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# Regulation of transcription of the RNA splicing factor *hSlu7* by *Elk-1* and *Sp1* affects alternative splicing

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## ABSTRACT

Alternative splicing plays a major role in transcriptome diversity and plasticity, but it is largely unknown how tissue-specific and embryogenesis-specific alternative splicing is regulated. The highly conserved splicing factor *Slu7* is involved in 3' splice site selection and also regulates alternative splicing. We show that *Slu7* has a unique spatial pattern of expression among human and mouse embryonic and adult tissues. We identified several functional Ets binding sites and GC-boxes in the human *Slu7* (*hSlu7*) promoter region. The Ets and GC-box binding transcription factors, *Elk-1* and *Sp1*, respectively, exerted opposite effects on *hSlu7* transcription: *Sp1* protein enhances and *Elk-1* protein represses transcription in a dose-dependent manner. *Sp1* protein bound to the *hSlu7* promoter in vivo, and depletion of *Sp1* by RNA interference (RNAi) repressed *hSlu7* expression. *Elk-1* protein bound to the *hSlu7* promoter in vivo, and depletion of *Elk-1* by RNAi caused an increase in the endogenous level of *hSlu7* mRNA. Further, depletion of either *Sp1* or *Elk-1* affected alternative splicing. Our results provide indications of a complex transcription regulation mechanism that controls the spatial and temporal expression of *Slu7*, presumably allowing regulation of tissue-specific alternative splicing events.

**Keywords:** *Slu7*; alternative splicing; spliceosome; *Elk-1*; *Sp1*; transcription

## INTRODUCTION

Human and mouse possess a similar number of protein-coding genes (~24,000) (Lander et al. 2001; Waterston et al. 2002). Current estimates indicate that the total number of human proteins exceeds the number of genes. Research is therefore needed to determine the mechanisms that underlie this discrepancy. Alternative splicing is known to significantly expand the transcriptomic potential and genetic diversity (Graveley 2001; Ast 2004; Xing and Lee 2006). Alternative splicing varies among tissues (Hanamura et al. 1998; Yeo et al. 2004; Ule et al. 2005), either as a function of different developmental stages (Wang and Grabowski 1996; Cooper 2005) or due to different physiological conditions (van der Houven van Oordt et al. 2000; Pelisch et al. 2005;

Shomron et al. 2005; Guil et al. 2006). There is also a link between aberrant splicing and human diseases, including cancer (Philips and Cooper 2000; Zhang et al. 2006).

Splicing is a highly conserved process from yeast to human, in which introns are removed from mRNA precursor and exons are ligated to generate mature mRNA. Four short sequences direct the splicing machinery to the splice junctions: the 5' and 3' splice sites (5'ss and 3'ss), the branch-site (BS) sequence, and the polypyrimidine tract; the latter two regions are located upstream of the 3'ss. The splicing reaction consists of two consecutive catalytic steps and is facilitated by a dynamic protein-RNA complex, known as the spliceosome. The spliceosome is composed of five small nuclear ribonucleoprotein particles (U1, U2, U4, U5, and U6 snRNPs) and more than 150 proteins (Black 2003).

The splicing factor *Slu7* was originally identified in yeast as a ubiquitous protein that was found to be synthetically lethal with U5. It is involved in the second step of splicing and is dispensable for in vitro splicing of introns with less than 12 nucleotides (nt) between the BS and the 3'ss (Frank et al. 1992; Zhang and Schwer 1997).

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In vitro, the human ortholog (*hSlu7*) affects the fidelity of 3' splice site selection when an incorrect 3' splice site sequence is adjacent to the functionally correct site. In the absence of *hSlu7* the incorrect 3' splice site is activated. This activation only occurs when the distance between the 3' splice site and the branch site is not more than ~30 nt (Chua and Reed 1999b). Recently, *hSlu7* was shown to regulate alternative splicing by a sensitive nucleo-cytoplasmic shuttling. This shuttling controls the nuclear concentration of *hSlu7* following specific physiologic stress conditions (Lev-Maor et al. 2003; Shomron et al. 2004, 2005).

Although the conservation of *hSlu7* from yeast to human suggests that it is a ubiquitous spliceosomal protein, we demonstrated that *hSlu7* is not required for cell viability in the examined cell lines (Shomron et al. 2005). This observation raises the question of whether *Slu7* is indeed a constitutively expressed protein. Here, we show that the mammalian *Slu7* is differentially expressed in various tissues and cell lines and also in developing embryonic tissues. We have begun to unravel the elaborate regulatory mechanism of *hSlu7* transcription. A complex promoter arrangement that controls *hSlu7* temporal and spatial expression via several potential Ets-like transcription factor binding sites (also called EBS) was identified. Some of these sites function as positive and others as negative regulatory elements. Also, two functional regions rich in GC-boxes that may be recognized by the zinc finger transcription factor Sp1 were identified. The experiments described suggest that Sp1 protein elevates transcription of *hSlu7*, whereas *Elk-1*, a member of the ETS transcription factor family, represses *hSlu7* transcription. Both Sp1 and *Elk-1* proteins bound the *hSlu7* promoter in vivo. Consistent with a repressive role, depletion of *Elk-1* in HeLa cells induced *hSlu7* endogenous expression. In contrast, depletion of *Sp1* repressed *hSlu7* expression. Silencing of *Elk-1* or *Sp1* proteins affected alternative splicing of specific exons. The expression pattern of *Slu7* appears to be controlled by a complex promoter arrangement and is activated or repressed by specific regulatory genes. Our data imply that *Slu7* is a splicing factor that regulates tissue- and embryonic-specific alternative splicing events.

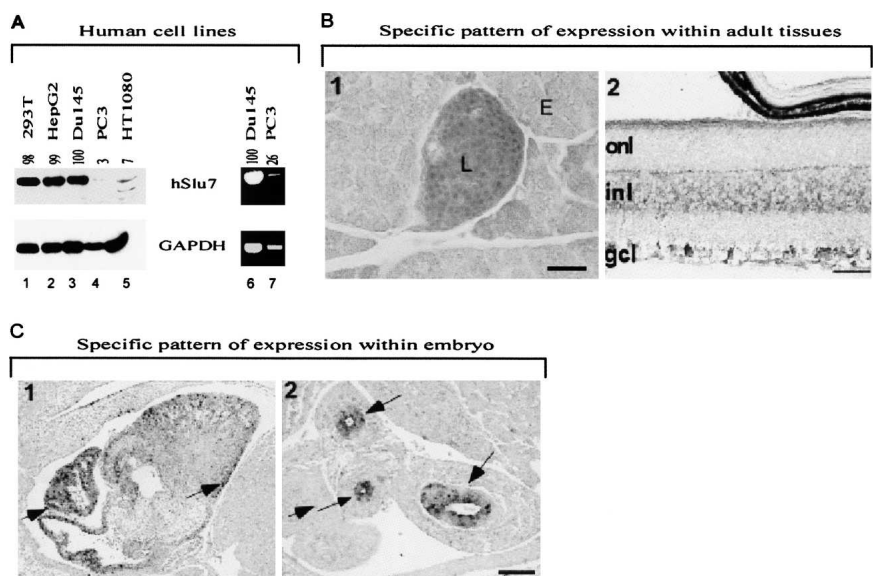
## RESULTS

### *Slu7* is differentially expressed in tissues and cell lines

We have shown previously that *hSlu7* is not required for cell survival (Shomron

et al. 2005). This observation raised the question of whether *Slu7* was expressed ubiquitously in all tissues and conditions. Thus, we analyzed *Slu7* protein and mRNA expression within various tissues and cell lines (Fig. 1). High levels of *hSlu7* protein were detected in 293T (human kidney embryonic cells), HepG2 (liver carcinoma), and Du145 (prostate carcinoma brain metastasis) cell lines (Fig. 1A, lanes 1–3). Low protein levels were detected in PC3 (prostate adenocarcinoma) and HT1080 (fibrosarcoma) cells (Fig. 1A, lanes 4,5). The mRNA level of *hSlu7* is correlated with that of the protein: high levels of both the protein and mRNA in Du145 cells and very low levels in PC3 cells (Fig. 1A, lanes 6,7). *Slu7* transcripts were also differentially expressed in various healthy adult tissues in both human and mouse (data not shown).

