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Unbalanced X;autosome translocations provide evidence for sequence specificity in the association of XIST RNA with chromatin

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Whether XIST RNA is indifferent to the sequence content of the chromosome is fundamental to understanding its mechanism of chromosomal inactivation. Transgenic Xist RNA appears to associate with and inactivate an entire autosome. However, the behavior of XIST RNA on naturally occurring human X;autosome translocations has not been thoroughly investigated. Here, the relationship of human XIST RNA to autosomal chromatin is investigated in cells from two patients carrying X;autosome translocations in the context of almost complete trisomy for the involved autosome. Since trisomies of either 14 or 9 are lethal in early development, the lack of serious phenotypic consequences of the trisomy demonstrates that the translocated autosomes had been inactivated. Surprisingly, our analyses show that in primary fibroblasts from adult patients, XIST RNA does not associate with most of the involved autosome even though the bulk of it exhibits other hallmarks of inactivation beyond the region associated with XIST RNA. While results show that XIST RNA can associate with human autosomal chromatin to some degree, several observations indicate that this interaction may be unstable, with progressive loss over time. Thus, even where autosomal inactivation is selected for rather than against, there is a fundamental difference in the affinity of XIST RNA for autosomal versus X chromatin. Based on these results we propose that even autosomal chromatin that had been inactivated earlier in development may undergo a stepwise loss of inactivation hallmarks, beginning with XIST RNA. Hence compromised interaction with XIST RNA may be a primary cause of incomplete or unstable autosomal inactivation.

INTRODUCTION

Inactivation of one of the two X chromosomes in mammalian females provides equivalent gene dosage between males and females (reviewed in 1). The *XIST* gene, expressed only on the inactive X (Xi), initiates the X inactivation process. *XIST* produces a novel functional nuclear RNA that does not encode a protein (2,3) and has been shown to be necessary to inactivate chromatin *in cis* (4–7). Stable XIST RNA accumulates in the nucleus and 'paints' the inactivated X chromosome, indicating a functional association with the inactive chromatin (8). Although previous results have shown that the loss of the *XIST* gene after differentiation does not directly affect X inactivation (9,10), it has been recently shown that there is a synergistic relationship between XIST RNA and DNA methylation in maintaining transcriptional inactivation (11).

Studies using ectopic mouse *Xist* integrated onto an autosome report proper localization and 'painting' of the ectopic RNA *in cis* (6,7,12) and long-range hallmarks of autosomal inactivation (6,13,14). These studies suggest that autosomal chromatin is fully competent to inactivate in response to XIST RNA. However, the question of whether XIST RNA is completely indifferent to the sequence composition of the chromosome is fundamental to understanding the mechanism by which this novel RNA induces heterochromatinization of an entire chromosome.

Naturally occurring X;autosome translocations circumvent the complexities of ectopic autosomal *Xist* expression, by allowing a direct comparison of the relationship of XIST RNA to autosomal versus X chromatin in individual cells where the *XIST* gene is present in a single copy and expressed in its native context. Although mouse transgene studies demonstrated that Xist RNA is capable of coating autosomal chromatin and inducing inactivation in mice, previous translocation studies found that Xist/XIST RNA was excluded from the autosomal segments of the translocated chromosomes (15,16). However, these translocations were reported not to have been inactivated, so the absence of the XIST RNA was not surprising. Indeed, in

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the majority of individuals with balanced X;autosome translocations, inactivation of the translocation is selected *against* due to the deleterious monosomy that would result. It is therefore essential to examine cells where there is no selection against autosomal inactivation. In cases of *unbalanced* X;autosome translocations, where all or a large part of the translocated autosome is trisomic, a highly deleterious functional trisomy is avoided *only* if the translocated chromosome is inactivated. Importantly, in these cases there would be no selective pressure against XIST painting and inactivation of the translocated autosomal material.

In this study, we investigate two such X;A translocations from patients where inactivation of the translocated chromosome would avoid an ordinarily lethal or devastating trisomy. The primary cells lines studied here were derived from patients with unbalanced (X;14) or (X;9) translocations, hence they contain almost three *complete* copies of chromosome 14 or 9, respectively (Fig. 1A and B). For both patients the lack of severe clinical consequences, which would result from functional trisomy of even part of these chromosomes, as well as the earlier replication timing studies, showed that the translocated autosome had been inactivated (17–19). As the cells are derived from human patients, they allow us to study the behavior of XIST RNA in a system other than mouse. This is important, as there may be species differences in the role of XIST RNA in X inactivation.

Here we demonstrate that XIST RNA clearly shows a deficient relationship to autosomal chromatin, even where autosomal inactivation is favored. This difference in the relationship of XIST RNA with X and autosomal sequences may result in a tenuous and unstable affinity of XIST RNA for autosomal sequences. Results presented here suggest that it is this deficient interaction of XIST RNA with autosomal chromatin that is responsible for the compromised inactivation of autosomal material.

