Substrate-dependent Differences in U2AF Requirement for Splicing in Adenovirus-infected Cell Extracts*§

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U2AF has been characterized as an essential splicing factor required for efficient recruitment of U2 small nuclear ribonucleoprotein to the 3′-splice site in a pre-mRNA. The U2AF65 subunit binds to the pyrimidine tract of the pre-mRNA, whereas the U2AF35 subunit contacts the 3′-splice site AG. Here we show that U2AF35 appears to be completely dispensable for splicing in nuclear extracts prepared from uninfected late-infected cells (Ad-NE). As a consequence, the viral IIIa and cellular IgM introns, which both have suboptimal 3′-splice sites and require U2AF35 for splicing in nuclear extracts from uninfected cells, are transformed to U2AF35-independent (13, 15).

Several sequence elements help to define the 3′-splice site: the branchpoint sequence, which is followed by a pyrimidine-rich sequence (pyrimidine tract) and a conserved AG dinucleotide at the 3′-end of the intron. The critical step in 3′-splice site definition is the stable recruitment of U2 snRNP to the 3′-splice site. U2 snRNP binds to the branchpoint sequence through a base pairing interaction with this sequence and U2 snRNA (reviewed in Ref. 4). Binding of U2 snRNP requires auxiliary splicing factors such as SF1/mBBP and U2AF (5). U2AF is a heterodimer consisting of a 65- and a 35-kDa subunit. The U2AF65 subunit binds specifically to the pyrimidine tract through its RNA recognition domains (6), whereas U2AF35 has been shown to make contact with the AG dinucleotide at the 3′-splice site (7–9).

In fact, introns appear to fall into one of two classes: AG-dependent introns and AG-independent introns (10). The AG-dependent introns typically have weak pyrimidine tracts, which make unstable interactions with U2AF65. In such introns, the U2AF35 interaction with the 3′-splice site AG dinucleotide becomes important, by stabilizing the U2AF65 interaction with the pyrimidine tract (11). In contrast, AG-independent introns do not require U2AF35 for activity. In such introns, binding of U2AF65 to the strong pyrimidine tract is usually sufficient to aid in the recruitment of U2 snRNP to the branchpoint sequence (7).

We are using the adenovirus major late region 1 (L1) as a model pre-mRNA to study the mechanisms controlling alternative splice site usage in adenovirus-infected cells. In the L1 unit, a common 5′-splice site can be joined to two alternative 3′-splice sites, resulting in the formation of the so-called 52,55K (proximal 3′-splice site) or IIIa (distal 3′-splice site) mRNAs. Early during virus infection, the 52,55K 3′-splice site is used exclusively, whereas the IIIa splice site becomes the preferred site in late virus-infected cells (reviewed in Ref. 12). Our previous work has demonstrated that regulated expression of the IIIa mRNA is, to a large extent, controlled by two sequence elements: a 49-nucleotide-long repressor element (the 3RE), which binds the SR family of splicing factors; and a 28-nucleotide-long virus infection-dependent splicing enhancer (the 3VDE).

Although we know much about the function of the 3RE (reviewed in Ref. 12), it appears clear that the 3VDE is the major element responsible for the enhanced splicing phenotype of the IIIa mRNA late during virus infection (13). The 3VDE is the minimal element required to confer an enhanced splicing phenotype to a heterologous transcript in nuclear extracts prepared from late virus-infected cells (Ad-NE). The 3VDE consists of the IIIa branch point sequence, pyrimidine tract, and AG dinucleotide. A limited mutational analysis of the 3VDE further revealed that the IIIa pyrimidine tract is a critical element required for the activated splicing of the IIIa pre-mRNA in Ad-NE (13, 14). However, much to our surprise, the increase in IIIa splicing did not correlate with an increased binding of U2AF65 to the IIIa pyrimidine tract. Collectively, our results have suggested that the splicing enhancer activity of 3VDE may operate through a mechanism that might, in fact, be U2AF-independent (13, 15).

