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A human U2 RNA mutant stalled in 3′ end processing is impaired in nuclear import

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ABSTRACT

The biosynthesis of U1, U2, U4 and U5 spliceosomal small nuclear RNAs (snRNAs) involves the nuclear export of precursor molecules extended at their 3′ ends, followed by a cytoplasmic phase during which the pre-snRNAs assemble into ribonucleoprotein particles and undergo hypermethylation of their 5′ caps and 3′ end processing prior to nuclear import. Previous studies have demonstrated that the assembly of pre-snRNAs into ribonucleoprotein particles containing the Sm core proteins is essential for nuclear import in mammalian cells but that 5′ cap hypermethylation is not. In the present investigation we have asked whether or not 3′ end processing is required for nuclear import of U2 RNA. We designed human pre-U2 RNAs that carried modified 3′ tails, and identified one that was stalled (or greatly slowed) in 3′ end processing, leading to its accumulation in the cytoplasm of human cells. Nonetheless, this 3′ processing arrested pre-U2 RNA molecule was found to undergo cytoplasmic assembly into Sm protein-containing complexes to the same extent as normal pre-U2 RNA. The Sm protein-associated, unprocessed mutant pre-U2 RNA was not observed in the nuclear fraction. Using an assay based on suppression of a genetically blocked SV40 pre-mRNA splicing pathway, we found that the 3′ processing deficient U2 RNA was significantly reduced in its ability to rescue splicing, consistent with its impaired nuclear import.

INTRODUCTION

The U1, U2, U4 and U5 spliceosomal small nuclear RNAs (snRNAs) of eukaryotic cells undergo maturation in the cytoplasm and then return to the nucleus to function in pre-mRNA splicing (1). Studies of U2 RNA biosynthesis in human cells have revealed that both 5′ cap hypermethylation and assembly with Sm proteins in the cytoplasm temporally precede the precursor's 3′ end processing (2). Moreover, when human pre-U2 RNA is incubated in a HeLa cell cytoplasmic extract, it assembles into an Sm protein-containing particle before 3′ processing occurs (3). However, subsequent studies employing more purified systems have demonstrated that accurate and efficient 3′ processing of pre-U2 RNA *in vitro* does not require that the precursor be assembled into a ribonucleoprotein particle (4,5).

Major differences have been observed with respect to the nuclear import of the U1, U2, U4 and U5 RNAs in mammalian cells versus *Xenopus* oocytes. Studies in *Xenopus* oocytes have revealed that nuclear import requires the cytoplasmic assembly of the snRNAs into ribonucleoprotein complexes containing the Sm proteins (6). Cap hypermethylation is also required for U2 snRNP nuclear import in *Xenopus* oocytes (7) but not in mammalian cells (8,9). However, the role of 3′ processing in snRNP nuclear import in mammalian cells has not been investigated. As part of our continuing studies of human U2 snRNA 3′ processing (3–5,8,10), we have now asked whether this step is required for nuclear import.

The strategy we employed in this investigation was to construct a mutant pre-U2 RNA that undergoes 3′ end processing inefficiently *in vivo*. We obtained such a mutant and found that it accumulates in the cytoplasm as an unprocessed precursor molecule. We then investigated its cytoplasmic assembly into Sm protein-containing snRNP particles and its capacity to rescue a genetically blocked pre-mRNA splicing pathway. Our results indicate that although this 3′ processing deficient pre-U2 RNA molecule assembles into an Sm protein-containing snRNP particle, it does not undergo efficient nuclear import.

MATERIALS AND METHODS

Plasmid constructions

Plasmids pSTER, SV40-AGU and U2-UCA (11,12) were kindly provided by James Manley (Columbia University). The U2-UCA double mutants used in this study (U2-UCA III and U2-UCA V; Fig. 1) were constructed by oligonucleotide-directed mutagenesis of plasmid U2-UCA employing the Altered Sites® II *in vitro* Mutagenesis System (Madison, WI). These mutants were verified by DNA sequencing in both orientations of double-stranded DNA templates. The mutants generated in the pALTER-1 vector were subcloned into plasmid pBluescript SK+ (Strategene, La Jolla, CA).

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Transfection of human 293 cells

Human embryonic kidney 293 cells were grown in F12K medium (Sigma) supplemented with 10% fetal bovine serum (Biowhittaker, Walkersville, MD). Confluent cells were split 1:7 and grown at 37°C for 2 days until they reached 50–70% confluence. The cells were transfected using LipofectAMINE (Gibco BRL, Gaithersburg, MD) and the procedures suggested by the manufacturer. Plasmids used for transfection were purified twice by CsCl gradient centrifugation. Typically, 8 µg of plasmid DNA were mixed with 40–48 µl of LipofectAMINE (2 mg/ml) and then added to 293 cells growing in 100 mm dishes; a total of 18 µg of plasmid DNA pre-mixed with 100 µl of LipofectAMINE (2 mg/ml) was used when transfecting cells growing in 150 mm dishes. In co-transfection experiments, 4.5 µg of SV40-AGU plasmid DNA plus 13.5 µg of U2-UCA (wild-type or mutant) plasmid DNA were used in each 150 mm dish. Four hours after the addition of the plasmids, the extracellular DNA–LipofectAMINE precipitates and medium were replaced with fresh growth medium (see above).

