Original Paper

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Direct Detection of Non-Random X Chromosome Inactivation by Use of a Transcribed Polymorphism in the XIST Gene

Abstract

As a result of X chromosome inactivation, females are mosaic for cell lineages in which either the paternal or the maternal X chromosome is active, and, if inactivation were random, each lineage should be present at approximately the same frequency. Detection of instances of non-random X inactivation can be important both clinically and for the study of X chromosome inactivation. Identification of a single-base polymorphism in an expressed region of the human XIST gene has permitted the development of a direct PCR-based assay for randomness of X inactivation. Oligonucleotide primers were designed, incorporating the variant base, and conditions established that allowed allele-specific PCR amplification. As the XIST gene is expressed only from the inactive X chromosome, differential amplification of the alleles in cDNA from heterozygotes can be used as an indicator of non-random inactivation. Using this assay, non-random X chromosome inactivation has been demonstrated in chromosomally abnormal cell lines and in lymphocytes from heterozygous, normal females. Virtually complete non-random X inactivation was also shown in a mother and her daughter, suggesting the existence of some familial factor affecting X chromosome inactivation.

Introduction

X chromosome inactivation results in dosage equivalence between females and males for X-linked genes [1]. The initial selection of which of the two X chromosomes to inactivate is believed to be random, and once made, this decision is stably maintained and inherited such that all clonal descendants inactivate the same X chromosome [2]. Non-ran-

dom inactivation can arise either from a bias in the original decision as to which chromosome to inactivate or from subsequent selective pressures. The latter has been observed in females with X chromosome rearrangements [3] and in carriers of a number of X-linked diseases such as adrenoleucodystrophy [4], X-linked agammaglobulinaemia [5] and Lesch-Nyhan syndrome [6]. Selection may be constitutive in the case of chromosomal rearrangements or restricted to a specific cell lineage as observed for a number of the X-linked lymphoproliferative disorders such as Wiskott-Aldrich syndrome [7].

Bias in the initial X inactivation event has been observed in marsupials [8], rodent extra-embryonic tissues [9, 10], and in mouse strains carrying different alleles at the Xce locus [11]. Apparent skewing of inactivation may also result from stochastic variations in the X inactivation status of progenitor cells in tissues with a limited number of founder cells. This has been demonstrated in human T cells where 10% of the cell populations showed skewing of greater than 75% [12] and in cultured fibroblast cell lines in which several populations showed exclusive inactivation of either the maternal or the paternal X chromosome [13].

We have reported a gene, XIST, whose location at the X inactivation centre and expression from only inactivated X chromosomes suggest a role in X chromosome inactivation [14]. Comparison of the relative expression of XIST from the paternal or maternal X in a population of female cells would provide a direct measure of the randomness of X inactivation in that sample. To develop such an assay, we have identified an expressed polymorphism within XIST and have used it to generate two allele-specific primers which can be used in PCR amplification of specific allele (PASA) reactions. In cases in which X chromosome inactivation is random,

both primers will amplify cDNA prepared from a heterozygote. If inactivation is not random, only one primer (that which corresponds to the allele on the preferentially inactivated X chromosome) will amplify (fig. 1). This assay is an efficient and direct method to assess the randomness of X chromosome inactivation.

Materials and Methods

Sequencing
All nucleotide sequencing was performed on double-stranded DNA using either the universal forward or reverse primers or the internal primers [14–16]. Sequence data were stored and analysed using DNA-STAR sequence analysis software on MacIntosh computers.

DNA and cDNA Preparation
Genomic DNA was prepared from cultured cell lines by NaCl/SDS lysis [17] and from whole blood using a Non-Organic DNA Extraction Kit (Oncor Inc., Gaithersburg, Md., USA). Southern transfer and hybridization were performed by standard techniques. Filters were probed with a ^{32}P-labelled segment of DNA encompassing the region amplified in the PASA experiments. Both alleles were equally represented in the probe. Filters were exposed to Kodak X-Omat film at $-80\,°C$ with enhancing screens.

