCTAAAACGGCCAAA
pDV	TCCCTGGCTCCCCTCCT	CTATAAGTGCGGCGACGATA
TK	AGCAAGAAGCCACGGAAGTC	GTTGCGTGGTGGTGGTTTTC
P1	CAAGCCAATTCCTGGTCTTCTAA	TGGCAACAAGTAAGAAGTATTGGTTT
P2	GAAAAGTCTGCCTGAACATGATGT	TCCCCTTCCCCTAAAATATTCTAGA
P3	TTTTTAGTTCCACCCAAATGCTTT	GCAACTGCAGAGCCTGCAA
P4	TGCCCGGGAAATAATTCAAG	TGCTATACACTGCTGCTTCAATCA
P5	CAGGAATCAATGGAATGACATCTT	TTTTGAAGGATATTTTGGCTGGAT
P6	CTGCCTACCAGGTTCAAGCAA	GGTGGTGAACCTGTAATCTCAGCTA
P7	TCTCGAACTCCTGACCTCAAATT	TCACGCCTGTAATCTCAATGCT
P8	ATCAGGAACAAGGCAAGAATTTCT	TTAGTGAGTTGCTCTGGACCTTCA
X chromosome-A	CTCACAGTTCAGGAAAGCAGAAGTC	CAGAGCCTCCGGGAGGAA
X chromosome-B	TTTTTAATGCCTGGCAGATGTGT	AAGAACGAAAGGCACAAGATGTC

[a] qPCR, quantitative PCR.

unwinding in the $(ATTCT)_{11}$ repeat spread into the flanking AT-rich genomic sequence. The preferential initiation of replication near the DUE of the c-*myc* replicator in vivo (54) and in vitro (7) and the tendency of the ATX10 pentanucleotide repeat region to act as a DUE in vitro have led to the hypothesis that abnormal replication origin activity near expanded $(ATTCT)_n$ repeats may be responsible for repeat instability and ultimately the etiology of SCA10 (33). Here, we confirm that the ATX10 locus in cells from SCA10 patients displays 5- to 10-fold higher nascent strand abundance than the same locus in control cells. To test how expanded ATTCT repeats might contribute to replication origin activity, we replaced the DUE of the human c-*myc* replicator with ATX10 DUEs containing variable lengths of $(ATTCT)_n$ tracts.

The 2.4-kb region upstream of the human c-*myc* gene is active as a replicator; i.e., it stimulates replication in the flanking chro-

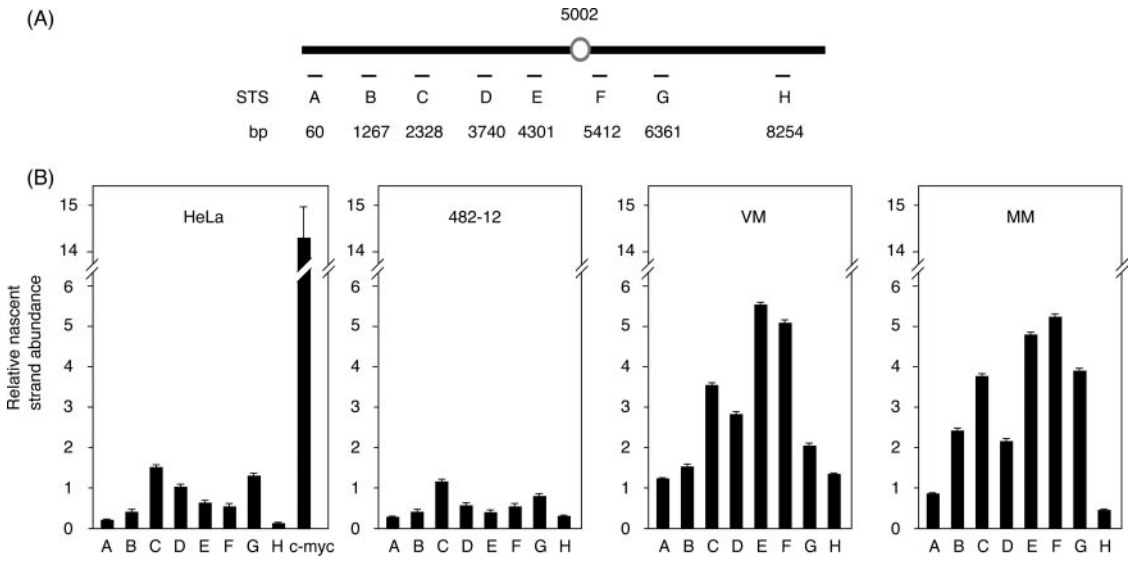


FIG. 1. Ectopic replication origin activity at the expanded $(ATTCT)_n$ tract in SCA10 cells. (A) Map of the ATX10 locus showing nucleotide coordinates of STSs used for quantitative PCR. Circle, position of $(ATTCT)_{13}$ repeats at the wild-type ATX10 locus in HeLa cells. (B) Relative nascent DNA strand abundance at the ATX10 locus in HeLa cells and lymphoblastoid 482-12 (DM1 myotonic dystrophy), VM (SCA10), and MM (SCA10) cells relative to that at the low abundance STS-54.8 in the β-globin locus (21). For comparison, the nascent strand abundance at STS-myc2 in the HeLa c-*myc* locus (24) is also shown.

