Association of Fragile X Syndrome with Delayed Replication of the FMR1 Gene

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Summary

The fragile X syndrome is commonly associated with mutant alleles of the FMR1 gene that are hypermethylated and have large expansions of CGG repeats. We present data here on the replication timing of FMR1 that confirm predictions of delayed replication of alleles from affected males. The normal FMR1 allele replicates late in S phase, while alleles from affected males replicate later, the major peak of replication occurring in the flow cytometry fraction usually referred to as G2/M. The delayed timing of replication is not the direct result of a single replication fork stalling at the expanded CGG repeat, because delayed replication was observed for regions on both sides of the repeat. The domain of altered replication timing includes sites at least 150 kb 5′ and 34 kb 3′ of the repeat, indicating that genes in addition to FMR1 may be affected.

Introduction

The gene associated with the fragile X syndrome, FMR1, has the properties of an X-linked housekeeping gene that is subject to X chromosome inactivation (Gartler et al., 1992). FMR1 transcripts have been detected in diverse tissues (Hinds et al., 1993); the 5′ region of the gene contains a CGG island that is unmethylated on the active X chromosome and methylated on the inactive X chromosome (Hansen et al., 1992; Oberlé et al., 1991). Mutant FMR1 alleles associated with the fragile X syndrome of mental retardation resemble the normal inactive X allele in that they are transcriptionally inactive (Peretti et al., 1991) and their 5′ CGG islands, including the CGG repeat (Hansen et al., 1992), are at least partially methylated (Bell et al., 1991; Dietrich et al., 1991; Hansen et al., 1992; Heitz et al., 1991; Oberlé et al., 1991). Two levels of sequence instability have been characterized at the FMR1 locus. There is a low level of expansion of CGG repeats, from a normal number of about 30 to between 50 and 175 repeats, that is not associated with phenotypic consequences; in pregnancy of females with the primary expansion, there is often a dramatic "secondary expansion" of the CGG repeat, to more than 200 repeats, that is associated with the fragile X phenotype (Fu et al., 1991; Heitz et al., 1992; Kramer et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Yu et al., 1992). It is not yet clear whether this secondary expansion is transmitted through the germ line or occurs early in embryonic development. It is also not known what role the CGG expansions have in establishing methylation patterns. A model that correctly predicted several molecular observations and is consistent with the maternal-specific mode of inheritance of the syndrome has been proposed (Laird, 1987). In this model, the fragile X mutation interferes with the normal reactivation of the inactive X allele during oogenesis, resulting in an abnormally inactive domain on an otherwise active X chromosome.

In the model for fragile sites that is complementary to the X inactivation imprinting model, the mutant locus on the active X chromosome is therefore predicted to be stably imprinted for transcriptional silence by a failure to reverse the normal process of X chromosome inactivation.

In a model for fragile sites that is complementary to the X inactivation imprinting model, it was predicted that fragile sites occur at alleles whose replication is delayed relative to nonfragile alleles (Laird et al., 1987). Cytogenetic data led to the prediction that a replication delay at the fragile site associated with fragile X syndrome (FXA5AX1q27.3) would prolong replication into what is usually referred to as G2 phase of the cell cycle (Laird et al., 1987).

Cytological studies of the inactivated X chromosome indicate that its replication is delayed relative to that of the active X homolog and the autosomes (Aldans et al., 1952; Lima-de-Faria et al., 1961; Taylor, 1950). In general, transcriptional inactivity is often associated with replication of genes late in S phase (Goldman et al., 1964; Hatton et al., 1986). Late replication of inactive X loci relative to active X loci has previously been confirmed at the molecular level for the factor IX (F9) and hypoxanthine phosphoribosyltransferase (HPRAT) genes (Schmidt and Migeon, 1990). Although there are cytological data suggesting that the chromosomal region containing the FMR1 gene is abnormally late replicating in affected fragile X males (Wells, 1982; Yu et al., 1990), there are no data on the replication timing of FMR1.