RNA isolation

Transfections were routinely terminated 48 h after the addition of the DNA. The following steps were carried out at 4°C. The medium was removed and the cells were washed with ice-cold PBS. PBS (2 ml for 100 mm dishes; 4 ml for 150 mm dishes) was added and the cells were scraped off using a rubber policeman. After the cells were pelleted (600 *g*, 3 min), they were suspended in 0.2–0.5 ml of ice-cold isotonic buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$). Triton X-100 was added to 0.5% (v/v), the suspension was incubated for 3 min on ice, and then centrifuged (1000 *g*, 3 min). The supernatant was taken for the isolation of cytoplasmic RNA. It is to be noted that this Triton-based method extracts the cytoplasmic pre-snRNAs which, unlike most of the ribosomal and mRNA, are not bound to cytoskeletal elements (13). The pelleted nuclei were washed once with isotonic buffer and resuspended in 0.1–0.3 ml of the same buffer, depending on the pellet size. Both cytoplasmic and nuclear fractions were extracted twice with phenol–chloroform–isoamyl alcohol (24:24:1, v/v/v) and precipitated with ethanol. The nuclear samples were then suspended in 20 µl of DNase digestion buffer (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl). Ten units of RQ1 DNase (Promega) were added. After a 30 min incubation at 37°C, the RNA was extracted with phenol–chloroform–isoamyl alcohol and precipitated with ethanol. Both cytoplasmic and nuclear RNA were resuspended in 20 µl of RNase-free water. Isolation of total RNAs from cotransfected 293 cells for S1 nuclease mapping was performed by using a phenol-free, filter based RNA isolation system (RNAqueous™, Ambion, TX).

Primer extension

Primer extension was performed essentially as described previously (12). To differentiate between the endogenous and wild-type and transfected mutant U2-UCA RNAs, a 5′-end-labeled synthetic oligodeoxynucleotide (5′-dAAACTGATAAGAACAG-3′; Fig. 2A) complementary to nucleotides (nt) 40–55 of the U2 RNA sequence was used as a primer. Typically, 10 µg of either cytoplasmic or nuclear RNA was hybridized with 20 fmol of ^{32}P-labeled primer in a 12 µl reaction consisting of 125 mM KCl and 83 mM Tris–HCl (pH 7.5) at 65°C for 10 min and at 37°C for 10 min. The hybridization reactions were allowed to cool to room temperature over a period of 30 min. The reaction mixture was then adjusted to 20 µl with final concentrations of 3 mM $MgCl_2$, 10 mM DTT, 0.1 mM each of dATP, dGTP, dTTP and dideoxyCTP, and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The reaction was carried out at 37°C for 1 h and stopped by extraction with phenol–chloroform–isoamyl alcohol (24:24:1, v/v/v), and the aqueous phase was precipitated with ethanol. The extended products were analyzed by electrophoresis in 16% polyacrylamide gels (19:1 ratio of acrylamide to bisacrylamide) containing 8.3 M urea, followed by autoradiography.

S1 nuclease mapping

S1 nuclease analysis was performed as described previously (12), with a 3′-end-labeled *Hin*dIII–*Bsm*I DNA probe (nt 5171–4528) isolated from pSTER. The use of this probe permitted resolution of unspliced pre-mRNA and undigested probe when SV-AGU was used in transfections (14). Typically, 100 µg of total RNA was hybridized with 20 ng of denatured double-stranded probe at 43°C overnight in 0.4 M NaCl, 1 mM EDTA, 80% formamide, 40 mM PIPES, pH 6.4. S1 nuclease digestion was then performed at 37°C for 1 h with 40 U of S1 nuclease (Promega). S1 nuclease-resistant products were resolved in 6% polyacrylamide (19:1 ratio of acrylamide to bisacrylamide) 8.3 M urea gels, followed by autoradiography.

RNase protection assays

RNase protection assays were performed as described previously (15). ^{32}P-labeled riboprobes complementary to the 3′ half of the pre-U2 UCA RNAs (including the precursor extension sequences) were transcribed from *Mse*I-digested wild-type or mutant U2-UCA encoding plasmids using SP6 RNA polymerase (Ambion). Typically, 10 µg of cytoplasmic or nuclear RNA was mixed with ~50 fmol of probes (~400 c.p.m./fmol) and the mixture was precipitated with ethanol. The pellet was redissolved in 30 µl of hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl and 1 mM EDTA) and denatured at 80°C for 10 min. The hybridization reaction was carried out at 43°C overnight. Ribonuclease digestion was performed at 37°C for 1 h with 5 U of RNase A and 200 U of RNase T1 (Ambion). The samples were then incubated with 200 µg of proteinase K (Sigma) at 37°C for 15–30 min. The ribonuclease-resistant products were extracted with phenol–chloroform–isoamyl alcohol (24:24:1, v/v/v) once and precipitated with ethanol, and then resolved by electrophoresis in 6% polyacrylamide (19:1 ratio of acrylamide to bisacrylamide) 8.3 M urea gels, followed by autoradiography.

