The timing of XIST replication: dominance of the domain

Stanley M. Gartler1,2+, Lester Goldstein2, Susan E. Tyler-Freer2 and R. Scott Hansen1

1Department of Medicine and 2Department of Genetics, Box 357360, University of Washington, Seattle, WA 98195, USA

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Contiguous replicons are coordinately replicated and may be organized in temporal–spatial domains with early replication domains containing expressed genes and late ones carrying silent genes. XIST is silent on the active, early replicating X chromosome and expressed from the inactive, late replicating homolog. These circumstances potentially deviate from the aforementioned generalization and make studies of replication timing for XIST of special interest. Although earlier investigations of XIST replication in fibroblasts based on analysis of extracted DNA from cells at different stages of the cell cycle suggested that the silent gene does replicate before the expressed allele, studies using FISH technology produced the opposite results. Because the FISH replication studies could not directly distinguish between the active and inactive X chromosomes in the same cell, we undertook a re-investigation of this question utilizing FISH analysis under conditions that allowed us to make that distinction using cells sorted into different cell cycle stages by flow cytometry. The findings reported here indicate that the silent XIST gene on the active X chromosome does replicate before the expressed allele on the inactive X, supporting the view that the time of a gene’s replication is determined by the large, multi-replicon domain in which it is located and not necessarily its expression state.

INTRODUCTION

Early observations in a number of laboratories suggested a relationship between chromosome condensation, late DNA replication and gene silencing (see for example refs 1,2). From more recent molecular studies, a general hypothesis emerged to the effect that expressed genes replicate early, silent genes replicate late and changes in expression status are accompanied by corresponding changes in the time of gene replication (3,4). While this rule holds for the great majority of genes that have been studied, a few examples of late replicating expressed genes have been observed (5–8). In such cases, however, if allelic differences (such as those produced by X inactivation or genomic imprinting) exist, the expressed allele replicates before the repressed allele (6,8,9), with one possible exception (see below).

The replicon is the basic unit of replication, with 50–330 kb of DNA replicating from a single origin in mammals (10). Contiguous replicons have coordinated replication patterns and may be organized in temporal–spatial domains of a mega-base or more, suggesting the existence of master control elements for replication timing (4,7,10–13). We will use the term ‘domain’ to refer to this multi-replicon concept and not to the (sub)domains defined by scaffold or matrix attachment sites as described by Gasser and Laemmli (14).

The shift of a silent, late replicating gene to an expressed, early replicating form (or vice versa) raises the question: does such a shift involve the entire multi-replicon domain or does the individual gene establish an independent time of replication? Limited evidence suggests that the multi-replicon domain is dominant (15–17). In the case of immunoglobulin \( \kappa \) light chain sequences, for example, non-transcribed genes replicate early because they are in proximity to transcribed ones (16). Further exploration of this question is called for because of conflicting reports in the literature (6,18–20).

The XIST gene offers a unique test of this question. It is silent on the active, early replicating X and expressed from the inactive, late replicating homolog (21). Because XIST is in relatively close proximity to genes that are subject to X inactivation, the expressed XIST allele may reside in a late replicating domain and the silent allele in an early one, making XIST replication timing studies of interest. Investigations of XIST replication in fibroblast cells utilizing analysis of extracted DNA from cells at different stages of the cell cycle by two groups [one using 5-bromo-2-deoxyuridine (BrdU) to label newly replicated DNA (6) and one using single nucleotide primer extension to differentiate active and inactive X alleles (20)] have reported that the silent gene (in the active X domain) replicates before the expressed allele (in the inactive X domain), whereas studies using FISH technology produced the opposite results (18,19). The FISH studies did not directly distinguish between the active and inactive X chromosomes in the same cell; comparisons were made between male cells (active X) and female cells (active and inactive Xs) and between hybrid cells with either an active or inactive human X. In addition, the FISH analysis was not restricted to S-phase cells and the percentage of XIST doublets could vary markedly between cell populations based only on differences in cell cycle parameters.

