MOLECULAR HYBRIDIZATION OF IODINATED 4S, 5S, AND 18S + 28S RNA TO SALAMANDER CHROMOSOMES

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ABSTRACT

4S, 5S, and 18S + 28S RNA from the newt *Taricha granulosa granulosa* were iodinated in vitro with carrier-free ^{125}I and hybridized to the denatured chromosomes of *Taricha granulosa* and *Batrachoseps wrighti*. Iodinated 18S + 28S RNA hybridizes to the telomeric region on the shorter arm of chromosome 2 and close to the centromere on the shorter arm of chromosome 9 from *T. granulosa*. On this same salamander the label produced by the 5S RNA is located close to or on the centromere of chromosome 7 and the iodinated 4S RNA labels the distal end of the longer arm of chromosome 5. On the chromosomes of *B. wrighti*, 18S + 28S RNA hybridizes close to the centromeric region on the longer arm of the largest chromosome. Two centromeric sites are hybridized by the iodinated 5S RNA. After hybridization with iodinated 4S RNA, label is found near the end of the shorter arm of chromosome 3. It is concluded that both ribosomal and transfer RNA genes are clustered in the genome of these two salamanders.

The location of specific DNA sequences on eukaryotic chromosomes can be determined by molecular hybridization of a homogeneous RNA species to denatured chromosomes in cytological preparations (13). RNA iodinated in vitro with carrier-free ^{125}I produces a hybrid that can be rapidly detected with a resolution comparable to that obtained with tritiated RNAs (33, 42, 43). Ribosomal and transfer RNAs can be easily purified, and their complementary sequences are repetitious in the genome of eukaryotes (4, 8, 35). Furthermore, in some organisms the gene sequences which code for rRNA (5S and 18S + 28S) are tandemly arranged at one or a few loci (1, 18, 23, 30, 42, 44), with untranscribed "spacers" separating adjacent transcribed sequences (3, 5). Recently, Clarkson et al. (7) have shown that, in the genome of *Xenopus*, isocoding tRNA genes are clustered and covalently linked to long spacer sequences of low G + C mole percent.

4S, 5S, and 18S + 28S RNA from the northern rough-skinned newt *Taricha granulosa granulosa* (Family Salamandridae) have been isolated and iodinated in vitro. Each labeled RNA was hydridized to spermatocyte chromosomes previously denatured with formamide. Distinct sites are hybridized by each RNA both in the chromosomes of *T. granulosa* and in the chromosomes of the Oregon slender salamander *Batrachoseps wrighti* (Family Plethodontidae). Thus, it is concluded that both t- and rRNA are clustered in the genome of these two amphibians.

MATERIALS AND METHODS

Animals

T. g. granulosa (Skilton) was collected from May to July in a pond at the Peavy Arboretum, Corvallis, Linn

County, Oregon. *B. wrighti* (Bishop) was collected in the Cascade mountains of west-central Oregon in the Willamette National Forest of Lane County during June. To obtain the required tissues the animals were killed by submerging them in a saturated solution of Chlorotone (1,1,1-trichloro-2-methyl-2-propanol), and then the body cavity was exposed by a mid-ventral cut.

Isolation of RNA

RNA from liver and ovaries was obtained according to the methods of Muramatsu (28). Frozen livers were minced and ground with dry ice in a mortar and pestle. The powder was suspended in cold TKM buffer (0.05 M Tris, pH = 7.4, 0.025 M KCl, and 0.005 M $MgCl_2$) with 0.25 M sucrose and 0.007 M 2-mercaptoethanol. Fresh oocytes were gently ruptured in the same medium with three strokes of a hand-driven glass homogenizer, as described by Sommerville (37). After being filtered through six layers of cheesecloth, the homogenate was centrifuged for 15 min at 12,000 *g* (4°C).

To obtain 4S and 5S RNA, sodium dodecyl sulfate to a final concentration of 0.5% was added to the supernate, and the mixture was repeatedly extracted with an equal volume of water-saturated redistilled phenol. The RNA in the aqueous phase was precipitated with 2-½ vol of ethanol, washed with ethanol, diethyl ether, and air dried. After the pellet was resuspended in 0.3 M NaCl, 0.01 M $MgCl_2$, 0.02 M $NaC_2H_3O_2$ (pH = 6.0), the RNA was loaded onto a DEAE-cellulose column equilibrated with the same buffer, washed extensively, and then eluted by raising the NaCl concentration to 1.0 M.

The RNA released from the DEAE-cellulose column was precipitated with ethanol as before and redissolved in 0.75 M NaCl, 0.02 M $NaC_2H_3O_2$ (pH = 5.0) with 1% (vol/vol) methanol. Chromatography on a Sephadex G-100 column (1.0 × 120 cm) equilibrated with the same buffer was done at 4°C as previously described (42). The central portion of the 4S and the 5S RNA peaks (Fig. 1) was pooled and precipitated with ethanol before it was resuspended in the acetate buffer for iodination. The $A_{260/280}$ ratio for each RNA in the acetate buffer was greater than 2.0.

18S + 28S RNA was obtained from ribosomes after the procedure of Muramatsu (28) except that the post-mitochondrial supernate obtained after the first centrifugation was layered over a 1.5 M sucrose cushion before the ribosomes were pelleted at 105,000 *g*. The RNA obtained from the ribosomal pellet was centrifuged through two consecutive 15–30% sucrose gradients in 0.04 M Tris (pH = 7.8), 0.02 M $NaC_2H_3O_2$, 0.002 M Na_2 ethylenediaminetetraacetic acid (EDTA) with 0.2% sodium dodecyl sulfate (25). After centrifugation at 152,000 *g* for 14 h at 18°C (SW40 rotor, L-2 Ultracentrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the gradients were fractionated by puncturing the bottom of the tube, and then the RNA under the shaded area of Fig. 2 was pooled and precipitated with ethanol before it was resuspended in the acetate buffer for iodination. The ratio of absorbance at 260/280 of this RNA was greater than 2.0.

