

The Element(s) at the Nontranscribed *Xist* Locus of the Active X Chromosome Controls Chromosomal Replication Timing in the Mouse

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Manuscript received March 8, 2005

Accepted for publication June 10, 2005

ABSTRACT

In female mammalian cells, the inactive X chromosome is replicated late in S phase while the active X chromosome is replicated earlier. The replication times of the X chromosomes reflect a general trend in which late replication is associated with gene repression and earlier replication with transcriptional competence. The X-linked *Xist* gene is expressed exclusively from the inactive X chromosome where it is involved in the initiation and maintenance of X-inactivation. In contrast, no biological activity has been assigned to the *Xist* locus of the active X chromosome where the *Xist* gene is transcriptionally silenced. Here, we provide evidence that the element(s) at the nontranscribed *Xist* locus of the active X chromosome controls chromosomal replication timing in *cis*.

X-INACTIVATION in female mammals is the formation of heterochromatin throughout one of two X chromosomes (GARTLER and RIGGS 1983). The X-linked *Xist* gene plays a central role in this process (PENNY *et al.* 1996) and *Xist* knockout mice die early in embryogenesis due to a failure to undergo X-inactivation (MARAHRENS *et al.* 1997). *Xist* encodes an untranslated RNA that is expressed from the inactive X chromosome (Xi) but not from the active X chromosome (Xa) (BROCKDORFF *et al.* 1991; BROWN *et al.* 1991). The *Xist* RNA is quite stable and colocalizes exclusively with the Xi (BROWN *et al.* 1992; CLEMSON *et al.* 1996).

Another feature that distinguishes the Xi from other chromosomes is that the Xi is replicated very late in S phase while the Xa is replicated earlier (TAYLOR 1968; TAYLOR and MINER 1968). In this regard, the replication timing of the X chromosomes reflects a general trend where late replication times are associated with gene repression and early replication with transcriptional competence (GILBERT 2002). The available evidence indicates that the source of the replication-timing differences between the two X chromosomes are the times in S phase that their origins are activated, with the same replication origins being utilized on the active and inactive X chromosome (COHEN *et al.* 2003; GOMEZ and BROCKDORFF 2004). It has been estimated that the human genome is replicated by ~30,000 origins (TODOROVIC *et al.* 1999). The X chromosome constitutes ~5% of the human (LANDER *et al.* 2001) and is therefore estimated to be replicated by ~1500 origins. The mouse

X chromosome is very similar in size to the human X (WATERSTON *et al.* 2002) and therefore may be replicated by comparable numbers of origins. We expect many of these origins to be activated earlier on the Xa than on the Xi. However, in view of evidence that at least a subset of adjacent origins are activated at approximately the same time in S phase in mammalian cells (HUBERMAN and RIGGS 1968), we expect considerably <1500 replication-timing domains on the Xa and on the Xi.

The elements responsible for maintaining the replication timing of the Xa and Xi have not been identified. Excision of 21 kb from the transcribed *Xist* allele from the Xi, after X-inactivation has been completed, led to a partial destabilization of X chromosomal gene silencing (CSANKOVSKI *et al.* 2001) but did not disrupt late replication (CSANKOVSKI *et al.* 1999). The destabilization of X-inactivation was accompanied by the loss of the *Xist* RNA and macroH2A from the Xi (CSANKOVSKI *et al.* 1999). The transcribed *Xist* allele on the Xi, therefore, functions in *cis* to maintain certain features of the Xi. In contrast, no biological activity has been assigned to the nontranscribed *Xist* locus on the Xa. Here we show that the excision of 21 kb from the *Xist* locus on the Xa alters the timing of Xa replication relative to the replication-timing program of the rest of the genome.

MATERIALS AND METHODS

Fibroblasts and growth conditions: All of the mice used in this study had a 129 genetic background. Mouse primary fibroblasts were obtained from crosses involving previously described mouse strains (CSANKOVSKI *et al.* 1999, 2001) by trypsinization of 13-day embryos, culture, and immortalization

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with SV40 T-antigen (JAT *et al.* 1986). Inherited *Xist*- Δ 21kb alleles were always from the mother, since paternal inheritance of the *Xist*- Δ 21kb allele leads to embryonic lethality (not shown) and in this regard resembles a *Xist*- Δ 16kb allele (MARAHRENS *et al.* 1997). Female mice carrying *Xist*- Δ 21kb alleles were maintained by breeding to 129 male mice. Subsequent to random X-inactivation, individually transformed fibroblast lines were obtained by plating cells at less than one cell per well of a multi-well plate (limiting dilution). To obtain $Xa^{Xist-2Lox}Xi^{WT}$ fibroblasts, mice that were homozygous for an X-linked GFP transgene (HADJANTONAKIS *et al.* 1998) and the *Xist*-2Lox allele were mated to 129 mice and fibroblasts were prepared from E13.5 embryos. Female fibroblasts were identified using PCR and subjected to cell sorting using flow cytometry to purify GFP-expressing cells (inferred to be $Xa^{Xist-2Lox, GFP^{tg}}Xi^{WT}$) from cells that do not express GFP (inferred to be $Xa^{WT}Xi^{Xist-2Lox, GFP^{tg}}$). $Xa^{Xist-21kb}Xi^{WT}$ fibroblasts were derived from the GFP-expressing $Xa^{Xist-2Lox, GFP^{tg}}Xi^{WT}$ fibroblasts by infection with adenovirus carrying *cre* recombinase (AdCre) (TAN *et al.* 1999). The infection was performed in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum for 1 hr. Primary and transformed mice fibroblast cell lines were grown in DMEM supplemented with 10% fetal bovine serum (GIBCO, Gaithersburg, MD), penicillin (100 μ g/ml), and streptomycin (100 μ g/ml). The PCR primers 5'-LoxF (5'-TTT CTG GTC TTT GAG GGC AC-3'), 5'-LoxR (5'-ACC CTT GCC TTT TCC ATT TT-3'), and Xint3R (5'-CAC TGG CAA GGT GAA TAG CA-3') were used to identify the *Xist*-2Lox (612-bp PCR product), *Xist*- Δ 21kb (513-bp), and *Xist*-WT (427-bp) alleles. $Xa^{Xist-21kb}Xi^{WT}$ fibroblasts were distinguished from $Xa^{WT}Xi^{Xist-21kb}$ fibroblasts using RNA FISH against the *Xist* RNA (see below).

