We have previously described human (HsSWAP) and mouse (MmSWAP) homologs to the *Drosophila* alternative splicing regulator *suppressor-of-white-apricot* (*su(wa)* or DmSWAP). DmSWAP was formally defined as an alternative splicing regulator by studies showing that it autoregulates splicing of its own pre-mRNA. We report here that mammalian SWAP regulates its own splicing, and also the splicing of fibronectin and CD45. Using an *in vitro* system of cell transfection, mammalian SWAP regulated 5′ splice site selection in splicing of its own second intron. SWAP enhanced splicing to the distal 5′ splice site, whereas the SR protein ASF/SF2 enhanced splicing to the proximal site. SWAP also regulated alternative splicing of the fibronectin IICS region by promoting exclusion of the entire IICS region. In contrast, ASF/SF2 stimulated inclusion of the entire IICS region. Finally, SWAP regulated splicing of CD45 exon 4, promoting exclusion of this exon, an effect also seen with ASF/SF2. Experiments using SWAP deletion mutants showed that splicing regulation of the fibronectin IICS region and CD45 exon 4 requires a region including a carboxyl-terminal arginine/serine (R/S)-rich motif. Since R/S motifs of various splicing proteins have been shown to interact with each other, these results suggest that the R/S motif in SWAP may regulate splicing, at least in part, through interactions with other R/S containing splicing factors.

Recent studies have identified several genes important in regulating alternative mRNA splicing. Members of the SR protein family, characterized by an arginine/serine (R/S)-rich domain in the carboxyl terminus and an RNA-recognition motif in the amino terminus were originally defined by their activity complementing HeLa cell S100 extracts for constitutive splicing, but SR proteins also have distinctive activities in regulating alternative splicing (1–3). ASF/SF2 (4–6) and SC35 (7), the two SR proteins most completely characterized as splicing regulators, can regulate alternative 5′ and 3′ splice site selection (7) and exon inclusion/exclusion (8). SR proteins likely differentially activate splice sites due to different affinity interactions with either 5′ splice sites or exon enhancers (9) and may similarly affect 3′ splice site selection (10). In addition to interacting with RNA, SR proteins interact with other constitutive splicing factors containing R/S-rich domains, including U2AF and the U1–70K protein through the R/S-rich region of each protein (11, 12). Several heterogeneous nuclear ribonucleoproteins (hnRNPs)3 can also influence alternative mRNA splicing. hnRNP A1 directly antagonizes the effect of ASF/SF2 on 5′ splice site selection both *in vitro* and *in vivo*: ASF/SF2 (as well as other SR proteins) increases splicing to proximal 5′ splice sites, while hnRNP A1 increases splicing to distal 5′ splice sites (8, 13–15). In some cases it also antagonizes the effect of ASF/SF2 on exon inclusion/exclusion (8). PTB also has activity in regulating alternative mRNA splicing (16). It binds to certain poly(A) tract sequences and by competing with U2AF65 represses splicing to the associated 3′ splice site (16).

Several splicing regulators have been identified in *Drosophila* through the study of phenotypic mutants. Three of these, *sex-lethal* (*sxl*), *transformer* (*tra*), and *transformer-2* (*tra-2*) determine *Drosophila* sex through a cascade of regulated splicing events (17). *Sxl*, similar to PTB, inhibits the binding of U2AF to a proximal, high affinity 3′ splice site of *tra* by competitive binding to the poly(A) tract, forcing U2AF to bind to a distal, lower affinity poly(A) tract and resulting in a female pattern of *tra* splicing (18, 19). *Tra* and *tra-2* regulate splicing of the female-specific *dsx* exon through several cis elements (enhancers) in the exon, referred to as the *dsx* repeat element (dsxRE, a 13-base pair element repeated six times in the exon) and the purine-rich enhancer (PRE, an 18-base pair element repeated once). *Tra-2* binds to the PRE, and both *tra* and SR proteins bind cooperatively to the dsxRE, resulting in retention of the female-specific exon (20–22). Exonic enhancers have now been identified in many mammalian pre-mRNAs and appear to be common cis-acting elements important in splicing regulation (23–30).

