Amphibian Ribosomal Ribonucleic Acids

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1. The larger rRNA molecules of different species of amphibia do not all have the same mobility on polyacrylamide gels, and the difference appears to be one between the Anuran and Urodele amphibia. 2. Alteration of conditions of electrophoresis so as to distinguish between differences of conformation and molecular weight does not affect the results. 3. The mobility on gel electrophoresis of the rRNA precursor in two species of amphibia is similar, although the intermediate processing products differ. 4. It is suggested that the mobility differences observed represent differences of molecular weight, and are an example of the variability of the products of transcription and processing of rRNA genes in two amphibian genera.

The size, as measured on polyacrylamide gels, of the mature rRNA species of animals and plants in relation to the size of the pre-rRNA* molecules has been extensively reviewed (Loening, 1970; Perry *et al.*, 1970; Grierson *et al.*, 1970). Two general points have emerged from these studies: first, that the size of the larger rRNA increases during evolution in animals, whereas it remains relatively constant in all plants; the size of the smaller rRNA is constant in both animals and plants; secondly, the size of the pre-rRNA molecule is consistently about 20–50% larger than the sum of its products in plants and animals other than birds and mammals, but becomes almost double the size of its products in the latter (Perry *et al.*, 1970; Grierson *et al.*, 1970).

Investigation of the structure and function of rRNA genes in amphibians began with the study of lampbrush chromosomes in amphibian oocytes (Gall, 1954; Callan & Lloyd, 1960), and led to the observation of large numbers of nucleoli in the germinal vesicle (Gall, 1954), and to the study of rRNA synthesis in oocytes (Gall, 1966). Discovery of the anucleolate mutant in *Xenopus laevis* by Elsedale *et al.* (1958) led to its characterization by Brown & Gurdon (1964) as an inability to synthesize rRNA. Wallace & Birnstiel (1966) showed that the cistrons coding for rRNA could be separated from the bulk DNA because of their high G+C content, and since that year much further work has been done on their structure (Birnstiel *et al.*, 1968; Miller & Beatty, 1969; Dawid *et al.*, 1970; Reeder & Brown, 1970).

*Abbreviation: 'pre-rRNA' refers to the primary product of transcription of the ribosomal genes; in the Amphibia it has previously been referred to as 40S RNA (Gall, 1966).

Throughout the work cited above, two genera of amphibians, namely *Xenopus* and *Triturus*, have been used to the virtual exclusion of others. It has been tacitly assumed that the process of rRNA synthesis is the same in these animals (cf. Gall, 1966; Landesman & Gross, 1969). Yet in plants polyacrylamide-gel analysis of rRNA synthesis has shown that the pre-rRNA molecules and their intermediate processing products are significantly different from one genus to another, and even from one tissue to another (Rogers *et al.*, 1970; Grierson & Loening, 1972). It is therefore possible that the greatly improved resolution of gel electrophoresis over sucrose-density-gradient analysis would reveal such differences between animal, and in this case amphibian, genera.

The work described in the present paper was begun as the result of the observation that the larger rRNA of the newt *Triturus cristatus carnifex* differed considerably in its mobility on polyacrylamide gels from that of the South African clawed toad *Xenopus laevis*. Since *Xenopus* has been used extensively for the study of the structure of the rRNA genes in amphibia (Birnstiel *et al.*, 1968; Loening *et al.*, 1969; Miller & Beatty, 1969; Dawid *et al.*, 1970; Reeder & Brown, 1970), it seemed important to extend this initial observation to see whether the mobility difference is a difference in molecular weight, and whether it is correlated with a difference in the size of the pre-rRNA and its processing products. We have investigated the rRNA species of a variety of amphibia, both Anurans and Urodeles, and have studied the synthesis of rRNA in *Triturus cristatus*, a Urodele, and in *Xenopus laevis*, an Anuran. Our results show that differences between

the molecular weights of the larger rRNA molecules of these two genera are accompanied by similar differences between the corresponding pre-rRNA processing products. The pre-rRNA itself is similar in *Triturus* and *Xenopus*, so that it is its processing and not its transcription that results in mature rRNA species with different molecular weights.

