Copyright © 1973 by Academic Press, Inc.
All rights of reproduction in any form reserved

Experimental Cell Research 80 (1973) 175–190

TRANSCRIPTION OF RIBOSOMAL RNA CISTRONS

Correlation of Morphological and Biochemical Data

U. SCHEER, M. F. TRENDELENBURG and W. W. FRANKE

Department of Cell Biology, Institute of Biology II, University of Freiburg, D-78 Freiburg i.Br., BRD

SUMMARY

Electron microscopic spread preparations of oocyte nucleoli (lampbrush stage) of various amphibians are quantitatively evaluated and the length distributions of repeat-, matrix-, and spacer-units along the rRNA cistron containing axes are given. The correlation of the matrix unit data with the gel electrophoretic pattern of labelled nuclear RNA from the same oocytes is examined. The mean value of the matrix unit corresponds fairly well to a 2.6 million D peak of pre-rRNA but the distribution of both matrix units and labelled pre-rRNAs shows an asymmetrical heterogeneity indicating the existence of some larger primary transcription products of rDNA. Novel structural aspects are described in the spacer regions which suggest that transcription does also take place in DNP regions between the matrix units. A special 'prelude piece' coding for approx. 0.5 million D of RNA is frequently visualized in the spacer segments at the beginning of a matrix unit. Possible artifacts resulting from the preparation, the relative congruence between the data obtained using both methods, and the functional meaning of the findings are discussed against the background of current concepts of structural organization and transcription products of nucleolar DNA.

The structural organization of the amplified ribosomal RNA (rRNA) cistrons has been extensively studied in amphibian oocytes with biochemical and physico-chemical methods (e.g. [1, 5, 11, 25, 43]). The DNA which contains the sequences coding for the 28S and 18S rRNA (rDNA) has been isolated from the oocytes of various amphibian species and its structure and the arrangement of repeating nucleotide sequences shown to be basically similar to that in somatic cells [11]. From these studies the following model has been derived. About 40% of the rDNA is composed of sequences coding for the 18S and 28S rRNAs [2, 15] which are transcribed in a common precursor molecule (pre-rRNA) with a mol. wt of 2.6×10^6 D. This pre-rRNA is about 18% larger than the combined rRNAs [2, 24, 34]. In addition, the rDNA contains 'spacer' sequences, i.e. sequences which do not code for the pre-rRNA [35]. The regions coding for the pre-rRNA and the spacer segments are highly repeated and alternate in a regular pattern [6, 43]. The entire repeating unit has a mol. wt in the range from 8.5 to 11 million D (see also [1, 11]). Since about 5 million D of double stranded DNA code for the pre-rRNA the mol. wt of the spacer should be between 3.5 and 6 million D.

Miller and his collaborators [28–33] identified the repeating units in electron micrographs of spread preparations of nucleolar cores and showed that each of them consists of two alternating regions with different transcriptional activities. In most of their

preparations the length of the 'matrix-covered' DNA segments is in good correspondence "to the B-conformation length of DNA necessary to code for the 40S pre-rRNA" [28]: 2.0 to 2.5 μm corresponding to 3.8 to 4.8 million D of DNA. However, their reported values for the non-transcribed spacer regions (at an average one-third of the matrix unit length, i.e. 0.75 μm corresponding to 1.44 million D of DNA) do not agree with the results quoted above [6, 43]. Moreover, Miller & Beatty [30, 31] emphasized a strong variability of the lengths of the spacer segments, sometimes resulting in spacers as long as ten times the matrix lengths. This discrepancy as well as discrepancies in the data for matrix unit lengths between earlier and more recent publications from Miller's laboratory (cf e.g. [28, 29]) and reports claiming the existence of rDNA transcripts larger than 2.6 million D [8, 9] prompted us to analyse the correlation between the structural and biochemical data in greater detail (see also [1, 23]).

Miller & Beatty [30–32] have combined, for their calculations, the structural data obtained from oocytes with biochemical values from somatic tissue. Since so far values for the molecular weight distribution of the pre-rRNA in the amplified oocyte nucleoli have not been reported, except for the early estimation from the sedimentation coefficient by Gall [13], we determined the size distributions of the pre-rRNA in the same oocyte material in which the structural measurements were made. Special attention was also focused onto the morphology of the 'spacer region'.

MATERIALS AND METHODS

Electron microscopy of extrachromosomal ribosomal RNA cistrons

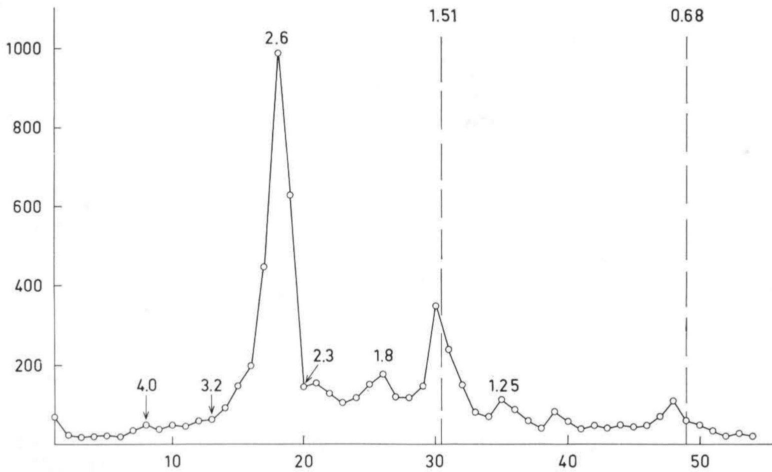
Triturus alpestris and *T. helveticus* females were collected during the breeding season (March until July) in the surroundings of Freiburg i.Br. (Black Forest area). *Xenopus laevis* and *Triturus cristatus carnifex* were kept in the laboratory. For excision of small pieces of the ovary the animals were anaesthesized in MS 222 (Sandoz, Basel). After sewing up, the newts were kept for several days in running tap water [7]. By this method it is possible to make several preparations from the same newt. Operated *Xenopus laevis* were kept in separate aquaria at 18°C. The excised ovary piece was transferred in Eagle minimal essential medium (1:1 diluted with distilled water) at about 18°C [40]. Nuclei were manually isolated from lampbrush stage oocytes (*T. alpestris* and *T. cristatus:* ca 800 μm; *X. laevis* and *T. helveticus:* ca 600 μm in diameter) in the '5:1-medium' (0.1 N KCl and 0.1 N NaCl in a ratio of 5:1) under a dissecting microscope and were washed several times in this solution until all adhering yolk material was completely removed. The procedure for visualization of the nucleolar cistrons was based on the method described by Miller & Beatty [30]. A detailed methodical description of this technique has recently been published by Miller & Bakken [28]. Two cleaned nuclei were transferred into a drop of water, adjusted to pH 9.0 with 0.01 M borate buffer, on a silicone-coated microscope slide. Herein the nuclear envelope was removed and the nucleoplasm was allowed to disperse for 10 to 20 min at 18°C. The microcentrifugation chamber was filled with a solution of 1% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sucrose. The quality of the final preparation appeared to be greatly improved when the time for glow-discharging the carbon-coated electron microscope grids was reduced to 1 min at 1 mA (using benzene in the Balzers Hochvakuum Bedampfungsanlage Mikro BA 3; Balzers, Liechtenstein). Centrifugation of the nuclear spread was performed at 2 200 g for 20 min at 10°C. The phosphotungstic acid (PTA) stock solution was centrifuged before diluting with ethanol.

Electron micrographs were taken with a Siemens Elmiskop 1A or 101 at 80 kV and 50 μm foil aperture or at 60 kV and 20 μm aperture. For every series of micrographs the magnification indicator was calibrated against two grating replicas of different spacing.

Morphological measurements were made from the micrographs or from calibrated positive prints projected at tenfold magnification onto a wall. The magnification was calibrated before and after each measurement series (deviations below 1%). The contours of the cistronic strands were drawn on large sheets of paper and lengths were traced with a 'geographer's tracer wheel' (curvometer) and compared with length standards. Each length value was the average from two (in some cases four) individual tracings. For definition of matrix-covered and spacer sections vide infra.

