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Genetics

Asynchronous replication of homologous loci on human active and inactive X chromosomes

(DNA replication/HPRT/factor 9/X chromosome inactivation)

MALGORZATA SCHMIDT* AND BARBARA R. MIGEON

Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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ABSTRACT **The two X chromosomes in mammalian females replicate asynchronously, the inactive later than the active one. Using BrdUrd-sensitive restriction and UV irradiation to identify newly synthesized DNA directly on Southern blots, and restriction fragment length differences to discriminate alleles on active and inactive human X chromosomes, we examined the replication of hypoxanthine phosphoribosyltransferase (HPRT) and clotting factor IX (F9) loci in clonal populations of mouse–human hybrids. We find that HPRT replicates at different times during the period of DNA synthesis (S phase), depending on its activity: It replicates in early S phase, when expressed (on the active X chromosome), and in late S phase when silent (on the inactive X chromosome). Furthermore, when reactivated, the derepressed locus is earlier replicating, supporting a relationship between replication and transcription. Neither F9 allele is expressed in these cells, and both replicate in the second half of S phase, (slightly earlier on active than on inactive X chromosome).**

The replication of the inactive human X chromosome differs from that of other chromosomes, as the onset is later (1), the duration is shorter (2), and there are tissue differences in the order (3–5) and rate (6, 7) at which chromosomal DNA is synthesized. While the basis for these cytogenetic observations is unknown, clearly, in this case, late replication is associated with repressed activity of most genes on the chromosome. As asynchronous replication occurs early in the inactivation process (8, 9), it may play a role in initiating the event. There is evidence for a relationship between the time when a gene replicates and its transcriptional state, based mainly on early replication of transcribed sequences in expressing cells (refs. 10–14; reviewed in ref. 15). Goldman *et al.* (12) showed that some single copy genes replicate early in the tissue in which they are expressed but either early or late in nonexpressing tissues. They also showed that hypoxanthine phosphoribosyltransferase (HPRT) on the active X chromosome is among the early replicating genes. However, studies of the replication of genes on the inactive X chromosome have not been reported, so the late replication of this chromosome has been a purely cytogenetic observation.

To explore the possibility that alleles with differential expression on the active and inactive X chromosomes replicate asynchronously, we examined replication of the HPRT locus, using the blood clotting factor IX (F9) locus for comparisons. We find that the time when HPRT replicates is related to its transcriptional activity and that the repressed gene is late replicating.

MATERIALS AND METHODS

Strategy. Mitotic cells from mouse–human hybrid clones containing active, inactive, or reactivated human X chromosomes were exposed to BrdUrd during various intervals of the S phase. BrdUrd-substituted DNA was selectively discriminated, based on its resistance to digestion by *Hin*dIII and sensitivity to UV light. Alleles on active and inactive X chromosome were distinguished by differences in length of restriction fragments on Southern blots probed with human HPRT and F9 sequences.

Cells. The human (G1 skin fibroblasts)–mouse (A9) hybrid cells have been described (16–18); the G1–A9 hypoxanthine/aminopterin/thymidine resistant (HAT^R) and G1–A9 rea3 hybrid cell lines are effectively subclones of the same original cell, and as X chromosome inactivation is stable through mitosis, they have the same inactive X chromosome. All G1–A9 HAT^R hybrid cells contain the active human X chromosome and ≈50% have the inactive X chromosome (18). The G1–A9 rea3 clone, isolated from a 6-thioguanine-resistant ($6TG^R$) derivative of G1–A9 HAT^R by treatment with 5-azacytidine (5azaC) and reselection in HAT, contains only the inactive X chromosome; the HPRT locus on this chromosome has been reactivated (17). Cytogenetic analysis of this reactivant showed a single late-replicating X chromosome with a localized area of earlier replication in the Xq26–27 region (18).

Synchronization and BrdUrd Labeling. Mitotic cells from 30 confluent 100-mm dishes were selectively detached by mitotic shake-off. The procedure was repeated three times at 3-hr intervals after the cultures were refed with warm conditioned medium. These mitotic cells were kept at 4°C, pooled, and replated in 6–10 100-mm dishes containing HAT medium. Ten hours later, the cells were placed in thymidine-free medium containing BrdUrd (0.1 mM) and methotrexate (0.4 μM) for 6–14 hr. Sixteen to 24 hr after replating, cells were rinsed with warm conditioned medium (to eliminate floating cells), and Colcemid was added for 1 hr before the final harvest by mitotic shake-off. The harvested cells were stored at −70°C until DNA extraction. The numbers of contaminating (nonmitotic) cells were usually <25%, based on cell morphology and/or cytogenetic analysis; samples with <15% contamination were used for replication analysis.