To examine further the possible role of X inactivation imprinting in fragile X syndrome and to test the late replication model for fragile sites, we determined replication timing of FMR1 DNA in cells derived from normal and fragile X males. In addition, several other X-linked loci were examined to determine whether or not fragile X mutations altered replication timing at large distances from FMR1.

Results

Replication Timing Method

We investigated the timing of replication of the FMR1 gene using a novel combination of previously described techniques for determining replication timing. Lymphoblastoid cell lines were pulse labeled with 5-bromodeoxyuridine (BrdU) (Furst et al., 1991) and fractionated into different phases of the cell cycle by flow cytometry, as shown...
Figure 1. Flow Cytometry Profile of BrdU-Labeled Cells
Lymphoblastoid cells were labeled for 1.5 hr in BrdU prior to harvesting. Cells were stained with propidium iodide and fractionated by flow cytometry into six fractions of DNA content corresponding to different compartments of the cell cycle: G1, two quadrants of S (S1, S2, S3, and S4), and G2/M.

The extent of replication for a particular locus was analyzed by quantitative polymerase chain reaction (PCR) of the fractionated DNA using locus-specific primers. The multiplex PCRs contained control primers to normalize replication fractions for differential recovery and amplification. PCR products were separated by gel electrophoresis, transferred to nylon membranes, hybridized to specific probes, and quantified by phosphorimager analysis. Five X-linked loci were examined for replication timing: FMR1, F9, phosphoglycerate kinase (PGK1), glucose-6-phosphate dehydrogenase (G6PD), and X-specific α-satellite sequences (Xα). The sites analyzed in the FMR1 gene included sequences flanking both sides of the CGG repeat in the 5′ CpG island (fmr78 and fmr910) and a region at the 3′ end of the gene (fmr3) (Figure 2). A sequence located 130 kb 5′ of FMR1 (fmr1648) at the DXS548 locus (Riggins et al., 1992) was also analyzed.

Our replication timing method produces results equivalent to other methods based on replication patterns previously established for PGK1 (Goldman, 1989), G6PD (Goldman, 1989), F9 (Goldman, 1989; Schmidt and Mege, 1990), and Xα (Ten Hagen et al., 1993). PGK1 and G6PD replicated early in S, while F9 and Xα replicated late in normal male cells (Figures 3A and 3B; data not shown).

Replication Timing of Normal and Mutant FMR1 Alleles
The cell lines derived from three normal males replicated the fmr910 sequence primarily in the second half of S phase. Two examples are shown in Figures 3A and 3B. The replication pattern of FMR1 is similar to that of the presumably inactive F9 gene (Figure 3B). A similar pattern of replication timing was observed for an FMR1 allele from another normal male whose cultured lymphocytes were examined (data not shown). The replication of an inactive X allele of FMR1 occurs even later than that of an active X homolog, as demonstrated by replication analysis of somatic cell hybrids containing either an active or an inactive X chromosome (data not shown).

The cell lines derived from affected males with methylated, secondary CGG expansions replicated the fmr910 sequence later than did cells from normal males, predominantly in the G2/M fraction (Figures 3A and 3B). The size of the CGG repeat and the methylation status of FmuHI sites in the CpG island were determined by Southern blot analysis (Hansen et al., 1992; data not shown). An allele with a primary expansion from a normal transmitting male was found to replicate in S3 and S4, like FMR1 replication in normal males (Figure 3B). An allele with a CGG repeat

Figure 2. Partial Map of FMR1 Region
Shown are cleavage sites of the specified restriction enzymes (vertical lines), the CGG repeat region (inverted boxes), selected exons (open boxes labeled α35M), and locations of FMR1 PCR products that were utilized for replication timing (fmr78, fmr910, and fmr3). Also shown is the FMR1 product that was used for analysis of the DXS548 locus (46F1R), located 150 kb 5′ of the FMR1 gene.
Abnormal Replication of **FMR1** Region in Fragile X