Gel electrophoresis of nuclear RNA

Usually 60 lampbrush stage oocytes were isolated from the freshly excised ovaries and incubated in a total volume of 1 ml TC 199 or Eagle minimal essential medium (both 1:1 diluted with distilled water) containing either ^{3}H-uridine alone at a con-



Figs 1, 2. Abscissa: slice no.; *ordinate:* (*fig. 1*) cpm; (*fig. 2*) cpm $\times 10^{-3}$.
Fig. 1. Separation of labelled nuclear RNA on acrylamide-agarose composite gel. Fifty nuclei were isolated from *Triturus alpestris* lampbrush stage oocytes incubated for 15 h at 22°C in TC 199 medium (1:1 diluted) containing 100 μCi/ml ^{3}H-uridine. Unlabelled ribosomal RNA isolated from *Xenopus laevis* ovaries was used as reference (1.51 and 0.68×10^6 D). The most prominent peak corresponds to a mol. wt of 2.6 million D. Note that some radioactivity is present on the heavy shoulder of this peak, in regions corresponding up to 4 million D. Intermediate products of processing are identified at about 1.8 and 1.55×10^6 D. The mature large rRNA of *T. alpestris* is smaller (ca 1.4×10^6 D) than that of the *X. laevis* marker.

centration of 100 μCi/ml or all four tritiated nucleosides (^{3}H-uridine, spec. act. 27 or 53 Ci/ml; ^{3}H-cytidine, 29 Ci/ml; ^{3}H-guanosine, 12.5 Ci/ml; ^{3}H-adenosine, 12.1 Ci/ml, each 100 μCi/ml). All radiochemicals were from The Radiochemical Centre, Amersham. After the incubation (10 to 48 h) the nuclei were isolated manually in the '5:1-medium', cleaned from adherent cytoplasmic material by several washings in the same medium and finally transferred into ice-cold ethanol–acetic acid (3:1). A batch of 50 nuclei, collected in this way, was washed 3 times in cold 70 % ethanol and freeze-dried. To the visible residue 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.4) was added containing 0.5 % SDS and 1 mg/ml pre-digested pronase (CalBiochem, RNAse-free). After 1 h at 25°C 20 μg of ^{14}C-uridine-labelled (intraperitoneal injection of 2×50 μCi after hormone-stimulated ovulation) or unlabelled *Xenopus laevis* oocyte rRNA (prepared according to 4 from ribosomes isolated after 12) was added as a marker and the RNA was precipitated by adding 50 μl of 1 N NaCl and 2.5 vol absolute ethanol. The solution was kept at −20°C for several hours, then centrifuged and the RNA pellet was suspended in 10 μl of electrophoresis buffer (0.02 M Tris, pH 8.0, 0.02 M NaCl, 0.002 M EDTA) with additional 0.2 % SDS, and applied to slabs of 0.5 % agarose, 2.25 % acrylamide composite gels. The separation conditions were identical to those described by Ringborg et al. [36]. After the run the two bands of the reference rRNA were marked under UV-light at 254 nm and the gel was cut into 54 slices using parallel razor blades with 1.1 mm spacing. Each slice was transferred into a counting vial, 10 ml of scintillation mixture (5 g PPO and 0.5 g dimethyl-POPOP per one liter toluene containing 50 ml NCS [Nuclear Chiacgo solubilizer]) was added and the vials were stored overnight at 30°C. The samples were counted in a Packard Tri–Carb scintillation spectrometer with efficiencies of 38 % (^{3}H) and 90 % (^{14}C). In the case of ^{14}C-uridine-labelled rRNA as marker double label counting was performed with the usual decrease in efficiency. All samples were corrected for the blank value. Quenching was very low and sufficiently constant in all samples so that correction was not necessary.

The mol. wts of the labelled nuclear RNA species were calculated by assuming a linear relationship between their electrophoretic mobility and the logarithm of the mol. wt. The mol. wts of the marker rRNAs were taken to be 1.51 and 0.68 million D [8, 22, 24].

RESULTS

Gel electrophoresis of labelled nuclear RNA

Fig. 1 shows in *Triturus alpestris* that after incubating selected lampbrush stage oocytes for 15 h in a medium containing ^{3}H-uridine, among the labelled nuclear RNAs a species with a molecular weight of 2.6 million D predominates (for a similar pattern in *Xenopus laevis* see [39]). The gel pattern of

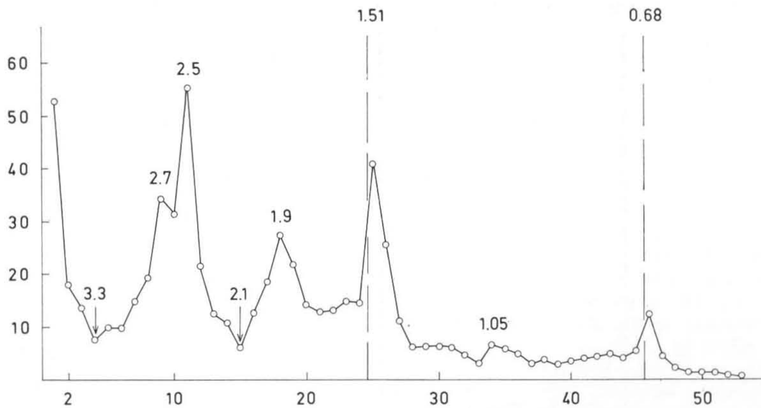


Fig. 2. Gel electrophoresis of the RNA from 50 nuclei isolated from *T. alpestris* lampbrush stage oocytes after an incubation for 2 days at 25°C in Eagle medium (1 : 1 diluted) with all four tritiated nucleosides (100 μCi/ml each). The heterogeneity of the precursor rRNA is evident; two chief components with a mol. wt of 2.5 and 2.7×10^6 can be distinguished in this separation. (Reference: *Xenopus laevis* rRNA.)

the nuclear RNAs also demonstrates that the processing of rRNA in the oocyte is essentially identical to that in somatic cells [24]. Results obtained after shorter and longer incubation times (10 to 48 h) were identical. We identify this high molecular weight RNA as the first nucleolar rRNA species which is sufficiently stable. The molecules appear to be slightly heterogeneous in size since there is always a shoulder on the heavy side of the peak. Sometimes even two components can be resolved (fig. 2). In no case was a major peak corresponding to molecular weights larger than 2.6 million D detected. There was, however, labelled RNA on the heavy side of the 2.6 million D peak which corresponds to a mol. wt of up to 4.0 million D. It should be emphasized, though, that such 'superheavy' pre-rRNA always constituted a very small amount of the total pre-rRNA region.

Structure of the repeating unit in rDNA

Spread preparations of the nucleolar cores reveal basically the same features as described by Miller & Beatty [30] for other species. An axial deoxyribonucleoprotein (DNP) fibril with a diameter of 70 to 100 Å is coated at intervals by 'matrix material' which consists of individual fibers about 170 Å thick of lengths progressively increasing to ca 0.5 μm (fig. 3). According to Miller & Beatty [29–32] one matrix unit represents a rRNA cistron with its primary transcription products attached in statu nascendi. The fibrils then are the pre-rRNA molecules complexed with protein. The region between neighbored matrix units usually appears to be free of such growing ribonucleoprotein (RNP) fibrils. Each one of these 'spacer' regions together with its neighboring 'matrix unit' constitutes a repeating unit of transcriptional activity along the rDNA.

Depending on the specific spreading conditions the matrix material can appear clumped into more or less electron-dense aggregates. This results in different aspects of the matrix units. Frequently differences in the structural aspect can even be seen within the same spread nucleolus (fig. 4). Some matrix material appears well stretched with the RNP fibrils individually discernible whereas in other matrix units the fibrillar material is clumped together (figs 13, 14).