RNP assembly and immunoprecipitation

Transfected cells were harvested and pelleted as described above. The cell pellets were suspended in 0.2–0.5 ml of ice-cold TSM-LLS buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5), and were incubated on ice for 10 min. The cell suspensions were transfered to a small ice-cold Dounce homogenizer and disrupted using 20–30 strokes. The homogenates were centrifuged at 800 *g* for 5 min and the supernatants were collected as cytoplasmic fractions for immunoprecipitation. The nuclei were washed once with TSM-LLS and suspended in 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$. The nuclei were disrupted

by brief sonication (20 s at setting 6, 4°C; Heat Systems Ultrasonics model W-375) and used for immunoselection as previously described (16,17). Briefly, ~100 µg of Y12 Sm monoclonal antibody or human non-immune IgG pre-bound to protein A–Sepharose was added to cytoplasmic or nuclear extract aliquots corresponding to 2×10^7 cells. After incubation at 4°C for 1.5 h, the protein A–Sepharose was pelleted by centrifugation, and washed twice with NET2 (0.15 M NaCl, 5 mM EDTA, 0.5% NP-40, 62.5 mM Tris–HCl, pH 7.5). The supernatant from the initial centrifugation and the subsequent washes were collected as the unbound fraction. The bound fraction was recovered from the pelleted protein A–Sepharose of the final centrifugation by boiling in 0.3 M sodium acetate, 1 mM EDTA, 0.1% SDS, 10 mM Tris–HCl, pH 7.4. RNA was extracted from both the unbound and bound fractions and was subjected to RNase protection assays as described above.

RESULTS

The first step in this investigation was to undertake the construction of mutant pre-U2 RNAs defective in 3′ end processing. Several mutants were constructed, of which two will be described here. One of these, termed 'U2-UCA III' (Fig. 1) has an extended 3′ tail relative to the normal pre-U2 ('U2-UCA' in Fig. 1). The extended 3′ tail of U2-UCA III is based on a previous study in which a very similar mutant pre-U2 RNA was found not to undergo 3′ processing in *Xenopus* oocytes (18). In the second mutant, 'U2-UCA V' (Fig. 1), the wild-type 3′ tail was replaced by a 33 nt long sequence designed to form a stable stem–loop structure. U2-UCA III and U2-UCA V, as well as the companion pre-U2 with the normal 3′ tail used as a control ('U2-UCA', Fig. 1), all contain the intron branch site recognition sequence mutation UAG→ACU. This mutation allows these U2 RNAs to function in the SV40 alternative pre-mRNA splicing suppression system (11).

Transfection of human 293 cells with the plasmids encoding the pre-U2 RNAs shown in Figure 1 resulted in expression of all three RNAs as shown by primer extension analysis using an oligonucleotide primer that, when extended by reverse transcription in the presence of chain-terminating dideoxyCTP affords discrimination between endogenous U2 RNA and the transfected U2-UCA RNAs (Fig. 2A). As can be seen in Figure 2B, expressed U2-UCA RNA was present in both cytoplasmic and nuclear fractions, as was U2-UCA III RNA. Note that the majority of U2-UCA III was in the nuclear fraction. In contrast, as shown in Figure 2C, the nuclear:cytoplasmic distribution of U2-UCA V was reversed, with the majority recovered in the cytoplasmic fraction. Although there was some preparation-to-preparation variation in the nuclear:cytoplasmic fractionation of U2 RNAs expressed from a given plasmid (e.g. note the variation between the U2-UCA experiments in Fig. 2B and C), the predominantly cytoplasmic distribution of U2-UCA V RNA (Fig. 2C) was consistently observed.

To investigate whether U2-UCA, U2-UCA III and U2-UCA V RNAs undergo 3′ processing, RNase protection experiments were carried out. The antisense RNAs used as probes for U2-UCA III and U2-UCA V RNAs sequences are illustrated in Figure 3A. Pre-U2-UCA RNA is expected to produce a 109 nt RNase protected fragment, whereas pre-U2-UCA III RNA should produce a 139 nt RNase protected fragment. Figure 3B illustrates the comparable situation for pre-U2-UCA V RNA,

