I. Supplementary Data for Motta-Mena et. al.

Figure S1: related to Figure 2

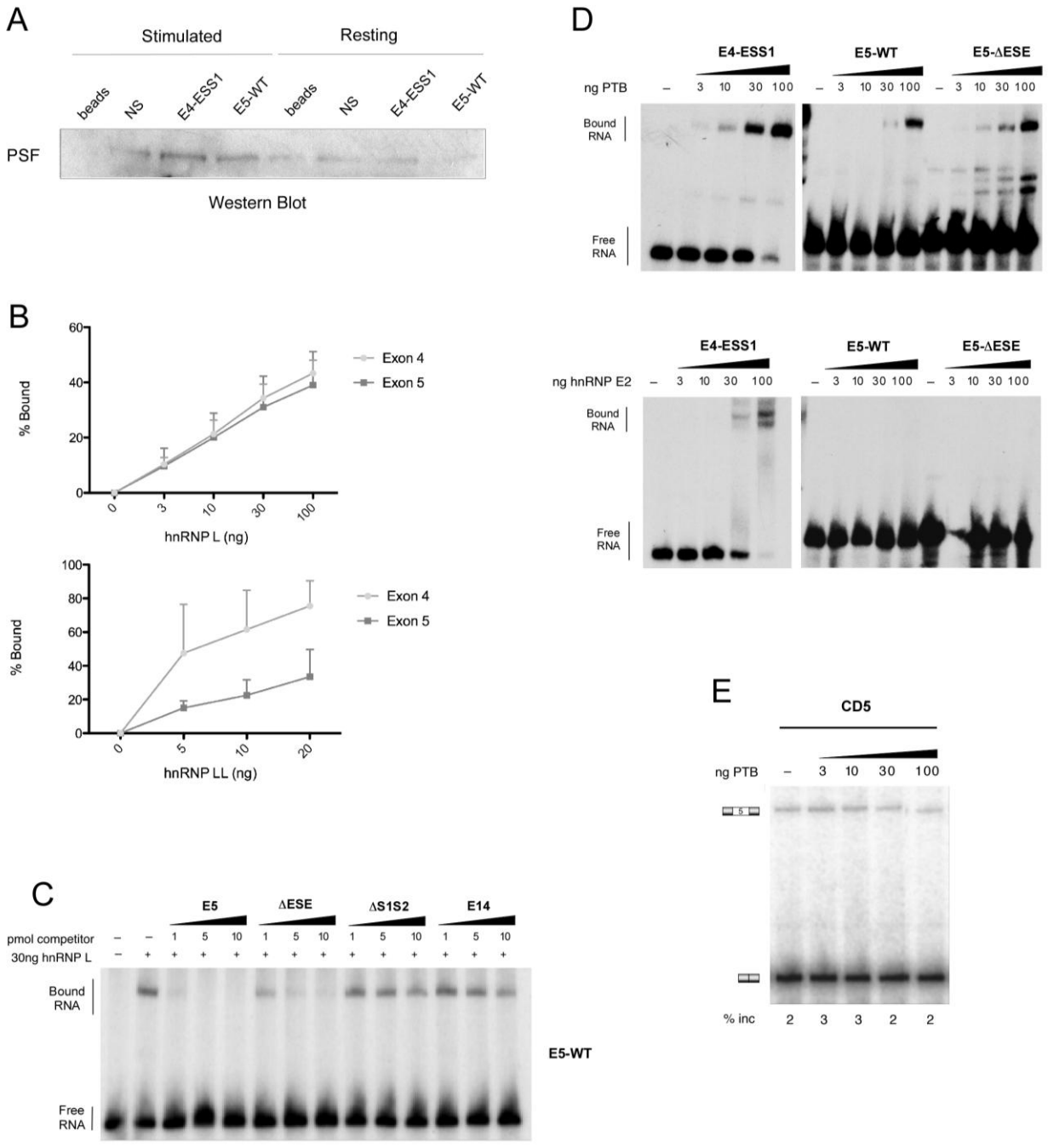