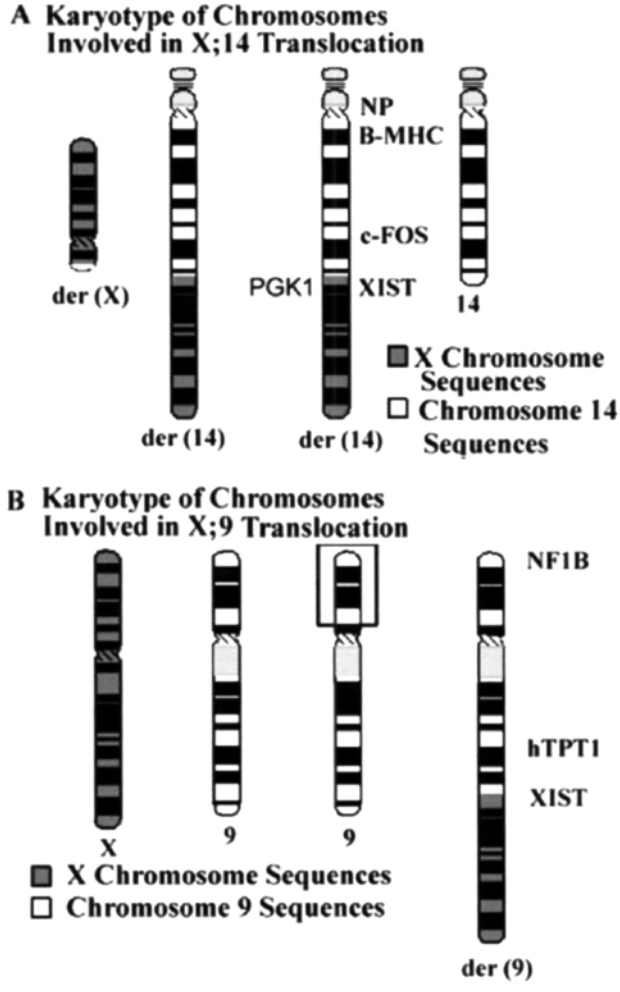

Figure 1.

RESULTS

XIST RNA fails to 'paint' all of the translocated autosome

The inactivation of the autosomal material in these translocation cell lines is selected for and the mild trisomy phenotype in the patients indicates that inactivation had occurred, which suggests that XIST RNA had inactivated the autosomal portion. We wanted to verify this and ascertain the *extent* of XIST RNA association with the autosomal portion of the translocated chromosome. Surprisingly, it was immediately clear from visual inspection that a large part of the autosomal material was consistently devoid of XIST RNA, in either translocation (Fig. 2A–H). In contrast, the X segment of the translocation was consistently covered by the XIST RNA signal (Fig. 2A–F). Because XIST RNA is not retained on human mitotic chromosomes (8), it was necessary to examine this in interphase nuclei, by comparing the distribution of XIST RNA with the autosomal and X chromosome segments. Quantitative analysis was performed by defining the boundaries of the XIST RNA and chromosome territories and determining, by digital imaging, the extent of overlap (examples, Fig. 2A–F). Although 85% of the X portion of the translocated chromosome was clearly painted by XIST RNA, as had been previously reported (8), surprisingly, the autosomal portion showed significantly less overlap: 51% for chromosome 14 and 43% for chromosome 9 (Fig. 3). The extent of interphase overlap was confirmed in 3-D verifying that the overlap between XIST RNA and the translocated autosomal segments was incomplete (Fig. 2I).

We emphasize, however, that the above percentages represent a maximal estimate of the extent of XIST RNA coverage of the autosomal material, due to apparent overlap between the X and autosomal portions when viewed in 2 dimensions. As shown in Figure 2J–K, when both segments of the translocated chromosome (X and autosomal) are viewed in 2-D there is some apparent 'overlap' seen in a few cells (~10%), which would make more of the autosomal material appear to be painted by XIST RNA than it actually is.

The partial coverage of the translocated autosomal chromatin was also verified on some mitotic chromosomes. Interestingly, while we have shown that XIST RNA is released from the human X chromosome early in mitosis (8), occasionally enough RNA signal remains in prophase to perform some analyses. The XIST RNA remaining in a small fraction of