RNA FISH: Fibroblasts were grown on coverslips for 24 hr and then fixed in 4% formaldehyde for 15 min at RT. The cells were permeabilized in PBS containing 0.5% Triton-X for 5 min on ice and washed in PBS and 2 \times SSC. RNA FISH hybridization was carried out as previously described (SPECTOR and GOLDMAN 1998.). The *Xist* probe was labeled by nick translation with biotin-21-dUTP. After overnight hybridization at 37 $^{\circ}$, posthybridization washes were done as previously described (SPECTOR and GOLDMAN 1998). The probe was detected with 500-fold dilution of avidin-FITC (Jackson ImmunoResearch, West Grove, PA) at RT for 1 hr. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Assay for late replication: Medium containing 30 μ M of bromodeoxyuridine (BrdU) was added to actively growing fibroblasts at 80% confluence. When lymphocytes were used, 1×10^6 B lymphocytes/ml from mouse spleens were cultivated in 10% fetal bovine serum/10 mM HEPES/RPMI 1640 under stimulation of 10 μ g/ml of lipopolysaccharide for 36 hr at 37 $^{\circ}$ and 5% CO₂ prior to adding 30 μ M of BrdU. A time course of BrdU pulse length was performed on multiple cell lines and these data were used to determine that 4.5 hr is the appropriate length of BrdU incorporation for each replication-timing experiment for both fibroblasts and activated lymphocytes (data not shown). After exponentially growing primary fibroblasts or activated lymphocytes were cultured for 4.5 hr in the presence of BrdU, 0.050 μ g/ml colcemid (Life Technologies, Grand Island, NY) was added 1 hr before harvesting. Cell suspensions were incubated 13 min in 0.4% KCl at 37 $^{\circ}$ followed by multiple changes of 3:1 methanol:acetic acid. Metaphase spreads were prepared by dropping the BrdU-treated cells onto coverslips followed by DNA denaturation in 70% formamide/2 \times SSC at 73 $^{\circ}$ for 2 min. Following preincubation with blocking buffer (1 \times PBS, 10% goat serum, 0.2% Tween 20), incorporated BrdU was detected using monoclonal anti-BrdU antibody (Sigma, St. Louis) followed by Texas-Red anti-mouse antibody (Jackson Immuno-

research) in blocking buffer. Images were captured with Quips mFISH software (Vysis). For a recorded image, the individual colors were stored separately by the Vysis Quips mFISH software and the representation of each color in the final image was determined by the software setting of the gain for that color. *Xist* RNA FISH was not done simultaneously with the BrdU incorporation studies because the *Xist* RNA is lost from the Xi during mitosis. The *Xist* RNA that can be seen on the Xi in mitotic cells is very fragile and the treatments entailed by the BrdU incorporation assay caused the *Xist* RNA to be lost from the Xi.

X chromosome paint: Ten microliters of mouse X chromosome-specific biotinylated probe (Cambio, Cambridge, UK) was used to detect the X chromosomes by *in situ* hybridization to ethanol-dehydrated cells according to the manufacturer's instructions. The probe was detected using the biotin (FITC) detection kit (Cambio). The chromosomes were counterstained with DAPI and viewed with a Leica DMR fluorescent microscope.

Quantitation: The proportions of BrdU signal in wild-type and mutant X chromosomes were compared using Fisher's exact test. The active and inactive X chromosomes were identified using X chromosome paints, and their relative levels of BrdU incorporation determined where the chromosome displaying the higher level of BrdU incorporation was always assumed to be the inactive one. Images were transferred from Quips mFISH to the National Institutes of Health (NIH) Image and the number of pixels occupied by the X chromosomes and the number of pixels occupied by fluorescently labeled BrdU were then calculated from images of mitotic chromosomes using NIH Image (<http://rsb.info.nih.gov/nih-image>). In Figures 1–4, “% area BrdU” represents the number of pixels of BrdU signal divided by the number of pixels of DAPI signal multiplied by 100. NIH Image also measured the intensity of each pixel. “BrdU area \times intensity” represents the number of pixels of BrdU signal multiplied by the average intensity of these pixels. BrdU quantitation was performed on several autosomes that displayed no BrdU according to visual inspection and also on five mitotic chromosomes from BrdU-untreated cells; in both cases we failed to record a single pixel of BrdU using the NIH Image program, indicating that the program was not recording a BrdU signal that could not be detected by eye nor was the program recording background noise. Differences in measurements were tested across categorical groupings by using the Kruskal-Wallis test (KRUSKAL 1964) and listed as *P*-values above the corresponding boxplots. Distributions of measurements across categorical groupings were visualized with boxplots. The statistical analyses were conducted using the software package R (IHAKA and GENTLEMAN 1996), which can be downloaded from <http://cran.r-project.org/>.

