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An Ancient Mechanism for Splicing Control: U11 snRNP as an Activator of Alternative Splicing

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SUMMARY

Alternative pre-mRNA splicing is typically regulated by specific protein factors that recognize unique sequence elements in pre-mRNA and affect, directly or indirectly, nearby splice site usage. We show that 5′ splice site sequences (5′ss) of U12-type introns, when repeated in tandem, form a U11 snRNP-binding splicing enhancer, USSE. Binding of U11 to the USSE regulates alternative splicing of U2-type introns by activating an upstream 3′ss. The U12-type 5′ss-like sequences within the USSE have a regulatory role and do not function as splicing donors. USSEs, present both in animal and plant genes encoding the U11/U12 di-snRNP-specific 48K and 65K proteins, create sensitive switches that respond to intracellular levels of functional U11 snRNP and alter the stability of 48K and 65K mRNAs. We conclude that U11 functions not only in 5′ss recognition in constitutive splicing, but also as an activator of U2-dependent alternative splicing and as a regulator of the U12-dependent spliceosome.

INTRODUCTION

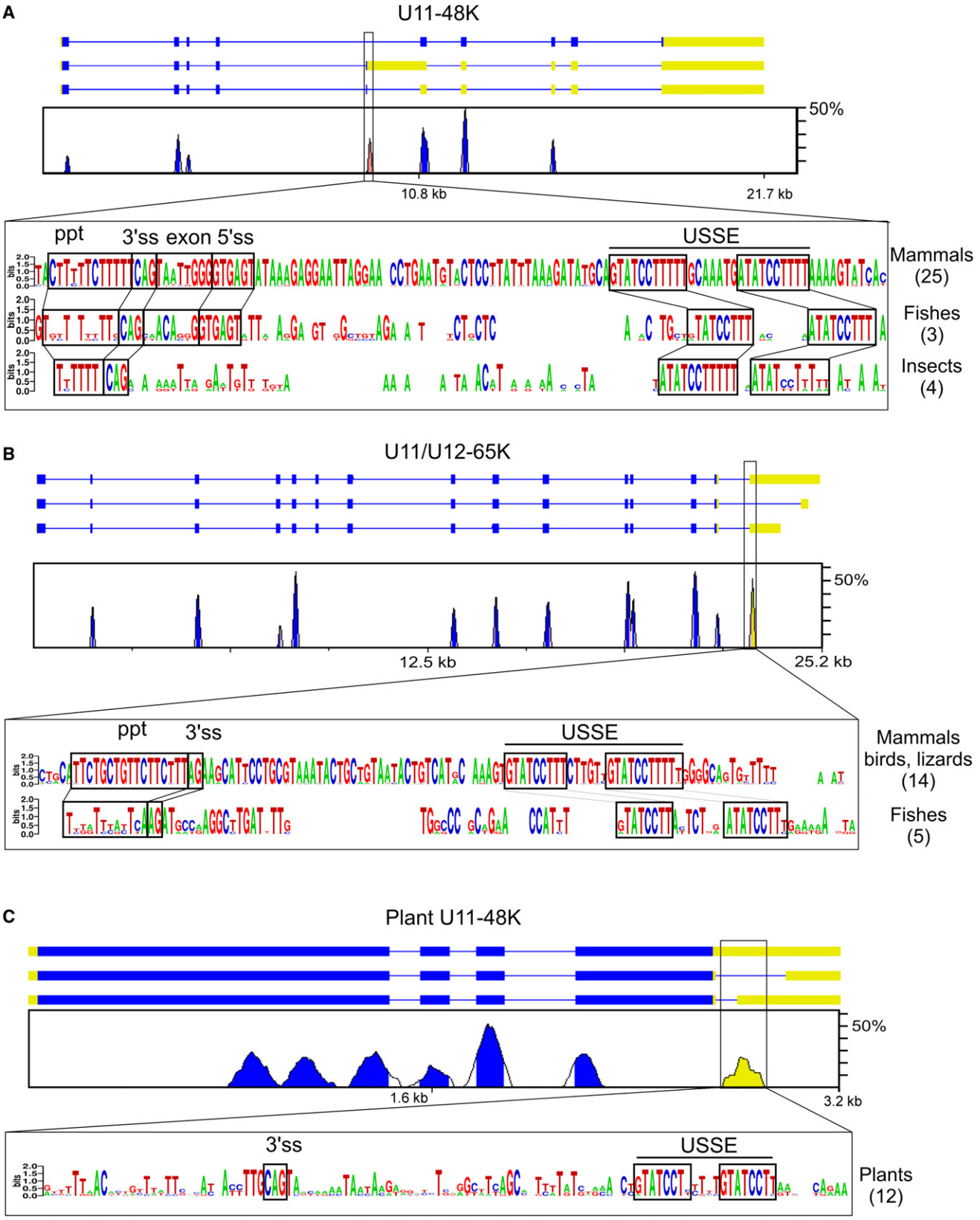
Alternative pre-mRNA splicing is a central process in eukaryotic gene expression that not only increases the diversity of the proteome but also provides an additional layer of posttranscriptional control of gene expression. Recent estimates indicate that over 92% of human genes produce at least two abundant (>15%) mRNA isoforms (Wang et al., 2008). The vast majority of alternative splicing is observed with U2-type introns, whereas alternative splice site usage is rarely seen with U12-type introns, possibly because of their more conserved splice site sequences (Levine and Durbin, 2001).

Alternative splicing is regulated by short sequence elements located in introns and/or exons of pre-mRNAs. These are recognized by regulatory proteins that either enhance or inhibit the stepwise assembly of spliceosomes. Canonical and well-characterized splicing regulators include splicing activators, in particular SR proteins, and the inhibitory hnRNP proteins. Splicing activators commonly act via protein-protein contacts that recruit constitutive splicing factors, such as U2AF and U1 snRNP (via the U1-70K protein) to the polypyrimidine tract (PPT) and the 5′ss, respectively. Alternatively, they can counteract the effects of inhibitory proteins (for review, see Wang and Burge, 2008). The combinatorial action of stimulatory and inhibitory factors affects the rate of spliceosome assembly at competing splice sites, and this ultimately determines splice site choice (Fu, 2004; Matlin et al., 2005).

In addition to producing different protein isoforms, alternative splicing has an important role in regulating gene expression. A well-characterized example is the coupling between alternative splicing and nonsense-mediated decay (NMD). Here, inclusion of poison cassette exons introduces premature termination codons (PTC) that lead to mRNA decay (Lewis et al., 2003). Such a mechanism has been shown to regulate, via negative feedback loops, both the core components of the U2-dependent splicing machinery and regulators of alternative splicing (Lareau et al., 2007; Ni et al., 2007; Saltzman et al., 2008). In many cases, such exons are flanked by long (>200 nt) sequence elements that are identical between human, mouse, and rat genomes, with high conservation also in other vertebrates. These sites are hypothesized to contain determinants for alternative splice site usage (Lareau et al., 2007; Ni et al., 2007).

In contrast to the major U2-dependent spliceosome, little is known about the regulation of minor spliceosome components or the splicing of U12-type introns. This spliceosome contains the U11, U12, U4atac, and U6atac snRNPs, the functional homologs of U1, U2, U4, and U6 in the U2-dependent spliceosome, respectively, whereas both spliceosomes contain the U5 snRNP (Hall and Padgett, 1996; Incorvaia and Padgett, 1998; Kolossova and Padgett, 1997; Tarn and Steitz, 1996a, b). The U12-dependent spliceosome catalyzes the removal of a specific subset of introns which comprise approximately 0.35% of human introns (~600) (Alioto, 2007; Burge et al., 1998; Sheth et al., 2006). The positions of U12-type introns are more conserved in metazoan genomes than those of U2-type introns, and their excision is slower; thus, removal of U12-type introns is a rate-limiting step of pre-mRNA processing (Basu et al., 2008; Patel et al., 2002; Pessa et al., 2006).

