

# Analysis of the Intracellular Localization and Assembly of Ro Ribonucleoprotein Particles by Microinjection into *Xenopus laevis* Oocytes

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**Abstract.** *Xenopus laevis* oocytes have been used to determine the intracellular localization of components of Ro ribonucleoprotein particles (Ro RNPs) and to study the assembly of these RNA-protein complexes. Microinjection of the protein components of human Ro RNPs, i.e., La, Ro60, and Ro52, in *X. laevis* oocytes showed that all three proteins are able to enter the nucleus, albeit with different efficiencies. In contrast, the RNA components of human Ro RNPs (the Y RNAs) accumulate in the *X. laevis* cytoplasm upon injection. Localization studies performed at low temperatures indicated that both nuclear import of Ro RNP proteins and nuclear export of Y RNAs are mediated by active

transport mechanisms.

Immunoprecipitation experiments using monospecific anti-La and anti-Ro60 antibodies showed that the *X. laevis* La and Ro60 homologues were cross-reactive with the respective antibodies and that both *X. laevis* proteins were able to interact with human Y1 RNA. Further analyses indicated that: (a) association of *X. laevis* La and Ro60 with Y RNAs most likely takes place in the nucleus; (b) once formed, Ro RNPs are rapidly exported out of the nucleus; and (c) the association with La is lost during or shortly after nuclear export.

THE Ro/SS-A and La/SS-B antigens are common targets of autoimmune responses occurring in a number of rheumatic disorders, of which systemic lupus erythematosus and Sjögren's syndrome are the most prominent (reviewed in Tan, 1989). Antibodies against these autoantigens and they also have some clinical interest in diagnosing different rheumatic diseases (reviewed in Slobbe et al., 1991a). The Ro/SS-A antigen consists of a set of ribonucleoprotein particles (Ro RNPs).<sup>1</sup> Ro RNPs consist of an RNA molecule, complexed with several proteins. The RNAs are transcribed by RNA polymerase III and, in humans, vary in length from 84 to 112 nucleotides (Hendrick et al., 1981). They are referred to as hY1, hY3, hY4, and hY5 RNA (hY2 RNA is a truncated version of hY1 RNA). Their sequences have been determined (Kato et al., 1982; Wolin and Steitz, 1983; O'Brien and Harley, 1990) and the predicted secondary structures are characterized by base pairing of the highly conserved 5' and 3' termini (reviewed by van Venrooij et al., 1993). The number of Y RNAs differs among species, vary-

ing from two in duck and mouse to four in several other mammals (Hendrick et al., 1981; Mamula et al., 1989a; Pruijn et al., 1993). In human tissues also a difference in the distribution of the four Y RNAs has been detected: in red blood cells only hY1 and hY4 RNA (Rader et al., 1989; O'Brien and Harley, 1990) and in human platelets only hY3 and hY4 RNA (Itoh and Reichlin, 1991) could be detected, while other human cells contain all four Y RNAs.

The protein part of a Ro RNP consists of at least three different proteins: the La protein (La) and the 52- and 60-kD Ro proteins (Ro52 and Ro60, respectively). One of the functions proposed for La is a role in the correct termination of transcription by RNA polymerase III (Gottlieb and Steitz, 1989a, b). La binds transiently to all RNA polymerase III transcripts, but is believed to bind to Y RNAs more stably, because Y RNAs don't lose their 3' U-tail which is the primary binding site for the La protein (Stefano, 1984; Mathews and Francoeur, 1984; Pruijn et al., 1991). For the Ro proteins different variants have been found in different cell types. While most human cells contain Ro60 and Ro52, red blood cells contain Ro proteins of 60 and 54 kD which are antigenically different from, but related to Ro60 and Ro52, respectively (Rader et al., 1989). Moreover, in human platelets only a 52-kD Ro protein can be detected that is also antigenically different from Ro52 (Itoh and Reichlin, 1991). The binding site for Ro60 is the terminal part of the conserved stem structure of the Y RNAs (Wolin and Steitz, 1984;

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1. *Abbreviations used in this paper:* NLS, nuclear localization signal; Ro RNP, Ro ribonucleoprotein particle.

Pruijn et al., 1991). Ro52 does not seem to bind directly to Y RNA, but most likely associates with the particle via protein-protein interactions with Ro60 (Slobbe et al., 1992). However, direct evidence for the association of Ro52 with Ro RNPs is still lacking and is complicated by the fact that some anti-Ro52 antibodies have been reported to cross-react with Ro60 (Itoh et al., 1992).

Although a number of functions for Ro RNPs have been proposed (reviewed in Pruijn et al., 1990), the actual function of these particles is still unknown. Also their intracellular localization is not clear yet. This subject has been addressed in several studies, but the results are controversial. By immunofluorescence Ro protein components were detected in only the nucleus (Harmon et al., 1984; Lopez-Robles et al., 1986; Xia et al., 1987; Mamula et al., 1989a), in both nucleus and cytoplasm (Ben-Chetrit et al., 1988; Slobbe et al., 1991b) or merely in the cytoplasm (Alspaugh and Maddison, 1979; Hendrick et al., 1981; Bachmann et al., 1986). We studied the intracellular localization and transport of Ro RNPs by microinjection of the different components into *Xenopus laevis* oocytes. This approach has proven to be very successful in studying transport of other RNPs, in particular U1 snRNP (reviewed in Nigg et al., 1991).