Here we extend these studies by examining the significance of
plasmids encoding for transcripts IIIa (pGDIIIa), AdML (pBSAd1), 52,55K (pGD52,55K), and IgM and IIa-GA/C transcripts. The conditions for splicing were as described above.

**Spliceosome Assembly**—Standard splicing reactions were set up using Ad-NE or Ad(ΔU2AF) and the 32P-labeled IgM-GA/C transcript. Reactions were incubated for 30 min at 30 °C, mixed with heparin (final concentration, 0.5 μg/μl), and resolved on a 4% (80:1 acrylamide: bisacrylamide) native polyacrylamide gel, which was cast in a buffer containing 50 mM Tris-glycine and 5% glycerol. The gel was run in a cold room at 350 V for ~2 h in the Tris-glycine buffer lacking glycerol.

**Western Blot**—The extent of U2AF depletion was tested for each detailed extract preparation by Western blotting comparing it with serial dilutions of 10 μl of HeLa-NE or AD-NE (both with a total protein concentration of 10 mg/ml). Extracts were subjected to SDS-PAGE on a 12.5% gel under reducing conditions and transferred to a nitrocellulose membrane using a semidy transfer apparatus (Bio-Rad). U2AF was detected using a polyclonal anti-U2AF35 antibody (11) or a monoclonal anti-U2AF65 antibody (MC3) (24) and visualized by chemiluminescence according to the manufacturer’s protocol (Fierce).

**RESULTS**

**IIIa Splicing Becomes AG-independent in Nuclear Extract Prepared from Adenovirus-infected HeLa Cells**—Introns containing weak pyrimidine tracts are typically AG-dependent. In such pre-mRNAs, the 3′-splice site AG dinucleotide must be recognized by U2AF35 prior to the first catalytic step of the splicing reaction. In contrast, removal of AG-independent introns requires the AG dinucleotide only during the second catalytic step. Therefore, AG-independent introns can still undergo the first catalytic step of splicing in transcripts where the 3′-splice site AG has been mutated to GA. Such interrupted splicing reactions accumulate the characteristic exon 1 and exon 2-intron lariat at splicing inefficiency sites (2).

The adenovirus IIIa splice site is considered to be a weak 3′-splice site because it has a short pyrimidine tract and is inefficiently spliced in HeLa-NE (26). According to the current model, this would suggest that the IIIa 3′-splice site should be classified as an AG-dependent intron. To test this hypothesis, we mutated the IIIa 3′-splice site from AG/A to GA/C (Fig. 1, IIa and IIa-GA/C). We also changed the first nucleotide of the second exon because previous work has shown that this nucleotide has a strong influence on U2AF35 binding, with cytidine being less efficient in recruiting U2AF35 compared with other nucleotides (7).
As a control for an AG-independent substrate, we used the adenovirus major late first intron (AdML), which is often used as a prototypical AG-independent intron (7, 16, 17). The major late intron contains a very strong pyrimidine tract (Fig. 1, AdML). For the same reason as described above, we changed the AdML 3’-splice site from AGA to GA/C (Fig. 1, AdML and AdML-GA/C). All four substrates were tested in in vitro splicing reactions using HeLa-NE or Ad-NE.

The experimental conditions were selected such that the IIIa and AdML wild type substrates should be spliced with a reasonable efficiency in both HeLa-NE and Ad-NE (Fig. 2, A and B, lanes 1 and 2). As has been shown for HeLa-NE (7, 16, 17), and as one would have expected for Ad-NE, the mutated AdML-GA/C substrate could still undergo the first catalytic step of splicing in both types of extracts (Fig. 2B, lanes 3 and 4).

