ARTICLE

Escape from gene silencing in ICF syndrome: evidence for advanced replication time as a major determinant

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Chromosomal abnormalities associated with hypomethylation of classical satellite regions are characteristic for the ICF immunodeficiency syndrome. We, as well as others, have found that these effects derive from mutations in the DNMT3B DNA methyltransferase gene. Here we examine further the molecular phenotype of ICF cells and report several examples of extensive hypomethylation that are associated with advanced replication time, nuclease hypersensitivity and a variable escape from silencing for genes on the inactive X and Y chromosomes. Our analysis suggests that all genes on the inactive X chromosome may be extremely hypomethylated at their 5′ CpG islands. Our studies of G6PD in one ICF female and SYBL1 in another ICF female provide the first examples of abnormal escape from X chromosome inactivation in untransformed human fibroblasts. XIST RNA localization is normal in these cells, arguing against an independent silencing role for this RNA in somatic cells. SYBL1 silencing is also disrupted on the Y chromosome in ICF male cells. Increased chromatin sensitivity to nuclease was found at all hypomethylated promoters examined, including those of silenced genes. The persistence of inactivation in these latter cases appears to depend critically on delayed replication of DNA because escape from silencing was only seen when replication was advanced to an active X-like pattern.

INTRODUCTION

ICF syndrome is a rare autosomal recessive disease characterized by immunodeficiency, centromeric decondensation, facial anomalies and restricted hypomethylation patterns (1–3). Recent data from our laboratory (4) and others (5,6) have shown that mutations in the DNMT3B DNA methyltransferase gene underlie the ICF syndrome. DNA methylation plays important roles in vertebrate genome structure and expression, and the ICF finding represents a significant advance toward the understanding of how genomic methylation patterns are determined. Our interest in X chromosome inactivation and reports of hypomethylation defects in ICF patients prompted us to examine in more detail the fidelity of silencing mechanisms on the inactive X chromosome in cells from ICF females as well as on the Y chromosome in cells from ICF males.

X inactivation is a very stable system of gene silencing in mammals, being composed of a multiplicity of repressive factors whose interactions have yet to be fully determined. Our studies of gene reactivation in somatic cell hybrids suggest that late replication of DNA plays an important role in X chromosome inactivation silencing that can operate independently of silencing by 5′ CpG island hypermethylation (7). ICF cells potentially provide an important model for determining the relationships between replication time, CpG island methylation, chromatin structure and gene silencing, because the hypomethylation defect in ICF is not as global as that following 5-azacytidine (5aC) treatment or in cells deficient in DNMT1 DNA methyltransferase (5,8,9). The inactive X appears to be particularly sensitive to the ICF defect as it is hypomethylated in several ICF females as determined by cytogenetic analysis of reactivity with an antibody to 5-methylcytosine (2,10). Hypomethylation at a limited number of specific sites has also been demonstrated by methylation-sensitive restriction site analysis (2,10–12).

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We report here the first examples of abnormal escape from X inactivation in untransformed human fibroblasts. We also describe an ICF male case with disruption of the normal silencing of SYBL1 on the Y chromosome. These phenomena occur in ICF cells in association with almost complete promoter demethylation and advanced replication time. Although promoter hypomethylation is widespread on the inactive X chromosome in ICF cells, escape from inactivation was only found for genes that replicate like active X alleles in early to mid-S phase, thus lending support to the hypothesis that late replication timing can play a critical role in gene silencing.

RESULTS

To examine the fidelity of X inactivation silencing in ICF syndrome, we studied methylation, chromatin structure, replication time and expression of several X-linked genes in cells derived from two ICF females, PT3 and PT4, that we found to carry mutations in both of their DNMT3B methyltransferase alleles (4). The cells studied include fibroblasts and lymphoblastoid cells derived from PT4 and fibroblasts from PT3. We also examined SYBL1 on the Y chromosome in lymphoblasts from two ICF males (PT1 and PT5). PT1 is homozygous for a DNMT3B missense mutation in the catalytic domain (4) and PT5 is a compound heterozygote for two other missense mutations in the catalytic domain (13). All cases studied had classical ICF features and the ICF defect of hypomethylated satellite sequences 2 and 3 were confirmed by Southern blot analysis in the cells that we examined (data not shown).

Hypomethylation of inactive X and Y chromosomal loci in ICF cells

To study X inactivation silencing in ICF, we first used methylation-sensitive restriction enzymes to examine in lymphoblasts and fibroblasts derived from the PT4 ICF female case the methylation patterns at the 5′ regions of 11 genes that are normally subject to X inactivation. These genes span much of the X chromosome and include PDHA1 (Xp22.1), AR (Xq12), XPCT (Xq13.2), PGK1 (Xq13.3), HPRT (Xq26.1), FMR1 (Xq27.3), FMR2 (Xq27.3), IDS (Xq28), G6PD (Xq28), MPP1/P55 (Xq28) and SYBL1 (Xq28). The 5′ regions of these genes are all hypomethylated in normal female lymphoblasts and fibroblasts as expected and are all hypomethylated in lymphoblasts and fibroblasts from PT4 (Fig. 1 and data not shown).

To examine the inactive X methylation patterns at G6PD, FMR1 and SYBL1 in more detail, we used the bisulfite conversion method of methylation analysis (14). Depicted in Figure 2 are the 5′ regions of these genes, as well as that of the XIST gene, that were examined for methylation status. The methylation patterns of individual strands are obtained with this method and it is expected that 50% of these strands would be derived from inactive X alleles if no bias was present in either the PCR or cloning steps of the procedure (15).

In the 5′ region of G6PD, we examined the methylation status of 52 CpG sites in a CpG-rich region encompassing the major start site of transcription (Fig. 2A). We found that this locus is essentially free of methylation on the active X alleles of normal males (data not shown) and females (Fig. 3B, unmethylated alleles) and is highly methylated on the inactive X in normal females (Fig. 3B, hypermethylated alleles). The approximately equal numbers of hypermethylated (inactive X) and hypomethylated (active X) clones derived from normal females suggests that the method is not heavily biased for either methylated or unmethylated strands at this locus. When comparing normal and ICF methylation patterns, however, it is evident that the inactive X allele of G6PD is hypomethylated both in fibroblasts from two different ICF cases (PT3 and PT4 in Fig. 3C and F) and in lymphoblasts from PT4 (Table 1) because all strands analyzed are hypomethylated. This extreme hypomethylation is also evident even when the data are summarized as the percentage of CpG sites methylated for all clones (Table 1). The G6PD methylation patterns for uncultured PT3 ICF lymphocytes, PT3 ICF fibroblasts, PT4 ICF lymphoblasts and PT4 ICF fibroblasts are very similar, with little methylation present. The overall percentage of CpG sites methylated in ICF clones is about one order of magnitude less than in normal clones. These differences underestimate the extent of hypomethylation on the inactive X in ICF cells because the unmethylated active X patterns are not subtracted. Similar analyses of bisulfite methylation patterns for FMR1, SYBL1 and XIST are also presented in Table 1.

