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Analysis of replication timing properties of human X-chromosomal loci by fluorescence *in situ* hybridization

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ABSTRACT **We have used fluorescence *in situ* hybridization on interphase nuclei of normal female cells to compare the replication timing patterns of genes on the human X chromosome that are known to escape X inactivation with those that are inactivated. By this procedure it was possible not only to determine the relative time of replication of the earlier-replicating allele for different loci but also to estimate the degree of asynchrony of replication of the two alleles for each individual locus. Loci such as *HPRT* and *FRAXA*, which are normally inactivated, displayed a high degree of replication asynchrony, whereas loci that are not inactivated (*ZFX* and *RPS4X*) were found to replicate very synchronously. Interestingly, examination of *XIST*, which is expressed only from the inactive X chromosome, by this procedure revealed that it also replicated asynchronously, with the expressed copy apparently replicating first. Therefore, by examining different loci from the X chromosome it was determined that there is a strict correlation between the expression and relative time of replication of individual genes.**

One of the distinctive hallmarks of X chromosomes in female mammalian cells is their asynchronous, or allocyclic, replication patterns during S phase, which was first noted >30 years ago (1-4). It was quickly recognized that the later-replicating chromosome was also the one that had undergone the process of X inactivation, which involved transcriptional silencing of the genes on this chromosome and was postulated to be a mechanism of gene dosage compensation between males and females (5, 6). This inactivated chromosome was also known to have an altered chromatin structure, associated with the formation of a heterochromatic Barr body in interphase cells (7). In recent years it has become evident that in human female cells not all the individual genes on the "inactive" chromosome actually undergo the inactivation phenomenon (reviewed in ref. 8). Thus, it is important to test the correlations between gene expression, replication timing, and chromatin structure at the subchromosomal level.

In the past, analysis of replication patterns of individual X-linked genes in female cells has been inhibited by the intrinsic asynchronous timing of the two homologs. However, recently a technique was developed by Selig *et al.* (9) that is not subject to this limitation. In this procedure unsynchronized cells are subjected to *in situ* hybridization with nonradioactive probes, with subsequent detection by fluorescence methods. By analyzing interphase nuclei one can rapidly determine the replication state of specific chromosomal regions; prior to replication in S phase single hybridization dots are detected, but after replication doublets are seen. Therefore, it is possible to determine the relative order of replication of different alleles and different loci by comparing the singlet/doublet ratios (9, 10).

We have used this technique to examine the replication properties of regions encompassing several X-linked genes that undergo the normal transcriptional inactivation patterns, as well as several that do not. The data are consistent with the conclusions that active alleles replicate earlier than inactive ones, regardless of their chromosomal position and that there is synchronous replication of alleles when both are transcriptionally active.

MATERIALS AND METHODS

Slide Preparation. Female RJK1267 lymphoblast cells (11) were grown by suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum. After addition of colchicine (0.06 μg/ml) for 20 min at 37°C, the cells were collected by centrifugation at 65× *g* in a clinical centrifuge for 8 min and the cell pellet was suspended in prewarmed 75 mM KCl for 17 min at 37°C. After the addition of 1 ml of freshly prepared fixative (methanol/glacial acetic acid, 3:1), the sample was subjected to centrifugation as before. The pellet was resuspended in 15 ml of fixative and then incubated at room temperature for 20–30 min. After pelleting at 65× *g* for 8 min, the cells were suspended in 15 ml of fixative and subjected to centrifugation again. This step was repeated five times in order to obtain very flat nuclei. The cells were then dropped onto dry microscope slides at room temperature and stored at 4°C.

Probe Preparation. Yeast artificial chromosomes (YACs) from regions of interest were isolated by a hierarchical PCR-based screening strategy (12, 13) from total human genomic YAC libraries (14, 15). The human *XIST* cosmid was identified by colony hybridization screening of an X-specific cosmid library (Lawrence Livermore National Laboratory) with a 200-bp PCR product from the 5′ end of the *XIST* gene. Cosmids from the *FRAXA* locus have been described (16). The X-specific centromere probe was obtained from Oncor.

