X inactivation-specific methylation of LINE-1 elements by DNMT3B: implications for the Lyon repeat hypothesis

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Lyon has proposed that long interspersed nuclear element 1 (LINE-1 or L1) repeats may be mediators for the spread of X chromosome inactivation. Cells from ICF patients who are deficient in one of the DNA methyltransferases, DNMT3B, provide an opportunity to explore and refine this hypothesis. Southern blot and bisulfite methylation analyses indicate that, in normal somatic cells, X-linked L1s are hypermethylated on both the active and inactive X chromosomes. In contrast, ICF syndrome cells with DNMT3B mutations have L1s that are hypomethylated on the inactive X, but not on the active X or autosomes. The DNMT3B methyltransferase, therefore, is required for methylation of L1 CpG islands on the inactive X, but not on the active X or autosomes. The DNMT3B methyltransferase, therefore, is required for methylation of L1 CpG islands on the inactive X, whereas methylation of the corresponding L1 loci on the active X, as well as most autosomal L1s, is accomplished by another DNA methyltransferase. This unique phenomenon of identical allelic modifications by different enzymes has not been previously observed. Apart from CpG island methylation, the ICF inactive X is basically normal in that it forms a Barr body, is associated with XIST RNA, mostly replicates late, and its X-inactivated genes are mostly silent. Because the unmethylated state of the ICF inactive X L1s probably reflects their methylation status at the time of X inactivation, these data suggest that unmethylated L1 elements, but not methylated L1s, may have a role in the spreading of X chromosome inactivation.

INTRODUCTION

A large portion of the human genome is riddled with interspersed repetitive elements, most of which appear to derive from transposition events driven by the L1 LINE (long interspersed nuclear element) machinery (reviewed in 1). L1 LINE elements themselves (L1s), including truncated versions, comprise about 17% of the draft human genome (1). Such elements would seem to be deleterious and not become fixed in the population, but their presence throughout mammalian genomes appears to derive from an interplay between their transpositional efficiency and the ability of host cells to limit their activity. One of the mechanisms involved in silencing L1s in somatic and germline cells is DNA methylation of the 5′ internal promoter region (2–4), thus providing direct repression of L1s with transposition potential and promoting heritable inactivation by methylation-induced mutation. Late replication may be another silencing mechanism used for these elements because they are frequently found in late-replicating cytogenetic bands (reviewed in 5,6).

ICF syndrome (immunodeficiency, centromeric instability and facial anomalies) is an autosomal recessive immunodeficiency disorder that can be caused by mutations in the DNMT3B DNA methyltransferase gene (7–9). The catalytic activity of the enzyme is compromised in most cases, thus leading to hypomethylation-related defects such as centromeric instability (10) and loss of gene silencing (11,12). The loci that are abnormally hypomethylated in ICF cells appear to be confined mostly to late-replicating CpG-rich heterochromatic regions such as pericentromeric satellites (10,13), certain subtelomeric repeats (12), and the CpG islands of the inactive X chromosome (11). Because L1s share the properties of 5′ CpG island methylation and late replication with loci affected in ICF syndrome, it seemed plausible that they might be another potential DNMT3B target that is abnormally hypomethylated in ICF cells, particularly if located on the inactive X chromosome.

The methylation status of L1s on the active and inactive X chromosomes in normal and ICF cells is of particular interest because L1s have been proposed by Lyon to possibly act as
booster elements in the spreading of X chromosome inactivation (14), and they have been confirmed to be enriched on the X chromosome (15). Such elements were originally proposed by Gartler and Riggs (16) to explain the cooperative spreading of the X inactivation signal from the X inactivation center, now known to be triggered by XIST RNA expression and localization (17). If the XIST-mediated spreading of silenced chromatin involves mechanisms such as L1-mediated looping by intrachromosomal pairing or RNAi-type chromatin silencing as a result of L1 expression, L1s are predicted to be unmethylated at the time of X inactivation.

