# Amplification of Ribosomal Ribonucleic Acid Cistrons in the Regenerating Lens of *Triturus*†

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ABSTRACT: The number of rRNA cistrons has been measured as a function of Wolffian lens regeneration in the adult newt (*Triturus viridescens*). Hybridization studies were performed with DNA from normal iris tissue and from iris tissue engaged in the transformation of iris into lens. The results indicate that the DNA of the iris in transformation contains 60% more rRNA cistrons. This suggests that the increase in the amount of rRNA synthesized, that accompanies lens regeneration, is facilitated by amplification of the rRNA genes.

When the lens is removed from the eye of the newt, *Triturus*, pigmented epithelial cells of the dorsal iris undergo profound morphological and biochemical alterations (for review, see Yamada, 1967). Among these changes are: an enhancement of RNA synthesis (Yamada and Karasaki, 1963; Yamada, 1966), an enhancement of protein synthesis (Yamada and Takata, 1963), entry into the S phase of the cell cycle (Eisenberg-Zalik and Yamada, 1967), depigmentation of the iris cells (Yamada, 1966), and the acquisition of lens-specific antigens (Takada *et al.*, 1964). These changes culminate in the formation of a regenerated lens, indistinguishable from the original one (Yamada, 1967). This metaplasia is all the more startling in that it involves the transformation of one fully differentiated tissue into another.

The earliest structural changes yet detected in cells of the transforming iris are an enlargement of the nucleoli, and the appearance of a granular component in the nucleoli (Dumont *et al.*, 1970). These changes occur within 2 days following the initiation of lens regeneration. There is evidence that the nucleoli are the site of rRNA synthesis (Birnstiel *et al.*, 1966; Brown, 1967). Accordingly, one might expect that nucleolar enlargement is an indication of increased rRNA synthesis. Such a correlation has been found by Reese *et al.* (1969), who report that the synthesis of 28S and 18S RNA is activated within 2 days following lens removal, subsequently enhanced, and remains at a high level during lens regeneration. They suggest that this initiation of rRNA synthesis is due to derepression of the iris cells. The onset of rRNA synthesis is the earliest biochemical event yet detected in lens regeneration, preceding the activation of DNA replication (Eisenberg-Zalik and Yamada, 1967), and protein synthesis (Yamada and Takata, 1963).

Proliferation of the nucleoli of transforming cells of the dorsal iris has also been observed (Karasaki, 1964; Dumont *et al.*, 1970). The number of nucleoli doubles within 2 days following lentectomy and remains constant for the next several days (Dumont *et al.*, 1970). As the nucleoli are the repository of the rRNA cistrons (Evans and Birnstiel, 1968), these two early structural alterations, proliferation and enlargement, might be indicative of an increase in the number of rRNA cistrons. This phenomenon is usually called gene amplification. The amplification of the genes which code for 28S and 18S rRNA has been shown by RNA–DNA hybridization experiments to occur in the oocytes of amphibians (Gall, 1968), including *Triturus* (Brown and Dawid, 1968) and fish (Vincent *et al.*, 1969). These oocytes exhibit nucleolar proliferation. It has also been detected in the oocytes of two marine invertebrates which show no nucleolar proliferation, but which undergo nucleolar enlargement (Brown and Dawid, 1968; Dawid and Brown, 1970). Gene amplification has recently been observed in human somatic cells in response to a hormone analog, under conditions whereby the nucleoli both proliferate and enlarge (Koch and Cruceanu, 1971). In all of these examples, gene amplification is a way to increase RNA synthesis, by increasing the number of templates, without mitosis.

On a theoretical basis the increased synthesis of rRNA by cells of the activated dorsal iris and the accompanying structural changes in the nucleoli could result from increased transcription of rRNA (Reese *et al.*, 1969), increased processing of the 40S rRNA precursor into 28S and 18S RNA (Papaconstantinou and Julku, 1968) or gene amplification (or some combination of these).

This communication describes DNA–RNA hybridization studies which indicate that a 1.6-fold increase in the number of rRNA genes occurs in cells of the dorsal iris within seven days after the initiation of lens regeneration. This is an amplification of 12,000 cistrons of these already redundant genes.