The *Drosophila suppressor-of-white-apricot* (*su(wa)*) or DmSWAP) protein regulates splicing of the *white-apricot* allele of the *white* gene (31). Mutation of DmSWAP reverses inhibition of proper splicing of the *white* gene caused by the *white-apricot* mutation, which is the result of a *copia* insertion in the second *white* intron (32). DmSWAP also negatively regulates splicing of its own pre-mRNA through inhibition of splicing of the DmSWAP pre-mRNA first and second introns (31, 33). These mRNAs contain in-frame stop codons in the retained introns and code for nonfunctional proteins. No other pre-mRNA splicing has been shown to be regulated by DmSWAP. Autoregulation of splicing by DmSWAP is dependent on an amino-terminal R/S-rich motif, which is necessary for targeting the protein to the nuclear speckled compartment (34); however, DmSWAP does not have an RNA-recognition motif or other consensus RNA binding site and its mechanism of action remains obscure.

We have recently described human and mouse homologs of...
the DmSWAP gene, referred to, respectively, as HsSWAP and MmSWAP (collectively referred to as SWAP in this manuscript), and provided data suggesting that this gene may be a mammalian splicing regulator (35). The SWAP protein has several distinct regions that are highly homologous to DmSWAP, including an amino-terminal homology, repeated homologies in the center of the protein called spur modules, and an RS-rich region in the carboxyl terminus (35). The spur modules in SWAP are also found in the constitutive splicing factor SF3a120 (identical to SAP114), one of three proteins that make up SF3a, a trimeric complex that associates with the U2snRNP and is required for prespliceosome formation (36). In addition to these conserved structural motifs, the pattern of alternative splicing of mammalian SWAP pre-mRNAs is also conserved with DmSWAP. Namely, similar to DmSWAP, SWAP alternatively splices its first and second introns, producing at least two transcript isoforms in addition to the properly spliced form: one in which the first intron is retained and one in which the second intron is spliced to an internal 5′ splice site that is out of frame with the upstream coding region (35). These data showed the evolutionary relationship between mammalian and Drosophila SWAP genes and suggested that SWAP might regulate splicing in mammals. We show here that SWAP regulates splicing in vivo of minigene constructs, including alternatively spliced regions of itself and two other mammalian genes, fibronectin and CD45. The regulation of multiple splicing events by SWAP suggests that, like the family of SR proteins, SWAP may have broad activity in regulation of alternative splicing.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Plasmid pSWAP12exp was prepared by cloning the EcoRI fragment of pSWAP12 (full-length mouse SWAP insert (35)) into pSG5 (Stratagene). Plasmids pSF2 and pHnRNP1 were prepared by amplifying ASF/SF2 and hnRNP A1 from human HeLa cell cDNA by PCR using oligonucleotides tailed with EcoRI (5′ primer) or BamHI (3′ primer), digesting the amplified fragments with EcoRI and BamHI, purifying the fragments after separation on an agarose gel (GeneClean, Bio-101), and ligating the fragments into the EcoRI/BamHI site of pSG5. The oligonucleotides used for ASF/SF2 amplification were, 5′ primer: TGAATTCTGCACGCGCATGTC, and 3′ primer: ATGGATCC AATATCATTTATTTGACAGA; and for hnRNP A1 amplification, 5′ primer: GGCGATATCGTCTAAGTCAGA, and 3′ primer: 5′-ACCGATCTAATAGTCGCTAC-3′.

SWAP expression deletion mutants pSWAP12expΔP, pSWAP12expH, pSWAP12expΔP2, and pSWAP12expΔBHI were obtained by restriction enzyme digestion of pSWAP12exp with, respectively, PstI, HindIII, BstEI, and HindIII, and BstEI, gel purification and religation of the plasmid. For construction of pSWAP12expP2 and pSWAP12expΔBHI, digested plasmids were treated with Klenow prior to ligation to maintain the downstream region in-frame. Plasmids pSV-mini-LCA18 and α1WF/NIIHCS were provided by Dr. Michel Streuli (37) and Dr. Helen J. Mardon (38), respectively.

