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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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Chiu-Jung Huang‡, Jan-Gowth Chang§, Shinn-Chih Wu¶, and Kong-Bung Choo‖**

From the ‡Department of Animal Science and Graduate Institute of Biotechnology, College of Agriculture, Chinese Culture University, Taipei 111, Taiwan 111, the §Department of Molecular Medicine, China Medical University Hospital, Taichung 404, Taiwan, the ¶Department of Animal Science, College of Bio-Resources and Agriculture, National Taiwan University, Taipei 106, Taiwan, and the ‖Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217, Taiwan

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 half-sections, *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 preimplantation development includes genes essential for the general maintenance of chromosomal stability, DNA repair, cell cycle control, and the tightly modulated transcription process (2). The expression profiles of some of these genes in the mouse embryo have been examined and found to be highly dynamic and complex throughout the preimplantation stages of the development (1, 3).

Upon fertilization, maternal transcripts in the one- and two-cell embryos are rapidly replaced by a new set of zygotic genome-derived transcripts that serve to support further development. Hence, appropriate zygotic genome activation is an important event that drives the initial phase of embryo development. An overview on possible mechanisms that control 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),[1] 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 carrying 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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** To whom correspondence should be addressed. Tel.: 886-2-2875-7400; Fax: 886-2-2872-1312; E-mail: kbchu@vghtpe.gov.tw.

[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 unfertilized 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. 1*A*) (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 (UCE) (14–18 and a list of other genes in Ref. 15). We have previously proposed that the bi-exonic and UCE structure was most likely acquired in multiple retrotransposition events in the course of evolution of the genes (15). 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 ubiquitous transcription 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 *CDP* gene harbored in the pMX139-MCH plasmid provided by Dr. A. Nepveu (McGill University, Quebec, Canada). This was done by first excising the full-length *CDP* gene from pMX139-MCH using NotI and XhoI, reversing these restriction recognition sites in the insert-free pMX139 vector using appropriate double-stranded oligonucleotides, and re-inserting the NotI-XhoI *CDP* sequence into the modified vector. The *CDP* expression plasmid was similarly constructed by inserting the gene into the expression plasmid pMX139-MCH under the control of the promoter of the adenovirus major late gene. All plasmid constructs generated were confirmed by sequence analysis. Transient transfection and SEAP assays were performed as previously described (11, 25). In short, the transfection experiments were done in duplicates or triplicates in 24-well culture dishes using a fixed total amount of plasmid DNA in the presence of the Lipofectamine Plus Reagent (Invitrogen). Forty-eight hours post-transfection, culture medium from each transfection sample was harvested and subjected to SEAP assay in duplicate using the Great EscAPe SEAP Reporter System 3 (DB Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. Each transfection experiment included triplicate samples of the pSEAP-Basic plasmid to determine the background SEAP value. Sample variations were normalization using the EGFP fluorescence values derived from the co-transfecting pEGFP-N2 control plasmid. All the transfection data presented in this work were derived from three or more independent transfection experiments.

Preparation of Recombinant CDP Polypeptides from E. coli—The plasmids encoding the CDP(CR1–CR2) and CDP(CR3-HD) subfragment polypeptides of the CCAAT-displacement protein (CDP) were kindly 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-β-D-thiogalactopyranoside for 3 h. Cells were harvested and resuspended in a lysis buffer (50 mM NaH_2PO_4, 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 using nickel-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 NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole) followed by elution using the elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole).

Electrophoretic Mobility Shift and Supershift Assays—The probes used in electrophoretic mobility shift assays (EMSAs) were generated by annealing complementary strands of oligonucleotides and labeled by standard Klenow fill-in reactions of the staggered ends in the presence of [α-^{32}P]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 $MgCl_2$, 5 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 3 μg of bovine serum albumin, and 30 μg/ml poly(dI-dC) at room temperature for 5 min followed by the addition of probe and a further 20-min incubation at 30 °C. For supershift assays of the His-tagged CDP polypeptides, 2 μg/μl 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 displayed on 6% polyacrylamide gels with a running time of 3.5 h for the CDP(CR3-HD) polypeptide or 5 h when CDP(CR1–CR2) was incubated.

