Negative Transcriptional Modulation and Silencing of the Bi-exonic Rnf35 Gene in the Preimplantation Embryo

BINDING OF THE CCAAT-DISPLACEMENT PROTEIN/Cux TO THE UNTRANSLATED EXON 1 SEQUENCE*

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Previous works have indicated promiscuous transcription from the zygotic genome immediately after fertilization. The mouse Rnf35 gene is bi-exonic in structure and is transcribed in the preimplantation embryo until it is permanently silenced at the blastocyst stage of development. We have previously shown that Rnf35 transcription is positively regulated by the nuclear factor Y. Using the uniquely permissive Chinese hamster ovary-K1 cell line in transient transfection assays, we demonstrate in this work that the Rnf35 promoter was negatively modulated by a cis-cognate repressor element, designated as the downstream exon 1 repressor, or DER, residing between +72 and +95 in the untranslated exon 1 of the Rnf35 gene. Simultaneous mutagenesis of the two halves, DER1 and DER2, of the DER sequence was required for derepression suggesting participation of multiple proteins in the DER-dependent transcriptional repression. Electrophoretic mobility shift assays demonstrated that the 3’-half of DER (DER2) was targeted by the repressor CCAAT-displacement protein (CDP)/Cux. Chromatin immunoprecipitation experiments further demonstrated in vivo CDP-DER association in the blastocyst and the 8.5-day embryo. Furthermore, the DER-dependent repression was partially relieved in vivo in co-transfection with an antisense CDP construct. Transcription of the Cdp gene was shown to first occur between the eight-cell and the blastocyst stages, correlating and possibly explaining the onset of Rnf35 silencing at the blastocyst stage. Taken together, our results suggest that the evolutionarily acquired exon 1 of Rnf35, and possibly exon 1 of other similarly structured bi-exonic early embryonic genes, contributes to transcriptional modulation and silencing in the developing mouse embryo.

It has been estimated that up to ∼15,000 genes of general functionality and temporal or spatial specificity are involved in different stages of development of the mammalian preimplantation embryo (1, 2). An inventory of null mutants lethal for the first bursts of transcription from the newly constituted zygotic genome in the late one-cell embryo is beginning to emerge (reviewed in Refs. 4 and 5). The initial phase of zygotic transcription is thought to be global and promiscuous: a considerable fraction of transcripts in the two-cell stage embryo is derived from repetitive sequences (1, 3). One way of achieving some order in transcriptional regulation at and soon after the two-cell stage is the participation of specific transcriptional activators or repressors. Typically, the Hsp70 gene is transcribed both maternally and from the zygotic genome (6, 7). At the one- and two-cell stages, Hsp70 transcription is dependent on a TATA-box promoter using Sp1 as an activator as evidenced by increases in Sp1 concentration in the embryonic nucleus between the one- and two-cell stages (8–10). A maternally derived GAGA-box-binding factor may be another contributor to Hsp70 transcriptional regulation (9). In a recent work, we have shown that the ubiquitous Y-box protein, the nuclear factor Y (NF-Y), is a positive transcriptional activator of the preimplantation embryo-restricted Rnf35 gene; Rnf35 transcription is driven by an initiator (Inr) core promoter element without the support of a TATA-box (11). The NF-Y acts on a Y-box sequence residing at −85 in the 5′-upstream regulatory region (5′-URR) of the Rnf35 gene. It is noteworthy that Sp1 and the NF-YA and NF-YC subunits of the NF-Y complex are structurally similar in encoding glutamine- and hydrophobic residue-rich activation domains (12, 13). The ubiquitous NF-Y and Sp1 may, therefore, be recruited as alternative activators in transcriptional control in the preimplantation development. Other transcription factors that may be involved are actively being pursued by different study groups.

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1 The abbreviations used are: NF-Y, nuclear factor Y; AS-CDP, antisense CDP; CDP, CCAAT-displacement protein; ChIP, chromatin immunoprecipitation; DER, downstream exon 1 repressor; EGFP, enhanced green fluorescence protein; EMSA, electrophoretic mobility shift assay; Inr, initiator; SEAP, secreted alkaline phosphatase; UCE, uninterrupted coding exon; 5′-URR, 5′-upstream regulatory region; CHO, Chinese hamster ovary; HD, homeodomain.
To investigate transcriptional regulation in the preimplantation development, we have been using the Rnf35 gene as a working model. Rnf35 is temporally transcribed in the unfer-tilized egg and in the preimplantation embryo up to the eight-cell stage; the gene is permanently silenced between the eight-cell and blastocyst embryonic stages (14–16). Rnf35 encodes a putative RING-finger protein with uncharacterized function. The Rnf35 gene is bi-exonic in structure in comprising a short and untranslated 133-bp exon 1, a 3.6-kb solitary intron, and an exon 2 containing the uninterrupted coding sequence and the rest of the gene (14, 15). Intriguingly, the Rnf35 exon 1 also doubles as the first exon of a minor population of transcript of a homologous and preimplantation embryo-restricted gene, Rnf33, that is located downstream of Rnf35 (Fig. 1A) (14, 15).

