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Differential ASF/SF2 activity in extracts from normal WI38 and transformed WI38VA13 cells

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ABSTRACT

The normal human fibroblast cell line WI38 and a transformed derivative, WI38VA13, differentially splice fibronectin pre-mRNA *in vivo*. As a first step to understand the molecular basis for this regulation of splicing, we examined the ability of WI38 and WI38VA13 nuclear extracts to splice model adenovirus and globin pre-mRNAs. Adenovirus RNA splicing was detected in WI38VA13 but not in WI38 extracts. Likewise, when supplemented with a HeLa post-nuclear supernatant (S100), human β-globin RNA splicing was detected in WI38VA13 but not in WI38 extracts. The splicing defect in WI38 extracts was associated with a reduced ability to form splicing complexes and with a corresponding decrease in the interaction of U2 small nuclear ribonucleoprotein (snRNP) with the branchsite. These defects did not correlate with a decrease in 65 kD U2AF binding since equivalent U2AF level and activity were detected in WI38 and WI38VA13 extracts. Rather, WI38 extracts displayed reduced ASF/SF2 activity and contained a low level of 30 and 40 kD SR phosphoproteins. Moreover, addition of purified ASF/SF2 dramatically increased splicing complex formation in WI38 extracts. These results raise the possibility that variations in the level and activity of ASF/SF2 and other SR proteins play a role in the regulation of fibronectin splicing.

INTRODUCTION

It is now well documented that a large variety of primary transcripts generate different mRNAs by the selective use of splice sites. This widespread process, named alternative splicing, is often regulated developmentally or in a tissue-specific manner (for a review, see references 10 and 25). The interaction of *trans*-acting factors with sequences residing near or in alternatively spliced exons likely constitutes the molecular basis of this regulation. It remains to be established whether regulation in mammalian cells is achieved by cell-specific factors or by subtle changes in the relative levels of constitutive splicing factors. It was shown recently that an increase in the relative concentration of the essential splicing factor ASF/SF2 promoted proximal 5′ splice site utilization *in vitro* (8, 16). Conversely, the hnRNP A1 protein antagonized the action of ASF/SF2 by increasing the use of distal 5′ splice sites (20). More recently, ASF/SF2 was shown to belong to a family of nuclear phosphoproteins that include the splicing factor SC35 (7, 27). These results suggest that the precise balance in the activities of distinct generic splicing factors will be an important regulatory determinant in splice site selection.

To understand the regulation of alternative splicing by *trans*-acting factors, we investigated the splicing behavior of frequently used model pre-mRNAs in nuclear extracts made from a pair of human lung fibroblast cell lines (WI38 and WI38VA13) that differentially splice fibronectin pre-mRNA *in vivo*. WI38VA13, a SV40-transformed derivative of the WI38 cell line, incorporates alternative exons ED-A and ED-B at higher frequency than normal WI38 fibroblasts (1, 3, 31). Likewise, the complete exclusion of the IIICS region in fibronectin mRNAs occurs more frequently in WI38 than in WI38VA13 cells (11).

Here we report that WI38VA13 and WI38 extracts assemble and splice model pre-mRNAs with different abilities. WI38 extracts display reduced U2 snRNP binding but equivalent 65 kD U2AF binding when compared to WI38VA13 extracts. Furthermore, ASF/SF2 activity was reduced in WI38 extracts and supplementing with purified HeLa ASF/SF2 considerably improved complex formation. Our results suggest that variations in the level and activity of ASF/SF2 and related SR proteins may contribute to the regulation of fibronectin alternative splicing.

MATERIALS AND METHODS

Cell lines

HeLa cells were a gift of J.A.Steitz (Yale University) and were grown in RPMI1640 (GIBCO) supplemented with 10% fetal bovine serum. WI38 and WI38VA13 (subline 2RA) cell lines were obtained from ATCC and were grown in Dulbecco's modified Eagle medium, 5% fetal bovine serum.

Transcripts and *in vitro* splicing

pSPAd (a gift of J.A.Steitz) and pHβΔ6 (a gift of A.Krainer) were cut with *Hinc*II and *Acc*I, respectively, and transcribed with SP6 RNA polymerase (Pharmacia). Transcription reactions contained 100 μCi of [α-^{32}P]UTP. Reactions were performed and transcripts were isolated as previously described (5).

HeLa cell nuclear extracts and S100 extracts were prepared according to Dignam et al. (6). WI38 and WI38VA13 extracts

were prepared according to Lee et al. (19) with an approximate yield, from 30 plates (100mm), of 300 μl at protein concentration of 3−5 mg/ml. Splicing reactions were set up according to Krainer et al. (17). The progress of splicing reactions was followed by taking aliquots at various times. Labeled RNA molecules were fractionated on gels as described in Chabot and Steitz (5) for the human β-globin RNA and as described in Konarska and Sharp (13) for the adenovirus RNA.

Electrophoretic separation of splicing complexes

For each splicing reaction a 4 μl aliquot was removed from the reaction mixture and added to 1 μl of 1 mg/ml of heparin (Calbiochem). The mixture was kept on ice until all samples were collected and 0.5 μl of loading buffer (50% glycerol, 1% bromophenol blue, 1% xylene cyanol) was added. The samples were loaded onto 4% polyacrylamide gels (acrylamide/bis-acrylamide = 80:1) in 50 mM Tris-glycine which had been pre-electrophoresed at 190 volts for 30 min. Gel electrophoresis was performed at 190 volts for 3−4 hours at room temperature. Following ^{32}P-labeled RNP complexes separation, the gels were autoradiographed.