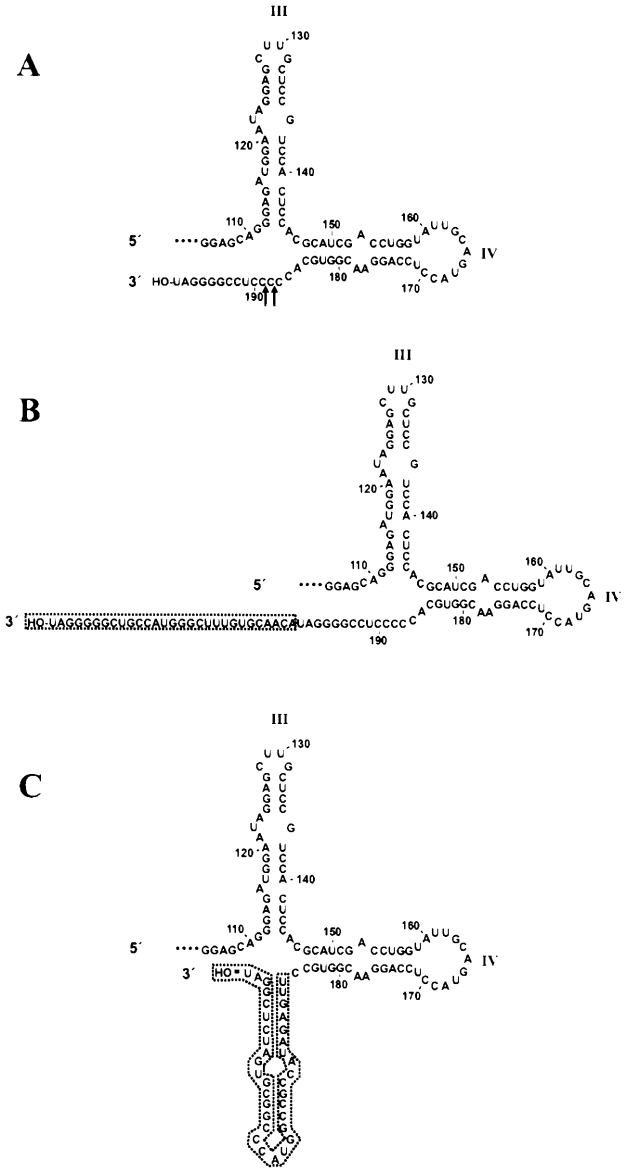

Figure 1. 3′ ends of wild-type and mutant human pre-U2 RNAs. (**A**) Pre-U2-UCA ('wild-type'); (**B**) pre-U2-UCA III RNA, a mutant designed with an extended, single-stranded 3′ tail; (**C**) U2-UCA V RNA, a mutant designed with an extended 3′ tail potentially forming a very stable stem–loop structure. All three of these RNAs contained the intron branch site recognition mutation UAG→ACU (11). The arrows in U2-UCA denote the positions corresponding to the 3′ ends of the mature RNA.

which should produce a 130 nt RNase protected fragment. Thus, if either U2-UCA III RNA or U2-UCA V RNA are deficient in 3′ end processing, RNase protected fragments of 139 and 130 nt, respectively, would be observed. Delayed processing kinetics might be anticipated to generate processing intermediates, which would appear in the RNase protection assays as bands longer than 109 nt and shorter than 139 or 130 nt in the cases of U2-UCA III and U2-UCA V RNAs, respectively.

Figure 4 shows the results of RNase protection analysis of nuclear and cytoplasmic RNA fractions from human 293 cells expressing U2-UCA RNA (lanes 2–5), U2-UCA III RNA (lanes 6 and 7) or U2-UCA V RNA (lanes 8 and 9). In the case

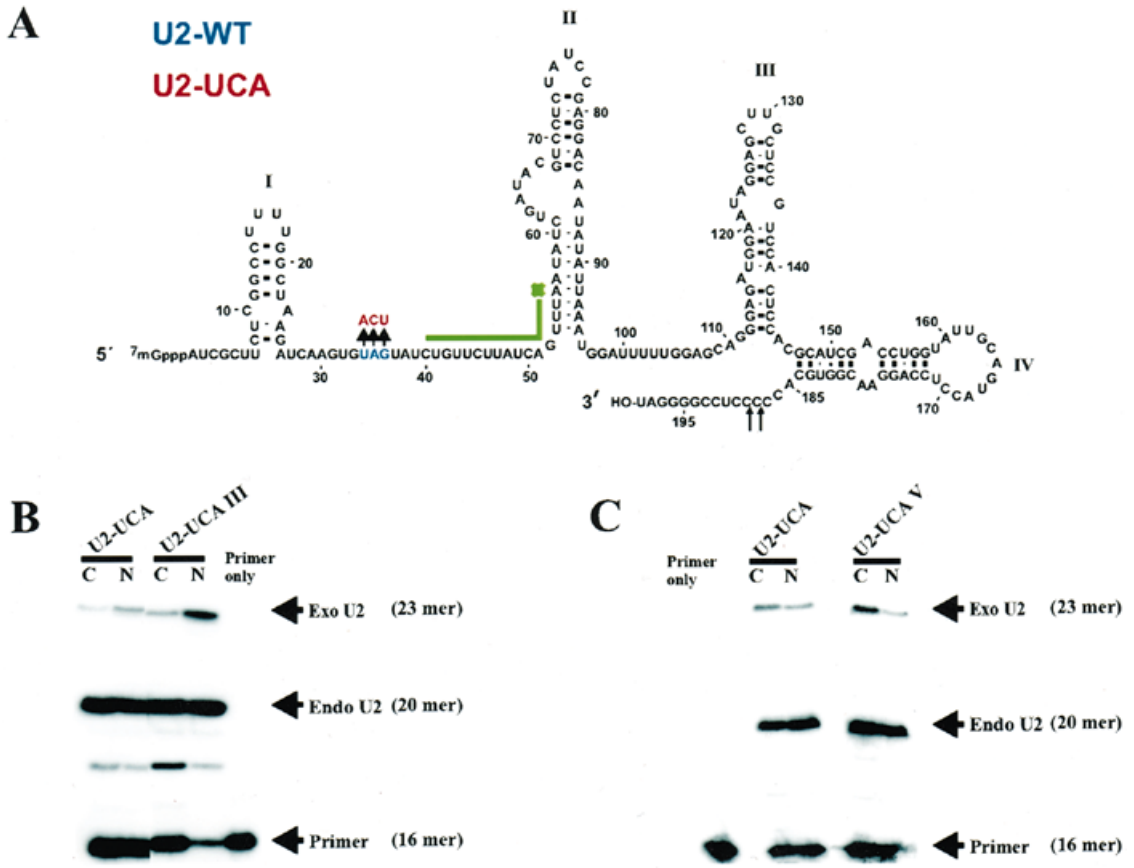

Figure 2. Primer extension analysis. (**A**) Schematic depiction of pre-U2-UCA RNA and the binding site of the 5′-end-labeled oligodeoxynucleotide (green) used to prime extension by reverse transcriptase. When the primer binds to wild-type U2 RNA (UAG at nt 34–36), transcription with dideoxyCTP generates a DNA product that stops at the G at position 36 (i.e. a 20 nt primer extension product). When the primer binds to U2-UCA RNA (ACU at nt 34–36), reverse transcription with dideoxyCTP generates a DNA product that stops at the G at position 39 (i.e. a 23 nt primer extension product). (**B**) Primer extension results for cells expressing U2-UCA RNA and U2-UCA III RNA. (**C**) Primer extension results for cells expressing U2-UCA RNA and U2-UCA V RNA.