In this study in vitro translated La, Ro52, and Ro60 were injected into the oocyte cytoplasm to elucidate their transport and localization. The Y RNAs, on the contrary, were injected into the nucleus of oocytes. The association of hY1 RNA with *X. laevis* La and Ro60 was examined by immunoprecipitation studies. Our results suggest a model in which the Ro and La proteins after their synthesis are transported to the nucleus, where they associate with the newly synthesized Y RNAs. The assembled Ro RNP particles are then rapidly exported to the cytoplasm.

## Materials and Methods

### In vitro Transcription/Translation

The transcription vectors for hY RNAs were derived from pHY1, pHY3, pHY4, and pHY5 (Pruijn et al., 1991; Peek et al., 1993). By PCR a T7 RNA polymerase promoter was inserted at a position that allowed wild-type 5'-end formation during transcription and a DraI site was created at a position corresponding to the 3'-end of the respective hY RNA. The PCR products were cloned into pUC18. The U1 snRNA clone in pUC19 has been described by Surowy et al. (1989). After linearization with DraI (hY1) and DdeI (U1) and transcription with T7 RNA polymerase, RNAs corresponding to the wild-type sequences were obtained. The U1 clone was transcribed in the presence of m7G(5')ppp(5')G cap. 0.5  $\mu$ g of linearized template DNA was transcribed in 10  $\mu$ l buffer, containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.1 mg/ml BSA, 1 mM NTPs, 2 U RNasin, 1 mM m7G(5')ppp(5')G (in case of U1 RNA), and 20  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]UTP with 10 U of T7 RNA polymerase. Unincorporated nucleotides were removed by gel filtration and proteins were removed by phenol-chloroform (1:1) extraction.

The cDNA clones for La and Ro60, used for in vitro transcription/translation, have been previously described by Pruijn et al. (1991). The Ro52 cDNA clone used is identical to the Ro52 cDNA clone described by Itoh and co-workers (1991) and was isolated from a human placental cDNA library and subcloned into pGEM-3Zf(+) (Bozic et al., 1993). In vitro transcription/translation was carried out as described by Scherly et al. (1989).

### Oocyte Injection

Oocytes were released from the ovary by treatment with collagenase B (2.5 mg/ml) in Barth medium without Ca<sup>2+</sup>. For protein studies 40 nl of the in

vitro translation mixture was injected into the cytoplasm of fully grown oocytes and subsequently incubated in Barth medium supplemented with cycloheximide at a concentration of 100  $\mu$ g/ml. After 18 h the oocytes were manually dissected in J buffer (20 mM Tris-HCl, pH 7.5, 70 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol). The nuclear fraction was precipitated immediately in ethanol, whereas the "total" and cytoplasmic fractions were first homogenized in TN<sub>150E</sub> (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). After removal of part of the yolk and insoluble components by centrifugation, proteins were precipitated by adding 5 vol of acetone. Pellets were resuspended in SDS sample buffer and portions of four oocyte equivalents were analyzed by 10% SDS-PAGE.

For RNA injections, oocytes were first centrifuged (600 g, 20 min, room temperature) to visualize the nucleus. 25 nl of the in vitro transcribed RNA was injected into the nucleus of oocytes. After incubation and dissection of the oocytes the fractions were homogenized in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl, 1.5 mg/ml proteinase K, and incubated at 56°C for 30 min. Proteins were extracted once with phenol/chloroform (1:1) and RNA was precipitated by adding 4 vol of ethanol. RNA of one oocyte equivalent was analyzed on a 10% denaturing polyacrylamide gel. For immunoprecipitation oocytes were homogenized in TN<sub>150E</sub>.

The *X. laevis* U1 RNA gene used for injection is described by Zeller et al. (1984). The hY RNA genes are located on a genomic DNA fragment described by Wolin and Steitz (1983). The hY1 and hY3 genes were first subcloned by ligating the EcoRI-SalI fragment (hY1) and EcoRI-BamHI fragment (hY3) into pGEM-3Zf(+). The DNA (1 mg/ml) was mixed with an equal volume of [ $\alpha$ -<sup>32</sup>P]UTP (10 mCi/ml) and injected (25 nl) into the oocyte nuclei. After 40 h, RNA was extracted from the oocytes and analyzed on polyacrylamide gels. Gel-purified RNA (Mattaj and Zeller, 1983) was dissolved in water and re-injected into the oocyte nucleus and analyzed as described.

### Antisera

The anti-La, anti-Ro60, and anti-Sm sera were from autoimmune patients. The anti-La and anti-Ro60 antibodies were affinity purified using bacterially expressed La and Ro60 (Slobbe et al., 1992). Their monospecificity was confirmed by Western blotting and by immunoprecipitation of in vitro translated proteins. The specificity of the anti-Sm serum was determined by Western blotting, counter-immunoelectrophoresis, and RNA precipitation.

