Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3074–3079, April 1996
Plant Biology

Pre-mRNA splicing in plants: Characterization of Ser/Arg splicing factors

(SF2/ASF splicing factor/complementation of splicing extracts/alternative splicing)

SERGUEI LOPATO*, AKILA MAYEDA†, ADRIAN R. KRAINER†, AND ANDREA BARTA*

*Institute of Biochemistry, Vienna Biocenter, Dr. Bohrgasse 9, A-1030 Vienna, Austria; †Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724-2208

Communicated by Jeff Schell, Max-Planck-Institut, Cologne, Germany, December 7, 1995

ABSTRACT **The fact that animal introns are not spliced out in plants suggests that recognition of pre-mRNA splice sites differs between the two kingdoms. In plants, little is known about proteins required for splicing, as no plant *in vitro* splicing system is available. Several essential splicing factors from animals, such as SF2/ASF and SC-35, belong to a family of highly conserved proteins consisting of one or two RNA binding domain(s) (RRM) and a C-terminal Ser/Arg-rich (SR or RS) domain. These animal SR proteins are required for splice site recognition and spliceosome assembly. We have screened for similar proteins in plants by using monoclonal antibodies specific for a phosphoserine epitope of the SR proteins (mAb104) or for SF2/ASF. These experiments demonstrate that plants do possess SR proteins, including SF2/ASF-like proteins. Similar to the animal SR proteins, this group of proteins can be isolated by two salt precipitations. However, compared to the animal SR proteins, which are highly conserved in size and number, SR proteins from *Arabidopsis*, carrot, and tobacco exhibit a complex pattern of intra- and interspecific variants. These plant SR proteins are able to complement inactive HeLa cell cytoplasmic S100 extracts that are deficient in SR proteins, yielding functional splicing extracts. In addition, plant SR proteins were active in a heterologous alternative splicing assay. Thus, these plant SR proteins are authentic plant splicing factors.**

The precise recognition of exon/intron boundaries is crucial for proper gene expression. The dominant recognition signals are sequences at the 5′ and 3′ splice sites and to a lesser extent the branch site. These signals are recognized by complex interactions of various protein factors and small nuclear ribonucleoproteins (snRNPs) involving dynamic rearrangments of RNA–RNA and RNA–protein interactions (1, 2).

Among the few well-characterized metazoan splicing factors are the proteins SF2/ASF, which is involved in selection of the 5′ splice site, and U2AF, which stimulates the interaction of U2 snRNP with the branch point region. It has been shown that these and other splicing factors contain a similar structural motif, termed the RS domain (3). These proteins are phosphorylated *in vivo* and share a serine phosphoepitope recognized by monoclonal antibody mAb104 (4, 5). They were termed SR proteins and comprise a family of evolutionary conserved nuclear phosphoproteins, which contain either one or two RNA recognition motifs and sequences of alternating serine and arginine (SR) residues (3, 5). The isolation procedure precipitated a set of SR proteins of similar molecular mass (ranging from 20 to 75 kDa) from various animal cells (5). The 30-kDa protein fraction contains the splicing factors SF2/ASF and SC-35, which are required for early spliceosome assembly and 5′ splice site selection (6–13). Any SR protein can complement splicing-deficient extracts that lack the entire family of SR proteins, and all of them have a distinct influence on alternative splicing (7, 10, 13–16). RS domains are also found in a variety of splicing factors from animals and are likely to have multiple roles, including promotion of protein–protein interactions, modulation of RNA binding, and subnuclear localization (17–20).

Much less is known about splicing in plants (21–23). Plant introns have well-conserved GU and AG sequences at their boundaries, surrounded by consensus sequences similar to their animal and yeast counterparts. However, neither a polypyrimidine tract nor a branch point consensus sequence appears to be essential for splicing in plants (24). Plants contain all five spliceosomal snRNAs, which are highly homologous to their mammalian counterparts. It is therefore generally assumed that the basic mechanism of splicing in plants is similar to that of yeast and mammals (22, 23, 25). Nevertheless, animal introns are not processed in any plant tissue so far examined (26, 27). The only exception is the simian virus 40 small t-antigen intron, which is very small and AU-rich (28). In contrast, plant introns are normally recognized in animal cells or extracts, suggesting the requirement of more specific sequences for intron recognition in plants (27, 29, 30). In this respect, it is interesting to note that plant introns have a pronounced elevation in the AU content compared to their respective exons (31). Using an artificial intron, it has been shown that efficient splicing in dicots requires introns that are AU-rich (24). Moreover, although *in vivo* experiments have shown that there are no substantial sequence or structural differences between the intron boundaries of animals and plants (32), several experiments have indicated that AU- or U-rich sequences are beneficial for splicing in plants (33–37). How these sequences function in intron recognition remains to be determined.

Studies of splicing in plants have been hampered by the lack of an *in vitro* splicing system. As all efforts to establish such a system by preparing nuclear extracts have so far been unsuccessful, we are pursuing the goal of reconstructing a functional *in vitro* system by isolating purified components. SR proteins are among the major players in the intron recognition process in animals but have not yet been isolated from plants and yeasts. Interestingly, yeast (*Saccharomyces cerevisiae*) cannot process animal introns, presumably because of differences in the recognition of the branch point region. Despite considerable knowledge about yeast splicing factors, no yeast protein with an extensive RS domain has been isolated to date. Although the general splicing mechanism seems to be quite similar in yeast and animal cells, there could be differences in the protein requirement during spliceosome assembly. Therefore, it was also of interest to test whether plants possess SR proteins or whether they might have developed alternative pathways for spliceosome assembly.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: snRNP, small nuclear ribonucleoprotein; DTT, dithiothreitol; hnRNP, heterogeneous nuclear RNP.

