**XIST RNA Associates with Specific Regions of the Inactive X Chromatin**

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Microscopy studies have shown that XIST RNA colocalizes with the inactive X chromosome (Xi). However, the molecular basis for this colocalization is unknown. Here we provide two lines of evidence from chromatin immunoprecipitation experiments that XIST RNA physically associates with the Xi chromatin. First, XIST RNA can be co-precipitated by antiserum against macroH2A, a histone H2A variant enriched in the Xi. Second, XIST RNA can be co-precipitated by antisera that recognize unacetylated, but not acetylated, isoforms of histones H3 and H4. The specificity of XIST RNA association with hypoacetylated chromatin, together with the previous finding that hypoacetylated histone H4 is enriched at promoters of X-inactivated genes, raises the possibility that XIST RNA may contribute to the hypoacetylation of specific regions of the Xi so as to alter the expression of X-linked genes.

X-chromosome inactivation in mammals is controlled by the Xist locus, which in humans encodes a 17-kilobase untranslated RNA that is expressed only from the inactive X (Xi) (4). Mouse X chromosomes with targeted deletions of the Xist gene lose the ability to undergo X inactivation (2, 3). In addition, ectopic expression of Xist RNA from multicopy transgenes during embryonic stem cell differentiation causes autosomes to exhibit features typically associated with X inactivation, such as late replication, histone H4 hypoacetylation, and transcriptional down-regulation (4, 5). These studies indicate that the Xist locus is required for X inactivation, but they do not distinguish between several possible modes of Xist action; one model is that the Xist genomic locus serves as a chromatin organizing region that, when transcribed, initiates changes in chromatin structure that are propagated in cis along the length of the Xi chromosome (6). An alternative model is that the noncoding RNA expressed from the Xist gene is functional and directly participates in the silencing process (1). One observation suggesting that the XIST transcript may directly participate in silencing comes from fluorescence in situ hybridization experiments that show that XIST RNA colocalizes with the inactive X chromosome (1, 7). However, the limited resolution of microscopy analysis does not provide any information on the molecular basis or the consequences of this colocalization. To examine the nature of the colocalization between XIST RNA and the Xi chromatin, the chromatin IP assay was used to precipitate chromatin from human female cells and then RT-PCR was performed to determine whether XIST RNA was co-precipitated. The results indicate that XIST RNA is indeed co-precipitated with chromatin by antibodies directed against several histone species. Significantly, XIST RNA was co-precipitated with antibodies against unacetylated, but not acetylated, histones H3 and H4. Given the previous finding that histone H4 is hypoacetylated at promoters of X-inactivated genes (8), these results raise the possibility that XIST RNA may be associated with promoters of silenced genes, as well as other hypoacetylated regions of the Xi.

MATERIALS AND METHODS

Chromatin Immunoprecipitations—10-cm plates of confluent cells were incubated in PBS containing 1% formaldehyde for 10 min at 37 °C. Chromatin immunoprecipitations were then performed using various polyclonal antisera as described previously (8, 9). Briefly, sonication of cross-linked nuclei was performed in a cup horn (Branson Sonifier 450) under conditions that gave a range in DNA fragments from 200–1000 bps. Sonicated nuclear lysates were precleared and incubated with the following antisera: anti-histone H3 or H4 antibodies (10 pm; Upstate Biotechnology), anti-AcH3 or -AcH4 antibodies (5 µl; Upstate Biotechnology), or affinity-purified, polyclonal anti-macroH2A1.2 antiserum (5 µl; see Ref. 10). The specificity of the anti-acetylated antibodies was confirmed in quality control testing by the manufacturer, as treatment of cells with the deacetylase inhibitor sodium butyrate greatly enhanced detection of the acetylated forms of histones H3 and H4 in immunoblot analysis of acid-extracted proteins. Further demonstration that the antibodies are specific in the chromatin IP assay comes from our previous work, in which antibodies against acetylated histone H4 were specifically unable to immunoprecipitate detectable levels of promoter-specific sequences of X-inactivated genes, whereas they were able to immunoprecipitate many other X-linked sequences (8). The specificity of the anti-macroH2A1.2 antibodies has been previously demonstrated by immunoblot analysis from a variety of cell types, including mouse liver cells (10), undifferentiated and differentiated mouse embryonic stem cells (11), and mouse blastocysts (12).