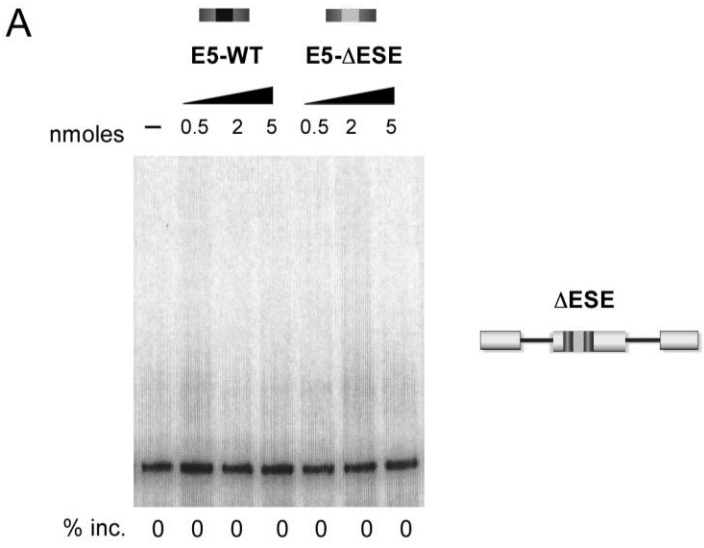
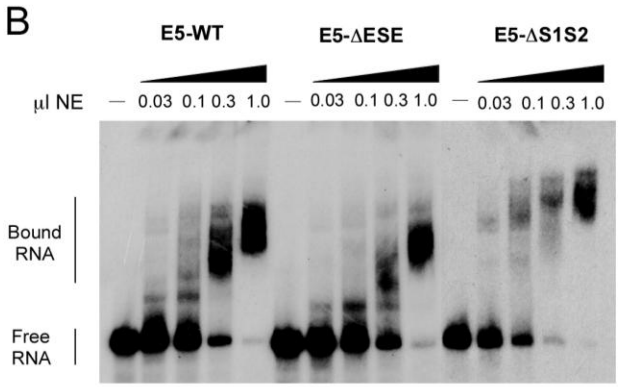


