Xenopus laevis ribosomal protein genes: isolation of recombinant cDNA clones and study of the genomic organization

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ABSTRACT
Poly-A+ mRNA from Xenopus laevis oocytes, partially enriched for r-protein coding capacity has been used as starting material for preparing a cDNA bank in plasmid pBR322. The clones containing sequences specific for r-proteins have been selected by translation of the complementary mRNAs. Clones for six different r-proteins have been identified and utilized as probes for studying their genomic organization. Two gene copies per haploid genome were found for r-proteins L1, L14, S19, and four-five for protein S1, S8 and L32. Moreover a population polymorphism has been observed for the genomic regions containing sequences for r-protein S1, S8 and L14.

INTRODUCTION
The eukaryotic ribosome biosynthesis is a complex process involving the coregulated expression of the two sets of genes for the structural components of the ribosome: rRNA genes and r-protein genes. While much information has been accumulated regarding the structure and expression of the rRNA genes, very little is known about the protein counterpart. To approach the study of the structure, organization and expression of r-protein genes in eukaryotes, we had first to prepare the suitable probes. This did not seem a simple task to realize because the r-protein mRNA consists of a heterogenous class of about 70 different sequences not highly represented in the cell. The X. laevis oocyte seemed to us a promising system from which to obtain an mRNA preparation fairly rich in sequences specific for r-proteins. In fact in consideration of the massive synthesis of ribosomes during oocyte growth one would have expected high levels of r-protein mRNA, probably more than in somatic tissues. Moreover the X. laevis
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oocyte together with the early embryonic developmental stages are interesting systems for studying the regulation of the synthesis of the ribosome components. In a previous work (1) we characterized such an mRNA fraction from X. laevis oocytes enriched for r-protein coding capacity. In the present paper we report the construction of recombinant plasmids containing cDNA to the above mentioned mRNA fraction and the selection of clones containing sequences for six r-proteins. The screening of the cloned sequences was carried out by the electrophoretic analysis of the proteins directed in vitro by the mRNA hybridized to each of them. Using these recombinant plasmids as probes it has been shown that in the X. laevis genome the r-protein genes are present in few copies (two to five per haploid genome) and that some of them are found in restriction fragments variable in the X. laevis population.

Recently the isolation and preliminary characterization of recombinant DNA molecules containing r-protein genes have been reported for yeast (2), Drosophila (3) and mouse (4).

MATERIALS AND METHODS

mRNA preparation

mRNA was prepared and fractionated as previously described (1).

Synthesis of double stranded DNA complementary to mRNA

Single stranded DNA complementary to the mRNA preparation was synthesized essentially as described by Efstradiatis (5). Synthesis of the second strand was performed in 0.12 M potassium phosphate pH 6.8, 10 mM MgCl₂, 10 mM DTT, 200 µM dGTP dATP dTTP, 180 µM dCTP, 2 nanomoles of ³H-dCTP (Amersham) and 50 units of E.coli polymerase I with a final yield of 95% after 6 h incubation at 15°C.

The product was isolated by Sephadex G75 gel filtration and concentrated by ethanol precipitation. Double stranded cDNA was resuspended in 0.2 M NaCl, 50 mM Na-acetate pH 4.6, 1 mM ZnSO₄, 150 µg/ml of 4S RNA and was digested with S1 nuclease to hydrolyze the hairpin loop connecting the two strands. After 30 min incubation at 37°C the mixture was
phenol-chloroform extracted and sedimented through 5-20% sucrose gradient in 0.1 M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA at 30,000 rpm for 16 h at 20°C in a Spinco SW 41 rotor. Material sedimenting more than 6S were collected and ethanol precipitated.

**Blunt end ligation to synthetic linkers**

Before ligation to linkers the double stranded cDNA was repaired with E.coli polymerase I in 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 50 μM ATP and 200 μM of the four dNTP. The reaction was allowed to proceed for 25 min at 15°C.

100 ng of double stranded cDNA were ligated in the same buffer with a 50 molar excess of EcoRI chemically synthesized linkers in 25 μl at 20°C for 4 h. The mixture was then brought to 100 μl of EcoRI digestion buffer heated at 80°C for 5 min and digested with 20 units of EcoRI for 1 h at 37°C.

