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Control of mouse U1 snRNA gene expression during *in vitro* differentiation of mouse embryonic stem cells

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ABSTRACT

Early in mouse development, two classes of U1 RNAs, mU1a and mU1b, are synthesized, but as development proceeds, transcription of the embryo-specific mU1b genes is selectively down-regulated to a barely detectable level. We show here that during *in vitro* differentiation of mouse embryonic stem (ES) cells, both exogenously introduced and endogenous U1b genes are subject to normal developmental regulation. Thus, ES cells represent a convenient isogenic system for studying the control of expression of developmentally regulated snRNA genes. Using this system, we have identified a region in the proximal 5′ flanking region, located outside the PSE element, that is responsible for differential transcription of the mU1a and mU1b genes in both developing cells and transiently transfected NIH 3T3 cells.

INTRODUCTION

U1 small nuclear (sn)RNA, which functions in the processing of pre-messenger RNAs (1,2), is synthesized in all cell types and at all stages of development (3,4). Frogs (5,6), mice (7,8), and several other organisms (9–11) have at least two families of U1 RNA genes. In mice, the constitutively expressed mU1a RNAs differ slightly in sequence from mU1b RNAs (7,8), which are expressed only in embryos and in adult tissues containing a high proportion of undifferentiated stem cells (7). The significance of the two types of RNAs is unclear, but the RNAs have different affinities for some snRNP proteins (12).

During mouse embryonic development, mU1b RNAs begin to accumulate at the 2–8 cell stage, reaching a level comparable with that of the constitutively expressed mU1a RNAs in blastocysts (13). As development proceeds, the level of mU1b RNA in differentiated tissues gradually decreases and is barely detectable at birth (7). Expression of mU1b RNA in many lines of cultured cells (7,8) apparently reflects the state of differentiation of the originating tissue. For example, mU1b RNA is present at a high level in pluripotent embryonic stem (ES) cells and embryonic carcinoma cells, but is almost absent in contact-inhibited, non-transformed fibroblasts (7). We have previously shown that the stage-specific accumulation of mU1b RNA during development is mediated by control of transcription rather than selective destruction of the RNA (14).

Mouse ES cells, derived from the inner mass of blastocysts (15,16), can undergo an elaborate *in vitro* differentiation (17) that resembles early embryogenesis up to the egg cylinder stage. During this process, many stage-specific genes, such as the cardiac myosin genes (18), exhibit correct onset of transcription. Since ES cells can be readily transfected with cloned DNA without losing their pluripotency (19), they provide a convenient isogenic system for studying the functions of different promoter regions of developmentally controlled genes (20).

Promoters of mU1a and mU1b genes, like other vertebrate snRNA genes transcribed by RNA polymerase II, consist of two *cis*-acting elements: an upstream enhancer DSE (distal sequence element) and an essential PSE (proximal sequence element). Both of these snRNA gene-specific elements are highly conserved in sequence and in distance from the site of transcription initiation (reviewed in 4,21,22). The DSE enhancer (23–25), located ~200–250 bp upstream of the initiation site, contains an octamer motif, the binding site for the Oct-1 factor (26) and, in some cases, binding sites for additional transcriptional activators (27–29). The PSE, encompassing a 10 bp core sequence around position –55 and a purine-rich region immediately downstream, interacts with the snRNA-activating complex (SNAPc) (30,31) and determines the site of transcription initiation (25,32–34).

We asked if the same regulatory elements are responsible for the differential expression of mU1a and mU1b genes in developing embryoid bodies and in fully differentiated fibroblast cells. We report that promoter sequences, located within the proximal 5′ flanking region but outside the PSE element, are responsible for the down-regulation of introduced U1b genes in both systems.

MATERIALS AND METHODS

Plasmid constructs and DNA templates

All U1 gene constructs were cloned between the *Kpn*I and *Bam*HI sites in the polylinker region of the pUC118 vector (35). For plasmids used in stable transfection, a neomycin/G418 resistance gene from pCGBPV9 (36) was inserted between the *Bam*HI and

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*Hin*dIII sites (cf. Fig. 5B). The mouse mU1a1, mU1b2 and mU1b6 gene sequences were derived from clones pU1a1-214, pU1b-136 and pU1b-453 respectively (37).

To generate mU1a1·800 and mU1b2·3000 reporter genes (cf. Fig. 1), a mini-U1 gene was first created, in which the U1 coding sequence from position +27 (the *Bcl*I site) to +150 were deleted and the remaining 15 bp of the coding region plus the downstream 3′-end signal were replaced by a PCR product of the corresponding sequence in the human hU1-1 gene (38). This PCR fragment (amplified by oligonucleotide primers 5′-GTA TGA TCA TCG CGC TTT CCC CTG ACT T-3′ and 5′-GTC CTG TGG ATC CGG TTA G-3′) had a *Bcl*I site attached at its 5′-end, thus restoring this unique restriction enzyme recognition site. A 142 bp DNA fragment of bacteriophage λ (between 5647 and 5505) was then inserted at the *Bcl*I site of the mini-U1 gene, to generate a template that would give rise to a 189 nt λ RNA transcript. mU1a1·800* and mU1b6·2600* were constructed in the same way except for the insertion of a different DNA fragment of bacteriophage λ (between 10 892 and 11 033).

