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The 5′ Repeat Elements of the Mouse Xist Gene Inhibit the Transcription of X-Linked Genes

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X chromosome inactivation in mammals requires the Xist gene, which is exclusively expressed from the inactive X chromosome (Xi). The large heterogeneous Xist nuclear RNA colocalizes with Xi, most likely through nuclear protein interactions. The 5′ region of the Xist RNA contains a series of well-conserved tandem repeats known to bind heteronuclear proteins in vitro and to enhance human XIST transcription. We show in an in vitro system that the conserved repeat element located in the 5′ region of the mouse Xist gene (Xcr) represses three X-linked genes but has no effect on the autosomal genes Aprt, Ins, and the viral SV40 gene. The repression effect is not mediated by the conserved core sequence (Ccs) of Xcr, but requires the presence of the complete Xcr. This Xcr effect on X-linked genes suggests that Xcr transcript recognizes the genes to be silenced and is involved in the spreading of X inactivation.

Xist gene X inactivation Transcriptional activity Conserved tandem repeats

X chromosome inactivation occurs in female mammals to transcriptionally inactivate one of the two X chromosomes, resulting in dosage compensation for X-linked genes between XX females and XY males (17). Developmental regulation of X inactivation has an absolute requirement for the presence, in *cis*, of a region defined genetically as the X inactivation center (Xic) (4,7,10,19,21,24,29,30). Xic, a several hundred kb region containing at least seven genes (22), is involved in initiating and spreading X inactivation. Among the genes located in it, Xist (X-inactive specific transcript) is so far unique in being expressed only from the inactive X chromosome (Xi) (3,5,6,14). The lack of any open reading frame of significant length in the Xist gene led to the suggestion that its action is mediated directly through its RNA product (5,9,14). Such a mechanism is supported by observations that Xist RNA not only remains in the nucleus but is also in direct association with the Xi in interphase nuclei, suggesting a role in the propagation of X inactivation (5,9,14). Xist RNA does not seem to be directly associated with chromosomal DNA itself (14), but rather interacts through specific factors available in terminally differentiated cells that do not normally express Xist (8,12).

An unusual feature of the Xist gene is the presence of several domains made of direct tandem repeats (5). The 5′ end of the transcribed mouse Xist gene, a 384-bp sequence from +329 to +713, contains eight copies of a repeat that consists of a 24-bp GC-rich core sequence highly conserved among eutherians (GCC CAWCGGGGCYNYGGATACCTG), separated by a variable length spacer region that is predominantly composed of T residues. The XIST conserved repeat sequence (XCR), arranged in a head-to-tail fashion, is present in nine copies at the equivalent position of the human XIST gene (9). The study of the XIST promoter by Hendrich, Plenge, and Willard (18) showed that XCR had a positive stimulatory effect on reporter gene activity in transient assays but only

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when positioned in the 5′-untranslated portion of the luciferase reporter gene, which suggested that this element acts posttranscriptionally.

We report that the mouse Xcr is sufficient to downregulate the promoter of Pgk1, Hprt, and G6pd, three classical X-linked genes, and that Xcr has no effect on the promoters of the mouse Ins and Aprt autosomal genes, and on the viral SV40 gene. Thus, Xcr represents a functional domain of Xist that might participate to the specific inactivation of X-linked genes.

MATERIALS AND METHODS

PCR Amplification, Cloning, and Sequencing

Specific primers were synthesized to amplify mouse Hprt promoter region (accession No. M12561) from −798 to +92 (Hprt-Kpn 49F: 5′-TCC AAG GTA CCA TGT GTC GCC ACA CCT GAC T-3′; Hprt-Mlu 937R: 5′-TCC AAA CGC GTA AGC GGT CTG AGG AGG AAG C-3′), mouse G6pd promoter region (accession No. X53617) from −576 to +119 (G6pd-Kpn 478F: 5′-TCC AAG GTA CCG TCT CCG AAC TCA GAG ATC TAC C-3′; G6pd-Mlu 1172R: 5′-TCC AAA CGC GTG CGT ACA AGC GTG AAG CAT GTC C-3′), mouse Aprt promoter region (accession No. M11310) from −198 to +92 (Aprt-Kpn 560F: 5′-TCC AAG GTA CCA AAG CAG GAC TGA A A AAG CGT G-3′; Aprt-Mlu 828R: 5′-TCC AAA CGC GTG GAG GAG GTA GGT ATC AGC ACG-3′), and mouse Ins promoter region (accession No. X04725) from −242 to +32 (Ins-Kpn 470F: 5′-TCC AAG GTA CCG TTC ATC AGG CCA TCT GGT C-3′; Ins-Mlu 698R: 5′-TCC AAA CGC GTA CCT GCT TGC TGA TGG TCT C-3′).

