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RESTRICTION MAPPING OF THE rRNA GENES FROM *Artemia* LARVAE

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SUMMARY

A restriction endonuclease analysis of the genes coding for the ribosomal RNA from *Artemia* larvae has shown that these genes consist of a repeat unit of 16.2 kilobase pairs (10.7 Mdaltons) and that the repeat unit seems to be homogeneous in size.

INTRODUCTION

The knowledge of the molecular structure of specific genes and their purification are important tools in studying the regulation of gene expression. Likewise, a current approach in the field of evolution is the comparison of the molecular structure of specific genes. The repeated gene families are the most widely used in studying these two problems due to their relatively simple identification and isolation.

We have chosen the rRNA genes from the crustacean *Artemia* (subclass branchiopoda, order anostraca) to study the regulation of their expression. *Artemia* is a very well suited system for this type of study due to its peculiar developmental program (1-3). *Artemia* is also interesting from the evolutionary point of view, since there is very little data on the rRNA gene structure of crustacea (4).

The overall structure of the rRNA genes is well known (4). It consists in a repeat unit that is mantained in all organisms studied from bacteria to higher eukaryotes: 5' - non-transcribed region - "18 S" RNA coding region - inner transcribed spacer - "28 S" RNA coding region - non-transcribed region - 3'. One way to approach the study of the structure of these genes is restriction endonuclease analysis, whereby a physical map of them can be obtained. We present here the physical map for the rRNA genes from *Artemia*.

MATERIALS AND METHODS

Artemia cysts were obtained from San Francisco Bay Brand Inc., Division of Metaframe Co., Menlo Park, CA 94025 (batches # 2018 and 1808). α-amylase,

Abbreviations: SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M trisodium citrate; kb, kilobase pairs.

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pancreatic ribonuclease and ribonuclease T1 were from Calbiochem. Proteinase K was from Merk. Restriction endonucleases Hpa I, Pst I, Sal I, Sma I and Xba I were from BioLabs and kindly supplied by Dr. E. Tabarés and Dr. V. Rubio; Eco RI, Hind III, Bam HI and polynucleotide kinase were from Boehringer Mannheim. All of them were used according to the manufacturer's instructions. (γ-^{32}P)ATP (>2,000 Ci/mmol) was from the Radiochemical Center, Amersham. Wild type λ DNA was the generous gift of Dr. V. Rubio.

Obtention of *Artemia* larvae. *Artemia* cysts were treated as previously described (5) and incubated in 0.25 M NaCl in a shaking incubator at 30°C for 20 hr. Nauplii were harvested in a separation funnel, filtered through a cloth, washed with distilled water, weighed and used immediately.

Preparation of high molecular weight DNA. New born nauplii were homogenized in 5 vol of 10 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 400 mM sucrose with 8 strokes in a glass hand-homogenizer. The crude extract was filtered through 8 layers of gauze and the filtrate was centrifuged at 500xg for 10 min at 4°C. The nuclear pellet was resuspended in 3 vol of 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA by vortexing and SDS was added to a final concentration of 0.25%. Proteinase K was added at 50 µg/ml and incubated with very gentle agitation for 30 min at 37°C. SDS concentration was raised to 2% and the incubation followed another 30 min at room temperature. This nuclear homogenate was chilled and extracted immediately with 1 vol of phenol:chloroform:isoamil alcohol (25:24:1) saturated with 500 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA for 30 min at 4°C with gentle agitation. The two phases were separated by centrifugation at 10,000 rpm for 15 min at 4°C in the HB-4 rotor of a Sorvall centrifuge. After another phenol-chloroform extraction, the aqueous phase was dialyzed against 500 vol of 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA for 24-36 hr at 4°C until the phenol was eliminated. The solution was then treated inside the dialysis bag with 50 µg/ml pancreatic ribonuclease and 100 U/ml ribonuclease T1 (both heated at 80°C for 30 min) for 3.5 hr at 37°C. α-amylase was added (25 µg/ml) and incubated 1.5 hr at 37°C. Finally, SDS was added at 0.25% and the solution digested with 50 µg/ml of Proteinase K for 3 hr at 37°C. After the last digestion, the solution was extracted at least 3 times with phenol-chloroform and dialyzed against 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA. DNA was stored at 4°C in this buffer. When needed, DNA was dialyzed against 10 mM Tris-HCl, pH 8.0. The molecular weight of the DNA preparations was analyzed by electrophoresis in 0.25% or 0.15% agarose gels (6) and was higher than 200 Mdaltons.