To generate deletion mutants in the 5′ flanking regions of mU1a1·800 and mU1b2·3000 reporter genes (cf. Fig. 1), the DNAs were cleaved with *Fok*I (mU1a1·412), *Dde*I (mU1a·125), *Ssp*I (mU1b2·861), *Pvu*II (mU1b2·396) or *Sac*II (mU1b2·150) and treated with the large Klenow fragment of DNA polymerase to create blunt ends. After redigestion with *Bam*HI, the truncated reporter gene fragments were isolated and recloned into the pUC118 vector. The A/B chimeric promoter was created by replacing the *Dra*I (at –116)–*Bam*HI fragment of the mU1a1·412 gene with a PCR fragment of the corresponding region of the mU1b2·396 gene (amplified with the internal primer U1b-D, 5′-GCA GAG CGC AAG AAT GTC G-3′, and the M13 forward sequencing primer that binds downstream vector sequences). To make the B/A chimeric promoter, a segment of the 5′ flanking sequence of the mU1a1·412 gene from the *Dra*I site to a unique *Kpn*I site in the polylinker region of vector pUC118 was replaced with another PCR fragment of the mU1b2·396 gene from position –117 to the same *Kpn*I site in pUC118 (amplified with the M13 reverse sequencing primer and the internal primer U1b-U, 5′-TGA CAC CCG CGT ATC CGC-3′). The mU1b2·$(PSE)_4$ mutant was created by PCR-based site-directed mutagenesis (39) using a mutagenic oligo 5′-GGG CTA ATT TAC CGT AAC TAT GAA ATG TAG AGG CGA CGG G-3′, in which the underlined sequences show the altered nucleotides of the PSE.

DNA templates for synthesizing RNA probes used in the detection of λ reporter RNA (probes A189 and B189 in Fig. 1) were generated by PCR amplification of DNA sequences extending from the 3′-end of the coding region (+189) to position –125 or –150 in the 5′ flanking regions of the mU1a1 or mU1b2 promoters respectively. The resulting two fragments were cloned into the pGEM-2 vector (Promega), so that the antisense RNA probes were transcribed from the T7 promoter. To generate a DNA template for the antisense mU1b2 RNA probe, the RNA coding region of clone pU1b-136 (37) was attached in reverse orientation to the T7 promoter of plasmid pGEM-2 at the *Hin*dIII site. This ligated DNA was amplified by PCR, using the U1b-D oligo and an oligo complementary to the T7 promoter.

Cell culture and transfection

NIH 3T3 cells were maintained in $DMEM^{HG}$ (Gibco), supplemented with 10% bovine calf serum (Hyclone). The mouse ES cells (E14TG2a) (40) were grown in $DMEM^{HG}$, 1× glutamine, 15% fetal bovine serum (Gibco) on a layer of mitotically inactivated feeder cells (STO cells) (19). Approximately $2–5 \times 10^6$ ES cells were added to each feeder dish, in which they normally grew in compact patches (Fig. 4A).

Transient transfection of NIH 3T3 cells was carried out with LipofectAmine™ (Gibco) according to the manufacturer's instructions. Each test DNA construct, mixed with 0.5 µg plasmid πSVHPα2 carrying the α-globin gene (34) was combined with LipofectAmine (at a fixed ratio of 1 µg DNA to 5–6 µl reagent) and applied to ~10^5 cells. After incubation in serum-free medium for 4 h the complete medium was added and the transfected cells were harvested 24 h later for RNA analysis (see below).

To generate stably transfected ES cell lines, ~2.5×10^6 undifferentiated ES cells were mixed with 10 µg DNA in 250 µl culturing medium, incubated at room temperature for 15 min and electroporated using a Gene Pulser (BioRad) at 250 V, 250 µF. These cells were plated into 10 cm Petri dishes coated with neo^r STO feeder cells (19) and after a 2–3 day recovery period, selective medium containing 0.25 mg/ml G418 was added. For each DNA, ~12 colonies of G418-resistant cells were isolated and expanded into individual lines.

Differentiation of ES cells *in vitro*

Pluripotent ES cells (E14TG2a) were induced to differentiate *in vitro* by culturing in suspension in the absence of STO feeder cells. Samples of $2–3 \times 10^7$ cells grown to confluency in a 60 mm dish were mildly trypsinized and dispersed by repeated pipetting before being evenly spread onto a small bacterial Petri dish. After 2 h incubation, when most feeder cells, but not the ES cells, were firmly attached, the ES cells were recovered, transferred to a siliconized glass Petri dish (at 0.5×10^6 cells/ml) and cultured for several weeks to generate embryoid bodies (Fig. 4A).

Metabolic labeling and immunoprecipitation

Embryoid bodies from various days of culturing were collected and allowed to attach on the plastic surface of 6-well microtiter plates. Each sample was incubated with 150 µCi [^{32}P]orthophosphate in 1 ml low phosphate medium (Gibco) supplemented with 10% dialyzed fetal bovine serum (FBS) for 12–16 h. The labeled embryoid bodies were then trypsinized, washed and resuspended in 400 µl IPP-500 buffer (10 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.1% NP-40 and 0.1% NaN_3) and whole cell extracts were prepared by sonication. The snRNPs present in the clarified cell extracts were immunoprecipitated using polyclonal anti-Sm antibodies (41) coupled to protein A–Sepharose beads (Sigma). The precipitated snRNAs were recovered from these beads by proteinase K treatment and phenol extraction. The U1a and U1b RNAs were separated on a 12% (30:0.8) polyacrylamide–7 M urea gel running under partially denaturing conditions (14 V/cm) at room temperature for 18 h.