RESULTS

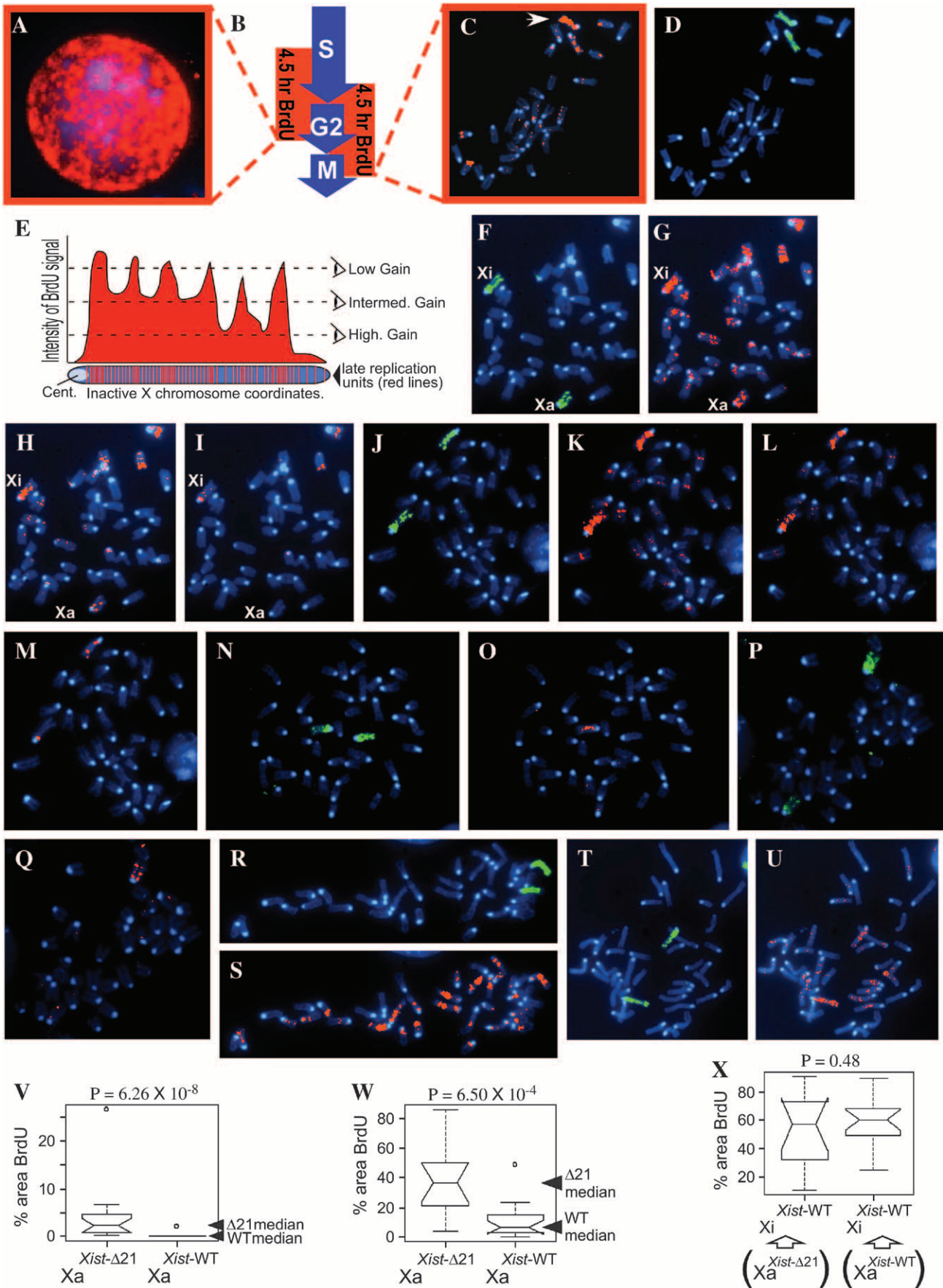
Altered replication times in transformed fibroblast line with 21 kb excised from the active X chromosome: DNA replication timing was examined in transformed mouse fibroblasts by pulse labeling growing cultures of wild-type female fibroblasts for 4.5 hr with BrdU and thereafter detecting the incorporated BrdU by fluorescent immunostaining. Cells that were in early or mid S phase at the beginning of the pulse failed to reach mitosis in 4.5 hr and were expected to display a decondensed nucleus with high levels of BrdU incorporation similar to

the image in Figure 1, A and B. However, cells in late S phase at the onset of the 4.5-hr incubation period reached mitosis and had their incorporated BrdU represented in mitotic chromosome spreads (Figure 1, B and C). Consequently, only BrdU incorporation in late S phase was detected in mitotic chromosome spreads using this procedure. The X chromosomes were identified using X chromosome paint (Cambio) (Figure 1D). This revealed that the latest replicating portions of the genome were disproportionately on one of the two X chromosomes in diploid cells (Figure 1, C and D). In female human cells the Xi is replicated considerably later in S phase than the Xa (PRIEST *et al.* 1967). In female mouse cells, the Xi is not replicated as late in S phase as it is in human cells (EVANS *et al.* 1965; GALTON and HOLT 1965; TIEPOLO *et al.* 1967). This has led to the murine Xi being distinguished by its absence of label incorporation in early S phase rather than by its being disproportionately replicated late in S phase (NESBITT and GARTLER 1970). Although there was always a consistent trend of the Xi displaying more BrdU incorporation late in S phase than the other chromosomes in both primary and transformed fibroblasts, the amount of incorporated BrdU observed on both X chromosomes varied somewhat among mitotic chromosome spreads when using this assay (not shown). One source of variability is that the mitotic cells were not all in the same position in S phase at the time of BrdU addition. A possible second source of variability is that the X chromosomes may not be replicated at the exact same time in every S phase but may be replicated in a distribution of times. Third, the number of fluorescently labeled antibody molecules binding a given amount of incorporated BrdU may not be perfectly even throughout a preparation of mitotic chromosome spreads. This potential source of variability can be reduced by carefully adjusting the setting of the gain used to obtain each image and by using the autosomes as a control.

The amount of fluorescent signal representing incorporated BrdU is dependent on the setting of the gain used to obtain the image. As mentioned in the Introduction, the X chromosomes are likely replicated by >1000 replication units and, at the low resolution afforded by mitotic chromosome spreads, differences in BrdU signal along the chromosome likely reflect differences in the concentration of late replicating units (Figure 1E, bottom). At low gain, only the most intense fluorescent signal emanating from the chromosome is recorded; this represents the regions on the X chromosome with the greatest amounts of BrdU incorporation and, most likely, the regions of the chromosome with the highest concentration of late replication units (Figure 1E). At intermediate gain, intermediate densities of BrdU incorporation also are recorded. At high gain, smaller amounts of fluorescent signal are also recorded, which means that BrdU incorporation will be detected in chromosomal regions where late replicating units are too few in number to be detected at intermediate or low

gain (Figure 1E). Other possible sources of BrdU signal in late S phase regions are elongating replication forks derived from early activated origins and possibly even scatter from neighboring fluorescence because of optical resolution issues. Another possibility is that the anti-BrdU antibody has uneven access to the BrdU-labeled DNA because of differences in chromatin structure or other variables. In primary mouse embryonic fibroblasts subjected to the aforementioned replication-timing assay and photographed at a relatively high gain, a considerable amount of BrdU incorporation was detected on both X chromosomes and most autosomes displayed BrdU incorporation late in S phase (Figure 1, F and G). In a photograph taken at a lower gain, BrdU incorporation could be detected in only roughly half of the chromosomes (Figure 1H). At a still lower gain, BrdU was detectable on the later-replicating X chromosomes and on four autosomes (Figure 1I). To reduce unwanted variation in BrdU signal among samples, all photographs were taken at "high gain" such that, in each photograph, BrdU signal is visible in all but five autosomes. In addition, all mitotic chromosome spreads were recorded at "low gain" such that, in each photograph, BrdU signal is visible in only five autosomes and only one pixel of BrdU is detected in one of these five autosomes. Note that even at disparate settings, the same X chromosome displays a greater amount of BrdU incorporation than the other X chromosome (compare Figure 1, G and I). Thus at either high or low gain the X chromosome with the greater amount of BrdU incorporation can be assumed to be the inactive one.

