Distinct molecular signals for nuclear import of the nucleolar snRNA, U3

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Export to the cytoplasm of U3 RNA transcribed from a rat U3 gene injected into the nucleus of *Xenopus* oocytes indicates that the biogenesis of U3 RNA, like that of the previously studied Sm-precipitable nucleoplasmic snRNAs (U1, U2, U4, and U5), includes a cytoplasmic phase. The regulation of import of the U3 snRNA into the nucleus has been analyzed by injection of synthetic human U3 transcripts into the cytoplasm of *Xenopus* oocytes. Binding of the major autoantigenic protein of the U3 snRNP, fibrillarin, and cap trimethylation can occur in the cytoplasm, but neither are required for import. The 3'-terminal 13 nucleotides are required for optimal import and cap trimethylation and participate in a phylogenetically conserved U3 structural element, a short 3'-terminal stem. An artificial construct containing the 3'-terminal 13 nucleotides, including the 3'-terminal stem, but only 56 nucleotides of the 217 nucleotides in U3, appears to be sufficient for import. The presence of the 3'-terminal stem in all snRNAs known to be imported suggests that it might be a universal element required for nuclear import.

[**Key Words:** Nuclear import; *Xenopus* oocytes; U3 snRNA; fibrillarin; trimethyl guanosine cap]

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All RNA polymerase II transcripts whose biogenesis has been well-studied, including the spliceosomal small nuclear RNAs (snRNAs) U1, U2, U4, and U5 and mRNA, exit the nucleus at some stage in their maturation (for review, see Parry et al. 1989; Lamond 1990; Andersen and Zieve 1991; Dingwall 1991; Goldfarb and Michaud 1991; Nigg et al. 1991). These RNAs acquire a 7-methyl guanosine cap cotranscriptionally, which acts as one of the signals for nuclear export (Hamm and Mattaj 1990). After export, mRNA remains in the cytoplasm to be translated into protein. In contrast, the spliceosomal snRNAs are reimported into the nucleus as ribonucleoproteins, where they participate in mRNA splicing.

DeRobertis et al. (1982) first showed that the human spliceosomal snRNAs (U1, U2, U4, U5, and U6) injected into the cytoplasm of *Xenopus* oocytes became concentrated in the nucleus and that binding of the common ribonucleoprotein polypeptides, called the Sm proteins, could occur in the cytoplasm. Subsequently, the binding of the Sm proteins was found to be required for import of *Xenopus* U1 and U2 (Mattaj and DeRobertis 1985; Mattaj 1986; Hamm et al. 1990) and of human U1 (Fischer and Lührmann 1990) by injection of mutant RNAs lacking the Sm-binding site into *Xenopus* oocytes. The binding of the Sm proteins is also required for hypermethylation of the 7-methyl guanosine cap to 2,2,7-trimethyl guanosine (TMG) (Mattaj 1986; Hamm et al. 1990), which occurs for each of the spliceosomal snRNAs (U1,

U2, U4, and U5) in the cytoplasm. It has been hypothesized that a bipartite signal, consisting of both the TMG cap and bound Sm proteins, is necessary for import of *Xenopus* U1 and U2 and human U1 snRNAs (Hamm et al. 1990; Fischer and Lührmann 1990). However, further investigation (Konings and Mattaj 1987; Neuman de Vegvar and Dahlberg 1990) suggests that this bipartite signal functions only on snRNAs with the correct structure. In contrast to the U1 and U2 snRNAs, the chicken U4 and *Xenopus* U5 snRNAs bind the Sm proteins in the cytoplasm but do not require a trimethylated cap structure to be imported (Fischer et al. 1991). These data suggest that there are at least two different pathways of import for trimethyl cap-containing snRNAs.

Another abundant vertebrate snRNA transcribed by RNA polymerase II (for review, see Dahlberg and Lund 1988), the U3 snRNA, participates in rRNA processing in the nucleolus (Kass et al. 1990; Savino and Gerbi 1990). It belongs to a group of three nucleolar snRNAs (U3, U8, and U13) that share sequence similarities (Tyc and Steitz 1989), including the trimethyl cap, and assemble into particles immunoprecipitable by anti-fibrillarin (α Fb) but not by anti-Sm (α Sm) autoantibodies. We refer to this group of nucleolar small nuclear ribonucleoproteins (snRNPs) as the Fb snRNPs. The U3 snRNP consists of one 217-nucleotide-long RNA and six proteins (74, 59, 34, 30, 13, and 12.5 kD; Parker and Steitz 1987). A sequence required for binding of its major autoantigenic protein, the 34-kD Fb, has been defined (Baserga et al. 1991). Otherwise, little is known about the biogenesis of the U3 snRNP.

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Here, we study the biogenesis of the mammalian U3 snRNP in *Xenopus* oocytes. We show that like the polymerase II-transcribed spliceosomal snRNAs (U1, U2, U4, and U5) the U3 snRNA is exported to the cytoplasm and reimported into the nucleus. We ask, by analogy to the binding of the Sm proteins to the spliceosomal snRNAs, whether binding of the common protein Fb or cap trimethylation is required for import of the U3 snRNA. We identify a novel structural motif, the 3'-terminal stem, which is required both for import and cap trimethylation of the U3 snRNA. A 56-nucleotide RNA containing the 3'-terminal stem appears to be sufficient for import. Inspection of all imported snRNAs studied so far reveals that a similar structural motif may be common among them.