## Materials and Methods

*Animals.* Adult newts, *Triturus* (*Notophthalmus, Diemictylus*) *viridescens viridescens*, were obtained from Mr. Glen Gentry of Donelson, Tenn., and maintained in aquaria for at least 1 week before use. They were fed minced beef heart and frozen brine shrimp three times a week.

*Surgical Procedure.* Lens regeneration was initiated by anesthetizing the newts in Tricaine and removing the lens through a corneal incision as described by Eisenberg-Zalik and Yamada (1967). Before lentectomy, each eye was washed with a steady stream of sterile distilled water, then 50% ethanol, and then sterile distilled water again. After lentectomy, each eye was washed with sterile distilled water and the dorsal eyelid was pulled down. The animals were kept overnight in a moist chamber at 21°, to allow the corneal incision to heal,

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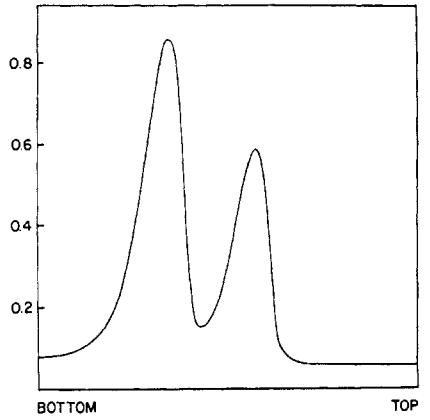


FIGURE 1: Sucrose density gradient analysis of purified rRNA. RNA was isolated from *Triturus* liver, purified by chromatography on methylated albumin kieselguhr and Sephadex G-100. This RNA was layered onto a 5–20% sucrose gradient containing 0.01 M Tris, 0.1 M KCl, 0.0015 M $MgCl_2$ (pH 7.4), and 200 μg/ml of poly(vinyl sulfate). This was centrifuged in a Spinco SW39 rotor at 20,000 rpm for 20 hr at 4°. The tube was punctured from the bottom and the gradient allowed to flow through an ISCO absorbance monitor.

then placed in aquaria. At the desired time after lens removal (7 days for the experiments described in this report) newts were anesthetized with Tricaine. Each eye was washed with 70% ethanol and then sterile distilled water. The upper one-third of the dorsal iris was carefully removed as described by Reese *et al.* (1969). At this stage of lens regeneration (stage II of Yamada and Roesel, 1964), cell replication has started and is continuing in the iris epithelium.

*Reagents.* [$^3$H]Uridine (5.4 Ci/mmole), generally labeled, and [$^3$H]sodium borohydride (4–7 Ci/mmole) were obtained from Amersham–Searle Corp. [$^3$H]Thymidine (1 Ci/mmole), generally labeled, was purchased from International Chemical and Nuclear Corp. Electrophoretically pure DNase was obtained from Worthington Biochemical Corp. Pancreatic RNase was obtained from Sigma. Bac-T-Flex B-7 (S-S) nitrocellulose filters were supplied by Schleicher and Schuell Co. RNase-free sucrose was obtained from Mann Research Laboratories. Cesium chloride was purchased from Fisher Scientific Co. Tricaine was purchased from Ayerst Laboratories.

*Isolation of RNA.* rRNA from liver was isolated by the method of Kirby (1968), treated with DNase (5 μg/ml), and passed through a methylated albumin kieselguhr column fitted with a Celite pad to remove impurities, as suggested by Brown and Weber (1968). The rRNA was precipitated with ethanol and further purified by chromatography on Sephadex G-100. Sedimentation of this RNA in a linear 5–20% sucrose gradient yielded two peaks, a 28S peak and an 18S peak. The RNA after Sephadex chromatography was subjected to the photochemical reduction described by Vincent *et al.* (1969) to insert a tritium label. Usually 1 mg of RNA was reacted with 100 mCi of sodium borohydride. The RNA was then precipitated three times with ethanol and passed through a Sephadex G-25 column to remove radioactive contaminants. Radioactivity was determined with a liquid scintillation counter. Quantitative determinations of nucleates were performed spectrophotometrically (an absorbance of 1.0 was taken to indicate 50 μg).

