A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5S rRNA precursors

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The Ro autoantigen is a 60-kD protein that is usually found in small cytoplasmic RNA-protein complexes known as Ro RNPs. Although the Ro RNPs are abundant and conserved components of a variety of vertebrate and invertebrate cells, their function is unknown. We have discovered that the Ro protein is also found complexed with certain variant 5S rRNAs in *Xenopus* oocytes. These RNAs contain one or more point mutations compared with the major oocyte 5S rRNA sequence as well as additional nucleotides at the 3' end. We demonstrate that the Ro protein binds specifically mutant 5S rRNAs containing 3' terminal extensions. These mutant RNAs are processed inefficiently to mature 5S rRNA and most eventually are degraded. The observation that the Ro autoantigen specifically associates with defective 5S rRNA precursors suggests that this protein may function as part of a novel quality control or discard pathway for 5S rRNA production.

[Key Words: Autoantigen; discard pathway; *Xenopus* oocytes; 5S rRNA processing]

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Patients suffering from systemic rheumatic diseases, such as polymyositis, systemic lupus erythematosus, and Sjögren's syndrome, often develop antibodies against highly conserved cellular components. Many of the autoantibodies recognize ribonucleoprotein particles (RNPs) and have been valuable probes for elucidating the function of these particles. The cellular RNPs recognized by these autoantibodies include the spliceosomal U snRNPs, ribosomes, and tRNA-synthetase complexes (for review, see Tan 1989; Chan and Andrade 1992).

In Sjögren's syndrome, a rheumatic disorder characterized by lymphocytic infiltration of the tear and salivary glands, the predominant autoantigens are the La and Ro RNPs. The La antigen is a 50-kD nuclear protein that binds to newly synthesized RNA polymerase III transcripts (Rinke and Steitz 1982; Chambers et al. 1988). Experiments using mammalian transcription extracts have led to the proposal that the La protein functions in the termination of transcription by RNA polymerase III (Gottlieb and Steitz 1989; Maraia et al. 1994). The La protein is highly conserved, as would be expected for a protein that plays a fundamental role in cellular metabolism. La protein homologs have been identified in a number of eukaryotes, including amphibians, flies, and yeast (Scherly et al. 1993; Yoo and Wolin 1994).

Ro RNPs consist of a 60-kD protein bound to one of

several small cytoplasmic RNA molecules known as Y RNAs (Wolin and Steitz 1984). Although the Ro RNPs are largely cytoplasmic, a fraction of the 60-kD Ro protein is also found in the nucleus (O'Brien et al. 1993; Peek et al. 1993; Kelekar et al. 1994). The 60-kD Ro protein is evolutionarily conserved, as it has been characterized in mammals (Deutscher et al. 1988), amphibians (O'Brien et al. 1993), and nematodes (D. Eisenberg, D. Van Horn, C. O'Brien, and S. Wolin, unpubl.). Both the Ro protein and the La protein are members of a family of RNA-binding proteins that contain an ~80-amino-acid sequence known as the RNA recognition motif (RRM) (Birney et al. 1993). A portion of the Ro RNPs in human cells may also contain a 52-kD protein, although this is controversial (Ben-Chetrit et al. 1988; Kelekar et al. 1994).

The number of distinct Y RNAs associated with the 60-kD Ro protein varies between different species. Human cells contain four discrete Y RNAs designated hY1, hY3, hY4, and hY5 (hY2 is a truncated version of hY1). However, many other vertebrate species contain only two or three distinct Y RNAs (Hendrick et al. 1981; Reddy et al. 1983; Mamula et al. 1989). Thus far, only the Y RNAs from human and *Xenopus laevis* cells have been sequenced (Kato et al. 1982; Wolin and Steitz 1983; O'Brien and Harley 1990; O'Brien et al. 1993). These RNAs range in size from 69- to 112-nucleotides and are transcribed by RNA polymerase III. All the sequenced Y RNAs can be drawn as structures containing a large internal loop and a long stem in which the 5' and 3' ends

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are base-paired (O'Brien et al. 1993). Ribonuclease protection experiments have revealed that the 60-kD protein binds to a conserved helix within the stem (Wolin and Steitz 1984). Each Ro RNP is present in $\sim 10^5$ copies per mammalian cell, which is $\sim 1\%$ the number of ribosomes. Despite their abundance and evolutionary conservation, the function of the Ro RNPs has long been elusive.

During our characterization of Ro RNPs in *X. laevis*, we found an unexpected small RNA in our anti-Ro immunoprecipitates from oocytes. We have analyzed this additional species and find that it consists of a population of variant 5S ribosomal RNA molecules. These 5S rRNAs contain additional nucleotides at the 3' end, consistent with the failure of RNA polymerase III to terminate at the first transcription termination signal. In addition, all these rRNAs contain one or more point mutations compared to the major oocyte 5S rRNA sequence. By microinjection of mutant and wild-type genes into oocytes, we demonstrate that the 60-kD Ro protein binds specifically mutant 5S rRNAs containing 3' terminal extensions. By following the fate of the newly synthesized mutant 5S rRNA precursors, we show that the mutant RNAs are processed inefficiently to mature 5S rRNA and eventually most are degraded. These results suggest that the 60-kD Ro autoantigen may function as part of a novel quality control or discard pathway for 5S rRNA production.

Results

Anti-Ro immunoprecipitates from ovary extracts contain an additional RNA

We showed previously that *Xenopus* tissue culture cells contained four distinct Y RNAs, which we named xY3, xY4, xY5, and xY α (O'Brien et al. 1993; also see Fig. 1, lane 2). As part of our analysis of *Xenopus* Ro RNPs, we examined the distribution of Y RNAs in germ cells by performing immunoprecipitations from ovary extracts. RNAs contained within the immunoprecipitates were visualized by labeling with [32 P]pCp (Fig. 1, lanes 3–8). Surprisingly, anti-Ro immunoprecipitates from ovary extracts contained, in addition to the four *Xenopus* Y RNAs, an RNA that migrated slightly slower than 5S rRNA (lanes 4,5, arrow). This RNA was not present in immunoprecipitates using a nonimmune patient serum (lane 3). Nine different patient anti-Ro sera that immunoprecipitated the *Xenopus* Y RNAs also immunoprecipitated the extra RNA (data not shown). This RNA was also present when a rabbit anti-bovine Ro serum was used for immunoprecipitation (lane 4). Because the efficiency of labeling RNAs with [32 P]pCp can vary, the immunoprecipitated RNAs were also examined by silver staining (lanes 9–14). This confirmed that the ovary-specific RNA was present in the anti-Ro immunoprecipitates at approximately the same level as the *Xenopus* Y RNAs characterized previously (lanes 10,11).