**Cell Culture, Transfection, and RNA Extraction**—COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD), and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine (culture medium). Cells were transfected at 70–80% confluence by the lipofectamine method (Life Technologies, Inc./BRL) with 25 μg of reaction buffer (Life Technologies, Inc./BRL), 2 μl of 25 mM MgCl2, 1 μl of 10 mM nucleotide triphosphates, and 2 μl of 0.1 M dithiothreitol and after a 5-min incubation at 42 °C, 1 unit of Superscript (Life Technologies, Inc./BRL) was added, and incubation continued at 42 °C for 50 min. The cDNA was then heated to 70 °C for 15 min and stored frozen.

For analysis of SWAP second intron splicing, reverse transcribed cDNAs were amplified by PCR using a 5′ primer overlapping the pSG5 vector and the SWAP second exon, 5′-GAGATTCCACGTGGAACAGAG-3′ and 3′ primer: 5′-ACCGATCTAATAGTCGCTAC-3′. The underlined regions of these two oligonucleotides are vector sequences; thus amplification was limited to SWAP mRNAs produced from the transfected SWAP minigene plasmid. 25-μl PCR reactions were composed of 2.5 μl of reaction buffer (Life Technologies, Inc./BRL), 8 μM MgCl2, 0.2 mM each nucleotide triphosphate (Pharmacia), 2.5 ng of each oligonucleotide, 0.625 units of Taq polymerase (Life Technologies, Inc./BRL), and 1 μl of template cDNA. Amplification conditions were: 96 °C, 45 s; 63 °C, 1 min; 30 cycles. For analysis of the fibronectin IIICS region, reverse

**FIG. 1. Autoregulation of splicing of the SWAP second intron.** A, diagram of the genomic region of SWAP, comprising the second intron and adjacent exons, cloned into pSG5 to produce pSWAPmini-G2. The pattern of alternatively spliced products, primers used for PCR, and resulting PCR product sizes are diagrammed below. B, RT-PCR products of alternatively spliced SWAP mRNAs from COS cells 2 days after transient co-transfection with pSWAPmini-G2 and either 3.0 μg or 1.0 μg of SWAP expression vector pSWAP12exp, or 3.0 μg of control plasmid pSG5. C, graph of alternatively spliced forms quantitated by scanning the photograph and densitometric analysis using NIH Image 1.5.2 software.
transcribed cDNAs were amplified using a 5’ primer in the fibronectin +1 exon, FN5–1: 5’-TTATGTCATTGCCCTGAAG-3’, and a 3’ primer in the downstream third exon of the human α1 globin gene found in the vector, FN3–1: 5’-AGCCAGAACTTGTCCAG-3’. Reaction mixtures and amplifications were identical as for amplification of SWAP transcripts except that reaction mixtures contained 2 mM MgCl2 and amplification conditions were: 96 °C, 30 s; 62 °C, 30 s; 30 cycles. For analysis of CD45, reverse transcribed cDNAs were amplified using a 5′ primer in the second exon, 45X2: 5′-CTTGGGACACGGCTGA-3′, and a 3′ primer oligo in the eighth exon, 45X8: 5′-CATAGGGACACGGCTGA-3′. Amplifications were as above except that reaction mixtures contained 6 mM MgCl2 and amplification conditions were: 96 °C, 45 s; 59 °C, 1 min; 30 cycles. All experiments shown of COS cell transfection and PCR are representative of at least three experiments showing similar results. Data were quantitated by scanning of photographs and densitometric analysis using NIH Image 1.5.2 software.

