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# The WW Domain-Containing Proteins Interact with the Early Spliceosome and Participate in Pre-mRNA Splicing In Vivo

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**A growing body of evidence supports the coordination of mRNA synthesis and its subsequent processing events. Nuclear proteins harboring both WW and FF protein interaction modules bind to splicing factors as well as RNA polymerase II and may serve to link transcription with splicing. To understand how WW domains coordinate the assembly of splicing complexes, we used glutathione *S*-transferase fusions containing WW domains from CA150 or FBP11 in pull-down experiments with HeLa cell nuclear extract. The WW domains associate preferentially with the U2 small nuclear ribonucleoprotein and with splicing factors SF1, U2AF, and components of the SF3 complex. Accordingly, WW domain-associating factors bind to the 3′ part of a pre-mRNA to form a pre-spliceosome-like complex. We performed both in vitro and in vivo splicing assays to explore the role of WW/FF domain-containing proteins in this process. However, although CA150 is associated with the spliceosome, it appears to be dispensable for splicing in vitro. Nevertheless, in vivo depletion of CA150 substantially reduced splicing efficiency of a reporter pre-mRNA. Moreover, overexpression of CA150 fragments containing both WW and FF domains activated splicing and modulated alternative exon selection, probably by facilitating 3′ splice site recognition. Our results suggest an essential role of WW/FF domain-containing factors in pre-mRNA splicing that likely occurs in concert with transcription in vivo.**

Gene expression in eukaryotic cells involves several steps, including transcription, mRNA processing, and export. Synthesis of mRNA by RNA polymerase II (Pol II) is coordinated with subsequent RNA processing events such as capping, splicing, and cleavage and polyadenylation (4, 12, 20, 23, 37). The largest subunit of RNA Pol II recruits mRNA processing activities during transcriptional elongation via its heptapeptide repeat-containing C-terminal domain (CTD) (4, 23). Truncation of the Pol II CTD results in reduced splicing in vivo (34). Under some circumstances, in vitro splicing of pre-mRNA can be stimulated by phosphorylated Pol II or CTD (22, 52). Several transcriptional activators or coactivators associate physically with small nuclear ribonucleoproteins (snRNPs) or serine/arginine-rich splicing factors (SR proteins) and thereby may modulate pre-mRNA splicing (18, 29, 35). Moreover, promoter structure affects alternative splice site selection during pre-mRNA splicing (11), and further analyses reveal that transcriptional activators differentially modulate the rate of transcriptional elongation, which in turn determines the outcome of two competing alternative splicing reactions (15, 26, 38). Nevertheless, several lines of evidence support the concept of a physical and functional coupling between transcription and splicing (12, 20).

Since the RNA Pol II CTD is implicated in promoting pre-mRNA splicing, proteins that associate with the CTD may mediate this function. A two-hybrid screen previously revealed a set of RS domain-containing proteins via their interaction with the CTD (51). Among these proteins, SR-like CTD-associated factor 8 (SCAF8) was shown to bind newly initiated Pol II and recruit processing factors to the elongation complex (39). In addition, several proteins containing both WW and FF domains, such as yeast Prp40p and mammalian CA150 proteins, interact directly with the phosphorylated CTD (2, 8, 9, 36, 43). Yeast splicing factor Prp40p may participate in cross-intron bridging via contact with branch-point binding protein and the U5 snRNP component Prp8p (49). Mammalian FBP21 may function similarly to its yeast counterpart, Prp40p, by interacting with U1 and U2 snRNPs in the pre-spliceosome A complex (2). The CA150 protein was previously identified as a transcriptional cofactor that regulates RNA Pol II elongation in a promoter-specific manner (43, 44), and it was recently found in active spliceosomes through independent proteomic analyses (25, 53). Indeed, CA150 binds to splicing factor SF1/mammalian branch-point binding protein (19). Nevertheless, the manner in which these WW-containing proteins participate in pre-mRNA splicing has not been well characterized.

The WW domain of ~40 residues is characterized by two strictly spaced tryptophan residues and folds as a triple-stranded β-sheet (32). This domain recognizes proline-containing ligands and mediates protein-protein interactions in many intracellular processes (32). CA150 binds directly to the proline-rich domain of SF1 via its WW domains (19). FBP21 associates with U2 snRNPs probably by interacting with proline-rich SmB or B′ protein (2). The FF domain harbors two highly conserved phenylalanine residues and often accompanies WW domains (1, 3). The repetitive FF domains of CA150 interact directly with the phosphorylated Pol II CTD, indicating that CA150 binds to elongation-competent Pol II (8). Thus, it has been postulated that the phosphorylated CTD stations CA150 such that it may participate in both transcriptional elongation and splicing (20).

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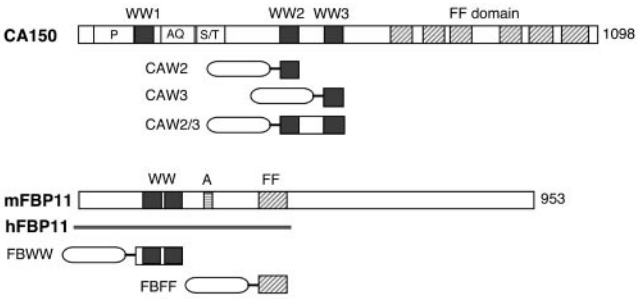



FIG. 1. Schematic representation of the domains of human CA150 (NCBI accession no. NP_006697) and mouse FBP11 (NP_061255) proteins. A human FBP11 homolog (AAC27506) is depicted by a gray line. The GST moiety (elongated oval) was fused to the N termini of human CA150 WW2, WW3, and WW2/3 and to that of human FBP11 WW and FF domains. P, proline-rich region; AQ, imperfect alanine-glutamine repeats; S/T, serine/threonine-rich region; A, alanine-rich region; WW, WW domain; FF, FF domain.

In the present study, we investigated whether WW domain-containing factors coordinate the functions of the transcriptional and pre-mRNA splicing machineries. First, we used an in vitro pull-down experiment to demonstrate that WW domains associate with a subset of splicing factors that are capable of binding the 3′ part of an intron to form a complex equivalent to the early spliceosome. We next tested the roles of CA150 in both in vitro and in vivo splicing. The data reveal that CA150 can activate splicing and influence alternative exon selection in vivo although it may not be critical for the splicing reaction per se.