At the FMR1 locus, we examined methylation of 22 CpG sites in the 5′ region (Fig. 2B) and found that all sites on the normal active X allele in males are hypomethylated (0.9% methylation) (Table 1). In normal females, we found a number of strands with heavy methylation that are likely derived from the inactive X and several strands with no methylation derived from the active X (28% methylation) (Table 1 and data not shown). As in the case of G6PD, all strands from PT4 ICF female cells were markedly hypomethylated (0.5% methylation) (Table 1), indicating that methylation of the inactive X allele is abnormal. The percentage of methylated CpG sites in the ICF female is similar to that in a normal male and is ~50-fold lower than the normal female value. Similar patterns of
hypomethylation were found in DNA from PT4 lymphoblastoid cells and PT4 fibroblasts (Table 1). The SYBL1 gene is located in the long arm X chromosome pseudoautosomal region and is subject to X inactivation in females. In addition, the Y allele is also silenced in males (16). Promoter methylation represses SYBL1 transcription from the inactive X and Y alleles as shown by reactivation in hybrids following demethylation by 5aC treatment and by studies of methylated and unmethylated reporter constructs (17). We used bisulfite methylation analysis to examine the 5′ region that includes exon 1 and contains 37 CpGs (Fig. 2C). An XhoI restriction site polymorphism in exon 1 was utilized to determine allele-specific expression patterns. These patterns can be correlated with allele-specific methylation status because the polymorphic nucleotide is preserved in the antisense DNA strand following bisulfite treatment.

In cells from a normal male, the expressed SYBL1 allele on the active X is fully unmethylated at all 37 CpG sites analyzed in 30 clones, whereas the repressed allele on the Y chromosome is hypermethylated on all clones (50% methylation) (Table 1; M.R. Matarazzo et al., manuscript in preparation). One ICF male, PT5, is heterozygous for the XhoI polymorphism in SYBL1. Lymphoblasts from this patient were examined for allele-specific methylation and, as shown below, for expression. The expressed X chromosome allele in PT5 is completely unmethylated as in normals and the Y allele is markedly hypomethylated (13.8% methylation) with almost two-thirds of the chromosomes showing fewer than one methylated site (Table 1 and data not shown).

Like the Y allele of SYBL1, the silent inactive X allele is also hypermethylated in female cells as seen in a culture in which the inactive X chromosomes can be distinguished from the active X chromosomes (58% methylation) (Table 1 and data not shown). In contrast, all strands from uncloned fibroblasts from the PT3 ICF female had an almost complete lack of methylation, indicating that both the paternal and maternal inactive X chromosomes are abnormally hypomethylated (1.9% methylation for the XhoI+ allele and 1.1% methylation for the XhoI– allele) (Table 1). These data are consistent with our methylation analysis of HpaII sites using standard Southern blot techniques; Southern analysis also revealed SYBL1 hypomethylation for an additional ICF male, PT1 (data not shown).

We infer from our restriction enzyme and bisulfite methylation analyses that most 5′ CpG islands associated with genes subject to X inactivation are hypomethylated in the ICF cells examined. To determine whether the active X chromosome may also be subject to abnormal hypomethylation in ICF females, we examined a small CpG cluster at the 5′ end of the XIST gene (Fig. 2D) that is normally methylated on the active
X and unmethylated on the inactive X (18). In contrast to inactive X loci, the silent \textit{XIST} locus on the active X appears to be methylated normally in PT4 ICF fibroblasts and lymphoblasts because the frequency of hypermethylated clones we obtained was similar in ICF and normal cells (Table 1 and data not shown). Methylation of this gene, however, is likely to be under strong selective pressure early in development so as to maintain an active X chromosome (see Discussion).

\section*{Advanced replication time of inactive X loci in ICF cells}

In addition to hypermethylation, the normal inactive X and satellite regions share the characteristic of late replication (7,19–21). Hypomethylation of such loci following 5aC treatment is known to bring about an advance in replication time (7,19,21). We compared the replication time, therefore, of a number of X-linked loci in normal and ICF cells. Replication time was determined by analysis of newly replicated DNA labeled with bromodeoxyuridine (BrdU) that was derived from cells sorted into specific cell cycle stages by flow cytometry (22,23). 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.

We analyzed replication time at \textit{PGK1}, \textit{G6PD}, \textit{AR}, \textit{MPP1}, \textit{IDS} and \textit{SYBL1} in PT4 ICF lymphoblasts and fibroblasts as

\begin{table}
\centering
\caption{Bisulfite methylation analysis of \textit{G6PD}, \textit{FMR1}, \textit{SYBL1} and \textit{XIST} in normal and ICF cells}
\begin{tabular}{llr}
\hline
Gene & Sample & CpG methylation (\% total CpG) \\
\hline
\textit{G6PD} & Normal female fibroblast (FB2) & 35.4 \\
 & ICF female fibroblast (PT4) & 3.1 \\
 & Normal female lymphoblast (GLP1) & 43.7 \\
 & ICF female fibroblast (PT4) & 4.2 \\
 & ICF female lymphocytes (PT3) & 6.5 \\
\textit{FMR1} & Normal male lymphocytes (II-4) & 0.9 \\
 & Normal female lymphocytes (L7) & 27.7 \\
 & ICF female fibroblast (PT4) & 0.5 \\
\textit{SYBL1} & Normal male lymphoblast & \\
 & Silent Y allele & 50.4 \\
 & Active X allele & 0.0 \\
 & Normal female lymphoblast & \\
 & Inactive X allele & 57.6 \\
 & Active X allele & 0.0 \\
 & ICF male lymphoblast (PT5) & \\
 & Expressed Y allele & 13.8 \\
 & Expressed X allele & 0.0 \\
 & ICF female fibroblast (PT3) & \\
 & \textit{Xho1+} allele & 1.9 \\
 & \textit{Xho1–} allele & 1.1 \\
\textit{XIST} & Normal female fibroblast (FB4) & 60.0 \\
 & ICF female fibroblast (PT4) & 37.5 \\
 & Normal female lymphoblast (194) & 32.5 \\
 & ICF female lymphoblast (PT4) & 37.5 \\
\hline
\end{tabular}
\end{table}

