UC Irvine
UC Irvine Previously Published Works

Title
The role of U2AF35 and U2AF65 in enhancer-dependent splicing.

Permalink
https://escholarship.org/uc/item/4rh2k3mp

Journal
RNA, 7(6)

ISSN
1355-8382

Authors
Graveley, BR
Hertel, KJ
Maniatis, T

Publication Date
2001-06-01

DOI
10.1017/s1355838201010317

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, availalbe at https://creativecommons.org/licenses/by/4.0/

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

RNA (2001), *7*:806–818. Cambridge University Press. Printed in the USA.
Copyright © 2001 RNA Society.

The role of U2AF^{35} and U2AF^{65} in enhancer-dependent splicing

BRENTON R. GRAVELEY,[1,2] KLEMENS J. HERTEL,[1,3] and TOM MANIATIS[1]

[1]Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA

ABSTRACT

Splicing enhancers are RNA sequence elements that promote the splicing of nearby introns. The mechanism by which these elements act is still unclear. Some experiments support a model in which serine-arginine (SR)-rich proteins function as splicing activators by binding to enhancers and recruiting the splicing factor U2AF to an adjacent weak 3′ splice site. In this model, recruitment requires interactions between the SR proteins and the 35-kDa subunit of U2AF (U2AF^{35}). However, more recent experiments have not supported the U2AF recruitment model. Here we provide additional evidence for the recruitment model. First, we confirm that base substitutions that convert weak 3′ splice sites to a consensus sequence, and therefore increase U2AF binding, relieve the requirement for a splicing activator. Second, we confirm that splicing activators are required for the formation of early spliceosomal complexes on substrates containing weak 3′ splice sites. Most importantly, we find that splicing activators promote the binding of both U2AF^{65} and U2AF^{35} to weak 3′ splice sites under splicing conditions. Finally, we show that U2AF^{35} is required for maximum levels of activator-dependent splicing. We conclude that a critical function of splicing activators is to recruit U2AF to the weak 3′ splice sites of enhancer-dependent introns, and that efficient enhancer-dependent splicing requires U2AF^{35}.

Keywords: recruitment; splicing enhancer; SR protein; U2AF

INTRODUCTION

The accurate removal of introns from pre-mRNA by RNA splicing requires specific recognition sequences located at the 5′ splice site, the branchpoint sequence (BPS), and the 3′ splice site (Burge et al., 1999). The latter includes a pyrimidine tract and AG dinucleotide. When these recognition sequences are a close fit to consensus they are efficiently recognized by the spliceosome, which is assembled in a stepwise manner on the intron. The initial events of spliceosome assembly include the binding of U1 small nuclear ribonucleoprotein particle (snRNP) to the 5′ splice site, and the U2 snRNP Auxiliary Factor (U2AF) to the pyrimidine tract. U2AF is a heterodimer consisting of 65- and 35-kDa subunits (Zamore & Green, 1989). The 65-kDa subunit (U2AF^{65}), which contacts the pyrimidine tract directly, contains an N-terminal arginine/serine (RS)-rich domain, a U2AF^{35} interaction domain, and three RRM-type RNA-binding domains (Zamore et al., 1992). U2AF^{35}, which contains a region with weak homology to an RRM-type RNA-binding domain (Zhang et al., 1992), has recently been shown to interact with the AG dinucleotide at the 3′ splice site (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999). Another protein, SF-1/mBBP, which recognizes the BPS, binds cooperatively with U2AF^{65} (Berglund et al., 1998). The U2AF/SF-1/mBBP complex then promotes the binding of U2 snRNP to the BPS, a process that involves direct interactions between U2AF^{65} and SAP155, a component of U2 snRNP (Gozani et al., 1998).

Deviation from the consensus recognition sequences results in a decreased affinity of the splicing machinery for the pre-mRNA (Smith & Valcárcel, 2000). For instance, U1 snRNP and U2AF bind weakly to nonconsensus 5′ and 3′ splice sites, respectively. Many introns that contain nonconsensus recognition sequences require additional RNA sequence elements, called splicing enhancers, for efficient splicing. Splicing enhancers are typically found within the exon downstream of the intron they act upon, and are recognized by serine/

Reprint requests to: Tom Maniatis, Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA; e-mail: maniatis@biohp.harvard.edu.

[2]Present address: Department of Genetics and Developmental Biology, University of Connecticut Health Center, School of Medicine, 263 Farmington Avenue, Farmington, Connecticut 06030-3301, USA.

[3]Present address: Department of Microbiology and Molecular Genetics, College of Medicine, B240 Medical Sciences I, University of California, Irvine, Irvine, California 92697-4025, USA.

arginine (SR)-rich proteins (Tacke & Manley, 1999; Blencowe, 2000; Graveley, 2000). SR proteins contain an N-terminal RNA-binding domain and a C-terminal RS domain that functions as a protein interaction domain. SR proteins bound to splicing enhancers have been shown to function by stimulating spliceosome assembly (Staknis & Reed, 1994; Zuo & Maniatis, 1996), although the precise mechanism by which they act is unclear.

Several studies support a mechanism whereby SR proteins bound to splicing enhancers interact with U2AF^{35}, thereby recruiting U2AF^{65} to the weak 3′ splice site (reviewed in Blencowe, 2000; Graveley, 2000). First, protein interaction assays have shown that certain SR proteins directly contact U2AF^{35} (Wu & Maniatis, 1993). Second, many regulated introns contain nonconsensus, or weak, pyrimidine tracts and therefore interact weakly with U2AF^{65} in vitro (Burtis & Baker, 1989; Tian & Maniatis, 1994; Lorson & Androphy, 2000). Thus, it is reasonable to postulate that U2AF^{65} binding to the pyrimidine tract could be the limiting step in spliceosome assembly on pre-mRNAs with nonconsensus pyrimidine tracts. Third, splicing enhancers have been shown to stimulate the binding of U2AF to the pre-mRNA in nuclear extracts (Wang et al., 1995; Zuo & Maniatis, 1996; Bouck et al., 1998). Fourth, purified recombinant SR proteins stimulate the binding of U2AF^{65} to pre-mRNA, and this binding requires U2AF^{35} (Zuo & Maniatis, 1996). Fifth, the RS domain of U2AF^{35}, which is required for interaction with SR proteins, has also been shown to be required for maximum levels of enhancer-dependent splicing of U2AF^{65} in vitro (Zuo & Maniatis, 1996). Finally, U2AF^{35}, in addition to U2AF^{65}, was required to restore splicing of the mouse IgM M1-M2 pre-mRNA in U2AF-depleted extracts (Guth et al., 1999). These studies all support a model in which enhancer-bound SR proteins interact with U2AF^{35} and recruit U2AF^{65} to the weak pyrimidine tract.

However, more recent studies are inconsistent with the U2AF recruitment model (reviewed in Blencowe, 2000; Graveley, 2000). First, the binding of U2AF^{65} to the pyrimidine tract of IgM and *dsx* pre-mRNAs did not differ in the presence or absence of an enhancer, even though splicing was not observed without the enhancer (Kan & Green, 1999). A second study concluded that U2AF^{65} binding is unaffected by the presence or absence of an enhancer (Li & Blencowe, 1999). However in this case, the assays employed did not directly measure binding of U2AF^{65} to the pyrimidine tract (Li & Blencowe, 1999). Second, although Guth et al. (1999) found that U2AF^{35} was required for IgM splicing, they also found that U2AF^{65} binding was unaffected by the presence or absence of U2AF^{35}. However, this study did not examine whether U2AF^{65} binding was affected by a splicing enhancer (Guth et al., 1999). Thus, in these studies, U2AF^{65} binding did not correlate with splicing activity, suggesting that some other step in splicing is enhancer dependent. In fact, the IgM pre-mRNA was found to contain a splicing inhibitor element downstream of the splicing enhancer (Kan & Green, 1999). This led to the proposal that the IgM splicing enhancer functions by overcoming the splicing inhibitor, rather than by recruiting U2AF.

Although many of the experiments described above support an important role for U2AF^{35} in enhancer-dependent splicing, other experiments are not consistent with such a role. First, U2AF^{65} was found to be sufficient to complement the splicing of the IgM pre-mRNA in U2AF-depleted extracts, suggesting that U2AF^{35} is not required for IgM splicing (Kan & Green, 1999). Second, although U2AF^{35} is an essential gene in *Drosophila*, deletion of the RS domain of *Drosophila* U2AF^{35} does not affect the splicing of the enhancer-dependent *dsx* pre-mRNA in transgenic flies (Rudner et al., 1998b). Thus, the precise function of U2AF^{35} in enhancer-dependent splicing is unclear.

Based on these apparent discrepancies we have re-examined the questions of whether U2AF binding is promoted by splicing enhancers and whether U2AF^{35} plays a role in enhancer-dependent splicing. Here we show that the IgM pre-mRNA requires a splicing enhancer for efficient splicing even in the absence of the splicing inhibitor, and that improving the pyrimidine tracts of both the *dsx* and IgM pre-mRNAs relieve the requirement for a splicing enhancer. In addition, we find that splicing activators stimulate the binding of U2AF^{65} and U2AF^{35} to both the *dsx* and IgM 3′ splice sites under splicing conditions. Finally, we show that U2AF^{35} is required for the efficient reconstitution of enhancer-dependent splicing in U2AF-depleted extracts. Together, these data support the view that at least one function of splicing activators and enhancers is to recruit U2AF to weak 3′ splice sites, and that U2AF^{35} plays an important role in enhancer-dependent splicing.

