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The U1 snRNP-associated factor Luc7p affects 5′ splice site selection in yeast and human

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ABSTRACT

yLuc7p is an essential subunit of the yeast U1 snRNP and contains two putative zinc fingers. Using RNA–protein cross-linking and directed site-specific proteolysis (DSSP), we have established that the N-terminal zinc finger of yLuc7p contacts the pre-mRNA in the 5′ exon in a region close to the cap. Modifying the pre-mRNA sequence in the region contacted by yLuc7p affects splicing in a yLuc7p-dependent manner indicating that yLuc7p stabilizes U1 snRNP–pre-mRNA interaction, thus reminding of the mode of action of another U1 snRNP component, Nam8p. Database searches identified three putative human yLuc7p homologs (hLuc7A, hLuc7B1 and hLuc7B2). These proteins have an extended C-terminal tail rich in RS and RE residues, a feature characteristic of splicing factors. Consistent with a role in pre-mRNA splicing, hLuc7A localizes in the nucleus and antibodies raised against hLuc7A specifically co-precipitate U1 snRNA from human cell extracts. Interestingly, hLuc7A overexpression affects splicing of a reporter *in vivo*. Taken together, our data suggest that the formation of a wide network of protein–RNA interactions around the 5′ splice site by U1 snRNP-associated factors contributes to alternative splicing regulation.

INTRODUCTION

Splicing of most polII transcripts is required prior to their export out of the nucleus (1). The spliceosome is the macromolecular complex responsible for this intricate process. Early spliceosome assembly events are target of regulatory factors that alter splice site choice and/or modulate splicing activity, constituting a widespread mechanism of gene expression regulation (2). Interestingly, processes as diverse as meiosis in yeast, dosage compensation in fruit flies or programmed cell death in humans are regulated in this manner (3–5).

Spliceosome assembly *in vitro* is initiated by formation of stable complexes containing U1 snRNP, pre-mRNA and non-snRNP factors (6). In yeast, the earliest known splicing complexes are called commitment complexes because their formation targets the pre-mRNA substrate to the splicing pathway (7). The mammalian E complex is the counterpart of the yeast commitment complexes (8). During the formation of commitment complexes, U1 snRNA base pairs with the intron and exon sequences flanking the 5′ splice site (9–11), whereas the cap-binding complex (CBC) binds to the pre-mRNA cap (12). In addition, several protein components of the U1 snRNP make contacts with the pre-mRNA (13,14). These protein–RNA contacts stabilize pre-mRNA–U1 snRNP interaction and affect 5′ splice site selection (13).

Like its metazoan counterparts, the yeast U1 snRNP contains two classes of proteins: the Sm proteins shared with the U2, U4 and U5 snRNPs and the U1 snRNP-specific proteins (15,16). Interestingly, homologs of all three mammalian U1 snRNP-specific proteins (U1-A, U1-C and U1-70K) can be found in the yeast complex (Mud1p, yU1-C and Snp1p, respectively). However, the yeast U1 snRNP contains in addition seven specific proteins (Snu71p, Snu65p, Snu56p, Prp39p, Prp40p, Nam8p and yLuc7p) (15,17,18). Among these proteins, only for Nam8p has been described a mammalian homolog, the apoptotic factor TIA-1 (4,19).

We identified yLuc7p as component of the U1 snRNP by means of biochemical purification (referred to as Snu30p in our previous study) (18) and through a genetic screen causing synthetic lethality with CBC (17). Analysis of yLuc7p mutants revealed that the composition of yeast U1 snRNP was altered in these strains and that the corresponding extracts were unable to support any of the defined steps of splicing unless supplemented with

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recombinant wild-type yLuc7p (17). In addition, splicing of introns with non-consensus 5′ splice site or branchpoint sequences was defective in yLuc7p mutant strains. For reporters containing two competing 5′ splice sites, a loss of efficient splicing of the cap proximal splice site was observed in yLuc7p-deficient cells, analogous to the defect seen in strains lacking CBC. These results lead Fortes *et al.* (17) to suggest that the loss of yLuc7p disrupts a U1 snRNP–CBC interaction normally contributing to 5' splice site recognition.

Using a combination of RNA–protein cross-linking, and a strategy we called Directed Site-Specific Proteolysis (DSSP), we have now shown that yLuc7p contacts specifically exon 1 of the pre-mRNA through the first of its two zinc finger motifs. Modification of the RNA sequence contacted by yLuc7p affects the pre-mRNA splicing efficiency in a yLuc7p-dependent manner. Our data suggest that interaction of yLuc7p with the upstream exon stabilizes the pre-mRNA–U1 snRNP interaction. This is reminiscent of the mode of action of Nam8p, which facilitates intron recognition by binding to intron sequences following the 5′ splice site (13). To assess whether the function of yLuc7p in splice site selection is conserved, we identified putative human homologs and cloned cDNAs encoding two of them (hLuc7A and hLuc7B2, respectively). Both proteins have RE and RS repeats characteristic of splicing factors. In addition, they have two zinc finger motifs similar to those present in yLuc7p and characteristic of RNA-binding proteins. By using antibodies raised against hLuc7A we show that this protein localizes in the nucleus of HeLa cells. Interestingly, these antibodies specifically precipitate U1 snRNA from HeLa extracts. Furthermore, overexpression of hLuc7A in HeLa cells affects splice site selection of an adenovirus E1A reporter, directing splicing selection towards the most 5′ distal site. Our results demonstrate that hLuc7A is a new U1 snRNP-associated splicing factor. In addition, our data support the formation of a wide network of protein–RNA interactions around the 5′ splice site by U1 snRNP associated-factors. This network allows for modulated 5′ splice recognition and contributes thereby to alternative splicing regulation.

MATERIALS AND METHODS

Strains and plasmids

All yeast strains derive from the wild-type MGD353-13D (9). BSY885 (yLuc7p-protA) and BSY761(Nam8p-TAP) were constructed following standard methodology (20,21). BSY747(yLuc7p-TAP) and BSY593(Nam8p-protA) have been previously described (13,18). The yLuc7p-HA-TEV-protA fusion inserted in a centromeric LEU2 plasmid was introduced by plasmid shuffling in a strain carrying a disrupted chromosomal copy of LUC7 to give strain BSY1069. An isogenic wild-type strain expressing the yLuc7p-protA fusion was constructed in parallel and named BSY1067. The deletion of the first zinc finger of yLuc7p (fusion yLuc7p-ΔZF-protA) was built following the same strategy, yielding strain BSY1077.

All the RP51A derivatives used in cross-linking reactions were synthesized with a specific activity of 2×10^7 Ci/mol as previously described (7). The constructs WT-B and 5′SSmut correspond to the plasmids pBS195 and pBS196, respectively (13,22) and were linearized with EcoRV prior to transcription. In order to obtain constructs Ex5′U (plasmid pBS1983), Ex3′U (pBS1982) and IntU (pBS1985), the sequence including from the T7 promoter to the EcoRV site from plasmid pBS195 was cloned by PCR between the EcoRI and XbaI sites of pUC19. The EcoRV site present in pBS195 was substituted by a PvuII site since this site lacks Ts upstream the site of cleavage and therefore no Us are incorporated upon transcription. These constructs were linearized prior to transcription with PvuII.

To analyze commitment complex assembled in mRNAs containing Ex5′U, Ex3′U and IntU sequences, we cloned by PCR the sequence between the EcoRV and the DdeI sites from plasmid pBS195 downstream of the XbaI site of each construct.

Plasmid pBS983 (23) was used to build the reporters used in splicing assays. It contains a synthetic intron inserted upstream of the lacZ coding sequence designed to include unique restriction sites in the intron and flanking exons. Sequences A, G and H (Figure 4A) were generated by PCR and introduced between the BamHI and KpnI sites of pBS983. Sequence H can potentially form a hairpin structure with $\Delta G = -8.2$ kcal/mol at 25°C. Duplications were obtained by cloning the XhoI–SacI fragment from each construct in the SalI–SacI sites of the same plasmids (13,22,24). Constructs AA, AG, AH, GA, GG, GH, HA, HG and HH correspond to the plasmids pOP708 to pOP716.

The sequence of hLuc7B2 is deduced from reconstructed cDNA sequence derived from the IMAGE clones 156683 and 897347. A full-length coding sequence was recreated by combining these two clones to give pBS1903. The sequence coding for hLuc7A was obtained by sequencing IMAGE clone 198596 and sequencing a 3′ RACE fragment to give plasmid pBS2101.

