

CHROMOSOMAL LOCALIZATION OF REPETITIVE DNA IN THE NEWT, *TRITURUS*

GIUSEPPINA BARSACCHI and JOSEPH G. GALL

From the Department of Biology, Yale University, New Haven, Connecticut 06520. Dr Barsacchi's present address is the Istituto di Zoologia e Anatomia Comparata, Università di Pisa, 56100 Pisa, Italy.

ABSTRACT

The repetitive DNA sequences of the newt, *Triturus viridescens*, have been studied by nucleic acid hybridization procedures. Complementary RNA was synthesized enzymatically from unfractionated newt DNA. This RNA hybridized strongly to the centromeric regions of both somatic and lampbrush chromosomes. It also bound to other loci scattered along the lengths of the chromosomes. The amplified ribosomal DNA in the multiple oocyte nucleoli was demonstrated by *in situ* hybridization.

INTRODUCTION

Hybridization of radioactive DNA or RNA with chromosomal DNA fixed in cytological preparations permits visualization and localization of those parts of the genome which are complementary to the radioactive nucleic acid employed in the assay (Gall, 1969; Gall and Pardue, 1969; John, Birnstiel, and Jones, 1969; Buongiorno-Nardelli and Amaldi, 1970).

This method is especially suitable for detecting repetitive DNA sequences. In particular, the localization of the ribosomal cistrons has been studied in the oogonia and oocytes of the toad *Xenopus laevis* (Gall and Pardue, 1969; Pardue and Gall, 1969) and in the polytene chromosomes of some *Diptera* (Pardue et al., 1970). Recently the 5S RNA genes have been located in the genome of *Drosophila melanogaster* (Wimber and Steffensen, 1970) and in Chinese hamster cells (Amaldi and Buongiorno-Nardelli, 1971).

The cytological localization of highly repeated DNA sequences has also been investigated by *in situ* hybridization. It has been shown that mouse satellite DNA is located in the centromeric heterochromatin (Pardue and Gall, 1969, 1970, Jones,

1970) and that centromeric regions are enriched in highly reiterated sequences in several species of *Drosophila* (Hennig, Hennig, and Stein, 1970; Jones and Roberston, 1970; Rae, 1970; Gall, Cohen, and Polan, 1971), in *Rhynchosciara hol-laenderi* (Eckhardt and Gall, 1971) and in *Plethodon c cinereus* (Macgregor and Kezer, 1971). In *Microtus agrestis*, repetitive DNA is located in the heterochromatin of the sex chromosomes (Arrighi et al., 1970).

It is of interest to investigate the localization of repetitive DNA in the genome of other organisms. Because of the occurrence of lampbrush chromosomes in the oocytes of salamanders, we chose to extend this study to these animals. The giant lampbrush chromosomes, because of their structural differentiations, represent an appropriate material for studies on the DNA constitution of chromosomes. This note reports experiments on the hybridization of radioactive complementary RNA, transcribed *in vitro* from the DNA of the newt, *Triturus viridescens* with interphase nuclei, mitotic, and lampbrush chromosomes from the same species.

MATERIALS AND METHODS

Animals

All experiments were performed on specimens of the newt, *T. viridescens* (Amphibia, Urodela) collected in Massachusetts.

DNA Isolation

DNA was extracted by a detergent-phenol procedure. The tissues (liver, spleen, testes) were first homogenized in $1 \times \text{SSC}^1$, then incubated for 2–4 hr at 37°C in a solution containing 0.5% Sarkosyl (Na-lauroyl-sarcosinate, Geigy Chemical Corp., Ardsley, N.Y.), 0.1 M EDTA, 0.05 M Tris pH 8.4, 100 $\mu\text{g}/\text{ml}$ of predigested pronase. The solution was extracted with an equal volume of water-saturated redistilled phenol. The nucleic acids were precipitated from the aqueous phase with two volumes of 95% ethanol. The precipitate was washed with 70% ethanol, redissolved in $0.1 \times \text{SSC}$ and treated with RNase-T₁ (330 units/ml), pancreatic RNase (100 $\mu\text{g}/\text{ml}$), and α -amylase (100 $\mu\text{g}/\text{ml}$) for 1 hr at 37°C to remove RNA and contaminating carbohydrates. The DNA was again extracted with phenol, precipitated with 95% ethanol, and then dissolved in a small volume of $0.1 \times \text{SSC}$.

DNA Centrifugation

DNA was centrifuged to equilibrium in preparative CsCl density gradients at 42,000 rpm and 15°C for 21–24 hr in a Spinco Al-50 or Ti-50 fixed angle rotor. Analytical CsCl gradient centrifugation was done for 24 hr at 44,770 rpm and 20°C in a Spinco model E centrifuge.

The initial density of the gradients was approximately 1.70 g/ml at 25°C . The volume of the preparative gradients was 4.5 ml, and each contained 25–50 μg of DNA. 30 fractions of 10 drops each were collected from the preparative gradients by puncturing the bottom of the centrifuge tubes. The optical density of each fraction was determined at 260 nm. The UV photographs taken in the analytical centrifuge were analyzed with a Joyce-Loebl micro-

¹ The following abbreviations were used in this paper: C, cytosine; cRNA, complementary RNA which has been transcribed in vitro using DNA-dependent RNA polymerase; C₀t, moles of nucleotide per liter \times seconds; EDTA, ethylenediaminetetraacetate; G, guanine; rDNA, the DNA which codes for rRNA; rRNA, ribosomal RNA; SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; T_m, melting temperature; toluene-PPO-POPOP, 4 g of 2,5 diphenyloxazole and 50 mg of 1,4 bis[2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of toluene, Tris, tris-(hydroxymethyl)-amino methane; tRNA, transfer RNA.

densitometer. The buoyant density of the *T. viridescens* DNA was determined using *Micrococcus lysodeikticus* DNA (buoyant density = 1.731 g/ml) as a marker. Heat-denatured DNA (5 min at 100°C in $0.1 \times \text{SSC}$) and renatured DNA (100 $\mu\text{g}/\text{ml}$ for 2 hr at 66°C in $2 \times \text{SSC}$) as well as DNase-treated preparations (30 min at 37°C with DNase at 20 $\mu\text{g}/\text{ml}$) were also analyzed by both preparative and analytical centrifugations.