Methods

Live animals

Anurans. *Xenopus laevis* females were kindly provided by Dr. P. Ford, Medical Research Council Epigenetics Unit, Edinburgh, U.K. *Rana pipiens* were provided by Dr. R. Kille, Department of Zoology, University of Edinburgh, Edinburgh, U.K. *Bufo bufo* and *Rana temporaria* were collected locally.

Urodeles. *Triturus cristatus carnifex* females were supplied by L. Haig, Newdigate, Surrey, U.K., and by Professor H. G. Callan, Department of Zoology, University of St. Andrews, St. Andrews, Fife, U.K. *Plethodon cinereus* was obtained from Professor H. Macgregor, Department of Zoology, University of Leicester, Leicester, U.K. *Pleurodeles waltlii* was provided by Miss M. Perry, Institute of Animal Genetics, University of Edinburgh, Edinburgh, U.K. RNA, prepared from *Triturus viridescens*, a Urodele, and precipitated in ethanol was kindly sent by Mr. Brian Spear, Department of Biology, Yale University, U.S.A.

Radioactive labelling of RNA in *Triturus* and *Xenopus*

Three types of tissue were used.

(1) *Isolated ovaries.* Ovaries were removed from anaesthetized decapitated animals under a sterile hood. Large mature oocytes were mostly rejected, because their yolk otherwise contaminated the RNA preparations. The remaining immature oocytes were washed in sterile amphibian Ringer solution containing magnesium acetate (2g/l), and incubated at room temperature in 1 ml of sterile Medium 199 (Flow Laboratories, Irvine, Ayrshire, U.K.) per ovary as described by Gall (1966). A 100 μ Ci portion of [5-³H]uridine (specific radioactivity about 20 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added per ml of culture medium.

(2) *Liver.* *Triturus cristatus* liver was labelled *in vivo* by intraperitoneal injection under anaesthesia (tricaine methanesulphonate; Kodak Ltd., Kirby, Liverpool, U.K.) of 15 μ Ci of [¹⁴C]orotic acid (specific radioactivity 60 mCi/mmol; The Radiochemical Centre). The animal was killed after 15 h and RNA was extracted from the liver.

(3) *Xenopus kidney cultures.* Cultures were maintained in the Medical Research Council Epigenetics Unit, Edinburgh, U.K., and cells were labelled as described by Loening *et al.* (1969).

Preparation of RNA

The technique was that of Parish & Kirby (1966), as modified by Loening *et al.* (1969). In all cases total cell RNA extracts were made. 'Somatic' RNA refers to that extracted from the liver, kidney or spleen. DNA was digested in all preparations by dissolving the nucleic acids in 0.2 ml of MES-Mg buffer [50 mM-2 - (N - morpholino)ethanesulphonic acid-2.5 mM - magnesium acetate, pH 7.0], and adding 50 μ g of deoxyribonuclease [electrophoretically purified, ribonuclease-free; Sigma (London) Chemical Co., London S.W.6, U.K.]/ml. Incubation was for 20 min in ice. The RNA was then reprecipitated by addition of dodecyl sulphate-acetate (0.5% sodium dodecyl sulphate-0.15 M-sodium acetate, pH 6) and ethanol. RNA samples were dissolved in an appropriate volume of half-strength electrophoresis buffer (referred to as 'E' buffer by Loening, 1969), containing sucrose (15%, w/v), before electrophoresis. RNA was stored frozen at -20°C in this buffer for subsequent use. Amphibian RNA is stable under these conditions, and could be kept for several weeks without degradation.

