An SC35-like Protein and a Novel Serine/Arginine-rich Protein Interact with Arabidopsis U1-70K Protein*†‡

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The U1 small nuclear ribonucleoprotein 70-kDa protein, a U1 small nuclear ribonucleoprotein-specific protein, has been shown to have multiple roles in nuclear precursor mRNA processing in animals. By using the C-terminal arginine-rich region of Arabidopsis U1-70K protein in the yeast two-hybrid system, we have identified an SC35-like (SR33) and a novel plant serine/arginine-rich (SR) protein (SR45) that interact with the plant U1-70K. The SR33 and SR45 proteins share several features with SR proteins including modular domains typical of splicing factors in the SR family of proteins. However, both plant SR proteins are rich in proline, and SR45, unlike most animal SR proteins, has two distinct arginine/serine-rich domains separated by an RNA recognition motif. By using coprecipitation assays we confirmed the interaction of plant U1-70K with SR33 and SR45 proteins. Furthermore, in vivo and in vitro protein-protein interaction experiments have shown that SR33 protein interacts with itself and with SR45 protein but not with two other members (SRZ21 and SRZ22) of the SR family that are known to interact with the Arabidopsis full-length U-70K only. A Clk/Sty protein kinase (afc-2) from Arabidopsis phosphorylated four SR proteins (SR33, SR45, SRZ21, and SRZ22). Coprecipitation studies have confirmed the interaction of SR proteins with AFC2 kinase, and the interaction between AFC2 and SR33 is modulated by the phosphorylation status of these proteins. These and our previous results suggest that the plant U1-70K interacts with at least four distinct members of the SR family including SR45 with its two arginine/serine-rich domains, and the interaction between the SR proteins and AFC2 is modulated by phosphorylation. The interaction of plant U1-70K with a novel set of proteins suggests the early stages of spliceosome assembly, and intron recognition in plants is likely to be different from animals.

A majority of plant protein coding genes (over 80%), like most eukaryotic genes, contain noncoding intervening sequences (introns) (1). Production of functional mRNAs from precursor mRNAs (pre-mRNAs)1 requires the removal of introns accurately in the nucleus (2). Some transcripts with multiple introns undergo alternative splicing to produce structurally and functionally different proteins from the same gene. Both constitutive and alternative splicing play an important role in the regulation of gene expression in eukaryotes. Nuclear pre-mRNA splicing occurs in spliceosomes by two consecutive trans-esterification reactions. The spliceosome assembly involves complex RNA-RNA, RNA-protein, and protein-protein interactions and is formed by the ordered interaction of four (U1, U2, U4/U6, and U5) small nuclear ribonucleoproteins (snRNPs) and a number of non-snRNPs proteins with the pre-mRNA (3–5). The formation of the commitment or early (E) complex, the first step in the spliceosome assembly, involves recognition of 5′ and 3′ splice sites by U1 snRNP and U2AF (U2 snRNP auxiliary factor), respectively (6–8). In metazoans, U1 snRNP contains one U1 mRNA molecule and at least 11 proteins, including three U1 snRNP-specific proteins (U1-70K, U1-A, and U1-C). The interaction of U1 snRNP with the 5′ splice site is facilitated by several proteins including the members of the serine/arginine-rich (SR) protein family and U1 snRNP-specific protein (8–10).

SR proteins, a large family of proteins, are one of the best characterized non-snRNPs proteins in the spliceosome. These proteins, with a molecular mass ranging from 20 to 75 kDa, contain one or two RNA binding domains (RBDs) and an arginine/serine-rich (RS) domain with multiple RS dipeptide repeats at the C terminus (9, 11). SR proteins from animals can individually complement splicing-deficient S100 extracts that contain all factors necessary for splicing except the SR proteins (9). In humans there are at least nine SR proteins and several SR protein-related polypeptides (11). SR proteins play central roles in both constitutive and alternative splicing as essential splicing factors and as specific splicing regulators at multiple stages in spliceosome assembly (9, 12–15). These proteins recruit other factors during spliceosome assembly through protein-RNA or protein-protein interactions involving their RS domain (14, 15). During early (E) complex formation, ASF (alternative splicing factor/SF2 (splicing factor 2), one of the SR proteins, recruits U1 snRNP to the 5′ splice by interacting simultaneously with the pre-mRNA and the U1-70K protein (16). SR proteins (e.g. SC35 and ASF/SF2) are also involved in bridging 5′ and 3′ splice sites by interacting concurrently with U1-70K and U2AF35 (U2 snRNP auxiliary factor 35) (8, 17, 18). Furthermore, SR proteins facilitate incorporation of the tri-snRNP complex (U4/U6.U5 snRNP) into the spliceosome and promote base pairing between U2 and U6 snRNA (19, 20).

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† The abbreviations used are: pre-mRNAs, precursor mRNAs; snRNP, small nuclear ribonucleoprotein; U1-70K, U1 small nuclear ribonucleoprotein; snRNA, small nuclear RNA; SR protein, serine/arginine-rich protein; RBD, RNA binding domain; RS domain, arginine/serine-rich domain; SRPR, SR protein-specific kinase; kb, kilobase pair; RT-PCR, reverse transcriptase-polymerase chain reaction; Ab, antibody; ASF, alternative splicing factor; SF, splicing factor.

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36428

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Recently, the RS domain of SR proteins has been found to modulate RNA-RNA interactions directly (21, 22). In addition to their role in constitutive splicing, SR proteins have been shown to play an important role in alternative splicing by influencing 5′ and 3′ splice sites selection in vitro and in vivo (9, 11, 13, 23–30). Recently, one of the SR proteins (SRP20) has been shown to regulate alternative splicing of its own pre-mRNA (31).

