Identification of a novel *cis*-acting RNA element involved in nuclear export of hY RNAs

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ABSTRACT

Ro RNPs are small cytoplasmic RNA–protein complexes of unknown function that have been found in all metazoan cells studied so far. In human cells, Ro RNPs consist of one of four small RNA molecules, termed hY RNAs and at least two well-characterized proteins, Ro60 and La. In previous *Xenopus laevis* oocyte microinjection studies, we showed that an intact Ro60 binding site (Stem-loop 1) is a prerequisite for efficient nuclear export of hY1 RNA, whereas an intact La-binding site promotes nuclear retention (Simons et al. *RNA*, 1996, 2:264–273). Here we present evidence that the distal half (Stem 2) of the conserved base-paired stem structure found in all hY RNAs also plays a critical role in the export process. A minimal RNA molecule containing this region, L1S2 RNA, competes effectively for the export of full-length hY1 RNAs and is itself exported very rapidly in a Ro60-independent and RanGTP-dependent manner. Mutational analyses of this RNA shows that a 5'/3' terminal double-stranded stem structure (>10 bp) of no specific nucleotide sequence constitutes a novel nuclear export element (NEE). Cross-competition studies indicate that this type of NEE may also be involved in export of other classes of RNAs. Like full-length hY1 RNA, L1S2 RNA also competes for export of ET-202 RNA, an RNA that was selected for its efficient nuclear export in the presence of the nuclear transport inhibitor, VSV Matrix protein (Grimm et al. *Proc Natl Acad Sci USA*, 1997, 94:10122–10127). However, export of L1S2 RNA is strongly inhibited by VSV-M protein, showing that these RNAs use partially overlapping, but not identical export pathways. We propose that export of Y RNAs is mediated by two contiguous *cis*-acting elements in the 5'/3' double-stranded stem region that is conserved between different Y RNAs.

Keywords: hY RNA; nucleocytoplasmic transport; RNA export; Ro RNP

INTRODUCTION

Ro RNPs are small cytoplasmic ribonucleoprotein particles of unknown function that are well conserved during evolution and are expressed in all types of cells. In human cells the RNA component of each Ro RNP consists of one of four different Y RNAs, which are approximately 100 nt long, hY1, hY3, hY4, and hY5 (Hendrick et al., 1981). The RNAs, transcribed by RNA polymerase III, are highly structured and characterized by base pairing of their 5' and 3' termini (5'/3' base-paired stem). The protein part of a Ro RNP consists of at least three different proteins: the La protein, the 60-kDa Ro protein (Ro60) and the 52-kDa Ro protein (Ro52; Hendrick et al., 1981; Wolin

& Steitz, 1984; Ben Chetrit et al., 1988). Recently, several additional hY1 and hY3 RNA-binding polypeptides have been identified (Fabini et al., 2000). Cell enucleation experiments and *Xenopus laevis* oocyte injection studies have shown that the Y RNAs accumulate primarily in the cytoplasm, most likely in the context of Ro RNPs (O'Brien et al., 1993; Peek et al., 1993; Simons et al., 1994), indicating that newly made Y RNAs must be efficiently exported to the cytoplasm.

Many nuclear export substrates are ribonucleoprotein particles (RNPs) that can be large and complex in composition. The exchange of macromolecules between the nucleus and the cytoplasm occurs mainly through nuclear pore complexes (NPCs). The NPC allows free diffusion of ions, metabolites, and small proteins, but large molecules must transit the NPC via active, energy-dependent mechanisms. Nucleocytoplasmic transport of most RNAs is saturable and thus receptor mediated (reviewed in Mattaj & Englmeier, 1998; Görlich & Kutay, 1999; Nakielnny & Dreyfuss,

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1999). The export factors are class specific as, for example, the saturable component(s) needed for export of tRNAs differs from those needed for mRNAs, 5S RNA, U snRNAs, or ribosomal subunits. It has been proposed that export of most RNAs is mediated by bound adapter proteins that contain appropriate export signals; the identification of transferable nuclear export signals (NESSs) of several RNA-binding proteins supports this model. The best characterized NES is a short leucine-rich sequence motif that was first identified in the HIV-Rev and PKI (protein kinase A inhibitor) proteins; different types of NESSs exist in shuttling hnRNP proteins (reviewed in Mattaj & Englmeier, 1998; Görlich & Kutay, 1999; Nakielnny & Dreyfuss, 1999). However, some RNAs like tRNAs bind directly to their export receptors (see below) and do not appear to require adapters.

The small GTPase Ran is essential for nucleocytoplasmic transport. Due to the specific intracellular locations of the effectors of the RanGTPase system, the concentration of RanGTP is predicted to be high in the nucleus and low in the cytoplasm (Izauralde et al., 1997). This asymmetry is essential for transport in and out of the nucleus (reviewed by Dahlberg & Lund, 1998). When import receptors bind to RanGTP in the nucleus, the bound cargo is released. Conversely, export receptors must associate with RanGTP to bind their cargo efficiently. Thus, the directionality of receptor-mediated nucleocytoplasmic transport is governed, at least in part, by binding or removal of RanGTP, which ensures that assembly and disassembly of receptor-cargo complexes occur in the appropriate cellular compartments.