We then characterized mouse *Slu7* (*mSlu7*) transcript distribution within complex adult tissues from mouse. Paraffin sections of adult mouse pancreas and eye (postnatal days 30 and 15) were hybridized with a specific RNA probe against *mSlu7* mRNA (see Materials and Methods). A unique pattern of expression of *mSlu7* within the pancreas and neuroretina was observed. In the pancreas, *mSlu7* transcripts were detected in the islets of Langerhans, but not in the acinar exocrine cells (Fig. 1B, panel 1). In the



**FIGURE 1.** Evidence for differential expression of the mammalian *Slu7*. mRNA and protein levels of human and mouse *Slu7* (*hSlu7* and *mSlu7*, respectively) were analyzed in adult and embryonic tissues and cell lines. (A) Protein and mRNA levels in different human cell-line lysates were analyzed by Western blot using an anti-*Slu7* antibody (lanes 1–5) or RT-PCR (lanes 6,7). (B) In situ hybridization using an *mSlu7* antisense probe was performed on paraffin sections of pancreas and eye from postnatal day 30 (P30) and day 15 mice (P15), respectively. The expression of *mSlu7* in the adult pancreas is detected in the islets of Langerhans (L) but not in the exocrine (E) tissue (panel B1). In the eye *mSlu7* transcript was detected in the neuroretina (panel B2). (gcl) Ganglion cell layer; (inl) inner nuclear layer; (onl) outer nuclear layer. Scale bar, 100  $\mu$ m. (C) In E12.5 embryo the expression of *mSlu7* is detected in the developing heart (panel C1) and in the epithelium of the developing lung buds (arrow heads) but not in the surrounding mesenchyme (panel C2). Regions of high *mSlu7* expression are marked by arrow heads. Scale bar, 50  $\mu$ m.

neuroretina, *mSlu7* was abundant in the inner nuclear layer and the ganglion cell layer but was weakly expressed in the outer nuclear layer (Fig. 1B, panel 2) and the cornea (data not shown). Furthermore, differential tissue expression was observed in mouse embryo; *mSlu7* transcripts were abundant in the developing heart and in the epithelium of the lung buds but not in the surrounding mesenchyme (Fig. 1C, panels 1 and 2, respectively). These results demonstrate that *Slu7* is expressed differentially among tissues and during development, thus implicating *Slu7* as a context-dependent modulator of the splicing machinery.

### Cloning and analysis of the *hSlu7* promoter structure

The observation that mammalian *Slu7* was expressed differentially led us to question its transcription regulation. A rapid amplification of 5' cDNA ends (5'-RACE) analysis was used to identify the transcription start sites (TSSs) of the human *Slu7* gene (see Supplemental Material). Four alternative TSSs were identified; the most abundant was selected in 11 of the 20 clones sequenced (see Fig. 2A; Supplemental Material). The most prevalent TSS was chosen as the reference point for the reporter construct assay and was named TSS(1). It is important to note that the use of a different TSSs does not affect the coding sequence but rather changes the length of the 5' UTR. We then cloned the human *Slu7* promoter from position -184 upstream to position +66 base pairs (bp) downstream from TSS(1) into firefly luciferase reporter plasmid (Fig. 2B). Comparative analysis of the *Slu7* promoter region (covering the first exon and 184 bp upstream) from a variety of mammals revealed 100% conservation of the TSS(1) region (Fig. 2C, marked by an arrow), which implies that this region is significant, probably for recruitment of basal transcription machinery.

We analyzed the construct for promoter activity and found it to be transcriptionally active by more than  $10^3$ -fold above the empty control pGL3 vector background signal (data not shown). Next, we searched for putative *trans*-acting factor binding sites, using several computational programs (see Materials and Methods). We also examined the conservation level within the promoter region using multispecies comparative analysis (Fig. 2C). Using this analysis, two types of putative transcriptional binding sites were identified. The first to be identified were two promoter regions rich in GC that contain potential binding sites for the transcription factor *Sp1* (Fig. 2C). We also identified five tandem putative Ets binding sites (marked EBS<sub>a-e</sub>) with a core sequence of GGAA (Fig. 2C; notice that EBS<sub>c</sub> is in the reverse orientation). The Ets family of transcription factors binds to a consensus sequence CCGGAA. Only EBS<sub>a</sub> and EBS<sub>c</sub> fully matched the conserved sequence in all mammals tested, whereas EBS<sub>d</sub> and EBS<sub>e</sub> are primate specific. The Ets family of transcription factors consists of several members that are

expressed in a tissue- and a cell-type-specific manner (Sharrocks 2001; Hsu et al. 2004).

### *hSlu7* promoter activity is regulated by EBS elements

To examine the functionality of the putative EBS elements, we generated specific point mutations within the GGAA elements (the mutations are shown in Fig. 2B). 293T cells were transfected with either the p*Slu7*-luc reporter construct or the mutant reporter constructs, and cell lysates were tested for luciferase activity 48 h post-transfection. Activities of the mutant reporter constructs were normalized with respect to the wild-type (WT) p*Slu7*-luc reporter activity, and results are shown as fold induction (Fig. 2D). Point mutations within the core GGAA elements revealed that all of the five elements were required for the normal transcriptional activity of *hSlu7*, and each of the elements uniquely regulates *hSlu7* transcription in 293T cells. Three of the EBS elements, EBS<sub>a</sub>, EBS<sub>c</sub>, and EBS<sub>e</sub>, functioned as positive regulatory elements, because mutations of these elements resulted in 40%, 50%, and 75% reduction in WT activity, respectively (Fig. 2D, a,c,e). The other two EBS sites, EBS<sub>b</sub> and EBS<sub>d</sub>, functioned as repressive elements; mutations in these elements resulted in >2.5- and >1.4-fold induction in the transcriptional activity, respectively (Fig. 2D, b,d). It is notable that two of the functional elements, EBS<sub>d</sub> and EBS<sub>e</sub>, are primate specific (Fig. 2C).

Double and triple mutants revealed a compensatory relationship between the EBS sites. For example, a point mutation in the core GGAA of the highly conserved enhancer EBS<sub>a</sub> abolished the effect of mutations in either one or both of the repressors EBS<sub>b</sub> and EBS<sub>d</sub> (Fig. 2D, ab,ad,abd). Induction of the activity by mutations that abolished the repression by EBS<sub>b</sub> or EBS<sub>d</sub> was partially reduced when combined with a mutation in the enhancer element EBS<sub>c</sub> (Fig. 2D, bc,cd). Finally, deletions within the two potential *Sp1* sites, GC-rich1 and GC-rich2, resulted in moderate effects of +20% and -20%, respectively, on the promoter activity (Fig. 2D, GC-1 Del and GC-2 Del). However, deletions in both regions resulted in more than 40% reduction in basal activity (Fig. 2D, GC-12 Del) implying an important role of these regions in mediating *Sp1* induction. The mutational analyses provide evidence for the functionality of all five EBS elements and each of the two GC-rich elements in both up- and down-regulation of the *hSlu7* promoter activity.

