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Absence of nucleosomes in transcriptionally active chromatin

Chromatin während der Transkription: das Fehlen von Nucleosomen

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Abstract

Chromatin structure – nucleosomes – transcription – electron microscopy

The ultrastructure of two kinds of transcriptionally active chromatin, the lampbrush chromosome loops and the nucleoli from amphibian oocytes and primary nuclei of the green alga *Acetabularia,* has been examined after manual isolation and dispersion in low salt media of slightly alkaline *p*H using various electron microscopic staining techniques (positive staining, metal shadowing, negative staining, preparation on positively charged films, etc.) and compared with the appearance of chromatin from various somatic cells (hen erythrocytes, rat hepatocytes, cultured murine sarcoma cells) prepared in parallel. While typical nucleosomes were revealed with all the techniques for chromatin from the latter three cell system, no nucleosomes were identified in either the lampbrush chromosome structures or the nucleolar chromatin. Nucleosomal arrays were absent not only in maximally fibril-covered matrix units but also in fibril-free regions between transcriptional complexes, including the apparent spacer intercepts between different transcriptional units. Moreover, comparisons of the length of the repeating units of rDNA in the transcribed state with those determined in the isolated rDNA and with the lengths of the first stable product of rDNA transcription, the pre-rRNA, demonstrated that the transcribed rDNA was not significantly shortened and/or condensed but rather extended in the transcriptional units. Distinct granules of about nucleosomal size which were sometimes found in apparent spacer regions as well as within matrix units of reduced fibril density were shown not to represent nucleosomes since their number per spacer unit was not inversely correlated with the length of the specific unit and also on the basis of their resistance to treatment with the detergent Sarkosyl NL–30. It is possible to structurally distinguish between transcriptionally active chromatin in which the DNA is extended

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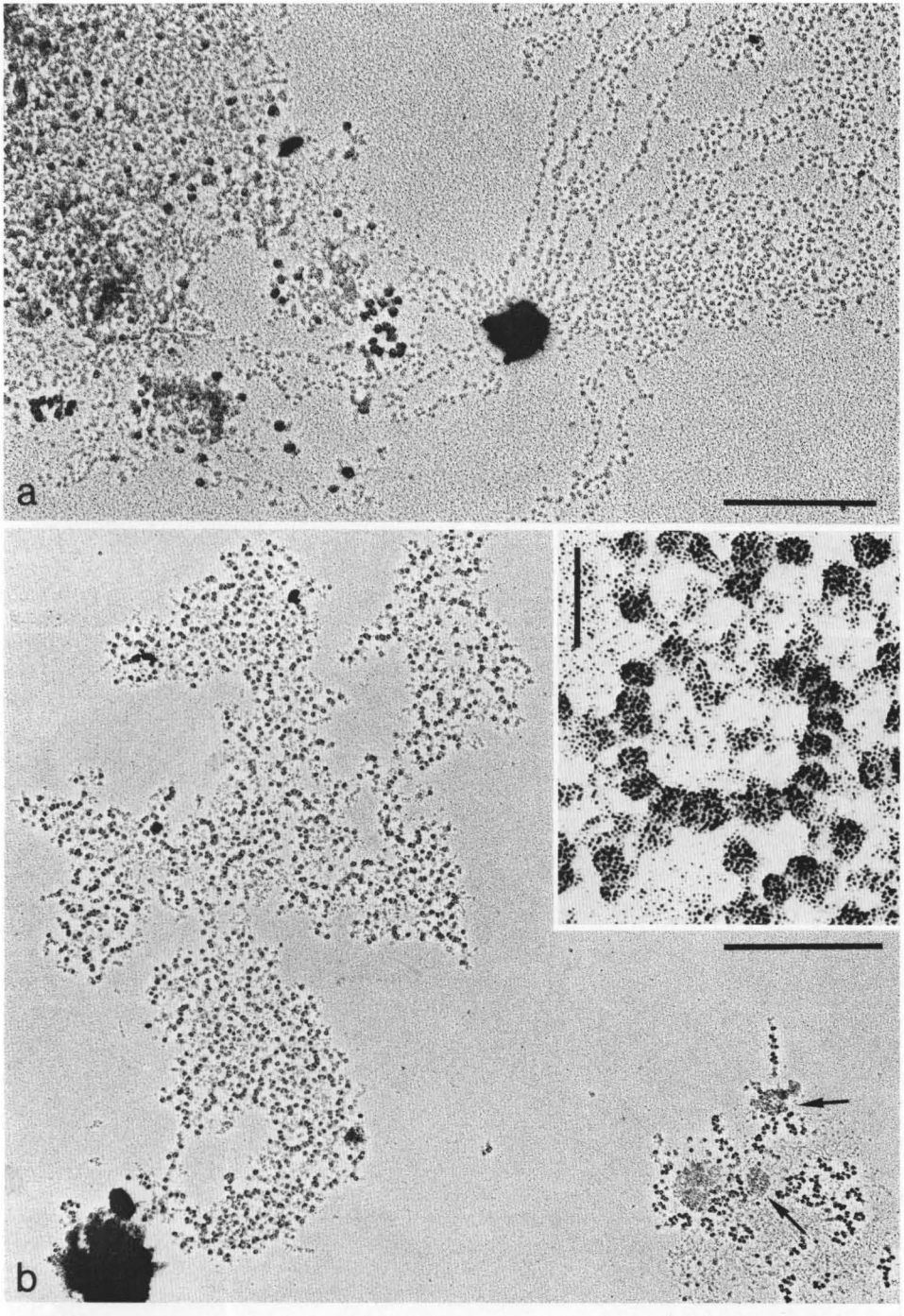
in a non-nucleosomal form of chromatin and condensed, inactive chromatin within the typical nucleosomal package. The characteristic extended structure of transcriptionally active chromatin is found not only in the transcribed genes but also in non-transcribed regions within or between ("spacer") transcriptional units as well as in transcriptional units that are untranscribed amidst transcribed ones and/or have been inactivated for relatively short time. It is hypothesized that activation of transcription involves a transition from a nucleosomal to an extended chromatin organisation and that this structural transition is not specific for single "activated" genes but may involve larger chromatin regions, including adjacent untranscribed intercepts.

Introduction

In the nuclei of most, if not all, eukaryotes, and possibly also in prokaryotes (e. g. [24]), large parts of the DNA are packaged in chromatin fibrils with a characteristic "beads on a string" appearance ([6, 7, 33, 45 to 47, 49, 53, 67, 71], for review see [12], for refs. on the appearance of chromatin fibrils in sections and in various preparation conditions see also [54]; an exception to this principle might perhaps be the dinoflagellates). Such repeating granular substructures, the nucleosomes (ν-bodies, [45]), have also been demonstrated after digestion of chromatin with various nucleases, including restriction endonucleases (see some of the refs. quoted above and also [13, 25, 27, 28, 31, 32, 36, 44, 48, 59, 63, 68, 72]). Their analysis has resulted in the construction of models of constitutive chromatin subunits containing about 200 base pairs of DNA that are associated with a defined complex of histone molecules (e. g. refs. [5, 31, 63], for critical evaluation of this concept see also [73]). Most of these studies, however, have used nuclei that are characterized by the predominance of transcriptionally inactive and condensed chromatin such as that from avian and amphibian erythrocytes, mammalian liver and thymus, and trout testis (see refs. indicated above; as to the general occurrence of nucleosomes in nuclei containing higher proportions of dispersed chromatin see also [71]). The nucleosome concept of the packing of DNA into units that contain only four of the histones and no non-histone proteins at all can hardly hold for all of the nuclear DNA since extranucleosomal histones, in particular those of the H 1 family, as well as non-histone proteins are well known components of chromatin in general (for recent review see [12]). Consequently, one might speculate that other, non-nucleosomal forms of chromatin exist which are enriched in these components and that the transcriptionally active chromatin is a candidate for the location of such non-nucleosomal chromatin proteins (for related refs. see [2, 22]). Therefore, we have examined the ultrastructural organization of chromatin strands of identifiable transcriptional activity in cytologically well defined situations, in particular in the nucleoli and the lampbrush chromosome loops of the oocytes of various amphibia and insects and of the primary nucleus of a series of dasycladacean green algae. The data presented strongly suggest that nucleosomes are absent not only from maximally transcribed chromatin strands but also from spacer regions between transcriptional complexes as well as from inactive gene regions that are located in the vicinity of transcribed genes.

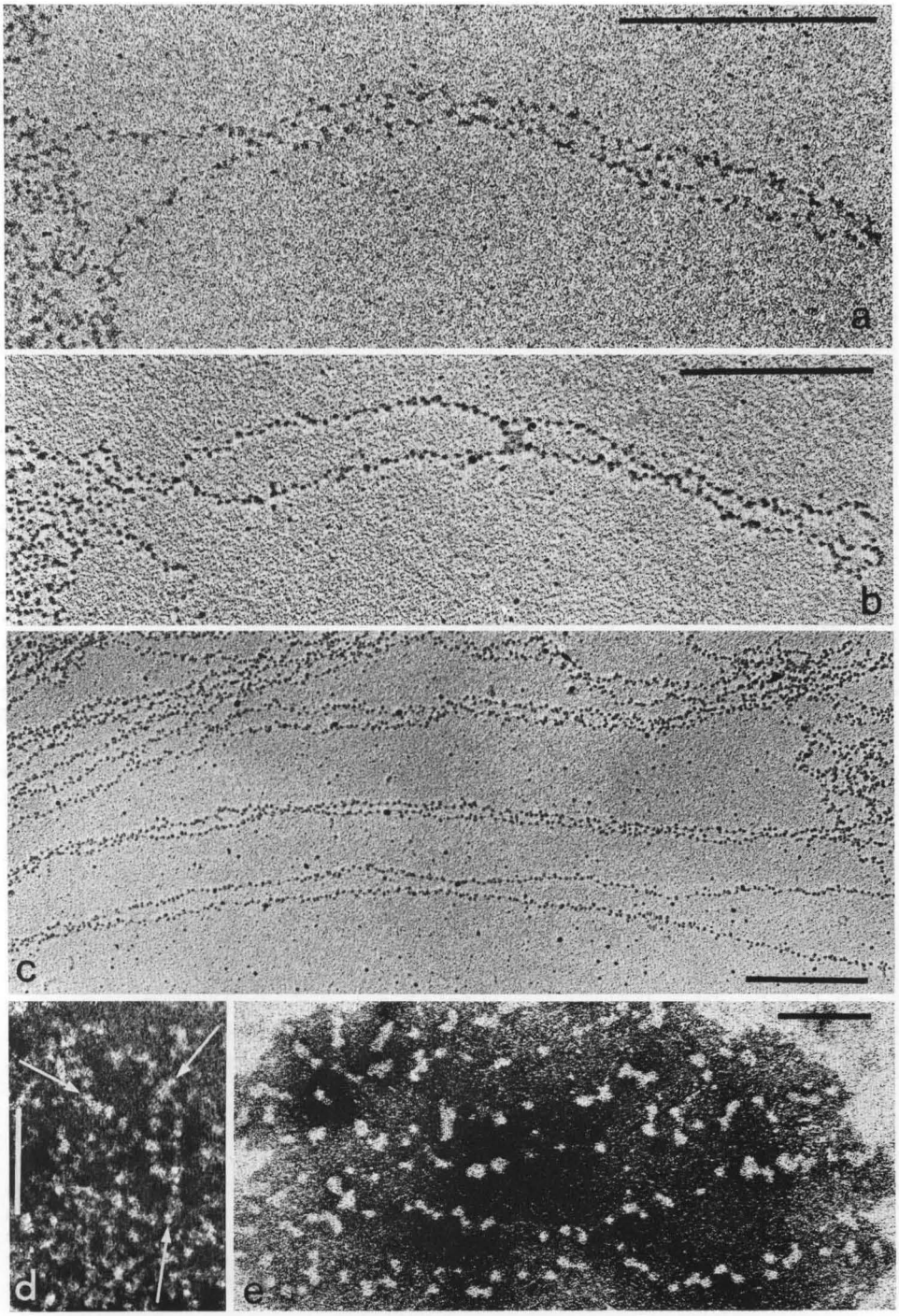
Figs. 1 a and b. Electron micrographs of chromatin-like structures in nuclei from murine sarcoma 180 cells that have been dispersed, spread, positively stained and metal shadowed (for details see Methods). Note the typical "beads on a string" structure (e. g. in the right part of **a**). In addition, larger globular particles are recognized which are especially frequent in association with fragments of the nuclear envelope (left part in **a** and **Fig. b**; for details see the insert in **b**); the arrowheads in **b** denote pore complexes. – **a.** 42 000 ×. – Scale 0.5 μm. – **b.** 22 000 ×. – Scale 1 μm. – Insert 135 000 ×. – Scale 0.1 μm.