Cosmid DNA was prepared with a column-based isolation kit (Qiagen, Chatsworth, CA) and was labeled for 3 hr with either dUTP-digoxigenin (Boehringer Mannheim) or dUTP-biotin (Oncor) by nick-translation. For YACs, *Alu*-PCR products were first prepared by using PCR on total yeast DNA from cells containing the YAC of interest, prepared by a standard method (17). Approximately 100 ng of this DNA was subjected to PCR in 50 μl containing 0.5 μM primer AGK34 (18), 1× GeneAmp buffer (Perkin–Elmer; 50 mM KCl/10 mM Tris Cl, pH 8.3/1.5 mM $MgCl_2$/0.001% gelatin), 200 μM each dNTP, and 2 units of Ampli*Taq* DNA polymerase (Perkin–Elmer). The sample was amplified for 30 cycles with denaturation for 1 min at 94°C, annealing for 45 sec at 55°C, and extension for 5 min at 68°C (18). A portion of the PCR mixture was analyzed by electrophoresis in a 1.5% agarose gel. After ethanol precipitation the pellet was dried and suspended in 24 μl of distilled water. Approximately one-third of this material was labeled as described above for cosmids.

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Abbreviations: FISH, fluorescence *in situ* hybridization; YAC, yeast artificial chromosome.