*DNA.* DNA was extracted from homogenized tissues essentially according to Marmur (1961). All samples were treated with pancreatic RNase (50 μg/ml, 3 hr, 37°), then Pronase (200 μg/ml, 3 hr, 37°), and precipitated with ethanol. DNA samples were sheared by 14 passes through a 27-gauge needle and fractionated by mixing with CsCl to a density of 1.70 g/cm$^3$ (determined with a Fisher refractometer) and centrifuging in a Spinco 50 Ti rotor at 34,000 rpm for 60–70 hr at 25° with a Spinco L3-50. The resulting gradients were punctured with a device designed by Salo (1965) and separated into fractions. In some cases the fractions containing DNA were recombined and immobilized on filters, in other cases, each fraction was immobilized on filters.

*Hybridization.* The procedure used was essentially that described by Brown and Weber (1968). After shearing, the DNA was denatured with alkali (0.15 N NaOH) for 10 min at room temperature, neutralized (0.15 N HCl), and brought to 6 × SSC.[1] The DNA was immobilized on a presoaked (6 × SSC) nitrocellulose filter by passage through a Fisher filtrator. The DNA-impregnated filters were dried at 80° overnight in a vacuum oven and exposed to rRNA in 6 × SSC. The final volume was 0.5 ml. After incubation at 70° for 24 hr the filters were washed with 6 × SSC and then 2 × SSC, and incubated with pancreatic RNase (50 μg/ml) for 1 hr at room temperature. The disks were washed in 2 × SSC, dried, and counted in toluene–fluors (2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene) in a liquid scintillation counter (Nuclear-Chicago, Mark I) at 35% efficiency for tritium. More than 95% of the DNA was retained by the filters. RNA absorption to blank filters was determined for each RNA input in each experiment and subtracted from the experimental values for that input.

*$^3$H-Labeled DNA.* Dorsal iris tissue from 50 irises was incubated under sterile conditions for 6 hr at 25° while shaking, in a sealed tube with 0.2 ml of [$^3$H]thymidine, 1.6 ml of amphibian culture medium (Wolf and Quimby, 1964), and 0.4 ml of sterile distilled water containing 600 units of penicillin and 600 μg of streptomycin. The pH was maintained at 7.6 throughout the incubation period. After incubation, the tissue was washed with cold 0.15 M NaCl–0.1 M EDTA (pH 8.0), homogenized, and the DNA was isolated. This DNA was purified by centrifugation in a CsCl gradient.

*Determination of the Molecular Weight of Triturus DNA.* The approximate sedimentation coefficient, *s*, of DNA was determined from a 5–20% sucrose gradient with 4S RNA as a marker (Martin and Ames, 1961). The molecular weight was calculated from the *s* value (Eigner, 1968), and found to be approximately 2.6 × $10^6$.

## Results

*Preparation of rRNA.* The sucrose gradient analysis of the purified rRNA before the tritium label was inserted revealed that very little degradation had occurred (Figure 1). After insertion of the radioactive label, sucrose gradient analysis revealed that there had been some degradation (Figure 2). The relative proportions of the 28S and 18S peaks had changed, and there were small amounts of 4S RNA.

In all experiments where the rRNA was to be used for hybridization with DNA, the RNA was filtered through three filters which had been soaked in 6 × SSC in order to remove any methylated albumin (Gillespie, 1968). The filters were then dried and counted. In all cases the third filter retained less than 4% of the counts filtered through it. When this RNA was

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: SSC, standard saline citrate, 1 × SSC = 0.15 M NaCl–0.015 M sodium citrate (pH 7.0).

