Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 225-233

TRANSCRIPTION OF THE MRP RNA GENE IN FROG STAGE I OOCYTES REQUIRES A NOVEL *CIS*-ELEMENT*

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Received May 25, 1994

SUMMARY: The RNA component of the mitochondrial RNA processing (MRP) enzyme is related to both replication of mitochondrial DNA and processing of 5.8S rRNA, which are accelerated in the frog earliest stage (stage I) of frog oocytes. Microinjection of the deleted genes into the stage I oocytes showed positive *cis*-elements in the upstream region of the gene. The specific binding of protein(s) to this region was detected in cell extracts from stage I oocytes and liver but not in extracts of stage II-IV oocytes and the concentration of this protein was 40 times higher in the extract of stage I oocytes than that in liver.

Marked accumulations of rRNA and mitochondrial DNA occur in the early stage of development of frog oocytes. The mitochondrial RNA processing (MRP) enzyme digests mitochondrial RNA transcripts at a site of transition from RNA to DNA synthesis in the leading-strand of mitochondrial DNA during replication and is considered to create an RNA primer for mitochondrial DNA replication (1,2). The RNA component of MRP (MRP RNA) is encoded by a nuclear gene, transcribed by RNA polymerase III, and some of it is imported into mitochondria, although most is present in the nucleoli. Its function is the

*The nucleotide sequence date reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number: D30745; description, *Xenopus laevis* gene for binding to the stage I-rich nuclear protein.

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Abbreviations: bp, base pairs; DNase, deoxyribonulease; kbp, kilo base pairs; MRP enzyme, mitochondrial RNA processing enzyme; PGC, primary germ cell; PMSF, phenylmethylsulfonyl fluoride.

0006-291X/94 $5.00

processing of 5.8S rRNA (3). MRP RNA is expressed at a high level in the earliest stage (stage I) of *Xenopus* oocytes (4). Thus analysis of transcription of the MRP RNA gene is important for understanding the molecular events in the earliest stage of oocytes.

In this study, we found a novel element required for transcription of the MRP RNA gene in stage I oocytes of *Xenopus laevis* (*X.l.*) and *Rana brevipoda porosa* (*R.b.p.*). We also found a specific factor(s) that binds to this element in cell extracts from *R.b.p.* oocytes in stage I, but not in later stages. This factor was also detected in liver cells, although at 40 times lower level than in stage I oocytes. These findings imply that this factor regulates the biogenesis of mitochondria in the earliest stage of oogenesis.

EXPERIMENTAL PROCEDURES

Cloning of the X.l. MRP RNA gene and plasmid construction for transcriptional experiments: Adult females of *Xenopus laevis* (*X.l.*) were purchased from Hamamatsu Seibutsu-Kyozai (Shizuoka, Japan). cDNA was synthesized from total RNA of stage I oocytes with a random hexamer primer, and was amplified by the polymerase chain reaction with 100 pmol of the primers GCCTAGGGGAAAGTCCCCGGATC and AGCCGCGCTGAGAATGAGCCCCG. The λDASHII clones carrying a MRP RNA gene were isolated by hybridization using amplified cDNA as a probe. The DNA fragments carrying the MRP RNA gene were subcloned and sequenced (5). The plasmids used for transcriptional experiments carried a series of deleted MRP RNA genes between the *PstI* and *HincII* sites of pBluescriptII SK(+). All plasmids were purified by CsCl density gradient centrifugation.

