Cell, Vol. 73, 1403–1409, July 2, 1993, Copyright © 1993 by Cell Press

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 CpG 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 (Pieretti et al., 1991) and their 5′ CpG 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 progeny of females with this "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; Kremer 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 germline 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 this "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 proposed 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 (FRAXA; Xq27.3) would prolong replication into what is usually referred to as the G2 phase of the cell cycle (Laird et al., 1987).

Cytological studies of the inactive X chromosome indicate that its replication is delayed relative to that of the active X homolog and the autosomes (Atkins et al., 1962; Lima-de-Faria et al., 1961; Taylor, 1960). In general, transcriptional inactivity is often associated with replication of genes late in S phase (Goldman et al., 1984; Hatton et al., 1988). 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 (*HPRT*) 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 (Webb, 1992; 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-bromo-2′-deoxyuridine (BrdU) (Furst et al., 1981) and fractionated into different phases of the cell cycle by flow cytometry, as shown

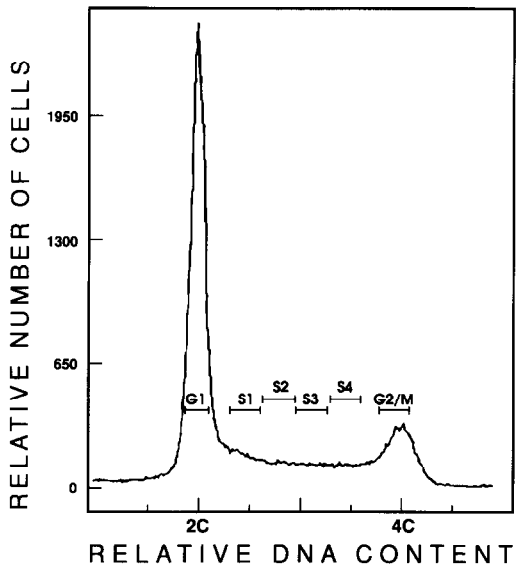

Figure 1. Flow Cytometry Profile of BrdU-Labeled Cells

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, four quarters of S (S1, S2, S3, and S4), and G2/M.

in Figure 1 (Gilbert and Cohen, 1987; Ten Hagen et al., 1990). Equal numbers of fractionated cells were collected for each of six DNA content ranges, including the G1 and G2/M peaks and four fractions of S phase. Our S phase fractions were similar in cellular DNA content to those of previously described methods (for example, see Furst et al., 1981). Replicated DNA was purified from these fractions by immunoprecipitation with an anti-BrdU antibody (Gale et al., 1992; Vassilev et al., 1990).

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 (X-α). 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 150 kb 5′ of *FMR1* (46F/R) 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, 1988), *G6PD* (Goldman, 1988), *F9* (Goldman, 1988; Schmidt and Migeon, 1990), and X–α (Ten Hagen et al., 1990). *PGK1* and *G6PD* replicated early in S, while *F9* and X-α 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 like 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 blood 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 Fnu4HI 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

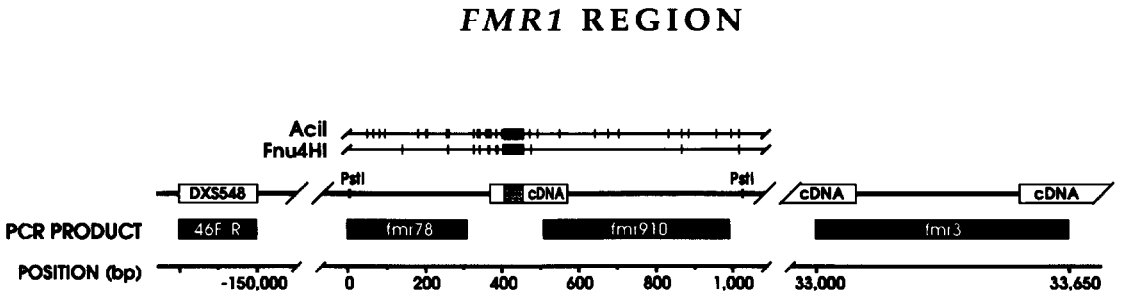

Figure 2. Partial Map of *FMR1* Region

Shown are cleavage sites of the specified restriction enzymes (vertical lines), the CGG repeat region (inverted boxes), selected exons (open boxes labeled cDNA), and locations of *FMR1* PCR products that were utilized for replication timing (fmr78, fmr910, and fmr3′). Also shown is the PCR product that was used for analysis of the DXS548 locus (46F/R), located 150 kb 5′ of the *FMR1* gene.