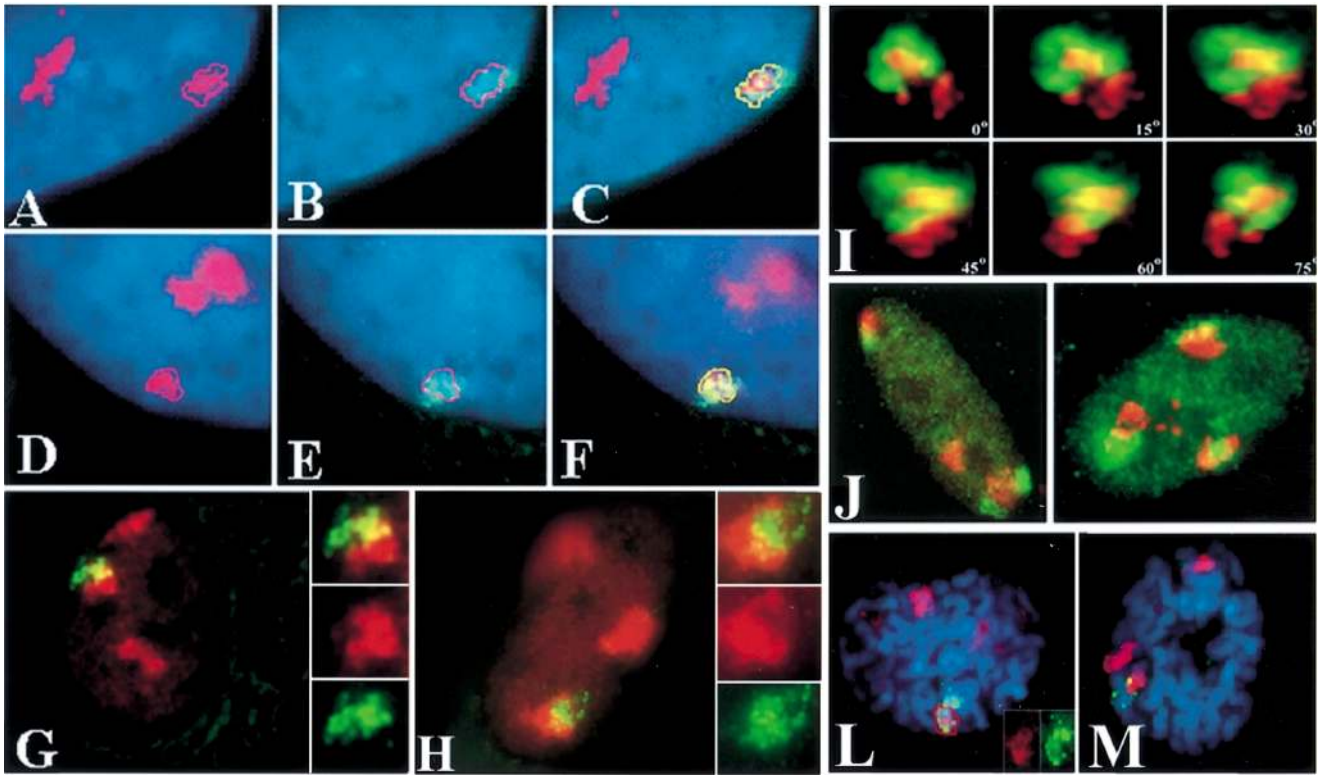

Figure 2. Limited XIST RNA localization to autosomal chromatin. (**A**–**F**) In t(X;14) fibroblast cells (*A–C*) or t(X;9) cells (*D–F*) X chromosome territories are *red*, XIST RNA is *green* (*B* and *E*) and overlap (*C* and *F*) appears yellow. The outlined regions delineate the Xi chromatin and reveal that the XIST RNA domain paints more than just the Xi. (**G**) Detection of XIST RNA (*green*) in t(X;14) fibroblast cells with 3 chromosome 14 territories (*red*). Overlap between the XIST RNA and chromosome 14 shows that the XIST RNA apparently associates with less than half of the t(14). Magnified color separations are shown as insets. (**H**) Similarly, the overlap between XIST RNA (*green*) and chromosome 9 (*red*) shows that even less of the t(X;9) translocation is painted. Magnified color separations are shown as insets. (**I**) Hybridization to XIST RNA (*green*) and t(9) chromosomal DNA (*red*) is visualized by deconvolution and 3-D reconstruction of optical sections (taken at 0.2 μ spacing). These projections of the volume rotated at 15° intervals around the Y axis confirm that part of t(9) (*red*) is painted by XIST RNA (*green*). (**J** and **K**) Chromosome paints are used to delineate the X (*green*) and 14 (*red*) segments of the translocated chromosomes at interphase. Some cells (~10%) show an apparent overlap of the X and autosomal domains in 2-D. (**L**) The XIST RNA (*green*) extends only slightly beyond the X territory (*red*) in the translocated mitotic chromosome (*counterstained with DAPI—blue*). The red outline delineates Xi. (**M**) At later stages of mitosis, the diminished RNA signal often hugs just the periphery of the chromosome. This example shows that the XIST RNA remnants (*green*) encircle t(Xi) and extend into only part of t(14) (*red*).

mitotic cells (Fig. 2L–M) hugs what appears to be the surface of the condensing chromosome (Fig. 2M). Morphometric analysis revealed that ~50% of the translocated chromosome 14 segment was painted by XIST RNA, while only ~35% of the translocated chromosome 9 segment was, thereby confirming only partial autosomal coverage.

The extent of other inactivation hallmarks along the autosomal segment is more complete than the XIST RNA coverage suggests

The partial XIST RNA coverage of the autosomal portion of the translocated chromosomes is not consistent with cell or patient survival with an almost complete trisomy. We therefore examined other hallmarks of inactivation to determine whether more of the autosomal portion was involved in the inactivation than the XIST coverage data indicates.

Few genomic probes were available for chromosome 14 and 9 linked genes that would be expressed at detectable levels in fibroblasts by FISH. However RNA signals from two detectable chromosome 14-linked genes, one gene (*NP*) distant from the breakpoint and the other adjacent (*c-FOS*), were scored relative to the XIST RNA signal (Fig. 4). Two c-FOS RNA signals were detected in most cells (Fig. 4A), which were not found associated with the XIST RNA signal and three NP RNA signals (Fig. 4B), with ~1/3 associating with the XIST RNA signal. Therefore, the *c-FOS* gene, which is closer to the breakpoint (and *XIST* gene), is inactivated, but the distant *NP* gene is not.