### Immunoprecipitation

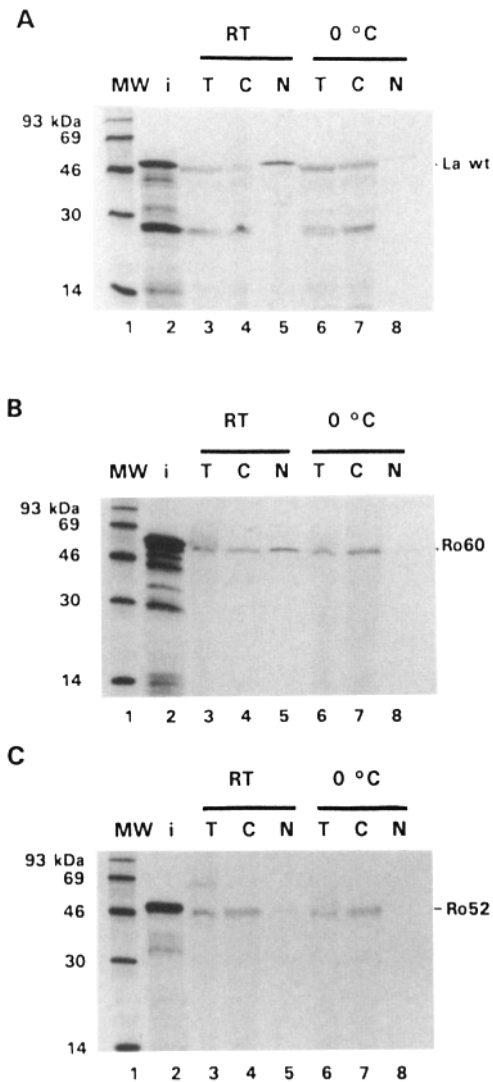
Antisera were coupled to protein A-agarose by head over head rotation at room temperature for 1 h in IPP<sub>500</sub> (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% NP-40). For each precipitation 50  $\mu$ l of affinity-purified anti-La or anti-Ro60 or 2  $\mu$ l of anti-Sm or normal human serum were used. Immunoprecipitations were carried out in IPP<sub>150</sub> (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40) for 2 h at 4°C. Immunoprecipitation with oocyte extracts was performed with three oocyte equivalents. The RNA was extracted and analyzed as described in the oocyte injection section.

For immunoprecipitation of endogenous proteins, oocytes were labeled in Barth medium with 0.5 mCi [<sup>35</sup>S]methionine. After 40 h the oocytes were homogenized in S100-buffer (25 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 M KCl, 0.01% NP-40, 20% glycerol), centrifuged to remove insoluble components and yolk, and then used for immunoprecipitation. The precipitated proteins were resuspended in SDS sample buffer and analyzed by 10% SDS-PAGE.

## Results

### Nuclear Import of Human La, Ro52, and Ro60 in *X. laevis* Oocytes

Because immunofluorescence led to controversial localization data, especially in the case of the Ro proteins, the intracellular localization of the Ro RNP proteins was studied by an alternative approach. In vitro translated La, Ro60, and Ro52 proteins were injected into the cytoplasm of *X. laevis* oocytes. After an overnight incubation the intracellular dis-



**Figure 1.** Nuclear import of La, Ro60, and Ro52 polypeptides. In vitro translated La (a), Ro60 (b), and Ro52 (c) were injected into the cytoplasm of *X. laevis* oocytes. After incubation at room temperature (RT) (lanes 3–5) or 0°C (lanes 6–8) for 18 h, oocytes (T) were dissected into cytoplasmic (C) and nuclear (N) fractions which were analyzed by 10% SDS-PAGE. (Lanes 1) Protein molecular weight markers. (Lanes 2) In vitro translation products used for injection (i). The positions of the full-length La, Ro60 and Ro52 polypeptides in the gel are indicated. The apparent difference in migration between nuclear and cytoplasmic La protein is due to the presence of some remaining yolk protein in the cytoplasmic fraction. When nuclear La is analyzed in combination with cytoplasmic La (lane T), i.e., in the presence of cytoplasmic material, it also migrates like cytoplasmic La. Note that migration of La in the nuclear fraction is identical to that of the in vitro translation product, which was used for injection.

tribution was determined by dissection of the oocytes and analysis of the cytoplasmic and nuclear fractions.

Injected <sup>35</sup>S-labeled recombinant proteins, obtained by translation in a wheat germ extract, became distributed among nucleus and cytoplasm after an overnight incubation. The La protein showed a strong nuclear accumulation leav-

ing only a minor part in the cytoplasm (Fig. 1 a, lanes 3–5). Also a substantial amount of Ro60 had entered the nucleus (Fig. 1 b, lanes 3–5), although the efficiency of nuclear entry appeared to be somewhat lower than that for La. In case of Ro52 most of the protein remained in the cytoplasm, although detectable amounts were reproducibly imported into the nucleus (Fig. 1 c, lanes 3–5). When La, Ro52, and Ro60 were injected into the nucleus of oocytes instead of into the cytoplasm the same distribution between cytoplasm and nucleus after an overnight incubation was obtained (data not shown), suggesting that the observed distributions reflect equilibrium situations.