### Discussion

**X Inactivation Imprinting Model of Fragile X Syndrome**

We have studied replication timing in cell lines derived from four males affected with fragile X syndrome that retain the properties associated with primary cells from such individuals: large CCG expansions in the 5' CpG island of the FMR1 gene and methylation of this island. Replication of the methylated, secondary expression allele of **FMR1** from the affected male occurs later in the cell cycle than does replication of alleles from four normal males that were studied. These data, and our previously pub-

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Figure 3: Replication Timing of **FMR1**, **FGK1**, and **F9** in Normal and Mutant Lymphoblastoid Cell Lines

(A) Replication patterns are shown for **FMR1** and **FGK1** from a normal male lymphoblast (homozygous) and from three different clones derived from affected males with methylated, secondary CCG expansions (C6H8, TL009, and IT17).

(B) Replication timing of **FMR1**, **FGK1**, and **F9** is compared between cells derived from another normal male (F9), from a normal transcribing male with a primary CCG expansion (H-5a), from an affected male with a methylated, secondary CCG expansion (TL016), and from an affected male that contains a predominant subpopulation of cells with an unmethylated, primary expansion-sized CCG repeat (TL007). The **FMR1** locus in the **FMR1** CpG island was assayed in both (A) and (B).

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Figure 4: Comparison of Replication Timing in Lymphoblastoid Cells at Locations 5' and 3' of the CCG Repeat

Replication timing is shown for F9 normal male and TL007 fragile X male at different regions of **FMR1** (lwr/2, lwr/10, and lwr/3) and of a sequence 150 kb 3' of the gene (46F10).

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number of about 200 was isolated in a lymphoblastoid cell line (TL007) from a population of fragile X lymphocytes that were mosaic in methylation and CCG repeat size. This allele was unmethylated at the EagI site and at all Fnu4HI and AciI sites assayed within the CpG island (V. M. L. et al., unpublished data). Although this allele was derived from a male classified as clinically affected (see Figure 2 in Yu et al., 1990), it has the methylation properties of a normal or primary expansion allele and has a CCG repeat size of a large primary expansion allele. Consistent with these properties, the replication timing for the TL007 allele was found to be like that of a normal or primary expansion allele (Figure 3B).

The replication timing of other X-linked genes was also examined to test for possible trans effects of the CCG expansion at distant loci. Replication patterns for **PGK1** in all of the cells containing mutant **FMR1** alleles were similar to those of cells of normal males (Figures 3A and 3B). Replication of **F9**, **G6PD**, and X-inactivation was also normal in the fragile X cells that were examined (Figure 3B; data not shown).

To determine whether the delayed replication observed for alleles with methylated, secondary CCG expansions was the result of a single replication fork stalling at the CCG repeat, we assayed replication at sites both 5' and 3' of the repeat. All four sites (46F10, lwr/2, lwr/10, and lwr/3) consistently replicated late, indicating that replication is delayed over a large region that extends at least 150 kb 5' and 34 kb 3' of the CCG repeat (Figure 4). The late replication of lwr/2 indicates that the delayed replication observed at the 5' sites is not simply the result of a fork traveling from the 5' direction that stalls at the expanded CCG repeat. Similarly, the late replication of lwr/10 and lwr/3 indicates that the late replication of lwr/2 is not the result of a fork traveling from the 3' direction that stalls at the repeat. Therefore, the stalling of a single replication fork at the expanded CCG repeat cannot account for the observed pattern of delayed replication. Furthermore, the delayed replication observed for the 46F10 and lwr/3 sequences, which are separated by 180 kb, suggests that the entire **FMR1** imprint may be altered in fragile X chromosomes.
lished data on the methylation patterns of FMR1 at the 5' CpG island (Hansen et al., 1992), indicate that mutant alleles from affected individuals retain properties of normal inactive X alleles, as predicted by the X inactivation imprinting model (Laible, 1987). It appears to be a novel phenomenon for mutations in a gene to be associated with delayed replication.