To determine whether the *Xist* locus on the Xa influences chromosomal replication timing, we examined a TAG-transformed fibroblast cell line derived from mouse embryos containing a *Xist* allele in which 21 kb of the gene was flanked by *Lox* sites (CSANKOVSKI *et al.* 1999). The 21-kb *Xist* sequence was excised using cre recombinase and clonal cell lines carrying the resulting *Xist*- Δ 21 allele on the Xa were established. Mitotic chromosome spreads prepared from one such cell line and from a wild-type fibroblast line were subjected to X chromosome paint to distinguish the X chromosomes from autosomes. The level of BrdU incorporation in the Xa appeared to be higher in mutant than in wild-type cells and rivaled the level of incorporation observed in the Xi across various gain settings (Figure 1, J–M). The levels of BrdU incorporation were compared at a low gain where BrdU signal is visible in only five autosomes. Using this methodology, little or no BrdU incorporation was detected on the earlier-replicating (active) X chromosome from Xa^{*Xist*-WT}Xi^{*Xist*-WT} cells (Figure 1, N and O, 17 diploid mitotic chromosome spreads examined). In contrast, BrdU signal was observed on the earlier-replicating X chromosome from Xa^{*Xist*- Δ 21}Xi^{WT} cells (Figure 1, P and Q, 12 diploid mitotic chromosome spreads examined). The level of BrdU incorporation in each X chromosome was quantitated using NIH Image



software and signals between the 17 $Xa^{Xist-WT}Xi^{Xist-WT}$ and 12 $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ mitotic chromosome spreads were compared using boxplots. This revealed that significantly higher levels of BrdU incorporation were detected on the active X chromosome in the $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ mitotic chromosome spreads than in the $Xa^{Xist-WT}Xi^{Xist-WT}$ spreads (Figure 1V) while little or no significant difference was seen for the Xi in the same spreads ($P=0.0344$) (not shown). In the same mitotic chromosome spreads, the levels of BrdU signal were also compared at high gain where the fluorescent signal representing BrdU incorporation was visible in all but five autosomes. At the high gain, the Xa also displayed significantly higher levels of BrdU signal in $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ (Figure 1, T and U) than in $Xa^{Xist-WT}Xi^{Xist-WT}$ chromosome spreads (Figure 1, R and S). At the high gain, the BrdU signal was quantitated using NIH Image software, revealing a significantly higher level of BrdU incorporation on the Xa in $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spreads (Figure 1W), while little or no significant difference was seen in the level of BrdU incorporation on Xi in these same spreads ($P=0.48$) (Figure 1X). This suggested that the 21-bp region of the Xa may be needed to maintain earlier replication of the Xa.

Altered replication timing in primary female fibroblasts: The higher levels of BrdU incorporation in the Xa in a $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ cell line than in a $Xa^{Xist-WT}Xi^{Xist-WT}$ cell line raised the issue of whether this difference in replication timing was caused by the 21-kb deletion on the Xa or by changes that occurred during the transformation of the two cell lines that were independent of the deletion. To distinguish between these two possibilities, primary fibroblasts were obtained from three

$Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ and three $Xa^{Xist-WT}Xi^{Xist-WT}$ embryos. Inheritance of the $Xist-\Delta 21$ kb allele results in nonrandom X-inactivation with the wild-type X chromosome being the inactive X chromosome (Xi^{WT}) and the mutant X chromosome being the active one ($Xa^{Xist-\Delta 21}$) in all cells (not shown) and in this regard resembles the nonrandom X-inactivation that had previously been reported for embryos that had inherited a 16-kb deletion in the $Xist$ gene (MARAHRENS *et al.* 1998). The six primary fibroblast lines were subjected to the aforementioned replication-timing assay. At a high gain setting where all but five autosomes displayed BrdU signal, the primary $Xa^{Xist-WT}Xi^{Xist-WT}$ mitotic chromosome spreads displayed less BrdU signal on the Xa (Figure 2, A and B) than did the primary $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spreads (Figure 2, C and D). At these settings, the signal representing BrdU incorporation was quantitated from 40 mitotic chromosome spreads from each of three primary $Xa^{Xist-WT}Xi^{Xist-WT}$ cell lines (120 mitotic chromosome spreads total) and three primary $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ fibroblast cell lines (120 mitotic chromosome spreads total) and the medians and distributions were compared using boxplots. A significantly larger proportion of the area of the Xa displayed BrdU incorporation in $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ than in $Xa^{Xist-WT}Xi^{Xist-WT}$ primary fibroblasts (Figure 2E). When, for each individual Xa, the area displaying BrdU signal was multiplied by the average intensity of BrdU signal, the significance of the difference between the $Xa^{Xist-\Delta 21}$ and the $Xa^{Xist-WT}$ in the primary fibroblasts increased (Figure 2F). In contrast, there was no significant difference in the level of BrdU signal on the $Xi^{Xist-WT}$ in the same mitotic chromosome spreads ($P=0.32$)