a
b

Material and methods

Spread preparations were made from manually isolated nuclei and/or nucleoli from oocytes of the following amphibia: *Triturus alpestris, Triturus cristatus, Triturus helveticus* (collected from various habitats in Baden-Württemberg, Federal Republic of Germany), *Xenopus laevis, Pleurodeles waltlii* (kindly provided by Dr. J. Brachet, Université Libre de Bruxelles, Belgium). For isolating nucleoli free from lampbrush chromosome material the peripheral membrane-attached nucleoli were manually isolated along with the nuclear envelope ghost (cf [15, 58]). Such nuclear envelope-nucleolar complexes were then used for further preparation. Different stages of oogenesis (cf [58]) were compared. In most experiments vitellogenic oocytes (mid-to-late lampbrush chromosome stage) were used, if not otherwise indicated. Similar preparations were made from primary nuclei isolated from growing vegetative stages (usually at about two thirds of maximal plant size) of the following dasycladacean green algae: *Acetabularia mediterranea* (for conditions of culture see [60]), *Acetabularia major* (kindly provided by Dr. H. G. Schweiger, Max-Planck-Institute for Cell Biology, Wilhelmshaven, Federal Republic of Germany; cf [60, 62, 66]), *Acetabularia cliftonii* (kindly provided by Dr. G. Werz, Free University, West-Berlin), and *Acetabularia calyculus* (also from Dr. Werz). In some experiments nuclei were taken from oocytes or *Acetabularia* cells that had been treated with actinomycin D for various times (for details see [57]). The nuclei were isolated in a NaCl and KCl containing medium buffered with 10 mM Tris-HCl (*p*H 7.2) specified in previous articles [56 to 58, 60]. The details of the spreading preparation, which was modified from the procedure originally described by Miller and coworkers [39 to 42] have also been described [56 to 58, 60, 64 to 66]. Various staining procedures were used: (*a*) the positive staining with ethanolic phosphotungstic acid (PTA); (*b*) as in (a) but with additional shadowing with gold : palladium (cf [57]) or platinum : palladium (80 : 20) either by rotary shadowing alone or by combined rotary and angle shadowing (7°); (*c*) negative staining with aqueous PTA (2 %) solution, adjusted to *p*H 7.2 with NaOH; or (*d*) nuclear contents were centrifuged onto carbon-coated collodion films which had been positively charged by glow discharge in methylamin according to Dubochet et al. ([11], cf [49]) and were then stained for 20 seconds with 0.1 % uranyl formate and dried [4]. Some of these preparations were additionally shadow-cast with platinum from different directions (angles of 7 to 10°; cf [49]). Whole hen erythrocytes (for source and purification see [74]) and hen erythrocyte nuclei [74, 75] were lysed in 0.1 to 0.5 mM sodium borate buffer (cf [60]) at various *p*H values (8.0, 8.5, 9.0) by shaking or by suspending with a few gentle strokes using a glass-Teflon homogenizer. Nuclei were isolated from rat liver as described [16] or by homogenization (as mentioned) and centrifugation (5 min at 1000 g) in a medium containing 0.083 M KCl and 0.017 M NaCl buffered with 10 mM Tris-HCl to *p*H 7.2 or in the very low salt borate buffer indicated above. The crude fractions of isolated nuclei were then processed for spread preparations. In addition, spread preparations were made from nuclei isolated from an established murine sarcoma cell line derived from the transplantable murine sarcoma 180 and grown in cell culture (Eagle's basal medium containing 20 % fetal calf serum), using the same isolation procedure. Before homogenization the cell layer was washed with the specific buffer and scraped off with a rubber policeman.

Figs. 2 a to e. Electron micrographs illustrating the appearance of hen erythrocyte chromatin after different contrasting procedures such as positive staining without (**a**), or with (**b** and **c**) metal shadowing, and negative staining of an aqueous suspension (**d**), or after fixation and drying in ethanol (**e**, see Methods). The characteristic nucleosomal organization of the chromatin strands is retained through all procedures. The white arrows in **Fig. 2 d** point to rows of nucleosomes in a rather closely packed form which may represent the native arrangement. The larger particles recognized in **e** represent either doublets of nucleosomes or correspond to the larger particles described in the previous figure. Note the occurrence of some stain particles in the background of the preparation (e. g. **Figs. 2 b and c**). – **a.** 72 000 ×. – **b.** 54 000 ×. – **c.** 33 000 ×. – **d.** 145 000 ×. – **e.** 130 000 ×. – Scales in **Figs. a to c** represent 0.5 μm, scales in **d** and **e** denote 0.1 μm.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c
d
e

In some experiments isolated nuclei of the material mentioned above were suspended in 0.5 mM sodium borate buffer (*p*H 9.0) containing 0.1 to 0.3 % of the anionic detergent Sarkosyl NL–30 (NL–30 is a 30 % aqueous solution of sodium lauryl sarcosinate; Ciba-Geigy, Basel, Switzerland) at 10° C. The resulting w/w ratio of detergent to DNA was at least 1000 : 1 in all experiments. After 10 min the dispersed material was centrifuged and processed as described above.

Electron micrographs were taken with a Zeiss EM 10 and a Siemens Elmiskop 101 at 60 kV. Quantitative evaluations were as described [56, 60, 66].

Results and discussion

Demonstrations of nucleosomes

All the various techniques used, particularly lysis of nuclei in slightly alkaline borate buffers (*p*H 8 to 9) of low ionic strength, demonstrated typical nucleosomal arrangements, i. e. fibrils with a "beads on a string" aspect (Figs. 1 and 2). Our findings confirm observations by others authors (for refs. see Introduction, in particular [45] and [71]). The sizes of the nucleosomal granules, however, varied according to the specific staining procedure. In hen erythrocyte chromatin, for example, the nucleosomes appeared somewhat smaller after positive staining alone (10 ± 1.2 nm) or after negative staining (10.8 ± 2 nm) than after positive staining plus subsequent metal shadowing or shadowing alone (12.8 ± 1.6 nm). While the data obtained with positive staining agree with those found by Woodcock et al. [71] with the same technique, our negative staining measurements show diameters larger than those reported, for example, by these authors, by Olins and coworkers [45, 46] and by Rattner et al. [53]. Our data, however, are quite similar to those reported by other authors [13, 33, 49] and also correspond to the 11 nm figure determined with X-ray techniques by Carlson and Olins ([7], see also [52]). In contrast to Woodcock et al. [71], we do not think that measurements of nucleosome sizes made in negatively stained preparations are more reliable than those made in positively stained preparations, especially since it usually remains unknown how much of the hydration sheath of such particles has been penetrated by the staining solution under the specific staining conditions (for a critical discussion of the problems of determining accurate nucleosomal sizes see [7]). The size distribution of the nucleosomal particles was not significantly different in the various cell types studied (hen erythrocytes, rat liver, murine sarcoma cells). Internucleosomal strands ("connecting strands" sensu Olins et al. [47]) were observed only rarely. They appeared more frequent in preparations in which the swelling of the chromatin had been prolonged and also seemed to be more clearly encountered in negative stain preparations in general (cf Figs. 2 d, e). In nuclei which were spread for relatively short times, we repeatedly noted another type of granular chromatin particle (mean diameter 26.2 ± 2.5 nm) that was especially frequent in the chromatin associated with the nuclear envelope. Such particles

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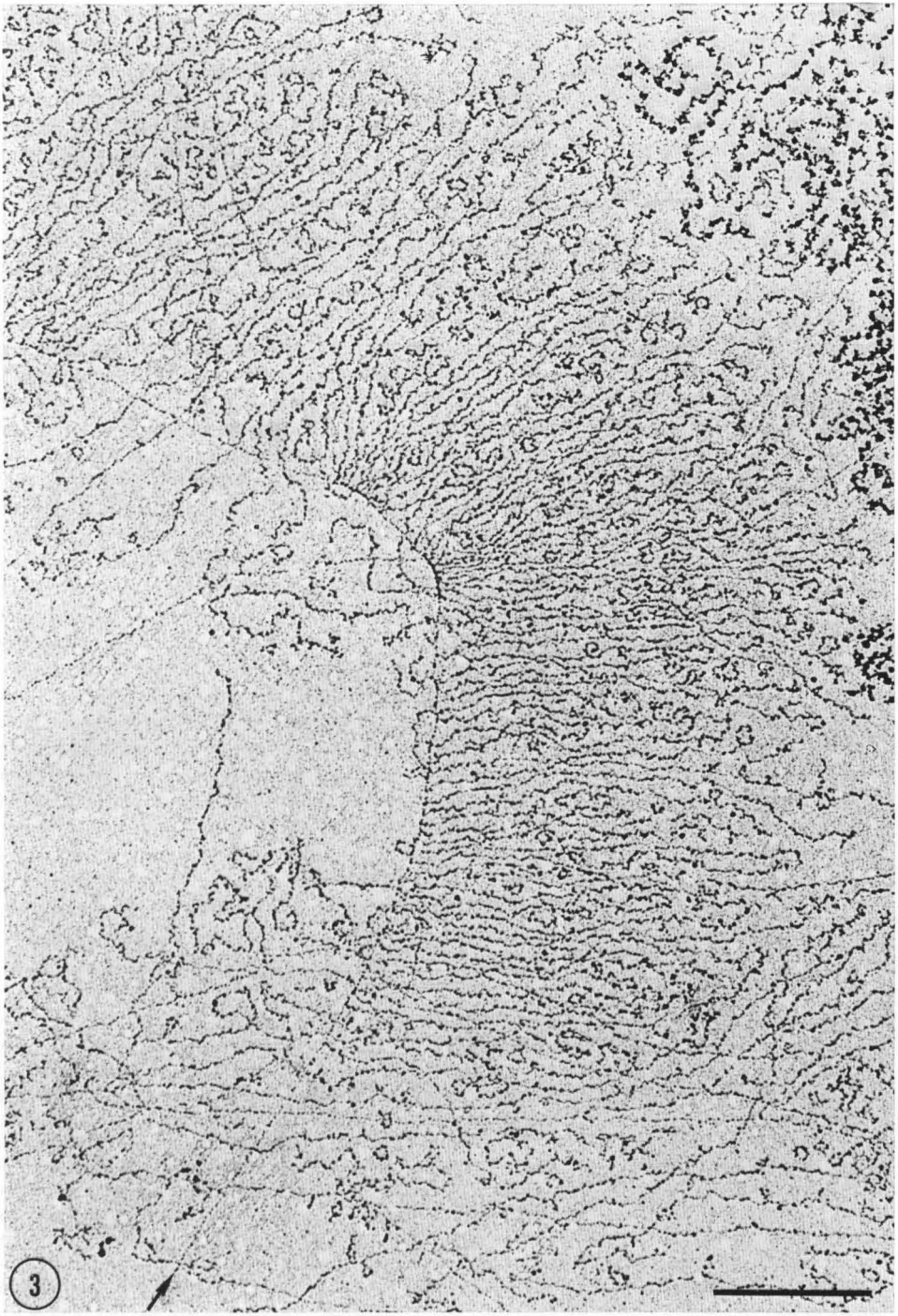
Fig. 3. Survey electron micrograph of a part of an extended transcriptional unit in a lampbrush chromosome loop from an oocyte of the newt, *Triturus cristatus*, as revealed after spreading and positive staining. The axial chromatin strand is denoted by the bottom arrow. Note the abundance of lateral fibrils which are very long (several micrometers) in this specific intercept of the transcriptional unit and show an almost closest packing in some regions. – 22 000 ×. – Scale 1 μm.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are interpreted as corresponding to the ca. 19 to 25 nm globular units described in sections through the peripheral chromatin of cells fixed in situ (Fig. 1; for refs. see [9, 14, 15, 75], for a description of 37 nm large particles in chromatin digests see [50]). It may well be that these larger chromatin globules represent further condensation and packing of the nucleosomal chromatin bead chains (cf [9]), characteristic for certain topological classes of inactive chromatin, including the most peripheral chromatin.

