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Structural Association of XIST RNA with Inactive Chromosomes in Somatic Cells : a Key Step in the Process that Establishes and Faithfully Maintains X-inactivation

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STRUCTURAL ASSOCIATION OF XIST RNA WITH INACTIVE CHROMOSOMES IN SOMATIC CELLS: A KEY STEP IN THE PROCESS THAT ESTABLISHES AND FAITHFULLY MAINTAINS X-INACTIVATION

A Dissertation Presented

By

Christine Moulton Clemson

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APPROVAL PAGE

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DEDICATION

For Conrad, my love and true companion. I am deeply grateful.
For Daniel, my child and ultimate joy. May you also have many dreams fulfilled.

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LIST OF ABBREVIATIONS

Xi	Inactive X chromosome
Xa	Active X chromosome
2-D	Two dimensional
3-D	Three Dimensional
BrdU	Bromodeoxyuridine
RT	Room Temperature
FBS	Fetal Bovine Serum
5-azadC	5-azadeoxycytidine

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PREFACE

X-inactivation is a preeminent example of global gene regulation as it culminates in the transcriptional silencing of thousands of X-linked genes. This process also has clinical importance, as inadequate and inappropriate establishment of X-inactivation patterns has devastating effects on an organism. Despite its significance, this process has remained a mystery for several decades. The discovery of the *XIST* gene (Brown et al., 1991a) was a seminal event for advancing understanding of X-inactivation. As the only gene known to be expressed exclusively from the X-inactivation center, *XIST* showed much promise for being a principle player in this process (Brown et al., 1991a; Brown et al., 1992).

My foray into this field occurred when the initial excitement was somewhat diminished as it was shown that *XIST* and its mouse counterpart *Xist* did not code for a protein (Brockdorff et al., 1992; Brown et al., 1992). Chapter I describes how using fluorescent *in situ* hybridization to examine the distribution of specific XIST transcripts in somatic cells, it was proven that the XIST RNA associates with the entire Xi (inactive X) chromosome territory which indicates that it functions as a structural element. This novel RNA displayed many unique characteristics relative to other protein-coding nuclear RNAs including being composed mostly of fully processed message. This work implicated *XIST* as being important for X-inactivation and was the first to provide evidence that the RNA was fully functional in the nucleus.

While studies subsequent to this work supported these findings and showed definitively that *Xist* was both necessary (Marahens et al., 1997; Penny et al., 1996) and sufficient

(Herzing et al., 1997; Lee and Jaenisch, 1997) for the process of X-inactivation in developing embryos, other work suggested that *XIST* was not required for X-inactivation in somatic cells (Brown and Willard, 1994; Rack et al., 1994). The work described in Chapter II helps explain these conflicting conclusions. By examining the distribution of XIST RNA in mouse/human somatic cell hybrids induced to express *XIST/Xist*, these studies proved that the localization and stability of Xist RNA are not sufficient for X-inactivation. The broader implications of these results are that Xist RNA acts in concert with developmental factors to affect transcriptional silencing of the X chromosome. Therefore, I have explained the differing requirements for *Xist* in terminally differentiated versus embryonic cells by showing that *Xist* is not competent to inactivate the X-chromosome in somatic cells devoid of these developmental factors.

If *XIST* is only required during development for initiation of X-inactivation, then the constitutive expression and localization in somatic cells shown in Chapter I is enigmatic. The work in chapter III begins to explain why *XIST* continues its high level of expression and comprehensive association with the X chromosome in terminally differentiated cells. By studying the relationship of XIST RNA with X and autosomal sequences in two separate X;autosome translocations, I have shown that XIST RNA has a greater affinity for X versus autosomal sequences. This lack of complete association with autosomes correlates with the unstable nature of autosomal inactivation to suggest that *XIST* is important for maintenance of X-inactivation in somatic cells. This work then is the first to provide evidence for a role for *XIST* after development.

In the course of the work described in this thesis I have used a unique molecular cytological approach to make contributions which likely would not have been possible using conventional molecular techniques. The work described in these three chapters has been instrumental in advancing our understanding of the X-inactivation process in general and the role of XIST RNA in particular.

CHAPTER I: XIST RNA PAINTS THE INACTIVE X CHROMOSOME AT INTERPHASE: EVIDENCE FOR A NOVEL RNA INVOLVED IN NUCLEAR/CHROMOSOME STRUCTURE

Abstract

The *XIST* gene is implicated in X-chromosome inactivation, yet the RNA contains no apparent open reading frame. An accumulation of XIST RNA is observed near its site of transcription, the inactive X chromosome (Xi). A series of molecular cytogenetic studies comparing properties of XIST RNA to other protein coding RNAs, support a critical distinction for XIST RNA; XIST RNA does not concentrate at Xi simply because it is transcribed and processed there. Most notably, morphometric and 3-D analysis reveals that XIST RNA and Xi are coincident in 2-D and 3-D space; hence the XIST RNA essentially paints Xi. Several results indicate that the XIST RNA accumulation has two components, a minor one associated with transcription and processing, and a spliced major component, which stably associates with Xi. Upon transcriptional inhibition the major spliced component remains in the nucleus and often encircles the extra-prominent heterochromatic Barr body. The continually transcribed *XIST* gene and its poly-adenylated RNA consistently localize to a nuclear region devoid of splicing factor/poly A RNA rich domains. XIST RNA remains with the nuclear matrix fraction after removal of chromosomal DNA. XIST RNA is released from its association with Xi during mitosis, but shows a unique highly particulate distribution. Collective results indicate that XIST RNA may be an architectural element of the interphase chromosome territory, possibly a component of non-chromatin nuclear structure that specifically associates with Xi. XIST RNA is a novel nuclear RNA which potentially provides a specific precedent for RNA involvement in nuclear structure and *cis*-limited gene regulation via higher-order chromatin packaging.

Introduction

Inactivation of the X chromosome in mammalian females as a means to achieve gene dosage compensation was hypothesized over 30 years ago (Lyon, 1961). Because of its enormous biological and clinical importance, this process has been studied extensively and many varying models have been proposed. However, the molecular mechanisms involved remain largely undefined (reviewed in Gartler et al., 1992; Gartler and Riggs, 1983; Rastan, 1994). It is generally agreed that the X-inactivation process involves at least three stages: 1) an initiation event which culminates in distinguishing the active X chromosome; 2) propagation of inactivation in *cis* throughout the X chromosome; and 3) maintenance of inactivation throughout the cell cycle and cell division. Inactive genes on the X chromosome are methylated, however some evidence indicates that methylation appears to follow rather than precede or be precisely concomitant with inactivation (Lock et al., 1987). The visibly condensed state of the Barr body, formed by heterochromatinization of all or part of the inactive X chromosome (Barr and Carr, 1962), indicates that a change in higher-level chromatin packaging is involved in the global transcriptional silencing *in cis*. Because of the exceptional stability of X-inactivation it has been postulated that multiple levels of control are involved to assure its maintenance (Brown and Willard, 1994).

A potential breakthrough for investigating regulation of this process is provided by the identification of a novel human gene, *XIST* (Brown et al., 1991a), and its mouse counterpart, *Xist* (Borsani et al., 1991; Brockdorff et al., 1991), which are likely candidates for involvement in X-inactivation. *XIST* maps to the X-inactivation center (XIC), the locus required in cis for inactivation to occur (Brown et al., 1991b; Mattei et al., 1981; Russell,

1963) and is expressed exclusively from the inactive X chromosome (Xi) (Brown et al., 1991a; Brown et al., 1992). While several human genes have been described that escape inactivation and are therefore expressed from both X chromosomes (reviewed in Disteche, 1995), *XIST* is the only known gene expressed solely from Xi, further suggesting a role in X-inactivation.

A body of work analyzing XIST/Xist RNA expression in different tissues during development further implicates *XIST* in the process of X-inactivation, as there exists an extremely close correlation between *XIST* expression and the timing and imprinting of X-inactivation. For example, *Xist* is expressed from the paternal X just prior to imprinted paternal X-inactivation in the trophectoderm; later, *Xist* is expressed coincident with random X-inactivation prior to primitive streak formation (Kay et al., 1994; Kay et al., 1993); and in males *XIST* is expressed concomitant with transient X-inactivation during spermatogenesis (McCarrey and Dilworth, 1992; Richler et al., 1992; Salido et al., 1992). While these developmental results suggest that *Xist* expression is not merely a consequence of X-inactivation, more recent results show that *Xist* is both necessary and sufficient for X-inactivation (Herzing et al., 1997; Lee and Jaenisch, 1997; Marahens et al., 1997; Penny et al., 1996).

Given its continual expression from the early embryo throughout the life of the animal (Kay et al., 1993), it is likely that *XIST*'s involvement may not be limited to a single stage in the process of X-inactivation. Although recent results in hybrid cells indicate that *XIST*, and the XIC region, may not be essential for maintenance of X-inactivation in culture, it may be essential for initiation or for maintenance of inactivation *in vivo*, where even a low

frequency of reversion to two active X's could have devastating effects on the organism (Brown and Willard, 1994). For example, phenotypic effects of X chromosome fragments in patients are markedly more severe if *XIST* is not expressed, presumably due to the deleterious affects of failed dosage compensation for genes on the extra chromosome fragment (Migeon et al., 1994; Migeon et al., 1993; Wolff et al., 1994).

In addition to its unique pattern of expression, the *XIST* gene revealed peculiar characteristics upon isolation and sequencing. The ~40 kb *XIST* gene produces a 17 kb transcript; however, the overall sequence contains no convincing open reading frame (Brown et al., 1992; Hendrich et al., 1993). The most conserved region between man and mouse is a tandem repeat of about 50 bp with no apparent coding potential (Brockdorff et al., 1992; Brown et al., 1992; Hendrich et al., 1993). XIST /Xist primary transcripts are spliced and polyadenylated, yet subcellular fractionation studies indicate they are not found in the cytoplasmic polysome fraction, (Brockdorff et al., 1992), further suggesting the RNA is not translated. Using *in situ* hybridization, concentrations of XIST transcripts are observed in the nucleus near the Barr body, with the number of XIST RNA accumulations correlating with the number of Xi's in aneuploid cells (Brown et al., 1992). These results further support that *XIST* is transcribed exclusively from Xi. Because the highest concentration of transcripts for protein coding RNAs are known to be near their site of transcription and splicing (Xing et al., 1993a), a concentration of XIST RNA at its transcription site on the Barr body is expected and does not in itself indicate that the RNA is morphologically, stably or functionally associated with Xi or nuclear structure (Brown et al., 1992; Rastan, 1994).

While there is good evidence that the *Xist* gene is important for X-inactivation, the role of the RNA is neither defined nor definitive. Hence, information as to whether the RNA constitutes the functional product is essential to establishing both the involvement of this gene and suggesting the nature of potential mechanisms. Global regulation of genes on an entire chromosome is likely to involve novel gene regulatory mechanisms. The apparent lack of coding potential in this large gene suggests the exciting possibility that the *XIST* gene product could represent a novel type of nuclear RNA, possibly involved in chromosome or chromatin architecture. This possibility could have implications not just for X-inactivation, but for the potential function of much hnRNA, the bulk of which is non-poly adenylated, not transported to the cytoplasm, and has no known function (reviewed in Harpold et al., 1981; Herman et al., 1978; Lewin, 1975; Lewin, 1990; Salditt-Georgieff et al., 1981).

To investigate the hypothesis that XIST RNA is a novel RNA involved itself in X-inactivation and chromosome/nuclear structure, a critical question is whether the spatial relationship to Xi exists simply because the RNA is transcribed and processed there, or whether the mature RNA shows a unique and more stable morphological association with the chromosome or nuclear structure, distinct from other known protein coding RNAs. To address this a series of *in situ* hybridization experiments were necessary to examine the detailed molecular cytology of XIST RNA relative to other pre-mRNAs and to Xi, using chromosome libraries and 2-D and 3-D analysis. Both mature RNA and intron-containing RNA were studied under normal conditions and after transcriptional inhibition. It's stability and behavior throughout mitosis are also pivotal to understanding the fundamental nature

of this RNA's potential role in X-inactivation and its structural relationship to the chromosome and nucleus, hence the behavior of the RNA during mitosis and in reforming G1 daughter cells was determined. For several of these parameters, Xist RNA was compared to other protein coding RNAs, particularly collagen RNA, providing new information on the relationship of these RNAs to nuclear structure. Analysis of the relationship of XIST RNA to the nuclear compartment enriched in poly A RNA and SC-35 (reviewed in Lawrence et al., 1993; Spector et al., 1993) provides information on both the role of XIST RNA and the relationship of RNA transcription and processing to this fundamental nuclear compartment.

Based on these results two testable models are proposed for the higher-level nuclear organization of active and inactive sequences on the X chromosome relative to the Barr body and XIST RNA.

Materials and Methods

Cells and Cell Culture

Female human diploid lung epithelial line ATCC CCL 75 (WI-38) and female human diploid skin epithelial line ATCC CCL 110 (Detroit-551) were grown according to recommendations of the American Type Culture Collection (Rockville, MD). Karyotypically abnormal female fibroblasts (47,XXX) were obtained from the Camden Cell Repository (Camden, New Jersey). Amniocytes were a gift from Dr. Lenny Sciorra (UMDNJ-Robert Wood Johnson Medical School. For inhibition of transcription, cells were incubated in either 5 μg/ml of actinomycin D (Sigma Chemical Co., St. Louis, MO) for 1 to 5 hr, 25 μg/ml DRB (5,6-dichloro-1-β-D-ribofuranosyulbenzimdidizole (Sigma) for 3 hr, or 50 μg/ml β-amanitin (Sigma) for 4.5 hr prior to fixation.

Cell Preparation

Our standard cell fixation procedure was used essentially as described previously (Lawrence et al., 1989b): Monolayer cells grown on glass coverslips were permeabilized on ice with triton X-100 in RNA-preserving CSK buffer (Carter et al., 1991; Fey et al., 1986a) prior to fixation in 4% paraformaldehyde for 5 min. and storage in 70% ethanol. Hybridizations were conducted and optimized for nuclear versus cytoplasmic RNA detection as previously described (Johnson et al., 1991). In general, longer triton extraction times (5 min.) were used for

maximum nuclear RNA detection, while shorter extraction times (30 seconds) afforded maximum retention of cytoplasmic RNA.

For nuclear matrix preparations, cells were fractionated according to Fey et al. (1986a). All steps were performed on ice except where noted. Coverslips with cells attached were washed in PBS and incubated in CSK plus 10 uM leupeptin, 10 uM VRC (Gibco BRL), and 0.5% triton X-100 for 10 minutes. Coverslips were then incubated in the above CSK extraction buffer plus 250 mM ammonium sulfate for 5 min. The chromatin was digested with 100 ug/ml bovine pancreas DNAse I in CSK buffer with only 50 mM NaCl for 30 minutes at RT. DNAse digestion was terminated by incubation in CSK plus 250 mM ammonium sulfate. Coverslips were then fixed in 4% paraformaldehyde as described above.

For analysis of XIST RNA during mitosis several procedures were used successfully: To separate mitotic chromosomes, a procedure adapted from Earnshaw (Earnshaw et al., 1989) was used. Coverslips with cells attached were swollen in 3 ml of prewarmed hypotonic (.85% Sodium Citrate) for 1 hr at 37°C. The cells were then spun using a Cytospin 2 (Shandon Southern Products, England) at 1100 rpm for 5 min., the cells were air dried, fixed in 4% paraformaldehyde for 5 minutes and dehydrated through a series of ethanols and stored at -80°C until further use. An optional triton extraction was included prior to fixation without affecting the distribution of the mitotic RNA. The standard cell fixation procedure and 5 min. triton extraction modification were also used and gave good mitotic RNA signal. Finally, a fixation protocol from Dirks et al., (1993) also revealed

mitotic RNA well: All steps performed at RT unless otherwise specified. Coverslips with cells attached were washed twice in PBS (pH 7.4), fixed in 4% paraformaldehyde/5% acetic acid in PBS for 20 min., rinsed in PBS , dehydrated through a series of ethanols, cleared in xylene 10 min., rehydrated through a series of ethanols to PBS, digested in .1% pepsin at 37°C for 7 min., post fixed in 1% paraformaldehyde, rinsed in PBS and dehydrated.

To harvest transcriptionally inactive early G1 daughter cells the protocol from Zieve et al. (1980) was adapted. Early passage WI-38 cells were blocked in 5 mM thymidine supplemented media for 16 hr, rinsed and the synchronized cells were grown in thymidine free media for 8 hr. The cells were metaphase arrested with .04 µg/ml nocodazole (Sigma) for 4.5 hr, released under transcriptional inhibition conditions by rinsing them into fresh media with 5 µg/ml actinomycin D for 80 min. Cells were then extracted and fixed following our standard protocol above. For RNAase treatment, cells were treated either prior to or after hybridization with RNAase H (Gibco BRL) at 8 U/ml for 1.5 hrs at 37°C in a buffer consisting of 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 50 mg/ml of BSA, 1 mM DTT, 0.7 mM EDTA, and 13 mM HEPES (Minshull and Hunt, 1986).

DNA Probes and Antibodies

For fluorescence *in situ* RNA hybridization, the following probes were used: pFh-1, a cDNA probe to fibronectin (provided by Dave Shapiro, St. Jude's Research Hospital, Memphis, TN); CG103, a 46 kb genomic cosmid to type IαI collagen;

and pHF67, a 1.8 kb cDNA clone to type 1α1 collagen (provided by B. Strauss, Whitehead Institute, Cambridge, Ma); a 143 bp PCR amplification product of intron 26 to type 1α1 collagen (provided by D. Rowe, The School of Medicine of the University of Connecticut Health Center, Farmington, CT); 15 λDMD, a probe spanning exon 44 of dystrophin (provided by C. Thomas Caskey, Baylor College of Medicine, Houston, TX); the *XIST* Probes used were: G1A, a ~10 kb genomic plasmid spanning from the 4th intron to 3' end of the *XIST* gene; pXISTHb-B, a plasmid spanning only intron 1; and pXistHb-E, a plasmid spanning only intron 2; pXistHbC1a, a 1.6 kb cDNA clone wholly contained within exon 1 and spanning the highly conserved repeat region; pXISTC4, a 2.3 kb cDNA clone spanning the 3' end of exon 6; and pXISTC14, a 2.5 kb clone spanning the 3' end of *XIST*. To detect another RNA that escapes inactivation besides *XIST*, A1S9T, a 40 kb cosmid that spans 18 kb of the *UBE-1* gene was used. A mouse monoclonal antibody reactive to the SC-35 spliceosome assembly factor was obtained from T. Maniatis (Harvard University, Cambridge, Ma).

Hybridization and Detection

Hybridization and detection of nick translated probes was performed according to previously established protocols (Johnson et al., 1991; Lawrence et al., 1988). DNA probes were nick translated using biotin-11-dUTP or digoxigenin-16-dUTP (Boehringer Mannheim). For exclusive RNA hybridization, conditions previously defined (Lawrence et al., 1989b) were used: Hybridization was to non-denatured

cells (such that cellular DNA was not accessible for hybridization) overnight at 37°C in 50% formamide, 2xSSC using a probe concentration of 5 µg/ml. Slides were rinsed and hybridization detected using anti digoxigenin antibody coupled to rhodamine or fluorescein-conjugated avidin (Boehringer Mannheim). For DNA detection of a single copy gene, as previously described (Lawrence et al., 1989b), cells were denatured at 75°C for 2 min. in 70% formamide, 2X SSC, dehydrated through ETOH and air dried and then hybridized and detected as described above for RNA. The Barr body was identified by staining nuclei with .1 µg/ml DAPI for 30 s and rinsing briefly twice in 1x PBS.

Simultaneous Detection of Whole Chromosome Library Hybridizations and RNA

Simultaneous chromosome library hybridization and RNA detection proved to be difficult because the rigors of RNA preservation and whole chromosome hybridization are somewhat incompatible; chromosome 'paints' are designed for use in standard cytogenetic preparation where the cells are fixed in methanol/acetic acid which preserves the RNA poorly. Additionally, sufficient permeability of the library to non methanol/acetic acid fixed cells was a major problem. Extracting cells in triton longer and denaturing at higher temperatures allowed adequate detection of the whole chromosomes in intact nuclei; detection and fixation of the RNA signal prior to denaturation allowed for preservation of the RNA signal. The cells were prepared and hybridized essentially as described above for our standard protocol with the following exceptions: the triton extraction step was extended to 7

min. The *in situ* RNA hybridization and fluorochrome detection was performed as described above, the RNA signal was "fixed" by incubation in 4% paraformaldehyde for 5 min. The cells were then denatured at 85°C for 5 min. in 70% formamide, 2X SSC, and immediately dehydrated as described above. For whole chromosome detection both the WCP-X and WCP-17 Direct Label Whole Chromosome Painting System (Imagenetics, Framingham. Ma) and the Coatasome Total Chromosome Probe, biotinylated X and 17 chromosomes (Oncor, Gaithersburg, Md) were used with similar results. 10 µl of the whole chromosome library probe mixture was denatured for 5 min. at 75°C and prehybridized at 37°C for 2 hours (prehybridization was only performed for the Oncor probe). Samples were then hybridized with the probes overnight at 37°C. All post-hyb washes were performed as described above for our standard hybridization at lower stringency than recommended in the included protocols. The Oncor Chromosome Paint was detected with fluorescein-conjugated avidin (Boehringer Mannheim). To control for maximal hybridization, metaphase chromosomes in the same cell preparations were used. Clearly the chromosome paints detect the bulk of the chromatin; however in light of the complexity of the probe, hybridization efficiency may not allow for 100% detection of all sequences on the chromosome. While we cannot rule out that the denaturation and hybridization conditions do not cause changes in the fine structure of chromatin packaging, the fact that the metaphase chromosomes in these cell preparations retained their morphology suggests that gross perturbations of the interphase chromosome morphology are unlikely.

Microscopy, 3D, and Morphometric Analysis

Images were obtained with a Zeiss Axiophot microscope equipped with multi-bandpass epifluourescence filters (Chroma, Brattleboro, VT). Images were recorded with a cooled charge-coupled device (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).

For these analyses correct registration of the color channels is very important, and was afforded by the use of a multi-band pass filter in the light path in conjunction with an excitation filter wheel. Since the filter wheel is not in the image path changing the filter does not cause an optical shift. To confirm X, Y and Z registration of color channels, we compared simultaneous point spread functions that were captured by using a bead that fluoresces at wavelengths of both fluorophores.

2-D morphometric analysis was performed by first selecting cells for 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 X 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 3D analysis, A Zeiss 100x, 1.4 NA Plan ApoChromat objective was used in combination with a 2.5x photo eyepiece and an optivar setting of 1.25x for a total

magnification of 312x at the camera (pixel size = 61 nm). Capture of the red and green channels was performed concurrently on each optical section, avoiding the need for reregistration. The deconvolution algorithm used was as described previously (Carter et al., 1993). To characterize the blurring introduced by the optics the point spread function of the imaging system was calculated by acquiring a series of optical sections of a 0.2 μM-diameter fluorescent bead, under similar optical conditions as those used to obtain the cell image. The blurring of the cell image was then reversed by combining the optical sections of the cell with this quantitative calibration of the microscope blurring and the *a priori* information that the dye density is non-negative (Fay et al., 1989). L^2 regularization with non-negativity constraint (Fay et al., 1989) was used to estimate the unknown dye density, *f*, as the nonnegative density that minimizes the expression

$$\Sigma \text{image}(i,j,k) - \text{blur}(f)|^2 + \alpha \iiint |f(x,y,z)|^2 \, dx\,dy\,dz$$

where blur $(f)(i,j,k)$ is the value at voxel (i,j,k) of the function $f(x,y,z)$ computationally blurred by the point spread function. The parameter, α, determines the smoothness setting of the restored image and is set at a level that avoids noisiness and graininess of the image. Volumetric rendering of the entire series of restored data set was performed using DAVE (Data Analysis and Visualization Environment) written by Lawrence Lifschitz and Jeff Collins in the Biomedical Imaging Group at the University of Massachusetts Medical Center. 3D images were then produced by digital image restoration of a series of digitized images (Z

axis spacing 0.2 μm). This procedure permits visualization of the entire data set in three dimensions with out-of focus light minimized.

Results

XIST RNA Is Unusual In Its Distribution Compared to Other Nuclear RNAs

Many protein coding RNAs accumulate in foci or more elongated tracks near their site of transcription and splicing (reviewed in Xing et al., 1995; Xing and Lawrence, 1993). An accumulation of XIST RNA is also observed near the site of transcription, the inactive X chromosome (Xi) or the Barr body, cytogenetically defined by dense DAPI staining (Figure 1-2, E and F). As a first step to address whether this accumulation of XIST RNA is analogous to that observed for protein coding RNAs or whether it shows a more unique distribution with Xi, the nuclear formation of the XIST RNA was directly compared to that of several protein coding RNAs. The XIST RNA was co-detected with collagen IαI, fibronectin RNA, and UBE-1 RNAs in fibroblasts, and dystrophin RNA in muscle cells. The distribution of XIST nuclear transcripts was unique relative to all protein coding RNAs investigated. The XIST RNA hybridization signal, detected only in the nucleus, was markedly larger and broader than the RNA accumulation for the protein coding RNAs studied. For example, fibronectin RNA, an abundant transcript from a larger gene, produced a smaller track-like formation in the nucleus, but in cells detected for cytoplasmic RNA, was clearly more abundant in the cytoplasm (Figure 1-1, A & B). Similarly, the average area occupied by the collagen nuclear RNA, a relatively large accumulation compared to many protein coding RNAs, was 0.8 μ^2, as contrasted with the XIST RNA average of 5.0 μ^2; (Figure 1-1, C and Figure 1-3, A).

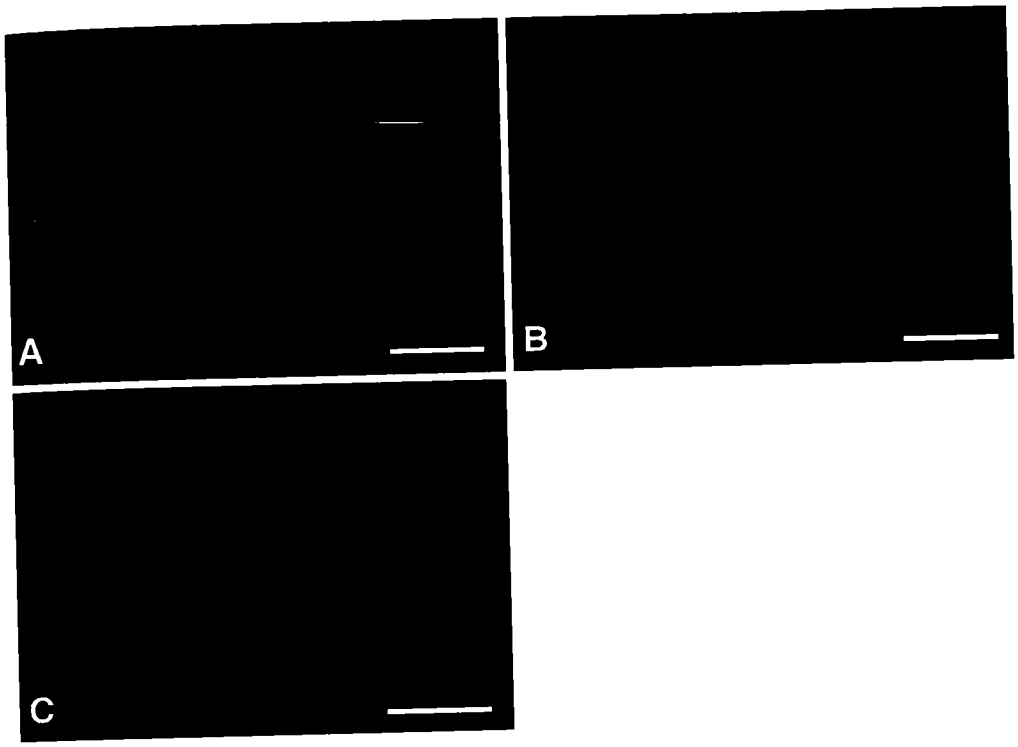


Figure 1–1: Fluorescence in situ Hybridization Comparison of XIST RNA with Protein Coding RNAs in Human Fibroblasts.
Digoxigenin and biotin–labeled probes were hybridized *in situ* to non–denatured cells and detected with fluorochrome–conjugated avidin or anti–digoxigenin antibody. Fluorochromes used were FITC (*green*), rhodamine (*red*) and DAPI DNA stain (*blue*). (A) Nuclear hybridization of XIST RNA (*red*) with fibronectin RNA (*green*) in 46,XX Detroit–551 diploid fibroblasts. XIST RNA produces a much larger, broader accumulation of XIST RNA relative to the "track" of fibronectin RNA, although fibronectin is a very abundant transcript which produces a larger primary transcript (B) XIST RNA (*green*) and fibronectin RNA (*red*) in 47,XXX cells containing 2 inactive X chromosomes. The cells were prepared for maximum retention of cytoplasmic RNA (see Materials and Methods), revealing abundant cytoplasmic fibronectin RNA and no XIST RNA cytoplasmic signal above background levels. (C) Collagen RNA (*green*) with XIST RNA (*red*) in 46,XX WI–38 cells. Notice the broad, apparently clustered nature of the XIST RNA. Bar, 5μm.

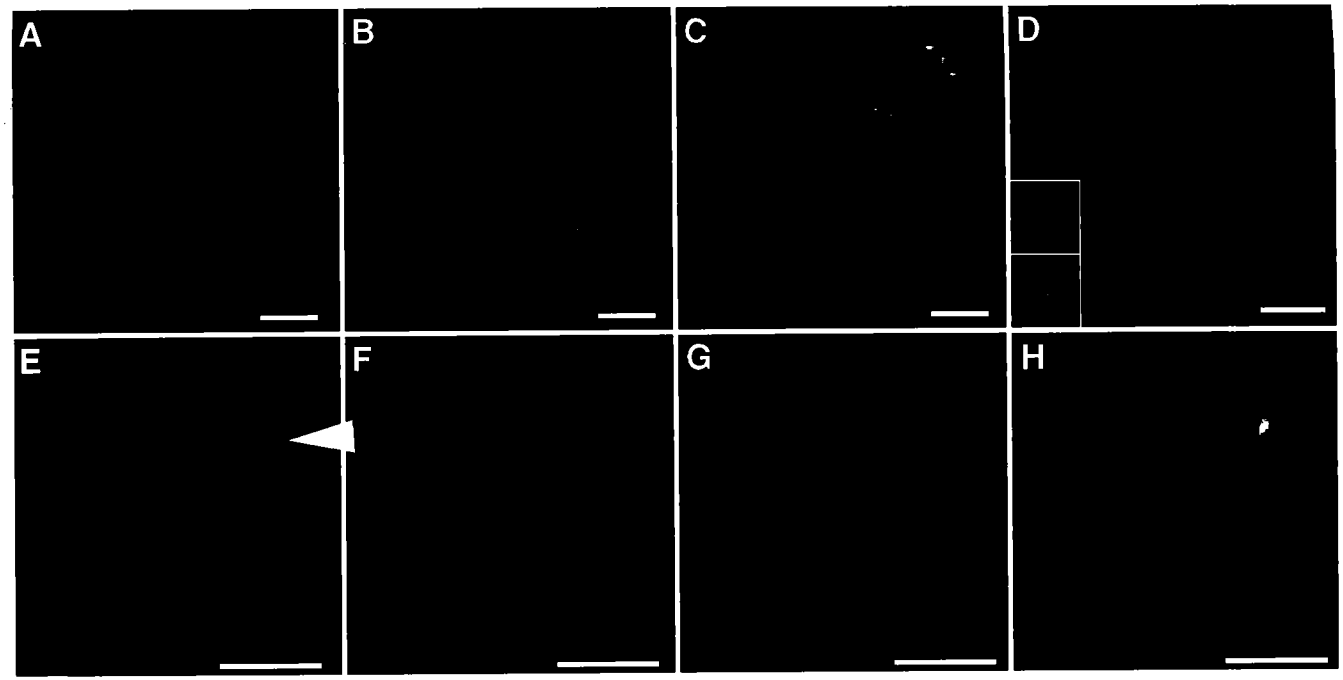


Figure 1–2: Co–localization of XIST or Collagen Nuclear RNAs with their Parent Interphase Chromosomes Detected with Whole Chromosome DNA Libraries.
Detection of chromosome libraries was carried out using either the Imagenetics WCP–X or Oncor Coatasome Painting systems and simultaneous hybridization to nuclear RNA was achieved as described (see Materials and Methods). (A) Detection of XIST RNA (*green*) in a 47,XXX amniocyte with two inactive X chromosomes. There is low level background fluorescence throughout the nucleoplasm under the detection conditions used. A similar level of background fluorescence is seen when no probe is added to the hybridization. (B) X chromosome detection using the WCP–X library directly labeled with spectrum orange in the same cell as (A). Note the striking similarity of the XIST RNA accumulation with the inactive X morphology, which shows a distinctive U shape and is more compressed than the active chromosome. (C) Overlap between the XIST RNA and the inactive X chromosomes appears yellow. DAPI DNA stain, blue. (D) Collagen nuclear RNA (*red*) does not overlap its parent chromosome 17 (*green*) but localizes at its periphery in most cells. However, when the RNA signal appeared within the chromosome 17 territory in many cells, it was found that the RNA actually colocalized with a void in the 17 library signal (*inset*). (E) Barr body (Xi) in a WI–38 nucleus revealed by DAPI staining (*arrow*). (F) XIST RNA occupies a similar spatial location as the Barr body in the same cell. (G) Detection of the two X chromosomes in the same cell using the Coatasome–X library detected with FITC. (H) Overlap between the XIST RNA and the inactive X chromosome (*yellow*). Bars, 5um.

The XIST RNA has an unusually punctate distribution, like an accumulation of many distinct, very bright clusters; the particulate nature of this RNA distribution is best revealed in Figure 1- 6 and Figure 1-10, A and C. This pattern was reproducible with several preparative techniques, and consistently contrasted with the less particulate, continuous accumulation of transcripts for large protein coding RNAs compared with XIST RNA (Figure 1-1, A-C).

XIST RNA "Paints" the Inactive X Chromosome

If XIST RNA itself has a direct role in X-inactivation, it might be expected that the RNA would be spatially coincident with all or most of Xi, the site of its purported function. The extent of the association of XIST RNA with Xi was investigated in detail using hybridization to a whole X chromosome library. The delineation of the Barr body (Figure 1-2, E) and the extent to which it encompasses Xi DNA are unclear, hence the X chromosome specific library was utilized to "paint" the entire X chromosome, in conjunction with simultaneous hybridization to XIST RNA. Development of procedures to accomplish this proved to be technically challenging, hence a variety of procedures and libraries were evaluated to achieve maximal hybridization of the chromosome and to simultaneously preserve and hybridize the XIST RNA (Materials and Methods).

Comparison of the XIST RNA signal with the library hybridization to Xi revealed a striking overlap of these two molecular entities. As illustrated in Figure 1-2 (A-H), the RNA occupied the same "territory" as the chromosome, viewed in human diploid

fibroblasts or amniocytes. This spatial coincidence was particularly well demonstrated in cells in which Xi had a distinctive bent shape. For example, the cell in Figure 1-2 (A-C) has three X chromosomes, exhibiting three red X-paint signals (Figure 1-2, B). In these cells, XIST RNA labels two nuclear sites in green (Figure 1-2, A), consistent with the notion that all X chromosomes in excess of one are inactivated (Grumbach et al., 1963). As viewed in two dimensions, the spatial distribution of XIST RNA is essentially identical to the inactive X paint signal (Figure 1-2, C and H).

For comparison to a protein coding RNA, the relationship of collagen nuclear RNA tracks to its parent chromosome, 17, was evaluated. This was also of interest in itself, since the question of how RNA tracks are distributed relative to the chromosome has implications for nuclear organization in general. The collagen RNA was much smaller than the signal from the chromosome 17 paint and the two signals never had similar shapes. While not directly investigated, the same results can be predicted for other protein coding RNAs based on the size and shape of their nuclear signals. The collagen RNA signal was at the periphery of the chromosome signal the majority of the time (>60%) (Figure 1-2, D). Interestingly, as illustrated in the inset of Figure 1-2 (D), when the collagen RNA signal was inside the Chromosome 17 territory, it was found that the RNA actually overlapped a distinct gap or "hole" in the chromosomal DNA signal. This contrasts sharply with the close and essentially complete coincidence of XIST RNA with the Xi chromosomal DNA. A global organization of the nucleus has been suggested in which nuclear RNAs and splicing components reside in freely diffusible channels between chromosome territories - the "interchromosome domain"(Cremer et al., 1993; Zachar et al., 1993; Zirbel et al., 1993).

Although collagen nuclear RNA tracks frequently localized at the chromosome periphery, a substantial fraction (40%) appeared to be within the territory; moreover, the XIST RNA was never observed in a distribution that fit a model in which nuclear RNA resides in spaces separating chromosomes.

The cytological association of XIST RNA with Xi was further analyzed by 2-D morphometrics. Measurements around the boundaries of the XIST RNA and X library signals were done in approximately 70 nuclei in 3 separate experiments. As summarized in Figure 1-3 (B), the ratio of the area occupied by XIST versus the chromosome library was determined for each cell, and on average was 0.99, indicating essentially identical sizes. This contrasts with an analogous value of 0.23 for collagen RNA and the Chromosome 17 library. Similarly, the average spatial overlap between XIST RNA and the X Chromosome library was 0.84, which contrasts with 0.13 for collagen versus Chromosome 17 (Figure 1-3, C). Where it was possible to discern discrete boundaries, the Barr body was measured and results suggested that its area was approximately 20% smaller than either the X paint or the XIST RNA (data not shown). Because of the difficulty in delineating the Barr body and the smaller sample size, this observation must be considered preliminary.