RESULTS

To examine global L1 methylation in normal and ICF syndrome cells, previously described amplification primers were used to target the 5' regions of 'younger' species of L1 that have retained CpG sequences in the internal promoter region (4). The primers were used to make probes for methylation analysis by Southern blot and to amplify bisulfite-converted DNA for analysis of single chromosome methylation patterns. Additional primers were designed to examine the methylation of particular X-linked L1s: (1) those that amplify unique 5' flanking sequence in bisulfite-untreated genomic DNA to make probes for Southern blot analysis; and (2) those replacing the 5' L1 primer with one from unique 5' flanking sequence for bisulfite methylation analysis.

Global L1 LINE methylation

The initial analysis of ICF syndrome cells for potential abnormalities in L1 methylation employed methylation-sensitive restriction enzymes and Southern blot analysis with an uncloned L1 probe amplified from genomic DNA (Fig. 1). Comparing MspI (methylation insensitive) to HpaII (methylation sensitive) digestion patterns, it appears that normal male and normal female L1s are resistant to HpaII digestion and are, therefore, hypermethylated. L1s are also hypermethylated in ICF samples, although ICF females appear to have a small hypomethylated fraction that is not seen in ICF males or normal DNAs. A previous study (8) also found L1s to be hypermethylated in ICF, but a hypomethylated component was not detected, perhaps because the ICF samples analyzed may have been male (gender was not given for the single ICF sample shown).

To determine L1 methylation patterns in more detail, a previously described protocol for L1s was followed (4) that is based on the bisulfite conversion method that modifies only unmethylated cytosines (18). In addition to allowing more CpG sites to be examined for methylation, this method reveals the cis methylation patterns (methylation haplotypes) for individual strands (epialleles) in a population. The primers used to analyze the bisulfite-converted top strand for methylation were also able to amplify untreated DNA. L1s were amplified from bisulfite-untreated 77120 fibroblast DNA, and 42 clones were sequenced to estimate the similarity among the L1 elements examined under the conditions used, with particular focus on the CpG site distributions at these loci. The average number of CpGs per clone should represent the maximal amount of average methylation (mCpGs/clone) that could be seen in the bisulfite converted clones if similar sequences are amplified following bisulfite conversion (4).

Most of the 'bisulfite-untreated' sequences analyzed were highly similar, containing an average of 25.6 ± 7.1 SD CpGs and 21 conserved CpG sites that were present in at least 70% of the clones (Supplementary Material Fig. 1). The Southern blot results shown in Figure 1 using the uncloned amplimer are consistent with the HpaII/MspI patterns present in the consensus sequence in that the major bands in the low size range correspond to those expected from different combinations of double cleavages at consensus sites in the probe region.

Sequences derived from bisulfite-treated DNA from 77120 fibroblasts suggest that L1s are highly methylated in normal adult skin fibroblasts (22.1 ± 5.1 SD mCpGs/clone), consistent with Figure 1 and previous data obtained from embryonic fibroblasts using this method (4). Because of sequence variations, these clones appear to represent methylation patterns at different genomic loci (data not shown). In contrast to the report by Woodcock et al. (4) that the antisense strand is hypomethylated in embryonic fibroblasts, this study of adult fibroblasts suggests that the two strands are equally hypermethylated (the antisense strand has 18.5 ± 5.5 SD mCpGs in bisulfite-treated clones compared with 20.5 ± 6.2 SD CpGs in 29 bisulfite-untreated clones that were also derived from
products of the antisense strand primer set). In addition, methylation observed by Woodcock et al. at non-CpG sites was absent in fibroblasts and other cell types examined here.

