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Nuclear retention of RNA as a mechanism for localization

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ABSTRACT

Two mutant RNAs, one derived from $tRNA_i^{met}$, the second from U1 snRNA, that are defective in export from the nucleus to the cytoplasm have been studied. In both cases, the RNAs are shown to be transport competent but prevented from leaving the nucleus by interaction with saturable binding sites. This contradicts previous hypotheses to explain the behavior of the tRNA mutant, and highlights a general problem in using mutant RNAs to study nuclear export. In the case of these mutants, it is argued that nuclear retention is likely to be artifactual. However, the additional example of U6 snRNA is described. In this case, nuclear retention appears to be a physiological mechanism by which intranuclear localization is achieved. Evidence that the site of interaction with the La protein in U6 snRNA is important for its nuclear retention is presented.

Keywords: La protein; nuclear transport; RNA export; tRNA; U snRNA

INTRODUCTION

The mechanisms by which particular RNAs achieve the correct intracellular localization are not understood in detail. In this paper, we investigate how certain RNAs come to be retained in the nucleus. Under normal conditions, a variety of RNAs accumulate in the nucleus and carry out their functions there. These RNAs include the small nuclear (sn) and small nucleolar (sno) RNAs that are involved in the processing of a variety of nuclear precursor RNAs including pre-mRNAs, pre-tRNAs, and pre-rRNAs (reviewed by Lührmann et al., 1990; Filipowicz & Kiss, 1993; Mattaj et al., 1993), and the polyadenylated mammalian Xist and *Drosophila* Hsr-omega-n transcripts (Brockdorff et al., 1992; Brown et al., 1992; Hogan et al., 1994).

In relation to their mode of nuclear accumulation, these RNAs fall into two categories. One class includes the U snRNAs involved in pre-mRNA processing that are transcribed by RNA polymerase II, i.e., U1, U2, U4, U5, and U7. These RNAs leave the nucleus after transcription. In the cytoplasm they each associate with the same group of common proteins, often called the Sm proteins. Following this interaction, hypermethylation of the U snRNA cap structure takes place. The partly assembled U snRNPs are then reimported into the nucleus (reviewed by Lührmann et al., 1990; Izaurralde & Mattaj, 1992). In contrast, U6 snRNA, which is involved in splicing but is transcribed by RNA polymerase III, the polyadenylated nuclear RNAs mentioned above, and the two snoRNAs that have been examined, U3 and U8, appear to remain in the nucleus after transcription (Vankan et al., 1990; Brockdorff et al., 1992; Brown et al., 1992; Hogan et al., 1994; Peculis & Steitz, 1994; Terns & Dahlberg, 1994). Whether nuclear retention in these cases is a default state or requires interaction between an RNA and other nuclear components is not well established, although Terns and Dahlberg (1994) observed that the production of large amounts of U3 snoRNA in *Xenopus* oocyte nuclei led to some of the RNA appearing in the cytoplasm. This led them to suggest that nuclear retention of U3 might be mediated by saturable nuclear binding sites.

A second, fundamentally different, category of RNA that is retained in the nucleus consists of mutant versions of RNAs that are normally exported to the cytoplasm. Mutants in tRNA (Zasloff et al., 1982; Tobian et al., 1985), 5S rRNA (Guddat et al., 1990), and U1 snRNA (this paper) that have this property have been described. Export of several different types of RNA from the nucleus has been shown to be energy dependent and to be mediated by saturable nuclear factors (Zasloff, 1983; Jarmolowski et al., 1994). The generally

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accepted explanation for the behavior of the export-defective RNA mutants is that they are unable to interact with mediators of RNA export (Zasloff et al., 1982; Tobian et al., 1985; Guddat et al., 1990; Singh & Green, 1993). However, the alternative possibility, that the mutant RNAs are retained via inappropriate interactions with nuclear components, has not been excluded.

In this manuscript we investigate the mechanism of nuclear retention of three RNAs, U6 snRNA and two mutant RNAs, one a tRNA and the other derived from U1 snRNA. In all three cases, we show that nuclear retention involves specific binding to nuclear components, and that saturation of these binding sites allows export of the RNAs from the nucleus.

RESULTS

Nuclear retention of a mutant initiator methionyl tRNA

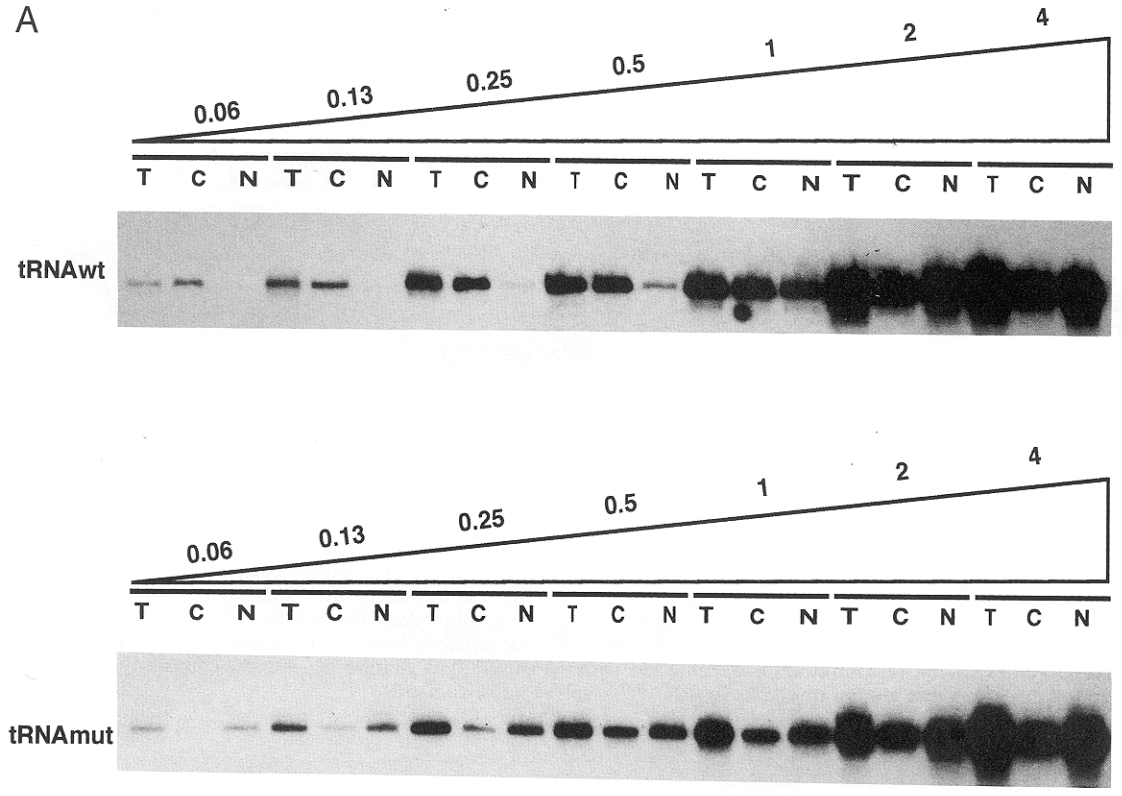
Among the first experiments carried out on RNA export from the nucleus (Zasloff et al., 1982; Zasloff, 1983; Tobian et al., 1985) were those showing that a particular mutant of human $tRNA_i^{met}$, in which G57 was mutated to U, was exported from the nucleus at a considerably reduced rate compared to the wild-type tRNA (Zasloff et al., 1982). This result has been interpreted as evidence that this and other similar mutants are defective in their interaction with components of the tRNA export machinery. An alternative explanation would be that the mutant RNAs bind tightly to some nuclear component and are thus prevented from leaving the nucleus. Consistent with the second possibility, we noted that when the nuclear concentration of $tRNA_i^{met}$ G57-U was increased, some of the RNA was exported to the cytoplasm. An example of such an experiment is shown in Figure 1A.

