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Mapping of an Origin of DNA Replication in the Promoter of Fragile X Gene *FMR1*

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Abstract

An origin of bidirectional DNA replication was mapped to the promoter of the *FMR1* gene in human chromosome Xq27.3, which has been linked to the fragile X syndrome. This origin is adjacent to a CpG island and overlaps the site of expansion of the triplet repeat (CGG) at the fragile X instability site, FRAXA. The promoter region of *FMR2* in the FRAXE site (approximately 600 kb away, in chromosome band Xq28) also includes an origin of replication, as previously described. *FMR1* transcripts were detected in foreskin and male fetal lung fibroblasts, while *FMR2* transcripts were not. However, both *FMR1* and *FMR2* were found to replicate late in S phase (approximately six hours into the S phase of normal human fibroblasts). The position of the origin of replication relative to the CGG repeat, and perhaps the late replication of these genes, might be important factors in the susceptibility to triplet repeat amplification at the FRAXA and FRAXE sites.

Keywords

Replication timing; nascent strand abundance; gene transcription; trinucleotide repeats; human fibroblasts

INTRODUCTION

Fragile X syndrome is an X-linked disorder that manifests most typically in males. Disease severity in symptomatic females seems to depend on the fraction of cells in which the X chromosome carrying the mutated FRAXA site remains active (Abrams et al., 1994;Rousseau et al., 1991). Pathophysiological conditions associated with the syndrome include mental retardation, autism or autistic-like behaviors, delays in speech and language development, postpubescent macroorchidism, long and prominent ears and jaws, high-pitched speech, hyperactivity, poor eye contact, and stereotypic hand movements, such as hand flapping and hand biting (Cummings and Zoghbi, 2000;Farzin et al., 2006;Garcia-Nonell et al., 2006;Hatton et al., 2006;Hou et al., 2006). Fragile X syndrome is the most common genetic disorder associated with mental retardation or autism in males, with an estimated prevalence of 1 in 4000 to 1 in 6000 males. Additional physiological conditions have been associated with or related to FRAXA, including tremor ataxia syndrome (Ennis et al., 2006;Greco et al., 2006;Iwahashi et al., 2006;Jacquemont et al., 2006) and premature ovarian failure (Fassnacht

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et al., 2006;Meskhi and Seif, 2006;Woad et al., 2006). As its name implies, FRAXA was originally identified through its association with a folate-sensitive fragile site at Xq27.3 in a fraction of affected patients. Sequence analysis of cosmids and cDNAs mapping to this fragile site revealed a polymorphic $(CGG)_n$ repeat in the 5′ untranslated region of the *FMR1* gene at Xq27.3, which is known as the FRAXA site (Verkerk et al., 1991). Triplet repeat amplification in excess of 200 copies (full mutation) leads to hypermethylation of the *FMR1* promoter region and gene silencing, hence a loss-of-function effect in affected males (Terracciano et al., 2005). In the pre-mutation stage, amplifications ranging from approximately 60 to 200 repeats, are associated with increased transcription of the mutant *FMR1* gene (Tassone et al., 2000). It appears, therefore, that the onset of disease is related to the length of the trinucleotide repeat. Interestingly, an intermediate length of the repeat (i.e., pre-mutation) makes the carrier susceptible to further expansion of the repeat region.

The cause of expansion of triplet repeats is not known, but this expansion is likely to occur during DNA replication in proliferating cells (Cleary and Pearson, 2005). Because of the antiparallel structure of double-stranded DNA and because DNA polymerases can only add nucleotides at the 3′ end of the growing strand, the two daughter strands are replicated by different mechanisms during semiconservative DNA replication. The leading strand is elongated in the same direction of the movement of the replication fork from an origin to a termination site; the lagging strand must be synthesized discontinuously, with new fragments initiated at Okazaki fragment Initiation Zones (OIZ) located approximately 150 - 300 nt apart (Cleary and Pearson, 2005;Pearson et al., 2005). CGG and CTG triplet repeats have been shown in vitro to slow or even stop DNA polymerases because of the formation of stable hairpins on template DNA (McMurray, 1999) and it has been reported that the extent of inhibition of fork progression due to this effect increases with the length of the repeat (Kang et al., 1995). The expansion of triplet repeats has been thought to be closely linked to the ability of these sequences to form alternative DNA structures, a degree of flexibility higher than non-repetitive DNA, and the difficulty that DNA polymerases encounter in progressing through repetitive DNA regions (Chastain et al., 1995;Chastain and Sinden, 1998;Pearson et al., 1998;Pearson and Sinden, 1996;Pearson and Sinden, 1998;Pearson et al., 1998;Sinden, 1999). If the newly synthesized DNA strand in these repeat regions forms unusual structures (such as hairpins), mispriming followed by elongation may lead to the expansion of the repeat tract. It has been reported that the CGG repeats on lagging strand DNA are more likely to form stable secondary structures (e.g. hairpins) that may facilitate amplification (Siyanova and Mirkin, 2001). Therefore, the location of the origin of replication with respect to the triplet repeat may determine the probability that expansion during DNA replication will occur in one of the daughter cells.