Amplifications were performed in 50-μl reaction mixtures containing 100 ng of DNA, 200 μM dNTPs, 1 μM primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 units AmpliTaq DNA polymerase (Perkin Elmer) in a Perkin Elmer Cetus 9600 thermal cycler. PCR conditions for Hprt and G6pd were as follows: denaturation at 94°C for 5 min, 30 cycles of 94°C/30 s, 60°C/30 s, 72°C/60 s; extension at 72°C for 10 min. Aprt and Ins amplification was carried out in the same conditions except for the annealing temperature (55°C). Formamide was added to a final concentration of 2% and 4% for the optimal amplification of Hprt and Aprt, respectively.

Amplified DNA was ligated into pGL3-basic vector (Promega) and transformed into competent *E. coli* (XL1-Blue). The purified pGL3/G6pd, pGL3/Hprt, pGL3/Aprt, and pGL3/Ins constructs were sequenced using an ABI PRISM 310 Genetic Analyzer.

The Xist and the Pgk1 promoter regions (−231/+20 and −319/+226, respectively) were subcloned into pGL3-basic vector from the previously described pCAT/Xist and pCAT/Pgk1 (1). The repeat elements (+241/+894) were subcloned from the pBS/4844B (27) using the *Ssp*I and *Hin*dIII restriction enzymes into the pGL3-basic vector and other derived constructions.

The double-stranded wild-type Ccs DNA TCTTG CCCATCGGGGCCACGGATACCTGTGTGT was concatenated 4 and 8 times and cloned downstream the Pgk1 promoter to give the pGL3/Pgk1 + 4xCcs and pGL3/Pgk1 + 8xCcs constructs.

Cell Culture

The BALB/3T3 cell line was grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere.

Transfection, Luciferase, and CAT Assays

BALB/3T3 ($1–2 \times 10^5$) were transiently transfected by liposome-mediated DNA transfection (DOTAP, Boehringer Mannheim) using 2.5 μg of the construct. Relative transfection efficiency was determined by cotransfection with a pRL/SV40 and a pSV/β-galactosidase reporter genes for the luciferase and CAT assays, respectively.

For luciferase assays, transfected cells were harvested using Cell Lysis Reagent (Promega) according to the manufacturer's instructions. Aliquots of 20 μl cell lysate were used to measure luciferase activity by addition of 50 μl Luciferase Assay Reagent (Promega), followed by luminescence quantitation in a E&G Berthold Lumat LB9507. The results were normalized to the value of Renilla luciferase activity measured from the cotransfected pRL/SV40 and indicated as rlu (relative light unit). The luciferase experiments were carried out 6 to 30 times depending on the constructs and a mean activity was reported with 1 SD.

CAT assays were carried out as previously described (1).

RNA Isolation and Semiquantitative RT-PCR

RNA was prepared from cultured cells by the guanidium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (11). Approximately 0.8 μg of RNA was transcribed in a total volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM dNTP, 20 units of RNasin, 50 units of MuLV reverse transcriptase, and 2.5 μM of random hexamers. The reaction was incu-

bated 10 min at room temperature, 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C. First strand synthesis (5 μl) was amplified by PCR in a total volume of 25 μl. PCR reaction contained 50 mM KCl, 10 mM Tris at pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of each primer, 1 μCi of [α-^{32}P]dCTP, and 1.5 units of Taq polymerase. The Luciferase, Renilla, and Ins genes amplification consisted of a denaturation step at 94°C for 5 min, followed by 20, 25, and 22 cycles, respectively, of PCR amplification at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a 10-min extension at 72°C. Products were analyzed by electrophoresis on an 8% polyacrylamide gel. Signals were quantified using a Bio-Rad phosphorimager (GS-363).

Preparation of Nuclear Extracts

Cells from 10-cm dishes were used for each preparation. Cell were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in 5 packed cell volume of buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)] for 10 min at 4°C. Cells were collected by microcentrifugation, resuspended in 3 volumes buffer A, and homogenized after addition of Nonidet P-40 (NP40) to 0.05%. The crude nuclei released were collected by microcentrifugation and resuspended in 1 ml of buffer C [5 mM HEPES, pH 7.9, 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF]. NaCl was added to a final concentration of 300 mM. Nuclei were incubated at 4°C for 30 min and clarified by microcentrifugation for 20 min. Nuclear extracts were frozen on dry ice and stored at −70°C.

