Stress alters the subcellular distribution of hSlu7 and thus modulates alternative splicing

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Summary

During pre-mRNA splicing, introns are removed and exons are ligated to form an mRNA. Exon choice is determined by different nuclear protein concentrations varying among tissues and cell types or by developmental stage. These can be altered by different cellular circumstances such as physiological stimuli, environmental effects and phosphorylation state. The splicing factor hSlu7 plays an important role in 3′ splice site selection during the second step of splicing in vitro and has been suggested to affect alternative splicing in vivo. Our results indicate that an ultraviolet-C (UV-C) stress stimulus triggers changes in the alternative splicing patterns of cellular genes by decreasing the nuclear concentration of hSlu7 through the modulation of its nucleus-to-cytoplasm transport. This shift is mostly dependent on the Jun N-terminal kinase (JNK) cascade. Although we found by RNAi knockdown that hSlu7 is not essential for cell viability, its nuclear concentration effects exon choice and inclusion:skipping ratio of alternative splicing. A possible spatial and temporal regulatory mechanism by which hSlu7 protein levels are regulated within the nucleus is suggested, thus implying a broad effect of hSlu7 on alternative splicing.

Key words: hSlu7, mRNA splicing, Nuclear-cytoplasmic shift, Stress, Cellular localization, Alternative splicing, Spliceosome, snRNP

Introduction

Most RNA-polymerase-II transcripts of higher eukaryotes are synthesized as precursor mRNA (pre-mRNA) containing exons and introns. During the splicing reaction, introns are removed and exons are ligated to form an mRNA, which is transported to the cytoplasm and translated to form a protein (Burge et al., 1999; Jurica and Moore, 2003; Rappsilber et al., 2002). Four short degenerate sequences are essential for splicing; the 5′ and 3′ splice sites (5′ss and 3′ss) (GU and AG, respectively) located at the 5′ and 3′ ends of the intron, and the polypyrimidine tract and branch-site sequence, which are located upstream of the 3′ss (Brow, 2002). Alternative splicing, in which different subsets of exons or splice sites in a given pre-mRNA are chosen, is a major mechanism that diversifies the genetic information by producing more than one type of mRNA from a single gene (Graveley, 2001). Exon choice is determined by different nuclear protein concentrations that vary among tissues (Hanamura et al., 1998) and cell types (Jensen et al., 2000) or during development (Lallena et al., 2002; Mahe et al., 2000; Smith and Valcarcel, 2000; Wang and Manley, 1995), and these can be altered by various cellular circumstances such as physiological stimuli (van der Houven van Oordt et al., 2000), environmental effects (Meshorer et al., 2002) or phosphorylation state (Xie et al., 2003). Aberrant regulation of splicing has been implicated in an increasing number of human diseases (Philips and Cooper, 2000; Stamm, 2002; Stoilov et al., 2002), including cancer (Hastings and Krainer, 2001; Modrek and Lee, 2002; Stoilov et al., 2002).

The splicing protein Slu7 (Frank and Guthrie, 1992) (synergistic lethal with U5 snRNA) and its human homolog hSlu7 (Chua and Reed, 1999a) have been shown to play important roles in 3′ss selection during step two of splicing. Human nuclear extract immunodepleted of hSlu7 reduces the affinity of the 5′ exon to the spliceosome, leading to different selection of AGs at the 3′ss. This suggests a role for hSlu7 in holding the 5′ exon within the spliceosome for the correct 3′ss attack (Chua and Reed, 1999b). Both Slu7 and hSlu7 act in selection of the distal 3′ss when the distance between the branch point and the 3′ss is greater than 7 or 23 nucleotides, respectively (Chua and Reed, 1999b; Zhang and Schwer, 1997). Although many attempts have been carried out to define the list and function of each protein involved in the splicing reaction, the comprehensive picture is still far from understood.

We have recently identified by bioinformatics and mutational analyses three functional domains of the hSlu7 protein that have distinct roles in its subcellular localization – a nuclear localization signal (NLS), a zinc-knuckle motif and a lysine-rich region (Shomron et al., 2004). The zinc-knuckle motif overlaps the NLS to form a unique functional structure that is required not for hSlu7's entrance into the nucleus but rather to maintain hSlu7 inside it, preventing its shuttle back to the cytoplasm via the chromosomal region maintenance 1 (CRM1) pathway. The selective synchronized translocation of proteins between the nucleus and the cytoplasm by eukaryotic cells provides a powerful mechanism for the regulation of gene expression and for many other cellular processes (Gorlich and Kutay, 1999; Mattaj and Englmeier, 1998; Weis, 1998), including the regulation of alternative splicing (van der Houven van Oordt et al., 2000; Xie et al., 2003). Identifying the signals causing this nuclear-cytoplasmic translocation is a key step in

understanding the role of these proteins and the regulation of their substrates. We have shown that the zinc-knuckle motif of hSlu7 determines the cellular localization of the protein through a nucleocytoplasmic-sensitive shuttling balance (Shomron et al., 2004). At this point, we asked whether physiological conditions can shift the endogenous nuclear hSlu7 concentrations towards cytoplasmic accumulation. Therefore, we examined the effect of stress on hSlu7 cellular localization and whether these concentrations are crucial for alternative splicing or 3′ss selection during the splicing reaction. We found that two stress conditions, ultraviolet-C light (UV-C) and heat, alter the cellular distribution of hSlu7, which is dependent mostly on the Jun N-terminal kinase (JNK)-kinase cascade. The differential cellular distribution of hSlu7 affects alternative splicing and 3′ss selection, although the protein is not essential for cell viability. Altogether, these results suggest that the stress-induced nuclear-cytoplasmic shift might be a spatial and temporal regulatory mechanism by which hSlu7 protein levels are regulated within the nucleus. This process affects the alternative splicing inclusion/skipping ratio of target exons.

Materials and Methods

Cell-line maintenance, transfection and treatments

HeLa, 293T, HepG2 and U2OS cells were grown on cover slides in 24-well plates or 100 mm culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 0.29 mg ml^{-1} L-glutamine, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 1 U ml^{-1} nystatin at 37°C in a humidified atmosphere with 5% CO_2. HeLa, HepG2 and U2OS cells were used for immunofluorescence studies and 293T cells were used for transfection experiments owing to their high transfectability (>95%). Transfection with ADAR minigenes (Lev-Maor et al., 2003) and a *JNK1*-cDNA-containing plasmid (provided by R. Seger, Weizmann Institute of Science, Rehovot, Israel; a mutant JNK1-containing plasmid was provided by T. Kallunki, Institute for Cancer Biology, Copenhagen, Denmark) was performed using FuGENE6 (Roche) according to the manufacturer's protocol. Transcription inhibition was carried out by administering 5 μg ml^{-1} actinomycin-D (Sigma) in serum-free DMEM for 3 hours at 37°C. Similarly, puromycin (Sigma) was used at 300 ng ml^{-1} to inhibit protein synthesis, and SB203580 or SP600125 (Sigma) to inhibit the p38- or JNK-mediated phosphorylation pathways, respectively. The latter reagents were added for 1 hour before UV-C treatment. For stress analysis, exponentially growing cells were mock-treated or treated with heat (42°C for 1 hour), 0.3 J cm^{-2} or 0.9 J cm^{-2} UV-C light (Vilbert-Lourmat, Marne La Vallée, France) (except for JNK1-activation experiments, which were irradiated at 0.01 J cm^{-2}), H_2O_2 (up to 100 mM), sorbitol (up to 600 mM), cisplatin (up to 1 mM), or Neocarzinostatin (NCS; up to 200 ng ml^{-1}). Cells were left to recover at 37°C for 3 hours and then fixed for immunofluorescence studies. The UV-C irradiation was administered without cell medium, which was subsequently added for the recovery period.

