Transcription in Amphibia in relation to the C-value Paradox. An electron microscopic study.

by

SANDYA NARAYANSWAMI

Department of Zoology

University of St. Andrews

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION

I hereby declare that this thesis is my own composition, and that the experimental work was performed by me alone. None of the material in this thesis has been submitted for any other degree

SANDYA NARAYANSWAMI.

12th November, 1979.

CERTIFICATE

I certify that Ms. Sandya Narayanswami has spent 12 terms at research work on transcription in Amphibia in relation to the C-value Paradox, that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

20th November, 1979. H.G. CALLAN.

UNIVERSITY CAREER

I began my university career in the School of Biological Sciences at the University of Leicester in October 1973, and graduated with a first class Honours B.Sc. in June 1976. I entered the Zoology Department of the University of St. Andrews in October 1976, and spent three years doing post-graduate research into the characteristics of transcription in amphibians of different C-value. The results of my work are presented here for the degree of Doctor of Philosophy.

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GENERAL INTRODUCTION

One of the most puzzling problems in molecular biology, one that touches upon many aspects of cell function, is the wide variation in nuclear DNA content present in many different groups of eukaryotic organisms, and known as the C-value Paradox. The paradox arises because variation in C-value, the amount of DNA in the haploid genome, is not correlated with complexity of organization or position on an evolutionary scale.

Surveys of DNA content in different taxonomic groups (Mirsky & Ris, 1951; Rees & Jones, 1972; Sparrow et al., 1972) show that the lowest nucleic contents are present among viruses, and that with increasingly complex organization the lowest C-values in the major phyla tend to increase. Among multicellular organisms, C-values increase gradually through the sponges and coelenterates, and data from nematodes, echinoderms and molluscs also fall into this pattern. Few C-values are known for the annelids, but the known ones are similar to the values for echinoderms and molluscs (Sparrow et al., 1972). Phylogenetic data are most extensive for the chordates, although the more primitive mammals have been only poorly characterized, and the data suggest that for higher vertebrates, evolutionary development was preceded by a substantial increase in DNA content. Both the minimum values and ranges of C-value for the urochordates, cephalochordates and agnathans, where known, reveal this trend, increasing gradually from 0.19 - 0.2 pg, to about 0.6 pg, to a value of 1.2 - 2.9 pg, respectively (Sparrow et al., 1972). However, one qualifying factor is that the full range of C-values for these groups is not yet known. It is true, however, that minimum C-values within a group show a slight increase through the higher vertebrates, although this increase levels off among birds and mammals. This general increase can be most easily explained by

postulating that with increasing evolutionary advancement, there is an increase in the amount of information encoded in the genome.

However, two types of evidence suggest that there is no rigid correlation between C-value and evolutionary advancement. The first of these is the observation that there is a wide spread of C-value in some groups of organisms, and even among the species of one genus, which can be expected to be closely related and of a similar degree of complexity. C-values range over nearly two orders of magnitude among the arthropods, and over four orders of magnitude among the algae. Among diploid flowering plants, haploid values range from 0.7 pg in Lilium usitatissimum to 36.1 pg in Lilium longiflorum (Rees & Jones, 1972). The significance of this variation within groups is still largely obscure.

The second line of evidence consists of the fact that in a large number of cases, though not all, primitive species have a higher DNA content than more advanced ones. In the case of the fish, for example, the initial increase in DNA content among the agnathans was probably followed by DNA loss, such that some of the more advanced teleost fish have C-values lower than some agnathans (0.6 - 4.8 pg as opposed to 1.2 - 2.9 pg for the agnathans) (Goin & Goin, 1968). The Holostei have a similar though slightly narrower range of C-values, at 1.2 - 1.8 pg, again suggesting some DNA loss. Other fish have intermediate values, 1.8 - 4.2 pg, but the major exception to this is the relatively primitive group, the Dipnoi, whose C-values can be as high as 60 pg.

The high C-values of the Dipnoi are similar to those of the urodele Amphibia, which may share a common ancestor with these primitive fish. At first sight this trend appears to be in opposition to the idea that primitive forms have high C-values. However, the more advanced amphibians,

such as the anurans, have relatively low C-values. Low C-values (1.8 - 3.0 pg) are also characteristic of reptiles, so that it may be true to say that although minimum C-value within a group increases slightly with complexity of organization, the emergence of the reptiles and possibly also birds and mammals, involved a dramatic drop in C-value. Birds are characterized by C-values of the order of 1.2 - 1.8 pg, appreciably lower than those of more primitive groups such as reptiles and amphibians. The range of variation is somewhat greater among the mammals, but the highest mammalian C-value is that of a marsupial. Marsupial C-values range from 1.8 - 5.4 pg, and together with the monotremes, they are the most primitive mammalian group. The C-values of monotremes are in the region of 3.6 pg, whereas those of the Eutheria, the most advanced group, range from a minimum of 3 pg to a maximum of 5.9 pg for the Aardvark (Bachmann et al., 1972; Sparrow et al., 1972).

This correlation of evolutionary advance with decrease in C-value is also seen among plants, where angiosperms in general have lsss DNA than either gymnosperms or ferns; however Trillium and Lilium, both higher plants, have the highest recorded plant C-values. It has been suggested therefore, that low C-values mark the ends of evolutionary lines, which become extinct because less DNA is available for mutation to act upon, thus allowing for the creation of fewer "new" genes (Hinegardner, 1968). The more advanced birds show the lowest C-values among advanced vertebrates and their development seems to have been confined to a type of speciation perhaps best described as "variation on a theme" inasmuch as no two bird species are as divergent as the bat and the whale among mammals, a group characterized by C-values slightly higher than those of birds (Goin & Goin, 1968).

In a few cases, there does appear to be a link between high C-value and evolutionary advancement within a group. Among the molluscs, C-values follow the general trend of C-value, inasmuch as the highly evolved squid has a higher C-value than more primitive molluscs.

Although C-values have been determined for many members of many groups, and their patterns exhaustively studied, few conclusions have been drawn about the origin and significance of this variation. Though higher organisms in many cases have more DNA per cell than lower organisms, the variations observed cannot be explained by postulating that all species with high C-values have more genes. Evidence from genetic analysis and reassociation experiments in a variety of species supports the idea that most eukaryotes have only between 4,000 and 20,000 genes, and that the greater part of the DNA does not code for proteins (Lodish et al., 1973; Galau et al., 1974; Lewin, 1975; Davidson, 1976; Hereford & Rosbash, 1977; Garcia-Bellido & Ripoll, 1978). Range in C-value occurs over a greater number of orders of magnitude than does the current estimate of range in gene number.

Related to the data on gene number are observations on such morphological characteristics of chromosomes as lampbrush chromosome loop length, and recombination rate. The lateral loops of amphibian lampbrush chromosomes, the extended transcriptionally active chromosomes found in oocytes, are much longer in high C-value species, i.e. the lateral loops of Axolotl chromosomes are longer than those of Triturus, which in turn exceed in length those of either Rana or Xenopus. If, as has been suggested, lampbrush chromosome loops comprise functional units, then high C-value organisms must have the same number of such units as low C-value organisms.

C-value does not appear to correlate with recombination rate as measured by recombination indices. The recombination index, obtained by

adding together the haploid number of chromosomes and the mean number of chiasmata, represents the mean number of blocks of genes segregating at meiosis. A ten-fold increase in C-value is not accompanied by a ten-fold increase in recombination rate (White, 1973), which again suggests that high C-values cannot be attributable solely to increase in gene number.

Much variation in C-value, especially among plants, can be explained by invoking polyploidy (Rees & Jones, 1972; Nagl, 1978) and, in some cases, polyteny (Brady & Clutter, 1974). However, polyteny has never been observed in the germ line chromosomes of any species, although for many years it was assumed in order to explain gross variation in chromosome size (Rothfels et al., 1966).

The C-value paradox itself only emerged when it became clear that the chromatids of all organisms are "unineme", that is, they contain only one DNA duplex. Evidence from a variety of sources has led to this idea (see Callan; 1972 for review). The experiments of Taylor, Woods and Hughes (1957) provided the first evidence for the semiconservative replication of eukaryote DNA. Root tip cells of Vicia faba were allowed to incorporate ^{3}H-thymidine during an S-phase and the distribution of label in fixed metaphase chromosomes, after various times, was examined by autoradiography. It was found that metaphase chromosomes which had incorporated label during the immediately preceding S-phase were labelled in both chromatids. After two rounds of replication, in the presence and then in the absence of label, the chromosomes were labelled in one chromatid only. These observations have been repeated in other systems (Callan, 1972) and also provide circumstantial evidence for uninemy, inasmuch as it is difficult to visualise the orderly segregation of labelled from unlabelled DNA duplexes in a polyneme chromosome labelled in this way.

Further evidence for the uninemy of the chromatid comes from the digestion experiments of Gall (1963). The kinetics of digestion, by pancreatic NDase, of lampbrush chromosome loop axes and interchromomeric axes are consistent with the chromatid consisting of only one double helix, and the chromosome comprising two duplexes. If newt lampbrush chromosomes are digested with trypsin and examined by electron microscopy, it is seen that the DNAse-sensitive trypsin-resistant lateral loop axis is some 2 - 3 nm wide, whereas the main axis between chromomeres is some 3 - 5 nm wide (Miller, 1965). These dimensions also allow for there being only one DNA duplex per chromatid.

More recent work on the viscoelastic properties of DNA (Kavenoff et al., 1973) also supports the contention that the eukaryote chromatid is unineme. Viscoelastic measurements indicate the presence of "chromosome-sized" DNA molecules in many species.

The only serious evidence against the idea of uninemy concerns the phenomenon of "isolabelling", where, in instances where only one chromatid of a chromosome should be labelled, both chromatids appear to be labelled. However, isolabelling is now known to be a consequence of multiple sister-chromatid-exchanges, plus poor autoradiographic resolution, as shown by the fact that BUdR incorporation followed by "fluorescence Giemsa" staining, which is independent of labelling does not show this phenomenon (Perry & Wolff, 1974).

Evidence exists in Lathyrus spp. (Narayan & Rees, 1977) and amphibians (Macgregor et al., 1976; Baldari & Amaldi, 1976, 1977) that variation in nuclear DNA amount may be attributable to differences in the amount of repetitive DNA. However, as the function of repetitive sequences is still largely obscure, the significance of the observed variation in C-value is still no clearer.

Many explanations have been proposed to account for the observed wide differences in DNA content. It has been suggested that non-coding DNA is largely without function, the result of genetic drift and the random accumulation of sequences in the genome (Ohno, 1972).