Figure S2: related to Figure 4





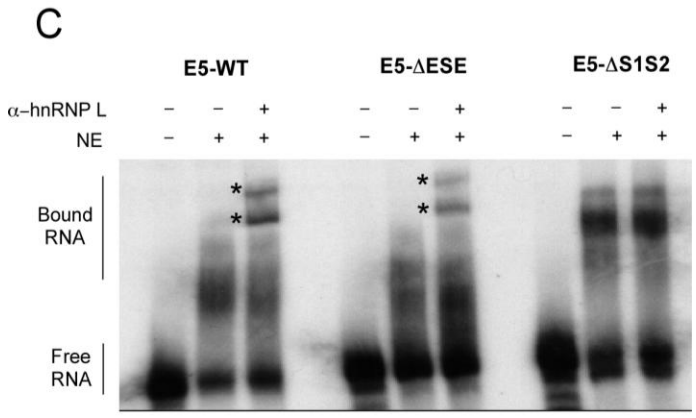


Figure S3: related to Figure 5

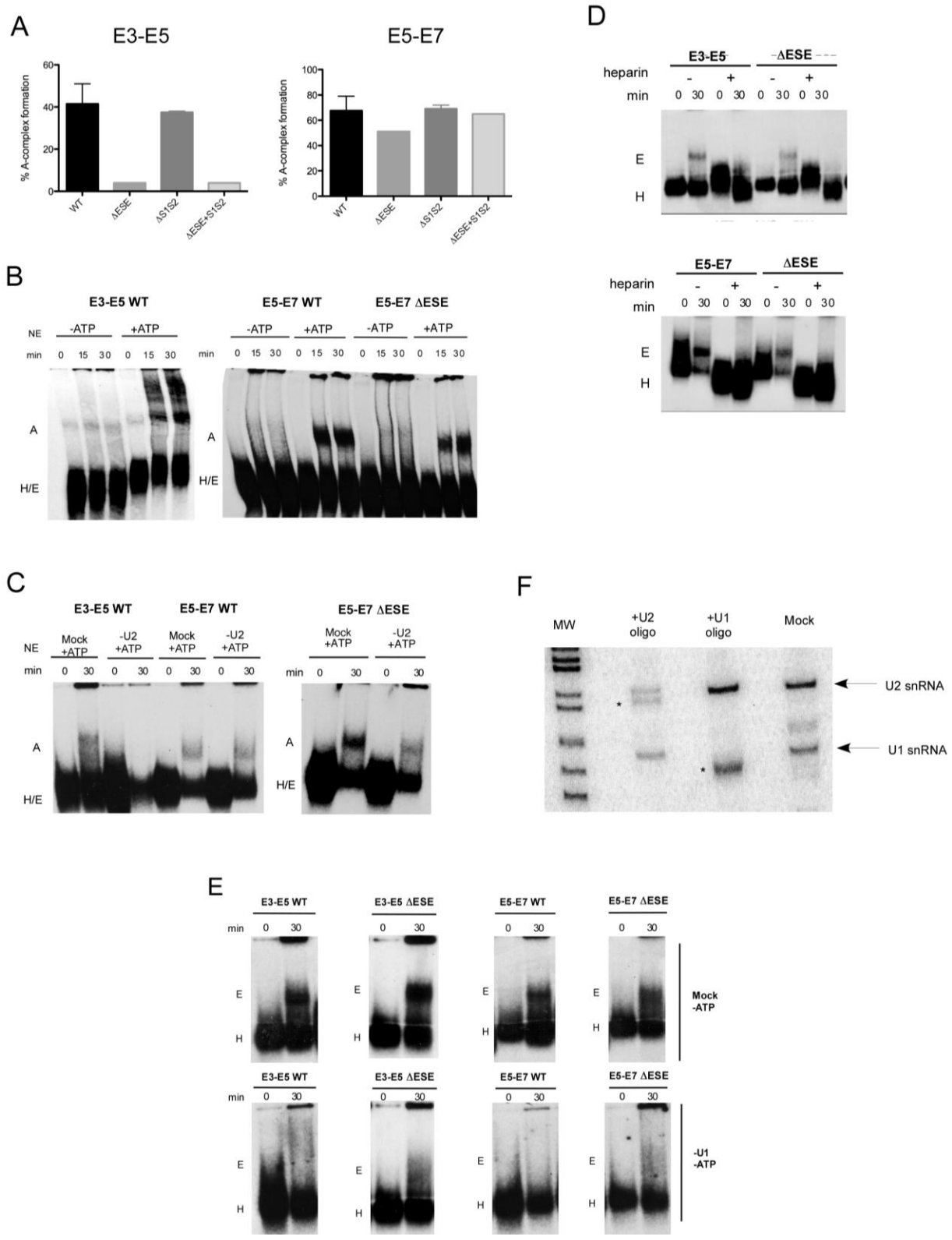
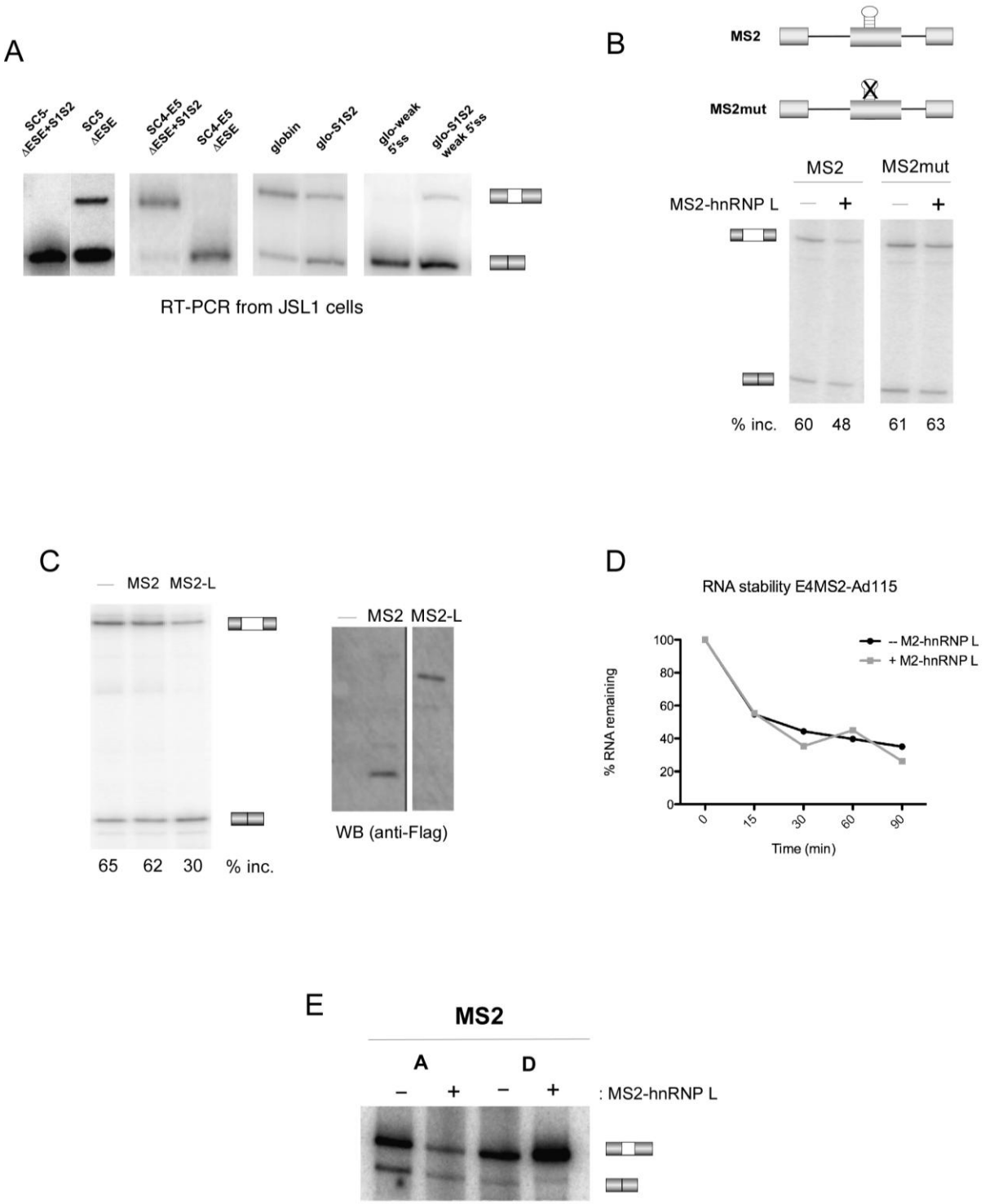