RESULTS

Pyrimidine tract up mutations relieve the requirement for a splicing enhancer

Regulated introns typically contain nonconsensus 3′ splice site pyrimidine tracts that are interrupted by purine bases. Previous studies have shown that increasing the pyrimidine content of the *dsx* pyrimidine tract can relieve the requirement for a splicing enhancer (Hoshijima et al., 1991; Tian & Maniatis, 1994). By contrast, similar experiments with the IgM pyrimidine tract revealed that replacing the weak IgM pyrimidine tract with a consensus U2AF binding site did not relieve the requirement for a splicing enhancer (Kan & Green, 1999). However, these experiments are complicated by the fact that they were performed in the presence of the IgM splicing inhibitor (Kan & Green, 1999). The

splicing inhibitor is a dominant element, because it can block the splicing of the constitutively spliced β-globin pre-mRNA (Kan & Green, 1999). Thus, the presence of the splicing inhibitor in the IgM pre-mRNA would be expected to mask the effect of the improved pyrimidine tract on the requirement for the splicing enhancer.

To test this possibility, we examined the effect of splicing activators on IgM pre-mRNA lacking the splicing inhibitor, but containing the wild-type or improved pyrimidine tract. To accomplish this we used a MS2-RS hybrid activator protein system (Graveley et al., 1998; Graveley & Maniatis, 1998). Specifically, the normal splicing enhancer of an enhancer-dependent pre-mRNA is replaced by a binding site for the bacteriophage MS2 coat protein. Hybrid proteins consisting of the MS2 bacteriophage coat protein and the RS domain of an SR protein can bind to the MS2 site on these pre-mRNAs and activate splicing in vitro. The ability of the MS2-RS proteins to activate splicing or promote spliceosome assembly is completely dependent on the presence of an MS2 binding site in the pre-mRNA substrate (Graveley & Maniatis, 1998; B.R. Graveley, unpubl. data). Thus, the MS2 binding site can functionally replace a splicing enhancer (Graveley et al., 1998; Graveley & Maniatis, 1998).

To study the effects of pyrimidine tract changes on *dsx* splicing, we used a pre-mRNA designated *dsx*(70)M1 (Graveley et al., 1998), which contains a single MS2 binding site 70 nt downstream of the 3′ splice site. The *dsx*(70)M1 pre-mRNA containing the wild-type pyrimidine tract was not spliced when incubated under splicing conditions in HeLa cell nuclear extract alone (Fig. 1A, lane 1), or when supplemented with the MS2 protein lacking an RS domain (Fig. 1A, lane 2). However, the

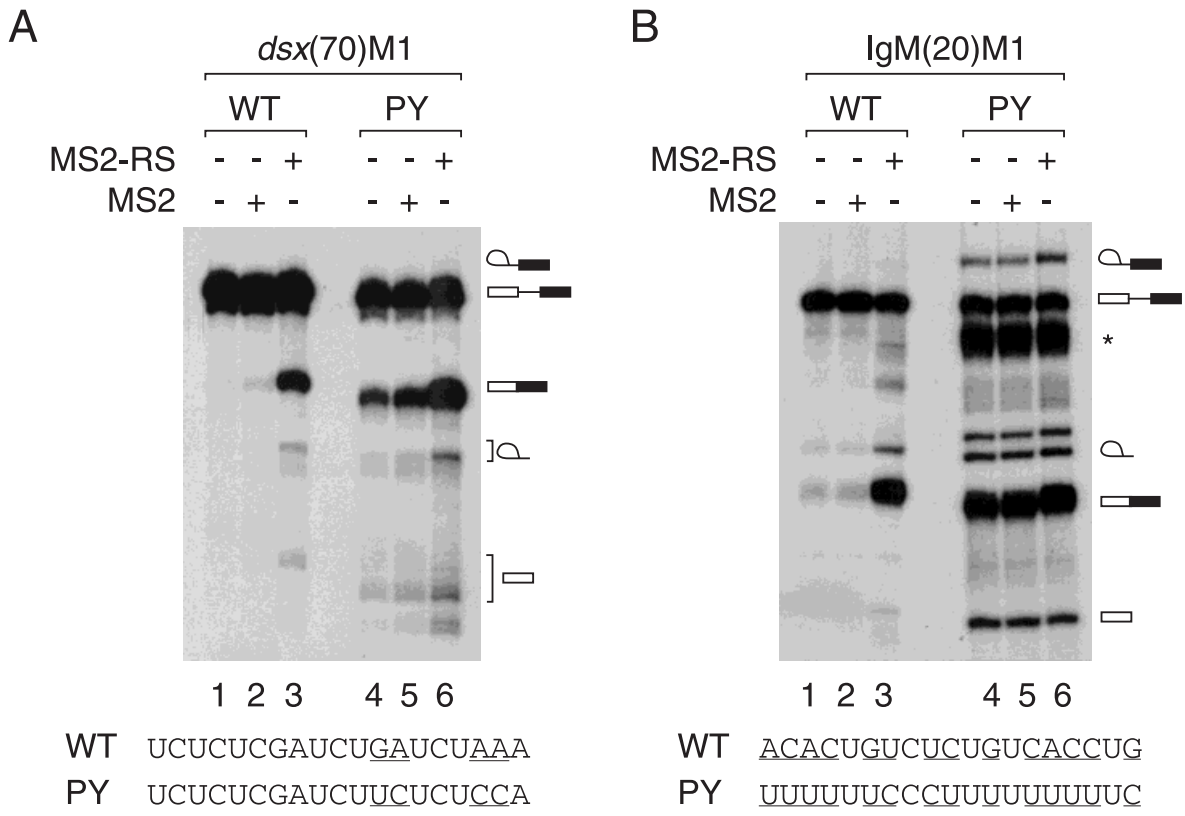

FIGURE 1. Comparison of the splicing efficiencies of *dsx* and IgM pre-mRNAs containing wild-type and improved pyrimidine tracts. **A:** Analysis of *dsx*(70)M1 splicing. Splicing reactions were carried out in HeLa nuclear extract for 2 h at 30 °C with pre-mRNAs containing a single MS2 binding site 70 nt downstream of the *dsx* 3′ splice site and either the wild-type (WT; lanes 1–3) or improved (PY; lanes 4–6) pyrimidine tract. The reactions contained either no added protein (lanes 1 and 4), MS2 protein (lanes 2 and 5), or 200 nM MS2-RS^{p55} (lanes 3 and 6). The reaction products were separated on a 10% denaturing polyacrylamide gel. The identities of the input, intermediates, and products are indicated. The nucleotide sequences of the two pyrimidine tracts are shown below the panel. The nucleotides that differ between the two sequences are underlined. **B:** Analysis of IgM(20)M1 splicing. Splicing reactions were carried out in HeLa cell nuclear extracts for 2 h at 30 °C with pre-mRNAs containing a single MS2 binding site 20 nt downstream of the 3′ splice site and either the wild-type (WT; lanes 1–3) or improved (PY; lanes 4–6) pyrimidine tract. The reactions contained either no added protein (lanes 1 and 4), MS2 protein (lanes 2 and 5), or 200 nM MS2-RS^{p55} (lanes 3 and 6). The reaction products were separated on a 10% denaturing polyacrylamide gel. The identities of the input, intermediates, and products are indicated. The nucleotide sequences of the two pyrimidine tracts are shown below the panel. The nucleotides that differ between the two sequences are underlined.

addition of the MS2 protein containing an RS domain (MS2-RS) activated splicing of this RNA (Fig. 1A, lane 3). Replacing four purines in the *dsx* pyrimidine tract with pyrimidines (UCUCUCGAUCU**GA**UCU**AA**A (WT) to UCUCUCGAUCU**UC**UCU**CC**A (PY)), led to efficient splicing in the absence of the hybrid protein (Fig. 1A, lane 4) and in the presence of an MS2 protein lacking an RS domain (Fig. 1A, lane 5). This RNA, however, was still responsive to the addition of MS2-RS (Fig. 1A, lane 6). The relatively subtle improvement of the pyrimidine tract is likely to be the reason that this pre-mRNA is still responsive to the splicing activator. Consistent with previous reports (Hoshijima et al., 1991; Tian & Maniatis, 1994), we conclude that replacement of purines by pyrimidines in the 3′ splice site of the *dsx* pre-mRNA can relieve the requirement for a splicing enhancer.

To study the effect of improving the pyrimidine tract on IgM splicing, we used the IgM(20)M1 pre-mRNA (Graveley et al., 1998), which contains a single MS2 binding site 20 nt downstream of the 3′ splice site. Importantly, the IgM(20)M1 pre-mRNA does not contain the splicing inhibitor element (Kan & Green, 1999). The IgM(20)M1 pre-mRNA containing the wild-type pyrimidine tract was inefficiently spliced in the absence of added protein, and in the presence of the MS2 protein lacking an RS domain (Fig. 1B, lanes 1 and 2). However, efficient splicing was observed when the MS2-RS protein was added to the splicing reaction (Fig. 1B, lane 3). Thus, in the absence of the splicing inhibitor, splicing of the IgM M1-M2 intron requires a splicing enhancer.

We next tested the effect of replacing the weak IgM pyrimidine tract with a U2AF consensus binding site, changing the sequence from ACACUGUCUCUGUCAC CUG (WT) to UUUUUUCCCUUUUUUUUUC (PY). We found that this substitution resulted in efficient splicing regardless of whether a splicing activator was added to the reaction (Fig. 1B, lanes 4–6). These results demonstrate that in the absence of the splicing inhibitor, the requirement for a splicing enhancer can be completely relieved by improving the pyrimidine tract of the IgM pre-mRNA.

These observations are consistent with the proposal that splicing activators function by recruiting U2AF to the 3′ splice site. However, this is only true if the binding affinity of U2AF to the improved pyrimidine tracts is higher than the affinity of U2AF to the respective wild-type pyrimidine tracts. We therefore used UV crosslinking to measure the affinity of recombinant U2AF^{65} for each of the variant pyrimidine tracts used in the experiments shown in Figure 1. Table 1 shows that the affinity of U2AF^{65} for each of the improved pyrimidine tracts is higher than for the wild-type pyrimidine tracts. In the case of the *dsx* pyrimidine tract, there is approximately a ninefold effect, whereas there is about a fivefold effect in the case of IgM. Taken together our data show that improved U2AF binding can relieve the requirement for a splicing enhancer.

TABLE 1. Binding affinities of U2AF^{65} to various pyrimidine tracts.