SDS-Polyacrylamide Gel Electrophoresis (PAGE), Immunoblotting, and in Vitro Transcription/Translation—Samples for SDS-PAGE were prepared from transfected cell cultures by direct lysis into 500 μl of 1.2 X PAGE sample buffer, or from in vitro transcription/translation products (produced using the TNT T7 coupled reticulocyte lysate system, Promega) by mixing 1:1 in 2 X PAGE sample buffer (2 X PAGE sample buffer: 0.125 M Tris, 20% (w/v) glycerol, 4.6% SDS). Samples were heated to 95 °C for 5 min, loaded on a 10% SDS-PAGE gel, and run at 40 mA for 3 h (39). For immunoblotting, proteins were transferred to nitrocellulose (Trans-blot cell, Bio-Rad; transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol) overnight at 15 mA. Nitrocellulose filters were blocked in TNT buffer (10 mM Tris, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) plus 5% low-fat milk for 1–3 h at 4 °C. Blotted proteins were incubated with the primary antibody (1:200 dilution of synovial fluid) diluted in TNT buffer plus 0.5% low-fat milk for 1–3 h, washed with TNT buffer 5 times for 5 min, incubated with the secondary antibody (goat anti-human IgG γ-chain specific/alkaline phosphatase, Zymed) diluted 1:1000 in TNT buffer plus 0.5% low-fat milk for 1 h at room temperature, and developed with 5-bromo-4-chloro-3-indoyl phosphate-toluidine salt/p-nitro blue tetrazolium chloride in developing buffer (100 mM NaCl, 5 mM MgCl2 100 mM Tris, pH 9.5).

RESULTS

Splicing Autoregulation of the Second SWAP Intron by SWAP—In order to determine whether SWAP functions as a regulator of alternative splicing, we developed a system to study its activity in cultured COS cells (14). The full-length MmSWAP cDNA was cloned into an eukaryotic expression vector and transiently transfected into COS cells along with one of several minigene constructs. The effect of SWAP overexpression on splicing of RNAs transcribed from the co-transfected minigene plasmid was assayed by RT-PCR using specific primers that permit selective amplification of the expressed minigene mRNAs. Autoregulation of DmSWAP splicing in Drosophila suggested that SWAP might autoregulate SWAP pre-mRNA splicing. Therefore, for initial studies a minigene expression plasmid containing the second SWAP intron was constructed as a target to study the effect of the SWAP protein on splicing of the second SWAP intron (diagrammed in Fig. 1A). This minigene construct (pSWAPmini-G2) was cloned by amplifying a genomic fragment of HsSWAP with primers from the second to the third exon.

The second SWAP intron is alternatively spliced using either of two 5′ splice sites. Transfection of pSWAPmini-G2 alone or with a control expression plasmid into COS cells produced two transcripts representing the two alternatively spliced forms of SWAP (Figs. 1, B and C, control lanes). A band representing unspliced SWAP RNA was also seen, suggesting relatively inefficient splicing of RNA transcribed from this construct. This band persisted after treatment of RNA samples with DNase before reverse transcription, confirming that this band represents unspliced RNA. Consistent with other studies, unspliced forms were not seen using a similar minigene plasmid that included the downstream 5′ splice site (data not shown). Although endogenous splicing of the SWAP second intron in COS cells could not be examined because the monkey cDNA sequence is not known, splicing of this intron in a variety of adult mouse tissues has shown that the alternatively spliced form 2A generally represents between 50 and 80% of the total transcripts.

