Multiple conserved segments of E1 small nucleolar RNA are involved in the formation of a ribonucleoprotein particle in frog oocytes

Donald D. RUHL, Mary Ellen PUSATERI and George L. ELICEIRI[1]

Department of Pathology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104-1028, U.S.A.

E1/U17 small nucleolar RNA (snoRNA) is a box H/ACA snoRNA. To identify E1 RNA elements required for its assembly into a ribonucleoprotein (RNP) particle, we have made substitution mutations in evolutionarily conserved sequences and structures of frog E1 RNA. After E1 RNA was injected into the nucleus of frog oocytes, assembly of this exogenous RNA into an RNP was monitored by non-denaturing gel electrophoresis. Unexpectedly, nucleotide substitutions in many phylogenetically conserved segments of E1 RNA produced RNPs with abnormal gel-electrophoresis patterns. These RNA segments were at least nine conserved sequences and an apparently conserved structure. In another region needed for RNP formation, the requirement may be sequence(s) and/or structure. Base substitutions in each of these and in one additional conserved E1 RNA segment reduced the stability of this snoRNA in frog oocytes. Nucleolar localization was assayed by fluorescence microscopy after injection of fluorescein-labelled RNA. The H box (ANANNA) and the ACA box are both needed for efficient nucleolar localization of frog E1 RNA.

Key words: nucleolar localization, snoRNA element, snoRNA stability, snoRNP asssembly, *Xenopus laevis*.

INTRODUCTION

There are many small nucleolar RNAs (snoRNAs) in the eukaryotic cell that function in the maturation of ribosomal RNA (reviewed in [1–4]). Most snoRNAs belong to one of two families. Box C/D snoRNAs have conserved sequence elements named box C (UGAUGA) and box D (CUGA) [1,5]. Some box C/D snoRNAs function in pre-rRNA cleavage processing; most guide 2-*O*-ribose methylation of pre-rRNA [6,7]. In the second family, box H/ACA snoRNAs have the conserved motif ACA and box H (ANANNA) [8,9]. Some box H/ACA snoRNAs have roles in pre-rRNA cleavage; most direct pseudouridine synthesis in pre-rRNA [10,11]. E1/U17 is an H/ACA snoRNA [12–16]. E1 RNA differs from most H/ACA snoRNAs in several aspects. First, it is required for the accumulation of mature 18 S rRNA [17]. Secondly, an rRNA 'pseudouridylation pocket', present in all the rRNA pseudouridylation guide snoRNAs, has not been found in E1 RNA [11]. (An rRNA pseudouridylation pocket consists of two sequences, one on each side of an internal loop structure of a snoRNA, that are complementary to two pre-rRNA sequences that flank a pseudouridylation site [11].) Thirdly, E1 RNA interacts directly (psoralen-photo-crosslinks) *in vivo* with two segments of pre-rRNA [13] which do not have any pseudouridylation sites that could be potentially guided by E1 RNA through the base-pairing mechanism of the known rRNA pseudouridylation guide snoRNAs.

Assembly into its ribonucleoprotein (RNP), usually its functional form, is an important process in the life cycle of most RNA species. Identification of the necessary RNA *cis*-acting elements is a valuable step towards studying RNP biogenesis. For each box C/D snoRNA species studied thus far, few RNA elements required for RNP assembly have been identified [18,19], and less is known about these elements in box H/ACA snoRNAs. We set out to identify the E1 RNA *cis*-acting elements needed for formation of its RNP. In the present study, an unexpectedly high number of phylogenetically conserved segments of E1 RNA was found to be involved in the formation of its RNP *in vivo*.

EXPERIMENTAL

Synthesis of mutants

Sequence *f* of *Xenopus laevis* E1 RNA was used, since it is expressed [16] and functional [17]. Site-specific base-substitution mutants were made by PCR-based *in vitro* mutagenesis. In the conserved sequences, nucleotide substitutions were G to C, C to G, A to U and U to A. In segments where sequence phylogeny indicated that the structure was conserved but the sequence was not [20], base substitutions were designed to prevent base pairing to the putative partner nucleotide on the opposite strand. For example, substitution was to G when the apparent base-pairing partner was a G, etc. The RNA sequence of the segment substituted in each mutant is shown in Table 1. A bacteriophage T7 RNA polymerase promoter was added to each product during PCR amplification so that each mutant RNA was made directly from a PCR product. Internal snoRNA mutations were made by a modification of the method for 'megaprimer' PCR-based mutagenesis [21]. The mutants were sequenced after the last PCR amplification. Their sequencing ladders were sufficiently clean, so that it was not necessary to clone the final PCR products.

Synthesis of RNA

Uncapped RNAs were synthesized *in vitro* with bacteriophage T7 RNA polymerase, using PCR products as templates. The synthetic (wild-type and mutant) E1 RNAs had the same 3′ end as natural E1 RNA, and two extra G residues at the 5′ terminus. The final concentrations of the four nucleotides were 0.5 mM to synthesize unlabelled RNA. To make ^{32}P-labelled RNA, [α-^{32}P]UTP was added and the final concentration of UTP was 25 μM. Fluorescein-labelled RNA was made using 12 μM fluorescein-UTP and 38 μM UTP. After PAGE, the level of fluorescein incorporation was quantified by scanning with 450-nm excitation light in a Storm 860 scanner (Molecular

Abbreviations used: snoRNA, small nucleolar RNA; RNP, ribonucleoprotein; snoRNP, small nucleolar RNP.

[1] To whom correspondence should be addressed (e-mail eliceiri@slu.edu).

Table 1 RNA sequences of the segments substituted in each E1 RNA mutant

Nucleotide positions correspond to those shown in Figure 2(A). Upper-case letters in the sequences denote substituted nucleotides, lower-case letters are unchanged bases.

Mutant number	Nucleotide positions	RNA sequence
1	2–7	GGUUGC
2	7–13	CACCUuU
3	15–22	GAcUACUC
4	20–22	CUC
5	27–29	GAG
6	35–43	CCGAGACAG
7	47–51	CACCG
8	56–61	CCCUCG
9	67–69	GGA
10	85–87	CAU
11	91–93	GGG
12	97–99	GGU
13	109–114	UgUacU
14	114–117	UUCG
15	153–155	GAC
16	153–158	GACGUA
17	157–164	UAUAAGGAU
18	168–171	GAAG
19	173–176	GUgU
20	196–199	CCUC
21	202–210	UUUGGUACG
22	216–218	UGU

Dynamics, Sunnyvale, CA, U.S.A.). To measure the amount of RNA made, the gel was then stained with SYTO 61 (Molecular Probes, Eugene, OR, U.S.A.) and the gel was scanned with 635-nm excitation light in the same apparatus.