Plasmids overexpressing SF2 and hnRNPA1 in pCGT7 (tagged with a T7 epitope) and the expression reporter for adenovirus E1A were gifts from J. Caceres (25). To overexpress hLuc7A in eukaryotic cells, the hLuc7A ORF was subcloned between the BamHI and XbaI sites of pCGT7. All the constructs were confirmed by sequencing.

Splicing analysis, cross-linking and DSSP

β-Galactosidase assays were performed twice independently and in duplicate. Variation between duplicate samples was at most 20%. Standard deviation is represented.

Extract preparation, commitment complex formation and cross-linking analysis were performed as described (13). All pre-mRNAs used in cross-linking reactions were internally labeled with αP^{32}-UTP, and 4-thio-UTP substituted for cold UTP. (13). Wash buffer in immunoprecipitations was IPP150 (10 mM Tris–Cl pH8.0, 150 mM NaCl, 0.1% NP-40). For TEV digestion, after immunoprecipitation the beads were sedimented in a picofuge and 40 μl of TEV cleavage buffer (IPP150 with

1 mM DTT and 1 mM EDTA) was added to the beads. Subsequently, samples were split in two and 1 µl of TEV enzyme (Gibco) was added to one of them. Samples were incubated 30 min at 37°C. Ten microliters of 3× SDS loading buffer was added directly after cleavage and proteins were analyzed by SDS–PAGE.

In the DSSP reactions, commitment complexes were assembled using extracts derived from the yLuc7p–HA-TEV–protA strain and from the parental yLuc7p–protA strain. Samples were UV irradiated, treated with RNaseT1, immunoprecipitated with IgG-coupled beads, and split in two. One half was treated with TEV protease while the other half was mock-treated. Subsequently, proteins were analyzed by SDS–PAGE.

Antibody production and western blot

Fragments coding for most of the hLuc7A protein, the N-terminal part containing the region of homology with yLuc7p, or part of the C-terminal extension containing the RS and RE repeats, were inserted in the pGEX2T′-6 plasmid (gift from V. Baldin) yielding plasmids pBS1905, pBS1907 and pBS1909, respectively. All three constructs were expressed in *Escherichia coli* codon plus RIL cells (Stratagene). Rabbits (New Zealand White females) were immunized following standard procedures.

Western blot to detect protein A tagged proteins was performed with peroxidase-conjugated antiperoxidase antibody (Sigma). Western blot anti-hLuc7A was performed with antibody 0J38 (polyclonal against the N-terminal part of hLuc7p) diluted at 1:1000. SF2, hnRNPA1 and hLuc7A were detected in western blots with specific antibodies against the T7 epitope tag (Chemicon International) at 1:1000 dilution.

Immunoprecipitation of HeLa nuclear extracts

HeLa cell nuclear extracts were prepared as described (26). Fifty microlitres of beads were washed three times in IPP150 and incubated with 100 µl of serum (0J38 for N-terminal, 0410 for C-terminal) and 300 µl of IPP150 for 2 h at 4°C. The volume equivalent to 10 µl of beads was used for each immunoprecipitation experiment. The beads were sedimented in a picofuge and 75 µl of IPP150 plus 25 µl of nuclear extract were added. After 2 h at 4°C, the beads were extensively washed with IPP150. RNA was recovered and analyzed by primer extension using primers specific for U1 snRNA (CTGGGAAAACCACCTTCG TGATC) and U2 snRNA (AGGACGTATCAGATAT TAAACTG).

Immunolocalization

HeLa cells grown to 70% confluence in DMEM medium supplemented with 10% fetal calf serum were transferred to coverslips and incubated overnight to allow for attachment. Cells were washed with PBS, fixed with 2% formaldehyde in PBS for 5 min at 37°C and permeabilized by incubation in 0.1% NP-40 in PBS for 10 min at 37°C. After washing with PBS, cells were incubated in presence of hLuc7A antibody 03J0 (raised against the N-terminal fragment of hLuc7A) at 1:5000 dilution and anti-tubulin (T9026, Sigma) at 1:1000 dilution for 2 h at RT. Cells were extensively washed with PBS and incubated with the secondary antibody (anti-rabbit Alexa 488 and anti-mouse Alexa 568, both from Molecular Probes) at 1:100 dilution for 1 h at RT. Cells were extensively washed with PBS, stained with DAPI (at 5 µg/ml) and coverslips were mounted for observation under a confocal microscope (Axioplan, Zeiss) equipped with a digital color video camera (Leica, Model LEI-750TD).

In vivo splicing assays

HeLa cells were grown in 60 mm Ø plates to 50% confluence in DMEM medium supplemented with 10% fetal calf serum. Cells were transfected with effectine reagent (Qiagen) using 300 ng of adenovirus E1A reporter, 170 ng of SF2 expression plasmid and 90 or 130 ng of hnRNPA1 or hLuc7A expression plasmids. pBluescript (Stratagene) was added to normalize the amount of DNA. Forty-eight hours post-transfection RNA was purified (Trizol, Gibco) and RT–PCR was performed as described (27).

RESULTS

yLuc7p specifically cross-links the pre-mRNA

yLuc7p has two zinc finger motifs of the types CCCH and CCHH, respectively. These motifs have been shown to act in other proteins as RNA-binding modules. Thus we tested whether yLuc7p binds the pre-mRNA in commitment complexes. We synthesized P^{32}-internally-labeled WT-B pre-mRNA, a derivative of the RP51A intron (22) with 4-thio-U substituted for normal U. This RNA was used to assemble commitment complexes in an extract prepared from a yeast strain expressing yLuc7p fused at its C-terminus with the *Staphyloccocus aureus* protein A (yLuc7p-protA, this modification has no phenotypic consequence). Subsequently, the reactions were UV irradiated, treated with RNase T1 and immunoprecipitated by using IgG-coupled beads. Cross-linked proteins present in the precipitate were analyzed by SDS–PAGE and identified by autoradiography. As a positive control we used Nam8p-protA, a protein known to contact the pre-mRNA in the intron downstream from the 5′ splice site (13). Nam8p–protA cross-linked to the functional WT-B pre-mRNA (Figure 1, lane 1). The corresponding signal was strongly reduced when we used the 5′SSmut pre-mRNA (lane 2), which contains a mutation in the 5′ splice site sequence (GUAUaU instead of GUAUGU) known to impair commitment complex formation (9). Figure 1, lane 3, shows the pattern of cross-linking obtained when an extract from a yLuc7p–protA tagged strain was used in commitment complex formation with the WT-B pre-mRNA. A protein migrating with the expected size of yLuc7p–protA cross-links to the pre-mRNA. This band was only detected when 4-thio-U substituted RNA was used for cross-linking (data not shown). Further experiments confirmed the identity of this protein as yLuc7p–protA (data not shown). The cross-linking signal is reduced to background level when the 5′SSmut substrate is used (Figure 1, lane 4) suggesting that it is meaningful in terms of splicing. These results indicate

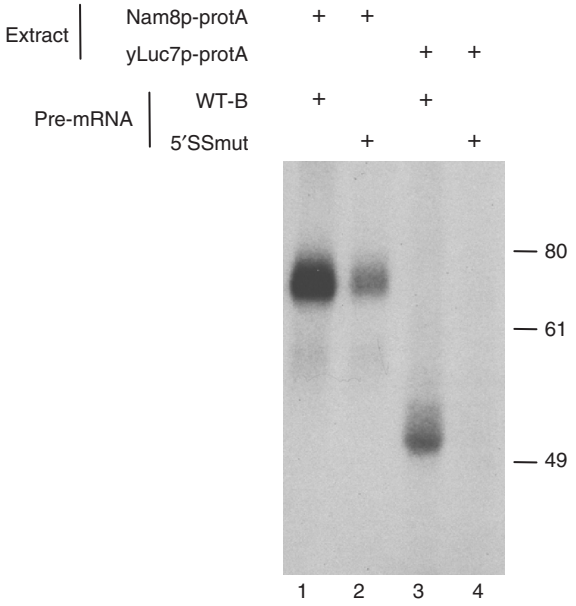

Figure 1. yLuc7p cross-links the pre-mRNA. Yeast extracts from Nam8p–protA (lanes 1 and 2) or yLuc7p–protA (lanes 3 and 4) tagged strains were incubated with WT-B (lanes 1 and 3) or 5′SSmut (lanes 2 and 4) pre-mRNAs. After cross-linking and immunoprecipitation, proteins were separated by SDS–PAGE.

that yLuc7p contacts the pre-mRNA during commitment complex formation. Similar results have been reported for other yeast U1 snRNP proteins such as Nam8p. Strikingly, yLuc7p had not been previously identified in a general analysis as a subunit of U1 snRNP contacting the pre-mRNA (14).

yLuc7p contacts the pre-mRNA in the 5′ exon

To determine the region of the pre-mRNA cross-linking to yLuc7p, we used a truncated version of the WT-B pre-mRNA (22) (Figure 2A). The pre-mRNA branchpoint region and downstream sequences were found to be dispensable for this reaction, indicating that yLuc7p contacts the pre-mRNA during formation of the first commitment complex (CC1) in the vicinity of the 5′ splice site (data not shown).

