Transcriptional modulation of the pre-implantation embryo-specific *Rnf35* gene by the Y-box protein NF-Y/CBF

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Maternal-to-zygotic transition of a fertilized egg and the subsequent pre-implantation development of the embryo involve zygotic genome activation and reprogramming of gene expression. The goal of the present study is to establish a model suitable for the characterization of transcriptional modulation of mammalian pre-implantation development. *Rnf35* is a mouse RING-finger protein gene that is temporally transcribed in the early embryo, but is permanently silenced before the blastocyst stage of development. We first show that the Chinese-hamster ovary-K1 cells are unique in supporting *Rnf35* promoter activities in transient transfection assays. Using the permissive Chinese-hamster ovary-K1 cell line, we show that *Rnf35* transcription is driven by an *Inr* (initiator) core promoter element in the absence of a TATA box; the *Inr* promoter function is confirmed by direct microinjection of mouse one-cell embryos. This is the first demonstration of the involvement of an *Inr* core promoter element in transcription in pre-implantation development. We show that the *Rnf35* promoter is regulated by three obligatory Y-box (CCAAT-box) elements: two Y boxes (Y_I and Y_{II}) located at -81 are coupled in a palindrome and act synergistically in contributing to *Rnf35* transcription; the third Y box (Y_{III}) is situated at -13, just upstream of the *Inr* element, and may be an integral part of the *Inr* function. Electrophoretic mobility-shift assays and competition experiments further reveal that the Y_I box is bound by the ubiquitous NF-Y (nuclear factor-Y)/CBF (CCAAT-binding factor) and that Y_{II} is targeted by an unidentified protein(s) that acts synergistically with the NF-Y. We suggest that the NF-Y, targeting at a Y-box sequence, may function as an important activator in transcriptional regulation of the *Rnf35* gene in the pre-implantation embryo.

Key words: CCAAT-binding factor (CBF), mammalian embryo, nuclear factor-Y (NF-Y), pre-implantation development, TATA-less promoter, transcriptional modulation.

INTRODUCTION

On fertilization, maternal transcripts in the fertilized egg are rapidly degraded and replaced by a new set of transcripts earmarked to support continued development. On the basis of the observation that a significant portion of transcripts in the two-cell stage embryo is derived from repetitive sequences, it has been proposed that the initial phase of embryonic transcription may be indiscriminate [1,2]. To impose some order on the transcription process, earlier studies have unveiled a number of check points unique to transcription in the developing embryo [3,4]. Initiation of transcription in the one-cell embryo is tightly coupled with DNA replication; disruption of nucleosomes during the replication process opens up windows of opportunity for the existing maternal transcription machinery to gain access to the otherwise cloaked promoters (reviewed in [3–6]). Transcriptional suppression is evident between S-phase of the first cell-cycle events in the one-cell embryo and as development progresses; it is now clear that transcriptional repression involves changes in the chromatin structure [3–7]. For continued and efficient transcription, there is now a preferential deployment of TATA-less promoters [8,9]. When the embryo enters the two-cell and later stages of development, there is an increased dependence on enhancers, presumably as a means to alleviate chromatin-mediated repression [3,4,9–11].

To elucidate transcriptional regulation of early embryonic genes, pre-implantation embryo-active promoters of such genes as *Hsp70* and *eIF-1A* and other artificially constructed promoter sequences have variously been analysed [8,10,12–14]. As an example, embryonic *Hsp70* transcription is initiated at the late one-cell stage and continues through the two-cell stage until the end of the second round of DNA replication when repression sets in [14,15]. At the one- and two-cell stages, *Hsp70* transcription is dependent on a TATA-box promoter recruiting Sp1 as an activator [2,13]. Furthermore, a maternally derived GAGA-box-binding factor has been proposed to contribute to the enhancer-dependent derepression of *Hsp70* transcription [13], but the binding factor has not yet been identified. The transcription factors other than Sp1 that may contribute to early transcription remain to be elucidated.

We have described previously a RING-finger protein gene *Rnf35* that is temporally transcribed in the unfertilized egg and in the early embryo, but is permanently silenced before the blastocyst stage of development [16–18]. The biological role of the putative RNF35 protein in early development is not clear. The *Rnf35* gene is bi-exonic in structure: it comprises a short exon 1 approx. 133 bp long having multiple but clustered mRNA start sites, a 3.6-kb solitary intron and an exon 2 that accommodates the rest of the gene, including the uninterrupted coding region [16,17]. The *Rnf35* exon 1 sequence is also exploited as the exon 1 in a minor population of transcripts of a downstream and similarly bi-exonic homologue, *Rnf33* [16,17]. To investigate further how gene expression is regulated in the mammalian pre-implantation

Abbreviations used: α-AM, α-amanitin; CBF, CCAAT-binding factor; CDP, CCAAT-displacement protein; CHO-K1 cells, Chinese-hamster ovary-K1 cells; DPE, downstream promoter element; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility-shift assay; hCG, human chorionic gonadotrophin; NF-Y, nuclear factor-Y; RT, reverse transcriptase; SEAP, secreted alkaline phosphatase; TNT, *in vitro* transcription and translation; 5′-URR, 5′-upstream regulatory region.

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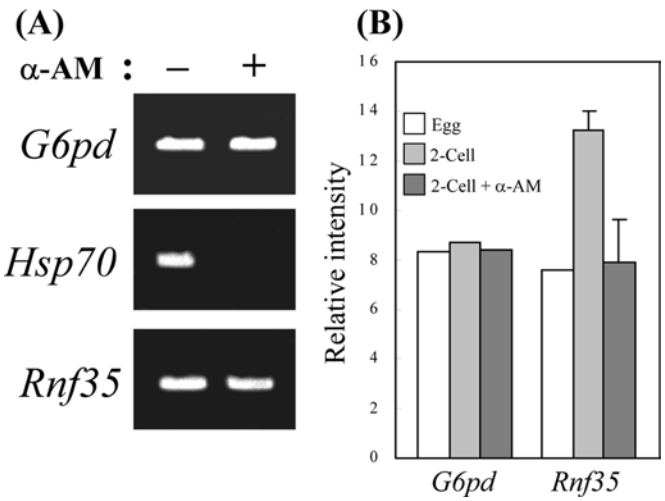

Figure 1 Active *Rnf35* transcription from the embryonic genome

(**A**) Effects of α-AM on *Rnf35* transcription in the early embryo. A total of 50 late one-cell embryos were cultured overnight to the two-cell stage without (denoted by '$-$') or in the presence ('+') of 50 μg/ml α-AM. The embryos were harvested for RNA preparation and RT–PCR analysis using *Rnf35*-specific primers. Controls included in the experiment were the *G6pd* and *Hsp70* genes (see text). (**B**) Quantification of the *G6pd* and *Rnf35* mRNA levels in the presence or absence of α-AM by real-time RT–PCR. RNAs prepared from pools of 50 unfertilized eggs and 50 one-cell embryos cultured to the two-cell stage without or in the presence of α-AM were used. The relative abundance was computed relative to the RT–PCR product of the β-actin mRNA in the same RNA preparation. The results shown were derived from two independent experiments.

embryo, we aimed in the present study to characterize the *Rnf35* promoter and to identify the transcription factors or activators that may contribute to *Rnf35* promoter activities. We demonstrate that an initiator sequence is used as a core promoter element without the benefit of a TATA box and that the ubiquitous NF-Y (nuclear factor-Y)/CBF (CCAAT-binding factor) acts on an upstream Y box to contribute to *Rnf35* transcription.