When increasing amounts of wild-type $tRNA_i^{met}$ are microinjected into the nucleus of *Xenopus* oocytes, the previously described saturation of the export pathway (Zasloff, 1983; Jarmolowski et al., 1994) is seen. This results in an increasing proportion of the RNA being retained in the nuclear fraction at higher concentrations (Fig. 1A, upper panel; T, C, N are total oocytes, cytoplasmic, and nuclear fractions, respectively). The behavior of the G57-U mutant RNA is shown in the lower panel of Figure 1A. When a mutant gene encoding this RNA is injected (Zasloff et al., 1982), or when in vitro-transcribed mutant RNA is injected at low concentration, this RNA is almost quantitatively retained in the nucleus (Fig. 1A, lower panel, first three lanes). However, when larger amounts of the RNA are injected, a significant fraction is exported to the cytoplasm (Fig. 1A, lower panel). The fraction of $tRNA_i^{met}$ G57-U in the cytoplasm as a function of the amount of RNA injected was found to be somewhat variable from experiment to experiment. Consistently, however, the maximal percentage of cytoplasmic RNA was found at an intermediate concentration of the G57-U mutant RNA. Further increasing the concentration of injected RNA caused the cytoplasmic fraction to decrease once more. This is more easily seen in the experiment shown in Figure 1B, where the fraction of either wild-type or mutant $tRNA_i^{met}$ exported to the cytoplasm has been quantified and plotted as a function of the amount of RNA injected. Note that in this experiment a wider concentration range of RNA was employed than in Figure 1A.

In the case of the wild-type RNA, the percentage of exported RNA is high over a wide range of concentration, then drops as levels saturating for export are reached (Fig. 1B). The behavior of the G57-U mutant RNA is more complex. At low concentration, virtually all the RNA is retained in the nucleus. However, the cytoplasmic fraction increases continually up to the point at which 0.5 pmol per oocyte was injected, at which time roughly 40% of the injected RNA is exported. Further increasing the amount of mutant tRNA injected led to a decrease, rather than an increase, in the cytoplasmic fraction, showing that the mutant tRNA leaves the nucleus by an export pathway that is saturable. These results suggest that $tRNA_i^{met}$ G57-U can interact with components of the export machinery, but that it is retained in the nucleus by interaction with some other saturable factor.

To demonstrate that the mutant tRNA is capable of interaction with the same export factors as the wild-type RNA, the ability of the mutant to inhibit export of wild-type $tRNA_i^{met}$ was tested. In this experiment, radioactively labeled $tRNA_i^{met}$ was mixed with an internal control RNA (U1ΔSm RNA) to monitor RNA recovery and microinjected into *Xenopus* oocyte nuclei either without further addition or after mixing with 2.5 pmol or 5 pmol of either wild-type or mutant $tRNA_i^{met}$. Both tRNAs competitively inhibited the export of the radiolabeled wild-type RNA (Fig. 2, compare the control lanes 4–6 with lanes 7–18), although the mutant RNA was a two- to threefold less efficient competitor (Fig. 2 and data not shown). Because other classes of RNA, such as 5S RNA, U snRNA, or mRNA, do not inhibit the export of tRNA in similar experiments (Jarmolowski et al., 1994), this provides good evidence that the mutant tRNA, when present in sufficient quantity, is exported by the standard tRNA export machinery.

The combination of results presented in Figures 1 and 2 rules out a third alternative explanation for the behavior of the mutant RNA, that it would have reduced, rather than no, affinity for export factors. The amount of the mutant tRNA required to saturate export (Figs. 1B, 2, and data not shown) is roughly twofold more than the wild-type RNA. Even if this were due to a difference in affinity for a component of the export machinery between the mutant and wild-type RNAs,



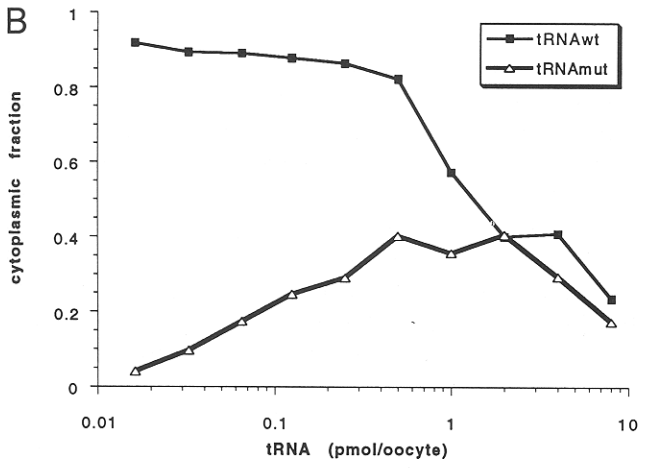


FIGURE 1. $tRNA_i^{met}$ mutant G57U is actively retained in the nucleus by a saturable factor. **A:** Increasing amounts of T7-transcribed wild-type $tRNA_i^{met}$ (upper panel) or mutant $tRNA_i^{met}$ with a G57 to U substitution (lower panel) were microinjected into the nuclei of *Xenopus* oocytes (amount indicated in pmol per oocyte). Directly after injection (three lefthand lanes) or after 70 min incubation at 19 °C the oocytes were dissected. RNA was reextracted from either total oocytes (T), cytoplasmic (C), or nuclear (N) fractions, and visualized by autoradiography after separation on denaturing polyacrylamide gels. **B:** Graphic representation of a similar experiment to that shown in A. Amounts of RNA in the cytoplasmic and nuclear fractions were quantified with a phosphoimager. Fraction of RNA exported into the cytoplasmic compartment is plotted as a function of total amount of RNA injected.

it would be insufficient to explain the large difference in export behavior seen over a wide range of concentrations (Fig. 1B). It is plausible that the difference in competitive efficiency might instead be due to a reduction in the effective concentration of the mutant RNA due to its binding to the sites that retain it in the nucleus.

