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A variable domain of delayed replication in *FRAXA* fragile X chromosomes: X inactivation-like spread of late replication

(replication timing/*FMR1*/*FMR2*/*FRAXE*)

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ABSTRACT **The timing of DNA replication in the Xq27 portion of the human X chromosome was studied in cells derived from normal and fragile X males to further characterize the replication delay on fragile X chromosomes. By examining a number of sequence-tagged sites (STSs) that span several megabases of Xq27, we found this portion of the normal active X chromosome to be composed of two large zones with different replication times in fibroblasts, lymphocytes, and lymphoblastoid cells. The centromere-proximal zone replicates very late in S, whereas the distal zone normally replicates somewhat earlier and contains *FMR1*, the gene responsible for fragile X syndrome when mutated. Our analysis of the region of delayed replication in fragile X cells indicates that it extends at least 400 kb 5′ of *FMR1* and appears to merge with the normal zone of very late replication in proximal Xq27. The distal border of delayed replication varies among different fragile X males, thereby defining three replicon-sized domains that can be affected in fragile X syndrome. The distal boundary of the largest region of delayed replication is located between 350 and 600 kb 3′ of *FMR1*. This example of variable spreading of late replication into multiple replicons in fragile X provides a model for the spread of inactivation associated with position-effect variegation or X chromosome inactivation.**

FMR1, the gene responsible for fragile X syndrome when mutated, is a widely expressed gene that replicates in the second half of S in at least two expressing cell types (1, 2) and is located in Xq27.3, a late-replicating G band. Thus, *FMR1* does not conform to the rule that widely expressed genes are located in R bands (3), and it is also an exception to the general finding that expressed genes replicate in the first half of S (4, 5). In most males affected with fragile X syndrome, the 5′ CpG island of *FMR1* is hypermethylated and there is a large expansion in the number of CGG repeats in this region; these conditions are associated with gene repression and the presence of the *FRAXA* fragile site (6, 7). The transcriptional silencing by methylation of mutant fragile X alleles of *FMR1* on the active X (8, 9) is quite similar to the normal silencing of the inactive X allele in female cells (10, 11). Mutant alleles in affected individuals also share with the normal inactive X allele the feature of delayed replication compared with the normal active X allele (1, 12). We previously found the domain of delayed replication on the active X chromosome in fragile X patients to be quite large (at least 180 kb), but the borders of this domain were not known (1). A large domain of delayed replication was also inferred from fluorescence *in situ* hybridization (FISH)-based replication studies (12, 13).

To investigate this replication delay further, we studied replication timing, using 5-bromo-2-′deoxyuridine (BrdUrd) incorporation, for multiple sequence-tagged sites (STSs) that span over 6 Mb of the region in normal and fragile X cells. This method of assessing replication timing provides a sensitive molecular approach that is generally applicable to the study of large chromosomal domains.

MATERIALS AND METHODS

BrdUrd Labeling, Flow Cytometry, Immunoprecipitation, and DNA Isolation. Epstein–Barr virus-transformed lymphoblastoid cell lines, fibroblasts, and mitogen-stimulated lymphocytes obtained from human individuals were used for this study. Fragile X cell lines were described previously (1) or were obtained similarly. Fragile X fibroblast cultures (4026 and 7730) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository. Standard procedures for cell culture, BrdUrd labeling of newly replicated DNA, cell cycle fractionation by flow cytometry, and isolation of newly replicated DNA were previously described (1, 2, 14). Peripheral lymphocyte cultures (10^6 cells per ml of RPMI medium 1640 plus 15% fetal bovine serum) were stimulated to divide by the addition of phytohemagglutinin to the medium (15) 3 days prior to labeling with BrdUrd. In higher-resolution replication profiles, cells from eight fractions of S were collected instead of four (16).