Pyrimidine tract	Binding affinity (K_D)
dsx WT: UCUCUCGAUCUGAUCUAAA	540 nM
dsx PY: UCUCUCGAUCUUCUCUCCA	60 nM
IgM WT: ACACUGUCUCUGUCACCUG	12 nM
IgM PY: UUUUUUCCCUUUUUUUUUC	2.6 nM

Splicing activators stimulate spliceosome assembly

Previous studies have shown that splicing enhancers promote spliceosomal E complex assembly (Staknis & Reed, 1994; Wang et al., 1995; Zuo & Maniatis, 1996). We have extended these studies by using native gel electrophoresis to determine whether splicing activators enhance the formation of pre-spliceosomal A complexes on the *dsx* and IgM pre-mRNAs. In previous studies significant levels U2AF were not detected in H complex (Bennett et al., 1992). By contrast, U2AF is present in (Bennett et al., 1992) and is required for the formation of A complex (Zamore & Green, 1991). Thus, based on the absence of U2AF in H complex and its presence in A complex, the presence of the latter complex provides an indirect assay for U2AF binding.

The *dsx*(70)M1 and IgM(20)M1 pre-mRNAs were incubated in nuclear extract in the absence of added protein, or in the presence of MS2 or MS2-RS for 15 min and then resolved on native polyacrylamide gels. Only H complex forms on *dsx*(70)M1 in the absence of added protein (Fig. 2A, lane 1) or in the presence of MS2 lacking an RS domain (Fig. 2A, lane 2). In contrast, in the presence of MS2-RS, a significant amount of A complex was formed on the *dsx*(70)M1 pre-mRNA (Fig. 2A, lane 3). Similar results were obtained with the IgM(20)M1 pre-mRNA. In this case, however, addition of the MS2 protein alone led to an increase in the amount of A complex formation when compared to low level of A complex formed in the absence of added protein (Fig. 2B, lanes 1 and 2). This effect is likely to be the result of an increase in RNA stability caused by the binding of MS2 to the IgM pre-mRNA. Addition of the MS2-RS protein, however, further stimulated the amount of A complex formed on the IgM(20)M1 pre-mRNA (Fig. 2B, lane 3). In conjunction with earlier studies (Staknis & Reed, 1994; Zuo & Maniatis, 1996) we conclude that splicing activators promote the formation of both the E and A spliceosomal complexes on enhancer-dependent pre-mRNAs. Thus, these splicing complex assembly studies provide additional indirect evidence that splicing activators function by recruiting U2AF to weak 3′ splice sites.

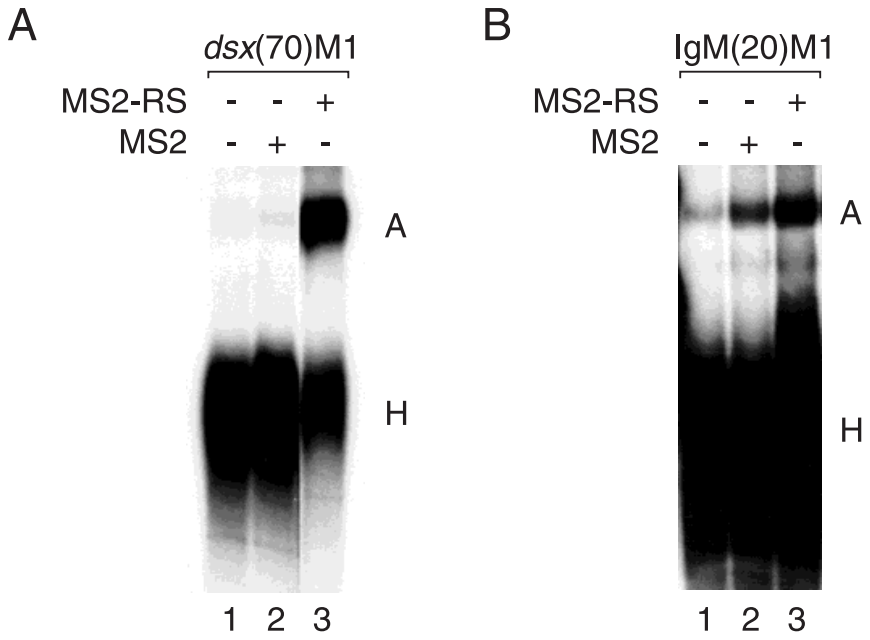

FIGURE 2. Analysis of pre-spliceosomal complexes formed on *dsx* and IgM pre-mRNAs. **A:** Formation of pre-spliceosomal complexes on the *dsx* pre-mRNA. The *dsx*(70)M1 pre-mRNA was incubated in HeLa cell nuclear extracts at 30 °C for 15 min under splicing conditions. The reactions contained either no added protein (lane 1), 200 nM MS2 (lane 2), or 200 nM MS2-RS^{p55} (lane 3). Heparin was added to a final concentration of 0.5 mg/mL and the reactions were resolved on 4% (80:1) Tris-glycine polyacrylamide gel. The identities of the H and A complexes are indicated. **B:** Formation of pre-spliceosomal complexes on the IgM pre-mRNA. The IgM(20)M1 pre-mRNA was incubated in HeLa cell nuclear extracts at 30 °C for 15 min under splicing conditions. The reactions contained either no added protein (lane 1), 200 nM MS2 (lane 2), or 200 nM MS2-RS^{p55} (lane 3). Heparin was added to a final concentration of 0.5 mg/mL and the reactions were resolved on a 4% (80:1) Tris-glycine polyacrylamide gel. The identities of the H and A complexes are indicated.

Splicing activators recruit splicing components to the 3′ splice site

The results presented above do not rule out the possibility that splicing activators recruit U2AF to the 3′ splice site by an indirect mechanism that requires components bound to the upstream exon or 5′ splice site. To test this possibility, we generated 3′ half substrates for both *dsx* and IgM that lacked the first exon and 5′ splice site, and contained only the branch point, pyrimidine tract, 20 nt of exon 2, and a single MS2 binding site. We designated these RNAs *dsx*(20)M1-3′ and IgM(20)M1-3′. These 3′ half substrates were incubated in nuclear extract in the absence of added protein or in the presence of MS2 or MS2-RS for 15 min and the substrates were resolved on a native polyacrylamide gel. Similar to the results with the full-length substrate, only H complex was formed on the *dsx*(20)M1-3′ RNA in nuclear extract alone (Fig. 3A, lane 1) or in reactions supplemented with MS2 (Fig. 3A, lane 2). However, the addition of MS2-RS resulted in the formation of A3′ complex (Fig. 3A, lane 3). The formation of A3′ complex requires ATP, incubation at 30 °C, and is significantly reduced by the addition of an oligonucleotide complimentary to U2 snRNA (data not shown). Similar results were obtained with the IgM(20)M1-3′ RNA. Very little A3′ complex was formed in nuclear extract in the absence of protein (Fig. 3B, lane 1). The addition of MS2 protein resulted in an increase in the amount of A3′ complex formed on the IgM(20)M1-3′ RNA (Fig. 3B, lane 2). Again, this effect is most likely due to an increase in RNA stability caused by the binding of the MS2 protein to the IgM pre-mRNA. However, the addition of MS2-RS further stimulated the formation of A3′ complex (Fig. 3B, lane 3). Thus, splicing activators stimulate the binding of spliceosomal components to the BPS and/or 3′ splice site.

We next tested whether the improved pyrimidine tracts would allow for A3′ complexes to form in the absence of a splicing activator. We therefore generated *dsx*(20)M1-3′ and IgM(20)M1-3′ pre-mRNAs that contained the improved pyrimidine tracts used in Figure 1. When the *dsx* pyrimidine tract was improved, A3′ complex formed in the absence of added protein (Fig. 3A, lane 4) or in the reactions supplemented with MS2 (Fig. 3A, lane 5). The addition of MS2-RS, however, resulted in a further stimulation of A3′ complex formation (Fig. 3A, lane 6). These results parallel the splicing results shown in Figure 1 using full-length substrates. When we tested the IgM(20)M1 RNA containing the improved pyrimidine tract, we found that in nuclear extract alone (Fig. 3B, lane 4) or supplemented with MS2

