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Same Origins of DNA Replication Function on the Active and Inactive Human X Chromosomes

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Abstract We previously characterized a functional origin of DNA replication at the transcriptional promoter of the human hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene (Cohen et al. [2002] J. Cell. Biochem. 85:346-356). This origin was mapped using a quantitative PCR assay to evaluate the relative abundance of HPRT markers in short nascent DNA strands isolated from asynchronous cultures of male fibroblasts. The *HPRT* gene on the X chromosome is transcriptionally active in male human fibroblasts. It is known that on the heterochromatic X chromosome in female cells the *HPRT* gene is transcriptionally silenced and its replication timing changes from early to late in S phase. This change in replication timing could indicate that replication of the *HPRT* gene is under the control of different origins of DNA replication in the active (euchromatic, early replicating) and the inactive (heterochromatic, late replicating) X chromosomes. In the present study, we identified the location of the origin of replication of a second X chromosome gene, glucose-6-phosphate dehydrogenase (*G6PD*), which we mapped to its transcriptional promoter, in normal male human fibroblasts. Then, we determined the activity of the previously identified HPRT and the G6PD human origins in hybrid hamster cells carrying either the active or the inactive human X chromosome. The results of these studies clearly demonstrated that the human HPRT and G6PD origins of replication were utilized to the same extent in the active and the inactive X chromosomes. Therefore, transcription activity at the *HPRT* and *G6PD* genes is not necessary for initiation of DNA replication at the origins mapped to these chromosomal loci. J. Cell. Biochem. 88: 923–931, 2003.

Key words: replication origin; G6PD; HPRT; X chromosome; quantitative PCR

In eukaryotic cells, the process of DNA replication occurs once per cell cycle and begins at sites called DNA replication origins. Complete duplication of the human genome is estimated to require the activation of approximately 30,000 DNA replication origins [Todorovic et al., 1999]. To date, only a fraction of these origins has been identified and no consensus sequence has emerged as a structural/functional signature of a replication origin in higher eukaryotes. It is interesting however, that several of the identified origins of DNA replication are associated with CpG islands/transcriptional promoters [Biamonti et al., 1992; Delgado et al., 1998; Tao et al., 2000; Cohen et al., 2002], suggesting that there may be a dual function associated with these GC-rich regions.

The order in which specific sequences are replicated is lineage specific and is maintained from one cell generation to the next [Hatton et al., 1988; Jackson and Pombo, 1998]. Lineage specific differences in the timing of replication have been linked to gene expression [Holmquist, 1987]. In general, a tissue specific gene replicates earlier in the S phase in cells where it is expressed than in cells where it is transcriptionally silent. In female cells, a difference in timing of gene replication also occurs between the inactive X chromosome, where there is widespread heterochromatization, and the active X chromosome. Most genes on the heterochromatic X chromosome replicate later than their counterparts on the transcriptionally active euchromatic X chromosome. The process of heterochromatization involves modifications in both DNA and chromatin-associated

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proteins. These changes are especially evident at transcriptional promoters and include methylation of DNA and hypoacetylation of histones [Gilbert and Sharp, 1999]. It has been suggested that such chromatin modifications lead to transcriptional silencing by inhibiting necessary changes in DNA secondary structure [Ackerman et al., 1993]. Since some replication origins coincide with transcriptional promoters, it is possible that transcriptional silencing may also result in the inability of an origin to be activated. For example, the shift in timing of replication of the *HPRT* gene in the heterochromatic X chromosome could represent the inactivation of its promoter-associated origin and replication of this gene could result from replication originated at adjacent or alternative replication origins. It has been reported that the origin of replication associated with the β-globin gene promoter is functional even in cells where the gene is not expressed [Kitsberg et al., 1993]. The *β-globin* gene, however, is found on chromosome 11, a chromosome that is not subjected to near complete heterochromatization as it occurs in one of the X chromosomes in female cells.

In the present study we examine the activity at two origins of replication on the X chromosome. As previously reported [Cohen et al., 2002], quantitative PCR of short nascent DNA strands was used to map an origin of DNA replication at the transcriptional promoter for the X-linked human *HPRT* gene in male fibroblasts. Here we report the mapping of a second origin of DNA replication on the X chromosome, this one associated with the transcriptional promoter of the *G6PD* gene. The activities of both origins were tested in hamster cells, which contained either the active or the inactive human X chromosome. We found that the HPRT and G6PD origins of replication that are functional in the active human X chromosome are utilized even when the two genes are transcriptionally silent in the inactive human X chromosome.