PCR Analysis of Replicated DNA. Replication timing was determined for different regions of Xq27 using several STSs described previously, including those for VK14/*DXS292* (18), VK23/*DXS297* (18), sWXD1449 (Genome Data Base accession number 1296921), sWXD616/*DXS7857* (Genome Data Base accession number 600868), M759/*DXS532* (21), 46F:R/*DXS548* (1, 22), G9L (23), 5′ *FMR1* (1, 2), 141R/*DXS465* (23), M749/*DXS533* (21), sWXD1208/*FMR2* (Genome Data Base accession number 1238739), and VK21C/*DXS296* (24). The STS for AFM224/*DXS998* (20) was modified by replacing the forward primer, AFM224zg11a, with dxs998-1U, whose sequence is 5′-CATCACAGCAATTTTTCAAAGG-3′ (from GenBank accession no. Z17020). Hamster (28S rRNA and *Aprt* genes) STSs used to analyze added control BrdUrd-labeled DNA (BrdUrd-DNA) were described previously (1, 2). Because mitochondrial DNA replication is independent of the cell cycle (17), a mitochondrial STS was used as an internal control for recovery and amplification in some cases: mto8000–8024, 5′-GACAATCGAGTAGTACTCCCGATTG-3′:mto8369–8345,

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Abbreviations: STS, sequence-tagged site; FISH, fluorescence *in situ* hybridization; BrdUrd, 5-bromo-2′-deoxyuridine; L, late; VL, very late.

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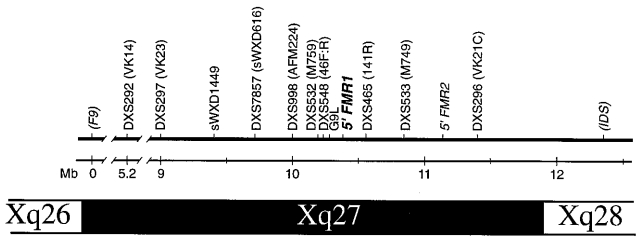

FIG. 1. Physical map of the late-replicating G band, Xq27. STSs used for replication timing were placed according to contig mapping data (ref. 25 and S.M., unpublished observations).

5′-GCATTTCACTGTAAAGAGGTGTTGG-3′ (designed from GenBank sequence HUMMTCG, accession no. J01415). PCR mixtures contained 2.5 units of AmpliTaq (Perkin–Elmer Cetus) and a portion of antibody-purified BrdUrd-DNA corresponding to 1000 sorted cells in 100 μl of standard reaction buffer (Perkin–Elmer Cetus; 1.5 mM $MgCl_2$). Reaction components were assembled on ice and thermocycling was initiated by placing the tubes into a water-cooled thermocycler (Ericomp) maintained at the denaturation temperature (94°C or 95°C). General PCR parameters for STSs have been described previously (1, 2, 14). Annealing temperatures used for specific primer sets, variant reaction buffer components, primer sequences, and STS sizes are available from the corresponding author upon request. Cycling parameters for mto8000–8024:mto8369–8345 are as follows: 94°C for 5 min, 21 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 2 min, and final extension at 72°C for 7 min. Agarose gel electrophoresis of PCR products, Southern transfer, probe isolation, probe labeling, hybridization conditions, and membrane washing procedures were as previously described (1, 10).

RESULTS

To better understand the replication defect in fragile X cells, replication timing was examined in normal and fragile X cells at several STSs mapped previously within the Xq27 region (Fig. 1). The time of replication was determined by isolation and analysis of BrdUrd-DNA obtained from cells that had been pulse-labeled *in vivo* and sorted by flow cytometry into different cell cycle stages (1, 2). Cells are sorted into G_1, G_2/M, and four fractions of S or, for higher resolution analyses, into