Figure S4: related to Figure 6



II. Supplementary Figure Legends

Supplementary Figure S1.

(A) Western blot for PSF from RNA-affinity isolations done with the RNAs and extracts noted. (B) The percent RNA bound for exon 4 (light grey) and exon 5 (dark grey) was calculated from RNA-mobility shift binding data derived from at least 3 different experiments done with recombinant hnRNP L (top) or hnRNP LL (bottom). (C) RNA mobility-shift experiment using 1 pmol E5-WT probe incubated with recombinant hnRNP L in the absence or presence increasing amounts of the indicated competitor RNAs. (D) RNA mobility-shift of radiolabeled ESS1, E5-WT and E5-ΔESE probes incubated with increasing amounts of recombinant PTB (top) or hnRNP E2 (bottom). (E) *In vitro* splicing of CD5 minigene in the absence or presence of increasing amounts of recombinant PTB. Percent inclusion for representative experiment is shown below.

Supplementary Figure S2.

(A) In vitro splicing of CD5ΔESE minigene done in JSL1 nuclear extract in the absence or presence of either E5-WT or ΔESE competitor RNAs. (B) RNA-mobility shift experiment done with radiolabeled exon 5 probes (WT, ESE, S1S2) and increasing amounts of JSL1 nuclear extract. (C) Same as in panel B except reactions were incubated in the absence (-) or presence (+) of an antibody against hnRNP L (α-hnRNP L). Super-shifted complexes are indicated by asterisk.

Supplementary Figure S3.

(A) Quantification of assembly gels from Figure 5B, and replicate experiments, done by phosphorimager. (B) Assembly done in nuclear extract depleted of ATP (-ATP) or left untreated (+ATP). The substrates used are indicated above. (C) Assembly done with nuclear extract depleted of U2snRNA (-U2) or untreated (mock/+ATP). (D) Assembly done as in Figure 5B except nuclear extract was depleted of ATP prior to use. (E) Assembly done with nuclear extract depleted of U1 snRNA and ATP (-U1/-ATP) or untreated and ATP-depleted (mock/-ATP). The substrates used are indicated above each picture, E3-E5 WT is shown on the right. (F) Primer extension of oligo/RNase H-treated (-U2 or –U1) and untreated (mock) nuclear extracts used in the experiments shown in panels C and E. A mix of radiolabeled primers against both U1 and U2 snRNA was used and products were resolved on denaturing polyacrylamide gel.

Supplementary Figure 4. MS2-hnRNP L specifically regulates alternative splicing when tethered to RNA.

(A) Representative gel for data used for quantitation shown in Figure 6A. (B) Substrates containing either the correct MS2 stem loop sequence or a mutated (reversed) version (MS2mut) were used in *in vitro* splicing assays in the presence or absence of MS2-hnRNP L fusion protein as described in Material and Methods. Resulting products were quantified using phosphoimager analysis; a result representative of 3 experiments is shown. (C) *In vitro* splicing as in B comparing the effects of nuclear extract alone, or nuclear extract containing MS2 or MS2-hnRNP L. MS2 alone does not influence alternative splicing; numbers represent mean %

inclusion of 3 experiments (left). Western blot analysis shows similar expression of MS2 and MS2-hnRNP L (right). (D) Uniformly ^{32}P labeled pre-mRNA containing the MS2 stem loop was incubated with nuclear extract in the presence or absence of MS2-hnRNP L under conditions that do not support splicing. After the indicated time points samples were analyzed by denaturing PAGE and remaining RNA was quantified using a phosphoimager. (E) To confirm that the data shown in Figure 6B were not an artifact of detection of weak inclusion, we optimized the RT-PCR for detection of the included product. This skews the ratio of detection in favor of the 3 exon product such that we no longer get a true reflection of the efficiency of splicing. However, we still get an accurate measure of the relative amount of included and excluded product between two samples. Shown is data demonstrating a MS2-hnRNP L-dependent increase in exon inclusion for construct D (see Figure 6B), but a decrease in exon inclusion for construct A (see Figure 6B).