Figure 2 summarizes the bisulfite analyses of global L1 methylation for all samples examined (top strand only). Similar to normal fibroblasts, L1s are extensively hypermethylated in normal lymphoblasts (24.0±5.7 SD mCpGs/clone) and in normal sperm (21.6±7.0 SD mCpGs/clone). Consistent with Southern blot analyses, L1 methylation in lymphoblasts and fibroblasts from DNMT3B-deficient ICF patients mostly appears normal. The ICF cells, however, appear to differ somewhat from normal cells in that they have a higher frequency of L1s with lower methylation levels, particularly the ICF female cells (see Supplementary Material Fig. 2 for clone distributions). For example, the percentage of clones with less than 17 mCpG sites is 34.9 for PT3 ICF fibroblasts, 36.4 for PT4 ICF fibroblasts, and 12.8 for 77120 normal fibroblasts; this difference in clone distribution between the 77120 normal fibroblast and the two ICF fibroblasts is significant (P < 0.01), and is consistent with the increased sensitivity of ICF fibroblasts to HpaII digestion (Fig. 1).

X-linked L1 methylation

A possible explanation for the hypomethylated clones from ICF cells is that they derive from heterochromatic regions known to be affected by DNMT3B deficiency such as the pericentromeric satellite regions or, in the case of female cells (PT3 and PT4), the inactive X chromosome. It was of particular interest to determine whether or not the L1 CpG islands on the inactive X are treated like the gene-associated CpG islands in being targeted for methylation by DNMT3B (11). Studying L1 methylation on the active and inactive X chromosomes might also provide data relevant to the question of whether L1s have a role in promoting the XIST-mediated spread of silencing during X inactivation (14,15).

Global X-linked L1 methylation was first examined in rodent-human somatic cell hybrids that contain either the active X or inactive X chromosome as the predominant human L1 source (Fig. 2). Although the L1 methylation levels are somewhat lower than in normal human cells, it is clear that L1s are hypermethylated in active X-only hybrids (GM06318 and t60-12), an inactive X-only hybrid (Lt23-1E2), and inactive X hybrids with contributions from chromosomes with lower L1 content (HY70C and t86-B1). Although both the active and inactive X L1s are hypermethylated, the methylation levels appear to be somewhat lower on the inactive X when the allelic distributions of just the active X-only hybrids to the inactive X-only hybrid are compared (Fig. 2 and data not shown).

To examine X-linked L1 methylation in normal and ICF cells, six L1s were chosen from X chromosome sequences with the criteria that they are potentially amplifiable with the 5’ L1 primers and that they contain enough unique 5’ flanking sequence to allow for design of a single-copy amplimer for probing Southern blots of genomic DNA. Because the HpaII sites of interest are 3’ to the probe, only those sites up to and including the first cleaved site could be evaluated for methylation status. In most cases, therefore, comparing methylation...
patterns between samples required evaluation of only a limited number of sites. One or two BstUI sites are also present in the L1s analyzed (CGCG recognition sequence; cuts if both sites are unmethylated). An example of such methylation analyses is shown in Figure 3 for a full-length L1Hs element on Xq22 (Xq22L1; 6 kb; in GenBank AL590306). HpaII digestion of DNAs from normal male and female cells indicates that the normal active and inactive X alleles are heavily methylated at a number of sites. For example, the main band seen in the complete digest with MspI (a 584 bp HpaII: HpaII fragment) is absent in normal male and female samples, but is quite prominent in ICF female fibroblasts. The single BstUI site is fully methylated in male cells, and partially hypomethylated in female cells. Like the HpaII sites, the BstUI site is more extensively hypomethylated in ICF female cells. Active-X and inactive-X hybrid cells derived from normal cells are both heavily methylated at a number of sites within Xq22L1 and at the other X-linked L1s examined (Fig. 3 and data not shown).

A summary of the Southern blot analyses of X-linked L1 methylation in human cells for six loci scattered throughout the X chromosome strongly suggests that ICF females are abnormally hypomethylated on the inactive X (Table 1).