Estimation of S Phase Occurring in BrdUrd. Based on previous studies (5, 7, 18) and cytogenetic analysis of harvested mitotic cells that revealed the length of S phase and G_2 phase in these cells, we estimated the subset of the S phase taking place in the presence of BrdUrd. Although this period is delineated in figures as if discrete, in fact, progression through S phase was influenced by experimental manipulation and 1-hr Colcemid treatment; hence, the diagrams represent our best estimate of the mean time in BrdUrd for each specimen.

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Abbreviations: HAT^R, hypoxanthine/aminopterin/thymidine resistant; $6TG^R$, 6-thioguanine resistant; 5azaC, 5-azacytidine; RFLP, restriction fragment length polymorphism.

*Present address: Murdoch Institute, Royal Children's Hospital, Flemington Road, Melbourne 3052, Australia.

DNA Purification. DNA was purified by proteinase K and RNase treatment, double phenol and chloroform extraction, concentration with 1-butanol, and dialysis.

Restriction Enzyme Digests. DNA was digested with *Hin*dIII (0.25–1.0 unit/μg) or *Nde* I (2 units/μg) for 15 hr. These enzymes were the only ones among 27 restriction endonucleases tested that could detect single-stranded BrdUrd substitution based on gels stained with ethidium bromide (data not shown). With 0.5–1.0 unit of *Hin*dIII (BRL) per μg, native DNA was usually completely digested, whereas BrdUrd-substituted DNA was not cleaved or incompletely digested (see Fig. 1).

To distinguish alleles on active and inactive X chromosomes, DNA first digested with the BrdUrd-sensitive endonucleases was precipitated and digested to completion with *Bam*HI to detect a restriction fragment length polymorphism (RFLP) in F9 or with *Sma* I to detect differences in methylation of HPRT. Analysis of HAT^R hybrid cells having only the active X chromosome or $6TG^R$ hybrids containing only the inactive X chromosome showed that the 3.0-kilobase (kb) *Hin*dIII/*Bam*HI F9 fragment and the 2.7-kb *Hin*dIII/*Sma* I HPRT fragment were from the active X chromosome, whereas the 1.3-kb *Hin*dIII/*Bam*HI F9 fragment and 5.0-kb *Hin*dIII/*Sma* I HPRT fragments were from the inactive X chromosome.

UV Irradiation. Samples for experiments shown in Figs. 2, 4, 5, and 6 were electrophoresed on a 1% agarose gel, stained with 0.1 μg of ethidium bromide per ml for 30 min, and irradiated on a standard 302-nm UV transilluminator for 5–15 min, depending on the sequence to be probed and the UV source. The time needs to be empirically determined as overtreatment damages native DNA as well.

Southern Blots. Blots were transferred under denaturing conditions to GeneScreen*Plus* membranes (19). The filters were hybridized to oligonucleotide-labeled probes in 1 M NaCl/50 mM Tris·HCl, pH 7.4, at 65°C overnight and washed at high stringency (final wash, 0.1× SSC, 65°C, twice for 30 min; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

Probes. HPRT:pPB1.7, a 1.7-kb *Bam*HI/*Pst* I human genomic fragment from the first intron (20) detects differences in methylation of the 5′ CpG island in *HPRT* alleles on active and inactive X chromosomes (see map in Fig. 4).

F9: pXI, a genomic fragment that includes the first exon and part of the first intron of the *F9* gene (21), detects a *Bam*HI RFLP, which we previously reported (22) (see map in Fig. 5).

RESULTS

Detection of BrdUrd DNA by *Hin*dIII Restriction and UV Irradiation. To establish suitable conditions, initial studies were carried out with hybrid cells having a single X chromosome and, hence, a single copy of HPRT and F9. Fig. 1 shows that DNA, synthesized in the presence of BrdUrd for the entire S phase, is not cleaved by BrdUrd-sensitive restriction enzymes *Hin*dIII (lane 2) and *Nde* I (lane 4) under the conditions specified. When blots were probed for HPRT, the DNA that was BrdUrd substituted (lanes 2 and 4) gave none of the hybridizing fragments found in digests of native DNA (lanes 3 and 5). While the results with *Nde* I were complicated by instability of the enzyme at 37°C, the effects of *Hin*dIII digestion were highly reproducible; if used in low concentration (<1 unit per μg of DNA), this enzyme has markedly less affinity for the BrdUrd substrate, even if BrdUrd is present in only a single strand. Lane 6 shows that restriction with *Hin*dIII also discriminates BrdUrd-containing sequences in a mixture of native and substituted DNA.