In the course of the analysis of other preimplantation embryo-restricted genes uncovered in our other laboratories, we have noted that numerous members of this family of genes are similarly bi-exonic and carry an uninterrupted coding exon (11). The UCE configuration of the gene confers obvious advantages in gene expression in circumventing splicing errors in the coding region and in expending less metabolic energies. The solitary intron in the bi-exonic structure could have contributed a further advantage to the expression of Rnf35: Matsumoto et al. (19) have unequivocally demonstrated that transcription of intron-less mRNA leads to translational silencing, an event alleviated by the presence of an intron at the 5‘-end of the transcript. Hence, 5‘-introns are important for regulating the coupled transcription-translation machinery required for efficient translation (20, 21). The first indication that the noncoding exon 1 may be biologically significant comes from the finding that Rnf35 uses an initiator (Inr) as the sole core promoter element in transcription (11). The Inr overlaps with the 5‘-terminus of the exon 1 sequence and within which resides the transcription start site. In the absence of an upstream TATA-box, other general or specific regulatory factors of the transcriptional machinery bind to Inr and flanking sequences to properly initiate transcription (reviewed in Refs. 22–24). In this work, we demonstrate that the 133-bp exon 1 of the Rnf35 gene harbors a cis-cognate repressor element that is targeted by the ubiquitously transcribed repressor CCAAT-displacement protein, CDP, also called Cux. Our data further suggest that CDP/Cux contributes to the initial phase of silencing of the Rnf35 gene in early mouse development.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction, Site-directed Mutagenesis, Transient Transfection, and SEAP Assay**—For construction of the SEAP reporter plasmids used in this work, various segments of the Rnf35 5‘-URR and exon 1 sequences were PCR-derived and cloned into the NheI and XhoI sites of the promoter and enhancer-free pSEAP2-Basic vector as described in previous works (11, 25). For site-directed mutagenesis, oligonucleotide primers encompassing the mutations and containing appropriate restriction cloning sites were used in the PCR amplification reactions. The CDP antisense construct was generated by inverting the human CDPCD gene harbored in the pMX139-MCH plasmid provided by Dr. A. Nepveu. The expression plasmids were introduced into the BL21(DE3) strain of *Escherichia coli*. When the bacterial culture reached an *A*~600~ value of 0.6, induction was typically performed with 1 mM isopropyl-p-D-thiogalactopyranoside for 3 h. Cells were harvested and resuspended in a lysis buffer (50 mM NaHPO4, pH 8.0, 300 mM NaCl, 10 mM imidazole) for homogenization with glass beads. After centrifugation, the supernatant was transferred to a fresh tube, and the His-tagged polypeptides were purified with Ni2+ nitrilotriacetic acid-agarose beads (Qiagen) according to the manufacturer's instructions. Binding was carried out at 4 °C overnight, and the polypeptide-bound beads were washed four times with a wash buffer (50 mM NaHPO4, pH 8.0, 300 mM NaCl, 20 mM imidazole) followed by elution using the elution buffer (50 mM NaHPO4, pH 8.0, 300 mM NaCl, 250 mM imidazole).

**Electrophoretic Mobility Shift and Supershift Assays**—The probes used for the electrophoretic mobility shift assays (EMSAs) were mobility shift assay (MSA) oligonucleotides (EMSA Oligos) labeled by annealing complementary strands of oligonucleotides and labeled by standard Klenow fill-in reactions of the staggered ends in the presence of [α-32P]dCTP. For binding reactions using the bacteria-derived CDP polypeptides, 1-μg aliquots of the polypeptides were first incubated in 25 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM MgCl2, 5 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 3 μg of bovine serum albumin, and 30 ng of DNA fragment (CDP) from the temperature of 5 min. The addition of probe and a further 20-min incubation at 30 °C. For supershift assays of the His-tagged CDP polypeptides, 2 μg/ml anti-His antibody (catalog no. MCA 1396, Serotec, Raleigh, NC) was added to the CDP polypeptide binding reactions as described above and the incubation was performed at room temperature for 15 min. Probe was then added, and the incubation was extended for 20 min at 30 °C. Binding complexes were analyzed by gel electrophoresis on 10% polyacrylamide gel containing 0.35% for 3 h for the CDP(CR3-HD) polypeptide or 5 h when CDP(CR1–CR2) was used.