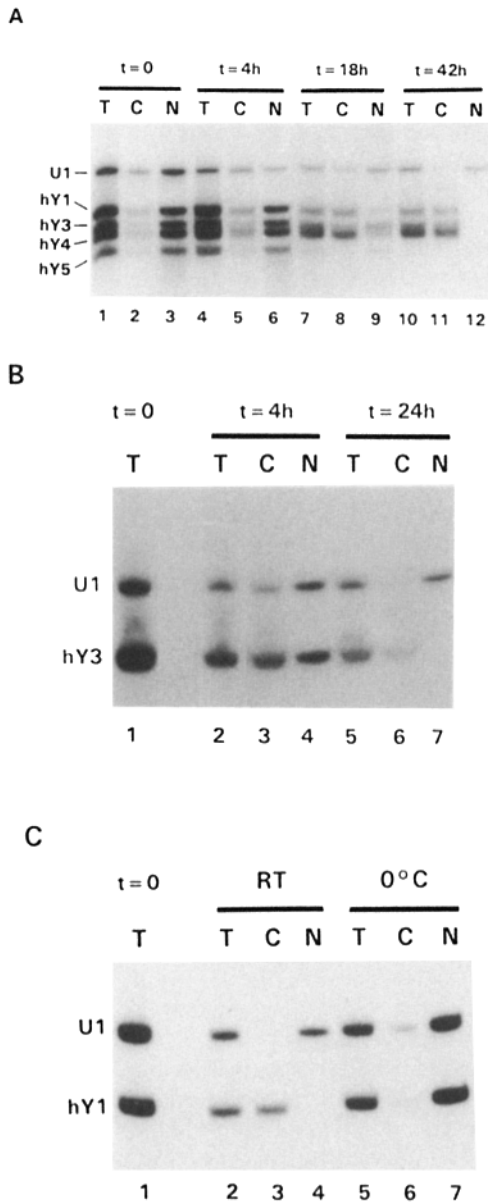
In the La translation mixture (Fig. 1 a, lane 2) the full-length protein is accompanied by a prominent smaller polypeptide. This represents a characteristic degradation product of the La protein (Habets et al., 1983; Chan and Tan, 1987), caused by the presence of a so-called PEST region in the middle of the protein (Chan et al., 1989a), which is known to be very susceptible to proteolytic degradation. This degradation product represents the NH<sub>2</sub>-terminal half of the La protein, because only this part contains methionines and thus is the part of the protein that will be radioactively labeled during in vitro translation by [<sup>35</sup>S]methionine. The observation that this truncated variant of La completely remained in the cytoplasm (Fig. 1 a, lanes 3–5) strongly suggests that transport of the full-length La protein to the nucleus is not caused by diffusion.

To investigate the mechanism of nuclear import of all three proteins the oocytes were, after injection, incubated at 0°C instead of at room temperature. This approach allows us to distinguish between diffusion and active transport because incubation at 0°C severely reduces the rate of energy-dependent transport while diffusion is hardly influenced (Newmeyer et al., 1986; Breeuwer et al., 1990). As shown in Fig. 1, a–c, transport to the nucleus of La, Ro60, as well as Ro52 was drastically reduced when oocytes were incubated at 0°C (lanes 6–8) in comparison with incubations at room temperature (lanes 3–5). Furthermore, this effect appeared to be reversible: oocytes, that were first incubated at 0°C for 18 h and subsequently at room temperature for 24 h, displayed protein distributions similar to oocytes that had been incubated at room temperature only (data not shown).

As a control, the UIA protein which previously has been shown to be actively transported to the nucleus (Kambach et al., 1992), was included in these types of experiments. Nuclear import of the UIA protein was also severely inhibited at 0°C and restored when oocytes were subsequently incubated at room temperature (data not shown). From these results we conclude that the La, Ro52, and Ro60 proteins are transported to the nucleus via an active process.

### Nuclear Export of Y RNA in *Xenopus laevis* Oocytes

Having observed active nuclear import of the three different Ro RNP proteins, the localization of the RNA components of Ro RNPs in *X. laevis* oocytes was studied. For this purpose, in vitro transcribed hY1, hY3, hY4, and hY5 RNA were injected into the nucleus of oocytes, i.e., in the cellular compartment where they are synthesized, and the distribution of the RNAs 4 and 18 h after injection was studied. In vitro transcribed U1 snRNA was co-injected as a control.



**Figure 2.** Nuclear export of hY RNAs. (a) In vitro transcribed hY1, hY3, hY4, hY5, and U1 RNA were co-injected into oocyte nuclei. RNA was extracted from total oocytes (T), cytoplasmic (C), and nuclear (N) fractions immediately (lanes 1–3), 4 h (lanes 4–6), 18 h (lanes 7–9), and 42 h (lanes 10–12) after injection and analyzed on a denaturing polyacrylamide gel. (b) In vivo transcribed hY3 RNA and U1 RNA were gel purified and re-injected into the oocyte nucleus. Immediately (lane 1), 4 h (lanes 2–4), and 24 h (lanes 5–7) after injection, oocytes were dissected and the extracted RNAs analyzed on a denaturing polyacrylamide gel. (c) In vitro transcribed hY1 RNA and U1 RNA were co-injected into the oocyte nucleus and subsequently incubated at room temperature (RT) (lanes 2–4) or 0°C (lanes 5–7) for 18 h. Thereafter RNA was extracted from the fractions and analyzed. (Lane 1) RNA extracted from oocytes immediately after injection.

Fig. 2 a shows that, upon injection into the nucleus, hY1, hY3, and hY4 RNA gradually moved to the cytoplasm. The distribution of hY5 RNA was not clear since it was not stable upon injection into the oocyte. After ~8 h nuclear export of hY1 RNA was completed (data not shown). The control RNA, U1 snRNA, was, as expected, first transported to the cytoplasm

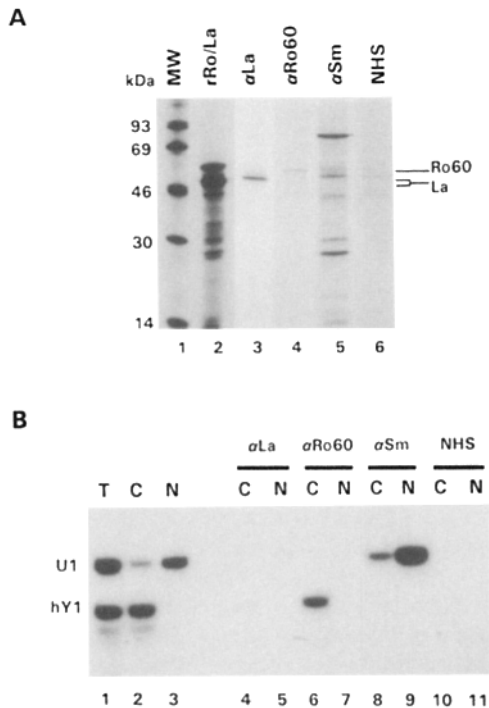
before returning to the nucleus (Fig. 2 a) (see Nigg et al., 1991).