In Vitro Transcription of cRNA

The gradient fractions, except for those containing the cistrons coding for rRNA, were pooled and the DNA was recovered by ethanol precipitation. Highly radioactive RNA was transcribed from this DNA using RNA-polymerase prepared from frozen cells of *Escherichia coli*, strain A-19 (Burgess, 1969).

The transcription and purification of the cRNA were carried out as previously described (Pardue et al., 1970). Approximately 2 μg of complementary polynucleotide was produced. It had a calculated specific activity of 1.1×10^8 dpm/ μg (about 1.1×10^7 cpm/ μg as counted on nitrocellulose filters in toluene-PPO-POPOP).

Cytological Hybridization

Slides of interphase nuclei, mitotic chromosomes, and lampbrush chromosomes from *T. viridescens* were prepared. Whole larvae were fixed in ethanol-acetic acid (3:1); small fragments of liver or tail tip were transferred into 45% acetic acid on a silicized cover slip and then squashed. The slides had been previously coated with gelatin by dipping them into a solution of 0.1% gelatin in 0.01% chrome alum. The preparations were frozen on dry ice, and the cover slips removed with a razor blade (Conger and Fairchild, 1953). After 5 min in 95% alcohol, the slides were air dried and used for cytological hybridization. Lampbrush chromosome preparations were made according to the procedure described by Gall (1966), fixed in acetic acid vapors, dehydrated in an alcohol series, and air dried. The cytological hybridization was carried out essentially as described by Gall and Pardue (1971). Each slide received 50 μl of $6 \times \text{SSC}$ or $3 \times \text{SSC}$ containing cRNA at concentrations from 1.3 to 3.1×10^6 cpm/ml. The slides were coated with Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water. They were developed after periods ranging from 3 days to 12 wk (2 min in Kodak D19, rinsed in 2% acetic acid, and 3 min in Kodak Fixer). After being rinsed several times in distilled water, slides were stained with Giemsa's and mounted in Permount.

Preparations of lampbrush chromosomes were also annealed with cRNA copied in vitro from the rDNA of *Xenopus*. The cRNA was used at concentrations

from 1.7 to 7.0×10^6 cpm/ml in either $2 \times$ SSC or $6 \times$ SSC.

Control experiments were performed in which the cytological hybridization was carried out without the alkali denaturation step.

Filter Hybridization

Each fraction (0.14 ml) collected from the CsCl gradient was treated with 1 ml of 0.1 N NaOH and neutralized with 2 ml of 0.05 N HCl in $9 \times$ SSC. The samples were loaded onto nitrocellulose membrane filters and the hybridization was carried out essentially as described by Gillespie and Spiegelman (1965). The filters were incubated with different amounts of cRNA ranging from 0.0045 μ g/ml (5×10^4 cpm/ml) to 0.045 μ g/ml (5×10^5 cpm/ml) for 15–17 hr at 66°C. Filter hybridization of gradients of heat-denatured DNA (5 min at 100°C in $0.1 \times$ SSC) or renatured DNA (2 hr at 66°C in $2 \times$ SSC) was also carried out. In some instances the DNA was sheared before the centrifugation by passing it several times through a 27 gauge needle.

Gradient fractions were also annealed with cRNA copied in vitro from the rDNA of *Xenopus*. The cRNA was used at either 2.5×10^5 or 5×10^5 cpm/ml. Competition experiments were performed with a 3000-fold excess of nonradioactive *Xenopus* rRNA. The radioactivity of filters was counted in toluene-PPO-POPOP in a Liquid Scintillation Counter, Mark I (Nuclear-Chicago, Des Plaines, Ill.).

Melting Profile

The thermal dissociation of the double-stranded DNA was monitored in a Beckman DU spectrophotometer equipped with a Gilford model 2000 multiple sample absorbance recorder. The melting temperature determination was carried out as described by Mandel and Marmur (1968). The DNA (15.5 μ g) had been collected from a CsCl gradient precipitated with ethanol and redissolved in $0.1 \times$ SSC. The fractions containing the rDNA cistrons had been discarded.

RESULTS

Analytical Centrifugation of

T. viridescens DNA

The buoyant density of the main peak of *T. viridescens* DNA as calculated by CsCl centrifugation is 1.704 g/ml (Fig. 1 a).

Analytical centrifugation of a greater amount of DNA demonstrated three minor peaks on the lighter side of the main band and a shoulder on the

heavy side (Fig. 1 b). The buoyant densities of the lighter peaks are 1.691, 1.688, and 1.681 g/ml. One of the minor bands (probably the one having a buoyant density of 1.688 g/ml) is resistant to DNase treatment and also is prominent when viewed by Schlieren optics. This band is probably a carbohydrate contaminant. The heavier shoulder has a buoyant density of 1.717 g/ml. It may include the cistrons complementary to rRNA (see Fig. 16). Using the formula of Schildkraut, Marmur, and Doty (1962), we can estimate the G + C content of the main peak as 44.8%.