Anti-hSlu7 antibody production

A recombinant fusion between glutathione-*S*-transferase (GST) and hSlu7 was produced as described (Shomron and Ast, 2003). This protein was used for polyclonal antibody production in rabbits according to standard procedures (Green and Manson, 1998).

Immunofluorescence

Following stress treatment, cells were washed with PBS and fixed for 10 minutes in 4% paraformaldehyde (PFA). Permeabilization was performed with 0.5% Triton X-100 in PBS for 10 minutes, then cells were incubated for 5 minutes with 0.1 mg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI) to mark the nucleus. To observe hSlu7, Sm or JNK1 proteins, the cells were incubated with anti-hSlu7 (Shomron and Ast, 2003), anti-Sm (provided by J. Steitz, Yale University, New Haven, CT) or anti-hemagglutinin (anti-HA) (fused to *JNK1* cDNA; provided by R. Seger), respectively, for 1 hour and then with rhodamine-conjugated anti-IgG or FITC-conjugated anti-IgG (Jackson ImmunoResearch) in blocking solution [5% FCS, 5% bovine serum albumin (BSA) in PBS] for half an hour. Fluorescent images were observed under a 410 Carl Zeiss confocal laser-scanning microscope (CLSM410) at the 365 nm, 488 nm and 568 nm laser lines. Images were obtained using the same parameters.

Image processing

For determination of hSlu7 localization, multiple fields were examined to count at least 200 cells. Images were acquired under identical conditions, ensuring that the maximal signal was not saturated. Acquisition of images and measurement of speckles intensity was performed using Adobe Photoshop, TINA and analySIS (Soft Imaging System) software. Most of the results represent values obtained from at least three separate experiments, and the results are average values. Small nuclear ribonucleoprotein (snRNP) localization was determined similarly.

RNA isolation and RT-PCR amplification

Cells were grown in 100 mm culture dish and transfection was performed using 4 μg indicated plasmid DNA. After 48 hours, total cytoplasmic RNA was extracted using TRI reagent (Sigma) followed by treatment with 2 U DNase (RNase-free; Ambion). First-strand oligo(dT)-primed cDNA synthesized with reverse transcriptase (RT) from avian myelobstosis virus (RT-AMV, Roche) from 2 μg total RNA was amplified with High Fidelity Taq polymerase (Roche) and *ADAR2*, *DDO*, *GAPDH* and *E1A* primers for 30 cycles, consisting of 94°C for 30 seconds, 60-65°C for 45 seconds and 72°C for 1 minute. The products were separated on 2% agarose gels and confirmed by sequencing.

Western blotting

Lysis buffer (50 mM Tris 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 to dissolve the total proteins extracted from the cells, which were then collected after cold centrifugation for 30 minutes at 17,000 ***g***. Total protein concentrations were measured using BioRad Protein Assay (BioRad). Proteins were separated in 12% SDS-PAGE and then electroblotted onto a Protran membrane (Schleicher and Schuell). The membranes were probed with anti-hSlu7, anti-hPrp18 (provided by D. Horowitz, Uniformed Services University of the Health Sciences, Bethesda, MD), anti-hPrp8 (p220; provided by M. Moore, Brandeis University, Waltham, MA), anti-glyceraldehyde-3-phosphate-dehydrogenase (anti-GAPDH) (provided by I. Yron, Tel Aviv University, Tel Aviv, Israel), anti-phospho-p38-specific (Santa Cruz Biotechnology) or anti-phospho-Jun-specific (US Biological) antibody, followed by the appropriate secondary antibody. Immunoblots were visualized by enhanced chemiluminescence (Lumi-Light Western Blotting Substrate; Roche) and exposure to X-ray film.

RNA interference

RNA interference (RNAi) was performed using the pSUPER plasmid (provided by R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) as described in (Brummelkamp et al.,

2002) with the following modifications. 21-nucleotide double-stranded DNA with a sequence homologous to the zinc-knuckle region of *hSlu7* mRNA, containing sticky-end restriction enzymes on either side, was chemically synthesized and purified by Sigma. Transfection of 3 μg cloned pSUPER plasmids was performed using FuGENE6 (Roche) in 100 mm culture as described above.

Results

We produced polyclonal antibodies directed against hSlu7 and used them to detect the cellular localization of hSlu7. The hSlu7 protein is localized to the nucleus in a distinct speckled pattern (Shomron et al., 2004), which is similar to other splicing proteins. However, the absence or presence of Slu7/hSlu7 was reported to affect 3′ss choice in vitro (Chua and Reed, 1999a; Frank and Guthrie, 1992) and our previous observations indicated that hSlu7 is an alternative splicing regulatory protein that can also cause an effect on 3′ss selection in vivo (Lev-Maor et al., 2003; Shomron et al., 2004). As a result, we wanted to determine whether altering the physiological environment of the cell changes hSlu7 nuclear concentrations [as reported for other splicing proteins (van der Houven van Oordt et al., 2000; Xie et al., 2003)] and whether a different subcellular distribution of hSlu7 can affect alternative splicing.

UV-C irradiation and heat alter hSlu7 subcellular localization

HeLa cells were exposed to various stress treatments – heat shock or UV-C irradiation, followed by a recovery period of 3 hours. Cells were then fixed, stained with anti-hSlu7 and DNA stained with DAPI (Fig. 1Ai-v). Both heat shock and UV-C irradiation (at two dose levels) caused a shift of hSlu7 proteins from exclusively nuclear to nuclear and cytoplasmic staining. The speckled nuclear pattern observed without stress (Fig. 1Ai) differs from the dispersed nuclear pattern after stress (Fig. 1Aii-v). The latter is composed of many small hSlu7 centers scattered evenly around the nucleus, similar to those seen in other proteins following stress (Dye and Patton, 2001). As a