Immunoprecipitation/T1 RNase digestion

Mouse monoclonal anti-Sm (Y12) antibodies were obtained by ammonium sulfate precipitation of hybridoma supernatants (12). The hybridoma cell line was obtained from J.A.Steitz. T1 ribonuclease was purchased from Calbiochem. Conditions for immunoprecipitation, digestion and washes were as previously described (2, 4, 5).

Ultraviolet cross-linking and immunoblot assays

The UV cross-linking assay was performed as described in Zamore and Green (28). Substrate RNAs were synthesized in the presence of 5-bromouridine (BrUrd, Boehringer Mannheim) and 120,000 counts/minute (Cerenkov; ≈7.5 fmoles) were used for each assay.

Immunoblot analysis was performed by incubating nitrocellulose filters in buffer M (0.5% Tween-20, 5% dry milk, 130 mM NaCl and 40 mM Tris pH 7.4) with anti-U2AF antibodies (anti-pep D; 29) or directly in hybridoma supernatant (mAb104; 27). Following three 5 min washes in buffer M, antibody binding was detected with 1 μCi/ml of ^{125}I-Protein A (U2AF blot) or 1 μCi/ml of ^{125}I-labelled anti-mouse antibody (SR proteins blot) in buffer M for 1 hour. Filters were washed and exposed on X-ray films.

U2AF depletion

Small-scale U2AF depletion was performed using the procedure of Zamore and Green (29). Seventy-five μl of extract was adjusted to 1M KCl, 3mM $MgCl_2$, 0.05% NP40 and loaded on 0.15 ml (column volume) of poly(U)-Sepharose. The flow-through was collected and dialyzed against buffer B + 100mM KCl (29). The

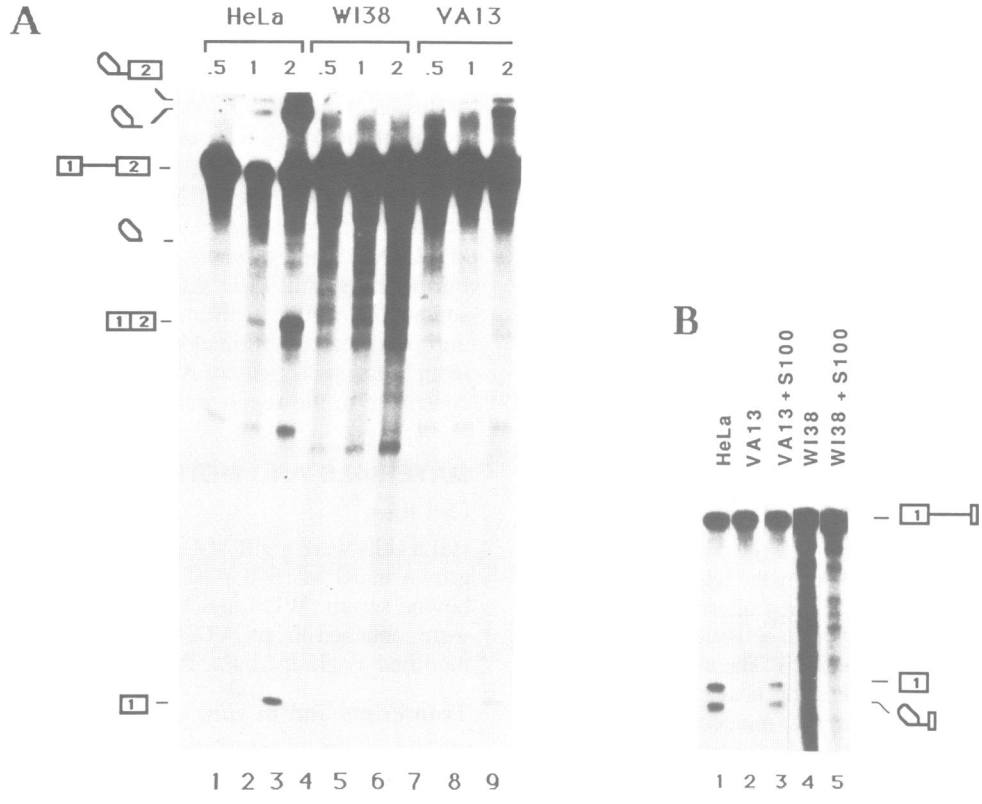

Figure 1. Adenovirus and human β-globin splicing in WI38 and WI38VA13 extracts. (A) The ^{32}P-labeled adenovirus substrate (Ad/*Hin*cII) was incubated for the indicated time (0.5, 1 or 2 hours) in 25 μl mixtures containing 20 μg (total protein) of HeLa extract (lanes 1−3), 25 μg of WI38 extract (lanes 4−6) or 25 μg of WI38VA13 extract (lanes 7−9). Reaction products (illustrated on the left) were analyzed in a 10% denaturing polyacrylamide gel. (B) The ^{32}P-labeled human β-globin substrate (HβΔ6/*Acc*I) was incubated for 60 minutes in mixtures prepared as in (A) or supplemented with 25 μg of HeLa S100 extract. Splicing products were separated on a 5% acrylamide/7M urea gel.

absence of U2AF from depleted extracts was confirmed by immunoblots. Complex formation assays containing purified U2AF of U2AF-depleted extracts were performed according to Zamore and Green (29).