Most protein components are shared between the two spliceosomes, but a set of seven unique proteins is associated


A
U11-48K
50%
10.8 kb
21.7 kb
ppt
3'ss exon 5'ss
USSE
Mammals (25)
Fishes (3)
Insects (4)
B
U11/U12-65K
50%
12.5 kb
25.2 kb
ppt
3'ss
USSE
Mammals birds, lizards (14)
Fishes (5)
C
Plant U11-48K
50%
1.6 kb
3.2 kb
3'ss
USSE
Plants (12)

with the U11/U12 di-snRNP, which is responsible for the recognition of 5′ss and branch-point sequences (BPS) of U12-type introns (Will et al., 2004; Will et al., 1999). Of the seven proteins, the function of only a few has been deduced. Sequence similarity suggests that the U11-35K protein is a functional homolog of the U1-70K protein (Will et al., 1999). Additionally, recent work demonstrated that the U11-48K protein is required for 5′ss recognition (Tidow et al., 2009; Turunen et al., 2008), and that the U11/U12-65K and U11/U12-59K proteins form a molecular bridge connecting the U11 and U12 snRNPs (Benecke et al., 2005; Wassarman and Steitz, 1992).

Here, we show that the levels of two specific protein components of the minor spliceosome, U11-48K and U11/U12-65K, are regulated via alternative splicing, during which the U11 snRNP acts as a positive regulatory component of the U2-dependent spliceosome. The sequence element recognized by U11 is a tandem repeat of the U12-type 5′ss that is not used for splicing. It is found in *U11-48K* and *U11/U12-65K* genes and is evolutionarily conserved, being present in either one or both genes in almost all animal and plant species studied. Our data suggest that the U11 snRNP, when bound to this element, activates an upstream U2-type 3′ss to promote exon or 3′ UTR inclusion during splicing. As a result, the levels of U11-48K and U11/U12-65K mRNA are downregulated through NMD or other destabilizing mechanisms, respectively.

RESULTS

Identification of Evolutionarily Conserved, Nonproductive U12-Type 5′ss in Vertebrate Genomes

An earlier study revealed that the U11-48K protein is required for recognition of the U12-type 5′ss and for stability of the U11/U12 di-snRNP (Turunen et al., 2008). We examined the *U11-48K* gene to identify regulatory motifs and found within the fourth intron a ~110 bp element that was >90% conserved in the 25 mammalian species examined (Figure 1A and Figure S1A available online). EST data from various mammals identified two mRNAs in addition to the major U11-48K mRNA: one containing an 8 nt exon from the 5′ end of the highly conserved region (termed exon 4i hereafter) and another containing the region between exons 4i and 5 (Figure 1A and data not shown). Both variants cause a frameshift and incorporation of PTCs, suggesting that the expression of U11-48K may be regulated by the NMD pathway.

Comparison of *48K* genes from mammalian, amphibian, and fish species provided insight into the possible regulatory mechanism. Even though the ~110bp intronic element as a whole is highly conserved in mammals (Figure 1A; Figure S1A), in phylogenetically distant organisms, such as human and fish, the conservation is limited to only two regions. One contains exon 4i and the surrounding splice sites, whereas the other is at the 3′ end of the element (Figure 1A). Intriguingly, the latter contains a tandem repeat of the 5′ss consensus sequence of U12-type introns and is also present in several insect *U11-48K* genes (Figure 1A; Figure S1A). EST evidence from multiple species and our own RT-PCR analyses indicate that the U12-type 5′ss sequences are not used for splicing, further suggesting a regulatory function. For this reason, we refer to the tandem 5′ss sequence as the **U**11 **S**nRNP-binding **S**plicing **E**nhancer (USSE).

A search of 29 vertebrate genome alignments revealed that the 3′ UTR of the *U11/U12-65K* gene contains the only additional conserved USSE. In the *U11/U12-65K* gene, the USSE is associated with an upstream U2-type 3′ss (Figure 1B; Figure S1B), which is subject to alternative splicing that affects the length of the 3′ UTR (Figure 1B). Similar to the 48K USSE, EST data indicate that the U12-type 5′ss motifs in the 65K USSE are not used for splicing.

Surprisingly, in plants, USSE-like sequences were detected in the 3′ UTR of the *U11-48K* gene, but not in *U11/U12-65K* or other genes encoding proteins of the U12-dependent spliceosome (Figure 1C; Figures S1C and S1D). RT-PCR analysis of *Arabidopsis thaliana* or *Populus trichocarpa* RNA samples revealed several alternatively spliced U11-48K mRNAs differing in 3′ UTR length, similar to the mammalian U11/U12-65K mRNAs (Figure 1C; Figure S1D). Together, sequence data (Figure S1) indicate that the 5′ss-like motifs within the USSEs are highly similar to the U12-type 5′ss consensus sequence in all organisms. All variations detected are conservative (e.g., either G or A as the first nucleotide, or C to U changes in positions +5 or +6) (Figure 1; Figure S1), similar to those observed in bona fide U12-type 5′ss.

U11/U12 di-snRNP Binds the USSE In Vitro

We next tested whether a specific complex forms on the USSE and whether it can be resolved in a nondenaturing gel. Indeed, at 30°C, two complexes that migrate similar to spliceosomal complexes are formed in vitro on RNA containing the WT, but not a mutated USSE (Figure 2B). In contrast, stable complexes fail to form on a splicing substrate containing a single U12-type 5′ss but no BPS (Figure 2C). To identify components binding to the USSE in HeLa nuclear extract, we performed pull-downs with biotinylated RNAs containing the 48K or 65K USSE and upstream sequences (see Figure 2A for details), followed by northern analysis. Substrate RNAs containing WT 48K and 65K USSEs clearly bound U11 and U12 snRNAs (Figures 2D–2F). Complete loss of binding was observed upon mutation of the binding sites for the 48K protein (A+3G) or U11 snRNA (CC+5/6GG) in both motifs of the USSE. When only one of the motifs was mutated, an intermediate level of binding was observed, suggesting that the motifs can be recognized independently of each other in vitro.

Figure 1. Conserved Sequence Elements in Animal or Plant U11-48K and Animal U11/U12-65K Genes

Isoforms depicted in (A) and (B) are present in vertebrates, and evidence for the three isoforms in (C) is provided in Figure S1D. In each panel, the upper part depicts the genomic organization and the splice variants of each gene with a "phylogenetic conservation plot" generated with MULAN (http://mulan.dcode.org) below. The conservation percentage in this plot is lower than in the sequence level comparison (Figure S1) because of the 100 bp sliding window used by the algorithm. Exons are indicated as boxes and introns as horizontal lines. Protein-coding sequences are in blue, and UTRs are in yellow. A blow-up showing the residue-level conservation of the intronic or 3′ UTR elements was generated with WebLogo (Crooks et al., 2004). The number of species used to generate each sequence logo is indicated in parentheses. See also Figure S1.