In some cases it is possible to reveal rapidly renaturing fractions which have the same buoyant density as the main peak, or to accentuate the density difference between the main peak and a light satellite by equilibrium centrifugation of DNA which has been denatured and then renatured (Waring and Britten, 1966). Under such circumstances the rapidly renaturing sequences display the density characteristic of double-stranded sequences, whereas the main peak retains the heavier density of single-stranded material. Such an experiment with sheared, denatured and renatured *T. viridescens* DNA showed only a single peak at $\rho = 1.716$ g/ml in the analytical ultracentrifuge. This experiment is relatively insensitive and would not detect a small fraction of rapidly reassociating DNA, especially if it did not return completely to its native density.

Hybridization of cRNA with CsCl

DNA Gradients

Hybridization experiments on nitrocellulose filters have been carried out in order to determine the buoyant density of the repetitive DNA sequence, i.e., those which hybridize with the cRNA at low C_{0t} values (Britten and Kohne, 1966). Fig. 2 shows the result of one of these experiments: the major hybridization occurs with main peak sequences. After denaturation alone or denaturation followed by renaturation, the hybridizing sequences remain with the main peak and the hybridized gradients are similar to the one shown in Fig. 2. In the latter experiments the DNA was sheared to less than 5×10^6 daltons before denaturation.

Annealing gradient fractions with cRNA made from the rDNA of *Xenopus* enabled the position of the cistrons coding for rRNA to be visualized in the CsCl gradients (Fig. 3). After competition

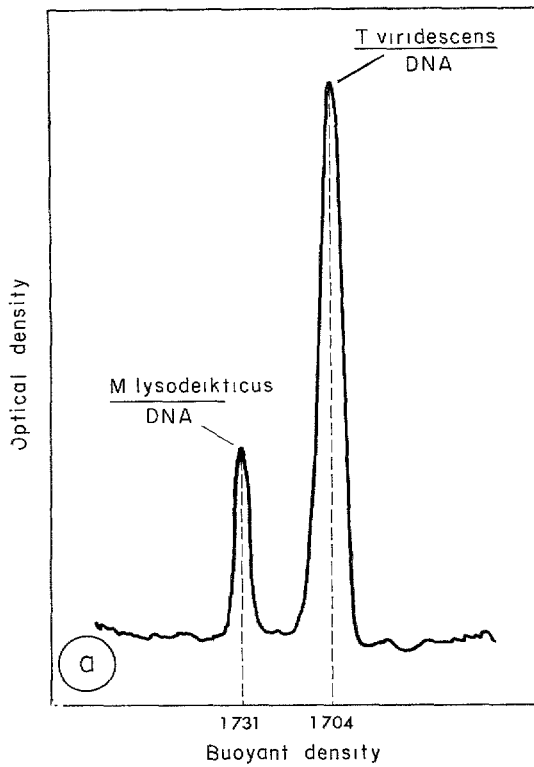
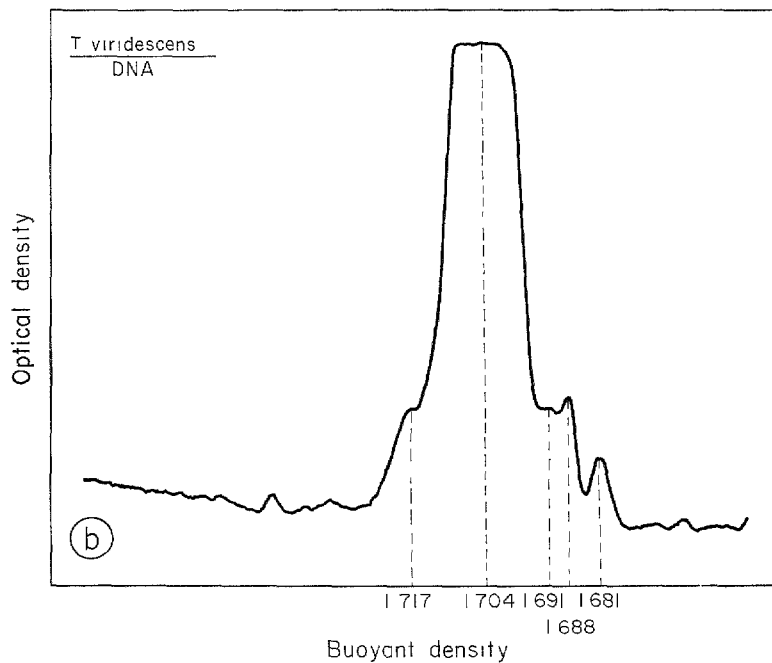


FIGURE 1 a Microdensitometer tracing of *T. viridescens* DNA centrifuged to equilibrium in neutral CsCl. 3.5 μ g of native DNA was centrifuged at 44,770 rpm and 20°C for 24 hr. The marker DNA is from *M. lysodeikticus* and has a buoyant density of 1.731 g/ml. The buoyant density of the *T. viridescens* main peak DNA is 1.704 g/ml, corresponding to a G + C content of 44.8%.

FIGURE 1 b Microdensitometer tracing of *T. viridescens* DNA centrifuged to equilibrium in neutral CsCl. 35 μ g of native DNA was centrifuged as in a. Several minor peaks are now visible. One of the low density peaks is probably carbohydrate



experiments, performed with a 3000-fold excess of nonradioactive rRNA from *Xenopus*, there was no significant radioactivity on the filters

Melting Profile

The melting profile of *T. viridescens* DNA is shown in Fig. 4. The T_m is 72°C in 0.1x SSC cor-

responding to a calculated G + C content of 44.1% (Mandel and Marmur, 1968)

Hybridization of cRNA with Cytological Preparations

INTERPHASE NUCLEI: After *in situ* hybridization with cRNA copied from whole DNA the interphase