Recently, two export receptors have been identified that are specific for different classes of RNA. CRM1 (or Exportin-1), which recognizes proteins that carry a leucine-rich NES, is required for export of both U snRNAs and unspliced HIV mRNAs (Fornerod et al., 1997; Stade et al., 1997). In contrast, exportin-t (Xpo-t), which is specific for export of tRNA, binds its RNA cargo directly (Arts et al., 1998; Kutay et al., 1998). In both cases, the stable interactions between receptor and cargo are dependent on the concomitant binding of RanGTP. Like other transport receptors of the importin β family (Görlich et al., 1997), both CRM1 and Xpo-t interact with components of the NPC and shuttle continuously between the nucleus and cytoplasm (reviewed by Görlich & Kutay, 1999).

We have previously shown that an intact Ro60-binding site is a prerequisite for efficient nuclear export of hY1 RNA and that this export is unlikely to involve CRM1 or Xpo-t (Simons et al., 1996). Here, we present evidence that in addition to Ro60 protein, another titratable nuclear factor is necessary for export of hY1 RNA. This factor apparently recognizes a second *cis*-acting element that is located in the upper part of the conserved 5'/3' base-paired stem of hY RNAs. A minimal RNA molecule containing (only) this region is exported very

rapidly, in a RanGTP-dependent manner, and is an effective competitor of the export of full-length hY1 RNA. Surprisingly, the nuclear export element (NEE) of this minimal RNA appears to consist simply of a double-stranded RNA region (>10 bp) of unspecified nucleotide sequence.

RESULTS AND DISCUSSION

A common nuclear export pathway for different human Y RNAs

Previous experiments have shown that hY RNAs injected in the nuclei of *X. laevis* oocytes accumulate in the cytoplasm in a time- and energy-dependent manner (O'Brien et al., 1993; Simons et al., 1994). At least two proteins, Ro60 and La, can bind in the nucleus to hY RNAs (Simons et al., 1994) and modulate nuclear transport of hY1 RNA. Using various mutants of hY1 RNA, we demonstrated that an intact Ro60-binding site is required for efficient nuclear export, whereas an intact La binding site promotes strong nuclear retention (Simons et al., 1996).

To determine if other human Y RNAs utilize the same export pathway as hY1 RNA, as would be expected from its requirement for an intact Ro60-binding site, we co-injected 1 pmol of either hY1, hY3, or hY4 RNA (Fig. 1A) as competitor for the export of ³²P-labeled hY1Xba RNA. We used hY1Xba RNA (see Fig. 2A) rather than wild-type hY1 RNA to monitor nuclear export because this molecule lacks the La-binding site and thus is not subject to the process of nuclear retention (Simons et al., 1996), which could complicate interpretation of the competition experiments. As a control, we included radiolabeled initiator tRNA^{Met}, which is rapidly exported. Ninety minutes after RNA injection into the nucleus, the oocytes were manually dissected and the nucleocytoplasmic distributions of the RNAs were analyzed by denaturing gel electrophoresis. As shown in Figure 1B, nuclear export of hY1Xba (lanes 1–4) was impaired to similar extents in the presence of the different competitor hY RNAs (lanes 5–10). As expected, the export of the control RNA was unaffected. These results indicate that the export pathways for different hY RNAs are overlapping, and probably identical.

Identification of an additional *cis*-acting element involved in nuclear export of hY1 RNA

Our previous competition studies (Simons et al., 1996) showed that export of hY1Xba RNA is also reduced when the competitor is a mutant RNA, Δ S1L1 (Fig. 2A) that lacks a Ro60-binding site (Fig. 2B, lane 11; Green et al., 1998). However, Δ S1L1 RNA itself is not exported (Simons et al., 1996), nor was export restored

FIGURE 1. Competition for hY1 RNA export by other hY RNAs. **A:** Secondary structure models for hY1, hY3, and hY4 RNA. The stem regions formed by base pairing of the 5' and 3' ends of these molecules contain the most highly conserved elements, which are boxed. The central parts of the hY RNAs contain the most divergent sequences and display a high degree of structural flexibility (Van Gelder et al., 1994; Teunissen et al., 2000). **B:** A mixture of in vitro-transcribed, ³²P-labeled hY1Xba RNA and tRNA^{Met} was injected into *X. laevis* oocyte nuclei alone (lanes 1–4) or together with 1 pmol of unlabeled hY1 RNA (lanes 5, 6), hY3 RNA (lanes 7, 8), or hY4 RNA (lanes 9, 10). RNA was extracted from total oocytes (T), cytoplasmic (C), and nuclear (N) fractions immediately (lane 1) or 90 min (lanes 2–10) after injection and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

by mutation of the strong La-binding site at the 3' end of this RNA (data not shown). This suggested that hY1 RNA contains an additional export element (other than the Ro60-binding site) that is necessary, but not sufficient for export.

To localize this additional element, several other deletion/substitution mutants of hY1 RNA were constructed (see Fig. 2A for their predicted secondary structures) and assayed for their abilities to be exported from oocyte nuclei. Deletion or alteration of the se-

FIGURE 2. (Legend on facing page.)

