UNDER-REPLICATION OF RIBOSOMAL CISTRONS IN POLYTENE CHROMOSOMES OF *RHYNCHOSCIARA*

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ABSTRACT

DNA preparations obtained from several tissues of *Rhynchosciara americana* and two related species, *R. milleri* and *R. papaveroi*, were hybridized to *R. americana* rRNA. The percentage of hybridization was found to be higher in tissues with low polyteny than in tissues with high polyteny, suggesting a relationship between the amount of rDNA and the tissue polyteny. This could be explained by under-replication of ribosomal cistrons in polytene cells, such as those from the salivary gland. Only slight tissue-dependent changes in the percentages of hybridization can be observed in heterologous hybridization using *Xenopus laevis* rRNA. The possibility that these experiments could not detect differences in the amount of ribosomal cistrons among tissues is discussed. The female:male ratio for the percentages of hybridization in the salivary gland of *R. americana* agrees with the results obtained by *in situ* hybridization experiments (16, 17) which have shown that the rRNA cistrons are distributed among chromosomes other than chromosome X.

INTRODUCTION

On the basis of cytological observations, Heitz (1, 2) proposed that the heterochromatic regions of *Drosophila* chromosomes replicate less than other regions. Subsequently, this hypothesis was confirmed by cytophotometric (3–5) and autoradiographic studies (6–8). Recently, evidence was obtained at the molecular level showing under-replication in highly repeated DNA sequences (9–12), and in ribosomal cistrons (13–15).

The ribosomal cistrons are a good model for the study of under-replication at the molecular level, due to their localization at the heterochromatic regions. The *in situ* hybridization of rRNA[1] with polytene chromosomes of the *Rhynchosciara* salivary glands showed that the ribosomal cistrons are located mainly in the heterochromatic regions near the centromeres of chromosomes X and C (16–17). However, Gerbi (18) did not detect under-replication of ribosomal cistrons in polytene tissues by hybridization experiments using *Xenopus* rRNA with *R. hollaenderi* DNA. Also, Gambarini and Meneghini (19), using homologous rRNA, found that the number of ribosomal cistrons did not change, in spite of an enlargement of the polyteny of the *R. americana*[2] salivary gland chromosomes during larval development. This latter result might be due to the shortness of the time interval in the

[1] *Abbreviations used in this paper:* rRNA, ribosomal RNA; rDNA, the DNA which codes for rRNA; SSC, 0.015 M sodium citrate, 0.15 M sodium chloride; EDTA, ethylenediaminetetraacetate.

[2] *Rhynchosciara americana* (Wiedemann, 1821) was redescribed by Nonato and Pavan (1951) as *angelae* and the synonymy was demonstrated by Breuer (20).

development of the larvae covered by these studies. Therefore, we expanded these studies to a longer time interval, and also measured the proportion of rDNA in several tissues of *R. americana* and other related species. We found that the proportion of rDNA is higher in tissues with low polyteny than in tissues with high polyteny. This suggests a relationship between the proportion of rDNA and tissue polyteny which could be explained by underreplication of ribosomal cistrons in polytene cells such as those from the salivary gland.

MATERIALS AND METHODS

Animals

Rhynchosciara americana was raised in the laboratory as previously described (21). We used larvae at the fourth instar, and adult virgin females 1 day after emergence. Larval age determination was made according to Terra et al. (22). *R. milleri* was maintained in the laboratory under the same conditions as *R. americana*. We used late fourth instar larvae and adult virgin females 1 day after emergence. Larvae of *R. papaveroi*, collected in nature, were grown in the laboratory until the beginning of the communal cocoon formation, when they were used.

We worked with the salivary gland and fat body from the larvae. The adult organs and tissues used were ovary and carcass. The carcass corresponds to all the somatic tissues remaining after ovary extirpation.

DNA Preparation

DNA was prepared in essentially the same way (19) for all tissues. The main difference in each case was the first homogenization step. Approximately 600 glands, or 600 fat bodies, or 300 ovaries were homogenized in a Dounce homogenizer with a tight pestle, in 10 ml of saline-EDTA (10 mM EDTA, 100 mM NaCl, pH 8.3) containing 0.5% Nonidet P 40 (Shell do Brasil S.A.). The carcasses were broken first in a Waring blender for 45 s (about 50 carcasses per 10 ml of saline-EDTA) and Nonidet P40 was added to a final concentration of 0.5%. The resulting suspension was homogenized in a Dounce homogenizer with a loose pestle and passed through cheesecloth. In each case, the chromatin was then collected by centrifugation, treated with RNase, α-amylase and pronase, and finally deproteinized (19). The DNAs so obtained were further purified before use by CsCl gradient centrifugation according to Flamm et al. (23), as previously described (19).