\begin{figure}
(A) Methylation patterns from a normal female fibroblast culture (FB2). CpG sites are represented as either methylated (M) or unmethylated (o). Each clone represents the methylation pattern of a single chromosome. Hypomethylated alleles correspond to the inactive X chromosome and are grouped together. (B) Methylation patterns from a PT4 ICF female fibroblast culture. (C) Methylation patterns from a PT3 ICF female fibroblast culture. The ICF female patterns are extensively hypomethylated, similar to the active X-only patterns of a normal male (data not shown), indicating that the inactive X alleles are abnormally undermethylated.
well as PT3 fibroblasts. Except for AR and IDS in fibroblasts, we found a clear association between hypomethylation in ICF and advanced replication time, but the magnitude of the shift to early replication is variable. Several inactive X loci in ICF females and the Y-linked SYBL1 locus in ICF males are advanced from S2 and G2/M to S4 and G2/M (Fig. 5A). In PT4 fibroblasts, however, G6PD replication on the inactive X is advanced even further, overlapping extensively with replication of the active X allele. This replication advance was seen at both the 5′ (Fig. 4B) and 3′ (data not shown) regions of G6PD.

In contrast to these examples, replication of AR appears normal in PT4 ICF fibroblasts (Fig. 4C); the active and inactive X alleles are not well resolved, however, as both replicate quite late in S phase. Like FMR1 (23), AR appears to be an example of a gene that is expressed from the active X even when it replicates quite late in S phase. Similar to AR, the active and inactive X alleles of IDS replicate in the latter half of S phase in normal fibroblasts and no difference from normal replication was seen in PT3 or PT4 ICF female fibroblasts (data not shown). Replication of MPP1 is biphasic in normal lymphoblasts and fibroblasts and the inactive X allele replicates in the second half of S phase in both normal and PT4 ICF fibroblasts and lymphoblasts (though somewhat advanced from normal; data not shown). Marked advances in replication time were found, however, for the Y allele of SYBL1 in PT1 and PT5 lymphoblasts, similar to the advance seen for G6PD in PT4 fibroblasts (Fig. 4D; alleles are not separated in the case of PT1 who is homozygous for the Xho allele).

**Escape from silencing on the inactive X and Y chromosomes in ICF cells**

From our studies of 5aC-induced reactivation of X-inactivated genes, we know that an unmethylated 5′ CpG island and replication in the first half of S phase is strongly associated with transcription (7). We examined cells from ICF cases, therefore, for expressed polymorphisms in X-linked or X/Y-linked genes to determine whether any genes escape inactivation on the inactive X or the Y. Heterozygosity was found at such sites for AR, G6PD, IDS, MPP1 and SYBL1 in various ICF cells, thus permitting allele-specific RT–PCR analysis.

Because the replication time of G6PD is markedly advanced in PT4 fibroblasts and the promoter is extremely undermethylated, we predicted that escape from inactivation would be found in this case. This prediction was verified as all 9 clones and 18 single cells examined expressed both alleles, whereas all 22 single normal fibroblasts showed monoallelic expression (Fig. 5A). In all 16 single PT4 lymphoblasts examined, however, G6PD expression was monoallelic. This normal silencing of the inactive X allele in the majority of PT4 lymphoblasts correlates with its later replication in these cells relative to PT4 fibroblasts (Fig. 4B). The stability of silencing in PT4 lymphoblasts appears to be compromised, however, for we found that 5aC treatment resulted in a high proportion of cells (5 of 14) that showed some evidence of biallelic expression (Fig. 5A). We also found that spontaneous reactivation of G6PD had occurred in each of two somatic cell hybrid clones that contain an inactive X derived from PT4 lymphoblasts (Fig. 5A). These cells presumably escape inactivation because of a further advance in replication time, but it is difficult to clone such lymphoblasts for replication analysis and we have not yet examined replication in the hybrid cells.

The Y allele of SYBL1 is markedly hypomethylated in PT5 ICF lymphoblasts (Table 1) and its replication is advanced to a time that is similar to the expressed, active X allele (Fig. 4D). Based on the reasoning discussed above for G6PD, our finding of biallelic SYBL1 expression in these cells was not surprising (Fig. 5B). SYBL1 also escapes silencing on the inactive X, as we found biallelic expression in 7 of 17 PT3 ICF fibroblast clones examined (Fig. 5B).

Monoallelic expression of IDS was seen in all five PT4 fibroblast clones examined (Fig. 5C). The inactive X allele of IDS appears to replicate normally in PT4 fibroblasts, i.e. in...
Figure 5. RT–PCR analysis of escape from silencing. (A) Allele-specific G6PD expression was analyzed in normal fibroblasts (GM01871), PT4 ICF female fibroblast clones, single PT4 lymphoblasts (PT4LB), single PT4 lymphoblasts treated with the demethylating agent, 5aC (PT4LB-5aC) and human–hamster hybrid subclones derived from PT4 lymphoblasts (hdA1s12, ID4s3 and ID4s5). Controls for the specificity of the analysis include FB2 and FB4 normal fibroblast patterns that are homozygous for different alleles. Both alleles are expressed in uncloned cultures of PT4 lymphoblasts, as would be expected for two populations of cells with normal, random X chromosome inactivation. Escape from inactivation occurs in all PT4 fibroblast clones (2A3, 2A5, 2A7, 2C6 and 2D9) and single cells (data not shown), but not in PT4 lymphoblasts, unless treated with 5aC or fused to Chinese hamster ovary cells. (B) Allele-specific expression analysis of SYBL1 in LB1, a normal male lymphoblast, LB1 treated with 5aC (LB1-5aC), two ICF male lymphoblasts (PT5 and PT12) and single PT3 ICF female fibroblasts. Escape from inactivation occurs in PT5 ICF male lymphoblasts and some PT3 ICF female fibroblasts, but not in normal LB1 male lymphoblasts or PT12 ICF male lymphoblasts. (C) Allele-specific expression analysis of IDS in PT4 ICF female fibroblast clones. M4 is the mother of PT4 and is homozygous for the Xho repeat unit; P4 is the father of PT4 and is Xho; PT4 and PT3 cells are heterozygous for the Xho polymorphism. Uncloned cultures of PT4 ICF female lymphoblasts and PT3 ICF female fibroblasts (PT3FB) express both alleles, as would be expected from normal, random X chromosome inactivation. PT4 ICF fibroblast clones that escape inactivation at G6PD (A) do not escape at IDS. No escape at IDS was also found in a few PT3 ICF fibroblast clones that were examined (data not shown). (D) Allele-specific expression analysis of AR expression in PT4 ICF female fibroblast clones. PT4 cells are heterozygous for a CAG repeat polymorphism in the 5′ coding region of AR, the alleles differing by one repeat unit. Both alleles are expressed in the uncloned fibroblast culture, consistent with normal random inactivation. Because of shadow bands, only clones with the smaller repeat are informative for partial expression of the inactive X allele. No evidence for such escape from inactivation was seen in any of the eight informative clones examined.