TABLE I

Quantitative Data from Five Selected Haploid Sets of T.g. granulosa and B. wrighti

Taricha granulosa				*Batrachoseps wrighti*			
Chromosome no.	Length of haploid genome	Arm ratio*	Centromere position*	Chromosome no.	Length of haploid genome	Arm ratio	Centromere position
	%				%		
1	12.2	1.19	m*	1	11.5	1.25	m
2	11.4	1.23	m	2	10.8	1.05	m
3	10.2	1.81	sm*	3	9.8	1.68	m
4	9.8	1.15	m	4	9.8	1.03	m
5	9.7	1.67	m	5	8.9	1.53	m
6	9.6	1.16	m	6	8.6	4.45	st*
7	9.3	2.32	sm	7	7.1	1.51	m
8	7.9	2.14	sm	8	7.0	1.37	m
9	7.6	1.23	m	9	5.8	1.20	m
10	6.5	1.17	m	10	5.6	2.63	sm
11	5.7	1.49	m	11	5.5	1.47	m
				12	5.1	1.24	m
				13	4.6	2.27	sm

* Arm ratio = length of long arm/length of short arm. Centromere position according to the definitions by Levan et al. (21). m = centromere in the median region, sm = centromere in the submedian region, and st = centromere in the subterminal region of the chromosome.

Acrylamide Gel Electrophoresis

The mobility of the peaks obtained from the Sephadex G-100 column. and the sucrose gradients was compared to commercial RNA standards by electrophoresis in 4% acrylamide gels (22) as previously described (42).

Iodination of RNA

RNA was iodinated with carrier-free ^{125}I (New England Nuclear, NEZ 033H, Boston, Mass.) according to Prensky et al. (33) and Wen et al. (42) with the following modifications. After iodination, the reaction mixture was layered on a Sephadex G-50 column (0.5 × 20 cm) equilibrated with 0.1 M NaCl, 0.05 M Tris (pH = 8.4), and 0.001 M Na_2EDTA. To the material eluting at the void volume of the column, 0.1 ml of 0.01 M Na_2SO_3 (in distilled water) was added, and the mixture was immediately reheated for 30 min at 60°C to remove unstably bound iodine (10, 14).

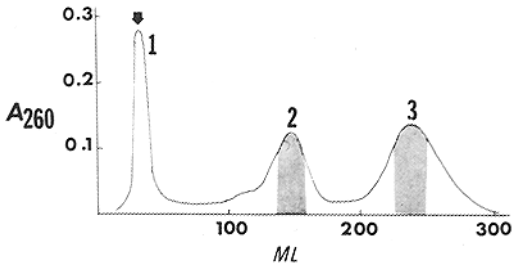

FIGURE 1 Elution profile of *T. granulosa* RNA from Sephadex G-100. The void volume of the column is indicated by the arrow. Peak *1* was identified as mostly 18S RNA on acrylamide gels and sucrose gradients, and peaks *2* and *3* as 5S and 4S, respectively, on acrylamide gels. The RNA under the shaded portion of peaks *2* and *3* was pooled for iodination.

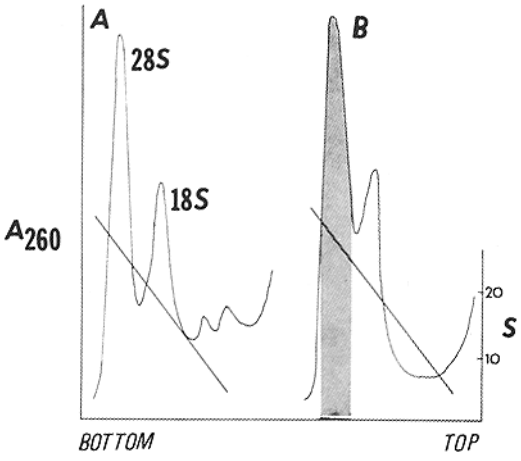

FIGURE 2 Sucrose gradients (15–30%) of *T. granulosa* RNA obtained from ribosomes. The first two peaks on the left (A) were pooled and recentrifuged through a second 15–30% sucrose gradient (B), and then the RNA under the shaded area was pooled for iodination.

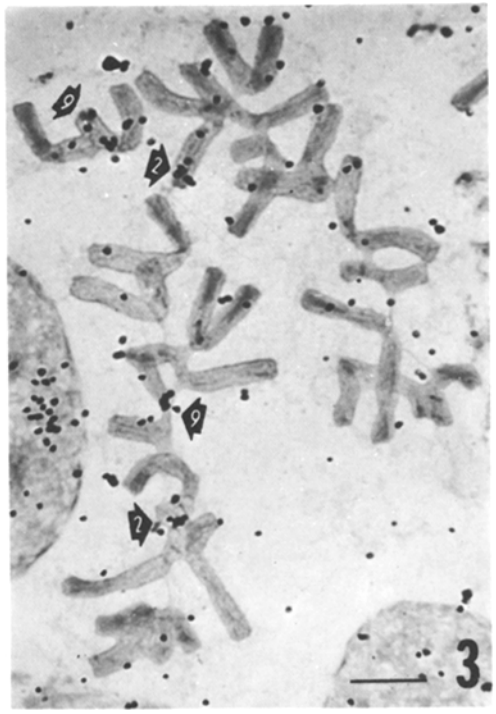

FIGURE 3 Gonial mitotic chromosomes of *T. granulosa* hybridized with homologous iodinated 18S + 28S RNA. Chromosomes no. *2* were consistently labeled near the tip of the shorter arm, and chromosomes no. *9* near the centromere on the shorter arm. Exposure time, 10 days. Scale = 5 μm.