### *hSlu7* promoter activity is up-regulated by *Sp1* in vivo

We next asked whether specific transcription factors can regulate *hSlu7* expression in vivo. Thus, 293T cells were cotransfected with the p*Slu7*-luc reporter construct and with increasing amounts (0.25, 0.5, and 0.75  $\mu$ g) of a vector expressing *Sp1* protein (pcDNA4-*Sp1*; Fig. 3A), a vector expressing Elk-1 protein (pCAG-*Elk-1*; Fig. 4A,B), or the

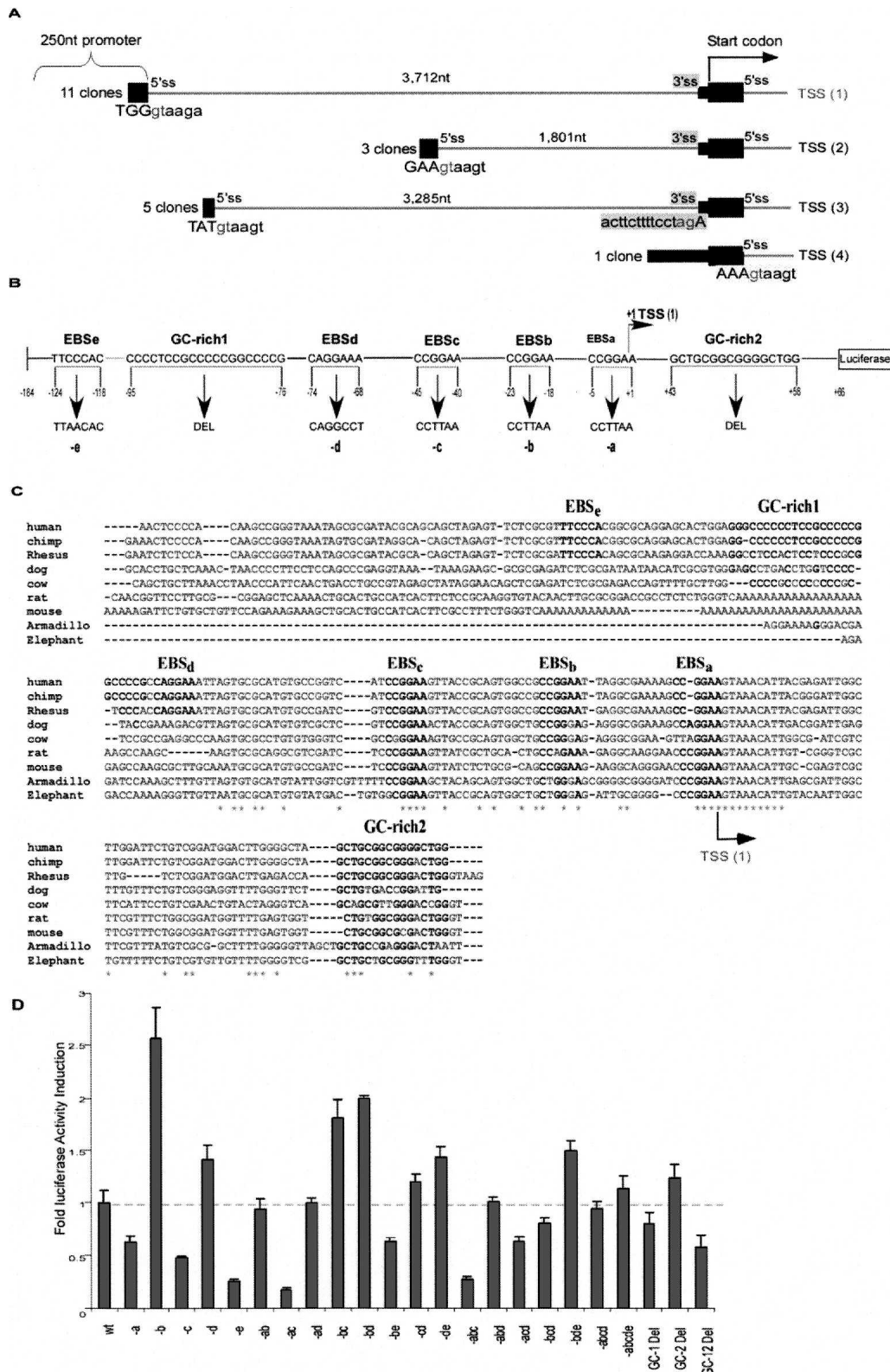


FIGURE 2. (Legend on next page)

relevant empty control vectors (pcDNA4 and pCAGGS, respectively). Cells were harvested 48 h post-transfection and cell lysates were tested for luciferase activity (Figs. 3A, 4A,B). Luciferase reporter activities were measured and normalized to the relevant empty control vectors (WT activities). All activities were also normalized to an internal control construct to normalize for transfection efficiency (see Materials and Methods). Cotransfection of Sp1 and p*Slu7*-luc vectors revealed specific and dose-dependent regulation of the *hSlu7* promoter activity, with ~1.5-fold induction at the highest dose (Fig. 3A, left panel). This induction was also observed in two other cell lines, HT1080 fibrosarcoma and U2OS osteosarcoma cells (data not shown). Deletions of either of the GC-rich elements (GC-rich1 or GC-rich2) did not abolish the Sp1-mediated activation of the *hSlu7* promoter. However, deletions in both regions significantly reduced Sp1-mediated induction from about twofold to about 1.3-fold (Fig. 3A, right panel; GC-12 Del-luc represent deletions in both GC-rich1 and GC-rich2). This suggests that these regions have important, but compensatory, functions in Sp1-mediated activation of *hSlu7* transcription. The moderate induction by Sp1 may be explained by the fact that the GC-rich regions serve as targets for many other transcription factors that might play a role in *hSlu7* transcription. These results highlight the complexities of regulation of this promoter.

We also demonstrated that Sp1 binds to *hSlu7* promoter in vivo. U2OS cells were transfected with HA-*Sp1* (in pcDNA4) using an empty pcDNA4 as a control, and cells were grown for 48 h. Chromatin immunoprecipitation (ChIP) was then performed using two sets of primers. The first targeted the *hSlu7* promoter (−140 to +44, relative to TSS(1); see Materials and Methods) and the second *hSlu7* intronic sequence (Fig. 3B, left panel intron 6, +6804 to +7002). The primers were designed to detect specific chromatin fragments immunoprecipitated by either hemagglutinin (HA) or nonspecific IgG antibodies (Fig. 3B, right panel). A specific and intense signal from the *hSlu7* promoter region was detected by PCR after immunoprecipitation with the HA antibody but not with nonspecific antibodies (Fig. 3B, right panel, cf. upper and lower

panels). This result showed that Sp1 binds the *hSlu7* promoter in vivo.

Furthermore, in situ hybridization analysis revealed that *Sp1* and *Slu7* were expressed in partially overlapping patterns in the mouse eye at postnatal day 15 (P15) within specific layers of the retina and cornea (data not shown). Altogether our results support the hypothesis that *Slu7* transcription is regulated by the zinc finger transcription factor Sp1.

