The inactive X (Xi) differs from its active homologue (Xa) in a number of ways, including increased methylation of CpG islands, replication late in S phase, underacetylation of histone H4 and association with XIST RNA. Global changes in DNA methylation occur relatively late in development, but the other properties all change during or shortly after the establishment of Xi and may play a role in the mechanism by which an inactive chromatin conformation spreads across most of the chromosome. In the present report, we use two human X;autosome translocation chromosomes to study the spreading of inactive X chromatin across X;autosome boundaries. In one of these chromosomes, t(X;6), Xp distal to p11.2 is replaced by 6p21.1–6pter and, in the other, ins(X;16), a small fragment derived from 16p13 is inserted into the distal third of Xq. In lymphoid cells from patients carrying these translocations in an unbalanced form, Xi was shown by HUMARA assay to be derived exclusively [t(X:6)] or predominantly [ins (X;16)] from the derived X chromosome. We used a combination of immunolabelling and RNA/DNA fluorescence in situ hybridization to define the distribution of XIST RNA, deacetylated H4 and late-replicating DNA across the two derived X chromosomes in inactive form. Within the limits of the cytogenetic techniques employed, the results show complete coincidence of these three parameters, with all three being excluded from the autosomal component of the derived X chromosome.

INTRODUCTION

Dosage compensation in mammals requires the stable genetic silencing, during early embryogenesis, of one X chromosome in each female cell (1,2). This process has an absolute requirement for the presence, in cis, of a region defined genetically as the X inactivation centre (XIC) (3). Establishment of the inactive X (Xi) requires the relatively rapid (i.e. 1–2 cell generations) dissemination of the silencing signal across an entire chromosome, a process that is often described as spreading and seen as analogous to position effect variegation (PEV) in Drosophila (4).

The XIC is required for both initiation and spread of X inactivation (3). A gene designated XIST (in human) and Xist (in mouse) has been mapped to the XIC region (5–7) and has been shown to be both necessary and sufficient for X inactivation to occur (8–12). The XIST/Xist gene produces large RNA molecules that lack protein-coding potential, but which coat the inactive X chromosome in interphase (13–15). Multiple copies of Xist transgenes inserted into mouse chromosome 12 produced an RNA transcript which coated the autosome (10). This chromosome then became hypoacetylated and late-replicating. Experiments in mouse embryonic stem cells (ES cells) have demonstrated that Xist is expressed from both X chromosomes in undifferentiated cells, which have two active X chromosomes (16). However, there is an up-regulation of Xist prior to the inactivation of one X chromosome (17). This developmental up-regulation is a consequence of the stabilization of Xist RNA (18,19), due to a promoter switch resulting in a stable transcript (20). The manner in which inactivity spreads along the selected X chromosome remains unclear.

The inactive X chromosome is stably inherited through mitosis; it is heterochromatic (21), late-replicating in S phase (22), has regions which are highly methylated (23) and is underacetylated at histones H3 and H4 (24). In this report, we have used these cytogenetic characteristics of the inactive X to ask whether there is a spread of inactive chromatin into translocated autosomal material. Previous studies of mouse X;autosome translocations have revealed a variable and reversible spread of silencing into the translocated autosomal regions (25). Many studies of X;autosome translocations have been based on replication timing as a measure of chromosome inactivity and have shown that the translocated autosomal material can become later replicating than its non-translocated counterpart (26–29), though the spreading may be incomplete (28,30,31). We compared the distribution of XIST RNA, H4 deacetylation and late-replicating DNA in cell lines carrying either an X;6
translocation or an X;16 insertion. We found that all three markers are coincident and are excluded from the autosomal component of the derived X chromosomes.

RESULTS

Patient 1 presented with learning difficulties (IQ = 75) and a developmental delay of 2 years at age 7 years 6 months. By a combination of conventional banding analysis and fluorescence in situ hybridization (FISH), the p-arm of the abnormal X, distal to p11.2, was shown to be derived from the 6p21.1–6pter portion of chromosome 6 (data not shown). The karyotype is 46X,der(X)t(X;6)(p11;p21.1)mat. The phenotypically normal mother of this patient carries the same X;6 translocation, but in a balanced form. This is summarized diagrammatically in Figure 1a.