The primary expansion allele that was examined appears to be replicated like alleles from normal males. The FMR1 gene in TL007 had properties characteristic of a primary-expansion allele and replicated like a normal or primary expansion allele, although TL007 was derived from an affected fragile X individual. Somatic mosaicism is often observed for CGG repeat size, Cpg island methylation, and FMR1 expression in fragile X patients (Fu et al., 1991; Hansen et al., 1992; Osterre et al., 1991; Pieretti et al., 1991; Yu et al., 1992). Because the lymphocyte DNA of this individual was mosaic in CGG repeat size and methylation (M. M. L. et al., unpublished data), we infer that this allele was derived from a subpopulation of lymphoblasts that was selectively selected by establishment and growth in tissue culture. The number of CGG repeats within this subpopulation, about 200, borders the number found for primary and secondary expansions (Fu et al., 1991; Holtz et al., 1992; Yu et al., 1992). The normal replication of this allele and of one containing a primary expansion from a normal transmitting male suggest that the presence of an unmethylated CGG repeat expansion of up to 600 bp in size is not sufficient for the maintenance of the normally delayed replication that can be detected with our assay. Similarly, an expanded but unmethylated CGG repeat is not sufficient for transcriptional inhibition of FMR1 (Sutcliffe et al., 1992).

It is probable that hypomethylation of the FMR1 CpG island is sufficient to lead to stable transcriptional repression (Garli et al., 1992; Pieretti et al., 1991; Razin and Cedar, 1991; Sutcliffe et al., 1992) and is therefore involved in the etiology of the fragile X syndrome. It is not known, however, whether the methylated state is the initial imprinting signal or whether it is a consequence of another imprinting mechanism, such as the switch from early to late replication. The determination of methylation status and replication timing of FMR1 in the human germline will be important for understanding the mechanism by which the imprinted state of the fragile X mutation is transmitted by females.

Although our data are consistent with predictions at the X inactivation imprinting model, other models are not yet excluded. For example, the CGG expansion could be considered as an abnormal insertion with properties similar to those of an imprinted transgene (Rekhi et al., 1990; Gasaki et al., 1991). The CGG expansion would therefore introduce a recognition site for an imprinting mechanism that is independent of X inactivation imprinting yet results in the methylation of the FMR1 CpG island. Further experiments are necessary to distinguish among these and other hypotheses.

Fragile Sites and Delayed Replication
Our finding of delayed replication at the mutant FMR1 locus also supports the hypothesis that the fragile X site occurs at a region of delayed replication (Lalict et al., 1987). It was predicted that the fragile site could result from a replication-based disturbance in normal chromosome condensation during G2. The replication of secondary expansion alleles of FMR1 occurs later than that of any other locus that we have examined thus far, including the X-autosome sequences that have substantial replication in the G2/M fraction (data not shown; Ton and Hagen, 1990).

The replication timing of four other X-linked loci, PGK1, F9, G6PD, and Xq27, was not affected by the fragile X mutation. It will be of interest to determine the relationship between replication of mutant FMR1 alleles and chromosome condensation, in particular, under conditions of fragile site induction (see Figure 2 in Laird et al., 1987). In addition, our data should encourage future studies of replication timing at other fragile sites.

Late Replication and the Expanded CGG Repeat
Replication of the highly expanded CGG repeat is known to be difficult, resulting in errors in vitro and in vivo with bacterial polymerases (Emera et al., 1992; Fu et al., 1991; Kremer et al., 1991). Difficulty in passage of replication fork through this 100% CGG repeat may explain the cytological manifestation of the fragile site (Fu et al., 1991; Kremer et al., 1991). We have recently shown that the expanded repeat can be highly methylated, particularly in lymphoblastoid cell lines (Hansen et al., 1993). The higher melting temperature of the methylated repeat (Collins and Myers, 1987) would be expected to cause even more difficulty for the passage of replication forks. The normally late replication of FMR1 alleles with methylated, secondary CGG expansions was not, however, the direct result of a stalling at the expanded CGG repeat of a replication fork traveling from a distal or proximal origin. This conclusion follows from the observation that replication timing was delayed on both sides of the repeat, including sequences 150 kb 5' and 34 kb 3' of the expansion.