RNA stability *in vivo*

Isolation of *X. laevis* oocytes, nuclear injections (9 or 18 nl per oocyte), and oocyte incubations were as described earlier [22]. ^{32}P-Labelled wild-type or mutant E1 RNA was mixed with ^{32}P-labelled yeast tRNA. Each mixture had Blue Dextran at a final concentration of 20 mg/ml, to verify which injections had reached the nucleus [23]. Some aliquots of each mixture were injected into oocytes and other aliquots were loaded on to electrophoresis gels. Oocytes were incubated for 20 h at 18 °C, and then their nuclei were isolated manually in oil [24]. Only oocytes with blue nuclei were analysed. RNA was extracted from whole oocytes with proteinase K, phenol and chloroform, and was fractionated by 10% PAGE in the presence of 7 M urea. Incorporation of ^{32}P was quantified by PhosphorImaging, using the scanner mentioned above.

Formation of RNA–protein complexes *in vivo*

A mixture of ^{32}P-labelled RNA and Blue Dextran, with and without a 10- or 100-fold molar excess of competitor unlabelled RNA, was injected into the nuclei of frog oocytes. After 4 or 20 h of incubation at 18 °C, nuclei were isolated. Each blue nucleus was mixed with 10 μl of 50 mM Tris/50 mM boric acid/1 mM EDTA, and broken up by pipetting briefly with a pipettor disposable tip. The nuclear contents were kept on ice and were loaded on to electrophoresis gels as quickly as possible. Non-denaturing 5% PAGE was in 50 mM Tris/50 mM boric acid/1 mM EDTA, at 4 °C.

Nucleolar localization of RNA

Fluorescein-labelled, wild-type or mutant E1 RNA (0.2 ng per oocyte), mixed with ^{32}P-labelled, wild-type frog E1 RNA (0.02 ng per oocyte), was injected into the nucleus of oocytes. The nucleus was isolated after 2 h of oocyte incubation. The nuclear contents of each oocyte were dispersed on a glass slide as described in [25]. The amount of ^{32}P in each nuclear-contents spread was quantified by PhosphorImaging to monitor the extent of injection delivery into the nucleus. Only the nuclear spreads with high levels of ^{32}P were analysed further. The nuclear contents were visualized by fluorescence microscopy [26] and phase microscopy.

RESULTS

RNP formation *in vivo*

The presence of endogenous E1 RNA in a small nucleolar RNP (snoRNP) particle *in vivo* has been observed by glycerol-gradient sedimentation [13]. In previous studies, the formation of RNPs

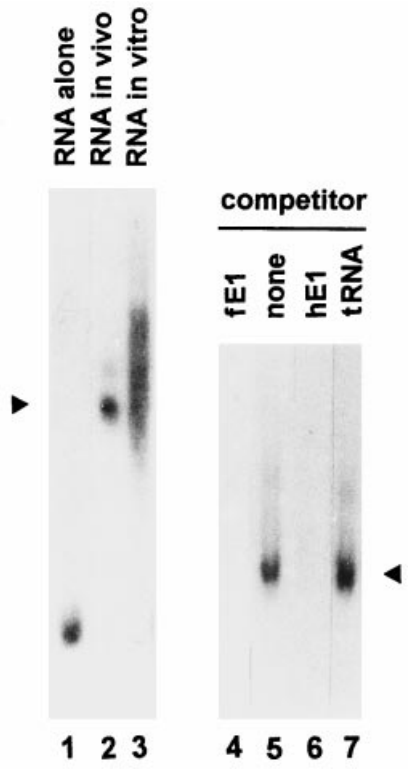

Figure 1 Exogenous E1 RNA assembles into a specific RNP *in vivo*

A mixture of ^{32}P-labelled, wild-type frog E1 RNA and Blue Dextran was injected into the nuclei of frog oocytes, followed by 4 h of oocyte incubation. Nuclei were isolated and, if blue (indicating that the injection had reached the nucleus), their contents were fractionated by non-denaturing PAGE (lane 2). As a control, E1 RNA was loaded directly on to the same gel (lane 1). As another control, E1 RNA was mixed with the contents of a nucleus from a non-injected oocyte (same amount of E1 RNA, nuclear contents and buffer, and buffer composition as in lane 1) and loaded directly on to the gel (lane 3). Frog oocytes were injected into the nucleus with ^{32}P-labelled, frog E1 RNA alone (lane 5), and mixtures of this RNA with a 10-fold excess of unlabelled frog E1 RNA (fE1, lane 4), a 10-fold excess of unlabelled human E1 RNA (hE1, lane 6), and a 100-fold excess of unlabelled tRNA (lane 7). The migration of the RNP formed *in vivo* is indicated by the arrowheads. Lanes 4–7 and 1–3 are from long and short electrophoretic runs, respectively.