III. Supplementary Experimental Procedures for Motta-Mena et al.

Minigenes

Construct SC5 was previously described in Tong et al. (2005). Briefly, the minigene consists of CD45 exon 5 and surrounding intron flanked by intron and exon sequence from the human β–globin gene. SC5-ΔESE, SC5-ΔS1S2, and SC5-ΔESE+S1S2 were made using PCR to replace the corresponding sequence within exon 5 to TCAGTATGACTCTCAGTATG . This sequence was originally identified as a sequence with no splicing regulatory activity (Schaal and Maniatis, 1999), moreover, we have used it extensively in previous studies with no discernable effects on splicing in any context (Tong et al., 2005; Lynch and Weiss, 2001; Rothrock et al., 2003). Oligonucleotides encoding the 100 nt E5-WT, -ΔESE, and -ΔS1S2 were cloned directly downstream of a T7 polymerase promoter and served as minigene templates for transcription of competitor RNAs and RNA probes. The RNAs were transcribed with T7 polymerase (Promega) in the absence or presence of ^{32}P-CTP to radioactively label probes. The CD4 and CD5 minigenes, used for *in vitro* splicing, were previously described in Rothrock et al. (2003) and Tong et al. (2005). Single-intron constructs (E3-E5 and E5-E7) were cloned into CD5 using Mlu I and Hind III. The exon 4/exon 5 chimeras (SC4-E5 ΔESE and SC4-E5 ΔESE+S1S2) were generated by inserting the 100 nt regulatory region of exon 5 into the PstI site of SC4 (Rothrock et al., 2003), resulting in exon 5 flanked by the splice sites and intron sequence from exon 4. Substrates for *in vitro* splicing that contained the MS2 binding site were generated by inserting a single MS2 hairpin into the MluI site of SCglo (Rothrock et al., 2003). A fragment of this construct with the MS2 containing exon and 110 bp intron on both sides was PCR amplified with primers generating XhoI and HindIII sites and ligated into an XhoI/HindIII cut AdML vector

(kind gift of K. Hertel) yielding a glo-MS2 exon flanked by 2 AdML exons. Splice site mutations were introduced by PCR and confirmed by sequencing.

Nuclear extract and recombinant proteins

Nuclear extract was purified from JSL1 cells using a standard protocol previously described in Lynch and Weiss, 2001. Recombinant hnRNP L and PTB were expressed as a GST fusion proteins in SF9 cells and were purified using glutathione sepharose 4B resin (GE Biosciences) as described previously in (Rothrock et al., 2005). MBP-hnRNP E2 was expressed and purified from *Escherichia coli* using a protocol previously described by Gamarnik and Andino (1997). PSF and hnRNP LL cDNAs were cloned directly downstream of a EF promoter and modified with an N-terminal Flag tag. JSL1 cells stably expressing either Flag-PSF or Flag-hnRNP LL were grown under resting or stimulated conditions and then lysed to prepare nuclear extract. Tagged proteins were purified from nuclear extract with EZ-View Red FLAG-conjugated resin (Sigma) in GFB100 (20mM Tris-Cl, pH 7.5, 100mM KCl, and 0.2mM EDTA, pH 8.0). Following extensive washing in GFB100, the proteins were eluted with 500 ng/ul of 3X Flag peptide (Sigma). SF2/ASF protein was expresses in SF9 cells and was purified as previously described by Lynch and Maniatis, 1996.

Cell culture

JSL1 cells (Lynch and Weiss, 2000) were cultured in RPMI+ 5% fetal calf serum at 37C in 5% CO_2. Sub-lines of JSL1 cells that stably express the minigenes described were created by transfecting 10 million cells with 10ug of minigene plasmid by electroporation and grown under drug selection as described (Rothrock et al., 2003). For splicing analysis, three independent

clones of each minigene were either left untreated or treated with 20 ng/ml of PMA for 60 h, after which cells were harvested and total RNA extracted using RNABee (Tel-Test). Minigene derived spliced products were analyzed by RT-PCR using vector-specific primers (see below).

RT-PCR

RT-PCR and analysis was carried out as previously described in detail by Rothrock et al. 2003. In brief, a low-cycle PCR protocol was used, such that the signal detected is linear with respect to input RNA. Minigenes were analyzed using the vector-specific primers ACT and GE3R (sequence published in (Rothrock et al., 2003)) for SC5 constructs and T7Mlu and E7R1 primers for CD4 and CD5 minigenes (sequence published in (House and Lynch, 2006)). Quantitation was done by densitometry using a Typhoon Phosphoimager (Amersham Biosciences).