To investigate these contradictory findings, we re-examined XIST replication in female fibroblasts by FISH analysis under conditions in which the two Xs could be distinguished in the same cell whose cell cycle stage was known. The findings we...
Figure 1. FISH and BrdU analysis of XIST replication time in male fibroblast strain 71-81. For FISH analysis (open circles), ~200 cells were scored for each cell cycle fraction. For BrdU analysis (closed circles), quantification of a replication profile described previously (6) was used to obtain cumulative replication values for XIST in different cell cycle phases.

Figure 2. FISH replication analysis in i(Xq) female fibroblasts. Inactive X alleles of XIST were identified in GM00088 cells by two FISH signals that were in relatively close proximity [representing the two alleles on i(Xq)] and the active X allele was identified as the other, well-separated signal. In the example shown, the cell is in S2 and XIST is replicated (D) on the active X and unreplicated (S) at the i(Xq) loci. The two i(Xq) loci are much further apart than a duplicated single locus.

RESULTS

Replication of the silent XIST gene in normal male fibroblasts

Before undertaking a direct examination of the time of replication on the active and inactive Xs in the same nucleus, we examined replication of the XIST locus in human male fibroblasts by FISH analysis of cells sorted by flow cytometry into different portions of the cell cycle: G1, four stages of S and G2. Nuclei with single XIST hybridization signals were scored as unreplicated whereas those with closely spaced doublet signals were scored as replicated. As shown in Figure 1, the results of such analyses reveal that the silent XIST gene replicates in the first half of S in the majority of cells. A similar pattern of XIST replication was observed when these fibroblasts were analyzed by examination of newly replicated DNA identified by BrdU labeling in flow-sorted cells (Fig. 1).

Replication of the expressed and silent XIST alleles in isochromosome X-containing fibroblasts

To microscopically distinguish between the active and inactive X chromosomes in the same cell during FISH analysis, we utilized female fibroblasts with an inactive isochromosome X, i(Xq), which contains two closely linked XIST loci. The i(Xq) is always inactive in these cells because of early selection against those cells in which the normal X is inactivated (22). This selection presumably occurs because a cell with an inactive normal X has a deficiency in Xp gene dosage and an excess of Xq dosage for those genes that are subject to X inactivation.

Replication patterns were scored in these cells only if the signals from the two XIST loci of the i(Xq) chromosome were distinctly separate from the single locus signal of the active X (Fig. 2). Figure 3A shows the results of one such analysis of XIST replication on the active and inactive X chromosomes in these cells. The replication pattern of XIST on the active X chromosome is quite similar to that seen in male cells (Fig. 1), whereas replication of XIST on the inactive X is noticeably later. The earliest that an XIST allele can replicate on the inactive X is represented by the proportion of cells with doublets on either or both i(Xq) loci. On the active X, the frequency of doublets at S2 is 58%, a level not reached on the inactive X until S3. The differences between the frequency of replicated signals for the active and inactive X chromosomes at S2 and S3 are highly significant (P < 0.001; see Fig. 3).

A FISH replication analysis on another X,i(Xq) strain (GM02595) in which the four sorted S-phase cell fractions were pooled gave 219 of 381 cells (57%) showing the XIST gene on the active X to be replicated, whereas 162 of 381 cells (42%) had replicated either one or both XIST loci on the inactive X. This difference is highly significant (P < 0.001). If the four S-phase fractions from Figure 3A (GM00088) are pooled, the results give 487 of 880 cells (55%) with XIST on the active X replicated and 385 of 880 cells (44%) with either one or both XIST loci replicated on the inactive X. These results are very similar to those from the GM02595 X,i(Xq) fibroblasts, suggesting the generality of these patterns and the repeatability of our assay.

These FISH replication data are consistent with those for XIST replication in a normal female fibroblast using a timing method based on the isolation of newly synthesized sequences (BrdU-labeled) from cells sorted into different fractions of the cell cycle by flow cytometry (Fig. 3B). In the BrdU analysis, the active and inactive alleles were distinguished by digestion with a methylation-sensitive restriction enzyme, SacII, and the resulting profile was quantified by phosphorimager analysis (6). The results reported in this section, therefore, strongly support the hypothesis that the silent XIST gene on the active X chromosome replicates before the expressed allele on the inactive X.