It was of interest to identify the factor responsible for the retention of the mutant tRNA in the nucleus. We reasoned that the G57-U mutant might bind abnormally tightly to one of the components responsible for tRNA processing. If this were true, the mutant tRNA should have been an effective inhibitor of pre-tRNA processing. This hypothesis was tested using the *Xenopus* tRNAPhe precursor (Lin-Marq & Clarkson, 1995). This pre-tRNA is efficiently processed in oocyte nuclei to produce the mature tRNA and the cleaved 5′ and 3′ extensions (Fig. 3, lanes 1–4, only the 3′ extension is long enough to be retained on the gel shown). Only the mature tRNA is exported from the nucleus. The small amount of pre-tRNA in the cytoplasmic fraction is also seen immediately after injection (lane 1), and thus must reflect either inaccurate injection or reflux of some RNA to the cytoplasm on injection. Wild-type $tRNA_i^{met}$ was a more efficient inhibitor of pre-tRNAPhe processing than the G57-U mutant (Fig. 3, compare the control

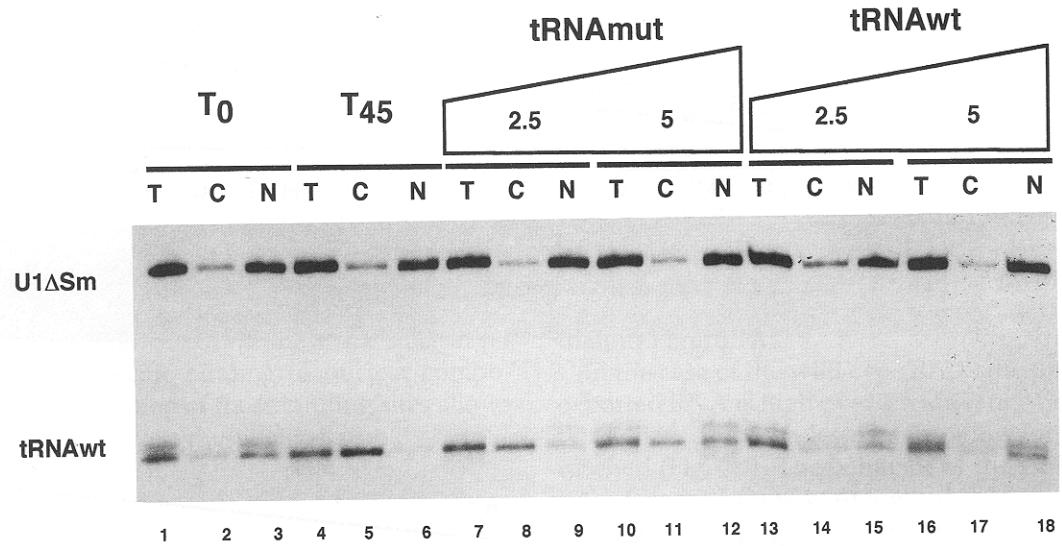


FIGURE 2. Transport-defective $tRNA_i^{met}$ mutant inhibits export of tRNAwt. Distribution between the nucleus and cytoplasm of 0.01 pmol of α-^{32}P-labeled wild-type $tRNA_i^{met}$ transcripts immediately after their injection into the nuclei of oocytes (lanes 1–3) or 45 min later (lanes 4–18). tRNA was injected either alone (lanes 4–6), together with 2.5 or 5 pmol per oocyte of unlabeled G57-U mutant $tRNA_i^{met}$ (lanes 7–12), or together with 2.5 or 5 pmol per oocyte of unlabeled tRNAwt (lanes 13–18). U1ΔSm RNA was coinjected with the $tRNA_i^{met}$ as an internal recovery control. In some experiments, for reasons that are not understood, $tRNA_i^{met}$ resolves into two bands.

lanes 3 and 4 with lanes 5–8). This argues against the retention factor being a component whose concentration is limiting for pre-$tRNA^{Phe}$ processing, but does not rule out the possibility that a nonlimiting processing factor might be involved in retention.

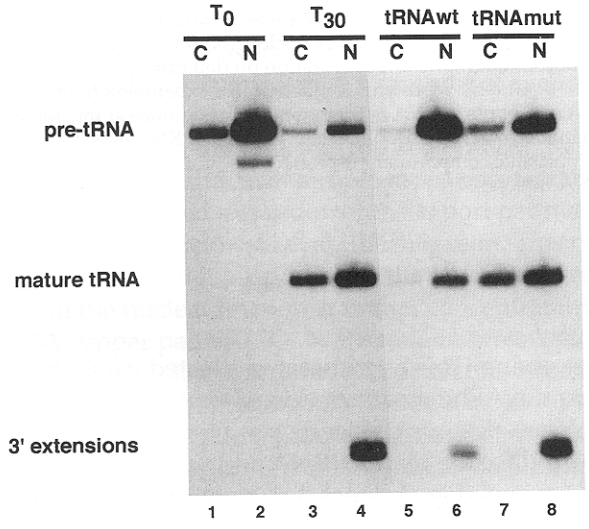


FIGURE 3. Transport-defective $tRNA_i^{met}$ does not inhibit pre-tRNA processing. Pre-$tRNA^{Phe}$ transcripts were microinjected into the nuclei of oocytes (0.1 pmol per oocyte) either in the absence of coinjected RNA (lanes 1–4) or together with 5 pmol per oocyte unlabeled tRNAwt (lanes 5, 6) or unlabeled G57U tRNAmut (lanes 7, 8). Oocytes were either dissected directly after injection (lanes 1, 2) or after 30 min incubation at 19 °C. RNA was extracted from cytoplasmic (C) and nuclear (N) fractions and visualized by autoradiography after separation on denaturing polyacrylamide gels.

A mutant U1 snRNA that is retained in the nucleus

To determine whether the retention behavior described above was confined to mutant tRNAs, a number of mutant derivates of U1ΔSm snRNA were tested. Like U1ΔSm, these RNAs are unable to bind the common U snRNP proteins and are therefore not reimported into the nucleus (Hamm & Mattaj, 1990). This makes their export easier to study. One mutant that differed from U1ΔSm by the presence of a 20-nucleotide 3′ extension was found to be defective in export from the nucleus. This is seen by direct comparison of the export rate of U1ΔSm and U1ΔSm+20 (Fig. 4A). When 0.01-pmol samples of both RNAs were coinjected, U1ΔSm was exported, whereas no U1ΔSm+20 was detected in the cytoplasm even 400 min after injection (Fig. 4A, lanes 5, 8).