Total RNA was prepared from cultured cell lines as described [18]. Total RNA from whole blood was prepared as follows: 7–10 ml of blood was drawn into heparin vacutainers and allowed to settle at room temperature for about 1 h. The top layer (from 2 to 4 ml) was drawn off and spun down at 325 g and room temperature for 10 min. The pellet was resuspended in 1.0 ml RNAzolB (Biotecx Laboratories Inc., Houston, Tex., USA). Chloroform (0.1 vol) was added and mixed vigorously, and the preparation was incubated on ice for 5 min. Following the incubation, the preparation was centrifuged at 12,000 g for 15 min at 4°C and the upper phase containing the RNA was drawn off. RNA was precipitated on ice with 1 vol isopropanol for 15 min and collected by centrifugation at 12,000 g for 15 min at 4°C. The RNA pellet was rinsed with 75% ethanol, centrifuged at 7,500 g for 8 min and resuspended in 50 μl water which had been pretreated with the RNAse inhibitor diethyl pyrocarbonate.

Fig. 1. Schematic diagram of PASA analysis of females heterozygous for the XIST alleles. The sample on the left, in which inactivation is completely random, will be mosaic for XIST expression, and her cDNA will amplify with either of the allele-specific primers equally. The sample on the right, in which inactivation is completely non-random, will have the same X inactivated in all cells. Her cDNA will amplify with only one of the allele-specific primers (in this case, the primer corresponding to allele 1). When the amplification products are analysed by electrophoresis (bottom), the amplification products from both allele-specific primers will be detected from the randomly inactivated female, whereas only the product from one allele will be observed from the non-randomly inactivated female.
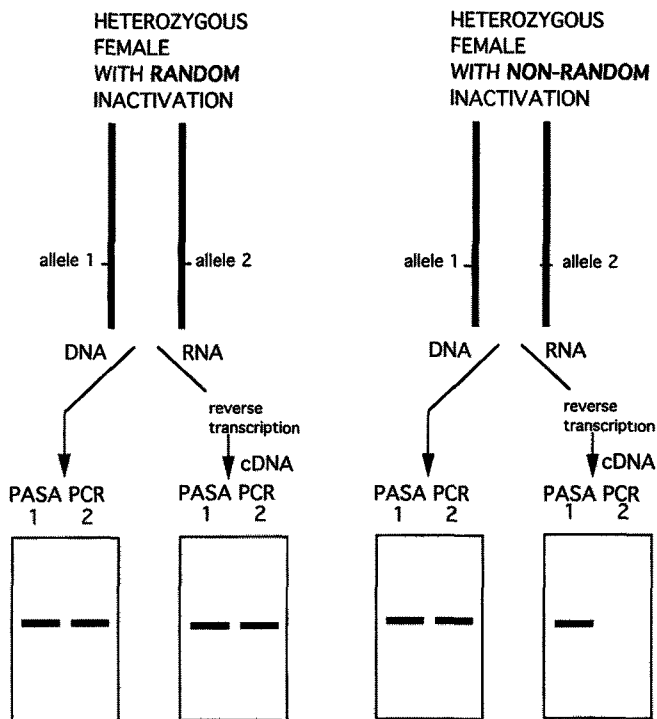
All cDNAs were prepared by reverse transcription of 1.0 μg total RNA primed with random hexamers with 5 U of reverse transcriptase, as previously described [19].

Cell Lines

Cell lines were established from members of a family ascertained through a boy with apparent X-linked ichthyosis. Both a lymphoblast cell line (HSC 593) and a fibroblast cell line (60) were established from the affected child's maternal grandmother, and a fibroblast cell line (59) was established from his aunt.

All lymphoblast cell lines, including SA70 [rec(X) dup p, inv(X)(p11.4q13)] [20] and those obtained from National Institute of General Medical Sciences (USA) Human Genetic Mutant Cell Repository (Camden, N.J., USA), were grown at 37 °C in RPMI media (Gibco) supplemented with 15% fetal calf serum, glutamine, penicillin and streptomycin (Gibco). BrdU incorporation has shown the rearranged X in lymphocytes from SA70 to be late-replicating and therefore

inactive [20]. The fibroblast cell line 67 (GM0705) [46,X,t(X;9)(q13.1;p24)] [21] was grown in α-MEM (Gibco) supplemented with 15% fetal calf serum, glutamine, non-essential amino acids (Gibco), penicillin and streptomycin. This line carries a balanced X;9 translocation, with the normal X chromosome preferentially inactivated [21]. The mouse/human somatic cell hybrid tSA70-D1-34azlf [22] carries the inactive X from SA70 and was grown in α-MEM (Gibco) supplemented with 7.5% fetal calf serum, glutamine, penicillin and streptomycin.