It can be demonstrated that there is a positive correlation between C-value and nuclear size, cell volume and cell-cycle length in a large number of different cell types (Defendi & Manson, 1963; Zalik & Yamada, 1967; Baserga & Wiebel, 1969; Nagl, 1974; Szarski, 1976; Olmo & Morescalchi, 1978). In both plant and animal species, life-cycle time correlates positively with C-value. These observations suggest that C-value may be subject to strong selection pressure, although it is difficult to prove a causal relationship. For these reasons, it has been proposed that DNA has two major functions unrelated to its coding capacity. The first of these is the control of nuclear volume by the number of replicon origins, in a manner similar to that which is postulated to occur in bacteria. The general correlation that exists between the size of actively dividing cells and their DNA content, in both pro- and eukaryotes may imply that all cells have a common mechanism of size determination. Among prokaryotes, cell division can take place only after the cell has completed a cycle of replication and has an appropriate content of DNA for its mass. New cycles of replication can be initiated only when the cell achieves an appropriate and constant ratio of cell mass to the number of origins.

This suggests that the cell can in some way titrate its mass to judge when it is ready for initiation, because the greater the number of origins, the greater the cell mass required before replication can begin.

Cells could titrate their mass if a critical amount of some protein controls initiation of replication. This protein could be an initiator synthesized by the cell and whose accumulation triggers initiation, or an inhibitor whose dilution by cell growth is necessary for replication to begin (Lewin, 1974).

The second major function of DNA is the determination of nuclear volume by its overall bulk. Cell and organismic growth rates are determined by cell volume and by the area of nuclear envelope available for transport, and these parameters in turn are dependent upon nuclear volume and therefore DNA content. The great diversity of cell volumes and growth rates, and therefore of DNA contents, among eukaryotes, is postulated to arise from a varying balance between selection for small cells, rapid growth rates, and therefore low C-values, and selection for large cells, slow growth rates and therefore high C-values (Cavalier-Smith, 1978).

Evidence also exists showing that cell generation times and other cell cycle parameters can vary with cell type within an organism, however (Brown & Oliver, 1968; Callan,1972; Balls & Godsell, 1973). It is proposed (Cavalier-Smith,. 1978) that polyteny, endopolyploidy and the synthesis of nucleoskeletal RNA (as opposed to heterogeneous nuclear RNA or messenger RNA, which contain protein coding regions) constitute a more finely tuned mechanism of modulating nuclear and hence cell size, and of allowing variation in cellular parameters over and above that permitted by varying the C-value.

The C-value Paradox lies at the very heart of all speculation about genome structure. Its elucidation will affect ideas on gene organization and regulation, the evolution of genomes and species, chromosome structure, and therefore on cellular events further removed from these levels of organization.

My thesis describes experiments designed to elucidate some aspects of the C-value Paradox. The systems used were established cell lines from two amphibian species Triturus cristatus carnifex (Rudak, 1976) and Xenopus laevis (Rafferty, 1969), closely related on the evolutionary scale and showing a difference in C-value of about tenfold. Both genera come from a group of animals showing the largest variation in C-value, and they are two members of a series of increasing C-value running from 3.2 pg per haploid nucleus in Limnodynastes tas to 52 pg in Necturus (Rees & Jones, 1972). As such, they were considered ideal for this study.

Both Triturus and Xenopus have been the subject of a large body of experimental work having a direct or indirect bearing on the work described below. The lampbrush chromosomes of both species, the extended and transcriptionally active chromosomes found in the oocytes, have been studied in great detail, using a variety of techniques (Gall, 1963; Snow & Callan, 1969; Sommerville, 1973; Malcolm & Sommerville, 1974; Muller, 1974; Mott & Callan, 1975; Macgregor & Andrews, 1977; Old et al., 1977; Hill, 1979, in press). In addition to morphological studies, biochemical data on sequence arrangement (Rosbash et al, 1974; Chamberlin, 1975; Baldari & Amaldi, 1976, 1977) DNA replication (Callan, 1972, 1973, 1976) and transcription (Malcolm & Sommerville, 1974; Sommerville, 1977) also exist, so that these species offer an unique opportunity to correlate morphological and biochemical data with studies of possibly C-value dependent events.

The C-value Paradox can be investigated in a number of ways. One approach is biochemical, involving the use of techniques such as nucleic acid hybridisation, direct study of DNA and RNA, and nucleic acid restriction and sequencing, all of which yield information on the fine structure of the hereditary material. On the other hand, direct morphological studies of the chromatin, relying upon such techniques as chromosome analysis, microdensitometry and autoradiography, and giving a more general overall view of genome organization, can be done.

The approach used here is mainly morphological and involves the use of the Miller spreading technique for visualising actively transcribing regions of chromatin (Miller, 1966; Miller & Beatty, 1968; Miller & Beatty, 1969a, b; Miller et al., 1970; Miller & Bakken, 1972; Miller & Hamkalo, 1972). The C-value Paradox has therefore been investigated at the most fundamental level of organization next to that revealed by DNA sequencing studies, namely transcription, the process whereby the information encoded in the chromatin is made available to the cell.

In this investigation, the results of morphological experiments could be compared to biochemical analyses because both tissue cultures and live animals of each species were available, allowing more refined study using sensitive biochemical techniques. Moreover, tissue-culture cells permit easy quantification of results, facilitating both morphological and biochemical approaches. To date only oocytes, somatic cells, primary cell lines of limited lifetime, and HeLa cells have been exploited, whereas chromatin is more easily manipulable in tissue culture cells, and it is possible to optimize spreading conditions in a way not possible in many cell types.

One advantage that arose, of having both tissue cultures and live animals available, was that both species-specific and tissue-specific patterns of transcription could be compared. Extensive data exist concerning both ribosomal and non-ribosomal transcription in a large number of plant and animal species. Ribosomal RNA synthesis has been described in newt and frog oocytes (Miller & Beatty, 1969a, b; Jordan & Loening, 1977; Scheer et al., 1973, 1977) bacteria, (Miller et al., 1970; Hamkalo & Miller, 1973), the house cricket (Trendelenburg et al., 1973), the algae Acetabularia and Chlamydomonas (Berger & Schweiger, 1975a, b, c; Spring et al., 1976), rat and Chinese hamster cells (Puvion-Dutilleul et al., 1977a, b), Oncopeltus fasciatus, a hemipteran (Foe, 1977), the slime mold Physarum polycephalum (Grainger & Ogle, 1978) and Chironomus (Derksen et al., 1975). Nonribosomal transcription has been characterized in Drosophila, for both nonpolytene (Hamkalo et al. 1973, Glätzer, 1975) and polytene (Derksen, 1973) nuclei, as well as in Chironomus polytene chromosomes (Lamb & Daneholt, 1979), Trichosia pubescens, a sciarid fly (Amabis & Nair, 1976), O. fasciatus (Foe et al., 1976), the silk-worm Bombyx mori (McKnight et al., 1976) rat liver (Puvion-Dutilleul et al., 1978) and newts (Angelier & Lacroix, 1975; Franke et al., 1976a). In addition a few quantitative studies have been performed (Mcknight et al., 1976; Foe, 1977) but to my knowledge no direct results of a comparison between species, using the Miller spreading technique, have so far been published, although some biochemical data are available (Rosbash et al., 1974; Lengyel & Penman, 1975). The Miller technique is admirably suited to direct analysis of transcription patterns and is thus an unique tool with which to study the C-value Paradox, because it reveals a level of organization immediately above that of the chromatin itself.

C H A P T E R I.

TRANSCRIPTION PATTERNS IN CULTURED CELLS OF *Xenopus laevis* AND *Triturus cristatus carnifex*

INTRODUCTION

A fundamental requirement for an understanding of the control of gene expression is a knowledge of the function of the "noncoding" sequences in the eukaryote genome. This problem is intimately connected with that of the C-value Paradox, the lack of obvious correlation between complexity of organization and genomic DNA content.

Evidence from genetic analyses, reassociation and recombination experiments indicates that most organisms possess only some 4,000 - 20,000 different genes (Davidson & Hough, 1971; Lodish *et al.*, 1973; Galau *et al.*, 1974; Lewin, 1975; Davidson, 1976; Hereford & Rosbash, 1977; Garcia-Bellido & Ripoll, 1978). Although gene numbers as measured by single-copy DNA saturation with mRNA are routinely overestimates (Kiper, 1979), the corrected figure of 10 - 15,000 different genes still varies over less than one order of magnitude, whereas C-values vary over several orders of magnitude.

Analysis of amino-acid and nucleotide sequences indicates that strict constraints apply to both genes and their products, if by "gene" is meant the coding sequence on the genome. The histones and cytochromes of species evolutionarily far apart are almost identical in both amino-acid sequence and overall length, but the composition of proteins whose function can tolerate more change can vary more between species. Although the redundancy of the genetic code allows some substitution of nucleotides in the sequences coding for those proteins whose amino-acid sequences are highly conserved, protein size and coding sequence length tend to remain similar between species, in marked contrast to C-value.

The variation in C-value among related species of similar complexity, must therefore be largely due to the presence of noncoding sequences in the DNA. Observations indicate that non-coding DNA is interspersed with the coding sequences, and the interspersion patterns fall into three main classes. The noncoding sequences themselves are either repeated throughout the genome, or unique, and the patterns of their interspersion have suggested that they may be involved in gene regulation. The so-called "Drosophila" or long-period interspersion pattern is characterized by alternating repetitive and nonrepetitive sequences both over 5 kilobases long. The "Xenopus" or short-period interspersion pattern characteristic of most moderately large genomes, consists of repetitive sequences only a few hundred base-pairs long, while the interspersed unique DNA is from one to several kilobases in length (Angerer et al., 1975; Davidson et al., 1975b; Angerer et al., 1976; Efstratiadis et al., 1976; Davidson et al., 1977). The third interspersion pattern is characteristic of large genomes, such as that of wheat, where it was first described (Flavell & Smith, 1976). In wheat, about 50 - 65% of the genome is cmmposed of repeated sequence DNA with short repetitive sequences of 350 - 650 base pairs in length interspersed with long repetitive sequences of 6 - 10 kilobases.

In all higher eukaryotes, a high molecular weight RNA fraction has been observed (Attardi et al., 1966; Penman et al., 1968; Soeiro et al., 1968; Getz et al., 1975; Lewin, 1975). This RNA shows a very high rate of turnover, as much as 90% in HeLa (Soeiro et al., 1968), and is mostly confined to the nucleus. Of short half-life (30 min - 10 h) and a heterodisperse size distribution, it is generally known as heterogeneous nuclear RNA (hnRNA). The sequence complexity of hnRNA is generally greater than that of messenger RNA (Getz et al., 1975; Lewin, 1975) and its base-composition is DNA-like. Recent work (Davidson et al., 1977) has shown

that the hnRNAs of many species consist largely of interspersed repetitive and nonrepetitive sequence transcripts. In the sea-urchin gastrula, 30% of the total unique sequence DNA is represented in hnRNA, whereas only 2.7% is represented in polysomal structural gene transcripts, so that many more individual sequence elements are present in hnRNA than are contiguous with structural genes. Current estimates of gene number, and the fact that the rate of divergence of unique sequences in hnRNA is greater than for structural gene sequences, suggest that only a small proportion of the unique sequences in hnRNA actually code for protein.