of U2-UCA RNA, which has the normal 3′ tail (Fig. 1), no pre-U2 RNA is detected (lanes 4 and 5), presumably because its 3′ processing is fast and no precursor is detectable at this level of sensitivity, involving steady-state RNA. Results similar to those shown in lanes 2–5 were obtained when RNA from non-transfected cells was analyzed similarly (data not shown). The endogenous U2 RNA precursor observed in previous [^{3}H]uridine pulse-labeling experiments (2,19–25) would be expected to produce a 109 nt fragment in the RNase protection assay (Fig. 3A). The absence of a band of this size (Fig. 4, lanes 4 and 5) indicates that both endogenous and transfected (U2-UCA) pre-U2 RNAs are not detected in steady-state RNA at the level of sensitivity afforded by the RNase protection assay, which is not surprising.

In cells expressing U2-UCA III, the expected 139 nt RNase protected fragment was also not observed (Fig. 4, lanes 6 and 7). (The multiple bands present at the top of lanes 6 and 7 are non-specific, seen also in lane 2 in which the cells were expressing U2-UCA rather than U2-UCA III. Moreover, the overall high autoradiographic intensity of lanes 6 and 7 reflects a much larger amount of RNA loaded than the other lanes.) This result suggests that the U2-UCA III pre-U2 RNA is either processed with kinetics similar to U2-UCA pre-U2 RNA and endogenous pre-U2 RNA (i.e. the precursor form is not detected in the RNase protection assay) or, alternatively, that this particular

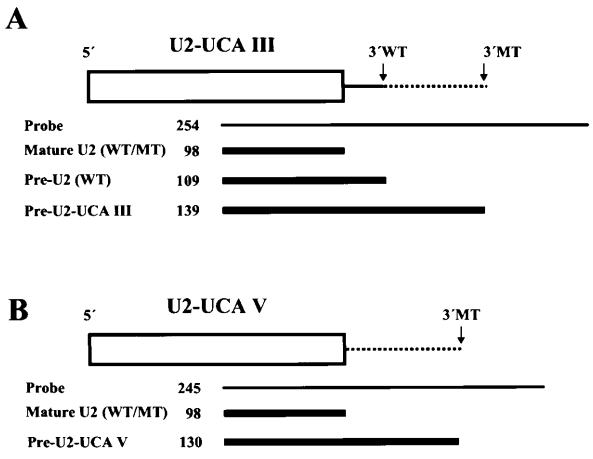

Figure 3. Design of RNase protection analysis. (**A**) Diagram of pre-U2-UCA III, the antisense RNA probe and the expected RNase protection products. '3′ MT' denotes the 3′ end of pre-U2 UCA III RNA and '3′ WT' denotes the 3′ end of wild-type pre-U2 RNA (endogenous and transfected U2-UCA). (**B**) Diagram of pre-U2 UCA V, the antisense RNA probe and the expected RNase protection products.

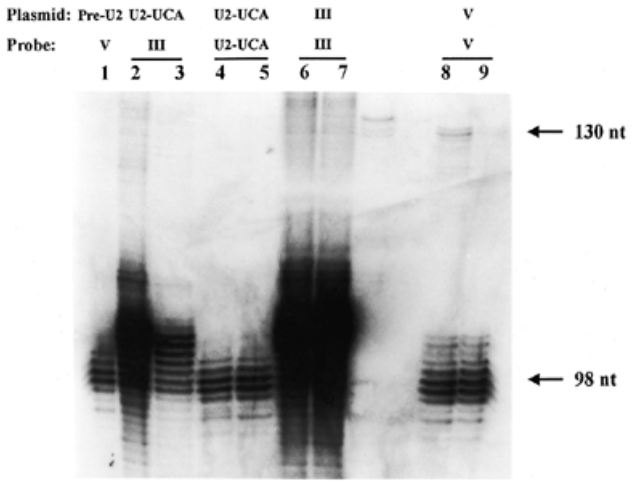

Figure 4. RNase protection analysis of U2 RNA 3′ ends. 293 cells were transfected with the plasmids indicated. Nuclear and cytoplasmic RNA was subjected to RNase protection analysis with the ^{32}P-labeled riboprobes indicated (see also Fig. 3). Lane 1, *in vitro* transcribed wild-type pre-U2 RNA subjected to RNase protection using probe V to provide a standard for the position of mature length U2 RNA; lanes 2 and 3, cytoplasmic (lane 2) and nuclear (lane 3) RNA from cells expressing U2-UCA RNA, using probe III; lanes 4 and 5, cytoplasmic (lane 4) and nuclear (lane 5) RNA from cells expressing U2-UCA RNA using probe U2-UCA; lanes 6 and 7, cytoplasmic (lane 6) and nuclear (lane 7) RNA from cells expressing U2-UCA III RNA, using probe III; lanes 8 and 9, cytoplasmic (lane 8) and nuclear (lane 9) RNA from cells expressing U2-UCA V RNA, using probe V. (The two unnumbered lanes between lanes 7 and 8 contained samples unrelated to the present report.)