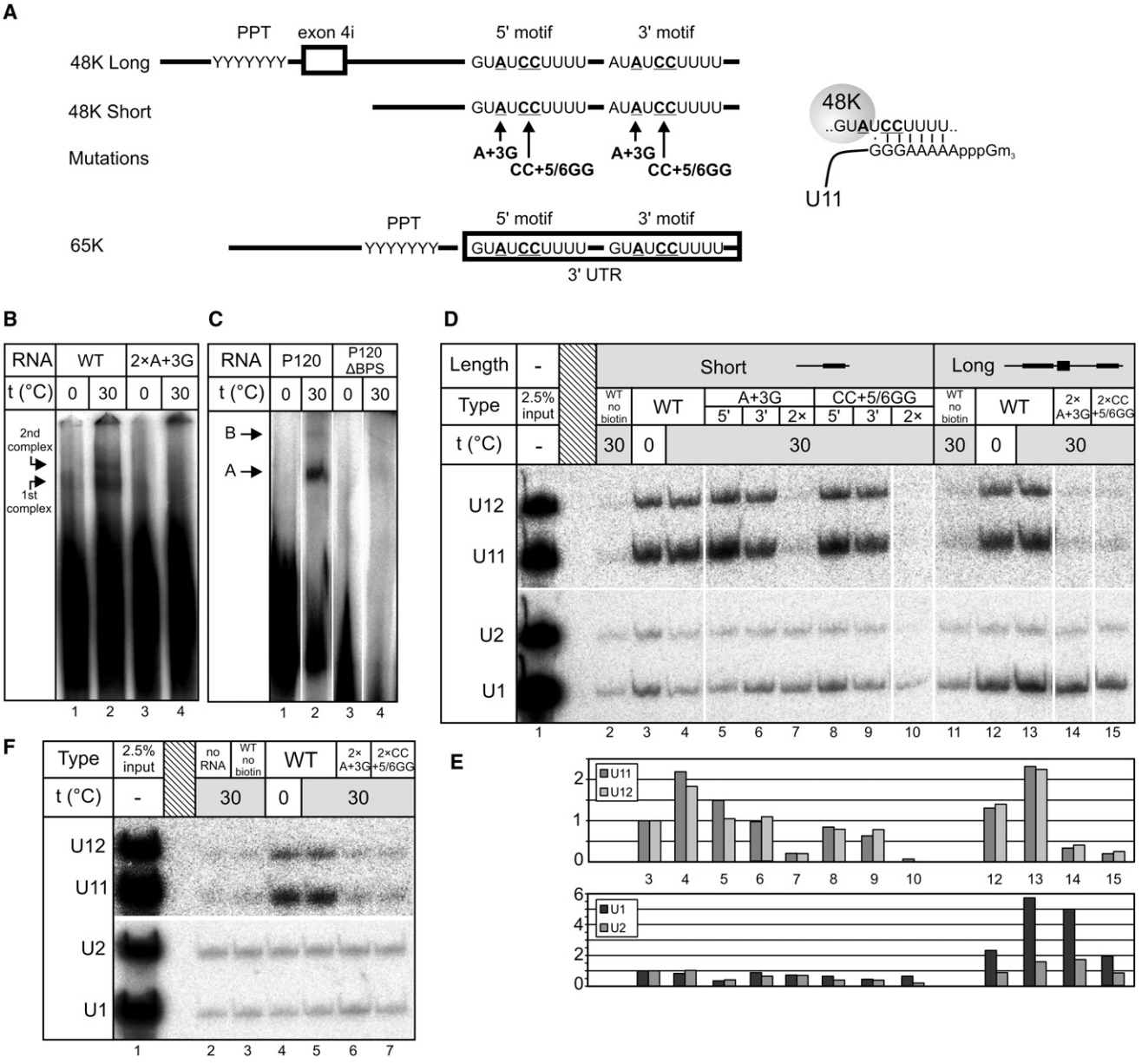


Figure 2. The USSE Binds U11/U12 di-snRNPs In Vitro

(A) Schematic representation of the RNA substrates used. The PPT, exon 4i (in 48K substrates), the 5′ and 3′ motifs of the USSE, and the 3′ UTR (65K substrate) are indicated. Arrows indicate sites mutated within the USSE. On the right, potential interaction sites for U11 snRNA and U11-48K within a 5′ss-like motif are shown.

(B) Complexes formed on long 48K substrate RNAs (uniform ^{32}P label) were separated by nondenaturing PAGE and were visualized by autoradiography. The position of the complexes is indicated on the left.

(C) Complex formation on a ^{32}P-labeled P120 or truncated P120-ΔBPS splicing substrate (Turunen et al., 2008) analyzed by native PAGE as in (B). To reduce background, a DNA oligo against the 5′ss was used after 15 min of incubation as described elsewhere (Turunen et al., 2008). The positions of the spliceosomal A and B complexes are indicated on the left.

(D) Biotinylated 48K USSE substrates were incubated as in (B), and a pull-down with streptavidin beads was performed, followed by northern blotting. The substrates and incubation temperature are indicated above the panel and the specificity of the probes on the left. See also Figure S2.

(E) Quantification of the indicated snRNAs in the identically numbered lanes in (D). The signal from each snRNA was normalized against the signal from the 48K substrate RNA (data not shown), and the value of the sample with short WT RNA at 0°C was set to 1.

(F) Streptavidin pull-down of 65K USSE substrates performed as in (D).

The long 48K substrate that contained exon 4i and the surrounding splice sites also bound U1, in addition to U11 and U12, but no other spliceosomal snRNA (Figures 2D and 2E and data not shown). Mutation of both USSE motifs, which prevented U11/U12 binding, did not completely abolish U1 binding (Figure 2D, lanes 14 and 15). This finding suggests that U1 can bind to the RNA independently of U11/U12, but its binding may be enhanced by interactions with the USSE-recognition complex. In our in vitro experiments, U2 binding does not appear to be stimulated by the USSE, which is somewhat surprising considering the role of the USSE in U2-type splicing in vivo (see below). However, the intronic sequence upstream of exon 4i does not contain a BPS that closely matches the consensus, suggesting that U2 binding is not very efficient in vitro and may require additional sequence elements not present in the short RNAs used here.

To identify interactions between the snRNAs and the USSE RNA, we performed psoralen crosslinking with the long 48K substrate. Northern hybridization revealed crosslinks specific for U11 and U1 snRNA (Figures 3A and 3B), but not for U12 snRNA (data not shown). Two major crosslinks between the WT substrate and U11 snRNA were observed even on ice, with four major crosslinks detectable at 30°C (Figure 3A). Crosslink formation was enhanced by, but not dependent on, the presence of ATP (Figure 3C). RNase H cleavage after crosslinking revealed that each crosslink contains 48K RNA and U11 (Figure 3A, lanes 6 and 7). Consistent with this, the crosslinks were sensitive to mutations that abolish interactions between the 5′ss and either U11-48K (A+3G) or U11 snRNA (CC+5/6GG). Mutations in either the 5′ or the 3′ USSE motif revealed that crosslinks 1 and 2 are U11 crosslinked to the 3′ USSE motif or 5′ USSE motif, respectively. In addition to 48K RNA and U11, crosslink 3 contains U1 snRNA, as revealed by RNase H digestion and a U1-blocking oligo (Figure 3A, lanes 8 and 9). The identity of crosslink 4 has not been verified, but it could result from internal crosslinking within crosslinks 1 and/or 2.

Only one major U1 crosslink was observed (Figure 3B), migrating slightly faster than U11 crosslink 3 in Figure 3A. Crosslink 3 was not detected with a U1-specific probe as a result of unspecific crosslinks in the same region and/or low abundance. Consistent with the pull-down results, the U1/48K substrate crosslink is only slightly affected by USSE mutation, again suggesting that U1 can bind to the substrate on its own. Together, our in vitro results suggest that the U11/U12 di-snRNP is recruited to the USSE in both 48K and 65K pre-mRNA and that 5′ss nucleotides known to interact with the 48K protein and U11 snRNA are required for formation of the USSE RNP complex. U12 snRNP does not appear to directly contact the USSE, and thus may instead be present in the complex solely due to its association with the U11 snRNP. RNase H cleavage of U12 snRNA nucleotides that recognize the BPS and U6atac snRNA does not affect U11 binding to the USSE (Figure S2). Thus, U11 snRNP alone may be sufficient for USSE recognition.

USSEs in the *48K* and *65K* Genes Regulate Alternative Splicing In Vivo

To study whether the USSE can affect the selection of upstream U2-type splice sites, the 48K USSE, together with exon 4i and the surrounding splice sites, was cloned into an intron of a reporter plasmid, whereas the unspliced 3′ UTR of 65K was cloned into a luciferase construct. RT-PCR analysis of RNA from transfected cells revealed that inclusion of exon 4i or the long 3′ UTR is observed with the WT 48K and 65K USSE reporters (Figures 4A and 4B, lane 1). Mutations in the USSE that interfere with 48K protein or U11 snRNA binding, lead to exclusion of 48K exon 4i and loss of the 65K long 3′ UTR isoform (Figure 4A, lanes 2 and 3; Figure 4B, lanes 2 to 5).