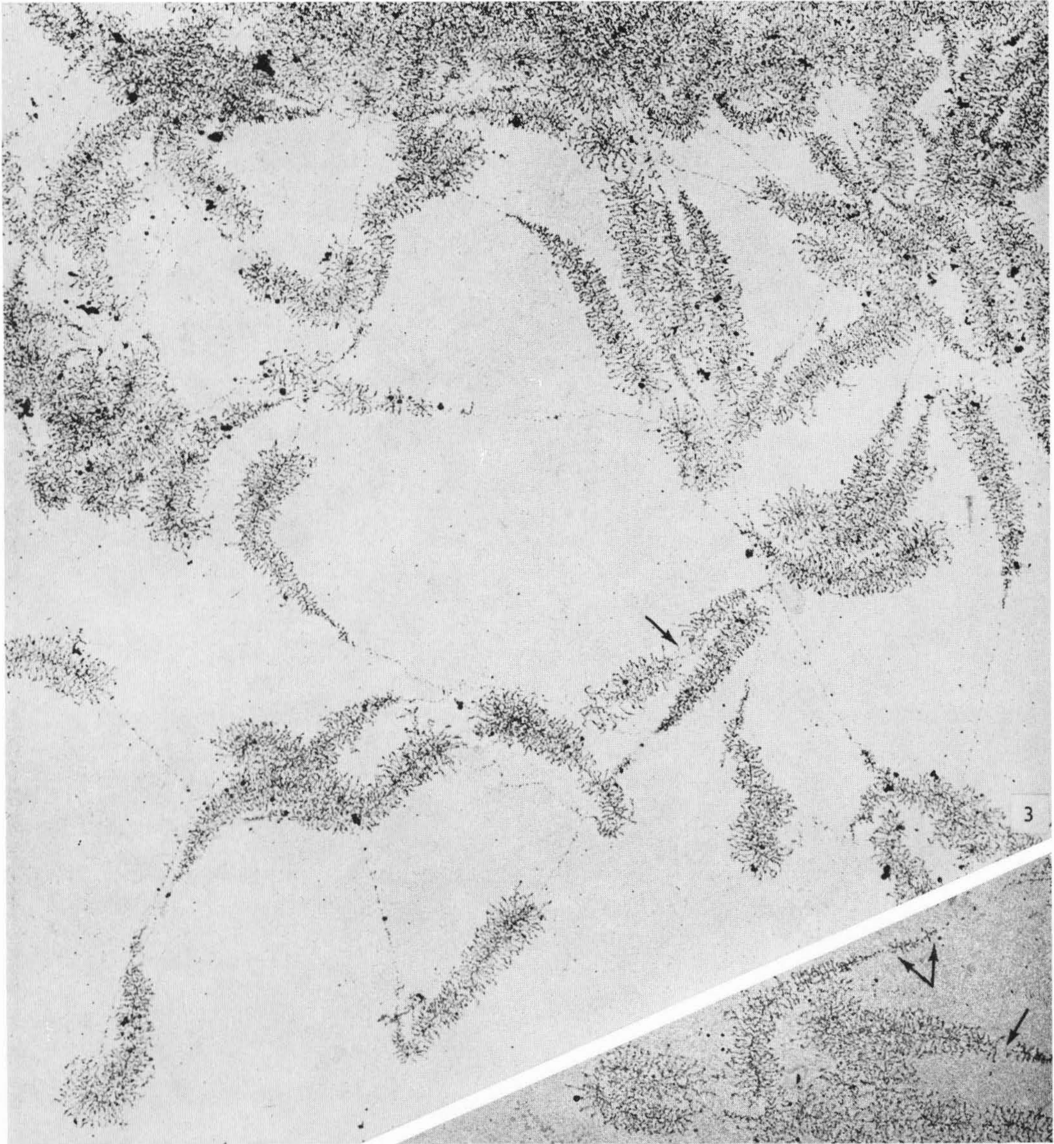


Fig. 3. Typical survey electron micrograph of a spread nucleolar core from a *T. alpestris* lampbrush stage oocyte. The DNP axis is coated in intervals by lateral rRNP fibrils which continuously increase in length thus constituting a 'matrix unit'. Intermatrix segments represent 'spacer regions'. Sometimes a short section of the spacer DNP axis close to the beginning of a matrix unit is covered by RNP fibrils (*inset*, *pair of arrows*). The gap between such a small 'prelude' region and the subsequent matrix unit is clearly different from the (perhaps artificial) 'gaps' within matrix units since the latter interrupt the length gradient of the RNP fibrils (e.g. single arrows in survey and inset). $\times$ 8 800; inset $\times$ 13 500.

This indicates that the appearance of active rRNA cistrons visualized with the 'Miller-technique' is not fully controllable by the investigator. Even within one matrix unit the aspect may change. A matrix unit may show 'clumped' fibrils at the beginning and dispersed fibrils in the posterior part (fig. 4). Vice versa, it may begin with dispersed RNP

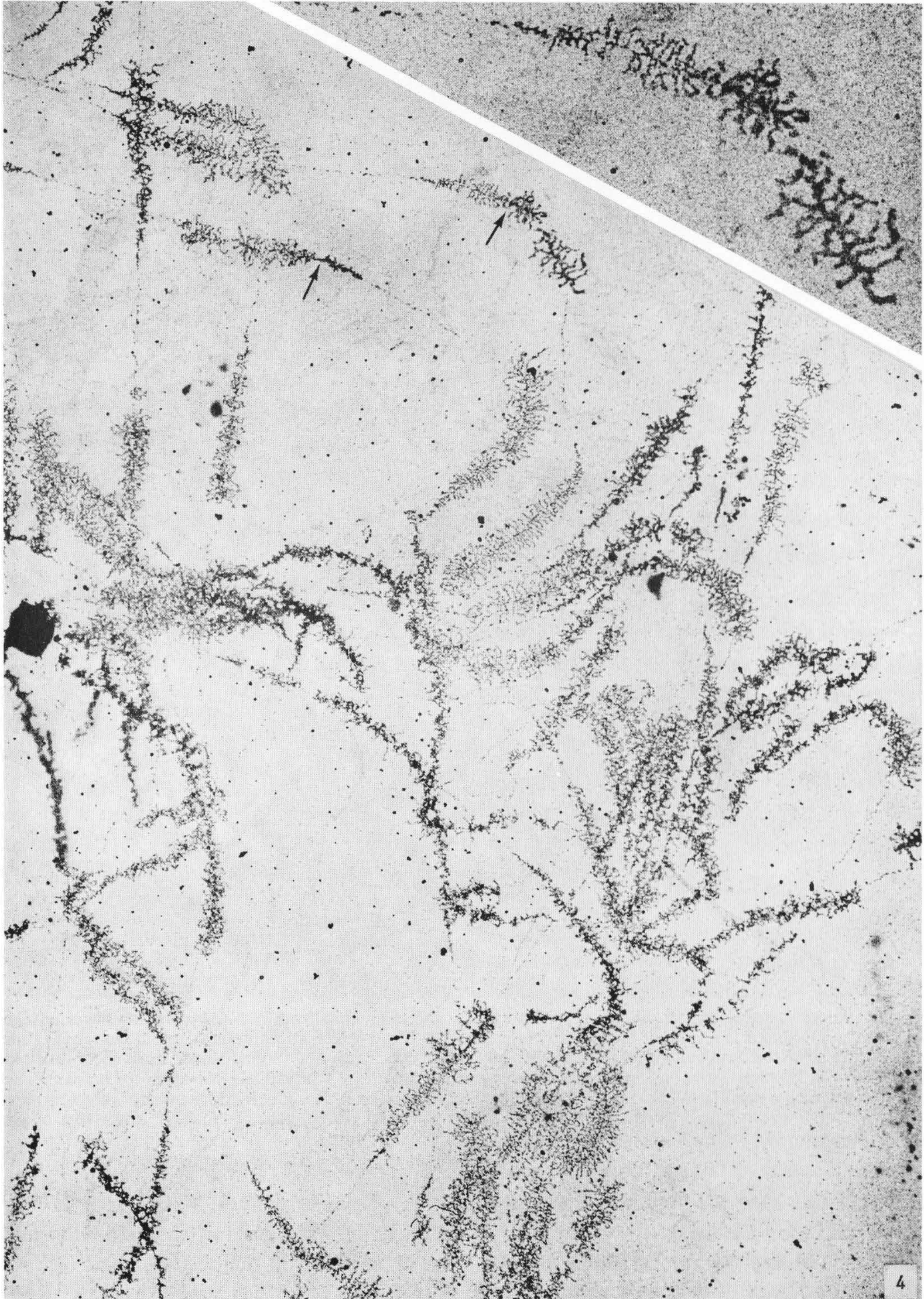


Fig. 4. Diversity of structural aspects of matrix units within one spread nucleolar core of a *T. alpestris* oocyte. In some matrix units all rRNP fibrils are well stretched and individually discernible, whereas in others the fibrils are clumped to variable extents. Moreover, the structural aspect of the matrix fibrils can even change within the same matrix unit (arrows and inset). ×10 000; inset ×23 000.