In this paper, we describe experiments in which procedures similar to those used for purification of SR proteins in animal cells or tissues were applied to plants. Analysis of these plant proteins showed that this family of splicing factors is indeed highly conserved, as the plant proteins are recognized by antibodies to the mammalian factors. Furthermore, plant SR proteins are able to complement splicing-defective human cytoplasmic S100 extracts and are therefore identified as true plant splicing factors.

MATERIALS AND METHODS

Cultivation of Plants and Suspension Cultures. Carrot suspension culture was diluted 1:10 every 7 days with a medium containing Moorashige and Skoog basal salt mixture (4.3 g/liter) (Sigma), sucrose (20 g/liter), myoinositol (100 mg/liter), thiamine (4 mg/liter), 2,4-dichlorophenoxyacetic acid (0.4 mg/liter), kinetin (0.1 mg/liter) (pH 5.8). Tobacco BY-2 suspension cell culture (38) was diluted 1:20 every 5 days with the same medium as described for carrots but without kinetin and the addition of KH_2PO_4 (200 mg/liter). All suspension cultures were maintained in the dark at 28°C and shaken at 100 rpm. *Arabidopsis* seeds were sterilized and incubated for 2 weeks exposed to light at 25°C in 250-ml flasks rotating at 50 rpm. The medium for cultivation of *Arabidopsis* contained Gamborg's B-5 basal salt mixture (1 g/liter) (Sigma) and sucrose (30 g/liter).

Purification of SR Proteins. Protein purification started with 100 g of cells from suspension cultures of tobacco or carrot or with the same weight of *Arabidopsis* plants. Suspension cultures were usually harvested 4–5 days after dilution. Protein purification was performed basically according to the method of Zahler *et al.* (5). Magnesium pellets were thawed and resuspended in 20 mM ATP (pH 7.5) on ice and centrifuged 5 min at 4°C in an Eppendorf centrifuge, and the supernatant was used for the S100 extract complementation assays in Fig. 3. For the complementation assays described in Figs. 4 and 5 the magnesium pellets were resuspended in 0.2 ml of buffer D [20 mM Hepes·KOH, pH 8.0/0.1 M KCl/0.2 mM EDTA/5% (vol/vol) glycerol/1 mM DTT] containing 4 mM ATP (pH 7.5), and the protein concentrations were estimated by the dye-binding method (Bio-Rad) and standardized with bovine serum albumin. Each SR protein solution was adjusted to 250 μg/ml with buffer D containing 4 mM ATP prior to use in the *in vitro* splicing assays. HeLa cell SR proteins were purified as described (39). SF2/ASF protein was purified from HeLa cells according to published procedures (6, 39).

Nuclei Isolation from Tobacco BY-2 Cells. Four-day suspension cultures of BY-2 tobacco cells were used for nuclei isolation. The isolated protoplasts (32) were resuspended in nuclei isolation buffer and were broken in an Ultra Turrax grinder in 20-ml aliquots in 50-ml Falcon tubes for 10 sec using a middle setting (40). The debris was removed by filtration through two layers of nylon membrane (120 and 20 μm). Nuclei were finally collected by centrifugation at 600 rpm for 10 min and washed with nuclei isolation buffer without Triton X-100. Nuclei were ≈90% pure and were used as starting material for the purification of SR proteins.

Immunoblot Analysis. After electrophoreses in a SDS/12.5% polyacrylamide gel, proteins were transferred to either nitrocellulose (Schleicher & Schuell) or PVDF (Millipore) membranes. The mouse monoclonal antibody mAb104, which is IgM, (4, 41), was a gift from A. M. Zahler; the mouse monoclonal anti-SF2/ASF antibody is IgG 2B (A. Hanamura and A.R.K., unpublished data). The secondary antibodies were anti-mouse IgM (Axell) and anti-mouse IgG (Sigma) conjugated to alkaline phosphatase.

Preparation of ^{32}P-Labeled Pre-mRNA. A part of the pea legumin gene containing one intron (E1, 48 bp; I, 138 bp; E2, 150 bp) has been cloned behind the phage SP6 promoter (30). The pSP65-LegJi plasmid was linearized with *Pst* I and used for transcription *in vitro*. Template plasmids pSP64-HβΔ6 for β-globin pre-mRNA (42) and pSP64-5′D16X for a β-globin pre-mRNA derivative containing a duplicated 5′ splice site (14, 43) were linearized at the *Bam*HI site near the end of exon 2. ^{7m}GpppG-capped ^{32}P-labeled RNA transcripts were synthesized using SP6 RNA polymerase (New England Biolabs) essentially as described (44, 45).

***In Vitro* Splicing Reactions.** The HeLa cell nuclear extract and S100 cytoplasmic fraction used for splicing reactions were prepared according to a protocol of Kramer and Keller (46) or of Krainer *et al.* (6). *In vitro* splicing reactions were carried out at 30°C for 1.5 h in 25 μl with the indicated amounts of HeLa cell nuclear extract, S100 extract, purified SF2/ASF, and recombinant heterogeneous nuclear (hn) RNP A1 as described (13, 15), except that 0.64 instead of 0.5 mM ATP was used. After phenol/chloroform extraction and precipitation with ethanol, the RNA was analyzed on a 5.5% (for β-globin constructs) or 12.5% (for legumin pre-mRNA) polyacrylamide/8 M urea gel followed by autoradiography.