To narrow down the pre-mRNA region cross-linking to yLuc7p we used derivatives of the WT-B carrying substitutions such that non-essential U residues were replaced by A residues in specific regions of the pre-mRNA (14) Three substrates covering the exon 1 (the 5′ exon) and the intron upstream of the branchpoint were used for these experiments (Figure 2A). Each substrate contained essential U residues in the 5′ splice site (GUAUGU). In addition, Ex5′U contained U residues in the 5′ half of exon 1 (nucleotides −42 to −19, where −1 is the nucleotide adjacent to the 5′ splice site, Figure 2A), Ex3′U contained U residues in the 3′ half of exon 1 (nucleotides −18 to −1) and IntU contained U residues in the intron region (nucleotides +1 to +66) (Figure 2A). Because 4-thio-U residues are essential for the detection of yLuc7p cross-linking to the pre-mRNA, appearance of a cross-linked species with one of these

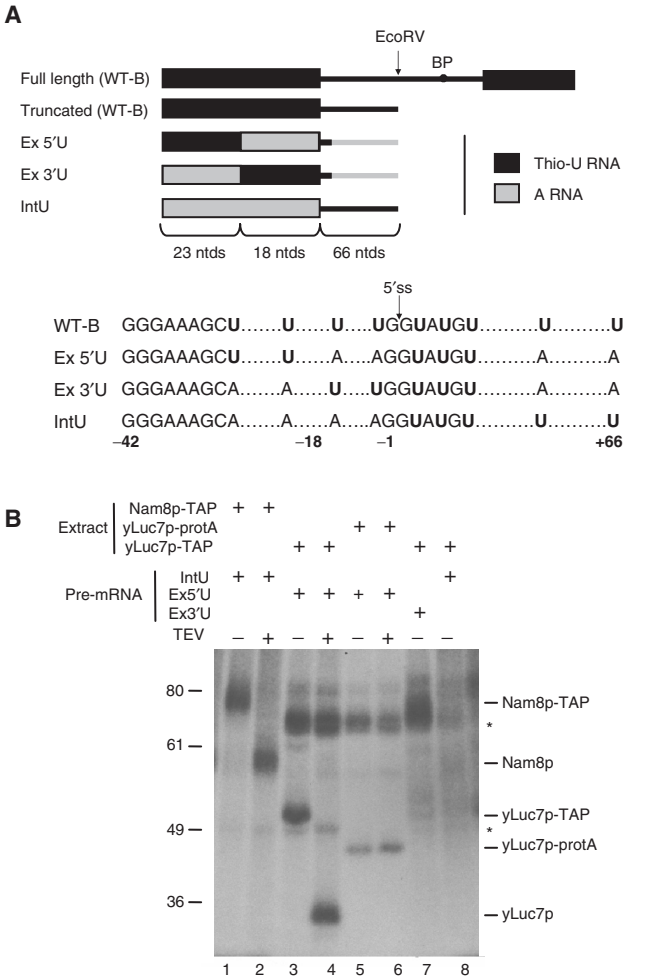

Figure 2. yLuc7p cross-links the WT-B pre-mRNA in a region close to the 5'cap. (**A**) Substrates used in cross-linking. WT-B full length, or truncated at the EcoRV site, were internally labeled with αP^{32}-UTP and 4-ThioU all over their sequence. Ex5′U contains 4-ThioU only in the 5′ half of exon 1 (nucleotides −42 to −19, where −1 represents the residue adjacent to the 5′ splice site) and in the 5′ splice site sequence (GUAUGU, necessary to form commitment complexes). Ex3′U contains 4-ThioU only in the 3′ half of exon 1 (nucleotides −18 to −1) and also in the 5′ splice site sequence. IntU contains 4-ThioU only in the intron (nucleotides +1 to +66). (**B**) Cross-linking to different substrates. Nam8p–TAP (lanes 1 and 2), yLuc7p–protA (lanes 5 and 6) and yLuc7p–TAP (lanes 3, 4, 7 and 8) tagged extracts were incubated with IntU (lanes 1, 2 and 8), Ex5′U (lanes 3 to 6) or Ex3′U (lane 7) P^{32}-labeled pre-mRNAs, cross-linked, RNaseT1 treated and immunoprecipitated. Proteins were separated by SDS–PAGE. Asterisks denote unspecific protein cross-linking. For unknown reasons, the cross-linking signal observed with yLuc7p–protA was weaker than yLuc7p–TAP in this experiment.

substrates would identify the region of contact between yLuc7p and the pre-mRNA. Cross-linking to all three substrates would indicate that yLuc7p contacts the pre-mRNA at the 5′ splice site itself. Control experiments demonstrated that these substrates, in the context of wild type, branchpoint and 3′splice site sequences (Materials and Methods section) formed commitment complexes with the same efficiency (Supplementary Figure 1). Commitment complexes were assembled in extracts containing yLuc7p–ProtA, yLuc7p–TAP and Nam8p–TAP

(the latter containing fusions of yLuc7p or Nam8p to the TAP tag) (18) and cross-linking reactions were carried out as before. yLuc7p–TAP cross-linked specifically to the Ex5′U substrate but not to the Ex3′U or IntU pre-mRNA (Figure 2B, compare lanes 3, 7 and 8). Consistently, yLuc7p-ProtA also cross-linked to the Ex5′U substrate (Figure 2B, lanes 5 and 6). In contrast, Nam8p–TAP was only cross-linked to the IntU substrate (Figure 2B, lanes 1 and 2), which was consistent with previous analyses (13), and demonstrated that this substrate was competent for cross-linking.

In order to confirm the identity of the cross-linked protein in the bands detected, half of the pellets in each cross-linking reaction were cleaved with the TEV protease. This enzyme specifically cleaves the TAP tag (18) releasing a ~15-kDa ProtA fragment. As expected, the cross-linked species detected when we used Nam8p–TAP or yLuc7p–TAP tagged extracts were cleaved with the TEV protease (compare lanes 1 with 2, and 3 with 4). However, there was no difference in mobility between TEV-treated and untreated samples when extracts from yLuc7p–protA were used (lanes 5 and 6), indicating that the cleavage observed was specific for the TAP tag and there are no other TEV sites within yLuc7p. Similarly, non-specific background bands were not affected by TEV protease treatment (Figure 2B, bands indicated with an asterisk). yLuc7p–TAP, yLuc7p–TAP cleaved by TEV protease, and yLuc7p–ProtA migrated at different positions, consistent with their predicted relative molecular weights (compare lanes 3, 4 and 5). These data confirm unequivocally the identification of the cross-linked species as yLuc7p. Moreover, this experiment demonstrates that, during commitment complex formation, yLuc7p contacts the 5′ half of exon 1 in the WT-B pre-mRNA, a region that in this construct is within 23 nt from the 5′ cap.