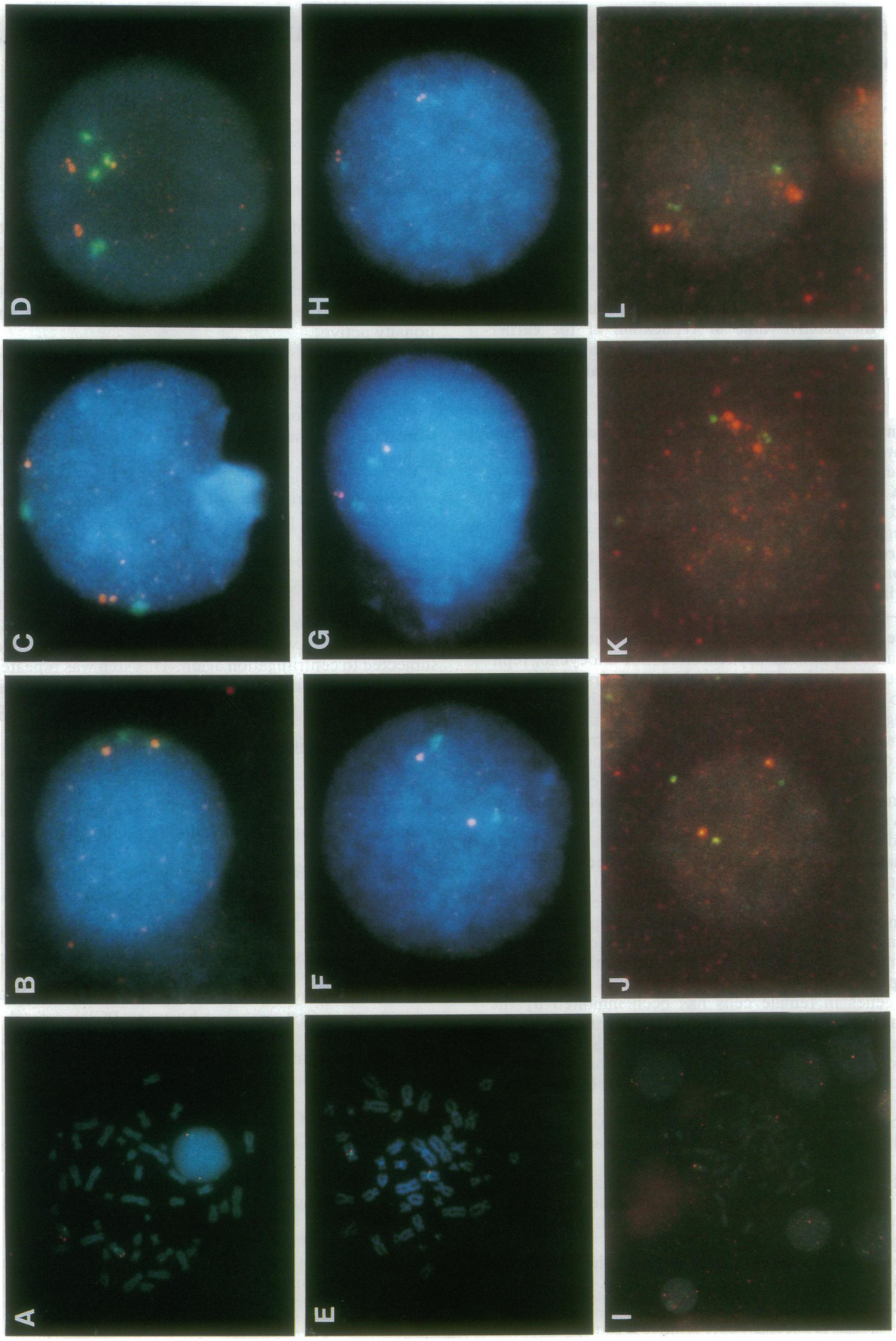

FIG. 1. *(Legend appears at the bottom of the opposite page.)*

Fluorescence *in Situ* Hybridization (FISH). Labeled YAC *Alu*-PCR products or cosmid DNA was ethanol-precipitated and suspended in 20 μl of hybridization solution [50% formamide/10% SDS/1× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7)]. After denaturation of the probe for 10 min at 72°C the labeled DNA was preassociated with COT-1 DNA (GIBCO/BRL) for 1 hr at 37°C. The slides were baked at 65°C for 30 min before being treated at 37°C for 30 min in 2× SSC/1× RNase (Oncor). The slides were then washed three times in 2× SSC for 5 min each. After a 70% and a 95% ethanol wash for 5 min each, the slides were air-dried. To denature the DNA the slides were heated at 72°C for 2 min in 70% formamide/2× SSC. The DNA was then dehydrated in 70% and 95% ethanol for 5 min each at −20°C. After the slides were quickly dried the probe was applied and the coverslip was sealed with rubber cement. After overnight incubation at 37°C, the slides were washed in 2× SSC for 5 min at room temperature, in 50% formamide/2× SSC for 20 min at 42°C, and in 2× SSC for 5 min at 42°C. Depending on the stringency needed for a particular YAC or cosmid, the slides were placed in 0.1–1.0× SSC for 15 min at 60°C. After a 5 min wash in 1× PBD (Oncor) at room temperature the digoxigenin and biotin signals were amplified by a series of secondary antibodies as follows. For digoxigenin the primary antibody was detected first by a monoclonal anti-digoxin antibody, mouse affinity (Sigma); followed by an anti-mouse immunoglobulin-digoxigenin, F(ab')$_2$ fragment (Boehringer Mannheim); and finally with rhodamine conjugated to anti-digoxigenin (Boehringer Mannheim). The biotin signal was first detected with anti-biotin, goat affinity (Calbiochem), followed by anti-goat IgG (Fc)-biotin (Calbiochem) and finally by fluorescein conjugated to anti-biotin (Calbiochem). The secondary antibody incubations were for 30 min each at 37°C in a humid chamber with three 5-min washes with 1× PBD (Oncor) between each incubation. The cells were counterstained with (4'6'-diamidino-2-phenylindole) (DAPI) and viewed with an Axiophot fluorescence microscope, using a Zeiss triple bandpass filter (Texas Red/DAPI/fluorescein isothiocyanate).

RESULTS

Determination of Replication Timing by FISH. To determine the replication timing of a number of X-linked loci with different expression patterns, DNA from either cosmids or YACs was labeled with nucleotide analogs (dUTP-digoxigenin or dUTP-biotin). These probes were then hybridized to metaphase spreads and interphase nuclei that were prepared from an unsynchronized female cell line (RJK1267). Examples of the results are shown in Fig. 1, where *A–D* show a typical metaphase spread and interphase nuclei hybridized with a cosmid from the fragile X locus (*FRAXA*). The *FRAXA* cosmid (244H3) was detected by anti-digoxigenin antibodies conjugated to rhodamine (red signal) and a human X centromere probe was detected by anti-biotin antibodies conjugated to fluorescein isothiocyanate (green signal). The presence of strong, specific hybridization signals on metaphase chromosomes, such as shown in Fig. 1*A*, was considered to be a prerequisite for analysis of hybridization signals on interphase nuclei. The singlet hybridization signals (1:1) shown in Fig. 1*B* are typical of nuclei in which neither copy of the *FRAXA* locus has replicated. An asynchronous pattern of one singlet and one doublet hybridization signal (1:2), such as is shown in Fig. 1*C*, indicates that only one copy has replicated. Finally, when both copies of the *FRAXA* locus have replicated, a 2:2 hybridization signal, such as is shown in Fig. 1*D*, is observed. Of ≈100 nuclei counted at the *FRAXA* locus, 60% showed unreplicated patterns (1:1), 32% gave an asynchronous pattern (1:2), and 7% showed complete replication (2:2). Thus, the *FRAXA* locus replicates relatively late and displays an asynchronous pattern of replication, as predicted for a typical locus on the mammalian X chromosome.