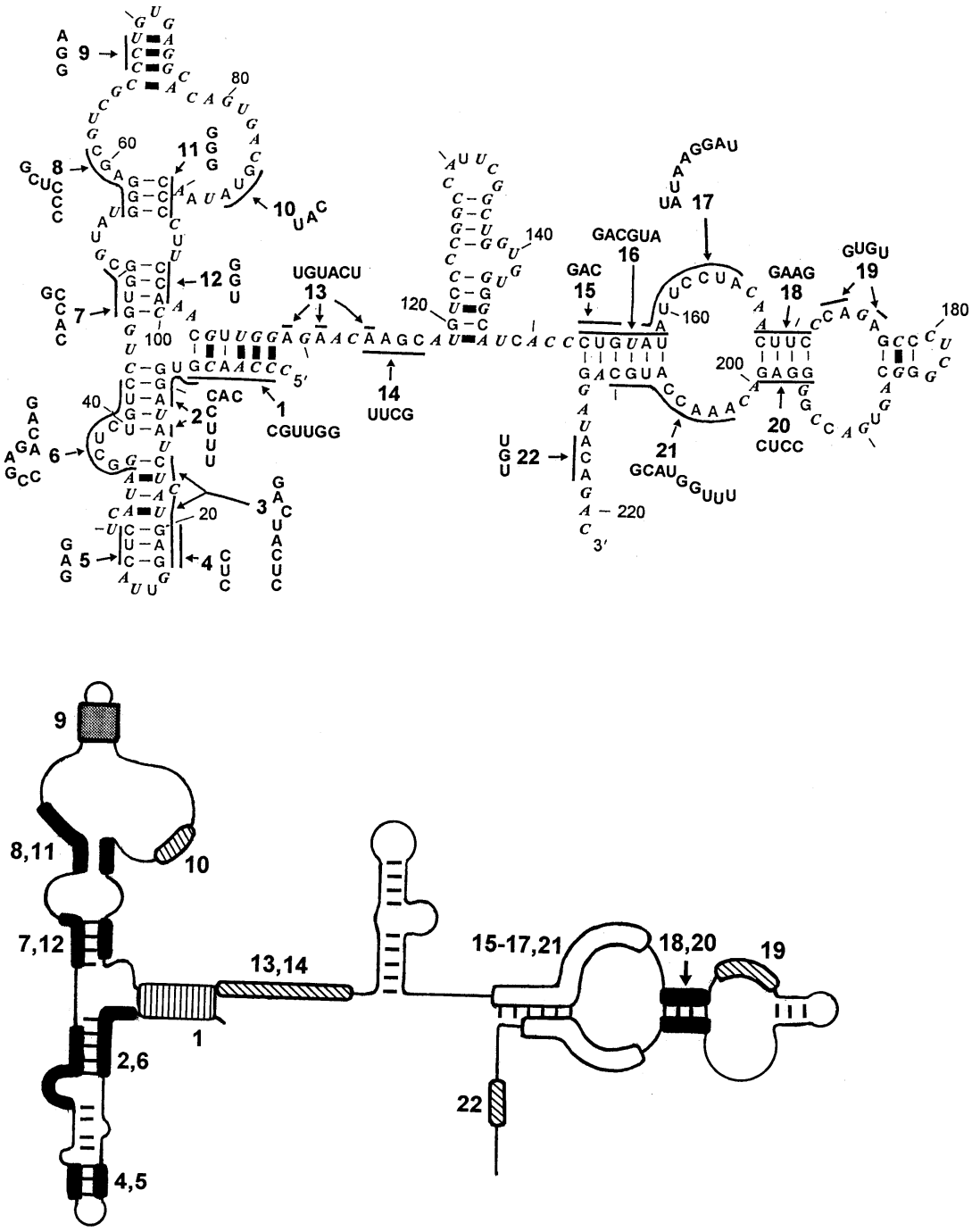

Figure 2 Substitution mutants of frog E1 RNA (top panel) and summary of segments conserved in E1 RNA that are involved in the RNP formation and/or stability *in vivo* of E1 RNA (bottom panel)

(Top panel) Vertebrate E1 RNA consensus sequence and consensus secondary structure are based on comparative sequence phylogeny, including co-variation analysis [20]. Nucleotides conserved in E1 RNA are in standard print. Non-conserved nucleotides of sequence *f* of *X. laevis* E1 RNA [16] are in italics. The 22 mutants were as long as the wild-type sequence. A number both identifies each mutant and the segment substituted in that mutant. The substituted segment of each mutant is indicated, both by a line next to each changed nucleotide and by showing the sequence of the segment substituted in each mutant, next to the wild-type full-length sequence. Sequence co-variations [20] are indicated by black rectangles. (Bottom panel) Conserved segments that are involved in E1 RNP formation and snoRNA stability *in vivo* are apparently (i) sequences in four regions (hatched boxes), (ii) one sequence in each of five regions, plus either the other sequence or the structure in that region (black boxes), (iii) a structure (grey box) and (iv) sequence(s) and/or structure in one region (white boxes). An additional conserved segment whose mutation decreases E1 RNA stability is apparently a structure (box with vertical lines). For simplicity, these segments are labelled with the same numbering as the mutants (see top panel).

was analysed by immunoprecipitation. Immunoprecipitation would fail to detect (i) RNA elements needed for those interactions with proteins that are not required, directly or indirectly, for association of the RNA of interest with the protein targeted by the antibody, and (ii) RNA elements needed only for RNP conformation. In addition, there are no known antibodies that