In vitro produced hY RNAs are structurally identical to hY RNAs isolated from human cells, except for the 3' uridine stretch which is 4-nucleotides long rather than 2 or 3. Apparently, these in vitro produced molecules are quite stable in oocytes. However, the possibility that the behavior of an injected in vitro produced hY RNA is different from that of an endogenously transcribed equivalent can not be excluded. Therefore, hY RNA genes (encoding hY1 and hY3 RNA) were injected in the oocyte nucleus together with [ $\alpha$ - $^{32}$ P] UTP. A *X. laevis* U1 RNA gene was injected as a control. Both hY3 RNA and U1 RNA were transcribed efficiently in oocytes, in contrast to hY1 RNA which was transcribed very poorly (data not shown). Therefore, the endogenously transcribed hY3 and U1 RNAs were isolated and re-injected. Fig. 2 b shows that the distribution of these RNAs was similar to that of the in vitro transcribed RNAs (Fig. 2, compare a with b). Also in this case the hY RNA was predominantly located in the cytoplasm while U1 RNA localized primarily in the nucleus 24 h after injection. The distribution of in vivo transcribed hY3 RNA was also studied directly after injection of the hY3 gene (and the U1 gene as a control) into the oocyte nucleus along with radioactive label and analysis of the cytoplasmic and nuclear localization of transcribed RNAs after a relatively long period of time (40 h). Since both Y RNAs and U RNAs are rather stable in *X. laevis* oocytes, one would expect a distribution that strongly resembles the real distribution of in vivo transcripts. Indeed, also in this experiment hY3 RNA was found to be primarily cytoplasmic in contrast to U1 RNA, which was, as expected, mainly nuclear (data not shown). We conclude that the intracellular transport of the in vitro transcribed RNAs is indistinguishable from that of the in vivo transcribed RNAs.

To investigate whether transport of hY RNA to the cytoplasm was an energy-dependent process, injected oocytes were incubated at 0°C. Virtually no export to the cytoplasm of hY1 RNA nor of U1 RNA could be observed (Fig. 2 c, compare lanes 2–4 with 5–7), strongly suggesting that nuclear export of hY1 RNA to the cytoplasm is an active process. We conclude that hY RNA, after synthesis in the nucleus, is actively transported to and accumulates in the cytoplasm.

#### Formation of Ro RNPs in *Xenopus laevis* Oocytes

The observations that the Y RNAs accumulate in the oocyte cytoplasm and that the Ro proteins at least in part move to the nucleus, prompted us to investigate whether the injected hY1 RNA associates with *X. laevis* proteins leading to the formation of a ribonucleoprotein particle. We hypothesized that such proteins would be homologous to the human Ro and La proteins. Recently, the presence of two variants of the La protein as well as a Ro60 homologue in *X. laevis* have been reported and cDNAs encoding these proteins have been cloned (Scherly et al., 1993; O'Brien et al., 1993). To investigate the association of hY1 RNA with *X. laevis* Ro60 and La proteins, affinity-purified monospecific human anti-Ro60 and anti-La antibodies were used. One of the prerequisites for the use of these antibodies is cross-reactivity of the antibodies with their *X. laevis* homologues. To study this, immunoprecipitations with a radiolabeled *X. laevis* oocyte extract were performed. Precipitation with monospecific anti-La antibodies showed, that these antibodies recognized



**Figure 3.** hY1 RNA association with *X. laevis* proteins. (a) Proteins from an  $^{35}\text{S}$ -labeled *X. laevis* oocyte extract were immunoprecipitated using antibodies with various specificities and analyzed by SDS-PAGE. (Lane 1) Protein molecular weight markers. (Lane 2) Mixture of in vitro translated recombinant human La and Ro60 (rRo/La). (Lane 3) Anti-La precipitation ( $\alpha$ -La). (Lane 4) Anti-Ro60 precipitation ( $\alpha$ -Ro60). (Lane 5) Anti-Sm precipitation ( $\alpha$ -Sm). (Lane 6) Normal human serum (NHS) precipitation. (b) In vitro transcribed hY1 RNA and U1 RNA were co-injected into oocyte nuclei and after 18 h RNA from total oocytes (T), cytoplasmic (C), and nuclear (N) fractions were extracted and analyzed by polyacrylamide gel electrophoresis (lanes 1–3). Cytoplasmic and nuclear fractions were used to perform immunoprecipitations with anti-La (lanes 4 and 5), anti-Ro60 (lanes 6 and 7), anti-Sm (lanes 8 and 9), and normal human serum (lanes 10 and 11). C and N correspond to the cytoplasmic and nuclear fraction, respectively.