FIGURE 2. Secondary structure models for wild-type and mutant hY1 RNAs and their Ro60-binding properties. **A:** The structure of hY1wt RNA (as proposed by van Gelder et al., 1994) was used for prediction of the structures of the deletion mutant RNAs. The behavior of the mutants after microinjection in the nuclear compartment of *X. laevis* oocytes is indicated. The export rates of Δ S3L3, Δ S4L4, Δ L2b, and L2bs were indistinguishable from that of hY1wt. Nucleotides in hY1Xba RNA (AG) that are different from the wild-type sequence (UU) and the altered Loop 2b in L2bs are boxed. **B:** Ability of hY RNA mutants to bind the Ro60 protein. In vitro-transcribed hY RNA mutants and U1 Δ Sm RNA were incubated in HeLa S100 extract and subsequently precipitated with monoclonal anti-Ro60 (lanes 9–16) and anti-U1 snRNP specific A protein (lanes 17–24) antibodies. RNA isolated from the precipitates (lanes 9–24) and 10% of the input RNAs (lanes 1–8) was analyzed by denaturing gel electrophoresis followed by autoradiography. The positions of some of the RNAs are indicated on the left. hY1Xba RNA comigrated with hY1wt RNA, Δ L1S2 comigrated with Δ S1L1 and Y3-L1S2, and S2 comigrated approximately with L1S2.

quences that are specific to hY1 RNA (Fig. 2A, mutants Δ S3L3, Δ S4L4, Δ L2b, and L2bs RNAs) did not interfere with RNA export (data not shown), consistent with the idea that the additional RNA export signal is likely to be conserved between different hY RNAs (cf. Fig. 1B). Mutant Δ L1S2, which lacks sequences that are common to hY RNAs and which binds the Ro60 protein inefficiently (Fig. 2B, compare lanes 9 and 12), was unstable in oocyte nuclei (data not shown) and therefore could not be reliably assayed for export. Presumably this instability reflects the lack of interaction with a stabilizing nuclear factor that recognizes hY1 Stem 2 sequences.

Taken together, these results show that in addition to the highly conserved Ro60-binding site (Stem 1) a second conserved sequence element of hY RNAs, most likely Stem 2 (perhaps in combination with Loop 1), is required for export of hY1 RNA and possibly other hY RNAs.

Stem 2 of hY RNA contains a *cis*-acting element for hY RNA export

To investigate this requirement, we made an RNA molecule, L1S2, that contained only Loop 1 and Stem 2 of hY1 RNA (Fig. 3A; for stability these sequences were connected with a tetraloop and extended by nonspecific 5'/3' stem sequences). In vitro binding experiments showed that, as expected, L1S2 RNA bound neither the Ro60 protein (Fig. 2B, lanes 5 and 13) nor

the La protein (data not shown). However, upon microinjection into oocyte nuclei, L1S2 RNA not only was stable but also was exported to the cytoplasm at an even faster rate than that of hY1Xba RNA (Fig. 3A). This Ro60-independent export appeared to be an active process, as it was reduced about 10-fold in rate when the injected oocytes were incubated at 0°C (data not shown). The slow but significant accumulation of L1S2 RNA in the cytoplasm under these conditions apparently reflects the very efficient export of this RNA (rather than diffusion) because transport at the normal temperature (18°C) was effectively blocked upon depletion of the nuclear pool of RanGTP (see below; Fig. 7B). As controls, we included radiolabeled initiator tRNA^{Met} and U1 Δ Sm RNA (a mutant U1 snRNA that is exported to the cytoplasm but is not imported back into the nucleus; Hamm et al., 1987) in these experiments.

The structural elements equivalent to Loop 1 and Stem 2 of hY1 RNA are also present in other hY RNAs (Prujn et al., 1997; Green et al., 1998); therefore, we tested if this region of hY3 RNA harbored a similar nuclear export element. The comparable RNA molecule, Y3-L1S2, (Fig. 3B) was indeed exported with kinetics very similar to those of L1S2 RNA (compare Fig. 3A and B). Because the Stem 2 regions (but not the Loop 1 regions) of the different hY RNAs contain a number of conserved residues (Prujn et al., 1997), we next asked if the Stem 2 sequences of hY1 RNA were sufficient to promote nuclear export. S2 RNA (Fig. 3C) also was exported rapidly, and like the other Stem 2

FIGURE 3. A nuclear export element (NEE) in Stem 2 of hY RNAs. A mixture of in vitro-transcribed, ³²P-labeled U1ΔSm RNA, hY1Xba RNA, and tRNA^{Met} was injected into *X. laevis* oocyte nuclei together with labeled L1S2 RNA (A), Y3-L1S2 (B), or S2 (C) RNAs. The predicted secondary structures of the Stem 2 derivatives are depicted above the autoradiograms, with the sequences corresponding to hY1 or hY3 Stem 2 regions shown in bold face; the most conserved sequence element in Stem 2 is boxed. RNA export was analyzed as in Figure 1B at 30 min (lanes 2 and 3) and 60 min (lanes 4 and 5) after injection D: A mixture of in vitro-transcribed, ³²P-labeled U1ΔSm RNA, hY1Xba RNA, and tRNA^{Met} was injected into *X. laevis* oocyte nuclei alone (lanes 1–5) or together with 1 pmol of unlabeled L1S2 (lanes 6–9), Y3-L1S2 (lanes 10–13), or S2 (lanes 14–17) competitor RNAs. RNA export was analyzed at 30 min (lanes 2–3, 6–7, 10–11, 14–15) and 60 min (lanes 4–5, 8–9, 12–13, 16–17) after injection.

derivatives, its export was strongly reduced at 0°C, again indicating that this transport was active (data not shown). Because these RNAs were all unable to bind the Ro60 protein (Fig. 2B, lanes 5–7 and 13–15) and

still were exported very efficiently, we conclude that this Stem 2-mediated transport is independent of Ro60.

