Most pre-mRNAs in higher eukaryotes contain multiple introns that are excised by the spliceosome to generate mature mRNA (Konarska and Query 2005; Smith et al. 2008; Wahl et al. 2009). The spliceosome, composed of five snRNAs (U1, U2, U4, U5, and U6) and many proteins, undergoes a series of dynamic changes during the splicing pathway (Staley and Guthrie 1998; Smith et al. 2008; Wahl et al. 2009). In the early steps, U1 small nuclear ribonucleoprotein (snRNP) binds to the pre-mRNA, and U1 snRNA base-pairs to the 5’ splice site to form the ATP-independent early [E] complex (Reed 1996; Smith et al. 2008; Wahl et al. 2009). U2 snRNP is also recruited to the E complex, but is not tightly bound (Das et al. 2006). We conclude that E7 results in formation of a defective spliceosome in which U2 snRNP fails to bind tightly to pre-mRNA. Our data indicate that E7 does not disrupt U2 snRNP or its association with SF3b, but does block an ATP-dependent remodeling event that exposes the BBR of U2 snRNA. We confirm that SF3b is required to remodel U2 snRNP to expose the BBR, and this event may allow the SF3b- and ATP-dependent tight binding of U2 snRNP to the pre-mRNA. These results provide important new insights into the mechanism of action of E7, and should aid in elucidating the novel antitumor properties of this drug.

Results and Discussion

E7 results in formation of a defective, labile spliceosome

To determine how E7 blocks splicing, we used a coupled RNA polymerase II [RNAP II] transcription/splicing system (Das et al. 2006). When a CMV-Ftz DNA template encoding one intron and two exons (Fig. 1A) was incubated in the presence of E7, transcription occurred normally, but splicing was blocked in a dose-dependent manner (Fig. 1B, lanes 1–8). Splicing was not affected by a negative control compound, pladienolide F (PlaF), and was the same as with no drug treatment (Fig. 1B, lanes 9–18). Significantly, however, when CMV-Ftz was first transcribed by RNAP II, followed by addition of the drug, splicing was not affected by E7 (or PlaF) (Fig. 1C). Previous work showed that the spliceosome assembles immediately on pre-mRNA when synthesized in the coupled system (Das et al. 2006). Thus, the data in Figure 1C indicate that E7 inhibits spliceosome assembly. We obtained the same results with CMV-AdML, indicating that the effects of E7 are general (Supplemental Fig. 1).

We next directly assayed for the spliceosome by transcribing CMV-Ftz in the coupled system and analyzing the reactions on a native gel. Surprisingly, the spliceosome had the same mobility whether assembled in E7 or PlaF (Fig. 2A). Moreover, the spliceosome assembled in E7 was splice site-dependent, as it did not form on a CMV-Δss transcript, which lacks functional splice sites and assembles into a nonspecific hnRNP complex (Fig. 2B; Das et al. 2006). We conclude that E7 results in formation of a complex that requires splicing signals and comigrates with no drug treatment (Fig. 1B, lanes 9–18). Significantly, however, when CMV-Ftz was first transcribed by RNAP II, followed by addition of the drug, splicing was not affected by E7 (or PlaF) (Fig. 1C). Previous work showed that the spliceosome assembles immediately on pre-mRNA when synthesized in the coupled system (Das et al. 2006). Thus, the data in Figure 1C indicate that E7 inhibits spliceosome assembly. We obtained the same results with CMV-AdML, indicating that the effects of E7 are general (Supplemental Fig. 1).

We next directly assayed for the spliceosome by transcribing CMV-Ftz in the coupled system and analyzing the reactions on a native gel. Surprisingly, the spliceosome had the same mobility whether assembled in E7 or PlaF (Fig. 2A). Moreover, the spliceosome assembled in E7 was splice site-dependent, as it did not form on a CMV-Δss transcript, which lacks functional splice sites and assembles into a nonspecific hnRNP complex (Fig. 2B; Das et al. 2006). We conclude that E7 results in formation of a complex that requires splicing signals and comigrates with no drug treatment (Fig. 1B, lanes 9–18). Significantly, however, when CMV-Ftz was first transcribed by RNAP II, followed by addition of the drug, splicing was not affected by E7 (or PlaF) (Fig. 1C). Previous work showed that the spliceosome assembles immediately on pre-mRNA when synthesized in the coupled system (Das et al. 2006). Thus, the data in Figure 1C indicate that E7 inhibits spliceosome assembly. We obtained the same results with CMV-AdML, indicating that the effects of E7 are general (Supplemental Fig. 1).