yLuc7p binds the pre-mRNA with its first zinc finger

Zinc finger motifs have been shown to act as DNA or RNA-binding modules (28). Since yLuc7p has two zinc finger motifs, we wanted to know whether yLuc7p contacts the pre-mRNA through any of its zinc finger motifs. For this purpose, we adapted a strategy originally described for topological studies of membrane proteins (29). We reasoned that insertion of a TEV protease cleavage site in a non-conserved loop at the surface of the yLuc7p protein would result in the synthesis of a functional protein. This would then allow DSSP of this polypeptide after cross-linking and identification of the peptide covalently linked to the radiolabeled RNA fragment. We first inserted the sequence coding for an HA epitope tag and a TEV site in regions predicted from phylogenetic comparison to be in a variable loop exposed on the surface of the yLuc7p protein and located between the two putative zinc fingers. These fusions were engineered in the context of a yLuc7p–ProtA fusion and next tested to determine whether they were functional and if they fully complemented a yLuc7p deletion. One such fusion, named here yLuc7p–HA-TEV–protA, was selected for further studies (Figure 3A). Western blot of extracts obtained from this strain showed that yLuc7p–HA-TEV–protA is stable in yeast cells (Figure 3B, lane 2). Commitment complex reactions were assembled using extracts derived from this strain and from the parental yLuc7p–protA strain. Samples were UV irradiated, immunoprecipitated with IgG-coupled beads and split in two. One half was treated with TEV protease while the other half was mock-treated. Proteins were analyzed by SDS–PAGE. Both yLuc7p–protA and yLuc7p–HA-TEV–protA cross-linked efficiently to pre-mRNA (Figure 3C, lanes 5 and 6). No effect was observed after TEV treatment of the yLuc7p–protA sample (Figure 3C, compare lanes 5 and 9). However, after TEV treatment, yLuc7p–HA-TEV–protA releases a fragment which is cross-linked to the pre-mRNA and has a mobility of ~18 kDa, corresponding to the size of the N-terminal half of yLuc7p plus the HA tag (Figure 3C, lane 10). Reproducible cross-linking to the C-terminal half (approximate MW 30 kDa) was not observed even though a weak and diffuse band with slower mobility (possibly a degradation product, shown in Figure 3 with an asterisk) was sometimes detected. This result demonstrates that the N-terminal part of yLuc7p cross-links to the pre-mRNA. Although our results suggest that in these conditions no interaction occurs through the C-terminal half of yLuc7p, due to the presence of the spurious band migrating close to 30 kDa in some of our experiments, we cannot exclude the possibility that the second zinc finger may bind cooperatively aiding in the U1snRNP–pre-mRNA interaction.

To further demonstrate that yLuc7p makes contacts with the pre-mRNA through its first zinc finger we generated a deletion mutant of yLuc7p HA-TEV–protA (named yLuc7p-ΔZF), which lacked its first zinc finger (Figure 3A). Cells expressing this protein as the sole source of yLuc7p were viable, indicating the first zinc finger is not essential. Western blot experiments showed that the protein lacking the first zinc finger was stable in yeast extracts (Figure 3B, lane 3). Interestingly, cross-linking of the pre-mRNA to this protein was completely abolished (Figure 3C, lanes 8 and 12). This lack of cross-linking was not due to problems with that particular sample since all samples cross-linked with similar efficiency as shown before immunoprecipitation (Figure 3C, lanes 1–4). Altogether these results demonstrate that yLuc7p makes contacts with the pre-mRNA through its first zinc finger.

The sequence of the pre-mRNA region where yLuc7p binding affects the efficiency of splicing in a yLuc7p-dependent way

Non-conserved sequences surrounding the 5′ splice site have been shown to be very important for efficient splicing (13). Given that yLuc7p makes contacts with the pre-mRNA in a non-conserved region, we wanted to know whether the sequence in that region affected splicing efficiency and whether yLuc7p was involved in this process. For this purpose we turned to a sensitive assay based on the alternative choice between two 5′ splice sites competing for a single 3′ splice site (Figure 4A) (10,13,24). The reporters used in this experiment had two duplicated

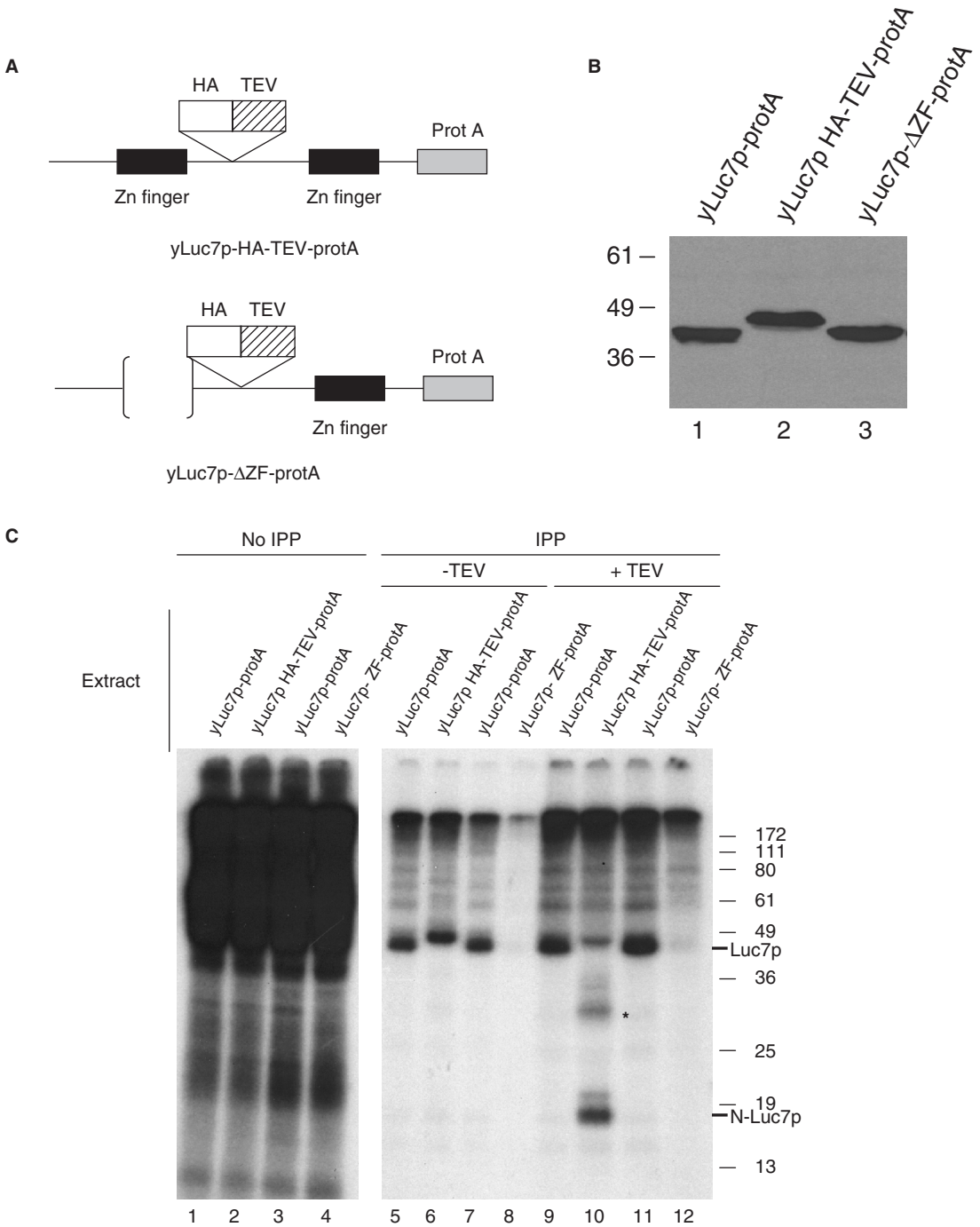

Figure 3. yLuc7p cross-links the pre-mRNA through its first zinc finger. (**A**) Scheme representing the structure of yLuc7p–HA-TEV–protA. (**B**) Western blot performed to detect protein A tagged proteins. (**C**) Extracts from yeast strains harboring yLuc7p–protA (lanes 1, 3, 5, 7, 9 and 11), yLuc7p–HA-TEV-protA (lanes 2, 6 and 10) or yLuc7p–ΔZF (lanes 4, 8 and 12) were incubated with WT-B pre-mRNA. After cross-linking, samples were treated with RNaseT1 and immunoprecipitated with IgG-coupled beads. TEV digestion was performed in samples 9–12. Lanes 1–4, no immunoprecipitation. The position of yLuc7p–protA and its N-terminal half are indicated. The intense band above 172 kDa is a non-specific cross-linking product. The band marked with an asterisk migrating above 30 kDa does not appear reproducibly in all experiments.