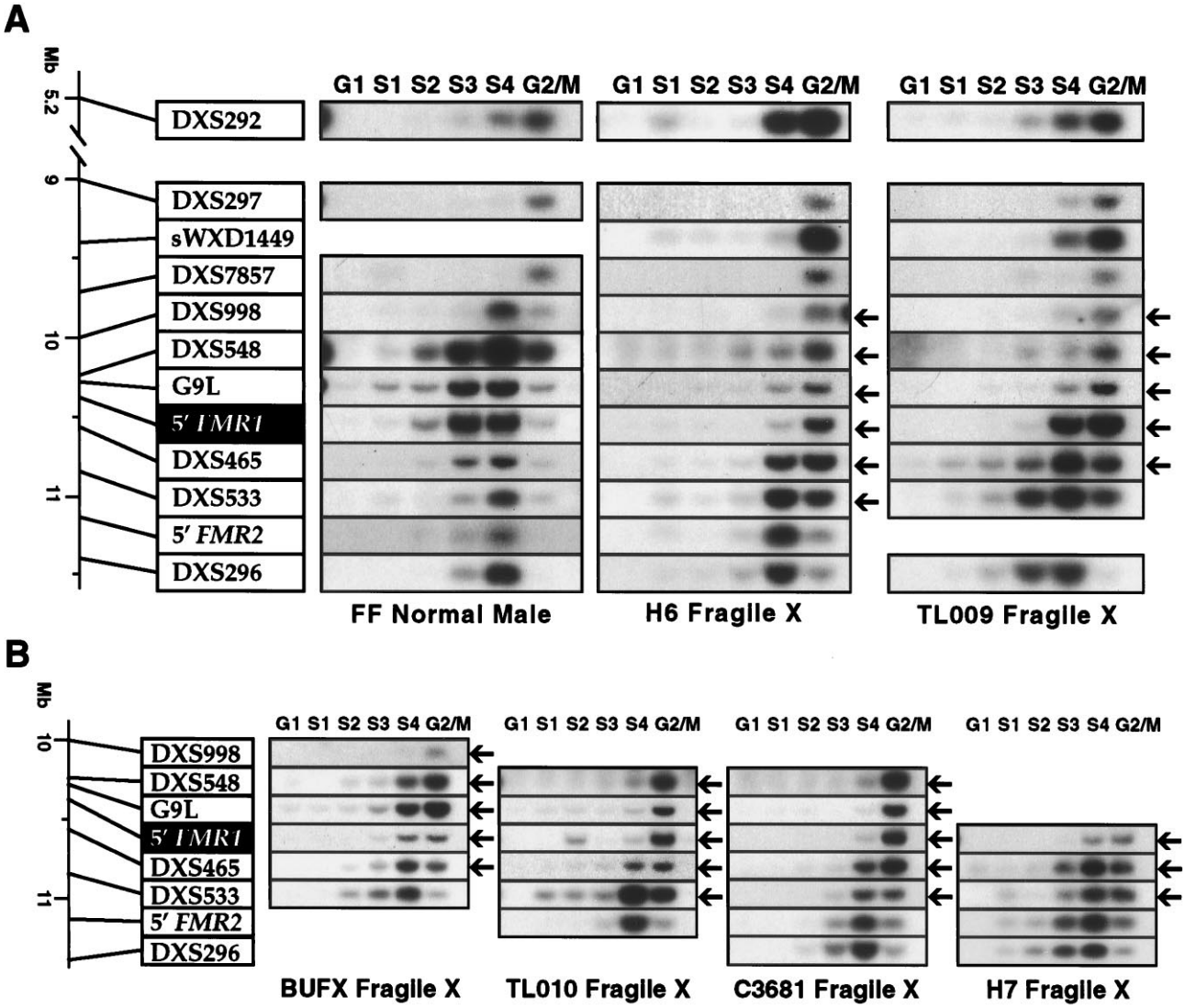

FIG. 2. Replication timing within Xq27 in normal and fragile X males. Replication patterns of several STSs within Xq27 are shown for a normal male lymphoblastoid cell line (FF) and two fragile X lymphoblasts (H6 and TL009). (*A*) Membrane hybridization signals are derived from BrdUrd incorporation into the analyzed locus at different portions of the cell cycle that correspond to flow cytometry fractions G_1, four fractions of S (S1, S2, S3, and S4), and G_2/M. Arrows designate regions that have a strong delay in replication. (*B*) Other examples of variability in the distal border of delayed replication in fragile X lymphoblasts.

eight fractions of S. Newly replicated DNA from the sorted cells is separated from the unlabeled DNA by using an anti-BrdUrd antibody; this BrdUrd-DNA is then analyzed by semiquantitative PCR to determine replication profiles for various STS loci. The cell types studied were derived from males and included mitogen-stimulated peripheral lymphocytes, Epstein–Barr virus-transformed lymphoblastoid cells, and cultured skin fibroblasts.