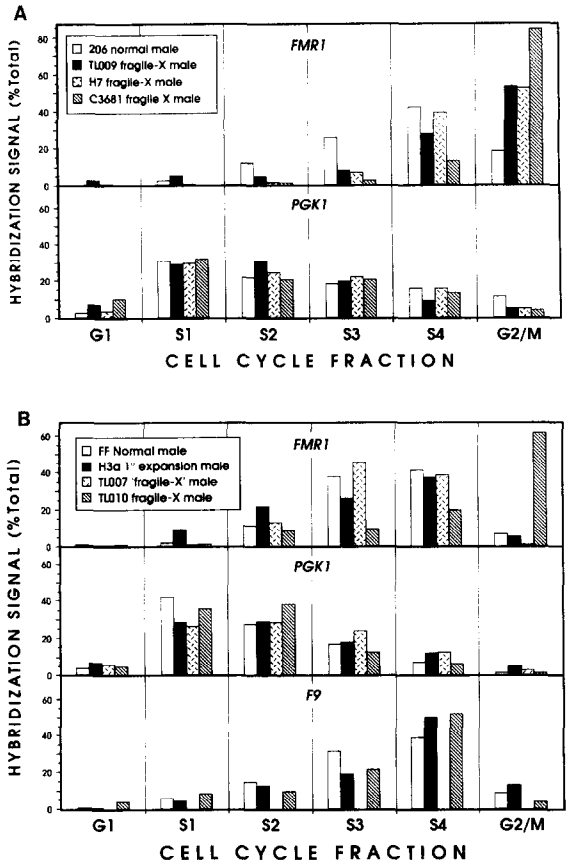

Figure 3. Replication Timing of *FMR1*, *PGK1*, and *F9* in Normal and Mutant Lymphoblastoid Cell Lines

(A) Replication patterns are shown for *PGK1* and *FMR1* from a normal male cell line (hcm206-5) and from three cell lines derived from affected males with methylated, secondary CGG expansions (C3681, TL009, and H7).

(B) Replication timing of *FMR1*, *PGK1*, and *F9* is compared between cells derived from another normal male (FF), from a normal transmitting male with a primary CGG expansion (H3a), from an affected male with a methylated, secondary CGG expansion (TL010), and from an affected male that contain a predominant subpopulation of cells with an unmethylated, primary expansion-sized CGG repeat (TL007). The fmr910 locus in the *FMR1* CpG island was assayed in both (A) and (B).

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 CGG repeat size. This allele was unmethylated at the EagI site and at all Fnu4HI and AciI sites assayed within the CpG island (M. 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 CGG 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 CGG 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-α 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 CGG expansions was the result of a single replication fork stalling at the CGG repeat, we assayed replication at sites both 5′ and 3′ of the repeat. All four sites (46F/R, fmr78, fmr910, and fmr3′) 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 CGG repeat (Figure 4). The late replication of fmr78 indicates that the delayed replication observed at the 3′ sites is not simply the result of a fork traveling from the 5′ direction that stalls at the expanded CGG repeat. Similarly, the late replication of fmr910 and fmr3′ indicates that the late replication of fmr78 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 CGG repeat cannot account for the observed pattern of delayed replication. Furthermore, the delayed replication observed for the 46F/R and fmr3′ sequences, which are separated by 180 kb, suggests that the entire *FMR1* replicon may be altered in fragile X chromosomes.

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 CGG expansions in the 5′ CpG island of the *FMR1* gene and methylation of this island. Replication of the methylated, secondary expansion alleles of *FMR1* from the affected males occurs later in the cell cycle than does replication of alleles from four normal males that were studied. These data, and our previously pub-

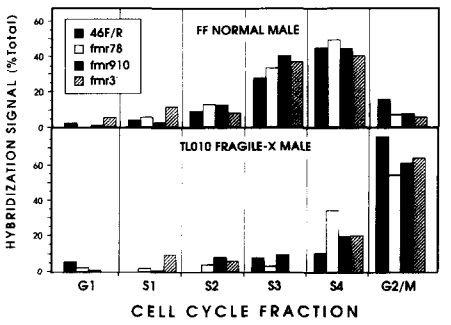

Figure 4. Comparison of Replication Timing in Lymphoblastoid Cells at Locations 5′ and 3′ of the CGG Repeat

Replication timing is shown for FF normal male and TL010 fragile X male at different regions of *FMR1* (fmr78, fmr910, and fmr3′) and at a sequence 150 kb 5′ of the gene (46F/R).