RNA Mobility Shift Assay

Synthesized RNA oligonucleotide Ccs (UCU UGC CCA UCG GGG CCA CGG AUA) was end-labeled with [γ-^{32}P]ATP and the T4 kinase. Typical mixtures (20 μl) for in vitro binding reactions contained 1 μg of poly(dI-dC), 1× binding buffer (5 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 5% glycerol), 10 U RNasin, 2–5 μg of nuclear extract proteins, and 2×10^{-2} pmol of the ^{32}P-labeled probe. Reaction mixtures were incubated for 15 min on ice and analyzed by electrophoresis on a 6% native polyacrylamide gel at 4°C and 350 V for 1 h in 0.5× Tris-borate/EDTA buffer (TBE). Competition reactions were performed by adding excess of competitor oligonucleotides to the binding reaction mixture prior to the addition of labeled probe. Mutants were synthesized and used in competition reactions. The Rep2 mutant contains a 5-bp substitution at the 5′ extremity (GCCCA→AUUUG), the Rep3 has a 6-bp substitution (CGGGGC→UAAAAU) in the core element, and Rep4 was modified at its 3′ extremity (GGAUACCUG→AAGCGUUCA). Gels were then dried under vacuum at 80°C for 30 min and exposed to X-OMAT AR films.

UV Crosslinking Reaction

The product of in vitro binding reaction was irradiated on ice for 30 min under an UV transilluminator of 254 nm, denatured 10 min at 95°C in 1× denaturing buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 3% SDS, 0.005% bromophenol blue, 5% β-mercaptoethanol), and loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis at 200 V for 90 min, the gel was dried and autoradiographed overnight. For size estimation, prestained molecular weight markers (BioRad) were included in each gel.

Northwestern Analysis

Nuclear proteins (50–100 μg) were denatured, separated on a SDS-polyacrylamide gel, and transferred onto nitrocellulose. The transfer was run at 4°C and 0.5 mA overnight in the electroblotter filled with buffer [25 mM Tris-HCl, pH 8.3, 190 mM glycine, and 20% methanol (v/v)]. After transfer, the membrane was washed and blocked on ice for 3 h in TNE-50 (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT) and 5% dried milk powder. The washed membrane was immersed on ice for 2–3 h in TNE-50 containing 10 μg/ml nonspecific competitor DNA and 2×10^6 cpm/ml labeled RNA probe and 4 U/ml RNasin. The filter was finally washed several times in TNE-50, for 5 min each time, until the counts corresponding to the nonspecific binding disappeared. After air drying, the filter was autoradiographed overnight.

RESULTS

Xcr Inhibits the Transcription of Pgk1, Hprt, and G6pd

Hendrich et al. (18) observed that the 5′ repeat element of XIST acts as a modulator of the human XIST expression. We verified the effect of the similar repeats on the mouse Xist promoter by transient expression assays. We generated the pGL3/Xist and pGL3/Xist+Xcr constructs, which contain the P1 Xist promoter (−231/+20) driving the expression of the luciferase gene, with or without Xcr downstream of the promoter. The promoter without Xcr induced efficient transcription when transiently transfected into female BALB/3T3 murine cells as already shown

(27) (Fig. 1a). Adding Xcr in the construct boosted luciferase activity by 750%. We then investigated the possible effect of Xcr on the promoter of genes known to be X inactivated and introduced the Xcr region downstream of the mouse Pgk1, G6pd, and Hprt promoters. The presence of Xcr in *cis* resulted in a decrease in luciferase activity from 100% to 28%, 42%, and 33%, respectively (Fig. 1b). In order to test whether this effect was linked to the use of the luciferase reporter system, the Pgk1 promoter, with or without Xcr, was introduced in front of a CAT reporter gene. In transient transfection experiments, even stronger decrease was obtained showing a decline of the Pgk1 promoter activity from 100% when no Xcr was present to 3% with Xcr (Fig. 1c). When Xcr was subcloned downstream of the promoter of the murine autosomal genes Aprt and Ins (Fig. 1d), or after the simian virus 40 (SV40) promoter, no effect on their transcriptional activity were observed. These transient transfection assays suggest that Xcr modulates differently the transcription of genes subjected to X inactivation from those that are on autosomes and the Xist gene.

As the luciferase activity assay is based on protein level, we wanted to investigate whether Xcr affects the RNA level. Female BALB/3T3 murine cells were transiently transfected and RNA produced from both the Luciferase and Renilla genes was quantified by semiquantitative RT-PCR. The luciferase transcript levels were in accordance with the luciferase activities concerning the X-linked Pgk1 and the autosomal Aprt promoter constructs (Fig. 2). When Xcr was subcloned downstream of the Pgk1 promoter, the luciferase RNA was decreased from 100% to 44%, whereas Xcr had no effect on the luciferase transcript level when localized 3′ to the Aprt promoter.