Several recent studies suggest that phosphorylation of SR proteins is required for spliceosome assembly and splice site selection (32, 33). Furthermore, dephosphorylation of SR proteins is also necessary for the later stages of splicing, suggesting that the phosphorylation and dephosphorylation cycles of SR proteins play a critical role in splicing (32–34). All SR proteins in animals are phosphoproteins and can be detected by monoclonal antibody 104 which recognizes a phosphoepitope within the RS domain (9, 35, 36). Several protein kinases that are capable of phosphorylating serine residues in the RS domain of SR proteins have been identified (37–41). Of these, two families of protein kinases, SRPK (SR protein-specific kinase) and Clk/Sty, that differ in their substrate specificity have been extensively characterized (38, 42). SRPKs are present in both the cytoplasm and the nucleus, whereas Clk/Sty is exclusively in the nucleus. Several studies indicate that phosphorylation of SR proteins affects their mode of interaction with other proteins. Phosphorylation of ASF/SF2 in the RS domain enhances its interaction with U-70K protein and affects its splicing activity (43, 44). Recruitment of SR proteins to sites of transcription is also mediated by phosphorylation (13, 39, 42, 45, 46). SRPK and Clk/Sty can influence the distribution of SR proteins within the nucleus, and an excess of SRPK can inhibit splicing (42, 45). Furthermore, nuclear import of SR proteins is also regulated by phosphorylation (47).

Although considerable progress has been made in understanding splicing in yeast and animals, little is known about splicing and splicing factors in plants. The lack of a plant in vitro splicing system is one of the main reasons for limited understanding of splicing in plants. Plant introns do share some structural features with animal introns. Plant introns, like non-plant introns, have canonical GU and AG dinucleotides at the 5′ and 3′ ends of the introns. However, there are many differences in cis-elements involved in splicing plant and vertebrate introns (1, 48–50). For example, 5′ and 3′ splice sites of plant introns are more loosely conserved than those found in yeast and vertebrates (48). The branch point consensus sequence in plants is also variable. The upstream region of the 3′ splice site has a concentration of uridines in place of a well defined polypyrimidine tract in animals (48, 51). In addition, plant introns are short and richer in uridines and adenosines than flanking sequences of exons (48, 52). Several reports have shown that intronic UA or U richness is important for recognition of 5′ and 3′ splice sites and for efficient splicing of introns in plants (49, 53–57). In vitro splicing studies in HeLa cell extracts and in vivo splicing assays with plant and animal pre-mRNAs indicate that significant differences exist in the mechanisms involved in the recognition of plant and animal introns (1, 49). Most plant intron-containing transcripts, with some exceptions, are either not processed or processed inaccurately in mammalian nuclear extracts (1, 49). Furthermore, animal introns are not excised from pre-mRNA transcripts in vivo in plant nuclei (49, 58, 59). Also, non-intron sequences of animal or bacterial origin are sometimes cryptically spliced in plants (49). These studies indicate that at least some of the proteins involved in splicing of plant pre-mRNAs splicing and the mechanisms involved in intron recognition are likely to be different from animals. Some recent reports on plant SR proteins with some novel motifs lend support to this notion (60–62). So far seven SR proteins have been reported from Arabidopsis (60–63). Of these, some have no real homologs in animals, and some are similar to SR proteins of animals with novel features (60, 62, 63). Two plant SR proteins have been shown to complement splicing-deficient S100 extract (61, 63), whereas Arabidopsis ASF/SF2-like protein did not (60). Overexpression of one of the ASF/SF2-like proteins in transgenic plants modulated splice site choice of several pre-mRNAs including its own pre-mRNA (61, 63).

Recently, we isolated a plant homolog of U1-70K gene from Arabidopsis, the only plant U1-70K gene that has been characterized thus far (64). Although the plant U1-70K protein has several structural features that are present in animal U1-70K protein, it differs from its animal counterpart in some structural features (64, 65). The C-terminal arginine-rich region in plant U1-70K contains mostly RD/RE dipeptides, whereas the plant protein contains, in addition to RD/RE dipeptides, several RS dipeptides. In animals, U1 snRNP participates in splice site selection, by interacting with two SR proteins (SC35 and ASF/SF2), and this interaction takes place through a specific association of the RS domain in SR proteins and a similar region in U1-70K protein in the U1 snRNP (9, 16, 17, 43). These, together with the reports of variations between plant and animal pre-mRNA splicing, have prompted us to identify the proteins that interact with the arginine-rich domain of plant U1-70K. In this study we have used the arginine-rich region of plant U1-70K in the yeast two-hybrid screen, which resulted in isolation of two proteins that contain modular domains typical of splicing factors in the SR family of proteins. One of the proteins (SR33) showed strong sequence similarity to SC35. Although the second protein (SR45) has some structural features of SR proteins, it has no sequence similarity to known SR proteins and contains two RS domains separated by an RBD. This is the first report to demonstrate an interaction between U1-70K protein and a double RS domain-containing protein. We further showed that an Arabidopsis Clk/Sty protein kinase phosphorylates plant SR proteins and modulates their interaction with AFC2.

**Experimental Procedures**

**Yeast Two-hybrid Analysis**—The C-terminal region of the U1-70K, a full-length U1-70K (U1-70K), N-terminal region, and an alternative transcript from Arabidopsis were fused to Ga4 DNA binding domain in pAS2 vector (62). The CDNA library prepared in pACT vector (62) was converted into a plasmid library, and the plasmid DNA was prepared and purified using CsCl density gradient (66). The yeast Y190 strain with the pAS2 containing the C terminus of U1-70K was transformed with the cDNA library and plated on selection (Leu−, Trp−, and His−) plates. Colonies that grew on selection plates were further assayed for β-galactosidase activity (67). DNA from the positives was electroporated into Escherichia coli HB101 (Amp selection), and pACT-containing colonies were identified by polymerase chain reaction (PCR) amplification with pACT 5′ and 3′ primers. To verify the interaction, the pACT plasmids were transformed back into either yeast Y190 strain or Y190 strain containing the pAS2/C-terminal plasmid and tested for β-galactosidase activity. The positive pACT clones were also tested for their interaction with other U1-70K constructs (full-length U1-70K, N-terminal, or alternative transcript) as described above.

The SR33 cDNA (1.1 kb) fragment from pET28a construct was excised by NdeI/blunt/XbaI (see “Expression and Detection of SR Proteins” below) and cloned into pAS1 vector as a fusion to Gal4 DNA binding domain. The fusion junction was verified by sequencing. To test the interaction of SR33 with plant SR proteins, the yeast Y190 strain with pAS/SR33 was then transformed with either SR33, SR45, SR22, or SR22 in pACT plasmid. Selection of transformants and β-galactosidase assays were performed as above.