EXPERIMENTAL

Collection of mouse embryos, RNA preparation and RT (reverse transcriptase)–PCR analysis

Cumulus cell-free unfertilized eggs or pre-implantation embryos were collected from 6–8-week-old ICR donors after injecting 10 units of pregnant mare serum gonadotrophin followed 48 h later by the injection of 10 units of hCG (human chorionic gonadotrophin). Unfertilized eggs were collected from hormone-treated but unmated donors 21 h after the hCG injection. One- or two-cell embryos were collected from mated donors 24 or 44 h post-hCG injection. The collected eggs or the embryos were snap-frozen in liquid nitrogen or were cultured in small drops of M16 medium under oil in an organ culture dish in an incubator with 5 % CO_2 at 37 °C. For the drug inhibition experiments described in Figure 1, groups of 50 one-cell embryos were cultured in the presence or absence of the transcription inhibitor α-AM (α-amanitin) at a concentration of 50 μg/ml [19]. When the embryos developed to the two-cell stage after culturing overnight, they were collected for RNA preparation and RT–PCR analysis as described in [16,17]. The RT–PCR primers used are listed in Table 1. To determine the relative levels of specific transcripts in the unfertilized eggs or embryos cultured in the presence or absence of α-AM, real-time RT–PCR was performed using the mouse β-actin mRNA as the quantification reference as described previously [20].

Table 1 Oligonucleotides used as primers in the RT–PCR and α-AM inhibition experiments

Gene	Sequence (5′–3′)
G6pd	Sense: TGAGGGTCGTGGGGGCTATTTTGA
	Antisense: GCATCAGGGAGCTTCACATTCTTG
Hsp70	Sense: GAAGGTGCTGGACAAGTGC
	Antisense: GCCAGCAGAGGCCTCTAATC
Rnf35	Sense: ACAGAATTATCAAGCGATTTCAAG
	Antisense: GGCTCGTAGTCATATCCAG TGTAG
β-actin	Sense: CCCTAAGGCCAACCGTGAAAAGAT
	Antisense: ACCGCTCGTTGCCAATAGTGATGA

Construction of *SEAP* (secreted alkaline phosphatase) and *EGFP* (enhanced green fluorescent protein) plasmids and site-directed mutagenesis

For the construction of the *SEAP* reporter plasmids, segments of the *Rnf35* 5′-URR (5′-upstream regulatory region) and the exon 1 sequence were amplified by PCR using sequence-specific oligonucleotide primers flanked by the *Nhe*I (upstream) and *Xho*I (downstream) restriction enzyme recognition sequences. The amplified segments were digested with the said restriction enzymes, column-purified and cloned into the *Nhe*I and *Xho*I sites of the promoter-enhancer-free pSEAP2-Basic vector (GenBank® accession no. U89937) [20]. For the construction of *EGFP* plasmids, the *EGFP* gene was first cloned into the *Eco*RI site of the pGEM7 vector to generate pGEM7-EGFP. Selected *Rnf35-SEAP* plasmids used in the transient transfection experiments were first digested with *Hin*dIII and *Xba*I to remove the *SEAP* gene. The *EGFP* gene was excised from pGEM7-EGFP by digestion with the same restriction enzymes, column-purified and cloned into the *SEAP* gene-depleted *Rnf35* plasmids. Note that the corresponding *SEAP* or *EGFP* plasmid constructs carrying the same *Rnf35* sequences share the same prefix designations. For site-directed mutagenesis, oligonucleotide primers encompassing the desired mutations and appropriate restriction cloning sites were used in PCR amplification. All plasmid constructs generated and used in the present study were confirmed by sequence analysis.

Transient transfection and SEAP assay

For transient transfection experiments, the somatic cell lines tested were cultured in Dulbecco's modified Eagle's medium, supplemented with either 10 % (v/v) fetal bovine serum or 10 % (v/v) fetal calf serum in 24-well culture plates. Cells were co-transfected in duplicate or triplicate with 0.5 μg of each construct being tested and 0.2 μg of the pEGFP-N2 plasmid (GenBank® accession no. U57608) using the LIPOFECTAMINE™ Plus reagent (Life Technologies, Rockville, MD, U.S.A.). The *EGFP* gene in pEGFP-N2 is under the control of the cytomegalovirus immediate early promoter; pEGFP-N2 was included in all transfection experiments to normalize plate-to-plate and transfection variations by way of the EGFP assay. Fresh medium was added 3 h after transfection, and the transfected cells were cultured for 48 h before harvesting the supernatant for SEAP activity assays, in duplicates, using the Great EscAPe SEAP Reporter System 3 (BD Biosciences ClonTech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. To evaluate fluorescence emission derived from the co-transfected pEGFP-N2 plasmid, transfected cells were washed three times with chilled PBS and lysed in 200 μl of 1 % Triton X-100 with vigorous horizontal shaking at room temperature (20 °C) for 30 min. The supernatant was harvested by a brief centrifugation and transferred on to a 96-well

microtitre plate. Fluorescence was detected in a Fluoroskan Ascent 1.6 fluorimeter (Labsystems, Bornheim-Hersel, Germany) at an excitation wavelength of 485 nm and an emission peak at 538 nm. Each transfection experiment also included duplicate samples of the pSEAP-Basic plasmid that did not carry any active promoter or enhancer element. Relative SEAP activities were computed by subtracting the background values obtained with the pSEAP-Basic transfection samples and normalization using the EGFP fluorescence data derived from the co-transfecting pEGFP-N2 control plasmid. For each *SEAP* construct assessed, three or more independent experiments were performed.

Microinjection of mouse embryos

Plasmid DNA samples used for microinjection were prepared and purified on QIAprep® columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For microinjection of one-cell mouse embryos, 2 pl (picolitre) aliquots of DNA diluted to 5 ng/μl were microinjected into the male pronuclei of mid-to-late one-cell embryos harvested from superovulated ICR mice 24–25 h after hCG injection. The cumulus cells were removed by treating for a brief period (30 s) with a hyaluronidase solution (100 units/ml) in standard M2 medium. Microinjection was performed using a micromanipulator and a continuous flow system mounted on an inverted microscope with differential interference contrast. After microinjection, embryos were cultured in an amino acid-supplemented KSOM medium at 37 °C in a humidified atmosphere of 5 % CO_2 for 10–12 h before the first evaluation of *EGFP* expression in the injected embryos under a fluorescence microscope.