## MATERIALS AND METHODS

**Plasmids.** The cDNAs encoding human CA150 and FBP11 fragments were obtained from a HeLa cell cDNA library constructed in pAS2-1 (Clontech). The cDNA fragments encoding WW or FF domains were amplified and subcloned in frame into pGEX-2TK (Amersham Pharmacia Biotech). Therefore, glutathione *S*-transferase (GST) fusions were constructed for CA150 amino acid residues 427 to 461 (CAW2), 526 to 566 (CAW3), and 427 to 566 (CAW2/3) and for FBP11 residues 137 to 221 (FBWW; corresponding to residues 133 to 217 of the mouse homolog; Fig. 1) and 375 to 441 (FBFF; residues 377 to 443 of the mouse homolog). Plasmid pEFBOST7-CA150 containing a near-full-length CA150 cDNA except for the first 60 nucleotides was kindly provided by Mariano A. Garcia-Blanco (Duke University, Durham, N.C.) and was used as a PCR template for the following constructs. The entire CA150 cDNA of pEFBOST7-CA150 was amplified by PCR and cloned in frame with the hemagglutinin (HA)-epitope tag in pCEP4 (Invitrogen) to generate pCEP4-CA150-HA. Analogously, the DNA fragment encoding amino acid residues 427 to 1098 of CA150 was cloned to create a HA-tagged CAΔN expression vector. Alanine was substituted for three consecutive tyrosine residues (residues 446 to 448) of the WW2 domain (19) of CAΔN using the QuickChange site-directed mutagenesis system (Stratagene). To construct pCEP4-CAΔAQ-HA, an internal region of CA150 was deleted by ligation of two fragments encoding the N-terminal 174 residues and C-terminal 672 residues, respectively, with an engineered NheI site. Finally, wild-type and mutant CAΔN DNA fragments were each subcloned in frame with the FLAG tag in pcDNA3.1.

**In vitro pull-down.** The GST fusion proteins were overproduced in *Escherichia coli* strain BLR, purified over glutathione-Sepharose beads (Amersham Pharmacia Biotech), and dialyzed against a buffer containing 20 mM HEPES (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM dithiothreitol, 1 mM EGTA, 8.7% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The GST-WW protein (2 μg) was incubated with 50 μl of HeLa cell nuclear extract (NE) (33) in a 100-μl mixture for 30 min at 30°C. The reaction was then supplemented with an equal volume of NET-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40) and subsequently incubated with 10 μl of glutathione-Sepharose for 2 h at 4°C. The beads were then washed extensively with NET-2 buffer. When treated with RNase, the beads were further incubated in the presence of 20 μg of RNase A for 30 min at 37°C. Bound fractions were either boiled in sodium dodecyl sulfate sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting or were extracted with phenol-chloroform for Northern blot analysis of snRNAs. Antibodies against SF1 and U2AF$^{65}$ were kind gifts from Angela Krämer (Geneva, Switzerland) and Juan Valcárcel (Barcelona, Spain), respectively; anti-polypyrimidine tract-binding protein was purchased (Oncogene). Northern blotting was performed with antisense U1 and U2 snRNA riboprobes as described previously (46). In addition, bound fractions could be eluted with 15 mM reduced glutathione for use in in vitro splicing or sedimentation analysis or with 9.8 M urea for two-dimensional gel electrophoresis using the PROTEAM II Xi Cell system (Bio-Rad).

**Identification of WW domain-associating proteins.** For mass spectrometry (MS) analysis, samples were prepared from a 10× scale of the above pull-down reaction. After two-dimensional gel electrophoresis, samples were stained with SYPRO-Ruby (Bio-Rad) and visualized using Typhoon 9410 (Amersham Biosciences). The spots of interest were excised and subjected to in-gel trypsinization and followed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS analysis using a Voyager-DE STR Biospectrometry Workstation (Applied Biosystems). MS-Fit (prospector.ucsf.edu) and Mascot (www.matrixscience.com) software were used for protein identification. Several proteins were also analyzed by liquid chromatography-coupled tandem mass spectrometry (LC MS/MS) on a LCQ Deca XP mass spectrometer (ThermoFinnigan). All tandem spectra were searched against the protein database of the National Center for Biotechnology Information using the SEQUEST algorithm.

**Preparation of antibodies against CA150.** To raise antisera to CA150, rabbits were immunized with recombinant nontagged CAW2/3 protein. To affinity purify the antibodies, antisera were incubated with nitrocellulose containing immobilized GST-CAW2/3 overnight at 4°C. Bound antibodies were eluted from the filters followed by neutralization according to the method of Lai et al. (30).

**In vitro splicing assay.** Preparation of $^{32}$P-labeled pre-mRNA substrate was as described previously (45). In vitro splicing was also performed as described previously (45) in 15-μl reaction mixtures containing 60% HeLa cell NE (33) or GST-WW eluate. For RNP complex analysis, 0.2 mg of heparin/ml was added to the reactions that were then loaded onto a 4% nondenaturing polyacrylamide gel and electrophoresed as described previously (45).

**Immunoprecipitation and immunodepletion.** For immunoprecipitation of the spliceosome, the mixture of a 25-μl splicing reaction was incubated with 10 μl of antibody-bound protein A-Sepharose (PAS) for 2 h at 4°C. For each 10 μl of PAS, 0.15 μg of purified anti-CA150 or 10 μg of anti-mouse immunoglobulin G (IgG) (as a control), anti-Sm from 2.5-μl ascites (gift of Joan Steitz, Yale University, CT), or 10 μg of anti-hnRNP A1 (Abcam) was immobilized. After incubation, the beads were washed extensively with NET-2 buffer containing 0.05% NP-40. RNA was extracted from the immunoprecipitates with phenol-chloroform for electrophoresis on a 5.6% denaturing polyacrylamide gel. To deplete CA150, ~0.3 μg of purified anti-CA150 was immobilized on 10 μl of PAS, and 20 μl of NE was then incubated with antibody-bound beads for 2 h at 4°C. For mock depletion, 10 μg of anti-mouse IgG was used. The unbound fraction was analyzed by Western blotting or subjected to in vitro splicing assay with the adenovirus major late (AdML) pre-mRNA substrate (45, 46). An antibody against lamin was purchased from NeoMarkers.

**Sedimentation analysis.** Sedimentation analysis of WW domain-associating complexes was performed using a 15 to 40% (wt/vol) glycerol gradient in a buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, and 150 mM KCl. The glutathione-Sepharose column eluate was applied to a 12.5-ml gradient and centrifuged in a Beckman SW41 rotor at 36,000 rpm at 4°C for 22 h. Fractions of 0.5 ml were manually collected and RNA was recovered for Northern blot analysis using an antisense U2 snRNA riboprobe (46).

**In vivo splicing assay, antisense oligonucleotides, and immunoprecipitation.** Cell culture and transient transfection were performed as described previously (31). The reporters used for in vivo splicing assays included pSV40-CAT(In1), in which human β-globin intron 1 was inserted into the chloramphenicol acetyltransferase (CAT) gene (29), the β-tropomyosin (β-TM) minigene construct pSV40-p2 (49), and the E1A expression vector pCEP4-E1A (7). Each reporter (0.5 μg) was cotransfected with an expression vector encoding one of the HA-tagged ASF/SF2 or CA150 fragments (4 μg) into $8 \times 10^5$ HEK 293 cells. At 48 h posttransfection, RNA was isolated and amplified by reverse transcription-PCR (RT-PCR). For pSV40-CAT(In1), oligonucleotides 5′-TTTTGGAGGCCTAG GCTTTT-3′ (forward; simian virus 40 [SV40]) and 5′-GCAAGCTTCACTCCA GAGCGATG-3′ (reverse) were used as primers in RT-PCR; for two other reporters, the primers used were as described previously (31). The RT-PCR products of CAT transcripts were analyzed by hybridization on blots using the

SV40 probe, followed by quantification using Typhoon 9410 (Amersham Biosciences). Splicing efficiency was measured by the arbitrary unit of spliced CAT mRNA, which was normalized with that of CAT pre-mRNA. Transcripts of β-TM and E1A were analyzed as described previously (31).

