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# XIST RNA Paints the Inactive X Chromosome at Interphase: Evidence for a Novel RNA Involved in Nuclear/Chromosome Structure

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***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 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- 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 polyadenylated 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 nonchromatin 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.

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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 and Riggs, 1983; Gartler et al., 1992; Rastan, 1994). It is generally agreed that the X inactivation process involves at least three stages: (*a*) an initiation event which culminates in distinguishing the active X chromosome; (*b*) propagation of inactivation in *cis* throughout the X chromosome; and (*c*) 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., 1991*a*), 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),[1] the locus required in *cis* for inactivation to occur (Russell, 1963; Mattei et al., 1981; Brown et al., 1991*b*) and is expressed exclusively from the inactive X chromosome (Xi) (Brown et al., 1991*a*, 1992). While several human genes have been described that escape inactivation and are

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1. *Abbreviations used in this paper*: CSK, cytoskeleton; Xi, inactive X; XIC, X inactivation center; 2-, and 3-D, two- and three-dimensional.

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therefore expressed from both X chromosomes (reviewed by 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., 1993, 1994); 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). Collectively, these results suggest that *Xist* expression is not merely a consequence of X inactivation, but may play an important role in its control. 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 effects of failed dosage compensation for genes on the extra chromosome fragment (Migeon et al., 1993, 1994; 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., 1993), 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).

Despite strong evidence suggesting an involvement of *XIST* in X inactivation, the role of this gene is neither defined nor definitive, particularly since no protein product has been described. 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 nonpoly 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 (two-dimensional) 2-D and (three-dimensional) 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, NJ). Amniocytes were a gift from Dr. Lenny Sciorra (University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Brunswick, NJ). For inhibition of transcription, cells were incubated

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in either 5 μg/ml of actinomycin D (Sigma Chemical Co., St. Louis, MO) for 1 to 5 h, 25 μg/ml DRB (5,6-dichloro-1-β-D-ribofuranosyulbenzimdidizole (Sigma Chemical Co.) for 3 h, or 50 μg/ml β-amanitin (Sigma Chemical Co.) for 4.5 h before fixation.

## Cell Preparation

Our standard cell fixation procedure was essentially as described previously and therefore will only be summarized in brief here (Lawrence et al., 1989). Monolayer cells grown on glass coverslips were permeabilized on ice with triton X-100 in RNA-preserving cytoskeletal (CSK) buffer (Fey et al., 1986*a*; Carter et al., 1991) before 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 s) afforded maximum retention of cytoplasmic RNA.

For nuclear matrix preparations, cells were fractionated according to Fey et al. (1986*a*). All steps were performed on ice except where noted. Coverslips with cells attached were washed in PBS and incubated in CSK plus 10 mM leupeptin, 10 mM vanadyl ribonucleoside complex (VRC; GIBCO BRL, Gaithersburg, MD), and 0.5% triton X-100 for 10 min. Coverslips were then incubated in the above CSK extraction buffer plus 250 mM ammonium sulfate for 5 min. The chromatin was digested with 100 mg/ml bovine pancreas DNAse I in CSK buffer with only 50 mM NaCl for 30 min at room temperature. 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 compared and yielded similar results. One procedure was adapted from Earnshaw et al. (1989). Coverslips with cells attached were swollen in 3 ml of prewarmed hypotonic (.85% sodium citrate) for 1 h at 37°C. The cells were then spun using a Cytospin 2 (Shandon Southern Products, Astmoor, Runcorn, Cheshire, England) at 1,100 rpm for 5 min, the cells were air dried, fixed in 4% paraformaldehyde for 5 min and dehydrated through a series of ethanols, and stored at −80°C until further use. An optional triton extraction was included before 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. Briefly: (all steps performed at room temperature 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, postfixed 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. Briefly, early passage WI-38 cells were blocked in 5 mM thymidine supplemented media for 16 h, rinsed, and the synchronized cells were grown in thymidine-free media for 8 h. The cells were metaphase arrested with .04 μg/ml nocodazole (Sigma Chemical Co.) for 4.5 h, 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 h 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 Ial collagen; and pH F67, 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 (Lawrence et al., 1988; Johnson et al., 1991; Xing et al., 1993). DNA probes were nick translated using biotin-11-dUTP or digoxigenin-16-dUTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN). For exclusive RNA hybridization, conditions previously defined (Lawrence et al., 1989) were used. Briefly, hybridization was to nondenatured cells (such that cellular DNA was not accessible for hybridization) overnight at 37°C in 50% formamide, 2× SSC 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 Biochemicals). For DNA detection of a single copy gene, as previously described (Lawrence et al., 1989), cells were denatured at 75°C for 2 min in 70% formamide, 2× 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 1× 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, 2× 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. Briefly, 10 μl of the whole chromosome library probe mixture was denatured for 5 min at 75°C and prehybridized at 37°C for 2 h (prehybridization was only performed for the Oncor probe). Samples were then hybridized with the probes overnight at 37°C. All posthybridization washes were performed as described previously for our standard hybridization (Lawrence et al., 1989) at lower stringency than recommended in the included protocols. The Oncor Chromosome Paint was detected with fluorescein-conjugated avidin (Boehringer-Mannheim Biochemicals). 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, 3-D, and Morphometric Analysis