**Chromatin Immunoprecipitation**—Mouse blastocysts and 8.5-day post-coitus embryos were collected from ICR mice and washed briefly with ice-cold phosphate-buffered saline before being frozen at −70 °C. To assay ChIP experiments were performed on pools of 50–60 blastocysts or ten 8.5-day embryos essentially as previously described (26) with a few modifications to accommodate fewer cells available in the embryos. In brief, protein-DNA complexes were cross-linked in 1% formaldehyde in 400 μl of phosphate-buffered saline, and the reaction was terminated by adding glycerine to a final concentration of 125 mM. Upon cell lysis, sonication was performed using an XL2020 sonicator (Misonix Inc., Farmingdale, NY) using a tapered tip with an output setting of 4.5 for six 10-s bursts on ice. For each ChIP sample, 100 μl of the sheared chromatin was diluted to 1 ml with an immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). After preclearance in the PASDT solution (50% v/v of packed protein A-Sepharose beads, 240 μg/ml salmon sperm DNA, 240 μg/ml tRNA), lysate was collected and incubated with 5 μl of an anti-CDP antisem (a gift from Dr. Ellis J. Neufeld, Harvard Medical School, Boston, MA) (27, 28). For controls, a mock sample without antisem treatment and a sample treated with a preimmune serum were also included in each experiment. The pulled-down immune complexes were washed and eluted; reversal of the cross-links and subsequent digestions with RNase and proteinase K were performed as described (26). The mix was finally dissolved in 10 μl of TE buffer (10 mM Tris, pH 8.1, 1 mM EDTA). Three microliters of each DNA sample was used in PCR analysis in the presence of 40 pm of each of the DFR-specific primers in 15-μl PCR reaction volumes. Due to scarcity of the embryonic cells, two rounds of nested PCR were performed. The first round of PCR was performed for 42 cycles using the primers 2406F (5’-AAGCCTCAGAAGACAACAGTT-C3’ and 287TR (5’-AAACATTCTACACTCACCCAG-3’). One
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Fig. 1. Identification of a cis-cognate downstream repressor element, DER, in the Rnf35 exon 1 sequence. A, a scheme showing the relative positions of the bi-exonic Rnf35 gene and the Rnf33 homologue (in approximate scale). The exons (denoted by boxes) and introns (dashed bent lines) are as previously determined (GenBank™ accession no. AY063497) (15). The −210 to +133 promoter segment encompassing the Rnf35 5′-URR and exon 1 analyzed in this work is denoted a thick bar. DER, downstream exon 1 repressor. B, preliminary mapping of DER between +72 and +133 in the Rnf35 exon 1 (box). In the experiment, SEAP gene constructs carrying different lengths of 5′-URR and partial (up to +71) or full-length exon 1 (up to +133) sequences were used in transient transfection of the CHO-K1 cells in triplicates. The relative SEAP activity data shown were derived from three or more independent experiments. Hatched vertical bars denote the presence of the initiator (I) element as previously elucidated (11). C, the DER element is functional predominantly in the CHO-K1 cells. Equal amounts of the N6–51 and N6–52 plasmid DNA were transfected into the indicated cell lines, and SEAP assays were performed 48 h post-transfection. The cell lines tested were CHO-K1 (Chinese hamster ovary), BHK-21 (hamster kidney cells), NIH3T3 (mouse embryo fibroblasts), COS7 (African Green Monkey kidney cells), Huh7 (human hepatocellular carcinoma), and HeLa (human cervical cancer). D, the DER element is also cis-repressive in transcription driven by a TATA-box promoter. The Rnf35 exon 1 sequence from +4 to +133 devoid of the Inr core promoter was inserted at the HindIII (H3) and EcoRI (R1) sites downstream of the SV40 promoter-enhancer (SV40k/p) sequence of the pSEAP-Control plasmid to generate pSEAP-Ex1. To generate pSEAP-Ex1AR, the sequence (gray box) between +72 and +133 was deleted. The relative transcription activities were determined in transient transfection of the CHO-K1 cells. E, fine mapping of DER (horizontally hatched bars) performed using serially deleted constructs N10gA through N10gC derived from construct N10g1 in CHO-K1 transient transfection experiments.