In contrast, mutating the AG dinucleotide at the IIIa 3’-splice site abolished all splicing activity in HeLa-NE (Fig. 2A, compare lanes 1 and 3), suggesting that IIIa, as predicted because of its weak sequence context (26), is an AG-dependent substrate. Interestingly, incubation of the IIIa-GA/C transcript in Ad-NE resulted in a very efficient accumulation of the first exon and the exon 2-lariat splicing intermediate (Fig. 2A, lane 4). This result was surprising because it suggests that the IIIa pre-mRNA was converted from an AG-dependent intron in HeLa-NE (Fig. 2A, lanes 1 and 3) to an AG-independent intron in Ad-NE (Fig. 1A, lane 4). It is noteworthy that the accumulation of splicing intermediates did not result from a general increase of the splicing activity in Ad-NE, as shown by the reactions using the wild type AdML substrate (Fig. 2B, compare lanes 1 and 2). In some experiments, splicing of the IIIa-GA/C transcript produced faint bands migrating at the position of the authentic spliced mRNA and one band migrating slightly faster (Fig. 2A, lane 4). Reverse transcription-PCR analysis showed that the faster migrating species was a minor aberrant product resulting from the use of a cryptic AG dinucleotide located 10 nucleotides downstream of the authentic IIIa 3’-splice site (data not shown). No cDNA product corresponding to an authentic splicing event was observed with the IIIa-GA/C transcript, suggesting that the slower migrating species most likely was a breakdown product.

To investigate whether the weak pyrimidine tract of IIIa (26) was the critical element in the IIIa pre-mRNA responsible for the AG-dependent splicing in HeLa-NE, we replaced 3VDE with the branch site and pyrimidine tract from the rabbit β-globin second intron. In addition, the globin 3’-splice site was mutated from AGA to GA/C. As shown in Fig. 2C, splicing intermediates were visible in both HeLa-NE and Ad-NE with the IIIa(-3VDE)GA/C transcript, indicating, as one would expect, that a strong pyrimidine tract can convert the IIIa transcript into an AG-independent substrate also in HeLa-NE.

IgM Becomes an AG-independent Substrate in Nuclear Extract Prepared from Adenovirus-infected Cells—Hypothetically, the AG-independent splicing of the IIIa pre-mRNA in Ad-NE might be part of a viral strategy to enhance splicing of introns with a weak 3’-splice site context, a feature typical for many of the late viral 3’-splice sites. To test this hypothesis, we used the well-characterized immunoglobulin μ pre-mRNA substrate in our assay system (17, 27, 28) (Fig. 1, IgM and IgM-GA/C). This pre-mRNA has been characterized as an AG-dependent substrate, which requires binding of U2AF35 to its 3’-splice site for activity (17, 28). As expected from previous results, splicing of the IgM transcript in HeLa-NE was abolished when the 3’-splice site AG was mutated to GA (Fig. 3, lane 3). In contrast, incubating the IgM-GA/C transcript in Ad-NE resulted in an efficient accumulation of exon 1 and the exon 2-intron lariat splicing intermediates characteristic of the first catalytic step of splicing (Fig. 3, lane 4). We conclude that the IgM pre-mRNA, like the IIIa pre-mRNA, was converted to an AG-independent substrate in Ad-NE. Collectively, our results suggest that the cellular splicing machinery may have undergone a major shift in activity during virus infection, a shift that is not specific to viral transcripts.

U2AF Is Required for Splicing of the Strong 52,55K Intron in Nuclear Extracts Prepared from Infected Cells—We have previously shown that introns with a weak 3’-splice site context are activated in Ad-NE, whereas introns with a strong pyrimidine content are slightly repressed (14). The results presented here suggest that splicing of both types of introns becomes U2AF35-independent in Ad-NE. This finding prompted us to investigate whether the U2AF heterodimer was necessary for splicing in Ad-NE. For this experiment, U2AF was depleted from HeLa-NE and Ad-NE by oligo(dT)-cellulose chromatography (6, 29). This protocol has previously been shown to remove both U2AF subunits almost to completion (21). The bound
U2AF-independent Splicing

FIG. 4. Efficient depletion of U2AF65 and U2AF35 from HeLa-NE (NE) and Ad-NE by oligo(dT)-cellulose chromatography. A, a Western blot in which the amount of U2AF subunits in the depleted extracts (lanes U2AF) is compared with serial dilutions of HeLa-NE (100% to 1%) and Ad-NE (100% to 1%), the 2.5 M KCl wash fraction (Wash), and the 2 M guanidine-HCl eluate (Eluted U2AF). The different fractions were separated on a 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with antibodies against U2AF65 and U2AF35. B, a longer exposure of the Western blot shown in A.