Since it was difficult to obtain probes for chromosome 9-linked genes expressed in fibroblasts, we relied on an assay of chromatin acetylation to assess the X;9 translocation, which gives a broader overview of the chromosome. In normal female cells the Xi is clearly hypoacetylated in comparison to the autosomes, which is indicative of its inactive state. In metaphase spreads of the X;9 cells the H4 acetylation signal was essentially absent from the entire long arm of the X;9 chromosome (Fig. 4C), which contains both X chromatin and most of the chromosome 9. This autosomal material appears consistently hypoacetylated to a level equal to that of the X chromosome. Some acetylated H4 was detected on the X;9 chromosome, on the opposite side of the centromere and far from the breakpoint, although the size of this acetylated region was small and variable (see insets, Fig. 4C). These results

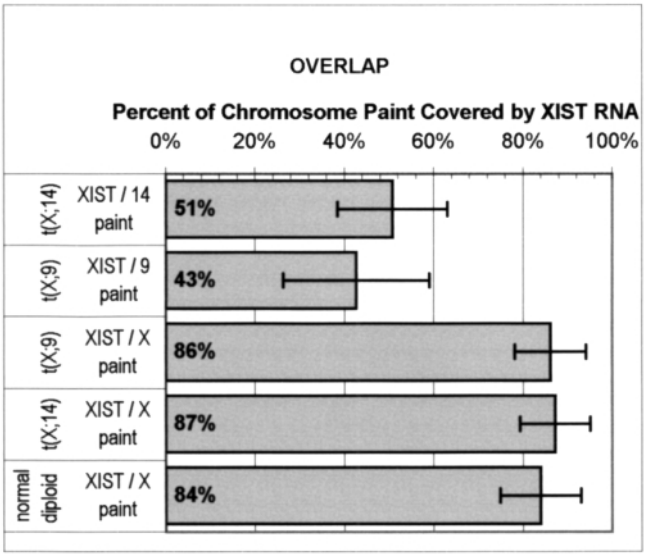

Figure 3. Morphometric analysis of spatial overlap of XIST RNA and chromosome territories. Morphometric analysis was performed on 2-D digital images as described (Materials and Methods). Summaries of measurements on ~100 t(X;14), t(X;9) and normal diploid fibroblast (WI-38) cells are presented for cells hybridized simultaneously for XIST RNA and the indicated chromosomal DNA. Percentage of chromosome overlapped by XIST RNA: 84–87% of the inactive X chromosome is, on average, covered by XIST RNA in diploid fibroblasts (as reported in 8), t(X;9)'s and t(X;14)'s, while only 51% of t(14) and 43% of t(9) is painted by XIST RNA in the t(X;14) and t(X;9) cells, respectively. The error bars represent +/− one standard deviation.

demonstrate that *the majority* of the translocated chromosome 9 (which includes most of the intact chromosome 9) consistently exhibits hallmarks of an inactive state, even though only ~1/3 of the autosome was typically associated with XIST RNA (Fig. 3).

Although DNA replication timing had been previously reported for these patient cells and was shown to be consistently late over the vast majority of the autosomal segments (17–19), due to the above results we decided to reassess replication timing in these cells. Cell cultures were terminally labeled with BrdU (see Materials and Methods), and then mitotic chromosomes were hybridized with either an X, 14 or 9 chromosome library. Because we used terminal labeling of non-synchronized cells and examined them at mitosis, all labeled cells contained late replicating DNA, with the least labeled samples revealing the latest replicating patterns. Although not in the published phenotype, one of the 'normal' 9 chromosomes was consistently deleted for the distal tip, (see box in Fig. 1B). In general, one of the two t(X;14) and the single t(X;9) showed later replication than the other chromosomes (Fig. 4D–G). The X portion of both translocated chromosomes was consistently late replicating, however the extent and timing of replication along the autosomal portions was highly variable. For example, some cells reveal only the autosomal bands close to the breakpoint having replication timing similar to the X material (Fig. 4G), while others showed significantly more (Fig. 4E–F) or almost all (Fig. 4D) of the translocated chromosome, except for the distal tip, was late replicating. Some examples of cells with lighter label on the autosomal portion (Fig. 4E) compared to the X portion, may also suggest that the autosomal chromatin exhibited a more *intermediate* late replication timing (i.e. later than the autosomal homolog, but earlier than the X segment). Therefore, while the X segment of the translocated chromosomes was consistently late replicating, the autosomal material showed a variable or intermediate degree of late replication.

Thus, these translocated autosomes are not as faithfully late replicating as the X material and are clearly different from what was previously reported (17–19). This data also demonstrates that much more of the autosomal material exhibits evidence of inactivation hallmarks than is evidenced by the XIST coverage data.

DISCUSSION

XIST RNA does not paint most of the autosomal segment which shows hallmarks of inactivation

To investigate the relationship between XIST RNA and autosomal chromatin in a natural physiological context, primary fibroblasts from two patients with unbalanced X;autosome translocations were investigated. In each case, prior phenotypic evidence had demonstrated that the large translocated autosomal segment had been inactivated (17–19). Without effective inactivation of the translocation, both patients would have been functionally trisomic for almost all of chromosome 14 and 9, respectively. These trisomies are not seen in live-borns and even much smaller segments would be incompatible with the lack of severe consequences from the trisomies seen in these patients. Hence, it is surprising that the XIST RNA painted only about 30–50% of the translocated autosomal material. In contrast, our analysis of other inactivation hallmarks reveals that *most if not all* of the autosomal material typically still showed some signs of inactivation. Thus, the hallmarks of inactive chromatin extend further along the translocated autosome than the association of XIST RNA.