After this reheating step, unlabeled commercial *Escherichia coli* tRNA (Schwartz/Mann Div., Becton, Dickinson & Co. Orangeburg, N.Y.) was added to the 5S and to the 18S + 28S RNA, or *Micrococcus lysodeikticus* DNA (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.) to the iodinated 4S RNA preparation. The nucleic acids were then dialysed overnight against 4 liters of distilled water and 1 drop of diethyl pyrocarbonate. Further purification of the iodinated RNA was done on Sephadex G-50 or G-100 columns (1 × 25 cm) equilibrated with 0.75 M NaCl, 0.01 M $NaC_2H_3O_2$ (pH = 5.0) and 1% (vol/vol) methanol. Before hybridization the RNA was precipitated with absolute ethanol and pelleted by centrifugation at 12,000 *g* for 15 min. The pellet was washed with 70% ethanol, absolute ethanol, and air dried. The RNA was resuspended in 0.5–1.0 ml of freshly prepared 50% formamide in 2 × standard saline citrate (SSC) (pH = 6.8).

After the RNA solution is reheated, approximately

three-fourths of the counts are TCA-precipitable, increasing to about 95% after overnight dialysis. The second gel filtration step renders all counts TCA-precipitable, indicating that all free iodine has been removed. In the presence of large amounts of carrier RNA, the iodinated molecules can be repeatedly precipitated with ethanol without loss of counts in the soluble fraction. Thus, ethanol precipitation was routinely done to concentrate the RNA before it was dissolved in the hybridization mixture. Rapid changes in the pH of the SSC-formamide solution at room temperature have prompted the preparation of fresh solution for every hybridization. By measuring the absorbance of the RNA before and after iodination, we have estimated the percent recovered; usually 80–90%. The specific activities of the 4S, 5S, and 18S + 28S RNA used in these experiments were 2×10^7, 1×10^7 and 3×10^6 dpm/μg, respectively, determined by counting aliquots in 0.4% Omnifluor (New England Nuclear) in toluene with the tritium channel of a Packard Tricarb 3380 spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

In Situ Hybridization

The hybridization reaction was done as previously described (42) according to the procedure of Gall and Pardue (13) modified by Wimber and Steffensen (44). Chromosome preparations were made from testis fixed in absolute ethanol: glacial acetic acid (3:1 vol/vol), squashed in 45% acetic acid, and made permanent by the dry-ice method of Conger and Fairchild (11). The dry slides were stored in vacuo at 4°C until they were treated with RNase and denatured for hybridization.

Denaturation was accomplished by incubating the preparations at 60°C for 2½ h in 90% formamide and 0.1 × SSC (pH = 6.8). Then the slides were repeatedly rinsed in ice-cold 2 × SSC, cold 70% ethanol, cold 95% ethanol, and air dried. Approximately 20 μl of the iodinated RNA in 50% formamide and 2 × SSC (pH = 6.8) was applied to each slide. The preparations were first incubated at 60°C for 2 h and then at 40°C for 2 additional hours, after a modification proposed by Wimber et al. (43). After hybridization the slides were

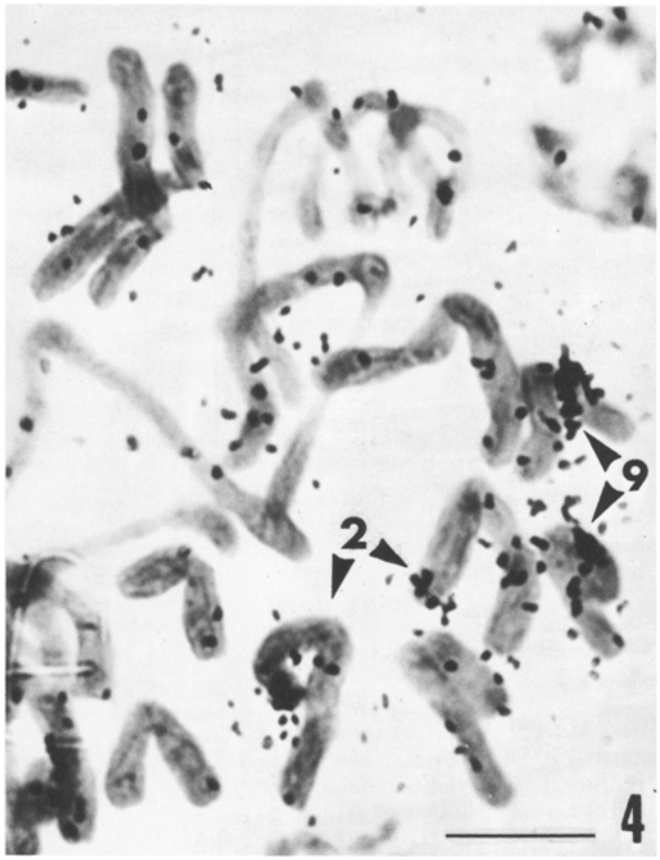

FIGURE 4 Gonial mitotic chromosomes of *T. granulosa*, as in Fig. 3, showing the two pairs of chromosomes that are labeled by the 18S + 28S RNA. Exposure time, 10 days. Scale = 5 μm.