Mechanisms other than polymerase stalling can be envisioned for the possible involvement of the methylated, secondary CGG expansion in causing a delay in replication over the entire replicon. The CGG expansion could directly inactivate a nearby replication origin, thus switching replication timing against to another, later, origin; the delayed replication could also result from a switch in timing of activation of the normal origin for the active X locus (Laird et al., 1987). Direct repeats have been observed in fractions enriched in eukaryotic replication origins (Opstun et al., 1989). Although most such sequences are A-T rich, repeats with 90% GC were also observed; it is therefore possible that the normal CGG repeat functions as an origin of replication. It will be of interest to determine the location of the replication origin for both the normal and the fragile X FMR1 replicon.

Other than a direct interaction with the replication origin, the CGG repeat could indirectly affect its activity by interaction with a "locus control region." For precedent for this possibility, see in the example of the human beta-globin gene, where deletion of the locus control region, located 60 kb 5' of the gene, results in gene repression, a change in chromatin structure, and a switch to late replication of a region spanning at least 220 kb (Forrester et al., 1989).
The large domain that undergoes switching to late replication on the fragile X chromosome is consistent with the large sizes of other domains that undergo changes in replication timing and transcriptional activity (Brown et al., 1987; Dhar et al., 1988; Forro et al., 1990; Halttunen and Schlipkraut, 1990; Seig et al., 1992; Speck et al., 1992). It is apparent from our data that the minimal domain of delayed replication includes the 180 kb region spanned by the 46F11/F12 and fmr1 sequences. It will be important to map the borders of the abnormal replication domain to determine whether or not multiple replications might be affected.

Our demonstration of delayed replication of a candidate gene for fragile X syndrome adds a new dimension to the genetic and epigenetic basis of this disease. For example, the finding of a large domain of late replication raises the possibility that inactivation of genes in addition to FMR1 could be associated with fragile X syndrome. In some cases of fragile X syndrome, it has been suggested that the duration of replication may extend several Mb to the iduronate sulphatase locus (Clarke et al., 1992). Reports of phenotypic variation support the involvement of other genes in the syndrome (Laird, 1987; Lowery et al., 1982). The possibility should also be considered that other hereditary diseases exist in which transcriptional inhibition is associated with a replication delay.

Experimental Procedures

Cell Culture and BrdU Labeling

Standard growth conditions for cultured cells were as previously described (Hansen et al., 1988). CHO-YH21 is a Chinese hamster ovary cell line (Reichouf and Chaisson, 1975). X0-OS is a Choriogonadotropin-resistant human-hamster hybrid cell line that contains a human inactive X chromosome (Hansen et al., 1988; Hansen and Baer, 1990; Hansen et al., 1989). GM005186 is a human-hamster hybrid cell line containing an inactive human X chromosome that was obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository. Lymphoblastoid cell lines were established by Epstein-Barr transformation of peripheral lymphoblasts, TLO99 and TLO102, derived from a healthy subject with fragile X syndrome, tested positive for the fragile X chromosome (Yu et al., 1985), and were obtained from Dr. S. Wengert (Division of Medical Genetics, Children's Hospital of Philadelphia). Dr. W. B. Brown (Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities) previously provided T25A1, a lymphoblastoid cell line that was derived from a fragile X male who tested positive for the fragile X chromosome (previously refereed to as YU85 by Huang, et al., 1992). Other cell lines were derived from the following individuals: FF and hcm206-5, normal males; TLO97, affected male who tested positive for the fragile X chromosome (Yu et al., 1985); and Dr. B. Rech, an affected male, and H3a, a normal transmitting male carrier.