Polyacrylamide-gel electrophoresis

RNA was analysed on 2.2% polyacrylamide gels, prepared as described by Loening (1967, 1968). The gels measured 0.9 cm $\times$ 9 cm. The buffers used were 'E' buffer, 'low-salt' buffer or 'magnesium' buffer (Loening, 1969). E buffer contained 0.2% and the other two buffers 0.1% of sodium dodecyl sulphate, and electrophoresis was carried out at room temperature (20-25°C) for up to 5 $\frac{1}{2}$ h at 50 V. Gels were pre-run for 30 min before application of RNA, which was layered on to the gels in 50-100 μ l of solution. Larger volumes tended to diminish resolution, owing to widening of the RNA bands. The gels were scanned at 265 nm in a Joyce-Loebl u.v. gel scanner.

Gels containing radioactive RNA were frozen and sliced as described by Loening (1967). Measurement of molecular weights of radioactive RNA species requires precise alignment of counts in each slice with the absorbance scan. The position of absorption bands was marked with Indian ink. When the gels were sliced the position of the markers was noted, and it was thus possible to allow for distortion of the gel during freezing.

RNA was hydrolysed in a glass vial in 0.2-0.3 ml of 0.5 M-NH₃-0.5 mM-EDTA, and the vials were placed on a hot-plate until all liquid had evaporated. Then 0.2 ml of water was added, which caused the dry gel to swell considerably, followed by 10 ml of scintillation fluid [methoxyethanol-A.R. toluene (2:3, v/v) containing 0.5% (w/v) of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD)]. Radioactivities of samples were counted in a Packard Tri-Carb liquid-scintillation counter.

Table 1. *Molecular weights of rRNA species*

Sedimentation coefficients listed are nominal values used as names to identify rRNA species and are not precisely determined.

Source	$10^{-6} \times$ Molecular weights	Nominal sedimentation coefficients (S)
<i>Escherichia coli</i>	1.08 and 0.56	23 and 16
Pea (<i>Pisum</i>)	1.3 and 0.7	25 and 16–18
Mung bean (<i>Phaseolus</i>)	1.3 and 0.7	25 and 16–18
Mouse myeloma	1.75 and 0.7	28 and 18
<i>Xenopus</i>	1.55 and 0.7	28 and 18
<i>Triturus</i>	1.39 and 0.7	28 and 18
<i>Plethodon</i>	1.45 and 0.7	28 and 18
<i>Xenopus</i> pre-rRNA	2.6–2.8 (2.7)	40
<i>Triturus</i> pre-rRNA	2.8–3.0 (2.9)	40
Predominant processing products:		
<i>Xenopus</i> (major)	1.66	30
(minor)	1.9	—
<i>Triturus</i> (major)	1.55	30
(minor)	1.6, 1.8	—

Molecular-weight determinations

Molecular weights were measured according to the linear relationship between electrophoretic mobility and $\log(\text{molecular weight})$ described by Bishop *et al.* (1967) and Loening (1968). The use of this relationship for determining relative molecular weights under various conditions has been analysed by Loening (1969). We have tested our values under different conditions of electrophoresis, and find them to be reproducible to within about $\pm 5\%$.

A summary of relative molecular weights used in the text and corresponding nominal sedimentation coefficients is given in Table 1. The molecular weights are relative to rRNA markers and should not be taken as absolute values. The markers were *Escherichia coli* rRNA, whose molecular weights were taken as those given by Stanley & Bock (1965), and rRNA from peas, mung beans and mouse cells. The values for the latter were those given by Loening (1968, 1969). Molecular weights of the pre-rRNA species were obtained by extrapolation. This method introduced small errors; averages of 20–30 determinations were used.

When two or more RNA samples were being compared, they were pre-mixed before applying to a gel, and were also run separately on other gels in the same tank. It is important to continue electrophoresis for at least 3 h to ensure resolution of peaks with similar mobilities, and to allow the RNA to move beyond the first centimetre of the gel, which is often more dilute.