To determine whether this behavior was due to a defective interaction with export factors or to nuclear binding and retention of U1ΔSm+20, we next increased the quantity of U1ΔSm+20 injected. As described above for mutant tRNA, increasing the quantity of U1ΔSm+20 injected allowed export of a fraction of the RNA to the cytoplasm (Fig. 4B, compare lanes 4–6 with lanes 7–12, U6Δss RNA is an internal recovery control). This export was via the normal U snRNA export pathway (Jarmolowski et al., 1994) because U1ΔSm+20 was able to compete with the export of U1ΔSm RNA, although it was slightly less efficient as a competitor than U1ΔSm itself (data not shown). Presumably a fraction of the +20 RNA associates with the nuclear factor re-

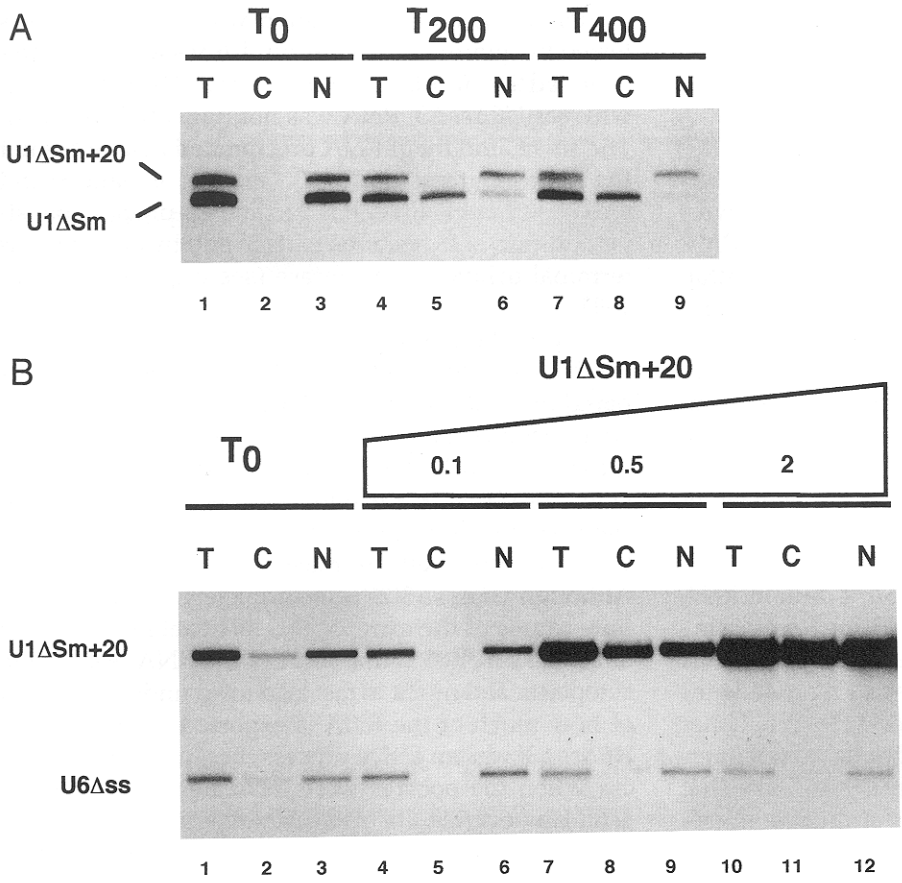


FIGURE 4. U1ΔSm+20 is actively retained in the nucleus by a saturable factor. **A:** U1ΔSm and U1ΔSm+20 transcripts (both 0.01 pmol per oocyte) were coinjected into oocyte nuclei and reextracted from total (T), cytoplasmic (C), or nuclear (N), fractions either immediately (lanes 1–3) or after 200 min (lanes 4–6) or 400 min (lanes 7–9) incubation at 19 °C. **B:** U1ΔSm+20 transcripts were microinjected in increasing amounts (indicated in pmol per oocyte) into *Xenopus* oocyte nuclei and reextracted from total oocytes (T), cytoplasmic (C), or nuclear (N) fractions either immediately (lanes 1–3), or 200 min later (lanes 4–15). U6Δss RNA was coinjected as an internal recovery control.

sponsible for its retention and is thus unavailable to act as a competitor of export.

Nuclear retention of U6 snRNA

We next investigated whether U6 snRNA, which normally exhibits nuclear localization, might also utilize a retention mechanism. As in the case of U1ΔSm, we used a derivative of U6 (U6Δss) that is, unlike wild-type U6 snRNA, unable to migrate from the cytoplasm to the nucleus. This was necessary because re-entry into the nucleus would prevent the detection of any U6 snRNA exported to the cytoplasm. It should be noted that U6Δss is able to functionally complement pre-mRNA splicing in oocytes lacking wild-type U6 snRNA (Vankan et al., 1990).

Injection of 0.5 pmol or less of U6Δss RNA resulted in nuclear retention of the RNA (Fig. 5, lanes 4–6; see also Fig. 4B, lanes 4–12). When more U6Δss was injected, however, a fraction of the injected RNA appeared in the cytoplasm (Fig. 5, lanes 7–15). As in the case of the G57-U $tRNA_i^{met}$ mutant, the ratio of nuclear:cytoplasmic RNA as a function of the concentration of U6Δss varied from experiment to experiment, but the appearance of a fraction of the RNA in the cytoplasm when large amounts were injected was con-

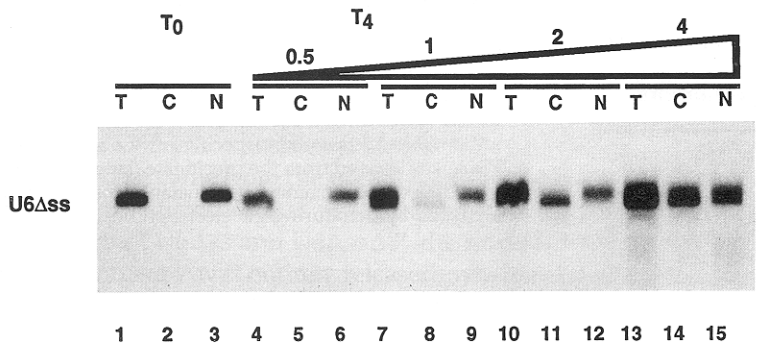


FIGURE 5. U6Δss snRNA is actively retained in the nucleus by a saturable factor. U6Δss transcripts were microinjected in increasing amounts (indicated in pmol per oocyte) into *Xenopus* oocyte nuclei and reextracted from total oocytes (T), cytoplasmic (C), or nuclear (N) fractions either immediately (lanes 1–3) or 240 min later (lanes 4–15) and visualized by autoradiography after separation on a denaturing polyacrylamide gel.

sistently observed and was suggestive of the existence of a saturable retention system.

