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Different modes of regulation of transcription and pre-mRNA processing of the structurally juxtaposed homologs, *Rnf33* and *Rnf35*, in eggs and in pre-implantation embryos

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ABSTRACT

Molecular events involved in gene expression in unfertilized eggs and pre-implantation embryos are beginning to be understood. In this work, we investigated the transcription and processing of two structurally juxtaposed mouse RING finger protein genes, *Rnf33* and *Rnf35*. Transcripts of these genes are detected only in eggs and in pre-implantation embryos. Both genes are intronless except for a solitary intron in the 5′-untranslated region. Here, we showed by rapid amplification of cDNA ends (RACE) and reverse transcription experiments that *Rnf35* transcription uses a single promoter and a terminating site. On the other hand, *Rnf33* is transcribed using multiple promoters. At the four-cell stage, however, *Rnf33* mRNA with a single transcription start site derived from the proximal promoter is detected, indicating that it is the major promoter. Sequences upstream of the *Rnf35* and the major *Rnf33* transcription start sites carry no TATA boxes but a putative transcription initiator (*Inr*) element is discernible in each case. The processing of the 3′-end of the *Rnf33* mRNA is also in disarray with multiple 3′-ends, an event that may be related to the absence of the AAUAAA element and the utilization of AAUAAA-like proxies. The multiplicity of the 3′-untranslated region is partially amended at the four-cell stage when only two major 3′-ends are in use. This work demonstrates that expression of some maternal and early zygotic genes may be opportunistic until a stringent transcriptional regulation mechanism is imposed.

INTRODUCTION

In mammals, fertilization of an egg marks the initiation of the development of a new life. We are now beginning to understand molecular events involved in oocyte maturation, the egg-to-zygote transition following fertilization and early zygotic gene expression (reviewed in 1–4). Each of these stages is characterized by a unique pattern of gene expression with far-reaching effects. In the mouse, zygotic genes are first activated in the late one-cell zygote; zygotic genome activation (ZGA) is in full swing by the late two-cell stage (1–3). Transcription that drives ZGA occurs in two phases. The early phase occurs in the late one-cell zygote; the late and major phase occurs at the two-cell stage (3,4). Using microinjected reporter genes, it has been shown that the early phase of transcription is regulated from proximal promoters; the distal enhancer elements are not utilized at this stage (1,5,6). After the first mitosis and in the early two-cell stage, enhancer-less promoters are repressed. It has been hypothesized that ZGA is global and relatively promiscuous, and that a balance between activation and repression dictates the observed stage-specific gene expression profile in pre-implantation embryos (7). However, direct evidence is still lacking. Another interesting feature of transcription of early embryonic genes is that TATA-less promoters are more efficiently utilized (8). The switch to the utilization of TATA-containing promoters and the activation of TATA-dependent enhancer activities are acquired as the embryo further develops (8,9). Such a mode of transcription introduces another regulatory element in the post-fertilization reprogramming of gene expression in pre-implantation embryo development.

To date, only a handful of maternal and early embryonic genes have been described. We have previously derived two mouse RING finger protein gene homologs, *Rnf33* (former name *2czf45*, GenBank accession no. AF290197) and *Rnf35* (GenBank accession no. AY063495) (10,11) that are new members of the RBCC (RING finger–B box finger–coiled coil) protein family. RING finger proteins (RNFs) form a unique subgroup of zinc finger proteins (12). RNFs often form components of multiprotein complexes present in nuclear or cytoplasmic structures, possibly in association with cytoskeletal or nuclear matrix proteins (13). A few RNFs are involved in ubiquitination or ubiquitin-like events (14,15).

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Most known RNFs carry only a single RING domain. One major RNF subclass is the RBCC proteins, so-called because they carry with them distinctive (from N- to C-terminus) RING, B box zinc finger and one or two α-helical coiled coil tripartite domains (16,17). Functionally, RBCC proteins are involved in cell growth, differentiation and transformation (18–24). The RBCC oncoproteins PML and T18 are activated by chromosomal translocation (21,22,25). More recently, a few RBCC proteins, including the well characterized XNF7 and RET, have been shown to contribute to development (18,24). The RBCC SS-A/Ro protein is, however, a ribonucleoprotein (26).

Transcripts for both the *Rnf33* and *Rnf35* genes are detected in unfertilized eggs and in pre-implantation embryos up to the eight-cell stage (10,11). The genes are encompassed in a 22 kb genomic fragment (GenBank accession no. AY063497) with *Rnf35* located 11.5 kb upstream of the *Rnf33* gene (11). Alignment of the *Rnf33* and *Rnf35* cDNA sequences with the genomic sequence has revealed that both the *Rnf33* and *Rnf35* genes are intronless in the coding region. Based on an analysis of *Rnf33* and *Rnf35* cDNA clones derived by *in silico* mining of the mouse dbEST database, we further observed that the genes share a common exon 1. We postulated that the *Rnf35* promoter was used in transcription of both the *Rnf33* and *Rnf35* genes (11). In this work, we validated the supposition and further showed that the previous observation is far from being the complete picture. The upstream *Rnf35* gene utilizes a single promoter in transcription. However, transcription and pre-mRNA processing of *Rnf33* are a lot more complex.