5′ splice sites and upstream of each one (nucleotides −15 to −35, Figure 4A) we introduced three different sequences: a sequence rich in Gs (G sequence), a sequence that could potentially form a hairpin, (H sequence) and a sequence with high content in As (A sequence). The resulting reporters were named using a two-letter code, where the first letter represents the sequence preceding (nucleotides −35 to −15, where −1 is the last nucleotide of exon 1, Figure 4A) the upstream 5′ splice site, and the second letter represents the sequence preceding the downstream 5′ splice site (Figure 4A). According to this nomenclature the construct named GH has a G-rich sequence preceding the upstream 5′ splice site and a potential hairpin sequence preceding the downstream

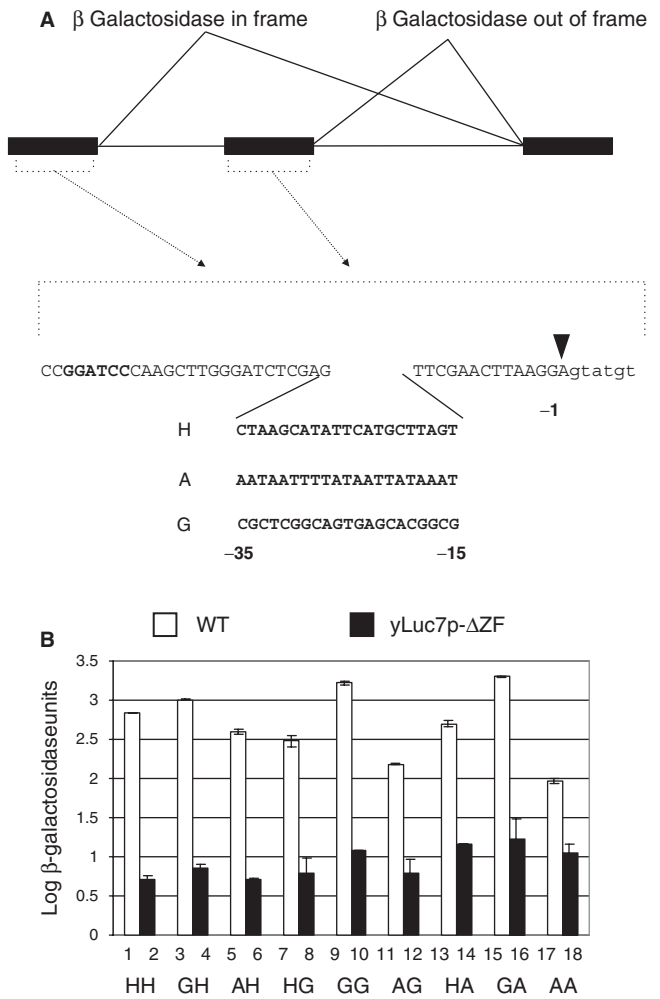

Figure 4. yLuc7p affects 5′ splice site selection. (**A**) Representation of the reporter constructs used to analyze splicing. β-Galactosidase activity reports splicing from the upstream versus the downstream 5′ splice. (**B**) Plasmids carrying A, G and H sequences in all possible combinations in both splice sites were transformed in the yLuc7p–protA (white bars) or yLuc7p–ΔZF (black bars) strains.

5′ splice site (Figure 4A). These constructs were introduced into isogenic yLuc7p–protA and yLuc7p–ΔZF strains. Splicing efficiency was then examined by analyzing β-galactosidase activity that reports usage of the upstream versus the downstream 5′ splice site (10,13,24). As shown in Figure 4B, changing the sequence of the pre-mRNA had important effects in the efficiency of splicing. The G-rich sequence was preferred over the A-rich sequence (10-fold difference, Figure 4B, compare lanes 9 to 11 and 15 to 17, note the logarithmic scale), and the potential hairpin H-sequence had an intermediate effect (compare, for example, lanes 7, 9 and 11). These effects are lost in the yLuc7p–ΔZF background (Figure 4B, compare odd with even lanes for each construct) indicating that activation was mediated, at least to a significant extent, through the first zinc finger of yLuc7p. We conclude that the non-conserved sequence contacted by yLuc7p has an effect in splicing efficiency and that yLuc7p, through interaction with its first zinc finger, can modulate this effect. Although these results suggest that the primary factor determining splice site selection in this context is yLuc7p, we cannot exclude contribution of yLuc7p-independent effects to the observed splicing phenotype. Thus it is possible that additional factors, by contacting the pre-mRNA in the same region, could act together with Luc7p to modulate splice site selection.

Two proteins with extended sequence homology to yLuc7p are expressed in human cell lines

Database searches revealed expressed sequence tags (ESTs) corresponding to three human proteins sharing extended homology with yLuc7p (hLuc7A, hLuc7B1 and hLuc7B2) (17). We cloned cDNAs for two of them, hLuc7B2 and hLuc7A (Figure 5A). Both proteins have two zinc finger motifs similar to the yeast protein. Interestingly, the human proteins have extended C-terminal domains rich in arginine, serine and glutamate (and to a lesser extent lysine and aspartate) as independently noted by others (30,31). Similar repeats are present in a number of known splicing factors, the SR proteins (32). Computer analysis using the cDNA sequence of hLuc7A also revealed several isoforms resulting from alternative splicing (data not shown) (Figure 5B).

N-terminal or C-terminal fragments of hLuc7A were expressed in *E. coli* and used to raise rabbit polyclonal antibodies. Antibodies against hLuc7A recognized a single band in HeLa nuclear extracts migrating in SDS polyacrylamide gels with an apparent MW of 58 kDa (Figure 5C). The same protein was detected in extracts from a wide variety of human cell lines (lanes 1–5 and data not shown). In addition, faint signals corresponding to shorter proteins were detected with some cells (e.g. lanes 1 and 3). These smaller products may correspond to a splice variant or to post-translational modification (i.e. phosphorylation) or weakly cross-reacting polypeptides expressed from the related hLuc7A and/or hLuc7B2 genes. Antibodies against hLuc7A also cross-reacted with a protein present in *Xenopus* egg extracts with similar mobility than hLuc7A (Figure 5C, lane 7), and weakly with a protein present in *Drosophila* S2 cell line extracts which has higher mobility. These results suggest that hLuc7A is expressed in different tissues and that it is present in metazoans from flies to humans.

hLuc7A is a nuclear protein

In order to know the subcellular localization of hLuc7A we used the antibodies raised against the N-terminal part of hLuc7A in immunolocalization studies. Figure 6 shows that anti-hLuc7A antibodies recognize a protein that co-localizes in HeLa cells with the DAPI signal for DNA. The hLuc7A signal does not overlap with the cytoplasm stained with an antibody specifically recognizing tubulin. Interestingly, the hLuc7A signal is not uniform in the nucleus. This is consistent with the speckled staining detected for other splicing factors in mammalian cells (33). This result demonstrates that hLuc7A is a nuclear protein consistent with a role in pre-mRNA splicing.