As these immunoprecipitates were performed on ex-

Figure 1. Immunoprecipitation of small RNPs from *Xenopus* ovary extracts and *Xenopus* tissue culture (XTC) cells. Extracts of XTC cells (lanes 1,2) and *Xenopus* ovary extracts (lanes 3–14) were subjected to immunoprecipitation as described in Materials and methods. RNAs contained in immunoprecipitates were either labeled with [32 P]pCp (lanes 1–8) or silver stained (lanes 9–14). Total and anti-Ro-immunoprecipitated RNA from XTC cells are shown in lanes 1 and 2, respectively. Immunoprecipitations from ovary extracts were performed using nonimmune human serum (lanes 3,9), rabbit anti-bovine Ro serum (lanes 4,10), human anti-Ro sera (lanes 5,11), human anti-La sera (lanes 6,12), or human anti-Sm serum (lanes 7,13). Lanes 8 and 14 contain total ovary RNA. The additional RNA found in the anti-Ro immunoprecipitates is designated (→). The presence of mature 5S rRNA in all the immunoprecipitates is nonspecific; it is attributable to the very high amounts of mature 5S rRNA that are stored in *Xenopus* oocytes [cf. the relative amounts of 5S rRNA and tRNA in the total RNA populations from XTC cells and ovary (lanes 1,8, respectively)]. The apparent enrichment of mature 5S rRNA in the human anti-Ro immunoprecipitates (lanes 5,11) is not reproducible. In lanes 6 and 7 only one-twentieth of the immunoprecipitates, and in lanes 12 and 13, one-fortieth of the immunoprecipitates, were loaded on the gel. In the case of the anti-La immunoprecipitates (lanes 6,12), less sample was loaded to examine the distribution of 5S rRNA species optimally. Note that the majority of 5S rRNA in this immunoprecipitate is 2–3 nucleotides longer than the mature 5S rRNAs and probably contains additional Us at the 3' end. When more of the immunoprecipitate is loaded (or on longer exposures of lane 6), the pre-tRNAs bound to the La protein are also apparent.

tracts of whole ovaries, the additional RNA could have been derived from either oocytes or somatic follicle cells. However, this RNA was also present in immunoprecipitations from extracts of defolliculated oocytes, indicating that it was probably an oocyte-derived RNA (data not shown).

The additional RNA in anti-Ro immunoprecipitates is present in both the nucleus and the cytoplasm of Xenopus oocytes

To determine the subcellular location of the new Ro RNA, we performed immunoprecipitations on fractions from manually enucleated oocytes. As expected, the four previously characterized *Xenopus* Y RNAs were present entirely in the cytoplasmic fraction (Fig. 2, lane 5). In contrast, the additional RNA was present in both the nuclear and cytoplasmic fractions (lanes 5,8). This indicated that the association of the additional RNA with the Ro protein does not require the Y RNAs, as they are absent from the nuclear complexes that contain this RNA.

As a control for nuclear leakage during the enucleation procedure, we incubated the fractions with anti-Sm antibodies to immunoprecipitate the U RNA-containing small nuclear RNPs. Although at least half of the Sm-precipitable RNAs were present in the nuclear fraction (lane 9), the cytoplasmic fraction also contained a significant amount of these RNAs (lane 6). Because follicu-

lated oocytes were used for the enucleation procedure, the U RNAs in the cytoplasmic fraction could have been derived from the follicle cells that surround the oocyte. To test this possibility, we used partially denaturing gel electrophoresis, followed by Northern blotting, to resolve the somatic and germ-line forms of U1 RNA (Forbes et al. 1984). This analysis revealed that the U1 RNA in the cytoplasmic fraction was derived from the somatic follicle cells (data not shown) and that very little nuclear leakage occurred in our enucleation experiments. Thus, the novel Ro RNA exists in both the nucleus and the cytoplasm of *Xenopus* oocytes.

The additional Ro RNA represents a collection of variant 5S rRNA transcripts containing 3' extensions

To identify the novel Ro RNA, we partially sequenced it using base-specific ribonucleases. This analysis indicated that it was a form of the *Xenopus* oocyte-specific 5S rRNA containing ~eight additional gene-encoded nucleotides at the 3' end (Fig. 3A). We therefore refer to this species as 5S+ rRNA. These 5S+ rRNA transcripts are apparently generated by the failure of RNA polymerase III to terminate at the first of three termination signals (underlined in Fig. 3A).

To characterize the interaction of 5S+ rRNA with the 60-kD Ro protein, we attempted to reconstitute the complex in *Xenopus* egg extracts containing the Ro protein. We used this approach previously to demonstrate that the *Xenopus* Ro protein binds the human Y3 RNA (O'Brien et al. 1993). Using the same strategy, we were able to reconstitute binding of the *Xenopus* Ro protein to 5S+ RNA that was extracted from the anti-Ro immunoprecipitates. However, we were unable to detect specific binding of the 60-kD Ro protein to 5S+ RNA that was synthesized in vitro from a DNA template encoding the major oocyte-specific 5S rRNA (data not shown). This result suggested that the endogenous 5S+ rRNA present in the anti-Ro immunoprecipitate differed slightly in sequence from the major oocyte-specific 5S rRNA. In fact, the sequence of the 5S+ RNA derived by direct RNA sequencing was ambiguous in several positions, perhaps indicating that we had sequenced a mixed population of RNAs. Therefore, we devised a cDNA synthesis and cloning strategy that allowed us to determine the exact sequence of individual transcripts of the 5S+ rRNA (see Materials and methods).