Microinjection into Stage I oocytes: Rana brevipoda porosa (*R.b.p.*) is a common species in Japan. Females were collected from rice fields in Mibu-machi, Tochigi-Prefecture, Japan. One-year-old females have only stage I oocytes, whereas those of 4-7 years old females have stage II - VI as well as stage I oocytes. Oocytes were isolated from the ovaries of female *X.l.* (8 - 10 cm) and *R.b.p.* (2.5-3.5 cm length to obtain stage I oocytes, and 7-8 cm to obtain stage II-IV oocytes), by gentle swirling for 2 hours at room temperature in modified Barth solution [10 mM HEPES-KOH, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, pH 7.6] containing 100 units/ml of penicillin G , 100 mg/ml of streptomycin sulfate and 0.2 (w/v) % collagenase (Wako Pure Chemicals, Osaka, Japan). The isolated oocytes were passed through a ϕ 300 mm iron mesh, collected on a ϕ 70 mm iron mesh, washed with modified Barth solution, and staged manually on ice-cold plates as described (6). Then 2 nl of 10 mM Tris-HCl, 0.5 mM EDTA (pH 7.5), containing 100 pg of test construct, 100 pg of control template and 0.37 kBq of [α-^{32}P]GTP were injected into stage I oocytes using an AIS auto-microinjection system (Zeiss, Germany). pEF-BOS/βGAL (7) was

used as a control template. After the injection, oocytes were incubated for 12 to 18 hours at 18 ℃. Then total RNA was prepared by homogenizing 25 viable oocytes in 0.3 ml of 10 mM Tris-HCl, 1 mM EDTA, 1% sodium lauryl sulfate (SDS), and 0.3 M sodium acetate, pH 5.0, followed by two extractions with phenol/ chloroform (1:1) and precipitation with 3 volumes of 98 % ethanol. This RNA fraction was dissolved in 95 % formamide, 0.1 mM EDTA-NaOH, (pH 8.0) 0.025 % bromophenol blue, 0.025 % xylene cyanol FF and separated by electrophoresis in 6 % polyacrylamide gel containing 50 % urea in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2). Bands were visualized and their radioactivities were measured using an Imaging System BAS2000 (Fuji Photo Film Co., Tokyo, Japan). The sizes of transcripts were calculated by comparison with radiolabeled DNA fragments of known sizes.

Preparation of cell extracts and heparin-Sepharose binding proteins from oocytes and livers: Whole cell extracts were prepared from stage I oocytes, stage II-IV oocytes and the liver of *R.b.p.*. All procedures were performed on ice or at 4 ℃. Oocytes were isolated as described above and livers were isolated from the females of 3.5 cm length. The oocytes and liver were homogenized in a Teflon homogenizer with 3 volumes of Buffer C [20 mM HEPES-KOH, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF, pH 7.8]. The supernatants obtained by two centrifugation for 10 minutes at 65,000 rpm in a Beckman TLA100 rotor were dialyzed against Buffer E (20 mM HEPES-KOH, 25 % glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, pH 7.8) until the NaCl concentration was decreased to 100 mM. DNA binding factors were partially purified by heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column chromatography by elution with Buffer E containing 400 mM NaCl after washing with Buffer E containing 100 mM NaCl. Eluates were dialyzed against Buffer E containing 100 mM NaCl, frozen in liquid nitrogen and stored at -80 ℃. Protein concentrations were determined with a Biorad protein assay kit.

Electro-mobility shift assay (EMSA) and footprinting assay: The probe and specific double strand competitor used for EMSA corresponded to the region from -383 to -360. The region from -445 to -422 was used as a non-specific competitor. Double strand oligonucleotides were prepared as described previously (8). A portion of the eluate from heparin-Sepharose CL-6B was mixed with 10 fmol of the probe and 500 fmol of the competitor in Buffer E containing 100 mM NaCl, 1 μg of poly (dIdC)(dIdC) and 100 pmol of unrelated oligonucleotide. After incubation at room temperature for 20 minutes, the binding mixtures were subjected to 4 % polyacrylamide gel electrophoresis in 1X TBE as described previously (8). Probe DNA fragments for footprinting assay were prepared by the polymerase chain reaction using the oligonucleotides corresponding to the regions from -494 to -474 and from -211 to -194, respectively (9). Then 10 fmol of the resultant 301 bp DNA fragment was partially digested with 15 units of DNase I in 200 μl of Buffer E containing 40 μg of poly (dIdC)(dIdC), 3 mM $MgCl_2$, and 100 μg of the heparin-Sepharose binding fraction for 60 seconds on ice. DNA was extracted and subjected to electrophoresis by a reported method (9). Bands were visualized with the Imaging System BAS2000.