Is La protein binding involved in U6 retention?

The involvement of nuclear proteins known to interact with U6 snRNA in its nuclear retention was investigated. Two proteins that interact with U6 snRNA have been described in metazoans (Hamm & Mattaj, 1989; Terns et al., 1992), but only one, the La protein, has been identified (Terns et al., 1992). La protein associates with RNAs primarily via 3′-terminal oligouridylate stretches (Stefano, 1984). We therefore constructed a derivative of U6Δss RNA that lacked the terminal U residues (U6ΔssΔ3′) and tested whether this RNA would still interact with the La protein either in vitro or in vivo.

Addition of increasing amounts of La protein to either wild-type U6 RNA or to the U6Δss mutant RNA led to the production of slower-migrating La protein–RNA complexes that could be separated from free RNA by nondenaturing gel electrophoresis (Fig. 6A, lanes 1–8). Deletion of the 3′-terminal U residues from either wild-type or mutant U6 drastically decreased the interaction with La protein (Fig. 6A, lanes 9–16; note that lanes 9 and 13 do not contain La protein, and the slower migrating bands in these lanes are most likely due to structural isoforms of the U6 mutant RNAs). To test for La interaction in vivo, U6ΔssΔ3′ was injected into *Xenopus* oocyte nuclei either alone (Fig. 6B, lanes 4, 8) or mixed with U6Δss RNA (Fig. 6B, lanes 1, 2, 5, 6). After a short incubation, association with La protein was tested by immunoprecipitation. U6Δss RNA was precipitated by anti-La antibodies (Fig. 6B, lanes 2, 3), whereas U6ΔssΔ3′ RNA was not (Fig. 6B, lanes 2, 4). The small amount of RNA precipitated in lane 4 is not the same length as U6ΔssΔ3′ (see Fig. 6B, lane 8) and we presume this RNA has become La-precipitable after addition of U residues to its 3′ end by endogenous terminal uridylate transferase (see e.g., Terns et al., 1993).

To test whether removal of the La binding site from U6 RNA would affect its nuclear retention, we compared the distribution of U6ΔssΔ3′ and U6Δss RNAs after microinjection of 0.01 pmol of each RNA into *Xenopus* oocyte nuclei. In contrast to U6Δss RNA (Fig. 7A, lanes 10–15), which is effectively retained in the nucleus at this concentration, a significant fraction of U6ΔssΔ3′ RNA left the nucleus (Fig. 7A, lanes 7–9). Although U6ΔssΔ3′ is not completely stable over the time course of the experiment, its instability does not affect the conclusion that some of the RNA reaches the cytoplasm and might in fact lead to an underestimation of how much of the RNA is exported. The export of U6ΔssΔ3′ was an active process, because it did not occur when the oocytes were incubated at 0 °C (lanes 4–6). However, in this and other experiments (data not shown) export of U6ΔssΔ3′ was not complete even after these relatively long incubation times, and no more than roughly 50% of U6ΔssΔ3′ was ever seen in the cytoplasm. This raised the possibility that removal of the La binding site might only partially relieve nuclear re-

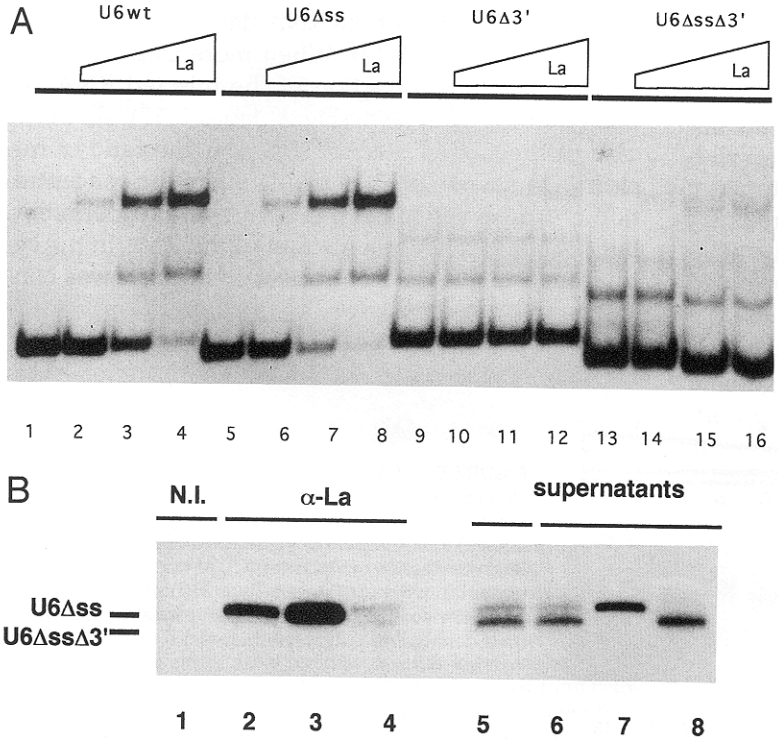


FIGURE 6. Removal of the 3′ end of U6Δss strongly reduces La protein binding. **A:** Increasing amounts of purified recombinant La protein (0, 0.4, 1.6, or 4 pmol) were incubated with 1.5 pmol U6wt RNA (lanes 1–4), U6Δss RNA (lanes 5–8), U6Δ3′ RNA (lanes 9–12), or U6ΔssΔ3′ RNA (lanes 13–16), and analyzed on a nondenaturing gel as described in the Materials and methods. Lanes 1, 5, 9, and 13 do not contain La protein. The retarded species in the U6Δ3′ and U6ΔssΔ3′ lanes presumably represent structural isoforms of the naked RNAs. **B:** U6Δss and U6ΔssΔ3′ transcripts were injected into the nuclei of 10 oocytes (0.25 pmol per oocyte) either as a mixture (lanes 1, 2) or separately (lanes 3, 4, respectively). After 30 min incubation at 19 °C, the oocytes were homogenized, and RNAs were immunoprecipitated from the extracts with either control antibody (lane 1) or monospecific anti-La antibodies. RNA was isolated from the precipitate (lanes 1–4) and from 10% of the remaining supernatant (lanes 5–8) and analyzed by denaturing gel electrophoresis.