PCR Conditions

Conditions for allele-specific PCR were based on parameters discussed by Sommer et al. [23]. Optimum specificity was obtained using the following conditions: assay buffer consisting of 50 mM KCl/10 mM Tris/HCl (pH 9.0), 0.1% Triton X-100 (BRL) or 50 mM KCl/20 mM Tris/HCl (pH 8.4) (Promega), 1.5 mM MgCl$_2$, 0.05 mM dNTPs and 2.5 U of *Taq* DNA polymerase. These conditions were used to am-
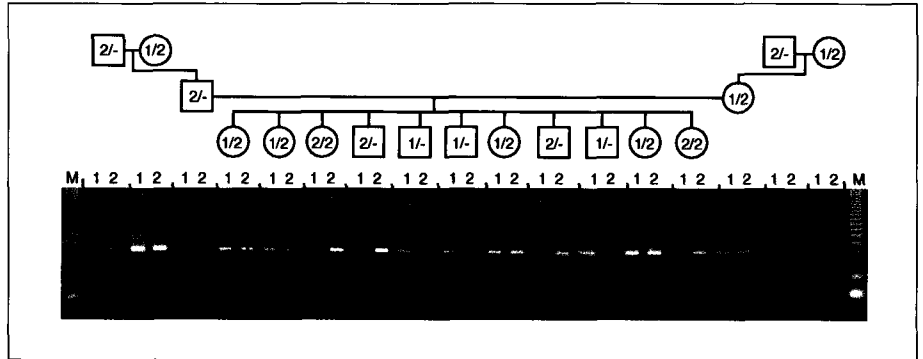
Fig. 2. Pedigree demonstrating mendelian transmission of the XIST alleles. DNAs were obtained from the Centre d'Etude du Polymorphisme Humain and was typed using the PASA assay. Each genotype is represented by PCR amplification products visualized in two lanes of an agarose gel. Presence of a band indicates presence of the allele in the individual. Transmission of the polymorphism is consistent with mendelian inheritance of an X-linked trait.

plify a sample of approximately 0.1 µg DNA, using 7.5–40.0 pmol of one of the allele-specific primers and an equivalent amout of a second non-specific primer. Reactions were 100 µl total volume overlaid with ~ 25 µl mineral oil. Amplification consisted of 30 cycles of a 1-min denaturation of 94°C, followed by a 2-min annealing at 59°C and a 3-min elongation at 72°C, with a final 7-min elongation at 72°C following cycle 30. As the amplification products from the PASA primers do not span an intron, controls without reverse transcriptase were required to exclude the possibility of DNA contamination in the RNA preparations.

All PCRs were performed using an Ericomp Twin-block thermocycler. The amplified products were analyzed by running 15- to 20-µl samples on a 2% agarose gel stained with ethidium bromide.

Results

Identification of a Polymorphism in XIST

A single-base polymorphism (G:A at base 15944) was detected during the initial sequencing of XIST [16] and confirmed by heteroduplex analysis (data not shown). Primers were designed incorporating the variant base at the 3′ terminal position, with matching melting points of 54°C. These primers, here-

after referred to as 1-1 (5′GTATAGAAC-TGTAGGCTTC3′) and 1-2 (5′TGTATAGA-ACTGTAGGCTTT3′), were used in conjunction with a non-polymorphic primer (5′CTC-ACTGTTAAAGGCACTGA3′) to amplify a 533-bp product from DNA and cDNA in an allele-specific manner. The genotype of any sample can be determined by performing a pair of PASA reactions, one with each of the specific primers. The combination of the results of the two reactions provides an unambiguous genotype (fig.1, 2).