Multiple Replication Timing Domains in Xq27. Xq27 STS replication in normal male lymphoblasts occurs in the second half of S, consistent with the observation that Xq27 is a late-replicating band (Fig. 2*A*: FF normal male). In this region, however, two different replication timing zones were observed: a centromere-proximal zone of about 4.5 Mb [*DXS292* to *DXS7857*; called the VL (very late) zone], in which replication occurs in the last quarter of S and in the G_2/M cytometry fractions, and an adjacent, distal zone of about 1.5 Mb [*DXS998* to *DXS296*; called the L (late) zone] that replicates somewhat earlier (more S3 and less G_2/M replication). The L zone contains *FMR1* and *FMR2*; *FMR2* contains a 5′ CGG repeat that is expanded and methylated in individuals with the *FRAXE* fragile site. Similar replication patterns were observed in mitogen-stimulated lymphocytes from two normal males and in lymphoblast cell lines from three other normal males (Fig. 3*B* and data not shown). Assuming that replicon sizes in the Xq27 region are in the range of 50–330 kb (26), the proximal and distal replication time zones must each contain several coordinately regulated replication origins.

Xq27 Replication in FRAXA Fragile X Lymphoblasts, Lymphocytes, and Fibroblasts. Cells derived from fragile X patients were previously found to have delayed replication in a 180-kb region of Xq27.3 containing the *FMR1* gene (1). Although present on the active X chromosome, this region replicates in very late S and in G_2/M in fragile X cells at about the same time as the normal inactive X allele in female cells (1, 2). To better understand the nature and origins of this phenomenon in fragile X, we have mapped further the region of delayed replication in cells from affected males. The replication profiles from two lymphoblastoid cell lines derived from fragile X males, H6 and TL009, are shown in Fig. 2*A*. Arrows mark the regions that are delayed relative to FF and other normal males. The border between the VL and L zones has been shifted in the two fragile X cases such that the VL zone now includes the *FMR1* locus. The location of the new distal border of the VL zone differs between H6 and TL009 by approximately 200 kb in that the VL zone of H6 includes *DXS533*, whereas that of TL009 does not. Such variability was also found among several other fragile X lymphoblasts (Fig. 2*B*). In BUFX, the VL zone includes *DXS465* (as in TL009), whereas the VL zone extends to include *DXS533* in TL010, C3681, and H7 (as in H6).

The fragile X replication delay was also examined in untransformed cell cultures derived from affected fragile X males. In two fragile X fibroblast cultures, 4026 and 7730, replication was delayed at *FMR1* relative to normal cells (Fig. 3*A*). The difference in replication timing between affected and normal alleles is less obvious in fibroblasts compared with lymphoblastoid cells, although these differences were reproducible in multiple assays. As in fragile X lymphoblasts, the VL domain expands to include the *FMR1* locus and extends variable distances beyond it. In both fibroblasts and lymphoblasts, therefore, the difference between normal and fragile X replication timing appears to involve the spread of late replication from the VL zone into the L zone even though the difference in timing between the two zones is smaller in fibroblasts than in lymphoblasts. Another aspect of the fragile X replication delay that fibroblasts share with lymphoblasts is the variability of the VL–L border; the VL zone extends