To knock down CA150, phosphorothioate oligonucleotide (14) complementary (antisense; 5′-GTCCCCGCCACGCTCGCCAT-3′) or identical (sense control) to nucleotide 1 to 21 of the CA150 coding region was used. HEK 293 cells ($\sim 8 \times 10^5$) were first transfected with 4 μg of oligonucleotide for 24 h and then with 2 μg of oligonucleotide, 2 μg of the CAΔN expression vector, and 0.5 μg of pSV40-CAT(In1) for another 36 h. Cell lysates were harvested for Western blotting using anti-CA150 and anti-tubulin (NeoMarkers) antibodies and total RNAs for RT-PCR analysis.

For immunoprecipitation, an expression vector encoding FLAG-tagged CAΔN or CAmΔN was transfected into HEK 293 cells for 48 h. Cell lysates were prepared and immunoprecipitation was performed with M2-agarose (Sigma). Immunoprecipitated proteins were detected by Western blotting using antibodies against RNA Pol II (Santa Cruz Biotechnology), SF1, and CA150.

## RESULTS

**Interaction of WW domains with splicing factors.** To explore how WW domains mediate interactions between splicing factors, we performed in vitro pull-down experiments with HeLa cell nuclear extract using GST fusions of CA150 or FBP11 WW domains (Fig. 1) as bait. Since FBP21 has been previously shown to interact with U2 snRNPs (2), the GST-WW and FF column eluates were first subjected to Northern blotting with a probe against snRNAs. The CAW2 and FBWW fragments interacted preferentially with the U2 snRNP but also bound with lower affinity to four other splicing snRNPs (Fig. 2A, lanes 3 and 6; data not shown for U4, U5, and U6). The CAW2/3 fragment bound weakly to all snRNPs but still had preference for U2 (lane 5). However, neither CAW3 nor FF domains pulled down detectable levels of snRNPs (lanes 4 and 7). Therefore, certain WW domains, but not FF domains, bind to the splicing snRNPs or perhaps to the spliceosome.

A previous report showed that CA150 interacts with splicing factor SF1 via its WW domains (19). SF1 binds cooperatively with $U2AF^{65}$ to the pre-mRNA intron at an early stage of spliceosome assembly (5, 24, 40, 42). Western blotting showed that CAW2 and FBWW could interact with both SF1 and $U2AF^{65}$ (Fig. 2B, lanes 3 and 4). Neither of these interactions was disrupted by RNase treatment (lanes 6 and 7), suggesting direct protein-protein contact. In contrast, neither polypyrimidine tract-binding protein (Fig. 2B, bottom panel) nor the U1 snRNP-specific 70K protein (data not shown) could be detected in the eluates of the WW domain columns.

**Mass spectrometry of WW domain-associating proteins.** To obtain a clearer picture of how the WW domains coordinate interactions between splicing factors, we used mass spectrometry to determine proteins that associate with CA150 WW2 or FBP11 WW domains. The eluates of the GST-CAW2- and FBWW-bound columns yielded 66 and 47 protein spots, respectively, on two-dimensional gels (data not shown; results were generally consistent across several independent experiments). The two WW domain baits pulled down ~27 common spots. The spots of relatively high abundance detected in the CAW2 eluate were first identified by MALDI MS. Several of these proteins were further characterized by LC MS/MS and/or the equivalent proteins selected from the FBWW eluate were subjected to MALDI analysis. As shown in Table 1, at least five components of SF3a and SF3b were detected, which

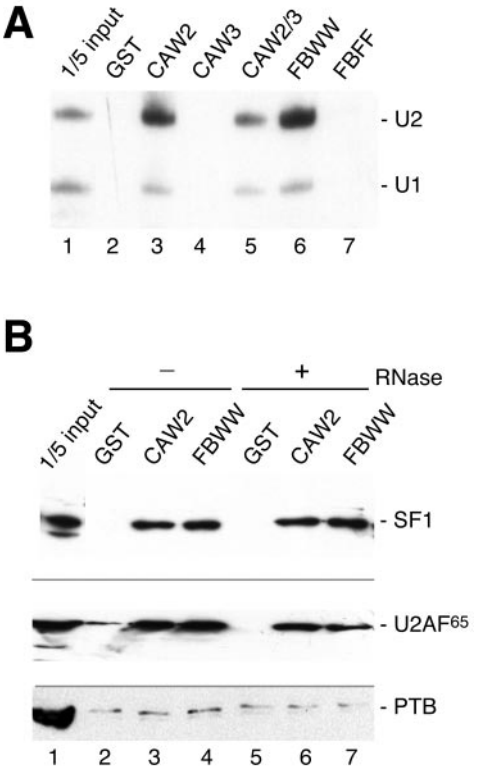



FIG. 2. CA150 and FBP11 WW domains associate with splicing factors. (A) GST alone (lane 2) or GST fusions to WW or FF domains (lanes 3 to 7) were used as bait in pull-down experiments with HeLa cell nuclear extract. RNA extracted from bound fractions was analyzed by Northern blotting with antisense probes against U1 or U2 snRNA. (B) The pull-down experiment was performed analogously to that in panel A, by using GST alone or GST fusions to CAW2 or FBWW as bait. Bound fractions were treated (lanes 5 to 7) or mock treated (lanes 2 to 4) with RNase and then analyzed by Western blotting with the antibodies indicated at the right. Lane 1 of both panels shows one-fifth of the input of nuclear extract. PTB, polypyrimidine tract-binding protein.

are associated with the core U2 snRNP in a 17S particle (6, 28). Consistent with the Western blotting data, $U2AF^{65}$ was also detected (Table 1). Moreover, this analysis revealed the potential association of WW domains with several RNA binding proteins and three HSP70 domain-containing proteins that have been detected in the pre-mRNA-associated complex H (53) (Table 1). Two additional proteins that were identified, KIAA1564 and CAF-1 subunit A, may be involved in chromatin remodeling (17, 41). The significance of their interaction with WW domains will require further experimentation. However, SF1 was not identified in this analysis even though it was observed in Western blots (Fig. 2B). In total, the MS analysis showed that the WW domains associate with the 17S U2 snRNP and the splicing factors involved in early spliceosome assembly.