A

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xLuc7A   -------MSAQAQMRALLDQLMGTSR-----DGDETRQRIKFTDDRVCKSHLLDCCPHDILAGTRMDLGECTKNHDLALRADYEIASKERDLF--FELDAMDHLESFIAECDRRTELAKK 106
xLuc7B   -------MSAQAQMRAMLDQLMGTAR-----DGDETRQRVKFTDDRVCKSHLLDCCPHDILAGTRMDLGECTKIHDLALRADYEIDRKERDLF--FELDAMDHLESFIAECDRRTELAKK 106
hLuc7B1  -------MSAQAQMRALLDQLMGTAR-----DGDETRQRVKFTDDRVCKSHLLDCCPHDILAGTRMDLGECTKIHDLALRADYEIASKERDLF--FELDAMDHLESFIAECDRRTELAKK 106
hLuc7B2  -------MSAQAQMRAMLDQLMGTSR-----DGDTTRQRIKFSDDRVCKSHLLNCCPHDVLSGTRMDLGECLKVHDLALRADYEIASKEQDFF--FELDAMDHLQSFIADCDRRTEVAKK 106
xLuc7C   -------MSAQAQMRALLDQLMGTSR-----DGDSTRQRIKFNDERVCKSHLLNCCPHDILLGTRMDLGECLKVHDLALRADYEIASKQQDFF--FELDAMDHLQSFIADCDRRTEIAKK 106
dLuc7    MSAPSNKMSATDQMRAMLDQLMGTTR-----NGDE--RQLKFSDPRVCKSFLLDCCPHDILASTRMDLGECPKVHDLAFRADYESAAKTRDYY--YDIEAMEHLQAFIADCDRRTDSAKQ 111
hLuc7A   ----------MISAAQLLDELMGRDRNL---APDEKRSNVRWDHESVCKYYLCGFCPAELFTNTRSDLGPCEKIHDENLRKQYEKSSRFMKVG--YERDFLRYLQSLLAEVERRIRRGHA 105
yLuc7p   ---MSTMSTPAAEQRKLVEQLMGRDFSFRHNRYSHQKRDLGLHDPKICKSYLVGECPYDLFQGTKQSLGKCPQMHLTKHKIQYEREVKQGKTFPEFEREYLAILSRFVNECNGQISVALQ 117
                    .   ::::***          .     :   .  :** .* . ** :: : .*: .*** : *    : :**   :  .    :: : :  *. :: : : :   .
xLuc7A   RLAETQEEISAEVS----VKAEKVHELNEEIGKLLAKAEQLGAEGNVDEAQKILMEMENVKGRKREAEEEYRNSMPAS-SFQQQKLRVCEVCSAYLGLHDNDRRLADHFGGKLHLGFILI 221
xLuc7B   RLAETQEEISAEVS----VKAEKVHELNEEIGKLLAKAEQLGAEGNVDEAQKILMEMEKVKGRKREAEDEYRNSMPAS-SFQQQKLRVCEVCSAYLGLHDNDRRLADHFGGKLHLGFILI 221
hLuc7B1  RLAETQEEISAEVS----AKAEKVHELNEEIGKLLAKAEQLGAEGNVDESQKILMEVEKVRAKKKEAEEEYRNSMPAS-SFQQQKLRVCEVCSAYLGLHDNDRRLADHFGGKLHLGFIQI 221
hLuc7B2  RLAETQEEISAEVA----AKAERVHELNEEIGKLLAKVEQLGAEGNVEESQKVMDEVEKARAKKREAEEEVYRNSMPAS-SFQQQKLRVCEVCSAYLGLHDNDRRLADHFGGKLHLGFIEI 221
xLuc7C   RLADTQEEISAEVA----VKAEKVHELNEEIGKLLAKAEQLGAEGNVEESQKVMDEVEKTRVRKREAEDIYRNSMPAS-SFQQQKLRVCEVCSAYLGLHDNDRRLADHFGGKLHLGFIEI 221
dLuc7    RLKETQEELTAEVA----EKANAVHGLAEEIGKKLAKAEALGEAGEVEDSMELMKEIEELRAKKIKAEHEYRTSMPAS-TYQQQKLRVCEVCSAYLGIHDNDIRLADHFGGKLHLGFLTI 226
hLuc7A   RLALSQNQQSSGAAGPTGKNEEKIQVLTDKIDVLLQQIEELGSEGKVEEAQGMMKLVEQLK-EERELLRSTTSTIESF-AAQEKQMEVCEVCGAFLIVGDAQSRVDDHLMGKQHMGYAKI 223
yLuc7p   NLKHTAEERMKIQQ-----VTEELDVLDVRIGLMGQEIDSLIRADEVSMGMLQSVKLQELISKRKEVAKRVRNITENVGQSAQQKLQVCEVCGAYLSRLDTDRRLADHFLGKIHLGYVKM 232
         .*  : ::             : :. *  .*.    : : *   .:*. .      :::   .. :      .         :::::.*****.*:*   * : *: **: ** *:*:  :
xLuc7A   REKLEMLRRTVAE-KQEKRNQDRLKRREEREKDDR-TGRRSKSKSRDRRRSRSKERRRRRSQSSSRDRRR-SRSRSR-------------DRHRRHRSRSGSRNRS--HHQS-------- 315
xLuc7B   REKLELLRRTVAE-KQEKRNQDRLKRREERERDDH-LGRRSKSKSKDRRRSRSKERRRRRSQSSSHDRRR-SRSRSR-------------DRHRRHRSRSGSRNRS--HYRS-------- 315
hLuc7B1  REKLDQLRKTVAE-KQEKRNQDRLRRREEREREER-LSRRSGSRTRDRRRSRSRDRRRRRSRSTSRERRKLSRSRSR-------------DRHRRHRSRSRSHSRG--HRRA-------- 316
hLuc7B2  REKLEELKRVVAE-KQEKRNQERLKRREEREREEREKLRRSRSHSKNPKRSRSREHRRHRSRSMSRERKRRTRSKSR-------------EKRHRHRSRSSSRSRSRSHQRSRHS----- 322
xLuc7C   REKLDELKRLVAE-KQEKRNQDRLKRREERDREEREKLK---------KRSRSREKRRHRSRSSSRDRKRRTRSKSR-------------EKRHRHRSRSGSRSRSHSHQRS-------- 310
dLuc7    REKLIELEKTAAPRKAELKRTGKMTDREDEGRGRNRYFVGGRELDRRSRVHRSRSRERQRNRDGDRERPNNGRGPEEKGSERPKEAADGPERAERAPDRGGRRDDRDNHGRDHRERERDG 346
hLuc7A   KATVEELKEKLRKRTEEPDRDERLK-KEKQEREER-EKEREREREEREKRRREEEEREKERARDRERRKRSRSRSRHSSRTSDRRCSRSRDHKRSRSRERRRSRSRDRRRSRSHD-RSE 340
yLuc7p   REDYDRLMKNNRTTNASKTATTLPGRRFV-------------------------------
         :    * .     . .         :
xLuc7A   --SRDRGSKHK-------------------------------------------------------------------------------
xLuc7B   --SRDRGSKHK-------------------------------------------------------------------------------
hLuc7B1  --SRDRSAKYKF----------------SRERASREESWESGRSERGPPDWRLESSNGKMASRRSEEKEAGEI------------------- 371
hLuc7B2  --SRDRSRERSKRRSSKERFRDQDLASCDRDRSSRDRSPRDRDRKDKKRSY-ESANGRSEDRRSSEEREAGEI------------------- 392
xLuc7C   ---RDRSR----------------------DRSSRTK----------------------------------------------------
dLuc7    RRDRDRHGRNDRGRFGDRGGGGGGGGHHRDDRRRSRSRERSPRERRNFNFHFRDGGGGGNGQRKRSYSRERNYRR------------------ 420
hLuc7A   RKHRSRSRDRRRSKSRDRKSYKHRSKSRDREQDRKSKEKEKRGSDDKKSSVKSGSREKQSEDTNTESKESDTKNEVNGTSEDIKSEGDTQSN 432
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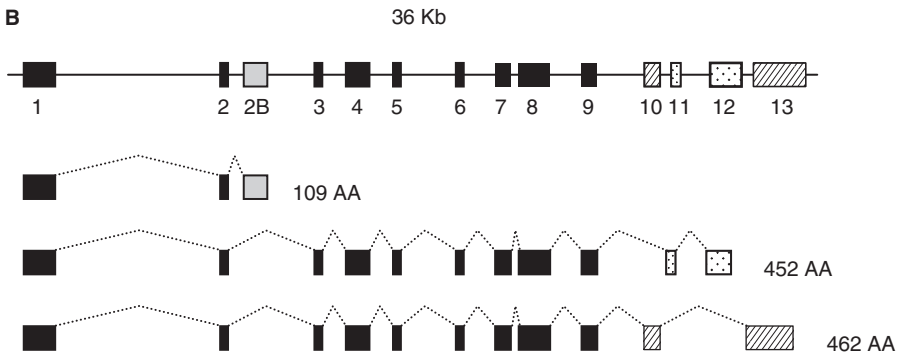

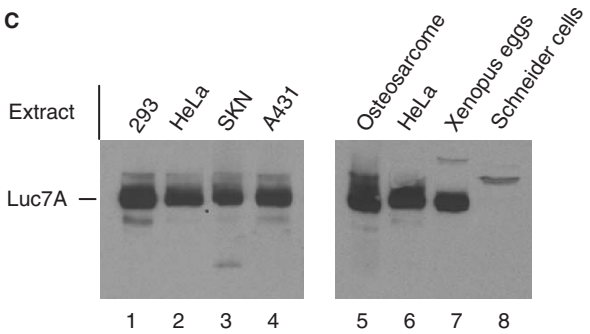

Figure 5. Metazoan Luc7 proteins. (**A**) Alignment of the yeast, *Xenopus*, *Drosophila* and human Luc7 proteins. The multiple sequence alignment of yLuc7p and the two human proteins deduced from the cDNA sequences underlines the two conserved zinc finger motifs and the human-specific C-terminal extensions with sequence composition typical of a splicing factor. (**B**) Structure of the hLuc7A gene showing the different alternative splice site variants. (**C**) Western blot with anti-hLuc7A antibodies in cell extracts from several cell types. Thirty micrograms of protein extract were loaded in each lane.