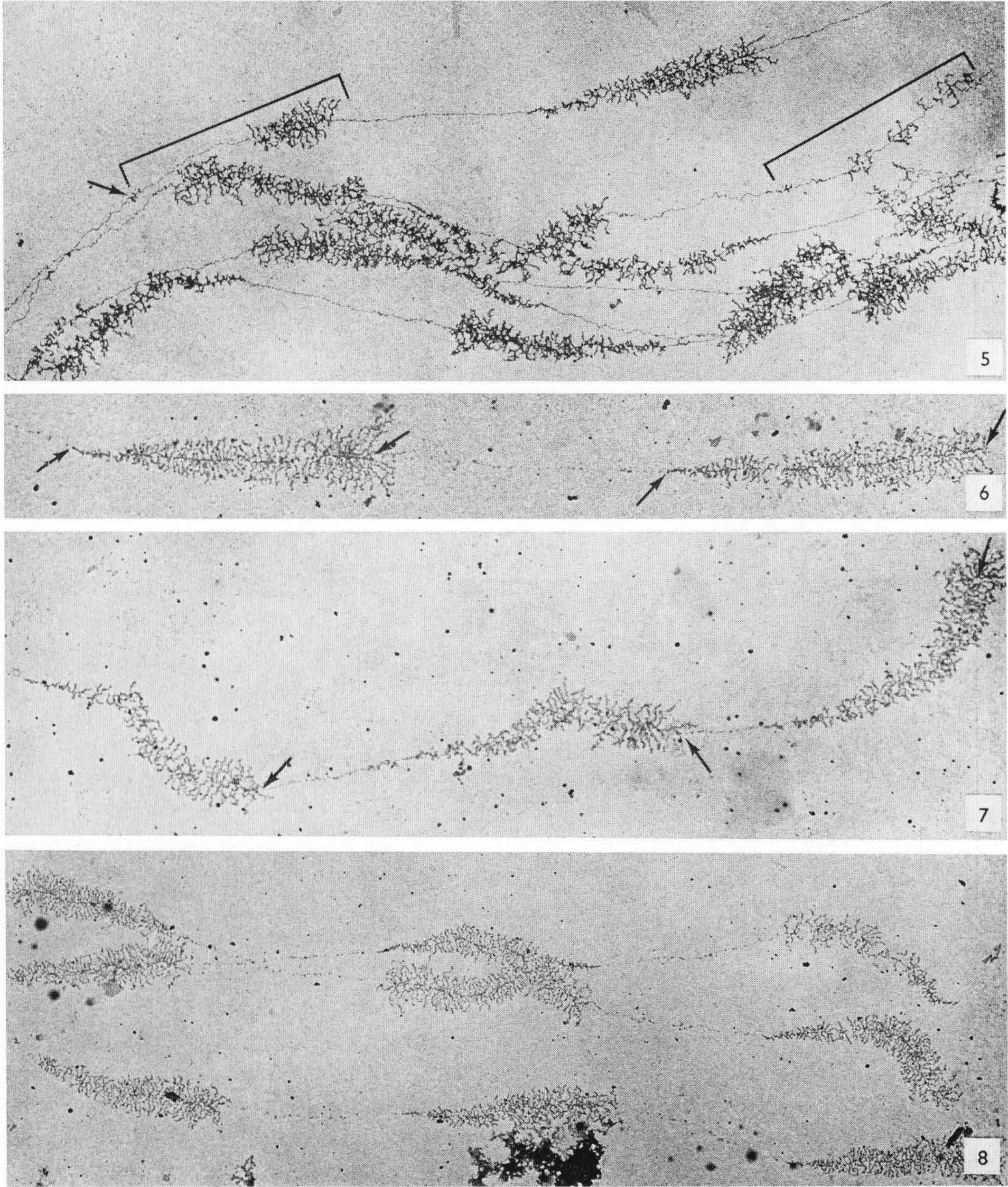


Fig. 5. Detachment of rRNP fibrils from the DNP axis can alter the appearance of the matrix units (here in an example from *T. helveticus*). The left arrow points to a group of short fibrils indicative of the beginning of a matrix unit (indicated by the left bar). Between the start and a terminal group of fibrils the matrix material is completely removed from the axis. The right bar denotes a matrix unit which contains only a few fibrils. ×17 800.

Figs. 6–8. Series of matrix units in spread oocyte nucleoli of *T. alpestris*. The two pairs of arrows in fig. 6 denote the beginning and the end of a matrix unit as defined in the text. Repeating units, consisting of a spacer and a matrix segment, are marked by the arrows in fig. 7. In fig. 8 three DNP axes, nearly parallel to each other, show the frequently observed lateral alignment of matrix units. Fig. 6, ×14 600; fig. 7, ×13 100; fig. 8, ×11 000.

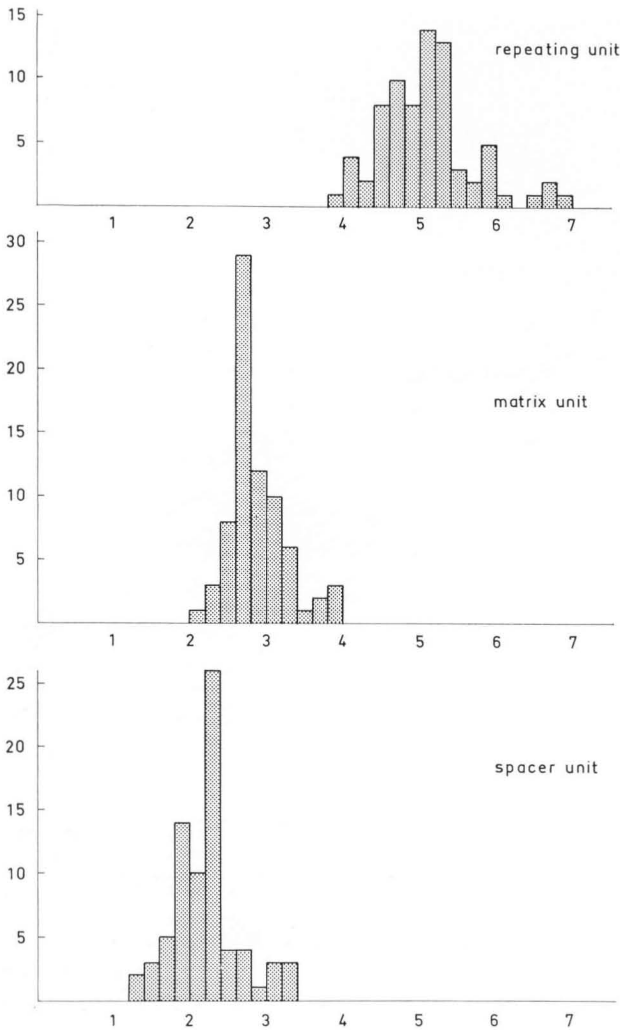


Fig. 9. Abscissa: μm; *ordinate: n.*
Length distribution of the matrix units, the spacer regions adjacent to the beginning of each matrix unit, and the total repeating units. The values were obtained from spread nucleoli of *T. alpestris* lampbrush stage oocytes.

and terminate with coarser and thickened RNP fibrils (fig. 4, inset). Frequently incomplete matrix units are encountered which do not show a 'beginning', i.e. the short RNP fibrils are lacking, perhaps stripped off during the preparation (fig. 5). Another anomalous appearance of matrix units is sometimes recognized in the form of 'gaps' between the individual growing RNP fibrils (fig. 5). This sparsity of fibrils in matrix units might also have been caused by partial loss of RNP fibrils from the axis during the preparation. While such anomalies and artifacts can be easily recognized, any artificial removal of terminal matrix material would not be detected and thus could result in underestimates of the matrix unit lengths.