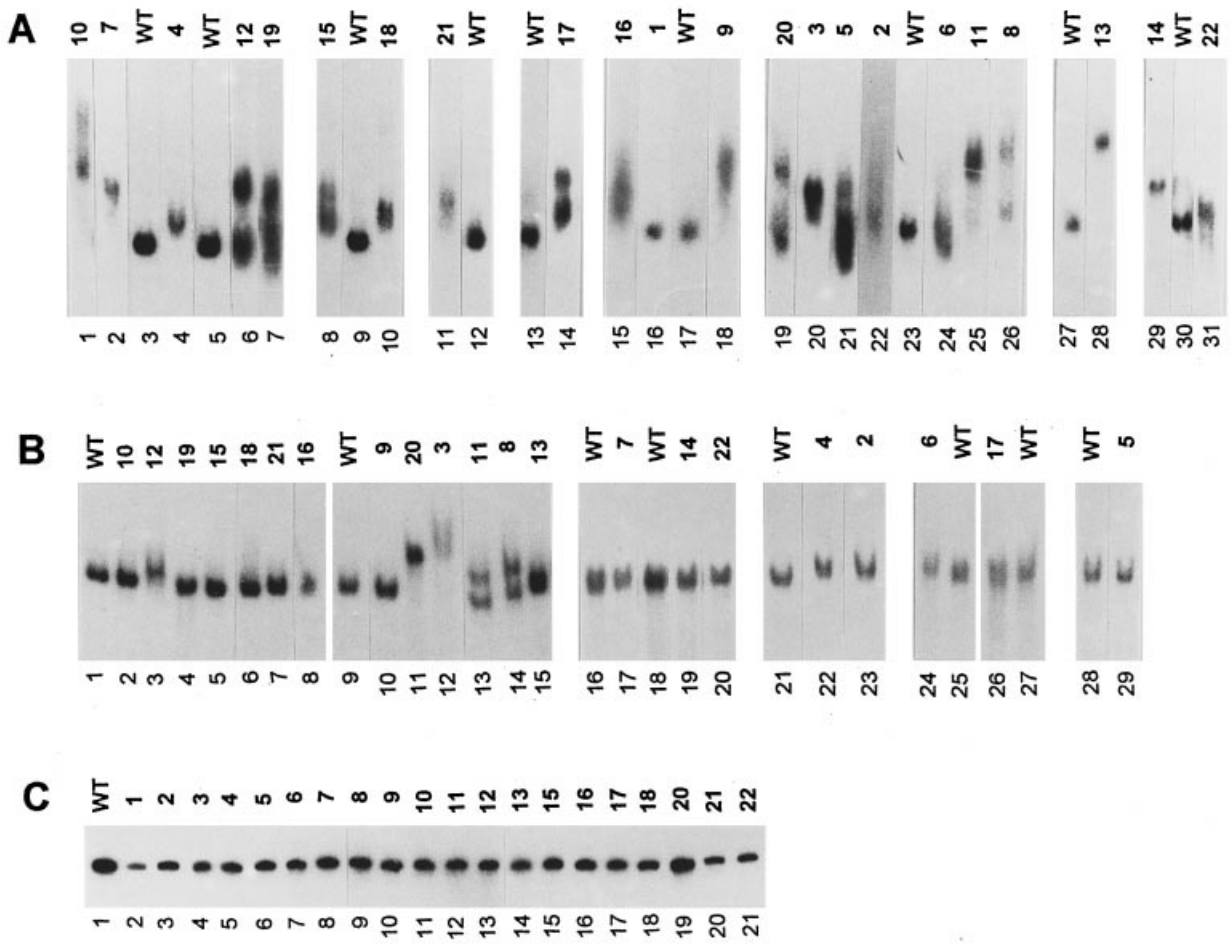

Figure 3 RNP assembly of different E1 RNA mutants *in vivo*

(**A**) ^{32}P-Labelled, wild-type E1 RNA (WT) or mutant E1 RNA (numbered above each lane as in Figure 2, top panel), mixed with Blue Dextran, was injected into the nuclei of frog oocytes that were then incubated for 4 h (lanes 1–7 and 11–28) or 20 h (lanes 8–10 and 29–31). The nucleus of each oocyte was isolated and, if blue, its contents were fractionated by non-denaturing PAGE. Gaps between lanes indicate separate electrophoreses. (**B**) Non-denaturing PAGE of wild-type and mutant E1 RNAs that had been synthesized *in vitro* and deproteinized, without injection into oocytes. (**C**) Same as (**B**), but analysed on denaturing (7 M urea) 10% PAGE.

recognize only the E1 snoRNP. It appeared that the non-denaturing gel electrophoresis typically used for gel mobility-shift analysis would be a more general assay to monitor RNP formation in whole cells. Potentially it could detect (i) the failure of the RNA to associate with any of the protein components of the RNP, and (ii) changes in RNP conformation. We chose to do this assay *in vivo* rather than *in vitro* because E1 RNA function, including snoRNA 'add-back' experiments, can be monitored in whole cells [17] but not in cell-free systems ([27], but see [27a]). We injected uncapped RNAs because natural (endogenous) E1 RNA is not capped and is metabolically long lived, and uncapped, synthetic, wild-type E1 RNA is also long lived *in vivo* (see below). After being injected into frog oocytes, *in vitro*-synthesized, wild-type E1 RNA assembled into an RNP, since its electrophoretic mobility was much lower than that of free E1 RNA (Figure 1, lanes 1 and 2). This particle, made of exogenous RNA and endogenous proteins, is functional, since it reverses a blockage in pre-rRNA processing in E1-depleted oocytes and this reversal is RNA-sequence-specific [17]. In contrast, naked E1 RNA does not reverse blocked pre-rRNA processing *in vitro* ([27], but see [27a]). E1 RNA, mixed with the contents of an isolated oocyte nucleus, generated a smear on gel electrophoresis (Figure 1, lanes 2 and 3), indicating that the RNP assembly of injected E1 RNA requires incubation *in vivo*. This smear could not be caused by testing overly concentrated RNA because that sample had the same RNA concentration as the samples in the other lanes, and the smear was reproducible. Exogenous E1 RNA assembles into an RNA-sequence-specific RNP particle, since this assembly was blocked by an excess of frog E1 RNA, but not tRNA (Figure 1, lanes 4, 5 and 7). For higher resolution, these were long electrophoretic runs in which free E1 RNA ran off the gel. However, these results represent competition because they were very reproducible. This RNP assembly depends on conserved segments of E1 RNA, since human E1 RNA competed with frog E1 RNA (Figure 1, lanes 5 and 6). This suggests that at least some of the proteins that bind E1 RNA specifically do so by recognizing conserved segments of this snoRNA. The high mobility of this RNP particle in 5% PAGE indicates that this complex is small, as expected from the sedimentation rate of the endogenous E1 snoRNP [13]. This RNP is nucleolar, since the injected E1 RNA localized in nucleoli (see below).

Evolutionarily conserved E1 RNA sequences and secondary structures were identified before, based on comparative sequence phylogeny, including co-variation analysis [20]. To identify the

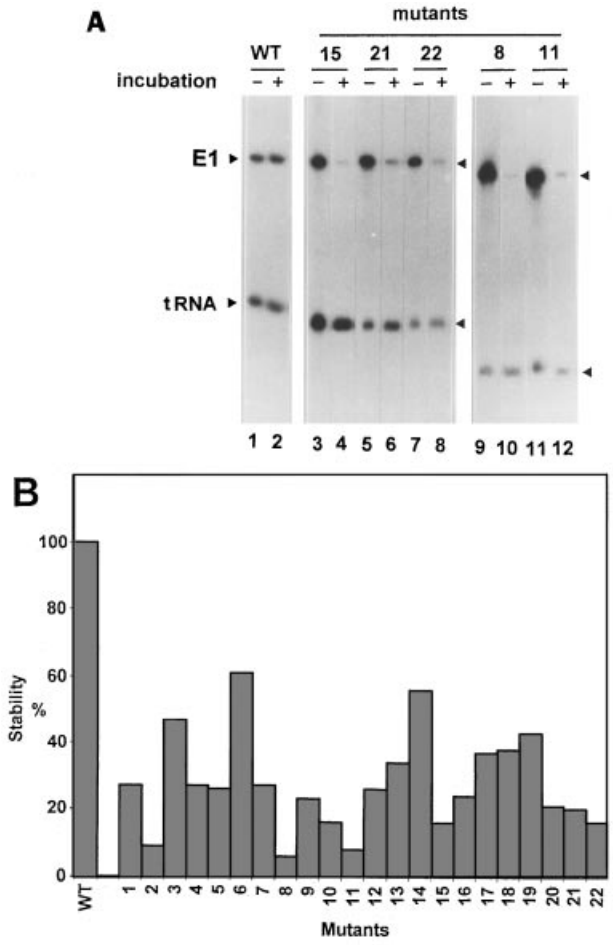

Figure 4 Stability of E1 RNA mutants *in vivo*

(**A**) Stability of some E1 RNA mutants, relative to tRNA. A mixture of ^{32}P-labelled tRNA, Blue Dextran and ^{32}P-labelled wild-type (WT) or mutant (numbered as in Figure 2, top panel) E1 RNA was injected into the nuclei of oocytes that were then incubated for 20 h. Only oocytes whose nuclei were blue (indicating a successful nuclear injection) were harvested. RNA extracted from whole oocytes was analysed by denaturing 10% PAGE, and then exposed to X-ray film. RNA mixtures before injection (—) and after oocyte incubation (+) are shown. Gaps between lanes indicate separate electrophoreses. (**B**) Stability of E1 RNA mutants, relative to (tRNA and then to) wild-type E1 RNA. After oocyte injection and incubation, and gel electrophoresis as in (**A**), the radioactive bands were quantified by PhosphorImaging. In each sample, the amount of radiolabelled E1 RNA was normalized by the level of radioactive tRNA. Then, the remaining amount of each mutant E1 RNA was corrected in reference to the remaining level of wild-type E1 RNA (WT).