![Figure 3. Southern analysis of X-linked L1 methylation. (A) Shown are features of the L1 element region on Xq22 analyzed for methylation at HpaII and BstUI sites. The only CpG island detected in the NdeI: NdeI fragment analyzed was that of the L1 element (length is 633 bp; %GC is 57.3; observed CpG/expected CpG is 0.677), according to the CpG Island Searcher program (43) implemented at the www.uscnorris.com/cpgislands/web site using default parameters. Positions for the CpG island, L1 element (subfamily L1Hs), probe, restrictions sites, and low complexity repeat regions are indicated. Also shown is the location of the amplimer, Xq22L1bs, used in subsequent bisulfite methylation analysis. (B) Examples of Southern blot analyses of various normal (norm) and ICF samples using methylation-sensitive restriction enzymes. Fibroblasts (FB), lymphoblastoid cells (LB), active X hybrid cells (Xa), or inactive X hybrid cells (Xi) were digested with either NdeI alone (N), NdeI and HpaII (H), NdeI and BstUI (B), NdeI and MspI (M), or MspI alone (M). Shown at the left are the fragments deduced to correspond to major bands observed. Normal males and females have similar patterns of hypermethylation, whereas ICF females appear to have a large unmethylated component that is absent in normal cells (filled arrows point to relevant NdeI-1 : BstUI and HpaII-0 : HpaII-1 fragments). ICF females also have a marked decrease in fully methylated NdeI-1 : NdeI-2 fragments (open arrow).](image)

Table 1. Summary of Southern blot analyses of X-linked L1 methylation.a

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sites methylated</th>
<th>Sites unmethylated</th>
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</tr>
<tr>
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<td>8.5</td>
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<tr>
<td>ICF Xi</td>
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<td>26.0</td>
<td>72.2</td>
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</tbody>
</table>

*aMethylation status was summarized for all informative methylation-sensitive restriction sites (HpaII, BstUI, and FspI) analyzed at several X-linked L1 loci (Xp22L1, Xp11L1, Xq22L1, Xq26L1, Xq27L1, and Xq28L1). Three normal male, five normal female, and three ICF female samples were included in the analysis (male ICF samples were not included).

**Active X (Xa) patterns from male samples were used to deduce inactive X (Xi) patterns in female samples (active X sites in females were not counted). Fibroblast and lymphoblast patterns were compared separately.

Because the probes are 5’ to the first HpaII site in the L1s, only sites up to and including the first cut site in any sample could be used for comparison of HpaII site methylation between samples. Sites are characterized as either fully or mostly methylated (value of 1), partially methylated (value of 0.5), or fully or mostly unmethylated (value of 0). Although BstUI has two CpG sites, only one was counted because uncut sites may contain an unmethylated CpG in addition to a methylated one. Similarly, the two FspI sites in Xq27L1 could not be resolved and were counted as one site.
Bisulfite methylation analysis of the Xq22L1 LINE element

To analyze X-linked L1 methylation patterns in more detail, an L1 bisulfite methylation assay was developed that uses a specific forward primer derived from the unique 5’ flanking sequence of Xq22L1 in conjuction with the L1 reverse primer used above (L1t-505r). The region covered by the Xq22L1 amplifier contains 32 CpG sites. The CpG methylation patterns of epialleles from representative DNAs are shown in Figure 4 (single chromosome methylation haplotypes as deduced from bisulfite-derived clones). Methylation levels for CpG sites 1–23 are consistently very high for nearly all epialleles in normal male and female lymphoblasts, uncultured female blood leukocytes, and normal male and female fibroblasts, suggesting that both the active- and inactive-X alleles are hypermethylated. In ICF female fibroblasts and lymphoblasts, however, there is a bimodal distribution of epialleles, with about half being extensively hypomethylated and the other half with normal hypermethylation. The hypomethylated ICF epialleles appear to correspond to those derived from the inactive X because an ICF male lymphoblast (PT5) has normal methylation patterns. The hypomethylated epialleles in PT4 lymphoblasts appear to be more methylated than those of PT4 fibroblasts, consistent with the Southern blot analyses showing greater \( HpaII \) and \( BstUI \) digestion of the fibroblast L1s (Fig. 3). The increased levels of methylation in ICF female lymphoblasts may derive from their transformation and/or more extensive in vitro culturing.

In normal cells, CpG sites near the 3’ end of the CpG island (sites 24–32) are methylated to a lower extent than in the 5’ region. This hypomethylation does not appear to reflect differences between the active and inactive X alleles because it is present in the majority of clones in female cells, and the epiallele distribution of methylation in this region is similar in male and female cells (Fig. 4 and data not shown).