It was important to address whether the XIST RNA overlapped the Xi territory in the Z axis; therefore we performed a 3-D analysis of several cells. Since Xi often abuts the nuclear periphery, it was of particular interest to determine whether the XIST RNA might lie at one surface of the chromosome, such as near the nuclear envelope. Optical sections through nuclei at 0.2 micron spacing were taken of several cells, creating a series of digitized images, which indicated that the XIST RNA and the chromosome library signal

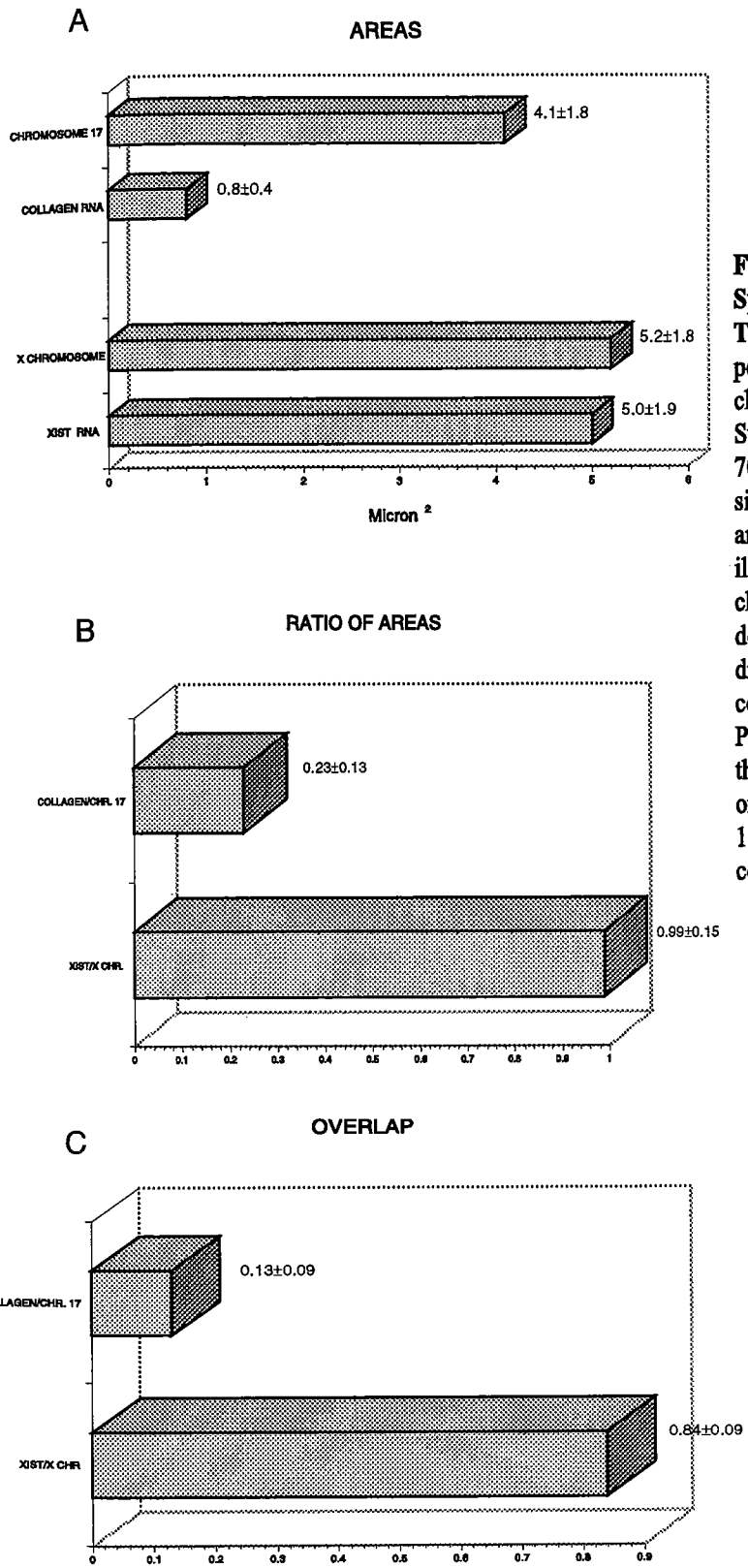


Figure 1-3: Morphometric Data on Size and Spatial Overlap of RNA and Chromosome Territories. Morphometric analysis was performed on 2D digital images in separate color channels as described (Materials and Methods). Summaries of measurements on approximately 70 WI-38 cells are presented for cells hybridized simultaneously for XIST RNA or collagen RNA and the corresponding chromosomal DNA, as illustrated in Fig. 1-2. (A) Average areas for chromosome and RNA territories, with standard deviations. (B) Ratio of areas occupied by RNA directly compared within the same nucleus to corresponding parent chromosome. (C) Percentage of chromosome that is overlapped by the RNA: 84% of the inactive X chromosome is, on average, covered by XIST RNA, while only 13% of chromosome 17 is overlapped by collagen RNA.

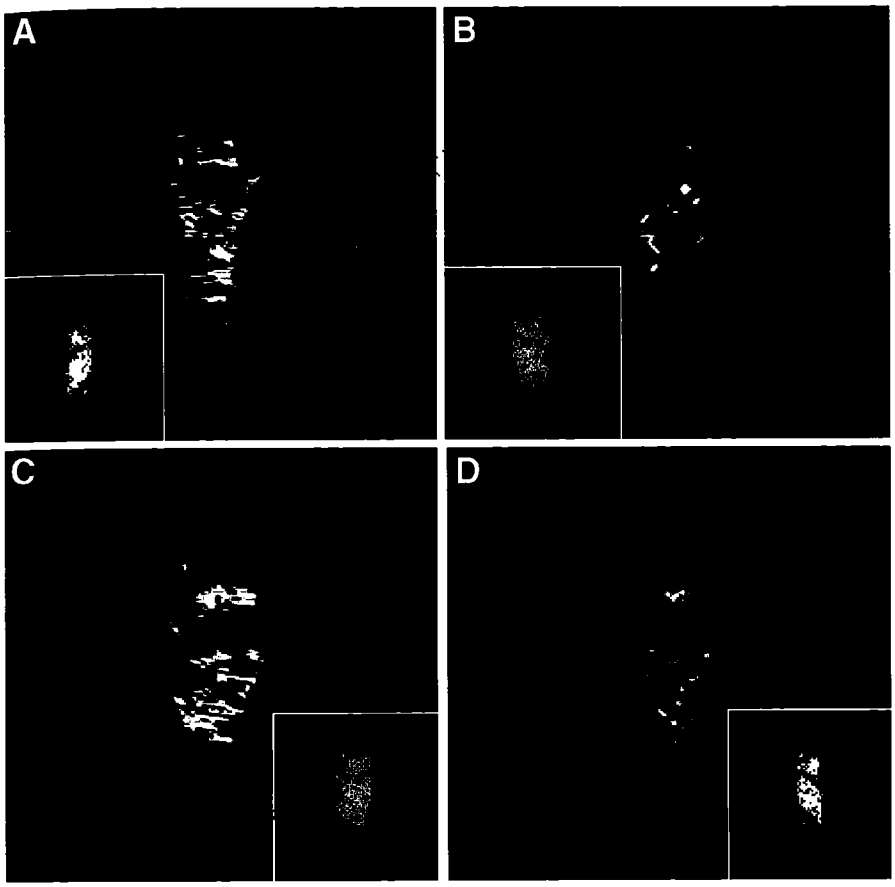


Figure 1–4: Three–dimensional Distribution of XIST RNA and the Inactive X Chromosome.
Hybridization to XIST RNA (*red*) and whole X chromosomal DNA (*green*) were visualized simultaneously and data sets were acquired and restored as described in Materials and Methods. (A–D) A binary volumetric rendering of an entire optical section data set after image restoration; this is a straight projection rotated at 90 degrees along the X axis. Thresholding of the volumetric projection was set to show the 5% most intense voxels. The data was treated as being opaque. The white (*inset*) represents voxels that contain both red and green and thus defines the overlap. This figure shows that the XIST RNA and Xi occupy the same 3–D spatial location and that XIST RNA is not restricted to one surface of the chromosome but overlaps the X library in every plane. Notice that while there is a significant degree of overlap between the XIST RNA and Xi it is not always a point for point correlation.

occupied the same planes of focus (Figure 1-4). The optical sections were deconvoluted to remove out of focus light and as shown in Figure 1-4, a 3-D rendered image of one cell is used to create a composite of 3-D spatial relationship between XIST RNA and the X chromosome library. This figure definitively shows that XIST RNA occupies the same 3-D nuclear territory as the chromosome library. The distribution of white pixels in the insets of Figure 1-4 shows points of precise overlap, indicating that there is a very intimate morphological association between the RNA and the Xi DNA. However, since not all pixels show overlap, these results suggest that the fine detail of the distributions are not point for point identical. These results indicate that the relationship between XIST RNA and the inactive X chromosome is not surface limited but appears to involve the entire chromosome.

That such a unique, extensive, and consistent morphological association of XIST RNA exclusively with Xi would occur randomly or coincidentally is extremely unlikely. Therefore, these findings alone strongly support a direct involvement of this RNA with the chromosome and/or with nuclear structure related to X-inactivation. Several other properties of this unusual RNA were then examined, which provide further support for this idea and insight into the nature and properties of the RNA.

XIST RNA is Stable Upon Transcriptional Inhibition

If Xi represents the site of functional deposition for XIST RNA, the RNA may well show a long nuclear half-life. XIST RNA and collagen RNA were both examined under conditions of transcriptional inhibition. Because of the impact of inhibition on RNA

transport (Herman and Penman, 1977) and the evidence from *in situ* hybridization studies that in many cells very little poly A RNA exits the nucleus after transcriptional inhibition (Huang et al., 1994; Lawrence et al., 1993), the impact of inhibition on either RNA could not be predicted, hence results for collagen nuclear RNA were of interest in themselves. In cells treated with actinomycin D, collagen nuclear RNA signals are substantially diminished after 1 hr and undetectable by 3 hours (Figure 1-5, A). Similar results were also observed for fibronectin RNA (Xing, 1993). In contrast, XIST RNA largely remained for up to 5 hours in actinomycin D in most cells (Figure 1-5, A), with similar nuclear stability observed after inhibition with β-amanitin and DRB (data not shown). With the caveat that transcriptional inhibition can induce stabilization of some RNAs and not others (Hogan et al., 1994), these results are most consistent with the idea that XIST RNA is long-lived within the nucleus, supporting its role as an RNA which may function in nuclear/chromosome structure. These results also provide the important confirmation that the collagen RNA localized in SC-35 rich domains is pre-mRNA (Xing et al., 1995) and contribute new insights into the interpretation of transcriptional inhibition effects on nuclear poly A RNA (see Discussion and Huang et al., 1994; Lawrence et al., 1993; Xing et al., 1995).

Two aspects of the RNA distribution are accentuated in inhibited cells. The particulate nature of the XIST RNA signal is more readily evidenced (Figure 1-6, C) as the clusters appear to break up and become more discrete and dispersed through the nucleoplasm (Figure 1-6, A and C). Another potentially important observation derives from the fact that transcriptional inhibition with actinomycin D results in overall chromatin condensation

(Bernhard, 1971; Nickerson et al., 1989) which produces a highly distinct Barr body. In approximately 10% of these cells it was apparent that XIST RNA clusters clearly encircle this prominent Barr body (Figure 1-5, B). This phenomenon may be a result of limited penetrability of probe to these highly condensed chromosomes; however, we have also occasionally seen this in untreated cells. These details of the distribution have potentially important implications which will be considered in the Discussion.

Transcription, Splicing, and Stability of XIST RNA

To determine the extent to which the XIST RNA was comprised of mature spliced RNA, the relative distribution of specific intron sequences was examined. This also provides insight into transcriptional status within individual nuclei, since intron signal would be primarily associated with newly synthesized transcripts. We cohybridized intron 1 and/or intron 2 probes (Brown et al., 1992) simultaneously with a cDNA or genomic probe (see Materials and Methods). Digoxygenin was used to label the intron, since this affords somewhat greater sensitivity than biotin labeling. Intron signal was detectable in cells ranging from early G1 to early prophase, in a non-synchronized population, suggesting that transcription is ongoing from early G1 to mitosis. The intron signal consistently occupied only a portion of the accumulation defined by the cDNA, indicative that the bulk of the RNA was spliced. The intron signal generally comprised a very small bright focus (Figure 1-6, B). Often there was a more dispersed distribution of very faint signal through a portion of the broader cDNA signal which may represent unspliced RNA or excised introns (Figure 1-6, A and B).

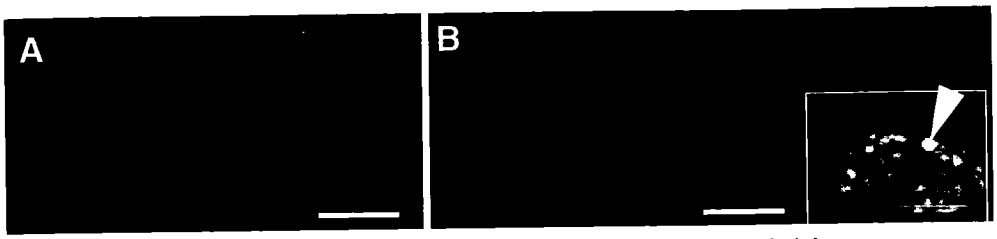


Figure 1–5: XIST RNA is Stable Upon Transcriptional Inhibition.
A) WI–38 cells treated for 3 hours with Actinomycin D. XIST RNA (*green*) remains whereas collagen RNA (*red*) is no longer detectable in the nucleus. (B) In actinomycin D treated cells, the XIST RNA (*red*) encircles the extra–prominent Barr body (*inset–arrow*). This phenomenon is apparent in approximately 10% of treated cells, and is also occasionally apparent in untreated cells.

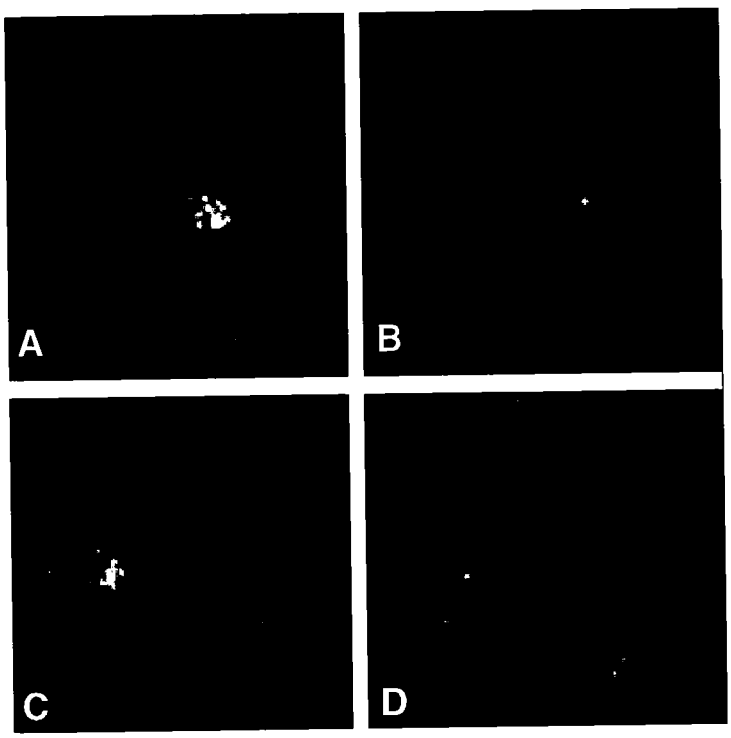


Figure 1–6: Detection of XIST RNA with Intron and cDNA Probes with and without Transcriptional Inhibition.
Probes to intron 1 and 2 of *XIST* were labeled with digoxigenin and cohybridized with a *XIST* cDNA probe labeled with biotin in WI–38 cells treated for 0 and 3 hours with actinomycin D. (A) XIST cDNA signal in an untreated cell (B) intron RNA signal in the same cell as (A). This cell was chosen and the photograph exposed to emphasize that, in addition to a prominent small focus of intron signal (see Fig 1–7) there is often a less intense, more dispersed intron signal which may represent either unspliced RNA or excised introns. However, even when this more dispersed intron is present, the intron accumulation still occupies only part of the cDNA accumulation and is much less intense. (C) cDNA signal after transcriptional inhibition, showing that the mature XIST RNA remains. (D) Intron hybridization in the same cell as (C) after actinomycin D treatment, showing that the intron signal is gone. We have overexposed this image to show that no intron containing RNA is detectable, with the possible exception of a small spot of intron signal that could represent intron–containing nascent transcripts stabilized on the gene (see Xing et al., 1995).

These results show that some of the XIST RNA is associated with transcription and splicing, and other results indicate that the bulk of it is not. This was first suggested by the fact that unspliced and fully spliced XIST message had no reproducible spatial relationship, unlike other pre-mRNAs we have studied. Fibronectin (Xing et al., 1993a) and collagen nuclear pre-mRNAs (Xing et al., 1995) have a predictable spatial relationship in that tracks or foci consistently show a bipolar distribution of intron+ and intron- sequences (Figure 1-7). This reflects an ordered structural arrangement most consistent with the vectorial progression of unspliced to spliced transcripts, after which the spliced transcripts are transported by an unknown route (reviewed in Xing and Lawrence, 1993). In contrast, the foci of XIST intron shows no such reproducible spatial configuration; no bipolar relationship to the larger RNA accumulation was apparent, such that RNA lacking the introns accumulated all around the small focus of RNA containing intron sequences. This unique distribution is consistent with the possibility that surrounding or associated with the transcription and splicing site of the XIST RNA is a larger accumulation of RNA that is a mature stable component. The latter interpretation was most clearly supported by repetition of experiments with actinomycin D using simultaneous hybridization to intron and exon sequences in two colors. It was consistently observed in multiple experiments that the signal with a genomic or cDNA probe was only slightly diminished after this treatment, whereas significant intron signal was not detectable even after prolonged exposures and computer enhancement (Figure 1-6, C and D). In a small fraction of cells a very dim, tiny spot of fluorescence was observed which could represent primary transcripts still associated with

the gene (Figure 1-6, D), however in most cells no residual transcripts were detected. Therefore the intron signal in uninhibited cells represents a minor fraction of the RNA that is related to the ongoing transcription of the gene. The bulk of XIST RNA is mature RNA that is resistant to transcriptional inhibition and morphologically associated with the body of the inactive X chromosome.

XIST RNA is Not Associated with SC-35 Domains

It was important to determine whether the apparently long-lived poly-adenylated XIST RNA was associated with discrete nuclear domains greatly enriched in splicing components and poly A RNA, for which the splicing assembly factor SC-35 is a marker (reviewed in Carter et al., 1993; Fu and Maniatis, 1990; Lawrence et al., 1993; Spector et al., 1993). This impacts upon the function of XIST RNA since it has been proposed, based on transcriptional inhibition studies, that long-lived structural RNAs preferentially localize in these domains which are putatively involved with splicing factor storage (see Discussion and Huang et al., 1994). As shown in Figure 1-8 (A), the large XIST RNA accumulation essentially never overlapped these regions, nor was there a close spatial association. Most cells showed no spatial contact at all, and the most association observed was in a fraction of cells where a tiny portion of the XIST RNA signal appeared to "contact" a SC-35 domain, as contrasted with some "associated" pre-mRNAs for which the body of the pre-mRNA signal was in intimate contact with the domain (Xing et al., 1993a; Xing et al., 1995).

To confirm that neither the *XIST* gene nor a small portion of the XIST RNA related to transcription/splicing was more closely associated with the domains, the position of *XIST*

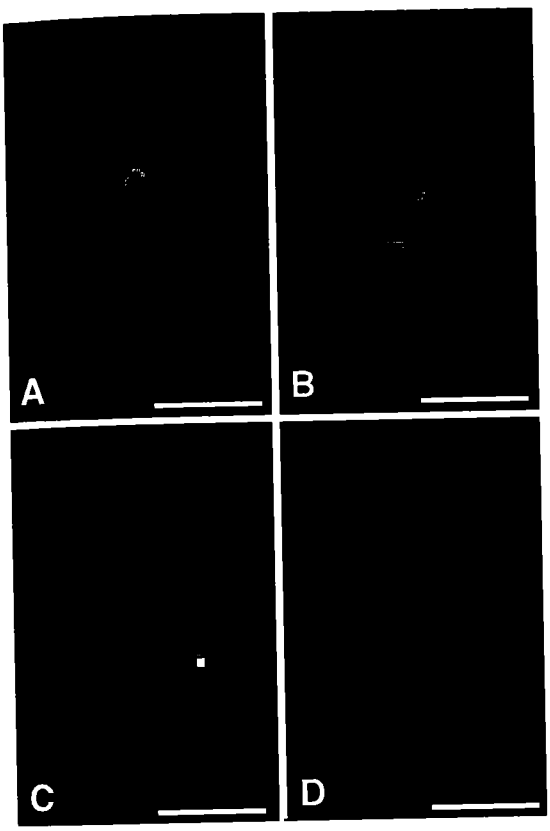


Figure 1–7: Comparison of *XIST* Intron/cDNA Distribution to the Collagen Intron/cDNA Distribution.
Probes to intron 1 and 2 of *XIST* were labeled with digoxigenin and cohybridized with *XIST* cDNA probe labeled with biotin in WI–38 cells. (A) and (B) show representative examples of the random localization of intron–containing XIST RNA (*red*) among the larger accumulation of more mature cDNA lacking the intron (*green*). (C) and (D) show representative examples of the polar localization of the collagen intron–containing RNA (*red*) among the processed cDNA (*green*). The differences in the relative distribution of spliced and unspliced RNA are consistent with collagen pre–mRNA being transcribed and processed in an ordered linear configuration, while there may be two populations of RNA for *XIST*; a smaller accumulation related to transcription and processing, and a larger accumulation consisting of spliced RNA which remains in the nucleus and likely functions in association with Xi. Bars, 2um.

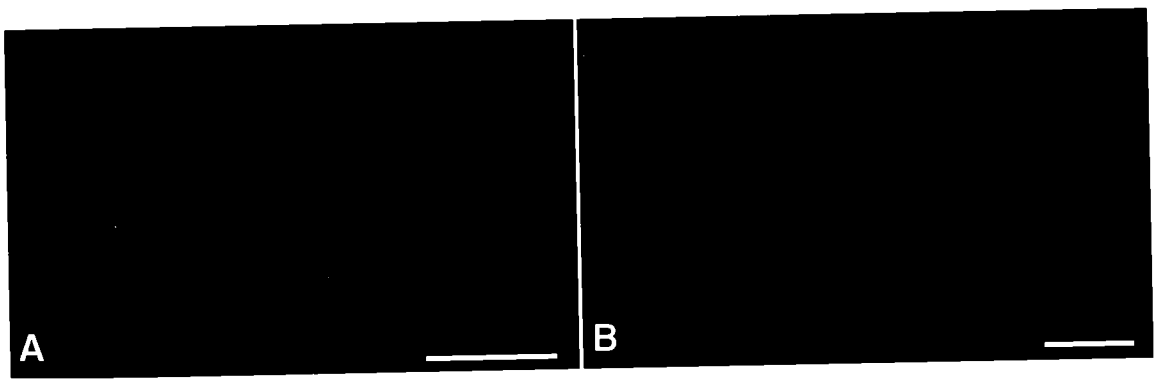


Figure 1–8: XIST RNA Does not Colocalize with SC–35 Domains.
Nuclear domains enriched in splicing components and poly A RNA were labeled with an anti–SC–35 antibody and detected with a FITC–conjugated secondary antibody. (A) Despite the large accumulation of the poly adenylated XIST RNA (*red*), it does not overlap nor show substantial association with these discrete SC–35 domains (*green*). (B) DNA hybridization to the *XIST* gene (*red*) relative to SC–35 domains (*green*), illustrating that the gene itself does not associate. Bars, 5um.

was localized by hybridization under conditions which remove RNA. As illustrated in Figure 1-8 (B), the *XIST* gene distribution analyzed in 30 cells showed no specific contact with SC-35 domains, with 80% of signals clearly separate from domains. While most pre-mRNAs or active genes which we have studied are preferentially associated with domains, some clearly are not (Xing et al., 1993a; Xing et al., 1995). Hence this in itself is not peculiar to *XIST* and cannot be the explanation for the unusual distribution of XIST RNA with Xi, but clearly shows that association of the *XIST* gene with a large "domain" or "speckle" of SC-35 is not necessary for its transcription and splicing.

The fact that this broad accumulation of RNA did not overlap any of the 30-50 discrete SC-35 rich regions indicates that the RNA is specifically positioned in a nuclear region devoid of such domains. These results have further implications which will be considered in the Discussion.

XIST RNA is Not Associated with the X Chromosome During Mitosis

The stable morphological association of mature XIST RNA with Xi and/or nuclear structure in interphase cells led us to investigate whether this association extended to the mitotic chromosome. This addresses a key point relevant to the nature of XIST RNA's relationship with the Xi and impacts upon the potential mechanisms whereby it may have a role in inactivation. Since the mitotic chromosome is not transcriptionally active and the *XIST* gene on Xi is unmethylated and hence programmed for clonal expression (Hendrich et al., 1993; Norris et al., 1994), its continued association with the chromosome during mitosis is not necessary for a role in inactivation. Traditional cytogenetic metaphase

preparations do not leave XIST RNA wholly intact, so the technical challenge was to prepare cells in such a way that the mitotic chromosomes were revealed sufficiently while preserving the RNA. Several hundred mitotic and postmitotic amniocytes and fibroblast cells were examined, and four fixation methods were successful in revealing a similar pattern of XIST RNA distribution throughout the cell cycle, although with one procedure, the XIST RNA clusters were more disaggregated at interphase and a few were occasionally detected in the cytoplasm; (see Materials and Methods and Dirks et al., 1993). Surprisingly, the XIST RNA clusters seen at interphase separate during mitosis, revealing a highly particulate nature and a unique mitotic progression. In prophase, the particles or clusters of XIST RNA suggested at interphase become much more distinct, as they apparently release from their constraints and disaggregate from one another, but remain close to Xi (Figure 1-9, B). In prometaphase and metaphase, the clusters become more separate and often distribute across many chromosomes; ultimately moving into the cytoplasm, (Figure 1-9, C and D). At telophase, no signal is seen over the chromatin, and the clusters are most often concentrated between the cleavage plane (Figure 1-9, E); in early G1, XIST RNA clusters are found in the cytoplasm and a small focus of signal is observed in the nucleus (Figure 1-9, F). Therefore, during mitosis we did not see the association between XIST RNA and Xi that was evident in interphase; while it is possible that the extraction procedure disrupts the mitotic association, this seems unlikely since we used four different preparative techniques (see Materials and Methods).

We examined collagen RNA under the same conditions and observed no signal associated with the chromatin and diffuse, granular signal in the cytoplasm (Figure 1-9, A),

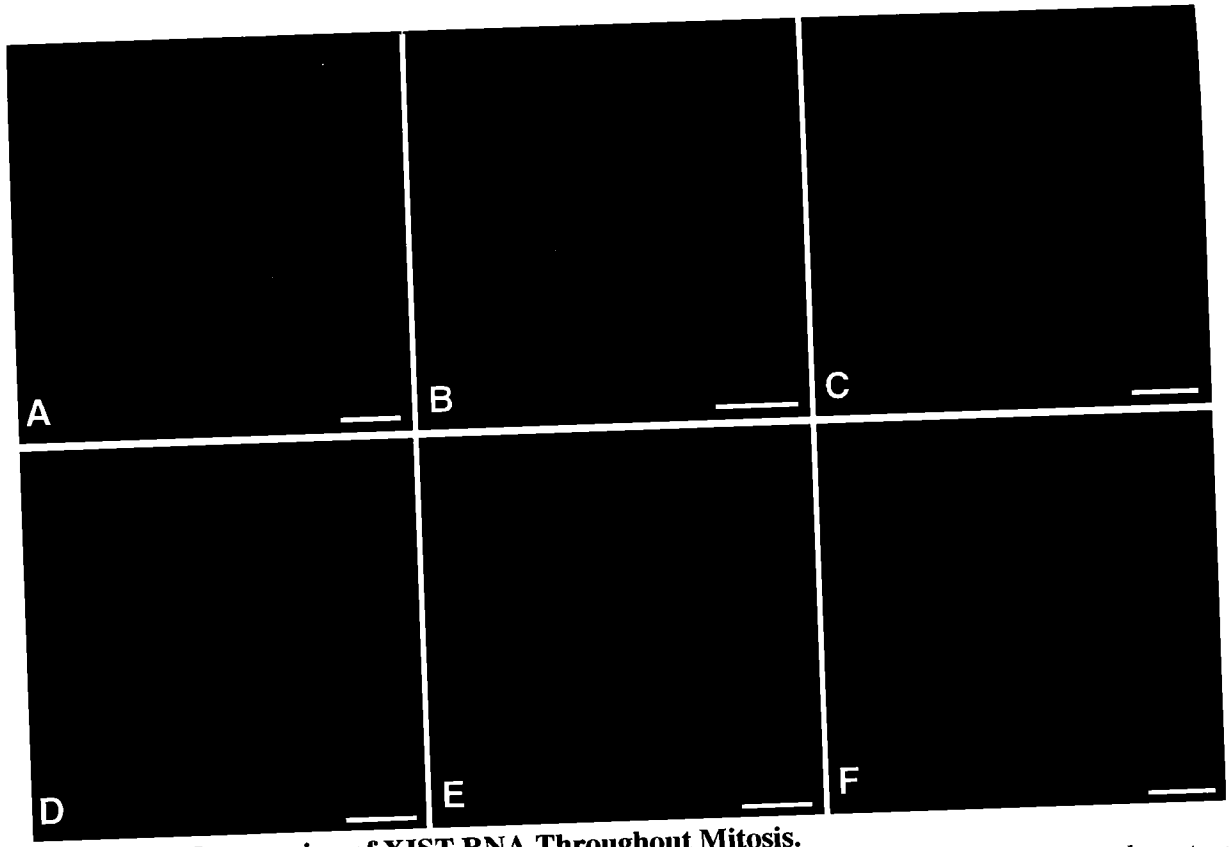


Figure 1–9. Progression of XIST RNA Throughout Mitosis.
The unique behavior of XIST RNA during mitosis is illustrated in cells ranging from prophase to early G1, and compared to collagen RNA. Results were reproduced with both digoxigenin or biotin–labeled *XIST* probes in fibroblasts and amniocytes prepared with 4 different fixation conditions (see Materials and Methods). Nuclei were counterstained with DAPI (*blue*). (A) For comparison, we examined collagen RNA (*green*) under identical conditions and observed no signal associated with the chromatin and diffuse, non–particulate signal in the cytoplasm, similar to results for other protein coding mRNAs. (B) At prophase the punctate nature of the XIST RNA (*green*) becomes more apparent as the clusters break up and spread across the chromatin and into the cytoplasm in metaphase (*C–red*) to anaphase (*D–red*). (E) At telophase the XIST RNA clusters (*red*) appear to concentrate between the cleavage plane. (F) Finally, at early G1, as evidenced in these recently divided daughter cells, the red XIST RNA clusters can still be detected in the cytoplasm as a small focus of RNA again appears in the nucleus. Bars, 5 um.

as previously described for bulk poly(A) RNA in mitotic cells (Carter et al., 1991). Hence, the presence of distinct clusters of RNA and their movement through mitosis is unique for XIST RNA compared to other, protein-coding RNAs.

Additional experiments were done in an attempt to address whether XIST RNA reenters the nucleus after mitosis or whether accumulation of XIST RNA in the subsequent interphase requires new RNA synthesis. Cells were synchronized, reversibly arrested in metaphase, released into actinomycin D-containing media, and fixed at a timepoint in which the bulk of the population was in early G1 (see Materials and Methods). XIST RNA was not detected in the nucleus of these cells, although there was still signal detectable in the cytoplasm. In contrast, in uninhibited early G1 cells, there were very small foci of nuclear XIST RNA detectable in many cells (data not shown). These results do not support the idea that XIST RNA re-enters the nucleus after mitosis. However they do not conclusively eliminate this possibility either, since other explanations are possible; e.g. inhibition of transcription may interfere with the transport of XIST RNA back into the nucleus, as suggested by previous work indicating that the processing and transport of hnRNA is altered by high concentrations of actinomycin D (Levis and Penman, 1977; Pinol-Roma and Dreyfuss, 1991).

XIST RNA is Retained after Chromosomal DNA Digestion during Matrix Preparation

A structural association of XIST transcripts in the nucleus could be via protein or nucleic acid interactions, or both. To investigate if the RNA is attached along the length of

the X chromosome by hybridization to DNA, RNase H digestion which specifically degrades RNA:DNA hybrids (Minshull and Hunt, 1986) was performed. In preparations treated with RNAase H prior to hybridization, the nuclear XIST RNA signal remained abundant. In contrast the XIST RNA signal was completely abolished in control cells treated with RNAase H after hybridization with the *XIST* DNA probe. This suggests that a large proportion of the RNA is not involved in an RNA-DNA hybrid (data not shown).

XIST RNA is highly resistant to detergent extraction (see Materials and Methods). To investigate whether the XIST RNA remains after removal of bulk chromatin, it was examined after biochemical fractionation procedures previously shown to remove approximately 90-95% of cellular DNA, protein and phospholipid. The insoluble fraction which remains has been extensively studied and is termed the nuclear matrix (reviewed in Berezney and Coffey, 1974; Fey et al., 1991). While some types of snRNAs and snRNPs are removed from the matrix (Vogelstein and Hunt, 1982; Zieve and Penman, 1976), most major classes of snRNAs and snRNPs as well as newly synthesized pre-mRNA are retained (for example, Fey et al., 1986b; Herman et al., 1978; Jackson et al., 1981). This is shown in Figure 1-10 (E and F), which demonstrates that foci or tracks of collagen nuclear RNA are strongly associated with the insoluble matrix. Interestingly, the fainter dispersed collagen signal often observed, which presumably represents more mature mRNA or free introns (Xing et al., 1995), is not detected in the matrix preparation. This is in keeping with previous reports of the selective matrix association of both globin pre-mRNA (Zeitlin et al., 1987) and pre-ribosomal RNA (He et al., 1990) in contrast to the release of more mature mRNA into the soluble fraction.

Since collagen pre-mRNA localizes within SC-35 domains (Xing et al., 1995), several protein and RNA components of which are selectively retained in the matrix (Blencowe et al., 1994; Gerdes et al., 1994; Huang et al., 1994; Smith et al., 1986; Spector et al., 1983), and because these regions are known to contain little DNA (Carter et al., 1991; Spector, 1990), the retention of collagen RNA after DNAse digestion and matrix fractionation is not unexpected. In the case of XIST RNA, however, the fractionation has a different significance, because rather than localize with SC-35 domains, the RNA localizes with Xi. Hence removal of chromosomal DNA and chromatin proteins from the matrix preparation impacts upon the nature of the structural relationship with Xi. As shown in Figure 1-10 (A and C), in cells treated to remove histones and DNA, the XIST RNA signal remained intense and essentially undiminished, even though the very weak DAPI staining confirmed that little DNA remained (Figure 1-10, B and D). The retention of XIST RNA is not specific to the extraction procedure as we have seen a similar result in a high salt (2M NaCl) 'halo' preparation (K. Wydner, unpublished observation). The fact that Xi DNA can be removed and XIST RNA remains shows that XIST RNA is not localized by virtue only of binding to Xi DNA or chromatin. Although XIST RNA closely associates with Xi and is confined to the same nuclear territory (Figures 1-2, 1-3 and 1-4), these results together with the above results on mitosis indicate that it is not necessarily an integral component of the chromatin or chromosome itself. The RNA apparently binds or comprises insoluble nuclear material and could, for example, serve as a bridge between the chromatin and other nuclear components involved in chromatin/chromosome packaging. These results, in conjunction with its unique nuclear abundance, localization, stability, constitution and lack of coding

potential, implicate XIST RNA as the first specific RNA whose primary function may be structural, and a likely component of the non-chromatin nuclear substructure or matrix.

Two models for the higher-level organization of Xi and its potential relation to XIST RNA, consistent with the collective body of data presented in this work, is provided in Figure 1-11 and considered in the discussion.