Preparation of nuclear extracts

Nuclear extracts were prepared as described by Schreiber et al. [21] with modifications. In brief, 2×10^7 CHO-K1 cells (Chinese-hamster ovary-K1 cells) were harvested by trypsinization. The cell pellet was washed once with PBS and twice with a Tris-buffered saline before being gently resuspended in 500 μl of cold buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT (dithiothreitol) and 0.5 mM PMSF]. The cell suspension was kept on ice for 15 min, followed by the addition of 25 μl of 10 % (v/v) Nonidet P40. The mixture was vigorously vortex-mixed for 10 s and then spun down at 16000 ***g*** for 5 min at 4 °C. The supernatant was discarded; the pellet was resuspended in 120 μl of ice-cold buffer C [20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF]. The suspension was vigorously rocked at 4 °C for 15 min on a shaking platform. The nuclear extracts were cleared by centrifugation at 16000 ***g*** for 10 min at 4 °C. Aliquots of the supernatant were kept at − 70 °C until use.

TNT (*in vitro* transcription and translation) reactions

TNT was performed using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI, U.S.A.). The TNT-competent plasmids, containing genes encoding the NF-YA, -YB and -YC subunits and the dominant-negative NF-YA mutant YA29 [22] were kindly provided by Dr R. Mantovani (Universiy of Milan, Milan, Italy). Plasmid DNA (1 μg) was used in each TNT reaction containing the TNT rabbit reticulocyte lysate, T7 RNA polymerase, amino acid mixture without methionine, RNasin RNase inhibitor and the reaction buffer included in the TNT kit. Methionine stock solution (1 μl of 1 mg/ml) was added and the reaction was performed at 30 °C for 2 h as recommended by the manufacturer. For binding assays, 3 μl aliquots of each TNT reaction were used.

EMSAs (electrophoretic mobility-shift assays) and supershift assays

The probes used in EMSAs were first prepared by annealing 0.4 pmol complementary strands of the oligonucleotides as follows: 94 °C, 5 min; 85 °C, 10 min; 65 °C, 10 min; 45 °C, 10 min; 37 °C, 10 min; and 10 °C, 10 min. All annealed double-stranded oligonucleotides contained staggered ends for end-labelling by standard Klenow fill-in reactions in the presence of [α-^{32}P]dCTP. For binding reactions using the $Y_{I\text{-}II}$ probe, the probe (1×10^5 c.p.m.) and 5 μl of the nuclear extracts were incubated in 20 mM Hepes (pH 7.9), 14.5 % (v/v) glycerol, 100 μg/ml BSA, 0.1 mM EDTA, 1 mM DTT, 30 mM KCl, 5 mM $MgCl_2$ and 1 mg/ml poly(dI-dC) · (dI-dC) at 4 °C for 30 min. When TNT-generated polypeptides were used in the incubation mixture, the binding buffer was 20 mM Hepes (pH 7.9), 50 mM NaCl, 5 % glycerol, 1 mM DTT, 5 mM $MgCl_2$ and 1 mg/ml poly(dI-dC) · (dI-dC) [1]. The incubation mixture was applied to a non-denaturing 6 % (w/v) polyacrylamide gel (30:1) and electrophoresis was performed in 0.5 × Tris/borate/EDTA buffer at 150 V for 3.5 h at 4 °C. For supershift assays, the anti-NF-YA subunit antibody was obtained from a commercial source (catalogue no. 100-401-100; Rockland, Gilbertsville, PA, U.S.A.). Anti-NF-YA antibody was added at a concentration of 90 μg/ml as recommended by the manufacturer; incubation conditions were similar to the $Y_{I\text{-}II}$ probe binding method described above.

RESULTS

Rnf35 is actively transcribed from the embryonic genome in the one-cell embryo

We have shown previously that the *Rnf35* mRNA is detected in the unfertilized egg and in the early embryo up to the eight-cell stage [15,16]. To ensure that the embryonic *Rnf35* transcript is not a residue of maternal origin but is derived from active transcription from the embryonic genome, fertilized eggs developed to the late one-cell embryonic stage were harvested and cultured overnight in the presence or absence of the RNA polymerase II inhibitor α-AM. RNA was prepared from the drug-treated or untreated two-cell embryos and used in RT–PCR analysis to determine gene expression (Figure 1A). For quantification, real-time RT–PCR was also performed, in which case an evaluation of the relative mRNA levels in the egg and in the drug-treated or untreated two-cell embryo was included (Figure 1B). As controls and to validate our approach, transcription of the *G6pd* and *Hsp70* genes was monitored. The *G6pd* gene has been shown previously to be transcribed in the unfertilized egg, but not from the embryonic genome on fertilization; however, *G6pd* mRNA has also been shown to be intrinsically stable enough to survive up to the two-cell stage [18]. In our experiments, we detected a similar level of the *G6pd* mRNA in the egg and in the untreated or drug-treated two-cell embryos (Figures 1A and 1B) as anticipated. The *Hsp70* gene, on the other hand, has been reported to be transcribed actively from the newly constituted embryonic genome between the late one-cell and the two-cell stages [15,18]. In our experiment, the *Hsp70* mRNA was detected in the untreated two-cell embryo; in the presence of α-AM, the *Hsp70* mRNA was totally ablated (Figure 1A), recapitulating the previous observation. *Hsp70* was, therefore, not included in the subsequent real-time RT–PCR quantification experiments. On a similar analysis of the *Rnf35* gene, we observe that the *Rnf35* transcription profile resembles that of *G6pd* in that the *Rnf35* mRNA was detected both in the presence or absence of α-AM (Figure 1A). However, real-time RT–PCR results indicate that the *Rnf35* mRNA level was doubled in the two-cell embryo when compared with that

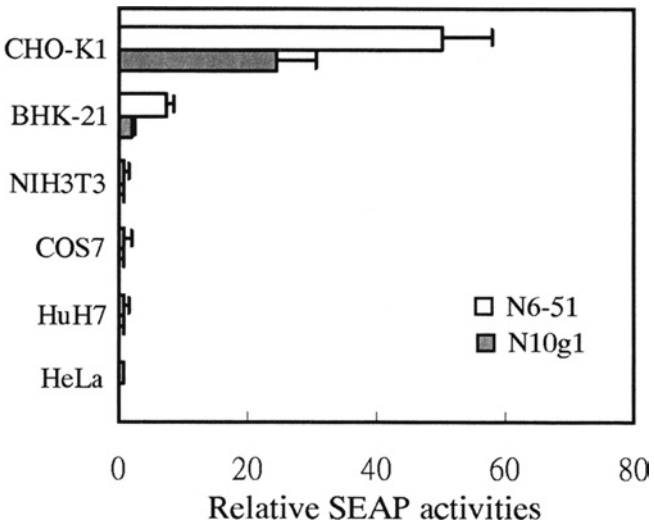

Figure 2 The CHO-K1 cells uniquely support *Rnf35* promoter activities

The two constructs N6-51 (open bars) and N10g1 (closed bars) carried *Rnf35* 5′-URR sequences between nt −210 or −13 and +71 within the exon 1 respectively (see Figure 3B below for a schematic representation of the constructs). The constructs were used in transient transfection assays in duplicate to different somatic cell lines as described in the Experimental section. The results shown were derived from three or more independent transfection experiments.

in the egg (Figure 1B); the increased mRNA level in the two-cell embryo reflects newly synthesized *Rnf35* mRNA transcribed from the embryonic genome. In the presence of α-AM, the *Rnf35* mRNA level in the two-cell embryo was similar to that found in the egg, consistent with inhibition of genomic transcription. Moreover, the results also indicate that the maternal *Rnf35* mRNA is highly stable and a large proportion of the transcript survives up to the two-cell stage, similar to the case of the *G6pd* mRNA. Hence we show that the *Rnf35* gene is also actively transcribed from the newly formed embryonic genome between the late one-cell and the two-cell stages of early development.