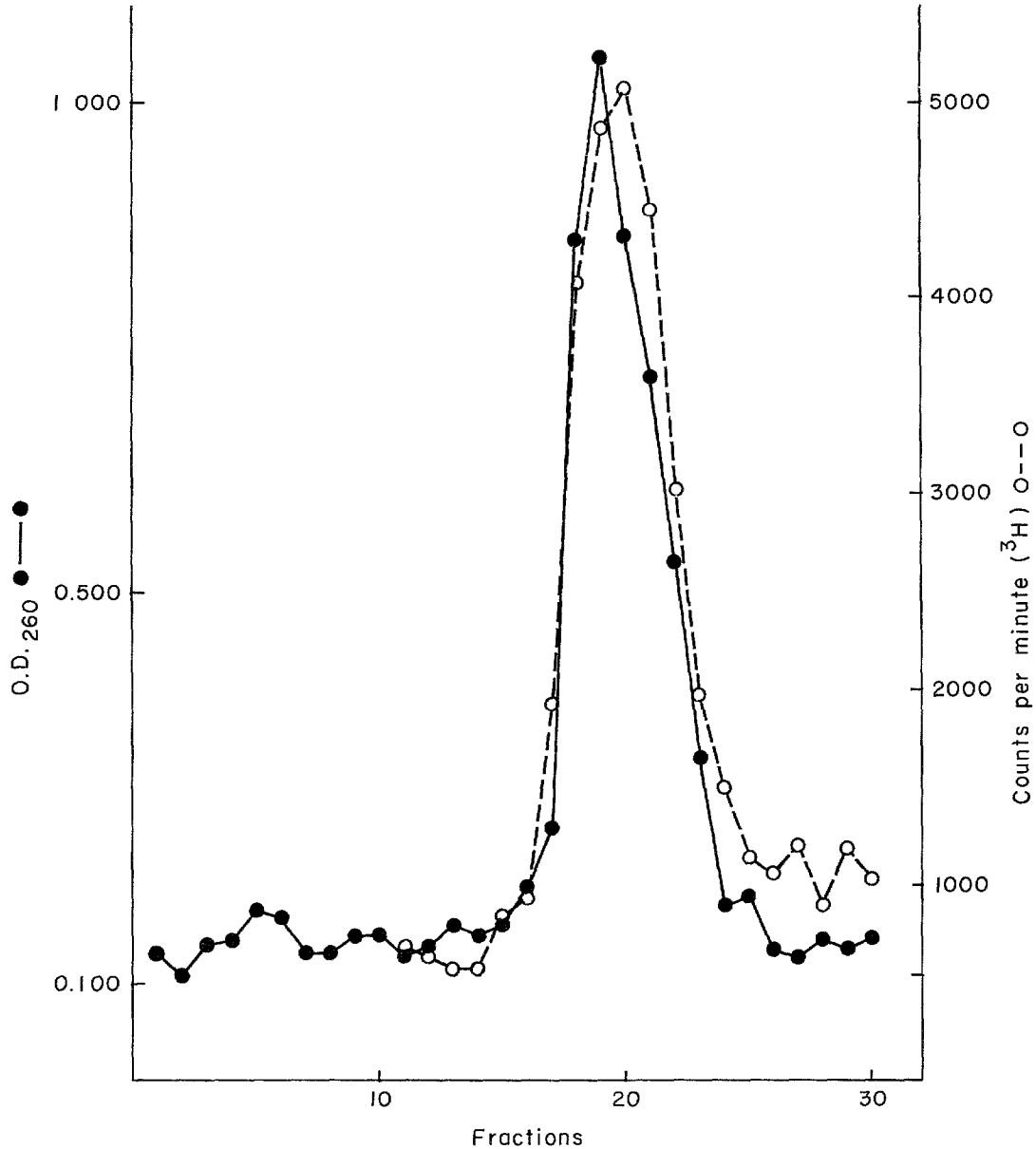


FIGURE 2 Hybridization of radioactive cRNA transcribed *in vitro* from *T. viridescens* DNA (rDNA excluded) to a *T. viridescens* DNA gradient immobilized on nitrocellulose filters. The hybridization (○---○) and optical density (●—●) profiles are very similar. The cRNA was used at a concentration of 4.4×10^5 cpm/ml. The hybridization was carried out in 2x SSC for 12 hr at 66°C.