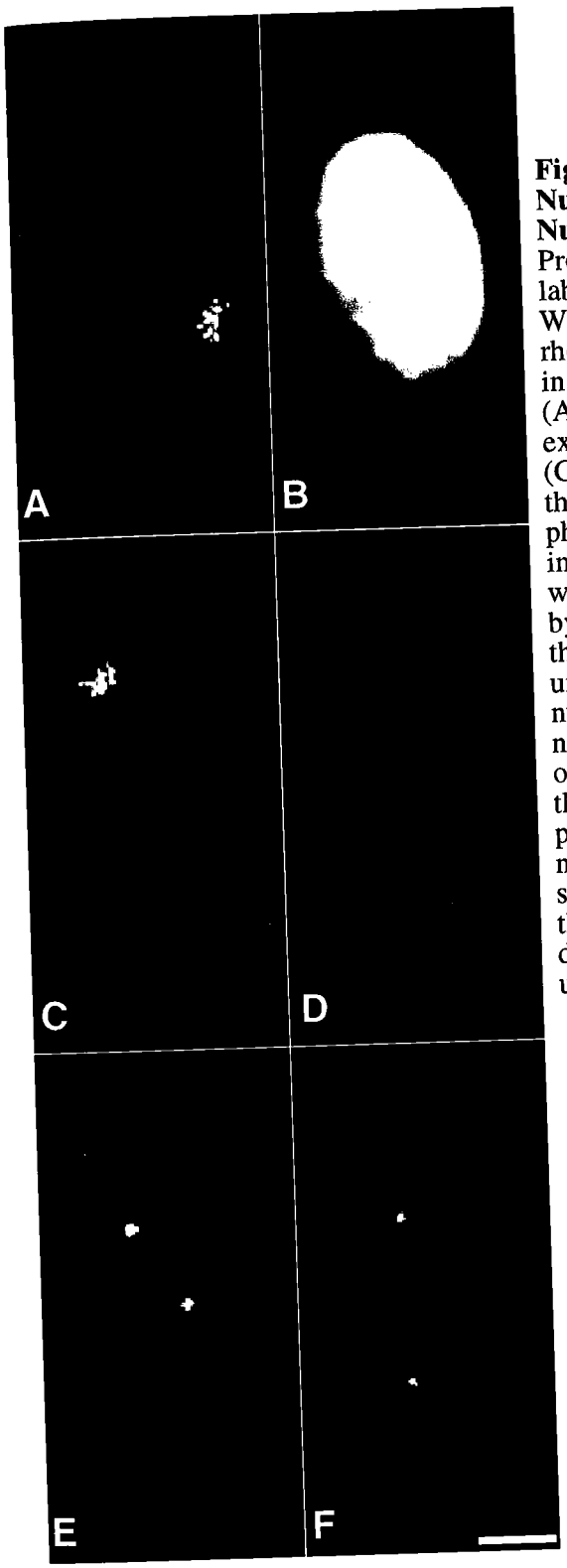


Figure 1–10: Fluorescent Detection of Nuclear RNA Within Intact Cell and Nuclear Matrix Preparations.
Probes for XIST and collagen RNA were labeled with digoxigenin, hybridized *in situ* to WI–38 cells, and hybridization detected with rhodamine anti–digoxigenin. (A) XIST RNA in an intact cell. (B) DAPI image of nucleus in (A). (C) XIST RNA signal in a nuclear matrix extracted cell. (D) DAPI signal of nucleus in (C). Note, the DAPI signal was so diminished that the intensity was artificially increased for photography. For quantitation the DAPI intensity of 18 unextracted and extracted nuclei was measured, the DAPI signal was diminished by 98% after nuclear matrix extraction. Notice that the RNA signal remains largely undiminished, despite extensive digestion of nuclear DNA. (E) Collagen RNA signal in a non–fractionated cell. The bright foci or tracks of collagen RNA are prominent. The images in this figure were enhanced to highlight the presence of weak dispersed signal in the nucleus and cytoplasm. (F) Collagen RNA signal after nuclear matrix extraction. Notice the bulk of the dispersed signal is no longer detectable while the bright nuclear foci remain undiminished. Bar, 5 um.

Discussion

This work provides insight into the mechanisms governing dosage compensation and X-inactivation in mammalian females, and potentially provides a specific precedent for RNA involvement in nuclear/chromatin packaging. Given extensive evidence implicating the *XIST* gene in X-inactivation, it was surprising when the large poly-adenylated RNA was found to lack a convincing open reading frame (Brockdorff et al., 1992; Brown et al., 1992). However, this finding makes sense in light of results presented here which support a direct role for the RNA with the inactivated interphase chromosome, but not the active homolog. To address the potential role of the RNA, it was critical to distinguish whether it localizes near Xi because it is transcribed and processed there, as expected, or whether the mature RNA has a more extensive, stable relationship to all or most of Xi (Brown et al., 1992). A collection of results presented here support the latter hypothesis, however the single most compelling is the demonstration that XIST RNA consistently "paints" the whole inactivated interphase chromosome, delineating the same nuclear territory. The findings that after transcriptional inhibition spliced XIST RNA remains and frequently circumscribes the Barr body, yet shows no association with poly A RNA/SC-35 rich domains implicated in splicing, further supports that this apparently long-lived nuclear RNA functions via a morphological relationship with Xi. Such a striking, extensive, and unique spatial organization of stable XIST RNA specifically with the heterochromatic interphase chromosome strongly supports a role for the RNA itself directly in X-inactivation and points to a mechanism involving RNA in chromatin/chromosome packaging.

The cis-limited global inactivation of genes on an entire chromosome is a novel phenomenon of vast biological and clinical significance which likely requires novel mechanisms, such as that suggested here. However, other possible explanations of these results should be considered. It cannot be ruled out entirely that the RNA encodes a small peptide (Hendrich et al., 1993); however this would not account for the RNA's unique spatial relationship to Xi, accumulation in the nucleus rather than the cytoplasm, the large size of the mature RNA, and the fact that even the small ORF's are not conserved between man and mouse, whereas a non-coding tandem repeat sequence is (Brockdorff et al., 1992; Brown et al., 1992). These observations, together with evidence that shows that *Xist* is both necessary and sufficient for X-inactivation, the imprinting of gene expression only from Xi, and the abundance of nuclear RNA, also argue against *XIST* being an expressed pseudogene uninvolved in X-inactivation. The possibility that *XIST* transcription is required for, or coincidental with, some other locus in the XIC region (Rastan, 1994) has thus far not been supported by attempts to find other genes in that region solely expressed from Xi and again would not explain the unique morphological association of abundant XIST RNA with Xi nor its apparent nuclear stability, as documented here.

X-inactivation is generally thought to be a multi-step process including steps of marking (Xa versus Xi), initiating, spreading and maintaining the inactive state. Given the critical need to avoid loss of dosage compensation by reversion of genes on Xi, the integrated roles of several factors may cooperate to maintain an inactive state and render it exceptionally stable *in vivo,* where even a low frequency of reversion could be devastating to the organism (discussed in Brown and Willard, 1994). There is an abundance of

evidence linking methylation with the inactivation status of X linked genes as many of the genes on Xi are associated with hypermethylated CpG islands (reviewed in Eden and Cedar, 1994; Grant and Chapman, 1988; Monk, 1986). However, evidence indicates that X-inactivation can occur in the absence of methylation (Driscoll and Migeon, 1990; Grant et al., 1992; Singer-Sam et al., 1992) and that methylation follows rather than precedes inactivation (Lock et al., 1987); suggesting that methylation may be important for maintenance but not establishment of X-inactivation. Additionally, the acetylation status of histone H4 has been implicated in maintenance of X-inactivation as it is acetylated on Xa but not Xi (Jeppesen and Turner, 1993). Brown and Willard (1994) have found that continued presence of XIST RNA or the XIC is not mandatory to prevent detectable reversion of genes on Xi in culture. However, *XIST*'s expression throughout the life of the organism (Kay et al., 1994; Kay et al., 1993) and the distribution of the RNA across Xi is consistent with a role in maintenance; but it may also be involved in initiation as well. Because XIST RNA is not retained on the chromosome through mitosis (Figure 1-9), the RNA cannot be the mark which distinguishes Xi from Xa through cell generations; however the *XIST* gene itself may constitute such a mark since the active and inactive copies of *XIST* are distinguished by differential DNA methylation (Hendrich et al., 1993). XIST RNA has a unique qualification as a primary component of cis-limited chromatin packaging, since it can be localized to a certain nuclear site by virtue of its synthesis, unlike protein which must enter from the cytoplasm. For example, XIST RNA's restriction to Xi versus Xa could result from imprinted transcription from Xi (Norris et al., 1994) followed

by "spreading" along the chromosome, conceivably laying the foundation for subsequent events.

XIST RNA May be a Structural Element of the Xi Interphase Territory

As illustrated here for the X, interphase chromosomes occupy discrete "territories" (Lichter et al., 1988; Pinkel et al., 1988), yet the architectural elements that constrain the DNA of a specific chromosome to a nuclear territory are not known. This is particularly important for Xi, which as demonstrated here appears smaller than its active counterpart (Figure 1-2), has a rounder morphology (Bischoff et al., 1993; Cremer et al., 1993), and has been observed to adopt a distinctive U-shape, with telomeres closely spaced (Walker et al., 1991); (also see Figure 1-2, B). We suggest that XIST RNA may be an architectural element of the chromosomal territory, possibly involved in delimiting it and defining the overall packaging of this structurally unique, largely heterochromatic chromosome. XIST RNA could possibly serve as a bridge between chromatin and other underlying structural components. Several observations suggest that XIST RNA is associated with insoluble nuclear components closely aligned with Xi, but it need not be an integral part of the chromatin itself. These include the finding that XIST RNA is clearly not a component of the mitotic chromosome and also does not appear to hybridize substantially to Xi DNA, yet is resistant to prolonged triton extraction and retained after digestion of chromosomal DNA. Imaging analysis clearly shows the RNA and Xi DNA share closely a common 3-D territory, but XIST RNA has a particulate, fine distribution unlike the distribution of Xi DNA.

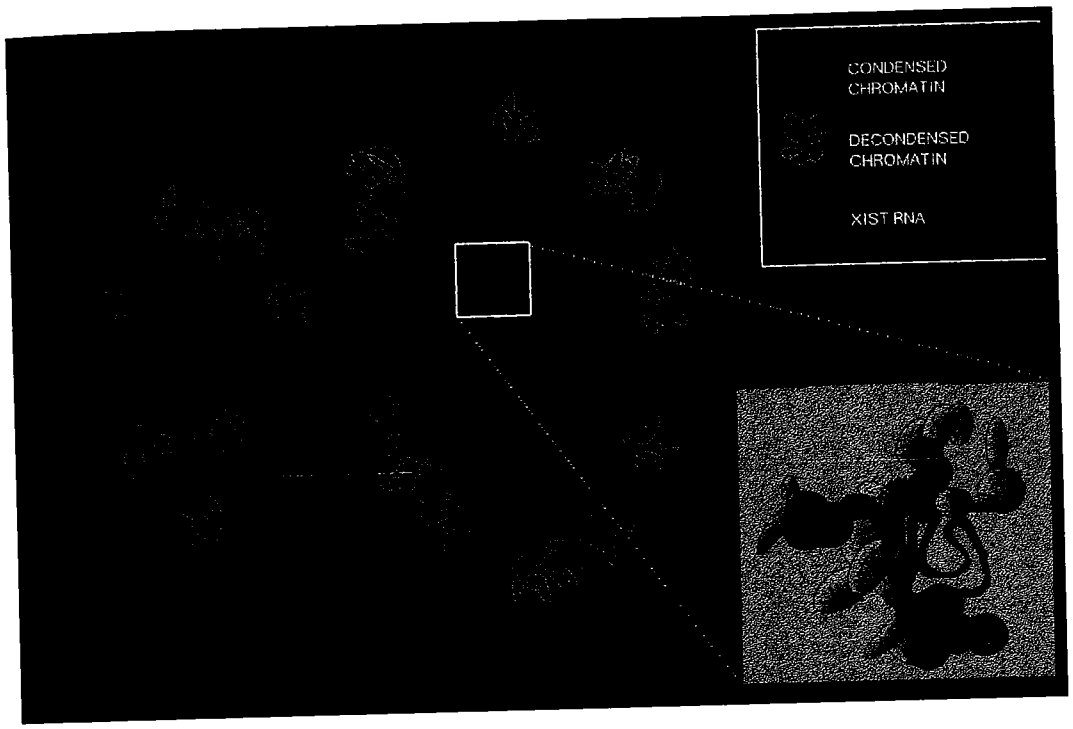


Figure 1-11: Models of Hypothetical Higher-level Organization of the Inactive X Chromosome relative to the Barr Body and XIST RNA. In two alternative views, the heterochromatic Barr body encompasses all (*left*) or a large part of (*right*) the inactivated X chromosome. In the latter case, genes which escape X inactivation would be organized at the chromosome periphery, outside of the Barr body, whereas in the model on the left the active chromatin on Xi is interspersed rather than cytologically separate from the body of the heterochromatic chromosome. The XIST RNA is shown clustered, to reflect the fact that the unusually large bright particles formed by the RNA are most likely composed of clusters of more than one mRNA molecule. In the model proposed on the left, XIST RNA associates with the heterochromatic part of the chromosome, although its association with the entire interphase territory would also be possible. The inset is intended to convey that the clusters of XIST RNA may function in higher-level chromatin packaging by serving as a junction or bridge with other nuclear components involved in nuclear structure, e.g. nuclear matrix (*gray*).

In Figure 1-11, aspects of these results are incorporated into two testable models for the global organization of Xi and the Barr body, and their relationship to XIST RNA. Based on these results, we do not propose that XIST RNA has a one to one stoichiometry with Xi DNA and blocks access to polymerases at each site by its direct binding, a model which would also not be supported by estimates of the amount of XIST RNA within the cell (Buzin et al., 1994). Rather, we favor a model in which the XIST RNA has a role in higher level packaging of the chromatin and chromosome, impacting upon gene expression. As illustrated in the inset to Figure 1-11, XIST RNA need not directly bind all along the DNA; and the apparent propensity of this RNA to cluster may be important to for function; i.e. clusters of XIST RNA molecules (red) may distribute close to Xi chromatin (blue) and possibly form junctions with other non-chromatin nuclear components involved in interphase chromosome architecture (gray). The change in higher-level packaging of Xi is evidenced by the cytologically obvious heterochromatic Barr body and the unusual shape of the interphase chromosome. Preliminary results suggest that the Barr body does not encompass the entire X chromosome. It will be important to rigorously determine if the Barr body represents a subset of Xi and whether XIST RNA associates with just the Barr body or the entire Xi. Adequate investigation of this will require precise definition of the Barr body and localization of specific active and inactive genes relative to the Barr body and XIST RNA. The highly conserved tandem repeat (Brockdorff et al., 1992; Brown et al., 1992; Hendrich et al., 1993) may be incorporated into this model as localizing the XIST RNA to Xi and maintaining its cis-limited restriction much like the homologous sequence

in xlsirt RNA is responsible for localizing RNAs in Xenopus laevis oocytes (Kloc et al., 1993).

XIST RNA Localizes Separately from SC-35/poly A RNA Rich Domains

Even though most of the XIST RNA apparently represents structural RNA that is stable after transcriptional inhibition, it consistently avoids poly A RNA rich SC-35 domains. This result contrasts with the interpretation that poly A RNAs which remain after transcriptional inhibition preferentially localize to these domains (Huang et al., 1994). In keeping with a role in chromatin packaging, XIST RNA is clearly not a structural component of these regions that are implicated in some aspect of splicing. In fact, despite clear evidence that RNAs containing splice junction sequences have an affinity for these regions (Wang et al., 1991), XIST RNA, which undergoes splicing (Brown et al., 1992), remains restricted to a nuclear territory devoid of these large discrete domains enriched in splicing factors. Although the XIST RNA is polyadenylated and forms a large nuclear accumulation, it is unlikely that it exists in a high enough quantity to be detectable as a discrete "domain" (Carter et al., 1991) with an oligo dT probe; additionally, evidence indicates that polyA domains represent more than transcripts and splicing factors associated with individual pre-mRNAs (Xing et al., 1995). Therefore it is doubtful that XIST RNA would form its own polyA domain. Since there are lower levels of both polyA RNA and splicing factors throughout the nucleoplasm (Carter et al., 1993; Carter et al., 1991; Spector, 1990), it is likely that splicing of this RNA is effected by low levels of splicing components that do not form visible 'domains'.

We have found that some pre-mRNAs show a highly non-random association with poly A RNA/SC-35 rich domains (Xing et al., 1993a; Xing et al., 1995). The finding that the active *XIST* gene is not associated with SC-35 illustrates the important point that transcription and processing for some genes does not require nor correlate with large spatial accumulations of SC-35. This is in keeping with uridine labeling studies which show transcription occurs throughout the nucleoplasm (for example, (Fakan and Bernhard, 1973; Fakan and Puvion, 1980). However, as illustrated here, much of this transcription may not be for messenger RNA and could represent longer-lived structural species. Furthermore, the fact that this intron-containing RNA does not co-localize with SC-35 domains, supports the idea that localization with these domains is gene specific and not simply the accumulation of splicing components with accumulated pre-mRNA, (discussed in Lawrence et al., 1993; Xing et al., 1995). Since XIST RNA is coincident with Xi and is not associated with SC-35 domains, this raises the possibility that most or all Xi genes could be sequestered in a region of the nucleus away from these domains enriched not only in splicing factors but hyperphosphorylated RNA polymerase II (Bregman et al., 1995). It is tempting to speculate that, if so, this could potentially contribute to a variable or reduced expression of genes which escape inactivation from Xi relative to Xa, as has been suggested (Disteche, 1995).

XIST May Provide a Precedent for RNA Involvement in Chromatin Packaging

The implications of these findings may well extend beyond X-inactivation. Much evidence shows that the bulk of hnRNA is non-polyadenylated and turns over within the

nucleus with no known function; whereas at least the vast majority of poly A RNA encodes pre-mRNA (Harpold et al., 1981; Herman et al., 1978; Lewin, 1990; Salditt-Georgieff et al., 1981). Some studies have suggested a fundamental role of RNA in nuclear architecture (for example Nickerson et al., 1989). Surprisingly, Hogan et al. (1994) found that the poly adenylated Hsr-Omega-n transcript remains in the nucleus, though no functional role has yet been described. Hence XIST and Hsr-Omega-n transcripts provide precedents and direct evidence that some subfraction of poly A RNA does not encode pre-mRNA.

The XIST RNA could represent a specialized case of structural nuclear RNAs, more widely involved in nuclear structure. This could be analogous to DPY-27, a protein involved in X chromosome down-regulation in C. elegans, which has been shown to be a specialized member of a family of proteins involved in generic chromatin condensation (Chuang et al., 1994). Although speculative, there are hints from other work that specific RNAs may be more broadly involved in chromatin organization. For example, the *maleless* protein which binds to and up-regulates transcription of the Drosophila X chromosome has homology to an RNA helicase (Kuroda et al., 1991); in addition, non-coding germline transcripts from the immunoglobulin gene locus appear to be necessary for class switch recombination of that locus to occur (Lutzker and Alt, 1988; Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos et al., 1986) . As with *XIST*, we suggest that RNA may be uniquely qualified for *cis*-limited gene control, since it can be localized to its site of function via clonal or imprinted expression from just one homolog, a phenomenon now know to occur for several genes (reviewed in Pfeifer and Tilghman, 1994).

Work presented here provides a foundation for much future work required to clearly establish and precisely define the mechanism of XIST RNA's role in X-inactivation and to explore the potential ramifications of RNA involvement in chromatin packaging within the nucleus.

CHAPTER II: XIST RNA STABILIZATION AND LOCALIZATION IS NOT SUFFICIENT FOR X- INACTIVATION IN SOMATIC CELLS

Abstract

These studies address whether XIST RNA is properly localized to the X chromosome in somatic cells where *XIST* expression is reactivated but fails to result in X-inactivation. Despite a nuclear RNA accumulation of normal abundance and stability, XIST RNA does not localize to reactivated or *inactive* human X chromosomes in mouse/human hybrid cells. The XIST transcripts are fully stabilized despite their inability to localize, hence XIST RNA localization can be uncoupled from stabilization, indicating that these are separate steps controlled by two distinct mechanisms. Mouse Xist RNA tightly localized to an *active* X chromosome demonstrating for the first time that the active X chromosome in somatic cells is competent to associate with Xist RNA. These results imply that species specific factors apparently present even in mature, somatic cells that do not normally express *Xist,* are necessary for localization. When Xist RNA is properly localized to an active mouse X chromosome, inactivation of the X chromosome does not result. Therefore, there is not a strict correlation between *Xist* localization and chromatin inactivation; moreover, expression, stabilization and localization of Xist RNA are not sufficient for X-inactivation.

Introduction

Recently it has been demonstrated that *Xist* is necessary for X-inactivation during development as mouse X chromosomes deleted for *Xist* failed to inactivate (Marahens et al., 1997; Penny et al., 1996). Transfection of ES cells further showed that *Xist* is sufficient for X-inactivation as an *Xist* containing cosmid or YAC introduced onto an autosome causes the chromosome to manifest signs of inactivation (Herzing et al., 1997; Lee and Jaenisch, 1997).

The transcript from *XIST* is a large untranslated RNA, which we have shown to have a unique and highly specific location in the nucleus (see Chapter I and Brown et al., 1992; Clemson et al., 1996). Unlike other nuclear RNAs, the bulk of the signal is fully processed message that is stably maintained in a specific site in the nucleus, and is not detected in the cytoplasm. This abundant message maintains a tight association with the X chromosome, mirroring its shape and size as if 'painting' the chromosome (see Chapter I and Clemson et al., 1996). When *Xist* is expressed ectopically through transfection of ES cells, the RNA shows the same association with autosomal chromatin after differentiation, which subsequently exhibits hallmarks of inactivation such as heterochromatinization, loss of acetylated histone H4 antibody staining and inactivation of sequences close to the insertion site (Herzing et al., 1997; Lee and Jaenisch, 1997). The apparent structural association of *XIST/Xist* with the inactive X chromosome (Clemson et al., 1996; Lee and Jaenisch, 1997; Lee et al., 1996) coupled with the evidence revealing *Xist's* importance in the process of X-inactivation (Herzing et al., 1997; Lee and Jaenisch, 1997; Lee et al., 1996; Penny et al., 1996) suggests that this RNA is a functional nuclear RNA that is most likely contributing to,

by the act of its association, inactivation of chromatin. As such, it is predicted that the precise localization of XIST RNA is critical for its function.

Although a spate of recent evidence has clearly demonstrated the absolute requirement for *Xist* in X-inactivation during mouse development, studies indicate that its human counterpart, *XIST*, is not essential for X-inactivation in cultured somatic cells. Brown and Willard (1994) showed that *XIST* expression is apparently not necessary for maintaining X-inactivation in mouse/human somatic cell hybrids, and Rack et al. (Rack et al., 1994) showed that inactive X chromosomes in human leukemic cells which had lost the X-inactivation center maintained their inactivation. These results are surprising in light of the fact that XIST RNA is constitutively expressed in differentiated female cells, suggesting that it is functioning in the maintenance of chromosome inactivation. Two recent papers show that expression of XIST RNA is not sufficient to cause inactivation (Hansen et al., 1998; Tinker and Brown, 1998). In these reports rodent/human somatic cell hybrids retaining the human active X chromosome were treated with several rounds of 5-azadeoxycytidine (5azadC), a demethylating agent, to create clones that reactivated the dormant *XIST* gene. Using mouse/human hybrids, Tinker and Brown analyzed eight X-linked genes by RT-PCR for expression, with none of the genes showing signs of inactivation as a result of *XIST* expression, while Hansen et al. reactivated *XIST* expression in hamster-human hybrids, and detected no silencing of two X-linked genes. These results contrast with those that show that forced *Xist* expression in differentiating male ES cells and developing mice embryos, caused by mutant methyltransferase, results in silencing of X-linked genes (Panning and Jaenisch, 1996).

The requirement for *Xist* in X-inactivation in developing mice embryos coupled with the precise spatial positioning of the RNA to inactivated chromatin (see Chapter I and Clemson et al., 1996; Lee et al., 1996; Panning and Jaenisch, 1996), strongly suggests that there is a correlation between XIST RNA binding and inactivation of chromatin; however, it is not known conclusively whether *Xist* localization results in X-inactivation. Somatic cell hybrids where *XIST* is apparently neither necessary (Brown and Willard, 1994), nor sufficient (Tinker and Brown, 1998) for X-inactivation allow us to rigorously investigate whether association of XIST RNA with the X chromosome corresponds with transcriptional silencing. Interestingly, our results reveal that the human XIST RNA shows aberrant positioning in mouse/human hybrid cells that express the normally dormant *XIST* gene by means of demethylation, in that the RNA is more disperse and spread across the entire nucleus. Even in hybrids that contain a naturally inactive human X chromosome, the XIST RNA does not show the normal tight localization to the X chromosome. These results suggest that the impotence of XIST RNA in this system may be due to a failure of the RNA to localize. Furthermore these results imply that the ability to localize to the chromosome is not an innate feature of the XIST RNA, but is afforded by specific factors that are not fully substituted by mouse homologues. We also show that when the RNA is not restricted to the chromosome, it is still found as a large and stable entity, indicating that stabilization and localization of XIST RNA are uncoupled. Using demethylation, we then forced expression of Xist RNA from the active *murine* X chromosome and examined the relationship of the RNA to the X chromosome. Unlike its human counterpart, the murine Xist RNA strictly localized to the X chromosome in the mouse background. Since the reactivated Xist RNA

correctly localized in a somatic cell, we conclude that its association to the X chromosome does not have to occur during development to mark the chromosome for Xist RNA binding. Furthermore, Xist RNA does not discriminate between active and inactive chromatin as it paints the active X chromosome completely. Additionally this result shows that the factors required to deposit the RNA at the X chromosome are present and available in terminally differentiated cells that do not normally express *Xist.* Finally, we show that when Xist RNA is properly localized to an active mouse X chromosome, inactivation of the X chromosome does not result. This important result clearly demonstrates a lack of correlation between Xist RNA localization and inactivation, and also indicates that removed from the normal developmental context, even with proper expression, stabilization and localization, *Xist* is not able to cause X-inactivation.

Materials and Methods

Cells, cell culture and treatment with 5-azadeoxycytidine

Derivatives of the AHA-11aB1 active X chromosome-containing human/mouse somatic cell hybrid which expressed human XIST RNA were isolated after treatment with successive rounds of the demethylating agents 5-azadeoxycytidine or 5-azacytidine (Tinker and Brown, 1998). To create additional hybrid subclones which expressed the murine *Xist* gene, AHA-A5-2b cells (which stably expressed human *XIST* from the active X chromosome) or subclones which had undergone one round of 5-azadeoxycytidine treatment but did not stably express human *XIST* were plated at 10^4 cells/60 mm culture dish and allowed to attach for 4-6 hours, before being treated with 5-azadeoxycytidine (0.2 ug/ml) or 5-azacytidine (4 uM) for 24 hours. Individual colonies were isolated by trypsinization in cloning cylinders and plated in 60 mm dishes. Cells were grown to confluence, subcloned and RNA was isolated for RT-PCR examination of *Xist* expression using primers MX23b and MIX20 (Kay et al., 1993). Reverse transcription was as described previously (Brown et al., 1990). *Xist* positive clones were grown for *in situ* hybridization (see below) and were subjected to one to four additional rounds of treatment with demethylating agents. Cells were maintained in alpha-MEM with 7.5% fetal calf serum, supplemented with penicillin/streptomycin (Gibco/BRL).

Cell Preparation for *in situ* hybridization

Our standard cell fixation has been described previously in Chapter I and (Lawrence et al., 1989a) and therefore will be summarized briefly here. Monolayer cells grown on glass coverslips were extracted with 0.5 % triton X-100, 5% Vanadyl Ribonucleoside Complex (VRC - Gibco/BRL) to preserve RNA, in CSK buffer (Fey et al., 1986a) for 2 minutes on ice. Cells were then fixed in 4% paraformaldehyde for 10 minutes at room temperature and then stored in 70% ethanol at 4°C.

DNA Probes

For fluorescence *in situ* RNA hybridization the following probes were used: Human *PGK1*: a genomic lambda clone for *PGK1* isolated from the ATCC X chromosome library LAOXNL01. Human *XIST*: XIST G1A, a ~ 10-kb genomic plasmid spanning from the 4th intron to the 3' end of the human *XIST* gene; and pXISTHbC1A, a 1.6 kb cDNA clone wholly contained within exon 1 of *XIST*. Mouse *Xist*: a genomic clone spanning exons 5 and 6 (generously provided by Barbara Panning) (Panning et al., 1997); mouse *Zfx* : PDP1115, a 6.8 kb cDNA obtained from ATCC (cat. # 63069); mouse *Pgk1* a 1.8 kb cDNA clone from ATCC (cat # 57222)

In situ hybridization and detection

Hybridization and detection was performed as described previously in Chapter I and (Johnson et al., 1991; Lawrence et al., 1988; Xing et al., 1993b). Simultaneous detection of

whole X chromosome library and RNA was done as described previously (see Chapter I and Clemson et al., 1996) with minor modifications. Cells were extracted and fixed as described in Chapter I. The cells were then denatured at 80°C for 5 min in 70% formamide, 2X SSC and immediately dehydrated through a ice cold ethanol series. The Total Chromosome Library Coatasome X-digoxigenylated (Oncor) was used for human chromosome detection, while biotinylated Mouse Chromosome X Paint Probe (Oncor), was used for mouse X detection. 10 ul of the whole chromosome probe mixture was denatured for 10 min at 75°C, and allowed to preanneal for 30 min at 37°C. Samples were then hybridized with the probes overnight at 37°C. Posthybridization washes, detection and postdetection washes were performed as described in Chapter I.

XIST Stability

To determine the stability of the XIST RNA in hybrid and lymphoblast cells, recently expanded cultures were treated with 2 ug/ml actinomycin in DMSO. Control cultures received only the DMSO. Whole cell RNA was harvested by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and reverse-transcribed as above. cDNA was diluted 1/100 and RT-PCR was performed with primers for *XIST* (Duncan et al., 1993) and actin (Chariot and Castronovo, 1996). PCR was demonstrated to be in a linear range of amplification, then products were quantitated with NIH Image on scanned images of ethidium bromide stained agarose gels. To determine the half-life of the *XIST* message the ratio of *XIST* product to actin product was divided by the average ratio of *XIST* to actin in the DMSO treated samples. The half-life of actin cytoplasmic mRNA has

previously been estimated at 25 hours (Sympson and Geoghan, 1990), and therefore should not have varied substantially over the 8 hour time period used here. Determinations of the XIST RNA stability by hybridization of slot blots yielded similar estimates (not shown).

For quantitation of relative amounts of XIST RNA, hybridization of slot blots was performed as described previously (Brown et al., 1991a). Briefly, total cellular RNA was prepared using the guanidinium thiocyanate method. 5 ug of RNA from an inactive X containing somatic cell hybrid (Willard et al., 1993) and the AHA-11aB1 active X-containing hybrid as well as its demethylated $XIST^{+}$ derivative was denatured in formaldehyde/formamide and transferred to a nitrocellulose membrane. The blots were hybridized with ^{32}P labeled XIST14A cDNA probe (Brown et al., 1991a). To control for loading equal amounts of the RNA, the blots were stripped and reprobed for the MIC2 gene.

Imaging

All images were captured with a Zeiss Axiophot microscope equipped with Zeiss 100X 1.4 NA Plan ApoChromat objective with a 2.5X photo eyepiece and multi-bandpass epifluorescence filters (Chroma, Brattleboro, Vt) . Images were recorded with a CCD camera (200 series; Photometrics, Inc., Tucson, AZ) with a pixel size of 19 uM and a 14 bit A/D converter (data acquisition system by G.W. Hannaway and Associates, Boulder, CO).

Results

Human XIST Transcripts in Hybrid Cells Show Abnormal Nuclear Localization

In other recent work (Tinker and Brown, 1998), subclones of AHA-11aB1 mouse/human somatic cells (AHA hybrids) were treated with 3 rounds of demethylation, each of which involved culturing cells for 24 hours with 5azadC. After removing the 5azadC and culturing cells in normal media, many clones were isolated that stably expressed RNA from the normally dormant *XIST* gene on the active human X chromosome. Subsequent RT-PCR analysis showed that none of eight X-linked genes examined were inactivated as a result of *XIST* expression from the active X chromosome. As it was possible that some of the cells in the population examined might have lost *XIST* expression and that the gene expression detected by RT-PCR could have been derived from this subset of cells, here we used *in situ* hybridization to confirm the lack of inactivation at a single cell level. Using genomic probes to the human genes, we examined expression of PGK-1 and XIST RNA in the AHA-A5-2b mouse/human hybrid cells, that contain a *human* X chromosome that expresses RNA from the hypomethylated *XIST* gene. PGK-1 RNA was detected in the majority of the cells that also expressed XIST RNA, confirming the PCR data from Tinker and Brown that the X chromosome is not inactivating as a result of reactivated *XIST* expression.

To begin our analysis of the correlation between *XIST* localization and inactivation of chromatin, we examined the distribution of the XIST RNA in AHA mouse/human hybrids that had previously been identified as stable *XIST* expressers by RT-PCR. Using *in*

situ hybridization we analyzed a total of 6 different hybrid clones, and found that almost every cell in each population was expressing XIST RNA. Furthermore, the hybrids continued to show stable *XIST* expression for over 2 years after the original demethylation, confirming that transcriptional reactivation of the human *XIST* locus is quite stable (Tinker and Brown, 1998). The XIST RNA signal in the reactivated hybrid cells was similar in brightness and apparent abundance to normal diploid fibroblasts (Figure 2-1, A and D), and the particulate nature of XIST RNA, which we have reported previously in normal fibroblasts (see Chapter I and Clemson et al., 1996), is highly apparent in the AHA hybrids (2-1, D-F). However, while the distribution of the XIST RNA in normal diploid fibroblasts is discrete and highly contained in a small area in the nucleus (2-1, A), the reactivated XIST RNA in the hybrids was distinctly different (Figure 2-1, D-F) in that it was more spread out, occupying a much larger area of the nucleus. This less contained pattern revealed numerous tiny distinct particles which spread over a significant portion of the nucleus. In normal diploid fibroblasts, the XIST RNA associates closely with the inactive X chromosome, in a pattern that is similar in both size and shape (Figure 2-1, A-C see also Figure 1-2). While the dispersed pattern of XIST RNA in the hybrid cells suggested that it was not closely associating with the X chromosome, it is possible that the human X chromosome in a mouse background also occupies a broad, more disperse territory. To examine the specific relationship of the XIST RNA to the X chromosome in the AHA hybrid cells, the RNA signal was directly compared to that from a whole X chromosome library hybridization in 100 cells in three separate experiments. In >90% of the AHA reactivants, the RNA signal was obviously not strictly associated with the X chromosome (Figure 2-1, E). While there

generally was some density of XIST RNA signal over the chromosome, a large quantity of signal was usually seen spreading out away from the X chromosome.

In order to determine if the lack of localization is due to artifacts created by reactivating *XIST* expression from the active X chromosome, (e.g., the active X chromosome may be refractory to *XIST* binding), we examined the distribution of XIST RNA in t86-B1maz1b-3a mouse-human hybrid cells that retain a normal human *inactive* X chromosome (Willard et al., 1993). In over 100 cells examined in four separate experiments, the distribution of the XIST RNA in the hybrid cells was identical to that in the AHA-A5 hybrids; the XIST RNA was disperse and spreading into the nucleoplasm with no strict limitation to the X chromosome library signal (Figure 2-1, F). This result indicates that the lack of localization is not an artifact of global demethylation as these cells were not treated with demethylating agents and still show no detectable localization of the XIST RNA to the X chromosome. It also strongly suggests that the aberrant localization is not due solely to inherent differences between active and inactive X chromosomes as the human X chromosome in this cell line is an inactive X chromosome known to remain transcriptionally inert (Brown et al., 1997).

Since the signal intensities in the different cell lines seemed equivalent (Fig 2-1, A, D and F), it appeared that hybrid cells that ectopically express *XIST* produce similar amounts of RNA as do cells that express *XIST* endogenously However, it is possible that aberrations in expression from the reactivated *XIST* gene causes the mislocalization. For example, demethylation could produce an overabundance of XIST RNA, causing the transcripts to spill from the site of transcription into the surrounding nuclear space.