To confirm the role of the USSE in the splicing of endogenous 48K and 65K pre-mRNAs, we cotransfected antisense 2ome or morpholino oligos, respectively, designed to bind to the USSEs, and thus block U11 binding. The effect on splice site choice was followed by RT-PCR. Transfection of the morpholino oligo complementary to the 65K USSE ("block" oligo) completely inhibited formation of 65K mRNA with a long 3′ UTR (Figure 4D, lane 2). In contrast, a "mock" morpholino, targeting an unconserved region downstream of the USSE, showed a WT splicing pattern (compare lanes 1 and 3). Similarly, blocking the 48K USSE while overexpressing the 48K protein (to enhance the detectability of exon 4i, see below) led to decreased exon 4i inclusion, compared with cotransfection of a "mock" oligo (Figure 4C).

To confirm in vivo binding of the U11 snRNP to the 65K USSE, we cotransfected constructs carrying a WT USSE and a U11 snRNA mutant, U11 GG6/7CC, impaired in its ability to bind the WT U12-type 5′ss. RT-PCR revealed a significant decrease in the 65K long 3′ UTR isoform and a concomitant rise in the short isoform (Figure 4E, compare lanes 1 and 2). This suggests that the overexpressed mutant U11 snRNA functions in a dominant negative (DN) manner and impairs USSE recognition. In contrast, overexpression of WT U11 did not change the isoform ratio (Figure 4E, lane 3).

We next tested whether mutations in either USSE motif in the 65K construct could be rescued by expression of U11 snRNA carrying compensatory mutations. We were able to rescue the CT+6/7GA mutations that disrupt U11/5′ss base pairing at the first and/or second motif by coexpressing U11 snRNA carrying compensatory AG5/6TC mutations (Figure 4F). Thus, we conclude that recognition of the USSE by U11 snRNP is necessary for the activation of the upstream U2-type 3′ss in both 48K and 65K pre-mRNAs in vivo.

USSE Activation Destabilizes 48K mRNA via Nonsense-Mediated Decay

48K overexpression experiments presented in Figure 4C indicated that the inclusion of exon 4i is sensitive to cellular levels of 48K. Because the 48K protein is involved in the recognition of the U12-type 5′ss (Tidow et al., 2009; Turunen et al., 2008), this result suggested the existence of a negative feedback loop, in which retention of either exon 4i or the entire intron downstream of exon 4i in the mRNA causes inclusion of PTCs and targets these mRNAs for NMD. To confirm the role of NMD, we tested whether exon 4i inclusion is sensitive to interference with the NMD machinery or, because NMD requires ongoing translation, inhibition of translation. Following treatment with the translation elongation inhibitor cycloheximide (CHX), the level of mRNA containing exon 4i increased, indicating it was

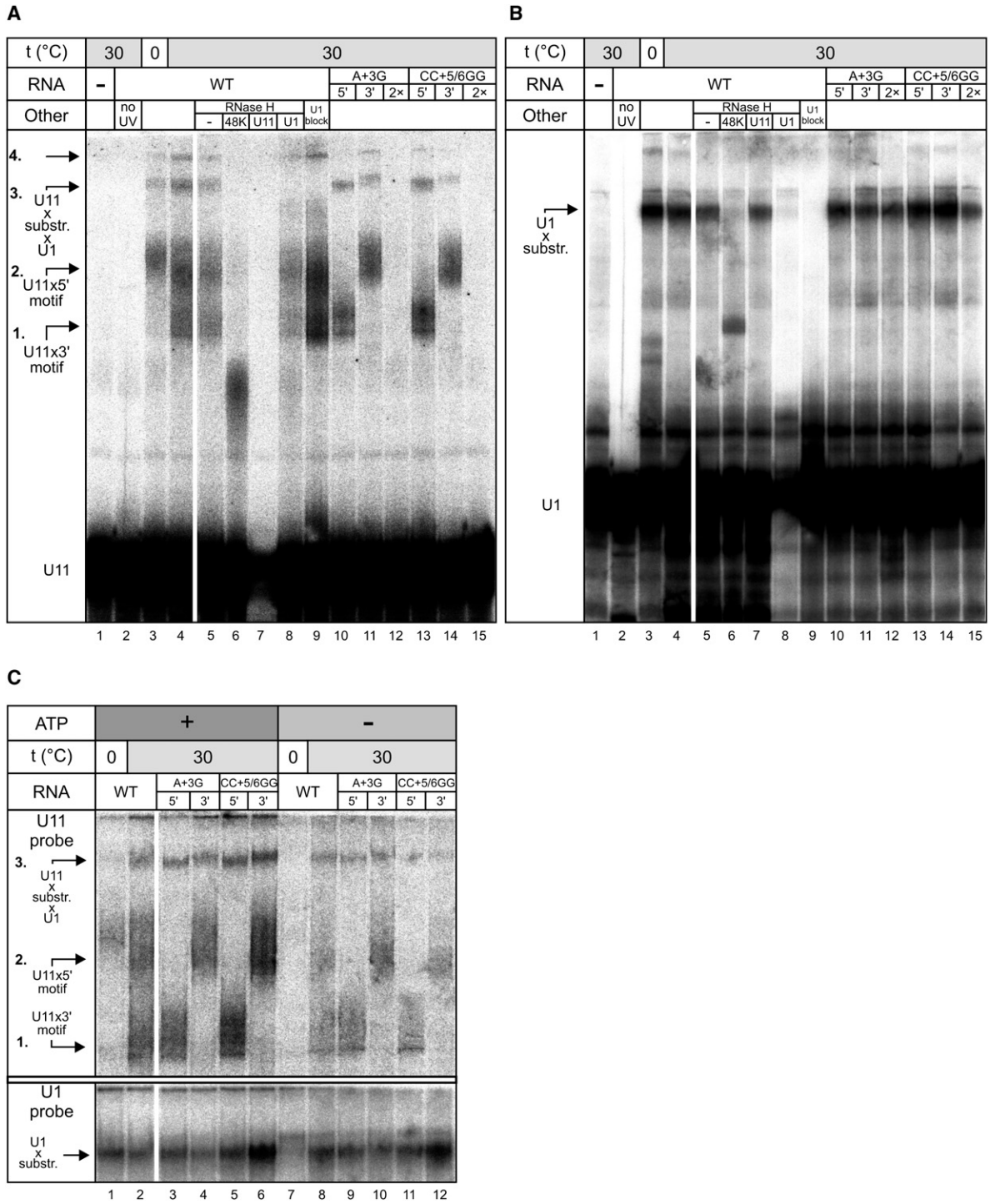


Figure 3. U11 snRNA Base Pairs to the USSE

(A and B) Splicing reactions were incubated with the long 48 K substrate RNA or mutants thereof, as indicated. "U1 block" indicates addition of the $U1_{1-14}$ oligo. Samples were crosslinked with psoralen, and RNase H cleavage of specific RNAs was then performed, as indicated. Northern blotting was performed with probes for U11 (A) or U1 (B). snRNAs and crosslinked species are indicated on the left.

(C) Samples were incubated in the presence or absence of ATP, as indicated, followed by crosslinking and northern blotting. Probes for U11 or U1 were then used as indicated.