MATERIALS AND METHODS

Collection of unfertilized eggs and pre-implantation embryos

Unfertilized eggs and pre-implantation embryos were collected from 6–8-week-old ICR donors that were superovulated by injection of 10 IU of pregnant mare serum gonadotrophin, followed 48 h later by 10 IU of human chorionic gonadotrophin (hCG). For the collection of unfertilized eggs, superovulated but unmated female mice were killed 21 h after hCG injection. For zygotes, hormone-treated ICR females were mated with ICR males. One-, two-, four- and eight-cell embryos and blastocysts were collected 24, 44, 64, 72 and 98 h post-hCG injection, respectively. The collected eggs or embryos were used for RNA preparation or were snap frozen in liquid nitrogen until used.

RNA preparation, RT–PCR, 5′- and 3′-RACE and molecular cloning

To ensure the generation of enough RNA for subsequent studies, RNAs were routinely prepared from pools of 100 unfertilized eggs, 100 one- or two-cell embryos, 50 four-cell embryos, 25 eight-cell embryos or 15 blastocysts (10,11). In brief, the biomaterial samples were transferred to 100 µl of lysis buffer (6.2 M guanidinium thiocyanate, 40 mM sodium acetate, pH 7.0, 0.8% sarkosyl and 1% 2-mercaptoethanol). For lysis, 20 µl of 2 M sodium acetate, pH 4.0, 100 µl redistilled phenol and 40 µl of chloroform/isoamyl alcohol (49:1) mixture were added. After a brief vortex mixing, the mixture was incubated at 4°C for 15 min and then centrifuged at low speed to obtain phase separation. The aqueous phase was transferred to a fresh tube to which an equal volume of isopropanol was added in the presence of 3 µl of glycogen. The mixture was kept at –20°C for 30 min, followed by centrifugation. The RNA pellet thus obtained was washed with 70% chilled ethanol, re-pelleted and air dried. The final RNA pellet was dissolved in 20 µl of DEPC-treated distilled water and was used in at least two reverse transcription reactions.

Reverse transcription (RT) was performed using the Superscript II RT kit obtained from Life Technologies (Rockville, MD) using oligo(dT)$_{12–18}$ as a primer. The RT products were dissolved in 20 µl of sterile distilled water. In subsequent PCRs, 1 µl aliquots of the RT products were used. For 5′ or 3′ rapid amplification of cDNA ends (5′- or 3′-RACE), first strand synthesis using the lock-docking oligo(dT) primer (27,28) and PCR amplification were performed using the SMART RACE cDNA Amplification Kit purchased from Clontech (Palo Alto, CA) using a 35-cycle mode of amplification as previously described (10,11,27). The *Rnf33*-specific primers used in this study are listed in Table 1. All the RACE-derived PCR products were subcloned into the pGEM-T vector (Promega, Madison, WI) using a standard cloning procedure (29).

Table 1. *Rnf35*- and *Rnf33*-specific oligonucleotide primers used in this study

Experiment	Primer	Sequence (5′→3′)
Rnf35		
5′-RACE	CZ8AR2	GGCTCGTAGTCATATCCAGTGTAG
Nested PCR	CZ8R	TGGTGCAGGACAGACAGACTTTAT
3′-RACE	CZ8F	TGAGGCACCTTACTTCTATTGACT
Nested PCR	CZ8AF2	ACAGAATTATCAAGCGATTTCAAG
Rnf33		
5′-RACE	CZ45R4	GTGTGTTCTTCCTGCCTCTTCCTC
Nested PCR	CZ45R6	GTTTGACAGACGGGACAGAAGATG
3′-RACE	CZ45-1	AGAGGAAGAGGCAGGAAGAACACA
Nested PCR	CZ45	CACATGGAGGAGCTGCGGAGTT
RT–PCR	CZ45A	GCAGGACCAGCCACAGCAGGAATG
	CZ45B	TTGCATCAGGTGTAGATTTCTCTC
	CZ45C	GCTGAAACTGACAACCATCAACAA
	CZ45D	GTCTGCAACTGAGAGGTCTGTGTG
	CZ45E	CACGCCTTCAATTCCACCACTTGG
	CZ45R4	GTGTGTTCTTCCTGCCTCTTCCTC

Sequencing analysis

Sequencing reactions were performed using the Prism DNA Sequencing Kit obtained from ABI (Foster City, CA) according to the user's manual, and the reaction products were analyzed in an ABI 377 automatic sequencer. Putative transcription factor binding sites were derived using version 1.3 of the TFSEARCH algorithms (http://www.cbrc.jp/research/db/TFSEARCH.html).

RESULTS

Analysis of the structure of *Rnf35* transcripts in two-cell embryos

Based on the analysis of an *Rnf35* cDNA clone (GenBank accession no. AY063495) derived by *in silico* mining (11), we previously determined that exon 1 of the *Rnf35* gene was 91 bp