Considering the limited sequence conservation between the short Stem 2 derivatives and wild-type hY

RNAs we questioned whether these small, structured RNAs were accessing the same export pathway as full-length hY1 RNA (cf. Fig. 1). As illustrated in Figure 3D, the export of hY1Xba RNA was strongly competed by 1 pmol of either of the small competitor RNAs, L1S2, Y3-L1S2, or S2, whereas the export of the efficiently exported control RNA, tRNA^{Met}, remained unaffected. Additional competition experiments revealed that L1S2, S2 and Y3-L1S2 RNAs also displayed mutual competition (data not shown). Thus, L1S2, S2, and Y3-L1S2 RNAs share at least one saturable export factor with full-length hY1 RNA.

We conclude that Stem 2 of hY RNAs functions as an NEE, which is sufficient for directing nuclear export of relatively small molecules and which is necessary, but not sufficient for nuclear export of intact hY RNAs. The combined competition studies (Figs. 1 and 3; Simons et al., 1996) suggest that hY RNA export is dependent on at least two *trans*-acting factors, the Ro RNP-specific protein Ro60 and the yet unidentified factor(s) that interacts with the novel NEE (Stem 2) of hY RNAs. The Stem 1 and Stem 2 regions of Y RNAs that contain the binding sites of the respective factors are the most highly conserved elements of Y RNAs, not only among the human Y RNAs, but also across species (Prujin et al., 1997). Ro60 protein remains stably associated with Y RNAs in the cytoplasm (O'Brien et al., 1993; Peek et al., 1993), but the other factor probably dissociates after transport, as no stable interaction of a protein with Stem 2 of Y RNAs has been observed in the cytoplasm (Fabini et al., 2000; S. Rutjes, unpubl.).

Specificity of the L1S2 RNA export pathway

To further delineate the novel NEE, we examined its nucleotide sequence requirements more closely. Several variant RNAs derived from S2 RNA were tested both for their own export and for their abilities to compete for the export of full-length hY1 RNA (Fig. 4). In both of these assays, S2swap RNA (in which the 5' and 3' sequences of Stem 2 were interchanged; Fig. 4A) behaved just like S2 RNA (Fig. 4B, compare lanes 2–5 and 7–10; data not shown), suggesting that the structure rather than the sequence of Stem 2 is of importance for NEE function. However, because the highly conserved sequence element of Stem 2 is still present in S2swap, albeit in the reverse orientation (boxed sequence in Fig. 4A), we also tested two other variants in which the conserved Stem 2 element had been eliminated. In S2M RNA, four consecutive nucleotides in one strand of Stem 2 were replaced by nucleotides that would prevent Watson–Crick base pairing and lead to formation of an internal loop structure in place of the conserved element, whereas in Stem Z RNA, the complete Stem 2 sequence was changed but base pairing was maintained (Fig. 4A). Again, both RNAs were rapidly

exported and were able to compete efficiently and specifically for export of hY1Xba RNA (Fig. 4B,C, lanes 12–15 and 17–20). Thus, recognition by the saturable export factor apparently depends only on the presence of a stable RNA stem structure. We note that S2M RNA contains an uninterrupted 11-bp terminal stem structure despite the partial disruption of its Stem 2 structure (Fig. 4A). In contrast, S2s RNA, a small variant molecule that can fold into a hairpin structure containing merely 9 bp (Fig. 4A), functioned very poorly as a competitor for hY1 RNA export (Fig. 4C, lanes 22–25). We conclude that this novel type of NEE contains no specific nucleotide sequences, but requires a terminal double-stranded region of a certain minimal size (10–11 bp), at least when assayed in the context of small hairpin RNAs.

Interestingly, nuclear export of the adenovirus encoded VAI RNA has recently been shown to depend on a largely dsRNA structure formed by base pairing of the 5' and 3' ends of the molecule; furthermore, VA1 RNA competes for export of hY1 RNA, and vice versa, thus indicating that this viral RNA PolIII transcript gains access to the same export pathway as that used by the cellular Y RNAs (E. Bertrand, pers. comm.). Although our data do not allow a precise prediction of the minimal length of the double-stranded RNA region required for NEE function, the size of Stem 2 of hY1 RNA (9 bp) and the observed export of and competition by S2M (Fig. 4) suggest that approximately 10 bp are sufficient. However, we cannot rule out that the formation of an extended helical region (due to helical stacking) in Y (or VA) RNAs is required for NEE function in the context of these larger RNAs.

