Characterization of a *Xenopus laevis* Ribonucleoprotein Endoribonuclease

**ISOLATION OF THE RNA COMPONENT AND ITS EXPRESSION DURING DEVELOPMENT**

(Received for publication, April 30, 1992)

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In order to facilitate studies of the assembly and transport of the site-specific RNase mitochondrial RNA processing (MRP) ribonucleoprotein, we have characterized it from *Xenopus laevis* cells. *X. laevis* RNase MRP displayed a similar spectrum of cleavage activity to that produced by previously isolated mammalian nuclear enzymes. A 277-nucleotide RNA component of the ribonucleoprotein was identified; the gene for the RNA was isolated, sequenced, and found to be 66 and 63% similar to mouse and human RNase MRP RNAs, respectively. Despite the evolutionary distance from its mammalian counterparts, *X. laevis* RNase MRP RNA contains five regions of homology to the mammalian RNase MRP RNA. Four of these regions correspond to those previously identified as conserved between RNase MRP and RNase P RNAs; the fifth encompasses nucleotides recently discovered to be sufficient for autoantigen binding. The expression and assembly of *Xenopus* RNase MRP RNA were examined in frog oocytes and developing embryos. RNase MRP RNA was expressed throughout oogenesis; it started to accumulate at stage I and reached a maximum in stage IV. During embryogenesis RNase MRP RNA expression began to elevate at approximately stage 22 and continued to rise through the swimming tadpole stage. When injected into the nucleus of mature oocytes, the *X. laevis* RNase MRP RNA gene was expressed accurately, and transcripts were packaged into immuno-precipitable particles.

RNase mitochondrial RNA processing (MRP) is a ribonucleoprotein (RNP) endoribonuclease that processes mtRNA transcripts at a site of transition from RNA to DNA synthesis in leading-strand mtDNA replication (Chang et al., 1985; Chang and Clayton, 1987a). However, the majority of the enzymatic activity is present in the nucleus where its role remains undetermined. Given the similar RNA processing activities of the nuclear and mitochondrial forms of the enzyme (Karwan et al., 1991) and the nucleolar localization of the nuclear activity by immunocytochemistry (Reimer et al., 1988), it has been suggested that nuclear RNase MRP might play a role in rRNA processing.

The African clawed toad *Xenopus laevis* provides an ideal system to investigate further the structure, cellular distribution, and function of RNase MRP. First, any role of RNase MRP in mtRNA processing is likely to be conserved in *X. laevis* mitochondria since the organization of *X. laevis* mtDNA is identical to that of mammalian mtDNA (Roe et al., 1985) and CSB II and CSB III, the two sequence blocks in mammalian mtDNA previously shown to be critical for efficient cleavage by RNase MRP (Bennett and Clayton, 1990), are conserved in *Xenopus* mtDNA. Second, the structure and distribution of RNPs can be readily investigated through the reconstitution of their individual RNA and protein components in *Xenopus* oocytes. This system has been successfully exploited for the dissection of the mode of assembly and nuclear import of U-series small nuclear ribonucleoproteins (see Mattaj (1988) for review). Third, the *Xenopus* system provides a unique environment in which it is possible to investigate the developmental control of a variety of cellular entities and processes: RNP accumulation and distribution, mtDNA replication and transcription, and rDNA amplification and ribosome biogenesis.

Mitochondrial DNA and rDNA replication are greatly accelerated during early *Xenopus* oogenesis (Brown and Dawid, 1968; Gall, 1968; Webb and Smith, 1977), enabling the *Xenopus* oocyte to accumulate the reservoir of ribosomes and mitochondria necessary to support the protein synthetic and energetic needs of the developing embryo through the swimming tadpole stage. During embryogenesis, however, transcription and replication of mtDNA take a distinct course from that of nuclear DNA. Contrary to the regular and rapid nuclear DNA replication in the early embryo, mtDNA replication is relatively nonexistent during early embryogenesis and does not reinitiate until late in organogenesis near the onset of the early tadpole stage, stages 30–32 (Chase and Dawid, 1972). A similar, although slightly offset, developmental profile is evident for mitochondrial transcription. RNA synthesis during early embryogenesis is virtually nonexistent and the bulk of new transcription initiates in stages 22–24, prior to the onset of mtDNA replication (Chase and Dawid, 1972; El Meziane et al., 1989). In contrast to mtRNA transcription, the activation of rRNA transcription begins early in embryogenesis at gastrulation (Brown and Litton, 1964). The controlled timing of replication and transcription in the nucleus and mitochondria during *Xenopus* oogenesis and embryogenesis therefore provides an exquisite system for studying RNase MRP with its potential dual roles in nuclear and mitochondrial nucleic acid metabolism.