Results

Export of the U3 snRNA from the nucleus to the cytoplasm

It was first necessary to establish whether the U3 snRNA, like the spliceosomal snRNAs, is exported to the cytoplasm and undergoes cap hypermethylation there to yield a trimethylated snRNA (2,2,7-TMG). A rat U3 gene cloned into pUC13 (called -375 U3D; Stroke and Weiner 1985; Ach and Weiner 1991) was injected into the nucleus of *Xenopus* oocytes, and transcription was monitored by coinjection of [³²P]GTP. RNA was harvested from the nucleus and cytoplasm and compared with total RNA and with RNA from oocytes injected only with [³²P]GTP. Time points at 10 and 30 min, 1, 3, and 18 hr were taken. Figure 1A shows the results with the 18-hr time point. The U3 transcript appears in total, cytoplasmic, and nuclear RNA (lanes 2–4). The level of transcription from this plasmid is comparable to that of the endogenous 5S RNA. The ratios of 5S RNA to U3 transcript are similar in both the total and cytoplasmic lanes. U3 is not seen in oocytes injected with [³²P]GTP only (lane 1). More U3 transcript appears in the nuclear lanes than in the cytoplasmic lanes at all time points (data not shown), as would be expected. As shown in the subsequent figures, our fixation and dissection procedure cleanly separates cytoplasmic and nuclear RNAs. The appearance of U3 in the cytoplasmic fraction therefore allows us to conclude that U3 is exported to the cytoplasm after transcription.

To investigate whether U3 snRNA can undergo cap trimethylation in the cytoplasm of *Xenopus* oocytes, synthetic ³²P-labeled GpppG-capped human U3 snRNA was injected into the cytoplasm of whole *Xenopus* oocytes or into enucleated oocytes. Soon after injection (0 hr) and at 18 hr, RNA was purified and trimethylation was assessed by immunoprecipitation with anti-trimethyl guanosine (αTMG) antibodies. As shown in Figure 1B, lanes 1 and 3, newly injected GpppG-capped U3 RNA is not immunoprecipitated by αTMG antibodies, whereas U3 RNA that has been incubated in either whole oocytes (lane 2) or in enucleated oocytes (lane 4)

for 18 hr becomes immunoprecipitable. Thus, the U3 cap can become trimethylated in the cytoplasm.

Injected U3 RNA can also bind Fb in the cytoplasm, as shown in Figure 1C. U3 RNA was injected into either enucleated or whole oocytes and then assayed for Fb binding by immunoprecipitation with αFb antibodies immediately after injection (0 hr) or at 2 hr. The amount of U3 bound to Fb at 0 hr reflects background binding and is proportional to the quantity of RNA injected. Figure 1C, lanes 2 and 4, reveals that the binding of Fb to U3 in enucleated oocytes is about equivalent to that in whole oocytes (lanes 6,8). The percentage of RNA immunoprecipitable with αFb antibodies with respect to the RNA in the supernatant was calculated; there is approximately threefold more U3 RNA immunoprecipitable in the enucleated oocytes than in the whole oocytes.

To ensure that the binding of Fb to U3 was specific, we took advantage of our previous demonstration that an intact box C sequence (see Fig. 3, below) is necessary for Fb binding in HeLa cell extract (Baserga et al. 1991). Equal amounts (cf. the 0-hr time points for each; lanes 6,10) of wild-type U3 and a U3 with a point mutation in box C (U3 G159A), which severely reduces binding of human Fb in vitro, were injected, and the resulting extracts were immunoprecipitated with αFb antibodies. The percentage of RNA immunoprecipitable with αFb antibodies with respect to the RNA in the supernatant was calculated; there is approximately eightfold less U3 G159A RNA immunoprecipitable when compared with wild-type U3. This suggests that box C also directs Fb binding in vivo in *Xenopus* oocytes.

Binding of the major autoantigenic protein Fb is not necessary for U3 snRNA import

Because Fb can bind to U3 snRNA in the cytoplasm, we asked, by analogy to the Sm proteins, whether Fb binding was necessary for nuclear import. Increasing amounts of purified αFb monoclonal antibody (Reimer et al. 1987) were coinjected with U3 snRNA into the cytoplasm of *Xenopus* oocytes. Even at antibody concentrations as high as 0.8 mg/ml, no inhibition of nuclear transport is seen after an 18-hr incubation (Fig. 2, lanes 7–10). This amount of antibody (50 nl of 0.8 mg/ml) is sufficient to bind all of the available U3 snRNPs in a *Xenopus* oocyte (data not shown). We conclude that although Fb can bind U3 snRNA in the cytoplasm its binding is not required for import. This conclusion is confirmed in experiments described below assessing the import of U3 mutated in box C.