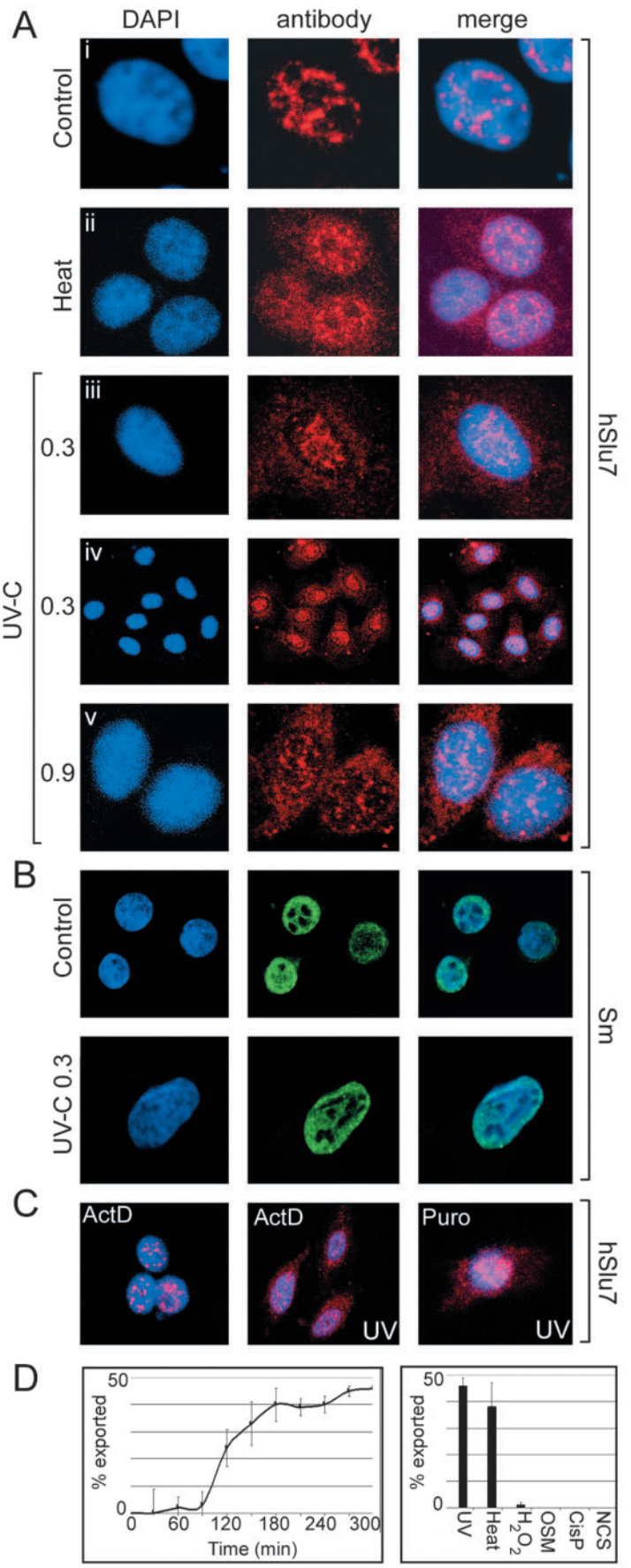

Fig. 1. Stress induces cytoplasmic hSlu7 accumulation. (A) HeLa cells were mock treated (i), incubated at 42°C for 1 hour (ii) or irradiated with UV-C (0.3 J cm^{-2} or 0.9 J cm^{-2} as indicated on the left; iii-v). Following a 3 hour recovery period under optimal conditions, cells were fixed and hSlu7 cellular localization was observed by indirect immunofluorescence using anti-hSlu7 antibody. (left) Nuclear DNA DAPI staining; (center) anti-hSlu7 staining; (right) merged image of the two. (B) As described in A except that the cells were fixed and snRNP cellular localization was observed by indirect immunofluorescence using anti-Sm antibody. Treatments are indicated on the left. (C) As described in A except that the cells were incubated in the presence of actinomycin-D (ActD; 5 μg ml^{-1}) or puromycin (Puro; 300 ng ml^{-1}) for 3 hours before UV-C irradiation (UV-C). (D, left) Time-dependent export of hSlu7 out of the nucleus following irradiation at 0.3 J cm^{-2}. The *y*-axis indicates the proportion of hSlu7 exported out of the nucleus. (D, right) The effects of various stress conditions on the export of hSlu7 out of the nucleus. HeLa cells were subjected to various stress conditions [UV-C (0.3 J cm^{-2}), heat (42°C for 1 hour), H_2O_2, sorbitol (OSM), cisplatin (cisP) and Neocarzinostatin (NCS)]. 3 hours later, the cellular localization of hSlu7 was observed by indirect immunofluorescence using anti-hSlu7. The error bars represent s.d.

control, staining with anti-Sm antibody against snRNPs and with anti-hPrp8 antibody (U5-specific protein – p220) demonstrated that this effect was specific and not caused by the generation of nuclear leakage, because no cytoplasmic accumulation of Sm and hPrp8 proteins were observed at either dose level (Fig. 1B and data not shown). Similar experiments using only the second antibodies gave no background fluorescence (data not shown). Addition of transcription and protein-synthesis inhibitors (actinomycin-D and puromycin, respectively) before the irradiation had no effect on hSlu7 cytoplasmic accumulation (Fig. 1C). This demonstrates that the accumulation of hSlu7 in the cytoplasm does not represent newly synthesized protein concomitant with nuclear import blockage. A time-scale analysis of hSlu7 export out of the nucleus following 0.3 J cm^{-2} UV-C irradiation shows that the effect begins 120 minutes after irradiation and reaches its maximum level (48%) after 300 minutes (Fig. 1D). Recovery is not seen even after 24 hours (data not shown).

The slightly different subcellular distribution observed after 0.3 J cm^{-2} and 0.9 J cm^{-2} UV-C irradiation suggests that it is a UV-dose-dependent response and not a general failure of any nuclear localization signals. This implies that the UV response is a unique effect of individual proteins to a stress-induced environment. The cytoplasmic accumulation is not cell-type specific, because UV-C irradiation caused similar subcellular distribution in three other cell lines (293T, HepG2 and U2OS; data not shown). The above results indicate that stress causes a shift in the cellular localization of hSlu7 – from nuclear to cytoplasmic accumulation. Other stress conditions, such as peroxidase, osmotic shock, cisplatin and neocarzinostatin, had no or very little effect on hSlu7 cytoplasmic accumulation (Fig. 1D and data not shown).

Functional role of hSlu7 nuclear concentrations on alternative splicing and 3′ss selection