RNase protection assay

Total nucleic acids of either transiently or stably transfected cells were isolated by the rapid urea lysis method (42). Typically, 1.5–5 $\times 10^6$ cells from a confluently grown 6 cm dish were lysed with 4 ml urea lysis buffer containing 7 M urea, 1% SDS, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA at room, temperature for 5–10 min. After extraction with an equal volume of phenol:chloroform (1:1), total

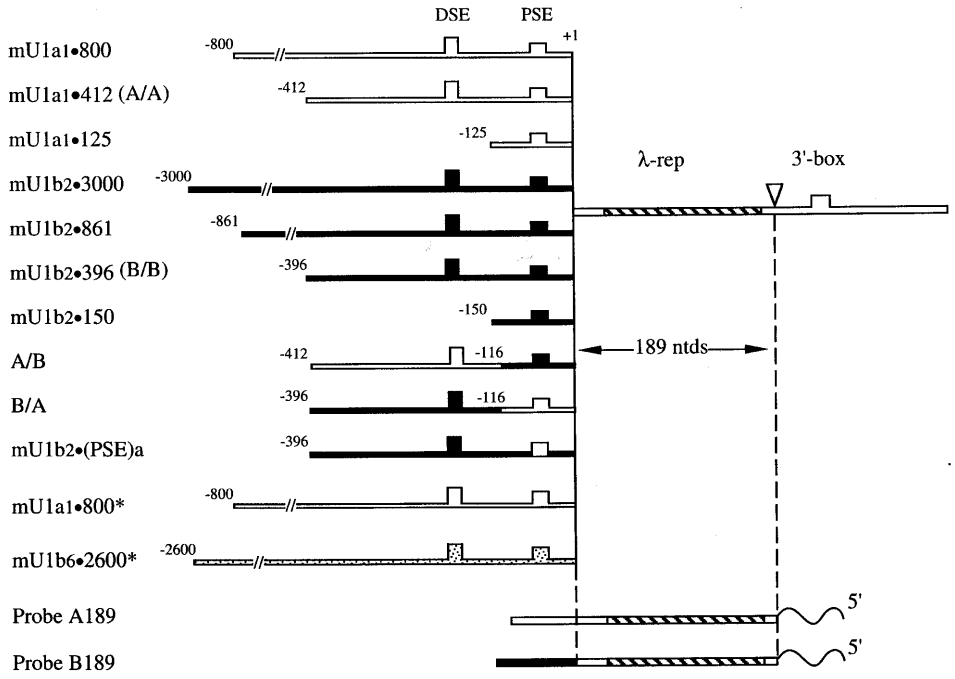

Figure 1. Schematic representation of U1 reporter constructs. The 5′ flanking sequences of mU1a1, mU1b2 and mU1b6 genes are indicated by open, filled and spotted bars respectively. The hatched zone in the coding region shows the inserted sequences derived from bacteriophage λ, whereas the inverted triangle indicates the site corresponding to the 3′-end of the transcript, directed by the U1 3′-end box; at least 60% of transcripts initiated from the +1 start sites formed correct 3′-ends in response to this 3′-end signal (data not shown). A different λ DNA fragment was inserted in the coding regions of the mU1a1·800* and mU1b6·2600* constructs. The wavy lines in the antisense RNA probes (A189 and B189) indicate sequences derived from the pGEM-2 plasmid vector.

nucleic acid was precipitated with 2 vol cold ethanol at –20°C. The RNA samples for RNase protection assays were prepared by treatment with RQ DNase I (Promega).

Antisense RNA probes for detection of λ reporter RNA and α-globin RNA were generated by *in vitro* transcription with T7 RNA polymerase (Promega) in a 10 µl reaction containing 250 µM each of ATP, GTP and CTP plus 20 µM UTP and 40 µCi [α-^{32}P]UTP (>800 Ci/mmol prior to dilution) and purified by gel electrophoresis. The antisense mU1b2 RNA probe was labeled under similar conditions except that 250 µM UTP and only 1.25 µCi [α-^{32}P]UTP were used. To quantitate the λ reporter RNA and the α-globin RNA in transiently transfected cells by the RNase protection assay (35), one tenth of the total RNA isolated from cells in a confluent 6 cm dish was mixed with ~15 fmol radioactively labeled antisense RNA probes. After hybridization at 45°C for 12–18 h, the mixture was treated with 66 µg/ml pancreatic RNase A at 30°C for 1 h. The protected probe fragments were separated on a 6% (30:0.8) polyacrylamide gel containing 7 M urea. To quantitate the λ reporter RNA in differentiated embryoid bodies, total RNA isolated from roughly 5×10^4 cells was used.

RESULTS

Differential transcription of mU1a and mU1b genes in transiently transfected NIH 3T3 cells

We previously showed that transcription of the embryonic mU1b gene (mU1b2) was very low in mouse NIH 3T3 cells, a fibroblast cell line that normally accumulates only the adult U1a RNA (7). That is true both for the endogenous genes and for stably transfected mU1b2 genes containing a 3 kb 5′ flanking region (14). To identify the sequences that are sufficient to control the stage- and tissue-specific expression of a reporter U1b gene in transgenic mice (14), we transfected various deletion constructs into cells that were at different stages of development.

We first asked if differential transcription of mU1a and mU1b genes could be observed during transient transfection. Three U1 reporter constructs were used, in which the 5′ flanking regions of the mU1b2 or mU1a1 genes were subcloned in front of a coding region composed primarily of a DNA sequence derived from bacteriophage λ (Fig. 1). Expression of the λ reporter RNA in transfected cells was analyzed by an RNase protection assay using antisense RNA probes with complementary sequences extending into the 5′ flanking regions of the two genes (Fig. 1, probes A189 and B189). In this assay, all transcripts initiated at position +1 protected a 189 nt piece of probe RNA, which was well separated by electrophoresis from the longer probe fragments that were protected by transcripts initiated at upstream sites (Fig. 2A, lane 13). Since the λ reporter RNA was unstable in these cells, (34,43), the steady-state level of the reporter RNA was proportional to the promoter activities.