Replication time within XIST

The suggestion of Torchia and Migeon (19) that discrepancies between their studies using the FISH replication assay and those using BrdU labeling may reflect differences in replica-
pare the replication pattern at the 5'-end of the XIST region ~25 kb away near the 3'-end of the gene. We found that the silent allele replicates before the expressed allele, though later replicating than its homologous domain, does not replicate as late as several other inactive X domains, such as those containing the inactive alleles of housekeeping genes (9). Such ‘earlier’ late replication may be required for transcriptional permissiveness in an otherwise repressive environment.

These replication data are difficult to reconcile with those of Torchia et al. (18) and Torchia and Migeon (19). In the latter study, the authors suggest that the use of BrdU in our initial study may have led to an artifactual reversal of XIST replication. That suggestion, however, can explain neither the studies of Xiong et al. (20), that did not involve BrdU labeling, nor the FISH replication results reported here.

Figure 4. BrdU analysis of replication time for the 5'- and 3'-regions of XIST in normal fibroblasts. Duplex PCR reactions for the 5'- and 3'-regions of XIST in a male fibroblast strain were performed on the newly synthesized DNA (BrdU DNA) isolated from cells at different stages of the cell cycle.

**DISCUSSION**

Our FISH analysis revealed patterns that are concordant with our studies of BrdU-labeled DNA from flow-sorted fibroblasts (6; this study). That the silent XIST allele replicates before the expressed allele in such cells is also supported by the studies of Xiong et al. (20), who determined XIST allelic ratios in unlabeled murine fibroblasts as they progressed through the cell cycle. Because the silent XIST gene is on the early replicating X, these observations further supported the idea of ‘dominance of the domain’ in establishing replication time. The boundaries of the XIST domain have not been completely mapped, but from our replication timing studies of STSs in the region (data not shown), the domain extends at least 150 kb centromeric (p10-Eg) and 200 kb telomeric (p4565-H8) and appears to contain at least two genes, BPX and CDX4, that are probably subject to X inactivation in humans, as they are in mice (23,24). The domain containing the expressed XIST allele, though later replicating than its homologous domain, does not replicate as late as several other inactive X domains, such as those containing the inactive alleles of housekeeping genes (9). Such ‘earlier’ late replication may be required for transcriptional permissiveness in an otherwise repressive environment.

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Torchia and Migeon (19), noting that our PCR analysis dealt with only a small part of the gene (500 bp), raised the possibility that the time of replication for that part could be quite different from that in another part of this 28 kb gene. They report marked variation in doublen frequency in unsynchronized cells using different probes (10–35% for XIST and 35–60% for FMR1 on the active X chromosome) and suggest that these variations result from different times of replication for the regions probed (19). A 25% range in doublet frequency represents about one-quarter of the S period or 2 h of replication for an 8 h S period.

Given a rate of replication fork movement of no greater than ~2 kb/min (10), 2 h is an order of magnitude longer than needed to replicate the XIST gene. Thus, the differences in the number of the doublets with different probes reported by Torchia and Migeon (19) must be unrelated to replication time differences. Furthermore, as we show in Figure 4, the 5'- and 3'-ends of XIST have very similar replication patterns with a predicted difference in FISH doublet frequency for the two regions being much less than the range reported by Torchia and Migeon, as discussed above. The similarity in replication patterns for the 5'- and 3'-regions of XIST was also found for the 5'- and 3'-regions of FMR1 in an earlier study (5). Some of the variability in the Torchia and Migeon (19) studies may be related to scoring all cells rather than only S-phase cells because the different cell cultures examined may have differed in their cell cycle profiles; a higher percentage of doublet signals is expected from cultures with a higher proportion of cells in S.
In their earlier study, in which XIST replication was studied by FISH in normal female fibroblasts, Torchia et al. (18) found an abnormally high frequency of doublet signals. In one case, for example, the frequency of cells with one doublet and one singlet signal (D/S) was 71%. Because the proportion of D/S cells is a minimum measure of the S phase of the cell cycle (very early S-phase cells will be seen as S/S and some D/D cells should still be in S phase), this large proportion of D/S cells raises serious questions about what a doublet signal reflects in their work. For cycling human fibroblasts, the S phase is usually found to be 30–40% of the cell cycle (25,26). Thus, the proportion of D/S cells observed by Torchia et al. (18) appears to be ~2-fold greater than expected, which implies that a significant fraction of the doublet signals they detected by FISH reflects something other than replication. It is difficult to account for such a high proportion of doublets, but these could derive in part from technical quirks such as artifactual doublets produced either by large probes or by excessive signal amplification for small probes.