This paper reports the initial study of RNase MRP from...
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X. laevis. An RNase MRP activity was isolated which displays an almost identical cleavage activity to that of mouse and human nuclear RNase MRPs on the standard mouse mtRNA substrate. The gene for the RNA component (MRP RNA) of this activity was cloned and found to have significant sequence homology to mammalian MRP RNA genes (Chang and Clayton, 1989; Topper and Clayton, 1990a; Yuan et al., 1989). Upon injection into the nucleus of stage VI oocytes, the X. laevis MRP RNA gene was accurately expressed, and MRP RNA was assembled as an immunoprecipitable particle. The expression of Xenopus MRP RNA was followed through oogenesis and embryogenesis and found to be consistent with its proposed role in mtDNA replication.

MATERIALS AND METHODS

Purification of Xenopus RNase MRP—Xenopus XTC tissue culture cells (a kind gift of Sandya Narayanswami, University of California, Irvine) were grown as monolayers in Dulbecco's minimal essential medium (GIBCO) supplemented with 10% fetal calf serum. Nuclear extracts were prepared essentially as described by Karwan et al. (1991) with slight modifications. Cleared nuclear extracts were applied directly onto 15-30% glycerol gradients without prior batch chromatography on DEAE-Sephacel. Thirty-six 300-μl fractions (50 μg/ml total protein) were collected, aliquoted, and stored frozen at −80 °C.

Immunoprecipitation and RNase MRP Cleavage Assay—Immunoprecipitations from XTC nuclear extracts were performed essentially as described by Tollervey and Mattaj (1987) except that no heparin was added during the initial immunoprecipitation step. Patient sera were used the same as in Karwan et al. (1991). RNPs bound to Sepharose were washed as described with either IPB-500 (500 mM NaCl, 10 mM HEPES (pH 7.5), 0.1% Nonidet P-40) or IPB-150 (150 mM NaCl, 10 mM HEPES (pH 7.0), 0.1% Nonidet P-40). For immunoprecipitation from oocytes, the basic protocol was similar to the above except antibody was added first. Oocytes were homogenized in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and centrifuged at 14,000 rpm for 15 min at 4 °C to remove cell debris. Before immunoprecipitation, extracts were precleared with Pansorbin (Calbiochem) to reduce nonspecific binding. Initial cleared extracts were incubated with 10 μl of human serum with slow rotation at 4 °C for 1 h, followed by an additional incubation with Pansorbin for 15 min to precipitate immune complexes. Supernatants were separated from pellets by centrifugation for 30 s at 10,000 rpm and saved for gel analysis. Pellets were washed three times with IPB (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium azide) (Hamm and Mattaj, 1990). RNase MRP cleavage reactions were performed on standard mouse mtRNA substrate (Chang and Clayton, 1987a) as described by Karwan et al. (1991).

Isolation of the Xenopus RNase MRP RNA Gene—Th immunoprecipitations from Xenopus XTC cell nuclear extracts were pooled, end-labeled with [32P]cytidine 3',5'-bis(phosphate), separated on a 21766 nucleotide cDNA was amplified using polymerase chain reaction and labeled with [32P]cytidine 3',5'-bis(phosphate), separated on a 6% polyacrylamide-7 M urea gel, and recovered from isolated gel fragments. In order to simplify the screening process, an enriched genomic library was constructed in λ ZAPII (Stratagene) using 3.0-4.0-kb EcoRI fragments electroeluted from an agarose gel (Ehrat, Schleicher & Schuell). The recombinant phage was packaged using Gigapack Plus commercial packaging extract (Stratagene). The resulting phage library was amplified, and individual phage containing the MR RNA gene were identified after hybridization at 42 °C with 32P-labeled pXLMU probe. PCR using SSPE containing 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA (pH 7.2), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, 50% formamide, and 200 μg/ml Escherichia coli tRNA. Filters were washed stringently at 60 °C in 0.2 × SSPE, 0.1% SDS. Six identical phage were obtained, each containing a 3.2 kb EcoRI insert. The gene encoding MR RNA was further localized to a 1.3-kb EcoRI fragment of the original insert. Insert was obtained from overlapping deletion clones constructed using the Erase-A-Base system (Promega) by the method of diodeoxy chain termination.

Oocytes and Embryos—Adult female X. laevis were purchased from Xenopus 1 (Ann Arbor, MI). Ovary segments were surgically removed from females after anesthesia with 0.3% methanesulfonate salt of 3-aminobenzoic acid ethyl ester (Sigma). Oocytes were dissociated from ovary segments by digesting in a 2 mg/ml solution of Collagenase (Sigmod type IA) in OR-2 medium (Wallace, 1973) for 1-2 h, then rinsed extensively with OR-2. Dissociated oocytes were manually staged according to Dumont (1972). Total RNA was prepared from oocytes by homogenizing in 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% SDS, Carrier tRNA (50-100 μg) was added to prevent oligo(dT)12-18 from binding, and Dnase I-treated as above.