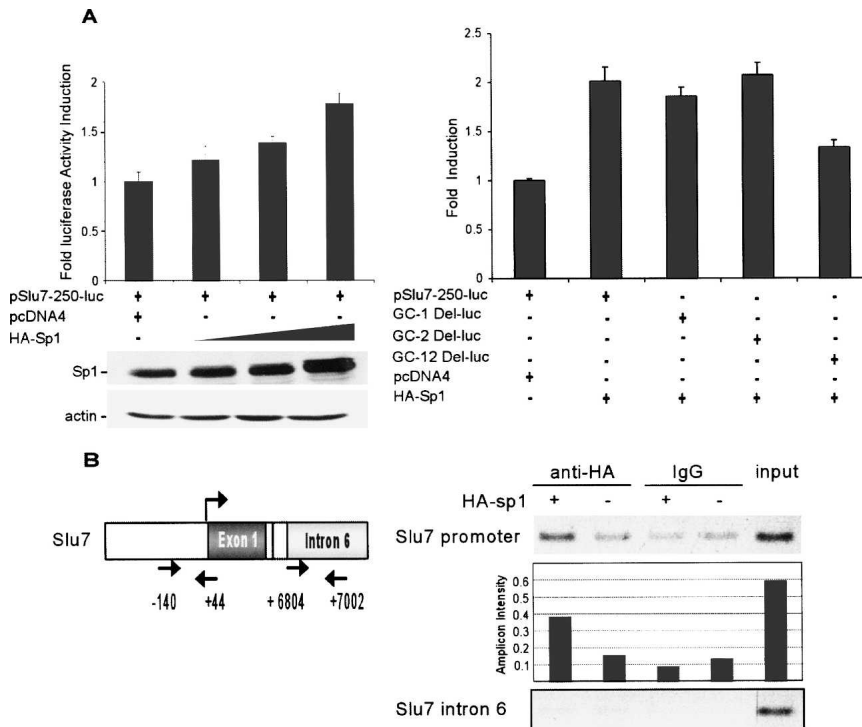
### Elk-1 binds to the *hSlu7* promoter in vivo and represses *hSlu7* expression

We then looked for a transcription factor that could serve as cellular guard that represses *hSlu7* expression. We found that Elk-1 down-regulates *hSlu7* promoter activity more than 90% in a dose-dependent manner (Fig. 4A). Another Ets transcription factor, *ERF*, which is known to have a strong transcriptional repressor activity (Sgouras et al. 1995), did not affect *hSlu7* promoter activity (not shown). This suggests that the repression activity mediated by Elk-1 is specific within the Ets family. The repression of *hSlu7* transcription by Elk-1 is presumably a general effect, as this repression was observed in all of the investigated cell lines with only minor differences (Fig. 4B). Also, repression was observed even at very low levels of transfected plasmid (50 ng), implying high sensitivity of Elk-1 to *hSlu7* promoter.

To validate the regulatory effect of Elk-1 on the *hSlu7* promoter, we examined binding of Elk-1 to the *hSlu7* promoter in vivo. ChIP assays were performed in HeLa cells using the same set of primers described for analysis of Sp1 binding to detect specific chromatin fragments immunoprecipitated by either *Elk-1* antibody or nonspecific antibodies (Fig. 5A). A specific signal from the *hSlu7* promoter region was detected by PCR after immunoprecipitation with the Elk-1 antibody but not with nonspecific antibodies (Fig. 5B). This result shows that Elk-1 protein binds to the *hSlu7* promoter in vivo.

To further validate this result, Elk-1 expression was reduced by treatment of HeLa cells with a small interfering RNA (siRNA) that targets *Elk-1*; nontargeting control

**FIGURE 2.** Comparative genomic and nucleotide sequence analysis of the *Slu7* promoter region predicts functional *Sp1* and EBS elements within the *hSlu7* promoter. (A) Alternative transcription start sites of human *Slu7* gene. Four alternative TSSs were identified using a 5'-RACE analysis. Black boxes represent exons and gray lines introns. The 250-nt promoter region is marked on the left with a brace. The distribution of the 20 clones is indicated near each TSS. The genuine start codon of *hSlu7* protein is indicated with an arrow. (B) Schematic representation of the cloned *hSlu7* promoter fused to a luciferase reporter construct. Seven putative transcription factor binding sites are indicated at the top. Arrows indicate specific mutations at the EBS and *Sp1* sites (below). Transcription start site of the most prevalent TSS [TSS(1)] was used as a reference point and is indicated with an arrow. (C) Multiple sequence alignment of the 5' region (including the first exon) of the *Slu7* gene from several mammalian organisms is shown. Alignments were generated using the ClustalW algorithm (Thompson et al. 1994). Putative Ets (GGAA core element; EBS<sub>e</sub> is in an inverted orientation) and GC-rich/*Sp1* putative binding sites are marked above the alignment. (D) Functionality of the EBS elements and regulation of *Slu7* promoter activity. 293T cells were cotransfected with either the wild type (wt) p*Slu7*-luciferase (p*Slu7*-luc) reporter plasmid [containing the −184 to +66 promoter region, with respect to TSS(1)] or various mutant reporter constructs containing single point mutations or combinational point mutants in the core GGAA elements (see also Materials and Methods) or deletions in the GC-rich sites (panel B shows the mutations). Relative luciferase activity was examined 48 h post-transfection and normalized to the wt promoter activity (shown by a broken line). All results are represented as the mean of at least three independent experiments (standard error bars are shown; *n* = 3).



**FIGURE 3.** Sp1 up regulates *hSlu7* promoter activity with 750 ng of pcDNA4-*Sp1*. (A, left panel) Induction of *hSlu7* promoter activity by Sp1. 293T cells were cotransfected with 250 ng of reporter construct (pSlu7-luc) together with 250 ng, 500 ng, or 750 ng of either the expression plasmid pcDNA4-*Sp1* or the corresponding pcDNA4 empty control vector. The fold induction represents the fold increase in luciferase activity 48 h post-transfection relative to that obtained following cotransfection of the reporter construct with control vector (pcDNA4). (Lower panel) Western blotting of 293T transfected cells with either anti-*Sp1* or anti-actin antibodies. (Right panel) Sp1 mediated induction is significantly impaired only after deletion of the two GC-rich regions. Similar analysis as showed in the left panel using mutants with deletion of GC-rich1 or -2 (GC-1 Del-luc and GC-2 Del luc, respectively) or deletions of both GC-rich regions (GC-12 Del-luc) (see also Fig. 2B) with 750 ng of pcDNA4-*Sp1*. All luciferase activities are normalized to the wt activity (reporter and empty pcDNA4 vector). Results are represented as the mean of at least three independent experiments (standard errors bars are shown;  $n = 3$ ;  $P$ -values represent  $T$ -test for each DNA dose group; 0.046, 0.020, and 0.007 for 250 ng, 500 ng, and 750 ng, respectively). (B) Sp1 binds to the *Slu7* promoter in vivo. Schematic diagram of the *Slu7* gene showing the location of the oligonucleotides used in the chromatin immunoprecipitation assay (left panel). U2OS cells were transfected with HA-*Sp1* and an empty pcDNA4 and grown for 48 h. Chromatin was precipitated using anti-HA or normal mouse IgG antibodies and used as a template for PCR analysis using primers spanning the promoter region of *hSlu7* (right panel; upper panel) and intron 6 (right panel; lower panel) as a control (see Materials and Methods for primers). Quantitative analysis of PCR amplicon intensities is shown in the lower part of the upper gel. Input sample comprised 5% of the total precipitated DNA.