FIG. 2. Regulation of SWAP second intron splicing by SWAP and ASF/SF2. A, RT-PCR products; and B, graphical representation of alternatively spliced SWAP mRNAs from COS cells 2 days after transient co-transfection with variable concentrations (0.5, 0.25, or 0.125 μg) of pSWAPmini-G2 and 3.0 μg of either SWAP expression vector (pSWAP12exp, lanes labeled SWAP), 3.0 μg of ASF/SF2 expression vector (pSF2, lanes labeled SF2), or 3.0 μg of control plasmid (pSG5, lanes labeled C).
spliced products. Basal splicing of the transfected pSWAP-mini-G2 therefore correlates approximately with the anticipated pattern of endogenous SWAP second intron splicing. Co-transfection of the SWAP expression vector with mini-G2 caused a shift in the pattern of splicing, favoring use of the upstream 5' splice site (form 2B). Form 2B increased from 16 to 27.2% of the total spliced products at one day and from 20.5 to 32.9% at 2 days after transfection (Fig. 1, B and C: control (C) compared to SWAP 3.0 mg). This effect is in contrast to that previously described for SR proteins, which generally enhance use of proximal 5' splice sites. In order to directly compare these two effects, ASF/SF2 was cloned by PCR into the same vector used for overexpression of SWAP. Co-transfection of this plasmid with mini-G2 caused the opposite effect as SWAP: use of the proximal 5' splice site was enhanced (Fig. 2, A and B). As seen before, overexpression of SWAP enhanced production of form 2B from 15.3 to 29% of the total spliced products (Fig. 2, A and B: control (C) compared to SWAP; 0.5 mg of transfected mini-G2). The effects were not dependent on the amount of transfected minigene construct (Fig. 2, A and B). hnRNP A1 has previously been reported to antagonize the effect of ASF/SF2 on 5' splice site selection and therefore might be expected to have an effect similar to the effect of SWAP. This cDNA was also cloned into the same expression vector and tested for activity on splicing of pSWAPmini-G2, however, no effect was seen (data not shown).

Splicing Regulation of the Fibronectin IIICS Region by SWAP and ASF/SF2—The SR family of proteins have been shown to have activity on a rather broad variety of target pre-mRNAs. In light of these observations, we decided to look at the potential for SWAP to regulate splicing of other alternatively spliced genes. We chose to study splicing of the IIICS region of the fibronectin gene because this region is alternatively spliced in a complex pattern (see Fig. 3A), increasing the likelihood of detecting an effect. In addition, this alternative splicing has well defined physiologic importance: the alternatively spliced 75 bp region at the 5' end of this exon encodes the high affinity binding site of fibronectin (CS1) for the $\alpha_4\beta_1$ integrin (or VLA-4).

A previously characterized minigene construct, $\alpha_{1w/IIICS}$, containing the fibronectin IIICS region (38) was transfected into COS cells with control, SWAP, or ASF/SF2 expression plasmids. Splicing of the fibronectin IIICS region was analyzed by RT-PCR using a 3' primer within the vector region so that only mRNAs produced from the transfected plasmid were amplified (diagrammed in Fig. 3A). Transfected fibronectin minigene construct without co-transfected plasmid (data not shown) or with control expression vector (Fig. 3, B and C) resulted primarily in the production of IIICS forms A and C, and also small amounts of form D (Fig. 3, B and C). Although forms B and C could not be distinguished by PCR because they are only 18 bp different in size, >90% of the amplified product at this size under basal splicing or after treatment with SWAP or ASF/SF2 represents form C as determined by restriction enzyme digestion of the PCR products and Southern blot (data not shown). Although qualitatively the forms of fibronectin mRNA produced using the $\alpha_{1w/IIICS}$ minigene construct are...
FIG. 4. Kinetics and dose-response of splicing by SWAP on fibronectin IIICS region splicing. A, RT-PCR products of alternatively spliced IIICS mRNAs from COS cells transiently co-transfected 1 or 2 days with 0.5 μg of α1w/FNIICS and 3.0 μg of SWAP expression vector (pSWAP12exp, lanes labeled SWAP), or 3.0 μg of control plasmid (pSG5, lanes C); C, RT-PCR products of alternatively spliced IIICS mRNAs from COS cells transiently co-transfected 2 days with 0.5 μg of α1w/FNIICS, and 3.0, 1.0, or 0.3 μg of SWAP expression vector (pSWAP12exp, lanes labeled SWAP), or 3.0 μg of control plasmid (pSG5, lane C). Total transfected DNA was kept constant by the addition of control expression vector, pSG5. B and D, graphs of alternatively spliced forms from A and C quantitated by scanning and densitometric analysis. E, RT-PCR products of alternatively spliced IIICS mRNAs from HT1080 cells transiently co-transfected 2 days with 0.5 μg of α1w/FNIICS and 3.0 μg of SWAP expression vector (pSWAP12exp, lanes labeled SWAP), 3.0 μg of control plasmid (pSG5, lanes C), or 3.0 μg of ASF/SF2 expression vector (pSF2, lane SF2).

