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Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors

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ABSTRACT

The SR proteins are essential metazoan pre-mRNA splicing factors that can also influence the selection of alternative 5' splice sites in a concentration-dependent manner. Their activity in alternative splicing *in vitro* is antagonized by members of the hnRNP A/B family of proteins. The opposite effects of members of these two families of antagonistic splicing factors *in vitro* and upon overexpression *in vivo* suggest that changes in their relative levels may be a natural mechanism for the regulation of alternative splicing *in vivo*. One prediction of this model is that the ratios of these antagonists should vary in different cell types and in other situations in which cellular or viral transcripts are differentially spliced. We raised monoclonal antibodies specific for SF2/ASF and used them to measure the abundance of SF2/ASF protein and its isoforms, its phosphorylation state *in vivo* and during splicing *in vitro*, and its association with the spliceosome. SF2/ASF exists predominantly or exclusively in a highly phosphorylated state *in vivo* in all cell types examined, and unphosphorylated protein was not detectable. Unphosphorylated recombinant SF2/ASF becomes rapidly phosphorylated under splicing conditions in HeLa cell extracts and associates stably with one or more exons of β -globin pre-mRNA. This interaction appears to persist through the splicing reaction and SF2/ASF remains bound to spliced mRNA. We compared the distribution of SF2/ASF to that of its antagonist, hnRNP A1, in different rat tissues and in immortal and transformed cell lines. We found that the protein levels of these antagonistic splicing factors vary naturally over a very wide range, supporting the notion that changes in the ratio of these proteins can affect alternative splicing of a variety of pre-mRNAs *in vivo*.

Keywords: hnRNP A1; monoclonal antibodies; phosphorylation; pre-mRNA splicing factors; SF2/ASF; SR proteins

INTRODUCTION

Alternative splicing is a major mechanism for controlling the expression of cellular and viral genes, and its regulation is probably accomplished by subtle variations of the general splicing mechanism. Consistent with this notion, certain components of the general splicing machinery, such as the SR proteins, can affect alternative splice-site selection in a dose-dependent manner (for reviews see Fu, 1995; Valcarcel & Green, 1996; Cáceres & Krainer, 1997). SF2/ASF and other members of the SR protein family regulate alternative splicing with a variety of pre-mRNAs, usually by promoting the use of proximal 5' splice sites. This activity of SR proteins is counteracted by hnRNP A1 and related proteins, which generally favor the use of distal 5'

splice sites (Mayeda & Krainer, 1992; Mayeda et al., 1994). Therefore, the antagonistic activities of SR proteins and hnRNP A1-like proteins are important determinants of alternative 5' splice-site selection *in vitro* and in transfected cells (Cáceres et al., 1994; Yang et al., 1994). These experimental observations suggest that these two families of RNA-binding proteins play prominent roles in the regulation of alternative splicing of a wide variety of transcripts *in vivo*, although the identity and number of these natural targets remain unknown. This hypothetical mechanism of alternative splicing regulation requires that the relative abundances or activities of these antagonistic factors vary under conditions in which one or more transcripts undergo differential splicing, e.g., in different cell types or during development.

All SR proteins are phosphorylated *in vivo*, as shown, e.g., by the fact that a monoclonal antibody, mAb104, which recognizes a shared phosphoepitope within their C-terminal RS domains, reacts with all the family members (Roth et al., 1991). However, because mAb104

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recognizes the SR proteins only when they are highly phosphorylated, analyses with this antibody do not reveal the extent of their phosphorylation in vivo or the overall abundance of all the forms of these proteins. It is presently unclear whether phosphorylated and/or partially or fully dephosphorylated RS domains are the active form of this domain in SR proteins. A role for reversible protein phosphorylation in the regulation of both constitutive and alternative splicing has been postulated (Mermoud et al., 1992, 1994), and it has also been shown that changing the levels of active PP1 phosphatase in nuclear extracts results in changes in alternative 5' splice-site selection (Cardinali et al., 1994). Several protein kinases capable of phosphorylating SR proteins on serine residues in vitro have been described, of which the best characterized are SRPK1 (Gui et al., 1994a, 1994b) and Clk/Sty (Colwill et al., 1996a, 1996b). Recent experiments showed that, at least under certain in vitro conditions, both phosphorylation and dephosphorylation cycles may be important for constitutive splicing (Cao et al., 1997; Xiao & Manley, 1997). It has also been proposed that the state of phosphorylation may control the subnuclear distribution of SR proteins in interphase cells and the reorganization of nuclear speckles during mitosis (Gui et al., 1994a; Colwill et al., 1996b; Misteli & Spector, 1997).

The observed regulation of alternative splicing by SF2/ASF and hnRNP A1 levels, both in vitro and in transfected cells, suggests that changes in the intracellular ratio of these proteins may be sufficient to cause differential regulation of certain alternative splicing events in a tissue-specific manner and during development. Any individual SR protein can complement a splicing-inactive HeLa S100 extract lacking all the SR proteins, suggesting functional redundancy for constitutive splicing of at least some pre-mRNAs. In contrast, some differences have been detected in the ability of SR proteins to regulate alternative splicing of different pre-mRNAs in vitro and in vivo (Zahler et al., 1993; Sreaton et al., 1995; Wang & Manley, 1995; for reviews see Fu, 1995; Manley & Tacke, 1996; Cáceres & Krainer, 1997). Regulation of the expression of many genes by alternative splicing may be accomplished at least in part by tissue-specific, developmentally regulated, physiological state-dependent, or virus-induced variations in the relative levels of one or more SR proteins and the antagonizing hnRNP A/B proteins. Tissue-specific variations in the total and relative amounts of phosphorylated SR proteins, or of mRNAs coding for SR proteins, have been described (Ayane et al., 1991; Fu & Maniatis, 1992; Vellard et al., 1992; Zahler et al., 1993; Sreaton et al., 1995). Likewise, cell-type specific variations in hnRNP A/B protein or mRNA levels have been reported (Faura et al., 1995; Kamma et al., 1995; earlier work reviewed in Dreyfuss et al., 1993). However, whether these changes occur in parallel—

which would not be expected to affect alternative splicing—or whether the relative abundances of these antagonists vary under natural conditions, was not known. To address the mechanisms of alternative splicing regulation by SF2/ASF and hnRNP A1, we raised monoclonal antibodies specific for SF2/ASF and used them to determine if the ratio of these proteins is constant or variable in normal and transformed cell lines, in different rat tissues, and during different stages of adenovirus infection.

RESULTS

Production of anti-SF2/ASF monoclonal antibodies

Human SF2/ASF is a 248-amino acid protein composed of four domains, based on the primary sequence and on motifs shared with other proteins (Fig. 1): an N-terminal RNA-recognition motif (RRM1), a poly-glycine hinge domain, a central atypical RRM (RRM2), and a C-terminal arginine/serine-rich (RS) domain (Ge et al., 1991; Krainer et al., 1991). The RRM is a conserved 80–90-amino acid sequence common to a very large group of RNA-binding proteins (Kenan et al., 1991; Birney et al., 1993). It consists of two short stretches of predominantly aromatic and hydrophobic residues, the octamer RNP-1 and the hexamer RNP-2 submotifs, and of several additional conserved positions that constitute a hydrophobic core. The domain folds into a four-stranded antiparallel β -sheet on the side facing RNA, with two α -helices lying on the opposite side. The RS domain is a region of low compositional complexity, rich in arginine and serine residues, which is characteristic of several metazoan splicing factors and splicing regulators (Birney et al., 1993). The RS domains of SF2/ASF, and of related members of the SR protein family, are characterized by arginine–serine periodicity and by serine phosphorylation at so far undetermined positions of the domain.