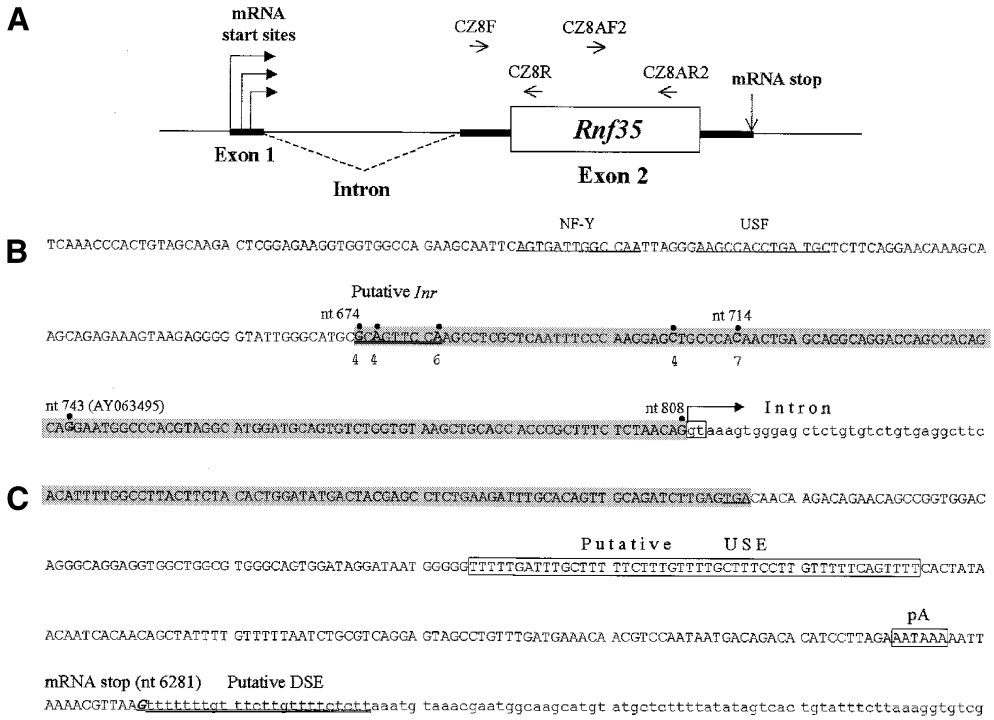

Figure 1. Transcription and processing of the *Rnf35* gene. (**A**) Schematic representation of the genomic structure of the *Rnf35* gene (11). In the scheme, horizontal thick bars represent the 5′- or 3′-UTR of the gene. The short exon 1 that forms part of the 5′-UTR is indicated. The intronless coding sequence of the gene is boxed. The intron in the 5′-UTR is shown by slanting dashed lines (not to scale). The primers used in the 5′- and 3′-RACE experiments are indicted above. Note that only approximate scaling is used in the scheme. (**B**) The exon 1 and the upstream sequence of the *Rnf35* gene are based on the *Rnf33/35* genomic sequence (GenBank accession no. AY063497). The exon 1 sequence is shown on a gray background and the intron sequence is shown in lower case. The transcription start sites of the gene as determined by 5′-RACE are indicated by bold letters capped with a dot; the number of 5′- RACE clones obtained for each site is shown below. The putative transcription factor binding sites are underlined; the proposed *Inr* element is double underlined. (**C**) Transcription stop site (at nucleotide 6281, underlined bold letter) and other salient features of the 3′-UTR of *Rnf35*. The coding sequence is shown on a gray background. The 3′-UTR sequence is shown in upper case and the transcribed genomic sequence that is removed on 3′-processing is shown in lower case. The putative upstream sequence element (USE) and the polyadenylation signal (pA) are boxed; the putative downstream sequence element (DSE) is underlined.

long. The 5′-end of the *Rnf35* mRNA was then mapped at nucleotide position 743 of the 22-kb *Rnf35/33* genomic sequence (AY063497). To authenticate and to search for other 5′-ends of the *Rnf35* mRNA, we prepared RNA from two-cell embryos for use in 5′-RACE analysis. To increase sensitivity and specificity, two 5′-RACE primers, CZ8AR2 and CZ8R, were used in two successive rounds of nested PCR (Fig. 1A and Table 1). Twenty-five 5′-RACE clones were obtained for sequencing analysis. Multiple 5′-ends were mapped upstream of the previously mapped 5′ site in a 41 bp cluster at nucleotides 674–714 (Fig. 1B). The previously determined 5′-splice site at nucleotide 808 and the 3′-splice site at nucleotide 4367 were found in all the 5′-RACE clones (Table 2), confirming the existence and the splicing of a 3.56 kb intron located in the 5-untranslated region (5′-UTR) in the *Rnf35* pre-mRNA.

Examination of the 5′-UTR of the *Rnf35* gene does not reveal the existence of any TATA box or CCAAT element in the putative promoter region (Fig. 1B). Putative binding sites for transcription factors are discernible but their involvement in *Rnf35* transcription will need to be experimentally established. However, the CGCA^{+1}GTTCC between nucleotides 673 and 681 that overlaps with a mRNA start site (A) at nucleotide 676 agrees very well with the consensus core sequence, YYYA^{+1}N(A/T)YYY, of a transcription initiator (*Inr*) (30). With the exception of a residue in the –2 position which is not a pyrimidine, the rest of the sequence agrees with the highly conserved features of an *Inr* element (31,32). Hence, in the absence of a TATA-like promoter, it is highly likely that the discerned *Inr* is used as a promoter element in the transcription of *Rnf35*.