Our systematical characterization of this novel NEE also provides an explanation for the previous observation that several small double-stranded RNAs (V_{GG} and V_{TL} RNAs) of structure similar to S2 RNA are exported very efficiently from oocyte nuclei (Hamm et al., 1997). In that study, the short stem-loop RNAs were used as control RNAs for the export of anti-idiotypic RNAs that had been selected as mimics of the leucine-rich NES of HIV-Rev protein.

Utilization of the L1S2 export pathway by other RNAs

To determine if the export factors used by L1S2 RNA might also be required for the export of other cellular RNAs, we performed additional competition experiments, using both "Y" (hY1, L1S2, and Δ S1L1 RNAs) and several "non-Y" RNAs (Th RNA [the RNA component of RNase MRP], 5S rRNA, tRNA^{Met}, U1 Δ Sm RNA) as competitors (Fig. 5). As expected from our previous results, the "Y1" competitors reduced export of both hY1Xba and L1S2 RNAs in a dosage-dependent manner (Fig. 5B–D, lanes 2–7) whereas export of both tRNA^{Met} and U1 Δ Sm was virtually unaffected (cf. Si-

FIGURE 4. (Legend on facing page.)

FIGURE 5. Cross-competition for hY1-Stem 2 mediated export by various RNAs. A mixture of in vitro transcribed, 32 P-labeled U1 Δ Sm RNA, hY1Xba RNA, tRNA^{Met}, and L1S2 RNA was injected into *X. laevis* oocyte nuclei alone (A) or together with 0.3 (lanes 2 and 3), 0.9 (lanes 4 and 5), or 2.7 (lanes 6 and 7) pmol of unlabeled hY1wt (B), L1S2 (C), Δ S1L1 (D), Th (E), 5S rRNA (F), tRNA^{Met} (G), or U1 Δ Sm (H) competitor RNAs. RNA was extracted from total oocytes (T) immediately (lanes 1) or 45 min (A, lane 4) after injection and from cytoplasmic (C) and nuclear (N) fractions 45 min (lanes 2–3 in A and lanes 2–7 in B–H) after injection.

mons et al., 1996; Figs. 1 and 3). Of the “non-hY1” competitors, only Th RNA (Fig. 5E) competed for export of L1S2 and hY1Xba RNA as efficiently as wild-type hY1 RNA (Fig. 5B); in contrast, 5S rRNA (Fig. 5F) or tRNA^{Met} (Fig. 5G) had little, if any, effect on this export, and, as expected, competitor U1 Δ Sm RNA was also without effect (Fig. 5H). Thus, only a specific subset of small cellular RNAs that are known to contain double-stranded structures are likely to access the export pathway used by L1S2 and hY1Xba RNAs.

Relationship between L1S2 and ET-RNA export

Previously, another RNA molecule, ET-202 RNA, was identified that is able to compete for export of hY1 RNA, and vice versa (Grimm et al., 1997; see below; Fig. 6). ET-202 RNA was isolated from a randomized pool of RNA molecules by virtue of its ability to be exported from oocyte nuclei under conditions where most RNA export, including that of hY1Xba RNA, is

FIGURE 4. Structure and nucleotide sequence requirements of the Stem 2 NEE. **A:** Structure of S2 mutants. The parts of these molecules that correspond to hY1 Stem 2 regions are shown in bold face. The most conserved sequence element in Stem 2 is boxed. **B:** A mixture of in vitro-transcribed, 32 P-labeled U1 Δ Sm RNA, hY1Xba RNA, and tRNA^{Met} was injected into *X. laevis* oocyte nuclei together with labeled S2 RNA (lanes 1–5), S2swap (lanes 6–10), S2M (lanes 11–15), Stem Z (lanes 16–20), or S2s (lanes 21–25) RNAs. RNA was analyzed at 30 min (lanes 2, 3, 7, 8, 12, 13, 17, 18, 22, and 23) and 60 min (lanes 4, 5, 9, 10, 14, 15, 19, 20, 24, and 25) after injection. **C:** A mixture of in vitro-transcribed, 32 P-labeled U1 Δ Sm RNA, hY1Xba RNA, and tRNA^{Met} was injected into *X. laevis* oocyte nuclei alone (lanes 1–5) or together with 1 pmol of unlabeled S2 (lanes 6–10), S2M (lanes 11–15), Stem Z (lanes 16–20), or S2s (lanes 21–25) competitor RNAs. RNA was analyzed at 30 min (lanes 2–3, 7–8, 12–13, 17–18, 22–23) and 60 min (lanes 4–5, 9–10, 14–15, 19–20, 24–25) after injection.

FIGURE 6. Inhibition of hY1Xba and L1S2 RNA export by ET-202 RNA. A mixture of in vitro transcribed, 32 P-labeled hY1Xba RNA, ET-202 RNA, and L1S2 RNA was injected into *X. laevis* oocyte nuclei alone (A) or together with 0.3 (lanes 2 and 3), 0.9 (lanes 4 and 5), or 2.7 (lanes 6 and 7) pmol of unlabeled hY1wt (B), L1S2 (C), or ET-202 (D) competitor RNAs. RNA export was assayed as in Figure 5.