- Microgram of the first-round PCR products was used in a second-round amplification step using the nested primers 2465F (5′-TAAACGCCCATTCTAAACG-3′) and 2746R (5′-TTCCGTTGGCAGCTTCTC-3′) for 36 cycles of amplification. The amplification product (282 bp in size) was analyzed in a 1.5% agarose gel and visualized after ethidium bromide staining.

- Western Blot Analysis—CHO-K1 cells transfected with the CDP expression plasmid were harvested 36 h after transfection and lysed in 1× SDS-sample buffer (50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol; 2% SDS; 0.1% bromphenol blue; 10% glycerol). The lysate was incubated at 95 °C for 15 min before being subjected to electrophoresis in a 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences), and the membrane filter was blocked in 5% skimmed milk in TBS (20 mM Tris-HCl, pH 7.6; 150 mM NaCl) at room temperature for 1 h. The anti-CDP antibody (as described above, in 1,000× dilution), or the control anti-NF-Ya subunit antibody (Rockland, Gibilsville, PA, catalog no. 100-401-100, in 1,000× dilution), was applied at room temperature for 1 h before filter was washed with 0.1% Tween 20 in TBS. An anti-guinea pig-horseradish peroxidase antibody (Chemicon, Temecula, CA, in 20,000× dilution) was added, and the mixture was incubated at room temperature for 1 h after which the filter was washed with 0.1% Tween 20 in TBS. Signals were visualized by chemiluminescence after treating the blot with a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer’s recommendations.

- PCR Detection of the Cdp Sequence in cDNA Libraries—DNA preparations from plate-amplified bacterial cultures of mouse cDNA libraries were used as templates for PCR detection of mouse Cdp sequences as previously described (15, 16, 29). The Cdp primer sequences were 5′-CAGGACAGCCAGCGGTAAGAGC-3′ and 5′-GAACTCAGCTTGGGAGGCTCCT-3′. PCR was carried out for 35 cycles using an annealing temperature of 61 °C and an extension time of 1 min for each cycle. The PCR product was 386-bp long covering nucleotides 4179–4565 of the 3′-untranslated sequence of the mouse Cdp gene (GenBank™ accession number AY037867). For control, mouse β-actin primers were used. The PCR products were analyzed on 2% agarose gels.

RESULTS

Identification of a cis-Cognate Downstream Repressor Element, DER, in the Rnf35 Exon 1 Sequence—A schematic representation of the bi-exonic Rnf35 and a downstream homologue, Rnf33, is shown in Fig. 1A. It is noted here that the exon 1 of Rnf35 also serves as the exon 1 of a minor population (<10%) of the Rnf33 transcript; the major promoter of Rnf33 is shown in Fig. 1A (14, 15). In a previous work, we demonstrated that a segment of the 5′-upstream regulatory region (5′-URR) of the Rnf35 gene up to nucleotide −210 in the plasmid construct N6–51 was sufficient in activating transcription of the SEAP reporter gene in the uniquely permissive cell line, CHO-K1 (11; see also Fig. 1B). A shorter 5′-URR segment terminating at −13 in construct N10g1 was also transcriptionally active albeit with a slight decrease in transcriptional activity (Fig. 1B). Deleting the previously identified Inr core...
promoter sequence in construct N10–141ΔI resulted in a 5-fold derepression in SEAP activity.