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 until use. ChIP experiments were performed using pools of 56–60 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 glycine 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 antiserum (a gift from Dr. Ellis J. Neufeld, Harvard Medical School, Boston, MA) (27, 28). For controls, a mock sample without antiserum 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 previously (26). The DNA pellet 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 pmol each of the *DER*-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′-AAGGCCCAGAGAACAAAGATTC-3′) and 2877R (5′-AAAACAATTCATCTCAACCCAG-3′). One

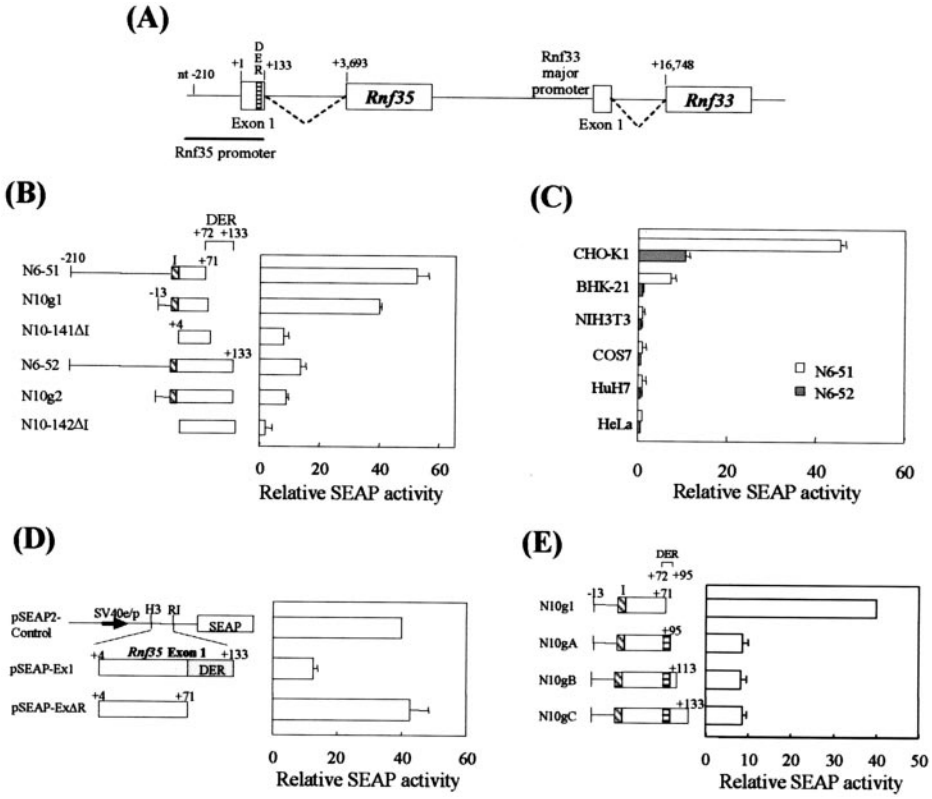

FIG. 1. **Identification of a *cis*-cognate downstream repressor element, *DER*, in the *Rnf35* exon 1 sequence.** *A*, a scheme showing the relative positions of the bi-exonic *Rnf35* gene and the *Rnf33* homologue (in approximate scale). The exons (denoted by *boxes*) and introns (*dashed bent lines*) 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 or 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 carcinomas), 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 (RI) sites downstream of the SV40 promoter-enhancer (*SV40e/p*) sequence of the pSEAP-Control plasmid to generate pSEAP-Ex1. To generate pSEAP-Ex1ΔR, the *DER* 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.

microliter of the first-round PCR products was used in a second-round amplification step using the nested primers 2465F (5′-TGAAAACGGCCATTCTAAACG-3′) and 2746R (5′-TTCCTGCTGTGGCTGGTCCTG-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, Gilbersville, PA, catalog no. 100-401-100, in 1,000× dilution), was applied at room temperature for 1 h before filter was washed three times 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′-CAGGACAGGACGACGGTGAAGACG-3′ and 5′-GAACTCCCATTCGATGGGCTCCTC-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 AY037807). 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. 1*A*. 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. 1*A* (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. 1*B*). A shorter 5′-URR segment terminating at −13 in construct N10g1 was also transcriptionally active albeit with a slight decrease in transcriptional activity (Fig. 1*B*). Deleting the previously identified *Inr* core

promoter sequence in construct N10–141ΔI resulted in a 5-fold depreciation in SEAP activity.