MATERIALS AND METHODS

Cell Culture and Isolation of Short Nascent DNA Fragments

Two sources of diploid human male fibroblasts were used in this study: hTERT-GM01604 (1604 cells), telomerase-immortalized fibroblasts from fetal lung [Ouellette et al., 2000] and NHF1, a strain of fibroblasts previously isolated in this laboratory from neonatal foreskin [Boyer et al., 1991]. The two hamster/human hybrid cell lines used were GM06318 (NIGMS Human Genetic Mutant Cell Repository) and X8-6T2S1 (a gift from Dr. R. Scott Hansen, Department of Medicine, University of Washington, Seattle, WA), which contain a single active or inactive human X chromosome, respectively. Human fibroblast and GM06318 cells were cultured in Eagle's minimal essential medium (MEM; GIBCO-BRL, Grand Island, NY) supplemented with 2 mM L-glutamine (GIBCO-BRL) and 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). X8-6T2S1 were grown in RPMI 1640 medium containing 2 mM L-glutamine and 10% fetal bovine serum. GM06318 cells were grown for ten days in complete MEM medium supplemented with 1 × HAT solution (hypoxanthine/aminopterin/thymidine; GIBCO-BRL) to remove from the cultures any cells that had lost the human *HPRT* gene. X8-6T2S1 cells were grown for ten days in complete RPMI 1640 medium supplemented with 30 μM 6-thioguanine (2-amino-6-mercaptopurine; Sigma-Aldrich Corp., St. Louis, MO), to select against HPRT-positive cells.

Logarithmically growing cultures of human fibroblasts or hamster hybrid cells were harvested by trypsinization and washed twice with phosphate buffered saline (PBS). Nascent DNA preparations were obtained as described by Cohen et al. [2002]. The nascent DNA samples from male human fibroblasts used in the present study were previously analyzed for origin activity at the HPRT origin [Cohen et al., 2002; preparations 1604a and NHF1a]. Two preparations from each of the hamster cell lines were used in this study; one preparation was obtained from cells harvested while in selection medium, the second from cells cultured in normal growth medium after the selection period. There were no significant differences in results from nascent strand abundance assays between preparations from the same cell line.

Preparation of Standard DNA for Quantitative PCR

DNA was purified from nuclei of logarithmically growing cells (an individual preparation for each cell type). After lysis with detergent and proteinase K treatment, DNA was extracted

with organic solvents, precipitated with isopropanol, and resuspended in TE (100 mM Tris, pH 8.0, 1 mM EDTA). DNA was sonicated on ice with a Branson model W140D sonifier for 21 s (seven pulses of 3 s) at 32% power. This treatment resulted in fragments with a size distribution between 500 and 3,000 bp (peak at approximately 1,500 bp), as estimated by agarose gel electrophoresis and ethidium bromide staining.

Primer Design and PCR

Primers for human HPRT were previously described [Cohen et al., 2002]. Human G6PD primers were designed using Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and GenBank sequence AF277315 (Table I). A primer set for the *DHFR* gene, corresponding to bases 5475-5630 in X94372 (forward primer CACTACCTGATGCCTCTTTC; reverse primer AATACATCCTTCTTCCCCTG) was used to amplify a region with no replication origin activity [Pelizon et al., 1996]. Amplification of nascent DNA with this primer set gave baseline values that were used to normalize results from all hamster hybrid nascent DNA preparations, so that data between preparations and different cell lines could be compared.

PCR was performed using the Omn-E PCR or the PCR-Express thermocycler (Hybaid Ltd., Ashford, Middlesex, UK). PCR reaction conditions were previously described [Cohen et al., 2002]. A negative control without template DNA was included with each set of PCR reactions. PCR products were separated by gel electrophoresis in 2% agarose in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3), and visualized with ethidium bromide. No extraneous bands were generated with any of the primer sets, when using either genomic or nascent DNA as template. All PCR reactions were run in the linear range of amplification. This was confirmed in each experiment by performing a PCR standard curve with increasing amounts of sonicated genomic DNA of the same size range as the nascent DNA. This standard curve was also used to quantify the amount of PCR products generated with each of the PCR primer sets. Images of ethidium bromide gels were captured and band densities analyzed with the AlphaInnotech imaging system and AlphaEase software (San Leandro, CA, version 5.5). Standard curve results were graphed using Microsoft Excel 2000. The relative abundance (ng equivalent) of each sequence marker in nascent DNA was determined from the standard curve that was generated in parallel using the same primer set but with sonicated genomic DNA.