RESULTS

Isolation and Immunostaining of Plant SR Proteins. A simple two-step method for isolation of SR proteins was first introduced by Roth *et al.* (41) using precipitation with ammonium sulfate and then with $MgCl_2$. With this method, SR phosphoproteins from *Drosophila*, HeLa cells, calf thymus, and *Xenopus* immature ovaries have been purified (5). Similar proteins have not yet been reported from yeast and plants, although >30 yeast splicing factors have already been identified. Except for the snRNP protein U2B″ (47), no plant protein splicing factors have been identified. We therefore analyzed proteins from magnesium pellets of different ammonium sulfate fractions prepared from extracts of *Nicotiana tabacum* BY-2 cell lines. These fractions were separated on a polyacrylamide gel (Fig. 1, lanes 1–4) and transferred to membranes. The proteins were immunostained with either the mAb104 antibody, which is specific for a phosphoepitope of SR proteins (Fig. 1*B*) or an antibody specific for the splicing factor SF2/ASF (Fig. 1*C*). As has been found in animal extracts, tobacco SR proteins could be detected only in the 65–90% ammonium sulfate fraction as two proteins of about 50 and 38 kDa (Fig. 1*B*, lane 4). SR proteins from rabbit (lanes 9) and calf thymus (lanes 10) show at least five characteristic main bands (5). Surprisingly, the 50-kDa band of tobacco also crossreacted with a monoclonal antibody specific for SF2/ASF (Fig. 1*C*, lane 4), indicating that not only the phosphoepitope has been conserved between animals and plants, but also a much more specific nonphosphorylated epitope present in mammalian splicing factor SF2/ASF and not in other mammalian SR proteins (A. Hanamura and A.R.K., unpublished data). To determine whether this is a general feature in plants, and to test the reproducibility of the isolation procedure, we examined five preparations of SR proteins from a carrot suspension cell line (Fig. 1 *A–C*, lanes 5–8, and *D*, lane 11). Although the same amount of starting material was used for all the preparations shown in Fig. 1 (except in lane 11), we reproducibly isolated greater amounts of SR proteins from carrot than from the tobacco BY-2 cell line. This might be due to the smaller cell size of the carrot culture. In the case of carrots, four broader bands in the range of 55–35 kDa crossreacted with the mAb104 antibody, whereas only the two larger ones crossreacted with the SF2/ASF antibody.

In addition, SR proteins from *Arabidopsis* plant tissue were isolated. With the mAb104 antibody two strong crossreacting bands of around 48 and 30 kDa could be identified (Fig. 1*D*, lane 12). The 48-kDa band was also immunostained by the SF2/ASF antibody (data not shown). It is interesting to note that the antibody against human SF2/ASF crossreacted with

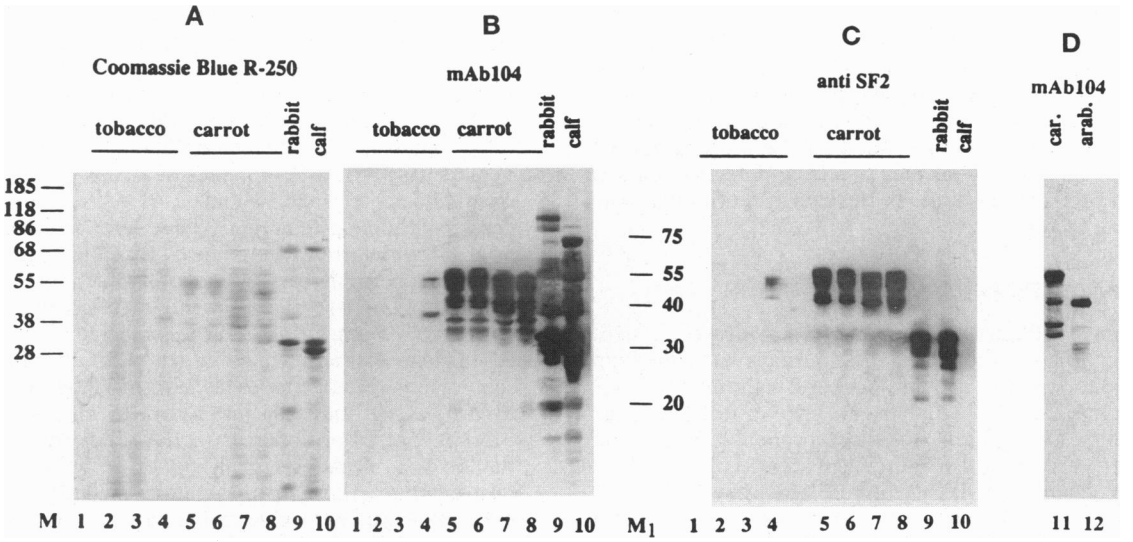

FIG. 1. Western blots of plant SR proteins either stained with Coomassie blue R-250 (*A*) or immunostained with mAb104 (*B* and *D*) and anti-SF2/ASF (*C*). Lanes 1–4, proteins from magnesium pellets of different ammonium sulfate fractions of *N. tabacum* BY-2 cell extract. Lane 1, 0–20%; lane 2, 20–40%; lane 3, 40–65%; lane 4, 65–90% ammonium sulfate. Lanes 5–8 and 11, five independent preparations of carrot SR proteins. Lanes 8 and 9, preparations of SR proteins from rabbit and calf thymus, respectively. Lane 12, SR proteins from *Arabidopsis* plants. M, prestained molecular markers (Sigma); M_1, molecular weights of the main calf thymus SR proteins.

larger plant SR proteins but not with the 30- to 35-kDa fractions. Human SF2/ASF is a 28-kDa polypeptide, but it migrates as a 33-kDa polypeptide in its maximally phosphorylated form. The magnesium pellets also contained other proteins that did not crossreact with either mAb104 or SF2/ASF antibodies. In the case of tobacco and *Arabidopsis*, the SR proteins constituted ≈10% of the precipitated protein, while in the case of carrot they accounted for >60% of the magnesium-precipitated protein stained by Coomassie blue. It is interesting that the complexity and molecular weights of SR proteins are different in different species of plants and also differ from the animal SR proteins, which are more conserved in size (5).