Chromatin sensitivity to nuclease digestion in the S′ regions of G6PD and PGK1

Another feature of expressed genes on the active X and 5aC-reactivated genes on the inactive X is that the chromatin at the promoter region is hypersensitive to nucleases. For example, we found previously that sensitivity in the PGK1 promoter to MspI nuclease readily discriminates between the active and inactive X alleles (24). When we applied this assay to G6PD in normal fibroblasts, we found a similar MspI discrimination between the active and inactive X alleles in that the active X in males is readily digested at the lowest MspI concentration examined, whereas the inactive X in females is resistant to digestion, even at the highest MspI concentration (Fig. 6). In contrast to the normal female pattern of MspI sensitivity, the PT4 ICF fibroblast pattern resembles that of an active X, consistent with our finding of biallelic expression of G6PD in PT4 fibroblasts. PT3 fibroblasts also have a pattern of increased chromatin sensitivity, but the transcriptional status of the inactive X allele in PT3 is unknown as this case is not informative for the expressed polymorphism studied.

Surprisingly, PT4 lymphoblasts also show a pattern of enhanced MspI sensitivity even though the inactive X allele of G6PD is not expressed in these cells (Fig. 5A). Similarly, PT4 fibroblasts show a pattern of increased MspI digestion of the inactive X allele of AR (data not shown), although it is silenced normally in these cells (Fig. 5D). PGK1 analysis of these blots also revealed enhanced sensitivity for the inactive X in ICF cells; transcriptional status of the inactive X was not, however, determined for this gene.

XIST RNA localization in ICF cells

Features other than methylation, replication time and nuclease sensitivity that distinguish the active and inactive X chromosomes include the inactive X-specific property of XIST RNA association. We examined XIST RNA in PT3 and PT4 ICF fibroblasts by fluorescence in situ hybridization (FISH) analysis and found that it is expressed and localized normally (data not shown). Because of the redundant silencing systems for maintaining X inactivation, it is not surprising that cells lacking XIST RNA still maintain inactivation (25,26). Observations that XIST expression is not sufficient for silencing in the absence of other X inactivation mechanisms (27–29) raise...
questions, however, about the role of XIST RNA in somatic cells. A major caveat to the conclusion that XIST RNA does not contribute to silencing in somatic cells is that the previous studies were performed on transformed cells in which XIST RNA localization is abnormal (29,30). Our findings for ICF fibroblasts appear to provide the first evidence that normal XIST expression in an untransformed cell type is not sufficient to silence a gene on the inactive X that is normally subject to X inactivation.

**DISCUSSION**

Our studies of ICF patients suggest marked hypomethylation of the CpG islands of X-linked genes that are normally subject to X inactivation. In addition, significant advances in replication time were found for five of six such loci studied. Heterozygosity at four loci permitted RT–PCR analysis of possible transcription from the normally silenced alleles; biallelic expression was detected at two loci. Clearly, mutations in DNMT3B have led to significant disturbances in various aspects of the X inactivation system in these ICF cases. Escape from X inactivation in untransformed fibroblasts is a novel finding in light of many previous attempts to detect spontaneous or induced reactivation of such genes. Further study of ICF and other DNMT3B-deficient cells should lead to the better understanding of the interplay between silencing factors in the X inactivation system.

**Late replication, promoter methylation and silencing on the Y and inactive X chromosomes**

Late replication and promoter methylation are two such factors whose roles in silencing are explored in our study. Examination of data summarized in Table 2 for ICF and normal cells suggests that advanced replication time is a critical factor for escape from inactivation in ICF cells. In the case of G6PD, escape from silencing occurs in all PT4 fibroblasts, but not in PT4 lymphoblasts even though the gene is extensively hypomethylated in both cultures. For the fibroblasts, replication time is advanced in most cells to an active X-like pattern, whereas G6PD still replicates in the last half of S phase in the lymphoblasts. An advance in replication time to an active X-like pattern was also seen for the Y allele of SYBL1 in lymphoblasts from each of two ICF males, PT1 and PT5 (Fig. 4D), and strong escape from silencing was demonstrated in the case of PT5 that was informative for the expressed poly-

Table 2. Summary of selected methylation, replication and gene expression data

<table>
<thead>
<tr>
<th>Gene</th>
<th>ICF cells</th>
<th>Hypomethylation</th>
<th>Replication advance</th>
<th>Biallelic expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD</td>
<td>PT4 fibroblasts</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>PT4 lymphoblasts</td>
<td>++++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>SYBL1</td>
<td>PT5 lymphoblasts</td>
<td>++++</td>
<td>++++</td>
<td>n.i.</td>
</tr>
<tr>
<td></td>
<td>PT1 lymphoblasts</td>
<td>++++</td>
<td>++++</td>
<td>n.i.</td>
</tr>
<tr>
<td></td>
<td>PT3 fibroblasts</td>
<td>++++</td>
<td>++++</td>
<td>n.i.</td>
</tr>
<tr>
<td>AR</td>
<td>PT4 fibroblasts</td>
<td>++++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IDS</td>
<td>PT4 fibroblasts</td>
<td>++++</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>PT4 lymphoblasts</td>
<td>++++</td>
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<tr>
<td></td>
<td>PT3 fibroblasts</td>
<td>++++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MPP1</td>
<td>PT4 fibroblasts</td>
<td>++++</td>
<td>+</td>
<td>n.i.</td>
</tr>
<tr>
<td>PGK1</td>
<td>PT4 fibroblasts</td>
<td>++++</td>
<td>+</td>
<td>n.i.</td>
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<tr>
<td></td>
<td>PT4 lymphoblasts</td>
<td>++++</td>
<td>+</td>
<td>n.i.</td>
</tr>
</tbody>
</table>