BrdUrd-substituted DNA is preferentially subject to photodegradation induced by UV irradiation (23, 24). Therefore,

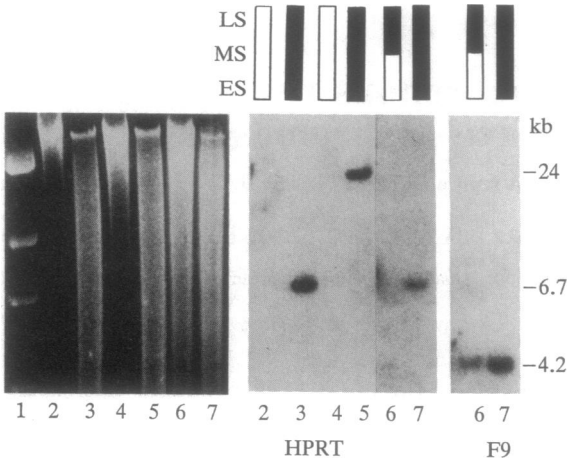

FIG. 1. Agarose gel stained with ethidium bromide (*Left*), and Southern blots (*Right*) showing that BrdUrd inhibits cleavage by 1 unit of *Hin*dIII per μg (all lanes but 4 and 5) and 2 units of *Nde* I per μg (lanes 4 and 5). Lanes: 1, λ marker cut with *Hin*dIII; 2–7, DNA (5 μg) from G1–A9 rea3 hybrid exposed to BrdUrd during the interval of the S phase (ES, MS, and LS denote early, middle, and late S phase, respectively) indicated above each lane. Rectangles show the proportion of the S phase in the presence (▭) or absence (▬) of BrdUrd. Blots hybridized with HPRT probe pPB1.7 were rehybridized with F9 probe pXI (*Right*). The G1–A9 hybrid has a single human X chromosome, the inactive one, with the reactivated *HPRT* gene. The lack of labeled HPRT fragment in DNA with one strand maximally substituted with BrdUrd (lanes 2 and 4) is due to lack of cleavage. The expected 6.7-kb *Hin*dIII fragment was also missing when BrdUrd was present only in the first half of the S phase (HPRT, lane 6), indicating that the reactivated *HPRT* gene replicates during early S phase. In contrast, the presence of the expected 4.2-kb F9 fragment (F9, lane 6) indicates that *F9* replicates during the last half of S phase.

as shown in Fig. 2, UV irradiation of agarose gels prior to transfer can select against DNA fully substituted with BrdUrd in one strand. This was also true for samples that were partially BrdUrd substituted (exposed to BrdUrd for only part of the S phase) (lane 6); however, the degree of degradation was

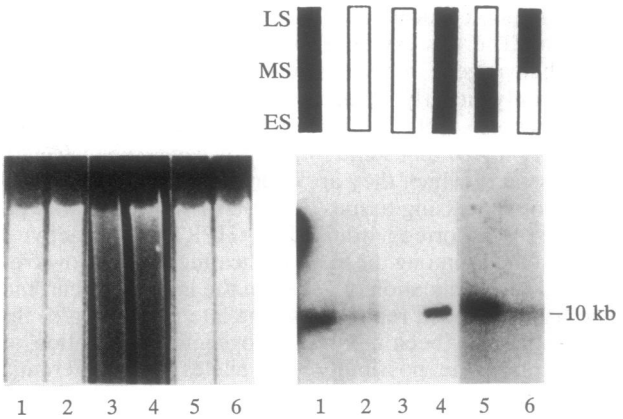

FIG. 2. Effect of UV irradiation of agarose gels containing independent samples of native and BrdUrd-substituted DNA. Symbols and abbreviations are the same as in Fig. 1. DNA from G1–A9 rea3, with the reactivated *HPRT* gene, was digested to completion with *Nsi* I (which is not BrdUrd sensitive). Ethidium bromide-stained DNA was photographed (*Left*), and the gel was left on the UV transilluminator (302 nm) for an additional 10 min. Filters hybridized with human HPRT probe pPB1.7 (*Right*) hybridize to native DNA (lanes 1 and 4) and show only trace hybridization to maximally BrdUrd-substituted DNA (lanes 2 and 3). The presence of the strong band in lane 5, but not in lane 6 (having similar amounts of DNA), indicates that the reactivated *HPRT* gene replicates during the first half of S phase.