The sequences of 36 5S+ cDNAs derived from anti-Ro immunoprecipitated 5S+ rRNAs are shown in Figure 3B. Surprisingly, all the transcripts contained at least one point mutation relative to the major oocyte-specific 5S rRNA sequence (top line, Fig. 3B). These mutations varied in position and did not correspond to the nucleotide changes present in the other two major families of *Xenopus* 5S genes, the somatic and trace oocyte 5S rRNA sequences (Miller et al. 1978; Peterson et al. 1980). However, certain mutations were present more frequently and were used to group some of the sequences in Figure 3B. Notably, 21 sequences (58%) contained a mutation at

Figure 2. Immunoprecipitations from nuclear and cytoplasmic fractions of *Xenopus* oocytes. Lysates prepared from equal numbers of whole oocytes, enucleated oocytes, or germinal vesicles were subjected to immunoprecipitation with either nonimmune human (lanes 1,4,7), human anti-Ro (lanes 2,5,8), or human anti-Sm sera (lanes 3,6,9). RNAs contained within immunoprecipitates were labeled with [³²P]pCp and fractionated on a denaturing polyacrylamide gel. Total RNA profiles (lanes 10–12) were prepared by phenol extraction of a small fraction of each lysate. The U RNAs in the cytoplasmic fraction (lane 6) are derived from the follicle cells that surround the oocyte (see text).

one of three positions in the last 10 nucleotides of the mature 5S rRNA (clones 16–36 in Fig. 3B). In addition, 28 of 36 sequences (78%) contained at least one mutation

that would destabilize stem I of the conserved 5S rRNA secondary structure (shown in Fig. 4A).

The ~20,000 oocyte-specific 5S rRNA genes have been

Figure 3. (See facing page for legend.)

shown previously to contain numerous sequence variations, with nucleotide changes in both the coding and noncoding regions (Fedoroff and Brown 1978; Miller and Brownlee 1978; Miller et al. 1978). To examine the natural heterogeneity present in oocyte 5S rRNA sequences and as a control for the cDNA synthesis and cloning procedures, we synthesized cDNA using 5S rRNA purified from 80S ribosomes (see Fig. 3C). Of 30 clones sequenced, 13 were identical to either the major oocyte 5S rRNA or trace oocyte 5S rRNA sequences. (The changes in the trace oocyte 5S rRNA genes are shown above the major oocyte 5S rRNA sequence in Fig. 3B,C.) The remaining clones contained a number of changes from the consensus major oocyte and trace oocyte 5S rRNA sequences. Although many of the ribosome-associated 5S rRNAs contained approximately the same number of nucleotide changes per transcript as the Ro-associated 5S+ rRNAs, the distribution was different.

As an additional control for the cDNA synthesis procedure, we also prepared cDNAs from *Xenopus* Y3 RNA and sequenced 12 of the resulting clones. Six clones were identical to our previously published genomic clone encoding xY3 RNA (O'Brien et al. 1993). The remaining six clones all contained six changes from the published sequence. As these six changes can be detected as nucleotide ambiguities when xY3 RNA is subjected to direct RNA sequencing (C. O'Brien and S. Wolin, unpubl.), probably they represent the product of a second gene encoding xY3 RNA. Only one of the 12 sequenced clones contained a likely artifact, the deletion of a single nucleotide in the xY3 RNA sequence. Thus, only a very small number of the nucleotide changes we observed in the 5S+ and ribosomal 5S rRNA populations were likely to be generated during the cDNA synthesis procedure.

The 60-kD Ro protein associates specifically with mutant readthrough 5S rRNAs

The finding that all 5S+ rRNAs in the anti-Ro immunoprecipitate contained mutations suggested that the Ro protein might associate specifically with mutant, but not wild-type, longer transcripts of 5S rRNA. To test this idea, we constructed mutant 5S genes that corresponded to three of the mutant sequences we identified in the anti-Ro immunoprecipitates. The three mutants chosen corresponded to clone 32 (mutant 1), clone 26 (mutant 2),

and clone 18 (mutant 3) in Figure 3B. We also constructed a 5S gene that contained a single T → C transition in the first termination site (wt-T). A mutation in this site was demonstrated previously to decrease significantly termination at the first site (Bogenhagen and Brown 1981). This wt-T construct allowed us to produce large amounts of essentially wild-type 5S+ rRNA upon microinjection to serve as a negative control for Ro protein binding. Figure 4A shows the predicted secondary structure of the major oocyte 5S rRNA and the positions and identities of nucleotide changes present in these mutants.

To examine the interaction of the transcribed RNAs with the Ro protein, we injected each of these genes into oocytes. The total transcription reactions are shown in Figure 4B (lanes 1–4). Transcription of the wild-type 5S rRNA gene containing a mutation at the first termination site (wt-T) yielded primarily the longer form of 5S rRNA during short incubations (data not shown; see Fig. 7, below). Thus, the mature 5S rRNA in lane 1 (5Sm) resulted largely from processing of the longer primary transcripts. The three mutant 5S rRNA genes contain all three termination sites (indicated in Fig. 3A); therefore, the mature 5S rRNA in lanes 2–4 can be generated either by termination at the first site or by processing of the longer RNAs. As a positive control for binding of the Ro protein, we coinjected a plasmid containing the gene for hY3 RNA with each plasmid containing a 5S rRNA gene.

The RNAs contained in the anti-Ro immunoprecipitates are shown in Figure 4B (lanes 13–20). Upon short exposures of the autoradiograph, 5S+ rRNA transcribed from only one of the 5S mutants, mutant 3, was detected in the anti-Ro immunoprecipitates (lanes 13–16). This mutant 5S+ rRNA bound to the Ro protein as efficiently as the control hY3 RNA (lane 16). Upon longer exposure, 5S+ rRNA from the remaining two mutants could also be detected in the anti-Ro immunoprecipitates (lanes 17–20). In each case, only the readthrough 5S rRNA transcripts were bound by the Ro protein, although large amounts of mature or properly terminated 5S rRNA were present (Fig. 4B, cf. lanes 17–20 with lanes 1–4). In addition, the longer wild-type transcripts were not bound by the Ro protein (lanes 13, 17). Thus, the Ro protein associated only with the readthrough transcripts of mutant 5S rRNA genes.