In studies to map the replication origin associated with the *HPRT* gene [Cohen et al., 2002], we showed that quantification of PCR products by comparison with a standard curve, as described above, yielded results that were not different from those using competitive PCR analyses. The latter uses an internal competitor molecule, which is amplified by the specific primer set added to each reaction, but generates PCR products of a different size than those originating from nascent DNA. This approach is typically used in origin mapping studies using quantitative PCR of short nascent DNA strands [Giacca et al., 1997]. However, since we validated the standard curve method by direct comparison with the competitive PCR method [Cohen et al., 2002], and the former offered the advantage of being easier and faster to perform, we decided that it was not necessary to use internal competitors in the present study.

TABLE I. Primers Used for PCR Analysis of G6PD Sequences[a]

	Location in AF277315 (nt)		
Primer set	Forward primer	Reverse primer	Annealing temp. (°C)
G6PD1	24008-24027	24169-24150	50
G6PD2	26035-26054	26159-26140	52
G6PD3	27064-27083	27204-27185	52
G6PD4	27261-27279	27429-27410	52
G6PD5	28351-28369	28509-28491	54
G6PD6	28513-28530	28662-28643	50
G6PD7	28959-28978	29124-29105	52
G6PD8	29122-29142	29254-29235	53
G6PD9	30367-30386	30530-30511	52

[a]See Cohen et al. [2002] for information on HPRT primers.

RESULTS

The primary objective of this study was to examine whether the utilization of origins of replication mapped to the 5′ end of specific genes was linked to their transcriptional activity. Origins of DNA replication on the X chromosome were considered particularly suitable for these analyses because of the transcriptional silencing of most genes in this chromosome that follows its heterochromatization. We previously mapped a functional origin of replication near the HPRT promoter [Cohen et al., 2002] and sought to find another origin on the X chromosome before addressing the biological link stated above. In addition to HPRT [Cohen et al., 2002], several origins of replication have been found in GC rich promoter regions of other genes. These include the hamster adenine phosphoribosyl transferase (*APRT*), growth arrest and DNA damage inducible (*GADD*), and thymidine kinase (*TK*) genes [Delgado et al., 1998], and the human c-myc [Tao et al., 2000], Trk A [Delgado et al., 1998], lamin B2/ppv1 [Biamonti et al., 1992]. This association motivated us to look for a functional origin of replication at the transcriptional promoter of the X-linked *G6PD* gene. A large CpG island is found in the G6PD gene promoter, which is also utilized by the NF-κB modulator protein gene (*NEMO*) [Galgoczy et al., 2001]. A schematic representation of the region is shown in Figure 1, including the position of the transcriptional promoter and exons 1 and 2 of G6PD. The first exon of NEMO is out of range of the sequence shown.

Analysis of origin activity by quantifying specific sequence markers in short nascent DNA strands has been used to map several origins of replication (i.e., those in the human *lamin B2* [Giacca et al., 1994], *β-globin* [Aladjem et al., 1995], *c-myc* [Tao et al., 2000], DNA methyl transferase (*DNMT1*) [Araujo et al., 1999] genes, and ori-β and ori-β′ in the hamster dihydrofolate reductase (*DHFR*) gene [Pelizon et al., 1996; Kobayashi et al., 1998]). This technique is based on the premise that nascent DNA strands increase in length bidirectionally from the origin. In short nascent fragments (from 900 to 1,700 bases in length) sequences that are closer to the origin will be more abundant than sequences further away from the origin.

Figure 1 shows results from the quantitative PCR analysis of a short nascent DNA preparation from hTERT-GM1604 diploid human fibroblasts (preparation 1604a). The position and relative abundance of the G6PD primer sets are displayed relative to the sequence elements shown. The exact location of primer sets for the G6PD region in AF277315 is listed in Table I, along with PCR annealing temperatures. Each of the nine primer sets was tested at least three times. The peak of abundance is found around the promoter/CpG island region, between primer sets G6PD4 and G6PD5. The 950 bp gap between G6PD primer sets 4 and 5 corresponds to a region of extremely high GC content. Primer sets designed to sit in this CpG region were either difficult to amplify or failed to amplify in a reproducibly linear manner. A preparation of short nascent DNA from a second normal fibroblast strain (NHF1a) was also analyzed (Fig. 2). The data from both preparations indicated that the center of origin activity was around the G6PD promoter. This is the first report of a functional origin of replication in association with the G6PD promoter. A previous study [Rivella et al., 1999] used hybridization of amplified DNA to investigate the presence of