blocked by the VSV Matrix protein (M protein; Grimm et al., 1997). To determine if the Stem 2 element of hY RNA might represent an NEE functionally equivalent to that of ET RNAs (thereby accounting for the mutual competition for export between full-length hY1 RNA and ET-RNAs), we performed cross-competition experiments between L1S2 and ET-202 RNAs and assayed for export of L1S2 RNA in the presence of VSV M-protein. In both respects, the behavior of L1S2 RNA was indistinguishable from that of full-length hY1 RNA: L1S2 RNA competed effectively for the export of ET-202 RNA, and vice versa, (Fig. 6B–D), and its export was strongly inhibited in the presence of the M protein (Fig. 7A, compare lanes 6–9 and 14–17). It should be noted that ET-202 RNA is a less efficient competitor of the export of L1S2 RNA than of hY1Xba RNA. This difference might be due to differences in the rates of export of these two RNAs. So although Y1 and ET-202 RNAs compete for a limiting nuclear export factor(s), their export pathways are likely to be only partially overlapping. However, export of both L1S2 and ET-202 RNAs was strongly inhibited when the nuclear pool of RanGTP was depleted by treatment with RanT₂₄N (Fig. 7B,C, compare lanes 2–7 and 8–13; RanT₂₄N disrupts the RanGTPase system through inactivation of RCC1, the nuclear RanGEF; Izaurralde et al., 1997; Richards et al., 1997).

These results suggest that more than one transport factor is involved in export of L1S2, one that is shared with ET-202 RNA and which is unaffected by M protein, and one that is sensitive to this inhibitor (Grimm et al., 1997). The simplest explanation would be that the same export receptor is involved (which is sensitive to depletion of nuclear RanGTP, but fully functional in the presence of M protein) whereas the function of the Stem 2 NEE binding factor (and maybe also that of Ro 60 protein) is abrogated by M protein. ET-202 RNA may either interact directly with the export receptor or

use a different adapter protein that is insensitive to M protein. The predicted secondary structure of ET-202 RNA (our unpubl. results, using the M-Fold program; Mathews et al., 1999) indicates this RNA contains only a 4 bp terminal stem structure, which would be unlikely to function like a Stem 2-type NEE.

Nuclear export of Y RNAs

The data presented here support the following model for Y RNA export. After synthesis by RNA polymerase III, nuclear Y RNA associates with La and Ro60 proteins and the putative Stem 2 binding factor (and likely additional “packaging” proteins that associate with other regions of the RNA, i.e., the central loop and Stem-loops 3 and 4). Subsequently La protein is either released from the Y RNP or modified in such a way that its nuclear retention function is turned off. At this stage, Ro60 protein and/or the Stem 2 binding factor are required as adapter molecules that mediate the formation of a Y RNP•export receptor complex; this complex is likely to contain RanGTP but not CRM1 or exportin-t. The resulting complex is translocated through the NPC and upon delivery in the cytoplasm both the export receptor (and Ran) and the Stem 2 binding factor dissociate from the complex and are imported back into the nucleus.

The interactions of both factors seem to be required for export of full-length Y RNA, but the Stem 2 binding factor can function independently of Ro60 protein, as demonstrated by the efficient export of small Stem 2-containing molecules derived from hY1 and hY3 RNA. However, the presence of Stem 2 in a larger molecule like Δ S1L1 RNA is insufficient for export. Because it is unlikely that the binding of “Stem 2 factor” is prevented in Δ S1L1 RNA (this RNA is still an effective competitor), it is possible that interactions of this factor with full-length Y RNA (e.g., formation of the export receptor

FIGURE 7. Inhibition of L1S2 RNA export by VSV M-protein or depletion of nuclear RanGTP. **A:** A mixture of in vitro-transcribed, ³²P-labeled U3 RNA, U1ΔSm RNA, ET-202 RNA, and L1S2 RNA was injected into the nuclei of *X. laevis* oocytes that had been preinjected with VSV M-mRNA (lanes 10–17), or control antisense RNA (lanes 2–9; cf. Grimm et al., 1997; Her et al., 1997). RNA was assayed at 30 min (lanes 2, 6, 10, and 14), 60 min (lanes 3, 7, 11, and 15), 120 min (lanes 4, 8, 12, and 16), and 210 min (lanes 5, 9, 13, and 17) after injection; lane 1 shows the injected RNAs. **B:** A mixture of in vitro-transcribed, ³²P-labeled U3 RNA, tRNA^{Met}, and L1S2 RNA was injected into the nuclei of control oocytes or oocytes that had been preinjected in the cytoplasm with RanT₂₄N to deplete nuclear RanGTP (Izaurralde et al., 1997; E. Lund, unpubl.). RNA export was assayed at 30 min (lanes 2, 5, 8, and 11), 60 min (lanes 3, 6, 9, and 12) and 4.5 h (lanes 4, 7, 10, and 13) after RNA injection. **C:** A mixture of in vitro-transcribed, ³²P-labeled U6 and ET-202 RNAs was injected into the nuclei of control oocytes and RanT₂₄N-treated oocytes as in **B**. RNA export was assayed 45 min (lanes 2, 5, 8, and 11), 200 min (lanes 3, 6, 9, and 12), and 21 h (lanes 4, 7, 10, and 13) after RNA injection. U3 and U6 snRNAs, which are not exported to the cytoplasm, served as controls for the accuracy of nuclear injections and dissections.

complex) depend on the presence of Ro60 protein. In addition, Ro60 protein may be required for the recruitment of other “RNA-packaging” factors (to cover Loop 2/Stem-loop 3/Stem-loop 4 regions of Y RNAs) whose binding, in turn, may be a prerequisite for efficient export of molecules that contain these sequences.