**WW domain-associating factors constitute a 17S U2 snRNP and form a stable complex with a pre-mRNA.** To test whether factors associated with the WW domains assemble as a 17S U2-containing particle, the CAW2 eluate from glutathione-Sepharose was fractionated on a glycerol gradient. RNA was isolated from gradient fractions and analyzed by Northern

TABLE 1. CA150 WW2-associating proteins identified by mass spectrometry

| Protein name | Acc. no.[a] | Feature(s) | Peptide no./% matched[b] |
|---|---|---|---|
| U2 snRNP | | | |
| SAP114 (SF3a120)[c] | Q15459 | 17S U2 | 7/14 |
| SAP61 (SF3a60)[c] | Q12874 | 17S U2 | 6/13 |
| SAP145 (SF3b145)[c] | Q13435 | 17S U2 | 13/39 |
| SAP155 (SF3b155) | O75533 | 17S U2 | 30/10 |
| SAP49 (SF3b50)[d] | Q15427 | 17S U2 | 4/3 |
| U2-A′[d] | P09661 | U2 protein | 5/4 |
| hnRNP and SR | | | |
| U2AF$^{65}$[c] | P26368 | SR | 3/9 |
| hnRNP H | P31943 | hnRNP | 9/11 |
| hnRNP M | P52272 | hnRNP | 8/11 |
| hnRNP E1[d] | Q15365 | hnRNP | 7/8 |
| ASF[d] | Q07955 | SR | 11/13 |
| H complex components | | | |
| HSP70 | P08107 | HSP70 | 10/12 |
| HSP71 | P11142 | HSP70 | 11/13 |
| GRP78 | P11021 | HSP70 | 7/8 |
| Miscellaneous | | | |
| Nucleophosmin (B23) | P06748 | Nucleolar phosphoprotein | 6/7 |
| Tropomyosin 4 | P07226 | Associated with the actin filaments | 7/7 |
| KIAA1564 | Q9HCK8 | Chromodomain-helicase-DNA binding | 17/17 |
| CAF-1 subunit A[d] | Q13111 | Chromatin assembly factor 1 subunit A | 52/21 |
| DnaJ B9 (Mdg-1)[d] | Q9UBS3 | DnaJ | 7/6 |

[a] SwissProt accession number.
[b] Number of unique peptides identified/percentage of masses matched.
[c] Also detected by LC MS/MS.
[d] Not detected in the FBP11-WW domain pull-down.

blotting with a probe for U2 snRNA. The U2 snRNP in the CAW2 eluate peaked at both the 12S and 17S regions of the gradient, consistent with the results using nuclear extract (Fig. 3A). Thus, at least a part of U2 snRNP that associates with the WW domains formed a 17S particle, consistent with the presence of several SF3 components.

We next investigated whether the WW domain-associating factors could function to recognize a pre-mRNA intron. The WW domain column eluates were incubated with a 3′-half splicing substrate derived from the AdML pre-mRNA. This AdML substrate bearing the branch site and 3′ splice site formed stable complexes when incubated in nuclear extract, of which a major one contains U2 but not U4/6-U5 snRNP and is equivalent to pre-spliceosome complex A (46) (Fig. 3B, lane 1). The pre-spliceosome-like 3′-half complex could be detected with the eluate from either the CAW2 or FBWW columns, albeit with slightly higher mobility (lanes 3 and 4). A Northern blot confirmed the presence of U2 snRNA in the complex (data not shown). We also observed the formation of complex A when using the full-length pre-mRNA as substrate (data not shown), emphasizing the stable binding of U2 snRNP to the intron. Note that neither the 3′-half nor A complexes form in cytoplasmic S100 extract except when supplemented with SR proteins (46). Nevertheless, the WW eluates may contain SR proteins (such as ASF [Table 1]) for stable assembly of the U2-containing complex near the 3′ end of the intron.

**CA150 is associated with the spliceosome but is dispensable for splicing in vitro.** We next investigated the role of the WW domain-containing proteins in pre-mRNA splicing. We raised polyclonal antibodies against the WW2/3 fragment of CA150. The anti-CA150 antiserum specifically recognized a band of ~170 kDa on a Western blot of HeLa cell nuclear and S100 extracts (Fig. 4A, lanes 1 and 2). Purified antibodies also immunoprecipitated this ~170-kDa protein from the nuclear extract (lane 3). Next, the splicing reaction was performed with the AdML pre-mRNA substrate and subsequently subjected to immunoprecipitation with anti-CA150. As with an antibody against snRNP Sm protein, anti-CA150 precipitated the pre-mRNA as well as the splicing intermediates and products (Fig. 4B, lanes 3 and 4). However, CA150 precipitated insignificant levels of the excised and debranched intron in comparison with hnRNP A1 (lane 5), suggesting that CA150 is an integral component of the spliceosome. To determine whether CA150 is essential for splicing, we depleted it from nuclear extract by using purified antibodies. While CA150 was reduced to an undetectable level in the depleted extract (Fig. 4C, upper panel, lane 3), in vitro splicing of the AdML pre-mRNA was not significantly affected (Fig. 4C, lower panel, lane 4). These data argue that CA150 is not critical for pre-mRNA splicing in vitro, although it is possible that minute quantities of CA150 are sufficient to drive the reaction.

To test whether exogenous CA150 WW domains have any effect on pre-mRNA splicing, we added recombinant proteins to the splicing reaction performed in either nuclear extract or cytoplasmic S100 extract with or without additional SR proteins. Splicing of AdML or β-globin pre-mRNA was not enhanced by WW domains under any of these conditions (data not shown). Therefore, we next examined the role of CA150 in splicing in vivo.