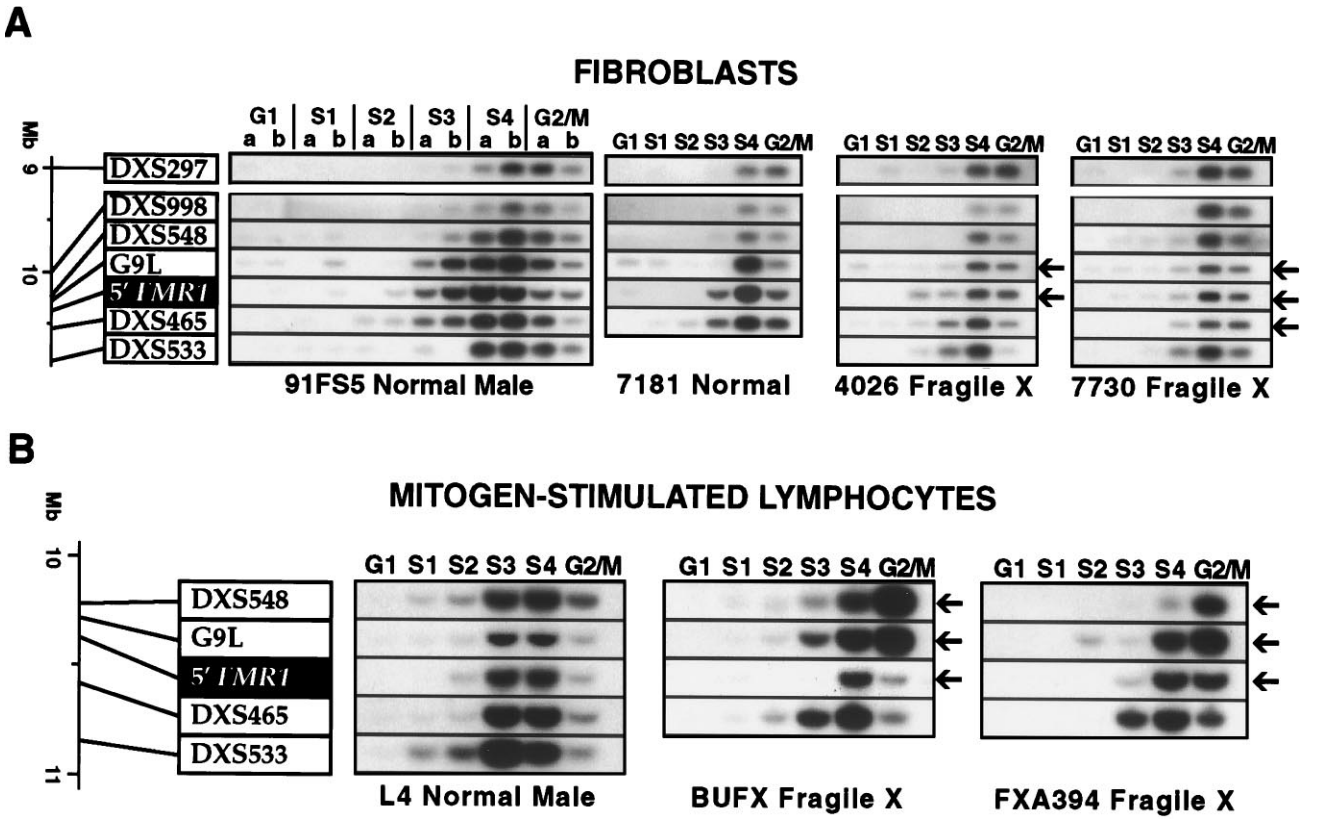

FIG. 3. Delayed replication is present in untransformed cells derived from fragile X patients. Replication patterns of Xq27 STSs are shown for fibroblasts (*A*) and mitogen-stimulated peripheral lymphocytes (*B*) that were derived from normal and fragile X males to examine further the delayed replication domain on fragile X chromosomes. Delayed replication was observed in all the fragile X cultures (arrows indicate strong replication delay).

beyond *FMR1* to include DXS465 in the case of 7730 fibroblasts, but not in the case of 4026 fibroblasts, reflecting a difference in location of about 150 kb.