Images were obtained with a Zeiss Axiophot microscope equipped with multi-bandpass epifluorescence filters (Chroma, Brattleboro, VT). Images

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were recorded with a cooled charge-coupled device (CCD) camera (200 series; Photometrics, Inc., Tucson, AZ) with a pixel size of 19 μM and a 14 bit A/D converter (data acquisition system by G. W. Hannaway and Associates, 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 G. W. Hannaway and Associates, the images were thresholded at the same level and the borders of the XIST 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 3-D analysis, A Zeiss 100×, 1.4 NA Plan ApoChromat objective was used in combination with a 2.5× photo eyepiece and an optivar setting of 1.25× for a total magnification of 312× at the camera (pixel size = 61 nm). Capture of the red and green channels was performed concurrently on each optical section, avoiding the need for reregistration. The deconvolution algorithm used was as described previously (Carter et al., 1993). Briefly, 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-diam 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 nonnegative (Fay et al., 1989). $L^2$ regularization with nonnegativity constraint (Fay et al., 1989) was used to estimate the unknown dye density, $f$, as the nonnegative density that minimizes the expression

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

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, $\alpha$, 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 data analysis and visualization environment (DAVE) written by Lawrence Lifschitz and Jeff Collins in the Biomedical Imaging Group at the University of Massachusetts Medical Center (Worcester, MA). 3-D 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 and Lawrence, 1993; Xing et al., 1995). An accumulation of XIST RNA is also observed near the site of transcription, the Xi chromosome or the Barr body, cytogenetically defined by dense DAPI staining (see Fig. 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 Iαl, 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 (Fig. 1, *A* and *B*). Similarly, the average area occupied by the collagen nuclear RNA, a relatively large accumulation compared to many protein coding RNAs, was 0.8 $\mu^2$, as contrasted with the XIST RNA average of 5.0 $\mu^2$; (Figs. 1 *C* and 3 *A*).

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 Figs. 6 (*A–C*) and 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 (Fig. 1, *A–D*). Many other examples of the contrasting nature of other pre-mRNAs can be found in our recent publication on the colocalization of collagen RNA with splicing factors (Xing et al., 1995). Even a very large pre-mRNA such as dystrophin, does not show a particulate distribution and produces a signal much smaller than XIST RNA (Coleman, J. and J. B. Lawrence, unpublished observation). To address whether the pattern of nuclear XIST RNA could be common to transcripts generated from the inactive X chromosome, the distribution of XIST RNA was compared to the nuclear transcripts from *UBE-1*, another gene that escapes inactivation (Brown and Willard, 1989). RNA from this gene formed a very small intense focus at two nuclear sites, extremely different from XIST RNA, even though the *UBE-1* gene is also a large gene (25 kb) (Fig. 1 *D*).

### 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 (Fig. 2 *E*) and the extent to which it encompasses Xi DNA are unclear, hence, the X chromosome specific library was used 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 Fig. 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 Fig. 2, *A–C* has three X chromosomes, exhibiting three red X-paint signals (Fig. 2 *B*). In these cells, XIST RNA labels two nuclear sites in green (Fig. 2 *A*), consistent with the observation that all X chromosomes in