Ribosomal RNA Preparation

About 500 newly hatched larvae were placed on agar in petri dishes, with 1 g of food (21) containing 1.5 mCi of [5-^{3}H]uridine (25 Ci/mmol), and 1.5 mCi of [5-^{3}H]orotic acid (10 Ci/mmol). The larvae were maintained in this mixture for 14 days, new mixture being given to the larvae on the seventh day. The larvae were then starved for 1–2 days before being used for RNA preparation. The extraction of RNA, as well as the sucrose gradient centrifugation to separate 28S and 18S RNA, was accomplished as previously described (19). The only modification introduced in the process was the dialysis of the solution of 28S + 18S RNA, solubilized in 10 mM Tris-HCl buffer, pH 7.4, 50 mM NaCl, against this buffer, at 4°C, and its subsequent passage through nitrocellulose filters (HA or GS 13-mm diameter, Millipore Corp., Bedford, Mass.), under pressure two or three times. This procedure reduces the background in hybridization experiments (18).

In hybridizing rRNA thus obtained to *Rhynchosciara* ovary DNA fractionated in a CsCl gradient, we found a hybrid peak at higher density than main band DNA, which is similar to previously reported results (19). The density of the hybrid was found to be the same in similar experiments in which salivary gland DNA was used. This pattern of hybridization is the same as that obtained by hybridizing *Xenopus* rRNA to *Rhynchosciara* DNA (see references 16 and 24; and Gambarini et al., unpublished observations). In contrast, *Rhynchosciara* nonribosomal RNA hybridizes to the main DNA peak (24). Furthermore, in hybridizing our *Rhynchosciara* [^{3}H]-rRNA (2 μg/ml) to gland DNA in the presence of *Escherichia coli* rRNA (50 μg/ml) as a competitor, the hybrid peak is not affected. These results are a good indication that *Rhynchosciara* rRNA is a satisfactorily pure preparation for the experiments reported in this paper.

Hybridization Experiments

We used the filter hybridization technique developed by Gillespie and Spiegelman (25), with the modifications introduced by Birnstiel et al. (26), under conditions previously described (19). DNA was denatured by adding an equal volume of 1 M NaOH and maintained for at least 10 min at room temperature before being neutralized with 4 vol of a solution containing 0.25 M Tris-HCl (pH 8.0), 0.25 M HCl, and 1 M NaCl. About 10 μg of denatured DNA were applied to each nitrocellulose membrane filter (Millipore, GS 13-mm diameter), and dried overnight in a desiccator and then for 2 h in an oven at 80°C. Incubation of a duplicate filter holding denatured DNA and of a blank filter was carried out in 2.0 ml of 6 × SSC containing increasing amounts of *Rhynchosciara* [^{3}H]rRNA at 68°C for 4 h. The rRNA preparation was previously heated in a boiling water bath for 3 min, and immediately chilled in an ice-bath. At the end of the reaction, the filters were rinsed with 6 × SSC, and then with 2 × SSC by swirling in a beaker. RNase treatment was accomplished with 20 μg/ml pancreatic RNase (Worthington Biochemical Corp., Freehold, N.J.), in 2 × SSC, at room temperature, for 1 h. The filters were then rinsed with 2 × SSC and dried. The radioactivity

was determined in a liquid scintillation spectrometer model L 250 (Beckman Instruments, Inc., Fullerton, Calif.). After this, the filters were washed with chloroform, dried, and incubated in 1 N HCl for 20 min at 98°C. DNA retention was determined by reading the absorbance of the acid solution at 268 nm (27.8 units of absorbance corresponding to 1 mg/ml DNA).