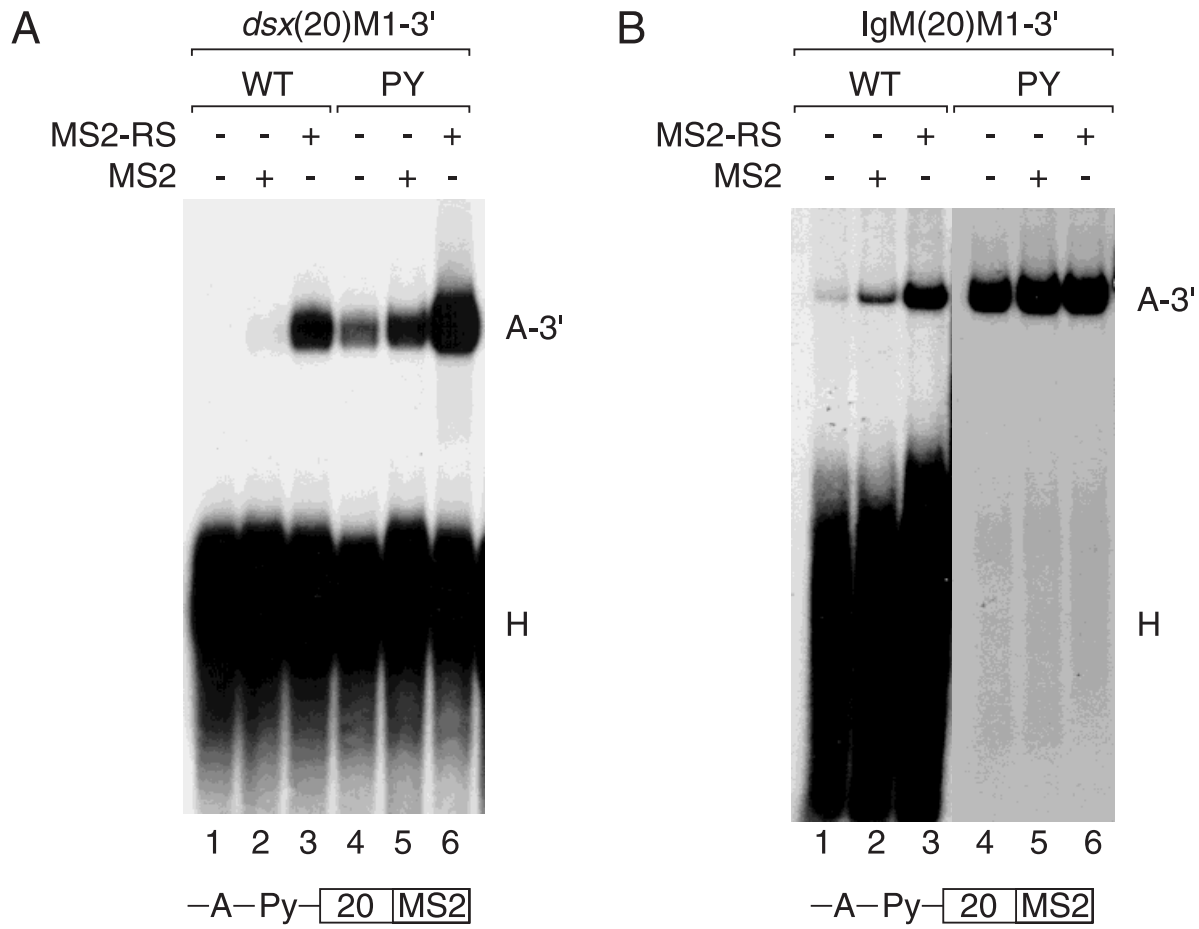

FIGURE 3. Analysis of spliceosomal complexes formed on minimal RNA substrates. **A:** Formation of spliceosomal complexes on *dsx* minimal substrates. RNA substrates were generated that contained the *dsx* branchpoint, pyrimidine tract, 20 nt of exon, and a single MS2 binding site (*dsx*(20)M1-3′). These substrates contained either the wild-type (WT; lanes 1–3) or improved (PY; lanes 4–6) pyrimidine tract. Because complexes formed on dsx(70)M1 3′ substrates were fairly unstable (data not shown), we decreased the distance between the 3′ splice site and the MS2 binding site in the *dsx* substrate to 20 nt. The splicing of full-length RNAs containing the MS2 site at this distance are activated by the addition of MS2-RS hybrid proteins, but not MS2 alone (data not shown). The RNAs were incubated in HeLa cell nuclear extracts at 30°C for 15 min under splicing conditions. The reactions contained either no added protein (lanes 1 and 4), 200 nM MS2 protein (lanes 2 and 5), or 200 nM MS2-RS^{p55} (lanes 3 and 6). Heparin was added to a final concentration of 0.5 mg/mL and the reactions were resolved on a 4% (80:1) Tris-glycine polyacrylamide gel. The identities of the H and A-3′ complexes are indicated. **B:** Formation of spliceosomal complexes on IgM minimal substrates. RNA substrates were generated that contained the IgM branchpoint, pyrimidine tract, 20 nt of exon, and a single MS2 binding site (IgM(20)M1-3′). These substrates contained either the wild-type (WT; lanes 1–3) or improved (PY; lanes 4–6) pyrimidine tract. The RNAs were incubated in HeLa cell nuclear extracts at 30°C for 15 min under splicing conditions. The reactions contained either no added protein (lanes 1 and 4), 200 nM MS2 protein (lanes 2 and 5), or 200 nM MS2-RS^{p55} (lanes 3 and 6). Heparin was added to a final concentration of 0.5 mg/mL and the reactions were resolved on a 4% (80:1) Tris-glycine polyacrylamide gel. The identities of the H and A-3′ complexes are indicated.

(Fig. 3B, lane 5), all of the RNA was present in the A3′ complex. In addition, the presence of MS2-RS did not affect the amount of A3′ complex formed (Fig. 3B, lane 6). Again, these results parallel the splicing results shown in Figure 1B. Thus, the requirement for an enhancer in spliceosome assembly can be relieved by improving the pyrimidine tracts of both IgM and *dsx* RNAs, again pointing to U2AF as the limiting component in spliceosome assembly on these RNA splicing substrates.

Splicing activators stimulate the crosslinking of U2AF^{65} and U2AF^{35} to the pre-mRNA

To directly test whether the splicing activators recruit U2AF to weak 3′ splice sites under splicing conditions, we performed UV crosslinking experiments with the *dsx* and IgM 3′ half substrates used in Figure 3. Briefly, uniformly radiolabeled RNAs were incubated in HeLa nuclear extract in the presence or absence of MS2-RS,

irradiated with UV light, and treated with RNase. To detect U2AF binding, we then immunoprecipitated U2AF with antibodies against either U2AF^{35} or U2AF^{65} and resolved the precipitates by SDS-PAGE and visualized them by autoradiography.

We found that the binding of either subunit of U2AF to the *dsx* RNA was almost undetectable in the absence of added protein (Fig. 4A, lanes 1 and 3). However, the addition of MS2-RS strongly stimulated U2AF^{65} crosslinking as well as a small amount of U2AF^{35} crosslinking (Fig. 4A, lanes 2 and 4). When the pyrimidine tract was improved, a low level of U2AF^{65} crosslinking was observed in the absence of added protein (Fig. 4A, lanes 5 and 7). However, the addition of MS2-RS stimulated both U2AF^{65} and U2AF^{35} crosslinking (Fig. 4A, lanes 6 and 8). These results show that splicing activators stimulate the binding of both U2AF subunits to the *dsx* 3′ splice site.

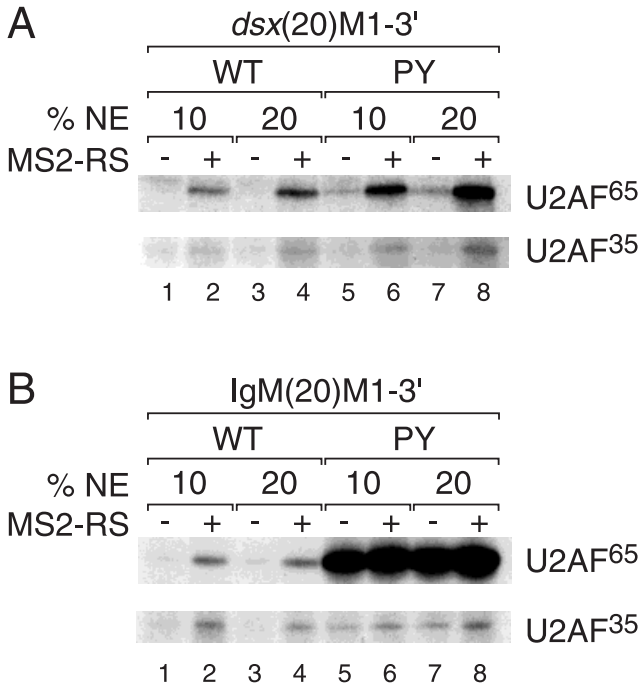

FIGURE 4. Analysis of U2AF binding to *dsx* and IgM minimal substrates. **A:** U2AF binding to the *dsx* RNA. The *dsx*(20)M1-3′ substrates containing either the wild-type (WT; lanes 1–4) or improved (PY; lanes 5–8) pyrimidine tracts were incubated in either 10% (lanes 1, 2, 5, and 6) or 20% (lanes 3, 4, 7, and 8) HeLa nuclear extract at 30 °C for 15 min under splicing conditions. The reactions contained either no added protein (lanes 1, 3, 5, and 7) or 200 nM MS2-RS^{9G8} (lanes 2, 4, 6, and 8). Following the incubation, the reactions were irradiated with UV light for 10 min at a distance of 0.5 cm. The reactions were then treated with RNase A. The reactions were then immunoprecipitated with antibodies against either U2AF^{65} (top) or U2AF^{35} (bottom) and then resolved by SDS-PAGE. **B:** U2AF binding to the IgM RNA. The IgM(20)M1-3′ substrates containing either the wild-type (WT; lanes 1–4) or improved (PY; lanes 5–8) pyrimidine tracts were incubated in either 10% (lanes 1, 2, 5, and 6) or 20% (lanes 3, 4, 7, and 8) HeLa nuclear extract at 30 °C for 15 min under splicing conditions. The reactions contained either no added protein (lanes 1, 3, 5, and 7) or 200 nM MS2-RS^{9G8} (lanes 2, 4, 6, and 8) and were treated as in **A**.

Similar results were obtained with the IgM RNAs. In the presence of nuclear extract alone, very low levels of U2AF^{65} and U2AF^{35} crosslinking were detected on the IgM RNA containing the wild-type pyrimidine tract, but lacking the inhibitor sequence (Fig. 4B, lanes 1 and 3). However, the addition of MS2-RS stimulated both U2AF^{65} and U2AF^{35} crosslinking (Fig. 4B, lanes 2 and 4). When the pyrimidine tract was improved, the amount of U2AF^{65} and U2AF^{35} crosslinking was identical regardless of whether MS2-RS was added to the reaction (Fig. 4B, lanes 5–8). These results indicate that splicing activators stimulate the binding of both U2AF subunits to the IgM 3′ splice site.

The U2AF crosslinking data are in complete agreement with both the complex formation and splicing data presented earlier. Taken together, these results provide strong evidence that splicing activators (and enhancers) can recruit both U2AF subunits to the *dsx* and IgM 3′ splice sites.