Fig. 1 *E–H* show a metaphase spread and interphase nuclei hybridized with a cosmid (D6122) containing the *XIST* gene, labeled with digoxigenin and detected by rhodamine fluorescence and an X centromere probe labeled with biotin and detected by fluorescein fluorescence. When the hybridization signals detected with this probe were counted, 66% of the interphase nuclei gave an unreplicated pattern, 25% showed an asynchronous replication pattern, and 9% showed complete replication.

A number of other X-linked loci were examined in a similar manner by using either YAC or cosmid clones mapping to these positions. For each of the probes, the ratio of singlet and doublet hybridization signals was determined for ≥100 interphase nuclei. On the basis of these data, it was possible to establish the relative order of replication of these regions, and this is presented schematically in Fig. 2. As diagrammed, the relative times of replication of the earlier-replicating copy of each complementary region is represented, regardless of the time of replication of the second copy. In most cases, as indicated, either the same probe was used multiple times or different probes from the same region were compared; for analysis of the *XIST*, both YACs and cosmids were used as probes. In all cases, the percentage of cells showing specific patterns was found to be reasonably reproducible. One might anticipate that both intrinsic imprecision in the biological system and technical limitations of the assay would preclude statistically significant analysis of absolute replication times. Nevertheless, this would appear to be a reliable method for determining an approximate relative order of replication of different sequences. As indicated, *PHKA1* was the first of the sequences examined to replicate, whereas *XIST* was the last. It appeared that there was a continuum of replication throughout S, with no apparent clustering of replication in early S phase.

Synchrony vs. Asynchrony. Another goal of this work was to determine the amount of asynchrony between the two X chromosomes at individual loci during replication. Most X-linked genes appear to inactivate the copy on the inactive chromosome and might be expected to show the asynchronous replication pattern that is characteristic of the chromosome as a whole. However several genes, such as *ANT3*,

FIG. 1. Representative patterns obtained by FISH. (*A–D*) Cosmid 244H3 was labeled with dUTP-digoxigenin, hybridized to metaphase chromosomes and interphase nuclei, and detected by rhodamine (red) fluorescence. To identify X chromosomes, an X-specific, α-satellite probe was labeled with biotin, hybridized, and detected by fluorescein (green) fluorescence. *A* shows hybridization to metaphase chromosomes and *B–D* show interphase nuclei exhibiting 1:1, 1:2, and 2:2 signals, respectively, for the *FRAXA* region. (*E–H*) Metaphase chromosomes and interphase nuclei were cohybridized with cosmid D6122 from the *XIST* region labeled with dUTP-digoxigenin and the biotin-labeled X centromere probe and fluorescent signals were detected. *E* shows hybridization to metaphase chromosomes and *F–H* show interphase nuclei exhibiting 1:1, 1:2, and 2:2 signals, respectively. (*I–L*) Metaphase chromosomes and interphase nuclei simultaneously probed with *FRAXA* and *XIST*. The *FRAXA* signal was detected by rhodamine (red) fluorescence after hybridization with dUTP-digoxigenin-labeled cosmid 244H3 and the *XIST* signal was detected by fluorescein (green) fluorescence following hybridization with dUTP-biotin-labeled cosmid E431. *I* shows hybridization to metaphase chromosomes; *J* shows a nucleus in which neither locus has replicated; *K* shows a nucleus in which the *FRAXA* locus, but not the *XIST* locus, has replicated on one chromosome while the *XIST* locus, but not the *FRAXA* locus, has replicated on the other; and *L* shows a situation where there has been complete replication of all alleles.