RESULTS AND DISCUSSION

We isolated a gene encoding *X.l.* MRP RNA from a non-selected genomic library and determined its nucleotide sequence from -2842 to +407 (Fig. 1). The our nucleotide sequence from -494 to +407 was 96 % homologous with the corresponding one reported from a *Xenopus* tissue culture (XTC) cell line (4). No discrepancy was found in the octamer-like sequences, a proximal sequence element or Box A except for the absence of a GC box-like sequence from -96 to -92 and different sequences of the TBP binding site. Search of the GenBank database using a GENETYX program (Software Co., Tokyo, Japan) revealed REM repeats and two putative repetitive sequences in the upstream region and a putative gene encoding a protein kinase C about ten kbp downstream from the MRP RNA gene (Fig. 1). The former finding implies that elements essential for transcriptional regulation of the MRP RNA gene are located downstream of -694.

To identify the region necessary for transcription of the MRP RNA gene in stage I oocytes, we developed a rapid and exact system for injection of DNA

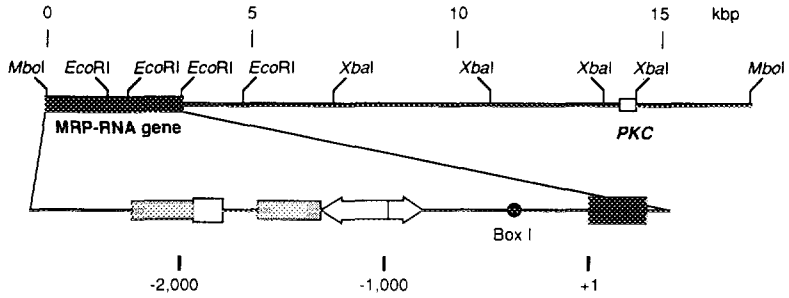

Fig. 1. Restriction map of λMRP13 carrying the *Xenopus l.* MRP-RNA gene. The upper bar shows the restriction map and genes encoding MRP RNA (filled box) and a putative protein kinase C (open box) with the scale numbered from a *MboI* site upstream of the MRP RNA gene, adjacent to the λ phage DNA arm. The lower bar shows the detailed structure of the MRP RNA gene. The transcribed region (filled box), two REM repeat relatives (arrows), the putative repetitive sequence A (dotted boxes) from -2310 to -2018 and from -1737 to -1400 of the MRP RNA gene 75 % homologous to that in the fifth intron of the *X.l.* ribosomal protein L14 gene (13), the sequence B (open box) from -2017 to -1903 of the MRP RNA gene 72 % homologous to one in the 3' noncoding region of the human hypoxanthine phosphoribosyltransferase (HPRT) (14) and 75 % homologous to one in the promoter region of human formylpeptide receptor gene (Perez, H. (1992) unpublished) were shown with the scale numbering from the transcription start of the MRP RNA gene. The position of the putative *cis*-element, Box I, is indicated by a closed circle.

into the oocytes. More than 99 % of the oocytes survived for 24 hours after injection of DNA, and the amount of template DNA injected (200 pg) was shown to be within the linear range for expression of the genes. We used mainly oocytes of *R.b.p.* , which is a common species in rice fields in Japan and in which development of oocytes shows a similar time course to that in *X.l.*. After microinjection of plasmid pMRP64 carrying the region from -839 to +407 of the MRP RNA gene, a transcript of the 277 bases was detected (Fig. 2). This result indicates that the region from -839 to +407 of the MRP RNA gene is enough for transcriptional initiation and termination in stage I oocytes of *R.b.p.*. A series of 5'-deletion constructs was introduced into stage I oocytes of *R.b.p.* to determine the elements required for transcription of the MRP RNA gene (Fig. 2). pMRP64d3 carrying the region from -403 and pMRP64d4