RESULTS

Proportion of rDNA in Tissues with Different Polyteny in R. americana

DNA preparations from tissues with different degrees of polyteny were hybridized with *R. americana* rRNA. The tissues used were: (*a*) salivary glands (high polyteny) and fat bodies (low polyteny) from female larvae at the third period of the fourth instar; (*b*) ovaries (polyploid and polytenic) and carcasses (low polyteny) from adult females. We have chosen salivary gland cells as typical polytene cells.[3] The cytology of fat body cells has not been worked out. However, their nuclei are much smaller than those of salivary gland cells (M. Marques, personal communication) and consequently, the polyteny must be much lower than that in glands. This must also be the case of ovary (27) and carcass, both having mixed populations of cells.

The results are presented in Fig. 1 which shows the hybridization saturation curves. According to these results, DNA preparations obtained from tissues with low polyteny give higher percentages of hybridization than those obtained from salivary gland. The patterns of the results shown in Fig. 1

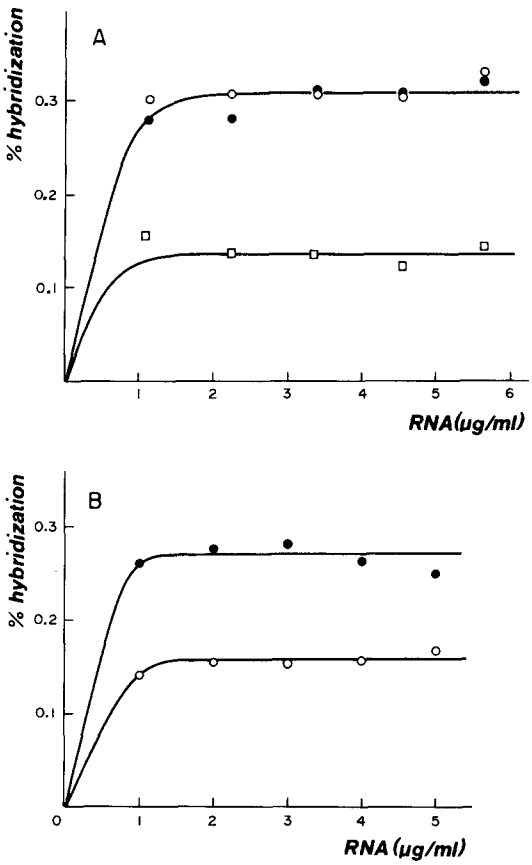

FIGURE 1 Saturation hybridization experiments between *R. americana* rRNA and DNA obtained from several *R. americana* tissues and organs. Preparations of ovary and carcass DNA from adult females, and of fat body and salivary gland DNA from third period larvae were denatured by alkali, immobilized on nitrocellulose filters (about 10 µg DNA/filter) and hybridized with increasing amounts of 28S + 18S [^{3}H]rRNA (42,000 cpm/µg), as described in Materials and Methods. The radioactivity was determined in a liquid scintillation spectrometer model L 250 (Beckman Instruments). The mean background of radioactivity measured 0.1% of the total radioactivity in the reaction tubes. DNA retention averaged 97% of the DNA input. Fig. 1 A ●——● ovary DNA; ○——○ carcass DNA; □——□ gland DNA. Fig. 1 B ●——● fat body DNA; ○——○ gland DNA.

were reproduced under different conditions, for example: (*a*) using the same rRNA preparation used in the experiment of Fig. 1 and varying the DNA preparations; (*b*) varying both the rRNA and DNA preparations; and (*c*) using an rRNA preparation not heated before the reaction. This

[3] The polyteny of salivary gland chromosomes can be estimated as follows: one larval female gland has about 300 cells (28). This number remains constant since the cells do not divide during development. In the fifth period of the fourth instar, one female gland has 0.89 µg of DNA (29). This gives about 3×10^{-3} µg or 1.8×10^{15} Daltons of DNA per cell. Dividing this by 1.3×10^{11} Daltons, which is the DNA content of the haploid genome (30), one arrives at a polyteny of 1.4×10^4. The DNA contents per gland in the third period and in the transition from the first to the second period are 0.50 µg (29) and 0.15 µg, respectively (A. G. Gambarini, unpublished observations). Using the same types of calculations described above, it is possible to estimate the polytenies of 7.7×10^3 for the third period and 2.3×10^3 for the transition from the first to the second period. These figures are mean degrees of polyteny for the whole gland since chromosomes of the proximal region of the gland are larger than those of the distal region.

