Association of Fragile X Syndrome with Delayed Replication of the \textit{FMR1} Gene

R. Scott Hansen,* Theresa K. Canfield,* Mary M. Lamb,† Stanley M. Gartier,* and Charles D. Laird†‡
*Department of Medicine
†Department of Zoology
‡Department of Genetics
University of Washington Seattle, Washington 98195
‡Program in Molecular Medicine
Fred Hutchinson Cancer Research Center Seattle, Washington 90104

Summary

The fragile X syndrome is commonly associated with mutant alleles of the \textit{FMR1} gene that are hypermethylated and have large expansions of CGG repeats. We present data here on the replication timing of \textit{FMR1} that confirm predictions of delayed replication of alleles from affected males. The normal \textit{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 \textit{FMR1} may be affected.

Introduction

The gene associated with the fragile X syndrome, \textit{FMR1}, has the properties of an X-linked housekeeping gene that is subject to X chromosome inactivation (Gartier et al., 1992). \textit{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 \textit{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 loci of sequence instability have been characterized at the \textit{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 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 predicts 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 \textit{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 \textit{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 \textit{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 \textit{FMR1}.

Results

Replication Timing Method

We investigated the timing of replication of the \textit{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.
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.

Our replication timing method produces results equivalent to other methods based on replication patterns previously established for PGK7 (Goldman, 1988), G6PD (Goldman, 1988), F9 (Goldman, 1988, Schmidt and Migeon, 1990), and X-a (Ten Hagen et al., 1990). PGK1 and G6PD replicated early in S, while F9 and X-a 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

**FMR1 REGION**

![Figure 2. Partial Map of FMR1 Region](image)

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.
Abnormal Replication of FMR1 Region in Fragile X

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 (C3661, 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 contains 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).

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-a 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-
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 (Gartier 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-a 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-a, 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 sequenced 160 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 euchromatic 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; Seil 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 idurionate 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 (Rosenstrauss and Chasin, 1975). X-4B2 is a 6-thioguanine-resistant human–hamster hybrid cell line containing a human inactive X chromosome (Hansen et al., 1988; Hansen and Gartner, 1990; Hansen et al., 1992). GM0631B 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, male; and H3a, a normal transmitting male carrier.

For BrdU labeling of lymphoblastoid cells, about 3 × 10^6 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. Manipulation of labeled cells and DNA extracts were done under yellow lights or in the dark ambient light to prevent photolysis of incorporated BrdU. Fibroblasts (CHO-YH21, X-4B2, and GM0631B) were similarly labeled with BrdU and harvested by trypsinization before they were 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 ethanol precipitated. 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. Anti-labeled CHO-YH21 DNA was added to extracts of five 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' 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 stored in a dark box at -20°C.

BrdU-labeled DNA from about 4 × 10^6 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.

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'ggtgctccgctgggtcc-3') and D2 (5'acccggcaacccagcggcgtc-3') (233 bp); human factor IX (sequences from HUMFIX, GenBank accession number K02402), IXUS (5'gtgctcgtcgtctggctcctcct-3') and IXUR (5'cgtctcgtcgtcgtcgtcgtcgtc-3') (46F/R: about 200 bp); human normal X-α (Warburton et al., 1991), X-3A (5'tgtaaatctgtactacttaaacta-3') and X-4A (5'tgtggataatctatcagagcggc-3') (533 bp); human G6PD gene (sequences from HUMG6PDGEN, GenBank accession number X54446), G6PDD (5'gcagccagggctgatcatac-3') and PDR (5'gcagggcaatcagcgtggtccta-3') (884 bp); DXS548, located 150 kb 5' of FMR1 (Riggins et al., 1990), and 46F/R: about 200 bp; human FMR1 Cpg island (sequences from Fu et al., 1991), C7U1 (5'gcagagctgtgctgctgct-3') and fmr1L1 (5'ggttcatcaggtagtaacc-3') (786 bp); and as fmr9U1 (5'gcagatgctgctgctgctgctgct-3').
and fmr10L.2 (5'-gacctcggatctttgtatga-3') and 28S1U.2 (5'-tagccc-3') (human, 301 bp; hamster, 200 bp); hamster adenine phosphoribosyltransferase gene (sequences from HAMAPRT1, GentBank accession number X03863), HAPRT-1 (5'-gacgatcagacccaggtggc-3') and HAPRT-1R (5'-tcccaggtggatgtaccgc-3') (HAPRT-1/R: 282 bp).

PCR parameters for multiplex reactions were one of the following: HAPRT-1/HAPRT-1R and C2-D2 or fmr7U.1-fmr8618 and 28S1U.2-28S2L.2; 95°C for 5 min, 25 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 1.5 min; or X3A-X4A and HAPRT-1/HAPRT-1R or 46F-46R and HAPRT-1/HAPRT-1R or fmr6904-fmr8616 and G6PD-PDR: 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) and the uncured DNA equivalent of 2000 sorted cells in 100 μl of standard reaction buffer (Perkin-Elmer Cetus).

Quantiﬁcation 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 fmr78 and fmr10 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, fmr6904-fmr8616 and G6PD-PDR: 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) and the uncured DNA equivalent of 2000 sorted cells in 100 μl of standard reaction buffer (Perkin-Elmer Cetus).

Total FMR1 replication signals (the sum of Gl, S1, S2, S3, and 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 replication in the G2/M fraction is likely to be the result of dilution with cells that completed S phase before the Brdu labeling period. This extent of underreplication is consistent with computer-generated profiles of the S and G2/M components of the G2/M peak (Multiplicity program, Phenix Flow Systems) that indicate a 2- to 3-fold greater number of cells in G2/M than in S (data not shown). Underreplication of replication was also apparent in the G1 and S1 fractions for cell lines that had predominant peaks of replication in early S/M, but those that did not show this. Therefore, this difference may be due to 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 S 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

We thank Alan Fjeld for preparation of primers and probes and for performance of gel electrophoresis and Southern hybridization analysis of PCR products from replicating DNA. S. Chong Kim for cell fractionation by flow cytometry, and Peter Rabínovitch for helpful discussions. PCR primers fmrd904 and fmrd8616 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.

References


mids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. Cell 50, 59–68.