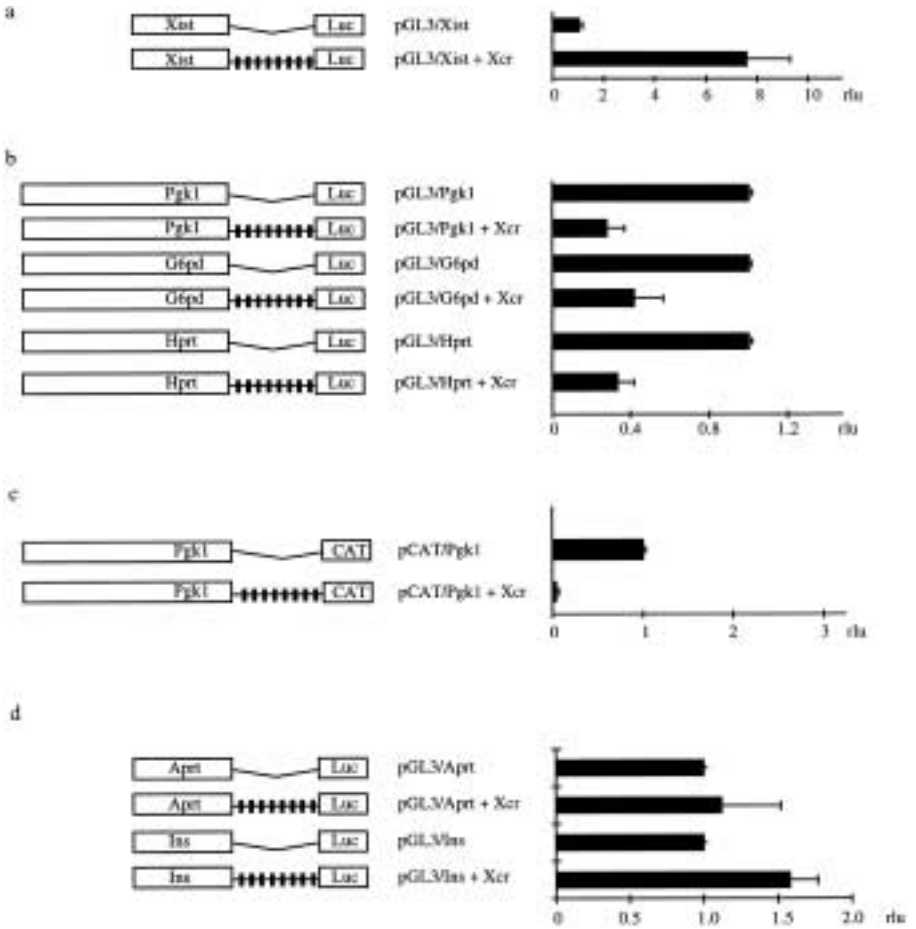

Figure 1. Xist promoter activity is enhanced by the repeat elements (Xcr), whereas Xcr reduces the transcription of other X-linked genes. Relative luciferase activity (rlu, relative light unit) is plotted after each construct with bars indicating 1 SD. (a) BALB/3T3 cells transfected with Xist and Xist+Xcr constructs. The black boxes indicate the 8 conserved core sequences spaced by T/U-rich elements.(b) BALB/3T3 cells transfected with the promoter of three X-linked genes (Pgk1, G6pd, and Hprt), with or without Xcr. (c) BALB/3T3 cells transfected with Xcr cloned 3′ of the Pgk1 promoter. CAT activity is plotted. (d) BALB/3T3 cells transfected with Xcr cloned 3′ of the autosomal Aprt and Ins genes promoter, with and without Xcr.