Requirements for U3 snRNA import from the cytoplasm to the nucleus

Modifications of the U3 snRNA were made either by truncation or by site-directed mutagenesis to establish what regions or structures are necessary for import and cap trimethylation. The sequence and structure of U3 snRNA is shown in Figure 3. U3 snRNAs truncated at

Figure 1. (A) U3 snRNA exits the nucleus after transcription. *Xenopus* oocytes were coinjected with the rat U3 gene (–375 U3D in pUC13) and [α -³²P]GTP or with [α -³²P]GTP alone. After 18 hr, total (10 oocytes) or nuclear and cytoplasmic (10 oocytes) RNAs were analyzed on an 8% denaturing polyacrylamide gel. (B) U3 snRNA undergoes 5' cap trimethylation in the cytoplasm of *Xenopus* oocytes. In vitro-transcribed GpppG-capped U3 snRNA was injected into the cytoplasm of whole or enucleated *Xenopus* oocytes. RNA was harvested immediately after injection (10 oocytes) or at 18 hr (10 oocytes) and assayed for trimethylation by immunoprecipitation with α TMG antibodies. Pellet and supernatant were analyzed on an 8% denaturing polyacrylamide gel. Some RNA degradation (lanes 5–8) occurs after isolation of the total RNA during the immunoprecipitation. (C) U3 snRNA can bind Fb in the cytoplasm of *Xenopus* oocytes, and such binding is affected by a single point mutation in box C (G159A). In vitro-transcribed U3 RNA was injected into either whole or enucleated oocytes. U3G159A was injected into whole oocytes. Oocyte extracts were made either immediately after injection (10 oocytes) or at 2 hr (10 oocytes). Binding of Fb was assayed by immunoprecipitation with α Fb autoantibodies and was compared with immunoprecipitation with normal human serum (NHS). RNA was analyzed on an 8% denaturing polyacrylamide gel. These results were quantitated on a Vision 200 densitometer.

the 3' end were synthesized by in vitro transcription of the U3 plasmid or replicative form (RF) DNA that had been digested with *Bst*UI (transcript of 204 nucleotides), *Fnu*4HI (transcript of 138 nucleotides) or *Dde*I (transcript of 104 nucleotides), as indicated in Figure 3. Mutations were also introduced into boxes B, C, and D; these regions have been deduced to be involved in protein binding to U3 snRNA (Parker and Steitz 1987) and are highly

conserved from yeast to human U3 snRNAs (Parker and Steitz 1987; Jeppesen et al. 1988; Porter et al. 1988; summarized in Tyc and Steitz 1989). Because simple deletion of the box B, C, or D sequences would be expected to alter the structure of the RNA drastically, substitution/deletion of each of these regions was designed instead. They are called Δ B, Δ C, and Δ D and are indicated in Figure 3 (β B = nucleotides 106–114; Δ C = nucleotides

Figure 2. Prior injection of α Fb antibody does not affect U3 RNA nuclear import. Oocytes were injected with buffer (lanes 1, 2, 6, 7) or with increasing concentrations of α Fb antibody (lanes 3–5, 8–10) 1 hr before injection with in vitro-transcribed human U3 RNA. Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). The maximum concentration of α Fb antibody injected is 0.8 mg/ml (lanes 5, 10). Lanes 4 and 9 represent a 1 : 10 dilution; lanes 3 and 8 represent a 1 : 100 dilution of the concentrated antibody.

158–166; Δ D = nucleotides 207–212). Finally, mutations predicted to perturb the U3 RNA secondary structure were introduced into the 5 terminal nucleotides of U3 (nucleotides 213–217). These mutations affect the 3'-terminal stem and are shown in the boxed sequence identified as the stem mutation in Figure 3. Compensatory mutations were also made in the U3 RNA (nucleotides 75–79) to restore base-pairing. These are indicated in the boxed sequences identified as the stem mutation suppressor in Figure 3.

Figure 4 shows that injected wild-type U3 snRNA is both imported into the nucleus (Fig. 4A, lanes 1–4) and cap trimethylated (Fig. 4B, lanes 1, 2). In contrast, U3 *Bst*UI (104 nucleotides; see Fig. 3), which is truncated only 13 nucleotides from the 3' end, is neither imported (Fig. 4B, lanes 5–8) nor trimethylated (Fig. 4C, lanes 3, 4). The absence of U3 *Bst*UI RNA in the nucleus is not the result of preferential increased nuclear degradation of this RNA because the overall stability of the wild-type and truncated RNAs is very similar. Two other truncated U3 snRNAs, U3 *Fnu*4HI and U3 *Dde*I, which are even shorter (see Fig. 3), exhibit similar behavior (Fig. 4D, and data not shown). Quantitatively, import of the truncated U3 snRNAs (U3 *Bst*UI, U3 *Fnu*4HI, and U3 *Dde*I) is $\sim$ 100-fold less than full-length U3 snRNA (Fig. 4D). These experiments suggest that a sequence or structure in the U3 snRNA itself is necessary for import. Deletion of 13 nucleotides from the 3' end might affect either direct binding of a protein to box D or a particular structure required for import.

Figure 4C compares the import into the nucleus of three substituted/deleted U3 snRNAs with that of wild-type after injection into the cytoplasm of *Xenopus* oocytes. Surprisingly, Δ B, Δ C, and Δ D are all imported to a significant extent (Fig. 4B, lanes 5–16). Quantitation of the results from Figure 4, A and B, and other experiments indicates that U3 snRNA import is lowered only two- to

threefold by the substitution/deletion mutations (Fig. 4D). We therefore consider that the substituted/deleted U3 snRNAs are subject to active, albeit somewhat impaired, import compared with the truncated RNAs, whose import is reduced to background levels (Fig. 4D).