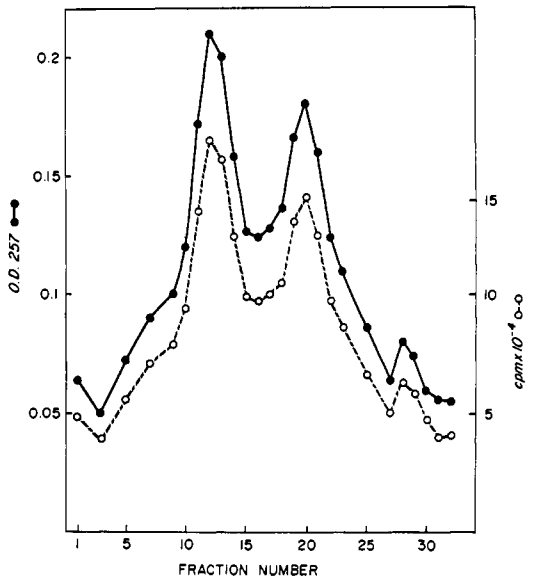


FIGURE 2: Sucrose density gradient analysis of $^3$H-labeled RNA. Purified rRNA (Figure 1) was labeled by [$^3$H]$NaBH_4$ treatment. This RNA (specific activity 15,700 cpm/$\mu$g) was layered onto a 5–20% sucrose gradient containing 0.01 M Tris, 0.1 M KCl, 0.0015 M $MgCl_2$ (pH 7.4), and 200 $\mu$g/ml of poly(vinyl sulfate). This was centrifuged in a Spinco SW39 rotor at 20,000 rpm for 17 hr at 4°. The tube was punctured from the bottom, and 12-drop fractions were collected. All fractions were diluted to 1 ml for determinations of optical density (●) and $^3$H (○). Tritium was determined by counting aliquots which had been applied to Whatman No. 3MM filter paper disks.

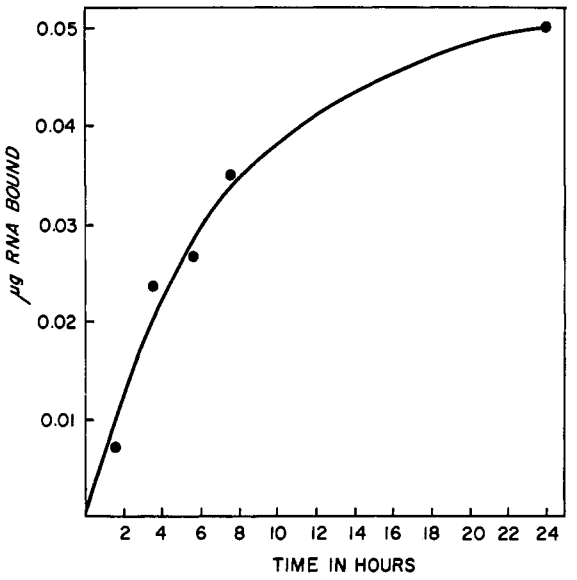


FIGURE 3: Time dependence of hybridization of rRNA with DNA. Filters impregnated with approximately 100 $\mu$g of sheared liver DNA were incubated with [$^3$H]rRNA, specific activity 11,500 cpm/$\mu$g, 4 $\mu$g/filter, in 6 × SSC, at 70° for the times indicated. The filters were washed in 2 × SSC, treated with RNase (50 $\mu$g/ml), washed again in 2 × SSC, and dried.

subsequently used in hybridization experiments, blank values of 0.016% were obtained after processing. Blank values were determined for each experiment and subtracted from the experimental values. These blank values were slightly above background, and amounted to 0.016% of the total input and less than 1.3% of the resulting hybrids. This is well within the acceptable limits for hybridization (Gillespie, 1968).

Figure 3 shows the time dependence of hybrid formation between DNA which had been immobilized on filters and a typical preparation of rRNA. The time required to achieve saturation appears to be longer than that usually reported for rRNA, which is generally from 3 to 8 hr. In any event, all subsequent hybridizations were performed for 24 hr.

*Saturation Curve for Triturus DNA.* A typical saturation experiment of liver DNA with rRNA is shown in Figure 4. The curve was obtained under what were determined to be the optimum conditions for this system. The amount of DNA which is complementary to a mixture of 28S and 18S rRNA is 0.051%. As only one strand of DNA forms hybrids with rRNA the amount of liver DNA which constitutes the rRNA genes is 0.1%. It can be seen (Figure 4) that 2 $\mu$g of rRNA saturates 100 $\mu$g of DNA. In all subsequent hybridizations, 4 $\mu$g/filter of rRNA were used, and the amount of DNA never exceeded 100 $\mu$g/filter. The standard conditions for all hybridizations are given in Methods. In all cases, only RNase-resistant hybrids were scored.