Isolation of Plant SR Proteins from Nuclei. Since all of the animal SR proteins described to date are located in the nucleus, we were interested in determining how many of the plant SR proteins are actually nuclear proteins. We therefore prepared magnesium fractions from 0–65% (Fig. 2, lanes 1 and 4) and from 65–90% ammonium sulfate fractions (lanes 2 and 3) either from intact tobacco BY-2 cells (lanes c) or from isolated BY-2 nuclei (lanes n). Immunoblots were stained with either mAb104 (Fig. 2*B*) or SF2/ASF antibody (Fig. 2*C*). Surprisingly, the mAb104 antibody crossreacted with four proteins from the nuclear extract but with only two SR proteins of 55 and 38 kDa from the whole cell extract. Closer inspection of both immunoblots revealed that the 38-kDa protein, which crossreacts only with the mAb104 antibody, did not change in size, whereas the 55-kDa protein seems to split into three bands of approximately 50, 33, and 30 kDa; all three bands also crossreacted with the SF2/ASF antibody (Fig. 2 *B* and *C*, lanes 2 and 3). As both preparations were obtained from the same amount of starting material, it is likely that the three new bands in the nuclear extract represent either processing or breakdown products of the 55-kDa tobacco SF2/ASF-like protein. All three new nuclear proteins reacted strongly with the mAb104 antibody. It therefore seems unlikely that the large size differences are due to differences in the phosphorylation state only. Interestingly, the smaller SF2/ASF-like proteins of tobacco have a size similar to the mammalian SF2/ASF protein (compare Fig. 2*C*, lane ct; this calf thymus preparation also contains several degradation products of the 33-kDa SF2/ASF protein). Although care was taken to avoid proteolytic activity during preparation of nuclei, smaller molecular weight peptides seem to accumulate in the nuclei fraction (compare Fig. 2*A*, lane 2 and lane 3). As the 38-kDa protein was unaffected by this procedure, it remains to be determined whether this specific breakdown of the 55-kDa protein is an artifact of the nuclear isolation procedure or whether it represents a physiological degradation step induced by manipulations during protoplast and nuclei preparation.

Complementation of HeLa S100 Extracts with Plant SR Proteins. Previous experiments have shown that SR proteins, and in particular SF2/ASF, can functionally complement HeLa cell S100 extracts or nuclear extracts, which are deficient in SR proteins (13–15, 48). To test whether the plant SR proteins isolated in the magnesium pellet are functional splic-

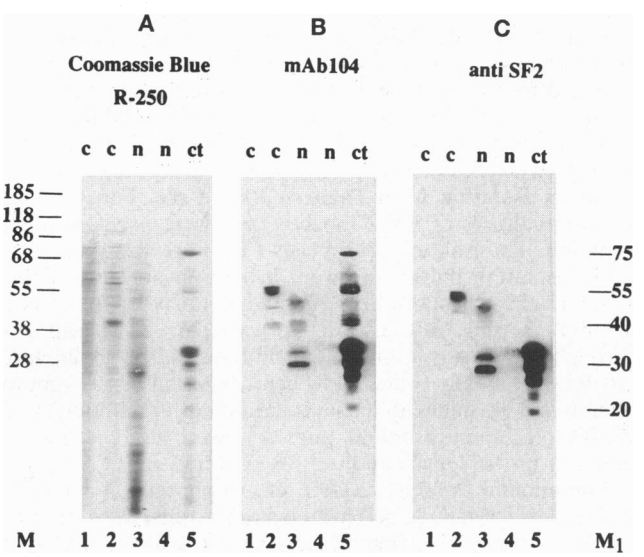

FIG. 2. Comparison of tobacco SR proteins from whole cell (lanes c) or nuclear extracts (lanes n). Western blots were either stained with Coomassie blue R-250 (*A*) or immunostained with mAb104 (*B*) or SF2/ASF (*C*) antibody. Lanes 1 and 4, proteins from magnesium pellets of 0–65% ammonium sulfate fractions. Lanes 2 and 3, proteins from magnesium pellets of 65–90% ammonium sulfate fractions. Lane 5, SR protein preparation from calf thymus (ct). M, prestained molecular markers (Sigma); M_1, molecular weights of the main calf thymus SR proteins.