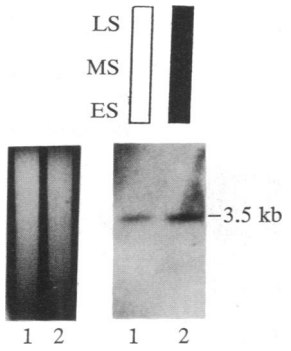

FIG. 3. Agarose gel stained with ethidium bromide (*Left*) and autoradiograph (*Right*) showing that DNA replicating in BrdUrd for two cell cycles (lanes 1) as well as native DNA (lanes 2) digested with non-BrdUrd-sensitive enzyme (*Pst* I) and hybridized with HPRT probe pPB1.7 gives the expected 3.5-kb *Pst* I fragment. Thus, the probe hybridizes to DNA, which is substituted in both strands with BrdUrd. Symbols and abbreviations are the same as in Fig. 1.

variable, perhaps a function of dose of effective UV and nature of the sequences probed.

Consistent with previous observations, Fig. 3 shows that genomic DNA that has been substituted with BrdUrd in both strands does not significantly inhibit hybridization of the probe, so that missing fragments in Figs. 1 and 2 can be attributed to poor digestibility and/or increased UV sensitivity of BrdUrd-substituted DNA. As BrdUrd-sensitive restriction (*Hin*dIII digestion) and UV irradiation of Southern gels both discriminate against single-stranded BrdUrd substitution in a mixture of native and substituted DNA, we combined these methods to carry out the replication studies shown in Figs. 4–6. In interpreting these results, we consider the absence of an expected fragment to mean it was synthesized in BrdUrd, and the presence of the fragment to indicate that it replicated in the absence of BrdUrd. Fragments, larger than expected, observed commonly in DNA samples exposed to BrdUrd for only part of the S phase are the products of incomplete digestion and indicate sequences that are at least partially BrdUrd substituted.

Replication of the Reactivated HPRT Locus on the Inactive X Chromosome. The HPRT locus on the inactive X chromosome in G1–A9 rea3 had been reactivated by 5-azaC (17, 18). Fragments expected from hybridization with HPRT probe pPB1.7 were not seen if cells were maintained in BrdUrd