The structure and characteristics of messenger RNA are very different. Most eukaryote messengers code for only one protein and are from 400 - 16,000 base-pairs in length, protein and messenger sizes being closely parallel (Lewin, 1975), and much smaller than hnRNA. The presence of 3' poly A tracts and a small proportion of noncoding sequences included within messenger RNA have recently been demonstrated, but in general, messenger RNA is derived from unique sequences (Jelinek *et al.*, 1973; Furuichi *et al.*, 1976). HnRNA and polysomal messenger hybridise strongly, indicating that hnRNA must include coding sequences, but it is only very recently that a precursor-product relationship has been established between the two (Giorno & Sauerbier, 1976; Bastos & Aviv, 1977; Egyhazi, 1978).

From these various data, it would seem reasonable to suppose that increase in C-value is accompanied by a corresponding increase in the length of heterogeneous nuclear RNA, i.e. that more adjacent noncoding DNA is transcribed along with the coding sequences in organisms with higher C-values.

A number of lines of evidence are consistent with this idea, but the great bulk comes from RNA extraction experiments, where hnRNA is extracted from nuclei, denatured, and its size determined by sedimentation on gradients, or movement on gels. In the case of *Aedes* and *Drosophila* (Lengyel & Penman,

1975), where the difference in C-value is between 5 and 6, Aedes, the higher C-value organism, makes hnRNA from 2 - 2.5 times longer than Drosophila. A result leading to a similar conclusion was obtained for yeast, by Hereford & Rosbash (1977), who showed that the primary transcript is the same size, or only slightly longer, than the messenger. Given the small genome size (4.8×10^7 b.p. haploid value) of yeast, this result is in accord with the idea that hnRNA length increases with C-value (Lodish et al., 1973)

A number of other results also demonstrate that the mean molecular weight of hnRNA shows a positive correlation with C-value. There is a progressive increase in C-value from the slime mould Dictyostelium discoideum, through the echinoderm Lytechinus pictus, chicken and rat ascites cells, which is accompanied by an increase in the molecular weight of hnRNA from 0.5×10^6D, through 2.0×10^6D, 1.6×10^6D to a value for rat of $5 - 10 \times 10^6$D (Chung, 1974; Williamson & Tobin, 1977).

Recent results (Scheer et al., 1979) demonstrate that primary RNA transcript molecules can be processed even when still attached to the DNP (Derman et al., 1976; Giorno & Sauerbier, 1976). The relationship of the primary transcript to hnRNA is still obscure so that it is not possible to conclude that the two are homologous. However, the molecular weight distribution of some hnRNAs does reflect that of the primary transcripts (Scheer et al., 1979) so that it may be that in some cases at least, primary transcript and hnRNA are directly related.

Bearing these considerations in mind I decided that it would be interesting to look at primary transcript size in Xenopus laevis and Triturus cristatus carnifex. The C-values of these species are 3.1 pg and 26.4 pg respectively, a difference larger than any so far employed in

investigations of C-value dependent differences in RNP size. If the above data are correct, one might expect to find a large difference in primary transcript size between these two species. Furthermore, the Amphibia have been the subject of a large body of biochemical investigation, so that it would be quite easy to relate my findings to previous work.

However, evidence of various types suggests that the RNA extraction results mentioned above are to a large degree unreliable. Most of the routine extraction and sizing procedures (Federoff et al., 1977) result in the production of large persistent aggregates of RNA that cannot be dissociated unless by the most extreme denaturation conditions (Spohr et al., 1976). The phenol-extraction procedure used in many investigations (Holmes & Bonner, 1973; Lengyel & Penman, 1975; Hereford & Rosbash, 1977) accelerates reannealing and aggregation of transcripts.

In addition, indirect evidence of another sort casts doubt upon the hypothesis that hnRNA size and C-value are positively correlated. In the last two or three years it has become clear that many of the genes of eukaryotes, such as the β, γ, and δ globins, chicken ovalbumin genes, mitochondrial genes, ribosomal genes, and transfer RNA genes, contain noncoding sequences inserted within the coding region of the gene (Breathnach et al., 1977; Jeffreys & Flavell, 1977; Bos et al., 1978; O'Farrell et al., 1978; Valenzuela et al., 1978; Hahn et al., 1979; Lai et al., 1979; Royal et al., 1979). These "introns" are transcribed into hnRNA, but later excised, giving rise to a messenger RNA molecule which is much smaller than the primary transcript (Roop et al., 1978). The position and length of these introns has been exhaustively studied for the β globin gene in man, rabbit, and mouse (Jeffreys & Flavell, 1977; Mears et al., 1978; Miller et al., 1978; Tilghman et al., 1978; Little et al., 1979). The results

show that between amino-acid positions 101 and 120, for rabbit β globin, 104 and 105 for mouse β globin, and for a similar position in human β globin, an intron of from 600 - 1,000 base-pairs in length exists. The longest intron is found in the human β-globin gene, and of these three species, the human genome is the largest, at 3.65 pg haploid value (Bachmann, 1972b). Both rabbit and mouse have a C-value of about 2.5 pg. Thus, although there is a slight increase in intron length with C-value, it is probably not great enough to affect hnRNA size appreciably. However, it could be argued that a larger difference in C-value than the one that exists between these three species, is necessary to demonstrate a significant difference in "intron" length.

It seemed to me, therefore, that to study primary transcript size by means of the Miller spreading technique would be a good way of checking the work done to date. This spreading technique, devised by Oscar Miller and his co-workers (Miller, 1966; Miller & Beatty, 1969a, b), consists essentially of the manual opening, or lysis, of cells or nuclei in "pH 9.0 water" or a weak alkaline solution of the commercial dishwashing liquid "Joy". The released chromatin is allowed to spread in these hypotonic solutions before being centrifuged through sucrose-formalin fixative on to an electron microscope grid.

This technique is more reliable than extraction, as a means of determining hnRNA length, because it is direct and rapid, and although the degree of stretching of the primary transcripts can vary, by using ribosomal genes as an internal control, one can assess this. Contamination with cytoplasmic RNA is eliminated because only those molecules still attached to the chromatin are considered. Finally, the problems due hnRNA aggregation are removed, because aggregates are easy to spot in these spread preparations, and only clear single RNP molecules need be measured.

The Miller technique has not been used to invessigate transcriptional patterns in related species with widely different C-values, where the lack of correlation between C-value and organizational complexity is particularly clear. To date, the organisms studied include Trichosia pubescens, a sciarid fly (Amabis & Nair, 1976), Strongylocentrotus purpuratus (Busby & Bakken, 1979), various species of Drosophila (Derksen, 1975; Glätzer, 1975; Laird & Chooi, 1976; McKnight & Miller, 1976), the hemipteran Oncopeltus fasciatus (Foe et al., 1976), yeast, HeLa cells (Hamkalo & Miller, 1973), the silkworm Bombyx mori (McKnight et al., 1976) and various tissues of the rat (Puvion-Dutilleul & Bernadac, 1976; Puvion-Dutilleul et al., 1977a). The C-values of these organisms are in the main quite small, ranging from haploid values of about 0.03 pg for yeast to about 3.0 pg in the case of HeLa cells (Sparrow et al., 1972). Although a positive correlation has been demonstrated to exist between C-value and transcription unit size in Drosophila, Oncopeltus and S. purpuratus (Busby & Bakken, 1979), in most cases the rarity and low ribonucleoprotein fibril density of transcription figures precludes all but descriptive studies of RNA synthesis.

For this reason, therefore, I decided to use two amphibian species which differ greatly in C-value, and I extended the scope of my investigation by including Necturus maculosus, with a C-value of 52 pg, as well as Xenopus laevis and Triturus cristatus carnifex. I felt that this together with the close relatedness of these three genera, and their similarity in organizational complexity, would increase the likelihood of the detection of substantial differences in transcription pattern. Both cell cultures (Rafferty, 1969; Rudak, 1976) and live animals of the species X. laevis and T.c. carnifex were available, as were specimens of N. maculosus, so that both morphological and biochemical experiments, on a variety of cell types, could be performed. I felt that this advantage outweighed the fact that

established cell lines such as HeLa, and the cells used by me (Hamkalo et al., 1973), unlike primary cultures or somatic cells (Puvion-Dutilleul et al., 1978), tend to show only low levels of transcriptional activity.

The Miller technique yields information about the type and length of gene being expressed. RNA polymerase density (Puvion-Dutilleul et al., 1978), the relationship of transcription to DNA replication, if any (McKnight & Miller, 1977), the degree of activity of the chromatin (McKnight & Miller, 1976), have been investigated, and electron microscopic autoradiography (Bouteille et al., 1974a, b, 1976; Bouteille, 1976; Angelier et al., 1976, 1979; Villard & Fakan, 1978) has considerably extended the range of this technique. However, the sample of cells that can be examined is necessarily very small, its size being limited by the amount of chromatin on an electron microscope grid, which, if too great, obscures the transcripts. Biochemical techniques, with their much higher sensitivity and sample size, form a valuable means of checking evidence from spread preparations.

It was therefore necessary to examine the rate of incorporation of ^{3}H-uridine by these cells, which would yield information about rate of RNA synthesis with time and with percent cell cycle time, which in its turn could be used to derivee mean transcription unit size, and so supplement the data from Miller spreads. Although the results of RNA extraction experiments alone are inadequate as an index of hnRNA size, I felt that by combining these with Miller spreads, the resulting data would be more reliable that what has hitherto been provided.

MATERIALS AND METHODS

Part 1.

(i) Tissue culture techniques

The techniques described here are modifications of those used by Rudak (1976).

(a) Cell cultures

The cell cultures used in these investigations were established lines from *X. laevis* and *T.c. carnifex*. The *X. laevis* line was originally derived from Dr. K.A. Rafferty's culture A6, an aneuploid line of epitheloid morphology derived from kidney cells (Rafferty, 1969). It had been in continuous culture since 1965. The *T.c. carnifex* cell line was a predominantly diploid culture, of fibroblastic morphology, originating from the abdominal skin of a female newt. It was established by Dr. Edwina Rudak (1976) in this department and had been in culture since 1976. Neither culture showed signs of phase III degeneration (Rudak, 1976).

(ii) Sterilization procedures

(a) Tissue culture room

All manipulations requiring a sterile environment were carried out in a sterile room specially designed for this purpose. The room and its contents could be irradiated with UV light from 2 wallmounted bactericidal lamps (Hanovia, Model 13A), which were switched on overnight or immediately before the room was to be used.

Actual subculturing of cells, and all other processes requiring a sterile environment, took place in the tissue culture room under a sterile

hood (Bassaire) fitted with a fan and light. Before use, all working surfaces were swabbed with 70% ethanol on a cotton wool swab, and this was repeated at intervals during use. When the fan was turned on, a bunsen burner with a small blue flame was placed inside the hood and used for flame sterilization.