The CHO-K1 cells uniquely support *Rnf35* promoter activities

Expression of the *Rnf35* gene is restricted to early embryonic stages. To elucidate promoter elements in the 5′-URR of *Rnf35*, a somatic cell line that supports *Rnf35* promoter functions in transient transfection assays would be most useful. To search for such a permissive cell line, we first ligated 5′-URR segments of the *Rnf35* gene to the human *SEAP* gene in the promoter- and enhancer-free pSEAP-Basic plasmid for use as a reporter gene [20]. SEAP encodes a secreted form of the placenta alkaline phosphatase and permits the use of a fluorescent substrate, 4-methylumbelliferyl phosphate, in quantitative enzymic assays [23]. Constructs N6-51 and N10g1 carried the *Rnf35* 5′-URR segment between nt −210 or −13 and +71 within the exon 1 respectively (see Figure 3B for a schematic representation of the constructs). Promoter activities of these constructs were tested in transient transfection assays in six cell lines derived from different cell types, including CHO-K1, BHK-21 (hamster kidney cells), NIH3T3 (mouse embryo fibroblasts), COS7 (African Green Monkey kidney cells), Huh7 (human hepatocellular carcinoma cells) and HeLa (human cervical cancer cells). The CHO-K1 cells consistently supported transcription of the reporter gene in the presence of the *Rnf35* 5′-URR sequences; BHK-21 showed only limited transcription activities, and all other cell lines did not show appreciable levels of transcriptional activation of the *SEAP* gene in the transient transfection assays (Figure 2). The results indicate that the CHO-K1 cells are uniquely permissive for *Rnf35* promoter functions; CHO-K1 cells have been shown previously to support the promoter activities of another pre-implantation embryo-specific gene, *Zfp352* [20]. Subsequent analysis of the *Rnf35* 5′-URR and the exon 1 sequences for promoter functions was performed using the CHO-K1 cells and the results thus derived were confirmed in microinjection of the embryo.

Rnf35 is transcribed from an *Inr* (initiator) core promoter element

The sequence covering the 5′-URR and the short exon 1 of the *Rnf35* gene has been previously determined (GenBank® accession no. AY063497) [15,16]. In the 5′-URR, no TATA box is discernible. A previously mapped *Rnf35* mRNA start site in the two-cell embryo is initiated from an adenine (underlined) in the sequence 5′-GCAGTTC-3′, which resembles the consensus sequence 5′-YYANWYY-3′ of an *Inr* element [24]. We have assigned +1 to the adenine in the putative *Inr* motif as the transcription start site (Figure 3A, middle line, double underlined). It is further noted that the 5′-GGAGCTG-3′ situated between +28 and +34 (Figure 3A, bottom line, underlined) shows a close resemblance to the consensus sequence 5′-(A/G)G(A/T)(C/T)-(G/A/C)TG-3′ of the mammalian *DPE* (downstream promoter element) and in the anticipated location (see [25] for a review).

To verify the contribution of the putative *Inr* motif and *DPE*-like sequence to *Rnf35* transcription, various segments of the *Rnf35* 5′-URR and the exon 1 sequences were ligated to the promoter- and enhancer-free pSEAP-Basic plasmid for transient transfection using the permissive CHO-K1 cells as described in the Experimental section [20]. We first showed that the 5′-URR fragment from −210 to −8 in the construct N6-53 reported little promoter activity (Figure 3B) owing to the absence of a TATA box in the sequence. The inclusion of the segment up to +71 of the exon 1 sequence in the construct N6-51, however, activated the transcription of the reporter gene, signifying that a core promoter element lies between −8 and +71. Trimming the 5′-URR to −13 in N10g1 resulted in the retention of approx. 72 % of the N6-51 promoter activity, further mapping the core promoter element to −13 and +71. On further deletion of the sequence between −13 and +4, which removed the putative *Inr* in the construct N10g-141ΔI, only residual promoter activity could now be detected, providing the first evidence that the discerned *Inr* sequence indeed acts as an active core promoter element.

To dissect further the involvement of *Inr* and possible contribution of the *DPE*-like sequence in *Rnf35* transcription, mutations were introduced into these sequences (depicted in Figure 3A) in the N10g1 construct that carried the shortest 5′-URR sequence required for *Rnf35* promoter activity. Subsequent transient transfection assays were performed using N10g1 as a reference plasmid. A 4 bp mutation in the *Inr* element in the construct N10g1-MutI virtually abolished the promoter activity (Figure 3C). On the other hand, mutating the *DPE*-like sequence in N10g1-MutD did not result in any appreciable effect on the N10g1 promoter activity. In the double-mutant N10g1-MutI/D, there was little measurable promoter activity due to the mutated *Inr*. Interestingly, shortening the 5′-URR from −13 to −6 in the construct N10g1ΔY_{III} resulted in a 5-fold suppression of the promoter activity when compared with that of N10g1. In N10g1, a 5′-ATTGG-3′ pentanucleotide (designated as Y_{III} box) is present between −13 and −9 (Figure 3A, middle line); the Y_{III} box is deleted in N10g1ΔY_{III}. The results unequivocally indicate that the *Inr* acts as the obligatory *Rnf35* core promoter. On the other hand, the *DPE*-like sequence contributed little to *Rnf35* promoter activity and does not constitute a DPE. Furthermore, a Y box located immediately upstream of the *Inr* seems mandatory to the *Rnf35* promoter function (see below).