After digestion the mixture was loaded on a 5-20% sucrose gradient in 0.1 M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA and run at 30,000 rpm for 17 h in a Spinco SW 41 rotor. Material sedimenting more than 6S were collected and ethanol precipitated.

**Preparation of recombinant DNA**

pBR322 DNA was linearized with EcoRI, monitored by electrophoresis on agarose gel and by direct visualization under the electron microscope. The restriction endonuclease digestion was followed by incubation with bacterial alkaline phosphatase (Warthington, BAPF) for 60 min at 54°C. Reaction mixtures were phenol and chloroform extracted and ethanol precipitated.

23 ng of double stranded cDNA attached to linkers were incubated with 250 ng of EcoRI digested and alkaline phosphatase treated pBR322 DNA in 30 μl of a mixture containing 50mM Tris-HCl pH 7.5, 10mM MgCl₂, 1mg/ml bovine serum albumine, 10mM DTT, 70 μM ATP and 0.3 units of T₄ DNA ligase. The reaction was allowed to proceed for 2 h at 0°C.

**Transformation**

Recombinant DNA was used to transform the E. coli K-12 strain HB101 essentially as previously described (6).
The mixture containing the transformed bacteria was plated on ampicillin plates (50 μg/ml) and the resistant colonies were picked onto Millipore filters (HAW604700) for the colony hybridization according to Grunstein and Hogness (7).

**Colony hybridization**

Colony hybridization was carried out in 2XSSC, 0.2% SDS, 10 μg/ml E. coli 4S RNA at 65°C for 12 h in the presence of 5X10⁶ cpm/ml of ²³P-cDNA (specific activity 50 x 10⁶ cpm/μg). The hybridization was performed in plastic bottles rotating in a 65°C incubator allowing the utilization of very small volumes of hybridization mixture.

**Recombinant plasmid purification**

Plasmid purification was performed with the acidic phenol extraction essentially as described by Zasloff et al (8). Cells were suspended in saline EDTA (0.1M EDTA, 0.15M NaCl pH 7.8) + 25% sucrose and lysed with 2 mg/ml Lysozyme at 0°C for 15 min. Sodium-N-laurylsarcosinate to 0.2% was added and the lysate incubated at 60°C for 15 min. The samples were diluted and made 1M in NaCl and extracted twice with pH 7.5 saturated phenol-chloroform (2:1) and once with chloroform-isoamylic alcohol (24:1).

Ethanol precipitated DNA was collected on glass rods, dissolved in water and brought to 75 mM NaCl and 50 mM Na-acetate pH 4.0. 2-3 extractions with 50 mM Na-acetate pH 4.0 saturated phenol completely eliminated the chromosomal DNA from the water phase. After neutralization, DNA was precipitated and dissolved in 0.3 M Na-acetate pH 7.0; 0.54 volumes of isopropllic alcohol selectively precipitated DNA leaving contaminating RNA in the supernatant.

When necessary plasmids were further purified by CsCl ethidium bromide centrifugation procedure.

**Hybridization and elution of mRNA**

20 μg of DNA from single recombinant plasmids were boiled 5 min in 0.15 N NaOH, diluted 20 times in 2M NaCl and loaded onto 0.8 X 4.0 cm preboiled Millipore filters. The filters were dried 2 h at 80°C and placed in groups of 10 into plastic scintillation vials and allowed to hybridize in a
rolling apparatus at 37°C for 20 h with 12 µg of mRNA in 600 µl of 50% formamide, 75 mM NaCl, 100 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS. At the end of the incubation the filters were washed 30 min at 37°C in the same hybridization buffer, 1 h in 0.1XSSC, 0.2% SDS at 52°C and 4 times in cold 0.1XSSC to avoid SDS presence on the filters. These were minced in Eppendorf tubes and the hybridized RNAs were eluted by two sequential washes at 100°C for 1 minute in 200 µl of 10 mM KCl. Each sample was ethanol precipitated after adding 0.4 µg of X. laevis tRNA and 0.3 M sodium acetate. tRNA was shown not to contain any coding capacity.