(b) Instruments

Rubber policemen, used for scraping cells for Miller spreads off plastic culture dishes, were first washed in distilled water, dried and wrapped in aluminium foil, and dry-air sterilized at 100°C. For purposes of subculture, sterile trypsin rather than a rubber policeman was used, and trypsinisation was carried out under sterile conditions.

(c) Glassware

All tissue-culture glassware to be sterilized was soaked for at least 8 hours in a 2% solution of the detergent RBS 25. All labels were removed and the glassware rinsed seven times in tap water and twice in distilled water. After drying in a heated drying cabinet, the mouths and necks of all bottles were sealed securely with a double-layer cap of aluminium foil. Bottles were sterilized by dry-heat, for 2 hr. at 160°C. Bottle caps were placed in deep petri dishes or small beakers, wrapped in 2 - 3 layers of aluminium foil and sterilized by dry heat for 2 hrs at 100 - 105°C. Bottles were capped in the sterile hood, and protected by foil caps.

Glassware used for Miller spreads or RNA extraction was soaked in distilled water overnight, washed in the same manner as for tissue-culture glassware, and dried in a heated drying cabinet, before being capped with foil and sterilized by dry heat for 4 - 5 hrs at 180°C, to inactivate nucleases. All other equipment was washed as above, and dried before use. Gloves were worn at all times.

(iii) Solutions.

Triple distilled water was sterilized either by Millipore filtration into sterile bottles or autoclaved in 100 ml aliquots in sterile bottles, for 15 min at 15 lb/sq in. pressure.

(a) Amphibian wash solution

		Grams
per litre	NaCl	6.100
	KCl	0.58
	Na_2HPO_4	1.065
	α-D glucose	0.400
(Armour)	BSA (30%)	10.0 ml
(Gibco) sterile	phenol red (w/v 0.5%)	2.0 ml
	triple distilled water	to 1000 ml

Sterilize by Millipore filtration into sterile bottles.

Store: 100 ml aliquots at +4°C.

To use: add 1.0 ml penicillin/streptomycin per 100 ml aliquot.

(b) Versene solution

per litre	Na_2EDTA	0.200 g
	NaCl	6.000 g
	KCl	0.200 g
	Na_2HPO_4	1.065 g
	sterile phenol red (w/v 0.5%)	1.0 ml
	triple distilled water	to 1000 ml

Sterilize by Millipore filtration into sterile bottles.

Store: at room temperature.

(c) Stock Eagle's MEM purchased sterile in 500 ml bottles.

Store: at +4°C.

To use: Cells cultured in medium comprising

88 ml Eagle's MEM
10 ml foetal calf serum
1 ml glutamine
1 ml penicillin/streptomycin

} referred to as "MEM"

(a) Eagle's MEM (IX) with Earle's salts, with l-glutamine. Gibco Cat. no. 109.

(b) Sterile foetal calf serum, in 100 ml bottles. Gibco Cat. no. 629 stored in 10.0 ml aliquots in sterile plastic centrifuge tubes (Falcon) at -20°C.

(c) l-glutamine (200 mM 100x) Sterile in 100 ml bottles. Gibco Cat. no. 503. Stored in 1.0 ml aliquots in sterile plastic tubes (Falcon) at -20°C.

(d) Eagle's basal medium (BME) in amphibian salts, with 0.5% lactalbumin hydrolysate.

per litre			
	NaCl	5.15 g	500 ml
	KCl	0.075 g	
	$MgSO_4 7H_2O$	0.204 g	
	$Ca(NO_3)_2\ 4H_2O$	0.078 g	
	$CaCl_2$		
	Na_2HPO_4	0.03 g	250 ml
	KH_2PO_4	0.037 g	
	$NaHCO_3$	0.750 g	250 ml
	lactalbumin hydrolysate (Difco)	5.0 g	
	sterile phenol red (w/v 0.5%)	1.0 ml	
	triple distilled water	to 1000 ml	

$NaCl$, KCl, $MgSO_4 7H_2O$, $Ca(NO_3)_2 4H_2O$ and $CaCl_2$ were dissolved in 500 ml of distilled water. The phosphates were dissolved in 250 ml distilled water. $NaHCO_3$ lactalbumin hydrolysate were dissolved in a further 250 ml distilled water. These three solutions were then combined, and phenol red was added. This procedure was found to prevent precipitation, which occurred when all the components of the medium were dissolved together in 1 litre of distilled water.

Sterilize by Millipore filtration into sterile bottles

Store: at +4°C.

To use:

- 86 ml medium
- 10 ml foetal calf serum
- 1 ml BME vitamins (100x)
- 1 ml BME amino-acids (100x)
- 1 ml penicillin/streptomycin
- 1 ml l-glutamine

) referred to as "BME"

(a) BME (100x) amino-acids without l-glutamine, 100 ml aliquots
Gibco Cat. No. 1053

Stored at +4°C.

(b) BME (100x) vitamin solution, 100 ml aliquots
Gibco Cat. no. 104.

Stored at -20°C.

(e) Penicillin-streptomycin. Difco 6 x 10 ml vials (5854-60)

Obtained in sterile desiccated form in vials, each containing 100,000 units of penicillin G-potassium, and 100,000 µg streptomycin sulphate. Each vial was rehydrated with 10.0 ml sterile triple distilled water, and stored as 1 ml aliquots in sterile plastic tubes, at -20°C. Unreconstituted vials were stored at +4°C.

(f) Trypsin. Difco Bacto-trypsin 6 x 10 ml vials, code 0153-60

Obtained in sterile desiccated form in vials. Each vial was rehydrated with 10 ml sterile triple distilled water, giving 10.0 ml of a 5% solution of trypsin. This was stored in 1 ml aliquots in sterile plastic centrifuge tubes at -20°C. Unreconstituted vials were stored at +4°C.

To use: Xenopus

1 ml of 5% trypsin was added to 99 ml versene to give 100 ml of a 0.05% solution, which was stored either as 10 ml or 2 ml aliquots, in sterile plastic centrifuge tubes, at -20°C.

Triturus

1 ml of 5% trypsin was added to 99 ml amphibian wash solution, and divided into 10 ml aliquots. These were stored at -20°C.

(iv) Tissue culture vessels (Falcon)

		Cat. no.
(a) Tissue culture flasks		
25 cm^2 growth area.	20 per pack, 500/case	3013
75 cm^2 growth area.	5 per pack, 100/case	3024
(b) Petri dishes		
60 x 15 mm	20 per pack, 500/case	3002
(c) Tubes with caps		
17 x 100 mm		2001
12 x 75 mm		2003F
(d) Pipettes (sterilin)		
1.0 ml serological	individually wrapped	106B
5.0 ml serological	10 per pack	134
10.0 ml serological	10 per pack	133

(v) Culture methods

(a) Procedure used to subculture cells of Xenopus laevis

To subculture cells, old medium was decanted and the monolayer washed twice with 5 ml versene. The wash solution was decanted and 5 ml fresh versene, together with 1.5 ml 0.05% trypsin, was added. Trypsinization was allowed to proceed for 5 mins, at 25°C, after which the culture vessel was gently rocked, to promote detachment of the cells. 1.5 ml of BME was added to the cells, in order to inactivate the trypsin, and the cell suspension was pipetted into a sterile capped tube, before being centrifuged for 5 - 10 min at 900 rpm (MSE bench centrifuge). The supernatant was discarded and the cell pellet gently resuspended in 8 ml BME. 1 ml of the cell suspension was added to each of the two culture vessels (75 cm^2 growth area) containing 14 ml BME. If small culture vessels (25 cm^2 growth area) were used, volumes were scaled down accordingly. Inoculation density was also varied, for particular experiments. Cells were checked under an inverted phase contrast microscope.

For routine purposes, a culture of X. laevis cells was maintained at an initial inoculation density of about 5 x 10^6 cells per large (75 cm^2 growth area) culture vessel. This culture was allowed to grow for one week, until confluent, and every Monday a 1:8 cubculture was made, into two new culture vessels. The cells were incubated at 25°C, in a water-cooled incubator, until the following Friday, when the old medium was discarded, and 15 ml fresh medium added. A haemocytometer was used to count cells. Growth rate experiments showed that at the above inoculation density the cells attained stationary phase in 7 days (Appendix II).

(b) Procedure used to subculture cells of Triturus cristatus carnifex (Rudak, 1976)

When a monolayer (large culture vessels were used) had attained confluence, the old medium was decanted and the cells washed twice with 6 ml aliquots of amphibian wash solution (AWS). The AWS was discarded, and 10.0 ml of 0.05% trypsin in AWS added to the cells. After 5 min trypsinization at 25°C, the cells were detached by gentle shaking, and 1 ml MEM added, to inactivate the trypsin. The cell suspension was pipetted into a sterile capped tube and centrifuged for 10 min at 900 rpm. The supernatant was discarded and the cells gently resuspended in 6 ml MEM. 2 ml of cells was added to each of 3 large (75 cm^2 growth area) culture vessels, each containing 13 ml MEM. Two separate cultures of the newt cells were maintained, and used interchangeably. Both were grown at 25°C, in a water-cooled incubator, and a 1:3 subculture was made of each, at intervals of three weeks.

Part 2. Visualisation of actively transcribing regions of the chromatin.

(i) Hardware required for Miller spreading

(a) Carbon-coated grids

For all Miller spreads I used grids coated with a thin (15 - 25 nm) carbon support film, in preference to carbon-Formvar. The generally low contrast of spread preparations necessitated the use of as thin a film as possible.

Two pieces of freshly cleaved mica (2.5 cm x 4.5 cm) were placed, cleaved side up, in the chamber of a vacuum coating unit (Balzers Mikro BA3, Balzers, Liechtenstein), and secured in position by porcelain tabs. The vacuum was reduced and carbon slowly evaporated on to the pieces of mica

The thickness of the carbon layer was judged by the colour of the porcelain tabs, on to which evaporation also occurred. Carbon films were used at once. The mica was held carefully in a pair of watchmakers' forceps, and about 2 mm was cut off round one short edge and two long edges.

A black Langmuir trough with a tap at the bottom was filled with distilled water. On a triangular glass support in the centre of the trough was placed a copper gauze, previously cleaned by rinsing in glacial acetic acid followed by distilled water, and about the same size as the carbon film. Gauzes were kept submerged in distilled water until needed. The water surface in the trough was cleaned by placing a few drops of pyroxalin in butyl acetate on it, allowing these to form a thin film, and removing the film, together with any adherent dust, with watchmakers' forceps.

Copper grids (Athene 400's, diameter 3.05 mm) were cleaned by rinsing in glacial acetic acid, washing twice in distilled water and twice in acetone, before drying on filter paper. Cleaned grids were placed dull side up on the copper gauze in the trough.