Mice were immunized with purified recombinant protein consisting of full-length SF2/ASF fused downstream of the *Escherichia coli* malE protein. Positive hybridomas were screened by western blotting with the same protein and with partially purified human SF2/ASF. Two clones, mAb96 and mAb103, reacted strongly and specifically, and were used in further experiments. A third monoclonal, mAb105, which recognizes the bacterial maltose-binding protein portion of the fusion protein, was also obtained in this screen, and was used as a negative control in some experiments.

To map the epitopes recognized by mAb96 and mAb103, we analyzed their reactivity by western blotting with several domain-deletion mutants of SF2/ASF (Cáceres & Krainer, 1993). The intact protein or the domain fragments were expressed in *E. coli* with T7 His-tag vectors and affinity purified on nickel-NTA res-

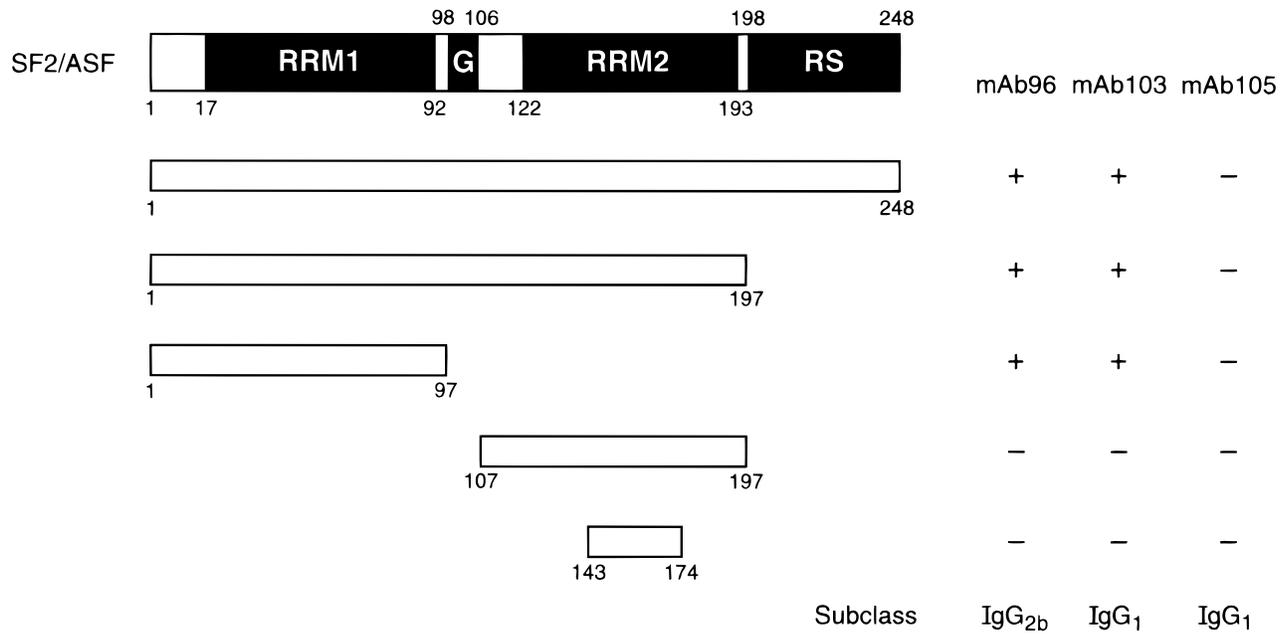


FIGURE 1. Epitope mapping of anti-SF2/ASF monoclonal antibodies. The domain structure of SF2/ASF is shown schematically; the main features include two RNA-recognition motifs (RRM1 and RRM2), a poly-glycine hinge (G), and an arginine/serine-rich domain (RS). Full-length and deletion constructs expressed in *E. coli* (in some cases as fusions with bacterial proteins or His-tagged) are shown below. The reactivity of each monoclonal antibody with the different proteins and fragments, as determined by immunoblotting, is shown on the right. The subclass of each antibody heavy chain is also shown. mAb105 was obtained in the same hybridoma screen and recognizes the maltose-binding protein portion of the malE-SF2/ASF fusion protein used for immunization. Four additional recombinant proteins consisting of full-length SF2/ASF with mutations within RRM1 (residues 54–55, 56 and 58, 60–67, or 51–55) were also tested and showed the same reactivity as the wild-type protein.

ins, as described previously. As summarized in Figure 1, both monoclonal antibodies, mAb96 and mAb103, specifically reacted with the N-terminal portion of the protein comprising RRM1. We also tested the following mutants with amino acid substitutions or small deletions within RRM1: (1) PP → RG beginning at residue 54; (2) FAF → DAD beginning at residue 56; (3) EFED PRDA → TFQNPANV beginning at residue 60; and (4) RGGPP → RGSGKKRG beginning at residue 51 (Cáceres & Krainer, 1993). None of these mutations affected the reactivity of mAb96 and mAb103, whereas other anti-SF2/ASF monoclonals were affected by several of these mutations and appeared to recognize discontinuous epitopes, but were not characterized further. We conclude that mAb96 and mAb103 recognize either one of the short regions flanking RRM1 within the first 97 amino acids of SF2/ASF, or a portion of the RRM1 itself, other than loop3, β_3 , loop4, and the first half of α_2 (Birney et al., 1993). mAb 103 was also used to study the localization of endogenous SF2/ASF in HeLa cells by indirect immunofluorescence. A typical nuclear speckled pattern and also a diffuse nuclear staining was observed (Cáceres et al., 1997). Although the two monoclonals have similar properties and reactivities, mAb96 gave stronger signals in western blotting, whereas mAb103 gave clearer staining in immunofluorescence assays (data not shown).

Phosphorylation state of SF2/ASF

The anti-SF2/ASF monoclonal antibodies (mAbs) were used to detect the SF2/ASF protein present in HeLa cell extracts used for splicing. A band or frequently a doublet of approximately 33 kDa was detected in nuclear extract, as expected (Ge et al., 1991; Krainer et al., 1991); in contrast, the same volume of S100 had almost no SF2/ASF, as expected from the complementation assay originally used to purify SF2/ASF (Krainer et al., 1990; see below). The mobility of SF2/ASF on SDS-PAGE is affected by its phosphorylation state (Fig. 2A), as seen previously with epitope-tagged and fusion proteins (Roth et al., 1991; Xiao & Manley, 1997). Treatment of SF2/ASF purified from HeLa cells with bacterial alkaline phosphatase resulted in a marked increase in mobility, such that the dephosphorylated protein co-migrated with untagged recombinant SF2/ASF (rSF2/ASF) purified from *E. coli* (Fig. 2A, lanes 1–3). A similar increase in mobility upon dephosphorylation was observed for another SR protein, SC35, as expected (Fig. 2A, lanes 4 and 5). SR proteins are phosphorylated at multiple serine residues within their RS domains, as determined by their reactivity with mAb104, which recognizes a phosphoepitope (Roth et al., 1991), and by studies of *in vitro* phosphorylation with several kinases (Gui et al., 1994b; Colwill et al., 1996a).

ASF was present after 1 h (Fig. 2C, lane 6). The rapid kinetics of phosphorylation precluded a simple experiment to determine if phosphorylation of SF2/ASF is essential for complementation activity. However, an apparent requirement for SF2/ASF phosphorylation when nonspecific RNA competitors are included in excess (Xiao et al., 1997), or when pre-mRNA is first pre-incubated with excess SF2/ASF (Cao et al., 1997), has recently been reported. The present observations, which confirm and extend previous studies, show that untagged, unphosphorylated recombinant SF2/ASF is rapidly phosphorylated at multiple sites under splicing conditions, such that its electrophoretic mobility is the same as that of the endogenous protein.