MATERIALS AND METHODS

DNA constructs

The plasmid for hY1wt RNA transcription has been described (Simons et al., 1994). The mutant hY1 RNA constructs were derived from this hY1 RNA construct using PCR-based strategies with 5′ primers containing the T7 RNA polymerase promoter sequence.

Using the appropriate primers, the hY1 RNA mutants ΔS1L1 and ΔL1S2 were amplified and cloned into the *Eco*RI-*Hinc*II sites of pUC19. The mutants ΔS3L3, ΔS4L4, ΔL2B, and L2Bs were created using the approach described by Picard et al. (1994). This procedure requires three oligonucleotide primers: two flanking primers complementary to regions upstream and downstream of the mutation site, respectively, and a mutagenic primer. As flanking primers, the 5′ and 3′ primers of hY1wt RNA were used. The resulting PCR products were digested with *Eco*RI and *Hind*III and cloned into the corresponding sites of pUC19. The plasmids encoding the hY RNA mutants L1S2, S2, Y3-L1S2, S2swap, S2M, S2s, and Stem Z were constructed by annealing the appropriate 5′ and 3′ primers followed by filling in the single-stranded overhangs using Klenow enzyme. The resulting dsDNAs were digested with *Eco*RI and *Hind*III and cloned into pUC19. Plasmids for expression of *Xenopus* U1ΔSm RNA and 5S rRNA, human hY1Xba, tRNA^{Met}, and Th RNA, and ET-202 have been described previously (Hamm et al., 1987; Hamm & Mat-taj, 1989; Chow et al., 1992; Verheijen et al., 1994; Jar-molowski et al., 1994; Simons et al., 1996; Grimm et al., 1997).

For transcription, the clones were linearized with *Dra*I (hY1wt, ΔS1L1, ΔL1S2, ΔS3L3, ΔS4L4, ΔL2B, L2Bs, and 5S), *Xba*I (hY1Xba), *Sty*I (S1L1, S2, Y3-L1S2, S2swap, S2M, S2s, Stem Z), *Bam*HI (U1ΔSm), *Bfa*I (tRNA^{Met}), or *Hind*III (Th RNA) and gel purified. The ET-202 template was generated by PCR using the appropriate primers (Grimm et al., 1997).

In vitro transcription

In vitro transcription of export substrates and VSV M-protein mRNA and purification of the ³²P-labeled RNA was performed as described previously (Simons et al., 1996; Grimm et al., 1997; Her et al., 1997; Pasquinelli et al., 1997). Competitor RNAs were prepared essentially as described by Jar-molowski et al. (1994).

Oocyte injections

RNAs were microinjected into the nucleus of *X. laevis* oocytes and analyzed as described elsewhere (Simons et al., 1994, 1996; Grimm et al., 1997). For these analyses, only oocytes that were successfully microinjected into the nucleus

(as monitored by co-injection of dextran blue; Jarmolowski et al., 1994) were selected. For each analysis, RNAs from pools of three or more oocytes were used.

Immunoprecipitation

Monoclonal antibodies reactive with Ro60 (2G10) and the U1 small nuclear RNP specific A protein (9A9) were coupled to protein A-agarose beads by incubation at room temperature for 1 h in IPP500 (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% NP-40). HeLa S100 extracts were mixed with ³²P-labeled, in vitro transcribed RNAs and incubated for 30 min at 0 °C. Immunoprecipitations were performed in TKED (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM DTE, 1 mM EDTA, 0.05% NP-40) by head over head rotation for 2 h at 4 °C. The immunoprecipitate was washed three times with TKED and subsequently coprecipitated RNA was extracted and analyzed by 10% denaturing polyacrylamide gel electrophoresis followed by autoradiography.