The mica bearing the carbon film was picked up with a pair of watchmakers' forceps and its trimmed short edge touched to the meniscus at an angle of about 30°. This caused the edge of the carbon film to float away from the mica, and once this had occurred, the piece of mica was gently lowered into the trough, causing the entire film to float off onto the water surface.

The film was now gently manouevred into position over the grids and water slowly emptied from the trough, thus lowering the film onto the grids. When the water level had dropped below that of the grids, the gauze was picked up, touched to filter paper (Whatman) to remove excess water, and placed in a Petri dish before being dried in air overnight.

The amount of film on each grid was checked either by eye or under a dissecting microscope. Only those grids with at least 50% coverage were used. These carbon films were very stable and grids could be used for a long period after they were made, although in practice, a batch of grids was used up in about two weeks.

(b) Microcentrifugation chambers

Microcentrifugation chambers were made of Perspex into which two wells, each just large enough to admit an electron microscope grid, had been bored. The dimensions are given in Fig. I-1. For centrifugation each chamber was placed on top of a solid Araldite plug (Fig. I-2). Each plug fitted snugly into a balanced 50 ml polypropylene centrifuge tube (26 x 104 mm) adapted to fit into the 5.75 inch swing-out rotor of a Sorvall enclosed superspeed bench centrifuge.

(ii) Miller spreading technique

For visualisation of transcription in a number of different cell types I used various modifications of the technique originally devised by Miller and co-workers (Miller & Beatty, 1969a, b; Miller & Hamkalo, 1972). These will be described below or in the appropriate chapters.

Solutions required

All solutions were made up with either glass distilled triple or single distilled water. Solutions were made up freshly for each preparation using water that had been boiled for 5 min, or autoclaved previously to remove carbon dioxide, and then cooled. All solutions were Millipore filtered (type GS. 0.22 μm pore size) into clean plastic bottles, and kept on ice until needed.

(a) 0.1 M sucrose plus 10% formaldehyde (sucrose formalin fixative, S/F)

During my experience with the spreading technique I used a number of different methods of making up the sucrose-formalin fixative. I found that the condition of the fixative was critical for my preparations, and the final method given here is the one that I found gave the best results.

1. For a final volume of 20 ml, 0.6846 g of sucrose (Analar) was dissolved in 18 ml distilled water. 2 ml formalin (Analar) was added, and the pH adjusted to 8.5 with either borate buffer or 0.1 N NaOH.

2. I noticed that formalin grew acid very quickly, causing deterioration in the quality of the preparations. Chromatin disperses best under conditions of high pH and low ionic strength. It was therefore necessary to use a formalin solution that had been made relatively alkaline.

5 g of paraformaldehyde (BDH) was suspended in 25 ml distilled water. 2 drops of 1 N NaOH were added. The suspension was heated gently until the paraformaldehyde dissolved. 10 ml of this 20% solution was added to 0.6846 g sucrose (RNAse-fre, Serva) together with 10 ml distilled water. The whole was brought to pH 8.5 with 20 µl of 0.1 N NaOH. If more than 20 µl was required, the fixative was discarded. This method of preparation gave the best results.

(b) Dispersal medium "Joy"

As for the fixative, a variety of modifications of the Joy solution used to disperse chromatin, were employed. For tissues of T.c. carnifex 0.2% Joy was used, whereas 0.1% was used for tissues of X. laevis. It was later found that this variation was not necessary (see below), and all tissues, including those of Necturus maculosus were dispersed in 0.1% Joy.

Method (3) was the most efficacious. Joy was a gift to H.G.C., from Professor Oscar Miller, Jr.

(1) 0.1 ml Joy was added to 9.9 ml of distilled water. Of this solution, 2 ml was added to 18 ml distilled water, giving a 0.1% solution, which was adjusted to pH 8.7 with stock borate buffer.

(2) Slightly better preparations were obtained when the modification of Grainger and Ogle (1978) was used. 0.3423 g sucrose (RNAse free, Serva) was added to 20 ml of a 0.1% or 0.2% solution of Joy, and adjusted to pH 8.7 as before.

(3) Chromatin preservation and contrast were greatest when the additional modification of Foe (Foe et al., 1976; Foe, 1977) was used. Small RNA from yeast was included in the spreading solution. The yeast RNA competes as a substrate for endogenous nucleases, and may also remove histone H1 from the chromatin (Foe et al., 1976) resulting in a reduction of the dispersal time.

0.3423 g sucrose (RNAse free, Serva) was added to 20 ml of a 0.1% solution of Joy, containing 100 µg/ml small RN^A from yeast (Sigma). pH was adjusted to 8.7 as before.

(c) Photoflo solution

0.1 ml of Kodak Photoflo was added to 24.9 ml distilled water to give a 0.4% solution, which was adjusted to pH 8.6, with 20 µl borate buffer.

(iii) Stains

All stains were made up freshly for each preparation.

(a) Phosphotungstic acid (PTA)

1 part of a 4% solution of PTS (BDH) in distilled water was added to 3 parts of filtered 95% ethanol. The whole was Millipore filtered.

(b) Uranyl acetate (UA)

1 part of a 4% solution of uranyl acetate (BDH) in distilled water was added to 3 parts of filtered 95% ethanol. The whole was Millipore filtered.

(iv) Glow discharging of grids

Immediately before use, grids were placed in the chamber of a vacuum coating unit and glow discharged for 1 - 1½ mins. They were then placed on a filter paper in a plastic petri dish, covered, until required. Grids were freshly glow discharged for each preparation.

(v) Visualization of transcription in cultured cells

For the visualization of transcription in tissue-culture cells, a spreading technique different from the one normally used for oocyte nuclei was devised. The germinal vesicles of oocytes are large and can be isolated manually. Tissue culture cells, on the other hand, are so small that they cannot be broekn open manually. I used a technique modified from Miller's method (Miller & Hamkalo, 1972) for cells other than oocytes. This involves lysis of the cells and dispersal of the released chromatin in weak alkaline solutions of the commercial dishwashing liquid "Joy" (Procter & Gamble, Cincinnati, Ohio). Dispersal of chromatin in this detergent has been used for a number of systems (Hamkalo et al., 1973; Glätzer, 1975; Amabis & Nair, 1976; McKnight & Miller, 1976; McKnight et al., 1976; Puvion-Dutilleul & Bernadac, 1976; Busby & Bakken, 1979). The ultrastructure of transcription is not affected by either the detergent (Miller & Bakken, 1972; Glätzer, 1975) or its perfume (Puvion-Dutilleul et al., 1978)!!

The DNA content of T.c. carnifex cells is high, and it has been generally noted that cultured cells show low transcriptional activity. In order to increase the number of transcription figures in preparations of tissue culture cells, I decided to try to eliminate chromatin from cells in mitosis although these were rare, the Mitotic Index being 0.2% for confluent cultures of both lines. To this end, I treated tissue culture cells of both species with low concentrations of the detergent Nonidet P-40, which breaks open cell membranes, but leaves nuclear membranes intact (Tres, 1977). After gentle centrifugation the pellet of nuclei was then dispersed in Joy. As before, a number of modifications were tried of which the last, due to its rapidity, gave the best results.

(a) Nonidet P-40 treatment

A 0.3% solution in distilled water was adjusted to pH 8.6 with stock borate buffer. The solution was Millipore filtered into plastic bottles and made up freshly for each preparation.

Cell monolayers were trypsinized routinely, centrifuged, and the supernatant discarded. The cell pellet was suspended in 8 ml of cold 0.3% Nonidet P-40. The cells were plunged into ice and detergent treatment was allowed to proceed for 5 min with frequent shaking to ensure complete removal of cytoplasm. The suspension was spun at 1000 rpm for 10 min and the supernatant discarded. The pellet of nuclei was suspended in pH 9 water to the desired concentration (6×10^5 nuclei/ml) before spreading.

For each preparation, the extent of removal of cytoplasm was estimated by examining the nuclei under phase contrast, after suspension in either AWS or versene. There were generally less than 1% whole cells in any preparation.

(b) Lysis of nuclei in Joy

Nuclei, suspended in pH 9 water, were lysed and their chromatin dispersed in Joy. The lowest possible Joy concentration required to cause lysis of nuclei was assessed for each tissue culture line by injecting 50 µl of a suspension of nuclei into a well slide containing 50 µl of various Joy concentrations. Lysis was monitored using an inverted phase contrast microscope, and those concentrations were chosen that effected complete lysis of the nuclei in 1 - 2 min. These were 0.2% for T.c. carnifex and 0.1% for X. laevis, very similar to the concentrations used for other systems. I later observed that 0.1% Joy would cause lysis and dispersal of both X. laevis and T.c. carnifex whole cells, and also those of N. maculosus, which suggests that Nonidet may in some way make nuclei resistant to attack by Joy.

The suspension of nuclei was diluted twentyfold into Joy in a clean Eppendorf tube, to give a final concentration of nuclei of about 3×10^4/ml. The tubes were covered with a clean square of Parafilm and the chromatin allowed to disperse for up to 75 min. Dispersal for shorter periods did not give adequate spread. Loading of grids was controlled empirically by the amount of Joy layered onto the sucrose-formalin fixative.

(c) Lysis of whole cells

To reduce the preparation time necessary for spreads of cultured cells, it was decided to eliminate the Nonidet treatment. The greater rapidity of the technique for lysis and dispersal of whole cells outweighed the advantages of removing the DNA of cells in mitosis. Preparation time was thus reduced to 15 - 20 min from 1½ hr. Transcription in preparations of spread nuclei was sparse, and it did not look as if removal of mitotic DNA had increased the relative abundance of transcription figures.

Cells were trypsinized and suspended in pH 9 water to a concentration of about 8 x 10^5 cells/ml. They were then either diluted into Joy as for nuclei or diluted twentyfold into a 95 µl droplet of Joy on a clean square of Parafilm in a sterile Petri dish. The second method gave the better and more repeatable results. Dispersal was for 75 min.

The best preparations of cultured cell chromatin with regard to contrast, and preservation of chromatin structure were obtained by a modification of the spreading method of Puvion-Dutilleul et al.(1978) together with the use of sucrose/Joy containing RNA.

Cells were grown at 25°C in 5 ml medium in small sterile plastic Petri dishes (Falcon) instead of closed culture vessels. After 2 days growth to ensure attainment of log phase the medium covering the cells was decanted and the cells washed with 5 ml pH 9 water. The cells were scraped off the substrate in 0.5 ml Joy, using a sterile plastic policeman, and immediately diluted twentyfold into Joy droplets as for whole cell preparations. In a few preparations, dispersal was for 1 hour, but this was later reduced to between 10 and 15 min. The whole procedure took some 20 min.

(vi) Preparation of microcentrifugation chambers

The preparation method for microcentrifugation chambers was the same for all cell types investigated, and is modified from Miller & Bakken (1972).