**Sequence Analysis**—Complete sequences of both strands of cDNAs were obtained by the dideoxy nucleotide chain termination method using double-stranded DNA as a template. Analysis of nucleotide and amino acid sequences was performed using Sequencer, Lasergene, and
MacVector sequence analysis software. Data base searches were performed at the National Center for Biotechnology Information and the Expert Protein Analysis System.

DNA and RNA Gel Blot Analyses—Arabidopsis genomic DNA blots were prepared and hybridized with [32P]cDNA essentially as described. RNA blots containing 5 μg of poly(A) RNA from each tissue were prepared and probed as described (62).

 Amplification of SR33 and SR45 Transcripts by RT-PCR—One microgram of 32P-labeled total RNA from different organs was used to synthesize first strand cDNA with an oligo(dT) primer in a 20-μl reaction volume using the Promega "Reverse Transcription System" (Madison, WI). Primers corresponding to 5' and 3' ends of U1-70K cDNAs were used to amplify the cDNA in all reactions were verified using the primers corresponding to the constitutively expressed cyclophilin gene (62).

 Expression and Detection of SR Proteins—A 1.1-kb XhoI fragment of SR33 cDNA was cloned in-frame into PET28a. To express SR33 in PET28a, a BamHI fragment from pAS1-SR33 construct (see "Yeast Two-hybrid Analyses") was cloned into PET28a. A 1.3-kb NcoI-XhoI fragment of SR45 cDNA was cloned in-frame in PET28c. The orientation of the cDNAs was confirmed by restriction analysis. Preparation of pET28a-U1-70K and PET28b-U1-70K expression constructs and expression and purification of SRZ proteins were described previously (62, 64).

 The plasmid constructs were transformed into E. coli BL21(DE3)-pLysS host cells for expression of fusion protein. Bacterial cultures were grown at 37 °C to an A600 of 0.6. Fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM, and the culture was allowed to grow further for about 3–4 h at 30 °C. Soluble proteins were prepared, separated on 12% SDS-polyacrylamide gels, and transferred onto a nitrocellulose membrane, as described earlier (62, 63). After blocking, the filters were incubated with either SR33 or SR45 proteins that are expressed as S.tag fusions in pET32) at room temperature for 2 h in the buffer described above (see "Expression and Detection of SR Proteins"). The yeast Y190 strain with two reporter genes (lacZ and HIS3) was transformed first with the plasmid containing the C-terminal region of U1-70K, the full-length U1-70K, and a fusion protein was induced by adding 0.5 mM 3-aminotriazole. Yeast colonies that grew on these selective media and showed no β-galactosidase activity (Fig. 1A, C-term) was expressed in a yeast expression vector (pAS2) as a fusion to the DNA binding domain of the yeast transcription factor Gal4 and used as a bait. The yeast Y190 strain with two reporter genes (lacZ and HIS3) was transformed first with the plasmid containing the C-terminal region of U1-70K and then with the Arabidopsis cDNA library in pACT vector. Over a million transformants from 16 independent transformations were plated on selection plates (synthetic complete (SC) media minus His, Leu, and Trp) containing 50 mM 3-aminoatrazole. Yeast colonies that grew on these plates were then screened for β-galactosidase activity (62).

 Screening for Proteins That Interact with the C-terminal Arginine-rich Region of Arabidopsis U1-70K Using the Yeast Two-hybrid System—To identify the proteins that interact with the C-terminal arginine-rich region of plant U1-70K, we used the yeast two-hybrid system (69). The C-terminal region (amino acids 179–427) of Arabidopsis U1-70K containing the arginine-rich domain (Fig. 1A, C-term) was expressed in a yeast expression vector (pAS2) as a fusion to the DNA binding domain of the yeast transcription factor Gal4 and used as a bait. The yeast Y190 strain with two reporter genes (lacZ and HIS3) was transformed first with the plasmid containing the C-terminal region of U1-70K and then with the Arabidopsis cDNA library in pACT vector. Over a million transformants from 16 independent transformations were plated on selection plates (synthetic complete (SC) media minus His, Leu, and Trp) containing 50 mM 3-aminoatrazole. Yeast colonies that grew on these plates were then screened for β-galactosidase activity (62).

 RESULTS

Salt Precipitation of SR Proteins—Soluble proteins from induced cultures were first precipitated by 65% ammonium sulfate, and the proteins in the resulting supernatant were then precipitated with 90% ammonium sulfate (36). Alternatively, SR proteins in the soluble fraction were precipitated directly with 20 mM MgCl2. Protein pellets obtained with 65 and 65–90% ammonium sulfate and MgCl2 were dissolved in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and separated in SDS-containing polyacrylamide gels. The gels were either stained with Coomassie Brilliant Blue or blotted and detected with T7.tag monoclonal antibody conjugated to alkaline phosphatase as described above (see under "Expression and Detection of SR Proteins").

 Coprecipitation Assays—To determine the interaction of U1-70K with SR proteins, pET28a-expressed U1-70K carrying the T7 tag peptide was first bound to T7-Tag antibody-agarose beads according to the protocol provided by Novagen. The agarose beads with bound U1-70K were then incubated with either SR33 or SR45 proteins (expressed as S.tag fusions in pET32) at room temperature for 2 h in the buffer containing 4.3 mM NaH2PO4, 1.5 mM KH2PO4, 2.7 mM KCI, 137 mM NaCl, and 20 mM Tris-HCl, pH 7.5. Following the incubation, the beads were extensively washed with the same buffer at room temperature and boiled for 2 min in SDS-containing buffer. The samples were separated on duplicate SDS-polyacrylamide gels and transblotted onto a nitrocellulose membrane. One blot was probed with T7.tag antibody and the other one with S.tag protein. The interaction of SR33 with itself and SR45 was determined by incubating SR33 bound T7-tag antibody-agarose beads with SR33 or SR45 proteins that are expressed as S.tag fusions essentially as above except that the blots were probed with SC35 antibody. T7.tag-agarose beads that were directly incubated with pET32a expressed proteins were used as controls.