To test whether the cellular localization of hSlu7 has a functional significance in alternative splicing, we used two minigenes containing the genomic sequence of the gene encoding adenosine deaminase, *ADAR2*, from exon 7 to exon 9, in which exon 8 is an alternatively spliced *Alu* exon (Lev-Maor et al., 2003). The 3′ end of the intron preceding exon 8 is composed of a polypyrimidine tract of 18 bases, followed by a 3′ss motif of G<u>AG</u>AC**AG**. In the 3′ss motif, the distal AG is selected (bold), and there is a delicate interplay between the two AGs. The proximal AG (which is not selected as a 3′ss; underlined) is essential to weaken the selection of the distal AG, thus maintaining alternative splicing. The 4-nucleotide distance between the proximal and distal AGs also ensures the mode of alternative splicing: Increasing that distance leads to exon 8 skipping, and exon 8 inclusion can be restored by a high concentration of hSlu7 when the distance between the two AGs is over 8 nucleotides (Lev-Maor et al., 2003). We therefore reasoned that, if hSlu7 proteins partially shift from the nucleus to the cytoplasm, this might alter the alternative splicing pattern. Thus, we transfected cells with the plasmids containing the *ADAR2* or *ADAR2+10* minigene. After 48 hours, UV-C stress was induced followed by a 3 hour recovery period and total RNA extraction. The splicing pattern was examined by RT-PCR using specific primers to the flanking exons (Fig. 2A, arrows). In the absence of stress conditions, the average inclusion/skipping ratio of the alternatively spliced *Alu* exon was 35%:65% (and the distal AG 3′ss was selected; this was confirmed by sequencing). In the *ADAR2+10* minigene, alternative exon inclusion was a rare event observed in 11% of the splicing events and the proximal 3′ss was selected [average of three experiments (Lev-Maor et al., 2003)]. The alternative splicing pattern of both *ADAR2* and *ADAR2+10* minigenes was affected by UV-C irradiation, 21% and only 2% *Alu*-exon inclusion was observed, respectively (average figures of three experiments; Fig. 2B, lanes 2-5, quantified in Fig. 2C). This suggests that native hSlu7 nuclear levels are required for alternative splicing and 3′ss selection of some genes (which still remain to be elucidated). It also suggests that lower concentrations of hSlu7 in the nucleus (and probably in the spliceosome) leads to suboptimal 3′ss selection and thus to exon skipping.

To test whether the different alternative splicing patterns were caused by reduced nuclear hSlu7 levels we used RNAi (for a review, see Novina and Sharp, 2004) to knockdown hSlu7 cellular concentrations. Cells with reduced levels of hSlu7 (Fig. 2D) showed a similar effect on alternative splicing of the *ADAR2* and *ADAR2+10* minigenes, thus supporting the notion that the alternative splicing pattern caused by UV-C irradiation is attributed to the reduced levels of nuclear hSlu7 concentrations (Fig. 2B, lanes 6-9; quantified in Fig. 2C; average figure of three experiments). We note that a control plasmid and other unrelated RNAi plasmids had no or little effect on the alternative splicing pattern (Fig. 2B,C and data not shown). In addition, reduced levels of hSlu7 had no effect on hPrp18 protein levels (Fig. 2D; see also Discussion).

To test whether the effect on splicing acts on an endogenous gene as well, we assayed the alternative splicing of the D-aspartate-oxidase (DDO) gene. DDO has a weak 3′ss upstream of its alternative exon 3 (see Fig. 2A for the sequence of the 3′ss). UV-C irradiation and RNAi treatment leading to reduction in hSlu7 nuclear concentration affect the inclusion/skipping ratio, changing it from mostly exon inclusion to mostly exon skipping (Fig. 2E, quantified in Fig. 2F). These results indicate that the reduced nuclear levels of hSlu7 are not restricted to the mutated minigene and might imply a broad effect of hSlu7 levels on alternative splicing of other exons as well.

To ensure that the effect of hSlu7 is on alternative 3′ss selection, we looked at the splicing pattern of the adenovirus *E1A* gene, which uses three alternative 5′ss spliced to a single 3′ss (Fig. 3A). If UV-C irradiation caused hSlu7 partial shift to the cytoplasm and this subsequently caused a different alternative splicing pattern, 5′ss selection should not be altered. In fact, *E1A* spliced products were selected differently only as a response to UV-C irradiation and not following hSlu7 knockdown or transfection of a control plasmid (Fig. 3B,C). We note that the effects on *E1A* splicing can be explained by stress-induced relocalization of hnRNP A1 (van der Houven van Oordt et al., 2000).

JNK signaling pathway is possibly involved in the subcellular distribution of hSlu7

The signaling pathways activated by stress have been extensively studied. Stress stimuli such as UV-C irradiation activate two stress-response kinase cascades: JNK and p38

(Canman and Kastan, 1996; Karin and Delhase, 1998; Kyriakis and Avruch, 1996). We therefore analysed the potential role of the JNK (SAPK) and p38 kinase (MKK) pathways in controlling hSlu7 subcellular redistribution after stress signaling. We used two specific inhibitors, SB205823 to inhibit the p38 pathway and SP600125 to inhibit the JNK pathway. Following a 1 hour incubation with the inhibitor, cells were UV-C irradiated and then allowed to recover for 3 hours, followed by fixation and staining with anti-hSlu7 (Fig. 4A, quantified in Fig. 4B). Blocking of both pathways was effective