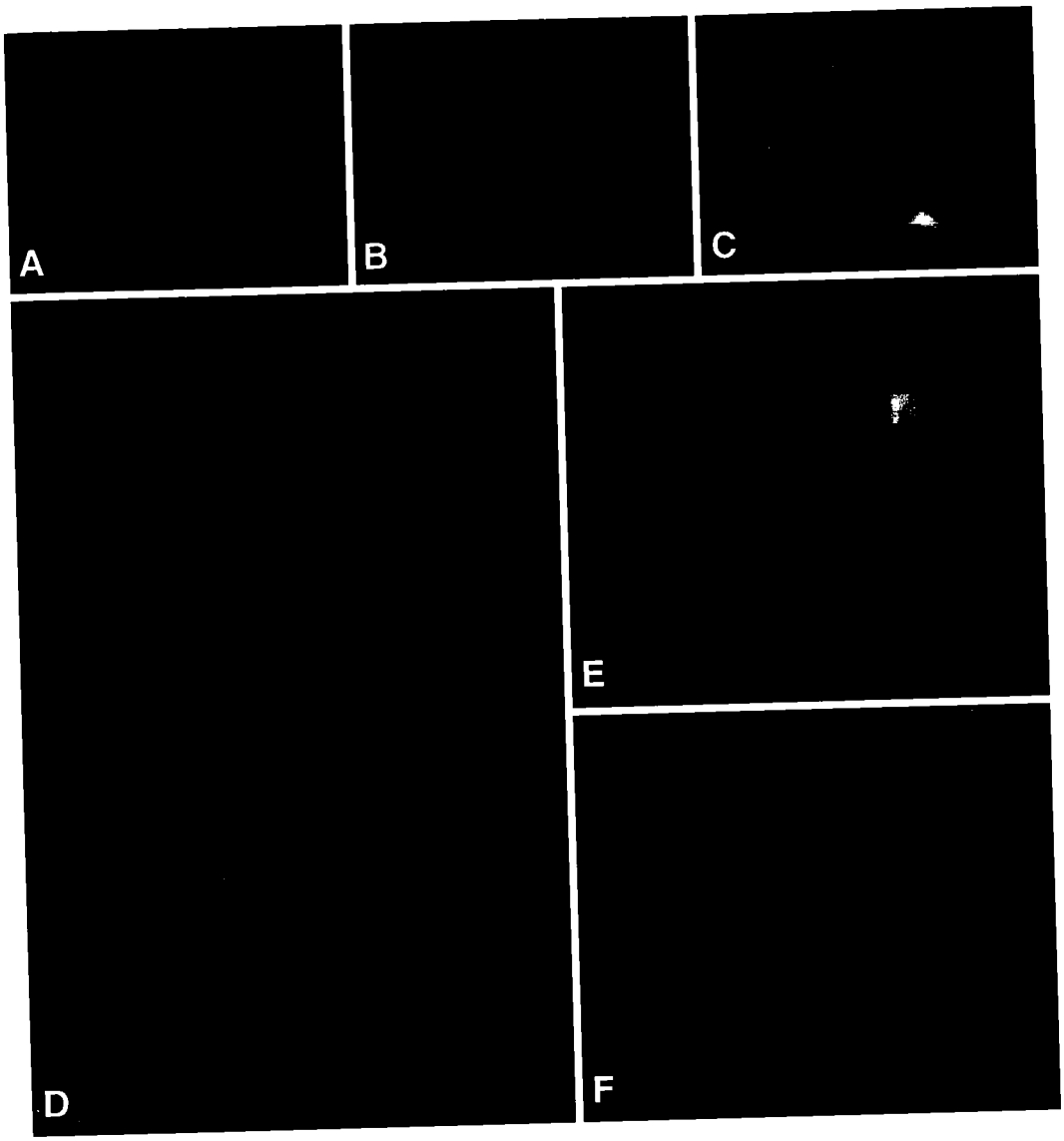


Figure 2–1: Fluorescence *in situ* hybridization detection of XIST RNA in normal human and mouse/human hybrid cells. Digoxigenin and biotinylated probes were hybridized *in situ* and detected with fluorochrome conjugated avidin or anti–digoxigenin antibody. Fluorochromes used were FITC (*green*), rhodamine (*red*) and DAPI (*blue*). A) In normal human diploid fibroblasts (46, XX WI–38 cells) the XIST RNA (*green*) occupies a discrete location in the nucleus, B) the XIST RNA has a similar shape to the X chromosome territory defined by the whole X chromosome library signal (*red*). (The weak red signal in the middle of this cell is the active X chromosome which is out of the plain of focus) C) the XIST RNA essentially paints the X chromosome in these normal nuclei (*overlap is yellow*) . D) In mouse/human hybrids containing a single *active human* X chromosome (AHA–A5) that expresses *XIST* through treatment with the demethylating agent 5azadC, the XIST RNA (*red*) is disperse and spreading throughout the nucleus. E) The XIST RNA (*red*) does not associate or strictly localize to the human whole X chromosome library signal (*green*) in the hybrid cells. F) In mouse/human hybrid cells (t86–B1maz1b–3a) that contain a single human inactive X that expresses *XIST* endogenously (no 5azadC treatment), the human XIST RNA (*red*) also shows aberrant localization to the whole X chromosome library signal (*green*).

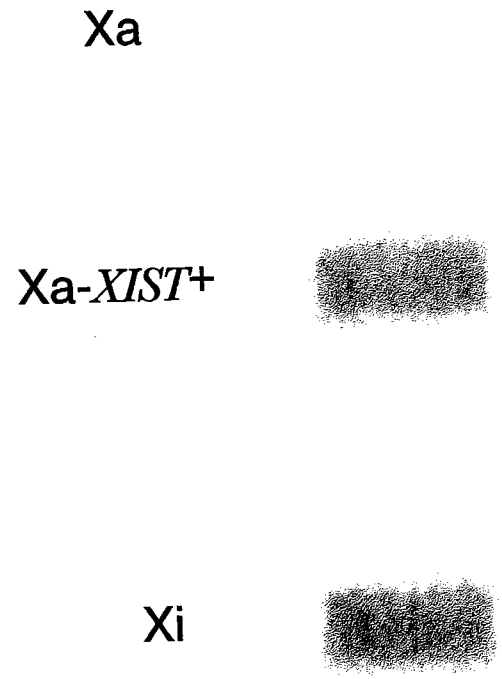


Figure 2-2: Reactivated and Endogenous *XIST* Expression is Similar in Hybrid Cells. Total cellular RNA from the following cells were transferred to nitrocellulose and hybridized with an XIST cDNA probe: AHA-11aB1, the active X-containing hybrid cells (Xa); AHA-2C-5C-9, the demethylated derivative that ectopically expresses *XIST* (Xa-*XIST*$^{+}$); and t11-4Aaz5 cells containing a human inactive X chromosome that normally expresses *XIST* (Xi). The blots were scanned and the density of signal from the Xi and Xa-XIST^{+} clones were essentially identical, demonstrating that the RNA is produced in similar quantities from endogenously expressing and demethylated *XIST* genes.

Alternatively, reactivation could produce quantities of RNA that are insufficient to paint the chromosome. This was addressed through a slot blot hybridization assay (Figure 2-2). XIST RNA from a hybrid cell line containing an active X (Xa), reactivated X (Xa-XIST$^+$), and normally expressing inactive X chromosome (Xi) were compared. No XIST RNA was detected from the active X chromosome, while a similar abundance of RNA was observed in the Xa-XIST$^+$ and Xi cell lines. This confirms the *in situ* hybridization data which suggested similar amounts and clearly shows that *XIST* reactivation by demethylation produces essentially the same levels of XIST RNA as the *XIST* gene on the 'naturally" inactivated X chromosome. This further suggests that lack of localization is not due to artifacts created by demethylation.

Stability and Localization of XIST RNA are Uncoupled

Next we examined the stability of the XIST RNA in hybrid cells, as previous results indicate that this nuclear RNA is highly stable (see Figure 1-5 and Clemson et al., 1996), and that nuclear stability is an important, developmentally regulated feature of this RNA (Panning et al., 1997; Sheardown et al., 1997). In XX somatic cells, only the inactive X chromosome expresses *XIST* (Brown et al., 1992; Clemson et al., 1996; Lee et al., 1996), and the transcripts which coat the chromosome form a nuclear accumulation much larger than other mRNAs examined (see Figure 1-1 and Clemson et al., 1996). This large XIST RNA accumulation was shown to be composed of two populations: a small focus of short-lived nascent transcripts found at the inactive X chromosome simply because it is

transcribed there, and a large, spliced and stable population that represents the mature, likely functional RNA (see Figures 1-6 and 1-7 and Clemson et al., 1996). Prior to X-inactivation in female cells or undifferentiated ES cells, XIST RNA is observed as tiny dots on both X chromosomes, or as a single dot in male cells (Lee et al., 1996; Panning et al., 1997; Panning and Jaenisch, 1996; Sheardown et al., 1997). It has been shown that during early development XIST RNA is initially expressed in an unstable form from both active and inactive X chromosomes, and becomes stabilized from the inactive X as development proceeds. The transition from single dot to large accumulation is not regulated by increased transcription, but by stabilization of the XIST transcripts in *cis* from the inactive X chromosome (Panning et al., 1997; Sheardown et al., 1997).

It is not known whether the deposition and stabilization of XIST RNA arise from a single mechanism or if separate mechanisms are involved in stabilizing and positioning the RNA. Analysis of XIST RNA in hybrids provides important insights in this regard. In all of 6 hybrid lines examined, it was clear that the XIST RNA comprised a large accumulation, with the quantity of signal indistinguishable from that observed in normal cells (Figure 2-1 and 2-2) suggesting that the XIST RNA represents a unique *XIST* intermediate, one in which the transcripts are expressed and accumulating, but not localizing to the chromosome. These results are the first to indicate that association of XIST RNA with the X chromosome does not automatically arise from the increased stability that occurs after differentiation.

To examine stability by another approach, we compared the half-life of XIST RNA after actinomycin D treatment in unsynchronized human female lymphoblasts (GM07350), where the transcripts are expected to be stable, and in the mouse/human hybrid cell line t75-

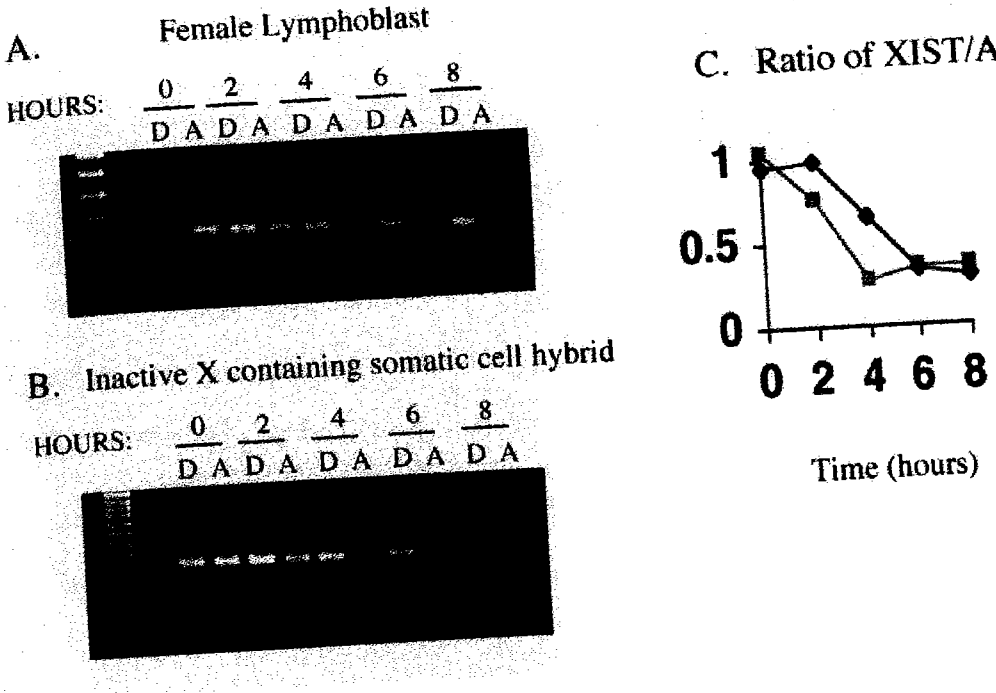


Figure 2-3: Stability of the XIST RNA after treatment of cells with actinomycin. Cells were harvested after 0-8 hours of actinomycin treatment (A) or control DMSO treatment (D) as the actinomycin is dissolved in DMSO. RT-PCR was performed on the RNA isolated from these cells with primers for *XIST* (*shown in panels A and B*) and Actin (*not shown*). The RT-PCR products for a female lymphoblast (GM07350; panel A) and an inactive X-containing human/mouse somatic cell hybrid (t75-2maz34-1a; panel B) are shown, and the ratio of *XIST* product to actin product (normalized to the ratio of *XIST* to actin for the control DMSO treatments) is plotted in panel C. The black line shows the ratio for the female cells while the grey line shows the ratio for the hybrid cell line.

2maz-34-1B that expresses *XIST* from the inactive human X chromosome. Figure 2-3 shows the results of RT-PCR with primers for human *XIST* from RNA isolated from cells after up to eight hours of actinomycin treatment. By normalizing to RT-PCR products for actin mRNA (which has a cytoplasmic half-life of approximately 25 hours (Sympson and Geoghan, 1990) the half-life of the RNA from the two cell lines could be determined. Both the hybrids and the lymphoblasts showed a half-life for XIST RNA of approximately 5 hours (Figure 2-3, A and C), which is similar to the half-life reported for stable XIST transcripts in diploid fibroblasts (Sheardown et al., 1997). The similarity of the half-life in the two cell lines confirms that the transcripts in the hybrids are stable. The similar half-lives, but very different patterns of XIST RNA localization in the nucleus strongly suggest that stabilization of XIST RNA does not arise from chromosomal localization, and vice versa. Therefore, some additional mechanism other than stability is responsible for localizing the XIST transcripts to the X chromosome.

We speculate that localization of this RNA to the X chromatin is brought about by specific localization factors that allow deposition of the human XIST transcripts along the chromosome. As there is approximately 30% sequence divergence between mouse and human *Xist/XIST* sequences (Brockdorff et al., 1992; Brown et al., 1992), it is not surprising if the homologues required for localization of Xist RNA are unable to completely substitute for the human factors. In the hopes of finding a human autosome that would allow for XIST RNA localization, and to ultimately identify potential localization factors, we examined the distribution of XIST RNA in six different hybrids containing a human inactive X chromosome. As these hybrids contained various other human chromosomes, we hoped to

identify a hybrid which contained the necessary 'human' factor to allow localization of human XIST RNA. None of the hybrids showed localized XIST RNA although all exhibited large apparently stable XIST RNA accumulations. The consistent failure of hybrid cells to correctly localize the RNA suggests that either the necessary locus was missing from these hybrids, or that more than one factor is required concurrently and that these hybrids did not have the right combination of human chromosomes. We favor the latter possibility as these hybrids have been shown by PCR or karyotyping to collectively include most of the human chromosome complement.

Reactivated Mouse Xist RNA Localizes to the Active X Chromosome

If the failure of the human XIST RNA to localize is the result of mouse/human species differences, then it follows that if the murine *Xist* were reactivated, the RNA would be properly localized as the full complement of murine factors would be present. To test this hypothesis, we isolated clones that expressed not only human XIST RNA but also mouse Xist RNA after additional rounds of demethylation. We studied the nuclear Xist/XIST RNA expression pattern in AHA-A5-2b-g cells, AHA hybrids expressing human *XIST* that had been treated with additional rounds of demethylating agents to induce *mouse* Xist expression, providing an opportunity to directly compare mouse and human XIST RNA in the same cell. The mouse cells used in the generation of these hybrids (A9) do not express Xist RNA without treatment; and these cells maintained ectopic murine *Xist* expression less consistently than human *XIST* expression (see below). In our analysis of 80 cells expressing both human and mouse *Xist*, >70% showed the following pattern of

expression: the human XIST RNA showed a small focal concentration, with particles apparently drifting away from the site of transcription into the rest of the nucleoplasm; in contrast, the murine Xist RNA signal was a large but discrete pattern that was much more confined in the nucleus than its human counterpart (Figure 2-4, A and B). We directly compared the distribution of the murine Xist signal to the mouse chromosome library signal in 50 different cells. Like the human XIST RNA in its normal human diploid environment, the murine transcripts formed a punctate accumulation very similar in size and shape to the mouse X chromosome paint, revealing a close association with the active murine X chromosome (Figure 2-4, C-E). Clearly, murine Xist RNA can properly localize in the mouse background, while in the same cell the human XIST RNA cannot.

These results support the hypothesis that the aberrant human XIST RNA location is due to a lack of species-specific factors necessary for localization. Interestingly, it is apparent that the factors required to localize the Xist RNA are present in terminally differentiated cells which normally do not express Xist RNA. This important result is informative on several other levels. First, it shows that the lack of localization of the human XIST was not due to 5 azadC treatment, as the localization of the murine Xist was observed after 5 azadC treatment; hence demethylation does not disallow localization of the transcripts. Second, the mislocalization is not due to general disruption of nuclear structure in hybrid cells. Perhaps most importantly, it shows that the XIST RNA does not discriminate between active and inactive X chromosomes. In these mouse cells the murine X chromosome has already been selected in early embryogenesis to remain active, but is

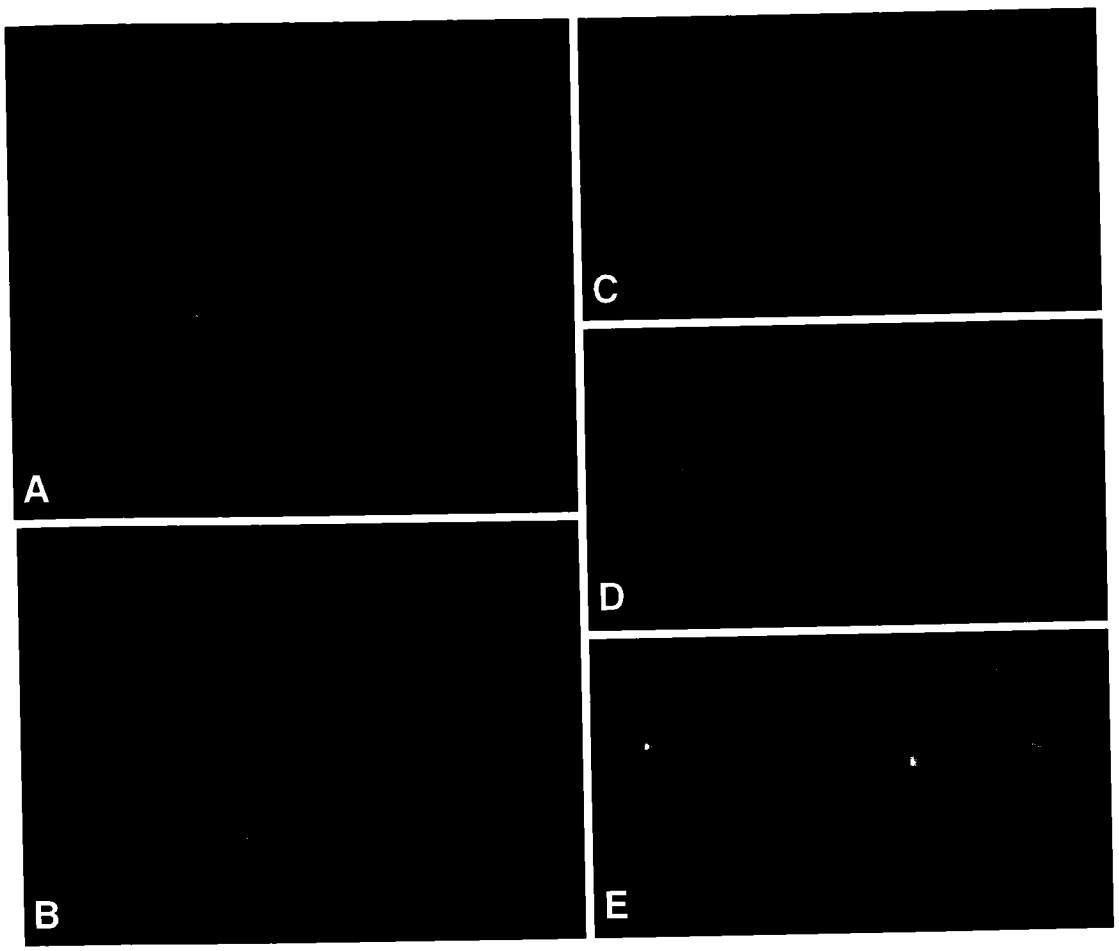


Figure 2–4: In situ RNA and murine X whole chromosome library detection in AS–2B mouse/human hybrid cells that contain both an active human and mouse X chromosome. Both human and murine Xist RNA is expressed from these chromosomes as a result of 5azadC treatment. A and B). The difference between the reactivated murine Xist RNA and human XIST RNA is illustrated in these cells, with the murine RNA appearing highly localized (red) and the human XIST RNA (*green*) showing a focus of expression (most likely representing the site of transcription), with particles that drift away into the nucleoplasm. **Reactivated murine Xist RNA localizes to the active X chromosome (C–E).** C) The murine Xist RNA (*red*) is discretely contained in the hybrid cell nucleus, D) in a pattern similar to the murine X chromosome signal (*green*) (the mouse X chromosome library cross reacts slightly with the human X chromosome, giving a second green signal apparent in the middle cell in D); E) the murine Xist RNA colocalizes with the active murine X chromosome (*overlap is yellow*).

able to stably express and bind the Xist RNA. Hence active X chromosomes are not recalcitrant to Xist RNA binding. The fact that the reactivated murine Xist RNA localizes unequivocally shows that the proper deposition of this RNA at the X chromosome can occur outside the realm of early development, and therefore, that no developmental factors are required to set up a pattern of Xist chromosome binding. Apparently all the requirements for expression, stability and localization of Xist RNA are available in somatic cells.

The Mouse X Chromosome Painted by Xist RNA Does Not Inactivate

Studies in transgenic mice have definitively demonstrated that *Xist* is not only necessary (Marahens et al., 1997; Penny et al., 1996) but also sufficient (Herzing et al., 1997; Lee and Jaenisch, 1997) to initiate X-inactivation in developing mice embryos. In our analysis, we have circumvented development by recreating, in terminally differentiated cells, several of the steps known to correlate with X-inactivation in embryonic cells: expression of Xist RNA (Kay et al., 1994; Kay et al., 1993), transition from a single dot of expression to stabilization of a large nuclear RNA accumulation (Panning et al., 1997; Sheardown et al., 1997); and deposition of these stable Xist transcripts along the entire length of the chromosome (Clemson et al., 1996). The obvious next question is: Does inactivation of the chromatin result from properly expressed, stabilized and localized Xist RNA?

While we were able to isolate stable, long term *human XIST* reactivants after three rounds of treatment with 5azadC, we were not able to isolate a clone that expressed *mouse Xist* long term, despite multiple serial treatments with demethylating agents. After selection

and culture of single cell isolates, the level of Xist RNA declined over a period of several weeks as detected by RT-PCR. By *in situ* techniques we saw that initially almost every cell in the population expressed *Xist*, then gradually the numbers of cells that expressed *Xist* declined (Figure 2-4, B), until eventually no more cells expressed Xist RNA.

While we initially thought that this reduction was due to some selective disadvantage conferred by mouse *Xist* expression, (for example the cells could be dying because the mouse Xist RNA inactivated the sole murine X chromosome), several considerations make this less likely. First, we detected no change in cell growth rate or evidence of large scale cell death in the reactivated hybrids, both of which would be expected if *Xist* expression was causing large scale toxicity. Second, we have isolated clones that continued to express mouse *Xist* in greater than 30% of the population for as long as 2 months after the initial demethylation. Additionally, we were able to subclone pure populations of Xist RNA expressors from clones that had reduced expression of *Xist* to only a few cells, which would not be expected if the cells were gradually dying. While it is possible that the reactivants were selected against, we consider it more likely that *Xist* was being remethylated gradually, much like what was seen for the unstable human reactivants (Tinker and Brown, 1998). Our failure to obtain a clone which expressed mouse *Xist* longer than several months, in contrast to the human gene, may reflect differences between the methylation of the two genes; for example the murine gene may have more methylation which must be completely removed before remethylation (and loss of *Xist* expression) is stopped. Ultimately the difficulty we had in creating long-term mouse *Xist* reactivants did not cause a problem for our experiments as any cells that showed detectable mouse *Xist*,

always showed the same large intense pattern allowing us to perform analysis of individual cells, even when as little as 10% of the population expressed Xist RNA.

The definitive way to assess whether the mouse X chromosome painted by reactivated Xist RNA is inactivated is to examine expression of individual genes in single cells. Because of the heterogeneity of *Xist* expression in our cell population, RT-PCR analysis could not address the critical question of whether individual genes were still expressed. We therefore used dual *in situ* detection of murine *Xist* with X-linked genes to examine directly X-inactivation in individual cells. *Pgk1* is subject to X-inactivation in humans and mice (Disteche, 1995), while *Zfx* is an X-linked gene that escapes inactivation in humans but is subject to X-inactivation in the mouse (Adler et al., 1991). Zfx and Pgk1 nuclear RNA expression were examined in single cells that expressed Xist RNA (Figure 2-5, A-C), using cDNA clones. In multiple experiments encompassing over 200 hybrid cells, these two genes were clearly transcribed in cells that were also expressing and localizing Xist RNA. While we were also able to detect the human RNA with the *Pgk-1* probe, we included in our analysis only those signals that were closely associated with the mouse Xist RNA signal, confirming that it was transcribed from the mouse X.

To evaluate whether the Xist RNA localization resulted in a more subtle transcriptional down regulation of the genes versus a complete silencing of the X chromosome, we compared the distribution of both Pgk1 and Zfx RNA from cells either expressing or not expressing murine Xist RNA. As exemplified in Figure 2-5 (A and C), the Pgk1 and Zfx RNA signals were not discernibly different in cells devoid of *Xist* expression versus those in which the mouse X is painted by Xist RNA, suggesting that the

murine X chromosome has not undergone detectable transcriptional down regulation as a result of *Xist* expression and localization. While we cannot rule out the possibility that, over the long term, progressive inactivation will occur, the expression of *Zfx* and *Pgk-1* was analyzed in subclones that expressed and localized murine Xist RNA for over two months. Therefore, our results show that Xist RNA can localize to the mouse X chromosome without inactivation of specific genes for many generations. We conclude that the association of Xist RNA with chromatin is not strictly correlated with transcriptional silencing. Furthermore, these results indicates that expression, stabilization and proper localization of Xist RNA in somatic cells is not sufficient to cause inactivation.

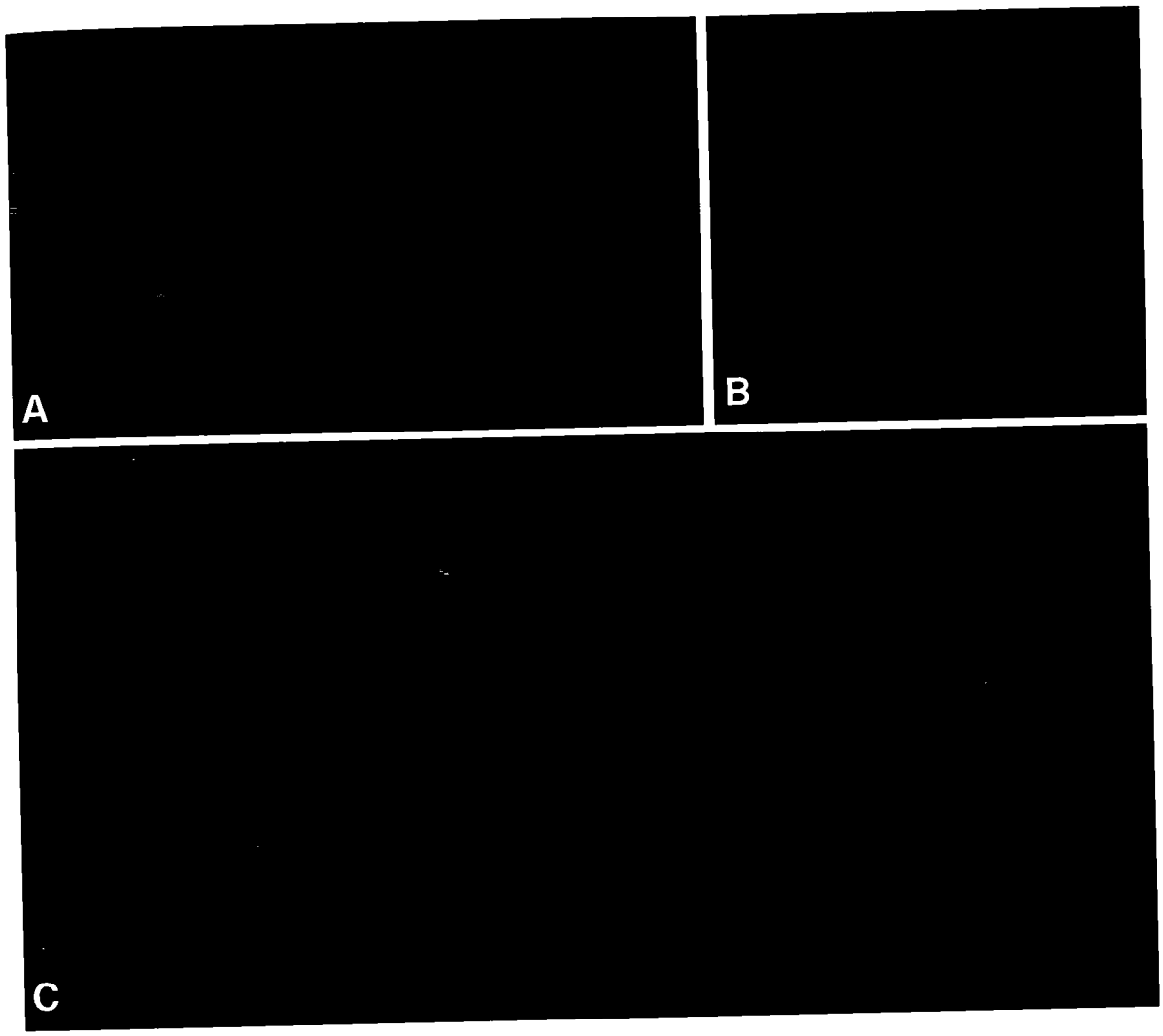


Figure 2–5: The mouse X chromosome painted by the Xist RNA does not inactivate. Codetection of murine Xist RNA (*green*) and Zfx RNA or Pgk1 RNA (*red*) in undenatured 1B–5C–3B cells. A and B) Zfx RNA (*red*) is expressed from cells that also express mouse Xist RNA (*green*), C) Pgk1 RNA expression (red) is also expressed from chromosomes that express Xist RNA (*green*), suggesting that the active X chromosome is not inactivated as a result of *Xist* localization. **Expression of mouse genes appears to be unaffected in cells that express mouse *Xist*.** A) There is a similar pattern and intensity of expression of Zfx RNA in cells that express mouse *Xist* (*left*), and those that don't (*right*). C). Similarly, there is comparable *Pgk1* expression in cells that express mouse *Xist* (*left*) and cells that don't (*far right*).

Discussion

Understanding the relationship of XIST RNA localization to X-inactivation and the mechanisms governing localization to the chromosomes is likely key to understanding the process of X-inactivation itself. Results presented here show that despite a nuclear RNA accumulation of normal abundance and stability, XIST RNA does not localize to reactivated or *inactive* human X chromosomes in mouse/human hybrid cells. In order to determine if the improper location of the RNA in these hybrids explained the lack of subsequent inactivation, we created clones that stably expressed not only the human *XIST*, but the *mouse* Xist RNA as well. The distribution of the mouse Xist RNA in these hybrids clearly showed a tight association with the active murine X chromosome, in marked contrast to the non-localized human XIST RNA in the same cells. This important result also allows several conclusions: 1) demethylation with 5azadC does not affect the ability of human XIST RNA to localize since the murine Xist RNA does localize properly in the same cells, 2) expression of this RNA does not have to begin in early development to allow localization, 3) Xist RNA does not discriminate between the developmentally selected active and the inactive X chromosome. Collectively these results indicate that the most likely reason for the lack of localization to the human X chromosome is mouse/human species differences. Since completion of this work it was shown that inactive human X chromosomes, when inserted into mouse embryonal carcinoma cells, activate expression of many genes, yet fail to stop expressing XIST RNA (Yoshida et al., 1997). Results presented here raise the

possibility that the XIST RNA may not closely associate with the chromosome in the mouse environment.

As many pre-mRNAs are most concentrated near their site of transcription; (reviewed in Xing et al., 1995; Xing and Lawrence, 1993), it is not clear to what extent the limited localization of the XIST RNA with the X chromosome in the hybrids is simply a consequence of its transcription or reflects a weak affinity for the chromosome. For example, the XIST RNA may be transcribed, but be unable to localize to the chromosome at all, causing the transcripts to gradually drift away into the nucleoplasm. Alternatively, the murine factors may be able to localize the human transcripts, but because of either reduced affinity or availability, allow only a small fraction of the transcripts to associate with the X chromosome.

Localization of XIST RNA Does Not Arise from Stability

Recent results showed the transition of Xist RNA from a small dot to an abundant accumulation in early development and suggested that the increased stability allowed the transcripts to localize to the inactive X chromosome (Panning et al., 1997; Sheardown et al., 1997). Our examination of the stability of the human RNA in human/rodent hybrids indicates that the transcripts are fully stabilized despite the inability to localize to the X chromosome, hence localization of XIST RNA does not result from increased stability. Our results make it clear that the process required for transcript stabilization can occur post development as the half life of XIST RNA in these hybrids is similar to that recently reported in normal diploid fibroblasts (Sheardown et al., 1997).

Differing Requirements for *XIST/Xist* Throughout Development

The observation that the chromosome is completely painted by the Xist RNA but still shows normal expression of both Zfx and Pgk1 RNA in the same cell shows that the chromosome is still transcriptionally active and that there is no strict correlation between Xist RNA chromosomal localization and inactivation of chromatin. We conclude that expression, stabilization and localization of Xist RNA is not sufficient for X-inactivation. Importantly, these results also suggest that there is a difference between the role of *Xist/XIST* in immature versus fully differentiated cell types. In creating a system where a single copy of the *Xist* gene produces RNA that is stably localized in fully differentiated cells, we are able to compare our results with those that show the absolute requirement for *Xist* in X-inactivation (Marahens et al., 1997; Penny et al., 1996) and the sufficiency of *Xist* with limited surrounding sequences to cause silencing (Herzing et al., 1997; Lee and Jaenisch, 1997). The critical difference is that in these previous studies, *Xist* is ectopically expressed in embryonic cells prior to differentiation, while in experiments reported here, *Xist* expression is forced in fully differentiated somatic cells. The contrasting results suggest that XIST RNA is fully competent to enact chromatin inactivation during embryogenesis, but later in development loses this ability either through lack of contributing factors, or a change in the RNA itself.

Potential Role of XIST RNA Localization during Development

Although we have demonstrated in this paper that association of Xist RNA with the X chromosome is not *sufficient* for transcriptional silencing, the supposition that the localization is *required* for X-inactivation is most likely still correct. The process of X-inactivation likely involves several steps, including the expression, stabilization and localization of Xist RNA. Our results not only demonstrate that the localization of XIST RNA with the chromosome does not necessarily culminate in transcriptional silencing, but also suggest that an additional step or mechanism is required to complete the process of X-inactivation.

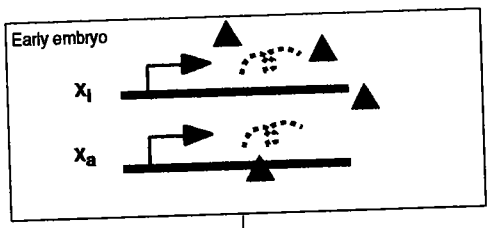
Based on our observations, we postulate a model in which the XIST RNA is involved in the process of X-inactivation but not directly responsible for transcriptional down regulation (Figure 2-6, A). In this model, we speculate that the Xist RNA serves to mark or potentiate the chromosome for a subsequent step or process. For example, the RNA, by virtue of its binding along the length of the chromosome and its affinity for certain factors, could recruit processes that ultimately result in transcriptional silencing. While there are many examples of processes that could act in concert with Xist RNA, we consider two for discussion: methylation and histone deacetylation. Both are established mechanisms of epigenetic imprinting strongly linked with inactive X chromatin; they are stably maintained and transferred to daughter cells and are involved with sites along the entire inactive X chromosome. Furthermore, methylation (Keohane et al., 1996; Lock et al., 1987) and overall deacetylation of the inactive X (Keohane et al., 1996) occur following *Xist* expression early in embryogenesis. Additionally, this idea may explain why *Xist* is not

necessary for maintenance of X-inactivation in somatic cells, while other results show that it is required during development. XIST RNA could be absolutely required during embryogenesis to establish the pattern of methylation, or histone deacetylation by recruiting factors like methyltransferases or deactelyases to the inactive X chromosome; however, once such a pattern was established on the inactive X, then the XIST RNA would no longer be rigorously required. The constitutive expression and localization of XIST RNA (see Chapter I and Clemson et al., 1996) in adult cells may serve as a back-up mechanism to ensure that epigenetic silencing is rigorously maintained. Ultimately this could explain why the *XIST* gene could be deleted *in vitro* from somatic cells with no obvious effect (Brown and Willard, 1994); i.e. if the primary source of inactivation maintenance was a process prone to little error, (such as maintenance methyltransferase which has an estimated efficiency of 99.9% (Pfeifer et al., 1990), then as a backup mechanism XIST RNA could be deleted without obvious phenotype in cultured cells. In contrast, *in vivo*, where even a slight failure of X-inactivation would be deleterious, XIST RNA would be needed as a constant fail safe mechanism. The model we present in 2-6A is consistent with the data on XIST RNA expression, stability and localization reported here, and we interpret our results in light of this model in Figure 2-6, B.

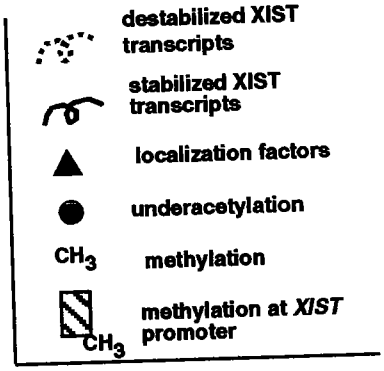
Of course, if *XIST/Xist* initiates subsequent events which ultimately silence the chromosome, then why didn't our reactivated mouse/human hybrid system succumb to transcriptional silencing? It is possible that cells that did inactivate were selected against, allowing us only to detect cells that managed to circumvent the X-inactivation. This appears less likely because we did not observe any evidence of cell death or change in growth rate,

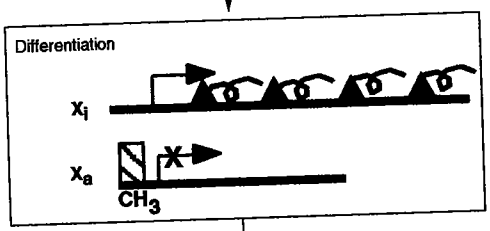
Figure 2-6

A. Potential Role of XIST RNA Localization During Development

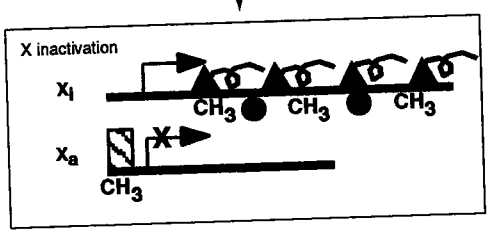


Prior to X-inactivation in the early embryo XIST RNA (red) is expressed from both chromosomes and is not stable, therefore the transcripts can't interact with localization factors.