 Protein Phosphorylation Assays—1.4-kb EcoRI/XhoI and EcoRI fragments of two cDNAs encoding Arabidopsis CIK/Sty-type kinases, AFC1 and AFC2, respectively, were cloned into PET32a. The clones were verified by restriction analysis, and the fusion proteins were expressed in E. coli as described above (see under "Expression and Detection of SR Proteins"). Native protein was purified using CLONTECH "Talon" cobalt-based affinity resin according to the manufacturer's protocol. Purified proteins in the supernatant were then precipitated by 90% SDS-containing gels, blotted onto a membrane exposed to x-ray film, and then used to detect the protein kinase and SR proteins using appropriate (T7.tag antibody or S protein) probes. The influence of phosphorylation of SR proteins and AFC2 on their interaction was studied by incubating the T7.tag antibody-agarose beads bound to phosphorylated and nonphosphorylated SR protein with phosphorylated and nonphosphorylated AFC2 kinase. After the incubation the beads were processed as described above under "Coprecipitation Assays."
70K takes place via C-terminal arginine-rich region.

Structural Features and Domain Organization of SR Proteins—SR33 and SR45 have open reading frames that encode proteins of 287 and 414 amino acids with a calculated molecular mass of 33 and 45 kDa, respectively. The nucleotide and deduced amino acid sequences of SR33 and SR45 are shown in Fig. 1B. These proteins have two well defined modular domains (an RBD and an RS domain) characteristic of proteins of the SR family. Both proteins are rich (39–43% of total amino acids) in serine and arginine amino acids (Table I). In addition, proline content is also high (12–17%) in these SR proteins. The SR33 protein contains one RBD at the N terminus, and an arginine/serine-rich (RS) region at the C terminus (Fig. 1C). However, SR45, unlike most known SR proteins, contains two distinct RS domains, one in the N terminus and one in the C terminus, separated by an RBD (Fig. 1C and Table I). There is also a stretch of seven proline residues at the C terminus of SR45 protein. RNP2 and RNP1 consensus sequences in RBD are present in both proteins. The RS domains of both protein have several repetitive sequences that are rich in arginine, serine, and proline (Fig. 1B and Table II).

A BLAST search of the protein data bases with the deduced amino acid sequence of SR33 revealed sequence similarity to animal serine/arginine-rich splicing factor, SC35. A similar search with SR45 sequence revealed some similarity between the RBD of SR45 and other RNA binding proteins, whereas the
by RT-PCR with the genomic sequence indicated that the small abundant 1.1-kb transcript corresponds to the SR33 cDNAs isolated in the yeast two-hybrid system, whereas the larger transcripts (above 1.25 kb) contain an additional 163 nucleotide sequence between exon 2 and exon 3 that corresponded to a part of intron 3 (Fig. 3D and Table III). However, the longer transcripts have an in-frame translation termination codon within the additional 163 nucleotides, resulting in a short protein containing only 44 amino acids with truncated RBD with only RNP2. In alternative splicing mode, there are two introns (3′-440 bases and 3′-161 bases) within the intron 3. Of these two, intron 3′ does not have the canonical splice site (GC..AG in place of GU..AG). Like most plant introns, both 3′ and 3″ introns as well as the 163 nucleotides included/excluded exon are rich in AU (about 64%) content. Hybridization of RNA blots containing poly(A) RNA with labeled SR45 did not show hybridization signal, suggesting that it is expressed at very low levels. However, a single transcript of expected size (1.3 kb) was amplified by RT-PCR using the primers corresponding to the 5′ and 3′ ends of the SR45 cDNA. The 1.3-kb product hybridized to SR45 cDNA (Fig. 3B), confirming that the amplified product is derived from the SR45 gene. Although both genes were found to be expressed in all tissues tested, the level of expression in different tissues varied.

Expression and Purification of SR Proteins—To characterize plant SR proteins and further confirm the interaction of U1-70K with SR proteins, we expressed SR33 and SR45 proteins as His-T7.tag fusions using the pET 28 system or Stag fusions in pET32, respectively. The gene fusion from the cDNA clones was expected to produce polypeptides of about 38 kDa for SR33 and 56 kDa for SR45. Total soluble proteins from induced cultures were tested for the presence of SR33 and SR45 proteins by using either T7.tag antibody (for SR33) or S.tag protein (for SR45). With the T7.tag monoclonal antibody, we detected a polypeptide of about 35 kDa in soluble proteins from cells containing SR33 (Fig. 4A, top), and S protein detected SR45 fusion protein of expected size in the soluble proteins from cells containing SR45 cDNA (Fig. 4A, bottom). In addition, SR33 protein was also expressed in pET32 vector as a fusion to S. tag (data not shown). Both SR proteins were precipitated by 20 mM magnesium chloride (Fig. 4A, lane 2), which is a characteristic of SR proteins (11, 36). However, unlike most animal SR proteins that are soluble in 65% ammonium sulfate (36), plant SR33 and SR45 proteins were completely precipitated by 65% ammonium sulfate (Fig. 4A, lane 3), leaving no fusion protein in the 65–90% ammonium sulfate precipitate (Fig. 4A, lane 4). A monoclonal antibody raised against the human SC35 splicing factor recognized SR33 and SR45 proteins (see under “Interaction between SR Proteins”), suggesting plant SR proteins are related to animal SR proteins.

Coprecipitation of SR Proteins with U1-70K Protein—To demonstrate that the interaction between the SR proteins and U1-70K is direct, we performed coprecipitation experiments. The U1-70K protein expressed as a fusion to T7.tag was bound to T7.tag antibody-agarose beads and incubated with either SR33 or SR45 protein expressed as a S.tag fusion. For each protein, duplicate blots were prepared, and one blot was probed with T7.tag antibody and the second one with S protein. As shown in Fig. 4B, SR33 and SR45 coprecipitated with U1-70K. T7.tag antibody-agarose beads did not bind to S.tag-fused SR33 or SR45 (data not shown). These results confirm the in vivo interaction data in yeast and the specificity of interaction between SR proteins and U1-70K.

Phosphorylation of SR Proteins by AFC2, a Ck1/sty (LAMMER-type) Kinase from Arabidopsis—The members of the SR family of proteins in animals are known to undergo extensive

### Table I

| Name (Accession no.) | Human homolog | RBD % | RS % | Gly % | Pro % | Alt.sp. | Intron % |
|----------------------|---------------|-------|------|-------|-------|---------|----------|
| SRp34 ASF/SF2       | 2             | 1     | 35   | 10    | 7     | ND      | S71185   |
| SRp30 ASF/SF2       | 2             | 1     | 34   | 6     | 7     | ND      | AJ131214 |
| SRp31               | 2             | 1     | 24   | 7     | 8     | ND      | E599345  |
| RSp40               | 2             | 1     | 29   | 8     | 7     | ND      | X90437   |
| RSp41               | 2             | 1     | 30   | 8     | 8     | ND      | X90436   |
| SRZ22 96           | 2             | 1     | 32   | 10    | 10    | ND      | AF033586 |
| SRZ22 98           | 2             | 1     | 31   | 12    | 10    | ND      | AF033587 |
| SR33 SC35          | 1             | 1     | 39   | 8     | 12    | +       | AF099940 |
| SR45               | 1             | 2     | 43   | 7     | 17    | −       | AF151366 |

* Alternative splicing was suggested.