U2AF was subsequently eluted from the column and used for reconstitution experiments. As shown in Fig. 4A, the depletion protocol was effective and removed U2AF65 and U2AF35 to undetectable levels in the NE(U2AF) and Ad(U2AF) extracts, respectively (i.e. far below 1% of the initial amount; see Fig. 4B for a longer exposure).

In the first set of experiments, we tested whether U2AF was necessary for splicing of an intron containing a consensus type of 3′-splice site. For this experiment, we choose the 52.55K pre-mRNA, which has an extended polypyrimidine tract (Fig. 1) that binds U2AF65 efficiently (14). As shown in Fig. 5, depletion of U2AF from either HeLa-NE or Ad-NE abolished 52.55K splicing completely (Fig. 5, lanes 2 and 6). Re-addition of U2AF eluted from the oligo(dT) column restored 52.55K pre-mRNA splicing in both the depleted HeLa-NE and Ad-NE (Fig. 5, lanes 3 and 7). Taken together, these results are in agreement with the conclusion that splicing of an intron containing a strong polypyrimidine tract (Fig. 1) requires U2AF for activity in both virus-infected and uninfected extracts. However, we found one interesting difference: supplementing HeLa-NE depleted of U2AF with a recombinant U2AF65 protein did not restore 52.55K splicing. In contrast, the U2AF65 protein was able to restore 52.55K splicing in U2AF-depleted Ad-NE (Fig. 5, lane 8). This finding adds further support to our conclusion that 3′-splice site recognition is altered in Ad-NE.

The weak IgM Intron Is Spliced in U2AF-depleted Ad-NE—We have previously observed an inverse correlation between the efficiency of IIIa splicing and the stable recruitment of U2AF65 to the weak IIIa pyrimidine tract (13, 14). This result may suggest that splicing of introns with a weak sequence context, as in the viral IIIa and also the cellular IgM pre-mRNAs, might occur in the absence of U2AF in Ad-NE. To test this hypothesis, our preferred transcript would have been the IIIa pre-mRNA. However, for unknown reasons, we have not been able to recover IIIa splicing in oligo(dT)-depleted extracts by any combination of reconstitution experiments (see Supplementary Fig. 1). Much additional work is needed to uncover what IIIa-specific factor(s) is lost/inactivated during the oligo(dT) fractionation of nuclear extracts.

Because reconstitution of IIIa splicing was not possible in the depleted extracts, we continued our analysis using the IgM pre-mRNA. The IgM intron resembles a typical late viral intron in that it has a 3′-splice site with a poor pyrimidine tract (Fig. 1). As shown in Fig. 6A, depletion of U2AF from HeLa-NE resulted in a complete loss of IgM splicing (lane 2). However, IgM splicing was fully restored by addition of the eluted NE-U2AF to the depleted NE(U2AF) extract (Fig. 6A, lane 3). In contrast, in splicing reactions supplemented with the IgM-GA/C mutant transcript, no splicing intermediates were observed even in the presence of native U2AF eluted from the oligo(dT)-cellulose column (Fig. 6A, lanes 5–8). As reported previously (17), and consistent with our results, this finding indicates that IgM splicing is AG-dependent and requires the U2AF heterodimer for activity in HeLa-NE.

Interestingly, incubation of the IgM pre-mRNA in Ad(U2AF) resulted in a significant accumulation of the free exon 1 splicing intermediate (Fig. 6B, lane 2). This result is noteworthy because it suggests that neither U2AF65 nor U2AF35 is required for the first catalytic step of IgM splicing in Ad-NE. Supplementing the reactions with purified U2AF fully restored IgM splicing (Fig. 6B, lane 3). This result indicates that U2AF is capable of stimulating IgM splicing in the presence of a functional 3′-splice site, even if it appears not to be essential for the first catalytic step of splicing in Ad-NE (Fig. 3B, lane 4).