Results

Comparisons of apparent molecular weights of rRNA species from different amphibia

Fig. 1(a) shows a scan of a gel containing a mixture of rRNA species from *Xenopus* and *Triturus*. If the molecular weight of the larger *Xenopus* rRNA species is taken as 1.52×10^6 , then that of the *Triturus* component is 1.4×10^6 . Mixtures of rRNA species from two Anurans, or two Urodeles, on the other hand, show no such difference. Exactly the same results were obtained with rRNA from all the species named in the Methods section, except for the salamander *Plethodon cinereus*, in which the large rRNA species has a molecular weight between 1.52×10^6 and 1.4×10^6 (Fig. 1b), which is higher than that of other Urodeles tested, but lower than that of all the Anurans investigated. None of the Anuran large rRNA species tested could be resolved from each other. The results were similar for rRNA isolated from somatic tissues and from ovaries.

Effects of changes in salt concentration and of heat on apparent molecular weights

In certain cases where large differences in base composition exist between two RNA species changes in salt concentration of the electrophoresis buffer cause changes in mobilities, which are due to conformational differences. An example of this is *Drosophila* rRNA, whose mobility decreases in 'low-salt' buffer (see the Methods section) relative to that of

Fig. 1. Electrophoresis of rRNA species from different Amphibia

(a) Electrophoretic pattern of a mixture of total RNA extracted from *Triturus* ovaries (T) and *Xenopus* kidney culture cells (X). The RNA mixture was layered on a 2.2% polyacrylamide gel and electrophoresis was continued for 5 h at 50 V at 12 mA/gel. (b) Electrophoretic pattern of a mixture of somatic RNA species from *Bufo* (B), *Xenopus* (X), *Plethodon* (P) and *Triturus* (T). The RNA samples were mixed before being layered on to a 2.2% polyacrylamide gel. Electrophoresis was continued for 6 h. The first 2–3 cm of the gels are not shown.

other higher-G+C rRNA species (Loening, 1969). Although all amphibian rRNA species that have been tested are high-G+C, it was possible that the observed differences in mobility were due to conformational differences that would be enhanced or decreased by changes in salt concentration. No such effect was observed when either 'low-salt' or 'magnesium' electrophoresis buffers were used (see the Methods section). This indicated that true differences of size were involved.

Heat treatment, either during or after purification, of rRNA can cause changes in electrophoretic mobility. This has been shown in mouse and human rRNA (Eliceiri & Green, 1969). Heat causes the loss of a small piece of RNA from the larger rRNA molecule from mammals (Pene *et al.*, 1968). Loss of this piece may or may not lead to a change in electrophoretic mobility.

Samples of rRNA from *Xenopus*, *Bufo*, *Triturus cristatus* and *Plethodon* were either subjected to two hot-phenol-cresol extractions at 55°C for 3 min or were extracted in the cold as usual, and were heated in half-strength E buffer-sucrose (see the Methods section) at 55°C for 3 min immediately before electrophoresis. In both cases some specific breakdown products and presumed aggregates were seen, but most of the rRNA from all the animals remained unchanged. There was no evidence of changes in relative mobility when heated rRNA from a particular species was mixed with rRNA that had not been heated at any stage. These experiments suggest that stable differences in electrophoretic mobility, and hence in molecular weight, exist between the rRNA molecules of different amphibia. Investigation of lower-molecular-weight RNA species on 5% gels showed no evidence of small pieces of RNA released by heat. Heating of *Xenopus* '28S' rRNA under the conditions used by Pene *et al.* (1968) to release mammalian '7S' RNA did not cause the release of a comparable molecule, or any significant degradation. The mobility of the heated '28S' rRNA was not changed. A '7S' RNA molecule is released from *Xenopus* 28S rRNA in 6M-urea, or on heating to higher temperatures than were used in our experiments (P. Ford, personal communication). This suggests that 7S RNA is more tightly bound in *Xenopus* than in mammalian rRNA.

The temperature of electrophoresis can affect the relative mobilities of RNA molecules, as was demonstrated with mitochondrial rRNA species (Groot *et al.*, 1970; Montencourt *et al.*, 1970). In all our experiments electrophoresis was carried out at 20–25°C, so that the mobility differences we observed cannot be due to large variations in the temperature of electrophoresis.