Table 2. Splice junctions of the *Rnf35* and *Rnf33* pre-mRNA

Designation[a]	Map position (nt)[b]	Nucleotide sequence[c]
Rnf35		
5′ ss	808	UAACAG/**gu**aaagugggagcucug
3′ ss	4367	ucuuaugucacccac**ag**/GAAGGA
Rnf33		
5′ ss (P1-sv1)	14 311	CUACAG/**gu**aaaaugugggggugg
5′ ss (P1-sv2)	14 474	AGGCAG/**gu**agaucucugggaguu
5′ ss (P2)	13 301	UUGGAU/**gu**gagucccugccacau
5′ ss (P3)	11 518	AGGCAG/**gu**ucuuuaccuacuccu
5′ ss (P4)	808	UAACAG/**gu**aaagugggagcucug
Common 3′ ss	17 421	uuuuuucucauccac**ag**/GAACCC

[a]For a definition of the 5- and the 3′-splice sites (5′ or 3′ ss), see text.
[b]The nucleotide positions are based on the *Rnf33/35* genomic DNA (GenBank accession no. AY063497) (11).
[c]Exon sequences are in upper case; intron sequences are in lower case. M is A or C; R is A or G; Y is C or T; N is any of the four nucleotides. Slashes demarcate the exon–intron junctions.

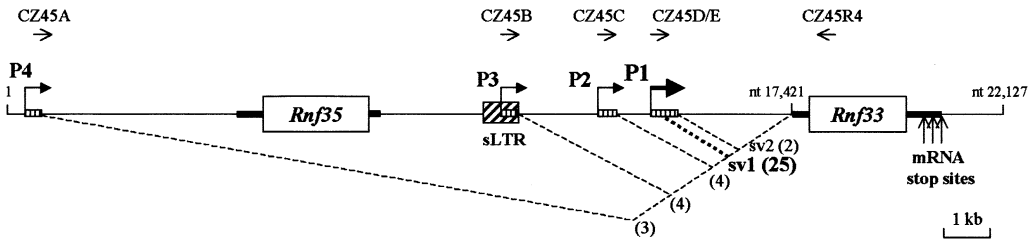

Figure 2. Transcription and splicing of the *Rnf33* mRNA. The relative positions of the intronless coding regions of the *Rnf33* and *Rnf35* genes (shown in boxes) are as previously determined (GenBank accession no. AY063497) (11). The horizontal thick bars represent the 5′- or 3′-UTR sequences of the genes. The relative positions of the putative *Rnf33*-P1 to *Rnf33*-P4 promoters are shown with the associated exon 1 sequences (as vertically hatched bars). The genomic map positions of the exon 1 sequences are as follows: P1 exon 1 (sv1), nucleotides 14 228–14 311 (84 bp in size); P1 exon 1 (sv2), nucleotides 14 228–14 474 (247 bp); P2 exon 1, nucleotides 13 301–13 323 (23 bp); P3 exon 1, nucleotides 11 345–11 518 (174 bp); P4 exon 1, nucleotides 676–808 (113 bp). All splicing events are shown as slanting dashed lines; the number of 5′-RACE clones associated with each splicing event is shown in parentheses. In the case of the P1 promoter-derived pre-mRNA, there are the splice variants sv1 and sv2, as defined in the text. The arrows at the top show the approximate position of the primers used in the RT–PCR experiment presented in Figure 4. The P3 promoter coincides with a solitary long terminal repeat (sLTR, cross-hatched box) (11). Only approximate scaling is used in the illustration.

3′-RACE experiments were similarly performed using the CZ8F and CZ8AF2 primers (Fig. 1A and Table 1). Fourteen independent 3′-RACE clones were obtained for sequence analysis. In all cases, the 3′-end of the *Rnf35* mRNA is mapped at the same site at nucleotide 6281 (Fig. 1C), in agreement with the previously mapped 3′-end of the *Rnf35* cDNA clone (11). An AATAAA hexamer representing the highly conserved polyadenylation signal is found 15 bp upstream of the mRNA stop site. Located immediately downstream of the mRNA stop site is a GT-rich sequence (Fig. 1C) that could be a *cis*-acting downstream sequence element (DSE). DSE sequences are transiently present in pre-mRNA and are involved in the tightly coordinated processes of transcription termination, pre-mRNA cleavage and polyadenylation processes (33–35). The 3′-UTR of the *Rnf35* pre-mRNA would also have carried a long U-rich segment (Fig. 1C), which may act as a putative upstream sequence element (USE) for transcription termination in some mammalian genes (33–35).

Taken together, the data indicate that in eggs and in early embryos, the mode of transcription and processing of the *Rnf35* gene is simple in the use of a single promoter and a termination site. The *Rnf35* pre-mRNA also carries a single 5′-UTR intron that is accurately removed.