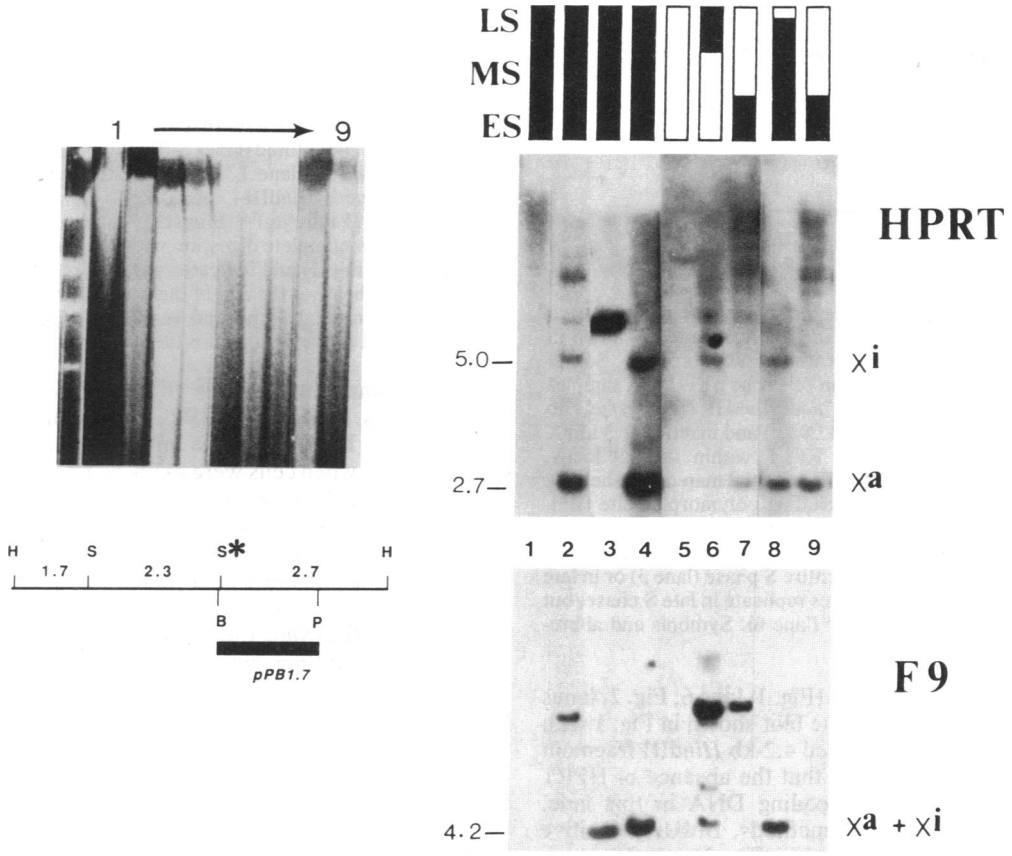

FIG. 4. Asynchronous replication of *HPRT* alleles on human active (X^a) and inactive (X^i) X chromosome in G1–A9 HAT^R. Gel stained with ethidium bromide (*Left*) and Southern blots (*Right*) hybridized with pPB1.7 (*Upper*) and rehybridized with F9 probe pXI (*Lower*). Partial map of human HPRT locus, showing *Hin*dIII (H) and *Sma* I (S) sites. S*, *Sma* I site that is differentially methylated on X^a and X^i. Map of the F9 locus is shown in Fig. 5. DNA from untreated cells (lanes 1–4) and five independent samples of cells that had incorporated BrdUrd as indicated (lanes 5–9). DNAs were digested as follows: lane 1, *Sma* I (5 units/μg); lane 2, *Hin*dIII (0.25 unit/μg) plus *Sma* I (5 units/μg); lane 3, *Hin*dIII (1 unit/μg); lanes 4–9, *Hin*dIII (1 unit/μg) plus *Sma* I (5 units/μg). HPRT: Control lanes (lanes 1–5) show that the two *HPRT* alleles can be distinguished by differential methylation of a *Sma* I site that gives 2.7- and 5.0-kb *Hin*dIII/*Sma* I fragments (lanes 2 and 4). The intensity of 2.7-kb fragments (X^a) is greater than 5.0-kb fragments (X^i) because all cells contain X^a, but at least half do not have X^i. The partial digest of native DNA in lane 2 shows that incomplete digestion with *Hin*dIII (0.25 unit/μg) does not change the relative intensity of 5.0- and 2.7-kb bands, as it is the same as in complete digests (lane 4). F9: In this digest, both *F9* alleles are contained on a 4.2-kb *Hin*dIII fragment. (*Hin*dIII digestion alone gives 4.2-kb fragments.) The *Sma* I sites that have not been mapped are far outside the *Hin*dIII sites, yielding fragments too large to be resolved by this gel, as there are no bands in *Sma* I digests (lane 1). Completely digested bands at 4.2 kb indicate replication in the absence of BrdUrd. The higher molecular weight bands consist of incompletely digested fragments. Symbols and abbreviations are the same as in Fig. 1.

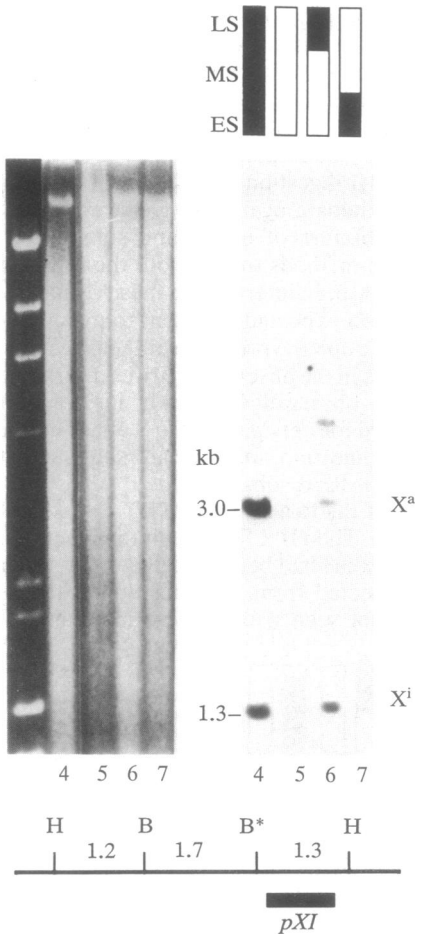

FIG. 5. Replication of *F9* alleles on human active (X^a) and inactive (X^i) X chromosomes in the same DNA samples shown in Fig. 4 (lanes 4–7). Gel (*Left*) stained with ethidium bromide. Southern blot (*Right*) hybridized with human F9 probe pXI. DNA samples were cut with *Hin*dIII (1 unit/μg) and *Bam*HI (5 units/μg) to distinguish between alleles on active (3.0 kb) and inactive (1.3 kb) X chromosomes, based on a *Bam*HI RFLP within the F9 locus. Markers: λ (*Hin*dIII) + ϕX174 (*Hae* III). Partial map of F9 showing *Hin*dIII (H) and *Bam*HI (B) sites including polymorphic site (B*). The greater intensity of the 3.0-kb fragment in unsubstituted DNA (lane 4) reflects the greater frequency of X^a. Both bands are absent in DNA treated with BrdUrd for the entire S phase (lane 5) or in late S phase (lane 7). Therefore, both alleles replicate in late S phase, but X^i replicates somewhat later than X^a (lane 6). Symbols and abbreviations are the same as in Fig. 1.