We also examined the binding of the mutant and wild-

Figure 3. The additional RNA in anti-Ro immunoprecipitates consists of readthrough mutant transcripts of oocyte 5S rRNA. (A) The sequence of the 3' terminus and flanking region of the *Xenopus laevis* major oocyte 5S rRNA gene is shown. The three termination sites are underlined. The 3' ends of mature 5S rRNA and 5S+ rRNA are indicated by arrows. (B) The sequences of 36 independent cDNA clones prepared from Ro-associated 5S+ rRNA are shown below the major oocyte 5S rRNA gene. Only nucleotides that differ from the 5S rRNA sequence are shown. Nucleotides corresponding to changes present in the trace oocyte 5S rRNA are indicated above the 5S rRNA gene. The 3' end of the mature 5S rRNA is indicated by the arrow. The dots at the beginning of clone 33 indicate missing nucleotides at the 5' end. Several of the cDNA clones (such as clones 2, 4, 21, 24, and 31) contain additional nucleotides at the 5' end. These extra nucleotides are probably added by reverse transcriptase during hairpin loop formation (O'Brien and Harley 1990). In addition, many of the cDNA clones contain an adenosine residue at the 3' end. This was observed on a similar fraction of clones when the same technique was performed on xY3 [which ends in GCU_{OH} (O'Brien et al. 1993)], and, therefore, probably represents an anomalous product that occurs during the ATP-dependent ligation of the DNA oligonucleotide to the RNA. (C) The sequences of 30 independent cDNA clones prepared from 80S ribosome-associated 5S rRNA are presented as in B.

Figure 4. Ro protein associates specifically with mutant 5S rRNA in vivo. (A) The predicted secondary structure of the oocyte-specific 5S+ rRNA is presented. Helices are indicated with roman numerals, and loops are indicated with letters. Nucleotide changes corresponding to mutants 1, 2, and 3 are shown in parentheses. The additional C → T change in mutants 1-T, 2-T, and 3-T and the single mutation in wt-T are enclosed by brackets. (B) Plasmids containing mutant oocyte 5S rRNA genes and the hY3 gene were coinjected with [³²P]GTP into mature oocytes and incubated overnight. Lysates prepared from groups of oocytes were incubated with either patient anti-La sera (lanes 5–8), mouse monoclonal anti-TFIIIA antibodies (lanes 9–12), or anti-Ro antibodies (lanes 13–20). (Lanes 1–4) Total RNA extracted from oocytes. Exposure times for the different panels were 3 hr (lanes 1–4), 48 hr (lanes 5–16), and 240 hr (lanes 17–20).

type 5S rRNA transcripts to two known 5S rRNA-binding proteins, the La protein and the 5S-specific transcription factor TFIIIA. The La protein binds to nascent RNA polymerase III transcripts by the 3' UUU_{OH} (Rinke and Steitz 1982; Stefano 1984). Newly synthesized 5S rRNA transcripts derived from each of the 5S plasmids were bound by the La protein (Fig. 4B, lanes 5–8). Note that transcripts derived from termination at both the first and second termination sites are present in the La immunoprecipitations from all three mutants (lanes 6–8). As expected, only transcripts corresponding to termination at the second site are present in the immunoprecipitate from the wild-type gene containing a mutation in the first site (Fig. 4B, lane 5). Although mature 5S RNAs transcribed from all the plasmids were bound by TFIIIA

(lanes 9–12), binding was most efficient to the wild-type mature 5S rRNA and to the shorter form of mutant 2. Of the 5S rRNAs containing 3' extensions, only the wild-type RNA bound TFIIIA efficiently (lane 9).

To determine whether the Ro protein could bind the mutant 5S+ rRNAs in vitro, we synthesized ³²P-labeled wild-type and mutant 5S+ rRNAs using T7 RNA polymerase and added these RNAs to two different sources of the Ro protein. First, we incubated the RNAs in *Xenopus* egg extracts (Fig. 5A). As controls, we included the nucleolar U3 RNA and the human Y3 RNA. The three mutant 5S+ rRNAs, but not the wild-type 5S+ rRNA, assembled with the *Xenopus* Ro protein to form immunoprecipitable RNPs (Fig. 5A, lanes 5–8). Note that the mutant 3 RNA bound the Ro protein more efficiently than the other two mutant 5S+ rRNAs (cf. lane 8 with lanes 6 and 7), although the difference was less striking than that seen in vivo (Fig. 4B, cf. lanes 18–20).

We also examined the human 60-kD Ro protein, translated in a rabbit reticulocyte lysate, to determine whether it could discriminate between mutant and wild-type *Xenopus* 5S+ rRNAs. As shown in Figure 5B (lane 5), the mutant 5S+ rRNA, but not the wild-type 5S+ rRNA, was bound by the Ro protein. Because the assembly of immunoprecipitable Ro RNPs was dependent on translation of mRNA encoding the human 60-kD Ro protein (Fig. 5B, cf. lanes 5 and 11), this result also demonstrated that binding by this protein (rather than some other protein recognized by the patient autoantibodies) is required for the assembly of mutant 5S+ rRNAs into Ro RNPs.

The mutant 5S+ rRNA exists in a complex containing both Ro and La proteins

Newly synthesized 5S rRNA precursors are bound normally by the La protein (Rinke and Steitz 1982; Guddat et al. 1990; Yoo and Wolin 1994), therefore, we wanted to determine whether the La protein was present in the complex containing mutant 5S+ rRNAs and the Ro protein. Sequential immunodepletion experiments were performed using lysates from oocytes microinjected with the mutant 5S and human Y3 plasmids (Fig. 6). In control experiments, we showed that two sequential rounds of immunoprecipitations with either anti-Ro (lanes 1–3) or anti-La antibodies (lanes 8–10) were sufficient to deplete the extract of the respective RNPs. We then depleted Ro RNPs from the lysate by two consecutive anti-Ro immunoprecipitations (lanes 4–5) and examined whether a population of 5S+ rRNA remained that was immunoprecipitable by anti-La antibodies (lane 6). This analysis revealed that the subsequent immunoprecipitation of 5S+ rRNA by anti-La antibodies was reduced significantly compared with a mock-depleted lysate (Fig. 6, cf. lanes 6 and 7). Conversely, depletion of the lysate with anti-La antibodies (lanes 11,12) resulted in a large decrease in the amount of 5S+ rRNA immunoprecipitated by anti-Ro antibodies, whereas the amount of immunoprecipitated hY3 RNA was unchanged (lanes 13,14). Taken together,