For BrdU labeling of lymphoblastoid cells, about 3 × 10⁵ cells in exponential growth were incubated with 50 μM BrdU for 60 min and washed with cold phosphate-buffered saline. Cell pellets were kept on ice until stained with propidium iodide for cell sorting. Manipulations of labeled cells and DNA extracts were done under yellow lights or dark ambient light to prevent photolysis of incorporated BrdU. Labeled cell blasts (C10-1H11, X0-OS2, and GM005186) were similarly labeled with BrdU and harvested by trypsinization before they were washed in phosphate-buffered saline; they were then fixed in 70% ethanol for 1 hr, pelleted, and resuspended in 70% ethanol for storage overnight at -20°C before flow cytometry.

Cell Cycle Fractionation

BrdU-labeled cells were separated into different phases of the cell cycle on an EPICS Elite cell sorter. Cells were resuspended in propidium iodide buffer (0.1% Triton X-100, 0.8% NaCl, 21 mM NaCl, 0.6% Nonident P-40, 50 μg of propidium iodide to 1 ml of 19 mg/ml RNase A in 0.8% NaCl at a concentration of 5 × 10⁶ to 1 × 10⁷ cells/ml of buffer and incubated for 30 min at room temperature just prior to sorting. Equal numbers of labeled cells were collected for each of the DNA content ranges by light microscopy and flow cytometry. The DNA content was considered to be 2C. Cells were collected in 1.5 ml microcentrifuge tubes containing 400 μl of 1% propidium iodide buffer (10 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% SDS, 0.2 mg/ml proteinase K, 0.25 mg/ml sheared and denatured salmon tester DNA (according to Vassilev et al., 1990). The fractions were incubated for 2 hr at 20°C and stored at 4°C.

BrdU-labeled DNA from about 4 × 10⁶ exponentially growing CHO-YH21 cells was isolated without cell fractionation. DNA was purified from the cell lysate by phenol and chloroform extraction and ethanol precipitation. DNA concentrations were determined by fluorescence dye binding (Lithner and Pehrson, 1980).

Isolation of Replicated DNA by Immunoprecipitation

Immunoprecipitation of BrdU-labeled DNA was carried out as described previously (Vassilev et al., 1990) with some modifications. BrdU-labeled CHO-YH21 DNA was added to extracts of flow-sorted cells prior to phenol extraction, to control for differential recovery during DNA purification procedures and subsequent PCR efficiency. Thirty nanograms of CHO-YH21 DNA were added for every 10⁷ flow-sorted cells; the same batch of BrdU labeled CHO-YH21 DNA was used for all experiments. The samples were extracted with phenol and chloroform and then precipitated with ethanol. After centrifugation, pellets were washed with 70% ethanol and dried. DNA was dissolved in 400 μl of TE (10 mM Tris- HCl (pH 7.5), 1 mM EDTA) and 10 pl of 3 mg/ml sheared and denatured salmon tester DNA was added. Samples were incubated for 15 to an average size of about 700 bp (range, 250 to 2500) in control experiments) and stained with ethidium bromide for 3 min at 37°C before loading on a gel. Samples were adjusted to 10 mM sodium phosphate (pH 7.0), 0.14 M NaCl, 0.05% Triton X-100 and then incubated with 2 μg of anti-BrdU monoclonal antibody (Hylen, D1582) (LUA) was precipitated by addition of 35 μg of rabbit immunoglobulin G directed against mouse immunoglobulin G (Sigma) and incubation at room temperature for 60 min with constant rotation. Samples were centrifuged for 5 min in a microcentrifuge. Pellets were washed with 750 μl of 10 mM sodium phosphate buffer (pH 7.0), 0.14 M NaCl, 0.25% Triton X-100, resuspended in 250 μl of 60 mM Tris- HCl (pH 0.1), 10 mM EDTA, 0.1% SDS, 0.25 mg/ml proteinase K, and incubated overnight at 37°C. An additional 100 μl of this lysate buffer was added, and samples were incubated 1 hr at 37°C. Yeast RNA (10 μg) was added, and the samples were extracted with phenol and chloroform and ethanol precipitated. The pelleted and dried BrdU DNA was dissolved in 80 μl of TE and stored in a dark box at -20°C.