Because we showed previously that a single-base substitution in box C abolishes Fb binding in vitro (Baserga et al. 1991) and now in vivo (Fig. 1C), the results with U3 Δ C (Fig. 4, lanes 9–12) argue further that Fb binding is not required for import. Additional experiments (data not shown) confirmed that several different point mutations in box C, which affect Fb binding in vitro, also have no effect on import or cap trimethylation.

Truncation of U3 snRNA by as little as 13 nucleotides abolishes import (100-fold less than wild type). Within these 13 nucleotides are two phylogenetically conserved motifs: (1) the box D sequence, and (2) the 3'-terminal stem (Fig. 3; Parker and Steitz 1987; Jeppesen et al. 1988). Because substitution of the box D sequence reduces import only about threefold (Δ D in Fig. 4D) and cap tri-

Figure 3. Sequence and structure of human U3 snRNA (Parker and Steitz 1987). Indicated are restriction sites used to cleave either plasmid or M13 RF DNA to make truncated U3 RNA transcripts (*Bst*UI, *Fnu*4HI, *Dde*I). The substitution/deletion mutations of boxes B, C, and D are indicated (Δ B, Δ C, Δ D). The stem mutation [nucleotides 213–217] and stem mutation suppressor are indicated.

Figure 4. (A) U3 snRNA import from the cytoplasm to the nucleus of *Xenopus* oocytes is regulated. In vitro-transcribed U3 RNA or U3 RNA truncated at the *Bst*UI site (see Fig. 3) was injected into the cytoplasm of *Xenopus* oocytes. Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). RNA from each compartment was analyzed on an 8% denaturing polyacrylamide gel. (B) Truncation of U3 snRNA abolishes 5' cap trimethylation. In vitro-transcribed U3 RNA or U3 RNA truncated at the *Bst*UI site was injected into *Xenopus* oocytes. Total RNA was harvested either immediately after injection or at 18 hr. Equal amounts of 32 P-labeled RNA were immunoprecipitated with α TMG antibodies and analyzed on an 8% denaturing polyacrylamide gel. (C) Substitution/deletion of three conserved regions in U3 snRNA has only a small effect on import. In vitro-transcribed U3 RNA and three substitution/deletions of U3 (Δ B, Δ C, Δ D; Fig. 3) were injected into the cytoplasm of *Xenopus* oocytes. Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). RNA from each compartment was analyzed on an 8% denaturing polyacrylamide gel. U3 Δ C (lanes 9–12) and U3 Δ D (lanes 13–16) are from the same experiment as U3 and U3 Δ B but were exposed for one-fifth the time. (D) Quantitation of import of U3 RNA, three truncated U3 RNAs (U3 *Bst*UI, U3 *Fnu*4HI, and U3 *Dde*I; Fig. 3), and three substituted/deleted U3 RNAs (Δ B, Δ C, Δ D; Fig. 3). The experiments in A and C, as well as others, were quantitated on a Molecular Dynamics PhosphorImager. The percent import is calculated as $N18/N18 + C18$. U3 snRNA import is set at 100%. Each bar represents the average of two experiments. In two experiments, the U3 Δ D was imported at 15% (C, lanes 13–16) or 52%, to make an average of 34%. This has been confirmed in a third trial.

methylation to about the same extent (data not shown), we next investigated whether nuclear import and cap trimethylation were sensitive to alterations in RNA structure in the 3'-terminal stem. Mutations were made that abolished the base-pairing (stem mutation; Fig. 3); base-pairing was then reestablished by compensatory mutations in the paired strand (stem suppressor mutation; Fig. 3). As shown in Figure 5A, absence of the 3'-terminal stem greatly reduces (4- to 10-fold) import when compared with wild type (lanes 4,5,10,11). When the stem is reestablished by compensatory mutations in the paired strand, import is restored to wild-type levels (lanes 4,6,10,12). Figure 5B shows similar results with respect to cap trimethylation. U3 snRNAs that lack the 3'-terminal stem are not detectably trimethylated (lanes 2,5). When the 3'-terminal stem is reestablished, cap trimethylation is restored to wild-type levels (lanes 3,6). These results suggest that maintenance of the 3'-terminal stem is necessary for nuclear import and cap trimethylation of the U3 snRNA.

Is the structure at the 3' end of the U3 RNA sufficient for nuclear import? A fragment of U3 RNA, containing the 3'-terminal stem and the box D sequence, was constructed (Fig. 6A). When this RNA is injected into *Xenopus* oocytes, it is highly unstable, and after an 18-hr incubation, only ~1% remains (Fig. 6, cf. lanes 5 and 6). Surprisingly, in spite of this degradation, some U3 3'-end fragment RNA was observed to be imported into the nucleus (Fig. 6B, cf. lanes 7 and 8). If percent import is calculated using the C0 value in the denominator (instead of C18 because of the extensive degradation), import of the U3 3'-end fragment is ~60- to 100-fold greater than that of the truncated U3 *Bst*UI RNA (Fig. 4A, and data not shown). These results suggest that this 56-nucleotide fragment of the U3 RNA is sufficient for import, although it appears not to be sufficient for maintenance of RNA stability.