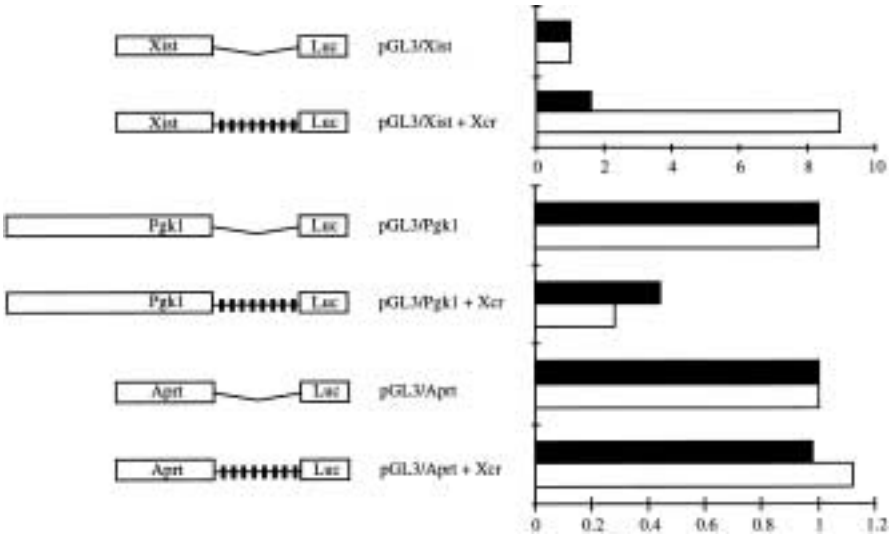

Figure 2. Quantification of RNA showed that Xcr reduces the transcription of X-linked genes. BALB/3T3 cells were transfected with the promoter of Xist, Pgk1, and Aprt, with or without Xcr, and relative luciferase activity (white bars) was compared to relative luciferase transcript level (black bars).

The study of these transient transfected cells indicated that Xcr modulates differently the transcription of the genes subject to X inactivation from those that are on autosomes. This suggests that Xcr recognizes, directly or indirectly, differences on the promoter of these genes.

Xcr Acts in cis, *Not in* trans

The propagation of X chromosome inactivation is thought to be mediated by *cis*-limited spreading of the nonprotein coding Xist transcript. It would therefore be expected that, if Xcr is involved in spreading X inactivation, its activity would be restricted to a *cis* effect and would have no *trans* activity. To test this hypothesis, the pGL3/Pgk1 construct was cotransfected with the pCAT/Xist+Xcr, a plasmid transcribing Xist and Xcr. Two molar ratios of both pGL3 and pCAT constructs were tested 1:1 and 1:4, respectively. As measured by luciferase activities, the presence of Xcr transcripts in *trans* had no significant effect on Pgk1 promoter, supporting the *cis*-limited action of the Xcr RNA (Fig .3).

Xcr Activating/Repressing Activity Is Position Dependent

To investigate whether Xcr activity was position dependent, Xcr was cloned upstream of the Pgk1 promoter in a region where it is not transcribed. Luciferase activity was then measured. No significant variation in promoter activity was observed (Fig .4). These data indicate that Xcr is not a classical enhancer element that shows position-independent effects. Rather, the strong action observed when it is part of the transcript itself is consistent with a posttranscriptional effect.

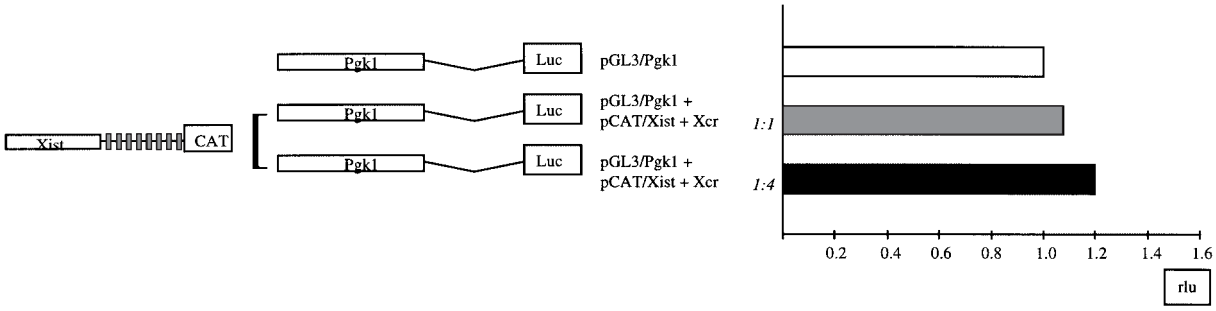

Figure 3. The Xcr transcript in *trans* has only few effects on the Pgk1 promoter. The pGL3/Pgk1 constructs was cotransfected with the pCAT/Xist+Xcr. The luciferase activity was measured using two molar ratios of both pGL3 and pCAT constructs (1:1 and 1:4).

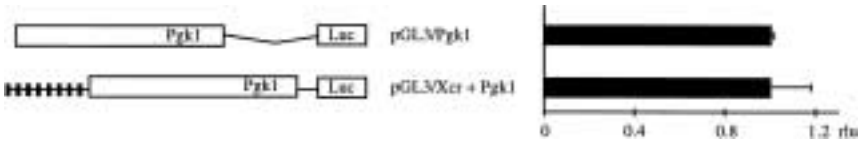

Figure 4. Xcr repressing activity is mediated through RNA rather than DNA. BALB/3T3 cells transfected with Xcr cloned 5′ of the Pgk1 promoter.