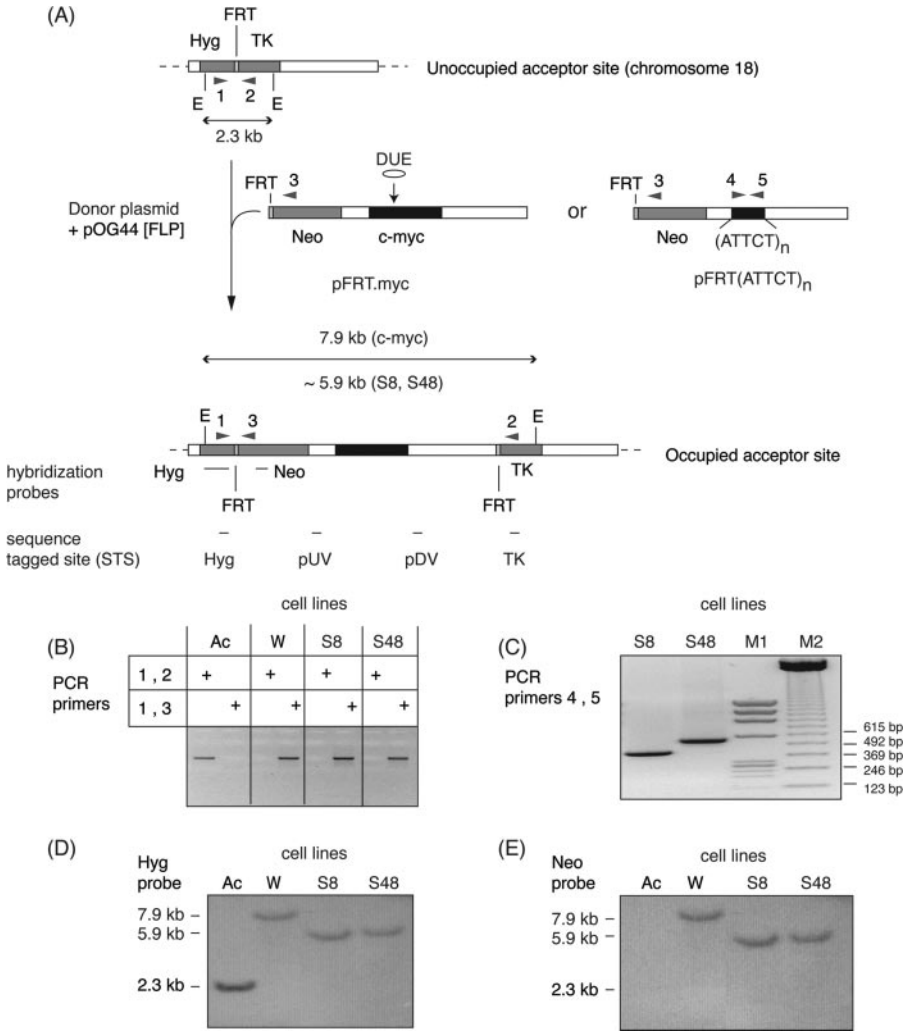


FIG. 2. Targeted integration of $(ATTCT)_8$ and $(ATTCT)_{48}$ DUEs in clonal cell lines. (A) The HeLa cell acceptor subline HeLa/406 contains a single chromosomal copy of the FRT plasmid pHyg.FRT.TK. TK, herpes simplex virus TK gene; unfilled rectangles, vector. Donor plasmids (shown linearized) contain a G418 resistance gene (Neo) with its promoter replaced by the FRT, plus the 2.4-kb HindIII/XhoI fragment of the c-*myc* replicator (pFRT.myc) or $(ATTCT)_n$ pentanucleotide repeats plus 20 bp of 5′ genomic flanking DNA and 104 bp of 3′ genomic flanking DNA (Table 1). pOG44 is a FLP recombinase expression plasmid. Accurately targeted cells are resistant to hygromycin, G418, and ganciclovir. PCR primers (Table 1) 1, 2, and 3 (horizontal arrowheads) give products diagnostic for the unoccupied acceptor site (primers 1 and 2) or the occupied acceptor site (primers 1 and 3). STSs Hyg, pUV, pDV, and TK used in real-time quantitative PCR quantitation are also indicated. The positions of restriction sites (E, EcoRI) and probes (solid bars; Hyg and Neo) relevant to Southern analyses are shown. (B) Diagnostic PCR using primers 1 and 2 or 1 and 3 with DNA from HeLa/406 acceptor cells (Ac) with an unoccupied FRT acceptor site and DNA from cells containing the wild-type c-*myc* origin fragment (pFRT.myc; W) or ATX10 DUEs $pFRT(ATTCT)_8$, (S8) or $pFRT(ATTCT)_{48}$ (S48) at the acceptor site. (C) PCR across the S8 or S48 integrated ATX10 DUEs using primer set 4 and 5. Lanes M1 (HaeIII-digested ΦX174) and M2 (123-bp ladder) are size markers. (D and E) Hybridization with the Hyg or Neo probe to EcoRI-digested DNA from HeLa/406 acceptor cells (Ac) or from 406.myc cells containing the wild type c-*myc* origin fragment (W), $pFRT(ATTCT)_8$ (S8), or $pFRT(ATTCT)_{48}$ (S48) at the acceptor site.

mosomal DNA when integrated at an ectopic FLP recombinase target (FRT) site at chromosome 18p11.22 (15, 35, 36). Chromosomal replication also initiates in this region of the c-*myc* gene in human (15, 31, 53, 54), mouse (16), and chicken cells (43) and within 4 kb 5′ of the c-*myc* gene in frog cells (16). The 2.4-kb c-*myc* replicator displays a nonrandom arrangement of nucleosomes (27, 28) and contains multiple transcription factor binding sites (17), as well as a predicted DUE also termed the far-upstream element that is sensitive to single-strand DNA-directed reagents in vivo and in vitro (6, 13, 35, 41). The DUE interacts with at least two proteins, the far-upstream element binding protein (13, 18) and the DUE binding protein DUE-B that was identified in a yeast one-hybrid screen using the c-*myc* DUE as bait (9, 23). At the ectopic FRT locus, deletion of a short DNA fragment containing the DUE eliminated replicator activity and DUE-B binding (14), and replacing the DUE region with a sequence of identical size and AT content, but greater predicted helical stability, could not restore c-*myc* replicator activity (35), suggesting that a structure involved in DNA unwinding is essential for origin activity.

Clonal cell lines were constructed containing ATX10 DNA comprising $(ATTCT)_n$ repeats plus 124 bp of flanking genomic

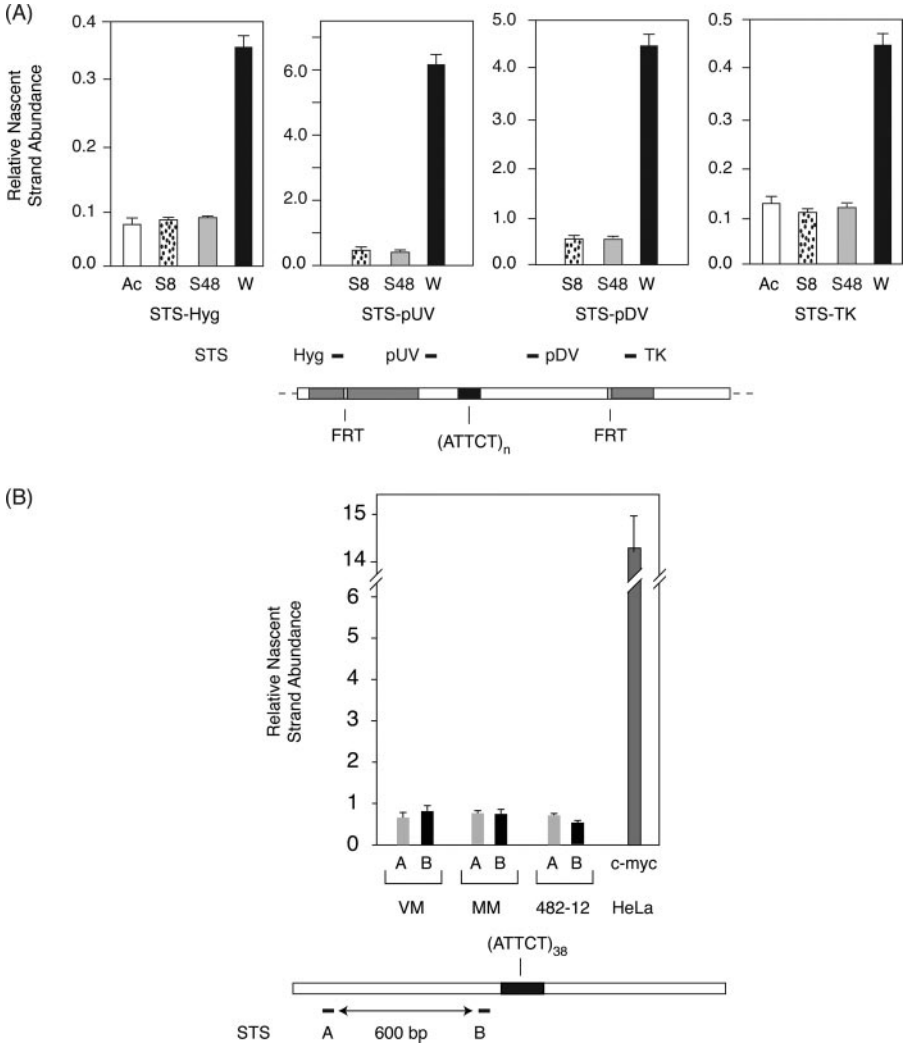


FIG. 3. Expanded $(ATTCT)_n$ tracts are not autonomous chromosomal replicators. (A) Short nascent DNA was isolated from asynchronously growing S8 and S48 cell lines and quantitated at STS-Hyg, -pUV, -pDV, and -TK sites. For comparison, origin activity at these sites is shown for the integrant containing the wild-type 2.4 kb c-*myc* replicator. (B) Short nascent DNA was isolated from asynchronously growing VM, MM, and 482-12 cells and quantitated at STS-A and -B flanking the $(ATTCT)_{38}$ repeat at X chromosome locus p22.2.

DNA (collectively referred to as ATX10 DUEs) integrated at the chromosomal FRT acceptor site without c-*myc* DNA; alternatively, the ATX10 DUEs were substituted for the DUE of the c-*myc* 2.4-kb replicator. Our data show that $(ATTCT)_n$ repeats of increasing length restore origin activity to the DUE-deficient c-*myc* replicator and suggest a mechanism by which expanded $(ATTCT)_n$ repeats may contribute to aberrant origin activity and genomic instability at the expanded ATX10 locus in the cells of SCA10 patients.