We also observed delayed replication in another untransformed cell type derived from fragile X males, mitogen-stimulated peripheral lymphocytes (Fig. 3*B*). As in the other cell types, the VL zone appears to extend through adjacent sequences to include *FMR1*. Note that lymphocytes from patient BUFX (Fig. 3*B*) have a different VL–L border than do lymphoblastoid cells derived from the same individual (Fig. 2*B*). This difference suggests that the establishment of the border may be different in T and B cell lineages, although it is possible that the lymphoblastoid cell line is a clonal derivative of a population of lymphoid cells that are mosaic with respect to the location of this border. The different regions of delayed replication that we found in fragile X cells are summarized in Fig. 4.

DISCUSSION

Our observations of replication timing on the active X chromosome have established that there are at least two replication zones in Xq27, each likely to contain multiple replicons. In addition, the replication delay characteristic of the *FRAXA* mutation was found to extend well beyond the *FMR1* domain to constitute a region that varies in size, encompassing greater than 750 kb of the chromosome in some cases.

Replication Domain Structure of Xq27. In addition to early autoradiographic data indicating synchronous initiation of adjacent replicons (26), several molecular replication studies of multiple markers within cytologically defined replication bands have indicated coordinate activation of contiguous replicons. Synchronous replication of several replicons covering 2 Mb was shown for the mouse H-2 major histocompatibility complex locus (27), and the same pattern was shown for several megabases of the mouse immunoglobulin κ light chain locus (28). One potential exception is a relatively sharp discontinuity in replication time that was reported for a 50- to 150-kb region of early replication in an otherwise uniform zone of late replication in the Prader–Willi/Angelman syndrome region of maternally derived chromosome 15 (29). This study, however, utilized a FISH-based replication assay; it is possible that this inferred discontinuity reflects structural rather than replication-time differences because the FISH-based method can be subject to such nonreplication effects (2, 16, 30). In contrast, we have observed a relatively uniform pattern of late replication for the Prader–Will/Angelman syndrome region on both maternally and paternally derived chromosomes when we used the molecular replication assay described here (16).

Our Xq27 replication data reported here also support the general model of coordinated replication of contiguous replicons within each of the two replication zones in this region: a centromere-proximal one (VL) replicating predominantly in the last quarter of S, and a distal one (L) replicating earlier, yet still in the last half of S. Each zone is larger than a megabase and should, therefore, consist of multiple replicons according to estimates of replicon sizes (50–330 kb) obtained from autoradiographic images of replicating DNA (26). Although we have sampled only a small part of the two zones, the similarity of replication patterns among widely spaced STSs leads us to expect that further replication studies in the region will support the general patterns we have observed.

Delayed Replication at *FRAXA* in Fragile X Syndrome. We interpret our data as indicating that delayed replication in fragile X syndrome can involve a different number of replicons in different individuals. The largest domain of delayed replication we observed in fragile X cells extends the VL replication zone to include *DXS533* but not *FMR2*, a distance of 750–1,250 kb from the normal VL–L border (Fig. 4). This size suggests that at least three replicons could be affected. A more direct indication that at least three replicons can be delayed is that we observed three different VL–L borders in fragile X cells: in BUFX and FXA394 lymphocyte cultures and in 4026 fibroblasts, the region of replication delay observed at *FMR1* did not extend to include the 141R STS; in TL009 and BUFX lymphoblasts and in 7730 fibroblasts, *FMR1* and 141R are delayed whereas M749 is not; in H6, C3681, TL010, and H7 lymphoblasts, the delayed region includes *FMR1*, 141R, and M749, but not *FMR2*. The approximate sizes of these putative replicons are consistent with those of replicons observed by autoradiography (26): 400 kb for the *FMR1* domain, 300 kb for the 141R domain, and 300 kb for the M749 domain (Fig. 4).