Pgk Inactivation Is Mediated by the Complete Xcr and Is Not Limited to the Xcr Conserved Core Sequence

In order to investigate the functional importance of the 24-bp conserved core sequence (Ccs) in transcriptional repression, this sequence was concatenated 4 and 8 times and subcloned 3′ of the Pgk1 minimal promoters. BALB/3T3 cells were transfected with the resulting constructs. Luciferase assays revealed that 4 and 8 concatenated Ccs had no significant effect on the Pgk1 promoter, suggesting that the T/U-rich spacers were necessary to modulate Pgk1 transcriptional activity (Fig. 5).

A Nuclear Protein Binds to the RNA of Ccs

Experiments shown above indicate that repression of the Pgk1 promoter activity requires the complete Xcr, including the core elements and the T/U-rich spacers. Two heteronuclear proteins, hnRNPC1 and C2, have been shown to have an affinity for the T/U-rich spacer between the conserved repeats (8). However, they do not have any known role in chromatin structure or stability. It should be interesting to further investigate the role of hnRNPC1 and C2 and their potential interaction with other proteins likely to bind the Xcr. We therefore examined whether a nuclear protein was able to interact with the Ccs RNA. Electrophoretic mobility shift assays (EMSA) performed using a radiolabeled Ccs RNA oligonucleotide in combination with nuclear extracts prepared from BALB/3T3 cells identified a delayed migrating complex. Competition experiments with cold homologous Ccs RNA and heterologous RNA oligonucleotides confirmed the sequence specificity of the observed complex (Fig. 6a). The specificity of this complex for RNA rather than for single- or double-stranded DNA was verified by competition with unlabeled single- and double-stranded Ccs DNA (Fig. 6a). This RNA binding protein was observed in human fibroblasts, mouse XX and XY fibroblasts, undifferentiated mouse ES cells, and rat pancreatic cells, suggesting an ubiquitous expression. Mutants were designed with alteration in those regions that were conserved in both mouse and human. As shown in Figure 6b, the mutant oligonucleotide Rep2+3+4, in which the mutation at the 5′ and the 3′ extremities and in the core element are combined, abolished the binding. In contrast, the other mutations did not influence the protein–RNA complex formation.

RNA–Protein Interaction Detected in UV-Crosslinking and Northwestern Experiments

We conducted both UV crosslinking and Northwestern experiments to further characterize the factor interacting with Ccs. Protein–RNA complex formed between BALB/3T3 nuclear extracts and the labeled RNA oligonucleotide Ccs was induced to crosslink under UV and was separated on SDS polyacrylamide gel. The molecular mass of the proteins was interpolated from prestained proteins standards at 60 kDa. A second protein was observed, whose 120 kDa size suggests a dimerization of the 60-kDa protein (Fig. 6c). Northwestern experiments identified a protein of 120 kDa (Fig. 6d).

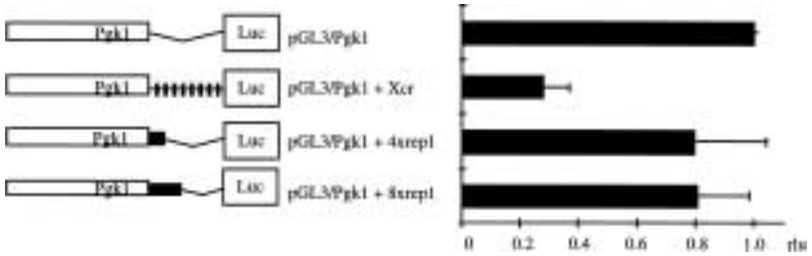

Figure 5. Eight copies of the conserved core element have no effect on the Pgk1 transcription. Relative luciferase activity is plotted after each construct with bars indicating 1 SD. BALB/3T3 cells transfected with control constructs and constructs containing the Ccs concatenated 4 and 8 times downstream of the Pgk1 promoter.