To test this hypothesis further, we analyzed the splicing phenotype of the IgM-GA/C transcript in Ad(U2AF). As shown in Fig. 6B (lane 6), the exon 1 splicing intermediate accumulated essentially with the same efficiency as in the non-depleted Ad-NE (lane 5). Interestingly, the IgM splicing activity did not increase in reactions supplemented with isolated U2AF (Fig. 6B, lane 8). This result is important because it supports the conclusion that U2AF is not required for the first catalytic step of IgM splicing in Ad-NE. Furthermore, this result makes it unlikely that the first step of splicing observed with the IgM-GA/C pre-mRNA is caused by residual amounts of U2AF in the depleted extract. It is noteworthy that the lariat exon 2 intermediate diminished significantly in Ad(AU2AF)
compared with Ad-NE (compare lane 5 and lanes 6–8 in Fig. 6B). The most likely explanation is that U2AF-depleted extracts have a higher debranching activity. However, it is important to note that low levels of the lariat exon 2 intermediate are detectable in Ad(ΔU2AF) in a splicing-dependent manner (see below and Supplementary Fig. 2).

Requirement of a Functional U2 snRNP for IgM Splicing in U2AF-depleted Ad-NE—The finding that IgM splicing appears to be U2AF-independent in Ad-NE was intriguing. Therefore, we tried to find evidence that the accumulation of the band with a size of the IgM exon 1 in Ad(ΔU2AF) extracts was due to splicing and was not a breakdown product that for unfortunate reasons had the same size as exon 1. To test this, we used oligonucleotide-directed cleavage of U2 snRNA to determine whether accumulation of the first exon band was dependent on splicing and was not a breakdown product that for unfortunate reasons had the same size as exon 1. To test this, we used oligonucleotide-directed cleavage of U2 snRNA to determine whether accumulation of the first exon band was dependent on splicing and was not a breakdown product that for unfortunate reasons had the same size as exon 1.

This conclusion is further supported by the observation that U2AF is not functionally inactivated as a general splicing factor during an adenovirus infection. This conclusion is further supported by the observation that U2AF is not functionally inactivated as a general splicing factor during an adenovirus infection. To address the question of whether U2AF is post-translationally inactivated during virus infection, we compared the capacity of NE-U2AF and Ad-U2AF to activate IgM splicing in the two types of depleted extracts (NE(ΔU2AF) and Ad(ΔU2AF)). As shown in Fig. 6, both U2AF fractions showed an essentially indistinguishable capacity to activate splicing. Thus, NE-U2AF and Ad-U2AF were efficiently activating IgM wild type splicing in both NE(ΔU2AF) and Ad(ΔU2AF) (Fig. 6A, 6B, lanes 1 and 2). Also, NE-U2AF and Ad-U2AF were both unable to activate IgM-GA/C splicing in NE(ΔU2AF) (Fig. 6A, lanes 5 and 6). Similarly, NE-U2AF and Ad-U2AF were both unable to stimulate the first catalytic step of IgM-GA/C splicing in Ad(ΔU2AF) (Fig. 6B, lanes 7 and 8). Collectively, these results suggest that U2AF is not functionally inactivated as a general splicing factor during an adenovirus infection. This conclusion is further supported by the observation that both HeLa-U2AF and Ad-U2AF are able to activate splicing of the 52,55K pre-mRNA that contains a consensus type of 3′-splice site (Fig. 5).
observed the opposite, a reduced binding of U2AF in Ad-NE. This and other observations have led us to speculate that splicing of certain suboptimal pre-mRNAs may operate without efficient U2AF recruitment and potentially may be U2AF-independent (13, 15).