15 min before the end of dispersal, microcentrifugation chambers were cleaned by compressed air (EMscope, London) and the wells filled with sucrose-formalin fixative until the meniscus was convex. A fine-bore Pasteur pipette was used.

Freshly glow-discharged grids were picked up with plastic forceps, and rinsed in 95% ethanol, followed by sucrose-formalin fixative. The grids were then carefully slid just under the meniscus of fixative and dropped so that they fell in film side up. Excess fixative was removed from the chambers until only 2.5 mm remained over the grids.

Chambers were prepared no more than 15 min before they were required in order to prevent loss of hydrophilic charge from the grids, and changes in pH of the fixative.

(vii) Centrifugation and drying of grids

At the end of dispersal, chromatin was carefully layered on to the cushion of S/F in the chamber, using a finely pulled Pasteur pipette (1.0 mm diameter). In my hands, dispersal of the chromatin in the centrifugation chamber did not yield good results, and I used the above pipætting procedure in preference.

The preparation was covered with a clean 3/8" circular coverslip and the chambers were placed in centrifuge tubes. The tubes were placed in the swing-out rotor of a Sorvall superspeed bench centrifuge and brought slowly up to 3500 rpm (2000 g). The preparation was spun at 2000 g for 6 min at room temperature.

The preparations were removed, and the coverslips discarded. The two wells of each centrifugation chamber were filled up with sucrose-formalin until the meniscus of each well was convex. The chambers were then inverted so that each grid fell into the meniscus. The grids were picked up with curved watchmakers' forceps. The grids were rinsed, in Photoflo for 10 sec, and placed on filter paper to dry in air at room temperature.

(viii) Staining of preparations

Dried grids were stained for 1½ min. in PTA, rinsed for 15 sec in 95% ethanol, stained 1½ min in UA, rinsed again in 95% ethanol, washed in 100% ethanol and finally in isopentane. They were then dried in air.

Uranyl acetate staining only resulted in a slight increase of contrast over that resulting from the use of PTA. Later preparations stained with PTA alone were of a high enough degree of contrast to make the use of uranyl acetate unnecessary. At this point, preparations could also be rotary shadowed, at an angle of 5 - 8° with 5 mm of Pt. Pd wire. For each preparation, eight grids were made, originating from four centrifugation chambers, each containing two wells.

(ix) Examination of preparations

Spread preparations of chromatin were examined on a Phillips EM301 operated at 60 kV. Magnifications were calibrated using catalase, and length measurements were made either with a calibrated map measurer or a digitizer (Leicester University; St. Andrews University).

Part 3.

Labelling of tissue culture cells with ^{3}H-uridine

Confluent cultures of T.c. carnifex and X. laevis cells were trypsinized at the same time and both were inoculated into small 25 cm^2 culture vessels (24 vessels per line), at a cell density of about 6.5 x 10^5 cells/vessel (total volume 5 ml). Previous experiments had shown that at this cell density (rather less than the usual subculture density for X. laevis) RNA synthesis in X. laevis was maximal. At higher densities it was depressed. T.c. carnifex showed little difference in the rate of RNA synthesis at different but comparable densities.

The cells were incubated at 25°C for 24 hr. to allow attachment of the cells to the substrate.

(i) Labelling

^{3}H-uridine (nonsterile Sp-Act 25 ci/mMol) was used to label RNA at 5 µci/ml/culture vessel (25 µCi/vessel). To each batch of 59.7 ml of either MEM or BME (fresh, prewarmed to 25°C) 300 µl of isotope was added (total volume 60 ml). The radioactive medium was sterilized by Millipore filtration into sterile bottles.

About ½ hr before labelling was due to begin, old medium was decanted from the control cells (12 vessels per line), which were given 4.9 ml each of fresh unlabelled medium, and replaced in the incubator.

The vessels containing cells to be labelled were decanted, and 5.0 ml labelled medium placed on the side of the vessel opposite to the monolayer. The vessels were sealed tightly and the medium gently shaken to mix well with any dregs of the old medium. 0.1 ml of medium was removed from each

culture vessel (final volume in vessle 4.9 ml) and placed on a filter (Whatman grade 3MM 2.5 cm diam) in order to assess the variation in input of label between culture vessels. When dry, the filters were counted in toluene scintillant in a Nuclear Enterprises 8312 scintillation counter.

At zero hour, all the culture vessels were turned over and reincubated at 25°C. Samples of T.c. carnifex were taken at 15 min, 30 min, 1 hr, 2 hr, 4 hr, 10 hr, 22 hr, 34 hr, 46 hr, 58 hr. Labelling reached a peak at 58 hr but samples of cells were counted at 82 hr, 106 hr, 130 hr, 154 hr, in order to plot an accurate growth rate. For X. laevis the last two samples were taken at 70 hr and 82 hr, as the increase in cell number is much faster than for T.c. carnifex. Cells were checked for contamination under the inverted phase contrast microscope.

(ii) Sampling of cells

For each sample, culture vessels were removed from the incubator, the radioactive medium decanted and the cells washed with ice cold versene or AWS. The cells were trypsinized routinely, on ice, centrifuged and the cell pellet suspended in a known volume of AWS or versene. The cells were counted at each time point, in order to calculate Tau, the mean generation time. Using an Eppendorf tip 100 µl of each sample was dried on to each of 2 filters in order to measure the rate of incorporation of ^{3}H-uridine. In a previous experiment, the effect of SDS lysis of the cells on the incorporation figures was examined. No difference in the values for incorporated label between cells lysed in SDS before being dried and counted, and unlysed cells, was observed, indicating that the counting method described below releases incorporated label from the cells effectively.

One dried filter from each time point was left in ice-cold 5% TCA at 0 - 4°C overnight, in order to remove unincorporated label. The filters were rinsed 8 - 9 times in ice-cold 5% TCA, rinsed twice in ice-cold absolute ethanol and air-dried. The filters were counted in toluene scintillant. For each time point, 100 µl of labelled medium was placed on each of two filters. One was treated with TCA and the other counted without TCA treatment, in order to assess the efficiency of removal by TCA of unincorporated label. This demonstrated that 98 - 99% unincorporated label was removed.

The ratioactivity in 100 µl of the medium from each culture vessel was counted in order to determine the point at which the label in the medium was exhausted.

(iii) Data

Cell number for each line at each sampling time was plotted and Tau determined by measuring the time taken for the cells to double in number.

Incorporation was plotted as counts per minute per cell against percentage cell cycle time (each time point expressed as a percentage of Tau), and as counts per minute per cell against absolute time.

Part 4.

Determination of RNA size on sucrose gradients

Confluent cultures of X. laevis and T.c. carnifex were trypsinized and one large culture vessel (75 cm^2 growth area) of each set up at an inoculation density of about 1.63 x 10^6 cells/vessel. The cultures were grown for 3 days at 25°C.

(i) Labelling

Medium was decanted from the culture vessels, and 9.5 ml fresh pre-warmed medium, together with 0.5 ml ^{3}H-uridine (0.5 mCi, Sp. Act 29 ci/mMol) was added to each culture vessel, on the opposite side to the cells. 0.5 ml medium was removed and 50 µl of this was counted to assess variation in label input. At zero hour, the culture vessels were turned over and the cells labelled for 1 hour at 25°C.

After 1 hour of labelling, the labelled medium was decanted, the cells washed twice in ice-cold versene or AWS, trypsinized, and the RNA extracted.

(ii) Extraction of RNA (Penman 1966)

All solutions were treated by autoclaving for 15 min at 15 lb/sq in with 0.1% diethylpyrocarbonate to destroy nucleases. The cells were counted and each pellet washed twice in 10 ml isotonic salt solution (0.14 M NaCl. 10 mM Tris. pH 7.5. 1 mM $MgCl_2$). Cells and solution were centrifuged for 10 min at 1250 rpm to pellet the cells, and the pellet carefully resuspended in 1.25 ml RSB buffer (0.01 M NaCl. 0.01 M Tris 7.4. 0.0015 M $MgCl_2$). 100 µl of a 1 mg/ml solution of RNAse-free DNAse (Sigma) in RSB was added and the cells incubated 10 min at room temperature. DNAse treatment of

whole cells was found to be necessary to break up cell clumps held together by DNA released from broken cells. Even in a young culture, especially of T.c. carnifex, there are some broken cells, and if this step is omitted, most of the labelled RNA becomes trapped in clumps of DNA that are difficult to disperse.

250 µl of an 8 mg/ml solution of proteinase K (Sigma) was added to the cells, together with 0.4 ml 5% SDS (final concentration 1%). After incubation for 30 min at room temperature 0.87 ml of 1 M NaCl (final concentration of NaCl is 0.3 M in a final volume of 2.6 ml) was added to each suspension and the RNA extracted twice with a mixture of equal volumes of phenol: cresol, and chloroform:isoamyl alcohol.

The aqueous phase containing the RNA was placed in a clean tube and given a second treatment with DNAse (100 µl of 1 mg/ml DNAse). Protein breakdown-products were removed and the RNA further purified by three extractions with phenol cresol-chloroform isoamyl alcohol. After two further extractions with chloroform:isoamyl alcohol alone, to remove excess phenol, the RNA was precipitated overnight in 2 volumes ice-cold absolute ethanol.

(iii) Removal of contaminating DNA and protein from the RNA

To remove any residual networks of DNA, which were especially persistent in T.c. carnifex preparations, the precipitated nucleic acids were spun for 40 min at 4000 rpm (Sorvall superspeed centrifuge, swing-out rotor). The ethanol was decanted and the tubes dried and inverted on tissue pads.

1.76 ml RSB buffer was added to each pellet to redissolve the RNA, which was digested for 15 min with 10 µg/ml DNAse (RNAse-free. Sigma) and for 15 min with 10 µm/ml proteinase K, 0.5% SDS.

The nucleic acids were extracted once with phenolcresol/chloroform/isoamyl alcohol, twice with chloroform/isoamyl alcohol, and precipitated overnight in two volumes of ice-cold absolute ethanol.

(iv) Running of gradients

Precipitated dried RNA was denatured in SDS at 65°C for 2 min, in a water bath. The denatured RNA was carefully layered on to 14 ml gradients of 5 - 20% sucrose, 0.5% SDS, 2 mM EDTA, 10 mM Tris 7.4 and centrifuged for 2 hr at 36000 rpm, at 22°C in a superspeed 65 centrifuge, using the 6 x 14 ml Ti swingout rotor. Gradients were fractionated and a trace of ultra-violet absorbance taken. 50 µl of each 0.5 ml sample was dried on to a filter with 50 µl 5 mg/ml BSA (Bovine serum albumin) as carrier. The filters were then washed four times in ice-cold 5% TCA to precipitate the RNA, twice in 95% ethanol, and counted in toluene scintillant. Incorporations were plotted as percentages of the total radioactivity on the gradient.