ing factors, we tested them for their ability to complement a heterologous HeLa cell S100 extract. The plant SR magnesium pellets could not be easily redissolved in buffer D, which is normally used for the splicing complementation assay (48); instead, an ATP solution turned out to be optimal for solubilization of plant SR proteins (see *Materials and Methods*). Fig. 3 shows splicing reactions using a pea legumin pre-mRNA (30) in a HeLa cell S100 extract (lane 2). Compared to a control lane without ATP (lane 1) only trace levels of splicing were observed. Addition of calf thymus SR proteins (lane 3) or purified SF2/ASF protein (lane 4) enhanced splicing considerably, as expected; addition of carrot SR proteins resulted in a pronounced stimulation of splicing (lane 5). To optimize the amount of SR proteins, we titrated S100 extract with SR proteins from carrot (Fig. 4, lanes 2–4), tobacco (lanes 5–7), and HeLa cells (lanes 8–10). In addition, we sought to determine whether plant SR proteins can support constitutive splicing of a simple animal pre-mRNA in HeLa cell S100 extracts. Fig. 4 shows a comparison of splicing activities of the legumin pre-mRNA and unmodified human β-globin pre-mRNA with plant and HeLa SR proteins. With the legumin pre-mRNA (Fig. 4*A*) both carrot and HeLa SR proteins stimulated splicing equally well, whereas tobacco SR proteins were somewhat less active. In contrast, β-globin pre-mRNA (Fig. 4*B*) was stimulated best by HeLa SR proteins but much less well by the carrot proteins and not at all by the tobacco SR proteins. The tobacco SR proteins show consistently lower activities as carrot SR proteins in the complementation assays,

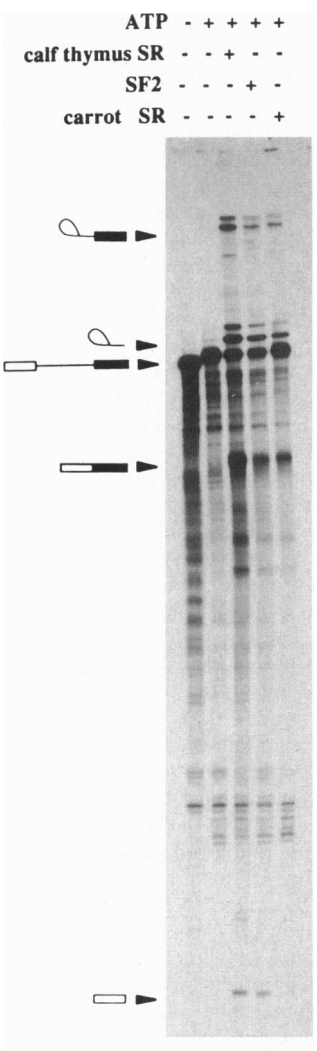

FIG. 3. Complementation of HeLa S100 cytoplasmic extract with SR proteins. Lane 1, splicing reaction without ATP; lane 2, splicing reaction with ATP. Splicing reactions were complemented by addition of either calf thymus SR proteins (lane 3), purified human SF2/ASF (lane 4), or carrot SR proteins (lane 5). Pre-mRNA, spliced products, and intermediates are shown as diagrams on the left.

at least in part because they extract less efficiently and have more non-SR protein contaminants (see Fig. 1). Experiments with another animal pre-mRNA (metallothionein pre-mRNA) also showed better splicing with animal SR proteins than with plant SR proteins (in contrast to legumin pre-mRNA; data not shown). Therefore, these differences in splicing activities are most probably due to species-specific differences in the SR proteins. These data clearly show that one or more proteins in the plant SR protein fraction are genuine splicing factors, as bovine serum albumin or other protein fractions do not complement for splicing in this highly specific assay (data not shown).

Addition of plant or animal SR proteins to plant nuclear extract did not show any splicing (data not shown), indicating that these plant nuclear extracts lack additional essential factors or that they contain inhibitors of splicing (see *Discussion*).

Alternative Splicing Activity of Plant SR Proteins in HeLa Cell Extracts. SR proteins have been shown to possess distinct functions in alternative pre-mRNA splicing *in vitro* and *in vivo*. In particular, the antagonistic activities of SR proteins and hnRNP A1 and A2 are key determinants of alternative 5′ splice site selection. It was therefore of interest to determine the ability of plant SR proteins to perform similar functions in a heterologous HeLa cell system. The construct normally used for this assay consists of a β-globin pre-mRNA with duplicated 5′ splice sites (14, 43). As we did observe splicing stimulation with the carrot SR proteins, we used them to perform the alternative splicing assay with this β-globin construct (Fig. 5). The conditions had to be optimized for the carrot SR proteins, whereby substituted S100 extract proofed better in this case than nuclear extract. As the ratio of the protein SF2/ASF to hnRNP A1 is crucial for selecting either 5′ splice site, HeLa cell S100 extract was supplemented with the appropriate amount of both proteins to obtain splicing of the distal splice site. Adding increasing amounts of HeLa SR proteins promoted usage of the proximal splice site (Fig. 5, lanes 8–10) as demonstrated (13, 15). We note that stimulation of the distal 5′ splice site was also observed rather than the usual switch toward the proximal site (10, 14), because these conditions were optimized to obtain an effect with the plant proteins. A switch toward the proximal 5′ splice site was seen with the carrot SR proteins (lanes 2–4), whereas the tobacco SR protein preparation inhibited splicing (lanes 5–7). From these experiments, we conclude that plant (carrot) SR proteins can functionally replace human SR proteins *in vitro* and thus can interact with other human splicing factors to catalyze splicing and to influence alternative 5′ splice selection.