MATERIALS AND METHODS

Plasmid construction. ATX10 DUEs containing $(ATTCT)_8$, $(ATTCT)_{13}$, $(ATTCT)_{27}$, or $(ATTCT)_{48}$ were amplified from genomic DNA or plasmid clones by PCR and substituted for the c-*myc* DUE in the vector pFRT.mycΔ5 (35). $(ATTCT)_n$ flanking sequences consisted of a 20-bp 5′ sequence (AGAGA GACTTCATCTCAAAA) and a 104-bp 3′ sequence (CCATTCTAGTAGTCT TTTAGTTGGATATTTAAGCCATTTACATTTAATATATTTATCAACAT GATTGAGTTTATCATTCTGCCATCTGTTTTCTATTTGTCTTC). Primer sequences are given in Table 1. Plasmids containing ATX10 DUEs of $(ATTCT)_8$ or $(ATTCT)_{48}$ without c-*myc* DNA sequences were constructed by substituting the corresponding ATX10 DUE for the entire 2.4 kb c-*myc* replicator in plasmid pFRT.myc (36).

Cell culture. Acceptor cells containing a single chromosomal FRT were constructed, grown, and transfected as described previously (35). Southern analysis was performed by standard methods using either the 756-bp EcoRI/XbaI fragment of the Hyg (hygromycin) gene from plasmid pFRT.Hyg.TK (where TK is thymidine kinase) or the 445-bp NcoI/SmaI fragment of the Neo gene from pFRT.myc (35). Lymphoblastoid cells were the generous gift of T. Ashizawa and were grown at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum.

PCR. Analytical PCR, short (0.6 to 2 kb) nascent DNA isolation, and quantitative PCR have been described previously (35). Small-pool PCR (spPCR) was performed on ~130 to 260 pg of genomic DNA, which corresponds to 5 to 10 copies of the ectopic site in pseudotetraploid HeLa-derived cells.

RESULTS

Replication initiation at the ATX10 locus containing expanded (ATTCT) repeats. The number of $(ATTCT)_n$ repeats at the ATX10 locus in HeLa cells and lymphoblastoid 482-12 cells is in the normal range of 10 to 22 (40). As a measure of

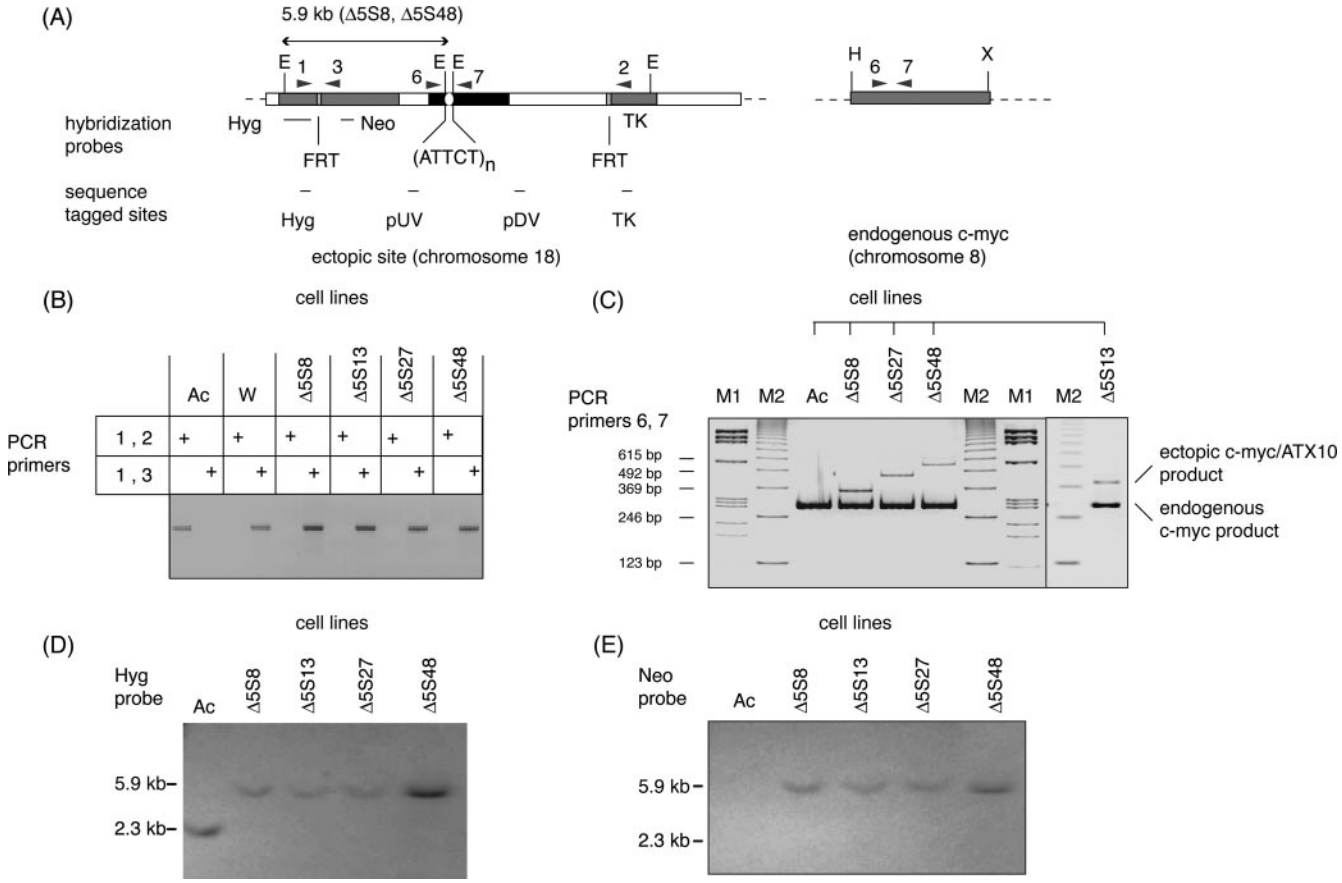


FIG. 4. Targeted integration of chimeric c-*myc*/ATX10 replicators. (A) The DUE region was deleted from the c-*myc* replicator (cell line Δ5) and replaced with ATX10 DUEs containing $(ATTCT)_8$, $(ATTCT)_{13}$, $(ATTCT)_{27}$, or $(ATTCT)_{48}$ to generate the clonal cell lines Δ5S8, Δ5S13, Δ5S27, and Δ5S48, respectively, as shown in Fig. 2. (B) Diagnostic PCR with DNA from HeLa/406 acceptor cells (Ac) and DNA from cells containing the wild type c-*myc* origin fragment (pFRT.myc; W) or chimeric replicators. (C) PCR using primers 6 and 7, which amplify the endogenous c-*myc* DUE and the ectopic ATX10 DUEs. The different intensities of the products from the single-copy ectopic c-*myc* site and the endogenous c-*myc* locus are due to the pseudotetraploid HeLa/406 genome. Lanes M1 and M2, size markers. Hybridization with the Hyg (D) or Neo (E) probe to EcoRI-digested DNA from HeLa/406 acceptor cells (Ac), 406.myc cells (W), and Δ5S8, Δ5S13, Δ5S27, and Δ5S48 cells.

origin activity at the ATX10 locus in these cells, the abundance of short (0.6 to 2 kb) nascent DNA strands was quantitated. The level of nascent strands at primer sites more than 3 kb from the $(ATTCT)_n$ microsatellite was similar to that at an inefficient initiation site in the β-globin locus (21, 35) (Fig. 1), indicating that replication rarely initiates within 3 kb of the $(ATTCT)_n$ tract in non-SCA10 cells.

In culture, lymphoblastoid cells derived from SCA10 patients exhibit instability at the ATX10 locus (34). In contrast to the low efficiency of replication initiation at the ATX10 locus in non-SCA10 cells, lymphoblastoid cells from two SCA10 patients with expanded microsatellite regions of $(ATTCT)_{>1000}$ (data not shown) displayed ~5-fold higher abundance of short nascent DNA over the ATX10 domain and ~10-fold higher nascent strand abundance at sites flanking the expanded $(ATTCT)_n$ microsatellite (sequence-tagged sites [STS] E and F) (Fig. 1B, VM and MM cells). Since the distance separating STS-E and STS-F in SCA10 cells (>10 kb) is greater than the largest (2 kb) nascent strand quantitated, this is a lower estimate of the frequency of replication initiation at the expanded ATX10 loci, which suggests that the expanded ATX10 region represents a zone of initiation where many sites, separated by more than the length of the 2-kb nascent strands, are efficiently used to begin replication.