In recent experiments we directly spotted on 4 X 8 cm Millipore filters 32 different recombinant plasmid native DNAs; denaturation and neutralization were performed in situ according to the methods used for colony or plaque hybridization. After hybridization and washings the filters were cut in the 32 parts and the RNAs singularly eluted as above described. We found that 4 µg of DNA spotted on the filters were still sufficient to give good results on the positive translation analysis.

In vitro translation and analysis of products

mRNAs eluted from filters were translated in a rabbit reticulocyte lysate system (NEN translation kit) containing 35S-methionine (1000 Ci/mmole). RNA was dissolved in 12.5 µl incubation mixture and incubation was carried out for 60 min at 33°C. Stimulation above the endogenous synthesis was not detectable. After incubation samples were extracted with 66% acetic acid and analyzed on a two dimensional acrylamide gel electrophoresis as previously described (1). Purified X. laevis ribosomal protein markers (100 µg) were added to each sample before electrophoresis. Usually 2/3 of the incubation mixture were loaded on the 1st dimension gel. Gels were stained, destained and fluorographed either in a DMSO-PPO mixture according to Laskey (9) or in 8 volumes of 1 M sodium salicylate solution shaking for 1 h. Dried gels were exposed to preflashed Kodak-X-Omat films at -70°C.

Peptide mapping

For analysis of peptides, acid extracts of in vitro synthesized r-proteins
were loaded on preparative two dimensional gels together with 1 mg of purified r-protein markers. The stained spots, containing the \textsuperscript{35}S in vitro synthesized products were punched out from the gel, equilibrated for 15 min and placed in wells of a 17% acrylamide SDS gel for digestion with Staphylococcus aureus V8 protease (14). After staining, gels were fluorographed as described above.

**Restriction endonuclease digestion and electrophoresis of DNA**

*X. laevis* DNA was extracted from erythrocytes of single individuals and purified by CsCl density gradient centrifugation.

Restriction endonucleases were either purchased from Boehringer (EcoRI, Hind III) or prepared in our lab (BamHI, PstI, BspI, Bgl II). The digestions were performed at 37°C with the appropriate amount of enzyme necessary to achieve complete digestion at a DNA concentration of 200 \(\mu g\) /ml. The digested DNA was run on a 1% agarose gel in Loening buffer (10) for 14 h at 40 volts. The gels were stained with ethidium bromide and photographed under UV lamp through a red filter; the DNA was transferred to nitrocellulose paper essentially as described by Southern (11).

For preparative purification of the inserted sequences from the recombinant plasmids, the EcoRI digested DNA was electrophoresed on 4% acrylamide gel for 3 h at 150 volts. After ethidium bromide staining the lower molecular weight band was cut and the DNA eluted.

**DNA labelling**

DNA was radioactively labelled in vitro by nick translation (12) to specific activities in the order of \(10^8\) cpm/\(\mu g\).

Radioactive cDNA complementary to mRNA was synthesized according to Efstradiatis (5) in the presence of \(\alpha^32\text{P}-dATP\) to specific activities of 2-5 \(\times 10^7\) cpm/\(\mu g\).

**DNA hybridization**

For DNA-DNA hybridization the filters were preincubated 3-6 h in Denhardt's medium (0.02% each Ficoll, Polyvinylpirrolidone, BSA in 2XSSC) at 65°C. The hybridizations were performed in rolling bottles at 68°C in 2XSSC, 0.2% SDS, 10 \(\mu g/ml\) of E. coli 4S RNA for 15 h. At the
end of the incubation the filters were washed for 30 min at 68°C in 2XSSC and 2 h at 54°C in 0.1XSSC. Filters were exposed to preflashed Kodak X-Omat films at -70°C.

RESULTS

Isolation of a poly(A)⁺RNA fraction enriched for r-protein mRNA

In a previous paper (1) we showed that the poly(A)⁺RNA prepared from X. laevis oocytes (stage II and III) has a good template activity for r-proteins. This activity was tested both in wheat germ and rabbit reticulocyte lysates; r-proteins were identified among radioactive products on the basis of comigration with purified markers (Fig. 1) in a two dimensional acrylamide gel electrophoresis optimized for basic proteins (13). This mRNA preparation was enriched in r-protein activity by subsequent passages on oligo (dT)-cellulose columns and by selection of a 10-16 S fraction by sucrose gradient centrifugation. It has been estimated that all together the 70 r-proteins account for 10-20% of the products coded in vitro by this mRNA fraction.