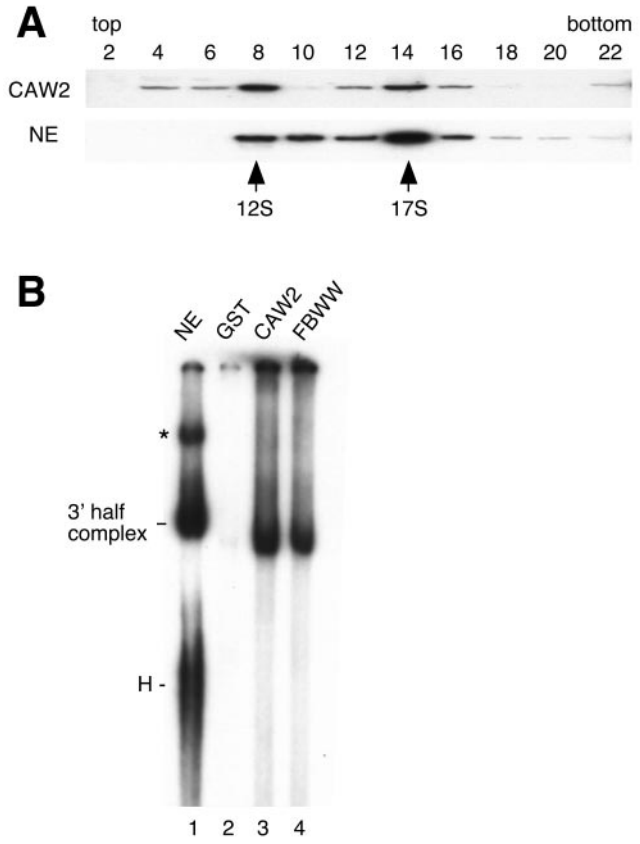



FIG. 3. WW domain-associating factors constitute a 17S U2 snRNP and bind stably to the 3′ half of a pre-mRNA. (A) The GST-CAW2 column eluate and HeLa cell nuclear extract were each fractionated on a 15 to 40% glycerol gradient. RNA was recovered and analyzed by Northern blotting using a probe for U2 snRNA. The U2 peaks at 12S and 17S are indicated by vertical arrows. (B) Nuclear extract (lane 1) or the eluate of the GST (lane 2), GST-CAW2 (lane 3), or GST-FBWW (lane 4) column was incubated with the AdML 3′-half RNA under splicing conditions for 30 min, and the assembled complexes were fractionated on a nondenaturing polyacrylamide gel. The 3′-half complex is equivalent to the pre-spliceosome complex A (46). In lane 1, the slowest migrating complex (asterisk) contains not only U2 snRNA but also U6 snRNA (data not shown), whereas H represents nonspecific hnRNP complexes.

**CA150 fragments containing WW and FF domains activate pre-mRNA splicing in vivo.** We initially attempted to overexpress full-length CA150 protein to investigate its role in splicing in vivo. Although a number of cell lines have been tested, no significant levels of exogenous CA150 could be detected. Therefore, we examined two truncated forms of CA150, CAΔAQ and CAΔN. CAΔAQ lacks an internal sequence corresponding to the alanine-glutamine (AQ) dipeptide-rich region and adjacent serine/threonine-rich stretch, whereas CAΔN lacks the entire N-terminal region; both truncations contain at least two WW domains plus the entire array of FF domains in the C terminus (Fig. 5A). Their expression in HEK 293 cells was confirmed by Western blotting (Fig. 5B) and their nuclear localization by indirect immunofluorescence (Fig. 5C).

Expression vector of CA150 fragments was cotransfected with a splicing reporter in which the CAT coding region has an insertion of human β-globin intron 1 (Fig. 5D, upper panel) (29). RT-PCR was performed to examine the splicing of CAT transcripts. As shown in Fig. 5D, both CAΔAQ and CAΔN fragments can activate splicing by approximately twofold. In contrast, a mutant CAΔN, in which three tyrosine residues of the conserved central aromatic block in WW2 were substituted by alanine (19), considerably lost its ability to activate splicing (Fig. 5D, CAmΔN). This mutant CAΔN was expressed and properly localized to the nucleus (Fig. 5B and C, CAmΔN). However, immunoprecipitation of FLAG-tagged CA150 fragments showed that the mutant CAΔN failed to interact with SF1 although it remained bound to RNA Pol II (Fig. 5E, lane 4). Therefore, the WW2 domain is critical for CA150's activity on splicing activation, probably via its interaction with SF1.

To further investigate whether CA150 is essential for splicing in vivo, phosphorothioate antisense oligonucleotides (14) were applied to down-regulate CA150 expression in HEK 293 cells. Western blot analysis showed that the level of CA150 was considerably lowered by the antisense, but not by the sense, oligonucleotide (Fig. 5F, left panel); residual amounts of CA150 observed could be in part from nontransfected cells. Reduction of CA150 protein expression led to a partial inhibition of CAT pre-mRNA splicing, whereas overexpression of the CAΔN protein can rescue the splicing (Fig. 5F, right panel). This result suggests a critical role of CA150 in splicing in vivo.

**WW and FF domain-containing CA150 facilitates 3′ splice site utilization in vivo.** To examine whether CA150 could modulate splice site selection, the rat β-TM reporter was used, in which tissue-specific exons can be differentially spliced (47). As observed with the SV40 probe, inclusion of exon 6 or 7 was enhanced by CAΔN in a dose-dependent manner (Fig. 6A). CAΔAQ could also activate exon inclusion whereas mutant CAΔN had no effect (Fig. 6B, lanes 3 and 5). These data demonstrated that CA150 protein can likely participate in alternative exon selection, albeit with lower efficiency than ASF (lane 2), and that the WW2 domain is critical for this activity. It was reported that inefficient exon 6 utilization is in part due to the poor polypyrimidine tract near the 3′ splice site of intron 5 (47). A specific probe was then exploited to confirm the inclusion of exon 6 by CA150 protein fragments (Fig. 6B, exon 6 probe). CA150-induced exon 6 inclusion coincides with the preferential association of its WW domains with splicing factors that bind to the 3′ part of an intron (Table 1 and Fig. 3). Thus, the result indicates that CA150 may facilitate 3′ splice site utilization.

We also tested the effect of CA150 protein fragments on alternative 5′ splice site selection. By contrast to ASF, neither CA150 fragment was capable of altering 5′ splice selection in the adenovirus E1A pre-mRNA (Fig. 6C). Therefore, CA150 and ASF probably act via different pathways to modulate alternative splicing.

From the above observations, we assume that the CA150 protein activates exon inclusion by recruiting splicing factors that enhance the recognition of the 3′ splice site relative to that for the 5′ splice site. Hence, protein fragments containing both WW and FF domains are possibly sufficient to activate the use of an inefficient 3′ splice site.