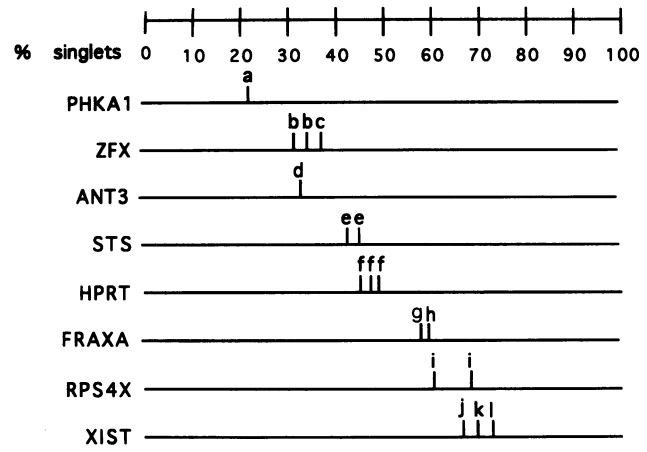

FIG. 2. Relative order of replication of X chromosome loci. Each labeled vertical line represents results from an independent experiment. The letters correspond to either YACs or cosmids used as probes as follows: a, YAC 120B3 (*PHKA1*; phosphorylase kinase α_1 polypeptide); b, YAC 460A9 (*ZFX*; zinc finger protein, X-linked); c, YAC 456A6 (*ZFX*); d, YAC 261G10 (*ANT3*; ADP/ATP translocase); e, YAC A223C9 (*STS*; steroid sulfatase); f, YAC A153A3 (*HPRT*; hypoxanthine phosphoribosyltransferase); g, cosmid 22.3 (*FRAXA*; fragile site, folic acid type, Xq27.3); h, cosmid 244H3 (*FRAXA*); i, YAC yWXD3933 (*RPS4X*; ribosomal protein S4, X-linked); j, cosmid D6122 (*XIST*; X-inactive-specific transcript); k, cosmid E431 (*XIST*); l, YAC B60D7 (*XIST*).

ZFX, and *RPS4X*, have been identified that escape inactivation and are expressed from both the active and the inactive X chromosome (19–21), and it was of particular interest to determine the relative time of replication of the two copies of these regions. It is possible to approximate the degree of asynchrony by comparing the percentage of cells showing replication of only one allele (singlet/doublet fluorescence signal) or both alleles (doublet/doublet signals) when analyzed with different probes. Representation of the data in this manner is shown in Fig. 3. Both *HPRT* and *FRAXA* undergo X inactivation and display a relatively high amount of asynchrony during replication between their two X chromosomes (Fig. 3). This also appeared to be true for *PHKA1*, although it was less obvious because its very early time of replication resulted in a large percentage of cells that demonstrated complete replication patterns. A sequence from the pseudoautosomal region, *ANT3*, displayed a relatively synchronous pattern of replication, as may be expected from the fact that its expression and inheritance patterns are similar to those of autosomal sequences (22, 23). An intermediate pattern was found for the *STS* locus, which may reflect the fact that there is a variable level of expression of this locus in females that is less then twice that found in males, suggesting partial escape from inactivation (19). Interestingly, when other sequences outside the pseudoautosomal region that are expressed on both copies of the X chromosome, such as *RPS4X* and *ZFX*, were analyzed, they were also found to exhibit relatively synchronous replication. On the other hand, *XIST*, which is expressed only from the inactive X chromosome (24), replicates very late and displays an asynchronous pattern of replication. Therefore, the replication synchrony of a particular locus is strictly correlated with its expression.

Replication Timing of the *XIST* Locus. The unusual expression pattern of *XIST* (exclusively from the inactive X chromosome) coupled with the asynchronous pattern of replication led to the interesting question of which X chromosome, active or inactive, replicated *XIST* first. To address this question we used simultaneous hybridization with *XIST* and *FRAXA* probes labeled with different fluorescent tags (Fig. 1 *I–L*). Both of these loci replicate relatively late, with *FRAXA* apparently replicating somewhat earlier than *XIST* (Fig. 3). We reasoned that when paired sets of signals from individual chromosomes were analyzed, if the earlier-replicating copy of each locus mapped to the same chromosome, the percentage of cells showing paired signals representing replication of *FRAXA* but not *XIST* should be very low, and there should be essentially no examples of the opposite pattern—i.e., *XIST* replicated and *FRAXA* not. Conversely, if the earlier-replicating copy of each of the two loci were located on different chromosomes, one would expect to see a significant number of situations where either doublet (replicated) *FRAXA* signals were linked to singlet (unreplicated) *XIST* signals or vice versa. Examples of data from the double-label hybridization experiment are given in Fig. 1 *I–L*. The results for this experiment, summarized in Table 1, supported the latter scenario. Therefore, if one assumes that the earlier-replicating copy of *FRAXA* maps to the active chromosome, then the earlier-replicating copy of *XIST* must map to the other, inactive chromosome, which is also where it is transcribed.