The lack of XIST RNA coverage along most of the autosome suggests that XIST RNA demonstrates a preference for X chromatin over autosomal chromatin. However, before considering the fundamental implications of this for XIST RNA's interaction with the chromosome, we first consider why XIST RNA is not found on chromatin that shows hallmarks of inactivation.

XIST RNA may have a tenuous relationship with autosomal chromatin

The above findings may be interpreted to suggest that inactivation propagated beyond the spread of XIST RNA and silenced chromatin that had not interacted with the RNA. However, we suggest an alternative explanation, which we believe is much more likely and consistent with both the current literature and our observations. All evidence from published literature indicates that XIST RNA painting is necessary for inactivation to initially occur, including two recent studies that provide direct demonstrations of this (20–22). Therefore, we propose that XIST coverage of the autosomal portion of these translocations was initially more extensive and coincident with the other inactivation hallmarks,

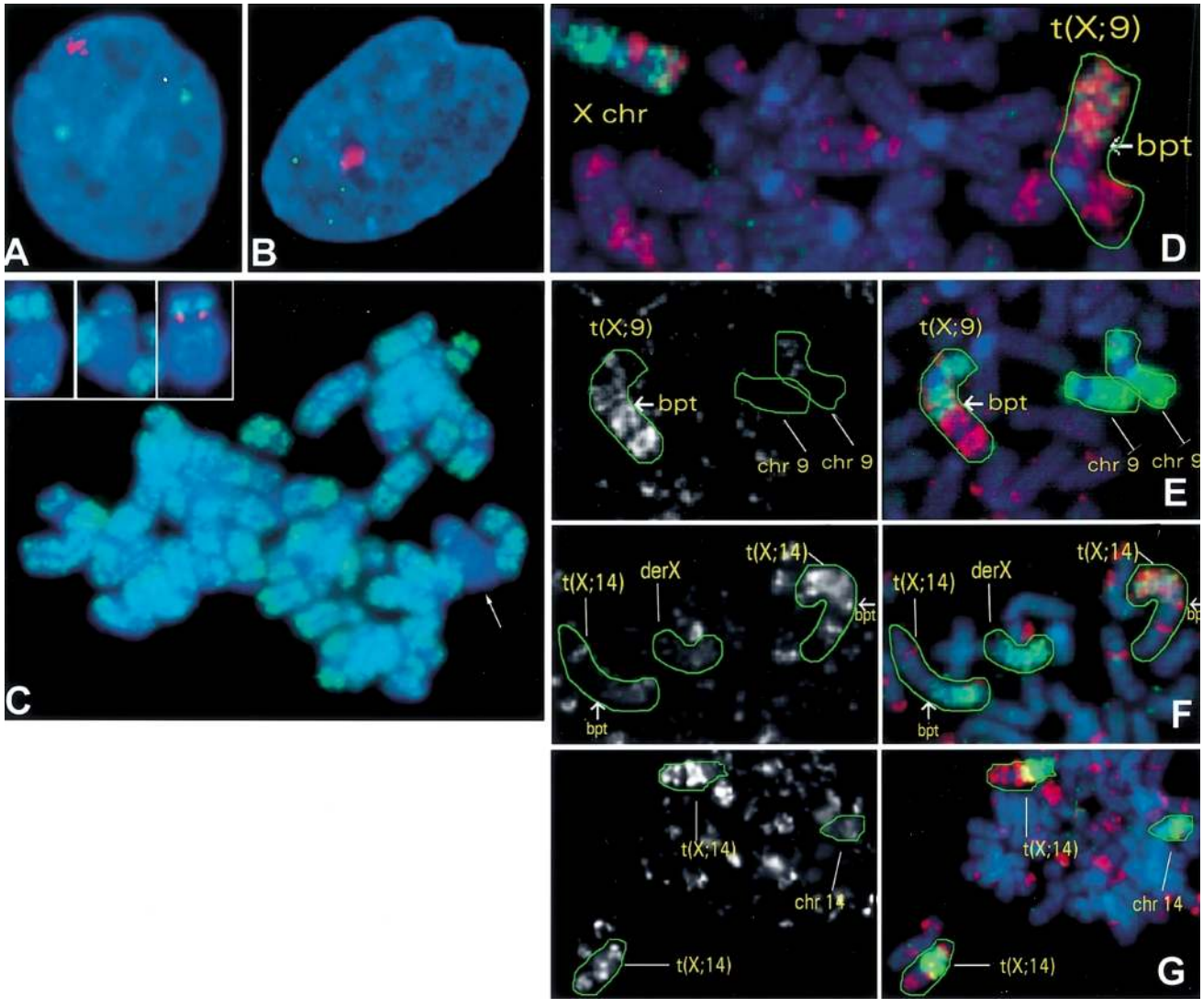

Figure 4. Hallmarks of autosomal inactivation. (**A** and **B**) Expression of genes adjacent and distant to the breakpoint in t(X;14) cells. (A) The *c-FOS* gene is subject to inactivation as it is not expressed from the t(X;14) chromosome associated with the XIST RNA signal (*red*) but is expressed (*c-FOS RNA is green*) from the two other alleles distant from the XIST RNA. (B) Three NP RNA signals (*green*) were consistently seen in t(X;14) cells, showing that NP escapes inactivation. (**C**) Staining for acetylated histone H4 (*green*) shows that the t(X;9) chromosome is almost entirely hypoacetylated in comparison to other chromosomes (*see arrow*). A variable amount of H4 acetylation is seen at the tip of the p arm (*see insets*). A DNA centromere probe (*red*) was used (*see inset at right*) to locate the chromosome 9 centromere. (**D**–**G**) BrdU (*red*) was incorporated in late S phase cells (see Material and Methods) and subsequent detection on mitotic chromosomes identified by chromosome paints (*red*) to ascertain replication timing status. Representative cells are presented. Black and white panels show only the BrdU signal for each three-color merged image. DNA is counterstained with DAPI (*blue*). The t(X;9) breakpoints and one t(X;14) breakpoint (bpt) are indicated with arrows. (D) BrdU incorporation (*red*) along the majority of the t(X;9), indicates almost full-length late replication, while the active X chromosome at left (*X chromosomes are green*) has incorporated very little label. (E) One t(X;9), and two chr9 domains (*green*) are painted. The 9 portion of the t(X;9) is later replicating than the other chromosomes 9, but is not as intensely labeled as the X segment. The boundary between the late and intermediate replicating sequences coincides with the breakpoint (*arrow*). (Although not in the published phenotype, the distal tip of one of the 'normal' 9 chromosomes was consistently deleted, see box in Figure 1B). (F) Two t(X;14) and one der(X) are defined by X chromosome paint (*green*). Much of the t(14) chromatin adjacent to the breakpoint is late replicating on the inactivated t(X;14). (G) Two t(X;14) and one der(14) are defined by a chromosome 14 paint (*green*). The normal 14 and a single t(X;14) (*bottom left*) incorporated little BrdU signal, while all of the Xi portion and only a small band within the chromosome 14 paint is late replicating in the other t(X;14) (*center top*).