a++++, a reduction in methylation of <30% of normal.
b++++, replication of >50% of the inactive X allele occurs when the active X allele replicates; ++, replication of the inactive X allele is advanced, but <50% overlaps with replication of the active X allele; +, replication of the inactive X allele is advanced, but does not overlap with replication of the active X allele more than normal; –, no evidence of a change in replication time.
c++++, all single cells and/or clones analyzed exhibited biallelic expression; ++, 40% of single cells and/or clones analyzed exhibited biallelic expression; +, no evidence of biallelic expression was observed; n.i., not informative for allelic differences in expression.
dOnly three clones tested.
mechanisms such as histone hypoacetylation (38). The importance of advanced replication for escape from silencing is also supported by our observations of late replication and normal silencing in ICF cells for the inactive X alleles of AR, IDS and MPP1 that are abnormally hypomethylated.

These data suggest that late replication may be sufficient for silencing in the absence of promoter methylation, emphasizing the importance of replication advance to an active X-like pattern for escape from inactivation. In marsupials, where 5' CpG island methylation does not appear to play a role in X inactivation (31,32), late replication may also be a dominant silencing factor (33). Advanced replication time, however, is not sufficient for escape from silencing in mammalian cells. We previously described an inactive X somatic cell hybrid, X8-6T2, with spontaneous replication advances at G6PD and HPRT that are similar to those seen for G6PD in PT4 fibroblasts and SYBL1 in PT1 and PT5 lymphoblasts. These genes are silenced in this hybrid (7), undoubtedly due to the hypomethylated state of their promoters (34, and data not shown).

One might argue that the replication advances seen for genes that escape transcriptional silencing in ICF cells occurred following escape rather than providing a primary role. Several silenced genes and non-coding satellites, however, were also found to have moderately advanced replication in ICF cells (Table 1 and data not shown) and in 5aC-treated inactive X hybrids (7). The apparent independence of promoter methylation, replication time and expression in the instances described above argues against the notion that expression, brought about by some unknown mechanism, would then lead to hypomethylation and advanced replication. Our analysis of 5aC-induced reactivation of HPRT in the X8-6T2 hybrid system indicates that with replication time already advanced, transcription factor binding and reactivation occur many hours later than hypomethylation and chromatin remodeling (35,36). The variability in replication timing in ICF cells suggests that methylation by DNMt3B contributes to the establishment and/or maintenance of late replication on the inactive X and Y chromosomes and that other factors can bring about late replication in the absence of this activity, albeit with less fidelity.

These ICF data are consistent with our previous observations of advanced replication time and transcriptional reactivation for X-inactivated genes following 5aC-induced demethylation (7). Although the mechanism for replication timing control by methylation is not known, one possibility is that there are specific, methylation-sensitive control loci that also promote the stable organization of the early- and late-replicating subnuclear compartments. The ICF hypomethylation defects that we observed would suggest that such control regions are likely to be CpG islands. Such regions could be the replication origins themselves and there is some evidence for CpG islands being common loci for replication initiation (37). ICF cells should, therefore, provide an excellent model system for exploring higher-order transcriptional control by replication timing, nuclear compartmentalization and other silencing mechanisms such as histone hypoacetylation (38).

Unexpected observations in ICF cells: normal XIST RNA localization with escape from X inactivation and chromatin hypersensitivity to nuclease with maintenance of X inactivation

Similar to studies of marsupial X inactivation (39), we found that silencing is unstable in ICF cells, leading to a variable escape from inactivation and spontaneous reactivation in somatic cell hybrids. It is possible that the abnormal XIST RNA association seen in such hybrids (29,30) may prevent efficient silencing in the absence of promoter methylation and late replication. Given our observations of escape from inactivation for G6PD and SYBL1 in ICF female fibroblasts, the finding that XIST RNA association with the inactive X is grossly normal in ICF fibroblasts is somewhat surprising. This association is clearly inadequate for continued repression of the two genes that we observed to escape inactivation, but it is consistent with an overall preservation of X inactivation. Additional genes should be analyzed for escape and more detailed XIST RNA localization studies are needed at escaping loci.

Our observations of increased nuclease hypersensitivity at silent, inactive X alleles of G6PD in PT4 lymphoblast nuclei and AR in PT4 fibroblast nuclei (Fig. 6 and data not shown) were also unexpected. How can we explain silencing in these cases if there appears to be an ‘open’ or accessible chromatin state in the promoter regions? Although hypomethylation of the 5’ CpG island is apparently sufficient to enable accessibility to Mxpl in isolated nuclei, it is possible that the late replication that we observe in the intact cell (even though somewhat advanced from normal) provides or reflects a temporal and/or spatial compartmentalization from the ubiquitous transcription factors that usually bind to the active X allele of this housekeeping gene. Such compartmentalization has been observed cytologically for early- and late-replicating chromosomal segments (40) and only the early-replicating domains were seen to be associated with nascent transcripts (41).

Variability in escape from silencing in ICF patients

A recent paper studying X inactivation in ICF females reported results quite different from ours (10). Bourc’his et al. (10) interpreted their data to infer that inactive X hypomethylation in ICF mainly involved the CpG sites outside the promoter regions. A cytological analysis of replication timing in one ICF case suggested normal late replication for the inactive X. Evidence against escape from inactivation included absence of 2-fold increases in enzymatic activity for five X-linked loci in three ICF cases, although such an increase was observed in one of eight observations. One possible explanation for our different results is that the methods that we employed are of higher sensitivity and resolution than the cytological and enzymatic assays used by Bourc’his et al. (10). However, the heterogeneity between the ICF cases studied is also a viable explanation as described for the genes studied here in PT3 and PT4. The characteristics of lymphoblasts from another ICF patient, PT12, suggest additional variability because this patient’s disease appears to have arisen from defects in another gene (13). Because different mutations or genes may contribute to the variability in silencing defects, more informed genotype–phenotype correlations may help to resolve these issues.
Escape from inactivation probably occurs in vivo

One might argue that some of the aberrant features that we have discovered regarding the inactive X in ICF are the results of cell culture. This seems unlikely to be the primary explanation because we found CpG island hypomethylation and escape from inactivation in two non-transformed ICF fibroblast cultures. Because both the paternal and maternal inactive X chromosomes are affected in these cultures, such a selection would require the unlikely events of escape from inactivation in at least two different cells occurring at about the same time so as to be equally represented in the final cultures. In addition, we also observed G6PD hypomethylation in vivo as detected in uncultured leukocytes from the PT3 ICF female that presumably occurs on both parental chromosomes (Table 1).