Transient transfection of NIH 3T3 cells with the mU1a1 construct resulted in efficient expression of the λ reporter RNA (Fig. 2A, lanes 4–8, and B, lane 1). However, the levels of reporter RNA made from the mU1b2 (Fig. 2A, lanes 9–13) or the mU1b6 promoters (Fig. 2B, lane 2) were much lower, in keeping with our previous results obtained with cells stably transfected with snRNA genes encoding complete U1 RNAs (14). After normalization for transfection efficiency using an α-globin RNA made from a

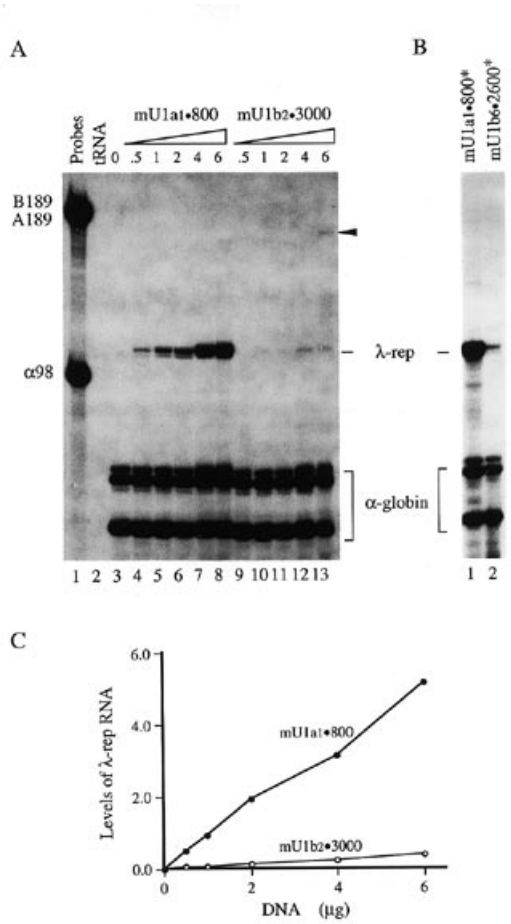

Figure 2. Promoter activities of U1 reporter constructs in NIH 3T3 cells. (**A**) Transient transfection of NIH 3T3 cells with increasing amounts of mU1a1·800 (lanes 4–8) or mU1b2·3000 (lanes 9–13) DNAs along with a plasmid carrying an α-globin gene as an internal control. The levels of λ reporter RNA expressed in transfected cells were analyzed by RNase protection assay. Lanes 1 and 2 show one tenth of the input antisense RNA probes and the background protection by 30 μg added carrier RNA respectively; lane 3 shows transfection with the α-globin control plasmid alone. The arrowhead indicates transcripts initiated from upstream cryptic start sites. (**B**) Transfection with 4 μg mU1a1·800* (lane 1) and mU1b6·2600* (lane 2) constructs. (**C**) Quantitation of λ reporter RNAs in (A). The level of each λ reporter RNA was quantitated by phosphorimager (Molecular Dynamics) analysis and adjusted for transfection efficiency according to the level of co-expressed α-globin RNAs.

co-transfected plasmid (34), the mU1a1 and mU1b2 promoters were found to differ by 10- to 12-fold in activity (Fig. 2C).

The difference in transcription levels of these two templates was specific for the type of cell used. Although the mU1b2 promoter was very inefficient in NIH 3T3 cells (Fig. 2), its activity was comparable with that of the mU1a1 promoter after introduction into undifferentiated ES cells (see below, Fig. 6 and data not shown), which express high levels of endogenous U1b RNAs (7). Thus, the mU1b promoters used here were not defective and the levels of expression of the mU1a and mU1b genes introduced into NIH 3T3 cells were regulated as the result of cell differentiation. We therefore used these transfected cells to probe sequences that might function in control of U1 promoter activity during normal development.

Sequences responsible for the low promoter activities of mouse mU1b genes

To identify *cis*-acting sequences that might regulate transcription from the mU1b2 promoter, we compared the promoter activities of various deletion mutants (Fig. 1) in transiently transfected NIH 3T3 cells (Fig. 3; see Table 1 for quantitation). Deletion of the distal (upstream) 2 kb from mU1b2 promoter resulted in an ~3-fold increase in the level of λ reporter RNA (Fig. 3A, compare lanes 4 and 5), indicating that sequences in the far upstream region reduced the level of expression. However, the resulting construct mU1b2·861 was still much less active than the comparable mU1a1 construct (Fig. 3A, compare lanes 1 and 5) and it retained developmentally regulated elements (see below, Fig. 6), suggesting that sequences closer to the coding region were also responsible for the differences in expression of the two classes of genes. Further deletion of ~400 bp from 5′ flanking regions of either the mU1b2·861 or mU1a1·800 promoters caused little change in the levels of transcription (compare lanes 1 and 2 or lanes 5 and 6). Thus, the difference in promoter activities of mU1a1 and mU1b2 genes can be accounted for by elements in the far upstream region and within the proximal 400 bp 5′ flanking sequences. Because the major snRNA promoter elements are located within 250 bp from the transcriptional start site, we concentrated our analysis on the proximal sequences.