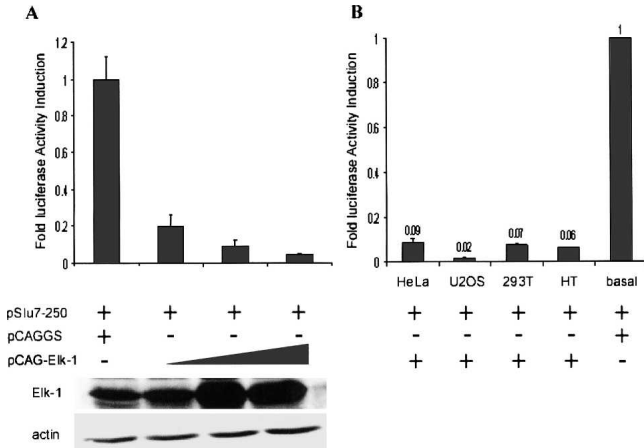
siRNA was used as a control (Materials and Methods). The reduction in the cellular concentration of Elk-1 was determined by Western blotting (Fig. 5C, left panel). ChIP analysis indicated that in the absence of Elk-1 protein the *hSlu7* promoter was not precipitated (Fig. 5C, right panel). To confirm that Elk-1 indeed repressed endogenous *hSlu7* expression, RT-PCR was used to determine the effect of knockdown of Elk-1 on the endogenous *hSlu7* mRNA, *Elk-1* mRNA, and on 18S rRNA expression. Reduction in Elk-1 levels caused a 3.5-fold induction of endogenous *hSlu7* (Fig. 5D, left graph), while *Elk-1* mRNA expression

was reduced >4-fold (Fig. 5D, middle graph). No significant change was observed in 18S rRNA expression (Fig. 5D, right graph). These results support the idea that *hSlu7* expression is tightly regulated by Elk-1 and also suggests a physiological role of Elk-1 in down-regulation of *hSlu7* expression.

### The effects of Sp1 and Elk-1 on *hSlu7* affect alternative splicing

To examine if the effects of Sp1 and Elk-1 on the transcription of *hSlu7* translate into effects on alternative splicing, we reduced the cellular concentrations of Sp1 and Elk-1 using RNA interference and examined the effects on alternative splicing. We previously showed that the nuclear concentration of *hSlu7* affects alternative splicing of exon 8 of the *ADAR10* mini-gene and of exon 4 of the *DDO* gene (Shomron et al. 2004, 2005). Figure 6 shows that treatment of cells with a short hairpin RNA (shRNA) specific for *Sp1* gene reduced Sp1 and *hSlu7* protein levels significantly and also reduced inclusion of exon 8 of *ADAR10* mini-gene (Fig. 6A, panel ii; Fig. 6B, panel i; cf. lanes 1 and 2 in all panels). To ensure specificity, the shRNA-*Sp1* treated cells were cotransfected with Sp1 cDNA containing a silent mutation that prevents shRNA-directed degradation (see Materials and Methods) or an empty control pcDNA4 construct (Fig. 6A,B lanes 3,4, respectively). The silent *Sp1* mutant restored both Sp1 and *hSlu7* expression levels (Fig. 6A, lanes 3,4) and also restore exon 8 inclusion (Fig. 6B, lanes 3,4). The siRNA treatment did not affect the transcription of *ADAR2* (data not shown). This indicates that the reduction in the inclusion

of exon 8 after knocking down Sp1 did not derive from a decrease in the steady-state expression of the reporter construct. Also, Elk-1 siRNA treatment reduced Elk-1 protein concentration and thus enhanced the inclusion level of exon 4 of the *DDO* gene (Fig. 5D,E, respectively). These results suggest that the cellular concentration of the transcription factors that regulate *hSlu7* transcription affects the level of exon inclusion or skipping in alternative splicing of specific exons. We demonstrated that two different transcription factors cause opposite effects on two different splicing events through regulation of *hSlu7*



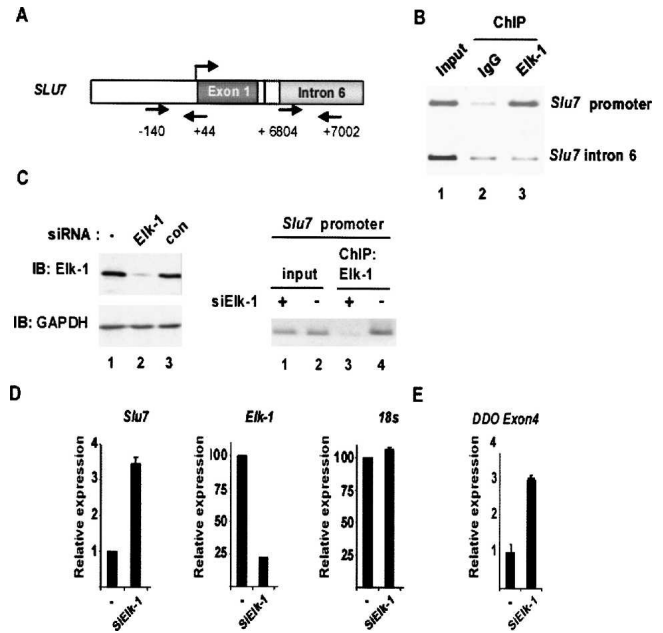
**FIGURE 4.** Repression of *hSlu7* promoter activity by Elk-1. (A) 293T cells were cotransfected with 250 ng of reporter construct (p*Slu7*-luc) together with 100 ng, 250 ng, or 500 ng of either the expression plasmid pCAG-*Elk-1* or the corresponding pCAGGS empty control vector. The fold induction represents the fold increase in luciferase activity 48 h post-transfection relative to that obtained following cotransfection of the reporter construct with a control vector (pCAGGS). Western blotting of 293T transfected cells with either anti-*Elk-1* or anti-actin antibodies are also shown (bottom panels). (B) Elk-1-mediated repression of the *hSlu7* promoter occurred in several cell lines. Cell lines (HeLa, U2OS, 293T, and HT1080) were cotransfected with 250 ng of reporter construct (p*Slu7*-luc) together with 250 ng of either the expression plasmid pCAG-*Elk-1* or the corresponding pCAGGS empty control vector. Results are represented as the mean of at least three independent experiments (standard errors bars are shown;  $n = 3$ ;  $P$ -values represent  $T$ -test for each DNA dose group: 0.0016, 0.00036, and 0.00031 for 100 ng, 250 ng, and 500 ng, respectively).

expression. We cannot exclude the possibility that the cellular concentrations of Sp1 and Elk-1 also affect transcription of other splicing factors involved in alternative splicing regulation of those exons. However, the splicing of the two genes analyzed was previously shown to be affected directly by the cellular concentration of hSlu7 (Shomron et al. 2004, 2005).

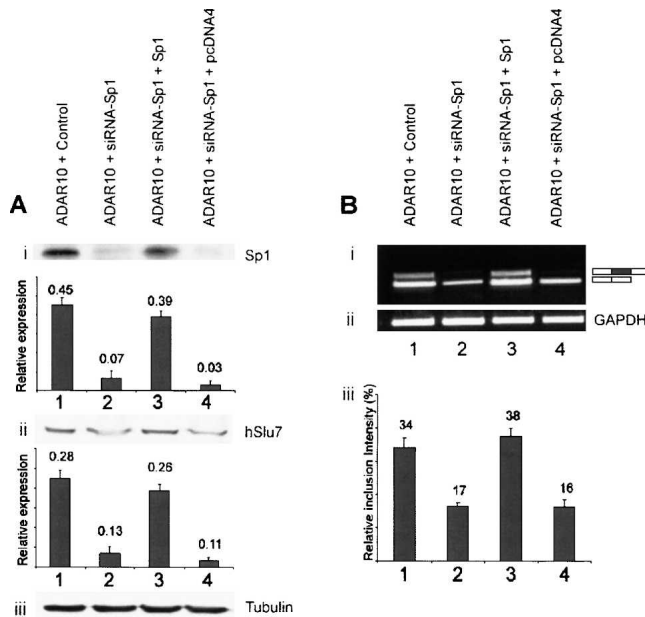
**DISCUSSION**

Previously, it was reported that the splicing factor hSlu7, a protein involved in the fidelity of the second step of splicing, is not required for cell viability but that the nuclear concentration of the protein regulates alternative splicing of certain genes (Shomron et al. 2004, 2005). This raised the question of whether *Slu7* expression is regulated among tissues. In this study, we demonstrated that *Slu7* is differentially expressed among various tissues and cell lines. Moreover, *Slu7* has unique patterns of expression within complex adult tissues and developing embryos, such as eye and pancreas. We also demonstrated that *hSlu7* transcription is regulated by at least two transcription factors, Sp1 and Elk-1, which might explain its differential expression.