Allele frequencies for this XIST polymorphism were established from 57 unrelated Caucasians (111 X chromosomes total), and the alleles were shown to be in equilibrium with frequencies of 0.31 (G; allele 1) and 0.69 (A; allele 2). Surprisingly, very different allele frequencies were observed in a small sampling of black females (0.75 for allele 1, 0.25 for allele 2, n = 28 X chromosomes). Transmission of the alleles was analyzed in several families from the Centre d'Etude du Polymorphism Humain and found to be consistent with an X-linked inheritance pattern. The pe-
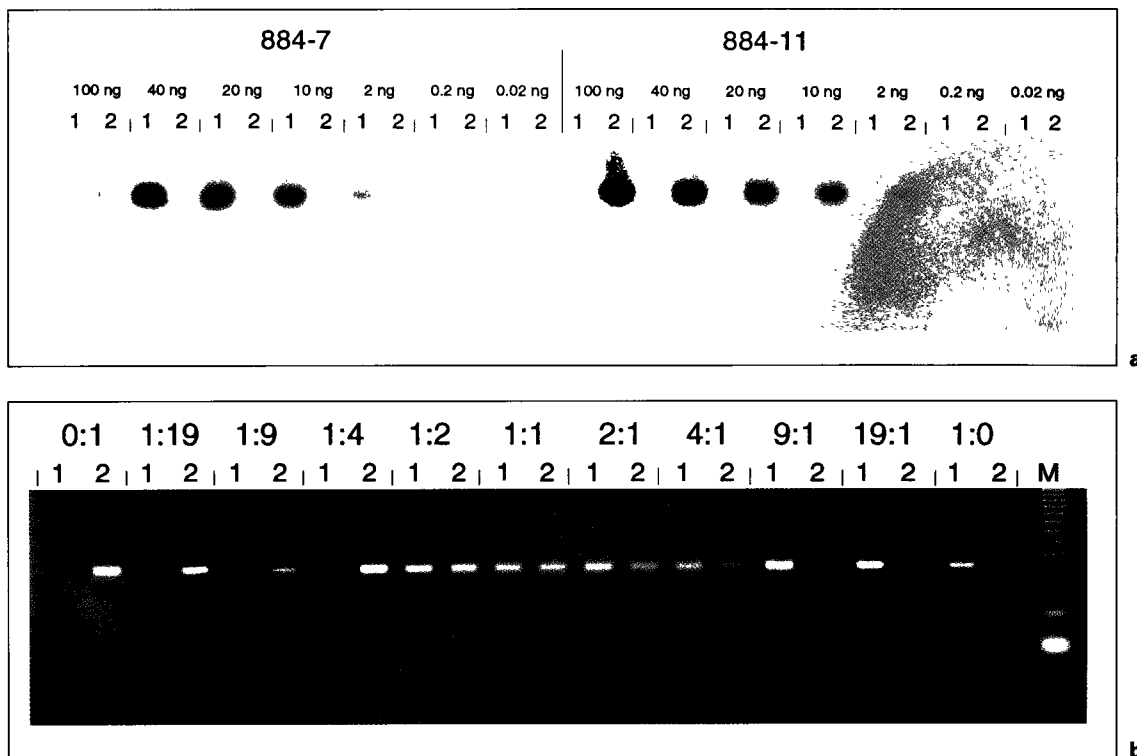
Rupert/Brown/Willard

Analysis of X Inactivation using XIST Polymorphism

Fig. 3. a Autoradiograph of a target sequence dilution experiment. Target DNA was prepared from 2 males (884-7 and 884-11) who were hemizygous for alleles 1 and 2, respectively. Samples ranging from 0.02 to 100 ng were amplified using the PASA primers, electrophoresed in 1% agarose and transferred to a nylon filter. The filter was hybridized with a labelled XIST probe spanning the polymorphic region and exposed for 3 h. Amplification is consistent with each male being hemizygous for a different allele, and no non-specific amplification can be seen. **b** Allele-specific amplification in the presence of excess non-target DNA. DNA was prepared from 2 females, each homozygous for a different XIST allele, and was mixed in the ratios shown. Each lane represents amplification of 125 ng of this mixture. Allele-specific amplification occurred even when the target DNA made up only 5% of the total DNA present. No non-specific amplification was seen in either of the pure homozygous DNAs (1:0, 0:1 lane pairs).

digree and representative data from one of these families (number 884) is shown in figure 2.

In order to determine the extent of which primer specificity was dependent on target availability, a dilution series was performed, in which 100 pg to 500 ng of male genomic DNA was amplified. Allele-specific amplification from as little as 10 ng target DNA was detectable on gels stained with ethidium bromide. After Southern transfer and hybridization, allele-specific amplification could be detected from as little as 2 ng of DNA (fig. 3a).