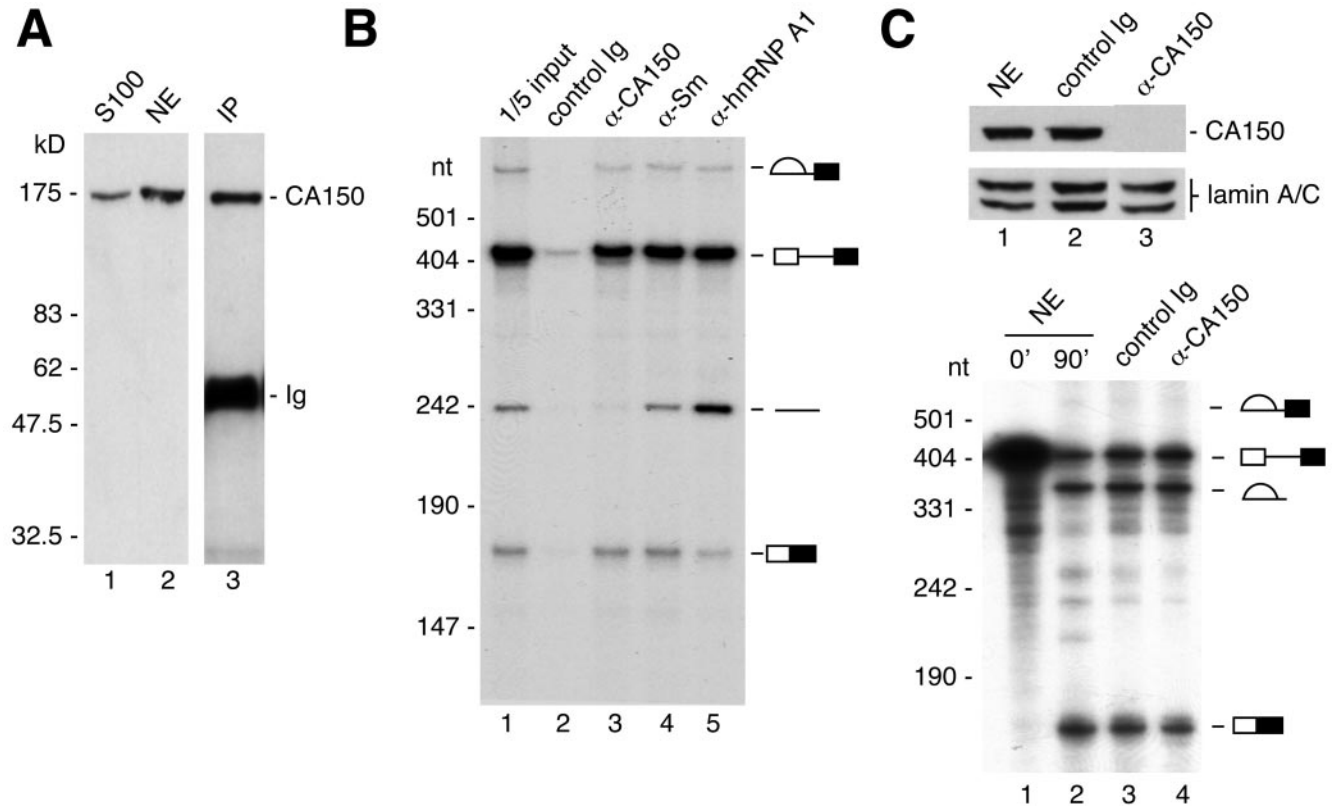


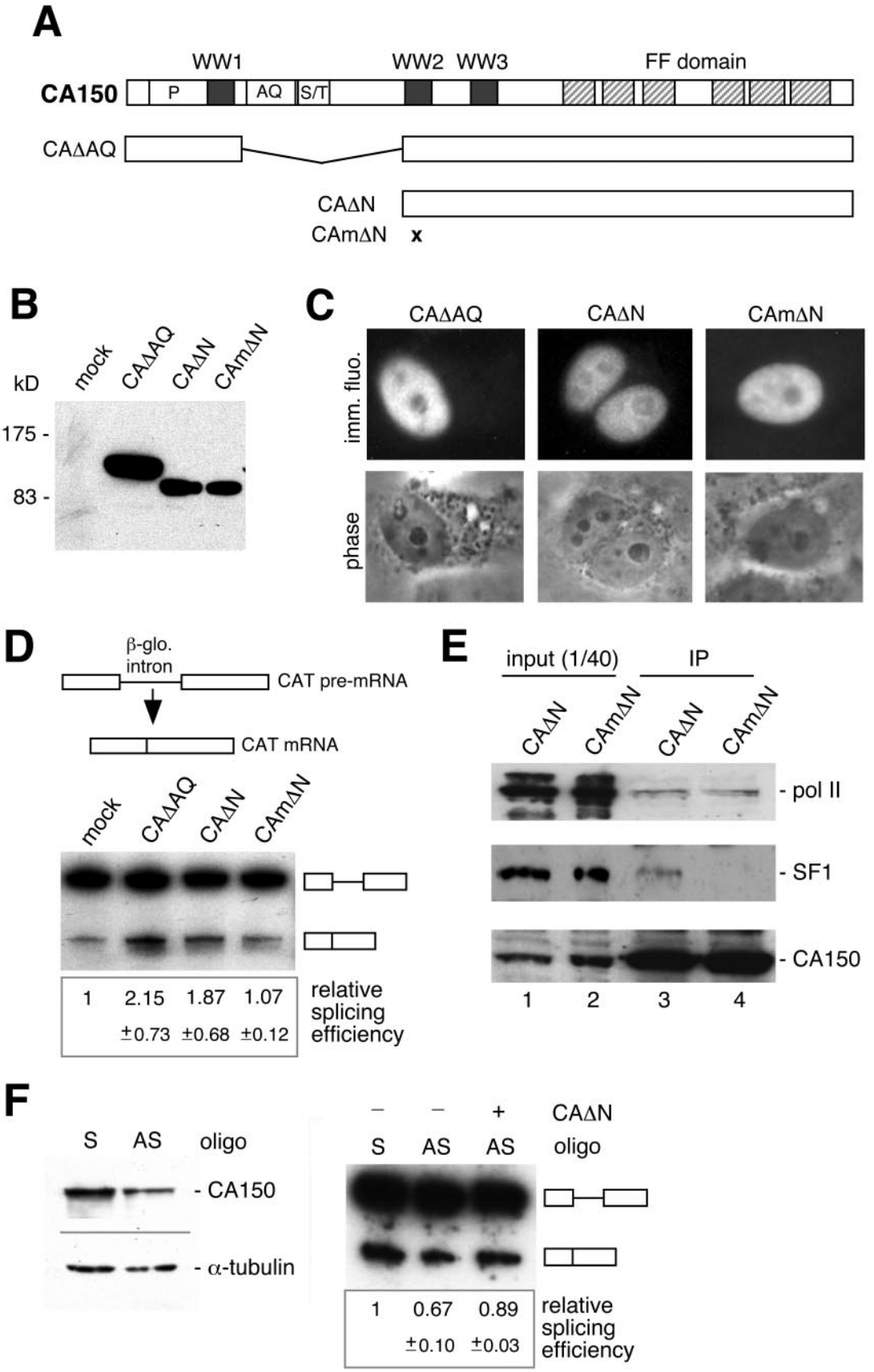

FIG. 4. CA150 is associated with the spliceosome but is not critical for splicing in vitro. (A) Western blotting was performed with anti-CA150 to examine HeLa cell cytoplasmic S100 (lane 1) and nuclear extracts (lane 2) and the anti-CA150 immunoprecipitate from nuclear extract (lane 3). Protein size markers are indicated at the left. (B) In vitro splicing reactions were performed with the AdML pre-mRNA for 45 min at 30°C. Immunoprecipitation was then performed with antibodies against mouse IgG (lane 2, control Ig), CA150 (lane 3), Sm (lane 4), or hnRNP A1 (lane 5). Lane 1 shows one-fifth of the splicing reaction used for immunoprecipitation. The band indicated by a line is the excised and debranched intron (241 nucleotides). (C) Upper panel, HeLa cell nuclear extract remained untreated (lane 1) or was subjected to immunodepletion with anti-mouse Ig (lane 2) or anti-CA150 (lane 3). Western blotting was performed with anti-CA150 and anti-lamin as control. Lower panel, in vitro splicing was performed with untreated (lanes 1 and 2), mock-depleted (lane 3), or CA150-depleted (lane 4) extract using the AdML pre-mRNA as substrate for 0 min (lane 1) or 90 min (lanes 2 to 4). In panels B and C, size markers (in nucleotides [nt]) are indicated at the left.