TABLE I

Percent Hybridization Values of rRNA and DNA obtained from Several Organs and Tissues of R. americana

Origin of DNA		Percent hybridization	Mean
Tissue	Stage		
Ovary	Adult	0.354	0.308
		0.300	
		0.312	
		0.264	
Carcass	Adult	0.308	0.319
		0.330	
Fat body	Larvae 3rd p	0.234	0.252
		0.270	
Salivary gland	Larvae 3rd p	0.154	0.153
		0.154	
		0.160	
		0.138	
		0.159	

P, period of fourth instar.
For experimental details see Materials and Methods.

latter finding removes the possibility that the result is an artifact due to the heating procedure which increases the formation of the hybrid by about 70% (19). The calculated percentages of hybridization for several experiments are presented in Table I. According to this table, the ovary DNA and carcass DNA have approximately the same percentage of hybridization, which is twice as great as that of gland DNA. The percentage of hybridization of fat body DNA is about 65–70% greater than that for gland DNA.

Changes in the Proportion of rDNA in the Salivary Gland and Fat Body of R. americana during Larval Development

The polyteny of the salivary gland chromosomes becomes progressively greater during larval development (see footnote 3). In an attempt to detect changes in the proportion of rDNA which could be parallel to an increase in polyteny in this organ, we prepared DNA from salivary glands of larvae at different periods of the fourth instar, which was then hybridized to rRNA. The time interval chosen covered the final half of the larval development. Similar measurements were also carried out with the fat body from larvae of the same periods of development, in order to verify the pattern of changes of rDNA in this tissue in which polyteny is much less than that of the gland. The results are shown in Fig. 2, from which the following conclusions can be drawn: (*a*) the percentages of hybridization obtained with DNA from fat body are greater than those obtained with DNA from salivary gland within the studied interval. The difference in the percentage of hybridization is about 70% in the third and fifth periods. (*b*) The percentage of hybridization in DNA from salivary gland practically does not change in the final periods of the fourth instar as previously described (19), while in the transition between the first and second periods it is about 30% greater than in the final periods. (*c*) The percentage of hybridization for fat body DNA seems to be the same from the first to the second period and up to the fifth period, as indicated by the interpenetration of the deviations.

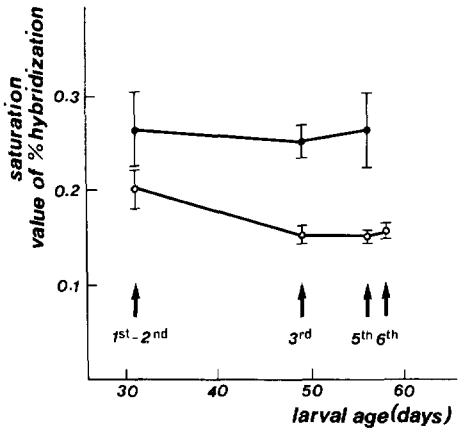

FIGURE 2 Changes in fat body and salivary gland rRNA-DNA hybridization percentages during larval development in *R. americana*. This figure shows the mean values for the percentages of hybridization for salivary gland and fat body DNAs from female larvae, calculated from saturation experiments as shown in Fig. 1 B. The period corresponding to each point is indicated by an arrow. The vertical line in each point represents the standard deviation of the determinations, except for the third period fat body where it represents the mean deviation. The means and respective deviations plotted, and also the number of determinations (indicated in parentheses) in each case were: salivary gland, first to second period, 0.202 ± 0.021 (three); third period, 0.153 ± 0.009 (five); fifth period, 0.151 ± 0.004 (three); sixth period, 0.157 ± 0.007 (three). Fatty body, first to second period, 0.265 ± 0.038 (three); third period, 0.252 ± 0.018 (two); fifth period, 0.264 ± 0.039 (four). O——O gland DNA. ●——●. fat body DNA.

Proportion of rDNA in Tissues with Different Polyteny in R. milleri and R. papaveroi

rRNA of *R. americana* was hybridized with *R. milleri* and *R. papaveroi* DNA, so that we could look for polyteny-dependent changes in the rDNA proportions in these species. The results obtained are shown in Table II. They are similar to those obtained with *R. americana*. The percentage of hybridization in fat body DNA of *R. papaveroi* is almost twice as great as that for salivary gland DNA. In *R. milleri* the differences found among the tissues were smaller than we expected on the basis of the above-mentioned results.