RESULTS

In vitro splicing in WI38 and WI38VA13 extracts

WI38 and WI38VA13 nuclear extracts were examined for their ability to splice model pre-mRNAs. The first splicing substrate used, Ad, was an SP6-transcript that contains the first two exons of the adenovirus major late transcription unit and a shortened first intron (2). Fractionation of splicing intermediates and products on a denaturing gel indicated that the first step of splicing occurred in WI38VA13 extracts (Fig. 1A, lanes 7–9). The lariat intron was also detected but not the mRNA suggesting that the second step of the splicing reaction took place but that the released mRNA was unstable. In contrast to WI38VA13 extracts, splicing of Ad RNA was not detected at all in WI38 extracts (Fig. 1A, lanes 4–6).

The second transcript used was a truncated human β-globin pre-mRNA (Glo) which contains only 14 nucleotides (nt) as a second exon. In HeLa extracts, this substrate gave rise to the expected splicing intermediates but inefficiently proceeded through the second step of splicing (Fig. 1B, lane 1; see reference 23). Glo RNA was not detectably spliced in WI38VA13 extracts but supplementing these extracts with a HeLa S100 fraction promoted splicing (Fig. 1B, lanes 2 and 3). Due to extensive RNA degradation in this particular WI38 extract, Glo splicing could not be detected (Fig. 1B, lane 4). Addition of a HeLa S100 fraction alleviated transcript degradation but the mixture did not splice Glo RNA (Fig. 1B, lane 5). We also failed to detect the production of globin lariat molecules in different percentage acrylamide gels when we used WI38 extracts containing less nuclease activity or a β-globin pre-mRNA containing a longer second exon (data not shown). Thus, splicing was always less efficient in crude or S100-supplemented WI38 nuclear extracts than in equivalent WI38VA13 extracts.

Complex formation in WI38 and WI38VA13 extracts

To determine at which step pre-mRNA splicing was deficient in WI38 extracts, we investigated splicing complex formation using a mobility assay on native gels. Incubation of model pre-mRNAs in HeLa extracts yields complex A which contains U2 small nuclear ribonucleoprotein (snRNP) bound to the branchsite sequences (13). Complex B is formed next and contains U2, U4, U5 and U6 snRNPs (14). As shown in Figure 2A (lanes 4–9), both WI38VA13 and WI38 extracts assembled complex A on Ad RNA but the efficiency of complex A formation was reduced in WI38 extracts.

Complex formation on Glo RNA was next investigated. Although splicing complexes were assembled in HeLa and

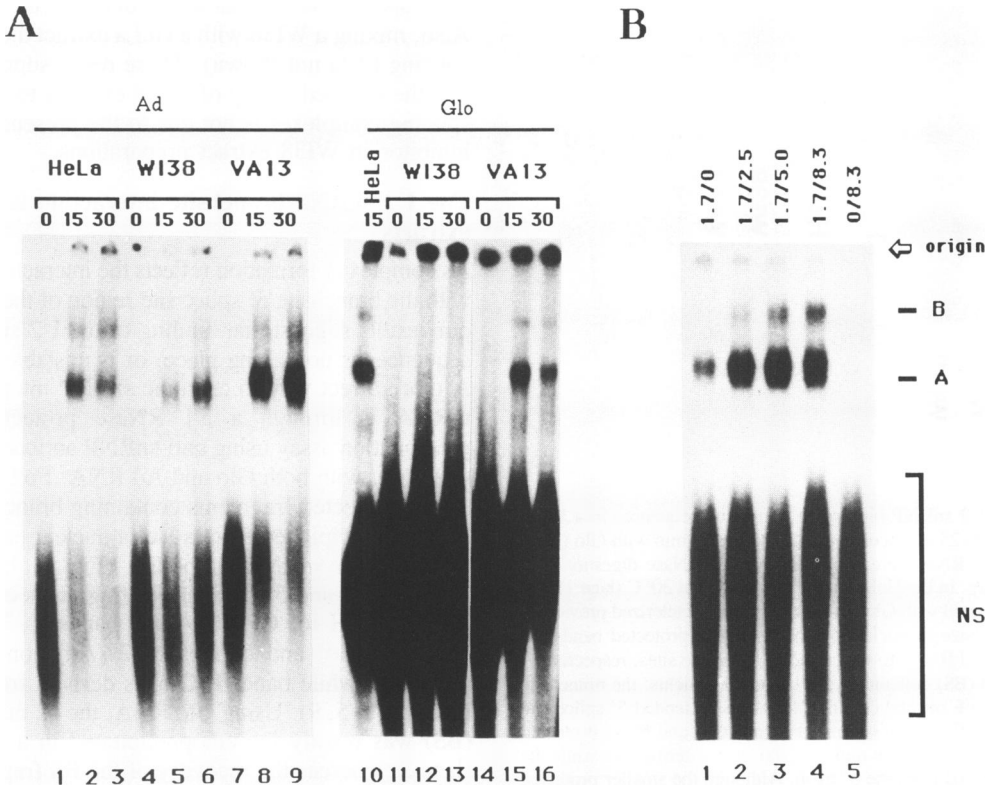

Figure 2. Splicing complex assembly in WI38 and WI38VA13 extracts incubated with adenovirus and human β-globin substrates. (A) Four μl aliquots were taken at the time indicated (0, 15 or 30 min) and processed as described in Materials and Methods from mixtures prepared as in Figure 1 legend and containing ^{32}P-labeled adenovirus (lanes 1–9) or β-globin (lanes 10–16) pre-mRNA. (B) WI38 extracts do not contain a factor that inhibits complex formation. In a separate experiment using different WI38 and WI38VA13 extracts, the indicated amounts (in μl) of WI38VA13 and WI38 extracts were mixed and incubated with adenovirus in 20 μl total reactions (lanes 1–5). For example, lane 4 contains 1.7 μl of WI38VA13 extract (8 μg total protein) and 8.3 μl of WI38 extract (27 μg total protein). The position of non-specific (NS) and A and B complexes are shown on the right.