E1 RNA segments involved in RNP formation, we made a series of base-substitution mutants of frog E1 RNA, in phylogenetically conserved sequences and secondary structures (Figure 2, top panel). Surprisingly, base substitutions in many conserved segments of E1 RNA resulted in abnormal RNP PAGE patterns (Figure 3A). The RNP smears were not caused by snoRNA degradation, since we verified that each of the E1 RNA mutants still migrated electrophoretically as a narrow, single RNA band after these oocyte incubations (results not shown). (A number both identifies each mutant and the segment substituted in that mutant.) The PAGE pattern differences between each mutant E1 RNA and wild-type E1 RNA, including the RNP smears, could not be caused by differences in the amount of E1 RNA injected per oocyte, or by differences in the amount of nuclear contents loaded per gel lane, because of the following. First, we injected the same amount of mutant E1 RNA and wild-type E1 RNA per oocyte, and monitored delivery into the nucleus by co-injecting Blue Dextran with each sample. Secondly, the PAGE pattern differences between each E1 RNA mutant and wild-type E1 RNA, including the RNP smears, were seen in multiple oocytes that were analysed individually. Thirdly, we saw the same PAGE patterns when loading either most of the nuclear contents of one oocyte per gel lane or one-tenth or less than that amount from the same oocyte per gel lane. In the examples shown in Figure 3(A), four mutants and their wild-type controls are shown after 20 h of post-injection oocyte incubation, and the other mutants and their wild-type controls are shown after 4 h of oocyte incubation. The differences in PAGE mobility between the RNP of each mutant snoRNA and the RNP of the wild-type snoRNA were seen both after 4 h and after 20 h of post-injection oocyte incubation. The differences in signal intensity among gel lanes do not necessarily represent recovery variations. Instead, these differences were caused (i) by loading on gels the contents of various percentages of one oocyte nucleus, to have wild-type controls in many sections of some gels, and (ii) by showing autoradiographs from different times of X-ray film exposure, to make the differences in electrophoretic migration patterns as visible as possible.

Deproteinized molecules of most mutant E1 RNAs migrated with or very close to wild-type, naked E1 RNA in non-denaturing PAGE; the exceptions were mutants 3, 8, 11 and 20 (Figure 3B). However, in these gels, the mobilities of deproteinized molecules of mutants 8, 11 and 20 relative to wild-type snoRNA (Figure 3B) were substantially different from the relative migrations of the corresponding RNPs (Figure 3A). Therefore, for 20 of these mutants the abnormal RNP PAGE patterns do not simply reflect a corresponding difference in migration of naked RNA. We cannot draw conclusions about mutant 3, since its mobility relative to wild-type was similar in RNPs (Figure 3A) and isolated RNA (Figure 3B). Deproteinized molecules of all these mutant snoRNAs co-migrated, as single bands, with wild-type E1 RNA in denaturing PAGE (Figure 3C). Only mutants 8 and 11 showed double bands of naked E1 RNA in non-denaturing PAGE (Figure 3B). Interestingly, segments 8 and 11 are on opposite strands of the same double helix in the phylogeny-based model of E1 RNA secondary structure (Figure 2, top panel) [20]. These results suggest that this double helix exists.

With regard to segments 10, 13, 14, 19 and 22, (i) they are evolutionarily conserved sequences in E1 RNA, (ii) they are in single-stranded regions in the phylogeny-based model of E1 RNA secondary structure (Figure 2, top panel) [20], and (iii) substitution of their nucleotides produced abnormal RNP patterns (Figure 3A). This suggests that, in these segments, their sequences are needed for E1 RNP formation. Segments 7 and 12 (i) are evolutionarily conserved sequences in E1 RNA, (ii) are on opposite strands of the same double helix in the phylogeny-based model of E1 RNA secondary structure (Figure 2, top panel) [20], and (iii) substitution of their bases generated abnormal RNP PAGE patterns that differed from each other (Figure 3A). If this double helix were the only requirement from this region for E1 RNP formation, mutants 7 and 12 would be expected to have the same RNP PAGE pattern. Therefore, these results suggest that E1 RNP formation requires both (i) the sequence of one of these two segments; and (ii) either the sequence of the other segment or this double helix. These observations, and therefore these conclusions, about the pair of segments 7 and 12 also apply to the four pairs of evolutionarily conserved segments 2 and 6, 4 and 5, 8 and 11, and 18 and 20 (Figures 2, top panel, and 3A).

Mutant 9 has an abnormal RNP PAGE pattern (Figure 3A). In segment 9, RNA structure appears to be involved in E1 RNP

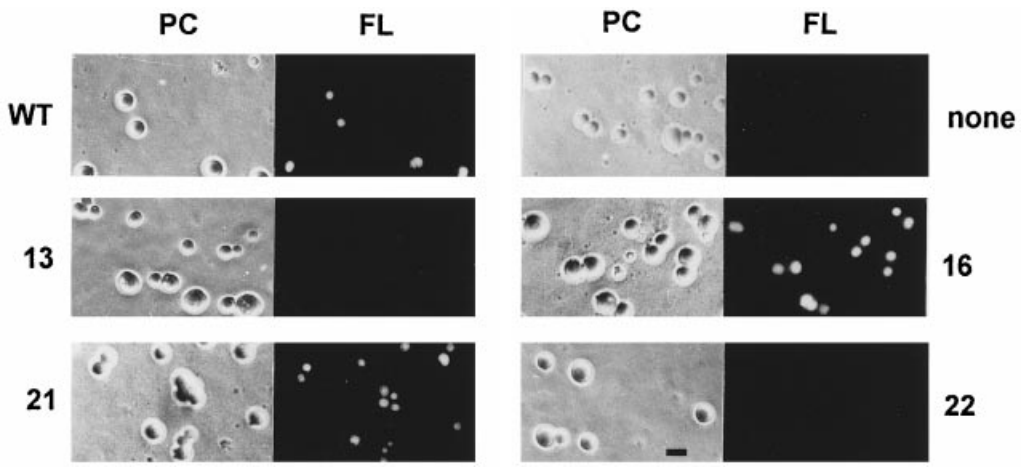

Figure 5 Nucleolar localization of exogenous wild-type and mutant E1 RNAs

Frog oocytes were injected into the nucleus with mixtures of ^{32}P-labelled, wild-type E1 RNA and a fluorescein-labelled RNA. In the examples shown, the fluorescein-labelled RNA was wild-type frog E1 RNA (WT), and E1 mutants 13, 16, 21 and 22. Some oocytes were not injected (none). After 2 h of oocyte incubation, nuclei were isolated and their contents were spread on glass slides. The level of ^{32}P in the nuclear contents (extent of nuclear injection) was quantified by PhosphorImaging. The nuclear contents were then photographed under phase microscopy (PC) and fluorescence microscopy (FL). Scale bar, 10 μm.