The Rnf35 exon 1 is 133 bp long (Fig. 1A) (14, 15). The three abovementioned constructs carried only the 5'–half of the Rnf35 exon 1 sequence up to +71. To explore if the 3'–half of the exon 1 sequence between +72 and +133 contributes to Rnf35 transcription, new constructs carrying the full-length exon 1 sequence and various lengths of the 5'-URR were generated (Fig. 1B). Transient transfection assays in the CHO-K1 cells indicate that when the exon 1 sequence was extended to its full length to +133 in construct N6–52, promoter activity was derepressed 4-fold relative to that of the truncated N6–51 (Fig. 1B). Likewise, in the N10g1/N10g2 and N10–141ΔI/N10–142ΔI pairs of constructs that shared similar 5' sequences but dissimilar 3' termini, about a 4-fold suppression of promoter activity was similarly observed. These data suggest the presence of a cis-acting repressor element located between +72 and +133 of the Rnf35 exon 1. We designate this cis-acting repressor as the downstream exon 1 repressor, or DER. When constructs N6–51 and N6–52 were tested in five other cell lines derived from different cell types besides CHO-K1, only the hamster kidney cells, BHK-21, showed an appreciable permissiveness for the Rnf35 promoter but at a level 5-fold lower than that found in the CHO-K1 cells (Fig. 1C). Nonetheless, the DER-dependent transcriptional repression conferred by construct N6–52 was clearly evident in BHK-21. Taken together, the repressive characteristic of DER in N6–52 was most prominent in the CHO-K1 cells, correlating well with the restricted permissiveness of CHO-K1 cells in supporting Rnf35 promoter function as previously described (11). To further verify the cis-repressor function of the DER sequence, a Rnf35 exon 1 sequence between +4 and +133 devoid of the Inr but retaining DER was inserted downstream of the SV40 promoter/enhancer in the plasmid pSEAP-Control (GenBank™ accession number U89938) generating construct pSEAP-Ex1 (Fig. 1D). Upon transfection, a 3-fold repression in the expression of the SEAP reporter gene was observed. When the Inr-less +4 to +71 sequence that is also devoid of the DER sequence (construct pSEAP-Ex1ΔR) was similarly tested, repression was abolished further supporting the presence of a cis-acting repressor element in the +72 to +133 exon 1 segment. The data further indicate that the cis-cognate repressor is also functional on a TATA-box promoter.

To further delineate the repressor sequence, we generated a series of deletion mutants, N10gA, N10gB, and N10gC, retaining increasing sequence lengths between +72 to +133 (Fig. 1E). N10g1 carrying the shorter but transcriptionally active 5'-URR sequence up to −13 was used to avoid interference that might arise from a longer 5'-URR sequence. All the three constructs showed approximately similar promoter activity on transient transfection indicating that the first increment between +72 and +95 in N10gA was sufficient to confer the observed repressor activity. We conclude that the DER sequence is contained within a 24-bp region between +72 and +95.

**The DER Sequence Is Functionally Partitioned into DER1 and DER2**—In a transcription factor binding sequence data base search, we found that the best match for the 5'-half of the DER sequence, 5'-TGGCCACGTTAG-3', was that of a binding site for a yeast PHO4-like protein (30–32). We call this segment DER1. The 3'-half of DER, 5'-GCATGGATGCA-3', which we now call DER2, aligned best with the binding sequence for the CCAAT-displacement protein (CDP)/Cux, a high molecular weight repressor protein containing three DNA-binding CUT repeats and a homeodomain (reviewed in Ref. 33).

To further delineate cis-contribution of the DER1 and DER2 sequences to Rnf35 transcriptional regulation, mutations were introduced into either one or both the DER1 and DER2 segments in the N10gA background (Fig. 2A). In the DER2 mutants, substitutions were introduced into the highly conserved 5'-ATGGAT-3' core sequence (mutated nucleotides are underlined) of the CDP-binding sequence (34, 35). Transient transfection experiments demonstrated that mutations in either DER1 or DER2 alone (constructs N10gA-MutR1 and N10gA-MutR2) had little effect on the Rnf35-repressed promoter activity (Fig. 2B); however, when both DER1 and DER2 were simultaneously mutated in the construct N10gA-MutR1/R2A, an appreciable derepression of the promoter activity was now observed. The promoter activity was restored to −81% of that of the construct N10g1 that was devoid of the DER sequence. Transfection of a second mutant N10gA-MutR1/R2B that contained a single-base deletion of a thymidine in DER2 besides two other substitutions in the highly conserved core sequence (Fig. 2A) not only fully restored the promoter activity, but a 50% enhancement relative to the repressor sequence-free N10g1 was observed (Fig. 2B). The transfection data indicate that the DER sequence between +72 and +95 in the Rnf35 exon 1 harbors two tightly coupled cis-cognate elements, DER1 and DER2, that act in synergy to down-regulate transcription of the Rnf35 gene.