FIGURE 1.—BrdU incorporation in late S phase in transformed female mouse embryonic fibroblasts that are wild type with respect to the $Xist$ locus ($Xa^{Xist-WT}Xi^{Xist-WT}$) or carry a 21-kb deletion in the $Xist$ locus on the active X chromosome ($Xa^{Xist-\Delta 21}Xi^{Xist-WT}$). In all mitotic chromosome spreads, the active X chromosome is always assumed to be the X chromosome displaying a lower proportion of BrdU incorporation. Following exposure of actively growing cultures to BrdU for 4.5 hr, mitotic chromosome spreads were prepared and immunostained using anti-BrdU (red), hybridized to X chromosome paint (green), and counterstained using DAPI (blue). The late-replicating inactive X chromosome in a mitotic chromosome spread may be marked by an arrow. (A–D) Diploid wild-type transformed fibroblast (A); possible times in the cell cycle that the indicated fibroblasts were inferred to be exposed to BrdU (B); diploid wild-type transformed fibroblast (C and D). (E) Simplified cartoon of a hypothetical mitotic telocentric chromosome (blue) with the centromere at the left end of the chromosome (“Cent.”) and late replication units along that chromosome (vertical red lines). Above the chromosome is a graph representing the hypothetical intensities of BrdU signal along the X chromosome from a replication-timing assay where BrdU had been incorporated into the chromosomal DNA late in the S phase and then subjected to red fluorescent immunostaining in the following mitosis. The graph illustrates that only the highest concentrations of late replication units on the chromosome would be visible at a low gain, while regions with lower concentrations of late replication units would also display a visible fluorescent signal at an intermediate gain, and even lower concentrations of late-replicating units would display a fluorescent signal at a high gain. (F–I) Mitotic chromosome spread from a diploid primary $Xa^{WT}Xi^{WT}$ fibroblast displaying X chromosome paint (F), BrdU incorporation at a higher gain (G), an intermediate gain (H), and a lower gain where only four autosomes display BrdU and one autosome displays one pixel of BrdU (I). (J–M) Mitotic chromosome spread from a transformed $Xa^{Xist-\Delta 21}Xi^{WT}$ fibroblast displaying X chromosome paint (J), BrdU incorporation at a higher gain (K), an intermediate gain (L), and a lower gain (M). Transformed $Xa^{WT}Xi^{WT}$ (N and O) and $Xa^{Xist-\Delta 21}Xi^{WT}$ (P and Q) fibroblasts subjected to the BrdU replication-timing assay and recorded at a gain where five autosomes display BrdU incorporation. Transformed $Xa^{WT}Xi^{WT}$ (R and S) and $Xa^{Xist-\Delta 21}Xi^{WT}$ (T and U) fibroblasts subjected to the BrdU replication-timing assay and recorded at a gain where all but five autosomes display BrdU incorporation. (V–X) Boxplots representing the quantitation of the proportion of area of the Xa or Xi displaying BrdU signal from the mitotic spreads of 17 $Xa^{WT}Xi^{WT}$ and 12 $Xa^{Xist-\Delta 21}Xi^{WT}$ transformed cells at a gain where five autosomes display BrdU signal and the BrdU signal in the Xa was quantitated (V) and at a gain where all but five autosomes display BrdU signal and the signal in the Xa (W) or the Xi (X) was quantitated. In V and W, the median of each set of measurements is indicated to the right of the boxplots. In V–X, P -values represent the significance of the difference between pairs of boxplots and were obtained using the Kruskal-Wallis test.

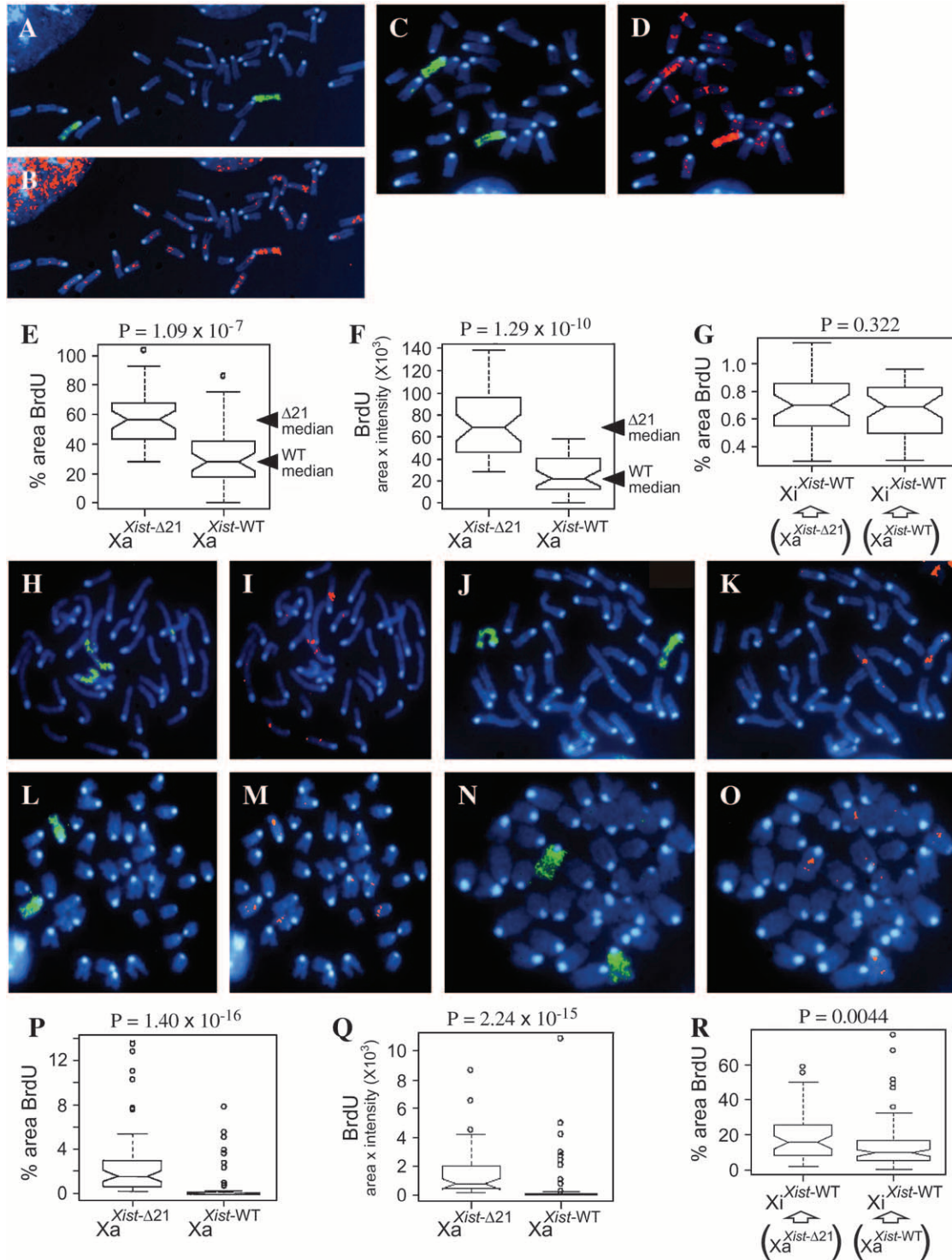


FIGURE 2.—Late S-phase BrdU incorporation into the X chromosomes in primary mouse embryonic fibroblasts. Mitotic chromosome spreads were prepared from exponentially growing cultures of primary mouse embryonic fibroblasts exposed to BrdU for 4.5 hr prior to immunostaining. Each spread is from a different embryo and each spread was subjected to X chromosome paint and fluorescent immunostaining using antibodies that recognize BrdU. Within each spread, the active X chromosome is always assumed to be the X chromosome displaying the lower level BrdU incorporation. (A–G) Data obtained from mitotic chromosome spreads of primary fibroblasts where the gain is adjusted such that, for each spread, all but five autosomes display BrdU signal; $Xa^{Xist-WT}Xi^{Xist-WT}$ spread displaying X chromosome paint (A) and BrdU incorporation (B); $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spread displaying X chromosome paint (C) and BrdU incorporation (D); quantitation of BrdU signal using NIH Image from 120 $Xa^{Xist-WT}Xi^{Xist-WT}$ and 120 $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spreads from six embryos (40 spreads from each embryo) summarized in boxplots that display the percentage of BrdU signal for the Xa (E), the percentage of BrdU signal multiplied by the average intensity of the BrdU signal for the Xa (F), and the percentage of BrdU signal for the Xi (G). (H–R) Data obtained from mitotic chromosome spreads where the gain is adjusted such that, for each spread, five autosomes display BrdU signal and one autosome displays one pixel of BrdU signal;