**PCR**—One-tenth of the chromatin immunoprecipitate was added to a 20-µl reaction mix containing 1.5 mM MgCl₂, 50 mM potassium chloride, 10 mM Tris, pH 9.0, 1% Triton X-100, 0.5 µM each primer, 0.1 mM dNTPs, and 1 Unit of Taq polymerase. After denaturation at 95 °C for 1.5 min, 30 cycles of PCR were performed where each cycle consisted of 1 min at 95 °C, 45 s at 60 °C, and 45 s at 72 °C. Products were resolved by agarose gel electrophoresis. Primers were as follows: ZFX forward (5′-TGGTCGTTGTTAAAAGGATAGCG-3′) and reverse (5′-AGGACCCCAATTTGGTGATGG-3′); SB1L3A forward (5′-AGGACATGATGGTACCTGT-3′) and reverse (5′-CGATGTTTGTGGAATCTCTGTC-3′), as described previously (13); RPSX forward (5′-TGATGATGTTAAGTGGC-3′) and reverse (5′-TAAAAGGATGGCCCA-3′).
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RESULTS

XIST RNA Can Be Co-precipitated with Antibodies against Histone macroH2A—To investigate whether XIST RNA directly associates with the chromatin of the Xi, we used the chromatin IP/RT-PCR (ChIP/RT-PCR) assay, which is a variation of the previously described chromatin immunoprecipitation (ChIP) assay (8, 14). Chromatin fragments of 200–1000 bp in length were generated by sonication and immunoprecipitated with antibodies against known components of the Xi chromatin. After DNase digestion, immunoprecipitates were subjected to RT-PCR to assay for the presence of XIST RNA.

The first antibodies used in the co-precipitation analysis were directed against macroH2A, a histone H2A variant. This protein is highly enriched in the Xi (10), and this enrichment is dependent on continued expression of the Xist locus in differentiated cells (15).

Immunofluorescence microscopy with polyclonal antibodies directed against the major splice variant of macroH2A confirmed that macroH2A was enriched in the two Xi chromosomes of 293 cells, a human female cell line that contains three X chromosomes (Fig. 1a). This pattern of staining, which is consistent with previous immunofluorescence reports (10), provides a measure of antibody specificity. Further specificity controls with these antibodies were provided previously by immunoblot analysis in a variety of cell types, including mouse liver cells (10), mouse embryonic stem cells (11), and mouse preimplantation embryos (12). When these anti-macroH2A antibodies were used in the chromatin IP/RT-PCR assay, they co-precipitated XIST RNA from 293 cells (Fig. 1b, lane 5). The primers used in this assay are capable of distinguishing XIST RNA from genomic DNA, because they span two introns that are alternatively spliced to generate cDNA products of either 179 or 242 bps in length. Fig. 1b, lane 4 confirms that the PCR products were dependent upon the presence of reverse transcriptase. This indicates that the signal is due to co-precipitation of spliced XIST RNA rather than XIST genomic DNA. The specificity of anti-macroH2A antibody in recovering XIST RNA was further demonstrated by control assays for the chromatin IP/RT-PCR, in which the abundantly expressed FIBRONECTIN mRNA was not co-precipitated with anti-macroH2A antisera, although this mRNA did survive the crosslinking and sonication procedure (data not shown).