† Interacts with the full-length of plant U1–70K.

‡ Interacts with the arginine-rich region of plant U1–70K.
phosphorylation in vivo, and the phosphorylation status of SR proteins is very important to their function. Hence, it would be of interest to determine whether the plant SR proteins are also regulated by phosphorylation. In animals, two groups of protein kinases (SRPKs and Clk/Sty kinases) that phosphorylate animal SR proteins, especially within their RS domains, have been extensively characterized (32, 46). To determine if plant SR proteins are phosphorylatable, we have used two recently isolated Arabidopsis kinases of the Clk/Sty group (AFC1 and AFC2) in phosphorylation studies with plant SR proteins (71).

We cloned and expressed AFC1 and AFC2 as S-tag fusions using the pET 32 system. AFC1 is expected to produce a protein of a molecular mass of 65 kDa, whereas the expected size of AFC2 protein is 61 kDa. We obtained expected size S-tag fusions of AFC1 and AFC2 protein kinases in soluble fraction prepared from induced cultures. Both proteins were purified to homogeneity using "Talon" affinity beads. Detection of purified AFC1 and AFC2 proteins by S protein is shown in Fig. 5A (lanes 1 and 3). We then used the purified proteins in in vitro phosphorylation assays in the presence and absence of histone. AFC2 showed autophosphorylation and phosphorylated histone (Fig. 5A, lanes 4 and 5), whereas AFC1 showed neither autophosphorylation (Fig. 5A, lane 2) nor phosphorylation of histone. Similar results were obtained with AFC1 and AFC2 using several different protein preparations (data not shown).

To test if the Arabidopsis U1-70K-interacting SR proteins (SR33, SR45, and recently reported SRZ21 and SRZ22 (62)) are the substrates for AFC1 or AFC2, in vitro phosphorylation assays were performed. Each plant SR protein was incubated with [γ-32P]ATP either in the presence or absence of protein kinase. Duplicate blots were made exposed to x-ray film and then probed with either T7.Ab or S protein. As shown in Fig. 5B, SR proteins are phosphorylated by AFC2, and the phosphorylation resulted in slower migration of proteins. Of the four SR protein tested, SR45, despite the presence of two RS domains, was most weakly phosphorylated.

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**TABLE II**

Repetitive sequences in SR33 and SR45 proteins

| Repeat consensus in SR33 | Location | Repeat consensus in SR45 | Location |
|--------------------------|----------|--------------------------|----------|
| RSRS | 157, 227, 251, 268 | SRSP | 58, 334, 383 |
| RSQS | 19, 229, 283 | RRRS | 229, 242, 304, 312, 331 |
| RGRS | 2, 16, 152, 199 | RGRS | 74, 275, 361 |
| SPPPSR | 148, 165, 214 | SPPPSR | 182, 280, 403 |
| SPFSSISRPR | 222, 246, 252 | SPFSSISRPR | 333, 343, 382 |
| SPRSISRSPSR | 216, 224, 240, 248 | SPRSISRSPSR | 333, 343, 382 |
| SRSLFSPRRSRSRS | 240, 248 | PSRGRSRS | 58, 334 |
| SPRSISRPSRSRRSR | 146, 210 | PSRGRSRS | 4, 273 |
| SPRSISRPSRSRRSR | 216, 240 | GLSPRRRSP | 225, 238 |

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**FIG. 2.** Comparison of predicted amino acid sequences of SR33 and SR45 with the proteins that showed the highest sequence similarity. A, alignment of SR33 with human SC35 splicing factor (Hs SC35, accession number Q01130) and mouse TLS-associated SR protein (Mm TASR, accession number AF042383). Conserved RNP2 and RNP1 regions are underscored. B, alignment of the RNA binding domain of SR45 with the RNA binding domain of a nematode (Ce RBP, accession number G45126) and rice GRP2 (Os G/RBP, accession number G26432) RNA-binding proteins. Conserved RNP2 and RNP1 motifs in the RBD are underscored. Identical amino acids are shown by reverse lettering. Dashes indicate gaps in alignment.
functions of SR proteins including protein-protein interactions are regulated by protein phosphorylation and dephosphorylation (13, 27, 34, 38, 39, 42, 45–47, 72). To analyze the effect of phosphorylation on the interaction between Arabidopsis SR proteins and AFC2, we used phosphorylated and nonphosphorylated SR proteins in coprecipitation assays with autophosphorylated and nonphosphorylated AFC2. T7.tag-expressed SR proteins were bound to T7.tag antibody-agarose beads. Half of the beads were mock-phosphorylated without adding AFC2 (Fig. 6, lanes 1 and 2), and the other half of the beads was phosphorylated with AFC2 (Fig. 6, lanes 3 and 4). Phosphorylated and nonphosphorylated beads were split into two equal halves, and one-half was incubated with nonphosphorylated AFC2 (Fig. 6, lanes 1 and 3) and the other half was incubated with autophosphorylated AFC2 (Fig. 6, lanes 2 and 4). Duplicate blots were prepared and probed with either T7.tag antibody to detect SR proteins or S protein to detect AFC2. As shown in Fig. 6A, the nonphosphorylated form of SR33 interacts very efficiently with autophosphorylated AFC2 (lane 2), and no binding was detected between nonphosphorylated AFC2 and SR33 (Fig. 6A, lane 1). The phosphorylated SR33 protein showed a weak interaction with both forms of AFC2 (Fig. 6A, lanes 3 and 4). These results demonstrate direct interaction