Table 1. Promoter activities of U1 genes in transiently transfected NIH 3T3 cells

U1 promoters	Relative levels of λ reporter RNA[a]
mU1a1·412	1.00
mU1a1·800	0.76
mU1a1·125	<0.02
mU1b2·3000	0.06
mU1b2·861	0.22
mU1b2·392	0.23
mU1b2·150	<0.02
B/A	0.92
A/B	0.31
mU1b2·396(PSE)$_a$	0.34

[a]The relative level of each λ reporter RNA in Figure 3A was normalized to that of the mU1a1·412 construct after being adjusted for transfection efficiency according to the level of co-expressed α-globin RNA.

Inspection of the mU1a1 and mU1b2 promoter sequences identified consensus DSE and PSE core elements at positions that are conserved in vertebrate snRNA genes (37). As expected, deletion of the DSE regions largely abolished transcription from both mU1a1 and mU1b2 constructs (Fig. 3A, lanes 3 and 7). To determine if the regulatory sequences could be delimited to either the DSE or PSE regions, we created two chimeric constructs by exchanging the distal segments between the mU1a1 and mU1b2 promoters at position –116 (Fig. 1, A/B and B/A). Similar levels of reporter RNA were made from B/A and mU1a1 (A/A) constructs (Fig. 3A, lanes 8 and 9) or from A/B and mU1b2 (B/B) constructs (lanes 11 and 10). Thus, the promoter activities of mouse U1 genes are determined primarily by sequences within

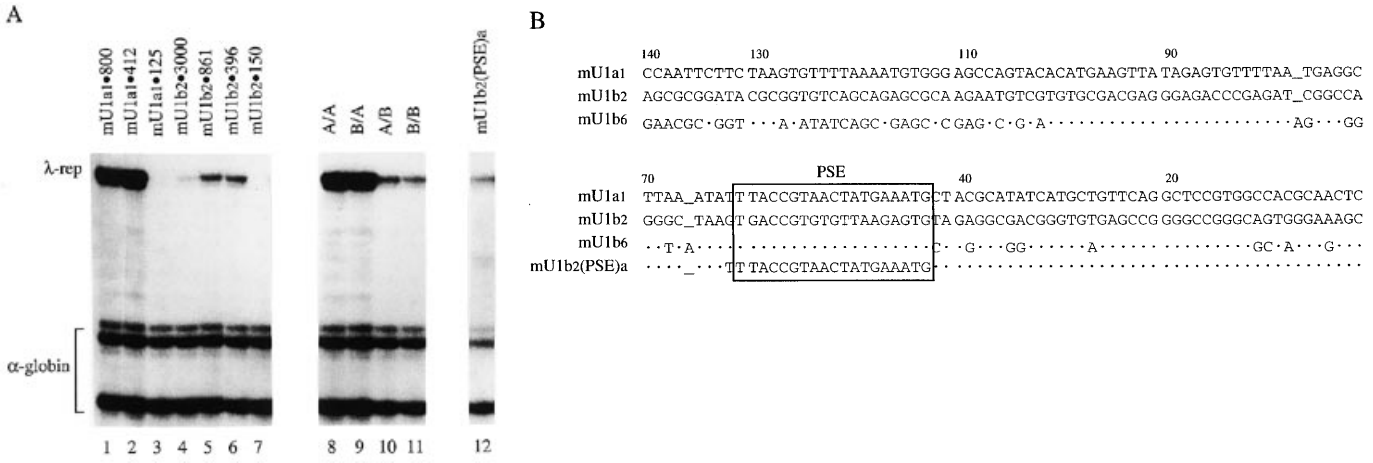

Figure 3. Mutational analysis of the 5′ flanking sequences of mU1a1 or mU1b2 genes. (**A**) RNase protection assay of the λ reporter RNA in NIH 3T3 cells transiently transfected with 4 µg each test DNA construct. Lanes 1, 6, 8 and 11 were loaded with identical RNA samples. The RNAs in lane 12 were from a separate experiment, in which controls were included (but not shown here). The results of quantitation by the phosphorimager are shown in Table 1. (**B**) Alignment of the proximal promoter sequences of mouse U1 genes. Dots represent nucleotides that are identical among different U1b genes. The boxed regions show the conserved PSE elements. The altered sequences in the mU1b2·$(PSE)_a$ mutant construct are shown.