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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 (Fig. 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%) (Fig. 2 *D*). Interestingly, as illustrated in the inset of Fig. 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. 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 three separate experiments. As summarized in Fig. 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 (Fig. 3 *C*). Where it was possible to discern discrete boundaries, the Barr body was measured and results suggested that its area was ~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 μm spacing were taken of several cells, creating a series of digitized images, which indicated that the XIST RNA and the chromosome library signal occupied the same planes of focus (Fig. 4). The optical sections were deconvoluted to remove out of focus light and as shown in Fig. 4, a 3-D rendered image of one cell is used to create a composite of the 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 Fig. 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 (Lawrence et al., 1993; Huang et al., 1994), 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 h and undetectable by 3 h (Fig. 5 *A*). Similar results were also observed for fibronectin RNA (Xing, 1993). In contrast, XIST RNA largely remained for up to 5 h in actinomycin D in most cells (Fig. 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 evidence supporting that the collagen RNA localized in SC-35 rich domains gives rise to mRNA (Xing et al., 1995) and contribute new insights into the interpretation of transcriptional inhibition effects on nuclear poly A RNA (Lawrence et al., 1993; Huang et al., 1994; see Discussion and 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 (Fig. 6 *C*) as the clusters appear to break up and become more discrete and dispersed through the nucleoplasm (Figs. 5 *A* and 6 *C*). An-

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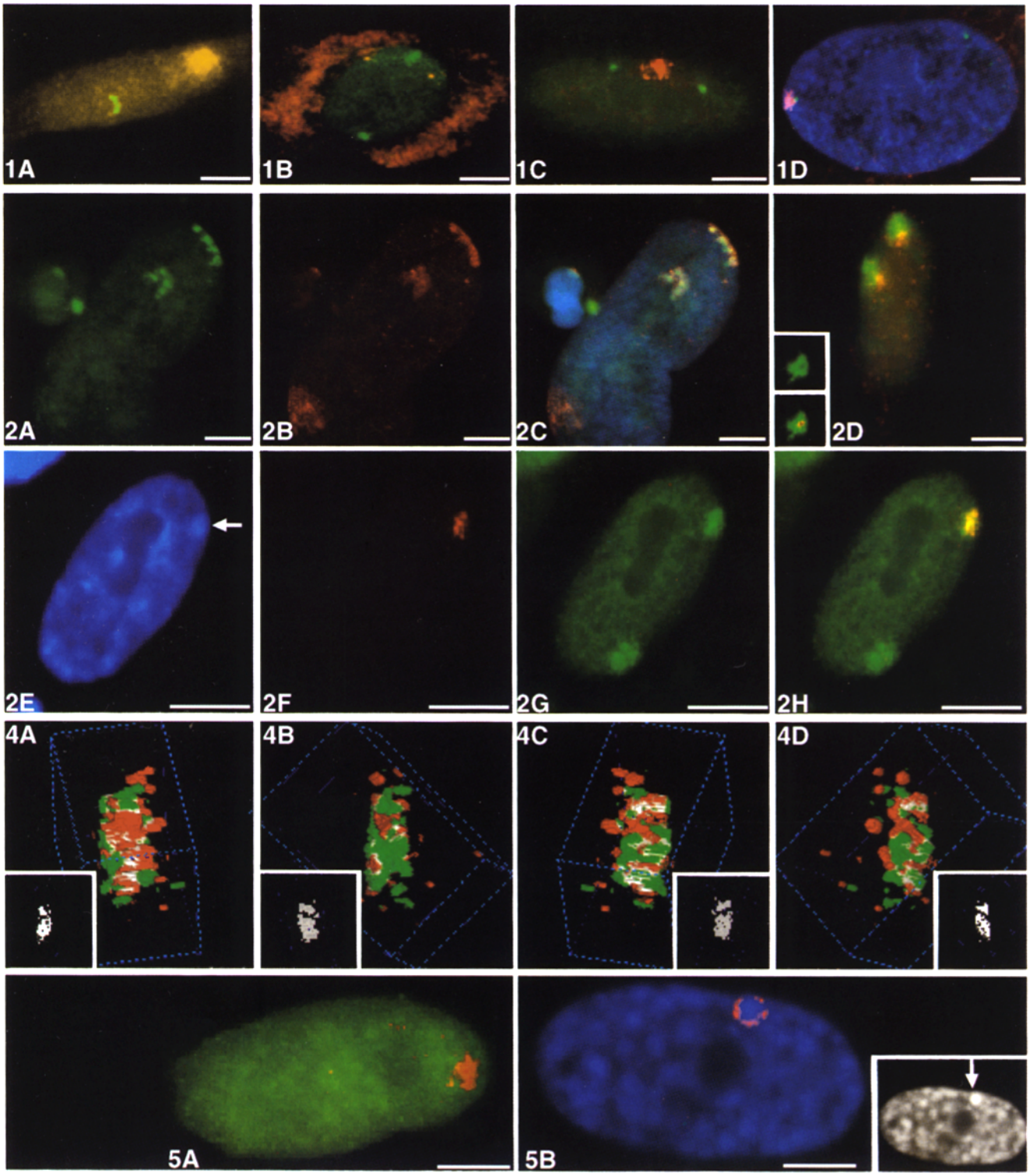



*Figure 1.* Fluorescence in situ hybridization comparison of XIST RNA with protein coding RNAs in human fibroblasts. Digoxigenin and biotin-labeled probes were hybridized in situ to nondenatured cells and detected with fluorochrome-conjugated avidin or anti-digoxigenin antibody. Fluorochromes used were 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. (*D*) XIST RNA (*red*) with nuclear RNA from UBE-1 (*green*), an X-linked gene that escapes X inactivation, in a WI-38 nucleus. Bars, 5 μm.