(Figure 2G). Fluorescent signal representing incorporated BrdU was also quantitated from the same 240 mitotic chromosome spreads at a low gain setting where only five autosomes display signal. Under these conditions, incorporated BrdU was undetectable on the Xa in most chromosome spreads from $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ cells (Figure 2, H–K) while all mitotic chromosome spreads from $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ cells displayed BrdU signal on the Xa (Figure 2, L–O). The increase in BrdU signal on the Xa in primary $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ over $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ cells was even more significant at these settings: compare $P = 1.40 \times 10^{-16}$ (Figure 2P) and $P = 2.24 \times 10^{-15}$ (Figure 2Q) at low gain to $P = 1.09 \times 10^{-7}$ (Figure 2E) and $P = 1.29 \times 10^{-10}$ (Figure 2F) at high gain. A slight difference in BrdU signal on the Xi between the $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ and the $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ chromosome spreads was detected at low gain settings (Figure 2R); however, this difference disappeared when BrdU area \times intensity was considered ($P = 0.0920$) (not shown). At both high and low gain our results showed that change in replication timing of the Xa in relation to the rest of the genome was caused by the 21-kb deletion. Because in primary fibroblasts the differences between the $Xa^{Xist-\Delta 21}$ and the $Xa^{Xist^{WT}}$ were most striking at a lower gain where BrdU incorporation can be detected on only five autosomes, all subsequent measurements were performed using only this approach.

Shift in replication timing is not unique to fibroblasts: We next set out to determine whether the increase in BrdU incorporation on the Xa in response to the *Xist*- $\Delta 21$ kb allele was unique to embryonic fibroblasts or also occurred in other cell types. To this end, activated lymphocytes were obtained from the spleens of two $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ mice and from two age-matched $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ 129 mice. The four lymphocyte preparations were subjected to the BrdU replication-timing assay and 80 mitotic chromosome spreads were photographed from each preparation at a gain where five autosomes display BrdU signal. The results for the activated lymphocytes closely resembled the earlier results for primary fibroblasts: significantly lower levels of incorporated BrdU were observed on the Xa in mitotic chromosome spreads from activated $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ lymphocytes (Figure 3, A–D, I–J) than from activated $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ lymphocytes (Figure 3, E–J) while measurements on the Xi in the same spreads were not significantly different ($P = 0.61$) (Figure 3K). Thus, the relatively late replication of the Xa in $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ cells was not unique to fibroblasts.

The finding that the $Xa^{Xist-\Delta 21}$ displays more BrdU incorporation than the $Xa^{Xist^{WT}}$ in an assay for late replication raised the possibility that the 21-kb deletion causes S phase and/or G_2 phase to be compressed. If the $S + G_2$ phase interval is compressed, then a low proportion of an asynchronous culture will be in these phases at any given time and the proportion of cells labeled by a pulse of BrdU will be lower. One hundred $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ and 100 $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ nonmitotic fibroblasts were scored for presence or absence of BrdU. No significant difference in the proportion of BrdU-containing cells was observed using Fisher's exact test ($P = 0.45$). One hundred $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ and 100 $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ nonmitotic lymphocytes also did not produce a significant difference ($P = 0.75$).

The deletion at the *Xist* locus alters replication timing in cells with one X chromosome: The finding that a deletion in the *Xist* locus on the Xa alters chromosomal replication timing raised the issue of whether a Xi was needed to be present in the cell to achieve this effect. The BrdU incorporation assay for late replication timing was performed on primary fibroblasts from a $X^{Xist^{WT}}O$ (Figure 4, A and B) and a $X^{Xist-\Delta 21}O$ (Figure 4, C and D) mouse (40 mitotic chromosome spreads from each). The X chromosome was found to display significantly more BrdU signal in the $X^{Xist-\Delta 21}O$ cells than in the $X^{Xist^{WT}}O$ cells (Figure 4E). The assay was also performed on mitotic chromosome spreads from primary fibroblasts from two $X^{Xist^{WT}}Y$ (Figure 4, F and G) and two $X^{Xist-\Delta 21}Y$ (Figure 4, H–K) embryos (40 spreads from each embryo). The XY chromosome spreads displayed significantly higher levels of BrdU signal on the X chromosome when in the presence of the *Xist*- $\Delta 21$ allele (Figure 4L).

DISCUSSION

We show that the active X chromosome displayed increased BrdU incorporation in an assay for late replication when 21 kb was deleted from the *Xist* locus of the Xa. This effect was observed in transformed fibroblasts, primary embryonic fibroblasts, and in activated lymphocytes. The presence of the 21-kb deletion also led to increased levels of BrdU incorporation in the late replication assay in male cells and in female XO cells. The element(s) residing at the *Xist* locus on the Xa therefore influences the timing at which regions on the Xa are replicated relative to the rest of the genome. The element(s) influences replication time independently

$Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ spread displaying X chromosome paint (H) and BrdU incorporation (I); $Xa^{Xist^{WT}}Xi^{Xist^{WT}}$ spread displaying X chromosome paint (J) and BrdU incorporation (K); $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ spread displaying X chromosome paint (L) and BrdU incorporation (M); $Xa^{Xist-\Delta 21}Xi^{Xist^{WT}}$ spread displaying X chromosome paint (N) and BrdU incorporation (O); quantitation of BrdU signal between 120 $Xa^{Xist-\Delta 21}$ cells and 120 $Xa^{Xist^{WT}}$ cells (40 spreads from each of six embryos) using boxplots that display the percentage of BrdU signal for the Xa (P), the percentage of BrdU signal multiplied by average intensity of the BrdU signal for the Xa (Q), and the percentage of BrdU signal for the Xi (R). *P*-values measure the significance of differences in measurements across the two categorical groupings using the Kruskal-Wallis test.