**FIG. 4.** SR protein expression and coprecipitation of SR proteins with U1-70K. A, expression, salt precipitation, and detection of SR proteins. Soluble proteins from induced cultures were prepared and precipitated with MgCl₂ or ammonium sulfate. The proteins were separated on duplicate gels. For each protein, one gel was stained with Coomassie Brilliant Blue R250 (stained gel), and a duplicate gel was blotted and probed with either T7.tag monoclonal antibodies (SR33/T7 Ab) or S.protein (SR45/SP). Lane 1, soluble proteins (Tot; lane 2, proteins precipitated with 20 mM MgCl₂ (MgCl₂); lane 3, proteins precipitated with 65% ammonium sulfate (65%); lane 4, proteins precipitated with 65–90% ammonium sulfate (65–90%). Arrowheads indicate SR33 and SR45 proteins. B, in vitro interaction of SR33 and SR45 proteins with the Arabidopsis U1-70K protein. The U1-70K protein expressed in pET28 was bound to agarose beads containing T7.tag antibody and incubated with either SR33 or SR45 proteins expressed as S.Tag fusions in pET32. The beads were washed, loaded onto SDS-containing gels in duplicate, and transferred onto a nitrocellulose membrane. One blot was used to detect SR33 (lane 1, top) or SR45 protein (lane 2, top) by S.protein (SP), and the second blot was probed with T7.tag antibody (T7 Ab) to detect U1-70K in both cases.

**FIG. 3.** Genomic DNA blot and expression analyses of SR33 and SR45 genes. A, Southern analysis. Duplicate blots were probed with either SR33 (left) or SR45 (right) cDNA. Numbers in the middle indicate the length of size markers in kb. B, expression analysis. SR33, the blot was probed with SR33 cDNA. The amount of mRNA in each lane was verified by probing a duplicate blot with a constitutively expressed ubiquitin cDNA (Ubq). SR45, transcript corresponding to SR45 in different tissues was amplified by RT-PCR using the primers corresponding to the 5' and 3' ends of the cDNA. The 1.3-kb product that was amplified by RT-PCR was resolved by electrophoresis, blotted, and hybridized with SR45 cDNA. Amplification of constitutively expressed SR45 genes. B, in vitro interaction of SR45 transcript corresponding to SR45 in different tissues was amplified by RT-PCR using the primers corresponding to the 5' and 3' ends of the cDNA. The 1.3-kb product that was amplified by RT-PCR was resolved by electrophoresis, blotted, and hybridized with SR45 cDNA. Amplification of constitutively expressed SR45 genes.

**TABLE III**

_Intron length, AU content, 5' and 3' splice junctions in SR33_

Nucleotides that are identical to consensus sequences of 5' and 3' splice sites in plants are underlined (5' consensus, AG GUAG, and 3' consensus, GCAG GU, according to Shuler (49)). Intron 3' and 3'' are the parts of alternatively spliced intron 3. Unconventional 5' splice site in intron 3' is shown in lowercase.

| Intron no. | Length | AU | 5' splice site | 3' splice site |
|-----------|--------|----|----------------|---------------|
|           |        |    | Exon | Intron | Exon | Intron | Exon |
|           |        |    |      |        |      |        |      |
| 1         | 522    | 63 | CG   | GUAG  | AUAG | A      |       |
| 2         | 230    | 66 | CG   | AACG  | UAAG | G      |       |
| 3         | 764    | 64 | CG   | GUAG  | GCAG | A      |       |
| 3'        | 440    | 64 | GG   | GUAG  | GCAG | U      |       |
| 3''       | 161    | 62 | AG   | GAAG  | GCAG | A      |       |
| 4         | 97     | 68 | AG   | GGGA  | UAAG | A      |       |
| 5         | 73     | 62 | AG   | GUGA  | ACAG | A      |       |

phosphorylate any of the four SR proteins (data not shown).

_The Interaction between AFC2 and SR33 Proteins Is Influenced by Their Phosphorylation Status—_In animals several
between SR33 and AFC2, and this interaction is modulated by the phosphorylation status of SR33 and AFC2. Similar studies with SRZ22 and SRZ21 also showed that direct interaction of phosphorylated and nonphosphorylated proteins takes place (Fig. 6B, data shown for SRZ22 only). However, phosphorylation did not significantly influence the interaction between the SRZ and AFC2 proteins.

Interaction between Plant SR Proteins—It is proposed that the major role of SR proteins is to facilitate a network of protein-protein interactions during the spliceosome formation (8, 9). We tested the interaction of SR33 with itself and three other plant SR proteins (SR45, SRZ21, and SRZ22) that interact with the plant U1-70K using the yeast two-hybrid system. The SR33 was cloned into the yeast expression vector, pAS1, and transformed into yeast cells containing either SR33, SR45, SRZ21, or SRZ22 cDNA in pACT vector. The transformants were preselected on selection plates, grown on YPD plates, and assayed for β-galactosidase activity. As shown in Fig. 7A, SR33 interacted with itself and SR45 but not with SRZ21 or SRZ22. These interactions were further confirmed by coprecipitation analysis. The SR33 on T7 tag antibody-agrose beads pulled down SR33 and SR45 proteins that are expressed as S tag fusions (Fig. 7B).

To obtain a rough indication of the relative strength of interactions between U1-70K and plant SR proteins, and among SR proteins, we quantified β-galactosidase activity (Table IV). The highest level of β-galactosidase activity was observed in the U1-70K/SRZ22 interaction whereas U1-70K/SR33 and U1-70K/SR45 interactions showed the same level of β-galactosidase activity. The interaction between SR33/SR45 was found to be stronger than SR33 self-interaction.

**FIG. 5.** Phosphorylation of plant SR proteins with Arabidopsis Clk/Sty kinases. A, *in vitro* phosphorylation with AFC kinase: AFC1 (left) and AFC2 (right) kinases were expressed as Stag fusion proteins using pET32. Blots containing the purified AFC1 (lane 1) or AFC2 (lane 3) proteins were probed with Stag protein (SP). AFC2 kinase showed autophosphorylation (lane 4, indicated by open arrowhead) and phosphorylated histone (lane 5, indicated by an arrow), whereas AFC1 did not show autophosphorylation (lane 2). B, AFC2 phosphorylates plant SR proteins: T7 tag fusion proteins of SR33, SRZ22, SRZ21, and Stag fusion protein of SR45 were purified and used in phosphorylation assays with AFC2 kinase. SR proteins treated with AFC2 kinase in the absence (−) or presence (+) of radiolabeled ATP were separated on duplicate gels and blotted onto a nitrocellulose membrane. The blots were probed with either T7 tag antibody (T7 Ab) or S protein (SP) and then exposed to x-ray film (•••P). Arrows indicate the phosphorylated plant SR proteins. Open arrowhead points to the autophosphorylated AFC2 kinase.