during the first half of the S phase (Fig. 1, lane 6; Fig. 2, lanes 2, 3, and 6). Rehybridization of the blot shown in Fig. 1 with the F9 probe revealed the expected 4.2-kb *Hin*dIII fragment (Fig. 1, F9 fragment, lane 6), so that the absence of HPRT fragments is not due to underloading DNA in this lane. Therefore, by two independent methods, BrdUrd-sensitive restriction (Fig. 1) and UV irradiation (Fig. 2), we show that hybridizing HPRT fragments are absent in DNA from cells exposed to BrdUrd during the first half of S phase. Therefore, the reactivated HPRT locus must replicate at that time.

Replication of HPRT Alleles on Early and Late Replicating X Chromosomes. The G1–A9 HAT^R hybrid with both active and inactive human X chromosomes was used to compare replication of HPRT alleles on the two chromosomes. To distinguish one allele from the other, we used differences in methylation of this locus on active and inactive X chromosomes (25, 26). Because a *Sma* I site in the 5′ CpG cluster is unmethylated in the active *HPRT* allele, but methylated in the inactive one, digestion with *Hin*dIII/*Sma* I produces fragments of 2.7 and 5.0 kb from active and inactive X

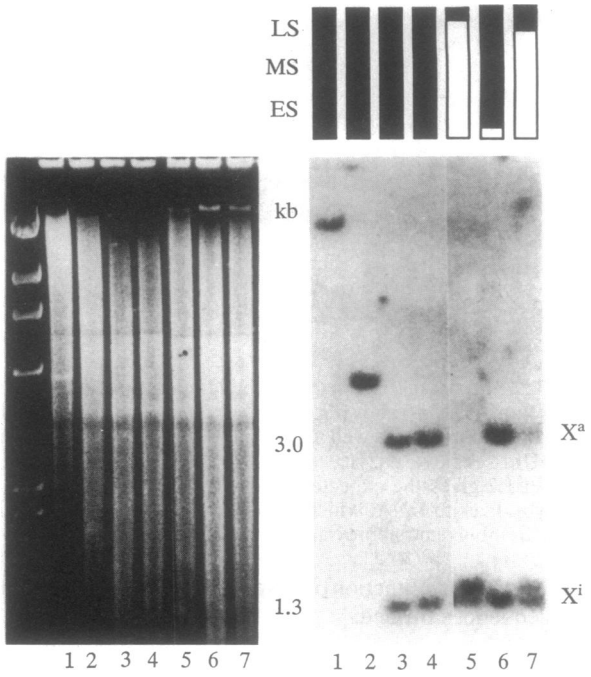

FIG. 6. Further evidence for slight asynchrony in replication of the unexpressed *F9* alleles from active (X^a) and inactive (X^i) X chromosomes. Agarose gel (*Left*) stained with ethidium bromide. Southern blot (*Right*) hybridized with pXI. DNA is either native (lanes 1–4) or substituted (lanes 5–7) as indicated by rectangles above each lane. Digests: lane 1, *Bam*HI (5 units/μg); lane 2, *Hin*dIII (5 units/μg); lane 3, *Hin*dIII (5 units/μg) + *Bam*HI (5 units/μg); lanes 4–7, *Hin*dIII (1 unit/μg) + *Bam*HI (5 units/μg). The 1.4-kb band is a product of incomplete digestion with *Hin*dIII due to a site 120 base pairs upstream (21) and indicates partial BrdUrd substitution. Both *F9* genes replicate at the end of the S phase, but not simultaneously, as the X^i-derived 1.3-kb band is the only one present in lane 5. Symbols and abbreviations are the same as in Fig. 1.

chromosomes, respectively (Fig. 4 *Lower Left*). Fig. 4 (*Upper*) shows the lack of HPRT fragments when cells were exposed to BrdUrd for the entire S phase (lane 5). On the other hand, when cells were exposed to BrdUrd during all but late S phase (lane 6), the only signal observed is from the inactive allele (5.0 kb), even though this gene is present in only 50% of the cells. Therefore, the inactive *HPRT* allele replicates significantly later than its active homolog (2.7 kb).