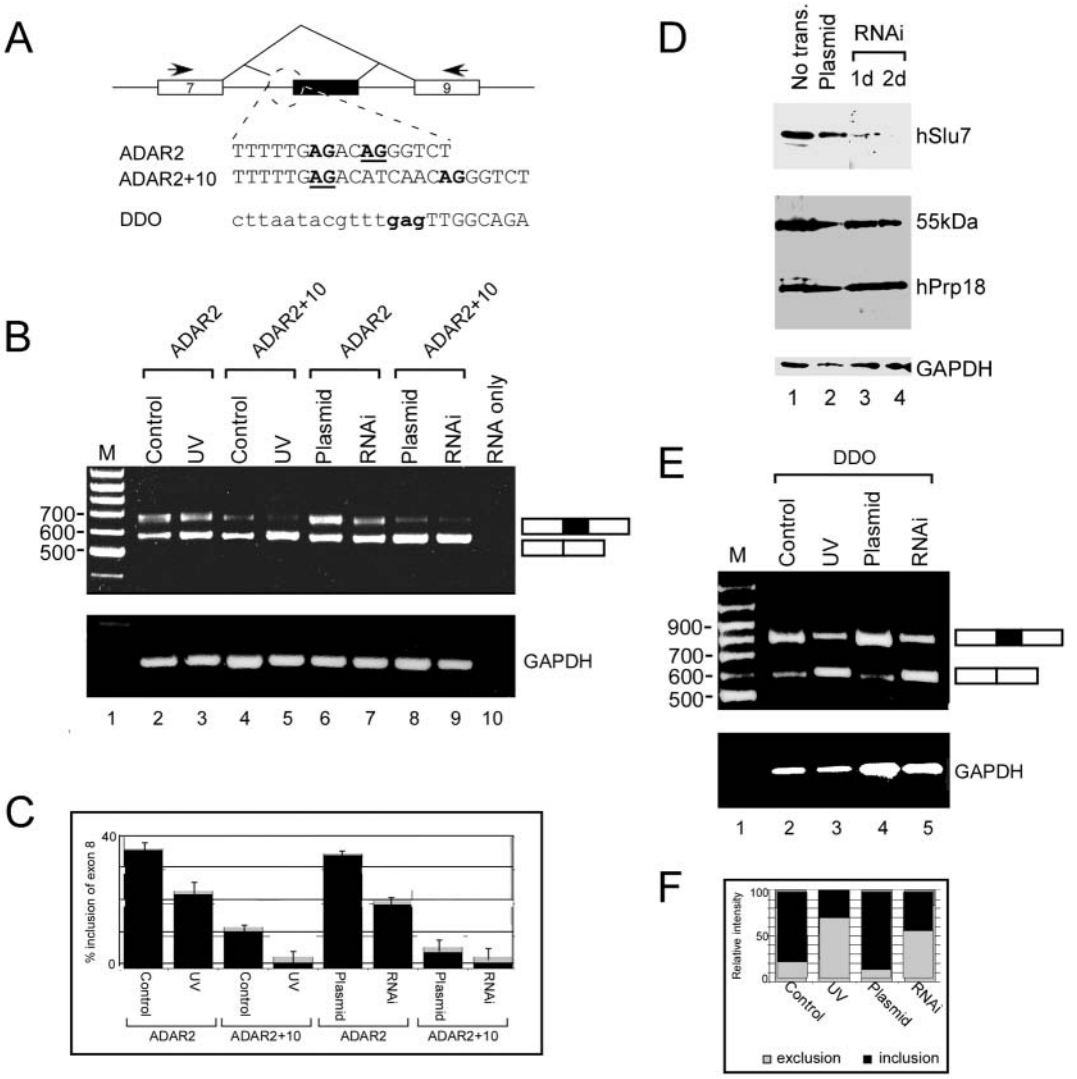

Fig. 2. Alternative splicing and 3′ss selection are affected by hSlu7 nuclear concentration. (A) The *ADAR2* minigenes and the sequence of the *Alu* exon 3′ss. The selected 3′ss is underlined. Proximal (left) and distal (right) AGs are indicated in bold. The 3′ss of the intron-2/exon-3 junction of the gene encoding D-aspartate oxidase (*DDO*) is shown at the bottom. Lower- and upper-case letters represent intron and exon sequences, respectively. (B) Cells were transfected with either of the *ADAR2* mini-genes (*ADAR2* and *ADAR2+10*) (Lev-Maor et al., 2003). Forty-eight hours after transfection, cells were either mock treated or UV-C irradiated and then incubated for 3 hours at optimal conditions. Then, cytoplasmic RNA was collected followed by RT-PCR using specific primers and separation on 2% agarose gel (lanes 2-9). Some of the cells were co-transfected with a plasmid for RNAi directed against hSlu7 (RNAi) or a control plasmid (Plasmid; lanes 6-9). A schematic representation of PCR products is indicated to the right of the gel. The alternative *Alu* exon is colored black. *GAPDH*, a house-keeping gene, was amplified from each sample to confirm that approximately the same amount of cDNA was used for each reaction. (C) A graph quantifying the spliced products, including exon 8 from B, expressed out of the total amount of spliced products in the same lane and experiment. The error bars represent s.d. (D) Cells were transfected with either an RNAi plasmid directed against hSlu7 or a control plasmid. After 1 day or 2 days, total proteins were extracted, separated by 12% SDS-PAGE, transferred to a membrane and probed for hSlu7, hPrp18 and GAPDH. Lane 1: 'No trans.' indicates cells that were not transfected. Lane 2: 'Plasmid' indicates cells transfected with a control plasmid. Lanes 3 and 4, 'RNAi' indicates cells transfected with an RNAi plasmid directed against hSlu7 for 1 day (1d) or 2 days (2d), respectively. The 55 kDa protein is a non-specific interaction of anti-hPrp18 antibody. (E) Cells were either mock treated (lane 2) or UV-C irradiated (lane 3), then incubated for 3 hours at optimal conditions followed by RNA isolation and RT-PCR. Some of the cells were transfected with an RNAi plasmid directed against hSlu7 (RNAi; lane 5) or a control plasmid (Plasmid; lane 4). A schematic representation of PCR products is indicated to the right of the gel. (F) A graph quantifying the spliced products including/excluding the alternative *DDO* exon from E expressed out of the total amount of spliced products in the same lane and experiment.

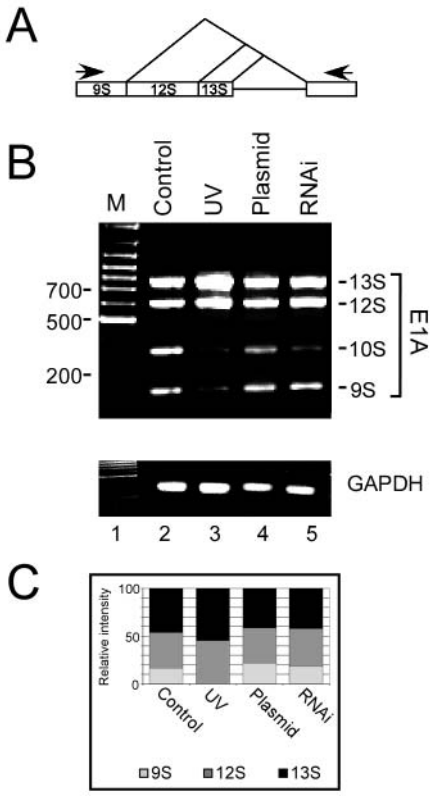

Fig. 3. hSlu7 effect is specific to 3′ss selection. (A) The *E1A* pre-mRNA containing three alternative 5′ss (9S, 12S and 13S, from distal to proximal). The 10S product is produced by aberrant splicing that does not involve simple competition between alternative 5′ss (Stephens and Harlow, 1987). (B) Spliced products were analysed following RT-PCR using primers on either side of the alternative exon (A, arrows). (bottom) RT-PCR carried out on a control *GAPDH* gene. (C) The relative amounts of 9S, 12S and 13S used in B expressed out of the total amount of spliced products in the same lane and experiment.

in reducing phosphorylated states of p38 and JNK kinases by ten- and sixfold after 1 hour, and nine- and fourfold after 3 hours, respectively (Fig. 4C). A considerable reduction in cytoplasmic localization of hSlu7 was observed upon treatment with the p38 pathway inhibitor (from an average of 45% to 18%), whereas the JNK pathway inhibitor had a greater impact on reducing cytoplasmic accumulation (from an average of 44% to 1%). Thus, the JNK pathway showed a stronger involvement than the p38 pathway in preventing the cytoplasmic accumulation of hSlu7 after UV-C irradiation (even at the lowest concentration of 100 nM; Fig. 4A). This suggests that the JNK signaling pathway might possibly be the significant player in the subcellular distribution of hSlu7.

To test whether the alternative splicing activity of hSlu7 (Fig. 2) can be reversed by inhibiting the JNK pathway and thus inhibiting the shift in hSlu7 localization, we assayed alternative splicing of the *DDO* and *ADAR+10* minigene, this time by also preventing hSlu7 shift to the cytoplasm using the JNK pathway inhibitor. As expected, preventing hSlu7 shift from the nucleus to the cytoplasm abrogates the effect caused by reduced hSlu7 nuclear concentrations (Fig. 4D and data not shown). This supports the link between stress induced by UV-C, activation of the JNK pathway and hSlu7 nuclear levels, which cause an effect on alternative splicing.