Fig. 2a shows the two dimensional pattern of radioactive products coded by this mRNA fraction which has been used in all the experiments described in the present paper and which we shall refer to as rpe-mRNA (r-protein enriched mRNA).

In the present work the rabbit reticulocyte has been preferred to the wheat germ lysate because there is no background due to endogenous synthesis in the gel region where r-proteins migrate. This is an important condition for the detection of faint signals expected in the positive translation procedure.

Cloning of the DNA complementary to the rpe-mRNA

Single stranded DNA complementary to the rpe-mRNA fraction was synthesized with AMV reverse transcriptase using oligo-dT as primer. cDNA was converted to its duplex form with E. coli pol-1 and S1 nuclease was used to open the terminal hairpin loop and to degrade any single stranded DNA.

To allow for the reversible insertion into a bacterial plasmid the ds-cDNA was given sticky ends by enzymatic blunt end ligation to chemically
Figure 1. Two dimensional gel electrophoresis of proteins from X.laevis oocyte ribosomes (a) and a numbered scheme for 40S and 60S components (b). For details see ref (1). Positions of MW standards are given.

synthesized EcoRI linkers and subsequent cleavage with the same enzyme.

The DNA was then inserted into the EcoRI site of plasmid pBR322 previously treated with bacterial alkaline phosphatase to remove the 5' terminal phosphate group.

These hybrid molecules were used to transform E. coli Hb101 and the transformed clones were selected on ampicillin plates.

Among the resistant colonies those containing sequences complemen-
Figure 2. Two dimensional analysis of radioactive products coded by: a) rpe-mRNA, b) no mRNA added, c-h) mRNA hybridized to recombinant plasmid pXom 62, 69, 78, 91, 92, 102. The large spot present in b to h is globin from residual endogenous synthesis of the reticulocyte system.
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tary to the rpe-mRNA preparation were selected by colony hybridization to $^{32}$P-cDNA. In this way we selected 127 recombinant clones (pXom 1-127). All of them were individually grown and the plasmids purified. The size of the inserted fragments was analyzed by EcoRI digestion. The average length of the insertions is about 450 bp with values ranging from 150 to 1100 bp.

Selection of clones containing sequences specific for r-proteins

Among the methods used for clone selection some involve the identification of the translation products encoded by the mRNAs either hybridized (positive translation) or not hybridized (negative translation) to single clone DNA. In our case, where the mRNA for each r-protein is not abundant, the negative translation has shown to be not suitable. In fact the hybridization kinetics, the translation efficiency variability and the intrinsic complexity of the mRNA population do not allow an unambiguous interpretation of the two dimensional gel electrophoresis. In consideration of all these facts a positive method seemed to be more reliable for our purpose.

Recombinant DNA from each clone was bound to Millipore filters and incubated with rpe-mRNA in conditions which allow hybridization also of low abundance mRNA species with minimal degradation of RNA. The selectively bound mRNA molecules were eluted from filters and translated in a reticulocyte cell free system. The products, after acid extraction, were analyzed on a two-dimensional gel electrophoresis. In order to be able to identify the synthesized products, non radioactive r-protein markers were added before electrophoresis. The majority of the 127 clones analyzed in such a way didn't give any radioactive spot besides the background due to endogenous synthesis. This was expected since the electrophoretic method is such to exclude some acidic products from entering the first dimension gel. Moreover some cloned sequences are expected to hybridize to rare mRNA with a reassociation kinetics too slow to give hybridized mRNA sufficient to direct the synthesis of a detectable product; proteins lacking methionine are also overlooked by this screening. 24 clones gave a positive result, namely a clear radioactive spot not due to endogenous synthesis. They were all checked at least twice with reproduc-
ble results. Out of the 24, 13 clones hybridized to mRNAs coding for products other than r-proteins. They were present among the translation products of the rpe-mRNA fraction but remain at the moment mostly unidentified. Only one of these clones (pXom 32) has been identified; it contains sequences corresponding to histone H3 as demonstrated by running the two dimensional electrophoresis with Xenopus histones as non radioactive markers. As summarized in Table 1, 11 clones gave positive results for identifiable r-proteins (Fig.2). Clones pXom 11, 58, 62 carry sequences coding for a protein comigrating with the ribosomal protein S8. Similarly pXom 92 corresponds to L14, pXom 69 and pXom 119 to S19, pXom 37 and pXom 91 to S1, pXom 97 and pXom 102 to L1. The identification has been confirmed comparing the peptide map obtained by digestion with S. aureus V8 protease (14) of the in vitro translation products and the corresponding r-protein markers (Fig.3).