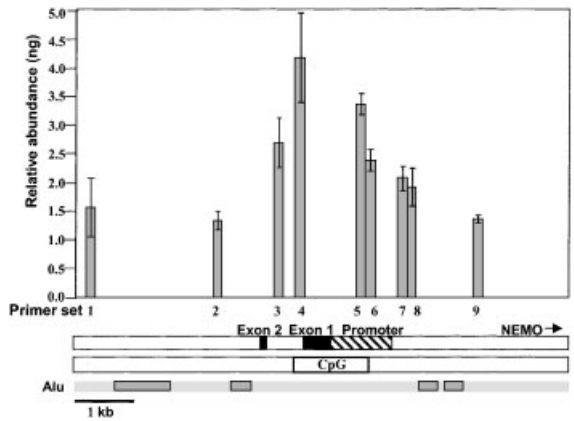

Fig. 1. Quantitative PCR mapping of the origin of DNA replication in the human G6PD region. The relative abundance of 9 PCR markers in a preparation of nascent DNA (1604a) is illustrated. PCR primer sets were generated using sequence data from GenBank accession number AF277315 and are listed in Table I. The position of each primer set in the chart is aligned with a schematic representation of sequence elements in the G6PD region. The relative abundance (ng equivalent) of each PCR marker in the nascent DNA preparation was determined by comparing the amount of product generated from a set amount of nascent DNA to a standard curve using sonicated genomic DNA as the template for PCR. Each primer set was tested at least three times and error bars indicate the standard deviation of the mean. The peak of nascent strand abundance coincides with the CpG island and transcriptional promoter, located between primer sets 4 and 5.

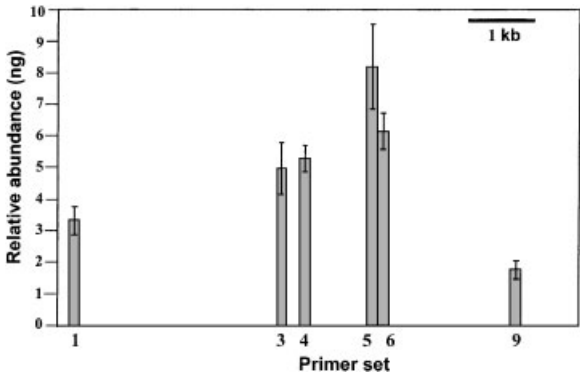

Fig. 2. Quantitative PCR results with an additional preparation of nascent DNA (NHF1a). In order to demonstrate reproducibility, an additional nascent preparation from a second fibroblast strain was tested. The relative abundance was determined as described in Figure 1. Each primer set was tested at least three times and error bars indicate the standard deviation of the mean. The peak of abundance is again seen in the promoter region of the gene, but sequences amplified by primer sets 5 and 6 are present in higher amounts than those amplified by primer set 4 in this preparation.

putative replication origins in a 500 kb sequence on the X chromosome that includes the *G6PD* gene. The authors found 15 putative origins that were located in a gene rich section of this region. However, none of the putative origins in the Rivella et al. [1999] study were associated with the *G6PD* gene. This may be due to a step in their origin isolation process that removed most repetitive and GC-rich sequences.

After the G6PD origin of replication was mapped, we evaluated replication activity at this origin and the HPRT origin of replication on the active and the inactive X chromosomes. A normal human female fibroblast cell strain could not be used for this study because it would still be difficult to clearly distinguish between PCR signal from the active and the inactive X, even if these cells were synchronized to a very high degree. Therefore, we used two hamster cell lines that contained either a single active or inactive human X chromosome. One primer set at the peak of activity for the G6PD origin (primer set G6PD4) and two primer sets flanking the origin (primer sets G6PD1 and G6PD9) were used to determine origin activity. Two preparations of nascent DNA from both cell lines were tested at least three times for each primer set. The results indicated that the previously discovered G6PD origin was functional in both the active and inactive human X chromosomes in the hamster hybrid cells (Fig. 3).