To asses whether JNK1 phosphorylation is accountable for the induction of hSlu7 relocalization and, as a consequence, affect alternative splicing, we increased the cellular levels of active JNK1 protein by transfecting the cells with a *JNK1*-cDNA-containing plasmid and then inducing its phosphorylation using mild UV-C irradiation (0.01 J cm^{-2}) (Hutter et al., 2002). By increasing active JNK1 levels we observed a shift in *DDO* alternative splicing towards the exclusion isoform, an opposite effect caused by the JNK inhibitor (Fig. 4D,E). Also, a significant boost in hSlu7 shuttling from the nucleus to the cytoplasm is observed (98% compared with 48%; Fig. 4Fi,iii) supporting the involvement of active JNK1 in hSlu7 localization. We note that the inclusion/skipping ratio of the *DDO* gene in these experiments is slightly different because of various UV-C doses used (compare lanes 3 in Fig. 4D,E) and that a mutant JNK1 protein had no effect (data not shown).

Discussion

In this study, we identify physiological conditions that alter the nuclear concentration of second-step splicing protein hSlu7. Our results strongly suggest that UV-C stress stimuli trigger changes in the alternative splicing patterns of cellular genes by decreasing the nuclear concentration of hSlu7 through the modulation of its nuclear to cytoplasmic transport. Therefore, this spatial and temporal regulatory mechanism might be the method by which hSlu7 protein levels are regulated within the nucleus upon exposure to environmental stimuli.

Two known mechanisms control the levels of splicing proteins within the nucleus: a regulation on the translational level, for SF2/ASF (Hanamura et al., 1998); and exclusion from the nucleus, for PTB (Xie et al., 2003) and hnRNP A1 (van der Houven van Oordt et al., 2000). All three proteins have been shown to affect alternative splicing of specific genes. The latter mechanism, which was also observed for hSlu7, seems to provide a finer regulation of nuclear protein levels that control splicing.

Several of the stress condition's effects might seem to be contradictory. Osmotic shock and UV-C irradiation, among other factors, stimulate the p38 and JNK stress-response kinase cascades (Canman and Kastan, 1996). In our experiments, we observed that UV-C irradiation and not osmotic shock resulted in cytoplasmic accumulation. However, the events upstream of these pathways are poorly defined and this inconsistent stress-induced localization effect has been reported elsewhere (van der Houven van Oordt et al., 2000). It is also intriguing that UV-C irradiation and not cisplatin elicits a change in hSlu7 cellular localization, because they both exert similar physiological effects (Hayakawa et al., 2003). UV-C light produces bulky adducts, pyrimidine dimers and other photoproducts in DNA, whereas cisplatin forms primarily 1,2-intrastrand cross-links between adjacent purines in DNA and also introduces DNA 1,3-intrastrand cross-links, interstrand cross-links and monoadducts (Kartalou and Essigmann, 2001). These DNA obstructions must be removed or bypassed to prevent arrest of the DNA polymerase during replication and thus are expected to activate the same repair cascade. However, a recent report showed that the mechanisms by which cells