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other 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 ~10% of these cells it was apparent that XIST RNA clusters clearly encircle this prominent Barr body (Fig. 5 *B*). While this phenomenon could possibly be a result of limited penetrability of probe to these highly condensed chromosomes 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 nonsynchronized 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 (Fig. 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 (Fig. 6, *A* and *B*).

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 implicated by the spatial relationship of intron and exon sequences for XIST RNA contrasted to two protein-coding mRNAs studied. Fibronectin (Xing et al., 1993) 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 (Fig. 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 intron signal was not detectable even after prolonged exposures and computer enhancement (Fig. 6, *C* and *D*). In a small fraction of cells a very dim, tiny spot of fluorescence was observed which could represent primary transcripts

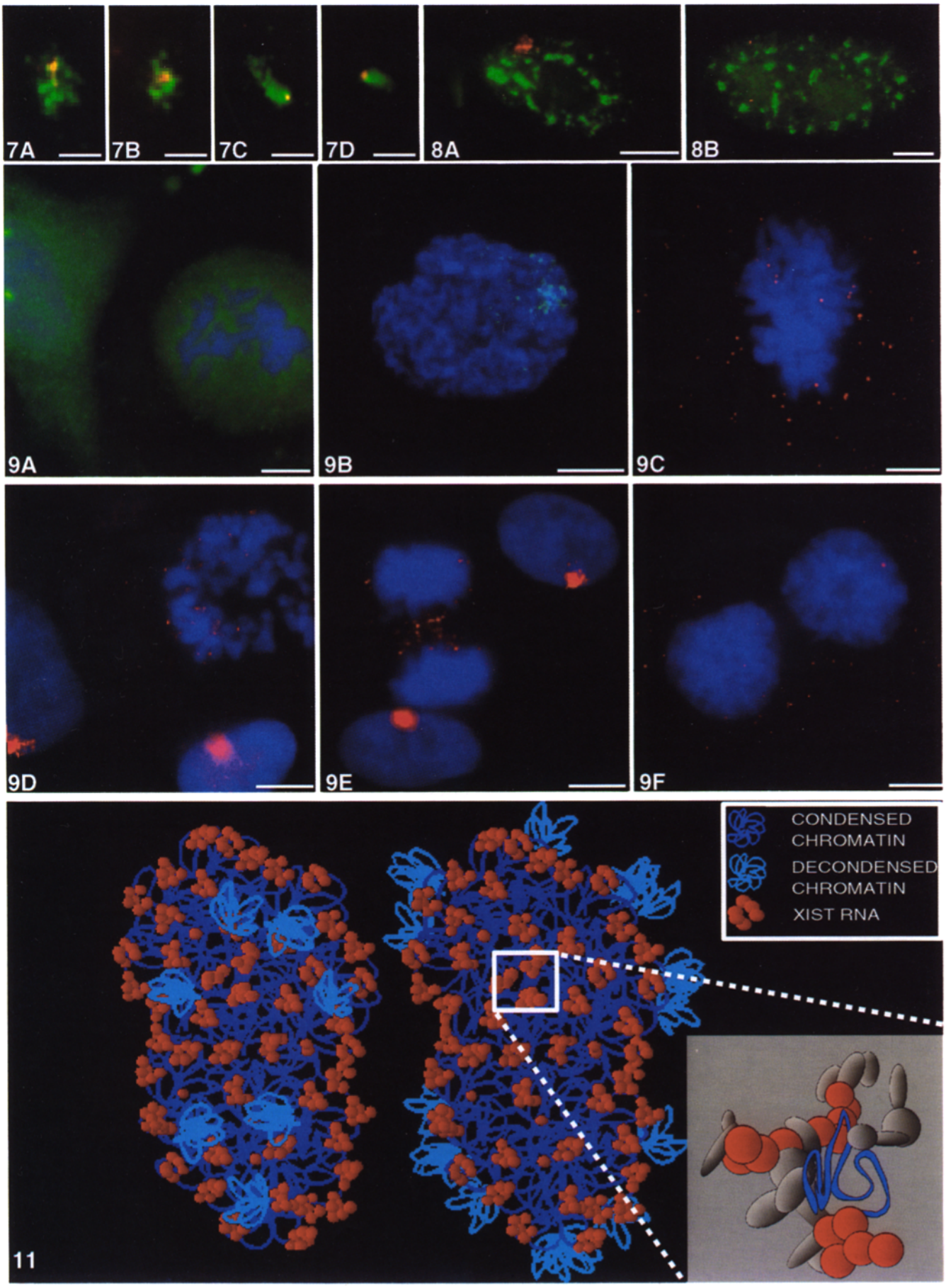
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*Figure 2.* Co-localization of XIST or collagen nuclear RNAs with their parent interphase chromosomes detected with whole chromosome DNA libraries. Detection of chromosome libraries was carried out using either the Imagenetics WCP-X or Oncor Coatasome 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, 5 μm.