These results indicate a minimum 5- to 10-fold increase in the frequency of SCA10 cells initiating replication near the expanded $(ATTCT)_n$ tract. To test whether abnormal replication initiation at the ATX10 locus could be because the expanded $(ATTCT)_n$ tracts function as DUEs, clonal cell lines were constructed in which $(ATTCT)_n$ repeats were either integrated at an ectopic FRT site without c-*myc* DNA or substituted for the c-*myc* replicator DUE (35) (Fig. 2A). $(ATTCT)_n$ pentanucleotide repeat tracts larger than ~45 repeats are unstable during growth in *Escherichia coli* (G. Liu, unpublished data; R. Sinden, unpublished data), and therefore the $(ATTCT)_{48}$ repeat tract was the largest construct that could be tested. Southern hybridization and PCR amplification confirmed that the $(ATTCT)_n$ donor plasmids had integrated uniquely at the FRT (Fig. 2B, D, and E) and that the original clonal integrant cell lines retained the input repeat lengths (Fig. 2C). To assess the replication origin activity of these constructs, short nascent DNA was quantitated from cells in

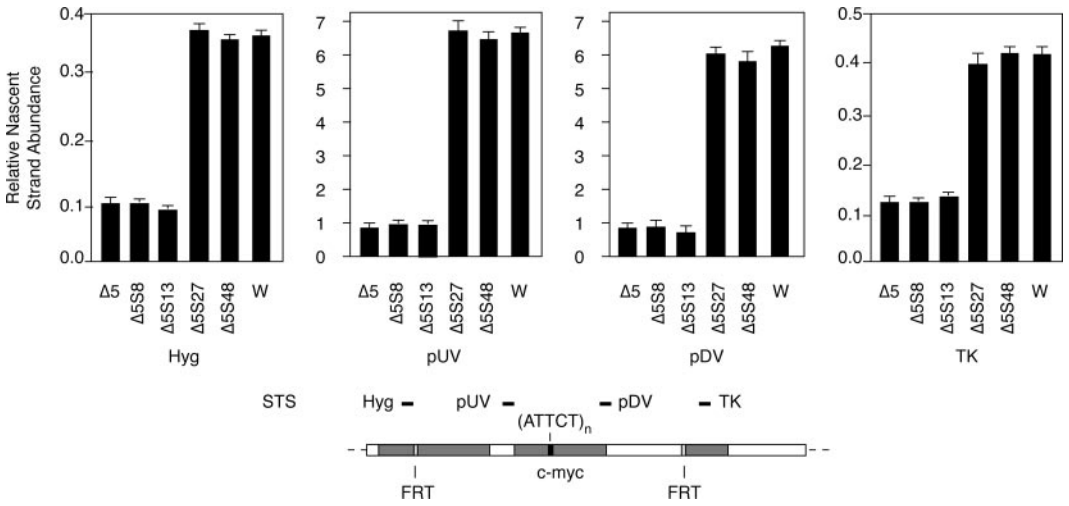


FIG. 5. Expanded $(ATTCT)_n$ tracts function as DNA unwinding elements in chimeric c-*myc*/$(ATTCT)_n$ replicators. Nascent DNA was isolated from asynchronously growing Δ5S8, Δ5S13, Δ5S27, and Δ5S48 cell lines and quantitated as described in the legend of Fig. 3.

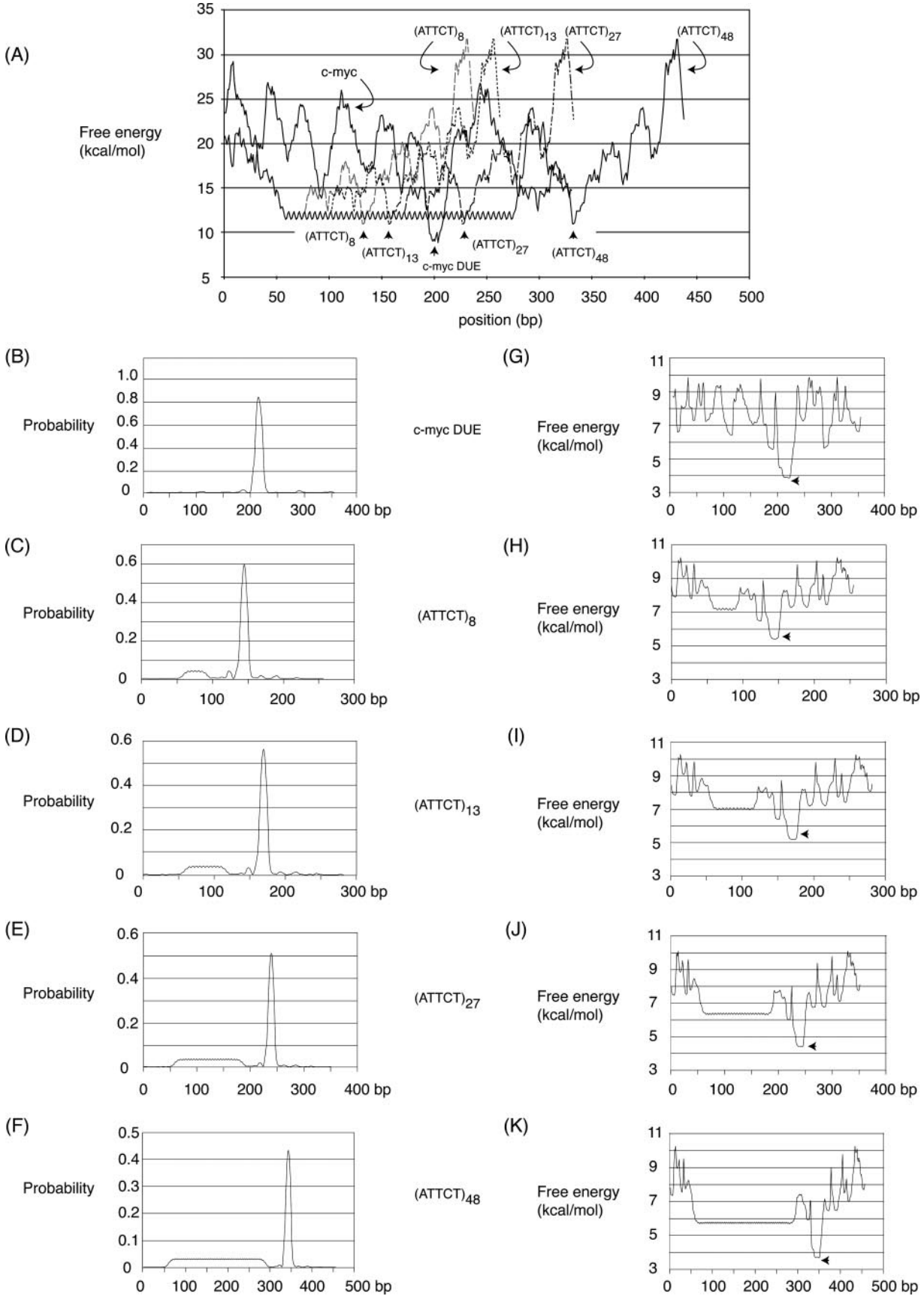
logarithmic growth. When the $(ATTCT)_8$ and $(ATTCT)_{48}$ repeats were integrated in the absence of flanking c-*myc* replicator sequences in S8 and S48 cells, respectively, the origin activity was no greater than that at the unoccupied FRT site in the acceptor cell line (Fig. 3A) (or at the same FRT site occupied by nonorigin control sequences) (35, 36). We also tested for origin activity near the $(ATTCT)_{38}$ repeat at the X chromosome locus p22.2 (1). As shown in Fig. 3B, only background nascent strand abundance was observed at this site. The absence of origin activity at chromosome X p22.2 is not explained by X chromosome inactivation, since the X chromosomes of HeLa cells are not bound by *Xist* RNA (44, 45). These results support the view that an extended $(ATTCT)_n$ repeat is not sufficient for autonomous replication origin activity in the contexts of these genomic sites.