Figure 5. Mutant 5S+ rRNAs assemble with the Ro 60-kD protein in vitro. (A) 32 P-labeled hY3, U3, and wild-type and mutant 5S+ rRNAs were synthesized in vitro with T7 RNA polymerase. A mixture of hY3, U3, and either wild-type 5S+ RNA (lanes 1,5) or mutant 5S+ rRNA (lanes 2–4 and 6–8) was incubated with *Xenopus* egg extracts. The extract was then aliquoted and either subjected to immunoprecipitation with anti-Ro antibodies (lanes 5–8) or extracted with phenol (lanes 1–4). The band designated with an asterisk (*) is a U3 RNA degradation product (O'Brien et al. 1993). (B) A synthetic mRNA encoding the human 60-kD Ro protein was synthesized in vitro and translated in a rabbit reticulocyte lysate. After translation, the reaction was incubated with a mixture of 32 P-labeled hY3, U3, and either wild-type 5S+ (lanes 1–3) or mutant 3 5S+ rRNA (lanes 4–6). The extract was then aliquoted and either extracted with phenol (lanes 1,4) or subjected to immunoprecipitation with either a patient anti-Ro serum (lanes 2,5) or a nonimmune serum (lanes 3,6). As a control, the translation was also performed in the absence of mRNA encoding the 60-kD Ro protein (lanes 7–12).

these results demonstrate that the majority of the mutant 5S+ rRNA complexes contain both the Ro and the La proteins.

Mutant 5S+ rRNAs are processed less efficiently than wild-type 5S+ rRNA and are less stable

Because the Ro protein selectively binds mutant longer transcripts of 5S rRNA, it seemed possible that these mutant RNAs might be processed inefficiently in oocytes and the Ro protein might function as part of a discard or quality control pathway for 5S rRNA processing. Therefore, we wanted to examine how efficiently the mutant 5S+ rRNA transcripts were processed to mature 5S rRNA. Because transcription of our mutant 5S rRNA genes could terminate at either the first or the second termination site, we were unable to use these constructs to determine directly the efficiency of processing of the longer form of 5S rRNA. Therefore, we created a T $\rightarrow$ C transition in the first termination site in each of these mutants (see Fig. 4A), so that only the longer form of 5S rRNA would be transcribed. We refer to these mutants as 1-T, 2-T, and 3-T. By transcribing each of these mutants in oocytes and performing immunoprecipitations, we verified that each of these

5S+ rRNAs was bound by the Ro protein (data not shown).

To measure the efficiency of processing, we injected these plasmids into *Xenopus* oocytes and allowed transcription to proceed for 2 hr. We then inhibited further transcription by injecting tagetitoxin, a specific inhibitor of RNA polymerase III (Steinberg et al. 1990). As an internal standard, we coinjected the hY3 gene, as the transcribed Y3 RNA is not processed. The efficiency of 5S rRNA processing was examined at various intervals after tagetitoxin injection (Fig. 7, lanes 13–16). We found that the wild-type 5S+ rRNA was slowly processed to mature 5S rRNA. After 20 hr, 35% of the initial 5S+ RNA was processed, and the remaining unprocessed RNA was apparently degraded. In contrast, two of the three mutant RNAs were processed very inefficiently to mature 5S rRNA and eventually were largely degraded (lanes 1–4, 9–12). The third mutant 5S+ rRNA (mt 2-T) did undergo processing, although less efficiently (6% conversion after 20 hr) and more slowly than the wild-type RNA (Fig. 7, cf. lanes 5–8 with 13–16). Note that the levels of mature mutant 5S RNAs remained stable or increased during the experiment, even after the bulk of the mutant precursors were degraded (e.g., cf. lanes 11 and 12). Thus, it is largely the defective 5S rRNA precursors that are unstable, rather than the mature forms of these RNAs.

Figure 6. The mutant 5S+ rRNA is complexed with both the 60-kD Ro protein and the La protein. Stage V and VI oocytes were coinjected with the mutant 3 5S+ rRNA gene, the hY3 gene, and [α - 32 P]rGTP. After an overnight incubation, extracts were prepared and subjected to either three sequential immunoprecipitations with patient anti-Ro sera (lanes 1–3), two sequential immunoprecipitations with anti-Ro sera followed by an anti-La immunoprecipitation (lanes 4–6), three consecutive immunoprecipitations using patient anti-La sera (lanes 8–10), or two sequential anti-La immunoprecipitations followed by an anti-Ro immunoprecipitation (lanes 11–13). The brackets enclose the multiple immunoprecipitations performed on the same aliquot of lysate. Two consecutive incubations with non-immune sera were performed before immunoprecipitation with anti-La (lane 7) or anti-Ro (lane 14) sera. In lane 15, nonimmune human serum was used for immunoprecipitation. RNAs present in immunoprecipitates were fractionated in a 5% polyacrylamide/8 M urea gel. The band labeled hY3** has been observed previously (Wolin and Steitz 1983) and apparently is generated by premature transcription termination from the hY3 gene. The band marked with an asterisk (*) is also derived from the hY3 gene. The identity of the large bands in the anti-Ro immunoprecipitates (lanes 1–5, 13, 14) are not known. Note that in contrast to Fig. 4B (lanes 5–8), the hY3 RNA is not bound to the La protein in this experiment (lanes 6–12). Although the injected oocytes were incubated overnight in both experiments, transcription may not always continue throughout this period. In this experiment, the transcription rate apparently declined during the incubation, so that only RNAs that bind the La protein stably are detected.

Discussion

The 60-kD Ro autoantigen is the major protein component of a class of small cytoplasmic RNA–protein complexes known as Ro RNPs. The RNA components of these particles, known as Y RNAs, are ~100 nucleotides

long. We have demonstrated that the 60-kD Ro protein also forms complexes with defective precursors of 5S rRNA in *Xenopus* oocytes. These mutant RNAs are processed inefficiently to mature 5S rRNA and are less stable than the wild-type 5S rRNA precursors. These results suggest that the 60-kD Ro protein may function as part of a novel discard, or quality control, pathway for abnormal 5S rRNA precursors.