We reported previously the presence of an origin of replication in the transcriptional promoter of the *FMR2* gene (FRAXE), also implicated in fragile X syndrome, located on chromosome Xq28, approximately 600 kb telomere-wise from the FRAXA site (Chastain et al., 2006). In this report, we present the identification of the origin of bidirectional replication associated with the *FMR1* gene, the determination of its time of replication, and the expression status of both *FMR1* and *FMR2* genes in normal human male skin and lung fibroblasts.

METHODS

Cell cultures

The human cells used in these studies were NHF1-hTERT, a cell line derived from normal neonatal foreskin fibroblasts (Boyer et al., 1991) and immortalized by ectopic expression of the catalytic subunit of telomerase (Heffernan et al., 2002); GM1604-hTERT, a male fetal lung fibroblast cell line also immortalized by telomerase expression (Ouellette et al., 2000); and CRL-1502 cells (ATCC), a strain of female fetal lung fibroblasts. The hTERT-immortalized

cell lines were grown in minimal essential medium (Invitrogen, Carlsbad, CA) containing 2X the concentration of MEM non-essential amino acids (Invitrogen). The CRL-1502 cells were cultured according to ATCC recommended conditions. Growth media were further supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

Cell synchronization and determination of replication timing

The protocol used to synchronize cultured human fibroblasts was described previously (Cordeiro-Stone et al., 1986). Cells arrested at confluence were replated at lower densities in medium containing 2 μg/ml of the DNA polymerase inhibitor aphidicolin, and incubated for 24 h (Brylawski et al., 2000;Tribioli et al., 1987). Once cells were released from the aphidicolin inhibition, they resumed DNA synthesis at a normal rate and transited through the S phase as a parasynchronous cohort. DNA synthesized in different 1-h windows of the S phase was isolated by CsCl gradient centrifugation (Cordeiro-Stone et al., 1990). Samples containing equal amounts of DNA were used for testing the timing of replication of the *FMR1* region by PCR, as described previously for the region of human chromosome X containing the *FMR2* gene (Chastain et al., 2006). DNA samples from two synchronization experiments were tested twice, each time using duplicate PCR reactions. PCR reactions were conducted under non-saturating conditions as determined by a standard curve generated with genomic DNA sheared to the same size as the test DNA (Cohen et al., 2002). Only when the correlation coefficient for the standard curve was equal to or greater than 0.9 were the PCR results taken into consideration.

Preparation of nascent DNA and mapping of the replication origin

Single-stranded nascent DNA ranging in size from ∼400-1000 nt in length was prepared from logarithmically growing cells as described previously (Cohen et al., 2002). Quantitative PCR was used to determine the relative copy number of selected genetic markers (Table I) from the *FMR1* gene region. A standard curve using sonicated genomic DNA (∼ 1500 bp) was amplified together with the nascent DNA sample for each primer set, so that differences in amplification efficiency would not affect the determination of relative abundance. Each preparation of short nascent DNA was also tested by measuring the relative abundance of sequences at the lamin B2 origin using previously described L5 (Cohen et al., 2002) and B13 (Giacca et al., 1994) primers.

PCR

Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the Prime program in the Wisconsin Package (Genetics Computer Group, Madison, WI, version 8) were used to design PCR primers for the amplification of DNA sequences within a length of DNA from nt 146697178 to nt 146701532 of chromosome band Xq27.3 as reported in Build 35 of human genomic sequences available at the UCSC Genome Bioinformatics site (www.genome.ucsc.edu). The primers are listed in Table 1 with their location, the size of the PCR product, and the annealing temperature used in the PCR reaction. PCR was performed in OmnE and PCR-Express thermocyclers (Thermo Electron Corp., Milford, MA) using Thermo-Start Taq DNA polymerase (ABgene, Rochester, NY). PCR reaction conditions were: 95°C for 15 min (once, for the activation of the polymerase), followed by 30-38 cycles of 94°C for 30 sec, annealing temperature (Table 1) for 30 sec, and elongation at 72°C for 30 sec. Some primer sets (marked with asterisks in Table 1) required a two-step PCR program: 95°C for 15 min, 30 cycles of 94°C for 1 min and annealing/elongation temperature (Table 1) for 2 min. For a few primer sets (as indicated in Table 1), 2% DMSO was also added to the reaction mixture. PCR products were separated by gel electrophoresis on 2% agarose and stained with ethidium bromide. Gel images were recorded with a digital imaging system (AlphaInnotech,