Preparation of rRNA. Ribosomes were obtained from new born nauplii. rRNA extraction and sucrose gradient separation of the 17 S and 25 S species were performed as described by Cruces *et al.* (manuscript in preparation). rRNAs were labeled in their 5' ends with polynucleotide kinase according to Maizel (7), except that the hydrolysis of both rRNAs was for 30 min at 90°C, to obtain fragments of 100 bases.

Transfer of DNA to nitrocellulose filters and hybridization. 5 µg aliquots of DNA were cleaved with different restriction endonucleases (or mixtures of them) at 37°C for 12 hr with enough enzyme to give complete digestion. Restricted DNA was electrophoresed in 0.5% agarose gels (23x14.5x0.3 cm) in slots of 24 mm^2 at 1 V/cm in Tris-borate buffer (6) until the marker dye was 15 cm from the origin. After staining with ethidium bromide and photographing, DNA was transferred to nitrocellulose filters (Millipore) as indicated by Southern (8).

Hybridization was carried out in plastic bags (Seal-N-Save, Sears) at 42°C for 36 hr with 50-100 µl/cm^2 of hybridization buffer (50% formamide, 4xSSC, 0.1% SDS, 25 mM EDTA, 20 mM sodium phosphate, pH 7.0) plus $2x10^5$ cpm/slot of (^{32}P)rRNA (specific activity, 10^7 cpm/µg) and 10-20 µg of the non-labeled rRNA. After hybridization the filters were washed twice at 42°C for 30 min with hy-

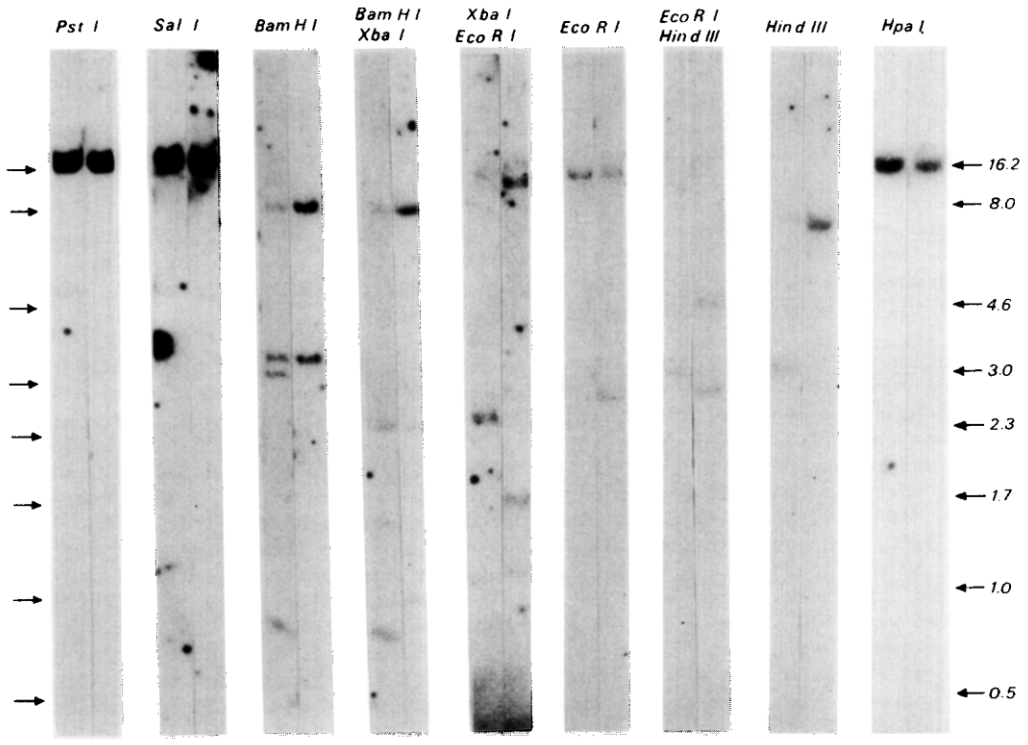

Figure 1. Pattern of hybridization or rRNA with restriction fragments obtained with different endonucleases on *Artemia* DNA.
In each digest, the left lane represents hybridization with 17 S and the right lane with 25 S rRNAs. Numbers representing the sizes of the fragments are expressed in kb.

bridization buffer (100 ml), twice with 2xSSC, 0.5% SDS (250 ml) and twice with 0.1xSSC, 0.5% SDS (250 ml). After air-drying, filters were exposed to sensitized X-ray film (Kodak X-omat XS-5) with an intensifying screen (Dupont Cronex Lightning-plus XL) at -70°C (9). In each gel (^{32}P)5' end-labeled λ DNA digested with Hind III was included as an internal molecular weight marker for hybridization.