Fig. 4 (*Lower Right*) shows the same blot reprobed with F9. In the *Hin*dIII/*Sma* I digest, both *F9* genes yield the same 4.2-kb *Hin*dIII fragments. The absence of the 4.2-kb fragment in lanes 7 and 9 shows that neither *F9* allele replicates in early S phase.

Replication of *F9* Alleles on Active and Inactive X Chromosomes. Whereas the *Hin*dIII/*Sma* I digest in Fig. 4 cannot distinguish between the two *F9* alleles, they can be distinguished by a *Bam*HI polymorphism within the *F9* gene. *Hin*dIII/*Bam*HI digests of the same DNA samples shown in Fig. 4 confirmed that both F9 genes replicate during late S phase (Fig. 5; note that expected bands are present in lane 6 but not in lane 7). Further studies (Fig. 6) show that F9 replicates somewhat later on inactive X than on active X chromosome. Only the fragments from the inactive X chromosome are seen if the last hours of the S phase occur in the absence of BrdUrd (Fig. 6, lanes 5 and 7).

DISCUSSION

Technical Considerations. Most replication studies rely on BrdUrd as a marker for newly synthesized DNA. The BrdUrd-substituted DNA is usually separated from native sequences by density-gradient centrifugation. We have used *Hin*dIII

restriction followed by UV irradiation to detect BrdUrd-substituted DNA directly on Southern blots with less mitotic cells than required otherwise. The method we used is based on methods that have been reported previously. That BrdUrd substitution inhibits *Hin*dIII-mediated cleavage of λ DNA was reported by Marchionni and Roufa (27). Preferential photodegradation of BrdUrd-substituted DNA was used by D'Andrea *et al.* (23) for studies of ribosomal gene replication.

More than 35 independently isolated DNA samples from cells exposed to BrdUrd for different segments of the S phase were used for these studies, and samples treated similarly gave consistent results. However, because synchronization of large numbers of cells is seldom complete, and because of difficulties in defining the S-phase intervals precisely, our results vary slightly from one specimen to another. Such variability is shown in Fig. 4 (lanes 7 and 9) in independent samples that were exposed to BrdUrd for approximately the same length of time. However, these samples were prepared from cells synchronized independently, exposed to BrdUrd independently, and digested independently. The variability most often affects the degree to which incompletely digested products are removed by UV light. Most important in this assay is the presence or absence of the expected completely digested *Hin*dIII fragments. Fig. 4 shows that these are consistent and provide evidence for the asynchronous replication of the loci studied. In addition, by rehybridization with a second probe, we have shown that absent signals are in fact related to the presence of BrdUrd—i.e., negative signal for HPRT, but positive for F9, which replicates at a different time (Fig. 1, lanes 2 and 6), and the reverse (Fig. 4, lanes 9); furthermore, the results are consistent when the same DNA is digested with different enzymes [compare *Hin*dIII/*Sma* I in Fig. 4 (lanes 4–7) and *Hin*dIII/*Bam*HI in Fig. 5 (lanes 4–7)], or when DNA samples were isolated independently (i.e., Fig. 4, lanes 7 and 9 both show the absence of the 5.0-kb band). That there is a higher molecular weight band in the F9 blot in lane 7 and not in lane 9 most likely reflects the sample variation discussed above, but in each sample the expected 4.2-kb F9 fragment is missing. Therefore, our methods are sufficiently sensitive to distinguish replication that takes place in early S phase from that occurring in late S phase and seem to be sensitive enough to detect consistent differences in replication within these periods.

Relationship Between DNA Replication and Gene Activity. Our observations that HPRT and F9 on the human active X chromosome replicate in early S phase and late S phase, respectively, agree with those of Goldman *et al.* (ref. 12; unpublished data cited in ref. 28) using cesium chloride separation of BrdUrd-substituted DNA from HeLa cells. In addition, we show that the *HPRT* gene on the human inactive X chromosome is not synchronous with its active homolog but replicates significantly later, providing evidence that single copy genes on the inactive X chromosome are late replicating. Furthermore, we also show that replication influences or is influenced by changes in the activity of the relevant gene. Our previous cytogenetic studies of G1–A9 rea3 (18) showed that 5-aza C-induced reactivation of the HPRT locus was accompanied by a change in replication of the Xq26 region of the chromosome (from late to earlier). Now we show that the change in replication also involves the reactivated gene located in this region. Although direct comparisons are not feasible (the two genes not being in the same cell), the reactivated gene replicates at approximately the same time as the locus on the active X chromosome [compare Fig. 1 (HPRT, lane 6) with Fig. 4 (HPRT, lane 6)]. It remains to be determined whether reactivation of a locus without detectable changes in replication at the cytogenetic level is always accompanied by a switch to early replication of the gene.