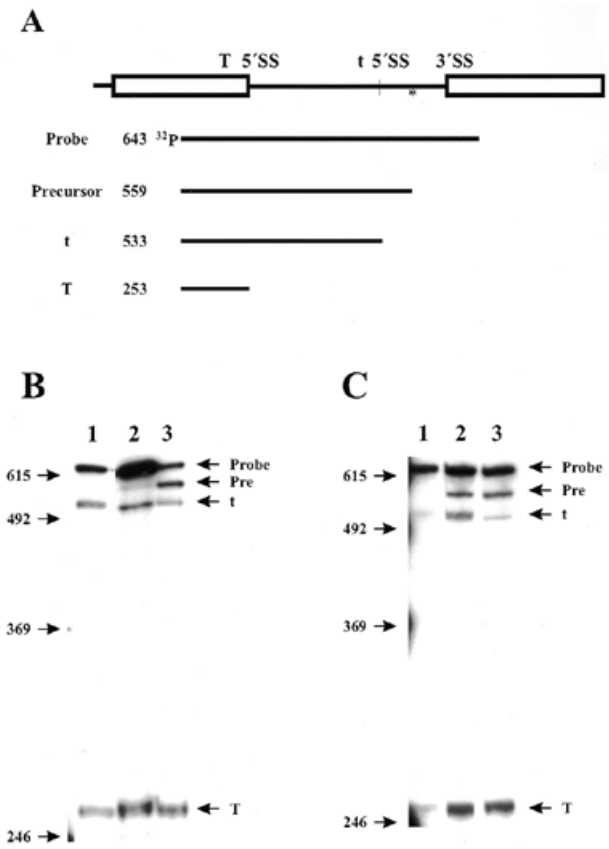

Figure 5. Rescue of pre-mRNA splicing by U2-UCA RNAs. (**A**) Diagram of SV40 pre-mRNA and the S1 nuclease protection strategy used to detect the SV40 AUG branch site mutant pre-mRNA and its two alternatively spliced mRNAs. The position of the SV40 pre-mRNA branch site mutation is indicated by an asterisk. 'Probe' represents a 3′-end-labeled DNA fragment (Materials and Methods). The probe is complementary to the wild-type SV40 pre-mRNA. Hence, hybridization to the branch site mutant pre-mRNA generates an S1 nuclease sensitive site at this position (see asterisk), producing the S1 digested probe fragments illustrated. (**B**) Lane 1, cells expressing wild-type SV40 pre-mRNA; lane 2, cells expressing SV40-AGU pre-mRNA and U2-UCA RNA; lane 3, cells expressing SV40-AGU pre-mRNA and U2-UCA III RNA. (**C**) Lane 1, cells expressing wild-type SV40 pre-mRNA; lane 2, cells expressing SV40-AGU pre-mRNA and U2-UCA RNA; lane 3, cells expressing SV40-AGU pre-mRNA and U2-UCA V RNA. The autoradiographic intensities of the S1 nuclease protected bands corresponding to spliced small t and large T antigen mRNA were determined by quantitative densitometry of the photographic prints. In (B), the ratios of small t:large T spliced mRNA were: lane 1, 0.88; lane 2, 0.82; lane 3, 0.71. In (C), the ratios of small t:large T spliced mRNA were: lane 1: 0.88, lane 2, 0.80; lane 3, 0.36.

RNA is metabolically unstable in the cell altogether. However, as will be shown below, U2-UCA III RNA is capable of rescuing SV40 small t antigen pre-mRNA splicing, hence the lack of detectable pre-U2-UCA III RNA in the RNase protection assays (Fig. 4) is not due to an instability of this RNA.

In contrast, a different result was obtained with U2-UCA V RNA. In this case the cytoplasmic fraction contained a distinct pair of bands corresponding to unprocessed U2-UCA V RNA (Fig. 4, lane 8). Therefore, relative to the 3′ processing kinetics of U2-UCA pre-U2 RNA, which does not reveal a steady-state precursor in this analysis, the 3′ processing of U2- UCA V RNA is apparently greatly slowed.

The observation that U2-UCA V pre-U2 RNA is significantly slowed in its 3′ processing, or perhaps even completely stalled, opened the door to its use as a vehicle to test the role of U2 RNA 3′ processing in nuclear import. Human 293 cells were transfected with either wild-type U2-UCA or mutant U2-UCA DNA and with mutant SV40 DNA containing a branch site mutation in an intron that produces small t antigen mRNA in the alternative splicing pathway of this pre-mRNA (11). Endogenous U2 RNA cannot splice this mutant SV40 pre-mRNA into small t antigen mRNA. Thus, one has an opportunity to test the ability of transfected U2 RNAs containing the compensatory mutation at the branch site recognition sequence to suppress the block of SV40 small t antigen mRNA splicing (11).

Figure 5A illustrates the S1 nuclease protection products expected for the SV40 pre-mRNA and the spliced large T antigen and small t antigen mRNAs. Because the 3′-end-labeled DNA probe is generated from the wild-type SV40 pre-mRNA, the mismatch at the mutant SV40 lariat branch site (indicated by the asterisk in Fig. 5A) results in an S1 nuclease cleavage site at this position in the probe. Thus, the unspliced SV40-AGU pre-mRNA generates a 559 nt S1 digestion fragment, whereas the spliced large T antigen mRNA and spliced small t antigen mRNA produce S1 digestion fragments of 253 and 533 nt, respectively. As shown in Figure 5B, lane 1, cells transfected with wild-type SV40 DNA displayed small t and large T spliced mRNAs in a ratio of ~1.0 as previously reported (11). (The actual densitometric small t:large T ratio in lane 1 was 0.88; see Fig. 5 legend.) When cells were co-transfected with the SV40-AGU and U2-UCA DNAs (lane 2), the amounts of small and large T spliced mRNAs were again similar (densitometric small t:large T ratio = 0.82). When cells were transfected with SV40-AGU DNA alone, no