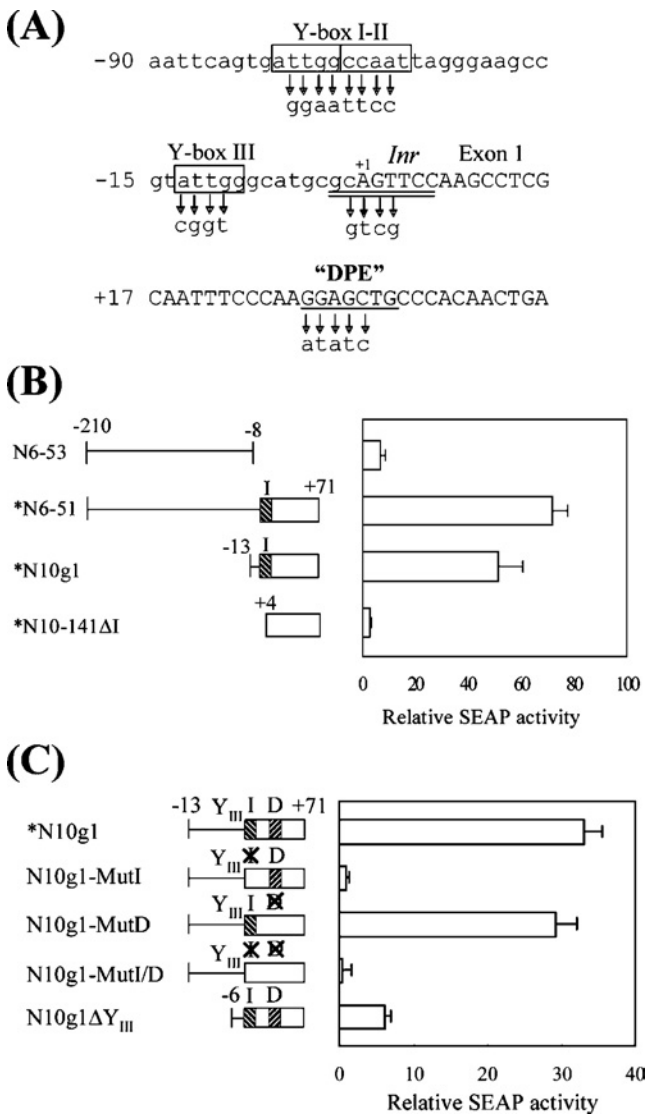

Figure 3 *Rnf35* transcription from an initiator under the regulation of Y-box elements

(**A**) Sequences of three short sections of the 5′-URR (lower-case letters) and exon 1 (upper-case letters) depicting the initiator (*Inr*; middle line, double underlined) and the *DPE*-like sequence (bottom line, underlined) in the *Rnf35* promoter. The three discernible Y boxes described in the present study are also shown (top and middle lines, boxed). Mutations are shown by downward-pointing arrows in lower-case letters. (**B**) An initial definition of *Inr* as the core promoter sequence. Plasmid constructs carrying different lengths of the *Rnf35* 5′-URR (horizontal line) and 71 bp of the exon 1 sequence (open box) were generated as shown schematically on the left (not drawn to scale). Hatched bar indicates the putative *Inr* (I) element. The numbers indicate termini of the *Rnf35* sequence in the constructs. The constructs were used in transient transfection of the CHO-K1 cells as described in the Experimental section. Culture media were harvested 2 days after transfection for alkaline phosphatase (SEAP) assays. The relative SEAP activities are displayed on the right panel. In each experiment, duplicates or triplicates were performed; the results shown were derived from three or more independent experiments; *, constructs used in the *EGFP* constructs for microinjection of the mouse embryos (see Table 1). (**C**) Mutational analysis of *Inr* (I) and the *DPE*-like (D) sequences. Mutations are the same as shown in (**A**); ×, mutated sites.

Obligatory involvement of Y-box elements in *Rnf35* transcription

Besides a core promoter, upstream binding sites specific for transcription factors are mandatory for the implementation of eukaryotic promoter functions. As first hinted in the transfection assay in Figure 3(C) above, deletion of a Y box in the construct 10g1ΔY_{III} had led to a significant depreciation of the *Rnf35*

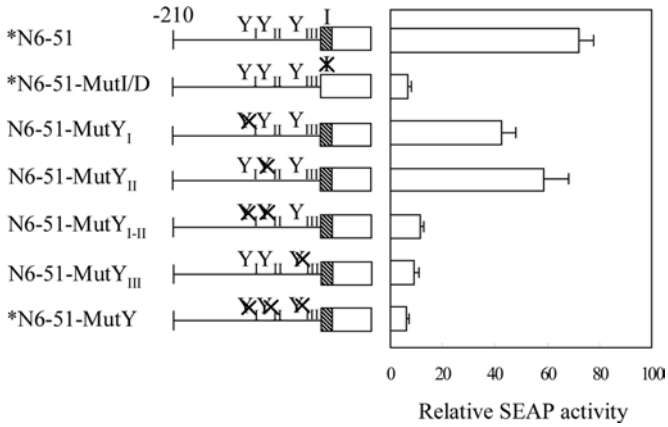

Figure 4 Involvement of the Y-box elements in the *Rnf35* promoter function

In the experiments, mutations (see Figure 3A, arrows) were introduced into one or more of the Y-box elements (designated Y_I–Y_{III}) in the parental plasmid N6-51. The constructs were used in transient transfection of CHO-K1 cells for SEAP assays as in Figure 3. See also the legend to Figure 3 for an explanation of the symbols used. The schematic diagram is not drawn to scale.

promoter activity, suggesting that the immediate-upstream Y-box element is obligatory. The Y-box sequence and its inverse complement the CCAAT box are found ubiquitously in the mammalian genome (see [26,27] for reviews). Besides the Y_{III} box at −13, two more palindromic Y boxes (5′-ATTGG/CCAAT-3′), designated Y_I and Y_{II}, are discernible between −81 and −72 (Figure 3A, top line). To evaluate further the possible contribution of the three Y-box elements to *Rnf35* transcription, mutational analysis was performed using the N6-51 plasmid as a reference (Figure 4). In this series of experiments, we first showed that mutating the *Inr* element (and the dispensable *DEP*-like sequence) in N6-51 virtually eradicated transcription activities as anticipated (the construct N6-51-MutI/D; Figure 4). When only the Y_I or Y_{II} box was mutated, the *Rnf35* promoter function was depreciated by approx. 40 or 20 % respectively. However, when both Y_I and Y_{II} were simultaneously mutated (N6-51-Mut$Y_{I\text{-}II}$; Figure 4), the *Rnf35* promoter function was now drastically curtailed; only 15 % of the wild-type promoter activity could now be detected. It is noteworthy to compare the N6-51-Mut$Y_{I\text{-}II}$ transcription activity with that of the $Y_{I\text{-}II}$-truncated plasmid N10g1, which showed only approx. 30 % decrease in transcription activity relative to N6-51 (Figure 3B). The disparity in the transcription activities presented by these $Y_{I\text{-}II}$ mutant plasmids suggests the presence of other *cis*-acting negative modulator element(s) between −210 and −13. When the Y_{III} box that lies immediately upstream of the *Inr* element was mutated in the construct N6-51-MutY_{III}, the *Rnf35* promoter activity was almost completely abolished, consistent with transfection data obtained with the Y_{III}-deleted mutant N10g1ΔY_{III} described above (Figure 3C). When all three Y-box elements were simultaneously mutated (construct N6-51-MutY), the promoter activity was almost completely abolished despite the presence of an intact *Inr* core promoter. Taken together, results presented in this section suggest that the three Y-box elements are indispensable contributors to *Rnf35* transcription: the Y_I and Y_{II} boxes in the $Y_{I\text{-}II}$ palindrome act synergistically and the Y_{III} box may act in association with the *Inr* core promoter.