*rRNA Cistrons in Normal Iris and 7-Day Iris.* Isopycnic gradient centrifugations of the DNA from normal iris and from iris which had been engaged in lens regeneration for 7 days were performed; the gradients were fractionated and each fraction was challenged with the ability to hybridize with rRNA (Figures 5 and 6). The DNA from 7-day iris has 60% more capacity to hybridize with rRNA than the DNA from normal iris. It is unclear at present why the optical density band and the hybridization band in Figure 6 are unsymmetrical. Such unsymmetrical bands are always observed with DNA from iris in regeneration (J. M. Collins, unpublished observations). It can be seen that isopycnic gradient centrifugation has resolved the DNA containing the ribosomal genes from the bulk of the DNA. From their relative densities, (G + C) contents of 72% and 40% can be estimated for the ribosomal genes and for bulk DNA, respectively (Schildkraut *et al.*,

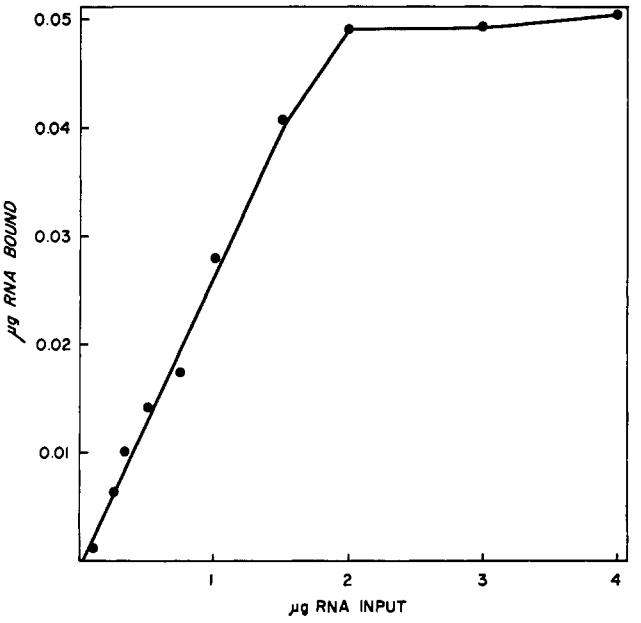


FIGURE 4: Saturation curve of rRNA with liver DNA. Increasing amounts of [$^3$H]rRNA, specific activity 28,500 cpm/$\mu$g, were incubated with 100 $\mu$g/filter of sheared liver DNA, which had been purified by CsCl centrifugation, in 6 × SSC for 24 hr at 70°. The filters were processed as in Figure 3.

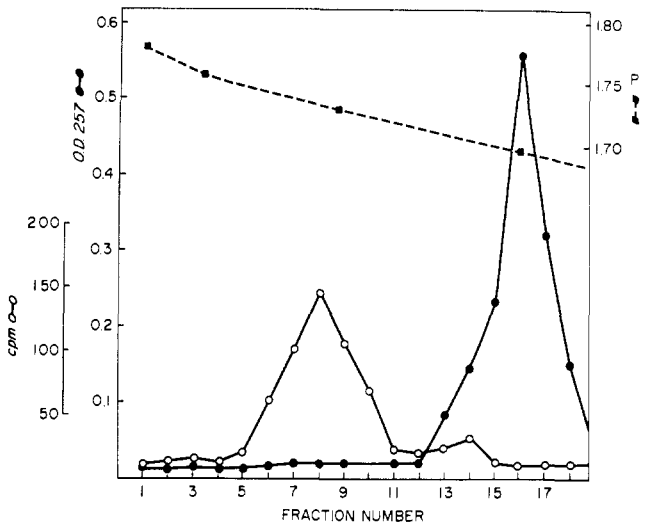


FIGURE 5: Hybridization of rRNA with DNA from normal iris. Approximately 75 μg of DNA extracted from the dorsal irises of 50 normal animals was sheared and centrifuged to equilibrium in CsCl (70 hr). The resulting gradient was separated ino 25 fractions. After refractive index and optical density measurements, all fractions were denatured and immobilized on nitrocellulose filters, then challenged with [$^3$H]rRNA, 4 μg/filter, specific activity 15,700 cpm/μg, in 6 × SSC for 24 hr at 70°. The filters were rinsed in 2 × SSC, treated with RNase (50 μg/ml) for 1 hr, rinsed again in 2 × SSC, and dried.