DISCUSSION

A number of studies have been conducted to determine the time of replication of individual genes in mammalian cells

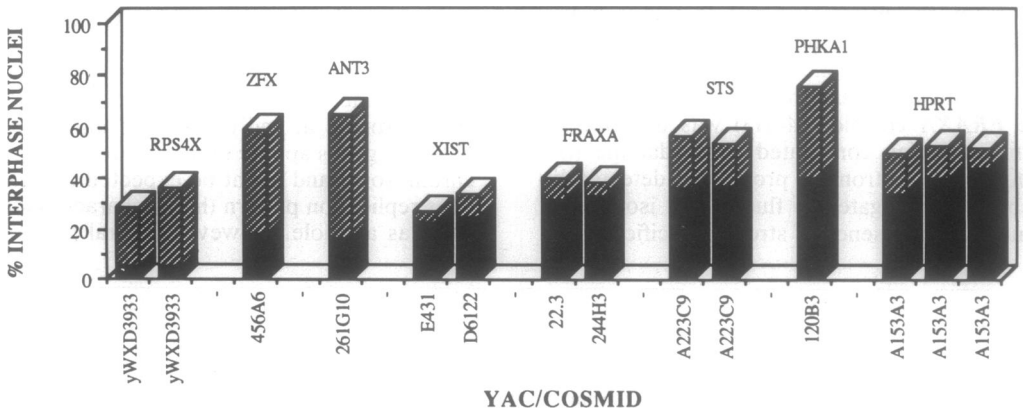

FIG. 3. Summary of replication timing data on the X chromosome. Hybridization probes used for each experiment are listed on the X axis (please refer to the legend to Fig. 2 for more details). Solid bars show the percent of interphase nuclei giving singlet/doublet patterns, representing nuclei in S phase in which only one of the two alleles has replicated. Hatched bars show the percent of nuclei giving doublet/doublet hybridization patterns, which includes all cells in S phase in which both alleles have replicated, as well as cells in G_2. *HPRT*, *PHKA1*, and *FRAXA* are transcribed only from active chromosomes, *XIST* is transcribed only from inactive chromosomes, and the other loci indicated are expressed fully (*RPS4X*, *ZFX*, and *ANT3*) or partially (*STS*) from both chromosomes.

Table 1. Results for dual-color FISH analyses of *FRAXA* and *XIST* replication patterns

FRAXA	*XIST*	%*
Not replicated	Not replicated	51
Replicated	Not replicated	21
Not replicated	Replicated	13
Replicated	Replicated	14

*Percent of individual chromosomes with linked fluorescence signals that exhibited the indicated patterns of replication.

and, on the basis of these studies, it has been proposed that there is a general correlation between transcriptional competence and early replication (25–27). However, only a few genes on the human X chromosome have been examined previously. Expressed copies of *PGK1*, *HPRT*, and *F9* have been shown to replicate early (28, 29), and in the latter two cases the copy on the inactive chromosome was found to have a delayed time of replication in mouse–human somatic cell hybrids (29); in contrast, expressed *FMR1* was found to replicate late in male cells (30). Using the FISH approach we were able to rapidly establish not only a relative order of replication of different loci but also to measure the degree of replication asynchrony between different alleles in normal female cells.

The data are consistent with the conclusion that there is an obligate correlation between replication properties and transcriptional activity but do not support models invoking simple temporal compartmentalization of replication into early versus late S phase. The times at which various regions first replicated appeared to be distributed throughout S phase. However, regardless of the time of replication or chromosomal position, it was found consistently that when only one allele was expressed, it was replicated earlier than the corresponding inactive allele, and when both alleles were expressed they replicated synchronously. One may speculate that these properties are due to a more open chromatin conformation for transcribed DNA or to its association with the nuclear matrix, either of which may facilitate accessibility to the DNA replication machinery. However, genetic analyses on the regulatory elements for both replication and transcription will be required to address these possibilities.

By pulse-labeling studies it has been shown that the late-replicating X chromosome still gives distinct, reproducible bands of incorporation at the cytogenetic level at various times in S phase (31). This result indicates that local control of replication timing has not been completely lost during the inactivation event. However, it offers no explanation for how the replication of the two chromosomes becomes uncoupled from the normally synchronous control observed with the other chromosomes. One of the first steps involved in trying to resolve this issue is to more precisely map the domains of replication control. The increasing availability of contigs of cloned sequences in a variety of vectors covering extensive regions of the genome and the ability to examine replication properties of relatively small regions at a single-copy level by FISH now provide the tools to accomplish this. It is apparent from our data that characteristic patterns of replication timing can be obtained for a variety of loci on the X chromosome, and by logical extension of this to flanking sequences, it should be possible to map transitional zones between domains differing in their time of replication, as well as domains differing in their degree of synchrony.

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