Analysis of the multiple *Rnf33* mRNA species in two-cell embryos

In the previous study, we observed in a cDNA clone (GenBank accession no. AF290197) derived by *in silico* mining that the *Rnf33* mRNA carried a 120 bp exon 1 (11). The start site of the cDNA was mapped at nucleotide 689 of the *Rnf35/33* genomic sequence, co-localizing with exon 1 of the *Rnf35* gene (11) (see Fig. 2). The data had led us to suggest that both the *Rnf33* and *Rnf35* genes were transcribed from the same *Rnf35* promoter. To search for other *Rnf33* mRNA transcription start sites, we performed 5′-RACE analysis using RNA derived from two-cell embryos. As in the 5′-RACE experiments for *Rnf35*, two successive rounds of PCR were carried out using the *Rnf33*-specific antisense primers CZ45R4 and CZ45R6 (Table 1). A total of 38 5′-RACE clones were obtained and sequenced. The 5′-end of the *Rnf33* mRNA was found to be heterogeneous, dispersing in four tight clusters over a 15-kb region upstream of the intronless *Rnf33* coding sequence as a result of the utilization of four widely spaced alternative promoters designated *Rnf33*-P1 to *Rnf33*-P4 (Fig. 2). Twenty-seven clones were associated with the putative P1 promoter and only three or four clones were mapped at each of the P2–P4 promoters (Fig. 2), suggesting that P1-derived mRNA is the major *Rnf33* transcript in the two-cell embryo. The previously determined mRNA start site in the *Rnf33* cDNA clone (AF290197) corresponds to that of a minor transcript derived from the P4 promoter as defined in this work. Intriguingly, each of the putative *Rnf33*-P1 to *Rnf33*-P4 promoters generates pre-mRNA that carries a short exon 1 of 247 bp or shorter and an intron sequence of variable size in the 5′-UTR. All the splice junctions uncovered conform to the GU-AG rule (Table 2). All the *Rnf33* mRNA species use a common 3′-splice site at nucleotide position 17 421 of the previously derived *Rnf33/35* genomic sequence (AY063497) (Fig. 2).

In the P1-derived mRNA, multiple RNA start sites are further found in the 24 clones examined dispersed over a 41-bp segment; a predominant site (17 clones) is, however, located at nucleotide 14 228 of the *Rnf33/35* genomic sequence (Fig. 3). Furthermore, there are two splice variants of the P1 promoter-derived *Rnf33* mRNA. The major splice variant (P1-sv1), represented by 25 5′-RACE clones, utilizes the 5′-splice site at nucleotide 14 311; the minor splice variant (P1-sv2), represented by only two clones, uses the 5′-splice site located further downstream at nucleotide 14 474 (Figs 2 and 3). Note that the sv2 splice junction is found within a short interspersed nuclear element (SINE) in the genomic sequence (Fig. 3).

Detection of *Rnf33* mRNA with only the P1-derived transcription start site in four- and eight-cell embryos

To further investigate *Rnf33* transcription in the unfertilized egg and in other developmental stages, RT–PCR was next performed using RNA preparations from eggs and different stages of embryos. In the experiments, primers specific for the alternative *Rnf33* mRNA species were used (see Fig. 2). mRNA derived from all four transcription start sites was detected in eggs and in one- and two-cell embryos (Fig. 4). At the four-cell stage, however, only the P1-sv1 mRNA was detected (Fig. 4). All other alternative transcripts were now undetectable. The P1-sv1 mRNA persisted, apparently in a much lower amount, up to the eight-cell stage; it was

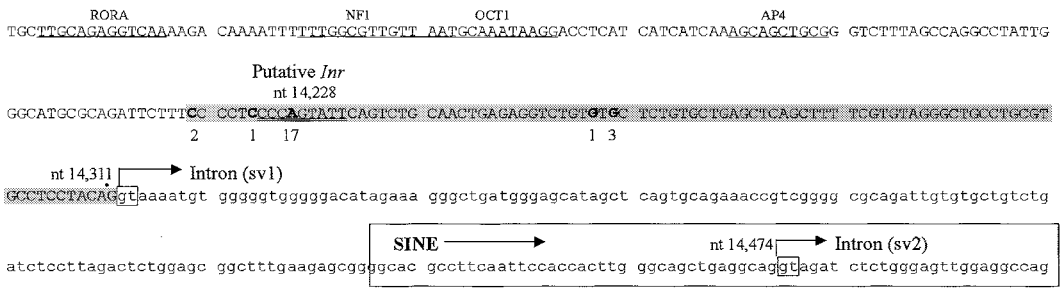

Figure 3. Exon 1 and the upstream sequence of the *Rnf33* gene, based on the *Rnf33/35* genomic sequence (GenBank accession no. AY063497). The exon 1 sequence is shadowed. The mRNA start sites mapped in the 5′-RACE experiments are indicated by bold letters capped with a dot; the number of 5′-RACE clones obtained for each site is shown below the letter. The intron sequence is shown in lower case. The putative *Inr* sequence is double underlined. The 5′ splice variants sv1 and sv2 are indicated; sv2 occurs within a short interspersed nuclear element (SINE). The putative transcription factor binding sites are underlined.

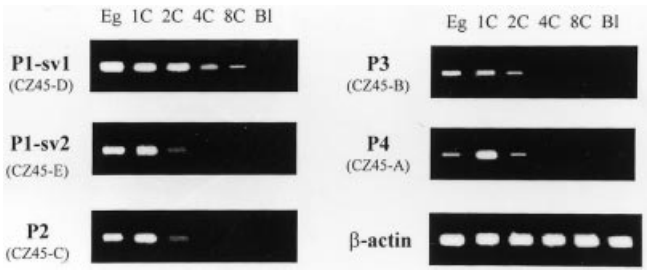

Figure 4. RT–PCR detection of *Rnf33* mRNA transcribed from the putative promoters P1–P4. In the experiments, RNA preparations from eggs (Eg), one-cell to eight-cell (1C–8C) embryos or blastocysts (Bl) were analyzed using the antisense primer CZ45R4 and the sense strand primers CZ45-A–CZ45-E (in parentheses) as shown in Figure 2. The presence of the two splice variants of the P1 transcript (P1-sv1 and P1-sv2) was also tested.