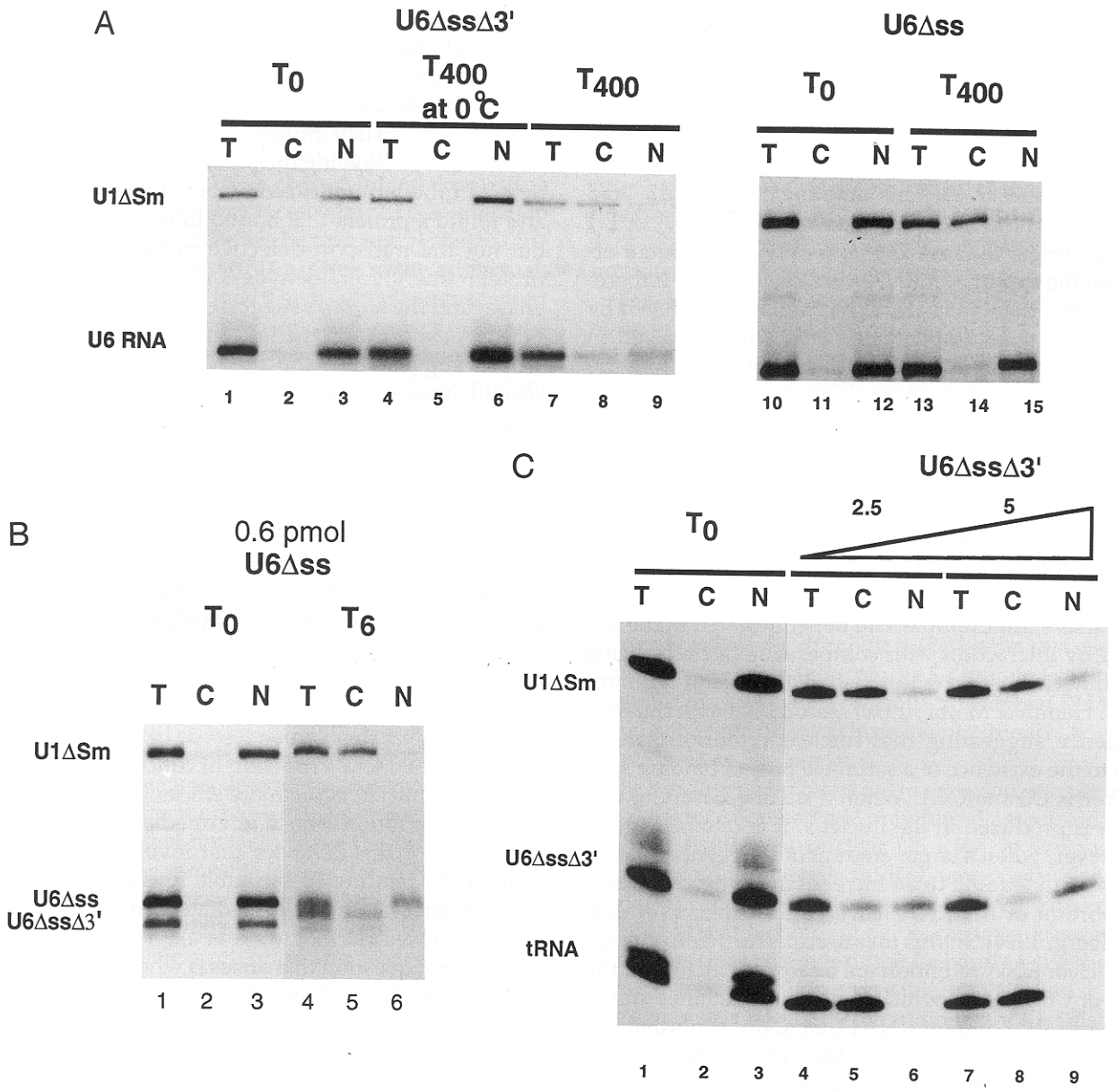


FIGURE 7. U6 snRNA lacking the La binding site is exported from the nucleus. **A:** 0.01 pmol of U6ΔssΔ3′ (lanes 1–9) or U6Δss (lanes 10–15) transcripts were injected into oocyte nuclei together with U1ΔSm RNA as an internal control. Oocytes were either dissected directly (lanes 1–3, 10–12), after 400 min incubation at 0 °C (lanes 4–6), or after 400 min incubation at 19 °C (lanes 7–9, 13–15). RNA was extracted from total oocytes (T), cytoplasmic (C), and nuclear (N) fractions, and analyzed by denaturing gel electrophoresis. **B:** A mixture of 0.01 pmol each of α-^{32}P-labeled U6ΔssΔ3′, U6Δss, and U1ΔSm RNA was injected into the nuclei of oocytes together with 0.6 pmol per oocyte of unlabeled U6Δss RNA. Oocytes were dissected either directly after injection (lanes 1–3) or after 6 h incubation at 19 °C (lanes 4–6). RNA was extracted from total (T), cytoplasmic (C), and nuclear (N) fractions and analyzed by denaturing polyacrylamide gel electrophoresis. **C:** A mixture of 0.01 pmol each of α-^{32}P-labeled U6ΔssΔ3′, U1ΔSm, and $tRNA_i^{met}$ transcripts was injected into oocyte nuclei together with 2.5 or 5 pmol per oocyte of unlabeled U6ΔssΔ3′ RNA, as indicated. The oocytes were dissected either directly after injection (lanes 1–3) or after 6 h incubation at 19 °C (lanes 4–9). RNA was extracted from total (T), cytoplasmic (C), and nuclear (N) fractions and analyzed by denaturing polyacrylamide gel electrophoresis.

tention of U6ΔssΔ3′. Further evidence that this was indeed the case came from the observation that when 0.6 pmol of unlabeled U6Δss was coinjected with 0.01 pmol each of U6Δss and U6ΔssΔ3′, the nuclear retention of U6ΔssΔ3′ was entirely released, whereas U6Δss itself was retained in the nucleus (Fig. 7B, lanes 4–6). This experiment suggests either that two separate factors cooperate to retain U6 RNA in the nucleus or alternatively that removal of the 3′ end reduces, but does not abolish, the affinity of U6 RNA for a single retention factor.