We next directly assayed for the spliceosome by transcribing CMV-Ftz in the coupled system and analyzing the reactions on a native gel. Surprisingly, the spliceosome had the same mobility whether assembled in E7 or PlaF (Fig. 2A). Moreover, the spliceosome assembled in E7 was splice site-dependent, as it did not form on a CMV-Δss transcript, which lacks functional splice sites and assembles into a nonspecific hnRNP complex (Fig. 2B; Das et al. 2006). We conclude that E7 results in formation of a complex that requires splicing signals and comigrates with no drug treatment (Fig. 1B, lanes 9–18). Significantly, however, when CMV-Ftz was first transcribed by RNAP II, followed by addition of the drug, splicing was not affected by E7 (or PlaF) (Fig. 1C). Previous work showed that the spliceosome assembles immediately on pre-mRNA when synthesized in the coupled system (Das et al. 2006). Thus, the data in Figure 1C indicate that E7 inhibits spliceosome assembly. We obtained the same results with CMV-AdML, indicating that the effects of E7 are general (Supplemental Fig. 1).

We next directly assayed for the spliceosome by transcribing CMV-Ftz in the coupled system and analyzing the reactions on a native gel. Surprisingly, the spliceosome had the same mobility whether assembled in E7 or PlaF (Fig. 2A). Moreover, the spliceosome assembled in E7 was splice site-dependent, as it did not form on a CMV-Δss transcript, which lacks functional splice sites and assembles into a nonspecific hnRNP complex (Fig. 2B; Das et al. 2006). We conclude that E7 results in formation of a complex that requires splicing signals and comigrates with no drug treatment (Fig. 1B, lanes 9–18). Significantly, however, when CMV-Ftz was first transcribed by RNAP II, followed by addition of the drug, splicing was not affected by E7 (or PlaF) (Fig. 1C). Previous work showed that the spliceosome assembles immediately on pre-mRNA when synthesized in the coupled system (Das et al. 2006). Thus, the data in Figure 1C indicate that E7 inhibits spliceosome assembly. We obtained the same results with CMV-AdML, indicating that the effects of E7 are general (Supplemental Fig. 1).
endogenous U6 snRNA and tRNA are indicated by PhosphorImager. The splicing intermediates and products were fractionated on a 5% denaturing polyacrylamide gel and detected in the reaction as in Fig. 3D, lanes 2,4. In contrast, the heparin-disrupted E7 spliceosome had the same mobility in cntl and U2 snRNA extracts [Fig. 3C, lanes 3,4]. These data indicate that U2 snRNPs is not a stable component of the heparin-disrupted, E7 spliceosome. In contrast, in low heparin, both the E7 and normal [PlaF] spliceosomes migrated with faster mobility in the U2 snRNA extract [Fig. 3D, lanes 2,4] versus the cntl extract [Fig. 3D, lanes 1,3], indicating that U2 snRP is a component of the low-heparin E7 (and PlaF) spliceosome.

To further verify that the low-heparin E7 spliceosome contains loosely bound U2 snRNA, we next asked whether this snRNA could be competed off of the pre-mRNA in the presence of normal nuclear extract. To do this, we carried out a chase assay in which 1 μM E7 or PlaF was added prior to transcription for 5 min to allow formation of the E7 or PlaF spliceosome, followed by a 10-fold dilution of these reactions into normal nuclear extract. At this dilution (100 nM), E7 has no effect on splicing [Fig. 3E, lanes 5–8]. However, when the E7 spliceosome was chased in the normal extract, splicing was inefficient [Fig. 3E, lanes 1,2]. In contrast, splicing was efficient when the PlaF spliceosome was chased in normal nuclear extract [Fig. 3E, lanes 3,4]. We conclude that the E7 spliceosome contains non-functional U2 snRNPs that is loosely bound to pre-mRNA but cannot exchange for the U2 snRNPs in the normal nuclear extract. Finally, antibodies to the U2 snRNPs protein with the spliceosome, but is incapable of splicing. To determine why the E7 spliceosome is defective, we examined it using different amounts of heparin, which affects spliceosome stability. Significantly, in the presence of high heparin, the E7 spliceosome was disrupted, whereas the PlaF spliceosome had similar mobility in low and high heparin [Fig. 2C]. These data indicate that the E7 spliceosome is an aberrant, labile complex.

To determine when along the spliceosome assembly pathway E7 exerts its effect, we made use of the uncoupled splicing system and a T7 transcript, because the kinetics of spliceosome assembly are so fast with the coupled system that the different spliceosomal complexes [e.g., A and B] are not detected. When T7 AdML was incubated in an uncoupled splicing reaction for 0, 4, or 25 min, the non-specific H, A, and B complexes were detected with no drug [Fig. 2D] or PlaF [data not shown]. In contrast, only the H complex was detected with E7 [Fig. 2D]. We conclude that E7 blocks assembly of the spliceosomal A complex.