**DER2 Is Targeted by the CDP/Cux**—The 5'-ATGGAT-3' core sequence of the Rnf35 DER2 domain aligns with only one mismatch (underscored) with a consensus core binding sequence, 5'-ATCGAT-3', of the human CDP/Cux gene (34, 35). Because mammalian CUT-like domains have a rather relaxed DNA-binding specificity (35), DER2 may be a binding site for CDP. To test this, we synthesized an oligonucleotide (2DER2) containing two copies of the DER2 sequence for CDP binding assays. An oligonucleotide (2DER2MutB) mutated in the core sequence was also generated for competition analysis (Fig. 3A, top panel). Truncated CDP polypeptide segments carrying either the DNA-binding Cut-repeat-homeodomain CDP(CR3-
A reaction that contained only the DER1/2 probe, which had run off the gel; lanes 3 specific DER2 mutant (DER1/2MutB) polypeptide. For use as competitors, 25-, 125-, or 250-fold molar excesses of either the wild-type (2DER2), CDP binding to electrophoresis for 3.5 h (lanes 1–7) respectively; dashes indicate unchanged nucleotides. In the competition experiments, the oligonucleotides were used in 25-, 125-, or 250-fold molar excesses. In lanes 1 and 8, no competitors were added (denoted by “—”). The binding reaction products were resolved on 6% polyacrylamide gel electrophoresis for 5.5 h (lanes 1–7) or 5 h (lanes 8–14). Arrows indicate specific oligonucleotide-protein binding complexes as discussed in the text. B, CDP binding to DER2 is DER1-independent. In the experiments, the DER1/2 probe was incubated in the presence of the CDP(CR1–CR2) polypeptide. For use as competitors, 25-, 125-, or 250-fold molar excesses of either the wild-type (DER1/2), DER1 mutant (DER1/2MutA), DER2 mutant (DER1/2MutB), or the DER1 and DER2 double mutant (DER1/2MutAB) oligonucleotide was included in the respective reaction. Lane 1, a reaction that contained only the DER1/2 probe, which had run off the gel; lane 2 displays the DER1/2-bound complex (indicated by the arrow) without competition. In lanes 3–14, different competitor oligonucleotides were used at 25-, 125-, and 250-fold molar excesses. C, confirmation of specific DER2-CDP binding in supershift analysis: His-tagged CDP(CR3-HD)- (lanes 1 and 2, arrows) or CDP(CR1–CR2)-bound 2DER2 complexes (lanes 3 and 4, arrow) without (denoted by “—”) or in the presence (denoted by “+”) of an anti-His tag antibody (Ab) are displayed. Arrowheads indicate the antibody-induced supershifted bands.

FIG. 3, DER2 is targeted by the CDP/Cux. A, EMSA binding and competition experiments. The wild-type (2DER2) and the mutant (2DER2MutB) oligonucleotides are shown in the top panel: wild-type and mutated sequences are shown in upper- and lowercase letters, respectively; dashes indicate unchanged nucleotides. In the competition experiments, the oligonucleotides were used in 25-, 125-, or 250-fold molar excesses. In lanes 1 and 8, no competitors were added (denoted by “—”). The binding reaction products were resolved on 6% polyacrylamide gel electrophoresis for 5.5 h (lanes 1–7) or 5 h (lanes 8–14). Arrows indicate specific oligonucleotide-protein binding complexes as discussed in the text. B, CDP binding to DER2 is DER1-independent. In the experiments, the DER1/2 probe was incubated in the presence of the CDP(CR1–CR2) polypeptide. For use as competitors, 25-, 125-, or 250-fold molar excesses of either the wild-type (DER1/2), DER1 mutant (DER1/2MutA), DER2 mutant (DER1/2MutB), or the DER1 and DER2 double mutant (DER1/2MutAB) oligonucleotide was included in the respective reaction. Lane 1, a reaction that contained only the DER1/2 probe, which had run off the gel; lane 2 displays the DER1/2-bound complex (indicated by the arrow) without competition. In lanes 3–14, different competitor oligonucleotides were used at 25-, 125-, and 250-fold molar excesses. C, confirmation of specific DER2-CDP binding in supershift analysis: His-tagged CDP(CR3-HD)- (lanes 1 and 2, arrows) or CDP(CR1–CR2)-bound 2DER2 complexes (lanes 3 and 4, arrow) without (denoted by “—”) or in the presence (denoted by “+”) of an anti-His tag antibody (Ab) are displayed. Arrowheads indicate the antibody-induced supershifted bands.
1.5% agarose gels. For each embryonic stage, two independent rounds of nested PCR were carried out for all the samples with appropriate dilutions before electrophoretic analysis of the PCR products in 1.5% agarose gels. For each embryonic stage, two independent experiments had been performed, which yielded the same displayed results.