We used the methylation status of the X-linked human androgen receptor (HUMARA) gene (32,33) in peripheral lymphocytes to show that both mother and daughter show complete, but opposite, skewing of X inactivation (Fig. 1b). In the mother, it can be concluded that Xi is derived exclusively from the normal X, while in the daughter Xi is derived from the translocated X. Lymphoblastoid cell lines gave the same results (data not shown). This situation is likely to have resulted from cell selection following random X inactivation. Inactivation of t(X;6) in the mother would result in functional disomy for Xp and monosomy for 6p, while inactivation of the normal X in the daughter would result in functional nullisomy for Xp.

Late-pulse bromodeoxyuridine (BrdU) labelling was used to examine the replication timing of the translocated X in a lymphoblastoid cell line (044) derived from this patient. Sequential detection of BrdU and chromosome 6 DNA shows that the X portion of the translocated chromosome is consistently late-replicating while the portion of 6p immediately distal to the breakpoint replicates early. Typical results are shown in Figure 2a. Of 26 chromosome spreads with late-replicating (i.e. BrdU-containing) chromatin, 23 showed clear labelling of the X component of t(X;6). In none of these spreads was there evidence for significant spreading of late replication into 6p.

Figure 2b shows part of a metaphase chromosome spread from the 044 cell line labelled with an antibody to acetylated histone H4. One chromosome is predominantly pale staining, with an acetylated region on the short arm (arrow). Simultaneous immunofluorescent labelling with R232 and DNA FISH with X-paints confirmed that this chromosome is t(X;6) and showed that the region of H4 underacetylation is confined to the X component (Fig. 2c). The normal X chromosome in these cells was never underacetylated, consistent with the complete skewing of X inactivation in this patient (Fig. 1b).

We used a combination of RNA FISH and DNA FISH to address the question of whether XIST RNA coated the autosomal component of t(X;6). In human cells, unlike those of the mouse, XIST RNA dissociates from Xi at prophase, so analysis of chromosome coating by XIST RNA must be carried out in interphase nuclei. Typical results are shown in Figure 2d. In 41 out of 67 nuclei, there was no overlap between the two signals, showing that, in these nuclei, XIST RNA was completely excluded from the translocated chromosome 6p. In the remaining 26 nuclei, there was some overlap of signal. However, this was to be expected, since some coincidence of signal will inevitably occur due to the impaction and collapse of cells onto the slide during the cytospin preparations. The RNA and DNA signals are detected sequentially and are visualized in two dimensions; thus, depending on the orientation of the nuclei, there will sometimes be overlap of signal. It is possible that analysis of the two signals by confocal microscopy may alleviate the overlap of signal due to nuclear orientation. Despite these technical limitations, these
Figure 2. (a) Chromosomes from cell line 044 t(X;6) treated with BrdU to demonstrate replication timing, followed by in situ hybridization with a paint for chromosome 6. Images of BrdU labelling were captured first, then slides were processed for DNA FISH. The BrdU and FISH images were merged. The chromosome 6 paint (red) identifies the two normal chromosome 6s (arrows) and the translocated 6p portion (arrowhead). Anti-BrdU antibodies (green) detect only the X portion of the translocated X chromosome. There is no overlap between red and green signals, showing that there is no detectable spread of late replication from the X into the autosomal portion of this chromosome. (b) Chromosomes from the t(X;6) cell line labelled with antibody R232 against acetylated histone H4 (green). The DAPI counterstain has been pseudocoloured red to aid visualization. One chromosome is predominantly pale staining, apart from an acetylated region on the short arm (arrow). (c) Simultaneous analysis of antibody labelling (red) and FISH using an X chromosome paint (green) identifies this chromosome as the t(X;6) (arrow). The autosomal portion of the chromosome retains its acetylation pattern (red) but does not hybridize with the X paint (green). The Xi portion of the t(X;6) chromosome is green (X paint only) as it is underacetylated. This simultaneous analysis of antibody labelling and FISH results in two clear regions of colour with no overlapping signals, showing that there is no detectable spread of underacetylation from Xi to the translocated region of chromosome 6. The active X chromosome in this spread (insert, bottom right) appears yellow (arrowhead) as it is both acetylated and hybridized with the X paint. In similar experiments (data not shown) where antibody labelling was combined with FISH using a chromosome 6 paint, there was complete coincidence of the FISH signal and acetylated H4 on the t(X;6) chromosome. [The chromosome which appears to be pale staining with antibody (red) in the lower part of the image is in a different focal plane.] (d) Sequential analysis of XIST RNA (by RNA FISH, green) and DNA FISH for chromosome 6 (red) in interphase cells from the t(X;6) cell line. Images of the localization of XIST RNA were captured. Slides were then hybridized with the chromosome 6 paint (red) and images of the same cells captured again. Red and green images were then superimposed. The red and green signals show little overlap, indicating minimal spreading of the XIST RNA (green) onto the autosomal region of the translocated chromosome, at least in interphase cells. The 6 paint also reveals the location of the normal chromosome 6s in these cells.