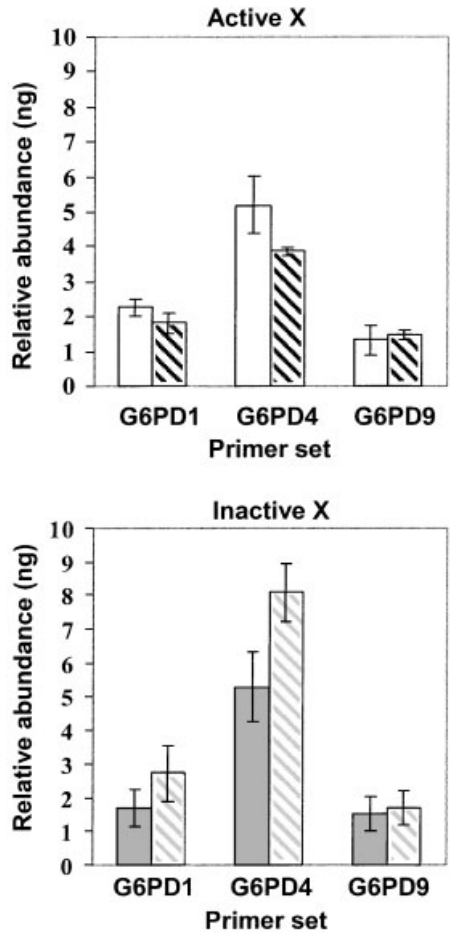

Fig. 3. Analysis of the G6PD origin of replication in the active and inactive X chromosome. The relative abundance of 3 PCR markers in two preparations of nascent DNA from CHO cells that contain either the active (cell line GM06318) or the inactive (cell line X8-6T2S1) human X chromosome is shown. Short nascent DNA was isolated from logarithmically growing cultures and used for quantitative PCR. The copy number of each PCR marker in the nascent preparation was determined as described in the legend to Figure 1. PCR results for the four nascent preparations were normalized using a site near the *DHFR* gene that has been shown not to have any origin activity [Pelizon et al., 1996]. A peak of nascent DNA abundance is found at primer set 4 in the G6PD in both cell types. This is consistent also with data from normal human fibroblasts.

Next, the HPRT origin of replication was tested. Figure 4A shows a schematic representation of the HPRT transcriptional promoter region, including the positions of the replication origin, an autonomously replicating sequence/nuclear matrix-attachment region (ARS/MAR) reported by Sykes et al. [1988], and the sequence amplified by the primer sets used in this study. Figure 4B shows the results of PCR analysis of the HPRT region with the same hamster/human hybrid nascent DNA preparations used