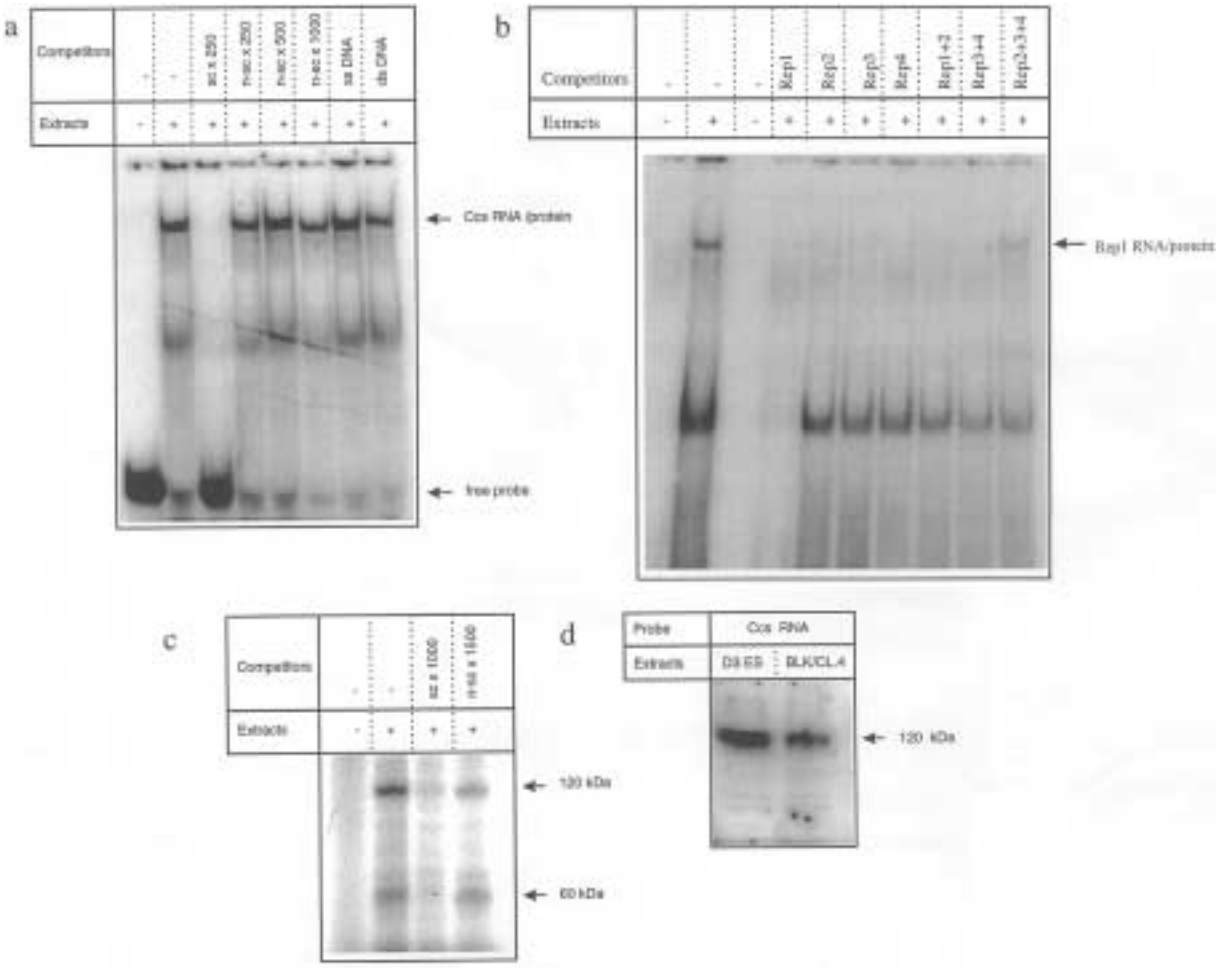

Figure 6. A 120-kDa nuclear protein interacts with Ccs RNA. (a) EMSA with or without BALB/3T3 nuclear extracts using radiolabeled Ccs RNA oligonucleotide. The nature of the competitors is indicated in each column (–: no competitor; sc × 250: unlabeled Ccs RNA; n-sc × 250, n-sc × 500, n-sc × 1000: different excess of nonspecific RNA; ss DNA: single-stranded DNA Ccs; ds DNA: double-stranded DNA Ccs). (b) EMSA with or without BALB/3T3 nuclear extracts using radiolabeled Ccs RNA oligonucleotide. The nature of the competitors is indicated in each column. (c) Radiolabeled Ccs RNA oligonucleotide was used in UV crosslinking. The nature of the competitors is indicated in each column (–: no competitor; sc × 1000: unlabeled Ccs RNA; n-sc × 1500: nonspecific RNA). (d) Northwestern experiments carried out with extracts from male mouse D3 ES and fibroblast BLK/CL.4 cell lines and a concatenated labeled Ccs RNA probe.