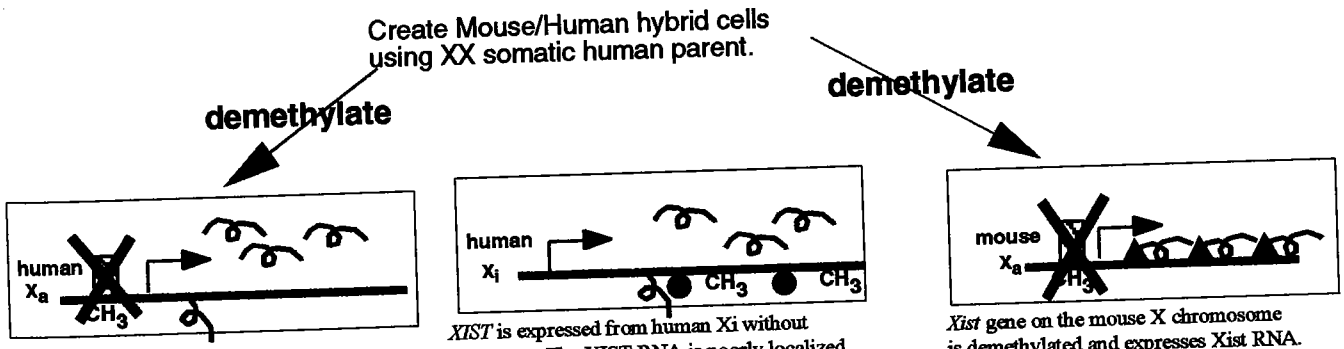


Developmental milestone causes XIST to be stabilized only on Xi, ultimately expression from active X (Xa) is silenced by methylation of the promoter. The long lived XIST transcripts are now available to localization factors (blue), and are deposited along the body of Xi.



XIST transcripts recruit other process(es) (e.g. methylation (CH_3) and deacetylation (green)), causing X-inactivation.

B. How Mouse/Human Hybrid Results are Explained by Above Model



XIST gene on the active X chromoosme is demethylated and expressed. Transcripts are stable because they are produced after differentiation, but with no human localization factors available the RNA is poorly localized.

XIST is expressed from human Xi without treatment. The XIST RNA is poorly localized becauze no human localization factors are available. Previously established genomic imprinting patterns (e.g. methylation and deacetylation) keep the chromosome transcriptionally silent.

Xist gene on the mouse X chromosome is demethylated and expresses Xist RNA. As murine localization factors are available, the RNA localizes along the body of Xa. Because this process happened outside of development, no recruitment of processes and no X-inactivation occurs.

combined with the fact that these cells retained an active human X chromosome, which could compensate for the loss of the single mouse X. Rather, we favor the possibility that the resistance to X-inactivation is due to the fact that the Xist RNA was not expressed prior to differentiation of these cells; e.g., it may be very difficult or impossible to establish *de novo* inactivation in cells after development as the required enzymes may not be available. For example the levels and location of *de novo* and maintenance methyltransferases differ in early embryonic versus terminally differentiated cells (reviewed in Razin and Shemer, 1995; Turker and Bestor, 1997).

To reactivate expression of *XIST/Xist*, the cells were treated with a demethylating agent which can impact cell viability as well as replication timing and condensation of the inactive X chromosome (Gregory et al., 1985 ; Haaf et al., 1988). Additionally, the new methylation patterns may be slowly incorporated into DNA (Toth et al., 1989). Although we were able to produce clones that expressed human *XIST* indefinitely, we could not isolate clones that expressed murine *Xist* longer than several months. Hence we cannot rule out the possibility that this was not enough time to set up adequate patterns of genomic imprinting that result in silencing of the previously active X linked genes like *Zfx* and *Pgk1*. However, the cells were only cultured in 5azadC for a short period of time (24 hours) to cause expression of the mouse or human *Xist*, and subsequently cultured without additional 5-azadC for as long as several months with a significant fraction (greater than 30%) still expressing mouse *Xist* . Expression of the X-linked genes, *Zfx* and *Pgk1* was analyzed as much as 8 weeks after 5azadC treatment, making it less likely that their continued expression is the result of demethylation. This is particularly true given that the

mouse/human hybrid cells which retain the full complement of murine chromosomes maintain the inactivation status of previously inactivated X chromosomes. Hence, it is unlikely that the lack of *de novo* X-inactivation is due merely to repression of X-inactivation in the mouse-human hybrid system studied here. It is clear that our reactivated hybrid system reconstitutes many steps known to be involved in normal X-inactivation, namely expression, stabilization and localization of Xist RNA. Therefore, it seems that we have created an intermediate to chromosomal inactivation that would not have been seen otherwise, one which makes it possible to analyze and dissect the separate steps involved in X-inactivation.

CHAPTER III: XIST RNA DOES NOT ASSOCIATE COMPLETELY WITH AUTOSOMAL CHROMATIN IN X;AUTOSOME TRANSLOCATIONS: IMPLICATIONS FOR A ROLE IN FAITHFUL MAINTENANCE OF X-INACTIVATION

While Chapters I and II represent work that has been completed and published, we are still in the process of refining the work described in this chapter and preparing it for publication.

Abstract

Recent studies of transgenic mice carrying multiple copies of *Xist* integrated onto an autosome have suggested that XIST RNA is sufficient for X-inactivation and fully capable of complexing with and inactivating an autosome. If so, this would indicate that the fundamental mechanisms whereby XIST RNA binds to and inactivates the chromosome may be indifferent to the sequence or protein composition of the chromosome itself. Here the relationship of XIST RNA to chromatin inactivation is investigated in cells derived from two patients carrying X;autosome translocations. This system was used to directly compare the binding of XIST RNA to X and autosomal sequences in an environment where *XIST* is present in *cis*, in a single copy, in its native context. In cells derived from both patients, XIST RNA showed some association with the translocated autosomal chromatin, extending over 30-50% of the autosomal chromatin closest to the breakpoint. This association was not due merely to proximity to the *XIST* gene, as XIST RNA showed no drift or promiscuous association with neighboring intact autosomes, and the translocated X and autosomal sequences are generally found as separate entities in interphase as well as metaphase. Although it was limited to the proximal portion of the autosome, the relationship between XIST RNA and autosomal sequences was indistinguishable by 3D analysis from that with the X chromosome. Sampling of two chr. 14 genes confirmed that autosomal material very distal to the breakpoint was active, but that material proximal to the breakpoint was inactive. This suggests that XIST RNA can complex with and potentially inactivate autosomal chromatin.

Importantly, however, the translocated autosomal material was readily distinguishable from translocated X chromatin. In contrast to the consistent, complete association of XIST RNA with intact Xi chromatin in the translocation or in normal cultured fibroblasts, only about half of the autosomal sequences were painted by XIST RNA. High-resolution analyses of replication timing demonstrated that, with the exception of early replicating distal material (known to remain active), the bulk of the translocated autosome was clearly later replicating than homologous autosomes in the same cell, but not as late replicating as the Xi chromatin involved in the translocation. These results combine with previous studies to suggest that the late replication and possibly inactivation status of the autosomal chromatin may regress due to instability of the association with XIST RNA.

Results are discussed in terms of the potential role of XIST RNA in the maintenance of chromosome inactivation over many cell generations.

Finally, a series of interphase gene mapping experiments were performed to assess the spatial relationship of specific genes relative to XIST RNA. These experiments provide evidence to support a novel model of chromosome organization as all genes on a normal X chromosome, regardless of activity, were found on the border of XIST RNA. These results also indicate that at a cytological level, genes that escape inactivation are not organized differently with respect to the XIST RNA territory. When a similar analysis was performed on the t(Xi;A) chromosomes, all Xi and proximal autosomal genes were also mapped to the border of XIST RNA, while distal autosomal genes were shown to be randomly arranged with respect to XIST RNA. These results suggest that the organization of the chromosome territory with respect to XIST RNA may correlate with or contribute to X-inactivation.

Introduction

In Chapter I it was demonstrated that XIST RNA is spatially coincident with most of the Xi, and that the bulk of the RNA is fully processed message, strongly suggesting that this RNA remains in the nucleus, functionally associated with the inactive chromatin (see also Clemson et al., 1996). The fact that this RNA does not code for a protein (Brockdorff et al., 1992; Brown et al., 1992) combined with evidence demonstrating that expression of the XIST gene alone is necessary and sufficient to inactivate the surrounding chromatin (Herzing et al., 1997; Lee and Jaenisch, 1997; Marahens et al., 1997; Penny et al., 1996) further suggests that XIST is a novel functional nuclear RNA. Results showing that *XIST* can be deleted from somatic cells without affecting X-inactivation (see Chapter II and Brown and Willard, 1994; Clemson et al., 1998; Tinker and Brown, 1998) suggest that the RNA is required only during embryogenesis in order for X-inactivation to occur. However, the fact that XIST RNA is constitutively transcribed and faithfully associates with the Xi in *adult somatic* cells (Clemson et al., 1996; Kay et al., 1993) suggests that it plays some role in maintaining X-inactivation.

In the model in Chapter II, it was proposed that XIST RNA acts in concert with other elements to affect X-inactivation (Figure 2-6), however it is not clear if these elements are specific to X chromatin or if all chromatin has the capability of forming a functional relationship with XIST RNA. Recent studies of transgenic mice carrying multiple copies of mouse *Xist* integrated onto an autosome have suggested that all chromatin is equally capable of forming an association with Xist RNA and being inactivated. In these experiments, it

has been reported that a complete 'painting' relationship is established between autosomal material and murine Xist RNA, when the RNA is ectopically expressed from an autosome (Herzing et al., 1997; Lee and Jaenisch, 1997; Lee et al., 1996). Furthermore, this relationship apparently causes the autosome to manifest hallmarks of inactivation such as late replication, underacetylation, heterochromatinization and transcriptional down regulation of two genes on the autosome (Lee and Jaenisch, 1997). These results preliminarily suggest that Xist RNA binds to an entire autosome and ultimately affects inactivation of autosomal sequences. If so, this would indicate that the fundamental mechanisms whereby Xist RNA binds and inactivates the chromosome may be indifferent to the sequence or protein composition of the chromosome itself.

However, there are several important caveats to transgenic experiments. First, multiple copies of the *Xist* gene (as few as 20 and as many as 120) were expressed from the autosome (Lee and Jaenisch, 1997; Lee et al., 1996). It is conceivable that transcription of 100 copies of *Xist* would produce so much RNA that the transcripts would appear to coat the autosome, when in reality they represent simply a large nuclear accumulation of newly synthesized RNA. Alternatively, it is possible that autosomal sequences have a reduced propensity for being inactivated due to DNA sequence or chromatin protein differences relative to Xi, but when confronted with the abnormally large quantities of Xist RNA in transgenic experiments, succumb to X-inactivation. If the Xist RNA were inactivating the entire autosome as it has been suggested, then one might expect to see large scale cell death due to rendering cells of the animal monosomic for most or all of a particular autosome; but no such negative selection was reported. Therefore, while it does appear that the autosome

is manifesting some signs of inactivation as these authors reported, it is possible that the entire autosome in these transgenic experiments is not inactivated since the comprehensive effect of ectopic *Xist* expression has been masked due to selective pressure.

Naturally occurring X;autosome translocations circumvent the pitfalls of ectopic autosomal *Xist* expression. By allowing an in-depth comparison of the relationship of XIST RNA to autosomal chromatin in an environment where the *XIST* gene is present in a single copy and expressed in its native context, the true affect of such a relationship can be ascertained. While in the majority of individuals with *balanced* X;autosome translocations, the normal X is non-randomly selected for inactivation and the translocated chromosome remains active, (in this manner monosomy of the autosome is avoided), there are many cases of *unbalanced* X;autosome translocations involving trisomy of all or part of the translocated autosome. In cells derived from such patients, a functional trisomy is avoided only if the translocated chromosome is inactivated; hence there exists strong selective pressure for *XIST* expression and X-inactivation.

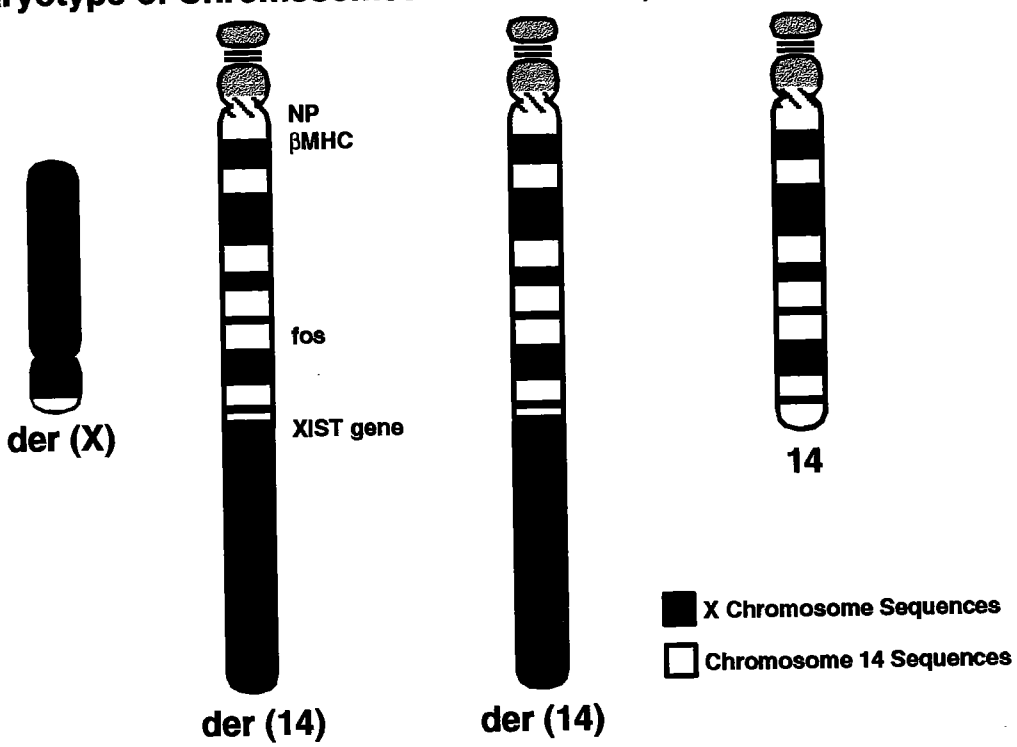
In this study, cells derived from patients with unbalanced X;autosome translocations were used to investigate the relationship of XIST RNA to autosomal sequences. These cells were derived from two patients with unbalanced (X;14) or (X;9) translocations, hence they contain three copies of chromosome 14 or 9 (Figure 3-1, A and B). For both of these patients, there is good evidence to suggest that most of the translocated autosome is inactivated. First, the clinical phenotype of the patients is relatively mild compared to what would be expected if the patients had a complete trisomy (Allderdice et al., 1978; Leisti et al., 1975; Pallister and Opitz, 1978). Secondly, traditional ^{3}H autoradiographic studies

suggested that the majority of the autosome in both of these Xi translocations was late replicating (Allderdice et al., 1978; Leisti et al., 1975). However, the evidence also suggests that some of the autosome escapes inactivation in both of these patients. While the clinical phenotype is mild relative to a complete duplication of chromosome 14 or 9, there does exist mild mental retardation, developmental delays and other minor anomalies in both patients, suggesting that complete silencing of the autosomal chromatin was avoided. Moreover, the distal tip of the X;14 did not appear to be late replicating all of the time (Allderdice et al., 1978), while the distal tip of the X;9 did not appear to be late replicating some of the time (Leisti et al., 1975).

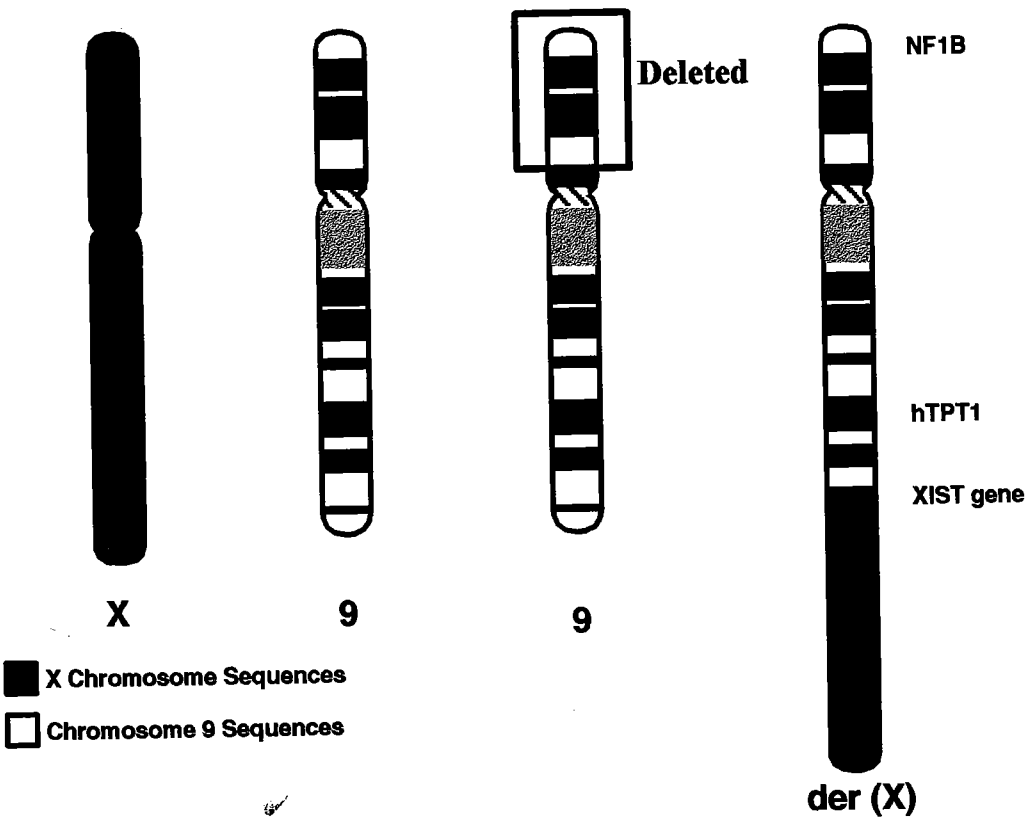
Here, cells from these two patients were used to determine whether XIST RNA binds to X and autosomal sequences equivalently by directly comparing the binding of XIST RNA to X and autosomal sequences on the same chromosome. The results show not only that there is no strict correlation between XIST RNA association and X-inactivation, but also suggest that X and autosomal sequences retain unique properties while on the same chromosome. The difference in the relationship of X and autosomal sequences indicates a weakened affinity of XIST RNA for autosomal sequences. This correlates with previous results showing that the inactivation of autosomal sequences is not rigorously maintained to suggest that it is an unstable relationship with XIST RNA that is the basis for this tendency towards reactivation. Ultimately, this may confirm that XIST RNA does have a role in fully differentiated somatic cells where patterns of inactivation have previously been established: to facilitate maintenance of X-inactivation.

FIGURE 3-1

A: Karyotype of Chromosomes Involved in X;14 Translocation



B: Karyotype of Chromosomes Involved in X;9 Translocation



Material and Methods

Cells and Cell Culture

The X;14 cell line is a 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 Figure 3-1, A). This translocation is also known as the KOP translocation (Pallister and Opitz, 1978). The X;9 cell line is a 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 Figure 3-1, B). Both the X;14 and X;9 lines were received from the NIGMS Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ), catalog number 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 (Greenberg et al., 1986; Huang and Spector, 1991). Briefly, cells were grown for several days to subconfluency, then cells were completely deprived of serum by washing and culturing in serum free medium for 24 hours. Cells were then stimulated with the addition of 15% FBS + 20 ug/ml of the protein synthesis inhibitor anisomycin for 15 or 60 minutes prior to fixation.

Histone deacetylase inhibitors like n-butyrate, have been used extensively to induce expression of specific genes; (see for example: Goldberg et al., 1992; Green and Shields, 1984; Ogryzko et al., 1996; Smith, 1987). To induce *NP* expression, cells were grown to

subconfluency on coverslips and then cultured in normal media supplemented with 1mM n-Butyric acid (Sigma) for 24 hours. Cells were then immediately prepared as described below.

Cell Preparation for *in situ* hybridization

Cells were generally prepared as described in Chapter I for in situ hybridization. An additional method, to ensure preservation of the 3-D structure of the cells (Eils et al., 1996), was used for comparison in several experiments. This technique afforded similar results to our standard fixation protocol. The cells were grown on coverslips and removed from the incubator and washed in PBS at 37°C. The cells were then fixed prior to extraction in 4% paraformaldehyde/PBS for 10 minutes at room temperature (RT). After a brief rinse in PBS, the coverslips were incubated in .1M HCl for 10 minutes at RT. Cells were then extracted in 0.5% triton-X-100/0.5% saponin in PBS for 10 minutes at RT and then equilibrated for 20 minutes in 20% glycerol/PBS at RT. Cells were further permeabilized through a freeze-thaw cycle consisting of dipping the coverslip quickly into liquid nitrogen. The cells were allowed only to thaw (not dry out) and then stored in 70% ethanol at 4°C.

RNA, DNA and simultaneous RNA/DNA hybridization and detection were performed as described in chapters I and II.

DNA Probes

For fluorescence *in situ* RNA and DNA detection the following probes were used: XIST G1A: a ~10kb 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; ICRFc100E0981 (UBE1), a cosmid containing ~13 kb of the genomic locus (Carrell et al., 1996); DXS128E (XPCT): cosmid containing genomic sequences to the XPCT locus; cIC1484.4 (PHKA): cosmid containing genomic sequences to the phosphorylase kinase alpha 1 (muscle) glycogenosis locus; (The 5 preceding probes were 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); p482.6 (F8C): containing 9.6 kb of genomic sequences to coagulation factor VIIIc, proagulant component hemophilia A (ATCC catalog number 57202); HM-1 (ß-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); λBER111 (ZFX): 18 kb genomic clone of the zinc finger protein locus (a generous gift of Dr. David Page, MIT); λHPTP: 15 kb of the human protein tyrosine phosphatase gene (a generous gift of Dr. Nicholas Tonks, Cold Spring Harbor); λNF1B1: containing a 10 kb genomic fragment of the nuclear factor 1B gene (Qian et al., 1995) (a generous gift of Feng Qian, Johns Hopkins University); D14Z1/D22Z1 chromosome 14/22 α-satellite: probe to 14 centromere (ONCOR, Gaithersburg, MD); DXZ1 X chromosome α-satellite: probe to X centromere sequences

(ONCOR, Gaithersburg, MD); Coatasome-X, -14 and -9 whole chromosome library probes (ONCOR , Gaithersburg, MD) were used to paint chromosome territories.

BrdU Antibody Technique

The BrdU banding procedure was adapted from the BAT technique (Vogel et al., 1986; Vogel et al., 1989). Translocation cells were grown to subconfluence on coverslips, and then cultured for a total of 6 hours in 25uM BrdU, after 4 hours colcemid was added to a concentration of 1.5 ug/ml, and 2 hours later metaphase preparations were produced directly on the coverslips. Cells were rinsed and swollen in 75mM KCl for 15 minutes at 37°C; cells were fixed with multiple additions of 3:1 methanol/acetic acid, metaphase cells were ruptured by allowing coverslips to airdry in a 37°C humid chamber. The cells were then denatured in 0.07N sodium hydroxide for 5 minutes at RT in order to facilitate detection of incorporated BrdU. BrdU was detected with mouse anti-BrdU (Partec) 1:500, and anti-mouse conjugated with FITC or rhodamine (Boehringer Mannheim). With this technique late replicating DNA produces a fluorescent G-band pattern.

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.

Morphometrics, 3-D Rendering and Image Analysis

Imaging, optics, morphometrics and 3-D deconvolution were as described in chapter I.

Results

XIST RNA "Paints" part of the Autosome

To examine whether XIST RNA associates with autosomal material that is physically connected to inactive X-linked chromatin, the first step was to compare the hybridization pattern of XIST RNA with a whole X chromosome library hybridization in the translocation lines. Comparison of the XIST RNA signal with the Xi library hybridization suggested that the RNA signal overlapped the X library signal completely and consistently. As illustrated for the X;14 cell line in Figure 3-2 (A-C) and the X;9 cell in Figure 3-2 (D-F), the X library hybridization is covered completely by the XIST RNA signal (as was shown in Figure 1-2 for normal diploid fibroblasts). In contrast to previous data, however, the XIST RNA signal is larger than just the X chromosome domain, suggesting that the XIST RNA is painting more than just X chromosome material. When the XIST RNA hybridization signal was compared to the autosomal sequences involved in the translocation, it was apparent that the RNA was spatially coincident with some, but not all of chromosome 14 and chromosome 9 library signal. As shown in Figure 3-2, G and H, a significant fraction of the autosomal sequences appear 'painted' by the XIST RNA, while some XIST RNA does not coincide with the autosomal signal and is presumably associated with the translocated X.

Measurements around the boundaries of the XIST RNA and library signals (see Figure 3-2, A and D for examples), were performed to determine the areas of

each of these domains in approximately 100 nuclei in 3 separate experiments (as described in Chapter I). To analyze the extent to which the chromosomal segments were painted by the XIST RNA, the percent of the pixels that were labeled with both XIST RNA and chromosome library signal were quantitated. As shown in Figure 3-3, about 85% of the Xi chromatin from the translocated chromosome was painted by XIST RNA, which is similar to the amount of overlap measured in diploid fibroblasts (Chapter I). As expected from the *in situ* hybridization experiments, the amount of the translocated *autosome* that was painted by XIST RNA was significantly less: 56% for chromosome 14 and 52% for chromosome 9.

Next, it was important to address whether the XIST RNA truly overlapped the translocated autosomal territory in the Z axis, so 3-D rendering was used to provide a higher-resolution analysis. Optical sections through nuclei at 0.2 micron spacing were taken of 5 cells from each translocation cell line. The optical sections were deconvoluted to remove out of focus light and as shown in Figure 3-4 (A: t(X;14) and B: t(X:9)), a 3-D rendered image of a representative cell is used to create a composite of 3-D spatial relationship between XIST RNA and the chromosome libraries. These 3D convolution results indicate that the overlap between XIST RNA and part of the translocated autosomal segments is not surface limited but occurs throughout many planes of focus, much like what is seen for the entire Xi in normal diploid fibroblasts (Figure 1-4). This result shows that the association between XIST RNA and the autosomal sequences is not an artefact of two-dimensional microscopy.

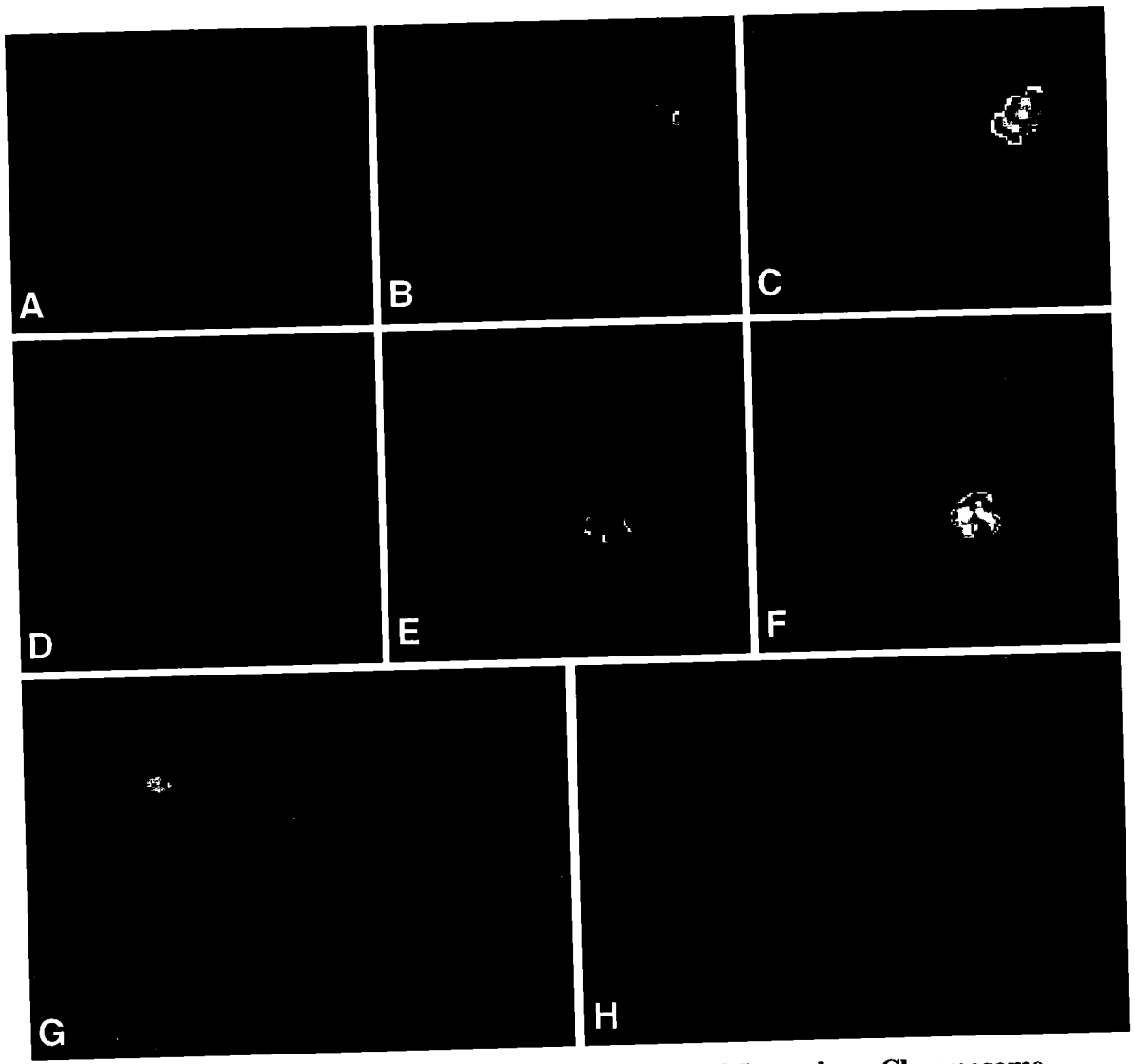


Figure 3–2: Co–localization of XIST RNA with X, 14 and 9 Interphase Chromosome Territories in Translocation cells. Detection of chromosome libraries was carried out using the Oncor Coatasome Painting systems and simultaneous hybridization to nuclear RNA was achieved as described (see Materials and Methods). (A): Two of the 3 X chromosome territories in t(X;14) fibroblast cells are shown (*red*). B) XIST RNA (*green*) in the same cell. C) overlap of XIST RNA (green) and the Xi chr (red) shows that the entire Xi segment is painted by XIST RNA. The outline in each of these panels delineates the Xi chromatin, and reveals that the XIST RNA domain is larger than Xi. (D–F): Detection of X chromosome territories (*red, panel D*), XIST RNA (*green, panel E*) in t(X;9) cells, overlap is yellow (*panel F*). Similar to panel C above, it is apparent that the XIST RNA signal is larger than just Xi. G): Detection of XIST RNA (*green*) in t(X;14) fibroblast cells with 3 chromosome 14 territories (*red*), one of which is inactivated (Figure 3–1). Overlap between the XIST RNA and chromosome 14 appears yellow. The XIST RNA apparently associates with some, but not all of the t(14). (H) Similarly, the overlap (*yellow*) between XIST RNA (*green*) and chromosome 9 (*red*) shows that only part of the autosome is painted in the t(X;9) translocation.

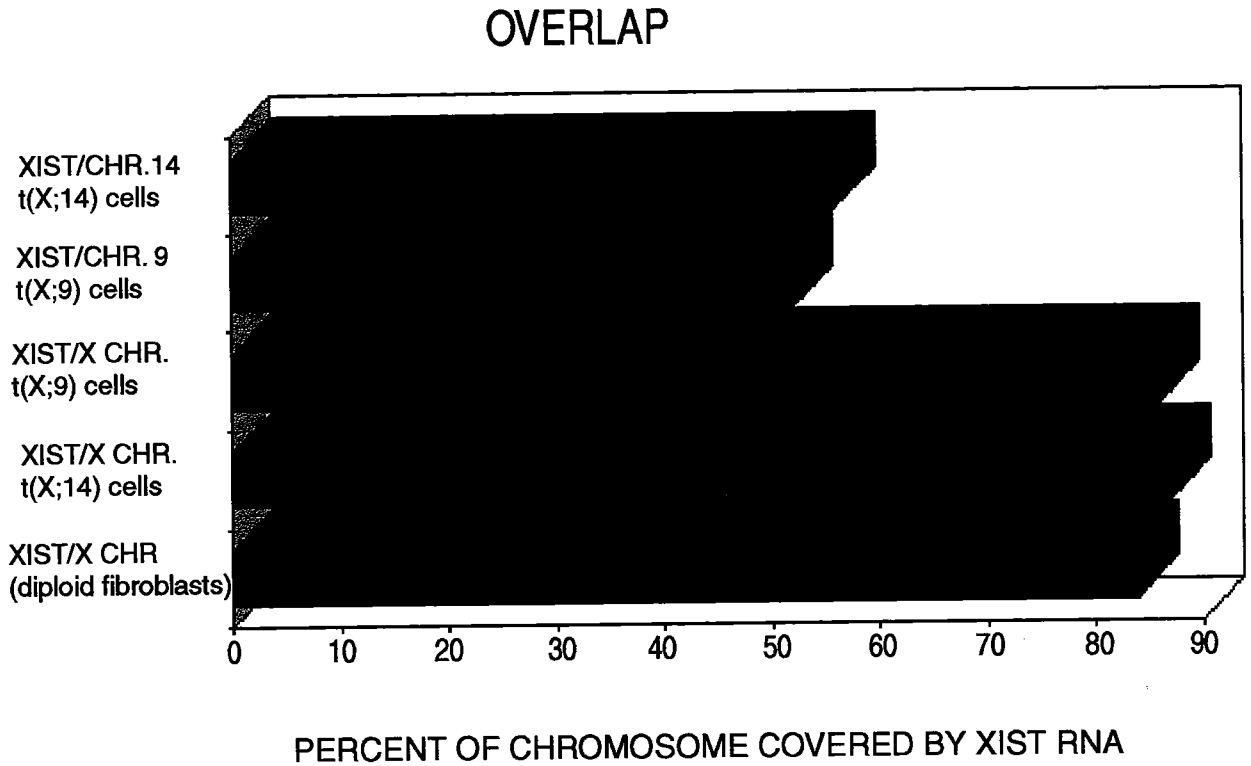


Figure 3-3. Morphometric Data on Spatial Overlap of RNA and Chromosome Territories. Morphometric analysis was performed on 2D digital images in separate color channels as described (Materials and Methods, Chapter I). Summaries of measurements on approximately 100 t(X;14), t(X;9) and normal diploid fibroblast (WI-38) cells are presented for cells hybridized simultaneously for XIST RNA and the corresponding chromosomal DNA. 85% of the inactive X chromosome is, on average, covered by XIST RNA in diploid fibroblasts, t(X;14) and t(X;9) cells, while only 56% of chromosome 14 and 52% of chromosome 9 is painted by XIST RNA in the t(X;14) and t(X;9) cells respectively.

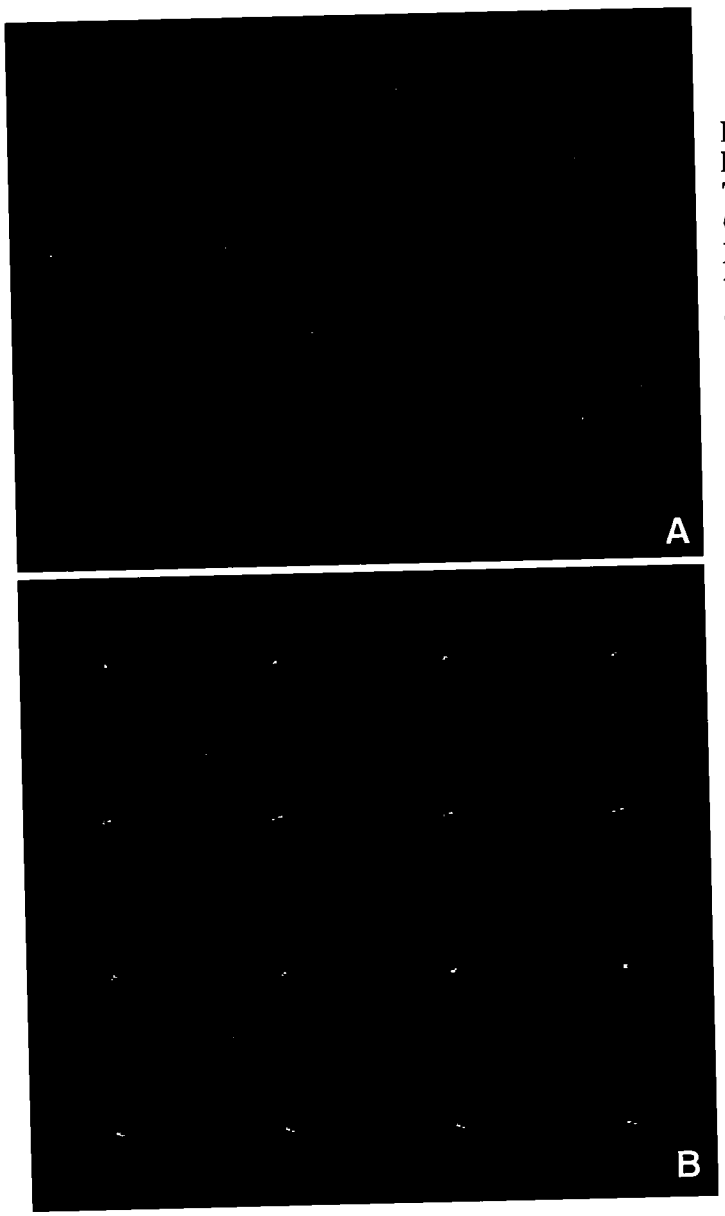


Figure 3–4: Three–dimensional Distribution of XIST RNA and Translocated Autosomal Chromatin. Hybridization to XIST RNA (*green*) and either t(14) (Panel A) or t(9) (Panel B) chromosomal DNA (*red*) were visualized simultaneously and data sets were acquired and restored as described in Materials and Methods, Chapter I. (A) A binary volumetric rendering of an entire optical section data set after image restoration; this is a straight projection rotated 180^{o} along the Y the X, and then again the Y axis. This figure shows that the XIST RNA (*green*) and part of t(14) (*red*) occupy the same 3–D spatial location. B) This is a straight projection rotated 180^{o} along the Y axis showing that part of t(9) (*red*) is painted by XIST RNA (*green*). (In this example the non–translocated chromosome 9 territory is also apparent.) There is XIST RNA signal that is not associated with the autosomal territories in both panel A and B , presumably this RNA is available for association with the t(Xi).