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 (Laird, 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; Oberlé 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 isolated 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; Heitz 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 suggests that the presence of an unmethylated CGG repeat expansion of up to 600 bp in size is not sufficient for the maintenance of the markedly 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 hypermethylation of the *FMR1* CpG island is sufficient to lead to stable transcriptional repression (Gartler 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 of 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 (Reik et al., 1990; Sasaki 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 (Laird 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-α sequences that have substantial replication in the G2/M fraction (data not shown; Ten Hagen et al., 1990). The replication timing of four other X-linked loci, *PGK1*, *F9*, *G6PD*, and X-α, 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, particularly 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 (Erster et al., 1992; Fu et al., 1991; Kremer et al., 1991). Difficulty in passage of replication forks through this 100% CG region 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., 1992). 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 abnormally 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 control 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 (Opstelten et al., 1989). Although most such sequences are AT-rich, repeats with 60% 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." Precedent for this possibility is found in the example of the human β-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., 1990).

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., 1989; Forrester et al., 1990; Hatton and Schildkraut, 1990; Selig et al., 1992; Spack et al., 1992). It is apparent from our data that the minimal domain of delayed replication includes the 180 kb region spanned by the 46F/R and fmr3′ sequences. It will be important to map the boundaries of the abnormal replication domain to determine whether or not multiple replicons 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 domain of repression 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; Loesch et al., 1992). 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 (Rosenstraus and Chasin, 1975). X8-6T2 is a 6-thioguanine-resistant human–hamster hybrid cell line that contains a human inactive X chromosome (Hansen et al., 1988; Hansen and Gartler, 1990; Hansen et al., 1992). GM06318B is a human–hamster hybrid cell line containing an active 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. TL009 and TL010, derived from males affected with fragile X syndrome, tested positive for the fragile X chromosome (Yu et al., 1990) and were obtained from Dr. S. Wenger (Division of Medical Genetics, Children's Hospital of Pittsburgh). Dr. W. T. Brown (Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities), provided C3681, a lymphoblastoid cell line that was derived from a fragile X male who tested positive for the fragile X chromosome (previously referred to as 3681 by Hansen et al., 1992). Other cell lines were derived from the following individuals: FF and hcm206-5, normal males; TL007, affected male who tested positive for the fragile X chromosome (I-1 in Figure 2C of Yu et al., 1990); H7, an affected male; and H3a, a normal transmitting male carrier.

For BrdU labeling of lymphoblastoid cells, about 3×10^7 cells in exponential growth were incubated with 50 μM BrdU for 90 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. Fibroblast cells (CHO-YH21, X8-6T2, and GM06318B) 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 4°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 staining buffer (9 vol of 40 mM Tris–HCl [pH 7.4], 0.8% NaCl, 21 mM $MgCl_2$, 0.05% Nonidet P-40, 50 μg of propidium iodide to 1 vol of 10 mg/ml RNAase A in 0.8% NaCl) at a concentration of 5×10^6 to 1×10^7 cells per ml of buffer and incubated for 30 min at room temperature just prior to sorting. Equal numbers of fractionated cells were collected for each of six DNA content ranges, including the G1 and G2/M peaks and four intermediate fractions of S (in different experiments, the number of cells collected per fraction ranged from 20,000 to 40,000). Typical ranges of cellular DNA content for each fraction are illustrated in Figure 1 and were estimated to be: G1, 1.86 C to 2.09 C; S1, 2.30 C to 2.60 C; S2, 2.62 C to 2.94 C; S3, 2.95 C to 3.26 C; S4, 3.29 C to 3.59 C; G2/M, 3.77 C to 4.07 C. DNA content was calculated relative to the fluorescent signal of cells at the G1 peak, whose DNA content was considered to be 2 C. Cells were collected in 1.5 ml microfuge tubes containing 400 μl of lysis buffer (1 M 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 testes DNA [according to Vassilev et al., 1990]). The fractions were incubated for 2 hr at 50°C and stored at −20°C.