One part of the signal for U1 and U2 snRNA import is the 5' TMG cap. To investigate whether a TMG cap structure is necessary for U3 snRNA import, U3 snRNA

Figure 5. (A) U3 RNA requires a structural motif, the 3'-terminal stem, for nuclear import. In vitro-transcribed U3 RNA and two RNAs with mutations in the 3'-terminal stem were injected into *Xenopus* oocytes. The U3 stem mutation (Fig. 3) abolishes the 3'-terminal stem; the U3 stem suppressor mutation restores the 3'-terminal stem with compensatory mutations in the base-paired strand (Fig. 3). Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). RNA from each compartment was analyzed on an 8% denaturing polyacrylamide gel. (B) U3 RNA requires a 3'-terminal stem for TMG cap formation. In vitro-transcribed U3 RNA or two U3 RNAs with mutations in the 3'-terminal stem (stem and stem suppressor; Fig. 3) were injected into *Xenopus* oocytes. Total RNA was harvested at 18 hr. Equal amounts of 32 P-labeled RNA were immunoprecipitated with α TMG antibodies and analyzed on an 8% denaturing polyacrylamide gel.

was synthesized with a cap (ApppG) that is not a substrate for trimethylation. The import of U3 containing an ApppG cap was then compared with that of the normal U3 transcript beginning with a GpppG cap, which is a substrate for trimethylation (Fig. 1B; Fischer and Lührmann 1990; Hamm et al. 1990). As controls, import of human U1 snRNA with or without ApppG and import of a truncated U1 snRNA lacking the Sm protein-binding site were examined in parallel. Both the U1 ApppG-capped RNA and the truncated U1 snRNA (GpppG capped) had been shown previously to be deficient in import (Fischer and Lührmann 1990). As shown in Figure 7, the U1 snRNA with a GpppG cap is imported (lane 4), whereas both the U1 snRNA with the ApppG cap (lane 8) and the U1 snRNA minus the Sm protein-binding site (lane 12) are not. The smaller size of the U1 snRNAs at 18 hr is the result of 3' trimming of the 25 extra nucleotides that are part of this runoff transcript. In contrast, the ApppG-capped U3 snRNA is imported, as well as the GpppG-capped U3 snRNA (Fig. 7, cf. lanes 16 and 20). This demonstrates that although trimethylation of the

U3 snRNA occurs, it is not required for import. We conclude that U3 RNA represents a unique class of import of trimethylated snRNAs that do not bind the Sm proteins and do not require trimethylation for import.

Discussion

Our studies have outlined the major steps in the biogenesis of the U3 snRNP leading to its accumulation in the nucleus. After transcription by RNA polymerase II, U3 is transported to the cytoplasm, where it can undergo cap trimethylation and bind its major autoantigenic protein Fb. Its subsequent import into the nucleus is dictated largely by a structural motif, the 3'-terminal stem, and requires neither cap trimethylation nor Fb binding. Mutation of boxes B, C, and D affects import to a lesser extent. U3 RNA import is therefore different from that described for the spliceosomal snRNAs (Mattaj and DeRobertis 1985; Mattaj 1986; Hamm et al. 1990; Fischer and Lührmann 1990; Fischer et al. 1991) in that it is not dependent on binding of its common autoantigenic protein.

U3 biogenesis includes a cytoplasmic phase

Whether the biogenesis of the U3 snRNP requires RNA transit through the cytoplasm has been controversial. In particular, steady-state analyses of U3 RNA and Fb during *Xenopus* oogenesis have localized both exclusively in the nucleus (Caizergues-Ferrer et al. 1991). Unlike the Sm proteins (Forbes et al. 1983; Zeller et al. 1983), Fb is not stockpiled in the *Xenopus* oocyte cytoplasm. However, as these investigators discuss, U3 and Fb synthesis correlates generally with rRNA synthesis. It therefore seems reasonable that Fb would not be stored because it is complexed with U3 in the U3 RNP, which is participating actively in rRNA processing in maturing oocytes. Furthermore, U3 snRNA transit through the cytoplasm may be brief and not detectable by Northern blot analysis (Caizergues-Ferrer et al. 1991).

The data we present here and experiments from other laboratories argue that U3 RNA does enter the cytoplasm after transcription. We have observed that U3 snRNA transcribed from a rat U3 gene injected into the nucleus of *Xenopus* oocytes is found in the cytoplasm (Fig. 1). In pulse-chase analysis of snRNA synthesis in mammalian cells with two different advanced techniques of cell fractionation (Zieve et al. 1988; Feeney et al. 1989), U3 snRNA was also detected in the cytoplasm. Furthermore, because the trimethylation reaction for the spliceosomal snRNAs is believed to be a uniquely cytoplasmic event (Mattaj 1986) and the U3 snRNA cap is trimethylated, U3 snRNA would be expected to undergo this reaction in the cytoplasm. Accordingly, we have shown that the U3 snRNA cap can be trimethylated in the cytoplasm, as is the case for spliceosomal snRNAs (Fig. 1). Finally, Stroke and Weiner (1985) observed that the primary transcript of rat U3 is slightly longer than the snRNA mature size (8–14 nucleotides). The trimming event that matures the 3' end of the snRNAs is

Figure 6. (A) Diagram of the U3 3'-end fragment RNA that contains the 3'-terminal stem. This RNA represents a fragment of the U3 RNA, including the box D sequence (boldface box), with these extra nucleotides: a 5' G and a tetraloop of UUCG between U3 nucleotides 101 and 198 (light-face box). (B) The U3 3' end fragment of snRNA is sufficient for import. In vitro-transcribed U3 RNA and the U3 3'-end fragment (A) were injected into the cytoplasm of *Xenopus* oocytes. Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). RNA from each compartment was analyzed on an 8% denaturing polyacrylamide gel.

thought to be almost completely cytoplasmic (for review, see Dahlberg and Lund 1988).