Microinjection of DNA into Oocytes—Closed circular plasmid DNA was prepared for injection by ethidium bromide-CsCl centrifugation and redissolution in 88 mM NaCl, 15 mM Tris-HCl (pH 7.5) at a final DNA concentration of 350–400 μg/ml. Microinjection of stage VI oocytes was similar to that of Nucleoplas (1981). Healthy looking, fully grown oocytes were selected and centrifuged for 10–20 min at 500 g (5 °C) to visualize the nucleus (germinal vesicle) just before injection (Kressmann et al., 1978). Twenty nI of DNA solution was injected into the nucleus. Injected oocytes were incubated in OR-2 medium for various times, and total RNA from oocytes was prepared as described above without Dnase I treatment. RNA was analyzed by Northern blot or primer extension as described below.

RNA Analysis—For a northern blot, RNA was purified as described above, fractionated in a 6% polyacrylamide-7 M urea gel, transferred to Duralon-UV nylon membrane (Stratagene) and cross-linked. Prehybridization was in 50% formamide, 5 × SSPE, 2 × Denhardt's, 0.1% SDS, and 200 μg/ml tRNA at 65 °C, then 5 × 106 cpm of 32P-labeled antisense RNA probe was added to the same solution, and incubation was continued at 65 °C overnight. Antisense RNA probes for mouse and Xenopus were prepared as described in the Genius nonradioactive labeling and detection kit (Boehringer Mannheim). Primer extension was performed with the XLPE oligonucleotide (5'AGCAGGGCGGATTATT-3') which is complementary to nucleotides 124-140 of Xenopus MRP RNA.

The resulting result was used to screen the mouse genomic DNA with pXLMU probe as described in the Genius nonradioactive labeling and detection kit (Boehringer Mannheim). Primer extension was performed with the XLPE oligonucleotide (5'AGCAGGGCGGATTATT-3') which is complementary to nucleotides 124-140 of Xenopus MRP RNA.
RESULTS

*Xenopus Tissue Culture Cells Contain an RNase MRP Processing Activity*—Dignam nuclear extracts of mammalian cells provide an ample source of RNase MRP (Karwan et al., 1991). To determine if *Xenopus* cells contain a similar processing activity, a nuclear extract was prepared from an *X. laevis* tissue culture line and fractionated over a 15–30% glycerol gradient (see “Materials and Methods”). Aliquots from individual fractions were assayed with the standard mtRNA substrate (Bennett and Clayton, 1990), and RNase MRP activity was assessed as described previously in Karwan et al. (1991) (Fig. 1). Consistent with its being an RNP, the RNase MRP activity from *Xenopus* cells sedimented in a broad peak near the bottom of the glycerol gradient (Fig. 1, fractions 1–9). Similar to mammalian RNase MRP, *Xenopus* nuclear RNase MRP displayed multisite processing on the mouse mtRNA substrate. Thus it is apparent that an endoribonuclease with grossly similar physical and biochemical characteristics to mammalian RNase MRP can be readily isolated from *Xenopus* tissue culture nuclear extracts.

*Xenopus Cells Contain an Immunoprecipitable RNA That Copurifies with RNase MRP Activity*—A discrete class of autoantiser (Th/To) immunoprecipitates two RNAs from human cells: H1 RNA, the RNA component of RNase P, and MRP RNA, the RNA component of RNase MRP (Gold et al., 1989). Doria et al. (1991) have previously used Th antisera in heterologous immunoprecipitation experiments to identify the RNA component of RNase P from *Xenopus* oocytes. In their study, immunoprecipitations on partially purified extracts identified a single RNA of 320 nucleotides which copurified with RNase P activity; no additional RNAs were noted. However, given the ability of Th antisera to precipitate both RNase P and MRP RNAs from mammalian cells, we reasoned that the Th epitope might be equivalently conserved in RNase MRP in *X. laevis* cells.

Immunoprecipitations using normal human sera and Th antisera were performed on crude *Xenopus* nuclear extracts (Fig. 2A). Using Th antisera, a single RNA species of ~280 nucleotides, similar in size to mammalian RNase MRP RNAs, was specifically immunoprecipitated (Fig. 2A, compare lanes 2 and 4 with lanes 1 and 3). Surprisingly, no RNA corresponding to *Xenopus* RNase P RNA was noted using either low or high salt washes (Fig. 2A, lanes 2 and 4; see “Discussion”). The lack of precipitation of RNase P RNA was not a result of a lack of specificity of the Th immune serum since control immunoprecipitations performed on mouse nuclear extracts produced equal amounts of murine RNase P and MRP RNAs (Fig. 2A, lane C).