BrdU-labeled DNA from about 4×10^7 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 fluorescent dye binding (Labarca and Paigen, 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 10^4 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 460 μl of TE (10 mM Tris–HCl [pH 7.5], 1 mM EDTA), and 40 μl of 5 mg/ml sheared and denatured salmon testes DNA was added. Samples were sonicated for 15 s to an average size of about 700 bp (range, 250 bp to 2 kb in control experiments) and then heat denatured for 3 min at 95°C before cooling on ice. 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 (Becton-Dickinson Immunocytometry) at room temperature for 20 min with constant end over end rotation. Antibody-bound BrdU DNA was precipitated by addition of 35 μg of rabbit immunoglobulin G directed against mouse immunoglobulin G (Sigma) and incubation at room temperature for 20 min with constant rotation. Samples were centrifuged for 5 min in a microcentrifuge. Pellets were washed once in 750 μl of 10 mM sodium phosphate buffer (pH 7.0), 0.14 M NaCl, 0.05% Triton X-100, resuspended in 200 μl of 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, 0.25 mg/ml proteinase K, and incubated overnight at 37°C. An additional 100 μl of this lysis buffer was added, and samples were incubated 1 hr at 50°C. Yeast tRNA (20 μg) was added, and the samples were extracted with phenol and chloroform and ethanol precipitated. The pelleted and dried BrdU DNA was dissolved in 50 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 *PGK1* (Pfeifer et al., 1990), C2 (5′-gggttggggttgcgccttttccaa-3′) and D2 (5′-acgccgcgaaccgcaaggaacct-3′) (223 bp); human factor IX (sequences from HUMFIXG, GenBank accession number K02402), IXU5 (5′-aggcctcactcttgctagttcct-3′) and IXU5R (5′-tggtgtttgggatgcctctccat-3′) (463 bp); human X–α (Warburton et al., 1991), X-3A (5′-ataatttcccataactaaacaca-3′) and X-4A (5′-tgtgaagataaaggaaaaggctt-3′) (533 bp); human *G6PD* gene (sequences from HUMG6PDGEN, GenBank accession number X55448), G6PDD (5′-gcagcagtggggtgaaaatacg-3′) and PD9 (5′-tgcaggccaacaatgtggtcct-3′) (864 bp); DXS548, located 150 kb 5′ of *FMR1* (Riggins et al., 1992), 46F (5′-gtacattagagtcacctgtggtgc-3′) and 46R (5′-agagcttcactatgcaatggaatc-3′) (46F/R: about 200 bp); human *FMR1* CpG island (sequences from Fu et al., 1991), fmr7U.1 (5′-gcagaaatgggcgttctggccct-3′) and fmr8L.1 (5′-cggccctccaccggaagtgaaac-3′) (fmr78: 323 bp), as well as fmr9U.1 (5′-ggctgaagagaagatggaggagc-3′)

and fmr10L.2 (5′-ggatcccgctgggagatgatgtttag-3′) (fmr910: 451 bp); human or hamster 3′ *FMR1* (sequences from Verkerk et al., 1991), fmr6904 (5′-tctgaccacagagacgaactcag-3′) and fmr8616 (5′-aagggatccatctgttgttcttcc-3′) (fmr3′: human, 650 bp; hamster, 1.3 kb); human or hamster 28S rRNA gene (sequences from HUMRGM, GenBank accession number M11167), 28S1U.2 (5′-aactcacctgccgaatcaactagccc-3′) and 28S2L.2 (5′-ctaggcttcaaggctcaccgcagc-3′) (human, 301 bp; hamster, 200 bp); hamster adenine phosphoribosyltransferase gene (sequences from HAMAPRTG, GenBank accession number X03603), HAPRT-1 (5′-gagccagaaatccaaaagggtgc-3′) and HAPRT-1R (5′-tcagcaggctggggtcatacca-3′) (HAPRT-1/1R: 282 bp).