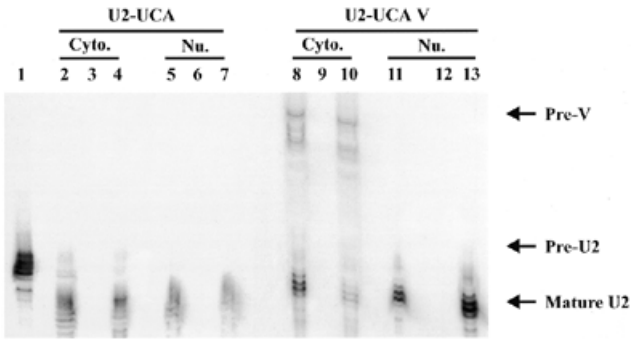

Figure 6. Sm antibody selection and RNase protection analysis of U2-UCA RNAs. Cells were transfected with U2-UCA, U2-UCA III or U2-UCA V plasmids and nuclear or cytoplasmic fractions were subjected to Sm antibody selection. The selected versus unselected RNAs were then assayed by RNase protection as in Figure 4. Lane 1, *in vitro* transcribed pre-U2-UCA RNA (no antibody selection), U2 UCA probe; lanes 2–4, cytoplasmic extracts from cells expressing U2-UCA RNA, probe UCA; lane 2, Sm antibody selection, non-bound fraction; lane 3, non-immune human IgG, bound fraction; lane 4, Sm antibody selection, bound fraction; lanes 5–7, nuclear extracts from cells expressing U2-UCA RNA, probe UCA; lane 5, Sm antibody selection, non-bound fraction; lane 6, non-immune human IgG, bound fraction; lane 7, Sm antibody selection, bound fraction; lanes 8–10, cytoplasmic extracts from cells expressing U2-UCA V, probe V; lane 8, Sm antibody selection, non-bound fraction; lane 9, non-immune human IgG, bound fraction; lane 10, Sm antibody selection, bound fraction; lanes 11–13, nuclear extracts from cells expressing U2-UCA V, probe V; lane 11, Sm antibody selection, non-bound fraction; lane 12, non-immune human IgG, bound fraction; lane 13, Sm antibody selection, bound fraction.

small t antigen spliced mRNA was observed (not shown). When cells were co-transfected with SV40-AGU DNA and U2-UCA III DNA (Fig. 5B, lane 3), the ratio of small t to large T spliced mRNA products was 0.71, indicating that U2-UCA III RNA is capable of restoring small t mRNA splicing although to a lesser extent than wild-type U2-UCA RNA. In contrast, when cells were co-transfected with SV40-AGU DNA and U2-UCA V DNA, the ratio of small t to large T spliced mRNA was greatly reduced, to 0.36 (Fig. 5C, lane 3; compare with lane 2 of Fig. 5B and lane 2 of Fig. 5C).

The accumulation of unprocessed U2-UCA V RNA in the cytoplasm of transfected human 293 cells (Fig. 4) suggests that 3′ processing is a prerequisite for nuclear import of U2 RNA. This is further supported by the observed reduced capacity of U2-UCA V RNA to restore small t antigen mRNA splicing (Fig. 5). Since nuclear import also requires the assembly of U2 RNA with the Sm group of snRNP proteins, it was important to determine whether pre-U2 UCA V RNA assembles into Sm protein-containing snRNP complexes *in vivo*. As shown in Figure 6, lane 10, pre-U2-UCA V RNA was detected in Sm antibody-selectable form in the cytoplasm, indicating that its reduced capacity to restore small t antigen mRNA splicing is not due to a failure to assemble into an Sm protein containing ribonucleoprotein particle. It can also be seen in Figure 6, lane 13, that no pre-U2-UCA V RNA was detected in the nuclear fraction.

DISCUSSION

In mammalian cells, pre-U2 snRNA is detected in the cytoplasm in Sm antibody-selectable form (2,24), indicating that its assembly with Sm proteins precedes 3′ end processing. Since mature-length U2 RNA is not detected in Sm antibody-selectable complexes in the cytoplasmic fraction of [^{3}H]uridine pulse-labeled HeLa cells (2,24), it is likely that nuclear import occurs quite promptly upon the completion of the 3′ processing reaction. In the present investigation we have asked whether the 3′ end processing of pre-U2 RNA is required for nuclear import. The first step in the present study was to construct a mutant human pre-U2 RNA that is inefficiently processed. We previously reported that a critical internal region of human pre-U2 RNA is required for accurate 3′ end processing (4). We initially contemplated using mutants in this region, but reasoned that these processing-deficient pre-U2 RNAs might also be impaired in their capacity to function in pre-mRNA splicing, the basis of our assay for nuclear import. This reservation was based on the fact that the internal region of pre-U2 RNA essential for its 3′ processing is part of the binding site for the U2 snRNP-specific protein B″, the presence of which in U2 snRNP is required for pre-mRNA splicing (12). We therefore turned our attention to the 3′ tail of pre-U2 RNA. Since we had observed in our most recent investigation (5) that the sequence of the tail was critical for accurate 3′ processing, we decided to construct mutants of pre-U2 RNA in which the 3′ tail was extremely modified. In U2-UCA III (Fig. 1), the normal 11 nt tail was extended with a random sequence designed to minimize intramolecular base-pairing. In U2-UCA V, which proved to be our desired processing-deficient mutant, a stable stem–loop structure was attached to the base of stem–loop IV and, in addition, the nucleotides at the site of the mature U2 RNA's 3′ end were mutated (Fig. 1).