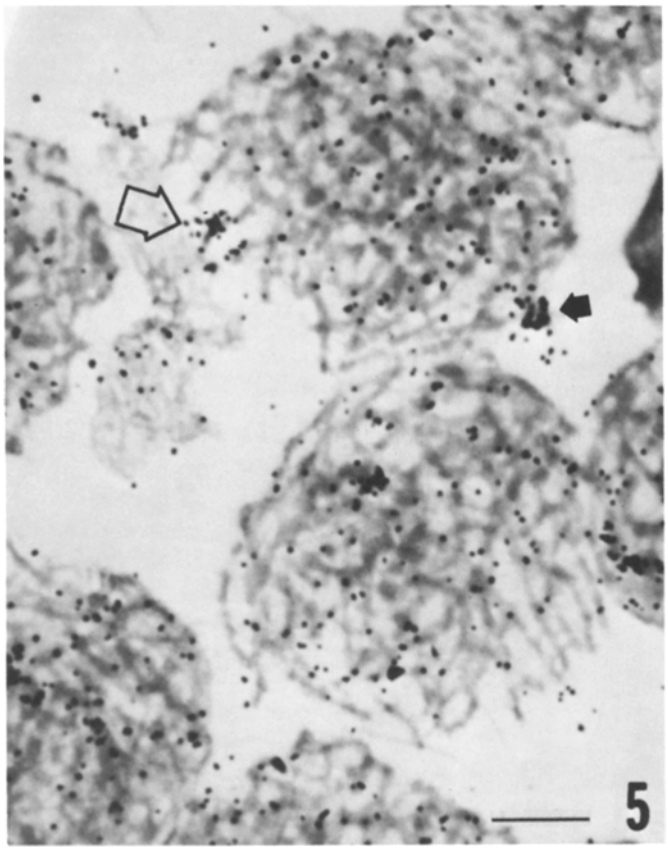

FIGURE 5 Pachytene spermatocytes from *T. granulosa* hybridized with 18S + 28S RNA. Two bivalents are consistently labeled, one of them is labeled interstitially (solid arrow), presumably chromosomes no. 9. The label on the other bivalent, presumably chromosome no. 2, is located near the end of one of the arms (open arrow). Exposure time, 10 days. Scale = 5 μm.

washed first with cold 2 × SSC, then with 2 × SSC at 60°C, digested with pancreatic RNase (200 μg/ml) and T_1 RNase (50 U/ml) for 1 h at 37°C, and dipped in NTB-2 liquid emulsion diluted 1:1 with distilled water. The slides were kept in black boxes at 4°C with dessication for the periods indicated in the figure legends, and then the preparations were developed and stained with Giemsa dye.

The standard karyotypes obtained from *T. granulosa* and *B. wrighti* (Table I) represent an average of five mitotic sets, considered to have a minimum of artifactitious distortion, and measured from enlarged photographs with an opisometer. Relative length and arm ratio, as defined by Levan et al. (21), were the two criteria utilized in identifying the chromosomes. Identification of the 11 chromosomes in the *T. granulosa* set is relatively easy since large differences (greater than 0.3) in the relative length and/or arm ratio are observed between similar chromosomes (Table I). The most ambiguous identifications involve chromosomes 4 and 6. In the chromosome set of *B. wrighti*, chromosomes 7 and 8 cannot be distinguished from each other since both the differences between their arm ratios, and their relative lengths are small (Table I).

For each gene localized, four hybridized karyotypes were also photographed, measured, compared to the standard karyotypes, and the location of the labeled sites was determined (Fig. 15). Many other sets were examined in order to ascertain the location of the hybrids.

RESULTS

RNA was separated by Sephadex G-100 into three distinct peaks (Fig. 1). The mobility of RNA from peaks *2* and *3* was repeatedly compared on acrylamide gel co-runs with *E. coli* tRNA and 5S RNA (Schwartz/Mann) before iodination. Peak *3* runs in coincidence with *E. coli* tRNA and remains as a single band even when 25 μg of RNA were loaded. 25 μg of RNA from peak *2* migrated in coinci-

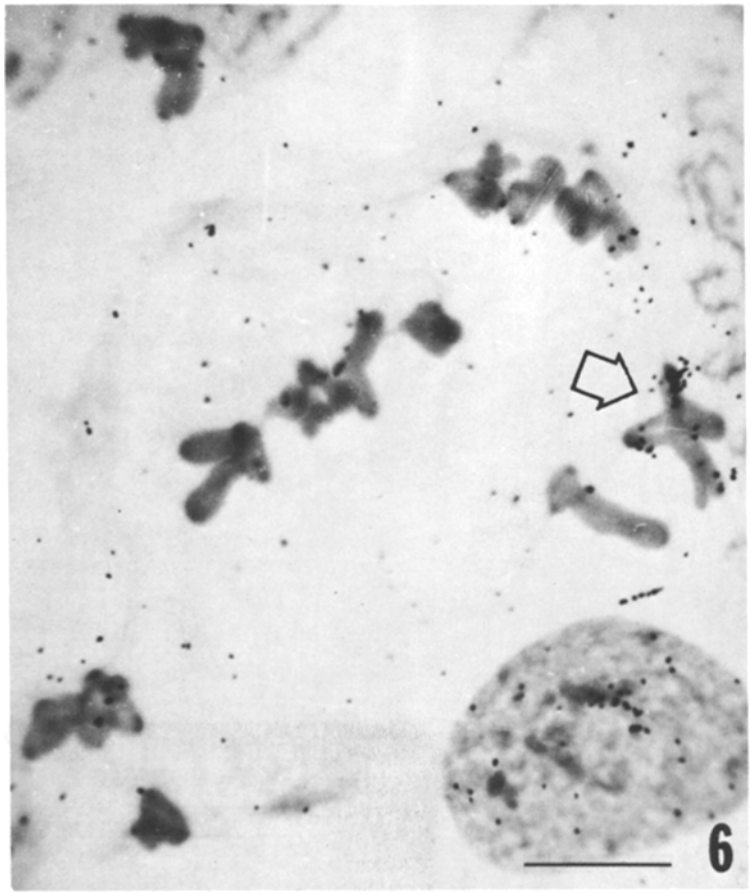

FIGURE 6 First meiotic metaphase chromosomes from *B. wrighti* testis, hybridized with iodinated 18S + 28S RNA from *T. granulosa*. The arrow points at the longest bivalent which is consistently labeled on the longer arm. Exposure time, 10 days. Scale = 5 μm.

dence with *E. coli* 5S RNA, also appearing as a single band. The RNA that elutes at the void volume of the column contains mostly 18S RNA, determined on acrylamide gels and sucrose gradients, and the rRNA from the shaded portion of Fig. 2 consists of mostly 28S, by these same criteria.