L1 replication timing

Although L1s have been shown to be present with higher frequency in late-replicating cytogenetic bands (6), the subset of L1s sampled for methylation analysis was examined to determine if they also replicate late in S phase. As shown in Figure 5A, the majority of amplifiable L1s replicate in the second half of S phase in both normal and ICF cells.

Replication timing for the Xq22L1 locus in normal cells is similar to that of bulk L1s, occurring primarily in the second half of S phase (S3 and S4 fractions; Fig. 5B). Normal male and female replication patterns are similar, suggesting that the active and inactive X alleles have a significant overlap in replication timing, although an increased G2/M replication signal in females probably derives from the inactive X. Although the G2/M signal is diminished in PT3 ICF fibroblasts, the extreme hypomethylation of the inactive X allele in these cells does not appear to result in a large shift in replication timing to early S phase (i.e. not to a time that is earlier than the methylated active X allele).

DISCUSSION

These studies suggest that most of the genomic L1 CpG islands in DNMT3B-deficient ICF cells are hypermethylated and late replicating, as they are in normal cells. Hypomethylation in ICF cells was found, however, for L1s on the inactive X chromosome. Based on global bisulfite methylation analyses in hamster–human hybrids (Fig. 2), and analyses of several X-linked L1s in human cells (Figs 3 and 4, and Table 1), L1s are normally hypermethylated on both the active and inactive X chromosomes. The essentially normal methylation of L1 sequences on the active X chromosome and autosomes in ICF cells suggests that DNMT3B is not required for methylation-dependent silencing of these sequences. Studies in murine ES cells mutant in different DNA methyltransferases indicate that DNMT3A and/or DNMT3B are responsible for bulk L1 methylation. The observation that active X and autosomal L1 methylation is basically normal in DNMT3B-deficient ICF cells suggests that it is DNMT3A that is responsible for the major portion of L1 methylation in normal and ICF cells. Thus, methylation of X-linked L1 elements in female cells is unique in that both the active and inactive X loci are methylated, but by different ‘allele-specific’ DNA methyltransferases.

The hypomethylation of the inactive X in ICF cells could have resulted either from the failure to establish methylation, maintain it, or both. A failure to establish methylation is an attractive idea because the DNMT3B methyltransferase is known to be important for de novo methylation early in development, and the levels of functional enzyme in somatic cells dramatically decrease following differentiation (9,19). Recent studies of DNMT3A and DNMT3B protein levels in the mouse suggest that de novo methylation at the time of X inactivation is largely due to DNMT3B (19), thus further implicating this enzyme in the CpG island methylation that is associated with X inactivation.

Available evidence points to methylation of CpG islands on the inactive X occurring as a secondary silencing event that follows XIST RNA spreading, late replication, and Polycomb association/histone H3 K9/K27 methylation (17,20–23). Studies in mice suggest that L1s are actively demethylated in the zygote and remain hypomethylated in the blastocyst (24,25). The 10–20% methylation levels observed in hypo- methylated L1 alleles on the ICF inactive X could be due either to maintenance of methylation established prior to X inactivation, or from residual activity of the mutant DNMT3B methyltransferases and/or other de novo methyltransferase activities. The ICF data suggest that the inactive X-linked L1s are protected from de novo methylation by the methyltransferases that normally initiate L1 methylation on the active X and autosomes. Such inaccessibility is consistent with the view that DNMT3B is specifically targeted to CpG-rich heterochromatic domains, including the newly-inactivated X chromosome early in development. Although the inactive X L1s are hypomethylated in ICF female somatic cells, their late replication (Fig. 5B) is likely to keep them repressed, similar to the hypomethylated genes on the ICF inactive X that are inactive (11).