Absence of nucleosomes in transcriptional units of lampbrush chromosome loops

When dispersed nuclear contents or chromosome material from amphibian oocytes or the giant nucleus of dasycladacean green algae were spread with the same method as used for the demonstration of nucleosomes in lysed somatic nuclei (see above), we noted extended chromosomal loops with highly active transcriptional units (Figs. 3 to 5; cf also [1, 21, 38, 40, 42, 60, 61]). In such preparations, the transcribed chromatin was identified in the form of matrix units, that is as long intercepts of chromatin strands covered with lateral fibrils which in initial regions reveal a typical length gradient. These matrix units are rather heterogeneous in length (some exceed ten micrometers) and can be arranged with alternating polarities (see refs. quoted; as to the limitation of the fibril length gradient to initial regions see [17]). The chromatin axis within such matrix units was usually very thin (4.5 to 7.5 nm) and this held for both, matrix units with densely packed lateral fibrils and matrix units with more or less reduced fibril density which might be indicative of a reduced transcriptional activity (Figs. 4 to 5; see also Figs. 7, 8 and 10 in [1], and Figs. 2 to 4 in [21]). The base of each lateral fibril was accentuated by an intensely stained, 13 ± 1.5 nm large granular particle (values taken from positively stained preparations) which most probably includes the RNA polymerase complex (cf [42]). The dimensions of these basal knobs correspond to those determined for the isolated RNA polymerase A and B molecules (ca. 12.5 nm, P. Oudet and P. Chambon, unpublished data; cf also [8], for dimensions of RNA polymerase from *Escherichia coli* see [18]). Since in regions of maximal packing of lateral fibrils the center-to-center distances between adjacent polymerase granules were in the range of 17 to 25 nm, it became obvious that nucleosomes of the dimensions described above would not have sufficient space in such intercepts (Fig. 5). In fact, frequently adjacent, putatively polymerase-containing basal granules of the transcription complexes appeared in direct contact in such regions (Fig. 5; cf [1, 40, 60]). Moreover, distinct, nucleosome-like granules were also not revealed in small fibril-free axial intercepts between more

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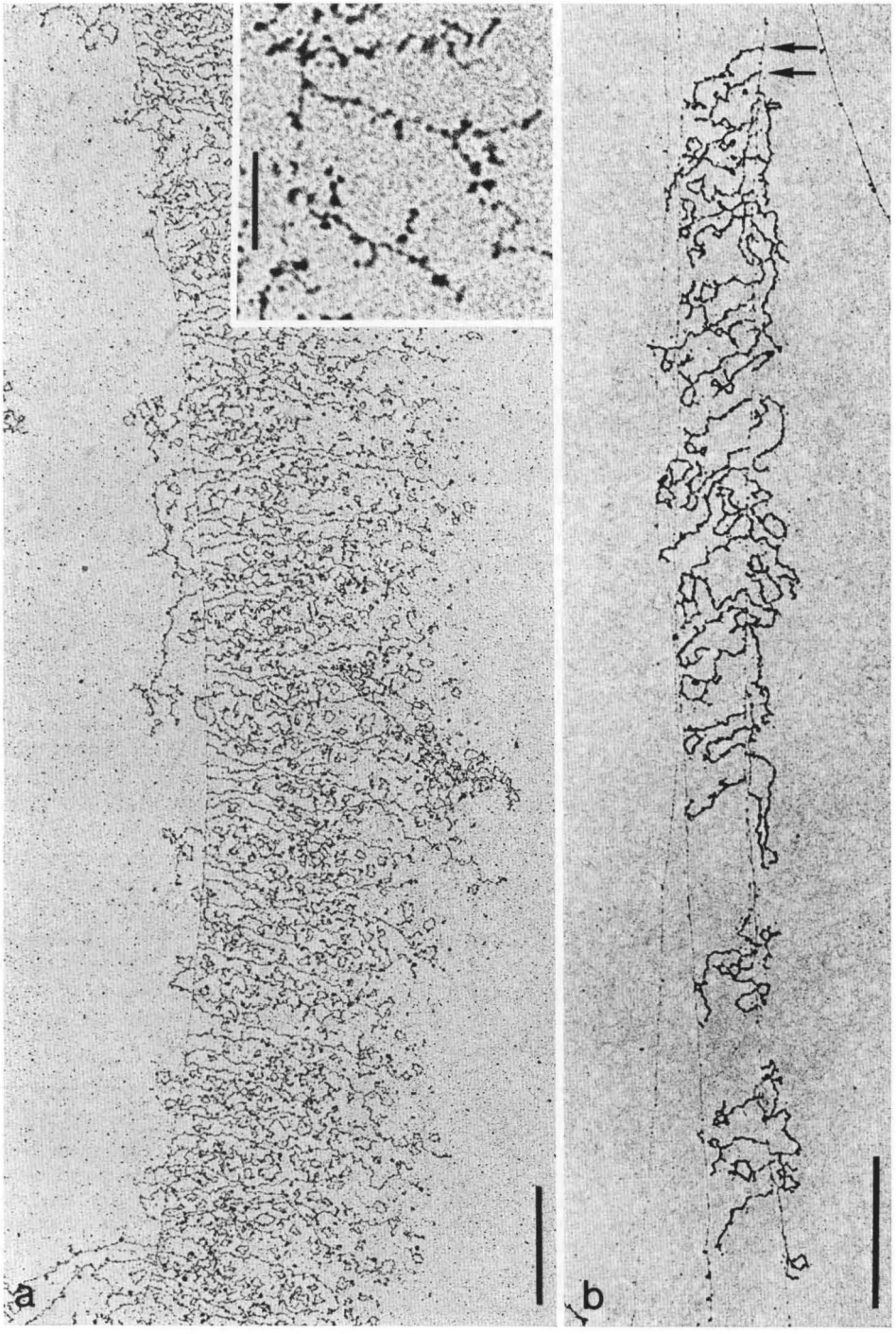
Figs. 4 a and b. Similar preparations of lampbrush chromosome loops of oocytes from *Pleurodeles waltlii* (**a**) and *Triturus cristatus* (**b**). These two micrographs represent different staining aspects (lateral fibrils in **a** appear more extended and thinner) of transcriptional units of slightly (**a**) or largely (**b**) reduced packing density of attached lateral fibrils (two are denoted by the arrowheads in **b**; for details see text). Note that the axial intercepts between adjacent growing fibrils, i. e. between adjacent functioning RNA polymerase molecules, are virtually free from granular substructures. Note also the absence of granular structures in fibril-free axes or axial intercepts. The **insert in Fig. a** presents details of the ultrastructure of a special type of "branched" lateral fibrils as revealed after swelling, spreading, and positive staining in lampbrush chromosome loops of *Triturus alpestris*. Note that granularities are identifiable not only at the bases of the lateral fibrils but also at their ends and elsewhere in the lateral fibrils. – **a.** 17 000 ×. – **Insert in a.** 73 000 ×. – **b.** 21 000 ×. – Scales 1 μm (**a** and **b**) and 0.2 μm (insert in **a**).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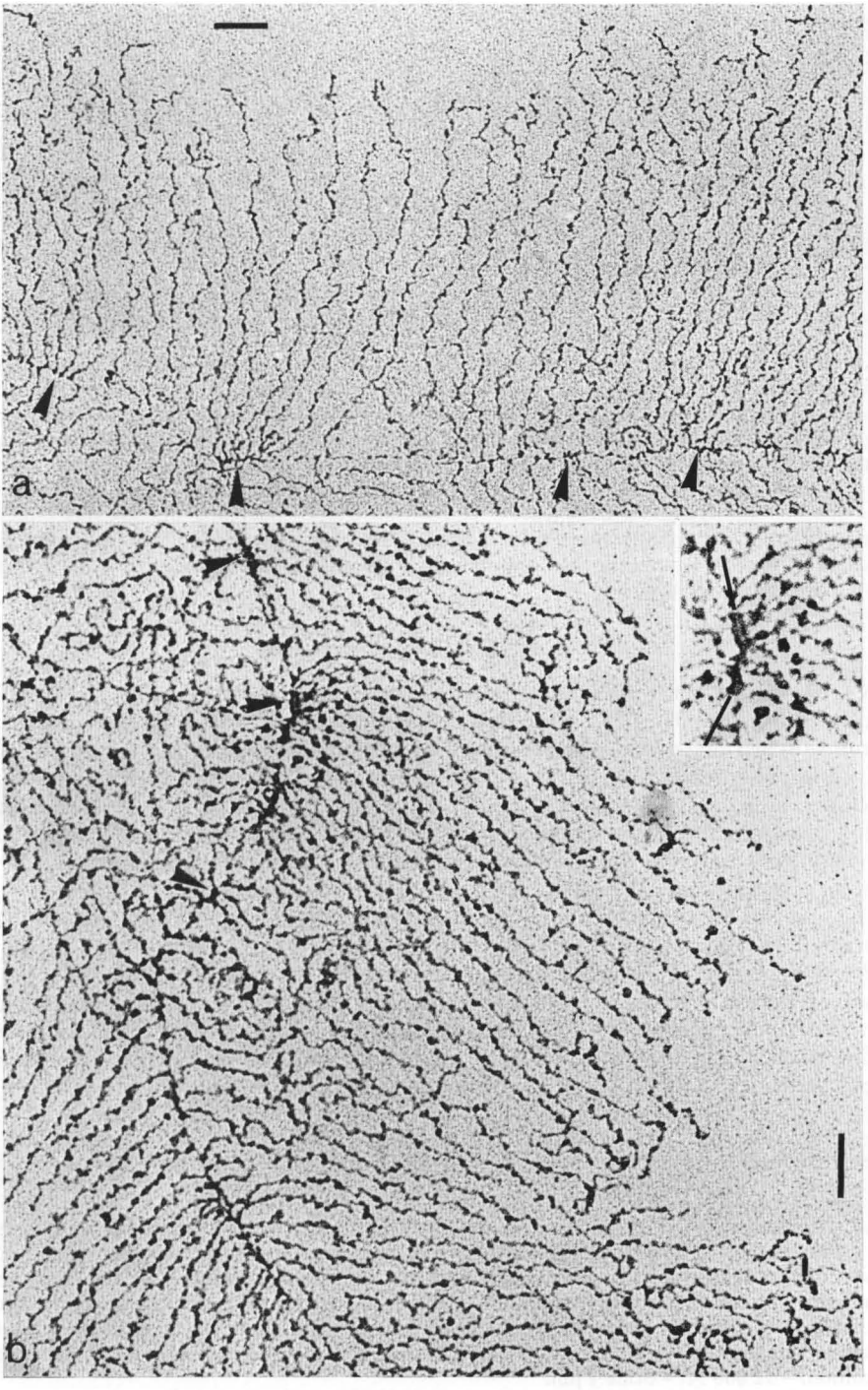
widely spaced transcriptional complexes within matrix units (Figs. 4 and 5 a). In some of our preparations as well as in some of the micrographs of other authors (e. g. Fig. 11 in [1]), we noted a certain granularity of fibril-free axial intercepts which, however, was a rather irregular and inconsistent feature. This coarse and variable granularity in axial intercepts was generally much more pronounced in shadow-cast preparations (see also below).

The type of matrix units of transcribed non-rDNA chromatin described above is somewhat different from the configurations of branched ("bush-like") lateral fibrils (e. g. insert in Fig. 4 a) arranged on chromatin axes at relatively large intervals without identifiable complete transcriptional units as described in spermatocytes from the fly, *Drosophila hydei* [21], and from the mouse ([29], cf also the preparations of HeLa cell chromatin described in [41]). Provided that these bush-like configurations do really contain nascent RNA molecules, our present interpretation would be at variance with that of Kierszenbaum and Tres [29] who interpreted the occurrence of small (6 to 9 nm) granules on such chromatin axes as indications of the presence of nucleosomes in partially transcribed chromatin strands.

Comparison of the length of repeating units of rDNA in isolated rDNA and in transcribed nucleolar chromatin

Genes coding for the common precursor molecules to 28 S, 18 S and 5.8 S rRNAs (as to the definitions used see [17]) of eukaryotic cells occur in chromosomal or extrachromosomal (amplified) clusters or in distinct, small, sometimes circular, extrachromosomal units (for refs. see [55]). These genes are arranged in repeating units each consisting of a region that is transcribed into an RNA molecule containing the pre-rRNA and an adjacent "spacer" region which does not contain sequences of the pre-rRNA. The length of the repeat unit of rDNA has been determined by cleavage with restriction nucleases in the two amphibian species *Xenopus laevis* and *X. muelleri* by Wellauer and colleagues [69, 70] and several, distinct size classes have been identified. The most frequent class was found to be of 7.3 million daltons molecular weight of double-stranded DNA, corresponding to a length of about 3.8 μm (see also [43]). Other minor size classes were found by these authors to be 3.6, 4.1, and 4.6 μm long. Such rDNA clusters, when maximally transcribed, appear in the form of morphologically characteristic repeating units that contain a fibril-covered intercept (matrix unit) and a usually fibril-free intercept, the "apparent spacer" (Figs. 6 to 15; see [1, 17, 21, 37, 39 to 42, 56, 58, 60, 62, 64 to 66]). We have analysed the nucleolar chromatin of *Xenopus laevis* oocytes in the electron microscope using the spreading technique (Fig. 6; cf [39]) and found size classes of repeating units of 3.3, 3.8, 4.3, and 4.6 μm length (an article on the detailed analysis of the morphology of transcriptional complexes of *Xenopus*