**Copy number of r-protein genes**

We used the selected clones to study the frequency with which r-protein genes are present in the X. laevis genome. For this purpose we followed two different approaches. pXom 62 and pXom 69 have been used as a probe in a Cot analysis of genomic DNA. Trace amount of highly radioactive inserted sequences from the clones have been added to a great excess of DNA purified from X. laevis erythrocytes. As shown in Fig.4 while genomic DNA has a complex reassociation profile including highly, middle repetitive and unique sequences, the radioactive trace is driven to reassociation by

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Figure 3. a, b and c show the stained pattern of digestion with S. aureus V8 protease of marker r-proteins S1, S19 and L14. a', b' and c' show the fluorography of digested radioactive proteins coded by mRNA hybridized to clones pXom 91, pXom 69 and pXom 92 respectively. Only peptides containing methionine are labeled.

Figure 4. Reassociation kinetics of trace amounts of $^{32}$P-labeled pXom 69 inserted sequence (o—o) driven by total genomic DNA of X. laevis erythrocytes (——).
the last ones.

These and the other r-protein clones have been used as probes for Southern blot analysis. As an example Fig. 5 shows the hybridization pattern of pXom 69 to genomic DNA from an adult X. laevis female. The hybridization pattern of the DNA digested with EcoRI, BamHI, HindIII, BglII, enzymes which do not cut the sequence inserted in the recombinant plasmid, give always two bands; on the other hand PstI, which does cut the insert (not shown), gives four hybridization bands. This result shows that the ge-

![Figure 5](image)

**Figure 5.** Southern blot analysis of DNA (5 μg), from a single individual digested with BglII, HindIII, BamHI, PstI, EcoRI and hybridized to $^{32}$P-pXom 69. Molecular weight standards were derived from EcoRI and HindIII digestions of λ DNA.

**Figure 6.** Southern blot analysis of DNA (5 μg) from six different individuals (3 males and 3 females) digested with BamHI and hybridized to $^{32}$P-pXom 69.
nes for r-protein S19 are contained in restriction fragments of two different sizes. These two classes of fragments do not represent allelic forms; in fact, hybridization patterns of BamH1 digested DNA from different individuals are identical (Fig. 6). At variance with the genes for protein S19 those for L14 appear to be located on genomic regions which differ in different individuals (Fig. 7). Similar heterogeneity has been found for S1 and S8 and not for L1 and L32 genes (not shown).

In a calibration experiment (Fig. 8a) we compared the intensity of the two HindIII bands with that of an internal standard of hybridization reaching the conclusion that each band corresponds to two genes per haploid genome. Two genes per haploid genome have been found also for r-proteins L1, L14 (pXom 102, 92). The gene copy number for proteins S8 (pXom 62) is higher than two. As shown in Fig. 8b, comparing the band intensity of the internal standard with that of X. laevis DNA it's evident that the reiteration of this specific sequences is about 4-5 copies per haploid genome. Similarly 4-5 copies have been found for S1 (pXom 91) and L32 (pXom 78).