For quantitative evaluations of repeat and matrix unit lengths only well spread preparations without any apparent aggregations and artificial removal of RNP fibrils were considered (e.g. figs 3, 6–8, 13). The matrix unit length was defined as the length of the central DNP axis between the first electron

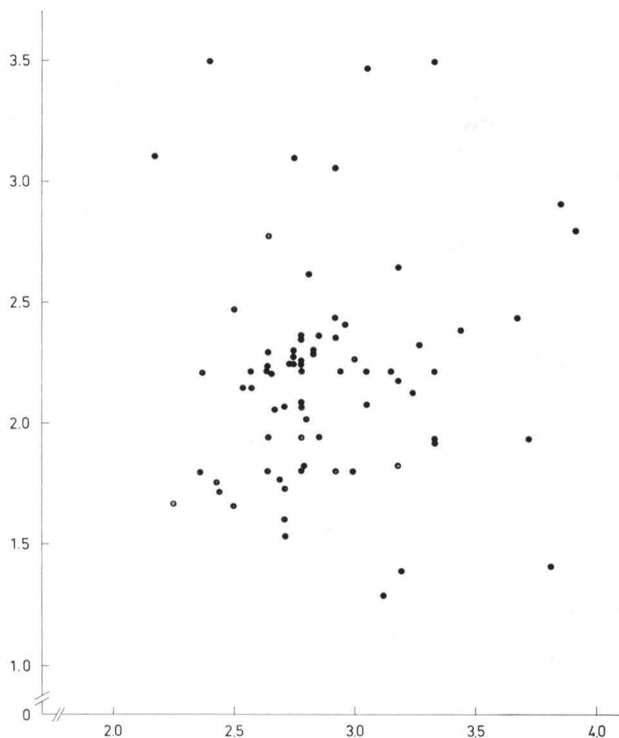
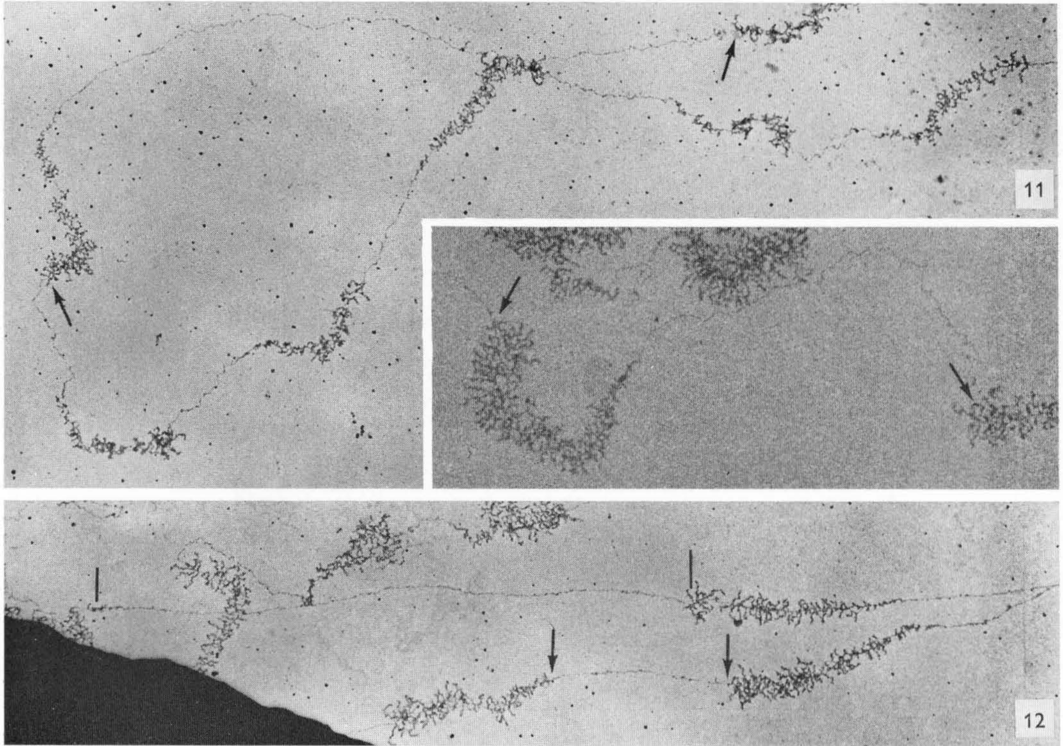


Fig. 10. Abscissa: matrix unit [μm]; *ordinate:* spacer unit [μm].
Correlation plot of pairs of matrix units and the adjacent spacer units (for details see text).

dense particle attached to the axis which had twice the size of the DNP axis width and the basal point of the terminal RNP fibril (e.g. fig. 6). Furthermore, between the start and the end of a matrix region there had to be one series of lateral fibrils of gradually increasing length. In accordance with Miller & Beatty [29] we define a repeating unit as the axial segment lying between the basal points of the terminal RNP fibrils of two neighbored matrix units, i.e. a period consists of the matrix covered region and the spacer adjacent to its origin (e.g. fig. 7). Measurements of the repeat unit length were performed only on preparations in which the spacer DNP axis could be clearly identified between the well spread matrix units.

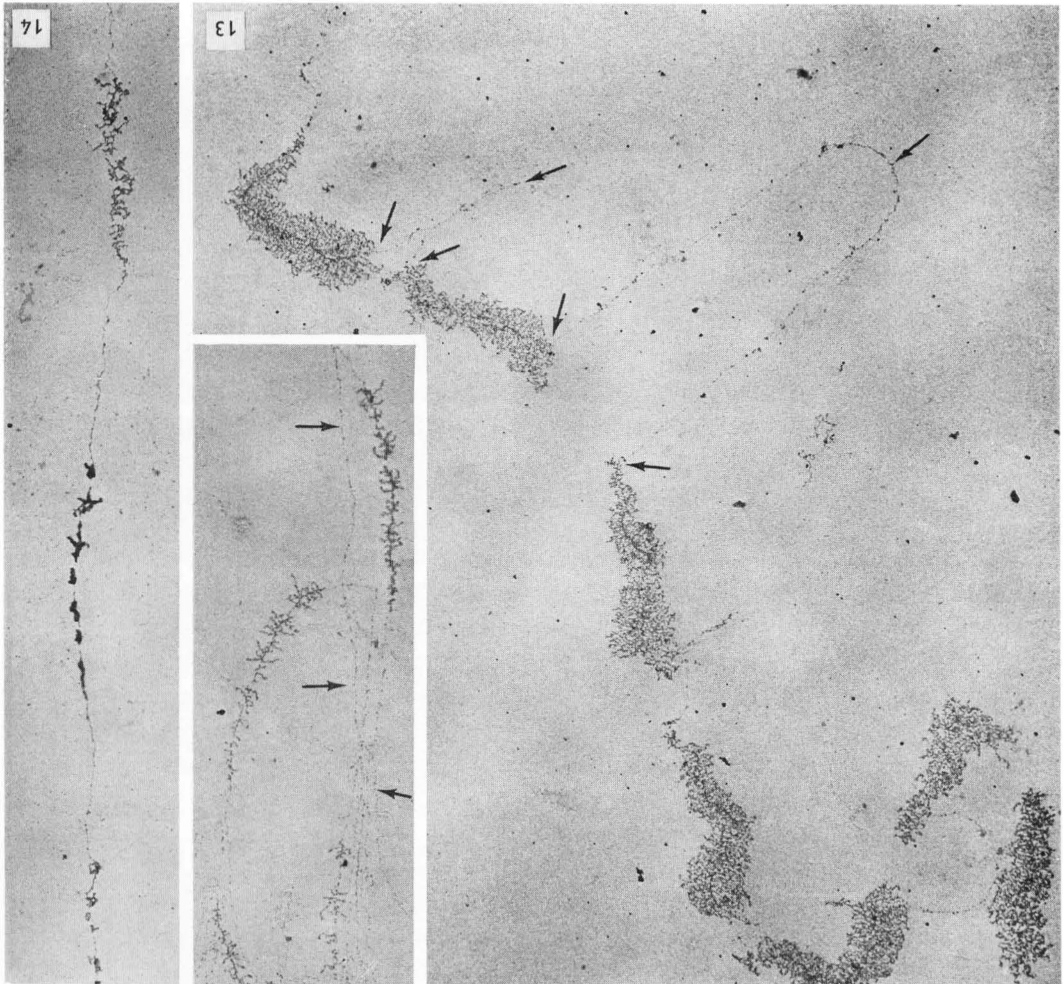
Measurements of repeat units, matrix units and spacer regions are presented in fig. 9 for *T. alpestris*. Most of the repeat units fall into the size class of 5.0 to 5.4 μm. A Gaussian transformation of the cumulative curve did not reveal a significant deviation from a normal distribution with a mean of 5.09 ± 0.6 μm (S.D.). Under the assumption that each micron of the DNP axis contains stretched double-stranded DNA equivalent to 1.92 million D (e.g. [3, 21, 37, 41]) the length of 5.0 to 5.4 μm corresponds to 9.6 to 10.4 million D of DNA (mean: $9.77 \pm 1.2 \times 10^6$ D). From fig. 9 one can further see that an average repeat unit consists of matrix and spacer segments of almost identical lengths. The majority of the spacer regions have lengths between 2.2 and 2.4 μm (mean: 2.20 ± 0.4 μm, equivalent to $4.22 \pm 0.8 \times 10^6$ D of DNA; this does not include the exceptionally long spacers described below). Most of the matrix units are distributed in the 2.6