Immunoprecipitation of spliceosomes

We next attempted to determine if SF2/ASF, which is required for the assembly of specific pre-spliceosome complexes (Krainer et al., 1990; Fu, 1993), is stably associated with pre-mRNA and/or with the intermediates and products of splicing. A splicing reaction with labeled β -globin pre-mRNA was incubated with mAb96, and the immune complexes were collected with protein G-Sepharose. RNA was extracted from the immunoprecipitate and the supernatant and analyzed by urea-PAGE (Fig. 3). The pellet was selectively enriched in spliced mRNA, although pre-mRNA, exon 1, and the lariat intermediate and product were also detectable (Fig. 3, lane 3). A control antibody against a bacterial protein (mAb105) precipitated only background levels of all RNA species (Fig. 3, lane 2), whereas an anti-hnRNP A1 mAb preferentially precipitated the excised lariat (Fig. 3, lane 4), and an anti-hnRNP C1/C2 mAb bound all RNA species with comparable efficiencies (Fig. 3, lane 5). We conclude that SF2/ASF associates stably with β -globin RNA through the splicing reaction and remains bound to exon sequences after splicing catalysis, although we cannot rule out the possibility that SF2/ASF first dissociates from the spliceosome and then reassociates with the spliced mRNA. Similar observations have been made with the SR-related proteins B1C8 and B4A11, which also remain bound to the mature mRNA after splicing is completed (Blencowe et al., 1994, 1995).

Quantitation of SF2/ASF and hnRNP A1 abundance in HeLa cells

The relative stoichiometries of SF2/ASF and hnRNP A1 influence alternative splicing *in vitro* and in co-transfection experiments (Mayeda & Krainer, 1992; Cáceres et al., 1994; Yang et al., 1994; Wang & Manley, 1995). To address the possible splicing regulatory role of these proteins *in vivo*, we measured their relative abundances in HeLa cells by quantitative western blotting using specific mAbs. Known amounts of puri-

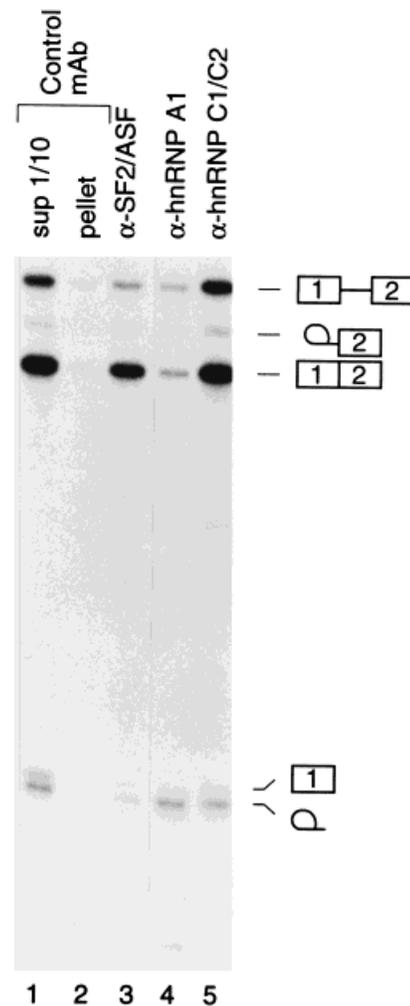


FIGURE 3. Immunoprecipitation of splicing reactions. Standard splicing reactions with ^{32}P -labeled β -globin pre-mRNA were incubated for 90 min and then rocked with monoclonal antibodies immobilized on protein G-Sepharose. Immunoprecipitates were washed and the RNAs extracted and analyzed by urea-PAGE and autoradiography. Lane 1, one tenth of the RNA recovered from the supernatant of an immunoprecipitation with the control mAb105 (against maltose-binding protein); RNAs recovered from the immunoprecipitates, using the anti-SF2/ASF mAb96 (lane 3), the anti-hnRNP A1 mAb9H10 (lane 4), and the anti-hnRNP C1/C2 mAb4F4 (lane 5). Structures and mobilities of the precursor, intermediates, and products of the splicing reaction are indicated on the right side.

fied HeLa SF2/ASF or hnRNP A1 proteins and crude lysates prepared from measured numbers of HeLa cells were loaded in parallel lanes of an SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes, which were probed with anti-SF2/ASF mAb96 (Fig. 4A) or anti-hnRNP A1 mAb9H10 (Piñol-Roma et al., 1988), respectively (Fig. 4B). mAb96 is highly specific for SF2/ASF and does not cross-react with other SR proteins that share significant sequence homology with SF2/ASF. It reacts with a single polypeptide band, or sometimes with a doublet in HeLa cell lysates or in purified SF2/ASF. The lower band is generated by partial dephosphorylation,

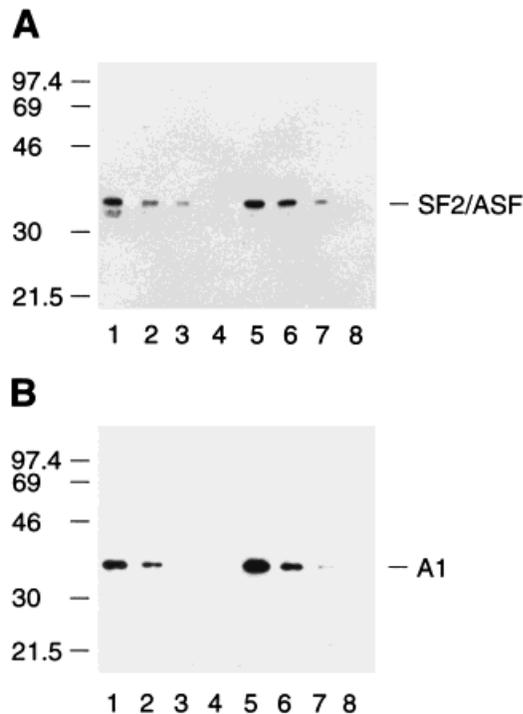


FIGURE 4. Abundance of SF2/ASF and hnRNP A1 in HeLa cells. Purified SF2/ASF or hnRNP A1, and HeLa cell lysates were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Blots were probed with mAb96 (A) or mAb9H10 (B) and the signals were detected by enhanced chemiluminescence. **A:** Lanes 1–4, 40, 20, 10, and 5 ng of purified human SF2/ASF plus 0.1 mg/mL carrier BSA. Lanes 5–8, total protein from 4×10^4 , 2×10^4 , 10^4 , and 5×10^3 HeLa cells. **B:** Lanes 1–4, 40, 20, 10, and 5 ng of recombinant hnRNP A1 plus 0.1 mg/mL carrier BSA. Lanes 5–8, total protein from 2×10^4 , 10^4 , 5×10^3 , and 2.5×10^3 HeLa cells. Mobilities of prestained molecular weight markers are indicated in kDa on the left side.

which probably occurs during sample preparation and/or storage, because this can be largely prevented by the inclusion of phosphatase inhibitors in the lysis buffer, or by lysis in hot SDS. mAb9H10 is also highly specific for hnRNP A1 and reacts predominantly with a single band in HeLa cell lysates (Fig. 4B). A less abundant form of lower mobility corresponds to hnRNP A1^B, which is generated by alternative splicing of hnRNP A1 pre-mRNA (Buvoli et al., 1990), but is not seen in HeLa cells with these short exposures (see below).