DISCUSSION

The antibody mAb104, which is specific for a shared phosphoepitope in animal SR proteins, has been used to identify SR proteins in plants. This antibody recognizes SR proteins from animals as diverse as mammals, *Drosophila*, and nematodes (5). The animal SR proteins consist of a set of at least six highly conserved proteins with apparent molecular masses around 20, 30, 40, 55, and 75 kDa. The 30-kDa fraction contains at least two proteins, the essential splicing factors SF2/ASF and SC-35. In contrast, plants have a less conserved pattern of SR proteins: in tobacco and *Arabidopsis* preparations, only two proteins were detected, whereas carrot apparently has four proteins. This is a conservative estimate, as some of these crossreacting bands might contain two or more SR proteins and/or there may be additional SR proteins of low abundance or poor antibody reactivity. As the mAb104 antibody detects a phosphoepitope specific for SR proteins, most probably one or more phosphoserines in an appropriate sequence context (41), we are confident that the plant proteins immunostained by this antibody possess the SR epitope. This assumption is further corroborated by the fact that the plant proteins exhibit

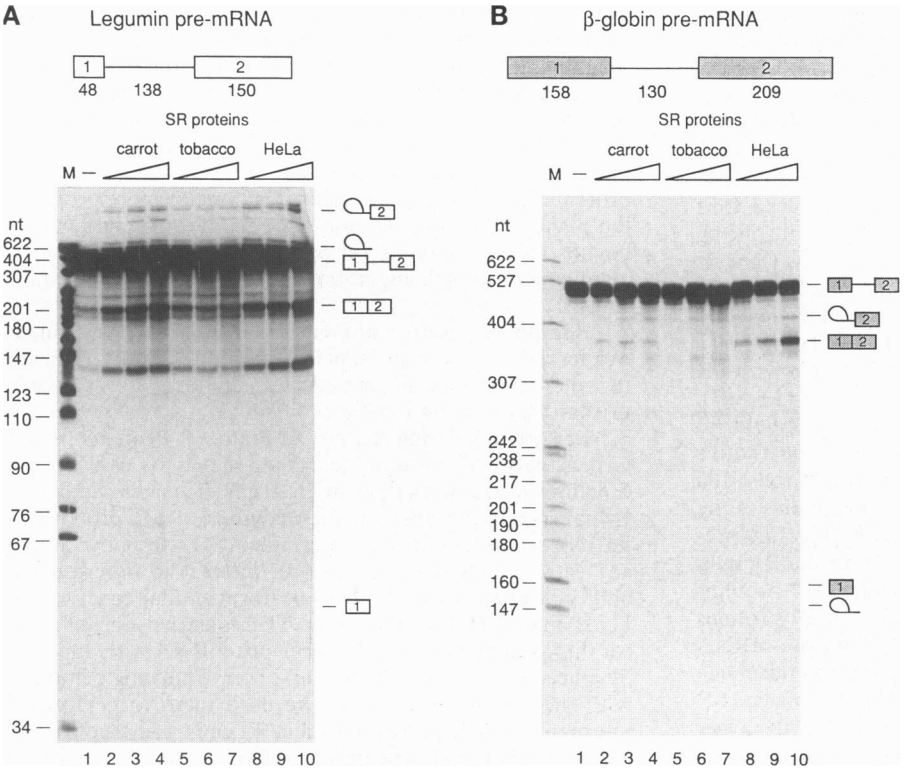

FIG. 4. Constitutive splicing assay *in vitro* with legumin pre-mRNA (*A*) and β-globin pre-mRNA (*B*). Structures of these pre-mRNAs are shown schematically at the top. Exon and intron sizes are indicated in nucleotides. Splicing reactions contained 8 μl of HeLa cell S100 extract complemented with increasing amounts of the indicated carrot, tobacco, and HeLa cell SR proteins [−, none (lanes 1); each set of three lanes (lanes 2–10) corresponds to 0.25, 0.5, and 1.0 μg of protein, respectively, from left to right]. Splicing products are shown schematically on the right. Prominent band of ≈140 nt may represent debranched lariat, which should be 138 nt (30). pBR322/*Hpa* II DNA markers are shown (M) with their sizes in nucleotides.

the same magnesium-dependent aggregation during purification as do animal SR proteins. These aggregates are thought to be mediated by salt bridging between phosphoserines on adjacent SR proteins.

Because of the general nature of the phosphoepitope recognized by mAb104, we anticipated that if SR proteins exist in plants, they would crossreact with this antibody, as long as the proteins are phosphorylated. We were quite surprised to find, however, that a subset of the SR proteins could also be immunostained by the more specific anti-human SF2/ASF antibody in all three plants tested. This antibody recognizes a discontinuous epitope in the N-terminal RNA recognition motif of SF2/ASF and does not crossreact with other human or rat SR proteins (A. Hanamura and A.R.K., unpublished data). Whether these proteins are true plant SF2/ASF homologs or related SR proteins with the same structural epitope remains to be determined by sequencing the proteins or genes. In this respect, it is interesting to note that there is already an *Arabidopsis* sequence in the data bank that is quite similar to the human SF2/ASF sequence (3). While this manuscript was being prepared, the expression of the *Arabidopsis* protein in *Escherichia coli* was reported (49). Although this protein did not complement HeLa cell S100 extract, it functioned in an alternative splicing assay.

To determine whether the SR proteins we isolated might indeed be splicing factors, we performed complementation experiments with HeLa cell S100 extracts. The animal proteins that crossreact with the mAb104 antibody all contain one or two RNA-binding domains and a region of repeating Ser-Arg (SR) dipeptides (3). The SR proteins isolated from mammalian tissues by the magnesium precipitation procedure are essential splicing factors required for spliceosome assembly and have both constitutive and alternative splicing activities *in vitro* and *in vivo* (5, 48, 50). At least five of them have been shown to complement SR protein-deficient HeLa cell S100 extract. At least in this *in vitro* system, several of the SR proteins are interchangeable. In other assays, however, differences in their activities have been observed (7, 10, 13, 15, 16, 48). Therefore, we chose this system to show that SR proteins from carrot and tobacco can indeed complement HeLa cell cytoplasmic S100 extracts. Furthermore, we could also demonstrate the ability of plant SR proteins leads to very efficient complementation of HeLa cell S100 extracts as has been also demonstrated for animal SR proteins (data not shown; ref. 16). Furthermore, we could also demonstrate the ability of plant SR proteins to function similarly to animal SR proteins in an *in vitro* alternative splicing assay—i.e., preferentially selecting the proximal 5′ splice site on a human β-globin construct.