**DISCUSSION**

The Arginine-rich Region of Arabidopsis U1-70K Interacts with a Novel SR Protein—The results obtained with the C terminus of U1-70K in the yeast two-hybrid screen and coprecipitation analysis show that the arginine-rich region of plant U1-70K interacts with two SR (SR33 and S45)
proteins. Both these proteins share several structural features with animal SR proteins. Like known SR proteins, SR33 and SR45 contain an RNA binding domain and an RS domain in the C terminus (Fig. 1, B and C). In addition, plant SR proteins are also precipitated by magnesium chloride (Fig. 4). One of the plant SR proteins (SR33) showed significant sequence similarity with animal SC35 and cross-reacted with human SC-35 monoclonal antibody (Fig. 2 and Fig. 7B). In animals, the RS domain of SC35 has been shown to interact directly with a similar domain in U1-70K. These data suggest that SR33 could be a plant homolog of animal SC35. Sequence of SR45, however, did not show significant similarity with any of the known animal SR proteins. In addition, SR45 contains two distinct RS domains, one at the N terminus and the second one in the C terminus, separated by an RBD. It has not been shown previously that U1-70K interacts with an SR protein containing two RS domains. The structural features of SR45 and the lack of sequence similarity with known SR proteins suggest that it could be a novel plant-specific SR protein. Although SR45 has no homolog in animals it is analogous to Drosophila Tra2, a splicing regulator involved in sex determination, in domain organization (73). Both these proteins have two RS domains separated by an RBD. Human Tra2 homologs described by Tacke and colleagues (74) possess similar double RS domain structure and function as sequence-specific activators of pre-mRNA splicing. These similarities suggest the SR45 may be a splicing regulator in plants.

**Plant U1-70K Interacts with a Different Set of SR Proteins**—We have used two different approaches to identify proteins that interact with the arginine-rich region of plant U1-70K. The *in vivo* analysis using the yeast two-hybrid system and *in vitro* coprecipitation studies demonstrate that the arginine-rich region of plant U1-70K specifically interacts with two SR proteins (SR33 and SR45). Isolation of only these two clones several times in screening over a million transformants indicates the specificity of interaction between SR proteins and the arginine-rich region of U1-70K. Previously we have shown that two 9G8-like splicing factors (SRZ21 and RZ22) interact only with the full-length U1-70K protein (62). Together these results indicate that the plant U1-70K interacts with at least four distinct SR proteins (Fig. 8). This is in contrast to animal U1-70K that is known to interact with two SR proteins (SC35 and ASF/SF2). Both of these animal SR proteins interact via the RS domain of U1-70K (16, 17). However, in plants SRZ21 and SRZ22 interact only with the full-length U1-70K, whereas SR33 and SR45 interact with the arginine-rich region of U1-70K. Of the four plant SR proteins that interact with U1-70K, one (SR33) appears to be similar to SC35, and the interaction of the other three proteins with the U1-70K is unique to plants. Among four plant U1-70K-interacting proteins, SRZ22 (also called atRSp22) has been shown to complement splicing-deficient HeLa cell S100 extract (61).

In animals there is single ASF/SF2 which interacts with U1-70K. Although there are at least two ASF/SF2-like proteins present in Arabidopsis (75), none of these was isolated in our yeast two-hybrid screening with either full-length or C-terminal U1-70K. Unlike its animal counterpart, Arabidopsis ASF/SF2 does not complement S100 extract (60). Since ASF/SF2 is expressed well in seedlings (75) (the source of mRNA for our cDNA library preparation), the representation of ASF/SF2 cDNA in our library is not likely to be the reason for not isolating ASF/SF2 in our screen. It would be interesting to test the interaction between plant ASF/SF2 proteins with plant U1-70K. The interaction of plant U1-70K with an SR45 protein containing double RS domains is also unique. These results demonstrate that plant U1-70K interacts with a different set of SR proteins including some novel SR proteins, suggesting that early stages of spliceosome formation or splice site selection may differ from animals. These data support recent observations which indicate that intron recognition in plant is likely to differ from animals (48, 49). Wu and Maniatis (17) have shown that two SR proteins (SC35 and ASF/SF2) that interact with U1-70K protein can simultaneously interact with U2AF35. At present it is not known if U1-70K-interacting SR proteins associate with a similar factor in plants. Two different forms of U2AF50 have been identified in plants (51). Recently, it has been shown that animal U1-70K interacts with Sip1 (SC35-interaction protein 1) which also contains an RS domain but does not contain an RNA-binding motif (76).

**Plant SR Proteins Possess Some Unique Structural Features**—Unlike known SR proteins, SR33 and SR45 proteins are rich in proline content (12% in SR33 and 17% in SR45) (Table I). The majority of these residues are located together with the serine and arginine residues within the RS domains (Table II). There is a long stretch of seven proline residues at the C terminus of SR45 (Fig. 2). A recently described human splicing coactivator, Smn160, whose entire open reading frame is an arginine-serine-rich domain without an RBD also contains a high amount of proline (18%) (77). In addition to proline richness, SR33 and SR45 proteins contain the highest percentage of serine and arginine residues (39–43%) among all plant SR proteins (see Table I). Of nine human SR proteins only three SR proteins (SRP20, SC35, and 9G8) contain one RBD, whereas the others have two such domains. Among SR proteins characterized in Arabidopsis, six of the eight contain one RBD.