DISCUSSION

Although several studies have shown that Xist is implicated in X chromosome inactivation, it is still not known how this is achieved and whether the complete Xist transcript is required for this effect. Alignment studies of the 15-kb Xist transcript with the human XIST gene identified 5 conserved repetitive sequences. We investigated whether the Xist conserved repeat (Xcr) located in the first repetitive sequence of the gene was active in the repression of X-linked genes.

Here, we report that Xcr inactivates the transcription of X-linked genes. When located downstream of the promoter of Pgk1, G6pd, and Hprt, three X-linked genes, Xcr decreases the luciferase activity by 72%, 58%, and 67% of the X-linked Pgk1, G6pd, and Hprt promoter activity, respectively. In similar conditions, Xcr has no modulatory effect on the promoter activity of the mouse Aprt and Ins autosomal genes or on the promoter of SV40, a viral gene with no inactivation. This repression action is measured at the protein level using both the luciferase and CAT expression systems and at the RNA level by RT-PCR. Although the exact mechanism by which this action is mediated is still unknown, it seems that Xcr acts through the production of its transcript as its insertion 5′ of the promoter does not induce any transcriptional modification.

Xist RNA does not bind directly to Xi DNA, but associates with nuclear proteins to influence higher order chromatin structure (13). Two heteronuclear

proteins, hnRNPC1 and C2 (41 and 43 kDa, respectively), have been shown to associate with a 1-kb Xist transcript containing Xcr (8) and to have a strong affinity for poly(U) sequences, which are found in the spacer of the repeats. The abundant hnRNPC1/C2 proteins (15) seem to be RNA chaperones involved in the annealing of RNAs and transfer of RNA to other proteins including the splicing machinery (28). However, they do not have any known role in chromatin structure or stability. Our transient transfection assays showed that Ccs alone had no effect on the Pgk1 promoter: 8 core copies concatenated downstream of the Pgk1 promoter did not induce any significant modification in transcriptional activity, suggesting that the T/U-rich spacers were needed for the inactivation process to occur. We have identified by EMSA a 120-kDa nuclear protein that binds specifically to Ccs RNA. Whether this protein interacts also with hnRNPC1 and C2 remains to be seen.

Noncoding RNAs may have various functional roles in both the cytoplasm and nucleus. In addition to the classical tRNA and rRNA capacities, RNAs can exhibit enzymatic function like the ribozymes or can act as regulators controlling gene expression. The transcripts of H19 (26) and IPW (32) have been shown to selectively silence neighboring genes (23,31). Interestingly, two noncoding RNAs, Rox1 and Rox2 (25), were recently shown to associate with the male X chromosome in *Drosophila* (2). This association was proposed to help the chromatin conformation modification and to achieve hypertranscription underlying the dosage compensation. The similarities between Xist and Rox1/2 are striking; they are both nuclear and localized to an X chromosome that is undergoing structural modification of chromatin as part of dosage compensation. Furthermore, the fact that an RNA molecule can specifically recognize and "paint" a single chromosome suggests a common feature of chromosome regulation. A family of *Xenopus* RNAs carrying small interspersed repeats, homologous to those present in Xist, has been implicated in ensuring cellular localization of other unique heterologous RNAs. The repeat sequences appear to play a critical role in this process (20).

The observed Xcr effect on Hprt, G6pd, Pgk1, Aprt, Ins, and SV40 lead to the proposal that the Xcr transcript specifically recognizes a common *cis* element on the promoter of X-linked genes, most likely through interactions with nuclear protein(s). There is still no indication as to how X inactivation spreading occurs. A hypothesis evokes a mechanism in which Xist RNA associates with high-affinity sites (boosters) on X chromatin via nuclear protein interactions. Because such booster elements are less frequent on autosomes, they would limit the spread of inactivation into autosomal material. A recent study suggests that these sites are not uniformly distributed over the X chromosome, but are preferentially associated with gene-rich, G-light regions and excluded from constitutive heterochromatin (16). Our results are compatible with the fact that a consensus sequence in the promoter of genes that are inactivated could act as booster elements interacting with the Xist transcript through nuclear protein binding. Analysis of sequential mutations of the Pgk1 promoter should allow the identification of the region that is necessary for inactivation through Xcr. This sequence could then be searched for in other X-linked genes subject to inactivation and in genes located on autosomes. We hypothesize that the majority of autosomal genes will be devoid of the consensus sequence, and will thus be protected from the inactivation process. Few autosomal genes may have Xcr binding domains, which, when located closely to Xist as in X/autosome translocations for example, may explain why inactivation could spread to these genes.

Our results indicate that Xcr is a functional domain of Xist and plays a role in the transcriptional regulation of X-linked genes.

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