formation, because (i) segment 9 has been shown to be in a double helix, since this section has had multiple base co-variations during evolution; and (ii) this segment does not have any conserved nucleotides (Figure 2, top panel) [20]. Segments 15–17 are base-paired to segment 21 in the phylogeny-based model of E1 RNA secondary structure (Figure 2, top panel) [20]. Mutants 15–17 and 21 each resulted in similarly abnormal RNP PAGE patterns (Figure 3A). Therefore, RNA sequence(s) and/or structure may be involved in these phenotypes. It might not be only RNA structure, since the RNP PAGE patterns of mutants 15–17 and 21 did not appear identical (Figure 3A). When mutated, not all conserved segments generated abnormal RNP PAGE patterns. For example, substitution of segment 1 produced an RNP that co-migrated electrophoretically with the RNP of wild-type E1 RNA (Figure 3A).

snoRNA stability *in vivo*

Metabolic stability is an important aspect of the life cycle of any RNA molecule. A few elements needed for snoRNA stability *in vivo* have been identified in box C/D snoRNAs [18,26,28–31]. Information about E1 RNA segments involved in snoRNA stability *in vivo* was also of interest both to correlate it with the preceding results on E1 RNP formation and to plan the search for E1 RNA nucleolar-localization elements (see below). The effect of the mutations shown in Figure 2 (top panel) on the metabolic stability of E1 RNA was tested next. We injected RNAs that were not capped because uncapped, *in vitro*-synthesized, wild-type E1 RNA is long lived *in vivo* (Figure 4A) and natural E1 RNA is not capped. A mixture of radioactive tRNA and wild-type or mutant E1 RNA was injected into oocytes, which were then incubated for 20 h. Base substitutions in all the regions of E1 RNA that produced abnormal RNP PAGE patterns also decreased the stability of this snoRNA *in vivo* (Figure 4B). RNA sequence is apparently involved in segment 2, since (i) mutant 6 was substantially more stable (Figure 4B), and segments 2 and 6 are on opposite strands of the same double helix in the phylogeny-based model of E1 RNA secondary structure and (ii) the six nucleotides in segment 2 are evolutionarily conserved in E1 RNA (Figure 2, top panel) [20]. In addition, mutant 1 was unstable in oocytes (Figure 4B), although its RNP PAGE pattern was apparently normal. In segment 1, RNA structure is apparently involved, because (i) at least four out of the six nucleotides in segment 1 are not evolutionarily conserved in E1 RNA, and (ii) segment 1 has been shown to be in a double helix, since this section has had multiple base co-variations during evolution (Figure 2, top panel) [20]. The instability of the mutants was not caused specifically by lacking a 5′ end cap, since the injected wild-type E1 RNA was also uncapped but was long lived *in vivo*.

snoRNA nucleolar localization

snoRNAs require nucleolar-localization elements, when nascent, to be transported from the nucleoplasm to the nucleolus, and when mature, to remain in the nucleolus between mitoses. The nucleolar-localization elements of box C/D snoRNAs have been identified [26,30–33]. For cellular-localization experiments, oocytes were incubated for 2 h after injection, since the levels of many of these mutants dropped appreciably after 20 h of oocyte incubation (Figure 4B). Fluorescein-labelled, wild-type E1 RNA injected into the nucleus migrated to nucleoli; the intensity of this signal can be compared with non-injected oocytes (Figure 5). Oocytes injected with fluorescein-labelled tRNA gave low background fluorescence images similar to those of non-injected oocytes (results not shown). The prominent structures in Figure 5 are the large nucleoli of *Xenopus laevis* oocytes, since the nuclei of these cells do not have any other structures of this size, morphology and abundance [34].

Mutants 13 and 22 (substituted in boxes H and ACA, respectively) localized poorly to nucleoli (Figure 5). These results were not due to instability of these mutants. After 2 h of oocyte incubation, in stability-quantification experiments similar to those in Figure 4(B), we measured similar stabilities in mutants 13 and 22 and in mutants that localized efficiently in the nucleolus (results not shown). Mutants 16 and 21 are shown as examples

of those positive controls (Figure 5). These data were not caused by low injection delivery into the nucleus, since each sample was co-injected with ^{32}P-labelled, wild-type E1 RNA, and nuclear delivery was quantified by PhosphorImaging.

DISCUSSION

Unexpectedly, we have identified 11 evolutionarily conserved regions in U17 RNA that are needed for U17 RNP formation *in vivo*. Similar results have not been reported in other H/ACA snoRNPs, any other snoRNPs, or apparently any other RNP. Figure 2 (bottom panel) summarizes the conserved sequences and structures of E1 RNA whose base substitution interfered with the RNP formation and/or metabolic stability of this snoRNA. At least some of the abnormal RNP PAGE patterns are expected to be caused by the failure of proteins to bind E1 RNA, which in some cases might be coupled to changes in RNP shape. Others might result instead from an alteration in the conformation of the RNP, preserving the number of bound polypeptides. Among the regions of E1 RNA that affected RNP formation, ten were detected by substitution of only three nucleotides each. The location of several of these three-base substitutions in E1 RNA suggests that they would not have major global effects on the conformation of E1 RNA. For example, three-base substitutions in four single-stranded segments (numbers 10, 13, 19 and 22) generated four different abnormal RNP PAGE patterns. Indeed, most of the 22 mutations had no detectable effect on the conformation of naked E1 RNA (Figure 3B). Many of the abnormal RNP PAGE patterns differed from each other, implying diverse changes in the RNP. The different RNP PAGE patterns generated by base substitutions in various conserved E1 RNA segments suggest that, in many E1 RNA elements, each element plays a different role in RNP formation. These different PAGE patterns, and the separation of these conserved E1 RNA regions by evolutionarily non-conserved sections, suggest that many of these segments are separate elements.