Figs. 5 a and b. Details of the structural organization in lampbrush-type chromosome loops of primary nuclei of the green alga, *Acetabularia major,* as revealed at higher magnification after dispersion, spreading and positive staining. Note that the density of the lateral fibrils on the transcribed chromatin axis sometimes reveals local differences (**a**) and that in some regions the basal granules of these fibrils are present in almost close packing (arrowheads). The **insert in Fig. b** illustrates a negative staining aspect of the thin chromatin axis of the transcriptional unit shown in **Fig. b.** – **a.** 37 500 ×. – **b.** 45 000 ×. – **Insert** 74 000 ×. – Scales 0.2 μm.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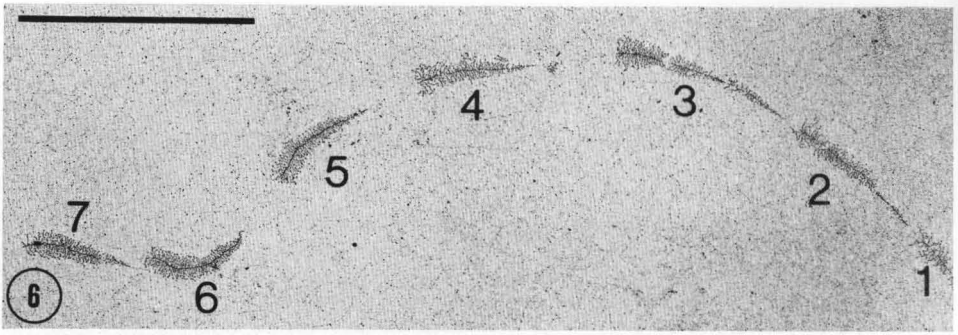
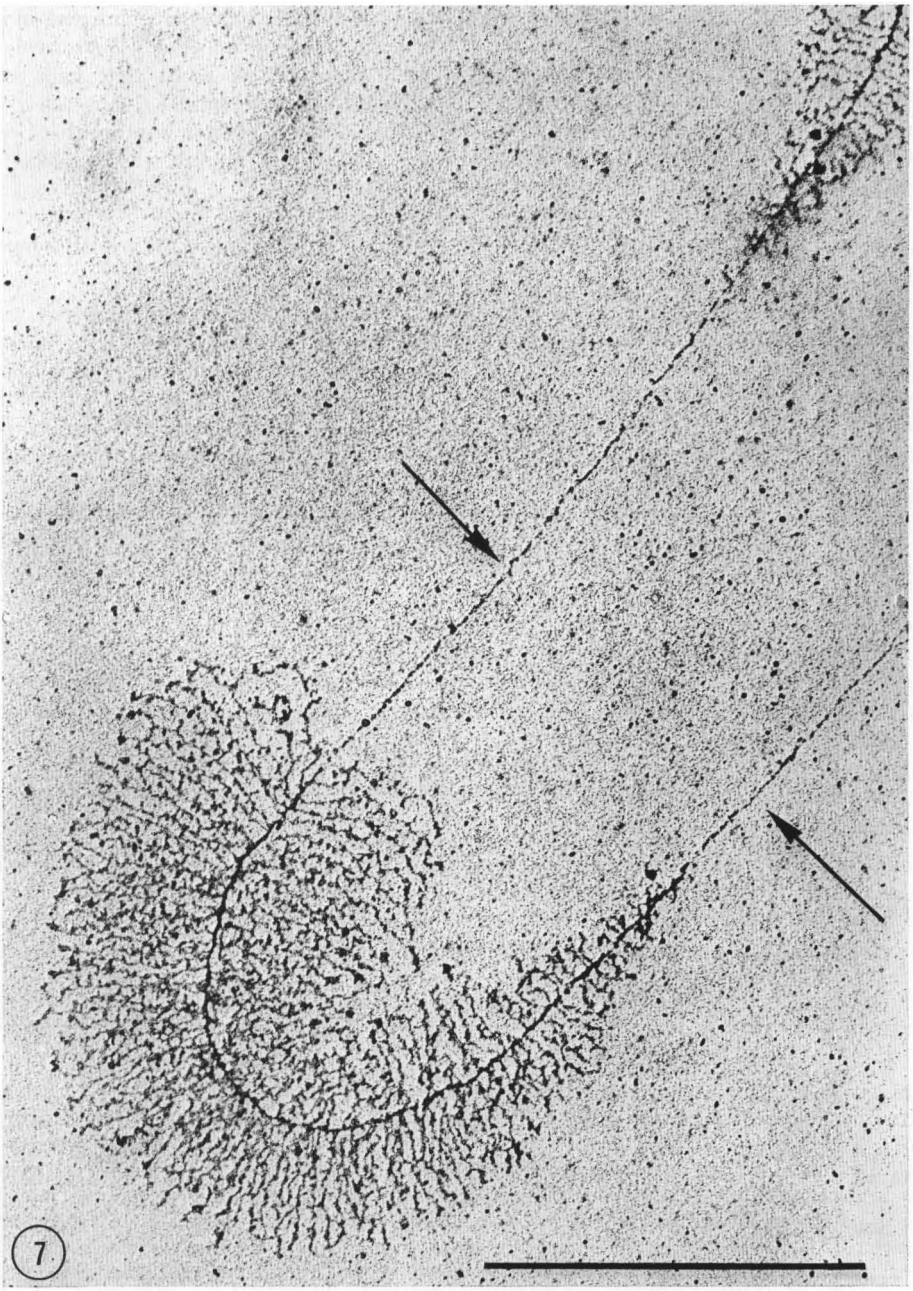


Fig. 6. Typical arrangement of transcriptional units of rDNA as revealed in nucleolar chromatin isolated from oocytes of the clawed toad *Xenopus laevis*, as revealed after swelling, spreading, and positive staining. These unigenic transcriptional units appear in the form of polar arrangements of repeating units each consisting of a transcribed region covered with lateral fibrils (matrix unit; the individual matrix units are numbered in sequence) and an adjacent region (apparent rDNA spacer) that is either free from lateral fibrils or may reveal some specific transcriptional complexes ("spacer transcripts", including the so-called "prelude complexes"; compare [56]). Note that the length of the repeating unit of the transcribed rDNA is in the same range as that determined in isolated rDNA by cleavage with restriction enzyme (for details see text). – 6200 ×. – Scale 5 μm.

rDNA by Scheer, Trendelenburg, and Franke is in preparation). This shows that a specific double-stranded DNA piece of a defined length and function is not significantly shortened when present in a fully transcribed chromatin strand. Since nucleosomes are defined as structures of a high DNA packing ratio (reported values of cm of DNA per cm chromatin strand range from 5 : 1 to 7 : 1; see [13, 20, 23, 49, 72]) our measurements exclude the occurrence of a significant number of nucleosomes in the transcribed rDNA chromatin, not only for the fibril bearing matrix (gene) region itself but also for the adjacent apparent spacer intercepts.

This conclusion seems to be in contrast to the report by Higashinakagawa and Reeder [26] who found distinct DNA fragments of about nucleosomal size upon nuclease digestion of nucleolar chromatin from immature *Xenopus laevis* oocytes. However, the early stages of oogenesis which were used by these authors are not fully active in rDNA transcription [58]. Therefore, their findings could well refer to inactive nucleolar chromatin (for morphological descriptions of inactive nucleolar chromatin in spread preparations see [57, 58]). Our finding of extended double-stranded DNA in the

Fig. 7. Similar nucleolar preparation as described in the previous figure from an oocyte of the crested newt, *Triturus cristatus*. The typical repeating units of maximal lateral fibril density of the matrix units might indicate high transcriptional activity. Note the background granularity of stained particles ("spottiness") which is variably revealed in such preparations. The irregularly "knobby appearance" of the 6 to 9 nm thick chromatin axis in the fibril-free sections ("apparent spacers"; arrows) is insignificant and may be the result of irregular stain deposition. – 52 000 ×. – Scale 1 μm.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nucleolar chromatin during transcription seems also to be at variance with the interpretations by Piper et al. [51], who, in the macronucleus of the ciliate, *Tetrahymena pyriformis*, found nucleosome-like sizes of nuclease-protected DNA that had been labelled during a nutritional shift up, i. e. a period of preferential, if not selective, synthesis of rDNA (see also [34]). Again, however, it is not known how much of this rDNA was actually transcribed in the cells from which the chromatin was isolated (for detailed discussion see [34]).

Comparison of the lengths of pre-rRNA molecules to the lengths of the transcriptional units of rDNA

When the sizes of the first stable products of rDNA transcription are compared with the mean lengths of the specific fibril-covered regions, the matrix units, a marked correspondence is found in some cell systems and organisms such as in amphibian oocytes (cf [56]), in spermatocytes of the fly *Drosophila hydei* [21, 37], and in the primary nuclei of the green alga *Acetabularia* [60, 62]. In the latter cell system, the size of the pre-rRNA molecules is only slightly less than the gene content of the whole repeating unit in the most predominant type of rDNA pattern (type I a [62]). In no cell system has a clear case been reported in which the pre-rRNA exceeds the size of the matrix unit or even the repeating unit. On the other hand, there are examples of matrix unit lengths that exceed the sizes of the pre-rRNA molecules by about 50 % as in the oocytes of the house cricket *Acheta domesticus* [65], or c. 30 % as in the water beetle *Dytiscus marginalis* ([64], Trendelenburg, Franke, Scheer, and Zentgraf, unpublished data). These observations of sizes of gene products of rDNA that correspond to, or are even smaller than both, the matrix units and the repeating units, provide further support to the concept that in the transcribed gene region the DNA is present in an extended form.

Absence of nucleosomes in matrix units and apparent spacer units of transcribed nucleolar chromatin

Figures 7 to 15 show the morphology of transcriptionally active nucleolar chromatin isolated from oocytes of various amphibia (Figs. 7 to 13) and from vegetative *Acetabularia* cells (Figs. 14 and 15) as revealed after the different electron microscopic procedures described under methods. The specific aspect of the appearance of the matrix

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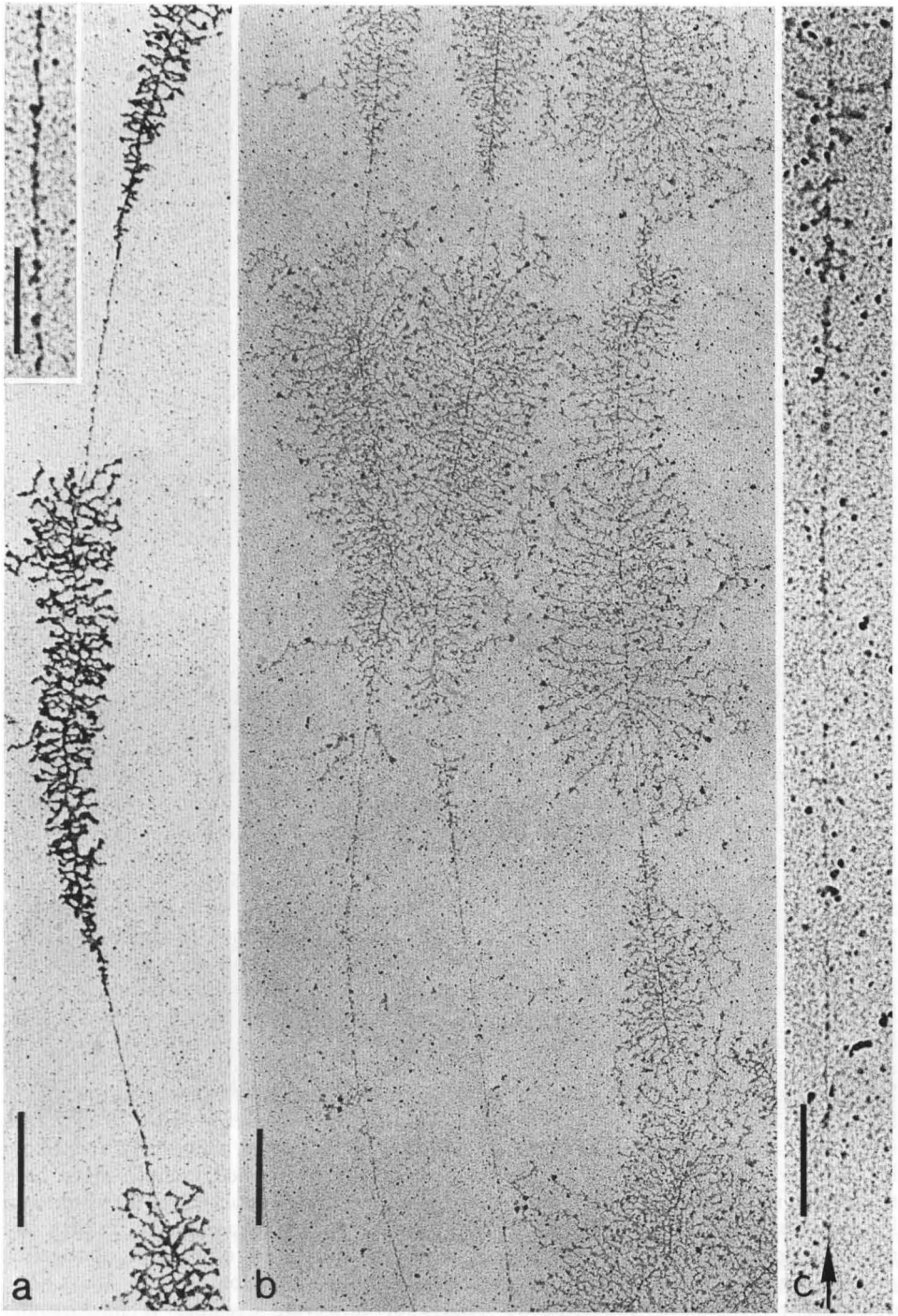
Figs. 8 a to c. Two extreme aspects of the appearance of nucleolar (rDNA) transcriptional units from amphibian oocytes as revealed after limited (**a**, *Triturus cristatus*) or extensive (**b** and **c**, *Pleurodeles waltlii*) dispersion and swelling in low salt medium. **Fig. a** presents a relatively condensed state (the **insert** shows the knobby appearance of the rDNA chromatin axis in the apparent spacer region) whereas **Fig. b** displays the maximally extended structure. The apparent width of the free chromatin axis is 5.5 to 7.5 nm in both preparations, the diameter of the knob structures varies from about 9 to 25 nm. Note that the mean length of the matrix units and the apparent spacer intercepts are not considerably different among the preparations of a different degree of dispersion. **Figs. b** and **c** have been selected in order to illustrate that in such fully extended, transcribed nucleolar chromatin, the axial chromatin strands can be traced readily even in the presence of a high density of background stain deposits (arrow in **c**). – **a.** 32 000 ×. – Scale 0.5 μm. – **Insert** 75 000 ×. – Scale 0.2 μm. – **b.** 27 000 ×. – Scale 0.5 μm. – **c.** 78 000 ×. – Scale 0.2 μm.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units varied somewhat with the degree of dispersion of the sample and was usually classified in terms of the degree of the extension and distinct visibility of the lateral fibrils (Figs. 8 and 14; cf [56]). In fully spread, fully fibril-covered rDNA genes (e. g. Figs. 7, 11 a, 14, 15), the packing density of the basal granules (13 ± 1.6 nm diameter) of the lateral fibrils was very close and regions of maximal packing density were frequently revealed (center-to-center spacings of 15 to 25 nm; e. g. Figs. 7 to 9, 11, 12, 14, 15; cf also [1, 37, 39 to 41, 56, 58, 60]). Assuming again diameters of the RNA polymerase molecules (here of A type; for nomenclature see [8]) of about 12.5 nm (cf also [39 to 41]), it is clear that there would be no space left for nucleosomal granules within such arrays.

