Nucleic Acids Research, Vol. 20, No. 16 4247–4254

Control of mouse U1a and U1b snRNA gene expression by differential transcription

Javier F.Cáceres[+], Debbie McKenzie[§], Rama Thimmapaya[φ], Elsebet Lund and James E.Dahlberg[*]
Department of Biomolecular Chemistry, University of Wisconsin–Madison, Madison, WI 53706, USA

Received May 13, 1992; Revised and Accepted July 24, 1992

ABSTRACT

The expression of mouse embryonic U1 snRNA (mU1b) genes is subject to stage- and tissue-specific control, being restricted to early embryos and adult tissues that contain a high proportion of stem cells capable of further differentiation. To determine the mechanism of this control we have sought to distinguish between differential RNA stability and regulation of U1 gene promoter activity in several cell types. We demonstrate here that mU1b RNA can accumulate to high levels in permanently transfected mouse 3T3 and C127 fibroblast cells which normally do not express the endogenous U1b genes, and apparently can do so without significantly interfering with cell growth. Expression of transfected chimeric U1 genes in such cells is much more efficient when their promoters are derived from a constitutively expressed mU1a gene rather than from an mU1b gene. In transgenic mice, introduced U1 transgenes with an mU1b 5′ flanking region are subject to normal tissue-specific control, indicating that U1b promoter activity is restricted to tissues that normally express U1b genes. Inactivation of the embryonic genes during normal differentiation is not associated with methylation of upstream CpG-rich sequences; however, in NIH 3T3 fibroblasts, the 5′ flanking regions of endogenous mU1b genes are completely methylated, indicating that DNA methylation serves to imprint the inactive state of the mU1b genes in cultured cells. Based on these results, we propose that the developmental control of U1b gene expression is due to differential activity of mU1a and mU1b promoters rather than to differential stability of U1a and U1b RNAs.

INTRODUCTION

U1 small nuclear RNA participates in the recognition of the 5′ splice sites of pre-messenger RNAs during splicing (1–8). The accumulation of U1 RNAs is controlled in both mice and frogs (Xenopus), with electrophoretically distinguishable forms being characteristic of cells or tissues at different stages of development (9–12). Adult forms (called U1a RNAs) are present in all cell types whereas embryonic forms (U1b RNAs) are present only in cells that are capable of further differentiation such as germ line or embryonic stem cells. Strain-specific sequence variants of these forms exist, with two adult (mU1a1 and mU1a2) and six embryonic (mU1b1–b6) species having been described for mice (10,11). The seven nucleotide differences that distinguish all mU1b RNAs from mU1a RNAs, clustered between positions 60 and 77 in stem-loop II (10,11), appear to influence the efficiency of binding of a U1 RNA-specific protein, the A protein (13). Recently, developmentally controlled U1 snRNA variants have also been described in sea urchins (14), fruit flies (15) and plants (16).

In mouse cultured cells, the levels of mU1b RNAs apparently reflect the developmental lineage of the original cells. Thus, fully differentiated fibroblasts like C127 and 3T3 cells synthesize little, if any mU1b RNAs (less than 2% of mU1a), whereas mouse L cells and embryonal carcinoma cells (EC cells) produce intermediate to high levels of these RNAs (30% and 55% of the total U1 RNAs, respectively) 10).

Although the different isoforms of U1 RNA might influence splice site utilization (17), they could also be functionally equivalent, as are the differentially expressed oocyte- and somatic-type 5S ribosomal RNAs of *X. laevis* (18). To date, there have been no reports of accumulation of high levels of embryonic forms of U1 RNA in cells that normally contain only the adult form. Thus, the consequences of accumulation of these RNAs in inappropriate cell types are unknown. Furthermore, the mechanism by which these RNAs accumulate in a differential manner in various cell types and tissues has not been determined. While a mechanism due to increased synthesis seems likely, one cannot rule out the possibility that stabilizing proteins are present only in embryonic tissues, and that U1b RNAs are preferentially degraded in 'non-expressing' cells.

Here, we show that expression of embryonic U1 RNA in mouse cells, both in culture and in intact animals is controlled

* To whom correspondence should be addressed

Present addresses: [+] Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, [§] Department of Veterinary Science, University of Wisconsin–Madison, Madison, WI 53706 and [φ] Department of Genetics, University of Illinois–Chicago, Chicago, IL 60612, USA

transcriptionally, rather than by degradation. Furthermore, we show that accumulation of embryonic RNA is not detrimental to cells that normally have only the adult form.

MATERIALS AND METHODS

DNA templates

All U1 gene constructs (Figure 1) were cloned between the PstI and HindIII sites of a modified pAT153 vector (19) containing the kanamycin/G418 resistance gene of pCGBPV9 (20) inserted between the BamHI and HindIII sites. The mouse mU1a1, mU1b2 and mU1b6 gene sequences were from clones pU1a1-214, pU1b-136 and -453 (21), respectively, and the human U1 coding plus 3′ flanking region sequences were from clone pHU1-1D (22). For generation of the chimeric genes, the various 5′ flanking and U1 coding region sequences were connected via the BclI site present at position +27 of all the U1 coding regions (cf. Figure 1). Restriction enzyme digestions, ligations and other DNA manipulations were performed according to Sambrook et al. (23).

Growth and Transfection of mammalian cells

Mouse NIH 3T3, C127, F9 and LT-Cl8 cells (10) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. For transfection of NIH 3T3 cells, 10 or 20 μg of plasmid DNA and 40 μg of Lipofectin (Bethesda Research Laboratories) were used per 60 mm dish of cells. The DNA and the Lipofectin reagent were combined as suggested by the manufacturer. 24 hours later the medium was changed and after additional 24 hours of growth the transfected cells were split (1:3) and subsequently maintained in selective medium containing 0.4 mg of G418 ('Geneticin', Sigma) per ml of medium. After 14 to 18 days of selection, individual clones of G418-resistant cells were isolated and expanded into cell lines. For transfection of C127 cells, the calcium phosphate precipitation method was used (24); selection of G418 resistant colonies was as above.

For preparation of ^{32}P-labeled RNA, exponentially growing cells (60 mm dish) were incubated for 16–20 hours with 5 ml of phosphate-free DMEM containing 1 mCi ^{32}P-orthophosphate (carrier-free, NEN-Dupont) and 10% dialyzed fetal bovine serum.