The above results suggest that the spatial association between the XIST RNA and the autosome is genuine; however, it is possible that the autosome is partially painted by the RNA simply because it is close to the site of *XIST* transcription. The fact that X-inactivation is severely *cis*-limited, combined with the fact that the size and shape of the XIST RNA and the X chromosome are identical (see Chapter I and Clemson et al., 1996) indirectly suggests that the RNA does not localize to non Xi-linked chromatin even when it is in close proximity. However, the possibility that XIST RNA can drift into neighboring chromosome territories has not been directly addressed. In order to rigorously determine if XIST RNA can 'leak' to parts of an adjacent autosome, the XIST RNA and chromosome 14 or 9 library signal were compared in normal diploid fibroblasts (Figure 3-5, A and B). From these experiments, it appeared that the XIST RNA did not overlap adjacent autosomes and this was confirmed through quantitative analysis. The relative positions of XIST RNA and chromosome 14 domains were scored in 200 normal diploid fibroblasts. XIST RNA was scored as adjacent to chromosome 14 in 11% of the cells. In this fraction of cells where the Xi and one of the 14 chromosomes were adjacent, there was no overlap 93% of the time (the other 7% of the time there appeared slight overlap of the two signals which is most likely attributed to the limits of optical resolution). This result shows that even when chromatin is near the site of *XIST* transcription, the RNA does not drift to the autosomal chromatin. This further supports that the association between the XIST RNA and part of the autosome in the two translocation cell lines is a significant *cis*-limited interaction.

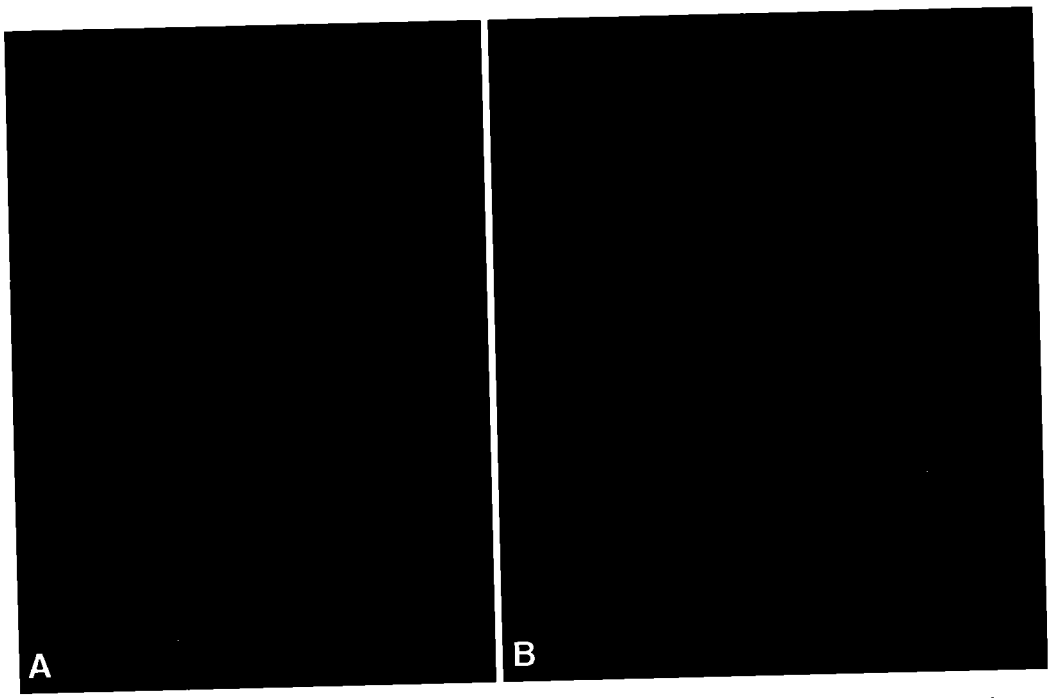


Figure 3–5: XIST RNA Does not Extend into Adjacent Autosomal Sequences in Diploid Fibroblasts XIST RNA and autosomal domains were compared in normal diploid fibroblasts (WI–38) A) XIST RNA (*red*) and chromosome 14 (*green*) show no significant overlap even when they are in close proximity in WI–38 cells. B) Similarly, XIST RNA (*red*) does not dribble into chromosome 9 (*green*) territories that are in close proximity.

The Autosomal and X Segments of the Translocated Chromosome Remain as Distinct Entities in Interphase

A plausible explanation for the above results is that the XIST RNA paints the X portion of the translocation completely, while only a portion of the translocated autosome is painted by the XIST RNA. However, it is possible that the XIST RNA really has an exclusive relationship with the Xi –linked chromatin, and that in interphase the X and autosomal portions of the translocated chromosome overlap. If the two segments of the translocation 'intermingle' as such in interphase, then the XIST RNA may only *appear* to associate with autosomal chromatin. A series of experiments were performed to determine the relative relationship of the two 'arms' of a translocated chromosome in interphase. These experiments are of interest themselves as they investigate a fascinating question: Do distant chromosome segments occupy separate space within the interphase chromosome territory, or does the decondensed chromatin become more interspersed? To examine this, dual chromosome library hybridizations were performed in the t(X;14) and t(X;9) cell lines (Figure 3-6). Using the morphometrics described above, similar results were seen in the two translocation lines. Generally, the chromosome domains of the autosome (14 or 9) in interphase were clearly distinct from the translocated X segment the majority (>84%) of the time with only a slight overlap of the two territories (Figure 3-6, A and C). While occasionally there appeared to be a large degree of overlap between the X and autosomal territories (Figure 3-6, B and D),

these examples were a minority (<10%), and this minor fraction was most likely caused by the limitations of our 2-D analysis. The t(X;14) cell line which contains two identical translocated chromosomes, one active and one inactive (Figure 3-1), showed similar results for both translocated chromosomes (Figure 3-6, A), suggesting that even active, presumably more spread out translocated chromosomes, maintain their borders between chromosomal segments. These results also show that much like the highly condensed mitotic chromosome, the two segments of a translocated chromosome remain distinct in the interphase nucleus; and therefore support that the overlap between the XIST RNA and translocated autosomal sequences is genuine.

The relationship between XIST RNA and the autosome was also examined in mitotic chromosomes where the two segments of the translocated chromosome could be precisely delimited. While it is known that the XIST/Xist RNA is progressively released from the X chromosome during mitosis in humans (Figure 1-9), and mice (C. Clemson and B. Panning unpublished data), enough RNA signal remains in prophase to perform the analysis. Interestingly, even at later stages of mitosis, the XIST RNA sometimes remains, hugging what appears to be the surface of the mitotic chromosome (Figure 3-7, B). In the highly condensed mitotic chromosome shown in Figure 3-7 (A), the XIST RNA very clearly paints the entire Xi sequence, and only a fraction of the autosomal sequences. Morphometrics was performed on 35 cells to quantitate the amount of the autosome painted by the XIST RNA in mitosis, revealing that 50% of the translocated chromosome 14 segment

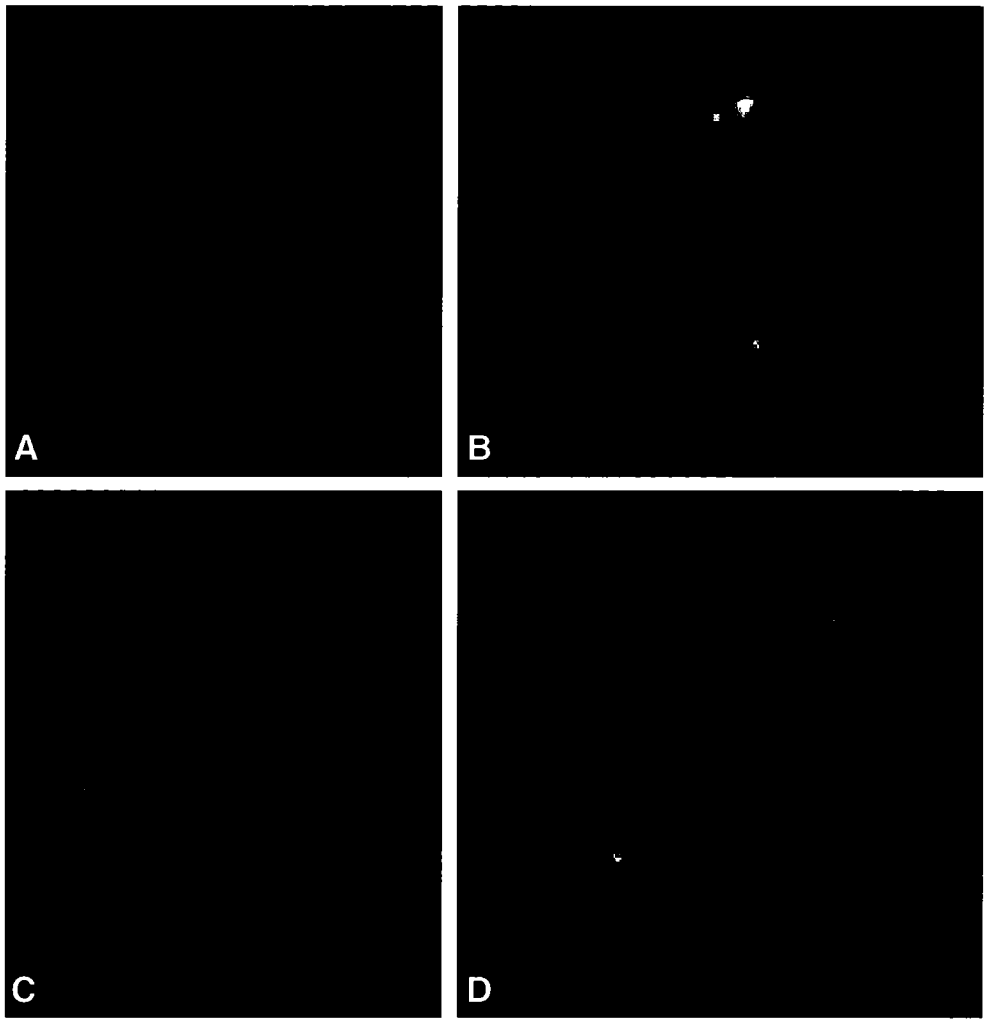


Figure 3–6: The X and Autosomal Chromatin in X;Autosome Translocations are Generally Found as Distinct Territories in Interphase Chromosome Paints were used to delineate the X and either 14 or 9 chromatin on the translocated chromosomes at interphase. A) the majority of the time the two t(X;14) chromosomes show separation of their t(Xi) (*green*) and t(14) (*red*) signals. C) Similarly, the t(Xi) and t(9) domains were generally not overlapping. B and D) Occasionally, some overlap of the X and autosomal domains was observed.

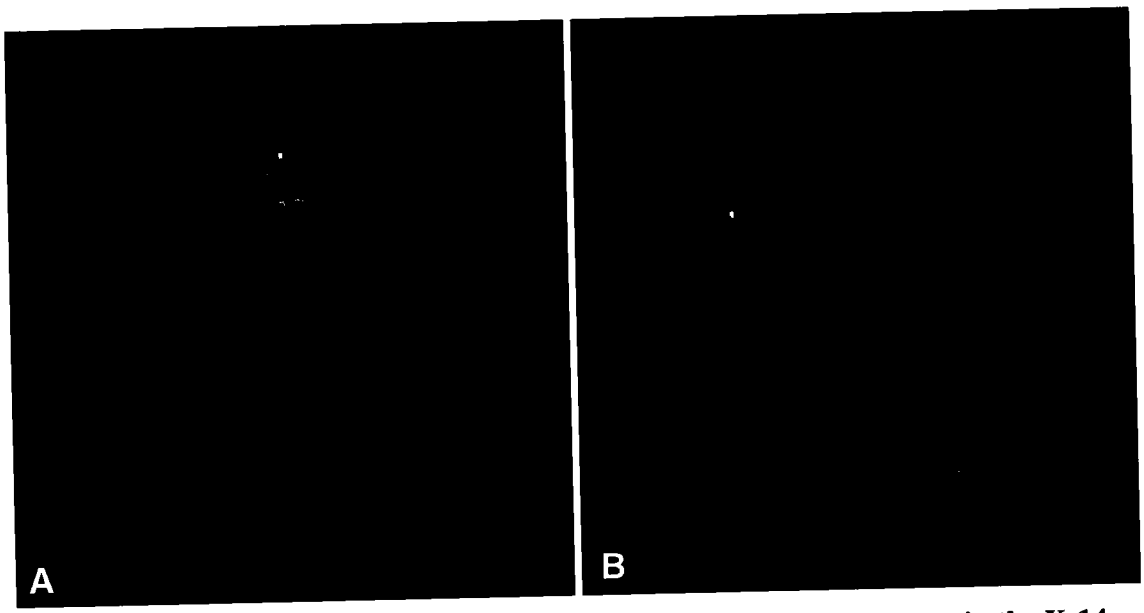


Figure 3–7: XIST RNA Paints All of t(Xi) and part of t(14) at Prophase in the X;14 translocation.
Chromosome paints were used to delineate the X (*panel A*) and 14 chromatin (*panel B*) on the mitotic t(X;14) chromosome. A) The XIST RNA (*green*) extends beyond the X paint (*red*) in the mitotic chromosome (*counterstained with DAPI – blue*). The linear order of the highly condensed mitotic chromosome reveals that the XIST RNA extends progressively beyond the X–linked chromatin. B) At later stages of mitosis, the RNA is clearly diminished, but the signal that remains oftentimes 'hugs' just the periphery of the chromosome. This example shows that the XIST RNA outline (*green*) encompasses all of t(Xi) and part of t(14) (*red*).

was painted by XIST RNA, while 35% of the translocated chromosome 9 segment was painted by XIST RNA. These results reinforce that the RNA does reproducibly associate with a large segment of the autosome. They also extend our interphase observations to demonstrate an important point: that the RNA associates linearly along the proximal half of the chromatin, rather than associating with selected segments throughout the translocated autosome.

c-FOS is Subject to and NP Escapes X-inactivation on the X;14 Chromosome

The next question is whether transcriptional silencing correlates with the spread of inactivation in the translocated autosome. The translocated chromosomes afford an ideal system for determining the functional ramifications of XIST RNA association as they contain large autosomal segments that show no such association with XIST RNA. To determine the transcriptional status of loci on the translocated autosome, dual RNA detection *in situ* hybridization experiments were performed to examine expression from the inactivated X;14 chromosome. RNA signals from two 14-linked genes distal (*NP* and *β-MHC*) and one gene proximal (*c-FOS*) to the breakpoint were scored relative to the XIST RNA signal (see Figure 3-8). Alleles from the inactive chromosome were distinguished from alleles on the active chromosomes on the basis of their proximity to XIST RNA. To control for erroneously scoring a gene as being from the inactive translocated chromosome, *PGK1* expression was analyzed in the X;14 cells. As expected (since there are two *PGK1* alleles in the X;14 cells and *PGK1* does not escape inactivation), only one

RNA signal was detected in most cells. This signal (presumably from the *active* X;14 chromosome) was erroneously scored as coming from the inactive chromosome about 8% of the time.

As expected from a muscle specific gene, no expression from the three *ß-MHC* alleles was detected under any conditions (data not shown). While initial experiments produced detectable RNA signals from *NP* and *c-FOS*, the weak signals were sporadically detected making quantification in a large cell number impractical. Serum has been shown to upregulate *c-FOS* expression, so serum induction (Greenberg et al., 1986; Huang and Spector, 1991) was used to increase both the signal intensity and number of cells exhibiting detectable *c-FOS* expression. The c-FOS RNA signals were analyzed in >200 cells in three separate experiments. Two c-FOS RNA signals were detected in most cells (Figure 3-8, A); 92% of these signals (by virtue of their distance from the XIST RNA domain) were scored as being produced from the alleles on the active 14 chromosomes, whereas *c-FOS* was not expressed from the inactive X;14 allele.

Since serum induction was not successful for inducing *NP* expression, butyrate induction was tried and found to effectively increase detectable *NP* expression. Approximately 200 signals were scored in 2 separate experiments. Unlike the two signals found for *c-FOS* and the one signal found for *PGK1*, three NP RNA signals were found in the majority of cells (Figure 3-8, B). Approximately 1/3 of the total NP RNA signals were scored as occurring near the XIST RNA signal and from the inactive chromosome allele. This was not due to inappropriate expression of

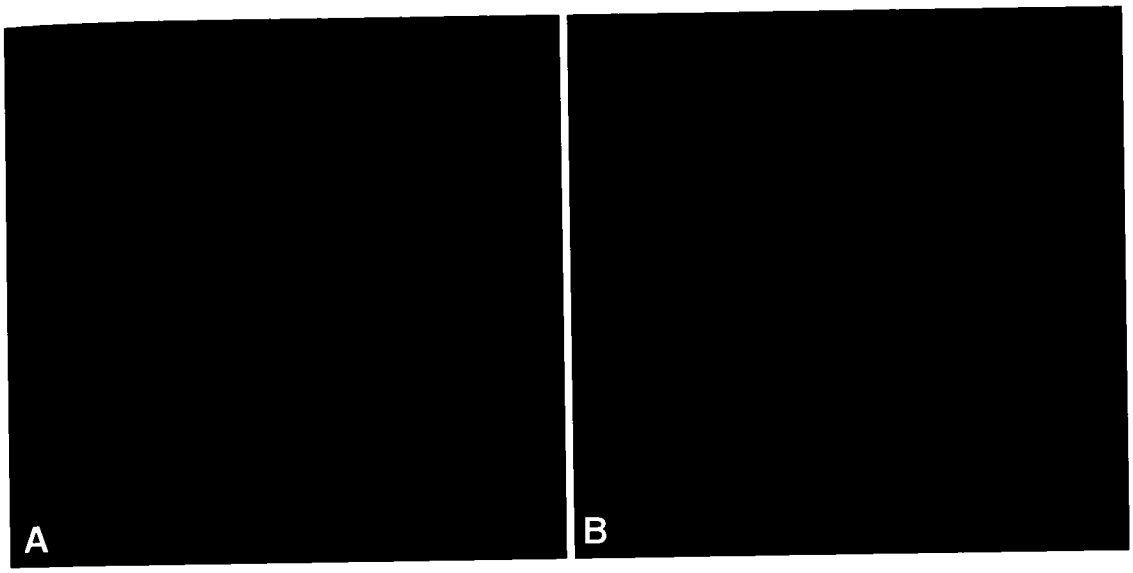


Figure 3–8: Analysis of Expression of Genes Proximal and Distal to the Breakpoint in t(X;14) Cells.
Using XIST RNA (*red*) as a marker for the inactive chromosome, the expression of two genes was analyzed under non–denaturing conditions in the t(X;14) cells (*nuclei counterstained with DAPI*). A) c–FOS RNA (*green*) is subject to inactivation as it is not expressed from the t(X;14) chromosome nearest the XIST RNA signal (red) but is expressed from the other two alleles. These cells were induced for increased expression with serum induction as described in Materials and Methods. B) Three NP RNA (*green*) signals were consistently seen in t(X;14) cells. 1/3 of the signals were close to the XIST RNA signal (*red*), showing that *NP* escapes inactivation. These cells were enhanced for *NP* expression via butyrate induction as described in Materials and Methods.

normally inactive genes due to butyrate induction, since single RNA signals were detected from *HPRT*, *PGK1* and *FMR1* (data not shown) under the same conditions. The RNA signals were rarely found near the XIST RNA indicating that butyrate induction did not induce inappropriate expression of the inactive alleles of these 3 genes. This result demonstrates that the NP gene escapes inactivation in the X;14 cell line.

The X and Autosomal Sequences Involved in the Translocation Replicate Asynchronously

Since *c-FOS* is subject to X-inactivation, while *NP* is not, these experiments provide direct proof that some but not all of the autosome is subject to X-inactivation. While this result also suggests that there may be a correlation between chromatin being painted by XIST RNA and the spread of inactivation, only two genes have been examined thus far. DNA replication timing allows an assessment of the general impact of the translocation throughout the chromosome, though the correlation with inactivation is less direct. Replication timing studies were performed in order to assess the transcriptional activity of large regions of the X;14 chromosome using immunofluorescent detection of incorporated bromodeoxyuridine (BrdU); this approach affords much higher resolution (Vogel et al., 1986; Vogel et al., 1989) than earlier radiographic or indirect staining methods. Cell cultures were terminally labeled with BrdU, and then hybridized with either an X, 14 or 9 chromosome library: in this manner the replication timing and,

correlatively, the transcriptional activity of the different parts of the translocation could be determined.

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. Examples of these are shown in Figure 3-9. Clearly, one of the t(X;14) and the single t(X;9) replicates later than other chromosomes. However, the translocated chromosome does not appear to replicate as synchronously as a normal Xi does. The chromosome 14 (Figure 3-9, A-C) and 9 (Figure 3-9, G-I) segments, while still later replicating than other autosomes, are not as late replicating as the *X portion*, producing an 'intermediate' replication pattern (best evidenced in Figure 3-9, G-I). With the cohybridization of the chromosome paint, it is possible to identify the translocation breakpoint, and it appears that the transition from the late replication to intermediate replication coincides with this breakpoint (Figure 3-9, G-I). However there were examples of some late replicating bands extending into the autosome (Figure 3-9, C), indicating that some small segments of the autosome, close to the breakpoint, may be later replicating than the rest of the translocated autosome. Occasionally, it was possible to identify cells in which only the X segment has incorporated heavy label (Fig 3-9, D-F), further confirming that the two segments of the translocation replicated somewhat asynchronously. Alternatively, it was possible to identify cells in which most of the translocated chromosome, except for the distal tip, had incorporated late label (Figure 3-9, J), showing that, while there are clear

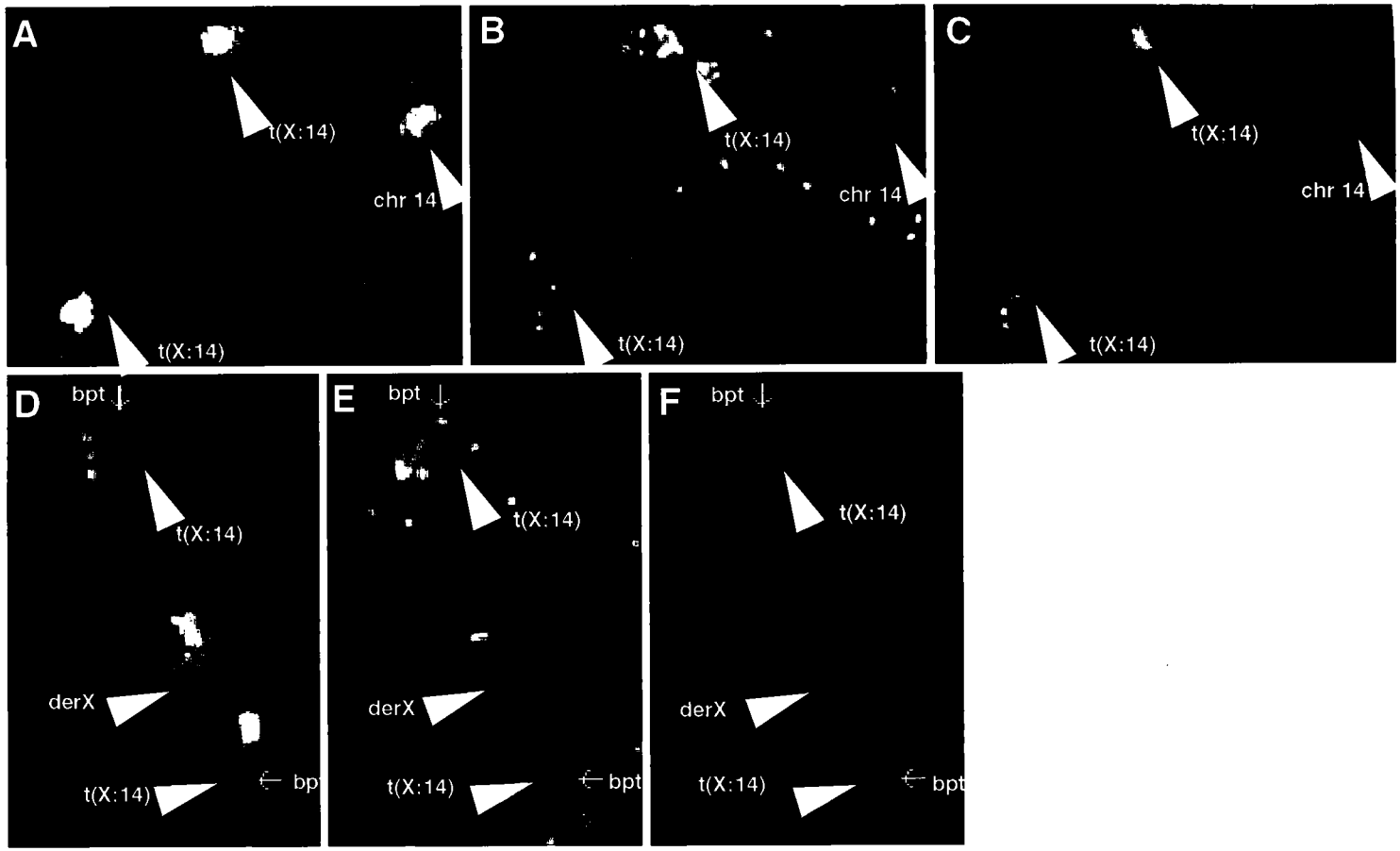


Figure 3–9: Replication Timing Studies on t(X;14) and t(X;9) using the BrdU Antibody Technique.
BrdU was incorporated in S phase and chromosome territories were delineated using chromosome paints. Representative cells with late replicating fluorescent patterns were analyzed (fluorescent bands are late replicating). (A–C): BrdU versus *chromosome 14* in t(X;14) cells. A) 3 chromosome 14 territories are outlined, showing two t(X;14) and one normal 14 chromosome (*arrowheads*). B) BrdU banding patterns showing that part of one t(X;14) is late replicating C) the overlap between the BrDU (*red*) and chromosome 14 domains (*green*). DNA is counterstained with DAPI (*blue*). The normal 14 and a single t(X;14) incorporated little signal, while the Xi portion of one t(X;14) is darkly stained. While most of the autosome is not late replicating, some of Chr. 14 is late replicating (*yellow*). D–F): BrdU banding versus *X chromosome* in t(X;14) cells. D) two t(X;14) and one der(X) (*arrowheads*) are defined by chromosome paint. E) BrdU banding patterns and F) the overlap between the BrdU bands (*red*) and the X chromosome domains (*green*) show that the X chromatin from one t(X;14) is late replicating while the other t(X;14) and der(X) has incorporated little label. The termination of the late replication pattern correlates with the t(X;14) breakpoint (*arrow*).

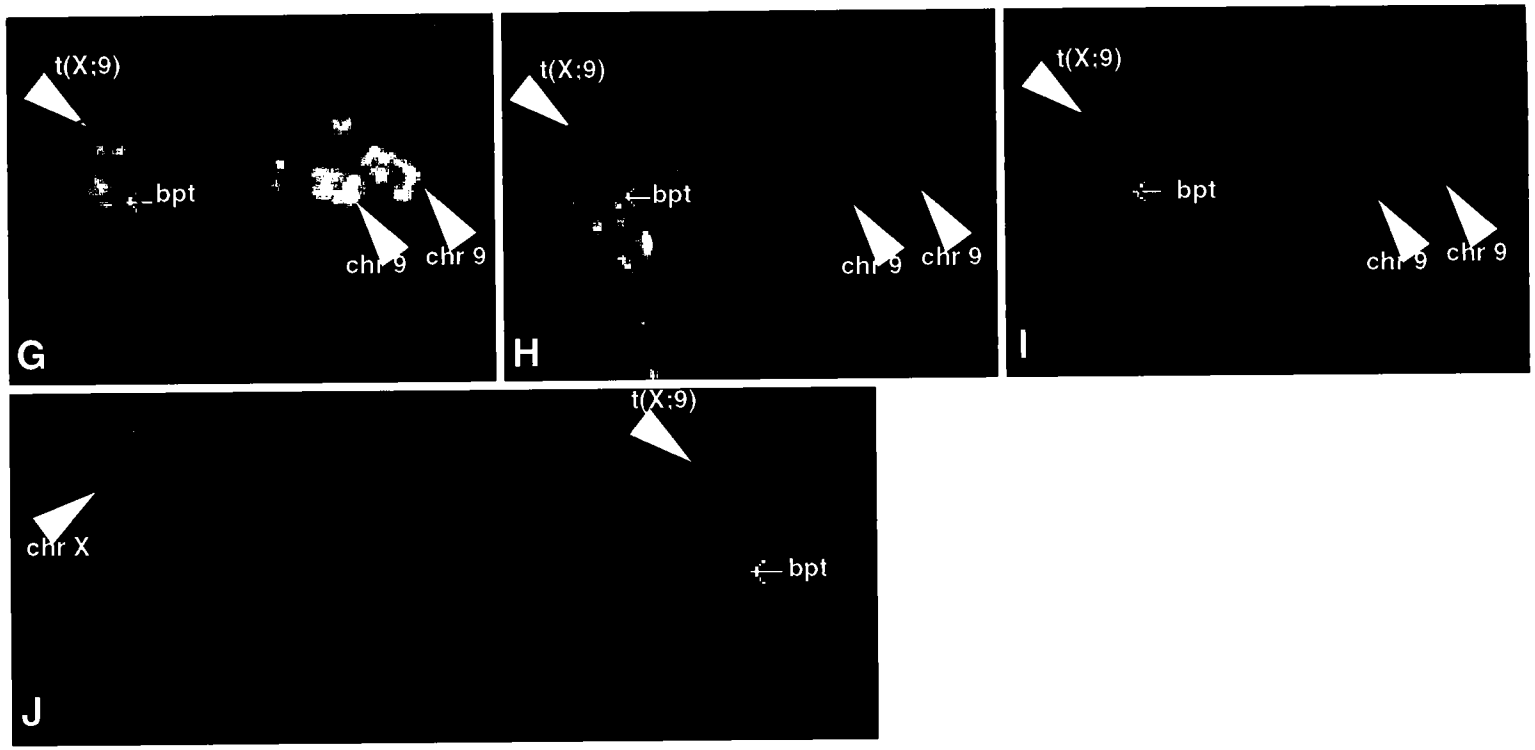


Figure 3–9 (G–I): Replication Timing in t(X;9) cells.
G) One t(X;9), and two chr 9 domains are painted (*arrowheads*), while the t(X;9) breakpoint is also shown (*arrow*). (Contrary to the published phenotype, I have found that one of the 'normal' 9 chromosomes was consistently deleted for the distal tip. See box in Figure 3–1,B. This did not occur in culture as the original passage from NIGMS showed this deletion.) H) BrdU banding pattern. The 9 portion of the t(X;9) is later replicating than the other 9 chromosomes, but is not as late replicating as the X segment. I) Overlap between chromosome 9 (*green*) and BrdU (*red*). The boundary between the late and intermediate replicating sequences coincides with the breakpoint (*arrow*). J): BrdU versus the *X chromosome* in the t(X;9) translocation. The normal X and t(X;9) are painted in green (*arrowheads*), the t(X;9) breakpoint is also shown (*arrow*). The t(X;9) is late replicating (except the distal tip) while the normal X has incorporated very little label.

differences between the different X and autosomal segments, almost the entire translocation is late replicating relative to other autosomes.

Since in our experiments a number of complicated BrdU patterns were observed, it is possible that the cells represent a mosaic population in which the autosomal replication patterns varied from cell to cell. However, the general theme gleaned from the most late replicating patterns is that the t(Xi) was later replicating than t(A) in both translocation cell lines. The results reported here are not inconsistent with previous autoradiographic replication timing studies in that most of both the t(X;14) and the t(X;9), except for the distal tip, appears later replicating than their homologs. The higher resolution afforded by the direct BrdU detection technique likely allowed discrimination of the subtle asynchrony between the autosomal and X-linked sequences.

There is a Reproducible Spatial Relationship between XIST RNA and Specific Genes

In the discussion of Chapter I, it was postulated, based on the fact that XIST RNA does not hybridize to DNA and is part of the nuclear matrix, that the RNA associates with interphase nuclear structural elements throughout Xi, thereby either exploiting or causing the unique Xi chromatin structure. It was further postulated that the inactivated X chromosome may have a higher level structure that is organized with respect to XIST RNA and that genes that escape inactivation may be organized differently (Figure 1-11). Since the above results show that Xi and

autosomal chromosome domains remain spatially distinct throughout interphase and show different affinity for XIST RNA, at a gross cytological level it is likely that these two domains are organized differently with respect to XIST RNA.

In order to compare the higher level organization of the individual arms of the X;14 and X;9 chromosomes, specific sequences from the proximal and distal segments on the translocated chromosomes were mapped relative to the XIST RNA at interphase. 6 X-linked genes (*PGK-1*, *XPCT*,*PHKA*, *HPRT*, *FMR-1* and *F8-C*); 4 chromosome 14-linked sequences (*NP*, *b-MHC*, *c-FOS*, and the centromere); and 2 chromosome 9-linked genes (*NFB1* and *hTPT-1*) were mapped relative to the XIST RNA signal in interphase (see Figure 3-11 for the position of these genes on the chromosome). Each gene was scored as occurring either inside, outside or on the border of the XIST RNA signal (see Figure 3-10 for details and examples of each category). Each gene was mapped in no less than 50 cells in at least 3 separate experiments, the results are summarized in Figure 3-11. It is apparent that the individual genes were not located randomly with respect to XIST RNA. All the X-linked genes, regardless of whether they were proximal or distal to the site of *XIST* transcription, were found adjacent to the XIST RNA signal a majority of the time. This bordering phenomenon was seen for the X-linked sequences in both the t(X;14) and t(X;9) cells (Figure 3-11, A and B). A similar pattern was seen for the autosomal-linked genes proximal to the breakpoint. c-FOS and hTPT-1, the genes closest to the site of *XIST* transcription were found to mostly border the XIST RNA in the X;14 and X;9 cells respectively. However, the sequences that

were more distal to the breakpoint deviated from this pattern. *NP*, β-*MHC*, and the 14-centromere were all consistently found well outside of the XIST RNA signal in the X;14 translocation (Figure 3-11, A); while the *NF1B* gene was well outside of the XIST RNA in the X;9 translocation (Figure 3-11, B).

While it is possible that the autosomal genes found outside of the XIST RNA represent a variation in global organization between autosomal and Xi-linked chromatin, it is also possible that genes from non-translocated Xi chromatin show similar deviations. In both of the X;14 and X;9 translocations, the entire short arm of the X chromosome, which contains the majority of the genes that escape inactivation, is deleted. In order to determine if XIST RNA maintains a consistent relationship with all the sequences on the X chromosome, two Xp-linked genes and the X-centromere were mapped relative to XIST RNA in normal diploid fibroblasts. As shown in Figure 3-11 (C), while the non-coding X-centromere α-satellite sequences have no reproducible relationship with respect to XIST RNA, the *ZFX* and *UBE-1* genes are found on the border of XIST RNA a majority of the time. This suggests that genes on Xi, regardless of the distance from the site of XIST transcription, have a similar cytological relationship to XIST RNA, while non-coding sequences have no such consistent relationship. Therefore, since all of the autosomal-linked genes do not maintain a bordering relationship with XIST RNA, it appears that there is an innate difference in the higher-level organization of autosomal chromatin with respect to XIST RNA. These results are considered in light of a model for overall interphase chromosome organization in the Discussion.