Figs 11, 12. Examples of 'extremely long' spacer regions in spread preparations of *T. alpestris* oocyte nucleoli. The corresponding 'apparent' repeat unit is the region between the two arrows in fig. 11. Note the unidirectional polarity of the matrix units along the DNP axis. These extremely long spacer regions exceed by far the maximal values of the distribution curve of fig. 9. The inset (*arrows*) shows, for comparison, one of the longest among the normal spacer regions. Fig. 12 allows a direct comparison between an extremely long spacer region (indicated by the bars) and a spacer region representative for the average spacer length (indicated by the arrows). Fig. 11, ×9 700; inset, ×16 800; fig. 12, ×10 500.

to 2.8 μm class. Since the distribution curve for the matrix unit length is skewed to the right, the mean (2.89 ± 0.3 μm) does not correspond to the frequency maximum. One matrix unit thus contains on the average $5.55 \pm 0.6 \times 10^6$ D of DNA. The extremely large matrix units fall into the size classes 3.8 to 4.0 μm (7.3 to 7.7 million DNA). It should perhaps be emphasized that the standard deviations of the matrix unit and the spacer length distributions are nearly identical. As can be seen from fig. 10 an increase in matrix unit length is not correlated with a decrease in the length of the adjacent spacer. In contrast, the ratio of the lengths of the two regions in a given repeat tends to be constant, as demonstrable by a correlation coefficient of +0.563 between the lengths of matrix unit and spacer pairs, i.e. within the same repeat unit. This suggests that both regions respond to stretching to a similar degree.

The average values for the lengths of repeat units, matrix units and spacer regions in other amphibia studied were: 4.57, 2.47, 2.22 (*T. cristatus carnifex*); 4.78, 2.63, 2.18 (*T. helveticus*); 4.66, 2.57, 2.11 μm (*X. laevis*). These data, however, are based on fewer determinations so that in these cases the standard deviation is higher.



Figs 13, 14. DNP axis of spread *T. alpestris* oocyte nucleolar core showing one 'superlong' spacer region (indicated by the left arrow-triplet). In this preparation spacer regions (*triple-arrows*) are drawn, in an almost perpendicular plane, from the matrix unit line, probably because of an orientation by streaming. *Inset:* Similar preparation from *Xenopus laevis*. The various aspects of lateral fibrils are seen. A long DNP stretch without matrix material is indicated by the arrows. *Fig. 14*, DNP axes of a spread nucleolus from a *T. cristatus* oocyte showing, side by side, two extreme aspects of the appearance of matrix fibrils, a dispersed one (below) and the clumped (aggregate) form (centre). Fig. 13, ×12 500; inset ×13 200; fig. 14, ×13 200.

In all the amphibia studied we found a limited number of extremely long spacers (e.g. in *T. alpestris* seven cases out of 170 preparations), comparable to those mentioned by Miller & Beatty ([30–32]; see our fig. 11: 9.6 μm; fig. 12: 7.3 μm; fig. 13: 8.3 μm). These values differ significantly from the maximal spacer lengths found in the repeat units of the distribution curve of fig. 9. The sum of the lengths of such extremely long spacer segments with that of the adjacent matrix units give values which are about twice the length of an average repeat unit (e.g. the above-mentioned three examples give 12.2, 10.1, and 10.8 μm). The explanation proposed by MacGregor [26]

that 'long spacers' may result from cistronic regions free from matrix material, be it artificially caused or representing non-transcribing cistrons, is in accord with our values. In addition, we occasionally observed aggregated DNP axes without any matrix material associated which could not be identified as 'spacer' due to the absence of clear matrix units (fig. 13, inset).

Spreading of the nucleolar cores often resulted in a conspicuous parallel association of certain regions of the DNP axis. In fig. 8, the matrix units within three parallel strands are arranged in register showing either the same or opposite polarity along the DNP axes. This array of parallel fibers may be caused by a sticking together of the matrix material of adjacent units.

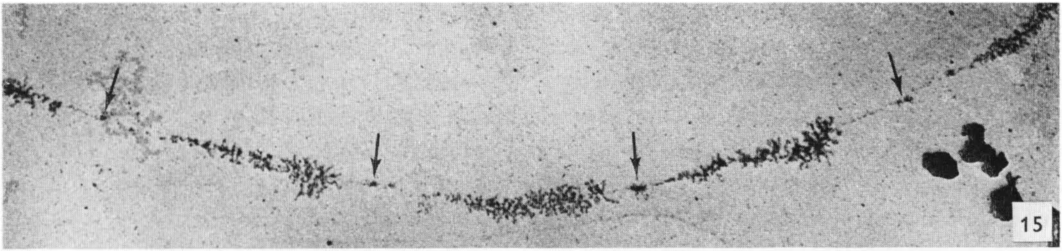
In various preparations some novel features were observed in the spacer regions. Figs. 15–19 demonstrate fibrillar material associated with DNP segments in between the matrix units. The dimensions of these spacer regions clearly rule out the possibility that this fibrillar material could be remnants of partially washed off matrix fibrils, such as are shown in fig. 4. The spacer DNP-attached material appears in the middle of the spacer regions, sometimes aggregated into a clump (fig. 15), and sometimes more disperse (figs 16, 17). Occasionally, two separate small stretches of lateral fibrils could be distinguished within one spacer region (fig. 18). Staining properties as well as widths of these spacer DNP-attached fibrils are indistinguishable from the matrix fibrils and there is nothing to contradict the conclusion that they represent RNP fibrils as well. The lengths of such spacer DNP-attached fibrils, however, do not approach those of the terminal matrix unit fibrils.

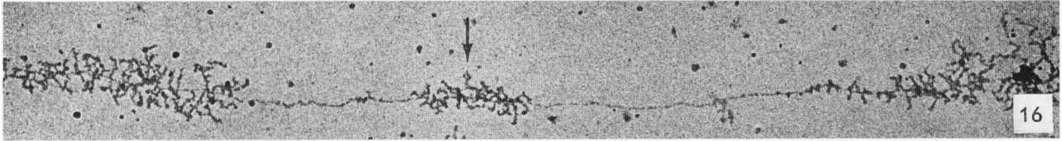
Frequently, special stretches of lateral fibrils are attached to the spacer segment close to the beginning of a matrix unit thus forming a 'prelude region' (figs 18, 19; see also inset in fig. 3). Such a prelude shows exactly the same structural feature as the beginning of a matrix unit including the gradient of increasing lengths of the laterally attached RNP fibrils. The prelude-sections are at an average 0.56 ± 0.09 μm long, corresponding to a minimal DNA content of 1.05 million D. The gap between the end of such a prelude region and the start of the adjacent matrix unit is very small, mostly about 0.2 μm. Since this following matrix unit shows the normal gradient of increasing lateral fibre length, starting from zero, the prelude piece cannot have been caused by artificial removal of matrix material.