The small differences in replication of the homologous *F9* genes may mean that this locus replicates asynchronously as well. Unlike the ubiquitously expressed *HPRT* gene, *F9* is expressed predominantly in liver and is not expressed in the G1–A9 hybrids (Northern blots not shown). Perhaps even unexpressed genes replicate earlier on active than on inactive X chromosomes. On the other hand, the asynchrony in replication of *F9* alleles is relatively subtle. In the absence of similar studies of homologous autosomal loci, it is not clear that homologous loci ever replicate simultaneously.

Relationship Between Replication and X Chromosome Inactivation. Asynchronous replication could provide the means to isolate one X chromosome from the other chromosomes, thus making it inaccessible to cell cycle-specific transcription factors. Perhaps it is not surprising that a number of inactive X chromosome loci that are expressed reside in a region (Xp22) that is relatively early replicating. On the other hand, late replication patterns that characterize the inactive X chromosome may simply result from the aggregation of many inactive genes. Further studies are needed to reveal whether replication changes precede the inactivation event or are merely a consequence thereof.

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1. Priest, J. H., Heady, J. E. & Priest, R. E. (1967) *J. Cell Biol.* **35**, 483–487.
2. Comings, D. E. (1967) *Cytogenetics* **16**, 20–37.
3. Willard, H. F. (1977) *Chromosoma* **61**, 61–73.
4. Latt, S. A., Barell, E. F., Dougherty, C. P. & Lazarus, H. (1981) *Cancer Genet. Cytogenet.* **3**, 171–181.
5. Schmidt, M., Stolzmann, W. M. & Baranovskaya, L. J. (1982) *Chromosoma* **85**, 405–412.
6. Takagi, N., Endo, S. & Sugawara, O. (1984) *Cytogenet. Cell Genet.* **38**, 62–69.
7. Schmidt, M. & Stolzmann, W. M. (1984) *Chromosoma* **89**, 68–75.
8. Sugawara, O., Takagi, N. & Sasaki, M. (1983) *Chromosoma* **88**, 133–138.
9. Takagi, N., Sugawara, O. & Sasaki, K. (1982) *Chromosoma* **85**, 275–286.
10. Furst, A., Brown, E. H., Braunstein, J. D. & Schildkraut, C. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1023–1027.
11. Calza, R. E., Eckhardt, L. A., DelGiudice, T. & Schildkraut, C. L. (1984) *Cell* **36**, 689–696.
12. Goldman, M. A., Holmquist, G. P., Gray, M. C., Caston, L. A. & Nag, A. (1984) *Science* **224**, 686–692.
13. Watanabe, N., Akasaka, K., Shinoya, T., Obinata, M. & Shimada, H. (1986) *Nucleic Acids Res.* **14**, 9509–9519.
14. Gilbert, D. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2924–2928.
15. Holmquist, G. P. (1987) *Am. J. Hum. Genet.* **40**, 151–173.
16. Pai, G. S., Sprenkle, J. A., Do, T. T., Mareni, C. & Migeon, B. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2810–2813.
17. Migeon, B. R., Johnson, G. G., Wolf, S. F., Axelman, J. & Schmidt, M. (1985) *Am. J. Hum. Genet.* **37**, 608–610.
18. Schmidt, M., Wolf, S. F. & Migeon, B. R. (1985) *Exp. Cell Res.* **158**, 301–310.
19. Reed, K. C. & Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221.
20. Jolly, D. J., Esty, A. C., Bernard, H. U. & Friedmann, T. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5038–5041.
21. Anson, D. S., Choo, K. H., Rees, D. J. G., Giarelli, F., Gould, K., Huddleston, J. A. & Brownlee, G. G. (1984) *EMBO J.* **3**, 1053–1060.
22. Ruta Cullen, C., Hubberman, P., Kaslow, D. C. & Migeon, B. R. (1986) *EMBO J.* **9**, 2223–2229.
23. D'Andrea, A. D., Tantravahi, U., Lalande, M., Perle, M. A. & Latt, S. A. (1983) *Nucleic Acids Res.* **11**, 4753–4774.
24. Murray, V. & Martin, R. F. (1989) *Nucleic Acids Res.* **17**, 2675–2691.
25. Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T. & Migeon, B. R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2806–2810.
26. Yen, P. H., Patel, P., Chinault, A. C., Mohandas, T. & Shapiro, L. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1759–1763.
27. Marchionni, M. A. & Roufa, D. J. (1978) *J. Biol. Chem.* **253**, 9075–9081.
28. Goldman, M. A. (1988) *Bioessays* **9**, 50–55.