*Figure 4.* 3-D 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° 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 and Xi it is not always a point for point correlation.

*Figure 5.* XIST RNA is stable upon transcriptional inhibition. (*A*) WI-38 cells treated for 3 h with Actinomycin D. XIST RNA (*red*) remains whereas collagen RNA (*green*) 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. Bars, 5 μm.

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still associated with the gene (Fig. 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 polyadenylated 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 (Fu and Maniatis, 1990; Carter et al., 1993; reviewed in Spector et al., 1993; Lawrence 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, rather than pre-mRNAs, preferentially localize in these domains (see Discussion; Huang et al., 1994). As shown in Fig. 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. This contrasts with some pre-mRNAs found "associated," for which the body of the pre-mRNA signal was in intimate contact with the domain (Xing et al., 1993; and 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 the *XIST* gene was localized by hybridization under conditions which remove RNA. As illustrated in Fig. 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 thus far are preferentially associated with domains, some clearly are not (Xing et al., 1993, 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

*Figure 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, 2 μm.

*Figure 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, 5 μm.

*Figure 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, nonparticulate 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 μm.

*Figure 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*).

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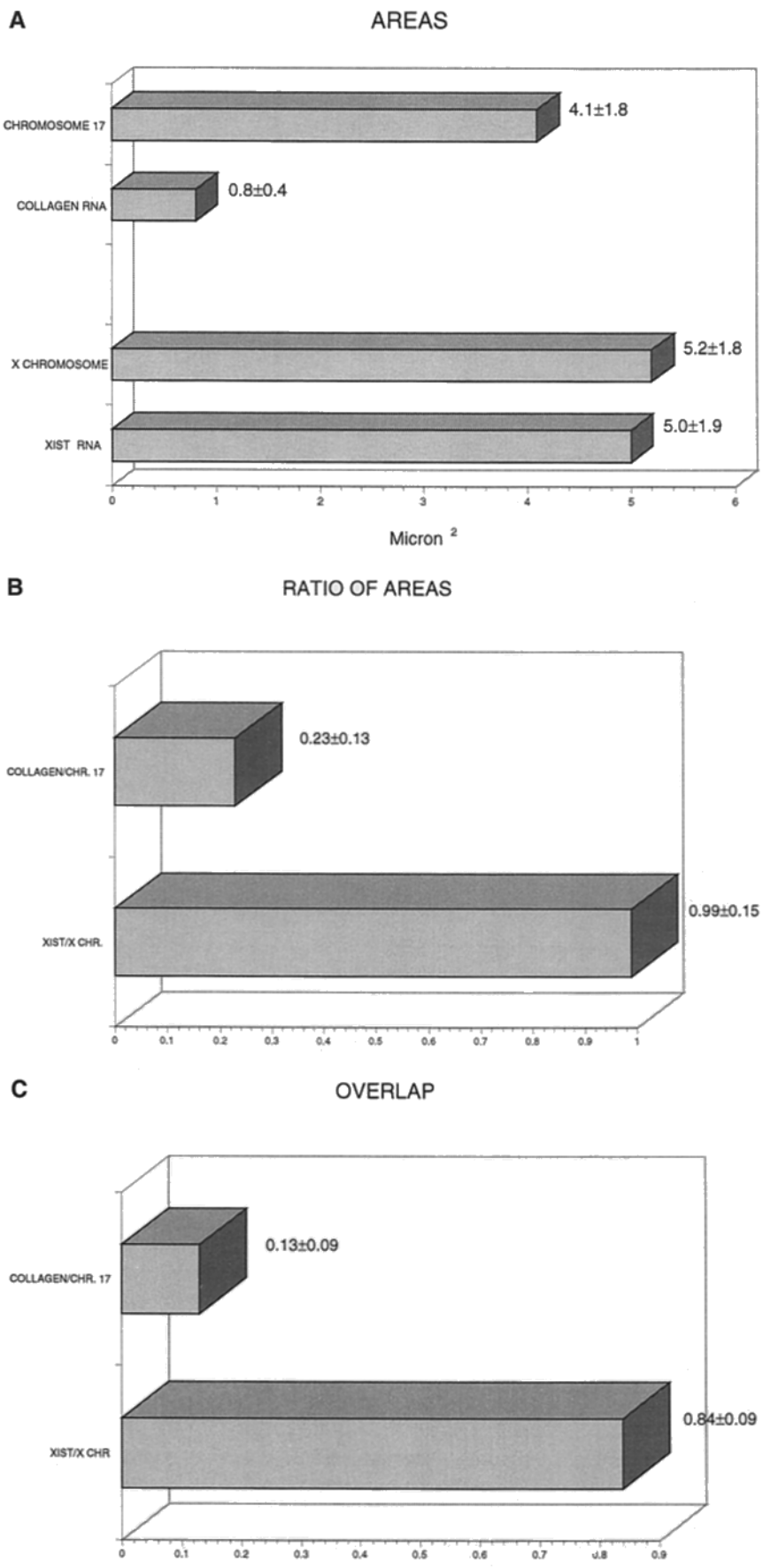