In order to assign this 280-nucleotide RNA with RNase MRP activity, fractions from the glycerol gradient of Fig. 1 were subjected to immunoprecipitation with Th antisera, and the precipitated RNAs were end-labeled and analyzed by gel electrophoresis. As previously seen in immunoprecipitations from crude nuclear extracts, Th immune serum precipitated RNase P RNA which cosediments with RNase MRP activity. A, *Xenopus* nuclear extracts were prepared as described under “Materials and Methods.” Extracts (100 µl) were mixed with either normal human serum or Th autoantiserum, washed extensively in either 150 or 500 mM NaCl, and the coprecipitating RNAs were 3'-end-labeled with pCP and separated on a 6% acrylamide-7 M urea denaturing gel. *Lane 1*, antibody: normal human serum, wash: 150 mM NaCl; *lane 2*, Th-150 mM NaCl; *lane 3*, normal human serum-500 mM NaCl; *lane 4*, Th-500 mM NaCl; *lane C*, Th immunoprecipitation of mouse nuclear extract. *Arrowhead* denotes the ~280-nucleotide RNA precipitated from *Xenopus* nuclear extracts with Th antiserum. Mouse RNase P and RNase MRP RNAs are denoted at left. B, 150-µl aliquots of individual fractions from the glycerol gradient in Fig. 1 were subjected to anti-Th immunoprecipitation, and the resulting RNAs were pCP-end labeled and separated on a denaturing gel; *lane L*, Th immunoprecipitation of the total nuclear extract; *lane M*, HpaII-digested pBR322 DNA markers. The direction of sedimentation in the gradient is depicted below. *Arrowhead* denotes the 280-nucleotide RNA.
the 280-nucleotide RNA as well as several contaminating RNA species (Fig. 2B, lane L). The profile of the 280-nucleotide RNA across the glycerol gradient corresponded closely to that of the RNase MRP activity (compare fractions 1–9 in Figs. 1 and 2B). The peak amount of the 280-nucleotide RNA migrated roughly one fraction ahead of the peak of RNase MRP activity, a phenomenon previously seen with the mouse 275-nucleotide MRP RNA and RNase MRP activity (Chang and Clayton, 1987b). In order to verify that the 280-nucleotide RNA was not a degradation product of Xenopus RNase P RNA, RNase P activity was measured across the glycerol gradient of Fig. 1 (data not shown). The peak of RNase P activity (fractions 7–9) sedimented slightly behind the peak of RNase MRP activity (fractions 5–7) with the majority of RNase P-containing fractions having little or no 280-nucleotide RNA; no 320-nucleotide RNA was evident. Therefore, the 280-nucleotide Th-immunoprecipitable RNA copurifies with RNase MRP through gradient purification, consistent with its being the RNA component of the Xenopus activity.

Sequence Analysis of the Xenopus MRP RNA Gene—The gene for *X. laevis* MRP RNA was isolated from an EcoRI size-selected Xenopus genomic library using a partial cDNA of the copurifying 280-nucleotide RNA as a probe (see “Materials and Methods” for details). In order to determine the copy number of the gene, a 1.6-kb SacI-EcoRI DNA fragment containing the MRP RNA gene was used to probe enzymatic digests of *Xenopus* genomic DNA. In BamHI, HindIII, Xbal, and EcoRI digests of genomic DNA, the probe hybridized to a single band indicating that *Xenopus* MRP RNA is likely encoded by a single-copy gene (data not shown).

The sequence of the gene and flanking regions is shown in Fig. 3. Nucleotide +1, the transcriptional start site, was mapped by primer extension analysis (see Fig. 6B) and confirmed by analysis of *in vitro* capped MRP RNA (data not shown). Similar to other MRP RNA genes, the 5' end of the 277-nucleotide Xenopus MRP RNA coding region is abutted by 4 consecutive T residues, which could serve as an RNA polymerase III transcription termination signal. Similar to mammalian MRP RNA genes, the 5' flanking sequence contains promoter elements common to both RNA polymerase III and RNA polymerase III transcription units. Polymerase II elements include SP1 sites at -253 and -92, distal sequence elements (octamer motif) at -200 and -166, and a TATA box at -24. A polymerase III A box promoter element (consensus: RRYNNARYGG) is present at +171. A proximal sequence element common to many Xenopus U-series snRNA genes (Parry et al., 1989) is located at position -56.