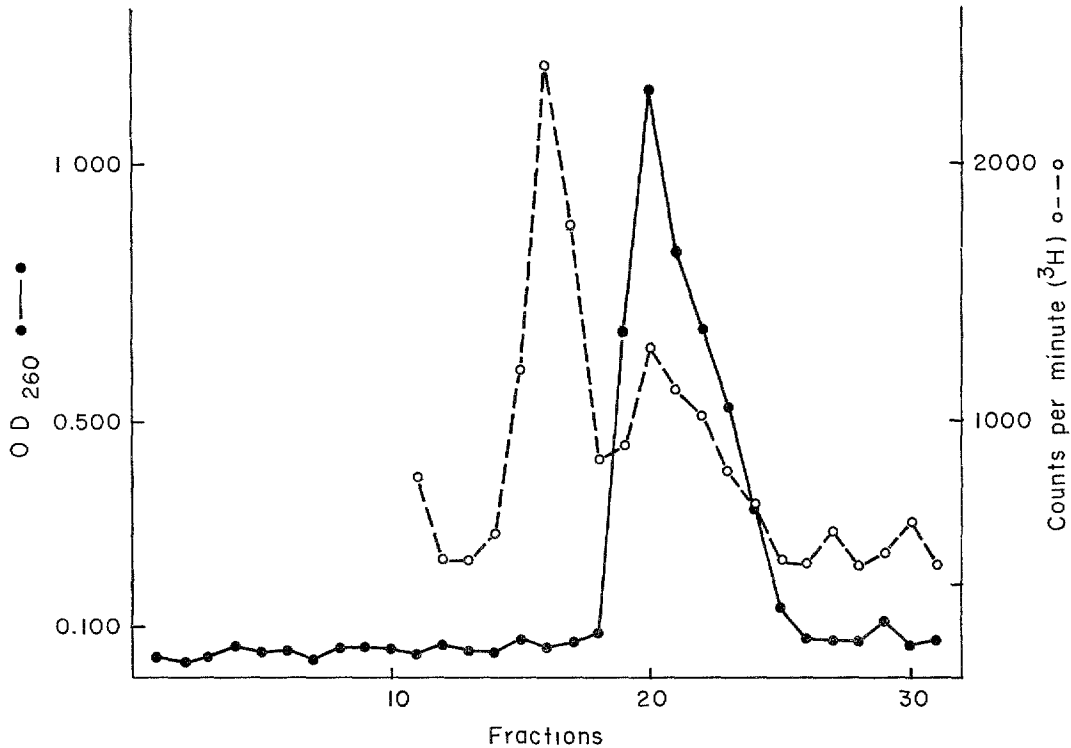


FIGURE 3 Hybridization of radioactive cRNA copied in vitro from rDNA of *Xenopus* to a *T. viridescens* DNA gradient immobilized on nitrocellulose filters. A prominent peak of hybridization occurs on the denser side of the DNA peak. The cRNA was used at a concentration of 2.5×10^5 cpm/ml. The hybridization was carried out in $2 \times$ SSC for 12 hr at 66°C . After competition experiments, performed with a 3000-fold excess of nonradioactive rRNA from *Xenopus*, no significant radioactive hybrid was detected. Annealing to main peak sequences, as in this example, also occurs when the experiment is carried out with labeled rRNA.

nuclei are more heavily labeled in the heterochromatic zones than in the euchromatic ones (Fig. 5). Heterochromatin is easily recognizable not only by its condensed state but also because, after the hybridization procedure, it stains more intensely with the Giemsa dye than the euchromatin (Pardue and Gall, 1970). In each nucleus some of the heterochromatic portions are not heavily labeled. Nuclei in prophase show the same pattern of labeling, although the heterochromatin is separated into more numerous parts (Fig. 6).

MITOTIC CHROMOSOMES: Mitotic chromosomes are labeled with cRNA throughout their lengths, but display a higher silver grain density near the centromeres, more precisely, the heterochromatin lying on each side of the centromeric constriction is the most heavily labeled part (Figs. 7 and 8). This chromatin, as well as that of the chromocenters of interphase nuclei and of the centromeres of lampbrush chromosomes, is the most intensely

stained after Giemsa staining in slides treated for cytological hybridization. Occasionally the telomeres show a number of grains exceeding the average (Fig. 8).

LAMPBRUSH CHROMOSOMES: The lampbrush karyotype of *T. viridescens* consists of 11 bivalents identifiable on the basis of their length, centromere position, and morphological landmarks (Gall, 1954). Two chromosomes bear a sphere in a sub-terminal position (numbers 5 and 10); one of the long chromosomes (number 7) carries the nucleolus-organizing region, close to the right telomere, and two peculiar enlarged bodies at the other extremity. The centromere region is represented by conspicuous Feulgen-positive bars up to 10μ long. These bars do not bear loops and so appear as short gaps in the generally fuzzy appearance of the chromosomes. A granule of varying size which stains blue with Giemsa's is often seen imbedded in the centromere bar or on one side of it.

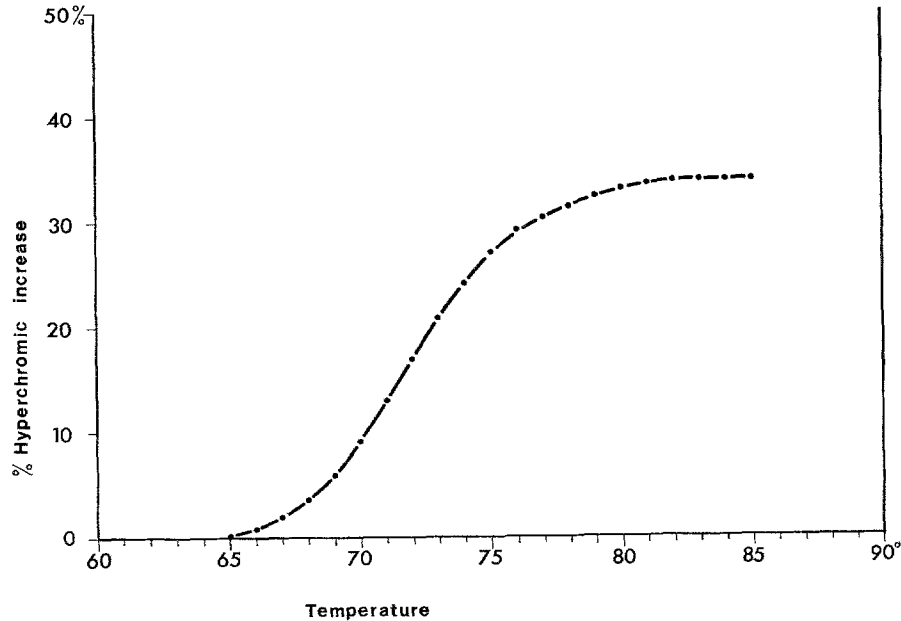


FIGURE 4 Ultraviolet absorbance-temperature profile of *T. viridescens* DNA (rDNA excluded). The hyperchromic increase of this preparation was 33.9%. The DNA was melted in 0.1X SSC. The T_m was 72°C, corresponding to a calculated G + C content of 44.1%.

After cytological hybridization with cRNA the lampbrush chromosomes show silver grains scattered along their axes. Some of the loops are labeled after long periods of exposure. The centromere regions show a preferential hybridization similar to that observed for the centromeric heterochromatin of mitotic chromosomes (Figs. 9 and 10). The granule inserted at the centromere bar is not labeled. In a few instances the telomeric regions and the chromosome axes on which the spheres are inserted show a number of grains exceeding the average (Figs. 11 and 12). Nucleoli, spheres, and globules are not labeled.