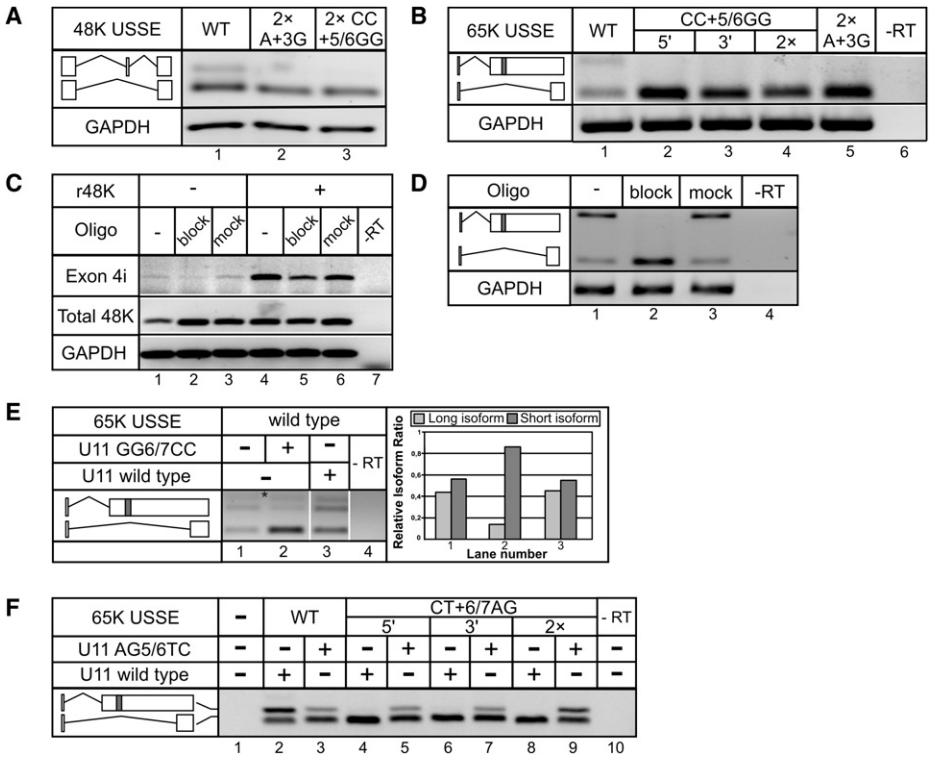


Figure 4. USSE Regulates 48K and 65K Alternative Splicing In Vivo

(A) Cells were transfected with an smEFP-splicing reporter with a WT or mutated USSE, and the mRNAs were analyzed by RT-PCR. Primers flank exon 4i and simultaneously detect both isoforms. Incorporation of exon 4i in the spliced mRNA was confirmed by sequencing.

(B) The unprocessed 3′ end of the 65K gene was cloned into a luciferase construct, and the effect of the indicated USSE mutations on long 3′ UTR formation was assessed by RT-PCR.

(C) Detection of exon 4i inclusion in 48K mRNA using RT-PCR after transfection with 2ome oligos. Primers detect the inclusion of 4i sequences (i.e., both NMD isoforms) (see Figure 1) and the 3′ UTR of all isoforms. Isoforms are detected in separate PCRs using primers targeting the 4i exon and endogenous 3′ UTR sequences of 48K because the intense signal from the 48K major isoform masks the less abundant 4i signal.

(D) Following transfection with morpholino oligos, the splicing pattern of the 65K gene was analyzed by RT-PCR. The control reaction was treated with transfection reagent only.

(E) Following overexpression of WT or mutated U11 snRNA, the splicing pattern of a WT USSE-containing reporter construct was analyzed by RT-PCR. The upper band (asterisk) is an unspecific PCR product based on DNA sequencing and was therefore not used for quantification.

(F) Constructs containing the indicated mutations in the 5′, 3′, or both motifs of the USSE were cotransfected with WT U11 or U11 snRNA with compensatory mutations. Expression of the 65K short and long 3′ UTR isoform was analyzed by RT-PCR.

stabilized (Figure 5A). This mRNA was also stabilized after cotransfection of a plasmid expressing a DN form of Upf1, an essential component of the NMD machinery, together with the 48K expression plasmid (Figure 5B). Expression of Upf1 DN mRNA greatly exceeded that of endogenous Upf1 mRNA (Figure 5B). Consistent with these results, U11-48K mRNA (c6orf151) is upregulated upon knockdown of the NMD factor eIF4AIII in mammalian cells (Giorgi et al., 2007). We conclude that mRNA containing exon 4i is degraded by the NMD pathway.

USSEs Regulate the Level of Cytoplasmic 65K mRNA

Transfection of 48K overexpression plasmids did not have any effect on the 65K mRNA isoform ratio (data not shown), suggesting differences in regulation between 48K and 65K. Translation inhibition by cycloheximide or expression of DN Upf1 (data not shown) also did not affect the stability of either 65K isoform, whereas known NMD targets, such as 9G8 and exon 4i of U11-48K, were clearly upregulated (Figure 5F). We next tested whether the isoform ratio of the 65K mRNA is altered upon siRNA knockdown of 48K or other U11/U12-specific proteins. qPCR analysis indicated that a ~90% knockdown of 48K resulted in a 4- to 5-fold upregulation of 65K mRNA, whereas mRNAs encoding the other U11/U12-specific proteins were unaffected (Figure 5C). Interestingly, a ~3.5-fold increase in 65K and 48K mRNA was observed also after knockdown of U11-35K. The latter shares homology with the U1-70K protein, which bridges the U1 snRNP with proteins at the 3′ end of an intron, and U11-35K is thus the most likely U11 protein to interact with factors recognizing the 3′ss. RT-PCR revealed that upon 48K or 35K knockdown there was a complete loss of the 65K mRNA with a long 3′ UTR and a concomitant increase in the 65K mRNA with a short 3′ UTR (Figures 5D and 5E), similar to the results of the morpholino blocking experiments (Figure 4D).

These results suggested that the length of the 3′ UTR may affect 65K mRNA stability. To test this idea, we cloned the 65K long and short 3′ UTR into a firefly luciferase construct and

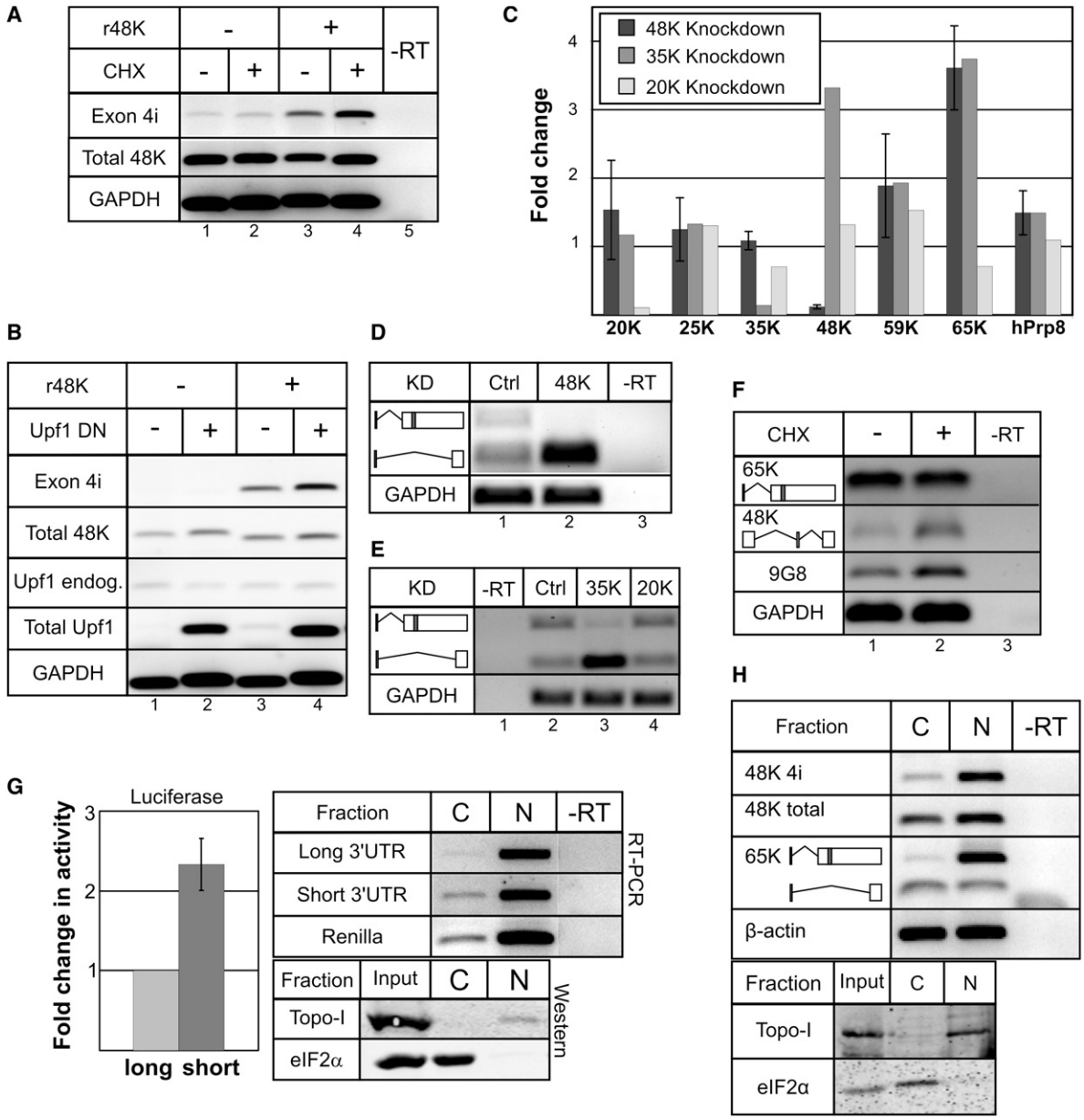


Figure 5. 48K and 65K mRNA Isoforms Are Destabilized via Different Pathways

(A) Cells were transfected with vector alone or expressing recombinant 48K protein and/or treated with CHX, as indicated. Inclusion of exon 4i and total 48K mRNA levels were determined by RT-PCR.