Preparation of nucleic acids

Total nucleic acids (DNA and RNA) of cultured cells and mouse tissues were isolated by the urea lysis method (10,25), using 4 ml of urea lysis buffer per $1-2\times10^6$ cells. After two extractions with phenol/chloroform/isoamylalcohol (24:24:1), the nucleic acids were precipitated with ethanol, washed with 67% ethanol, dried and resuspended in 10 mM Tris pH 8, 1mM EDTA.

Analysis of RNA

For northern blot analyses, total RNAs were separated by electrophoresis either in partially denaturing gels containing 12% (30:0.8) polyacrylamide (for resolution of mouse U1a and U1b RNAs [10]) or in non-denaturing gels containing 15% (19:1) (26) or 12% (19:1) polyacrylamide (27) (for separation of human and mouse U1a RNAs). After electrophoresis and staining with ethidium bromide, the RNAs in the U1 RNA region of the gel were transferred to Gene Screen Plus (New England Biolabs) or Zeta Probe (BioRad) nylon membranes by electroblotting using an LKB Transphor unit. For hybridization, blots were incubated at 68°C in 0.9 M NaCl, 1% SDS, 10 mM Tris (pH 7.5), 1 mM EDTA containing a ^{32}P-labeled U1-specific RNA probe as previously described (10).

Immunoprecipitaiton

^{32}P-labeled snRNPs were precipitated from clarified cell-sonicates by incubation with human polyclonal anti-Sm antibodies (28) coupled to protein A-sepharose. After proteinase K digestion, phenol extraction and ethanol precipitation, the immuno-precipitated RNAs were analyzed by polyacrylamide gel electrophoresis, as above, and individual RNAs were quantitated by Cerenkov counting of excised gel pieces. ^{32}P-labeled precursor snRNAs containing m^{7}G-caps were isolated from total RNA by immuno-precipitation with rabbit polyclonal anti m^{7}G-cap antibodies (29).

Analysis of DNA

Restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were according to standard procedures (23), using Gene Screen Plus (New England Biolabs) or Zeta Bind (Cuno) nylon membranes and alkaline transfer buffer (30). For Southern blots, hybridization conditions were as described for northern blots except the probes were ^{32}P-labeled DNA prepared by random priming using a 'Prime a gene' kit (Promega) according to instructions. The structures of the mU1b6- and mU1b2-specific probes are indicated in Figure 6. The mU1a1-specific probe (Fig. 2) corresponded to 5′ flanking region sequences from position −220 to −800 and the plasmid-specific probe (Fig. 4B) was the BamHI-HindIII fragment containing the kanamycin resistance gene.

Transgenic mice

To generate transgenic mice, the 3.6 kb PstI fragment containing the mouse mU1b2 promoter and the human hU1 coding region (plus 3′ end flanking sequences) was purified from the mU1b2/hU1 plasmid DNA (Fig. 1, bottom construct); in addition to the chimeric U1 gene, this fragment also contained 346 basepairs of pBR322 DNA sequences (corresponding to positions 29 to 375). This PstI fragment was introduced into fertilized mouse eggs (31,32) and transgenic mice were identified by Southern blot analyses of tail DNA using the pBR322 sequences as a transgene-specific probe. Homozygous transgenic mice were identified by quantitative slot blot hybridization. Tissues to be used for northern blot analyses of U1 RNAs were isolated from sexually mature mice as described previously (10).

RESULTS

Two general models could explain the low levels of U1b RNA in differentiated cells. On the one hand, the RNA could be unstable in such cells, perhaps due to the absence of a protecting protein. On the other hand, the U1b gene promoters might not be active in such cells. To differentiate betwen these models we have investigated both the stability of U1b RNA and the activity of U1b promoters in 3T3 and C127 fibroblast cells in which the level of U1b RNA is normally very low.

Stable expression of U1b RNA in 3T3 cells

To test the stability of U1b RNA in NIH 3T3 cells, we stably transfected the cells with a chimeric U1 gene that contained a mouse U1a1 promoter coupled to a mouse U1b6 coding region

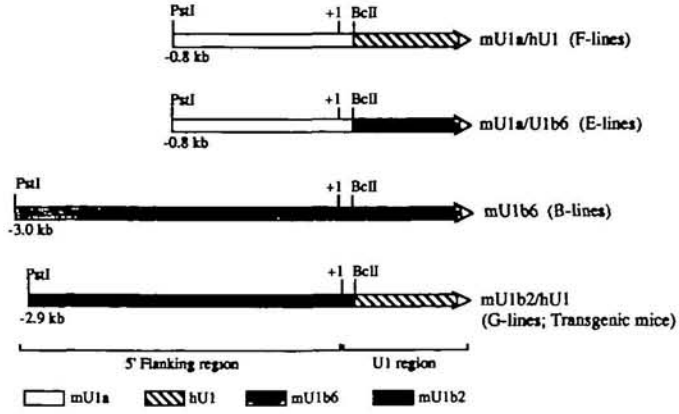

Figure 1. Chimeric and wildtype U1 genes used for transfection and generation of transgenic mice. The 5′ flanking regions of mouse mU1a or mU1b2 genes were joined to either a human U1 coding region (mU1a/hU1, mU1b2/hU1) or a mouse U1b6 coding region (mU1a/U1b6) via the BclI sites present at +27. U1 coding region sequences between positions +1 (transcription start site) and +27 (BclI site) are identical in the mU1a, mU1b2, mU1b6, and hU1 genes. The nomenclature of cell lines generated by transfection of mouse cells with chimeric U1 or wild type mU1b6 genes are indicated. 5′ flanking region sequences are not drawn to scale.

(mU1a/U1b6, Fig. 1). Accumulated U1 RNAs were analyzed from these cells and from control cells which had been transfected by another chimeric U1 gene, mU1a/hU1, that would direct the synthesis of a human U1a-type RNA (hU1). mU1b6 RNA, hU1 RNA and endogenous mU1a RNA can be distinguished by electrophoresis in partially denaturing (10) or non-denaturing gels 26,27).