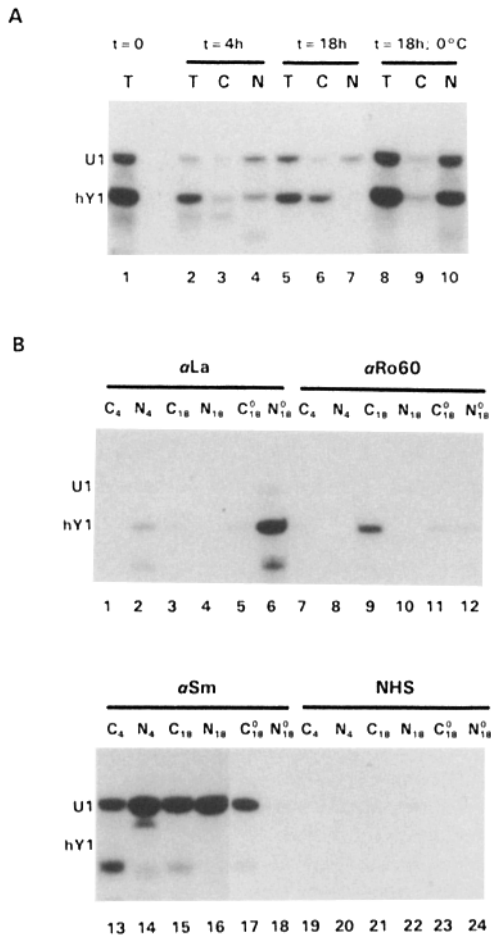
at least one of the two *X. laevis* La variants, which are not separated by SDS-PAGE (Fig. 3 a, lane 3). Precipitations with monospecific anti-Ro60 antibodies revealed that these antibodies were reactive with the *X. laevis* Ro60 homologue (Fig. 3 a, lane 4). When monospecific anti-Ro52 antibodies were used for similar immunoprecipitations no specific precipitation of a *X. laevis* polypeptide was observed (data not shown). This is in agreement with previous results indicating that immunoreactive Ro52 could only be detected in primate cells (Slobbe et al., 1991). As a control for the immunoprecipitations normal human serum and a polyclonal serum containing antibodies to the Sm proteins, which are associated with U snRNPs and are present in *X. laevis* oocytes (Zeller et al., 1983), were used (Fig. 3 a, lanes 6 and 5, respectively).

Using the monospecific affinity-purified anti-La and anti-Ro60 antibody preparations the association of *X. laevis* La and Ro60 with hY1 RNA was investigated. When hY1 RNA and U1 RNA were co-injected into the nucleus of oocytes the expected distribution after an overnight incubation was obtained (Fig. 3 b, lanes 1–3). Cytoplasmic as well as nuclear extracts of these oocytes were used for immunoprecipita-

tions. Anti-Ro60 antibodies efficiently precipitated labeled hY1 RNA from the cytoplasmic extract, while anti-La antibodies resulted in signals that were not or hardly above background (Fig. 3 b, lanes 4–7). Control antibodies from the anti-Sm or from a normal human serum did not precipitate hY1 RNA (Fig. 3 b, lanes 8–11). U1 RNA was, as expected, specifically precipitated by the anti-Sm antibodies, especially from the nuclear extract, in accordance with the nuclear accumulation (Fig. 3 b, lanes 8 and 9). The specificity of the immunoprecipitation was further confirmed by the lack of precipitation by normal human serum. From these results we conclude that in vitro transcribed hY1 RNA, which accumulates in the cytoplasm, is associated with the *X. laevis* Ro60 protein but apparently not or hardly with the *X. laevis* La protein.

The weak precipitation of hY1 RNA with anti-La antibodies as compared with anti-Ro60 antibodies was rather unexpected, since several studies performed in mammalian cells indicate that in such cells most, if not all hY RNA molecules are associated with La (Boire and Craft, 1990; Peek et al., 1993). A possible explanation for this phenomenon is that this affinity-purified anti-La antibody is poorly recognizing and precipitating the *X. laevis* La protein when associated with hY1 RNA. Differences in efficiency of precipitation of *X. laevis* La RNPs by various antibodies raised against human La have been reported before (Terns et al., 1992). Therefore, several monospecific anti-La sera were compared in immunoprecipitating capacity of *X. laevis* La from a [ $^{35}\text{S}$ ]methionine-labeled oocyte extract. The monospecific anti-La serum used in the experiments described above precipitated both human and *X. laevis* La with a high efficiency (data not shown). Furthermore, this antibody precipitated  $^{32}\text{P}$ -labeled hY1 RNA incubated in an oocyte extract at least as efficiently as antibodies from other sera. In fact, hY1 RNA precipitation with the monospecific anti-Ro60 serum used in the experiment depicted in Fig. 3 b was less efficient than precipitation with the monospecific anti-La serum (data not shown). In conclusion, the weak precipitation of hY1 RNA from the oocyte cytoplasm by anti-La antibodies does not seem to be caused by the quality or specificity of the antibody used.

To shed more light on this aspect and to investigate whether the association of the *X. laevis* La and Ro60 proteins with hY1 RNA takes place in the cytoplasm or the nucleus, the following experiment was performed. hY1 RNA and U1 RNA were co-injected into the nucleus of oocytes and, after 4 and 18 h of incubation, nuclear and cytoplasmic fractions were prepared. In parallel, extracts were prepared from similarly injected oocytes that were incubated for 18 h at 0°C. At the latter temperature, hY1 RNA and U1 RNA are not transported to the cytoplasm (Fig. 4 a, lanes 8–10). As observed before, after 4-h incubation at room temperature part of the hY1 and U1 RNAs had been transported to the cytoplasm (Fig. 4 a, lanes 2–4). 18 h after injection most of the hY1 RNA was present in the cytoplasm while most of the U1 RNA had returned to the nucleus (Fig. 4 a, lanes 5–7). Immunoprecipitations with various antibodies were performed on the different cytoplasmic and nuclear fractions. The precipitations with anti-La antibodies clearly indicate, that in the nucleus La efficiently associates with hY1 RNA and that the La-hY1 RNA complex was found almost exclusively in the nucleus (Fig. 4 b, lane 2). This is especially evident after an incubation for 18 h at 0°C (Fig. 4 b,