From the data in Figure 4 and from other comparable titration experiments, we conclude that 2×10^4 HeLa cells contain the equivalent of 30 ng of SF2/ASF, whereas 10^4 HeLa cells contain 37 ng of hnRNP A1. The molecular weights of SF2/ASF and hnRNP A1 are ~ 27.7 kDa and ~ 34.2 kDa, respectively; therefore, we estimate that a single HeLa cell grown under these conditions contains 3×10^7 SF2/ASF molecules and 6×10^7 hnRNP A1 molecules. The protein concentration of the purified SF2/ASF and hnRNP A1 preparations was determined using BSA as a standard, which may result in an overestimate of the actual protein con-

centration. Because both SF2/ASF and hnRNP A1 have large domains of low compositional complexity, which may have nonaverage dye-binding properties, we estimate that the above values are probably accurate only within a factor of 2–3. However, the value obtained for hnRNP A1 in HeLa cells is in excellent agreement with the previously reported value of 7×10^7 molecules per cell (Kiledjian et al., 1994).

The human and mouse SF2/ASF genes have been shown to encode at least three alternatively spliced isoforms (Ge et al., 1991; Tacke et al., 1992). The form first isolated on the basis of splicing activities is 248 amino acids long, and it was named SF2 or ASF-1 (Ge et al., 1991; Krainer et al., 1991). Two other cDNAs generated by alternative 3' splice-site use or by intron retention are designated ASF-2 (292 amino acids) and ASF-3 (201 amino acids). When these isoforms are synthesized in rabbit reticulocyte lysates, their SDS-PAGE mobilities correspond to approximately 40 kDa and 20 kDa, respectively (Ge et al., 1991). All three isoforms share the N-terminal 184 amino acids, and differ only in the C-terminal regions. Because mAb96 recognizes the N-terminal RRM, it should detect all the isoforms if they are expressed as stable proteins in vivo. In HeLa cells (Fig. 4A) and in other lines and tissues (see below), SF2/ASF was the only isoform expressed at detectable levels.

Tissue-specific expression of SF2/ASF, hnRNP A1, and SR proteins

A large number of pre-mRNAs are alternatively spliced in a tissue-specific manner, giving rise to isoforms with distinct structures and functions. The effects of SF2/ASF and hnRNP A1 on alternative splicing in vitro do not appear to be strongly substrate specific. We and others have shown that altering the ratio of SF2/ASF to hnRNP A1 causes drastic changes in splice-site utilization of several co-transfected splicing reporters, affecting different patterns of alternative splicing, such as alternative 5' splice-site selection and exon inclusion/skipping (Cáceres et al., 1994; Yang et al., 1994; Wang & Manley, 1995).

It is not known, however, whether SF2/ASF and hnRNP A1 regulate the in vivo alternative splicing patterns of large groups of genes or perhaps of only a few specific genes. Overexpression or disruption of related proteins in *Drosophila*, which in some cases lead to lethality or abnormal development, appear not to result in generalized alternative splicing defects, at least for the family members examined (Kraus & Lis, 1994; Ring & Lis, 1994; Zu et al., 1996). Tissue-specific changes in the levels or posttranslational modifications of either protein would be consistent with a regulatory role for these proteins in vivo. Systematic studies using monoclonal antibodies specific for hnRNP proteins have shown that the expression of major hnRNP proteins in

mouse (Kamma et al., 1995) and in rat (Faura et al., 1995) tissues varies among different tissues/cell types, but no studies so far have investigated the ratio of hnRNP/SR proteins.

For convenience, we analyzed rat tissues, rather than human ones. We expected to see cross-reactivity of rat proteins with mAb96 and mAb9H10, which were raised against human proteins, because of the exceedingly high conservation of these proteins. Rat and human hnRNP A1 proteins are 100% identical; conservative substitutions at two positions appear to be allelic, rather

than species specific (Buvoli et al., 1988). The sequence of rat SF2/ASF has not been reported, but human and mouse SF2/ASF proteins are 100% identical (Birney et al., 1993). Figure 5A shows western blots of equal amounts of total protein from 10 rat tissues probed with mAb96 (top) and mAb9H10 (middle). In the mAb9H10 blot, three bands of different molecular weights are visible, the lowest of which (34 kDa) is hnRNP A1. A1^B (38 kDa) is an alternatively spliced isoform of hnRNP A1, which has weaker alternative splicing activity (Buvoli et al., 1990; Mayeda et al., 1994;