1962). Performing hybridizations with DNA which has been previously fractionated in CsCl results in increased specificity, as pointed out by Brown and Weber (1968). From the data in Figures 5 and 6, assuming 89 pg of total DNA/nucleus in *Triturus* (Edstrom, 1964) and a molecular weight of 2.5 × $10^6$ for the 40S rRNA precursor (Loening *et al.*, 1969), the number of ribosomal genes per cell can be calculated for normal iris, and for regenerating lens. This is presented in Table I, which also includes liver DNA (Figure 4). The DNA from regenerating lens cells contain 1.6 times more rRNA genes (Table I). These data were confirmed by two repeat experiments with normal iris and two repetitions with the 7-day iris.

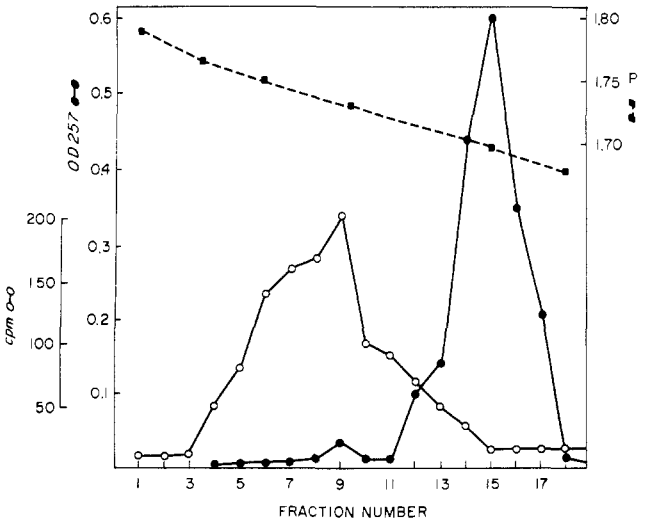


FIGURE 6: Hybridization of rRNA with DNA from activated iris. This experiment was performed as in Figure 5, except that approximately 90 μg of DNA from the dorsal irises of animals which had undergone lentectomy 7 days previously, were used.

TABLE I: The Number of rRNA Cistrons in Normal and Regenerating Tissue.[a]

| | Normal Iris | 7-Day Iris | Liver |
|---|---|---|---|
| Total DNA (μg) used in the experiment | 75 | 90 | 100 |
| Total cpm in hybrid | 590 | 1,170 | 785 |
| % rRNA cistrons[b] | 0.1 | 0.16 | 0.1 |
| Number of rRNA[c] cistrons | 19,900 | 31,800 | 19,900 |

[a] The numbers of rRNA genes were calculated from the data presented in Figures 4, 5, and 6. [b] Assuming that only one strand of DNA hybridizes with rRNA. Thus, if 0.05% of a DNA forms hybrids with rRNA, the per cent rRNA cistrons is taken to be 0.1. [c] Assuming 89 pg of DNA/nucleus (Edstrom, 1964) and a molecular weight of 2.5 × $10^6$ for the 40S rRNA precursor (Loening *et al.*, 1969).

## Discussion

There is no significant contamination of the rRNA used in these experiments with other RNAs due to the fact that the rRNA was labeled after isolation and purification, a procedure which eliminates the preferential labeling of mRNA. This is evidenced by the virtually identical specific activities of the 28S peak and the 18S peak in Figure 2. Furthermore, hybridization occurs predominantly with a high-density satellite which has a (G + C) content of 72% (Figures 5 and 6). This is further indication that only rRNA is present in the RNA preparation.