but that it receded with time due to a more tenuous interaction of XIST RNA with autosomal chromatin. The impact of a functional trisomy would be most deleterious during early development; therefore it is likely that the XIST RNA more fully painted the translocation at that time. However, there are clear indications that the inactivation state may not be fully maintained on the autosomal material. This is best illustrated by the replication timing analyses. Early autoradiographic studies of these same translocations showed that almost the entire autosomal segment appeared to replicate as late as the X chromosome segment (17–19). However, in our analyses major parts or all of the autosomal segment frequently replicated earlier than the inactive X segment, or showed a lighter label suggestive of intermediate replication timing. Although

variable or intermediate replication patterns might have been more difficult to detect by earlier autoradiographic techniques, a similar analysis did reveal an intermediate replication timing pattern in a different X;21 translocation (23). This suggests that the autosomal portions of the translocations studied here lost the late replication pattern over time. Similar somatic instability of replication timing for the autosomal portion of an X;14 translocation was directly demonstrated by clonal analysis of cells over successive generations (24). In addition to autosomal replication timing instability, a recent study of XIST transgenes demonstrated the loss of XIST RNA localization to autosomes over time in culture (25). This further supports our finding that XIST has an unstable relationship with autosomal chromatin in X;autosome translocations. As further considered below, loss of XIST RNA from the autosomal chromatin may result in a reduced level of maintenance of the inactivate state (including late replication) in adult somatic or cultured cells.

Deficient interaction with XIST RNA may be responsible for compromised spread and maintenance of autosomal inactivation

The analysis of XIST RNA on these translocations may provide a paradigm to help explain the unpredictable and variable behavior of autosomal translocations more generally and provide insights into the mechanism(s) that account for this. While prior studies clearly document cases in which X inactivation has spread through autosomal sequences (for example 6,13,14), there are many examples in which the inactivation of autosomal material (particularly in X;autosome translocations) appears less complete or potentially less stable even in the absence of any negative selection (e.g. balanced translocations) (6,12,13,26, reviewed in 27). These observations raise the possibility that autosomal material has a compromised capacity to undergo or maintain a fully inactive state. Our data demonstrating loss of both XIST RNA binding and late replication in autosomal material previously shown to be inactivated, suggests that this reflects instability of inactivation rather than merely failed spread of inactivation. While XIST/Xist RNA was generally not studied, the instability of inactivation is further supported by many other cases of unbalanced translocations in the literature, showing weak or variable inactivation hallmarks and often unexpectedly mild patient phenotypes (for example 16,23,26,28,29–37). Similar variability in inactivation hallmarks has also been reported for *mouse* X;autosome translocations (reviewed in 1,27). Finally we note that studies involving Xist autosomal transgenes mentioned that a 'significant portion' of the clonal population exhibited some subsequent signs of incomplete inactivation (gene transcription, late replication and hypoacetylation) (6,12,13).

Prior to this study, it was possible, *a priori*, that deficiency of autosomal inactivation could be due to any one of the chromatin remodeling steps in response to XIST RNA, or to some failure of XIST RNA itself to interact with autosomal chromatin. Results presented here provide evidence that deficiency of XIST RNA's interaction with autosomal chromatin is key. This may include two types of deficiency of the XIST RNA/chromosomal interaction: failure of XIST RNA to fully propagate across the autosomal segment, resulting in failed spread of the inactivation signal, or loss of XIST RNA from the autosomal material in successive cell generations, resulting in deficient maintenance of inactivation.