The *Rnf35 Inr* core promoter is functional in microinjected late one-cell embryos

To test if the *Inr* unveiled in the transfection studies was functional in the embryo, we selected 5′-URR and exon 1 constructs and

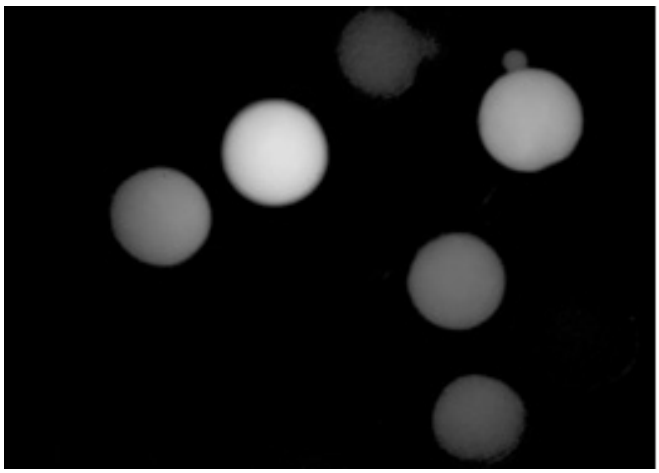

Figure 5 *Rnf35* promoter-driven *EGFP* expression in microinjected mouse embryo

The N6-51 plasmid harbouring the *EGFP* gene (Figure 3) was microinjected into the male pronuclei of one-cell mouse embryos and the microinjected embryos were cultured for 10–12 h before being scored for *EGFP* expression and photographed.

Table 2 Analysis of the *Inr* activities in microinjected one-cell embryos

	Number of embryos			
Plasmid*	Injected	Survived	Cleaved†	EGFP expression†
N6-51	84	74 (88%)	43 (58%)	13 (18%)
N10g1	142	141 (99%)	119 (84%)	26 (18%)
N6-51MutI/D	60	59 (98%)	54 (82%)	0
N10-141ΔI	31	30 (97%)	29 (97%)	0
N6-51MutY	58	56 (97%)	46 (82%)	0
pEGFP-Control	73	69 (95%)	65 (94%)	9 (13%)
pEGFP-Basic	52	46 (88%)	34 (74%)	0
No DNA	47	46 (98%)	32 (70%)	0

* The plasmid designations are the same as those used in the transient transfection experiments (* in Figures 3 and 4) except that the *SEAP* gene had been replaced by the *EGFP* gene. Typical examples of *EGFP*-expressing and fluorescing embryos are shown in Figure 5.

† The percentages were calculated relative to the number of embryos that had survived microinjection.

related mutants used in transient transfection assays (Figures 3 and 4, construct marked by *) for functional analysis in the mouse embryo (Figure 5 and Table 2). For this purpose, the *SEAP* gene in the selected transfection constructs was first replaced by *EGFP*. Highly purified plasmid DNA was microinjected into the male pronuclei of mid-to-late one-cell embryos. The injected embryos were cultured for 10–12 h before being inspected for *Rnf35* promoter-driven *EGFP* gene expression under a fluorescence microscope (Figure 5). At this time, the first sign of *EGFP* expression in the strongest fluorescing embryos had occurred. A time lag in *EGFP* expression in the injected one-cell embryos was anticipated, since plasmid-borne gene expression is linked to the onset of zygotic genome activation when endogenous zygotic gene expression also commences [11,15,28]. The green fluorescence increased in intensity 12–18 h after injection, before rapidly fading. By approx. 18 h after injection, all *EGFP*-expressing embryos fluoresced, albeit with different intensities (see Figure 5 for a representative example). The fluorescence was evenly distributed over the whole embryo in all positive cases. The bulk of fluorescing embryos injected with the *Rnf35* plasmids was, however, morphologically arrested at the one-cell stage for unknown reasons; only a very small fraction (approx. 5%) of the fluorescing embryos developed into the two-cell stage during the 24 h period of observation after injection. In these rare cases, the two-cell embryos rapidly lost their fluorescence. Embryos unequivocally fluorescing 12–18 h post-injection were scored as positive (Figure 5). In the microinjection experiments, the pEGFP-Control plasmid bearing the constitutive SV40 promoter and enhancer sequence and the pEGFP-Basic plasmid devoid of promoter and enhancer were used as positive and negative controls respectively (Table 2). These control plasmids were *EGFP* replacements of the pSEAP-Control and pSEAP-Basic plasmids used in the transfection work as described in Figures 2 and 3. In the microinjection experiments, 88% or more of the microinjected embryos remained viable on culture (Table 2). Cleavage rates, however, varied in the range 58–97%.

When the N6-51 *EGFP* plasmid carrying the −210 to +71 sequence and the *Inr* was tested, 13 (18%) of the 74 survived embryos were clearly fluorescing (Figure 5 and Table 2); when the 5′-URR was trimmed to −13 in construct N10g1, which still carried the *Inr* and the Y_{III} box, 26 (18%) of the survived 141 microinjected embryos were *EGFP*-positive. The positive rates of these two constructs were similar to that obtained with the pEGFP-Control, in which case 9 (13%) of 69 viable embryos were positive. When the initiator was mutated in the construct N6-51-MutI/D or deleted in N10-141ΔI, none of the viable embryos now expressed the *EGFP* gene. The results unequivocally indicate that the *Inr* element is obligatory and active as a core promoter element in the one-cell embryo as in the CHO-K1 cells. The positively expressing N6-51 *EGFP* construct carries a full complement of the upstream Y-box elements. To investigate if the Y-box elements are also needed for *Inr* promoter function in the embryo, we tested the Y-box mutant N6-51-MutY in which all three Y boxes had been mutated. None of the 56 surviving embryos expressed *EGFP*, indicating that the *Rnf35* promoter requires the participation of the Y boxes in the one-cell embryo. Since the positively expressing N10g1 carried only the Y_{III} box, the Y_{III} box may be an intimate part of the *Inr* promoter activity.

The NF-Y/CBF binds to the Y_I box

To investigate if the Y_I and Y_{II} boxes in the *Rnf35* promoter were targeted by activator protein(s), EMSAs were performed using a radiolabelled probe ($Y_{I\text{-}II}$) that carried the $Y_{I\text{-}II}$ palindrome (Figure 6A); competition was also performed using Y_I (Y_IMut) or Y_{II} mutant (Y_{II}Mut) or the $Y_{I\text{-}II}$ double mutant ($Y_{I\text{-}II}$Mut) oligonucleotide (Figure 6A) to verify binding specificity. Nuclear extracts prepared from the CHO-K1 cells were first tested in EMSAs, since the cell line was used in all transient transfection experiments described above to elucidate the *Rnf35* promoter function. On incubation of the $Y_{I\text{-}II}$ probe with the CHO-K1 nuclear extracts, two prominent $Y_{I\text{-}II}$–protein complexes were discernible in the EMSA gel (Figure 6B, lane 2, arrows). In competition experiments, molar excess of the unlabelled $Y_{I\text{-}II}$ oligonucleotide effectively competed for the complex represented by the upper band (Figure 6B, lanes 3–5, closed arrow), indicating that the higher molecular mass complex was $Y_{I\text{-}II}$-specific. In the presence of a competing Y_I or Y_{II} mutant oligonucleotide, only at high concentrations were the competitors able to compete effectively for this binding complex (Figure 6B, lanes 6–11), suggesting that both the Y_I and Y_{II} boxes contributed to protein binding. Furthermore, we note that the Y_I mutant oligonucleotide was less effective than the Y_{II} mutant oligonucleotide in the competition, suggesting that the Y_I box in the 5′-ATTGG-3′ configuration has a higher binding affinity compared with the Y_{II} box (5′-CCAAT-3′).