Expanded repeats support replication initiation in the context of the c-*myc* replicator. To test whether the putative ATX10 DUEs could act in place of DUEs in the context of an ectopic replicator, a second panel of clonal cell lines designated Δ5S8, Δ5S13, Δ5S27, and Δ5S48 was constructed containing ATX10 DUEs with $(ATTCT)_8$, $(ATTCT)_{13}$, $(ATTCT)_{27}$, or $(ATTCT)_{48}$ repeats, respectively, substituted for the c-*myc* DUE (Fig. 4A to C). Replacement of the c-*myc* replicator DUE with ATX10 DUEs containing 8 or 13 ATTCT repeats did not restore replication origin activity to the DUE-deficient Δ5 mutant replicator (Fig. 5). In contrast, origin activity was similar to that at the wild-type ectopic c-*myc* replicator (406.myc cells) when ATX10 DUEs of 27 or 48 ATTCT repeats replaced the c-*myc* DUE. Thus, in the presence of flanking replicator auxiliary sequences (35), the expanded ATX10 DUEs were able to substitute functionally for the c-*myc* DUE in a manner dependent on the length of the $(ATTCT)_n$ tract.

Analysis of the ease of unwinding of ATX10 DUEs. A DNA segment of identical length and AT content but which was predicted to be more difficult to unwind could not effectively replace the c-*myc* DUE (35). To determine whether the c-*myc* and the ATX10 DUEs have similar tendencies to unwind, they were analyzed using the WEB-THERMODYN algorithm (20) which calculates DNA helical stability based on the thermodynamic properties of the nearest-neighbor nucleotides in a DNA segment. The DUEs were also analyzed using WebSIDD (8) which predicts the probability and location of stress-induced duplex destabilization (SIDD) in double-stranded DNA based on the statistical mechanical distribution of a population of DNA molecules at equilibrium.

Because the nucleotide nearest-neighbor and junction sequences are the same regardless of $(ATTCT)_n$ repeat number, WEB-THERMODYN predicts the same free energy of melting (~12 kcal/mol) for ATTCT tracts of 8, 13, 27, or 48 repeats (Fig. 6A), as well as a short segment of slightly more negative free energy of melting approximately 55 bp downstream of each $(ATTCT)_n$ tract (Fig. 6A, arrowheads) in the flanking AT-rich genomic DNA. The sequence of this downstream segment matches 9 of 11 bp of the *S. cerevisiae* ARS consensus. This segment is also a preferred site of unwinding as calculated by WebSIDD (Fig. 6B to F), and it displays decreasing stability with increasing length of the neighboring $(ATTCT)_n$ tract (Fig. 6H to K). The calculated free energy of unwinding of the $(ATTCT)_{48}$ ATX10 DUE is comparable to that of the c-*myc* DUE (Fig. 6G), which also contains sequence matching 9 of 11 bp of the *S. cerevisiae* ARS consensus.

Expanded $(ATTCT)_n$ tracts that support replication initiation exhibit genomic instability. Instability at the ATX10 locus is observed primarily during male germ line transmission in SCA10 families, although mosaicism of repeat lengths is observed in somatic tissues (40). We next determined whether the ATX10 $(ATTCT)_n$ repeats were stable during the establishment of the respective clonal cell lines. As shown in Fig. 7A, at 10 weeks (~50 population doublings [PD]) after cloning, no expansion of the DUEs was observed. However, by passage of the culture to ~250 PD, instability of the ATX10 $(ATTCT)_{48}$ sequence resulted in expansions, evident as bands larger than those from the original clonal cell lines, and contractions (see below). The instability was dependent on the length of the $(ATTCT)_n$ tract, since instability was not observed in Δ5S8 or Δ5S13 cells (Fig. 7A) but was observed in Δ5S27 and Δ5S48 cells in which replication was restored to the c-*myc* replicator. The $(ATTCT)_{48}$ DUE that showed instability



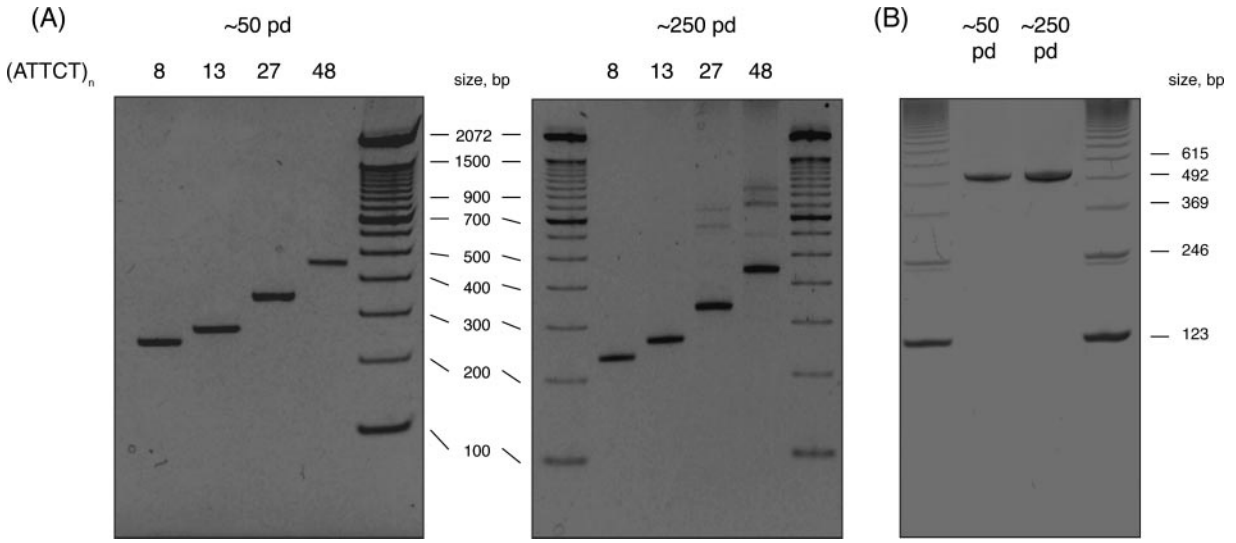


FIG. 7. Genomic instability at $(ATTCT)_n$ tracts correlates with ectopic origin activity. Genomic DNA from the Δ5S8, Δ5S13, Δ5S27, and Δ5S48 cell lines containing chimeric c-*myc*/$(ATTCT)_n$ replicators (A) or from S48 cells containing the ectopic $(ATTCT)_{48}$ DUE (B) was isolated at approximately 50 PD or approximately 250 PD and amplified using PCR primers 4 and 5 (Fig. 2).

in the context of the c-*myc* replicator in the Δ5S48 cell line did not show instability when integrated at the same ectopic location in the absence of flanking c-*myc* sequences in S48 cells (Fig. 7B), suggesting that proximal replication origin activity promotes instability in repeats longer than normal length. The absence of PCR products larger than those of the originally integrated $(ATTCT)_n$ DUE in Δ5S27 and Δ5S48 cells grown to ~50 PD or less (Fig. 4C and 7A) argues that the expanded bands seen after ~250 PD are not PCR artifacts.

To test at higher sensitivity for DNA instability, spPCR was used to amplify 5 to 10 genomic copies of the ectopic c-*myc*/ATX10 (c-*myc* replicator with ATX10 DUEs) replicators. In more than 20 independent spPCR amplifications each, instability was not detected in Δ5S8 or Δ5S13 cells, while expansions and contractions were readily detected in Δ5S27 and Δ5S48 cells (Fig. 8 and data not shown). Comparable ladder patterns of expanded spPCR products were obtained using Δ5S27 and Δ5S48 cell DNA. The reproducibility of the patterns obtained from independent spPCR amplifications suggests that the ATX10 DUE was expanded by a similar mechanism in many cells of the population. The spPCR products from Δ5S27 cells were offset by approximately 150 to 200 bp from those of Δ5S48 cells. The most prominent PCR products derived from expanded $(ATTCT)_{27}$ tracts in Δ5S27 cells were approximately 160 bp apart, while those from Δ5S48 cells were approximately 200 bp apart.