When we examined small lateral fibril-free regions ("gaps"; cf [56]) within such densely fibril-covered matrix units (e. g. Figs. 8 b, 11 b, 12; cf also Fig. 6 in [37]) we could readily identify the very thin axis (widths from 4 to 8.5 nm). In some preparations (e. g. Fig. 18 a), this axis appeared so thin that it could hardly be traced. This shows that such small "intramatrical" intercepts which are not associated with transcriptional complexes are also not organized into nucleosomes.

As to the morphology of the fibril-free apparent spacer intercepts that are regularly interspersed between the matrix units, we again recognized the thin chromatin axis with basal widths of 4 to 8 nm (Figs. 7 to 11, 14). Depending, however, on the specific preparation, one also sometimes notes a certain granularity ("knobby appearance") with granule diameters usually ranging from 6 to 12 nm and, occasionally, some isolated larger particles (up to 25 nm size). This "knobby appearance" was especially frequent in positively stained preparations that were characterized by a relatively high background of stain deposit granules on the supporting film (e. g. Figs. 7, 8 b, c) and was well distinguished from the typical nucleosomal "beads on a string" pattern afore described, even under conditions in which the nucleosomal organization was readily seen in parallel preparations of chromatin from the somatic cell types mentioned above (Figs. 1, 2). Only in a few preparations we noted a pronounced regular granularity in apparent spacer regions similar to that described by Miller and Beatty ([40], their Fig. 7; cf also Fig. 19 in [60]), but this was much more frequent in partly inactive chromatin (see below). Negatively stained preparations (Fig. 9) and preparations on positively charged films (Fig. 10) according to Dubochet et al. [11] also did not reveal the regular presence of nucleosome-type beads in the apparent spacer intercepts (for the appearance of condensed chromatin with the latter technique cf [49]). More frequently we noted distinct, somewhat irregularly spaced granules attached to apparent spacer axes (Figs. 11, 12, 14, 15; cf [58]), similar in appearance to those mentioned by other authors (e. g. [1, 37, 40]). The frequency and density of such spacer-attached granules, however, was highly variable from one preparation to another as well as among different chromatin strands and even in different intercepts on the same strand. They were usually separated by larger mean distances than the granules within the matrix units but revealed a tendency to be arranged in groups (e. g. Fig. 15). Two size classes could be distinguished. One relatively rare type of granule had diameters from 20 to 30 nm (Fig. 11 b), another, much more common type of granule ranged between 12 and 18 nm in diameter (Figs. 11, 12, 14, 15). Such apparent spacer-attached granules have been tentatively interpreted either as RNA polymerase molecules attached to the chromatin but not transcribing (e. g. [37, 40, 58, 60]), as RNA polymerase molecules that are transcribing but are associated with only very small amounts of nascent ribonucleoprotein material (see [17]), or as nucleosomes (for discussion cf also [53]). The quantitative evaluation presented in Figure 16, however, excludes a nucleosomal

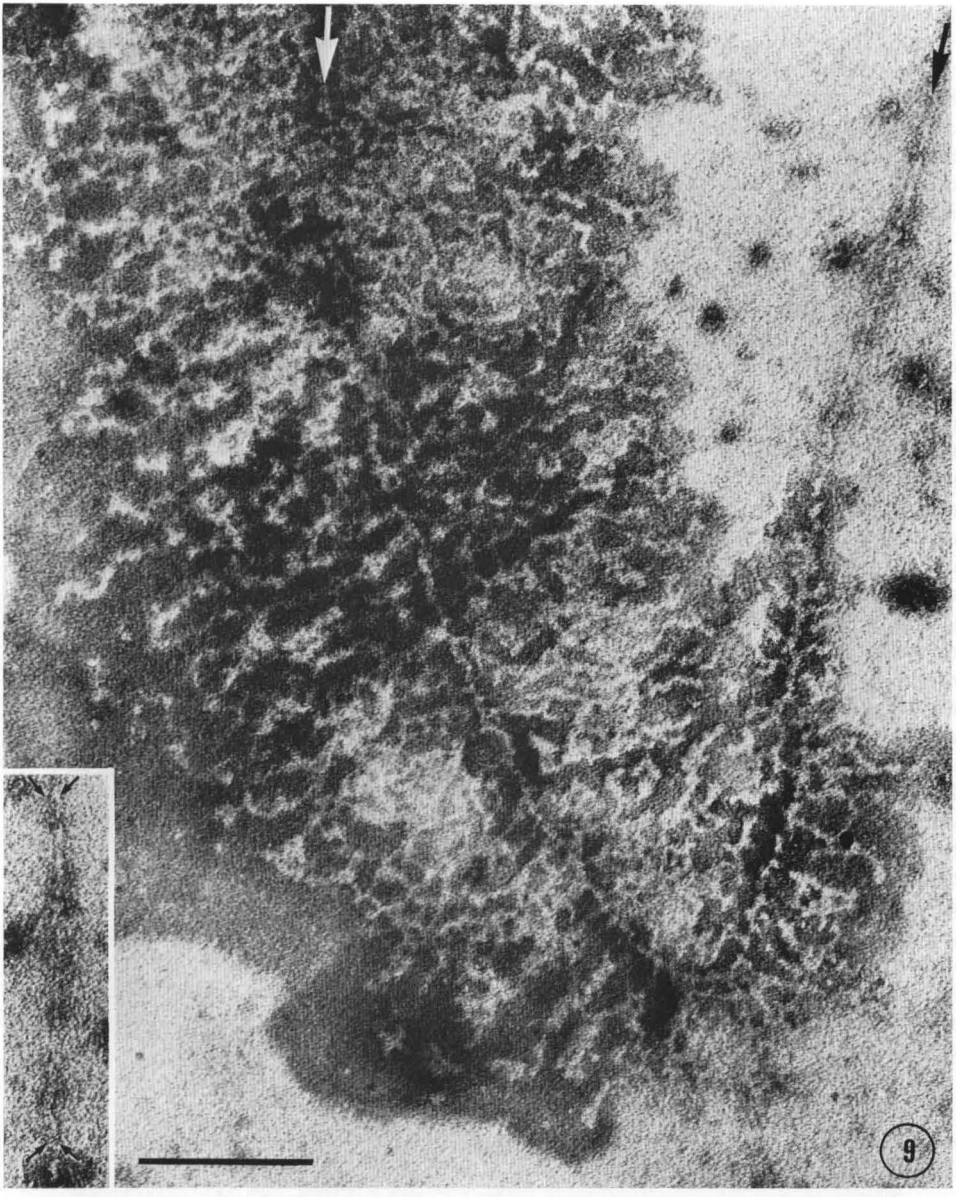


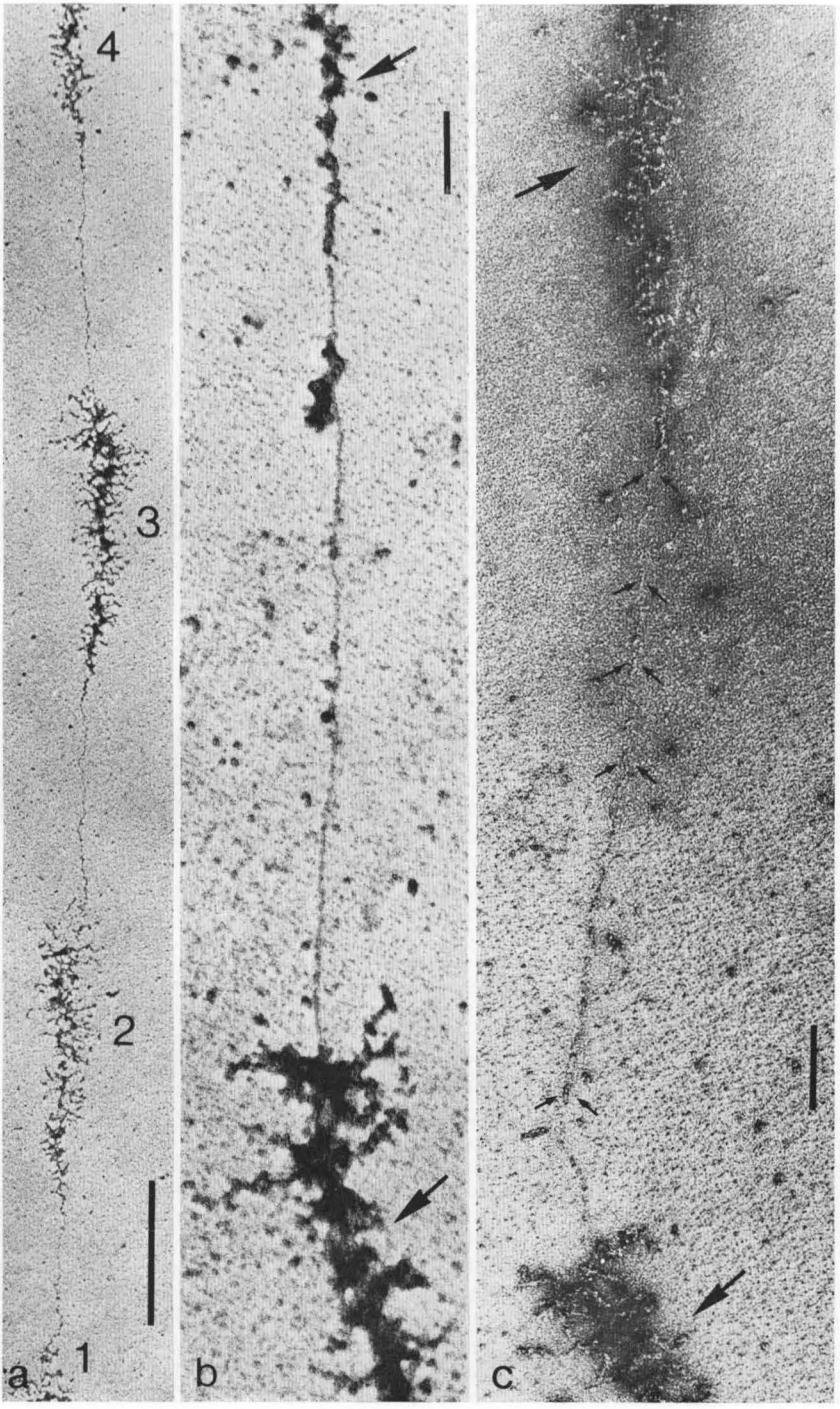
Fig. 9. Transcriptional unit of rDNA of a nucleolus from oocytes of *Triturus cristatus* as revealed after spreading and negative staining (see Methods). Note the rather uniform width of the chromatin axis (denoted by the arrows) which is 6 to 8 nm thick (shown with selectively higher contrast in the insert). – 115 000 ×. – Scale 0.2 μm.

character of such spacer-attached, granular particles. Increased numbers of granules with diameters larger than 8 nm in apparent spacer intercepts did not result in a corresponding foreshortening of the whole spacer length as one would have to expect from the condensing by the nucleosomal packing of a given piece of DNA (the expected correlation shown in Fig. 16 has been calculated assuming a condensing and shortening factor of 5.35 as taken from [49]). Even when only particles larger than 10 nm were considered in such evaluations, no inverse correlation of particle number and spacer length was found.