As illustrated in Figures 2A and B, very high levels of both U1b6 and hU1 RNAs could be achieved in such transfected cells (e.g., lanes 4). Thus, mU1b RNA is stable and can accumulate in differentiated mouse cells. We note that the amounts of accumulated mU1b or hU1 RNAs varied between different cell lines (for example, Fig. 2B, lanes 3−6), as would be expected if the transfected, exogenous genes were integrated in variable copy numbers and/or at different chromosomal locations. These data also show that the constitutive mU1a promoter is utilized efficiently in 3T3 cells.

The RNA products of the transfected genes were incorporated efficiently into U1 snRNPs (28). As illustrated in Figure 3, both mU1b6 RNA and hU1 RNA were precipitated to the same extent as mU1a RNAs using either polyclonal anti-Sm (lanes 2 and 4) or anti-RNP antibodies (not shown). Moreover, in E12 cells the relative levels of mU1a and mU1b RNAs that were precipitable as precursors (using anti-m^7G cap antibodies; 29) correlated with the levels of mature RNAs in U1 snRNPs (data not shown). Therefore, the control of accumulation of mU1b RNA in 3T3 cells apparently is not mediated through degradation of newly made RNAs.

Southern blot analyses of BamH1-digested DNAs isolated from the transfected cells (Figure 2C) showed that the number of copies of the exogenously introduced chimeric genes was significantly higher than that of the endogenous mU1a genes (e.g., lanes 2 and 3). However, since the endogenous mU1a genes detected by this 5′ flanking region probe represent only about ten per cent of all of the mouse U1 genes (21,33−35), we estimate that the numbers of introduced and endogenous genes are comparable.

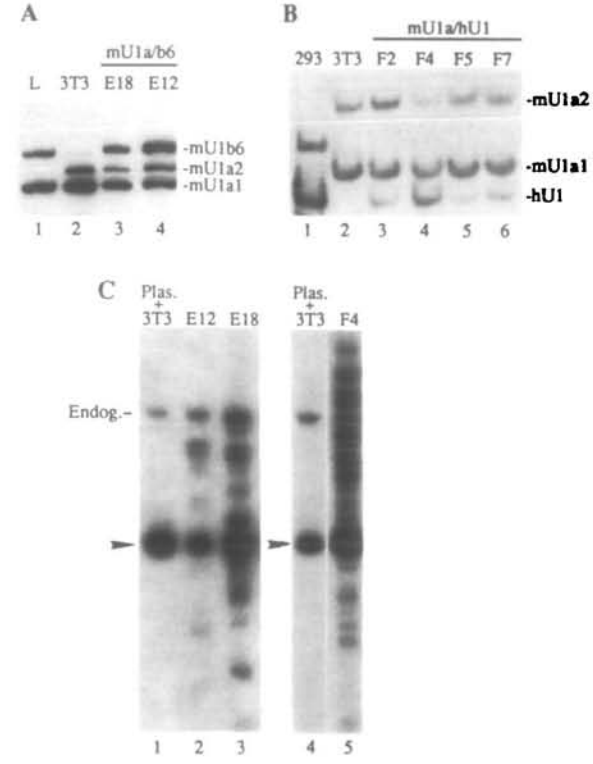

Figure 2. Stable expression of mU1b6 and hU1 RNAs in transfected NIH-3T3 cells. (A) Northern blot hybridization of U1 RNAs of 3T3 cells transfected with mU1a/U1b6 chimeric genes (E18 and E12 cell lines, lanes 3 and 4). Total RNAs, including RNAs of L cells (lane 1) and untransfected 3T3 cells (lane 2) as markers for mU1a and mU1b RNAs, were fractionated in a partially denaturing 12% polyacrylamide gel and probed with a U1-specific single stranded RNA probe. (B) Northern blot analysis of U1 RNAs of 3T3 cells transfected with mU1a/hU1 chimeric genes (F2, F4, F5 and F7 cell lines, lanes 3−6). Total RNAs, including markers of human 293 cells (lane 1) and untransfected 3T3 cells (lane 2), were separated in a non-denaturing 12% polyacrylamide gel. (C) Southern blot analysis of endogenous wildtype (Endog.) and transfected chimeric mU1a genes in E and F cell lines. BamHI-digested genomic DNA of untransfected 3T3 (lanes 1and 4), E12 and E18 (lanes 2 and 3) and F4 cells (F4, lane 4) were fractionated in a 1% agarose gel, transferred to a nylon membrane and probed with an mU1a specific probe. (Plas.) 50 pg of linearized mU1a/U1b6 (lane 1) or mU1a/hU plasmid DNA (lane 4) were mixed with genomic 3T3 DNA as markers of full-length transfected genes (arrowheads).

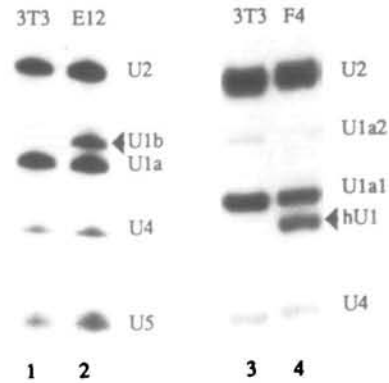

Figure 3. Anti-Sm precipitable snRNPs of transfected 3T3 cells. ^{32}P-labeled RNAs from transfected E12 (lanes 2) or F4 cells (lane 4) or untransfected 3T3 cells (lanes 1 and 3) were isolated by immuno-precipitation with anti-Sm antibodies and analyzed by polyacrylamide gel electrophoresis in a partially denaturing (lanes 1 and 2) or a non-denaturing gel (lanes 3 and 4, as in Fig. 2). The ratios of total U1 RNA to U2, U4 or U5 RNAs were determined by scintillation counting of excised gel pieces (Table 1 and data not shown).