Here, we have further investigated the significance of U2AF for splicing in Ad-NE. Although we have only used a few sub-,

strate RNAs, the evidence that is accumulating appears to

provide strong support for the hypothesis that 3'-splice site rec-

ognition differs substantially in Ad-NE compared with ex-

tracts prepared from uninfected cells. In fact, our results sug-

gest that two parallel pathways of 3'-splice site recognition

exist in late adenovirus-infected cells. The normal U2AF-de-

pendent recognition of the pyrimidine tract is functional, as
demonstrated by the fact that 52,55K splicing requires U2AF
for activity in Ad-NE (Fig. 5). This conclusion is further sup-

ported by the observation that U2AF isolated from Ad-NE is
fully functional, and it is as active as U2AF isolated from

uninfected HeLa cells in reconstituting IgM (Fig. 6) and 52,55K
splicing (Supplementary Fig. 1) in U2AF-depleted infected or

uninfected extracts. Thus, it seems unlikely that U2AF is a

target for virus-induced post-translational inactivation, as is
the case for the SR family of splicing factors (30). It appears
likely that pre-mRNAs with consensus-type introns continue
to use the U2AF pathway in Ad-NE.

More interestingly, our results provide evidence for a second alternative assembly pathway in Ad-NE, which appears to
have a relaxed U2AF requirement and, on certain transcripts,
may be U2AF-independent. For example, we show that the AG

dinucleotide, which appears to be a signature for the require-

ment of the U2AF55 subunit in 3'-splice site definition (7–9), is

dispensable for the first catalytic step of splicing in Ad-NE. A

virus-induced shift to AG-independent splicing was observed

on two substrates, the viral IIIa (Fig. 2A) and the cellular IgM

(Fig. 3) pre-mRNAs, which both contain 3'-splice sites with a

suboptimal sequence context (Fig. 1) and therefore are AG-de-
pendent in HeLa-NE (Figs. 2A and 3). As expected, splicing of

substrates containing strong pyrimidine tracts, which are al-
dready AG-independent in HeLa-NE, were also AG-independent

in Ad-NE (Fig. 2, B and C). Secondly, we show that the first
catalytic step of IgM splicing occurs in Ad-NE depleted of U2AF
(Fig. 6B). This observation was interesting because depletion
of U2AF from HeLa-NE completely abolished IgM splicing (Fig.
6A; see also Ref. 17). The step 1 splicing activity of the IgM-

GA/C mutant transcript was not stimulated by re-addition of
a fraction highly enriched in U2AF to the depleted Ad-NE, a
result that argues strongly against the hypothesis that trace
amounts of U2AF in the depleted Ad-NE are responsible for the
efficient first step of splicing in the depleted Ad-NE. Thirdly,
we show that the viral 52,55K pre-mRNA, which requires

U2AF for activity in both HeLa-NE and Ad-NE (Fig. 5), is also
activated by a recombinant U2AF65 protein. However, this
activation is only observed in Ad-NE (Fig. 5). Taken together,
these results provide strong support for the hypothesis that
3'-splice site recognition differs significantly in uninfected ver-
sus adenovirus-infected cells. Thus, a parallel, potentially
U2AF-independent splicesome assembly pathway appears to
exist in adenovirus-infected cells. This pathway appears to
favor splicing of introns with a weak 3'-splice site context, a
feature typical for many of the adenoviral introns that are
activated at late times of infection (14).

To the best of our knowledge, there are only two examples of
U2AF-independent splicing described in the literature. First,
MUD2, which is the yeast homolog of U2AF65 (31), is not an
essential gene (32). Second, splicing of a chimeric major late/β-globin hybrid transcript has been reported to take place in a
U2AF-depleted extract supplemented with high amounts of the
SR protein SC35 (33). Although formally possible, it appears
unlikely that an SR protein takes over the function of U2AF in adenovirus-infected cells because available data suggest that SR proteins are functionally inactivated as splicing factors late during an adenovirus infection through a virus-induced dephosphorylation (30, 34). In conclusion, our data support the hypothesis that an alternative 3′-splice site binding factor of viral or cellular origin may replace the classical U2AF heterodimer in spliceosome assembly on certain transcripts in Ad-NE. Despite extensive efforts, we have not been able to biochemically purify the elusive component that appears to substitute for U2AF in spliceosome assembly on the non-consensus type of 3′-splice sites in Ad-NE. However, this work is clearly at the center of our current interest.

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