U2AF^{35} promotes enhancer-dependent splicing

Based initially on protein–protein interaction data, U2AF^{35} was proposed to mediate interactions between SR proteins bound to splicing enhancers and U2AF^{65}, thereby recruiting U2AF^{65} to the pyrimidine tract (Wu & Maniatis, 1993). This model was supported by in vitro experiments with recombinant proteins showing that U2AF^{35} is required for SR protein-dependent crosslinking of U2AF^{65} to the *dsx* 3′ splice site (Zuo & Maniatis, 1996). However, more recent experiments carried out in nuclear extracts are inconsistent with these observations. Specifically, Kan and Green (1999) found that U2AF^{35} was not required for splicing of the IgM pre-mRNA. In contrast, Guth et al. (1999) found that although U2AF^{35} was essential for splicing of the IgM pre-mRNA, it had no impact on the binding of U2AF^{65}. We therefore tested the requirement for U2AF^{35} in enhancer-dependent *dsx* and IgM splicing. To do this, we compared the activities of recombinant U2AF^{65} alone and a recombinant U2AF$^{65/35}$ heterodimer in restoring enhancer-dependent splicing in U2AF-depleted extracts. We expressed His-tagged U2AF^{65} either alone or together with untagged U2AF^{35} in Sf9 cells and purified the recombinant proteins by Ni-chromatography. Figure 5A shows Coomassie-stained gels of these protein preparations. We next depleted HeLa nuclear extracts of U2AF by passing them over an oligo-dT column. Western blots of the depleted extract demonstrate that at least 99% of U2AF^{65} and U2AF^{35} were depleted by this method (Fig. 5B).

We next tested the ability of U2AF^{65} and U2AF$^{65/35}$ to restore enhancer-dependent splicing in the oligo-dT-depleted HeLa cell nuclear extract. This was done by incubating either the *dsx*(70)M1 or IgM(20)M1 pre-mRNAs in oligo-dT depleted extract in the presence or

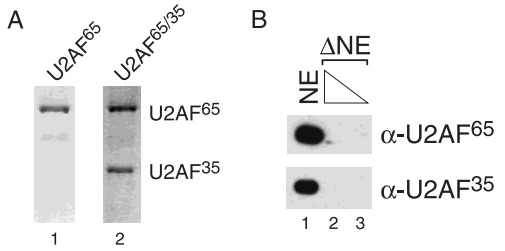

FIGURE 5. Reagents used to address the role of U2AF^{35} in enhancer-dependent splicing. **A:** Recombinant U2AF preparations. Preparations of N-terminal his-tagged U2AF^{65} (400 ng; lane 1) or U2AF heterodimer (500 ng; N-terminal his-tagged U2AF^{65} and untagged U2AF^{35}; lane 2) were separated by SDS-PAGE and stained with Coomassie Brilliant blue. The proteins were expressed in and purified from Sf9 insect cells using recombinant baculoviruses as described in Materials and Methods. **B:** Analysis of U2AF levels in oligo-dT depleted extract. A total of 0.1 μL of undepleted HeLa nuclear extract (lane 1) and 1 μL (lane 2) or 0.1 μL (lane 3) of oligo-dT-depleted HeLa nuclear extract were resolved by SDS-PAGE and transferred to a membrane. The blots were separately probed with antibodies directed against U2AF^{65} (MC3) or U2AF^{35} (rabbit polyclonal) and visualized by ECL and autoradiography.

absence of MS2-RS in combination with either U2AF^{65} or the U2AF$^{65/35}$ heterodimer. The *dsx*(70)M1 pre-mRNA was not spliced in the oligo-dT depleted extract alone (Fig. 6A, lane 3) or when supplemented with only MS2-RS (Fig. 6A, lane 4). Moreover, the addition of U2AF^{65} alone did not restore splicing of this RNA (Fig. 6A, lane 5). However, a low level of splicing was observed in the presence of high concentrations of U2AF^{65} and MS2-RS (Fig. 6A, lanes 6–9). Surprisingly, a low level of splicing was observed in the presence of ~400 nM U2AF$^{65/35}$ even in the absence of MS2-RS (Fig. 6A, lane 10). However, this result is consistent with the observation that low levels of *dsx*(70)M1 pre-mRNA splicing can be observed in the absence of MS2-RS when high concentrations of undepleted nuclear extract were used (data not shown). In addition, the efficiency of splicing in the presence of U2AF$^{65/35}$ is enhanced in the presence of MS2-RS. Quantitation of this data (Fig. 6B) shows that activator-dependent splicing in the presence of U2AF$^{65/35}$ is approximately threefold more efficient than in the presence of U2AF^{65}. These results show that U2AF^{35} is required for maximal levels of enhancer-dependent splicing.

We next compared the abilities of U2AF^{65} and U2AF$^{65/35}$ to restore IgM splicing. As with the *dsx* substrate, the IgM(20)M1 pre-mRNA is not spliced in the oligo-dT depleted extract alone (Fig. 6C, lane 3) or in the presence of MS2-RS (Fig. 6C, lane 4). In addition, splicing was not observed when the reactions were supplemented with U2AF^{65} alone (Fig. 6C, lane 6) or with U2AF$^{65/35}$ alone (Fig. 6C, lane 10). A very low level of splicing occurred at the highest concentration of U2AF^{65} and MS2-RS (Fig. 6C, lanes 6–9). However, efficient splicing occurred in the presence of U2AF$^{65/35}$ and MS2-RS (Fig. 6C, lanes 11–14). Quantitation of this data revealed that U2AF$^{65/35}$ was approximately fivefold more active than U2AF^{65} in restoring IgM splicing in the oligo-dT depleted extract (Fig. 6D). We conclude that U2AF^{35} is required for efficient enhancer-dependent splicing.

DISCUSSION

Evidence that splicing activators and enhancers can recruit U2AF to weak 3′ splice sites

Several previous studies are consistent with the model that splicing activators and enhancers function primarily by recruiting U2AF to weak 3′ splice sites (Wu & Maniatis, 1993; Tian & Maniatis, 1994; Wang et al., 1995; Zuo & Maniatis, 1996; Bouck et al., 1998). However, more recent studies have found that U2AF^{65} binding to weak splice sites does not change in the presence and absence of an enhancer (Kan & Green, 1999; Li & Blencowe, 1999). In addition, contradictory results regarding the requirement for U2AF^{35} have made the precise role for this splicing factor in enhancer-dependent splicing unclear (Zuo & Maniatis, 1996; Rudner et al., 1998b; Guth et al., 1999; Kan & Green, 1999). We have therefore carried out additional experiments, and here present several lines of evidence that are consistent with the U2AF recruitment model.

First, we have shown that improving the weak pyrimidine tracts of both the *dsx* and IgM pre-mRNAs increases the affinity of U2AF^{65}, and relieves the requirement for a splicing enhancer. The result with the *dsx* RNA confirms previously published studies (Hoshijima et al., 1991; Tian & Maniatis, 1994), but the results with the IgM pre-mRNA are not consistent with those of Kan and Green (1999). However, this apparent contradiction is simply a matter of experimental design. The earlier experiments (Kan & Green, 1999) were carried out with substrates containing the dominant-acting inhibitor sequence, which, in retrospect, masked the effect of changes in the pyrimidine tract sequence. Thus, in the absence of the inhibitor, an improved pyrimidine tract completely relieves the requirement for a splicing enhancer. This observation, in conjunction with measurements of the relative affinities of U2AF for wild-type and improved pyrimidine tracts supports the U2AF recruitment model.

Second, evidence consistent with the recruitment model was provided by the results of spliceosome assembly experiments. For both the IgM and *dsx* pre-mRNAs, we found that only low levels of A complex assemble on RNAs lacking a bound activator, whereas the addition of the MS2-RS activator protein dramatically increased the level of A complex formation. In fact, the level of spliceosome assembly in the presence and absence of an activator with both substrates correlated well with the level of splicing observed with the same