In *T. g. granulosa* there are 11 chromosomes in the haploid set. Their centromeres are in the median and submedian region, and they form a gradually decreasing relative length series (Table I). The data in Table I for the Corvallis specimens have been compared to the karyotype data obtained by Seto and Pomerat (36) from animals collected at another locality in Oregon. Although the relative lengths are similar, the arm ratios of chromosomes *2*, *4*, *7*, and *10* are considerably different. I am unaware of the reason for these discrepancies. In *B. wrighti*, (N = 13) with median and submedian region centromeres in 12 of the chromosomes and subterminal centromere in one of the chromosomes (Table I). As in *Taricha*, they form a gradually decreasing relative length series.

The iodinated 18S + 28S RNA hybridizes to two homologous pairs of *T. granulosa* mitotic metaphase chromosomes obtained from spermatogonia (Figs. 3 and 4). Chromosome *9* is the most heavily labeled pair. It is slightly submetacentric (arm ratio = 1.23), and the label is located on the shorter arm, near the centromere. Chromosome *2*, also with arm ratio = 1.23, is labeled near the distal end of the shorter arm. In the more extended pachytene chromosomes it is possible to observe that the label on chromosome 2 is not totally terminal (Fig. 5, solid arrow). Pachytene nuclei are also labeled near the centromeric region of one of

the U-shaped bivalents, presumably bivalent 9 (open arrow). In the elongate developing spermatids two groups of silver grains are repeatedly encountered (not shown): one close to the base of the head, and another close to the acrosome. When this RNA is hybridized to the chromosomes of the plethodontid salamander, *B. wrighti*, it labels the longer arm, close to the centromere (Fig. 6).

Most of the label produced after hybridization with iodinated 5S RNA is found over chromosome no. 7 of dividing spermatogonial cells in *Taricha* (Fig. 7). This chromosome is strikingly submetacentric with an arm ratio greater than 2.0, and the label is located very close to or on the centromere. The centromere region of a submetacentric bivalent, presumably chromosome no. 7, is labeled in the metaphase I of meiosis (Fig. 8, arrow). When the iodinated 5S RNA is not competed with unlabeled 18S + 28S RNA, some label can be detected on the nucleolus organizers of chromosomes 2 and 9. This label is abolished by including 50-fold competitor in the hybridization mixture. However, in addition to the major labeled site, a persistent centromeric locus is labeled in spermatocyte bivalents even in the presence of unlabeled 18S + 28S RNA (Fig. 9); clear identification of its location was not possible, but it may correspond to the centromere on chromosome no. 4 (Fig. 15). Spermatids are labeled close to the base of the head. This RNA also labels the centromeric region of bivalents 2 and 3 in the spermatocytes of *B. wrighti* (Fig. 10).

Iodinated 4S RNA hybridizes to chromosome no. 5 (arm ratio = 1.67) of *Taricha*, near the distal end of the longer arm (Fig. 11). The first meiotic metaphase chromosomes in Fig. 12 demostrate that the label is located over one bivalent, with no consistent label on other sites. In pachytene sper-

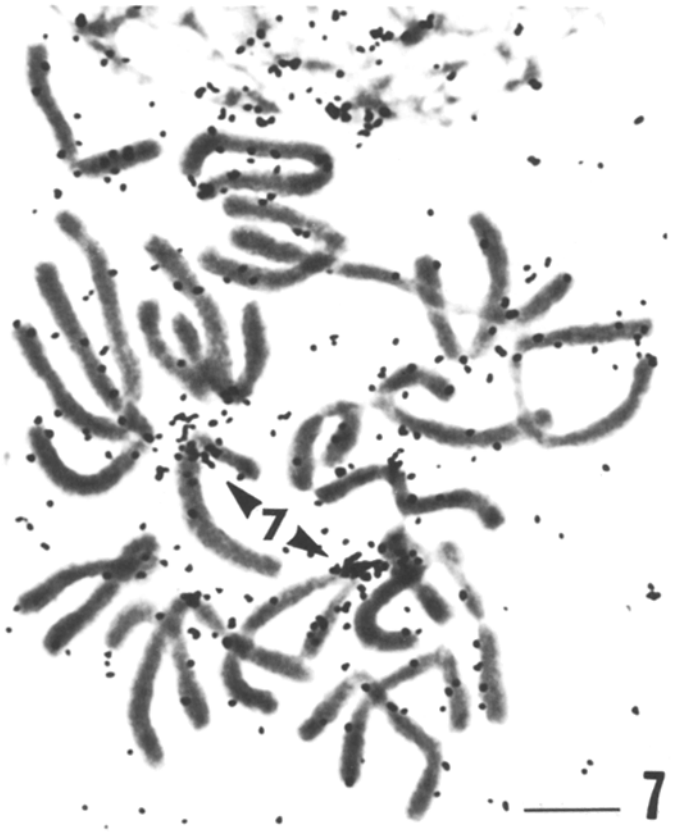

FIGURE 7 Gonial mitotic chromosomes from *T. granulosa* hybridized with iodinated 5S RNA. The label is located over the homologous chromosome pair no. 7, near or on the centromere (arrow). Exposure time, 10 days. Scale = 5 μm.

matocytes the genes hybridized on chromosome 5 are found on two adjacent sites separated by a short, unlabeled segment (Fig. 13). This condition was encountered in many pachytene spermatocytes. When the chromosomes of *B. wrighti* are hybridized with 4S RNA, the label is located over one locus near the tip of the longer arm of chromosome 3 (Fig. 14). The labeled sites produced by the 4S RNA on the chromosomes of *B. wrighti* and *T. granulosa* (Fig. 15) do not correspond to those labeled by the 5S and 18S + 28S RNA.