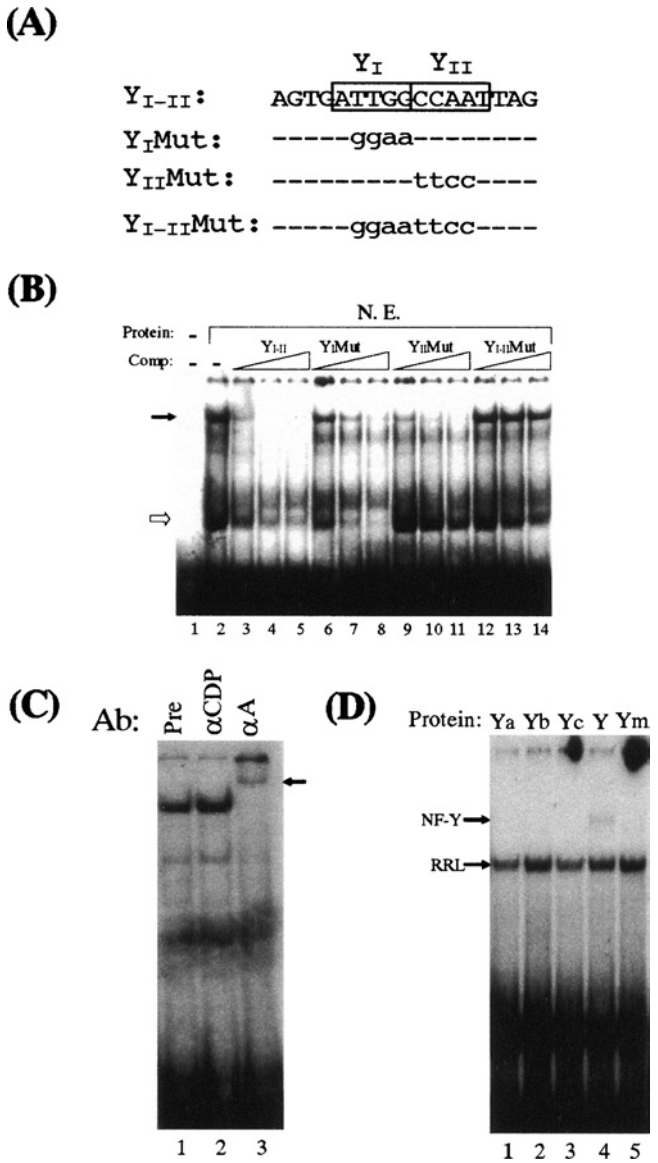

Figure 6 Binding of the NF-Y to the Y_I-box element

(**A**) Sequences of the wild-type Y_{I-II} and the Y_{I-II} mutant oligonucleotides used for the EMSAs. In the oligonucleotide sequences, the wild-type sequence is shown in upper-case letters. In the mutant sequences, dashes indicate unchanged nucleotides; mutated nucleotides are shown in lower-case letters. (**B**) EMSA experiments using nuclear extracts prepared from the CHO-K1 cells; radiolabelled wild-type Y_{I-II} was used as the probe. After incubation, the binding complexes were displayed on a 6 % non-denaturing polyacrylamide gel running for 3.5 h. In lane 1, neither nuclear extracts nor the competitor oligonucleotide was added (denoted by '–'); in lane 2, the Y_{I-II} probe was incubated with the nuclear extracts without competing oligonucleotides. In all other lanes, 25-, 125- or 250-fold molar excess of the unlabelled and double-stranded wild-type Y_{I-II} (lanes 3–5), Y_I mutant (Y_IMut; lanes 6–8), Y_{II} mutant (Y_{II}Mut; lanes 9–11) or Y_{I-II} double mutant (Y_{I-II}Mut; lanes 12–14) oligonucleotide was included as a competitor (Comp). The two major Y_{I-II}–protein binding complexes indicated by arrows are as discussed in the text. (**C**) Identification of the NF-Y-bound complex in the EMSA supershift assay using an anti-NF-YA antibody. The EMSA was performed as in (**B**) using the CHO-K1 nuclear extracts and the Y_{I-II} probe. In the control lanes, a pre-immune serum (Pre, lane 1) and a non-specific anti-CDP (αCDP, lane 2) antibody were used. In the presence of an anti-NF-YA antibody (αA, lane 3), only the high molecular mass band supershifted (lane 3, arrow), indicating NF-Y binding specificity. (**D**) Confirmation of NF-Y binding to Y_{I-II}. *In vitro* translated NF-YA (Ya), NF-YB (Yb) and NF-YC (Yc) subunits were used individually (lanes 1–3) or in a combined mixture (Y) of all three subunits in equal molar concentrations (lane 4) in binding reactions in the presence of the Y_{I-II} probe. The resulting NF-Y-binding complex is indicated; the RRL band was a non-specific product arising from the use of the rabbit reticulocyte lysate. In lane 5, a dominant-negative NF-YA mutant subunit (Ym) was used in the incubation mixture in the presence of the wild-type NF-YB and NF-YC subunits.

When the Y_I and Y_{II} double-mutant oligonucleotide was used as the competitor, no competition for the higher molecular mass complex was now evident at as high as 250-fold molar excess (Figure 6B, lanes 12–14), further supporting that the complex was Y_{I-II} palindrome-specific. Incubating the Y_{I-II} probe with the CHO-K1 nuclear extracts also resulted in a lower molecular mass binding complex in EMSA (Figure 6B, open arrow). High molar excess of the unlabelled Y_{I-II} or Y_I mutant oligonucleotide showed appreciable competition for the complex (Figure 6B, lanes 2–8); however, up to 250-fold molar excess of the Y_{II} mutant and the Y_{I-II} double-mutant oligonucleotides showed little or no competition for this complex (Figure 6B, lanes 9–14). The appearance and competition behaviour of the higher mobility complex are best explained by the binding of a different protein moiety to Y_{II}.

A major Y-box-binding protein that has an almost absolute requirement for each of the 5′-ATTGG-3′ pentanucleotides in either orientation is NF-Y (also called CBF; see [26] for a review). The NF-Y is an oligomeric protein complex composed of three subunits NF-YA, NF-YB and NF-YC. To investigate if the Y_{I-II}-bound protein in the nuclear extracts was NF-Y, a supershift assay was performed. In reaction mixtures that included the CHO-K1 nuclear extracts and the Y_{I-II} probe, the addition of an antibody directed against the NF-YA subunit resulted in the Y_{I-II} complex being retarded (Figure 6C, lane 3, arrow); the use of a pre-immune serum and a non-specific anti-CDP antibody (where CDP stands for CCAAT-displacement protein) had no effect on the Y_{I-II}-bound complex (Figure 6C, lanes 1 and 2). The results demonstrate that the protein that bound to the Y_{I-II} sequence was the NF-Y. The NF-Y binding to the Y_{I-II} sequence was further tested using *in vitro* translated NF-Y subunit polypeptides. When the three NF-Y subunits were used individually in the binding assays, no Y_{I-II} probe-specific complexes were observed (Figure 6D, lanes 1–3) other than a non-specific band arising from the incubation with the rabbit reticulocyte lysate (Figure 6D, the band labelled RRL). On mixing all three NF-Y subunits in the binding reaction, however, a retarded complex was now formed (Figure 6D, lane 4). When a dominant-negative NF-YA mutant subunit that has previously been shown to affect DNA binding (mutant YA29; [27]) was used in the subunit mixture in place of the wild-type NF-YA polypeptide, the complex was not formed (Figure 6D, lane 5), confirming NF-Y binding. In summary, our results show that the Y_{I-II} box in the *Rnf35* promoter is bound by the ubiquitous NF-Y in eliciting *Rnf35* promoter activities.