Part 5.

Bleomycin treatment of chromatin

Cells of both X. laevis and T.c. carnifex were inoculated into large (75 cm^2 growth area) culture vessels at 3 x 10^6 cells per vessel. Two cultures were set up for each line. This high cell density gave an adequate yield of DNA for loading onto gels. Cells were grown for 2 days at 25°C.

(i) Bleomycin treatment of spermidine-extracted chromatin

Cells of both lines were trypsinized routinely. Nuclei were prepared by homogenisation in buffer containing spermine and spermidine (Hewish & Burgoyne, 1973). Nuclei were checked under phase-contrast illumination and spun through sucrose to remove débris.

DNA was isolated after the method of Hewish & Burgoyne (1973). Chromatin concentration was checked spectrophotometrically and stock solutions were used. DNAs were centrifuged and taken up in 25 µl activation buffer together with 1 unit of Bleomycin (Hewish & Burgoyne, 1973; Kuo & Hsu, 1978). Chromatin samples were treated with serial dilutions of Bleomycin. Digestion was allowed to proceed for 10 min, and to stop the reaction, 5% SDS in 20 mM EDTA was added to the samples. Chromatin samples were phenol extracted, as was a control sample not treated with Bleomycin.

(ii) Bleomycin treatment of spread chromatin

Nonidet P-40 isolated nuclei of both lines were lysed and the chromatin spread (75 min in 0.1% or 0.2% Joy) as for a Miller preparation. Spread chromatin was centrifuged and treated with Bleomycin as above. After the reaction was stopped with SDS, chromatin samples were phenol extracted.

As before, different dilutions of Bleomycin were used, and one aliquot of the spread chromatin remained untreated for purposes of comparison. 5 µl of bromophenol blue in 10% Ficoll was added to each sample to give a final Ficoll concentration of 3%.

(iii) Gels

Samples were electrophoresed overnight at 50v (75 mA) on a 20 cm x 20 cm x 3 mm 1.4% agarose gel. The gels were stained with ethidium bromide and fluoresced under an ultraviolet light.

Part 6.

Determination of cellular and nuclear dimensions of tissue culture cells.

Measurements were made for each cell line at two different inoculation densities, because crowding of the cells at high concentrations causes them to be less well displayed. Cells were inoculated into small (25 cm^2) culture vessels, using routine culture techniques as described earlier in this section. Inoculation densities were 6.5×10^5 cells/vessel and 3×10^5 cells/vessel for X. laevis, the former density being that used for Miller spreads. T.c. carnifex low-density cultures were grown at 1.25×10^5 cells/vessel, as the cells were larger than those of X. laevis. High-density cultures were inoculated with 6.5×10^5 cells, as for X. laevis. Separate cultures were set up for cell volume and nuclear cross-sectional area determinations, and were grown for 2 d at 25°C to ensure attainment of logarithmic growth.

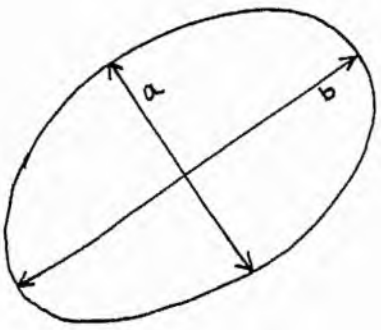
(i) Determination of cell volume

Cells growing as monolayers have many cell extensions, making it impossible to measure their volumes accurately. However, trypsinization results in the cells rounding off and withdrawing their processes, facilitating accurate measurement. Cultures were trypsinized and the cell pellet suspended in growth medium, which is isotonic to the cells and therefore not likely to cause swelling or shrinkage. Little or no clumping occurred.

A drop of medium containing cells was placed on a clean slide and sealed with a coverslip. A large excess of fluid was used to prevent compression of the cells. The slide was then placed on the stage of an inverted phase-contrast microscope. Cells were measured using a x20 objective and a x 10 eyepiece containing a calibrated micrometer scale. Under these conditions 1 eyepiece division was equivalent to 4.2 μm.

Cells were selected for measurement by moving the slide randomly over the stage. The cell nearest the centre of each of 50 random fields was selected. If more than one cell was at the centre, a new field was chosen.

The cells were assumed to be roughly spherical because they were seen to roll over when the coverslip was touched with a mounted needle. As I used a large droplet, I assumed the cells were not compressed. However, as cells are only roughly spherical, two diameters, at 90°, were measured. i.e



The mean of these measurements was calculated and half of this value, the mean radius, was used to determine the cell volume, expressed by the formula $4/3\ \pi r^3$.

For any determination cells were selected from two or more slides to minimise the effects of changes in cell size due to possible temperature variation and/or evaporation of the medium.

(ii) Determination of nuclear cross-sectional area

Both cultures grew in the form of monolayers. When the cell number in the culture is low the cells are well stretched, with many processes, and the nuclei are clearly visible.

Cultures were examined under the same conditions of microscopy and magnification as above, except that culture vessels containing untrypsinized monolayers were used. The sampling method was as above.

The nuclei appeared as ellipsoid bodies often containing a prominent nucleolus. Two diameters were measured and half the mean value, the radius, used to determine nuclear cross-sectional area. This area, as cells and nuclei are relatively transparent, and the latter assumed to be roughly spherical, really represents the maximum cross-sectional area, and as such may be affected by possible irregularities in the shape of the nucleus. However, this value, rather than nuclear volume was determined, as nuclei are much more elongated in shape than trypsinized cells, which generally approached the spherical. The "depth" of the nuclei was impossible to determine without fixation and sectioning of the cell layer and without accurate values for this parameter nuclear volume cannot be determined with accuracy. I did not have sufficient time to determine nuclear "depth".

RESULTS

(i) Xenopus laevis culture cells. Characteristics of transcription

During my investigation of transcription patterns in X. laevis cultured cells I experimented with a variety of modifications of the Miller technique. Most of these yielded data, but observations of transcription itself came largely from one preparation made at a late stage of this study.

Early preparations were made from cells grown at the usual subculture inoculation density of 5×10^6 cells per large culture vessel, and when they were in log phase to maximise the chances of finding transcription figures. The quality of these preparations was relatively poor with regard to contrast and the preservation of structural detail, and little material was present on the grids. When cells were grown at twice the usual subculture density, more material was present on the grids, although contrast was not improved, and I obtained slightly more data from these preparations. The poor quality of the preparations prevented my finding many transcription figures at either cell density.

During my studies of tritiated-uridine incorporation by these cells, it became clear that RNA synthesis was depressed if X. laevis cells were grown at high cell density. Using modifications of the Miller technique that resulted in an improved contrast and preservation of chromatin, I found that transcription figures were most frequent when the cells were grown at an inoculation density of 1.95×10^6 cells per large culture vessel (the cell density resulting in maximal rates of incorporation of ^{3}H-uridine), and only preparations of chromatin from cells grown at this concentration were analysed further. The frequency of transcription figures was higher than in the early preparations discussed above. Nonetheless, valuable information about other aspects of chromatin structure was obtained from my early preparations.

When preparations were made from Nonidet P-40 extracted nuclei the chromatin appeared predominantly in the form of clumps, roughly the size of an individual nucleus although smaller clumps were also seen. The clumps themselves had a granular structure in which no significant detail could be resolved (Fig. I-3a).

The degree of dispersal of the chromatin varied, but was in general almost complete after 75 min of spreading. In the majority of cases dispersed chromatin radiated from larger clumps (Fig. I-3a). The degree of dispersal varied between different preparations, different grids of the same preparation, and sometimes over the area of a single grid, under the same spreading conditions. I have found that the Miller technique is very subject to variation which I assume to be due to uncontrollable factors such as fixative pH, grid charge etc. In many cases, chromatin tended to lie close to grid bars or around breaks in the carbon films, perhaps due to differences in charge in these regions.

Most of the chromatin in preparations of X. laevis culture cells exhibited a clearly beaded appearance (Figs I-3b and c). These beads were, in later preparations especially, ubiquitous, and this, together with their size, which varied from between 9.7 nm and 14.7 nm $\pm$ 2.5 nm and periodicity, suggests that they are analogous to the "nu-bodies" or nucleosomes described by Olins and other authors (Olins, 1977). The variation in their size is probably due to my method of visualisation. In stained preparations the nucleosomes were significantly smaller than in rotary shadowed preparations, where progressive deposition of metal on the chromatin increases their dimensions.

In some cases the nucleosomes were seen to be interconnected by a fine

fibril about 5 - 6 nm wide, and varying in length depending upon the frequence of nucleosomes in the preparations. Later preparations, involving short dispersal times in the presence of yeast transfer RNA, sucrose and Joy, showed an improved preservation of chromatin secondary structure. Nucleosomes were more closely spaced and the interconnecting fibrils were seldom visible (Fig. I-13a - d).

Chromatin often had a "stretched" appearance, consisting of low contrast fibres with few if any nucleosomes (Fig. I-3a). These fibres were about 10 nm wide. Occasionally completely smooth fibres were present interspersed with nucleosomal chromatin (Fig. I-3c). The width of these fibres is such that the DNA must be covered in protein, although a beaded structure is not apparent. Naked DNA is only 2 nm in width. It is not clear whether such an unbeaded conformation occurs in vivo, or is merely a preparative artefact. My later observation that chromatin of these cells when spread under better conditions is almost entirely nucleosomal in appearance suggests that smooth chromatin strands are indeed a spreading artefact.

Transcription figures were rarely seen in preparations from these cells. Their frequency varied between preparations. It is not clear whether this is a real effect or because of the small proportion of a total cell population that is analysable with this technique, thus resulting in large differences in the origin of the chromatin on any preparation. Spreading conditions might also affect the amount of transcription seen. Of forty preparations only fourteen had transcription figures present.

Transcription occurred predominantly in the form of isolated RNP fibrils attached to the chromatin axis. These were generally of higher contrast than the DNP, and were identified as RNP by this criterion, and by the fact that they were generally of a more diffusely beaded structure, and

occasionally present as arrays. Replication forks were rare in preparations of cells grown at high inoculation densities, but much commoner in preparations of cells from sparser cultures. They always took the form of replication "bubbles" (Fig. I-4), which were never broken. Thus putative transcripts, with a single free end, were scored as such, rather than as broken replication forks.

In the majority of investigations made to date, (Amabis & Nair, 1976; McKnight & Miller, 1976; McKnight et al., 1976; Puvion-Dutilleul & Bernadac, 1976; Puvion-Dutilleul et al., 1978; Busby & Bakken, 1979) transcription units have been measured. If as in all the above cases, several adjacent RNP fibrils are considered, the data are much more reliable than if isolated fibrils alone are scored. In the case of X. laevis cultured cells, arrays of fibrils were rare, so that it became necessary to base my studies on the sizes of single RNP fibrils. To supplement these data I decided where possible either to measure the lengths of transcription units, or where this could not be done, the lengths of the terminal (longest) RNP fibrils (determined by Laird Analysis) of less well spread transcription units. The latter type of analysis was eventually done because few of the transcriptional units or arrays I found could be analysed in any other way. The low density of RNP fibrils made accurate determinations of overall transcription complex length difficult. Although foreshortening of the RNP occurs, so that an RNP fibril appears shorter than the DNP from which it is transcribed, measurements of terminal (longest) fibrils of arrays do approximate more closely to transcription unit size than do measurements of isolated RNP fibrils.