XIST RNA Can Be Co-precipitated with Antibodies against

Unacetylated, but Not Acetylated, Histones H3 and H4—We next examined whether XIST RNA could be recovered by antisera directed against other chromatin proteins. Histone hypoacetylation has been shown to feature prominently in X inactivation; microscopy studies have shown that histones H2A, H3, and H4 are underacetylated in the Xi as compared with other chromosomes (16–18). Furthermore, we have recently shown by chromatin IP analysis from somatic cell hybrids containing a human Xi that histone H4 hypoacetylation is restricted to promoter regions of X-inactivated genes, whereas regions downstream of these promoters show a moderate level of acetylation (8).

Antibodies capable of distinguishing between the acetylated and unacetylated isoforms of histones H3 and H4 were used to immunoprecipitate chromatin from 293 cells. The specificity of these antibodies was previously demonstrated in two ways. First, the manufacturer performed quality control testing, in which treatment of cells with the deacetylase inhibitor sodium butyrate greatly enhanced detection of the acetylated isoforms of histones H3 and H4 in immunoblot analysis of acid-extracted proteins. Second, our previous work showed that the antibodies against acetylated histone H4 were specifically unable to immunoprecipitate detectable levels of promoter-specific sequences of X-inactivated genes, whereas they were capable of immunoprecipitating many other X-linked sequences (8). After performing immunoprecipitations with these antibodies, the resulting immunoprecipitates were subjected to RT-PCR to examine whether XIST RNA was also recovered. The results demonstrate that spliced XIST RNA was co-precipitated by the antibodies against unacetylated H3 and H4 (Fig. 2a, lane 6 and lane 7). In contrast, XIST RNA was not co-precipitated by antibodies against acetylated H3 and H4 (Fig. 2a, lane 8 and lane 9). Sequencing analysis confirmed the identity of the 242-bp band in Fig. 2a, lane 6 and of the 179-bp band in Fig. 2b, lane 2 (data not shown). The precipitation profile suggests

FIG. 1. XIST RNA can be co-precipitated with antibodies against histone macroH2A. a, immunofluorescence staining with antibodies against histone macroH2A, showing its enrichment in the two macrochromatin bodies (red) of a typical 293 cell, which contains 2 Xi chromosomes. b, chromatin IP/RT-PCR analysis of XIST RNA recovery with antibodies against macroH2A. Immunoprecipitates were reverse transcribed and subjected to PCR using primers that detect two alternatively spliced XIST transcripts.
that this region of XIST RNA physically associates with unacetylated regions of the Xi.

The abundantly expressed FIBRONECTIN mRNA, as well as 18 S rRNA, provided specificity controls for the H4 immunoprecipitations. Antibodies against either acetylated or unacetylated histone H4 failed to recover FIBRONECTIN mRNA or 18 S rRNA, despite the fact that these transcripts were still detectable in total input nuclear RNA after sonication (data not shown). This suggests that the ability of antibodies against unacetylated histones to co-precipitate XIST RNA is specific and not an in vitro artifact due to nonspecific interactions between histones and RNA molecules.

Chromatin IPs performed in parallel with antibodies against histones H3 and H4 in their acetylated and unacetylated states indicated that the antibodies were indeed efficient in precipitating chromatin from 293 cells, which contain one active and two inactive X chromosomes. Although antibodies against unacetylated histone H4 immunoprecipitated X-linked chromatin with reduced efficiency (Fig. 2c, lane 5), these antibodies were able to recover XIST RNA, whereas the more efficient antibodies against acetylated H4 did not (Fig. 2b). This experiment shows that the recovery of XIST RNA by some antisera but not others cannot be attributed to differences in their efficiency of precipitating chromatin. Finally, we showed that, as expected, XIST RNA was not recovered from BJEH human male cells (19), which do not express this RNA, by any of the anti-histone antisera (data not shown). This control rules out the possibility that the RT-PCR signal is attributable to contaminating RNAs introduced by the antibodies.