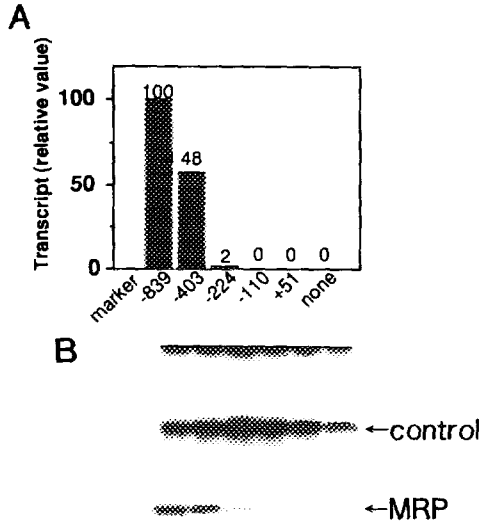

Fig. 2. Transcription of deleted MRP RNA genes in stage I oocytes.
A, The levels of transcripts from the series of deletion plasmids in stage I oocytes are shown by bars, normalized with control transcripts from pEF-BOS/βGAL. The numbers -839, -403, -224, -110 and +51 show the values for pMRP64, pMRP64d1, pMRP64d3, pMRP64d4 and pMRP64d5 corresponding to lanes 2 to 6 in panel B. 'None' shows the value for no plasmid carrying a MRP RNA gene, corresponding to lane 7 in panel B. The relative values in the figure are total densities of transcripts measured with BAS2000, taking the value for pMRP64 as 100 %. B, Autoradiogram of transcripts from the deletion plasmids and control plasmids with size marker DNA fragments in lane 1. MRP RNA and control RNA transcripts are indicated by arrows.

carrying the region from -110 showed 2 % and 0 % , respectively, of the transcriptional activity of pMRP64, whereas pMRP64d1 carrying the region from -403 showed 48 % (Fig. 2) of this activity. These results indicate the presence of positive elements in the region from -403 to -224, which contains no sequence homologous to any known elements for transcription by RNA polymerase III and of elements essential for transcription in the region from -224 to -110, which contains two octamer-like sequences. pMRP64d2 carrying the region from -311 had <1 % of the transcriptional activity of pMRP64, in *X.l.* stage I oocytes, indicating that the region upstream of -311 is necessary for transcription (data not shown). To determine the sites binding to nuclear proteins located from -403 to -224, we analyzed the upstream region interacting with DNA binding proteins by DNase I footprinting assay. Only one footprint was detected in the region from -375 to -368 (Fig. 3) using the heparin-Sepharose-binding fraction from *R.b.p.* stage I oocytes. The protected region corresponded to ACTTTATT and was designated as 'Box I'. Specific binding of protein(s) to Box I was also detected by EMSA (Fig. 3) using a 24 bp double strand oligonucleotide corresponding to the region from -383 to -360. The binding of the factor(s) to Box I was competitively inhibited by an excess amount of the oligonucleotide corresponding to Box I, but was not inhibited by a sequence corresponding to the region from -445 to -422 (Fig. 4). The binding of the protein(s) to Box I was not inhibited by an excess amount of DNA fragments corresponding to the region from -319 to -70 or from -70 to +117 of the MRP RNA gene or of the region corresponding to +11 to +110 of the *X.l.* somatic-type 5S RNA gene (10). These results suggest that Box I interacts with a specific DNA binding protein(s).

To determine the relationship between the level of the Box I-binding factor and mitochondrial biogenesis, we examined the specificity of the Box I-binding factor(s) in stage I oocytes, in comparison with stage II-IV oocytes and liver of *R.b.p.*. We detected a 4 times lower concentration of Box I-protein binding complex in the heparin-Sepharose-binding fraction of liver than in that stage I oocytes and none in the fraction from stage II-IV oocytes by EMSA (Fig.4, lanes 7-15). The heparin-Sepharose-binding fractions of stage I oocytes and liver contained 50 % and 5 % of the total protein and 50 % and 50 % of the Box I-binding activities, respectively. These results indicate that stage I oocytes contain 40 times more Box I-binding factor per total protein than liver. One stage I oocyte is estimated to contain >100 more Box I-binding