A collection of defective 5S rRNA precursors

Although 3' cleavage of a precursor molecule is required for 5S rRNA maturation in *Drosophila melanogaster* and *Saccharomyces cerevisiae*, the *Xenopus* 5S rRNA is not synthesized as an obligate precursor. However, a minor fraction of 5S rRNA containing extra nucleotides at the 3' end has been observed upon examination of endogenous RNA synthesis (Denis and Wegnez 1973) and upon microinjection of cloned genes into oocytes (Gurdon and Brown 1978; Xing and Worcel 1989). These elongated forms of 5S rRNA are processed to mature RNA (Xing and Worcel 1989; also see Fig. 7). Our data indicate that *Xenopus* oocytes also contain a population of defective 5S rRNA precursors that are processed inefficiently and eventually degraded.

The structural requirements for processing of 5S rRNA precursors have been studied most extensively in *Drosophila* cell extracts (Levinger et al. 1992; Vasisht et al. 1994). These experiments have revealed that many point mutations in stems I, II, III, and V as well as in loops B and C inhibit processing (Fig. 4A), indicating that the machinery that recognizes and processes pre-5S rRNA is extremely sensitive to structural perturbations. Although few studies have been performed in *Xenopus*, Xing and Worcel (1989) demonstrated that small perturbations in stem I (including a single mismatch in the 9-bp helix) decreased the stability of precursor 5S rRNA in vitro and in vivo. On the basis of this data, they proposed that this helix serves to prevent further degradation of the oocyte-specific 5S rRNA by a 3' exonuclease.

Our finding that the three mutant RNAs we have examined are processed inefficiently suggests that we have identified a family of natural processing mutants bound to the 60-kD Ro protein. The majority of these mutant 5S rRNA precursors contain alterations in stem I, consistent with the idea that this helix is important for the stability and processing of 5S rRNA precursors (Xing and Worcel 1989). However, the remainder of these RNAs contain alterations outside this region. This suggests that, as shown in *Drosophila*, a variety of nucleotide changes may affect processing of 5S rRNA precursors in *Xenopus*.

Because our cDNA synthesis procedure included a PCR amplification step, it is possible that some of the nucleotide changes that we observed in the 5S+ rRNA population are errors introduced during the PCR procedure. Because control reactions in which we prepared cDNA from *Xenopus* Y3 RNA did not exhibit this error rate, we believe that only a small number of changes could have been generated in this way. However, even if

Figure 7. Pulse-chase labeling of wild-type and mutant 5S+ rRNA transcripts. Mature oocytes were coinjected with [α - 32 P]rGTP, the hY3 gene, and either mutant (lanes 1–12), or the wt-T (lanes 13–20) 5S+ rRNA gene constructs. After a 2-hr incubation, the oocytes were either injected with tagetitoxin (lanes 1–16) or left uninjected (lanes 17–20). At either 0 (lanes 1,5,9,13,17), 4 (lanes 2,6,10,14,18), 8 (lanes 3,7,11,15,19), or 20 (lanes 4,8,12,16,20) hrs after tagetitoxin injection, aliquots (10 oocytes each) were removed and the RNAs extracted with phenol–chloroform and fractionated in a 5% polyacrylamide/8 M urea gel. The differences in the amount of transcription seen at the 0 timepoint between the mutant and the wild-type 5S genes are not reproducible.

some errors were introduced during cDNA synthesis, our conclusion that the Ro protein binds mutant 5S rRNA precursors is unaffected, as we have reconstituted binding of this protein to the mutant RNAs both in vivo and in vitro (Figs. 4B and 5).

A number of the mature 5S rRNAs present in ribosomes also contained changes from the consensus oocyte 5S rRNA sequence (Fig. 3C). *X. laevis* has been estimated to contain ~20,000 copies of the major oocyte-specific 5S genes and 1300 copies of the trace oocyte genes per haploid genome (Peterson et al. 1980). The fact that these genes contain microheterogeneities in the coding sequences has long been appreciated. The first several genomic DNA fragments to be analyzed contained several sequence substitutions in both coding and noncoding regions of the 5S rRNA gene tandem repeat (Fedoroff and Brown 1978; Miller and Brownlee 1978; Miller et al. 1978). From these analyses, it was estimated that individual oocyte-specific 5S rRNAs probably contained ~1% heterogeneity at any particular position (Miller and Brownlee 1978). Our analysis of the 5S rRNA is consistent with this estimate, as nearly half of the 5S rRNA was identical to the consensus major oocyte and trace oocyte RNAs, whereas the remainder exhibited an average of two changes per transcript (Fig. 3C).

Despite the fact that the structure of 5S rRNA is highly conserved, it has been observed previously that many nucleotide changes do not affect the ability of mature 5S rRNAs to assemble into 60S ribosomal subunits. In yeast, expression of a variety of mutant 5S rRNAs did not affect growth rates significantly, even when $\leq 80\%$ of the ribosomal 5S rRNA was replaced by mutant RNA (Van Ryk et al. 1990, 1992). In *Xenopus*, Allison et al. (1993) injected 31 in vitro-synthesized 5S rRNA mutants into oocytes. Surprisingly, only four of these mutants were completely defective in ribosome assembly.

It is curious that defective 5S rRNA precursors should be subject to a discard pathway, whereas mature 5S rRNAs with similar changes appear to assemble into ribosomes. It is possible that 5S rRNAs containing additional 3' nucleotides cannot be incorporated into the 60S subunit, and are therefore discarded. Alternatively, defective 5S rRNA precursors may actually be deleterious to some aspect of cell metabolism, such as ribosome biogenesis.

A novel discard pathway

It has long been recognized that abnormal mRNAs, such as unspliced mRNAs and mRNAs containing premature stop codons, are unstable. Genetic analyses in yeast and nematodes have identified components of the "mRNA surveillance" systems that recognize and degrade abnormal mRNAs (Leeds et al. 1992; Pulak and Anderson 1993). In addition, the yeast ATPase *PRP16* has been shown to regulate the entry of defective pre-mRNA splicing intermediates into a discard pathway (Burgess and Guthrie 1993). Our results indicate that inefficiently processed 5S rRNA precursors are similarly unstable. Thus, cells must also possess mechanisms for the recognition and degradation of defective small RNA molecules.