DISCUSSION

Microscopy studies have shown that XIST RNA colocalizes with the Xi (1, 7). Our results further demonstrate that this colocalization is likely the result of a physical association of processed XIST RNA with the Xi chromatin. Significantly, the data shows that XIST RNA preferentially associates with hypoacetylated histones H3 and H4. This strongly suggests that XIST RNA is associated with hypoacetylated regions of the Xi. This may include the hypoacetylated promoters of X-inactivated genes (8), as well as additional hypoacetylated regions of the Xi.

A previous report suggested that XIST RNA was not an integral component of the Xi chromatin, as it remained with the nuclear matrix after near-complete removal of chromosomal DNA (7). However, this observation was made after treating nuclei with a stringent set of conditions, including high salt and detergent, which may have dissociated XIST-containing complexes. Hence, these observations may not be inconsistent with the findings reported here, in which formaldehyde cross-linking had been used to stabilize protein-protein and protein-nucleic acid interactions.

The cross-linking step, if performed too extensively, could produce nonspecific communoprecipitations, but it appears that this was not the case. The specificity of the cross-linking is shown by the fact that whereas antibodies against both acetylated and unacetylated histones H3 and H4 were capable of recovering chromatin, only the antibodies against unacetylated histones recovered XIST RNA. Nevertheless, this result does not address the nature of the association between XIST RNA and chromatin, which may be either direct or mediated through bridging factors.

The finding that XIST RNA is associated with hypoacetylated regions of the Xi chromatin in human cells suggests the possibility that it may nucleate the assembly of ribonucleoprotein complexes that possess chromatin modifying activity. One apparent example of such a system is Drosophila dosage compensation. Here, X-linked gene expression is equalized by a converse strategy in which transcription is up-regulated from most genes on the single male X. This transcriptional up-regulation coincides with chromatin remodeling, whereby the male X acquires a rare acetylation of lysine 16 on histone H4, as well as a diffuse microscopic appearance (20, 21). Genetic analysis has identified several MSL (male-specific lethal) proteins that are essential for both dosage compensation and the site-specific histone acetylation (20, 22). One of the MSL proteins, Mof, is homologous to histone acetyltransferases, supporting the notion that the MSL complex may function to covalently modify nucleosomes on the regulated male X (23). These MSL proteins assemble into complexes that bind to hundreds of sites along the polytene male X in a reproducible manner.

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**Fig. 2. Profile of XIST RNA co-precipitation with various anti-histone antisera.** a and b, RT-PCR analysis of chromatin IPs using designated antisera. Chromatin was prepared from female cells by cross-linking and sonication of nuclear material. Immunoprecipitations were performed with antibodies against unacetylated and acetylated histone H3 (a) and antibodies against unacetylated and acetylated histone H4 (b). PCR primers are specific for XIST cDNA and detect two alternatively spliced transcripts. The genomic control and RT control demonstrate specificity for the RNA. c, PCR analysis of efficiency of various antisera in recovering DNA sequences of X-linked genes.
pattern (24). It has been proposed that localization of the MSL complex is mediated by the noncoding RNAs (roX1 and roX2) expressed from the male X that provide nucleation sites for assembly of the MSL complexes and their subsequent spreading in cis along the male X (25).

By analogy to the mechanism of dosage compensation employed by Drosophila, it is possible that expression of XIST RNA from the Xi nucleates the assembly of ribonucleoprotein complexes that possess chromatin modifying activity, as well as the spread of these complexes along the Xi in cis. The observation that macroH2A localization to the Xi is dependent upon XIST RNA expression suggests that XIST RNA may play a role in recruiting macroH2A to the Xi (15). Consistent with a possible interaction between XIST RNA and the macroH2A protein is the observation that macroH2A contains a putative RNA-binding domain (26). If the XIST-containing complexes indeed assemble on the Xi chromatin, one possible function of these complexes may be to mediate the promoter-specific hypoacetylation of histone H4, which correlates with X inactivation in somatic cell hybrids (8).

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