**Figure 7.** Southern blot analysis of DNA (5 μg) from six different individuals (3 males and 3 females) digested with BamH1 and hybridized to $^{32}$P-pXom 92.
Figure 8. Determination of gene copy number for r-proteins S19 (a) and S8 (b) in X. laevis genome. DNA from a single individual has been digested with Hind III (a) and Bam H I (b) and 5 μg run in each lane of the gel together with increasing amounts of linearized pXom69 (a) and pXom62 (b), as a calibrations standard, equivalent to 1, 3, 10, 30 copies per haploid genome (2.7x10^9 base pairs) the blot of this gel was hybridized to 32P-pXom69 inserted sequence (a) and to 32P-pXom62 inserted sequence (b).

DISCUSSION

In a previous work we characterized an mRNA fraction (rpe-mRNA) from X. laevis oocytes, enriched for r-proteins coding capacity (1). In the present paper we report the cloning in pBR322 of cDNA to the rpe-mRNA, the selection of 11 clones containing sequences specific for six different and identified r-proteins and the use of these clones as probes in some experiments designed to obtain information about the structure and organization of the corresponding genes in the X. laevis genome.

It has been estimated (1) that each r-protein accounts for approximately 0.2% of the products coded in vitro by the rpe-mRNA fraction utilized for cDNA synthesis and cloning. Thus we might have reasonably expected that about 10–20% of the recombinant clones should bear sequences specific for some r-proteins. This evaluation appears now to be not too far from
the results obtained: in fact we identified 11 clones, out of the 127 screened, as containing sequences specific for r-proteins.

Synthesis of double stranded cDNA and its cloning in pBR322 have been carried out following substantially the standard procedures. E. coli colonies, resistant to ampicillin, have been hybridized to $^{32}$P-cDNA, complementary to the rpe-mRNA. The positive colonies were very heterogenous as for the intensity of the autoradiographic spots. This was not unexpected since the rpe-mRNA fraction, used to prepare the radioactive probe for colony hybridization, is a heterogenous population of many different mRNAs present at different abundance levels. Colony hybridization will result thus in heavily labeled clones, if the corresponding cDNA are abundant, and in more or less faint ones for low abundance cDNA. The clones we chose for further screening belong to all classes of colonies, both heavily and faintly labeled. All the 127 clones chosen, even those barely visible in the autoradiography of colony hybridization, have been found to contain an inserted sequence. This suggests that probably many other clones resistant to ampicillin, which were discarded because of negative results in colony hybridization test, might have contained inserted sequences complementary to very rare mRNAs. A posteriori we can say that the clones containing r-protein sequences gave rather faint signals in the colony hybridization experiment. The average length of the sequences inserted in the 127 clones is about 450 base pairs ranging from 150 to 1100.

Because of the lack of any specific probe, the selection of the clones containing sequences specific for r-protein and their identification has been done analysing the in vitro translation products of the rpe-mRNA hybridized to individual recombinant plasmids. We had at first discouraging results with negative translation procedures. In fact we found (not shown) that the pattern of radioactive products in the two dimensional gel electrophoresis is not quantitatively reproducible: the relative intensity of the spots being variable in different experiments. These variations are slight but enough to make impossible to consider as a valid identification the lowering of the intensity of a spot in a pattern of translation products so complex as ours. On the contrary the positive method here utilized, that is the analy-
ysis of the translation product of the mRNA recovered from the hybrid, gave very good results, always reproducible.

12 of the 127 clones screened gave positive signals for proteins which remain unidentified, 1 showed to contain sequences specific for a histone protein and 11 for 6 different r-proteins. In fact some of them contain sequences for the same protein. This identification was at first based on the precise comigration of the in vitro translation products with purified r-protein markers and subsequently confirmed by peptide mapping with S. aureus V8 protease.

The inserts of the clones specific for r-proteins are all longer than 420 base pairs; although probably not representing the complete mRNA sequences, they are suitable for hybridization experiments.

The analysis of the organization of these sequences in the genomic DNA has shown that they are present in very few copies, namely two copies for L1, L14, S19 and four-five copies for L32, S1 and S8. Furthermore we found that some of these sequences (S1, S8, L14) are present in genomic regions which are variable, by restriction analysis, in different individuals. Such variability wasn't observed for L1, S19 and L32. This rather simple organization differs from the one described in the mouse genome (4) where the complex pattern of hybridization suggests the presence of a larger number of homologous sequences for individual r-proteins.

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