The *Xenopus* oocyte has been used widely to study RNA trafficking of both *Xenopus* snRNAs and snRNAs from other species because of the ease and precision of microinjection into the nuclear and cytoplasmic compartments. Localization studies of nascent and steady-state snRNAs in mammalian cells using conventional cell fractionation procedures are largely in agreement with data from *Xenopus* oocytes (Eliceiri 1974; Eliceiri and Sayavedra 1976; Zieve and Penman 1976; Gurney and Eliceiri 1980), as are more recent results (Zieve et al. 1988; Feeney et al. 1989). Taken together, the data argue that snRNP biogenesis in mammalian somatic cells and *Xenopus* oocytes is similar. Previously, conclusions concerning the biogenesis of the *Xenopus*, human, and chicken U snRNAs have been drawn from studies in the *Xenopus* oocyte (see references in introductory section). We anticipate that our findings concerning the human and rat U3 RNA will likewise be generally applicable.

U3 defines a unique class of snRNA import signals

Previously, it has been shown that import of the spliceosomal snRNAs (U1, U2, U4, U5, U6) falls into three distinct groups. Import of U1 and U2 requires both binding of the Sm proteins and cap trimethylation (Mattaj and DeRobertis 1985; Mattaj 1986; Fischer and Lührmann 1990; Hamm et al. 1990), whereas import of U4 and U5 requires only binding of the Sm proteins (Fischer et al. 1991). Although U6 is a spliceosomal snRNA, it is transcribed by RNA polymerase III (Kunkel et al. 1986; Das et al. 1988) and acquires a γ -methyl triphosphate cap (Singh et al. 1990). Unlike the other spliceosomal RNAs but consistent with its transcription by RNA polymerase III and function in the nucleus, U6 does not enter the cytoplasm after transcription (Vankan et al. 1990). If synthetic U6 is injected into the cytoplasm of *Xenopus* oocytes, however, it is imported in a sequence-dependent manner (Hamm and Mattaj 1989).

Here, we have shown that import of U3 snRNA de-

Figure 7. U3 snRNA is imported in a cap trimethylation-independent manner. In vitro-transcribed U1 snRNA (GpppG- or ApppG-capped), truncated U1 snRNA (U1 minus Sm, 115 nucleotides), and U3 snRNA (GpppG- or ApppG-capped) were injected into the cytoplasm of *Xenopus* oocytes. Oocytes were fixed and dissected into cytoplasm (C) and nucleus (N), either immediately after injection (C0, N0) or after 18 hr (C18, N18). RNA from each compartment was analyzed on an 8% denaturing polyacrylamide gel.

defines a fourth group that is not dependent on binding of the major autoantigenic protein, Fb, or cap trimethylation. Our results are consistent with studies in *Saccharomyces cerevisiae* (Tollervy et al. 1991), which showed that depletion of the yeast analog of Fb (NOP1) has no effect on the nuclear or nucleolar localization of U3 snRNA. Michaud and Goldfarb (1992) have also studied import of human U3 snRNA in *Xenopus* oocytes and found it to be kinetically distinct from U1 snRNA and protein import on the basis of competition assays using free TMG cap or a synthetic peptide analog of the nuclear localization signal of SV40 T antigen [p(Lys)-BSA]. Because it is TMG cap independent, the pathway of import of the U3 snRNP is formally similar to that of the U4 and U5 snRNPs (Fischer et al. 1991). Presumably, proteins of the U4 and U5 snRNPs are recognized by a cytosolic receptor protein before import. Although proteins of the U3 snRNP are also likely to be critical to import, the receptor would be expected to be different because no polypeptides are known to be shared between the U3 and the U4 or U5 snRNPs.

We have determined that the U3 snRNA requires a structural motif, an intact 3'-terminal stem of 5 bp, for both import and cap trimethylation. When a fragment of the U3 snRNA containing the 3'-terminal 13 nucleotides, which includes the 3'-terminal stem, is injected into oocytes, it appears to be sufficient for import, although we interpret these results cautiously because the fragment is subject to severe degradation. A 3'-terminal stem is conserved among U3 snRNAs from plants to mammals, although the sequences of the base-paired regions are not (Parker and Steitz 1987; Jeppesen et al. 1988). The sequences that comprise Box D and the 3'-terminal stem are likely to be a protein-binding site based on RNase protection analyses of native human U3 snRNPs (Parker and Steitz 1987). However, because the only U3 sequence known to be required for binding of a known protein is an intact box C sequence for Fb association *in vitro* (Baserga et al. 1991), further characterization of U3 snRNP proteins is necessary to establish whether the stem is important for RNA-protein interactions or only as a structure required for import.

This is not the first instance in which an RNA structural motif plays an important role in production of intact snRNPs. A terminal stem domain of 4–6 bp is required for accumulation of another nucleolar snRNA that binds Fb, the yeast U14 snRNA (Li and Fournier 1992). Our studies on the human U3 snRNA suggest that the presence of the 3'-terminal stem is required for biogenesis of the U3 snRNP; in contrast, the yeast U14 snRNA 3'-terminal stem seems to be crucial for RNA stability.