After annealing with RNA complementary to

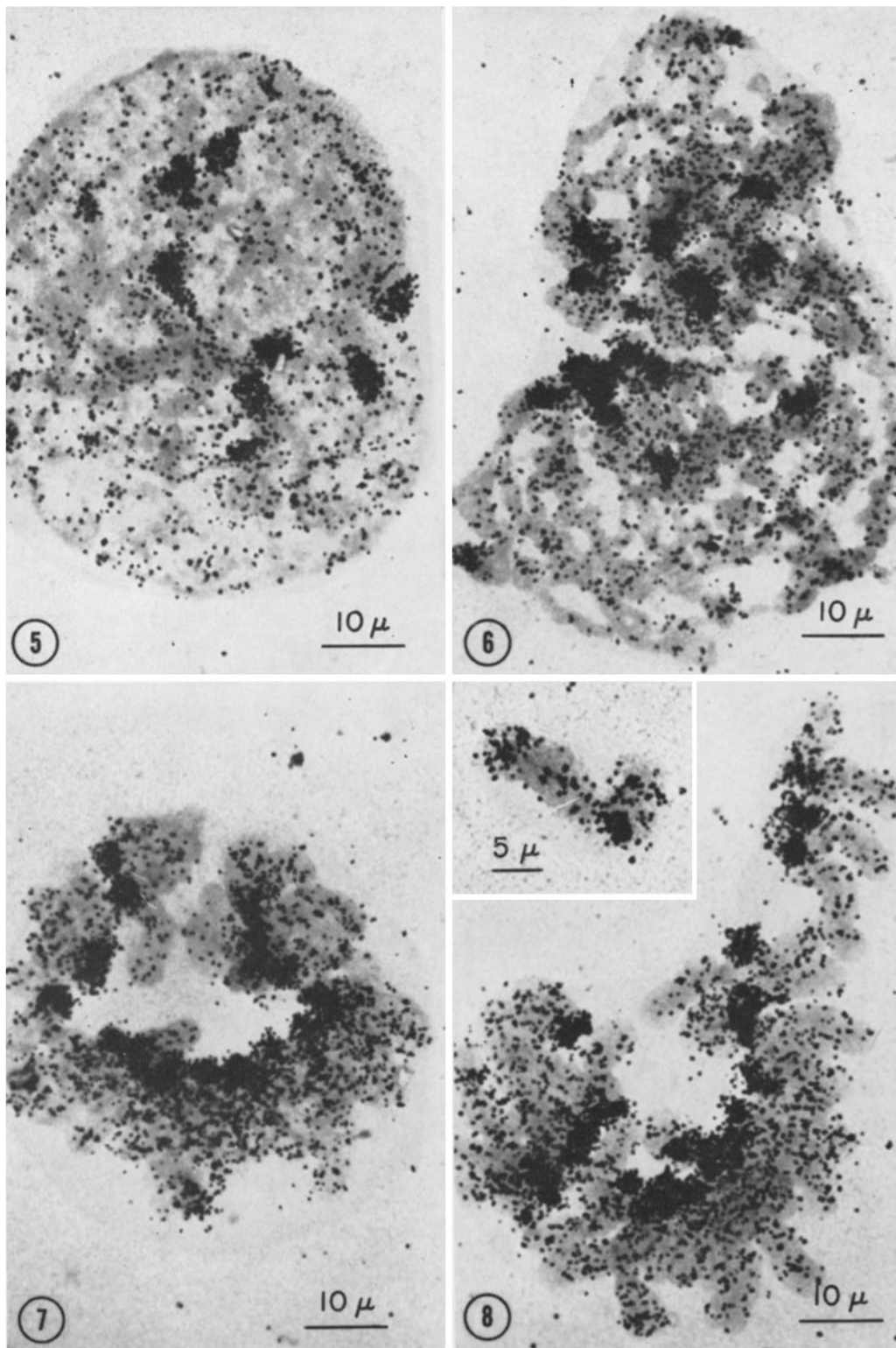
the rDNA from *Xenopus*, lampbrush chromosomes do not show any hybridization; however, nucleoli, both free in the nuclear sap or attached to the nucleolus-organizer region, are labeled. In some instances nucleoli exhibit a localized labeling (Figs. 13 and 14).