It has been proposed that the spread of X inactivation from the inactivation center to the ends of the chromosome arms may involve sequences along the X chromosome that act as ‘way stations’ for cooperative spreading of the silencing signal (16) and that L1 LINE elements are potential candidates for such way stations (14). Lyon (14) suggested that L1s might enable
Figure 4. Methylation patterns for active and inactive X alleles of the Xq22L1 LINE element in normal and ICF samples. A map of the 500 bp L1 region examined by bisulfite methylation analysis is shown at the top. Included are the positions of the amplification primers (xq22L1-1ubsf and L1t-505r), the predicted start site of L1 transcription, the 32 CpG sites, and the HpaII and BstUI restriction sites. Examples of epiallele methylation patterns for normal and ICF lymphoid cells and fibroblasts are shown below the map. Each row corresponds to the mCpG patterns of a single clone. Open squares represent unmethylated sites and filled squares represent mCpGs (methylated sites). Dashes indicate that the methylation status was not determined for that site. The number of mCpGs in each clone is given in the right column; clones are ordered from highest to lowest mCpG content. The normal active and inactive X alleles of this L1 appear to be hypermethylated to a similar extent because normal male and female cells are equally methylated. Although the ICF male (dashed box) has normal levels of methylation, ICF females (boxes) are hypomethylated on about half of their epialleles, suggesting a specific hypomethylation of the inactive X chromosome in these cases. Note that PT4 ICF lymphoblasts differ from PT4 ICF fibroblasts in that the levels of methylation are somewhat higher in the hypomethylated class of epialleles (i.e. the inactive X).
regions that are subject to X inactivation relative to those that escape, including autosomal regions in X:autosomal locations. The methylation status of specific X-linked Alus in ICF and normal cells is currently under investigation to evaluate them as potential targets of DNMT3B.

Because X inactivation in ICF cells is essentially normal at the cytological level and at the individual gene level for most genes examined (11 and Hansen, unpublished observations), the establishment and spread of X inactivation appears to be normal in a DNMT3B-deficient background. If L1s are involved in spreading the X inactivation signal, then the unmethylated state appears to be involved, and may be necessary. Lyon (14) suggested that XIST RNA might enhance intrachromosomal pairing of L1s for spreading of silenced chromatin (via looping), but the issue of methylation was not addressed in this context. An unmethylated state would be expected, however, because methylation is known to inhibit recombination and other processes that require pairing (28–30). It is also possible that nascent L1 transcripts could potentially be involved in attracting silencing complexes, perhaps via RNAi-related mechanisms similar to those described in yeast (31,32) and higher eukaryotes (33–35). Antisense L1 RNAs are likely to be expressed in most cells because gene-associated L1s are predominantly intronic, and in the antisense orientation to avoid abnormal splicing (36). The subsequent production of L1 siRNAs could then help spread the X inactivation signal by enhancing the establishment and spread of silencing through histone modification and heterochromatin protein association. ICF cells thus continue to provide a valuable model system for understanding genome methylation patterns and gene silencing mechanisms, particularly with respect to the multiple systems involved in X chromosome inactivation.

MATERIALS AND METHODS

Cell cultures and DNA samples

The cells examined in this study were cultured under standard conditions and included ICF and normal cultures that were previously described (7,11,13,37). GM06318 (active X chromosome as the only human chromosome) was also described previously (38). HY70C is an inactive-X hamster–human hybrid that was obtained from, and characterized for human chromosome content by, M. Rocchi (Sezione di Genetica, Bari, Italy; personal communication). DNAs from other rodent–human hybrids containing either the active or inactive X chromosomes were obtained from L. Carrel (see 39). Replication timing experiments were performed using bromodeoxyuridine incorporation as previously described (11).