In addition, there are several lines of evidence that suggest that the bipartite signal for nuclear import (Sm proteins and TMG cap) of the U1 and U2 snRNAs functions only in the presence of the correct RNA structure. More specifically, all of the snRNAs studied so far with respect to their nuclear import possess stems at or near their 3' ends. Results with human U1 and U2 snRNAs suggest that their 3'-terminal stems might be important

for nuclear import (Neumann de Vegvar and Dahlberg 1990). In general, elongated human U1 and U2 snRNAs are not efficiently imported without prior 3' end trimming. Surprisingly, two RNAs (hU2+8 and sp-hU1+15) are efficiently imported. Unlike the other elongated snRNAs, these RNAs can be folded so that they would have a base-paired stem at their 3' ends. *Xenopus* U2 snRNAs that are elongated at their 3' ends (Konings and Mattaj 1987) are not imported, although they do bind the Sm proteins. By computer analysis, these RNAs have a different structure than the wild type. However, deletion of a single nucleotide from the 3' end of human U1 snRNA, which would maintain the 3' stem, also curtails import, suggesting that for U1 and U2 the structural signal for transport is more complicated than the mere presence of a 3'-terminal stem (Neumann de Vegvar and Dahlberg 1990).

Kiss et al. (1991) have presented convincing evidence that the U3 snRNA in plants is transcribed by RNA polymerase III and does not possess a trimethyl cap structure. They argue that this conversion from synthesis by polymerase II to III probably occurred after the evolution of plants. It follows that the pathway for biogenesis of plant U3 must be different from what we have deduced here for mammalian U3 snRNA. Interestingly, when the plant U3 gene is manipulated so that it is transcribed by RNA polymerase II, although the U3 snRNA binds Fb and acquires a trimethylated cap structure, it does not become part of a larger complex that is thought to function in rRNA processing (Kiss et al. 1991). Clearly, therefore, the pathway of U3 snRNP biogenesis does affect its ability to be utilized correctly, and the alternate pathway for biogenesis evolved by mammalian cells is incapable of achieving functional U3 complexes in plants.

Unlike the spliceosomal snRNAs (U1, U2, U4, and U5), which are nucleoplasmic, the human U3 snRNA ultimately becomes localized in the nucleolus. It is not known whether the signals for nuclear import of the U3 snRNP and those for its targeting to the nucleolus are overlapping or discrete. Sequences necessary for nucleolar localization of the following proteins have been delineated: HSP70 (Munro and Pelham 1984; Dang and Lee 1989; Milarski and Morimoto 1989), the Tat and Rev proteins of human immunodeficiency virus (HIV) (Dang and Lee 1989; Cochrane et al. 1990), the Rex protein of human T-cell leukemia virus, type I (HTLV-I) (also called p27X-III; Siomi et al. 1988), and the NO38 protein (Peculis and Gall 1992). Only in the case of NO38 (Peculis and Gall 1992) has the sequence for nucleolar localization been shown to be nonoverlapping with the signal for nuclear localization. The truncated and mutated U3 snRNAs that we have constructed will be useful for determining the regions of U3 snRNA that are necessary for nucleolar localization.

Materials and methods

Reagents

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs, Pharmacia, and Boehringer

Mannheim. Nucleotides and dinucleotides (GpppG and ApppG) were purchased from Pharmacia. Female *Xenopus laevis* specimens were obtained from *Xenopus* I. Oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer by Dr. John Flory (Yale University School of Medicine, New Haven, CT).

Antibodies

α TMG antibodies (Krainer 1988) were obtained from Oncogene Science (Manhasset, NY). α Fb antibodies (no. 1875) were obtained from patients with scleroderma and were the gift of Joe Craft (Yale University School of Medicine). α Fb monoclonal antibody (Reimer et al. 1987) was a gift from Eng Tan and Michael Pollard (Scripps Clinic, La Jolla, CA). Anti-Sm antibodies were from the hybridoma cell line Y-12 (Lerner et al. 1981) and were prepared by Mei-Di Shu.

snRNA constructs and mutagenesis

The rat U3 gene (–375 U3D) was obtained from Rob Ach and Alan Weiner. It contains a clone of the rat U3D gene in pUC13 with –375 of the 5'-flanking region. This clone is transcribed at the same levels as the U3D gene with –538 of the 5'-flanking region (Ach and Weiner 1991; Stroke and Weiner 1985).

The human U3 cDNA cloned behind a T7 RNA polymerase promoter was constructed as described previously (Baserga et al. 1991). The U3 cDNA is cloned both into the pSP64 plasmid (Promega Biotech) and into M13 mp18. The construction of the U3G159A mutation was described previously (Baserga et al. 1991). Site-directed mutagenesis was performed to create substitution/deletions at conserved boxes B, C, and D (Δ B, Δ C, Δ D) and in the 3'-terminal stem (Fig. 3; Zoller and Smith 1983; Kunkel et al. 1987). The following oligonucleotides were used: for Δ B, 5'-GCCAGAAAGCCGCGCAAAAAAAAAAGGAGAA-AACGCTACC; for Δ C, 5'-TACGGAGAGAAGAAGGGGGG-GGGATGGCTGACGGCAGTTGC; for Δ D, 5'-ACCACTTT-TTTCGCGTTCTCTCCCTCTCACTCCC; for the stem mutation, 5'-CCCCGGGTACCGAGCTCCCGGTGTCAGACCG-CGTTCTCTCC; and for the stem mutation suppressor, 5'-CG-CTACCTCTCTTCCCTCCACCTTTTCGGTGCTCTAC. All clones were sequenced in their entirety. RF M13 DNA was prepared according to standard procedures.