**Figure 4.** Assembly and nuclear export of hY1 RNP in *X. laevis* oocytes. (a) In vitro transcribed hY1 RNA and U1 RNA were co-injected into the oocyte nucleus and incubated at room temperature (lanes 2–7) or 0°C (lanes 8–10). After 4 h (lanes 2–4) and 18 h (lanes 5–10) RNA from total oocytes (T), cytoplasmic (C), and nuclear (N) fractions were extracted and analyzed on gel. (Lane 1) RNA extracted from oocytes immediately after injection ( $t = 0$ ). The RNA in each lane corresponds to the contents of 0.5 oocyte or oocyte compartment. (b) Injected RNAs as in a were immunoprecipitated from the cytoplasmic (C) and nuclear (N) fractions with anti-La (lanes 1–6), anti-Ro60 (lanes 7–12), anti-Sm (lanes 13–18), and normal human serum (lanes 19–24) and then analyzed by polyacrylamide gel electrophoresis. Material from three oocytes (or oocyte compartments) was used for each immunoprecipitation. Subscripts indicate the incubation times and superscripts refer to the incubation temperature before preparation of the extracts (no superscript corresponds to incubation at room temperature).

lane 6). In the cytoplasmic fractions no or hardly any La-hY1 RNA complex could be observed (Fig. 4 b, lanes 1, 3, and 5). These results suggest that in *X. laevis* oocytes the association with La is lost during transport of hY1 RNA to the cytoplasm. The latter observations are in agreement with the weak immunoprecipitation signals observed before (Fig. 3 b). Similar precipitations with anti-Ro60 antibodies showed that hY1 RNA in the cytoplasm is associated with Ro60 while Ro60-hY1 RNA complexes in the nucleus are not detectable after incubations at room temperature (Fig. 4 b, lanes 7–10). In contrast, hY1 RNA in the nucleus is precipitable with anti-Ro60 antibodies when the oocytes are incubated at 0°C (Fig. 4 b, lane 12), suggesting that the binding of *X. laevis* Ro60

to hY1 RNA takes place in the nucleus, followed by rapid export to the cytoplasm. Control precipitations were performed with anti-Sm antibodies and normal human serum. It appears that only U1 RNA molecules, that have passed through or are in the cytoplasm, are immunoprecipitable with anti-Sm antibodies (Fig. 4 b, lanes 13–17). U1 RNA that did not leave the nucleus during the 0°C incubation and for that reason is not complexed with Sm proteins (Nigg et al., 1991) could not be precipitated by anti-Sm antibodies, as expected (Fig. 4 b, lane 18).

## Discussion

The intracellular localization of Ro RNPs has been studied by several groups primarily using the immunofluorescence technique. The results of these studies were not very conclusive. In a recent study (Peek et al., 1993) it became clear that the localization as determined by immunofluorescence critically depends on the cell fixation method. The contradicting results obtained with the immunofluorescence technique might be explained in part by leakage of intracellular components during fixation or by masking of epitopes due to fixation. Recently, cell enucleation studies have shed more light on the intracellular localization of Ro RNPs (O'Brien et al., 1993; Peek et al., 1993). In order to verify and extend these data a second approach was chosen: injection of Ro RNP components into *X. laevis* oocytes. This approach has also successfully been employed to elucidate the intracellular distribution and transport of different U snRNPs and their proteins (reviewed in Nigg et al., 1991). While this work was in progress Ro RNPs were identified and characterized in *X. laevis* (O'Brien et al., 1993), which allowed us to use the oocytes as a bona fide system to study the intracellular distribution and transport of Ro RNP components.

### Ro RNP Proteins Are Actively Transported to the Nucleus

When injected into the cytoplasm, the recombinant human La protein accumulates in the oocyte nucleus. The primarily nuclear localization is in agreement with the results obtained with immunofluorescence (Hendrick et al., 1981), cell fractionation (Habets et al., 1983) and immunoelectron microscopy (Carmo-Fonseca et al., 1989). The observation that Ro60 and Ro52 are also actively transported to the nucleus, although less efficiently, is in agreement with data obtained by cell enucleation of Jurkat and 3T3 cells (Peek et al., 1993). After cell enucleation La, Ro52, and Ro60 were found in both cytoplasm and nucleus. Minor quantitative differences obtained by different techniques as oocyte injection and cell enucleation might be explained by the different cell types that were used in these studies and/or by differences in the total amount of Ro or La protein per cell. Furthermore, one should realize that the binding to endogenous components, like Y RNAs, may influence the intracellular distribution of the injected molecules.

All three proteins are transported to the nucleus via an active transport mechanism. This probably implies that a nuclear localization signal (NLS) is involved. An NLS is a structural element that both is necessary and sufficient for transport of a protein to the nucleus. A nuclear protein will

either possess its own NLS or enter the nucleus by cotransport while associated with another protein containing an NLS (Silver, 1991; Garcia-Bustos et al., 1991). A sequence element matching the bipartite NLS (Dingwall et al., 1991), which is found in the majority of nuclear proteins, is present in La but not in Ro60 or Ro52. At present, studies are being performed to determine which sequences in La, Ro60, and Ro52 are involved in nuclear import of these proteins.