The *Rnf35* exon 1 is 133 bp long (Fig. 1*A*) (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. 1*B*). 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 depreciated 4-fold relative to that of the truncated N6–51 (Fig. 1*B*). 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. 1*C*). 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. 1*D*). 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. 1*E*). 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′-TGGCCCACGTAG-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*

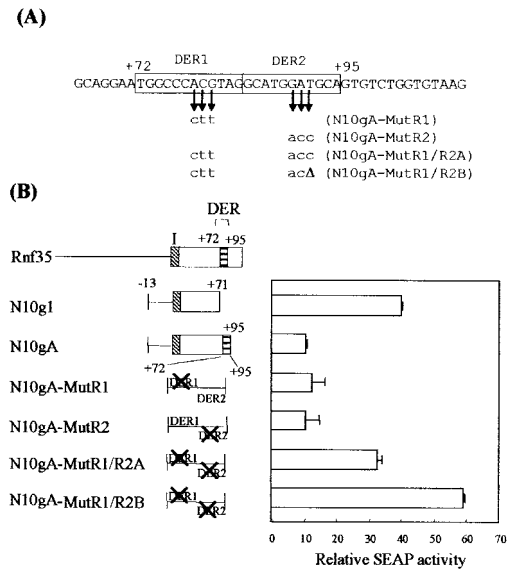

FIG. 2. **The *DER* sequence is functionally partitioned into *DER1* and *DER2*.** *A*, site-specific mutagenesis of *DER1* and *DER2* in the parental plasmid N10gA. The *top line* shows a partial *Rnf35* exon 1 sequence. The mutated nucleotides are shown by *downward arrows* and in *lowercase letters* below the parental sequence; designations of the mutant constructs are shown in *parenthesis* on the *right*. *B*, transcription analysis of the *DER* mutant constructs. In the schematic, the *DER1-DER2* segment of the mutant constructs between +72 and +95 are expanded and displayed. Transient transfection of CHO-K1 and SEAP assays were as described in the legend to Fig. 1. See also legend to Fig. 1 for an explanation of the symbols used.

sequences to *Rnf35* transcriptional regulation, mutations were introduced into either one or both the *DER1* and *DER2* segments in the N10gA background (Fig. 2*A*). In the *DER2* mutants, substitutions were introduced into the highly conserved 5′-ATCGAT-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. 2*B*); 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. 2*A*) not only fully restored the promoter activity, but a 50% enhancement relative to the repressor sequence-free N10g1 was observed (Fig. 2*B*). 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. 3*A*, *top panel*). Truncated CDP polypeptide segments carrying either the DNA-binding Cut-repeat-homeodomain CDP(CR3-