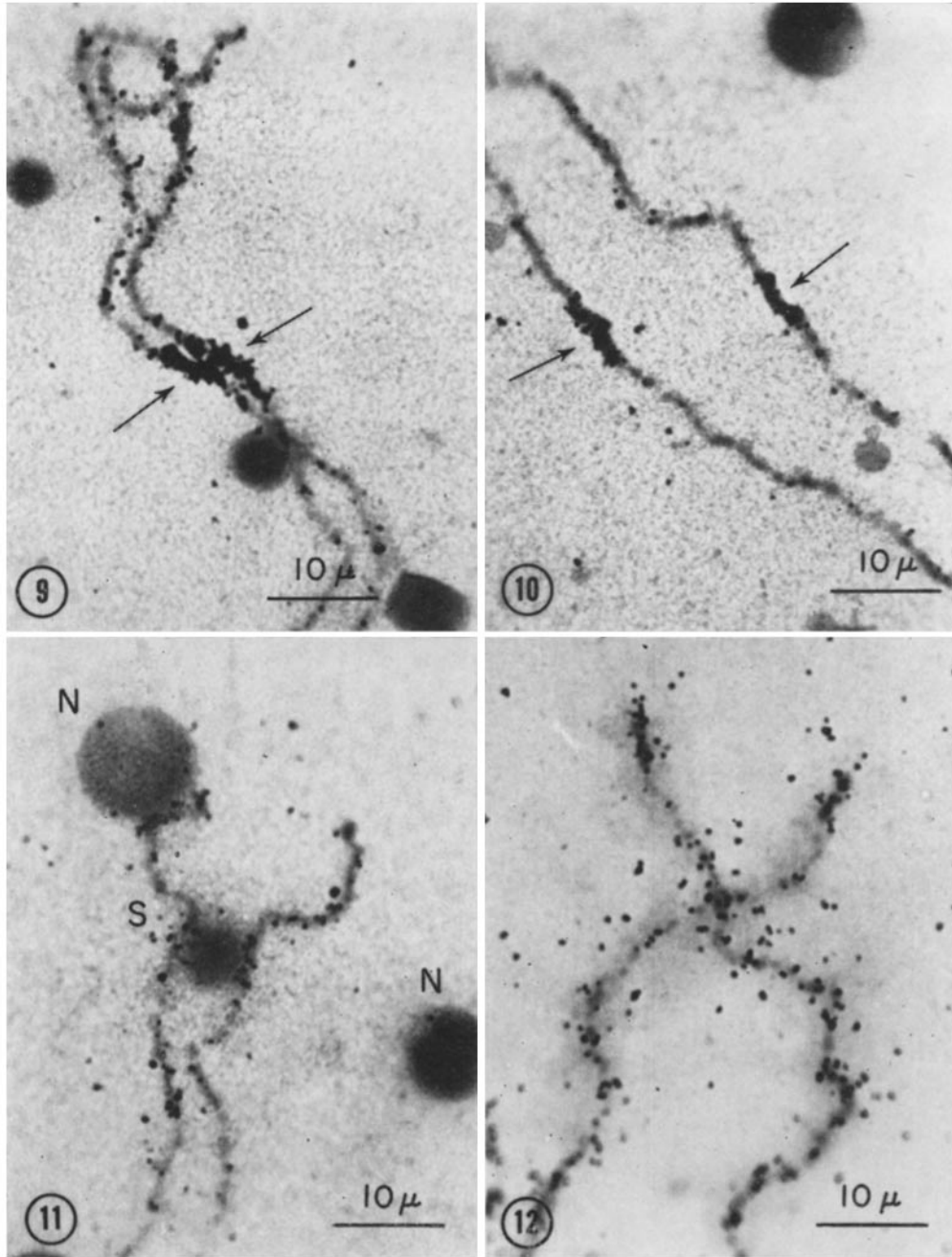
The control experiments, performed with cytological preparations not submitted to the DNA denaturation step, showed no detectable label.

DISCUSSION

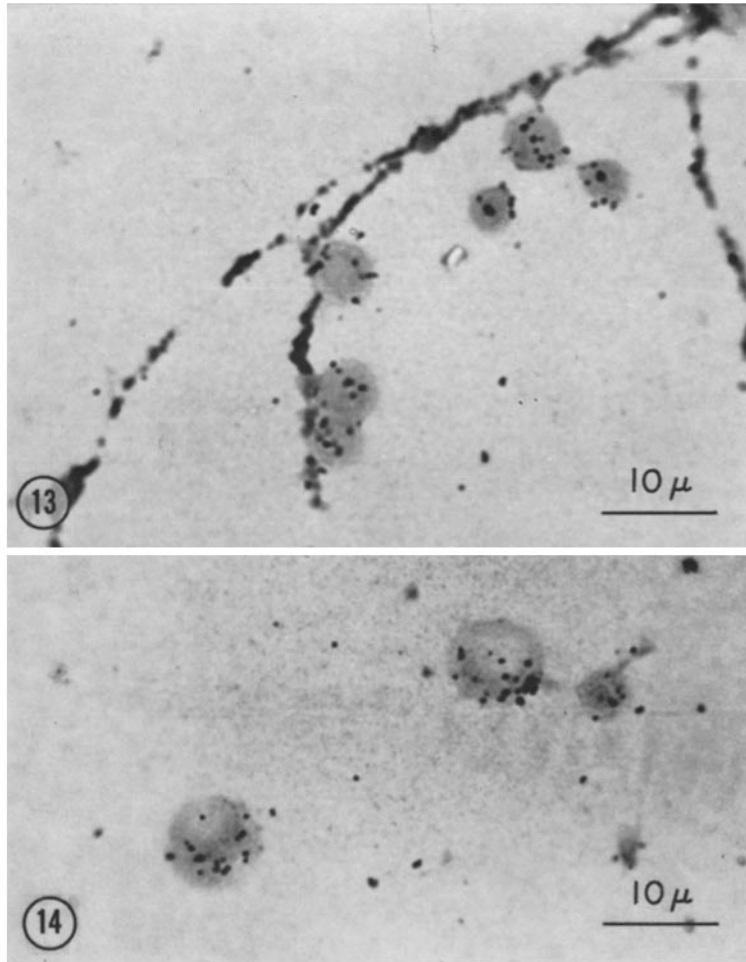
The annealing of cRNA to the DNA of cytological preparations from *T. viridescens* resulted in a widely distributed hybridization; there was, however,

FIGURES 5-8 Radioautographs of nuclei in interphase (Fig. 5), prophase (Fig. 6), and metaphase (Figs. 7 and 8) from a larva of *T. viridescens* after hybridization with radioactive cRNA transcribed in vitro from *T. viridescens* DNA (rDNA excluded). Fig. 5: There is a general distribution of silver grains over the nucleus; the chromocenters appear to be heavily labeled. Fig. 6: The prophase nucleus shows that the chromocenters correspond to the centromeric heterochromatin. Figs. 7 and 8: Silver grains are widespread all over the chromosomes; the most intense labeling is over the centromeric heterochromatin. The inset in Fig. 8 shows a heterobrachial chromosome with dense labeling over a telomeric region. The cRNA was used at a concentration of 1.3×10^6 cpm/ml in 6X SSC. The hybridization was carried out for 15 hr at 66°C. Exposure, 49 days. Giemsa stain. $10\mu = 12.2$ mm; $5\mu = 7.6$ mm. Figs. 5-8: $\times 1220$; inset in Fig. 8: $\times 1520$.