Restriction enzyme and bisulfite conversion methylation analysis

The bisulfite conversion conditions and the general methodology used were as described previously (11). For untreated genomic L1 amplification and global L1 bisulfite methylation analysis, the primers used were L1t-1f, 5′-GGGGGGAGGAGT-TAAGATGGT(C/T)G-3′ and L1t-505r, 5′-CTCCACCCAAT-TC(G/A)AACTTCCC-3′ that target the converted sense strand
as previously described (4). A consensus sequence was obtained from untreated DNA amplimer clones following Clustal sequence alignment (http://pir.georgetown.edu/pirww/search/multalin.html) and sequence LOGO depiction (http://weblogo.berkeley.edu/logo.cgi). Bisulfite methylation analysis of the antisense strand was performed with the previously described primers L1bsb-30, 5′-CAACTCCAATCTACAACCTCC-3′ and L1bsb-464, 5′-GTAAGTTTGGAATAGGTGGGCG-3′ (4). Amplification products were cloned and sequence was determined as previously described (11), except that products were generally cloned after a single round of amplification using the TOPO TA cloning system from Invitrogen (Carlsbad, CA, USA). CpG sites were identified in aligned sequences, manipulated in Microsoft Word, and counted in a Microsoft Excel spreadsheet using the COUNTIF function.

For Southern blot methylation analyses of specific X-linked L1s, amplimers were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) from unique L1s, amplimers were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) from unique L1s that were detected on the X chromosome by BLAT searching (http://genome.ucsc.edu/) with the consensus primers. The unique L1s are: Xq22L1—xq22L1-1f, 5′-CAGTCTGTGATGTTGGC-TGTGGA-3′ and xq22L1-2r, 5′-TCTGAGCCATCCTTTA-ACTT-3′ (544 bp product; in GenBank AL590306); Xp22L1-DXS255—xp22L1-1f, 5′-TGGGCGGATTTTAAATCTAT-3′ and xp22L1-2r, 5′-TGCGTTATGGAAATGATCGAG-AAA-3′ (535 bp product; in GenBank AC004554); Xp11L1—xq22L1-1f, 5′-TCAACCTGTGATCTGGTGCCTCA-3′ and xq22L1-2r, 5′-GCTCCTCTCCAAGGTGTTGCTTA-3′ (420 bp product; in GenBank AF198097); Xq26L1—xq26L1-1f, 5′-TTTGGCAGTAAGGATCAGTCAGG-3′ and xq26L1-2r, 5′-TTTAAATGACAGAAAGCCCCCATGA-3′ (544 bp product; in GenBank Z82195); Xq27L1—xq27L1-1f, 5′-CCAGTTCGACTAAGACCCCAAGG-3′ and xq27L1-2r, 5′-CCTTGACAAGGTTTACCTTGG-3′ (472 bp product; in GenBank AC007538); and Xq28L1—xq28L1-1f, 5′-TGGTCATCCACCGATTACAATTC-3′ and xq28L1-2r, 5′-CCAGTTCG-CCTTGCAC-3′ (420 bp product; in GenBank AL034384). Restriction enzyme digestion and Southern blot analyses were performed as previously described (40,41).

For bisulfite methylation analysis of Xq22L1, Primer3 was used to pick a forward primer from the converted sense strand sequence with L1t-505r as the fixed right primer. The primer chosen, xq22L1-1ubsf, 5′-ATG(C/T)GGTTAGAA/AG(T/G)GAAGTTGAAAG-3′, results in a 548 bp amplifier with L1t-505r under the same amplification conditions used for L1t-1f: L1t-505r.

Replication time analysis

Replication time was determined by analysis of newly replicated DNA, labeled with bromodeoxyuridine, derived from cells sorted into specific cell cycle stages by flow cytometry as previously described (38,42). Briefly, the BrdU-labeled DNA was isolated with an anti-BrdU antibody, and these newly replicated DNA fractions were then analyzed for the presence of specific loci by semi-quantitative PCR and Southern blot hybridization. The loci examined included general L1s amplified with the L1t-1f: L1t-505r primer set, and the specific L1 locus, Xq22L1. The amount of template used for L1t-1f: L1t-505r was the equivalent of 1000 flow-sorted cells, and 25 amplification cycles were performed using the cycling parameters previously described (4). Amplification with the xq22L1-1f: xq22L1-2r primer set was similar, except that the amount of template was the equivalent of 1000 flow-sorted cells and 27 cycles were performed.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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