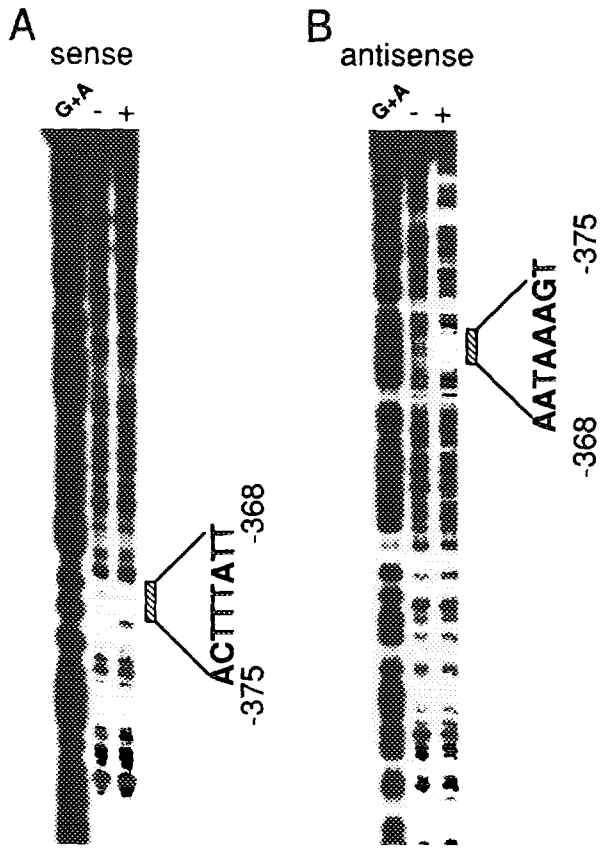

Fig. 3. Footprinting assay of the upstream region of the MRP RNA gene.
A 301 bp fragment covering the region from -403 to -224 was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at the 5' end of the sense strand (panel A) and antisense strand (panel B). The fragments were digested by DNase I without cell extract (middle lanes) and with 100 mg of the heparin-Sepharose-binding fraction from stage I oocytes (right lanes). Digested fragments were subjected to denaturing polyacrylamide gel electrophoresis with size markers generated by partial chemical digestion of the 301 bp fragment (left lanes). The footprint is indicated by a hatched bar in each panel with the corresponding sequences, designated as Box I.

factor than one liver cell. Quantification of the DNA-protein complexes from their radioactivities showed that 10^6 - 10^7 box-binding factor molecules are present in one stage I oocyte, consistent with accelerated biogenesis of mitochondrial DNA and 5.8S rRNA.

It is important to understand the molecular events involved in the earliest stage of oogenesis and to examine whether other genes require Box I-like sequences for transcription by RNA polymerase III in stage I oocytes. There are no comparable reports on transcriptional regulation by RNA polymerase III