(B) Cells were transfected with a dominant negative (DN) form of Upf1 and/or 48K expression plasmid as indicated. The inclusion of exon 4i and total 48K mRNA levels were assessed by RT-PCR.

(C) After 72 hr of 48K, 35K, or 20K knockdown, levels of mRNA encoding the indicated proteins were assayed by real-time PCR. Values represent averages from two (20K and 35K) or four (48K) independent knockdowns. Error bars indicate standard deviations for 48K knockdown samples.

(D) Following knockdown of 48K, RT-PCR was used to examine the 65K splicing pattern. The control knockdown was targeted against firefly luciferase.

(E) The effect of 35K knockdown on the 65K splicing pattern as analyzed via RT-PCR.

(F) The stability of the 65K long 3′ UTR mRNA, and NMD-targeted isoforms of 9G8 and 48K mRNAs were assessed by RT-PCR after CHX treatment.

(G) Cells were transfected with luciferase constructs containing either the 65K long or short 3′ UTR. Luciferase activities are shown with error bars representing the standard deviation of triplicate samples. After cell fractionation, the distribution of reporter mRNAs in cytoplasmic (C) and nuclear (N) fractions was monitored by RT-PCR, and the quality of cell fractionation was determined by western blotting; 1 μg and 0.5 μg of isolated total RNA were used in RT-PCR analysis of nuclear and cytoplasmic samples, respectively.

(H) The presence of endogenous 65K mRNA isoforms in cytoplasmic (C) and nuclear (N) fractions was analyzed by RT-PCR after cell fractionation using 1 μg total RNA from both fractions.

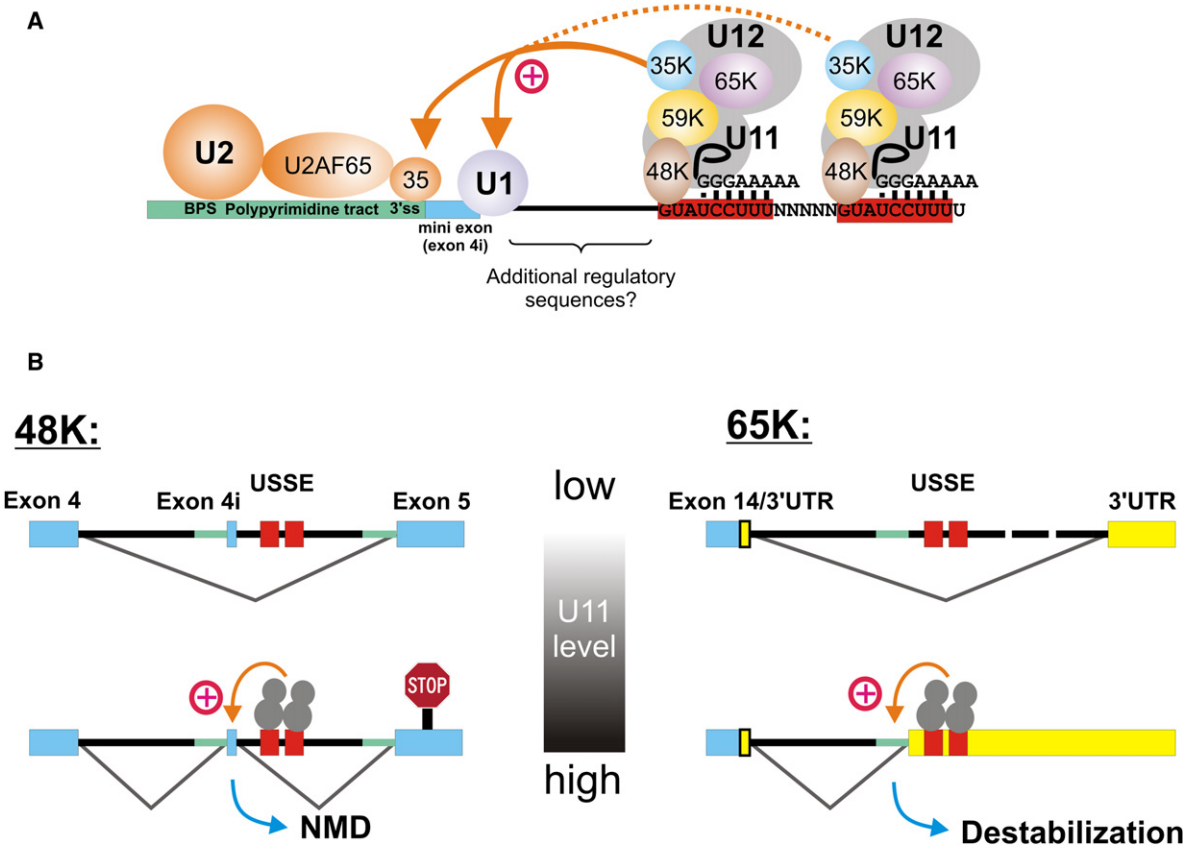


Figure 6. A Model of USSE Regulation of 48K and 65K mRNA Levels

(A) U11 snRNP binding at the USSE promotes the upstream 3′ss choice by recruiting U2-dependent factors, an interaction most likely mediated by the U11-35K protein. This leads to the inclusion of an 8 nt exon (4i) in the 48K mRNA or the formation of 65K mRNA with a long 3′ UTR. See also Figure S3.

(B) The level of U11 snRNP determines the isoform type of both the 48K and 65K mRNA. High levels of U11 lead to downregulation of 48K through NMD and formation of a less stable mRNA with a long 3′ UTR in the case of 65K.

transfected them into HEK293 cells. Constructs with the 65K short 3′ UTR exhibited two-fold higher luciferase activity, compared with those with the long 3′ UTR (Figure 5G). To provide additional evidence for reduced mRNA stability, equimolar amounts of the two plasmids were cotransfected into cells, and the cells were then fractionated into nuclear and cytoplasmic fractions, and nearly equal amounts of total RNA from both fractions were analyzed by RT-PCR. Although the level of long and short UTR RNA was almost identical in RNA isolated from whole cell lysates (data not shown), the cytoplasmic fractions contained 2–3-fold more RNA with a short 3′ UTR (Figure 5G). With each construct, a large fraction of mRNA was located in the nucleus, and, thus, cellular fractionation was necessary to determine the mRNA pool available for the translation machinery. The most likely explanation for this is the lack of introns in the expression constructs, which is known to reduce the efficiency of mRNA export (Valencia et al., 2008). Importantly, the endogenous 65K RNA showed a similar, but more pronounced nuclear versus cytoplasmic distribution (Figure 5H); the long isoform was nearly absent from the cytoplasmic fraction even though it predominates over the short isoform in the nuclear fraction. Thus, the results in Figures 4D and 5C–5H suggest rapid cytoplasmic decay of the 65K mRNA with a long 3′ UTR. Together, our data suggest that the USSE regulates 65K protein levels by influencing the stability of the 65K mRNA.