## DISCUSSION

In the present study, we show that a subset of pre-mRNA splicing factors including 17S U2 associate with WW domains derived from two spliceosomal proteins, CA150 and FBP11. We also demonstrate that CA150 is critical for in vivo splicing and can modulate exon selection. According to our observations, we assume that WW domain-containing proteins may nucleate the assembly of splicing factors at the 3′ splice site of the intron, which in turn facilitates splicing or exon selection (Fig. 7). Since these factors also interact with phosphorylated RNA Pol II mainly via their FF domains (19), they likely serve a role in regulating cotranscriptional pre-mRNA splicing (Fig. 7).

The WW domains of CA150 and FBP11 showed potential association with a number of factors in vitro, including spliceosomal components that recognize the intron elements near the 3′ splice site as well as several additional proteins (Table 1). In particular, we detected the functional 17S U2 snRNP (Fig. 3) consisting of the 12S U2 core and the heteromeric complex SF3a/b (6). Additionally, Western blotting revealed SF1 and U2AF$^{65}$ (Fig. 2). SF1 may bind directly to the WW domains via its proline-rich motif (19), whereas the nature of U2AF interactions with WW domains is unclear. U2AF$^{65}$ can interact via its noncanonical RRM3 with SF1 or component p155 of SF3b (42, 48), suggesting that the association between U2AF and WW domains can be mediated by either factor, although direct interactions still remain possible. At present, it is unclear whether the identified WW-associating factors concomitantly assemble into a large complex or only form subcomplexes that in turn interact individually with a WW domain. SF1 indeed interacts with U2AF in a transient complex to bind to the 3′ end of the intron (5, 24, 40, 42). Moreover, a report that the 17S U2 snRNP associates with substoichiometric levels of U2AF subunits (50) suggests preassembly of subcomplexes prior to splicing. Evidence of subcomplexes is also supported by the mutually exclusive interaction of SF1 with U2AF and a 17S U2 snRNP protein and by the sequential binding of SF1 and U2 to the branch site (48). Therefore, WW domain-containing proteins probably serve as a platform for exchange of these early splicing factors on 3′ intron sequences.

Depletion of CA150 had no effect on in vitro splicing but reduced efficiency of in vivo splicing (Figs. 4 and 5). This result implies that CA150 serves a role necessary for in vivo but not for in vitro splicing. The splicing of pre-mRNA in vivo occurs


A
WW1
WW2 WW3
FF domain
CA150
P
AQ
S/T
CAΔAQ
CAΔN
CAmΔN
x
B
kD
mock
CAΔAQ
CAΔN
CAmΔN
175 -
83 -
C
CAΔAQ
CAΔN
CAmΔN
imm. fluo.
phase
D
β-glo. intron
CAT pre-mRNA
CAT mRNA
mock
CAΔAQ
CAΔN
CAmΔN
1 2.15 1.87 1.07
±0.73 ±0.68 ±0.12
relative splicing efficiency
E
input (1/40)
IP
CAΔN
CAmΔN
CAΔN
CAmΔN
- pol II
- SF1
- CA150
1 2 3 4
F
S AS oligo
- CA150
- α-tubulin
– – + CAΔN
S AS AS oligo
1 0.67 0.89
±0.10 ±0.03
relative splicing efficiency

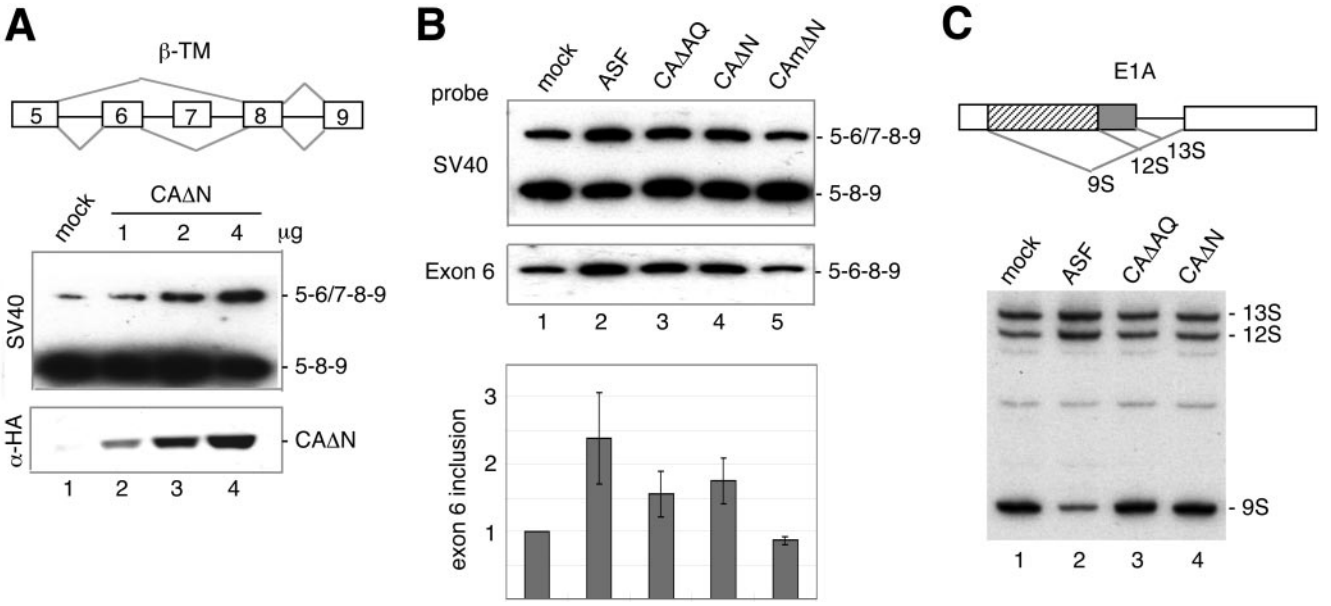



FIG. 6. WW and FF domain-containing CA150 proteins can modulate exon selection. (A) The β-TM minigene was cotransfected with empty vector (mock) or different amounts (1, 2, or 4 μg) of HA-tagged CAΔN expression vector into $8 \times 10^5$ HEK 293 cells. Splicing products were amplified by RT-PCR and analyzed by hybridization on blots using the SV40 probe as described previously (31). The SV40 probe cannot distinguish exon 6 or 7 inclusion; therefore, the upper band is labeled as 5-6/7-8-9. The Western blot was probed with anti-HA. (B) Transfection was as in panel A except that a variety of effector proteins as indicated above were tested. The blots were subjected to sequential hybridization with the SV40- and exon 6-specific probe (31), followed by quantitative analysis (bar graph). Averages and standard deviations reflect five independent experiments. (C) The E1A reporter was transfected with effector expression vector as indicated above the blot. The E1A mRNA products were detected by RT-PCR and hybridization (31).