Co-transfection of the SWAP expression vector with the fibronectin minigene construct resulted in the appearance of high levels of form E, a relatively decreased proportion of form A, and a relatively increased proportion of form D (Fig. 3, B and C). The effect of SWAP on the splicing of all these forms was relatively rapid and could be seen either 1 or 2 days after transfection (Fig. 4, A and B). The effect of SWAP on the splicing of form E was similar at 1 or 2 days after transfection, but its effect on forms C and D was greater at day 1 than day 2 after transfection (Fig. 4, A and B). The effect of SWAP on alternative splicing of all forms was dependent on the amount of transfected plasmid, and was barely detectable with less than 1.0 μg of transfected expression plasmid (Fig. 4, C and D).

Evolutionary sequence conservation suggests that several regions of the SWAP protein might be important for regulation of alternative splicing. To test the importance of different regions in splicing regulation by SWAP, a series of deletions were introduced into the SWAP expression vector, and the resulting plasmids co-transfected with the fibronectin minigene plasmid. Mutants deleted of the R/S region, pSWAPexp12ΔP and pSWAPexp12ΔH, had no effect on splicing as compared to control transfection; however, mutants that retained the R/S homologies were not necessary for SWAP regulation of fibronectin alternative splicing and suggest that the R/S region may be necessary.

In Vitro and in Vivo Expression of SWAP Deletion Mu-
In order to show that the deletion mutant plasmids were capable of making protein, each plasmid was used as a template for \textit{in vitro} transcription/translation and analyzed by SDS-PAGE. Full-length SWAP cDNA produces a protein of 180 kDa, which is much greater than the calculated molecular mass of 104.1 kDa (Fig. 6, A and B). The size of this protein is not affected by reduction (data not shown), and the same size protein is produced \textit{in vivo} after transfection of the plasmid into COS cells (Fig. 6B). The SR family of proteins also exhibit a markedly increased observed versus calculated \textit{M}_r, apparently due at least in part to phosphorylation of residues in the R/S region (40). Attempts at dephosphorylation of \textit{in vitro} transcribed/translated SWAP did not result in any shift in \textit{M}_r (data not shown). The difference in deleted SWAP proteins calculated versus observed molecular weights indicates that much of the increased observed \textit{M}_r is attributable to regions other than the R/S region (for example, compare the calculated versus observed \textit{M}_r of the protein resulting from \textit{in vitro} transcription/translation of pSWAP12expΔH, Fig. 6A). To further document that the mutant proteins were being produced in transfected cells, full-length SWAP expression plasmid and each of the mutant expression plasmids were transfected into COS cells, and after 2 days cell lysates analyzed by immunoblotting using a human synovial fluid that we have previously determined reacts primarily against epitopes in the region of the SWAP sup motifs (41). Full-length SWAP protein and the SWAPexpΔH mutant protein stained at comparable intensities.
Fig. 6. Showing that the lack of activity of this mutant is due to the deleted region and not due to differences in its level of intracellular expression. Very faint staining of the SWAPexpΔP mutant was also seen (band not visible after reproduction). Although the SWAPexpΔB mutant did not stain, this mutant had marked activity in altering splicing of the fibronectin minigene construct. Therefore, the lack of staining of SWAPexpΔB and possibly the low level staining of SWAPexpΔP is because the antibodies to SWAP in this synovial fluid require a region including the second surp homology and the adjacent carboxyl-terminal peptides for binding.