These patterns suggest that the expansion occurs by the local stepwise amplification of the region containing the ATX10 DUEs between the primer sites used for spPCR. To test this idea, expanded spPCR products from Δ5S27 cells (~500 to 900 bp) and Δ5S48 cells (~600 to 1,100 bp) were excised from polyacrylamide gels and cloned into *E. coli*. Sequencing of three clones from Δ5S27 cells and four clones from Δ5S48 cells revealed pure $(ATTCT)_n$ repeats and flanking DNA without substitution or interruption; however, the cloned ATX10 DUEs from Δ5S27 cells contained 25, 26, and 26 $(ATTCT)_n$ repeats and 18, 44, 45, and 47 $(ATTCT)_n$ repeats when cloned from Δ5S48 cells. Despite the quantitative loss of integral numbers of repeats during cloning in *E. coli*, these results argue that instability of the ectopic ATX10 DUEs results from a local amplification of $(ATTCT)_n$ repeats during prolonged growth of the Δ5S27 and Δ5S48 cells.

DISCUSSION

Binding sites for the ORC and minichromosome maintenance complex have been identified at several metazoan replication origins (2, 4, 5, 14, 22, 25, 29, 49, 50); however, these sites are not sufficient to specify a chromosomal region as an origin of replication in the absence of a DUE (14). Not surprisingly, local regions of predicted helically unstable DNA are common features of mammalian replication origins, including the c-*myc* replication origin (18). The replication origin activity associated with the easily unwound DNA elements at the mutant SCA10 locus and at the engineered ectopic c-*myc* replicator argue that, in addition to possible roles in the modification of chromatin structure or protein binding, DUEs facilitate

FIG. 6. $(ATTCT)_n$ tract expansion decreases the predicted free energy of supercoiling-induced DNA unwinding. Predicted helical stability of the c-*myc* and ATX10 DUEs. The genomic regions (~330 to 440 bp) containing the c-*myc* or ATX10 DUEs were analyzed by WEB-THERMODYN or WebSIDD. (A) Free energy of unwinding predicted by WEB-THERMODYN (step size, 10 bp; window size, 20 bp). (B to F) Probability of unwinding (WebSIDD). (G to K) Predicted free energy of unwinding (WebSIDD) (window size, 200 bp; σ, −0.055). Arrowheads indicate zones of lowest free-energy cost of unwinding.

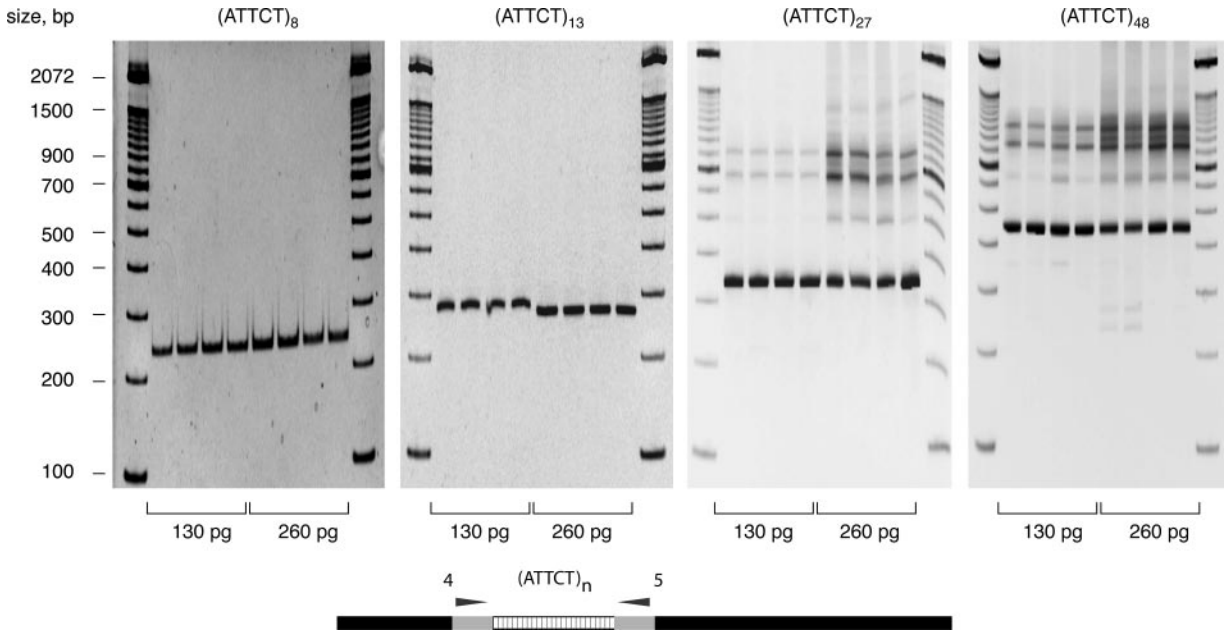


FIG. 8. spPCR confirms that genomic instability correlates with ectopic origin activity. Genomic DNA from Δ5S8, Δ5S13, Δ5S27, and Δ5S48 cell lines containing chimeric c-*myc*/$(ATTCT)_n$ replicators was isolated at approximately 250 PD and ~5 (130 pg) to ~10 (260 pg) copies of the ectopic $(ATTCT)_n$ sites were amplified using PCR primers 4 and 5 (Fig. 2).

the supercoiling-induced helix unwinding of the DNA template strands during replication initiation.

We have previously shown that $(ATTCT)_n$ repeat tracts longer than those in normal human alleles act as DUEs and support aberrant DNA replication initiation in vitro (46). These observations led to the hypothesis that expanded repeats may promote aberrant replication in human chromosomes and cause the instability of repeats for which disease is associated with increased repeat length. Here, we present data confirming these hypotheses. First, we show that the endogenous $(ATTCT)_n$ tract within the ataxin 10 gene in normal cells shows only background replication origin activity but that origin activity is elevated at least 5- to 10-fold in lymphoblasts from SCA10 patients with expanded $(ATTCT)_n$ tracts. Second, we show that the $(ATTCT)_n$ tracts for which *n* is 27 or 48 function as DUEs replacing the natural DUE within the c-*myc* replicator, which is essential for replication initiation. Repeat tracts where *n* is 8 or 13, that is, below or within the normal range of 10 to 22 repeats, fail to support DNA replication initiation. Third, we show that $(ATTCT)_{48}$ at the ectopic location but in the absence of c-*myc* replicator sequences does not support replication. Fourth, we show that longer-than-normal repeat tracts undergo two- to fourfold expansion, from 48 to ~170 and from 27 to ~125 repeats, during growth in human cells. The expansion, significantly, is dependent on the proximity of replication origin activity; expansion is not observed during replication from a distant origin.

$(ATTCT)_n$ tracts longer than the normal range are unstable when placed close to sites of replication initiation but appear to be stable when replicated from distal origins. The simplest explanation for expansion of the AT-rich pentanucleotide tracts between preserved ATX10 flanking sequences is replication slippage (51) in which greater phasing of Okazaki fragment initiation sites proximal to the origin favors the formation of metastable loops in the newly synthesized strand (11). Instability may also be a direct consequence of replication if $(ATTCT)_n$ sequences are unwound and recombinogenic when they constitute the majority of a newly synthesized strand near an origin but are stable when they comprise the 3′ end of a long nascent strand replicated from a distal origin (46). Local amplification of the ATX10 DUEs in the Δ5S27 and Δ5S48 cell lines would be consistent with such mechanisms. Alternatively, the length- and position-dependent instability of $(ATTCT)_n$ tracts may not be related to replication per se but could reflect a chromosome structure permissive for DNA unwinding and recombination at the origin or changes in the protein composition of replication forks as they progress.