PCR Amplification of Replicated DNA

The oligonucleotide primer pairs employed for PCR and the sizes of corresponding PCR products are as follows: human PSK1 (Pelletier et al., 1990), C2 (5'-gaggttgagtgtgtagccttac-3'), and D2 (5'-agaagaggcgaaccagacac-3') (253 bp), human factor IX (sequences from HUMF7X, GenBank accession number X24033, KX45 (5'-agccctagtgaagtct-3'), and X25 (5'-ctcaagtgctttgctgctgct-3') (633 bp); human X-gene (sequences from HUMX45, GenBank accession number X00229, GSP500 (5'-gaggagagacaaacgaggctaa-3') and GSP1180 (5'-cagggcagtaacggagtccgac-3') (551 bp); DSSX46 (located 150 bp 6' of FMR1 (Julien et al., 1992), 485 bp containing rctactgctccgtgctcgcg and 48N (5'-agcagctaggagctaggctggctg-3') (1467 bp, about 320 bp); human FMR1 5'TG isoform (sequences from Fu et al., 1991), fmr1 T (5'-tcaaggagagttgtgtgtagct-3') and fmr1 L (5'-cgaggtgtgagttgtagct-3') (fmr1 L) (5'-cgaggtgtgagttgtagcag-3') as well as fmr1 U (5'-cgaggtgtgagttgtagcag-3').
Quantitative PCR Products

Aguere gelet electrophoresis, Southern transfer, hybridization conditions, and membrane washing procedures were all performed as previously described (Harlow et al., 1988). Most probes were generated by PCR amplification. DNA products were obtained by gel electrophoresis in low melting agarose. PCR products in gels were labeled by the random hexanucleotide primer method (Feinberg and Vogelstein, 1984). The 170 bp and 610 bp probes were prepared by using HAPRTI/HAPRTI and HAPRTI/HAP1 with the 170 bp described in this paper and the 610 bp described in this paper, respectively (Hansen et al., 1985). Quantitation of hybridization signals was performed by phosphorimager analysis (Molecular Dynamics; University of Washington Microholography Facility). Band intensities were normalized using results from a standard DNA in the multiplex reactions (170, 610, 250, 500, 250, 500, 170, 610, 170, 2000). Total FAM171 replication signals (the sum of G1, S, G2, and G3/G2 hybridization signals) for cell lines that had predominant peaks of replication in G2 were 2- to 3-fold lower than those that did not. The difference indicates an underestimation of replication per cell in the G2 phase. There was no difference between normal and fragile cell lines in the efficiency of antibody precipitation or PCR amplification of the 170 bp region. Thirty to 50% underrepresentation of replication signals in the G3/G2 peak (170, 610, 250, 500, 250, 500, 170, 610, 170, 2000) was consistent with complete disappearance of signals of the S and G3/G2 components of the G3/G2 peak (Phoenix Flow Systems) that indicates a 2- to 3-fold greater number of cells in G2 than in G3/G2 (data not shown). Underrepresentation of replication signals was also apparent in the G1 and S fractions for replicons that replicate very early, probably because of the large amount of nonreplicating G1 cells in these fractions. These problems have been avoided in some studies by examining equal quantities of BrdU-label DNA in the cell lines for hybridization to specific probes (Brown et al., 1988; Dhar et al., 1989a, 1989b; Spack et al., 1992). However, this method overestimates replication in the early and late fractions because cells in early or late S phase would be expected to contain less total BrdU-label DNA per cell than in the other fractions. Our data are presented without correction because the problem of dilution of BrdU-label DNA by nonreplicating cells.

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