Our results provide further insight into the relationships between different hallmarks of inactivation. Because XIST RNA covers less of the autosome than the other inactivation hallmarks studied here, we suggest that it receded first. Histone deacetylation is also more consistently retained along the length of the autosomal segment, indicating that late replication is lost before deacetylation. Sharp *et al.*, reported that transcriptional inactivation can be present in the absence of late replication (30), which is consistent with our observation that some features of inactive chromatin can remain while others are lost. If eventually all of these features recede, this might explain why Keohane *et al.* observed absence of all hallmarks, including XIST RNA, late replication and deacetylation in an unbalanced translocation with an unusually mild phenotype (16). All of these results are consistent with hallmarks of inactivation receding in a stepwise fashion, with XIST RNA lost first. That the loss of XIST RNA post-inactivation could lead to compromised maintenance is supported by a recent study that demonstrated that XIST RNA acts synergistically with other silencing factors to maintain a strict inactive state on the Xi (11). Thus, autosomal chromatin that is capable of initial XIST RNA propagation and chromosomal inactivation, may not be fully competent to maintain the XIST signal, which may explain the variability of inactivation hallmarks in some unbalanced X;autosome translocations that exhibit complete inactivation.

Specificity in the interaction of XIST RNA with chromatin

The finding that the autosomal portion of the translocated chromosome, in both translocations studied, failed to retain the XIST RNA even where autosomal inactivation is favored, provides direct evidence that there is an innate *difference* in the affinity of autosomal and X-linked sequences for XIST RNA. The widespread instability of autosomal inactivation makes it unlikely that this is strictly a phenomenon of cell culture, but, regardless, it still indicates an inherent difference between autosomal and X chromatin. The lesser affinity of XIST RNA for autosomal chromatin *in cis* makes a very important point about the fundamental mechanism by which XIST RNA interacts with chromatin: it shows sequence specificity. This hypothesis is further supported by the observation of an Xist RNA banding pattern seen on mitotic murine chromosomes (15,38). This mouse banding pattern corresponds to R-band DNA (rich in SINE elements) and occurs during the disassociation of Xist from the chromatin at mitosis (Hall *et al.*, in preparation).

The possible existence of sequence elements along the X chromosome that facilitate X inactivation has long been a subject of interest (27,39). Recent reports show that young LINE elements are more abundant and distributed differently on the X chromosome than on autosomes, suggesting that X inactivation is more efficient along the X chromosome because of the presence and placement of these elements (40). Instead, recent data from our lab showing that LINE elements are even more abundant and distributed similarly along the Y chromosome, suggests that the abundance of

LINE elements on the X chromosome is not necessarily related to X inactivation but may be a consequence of its unusual properties and selective pressures as a sex chromosome (McNeil *et al.*, in preparation).

The evidence that XIST RNA is not indifferent to sequence content has profound implications for the mechanism of XIST RNA's interaction with the chromosome. Irrespective of the specific sequence(s) involved, evidence here indicates that XIST RNA differentiates between X and autosomal chromatin either directly through as yet unidentified sequence elements, or through chromosomal proteins/modifications that are differentially distributed between the X and among the various autosomes. Indeed, this may go beyond its implications for X inactivation, in that it provides evidence for interspersed genomic sequences that function in chromatin regulation. This may eventually implicate non-coding 'junk' DNA, the bulk of the human genome, with some role in higher-level chromatin folding and gene expression.

MATERIAL AND METHODS

Cells and cell culture

The X;14 cell line is a primary fibroblast line derived from a Klinefelter male with an unbalanced karyotype: 47 Y, t(X;14) (Xpter>Xq13::14q32>14qter; 14pter>14q32::Xq13>Xqter)mat (see Fig. 1A). The X;9 cell line is a primary fibroblast line derived from a Turner female with an unbalanced karyotype: 46,X,−X,+der(9) t(X;9)(9pter>9q34::Xq13>Xqter)mat (see Fig. 1B). Both the X;14 and X;9 lines were received from the NIGMS Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ, USA), catalog numbers GM00074 and GM01414A, respectively. The normal diploid fibroblast line used was ATCC CCL 75 (WI-38), from the American Type Culture Collection (ATCC). The cells were routinely cultured until senescence in DMEM high media with 15% fetal bovine serum (FBS).

c-FOS expression was induced via serum induction as described previously (41,42). *NP* expression, which was analyzed in ∼200 cells in two replica experiments, was induced with the histone deacetylase inhibitor, n-butyrate (1 mM n-butyric acid (Sigma) for 24 h). This is a common method to induce expression of specific genes (see, for example, 43,44–46). Control experiments (data not shown) indicated that butyrate treatment did not induce inappropriate expression of normally inactive genes (*HPRT*, *PGK1* and *FMR1*). The β-*MHC* gene on chromosome 14 is muscle specific and was not detected under any conditions tested.

Alleles from the inactive X;14 chromosome were distinguished from alleles on the active chromosomes (X;14 and 14) on the basis of their proximity to XIST RNA. To determine the accuracy of this approach for assessing gene transcription from the inactive X;14 chromosome, the X-linked *PGK1* gene was analyzed in the X;14 cells as a control. Since there are two *PGK1* alleles in the X;14 cells and *PGK1* does not escape X inactivation, only one RNA signal was detected in most cells, as expected (data not shown).