**MRP RNA Gene Expression in Development—Replication of mtDNA is known to be developmentally regulated during *Xenopus* oogenesis and embryogenesis (Webb and Smith, 1977; Chase and Dawid, 1972). While nuclear DNA is arrested in prophase I of meiosis during oocyte development, mtDNA is greatly amplified from the previtellogenic oocytes (stage I) to stage IV oocytes. During late oogenesis and early embryogenesis, the amount of mitochondria remains relatively constant (Webb and Smith, 1977); however, later in development, the content of mtDNA again increases from the young tadpole (stage 30) to the swimming tadpole (stage 45) (Chase and Dawid, 1972). In addition, mtDNA displacement-loop (D-loop) frequency, a potential indicator of mtDNA synthetic activity, is higher in small oocytes and in tadpoles when mtDNA replication is active (Callen et al., 1983). Together, these data suggest that developmental regulation of mtDNA replication might be effected by the initiation of leading-strand synthesis, conceivably by the modulation of RNase MRP activity.

Northern blot analysis was performed to assay the MRP RNA expression pattern throughout *Xenopus* development (Fig. 4A). Total RNA was prepared from various stages of oocytes and embryos (Fig. 4A, upper panel). An abundant single species of 277 nucleotides was detected with the MRP RNA probe throughout oogenesis and embryogenesis. An identical RNA species was also detected in Northern hybridizations of total RNA from tissue culture cells (data not shown). Membranes were rehybridized with 5.8S rRNA (lower panel) and 5S rRNA (data not shown) probes as controls. Autoradiographs such as those shown in Fig. 4A were scanned, and the relative expression levels (MRP/5.8S) of various stages were plotted compared to that of stage VI oocytes (Fig. 4B). Consistent with previously published data on rRNA synthesis, the level of rRNA was greatly increased during oogenesis, reaching a plateau at stage III and remaining at an almost constant level throughout embryogenesis (Fig. 4A; Davidson, 1986). Higher levels of rRNA were isolated from stage I oocytes in the present study than noted in previous works (Taylor and Smith, 1985).

The developmental profile of MRP RNA expression was reproducible among the different animals used in these experiments.
under “Materials and Methods” probes were similar; however, exposure times were 1-2 days for the

Total RNA was fractionated in a 6% polyacrylamide-7 M urea gel and blot-hybridized with MRP RNA antisense probe as described under “Materials and Methods” (upper panel). The full length MRP RNA is shown in most lanes; some degradation products are seen in oocyte lanes. The same membranes were rehybridized with the 5.8s rRNA antisense probe (lower panel). Specific activities of the two probes were similar; however, exposure times were 1-2 days for the MRP probe hybridization and 10-20 min for the 5.8s rRNA probe. Oocyte stages are according to Dumont (stages I thru VI) (Dumont, 1972); total ovary (O) was also loaded. Embryo stages are as follows: E, unfertilized eggs; 9, blastula; 11, gastrula; 13-16, neurula; 22, late neurula; 38 and 45, tadpoles. B, graphic representation of MRP RNA expression. Northern blots as shown in A were scanned with a densitometer. Relative amounts of MRP transcripts were compared to that of stage VI oocytes and plotted at various stages of development (MRP, △). The amount of MRP transcript was normalized to the amount of 5.8s rRNA in each stage from the same membrane, then compared with the value from stage VI oocytes (MRP/5.8S, ▲). In the oocyte graph, three independent experiments were pooled, and the mean values are plotted along with the standard deviation. A representative set of data from three different frog developments is shown in the embryo graph.

experiments. The MRP RNA was detected throughout oocyte development with a steady increase in amount from stages I to IV, followed by a slight decrease in stages V and VI. However, after normalization, it is evident that the relative amount of MRP RNA in early stage oocytes is much greater than in later stages (Fig. 4B, oocytes). A relatively lower level of MRP RNA was detected in the unfertilized eggs (E) and early cleavage embryos with a slight increase in expression in the blastula stage (stage 9) followed by a temporary decrease in the neurula stages (stages 13-16). This decrease during neurulation displayed minor variability among different animals (data not shown). However, in every animal tested, MRP RNA gene expression was significantly increased later in development, from the late neurula stage (stage 22) up to the swimming tadpole stage (stage 45). Thus, MRP RNA becomes very abundant in the tadpole stage when mtDNA replication reinitiates (Chase and Dawid, 1972). The elevation in MRP RNA level occurs earlier (stage 22) than mtDNA replication (stage 30), perhaps secondary to a time delay necessary for

the assembly and transport of a sufficient amount of functional RNase MRP particles. Interestingly, mitochondrial transcription starts near stage 22, suggesting a potential coordinate activation of mitochondrial and nuclear genes for mitochondrial biogenesis.