**SR33 Gene Produces Multiple Transcripts**—RNA gel blot analysis and RT-PCR data indicate the presence of multiple transcripts (at least four) of SR33 derived from the SR33 gene. We found that all transcripts that are larger than 1.25 kb are generated by alternative splicing of the SR33 pre-mRNA and...
contain an additional exonic sequence of 163 nucleotides which originates from the unusually large (764 nucleotides) intron 3. Some of these transcripts differ from others in their 3'-untranslated sequences. However, unlike SR33, three other genes (SR45, SRZ221, and SRZ222), whose protein products also interact with plant U1-70K, produce a single transcript. Studies with animal systems show that the expression of SR proteins is regulated at the transcriptional and/or post-transcriptional level. For example, SRp20 is highly expressed in thymus, testis, and spleen but not in liver of kidney of mice (78). The transcription of some SR proteins is regulated by hormones and mitogens (11). In addition, several SR protein pre-mRNAs are known to undergo alternative splicing to produce multiple isoforms (79–81). For example, alternative splicing of ASF pre-mRNA produces three isoforms of ASF (ASF-1, ASF-2, and ASF-3) of which ASF-2 and ASF-3 isoforms lack an RS domain (81). Multiple isoforms have also been detected in other SR proteins such as SRp55, SRp40, and SC35. In the case of SC35, alternative splicing events do not effect its coding region but alter 3'-untranslated region (11). The expression of some of the SR genes in plants is also regulated by alternative splicing (Table I) (60, 63, 75). However, in most cases, the function of alternatively spliced transcripts and their encoded proteins is not known.

Interaction between Plant SR Proteins—SR proteins are involved in a network of protein-protein interactions primarily through their RS domain. Some SR proteins interact with more than one protein simultaneously. SC35 and ASF/SF2, in addition to interacting with U1-70K and U2AF35 interact with each other as well as with Tra and Tra2 proteins (17, 76). In addition, self-interaction has also been shown for SC35, ASF/SF2, and U2AF35. Recently, a new splicing factor (Sip1) has been shown to interact with SC35, suggesting complex protein-protein interactions among RS domain-containing proteins play an important role in splicing (17). To investigate the interaction among SR proteins that interact with U1-70K, we used SR33 as a bait in yeast two-hybrid system and tested its interaction with itself and other U1-70K-interacting SR proteins (SR45 and SRZ22 and SRZ221). SR33 protein interacted with itself and SR45 protein. Surprisingly, no interaction between SR33 and SRZ proteins (SRZ21 and SRZ22) was detected. The results were further confirmed in vitro, suggesting that there is stringent specificity in the interaction among plant RS domain-containing proteins. Quantitative analyses of interaction strength indicate that SRZ22 and SRZ23 interact strongly with U1-70K as compared with SR33 and SR45. Furthermore, the interaction of SR33 with SR45 is stronger than self-association of SR33.

Modulation of Interaction by Phosphorylation—A number of recent studies indicate the importance of the phosphorylation status of SR proteins in the regulation of splicing process (41, 43, 44, 46, 47, 72, 82). Two groups of kinases (SRPK and Clk/Sty) that phosphorylate animal SR proteins have been well characterized. Clk/Sty is a group of dual-specificity protein kinases that can phosphorylate serine, threonine, and tyrosine residues (38, 42). The Clk/Sty kinases (also called LAMMER-type kinases) contain a unique sequence (EHLAMMERILG-DLA) in subdomain X of the kinase catalytic domain. The interaction of Clk/Sty with some of its target proteins involves an RS-rich region at the N terminus of Clk/Sty kinase.

The plant kinases that phosphorylate plant SR proteins have not yet been identified. Recently, Bender and Fink (71) isolated a Clk/Sty kinase (AFC1) from Arabidopsis that can suppress mating defect in fus3 and kss1 signal transduction mutant in Saccharomyces cerevisiae. Two other closely related Clk/Sty kinases (a full-length AFC2 and a partial AFC-3) were also isolated from Arabidopsis using primers corresponding to conserved regions in AFC1 kinase (71). However, AFC2, although closely related to AFC-1, did not suppress the mating defect in double mitogen-activated protein kinase mutant, suggesting a difference in their function. The substrates of plant Clk/Sty kinases have not been identified. Since mammalian Clk/Sty kinase phosphorylates SR proteins in animals, we tested two Arabidopsis Clk/Sty kinases for their ability to phosphorylate U1-70K-interacting plant SR proteins. One of the kinases (AFC-2) undergoes autophosphorylation and heavily phosphorylated four plant SR proteins. However, the plant Clk/sty kinase does not contain an RS-rich region in the N terminus, suggesting that structural features other than an RS domain are also important in the interaction of AFC2 with other proteins. AFC1 which rescues yeast double mitogen-activated protein kinase mutant does not phosphorylate SR proteins, suggesting that the ability to phosphorylate SR proteins is not likely to be a feature of all Clk/Sty kinases in Arabidopsis.

In animals, interaction among SR proteins, nuclear import of SR proteins, intranuclear movement of SR proteins, and splice site selection appear to be mediated by phosphorylation of the RS domain in SR proteins (13, 27, 38, 39, 42, 45–47, 72). By using the coprecipitation analysis, we have demonstrated that AFC2 kinase binding to one of the substrates (SR33) is dependent on its phosphorylation status. Since all U1-70K-interacting proteins bound to AFC2, our attempts to study the effect of phosphorylation of SR proteins on their interaction with each other in vitro were not conclusive. Further studies are necessary to understand the roles of AFC2-mediated phosphorylation in the interaction of SR proteins and the intranuclear movement of SR proteins. Localization of SR proteins in Arabidopsis cultures that are overexpressing AFC2 should allow us to answer some of these questions. In mammalian cells overexpression of active Clk/Sty causes dissociation of nuclear speckles and redistribution of SR proteins within the nucleus (42). The data on interaction of U1-70K with plant SR proteins and interaction among SR proteins is summarized in Fig. 8. Two plant SR proteins (SRZ21 and SRZ22) that share some features with animal 9G8-like protein interact with only the full-length U1-70K, whereas SR33 and SR45 interact with the C-terminal arginine-rich region of U1-70K. In addition, SR33 interacts with itself and SR45 but not with SRZ21 or SRZ22 proteins. A Clk/Sty (LAMMER-type) kinase (AFC2) binds and phosphorylates all four SR proteins. In animals, 9G8 splicing factor is not known to interact with U1-70K. Furthermore, the interaction of U1-70K with an SR protein containing two RS domains with U1-70K is also new. Our data demonstrate that the plant U1-70K interacts with a novel set of SR proteins and suggest that the early stages of spliceosome assembly, particularly splice site selection in plants, are likely to be different from animals.

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Note Added in Proof—The SR45 gene is a chromosome 1, and the coding region is interrupted by 11 introns (SR45 gene accession number AC0 11808).

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