U6 snRNA is not normally exported from the nucleus, and it was therefore of interest to characterize U6ΔssΔ3′ export. To do this, the effect of a further in-

crease in the amount of RNA injected was tested. When up to 2.5 pmol of U6ΔssΔ3′ was injected, roughly half the RNA was, as before, found in the cytoplasm after 400 min (Fig. 7A, lanes 7–9; 7C, lanes 4–6). Further increasing the amount injected to 5 pmol led to a reduction in the cytoplasmic:nuclear ratio (Fig. 7B, lanes 7–9). This amount of U6ΔssΔ3′ had no effect on the nuclear export of either tRNA or U1 snRNA (Fig. 7C, lanes 7–9) and only a very minor effect on the export of mRNA (data not shown). It therefore seems that U6ΔssΔ3′ export must be mediated by a specifically saturable factor that is not involved in the export of the other three classes of RNA from the nucleus.

DISCUSSION

We have analyzed the retention of various RNA species in the cell nucleus. Previous work had shown that different RNAs could be retained in the nucleus by interaction with specific nuclear factors. Messenger RNA precursors, for example, can be sequestered in the nucleus by interaction with components of the splicing machinery (Legrain & Rosbash, 1989; Chang & Sharp, 1989; Hamm & Mattaj, 1990). Additional experimental evidence suggesting that nuclear retention can be due to the existence of a saturable pool of binding sites concerns U3 snoRNA. When a small quantity of this RNA is produced in the nucleus, it is retained there. However, when a larger amount of DNA encoding U3 is injected, leading to an increase in U3 production, a proportion of the RNA leaves the nucleus (Terns & Dahlberg, 1994). In this manuscript, further examples of nuclear RNA retention are analyzed, including the case of U6 snRNA, which is normally retained in the nucleus. Two mutant RNAs that show abnormal nuclear retention were also studied. These RNAs are artifactually retained by acquiring the property of binding specifically to different saturable nuclear components.

Nuclear retention of mutant RNAs

Previous work had identified mutant versions of tRNAs and of 5S rRNA that exhibited drastically reduced rates of nuclear export (Zasloff et al., 1982; Tobian et al., 1985; Guddat et al., 1990). In this paper, we characterized a mutant version of U1 snRNA with a 3′ extension that has similar properties. Previously, the hypothesis advanced to explain the behavior of such mutants was that they were unable to interact with components of the RNA export machinery and thus became trapped in the nucleus by default. We now show that this is not the explanation for transport-defective mutants of $tRNA_i^{met}$ and U1 snRNA. The mutant RNAs appear to be transport competent but are retained in the nucleus by specific interactions with saturable binding sites. Once these sites are occupied, the unbound mutant RNAs are recognized by the transport machinery in a very similar manner to wild-type versions of the same RNAs.

This necessitates revision of hypotheses to explain the behavior of such mutants. For example, Singh and Green (1993), following the previous explanation of the defect in the mutant $tRNA_i^{met}$ behavior, searched for and found a protein that bound to wild-type $tRNA_i^{met}$ but not the transport-defective mutant G57-U. This protein was glyceraldehyde-3-phosphate dehydrogenase, and the authors suggested it might be involved in tRNA export from the nucleus. Clearly, our results would suggest that the mediator of tRNA export should interact similarly with both RNAs, making glyceraldehyde-3-phosphate dehydrogenase an unlikely candidate for this activity. On the basis of similar experiments, Guddat et al. (1990) suggested that either ribosomal protein L5 or transcription factor TFIIIA might mediate 5S rRNA transport out of the nucleus. It would obviously be desirable to carry out experiments with the 5S rRNA mutants analogous to those performed here with the U1 snRNA and tRNA mutants. Technically, however, such experiments are unlikely to be feasible because the export of 5S rRNA from the nucleus is not saturable within the range of concentration of RNA that can be tested. In addition the export of 5S rRNA as a function of concentration shows complex behavior, being more efficient at both low and high concentration than at intermediate levels. An explanation for this behavior that involved nuclear retention via binding to saturable low-affinity nuclear binding sites at the intermediate concentration has been proposed (Jarmolowski et al., 1994).

A general question that arises is whether the nuclear retention of the mutant RNAs studied is due to the chance generation of binding sites for nuclear factors, or whether it might reflect the existence of some kind of proofreading mechanism to prevent defective RNAs from reaching the cytoplasm. The 60-kDa Ro protein has, for example, recently been proposed to participate in the recognition and degradation of defective 5S rRNA transcripts (O'Brien & Wolin, 1994). The evidence argues strongly against the existence of a specific mechanism. Firstly, only a relatively small fraction of the many $tRNA_i^{met}$ mutants (Tobian et al., 1985) or 5S rRNA mutants (Guddat et al., 1990) tested exhibited nuclear retention. In the case of U1 snRNA, more than 20 mutant versions of RNA leave the nucleus perfectly normally (e.g., Hamm et al., 1987; Hamm & Mattaj, 1990). Secondly, the behavior of U1ΔSm+20 results from the specific sequence of the 3′ extension. U1 derivatives with other 3′ extensions (see, e.g., Konings & Mattaj, 1987; Terns et al., 1993), including normal precursors of U1 snRNA, behave differently. They can, for example, show normal export behavior, or be very unstable. Indeed, the addition of sequences to the 3′ end of U1ΔSm that include the same 20 nucleotides present

in the +20 mutant, but are extended further, results in the production of an RNA that can be exported from the nucleus (data not shown). The idiosyncratic behavior of these different U1 derivatives argues against the existence of a specific cellular mechanism to deal with aberrant U1 transcripts and suggests that the retention of U1ΔSm+20 is due to a chance interaction with a component that happens to recognize U1ΔSm+20 in a specific way. We therefore believe it is likely that the behavior of the mutant RNAs is due to the artifactual, albeit specific, generation of high-affinity sites of interaction with nuclear components. In the cases examined here, these novel interactions must be of sufficiently high affinity to interfere with export of the mutant RNAs in a dominant fashion.

Is there a "default" state for nuclear RNAs?

It might have been imagined that the "default state" for nuclear RNAs would be either nuclear retention, diffusion through nuclear pore complexes, or transport out of the nucleus by an active but nonspecific system. We have found no evidence that this is the case. The mutant U1 and tRNA species, at an appropriate concentration, are able to leave the nucleus in a manner similar to the corresponding wild-type RNAs. More surprisingly, we found that a derivative of U6 snRNA that leaves the nucleus does so by an active, saturable, apparently specific mechanism. The export of different classes of RNA that normally leave the nucleus is mediated by specific saturable factors (Jarmolowski et al., 1994), but none of those factors tested appear to be involved in U6ΔssΔ3′ export. Thus, for the examples studied, both nuclear retention and nuclear export require specific interactions and neither can be described as a default state.

The export of U6ΔssΔ3′ in a specific way raises a question of general interest. Because wild-type U6 RNA is not exported, and because the U6ΔssΔ3′ transcript is an unnatural one, the implication is that the RNA export machinery has the flexibility to recognize and transport RNAs that it has not evolved to deal with. We have no satisfactory idea of how this might occur, but note that the result again underlines the fact that if an RNA is not specifically retained in the nucleus, it will be exported.