The correspondence between our FISH replication data and the data obtained using extracted DNA from cells at different stages of the cell cycle (6,20; this study) suggests that using distinguishable X chromosomes along with flow cytometry to follow the progression of cells in S phase is advantageous for FISH analysis of XIST replication and should be useful for the analysis of other loci. Allele-specific replication time by FISH analysis of flow-sorted cells was described previously for several loci on chromosome 15 (27), although other replication timing methods were not used for comparison.

Our results emphasize that the rule ‘expressed genes replicate early and silent ones late’ should be replaced by ‘the replication time of a gene depends on the dominant mode of gene regulation within the multi-replicon domain’.

The temporal–spatial compartmentalization of a multi-replicon domain (28) is established by factors that promote early replication when the domain is dominated by expressed genes and late replication when dominated by genes that are repressed. The active X allele of XIST can be included in the known category of silent genes that reside in early replicating domains, such as the α-globin gene (29) and non-expressed members of immunoglobulin κ light chain family (16). The inactive X allele of XIST, however, represents an unusual example of what is probably a rare class of genes that retain transcriptional activity when embedded in a late replicating domain (6,7) and it is the only case known where a silent gene replicates before its expressed allele.

MATERIALS AND METHODS

Cell culture and flow cytometry

Normal human fibroblasts (71-81 and 78-18) were obtained from G. Martin (Department of Pathology, University of Washington, Seattle, WA) and X.i(Xq) fibroblasts (GM00088 and GM02595) were obtained from the NIGMS Human Genetic Mutant Cell Repository. The cells were grown in AminoMax-C100 (Gibco BRL, Gaithersburg, MD) and harvested in trypsin/EDTA (Gibco BRL). For FISH analysis, cells were labeled by the addition of 33 µM Hoechst 33342 to the cell culture for 1 h, harvested by trypsinization and stored in RPMI medium (Gibco BRL) with 2% fetal bovine serum and 33 µM Hoechst 33342. The cells were sorted as described previously (5), with the exception that they were sorted directly onto polylysine-treated (50 µg/ml) slides. For cultures labeled with BrdU, the procedures for flow cytometry and isolation of BrdU DNA were as described previously (5,7). For duplex PCR analysis of 5' and 3' XIST replications, primers for the 500 bp xst30:29r product (6) were combined with those for the 370 bp xst30:2r product (xst30, 5'-gggggctgatcatgtagaggg-3'; xst30r, 5'-tgcctctctctctctctctct-3') to amplify BrdU DNA using the following cycling parameters: 95°C for 1 min, 60°C for 1 min and 72°C for 2 min for 25 cycles; the amount of BrdU DNA per reaction corresponded to that isolated from 1000 cells.

FISH analysis

Ten thousand to 20 000 cells were sorted onto each slide and allowed to attach undisturbed for 15 min at 37°C in a humidified incubator. The slides were then flooded with 75 mM KCl for 15 min at 37°C and fixed in 3:1 methanol:acetic acid for 15 min. The slides were air dried, placed in 2× SSC at 37°C for 30 min, dehydrated through a standard alcohol series and air dried. Slides were denatured in 70% formamide at 72°C for 3 min and dehydrated through a cold alcohol series. The XIST probe (a gift from A.C. Chinault, Baylor College of Medicine, Houston, TX) was prepared and denatured as reported previously (30) and 5 μl was applied to the denatured cells, covered with a plastic coverslip and hybridized overnight at 37°C. Probe detection was accomplished as described previously (30). Slides were examined with a Nikon MicrophotFXA microscope.

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REFERENCES