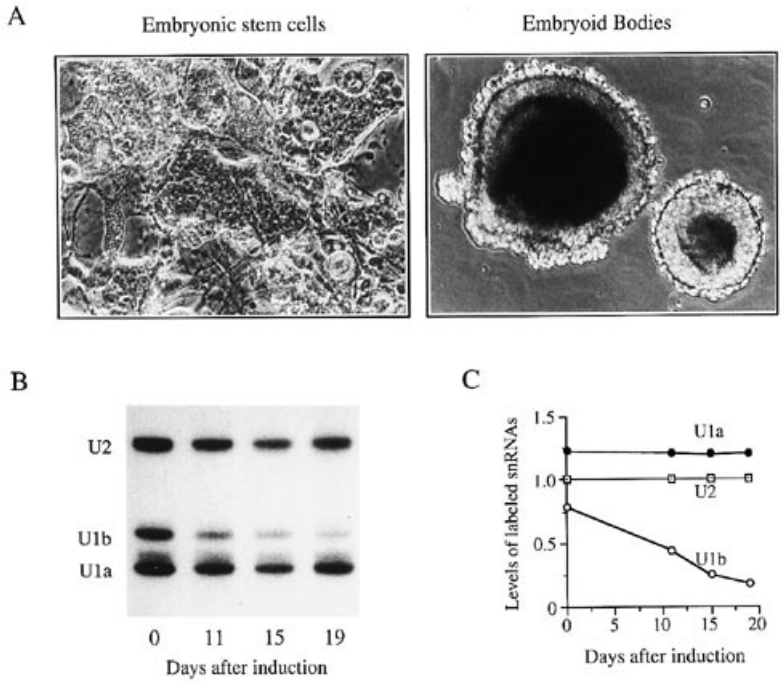

Figure 4. Expression of endogenous U1 RNAs during *in vitro* differentiation of embryonic stem (ES) cells. (**A**) Formation of embryoid bodies (EBs). The undifferentiated ES cells (E14TG2a) appear as densely grown colonies on the mitotically inactivated STO feeder cells (left). Cystic embryoid bodies at 14 days post-differentiation are shown on the right. Phase contrast microscope, ×180. (**B**) Synthesis of endogenous snRNAs in EBs as monitored by metabolic labeling with [^{32}P]orthophosphate. The labeled snRNAs were immunoprecipitated by anti-Sm antibodies from whole cell extracts and analyzed on a 12% partially denaturing gel under conditions that separate the mU1b and mU1a RNAs (7). (**C**) Quantitation of the labeled snRNAs. The autoradiograph of (B) was scanned and analyzed by Scan Analysis software. The levels of mU1a or mU1b RNAs were normalized to that of the U2 RNA and plotted against time after induction of *in vitro* differentiation.

116 bp from the transcription start site, regardless of differences in their DSE enhancers.

Different members of the mouse mU1b gene family are ~90% identical in sequence in the first 100 bp upstream of the site of transcription initiation (37). In contrast, the mU1a and mU1b genes share no extended sequence homology within this region except for the moderately conserved PSE elements (Fig. 3B). Variations in the PSE were shown to affect the promoter activities of snRNA genes tested *in vitro* (30), but the influence of neighboring sequences was not excluded. To distinguish between

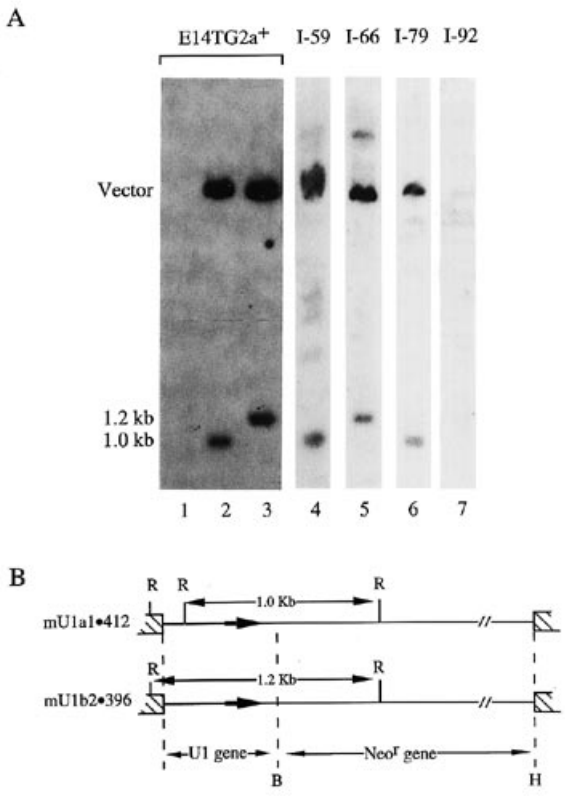

Figure 5. Southern blot analysis of transfected DNAs in various ES cell lines. (**A**) Genomic DNAs from untransfected E14TG2a cells (lanes 1–3) or stably transfected I-59 (mU1a1·412, lane 4), I-66 (mU1b2·396, lane 5), I-79 (A/B, lane 6) and I-92 (B/A, lane 7) ES cells were digested with *Eco*RI and analyzed on a 1% agarose gel. After transfer to a nylon membrane, these DNAs were probed for sequences of the Neo^r resistance gene. 100 pg *Eco*RI-digested plasmid DNAs carrying the mU1a1·412 or mU1b2·396 constructs were added to lanes 2 and 3 respectively. (**B**) Restriction maps of the relevant regions of mU1a1·412 and mU1b2·396 plasmid constructs. R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

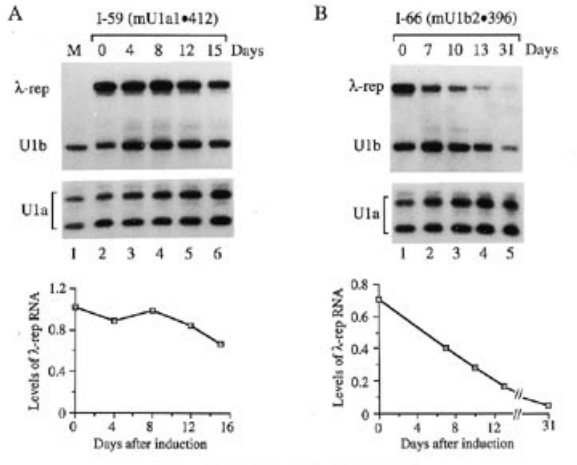

Figure 6. Promoter activites of mU1a1·412 and mU1b2·396 constructs in embryoid bodies. Total RNAs were prepared from I-59 (**A**) and I-66 (**B**) cells at the indicated days after induction of *in vitro* differentiation and used for the quantitation of λ reporter RNA by the RNase protection assay. As an internal standard for the adjustment of cell number, the endogenous mU1a RNAs were quantitated using an antisense RNA probe derived from sequences complementary to the mU1b2 RNA (lane 1 in A). This probe allows for the separation of mU1a and mU1b RNAs due to the sequence variations between the RNAs. Because U1 RNA is stable, the decrease in the level of total accumulated mU1b RNA is less obvious than the change in the rate of synthesis of new RNA (cf. Fig. 4B). The graph at the bottom of each panel was generated by plotting the normalized levels of λ reporter RNA against time.