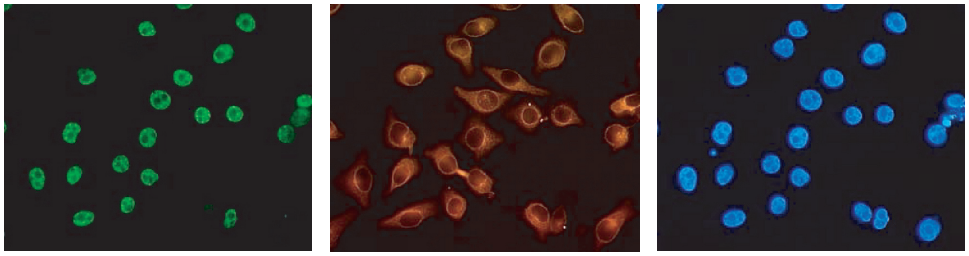

Figure 6. hLuc7A is a nuclear protein. *In situ* staining was performed in HeLa cells with antibodies against hLuc7A (green) and tubulin (red). DAPI appears in blue.

Antibodies against hLuc7p specifically precipitate U1 snRNA

In yeast, yLuc7p is tightly associated to the U1 snRNP. This allowed purification of U1 snRNP by using a tagged version of yLuc7p (18). Purified human U1 snRNP contains only three specific proteins U1-A, U1-C and U1-70k (34). However it is possible that some other proteins interacting loosely and/or non-stoichiometrically with the U1 snRNP are not detected after the purification process. In order to know whether hLuc7A associates to U1 snRNP, we performed immunoprecipitation of HeLa nuclear extracts with antibodies against hLuc7A and assayed for the presence of U1 snRNA in the pellets. As positive control we used antibodies against the U1 snRNP protein, U1-A. As negative control an antibody that recognizes POP1, an RNase MRP component, was used. U1-A antibodies precipitated U1 snRNA (Figure 7, lane 11). However, only background is seen with POP1 antibodies (Figure 7, lane 10). Antibodies raised against the N-terminal or the C-terminal regions of hLuc7A specifically precipitated a significant amount of U1 snRNA but not U2 snRNA (Figure 7, lanes 7 and 8). The pre-immune serum precipitated only background levels of U1 snRNA (Figure 7, lane 9). Analysis of the supernatant confirmed that lack of co-precipitation did not result from RNA degradation (lanes 2–6) Western blot analysis also demonstrated that anti-hLuc7p antibodies co-precipitated specifically the U1-A protein (data not shown). These results show that hLuc7A associates specifically to U1 snRNP despite the fact that it does not copurify with it.

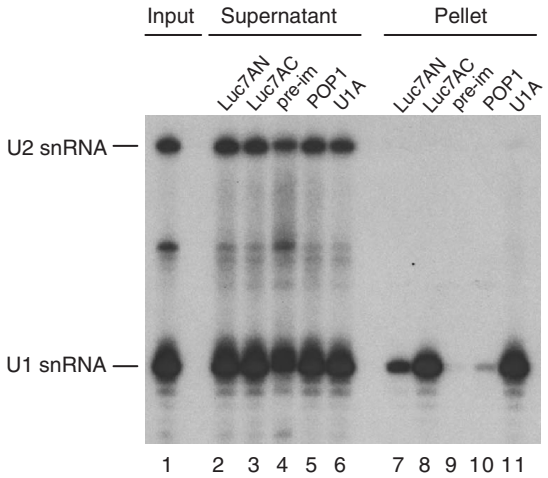

Figure 7. hLuc7A is associated to U1 snRNA. Immunoprecipitation experiments were performed with HeLa nuclear extracts and different sera. Lane 1, input, lanes 2–6 supernatants, lanes 7–11 pellets. Antibodies recognized: h-Luc7A N-terminal region (lanes 2 and 7) and C-terminal region (lanes 3 and 8); POP1 (lanes 5 and 10); U1-A (lanes 6 and 11). Lanes 4 and 9, pre-immune serum corresponding to C-terminal antibody.

hLuc7A affects 5′ splice site selection *in vivo*

hLuc7A can be depleted to 97% from HeLa nuclear extracts by using a mixture of antibodies generated against it. These extracts, when assayed in splicing *in vitro* by using several reporters (35) are 2- to 3-fold less active (data not shown). To further demonstrate a role for hLuc7A in pre-mRNA splicing, we decided to analyze the effect of overexpression of hLuc7A on splicing of a reporter *in vivo*. We used the system where HeLa cells are co-transfected with an adenovirus E1A reporter and overexpress SF2, which activates splicing from proximal (13s) sites (Figure 8B) (25,27). We then analyzed the effect of co-transfected hLuc7A on splicing. As a positive control we co-transfected hnRNPA1, which activates distal sites (9s) and counteracts the effect of SF2. Figure 8A and D, lane 1, shows the pattern of mRNAs obtained when E1A reporter is transfected with SF2 alone. The 13s, 12s and 9s mRNAs derived from splicing are shown. Co-transfection of hnRNPA1 activated splicing from the distal site detected as a reduction in 13s and a relative increase in 9s (compare Figure 8A and D, lanes 1–3). hLuc7A overexpression acted in a similar way to hnRNPA1, by preventing reproducibly the splicing from the proximal sites (12s and 13s). Therefore overexpression of hLuc7A switched 5′ splice site utilization towards the more distal site, an effect similar to that produced by hnRNPA1. Taken together, our results demonstrate that hLuc7A is a new splicing factor.

DISCUSSION

Our work provides insight in the role of yLuc7p in splicing, which we find remarkably similar to that of Nam8p. We previously showed (13) that Nam8p, a U1 snRNP component, binds the pre-mRNA during commitment complex formation in a region directly