Hybridization of R. americana DNA with Xenopus laevis rRNA

In an early paper, Gerbi (18) found identical levels of rDNA in carcass DNA from adult males, and of gland DNA from male larvae, in hybridization experiments using *Xenopus laevis* rRNA. We have carried out several similar experiments, using DNA prepared from female tissues, which agree with Gerbi's findings. The results of one of these experiments are shown in Table III. We could not detect differences greater than 13% and 24% in the rDNA levels between gland and fat body, and gland and ovary, respectively. However, using homologous rRNA we could detect differences of 70% and 100% between these pairs of tissues.

Chromosomal Distribution of rDNA

Early cytological observations (31, 32) indicated that the nucleolus of *R. americana* is organized by chromosome X. This should indicate that the nucleolar organizer of chromosome X is the site of the ribosomal cistrons. Later, this structure was demonstrated to be present in the four chromosomes of the salivary gland (33, 34). Thus, it should be reasonable to assume the presence of ribosomal cistrons in other chromosomes besides chromosome X. In order to clarify this point, we hybridized rRNA with DNA prepared from somatic tissues from females (XX), and males (XO). The data in Table IV are the results obtained by hybridizing DNA of salivary gland and fat body of female and male larvae of the fifth period of the fourth instar. The percentages of hybridization for gland and fat body of females are, respectively, 1.25- and 1.44-fold greater than the corresponding values in males. The results shown in Table IV essentially confirm earlier results obtained under different conditions, namely: (*a*) by hybridizing gland and fat body DNA from third period larvae to an rRNA preparation not heated before the reaction; (*b*) by hybridizing unheated rRNA to DNA prepared from whole second period larvae; and (*c*) by hybridizing DNA from whole second period larvae to *Xenopus* 28 S RNA (35). Assuming that chromosome X has the same number of cistrons in both females and males, the results described above suggest that ribosomal cistrons are localized in other chromosomes besides chromosome X.

Analyzing Table IV, we also see that the percentage of hybridization for DNA from the fat body of males is about 60–65% greater than that for DNA from the salivary gland of males. This indicates that in *Rhynchosciara*, the same variations in the proportion of ribosomal cistrons with the polyteny of the tissue presented above for females are valid also for males.

TABLE II

Percent Hybridization Values of R. americana rRNA and DNA obtained from Several Organs and Tissues of R. milleri and R. papaveroi

Species	Origin of DNA		Percent hybridization
	Tissue	Stage	
R. papaveroi	Salivary gland	4th instar larvae ♀	0.130
	Fat body	4th instar larvae ♀	0.250
R. milleri	Salivary gland	4th instar larvae ♀	0.145
	Fat body	4th instar larvae ♀	0.166
	Carcass	adult ♀	0.226
	Ovary	adult ♀	0.226

For experimental details see Materials and Methods.

TABLE III

Comparison of the Values of Percent Hybridization of R. americana DNA with R. americana or Xenopus laevis rRNA

Origin of DNA	Percent hybridization: *Xenopus* rRNA 28S	Percent hybridization: *R. americana* rRNA 28+ 18S
Salivary gland ♀ 5th p	0.038	0.151
Fat body ♀ 5th p	0.043	0.264
Ovary adult	0.047	0.308

Filters with salivary gland or fat body DNA from fifth period female larvae, and with adult ovary DNA, were incubated with increasing amounts of *Xenopus* 28S [^{3}H]rRNA (600,000 cpm/μg, not heated) in 6 × SSC, for 12 h at 66°C. The subsequent treatments were done as described in Materials and Methods.

The values of percentage of hybridization for *R. americana* rRNA were obtained from Fig. 2 (gland and fat body) and Table I (ovary).

TABLE IV

Percent Hybridization Values of rRNA and DNA obtained from Male and Female Tissues of R. americana Larvae at the Fifth Period of the Fourth Instar

Origin of DNA	Percent hybridization	♀:♂ Ratio
Salivary gland		
♀	0.153	1.25
♂	0.122	
Fat body		
♀	0.283	1.44
♂	0.197	

For experimental details see Materials and Methods.