San Leandro, CA) and the intensity of the bands was analyzed with AlphaEase software from the same manufacturer. Microsoft Excel was used to graph the standard curves, determine the equations of the linear regression lines, and calculate the genomic ng-equivalent value of the unknown samples.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from NHF1-hTERT, 1604-hTERT, and CRL-1502 cells using the SV Total RNA Isolation System (Promega, Madison, WI). RNA was quantified and aliquots were run on agarose gels to check for quality. First strand cDNA synthesis was performed on 200 ng of total RNA using Superscript III (Invitrogen, Carlsbad, CA) and a reverse primer for either *FMR1* or *FMR2*. PCR was performed with one tenth of the cDNA reaction using Thermo-Start DNA polymerase (ABgene, Rochester, NY) in an OmnE or Express thermocycler (Thermo Electron Corp., Milford, MA). PCR was carried out according to the 3-step protocol described above, with 35 cycles used for each primer set. Primer sequences were as follows: *FMR1* forward gctgaagagaagatggaggag, reverse acaggaggtgggaatctgac; *FMR2* forward atgggagcggaggaatcaag, reverse ttctggactcggttggcaag. The PCR product for *FMR1* extends from exon 2 to 4 and the primer set for *FMR2* extends from exon 2 to 3.

RESULTS

Mapping of an origin of DNA replication by measuring nascent strand abundance is based on the concept that newly synthesized strands of DNA are elongated bidirectionally away from a replication start. Therefore, within the population of nascent strands of different lengths purified from the bulk of genomic DNA, the sequences closest to the origin will be found at the highest relative abundance (Giacca et al., 1997), while sequences further from the origin will be progressively less abundant. Semi-quantitative PCR with primer sets targeting the region of interest (Cohen et al., 2002) is used to determine the relative abundance of specific sequence markers in a preparation of nascent DNA fragments and thus establish which are the closest to a functional origin of replication. This type of analysis on the sequence around the promoter region of the *FMR1* gene revealed a peak of abundance overlapping the triple repeat sequence previously reported to be linked to the onset of fragile X syndrome. Both cell lines studied displayed the peak of abundance with PCR marker E (Fig. 1 and 2). No other initiation zone was detected in the 5 kb of surrounding sequence.

In both the *FMR1* (this report) and *FMR2* (Chastain et al., 2006) regions the origin of replication was mapped within the transcriptional promoter. We analyzed the transcriptional activity of *FMR1* and *FMR2* in the cell lines that were tested for origin activity (NHF1- and 1604-hTERT cells; Fig. 3). *FMR1* was expressed in both of these cell lines, but there was no detectable expression of *FMR2*. These results indicate that even though the origins of replication associated with both the *FMR1* and *FMR2* genes are located at their transcriptional promoter, transcription itself is not necessary for origin firing. This is in agreement with previous reports that showed that origins of replication associated with the human *HPRT* and *G6PD* transcriptional promoters fire on both the active and inactive X chromosomes (Cohen et al., 2003).

We determined that the FRAXA region corresponding to the *FMR1* gene in chromosome Xq27.3 replicates in the second half of the S phase in two normal human fibroblasts cell lines (Fig. 4). We were interested in establishing precisely the timing of replication of the *FMR1* gene, previously reported to be late replicating (Hansen et al., 1997;Hansen et al., 1993) by using the same cells and the same method we applied previously to the *FMR2* region (Chastain et al., 2006). For a particular type of cell, genomic DNA is replicated according to a highly organized and reproducible temporal order. A correlation has been proposed between the time of replication and the expression of genes in metazoan cells, with expressed genes replicating