We next investigated whether MRP RNA is assembled with MRP proteins in various oocyte stages (Fig. 5). Immunoprecipitation with a Th antibody coprecipitated MRP RNA from both stages I and VI oocytes, whereas normal human serum (NHS) failed to do so. Therefore, the abundant MRP RNA found in stage I oocytes is assembled into Th-immunoprecipitable particles. Interestingly, MRP RNA from oocytes and Xenopus tissue culture cells was not completely precipitated with an amount of Th antibody capable of depleting MRP RNA from mouse tissue culture cells. Further quantitative immunodepletion experiments will be necessary to judge the relative specificity of Th antisera for mammalian and Xenopus RNase MRPs. It is possible that some MRP RNA in oocytes is not completely assembled with its cognate proteins, is assembled with other proteins, or is present as free RNA.

Injection of the MRP RNA Gene into Oocytes—The gene for MRP RNA was injected into the nuclei of stage VI oocytes to test whether it could be transcribed and subsequently assembled with endogenous frog RNase MRP proteins. After various times of incubation, total RNA was prepared from the injected oocytes, and the newly transcribed MRP RNA was analyzed by Northern blots (Fig. 6A). Transcription of the exogenous MRP RNA gene template was quite efficient in stage VI oocytes (Fig. 6A, compare 0 with 16 h). The smaller RNAs present could be either degradation products of the full length RNA or prematurely terminated transcripts. A longer exposure of the same gel shows only intact endogenous RNA in un.injected oocytes (0 h) (data not shown), suggesting that RNA degradation during the experimental procedure is unlikely.

Primer extension of MRP RNA (Fig. 6B) demonstrates that the MRP RNA from the exogenous gene starts at the same guanosine nucleotide as the endogenous RNA (in vitro cap analysis, data not shown). Because the level of MRP RNA transcripts from the cloned gene was significantly higher than that from the endogenous gene (Fig. 6A), extension products complementary to RNA isolated from injected oocytes reflect

FIG. 4. Expression of MRP RNA. A, Northern blot analysis of MRP RNA expression during Xenopus development. All lanes contain total RNA from 2.5 oocytes or 10 μg of total RNA from embryos. Total RNA was fractionated in a 6% polyacrylamide-7 M urea gel and blot-hybridized with MRP RNA antisense probe as described under “Materials and Methods” (upper panel). The full length MRP RNA is shown in most lanes; some degradation products are seen in oocyte lanes. The same membranes were rehybridized with the 5.8s rRNA antisense probe (lower panel). Specific activities of the two probes were similar; however, exposure times were 1-2 days for the MRP probe hybridization and 10-20 min for the 5.8s rRNA probe. Oocyte stages are according to Dumont (stages I thru VI) (Dumont, 1972); total ovary (O) was also loaded. Embryo stages are as follows: E, unfertilized eggs; 9, blastula; 11, gastrula; 13-16, neurula; 22, late neurula; 38 and 45, tadpoles. B, graphic representation of MRP RNA expression. Northern blots as shown in A were scanned with a densitometer. Relative amounts of MRP transcripts were compared to that of stage VI oocytes and plotted at various stages of development (MRP, △). The amount of MRP transcript was normalized to the amount of 5.8s rRNA in each stage from the same membrane, then compared with the value from stage VI oocytes (MRP/5.8S, ▲). In the oocyte graph, three independent experiments were pooled, and the mean values are plotted along with the standard deviation. A representative set of data from three different frog developments is shown in the embryo graph.

FIG. 5. Immunoprecipitation of the RNase MRP particle from stages I and VI oocytes and tissue culture cells. Whole cell extracts were prepared from 75 stage I and 10 stage VI oocytes and immunoprecipitated with normal human serum (NHS lanes) or anti-Th human autoimmune serum (Th lanes). Ten μl each of the nuclear extracts of Xenopus (XTc) and mouse (L9) cells were also immunoprecipitated with Th antisera as controls. Pansorbin was used to precipitate immune complexes in this experiment. Bound (P, pellet) and unbound (S, supernatant) fractions were extracted, and coprecipitating RNAs were electrophoresed in a 6% polyacrylamide-7 M urea gel and subsequently blot-hybridized with a MRP RNA probe. Arrowheads indicate the full length MRP RNA.
Activities-Nuclear RNase MRP isolates from human or antisense probe as described under “Materials and Methods.” Since a short exposure is shown here, a very low level of endogenous MRP by the with a MRP RNA probe (Fig. 6C). Immunoprecipitation with Th-antiserum and RNA transcrption from the exogenous MRP RNA gene. Total RNA was prepared from the oocytes 5 h after injection and subsequently extended with reverse transcriptase after hybridization to the XLPE oligonucleotide. Lane 1, 12 μg of XTC total RNA; lane 2, 2 μg of injected oocyte total RNA. Sequencing ladders were generated by primer extension using the oligonucleotide XLPE. The 5′-end guanosine nucleotide is indicated RNA transcribed from the injected DNA. Immunoprecipitation with Th antisera and mammalian cellular extracts. Doria et al. (1991) employed partially purified oocyte nuclear extracts which could have been depleted of MRP RNA. A more interesting possibility is that a subtle distinction has evolved between RNase MRP and RNase P in X. laevis which can be revealed with different Th antisera.