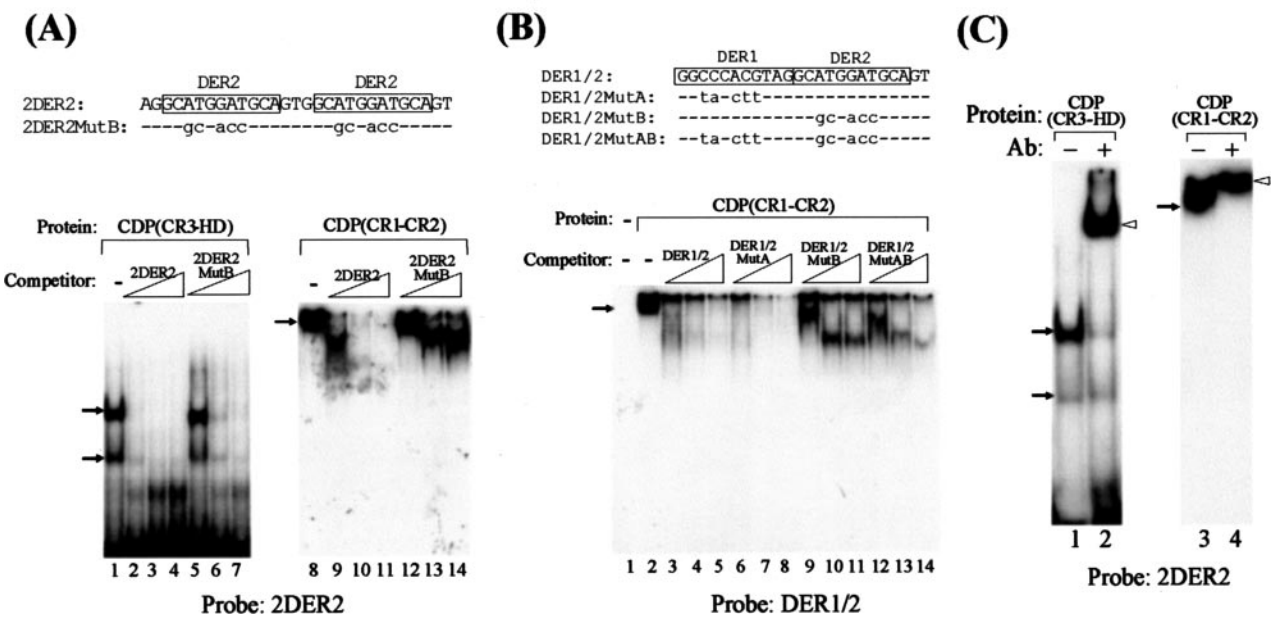

FIG. 3. ***DER2* is targeted by the CDP/Cux.** *A*, EMSA binding and competition experiments. The wild-type (2DER2) and the mutant (2DER2MutB) oligonucleotides used are shown in the *top panel*: wild-type and mutated sequences are shown in *upper-* and *lowercase letters*, respectively; *dashes* indicate 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 3.5 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.

HD) or the Cut-repeats CDP(CR1–CR2) were used in the analysis. Full-length CDP was not used, because the full-length protein has been shown to bind target sequences only transiently (36, 37) and may not produce stable binding complexes in *in vitro* assays. The CDP-segment polypeptides were expressed in *E. coli* and were harvested by purification via the polyhistidine tag added to the amino termini of the polypeptides. When the CDP(CR3-HD) polypeptide was used in the binding reaction co-incubating with the 2DER2 probe, two prominent bands were apparent (Fig. 3*A*, *lane 1*, *arrows*). The two 2DER2-bound complexes were probably formed by one or two CDP(CR3-HD) polypeptide molecules binding to one or both the *DER2* boxes in the dimeric 2DER2 probe (38). Specificity of the putative binding complex was verified by competition using the wild-type and mutant 2DER2 oligonucleotides in molar excesses. As little as 25-fold molar excess of the unlabeled 2DER2 oligonucleotide was sufficient to effectively compete out the binding complexes (Fig. 3*A*, *lane 2*). When the 2DER2MutB oligonucleotide was used, effective competition for the complexes was not evident at 25-fold molar excess; at 125- and 250-fold molar excesses, however, 2DER2MutB effectively competed for the 2DER2-bound complexes (Fig. 3*A*, *lanes 5–7*). The data indicate that the CDP(CR3-HD) polypeptide binds specifically to 2DER2 but with a relatively relaxed sequence requirement and dubious affinity. On the other hand, similar binding and competition experiments show that binding of the CDP(CR1–CR2) polypeptide to the 2DER2 probe (Fig. 3*A*, *lane 8*, *arrow*) had a stringent sequence requirement: unlabeled 2DER2 effectively competed for the binding complex generated by the 2DER2 probe (Fig. 3*A*, *lanes 9–11*), but the mutant 2DER2MutB oligonucleotide at up to 250-fold molar excess did not show appreciable competition for the binding complex (Fig. 3*A*, *lanes 12–14*). The result is consistent with the fact that the CR1–CR2 segment is a high affinity DNA-binding domain of the CDP (34, 35, 37).