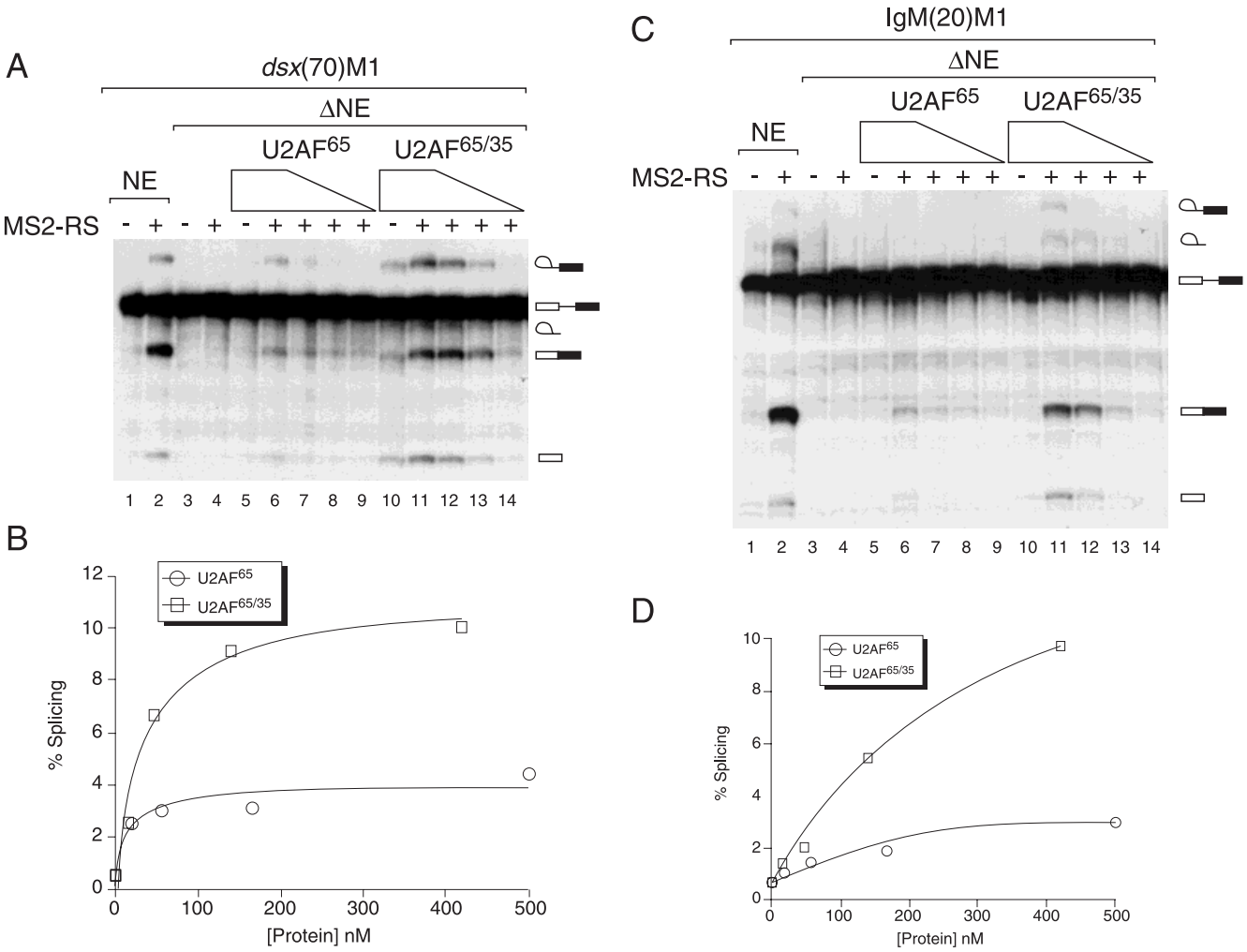

FIGURE 6. Reconstitution of *dsx* and IgM splicing with U2AF in oligo-dT-depleted extract. **A:** Reconstitution of *dsx* splicing. *dsx*(70)M1 pre-mRNA was incubated at 30 °C for 2 h in 15% undepleted nuclear extract (lanes 1 and 2) or 30% oligo-dT depleted extract (lanes 3–14). The reactions in lanes 2, 4, 6–9, and 11–14 were supplemented with 200 nM MS2-RS^{p55}. In addition, the oligo-dT-depleted extract was supplemented with U2AF^{65} (lanes 5 and 6, 500 nM; lane 7, 167 nM; lane 8, 55 nM; lane 9, 18 nM) or U2AF$^{65/35}$ (lanes 10 and 11, 420 nM; lane 12, 140 nM; lane 13, 47 nM; lane 14, 15 nM). The reactions were resolved on a 12% denaturing polyacrylamide gel. **B:** Quantitation of the data in **A**. The efficiency of splicing (% splicing) is plotted as a function of U2AF concentration (nM). ○: data with U2AF^{65}. □: data with U2AF$^{65/35}$. **C:** Reconstitution of IgM splicing. IgM(20)M1 pre-mRNA was incubated at 30 °C for 2 h in 15% undepleted nuclear extract (lanes 1 and 2) or 30% oligo-dT depleted extract (lanes 3–14). The reactions in lanes 2, 4, 6–9, and 11–14 were supplemented with 200 nM MS2-RS^{p55}. In addition, the oligo-dT-depleted extract was supplemented with U2AF^{65} (lanes 5 and 6, 500 nM; lane 7, 167 nM; lane 8, 55 nM; lane 9, 18 nM) or U2AF$^{65/35}$ (lanes 10 and 11, 420 nM; lane 12, 140 nM; lane 13, 47 nM; lane 14, 15 nM). The reactions were resolved on a 12% denaturing polyacrylamide gel. **D:** Quantitation of the data in **C**. The efficiency of splicing (% splicing) is plotted as a function of U2AF concentration (nM). ○: data with U2AF^{65}. □: data with U2AF$^{65/35}$.

substrates. Consistent with the notion that the splicing activators recruit spliceosomal components to the 3′ splice site, we obtained similar results when spliceosome assembly was analyzed on full-length substrates and RNAs containing only the 3′ splice site. In addition, improving the pyrimidine tracts of the 3′ half RNAs led to the formation of A complex in the absence of a splicing activator. Together, these results suggest that splicing activators function to stimulate the binding of splicing factors to weak 3′ splice sites.

Third, we found that splicing activators stimulate the binding of both U2AF^{65} and U2AF^{35} to the 3′ splice sites of both the IgM and *dsx* pre-mRNAs. These results are consistent with the previous results of some groups (Wang et al., 1995; Zuo & Maniatis, 1996; Bouck et al., 1998), but not others (Kan & Green, 1999; Li & Blencowe, 1999). Several studies have shown that splicing enhancers can stimulate the binding of U2AF. For example, it was shown that the troponin t and Adenovirus L2 splicing enhancers can stimulate U2AF^{65} binding in HeLa nuclear extracts (Wang et al., 1995). In addition, using only purified recombinant proteins, it was shown that SR proteins could stimulate the binding of U2AF^{65} to the pre-mRNA only in the presence of

U2AF^{35} (Zuo & Maniatis, 1996). Importantly, the identity of the splicing enhancer present on the pre-mRNA determined which SR protein could successfully recruit U2AF (Zuo & Maniatis, 1996). It has also been shown that deletion of an exonic splicing enhancer in avian sarcoma virus resulted in decreased U2AF^{65} crosslinking under splicing conditions (Bouck et al., 1998).

Although many experiments, including ours, support the U2AF recruitment model, some recent reports do not. First, using an UV-crosslinking/immunoprecipitation assay, it was shown that the binding of U2AF^{65} to the *dsx* and IgM pre-mRNAs does not change in the presence or absence of a splicing enhancer (Kan & Green, 1999). Second, the association of U2AF^{65} with the *dsx* pre-mRNA did not change in the presence or absence of a splicing enhancer when assayed by western blotting of affinity-purified spliceosomes (Li & Blencowe, 1999). However, due to the assay used in this study, it is not possible to determine whether the U2AF^{65} associated with the purified spliceosomes is bound in a functional manner. Nonetheless, these two studies raise the possibility that splicing enhancers do not function by recruiting U2AF.

We imagine that variations in experimental conditions and the highly cooperative nature of spliceosome assembly (Reed, 2000) may contribute the observed differences. First, although many of the experiments were performed using similar conditions, there are still many differences that could account for the discrepancies. For example, most other studies have compared the binding of U2AF to different RNAs (+/− enhancer), whereas we have used a single RNA and provided the enhancer activity in *trans* (MS2-RS proteins). In addition, some studies have examined the binding of U2AF directly (Wang et al., 1995; Zuo & Maniatis, 1996; Bouck et al., 1998; Kan & Green, 1999; our data) whereas others have indirectly assayed U2AF binding (Li & Blencowe, 1999). Second, the highly cooperative nature of spliceosome assembly may result in the observation of profound effects on the binding of one splicing factor caused by subtle differences in the concentrations of other splicing factors. Such effects could be caused by small variations in nuclear extract preparations and differences in the quantity of nuclear extract used in various studies. One explanation that is consistent with the results presented here, which show an enhancer-dependent binding of U2AF to weak 3′ splice sites, is that U2AF binding is necessary, but not sufficient for splicing. Additional critical components may be recruited to the complex by splicing enhancers or activators.

Protein–protein interactions required for U2AF recruitment

It has been proposed that U2AF recruitment is mediated by a direct interaction between SR proteins bound to the splicing enhancer and the RS domain of U2AF^{35} (Wu & Maniatis, 1993; Zuo & Maniatis, 1996). Consistent with this model, purified SR proteins can stimulate the binding of purified U2AF^{65} only in the presence of purified U2AF^{35} (Zuo & Maniatis, 1996). In addition, the RS domain of U2AF^{35} has been shown to be required for maximal levels of enhancer-dependent splicing in vitro (Zuo & Maniatis, 1996). Moreover, both the small (dU2AF^{38}) and large (dU2AF^{50}) subunits of U2AF have been shown to be essential for viability in *Drosophila* (Kanaar et al., 1993; Rudner et al., 1996), as is the interaction between the two subunits (Rudner et al., 1998c). However, the RS domain of dU2AF^{38} is dispensable for viability, and is not required for alternative splicing of *dsx* pre-mRNA in vivo. Thus, splicing activators either do not recruit U2AF by interacting with the RS domain of the small subunit, or in the absence of the small subunit RS domain it is possible to bypass the normal mechanism. In support of the latter, some SR proteins have been shown to interact with U2AF^{65} (Zhang & Wu, 1996). Moreover, we have found that the RS domain of U2AF^{65} can activate splicing when fused to MS2 (B.R. Graveley, unpubl. data) suggesting that it has the ability to both participate in protein–protein interactions and promote the annealing of U2 snRNA to the branchpoint (Valcárcel et al., 1996). Finally, the RS domain of the small U2AF subunit is only dispensable when the RS domain of the large subunit is present (Rudner et al., 1998b). Thus, although SR proteins within a splicing enhancer complex may preferentially interact with U2AF^{35} to recruit U2AF^{65}, in the absence of the U2AF^{35} RS domain, the splicing enhancer complex may recruit U2AF^{65} by interacting with its RS domain. Consistent with this idea, we have observed that U2AF^{65} alone can weakly complement enhancer-dependent splicing in U2AF-depleted extracts. An additional possibility is that the splicing enhancer complex can recruit U2AF indirectly through a mediator or coactivator. In fact, it has recently been shown that the SRm160/300 coactivator may be required for the function of exonic splicing enhancers (Eldridge et al., 1999). Further experiments will be necessary to determine which of these mechanisms is correct, or if there are redundant mechanisms.

The role of U2AF^{35} in enhancer-dependent splicing

The requirement for U2AF^{35} in pre-mRNA splicing has been unclear for many years. As mentioned above, dU2AF^{38} is required for viability (Rudner et al., 1996), as is its interaction with dU2AF^{50} (Rudner et al., 1998c). It is clear from in vitro assays that it can be dispensable for constitutive splicing under certain conditions (Zamore & Green, 1991; Zhu & Krainer, 2000), although it has also been shown to be required (Zuo & Maniatis, 1996; Zhu & Krainer, 2000). The recent discovery that U2AF^{35}

interacts with the 3′ splice site AG (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999) may resolve some of these issues. U2AF^{35} does not appear to be essential for the splicing of AG-independent introns (Wu et al., 1999)—introns containing a strong pyrimidine tract (Reed, 1989). However, U2AF^{35} is required for the splicing of AG-dependent introns (Wu et al., 1999)—introns that contain short or weak pyrimidine tracts (Reed, 1989).