Since one of our long-term goals is the establishment of a plant *in vitro* splicing system, it was of interest for us to check the presence of SR proteins in our inactive plant extracts. Indeed, no SR proteins could be detected in magnesium pellets from splicing deficient carrot nuclear extracts, and only minute amounts were present in tobacco nuclear extracts (data not shown). However, addition of plant or animal SR proteins did not result in active splicing extracts, indicating either that additional splicing factors are missing or that some inhibitory substances might be present in these extracts. Mixing experiments of plant and HeLa cell nuclear extracts show a rapid decline of splicing activity, indicating a problem with inhibitors of splicing (data not shown).

One of the main obstacles in research on plant splicing is the inability of plants to process animal introns, whereas animal *in vitro* and *in vivo* systems are competent in splicing plant pre-mRNAs. Many *in vivo* experiments in plants suggest that the block is due to an intron recognition problem. The results presented in this paper strongly suggest that SR proteins and most probably analogs of the splicing factor SF2/ASF exist in plants. The *in vitro* splicing complementation experiments suggest that the plant SR proteins behave similarly to the animal SR proteins and that at least one of the functions of SR proteins can be provided by the plant SR proteins. Even though plant SR proteins are similar to the animal proteins, the former might assemble differently and/or require additional factors to recognize introns precisely *in vivo*. Interestingly, our results indicate a species preference of plant SR proteins for plant pre-mRNA, as human metallothionein and human β-globin pre-mRNAs were much less efficiently spliced upon addition of plant SR proteins (this paper and unpublished results). In contrast, plant pre-mRNA was efficiently processed in the presence

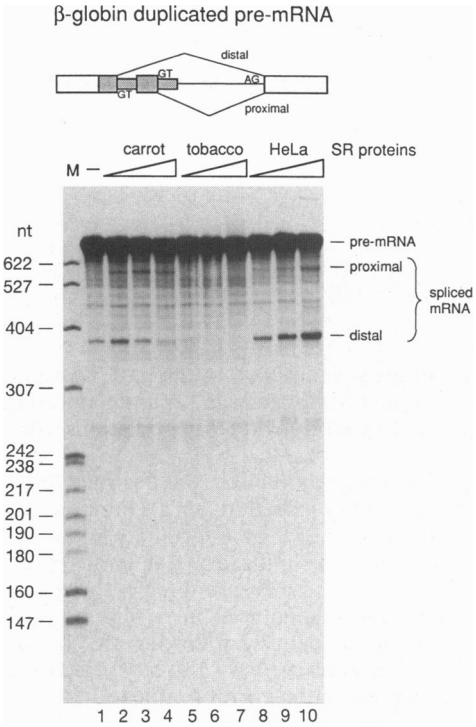

FIG. 5. Alternative splicing assay *in vitro* with model β-globin pre-mRNA. Structure of the pre-mRNA with duplicated 5′ splice sites and the two possible splicing paths are shown schematically at the top. Splicing reaction mixtures contained 7 μl of S100 extract complemented with 7.5 pmol of purified HeLa cell SF2/ASF and 15 pmol of recombinant hnRNP A1, plus increasing amounts of the indicated carrot, tobacco, and HeLa cell SR proteins [−, none (lanes 1); each set of three lanes (lanes 2–10) corresponds to 0.25, 0.5, and 1.0 μg of protein, respectively, from left to right]. Positions of the unspliced pre-mRNA and spliced mRNAs generated by selection of proximal or distal 5′ splice sites are indicated. pBR322/*Hpa* II DNA markers are shown (M) with their sizes in nucleotides.

of animal SR proteins. This correlates well with the *in vivo* data and suggests that differences in the SR proteins might be one of the reasons why plants cannot recognize animal introns.

One of the interesting features of plant introns is the pronounced difference in AU content of the introns compared to the exons (31). It has been shown that the spliceability of an intron can depend on AU-rich short sequences (24, 32–35, 37). Additional work is required to determine whether one of the plant SR proteins could provide such an AU binding activity that would help in intron recognition.

We would like to thank A. Zahler for providing the monoclonal antibody mAb104, J. Brown for plasmid pSP65legJi, and M. Matzke for critically reading the manuscript. We are indebted to K. Hartmuth and Z. Rattler for comments and invaluable discussions. A.R.K. is a Pew Scholar in the Biomedical Sciences. A.M. and A.R.K. were supported by grants from the National Institutes of Health and the National Cancer Institute and S.L. and A.B. were supported by a grant from the Österreichische Fonds zur Förderung der wissenschaftlichen Forschung.