Using a FISH-based replication assay, Subramanian *et al.* (13) recently observed a large domain of delayed replication that includes *FMR1* in two cell lines from fragile X males. The delayed region in these two lymphoblastoid lines is somewhat larger than any we have observed, extending from *DXS998* to

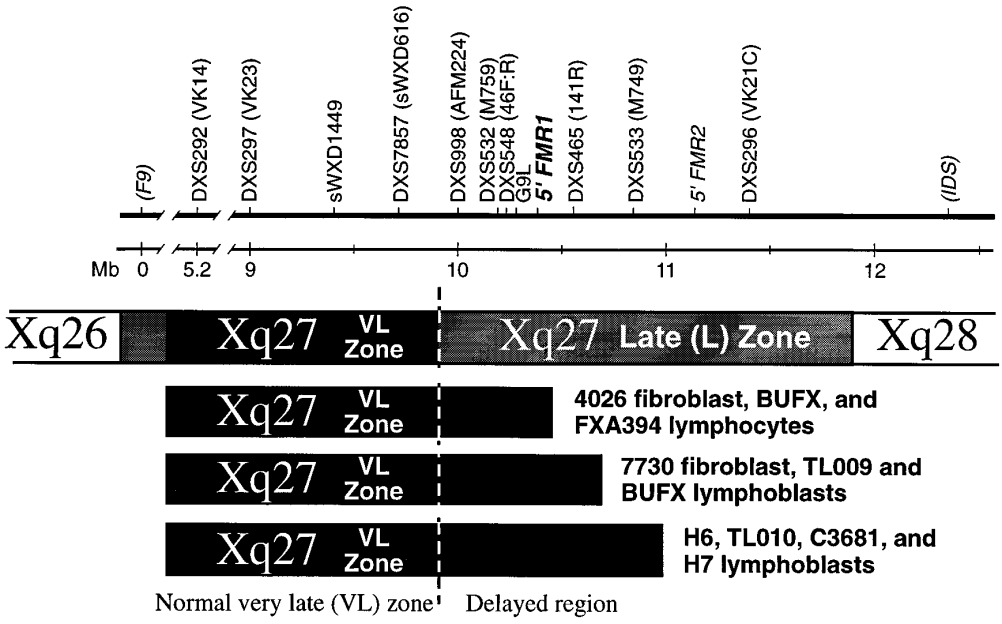

FIG. 4. Summary of fragile X-associated regions of delayed replication. A map of Xq27 replication time zones on the active X chromosome is shown for normal and fragile X cells to illustrate regions of marked replication delay found in different fragile X cell lines or cell cultures.

DXS296 in both cases. These cases also differ from the nine fragile X cases we studied in that they exhibited as strong a replication delay at *FMR2* as at *FMR1*. These data are suggestive of further variability in the telomeric border of the VL zone in fragile X. Alternatively, the observation of late replication for the more distal loci might have resulted from a tendency of the FISH-based method to assign late replication to earlier-replicating loci which are near late–early replication borders (30).

One explanation for the variable region of delayed replication in *FRAXA* fragile X cells is that during the developmental establishment of replication timing domains, the VL zone of very late replication on the active X chromosome spreads distally to include *FMR1*. The variability in the telomeric border of delayed replication in fragile X might be explained in part by the apparent absence of genes other than *FMR1* in the regions of delayed replication. The presence of such genes might have otherwise provided a selective pressure for the late domain to spread only to *FMR1* to avoid repression of these distal genes by late replication. This phenomenon may be similar to the spread of late replication along the normal X chromosome or into the autosomal portions of X-autosome translocation chromosomes during X chromosome inactivation. Position effect variegation also appears to have similar features (31–33).

Multiple Replicon Delay in Fragile X Because of Selection for Methylated CGG Repeats? Our observation that the fragile X mutation can affect the timing of replication initiation at several adjacent replicons is somewhat surprising, given the localized nature of this defect (CGG expansion and hypermethylation in the promoter region of *FMR1*). A plausible, direct effect of the CGG expansion could be an alteration of a master control locus that influences initiation at multiple replicons. The existence of replication control loci for *FMR1* and other X-linked genes is supported by our observations of 5-azacytidine-induced advances in replication time for inactive X alleles that were independent of gene expression (14). Alternatively, the affect of the expansion on replicon timing may be indirect, deriving from developmental selection. Positive selection for a VL zone of very late replication that includes *FMR1* might occur if cell viability is compromised by earlier replication of a large CGG repeat, particularly if the expanded repeat is unmethylated.