*Figure 3.* Morphometric data on size and spatial overlap of RNA and chromosome territories. Morphometric analysis was performed on 2-D digital images in separate color channels as described (Materials and Methods). Summaries of measurements on approximately 70 WI-38 cells are presented for cells hybridized simultaneously for XIST RNA or collagen RNA and the corresponding chromosomal DNA, as illustrated in Fig. 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.

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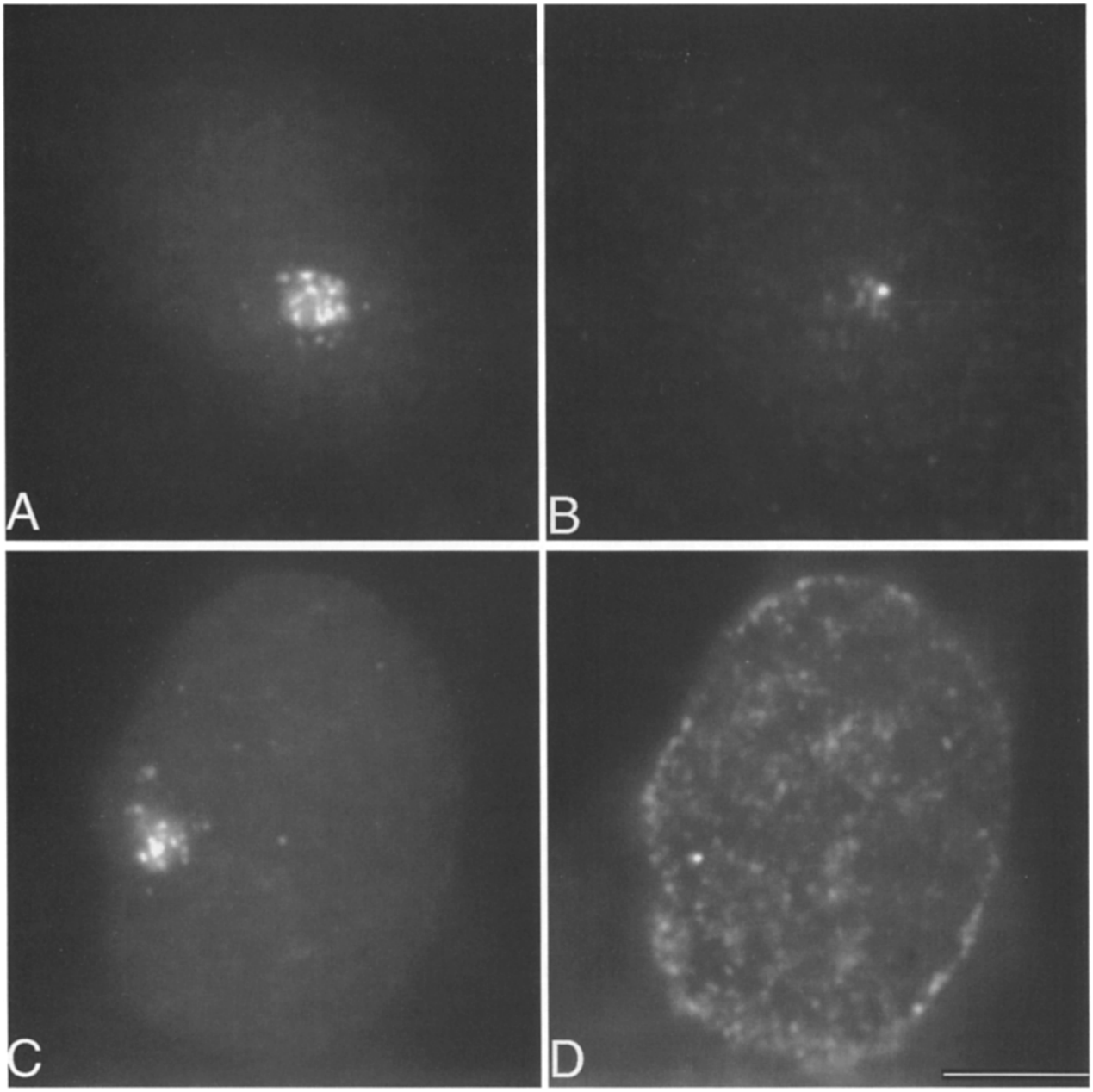