It is intriguing that a splicing factor, one component of a megacomplex, has such a sophisticated mechanism for transcription regulation. Although very little is known about transcription regulation of other splicing factors (for example, see Romanelli et al. 2005), regulation of transcription of other nonsplicing proteins has been characterized (see,



**FIGURE 5.** Elk-1 binds to the *Slu7* promoter in vivo and knocking down Elk-1 induces *hSlu7* expression. (A) Schematic diagram of the *Slu7* gene showing the location of the oligonucleotides used in the ChIP assay. (B) Elk-1 binds to *hSlu7* promoter in vivo. HeLa cells were starved in serum-free DMEM for 48 h before harvesting. Sonicated chromatin was immunoprecipitated with either an anti-*Elk-1* antibody or nonspecific IgG. PCR analysis of eluted DNA was performed using oligonucleotides specific for the *Slu7* promoter (top panel) or *Slu7* intronic sequence (lower panel). Five percent of input DNA is shown in lane 1. The panels shown are inverted images of ethidium bromide-stained gels. Results shown are representative of four independent experiments. (C) Chromatin immunoprecipitation of Elk-1 bound to the *Slu7* promoter in the presence or absence of a siRNA directed against Elk-1. HeLa cells were transfected with *Elk-1* siRNA or a negative control siRNA 48 h before harvesting. Sonicated chromatin was immunoprecipitated with either an anti-*Elk-1* antibody or nonspecific IgG. Right panel shows PCR analysis of eluted DNA using oligonucleotides specific for the *Slu7* promoter (5% of input DNA is shown in lanes 1 and 2). Left panel shows a Western blot of HeLa cells treated in parallel. Results shown are representative of two independent experiments. (D) Knocking down Elk-1 levels induces expression of *Slu7* mRNA. HeLa cells were transfected with *Elk-1* siRNA for 48 h before harvesting total RNA. RT-PCR was performed to detect endogenous *Slu7* mRNA (left graph), *Elk-1* mRNA (middle graph), and 18S rRNA expression (right graph). Results shown are representative of three independent experiments. (E) Knocking down Elk-1 levels results in increased inclusion of alternatively spliced exon 4 of the *DDO* gene relative to cells treated with control siRNA. HeLa cells were transfected with Elk-1 siRNA for 48 h before harvesting total RNA. Real time RT-PCR was performed to detect endogenous *DDO* mRNA containing alternatively spliced exon 4. Results shown are representative of three independent experiments.



**FIGURE 6.** Knocking down Sp1 represses *hSlu7* expression and thus affects alternative splicing. (A,B) 293T cells were transfected with *ADAR10* mini-gene and with control shRNA expressing pSUPER retro (lane 1), shRNA targeting Sp1 (lane 2), or shRNA targeting Sp1 and also Sp1 expression vector containing a silent mutation that eliminates the shRNA activity (marked \*Sp1, lane 3) or a control empty pcDNA4 vector (lane 4). The cells were incubated for 72 h before harvesting and analysis of total protein (panel A, i,ii,iii) or RNA (panel B). Samples were analyzed by Western blot with anti-Sp1, anti-tubulin antibodies (panel A, i,iii, respectively), or anti-*hSlu7* (panel A, ii). Band intensities were normalized to tubulin and quantitative analysis of both Sp1 and *hSlu7* are shown at the bottom of each blot. Experiments were repeated three times in duplicate (error bar are shown). The two mRNA products and the control GAPDH mRNA are indicated (panel B, i,ii, respectively). Quantitative analysis of the inclusion levels of exon 8 of the *ADAR10* for each experiment is shown (panel B, iii, in percentages). Experiments were repeated three times in duplicate (error bars are shown).

for example, Elkon et al. 2005) and exhibits patterns similar to the one described here. In addition, regulation of individual protein components of other large complexes, such as the DNA repair complex, is similar (Iwanaga et al. 2004). The results described here add an additional fascinating dimension to spliceosome assembly, namely, the timely expression of each component dictates the interplay with other proteins and ultimately dictates protein expression from specific genes. We cannot rule out that *Slu7* has roles in processes independent of its involvement with the spliceosome. Finally, *Slu7* transcriptional regulation is another layer in the finely tuned regulation already described in our previous study involving binding of zinc to protein (Shomron et al. 2004).

Our results indicate that there are cells and tissues that lack or express negligible levels of *Slu7*. Wide-scale studies of splicing complexes revealed that some spliceosomal complexes did not contain *Slu7* (for reviews, see Will and

Luhrmann 1997; Zhou et al. 2002; Jurica and Moore 2003; Deckert et al. 2006). Chau and Reed (1999a,b) have isolated a spliceosomal complex lacking *hSlu7* that loosely holds the free exon 1 (after the first step of mRNA splicing). The absence of *hSlu7* causes aberrant attachment on different 3' ss-AGs. The complex interplay among splicing factors during spliceosome assembly is demonstrated by a number of results. The requirement of certain splicing factors for specific steps of splicing can be compensated by other factors (Chen et al. 2001; Kistler and Guthrie 2001). For example, mammalian UAP56 is required for pre-spliceosome formation. In the absence of the *Mud2* gene (yeast homolog for *U2AF65*), which interacts with SUB2 (yeast *UAP56* homolog), SUB2 functionality becomes dispensable (Kistler and Guthrie 2001). In other cases, *Prp28*, a DEAD-box protein, is required to promote the exchange of U1 for U6 at the 5' ss, but becomes dispensable after knockout of the U1C snRNP protein, which stabilizes the U1-5' ss interaction (Chen et al. 2001). *Sub2* and *Prp28* may have roles in splicing regulation and fidelity maintenance (Kistler and Guthrie 2001). Redundancy of *Slu7* can bypass the need for another second-step splicing factor, *Prp18*, in vitro (Zhang and Schwer 1997).

It is also worth noting that most of the research on splicing assembly and factor requirements has been conducted in vitro and/or in specific spliceosomal complexes (for example, Adeno ML and  $\beta$ -globin driven transcripts incubated in HeLa nuclear extract). It may be that the essential functions attributed to *hSlu7* are restricted to those conditions. For example, knocking down specific splicing factors in mouse revealed tissue-specific alternative splicing abnormalities and functionality (Ladd et al. 2005; Ule et al. 2005). The tight regulation on the *hSlu7* promoter implies that the essential function of this protein might be restricted to certain cells/tissues or stages of embryonic differentiation. Although dispensable in cultured cells, *Slu7* is likely to be essential in the context of a whole organism. For instance, *Sp1* null/null mouse embryos die at 10 d of gestation (Marin et al. 1997; Pore et al. 2004). Redundancy of a related transcription factor, *Sp3*, may compensate for *Sp1* absence (Bouwman et al. 2000).

We also suggest that the *Slu7* promoter underwent changes throughout evolution. These changes may have served as a mechanism to shape *Slu7*'s unique pattern of expression. We show here possible mechanisms for up- and down-regulation of *hSlu7* expression. Sp1 binds to the *hSlu7* promoter in vivo and up-regulates *Slu7* promoter activity in a dose-dependent manner. Depletion of this transcription factor was also shown to repress *hSlu7* expression. Sp1 recognizes GC-rich promoter elements, and, although it was reported to be ubiquitously expressed, it is also found to regulate many tissue-specifically expressed genes, viral genes, and cell-cycle-regulated genes (Bouwman and Philipsen 2002). Sp1 expression is variable in adult and developing embryonic tissues (Saffer et al. 1991; Nakamura et al. 2005).