Fragile X syndrome is caused by a deficit in functional FMR1 protein (34, 35) that usually results from transcriptional silencing by promoter methylation (8, 9, 36). Although the CpG dinucleotides in the CGG repeat and surrounding 5′ CpG island of *FMR1* are heavily methylated on inactive X and fragile X chromosomes (10, 37), they are expected to be unmethylated in the early embryo because such X-linked CpG islands are unmethylated in gametes (38, 39). In addition, a recent study of *FMR1* methylation in ovaries of a fragile X carrier suggests that alleles with large CGG expansions are unmethylated in oocytes (40).

It has been suggested that methylation of the repeat has a stabilizing effect that allows mitotic division of cells with large repeat regions whereas, normal repeats are stable in the unmethylated state (41, 42). Such an effect would be particularly important in the early embryo when cell division is rapid. This idea is supported by the observation that large repeats found in cells from fragile X patients are methylated and mitotically stable (41). Increased stability by methylation is also suggested by the fact that the four other known cases of large CGG expansions (*FRAXE*, *FRAXF*, *FRA16A*, and *FRA11B*) are also associated with hypermethylation (24, 43–46). Potential influences of methylation on repeat stability include decreased recombination (47, 48), enhanced repair (42), and decreased tendency to form unusual structures that interfere with replication or are difficult to repair (49–52).

Deletion is another potential mechanism for human cells to avoid the detrimental effects of large, unmethylated CGG expansions; deletions are common in bacterial hosts containing such sequences (53, 54). Many fragile X individuals do have deletions that include the CGG repeat region (23, 55–65). Several of these deletions are known to be of mitotic origin because they were observed in mosaic populations in which some cells contain large methylated expansions. The deleted alleles are probably derived from large, unmethylated expansions, because much of the CpG island that flanks the CGG repeat is retained and is unmethylated (55, 56, 61, 64). Our previous study (1) indicated normal replication timing for *FMR1* in a fragile X cell line (TL007) that has a small unmethylated CGG expansion though it was derived from an affected male that appeared to have only a large methylated expansion in his leukocyte DNA. The TL007 allele may be another example of an unmethylated deletion allele. Replication timing was normal for TL007 at several additional STSs from Xq27 (data not shown). These data, therefore, are consistent with the hypothesis that large unmethylated CGG repeats are present in early development and are selected against during cellular expansion.

Methylation and/or late replication, rather than deletion, appears to be the most common method for stabilization of expanded CGG repeats in early development. Our data indicate that multiple replicons can be affected in such fragile X cases. The developmental establishment of a VL zone that contains the *FMR1* locus could be the initial event leading to repeat stabilization and gene silencing; methylation and stabilization of the expanded CGG repeat would occur as a consequence of very late replication. If methylation is sufficient to provide stability for large repeats, we predict that some cases of fragile X involve *de novo* methylation of the repeat without a replication delay. Such cases may be rare, however, because VL spreading into *FMR1* may be much more frequent than primary *de novo* methylation of the repeat due to the close proximity of the gene to the normal VL zone; the developmental establishment of this zone may involve a cooperative mechanism for spreading late replication into adjacent replicons.

Given the similarities in 5′ CpG island methylation and late replication between fragile X alleles of *FMR1* and normal inactive X alleles, it is interesting that an initial switch to late replication with subsequent CpG island methylation may also occur during the normal process of X chromosome inactivation (66, 67). The spread of very late replication into the *FMR1* region that apparently occurs in the early development of fragile X males may, therefore, be quite similar to the normal spreading of X chromosome inactivation that occurs in the early stages of female development. It is apparent that *XIST* plays an essential role in the establishment of X inactivation (68), but little is known about the regulatory action of its gene product, a spliced RNA that localizes to the inactive X chromosome (69). The apparent stochastic spread of late replication from proximal to distal Xq27 that we have observed in fragile X cases suggests the possibility that XIST RNA may promote the spread of X inactivation by enhancing the probability for the spread of late replication.

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