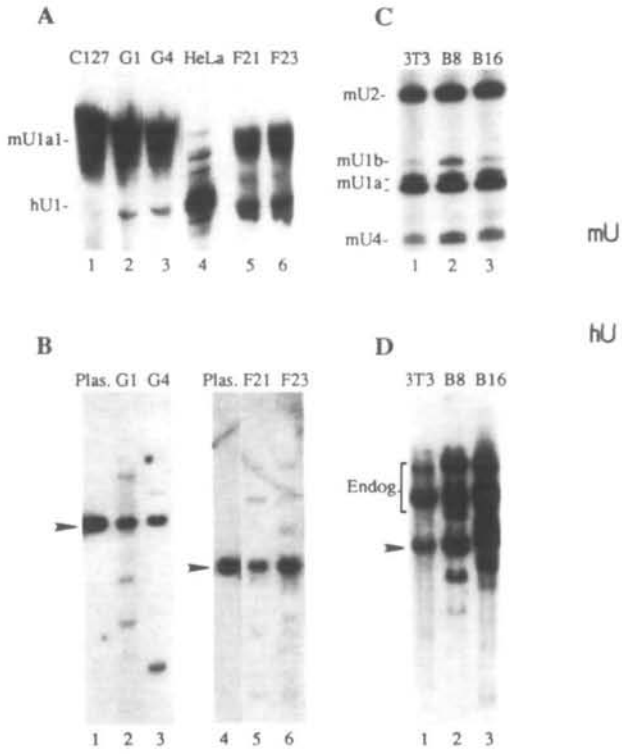

Figure 4. Differential activity of mU1a and mU1b promoters in transfected fibroblasts. (A) Differential expression of chimeric mU1b2/hU1 (G cell lines) and mU1a/hU1 genes (F cell lines) in transfected C127 cells. Total cellular RNAs of untransfected C127 cells (lane 1), transfected G1 (lane 2), G4 (lane 3), F21 (lane 5) or F23 cells (lane 6) and human HeLa cells (lane 4) were separated as in Figure 2B and assayed for U1 RNAs by northern blot hybridization. (B) Comparison of gene copy numbers of the transfected, chimeric genes in G and F cell lines. Southern blot of BamHI-digested genomic DNAs of G (lanes 2 and 3) and F cells (lanes 5 and 6) (Fig. 2C) was hybridized with a plasmid-specific kanamycin resistance gene probe (Plas.). Linearized mU1b2/hU1 (Lane 1) or mU1a/hU1 (lane 4) plasmid DNAs (arrowheads) were mixed with DNA of untransfected C127 cells. (C), (D) Stable transfection of 3T3 cells with wildtype mU1b6 genes (B cell lines). (C) Anti-Sm precipitable ^{32}P-labeled RNAs of untransfected 3T3 (lane1) and transfected B8 (lane 2) and B16 cells (lane 3) were analyzed in a partially denaturing gel as in Figure 3. (D) Copy number analysis of transfected mU1b6 genes in B cell lines. Southern blot of BamHI-digested genomic DNAs of 3T3 (lane 1), B8 (lane 2) and B16 cells (lane 3) was hybridized with an mUb6-specific probe (Fig. 6). Linearized mU1b6 plasmid DNA (arrowhead) was mixed with 3T3 DNA (lane 1); endogenous (Endog.) mU1b6 genes are indicated by the bracket.

Table 1.

Cell Line	U1 Gene Transfected	U1b RNA (as % of total U1 RNA)	hU1 RNA	U2 RNA / U1 RNA
3T3	–	1.5	–	1.0
E12	mU1a/U1b6	37	–	0.9
F4	mU1a/hU1	–	46	1.0

Gene dosage compensation in stably transfected mouse 3T3 cell that carry multiple copies of chimeric U1 genes. The relative levels of individual snRNAs were determined by scintillation counting of excised gel pieces (Figure 3 and data not shown).

Hence, the amount of U1 RNA made per gene is about the same for the endogenous and exogenous templates.

Although the U1 RNAs from the exogenous genes represent approximately 40% of the total U1 RNAs of the transfected cells

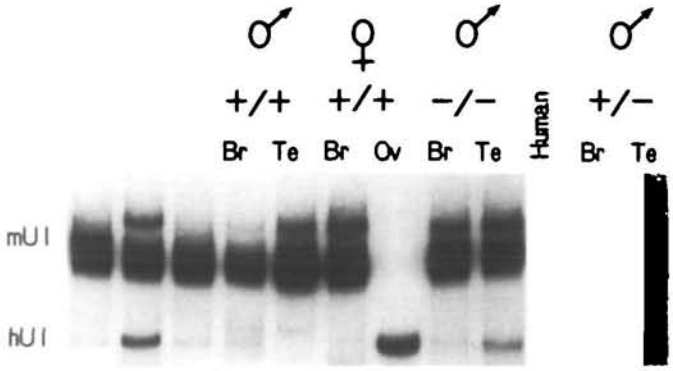

Figure 5. Expression of chimeric mU1b/hU1 genes in transgenic mice. Northern blot analysis of the U1 RNAs accumulated in brain (Br), testis (Te) or ovary (Ov) of mice that were either homozygous (+/+), heterozygous (+/−) or negative (−/−) for a transgene encoding human U1 RNA. This line of transgenic mice carries only 1 to 2 copies (per haploid genome) of the mU1b2/hU1 chimeric gene (Figure 1). A marker of human U1 RNA from Raji cells is included. The band of RNA above mU1a RNA in some samples contains a mixture of endogenous mU1b RNA of undifferentiated cells in the testis (10) and electrophoretic variants of mU1a RNA in some of the parental transgenic lines.

(cf. Fig. 3, lanes 2 and 4), the total amount of U1 RNA (i.e., the sum of endogenous mU1a1 and mU1a2 plus either mU1b6 or hU1 RNAs) did not increase relative to the other major (endogenous) snRNAs; this is evident from the constant ratio of U1 to U2 (or U4 and U5) RNAs (see Table 1 and data not shown). Thus, we conclude that gene dosage compensation is operating in transfected 3T3 cells, as has been described previously for bovine papilloma virus-transformed mouse C127 cells carrying multiple copies of an hU1 gene (27).

Differential Activities of mU1a and mU1b promoters in transfected cell lines

To determine if mU1b promoters could function in cells that normally do not express mU1b genes, we stably transfected C127 cells with a chimeric gene that contains the 5′ flanking region of an mU1b2 gene and the coding region of an hU1 RNA gene (mU1b2/hU1, Fig. 1; G cell lines). As a control, C127 cells were also transfected with the chimera mU1a/hU1 (F cell lines). Northern blot analysis of U1 RNAs from such cells (Fig. 4A) demonstrated that the accumulation of hU1 RNA was consistently lower when the cells received genes with the mU1b2 promoter (G cell lines, lanes 2 and 3) rather than the mU1a promoter (F cell lines, lanes 5 and 6). Southern blot analysis of the DNAs of the transfected cells (Figure 4B), showed that similar levels of chimeric genes were present in all cell lines (compare lanes 2, 3, 5 and 6). Likewise, low levels of mU1b promoter activity were also observed in NIH '3T3 cells transfected with the mU1b2/hU1 construct (data not shown).