Precursors to rRNA

Since mature rRNA species were found to differ in molecular weight, it was decided to test whether the pre-rRNA species also differed. Pre-rRNA species were identified by their radioactive labelling kinetics in relation to rRNA, by their base compositions and by their labelling with [Me^3H]methionine (Fig. 2), previously shown to be a specific label for pre-rRNA and rRNA (Weinberg *et al.*, 1967).

It might be predicted that Anuran pre-rRNA species would be larger than those of Urodeles,

Fig. 2. Methylation of *Xenopus* rRNA and rRNA precursors

Xenopus kidney culture cells were incubated at 25°C for 3 h in medium containing 1.5 mCi of [*Me*-³H]methionine. Total RNA was extracted and layered on to a 2.4% polyacrylamide gel. Electrophoresis was continued for 5 h at 50V at 12mA/gel. Analysis of radioactivity in the gel is described in the Methods section.

because one of their mature products is larger. Unfortunately, sufficient numbers of most of the species were not available to perform radioactive-labelling experiments on all those from which unlabelled rRNA had been obtained. Results are available only for *Xenopus laevis* and *Triturus cristatus*. They show that the *Xenopus* pre-rRNA is not larger, but slightly smaller, than that of *Triturus*, even though the combined molecular weight of its products is higher.

Fig. 3 shows the radioactive pre-rRNA molecules of *Xenopus* and *Triturus* run on the same gel. The proportions of the mixture were carefully controlled so that the amount of ³²P-labelled material was insufficient to cause overwhelming contamination of the ³H channel. RNA samples were also run separately alongside the mixed samples to provide additional control of any anomalies in the mixture. As shown, *Triturus* pre-rRNA is very slightly (approx. 0.1×10^6)

larger than *Xenopus* pre-rRNA. The mobility difference is reproducible. The molecular weights obtained by extrapolation were 2.7×10^6 – 3.0×10^6 for the former and 2.6×10^6 – 2.8×10^6 for the latter (see also Loening *et al.*, 1969).

In both animals the pre-rRNA peak of radioactivity is clearly not homogeneous. It includes two or three components in *Xenopus* (Loening *et al.*, 1972; M. L. Sartirana & U. E. Loening, unpublished work). Gels quite frequently show a double pre-rRNA peak of radioactivity, and the corresponding absorption peak is always double. The significance of the heterogeneity will probably only become clear when the chemistry of the molecules has been investigated. We have found no evidence for any pre-rRNA molecule heavier than 3.0×10^6 . This has been checked in somatic tissues, in diplotene oocytes and in oocytes that are in pachytene and that are therefore in the process of amplifying their ribosomal

Fig. 3. Comparison of rapidly labelled RNA from *Xenopus* and *Triturus*

Xenopus (X) kidney culture cells were incubated at 25°C for 1½ h in medium containing 1 mCi of [^{32}P]orthophosphate. Two ovaries were removed from a female *Triturus* (T) and incubated at room temperature in 1 ml each of medium containing 100 μCi of [^3H]uridine. Total RNA was extracted, mixed and applied to a 2.2% polyacrylamide gel. Electrophoresis was continued for 6 h at 50 V at 12 mA/gel, and radioactivity was analysed as described in the Methods section. The arrow indicates the position of a putative third processing product in *Triturus*.

DNA. It is, however, possible that a heavier but very short-lived molecule is formed but is undetected in our experiments.

The intermediate processing products are of some interest. It should be noted that the resolution of these has been improved in these experiments (cf. Gall, 1966; Rogers, 1968; Landesman & Gross, 1969; Loening *et al.*, 1969). Whereas the pre-rRNA of *Xenopus* has a slightly lower molecular weight than that of *Triturus*, the two intermediate processing products have a higher molecular weight (Fig. 3). These differences remain when the salt concentra-

tion is altered. Thus the first step(s) in processing of *Triturus* pre-rRNA involves the loss of more 'excess RNA' than in *Xenopus*.