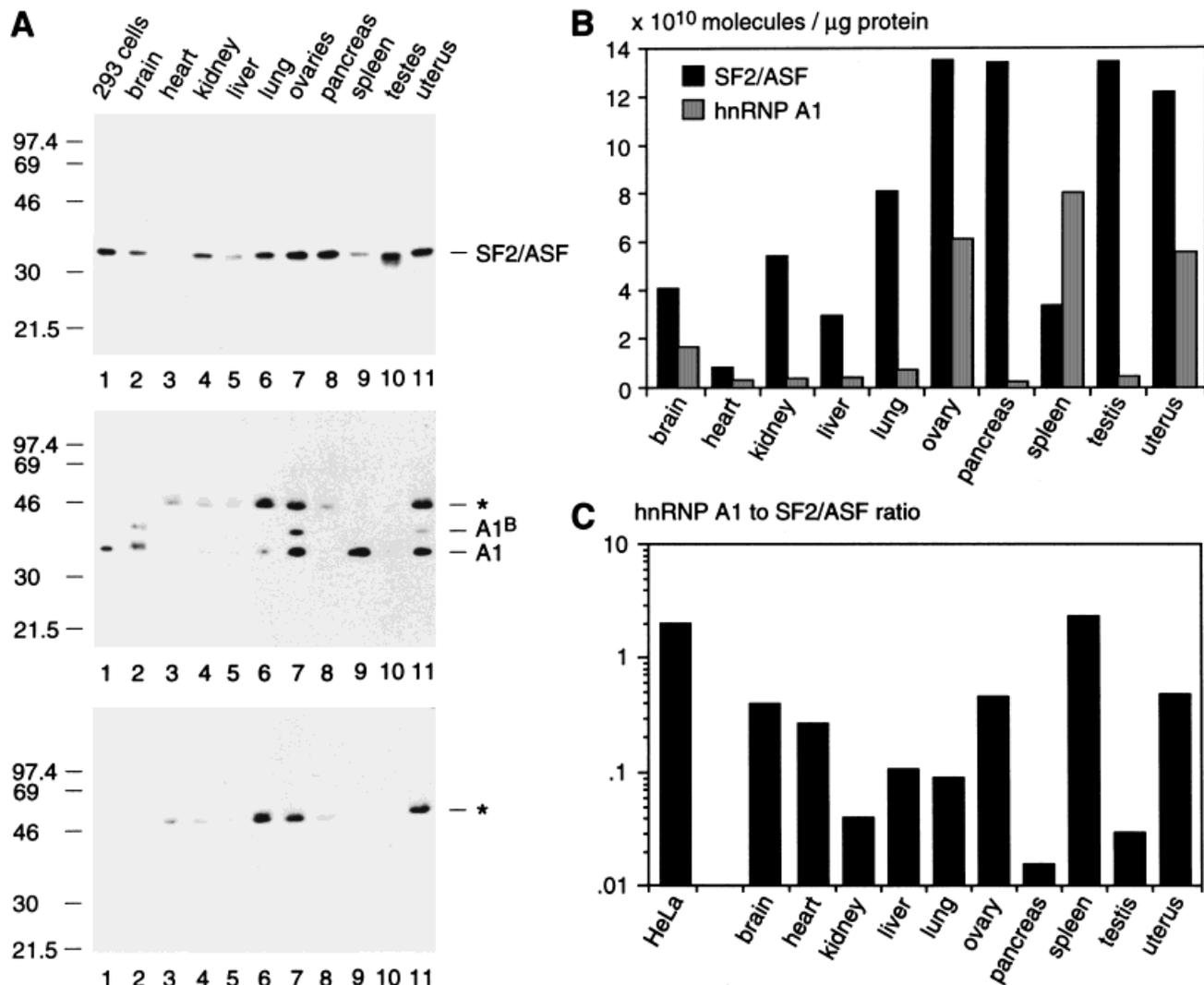


FIGURE 5. Differential expression of SF2/ASF and hnRNP A1 in rat tissues. **A:** Total proteins from 10 different rat tissues were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Top panel: 4 μg of total protein from each tissue was loaded and the membrane was probed with mAb96. Middle panel: 6 μg of total protein from each tissue was loaded and probed with mAb9H10. A1^B is an alternatively spliced isoform of hnRNP A1. Bottom panel: Samples were blotted and probed under the same conditions as in the middle panel, but omitting the primary antibody. Asterisk indicates a nonspecific band that cross-reacts with secondary antibody. **B:** Relative amounts of SF2/ASF and hnRNP A1 in rat tissues were determined by densitometric tracing of the immunoreactive bands on the films shown in the top and middle panels of A. The number of molecules of SF2/ASF or hnRNP A1 per microgram of protein in lung tissue (for SF2/ASF) or spleen tissue (for hnRNP A1) was extrapolated by comparison with known amounts of purified SF2/ASF or hnRNP A1 on the same blots (data not shown). Based on these values, the number of molecules of SF2/ASF and hnRNP A1 per microgram of protein in each tissue was calculated. **C:** Molar ratios of hnRNP A1 to SF2/ASF in each tissue (data from Fig. 5A) and HeLa cells (data from Fig. 4) are shown.

Tissue-specific ratio of antagonistic splicing factors

Yang et al., 1994). Interestingly, the ratio of A1^B to A1 proteins varies in different tissues (Fig. 5A). The band marked by an asterisk is due to cross-reactivity with the secondary antibody under the detection conditions optimized for mAb9H10 (middle and bottom). The intensity of each band was quantitated by densitometry. The number of SF2/ASF and hnRNP A1 molecules in 1 μ g of total extracted protein for each tissue was determined by comparison with purified standards, and is shown in Figure 5B. The level of SF2/ASF (black bars) varies widely among tissues, and reached as much as 0.6% of the total extracted protein. For example, it differs nearly 20-fold between heart and testis. In testis, bands of slightly higher mobility were seen, but we cannot distinguish whether these represent SF2/ASF that is underphosphorylated *in vivo*, or artifactual dephosphorylation during sample preparation. The mobility of SF2/ASF in other rat tissues is the same as in human 293 cells (Fig. 5A, lane 1) and HeLa cells (not shown). Therefore, in all of these tissues, SF2/ASF is highly phosphorylated, and no unphosphorylated protein was detected. The levels of hnRNP A1 (gray bars) also vary widely, with, e.g., an observed 45-fold difference between heart and spleen.

The observed differences in hnRNP A1 or SF2/ASF abundances between tissues may underestimate or overestimate the differences in the nuclear abundances of these proteins, because the concentration of nuclei varies considerably among different tissues (Kuehl, 1977), and normalization was on the basis of total protein (see Materials and Methods). However, the ratio of the two proteins is not affected by the normalization method. The calculated molar ratio of hnRNP A1 to SF2/ASF in each tissue is shown in Figure 5C. For example, in pancreas and testis, SF2/ASF is abundant, whereas hnRNP A1 expression is low, and the hnRNP A1 to SF2/ASF molar ratio in these tissues is roughly 0.02. In contrast, SF2/ASF is less abundant and hnRNP A1 is most abundant in spleen, with a molar ratio of 2.4. Greater than 100-fold differences in this ratio were observed among tissues. Because each organ is composed of multiple cell types, even greater differences between individual cell types may exist. In most tissues analyzed, SF2/ASF was present in a molar excess over hnRNP A1; an excess of hnRNP A1 was seen only in spleen and in HeLa cells.

In addition to SF2/ASF, the tissue-specific patterns of expression of other SR proteins were determined by western blotting with mAb3C5 (Fig. 6). Because this antibody recognizes a phosphoepitope whose sequence has not been defined precisely (Turner et al., 1985), the relative reactivities of different SR proteins are unknown. However, it is apparent from the data that each tissue expresses a characteristic pattern of SR proteins, of which some are ubiquitous (Fig. 6). This result is consistent with previous studies of bovine SR proteins using the mAb104 antibody (Zahler et al., 1993).

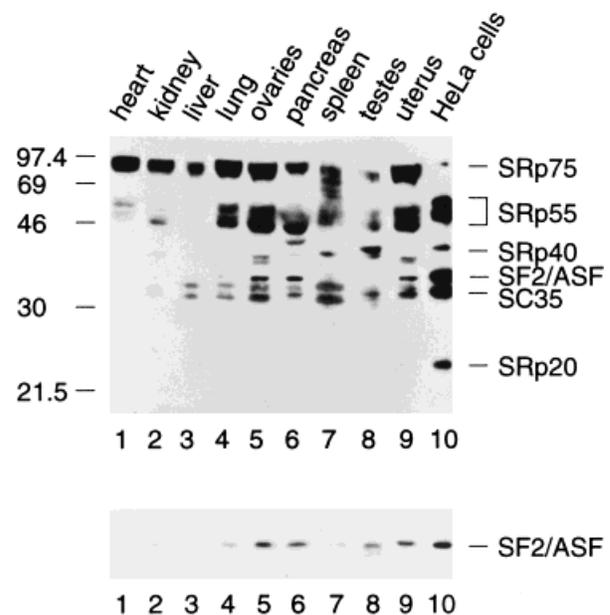


FIGURE 6. Differential expression of SR proteins in rat tissues. Rat tissue samples (6 μ g each) or 2×10^4 HeLa cells were analyzed by immunoblotting as in Figure 5, except that the filters were probed with mAb3C5, which recognizes a phosphoepitope shared by all known SR proteins. Bottom panel shows the same samples analyzed in parallel with mAb96. Because mAb3C5 but not mAb96 reactivity is lost upon dephosphorylation, the correspondence of the SF2/ASF signals in both panels is a measure of the phosphorylation state of the SR proteins in these samples.

The analysis of SR protein distribution with either mAb104 or mAb3C5 could give misleading results, if dephosphorylation occurs during sample preparation, which would lead to a reduction or loss of immunoreactivity. Comparison of the signals obtained in the same samples with mAb96 (Fig. 6, bottom panel) and the corresponding SF2/ASF band reacting with mAb3C5 (Fig. 6, top panel), suggests that little or no dephosphorylation of SF2/ASF—and by extension of other SR proteins—has occurred under the conditions used in the present study. One exception was in testes (Fig. 6, lane 8), for which SF2/ASF was not detected by mAb3C5, consistent with the slight increase in the mobility of SF2/ASF (see also Fig. 5A). In general, cell lysis in boiling SDS or in the presence of phosphatase inhibitors was essential to preserve 3C5 immunoreactivity and the correct electrophoretic mobilities (data not shown).