DISCUSSION

We describe here a mechanism for feedback regulation, in which components of both U12- and U2-dependent spliceosomes form a control circuit that regulates the mRNA levels of two key proteins of the U12-dependent spliceosome. The regulatory switch uses an atypical splicing enhancer element (termed the USSE) that is evolutionarily conserved and is present from humans to plants in genes encoding the U11-48K and/or U11/U12-65K proteins. It is composed of a tandem repeat of perfect U12-type consensus 5′ss motifs. The USSE is recognized by the U11 snRNP or U11/U12 di-snRNP, which activates an upstream U2-type 3′ss and leads to the formation of unstable versions of the 48K and 65K mRNAs. Because both proteins are necessary for U12-type intron recognition and U11/U12 di-snRNP stability, our results suggest that the USSE is part of a regulatory circuit that adjusts the levels of functional U11/U12 di-snRNPs (Figure 6).

U11 snRNP as a Positive Regulator of Alternative Splicing

Our in vitro (Figures 2 and 3) and in vivo (Figure 4) data indicate that the USSE is recognized by the U11 snRNP and that this recognition is necessary for the activation of an upstream U2-type 3′ss. The nearly complete loss of upstream 3′ss activity after any manipulation reducing U11 snRNP binding suggests a key role for U11 in the activation of the upstream 3′ss. This is reflected by the fact that solely the sequences of the USSE and upstream 3′ss are conserved in distantly related species such as human and fish (Figure 1). However, the high level of conservation of the sequences between the USSE and 3′ss among mammals suggests that additional factors binding to nearby regulatory sequences may modulate USSE activity in these organisms. Indeed, this region contains several putative binding sites for known splicing regulators (e.g., SR proteins; Figure S3) that could fine-tune the activity of the USSE.

Our data are consistent with a model in which U11 snRNP interacts with components of the U2-dependent spliceosome, such as U2 snRNP or U2AF, either directly or indirectly, similar to the classic exon definition model (Hoffman and Grabowski, 1992; Robberson et al., 1990; Wu and Maniatis, 1993). Alternatively, U11 snRNP could counteract the effect of non-snRNP inhibitory complexes (Kan and Green, 1999). Even though we cannot distinguish between these two possible mechanisms, our results clearly indicate that the U11 snRNP can function as an activator of alternative splicing. The most likely component within the U11 snRNP mediating the activation of the U2-type 3′ss is the U11-35K protein, a putative functional homolog of the U1-70K protein. U11-35K contains an "alternating Arg" domain rich in Arg-Asp and Arg-Glu dipeptides (Will et al., 1999), and was proposed to mediate communication between the two spliceosomes (Hastings and Krainer, 2001; Will et al., 1999; Wu and Krainer, 1996). Consistent with this model, knockdown of U11-35K led to a loss of the USSE-dependent isoform of the 65K mRNA (Figure 5E).

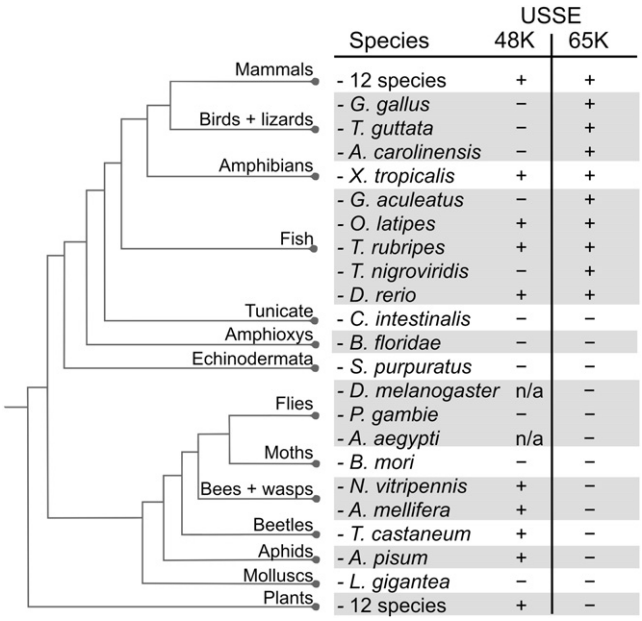
Duplication of the 5′ss within the USSE most likely compensates for the lack of a BPS sequence that is normally needed for stable U11/5′ss binding during intron recognition (Frilander and Steitz, 1999). The fact that either one of the 5′ss motifs within the USSE can base-pair with U11 snRNA alone is probably due to the saturating concentrations of USSE element-containing RNA used in our crosslinking studies (Figure 3). We suggest a model in which a duplicated 5′ss motif creates a sensitive regulatory element for measuring intracellular U11 snRNP concentrations. Consistent with this, a duplicated 5′ss motif has higher affinity for U11 than orphan 5′ss motifs, but lower affinity than the 5′ss of authentic U12-type introns (Figures 2B and 2C). Furthermore, a >90% knockdown of 48K completely inhibits use of an upstream 3′ss within the 65K mRNA (Figure 5D), but shows only relatively mild effects on U12-dependent splicing (Turunen et al., 2008).

There are several reports of negative regulation involving 5′ss-like sites. For example, U2-type 5′ss sequences were identified in a screen of exonic splicing silencers (Wang et al., 2004) and the U1-A protein is regulated by a single U1-binding site in the last exon of its mRNA, which inhibits polyadenylation and leads to mRNA destabilization (Guan et al., 2007). Rous sarcoma virus uses overlapping U1 and U11 binding sites to adjust the balance between spliced mRNAs and unspliced genomic RNAs (Hibbert et al., 1999; McNally et al., 2004). Similarly, duplicated U1 binding sites have been shown to suppress splice sites in the *Drosophila* P-element and in the human apoptotic regulator Bcl-x (Cloutier et al., 2008; Ignjatovic et al., 2005). Because these sites do not function as splicing donors themselves, it seems that duplication of the 5′ss sequence not only enhances U1 binding, but also suppresses splicing by strong mutual interference, as reported elsewhere (Cunningham et al., 1991). In the above examples, regulation affects either the last exon only, or functions through mutual interference via closely spaced sites. This may be a general property of regulatory systems based on U1, because the binding of U1 could otherwise lead to cryptic 3′ splice site activation. This would be less likely in the U12-dependent system, given the high conservation of U12-type splice sites.

USSE Downregulates 48K and 65K mRNAs Levels

Both U11-48K and U11/U12-65K mRNAs are actively downregulated by the USSE, as suggested by the 3–4-fold increase in mRNA levels after U11-35K knockdown (Figure 5C). With 48K, regulation resembles the classic negative feedback regulation in which poison exons that activate the NMD pathway are inserted into mRNA as a result of alternative splicing, as described for numerous other splicing factors (Hase et al., 2006; Lareau et al., 2007; Ni et al., 2007; Saltzman et al., 2008). Inclusion of both the 8-nt exon 4i and a longer stretch of intronic sequences is predicted to result in premature termination of translation. Indeed, our data show the increased inclusion of exon 4i by 48K overexpression, and the stabilization of the 4i-containing isoform by cycloheximide treatment and by expression of a DN form of Upf1 (Figures 5A and 5B). A negative feedback loop regulating 48K is also consistent with its function in U12-type 5′ss recognition (Tidow et al., 2009; Turunen et al., 2008). In contrast, even though 65K mRNA is also a target of this regulation, it is unclear whether the 65K protein, which is associated with the U12 snRNA and thought to play a role in U11/U12 di-snRNP formation (Benecke et al., 2005; Will et al., 2004), is needed for USSE recognition. It has not been possible to knock down the 65K protein (Benecke et al., 2005 and data not shown), and our in vitro data (Figures 2 and 3 and Figure S2) do not conclusively rule out a role for U12 in USSE recognition.

Even though our data indicate that the long 3′ UTR isoform of the 65K is a target for fast cytoplasmic decay (Figures 5C–5H), the mechanistic details of this decay appear different from the NMD of the 48K mRNA. The 5′ss in the 65K 3′ UTR is 15 nt downstream of the stop codon and therefore does not constitute a classic NMD target. Importantly, neither of the 65K mRNA isoforms is stabilized upon cycloheximide or DN Upf1 treatment (Figure 5F and data not shown), further arguing against the possibility that the NMD pathway would be induced by the long 3′ UTR isoform, as suggested recently for other transcripts (Eberle et al., 2008; Singh et al., 2008). Together, our data indicate that despite the mechanistic similarities of U2-type 3′ss regulation in the 48K and 65K pre-mRNAs, the downstream pathways used for USSE-mediated mRNA destabilization differ.