Discussion

In this paper we emphasize two characteristics of rRNA synthesis in different amphibia. First, the combined molecular weight of the mature rRNA species is not correlated with the molecular weight of the pre-rRNA. The increase in combined molecular weights between *Triturus* rRNA species and those of

Xenopus is 0.15×10^6 , yet *Xenopus* pre-rRNA is, if anything, smaller than *Triturus* pre-rRNA by about 0.1×10^6 . Thus higher combined weights do not result in correspondingly larger precursors. There are clearly other factors that determine how much of the non-ribosomal DNA, which acts as spacer between the ribosomal DNA (Birnstiel *et al.*, 1968; Brown & Weber, 1968), is transcribed. This conclusion is supported by observations on plant systems. Different tissues in one plant, namely *Phaseolus aureus*, have been shown to have different rRNA precursors, even though the mature rRNA species are, of course, the same. Preliminary experiments in *Xenopus* and *Triturus* show no obvious differences between the pre-rRNA species in somatic and ovarian tissue, although further work may reveal tissue-specific molecules.

The size of the pre-rRNA in relation to its mature products is greater in *Triturus* than it is in *Xenopus*, i.e. there is more 'excess RNA'. Recent estimates of the amount of 'excess RNA' in *Xenopus* pre-rRNA, which have been obtained from hybridization-competition experiments, suggest that there is up to 16% excess (R. Hecht & M. L. Birnstiel, personal communication). This is very similar to our own estimate of 16.6%, derived from a molecular-weight value for the pre-rRNA of 2.7×10^6 . Hybridization results are not available for *Triturus*.

The second feature of our experiments on rRNA synthesis concerns the intermediate molecules formed during processing of the pre-rRNA to its mature products, namely the two rRNA species. The number and size of the processing products in animal systems has not been extensively studied except in mammals (Weinberg *et al.*, 1967; Busch & Smetana, 1970) and in *Xenopus* (Loening *et al.*, 1969). In *Xenopus* there are two distinct processing products of about 1.9×10^6 and 1.65×10^6 – 1.7×10^6 molecular weight, both of which are methylated (Fig. 2). In *Triturus*, two intermediate processing products are also present, and a third is possibly present (Fig. 3). The approximate sizes of these molecules are shown in Table 1. It should be emphasized that the resolution of the closely spaced processing products is somewhat variable, and we do not regard the molecular-weight values given here as more than approximations. With this reservation in mind, however, it is still clear that all the processing products demonstrated in our experiments must be 'precursors' to the larger rRNA only. None is sufficiently large relative to the pre-rRNA, allowing for errors in measurement of molecular weight, to include the smaller rRNA sequences as well. Thus there is no equivalent in amphibia of the 41S intermediate of HeLa cells (Weinberg & Penman, 1970), unless the first processing product is only slightly smaller than the pre-rRNA itself and is not resolved from it. This suggestion could account for the heterogeneity of the pre-rRNA

peaks (M. L. Sartirana & U. E. Loening, unpublished work).

It is still unclear whether there is any equivalent in amphibia of the 20–23S precursor to 18S rRNA in HeLa cells (Weinberg *et al.*, 1967), rat liver (Egawa *et al.*, 1971) and *Chironomus* (Edström & Daneholt, 1967). Molecules of about the correct size have been reported by Rogers (1968) and C. Vesco (unpublished work, cited by Weinberg & Penman, 1970), but their relationship to 18S rRNA has not been proved. Peaks of radioactivity have been seen in the relevant regions of gels in our experiments, but they are not present consistently. They are labelled after incorporation of [^{3} H]methionine (Fig. 2). We cannot state definitely that a specific precursor to the 0.7×10^6 rRNA exists. If it does, we should expect a peak of radioactivity at about 1.0×10^6 – 1.3×10^6 in *Triturus*, and one at about 0.8×10^6 – 1.0×10^6 in *Xenopus*. But even after the shorter incubation times used, when the larger rRNA is not yet labelled, there is radioactivity in the smaller rRNA. This suggests that any intermediate is short-lived or present in very small amounts (cf. the 20S RNA in HeLa cells; Weinberg & Penman, 1970).

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