Effect of transformation on the hnRNP A1 to SF2/ASF ratio

There are numerous examples of changes in the patterns of alternative splicing of cellular pre-mRNAs as a result of oncogenic transformation: α -tropomyosin (Goodwin et al., 1991), fibronectin (Borsi et al., 1987; Zardi et al., 1987; Magnuson et al., 1991; Oyama et al.,

1993), the CD44 family of glycoproteins (Gunthert et al., 1991), and tenascin (Carnemolla et al., 1992), among many others. In addition, increased exon skipping of mRNAs encoding for tumor suppressor genes, such as the IRF-1 gene (Harada et al., 1994) and the WT1 gene (Haber et al., 1993), has also been associated with the transformed phenotype, and this could represent a novel mechanism of tumor suppressor inactivation. It is therefore likely that the machinery that regulates alternative splicing is modified during malignant transformation, leading to the production of particular protein isoforms that are associated with, and may be responsible for, the altered phenotype. As putative global regulators of alternative splicing, the SR and hnRNP proteins are excellent candidates for factors responsible for these extensive alterations in RNA processing patterns.

We measured the intracellular levels of SF2/ASF and hnRNP A1 in the rat embryo fibroblast cell line REF52 and in several derivative lines that were transformed by infection with adenovirus or SV40, or by transfection with cellular and viral oncogenes. These cell lines have been characterized extensively (Garrels & Franza, 1989a, 1989b). REF52 is a cloned immortal line of post-crisis Fisher rat embryo cells, and the T24 cell line was derived from REF52 by transformation with transfected adenovirus E1A and Ha-ras oncogenes (Franza et al., 1986). Cells were grown as monolayers and samples were prepared from exponentially growing cells (50–60% confluent) and from confluent cells (two days after confluence). As shown in Figure 7, the levels of SF2/ASF changed only very slightly between immortal and transformed cells (1.2-fold reduction between lanes 1 and 2). The levels of hnRNP A1 and A1^B both in-

creased fivefold, consistent with previous reports of increased hnRNP A1 expression in transformed and rapidly proliferating cells (Celis et al., 1986; Planck et al., 1988; Minoo et al., 1989; Biamonti et al., 1993; Zhang et al., 1997). This increase is comparable to the fourfold increase observed for PCNA (Fig. 7, lanes 5 and 6), a common marker for increased expression upon transformation (Garrels & Franza, 1989b). The overall increase in the hnRNP A1 to SF2/ASF ratio was calculated from the data in Figure 7 and from other measurements to be in the range of five- to sixfold. On the basis of *in vitro* experiments with model pre-mRNAs, a change of this magnitude would be expected to cause alterations in the alternative splicing patterns for at least some pre-mRNAs in the transformed T24 line.

SF2/ASF and hnRNP A1 levels during adenovirus infection

Many adenovirus transcripts are generated by alternative splicing. For example, the early region 1A (E1A) pre-mRNA is spliced into three predominant mRNA species termed 13S, 12S, and 9S mRNAs, through the use of three alternative 5' splice sites and a single 3' splice site (Berk & Sharp, 1978). The 13S and 9S mRNAs are spliced via the proximal and distal 5' splice site, respectively. The 13S mRNA predominates during the early stages of viral infection, whereas the 9S mRNA predominates during the late stages. There is evidence that virus-encoded proteins can directly alter the host splicing machinery to change the patterns of viral alternative splicing (Svensson & Akusjärvi, 1986; Delsert et al., 1989; Nordqvist et al., 1994). The selection of splice sites in E1A pre-mRNA has been shown to be modulated by changes in the abundance of hnRNP A1 or SF2/ASF both *in vitro* (Harper & Manley, 1992; Mayeda & Krainer, 1992) and in transfected cells (Cáceres et al., 1994; Yang et al., 1994; Wang & Manley, 1995). A decrease of SF2/ASF and/or an increase of hnRNP A1 during the early to late transition, at 6–8 h postinfection, might account for differential E1A splicing in virus-infected cells.

Seventy to eighty percent confluent HeLa cells were infected with adenovirus 2 (Ad2) at high multiplicity. Every 5 h after infection, cells were lysed and analyzed by western blotting (data not shown). Intracellular SF2/ASF levels increased slightly with time, up to 45 h postinfection. However, no significant differences were observed between mock-infected and Ad2-infected cells. Intracellular hnRNP A1 levels in mock-infected cells increased gradually during the first 20 h and then leveled off. In Ad2-infected cells, hnRNP A1 also increased gradually during the first 20 h postinfection, then decreased slightly. The ratio of hnRNP A1 to SF2/ASF did not differ significantly during the first 20 h between mock-infected and Ad2-infected cells. After 20 h, the ratio was slightly lower in infected cells. This is too late to

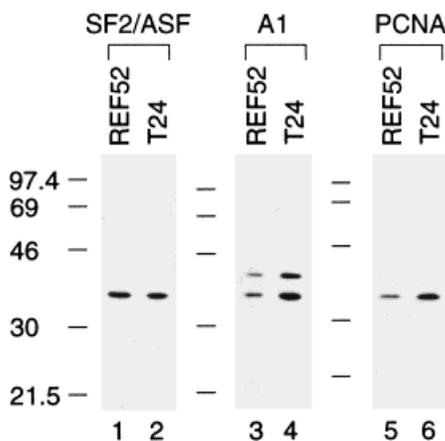


FIGURE 7. Differential expression of SF2/ASF and hnRNP A1 in immortal and transformed cell lines. REF-52 or T24 cells were grown to confluence and lysates from 5×10^4 cells were analyzed by western blotting. Identical pairs of samples were probed with mAb96 (anti-SF2/ASF), mAb9H10 (anti-hnRNP A1), or mAbPC-10 (anti-PCNA), as indicated. T24 is a REF-52 derivative transformed with ras and E1A oncogenes. These and similar data were quantitated using NIH Image software.

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account for the E1A early to late switch, and would be expected to promote the early rather than the late pattern of E1A splicing. No changes in electrophoretic mobility reflecting the state of phosphorylation were detected during the infection time course. Similarly, no significant changes in the levels or phosphorylation state of SR proteins were detected by western blotting with mAb3C5 (data not shown).

We conclude that a simple model involving changes in the abundance of SR proteins and/or hnRNP A1 does not account for the regulation of alternative splicing of adenovirus transcripts. However, recent experiments provided evidence that the transition from early to late patterns of alternative splicing is modulated by sequestration of SR proteins by adenoviral RNAs (Gattoni et al., 1991; Himmelspach et al., 1995). This mechanism does not involve changes in protein levels that can be detected by western blotting.

DISCUSSION

We developed monoclonal antibodies specific for the SF2/ASF splicing factor and used them to study the abundance of this SR protein in a variety of cell lines and animal tissues. In each case, we analyzed the ratio of SF2/ASF to its antagonist hnRNP A1, because *in vitro* splicing studies have suggested that it is the relative levels of these factors, rather than their absolute amounts, that determine the selection of alternative splice sites. We found that unphosphorylated recombinant SF2/ASF undergoes rapid phosphorylation when added to *in vitro* splicing reactions. We also found that highly or maximally phosphorylated SF2/ASF is the predominant steady-state form of the protein *in vivo*, and no unphosphorylated protein could be detected.