early and silent genes replicated later in S phase (Hatton et al., 1988;Schwaiger and Schubeler, 2006). This difference in replication timing has been attributed to the chromatin status in the genome: chromatin that is more gene-rich (euchromatin) is more open because of transcription and replicates early, while gene-poor, more compacted chromatin (heterochromatin) replicates later in S phase. In the region of the X chromosome we analyzed in these studies, however, we found two genes, one transcriptionally active (*FMR1*) and the other inactive (*FMR2*), both replicating towards the end of the S phase. Because of this atypical replication status, it is likely that the *FMR1* region displays an unusual chromatin structure, having both the epigenetic markings for active transcription and, perhaps, some yet to be determined cis-acting element determining the late replication timing (Hansen et al., 1997). A shift to replication even later in S phase (closer to the S/G_2 border), associated with the silencing of the gene, is found in fragile X syndrome (Hansen et al., 1997). Based on published studies on X inactivation, we would predict that this shift to later replication would occur during embryonic development and should precede methylation of the promoter (reviewed in Heard, 2004). Therefore, it is possible that triplet repeat expansion leading to the alteration of a cis-acting element that controls replication timing may be involved in the switching of this gene to the inactive status (i.e., DNA hypermethylated at the promoter) that is found in fragile X syndrome.

DISCUSSION

This study disclosed the presence of an active origin of replication in the promoter region of the human *FMR1* gene. Although the nascent DNA abundance method for origin mapping allows the positioning of an origin in a fairly precise location, it does not specify an initiation site at the nucleotide level. Because of the difficulty in amplifying certain sequences (particularly CGG repeats), when analyzing the *FMR1* origin region we could not generate PCR products between or overlapping markers D and E, and E and F (Fig. 1). Therefore, we cannot be certain that sequence markers in these areas are not represented at a relative abundance higher than E. Consequently, we defined an “initiation region” as spanning from the midpoint of the sequence between markers D and E to the midpoint of the sequence between marker E and F (Fig. 1). Accordingly, the initiation region for the *FMR1* origin spans 483 bp, and it overlaps the triplet repeat site (Fig. 5A). Since no other initiation zone was detected within the surrounding 5 kb, we assume that this is the origin responsible for the replication of the triplet repeat tract. With the origin of replication in this position, the repeat $(CGG)_n$ is located in the upper strand of the diagram (Fig. 5A), which would represent the template for the lagging strand. This arrangement is not considered to be conducive to amplification (Siyanova and Mirkin, 2001) because the strand containing the $(GCC)_n$ repeat would serve as the template to the leading strand, which is synthesized continuously and is less likely to form hairpin loops at the complementary $(CGG)_n$ repeat. In our study, however, the *FMR1* origin was mapped in neonatal (NHF1) and 12-week fetal cells (GM1604). In embryonic cells, where more origins of DNA replication are likely to be active, replication through this repeat may be controlled by another origin, not active in the adult cells, located on the 3′ side of the triplet repeat (Fig 5B). In that case, the $(GCC)_n$ template would direct the synthesis of the lagging strand, favoring the formation of secondary structures (e.g. CGG hairpins) in the nascent complementary strand, possibly leading to expansion. This event would explain expansion occurring in early development rather than in adult cells, as has been reported (Wohrle et al., 1993). Further, if either origin selection or amplification is a stochastic rather than deterministic process and expansion shows strong strand bias, then triplet repeat expansion mutations would not be found in all adult cells of fragile X syndrome patients, which is consistent with diagnoses of mosaicism for the triplet repeat expansion seen in affected patient. In fact, it has been reported that fragile X female fetal fibroblasts display dynamic instability in culture, whereas fragile X adult male fibroblasts do not (Sun and Han, 2004). Another explanation for the generation of instability in the *FMR1* region would be the presence of acquired changes in the location where Okazaki fragments are primed relative to the position of the repeat (Cleary and

Pearson, 2005). It has been reported that difficulty in the anchoring of primers in repeat regions (Lyons-Darden and Topal, 1999) may favor slippage in primer binding and lead to expansion.

Interestingly, in the origin of replication associated with the promoter/trinucleotide repeat of the *FMR2* gene that we reported recently (Chastain et al., 2006), the conditions are reversed. The initiation region (defined as above) is 373 bp and overlaps the triplet repeat as well (Fig. 5C). However, unlike the *FMR1* origin, the $(CCG)_n$ repeat is in the template for the lagging strand, potentially resulting in the expansion of the complementary $(GGC)_n$ repeat in the nascent strand that is synthesized discontinuously, consequent to the generation of unusual secondary structures, such as stable hairpins. For this origin configuration, the risk of amplification would be lessened if the repeat tract were replicated from another origin active on its 3′ side (Fig. 5D). According to this model for the amplification of triplet repeats, it would appear that the configuration at the FRAXE origin site is much more conducive to expansion than the one at the FRAXA site. This conclusion, however, is not supported by the observed prevalence of full mutation of 1 in 5530 for FRAXA and 1 in 23423 for FRAXE (Youings et al., 2000). Therefore, it is likely that different molecular mechanisms are responsible for expansion at the FRAXA and FRAXE sites, thus accounting for the differences in the incidence of these mutations among fragile X syndrome patients.