The unsymmetrical shape of the hybridization peak in Figure 6 deserves special comment. It has been found that the amplified rRNA genes of *Xenopus* oocytes have a bouyant density of 1.729 g/cm$^3$ whereas the unamplified rRNA genes of somatic cells have a bouyant density of 1.724 (Brown and Dawid, 1968). It is possible that a mixture of rRNA genes of different densities occurs in the regenerating lens cells. In this regard it has been reported that the only difference between the composition of the amplified and the somatic rRNA genes of *Xenopus* is in the methylation of the cytosine residues (Dawid *et al.*, 1970). The somatic genes contain 4.5% 5-methylcytosine whereas the amplified genes contain no detectable amounts. Preliminary experiments in this laboratory indicate that a preferential methylation of the heavy DNA satellite occurs in normal iris relative to the regenerating lens system (unpublished observations).

In view of the rather long S phase of 30 hr, exhibited by cells of the dorsal iris during lens regeneration (Eisenberg-Zalik and Yamada, 1967), the possibility arises that these cells might be in a part of the S phase where the rRNA genes have been replicated, but the bulk of the chromosomal DNA has not. If such a situation existed at day seven of regeneration, it might account for our results. However, it should be emphasized that during lens regeneration the cells enter the S phase at about day 5, whereas our measurements were performed on DNA from 7-day regenerates, which have almost completed the S phase (Eisenberg-Zalik and Yamada, 1967). This would indicate that amplification of the rRNA cistrons has occurred, not preferential synthesis.

The value of 0.1% rRNA genes obtained for normal iris and

liver (Table I) agrees well with that obtained by Brown and Dawid (1968) for *Triturus* somatic cells. It can be calculated that these cells contain an average of almost 20,000 genes coding for rRNA (Table I). The 1.6-fold increase in the number of rRNA genes in the lens regenerates thus amounts to an increase of approximately 12,000 genes.

In the ovary (germinal vesicle) of *Triturus* a 1500-fold amplification of rRNA occurs (Brown and Dawid, 1968). The smallest amplification thus far reported for an ovarian tissue (Urechis) is 6-fold; however, this value is probably minimal (Dawid and Brown, 1970). It has been suggested that in the ovaries of amphibians and several insects, amplification of rRNA cistrons is a "differentiated function," necessary to synthesize a large number of ribosomes which are stored in the egg and utilized several months later during embryogenesis (Brown and Dawid, 1968). This situation is thought to be in contrast to somatic cells, where the synthesis of ribosomes is a "maintenance function" which is controlled without a change in the number of rRNA genes. The cells of the transforming iris are unique among somatic cells in that they are undergoing metaplasia into progenitor cells which will ultimately differentiate into a lens. In this regard, amplification of the rRNA cistrons could serve a differentiative function in lens regeneration.

It is not clear at present whether gene amplification is a widespread phenomenon of somatic cells. In different tissues which vary considerably in the rate of rRNA synthesis, the same amount of DNA complementary to rRNA was found for chick (Ritossa *et al.*, 1966), *Xenopus* (Brown and Weber, 1968), and *Triturus* (J. M. Collins, unpublished observations). In the mouse prostate, where rRNA synthesis is elevated in response to testosterone, no amplification of rRNA genes was detected (Brown and Dawid, 1968). No changes in the number of rRNA genes were found in the livers of male and female *Xenopus laevis*, in response to estrogen (J. M. Collins, unpublished observations). The specific amplification of genes in somatic cells is not restricted to the regenerating lens. After the completion of the experiments described in this paper, it was reported by Koch and Cruceanu (1971) that the rRNA genes of cultured human liver cells (Chang) are amplified 1.8-fold in response to a hormone analog.

We believe the 1.6-fold increase in the number of rRNA cistrons in the dorsal iris of *Triturus*, reported here, at least partly accounts for the 4-fold increase in rRNA synthesis (Reese *et al.*, 1969) which is initiated by lens regeneration. Perhaps the 12,000 new genes are controlled by some extrachromosomal mechanism which allows them to escape the normal, chromosomal regulation of the 20,000 rRNA genes that are always present.

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