Sequence Conservation among MRP RNAs—The overall percent similarity between Xenopus MRP RNA and mouse and human MRP RNAs is 66 and 63%, respectively. Several blocks of high sequence homology are noteworthy and are depicted in Fig. 3 (regions I, II, III, IV, and V). Region II has recently been shown to be sufficient for the binding of the Th/To 40-kDa protein in vitro (Yuan et al., 1991). Regions I, III, IV, and V demarcate sequence blocks previously shown to be conserved between MRP and RNase P RNAs (Gold et al., 1988; Forster and Altman, 1990). Regions I and III adjacent to the Th-antigen binding region II share a high degree of homology with their mammalian counterparts (92%). These regions may interact directly with region II to bind the Th autoantigen or, alternatively, may energetically stabilize a critical RNA secondary structure.

Two models have been proposed for the secondary structure of MRP RNA (Topper and Clayton, 1990b; Forster and Altman, 1990). Topper and Clayton (1990b) used chemical modification of the intact MRP RNP to identify single-stranded and double-stranded regions in the secondary structure of MRP RNA, and assembled an energetically favorable model based on their results. Forster and Altman (1990), hypothesizing from the antigenic relationship of RNase MRP and RNase P, proposed applying the phylogenetically conserved secondary structure of RNase P (James et al., 1988) onto the primary sequence backbone of MRP RNA. The pseudoknot proposed by Topper and Clayton (1990b) (see asterisks in Fig. 3) has two compensatory G:C nucleotide changes in Xenopus MRP RNA; the pseudoknot proposed by Forster and Altman (1990) shows 100% conservation.

The 5′-flanking sequence of the Xenopus MRP RNA gene is distinct from mammalian MRP RNA genes and other U-

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**Fig. 6. Analysis of transcripts after injection of the MRP RNA gene into oocytes.** A, Northern blot of time-course injection. DNA was injected into the nuclei of oocytes, and total RNA preparations were isolated after the indicated times of incubation. In each case RNA from four oocytes was isolated, fractionated in a 6% polyacrylamide-7 M urea gel, and blot-hybridized with the digoxigenin-labeled MRP antisense probe as described under “Materials and Methods.” Since a short exposure is shown here, a very low level of endogeneous MRP RNA is visible in this figure (see 0-h time point). The full length MRP RNA is indicated by the arrowheads. B, primer extension of MRP RNA transcribed from the injected DNA. Total RNA was prepared from the oocytes 5 h after injection and subsequently extended with reverse transcriptase after hybridization to the XLPE oligonucleotide. Lane 1, 12 μg of XTC total RNA; lane 2, 2 μg of injected oocyte total RNA. Sequencing ladders were generated by primer extension using the oligonucleotide XLPE. The 5′-end guanosine nucleotide is indicated by the asterisk. C, immunoprecipitation of MRP RNA from injected oocytes. Either nucleus (Nuc), cytoplasmic (Cyt), or whole cell (WC) extracts from six oocytes were prepared 16 h after injection of the MRP RNA gene. Immunoprecipitation with Th-antiserum and RNA detection were as described for Fig. 5. MRP RNA is visualized by Northern hybridization. Both pellet (P) and supernatant (S) fractions are shown. Arrowheads indicate the full length MRP RNA.
series snRNA genes in that it contains two distal sequence elements upstream of the transcriptional start site. Both distal sequence elements contain a single base mismatch from the consensus octamer motif. Although such single base transversions have been shown to be detrimental to promoter function both in vitro and in vivo, using synthetic promoter constructs (Staudt et al., 1986; Wirth et al., 1987), such stringent conformation to the octamer consensus has not been required when these enhancer elements are analyzed in their natural context (Atchison et al., 1990). It is thought that within these natural sequences nonconsensus octamer motifs are aided by DNA conformational changes caused by neighboring sequence elements or auxiliary protein factors bound to adjacent control sequences (Currie and Roeder, 1989; LeBowitz et al., 1989).

In the case of the Xenopus MRP RNA gene, the two distal sequence elements may act in combination to bind stably an appropriate upstream activator protein. More interestingly, the two MRP RNA gene octamer sequences might be utilized in different stages of development by different octamer binding proteins (Hinkle et al., 1992).