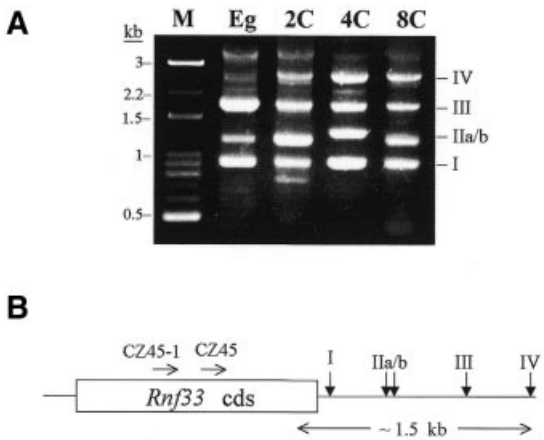

Figure 5. Detection of multiple transcription termination sites in the *Rnf33* mRNA in the egg (Eg) and the zygotes (2C–8C) by 3′-RACE. (**A**) Electrophoretic display of the 3′-RACE products. The band designation is as defined in (B) below and in the text. (**B**) Schematic summary of the 3′-end (approximation only; see Fig. 6 for details). The *Rnf33*-specific primers used in the 3′-RACE are indicated above the coding sequence (cds) depiction.

undetectable in the blastocyst, in agreement with the previously determined *Rnf33* transcription profile (10,11). Further sequencing analysis of nine 5′-RACE clones derived from four- and eight-cell embryos confirmed P1 usage and that in these developmental stages, the mRNA start site at nucleotide 14 228 and the sv1 5′-splice site (see Fig. 3) are exclusively used.

With the exception of *Rnf33*-P3, no TATA boxes are discernible in the sequences upstream of the mapped *Rnf33* transcription start sites. In the P1 region, the sequence CCCA^{+1}GTATT, which overlaps with the major mRNA start site at nucleotide 14 228 (Fig. 3) agrees completely with the consensus core sequence of an *Inr* element (30–32) and may, as in the case of *Rnf35*, be used as the core promoter element for *Rnf33* transcription. Putative binding sites for a number of transcription factors are discernible in the sequence preceding the P1 mRNA start site (Fig. 3), but their involvement in *Rnf33* expression remains to be determined.

The *Rnf33*-P4 promoter is the putative *Rnf35* promoter and may use an *Inr* element (see above). In the *Rnf33*-P2 promoter region, a short sequence with a weak resemblance to the *Inr* core could also be discerned (data not shown). On the other hand, the P3 promoter falls within a viral solitary long terminal repeat (sLTR) (36,37) that we have previously identified that lies between the *Rnf33* and *Rnf35* coding regions (11) (see Fig. 2). A TATA box and a CCAAT box are discernible in the sLTR and are probably used as the P3 promoter in the *Rnf33* transcription (11).

Taken together, our analysis of the *Rnf33* mRNA start sites indicates that *Rnf33* transcription recruits weak and therefore minor promoters in the egg and in the early phases of the formation of the zygotic genome. However, at the four-cell stage, the heterogeneity of transcription initiation site usage is resolved into the use of a single major start site. The lack of a TATA box further suggests that *Rnf33* is transcribed using an *Inr* as a promoter. Another interesting feature of the transcription of the *Rnf33* gene is that an intron is invariably retained in the 5′-UTR of the *Rnf33* pre-mRNA, implying that the solitary intron may be important for *Rnf33* expression.

Indiscriminate transcription termination of the *Rnf33* mRNA

To delineate the 3′-end of the *Rnf33* mRNA, 3′-RACE analysis was performed. The primers used for the nested PCR were CZ45-1 and CZ45, based on the *Rnf33* coding sequence (Table 1 and Fig. 5). The 3′-RACE clones obtained were