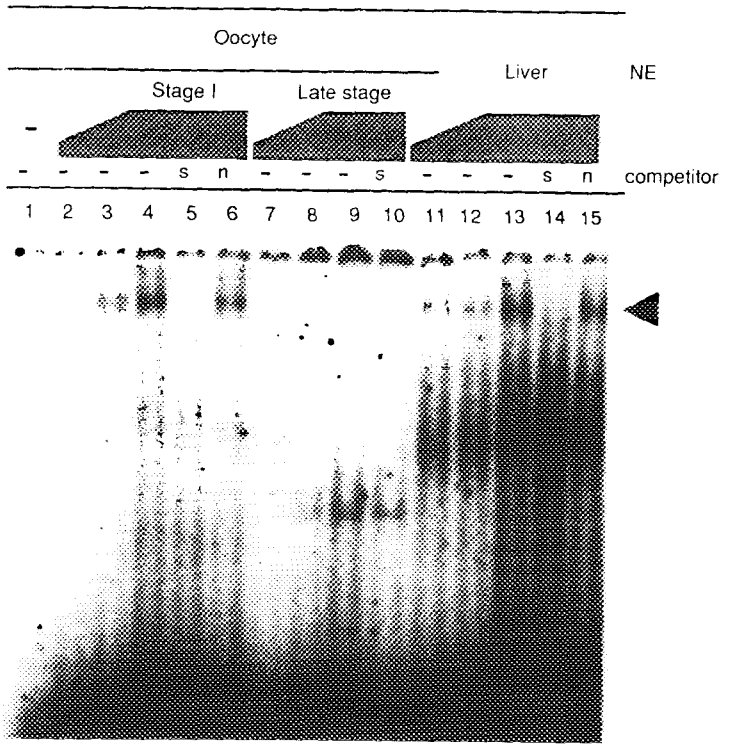

Fig. 4. Box I-binding factor highly specific to stage I oocytes. Preparation of cell extracts and EMSA were performed as described in **Experimental Procedures.** The binding mixtures contained no protein in lane 1 and 0.2, 0.4 0.8, 0.8 and 0.8 μg of heparin-Sepharose-binding fraction from stage I oocytes in lanes 2 to 6, respectively; 5, 10, 20 and 20 μg of the same fraction from stage II-IV oocytes in lanes 7 to 10, respectively, and 0.5, 1, 2, 2 and 2 μg of the same fraction from liver in lanes 11-15, respectively. The binding mixtures for lanes 5, 10 and 14 contained 50-fold levels of the unlabeled double strand oligonucleotide of the probe as a specific competitor (s), while those for lanes 6 and 15 contained 50-fold the corresponding region from -445 to -422 as a non-specific competitor (n). The specific DNA-protein complex is indicated by an arrowhead.

in stage I oocytes. The genes encoding oocyte-specific 5S rRNA and tRNAs were highly transcribed in stage II oocytes whereas no increase of the MRP RNA was detected in stage II-VI oocytes. The level of TFIIIA, which is essential for transcription of 5S rRNA genes, is regulated during oogenesis by the B3 site in earlier stages and by the B2 site in later stages (11,12). Protein binding to the B3 site was detected in extracts from stage III oocytes, whereas that to Box I was not detected in extracts from stage II-IV (12).

ACKNOWLEDGMENTS

The second author contributed as much to this work as the first author.
We thank Dr. Yuriko Suzuki-Yagawa for providing plasmid pEF-BOS/βGAL, Miss Tazuko Ono for technical assistance and Miss Eriko Ueno for oligonucleotide synthesis.

REFERENCES

1. Chang, D.D., Hauswirth, W.W., and Clayton, D.A. (1985) *EMBO J.* **4**, 1559-1567.
2. Chang, D.D., and Clayton, D.A. (1987) *EMBO J.* **6**, 409-417.
3. Schmitt, M.E. and Clayton, D.A. (1993) *Mol. Cell. Biol.* **13**, 7935-7941.
4. Bennett, J.L., Jeong-Yu, S., and Clayton, D.A. (1992) *J. Biol. Chem.* **267**, 21765-21772.
5. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
6. Dumont, J.N. (1972) *J. Morphol.* **136**, 153-180.
7. Suzuki-Yagawa, Y., Kawakami, K., and Nagano, K. (1992) *Mol. Cell. Biol.* **12**, 4046-4055.
8. Tomura, H., Endo, H., Kagawa, Y., and Ohta, S. (1990) *J. Biol. Chem.* **265**, 6525-6527.
9. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic, San Diego).
10. Peterson, R.C., Doering, J.L., and Brown, D.D. (1980) *Cell* **20**, 131-141.
11. Scotto, K.W., Kaulen, H., Roeder, R.G. (1989) *Genes Dev.* **3**, 651-662.
12. Pfaff, S.L., and Taylor, W.L. (1992) *Dev. Biol.* **151**, 306-316.
13. Beccari, E., and Mazzetti, P. (1987) *Nucleic Acids Res.* **15**, 1870-1872.
14. Kim, S.H., Morres, J.C., David, D., Respess, J.G., Jolly, D.J., and Friedmann, T. (1986) *Nucleic Acids Res.* **14**, 3103-3118.