A second dilution series was performed in order to determine whether allele specificity could be maintained in the presence of excess amounts of the other allele, as would be expected in a case of non-random X inactiva-

tion. DNAs from 1/1 or 2/2 homozygous females were mixed in ratios of 19:1, 9:1, 4:1, 2:1 and 1:1, and amplified. In all cases, amplification of both alleles could be seen on agarose gels stained with ethidium bromide (fig. 3b). Amplification was quantitative in that there was a clear correlation between the amount of amplification product and the relative amount of input DNAs.

Assay to Determine Randomness of Inactivation

The PASA primers were used to amplify cDNA prepared from blood taken from normal heterozygous and homozygous females (fig. 4a). In all cases, the amplification of cDNA was consistent with the genotype determined from the genomic DNA. As a further test of this assay, we also analysed 2 female cell lines with X chromosome rearrangements (SA70 [20] and 67 [21]), in which non-random X inactivation had previously been demonstrated. Both SA70 and 67 were heterozygous for the XIST polymorphism, as demonstrated by amplification of both alleles in genomic DNA, but expressed only a single XIST allele (fig. 4b). For SA70, the expressed allele was the same as that shown previously to be on the structurally abnormal, inactive human X chromosome [22]. This observation confirms, in human cells, the exclusive expression of XIST from the inactive X chromosome, previously demonstrated in somatic cell hybrids [14, 16].

Having established the specificity of the assay and its ability to detect non-random inactivation, we examined the randomness of X inactivation in a series of samples from normal females (fig. 1). Lymphocyte cDNAs from 11 unrelated heterozygous females were assayed (table 1); 7 of these showed equivalent amplification of each allele upon repeated analysis. Slight skewing was seen in 2 (each favouring a different allele), and strong

Table 1. PASA analysis of randomness of X inactivation in lymphocyte and lymphoblast RNAs

Sample	Allele 1	Allele 2
Lymphocyte		
F1	50	50
F2	50	50
F3	50	50
F4	50	50
F5	50	50
F6	50	50
F7	50	50
F8	40	60
F9	60	40
F10	70	30
F11	80	20
Lymphoblast		
GM 6988	90	10
GM 6999	20	80
GM 7002	95	5
GM 7005	80	20
GM 7023	0	100
GM 7059	90	10
GM 7345	30	70
GM 7348	95	5
GM 7350	30	70

PASA analysis of various cDNAs prepared from RNA extracted from whole blood (F1–F11) and from lymphoblast cell lines. F1–F11 are unrelated heterozygous females. The relative extent of expression of each allele is based on comparison to a dilution standard and is given as a percent of total expression of both alleles. Random inactivation will result in equal expression of each allele and will be scored as 50:50. Estimates are rounded to the nearest 5 or 10%, based on the standards (fig. 3).

skewing (>70:30) was observed in the remaining 2 cases. In all cases, equivalent amplification of the 2 alleles was observed when genomic DNA was amplified (data not shown). A similar analysis was performed on 9 lymphoblast cell lines (table 1). Some degree of skewing was observed in all cases, with extensive skewing (>90:10) in 5 lines, probably

XIST 1/1   XIST 1/2   XIST 2/2

┌ DNA cDNA   -RT ┐┌ DNA cDNA   -RT ┐┌ DNA cDNA   -RT ┐
  1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2   M