An important question raised by our work is whether similar discard pathways occur in other cell types. In the majority of species, 5S rRNA genes are located in clusters of tandemly repeated units, with copy numbers ranging from ~160 to several thousand. In all species examined, there appear to be numerous 5S rRNA gene variants and pseudogenes (Piper et al. 1984; Sharp et al. 1984; Reddy et al. 1986; Sorensen and Frederiksen 1991). At least some of these variant genes are transcribed in vivo, as heterogeneity has been detected by RNA fingerprinting of 5S rRNA from both yeast and *Xenopus* (Weg-

nez et al. 1972; Piper et al. 1984). Thus, discard pathways for variant 5S rRNA molecules may also exist in other species. We have never detected a population of defective 5S rRNA precursors in our anti-Ro immunoprecipitates from vertebrate somatic cells, possibly because these genes are at least 10-fold less numerous than the *Xenopus* oocyte-specific 5S rRNA genes. In addition, *Xenopus* oocytes synthesize ribosomes at a rate that is several thousand-fold higher than occurs in somatic cells (Brown 1981). It is possible that the far smaller number of mutant 5S rRNA precursors that would be bound to the 60-kD Ro protein in somatic cells might simply be below the level of detection. Further studies, such as the overexpression of mutant 5S genes in somatic cells, will be required to determine whether similar degradation pathways occur in these cells.

Discard pathways may also exist for other defective RNA precursors encoded by multigene families, such as the small nuclear U RNAs, tRNAs, and large rRNAs. In multigene families, there is less natural selection against mutations in individual gene copies. Mechanisms such as gene conversion and unequal crossing-over help to maintain the characteristic homogeneity of these gene families (Jinks-Robertson and Petes 1993). However, at any point in time, a number of mutant gene copies will exist, and some of these genes are likely to be transcribed. Thus, cells may need mechanisms to recognize and degrade these abnormal RNAs. It will be interesting to determine whether other mutant RNA precursors also bind the 60-kD Ro protein.

What role might the 60-kD Ro protein play in this discard pathway? One possibility is that the Ro protein interacts with all newly synthesized 5S rRNA precursors to facilitate folding into the proper conformation for processing. The Ro protein would function as an "RNA chaperone" (Herschlag et al. 1994; Portman and Dreyfuss 1994). Although all 5S rRNA precursors would bind the Ro protein, perhaps only the mutant precursors bind the Ro protein stably and are detectable by immunoprecipitation. An analogy would be to members of the Hsp70 family of chaperones, such as BiP. Members of this protein family associate transiently with newly synthesized proteins and are thought to facilitate proper folding (Beckman et al. 1990). However, abnormal or mutant proteins that are unable to fold properly and to assemble are bound more stably by these proteins (for review, see Gething and Sambrook 1992; Georgopoulos and Welch 1993). Interestingly, when the human 60-kD Ro protein is expressed at high levels in *S. cerevisiae*, it binds to wild-type precursors of yeast 5S rRNA (C. O'Brien and S. Wolin, unpubl.). One interpretation of this result is that the Ro protein normally binds nascent 5S rRNA precursors to facilitate their folding.

Alternatively, the Ro protein might recognize and bind specifically defective 5S rRNA precursors. Although the precise requirements for binding of the 60-kD Ro protein to the Y RNAs have not been determined, the bound protein protects a conserved helix from nuclease digestion (Wolin and Steitz 1984). Within this conserved helix is a single bulged cytidine. Experiments in which the

bulged cytidine was deleted or replaced with adenine have confirmed its importance as a recognition element for the Ro protein (Pruijn et al. 1991). Although we have not detected a similar structure in our mutant 5S rRNA precursors, the mutant RNAs may form a secondary structure that is recognized by the Ro protein. Because 78% of our mutants weaken stem I, one possibility is that an alternative stem forms. However, any hypothesis must take into account the wide variety of mutant 5S rRNA transcripts, some with very subtle changes, that are bound by the Ro protein. In addition, the 3' terminal extension must play a role in recognition, as only the longer RNAs are bound by the Ro protein. Further studies in which we define the binding site for the 60-kD Ro protein on the mutant 5S rRNA precursors will be required to resolve this question.

Although we do not yet know what role the Y RNA components of Ro RNPs play in this pathway, there are several intriguing possibilities. The Y RNAs may serve to sequester the 60-kD Ro protein in the cytoplasm when it is not needed, and thereby regulate the amount of free 60-kD Ro protein in the nucleus. In this scenario, they may have evolved from defective 5S rRNA precursors to bind the Ro protein stably and escape degradation. Alternatively, the Y RNAs may be an integral part of the discard or quality control pathway. A tantalizing possibility is that the Y RNAs are components of a cytosolic RNA degradation machinery.

Materials and methods

Antisera and immunoprecipitations

The human anti-Ro, Sm, and La sera used in this study have been described previously (O'Brien and Harley 1990; O'Brien et al. 1993). The rabbit anti-bovine Ro serum (Mamula et al. 1989) was a gift of M. Mamula (Yale University, New Haven, CT). Monoclonal anti-TFIIIA antibodies (Kramer and Roeder 1983) were a gift of B. Moorefield and R. Roeder (The Rockefeller University, NY).

Whole ovary tissue or groups of oocytes were prepared for immunoprecipitation by washing three times in modified Barth's solution [(MBS) 88 mM NaCl, 1 mM KCl, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES (pH 7.4)]. The ovaries or oocytes were resuspended in NET-2 [40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% NP-40] containing 10 mM vanadyl ribonucleoside complexes and disrupted by sonication (ovary tissue) or by pipetting (oocytes). Lysates were cleared by centrifugation at 100,000g in a Beckman TL100.3 rotor for 30 min at 4°C. The cleared lysates were incubated with antisera and processed as described previously (Wolin and Steitz 1984). RNAs present in immunoprecipitates were 3' end-labeled using [³²P]pCp and T4 RNA ligase (England et al. 1980) and fractionated in 5% polyacrylamide/8 M urea gels. Alternatively, RNAs were visualized by staining with silver after gel electrophoresis (Forman et al. 1985).