Absence of nucleosomes in individual untranscribed chromatin regions containing genes for pre-RNA

Sometimes we identified individual matrix units with very few lateral fibrils or completely fibril-free intercepts corresponding to the position of untranscribed pre-rRNA genes within a sequence of fibril-covered matrix units in nucleolar chromatin (e. g. Figs. 11 b and 13). These were especially frequent in stages of reduced transcriptional activity (cf [57, 58], see also [1, 37]). When we compared the mean length of such "naked" spacer-gene-spacer units with those of the corresponding, adjacent units that contained fully fibril-covered matrix units (micrographs not shown here), we found neither a significant shortening (limit of confidence 7 % of spacer-gene-spacer length) nor a considerable extension (12 % limit of confidence). This indicates that the structural changes of the conformation of the DNA that take place during transcription (for refs. see [3]) do not result in significant change in the length of the transcribed regions. In view of the above mentioned correspondence of the length of the rDNA repeating unit in transcribed nucleolar chromatin with that determined in isolated rDNA, this suggests that nucleosomes are likewise not present in non-transcribed rDNA chromatin regions which are located between transcribed regions, in concurrence with our observations on the absence of nucleosome-like structures in such intercepts (cf [58]). In some instances we identified relatively extended regions of chromatin axes with a nucleosomal, i. e. beaded, appearance. However, such observations were almost exclusive to nucleolar chromatin from cell stages characterized by a predominance of transcriptionally inactive rDNA as, for example, in amphibian oocytes from early previtellogenic and mature stages (Fig. 13; cf [58]) or after inhibition of transcription in vitellogenic oocytes with actinomycin D (cf [57]). Occasionally, we were able to trace an individual chromatin strand from a thin and smooth character in a matrix unit bearing intercept into a fibril-free, beaded intercept. These observations would be compatible with the notion of the appearance of a nucleosomal organization during inactivation of nucleolar chromatin.

Figs. 10 a to c. Appearance of transcriptional units of rDNA of nucleoli from *Triturus cristatus* oocytes as revealed after preparation on positively charged films [for details see the contrasting procedure (*d*) described in Methods]. **Fig. a** presents a survey showing four transcriptional units (numbered in sequence). **Fig. b** and **c** show both the positively (**b**) and negatively (**c**) stained aspects which are observed in such preparations (arrows denote matrix units). The chromatin axis, which is marked by small arrowheads in the apparent spacer intercept shown in **c**, is 5 to 7 nm thick. – **a.** 19 000 ×. – Scale 1 μm. – **b** and **c.** 54 000 ×. – Scales 0.2 μm.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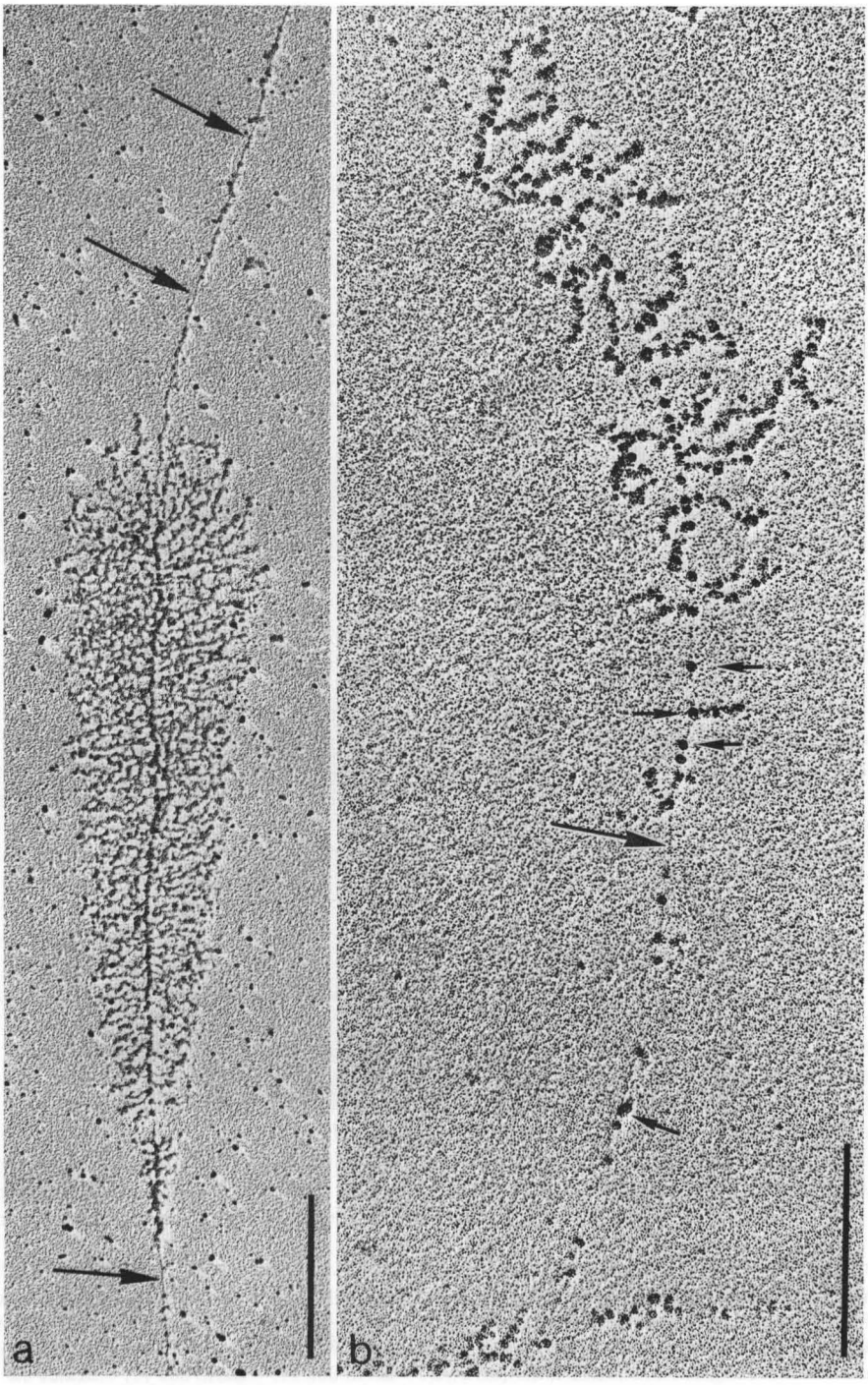
Persistence of the attachment of the 12 to 18 nm granules to chromatin strands after treatment with Sarkosyl

Recent biochemical work has shown that transcriptional complexes, i. e. the RNA polymerases plus the nascent RNAs, remain attached and functionally intact during treatment with the detergent, Sarkosyl NL–30 ([10, 19]; for details see Methods) whereas the bulk of the other chromatin proteins is removed. We have treated the transcriptionally active chromatin material of nuclei isolated from the amphibian oocytes and from *Acetabularia* primary nuclei with concentrations of this detergent which were effective in progressively disintegrating the beaded chain (nucleosome) arrangement of the hen erythrocyte chromatin shown in Figure 2. Under such conditions, the RNA polymerase containing granules that are recognized especially clearly in units with reduced lateral fibril densities (e. g. Fig. 18 a), remained attached to the chromatin axis (Figs. 17 and 18). Such persistent granules were recognized in both the regions of the matrix units and in the apparent spacer intercepts (Figs. 17 and 18). After this treatment, the basal granules of the lateral fibrils and the spacer-attached granules appeared rather dense and distinct whereas the lateral fibrils showed some reduction in stainability. The chromatin axis itself appeared uniformly thin with diameters from 3 to 7 nm (Fig. 18). This persistence of the axis-attached granules after Sarkosyl treatment supports the interpretation that the granules within apparent spacer regions were similar, if not identical, to the basal granules of the transcriptional complexes and therefore represented RNA polymerase molecules or transcriptional complexes (for discussion see above) and not nucleosomes.

Occurrence of granular structures in lateral fibrils containing nascent RNA

Granular structures in transcriptionally active chromatin were found not only on the chromatin axis but also in the lateral fibrils. This was especially apparent after incomplete spreading and in the special lampbrush loop-like chromosomal configurations with branched lateral elements somewhat resembling the bush-like structures mentioned above (cf [21, 29]). Except for the granules at the distal ends of the lateral fibrils (insert in Fig. 4 a; cf [39 to 42]) which have diameters ranging from about 14 nm (e. g., in *Acetabularia;* cf [17, 60, 66]) to ca. 30 nm (as in some insect oocytes and spermatocytes;

Figs. 11 a and b. Transcriptional matrix units of rDNA in nucleoli from a maturing oocyte of *Triturus alpestris* (**b**) and a vitellogenic oocyte of *Triturus cristatus* (**a**) at maximal (**a**) and reduced (**b**) lateral fibril density as revealed after spreading, positive staining and subsequent metal shadowing (see Methods). The reduced lateral fibril density (**b**) indicates a reduced transcriptional activity (for discussion see text). Note the high packing density of particles at the bases of the lateral fibrils in **Fig. 12 a.** This high density results in an almost uniform thickening of the matrix unit intercept of the chromatin axis, in contrast to the distinctiveness of the individual basal particles in the "diluted" matrix unit shown in **Fig. 12 b** (some basal granules are denoted by arrowheads). The basal width of the chromatin axis in fibril and particle free regions (examples are denoted by arrows) appears in such preparations as 4.5 to 7 nm. Note also that among the axis-attached particles, two size classes are apparent. One category is of 12 to 18 nm granules and the other is 20 to 30 nm granules; the latter may include some nascent material (an example of the comparison of these two size classes is denoted by the arrowhead in the bottom of **b**). – **a.** 44 000 ×. – **b.** 57 000 ×. – Scales 0.5 μm.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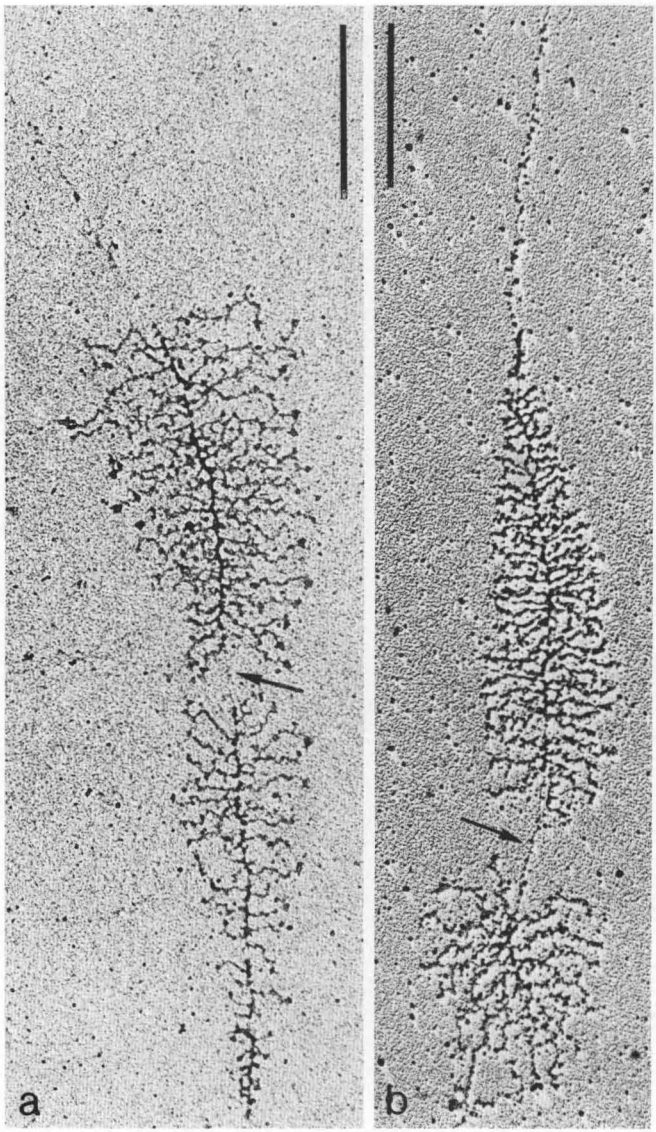

Figs. 12 a and b. Selected examples of transcriptional units of rDNA from ooccytes of *Xenopus laevis* (**a**) and *Triturus cristatus* (**b**) as revealed after positive staining (**a**) or metal shadowing (**b**) in order to demonstrate the thinness (4.5 to 8.5 nm) of the chromatin axis in small, fibril-free regions of matrix units (arrows). – **a.** 46 000 ×. – **b.** 43 000 ×. – Scales 0.5 μm.

cf [17, 37, 65]), we sometimes recognized granular formations (10 to 20 nm) within the lateral elements themselves which in some situations show a regular array (insert in Fig. 4 a; cf also [1]). This illustrates that "beads on a chain" aspects are not *per se* indicative of a nucleosomal chromatin structure but may also occur in ribonucleoproteinaceous material.