DISCUSSION

Proportion and Distribution of DNA Complementary to rRNA in Rhynchosciara

The results we have presented here show that according to the tissue of origin, different amounts of *R. americana* DNA hybridize to homologous rRNA. Tissues or organs with low polyteny have more DNA hybridizable to rRNA than a typical polytene tissue, such as the salivary gland. This has been observed for somatic (carcass or fat body) and germinative (ovary) tissues. It holds also for tissues having a homogeneous cell type (as seems to be the case of fat body), as well as for those having mixed populations of cells (carcass and ovary). Preliminary results obtained with *R. milleri* and *R. papaveroi* indicate a pattern of DNA-rRNA hybridization similar to that found for *R. americana*. These observations suggest that the difference in hybridizable DNA is related in some way to differences in polyteny among the tissues and organs. We will discuss in the following section a possible relationship between polyteny and rRNA cistron replication.

The fact that the percentage of hybridization of rRNA with ovary DNA is practically the same as with carcass DNA favors the hypothesis previously suggested (19), that the difference between the levels of rRNA cistrons in ovary and gland is a consequence of the difference in polyteny between these organs, since the cytology of the ovary does not suggest the occurrence of rDNA amplification (27).

The female:male ratio for the percentage of hybridization of salivary gland DNA should be a consequence of the distribution of rRNA cistrons between chromosomes X and C, the first of which is present singly in the somatic male cells (16, 17). The female:male ratio for the fat body (low polyteny) is similar to that found for the salivary gland (high polyteny). This indicates that such a ratio may not be a characteristic peculiar to tissues with high polyteny, as is the case of certain *D. melanogaster* strains (15). However, we do not have enough data to analyze the influence of factors other than chromosomal distribution on the female:male ratio. Differences in chromosome polyteny or mechanisms affecting cistron replication, could possibly determine a higher level of rRNA cistrons in male cells in order to compensate for the presence of just one X chromosome.

Under-Replication of Ribosomal Cistrons in Rhynchosciara

In other diptera, the heterochromatin of the polytenic chromosomes is not very active in replication (1–13). In *Rhynchosciara* the same seems to be the case. Recently, it was shown (30, 36) that only about one-third of the highly repeated DNA sequences assumed to belong to the heterochromatin (37) is normally replicated during the process of polytenization which occurs in the salivary gland. The results presented in this paper suggest that the rDNA is also under-replicated in

polytenic cells, which may be due to its localization in heterochromatin regions.

The rDNA proportion present in carcass DNA (low polyteny) is only twice as great as that present in late fourth instar gland DNA (Table I), indicating that the difference between the rDNA proportions present in polytene and truly diploid cells should be relatively small. In the time-interval covered by our studies on gland, the polyteny of the gland chromosomes enlarges about 10-fold (see footnote 3), with a parallel decrease of only about 30% in the rDNA proportion (Fig. 2), indicating that the process of under-replication should be slow, at least in the studied interval. These findings point in favor of the hypothesis previously proposed (10, 13), that the rDNA should participate in the major part of the replicative steps which occur during the process of polytenization.

The under-replication phenomenon could be explained by assuming an equally slower replication rate for the entire body of ribosomal cistrons, in relation to the rest of the DNA. Another explanation is the assumption that the rDNA is made up of cistron classes replicating differentially during polytenization. This second possibility seems to be supported, although circumstantially, by the differences of hybridization obtained between *Rhynchosciara* DNA and *Xenopus* rRNA (see Table III). The hybridization with heterologous rRNA suggests the possibility that it does not detect differences in the amount of rDNA among tissues. On the other hand, some results on rRNA structure (see, for example, references 38 and 39) indicate that in spite of their resemblance, the cistrons coding for this kind of RNA are not necessarily identical. Thus, *R. americana* ribosomal cistrons could not be perfectly identical, with part of them not being sufficiently homologous to *Xenopus* 28S rRNA to allow the formation of stable hybrids. If the cistrons not homologous to *Xenopus* rRNA were preferentially under-replicated, hybridization experiments with this RNA should not detect changes in the rDNA with the polyteny, while in those experiments with *R. americana* rRNA, the differences should be detected. In this connection, the conclusion of Hennig (12), indicating that the highly repeated DNA sequences in the *Drosophila hydei* genome could be differentially under-replicated in polytenic cells, is interesting.

We used only *Xenopus* 28S rRNA in our experiments but, since in eukaryotes the 18S and 28S cistrons occur in a 1:1 ratio (38), what happens to the 28S cistrons might also be true for the 18S cistrons. If so, we may surmise that under-replication of 18S cistrons might have been detected if investigated.

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