DISCUSSION

The focus of the present study was to characterize the *Rnf35* promoter and to elucidate *cis*- and *trans*-acting factors that may contribute to transcriptional regulation in the pre-implantation embryo. In the absence of appropriate early embryo-related cell lines, we found that the CHO-K1 cell line was uniquely permissive in supporting *Rnf35* promoter function (Figure 2), as for another pre-implantation-specific gene, *Zfp325* [20]. Promoter analysis results derived from the use of a somatic cell line must, however, be interpreted with caution; somatic cells and embryos predictably present different environments for gene expression. Nonetheless and notwithstanding subtle differences, we show in transient transfection of the CHO-K1 cells that the upstream sequence from −210 and the short exon 1 sequence combined to constitute the *Rnf35* promoter module and that an initiator acts as the core promoter element (Figure 3). The *Inr* element was functional when microinjected into the pronuclei of one-cell mouse embryos; mutating or deleting the *Inr* effectively abolished promoter function in the embryo (Table 2; constructs N6-51-MutI/D and N10g1-141ΔI). The present study is the first

demonstration that *Inr* may be used as a core promoter element in transcription in the pre-implantation embryo without the benefit of a TATA box, echoing earlier findings that TATA-less core promoters are preferred at the onset of zygotic genome activation [8,29]. In the absence of a TATA box, *Inr* is pivotal in recruiting the general transcription complex TFIID, the binding of which covers an extended region between the upstream location of the TATA box at approx. −25 to −30 and the transcription start site (see [30–32] for reviews). Contributions from activators binding to the sequence immediately upstream of *Inr* may now be obligatory for TFIID interactions.

We have identified three Y-box elements in the *Rnf35* promoter that are mandatory for *Rnf35* transcription in the CHO-K1 cell line and probably in the mouse embryo. A Y box (designated Y_{III}) is located immediately upstream of the RNA start site at −13 to −8; mutation or deletion of Y_{III} resulted in a 5-fold decrease in promoter activity in the CHO-K1 cells (Figures 3 and 4), suggesting significant Y_{III} contributions to the *Rnf35* promoter function. Protein(s) interacting with Y_{III} remain to be elucidated; however, it is not unlikely that Y_{III} contributes to TFIID interactions with the *Inr* core promoter element. Examples of other *Inr*-based promoters with active participation from close proximity Y boxes include *Timp-4* and the *RUSH*/*SMARCA3* gene [33,34].

Y boxes are generally located between −60 and −100 from the transcription start site [35,36]. We show in the present study that a transcriptionally active dual Y-box module, designated Y_{I-II}, is located at −81 of the *Rnf35* 5′-URR. On the basis of results obtained both from mutational analysis in transfection assays (Figure 4) and competition experiments in EMSAs (Figure 6), we obtained evidence to indicate that Y_I and Y_{II} are targeted by different proteins that act synergistically. Double mutations to both the Y-box elements are needed to abolish effectively contribution from the 10-bp element. We further show that the Y_I box is targeted by the ubiquitous NF-Y (Figure 6), a first demonstration of the participation of the NF-Y in transcriptional regulation in the mammalian pre-implantation embryo. NF-Y is a versatile protein that has a strict requirement for the 5′-ATTGG-3′ pentanucleotide for effective and specific binding; the protein is also a heteromeric complex composed of three subunits. An important feature of the NF-Y relevant to this work is that subunits NF-YA and NF-YC carry glutamine-rich and hydrophobic residue-rich activation domains that are structurally and functionally similar to those found in another ubiquitous transcription factor Sp1 [37,38]. Early investigations have variously demonstrated that Sp1 is involved in transcription in the early stages of development [12,13,39]. In the *Rnf35* 5′-URR up to −210 dissected in the present study, no Sp1-binding sites are discernible. Given the resemblance between Sp1 and NF-Y, results of the present study strongly suggest that the NF-Y may be recruited as an alternative activator in transcriptional control in pre-implantation development. This hypothesis correlates well with a recent report that examined conditional *Nfya* mutant mice [40]. The authors reported that no *Nfya*-null embryos as early as 8.5 days post-coitus in the development stage were ever obtained due to early embryo lethality as a result of a block in the S-phase of the cell cycle, leading to defective cell proliferation. Indeed, key cell-cycle-related genes frequently contain multiple and crucial Y boxes, many of which are controlled by NF-Y (see [26,41] for a review; see also [42] and references therein). It may be worth noting here our attempt to establish the expression profile of the NF-Y subunits in the pre-implantation embryo to correlate with the proposed involvement of NF-Y in *Rnf35* transcription. When mouse cDNA libraries constructed from unfertilized eggs and the two- and eight-cell embryos were used as templates in PCR-based detection of the three-subunit cDNA sequences as we had previously done for expression profiling of other pre-implantation embryo-specific genes [17,18,43], we were able to detect cDNA sequences of one or two of the three subunits but not all three subunits in each of these cDNA libraries (results not shown). However, when a similarly constructed blastocyst cDNA library and cDNA libraries constructed from other adult tissues were used, all three subunits were consistently detected (results not shown). Although differential expression of the NF-Y subunits has been documented [44,45], the physiological state of the NF-Y subunits in the egg and the early embryo will have to be carefully evaluated before drawing further conclusions.

Another versatile characteristic of the NF-Y is its ability to interact with other activator or repressor protein(s) bound in the vicinity of the same promoter (see [27,46] for a review). Our results confirm that the Y_{II} box in the Y_{I-II} module is bound by another protein(s) that inevitably has to interact with the NF-Y due to close proximity in eliciting transcriptional modulation (see [46] for a review). Examples of juxtaposed binding sites for NF-Y and other proteins are found in promoters of the liver-specific serum albumin gene, the MHC class II gene and the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene [36,47–50]. In the *CFTR* promoter, two Y boxes in inverted orientation are found separated by only 4 bp; the upstream 5′-AATTG-3′ box, targeted by the NF-Y, overlaps with the binding motif for the CDP/Cux, thus opening opportunities for protein–protein interactions and competition for occupying binding sequences [47,49]. Identification of the Y_{II}-binding protein(s) and elucidation of interactions between the candidate protein(s) and the NF-Y are subjects of ongoing studies.

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