observations are consistent with the proposition that, in the living cell, spreading of XIST RNA into the translocated 6p is infrequent and may not occur at all.

Patient 2 presented with premature ovarian failure, mental retardation and short stature. Cytogenetic analysis revealed the presence of additional material on one chromosome 16p and an extra G-dark band in the distal third of one Xq. The X-inserted material is detectable with the probe YAC55.5, which maps to 16p13 (ref. 34 and data not shown). This, together with the size...
Figure 3. (a) Chromosomes from the ins(X;16) cell line (027) labelled with antibody R232 against acetylated H4. One chromosome is pale staining apart from a small band of acetylation on the long arm (arrow). FISH analysis using X and 16 paints shows this acetylated band to be derived from chromosome 16 (data not shown). (b) Chromosomes from the ins(X;16) cell line analysed for replication timing and 16 paint. As previously, BrdU (green) images were captured first, then superimposed onto those for chromosome 16 paint (red). The inserted part of chromosome 16 is not as late-replicating as the inactive X chromosome into which it is inserted (arrow). The box in the right hand corner shows the BrdU signal alone for this same chromosome. A break in the BrdU signal is clearly seen (arrowhead). (c) Sequential detection of XIST RNA (green) and chromosome 16 DNA (red) in an interphase cell from the ins(X;16) cell line. XIST RNA images were captured first, then slides were hybridized with the chromosome 16 paint. The red and green images were superimposed. The 16 paint detects the two normal chromosome 16s (yellow arrows) and a smaller signal corresponding to the inserted material (white arrow). In this example, the XIST RNA (green, indicated by the white arrowhead) shows only very slight overlap (yellow) with adjacent chromosome 16 material.

and staining properties of the insertion, suggests that it is derived from the region 16p11.2–16p13.1 (Fig. 1a).

Metaphase spreads were prepared from a lymphoblastoid cell line derived from patient 2 (027) and labelled with antibodies against acetylated H4. One X chromosome was predominantly underacetylated, with the exception of an acetylated region on the q arm (Fig. 3a, arrow). The location of this strongly labelled region correlates exactly with that of the inserted chromosome 16 fragment. Of 30 spreads that were examined in detail, 20 (i.e. 67%) showed this staining pattern whilst the remainder had a completely pale-staining inactive X chromosome. This is consistent with the 30:70 ratio of inactivation of the normal X and ins(X;16) demonstrated by the HUMARA methylation assay (as above; data not shown). Replication timing of the inserted chromosome was investigated using late-pulse BrdU labelling. In 19 out of 22 spreads examined, the ins(X;16) chromosome was shown to be predominantly late-replicating. In most of these 19 spreads, an early-replicating (i.e. BrdU-free) band was detected in the long arm that corresponded exactly to the inserted chromosome 16 DNA (Fig. 3b). The absence of a clearly defined, BrdU-free band in the remaining spreads is likely to be due to chromosome compaction and masking of the small inserted region. However, sequential detection of BrdU and chromosome 16 DNA showed that the inserted chromosome 16 material had not incorporated BrdU (i.e. was red, not yellow, Fig. 3b) and was therefore early-replicating. Attempts to determine whether or not XIST RNA spreads over the inserted part of chromosome 16 in interphase cells [as above for t(X;6)] have been hampered by the small size of the autosomal insert. Despite this, in nine out of 15 nuclei, there is no co-localization of signal, indicating that the XIST RNA does not cover the inserted autosomal material in at least the majority of nuclei. Of the other six cells, four showed only partial overlap and two complete overlap of signal. This overlap may be an artefact of slide preparation as discussed above. A typical nucleus is shown in Figure 3c.