Most of the abnormal E1 RNP PAGE patterns were two bands or a smear (Figure 3A). This did not reflect an *in vivo*-induced change in the RNA, since after oocyte incubation each mutant E1 RNA, deproteinized, co-migrated with wild-type E1 RNA as a single, narrow band in denaturing PAGE (results not shown). In some of these cases, failure to bind a protein might generate two or more forms of the RNP. Except for the H and ACA boxes, the other snoRNA segments needed for E1 RNP formation and/or snoRNA metabolic stability are specific for E1 RNA, rather than generic for the box H/ACA snoRNA family [4,20]. It is likely that disruptions of RNP formation at various E1 RNA segments may then decrease E1 RNA stability *in vivo*, since both were abnormal after mutating each of 11 regions of this snoRNA. In contrast, segment 1 is involved in snoRNA stability but apparently is not essential for E1 RNP formation.

The H and ACA boxes, and the proximal stem structures in the 5′ and 3′ hairpins, are essential for accumulation of some H/ACA snoRNAs (yeast snR5, snR11 and snR36, and human U64) in experiments that could not distinguish RNA biosynthesis from RNA stability [8,9,35]. Our results indicate that RNA stability is affected in those segments, since the following frog E1 RNA mutants were very unstable: 13 (substituted in box H); 22 (changed in box ACA); 1 (mutated in the 5′-terminal stem); and 21 (altered in the 3′-terminal stem). These are among the most unstable E1 RNA mutants in frog oocytes (Figure 4). We also detected segments of the snoRNA involved in RNA stability that had not been detected before. There are several differences between the work in [8,9,35] and ours. Those studies (i) did not mutate some of the snoRNA segments that we mutated, (ii) did not monitor RNA stability itself, but assayed for RNA accumulation, the sum of RNA biosynthesis and RNA stability (then, a role of a given RNA segment in RNA biosynthesis might mask an additional participation of the same RNA region in RNA stability), (iii) focused primarily on yeast snoRNAs, and there are differences between yeast RNAs and their vertebrate orthologues and (iv) dealt with H/ACA snoRNA species other than E1 RNA, and there are differences among various members of a given snoRNA family [26,28,30,31,33].

Many snoRNA segments are involved in the stability of E1 RNA *in vivo* (this study), but few snoRNA stability elements have been reported for box C/D snoRNAs [18,26,28–31]. Another factor for this apparent difference might be various differences in experimental design in each study. We (i) incubated oocytes for 20 h after snoRNA injection, which makes it easier to detect decreased RNA stability, (ii) injected all snoRNA mutants into the nucleus (snoRNAs tend to migrate poorly from cytoplasm to nucleus; they might have abnormal stabilities in their non-physiological subcellular compartment), (iii) co-injected each E1 RNA mutant with a long-lived, heterologous RNA and (iv) mutated every evolutionarily conserved sequence and structure of the snoRNA.

While this manuscript was being revised, it was reported that (i) the nucleolar localization of human U67 and E1 H/ACA snoRNAs was fully eliminated when either only box H or only box ACA was mutated [36], (ii) in apparent contrast, the nucleolar localization of frog E1 RNA decreased partially when either only box H or only box ACA was mutated, and that it was completely abolished when both boxes were mutated simultaneously [37], (iii) disruption of the base of the 3′ hairpin decreased the nucleolar localization of human U65 H/ACA snoRNA [36] and (iv) box H and box ACA are elements for frog E1 RNA stability *in vivo* [37]. In the present work, substitution of either box H alone or box ACA alone resulted in a marked decrease in nucleolar localization of frog E1 RNA. We did not detect less nucleolar localization of frog E1 RNA after disruption of the base of the 3′ hairpin (mutants 16 and 21, Figure 5). We found that mutation of many additional individual conserved segments of frog E1 RNA lowered its stability *in vivo*. Some of the apparent differences in results might be related to the fact that the E1 RNA molecules tested were not capped in the present work and in [36], but were 5′-end-capped in [37] (natural E1 RNA is not capped). For example, a 5′-end-cap-binding protein (for other RNA species) might increase the stability of wild-type and mutant E1 RNAs, and might make it difficult to detect some snoRNA stability elements. There are also apparent differences in the nucleolar-localization element requirements of various snoRNA species that are members of the same snoRNA family [26,28,30,31,33].

In other small RNAs, very few *cis*-acting elements are known to be required for RNP assembly or RNA metabolic stability. The present study shows that for E1 RNA, the formation of the RNP and the stability of the RNA *in vivo* depend on a much larger number of phylogenetically conserved snoRNA segments. In view of this surprising complexity, it will be interesting to identify the proteins whose specific interactions with these snoRNA elements are necessary for E1 RNP formation.

We thank Rani Tanam and Jennifer Wu for preparing some of the mutants, Francesco Amaldi and Glauco Tocchini-Valentini for DNA clones, James W. Brown for the folded RNA structure, Sascha Lange, Wayne Speckmann and Joseph G. Gall for advice on methods, and Jan Ryerse, Clifford Bellone and Peter Zassenhaus for the use of equipment. We also thank Joyce Weber for photography and Anne Chau for assistance. This work was supported by grant R01 GM56301 from the National Institute of General Medical Sciences.