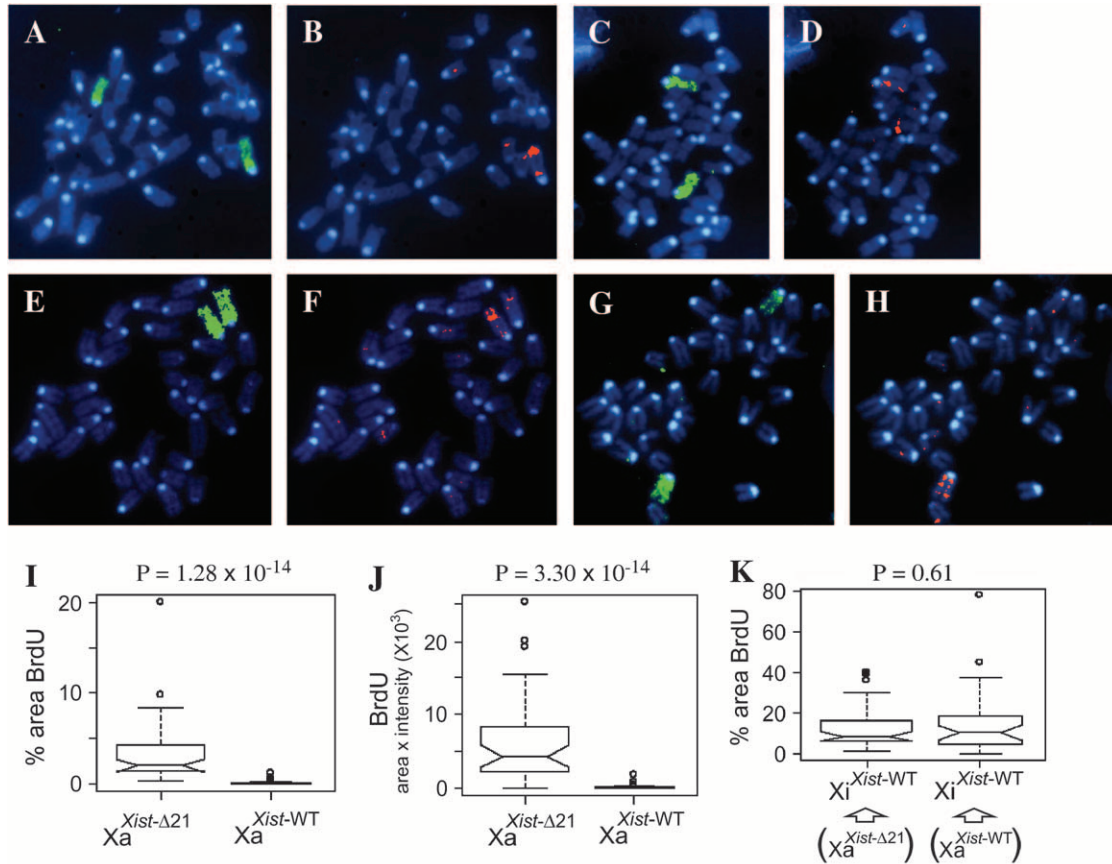


FIGURE 3.—BrdU signal observed in mitotic chromosome spreads from exponentially growing cultures of lymphocytes from adult mice. Following exposure of cultures to BrdU for 4.5 hr, mitotic chromosome spreads were prepared and immunostained using anti-BrdU (red), hybridized to X chromosome paint (green), and counterstained using DAPI (blue). Images were analyzed with the gain adjusted such that, for each spread, five autosomes display at least one pixel of BrdU signal (“low gain”). Each spread is from a different embryo. $Xa^{Xist-WT}Xi^{Xist-WT}$ spread displaying X chromosome paint (A) and BrdU incorporation (B); $Xa^{Xist-WT}Xi^{Xist-WT}$ spread displaying X chromosome paint (C) and BrdU incorporation (D); $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spread displaying X chromosome paint (E) and BrdU incorporation (F); $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ spread displaying X chromosome paint (G) and BrdU incorporation (H); box-plots comparing the amount of BrdU signal between 80 $Xa^{Xist-\Delta 21}$ cells and 80 $Xa^{Xist-WT}$ cells (40 mitotic chromosome spreads from each embryo) when considering the proportion of the area of the active X chromosomes occupied by BrdU (I), the area of the Xa occupied by BrdU signal multiplied by the intensity of the BrdU signal (J), and the area of the Xi occupied by BrdU (K). *P*-values measure the significance of differences in measurements across categorical groupings using the Kruskal-Wallis test.

of the presence of an inactive X chromosome. Regions located far away from the *Xist* locus were affected by the 21-kb deletion. For example, one or more regions located very close to the centromere, and therefore >90 Mb away from the *Xist* locus, which is 98 Mb distal to the centromere, frequently displayed a BrdU signal at low gain in $Xa^{Xist-\Delta 21}Xi^{WT}$ cells (Figure 1, M and Q; Figure 2O; Figure 3H; Figure 4D) but not at low gain in $Xa^{WT}Xi^{WT}$ cells (Figure 1, I and O; Figure 2, I and K; Figure 3, B and C; Figure 4, B and G).

The replication-timing data are most easily explained by a *cis*-acting model: Two ways in which our results can be interpreted are as follows. First, a *cis*-acting element at the *Xist* locus of the Xa may influence the replication timing of the Xa. A second explanation is that an element at the *Xist* locus on the Xa controls the time that it takes a cell to traverse S phase and/or G₂ phase of the cell cycle. In the absence of this element, S phase

and/or G₂ phase are compressed. However, compression of S and/or G₂ phase would be expected to result in an increase in BrdU incorporation on the Xi. Since no significant difference in BrdU incorporation was observed on the Xi between $Xa^{Xist-\Delta 21}Xi^{Xist-WT}$ and $Xa^{Xist-WT}Xi^{Xist-WT}$ cells, our data are more consistent with *cis*-regulation of replication timing on the Xa. Triplet-repeat expansions at the fragile X locus on the Xa in humans have previously been shown to influence replication time over megabase distances (HANSEN *et al.* 1997) and long-distance control of replication timing has also been reported on autosomes (FORRESTER *et al.* 1990; CIMBORA *et al.* 2000; SINGH *et al.* 2003; ENSMINGER and CHESS 2004). Since the *Xist* locus of the Xa is not transcribed, and there is precedent for long-distance *cis* influence of replication timing by a nontranscribed locus (HANSEN *et al.* 1997), we consider *cis*-regulation of the Xa replication timing an attractive