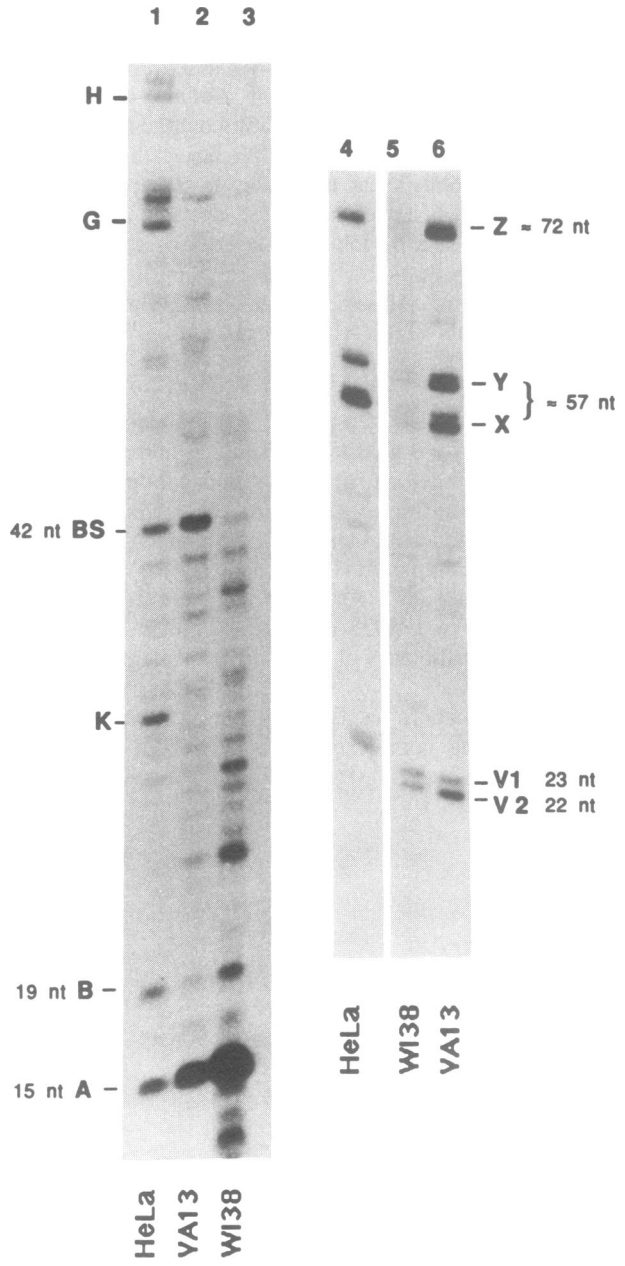

Figure 3. Reduction of U2 snRNP binding to branchsite sequences in a WI38 extract. Splicing reactions (25 μl) incubated at 30°C for 60 min with Glo (lanes 1–3) or Ad (lanes 4–6) RNA were subjected to a T1 RNase digestion/Y12 immunoprecipitation assay. In the HeLa mixture incubated at 30°C (lane 1), the protected fragments generated with Glo RNA have been characterized previously (2, 5). The position and size in nucleotides of the major protected bands are indicated. Fragments A and B map to the 5′ and the 3′ splice sites, respectively. The 42 nt protected band (BS) contains two protected fragments: the branchsite protected fragment (E, 90% of total counts [2]) and the extended 5′ splice site protected fragment (E', 10% of total counts). Fragments G and H are protected fragments derived from the branchsite region of the lariat intermediate while the K fragment (28 nt) is derived from the 5′ exon. Although the smaller protected fragments observed in lane 3 (WI38 extract) have not been characterized further in this experiment, their size are consistent with previously identified fragments mapping at the 5′ and 3′ splice sites (5). With Ad RNA, fragments V1 and V2 are derived from the 5′ splice site and contain the first 21 nt of the intron. The extremities of fragments X, Y and Z have not been mapped with precision but they all contain T1 oligonucleotides from to the branchsite, the 3′ splice site and the beginning of exon 2 (B.C. and J.A.Steitz, unpublished observations).

WI38VA13 extracts, most of Glo RNA was found in non-specific complexes (Fig. 2A, lanes 10, 14–16). This contrasted with Ad RNA which was majoritarily assembled into complex A in HeLa and WI38VA13 extracts (Fig. 2A, lanes 2, 3 and 8, 9, respectively). Thus, the difference in the efficiency of Glo and Ad RNA splicing was reflected in their respective ability to form splicing complexes. Stable globin complex A formation was dramatically low in WI38 extracts (Fig. 2A, lanes 11–13). WI38 extracts tested from over fifteen different extract preparation always displayed a lower ability to assemble complexes when compared to any WI38VA13 extract preparation. Some WI38 extracts yielded variable level of non-specific (NS) complexes for reasons that are unclear (for example see Fig. 6, lanes 1 and 7). However, in these extracts, the level of complex A formed remained equivalent. The difference in complex formation between WI38 and WI38VA13 extracts varied from 3-fold (Fig. 2A, compare lanes 6 and 9) to more than 15-fold (Fig. 2B, compare lanes 1 and 5; Fig. 6, compare lanes 7 and 9). Thus, the reduced ability of WI38 extracts to assemble splicing complexes is reproducible and suggests that WI38 extracts are deficient in a factor required for complex A formation.