REFERENCES

1 Maxwell, E. S. and Fournier, M. J. (1995) The small nucleolar RNAs. Annu. Rev. Biochem. **35**, 897–934
2 Tollervey, D. and Kiss, T. (1997) Function and synthesis of small nucleolar RNAs. Curr. Opin. Cell Biol. **9**, 337–342
3 Weinstein, L. B. and Steitz, J. A. (1999) Guided tours: from precursor snoRNA to functional snoRNP. Curr. Opin. Cell Biol. **11**, 378–384
4 Eliceiri, G. L. (1999) Small nucleolar RNAs. Cell. Mol. Life Sci. **56**, 22–31
5 Tyc, K. and Steitz, J. A. (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleus. EMBO J. **8**, 3113–3119
6 Kiss-László, Z., Henry, Y., Bachellerie, J.-P., Caizergues-Ferrer, M. and Kiss, T. (1996) Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell **85**, 1077–1088
7 Tycowski, K. T., Smith, C. M., Shu, M.-D. and Steitz, J. A. (1996) A small nucleolar RNA requirement for site-specific ribose methylation of rRNA in *Xenopus*. Proc. Natl. Acad. Sci. U.S.A. **93**, 14480–14485
8 Balakin, A. G., Smith, L. and Fournier, M. J. (1996) The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions. Cell **86**, 823–834
9 Ganot, P., Caizergues-Ferrer, M. and Kiss, T. (1997) The family of Box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation. Genes Dev. **11**, 941–956
10 Ni, J., Tien, A. L. and Fournier, M. J.. (1997) Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. Cell **89**, 565–573
11 Ganot, P., Bortolin, M.-L. and Kiss, T. (1997) Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell **89**, 799–809
12 Ruff, E. A., Rimoldi, O. J., Raghu, B. and Eliceiri, G. L. (1993) Three small nucleolar RNAs of unique nucleotide sequences. Proc. Natl. Acad. Sci. U.S.A. **90**, 635–638
13 Rimoldi, O. J., Raghu, B., Nag, M. K. and Eliceiri, G. L. (1993) Three new small nucleolar RNAs that are psoralen crosslinked *in vivo* to unique regions of pre-rRNA. Mol. Cell. Biol. **13**, 4382–4390
14 Kiss, T. and Filipowicz, W. (1993) Small nucleolar RNAs encoded by introns of the human cell cycle regulatory gene RCC1. EMBO J. **12**, 2913–2920
15 Nag, M. K., Thai, T., Ruff, E. A., Selvamurugan, N., Kunnimalaiyaan, M. and Eliceiri, G. L. (1993) Genes for E1, E2 and E3 small nucleolar RNAs. Proc. Natl. Acad. Sci. U.S.A. **90**, 9001–9005
16 Cecconi, F., Mariottini, P., Loreni, F., Pierandrei-Amaldi, P., Campioni, N. and Amaldi, F. (1994) $U17^{XS8}$, a small nucleolar RNA with a 12 nt complementarity to 18S rRNA and coded by a sequence repeated in the six introns of *Xenopus laevis* ribosomal protein S8 gene. Nucleic Acids Res. **22**, 732–741
17 Mishra, R. K. and Eliceiri, G. L. (1997) Three small nucleolar RNAs that are involved in ribosomal RNA precursor processing. Proc. Natl. Acad. Sci. U.S.A. **94**, 4972–4977
18 Peculis, B. A. and Steitz, J. A. (1994) Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved. Genes Dev. **8**, 2241–2255
19 Watkins, N. J., Newman, D. R., Kuhn, J. F. and Maxwell, E. S. (1998) *In vitro* assembly of the mouse U14 snoRNP core complex and identification of a 65-kDa box C/D-binding protein. RNA **4**, 582–593
20 Selvamurugan, N., Joost, O. H., Haas, E. S., Brown, J. W., Galvin, N. J. and Eliceiri, G. L. (1997) Intracellular localization and unique conserved sequences of three small nucleolar RNAs. Nucleic Acids Res. **25**, 1591–1596
21 Datta, A. K. (1995) Efficient amplification using 'megaprimer' by asymmetric polymerase chain reaction. Nucleic Acids Res. **23**, 4530–4531
22 Eliceiri, G. L. (1999) Reversible depletion of specific RNAs by antisense oligodeoxynucleotide-targeted degradation in frog oocytes. Methods Enzymol. **313**, 436–442
23 Jarmolowski, A., Boelens, W. C., Izaurralde, E. and Mattaj, I. W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. J. Cell Biol. **124**, 627–635
24 Lund, E. and Paine, P. L. (1990) Nonaqueous isolation of transcriptionally active nuclei from *Xenopus* oocytes. Methods Enzymol. **181**, 36–43
25 Gall, J. G., Murphy, C., Callan, H. G. and Wu, Z. (1991) Lampbrush chromosomes. Methods Cell Biol. **36**, 149–166
26 Lange, T. S., Borovjagin, A., Maxwell, E. S. and Gerbi, S. A. (1998) Conserved boxes C and D are essential nucleolar localization elements of U14 and U8 snoRNAs. EMBO J. **17**, 3176–3187
27 Enright, C. A., Maxwell, E. S., Eliceiri, G. L. and Sollner-Webb, B. (1996) 5 ETS rRNA processing facilitated by four small RNAs: U14, E3, U17 and U3. RNA **2**, 1094–1099
27a Erratum (1996) RNA **2**, 1318
28 Terns, M. P., Grimm, C., Lund, E. and Dahlberg, J. E. (1995) A common maturation pathway for small nucleolar RNAs. EMBO J. **14**, 4860–4871
29 Caffarelli, E., Fatica, A., Prislei, S., De Gregorio, E., Fragapane, P. and Bozzoni, I. (1996) Processing of the intron-encoded U16 and U18 snoRNAs: the conserved C and D boxes control both the processing reaction and stability of the mature snoRNA. EMBO J. **15**, 1121–1131
30 Lange, T. S., Borovjagin, A. and Gerbi, S. A. (1998) Nucleolar localization elements in U8 snoRNA differ from sequences required for rRNA processing. RNA **4**, 789–800
31 Lange, T. S., Ezrokhi, M., Borovjagin, A. V., Rivera-León, R., North, M. T. and Gerbi, S. A. (1998) Nucleolar localization elements of *Xenopus laevis* U3 small nucleolar RNA. Mol. Biol. Cell **9**, 2973–2985
32 Samarsky, D. A., Fournier, M. J., Singer, R. H. and Bertrand, E. (1998) The snoRNA box C/D motif directs nucleolar targeting and also couples snoRNA synthesis and localization. EMBO J. **17**, 3747–3757
33 Narayanan, A., Speckmann, W., Terns, R. and Terns, M. P. (1999) Role of the C/D motif in localization of small nucleolar RNAs to coiled bodies and nucleoli. Mol. Biol. Cell **10**, 2131–2147
34 Gall, J. G., Tsvetkov, A., Wu, Z. and Murphy, C. (1995) Is the sphere organelle/coiled body a universal nuclear component? Dev. Genet. **16**, 25–35
35 Bortolin, M.-L., Ganot, P. and Kiss, T. (1999) Elements essential for accumulation and function of small nucleolar RNAs directing site-specific pseudouridylation of ribosomal RNAs. EMBO J. **18**, 457–469
36 Narayanan, A., Lukowiak, A., Jády, B. E., Dragon, F., Kiss, T., Terns, R. M. and Terns, M. P. (1999) Nucleolar localization signals of Box H/ACA small nucleolar RNAs. EMBO J. **18**, 5120–5130
37 Lange, T. S., Ezrokhi, M., Amaldi, F. and Gerbi, S. A. (1999) Box H and box ACA are nucleolar localization elements of U17 small nuclear RNA. Mol. Biol. Cell **10**, 3877–3890

Received 12 January 2000/28 March 2000; accepted 12 April 2000