Because the cells that we have studied derive from adult tissues, it is difficult to determine whether the escape from silencing was an early embryonic event or one that occurred over several cell generations in vivo. The latter possibility is favored by our observation that fibroblasts and a lymphoblastoid cell line from the same patient differed in escape from silencing of G6PD. The normal pattern of XIST RNA association that we found in ICF fibroblasts is also consistent with a normal establishment of X inactivation. In addition, a secondary escape from inactivation is supported by our observations that G6PD silencing is less stable on inactive X chromosomes from PT4 ICF lymphoblasts than those from normal cells when placed into a hamster cell background. Escape from silencing subsequent to establishment of X inactivation has previously been shown to explain why a mouse gene normally escapes inactivation in adult cells (42). The observations described above for PT3 and PT4 fibroblasts, however, argue in favor of escape occurring early in the fibroblast lineage because both parental X chromosomes are represented in the abnormal inactive X alleles.

Whether or not escape from silencing occurred early or late in development, the MT3β-mediated de novo methylation defect in ICF probably compromised X inactivation early in development, when the enzyme is likely to have a major role in inducing promoter repression by CpG island methylation. MT3β may also have an early role in promoting late replication. ICF defects in these activities would, therefore, provide a less stable state of silencing for subsequent cell generations. A direct role for MT3β in the maintenance of inactivation is also possible and recent studies have implicated it as the enzyme that maintains CpG island methylation in cancer cell lines that are deficient in DNMT1 expression (43).

The alterations in DNA methylation within ICF cells appear to be limited to a small percentage of the genome because global methylation levels are in the normal range (9). Our results are in accordance with this conclusion as they indicate that the DNMT3B protein, MT3β, is targeted to certain heterochromatic regions, such as the inactive X and satellite 2 and 3 sequences. Similar observations were found in mouse cells deficient in MT3β (5). We found no consistent evidence for hypomethylation at the XIST gene on the active X, implying that a methyltransferase other than MT3β, such as MT3ε, may be responsible for methylation of this region. Studies of mice deficient in MT3ε and MT3β suggest that these proteins have distinct roles, with some overlap in function (5). Alternatively, the mutant ICF forms of MT3β may provide enough activity for at least partial methylation of this locus, especially given that there would be selection for such methylation to maintain an active X chromosome (44,45). The existence of residual methyltransferase activity in ICF patients is supported by their post-neonatal survival in contrast to mice with homozygous disruptions of Dnmt3b which all die before birth (5).

Escape from silencing and the ICF phenotype

Although genes other than G6PD and SYBL1 are likely to escape X inactivation in ICF females, this phenomenon is probably limited in terms of the number of genes and tissues involved in a given individual. This conclusion follows from the fact that ICF males and females have similar phenotypes (46,47). Widespread escape from X inactivation would almost certainly produce a female-specific phenotype. Genes that do escape, however, may suggest pathways that may be affected in ICF and point to candidate genes in the pathway that may be abnormally regulated. X-linked genes involved in cell growth and/or survival, for example, could have been under selective pressure for overexpression and/or overexpression may have compensated for more primary abnormalities involved in the ICF phenotype. G6PD overexpression, for example, is known to confer a growth advantage (48). In addition, the 5′ CpG island of G6PD is also shared by NEMO, the NF-xB essential modulator that is expressed in the opposite orientation and is mutated in incontinentia pigmenti patients (49). Biallelic expression of NEMO is likely to occur in PT4 ICF fibroblasts because replication of the 5′ region is advanced to an active X-like pattern (Fig. 4B) and the 5′ region is hypomethylated (Fig. 3E). If overexpressed, this gene is also likely to have had effects on cell growth and survival. In the case of SYBL1, biallelic expression in both females and males might reflect either primary or compensatory abnormalities in intracellular vesicle trafficking.

The disturbances that we observed in X and Y inactivation suggest that autosomal genes might also be affected in ICF syndrome. The facial anomalies and immune deficiencies in ICF might be explained by the inability to repress key genes by promoter methylation (50), although few genes with such regulation are known outside the inactive X and imprinted regions (51). Abnormal gene regulation could also be a secondary effect of hypomethylation at pericentromeric regions or other such repetitive, CpG-rich regions similar to the phenomenon of position-effect variegation found in yeast, Drosophila and mammalian systems (52,53). Genes of this type would be affected in cis because they are within or near repetitive regions that are normally hypermethylated and late replicating. A possible mechanism for ICF cytological abnormalities at these regions and disruption of silencing is that heterochromatin proteins normally available for assembly following replication late in S phase are unavailable earlier in the cell cycle when replication occurs in ICF cells. The Y allele of SYBL1 is a candidate for such a gene because it is in proximity to a large block of heterochromatin on the long arm (54). Further studies of this region will be necessary to determine whether replication time and methylation are altered in ICF cases in which SYBL1 escapes inactivation. ICF abnormalities in heterochromatin are also likely to have effects in trans because pericentromeric satellite regions appear to act as silencing centers for genes brought into proximity by protein
mediators such as IKAROS (55,56). Studies in lymphocytes, for example, suggest that IKAROS-directed localization of lineage-specific genes to satellite regions is needed for the stable maintenance of their silencing in subsequent cell generations (55).

Clearly, mutations in DNMT3B have led to significant disturbances in various aspects of the X inactivation system in these ICF cases. Escape from X inactivation in untransformed fibroblasts is a novel finding in light of many previous attempts to detect spontaneous or induced reactivation of such genes. Our observations of defective gene repression with a limited gene sampling suggests that screening a larger set of genes for escape from silencing may help in identifying genes that are important for the ICF phenotype. That other loci are affected in ICF is supported by a recent report of escape from silencing in ICF cells of a repetitive expressed sequence tag associated with an abnormally hypomethylated region (57). In addition to learning more about ICF, further studies of genes that escape inactivation in ICF, including the X- and Y-inactivated genes that are abnormally regulated, should also help us to better understand silencing in its various forms.