PCR parameters for multiplex reactions were one of the following: HAPRT-1–HAPRT-1R and C2–D2 or fmr7U.1–fmr8L.1 and 28S1U.2–28S2L.2: 95°C for 5 min, 25 cycles of 95°C for 1 min, 65°C for 1 min, 72°C for 2 min, and final extension at 72°C for 7 min; HAPRT-1–HAPRT-1R and IXU5–IXU5R or fmr9U.1–fmr10L.2 and 28S1U.2–28S2L.2 or fmr6904–fmr8616 and IXU5–IXU5R: 94°C for 5 min, 25 cycles of 94°C for 1 min, 60° for 30 s, 72°C for 2 min, and final extension at 72°C for 7 min; or X3A–X4A and HAPRT-1–HAPRT-1R or 46F–46R and HAPRT-1–HAPRT-1R or fmr6904–fmr8616 and G6PDD–PD9: 94°C for 5 min, 7 cycles of 94°C for 1 min, 60°C for 1.5 min, 72°C for 1.5 min, 18 cycles of 94°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min, and final extension at 72°C for 7 min. All reactions contained 2.5 U of AmpliTaq (Perkin–Elmer Cetus) and the uncorrected DNA equivalent of 2000 sorted cells in 100 μl of standard reaction buffer (Perkin–Elmer Cetus). X3A–X4A, 28S1U.2–28S2L.2, and IXU5–IXU5R reactions contained 0.5 μM primers; all other reactions contained 0.2 μM primers. Reactions with fmr7U.1–fmr8L.1 primers contained 5% dimethylsulfoxide; fmr9U.1–fmr10L.2 reactions contained 10% dimethylsulfoxide.

Quantitation of PCR Products

Agarose gel electrophoresis, Southern transfer, hybridization conditions, and membrane washing procedures were as previously described (Hansen et al., 1992). Most probes were obtained by PCR amplification of genomic DNA; products were isolated by gel electrophoresis in low melting agarose. PCR products in agarose were labeled by the random hexanucleotide priming method (Feinberg and Vogelstein, 1984). The fmr78 and fmr910 products were probed with the previously described fmr7/8 and fmr9/10 PCR products, respectively (Hansen et al., 1992). Quantitation of hybridization signals was performed by phosphorimager analysis (Molecular Dynamics; University of Washington Phosphorimager Facility). Band intensities were normalized using products from hamster DNA present in the multiplex reactions (28S1U.2/28S2L.2, HAPRT-1/1R, or fmr3′).

Total *FMR1* replication signals (the sum of G1, S1, S2, S3, S4, and G2/M hybridization signals) for cell lines that had predominant peaks of replication in G2/M were 2- to 3-fold lower than those that did not. This difference indicates an underestimation of replication per cell in the G2/M fraction. There was no difference between normal and fragile X alleles in the efficiency of antibody precipitation or PCR amplification of the fmr910 region. The 2- to 3-fold underrepresentation of G2 replication is likely to be the result of dilution with cells that completed S before the BrdU labeling period. This extent of underrepresentation is consistent with computer-generated profiles of the S and G2/M components of the G2/M peak (Multicycle program, Phoenix Flow Systems) that indicate a 2- to 3-fold greater number of cells in G2/M than in S (data not shown). Underrepresentation of replication was also apparent in the G1 and S1 fractions for loci 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-labeled DNA in the cell fractions for hybridization to specific probes (Brown et al., 1987; Dhar et al., 1989; Forrester et al., 1990; Hatton and Schildkraut, 1990; 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-labeled DNA per cell than in the other fractions. Our data are presented without correction for the probable dilution of BrdU-labeled DNA by nonreplicating cells.

Acknowledgments

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We thank Alan Fjeld for preparation of primers and probes and for performance of gel electrophoresis and Southern hybridization analysis of PCR products from replicated DNA, S. Chong Kim for cell fractionation by flow cytometry, and Peter Rabinovitch for helpful discussions. PCR primers fmr6904 and fmr8616 were kindly provided by David Nelson. We thank W. Ted Brown and Sharon Wenger for providing some of the lymphoblastoid cell lines.

This work was supported by grants from the National Institutes of Health (NIH) (HD16659 and GM19179), the National Science Foundation (Eukaryotic Genetics), and the Joseph P. Kennedy, Jr., Foundation. S. M. G. is a recipient of an NIH Career Award.

Received March 29, 1993; revised May 7, 1993.

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