the roles of PSE elements and surrounding sequences in controlling the activities of U1 promoters, we exchanged the 20 nt containing the PSE of the mU1b2 promoter, from position –62 to –43, with the corresponding sequence derived from the mU1a1 promoter [Fig. 1, mU1b2·(PSE)$_a$]. This substitution did not change the level of mU1b2 promoter activity (Fig. 3A, lane 12), showing that sequences outside the PSE, but within the proximal 116 bp, are responsible for the differential efficiencies of transcription of mU1a1 and mU1b2 genes.

Synthesis of endogenous U1 RNAs in mouse embryonic stem cells differentiated *in vitro*

We asked if the proximal promoter sequences responsible for the differential transcription of mU1a and mU1b genes in NIH 3T3 cells could also account for down-regulation of the U1b genes during early development. Since expression of many stage-specific genes shows correct onset during *in vitro* differentiation of mouse ES cells (18,20), we used this system to analyze the developmental control of U1 RNA accumulation. Undifferentiated, pluripotent ES cells (E14TG2a; 40) were induced to differentiate *in vitro* by being dispersed into a suspension of single cells which were then allowed to form aggregates. Over a 2 week period of culturing, these ES aggregates developed into highly structured embryoid bodies (EBs) (Fig. 4A), which in many ways resembled 8–10 day old mouse embryos at the egg cylinder stage (17).

To detect changes in the expression of endogenous U1 RNA genes, we monitored the synthesis of snRNAs by direct metabolic labeling with [^{32}P]orthophosphate. After preparation of whole cell extracts from labeled embryoid bodies at different stages of differentiation, the newly made snRNAs were isolated by immunoprecipitation with anti-Sm antibody and analyzed by electrophoresis under partially denaturing conditions that allow for the separation of embryonic mU1b and adult mU1a RNAs (7). As ES cells differentiated, the relative levels of labeled mU1b RNA gradually diminished (Fig. 4B), as compared with those of U2 RNA used as an internal standard for normalization (Fig. 4C). In contrast, the levels of labeled mU1a RNA remained constant. Thus, accumulation of embryonic mU1b RNA was down-regulated during *in vitro* differentiation of ES cells, as has been observed during normal embryonic development *in vivo* (7). Since these embryoid bodies are composed of a heterologous population of cells that have differentiated to various extents (43), we cannot determine whether the 3- to 4-fold reduction in the expression of endogenous mU1b RNAs resulted from complete inactivation of mU1b genes in a subpopulation of cells or from partial inactivation in all cells. In either case, the ES cells provide a reliable system for studying the mechanism of inactivation of embryonic mU1b genes during early development.

Sequences responsible for inactivation of mU1b genes during development

We next tested if exogenous U1 genes were subject to control like that observed for endogenous mU1b genes during *in vitro*

differentiation of ES cells. The λ reporter constructs (with 400 bp of 5′ flanking sequences from either the mU1a1 or mU1b2 genes) were stably introduced into E14TG2a cells and a dozen independent colonies from each transfection were isolated and subsequently expanded into individual cell lines. Among these ES cell lines, I-59 and I-66, which received mU1a1·412 and mU1b2·396 constructs respectively, expressed the highest levels of λ reporter RNA (data not shown). Comparable amounts of plasmid DNAs were integrated into the chromosomes of these cells, as shown by Southern blot analysis (Fig. 5, lanes 4 and 5). These two cell lines were selected for further analysis of the expression of transfected U1 genes during *in vitro* differentiation.

Prior to differentiation of I-59 and I-66 cells, the activities of exogenous mU1a1 and mU1b2 promoters were comparable, as shown by the levels of λ reporter RNA relative to that of endogenous mU1a RNA (Fig. 6A, lane 2, and B, lane 1). However, when these stably transfected ES cells were induced to differentiate by culturing in suspension, the relative levels of λ reporter RNA progressively decreased in I-66 cells, which contained the mU1b2·396 construct (Fig.6B), but they remained within ~20% of each other in I-59 cells, which contain the mU1a1·412 construct (Fig.6A). Thus, transcription of exogenous mU1b genes was specifically down-regulated during *in vitro* differentiation of embryoid bodies.

To test if the conserved 116 bp proximal 5′ flanking region around the PSE was responsible for down-regulation of the mU1b2 gene in differentiated ES cells, we generated two other stably transfected ES cell lines, I-79 and I-92, carrying the chimeric U1 constructs A/B and B/A respectively. When these two cell lines were induced to differentiate, only the I-79 cells showed changes in the expression of λ reporter RNA (Fig. 7), indicating that activity of the A/B chimeric promoter decreased as differentiation proceeded. Southern blot analysis showed that the amount of integrated plasmid DNA in the I-79 cells was comparable with that in the I-59 and I-66 cells (Fig. 5, compare lane 6 with 4 and 5), but it was much lower in I-92 cells (lane 7). This reduced amount of DNA accounted for the overall lower level of expression of the λ reporter RNA in I-92 cells than in I-79 cells (compare lanes 1 of Fig. 7A and B); because that level did not change upon differentiation, we conclude that sequences within the 116 bp of the proximal 5′ flanking region are responsible for inactivation of the mU1b2 gene during development.

