Differential replication timing of X-linked genes measured by a novel method using single-nucleotide primer extension

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ABSTRACT

The ratio of two differentially replicating alleles is not constant during S phase. Using this fact, we have developed a method for determining allele-specific replication timing for alleles differing by at least a single base pair. Unsynchronized cells in tissue culture are first sorted into fractions based on DNA content as a measure of position in S phase. DNA is purified from each fraction and used for PCR with primers that bracket the allelic difference, amplifying both alleles. The ratio of alleles in the amplified product is then determined by a single nucleotide primer extension (SNuPE) assay, modified as described [Singer-Sam,J. and Riggs,A.D. (1993) Methods Enzymol., 225, 344–351]. We report here use of this SNuPE-based method to analyze replication timing of two X-linked genes, Pgk-1 and Xist, as well as the autosomal gene Gabra-6. We have found that the two alleles of the Gabra-6 gene replicate synchronously, as expected; similarly, the active allele of the Pgk-1 gene on the active X chromosome (Xa) replicates early relative to the silent allele on the inactive X chromosome (Xi). In contrast, the expressed allele of the Xist gene, which is on the Xi, replicates late relative to the silent allele on the Xa.

For both X-linked and autosomal genes, early replication is strongly correlated with transcriptional activity and late replication with inactivity (1,2), but there are some apparent exceptions. For example, the Xist gene, which is expressed exclusively from the inactive X chromosome (Xi) and is essential for the initiation of X chromosome inactivation (3–5), has been reported to replicate late when it is transcriptionally active on the Xi (6). This result, however, has been controversial since others using FISH analysis have obtained opposite results (7,8). The controversy has been difficult to resolve because the techniques so far used, BudR incorporation followed by immunoprecipitation (6), and statistical analysis of fluorescent dots obtained using FISH (7–9), are each in their own way indirect and subject to possible artifacts. We describe here a simple, alternative method, shown schematically in Figure 1, that makes use of the PCR-based single nucleotide primer extension (SNuPE) assay for quantitative measurement of allelic ratios (10–12). The SNuPE-based method does not require BudR incorporation, immunoprecipitation, cell synchrony or statistical analysis, and it allows measurement of replication timing differences between alleles differing by a single nucleotide. The SNuPE method measures the extension of an oligonucleotide primer by a single nucleotide; since only one deoxyribonucleoside triphosphate is provided, it is incorporated only if it is complementary to the template strand at the position immediately adjacent to the primer at its 3′-end. We have used this SNuPE-based method to confirm the known order of replication timing of the expressed X-linked Pgk-1 allele relative to the silent allele, and the synchronous replication of the autosomal Gabra-6 gene. In addition, we have measured allele-specific replication timing of Xist; we find late replication of the expressed allele (present on the inactive X chromosome), as observed by Hansen et al. (6).

To analyze the X-linked genes Pgk-1 and Xist, the hybrid mouse cell line BML-2 was used (13,14). While this cell line is derived from congenic C57BL/6 mice, one of its two X chromosomes contains the Pgk-1a allele, varying from the sequence of the wild type (Pgk-1b) at several sites (15). The Pgk-1a-containing X chromosome also shows allelic variation at the Xist locus (4,16); we previously identified one such difference, and termed the allele Xist a (16). The X chromosome containing the Pgk-1a and Xist a alleles is the Xi in BML-2 cells, while the wild-type X chromosome is the Xa (13,14). To analyze the Gabra-6 gene, the hybrid HOBMSKI mouse cell line (17) was used. The cell line is derived from strains C57BL/6 and Mus spretus, allowing us to make use of the reported sequence difference between the two strains at the Gabra-6 locus (18), here termed Gabra-6mas and Gabra-6Spe, respectively.

Both cell lines were cultured as described (13), fixed in high-citrate buffer and stained with propidium iodide according to Klevecz et al. (19,20). Log phase, washed cells were suspended at 1 × 10⁶ cells/ml in 0.4 M sodium citrate (pH 2.35)

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Figure 1. Schematic of the DNA replication timing assay. Log phase cells are separated into G1, G2 and several S phase fractions on the basis of DNA content by fluorescence activated cell sorting (FACS). DNA is prepared from each fraction, and used for PCR amplification of fragments containing a region of heterozygosity. The ratio of alleles in the PCR product is then determined by use of the single nucleotide primer extension (SNuPE) assay (12).