FIGURES 9-12 Radioautographs of lampbrush chromosome preparations from *T. viridescens* after hybridization with radioactive cRNA transcribed in vitro from *T. viridescens* DNA (rDNA excluded) Figs. 9 and 10 Silver grains are distributed along the chromosome axes; the centromeric heterochromatin (arrows) is intensely labeled. Nucleoli are not labeled. Figs. 11 and 12 show respectively the chromosome axes where the sphere is inserted and two homologous telomeric regions more heavily labeled than other areas of the chromosomes Sphere (S) and nucleoli (N) are not labeled. The cRNA was used at a concentration of 3.1×10^6 cpm/ml in $6\times$ SSC. The hybridization was carried out for 17 hr at 66°C Exposure, 56 days (Figs. 9-11) and 70 days (Fig. 12). Giemsa stain. $10\mu = 15.2 \text{ mm.} \times 1520$.



FIGURES 13 and 14 Radioautographs of lampbrush chromosome preparations from *T. cristatus* after hybridization with radioactive RNA transcribed in vitro from *Xenopus* rDNA. Fig. 13 Several labeled nucleoli and unlabeled segments of lampbrush chromosomes. Fig. 14 Eccentrically labeled nucleoli. The cRNA was used at a concentration of 1.7×10^6 cpm/ml in $6\times$ SSC. The hybridization was carried out for 15 hr at 66°C . Exposure, 70 days. Giemsa stain. $10\mu = 15.2$ mm. $\times 1520$

preferential binding to the centromeric heterochromatin

Under our experimental conditions, only redundant DNA sequences should exhibit hybridization (Britten and Kohne, 1966, 1968). The labeling patterns of the mitotic and lampbrush chromosomes show, therefore, that repetitive DNA is widely distributed in the genome of *T. cristatus*.

Biochemical methods have demonstrated the presence of repetitive DNA in the genome of every eukaryotic species so far examined (Britten and Kohne, 1968). The amount of repeated sequences varies in different species from 20% to at least 80% of the total nuclear DNA under standard criteria

of measurement. Variable numbers of identical or similar repeated sequences are included in different "families," which are interspersed in the length of the genome. Some of these sequences are known to be transcribed, since in vivo-labeled RNA will hybridize to the DNA of higher organisms at low C_{0t} values. Furthermore, different families of repeated sequences seem to be transcribed in different tissues and embryonic stages (see Britten and Kohne, 1970).

The hybridization of cRNA to mitotic and lampbrush chromosomes has also shown that binding occurs preferentially to the DNA of the centromeric heterochromatin. The labeling ob-

served in interphase nuclei demonstrates that the centromeric regions are associated in chromocenters. Since the rate of hybridization is proportional to the concentration of complementary sequences, the high level of hybridization indicates that the centromeric heterochromatin contains highly repeated sequences. This interpretation is supported by the situation observed in other organisms in which repetitious DNA is included in the centromeric heterochromatin (see Introduction). In particular, in the mouse the light satellite DNA (Waring and Britten, 1966), formed of a sequence between 8 and 13 nucleotide pairs long and repeated up to millions of times (Southern, 1970), is located in the centromeric heterochromatin (Pardue and Gall, 1969, 1970; Jones, 1970). These results raise questions about possible relationships between highly repetitive DNA and centromere structure and function (see Brinkley and Stubblefield, 1970): there is, in fact, evidence that the centromeric heterochromatin may affect the behavior of the centromere (Lindsley and Novitski, 1958; Crouse, 1960). Moreover, the presence of highly repetitive DNA in the centromeric heterochromatin could provide a biochemical basis for phenomena involving centromeres: in *D. melanogaster*, somatic crossing-over takes place preferentially in the centromere region (Stern, 1936), and in human lymphocytes it has been demonstrated that sister chromatid exchanges occur also at the centromeres (Cuevas-Sosa, 1967). Centromeric fusions have been observed in lampbrush chromosomes (Gall, 1954; Callan and Lloyd, 1960); and the chromocenters of polytene nuclei (Heitz, 1934) and of interphase nuclei of some species (for instance mouse: see Pardue and Gall, 1970) are examples of centromere associations. The hybridization of cRNA at the telomere regions indicates that they also contain repetitive DNA and suggests a biochemical similarity between centromeres and telomeres (see Eckhardt and Gall, 1971). These aspects, however, require further study to be clarified.

Hybridization on lampbrush chromosomes showed that repetitive DNA is also located at the sphere loci. Although it is known that spheres probably contain acidic proteins (Gall, 1954) and incorporate radioactive amino acids but not RNA precursors (Mancino, Barsacchi, and Nardi, 1968), their functional meaning remains unknown. In lampbrush preparations made from oocytes undergoing maturation, after either a gonadotropic

injection to the females or an in vitro treatment of oocytes with progesterone, the chromosomes lack spheres but display instead prominent loops at the sphere loci (G. Barsacchi and A. A. Humphries, Jr., unpublished observation). After cytological hybridization with cRNA these newly formed loops showed a moderate number of grains, indicating that they contain repetitive DNA. These observations may induce one to speculate about the possible relationships between the sphere loci and phenomena connected with oocyte maturation: if such a relationship exists, the repetitive DNA located at the sphere loci could be functionally related to the need for a large amount of a particular gene product.

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