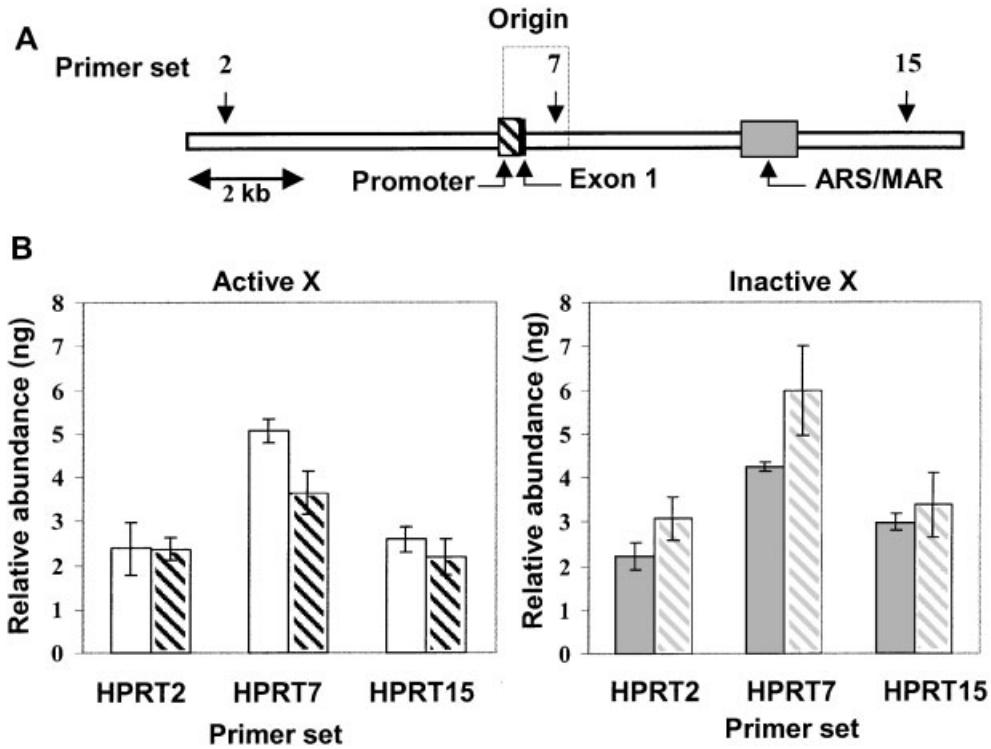

Fig. 4. Analysis of the HPRT origin of replication in the active and inactive X chromosome. **A**: The relative position of DNA regions amplified by the primer sets is indicated along with the positions of the promoter, exon 1 of the gene, and a previously reported ARS/MAR region [Sykes et al., 1988]. The position of the replication origin is based on data from nascent DNA abundance assays [Cohen et al., 2002]. **B**: The relative abundance of 3 PCR markers in two preparations of nascent DNA from CHO cells that contain either the active (cell line GM06318) or the inactive (cell line X8-6T2S1) human X chromosome is shown. Each primer set was tested at least three times and error bars indicate the standard deviation of the mean. PCR results for the four nascent preparations were normalized as described in the legend to Figure 3. A peak of nascent abundance is found for HPRT primer set 7, consistent with data from normal human fibroblasts.

to analyze the G6PD origin. Results indicate that the previously identified HPRT origin is also functional in both the active and inactive human X chromosomes in these hybrid cells.

DISCUSSION

The inactivation of one X chromosome in female mammalian cells is necessary to ensure expression of X-linked genes at equal doses in males and females. X chromosome inactivation is a multi-step process that occurs during differentiation and is thought to begin with activity from the X inactive-specific transcript (*XIST*) gene [Penny et al., 1996]. RNA from one of the *XIST* gene alleles coats the surface of its own X chromosome, thus marking it for heterochromatization. The coating of the X chromosome by XIST RNA leads to transcriptional silencing by a mechanism that is currently unknown. It has been proposed that XIST is only necessary for the initiation phase of X inactivation and that other factors are involved in the maintenance of the inactive state [Hansen et al., 1996; Avner and Heard, 2001]. A shift to replication at a later time and the appearance of a form of histone H3 methylated on lysine 9 (H3 Me/K9) is detectable on the inactive X at about the same time as the start of transcriptional silencing [Mermoud et al., 2002]. Hypoacetylated forms of histones H3 and H4 then begin to accumulate in promoter regions on the inactive X and are believed to be involved in the maintenance of transcriptional silencing [Keohane et al., 1996; Gilbert and Sharp, 1999]. DNA methylation on the X chromosome occurred well after genes were transcriptionally silenced; DNA methylation is also believed to play a role in maintenance of silencing [Keohane et al., 1996].

Although the mechanism for transcriptional silencing is not precisely known, it has been suggested that DNA methylation maintains transcriptional silencing on the inactive X

either by directly blocking the binding of transcription factors, or by altering chromatin structure, which indirectly prevents regulatory factors from binding [Litt et al., 1997]. It is unlikely that DNA methylation directly blocks transcription factor binding at the HPRT promoter. After treatment with the DNA-demethylating agent 5-aza-2′-deoxycytidine (5-aza-Cdr), Chen et al. [2001] have shown that there are three specific methylation sites at the HPRT promoter that are critical for maintaining transcriptional silencing. None of these are located in DNA footprints associated with transcription factor binding. However, there is evidence for an alteration of chromatin structure at promoters on the inactive X. Ackerman et al. [1993] have proposed that a stem-loop structure forms at the transcriptional start site of CpG-rich promoters. Furthermore, it has been reported that methylation at the CpG dinucleotides causes a reduction in the formation of stem-loop structures [Murchie and Lilley, 1989], resulting in inactivation of the gene [Jiralerspong and Patel, 1996]. Although there is no direct evidence for the formation of the stem-loop type of secondary structure at the HPRT and G6PD promoters, it has been reported that the alterations in chromatin structure found on the inactive X must be reversed before HPRT transcription can occur following 5-aza-Cdr treatment. In reactivation studies using 5-aza-Cdr, Litt et al. [1997] reported that an alteration in chromatin structure, as indicated by an increase in nuclease sensitivity, occurs at the HPRT promoter prior to transcription factor binding.