To confirm specific binding of the His-tagged CDP polypeptides to the DER2 probes, supershift assays were performed using an anti-His-tag antibody in the binding reactions. In the presence of the anti-His-tag antibody in the 2DER2-CDP(CR3-HD) binding reaction, a supershifted band appeared (Fig. 3C, lane 2, arrowhead); the presumptive dimeric binding complex was almost completely depleted confirming that it was an authentic 2DER2-CDP(CR3-HD) binding complex. Similarly, co-incubation with the anti-His antibody also resulted in the supershift of the 2DER2-CDP(CR1–CR2) binding complex (Fig. 3C, compare lanes 3 and 4, arrowhead). In summary, data present in Fig. 3 show that the DER2 sequence is targeted by the repressor protein CDP in vitro.

In Vivo Binding of CDP to the DER Sequence in the Mouse Embryo—To demonstrate in vivo binding of CDP to the DER sequence in the mouse embryo, chromatin immunoprecipitation (ChIP) experiments were performed (26). Due to the scarcity of embryos and on the basis of the expression profile of the mouse Cdp gene (see below), the blastocyst and the 8.5-day post-coitus embryo were analyzed (Fig. 4). Pools of 50–60 blastocysts or ten 8.5-day embryos were used in each ChIP experiment using an anti-CDP antibody. To identify the anti-CDP-bound DNA sequence, two rounds of nested PCR using DER-flanking primers were performed, and the identity of the PCR products thus derived was confirmed by cloning and sequencing. In the ChIP experiments, the input samples readily generated the predicted PCR product using the DER-specific primers. In the blastocyst and the 8.5-day embryo samples, the DER-specific sequence was also evident in nested PCR in the anti-CDP antiserum-precipitated chromatin. No PCR products were discernible in the mock or preimmune serum-treated samples. Taken together, in vitro and in vivo data presented in Figs. 3 and 4 show that the Rn35 DER element is targeted by the repressor protein CDP in the blastocyst and in the later stage embryo.

In Vivo Participation of CDP in Transcriptional Repression—To correlate the DER-CDP binding and transcriptional repression, an antisense construct of the entire human CDP gene was generated; the human CDP gene was used because the full-length Chinese hamster gene was unavailable. The specificity of the CDP antisense plasmid was first established in CHO-K1 co-transfection in the presence of a fixed amount of a CDP expression plasmid. Western blot analysis of cell lysates of the co-transfected cells using an anti-CDP antibody clearly showed a dose-dependent CDP repression in the presence of increasing amounts of the antisense DNA (Fig. 5A, lanes 3–6), indicating specific CDP antisense plasmid-induced CDP repression. The CDP antisense construct was tested in transient transfection of CHO-K1 cells in the presence of the DER-less N6–51 or the DER-carrying N6–52 construct. An empty vector was used as a negative assay control. The data show that co-transfecting the CDP antisense plasmid and N6–51 had little effect on the N6–51 SEAP activity (Fig. 5B). The relative SEAP activity of the construct N6–52 co-transfecting with the empty vector was about one-fourth that in the N6–51 transfection, consistent with previously obtained data (Fig. 1B). Upon co-transfecting N6–52 and the CDP antisense construct, however, N6–52 consistently showed a 2-fold increase in SEAP activities (Fig. 5B) suggesting that blocking the CDP function had resulted in a partial relief from CDP suppression; CDP derepression was only partial, probably because a human CDP sequence was used. Furthermore, transient transfection and EMSA data described above (Figs. 2 and 3) have hinted that both the DER1 and DER2, probably targeted by different proteins, act in synergy to elicit the observed transcriptional repression. Effective derepression may require simultaneous repression of the CDP binding to DER2 and the uncharacterized protein(s) targeting at DER1. Nonetheless, the CDP antisense transfection data shown in Fig. 5 are in line with our proposition that CDP contributes to transcriptional repression.