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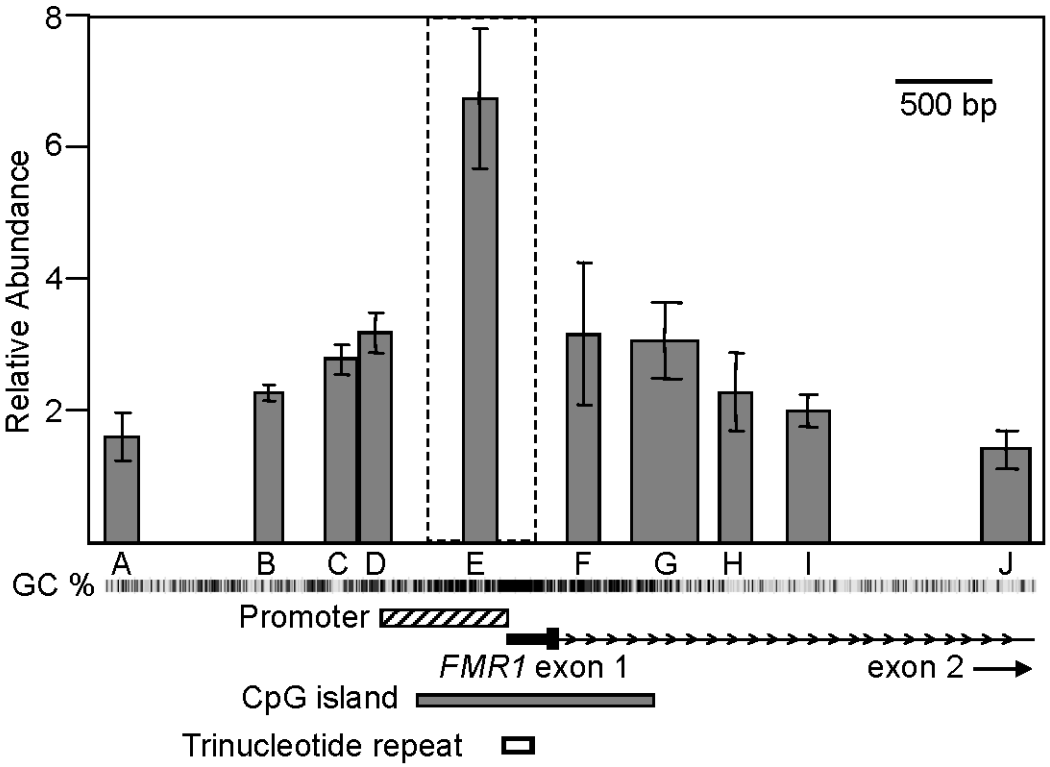

Figure 1.

Nascent strand abundance analysis in the human *FMR1* promoter region of NHF1-hTERT cells. Each marker shown in this histogram was tested at least three times using nascent DNA prepared from normal human fibroblasts (NHF1-hTERT cells). Error bars show the standard deviation. A second preparation of nascent DNA from these cells was also tested (each marker tested at least twice) with the same results. The width of each bar in the histogram is proportional to the size of the PCR product and is plotted based on sequence data available at genome.ucsc.edu. Below the histogram are indicated the percent GC in 5-base windows (dark areas: high GC content, light areas: low GC content), the position of the transcriptional promoter (Drouin et al., 1997) and *FMR1* exon 1, the associated CpG island and trinucleotide repeat (based on data also available at genome.ucsc.edu). The shaded box drawn around the peak of abundance delimits the boundary of the "initiation region" as defined in the text.