We wondered whether structural changes in chromatin, apparently responsible for transcriptional silencing on the inactive X, would also interfere with the firing of replication origins that are located at transcriptional promoters. Not all sites capable of origin function are utilized; there is a decrease in the number of origins used as cells differentiate [Hyrien et al., 1995; Sasaki et al., 1999]. It has also been shown that in a given population of cells the efficiency of utilization differs among origins. This can be seen with the DHFR locus in CHO cells where ori β is the origin activated in a much higher percentage of cells than ori β′ [Kobayashi et al., 1998]. Restriction of origin usage at transcriptionally silenced promoters on the inactive X, therefore, could lead to DNA synthesis through such regions by replication that begins at alternative origins. In the present work we investigated this possibility by analyzing origin function at replication origins associated with the transcriptional promoter of two X-linked genes, *HPRT* and *G6PD*. Quantitative PCR with preparations of nascent DNA from hamster cells containing either the active or inactive human X chromosome showed that the HPRT and G6PD origins were active in both cell types despite changes in chromatin structure at the transcriptional promoter. Although no alternative sites were tested, we were able to establish that the level of origin firing at the transcriptional promoters of HPRT and G6PD was not diminished on the inactive X. A direct comparison of the abundance of specific markers in nascent DNA prepared from two hamster cell lines was possible because the results were normalized to baseline values obtained for a hamster chromosome site in the DHFR region, away from the replication origin. Therefore, for these two gene promoter regions there seems to be neither a change in the initation site for replication nor a decrease in origin activity upon transcriptional inactivation in the heterochromatic X chromosome.

Although DNA methylation at transcriptional promoters has been shown to influence transcriptional activity, there appears to be no clear relationship between methylation status of CpG regions and the activity of origins of replication at these sites. Some origins are methylated at their CpG regions, such as the DHFR ori-β and ori-RPS14 in CHO cells [Rein et al., 1999]. Furthermore, retention of methylation status is important for the function of the hamster DHFR ori-β, where hypomethylation resulted in a loss of origin activity at the DHFR ori-β and ori-β′ [Rein et al., 1999]. Alternatively, the human c-myc and lamin B2 origins [Araujo et al., 1998; Rein et al., 1999] are not methylated at their associated CpG dinucleotide regions. For the *HPRT* and *G6PD* genes, the CpG dinucleotides at their promoters on active X chromosomes are not methylated, but are methylated in the inactive X [Toniolo et al., 1988; Hornstra and Yang, 1994]. Since the HPRT and G6PD associated origins are active on both the active and inactive human X chromosomes, activation of these origins must be independent of the methylation status of the DNA in these regions.

A connection between transcription activity and time of replication in S phase has long been

established [Holmquist, 1987]. However, the mechanistic link between replication timing and transcriptional silencing may not be as straightforward as previously thought. Hansen et al. [1996] have shown that in the hybrid cell line containing the inactive X chromosome (X8-6T2S1 cells), both the *HPRT* and *G6PD* genes replicate later in S phase than their transcriptionally active counterparts. However, the two genes do not replicate as late as they have been observed to do in the heterochromatic X chromosome in female human cells and they are more easily reactivated with 5-azacytidine (5-aza-C) than other genes tested on the inactive X chromosome [Hansen et al., 1996]. This may be indicative of an alteration in factors that are normally involved in the maintenance of the heterochromatic state. We know from published studies that the *HPRT* gene in X8-6T2S1 cells is inactive and hypermethylated [Hornstra and Yang, 1994]. These cells survive in 6-thioguanine, and preliminary data (not shown) from our laboratory indicate that the HPRT promoter in this hybrid cell line is also hypoacetylated on histone H3 lysine 9, as would be expected for an inactivated gene. In addition, Hansen et al. [1996] have shown that genes that are transcriptionally silent on the active X chromosome also replicate earlier in 5-aza-C treated cells. Therefore, the mechanisms involved in the regulation of the timing of activation of replication origins within the S phase remain obscure. The maintenance of late replication of genes on the inactive X chromosome may reside in a yet to be determined regulatory site, which might not coincide with the location of origins of replication [Hansen et al., 1996]. Nonetheless, the data presented in this report demonstrate that DNA and chromatin alterations associated with transcriptional silencing do not block the utilization of origins of replication. The potential role (or lack thereof) of chromatin alterations in determining when in S phase the origin is activated remains to be established.

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