and kept overnight at room temperature and then at 4°C for 2 days; cells were centrifuged, resuspended in 0.4 M sodium citrate (pH 4.5) and either used immediately for staining or stored at 4°C for up to 3 months. For staining, fixed cells were centrifuged and resuspended at 3 × 10^6 cells/ml in 10 mM HEPES, 0.1% BSA containing 100 mg/ml propidium iodide (PI) (Sigma, St Louis, MO). Staining was done at room temperature for 12–16 h just prior to flow sorting. The PI-stained cells were analyzed and sorted on either a FACS IV (Becton Dickenson, Mountain View, CA) or a MoFlo (Cytomation, Fort Collins, CO) flow cytometer, using separate laser excitation for scatter signals and fluorescence excitation. Cells were selected based on DNA content (PI intensity) as well as cell size (autogreen fluorescence for the FACS IV; sidescatter for the MoFlo flow cytometer). Between 4 × 10^4 and 1 × 10^5 cells were collected in each fraction of S-phase (generally six fractions), G1 and G2.

DNA was prepared from the fractions as described (14). For each gene, PCR was carried out with primers (see Fig. 3 legend) common to both alleles, and flanking the known allelic differences. The allelic ratio in each amplified product was then measured by quantitative SNuPE assay (12), using the appropriate SNuPE primers (Fig. 3 legend).

For each gene assayed, mixing experiments were done to confirm the linearity and reproducibility of the SNuPE assay for the range of allelic ratios measured; Figure 2 shows the standard curve obtained for Xist. Figure 3 shows results obtained for each gene after flow sorting and SNuPE assay. The ratio of the expressed \( \text{Pgk-1}^b \) allele to the silent \( \text{Pgk-1}^a \) allele increases from \( \sim 1:1 \) at G1 to 2:1 at S2, and returns to 1:1 at S6 (Fig. 3A). This pattern is consistent with the known early replication of the active \( \text{Pgk-1} \) allele and late replication of the inactive allele. In contrast, the \( \text{Xist} \) gene shows an inverse pattern of DNA replication timing with respect to transcriptional activity, with the silent \( \text{Xist}^b \) allele

Figure 2. Standard curve for Xist. Pure \( \text{Xist}^a \) or \( \text{Xist}^b \) PCR products were mixed at various ratios and used for quantitative SNuPE.

Figure 3. Replication timing assay of the \( \text{Pgk-1} \), \( \text{Xist} \) and \( \text{Gabra-6} \) genes. DNA isolated from the cell cycle fractions shown was assayed by SNuPE for the ratio of (A) \( \text{Pgk-1}^b/\text{Pgk-1}^a \); (B) \( \text{Xist}^b/\text{Xist}^a \); and (C) \( \text{Gabra-6}^\text{mus}/\text{Gabra-6}^\text{spe} \). All experiments were done in duplicate; bars show the range of the averaged values. \( \text{Gabra-6} \) PCR primers and \( \text{Xist} \) PCR primers 1994 and 2300 have been described (16,18); for \( \text{Pgk-1} \), PCR primers were GGGTAGGGGAGGCGCTTTTCCCAA, and CGGTGCTGTCCA TCTGCACGA. DNA from ~5000 cells was amplified by incubation at 95°C for 1 min, at 58°C (\( \text{Gabra-6} \)), 55°C (\( \text{Pgk-1} \)) or 53°C (\( \text{Xist} \)) for 2 min, then at 72°C for 2 min, for 35 cycles. PCR products were purified by band isolation from a 2% agarose gel using Wizard (Promega, Madison, WI) or QIExII (Qiagen Inc., Chatsworth, CA). The band-purified PCR products were quantified on 2% agarose gels by ethidium bromide fluorescence relative to DNA at known concentrations. The SNuPE primer for \( \text{Xist} \) has been described (16); for \( \text{Pgk-1} \), the primer CCCCTAGTCAGGAAGTT was used, adjacent to a T versus C mismatch (15); for \( \text{Gabra-6} \), the primer ATACAGTCATAGTAGATG was used, also adjacent to a T versus C mismatch (18). SNuPE assays were carried out with a single cycle of denaturation at 95°C for 1 min, annealing at 42°C (55°C for \( \text{Pgk-1} \)), and primer extension at 72°C, for 2 min. After denaturing polyacrylamide gel electrophoresis, the radioactive gels were analyzed by use of a phosphorImager and the ImageQuant software package (Molecular Dynamics Inc., Sunnyvale, CA).
replicating prior to the expressed Xistα allele (Fig. 3B). The autosomal Gabra-6 gene (21) was assayed as a control; as expected, no asynchrony in replication timing of the two alleles was seen (Fig. 3C). Our results demonstrate the usefulness of this new replication timing assay, which is limited only by the need for known sequence differences in the alleles. The results also confirm the reverse replication pattern that Hansen et al. (6) obtained for Xist using BrdU incorporation and immunoprecipitation. At least for this gene, early replication is not necessary for transcriptional activity.

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