RESULTS AND DISCUSSION

Figure 1 shows the results of the hybridization with 17 S and 25 S (^{32}P) rRNAs of the DNA fragments obtained with different restriction endonucleases and mixtures of them on total *Artemia* DNA. The size of the different fragments and the probe to which they hybridize are shown in Table I for all the digestions performed. Sal I, Pst I and Hpa I cut only once per repeat unit, being the fragment lengths of 17, 16.8 and 16.9 kb respectively. These small differences can be accounted for by the difficulty in assessing true molecular weights from the gels, due to the steepness of the log(MW) vs. mobility curve in this

Table I.

Summary of restriction fragments obtained from *Artemia* DNA complementary to rRNA.

Endonuclease	Fragments	Total length
Sal I	17.0 (25, 17)	17.0
Sal I + Pst I	11.1 (25); 5.2 (17)	16.3
Pst I	16.8 (25, 17)	16.8
Pst I + Sma I	14.6 (25, 17); 1.0 (25)	15.6
Sma I	14.7 (25, 17); 1.6 (25)	16.3
Sma I + Hpa I	13.5 (25, 17); 1.5 (25) 1.3 (17)	16.3
Hpa I	16.9 (25, 17)	16.9
Hpa I + Hind III	7.4 (25); 1.8 (17); 1.2 (17)	10.4
Hind III	7.4 (25); 3.0 (17)	10.4
Hind III + Eco RI	4.6 (25); 3.0 (17); 2.7 (25)	10.3
Eco RI	13.4 (25, 17); 2.8 (25)	16.2
Eco RI + Xba I	10.9 (25); 2.5 (17); 1.7 (25); 1.1 (25)	16.2
Xba I	12.4 (25); 3.8 (25, 17)	16.2
Xba I + Bam HI	8.0 (25); 2.3 (25, 17); 1.0 (25); 0.7 (17); 0.55 (25); 0.35 (25)	12.9
Bam HI	8.0 (25); 3.4 (25, 17); 3.1 (17); 0.7 (17); 0.55 (25); 0.35 (25)	16.1
Bam HI + Sal I	7.9 (25); 3.4 (25, 17); 3.1 (17); 0.7 (17); 0.55 (25); 0.35 (25)	16.0

Fragment size is expressed in kb. Numbers between brackets refer to which rRNA the fragment hybridizes.

range (6). To calculate a more accurate size for the repeat unit it is better to use enzymes that cut more than once per repeat. In this case, one obtains for the size of the repeat unit a value of 16.2 kb for Sma I, Eco RI and Xba I, and 16.1 kb for Bam HI. The only exception is Hind III, which gives a size of 10.4 kb. We believe that this is because Hind III generates a "silent" fragment within a non-transcribed region and therefore cannot be detected with either probe (see below). In conclusion, we estimate that the size of the repeat is 16.2 kb.

The repeat unit seems to be homogeneous in size, since no double bands of high molecular weight with the same hybridization pattern are detected in a given digest. The only other possible way to get the same size for the Sal I, Pst I and Hpa I digests would be if these three enzymes cut the repeat very