The possibility that WI38 extracts contained an inhibitor interfering with complex assembly was ruled out by examining the effect of adding increasing amounts of a WI38 extract to a WI38VA13 extract (Fig. 2B, lanes 1–5). Addition of a WI38 extract did not reduce the ability of the WI38VA13 extract to form splicing complexes with Ad RNA. Rather, it stimulated complex formation suggesting that the WI38 extract provided some splicing factors that were limiting in the WI38VA13 extract. Also, mixing a WI38 with a HeLa extract did not prevent globin splicing (data not shown). These result supports the conclusion that the reduced ability of WI38 extracts to splice and assemble splicing complexes is not due to the presence of a non-specific inhibitor in WI38 extract preparations.

The U2 snRNP/branchsite interaction is reduced in WI38 extracts

As complex A formation reflects the interaction of several factors with the branchsite/3′ splice site region of the primary transcript, our results suggest that binding of the U2 snRNP to branchsite sequences is not taking place, or is unstable, in WI38 extracts. A more direct way to examine snRNP interactions with a pre-mRNA is through a T1 RNase protection and immunoprecipitation assay using anti-snRNP antibodies. We performed this assay with both Glo and Ad RNA. Following incubation at 30°C, protected fragments containing branchsite sequences are immunoprecipitated from a HeLa nuclear extract using anti-Sm or anti-(U2) snRNP antibodies (Fig. 3, lanes 1 and 4; see references 2 and 5). Branchsite fragments correspond to bands BS, G and H for Glo RNA and bands X, Y, Z for Ad RNA. Band A (Glo) and band V1/V2 (Ad) contains 5′ splice site sequences while band B (Glo) is derived from U5 snRNP/IBP binding (2, 5, 9). Using Glo RNA, the 42 nt protected fragment (BS) was readily immunoprecipitated in a WI38VA13 extract (lane 2) whereas the recovery of the BS fragment from a WI38 extract (lane 3) was reduced by a factor of 20. In contrast, the immunoprecipitation of fragments containing only 5′ or 3′ splice site sequences (band A and band B, respectively) was not diminished in WI38 extracts. Similarly with Ad RNA, the recovery of fragments containing branchsite sequences (bands X, Y and Z) was strongly reduced in a WI38 extract (> 10-fold