DISCUSSION

The interpretation of these results is contingent upon the identity of the RNAs that gave rise to the hybrids observed in the autoradiographs. The identity of the peaks obtained from Sephadex G-100 was determined from previous work (27, 42) and confirmed by acrylamide gel electrophoresis. Acrylamide gel electrophoresis also indicated that the RNA obtained from the Sephadex G-100 peaks was at least 90% homogeneous in size. However, the 5S RNA preparation evidently contained breakdown products of 18S + 28S RNA that hybridized to the nucleolus organizers and that were effectively competed away by including nonradioactive 18S + 28S RNA in the hybridization mixture (42, 44). The 4S RNA preparation, when hybridized to *Taricha* and *Batrachoseps* chromosomes in the presence of nonradioactive 18S + 28S RNA, did not label the nucleolus organizers and barely labeled the sites complementary to 5S RNA. Thus, the RNA that gave rise to the hybrid is not ribosomal. The labeled site produced with the 4S RNA was not seen in the

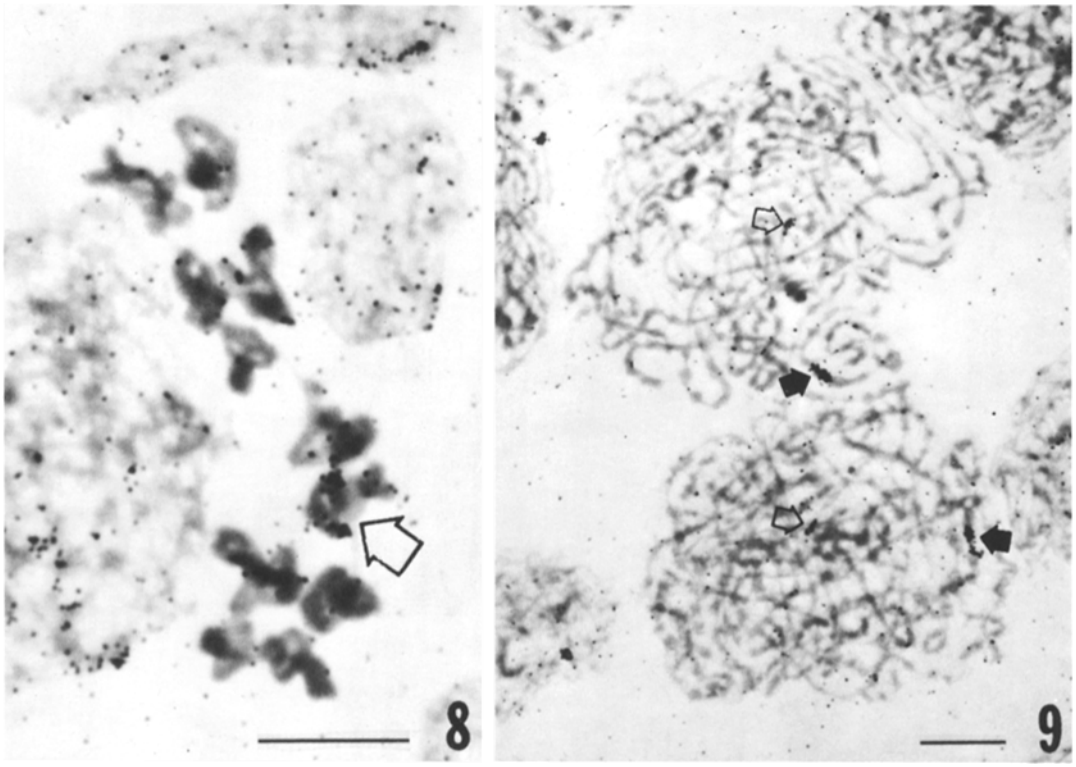

FIGURE 8 First meiotic metaphase chromosomes from *T. granulosa* testis hybridized with 5S RNA. The hybrid is localized on a submetacentric bivalent, presumably no. 7 (arrow). Exposure time, 10 days. Scale = 5 μm.

FIGURE 9 Pachytene spermatocytes hybridized with iodinated 5S RNA in the presence of unlabeled, homologous 18S + 28S RNA. One lightly labeled site (open arrow) can be seen in each nucleus, in addition to the heavily labeled site (solid arrow). Exposure time, 10 days. Scale = 5 μm.