DISCUSSION

Genetic and cytogenetic studies in mice (25,27,35–37) and humans (28,29,38–40) carrying X;autosome translocations have provided evidence that at least some characteristics of inactive X chromatin can spread, in cis, into the translocated autosome. However, the degree of spreading is extremely variable and, although this may be due, to some extent, to the use of low-resolution methods for detecting late-replicating DNA, it seems likely that other factors also play a part. Insertion into some mouse autosomes of multiple copies of Xist-containing transgenes has been shown to result in spreading of properties of Xi chromatin into the autosome (9). The finding that this was not seen for all transgene-carrying autosomes suggests that either the autosome itself, or the site of insertion, may be crucial, a factor that may also explain the variable properties of human X;autosome translocations (39). In the present report, we show that a large fragment of chromosome 6p translocated onto Xp and a small fragment of 16p inserted into Xq both retain their autosomal properties by retaining normal levels of H4 acetylation, by resisting XIST RNA binding and by replicating relatively early in S phase. Strikingly, we are able to show that within the limits of the cytogenetic techniques used, all three properties define the same sharp, cytological boundary and that this boundary...
corresponds to that between autosomal and X-DNA. Further, we see no evidence for the saltatory spreading of Xi-type chromatin (40), which one would detect as patches of H4 underacetylation embedded within the translocated autosomal region. While it is possible that these findings are dependent on the specific chromosome rearrangements studied, they show that at least some extensive autosomal regions are permissive for the formation of Xi-type chromatin.

The recent finding that XIST RNA is absent from Xi centric heterochromatin (41), which is both late-replicating and under-silencing in humans can be addressed directly by RNA FISH to detect transcription of genes on the X;autosome translocations. Such studies are underway.

Detection of late-replicating chromosome domains

To assess replication timing of chromosomes, cells were grown in medium containing BrdU [5 µg/ml for t(X;6) cells, 10 µg/ml for ins(X;16) cells] for 3 h prior to colcemid addition. t(X;6) cells were incubated with colcemid for 2 h, ins(X;16) cells for just 1 h. Late-replicating regions were detected using FITC–anti-BrdU antibodies as described previously (44).

Cultures were enriched for metaphase cells by the addition of colcemid (Gibco) at 0.1 µg/ml for 2 h. Chromosome spreads were then prepared according to methods described previously (24,43). Spreads were immunolabelled using antisera to acetylated H4 (24). Primary antibodies were detected using a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Sigma) diluted ×50 in KCM/1% bovine serum albumin (BSA) (KCM: 120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl pH 8, 0.5 mM EDTA, 0.1% Triton X-100). Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (0.2 µg/ml) in 50% glycerol/phosphate-buffered saline (PBS) + 2% DABCO, and viewed using a Zeiss Axioplan epifluorescence microscope. Images were captured with the Vysis Smartcapture image analysis system.

**MATERIALS AND METHODS**

**Cell culture**

Two Epstein–Barr virus (EBV)-transformed human B cell lymphoblastoid lines were derived from patient blood samples by the Centre for Applied Microbiology and Research (CAMR), Porton Down, Wilshire, and stored by the European Collection of Cell Cultures. One line, 044, derived from patient 1, carries an X;6 translocation, and the other, 027, from patient 2 carries an X;7 translocation, and the other, 027, from patient 2 carries an X;6 translocation, and the other, 027, from patient 2 carries an X;autosome boundary. Similarly, in an X;4 translocation, hypoacetylation of H4 was restricted to the Xi material and in most instances there was little spread of XIST RNA across the boundary. However, in both chromosome rearrangements, a limited spread of XIST RNA has been observed in a minority of chromosomes (41). These results are entirely consistent with those reported here for human cells.

We have shown previously that late replication is not an immediate and inevitable cause of H4 underacetylation (42), but the coincidence of the boundaries for all three properties examined here strongly suggests that they are functionally linked, though the nature of this link remains to be determined. The relationship between XIST RNA and spreading of transcriptional silencing in humans can be addressed directly by RNA FISH to detect transcription of genes on the X;autosome translocations described here. Such studies are underway.