in concert with transcription (4, 23, 37). RNA Pol II conducts cotranscriptional splicing possibly via the interaction of the CTD with splicing factors such as SR proteins and snRNPs (4, 23, 37). Our data indicate a critical role of CTD-interacting CA150 in splicing in vivo, emphasizing a very close relationship between transcription and pre-mRNA splicing. Moreover, WW and FF domain-containing CA150 polypeptides activated in vivo splicing or exon inclusion but failed to drive alternative 5′ splice site selection (Fig. 5 and 6). The critical role of the WW2 domain in activation of exon inclusion is apparently consistent with the assumption that WW domain proteins recruit the factors that facilitate recognition of elements near the 3′ splice site (Table 1 and Fig. 3). However, the possibility remains that WW-associating factors bind to exonic elements, thereby altering splicing outcome. Finally, CA150 has been implicated in transcriptional regulation via specific promoters (43, 44), and therefore it may be useful to investigate whether CA150 associates with the transcriptional machinery and concomitantly regulates splicing in a gene-specific manner.

We also identified several hnRNP proteins in association with WW domains (Table 1). The hnRNP M protein contains an unusual repeat region rich in methionine and arginine residues that resembles a component of the cleavage stimulation factor (CstF) involved in polyadenylation (13). Since hnRNP M is transiently associated with the pre-mRNA at early stages of spliceosome assembly (27), it may thus act concurrently with several other WW domain-associated splicing factors. We observed that hnRNP M overexpression enhances exon 6 inclu-

FIG. 5. CA150 plays an essential role in splicing in vivo and its fragments containing WW and FF domains activate splicing. (A) Schematic representation of CA150 protein fragments used for in vivo splicing assays. A point mutation was introduced into the WW2 domain of the CAΔN fragment as described in the text. CAΔAQ was C-terminally tagged with the HA-epitope, whereas two respective tag versions, HA and FLAG, of CAΔN and CAmΔN were made. (B) HEK 293 cells were transfected with empty (mock) or HA-tagged CA150 fragment-expressing vector. Total cell lysates were analyzed by Western blotting with anti-HA. (C) Indirect immunofluorescence was performed using anti-HA to detect the cellular localization of transiently expressed HA-tagged CA150 fragments. (D) HEK 293 cells were cotransfected with pSV40-CAT(In1) and an empty vector (mock) or expression vector of HA-tagged CA150 fragments. Splicing of CAT transcripts was analyzed as in Materials and Methods. The values below the blot represent relative splicing efficiency (CA150 proteins over the mock); an average with standard deviation was obtained from three independent experiments. (E) An expression vector encoding FLAG-tagged CAΔN or CAmΔN was transfected into HEK 293 cells. Immunoprecipitation of FLAG-CA150 fragments was performed. The lysates (1/40 of the amounts used for immunoprecipitation) and immunoprecipitates were analyzed with the antibodies indicated to the right. (F) HEK 293 cells were sequentially transfected with sense (S) or antisense (AS) oligonucleotide twice as described in Materials and Methods. Cell lysates were harvested for Western blotting with anti-CA150 and antitubulin antibodies (left panel). For splicing assays, pSV40-CAT(In1) and empty or CAΔN expression vector were introduced into the cells together with respective oligonucleotide during the second transfection (right panel). Relative splicing efficiency (AS over S) was obtained from three independent experiments.

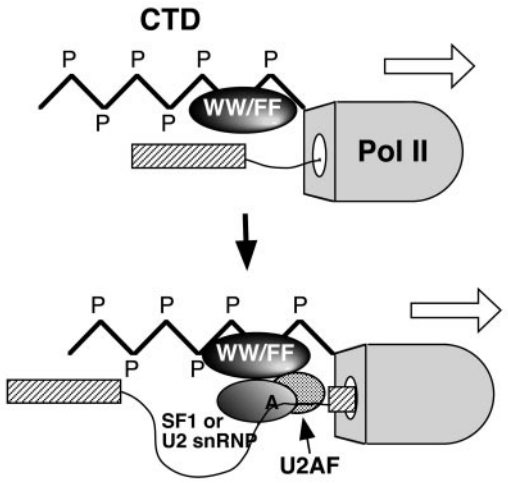



FIG. 7. Model for a role of WW/FF domain-containing factors in pre-mRNA splicing. WW/FF-proteins bind to the phosphorylated CTD of the largest subunit of RNA Pol II (20) and recruit splicing factors such as 17S U2 snRNP, SF1, and U2AF to facilitate 3′ splice site recognition of emerging mRNA precursors and/or to dictate splice site selection during alternative splicing. The nucleotide A (lower scheme) represents the branch site within the intron.

sion of the β-TM (data not shown), consistent with the role predicted for WW-associated splicing factors. Several different functions have been assigned to hnRNP F, including activation of c-*src* exon N1 inclusion (10). It will be interesting to investigate whether hnRNP F functions in conjunction with WW domains and/or their associating factors in alternative exon selection or in any other steps of mRNA processing.

Hsp70 proteins function as molecular chaperones to assist protein folding. Three members (HSP70, HSP71, and GRP78) were previously identified as H complex components that bind to pre-mRNA prior to spliceosome assembly (53). These three proteins were identified in the WW column eluates (Table 1), consistent with a role for WW-associating factors in early splicing complex formation. It is noteworthy that the DnaJ domain-containing protein SPF31 was recently shown to interact with the 17S U2 snRNP (50). Our data also revealed different DnaJ homologs that associate with CA150 (Table 1) or FBP11 (data not shown). The DnaJ cochaperones can regulate the activity of Hsp70 with respect to substrate binding and ATP hydrolysis (21). It is thus possible that these chaperone factors are involved in protein folding or remodeling during spliceosome assembly.

Finally, we identified two additional WW domain- or splicing factor-associated proteins with potential chromatin remodeling activities (Table 1). ATP-dependent chromatin assembly factor acts on histone deposition into periodic nucleosome arrays by hydrolyzing ATP (17). Another protein identified is a novel member of the family of chromatin remodeling, helicase, and DNA-binding proteins that is a part of the nucleosome remodeling and histone deacetylation (NuRD) complex (41). CA150 represses RNA Pol II transcription by inhibiting transcriptional elongation via its first two WW domains (44). Thus, it will be interesting to investigate whether the NuRD complex plays any role in CA150-mediated transcriptional inhibition. Moreover, the spliceosome-associated kinase hPRP4 was recently shown to form a complex with the N-CoR histone deacetylase complexes (16), suggesting a possible link between pre-mRNA splicing and chromatin remodeling during mRNA synthesis. Here, our data reveal a possibility that WW domains coordinate these two events; thus, whether WW-containing proteins influence exon selection also by modulating the rate of transcription remains to be determined.

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