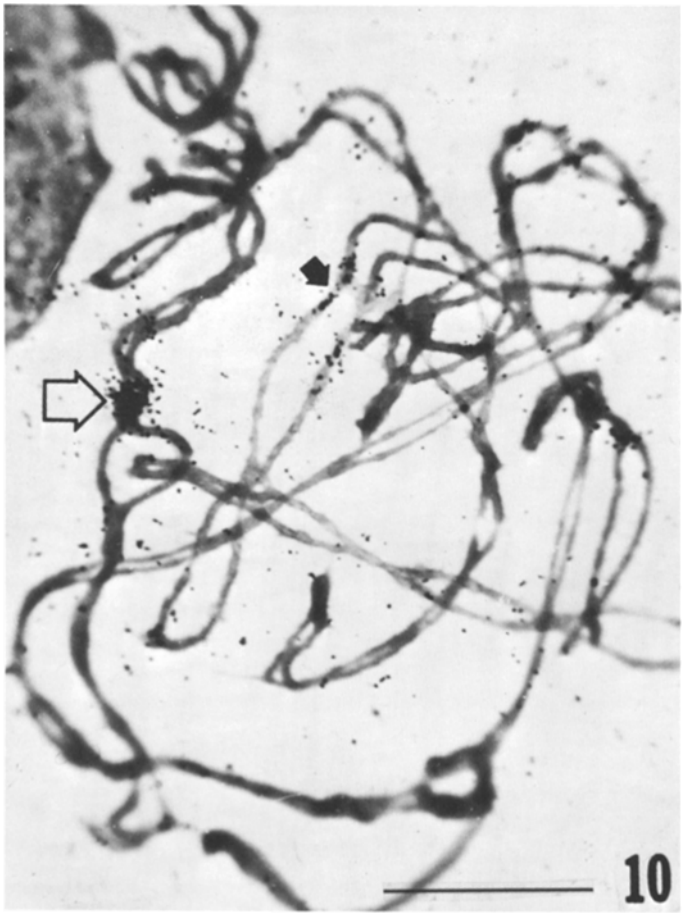

FIGURE 10 Diplotene bivalents from *B. wrighti* testis hybridized with 5S RNA from *T. granulosa*, in the presence of unlabeled 18S + 28S RNA. Bivalent no. 2 is heavily labeled near the centromere (open arrow) and a lightly site occurs on the centromere region of bivalent no. 3 (arrow). Exposure time, 10 days. Scale = 5 μm.

preparations annealed to 5S RNA. This indicated that the labeled 4S RNA site was not produced by fragments of a larger molecule since breakdown products would surely appear in the 5S RNA isolations. It is possible that the 4S RNA hybridization may not indicate tRNA genes. A 4S which is not a tRNA has been found in *Drosophila melanogaster* by P. Szabo (personal communication); this small RNA species hybridizes to a specific band on the X chromosome.

Regardless of the identity of the RNA, the results obtained with two distantly related salamanders indicate that the sequence is repetitious and conservative. The simplest assumption is that the label resulted from hybridization to clustered tRNA genes (cf. reference 7). Confirmation of this assumption would require comparing labeled sites obtained with several purified tRNA species.

Newts belong to the family Salamandridae, and are distributed throughout the Holartic region, although the Old World is generally accepted as their place of origin (29). All members of the family so far studied have 2N = 24, except for the Neartic genera *Taricha* and *Notophthalmus* (= *Triturus*) in which 2N = 22. Wake and Özeti (41) found evidence that these two genera are closely related, although they may have been separated since the Miocene (40). Also, they are presumably related to the European genus *Triturus* (41).

The molecular hybridization experiments demonstrated the presence of two nucleolus organizers

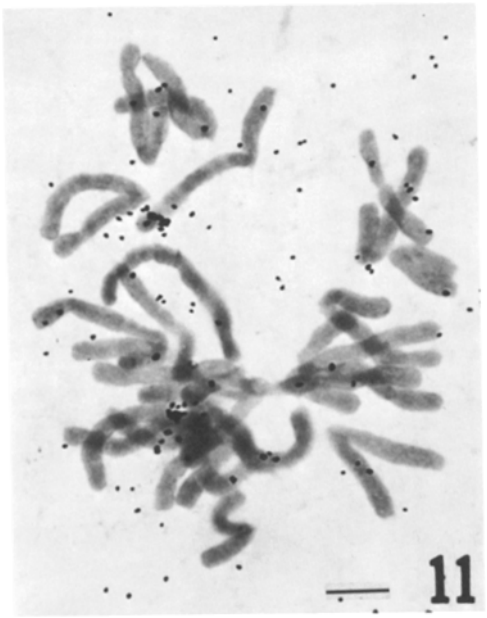

FIGURE 11 Gonial mitotic chromosomes from *T. granulosa* hybridized with 4S RNA. Chromosome no. 5 is labeled near the tip of the longer arm. Exposure time, 12 days. Scale = 5 μm.

in *Taricha*, in agreement with Berns and Cheng (2). The European newts *Triturus cristatus* and *Triturus marmoratus* present organizers on chromosomes 9 (6, 18) and 10 (1), respectively. In *Notophthalmus*, on the other hand, Gall (12) observed a nucleolus organizer on the second longest chromosome.

In contrast to the situation found in *Xenopus laevis* (30, 31) where 5S RNA gene clusters are probably present at one end of all of the chromosomes, the genome of *Taricha* presents one main site complementary to 5S RNA. A similar situation has been observed in other salamanders belonging to several families, including the primitive Cryptobranchidae[1] and also in *Triturus marmoratus* (1). In *Notophthalamus*, four chromosomes of the haploid set contain sequences near the centromeres, complementary to 5S RNA (34). As in *Taricha*, chromosome no. 7 is hybridized on or close to the centromere.

The localized concentration of label after hybridization with 4S RNA, presumably tRNA, is a most unexpected result, unlike those obtained when tRNA is hybridized to the chromosomes of dipterans (15, 39). If my interpretation of these results is correct, it indicates that the tRNA cistrons can be arranged in the genome of eukaryotes either clustered into one or a few sites, as in these salamanders, or dispersed throughout the genome as in *Drosophila*. Such is also the case with the 5S rRNA cistrons, which are found clustered on one site (1, 9, 44), a few sites (9, 39, 42), or a large number of sites (30, 31). Finally, the 18S + 28S RNA genes can be located on one (20, 32) or several loci (17, 42).