a

SA70   67
┌ DNA   cDNA   -RT   Xi DNA ┐┌ DNA   cDNA   -RT ┐ M
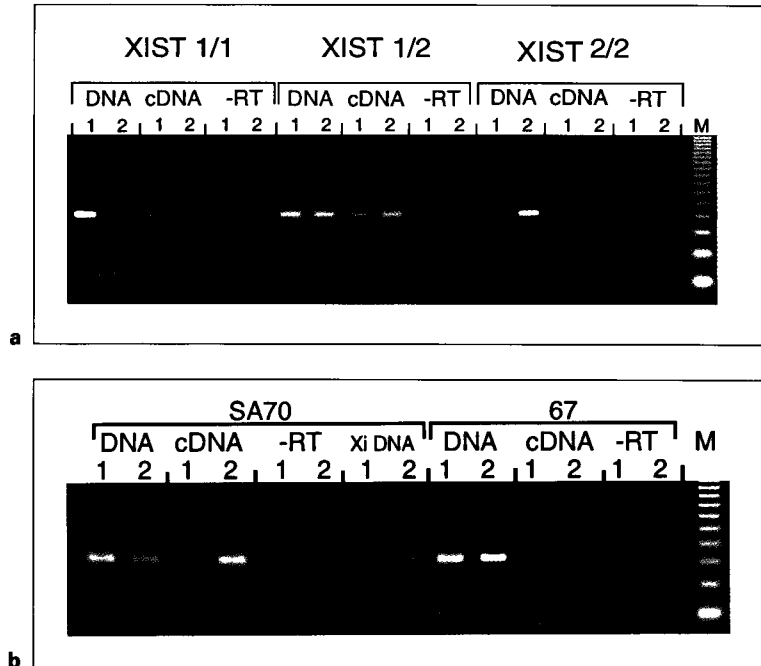 1 2 1 2   1   2 1   2   1   2 1   2 1   2

b

Fig. 4. a Specific amplification of alleles in DNA and cDNA from 3 unrelated females. The female on the left is homozygous for allele 1, and the female on the right is homozygous for allele 2. The female in the center is heterozygous. The amplification patterns in cDNA are consistent with the genotypes and random inactivation in the heterozygous female. Genomic DNA and total RNA was extracted from whole blood. Reverse transcriptase minus controls (-RT) are shown for each cDNA. b Nonrandom X chromosome inactivation in cell lines SA70 and 67. Amplification of genomic DNA indicates that both cell lines are heterozygous for alleles 1 and 2, whereas amplification of cDNA shows expression of only one allele (allele 2). The lane pair labelled 'Xi DNA' contains amplified DNA prepared from the somatic cell hybrid tSA70-D1-34az1f. This hybrid contains the inactive X (Xi) chromosome from SA70, but not the active X chromosome. Reverse-transcriptase minus (-RT) controls are shown for each cDNA.

reflecting at least in part the well-established clonality of many transformed lymphoblast lines.

Familial Non-Random X Inactivation

Having established that the assay could be used to determine the pattern of inactivation, we analysed a family ascertained through a male relative with X-linked ichthyosis. The mother and daughter in this family (grandmother and aunt of the proband) are carriers for the common deletion of the steroid sulphatase gene [24]. As part of the initial carrier detection studies, a total of 25 independent human-mouse hybrid lines were established from both the daughter and mother, under

Fig. 5. PASA analysis of a family in which both the mother and the daughter are carriers for steroid sulphatase deficiency. Amplification of DNA indicates that both mother and daughter are heterozygous and that the father is hemizygous for allele 1. Amplification of cDNA indicates that both mother and daughter express only the XIST-2 allele, which the daughter must have inherited from her mother. Reverse-transcriptase minus (-RT) controls are shown for each cDNA.
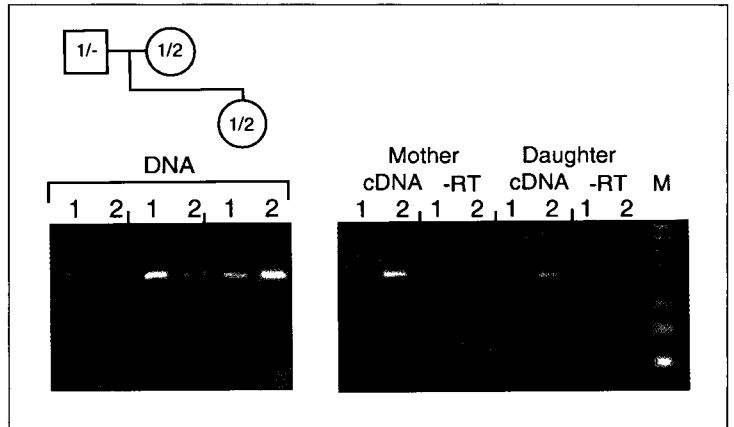
HAT selection to maintain the active human X chromosome. Surprisingly, 23 hybrids carried 1 X and only 2 the other X, based both on steroid sulphatase biochemical analyses and on DNA marker analysis (data not shown). These data suggested the possibility that both women had non-random inactivation.