To further test the binding of the CDP to *DER2* in the natural context, binding and competition experiments were further performed using a probe (DER1/2) that encompassed both the *DER1* and *DER2* sequences (Fig. 3*B*, *top panel*). Mutations were also introduced into the core sequence of *DER1* (DER1/2MutA) or *DER2* (DER1/2MutB), or in both *DER1* and *DER2* (DER1/2MutAB) in the DER1/2 oligonucleotide (Fig. 3*B*, *top panel*). In this experiment, the DNA-binding CDP(CR1–CR2) segment was used in the binding analysis. As in the case when the 2DER2 probe was used in the binding reaction (Fig. 4*A*, *lanes 8–14*), the DER1/2 probe and the CDP(CR1–CR2) polypeptide formed a slow migrating binding complex (Fig. 3*B*, *lane 2*, *arrow*), which was effectively competed out by excessive unlabeled DER1/2 oligonucleotide (Fig. 3*B*, *lanes 3–5*). The *DER1*-mutated DER1/2MutA oligonucleotide also effectively competed for the binding complex (Fig. 3*B*, *lanes 6–8*), but the *DER2* and the *DER1-DER2* double mutant oligonucleotides had little effect in competition for the probe-bound complex (Fig. 3*B*, *lanes 9–14*), reminiscent of the scenario when the 2DER2 probe was used (Fig. 3*A*, *lanes 8–14*). The results are consistent with the anticipation that the *DER2* sequence is targeted by CDP and further hint that the binding of CDP to *DER2* is independent of the *DER1* sequence. In a preceding transient transfection experiment, we showed that both *DER1* and *DER2* acted in synergy to achieve transcriptional derepression (Fig. 2). Taken together, the transfection and the EMSA data predict that *DER1* is targeted by one or more other protein(s) that act in concert with the CDP to elicit transcriptional repression.

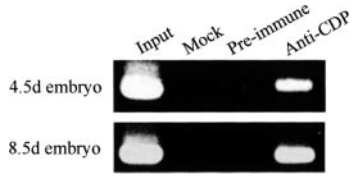

FIG. 4. ***In vivo* binding of CDP to the *DER* sequence in the mouse embryo.** The chromatin immunoprecipitation experiments were performed as described under "Experimental Procedures." In the experiments, pools of 50–60 blastocysts or ten 8.5-day embryos were used. The input samples contained total 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.

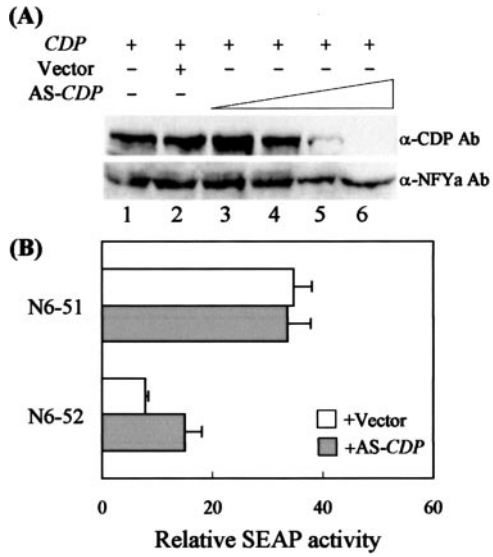

FIG. 5. ***In vivo* participation of CDP in transcriptional repression.** *A*, specificity of the *CDP* antisense plasmid (*AS-CDP*) used in the repression experiments. A *CDP* expression plasmid (1 μg of DNA) was co-transfected to CHO-K1 cells in a 6-well culture dish in the presence of the empty vector (*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.

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. 3*C*, *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. 3*C*, 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 chromatins. 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 *Rnf35 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* 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. 5*A*, *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. 5*B*). 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. 1*B*). Upon co-transfecting N6–52 and the *CDP* antisense construct, however, N6–52 consistently showed a 2-fold increase in SEAP activities (Fig. 5*B*) 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 *DER*2 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 *Rnf35* 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