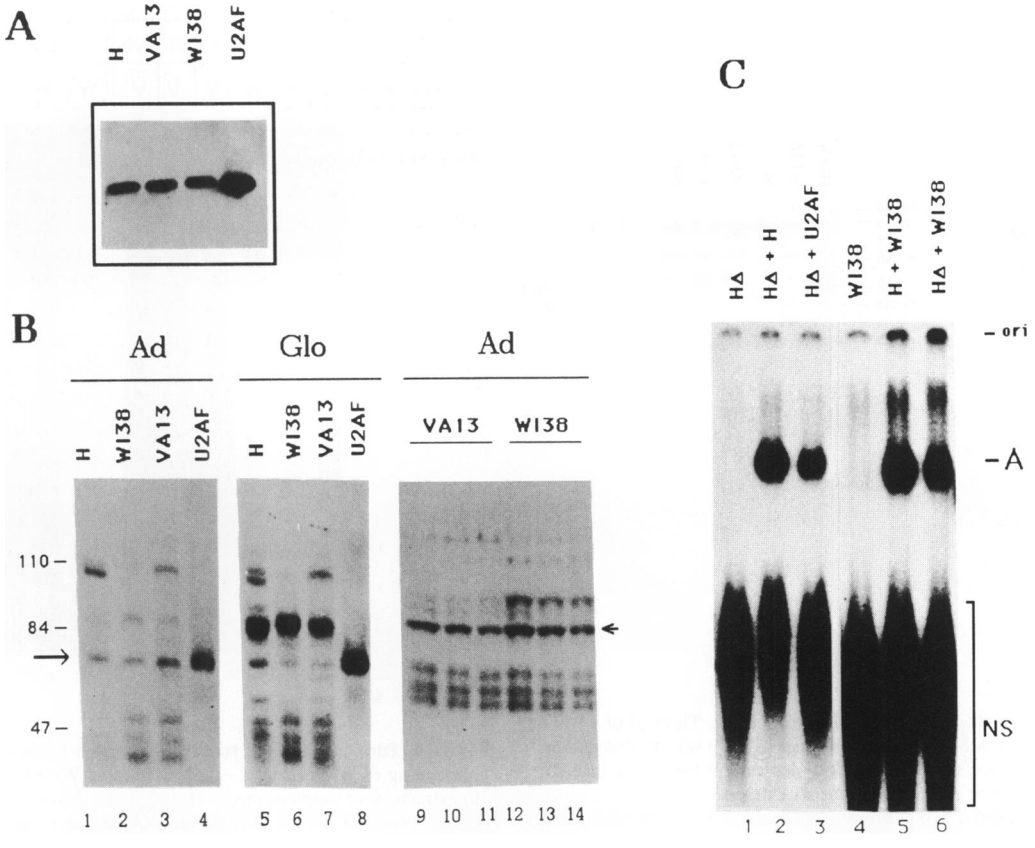

Figure 4. 65 kD U2AF levels and activity. (A) Immunoblot analysis using anti-U2AF antibodies. Five μg of HeLa, 7 μg of WI38VA13 and WI38 extracts and 2 μl of purified HeLa U2AF were run on a SDS/polyacrylamide gel. The proteins were transferred onto nitrocellulose and probed for U2AF proteins by using anti-65 kD U2AF antibodies (1:2700). (B) 65 kD U2AF binding. Cross-linking was performed with adenovirus (lanes 1–4 and lanes 9–14) and β-globin (lanes 5–8) BrUrd ^{32}P-labeled RNA substrates. Mixtures contained 0.5 μl of purified HeLa U2AF (lanes 4 and 8), 50 μg (total protein) of HeLa (lanes 1 and 5), 50 μg of WI38 (lanes 2 and 6) and 50 μg of WI38VA13 (lanes 3 and 7) extracts. The experiment presented in lanes 9 to 14 was performed with reduced amount of WI38 and WI38VA13 extracts; 30 μg (lanes 9 and 12), 15 μg (lanes 10 and 13) and 6 μg (lanes 11 and 14). Proteins were fractionated on a 9% acrylamide/SDS gel. Molecular weight markers are indicated. The position of the 65 kD U2AF band is indicated by an arrow. (C) Functional U2AF molecules in WI38 extracts. Ten μl reactions using the adenovirus pre-mRNA substrate and containing 10 μg (total protein) of HeLa nuclear extract depleted in U2AF (HΔ; lanes 1–3 and 6) were supplemented with 0.5 μl of HeLa extract (H; lanes 2), 1 μl of purified HeLa U2AF (lane 3), or 7 μg of WI38 extract (lane 6). Lane 4 represents a reaction set up with 7 μg of WI38 extract alone and lane 5 a reaction set up with 7 μg of WI38 extract plus 0.5 μl of HeLa extract. After an incubation at 30°C for 15 min, four μl aliquots were collected, adjusted to 250 μg/ml heparin and loaded on a native polyacrylamide gel as described previously. The position of A and non-specific (NS) complexes is indicated on the right.

decrease) whereas 5′ splice site binding was nearly unaffected (Fig. 3, lanes 4–6). These results strongly suggest that U2 snRNP binding to branchsite sequences is considerably and specifically reduced in WI38 extracts. Thus, the failure of WI38 extracts to efficiently assemble splicing complexes on model pre-mRNAs correlates with poor U2 snRNP binding to branchsite sequences.

Normal 65 kD U2AF levels and activity in WI38 extracts

Since U2 snRNP binding and complex A formation require U2AF activity (24), we wished to determine whether the differences in complex formation between WI38 and WI38VA13 extracts correlated with differences in U2AF levels and/or activity. U2AF activity co-purifies with two polypeptides of 65 kD and 35 kD (28). Although the 35 kD protein is not required for U2AF activity *in vitro* (29), the 65 kD protein is an essential splicing factor that tightly binds to the polypyrimidine tract/3′ splice site region of globin and adenovirus model pre-mRNAs (28–30). To determine the amount of 65 kD U2AF in our extracts, we performed an immunoblot analysis with anti-65 kD U2AF antibodies (kindly supplied by P.Zamore and M.Green). Since WI38 and WI38VA13 extracts contained similar amounts of 65 kD U2AF protein (Fig. 4A), reduced U2 snRNP binding in WI38 extracts is not simply due to a decrease in the level of 65 kD U2AF. To assess U2AF binding activity in WI38VA13 and WI38 extracts, a cross-linking assay was performed using BrUrd ^{32}P-labeled RNAs following a 10 min incubation at 30°C (28). In HeLa extracts, a 65 kD protein co-migrating with highly purified 65 kD U2AF polypeptide was efficiently labeled with Ad and Glo RNA (Fig. 4B, lanes 1, 4, 5 and 8). 65 kD U2AF binding to Ad and Glo RNA was detected in WI38 extracts and was not significantly different from the binding activity displayed by WI38VA13 extracts (Fig 4B, lanes 2, 3, 6, 7 and 9–14). Thus, the decrease in complex formation and in U2 snRNP binding does not correlate with a decrease in U2AF binding activity in WI38 extracts.

To further assess the function of U2AF molecules in WI38 extracts, we tested the ability of a WI38 extract to complement

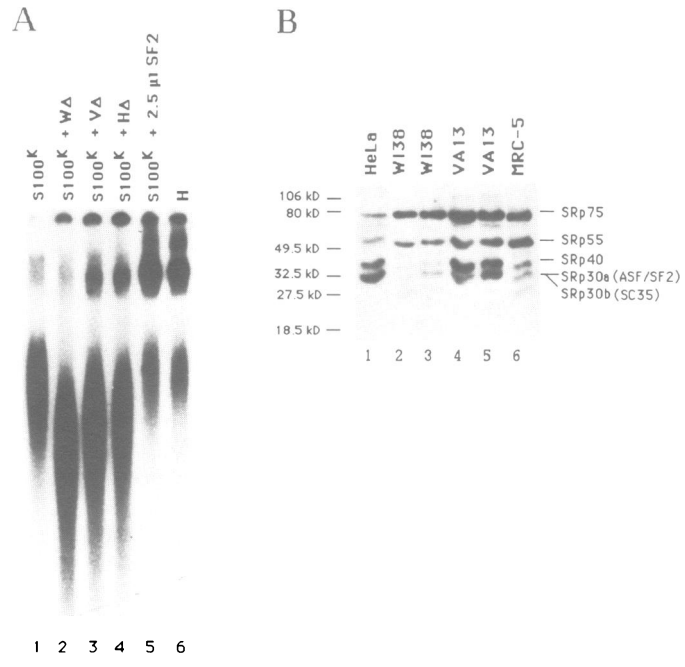

Figure 5. (A) WI38 extracts display low ASF/SF2 activity. Three µl of a HeLa S100 fraction provided by A.Mayeda and A.Krainer ($S100^K$) was incubated alone (lane 1) or in the presence of 3 µl of U2AF-depleted WI38 (WΔ; lane 2), WI38VA13 (VΔ; lane 3) or HeLa (HΔ; lane 4) extracts in 12.5 µl reactions. Control incubations included a $S100^K$ supplemented with 2.5 µl of purified HeLa ASF/SF2 (lane 5), and 3 µl of a HeLa extract (lane 6). Aliquots from the above incubations were processed for native gel analysis as described previously. (B) Detection of SR proteins using the mAb104 antibody (generously supplied by M. Roth). Twenty micrograms (total protein) of the indicated nuclear extracts were fractionated on a 10% acrylamide/SDS gel. The proteins were transferred onto nitrocellulose and probed for SR proteins using the supernatant of hybridoma producing mAb104 antibodies. Detection was performed using ^{125}I-labelled anti-mouse antibody from sheep (Amersham). Molecular weight markers and the position of the SR proteins are indicated.