The generality that seems to emerge from the *in situ* hybridization experiments discussed here is that ribosomal and perhaps tRNA genes can exist in the genome of eukaryotes clustered into one or a few sites or dispersed throughout many chromosomes. One might speculate that if the repetitious sequences arise tandem to each other, as by a rolling circle mechanism (19), then the dispersed state is the derived state. Dispersion of the genes might be selected during the evolution of the karyotype for regulatory reasons, for instance, if

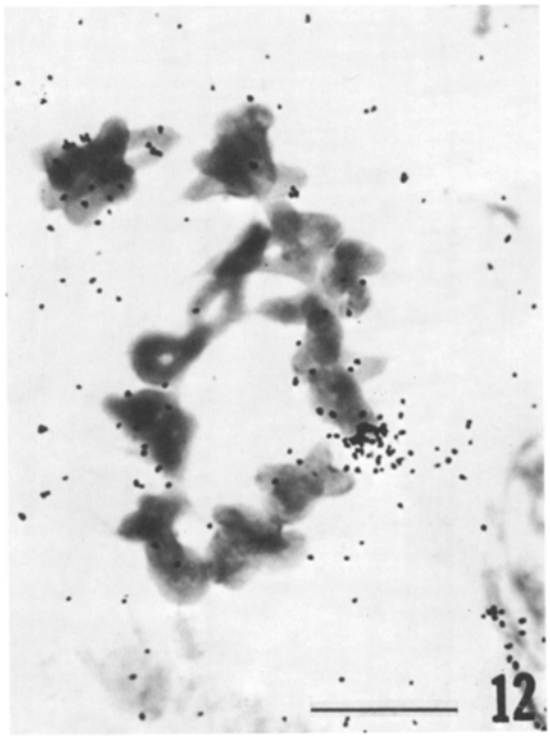

FIGURE 12 Meiotic metaphase chromosomes from *T. granulosa* primary spermatocytes hybridized with 4S RNA. One bivalent, presumably no. 5, is labeled near the tip of one of the arms. Exposure time, 12 days. Scale = 5 μm.

[1] León, P. E., and J. Kezer. Manuscript in preparation.

FIGURE 13 Pachytene spermatocytes from *T. granulosa* hybridized with 4S RNA. Spermatocyte bivalents were consistently labeled over two adjacent sites separated by a short unlabeled segment (arrow). Exposure time, 12 days. Scale = 5 μm.

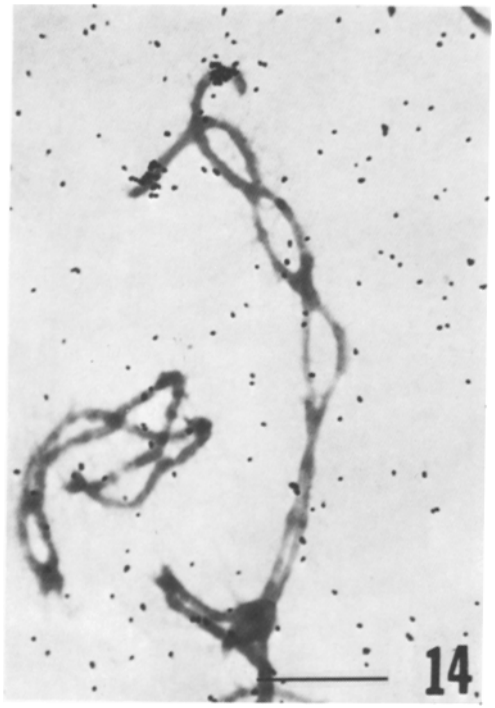

FIGURE 14 Diplotene bivalent from *B. wrighti* hybridized with *T. granulosa* 4S RNA. The label is located near the end of the longer arm of chromosome no. 3. Exposure time, 12 days. Scale = 5 μm.

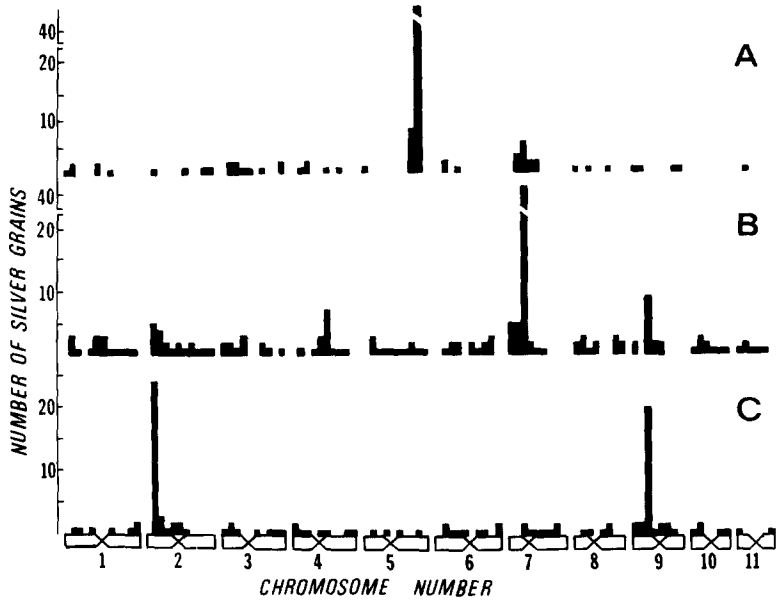

FIGURE 15 Graph showing the localization of label on mitotic chromosomes of *T. granulosa* hybridized with (A) 4S RNA, (B) 5S RNA, and (C) 18S + 28S RNA. The unit length of the chromosome is 1% of the total genome length, and the cumulative number of silver grains in each unit length was determined from four chromosome sets for each of the three RNA species hybridized.

each locus is under some sort of coarse control (16, 26). Also, differences between the clumped and dispersed arrangements might have an effect on cellular diffusion and transport phenomena and could be subject to selection. It would be interesting to study taxa in which "generalized" and "specialized" species have been clearly identified to determine if any informative patterns emerge.

Apparent differences in the quantity of label between half-bivalents hybridized with 5S and 18S + 28S RNA have been observed. These differences in label are being quantitatively analyzed, and the results will be published elsewhere.

Macgregor and Walker (24) have found that in the sperm heads of the red-backed salamander *Plethodon cinereus* the chromosomes are packaged in such a way that all the centromeres are located adjacent to the mid-piece, and the chromosome arms extend toward the acrosome. The localization of the hybrids produced by rRNA on developing spermatids of *Taricha* is in agreement with the findings of Macgregor and Walker (24).

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