Correlation of MRP RNA Expression and mtDNA Replication—Early Xenopus Development—Expression of MRP RNA was examined to mtDNA replication during Xenopus development. In early oogenesis, mtDNA is amplified and forms a distinctive cytoplasmic structure called the mitochondrial mass (Haesman et al., 1984). By stage IV oocytes, the amount of mtDNA in a single oocyte is amplified to a level equal to $10^6$ somatic cell equivalents (Webb and Smith, 1977). MRP RNA can be detected throughout oogenesis; it begins to accumulate rapidly from stage I to IV oocytes, then declines slightly in fully grown oocytes, consistent with a potential role of RNase MRP in mtDNA replication (Chang and Clayton, 1987a). During oogenesis, total RNA is increased at least 30-40-fold (Davidson, 1986), most of it ascribed to rRNA synthesis (Taylor and Smith, 1985). Considering the great increase in total RNA, the relative percentage of MRP RNA in early stage oocytes is much higher than in later stage oocytes. In fact, in situ hybridization of a MRP RNA probe to early stage I oocytes shows a very strong nuclear signal, suggesting a high concentration of MRP RNA.

Mammalian RNase MRP is highly abundant in the nucleolus (Heimer et al., 1986), suggestive of a role in ribosomal RNA metabolism. In Xenopus, the number of rRNA genes is greatly amplified in early oocytes in order to meet the need for massive transcription of rRNA in later oogenesis (Brown and Dawid, 1968; Bird and Birnstiel, 1971). If RNase MRP were involved in this extrachromosomal replication of rRNA, it might be expected that RNase MRP should be abundant in early oocytes, especially in amplified nucleoli. The expression of MRP RNA in oogenesis and embryogenesis is distinct from that of U3 snRNA (Caizergues-Ferrer et al., 1991), a small nucleolar RNA critical for rRNA processing (Savino and Gerbi, 1990; Hughes and Ares, Jr., 1991). The time course of ribosome assembly during embryogenesis is closely matched to U3 expression (Caizergues-Ferrer et al., 1991) but quite different from MRP RNA expression, which occurs later in development. In oogenesis, MRP RNA expression clearly precedes that of U3 snRNA.

Following maturation of the oocyte to the egg, MRP RNA rapidly decreases to its lowest level and remains depressed during the early cleavage and blastula stages. During this period of Xenopus development, nuclear transcription is turned off and the mitochondrial genome is completely inactivated (El Meziane et al., 1989). Around the midblastula transition (Newport and Kirschner, 1982), a slight increase in MRP RNA was detected. During the midblastula transition, resynthesis of some polymerase III (5S RNA, tRNAs, and 7S RNA) and polymerase II (mostly snRNAs) transcripts (Forbes et al., 1983) occurs. Even though the MRP RNA promoter has characteristics of both polymerase II and III promoters, mammalian MRP RNA transcription is known to be mediated by RNA polymerase III in vitro (Yuan and Reddy, 1991). It would be of interest to learn if the MRP RNA gene promoter is an RNA polymerase II transcription unit at any time during early development. Stimulation of MRP RNA transcription at the midblastula transition was not as strong as other major polymerase III RNAs, with just a slight increase over its low level in the cleavage stage embryos.

The most pronounced and reproducible elevation of MRP RNA abundance occurs later in development, starting around the tailbud stage (stage 22). This time corresponds to the start of mtRNA transcription, which reaches its maximum at stages 34-37. Mitochondrial DNA replication, on the other hand, initiates slightly later, in the young tadpole stages. An almost linear accumulation of mtDNA from stages 30 to 45 leads to the eventual doubling of the mtDNA content per embryo (Chase and Dawid, 1972). The level of MRP RNA continuously increases at the onset of organogenesis, from the tailbud to the feeding tadpole stages (32-45), resulting in at least a 3-4-fold increase in MRP RNA.

The accurate expression of the MRP RNA gene and subsequent assembly of the transcribed MRP RNA upon injection into stage VI oocytes indicates that the gene is functional. Transcripts from the exogenous MRP RNA gene are at least 10-fold more abundant than the endogenous transcripts. These newly transcribed RNAs are exported to the cytoplasm and assembled with RNase MRP proteins. Although immunoprecipitable MRPs were present both in the nucleus and the cytoplasm after injection, further work is needed to establish the exact location of assembly. Having a system with proper transcription, export and assembly of MRP RNA affords the opportunity to study various aspects of intracellular transport and assembly of the RNase MRP ribonucleoprotein particle in biochemical detail.

Acknowledgments—We thank John C. Gerhart for help with Xenopus ooplasm and fertilization steps and Richard M. Harland for Xenopus embryo RNA. We also thank K. L. Chao, D. J. Dairaghj, J. L. Paluh, M. A. Parisi, and M. E. Schmit for comments on the manuscript.

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