Both SF2/ASF and hnRNP A1 are extremely abundant proteins in the nucleus of exponentially growing HeLa cells. The nuclear concentration of these proteins can be estimated on the basis of: (1) the measured abundance of 3×10^7 SF2/ASF molecules per cell and 6×10^7 hnRNP A1 molecules per cell (Fig. 4); (2) the almost exclusively nuclear localization of both proteins at steady state (as seen by immunofluorescence and subcellular fractionation methods); and (3) an estimated diameter of 10 μm for the large, roughly spherical-shaped nucleus of a HeLa cell grown in suspension, corresponding to a volume of about 5×10^{-13} L. These calculations yield an estimated nuclear concentration of 0.1 mM (~ 3 mg/mL) for SF2/ASF and 0.2 mM (~ 7 mg/mL) for hnRNP A1. These very high concentrations suggest that most, if not all, pre-mRNAs, including those lacking high-affinity binding sites, can be bound by multiple SF2/ASF and hnRNP A1 molecules, at least in HeLa cells. This has been suggested previously to be the case for hnRNP A1 and other core hnRNP proteins (McAfee et al., 1997).

Antagonistic effects between SR proteins and hnRNP A/B proteins may serve to regulate alternative splicing events in living cells. This model is based on experiments showing that transiently increasing the levels of these proteins in HeLa cells can modulate the alternative splicing of co-transfected splicing reporters. For this model to hold in natural situations, one prediction is that individual SR and hnRNP A/B proteins should have unique tissue distributions and abundances. Most importantly, different ratios of these antagonists should be present in different *in vivo* settings.

We found that the abundances of both SF2/ASF and hnRNP A1 vary widely among rat tissues, and significantly, the molar ratio of hnRNP A1 to SF2/ASF showed variations of greater than 100-fold among certain rat tissues. Potentially even greater differences may exist among the individual cell types that make up these tissues. Such changes are expected to have profound consequences for alternatively spliced transcripts of genes that are expressed in these tissues, because a four- to eightfold change of the hnRNP A1 to SF2/ASF ratio is sufficient to elicit complete switches between alternative 5' splice sites *in vitro* (Fu et al., 1992; Mayeda & Krainer, 1992). However, it is not possible to predict at the present time which and how many specific cellular transcripts are spliced differentially in response to changes in the hnRNP A1 to SF2/ASF ratio. This, in part, is attributable to the fact that other family members, and potentially other families of splicing regulators with similar properties, must also have characteristic tissue-specific abundances. However, we believe that particular transcripts are alternatively processed preferentially in response to changes in the levels of particular sets of hnRNP A/B and SR proteins, with the necessary specificity arising combinatorially (Screaton et al., 1995).

Unique ratios of SR and hnRNP A/B proteins can be established in a tissue-specific or developmentally regulated manner by regulating the expression of their genes either at a transcriptional or posttranscriptional level. Analysis of the promoter regions of several SR genes has begun recently (reviewed in Cáceres & Krainer, 1997). It has been shown that genes coding for SR proteins, such as SC35, SRp20, and SRp40, are upregulated by mitogenic stimuli. This upregulation may reflect the requirement for increased RNA splicing as a result of an overall increase in gene expression during late G1 phase. Alternatively, those SR proteins whose expression is upregulated by mitogens may be involved in specific RNA processing events that may be required for cell cycle progression. Analysis of the hnRNP A1 gene revealed a complex promoter, consistent with the modulation of its expression in response to different cellular and physiological stimuli (Biamonti et al., 1993). The intracellular level of hnRNP A1 protein (and other hnRNP proteins) changes as a function of the proliferation state in certain cell types, and its

expression is also upregulated in transformed cell lines. For instance, hnRNP A2/B1 expression is highly increased in lung cancer cells compared to primary normal bronchial cells, and this increased expression of hnRNP A2 may serve as an early marker of lung epithelial transformation and carcinogenesis (Zhou et al., 1996). We observed that hnRNP A1 expression was significantly increased in REF52 cells that have been transformed with Ras and E1A (Fig. 7).

With respect to posttranscriptional control of SR protein gene expression, several SR protein genes express alternatively spliced mRNAs, and the corresponding cDNAs have been found in libraries prepared from various tissues (reviewed in Cáceres & Krainer, 1997). However, we found no evidence for the expression of the ASF-2 and ASF-3 isoforms of SF2/ASF as stable proteins *in vivo*. Therefore, it is likely that ASF-2 and ASF-3 mRNAs are generated as a way to downregulate the expression of SF2/ASF, but the presence of premature nonsense codons in these transcripts may render them unstable (for review see Maquat, 1995). The hnRNP A1 gene also encodes alternatively spliced isoforms, A1 and A1^B (Buvoli et al., 1990). Both isoforms were detected as stable proteins in this and in previous studies, although it has been shown that the A1^B isoform is much less active than the more abundant A1 isoform *in vitro* and *in vivo*, at least with the pre-mRNAs tested (Mayeda et al., 1994; Yang et al., 1994). The A1^B isoform arises from inclusion of an optional exon, resulting in an internal insertion within the C-terminal domain (Buvoli et al., 1990). The present analysis showed that the A1^B/A1 protein ratio is tissue specific (Fig. 5A). The hnRNP A/B proteins A2 and B1 are also isoforms encoded by a single gene; they differ by the inclusion of a short exon near the 5' end of the B1 mRNA (Burd et al., 1989; Biamonti et al., 1994). Both proteins have been detected *in vivo*, and so far it appears that both proteins have comparable activities in alternative splicing (Mayeda et al., 1994).

Additional levels of control of SR and hnRNP A/B protein expression or activity appear to occur at the posttranslational level. The SR proteins are phosphorylated *in vivo*, and, at least under certain conditions, this posttranslational modification is necessary for constitutive splicing activity *in vitro* (Cao et al., 1997; Xiao et al., 1997). The present study revealed, for the first time, that unphosphorylated SF2/ASF is not present at detectable levels *in vivo* in any of the cell types studied. Only highly or maximally phosphorylated SF2/ASF was detected; however, a higher-resolution analysis of the multiple phosphorylation sites will be required to determine if one or a few sites of phosphorylation are more dynamic, and to compare the activities of hypophosphorylated and/or hyperphosphorylated forms of the protein.

hnRNP A1 has also been shown to be phosphorylated *in vivo* (Cobianchi et al., 1993; Municio et al.,

1995). hnRNP proteins are nuclear proteins, but it has been demonstrated that a subset of them shuttles continuously between the nucleus and the cytoplasm (reviewed in Dreyfuss et al., 1993). We have recently shown that some, but not all, SR proteins also shuttle continuously between the nucleus and the cytoplasm (Cáceres et al., 1998). Nucleo-cytoplasmic trafficking of these RNA-binding proteins appears to be a regulated process; for example, modulation of hnRNP A1 shuttling is an early cellular response to genotoxic stress (J. Lozano, J.F. Cáceres, A. Monjas, M.T. Diaz-Meco, A.R. Krainer, & J. Moscat, *in prep.*). Likewise, overexpression of the Clk/Sty kinase causes a decrease in the steady-state levels of SF2/ASF in the nucleus, and an accumulation of this protein in the cytoplasm (Cáceres et al., 1998). This effect correlates well with the effect of Clk/Sty transient overexpression on alternative splicing of a co-transfected adenovirus E1A reporter gene (Duncan et al., 1997). Therefore, the regulation of nucleo-cytoplasmic shuttling of SR and hnRNP proteins may provide an additional mechanism for modulating the relative abundance of SR and hnRNP A/B proteins in the nucleus. It is therefore likely that signal transduction pathways can affect splicing through the regulation of phosphorylation and/or nucleo-cytoplasmic shuttling of splicing factors. Finally, the effective levels of SR proteins can be modulated through sequestration of these proteins by cellular or viral RNAs with high-affinity binding sites (Himmelspach et al., 1995).

In summary, a multiplicity of mechanisms appears to operate to control the levels and/or activities of specific SR and hnRNP A/B proteins in response to different physiological stimuli and in different cell types. Such changes provide the flexibility that would be required to allow these proteins to regulate the alternative splicing patterns of many different cellular or viral transcripts in a highly specific manner.