**E7 results in weak binding of U2 snRNA to pre-mRNA**

As E7 targets U2 snRNPs, and this snRNA first binds tightly in the A complex, we next asked whether U2 snRNA was present in the defective, heparin-sensitive E7 spliceosome. To do this, we inactivated U2 snRNPs using the endogenous RNase H in the nuclear extract with an oligonucleotide complementary to the 5’ portion of U2 snRNA [Fig. 3A]. U2 snRNA was efficiently cleaved in the presence of this oligo, whereas a negative control oligo [cntl] had no effect [Fig. 3B]. When CMV-Ftz was incubated in U2 snRNA or cntl extracts in the coupled system, the spliceosome was detected in the PlaF-treated cntl extract, but was disrupted in the U2 snRNA extract [Fig. 3C, cf. lanes 1 and 2]. In contrast, the heparin-disrupted E7 spliceosome had the same mobility in cntl and U2 snRNA extracts [Fig. 3C, lanes 3,4]. These data indicate that U2 snRNPs is not a stable component of the heparin-disrupted, E7 spliceosome. However, when the E7 spliceosome was chased in the normal extract, splicing was inefficient [Fig. 3E, lanes 1,2]. In contrast, splicing was efficient when the PlaF spliceosome was chased in normal nuclear extract [Fig. 3E, lanes 3,4]. We conclude that the E7 spliceosome contains non-functional U2 snRNPs that is loosely bound to pre-mRNA but cannot exchange for the U2 snRNPs in the normal nuclear extract. Finally, antibodies to the U2 snRNPs protein

**Figure 1.** E7 blocks splicing when added prior to but not after RNAP II transcription. (A) Schematic of CMV-Ftz DNA template showing the CMV promoter and sizes of the exons and intron. (B) CMV-Ftz DNA was incubated in coupled RNAP II transcription/splicing reaction mixtures for 5 min in the presence of the indicated amounts of E7, PlaF, or no drug. α-Amanitin was then added, and incubation was continued for 45 min. (C) CMV-Ftz DNA was incubated for 5 min, followed by addition of α-amanitin. (Lanes 1–6,11,12) After transcription, the indicated amounts of E7 or PlaF were added, and incubation was continued for 45 min. In lanes 7–10, 13, and 14, the indicated amounts of E7 or PlaF were added at the beginning of the coupled reaction as in B. No drug was added in lanes 15 and 16. Total RNA was fractionated on a 5% denaturing polyacrylamide gel and detected by PhosphorImager. The splicing intermediates and products and endogenous U6 snRNA and tRNA are indicated.

**Figure 2.** E7 results in formation of a labile spliceosome and blocks the A complex assembly. (A) CMV-Ftz was incubated in coupled transcription/splicing mixtures in the presence of E7 or PlaF for 8 min. CMV-Ftz was omitted in lane 3. Heparin [0.5 μL] was added to the reaction mixtures, and then an aliquot was fractionated on a 1.2% native low-melting agarose gel and detected by PhosphorImager. The spliceosome [ssosome] is indicated. (B) Same as A, except using CMV-Ftz and CMV-Δss DNA templates. The spliceosome and non-specific Δss complexes are indicated. (C) Same as A, except 0.5 μL and 1.5 μL of heparin was added. The white box to the right of lane 4 indicates the heparin-sensitive E7 spliceosome. (D) T7-AdML was incubated under standard splicing conditions in E7 or no drug for the indicated times, heparin was added, and spliceosomal complexes were separated on a 1.5% native low-melting agarose gel.
E7 blocks ATP-dependent remodeling of U2 snRNP that exposes the BBR

We next sought to determine why E7 inhibits tight binding of U2 snRNP to pre-mRNA. We first examined the integrity of U2 snRNP, which exists as a 17S particle containing a 12S core U2 snRNP particle associated with SF3b, and another multiprotein complex known as SF3a [Wahl et al. 2009]. When U2 snRNP was immunoprecipitated from nuclear extract after treatment with E7, PlaF, or no drug, the full set of 17S U2 snRNP proteins, including the SF3a/b proteins, was detected on a Coomassie-stained gel and by mass spectrometry of total proteins [Supplemental Fig. 2]. Consistent with these results, Western analyses showed that the levels of SF3a/b components were the same in immunopurified E7-U2 snRNP and PlaF-U2 snRNP [Supplemental Fig. 2]. Together, these data indicate that E7 has no apparent effect on the integrity of U2 snRNP or its association with SF3a/b.