The promoter of an mU1b6 gene, another variant of the mouse U1b genes, also had low activity in 3T3 cells. In two independent, stably transfected B cell lines, mU1b6 RNA accounted for only 5–7% of the anti-Sm precipitable or total U1 RNA of the cells (Fig. 4C, lanes 2 and 3; data not shown), although Southern blot analysis showed that the copy numbers of the transfected mU1b6 genes were high relative to the endogenous genes (Fig. 4D, compare lane 1 with lanes 2 and 3). We conclude that, in contrast to mU1a promoters, the mU1b promoters have little activity in differentiated cells.

Tissue-specific activity of an mU1b2 promoter in transgenic mice

The results in Figures 2–4 indicate that the differential control of mU1b gene expression in cultured cells is mediated by the 5′ flanking region of the genes, rather than by the structure of the RNA transcript. To test if this control reflects the situation in intact animals, we generated a line of transgenic mice carrying the chimeric mU1b2/hU1 gene; hence, accumulation of the reporter RNA, hU1 RNA, would indicate mU1b2 promoter activity.

Northern blot analysis of total RNA isolated from various tissues of homozygous and heterozygous mice (Fig. 5) shows that hU1 RNA accumulates in adult testis, which normally contains high levels of mU1b RNA (lanes Te, +/+ and +/−). In contrast, adult liver, brain and ovary, tissues that normally do not accumulate mU1b RNAs (Lund et al., 1985), show no detectable hU1 RNA (lanes Br, Ov and data not shown). Thus, the chimeric mU1b transgene was subject to normal tissue-specific controls, indicating that also in whole animals control is determined by sequences in the 5′ flanking region of the gene rather than by U1b RNA instability.

Methylation of Endogenous mU1b genes

As an independent test of whether the mU1b6 genes are indeed transcriptionally inactive in cultured cells, we analyzed the degree of methylation of CpG sequences in the 5′ flanking regions of these genes. In vertebrates, most of the CpG sequences in genomic DNA are methylated as m^5CpG; non-methylated sequences are confined to the so-called 'CpG islands' present in the 5′ flanking sequences of genes (36,37). CpG islands associated with transcriptionally inactive genes are unmethylated in tissues of intact animals, but they are frequently methylated in tissue culture cells (38,39).

The developmentally expressed variants of the mU1b genes studied here (21) and other isolates of mU1b2 genes (40) have CpG islands in their immediate 5′ flanking regions, but the constitutively expressed mU1a genes lack CpG islands. We analyzed the state of methylation of the mU1b genes in genomic DNA of cultured cells and tissues, after first separating the genes from other, irrelevant sequences by digestion with PstI. The resulting PstI fragments were further digested with either MspI (which recognizes the sequence C-C-G-G regardless of methylation) or its isoschizomer HpaII (which does not digest C-m^5C-G-G) and the redigestion products were characterized by Southern blotting, using probes specific for the 5′ flanking regions of mU1b6 or mU1b2 genes.

As shown in Figure 6A, PstI-digestion generated a major 5.5 kb fragment that corresponds to the cloned mU1b6 gene (panel A and data not shown). Additional digestion with MspI reduced the size of this mU1b6-specific fragment by several kilobasepairs (panel B). However, redigestion with HpaII left the 5.5 kb PstI fragments of DNA of 3T3 cells intact (panel C, lane 1), indicating that the MspI/HpaII sites of the mU1b6 genes were highly methylated in these cells. In contrast, not all of these HpaII sites were methylated in the DNA of L cells (lane 2), as demonstrated by the generation of some shorter fragments (cf. panel B). In the extreme case of embryonal carcinoma (EC) LT-Cl8 and F9 cells, the mU1b6 genes were unmethylated (panel C, lanes 3 and 4). The decreased methylation of mU1b6 genes in L cells and the lack of methylation in EC cells correlates with the fact that these cells synthesize moderate or large amounts of U1b RNA,