Although the mechanism of pre-U2 RNA 3′ end processing was not the focus of the present study, the results with the mutant pre-U2-UCA III and V RNAs bear on our previous studies of this reaction (4,5,10). In our most recent study, there were indications that the initial step in pre-U2 RNA 3′ end processing is the exonucleolytic removal of the last 2 nt of the precursor, promptly followed by an endonucleolytic cleavage at the mature 3′ end (5). These experiments were carried out *in vitro* with a highly purified pre-U2 RNA 3′ processing activity (5), and this must be borne in mind when considering the previous results in the context of the present *in vivo* investigations of pre-U2 RNA 3′ processing. Nonetheless, the finding that pre-U2 UCA III is apparently processed efficiently is of interest in the context of our previous work. A 3′ exonuclease might remove (the unstructured) nt +40 to +12 of pre-U2-UCA III, at which point the molecule would be identical to the normal pre-U2 RNA (Fig. 1). In contrast, pre-U2-UCA V has, by our design, a potential stem–loop structure at its 3′ end that might be more resistant to exonucleolytic attack. Moreover, even if the 3′ tail of pre-U2-UCA V was exonucleolytically trimmed, the mature 3′ site is itself mutated (Fig. 1), and therefore the site-specific endonucleolytic cleavage implicated in our previous work (5) might not occur. While the foregoing interpretations of the mechanistic basis for the 3′ processing-permissive versus 3′ processing-deficient phenotypes of pre-U2-UCA III versus pre-U2-UCA V are admittedly speculative, they are entirely consistent with our previous findings (4,5). Our results also indicate that the wild-type 3′ tail of pre-U2 RNA is not required for its nuclear *export*, since pre-U2-UCA V RNA, with its mutant 3′ tail, was observed in the cytoplasm (Fig. 4). Moreover, the fact that the processing-permissive pre-U2-UCA III RNA functions in pre-mRNA splicing demonstrates that a functional U2 RNA need not necessarily start out as a natural precursor molecule.

We note that our results do not necessarily establish that pre-U2-UCA V RNA is completely stalled in 3′ processing, as opposed to being processed very slowly. In fact, we noted that a low level of SV40-AGU small t antigen mRNA splicing occurred with U2-UCA V. It is also possible that a small amount of unprocessed pre-U2-UCA V RNA in the cytoplasm becomes internalized in nuclei when the nuclear envelope reforms after mitosis. (Our experiments examine RNA 48 h after transfection in cells growing with a population doubling time of ~18 h.) Moreover, we cannot presently rule out the possibility that a small amount of pre-U2-UCA V RNA, in its unprocessed form, enters the nucleus (escaping detection in our analyses) but is intrinsically deficient, due to its unprocessed 3′ tail, in supporting splicing of the reporter pre-mRNA. This latter possibility seems most unlikely since: (i) the known U2 RNA sites essential for splicing are not perturbed in pre-U2-UCA V; (ii) the 3′ tail of pre-U2-UCA V does not have significant base-pairing potential with any functional sites in the internal region of U2 RNA; and (iii) pre-U2-UCA V assembles into an Sm protein-containing complex, showing that this functionally essential aspect of U2 snRNP is not impaired by the unprocessed 3′ tail of pre-U2-UCA V RNA. It is also noteworthy in this respect that a yeast U2 RNA carrying an unnatural poly(A) tail nonetheless is functional in splicing (26). A final caveat is the possibility that pre-U2-UCA V RNA gets trapped in the cytoplasm not because of its 3′ processing deficiency *per se* but because its unprocessed 3′ tail has an affinity for some element of cytoplasmic structure. However, this possibility seems rather unlikely since we employed a Triton extraction procedure for the preparation of cytoplasmic RNA (Materials and Methods) that is known to selectively release RNAs that are not associated with cytoplasmic structural elements (27); in fact, the endogenous pre-snRNAs in the cytoplasm are known to be among these Triton-released RNAs (13).

Previous studies in *Xenopus* oocytes have revealed that U1 RNA molecules with unprocessed 3′ ends are not imported into the nucleus (28), and our present results (for U2 RNA) in cultured human cells are entirely compatible with the earlier oocyte findings. The *Xenopus* oocyte U1 RNA enters the nucleus as a +2 nt precursor molecule, suggesting that this distinctive 3′ end constitutes, or contributes to, the nuclear import signal (28). According to this model, longer, unprocessed U1 or U2 RNA precursors such as U2-UCA V, are not imported because a distinctive 3′ end signal is not revealed as a 3′ terminal structure. Alternatively, it is possible that the 3′ end secondary structure of some unprocessed U1 or U2 RNA mutants actually inhibits nuclear import (29). In our experiments we cannot distinguish between the possibilities that pre-U2 UCA V RNA is unable to enter the nucleus because it never attains a correctly processed 3′ end ‘signal’ versus a scenario in which its non-processed 3′ tail is itself directly inhibitory to import. It should also be borne in mind that because the 3′ end structures of mammalian U1 and U2 RNAs at the moment of nuclear import are not precisely known (+2, +1, +0?), it is unclear whether or not the *Xenopus* U1 RNA nuclear import model applies to mammalian cells. As indicated in the Introduction, numerous precedents exist for RNA transport mechanisms differing between *Xenopus* oocytes and mammalian cells, e.g. the necessity versus non-necessity, respectively, of cap hypermethylation for nuclear import of U2 snRNP (7–9). Indeed, these reported differences were among the considerations that prompted the present investigation.

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