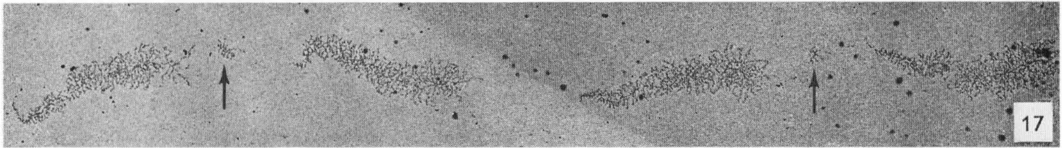
DISCUSSION

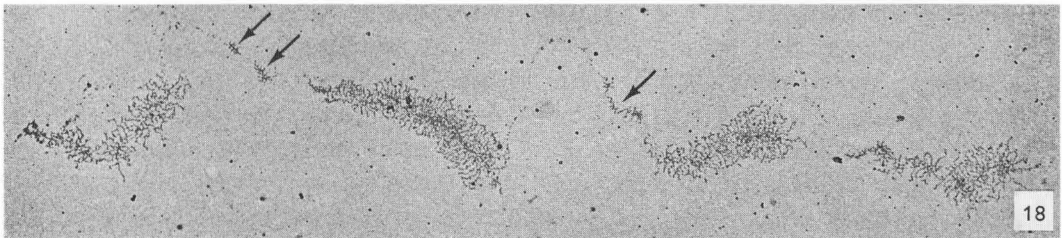
The morphological data show that the extrachromosomal rDNA and consequently the average repeating unit is divided into two distinct regions in a relatively constant proportion: 57% is covered by matrix material and 43% is free of it. According to the interpretation of Miller & Beatty [29–32] this means that at any time only 57% of the rDNA is being transcribed. This is in

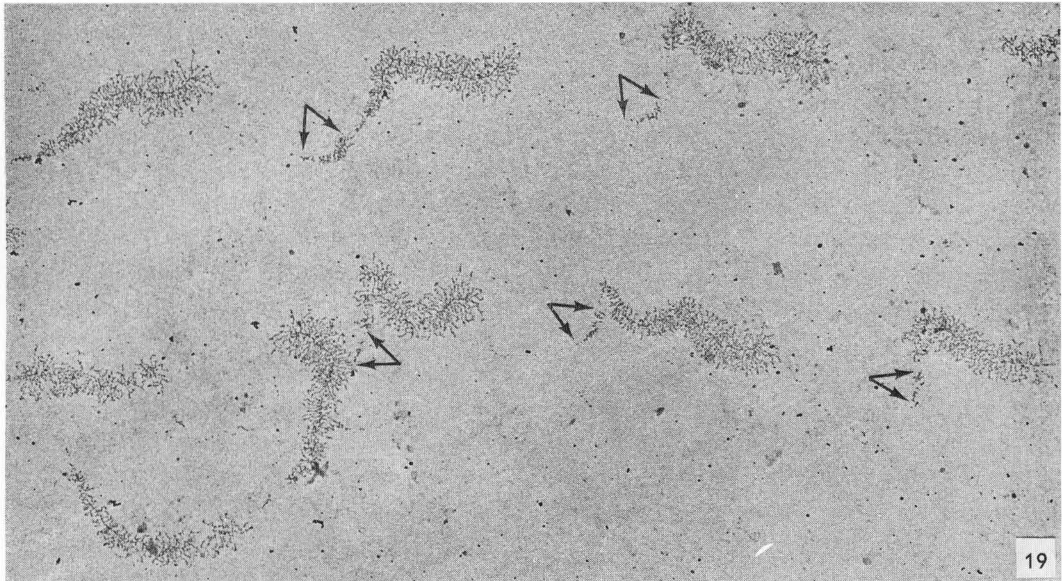
Figs 15–19. RNP fibrils associated with spacer regions. In the intermatrix region of fig. 15 electron-dense material is associated with the DNP axis in the central parts of spacer regions (*arrows*). Under more favourable spreading conditions the spacer axis-attached material is resolved into the individual fibrils (fig. 16, *arrow*). *Fig. 17* shows two spacer segments with fibrils attached to the middle region of their axes (*arrows*) separated by a spacer without such attached material. The two arrows in the left of fig. 18 point to two separate groups of spacer axis-attached fibrils. The right arrow denotes a stretch of RNP fibrils close to the beginning of a matrix unit ('prelude piece'). *Fig. 19* demonstrates the frequency and the structural aspects of such 'prelude regions' (*pairs of arrows*). The pair of arrows in the upper left points to a prelude piece which exhibits the length gradient of its RNP fibrils. The polarity of the prelude regions has the same direction as the matrix units. Fig. 15, ×10 500 fig. 16, ×15 500; fig. 17, ×10 000; fig. 18, ×10 500; fig. 19, 10 600.


15


16


17


18


19

good agreement with various physical and biochemical data from oocytes and somatic cells of *Xenopus laevis* [11, 43]. The most prominent peak in our electropherograms of labelled amphibian oocyte nuclear RNA corresponds to a mol. wt of about 2.6 million D. Figs 1 and 2 show that pre-rRNA molecules transcribed in the extrachromosomal nucleoli have mol. wts in the range from 2.5 to ca 3.2 million D. These values are in accord with corresponding estimations from hybridization data in *Xenopus* [2, 14, 15] and also with the sedimentation properties of the precursor in oocytes from *T. viridescens* and *Ambystoma mexicanum* [13, 38]. They furthermore correspond with determinations of the pre-rRNA size in somatic amphibian cells [19, 20, 24, 27, 34]. A RNA molecule of 2.5 to 3.2 million D is coded for by a double-strand DNA helix of a mol. wt between 5.0 and 6.2 million D, assuming that only one strand of the DNA is transcribed [35]. Thus one calculates that one rRNA cistron has a length of 2.6 to 3.2 μm. This estimation agrees well with our measured mean value of 2.9 μm of the matrix unit lengths.

The agreement between the biochemical data and the morphological measurements indicates further that the DNA (B-conformation) within the matrix unit is largely stretched. This conclusion is also supported by measurements of matrix unit lengths in nucleolar spreadings on the surface of a water drop, i.e. conditions similar to those of the 'Kleinschmidt technique' for spreading DNA-protein films. The absolute values as well as the relative length proportions of matrix units and matrix-free regions were not significantly different from those obtained after the normal 'Miller preparation'. The observed extreme values of matrix unit lengths (up to 4.0 μm) therefore are unlikely to be caused by special uncoiling of the DNA within these matrix DNP axes but rather indicate that true length differences exist in the matrix unit population. The present study demonstrates that matrix units can considerably exceed the 2.3 to 2.5 μm average values emphasized in the publications of Miller and co-workers [28, 30–33]. The existence of such long matrix units is not only documented in our communication but has also been noted by Miller in the earlier publications (e.g., [29–33]). Thus, one is faced with the fact that among a majority of ca 2.7 μm long matrix units (fig. 9) there also occur individual longer matrix units which maximally could code for even more than 4 million D of RNA. No conclusion can be drawn at the moment as to whether the matrix unit is the morphological equivalent of the transcription of only one defined pre-rRNA or whether some transcribed rDNA sections contain variable stretches in excess of DNA complementary to the pre-rRNA which thus leads to the heterogeneity of matrix unit lengths. On the other hand, the distribution pattern of matrix unit lengths is hardly compatible with the view that the primary pre-rRNA has an average molecular weight of 4.2 to 4.4 million D as reported in embryonic cells of *Rana pipiens* [8]. All the current concepts, however, presuppose that no (artifactual) shortening of the matrix unit regions occur during the preparation steps as, for instance, a preferential detachment of terminal RNP fibrils.