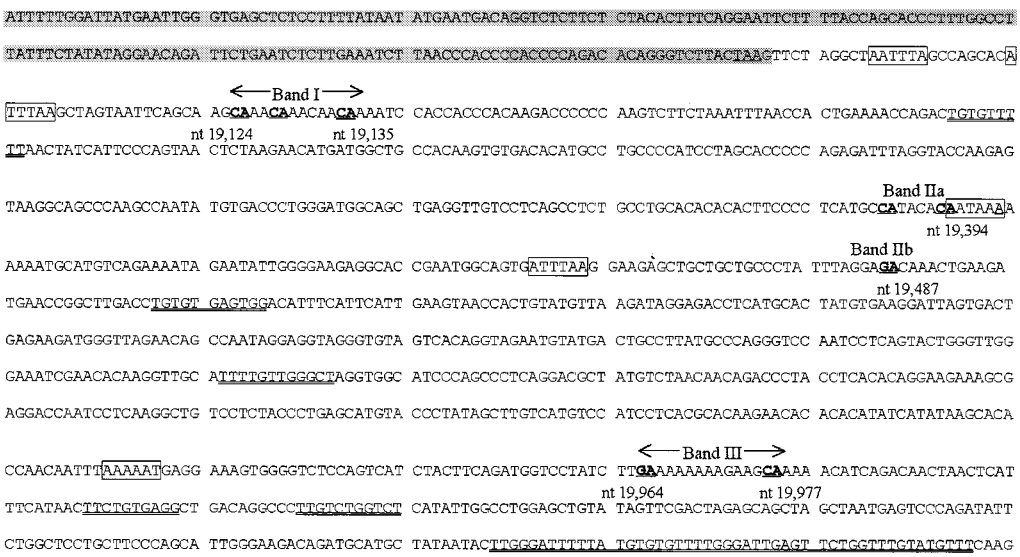

Figure 6. Features of transcription termination of the *Rnf33* mRNA. The coding sequence is shadowed. The CA or GA dinucleotides that form the 3′-ends of the mRNA are indicated by underlined bold letters. Putative AAUAAA-like proxies are boxed; the GU-rich segments that may act as downstream elements (DSE) are underlined. The sequence shown is from nucleotide 18 901 to 20 200 of the *Rnf33*/35 genomic sequence (GenBank accession no. AY063497).

qualified for further analysis by the presence of a poly(A) tract with a minimal length of 20 nt. Surprisingly, in the egg and the embryos tested, four major 3′-RACE products, designated I–IV, were consistently detected, suggesting multiple sites of transcription termination (Fig. 5A). Based on a minor difference in electrophoretic mobility, band II could further be resolved into IIa, found in the egg, the two-cell and the eight-cell embryos, and IIb, which was detected only in the four-cell embryo. In the two-cell embryo, a minor band with an electrophoretic mobility higher than that of band I was also detected (Fig. 5A). Subsequent analysis showed that it was a minor early transcription termination product (data not shown).

To map the 3′-termini, the RT–PCR bands in the egg and the two-cell and the four-cell embryos were excised from the gel and cloned. A total of 70 poly(A)-qualified clones were obtained for sequence analysis. Thirty-two clones generated from band I produced a cluster of three different termination sites mapping 50–60 nt downstream of the coding sequence (Figs 5B and 6). All three termini are mapped at a CA dinucleotide, the most frequently used site for pre-mRNA cleavage and polyadenylation in eukaryotic genes (38,39). Within the band I cluster, the CA at nucleotides 19 124 and 19 135 appear most preferred based on the number of clones (8 and 13, respectively) obtained. Moreover, the nucleotide 19 135 terminus is used exclusively in the four-cell embryo and may constitute a major 3′-end of the *Rnf33* mRNA. In band IIa, two 3′-termini are located at nucleotides 19 388 and 19 394; in band IIb, exclusively found in the four-cell embryo, all six RACE clones obtained are mapped at nucleotide 19 487, at a GA dinucleotide. The 3′-termini derived from band III are mapped at both GA and CA with no obvious preference. Since only four clones were obtained from band IV, the corresponding ends may not be representative and are not included.

Absence of the AAUAAA polyadenylation signal in the 3′-UTR of the *Rnf33* mRNA

A close scrutiny of the sequences associated with the 3′-end of *Rnf33* mRNA further reveals an unusual feature in the 3′ processing of *Rnf33* mRNA (Fig. 6). The highly conserved AAUAAA hexamer, used in a vast majority of genes as an important *cis* element for pre-mRNA cleavage and polyadenylation, is not found in the expected upstream position in all the mapped *Rnf33* mRNA termini, with a single exception. In the 3′-termini mapped at band IIa, an AAUAAA element is found immediately downstream of and overlapping with the mRNA 3′-end mapped at nucleotide 19 394 (Fig. 6). There are no other AAUAAA-like elements upstream of the IIa termini. In all other cases, proxies closely resembling AAUAAA are found upstream of the mapped 3′-termini clusters (Fig. 6). In cluster I, for example, two putative proxies of a polyadenylation signal in the form of AAUUUA and AUUUAA are discernible. Associated with IIb is a putative proxy AUUUAA located 26 nt upstream of the mapped 3′-end. In cluster III the closest resemblance is AAAAAU located 46 bp upstream. However, it remains to be elucidated if any of the suggested hexameric proxies is used in the 3′ processing of the *Rnf33* pre-mRNA in the egg and early embryos. It is further noted that GU-rich stretches are present downstream of each *Rnf33* mRNA termini cluster (double underlined in Fig. 6). Such GU boxes may act as the *cis*-acting DSEs. DSE elements are normally located 20–40 nt downstream of the endonucleolytic site of pre-mRNAs (34,35) and are involved in recruitment of cleavage stimulation factor (CstF) in the protein assemblage for transcription termination processing of pre-mRNA (33).

In summary, our data indicate that the 3′ processing of the *Rnf33* pre-mRNA is inaccurate in the egg and the early zygote, a phenomenon that may be associated with the absence of the AAUAAA *cis* element and the imprecise utilization of

AAUAAA-like proxies. It will be interesting to determine if the differentially terminated *Rnf33* mRNA bears any functional significance.