In the absence of the c-*myc* replicator, the ectopic FRT site is replicated primarily from a downstream origin (unpublished results). Thus, we cannot formally rule out the possibility that a change in replication polarity is responsible for the stability of the $(ATTCT)_n$ tracts in the absence of the proximal c-*myc* replicator. However, in similar experiments, $(CAG)_{102}$ tracts are unstable irrespective of orientation when flanked by the ectopic c-*myc* replicator but stable in the absence of a proximal origin (G. Liu, unpublished results).

After ~250 population doublings spPCRs of Δ5S27 and Δ5S48 cells show similar patterns of $(ATTCT)_n$ tract amplification, which we interpret to indicate expansion by a similar mechanism in many cells of the population. While it cannot be totally excluded that several subpopulations with favored repeat lengths have overtaken the culture, the similarity in the offset patterns from the independently derived Δ5S27 and Δ5S48 cells argues strongly that the distributions of amplified repeat lengths reflect the mechanism of expansion rather than mitotic drive.

The present data indicate that the expansion of $(ATTCT)_n$ tracts that leads to SCA10 causes abnormal replication origin activity and genomic instability. These results suggest a model in which sporadic replication origin activity at the ATX10 locus promotes increases in $(ATTCT)_n$ repeat length, which potentiate origin activity and the formation of larger tracts (46). The absence of origin activity at the X chromosome $(ATTCT)_{38}$ tract or the $(ATTCT)_8$ or $(ATTCT)_{48}$ repeats at the ectopic locus in the absence of c-*myc* replicator sequences implies that structures in addition to a DUE are required to specify a chromosomal origin, although it has not been possible to test whether highly extended, disease-length $(ATTCT)_n$ microsatellites by themselves are sufficient for origin activity. Nevertheless, a requirement for multiple replicator elements is consistent with previous results (14, 35, 55). Thus, the inefficient origin activity of the wild-type ATX10 locus may reflect the absence of appropriate ancillary elements or epigenetic structure. That expansion of the $(ATTCT)_n$ tract is sufficient to enable origin activity suggests that a change in DNA or chromatin structure is of primary importance. The sequence specificity of metazoan ORC binding is modest compared to its preference for binding to supercoiled DNA (48, 52). The demonstration that origin activity is low at the wild-type ATX10 locus but significantly elevated at the expanded ATX10 locus raises the question of whether prereplicative complex proteins are bound but inactive at the wild-type ATX10 locus or are recruited to the ATX10 locus as a result of expansion of the $(ATTCT)_n$ tract and a concomitant alteration of DNA topology and chromosome organization. Experiments are currently under way to address these questions.

These data present strong evidence validating models suggesting that expanded DNA repeats promote aberrant DNA replication initiation (33, 46) and that the activation of cryptic origins leads to genomic instability (12). Moreover, they suggest a molecular mechanism associated with $(ATTCT)_n$ repeat expansion: the initiation of DNA replication. In addition, the results suggest that the analysis of repeat instability in this ectopic location in HeLa cells provides a valuable model for studying *cis*- and *trans*-acting factors affecting repeat instability.

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REFERENCES

1. **Abdella, Z., et al.** 2004. Finishing the euchromatic sequence of the human genome. Nature **431:**931–945.
2. **Abdurashidova, G., M. B. Danailov, A. Ochem, G. Triolo, V. Djeliova, S. Radulescu, A. Vindigni, S. Riva, and A. Falaschi.** 2003. Localization of proteins bound to a replication origin of human DNA along the cell cycle. EMBO J. **22:**4294–4303.
3. **Ak, P., and C. J. Benham.** 2005. Susceptibility to superhelically driven DNA duplex destabilization: a highly conserved property of yeast replication origins. PLOS Comput. Biol. **1:**e7.
4. **Alexandrow, M. G., M. Ritzi, A. Pemov, and J. L. Hamlin.** 2002. A potential role for mini-chromosome maintenance (MCM) proteins in initiation at the dihydrofolate reductase replication origin. J. Biol. Chem. **277:**2702–2708.
5. **Austin, R. J., T. L. Orr-Weaver, and S. P. Bell.** 1999. *Drosophila* ORC specifically binds to ACE3, an origin of DNA replication control element. Genes Dev. **13:**2639–2649.
6. **Bazar, L., D. Meighen, V. Harris, R. Duncan, D. Levens, and M. Avigan.** 1995. Targeted melting and binding of a DNA regulatory element by a transactivator of c-myc. J. Biol. Chem. **270:**8241–8248.
7. **Berberich, S., A. Trivedi, D. C. Daniel, E. M. Johnson, and M. Leffak.** 1995. In vitro replication of plasmids containing human c-myc DNA. J. Mol. Biol. **245:**92–109.
8. **Bi, C., and C. J. Benham.** 2004. WebSIDD: server for predicting stress-induced duplex destabilized (SIDD) sites in superhelical DNA. Bioinformatics **20:**1477–1479.
9. **Casper, J. M., M. G. Kemp, M. Ghosh, G. M. Randall, A. Vaillant, and M. Leffak.** 2005. The c-myc DNA-unwinding element-binding protein modulates the assembly of DNA replication complexes in vitro. J. Biol. Chem. **280:**13071–13083.
10. **Chuang, R. Y., and T. J. Kelly.** 1999. The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. Proc. Natl. Acad. Sci. USA **96:**2656–2661.
11. **Cleary, J. D., and C. E. Pearson.** 2005. Replication fork dynamics and dynamic mutations: the fork-shift model of repeat instability. Trends Genet. **21:**272–280.
12. **Dominguez-Sola, D., C. Y. Ying, C. Grandori, L. Ruggiero, B. Chen, M. Li, D. A. Galloway, W. Gu, J. Gautier, and R. Dalla-Favera.** 2007. Non-transcriptional control of DNA replication by c-Myc. Nature **448:**445–451.
13. **Duncan, R., L. Bazar, G. Michelotti, T. Tomonaga, H. Krutzsch, M. Avigan, and D. Levens.** 1994. A sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif. Genes Dev. **8:**465–480.
14. **Ghosh, M., M. Kemp, G. Liu, M. Ritzi, A. Schepers, and M. Leffak.** 2006. Differential binding of replication proteins across the human c-*myc* replicator. Mol. Cell. Biol. **26:**5270–5283.
15. **Ghosh, M., G. Liu, G. Randall, J. Bevington, and M. Leffak.** 2004. Transcription factor binding and induced transcription alter chromosomal c-*myc* replicator activity. Mol. Cell. Biol. **24:**10193–10207.
16. **Girard-Reydet, C., D. Gregoire, Y. Vassetzky, and M. Mechali.** 2004. DNA replication initiates at domains overlapping with nuclear matrix attachment regions in the *Xenopus* and mouse c-myc promoter. Gene **332:**129–138.
17. **Hay, N., J. M. Bishop, and D. Levens.** 1987. Regulatory elements that modulate expression of human c-myc. Genes Dev. **1:**659–671.
18. **He, L., J. Liu, I. Collins, S. Sanford, B. O'Connell, C. J. Benham, and D. Levens.** 2000. Loss of FBP function arrests cellular proliferation and extinguishes c-myc expression. EMBO J. **19:**1034–1044.
19. **Huang, R. Y., and D. Kowalski.** 1993. A DNA unwinding element and an ARS consensus comprise a replication origin within a yeast chromosome. EMBO J. **12:**4521–4531.
20. **Huang, Y., and D. Kowalski.** 2003. WEB-THERMODYN: sequence analysis software for profiling DNA helical stability. Nucleic Acids Res. **31:**3819–3821.
21. **Kamath, S., and M. Leffak.** 2001. Multiple sites of replication initiation in the human beta-globin gene locus. Nucleic Acids Res. **29:**809–817.
22. **Keller, C., E. M. Ladenburger, M. Kremer, and R. Knippers.** 2002. The origin recognition complex marks a replication origin in the human TOP1 gene promoter. J. Biol. Chem. **277:**31430–31440.
23. **Kemp, M., B. Bae, J. P. Yu, M. Ghosh, M. Leffak, and S. K. Nair.** 2007. Structure and function of the c-myc DNA-unwinding element-binding protein DUE-B. J. Biol. Chem. **282:**10441–10448.
24. **Kemp, M. G., M. Ghosh, G. Liu, and M. Leffak.** 2005. The histone deacetylase inhibitor trichostatin A alters the pattern of DNA replication origin activity in human cells. Nucleic Acids Res. **33:**325–336.
25. **Kinoshita, Y., and E. M. Johnson.** 2004. Site-specific loading of an MCM protein complex in a DNA replication initiation zone upstream of the c-MYC gene in the HeLa cell cycle. J. Biol. Chem. **279:**35879–35889.
26. **Kong, D., and M. L. DePamphilis.** 2001. Site-specific DNA binding of the *Schizosaccharomyces pombe* origin recognition complex is determined by the Orc4 subunit. Mol. Cell. Biol. **21:**8095–8103.
27. **Kumar, S., and M. Leffak.** 1991. Conserved chromatin structure in c-myc 5′ flanking DNA after viral transduction. J. Mol. Biol. **222:**45–57.
28. **Kumar, S., and M. Leffak.** 1989. DNA topology of the ordered chromatin domain 5′ to the human c-myc gene. Nucleic Acids Res. **17:**2819–2833.
29. **Ladenburger, E. M., C. Keller, and R. Knippers.** 2002. Identification of a binding region for human origin recognition complex proteins 1 and 2 that coincides with an origin of DNA replication. Mol. Cell. Biol. **22:**1036–1048.
30. **Lee, J. K., K. Y. Moon, Y. Jiang, and J. Hurwitz.** 2001. The *Schizosaccharomyces pombe* origin recognition complex interacts with multiple AT-rich regions of the replication origin DNA by means of the AT-hook domains of the spOrc4 protein. Proc. Natl. Acad. Sci. USA **98:**13589–13594.
31. **Leffak, M., and C. D. James.** 1989. Opposite replication polarity of the germ line c-*myc* gene in HeLa cells compared with that of two Burkitt lymphoma cell lines. Mol. Cell. Biol. **9:**586–593.
32. **Lin, S., and D. Kowalski.** 1997. Functional equivalency and diversity of *cis*-acting elements among yeast replication origins. Mol. Cell. Biol. **17:**5473–5484.
33. **Lin, X., and T. Ashizawa.** 2005. Recent progress in spinocerebellar ataxia type-10 (SCA10). Cerebellum **4:**37–42.
34. **Lin, X., and T. Ashizawa.** 2003. SCA10 and ATTCT repeat expansion:

clinical features and molecular aspects. Cytogenet. Genome Res. **100:**184–188.
35. **Liu, G., M. Malott, and M. Leffak.** 2003. Multiple functional elements comprise a mammalian chromosomal replicator. Mol. Cell. Biol. **23:**1832–1842.
36. **Malott, M., and M. Leffak.** 1999. Activity of the c-*myc* replicator at an ectopic chromosomal location. Mol. Cell. Biol. **19:**5685–5695.
37. **Manto, M. U.** 2005. The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum **4:**2–6.
38. **Marz, P., A. Probst, S. Lang, M. Schwager, S. Rose-John, U. Otten, and S. Ozbek.** 2004. Ataxin-10, the spinocerebellar ataxia type 10 neurodegenerative disorder protein, is essential for survival of cerebellar neurons. J. Biol. Chem. **279:**35542–35550.
39. **Matsuura, T., P. Fang, X. Lin, M. Khajavi, K. Tsuji, A. Rasmussen, R. P. Grewal, M. Achari, M. E. Alonso, S. M. Pulst, H. Y. Zoghbi, D. L. Nelson, B. B. Roa, and T. Ashizawa.** 2004. Somatic and germ line instability of the ATTCT repeat in spinocerebellar ataxia type 10. Am. J. Hum. Genet. **74:**1216–1224.
40. **Matsuura, T., T. Yamagata, D. L. Burgess, A. Rasmussen, R. P. Grewal, K. Watase, M. Khajavi, A. E. McCall, C. F. Davis, L. Zu, M. Achari, S. M. Pulst, E. Alonso, J. L. Noebels, D. L. Nelson, H. Y. Zoghbi, and T. Ashizawa.** 2000. Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. Nat. Genet. **26:**191–194.
41. **Michelotti, G. A., E. F. Michelotti, A. Pullner, R. C. Duncan, D. Eick, and D. Levens.** 1996. Multiple single-stranded *cis* elements are associated with activated chromatin of the human c-*myc* gene in vivo. Mol. Cell. Biol. **16:**2656–2669.
42. **Okuno, Y., H. Satoh, M. Sekiguchi, and H. Masukata.** 1999. Clustered adenine/thymine stretches are essential for function of a fission yeast replication origin. Mol. Cell. Biol. **19:**6699–6709.
43. **Phi-van, L., C. Sellke, A. von Bodenhausen, and W. H. Stratling.** 1998. An initiation zone of chromosomal DNA replication at the chicken lysozyme gene locus. J. Biol. Chem. **273:**18300–18307.
44. **Plath, K., J. Fang, S. K. Mlynarczyk-Evans, R. Cao, K. A. Worringer, H. Wang, C. C. de la Cruz, A. P. Otte, B. Panning, and Y. Zhang.** 2003. Role of histone H3 lysine 27 methylation in X inactivation. Science **300:**131–135.
45. **Plath, K., S. Mlynarczyk-Evans, D. A. Nusinow, and B. Panning.** 2002. Xist RNA and the mechanism of X chromosome inactivation. Annu. Rev. Genet. **36:**233–278.
46. **Potaman, V. N., J. J. Bissler, V. I. Hashem, E. A. Oussatcheva, L. Lu, L. S. Shlyakhtenko, Y. L. Lyubchenko, T. Matsuura, T. Ashizawa, M. Leffak, C. J. Benham, and R. R. Sinden.** 2003. Unpaired structures in SCA10 $(ATTCT)_n \cdot (AGAAT)_n$ repeats. J. Mol. Biol. **326:**1095–1111.
47. **Ranum, L. P., and T. A. Cooper.** 2006. RNA-mediated neuromuscular disorders. Annu. Rev. Neurosci. **29:**259–277.
48. **Remus, D., E. L. Beall, and M. R. Botchan.** 2004. DNA topology, not DNA sequence, is a critical determinant for *Drosophila* ORC-DNA binding. EMBO J. **23:**897–907.
49. **Ritzi, M., M. Baack, C. Musahl, P. Romanowski, R. A. Laskey, and R. Knippers.** 1998. Human minichromosome maintenance proteins and human origin recognition complex 2 protein on chromatin. J. Biol. Chem. **273:**24543–24549.
50. **Schaarschmidt, D., E. M. Ladenburger, C. Keller, and R. Knippers.** 2002. Human Mcm proteins at a replication origin during the G1 to S phase transition. Nucleic Acids Res. **30:**4176–4185.
51. **Sinden, R. R., M. J. Pytlos, and V. N. Potaman.** 2006. Mechanisms of DNA repeat expansion, p. 3–53. *In* K. U. M. Fry (ed.), Human nucleotide expansion disorders. Springer, Berlin, German.
52. **Vashee, S., C. Cvetic, W. Lu, P. Simancek, T. J. Kelly, and J. C. Walter.** 2003. Sequence-independent DNA binding and replication initiation by the human origin recognition complex. Genes Dev. **17:**1894–1908.
53. **Vassilev, L., and E. M. Johnson.** 1990. An initiation zone of chromosomal DNA replication located upstream of the c-*myc* gene in proliferating HeLa cells. Mol. Cell. Biol. **10:**4899–4904.
54. **Waltz, S. E., A. A. Trivedi, and M. Leffak.** 1996. DNA replication initiates non-randomly at multiple sites near the c-myc gene in HeLa cells. Nucleic Acids Res. **24:**1887–1894.
55. **Wang, L., C. M. Lin, J. O. Lopreiato, and M. I. Aladjem.** 2006. Cooperative sequence modules determine replication initiation sites at the human beta-globin locus. Hum. Mol. Genet. **15:**2613–2622.
56. **Wilmes, G. M., and S. P. Bell.** 2002. The B2 element of the *Saccharomyces cerevisiae* ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. Proc. Natl. Acad. Sci. USA **99:**101–106.