MATERIALS AND METHODS

Cells and culture conditions

The cells examined in this study were all derived from classical ICF features. Three cases, PT1 male, PT3 female and PT4 female, were reported previously to have DNMT3B mutations (4). The cells used for analysis included lymphoblastoid cells from the PT1 male (PT1 LB), fibroblasts from the PT3 female (PT3 FB), lymphoblasts (PT4 LB or GM08714) and fibroblasts (PT4 FB or GM08747) from the PT4 female, lymphoblasts from PT4’s mother (M4 LB or GM08728) and lymphoblasts from PT4’s father (F4 LB or GM08729). Lymphoblasts were also examined from an unpublished male ICF case (PT5 LB) who has classical features and heterogeneous mutations in DNMT3B (13). The Coriell Cell Repositories (Camden, NJ) supplied the ICF family cell lines GM08747, GM08714, GM08728 and GM08729. Control fibroblasts were supplied by Coriell Cell Repositories (e.g. GM01871) and G. Martin (University of Washington); W. Raskind (University of Washington); W. Martin (University of Washington) provided normal control lymphoblasts. Lymphoblasts and fibroblasts were cultured as previously described (24,29). Fibroblast clones were obtained from culture dishes following limiting dilution and colony growth. Clonal human-hamster somatic cell hybrids derived from GM08714 cells were described previously (4). Hybrids that contain a human inactive X were identified by XIST RT-PCR (7).

Methylation-sensitive restriction digestion and nuclease hypersensitivity

Methylation analyses of satellite 2 and 3 sequences in fibroblasts and lymphoblasts were accomplished by restriction enzyme digestion and Southern blot analysis using probes derived from linear PCR of genomic DNA with S2 and S3 primers as described (4). Similar results were found for satellite 2 with a probe generated by exponential PCR of genomic DNA using the primers sat2-384U, 5’-ATGGAAATGAAAGGGTTCATCATCCT-3′, and sat2-781L, 5’-ATTCGAGTCATTTCGATGATTCAT-3′. Methylation-sensitive enzymes for these analyses included HpaII, BstBI and McrBC, and digestions were performed according to the manufacturer’s conditions (New England Biolabs, Beverly, MA).

Restriction enzyme digestion of X- and Y-linked genes was performed according to the manufacturer’s suggested reaction conditions (New England Biolabs) with some modifications as described previously (24,58,59). The following probes were used for methylation analysis: AR5p1U:1L for AR (PCR-generated from genomic DNA using AR5p1U, 5’-CTACCTGGTCTCCTGAGGAACAG-3′, and AR5p1L, 5’-GGCTTCTACGACCTTCTTAAGTCCTTTTGGTTAACCT-3′); fmr910 for FMR1 (59); OxE20 for FMR2 (60); g6p1L:2:4L:2 for G6PD (PCR-generated using g6p1u.2, 5’-GATAAGCAGCCGGCCACCTTGGCA-3’, and g6p4L:2, 5’-GGTGCAGCTCGCGTATGTCGCCACCT-3′); pB17.1 plasmid for HPRT (35); ids4:1C for IDS (PCR-generated using ids4L, 5’-GAGCGACGTCTAGAAAGCGCCACCAGACC-3’, and idsC, 5’-CTGCTAATGGCGGCCACCTG-3’); igf2r-mip:1L:2 for IGF2R (PCR-generated using igf2r-mip1U, 5’-AGCCGCGTGAACCTGGGAGATTTTGTCGC-3’; p555p1L:2L:2 for MPPI (PCR-generated using p555p1U, 5’-AGGAAGGCTTCACTGATTCTAGGATTT-3’, and p555p2L, 5’-TTGGATCTGTCAGTCATTGCCGAAA-3’); pdh5p1L:2U:2 for PDHAI (PCR-generated using pdh5p1U, 5’-GAGATCGACGAGCACTTGTGGGG-3’, and pdh5p2L, 5’-GAGCATCTCTCCTGATGACAGACG-3’); BI 2.20 for PKG1 (24); SYBL1, a plasmid containing a 450 bp EcoRI-BstHI insert in the 5′ region of SYBL1 [a subfragment of the 1.6 kb EcoRI insert described previously (17)]; alu5p6-928L:3L:2 (PCR-generated using alu5p6, 5’-GTTAGTTTTTGTTTTTYGGG-3’, and alu5p7-928L, 5’-GGTGCAGCGTTCTCATTGG-3’); and alu5L2L:2L:2 (PCR-generated using alu5U, 5’-GGTGCAGCTCCTCTACGAGCGATTTCC-3’, and alu5L2L:2, 5’-GAGACGAGGATGTTCAGCTCCTCTGTCGC-3’). Conditions for Southern transfer to nylon membranes, hybridization and washing were as previously described (24,58,59).

Chromatin sensitivity to MspI digestion was carried out essentially as described previously (24). Briefly, nuclei from normal or ICF cells were treated with 0.4, 120, 400, or 800 U/ml MspI; genomic DNA was then isolated, digested to completion with NcoI and subjected to Southern blot analysis with the g6p1u.2:4L:2 probe for G6PD, BI2.20 for PKG1 and sat2-384U:sat2-781L probe for satellite 2.

Bisulfite methylation analysis

Portions of the 5′ CpG-rich regions of FMR1, G6PD, SYBL1 and XIST were examined by sequencing of cloned PCR products derived from genomic DNA following the bisulfite-mediated conversion of unmethylated, but not methylated, C→U (14). The bisulfite conversion conditions and the general methodology used were as described previously (63). The primers and conditions for FMR1 analysis were as described (63). Similar conditions were used for analyzing methylation in the 5′ regions of G6PD, XIST and SYBL1.

For G6PD analysis, converted DNA was amplified with primers that target the antisense strand; the initial round of amplification was with gconL-f, 5’-GTCTATTTTTGTGGTTTTTYYGGGATTGTGG-3’, and gconL-1R, 5’-CTAAATCCRCACAAACTCCTACAACCC-3’, followed by a semi-nested secondary amplifi-
cation with gcconL-1F, 5'-GTTTGGTTTATATTTGTYGTG-3' and gcconL-1R (457 bp product). XIST analysis was done using the following primers: xist1F, 5'-GTTTGAAGGAATGTT-TATTITTAG-3', and xist2R, 5'-CTCAAAATTTATCCAA-TACTTTC-3', for primary amplification of the converted sense strand and xist2F, 5'-TGTTTTTATTGGTAAATTITTTG-3', and xist2R for semi-nested secondary amplification (366 bp product).