These observations imply that Sp1 might have a role in supporting *hSlu7* expression in a cell-type-specific manner.

Elk-1 repressed transcription from the *hSlu7* promoter and bound to the *hSlu7* promoter in vivo. Moreover, siRNA-induced reduction of Elk-1 increased endogenous *hSlu7* expression, suggesting that the *Slu7* promoter is down-regulated by Elk-1. Elk-1-mediated repression was shown to be dose dependent in several cell lines. However, Elk-1-mediated repression of *hSlu7* transcription was not completely abolished even after deletion of all five potential *Elk-1* sites (data not shown). We also did not detect any potential serum response elements (SREs) in the promoter (these elements are required for SRF-dependent Elk-1 DNA binding). The repressive effects of Elk-1 are consistent with previous observations that Elk-1 associates with repressive complexes (Yang et al. 2001; Yang and Sharrocks 2004). The exact repression mechanism remains to be elucidated.

Gene expression profiles from microarray data sets revealed that in the PC3 prostate cell line there is an extremely low expression level of Sp1 (GEO accession GDS1736), whereas Elk-1 is expressed. This further corroborates results from our Sp1 siRNA assay that showed a significant reduction in *hSlu7* expression when Sp1 expression was reduced. We could not use this method to confirm the effects of Elk-1 expression levels on *hSlu7* protein expression because no cell lines were found in which Elk-1 expression is very low or absent. We cannot rule out the possibility that other factors may contribute to the expression level of *Slu7*.

We have shown here that the temporal and spatial expression of the mammalian splicing factor *Slu7* is elaborately regulated among tissues and during embryogenesis. The involvement of *hSlu7* in regulation of alternative splicing of certain exons might indicate that *hSlu7* is essential for tissue- and differentiation-specific alternative splicing events.

## MATERIALS AND METHODS

### Isolating the 5' ends of human *Slu7* mRNA

5'-RACE was performed according to the circular, or concatemeric, RACE methodology (Maruyama et al. 1995) in extract from 293T cells with a human *Slu7* gene specific primer: 5'-TCCTCAGAGT TAACAATCTCCTTCC-3'. The first PCR primers were 5'-TGC TGGAGATAACTTTGTTAGGTACAC-3' and 5'-CTCATTTCCTT GGACCCCGATA-3'. The second, nested PCR primers were 5'-ACCATTTC AATGGCTCAGACAC-3' and 5'-TGTGGCTGACAT GGTATCTGG-3'. The PCR product was eluted and purified following gel electrophoresis. After cloning into TOPO TA cloning vector (Invitrogen), 20 colonies were sequenced.

### Reporter and effector constructs

The 5'-flanking regions of the human *Slu7* gene [−184 to +66 relative to TSS(1)] were amplified from genomic DNA and

inserted upstream of the firefly luciferase gene in the reporter vector pGL3-basic (Promega) to create p*Slu7*-luc. Mutations were introduced using overlapping oligonucleotide primers containing the desired mutation. PCR was performed using the high-fidelity DNA polymerase UltraPfu (Stratagene); then reaction products were digested with DpnI restriction enzyme (New England Biolabs) for 1 h at 37°C. A 5 μL aliquot of the reaction was used to transform the *Escherichia coli* DH5α strain, and DNA from positive colonies was extracted using a Mini-prep extraction kit (Qiagen). Primers were generated harboring the desired mutation (mutated nucleotide in bold) to abolish the GGAA core elements:

EBS\_a\_GG2TT\_Fw 5'-GCCGGAATTAGGCGAAAAGCCTTAAGT AAACATTACGAGATTGG-3';  
 EBS\_a\_GG2TT\_Rv 5'-CCAATCTCGTAATGTTTACTTAAGGCT TTTCGCCTAATTCCGGC-3';  
 EBS\_b\_GG2TT\_Fw 5'-TACCGCAGTGGCCGCTTAATTAGGC GAAAAGCC-3';  
 EBS\_b\_GG2TT\_Rv 5'-GGCTTTTCGCCTAATTAAGGCGGCC ACTGCGGTA-3';  
 EBS\_c\_GG2TT\_Fw 5'-CATGTGCCGGTCATCCTTAAGTTACC GCAGTGGC-3';  
 EBS\_c\_GG2TT\_Rv 5'-GCCACTGCGGTAACCTTAAGGATGACCG GCACATG-3';  
 EBS\_d\_AAA2CCT\_Fw 5'-CCCGCCCCGCCAGGCCTTTAGTG CGCATGTGC-3';  
 EBS\_d\_AAA2CCT\_Rv 5'-GCACATGCGCACTAAAGGCCTGGC GGGGCCGGG-3';  
 EBS\_e\_CC2AA\_Fw 5'-GAGTTCTCGCGTTTAAACACGGCGCAG GAG-3';  
 EBS\_e\_CC2AA\_Rv 5'-CTCCTGCGCCGTGTTAAACGCGAGAA CTC-3'.

The same primers were used to create all the combination mutants. Deletion mutants were created using two phosphorylated primers that flanked the desired regions followed by ligation of the clean PCR construct product. The pCAGGS (control vector) and pCAG-*Elk-1* constructs were kindly provided by Dr. Hiroshi Kubota (Kyoto University) (Yamazaki et al. 2003). *Sp1* mutated at the siRNA recognition site was cloned within the mammalian expression construct pcDNA4 (data not shown). Nucleotide sequences of the reporter and effector constructs were confirmed by sequencing.

### Cell line maintenance, transfection, and reporter gene assay

HeLa, 293T, HT1080, and U2OS cells were grown on six-well plates and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1 U/mL nystatin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were grown for 24 h and then transfected with 250 ng of reporter construct together with 250 ng (or other dose as indicated) effector construct, including 20 ng of internal control reporter vector (pRL-SV40; Promega) using FuGENE6 (Roche), as described in the manufacturer's protocol. Cell lysates were prepared and luciferase activities of transfected cells were determined using the dual luciferase assay system (Promega), according to the manufacturer's instructions, and the activity of firefly

luciferase was normalized against that of the sea pansy enzyme (*Renilla reniformis*).

### RNA isolation, RT-PCR analysis, PCR, and quantitative RT-PCR

Cells were grown in a 100-mm culture dish and were harvested 48 h after transfection. Total cytoplasmic RNA was extracted using TRI Reagent (Sigma), followed by treatment with 2 U DNase (RNase-Free; Ambion). Tissue samples from three adult mice were homogenized in TRI reagent (1 mL/30–100 mg tissue). First-strand oligo(dT)-primed cDNA synthesized with reverse transcriptase from avian myeloblastosis virus (RT-AMV; Roche) from 1000 ng of total RNA was amplified with High Fidelity Taq polymerase (Roche) and *DDO*, *GAPDH*, *mSlu7*, *mPrp18*, and *Tubulin β5* primers for 30 cycles, consisting of 94°C for 30 sec, 60–65°C for 45 sec, and 72°C for 1 min. The products were separated in 2% agarose gel and confirmed by sequencing. For quantitative RT-PCR, total RNA was harvested using an RNeasy kit (Qiagen). RNA (40 ng) was used in a one-step RT-PCR reaction using Quantitect SYBR green reagent (Qiagen) and the following primers:

*Slu7* forward, 5'-GTGGCCAAGAACATTTGGAT-3';  
*Slu7* reverse, 5'-CATCGGCCTTCTTCCAGTA-3';  
*Elk-1* forward, 5'-GGTGGTGAATTCAAGCTGGT-3';  
*Elk-1* reverse, 5'-ATTTGGCATGGTGGAGGTAA-3';  
 18S forward, 5'-TCAAGAACGAAAGTCGGAGGTT-3';  
 18S reverse, 5'-GGACATCTAAGGGCATCACAG-3'.