To examine this directly, we used the XIST PASA assay (fig. 5). Both XIST alleles were detected in their genomic DNA, demonstrating that both are heterozygous. However, only allele 2 was detected in the fibroblast cDNA from each woman. Therefore, both mother and daughter show extreme preferential inactivation of the same X chromosome. It is unlikely that this skewing is due to selection against the steroid sulphatase deletion as there is no evidence for non-random inactivation in similar heterozygotes [25, 26].

Discussion

X chromosome inactivation occurs early in development, resulting in dosage equity for X-linked genes between males, who only have a single X chromosome, and females, who have 2 [1, 2]. Inactivation is believed to be random, affecting either the maternal or the paternal X with equal probability. The choice, once made, appears to be stable such that females are mosaic for 2 somatic cell populations distinguished by the inactive X chromosome [27].

In order to analyse patterns of X inactivation directly, we have made use of a transcribed sequence polymorphism in the XIST gene to design allele-specific PCR primers. Allele-specific amplification could be detected visually from as little as 10 ng of DNA and from relative concentrations as low as 5% of the total target input. Allele specificity was maintained in cDNA, and therefore the assay can be used in heterozygotes to test for the degree of randomness of inactivation. The assay assumes that both alleles are transcribed and reverse-transcribed at equal rates and that the transcripts are equally stable; we believe these to be valid assumptions as neither allele was consistently underrepresented in blood or lymphoblast cDNA (table 1). Amplification of cDNA produced from a population of cells heterozygous for the alleles should therefore show each allele to be equally represented, and any reproducible variation from this should reflect skewing in X chromosome inactivation.

Early assays used to analyse randomness of X chromosome inactivation were based on isozyme analysis of relatively rare protein polymorphisms [28]. Subsequently, the correlation between X inactivation and DNA methylation [2, 29] provided an indirect DNA-based approach for assessing the randomness of inactivation. Initially, these assays involved Southern analysis of genomic DNA [30–32]; although a number of sensitive assays combining PCR amplification and methylation-sensitive restriction digests have been developed recently [33, 34], these methods are indirect and rely on complete digestion of the DNA and on the relationship between X inactivation and DNA methylation, which is not yet fully understood [13]. In contrast, analysis with the XIST PASA assay is expression-based and is, therefore, a direct test of inactivation status. The alleles are relatively common ($\sim$ 50% of females are heterozygous), and the assay requires a simple pair of PCR reactions analysed on an agarose gel.

When used on DNA from a small sample of unrelated females, the assay detected skewed inactivation in 4 (table 1). This frequency is consistent with previous estimates based on methylation analysis [13, 30, 32, 35]. A much more comprehensive study of the randomness of X inactivation in the general population is needed to test the commonly accepted notion that the degree of skewed inactivation will be randomly distributed about a mean of 50:50 [2]. The assay we have demonstrated here should be suitable for such studies.

We had previously identified a family in which non-random X inactivation was suspected, based on results from a series of somatic cell hybrids. When selected under conditions which maintain the human active X chromosome, >90% of mouse/human somatic cell hybrid lines established from the mother and daughter in this family maintained the same X chromosome. This highly non-random X inactivation was also seen using the PASA assay, which showed unilateral expression of XIST from both women (fig. 5).

Familial non-random inactivation has been reported for a number of families with different X-linked diseases [2], including Fabry disease [36], Hunter syndrome [37, 38], and hemophilia B [39]. It has been proposed that such cases may result from selection against an unknown mutation on the inactivated X chromosome [40]. An alternative possibility is that the familial selective inactivation of one of the X chromosomes is due to a mutation affecting the actual mechanism of inactivation [2]. Although no such locus has been documented in humans, the Xce locus in mice affects the probability of a chromosome being subject to X activation [11]. In mice, this locus is tightly to the XIST gene, both of which map within the X chromosome inactivation centre region [41]. In the family presented here, the mother and daughter share the same XIST allele and, therefore, likely share the same copy of the X chromosome inactivation centre. Further analysis in additional families using polymorphic loci along the X chromosome would be required to determine whether the X chromosome inactivation centre is the best candidate region for such a putative-X-controlling element in humans.

Acknowledgments

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