**HUMARA assay**

The methylation status of the two alleles of the polymorphic HUMARA gene was determined essentially as described previously (32,33) by digestion with the methylation-sensitive endonuclease HpaII (Gibco) and the methylation-dependent endonuclease MspI (New England Biolabs). HpaII only cuts unmethylated DNA, i.e. the active X for the HUMARA locus, whereas MspI only cuts at methylated sites, i.e. the inactive X at this locus. Only uncut DNA is a suitable substrate for PCR amplification.

**Preparation of metaphase spreads and immunolabelling**

Cultures were enriched for metaphase cells by the addition of colcemid (Gibco) at 0.1 µg/ml for 2 h. Chromosome spreads were then prepared according to methods described previously (24,43). Spreads were immunolabelled using antisera to acetylated H4 (24). Primary antibodies were detected using a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Sigma) diluted ×50 in KCM/1% bovine serum albumin (BSA) (KCM: 120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl pH 8, 0.5 mM EDTA, 0.1% Triton X-100). Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (0.2 µg/ml) in 50% glycerol/phosphate-buffered saline (PBS) + 2% DABCO, and viewed using a Zeiss Axioplan epifluorescence microscope. Images were captured with the Vysis Smartcapture image analysis system.

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**Sequential detection of replication timing and DNA FISH**

In some cells, DNA FISH with chromosome 6 or 16 paints was performed after the replication timing had been determined. In these experiments, replication timing was carried out as described above and images captured using the Vysis Smartcapture software. Slides were then dehydrated (70, 85, 100% ethanol 2 min each) then hybridized with the chromosome paints according to the manufacturer’s instructions. Images of the same chromosomes were then recaptured and the two images superimposed.

**Combined immunolabelling and in situ hybridization**

For these experiments, digoxigenin-labelled whole chromosome paints for chromosomes X, 6 and 16 were obtained from Oncor. For in situ hybridization alone, metaphase spreads were prepared as for immunolabelling but fixed for 5 min in ice-cold 3:1 methanol/acetic acid immediately after cytokinetification. After fixation, slides were allowed to air dry then stored at −20°C until required. In situ hybridization was then performed according to the manufacturer’s instructions. For combined immunolabelling and in situ hybridization, slides were prepared as for immunolabelling until the addition of second antibody. For the double labelling procedure, the FITC-conjugated antibody was substituted with a biotinylated anti-rabbit antibody diluted ×500 in KCM/1% BSA. After washing, slides were fixed in 3:1 methanol/acetic acid for 10 min in place of the formaldehyde fixation. Slides were then allowed to air dry and stored at −20°C until required. In situ hybridization with digoxigenin-labelled paints was then performed essentially according to the manufacturer’s instructions. Post-hybridization washes were as follows: 50% formamide/2× SSC, pH 7, at 43°C for 15 min followed by 0.1× SSC, pH 7, at 60°C for 15 min. Slides were then transferred to 4× SSC/0.1% Tween-20 at room temperature. Digoxigenin was detected with an FITC-conjugated anti-digoxigenin antibody (Sigma) diluted ×20 in 4× SSC/1% BSA. Then 30 µl of antibody was added to each slide, covered with paraffin and the slides were incubated overnight.
in the dark in a humid chamber at room temperature for 30 min. Slides were washed three times for 5 min each in 4x SSC/0.1% Tween-20. The biotinylated anti-rabbit antibody was detected using Texas red–streptavidin (Pierce) diluted 1:1000 in 4x SSC/1% BSA for 30 min at room temperature. Slides were washed (4x SSC/0.1% Tween-20), rinsed in distilled water then mounted as for immunolabelling alone.

**RNA FISH**

Cells for RNA FISH (1×10⁶/ml) were spun onto ethanol-washed slides using a Shandon cytocentrifuge (800 r.p.m., 5 min). Samples were fixed and processed using methods described (41,45), omitting the xylene wash. Cells were digested for 10 min in 0.1% pepsin at 37°C. RNA FISH for human XIST RNA was performed using the pXIST X-4 probe (generously donated by Professor H.F. Willard). The probe was labelled directly with fluorescein-12-dUTP by nick translation. Samples were hybridized (47x548).

**ACKNOWLEDGEMENTS**

We thank Jayne Lavender for expert technical assistance, Dr Trevor Cole and Professor Peter Filandorn for referring the patients, Professor Hunt Willard for the pXIST X-4 probe used in XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. J. Cell Biol., 132, 259–275.


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