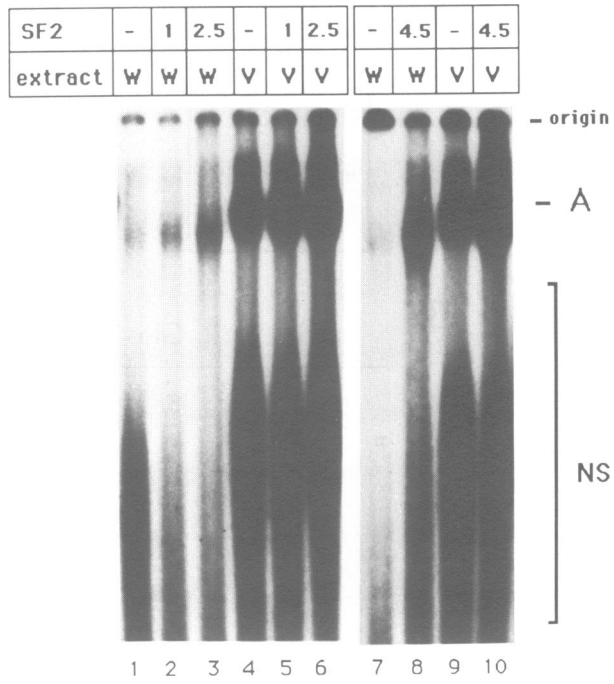

Figure 6. Purified ASF/SF2 stimulates complex formation in a WI38 extract. Fourteen µg of WI38 (lanes 1–3, 7 and 8) and WI38VA13 (lanes 4–6, 9 and 10) extracts were supplemented with 1, 2.5 or 4.5 µl of purified HeLa ASF/SF2 (kindly provided by A.Mayeda and A.Krainer), as indicated above the autoradiogram. The position of non-specific (NS) and A complexes are indicated on the right.

a HeLa extract depleted in U2AF (HΔ) by poly(U) chromatography (29). Whereas mixing a U2AF-depleted WI38 extract (WΔ) with a HΔ extract failed to stimulate complex formation (data not shown), a WI38 extract efficiently complemented a HΔ extract (Fig. 4C, lanes 1–6) indicating the presence of functional U2AF molecules in WI38 extracts. Complementation with purified HeLa U2AF (kindly provided by P.Zamore and M.Green) stimulated complex formation 5- and 2-fold in WI38 and WI38VA13 extracts, respectively (data not shown). We interpret this result as an indication that only minor differences in U2AF activity exist between WI38 and WI38VA13 extracts. Thus, U2AF is not responsible for reduced U2 snRNP binding and complex formation in WI38 extracts.

Reduced ASF/SF2 activity in WI38 extracts

The splicing factor ASF/SF2 is required for the assembly and/or the stabilization of the earliest detectable splicing complexes (15). Since purified HeLa ASF/SF2 rescues complex formation in a HeLa S100 fraction (15), we used the S100 complementation assay to evaluate the level of ASF/SF2 activity in WI38 and WI38VA13 extracts. U2AF-depleted WI38, WI38VA13 and HeLa extracts (WΔ, VΔ and HΔ, respectively) were added to a S100 extract (the HeLa S100 was generously provided by A.Mayeda and A.Krainer) and complex formation was monitored following incubation at 30°C. The level of complexes formed in these conditions should reflect the level of ASF/SF2 activity in each U2AF-depleted extracts. The results are shown in Figure 5A and indicate a very weak stimulation of complex formation with the WΔ extract (lane 2), whereas strong stimulation was observed with VΔ and HΔ extracts (lanes 3 and 4, respectively). We conclude that, in comparison to extracts from transformed HeLa and WI38VA13 cells, ASF/SF2 activity is low in extracts prepared from normal WI38 cells.

ASF/SF2 is identical to hSRp30a, a member of the family of SR phosphoproteins recognized by the monoclonal antibody mAb104 (27). Human members of this family also include the hSRp30b protein (identical to PR264 [26] and SC35 [7]) and three proteins of size 40, 55 and 75 kD. Gel-purified 30, 40, 55 and 75 kD proteins individually complement a HeLa S100 extract for splicing activity (21, 27). Each SR protein therefore displays the activity originally associated with ASF/SF2 in generic splicing (15). We used mAb104 antibodies (kindly provided by M.Roth) to examine the abundance of SR proteins in WI38 and WI38VA13 extracts. Immunoblot analysis revealed that, in WI38VA13 extracts, the abundance of the 30 and 40 kD proteins was equivalent to that of a HeLa extract, whereas the relative amount of the 75 and 55 kD proteins was increased considerably (Fig. 5B, compare lane 1 with lanes 4 and 5). In WI38 extracts (Fig. 5B, lanes 2 and 3), the 75 and 55 kD species were the most abundant species, although slightly reduced (less than 2-fold) when compared to WI38VA13 extracts. The reduction was more striking for the 30 kD and the 40 kD species. From the scanning of a film exposed without a screen, we estimate the extent of this reduction to be of the order of 15-fold for the 40 kD protein and 5-fold for the 30 kD species (comparing lane 3 with lane