1. Moore, M. J., Query, C. C. & Sharp, P. A. (1993) in *The RNA World*, eds. Gesteland, F. & Atkins, J. F. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 303–358.
2. Nilsen, T. W. (1994) *Cell* **78,** 1–4.
3. Birney, E., Kumar, S. & Krainer, A. R. (1993) *Nucleic Acids Res.* **21,** 5803–5816.
4. Roth, M. B., Murphy, C. & Gall, J. G. (1990) *J. Cell Biol.* **111,** 2217–2223.
5. Zahler, A. M., Lane, W. S., Stolk, J. A. & Roth, M. B. (1992) *Genes Dev.* **6,** 837–847.
6. Krainer, A. R., Conway, G. C. & Kozak, D. (1990) *Genes Dev.* **4,** 1158–1171.
7. Ge, H. & Manley, J. L. (1990) *Cell* **62,** 25–34.
8. Krainer, A. R., Mayeda, A., Kozak, D. & Binns, G. (1991) *Cell* **66,** 383–394.
9. Ge, H., Zuo, P. & Manley, J. L. (1991) *Cell* **66,** 373–382.
10. Mayeda, A. & Krainer, A. R. (1992) *Cell* **68,** 365–375.
11. Fu, X. D. & Maniatis, T. (1992) *Science* **256,** 535–538.
12. Fu, X. D. & Maniatis, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1725–1729.
13. Mayeda, A., Zahler, A. M., Krainer, A. R. & Roth, M. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1301–1304.
14. Krainer, A. R., Conway, G. C. & Kozak, D. (1990) *Cell* **62,** 35–42.
15. Fu, X. D., Mayeda, A., Maniatis, T. & Krainer, A. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11224–11228.
16. Fu, X. D. (1993) *Nature (London)* **365,** 82–85.
17. Li, H. & Bingham, P. M. (1991) *Cell* **67,** 335–342.
18. Wu, J. Y. & Maniatis, T. (1993) *Cell* **75,** 1061–1070.
19. Amrein, H., Hedley, M. L. & Maniatis, T. (1994) *Cell* **76,** 735–746.
20. Kohtz, J. D., Jamison, S. F., Will, C. L., Zuo, P., Luhrmann, R., Garcia Blanco, M. A. & Manley, J. L. (1994) *Nature (London)* **368,** 119–124.
21. Brown, J. W., Simpson, C. G., Simpson, G. G., Turnbull Ross, A. D. & Clark, G. P. (1993) *Philos. Trans. R. Soc. London B* **342,** 217–224.
22. Filipowicz, W., Gniadkowski, M., Klahre, U. & Liu, H-X. (1995) in *Pre-mRNA Processing*, ed. Lamond, A. (Landes, Georgetown, TX), pp. 66–77.
23. Luehrsen, K. R., Taha, S. & Walbot, V. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* **47,** 149–193.
24. Goodall, G. J. & Filipowicz, W. (1989) *Cell* **58,** 473–483.
25. Solymosy, F. & Pollak, T. (1993) *Crit. Rev. Plant Sci.* **12(4),** 275–369.
26. Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M. A. & Matzke, A. J. M. (1986) *Plant Mol. Biol.* **6,** 347–357.
27. Van Santen, V. L. & Spritz, R. A. (1987) *Gene* **56,** 253–265.
28. Hunt, A. G., Mogen, B. D., Chu, N. M. & Chua, N. H. (1991) *Plant Mol. Biol.* **16,** 375–379.
29. Hartmuth, K. & Barta, A. (1986) *Nucleic Acids Res.* **14,** 7513–7528.
30. Brown, J. W., Feix, G. & Frendewey, D. (1986) *EMBO J.* **5,** 2749–2758.
31. Wiebauer, K., Herrero, J.-J. & Filipowicz, W. (1988) *Mol. Cell. Biol.* **8,** 2042–2051.
32. Waigmann, E. & Barta, A. (1992) *Nucleic Acids Res.* **20,** 75–81.
33. Goodall, G. J. & Filipowicz, W. (1991) *EMBO J.* **10,** 2635–2644.
34. Simpson, C. G. & Brown, J. W. (1993) *Plant Mol. Biol.* **21,** 205–211.
35. Lou, H., Mccullough, A. J. & Schuler, M. A. (1993) *Mol. Cell. Biol.* **13,** 4485–4493.
36. Mccullough, A. J., Lou, H. & Schuler, M. A. (1993) *Mol. Cell. Biol.* **13,** 1323–1331.
37. Luehrsen, K. R. & Walbot, V. (1994) *Plant Mol. Biol.* **24,** 449–463.
38. Nagata, T., Nemoto, Y. & Hasezawa, S. (1992) *Int. Rev. Cytol.* **132,** 1–30.
39. Mayeda, A., Helfman, D. M. & Krainer, A. R. (1993) *Mol. Cell. Biol.* **13,** 2993–3001.
40. Saxena, S. K. & Ackerman, E. J. (1990) *J. Biol. Chem.* **265,** 17106–17109.
41. Roth, M. B., Zahler, A. M. & Stolk, J. A. (1991) *J. Cell Biol.* **115,** 587–596.
42. Krainer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. (1984) *Cell* **36,** 993–1005.
43. Reed, R. & Maniatis, T. (1986) *Cell* **46,** 681–690.
44. Krainer, A. R. & Maniatis, T. (1985) *Cell* **42,** 725–736.
45. Mayeda, A. & Ohshima, Y. (1988) *Mol. Cell. Biol.* **8,** 4484–4491.
46. Kramer, A. & Keller, W. (1990) *Methods Enzymol.* **181,** 3–19.
47. Simpson, G. G., Vaux, P., Clark, G., Waugh, R., Beggs, J. D. & Brown, J. W. S. (1991) *Nucleic Acids Res.* **19,** 5213–5222.
48. Zahler, A. M., Neugebauer, K. M., Lane, W. S. & Roth, M. B. (1993) *Science* **260,** 219–222.
49. Lazar, G., Schaal, T., Maniatis, T. & Goodman, H. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7672–7676.
50. Caceres, J. F., Stamm, S., Helfman, D. M. & Krainer, A. R. (1994) *Science* **265,** 1706–1709.