DISCUSSION

The *Rnf33* and *Rnf35* homologs share a similar genomic structure and transcription profile in the unfertilized egg and in early embryos. The transcripts of the genes are detected in the egg and up to the eight-cell embryonic stage (10,11). Since most maternal mRNAs are degraded by the two-cell stage (40,41), it is most likely that the *Rnf33* and *Rnf35* transcripts found in the embryos are zygotically transcribed. However, at this stage of our study, we cannot rule out absolutely the unlikely possibility that the maternally derived *Rnf33* and *Rnf35* transcripts, in particular the P1-derived *Rnf33* mRNA, are unusually stable and survive up to the eight-cell stage. Experiments designed to directly demonstrate translation of the genes in the egg and in early embryos are also in progress. Nonetheless, the genes provide an opportunity for the elucidation of transcriptional events in the egg and in early development. Despite their similarity and close physical proximity, the genes behave quite differently in transcription and pre-mRNA processing. The upstream *Rnf35* is probably transcribed using an *Inr* element as its core promoter and transcription is terminated at a single site (Fig. 1). On the other hand, transcription and processing of the downstream *Rnf33* gene is highly complex. We found that in the egg and in the early zygote, the *Rnf33* transcripts include a major mRNA species with the proximal P1 start site and three minor mRNA species derived from other distal promoters. At the four-cell stage, however, transcription initiation occurs with high fidelity with the use of only the major P1 promoter, a single start site at nucleotide 14 228 and a single splicing event. These observations strongly suggest that the *Rnf33*-P2–*Rnf33*-P4 promoters may be fortuitously activated (7), including utilization of the promoter of a sLTR (36,37).

The use of TATA-less promoters by the genes is consistent with the previous finding that TATA dependency is developmentally acquired and that TATA-less promoters are preferably utilized in the pre-implantation development of the mouse embryo (8,9). The *Rnf33* and *Rnf35* mRNAs carry multiple initiation sites, a phenomenon previously linked to the transcription of other TATA-less genes (42–44). In the upstream sequences of both the *Rnf33*-P1 and the *Rnf35* mRNA start sites, we have identified a transcription initiator (*Inr*) element that may be used as a core promoter in the egg and possibly in early embryos (45,46), in line with the fact that the ubiquitous Sp1 transcription factor has a strong preference for *Inr* elements (47,48). Sp1 has been shown to be associated with oocyte maturation and the development of early pre-implantation embryos in the mouse (49–51). It is possible that Sp1, or other early embryonic Sp1-like transcription factors, act to regulate *Inr* in early embryo development.

We notice that other genes that are intronless in the coding region share a similar genomic organization with *Rnf33* and *Rnf35* in possessing a short exon 1 and a solitary intron in the 5′-UTR. These genes include the *Xenopus* XFDL 141 and 156 genes and mouse *eIF-1A*, *Hsp70.1*, *Zfp352* and *2cpoz56* (10,27,52–54; K.-B.Choo, unpublished data). In a series of dissertations, Kozak (55,56) has postulated that the primary sequence and the secondary structure of exon 1 are important in translation initiation. Matsumoto *et al.* (57) have elegantly and unequivocally demonstrated that a 5′ intron preceding a coding sequence is required for efficient translation and that the nuclear history of pre-mRNA processing is critical in fulfilling such a requirement. The essentiality of 5′-UTR introns in significantly improving translation efficiencies has indeed been demonstrated in many reports involving expression of transgenes in both animals and plants (58–60). Taken together, we propose here that the naturally occurring exon 1 and the solitary 5′ intron found in *Rnf33*, *Rnf35* and other genes serve to regulate and to couple transcription to the translation machinery (61–63).

Transcription termination of *Rnf35* is simple in nature following well characterized structural features, including the use of the conserved AATAAA polyadenylation signal and utilization of the putative USE and DSE (Fig. 1C). On the other hand, transcription of the *Rnf33* gene in early zygotes gives rise to a heterogeneous population of polyadenylated mRNA displaying different lengths of the 3′-UTR region. The heterogeneity is partially resolved at the four-cell stage when selected 3′-ends are now used (Fig. 6). Since the highly conserved AAUAAA *cis*-acting element used for polyadenylation in most genes (39,64) is absent in the 3′-UTR of the *Rnf33* mRNA, the multiple 3′-ends observed may be a consequence of the use of unconventional hexameric proxies as in the transcription of the murine dihydrofolate reductase, the avian histone and the human D-type cyclin genes (65–67). In a bioinformatics-based survey carried out by Gautheret *et al.* (64), ~30% of mRNA in the 3′ EST clusters of the human genes analyzed does not contain AAUAAA or AUUAAA. In yeast, the AAUAAA sequence is also not obligatory (68). Despite the absence of the AAUAAA element, polyadenylation of the *Rnf33* mRNA occurs mostly 3′ to the CA dinucleotide, as is found for most genes (38,39). The GA dinucleotide is also a preferred site of polyadenylation. The fact that the *Rnf33* 3′-UTR sequence is heavily mined with CA and GA dinucleotides (Fig. 5) may be a structural feature that has contributed to the mischievous choices of polyadenylation site observed. It is also noted that numerous GU-rich segments could have been present in the 3′-UTR of *Rnf33* pre-mRNA that could further contribute to inaccurate endonucleolytic cleavage of the pre-mRNA.

The biological significance of the multiple 3′-UTRs in the *Rnf33* transcripts is open to speculation. One possibility is that these mRNAs have different stabilities (33). The translation of many maternal mRNAs is delayed (69,70) and is activated by cytoplasmic polyadenylation, a process that involves interactions between regulatory proteins and sequence elements such as the cytoplasmic polyadenylation element located in the 3′-UTR of targeted mRNAs (70,71). The different 3′-UTRs in the *Rnf33* transcripts thus offer ample opportunity for differential regulation of *Rnf33* expression at the translation level.

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