### Computational analyses

The *hSlu7* promoter sequence was scanned for binding sites for transcription factors using the following programs: TRANSPLORER (<http://www.developmentontheedge.com/transplorer.shtml>); Genomatix (<http://www.genomatix.de/>); NCITE (<http://www.softberry.com/berry.phtml>); Signal Scan (<http://bimas.dcrf.nih.gov/molbio/signal/>); and TFSEARCH (<http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>). Multiple alignment of the *hSlu7* promoter region alignment was done using the ClustalW algorithm (<http://www.ebi.ac.uk/clustalw/>).

### In situ hybridization

In situ hybridization analysis was performed as described by Yaron et al. (2006). For digoxigenin-labeled antisense RNAs, reverse transcription was performed on oligo(dT)-primed cDNA from mouse adult testis and brain tissues, and the resulting cDNA was used as a template for PCR. Standard PCR conditions were used with an annealing temperature of 68°C for 31 cycles. The PCR primers were *mSlu7\_973\_F*, 5'-GCTCAAACACAACCTGTTTGCTTGG-3' and *mSlu7\_3UTR\_R*, 5'-TAATACGACTCAC TATAGGGCAGAGGACTGACGGCATGTACAT-3'. For the sense negative control *mSlu7* probes, the same primers were used except that the T7 promoter tag was switched from the reverse to the forward primer. The resulting 1364-bp PCR products were analyzed and extracted from a 1% agarose gel, and the purified PCR products, including a 5'-T7 minimal promoter tag, were used to create the related digoxigenin-labeled antisense in vitro using DIG RNA labeling mix (Roche). The *Sp1* probes and

primers were used as reported before (Gray et al. 2004; Nakamura et al. 2005).

### RNA interference

HeLa cells were transfected with 70 nM *Elk-1* siRNA (Dharmacon) or nontargeting control siRNA (Santa Cruz Biotechnology) using oligofectamine (Invitrogen) according to the manufacturer's protocol. HEK 293T cells were transfected with pSUPER.retro (Oligoengine) *Sp1*, and *GFP* control vectors expressing short hairpin (shRNA) against *Sp1* corresponding to cDNA position 396 using fugene6 transfection reagent (Roche). For the *Sp1* rescue analysis cells were also cotransfected with pSUPER.retro *Sp1* and with either rescue construct expression *Sp1* (mutated at the siRNA recognition site) or an empty control vector (pcDNA4; Invitrogen). At 72 h post-transfection, cells were harvested for protein and RNA preparation. Primers for the real time RT-PCR of *DDO* were exon3\_Fw, 5'-CATTACACGCAGAAGCAGT-3', and *DDO*\_exon4\_Rv, 5'-GGGTTGTAAGCCTGACCA-3'. Primers for detection of the inclusion level of exon 8 of *ADAR10* transcript were described before (Lev-Maor et al. 2003).

### Chromatin immunoprecipitation

U2OS cells were transfected with HA-*Sp1* (in pcDNA4) for the immunoprecipitation of exogenous hemagglutinin (HA) tagged *Sp1* proteins and an empty pcDNA4 as a control and were grown for 48 h. HeLa cells were untransfected (for the immunoprecipitation of endogenous Elk-1 proteins). Cells were then treated with 1% formaldehyde for 10 min at room temperature before quenching with 0.125 M glycine for 5 min. Cells were harvested in ice-cold PBS with complete protease inhibitors (Roche), washed sequentially with BufferI (10 mM HEPES at pH 6.5, 0.5 mM EGTA, 10 mM EDTA, 0.25% Triton X-100) and BufferII (10 mM HEPES at pH 6.5, 0.5 mM EGTA, 1 mM EDTA, 200 mM NaCl), and then resuspended in SDS lysis buffer (50 mM Tris at pH 8.1, 10 mM EDTA, 1% SDS). Lysates were sonicated on ice to yield 200–800-bp DNA fragments. One quarter of a 10-cm dish was used per IP, diluted 1/10 in IP Dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris at pH 8.1, 167 mM NaCl), and incubated overnight at 4°C with either 1 μg of *Elk-1* antibody (Santa Cruz Biotechnology) for HeLa cells or anti HA F-7 (Santa Cruz Biotechnology) for U2OS cells or 1 μg non-specific IgG (Upstate Biotechnology) for both cell types. Immunocomplexes were precipitated by incubation for 30 min with protein A-conjugated magnetic beads (Dynal) that had been preblocked by incubation with 10 μg salmon sperm DNA. Immunoprecipitates were washed sequentially with TSEI (20 mM, Tris at pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), TSEII (20 mM Tris at pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), BufferIII (10 mM Tris at pH 8.1, 0.25 M LiCl, 1 mM EDTA, 1% NP40, 1% DOC), and TE before eluting in 1% SDS/0.1 M NaHCO<sub>3</sub>. Cross-links were reversed by heating to 65°C overnight, then treating with proteinase K for 1 h at 45°C. Chromatin was cleaned using QiaQuick PCR cleanup columns (Qiagen). PCR was performed using specific primers to the human *Slu7* promoter (−140 to +44, relative to TSS(1); forward, 5'-GCTAGAGTTCTCGCGTTTCC-3'; reverse, 5'-CCAAGTCCATCCGACAGAAT-3') or *Slu7* intronic sequence (intron 6, +6804 to +7002 relative to TSS(1); forward,

5'-TGCAGTCAGTTTGGGAACAA-3'; reverse, 5'-TTCCTGTTC CTGGACATTT-3').

## Western blotting

Lysis buffer (50 mM Tris at pH 7.5, 1% NP40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, protease inhibitor cocktail, and phosphatase inhibitor cocktails I and II; Sigma) was used for protein extraction. Lysates were cold centrifuged for 30 min at 14,000 rpm. Total protein concentrations were measured using BioRad Protein Assay (Bio-Rad). Proteins were separated in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto a Protran membrane (Schleicher and Schuell). The membranes were probed with either anti-*Elk-1*, anti-actin antibody (Santa Cruz Biotechnology), anti-*hSlu7* (Shomron et al. 2004; Abnova), or anti-*Sp1* (BL938, Bethyl Laboratories), and anti- $\alpha$ -tubulin (B512; Sigma) followed by the appropriate secondary antibody. Immunoblots were visualized by enhanced chemiluminescence (Lumi-Light Western Blotting Substrate; Roche) and exposure to X-ray film. For ChIP assay, Western blotting was performed using Supersignal West Dura Extended Duration Substrate (Pierce) and primary antibodies anti-*Elk-1* (Santa Cruz Biotechnologies) and anti-*GAPDH* (Abcam). Data were visualized using Bio-Rad Fluor-S MultiImager and Quantity One software (Bio-Rad).

## Image processing and microscopy

Acquisition of images and measurement of DNA intensity was performed using TINA, ImageJ, and analySIS software (Soft Imaging System). Most of the results represent values obtained from at least three separate experiments, and the results are average values. Fluorescent images were taken with a confocal laser-scanning system, consisting of an SLM 410 Zeiss confocal microscope with a 20 $\times$  or 40 $\times$  oil objective.

## SUPPLEMENTAL DATA

Supplemental Material is available at [http://www.tau.ac.il/~gilast/sup\\_mat.htm](http://www.tau.ac.il/~gilast/sup_mat.htm).

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