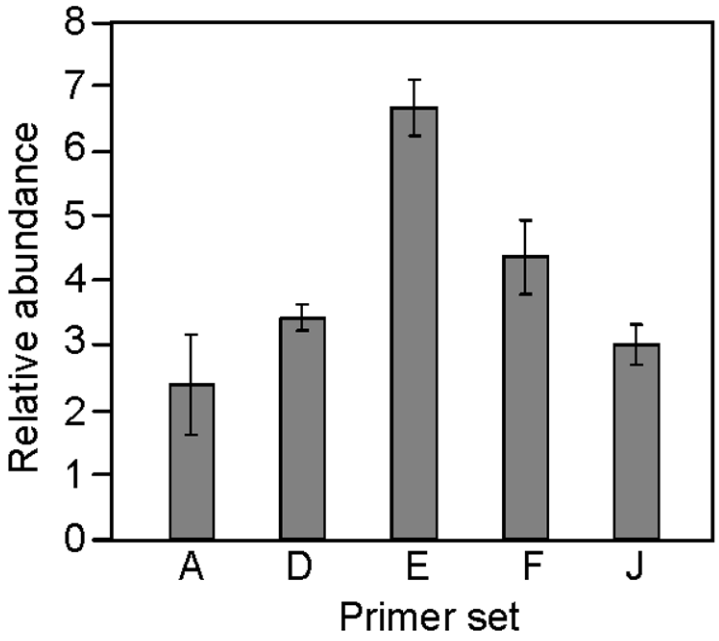

Figure 2.

Nascent strand abundance analysis in the human *FMR1* promoter region of GM1604-hTERT cells. The relative abundance of markers in a preparation of short nascent DNA from GM1604-hTERT, a fetal lung fibroblast cell line, was determined as described for NHF1-hTERT cells. Each primer set was tested at least twice and error bars show the standard deviation. These results indicate that the origin of replication associated with the FMR1 promoter is found in the same location in both the neonatal and fetal fibroblast cell lines that were tested.

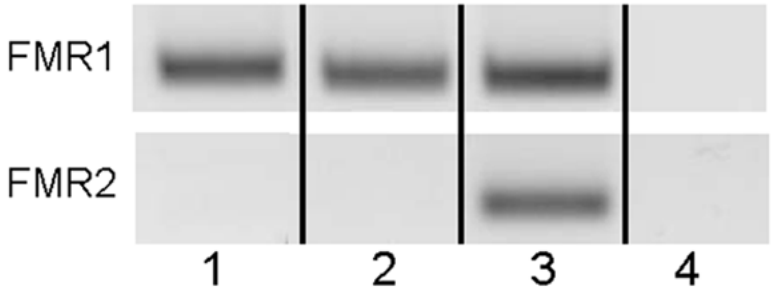

Figure 3.

Reverse Transcription PCR. RT-PCR of *FMR1* and *FMR2* messenger RNA in total RNA isolated from log phase NHF1-hTERT (lane 1), 1604-hTERT (lane 2), and CRL-1502 (lane 3) cells. Products were visualized on a 2% agarose gel stained with ethidium bromide (shown in reverse contrast). A negative control (no template DNA) was also included (lane 4). Results show that *FMR1* is expressed in both cell types analyzed for replication origin activity while *FMR2* is expressed in neither. We also analyzed cDNA from CRL-1502 cells (female fetal lung fibroblast cells) as a positive control. The resulting PCR product had the expected size, indicating that the *FMR2* primer set was working correctly.