MATERIALS AND METHODS

Proteins

SF2/ASF and hnRNP A1 were purified from HeLa cells as described previously (Krainer et al., 1990; Mayeda & Krainer, 1992; Mayeda et al., 1993). Protein concentrations were measured by the dye-binding method (Biorad), with BSA as a standard. MalE-SF2/ASF fusion protein expressed in *E. coli* was purified by amylose affinity chromatography (New England Biolabs). Recombinant SF2/ASF and hnRNP A1 wild-type or mutant proteins were purified from *E. coli* as described (Krainer et al., 1991; Mayeda & Krainer, 1992; Cáceres & Krainer, 1993; Sreaton et al., 1995). Purified SC35 expressed in baculovirus-infected SF9 cells (Fu & Maniatis, 1992) was a generous gift from X.-D. Fu and T. Maniatis.

Antibodies

For preparation of anti-SF2/ASF mAbs, mice were immunized with malE-SF2/ASF fusion protein purified to apparent

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homogeneity. Standard procedures were used for immunization and for generating hybridomas (Harlow & Lane, 1988). Antibodies reacting with SF2/ASF were screened in pools by western blotting with mAbE-SF2/ASF and with partially purified HeLa SF2/ASF. mAb96 is IgG_{2b}, and mAb103 is IgG₁. Anti-hnRNP A1 mAb9H10 and anti-hnRNP C1/C2 mAb4F4 were kindly provided by G. Dreyfuss, and are both IgG. Anti-SR protein mAb3C5 was a generous gift from B. Turner. It recognizes a family of nuclear phosphoproteins through binding to a shared phosphoepitope (Turner et al., 1985). This is apparently the same specificity as that of mAb104 (Roth et al., 1991), as also found by others (Bridge et al., 1995). Both antibodies are IgM, but in our hands mAb3C5 is more sensitive in western blotting. Anti-PCNA mAbPC-10 (IgG_{2a}) was obtained from B. Stillman. Rabbit anti-DNA binding protein (DBP) antiserum was a kind gift from E. Moran. HRP-conjugated anti-mouse IgG or IgM secondary antibodies were purchased from Sigma.

Preparation of cell lysates

HeLa cells were grown in liquid suspension culture in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 5% calf serum. Exponentially growing cells were collected by centrifugation, suspended in phosphate buffered saline (PBS) containing 2 mM EDTA, and the cell density was measured using a hemocytometer. In the meantime, the cells were pelleted and immediately resuspended in boiling 2× SDS sample buffer [63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue] and boiled further for 7 min. REF52 and T24 cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum. Confluent cells were rinsed in PBS, resuspended, and counted on a hemocytometer, and separate aliquots were pelleted and immediately resuspended in boiling 2× SDS sample buffer and boiled further for 7 min. Equal numbers of REF52 and T24 cells were used for western blot analysis.

Adenovirus infection

HeLa cells were grown as monolayers in DMEM with 5% fetal bovine serum. Confluent cells (70–80%) were infected with adenovirus serotype 2 (Ad2) at a multiplicity of infection of 60–300. The medium was replaced after 1 h and the cells were incubated further at 37°C. Every 5 h after infection, the cells were rinsed with PBS, scraped in boiling 2× SDS sample buffer, and boiled further for 7 min. The efficiency of infection was checked at 24 h postinfection by indirect immunofluorescence using anti-DBP antiserum at 1:200 dilution and FITC-conjugated goat anti-rabbit IgG (Sigma) at 1:50 dilution. Under these conditions, nearly all the cells became infected.

Rat tissue samples

Rapidly frozen rat organs were obtained from Pel-Freez Biologicals. Organs were derived from adult (200–300 gram weight), mixed sex (except for ovaries, testes, and uterus) Sprague-Dawley rats. Each batch of a specific organ type was homogenized with a Polytron (Brinkmann Instruments)

in boiling lysis buffer [50 mM Tris-HCl, pH 6.8, 5% (v/v) glycerol, 0.5% (w/v) SDS, 150 mM NaCl, 2.5% (v/v) β-mercaptoethanol, 1 mM EDTA]. Insoluble material was removed by centrifugation. An aliquot of each supernatant was diluted 10-fold to reduce the SDS concentration to 0.05%, and its protein concentration was determined using Coomassie Plus reagent (Pierce) with BSA as a standard. The total protein concentration in each sample was then adjusted to 1 mg/mL by dilution in lysis buffer.

Western blotting

Samples were separated by SDS-PAGE and electroblotted onto 0.2-μm nitrocellulose membranes (Schleicher & Schuell) in 37 mM Tris base, 140 mM glycine in a semi-dry apparatus (Owl Scientific). For probing SF2/ASF, the membrane was first treated with 0.4% Tween 20/PBS and washed in PBS prior to blocking. The membranes were blocked with 10% nonfat dry milk in TBST (0.1 M Tris-HCl, pH 8, 1.5 M NaCl, 0.5% Tween 20), followed by incubation with anti-SF2/ASF mAb96 hybridoma supernatant at 1:10 dilution (although much greater dilutions are also effective) or anti-hnRNP A1 mAb9H10 ascites fluid at 1:1,000 dilution. After washing five times with TBST, the membranes were blocked again, incubated with HRP-conjugated anti-mouse IgG at 1:16,000 dilution, and washed a further five times. Immunoreactive protein bands were detected using the ECL system (Amersham) according to the manufacturer's instructions. To detect total SR proteins, the membranes were pre-blocked and blocked as above, except that 2–5% dry milk was used. The mAb3C5 hybridoma supernatant and HRP-conjugated anti-mouse IgM were used at 1:6 and 1:20,000 dilutions, respectively. The phosphatase inhibitors sodium fluoride and β-glycerophosphate (10 mM each) were added to the blocking solution and wash buffer. To detect PCNA, blocking was with 10% dry milk, the primary IgG_{2a} mAbPC-10 as ascites fluid was diluted 1/2,000, and the secondary anti-mouse IgG antibody conjugate was diluted 1/16,000.

Quantitation of western data

ECL signals were quantitated by densitometry of the films on a Molecular Dynamics model 110A laser scanner, using ImageQuant version 3.2 software. For the data in Figure 7, the films were scanned on a UMAX PS-2400x scanner at 300 dpi and quantitated with NIH Image version 1.6 software using the built-in logarithmic calibration.

Dephosphorylation of SR proteins

Dephosphorylation reactions were performed in 30 μL with 1 μg of purified SR protein in 0.3 mM ZnCl₂, 0.5 M Tris-HCl, pH 8.0, and 0.1 units of bacterial alkaline phosphatase (4.8 mg/mL, 31 units/mg; Sigma P4151) for 2 h at 65°C. For the mock reactions, the same volume of enzyme storage buffer [5 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 0.5 mM ZnCl₂, 50% (v/v) glycerol] was added instead of phosphatase.

Immunoprecipitation of spliceosomes

Splicing reactions with radiolabeled β-globin pre-mRNA were performed in a volume of 250 μL, including 60 μL of HeLa

nuclear extract. After incubation for 1.5 h, the reactions were diluted with 500 μ L of IP buffer [20 mM HEPES/Na⁺, pH 8, 150 mM NaCl, 0.05% (v/v) Triton X100] and incubated with antibodies pre-immobilized on protein G-Sepharose (10 μ L packed resin). The tubes were rocked for 1 h in the cold room and the bound material was washed four times with IP buffer. The pellet was suspended in 1 \times PK buffer (Sambrook et al., 1989) and the RNA was recovered by phenol extraction and ethanol precipitation.

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