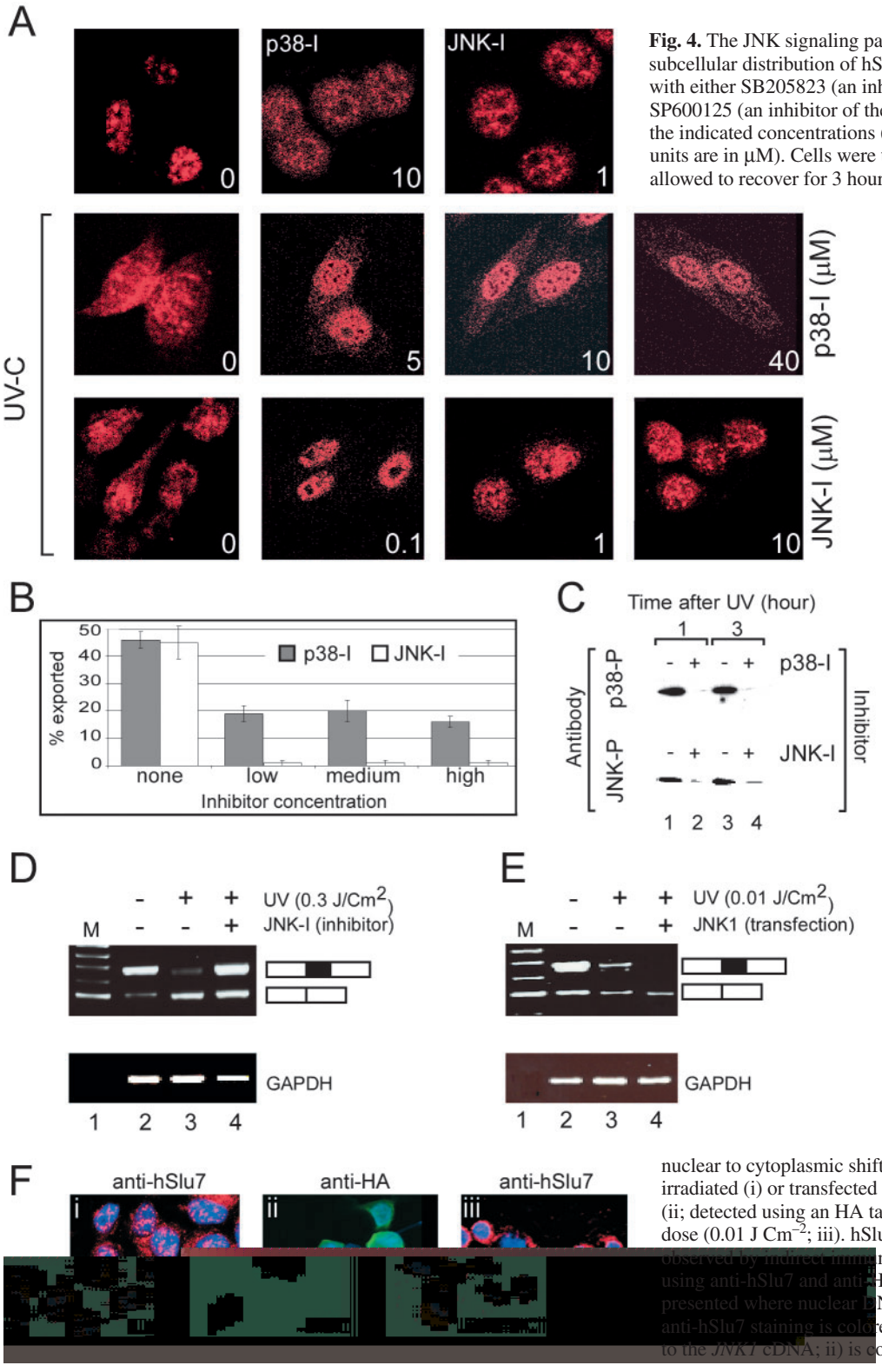

Fig. 4. The JNK signaling pathway might be responsible for the subcellular distribution of hSlu7. (A) HeLa cells were incubated with either SB205823 (an inhibitor of the p38 pathway; p38-I) or SP600125 (an inhibitor of the JNK pathway; JNK-I) for 1 hour at the indicated concentrations (bottom right corner of each image; units are in μM). Cells were then UV-C irradiated (0.9 J cm^{-2}) and allowed to recover for 3 hours, followed by fixation and staining with anti-hSlu7. (B) Proportion of hSlu7 exported out of the nucleus. Inhibitor concentration (low, high and medium) refers to 5 μM, 10 μM and 40 μM for SB205823 (p38 inhibitor; shaded bars) and to 0.1 μM, 1 μM and 10 μM for SP600125 (JNK inhibitor; white bars). (C) Inhibition of p38 and JNK phosphorylation by SB205823 and SP600125, respectively, is effective. HeLa cells were UV-C irradiated (0.9 J cm^{-2}) and then allowed to recover for 1 hour or 3 hours, as indicated (lanes 1,2 and 3,4, respectively). To some cells, either SB205823 (40 μM; p38-I; top) or SP600125 (10 μM; JNK-I; bottom) was added. Total proteins were extracted, separated by SDS-PAGE and probed with a monoclonal anti-phospho-p38-specific antibody (p38-P) or a polyclonal anti-phospho-Jun-specific antibody (JNK-P). (D) Inhibition of JNK phosphorylation reverses the UV-C irradiation effect on *DDO* alternative splicing. Cells were mock treated (lane 2) or UV-C irradiated (lanes 3,4) and then incubated for 24 hours at optimal conditions followed by RNA isolation and RT-PCR. One sample was pretreated with 1 μM SP600125 (JNK inhibitor; JNK-I) for 1 hour before irradiation (lane 4). (E) Active JNK1 affects *DDO* alternative splicing. Cells were mock treated (lane 2) or UV-C irradiated at a low dose (0.01 J cm^{-2}; lanes 3,4) in the absence or presence of a *JNK1*-cDNA-containing plasmid (lanes 3,4, respectively). Following 24 hours at optimal conditions, RNA was isolation and RT-PCR performed. (F) Active JNK1 increases the hSlu7 nuclear to cytoplasmic shift. 293T cells were either UV-C irradiated (i) or transfected with a *JNK1*-cDNA-containing plasmid (ii; detected using an HA tag) and then UV-C irradiated at a low dose (0.01 J Cm^{-2}; iii). hSlu7 and JNK1 cellular localization was observed by indirect immunofluorescence by fixing the cells and using anti-hSlu7 and anti-HA antibodies. Superimposed images are presented where nuclear DNA DAPI staining is colored blue (i-iii), anti-hSlu7 staining is colored red (i and iii) and anti-HA-tag (fused to the *JNK1* cDNA; ii) is colored green.

sensitize to UV and cisplatin are likely to be different (Bowman et al., 2000). In the case of UV, global genome nucleotide-excision repair (GG-NER) is enhanced without affecting transcription-coupled nucleotide-excision repair (TC-NER), which is activated by cisplatin. We could not find any reasonable explanation why only UV-C and heat shock (out of the treatments we tested) affected hSlu7 cellular localization, yet we should note that different splicing proteins react differently to different stress stimuli and this can change considerably in different cell lines (van der Houven van Oordt et al., 2000).

In a previous study, van der Houven van Oordt and

colleagues first showed that signal-transduction pathways regulate not only gene transcription but also the correct processing of mRNA (van der Houven van Oordt et al., 2000). These studies showed a remarkable link between a signaling cascade route (p38) and a splicing protein (hnRNP A1). They demonstrated that the subcellular localization of hnRNP A1 modulated by the p38 pathway in response to stress and caused an increase in cytoplasmic and a decrease in nuclear hnRNP A1 levels, leading to changes in alternative splicing activity. If the JNK signaling pathway is indeed involved in the subcellular distribution of hSlu7 (with or without p38), as suggested by our results, this represents a new facet to the hypothesis that the subcellular distribution of splicing factors is a regulated process (because, in the case of hSlu7, the JNK-kinase cascade appears to be mostly involved). The mechanism whereby hSlu7 cytoplasmic accumulation is regulated by JNK kinases is currently unknown, yet the observed considerable reduction in cytoplasmic localization of hSlu7 upon treatment with the p38 pathway inhibitor suggests a possible cooperation between these cascades in controlling hSlu7's cellular localization.

We assess the affect of hSlu7 nuclear concentration on alternative splicing of the endogenous gene *DDO*. In the presence of endogenous hSlu7, two mRNA isoforms are observed in 293T cell line. Following a decrease in hSlu7 nuclear concentrations, using either RNAi or UV-C, the amount of the inclusion isoform is reduced (Fig. 4D). Interestingly, following *hSlu7* cDNA transfection, the shift of the alternative isoforms is towards the inclusion isoform. The two forms of the alternatively spliced *DDO* mRNA (*DDO-1* and *DDO-2*) have been previously described (Setoyama and Miura, 1997). The inclusion isoform encodes the DDO-1 protein which consists of 341 amino acids. The skipped isoform, DDO-2, is identical to that of DDO-1, except for the absence of 59 amino acids covering residues 95-153 of DDO-1. Expression of human DDO-1 and DDO-2 showed that the 37-kDa DDO-1 protein is the active form and that the expression of DDO-2 produces an insoluble protein whose functional role could not be determined (Setoyama and Miura, 1997). It is suggested that the protein is involved either generally in animal development or specifically in the regulation of the central nervous system (Tedeschi et al., 1999). We have shown here that hSlu7 concentrations might have significant consequences on cellular processes.

A possible redundancy in function of hSlu7 with another protein might explain how the spliceosome can function in vivo under low levels or in the absence of hSlu7. The most probable candidate is hPrp18 (another second-step splicing protein) because, in yeast, these proteins have shown some redundant function in vitro (Zhang and Schwer, 1997). However, the fact that hPrp18 cellular concentration did not compensate for reduced hSlu7 nuclear levels after depletion of hSlu7 using RNAi suggests that their cellular levels are independent of one another. We should note that the anti-hPrp18 antibody identifies two bands: hPrp18 and a non-specific 55 kDa band that was reported by the producers of the antibody (Horowitz and Krainer, 1997) (Fig. 2D). Hence, there was no point in performing immunofluorescence experiments with these antibodies owing to the interaction with the two different proteins. HeLa and 293T cells knockdown of hSlu7 did not show any phenotype detectably different from wild-type and plasmid-only-transfected cells (data not shown), or any change in cell cycle (data not shown), indicating that cells with reduced levels of hSlu7 are viable. One might argue that using the RNAi mechanism might bias the splicing results because the RNAi system was shown to exert (in some cases) a broad, nonspecific downregulating effect (Jackson et al., 2003). However, the similar results obtained with UV-C irradiation argue against this idea.

Before this study, comprehensive research was performed on hSlu7 to elucidate its function during the second step of splicing and its 3′ss selection mechanism (Chua and Reed, 1999a; Chua and Reed, 1999b). Here, we have elucidated a possible mechanism by which hSlu7 can regulate alternative splicing or 3′ss selection in a specific environmental cellular context.

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References

Bowman, K. K., Sicard, D. M., Ford, J. M. and Hanawalt, P. C. (2000). Reduced global genomic repair of ultraviolet light-induced cyclobutane pyrimidine dimers in simian virus 40-transformed human cells. *Mol. Carcinog.* **29**, 17-24.

Brow, D. A. (2002). Allosteric cascade of spliceosome activation. *Annu. Rev. Genet.* **36**, 333-360.

Brummelkamp, T. R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553.