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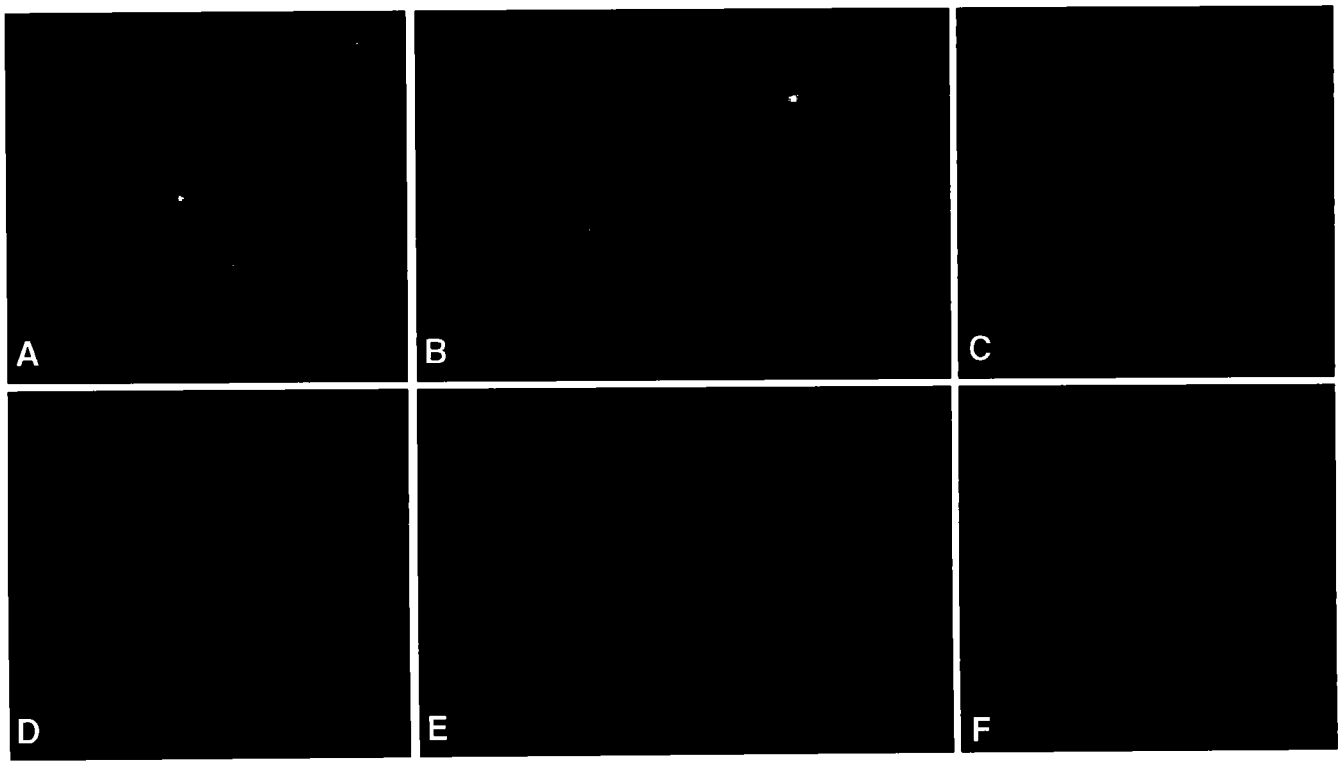
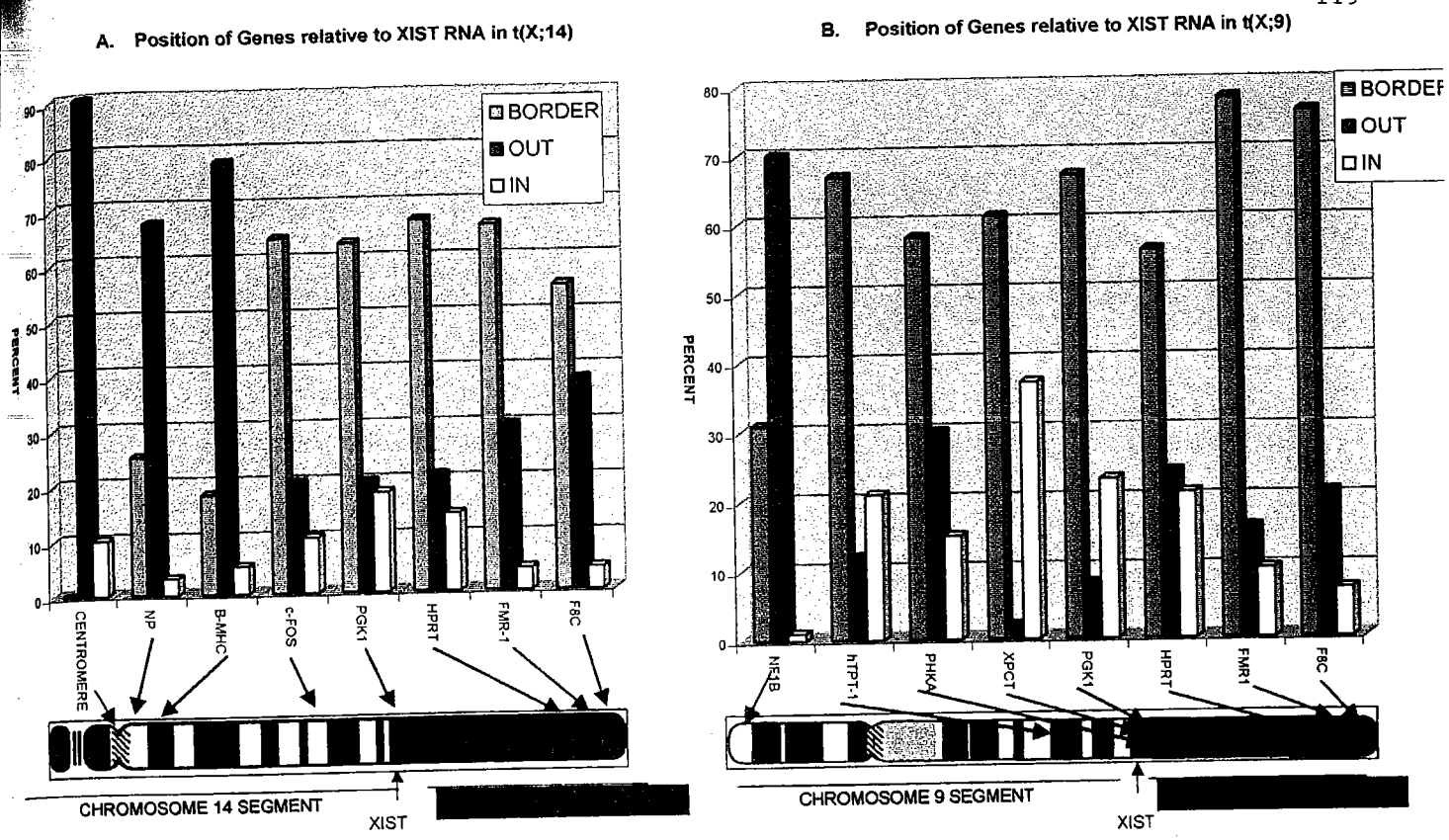


Figure 3–10: Representative Examples of Gene Positions Relative to XIST RNA
Specific sequences were mapped relative to XIST RNA at interphase in either normal diploid fibroblasts (WI–38), t(X;9) or t(X;14) cells; the gene signals were scored as occurring either Inside (A and B), Outside (C), or on the Border (D–F) of the RNA domain. (Oftentimes the signals from non Xi–linked alleles occur in different focal planes, so all gene signals from the active chromosome(s) are not apparent in each figure). **EXAMPLES OF EACH CATEGORY**: **IN:** A) *XPCT* (*red*), XIST RNA (*green*) in WI–38 cells. This X–linked gene that is subject to X–inactivation was found on the border of the XIST RNA a majority of the time, but it was found inside the RNA domain occasionally. B) X–centromere (*green*), XIST RNA (*red*) in WI–38 cells. The non–coding α–satellite sequences were found randomly positioned with respect to XIST RNA, occurring inside about 50% of the time. **OUT:** C) *NFIB* (*red*) was found outside of the XIST RNA (*green*) most of the time in the t(X;9) cells. **BORDER:** Most genes fell into this category. D)*HPRT* (*red*) predominantly occurs at the periphery of the XIST RNA domain (*green*) in WI–38 cells. E) *c–FOS* (*red*) is mostly found at the periphery of the XIST RNA domain (*green*) in t(X;14) cells. F) *HPTP1* (*green*) occurs a majority of the time at the border of the XIST RNA (*red*) in t(X;9) cells. Full results on each of these genes is shown in Figure 3–11.



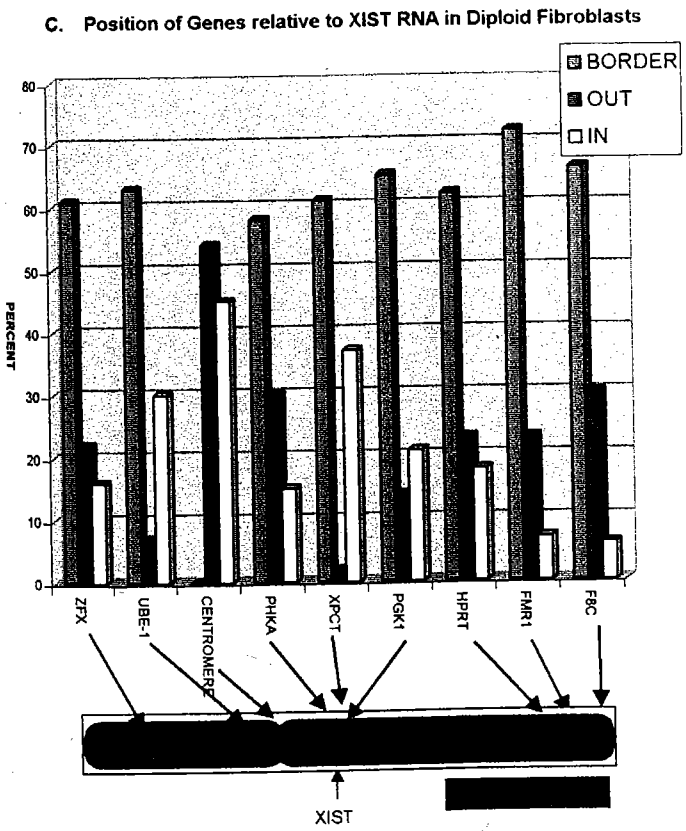


Figure 3-11: Summary of Data from Mapping Genes versus XIST RNA at Interphase Genomic probes for genes or centromere sequences were cohybridized with XIST RNA in A) t(X;14); B) t(X;9) and C) normal diploid fibroblast (WI-38) cells. The signals were scored relative to XIST RNA (see Figure 3-10 for examples of each category). Each bar represents the average score for no less than 50 cells in at least 3 separate experiments. A) Specific sequences were mapped in t(X;14) cells, all Xi-linked genes and *c-FOS* were scored as being on the Border of XIST RNA a majority of the time, while the non-coding α-satellite and two distal genes (*NP* and *β-MHC*) were found Outside of the XIST RNA signal most of the time. B) Similarly, all of the Xi-linked genes and *hPTP1* were found to Border the XIST RNA domain at interphase, while a distal gene (*NF1B*) was scored as Outside in the t(X;9) cells. C)All Xi-linked genes, regardless of activity, maintain a Bordering relationship with XIST RNA, while the α-satellite centromere sequences maintained no such ordered position in WI-38 cells.

Discussion

Limitation of X-Inactivation to the Chromosome is Not Due to the Specificity of XIST RNA

Recent evidence has suggested that ectopic autosomal expression of *Xist* causes the entire autosome to be painted by Xist RNA and exhibit X-inactivation (Herzing et al., 1997; Lee and Jaenisch, 1997; Lee et al., 1996). Full interpretations of these experiments is limited by the complexities of expressing multiple copies of *Xist* in it's non-native environment. To investigate the extent and functional consequences of a spatial association between XIST RNA and autosomal chromatin in a natural physiological context, two X;autosome translocation cell lines were investigated. Surprisingly, the XIST RNA structurally associates with about half, but not all of the autosome. Results show that XIST RNA associates with the autosomal material in 3-dimensions and that this relationship is not an artefact created by either intermingling of the translocated chromosome domains or 'drift' of XIST RNA onto adjacent autosomes. The fact that the XIST RNA consistently paints the proximal portion of the autosome shows that XIST RNA needs to propagate along a contiguous structure; hence it is not just free floating in the chromosome territory, consistent with its being part of the nuclear matrix. *A priori*, the *cis*-limitation to X-inactivation potentially could derive from the specificity of XIST RNA for only X-linked sequences. However, my results combine with the previous transgenic studies to clearly demonstrate that if given access to autosomal chromatin in *cis* (e.g. as part of the same chromosome structure), XIST RNA can complex with it. It

seems then that the primary restriction to the spread of XIST RNA, and hence to X-inactivation, resides in the rigorous maintenance of chromosomal boundaries or "territories" which persist at interphase.

Lack of Comprehensive XIST RNA Association: Potential Explanation for Instability of Autosomal Inactivation

The results presented here contrast with those from transgenic studies which show the entire autosome painted by Xist RNA, and also challenge any assumption that autosomal material translocated to Xi is equally competent to be inactivated. While it is commonly thought that such translocated autosomal material, in the absence of selection, is readily inactivated, an in-depth search of the literature on *unbalanced human* X;autosome translocations suggests that the spread of inactivation into autosomal segments is not definitive. In approximately half of the studies examined, no late replication timing (X-inactivation) was observed at all in autosomal sequences (Bettio et al., 1994; Caiulo et al., 1989; Camargo and Cervenka, 1984; Disteche et al., 1984; Garcia-Heras et al., 1997; Keitges and Palmer, 1986; Palmer et al., 1980; Preis et al., 1996). When late replication (X-inactivation) does appear to spread into autosomal sequences, it generally encompasses only a small part of the autosome (Camargo and Cervenka, 1984; Couturier et al., 1979 ; Ejima et al., 1982; Keitges and Palmer, 1986; Mohandas et al., 1982; Schanz and Steinbach, 1989; Taysi et al., 1982). Even when most of the translocated autosome does appear late-replicating, some small portion (generally the distal tip) remains early replicating (Allderdice et al., 1978; Hagemeijer et al., 1977; Leisti et al., 1975; Markovic et al., 1985)

and oftentimes the spreading is highly variable resulting in mosaicism (Caiulo et al., 1989; Couturier et al., 1979; Zuffardi et al., 1977). Clearly, in those cases where autosomal X-inactivation occurs at all, it is usually sporadic and highly variable.

In addition to the sporadic occurrence of autosomal inactivation, additional evidence suggests that autosomal inactivation is unstable relative to X-inactivation. Most recently, our replication timing experiments show that the inactive translocated chromosome is apparently segmented into late and intermediate replicating portions, with the X portion replicating the latest, and the autosomal sequences replicating a bit earlier, but later than their counterparts. A similar intermediate replication pattern was reported for the 21 segment of an X;21 translocation (Couturier et al., 1979). Since there is good evidence that late replication timing correlates with transcriptional silencing (Boggs and Chinault, 1994; Disteche et al., 1979; Gartler et al., 1992; Hansen et al., 1996; see for review: Holmquist, 1987; Schmidt and Migeon, 1990; see for specific examples: Taylor, 1960; Torchia et al., 1994), the replication timing asynchrony reported here implies that most of the autosomal sequences (the distal tip excluded) may not be as tightly inactivated as the translocated X segment.

Previous literature shows not only that autosomal inactivation is variable and sporadic, but also that it may be unstable relative to X-inactivation (reviewed in and Cattanach, 1974; Eicher, 1970; Krumlauf et al., 1986). A previous study of a (X;14) translocation similar to that studied here, has directly shown somatic instability of inactivation of the chromosome 14 segment (Schanz and Steinbach, 1989). Autosomal late replication banding patterns from this patient's cells show regression with progressive cell division *in*

vitro. While the authors initially proposed that this was due to a mosaic of inactivation patterns established in early embryogenesis, they showed through single cell clone analysis that this variation was due to instability of autosomal inactivation (Schanz and Steinbach, 1989). Finally, results of the transgenic studies are consistent with instability of autosomal inactivation. The distal portion of the purportedly inactivated mouse chromosome 12 became reacetylated with time, while a previously inactive distal gene (*Yy1*) showed signs of sporadic reactivation (Lee and Jaenisch, 1997).

There are many mechanisms that could give rise to a reduced inactivation of autosomal material. Our finding that there is an innate difference in the affinity of autosomal and X-linked sequences for XIST RNA localization makes the fundamental point that a deficiency in binding of XIST RNA is involved. This is important because *a priori* it was possible that some subsequent step such as methylation or deacetylation was the limiting factor. There are two general explanations for why XIST RNA is not seen spread through all autosomal chromatin. First, perhaps no relationship between XIST RNA and distal autosomal sequences was ever established. If so then there could be an obstacle to the spread of XIST RNA somewhere along the autosome (e.g. specific sequences) or there could be insufficient quantities of XIST RNA to associate with a chromosome the size of the t(X;14) or t(X;9). The second possibility is that the relationship between XIST RNA and autosomal material is not maintained as faithfully such that the extent of association regresses in later cell generations. While barriers to the initial spread may occur in some cases, we forward the hypothesis that compromised maintenance of the association of XIST RNA with autosomal chromatin is involved.

XIST May be Necessary for Faithful Maintenance of X-Inactivation in Somatic Cells

The variability and instability of autosomal inactivation reported here and in the literature makes it more likely that the XIST RNA originally associated with most of the autosome, but this association was progressively lost. For example, for the literature reviewed here, in almost half of the cases where somatic autosomal material was not inactivated (not late replicating), patients exhibited an inexplicably mild phenotype for what should be a severe or lethal trisomy (Disteche et al., 1984; Garcia-Heras et al., 1997; Keitges and Palmer, 1986; Zuffardi et al., 1977). Perhaps the trisomic autosomal segment was inactivated due to comprehensive autosomal XIST RNA localization in early development where the consequences of trisomy would be most severe, but subsequently this localization and inactivation was not maintained in adult cells with no significant impact. This correlates well with the absolute requirement for XIST RNA in the early embryo, and the apparent loss of this strict requirement in adult somatic cells.

Data showing that XIST RNA is not required for maintenance of X-inactivation in somatic cells (Brown and Willard, 1994; Tinker and Brown, 1998) but is absolutely required in embryogenesis (Penny et al., 1996) would suggest that XIST RNA localization would be required initially to establish patterns of chromatin organization, but could be progressively lost without obvious effect in somatic cells. However, XIST RNA ubiquitously maintains a tight structural association with Xi in terminally differentiated mammalian female cells, and this relationship is not progressively lost in culture (Chapter I

and Clemson et al., 1996). This inconsistency has led to the suggestion that XIST RNA is required after development not for principle maintenance of X-inactivation but as a safeguard to ensure that this process does not fail or become leaky over the life of the organism (see Discussion Chapter II and Brown and Willard, 1994). However evidence for such a role has not yet been reported. Our results suggest that the propensity for reactivation of the distal regions of autosomal segments correlates with the apparent inability of the XIST RNA to extend consistently to those regions. Therefore results presented here give indirect evidence for a specific role for *XIST* in facilitating faithful maintenance of X-inactivation.

A Novel Model of General Chromosome Organization

The interphase gene mapping results reported here support a novel model for general chromosome organization. Surprisingly, all of the X-linked genes in the X;14, X;9 and normal diploid cells were shown to map to the border of the XIST RNA accumulation at interphase; while the X-centromere does not show this specific relationship with respect to the XIST RNA. Since the XIST RNA accumulation has been shown to mimic the size, shape and position of the X chromosome territory in chapter I, this would suggest that all the genes, regardless of activity are on the outer surface of the inactive X chromosome. Similarly, a recent 3-D analysis of three genes and their parent chromosomes, shows that the genes are positioned at the surface of their parent chromosomes, while two non-coding sequences are positioned randomly within the chromosome territory (Kurz et al., 1996).

While the higher-level organization of individual chromosomes has been an interesting question and the source of speculation for many years, the arrangement of all or most genes on the surface of the chromosome, with non-coding sequences residing at the interior of the territory was not predicted for good reasons. First, it seems an inefficient use of space, with the bulk of the chromosome domain not containing genes. If so, then what would be inside the inner regions of the territory? Secondly, separating genes from non-coding sequences and positioning them on the surface of the chromosome territory would seem to present a logistical nightmare for chromosome packaging. However, there are considerations that mitigate the apparent impossibility of this hypothetical chromosome territory organization and suggest functional implications. First, the bulk of the chromosome (>90%) is composed of non-coding sequences. All of this 'junk' DNA could serve to provide a framework that positions the 'important' genes properly at the surface of the chromosome. Second, this model would mean that metabolic components would only need to access the chromosome periphery. Such a design would be efficient for maximizing limited resources as it would increase the probability that components of the transcriptional apparatus would find their target. In such a manner, the relatively large nuclear size would be reduced to the manageable spaces on the surface and between chromosomes. Consistent with this, other results show that genes border splicing factor rich interchromatin domains (Xing et al., 1995). Finally, this seemingly complicated interphase pattern could possibly be simplified by the preexisting organization of the metaphase chromosome, i.e. gene-rich light G bands could somehow align themselves on the chromosome surface, with dark G bands constituting the interior of the interphase territory. Clearly much more work is needed to

confirm the true organization of the chromosome territory, but the results presented here definitely provide a provocative indication that genes are highly ordered in the interphase chromosome territory.

XIST RNA Does not Exploit Organizational Differences At The Single Gene Level to Affect X-Inactivation

The gene mapping experiments are also informative on another level as they allow us to distinguish between the two models of potential Xi organization proposed in chapter I (figure 1-11). In the first model, the active and inactive genes on Xi assume cytologically indistinguishable positions, hence no differences in their gross location relative to XIST RNA would be detected. In the second model, active and inactive genes assume cytologically distinguishable positions with the heterochromatinized genes internal and active genes available for transcription in the outer chromosome territory. To distinguish between these two models, the transcriptional status of the two Xp-linked genes studied here is important. While *ZFX* has been shown to escape inactivation and hence is expressed from both human X chromosomes (Schneider-Gadicke et al., 1989), previous evidence that *UBE1* escapes inactivation was contradictory (Brown and Willard, 1989; Zacksenhaus and Sheinin, 1990). To directly demonstrate that *UBE1* is expressed from the inactive X chromosome, *UBE1* transcripts were detected in normal diploid fibroblasts (Figure 3-12 and Carrell et al., 1996). With XIST RNA as a control for Xi, it is apparent that *UBE1* is transcribed from both the active and inactive X

chromosome (Figure 3-12). As *UBE1* and *ZFX* both position on the border of the XIST RNA domain during interphase, this suggests that active and inactive genes are organized in cytologically indistinguishable positions within the territory, supporting the first model. Additionally, this implies that X-inactivation does not occur through sequesteration of inactive sequences to transcriptionally inert chromosomal regions.

Although within an inactive chromosome, genes which do and do not escape inactivation may have similar positioning on the border of the XIST RNA territory, it is still possible that bulk chromatin needs to be organized with respect to XIST RNA in order for inactivation of any genes to occur. Results showing that XIST RNA is part of the nuclear structure (Chapter I) and other evidence that residual XIST RNA often binds only to the surface of the X-chromosome (Figure 1-5, B and Figure 3-7, B) combine with the above model of chromosome organization to suggest that regions of enhanced XIST RNA association may be along the potentially gene-rich chromosome territory surface. Moreover, in the t(X;14) studied here it was shown that the early replicating distal autosomal segment was not painted by XIST RNA and that this segment contains genes that localize outside of the XIST RNA territory, at least one of which was shown to be transcriptionally active (*NP*). These results raise the possibility that there is a gross organizational difference between active and inactive chromatin segments with respect to the XIST RNA territory. Furthermore, results which show that translocated autosomal and X sequences remain as separate interphase entities combine with these results

to suggest that distal regions of the translocated autosome may not be considered by XIST RNA to be part of the chromosome territory. Ultimately this would imply that *XIST* may exploit or impact upon higher-level chromosome territory organization in order to affect X-inactivation.

While much progress has been made, the work presented in this chapter is yet to be fully completed for publication. Presently, I am investigating the positions of specific genes on metaphase chromosomes labeled with BrdU to more precisely define the boundary of late and intermediate replication. Specifically, I will investigate whether *c-FOS* is in a region of late replication (as opposed to intermediate). This distinction may be important in determining if there are specific differences in properties of autosomal chromatin bound by XIST RNA. I plan to examine the transcriptional activity and interphase position of more centrally located genes on both the X;14 and X;9. Additionally, analysis of clonal populations of early versus late passage translocation cells is planned to assess whether the relationship between XIST RNA and autosomal sequences is progressively lost, and if this has any long-term affect on transcriptional activity or replication status. Future studies directed at elucidating the organizational framework of the interphase chromosome will examine the position of known chromosome organizational motifs such as light and dark bands for their orientation in the chromosome territory.

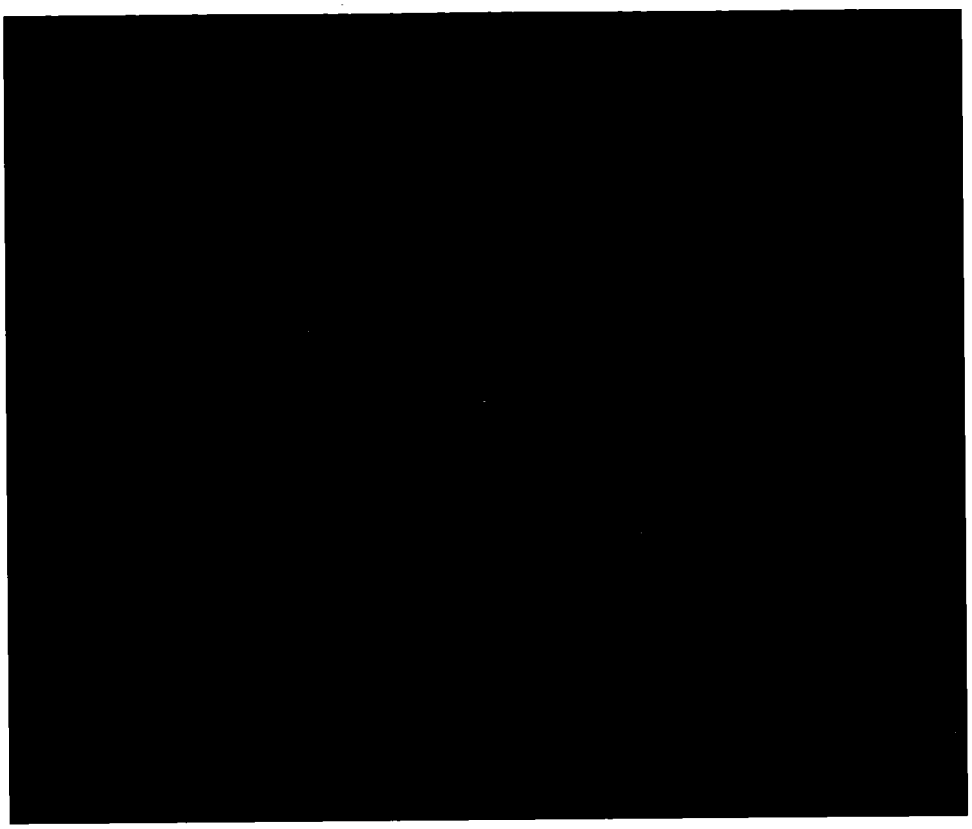


Figure 3–12: *UBE1* is Expressed from the Inactive X Chromosome in Diploid Fibroblasts
Using XIST RNA (*red*) as a marker for the inactive X chromosome, the expression of *UBE1* was analyzed in WI–38 cells under non–denaturing conditions (*nuclei counterstained with DAPI –blue*). UBE1 RNA signals (*green*) were apparent from both the active and inactive X chromosome, demonstrating clearly that *UBE1* escapes X–inactivation. The relative intensity of the two signals from each chromosome was variable from cell to cell, with no consistent difference between the two.

EPILOGUE

The work described in these three chapters has been instrumental in developing an understanding of the specific role of XIST RNA. Moreover, these results have increased our understanding of the process of X-inactivation, which has fundamental implications for development in a broader context. While the processes of differentiation and development are often narrowly conceptualized in terms of individual gene regulation, these processes are more globally characterized by a progressive restriction in the competence for expression of large segments of the genome. The progression from the totipotent zygote to the terminally differentiated cell involves lineage-specific packaging of many genomic sequences into inactivated heterochromatin. The most dramatic example of this global regulation is the heterochromatinization of one X chromosome during early embryonic development in mammalian females. The findings detailed here provide an exciting precedent for a novel and unanticipated role for RNA in this global silencing mechanism.

RNA may be uniquely qualified for *cis*-acting chromosome regulation, as it provides a mechanism of limiting regulation to specific homologs or alleles. Results in Chapter I reveal not only that XIST RNA structurally associates with architectural elements of the X chromosome territory, but also that it is most likely functional in the nucleus. These results have paved the way for the discovery of an entire class of RNAs that appear to play a role in chromatin regulation.

Several novel RNAs have been recently described that appear to be involved in imprinting, a process which culminates in haploid expression of diploid genes by some as yet unknown epigenetic process. Recently non-coding RNAs, that have a striking similarity

to XIST RNA, have been identified and strongly implicated in this epigenetic regulation. The incorrect expression of imprinted genes is often associated with a genetic disorder. *IPW*, an imprinted gene involved in Prader-Willi Syndrome, produces a stable, non-coding transcript (reviewed in Barlow, 1997). *UBE3A* is involved in Angelman Syndrome and expression of this maternally expressed gene is repressed by the expression of an oppositely imprinted antisense RNA that is embedded in its own intron (Rougeulle et al., 1998). Similarly, expression of *Igf2r* is regulated by the expression of *AS*, an intron-embedded antisense RNA (Wutz et al., 1997). The *H19/Igf2* genes are an oppositely imprinted pair of genes involved in Beckwith/Weidemann Syndrome. Like *XIST*, *H19* produces a large non-coding RNA that appears to play a role in gene silencing (Bartolomei et al., 1991). Expression of the H19 RNA apparently has long range *cis* affects as its expression inhibits the expression not only of *Igf2* which is 90 kb downstream of *H19*, but also *Insulin2* which is 100 kb downstream (Barlow, 1997). Therefore, imprinting provides other examples of genes like *XIST* which are imprinted, do not appear to code for a protein, and have long range *cis* effects on gene expression. It is interesting to speculate that the role of *XIST* has evolved from the regulation of a few imprinted genes, to the control of thousands of X-linked genes.

The recent discoveries of non-coding RNAs involved in regulation of imprinted gene expression has lead to the "expression-competition model" in which expression of an 'imprintor' disallows the expression of a 'target gene(s)' by virtue of limited transcription factors (Barlow, 1997; Bartolomei and Tilghman, 1992). However, it is difficult to extend this model to the role of *XIST* in the silencing of tens of thousands of X-linked genes. It

seems unlikely that the expression of this single gene would be able to compete for factors from thousands of genes, most of them far removed from *XIST* expression. The results in chapter I show that the inactive X chromosome in not associated with domains rich in splicing components. Therefore, perhaps competition for transcription factors is not necessary as XIST RNA may be involved in sequestering the bulk of the chromosome territory into an area depleted of metabolic factors.

Non-coding RNAs have also been implicated in Drosophila gene dosage compensation. *rox1* and *rox2* map to the X chromosome in Drosophila and are regulated by the gene dosage compensation system (Amrein and Axel, 1997; Meller et al., 1997). In contrast to X-inactivation, Drosophila gene dosage is accomplished by the up-regulation of the single X in males. Importantly, rox1 RNA specifically associates with the decondensed, up-regulated X chromosome, essentially painting it in a manner similar to the XIST RNA and the inactive X chromosome (Meller et al., 1997), providing another example of an RNA involved in chromatin remodeling.

While dosage compensation in flies involves up-regulation, this process is accomplished in nematodes by the down-regulation of the two X chromosomes in hermaphrodites to the level of the single X in males (reviewed in Cline and Meyer, 1996). Many genes are known to be required for and involved in the processes of dosage compensation in flies and worms (reviewed in Cline and Meyer, 1996), however only one gene, *XIST*, has been implicated in dosage compensation in mammals. Studies on X;autosome translocations have revealed that X-inactivation is a process that involves a single *cis*-acting switch that maps to the X-inactivation center. No other gene besides *XIST*

has been mapped to this region, and more importantly *XIST* has been shown to be necessary and sufficient for most of the steps in X-inactivation.

X-inactivation is thought to occur in several steps: 1) counting of X chromosomes, 2) choice as to which X chromosome remains active, 3) initiation of X-inactivation, 4) spreading of inactivation throughout the entire chromosome and 5) maintenance of X-inactivation. While it was originally thought that the single active X was chosen, recently it has been shown that *Xist* is the controlling element for choice in X-inactivation, and that the choice is actually which X chromosome is *inactivated.* In targeted *Xist* deletions, only those chromosomes that retain a 6 kb choice element are chosen for inactivation (Marahens et al., 1997) . In other *Xist* deletions, that don't remove the 6 kb choice element, the deleted chromosome can still be chosen for, but is not able to complete, X-inactivation (Penny et al., 1996). This finding not only confirms that *Xist* is responsible for choosing the inactive chromosome, but also shows that *Xist* is necessary for *initiation* of X-inactivation. Other results have shown that expression of *Xist* from an autosome in transgenic mice causes that chromosome to manifest signs of inactivation, demonstrating that *Xist* is sufficient for initiation of X-inactivation (Herzing et al., 1997; Lee and Jaenisch, 1997).

Methylation at the *Xist* locus has been shown to be important for the regulation of X-inactivation. In methyltransferase mutants, the *Xist* gene is active in male cells, causing inactivation of the single X chromosome and presumably death to the embryo (Panning and Jaenisch, 1996). In normal embryo development the paternal X is non-randomly inactivated in extraembryonic lineages. Imprinted inactivation of the paternal allele in extraembryonic lineages has been definitively shown to be caused by preemptive methylation of the

maternal *Xist* allele (Norris et al., 1994). The paternal *Xist* allele has no such methylation, so is expressed exclusively, causing inactivation of the paternal X. In the embryo proper either the maternal or paternal X is randomly chosen for inactivation such that the female is a mosaic composed of 50% of cells expressing the maternally contributed X and 50% expressing the paternal X. Since differential methylation of the paternal and maternal *Xist* alleles precedes X-inactivation in the developing embryo, Xist methylation also correlates with random X-inactivation (Norris et al., 1994).

These results combine with the fact that no other gene has been identified, to suggest that *Xist* is the master element for X-inactivation, and further suggest that it is the only element involved in this process. However, it is clear that the regulation of Xist expression relies on some other process. During embryogenesis, just prior to random X-inactivation, methylation imprints are erased, so the identities of the two *Xist* alleles are erased (Kay et al., 1994). Both alleles of *Xist* are expressed as unstable transcripts producing tiny foci from both X chromosomes. Ultimately one *Xist* allele is stabilized on what will be the inactive X, while the other allele on the active X continues to produce labile transcripts, until it is ultimately methylated and silenced (Panning et al., 1997; Sheardown et al., 1997). Therefore, some mechanism must provide for the stabilization or the lack of stabilization at the *Xist* locus. It is possible that some imprint remains, such that the active X chromosome produces a factor that disallows its Xist transcripts to be stable; alternatively, the inactive X could produce stabilization factors. It is clear that certain mutations in the Xist promoter can skew the randomness of X-inactivation (Plenge et al., 1997) suggesting that the *Xist*

sequence may control its stability. Perhaps variations in the 6 kb choice element can influence the chances that the Xist transcripts will be stable.

My results in Chapter II demonstrate that just stabilization of the Xist transcripts is not sufficient for localization, and further demonstrate that Xist RNA does not act alone to initiate or maintain X-inactivation. This result indicates that X-inactivation is a multi-step process that is initiated with XIST RNA at a specific time during development, but that absent other developmental factors, the RNA cannot establish *de novo* inactivation. This result further suggests that Xist RNA localization is not the true effector of gene silencing, that other processes like methylation and underacetylation actually work to silence the genes. The fact that there are not enough enzymes in somatic cells to establish large scale patterns of *de novo* methylation and underacetylation (reviewed in Razin and Shemer, 1995; Turker and Bestor, 1997), may explain why I did not detect X-inactivation in adultscells when the Xist RNA was properly localized.

It is interesting to speculate on how an RNA could be responsible for global transcriptional regulation. It is well established that candidate processes for transcriptional silencing such as methylation and underacetylation are established after, but temporally close to *Xist* expression and gene silencing (Keohane et al., 1996; Lock et al., 1987; Migeon, 1994). These results suggest that Xist RNA localization itself may result in some gene silencing. The fact that late replication timing patterns are established soon after *Xist* expression (Keohane et al., 1996) suggests that Xist RNA may bind to and suppress the firing of replication origins (Panning and Jaenisch, 1998). My results in Chapter II suggest that Xist localization serves to recruit other factors that play a critical role in transcriptional

silencing. Perhaps the RNA has an inherent affinity for enzymes such as *de novo* methlytransferases or deacetylases thereby recruiting them to Xi. Alternatively, perhaps Xist localization causes the change in chromatin structure that so well characterizes Xi, thereby allowing these factors access to critical regions.

Others have concluded that *Xist* is not required for maintenance, the final stage of X-inactivation (Brown and Willard, 1994), however my results in chapter III begin to dispel that notion. These results are the first to show that the autosomal chromatin shows less complete or weaker association with XIST RNA. They further suggest that the instability of autosomal X-inactivation may be caused by this limited association. This important result suggests that X-inactivation is maintained not only by processes such as methylation and underacetylation, but that XIST RNA may also play a role in ensuring that patterns of X-inactivation are rigorously maintained.

While the specificity of XIST RNA for X sequences might have appeared to explain the strict *cis*-limitation to X-inactivation, results in Chapter III showed that this clearly is not the case. XIST RNA can associate with autosomal sequences, showing that X-inactivation must be limited not by the discrimination of DNA sequences by XIST RNA but by strict maintenance of chromosomal boundaries. Future directions will involve expanding these studies into the area of human clinical genetics. My post-doctoral research will include further studies on translocations to determine rigorously whether XIST RNA is progressively lost from autosomal sequences and whether this correlates with reactivation in patients with unbalanced X;autosome translocations. This work may have significant

clinical impact if regression of autosomal inactivation correlates with onset of phenotypic consequences in later life.

Throughout these studies results also contribute to our knowledge of chromosomal organization in general. Most notably, Chapter III provides evidence for a novel model of chromatin organization, one in which genes, regardless of activity, are generally found on the periphery of the chromosome territory. This not only advances a theory of overall chromosome organization, but it also proves that individual inactive genes are not packaged in a separate interphase chromosome region at a gross cytological level. Rather, mechanisms of X-inactivation most likely involve overall organizational differences in active and inactive chromosome territories.