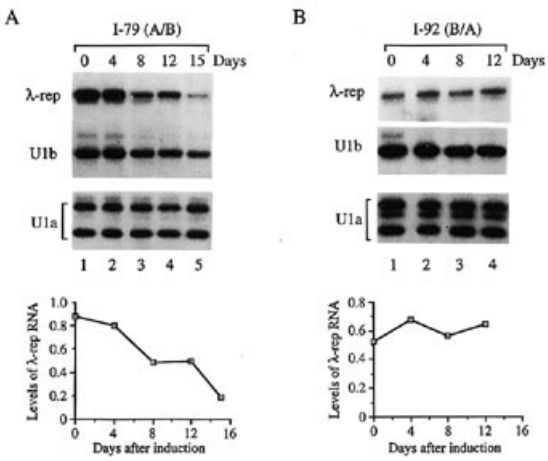

Figure 7. Promoter activites of B/A and A/B chimeric constructs in embryoid bodies. The levels of λ reporter RNA in I-79 (**A**) and I-92 (**B**) cells that were differentiated *in vitro* were analyzed as described in Figure 6. The autoradiograph in the region of λ reporter RNA in I-92 cells (B) was exposed longer than the U1a and U1b regions.

DISCUSSION

We have shown that the developmental control of U1 genes can be studied accurately in transiently transfected NIH 3T3 cells and in ES cells differentiated *in vitro*. In both systems, we find that sequences in the proximal 5′ flanking regions around the PSE element are responsible for differential transcription of the mU1a1 and mU1b2 genes (Figs 3 and 7). Our results are compatible with models in which sequences around the PSE of mU1b genes are bound by developmentally controlled repressor-like factors or transcriptional activators.

Together, the mU1a and mU1b gene families contribute ~20–40 copies of true U1 genes per haploid mouse genome. Embryonic mU1b genes are clustered at a single locus near the center of chromosome 3 (44), whereas the mU1a1 and mU1a2 genes are localized on chromosomes 11 and 12 (45,46) respectively. The proper stage- and tissue-specific expression pattern of a mU1b2 transgene (14) shows that accumulation of U1b RNAs is regulated at the level of transcription of individual genes. Here we have demonstrated that sequences around the PSE of mU1b2 genes are necessary for this control. This same region of the mU1b2 promoter shares extensive homology with the promoter region of another cloned member of the mU1b gene family, the mU1b6 gene (37). The fact that the mU1b6 gene also had reduced activity in NIH 3T3 cells suggests a common mechanism for the regulation of all members of the mU1b gene family during development.

The PSE element of snRNA promoters is necessary for transcription. Several PSE binding factors have been identified (30,31,47–49). Among these, the SNAPc complex (30 31), composed of the TATA box binding protein (TBP) and several unique TBP-associated factors (TAFs), is capable of activating transcription from pol II class snRNA promoters. The affinities of SNAPc for different PSE sequences correlate closely with the promoter activities of snRNA genes transcribed *in vitro* (30) and binding of this complex to the PSE, which is potentiated by the presence of the DSE enhancer (48), is required for recruitment of RNA polymerase II. However, differences in the DSE regions do not appear to determine the promoter activities of mouse U1 genes (Fig. 3A and Table 1), in keeping with the report that octamer binding factor, Oct-1, binds the DSEs of mU1a1 and mU1b2 genes with similar affinities (50).

The PSE sequences of the mU1a1 and mU1b2 genes resemble those showing strong and weak binding affinities respectively for SNAPc complexes in the *in vitro* assay (30). Thus, it was surprising that replacement of the mU1b2 PSE with the corresponding sequence of mU1a1 promoter failed to increase the expression level of λ reporter RNA in transiently transfected NIH 3T3 cells (Fig. 3A). Because differential transcription from the mU1a1 and mU1b2 promoters cannot be explained by differences between the respective PSE elements, sequences around the PSE must be involved. We propose that a developmentally controlled factor which specifically binds sequences around the PSE of mU1b genes modulates the occupancy of PSEs by SNAPc complexes, thereby controlling transcription during development.

The current study does not show whether such a factor acts as an activator of mU1b genes in embryonic tissues or a repressor in adult tissues.

Several lines of circumstantial evidence lead us to favor the repressor model. First, both the mU1a and mU1b genes are very active in other expression systems, such as HeLa cells (51) and *Xenopus laevis* oocytes (37), indicating that the low activity of mU1b genes in mouse cells is due to the presence of a factor that is absent from heterologous cells. Second, negative regulation by the large T antigen of SV40 virus has been observed with a mutant human U1 gene in which a binding site for the protein was inserted between the PSE element and the initiation site (52). Third, expression of an embryonic U4x gene was down-regulated during early chicken development (53) by a DNA binding protein, PPBF (proximal palindromic binding factor); the presence of PPBF in the liver, heart and kidney, but not in embryos, suggests that it may act as a repressor.

To date, few factors that regulate transcription of specific snRNA genes have been identified, partly because of the difficulty in obtaining active *in vitro* transcription systems. The ability of the same proximal 5′ flanking region to down-regulate U1b genes in transiently transfected NIH 3T3 cells and in developing embryoid bodies suggests that regulation might be mediated by a common mechanism in these two systems. Recent progress in developing systems for transcription of snRNA genes *in vitro* (30,31,54) raises the possibility that the factor(s) responsible for differential expression of mU1a and mU1b genes will soon be identified.

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