**Cdp Transcription First Occurs between the Eight-cell and Blastocyst Stages of Preimplantation Development**—To further correlate the established embryonic Rn35 transcription pattern and expression of the Cdp gene in the mouse, we next performed experiments to determine the expression profile of the mouse Cdp gene in different stages of preimplantation embryos. To do this, DNA samples prepared from cDNA libraries derived from the mouse unfertilized egg, the two- and eight-cell embryos, and the blastocyst were used (15, 16, 29). cDNA library derived from a liver of an adult mouse was also included as a positive control. In these cDNA libraries, the average cDNA insert size is 1 kb or more. The libraries also carried enough genetic complexity to permit detection of transcripts in

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**Fig. 4. In vivo binding of CDP to the DER sequence in the mouse embryo.** The chromatin immunoprecipitation experiments were performed as described under “Experimental Procedures.” In the experiments, pools of 50–60 blastocysts or ten 8.5-day embryos were used. The input samples contained total and untreated chromatins; mock samples were not treated with any antisera. After immunoprecipitation with a preimmune serum or the anti-CDP antiserum, two rounds of nested PCR were carried out for all the samples with appropriate dilutions before electrophoretic analysis of the PCR products in 1.5% agarose gels. For each embryonic stage, two independent experiments had been performed, which yielded the same displayed results.

**Fig. 5. In vivo participation of CDP in transcriptional repression.** A, specificity of the CDP antisense plasmid (AS-CDP) used in the repression experiment. A CDP expression plasmid (1 μg of DNA) was co-transfected to CHO-K1 cells in a 2-well culture dish in the presence of the empty vector (lane 2) or increasing amounts (0.5–3 μg of DNA) of the AS-CDP DNA (lanes 3–6). Thirty-six hours post-transfection, cell lysates were subjected to Western blot analysis using an anti-CDP antibody (α-CDP/Ab); an anti-NF-Ya subunit antibody (α-NFYa/Ab) was used as a Western control. Samples in lanes 1 and 2 were controls in which the CDP expression plasmid was co-transfected in the absence or presence of the vector DNA used in the AS-CDP construct. B, transcriptional repression by antisense CDP. N6–51 or N6–52 plasmid DNA was transfected into CHO-K1 cells in the presence of an equal amount of either an empty vector or AS-CDP DNA. Media were harvested for SEAP assays 48 h after transfection. The data were derived from three independent transfection experiments.
The mammalian CDP/Cux is a structurally complex multifunctional repressor that regulates differentiation, cell growth, and development (39–44; see also references in Ref. 33). The full-length CDP protein contains four DNA-binding domains in the order of the three highly conserved Cut repeats (CR1, CR2, and CR3) followed by a homeodomain (HD). None of the domains could act alone in effective DNA binding; the CR1–CR2 and CR3-HD combinations have been shown to exhibit high DNA binding affinities albeit with different binding kinetics and preferred binding sequences (34, 35, 37). In our work, we observed that both the CR1–CR2 and the CR3-HD polypeptides recognized and bound the Rnf35 DER2 sequence albeit with different sequence requirements and binding affinities. The CR1–CR2 segment has been correlated with the CCAAT-displacement activity of the CDP; for example, the binding of CDP(CR1–CR2), but not CDP(CR3-HD), has been shown to dislocate NF-Y from its binding site (37, 42). Coincidentally, we have demonstrated previously that the NF-Y participates in Rnf35 transcription (11). Besides playing an active role as a repressor when bound to DER2, it remains to be demonstrated if the CDP also displaces other protein(s) from occupancy of other Rnf35 promoter sequences to render passive CDP repression (43, 44).

We have previously shown that Rnf35 is temporally transcribed in the preimplantation embryo up to the eight-cell stage; Rnf35 transcription is then permanently silenced (14, 15). The restrictive transcription pattern of Rnf35 may now be explained by data presented in this work: the Cdp gene is not expressed prior to the eight-cell stage (Fig. 6) thus sparing the Rnf35 promoter from transcriptional repression. When the repressor protein CDP first appears between the eight-cell and the blastocyst stages, the CDP now binds to the Rnf35 DER2 sequence resulting in repression; the CDP-dependent mode of silencing persists in the developing embryo up to at least 8.5 days postcoitum. It remains to be determined when and if other silencing mechanism(s), in particular promoter hypermethylation, are subsequently involved to maintain the silenced state of Rnf35. Besides housing the Inr core promoter element, findings in this work attribute a further biological role to the Rnf35 exon 1, namely that of negative transcriptional modulation and the initial phase of permanent silencing of the gene. It may be noteworthy that, besides early embryonic genes, members of the exceptionally large olfactory receptor superfamily, which encode proteins that permit recognition of the huge number of odors, also carry a similar UCE gene structure at the 3’-end preceded by one or two short untranslated exons (45–47). The 5’-noncoding exons of the olfactory receptor genes are likely to harbor cis-acting transcriptional modulator sequences as in the case of the Rnf35 gene.

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