5). Since the 30 kD band represents a mixture of SC35 and ASF/SF2 proteins, we ran a higher percentage acrylamide gel to separate these proteins. An immunoblot from this gel revealed equivalent diminution of both ASF/SF2 and SC35 proteins in WI38 extracts (data not shown). Thus, three SR phosphoproteins (hSRp40, SC35 and ASF/SF2) are present in reduced amount in WI38 extracts. We do not know whether this reduction reflects a decreased in the amount of SR proteins or is due to a dephosphorylation of the epitope recognized by mAb104. Because SR proteins are known to display ASF/SF2 activity in vitro (27), the reduced level of specific SR proteins in WI38 extracts is most likely responsible for the low ASF/SF2 activity associated with WI38 extracts.

Addition of ASF/SF2 stimulates complex formation in a WI38 extract

We obtained purified HeLa ASF/SF2 (kindly provided by A.Mayeda and A.Krainer) which rescued Ad complex formation in a HeLa S100 fraction (Fig. 5A, lane 5). We used ASF/SF2 to complement WI38 extracts deficient in complex formation. Addition of purified ASF/SF2 to WI38 extracts stimulated complex formation up to 25-fold (Fig. 6, lanes 1–3, 7 and 8), whereas addition of equivalent amounts of SF2 to WI38VA13 extracts improved complex formation by no more than 2.5-fold (lanes 4–6, 9 and 10). With the addition of 4.5 μl of ASF/SF2, complex formation in the WI38 extract (lane 8) nearly equalled that of the WI38VA13 extract (lane 9). This result is therefore consistent with the conclusion that ASF/SF2 activity is altered in WI38 extracts. We noted that the complex A in WI38 and SF2-supplemented WI38 extracts was slightly smaller than the complex A formed in a WI38VA13 extract, although a tendency toward equivalent migration is observed in supplemented WI38 extracts (e.g. Fig. 6, lane 8). Because the degree to which an RNA is retarded in a native gel often depends on the concentration of the limiting factor (e.g. see ref. 18), faster migration may simply indicate an unsaturating amount of ASF/SF2 in the reaction.

DISCUSSION

The results presented here clearly show that WI38 and WI38VA13 extracts differ in their ability to splice model pre-mRNAs. WI38VA13 but not WI38 extracts spliced an adenovirus pre-mRNA. Also, splicing of a human β-globin pre-mRNA was observed in S100-supplemented WI38VA13 extracts but not in S100-supplemented WI38 extracts. Further analysis showed that WI38 extracts manifested a limited ability to assemble splicing complexes and displayed reduced U2 snRNP binding. Reduction in complex formation was not due to the presence of an inhibitor and defective U2 snRNP binding could not be attributed to inadequate U2AF activity. On the other hand, the activity of the splicing factor ASF/SF2 was found to be considerably reduced in WI38 extracts suggesting that a defect in ASF/SF2 activity is responsible for reduced U2 snRNP binding and inefficient complex formation in WI38 extracts. Correspondingly, the reduction in ASF/SF2 activity in WI38 extracts was associated with a dramatic reduction in some of the SR proteins. Specifically, the levels of SRp40 and, to a lesser extent, ASF/SF2 and SC35 were reduced in WI38 extracts. The recent demonstration that individually purified SR proteins restored splicing when added to a S100 extract (21, 27) suggests that the reduction in the 40 and 30 kD SR proteins is directly responsible for the defect in complex formation in WI38 extracts. Consistent with this interpretation is our observation that the addition of purified ASF/SF2 strongly stimulated complex formation in WI38 extracts. Future experiments will address whether each SR protein can stimulate complex formation in WI38 extracts. Restoration of splicing with purified SR proteins will also be investigated to verify whether reduced ASF/SF2 activity is the only splicing defect in WI38 extracts.

Differences in ASF/SF2 activity between cell lines were previously reported for extracts prepared from HeLa and 293 cells (8). These differences correlated with splicing profiles observed *in vivo* with the SV40 early pre-mRNA (22). We do not know whether reduced ASF/SF2 activity in WI38 extracts is a true reflection of the situation in WI38 cells. We are currently addressing this question by examining the alternative splicing of specific pre-mRNAs in WI38 and WI38VA13 cells. Assuming a correlation between our *in vivo* and *in vitro* observations, a difference in ASF/SF2 activity between a normal cell line and its transformed derivative is intriguing. The mechanisms that regulate splicing decisions are often altered in transformed cells. For example, the alternate exons of fibronectin are incorporated more frequently in transformed (e.g. HeLa and WI38VA13) than in normal (e.g. WI38 and MRC-5) fibroblasts (1, 3, 11, 31). Interestingly, MRC-5 extracts also display a defect in complex formation (data not shown) and contain reduced amount of the 40 and 30 kD species (Fig. 5B, lane 6). In contrast, extracts from spontaneously transformed WI38 cells assemble splicing complexes as efficiently as WI38VA13 extracts (not shown). The noted differences in ASF/SF2 activity and the differential expression of SR proteins could provide a molecular basis for the regulation of fibronectin alternative splicing in WI38, MRC-5, WI38VA13 and HeLa cells. We now plan to investigate more directly the contribution of the individual SR proteins to the process of fibronectin pre-mRNA splicing *in vitro*.

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