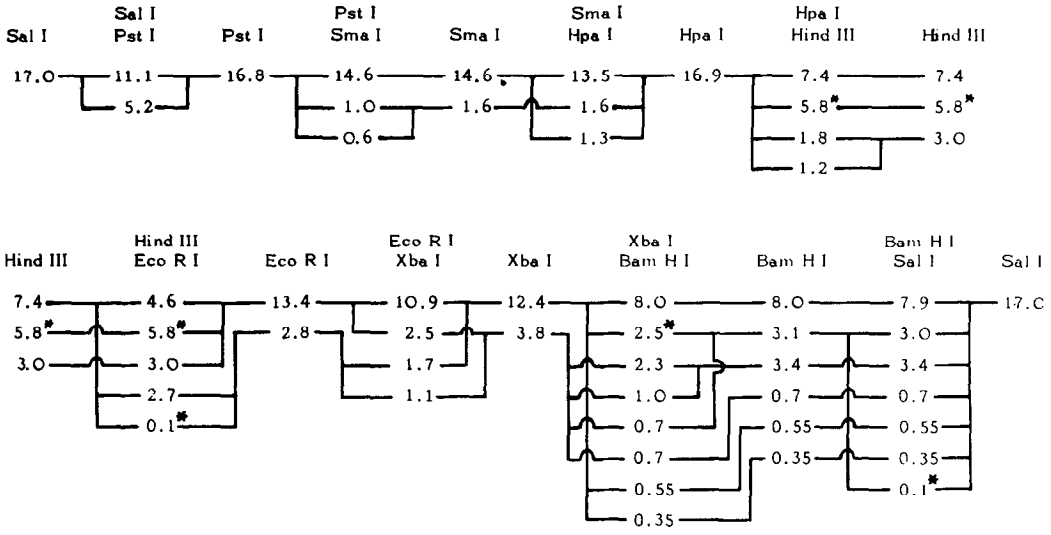

Figure 2. Generation and lengths (in kb) of the restriction fragments obtained from *Artemia* rDNA.

close to each other and on both ends of an extra DNA fragment in a non-transcribed region, and this is not the case, as different double digests with these enzymes give different patterns (compare, for instance, Sal I + Pst I and Pst I + Sma I).

Figure 2 shows a representation of all the fragments obtained and their relationship. This type of representation has been taken from Wild and Gall (10). An asterisk on a given fragment indicates that it is "silent". With all the data presented in Table I and Fig. 2, a physical map of the repeat unit has been constructed which accounts for every fragment obtained. The map is shown in Figure 3. It also explains the production of silent fragments in digestions with Hind III and in the Xba I + Bam HI double digest. The sizes of the different regions of the repeat have been deduced from the map: 17 S coding region, 1.9 kb; 25 S coding region, 3.9 kb, both in agreement with the molecular weight of 17 S and 25 S rRNAs (Cruces *et al.*, in preparation). The inner transcribed spacer is of about 1 kb and the distance between the 3' end of 25 S and the 5' end of 17 S coding regions of about 9 kb. A close examination of the map shows that almost all the cuts produced with the restriction endonucleases used are within the transcribed regions. This is because the enzymes used have hexanucleotide recognition sequences based mainly on A and T residues. In this respect, it is noteworthy that Hpa II (CCGG) and Hha I (GCGC) cut the repeat extensively, giving very small fragments (data not shown).

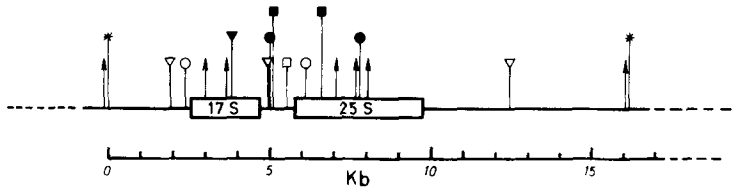

Figure 3. Physical map of the rRNA repeat unit from *Artemia*. (↑): Bam HI. (↑): Eco RI. (↑): Hind III. (↑): Hpa I. (↑): Pst I. (↑): Sal I. (↑): Sma I. (↑): Xba I.

Several characteristics of the structure of the rRNA genes in *Artemia* remain to be solved, including the characterization of the pre-rRNA molecule to define the size of the non-transcribed spacer, and the localization of the 5.8 S rRNA coding region, usually found in the inner transcribed spacer.

Some considerations can be made from the evolutionary point of view. Southern (8, 11) has suggested that the two Eco RI sites in the rRNA genes have been conserved since the divergence of amphibians and mammals, and this has been confirmed in the avian repeat (12): the large fragment hybridizes only with the 18 S rRNA and the small fragment with both 18 S and 28 S. The restriction pattern of Eco RI in *Artemia* rDNA is different. Although there are also two fragments, it is the large one which hybridizes with both rRNAs, while the small fragment hybridizes only with 25 S rRNA. It is not yet clear whether this difference is a general feature in the rDNA of crustacea and in the evolution of the rRNA genes.

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