The U3 3'-end fragment clone was constructed using two overlapping oligonucleotides containing U3 nucleotides 71–101 and 198–217, as well as a T7 RNA polymerase promoter, *Eco*RI and *Hind*III restriction sites on the ends, an *Rsa*I site at the end of the U3 RNA sequence, an extra G on the 5' end, and a stable tetraloop (Fig. 6A). The sequences of the oligonucleotides are 5'-AATTCTAATACGACTCACTATAGGAAAACACG-AGGAAGAGAGGTAGCGTTTTCTTCGGAGAACGCGGTC-TGAGTGGTACCA and 5'-AGCTTGGTACCACTCAGACCG-CGTTCTCCGAAGAAAACGCTACCTCTCTCCTCGTGGT-TTTCCTATAGTGAGTCGTATTAG. These oligonucleotides were cloned directly into plasmid pSP64 after kinasing and annealing.

pHU1 was originally obtained from Thoru Pederson (Patton et al. 1987) and contains a human U1 RNA under the control of an SP6 RNA polymerase promoter.

Transcriptions

U3 plasmid or RF DNA was linearized with *Rsa*I for generation of full-length U3 transcripts or with *Bst*UI, *Fnu*4HI, or *Dde*I for generation of truncated U3 transcripts. The U3 stem mutation and stem suppressor DNAs were linearized with *Sma*I. The U3

3'-end fragment was linearized with *Rsa*I and, upon transcription, yields an RNA of 56 nucleotides. The pHU1 plasmid was linearized with *Bam*HI, which upon transcription gives a U1 RNA 25 nucleotides beyond the correct 3' end. To produce U1 minus Sm, pHU1 was cut with *Taq*I, giving a transcript of 115 nucleotides, ending before the Sm site. Transcriptions were performed with 1 μ g of DNA template in 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol, 250 μ M each cytidine triphosphate and guanosine triphosphate, 100 μ M each uridine triphosphate and adenosine triphosphate, 2 mM GpppG or ApppG dinucleotide, 40 μ Ci [α - 32 P]uridine triphosphate and [α - 32 P]adenosine triphosphate (specific activity each of 3000 Ci/mmol; 10 μ Ci/ μ l), 80 units of RNase inhibitor (Boehringer Mannheim), and 120 units of T7 or SP6 RNA polymerase (Pharmacia).

Oocyte injections

Injections of the rat –375 U3D gene into the nucleus of *Xenopus* oocytes were performed according to Ach and Weiner (1991).

When RNA was injected, ~10 fmoles (0.5×10^6 cpm/ μ l) of either 32 P-labeled U1 or U3 RNA was injected into the vegetal pole of stage VI *Xenopus* oocytes. Oocytes were incubated at 18°C in Barth's modified saline (MBS) overnight or as indicated. Oocytes were dissected manually into nucleus and cytoplasm after transfer into ice-cold dissecting buffer [80% ethanol/3% acetic acid (vol/vol); de la Pena and Zasloff 1987]. Twenty oocytes were injected for each RNA, half were harvested at 0 time, and half were harvested after incubation. For analysis of import and cap trimethylation, RNA was harvested according to Hamm et al. (1990). All of the RNA from each injection was analyzed on an 8% polyacrylamide gel.

When α Fb monoclonal antibodies are injected, they are injected into the vegetal pole of stage VI oocytes 1 hr before injection of in vitro-transcribed U3 snRNA. Approximately 50 nl of purified α Fb antibody is injected; the highest concentration of antibody used is 0.8/mg/ml. Analysis of nuclear import proceeds as described above.

Enucleation

The enucleation of oocytes was performed using the procedure of Ford and Gurdon (1977). Oocytes were inspected for resealing, transferred to MBS buffer, and allowed to incubate for 3 hr before use.

Immunoprecipitations

RNAs were immunoprecipitated either from purified RNA (α TMG) or from *Xenopus* oocyte extracts (α Fb autoantibodies). RNA was purified from 10 oocytes at the indicated time points according to Hamm et al. (1990). Extracts were prepared from 10 oocytes at the indicated time points by homogenization in NET-2 buffer (150 mM NaCl, 50 mM Tris at pH 7.5, 0.05% NP-40), followed by a 10-min microcentrifuge spin. Except for the experiment in Figure 1C, equal amounts of radioactive RNA, as measured by a scintillation counter, were used for each set of precipitations. For Figure 1C, equal numbers of injected oocytes were used, and a 0-hr time point (background binding) was included that reflects the amount of RNA injected. Two microliters of α TMG and 10 μ l of α Fb antibody were used in the respective precipitations. Immunoprecipitations were performed as described in Steitz (1988).

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Distinct molecular signals for nuclear import of the nucleolar snRNA, U3.

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