We next asked whether E7 alters U2 snRNP conformation. The 5’ region of U2 snRNA, which associates with SF3b, plays a critical role early in spliceosome assembly [Kramer et al. 1999; Dybkov et al. 2006]. As E7 targets SF3b, we focused on this region of U2 snRNA. Using native gels, we assayed for binding of a 32P-labeled 12-nt oligonucleotide complementary to nucleotides 27–42 of U2 snRNA [designated BBR oligo] [Fig. 4A]. Consistent with previous work in yeast (Abu Dayyeh et al. 2002), the BBR oligonucleotide [Fig. 4B, lanes 1,3], but not a cntl [Fig. 4B, lanes 2,4], binds to 17S U2 snRNP in an ATP-dependent manner. In striking contrast, when nuclear extract is treated with E7, ATP-dependent binding of the BBR oligonucleotide to 17S U2 snRNP is abolished [Fig. 4C, lanes 5–8]. We conclude that E7 potently inhibits an ATP-dependent conformational change that exposes the BBR of U2 snRNA [Fig. 4D].

To further investigate the remodeling of U2 snRNP, we carried out an order-of-addition experiment in which nuclear extract was first preincubated in ATP, and then E7 or PlaF was added. Significantly, binding of the BBR oligonucleotide to 17S U2 snRNP was now completely resistant to E7 [Fig. 4E]. These data, together with the data in Figure 4C, suggest that, once the ATP-dependent, SF3b-dependent remodeling of U2 snRNP exposes the BBR [Fig. 4D], then binding of the BBR oligonucleotide can occur in an SF3b-independent manner [i.e., E7-resistant manner] [Fig. 4F]. We conclude that SF3b and ATP, but not the BBR oligonucleotide, are required to trigger remodeling of U2 snRNP to expose the BBR.
In this study, we showed that E7 results in formation of defective spliceosomes in which U2 snRNP cannot bind tightly to pre-mRNA. Consistent with this conclusion, our data show that E7 abolishes the A complex assembly, in which U2 first binds tightly to pre-mRNA. We did not detect any effect of E7 on the integrity of U2 snRNP, but showed that E7 does potently block an ATP-dependent remodeling event that exposes the BBR of U2 snRNP. As E7 targets SF3b, our data lead to a model in which SF3b is required for the ATP-dependent remodeling of U2 snRNP to expose the BBR, and this event may be required for the SF3b- and ATP-dependent tight binding of U2 snRNP to pre-mRNA. Previous studies in yeast suggested that splicing factors Prp5p, which is an ATPase/helicase, facilitates an ATP-dependent alteration in U2 structure that is required for the BBR of U2 snRNA to become accessible to the BPS region in the intron (Ruby et al. 1993; Abu Dayyeh et al. 2002). Our work showing that E7 blocks ATP-dependent remodeling of U2 snRNP to expose the BBR strongly suggests that E7 exerts its effects at this Prp5p-dependent remodeling step. Another study showed that, during the A complex assembly, SF3a/b components bind tightly to the pre-mRNA at a region known as the anchoring site, which is located immediately upstream of the BPS (Gozani et al. 1996). More recently, Query and colleagues (Newnham and Query 2001) showed that the anchoring site mediates the ATP requirement for stable U2 snRNP binding, and suggested that this could be due to remodeling of SF3a/b. Our studies using E7 provide direct evidence for this proposal, showing that SF3b is required for tight binding of U2 snRNP to pre-mRNA and for remodeling U2 snRNP.

The identification of specific roles for SF3b via the use of E7 and splicecostatin, as well as other recent work using small molecules to examine splicing, underscores the utility of these compounds for elucidating specific functions of the highly complex, dynamic factors in the spliceosome (Pitch et al. 2001; Muraki et al. 2004; Kaida et al. 2007; Kotake et al. 2007; O’Brien et al. 2008; Stoilov et al. 2008; Sumanasekera et al. 2008; Kuhn et al. 2009; Younis et al. 2010). Although the binding affinity of SF3b to U2 is highly correlated with its cell growth inhibition activity (Kotake et al. 2007), the mechanistic basis for the anti-tumor effect of E7 is not known. If the effect is due to splicing inhibition, then E7 may, for example, differentially affect splicing of pre-mRNAs containing weak splicing signals, and these pre-mRNAs may encode proteins involved in tumorigenesis. Our insights into the mechanism of action of E7 and its effects on SF3b/U2 snRNP will contribute to establishing the therapeutic potential of the drug, as well as the development of second-generation derivatives.