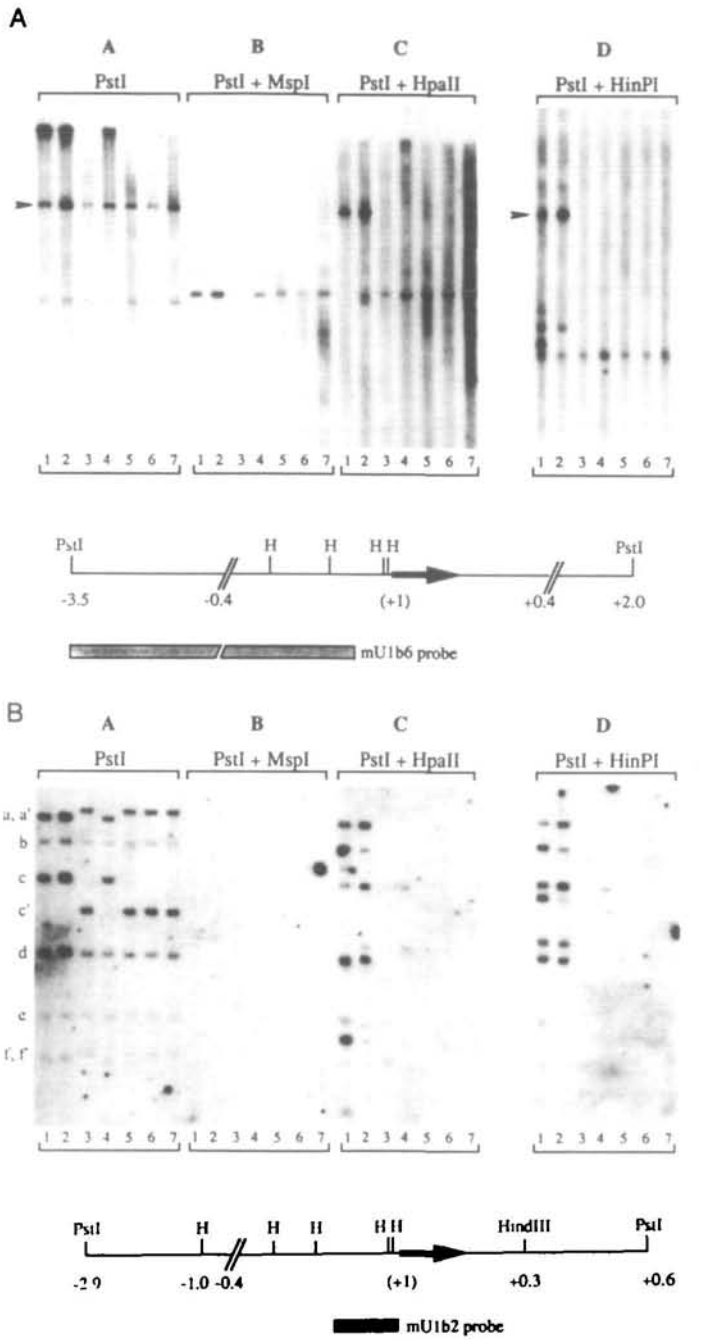

Figure 6. Differential methylation of the CpG islands in mU1b genes of cultured cells and tissues. Southern blot analysis of the methylation patterns of endogenous mU1b6 (A) and mU1b2 (B) genes. Genomic DNAs of 3T3 (lanes 1), L (lanes 2), LT-Cl8 (lanes 3) or F9 cells (lanes 4) or liver (lanes 5), brain (lanes 6) and testis (lanes 7) of a male LT mouse were digested with either PstI alone (panel A), PstI+MspI (panel B), PstI+HpaII (panel C) or PstI+HinPI (panel D). Line drawings at the bottom indicate the locations of the known HpaII sites (H) in the flanking regions of the cloned mU1b6 and mU1b2 genes and the extent of gene-specific DNA probes used for hybridization. (A) Arrowheads mark the migration of the 5.5 kb PstI-PstI fragment corresponding to the cloned mU1b6 gene. (B) Letters a, a′ to f, f′ indicate the family of polymorphic mU1b2 genes observed with the mU1b2-specific probe (see text).

respectively (10). However, no methylation of CpG sequences is apparent in the 5′ flanking regions of mU1b genes of whole animal tissues like liver, brain and testis (panels B and C, lanes 5–7), regardless of the levels of accumulated U1b RNA (10).

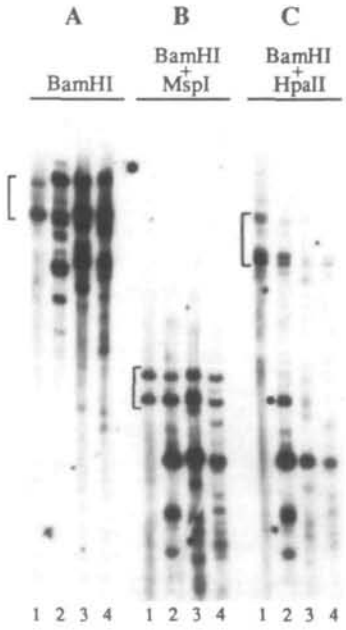

Figure 7. Lack of methylation of CpG islands in transfected mU1b6 genes. The methylation status of CpG sequences of transfected (B cell lines) and endogenous mU1b6 genes (brackets) was assayed by Southern blot hybridization using the mU1b6-specific probe (cf. Figure 6). Genomic DNAs of untransfected 3T3 cells (lanes 1) or transfected B4 (lanes 2), B8 (lanes 3) and B16 cells (lanes 4) were digested with either BamHI alone (panel A), BamHI+MspI (panel B) or BamHI+HpaII (panel C). Redigestion of the BamHI fragments with HpaII (or HinPI) trims the ends of the large endogenous mU1b6-specific fragments generating slightly faster migrating HpaII-resistant forms, as indicated by the brackets (compare panels A and C; data not shown). The amounts of B8 and B16 DNAs in panel C (lanes 3 and 4) were lower than those used in panels A and B. The dot (●) in panel C (lane 2) marks fragments derived from introduced mU1b6 genes that comigrate with fragments from endogenous genes in panel B.

These observations were extended to other potential methylation sites by redigestion of the PstI-generated fragments with HinPI, an enzyme that cuts DNA at GpCpGpC but not at Gpm^{5}CpGpC sequences (Fig. 6A, panel D). Again, the mU1b6 genes of 3T3 and L cells showed a significant level of methylation of the HinPI sites (lanes 1 and 2) whereas these sites were unmethylated in EC cells (lanes 3 and 4) and in adult tissues (lanes 5–7).

The members of another family of mU1b genes, the mU1b2 genes, were also methylated in cultured cells in which they were not expressed (Fig. 6B). Analysis of PstI-digested genomic DNAs was complicated slightly by polymorphisms in the U1b2 gene family that resulted in size variations in three of the six mU1b2-specific PstI fragments (a/a′, c,/c′ and f/f′; 35). Consequently, digests of DNAs of 3T3, L and F9 cells produced fragments a, c and f (panel A, lanes 1, 2 and 4) whereas those of LT-Cl8 cells and all tissues of LT mice yielded fragments a′, c′ and f′ (lanes 3 and 5–7). Redigestion with MspI produced small fragments of mU1b2 genes that were not retained on the blot (panel B). Again, only the DNAs of 3T3 and L cells produced mU1b2 PstI fragments that were resistant to redigestion with HpaII or HinPI (panels C and D, lanes 1 and 2).

Methylation of other mU1 genes

DNA methylation cannot account for the relatively low level of transcription of mU1b6 genes transfected into 3T3 cells (Fig. 4C). In BamH1-digested DNAs from such transfected cells (B cell lines) the exogenous genes were sensitive to digestion by either MspI or HpaII (Fig. 7, panels B and C, lanes 2–4). This difference in methylation between the endogenous (brackets; cf. Fig. 6A) and the introduced mU1b6 genes was confirmed by redigestion with HinPI (data not shown).

The mU1a gene studied here contains no CpG islands in the 5′ upstream region (21). As expected, no methylation of CpG sequences was detected in that region of these constitutively expressed genes either in cultured cells or in the tissues (data not shown).

The observed patterns of methylation of endogenous mU1b genes are consistent with control of their expression being mediated at the level of transcription. This conclusion is in complete agreement with our findings (Figs. 4 and 5) that the 5′ flanking regions of these genes are responsible for differential expression.