Concluding remarks

Our observations of nucleosomes in hen erythrocytes, rat hepatocytes and cultured murine sarcoma cells, in confirmation of the numerous demonstrations of these structures in other cells (for refs. see Introduction), support the idea that the bulk of the transcriptionally inactive chromatin in nuclei as well as in metaphase chromosomes (cf [53]) is arranged in nucleosomes. On the other hand, our observations in specific transcriptionally active chromosomal regions such as lampbrush chromosome loops and extrachromosomal genes such as amplified nucleoli, strongly suggest that the DNA of these species of chromatin is not packaged into nucleosomes (for related biochemical data see [2, 22]; for discussion see also [53]). Consequently, a transition of the organization of chromatin strands from the nucleosomal into the extended form should take place during the activation of genome parts that have been transcriptionally inactive for relatively long time spans (long-term inactive chromatin). The present data further indicate that not only transcribed gene regions but also intergenic spacer intercepts which contain sequences that are either never transcribed or are not read out at this specific moment are not packaged as nucleosomes. Moreover, gene regions that are untranscribed but interspersed between transcribed units, as well as genes which have been inactive for relatively short times (short-term inactive chromatin) are also not packaged as nucleosomes. This leads to the hypothesis that the transition from the nucleosomal to the extended chromatin state does not exclusively involve the specific "activated" gene region but larger chromatin portions and include adjacent untranscribed intercepts as well. In structural terms, we therefore propose to distinguish between (1) transcriptionally *inactive chromatin*, i. e. chromatin that is inactive for relatively long times and is organized in nucleosomes and (2) transcriptionally *active chromatin* which is not organized in nucleosomes and includes (*a*) the transcribed regions, (*b*) untranscribed regions located between transcriptional units ("spacer intercepts"), (*c*) untranscribed regions located between transcribed regions of the some transcriptional unit, (*d*) untranscribed whole transcriptional units located between transcribed ones of the same kind and (*e*) newly inactivated chromatin regions.

Of course, our data cannot rule out the possible occurrence of a few, occasional nucleosomes within such regions of generally extended, "active" chromatin but they clearly show that most of the DNA in this chromatin must be associated with proteins and RNA in other, non-nucleosomal forms. Obviously transcriptionally active chromatin should contain, besides perhaps of its specific histone pattern and arrangement, a relatively large amount of non-histone proteins, including those associated with the specific nascent RNAs (as to the protein patterns of nucleoli and lampbrush chromosomes in vitellogenic stages of amphibian oogenesis see also [35]).

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Note added in proof. Three recent reports have described experiments using molecular hybridization techniques in order to determine the amount of transcribed DNA sequences contained in nucleosomes. While two of them (Pospelov, V. A., A. A. Sokolenko, and G. L. Dianov: Investigation of DNA bound to histones in chromatin. Molekulyarnaya Biologiya **9**, 691–698, 1975; Kuo, M. T., C. G. Sahasrabuddhe, and G. F. Saunders: Presence of messenger specifying sequences in the DNA of chromatin subunits. Proc. Nat. Acad. Sci. USA **73**, 1572–1575, 1976) favour the notion that transcriptionally active DNA is packed into nucleosomes, the study by R. Reeves and A. Jones (Genomic transcriptional activity and the structure of chromatin. Nature **260**, 495–500, 1976) demonstrates "that there are fewer ribosomal gene sequences protected from nuclease digestion in the chromatin of transcriptionally active cells than in chromatin that is not active in synthesis of rRNA". It has to be stated, however, that the number of transcriptionally active genes and the specific degree of activity is unknown in the cells used in these studies.

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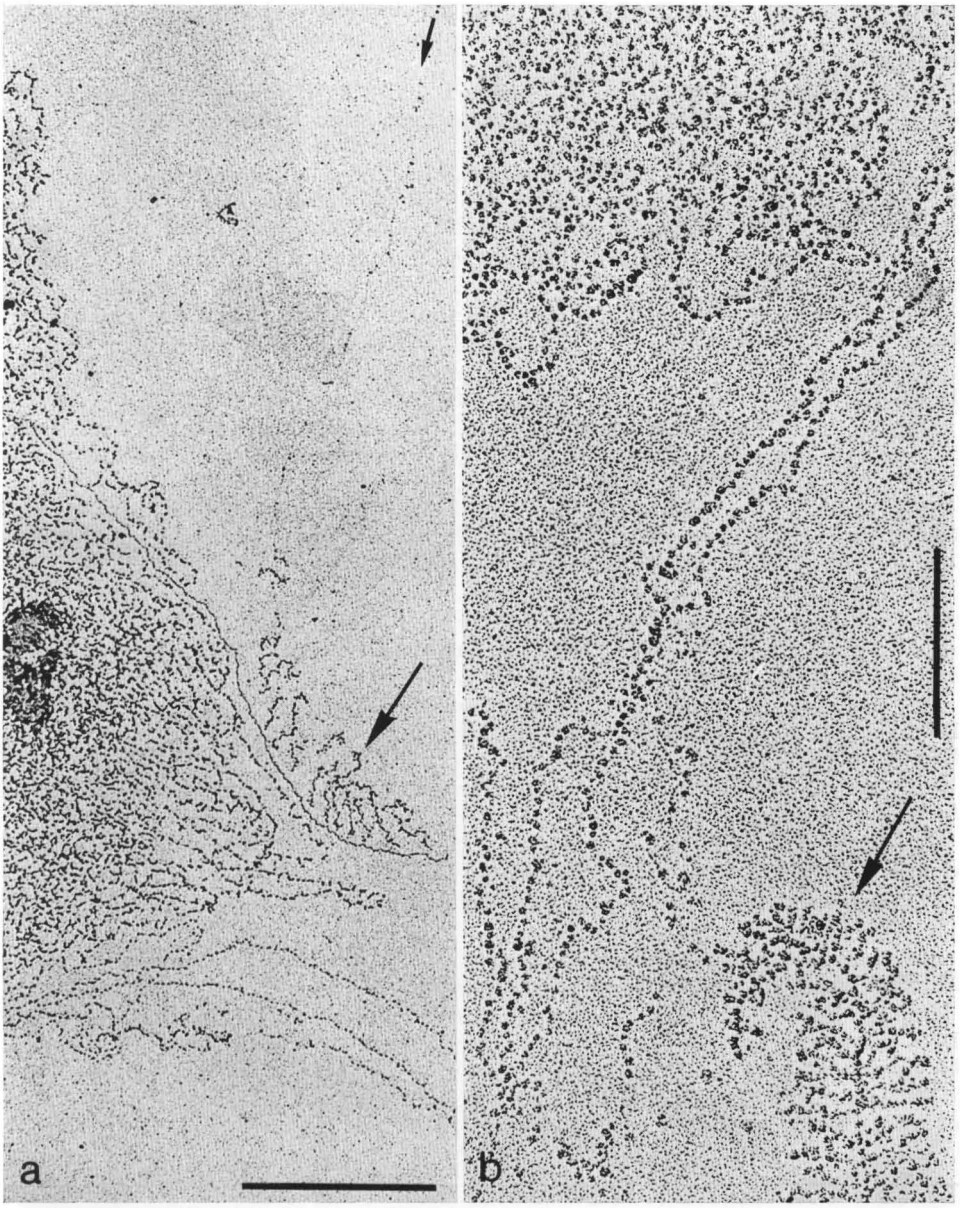
Figs. 13 a and b. Spread preparation of nucleolar chromatin from mature oocytes of the alpine newt, *Triturus alpestris*, as revealed after positive staining before (**a**) and after (**b**) metal shadowing. The nucleoli of this stage are characterized by a drastically reduced transcriptional activity (for details see text). Only a few matrix units with either high (arrow in **b**) or reduced (arrow in the lower right of **a**) fibril densities are present. One frequently gains the impression of a more pronounced granularity of the fibril-free, i. e. transcriptionally inactive, chromatin strands or chromatin strand intercepts (granule sizes from 12 to 20 nm under these conditions). This may indicate the appearance of nucleosome-like structures in such inactive nucleolar chromatin. The arrowhead in the upper right corner of **Fig. 14 a** points to an axis which reveals a long untranscribed intercept, followed by a matrix unit with reduced fibril density (arrow), then another untranscribed section, and finally immerges into tangles of nucleolar chromatin strands with a distinctly beaded appearance. – **a.** 26 000 ×. – Scale 1 μm. – **b.** 50 000 ×. – Scale 0.5 μm.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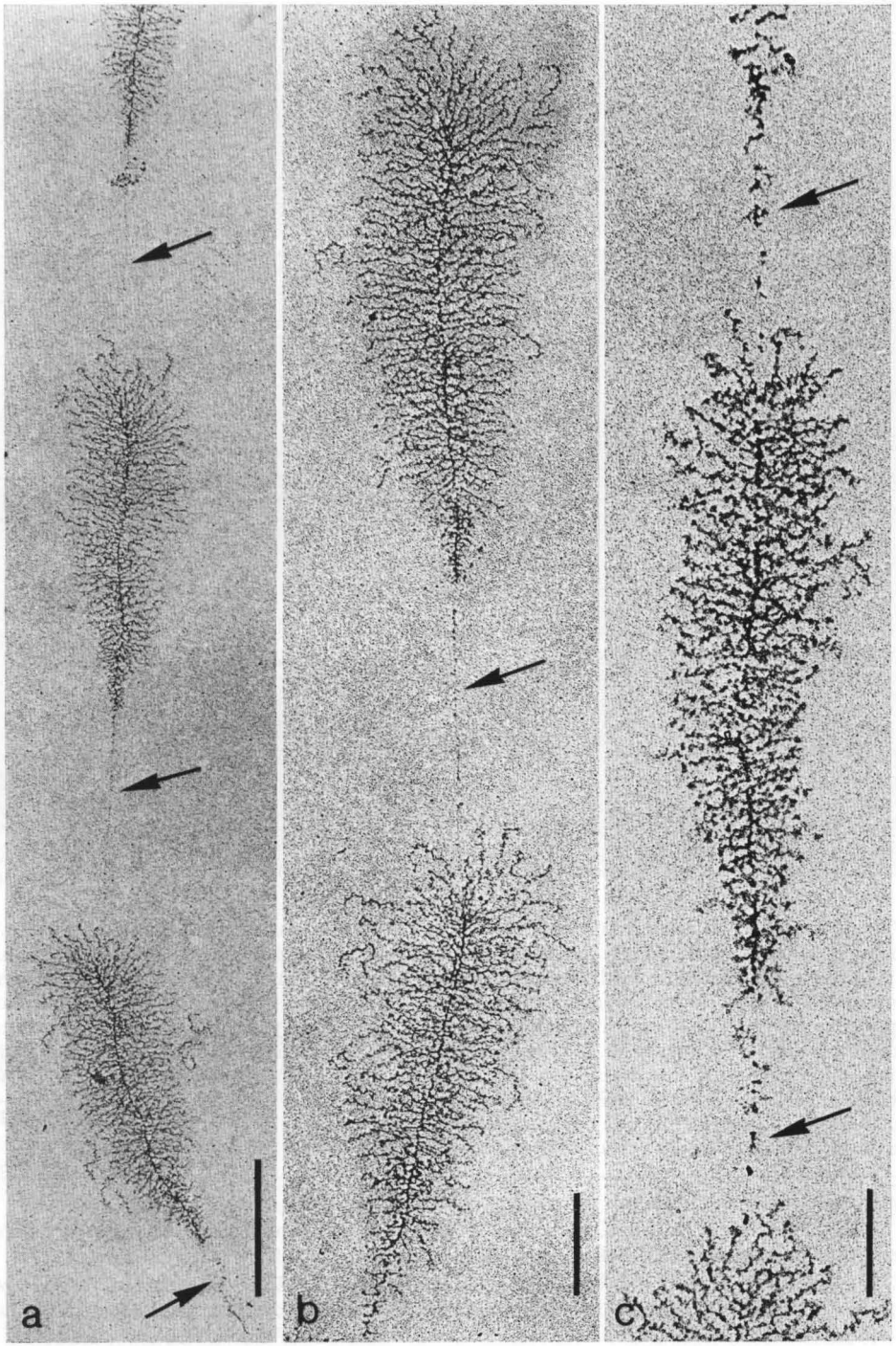
Figs. 14 a to c. Transcriptional units of rDNA of nucleoli isolated from primary nuclei of the algae *Acetabularia cliftonii* (**a** and **b**) and *A. mediterranea* (**c**). In maximally (**a, b**) as well as in moderately (**c**) spread preparations, the nucleolar chromatin axis is usually very thin (5 to 8.5 nm) as is especially conspicuous in apparent spacer regions (arrows). In the matrix units, the axis appears usually thickened, due to the close spacing of the lateral fibrils and their basal knobs, respectively. Granular particles are occasionally seen in the apparent spacer intercepts (**c**). – **a.** 19 000 ×. – Scale 1 μm. – **b.** 28 500 ×. – **c.** 49 000 ×. – Scales 0.5 μm.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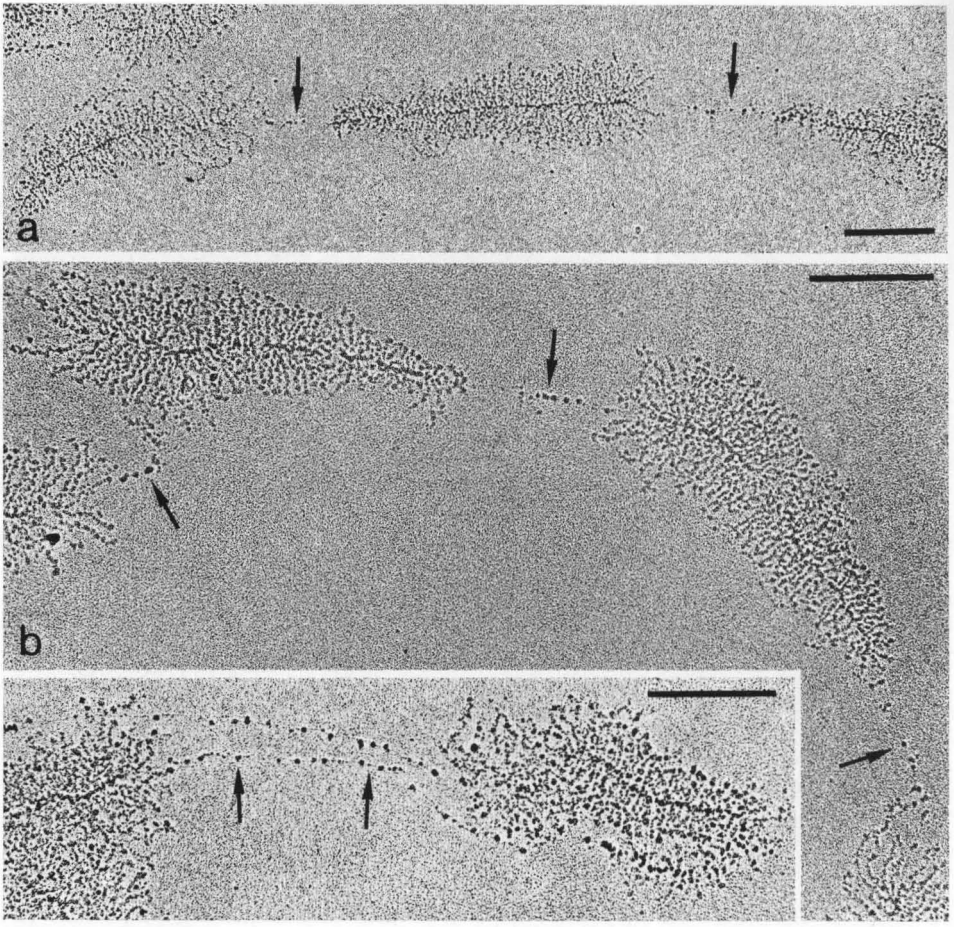