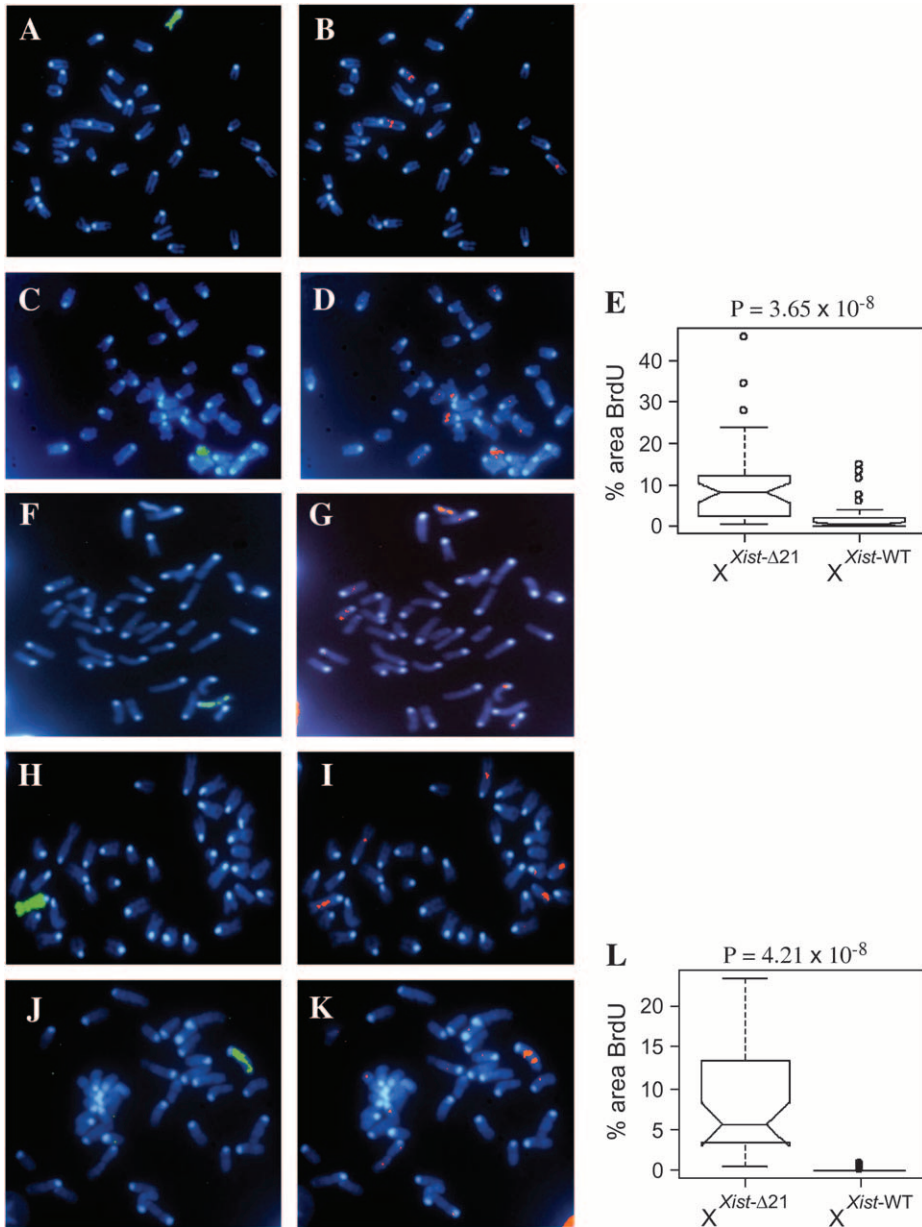


FIGURE 4.—BrdU signal observed in mitotic chromosome spreads from exponentially growing cultures of primary embryonic XO and XY fibroblasts. Following exposure of cultures to BrdU for 4.5 hr, mitotic chromosome spreads were prepared and immunostained using anti-BrdU (red), hybridized to X chromosome paint (green), and counterstained using DAPI (blue). Each spread is from a different embryo. $X^{Xist^{WT}O}$ spread displaying X chromosome paint (A) and BrdU incorporation (B); $X^{Xist^{\Delta 21}O}$ spread displaying X chromosome paint (C) and BrdU incorporation (D); boxplots of the data from 40 $X^{Xist^{WT}O}$ and 40 $X^{Xist^{\Delta 21}O}$ spreads (E); $X^{Xist^{WT}Y}$ spread displaying X chromosome paint (F) and BrdU incorporation (G); $X^{Xist^{\Delta 21}Y}$ spread displaying X chromosome paint (H) and BrdU incorporation (I); $X^{Xist^{\Delta 21}Y}$ spread displaying X chromosome paint (J) and BrdU incorporation (K); boxplots comparing the proportion of the area of the active X chromosomes occupied by BrdU signal between 80 $X^{Xist^{WT}Y}$ cells and 80 $X^{Xist^{\Delta 21}Y}$ cells (L) (40 mitotic chromosome spreads from each of four embryos). The P -value measures the significance of differences in measurements across categorical groupings using the Kruskal-Wallis test.

explanation for our results. It was previously proposed that LINE-1 elements play a role in X-inactivation (LYON 1998; MARAHRENS 1999; BAILEY *et al.* 2000; HANSEN 2003) and we previously proposed that the *Xist* locus controls the chromatin structure of the Xi via physical looping interactions with LINE elements (MARAHRENS 1999). We speculate that elements at the nontranscribed *Xist* locus of the Xa control replication at distant sites on the Xa by similar looping interactions with LINE-1 elements and other repetitive sequences.

Since late replication times are associated with gene repression, our findings raise the question of whether gene expression is affected by the later replication of the $Xa^{Xist^{\Delta 21}}$. The altered gene expression levels that may occur on the $Xa^{Xist^{\Delta 21}}$ are presumably not very severe since $Xa^{Xist^{\Delta 21}}Xi^{Xist^{WT}}$ mice are viable. We are currently

using gene expression arrays to search for gene expression changes on the $Xa^{Xist^{\Delta 21}}$.

We thank Sui Huang and Chen Wang for valuable technical help. Adenovirus AdCre (TAN *et al.* 1999) and adenovirus expressing GFP were kindly provided by Carol Eng and Arnold J. Berk. We thank Jennifer Salstrom and Elaine Wong for generating some of the primary fibroblasts and Tao Shi for performing some statistical analyses. This work was supported by March of Dimes Basil O'Connor Starter Scholar research award 5-FY99-819 (Y.M.) and National Institutes of Health grant R01 HD41451:01 (Y.M.).

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Communicating editor: J. A. BIRCHLER