RNA sequencing and cDNA synthesis

Direct RNA sequencing using base-specific ribonucleases was performed as described (O'Brien et al. 1993). To obtain the sequences of individual RNA transcripts, a procedure was devised to generate full-length cDNAs. The RNA was ligated to 200 ng

of a 5' ³²P-labeled deoxyoligonucleotide that was blocked at the 3' end with cordocypin [SP6Reco: ATAGTGTACCTAAATG-AATTCC(3'dA)] with T4 RNA ligase (England et al. 1980). The ligation products were fractionated in a 5% polyacrylamide/8 M urea gel and the desired bands excised. A second 5' ³²P-labeled oligonucleotide (SP6eco: GGAATTCATTTAGGTGACACT-AT), complementary to SP6Reco, was used for cDNA synthesis of the ligation product using reverse transcriptase. The reaction was digested with RNase A, and full-length first-strand cDNAs were gel purified. First-strand cDNAs were tailed with terminal deoxytransferase and dATP. Products of the tailing reaction were amplified by performing PCR with an anchored dT primer (CGCGCATGCCTGCAGAAGCTTTTTTTTTTTTTTTTTT) and the SP6eco primer. Before amplification, the samples were incubated at 85°C for 5 min and 95°C for 1 min. The reactions were then subjected to two amplification cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min, followed by an additional 35 cycles in which the incubation at 55°C was for 1 min. Amplification products were gel purified, ligated into the pCRII vector (Invitrogen), and completely sequenced on both strands.

Plasmids and mutagenesis

A plasmid containing the hY3 gene was prepared by subcloning the 800-bp *Bam*HI–*Eco*RI fragment of the genomic clone (Wolin and Steitz 1983) into pBluescript KS– (Stratagene). Plasmids containing the *X. laevis* oocyte 5S rRNA gene (pXloΔ3' + 176) and somatic 5S rRNA gene (pXls11) were a gift of D. Brown (Bogenghagen and Brown 1981). The oocyte 5S rRNA gene was prepared for in vitro mutagenesis by cloning the 530-bp *Eco*RI–*Hind*III fragment of pXloΔ3' + 176 into pBluescript KS–. Single-stranded DNA derived from this plasmid was used as template for site-directed mutagenesis as described by Kunkel et al. (1987). The 5S rRNA-coding region of plasmid pXloΔ3' + 176 contained 2-base changes relative to the consensus major oocyte 5S rRNA sequence. Therefore, we converted this gene to the consensus 5S rRNA sequence by performing site-directed mutagenesis before making further alterations. Mutations were verified by double-stranded DNA sequencing.

Microinjection and enucleation of oocytes

Mature *X. laevis* oocytes were coinjected with plasmids and [α-³²P]rGTP as described by Gurdon and Brown (1978). Fourteen nanoliters of a solution containing plasmid DNA (50 ng/ml) and [α-³²P]rGTP (5 mCi/ml) was microinjected into the germinal vesicle using a Nanoject micropipet (Drummond). Injected oocytes were maintained at 18°C in MBS supplemented with 0.1 mg/ml of bovine serum albumin. Enucleation of oocytes under mineral oil was performed as described by Lund and Dahlberg (1989). Somatic and germ-line forms of U1 RNA were resolved using the partially denaturing gel system described by Peck et al. (1987).

In experiments in which RNA polymerase III transcription was inhibited, oocyte nuclei were first injected with plasmids and [α-³²P]rGTP. After a 1- to 2-hr incubation, 18.4 nM of tagetitoxin (20 U/ml; Epicentre Technologies) was injected into the oocyte cytoplasm and the incubation continued for an additional 20 hr. Quantitation of 5S rRNA processing was carried out using a PhosphorImager (Molecular Dynamics). Each value was normalized to the level of hY3 RNA in the lane. Percent processing was calculated by dividing the increase in mature 5S RNA [(t = 20 hr) – (t = 0 hr)] by the amount of 5S+ RNA present at the start of the experiment.

RNP Reconstitutions

We used PCR to place the mutant and wild-type 5S rRNA coding sequences behind T7 promoters. The 5' primer for all four constructs contained 14 nucleotides of 5S rRNA-coding sequence preceded by a T7 promoter and an *Eco*RI site. The 3' primers contained 14 nucleotides complementary to the 3' end of each 5S sequence, preceded by a *Dra*I site and a *Bam*HI site. After PCR using the cloned 5S rRNA sequences as templates, the product was digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI–*Bam*HI sites of pSP64 (Promega). After cleavage with *Dra*I, transcription with T7 RNA polymerase yielded RNAs identical to the endogenous 5S+ sequences. The constructs that allow transcription of the U3 and hY3 RNAs have been described (Baserga et al. 1991; O'Brien et al. 1993).

Reconstitutions in *Xenopus* egg extracts were performed essentially as described (O'Brien et al. 1993). Briefly, ³²P-labeled U3, hY3, and 5S+ rRNAs were synthesized using T7 RNA polymerase as described previously. After transcription, each RNA was resuspended in 20 μl of water, and 0.3 μl of each was added to 10 μl of egg extract (Murray and Kirschner 1989; a gift of M. Solomon, Yale University, New Haven, CT). After a 30-min incubation at 22°C, extracts were immunoprecipitated as described above, except that 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 (HBS-T) was substituted for NET-2.

For in vitro translation, the *Eco*RI fragment containing the full-length cDNA encoding the human 60-kD Ro protein (Deutscher et al. 1988; a gift of J. Harley, Oklahoma Medical Research Foundation, Oklahoma City) was inserted into the *Eco*RI site of pGEM2. After linearization with *Bam*HI, the DNA was transcribed using SP6 RNA polymerase as described previously (Wolin and Walter 1988). Translations were performed using the Flexi Reticulocyte Lysate System (Promega) as described by the manufacturer. After translation, 2 μl of a mixture of the labeled RNAs (0.3 μl each RNA) was added to 100 μl of the translation reaction. After a 30-min incubation at 4 °C, the mixture was subjected to immunoprecipitation in HBS-T as described above.

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A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5S rRNA precursors.

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