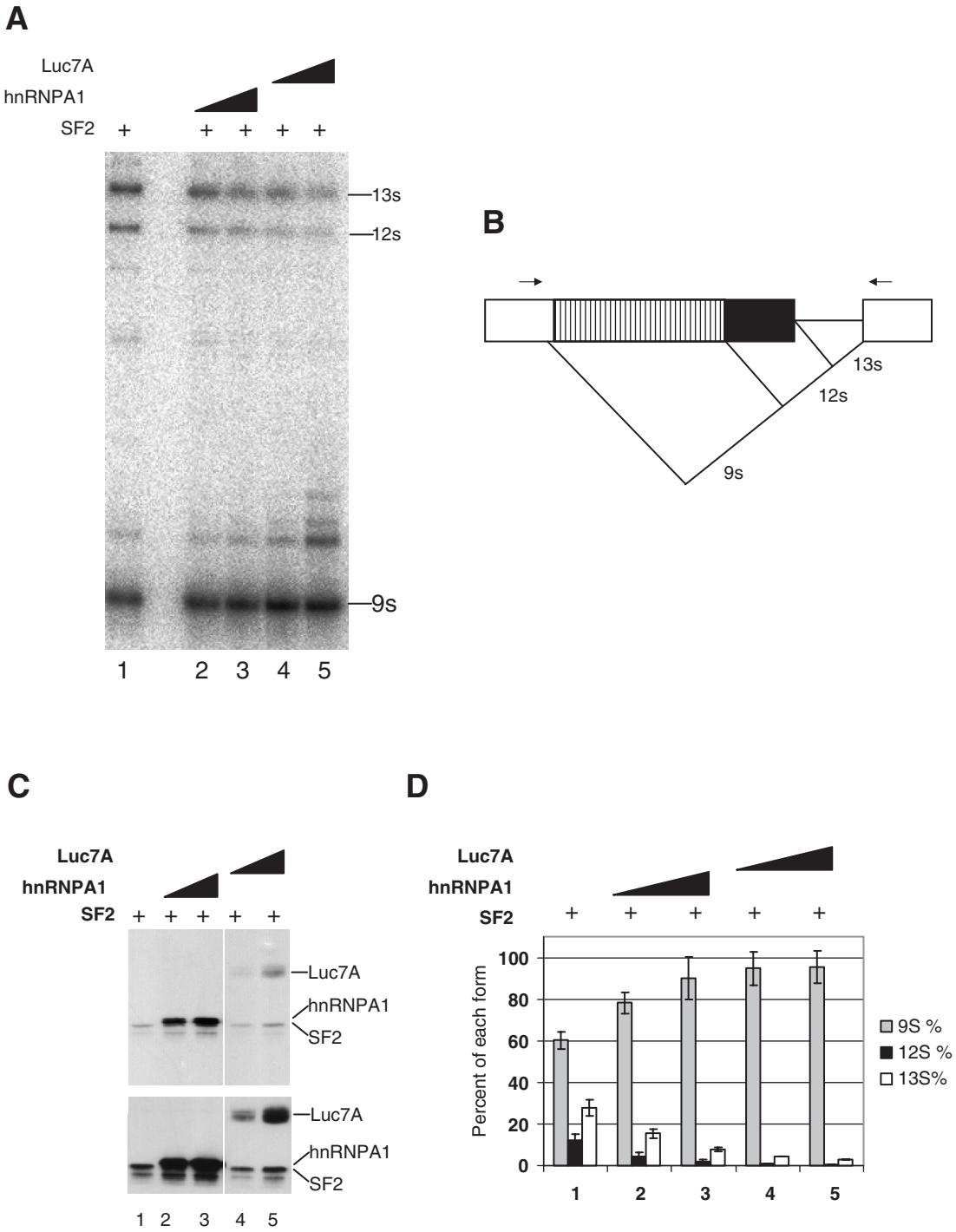

Figure 8. hLuc7A affects splicing *in vivo*. (**A**) A representative image of splicing analysis carried out in HeLa cells co-transfected with E1A reporter plasmid and expression plasmids for SF2, hnRNPA1 and hLuc7A. After transfection, total RNA was purified and radioactive RT–PCR was performed using primers specific for the exons. HeLa cells were transfected with 300 ng of E1A reporter and 170 ng of SF2 expression plasmid (lanes 1–5). In addition, 90 or 130 ng of hnRNPA1 (lanes 2 and 3) or hLuc7A (lanes 4 and 5) were co-transfected. The splicing products corresponding to the 13s, 12s and 9s forms are indicated. pBluescript was used to normalize the amount of DNA. We cloned and sequenced the band migrating just above the 9s splicing product and it contains a modified duplication of the 9s sequence, suggesting that it corresponds to a PCR artifact derived from primer–dimer pairing on the 9s splicing product. Its intensity correlates with the intensity of the 9s band (data not shown). (**B**) Schematic diagram representing the E1A reporter with the different splicing products (13s, 12s and 9s). The primers used for analysis of splicing activity by RT–PCR are marked with two arrows. (**C**) Western blot of the expressed proteins from panel A using the T7 epitope tag. Two different exposures are shown (above and below). Bands corresponding to SF2, hnRNPA1 and hLuc7A are indicated. (**D**) Quantification of data presented in panel A. The intensity of bands corresponding to 13s, 12s and 9s mRNAs from three independent experiments was quantified with a phosphorimager and represented as percentage for each form, ±SD.

downstream of the 5′ splice site, where it stabilizes pre-mRNA–U1 snRNP interaction and helps in the formation of commitment complexes. We also showed that changes in the pre-mRNA sequence introduced in the non-conserved region contacted by Nam8p produced a striking Nam8p-dependent effect in splice site recognition and selection. Thus, Nam8p by stabilizing U1 snRNP–pre-mRNA interaction modulates splicing (13). We now propose that yLuc7p acts in a similar way. yLuc7p contacts non-conserved sequences in the exon, and in the specific context of our synthetic constructs, in a region within 23 nt from the cap. We also showed that this contact occurs mainly through its first zinc finger. Interestingly, similarly to what happens with Nam8p, changes in the non-conserved pre-mRNA sequence bound by yLuc7p affect splice site selection, and this effect is dependent on the presence of its first zinc finger too. Fortes *et al*. (17) showed that extracts from yLuc7p mutant strains display defects in all steps of splicing *in vitro*, and these defects can be rescued by adding recombinant yLuc7p. They also showed that yLuc7p mutants exhibit reduced splicing activity *in vivo*, and that yLuc7p is required for CBC–U1snRNP interactions. Based on our data, and in agreement with the data from Fortes *et al* (17), we propose a model where yLuc7p contacts the pre-mRNA in a region close to the cap, where it is more likely to establish an interaction with CBC. yLuc7p acts by binding the pre-mRNA upstream the 5′ splice site and stabilizing pre-mRNA–U1 snRNP interaction in commitment complexes. Therefore, yLuc7p could directly or indirectly interact with CBC and mediate the CBC effect in splice site selection. Zhang and Rosbash (14) showed that another U1snRNP component, U1-C, contacts the pre-mRNA and stabilizes its interaction with the U1snRNA. Altogether, yLuc7p, U1-C, Nam8p, CBC and maybe other U1 snRNP proteins (ySnp1p/U1-70K, SmD1, SmD3 and Snu56p) (14) would interact with the pre-mRNA and produce a network of protein–RNA interactions keeping the pre-mRNA stably bound to the U1 snRNP.

yLuc7p has two zinc finger motifs. We show that the first one cross-links the pre-mRNA and it is required for yLuc7p splicing activity. What is then the role for the second zinc finger? The U1 snRNP particle purified from a yLuc7p mutant strain appears completely disrupted, missing several proteins and it is inactive in splicing (17). It is possible that yLuc7p acts as a bridge between the pre-mRNA and the U1 snRNA through its two zinc fingers, the first one binding to the pre-mRNA and the second one binding to U1 snRNA. We tried to generate a yeast strain lacking the second zinc finger but we did not succeed. Diploid cells integrated the mutation but after sporulation none of the spores harboring the deletion were viable. This indicates that the integrity of the second zinc finger is necessary for viability. Perhaps it is required to keep a minimal structure of the U1 snRNP. It is also possible that the second zinc finger contributes to stabilize globally the snRNP–pre-mRNA interaction by cooperatively helping the first zinc finger bind the pre-mRNA. Further experiments will be necessary to clarify this point.

Three U1 specific proteins co-purify with human U1 snRNP (U1-A, U1-C and U1-70k) and all three have counterparts in yeast U1 snRNP. In contrast, yeast U1 snRNP has seven additional proteins (Snu71p, Snu65p, Snu56p, Prp39p, Prp40p, Nam8p and yLuc7p). However, a human homolog has been described only for Nam8p, the apoptotic factor TIA-1 (19). Here we demonstrate that hLuc7A is a new splicing factor, homolog to yeast yLuc7p. Human hLuc7A is a nuclear protein expressed in several human cell lines (Figures 5 and 6). Importantly, antibodies against hLuc7A specifically precipitate U1 snRNA (Figure 7), indicating that it is a *bona fide* U1snRNP component. Interestingly, hLuc7A affects splice site selection by activating splicing from the distal 5′ splice site (Figure 8). Supporting our results, a recent report identified the new SR protein, SRrp53, as a protein interacting with hLuc7A (36). In addition, hLuc7A was purified with the supraspliceosome, a macromolecular complex involved in pre-mRNA splicing (37). Therefore, we conclude that hLuc7A is a new splicing factor.

hLuc7 has three isoforms (hLuc7A, hLuc7B1 and hLucB2) derived from different genes, suggesting that it could be tissue specific or developmentally regulated. ESTs analysis shows that the pre-mRNA for hLuc7A undergoes alternative splicing, although we do not know yet the biological meaning of this variability. It is possible that hLuc7B1 and hLuc7B2 are also regulated by alternative splicing. The abundance of isoforms present in mammalian cells is remarkable when compared to the simplicity of the yeast system. This underscores the degree of complexity in the regulation of splicing in mammalian systems.

hLuc7A has a C-terminal tail rich in Asp, Ser and Arg repeats. These repeats are present in the splicing factors known as SR proteins where they have been shown to act as protein–protein interaction modules, or to influence RNA–RNA interactions. The SR repeats in hLuc7A could act by recruiting other splicing factors to the pre-mRNA, or by stabilizing U1snRNP–pre-mRNA interactions. The fact that hLuc7A does not co-purify with human U1 snRNP (38) indicates that it remains loosely associated to this particle. This situation allows for more flexibility in the regulation of splice site selection. Thus, hLuc7A could first bind the pre-mRNA independently from U1 snRNP and, perhaps through interaction with U1-70k (via their RS domains) or by direct binding to U1 snRNA, could subsequently recruit the U1 snRNP to form the E-complex. In this sense, hLuc7A would act in a similar way than TIA-1, the human homolog of yeast Nam8p (19). Interestingly, human and yeast Luc7p share 50% identity in the zinc finger region suggesting that the mechanism of action might be similar. More experiments will be necessary to confirm this hypothesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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