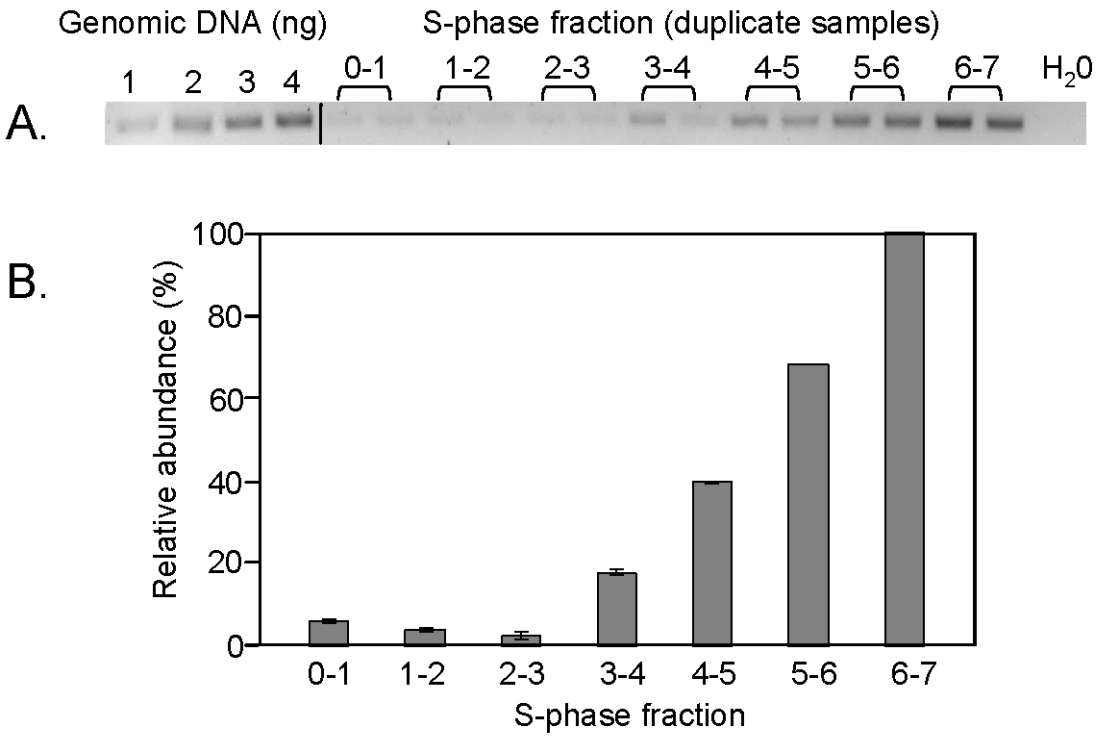

Figure 4.

Determination of the timing of replication of the *FMR1* gene in NHF1-hTERT cells. Newly synthesized DNA from seven different 1-h S phase intervals was tested by quantitative PCR to determine when the *FMR1* gene region replicates. **Panel A**: Image of PCR products separated on an ethidium bromide-stained agarose gel (shown in reverse contrast). S-phase fractions were each tested in duplicate. Images were scanned and bands quantified as described in the Materials and Methods section. **Panel B**: Bar graph illustrating the abundance of the marker in each of the seven 1-h S-phase samples. Relative abundance was calculated from the linear regression equation of the standard curve and expressed as a percentage of the highest value. Results are the average of two tests and error bars indicate the standard deviation. A second synchronization was tested with similar results. These data indicate that *FMR1* replicates very late in S phase, approximately at the same time as the *FMR2* gene region (Chastain et al., 2006).