Materials and methods

Plasmids

The plasmid encoding the 75S-nucleotide (nt) CMV-Fze transcript was described (Das et al. 2006). The plasmid encoding the 695-nt CMV-Dss transcript was a generous gift from H. Lei and encodes the naturally intronless HSBP3 gene. CMV-DNA templates used in the coupled RNAP II transcription/splicing system were amplified by PCR using a forward primer (5′-TGAGCCGTGCATGACTGTC-3′) and reverse primer (5′-TACAGCCACAGTCCAGGCT-3′). AdML pre-mRNA was transcribed with T7 RNA polymerase from a PCR product using the same primers and the DoaA plasmid (Das et al. 2006).

Coupled transcription/splicing

In vitro RNAP II transcription/splicing reactions were performed at 30°C in 25-μL reaction mixtures containing 200 ng of CMV-DNA template, 1 μL of α-32P-UTP (800 Ci/mmol, Perkin Elmer Life Sciences catalog no. BU5057x), 0.5 mM ATP, 3.2 mM MgCl2, 20 mM creatine phosphate (di-Tris salt), and 15 μL of HeLa nuclear extract (Das et al. 2006). The indicated amounts of E7 or PlaF were added to the reaction mixtures before or after RNAP II transcription, as designated. α-Amanitin (10 ng/25 μL) was added after transcription, and incubation was continued for the indicated times.

Spliceosome assays

For spliceosome assembly, CMV-DNA templates were incubated under RNAP II transcription/splicing conditions for 8 min. G-50 columns (GE Healthcare Life Sciences) were used to remove unincorporated α-32P-UTP. For low- or high-heparin samples, 0.5 or 1.5 μL of heparin (6.5 g/L), respectively, was added to 10 μL of G-50-column-purified reactions prior to loading on 1.2% low-melting-point agarose gels (Das and Reed 1999). For assembly of spliceosomal complexes using the uncoupled system, AdML pre-mRNA (2 ng) was incubated for 0, 4, and 25 min under standard splicing conditions (0.5 mM ATP, 3.2 mM MgCl2, 20 mM creatine phosphate).
tracts were used for spliceosome assembly assays as described above. For preparation of U2 snRNA and control extracts, 250 ng of oligonucleotide targeting U2 snRNA (5'-CCA AAAGGCCGAGAACGATGTC-3') or cntl (5'-GGGTGTAATCTTCGTCACG-3') was added to 15 mL of nuclear extract, followed by incubation for 5 min at 30°C for endogenous RNase H cleavage of the snRNA. Total RNA was prepared from these samples and fractionated on a 5% denaturing gel, and snRNAs were detected by ethidium bromide. The control extract and 7.5 mL of Splicing Dilution Buffer (20 mM Hepes at pH 7.6, 100 mM KCl). No drug or 1 mM E7 was included in the reactions. Aliquots (10 mL) of each reaction were mixed with 1 mL of heparin (6.5 g/L) and run on 250 ng of oligonucleotide complementary to the BBR [di-Tris salt] in a 25-mL reaction mixture containing slight activity. Reaction mixtures were incubated for 20 min at room temperature. Reaction mixtures in the orders indicated in Figure 4. For reactions carried out in the absence of ATP, water was used to bring the volume up to 25 mL.

U2 snRNP conformation assay

The BBR 2'O-methyl oligonucleotide complementary to the BBR (5'-mGmAmAmUmAmCmAmCmUmAmCmU mG-3') was 32P-labeled using γ-ATP (6000 Ci/mmol) and T4 polynucleotide kinase. For all assays, ATP was depleted from nuclear extract by incubation for 20 min at room temperature. Reaction mixtures (25 mL) containing the depleted nuclear extract (15 mL) were mixed with 1 mM ATP, 3.2 mM MgCl2, and 20 mM creatine phosphate [di-Tris salt] for the indicated times. BBR or control 2'O-methyl oligonucleotide (34 ng of each) and 1 mM E7 or Plaf were added to the reaction mixtures in the orders indicated in Figure 4. For reactions carried out in the absence of ATP, water was used to bring the volume up to 25 mL.

Acknowledgments

We are grateful to R. Das, K. Dufu, C.S. Lee, and members of the Reed laboratory for useful discussions, and to H. Lei and K. Dufu for critical comments on the manuscript. HeLa cells were obtained from the National Cell Culture Center [Minneapolis, MN]. This work was supported by a post-doctoral fellowship to E.F. from Eisai Pharmaceuticals and an NIH grant to R.R.

References