Burge, C. B., Tuschl, T. and Sharp, P. A. (1999). Splicing of precursors to mRNA by the spliceosome. In *The RNA World* (ed. R. F. Gesteland and J. F. Atkins), pp. 525-560. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Canman, C. E. and Kastan, M. B. (1996). Signal transduction. Three paths to stress relief. *Nature* **384**, 213-214.

Chua, K. and Reed, R. (1999a). Human step II splicing factor hSlu7 functions in restructuring the spliceosome between the catalytic steps of splicing. *Genes Dev.* **13**, 841-850.

Chua, K. and Reed, R. (1999b). The RNA splicing factor hSlu7 is required for correct 3′ splice-site choice. *Nature* **402**, 207-210.

Dye, B. T. and Patton, J. G. (2001). An RNA recognition motif (RRM) is required for the localization of PTB-associated splicing factor (PSF) to subnuclear speckles. *Exp. Cell Res.* **263**, 131-144.

Frank, D. and Guthrie, C. (1992). An essential splicing factor, SLU7, mediates 3′ splice site choice in yeast. *Genes Dev.* **6**, 2112-2124.

Gorlich, D. and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell. Dev. Biol.* **15**, 607-660.

Graveley, B. R. (2001). Alternative splicing: increasing diversity in the proteomic world. *Trends Genet.* **17**, 100-107.

Green, J. A. and Manson, M. M. (1998). Production of polyclonal antisera. *Methods Mol. Biol.* **80**, 1-4.

Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R., Jr and Krainer, A. R. (1998). Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* **4**, 430-444.

Hastings, M. L. and Krainer, A. R. (2001). Pre-mRNA splicing in the new millennium. *Curr. Opin. Cell Biol.* **13**, 302-309.

Hayakawa, J., Depatie, C., Ohmichi, M. and Mercola, D. (2003). The activation of c-Jun NH_2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *J. Biol. Chem.* **278**, 20582-20592.

Horowitz, D. S. and Krainer, A. R. (1997). A human protein required for the second step of pre-mRNA splicing is functionally related to a yeast splicing factor. *Genes Dev.* **11**, 139-151.

Hutter, D., Chen, P., Li, J., Barnes, J. and Liu, Y. (2002). The carboxyl-terminal domains of MKP-1 and MKP-2 have inhibitory effects on their phosphatase activity. *Mol. Cell. Biochem.* **233**, 107-117.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol*. **21**, 635-637.

Jensen, K. B., Dredge, B. K., Stefani, G., Zhong, R., Buckanovich, R. J., Okano, H. J., Yang, Y. Y. and Darnell, R. B. (2000). Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. *Neuron* **25**, 359-371.

Jurica, M. S. and Moore, M. J. (2003). Pre-mRNA splicing: awash in a sea of proteins. *Mol. Cell* **12**, 5-14.

Karin, M. and Delhase, M. (1998). JNK or IKK, AP-1 or NF-kappaB, which are the targets for MEK kinase 1 action? *Proc. Natl. Acad. Sci. USA* **95**, 9067-9069.

Kartalou, M. and Essigmann, J. M. (2001). Mechanisms of resistance to cisplatin. *Mut. Res*. **478**, 23-43.

Kyriakis, J. M. and Avruch, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem*. **271**, 24313-24316.

Lallena, M. J., Chalmers, K. J., Llamazares, S., Lamond, A. I. and Valcarcel, J. (2002). Splicing regulation at the second catalytic step by Sex-lethal involves 3′ splice site recognition by SPF45. *Cell* **109**, 285-296.

Lev-Maor, G., Sorek, R., Shomron, N. and Ast, G. (2003). The birth of an alternatively spliced exon: 3′ splice-site selection in *Alu* exons. *Science* **300**, 1288-1291.

Mahe, D., Fischer, N., Decimo, D. and Fuchs, J. P. (2000). Spatiotemporal regulation of hnRNP M and 2H9 gene expression during mouse embryonic development. *Biochim. Biophys. Acta* **1492**, 414-424.

Mattaj, I. W. and Englmeier, L. (1998). Nucleocytoplasmic transport: the soluble phase. *Annu. Rev. Biochem*. **67**, 265-306.

Meshorer, E., Erb, C., Gazit, R., Pavlovsky, L., Kaufer, D., Friedman, A., Glick, D., Ben-Arie, N. and Soreq, H. (2002). Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* **295**, 508-512.

Modrek, B. and Lee, C. (2002). A genomic view of alternative splicing. *Nat. Genet*. **30**, 13-19.

Novina, C. D. and Sharp, P. A. (2004). The RNAi revolution. *Nature* **430**, 161-164.

Philips, A. V. and Cooper, T. A. (2000). RNA processing and human disease. *Cell. Mol. Life Sci*. **57**, 235-249.

Rappsilber, J., Ryder, U., Lamond, A. I. and Mann, M. (2002). Large-scale proteomic analysis of the human spliceosome. *Genome Res.* **12**, 1231-1245.

Setoyama, C. and Miura, R. (1997). Structural and functional characterization of the human brain D-aspartate oxidase. *J. Biochem.* **121**, 798-803.

Shomron, N. and Ast, G. (2003). Boric acid reversibly inhibits the second step of pre-mRNA splicing. *FEBS Lett.* **552**, 219-224.

Shomron, N., Reznik, M. and Ast, G. (2004). Splicing factor hSlu7 contains a unique functional domain required to retain the protein within the nucleus. *Mol. Biol. Cell* **15**, 3782-3795.

Smith, C. W. and Valcarcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control. *Trends Biochem. Sci*. **25**, 381-388.

Stamm, S. (2002). Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome. *Hum. Mol. Genet*. **11**, 2409-2416.

Stephens, C. and Harlow, E. (1987). Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kD and 35 kD proteins. *EMBO J.* **6**, 2027-2035.

Stoilov, P., Meshorer, E., Gencheva, M., Glick, D., Soreq, H. and Stamm, S. (2002). Defects in pre-mRNA processing as causes of and predisposition to diseases. *DNA Cell Biol.* **21**, 803-818.

Tedeschi, G. A., Negri, G., Bernardini, Oungre, E., Ceciliani, F. and Ronchi, S. (1999). D-Aspartate oxidase is present in ovaries, eggs and embryos but not in testis of *Xenopus laevis*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **124**, 489-494.

van der Houven van Oordt, W., Diaz-Meco, M. T., Lozano, J., Krainer, A. R., Moscat, J. and Caceres, J. F. (2000). The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J. Cell Biol.* **149**, 307-316.

Wang, J. and Manley, J. L. (1995). Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA* **1**, 335-346.

Weis, K. (1998). Importins and exportins: how to get in and out of the nucleus. *Trends Biochem. Sci*. **23**, 185-189.

Xie, J., Lee, J. A., Kress, T. L., Mowry, K. L. and Black, D. L. (2003). Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proc. Natl. Acad. Sci. USA* **100**, 8776-8781.

Zhang, X. and Schwer, B. (1997). Functional and physical interaction between the yeast splicing factors Slu7 and Prp18. *Nucleic Acids Res.* **25**, 2146-2152.

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