I plan to utilize the unique pattern of residual XIST RNA on the mitotic chromosome to further advance knowledge of chromosomal organization. The fact that the minimal XIST RNA transcripts often adhere to just the surface of the chromosome (Figure 1-5, B and Figure 3-7, B), suggests that XIST RNA is either associating with or is a critical component of a cage around the chromosome territory. I have performed other experiments (not described here) which show that the residual mitotic murine Xist RNA not only hugs the periphery but oftentimes forms a banding pattern on mitotic mouse chromosomes (see cover photo). This result may for the first time point to critical sequence motifs involved in Xist RNA association, which in turn may correlate to motifs important for chromosomal organization.

References

Adler, D.A., S.L. Bressler, V.M. Chapman, D.C. Page, and C.M. Disteche. 1991. Inactivation of the Zfx gene on the mouse X chromosome. *Proc. Natl. Acad. Sci. USA*. 88:4592-4595.

Allderdice, P.W., O.J. Miller, D.A. Miller, and H.P. Klinger. 1978. Spreading of Inactivation in an (X;14) Translocation. *Am. J. Med. Genet.* 2:233-240.

Amrein, H., and R. Axel. 1997. Genes expressed in neurons of adult male drosophila. *Cell*. 88:459-469.

Barlow. 1997. Competition- a common motif for the imprinting mechanism? *EMBO*. 16:6899-6905.

Barr, M.L., and D.H. Carr. 1962. Correlation between sex chromatin and chromosomes. *Acta.Cytol.* 6:34-45.

Bartolomei, M.S., and S.M. Tilghman. 1992. Parental Imprinting of the mouse chromosome 7. *Semin. Dev. Biol.* 3:107-117.

Bartolomei, M.S., S. Zemel, and S.M. Tilghman. 1991. Parental Imprinting of the mouse H19 gene. *Nature*. 351:153-155.

Berezney, R., and D.S. Coffey. 1974. Identification of a nuclear protein matrix. *Biochem.Biophys.Res.Commun.* 60:1410-1419.

Bernhard, W. 1971. Drug-induced changes in the interphase nucleus. *In* Advances in Cytopharmacology. F. Clementi and B. Ceccarelli, editors. Raven Press, New York. 49-67.

Bettio, D., N. Rizzi, and D. Giardino. 1994. Familial translocation (X;3) (p22.3;p23): chromosomal *in situ* suppression (CISS) hybridization and inactivation pattern study. *Clinical Genetics*. 1994:360-363.

Bischoff, A., J. Albers, I. Kharboush, E. Stelzer, T. Cremer, and C. Cremer. 1993. Differences in size and shape of active and inactive X-chromosome domains in human amniotic fluid cell nuclei. *Micros.Res.Tech.* 25:68-77.

Blencowe, B.J., J.A. Nickerson, R. Issner, S. Penman, and P.A. Sharp. 1994. Association of nuclear matrix antigens with exon-containing splicing complexes. *J.Cell Biol.* 127(3):593-607.

Boggs, B.A., and A.C. Chinault. 1994. Analysis of replication timing properties of human X-chromosomal loci by fluorescence in situ hybridization. *Proc. Natl. Acad. Sci. USA*. 91:6083-6087.

Borsani, G., R. Tonlorenzi, M.C. Simmler, L. Dandolo, D. Arnaud, V. Capra, M. Grompe, A. Pizzuti, D. Musny, C. Lawrence, H.F. Willard, P. Avner, and A. Ballabio. 1991. Characterization of a murine gene expressed from the inactive X chromosome. *Nature*. 351:325-329.

Bregman, D.B., L. Du, s. van der Zee, and S.L. Warren. 1995. Transcription-dependent redistribution of the large subunit of RNA polymerase II to discrete nuclear domains. *J.Cell Biol.* 129(2):287-298.

Brockdorff, N., A. Ashworth, G.F. Kay, P. Cooper, S. Smith, V.M. McCabe, D.P. Norris, G.D. Penny, D. Patel, and S. Rastan. 1991. Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature*. 351:329-331.

Brockdorff, N., A. Ashworth, G.F. Kay, V.M. McCabe, D.P. Norris, P.J. Cooper, S. Swift, and S. Rastan. 1992. The Product of the Mouse Xist Gene is a 15 kb Inactive X-Specific Transcript Containing No Conserved ORF and Located in the Nucleus. *Cell*. 71:515-526.

Brown, C.J., A. Ballabio, J.L. Rupert, R.G. Lafreniere, M. Grompe, R. Tonlorenzi, and H.F. Willard. 1991a. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature*. 349:38-44.

Brown, C.J., L. Carrel, and H.F. Willard. 1997. Expression of genes from the human active and inactive X chromosomes. *Am. J. Hum. Genet.* 60:1333-1343.

Brown, C.J., A.M. Flenniken, B. Williams, and H.F. Willard. 1990. X chromosome inactivation of the human TIMP gene. *Nuc. Acids Res.* 18:4191-4195.

Brown, C.J., B.D. Hendrich, J.L. Rupert, R.G. Lafreniere, Y. Xing, J.B. Lawrence, and H.F. Willard. 1992. The Human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell*. 71:527-542.

Brown, C.J., R.G. Lafreniere, V.E. Powers, G. Sebastio, A. Ballabio, A. Pettigrew, D.H. Ledbetter, E. Levy, I.W. Craig, and H.F. Willard. 1991b. Localization of the X inactivation centre on the human chromosome in Xq13. *Nature*. 349:62-64.

Brown, C.J., and H.F. Willard. 1989. Noninactivation of a selectable human X-linked gene that complements a murine temperature-sensitive cell cycle defect. *Am. J. Hum. Genet.* 45(4):592-598.

Brown, C.J., and H.F. Willard. 1994. The human X inactivation center is not required for maintenance of X chromosome inactivation. *Nature*. 368:154-156.

Buzin, C.H., J.R. Mann, and J. Singer-Sam. 1994. Quantitative RT-PCR assays show XIST RNA levels are low in mouse female adult tissue, embryos and embryoid bodies. *Development*. 120:3529-3536.

Caiulo, A., B. Bardoni, G. Camerino, S. Guioli, A. Minelli, M. Piantanida, F. Crosato, T. Dalla Fior, and P. Maraschio. 1989. Cytogenetic and Molecular Analysis of an unbalanced translocation (X;7)(q28;p15) in a dysmorphic girl. *Hum. Genet.* 84:51-54.

Camargo, M., and J. Cervenka. 1984. DNA replication and inactivation patterns in structural abnormality of sex chromosomes. I.X-A translocations, rings, fragments, isochromosomes, and pseudo-isodicentrics. *Hum. Genet.* 67:37-47.

Carrell, L., C.M. Clemson, J.M. Dunn, A.P. Miller, P.A. Hunt, J.B. Lawrence, and H.F. Willard. 1996. X inactivation analysis and DNA methylation studies of the ubiquitin activating enzyme E1 and PCTAIRE-1 genes in human and mouse. *Hum. Mol. Genet.* 5:391-401.

Carter, K.C., D. Bowman, W. Carrington, K. Fogarty, J.A. McNeil, F.S. Fay, and J.B. Lawrence. 1993. A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science*. 259:1330-1335.

Carter, K.C., K.L. Taneja, and J.B. Lawrence. 1991. Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. *J.Cell Biol.* 115:1191-1202.

Cattanach, B.M. 1974. Position effect variegation in the mouse. *Genet. Res.* 23:291-306.

Chariot, A., and V. Castronovo. 1996. Detection of HOXA1 expression in human breast cancer. *Biochem. Biophys. Res. Comm.* 222:292-297.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Chuang, P.T., D.G. Albertson, and B.J. Meyer. 1994. DPY-27: A chromosome condensation protein homolog that regulates C. elegans dosage compensation through association with the X chromosome. *Cell.* 79:459-474.

Clemson, C., J.C. Chow, C.J. Brown, and J.B. Lawrence. 1998. Stabilization and Localization of Xist RNA are Controlled by Separate Mechanisms and are Not Sufficient for X-Inactivation. *J.Cell Biol.* submitted.

Clemson, C.M., J.A. McNeil, H.F. Willard, and J.B. Lawrence. 1996. 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.

Cline, T.W., and B.J. Meyer. 1996. Vive la difference: males vs females in flies vs worms. *Annu. Rev. Genet.* 30:637-702.

Couturier, J., B. Dutrillaux, P. Garber, O. Raoul, M. Croquette, J.C. Fourlinnie, and E. Maillard. 1979. Evidence for a Correlation Between Late Replication and Autosomal Gene Inactivation in a Familial Translocation t(X;21). *Hum. Genet.* 49:319-326.

Cremer, T., A. Kurz, R. Zirbel, S. Dietzel, B. Rinke, E. Schrock, M.R. Speicher, U. Mathieu, A. Jauch, P. Emmerich, H. Scherthan, T. Ried, C. Cremer, and P. Lichter. 1993. Role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold Spring Harbor Symp.Quant.Biol.* LVIII:777-792.

Dirks, R.W., F.M. van de Rijke, S. Fujishita, M. Van der Ploeg, and A.K. Raap. 1993. Methodologies for specific intron and exon RNA localization in cultured cells by haptenized and fluorochromized probes. *J.Cell Science.* 104:1187-1197.

Disteche, C.M. 1995. Escape from X inactivation in human and mouse. *TIG.* 11(1):17-22.

Disteche, C.M., E.M. Eicher, and S.A. Latt. 1979. Late replication in an X-autosome translocation in the mouse: correlation with genetic inactivation and evidence for selective effects during embryogenesis. *Proc. Natl. Acad. Sci. USA.* 76:5234-5238.

Disteche, C.M., K. Swisshelm, S. Forbes, and R.A. Pagon. 1984. X-inactivation patterns in lymphocytes and skin fibroblasts of three cases of X-autosome translocations with abnormal phenotypes. *Hum. Genet.* 66:71-76.

Driscoll, D.J., and B.R. Migeon. 1990. Sex difference in methylation of single-copy genes in human meiotic germ cells: implications for X chromosome inactivation, parental imprinting and origin of CpG mutations. *Somat.Cell Molec.Genet.* 16:267-282.

Duncan, A.M.V., A. Macdonald, C.J. Brown, D. Wolff, H.F. Willard, and B. Sutton. 1993. Characterization of a small supernumerary ring X chromosome by fluorescence in situ hybridization. *Am. J. Med. Genet.* 47:1153-1156.

Earnshaw, W.C., I. Ratrie, H., and G. Stetten. 1989. Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma.* 98(1):1-12.

Eden, S., and H. Cedar. 1994. Role of DNA methylation in the regulation of transcription. *Current Opinion in Genetics and Development.* 4(2):255-259.

Eicher, E.M. 1970. X-autosome translocations in the mouse: total inactivation versus partial inactivation of the X chromosome. *Adv. Genet.* 15:175-259.

Eils, R., S. Dietzel, E. Bertin, E. Schrock, M. Speicher, T. Ried, M. Robert-Nicoud, C. Cremer, and T. Cremer. 1996. Three-Dimensional Reconstruction of Painted Human Interphase Chromosomes: Active and Inactive X Chromosome Territories Have Similar Volumes but Differ in Shape and Surface Structure. *J.Cell Biol.* 135:1427-1440.

Ejima, Y., M.S. Sasaki, A. Kaneko, H. Tanooka, Y. Hara, T. Hida, and Y. Kinoshita. 1982. Possible inactivation of part of chromosome 13 due to 13qXp translocation associated with retinoblastoma. *Clinical Genetics.* 21:357-361.

Fakan, S., and W. Bernhard. 1973. Nuclear labeling after prolonged ^{3}H-uridine incorporation as visualized by high resolution autoradiography. *Exp.Cell Res.* 79:431-444.

Fakan, S., and E. Puvion. 1980. The ultrastructural visualization of nuclear and extranucleolar RNA synthesis and distribution. *Int.Rev.Cytol.* 65:255-299.

Fay, F.S., W. Carrington, and K.E. Fogarty. 1989. Three-dimensional molecular distribution in single cells analyzed using the digital imaging microscope. *J.Microscopy.* 153:133-149.

Fey, E.G., P. Bangs, C. Sparks, and P. Odgren. 1991. The Nuclear Matrix: Defining Structural and Functional Roles. *Eukar.Gene Exp.* 1(2):127-143.

Fey, E.G., G. Krochmalnic, and S. Penman. 1986a. The non-chromatin substructures of the nucleus: the ribonucleoprotein RNP-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J.Cell Biol.* 102:1654-1665.

Fey, E.G., D.A. Ornelles, and S. Penman. 1986b. Association of RNA with the cytoskeleton and the nuclear matrix. *J.Cell Sci.* Suppl.5:99-119.

Fu, X.-D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature.* 343:437-444.

Garcia-Heras, J., J.A. Martin, S.F. Witchel, and P. Scacheri. 1997. De novo der (X)t(X;10)(q26;q21) with features of distal trisomy 10q: case report of paternal origin identified by late replication with BrdU and the human androgen receptor assay (HAR). *Journal of Medical Genetics.* 34:242-245.

Gartler, S.M., K.A. Dyer, and M.A. Goldman. 1992. Mammalian X Chromosome inactivation. *Mol.Gen.Med.* 2:121-160.

Gartler, S.M., and A.D. Riggs. 1983. Mammalian X-chromosome inactivation. *Ann.Rev.Genet.* 17:155-190.

Gerdes, M.G., K.C. Carter, P.T. Moen, and J.B. Lawrence. 1994. Dynamic changes in the higher-level chromatin organization of specific sequences revealed by *in situ* hybridization to nuclear halos. *J.Cell Biol.* 126(2):289-304.

Goldberg, Y.P., V.D. Leaner, and M.I. Parker. 1992. Elevation of Large-T Antigen Production by Sodium Butyrate Treatment of SV40-Transformed WI-38 Fibroblasts. *J. Cell. Biochem.* 49:74-81.

Grant, M., M. Zuccotti, and M. Monk. 1992. Methylation of CpG sites of two X-linked genes coincides with X-inactivation in the female mouse embryo but not in the germ line. *Nature Gen.* 2(2):161-166.

Grant, S.G., and V.M. Chapman. 1988. Mechanisms of X-chromosome regulation. *Ann.Rev.Genet.* 22:199-233.

Green, R., and D. Shields. 1984. Sodium Butyrate Stimulates Somatostatin Production by Cultured Cells. *Endocrinology.* 114:1990-1994.

Greenberg, M.E., A.L. Hermanowski, and E.B. Ziff. 1986. Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Mol. Cell. Biol.* 6:1050-1057.

Gregory, P., C. Greene, E. Shapira, and E. Wang. 1985. Alterations in the time of X chromosome replication induced by 5-azacytidine in a patient with 48,XXXY/47,XXY. *Cytogenet. Cell Genet.* 39:234-236.

Grumbach, M.M., A. Morishma, and H. Taylor. 1963. Human sex chromosome abnormalities in relation to DNA replication and heterochromatinization. *Proc.Natl.Acad.Sci.USA.* 49:581-589.

Haaf, T., G. Ott, and M. Schmid. 1988. Inhibition of condensation in the late-replicating X chromosome induced by 5-azadeoxycytidine in human lymphocyte cultures. *Hum. Genet.* 79:18-23.

Hagemeijer, A., J. Hoovers, E.M. Smit, and D. Bootsma. 1977. Replication pattern of the X chromosomes in three X/autosomal translocations. *Cytogenet. Cell Genet.* 18:333-348.

Hansen, R.S., T.K. Canfield, A.D. Fjeld, and S.M. Gartler. 1996. Role of late replication timing in the silencing of X-linked genes. *Hum. Mol. Genet.* 5:1345-1353.

Hansen, R.S., T.K. Canfield, A.M. Stanek, E.A. Keitges, and S.M. Gartler. 1998. Reactivation of the XIST gene. *submitted.*

Harpold, M.M., M.C. Wilson, and J. Darnell, J.E. 1981. Chinese hamster polyadenylated messenger ribonucleic acid: relationship to non-polyadenylated sequences and relative conservation during messenger ribonucleic acid processing. *Mol.Cell Biol.* 1(2):188-198.

He, D., J.A. Nickerson, and S. Penman. 1990. Core filaments of the nuclear matrix. *J.Cell Biol.* 110:569-580.

Hendrich, B., C.J. Brown, and H.F. Willard. 1993. Evolutionary conservation of possible functional domains of the human and murine XIST genes. *Hum.Molec.Gen.* 2(6):663-672.

Herman, R., L. Weymouth, and S. Penman. 1978. Heterogeneous nuclear RNA-protein fibers in chromatin-depleted nuclei. *J.Cell Biol.* 78:663-674.

Herman, R.C., and S. Penman. 1977. Multiple Decay Rates of Heterogeneous Nuclear RNA in HeLa Cells. *Biochem.J.* 16:3460-3465.

Herzing, L.B.K., J.T. Romer, J.M. Horn, and A. Ashworth. 1997. Xist has properties of the X-chromosome inactivation centre. *Nature.* 386:272-275.

Hogan, N.C., K.L. Traverse, D.E. Sullivan, and M.-L. Pardue. 1994. The nucleus-limited Hsr-Omega-n transcript is a polyadenylated RNA with a regulated intranuclear turnover. *J.Cell Biol.* 125(1):21-30.

Holmquist, G.P. 1987. Role of Replication Time in the Control of Tissue-Specific Gene Expression. *J. Hum. Genet.* 40:151-173.

Huang, S., T.J. Deerinck, M.H. Ellisman, and D.L. Spector. 1994. In vivo analysis of the stability and transport of nuclear poly(A)+ RNA. *J.Cell Biol.* 126(4):877-899.

Huang, S., and D.L. Spector. 1991. Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes & Development.* 5:2288-2302.

Jackson, D.A., S.J. McCready, and P.R. Cook. 1981. RNA is synthesized at the nuclear cage. *Nature.* 292:552-555.

Jeppesen, P., and B.M. Turner. 1993. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell.* 74:281-289.

Johnson, C.V., R.H. Singer, and J.B. Lawrence. 1991. Fluorescent detection of nuclear RNA and DNA: Implication for genome organization. *Methods Cell Biol.* 35:73-99.

Kay, G.F., S.C. Barton, M.A. Surani, and S. Rastan. 1994. Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development. *Cell.* 77:639-650.

Kay, G.F., G.D. Penny, D. Patel, A. Ashworth, N. Brockdorff, and S. Rastan. 1993. Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell.* 72:171-182.

Keitges, E.A., and C.G. Palmer. 1986. Analysis of spreading of inactivation in eight X autosome translocations utilizing the high resolution RBG technique. *Hum.Genet.* 72:231-236.

Keohane, A.M., L.P. O'Neill, N.D. Belyaev, J.S. Lavender, and B.M. Turner. 1996. X-inactivation and histone H4 acetylation in embryonic stem cells. *Dev. Biol.* 180:618-630.

Kloc, M., G. Spohr, and L.D. Etkin. 1993. Translocation of repetitive RNA sequences with the germ plasm in Xenopus oocytes. *Science.* 262:1712-1714.

Krumlauf, R., V.M. Chapman, R.E. Hammer, R. Brinster, and S.M. Tilghman. 1986. Differential expression of alpha-fetoprotein genes on the inactive X chromosome in extraembryonic and somatic tissues of a transgenic mouse line. *Nature*. 319:224-226.

Kuroda, M.I., M.J. Kernan, R. Kreber, B. Ganetzky, and B.S. Baker. 1991. The maleless protein associates with the X Chromosome to regulate dosage compensation in Drosophila. *Cell*. 66:935-947.

Kurz, A., S. Lampel, J.E. Nickolencko, J. Bradl, A. Benner, R.M. Zirbel, and P. Lichter. 1996. Active and Inactive Genes Localize to the Periphery of Chromosome Territories. *J.Cell Biol.* 135:1195-1205.

Lawrence, J.B., K.C. Carter, and X. Xing. 1993. Probing Functional Organization within the Nucleus: Is Genome Structure Integrated with RNA Metabolism? *Cold Spring Harbor Symp.on Quant.Biol.* LVIII:807-818.

Lawrence, J.B., R.H. Singer, and L.M. Marselle. 1989a. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell*. 57:493-502.

Lawrence, J.B., K. Taneja, and R.H. Singer. 1989b. Temporal resolution and sequential expression of muscle-specific genes revealed by in situ hybridization. *Dev.Biol.* 133:235-246.

Lawrence, J.B., C.A. Villnave, and R.H. Singer. 1988. Sensitive high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell*. 52:51-61.

Lee, J.T., and R. Jaenisch. 1997. Long-range cis effects of ectopic X-inactivation centres on a mouse autosome. *Nature*. 386:275-279.

Lee, J.T., W.M. Strauss, J.A. Dausman, and R. Jaenisch. 1996. A 450kb Transgene Displays Properties of the Mammalian X-Inactivation Center. *Cell*. 86:83-94.

Leisti, J.T., M.M. Kaback, and D.L. Rimoin. 1975. Human X-Autosome translocations: Differential Inactivation of the X Chromosome in a Kindred with an X-9 Translocation. *Am.J.Hum.Genet.* 27:441-453.

Levis, R., and S. Penman. 1977. The metabolism of poly(A)+ and poly(A)- hnRNA in cultured Drosophila cells studied with a rapid uridine pulse-chase. *Cell*. 11:105-113.

Lewin, B. 1975. Units of transcription and translation: sequence components of heterogeneous nuclear RNA and messenger RNA. *Cell.* 4:77-93.

Lewin, B. 1990. Chromatin structure: the nucleosome. *In* Genes II. John Wiley & Sons.

Lichter, P., T. Cremer, J. Borden, L. Manuelidis, and D.C. Ward. 1988. Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum.Genet.* 80:224-234.

Lock, L.F., N. Takagi, and G.R. Martin. 1987. Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. *Cell.* 48(1):39-46.

Lutzker, S., and F.W. Alt. 1988. Structure and expression of germ line immunoglobulin gamma 2B transcripts. *Mol.Cell Biol.* 8(4):1849-1852.

Lyon, M.F. 1961. Gene action in the X chromosome of the mouse (Mus. musculus L.). *Nature.* 190:372-373.

Marahens, Y., B. Panning, J. Dausman, W. Strauss, and R. Jaenisch. 1997. Xist deficient mice are defective in dosage compensation but not spermatogenesis. *Genes & Dev.* 11:156-166.

Markovic, V.D., D.W. Cox, and J. Wilkinson. 1985. X;14 Translocation: An Exception to the Critical Region Hypothesis on the Human X-Chromosome. *Am. J. Med. Genet.* 20:87-96.

Mattei, M.G., J.F. Mattei, I. Vidal, and F. Giraud. 1981. Structural anomalies of the X chromosome and inactivation center. *Hum.Genet.* 56:401-408.

McCarrey, J.R., and D.D. Dilworth. 1992. Expression of *Xist* in mouse germ cells correlates with X-chromosome inactivation. *Nature Gen.* 2:200-203.

Meller, V.H., K.H. Wu, G. Roman, M.I. Kuroda, and R.L. Davis. 1997. rox1 RNA paints the X chromosome of male drosophila and is regulated by the dosage compensation system. *Cell.* 88:445-457.

Migeon, B.R. 1994. X-chromosome inactivation: molecular mechanisms and genetic consequences. *Trends Gent.* 10(7):230-235.

Migeon, B.R., S. Luo, M. Jani, and P. Jeppesen. 1994. The severe phenotype of females with tiny ring X chromosomes is associated with inability of these chromosomes to undergo X inactivation. *Am.J.Hum.Genet.* 55:497-504.

Migeon, B.R., S. Luo, B.A. Stasiowski, M. Jani, J. Axelman, D.L. VanDyke, L. Weiss, P.A. Jacobs, T.L. Yang-Feng, and J.E. Wiley. 1993. Deficient transcription of XIST from tiny ring X chromosomes in females with severe phenotypes. *Proc.Natl.Acad.Sci.(USA).* 90:12025-12029.

Minshull, J., and T. Hunt. 1986. The use of the single-stranded DNA and RNAse H to promote hybrid arrest of translation of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. *Nucl.Acids Res.* 14:6433-6451.

Mohandas, T., R.S. Sparkes, and L.J. Shapiro. 1982. Genetic Evidence for the Inactivation of a Human Autosomal Locus Attached to an Inactive X Chromosome. *Hum. Genet.* 34:811-817.

Monk, M. 1986. Methylation and the X chromosome. *BioEssays.* 4:204-208.

Nickerson, J.A., G. Krochmalnic, K.M. Wan, and S. Penman. 1989. Chromatin architecture and nuclear RNA. *Proc.Natl.Acad.Sci.USA.* 86:177-181.

Norris, D.P., D. Patel, G.F. Kay, G.D. Penny, N. Brockdorff, S.A. Sheardown, and S. Rastan. 1994. Evidence that random and imprinted Xist expression is controlled by preemptive methylation. *Cell.* 77:41-51.

Ogryzko, V.V., T.H. Hirai, V.R. Russanova, D.A. Barbie, and B.H. Howard. 1996. Human Fibroblast Commitment to a Senescence-Like State in Response to Histone Deacetylase Inhibitors is Cell Cycle Dependent. *Molecular and Cellular Biology.* 16:5210-5218.

Pallister, P.D., and J.M. Opitz. 1978. The KOP Translocation. *Birth Defects: Original Article Series.* XIV:133-146.

Palmer, C.G., T.W. Hubbard, G.W. Henry, and D.D. Weaver. 1980. Failure of X inactivation in the autosomal segment of and X/A translocation. *Am. J. Hum. Genet.* 32:179-187.

Panning, B., J. Dausman, and R. Jaenisch. 1997. X chromosome inactivation is mediated by *Xist* RNA stabilization. *Cell.* 90:907-916.

Panning, B., and R. Jaenisch. 1996. DNA hypomethylation can activate Xist expression and silence X-linked genes. *Genes & Dev.* 10:1991-2002.

Panning, B., and R. Jaenisch. 1998. RNA and the Epigenetic Regulation of X Chromosome Inactivation. *Cell.* 93:305-308.

Penny, G.D., G.F. Kay, S.A. Sheardown, S. Rastan, and N. Brockdorff. 1996. Requirement for XIST in X chromosome inactivation. *Nature*. 379:131-137.

Pfeifer, G.P., S.D. Steigerwald, R.S. Hansen, S.M. Gartler, and A.D. Riggs. 1990. Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: Methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. *Proc. Natl. Acad. Sci. USA*. 87:8252-8256.

Pfeifer, K., and S.M. Tilghman. 1994. Allele-specific gene expression in mammals: the curious case of imprinted RNAs. *Genes & Development*. 8(16):1867-1874.

Pinkel, D., J. Landegent, C. Collins, J. Fuscoe, R. Seagraves, J. Lucas, and J. Gray. 1988. Fluorescence in situ hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocations of chromosome 4. *Proc.Natl.Acad.Sci.,USA*. 85:9138-9142.

Pinol-Roma, S., and G. Dreyfuss. 1991. Transcription-Dependent and Transcription-Independent Nuclear Transport of hnRNP Proteins. *Science*. 253:312-314.

Plenge, R.M., B.D. Hendrich, D. Schwartz, J.F. Arena, A. Naumova, C. Sapienza, R.M. Winter, and H.F. Willard. 1997. A promoter mutation in the Xist gene in two unrelated families with skewed X-chromosome inactivation. *Nature Genet.* 17:353-356.

Preis, W., G. Barbi, S. Liptay, I. Kennerknecht, S. Schwemmle, and F. Pohlandt. 1996. X/autosome translocation in three generations ascertained through an infant with trisomy 16p due to failure of spreading of X-inactivation. *American Journal of Medical Genetics*. 61:117-121.

Qian, F., U. Kruse, P. Lichter, and A.E. Sippel. 1995. Chromosomal localization of the four genes (NFIA,B,C and X) for the human transcription factor nuclear factor I by FISH. *Genomics*. 28:67-73.

Rack, K.A., J. Chelly, R.J. Gibbons, S. Rider, D. Benjamin, R.G. Lafreniere, D. Oscier, R.W. Hendriks, I.W. Craig, H.F. Willard, A.P. Monaco, and V.J. Buckle. 1994. Absence of the XIST gene from late-replicating isodicentric X chromosomes in leukaemia. *Hum. Mol. Genet.* 3(7):1053-1059.

Rastan, S. 1994. X-Chromosome inactivation and the Xist gene. *Cur.Opin.Genet.Dev.* 4:292-297.

Razin, A., and R. Shemer. 1995. DNA methylation patterns in the mammalian genome. *Hum. Mol. Genet.* 4:1751-1755.

Richler, C., H. Soreq, and J. Wahman. 1992. X inactivation in mammalian testis is correlated with inactive X specific transcription. *Nature Gen.* 2:192-195.

Rougeulle, C., C. Cardoso, M. Fontes, L. Colleaux, and M. Lalande. 1998. An imprinted antisense RNA overlaps UBE3A and a second maternally epxressed transcript. *Nat. Genet.* 19:17-18.

Russell, L.B. 1963. Mammalian X chromosome action: inactivation limited in spread and in region of origin. *Science.* 140:976-978.

Salditt-Georgieff, M., M.M. Harpold, M.C. Wilson, and J.E. Darnell Jr. 1981. Large heterogeneous nuclear ribonucleic acid has three times as many 5' caps as polyadenylic acid segments, and most caps do not enter polyribosomes. *Mol.Cell.Biol.* 1(2):179-187.

Salido, E.C., P.H. Yen, K.M. Thuluvancheri, and L.J. Shapiro. 1992. Expression of the X-inactivation associated gene *XIST* during spermatogenesis. *Nature Gen.* 2:196-199.

Schanz, S., and P. Steinbach. 1989. Investigation of the "variable spreading" of X inactivation into a translocated autosome. *Hum. Genet.* 82:244-248.

Schmidt, M., and B.R. Migeon. 1990. Asynchronous replication of homologous loci on human active and inactive X chromosomes. *Proc. Natl. Acad. Sci. USA.* 87:3685-3689.

Schneider-Gadicke, A., P. Beer-Romano, L.G. Brown, R. Nussbaum, and D.C. Page. 1989. ZFX has a gene structure similar to ZFY, the putative human sex determinant, and escapes X inactivation. *Cell.* 57:1247-1258.

Sheardown, S.A., S.M. Duthie, C.M. Johnston, A.E.T. Newall, E.J. Formstone, R.M. Arkell, T.B. Nesterova, G.C. Alghisi, S. Rastan, and N. Brockdorff. 1997. Stabilization of Xist RNA mediates initiation of X chromosome inactivation. *Cell.* 91:99-107.

Singer-Sam, J., L. Goldstein, A. Dai, S.M. Gartler, and A.D. Riggs. 1992. A potentially critical Hpa II site of the X chromosome-linked *PGK-1* gene is unmethylated prior to the onset of meiosis of human oogenic cells. *Proc.Natl.Acad.Sci.,USA.* 89:1413-1417.

Smith, H.C., R.L. Ochs, E.A. Fernandez, and D.L. Spector. 1986. Macromolecular domains containing nuclear protein p107 and U-snRNP protein p28: further evidence for an in situ nuclear matrix. *Mol.Cell.Biochem.* 70:151-168.

Smith, T.J. 1987. n-Butyrate Inhibition of Hyaluronate Synthesis in Cultured Human Fibroblasts. *The Journal of Clinical Investigation, Inc.* 79:1493-1497.

Spector, D.L. 1990. Higher order nuclear organization: Three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc.Natl.Acad.Sci.,USA.* 87:147-151.

Spector, D.L., R.T. O'Keefe, and L.F. Jimenez-Garcia. 1993. Dynamics of transcription and pre-mRNA splicing within the mammalian cell nucleus. *Cold Spring Harbor Symp.on Quant.Biol.* LVIII:799-805.

Spector, D.L., W.H. Schrier, and H. Busch. 1983. Immunoelectron microscopic localization of SnRNPs. *Biol.Cell.* 49:1-10.

Stavnezer-Nordgren, J., and S. Sirlin. 1986. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J.* 5:95-102.

Sympson, C.J., and T.E. Geoghan. 1990. Actin gene expression in murine erythroleukemia cells treated with cytochalasin D. *Exp. Cell Res.* 189:28-32.

Taylor, J. 1960. *J. Biophys. Biochem. Cytol.* 7:455-464.

Taysi, K., R.S. Sparkes, T.J. O'Brien, and D.R. Dengler. 1982. Down's syndrome phenotype and autosomal gene inactivation in a child with presumed (X;21) de novo translocation. *Journal of Medical Genetics.* 19:144-148.

Tinker, A.V., and C.J. Brown. 1998. Induction of XIST expression from the human active X chromosome in mouse/human somatic cell hybrids by disruption of methylation. *submitted.*

Torchia, B.S., L.M. Call, and B.R. Migeon. 1994. DNA Replication analysis of FMR1, XIST and factor 8C loci by FISH shows non transcribed X-linked genes replicates late. *Am. J. Hum. Genet.* 55:96-104.

Toth, M., U. Lichtenberg, and W. Doerfler. 1989. Genomic sequencing reveals a 5-methylcytosine-free domain in active promoters and the spreading of preimposed methylation patterns. *Proc. Natl. Acad. Sci. USA.* 86:3728-3732.

Turker, M.S., and T.H. Bestor. 1997. Formation of methylation patterns in the mammalian genome. *Mut. Res.* 386:119-130.

Vogel, W., M. Autenreith, and G. Speit. 1986. Detection of bromodeoxyuridine-incorporation in mammalian chromosomes by a bromodeoxyuridine-antibody. I. Demonstration of replication patterns. *Hum.Genet.* 72:129.

Vogel, W., M. Autenrieth, and K. Mehnert. 1989. Analysis of chromosome replication by a BrDU antibody technique. *Chromosoma.* 98:335-341.

Vogelstein, B., and B.F. Hunt. 1982. A subset of small ribonucleoprotein particle antigens is a component of the nuclear matrix. *Biochem.Biophys.Res.Commun.* 105:1224-1232.

Walker, C.L., C.B. Cartgile, K.M. Floy, M. Delannoy, and B.R. Migeon. 1991. The Barr body is a looped X chromosome formed by telomere association. *Proc.Natl.Acad.Sci.(USA).* 88:6191-6195.

Wang, J., L. Cao, Y. Wang, and T. Pederson. 1991. Localization of pre-messenger RNA at discrete nuclear sites. *Proc.Natl.Acad.Sci.,USA.* 88:7391-7395.

Willard, H.F., C.J. Brown, L. Carrel, B. Hendrich, and A.P. Miller. 1993. Epigenetic and chromosomal control of gene expression: molecular and genetic analysis of X chromosome inactivation. *Cold Spring Harbor Symp. Quant. Biol.* 58:315-322.

Wolff, D.J., C.J. Brown, S. Schwartz, A.M.V. Duncan, U. Surti, and H.F. Willard. 1994. Small marker X chromosomes lack the X inactivation center: Implications for karyotype/phenotype correlations. *Am.J.Hum.Genet.* 55:87-95.

Wutz, A., O.W. Smrzka, N. Schweifer, K. Schellander, E.F. Wagner, and D. Barlow. 1997. Imprinted Expressiuon of the Igf2r gene depends on an intronic CpG Island. *Nature.* 389:745-749.

Xing, Y. 1993. Nuclear Structure Studied by Fluorescence Hybridization: Visualization of Individual Gene Transcription and RNA Splicing. *Thesis.*

Xing, Y., C.V. Johnson, P. Dobner, and J.B. Lawrence. 1993a. Differential nuclear distribution of intron and exon sequences for endogenous RNAs revealed by fluorescence in situ hybridization. *Science.* 259:1326-1330.

Xing, Y., C.V. Johnson, P.R. Dobner, and J.B. Lawrence. 1993b. Higher level organization of individual gene transcription and RNA splicing. *Science.* 259:1326-1330.

Xing, Y., C.V. Johnson, P.T. Moen, J.A. McNeil, and J.B. Lawrence. 1995. Nonrandom gene organization: Structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* 131:1635-1647.

Xing, Y., and J.B. Lawrence. 1993. Nuclear RNA tracks: structural basis for transcription and splicing? *Trends in Cell Biology*. 3:346-353.

Yancopoulos, G.R., R. DePhino, K. Zimmerman, S. Lutzker, N. Rosenberg, and F.W. Alt. 1986. Secondary rearrangement events in pre-B cells; VH· DJH· replacement by a LINE-1 sequence and directed class switching. *EMBO J.* 5:3259-3266.

Yoshida, I., Y. Nishita, T.K. Mohandas, and N. Takagi. 1997. Reactivation of an inactive human X chromosome introduced into mouse embryonal carcinoma cells by microcell fusion with persistent expression of XIST. *Exp. Cell. Res.* 230:208-219.

Zachar, Z., J. Kramer, I.P. Mims, and P.M. Bingham. 1993. Evidence for channeled diffusion of pre-mRNAs during nuclear RNA transport in metazoans. *J.Cell Biol.* 121:729-742.

Zacksenhaus, E., and R. Sheinin. 1990. Molecular cloning, primary structure and expression of the human X linked gene A1S9 gene cDNA which complements the ts A1S9 mouse Lcell defect in DNA replication. *EMBO J.* 9:2923-2929.

Zeitlin, S., A. Parent, S. Silverstein, and A. Efstratiadis. 1987. Pre-mRNA splicing and the nuclear matrix. *Mol.Cell.Biol.* 7:111-120.

Zieve, G., and S. Penman. 1976. Small RNA species of the HeLa cell: Metabolism and subcellular localization. *Cell.* 8:19-31.

Zirbel, R.M., U.R. Mathieu, A. Kurz, T. Cremer, and P. Lichter. 1993. Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. *Chrom.Res.* 1:92.

Zuffardi, O., L. Tiepolo, S. Scappaticci, D. Francesconi, C. Bianchi, and D. di Natale. 1977. Reduced phenotypic effect on partial trisomy 1q in a X/1 translocation. *Ann Genetics*. 20:191-194.