With regards to enhancer-dependent splicing, U2AF^{35} has been reported to be both dispensable (Kan & Green, 1999) and essential (Zuo & Maniatis, 1996; Guth et al., 1999). In addition, U2AF^{35} has been shown to be both required for (Zuo & Maniatis, 1996) and dispensable for (Guth et al., 1999; Kan & Green, 1999) enhancer-dependent U2AF^{65} crosslinking. Here we have shown that although U2AF^{35} is not absolutely required for enhancer-dependent splicing, it does enhance the activity of U2AF for both the *dsx* and IgM pre-mRNAs. We also have shown that the splicing activators stimulate the binding of U2AF^{35} to the pre-mRNA, presumably to the AG at the 3′ splice site (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999).

We believe that U2AF^{35} has two important functions that can increase the activity of U2AF in certain circumstances. First, because U2AF^{35} can bind the 3′ splice site (Merendino et al., 1999; Wu et al., 1999; Zorio & Blumenthal, 1999), its association with U2AF^{65} provides at least one additional protein–RNA contact when compared to U2AF^{65} alone. In fact, the binding affinity of the U2AF$^{65/35}$ heterodimer for RNA is greater than that of U2AF^{65} alone (Rudner et al., 1998a; Merendino et al., 1999; Wu et al., 1999). The second reason that U2AF^{35} can increase the activity of U2AF may be due to an increased number of protein interaction surfaces. SR proteins have been shown to interact with both U2AF^{35} (Wu & Maniatis, 1993) and U2AF^{65} (Zhang & Wu, 1996). The U2AF$^{65/35}$ heterodimer, therefore, contains more SR protein binding sites than U2AF^{65} alone. Further experiments should clarify the exact role of U2AF^{35} in enhancer-dependent splicing.

Splicing of the IgM pre-mRNA requires a splicing enhancer in the absence of the splicing inhibitor

The IgM pre-mRNA has previously been shown to require a splicing enhancer for efficient splicing (Watakabe et al., 1993; Graveley et al., 1998; Graveley & Maniatis, 1998). A splicing inhibitory sequence has been identified in the IgM pre-mRNA, downstream of the splicing enhancer (Kan & Green, 1999). When the inhibitor is removed, splicing occurred both in the presence and absence of the splicing enhancer (Kan & Green, 1999). Kan and Green therefore proposed that the primary function of the splicing enhancer is to counteract the splicing inhibitor. However, data presented by Kan and Green (1999) supports our observation that in the absence of the splicing inhibitor, the splicing enhancer significantly improves the efficiency of splicing. We observe a low level of splicing in the absence of a bound activator in our IgM constructs, and a significant positive effect of the splicing enhancer. Together these results suggest that the IgM splicing enhancer has an important function in activating splicing of the IgM pre-mRNA that is separate from its function in counteracting the splicing inhibitor. We therefore conclude that, as with the *dsx* pre-mRNA, efficient splicing of the IgM M1-M2 intron requires a splicing enhancer to recruit components of the splicing machinery. The naturally occurring IgM splicing enhancer may, however, be bifunctional—one function is to recruit U2AF and another is to overcome the splicing inhibitor.

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides were used in this study:

180-16b: 5′-ATTTAGGTGACACTATAGAATCCCTACAAGTGGACAGC-3′;
180-16c: 5′-ATTTAGGTGACACTATAGAACCAATCAACTGGCTGTGAAG-3′;
180-16e: 5′-GCGTACCCTGATGGTGTACGCGGGAGTACTCATTCACAACG-3′;
180-63c: 5′-CCGGTCCTCGAGCCACCATGGCGGAGTATCTGGCC-3′;
180-63d: 5′-GGGGTACCTCAGAATCGCCCAGATCCTTC-3′;
180-72: 5′-GCGTACCCTGATGGTGTACGCATAAGTTTACTAGGGTCAC-3′;
180-92a: 5′-TCGAGTGTTTTTTTCCCTTTTTTTTTCCAGGTG-3′;
180-92b: 5′-GATCCACCTGGAAAAAAAAAGGGAAAAAAACAC-3′;
180-92c: 5′-TCGAGTTCACACTGTCTCTGTCACCTGCAGGTG-3′;
180-92d: 5′-CATCCACCTGCAGGTGACAGAGACAGTGTGAAC-3′;
180-93a: 5′-TCGAGAATGTCTCTCGATCTCTTCTCTGCCAGGCG-3′;
180-93b: 5′-CATCCGCCTGGCAGAGAAGAGATCGAGAGACATTC-3′;
180-93c: 5′-TCGAGAATGTCTCTCGATCTGATCTAAACCAGGCG-3′;
180-93d: 5′-GATCCGCCTGGTTTAGATCAGATCGAGAGACATTC-3′.

Proteins

The expression and purification of MS2, MS2-RS^{9G8} and MS2-RS^{p55} were described previously (Graveley et al., 1998; Graveley & Maniatis, 1998). U2AF^{65} was expressed in and purified from baculovirus infected Sf9 cells (a gift from Rajesh Gaur).

To produce recombinant U2AF heterodimer, a single baculovirus was constructed that simultaneously expresses unmodified U2AF^{35} and N-terminal His-tagged U2AF^{65}. To do this, the cDNA encoding U2AF^{35} was amplified by PCR with primers 180-63c and 180-63d. The PCR product was digested with *Xho*I and *Kpn*I and inserted into pFASTBAC-Dual to generate pFB-Dual-U2AF^{35}. Next, a *Bss*HI-*Hin*DIII fragment from pHIS-BIVT-H_6U2AF^{65} was ligated into pFB-Dual-U2AF^{35} to generate pFB-U2AF^{35}/H_6U2AF^{65}. The complete nucleotide sequence of this plasmid is available at http://penguin.uchc.edu/~intron. This plasmid was used to generate recombinant baculovirus as described by the manufacturer. The U2AF heterodimer was expressed by infecting Sf9 cells for 3 days. A pellet of 3.0×10^8 cells was resuspended in 20 mL lysis buffer (50 mM Tris, pH 8.0, 1% NP-40, 1 mM PMSF, and 1 μg/mL leupeptin) and sonicated for 30 s on ice. The lysate was cleared by centrifugation and the supernatant mixed with 0.5 mL Ni-NTA agarose for 2 h at 4 °C. The solution was then applied to an Econo-column (Bio-Rad) and the unbound material collected by gravity flow. The column was then washed with 20 mL wash buffer (50 mM Tris, pH 8.0, 500 mM NaCl) followed by 20 mL of second wash buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole). The bound protein was eluted with 5 mL of elution buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.5 M imidazole) and fractions of 1 mL were collected. The eluted protein was dialyzed overnight against BC100 (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA). U2AF^{35} appears to be stoichiometric with respect to U2AF^{65} as judged by the staining intensity of the two proteins on an SDS gel (see Fig. 5A, lane 2). The identities of the proteins were verified by immunoblots with anti-U2AF^{35} and anti-U2AF^{65} antibodies.

RNAs

IgM(20)M1 and *dsx*(70)M1 were described previously (Graveley et al., 1998). *dsx*-Py(70)M1 was previously described as *dsx*-(PY)MS2 (Graveley & Maniatis, 1998). The IgM-Py(20)M1 was generated by PCR with the SP6 primer and primer 180-72 using pJKμMΔ15 (used in Fig. 3, lane 2, of Kan & Green, 1999) as a template. The 3′ half substrates were all generated by PCR. IgM(20)M1-3′ was generated with primers 180-16b and 120-145a and pμMΔ as a template. IgM-Py(20)M1 was generated with primers 180-16b and 180-72, and pJKμMΔ15 as a template. *dsx*(20)M1-3′ was generated with primers 180-16c and 180-16e, and p*dsx*(70)M2 (Graveley et al., 1998) as a template. *dsx*-Py(20)M1-3′ was generated with primers 180-16c and 180-16e, and p*dsx*-Py(70)M1 as a template. All of the templates encoding the 3′ half substrates were transcribed with SP6 RNA polymerase. Plasmid DNAs were linearized and transcribed with either SP6 or T7 RNA polymerase and the RNAs gel purified.

To generate the plasmids encoding the various pyrimidine tracts, oligonucleotide pairs were annealed and ligated into the *Xho*I and *Bam*HI sites of pSP72. Oligonucleotides 180-92a and 180-92b were used to generate pSP72-Py(UP)IgM, 180-92c and 180-92d for pSP72-Py(WT)IgM, 180-93a and 180-93b for pSP72-Py(UP)*dsx*, and 180-93c and 180-93d for pSP72-Py(WT)*dsx*. pSP72-Py(UP)IgM and pSP72-Py(WT)IgM were digested with *Sma*I prior to transcription with SP6 RNA polymerase. pSP72-Py(UP)*dsx* and pSP72-Py(WT)*dsx* were digested with *Bam*HI prior to transcription with SP6 RNA polymerase. The complete nucleotide sequences of these RNAs are available at http://penguin.uchc.edu/~intron.

Splicing

In vitro splicing assays were performed as described (Graveley & Maniatis, 1998). Splicing assays with the U2AF-depleted extract was performed with 30% depleted extract in a volume of 12.5 μL.

Splicing complex formation

Spliceosomal complexes were formed on 5 fmol of RNA in 10 μL reactions under splicing conditions (see above). After incubation at 30 °C for 15 min, heparin was added to a final concentration of 0.5 mg/mL. The reactions were separated on a 4% (80:1) polyacrylamide gel in 1× Tris-glycine for 3–4 h at 4 W at room temperature (Konarska, 1989). The gels were dried under vacuum and exposed to film.