Involvement of La protein in U6 snRNA retention

It is of interest to identify which nuclear components mediate the retention of RNAs under physiological conditions. We showed that removal of the binding site for La protein from the 3′ end of U6Δss snRNA partially overcame the nuclear retention of this RNA and allowed some export to the cytoplasm, implicating La in the normal retention process. La binding alone is, however, not sufficient to explain nuclear retention because evidence was presented that U6ΔssΔ3′ was still significantly retained in the nucleus in a way that could be overcome by coinjection of U6Δss RNA. This result suggests that either there is more than one factor involved in the retention of U6 in the nucleus, or that there is a single factor whose binding site is only partly removed by the Δ3′ deletion. Either of these possibilities could explain why Terns et al. (1993) failed to see export from the nucleus when they made a similar, but nonidentical, U6 truncation mutant.

There are arguments to suggest that the involvement of La in U6 retention may not be direct. La protein is thought to bind to the primary transcripts of all RNA polymerase III-transcribed genes (Rinke & Steitz, 1982) via their 3′-terminal oligouridylate stretches (Stefano, 1984). This means that La binds to many RNAs that leave the nucleus and provides an argument against it being a general retention factor. However, in some cases, like pre-tRNAs, the La binding site is removed before nuclear export, and in other cases, like 5S rRNA or hY1 RNA (Guddat et al., 1990; Simons et al., 1994), the cytoplasmic fraction of the RNA is not found in association with La. It might therefore be that dissociation from, or dislodging of, the La protein is a prerequisite for nuclear export of pol III-transcribed RNAs. In the absence of either of these events, the RNA would be retained in the nucleus.

If La is directly involved in U6 retention, its role must be transient because La is only found associated with a minor fraction of nuclear U6 RNA in various cell types, including the cells studied here, *Xenopus* oocytes (Rinke & Steitz, 1985; Terns et al., 1992). This makes it unlikely that La binding per se causes nuclear retention. Why does removal of the La binding site then affect U6 retention? One possible explanation is that there may be a bona fide retention factor that also recognizes the 3′ end of U6 snRNA. In this case removal of the La binding site would also prevent interaction with this factor. A more interesting possibility is that interaction with La would be a necessary step in the correct formation of the nuclear U6 snRNP (Hamm & Mattaj, 1989; Terns et al., 1992), with La performing the role of a chaperone in RNP formation. Without La, the U6 snRNA would be unable to efficiently form the nuclear RNP and would be free to enter the export pathway.

MATERIALS AND METHODS

Plasmids

The human $tRNA_i^{met}$, $tRNA_i^{met}$G57U, *Xenopus* T7 U1ΔSm (previously called ΔD), and U6Δss constructs with promoters for transcription by T7 RNA polymerase have been described previously (Hamm et al., 1987; Hamm & Mattaj, 1989; Jarmolowski et al., 1994). The U1ΔSm+20 transcript contains a 20-nucleotide extension at the 3′ end (CCGGGUACCGAG

CUCAAUU), encoded by the polylinker region of pUC19, and was made by transcription of the U1ΔSm plasmid linearized with *Eco*R I instead of *Bam*H I. U6ΔssΔ3′ RNA is transcribed from a PCR product of U6Δss plasmid synthesized with a 5′ oligonucleotide complementary to the T7 promoter and a 3′ oligo with the sequence GGGAACGCTTCAC GAATTTGCG. This results in a U6Δss transcript in which the 3′ sequence AUAUUUUU is replaced by a C. *Xenopus* pre-tRNA^{Phe} plasmid contains the *Sac* I–*Msp* I fragment of the *Xenopus* tRNA^{Phe} gene behind the T7 RNA polymerase promoter (Lin-Marq & Clarkson, 1995). For T7 transcription, this clone was linearized with *Dra* I.

RNA preparation and microinjection

To prepare ^{32}P-labeled RNA in vitro transcription was performed: a mixture of 0.1 μg/μL of linearized DNA, 40 mM Tris-HCl, pH 8.0, 8 mM MgCl, 10 mM DTT, 1 mM NTPs (Pharmacia), 10 U RNasin (Promega), and 2 U/μL T7 RNA polymerase (Stratagene) was incubated at 37 °C for 2 h. For U1 transcription, 1.5 mM m^{7}GpppG, and for U6 transcription, 5 mM γ-methyl-GTP, was present in the transcription mixture. To prepare high specific activity RNA, the reaction was done in a 10-μL transcription mixture containing 10 μCi [α-^{32}P]GTP and [α-^{32}P]UTP. To prepare low specific activity or nonradioactive RNA, transcription was done in a 100-μL reaction to which 10 μCi or a trace amount of [α-^{32}P]GTP was added. The concentration of the synthesized RNAs was determined with the help of the incorporated label. The transcripts were purified as described previously (Jarmolowski et al., 1994).

The RNA was microinjected into the nuclei of *Xenopus laevis* oocytes together with dextran blue (2,000,000 molecular weight; Serva Biochemicals) in order to control nuclear injection. The RNA was reextracted from the oocytes and analyzed by denaturing gel electrophoresis as described (Jarmolowski et al., 1994).

Immunoprecipitation of U6 RNAs from oocytes

The U6 RNA was injected into the nuclei of 10 oocytes and incubated for 30 min. The oocytes were homogenized on ice in 500 μL TNE (50 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 1 U/μL RNasin) and spun for 5 min in a benchtop microcentrifuge. The supernatant was added to 400 μL IPP150 (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP40) containing 20 μL protein A–agarose beads to which monospecific α-La antibodies isolated by affinity purification from the serum of an autoimmune patient were coupled (Simons et al., 1994). The mixture was rotated end over end at 4 °C for 90 min. The beads were collected by centrifugation and washed three times in 1 mL IPP150. The RNA bound to the beads was isolated and analyzed as described above.

Nondenaturing gel electrophoresis

The labeled transcripts and recombinant La protein were incubated at 30 °C in 10 μL incubation buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 2 mM EDTA, 5% glycerol, 0.1 μg/μL tRNA, 0.01 μg/μL BSA, and 2 mM DTT). The recombinant La protein used was purified to near homogeneity by chromatography from a lysate of bacteria overexpressing human La protein (Xiao et al., 1994). After 30 min incubation, 3 μL incubation buffer was added containing 25% glycerol. The samples were loaded on a 6% acrylamide/0.1% bis acrylamide gel containing 1× TBE and electrophoresed for 3 h at 10 V/cm at 21 °C.

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