Splicing Regulation of CD45 by SWAP and ASF/SF—The potential for SWAP to regulate alternative splicing was further assessed with a CD45 minigene construct, pSV-mini-LCA18. CD45 is a membrane-associated tyrosine phosphatase that is expressed on the surface of all leukocytes and is required for T-cell activation through the T-cell receptor. Alternative splicing of CD45 generates several isoforms with different extracellular domains, involving three of the 33 exons of the CD45 gene. These regulated exons (exons 4, 5, and 6) can be included or skipped independently (42). CD45 antibodies designated CD45RA are directed against exon 4, which is found in two of the five isoforms identified in humans (43), and a switch in expression from CD45RA to CD45R0 is a marker for T-cell memory (44, 45). The cis elements responsible for the cell type specific splicing of CD45 exon 4 have been previously defined through deletion mapping of CD45 minigene constructs and reside primarily in the alternatively spliced exon 4. pSV-mini-LCA18 contains the genomic region between exons 2 and 8, deleted of large regions, including exon 3, exons 5–7, and adjacent intron regions (Fig. 7A). Splicing of pre-mRNA produced from this construct is regulated in a tissue-specific manner: B-cells include and thymocytes exclude exon 4 in the mRNAs produced from transfected pSV-mini-LCA18 (37).

Transfection of pSV-mini-LCA18 into COS cells alone or with control plasmid resulted in production primarily of the CD45 form containing exon 4 (form RA) and approximately 20% of the CD45 form excluding exon 4 (form R0; Fig. 7, B and C). Splicing of this construct in COS cells, which normally do not express this gene, therefore reflects a pattern intermediate between that found in B cells and thymocytes, but is closer to that found in B cells. Overexpression of SWAP or ASF/SF2 resulted in an in-
increase in the R0 form (Fig. 7, B and C), although neither completely switched splicing to the R0 form. The effect of SWAP on CD45 exon 4 exclusion was dose dependent and maximal (47% form R0) at 3.0 \( \mu \)g of co-transfected expression plasmid (Figs. 8, A and B). SWAP deletion mutants co-transfected with the CD45 minigene construct showed the same pattern of activity as that seen when co-transfected with the fibronectin minigene construct: constructs pSWAP12expH and pSWAPexp12AP had no activity, but pSWAP12expAB showed activity higher than that of the undeleted construct (Fig. 8, C and D).

**DISCUSSION**

We show here that SWAP is a mammalian splicing regulator that can apparently target a wide variety of alternatively spliced pre-mRNAs. It has splicing activity that in some instances opposes the effect of ASF/SF2. For example, whereas SWAP enhances utilization of the distal 5′ splice site in splicing of the second SWAP intron, ASF/SF2 enhances the proximal 5′ splice site. In the fibronectin IIICS region SWAP enhances the production of a form lacking an internal exon, but ASF/SF2 enhances inclusion of the entire IIICS region. However, in splicing of the CD45 exon 4 it has activity similar to ASF/SF2: both SWAP and ASF/SF2 enhance exon skipping.

**SWAP and SF2 Have Opposing Effects on SWAP Second Intron Splicing**—Both the mammalian and Drosophila SWAP genes employ autoregulated splicing of the second intron to control production of mRNAs that have a full-length open reading frame. Mammalian SWAP autoregulation affects 5′ splice site selection, while DmSWAP affects intron retention; but the effect in both cases is to promote splicing events that result in transcripts containing termination codons in the amino terminus of the encoded protein. Truncated SWAP proteins likely have no functional activity as is supported by experiments with deletion mutants pSWAP12expΔH and pSWAP12expΔP, which were unable to regulate splicing of fibronectin IIICS or of CD45 exon 4.

Regulation of SWAP splicing by ASF/SF2 suggests that, as for Drosophila sex determination, mammalian splicing regulators can modulate the splicing of other splicing regulators. However, in contrast to the cascade of splicing regulators involved in Drosophila sex determination, which appear to have specific downstream targets resulting in a specific phenotype, both ASF/SF2 (for example, see Ref. 3 and 8) and SWAP (this manuscript) appear to regulate a broad variety of alternatively spliced genes. For SR proteins this has led to the proposal that relative levels of the various SR proteins may determine splice site selection (3). The functional effect of increased ASF/SF2 expression is to increase SWAP transcripts properly spliced at the second intron due to increased splicing to the proximal 5′ splice site.