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REFERENCES

- Arts GJ, Fornerod M, Mattaj IW. 1998. Identification of a nuclear export receptor for tRNA. *Current Biol* 8:305–314.
- Ben Chetrit E, Chan EK, Sullivan KF, Tan EM. 1988. A 52-kD protein is a novel component of the SS-A/Ro antigenic particle. *J Exp Med* 167:1560–1571.
- Chow CS, Hartmann KM, Rawlings SL, Huber PW, Barton JK. 1992. Delineation of structural domains in eukaryotic 5S rRNA with a rhodium probe. *Biochemistry* 31:3534–3542.
- Dahlberg JE, Lund E. 1998. Functions of the GTPase Ran in RNA export from the nucleus. *Curr Opin Cell Biol* 10:400–408.
- Fabini G, Rutjes S, Zimmermann C, Pruijn GJM, Steiner G. 2000. Analysis of the molecular composition of Ro ribonucleoprotein complexes. Identification of novel Y RNA-binding proteins. *Eur J Biochem* 267:2778–2789.
- Fornerod M, Ohno M, Yoshida M, Mattaj IW. 1997. CRM1 is an export receptor for leucine rich nuclear export signals. *Cell* 90:1051–1060.
- Görlich D, Dabrowski M, Bischoff FR, Kutay U, Bork P, Hartmann E, Prehn S, Izaurralde E. 1997. A novel class of RanGTP binding proteins. *J Cell Biol* 138:65–80.
- Görlich D, Kutay U. 1999. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* 15:607–660.
- Green CD, Long KS, Shi H, Wolin SL. 1998. Binding of the 60-kDa Ro autoantigen to Y RNAs: Evidence for recognition in the major groove of a conserved helix. *RNA* 4:750–765.
- Grimm C, Lund E, Dahlberg JE. 1997. Selection and nuclear immobilization of exportable RNAs. *Proc Natl Acad Sci USA* 94:10122–10127.
- Hamm J, Huber J, Lührmann R. 1997. Anti-idiotypic RNA selected with an anti-nuclear export signal antibody is actively transported in oocytes and inhibits Rev- and cap-dependent RNA export. *Proc Natl Acad Sci USA* 94:12839–12844.
- Hamm J, Kazmaier M, Mattaj IW. 1987. In vitro assembly of U1 snRNPs. *EMBO J* 6:3479–3485.
- Hamm J, Mattaj IW. 1989. An abundant U6 snRNP found in germ cells and embryos of *Xenopus laevis*. *EMBO J* 8:4179–4187.
- Hendrick JP, Wolin SL, Rinke J, Lerner MR, Steitz JA. 1981. Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: Further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells. *Mol Cell Biol* 1:1138–1149.
- Her LS, Lund E, Dahlberg JE. 1997. Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. *Science* 276:1845–1848.
- Izaurralde E, Kutay U, Vonkobe C, Mattaj IW, Görlich D. 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J* 16:6535–6547.
- Jarmolowski A, Boelens WC, Izaurralde E, Mattaj IW. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J Cell Biol* 124:627–635.
- Kutay U, Lipowsky G, Izaurralde E, Bischoff FR, Schwarzmaier P, Hartmann E, Görlich D. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol Cell* 1:359–369.
- Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* 288:911–940.
- Mattaj IW, Englmeier L. 1998. Nucleocytoplasmic transport: The soluble phase. *Annu Rev Biochem* 67:265–306.
- Nakielnny S, Dreyfuss G. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell* 99:677–690.
- O'Brien CA, Margelot K, Wolin SL. 1993. Xenopus Ro ribonucleoproteins: Members of an evolutionarily conserved class of cytoplasmic ribonucleoproteins. *Proc Natl Acad Sci USA* 90:7250–7254.
- Pasquinelli AE, Ernst RK, Lund E, Grimm C, Zapp ML, Rekosh D, Hammerskjöld M, Dahlberg JE. 1997. The constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV) accesses a cellular mRNA export pathway. *EMBO J* 16:7500–7510.
- Peek R, Pruijn GJM, Van der Kemp AJ, Van Venrooij WJ. 1993. Subcellular distribution of Ro ribonucleoprotein complexes and their constituents. *J Cell Sci* 106:929–935.
- Picard V, Erdsdal Badju E, Lu A, Bock SC. 1994. A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. *Nucleic Acids Res* 22:2587–2591.
- Prujn GJM, Simons FHM, Van Venrooij WJ. 1997. Intracellular localization and nucleocytoplasmic transport of Ro RNP components. *Eur J Cell Biol* 74:123–132.
- Richards SA, Carey KL, Macara IG. 1997. Requirement of guanosine triphosphate-bound Ran for signal-mediated nuclear protein export. *Science* 276:1842–1844.
- Simons FHM, Pruijn GJM, Van Venrooij WJ. 1994. Analysis of the intracellular localization and assembly of Ro ribonucleoprotein particles by microinjection into *Xenopus laevis* oocytes. *J Cell Biol* 125:981–988.
- Simons FHM, Rutjes SA, Van Venrooij WJ, Pruijn GJM. 1996. The interactions with Ro60 and La differentially affect nuclear export of hY1 RNA. *RNA* 2:264–273.
- Stade K, Ford CS, Guthrie C, Weis K. 1997. Exportin 1 (CRM1p) is an essential nuclear export factor. *Cell* 90:1041–1050.
- Teunissen SWM, Kruithof MJM, Farris AD, Harley JB, Van Venrooij WJ, Pruijn GJM. 2000. Conserved features of Y RNAs: A comparison of experimentally derived secondary structures. *Nucleic Acids Res* 28:610–619.
- Van Gelder CW, Thijssen JP, Klaassen EC, Sturchler C, Krol A, Van Venrooij WJ, Pruijn GJM. 1994. Common structural features of the Ro RNP associated hY1 and hY5 RNAs. *Nucleic Acids Res* 22:2498–2506.
- Verheijen R, Wiik A, De Jong BAW, Hoiermadsen M, Ullman S, Halberg P, Van Venrooij WJ. 1994. Screening for autoantibodies to the nucleolar U3- and Th(7-2) ribonucleoproteins in patients sera using antisense riboprobes. *J Immunol Methods* 169:173–182.
- Wolin SL, Steitz JA. 1984. The Ro small cytoplasmic ribonucleoproteins: Identification of the antigenic protein and its binding site on the Ro RNAs. *Proc Natl Acad Sci USA* 81:1996–2000.