DISCUSSION

At least two classes of U1 small nuclear RNAs exist in mice, the constitutively expressed mU1a RNAs, and the developmentally regulated mU1b RNAs (called the adult and embryonic forms, respectively). Here, we have investigated the mechanism of differential accumulation of mU1b RNA and examined whether control is exerted through stability of the RNA product or through inactivity of the promoter.

As model systems for these analyses we used primarily mouse tissue culture cells that showed low, intermediate or high levels of expression of their endogenous mU1b genes. By stably transfecting these cells with chimeric genes we were able to produce mU1b RNA in cells where it normally is not observed and to analyze the relative strength of mU1a and mU1b promoters in such cells. Furthermore, we used the state of methylation of the endogenous mU1b genes of cultured cells as an indicator of whether these genes were transcriptionally active. We find that mU1b RNAs are stable in cells that normally do not accumulate them, whereas mU1b promoters are largely inactive. Thus, we conclude that the level of mU1b RNA in differentiated cells is controlled primarily through modulation of mU1b promoter activity.

It is unclear whether mU1b promoter inactivation occurs by the action of a specific inhibitory protein that represses gene activity or by the inability of the promoter to bind sufficient amounts of transcription factors. While mU1a and mU1b promoters share elements that are characteristic of snRNA genes, such as the DSE and PSE (reviewed in references 41–43), the precise sequences of these elements are not identical; hence, they could differ in their abilities either to compete for a limiting transcription factor(s) or to function in the absence of such a factor. We have shown that the DSE acts as an enhancer that is specific for genes utilizing a PSE, such as U1 snRNA (44). This snRNA-specific activation is mediated by the Oct-1 transcription factor, which binds to octamer sequences within the DSE and interacts with other proteins, presumably at the PSE (45). Because of the promoter and factor specificity of the DSE, the significance of assays of mU1b octamer function that are based on mRNA transcription (46) remain unclear.

Sequences outside the shared elements differ significantly between mU1a and mU1b promoters (21). For example, the 5′ flanking region of the mU1b6 gene has three binding sites for the transcription factor Sp1 (47), whereas the promoter region

of the mU1a gene has no such sites (21). If binding of Sp1 to these sites is required for efficient transcription of U1b genes, then the decrease in the level of this factor during development (48) might lead to differential inactivation of these genes. Such a model does not rule out a possible role for a repressor-like factor that would specifically shut down mU1b transcription. We are currently testing these models using transiently transfected 3T3 cells, to determine whether a titratable inhibitor exists and whether the two classes of U1 genes compete equally in various types of cells (49).

Stably transfected genes with mU1b promoters are expressed only poorly in C127 or 3T3 fibroblast cells, which normally have almost no mU1b RNAs (10). It is unlikely that this is due to inopportune integration of the transfected genes into inactive chromatin, because the same result was obtained every time the experiment was repeated. The promoters themselves are functional in the right environment (or cell type), as evidenced by their activity when introduced into transgenic mice (Fig. 5) or when injected into *X. laevis* oocytes (data not shown). We did not test the activity of these genes in stably transfected L-cells, which express intermediate levels of mU1b RNAs, but Moussa et al. (50) did report transcription of exogenous mU1b 2 genes in such cells.

Methylation of CpG islands in promoter regions can be envisioned as a way of imprinting the inactivity of genes in cultured cells (38,51). When the CpG sequences of the endogenous mU1b genes of different cell lines were analyzed, a good correlation was apparent between low levels of mU1b RNAs and the extent of DNA methylation (Fig. 6). This result strongly supports our conclusion that mU1b genes are transcriptionally inactive in differentiated cells. The lack of methylation of these sequences in DNAs of differentiated tissues of adult animals does not contradict this conclusion since many genes that are transcriptionally inactive are unmethylated in whole animals (38,39).

In several cell lines that were stably transfected with DNA carrying the mU1a promoter and either hU1 or mU1b6 coding regions, up to half of the total accumulated U1 RNA were transcribed from the exogenous genes (Figs. 2 and 3). In spite of this new, additional source of active promoters, the total amount of U1 RNA per cell remained constant, relative to the levels of U2 or U4 RNAs (Table 1). Similar gene dosage compensation has been described before, in mouse cells transformed with recombinant bovine papilloma virus DNA containing a human U1 gene (27). It is unclear whether the intracellular level of U1 RNA is kept constant through a system that monitors total accumulated U1 RNA or through a mechanism that limits the total rate at which U1 synthesis can occur (for example by having a limited amount of a transcription factor). However, it is unlikely that gene dosage compensation is controlled by the same mechanism(s) that operate in the tissue- and stage-specific expression of mU1b genes during development.

The utility of having two independently controlled classes of mU1 RNA genes is unclear. In transfected 3T3 cells the mU1b6 RNA made from exogenous genes having mU1a promoters are incorporated into snRNPs and presumably can function in splicing of pre-mRNAs. Also, such stably transfected cells, in which up to 40% of the U1 RNA is the 'inappropriate' embryonic form, grow normally (J.C. and R.T., unpublished results). Thus, this control does not appear to be essential for cultured cells, but it could be important in whole animals at some stage of development. Therefore, it will be of interest to learn whether mice can develop normally with a high level of chimeric transgene(s) composed of the mU1a promoter and the coding region of an mU1b gene.

ACKNOWLEDGEMENTS

We thank Adrian Bird for stimulating discussions about DNA methylation, and members of the Dahlberg laboratory and Nouria Hernandez for critical comments. We thank Mel Simon for hospitality and enthusiastic suppport during our sabbatical leave (E.L. and J.E.D.) and Jessica Dousman for expert assistance with generation of transgenic mice. This work was supported by NIH grant GM 30220 to J.E.D. and E.L.