RNA affinity purification

500 pmol of *in vitro* transcribed RNA was incubated with sodium periodate and coupled to adipic acid beads overnight using a protocol adapted from that previously described (Caputi and Zahler, 2002). Beads were incubated with ~200μg of JSL1 nuclear extract in a 500μl binding reaction containing (final concentrations): 3.2mM $MgCl_2$, 20mM phosphocreatine, 1mM ATP, 1.3% polyvinyl alcohol, 25ng of yeast tRNA, 75mM KCl, 10mM Tris, pH 7.5, 0.1 mM EDTA, 10% glycerol. Binding reactions were incubated with gentle agitation for 30 min at 30°C. The protein-RNA-bead complexes were washed with GFB100+ 4mM $MgCl_2$, resuspended in 2X SDS loading buffer, denatured for 5 min at 95°C, analyzed under denaturing conditions on a 10% gel (Acrylamide/Bis 37.5:1, BioRad), and detected by silver staining (BioRad) or western blot.

Western blotting

Western blotting was carried out as previously described in Lynch and Weiss (2000). Antibodies for Western blots were as follows: anti-hnRNP L (4D11, Abcam), anti-PTB N-term (rabbit polyclonal, a gift from D. Black), anti-hnRNP E2 (rabbit polyclonal, a gift from R. Andino), anti-hnRNP K/J (3C2, Immunoquest), anti-hnRNP D (rabbit polyclonal; BioLegend), anti-PSF (6D7, Abnova), and anti-hnRNP LL (Aviva).

RNA mobility shift assay

In vitro transcribed RNAs were gel-purified and adjusted to 10^4 cpm/μl specific activity. Each RNA was incubated with JSL1 nuclear extract in a total volume of 10 μl and under standard binding conditions similar to that described for the RNA-affinity assays, but with the addition of (final concentration) 0.1 μl of RNasin (Promega, 40U/μl) and 0.8 μg of BSA. Reactions were incubated for 20 min at 30°C, after which heparin was added to a final concentration of 5 μg/ml and incubated for an additional 5 min at 30°C. Reactions were analyzed on a 4.5% native gel (Acrylamide/Bis 29:1 BioRad) and visualized by autoradiography.

***In vitro* splicing**

In vitro splicing reactions were carried out as detailed by Rothrock et al. (2005). In brief, 1 fmol of transcribed pre-mRNA template was incubated in 30% JSL1 nuclear extract in a total volume of 12.5 μl under standard binding conditions similar to those described above and in Rothrock et al. (2005). Reactions were incubated for 2 h at 30°C, after which the RNA was recovered by proteinase K treatment followed by phenol-chloroform extraction and precipitation. Analysis of

the resulting spliced products was done by RT PCR as described above. For competition studies, the specified amount of competitor (E5-WT, -M3, or –M2M4) was added to splicing reactions before addition of RNA substrate and reactions were processed as described above. For in vitro splicing experiments with MS2-tagged hnRNP L, reactions as above were supplemented with nuclear extract from 293 cells that were transiently transfected with an MS2-hnRNP L expression construct, or an empty vector control. Reactions were processed and analyzed as above.

UV Crosslinking

Radiolabeled RNA was incubated in JSL1 nuclear extract or with recombinant hnRNP L or SF2/ASF under similar conditions described for the RNA affinity purification assays. Reactions were incubated for 20 min at 30°C, crosslinked using UV light (254 nm) for 20 min on ice, and digested with 2ug (final concentration) of RNase T1 and RNase A each for 20 min at 37°C. Reactions were analyzed under denaturing conditions on a 12% gel (Acrylamide/Bis 37.5:1, BioRad), and visualized by autoradiography.

Spliceosome assembly assay

Spliceosome assembly on our pre-mRNA substrates was analyzed using standard protocols (Das and Reed, 1999; Konarska and Sharp, 1986). Radiolabeled pre-mRNA substrates were adjusted to 10^5 cpm/μl and incubated in nuclear extract under splicing conditions for the indicated amount of time. Spliceosome complexes were analyzed on nondenaturing polyacrylamide (4%; Acrylamide/Bis 40:1) or agarose (1.5%) gels as specified in each case. Gels were run at 250 V for 5-6 h for acrylamide gels and at 70 V for 3-3.5 h for agarose gels.

IV. References Cited in Supplement

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