Figs. 15 a and b. Selected examples of spread and positively stained preparations of nucleolar material isolated from primary nuclei of *Acetabularia mediterranea* as revealed after metal shadowing. This procedure facilitates the detection of granules attached to the apparent spacer intercepts of the nucleolar chromatin axes (arrows). Note some tendency of these particles to appear in groups. The **Figs. a** and **b** show examples of a rDNA pattern type with short spacer regions, whereas the **insert in b** presents an example from a rDNA chromatin strand with long spacer intercepts (for details and refs. see text). Note again the two size classes of axis-attached particles and the thinness of the axes themselves (5 to 7 nm). – **a.** 24 000 ×. – **b.** 33 000 ×. – **Insert** 34 000 ×. – Scale 0.5 μm.

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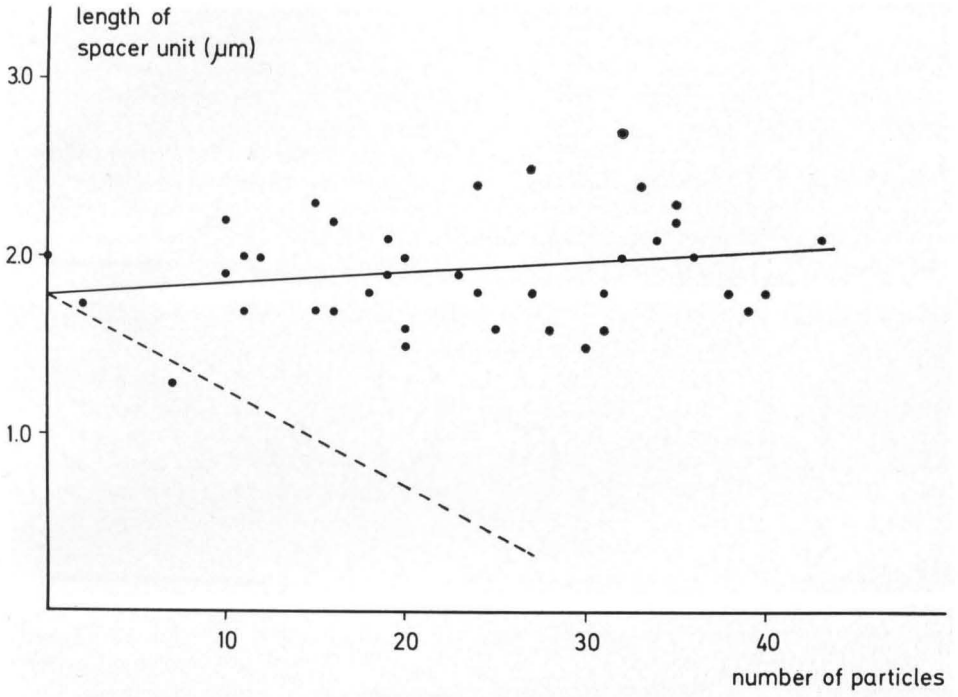

Fig. 16. The number of granular particles larger than 8 nm that are recognized in the chromatin axis of an apparent spacer intercept versus the length of the specific spacer unit. The values are cumulated data from transcriptional units of rDNA of oocytes of *Triturus alpestris* and *T. cristatus*. The solid line represents the computed regression line ($y = 1.79 + 0.006\,x$) which does not show an inverse correlation of particle number and intercept length in the spacer units. The dotted line represents the expected reduction of the apparent spacer length by the particles, if they were nucleosomes, assuming a condensation factor of 5.35 (for details see text).

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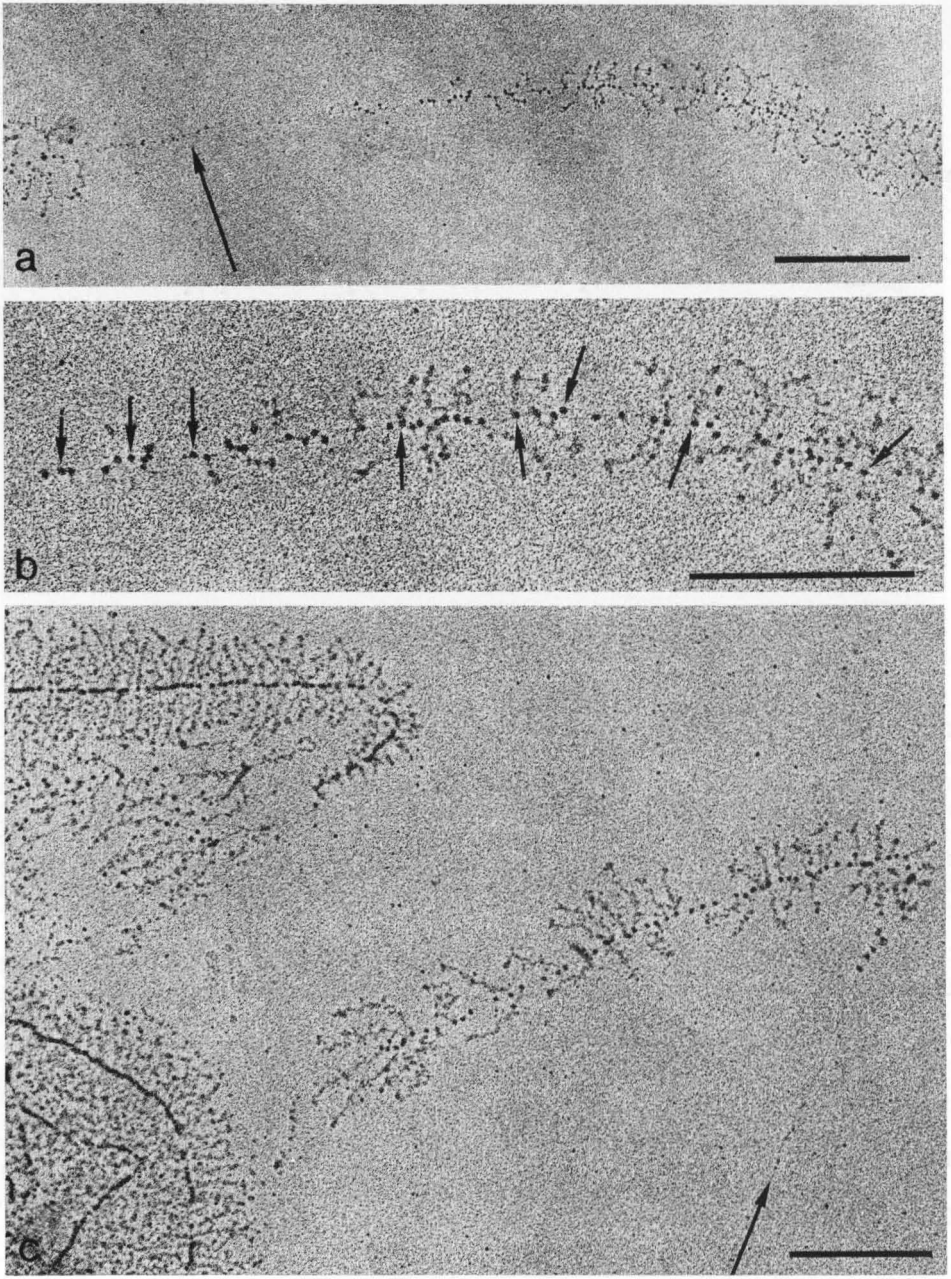
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Fig. 17 a to c. Appearance of transcriptional complexes of rDNA from nucleoli of *Triturus cristatus* oocytes after treatment with solutions containing 0.1 % Sarkosyl NL–30 (for details see Methods) and preparation by spreading, positive staining and metal shadowing. Note the persistence of the distinct granules attached to the chromatin axis in both regions, matrix units and apparent spacers. Most of these particles (denoted, e. g., by arrowheads in **b**; diameters from 13 to 15 nm) are still associated with lateral fibrils containing the nascent pre-rRNA. The close spacing of these granules within the fully fibril covered matrix units results in an indistinct, rather uniform thickening of the intramatrical axis (e. g. in the left part of **Fig. 17 c**). – **a.** 36 000 ×. – **b.** 60 000 ×. – **c.** 38 000 ×. – Scales 0.5 μm.


a
b
c

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Figs. 18 a to c. Details of the ultrastructure of matrix units of nucleolar material from a previtellogenic oocyte of *Triturus alpestris* (**a**), showing that in matrix units of reduced fibril density the individual lateral fibrils and their basal, RNA polymerase containing granules, can be readily identified. The arrowheads in **a** point to some of these basal granules which with this contrasting method (positive staining alone) appear at diameters ranging from 10.5 to 13.5 nm. **Figs. b** and **c** show survey micrographs of nucleolar chromatin from oocytes of *Triturus cristatus* as revealed after treatment with 0.3 % Sarkosyl NL–30 (see Methods), spreading and positive staining, followed by metal shadowing. Note the thinness of the chromatin axis in fibril-free regions (arrows; 5 to 7 nm) and the persistent association of some dense granules with such regions. – **a.** 67 000 ×. – **b.** 33 000 ×. – **c.** 23 000 ×. – Scales 0.2 μm (**a**) and 1 μm.

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