### *Y RNAs Are Located in the Cytoplasm*

In contrast to the Ro RNP-specific proteins, hY RNAs were found to be located exclusively in the cytoplasm after an overnight incubation of injected oocytes. Upon injection hY RNAs are actively exported out of the nucleus into the cytoplasm. These results are in agreement with the data obtained with cell enucleation studies (O'Brien et al., 1993; Peek et al., 1993) in which human and murine cells were used. In vivo transcribed hY3 RNA also accumulated in the cytoplasm, thereby justifying the use of in vitro transcribed RNAs. In accordance with our results, the endogenous *X. laevis* xY RNAs were recently also shown to be exclusively localized in the oocyte cytoplasm (O'Brien et al., 1993). It therefore seems unlikely that the use of a heterologous RNA might have influenced the results.

The hY1 gene was transcribed very poorly in *X. laevis* oocytes and produced several transcripts of approximately hY1 RNA size. A possible explanation for the inefficient transcription of the hY1 gene is that the upstream sequence present in this genomic clone was relatively small (~100 bp, instead of ~400 bp in the case of the hY3 gene). Probably some essential transcription regulatory sequence elements in the hY1 gene are lacking.

### *Interaction of X. laevis La and Ro60 with hY1 RNA*

La is evolutionarily reasonably well conserved, as has been shown by Western blotting data (Hoch et al., 1984; Slobbe et al., 1991b) and cDNA sequence comparisons (Chan et al., 1989b; Scherly et al., 1993; Topfer et al., 1993; Semsei et al., 1993). Two *X. laevis* La cDNA variants have been described. Both encode polypeptides that are about 20-amino acids longer (427 and 428 amino acids, respectively) than their human counterpart (Scherly et al., 1993). Monospecific anti-La antibodies immunoprecipitated at least one of these *X. laevis* La proteins which indeed had an apparent molecular weight somewhat higher than that of the human La protein. Like human La, *X. laevis* La was able to associate with hY1 RNA. The evolutionary conservation of Ro60 is also evident: 60-kD proteins from various species react on Western blots with antibodies raised against human Ro60 (Mamula et al., 1989a; Slobbe et al., 1991b). By immunofluorescence the existence of the Ro/SS-A antigen in frogs has been demonstrated (Xia et al., 1987). A cDNA encoding a 60-kD Ro protein sharing 78% amino acid sequence identity with human Ro60 has recently been isolated from a *X. laevis* cDNA library (O'Brien et al., 1993). Our immunoprecipitation experiments showed that *X. laevis* indeed contains a 60-kD protein which is reactive with anti-Ro60 antibodies and is able to interact with hY1 RNA (Fig. 3 a, lane 4 and b, lane 6).

Analyses of the association of *X. laevis* La with hY1 RNA strongly suggest that during or shortly after export from the nucleus La dissociates from hY1 RNA in oocytes. A similar

phenomenon has been observed with the transport of 5S rRNA from nucleus to cytoplasm in *X. laevis* oocytes (Gudat et al., 1990). The loss of association with La might be related to the fact that pre-5S rRNA loses its 3' U-stretch upon processing. At the moment it is not clear whether the human Y RNAs in *X. laevis* oocytes are also subject to processing events at their 3'-end. The apparent dissociation of La from hY1 RNA in *X. laevis* oocytes is, however, in contradiction to the results obtained by enucleation (Peek et al., 1993) and fractionation (Boire and Craft, 1990) of human cells because Y RNAs were found to be stably associated with the La protein in these cells. Future studies will show whether dissociation of La from Y RNAs is dependent on the type of cell studied.

In addition to La, *X. laevis* Ro60 was found to associate with hY1 RNA. The low level of association of Ro60 with nuclear hY1 RNA as detected at 4 h after injection suggests that the association with Ro60 might be the rate limiting step in nuclear export of hY1 RNA. An alternative explanation would be that the Ro60-hY1 RNA association in the nucleus is very inefficient and mostly takes place after export of hY1 RNA to the cytoplasm. Indeed, the Ro60-hY1 RNA association in the nucleus at 0°C incubation appears to be rather inefficient (Fig. 4 B, lane 12), which may, however, also be related to the smaller amount of Ro60 in the oocyte nucleus in comparison with the amount of La. Alternatively, the association of Ro60 with hY1 RNA in the nucleus might be temperature dependent. Preliminary results of experiments, in which we injected a hY1 RNA mutant that was unable to associate with Ro60, however, strongly suggest that Ro60 binding in the nucleus is required for efficient export of hY1 RNA to the cytoplasm (unpublished observations).

The results presented in Fig. 4 might suggest that the binding of endogenous Ro60 and La to hY1 RNA injected into oocytes is mutually exclusive. Although we can not completely exclude this possibility for the *X. laevis* proteins, several data obtained with the human and mouse proteins, however, indicate that both proteins can simultaneously bind to one hY RNA molecule (Mamula et al., 1989b; Boire et al., 1990; Peek et al., 1993).

The combination of the present results and the results obtained by enucleation of human and murine cells (Peek et al., 1993) leads to the following model for Ro RNP synthesis and assembly. Ro60 and La, after translation in the cytoplasm, are actively transported to the nucleus. In the nuclear compartment they associate with Y RNAs shortly after or even during transcription. Once such complexes have been formed, they are rapidly and actively exported out of the nucleus to the cytoplasm where they will fulfill their function(s). It should be noted that our results do not exclude the possibility that other, yet unidentified factors are also involved in the transport process.

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