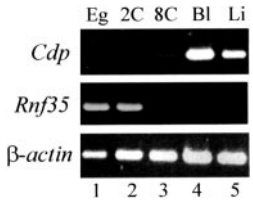

FIG. 6. ***Cdp* transcription first occurs between the eight-cell and the blastocyst stages of preimplantation development.** PCR was performed using a set of mouse *Cdp* gene-specific primers; the PCR templates were DNAs prepared from cDNA libraries derived from the egg (*Eg*), the 2- (*2C*) and 8-cell (*8C*) embryo, the blastocyst (*Bl*) and the adult liver (*Li*) of the mouse. The *Rnf35* transcription profile was similarly established (14, 15) and displayed. The mouse β-actin gene was included as a PCR control. The PCR products were analyzed on a 2% agarose gel.

low abundances (29). Because the said cDNA libraries were constructed using oligo(dT) in the initial reverse transcription reaction, mouse *Cdp*-specific primers were designed to detect the *Cdp* 3′-untranslated sequences. The *Cdp*-specific PCR bands obtained (Fig. 6) were confirmed by sequencing. The results show that the mouse *Cdp* transcript was not detected in the unfertilized egg, the two- and eight-cell stages of development (Fig. 6, *top panel*, *lanes 1–3*). In the blastocyst, however, *Cdp* transcription is evident, as in the adult liver (Fig. 6, *top panel*, *lanes 4* and *5*). For direct correlation with *Cdp* expression, the transcription profile of *Rnf35* was re-established using the same set of preimplantation embryo cDNA templates (Fig. 6, *middle panel*). The results confirm the previous observation that *Rnf35* silencing first occurs between the eight-cell and the blastocyst stages (14, 15).Our data, thus, show that *Cdp* transcription first occurs between the eight-cell and the blastocyst stages in the mouse, coinciding with *Rnf35* silencing. Although *Cdp* expression in the preimplantation embryo warrants further examination, our observation offers a plausible mechanism for the initial phase of silencing of the *Rnf35* transcription beyond the eight-cell stage when the CDP repressor is first being expressed. Involvement of other mechanism(s) such as hypermethylation may subsequently contribute to permanent silencing of the *Rnf35* gene.

DISCUSSION

In this work, we uncover a 24-bp downstream *cis*-cognate repressor sequence, called *DER*, located in the short and noncoding exon 1 of the bi-exonic *Rnf35* gene. The *DER* sequence is functional in the uniquely permissive somatic cell line, CHO-K1. The *DER* sequence may be partitioned into two subsections, *DER1* and *DER2*. Mutating either one of the *DER* subsection did not result in transcriptional derepression in transient transfection assays; however, when both *DER1* and *DER2* were simultaneously mutated, derepression was achieved with restoration and even an enhancement in transcriptional activities. EMSA experiments indicate that *DER2* was bound by the ubiquitous Y-box protein CDP/Cux (Fig. 3) and that the binding is independent of *DER1*. More importantly, we demonstrate in ChIP experiments *in vivo* CDP binding to the *DER* sequence in the blastocyst and in the 8.5-day post-coitus embryo (Fig, 4), and transcriptional derepression in the presence of a *Cdp* antisense construct in transfected cells (Fig. 5) further supporting *in vivo* participation of CDP in transcriptional modulation. Although CDP-binding sites are generally located upstream of transcriptional start sites, the CDP-binding sequence in the human histone *H4* gene is found downstream of the start site (39), as in the case of the *Rnf35 DER2*. Our data also hint that the *DER2*-coupled *DER1* sequence is targeted by one or more undetermined proteins. In a TRANSFAC data base search for possible transcription factor-binding sequences, the best match for the *DER1* sequence is the binding sequence for the yeast PHO4 protein (data not shown). PHO4 is a basic helix-loop-helix transcriptional activator of the yeast *Pho5* promoter. Upon phosphate starvation, the PHO4 protein is dephosphorylated. In tight interaction with another homeoprotein, PHO2, PHO4 subsequently binds to and regulates the *Pho5* promoter (30–32). It may not be entirely coincidental that the CDP that binds to the *Rnf35 DER2* sequence is also a homeoprotein. It remains to be shown if such a PHO4-like protein exists and participates in *Rnf35* transcriptional modulation in the preimplantation embryo.

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 *DER2* sequence resulting in repression; the CDP-dependent mode of silencing persists in the developing embryo up to at least 8.5 days post-coitus. 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 odorants, 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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