Species	USSE 48K	USSE 65K
- 12 species	+	+
- *G. gallus*	–	+
- *T. guttata*	–	+
- *A. carolinensis*	–	+
- *X. tropicalis*	+	+
- *G. aculeatus*	–	+
- *O. latipes*	+	+
- *T. rubripes*	+	+
- *T. nigroviridis*	–	+
- *D. rerio*	+	+
- *C. intestinalis*	–	–
- *B. floridae*	–	–
- *S. purpuratus*	–	–
- *D. melanogaster*	n/a	–
- *P. gambie*	–	–
- *A. aegypti*	n/a	–
- *B. mori*	–	–
- *N. vitripennis*	+	–
- *A. mellifera*	+	–
- *T. castaneum*	+	–
- *A. pisum*	+	–
- *L. gigantea*	–	–
- 12 species	+	–

Figure 7. Evolutionary Distribution of USSEs in 48K and 65K Genes in Eukaryotes

All organisms listed contain U12-type introns (Dávila López et al., 2008). "n/a" refers to cases in which the ortholog of 48K could not be identified.

Is the Evolutionary Fate of U12-Type Introns Linked to USSEs?

The high conservation of the USSE element and the upstream U2-type splice site in both animal and plant lineages suggests that this regulatory system is under strong selection pressure, further suggesting that its function in maintaining the levels of 48K and 65K protein is important at the cellular or organism level. This may reflect the need to control the activity of the U12-dependent spliceosome to provide the rate-limiting regulation of genes containing U12-type introns, as suggested by Patel et al. (2002) and Pessa et al. (2006). Interestingly, a recent study indicated that the half-life of the 65K mRNA is significantly shorter than that of other U11/U12 di-snRNP components (Friedel et al., 2009), further suggesting that its stability regulates the activity of the U12-dependent spliceosome. Additionally, the conservation between the animal and plant kingdoms supports the early origin of the U12-dependent spliceosome and might hint that it was regulated via the USSE already in the earliest ancestors of modern eukaryotes. However, the monophyletic origin of the USSE is somewhat open given the difference in *48K* gene structure and the location of the USSE (either intronic or 3′ UTR) between animals and plants, which is also consistent with convergent evolution. Loss of the USSE from either the *48K* or *65K* gene, as observed in the bird lineage, several insects, and plants (Figure 7), suggests that the processes driving the removal of U12-type introns also affect this regulatory system. The loss of both USSE elements from various animal lineages is more difficult to reconcile. An evolutionary scenario in which the deletion of USSEs from both genes could lead to the loss of the rate-limiting regulatory function of U12-type introns and, ultimately, to accelerated loss of U12-type introns is consistent with our results. The low number of U12-type introns in *Drosophila* and other *Diptera* species (Alioto, 2007) supports this conclusion, but currently the number of fully sequenced genomes is not sufficient to address this question. Nevertheless, the presence of a strong evolutionary selection pressure indicates an important function for the USSE and U12-type introns that will stimulate research efforts in the field in the near future.

EXPERIMENTAL PROCEDURES

Oligonucleotides, Plasmids, and Databases

For a list of oligos, see the Supplemental Experimental Procedures. Plasmids p48KUSSE and p65KJT were used as templates for in vitro transcription, pSmE-48KUSSE as a reporter, and pCIneo-48K for mammalian overexpression experiments, and pGL4.13L65K, pGL4.13S65K, and pGL4.13F65K for analysis of 65K 3′ UTR function. For details on the construction of plasmids, see the Supplemental Information. The DN form of Upf1 was expressed from pcDNA3-hUpf1 R844C (Lykke-Andersen et al., 2000), and WT and mutant forms of U11 plasmids were as by Incorvaia and Padgett (1998). Databases used are listed in the Supplemental Information.

In Vitro RNA Substrates

Templates for in vitro transcription were generated by PCR using oligos detailed in the Supplemental Information. Capped substrates were produced by in vitro transcription using T7 RNA polymerase. To biotinylate the RNA, transcription was done in the presence of aminoallyl-UTP, and the transcripts were subsequently coupled to biotin-succinimidyl esters (Biotin-XX-SE, Molecular Probes) according to manufacturer's instructions.

In Vitro Complex Assembly, Native Gel Analyses, and Crosslinking

USSE complexes were assembled in vitro under splicing conditions and resolved on native gels as described by Turunen et al. (2008), and ATP was depleted using a glucose-hexokinase mixture as described by Michaud and Reed (1991). Psoralen crosslinking and RNase H cleavage were performed essentially as described by Tarn and Steitz (1994), and blocking with 2ome oligos as described by Frilander and Steitz (1999).

Streptavidin Pull-Downs

Twenty-microliter samples of assembled USSE complexes were added to 10 μl streptavidin-agarose beads (Thermo Scientific) in 200 μl pull-down buffer (PDB; 20 mM HEPES [pH 7.9], 150 mM KCl, 3 mM $MgCl_2$, 0.05% NP-40, and 50 μM DTT) and incubated in an end-over-end rotator for 3 hr at RT. The beads were washed with PDB four times and treated with proteinase K, followed by phenol extraction and ethanol precipitation of the RNA.

Northern Blotting

Northern blot analyses were as described by Tarn and Steitz (1996b), except that for the LNA probes hybridization was performed at 50°C and the final wash at 80°C (U11 or U1 probes) or 65°C (other snRNA probes), and for the DNA probes the washes with 0.1 × SSC – 0.1% SDS were omitted. Blots were visualized with a phosphoimager (Fuji FLA-5010).

Cell Culture and RNAi

HEK293 and CHO cells were transfected using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. For oligo block experiments, 100 nM 2ome RNA oligos were cotransfected with pCIneo-48K as indicated. Morpholino oligos (10 μM) were transfected to HEK293 cells using the endoporter reagent (Gene Tools). To study the effect of NMD, cells were treated with 25 μg/ml cycloheximide for 2 hr prior to RNA extraction, and ethanol was used for the control reaction. To address the role of U11 in 65K 3′ UTR splicing, CHO cells grown in 12-well cell culture dishes were transfected with 80 ng of the 65K 3′ UTR reporter and 1520 ng of the U11 construct. RNAi-mediated knockdown of U11/U12 proteins was performed with HeLa cells and total

RNA was isolated as described elsewhere (Will et al., 2004; Turunen et al., 2008).

Cell Fractionation

HEp-2 cells (3–5 × 10^6) were used per fractionation. Cells were washed with cold PBS and resuspended in cold hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT). Cells were lysed in a Kontes tight pestle Dounce homogenizer. Lysate was then layered over 1 M sucrose in hypotonic buffer and centrifuged for 30 min at 2400 × *g*. Cytoplasmic supernatant was recovered directly. Nuclei that pelleted through the sucrose cushion were washed once in sucrose buffer and then resuspended in hypotonic buffer. All samples were sonicated using a Bioruptor bath sonicator and centrifuged for 10 min at 12 000 × *g*. For reporter assays, 3 × 10^6 cells were electroporated using a BioRad GenePulser II with equimolar amounts (totaling 15–20 μg) of the respective luciferase constructs 48 hr prior to fractionation. Fractionation quality was checked by western blotting using eIF2α as a cytoplasmic and Topoisomerase-I as a nuclear marker.

RT-PCR

RNA was extracted 24 or 48 hr after transfection using Trizol (Invitrogen) and was treated with DNase. cDNA was prepared using either oligo-$dT_{(20)}$ or random primers and SuperScript III reverse transcriptase (Invitrogen). To evaluate splicing of the 65K 3′ UTR, a multiplex PCR was performed using reverse primers targeting the long and short isoform.

Expression Analysis of Luciferase Constructs

For the 65K 3′ UTR dual luciferase expression assay (Promega), equimolar amounts of pGL4.13L65K and pGL4.13S65K were transfected into HEK293 cells. Luciferase activity was measured after 24 hr with a luminometer (GloMax 20/20, Promega) as instructed by the manufacturer and normalized against *Renilla* luciferase activity.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at doi:10.1016/j.molcel.2010.02.014.

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