Cell preparation for *in situ* hybridization

Cells were generally prepared for *in situ* hybridization according to our standard protocol (47,48). Hybridizations were conducted and optimized for nuclear versus cytoplasmic RNA detection (47). An additional method, to ensure preservation of the 3-D structure of the cells (49), was used for comparison in several experiments with similar results to our standard fixation protocol. The cells were fixed prior to extraction in 4% paraformaldehyde/PBS for 10 min at room temperature (RT), rinsed in PBS, incubated in 0.1 M HCl for 10 min at RT, extracted in 0.5% triton-X-100/0.5% saponin in PBS for 10 min at RT and then equilibrated for 20 min in 20% glycerol/PBS at RT. Cells were further permeabilized through a freeze-thaw cycle consisting of dipping the coverslip quickly into liquid nitrogen (49). The cells were allowed only to thaw (not dry out) and then stored in 70% ethanol at 4°C.

In situ hybridization and detection

Hybridization and detection was performed as described previously (47,48,50,51). Simultaneous detection of whole X chromosome library and RNA was done as described previously (8,52) with minor modifications. Cells were extracted and fixed as described above. The cells were then denatured at 80°C for 5 min in 70% formamide, 2× SSC and immediately dehydrated through an ice cold ethanol series. The Total Chromosome Library Coatasome X-digoxigenylated (Oncor) was used for human chromosome detection.

DNA probes

For fluorescence *in situ* RNA and DNA detection the following probes were used: *XIST* G1A: a ∼10 kb genomic plasmid spanning from intron 4 to the 3′ end of the XIST gene; PGK1 a genomic lambda clone isolated from the ATCC X chromosome library LAOXNLO1; (both a generous gift from Dr Hunt Willard, Case Western Reserve). pc-fos (human)-1 (*c-FOS*): a plasmid containing 9 kb of genomic FBJ murine osteosarcoma homolog sequences (ATCC catalog number 41042); Hulambda4x-8 (*HPRT*): containing 16.8 kb of genomic sequences to hypoxanthine phosphoribosyltransferase (ATCC catalog number 57236); HM-1 (*β-MHC*): cosmid containing 35 kb of genomic beta myosin heavy chain gene; lamdaPNP2 (*NP*): 46 kb of genomic sequences to the nucleoside phosphorylase gene (ATCC catalog number 59940); C22.3 (*FMR-1*): cosmid containing genomic sequences to the fragile X locus (a generous gift from Dr Steve Warren, Emory University); Coatasome-X, -14 and -9 whole chromosome library probes (ONCOR, Gaithersburg, MD, USA) were used to paint chromosome territories.

H4 acetylation assay

The assay used to detect the level of histone H4 acetylation on metaphase chromosomes has been previously described by Jeppesen and Turner (53). The X;9 cells were grown in the presence of 4 mM sodium butyrate for 16 h prior to harvest, to enhance the level of acetylation on all chromosomes. 1:100 dilution of α-acetylated histone H4 antibody (Upstate Biotech)

was used for detection. H4 acetylation data was not obtained for X;14 cells due to cellular senescence, and the resulting difficulty in obtaining sufficient cells and mitotic chromosome spreads for this primary patient cell line.

Morphometrics, 3-D rendering and image analysis

Imaging, optics, morphometrics and 3-D deconvolution were as described previously (8,52). Briefly, images were obtained with a Zeiss Axiophot microscope equipped with multi-bandpass epifluourescence filters (Chroma, Brattleboro, VT, USA). Images were recorded with a CCD camera (200 series, Photometrics, Inc) with a pixel size of 19 μm and a 14 bit A/D converter (data acquisition system by G. W. Hannaway Assoc., Boulder, CO, USA).

Cells were analyzed in two ways, using 2-D analysis to examine large cell numbers and 3-D analysis of a few individual cells to verify specific points. 2-D morphometric analysis was performed on ~35 cells from each line, showing strong RNA and chromosome hybridization signals. Using imaging software by Hannaway and Associates, the images were thresholded at the same level and the borders of the XIST RNA or chromosome domains in separate color channels were manually outlined. The number of pixels in each territory was then determined by integration of the combined registered channels.

For 3-D analysis, a Zeiss 100×, 1.4 NA Plan ApoChromat objective was used in combination with a 2.5× photo eyepiece and an optivar setting of 1.25× for a total magnification of 312× at the camera (pixel size = 61 nm). Capture of the red and green channels was performed concurrently on each optical section through 5 different nuclei from each cell line, avoiding the need for reregistration. The deconvolution algorithm used was as described previously (8).

Replication timing

The BrdU banding procedure was adapted from the BAT technique (54,55). Translocation cells were grown to subconfluence on coverslips and then cultured for a total of 6 h in 25 μM BrdU, after 4 h colcemid was added to a concentration of 1.5 μg/ml and 2 h later metaphase preparations were produced using standard procedures involving swelling in 75 mM KCl and fixing in 3 : 1 methanol/acetic acid. Because we used terminal label of non-synchronized cells and examined them at mitosis, all labeled cells contained late replicating DNA, with the least labeled samples revealing the latest replicating patterns. Cellular DNA was denatured in 0.07 N NaOH for 5 min at RT in order to facilitate detection of incorporated BrdU. BrdU was labeled with a polyclonal anti-BrdU antibody (Partec) 1 : 500 and detected with FITC or rhodamine anti-mouse antibodies (Boehringer Mannheim). When BrdU banding was performed in conjunction with *in situ* hybridization, probe hybridization, detection and fixation of signal in 4% paraformaldehyde was performed prior to the BrdU detection.

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