*Figure 6.* Detection of XIST RNA with intron and cDNA probes with and without transcriptional inhibition. Probes to intron 1 and 2 of *XIST* were labeled with digoxigenin and cohybridized with a *XIST* cDNA probe labeled with biotin in WI-38 cells treated for 0 and 3 h with actinomycin D. (*A*) XIST 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. 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). Bars, 5 μm.

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

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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 (Dirks et al., 1993; see Materials and Methods). 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 (Fig. 9 *B*). In prometaphase and metaphase, the clusters become more separate and often distribute across many chromosomes; ultimately moving into the cytoplasm (Fig. 9, *C* and *D*). At telophase, no signal is seen over the chromatin, and the clusters are most often concentrated between the cleavage plane (Fig. 9 *E*); in early G1, XIST RNA clusters are found in the cytoplasm and a small focus of signal is observed in the nucleus (Fig. 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 including ones that omitted hypotonic swelling and cell spinning (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 (Fig. 9 *A*), 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 the 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 (Pinol-Roma and Dreyfus, 1991; Levis and Penman, 1977).

### *XIST RNA Is Retained after Chromosomal DNA Digestion during Matrix Preparation*

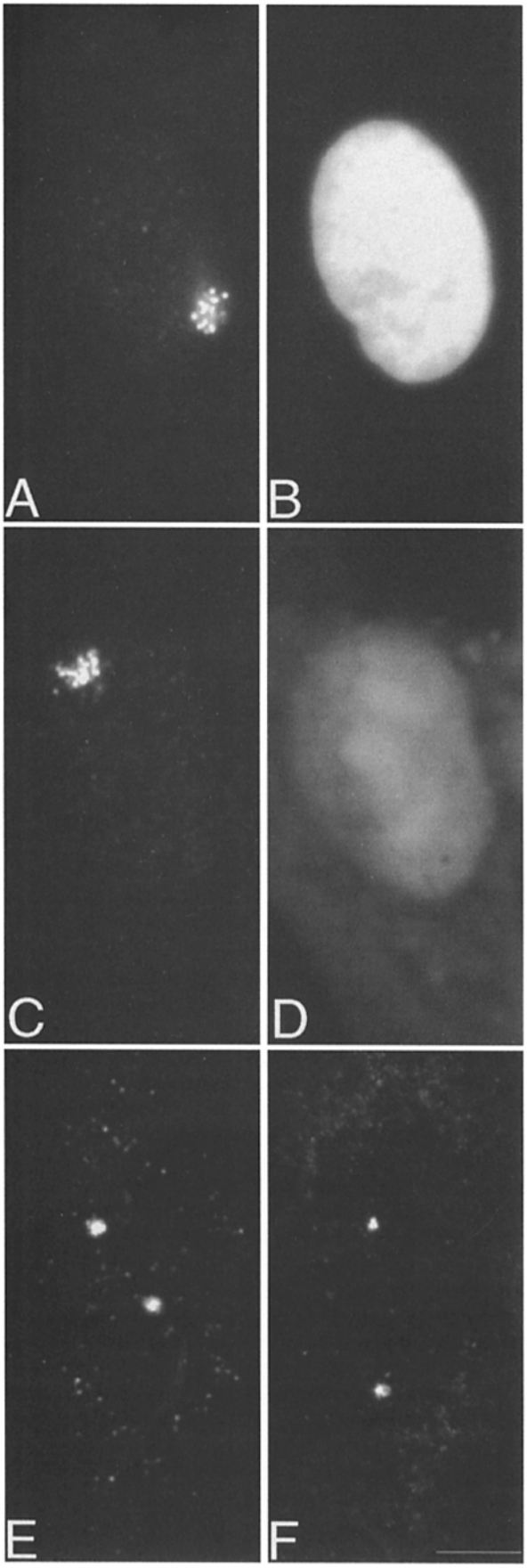
A structural association of XIST transcripts in the nucleus could be via protein or nucleic acid interactions, or both. To investigate if the RNA is attached along the length of the X chromosome by hybridization to DNA, RNase H digestion which specifically degrades RNA/DNA hybrids (Minshull and Hunt, 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 ~90–95% of cellular DNA, protein and phospholipid. The insoluble fraction which remains has been extensively studied and is termed the nuclear matrix (Berezney and Coffey, 1974; reviewed in Fey et al., 1991). While some types of snRNAs and snRNPs are removed from the matrix (Zieve and Penman, 1976; Vogelstein and Hunt, 1982), most major classes of snRNAs and snRNPs as well as newly synthesized pre-mRNA are retained (for example see Fey et al., 1986*b*; Jackson et al., 1981; Herman et al., 1978). This is shown in Fig. 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; Spector et al., 1983; Huang et al., 1994; Smith et al., 1986), 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 Fig. 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 (Fig. 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 (2 M NaCl) "halo" preparation (Wydner, K., and J. B. Lawrence, 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 (Figs. 2–4), these results together with the above results on