REFERENCES

1. Rogers, J. and Wall, R. (1980) *Proc. Natl. Acad. Sci.* **77**: 1877–1881.
2. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980) *Nature* **283**: 220–224.
3. Mount, S.M., Pettersson, J.A., Hinterberger, M., Karmas, A. and Steitz, J.A. (1983) *Cell* **33**: 509–518.
4. Zhuang, Y. and Weiner, A.M. (1986) *Cell* **46**: 827–835.
5. Maniatis, T. and Reed, R. (1987) *Nature* **325**: 673–678.
6. Seraphin, B., Kretzner, L. and Rosbash, M. (1988) *EMBO J.* **7**: 2533–2538.
7. Siliciano, P.G. and Guthrie, C. (1988) *Genes Dev.* **2**: 1258–1267.
8. Steitz, J.A., Black, D.L., Gerke, V., Parker, K.A., Kramer, A., Frendewey, D. and Keller, W. (1988) In Birnstiel, M.L. (ed), *Structure and function of major and minor small nuclear ribonucleoprotein particles*, Springer Verlag, Heidelberg, pp 115–154.
9. Forbes, D.J., Kirschner, M.W., Caput, D., Dahlberg J.E. and Lund, E. (1984) *Cell* **38**: 681–689.
10. Lund, E., Kahan, B. and Dahlberg, J.E. (1985) *Science* **229**: 1271–1274.
11. Kato, N. and Harada, F. (1985) *J. Biol. Chem.* **260**: 7775–7782.
12. Lund, E. and Dahlberg, J.E. (1987) *Genes Dev.* **1**: 39–46.
13. Bach, M., Krol, A. and Lührmann, R. (1990) *Nucleic Acids Res.* **18**: 449–457.
14. Santiago, C. and Marzluff, W.F. (1989) *Proc. Natl. Acad. Sci.* **86**: 2572–2576.
15. Lo, P.C.H. and Mount, S.M. (1990) *Nucleic Acids Res.* **18**: 6971–6979.
16. Hanley, B.A. and Shuler, M.A. (1991) *Nucleic Acids Res.* **19**: 6319–6325.
17. Kuo, H.-C., Nasim, F.H. and Grabowski, P.J. (1991) *Science* **251**: 1045–1050.
18. Wolffe, A.R. and Brown, D.D. (1988) *Science* **241**: 1626–1632.
19. Twigg, A.J. and Sheratt, D. (1980) *Nature* **283**: 216–218.
20. Matthias, P.D., Bernard, H., Scott, A., Brady, G., Hashimoto-Gotoh, T. and Schütz, G. (1983) *EMBO J.* **2**: 1487–1492.
21. Howard, E.F., Michael, S.K., Dahlberg, J.E. and Lund, E. (1986) *Nucleic Acids Res.* **14**: 9811–9825.
22. Lund, E. and Dahlberg, J.E. (1984) *J. Biol. Chem.* **259**: 2013–2021.
23. Sambrook, J., Frisch, E.F. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (Cold Spring Harbor, New York: Cold Spring Harbor laboratory).
24. Graham, F.L. and Van der Eb, A.J. (1973) *Virology* **52**: 456–467.
25. Ross, J. (1976) *J. Mol. Biol.* **106**: 403–420.
26. Lund, E., Bostock, C., Robertson, M., Christie, S., Mitchen, J.L. and Dahlberg, J.E. (1983) *Mol. Cell. Biol.* **3**: 2211–2220.
27. Mangin, M., Ares, M. and Weiner, A.. (1985) *Science* **229**: 272–275.
28. Lerner, M.R. and Steitz, J.A. (1979) *Proc. Natl. Acad. Sci.* **76**: 5495–5499.
29. Munns, T.W., Liszewski, K.M., Tellam, J.T., Sims, H.F. and Rhoads, R.E. (1982) *Bichemistry* **21**: 2922–2928.
30. Reed, K.C. and Mann, D.A. (1985) *Nucleic Acids Res.* **13**: 7207–7221.
31. Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980) *Proc. Natl. Acad. Sci.* **77**: 7380–7384.
32. Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Yagle, M.K. and Palmiter, R.D. (1985) *Proc. Natl. Acad. Sci.* **82**: 4438–4442.
33. Michael, S., Hilgers, J., Kozak, C., Whitney III, J.B. and Howard, E.F. (1986) *Somatic Cell and Mol. Genet.* **12**: 215–223.
34. Lund, E. and Nesbitt, M.N. (1988) *Somatic Cell and Mol. Genet.* **14**: 143–148.
35. Blatt, C., Saxe, D., Marzluff, W.F., Lobo, S., Nesbitt, M.N. and Simon, M.I. (1988) *Somatic Cell and Mol. Genet.* **14**: 133–142.

36. Bird, A., Taggart, M., Frommer, M., Miller, O.J. and Macleod, D. (1985) *Cell* **40**: 91–99.
37. Bird, A.P. (1986) *Nature* **321**: 209–213.
38. Antequera, F., Boyes, J. and Bird, A. (1990) *Cell* **62**: 503–514.
39. Jones, P.A., Wolkowicz, M.J., Rideout III, W.M., Gonzales, F.A., Marziasz, C.M., Coetzee, G.A., and Tapscott, S.J. (1990) *Proc. Natl. Acad. Sci.* **87**: 6117–6121.
40. Marzluff, W.F., Brown, D.T., Lobo, S. and Wang, S.S. (1983) *Nucleic Acids. Res.* **11**: 6255–6270.
41. Dahlberg, J.E. and Lund, E. (1988) In Birnstiel, M.L. (ed), *Structure and function of major and minor small nuclear ribonucleoprotein particles*, Springer Verlag, Heidelberg, pp. 38–70.
42. Parry, H.D., Scherly, D. and Mattaj, I.W. (1989)*TIBS* **14**: 15–19.
43. Reddy, R. and Singh, R. (1991) *Prog. Subcell. Biol.* **12**: 1–36.
44. Dahlberg, J.E. and Schenborn, E.T. (1988) *Nucleic Acids Res.* **16**: 5827–5840.
45. Tanaka, M., Lai, J.-S. and Herr, W.. (1992) *Cell* **88**: 755–567.
46. Lea, I., Moore, H.D.M. and Latchman, D.S. (1991) *Biochem. J.* **277**: 719–722.
47. Dynan, W.S. and Tjian, R. (1983) *Cell* **32**: 669–680.
48. Saffer, J.F., Jackson, S.P. and Annarella, M.B. (1991) *Mol. Cell. Biol.* **11**: 2189–2199.
49. Lund, E., Bostock, C. and Dahlberg, J.E. (1987) *Genes Dev.* **1**: 47–56.
50. Moussa, N.M., Lobo, S.M. and Marzluff, W.F.. (1985) *Gene* **36**: 311–319.
51. Shimada, T. and Nienhuis, A.. (1985) *J. Biol. Chem.* **260**: 2468–2474.