For methylation analysis of SYBL1, the converted antisense strand was targeted so as to preserve a common sequence polymorphism; primary amplification was performed with sybcon-L1F, 5'-TAATATTGAGAAAGATTTATAT-3', and sybcon-L2r, 5'-TACTAAACTAAACAAAAAA3'-3', followed by a nested amplification with sybcon-L3L, 5'-ATTGGGAATTGTTTAGATTAG-3', and sybcon-L4r, 5'-CATCCTCTCATTTTACTATGCT-3' (493 bp product). PCR products were ligated into a TA cloning vector (Invitrogen, Carlsbad, CA), transfected into Escherichia coli and cloned; the plasmid inserts were sequenced using vector primers as described previously (4).

Replication time analysis

Replication time was determined by analysis of newly replicated DNA labeled with BrdU that was derived from cells sorted into specific cell cycle stages by flow cytometry as previously described (7,22). 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. The sequence tagged site DNA fractions were then analyzed for the presence of specific IDS alleles by semi-quantitative PCR. The sequence tagged site DNA fractions were then analyzed for the presence of specific loci by semi-quantitative PCR. The sequence tagged site DNA fractions were then analyzed for the presence of specific loci by semi-quantitative PCR. The sequence tagged site DNA fractions were then analyzed for the presence of specific loci by semi-quantitative PCR.

Allele-specific RT–PCR analysis of escape from silencing

Replication time analysis was done using a mixture of 0.5 µM g6pdd and mutagenically separated allele-specific bases (1.5 mM MgCl2); this product was then amplified in the same tube using gene-specific primers as described below, except that two rounds of primary amplification were performed prior to allele-specific analysis.

Allele-specific expression analyses involved a primary amplification of cDNA across the polymorphic region of interest followed, in most cases, by either a restriction enzyme digestion or a nested, allele-specific amplification in which one of the primers was specific for the polymorphic base at its 3' end. IDS analysis was done with Bss/Hind digestion as described previously (64), or with a modification that allows allele-specific amplification in a single tube by using mutagenically separated primers of different length according to methods described previously (69).

Primary amplification of IDS cDNA was done with 0.5 µM ids-xn3u1, 5'-CTCTGGAAGGTTGACCGTCG-3', and 0.5 µM ids-xn4L2, 5'-TGCCCTCGACATCTGCTGGT-3', at 65°C annealing temperature (201 bp product); limited allele-specific amplification of the primary product (10 µl in a 50 µl reaction; 9 PCR cycles) was performed with a mixture of 0.5 µM ids-xn3u1, 0.5 µM ids-as2LT, 5'-CACCACGTATACCGGAATACCA-3' (141 bp product, mutagenized and allele-specific bases are underlined), and 0.1 µM ids-as1LC, 5'-ATATAAGGTGAAAAGACCGACGTATACCGGAATACCA-3' (156 bp product), at an annealing temperature of 60°C, all in standard AmpliTaq buffer (1.5 mM MgCl2). Allele-specific expression analysis for G6PD and MPP1/1P55 was performed similarly.

Primary amplification of G6PD cDNA was with 0.5 µM G6PD-F, 5'-TGTTCTCCAAACCGCAGG-3', and 0.5 µM g6pdd (7) at 59°C annealing temperature (177 bp product); secondary amplification was with a mixture of 0.5 µM g6pdd and mutagenically separated allele-specific primers: 0.2 µM g6pdd-3C, 5'-CTCTGGAAGGTTGACCGTCG-3' (120 bp product), and 0.1 µM g6pdms-5T, 5'-TGCCCTCGACATCTGCTGGT-3', at 64°C annealing temperature. Primary amplification of MPP1/1P55 was done with 0.5 µM p55-9J, 5'-CAGAGGCTGATACCGGAATACCA-3' (132 bp product), or 0.5 µM p55-2G, 5'-CAGAGGCTGATACCGGAATACCA-3' (134 bp product), allele-specific primers.

Escape from silencing for X- and Y-linked genes were examined in informative cases using expressed polymorphisms in IDS (64), G6PD (65–67), and MPP1 (67). AR (68) and SYBL1 (M.R. Matarazzo et al., manuscript in preparation). Female fibroblasts were examined as single cells or as clones and female lymphoblasts as single cells. DNA from clones and mass cultures was isolated by the acid guanidinium thiocyanate method as described previously (35). DNA from single cells was analyzed by RT–PCR similar to a method used previously (42). Briefly, cells were washed and diluted into isotonic buffer, 1 µl of the suspension was placed into 72 well MicroWell MiniTrays (Nalge Nunc, Rochester, NY) and then wells were scored by microscopic examination for the presence of single cells. Cells were lyzed by three cycles of freeze–thawing and 4 µl of reverse transcriptase reaction buffer, including random hexamers and SuperScript II (Life Technologies, Rockville, MD), was added to wells with single cells and to some control wells with no visible cells. The well contents were transferred to PCR tubes, covered with mineral oil and incubated for 1 h on ice, 10 min at room temperature, 1 h at 42°C and 10 min at 99°C. The entire reaction was then PCR-amplified in the same tube using gene-specific primers as described below, except that two rounds of primary amplification were performed prior to allele-specific analysis.

For AR expression analysis, primary amplification of cDNA was across the highly polymorphic CAG repeat region using arcAg1.1a, and 5'-GCGCTTTGAACTCTTCTGACG-3', and
arcag1.2s, 5′-GCTGTGAAGGTTGCTTTTC-3′, as previously described (68). As this region is co-linear with DNA, the RNA samples were treated with DNase I prior to cDNA synthesis (61). Efficient removal of DNA was verified by the lack of PCR amplification in templates treated without reverse transcriptase. Allele-specific analysis was performed similarly to previously described procedures (71), where the primary product was amplified with AR.PCR1.2 and fluorescently labeled AR.PCR1.1 and annealed at 65°C, the product was run on an ABI PRISM 377 automated sequencer (PE Biosystems, Foster City, CA) to separate alleles that differed in CAG repeat number and then analyzed using GenoTyper 2.1 software (PE Biosystems).

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