The degree of variability of the spacer lengths (for problems of defining the 'spacer' vide infra) is similar to that of the matrix units. Our measurements of the average length of spacer segments (in *T. alpestris*: 2.2 ± 0.4 μm, corresponding to 76% of the matrix length) differ somewhat from the results of Miller and co-workers [28, 30–33] in *T. viridescens* and *X. laevis*. They are consistent, however, with the data of

Wensink & Brown [43] and Brown et al. [6] for the more thermostable stretch in the rDNA repeat units in two *Xenopus* species.

The various definitions of 'spacer regions' in rDNA as (i) more thermostable regions in rDNA [6, 43], (ii) as carrying base sequences not contained in the ca 2.6 million D pre-rRNA (see [35]), or (iii) as stretches of nucleolar DNA axes not associated with RNP fibrils, i.e. non-transcribing DNA [29–32], are not necessarily congruent. The observations in the present study further complicate the problem of defining 'spacer segments' since we show that intersections between adjacent rRNP fibril cascades can themselves carry individual or grouped lateral fibrils. This finding that distinct regions within spacer segments in the sense of Miller (for references see above) can be associated with RNP fibrils indicates transcription of at least parts of the DNA in these regions. Therefore, we think that spacer segments should not be envisaged as being non-transcribed but should be defined, strictly functionally in the sense of [35], as those sections of the DNA within a rDNA repeating unit which do not code for the specific pre-rRNA.

It is not clear whether the ca 0.5 million D piece of RNA synthesized in the prelude regions has any relationship to the transcription of rDNA at all. At least it is evident that this RNA piece is not continuous with the long pre-rRNA made in the subsequent matrix unit. The existence of such prelude pieces demonstrates further that in rDNA two initiation sites for transcription can be relatively close together and that there is a preferential clustering of these initiation sites near the beginning of a rRNA cistron with a somewhat regular spacing corresponding to approximately 2 000 deoxynucleotide pairs. It is quite conceivable that the beginning of the prelude piece is no more than an 'erroneously early' initiation. On the other hand, it may also be that sometimes the transcription of the prelude piece runs through into the reading of the following matrix unit cistron and this would result in a somewhat longer primary pre-rRNA carrying the prelude piece of RNA at its 5′-region (cf [18]). Such a "read through transcript" is one of the alternatives explaining the occurrence of pre-rRNAs of molecular weights higher than 3 million D.

Although our data underline the predominance of pre-rRNA molecules corresponding to about 2.6 million D mol. wt, they also highlight the existence of a smaller, apparently heterogeneous subpopulation of longer pre-rRNAs. Therefore, it should be emphasized that they do not allow the conclusion that the RNA with a mol. wt of 2.6 million D represents the primary transcription product. The present data would be also consistent with the interpretation that some precursor rRNA molecules of higher molecular weights are rapidly processed to the relatively stable 2.6 million D size class during or shortly after their transcription (see e.g., [16, 17, 42]).

The authors gratefully acknowledge the support of the Deutsche Forschungsgemeinschaft (SFB 46 MOLGRUDENT) and the valuable technical assistance of Miss Marianne Winter. We also thank Drs. H. Falk (this institute). P. P. M. Rae (Max-Planck-Institut für Biologie, Tübingen), Susan Gerbi (Brown University, Providence, USA) and R. R. Kay (University College, London) for discussions on and corrections to the manuscript.

Note added in proof

Recently, M. E. Rogers & G. Klein (Biochem j 130 (1972) 281) have reported a similar mol. wt (2.9×10^6 D) for the pre-rRNA in *Triturus cristatus* by labelling isolated ovaries. Moreover, these authors as well as U. E. Loening, D. Grierson, M. E. Rogers & M. L. Sartirana (FEBS symp 23 (1972) 395) have shown the existence of a family of pre-rRNA molecules "on the heavy side of the peak", indicating heterogeneity.

A survey of the results obtained with the spreading technique is presented in the review by O. L. Miller & B. A. Hamkalo (Int rev cytol 33 (1972) 1).

REFERENCES

1. Birnstiel, M L, Chipchase, M & Speirs, J, Progress in nucleic acid research and molecular biol (ed J N Davidson & W E Cohn) vol. 11, p. 351. Academic Press, New York and London (1971).
2. Birnstiel, M L, Speirs, J, Purdom, I, Jones, K & Loening, U E, Nature 219 (1968) 454.
3. Biswal, N, Kleinschmidt, A K, Spatz, H C & Trautner, T A, Mol gen genetics 100 (1967) 39.
4. Brown, D D, Methods in developmental biology (ed F H Wilt & N K Wessels) p. 586, Crowell, New York (1967).
5. Brown, D D & Dawid, I B, Science 160 (1968) 272.
6. Brown, D D, Wensink, P C & Jordan E, J mol biol 63 (1972) 57.
7. Callan, H G & Lloyd, L, Phil trans roy soc London ser B, 243 (1960) 135.
8. Caston, J D & Jones, P H, J mol biol 69 (1972) 19.
9. Crippa, M & Tocchini-Valentini, G P, Proc natl acad sci US 68 (1971) 2769.
10. Davidson, E H, Allfrey, V G & Mirsky, A E, Proc natl acad sci US 52 (1964) 501.
11. Dawid, I B, Brown, D D & Reeder, R H, J mol biol 51 (1970) 341.
12. Ford, P J, Biochem j 125 (1971) 1091.
13. Gall, J G, Natl cancer inst monograph 23 (1966) 475.
14. — Proc natl acad sci US 60 (1968) 553.
15. — Genetics, suppl. 61 (1969) 121.
16. Grierson, D & Loening, U E, Nature new biol 235 (1972) 80.
17. Grierson, D, Rogers, M E, Sartirana, M L & Loening, U E, Cold Spring Harbor symp quant biol 35 (1970) 589.
18. Hecht, R M & Birnstiel, M L, Eur j biochem 29 (1972) 489.
19. Knowland, J & Miller, L, J mol biol 53 (1970) 321.
20. Landesman, R & Gross, P R, Dev biol 19 (1969) 244.
21. Lang, D, Bujard, H, Wolfe, B & Russel, D J, J mol biol 23 (1967) 63.
22. Loening, U E, J mol biol 38 (1968) 355.
23. — Symp soc gen microbiol 20 (1970) 77.
24. Loening, U E, Jones, K W & Birnstiel, M L, J mol biol 45 (1969) 353.
25. MacGregor, H C, J cell sci 3 (1968) 437.
26. — Biol rev 47 (1972) 177.
27. Miller, L, Biochem j 127 (1972) 733.
28. Miller, O L & Bakken, A H, Acta endocrin, suppl. 168 (1972) 155.
29. Miller, O L & Beatty, B R, Handbook of molecular cytology (ed A Lima-de-Faria) p. 605. North-Holland, Amsterdam and London (1969).
30. — Science 164 (1969) 955.
31. — Genetics, suppl. 61 (1969) 134.
32. — J cell physiol 74, suppl. 1 (1969) 225.
33. Miller, O L, Beatty, B R, Hamkalo, B A & Thomas, C A, Cold Spring Harbor symp quant biol 35 (1970) 505.
34. Perry, R P, Cheng, T Y, Freed, J J, Greenberg, J R, Kelley, D E & Tartof, K D, Proc natl acad sci US 65 (1970) 609.
35. Reeder, R H & Brown, D D, J mol biol 51 (1970) 361.
36. Ringborg, U, Daneholt, B, Edström, J E, Egyházi, E & Lambert, B, J mol biol 51 (1970) 327.
37. Ritchie, D A, Thomas, C A, MacHattie, L A & Wensink, P C, J mol biol 23 (1967) 365.
38. Rogers, M E, J cell biol 36 (1968) 421.
39. Scheer, U, Dev biol 30 (1973) 13.
40. Scheer, U, Berezney, R & Franke, W W. In preparation (1973).
41. Thomas, C A, J gen physiol 49 (1966) 143.
42. Tiollais, P, Galibert, F & Boiron, M, Proc natl acad sci US 68 (1971) 1117.
43. Wensink, P C & Brown, D D, J mol biol 60 (1971) 235.

Received October 30, 1972
Revised version received January 23, 1973