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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 nonchromatin 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, are provided in Fig. 11 and considered in the Discussion.

## *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 polyadenylated RNA was found to lack a convincing open reading frame (Brown et al., 1992; Brockdorff 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 further supports that this apparently long-lived nuclear RNA functions via a morphological relationship with Xi. Such a striking, extensive, and unique spatial

*Figure 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 we had to artificially increase the intensity for photography. For quantitation, we measured the DAPI intensity of 18 unextracted and extracted nuclei and found that 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. Bars, 5 μm.

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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 noncoding tandem repeat sequence is (Brown et al., 1992; Brockdorff et al., 1992). These observations, together with evidence for involvement of *XIST* during development, 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 Monk, 1986; Grant and Chapman, 1988; Eden and Cedar, 1994). However, evidence indicates that X inactivation can occur in the absence of methylation (Grant et al., 1992; Driscoll and Migeon, 1990; 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 (Jeppenson 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., 1993, 1994) 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 apparently not retained on the chromosome through mitosis (Fig. 9), the RNA is unlikely 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). Unlike other factors examined to date, the developmental regulation of XIST RNA suggests that it is involved in the establishment of X inactivation as its expression is found to precede inactivation in the developing mouse embryo, and the parental origin of expression correlates precisely with paternal and maternal X inactivation (Kay et al., 1993, 1994; and Norris et al., 1994). 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 (Pinkel et al., 1988; Lichter 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 (Fig. 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 Fig. 2 *B*). Based on these collective results 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 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.

In Fig. 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 Fig. 11, XIST RNA need not directly bind all along the DNA, and the apparent propensity of this RNA to cluster may be important for function, i.e., clus-

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ters of XIST RNA molecules (*red*) may distribute close to Xi chromatin (*blue*) and possibly form junctions with other nonchromatin 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. It will be of interest to determine the 3-D distribution of only the stable, intron lacking XIST RNA which remains after transcriptional inhibition. The highly conserved tandem repeat (Brown et al., 1992; Brockdorff et al., 1992; Hendrich et al., 1993) is a potential candidate for involvement in 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 implicated in a role related to 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 (Spector, 1990; Carter et al., 1991, 1993), 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., 1993, 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 see Fakan and Puvion, 1980; Fakan and Bernhard, 1973). 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 nonpolyadenylated and turns over within the nucleus with no known function; whereas at least the vast majority of poly A RNA encodes pre-mRNA (Herman et al., 1978; Harpold et al., 1981; Salditt-Georgieff et al., 1981; Lewin 1990). Some studies have suggested a fundamental role of RNA in nuclear architecture (for example see 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 a family 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 *Caenorhabditus 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, noncoding germline transcripts from the immunoglobulin gene locus appear to be necessary for class switch recombination of that locus to occur (Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos et al., 1986; Lutzker and Alt, 1988). 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 known to occur for several genes (reviewed in Pfeifer and Tilghman, 1994).

Work presented here provides a foundation for much

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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.

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