**SWAP Regulation of the Fibronectin IIICS Region**—The complex splicing of the fibronectin IIICS region involves several apparently independent splicing decisions. The response of this region to SWAP and ASF/SF2 suggests that one model for these decisions is: 1) 3′ splice site selection in splicing of the −1 exon to either the 5′ splice site of the 75-bp IIICS fragment (forms A and C), the 192-bp IIICS fragment (forms B and D), or the +1 exon (form E); and 2) retention (forms A and B) or deletion (form C and D) of the IIICS 93-bp internal exon fragment (refer to Fig. 3). This model of cell-type specific regulation of IIICS alternative splicing has been previously suggested on the basis of the tissue specific patterns of splicing (46). In this model SWAP would affect the first of these decisions by promoting use of the most distal 3′ splice site. An alternative model for the effect of SWAP on fibronectin IIICS splicing would be to view its primary effect as stimulating form E while inhibiting form C. In this model SWAP promotes skipping of the exon fragment.
that includes the 75-bp CS1 region and the adjacent 192-bp fragment. In either model, ASF/SF2 appears to affect a second splicing event, 2 as outlined above, by promoting form A over form C.

**SWAP and SF2 Enhance CD45 Exon 4 Exclusion**—In contrast to the differing effects of SWAP and ASF/SF2 on SWAP second intron and fibronectin IIICS splicing, both SWAP and ASF/SF2 enhanced exon 4 skipping of CD45. These data indicate that SWAP is not simply an antagonist of ASF/SF2, but that more complex interactions are responsible for its effect. ASF/SF2 has previously been shown to enhance exon inclusion (8, 14) by binding to purine-rich regions referred to as splicing enhancers within regulated exons (23, 24, 47). Binding to purine-rich regions by ASF/SF2 and other SR proteins is mediated by their RNA-recognition motifs, which appear to bind to similar or related sequences (24, 25). Purine-rich regions and other enhancer elements have been identified in a number of other alternatively spliced pre-mRNAs (23–30, 48, 49). Our data indicate that ASF/SF2 can also promote exon skipping, and that SWAP has a similar effect on CD45 exon 4. This negative splicing regulation could potentially occur through exonic silencer(s) similar to those recently described as regulating human immunodeficiency virus type 1 splicing (29, 30).

The Region Including the SWAP R/S Region Is Necessary for Splicing Regulation—Our data show that splicing of the fibronectin IIICS can be regulated by SWAP mutants deleted of a large central region of the protein, including both surp motifs and approximately half of the amino-terminal homology shared with DmSWAP. In contrast, deletion mutants lacking the carboxy terminus did not change the endogenous pattern of splicing. In particular, pSWAP Exp3H, which contains both surp homologies and the entire amino-terminal DmSWAP homology, and is expressed at levels similar to full-length SWAP in transfected COS cells, showed no activity in splicing regulation of fibronectin or CD45. Together these results indicate that the carboxy-terminal half of SWAP is necessary for splicing regulation. Since the R/S region is the only homology in the carboxy terminus conserved between the mammalian, insect, and nematode forms of SWAP (35, 51), this region is likely responsible, at least in part, for the observed effect on splicing.

Previous work has shown that the DmSWAP R/S region is necessary for regulation of alternative splicing by DmSWAP, but that its primary function is restricted to localizing DmSWAP protein to a speckled subnuclear compartment: the function of this region could be partially bypassed by its replacement with an SV40 nuclear localization sequence (34). In vitro work has shown that the R/S regions of ASF/SF2 are required for constitutive but not regulated splicing (52, 53), and that the R/S region in U2AF is also required for its constitutive activity (54). The work here supports a role for R/S regions in regulated as well as constitutive splicing. This might occur through interactions with the R/S regions of other splicing factors or through another undefined function of these regions.

In summary, we show that mammalian SWAP can regulate alternative splicing of several genes. It selectively activates splicing of a distal 5′ splice site and enhanced exon skipping.

This effect is mediated by a region that includes the carboxyterminal R/S motif that has been previously shown in other proteins to be important in mediating protein-protein interactions between splicing factors.

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