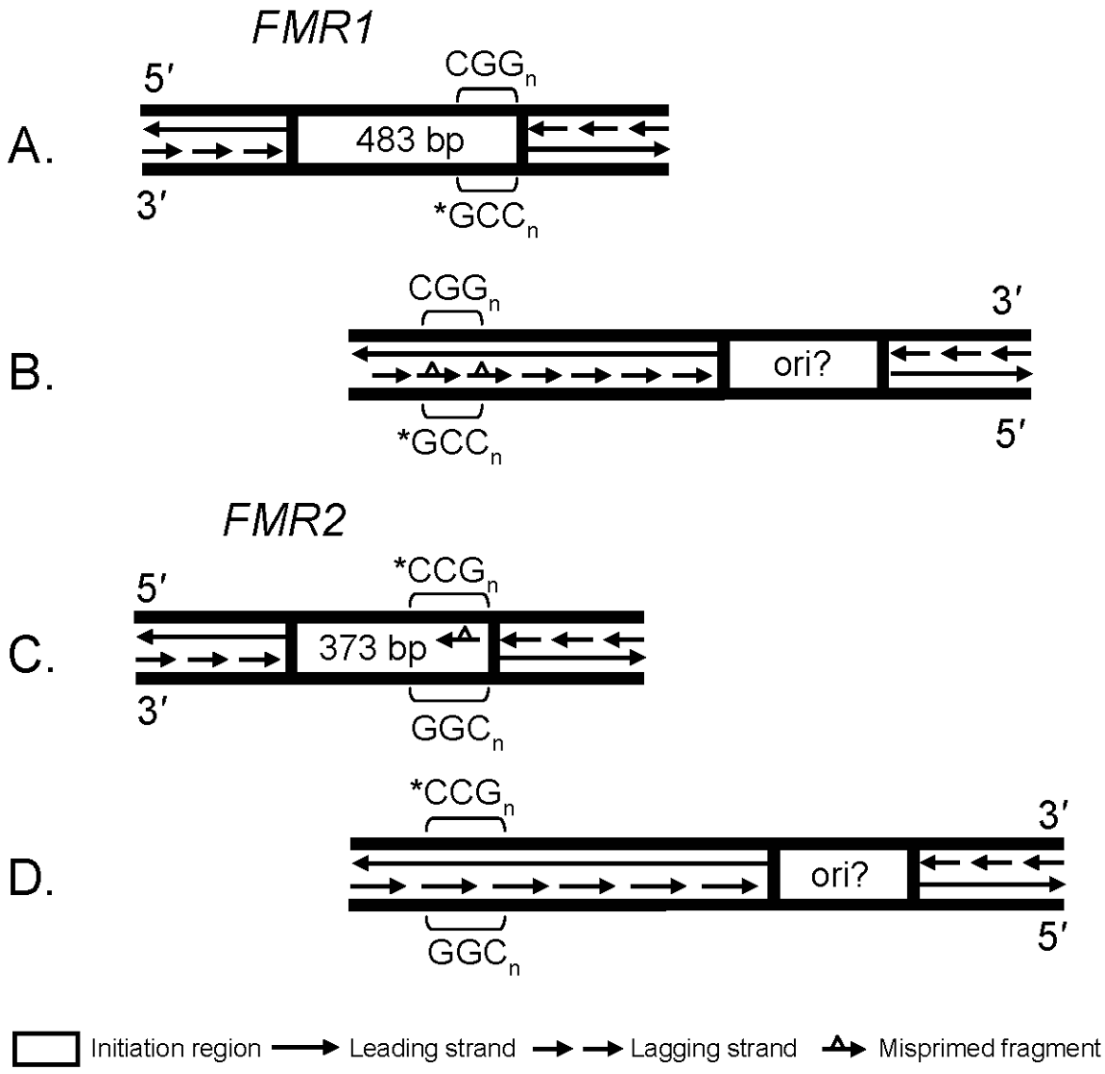

Fig 5.

Schematic representation of the relative position of initiation region and triplet repeats for the *FMR1* and *FMR2* origins. The boxed areas in A and C represent the "initiation region" for the found origins spanning from the midpoint of the sequence between markers D and E to the midpoint of the sequence between marker E and F (illustrated in Fig. 1). The position and type of triplet repeats are indicated for *FMR1* (A, B) and *FMR2* (C, D) (Chastain et al., 2006). The schematics in A and C illustrate the position of leading and lagging replicating strands with respect to the active origin and the triplet repeats. The schematics in B and D illustrate a hypothetical origin active on the 3′ site of the respective origin, and the consequent possible formation of misprimed fragments during replication. The asterisk indicates the triplet repeat configuration in the template that is more likely to cause mispriming in nascent fragments.

Table 1

Primer sets used for nascent strand abundance and replication timing analyses.

Primer Set	Sequence	Position in X Chromosome	Product Size	Annealing Temp.(° C)
FMR1A-F[#]	CTGAAAATTGAGGAGCAAAG	146697178-146697197	183	52
FMR1A-R[#]	ACTACAAGCCACACTCAACC	146697342-146697361		
FMR1B-F	TGTCCACCTACTTTGCTAGG	146697879-146697898	152	52
FMR1B-R	GCTGGTATCCAACTGAGAAG	146698012-146698031		
FMR1C-F	ATACAGTAGGGGCAGAAATG	146698212-146698231	181	52
FMR1C-R	TAAGGGACATGGATTGAGTC	146698358-146698377		
FMR1D-F	GACTCAATCCATGTCCCTTA	146698358-146698377	173	52
FMR1D-R	AGGGGTGAAGGATTAGACAG	146698512-146698531		
FMR1E-F[*&]	CCCAGGCCACTTGAAGAGAG	146698865-146698884	160	64
FMR1E-R	ACCGGAAGTGAAACCGAAAC	146699006-146699025		
FMR1F-F[*&]	CTCCCTTTTCTTCTTGGTG	146699337-146699355	178	57
FMR1F-R	GGTCTCTCATTTCGATAGGC	146699496-146699515		
FMR1G-F	TTATTCCCCCTTTCCTAAAC	146699642-146699661	330	52
FMR1G-R	GCTAGACCGGAAAAGAGAAG	146699953-146699972		
FMR1H-F	GCGTCTAAAACCATTAGCAG	146700051-146700070	224	52
FMR1H-R	AAAGCCTCAACAATTCAGTC	146700256-146700275		
FMR1I-F	TAGCAGTAGTGCGTTTGTTG	146700374-146700393	227	52
FMR1I-R	AAAGCTGTCCTGTCTGAAAC	146700582-146700601		
FMR1J-F[&]	GTGTGTGTCAGACAAATTGC	146701286-146701305	246	52
FMR1J-R	ACACTTCTCCACAGCAGTTC	146701513-146701532		

[#]Primer set used to determine the time of replication of the origin region.

[*]Primer sets requiring 2-step PCR amplification conditions.

[&]Primer sets requiring the addition of 2% DMSO in the amplification reaction.