UV crosslinking

The experiments conducted to examine enhancer-dependent U2AF recruitment were performed as follows. Uniformly ^{32}P-labeled RNAs were incubated in either 10% or 20% nuclear extract in the presence or absence of MS2-RS under splicing conditions for 20 min at 30 °C. The reactions were then placed on ice and irradiated with UV-light for 10 min. The crosslinked reactions were then treated with RNase A, and subsequently incubated for 3–4 h at 0 °C with either U2AF^{65} (Gama-Carvalho et al., 1997) or U2AF^{35} (Zuo & Maniatis, 1996) antibodies. The reactions were then incubated overnight at 4 °C with anti-mouse (U2AF^{65}) or anti-rabbit (U2AF^{35}) IgG agarose. The beads were washed, the bound proteins eluted with 1× SDS-PAGE buffer and separated on a 10% SDS gel. The crosslinked proteins were visualized by exposing the dried gel to film.

To determine the binding affinity of U2AF^{65} for the various pyrimidine tracts, RNAs containing each pyrimidine tract were incubated with increasing concentrations of purified U2AF^{65} for 10 min at 30 °C. Following this incubation, the reactions were irradiated with UV light, treated with RNase, and resolved by SDS-PAGE. The gel was dried and quantitated with a phosphorimager.

Depletion of U2AF from nuclear extract

One milliliter of nuclear extract was brought to 1 M KCl and incubated on ice for 30 min. The extract was passed over an oligo-dT column equilibrated in 1 M buffer D (1 M KCl, 20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.05% NP-40). Fractions of 0.5 mL were collected and the protein concentrations measured by the Bradford assay. Peak fractions were dialyzed overnight against 100 mM Buffer D.

Antibodies

The rabbit anti-U2AF^{65} and anti-U2AF^{35} polyclonal antibodies were described previously (Zuo & Maniatis, 1996). The hybridoma cell line expressing the monoclonal antibody MC3 that recognizes U2AF^{65} (Gama-Carvalho et al., 1997) was obtained from Juan Valcárcel.

ACKNOWLEDGMENTS

We thank Doug Melton for sharing laboratory space, Julie Kan and Michael Green for providing several IgM clones, and Rajesh Gaur for assistance in the U2AF depletion and the gift of purified recombinant U2AF^{65}. We also thank Juan Valcárcel, Sabine Guth, and Adrian Krainer for communicating results prior to publication. This work was supported by postdoctoral fellowships from the Jane Coffin Childs Memorial Fund for Medical Research (B.R.G. and K.J.H.) and a National Institutes of Health grant (GM42231) (T.M.).

Received March 5, 2001; returned for revision March 23, 2001; revised manuscript received April 3, 2001

REFERENCES

Bennett M, Michaud S, Kingston J, Reed R. 1992. Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes & Dev 6*:1986–2000.

Berglund JA, Abovich N, Rosbash M. 1998. A cooperative interaction between U2AF^{65} and mBBP/SF1 facilitates branchpoint region recognition. *Genes & Dev 12*:858–867.

Blencowe BJ. 2000. Exonic splicing enhancers: Mechanism of action, diversity and role in human genetic diseases. *Trends Biochem Sci 25*:106–110.

Bouck J, Fu X-D, Skalka AM, Katz RA. 1998. Role of the constitutive splicing factors U2AF^{65} and SAP49 in suboptimal RNA splicing of novel retroviral mutants. *J Biol Chem 273*:15169–15176.

Burge CB, Tuschl T, Sharp PA. 1999. Splicing of precursors to mRNAs by the spliceosomes. In: Gesteland RF, Cech TR, Atkins JF, eds. *The RNA world*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.

Burtis KC, Baker BS. 1989. *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell 56*:997–1010.

Eldridge AG, Li Y, Sharp PA, Blencowe BJ. 1999. The SRm160/300 splicing coactivator is required for exon-enhancer function. *Proc Natl Acad Sci USA 96*:6125–6130.

Gama-Carvalho M, Krauss RD, Chiang L, Valcárcel J, Green MR, Carmo-Fonseca M. 1997. Targeting of U2AF^{65} to sites of active splicing in the nucleus. *J Cell Biol 137*:975–987.

Gozani O, Potashkin J, Reed R. 1998. A potential role for U2AF-SAP 155 interactions in recruiting U2 snRNP to the branch site. *Mol Cell Biol 18*:4752–4760.

Graveley BR. 2000. Sorting out the complexity of SR protein functions. *RNA 6*:1197–1211.

Graveley BR, Hertel KJ, Maniatis T. 1998. A systematic analysis of the factors that determine the strength of pre-mRNA splicing enhancers. *EMBO J 17*:6747–6756.

Graveley BR, Maniatis T. 1998. Arginine/serine-rich domains of SR proteins can function as activators of pre-mRNA splicing. *Mol Cell 1*:765–771.

Guth S, Martinez C, Gaur RK, Valcárcel J. 1999. Evidence for substrate-specific requirement of the splicing factor U2AF^{35} and for its function after polypyrimidine tract recognition by U2AF^{65}. *Mol Cell Biol 19*:8263–8271.

Hoshijima K, Inoue K, Higuchi I, Sakamoto H, Shimura Y. 1991. Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in *Drosophila*. *Science 252*:833–836.

Kan JLC, Green MR. 1999. Pre-mRNA splicing of IgM exons M1 and M2 is directed by a juxtaposed splicing enhancer and inhibitor. *Genes & Dev 13*:462–471.

Kanaar R, Roche SE, Beall EL, Green MR, Rio DC. 1993. The conserved pre-mRNA splicing factor U2AF from *Drosophila*: Requirement for viability. *Science 262*:569–573.

Konarska MM. 1989. Analysis of splicing complexes and small nuclear ribonucleoprotein particles by native gel electrophoresis. *Methods Enzymol 180*:442–453.

Li Y, Blencowe BJ. 1999. Distinct factor requirements for exonic splicing enhancer function and binding of U2AF to the polypyrimidine tract. *J Biol Chem 274*:35074–35079.

Lorson CL, Androphy EJ. 2000. An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum Mol Genet 9*:259–265.

Merendino L, Guth S, Bilbao D, Martinez C, Valcárcel J. 1999. Inhibition of *msl-2* splicing by Sex-lethal reveals interaction between U2AF^{35} and the 3′ splice site AG. *Nature 402*:838–841.

Reed R. 1989. The organization of 3′ splice-site sequences in mammalian introns. *Genes & Dev 3*:2113–2123.

Reed R. 2000. Mechanisms of fidelity in pre-mRNA splicing. *Curr Opin Cell Biol 12*:340–345.

Rudner DZ, Breger KS, Kanaar R, Adams MD, Rio DC. 1998a. RNA binding activity of heterodimeric splicing factor U2AF: At least one RS domain is required for high-affinity binding. *Mol Cell Biol 18*:4004–4011.

Rudner DZ, Breger KS, Rio DC. 1998b. Molecular genetic analysis of the heterodimeric splicing factor U2AF: The RS domain on either the large or small *Drosophila* subunit is dispensable in vivo. *Genes & Dev 12*:1010–1021.

Rudner DZ, Kanaar R, Breger KS, Rio DC. 1996. Mutations in the small subunit of the *Drosophila* U2AF splicing factor cause lethality and developmental defects. *Proc Natl Acad Sci USA 93*:10333–10337.

Rudner DZ, Kanaar R, Breger KS, Rio DC. 1998c. Interaction between subunits of heterodimeric splicing factor U2AF is essential in vivo. *Mol Cell Biol 18*:1765–1773.

Smith CW, Valcárcel J. 2000. Alternative pre-mRNA splicing: The logic of combinatorial control. *Trends Biochem Sci 25*:381–388.

Staknis D, Reed R. 1994. SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. *Mol Cell Biol 14*:7670–7682.

Tacke R, Manley JL. 1999. Determinants of SR protein specificity. *Curr Opin Cell Biol 11*:358–362.

Tian M, Maniatis T. 1994. A splicing enhancer exhibits both constitutive and regulated activities. *Genes & Dev 8*:1703–1712.

Valcárcel J, Gaur RK, Singh R, Green MR. 1996. Interaction of U2AF^{65} RS region with pre-mRNA of branch point and promotion base pairing with U2 snRNA. *Science 273*:1706–1709.

Wang Z, Hoffmann HM, Grabowski PJ. 1995. Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. *RNA 1*:21–35.

Watakabe A, Tanaka K, Shimura Y. 1993. The role of exon sequences in splice site selection. *Genes & Dev 7*:407–418.

Wu JY, Maniatis T. 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell 75*:1061–1070.

Wu S, Romfo CM, Nilsen TW, Green MR. 1999. Functional recognition of the 3′ splice site AG by the splicing factor U2AF^{35}. *Nature 402*:832–835.

Zamore PD, Green MR. 1989. Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc Natl Acad Sci USA 86*:9243–9247.

Zamore PD, Green MR. 1991. Biochemical characterization of U2 snRNP auxiliary factor: An essential pre-mRNA splicing factor with a novel intranuclear distribution. *EMBO J 10*:207–214.

Zamore PD, Patton JG, Green MR. 1992. Cloning and domain structure of the mammalian splicing factor U2AF. *Nature 355*:609–614.

Zhang M, Zamore PD, Carmo-Fonseca M, Lamond AI, Green MR. 1992. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. *Proc Natl Acad Sci USA 89*:8769–8773.

Zhang W-J, Wu JY. 1996. Functional properties of p54, a novel SR protein active in constitutive and alternative splicing. *Mol Cell Biol 16*:5400–5408.

Zhu J, Krainer AR. 2000. Pre-mRNA splicing in the absence of an SR protein RS domain. *Genes & Dev 14*:3166–3178.

Zorio DA, Blumenthal T. 1999. Both subunits of U2AF recognize the 3′ splice site in *Caenorhabditis elegans*. *Nature 402*:835–838.

Zuo P, Maniatis T. 1996. The splicing factor U2AF^{35} mediates critical protein–protein interactions in constitutive and enhancer-dependent splicing. *Genes & Dev 10*:1356–1368.