I photographed and analysed all clear instances of transcription that I found. A transcription unit is defined here as two or more adjacent RNP fibrils closer together than an arbitrary distance, which I set at 1 μm.

Groups of two RNP fibrils spaced more than 1 μm apart were considered to belong to different transcription units. In general, transcription took the form either of isolated fibrils separated by several microns of chromatin, or of arrays of fibrils separated by fractions of a micron. In practice no transcription figures separated by distances of slightly under or slightly over one micron were observed.

In rare instances, arrays were well spread enough for a so-called "Laird Analysis" (Laird & Chooi, 1976; Laird et al., 1976) to be done. The point of initiation of transcription of a transcription unit, and hence its true length can be determined if the length of each RNP fibril of the array is plotted against its position in the array (Fig. I-5). A line drawn down the resulting slope will cut the x-axis at the presumptive initiation point. In practice only minimum transcription unit length can be determined, because the size of the longest fibril of the array will depend upon the stage of transcription at which the unit was "frozen". Thus, if a particular gene is being intensively transcribed from the very start of transcription, a certain interval will be required for polymerases to reach the termination point of the gene. If the chromatin is spread and fixed before this point, even a Laird Analysis will give an underestimate of transcription unit size.

In many of the systems studied, ribosomal transcription units appear as the classical "Christmas Tree" figures (Derksen et al., 1973; Hamkalo & Miller, 1973; Trendelenburg et al., 1973; Berger & Schweiger, 1975a, b, c; Franke et al., 1976b; Puvion-Dutilleul et al., 1977a; Reeder et al., 1977; Trendelenburg & Gurdon, 1978). Their tandem repetition, amplification to form free nucleoli, and biochemical analyses of gene length and product size all support the idea that these structures do indeed represent the genes for 28S and 18S ribosomal RNA. Determinations of initiation point are easy because of the clear fibril gradients and high transcriptional activity of these genes.

The majority of arrays found in many tissue types are of a different conformation and are thought to represent nonribosomal transcription (Angelier & Lacroix, 1975; Derksen, 1975; Laird & Chooi, 1976; Laird et al., 1976; McKnight & Miller, 1976; Puvion-Dutilleul et al., 1977a; Harper & Puvion-Dutilleul, 1979 in press). They are characterized by a low RNP fibril density, absence of a clear gradient of fibril lengths, and the fibrils themselves are often much longer than those of ribosomal transcription units. Laird Analysis gives much more equivocal results, and in many cases, the initiation point cannot be determined with any certainty. In my analysis, quantitative measurements included only those transcription units defined as nonribosomal by the above criteria. Laird Analyses were done where possible.

In Xenopus cultured cells, the lengths of isolated RNP fibrils fell around a median value of about 0.44 μm (Table I, p54 Figs I-6 and I-7a, b). The RNP lengths extended over a range of 2.1 μm (Table I, p54 Fig. I-6). In one case an isolated fibril, at least 2 μm long, and possibly consisting of two fibrils entangled, was found (Fig. I-7b). Unfortunately no arrays were found well enough spread for either their length or the length of their terminal fibril to be measured (Fig. I-7c).

In cells grown at a higher concentration, a few analysable arrays were photographed. Laird Analysis of one of these gave a length of 3.5 μm (Fig. I-8a). A second array about 2.8 μm long but not further analysable, was seen (Fig. I-8b). Arrays were too rare for any quantitative measurements to be made, although they were of a size order found in other cell types (Puvion-Dutilleul & Bernadac, 1976; Puvion-Dutilleul et al., 1978; Busby & Bakken, 1979).

TABLE I.

Median lengths of isolated RNP fibrils, terminal fibrils of multifibril transcription complexes, and transcription complex length in different cell types.

Cell type	Isolated fibrils (μm)	Range (μm)	Sample size	Terminal fibrils (μm)	Range (μm)	Sample size	Transcription complexes (μm)	Range (μm)	Sample size
X. laevis cultured cells	0.44	2.1	35	-	-	-	-	-	-
T.c. carnifex cultured cells	0.33	1.03	8	0.4	0.2	1	very long (10-20μm)	-	2
X. laevis liver	0.66	6.3	56	0.4	3.6	8	1.93	1.31	2
T.c. carnifex liver	0.60	6.5	50	0.95	9.6	22	3.21	-	1
N. maculosus liver	1.1	9.13	30	1.96	7.08	16	2.42	2.3	3
T.c. carnifex neurulae	0.82	2.3	43	1.3	1.4	6	2.86	-	1
X. laevis cultured cells plus cortisol	0.74	7.5	51	0.65	7.1	18	3.11	0.68	2

Ribosomal genes were found only on one or two preparations. It is not clear why this should be so, for phase contrast observations indicated that these cells have prominent nucleoli. However, the fact that the genes are tandemly arranged may lower the probability of their detection unless a large amount of chromatin is examined. Only smallish clumps of ribosomal genes were seen, presumably of unamplified cistrons (Fig. I-9). There was no indication that transcription of circular DNA was occurring. Only three measurable ribosomal transcription units were found, having a mean length of 3.2 μm, (Table IX) which is greater than Scheer's estimate (1977) ov 2.2 μm for X. laevis oocyte ribosomal genes. This may be due to stretching of the chromatin. These ribosomal genes had the characteristic "Christmas Tree" structure, with prominent polymerases. In my preparations clear RNA polymerases were only present on ribosomal genes, and were consistently larger and more contrasted than nucleosomes, being about 20 - 24 nm in diameter. This may be because ribosomal and nonribosomal genes are transcribed by different polymerases (Roeder et al., 1970; Biswas et al., 1975) although the size differences between these are probably not great enough to be visible in a Miller spread. On the whole, attached RNP was sparse and of low contrast, perhaps due to a high endogenous nuclease activity in these cells. No clearly traceable spacers were seen.

The appearance of the primary transcripts varied between different preparations, but most often they were compact and of higher contrast than their chromatin axes. Only in one case (Fig. I-7b) was a clearly beaded structure observed, the beads being about 14 nm in diameter and probably analogous to the RNP particles described by Sommerville (1973). In most of my preparations, the subunit structure of primary transcripts, though evident, was usually less discrete than that of the DNP axes. In the case above (Fig. I-7b) the beaded structure was very clear, suggesting that in some

TABLE X.

Lengths of ribosomal transcription complexes from culture cell chromatin.

Xenopus laevis.

Low inoculation density cultures

Treatment	Matrix unit length (μm)	Mean (μm)	Spacer length (μm)	Mean (μm)	Repeat unit length (μm)	Mean (μm)
Control	2.59	3.2	-	-	-	-
	3.70		-		-	
	3.2		-		-	
+ cortisol	3.3	2.7	1.44	2.01	-	4.9
	2.7		-		-	
	2.46		3.1		5.7	
	2.52		1.5		4.1	
	2.55				-	
	2.65				-	
	2.62				-	
	2.74				-	

TABLE XI.

High inoculation density cultures (after Laird Analysis)

Treatment	Matrix unit length (μm)	Mean (μm)	Spacer length (μm)	Mean (μm)	Repeat unit length (μm)	Mean (μm)
Control	-	-	-	-	-	-
+ cortisol	1.65	1.45	-	-	-	-
	2.26		-		-	
	0.54		-		-	
	0.82		-		-	
	1.96		-		-	

cases at least, broken replication forks might be mistaken for RNP molecules. Forks themselves were generally found in tandem arrays (Fig. I-4). of which even very long ones were never broken, which argues against this interpretation. The DNP itself appeared on the grid as very long strands, sometimes extending over several grid squares, which again indicates that the degree of breakage was probably very small, as does the observation that conformations perhaps originating from broken forks with 2 ends i.e were not seen.

Because all transcriptional figures seen were photographed, the percentage of different types of transcription figure will be a valid index of the relative amounts of different sequences being transcribed. It could further provide a new criterion with which to characterize stage specific transcription. In X. laevis culture cells, single RNP fibrils comprised 94.4% of the total transcription whereas arrays of two fibrils closer together than 1 µm were entirely absent. Arrays of three or more fibrils comprised a further 5.6% of the total transcription observed (Table II, p56). Such a distribution is similar to that obtained for Strongylocentrotus purpuratus (Busby & Bakken, 1979) although the small sample size makes my data rather incomplete. This low frequency of transcriptional figures is in agreement with the finding that tissue culture cells express only a minimum of genes, those determining the "household functions" common to every cell type. Both growth rate (Appendix II) and Mitotic Index of the cells were characteristic of a slowly growing and metabolising cell line.

Due to the low transcriptional activity of these cells I was not able to determine the percentage of the total chromatin comprising transcription complexes (McKnight & Miller, 1976).

TABLE II.

The frequency of different types of transcription complex expressed as percentage of the total number of transcription complexes found in different cell types.

Cell type	Number of isolated fibrils separated by > 1 μm	Groups of 2 fibrils separated by < 1 μm	Arrays of 3 or more fibrils	Total no. of complexes	A. % isolated fibrils	B. % groups of 2 fibrils	C. % groups of ≥ 3 fibrils	% all types of multifibril array (B + C)
X. laevis cultured cells	35	-	2	37	94.6	-	5.4	5.4
T.c. carnifex cultured cells	8	1	2	11	72.7	9.1	18.2	27.3
X. laevis liver	56	6	3	65	86.1	9.2	4.6	13.8
T.c. carnifex liver	50	18	6	74	67.6	24.3	8.1	32.4
N. maculosus liver	30	11	5	46	65.2	23.9	10.9	34.8
T.c. carnifex neurulae	43	5	4	52	82.7	9.6	7.7	17.3
X. laevis cultured cells plus cortisol	51	13	7	71	71.8	18.3	9.8	28.1

(a) Packing ratios

(1) DNA packing ratio (DNA P.R.) of transcriptionally inactive regions

In a further attempt to characterize stage-specific transcription I decided to examine DNA and DNA/RNA packing ratios in these cells (Laird & Chooi, 1976; Laird et al., 1976). In later preparations both transcriptionally active and inactive chromatin appeared beaded and the characteristics of these beads suggested that they were nucleosomes. Biophysical and biochemical data demonstrate that on average one nucleosome and its adjacent filament on one side contain some 200 base pairs of DNA. If so, then the average number of beads ($\bar{x}$) per μm of chromatin will contain $\bar{x} \times 200$ base pairs of DNA. The DNA packing ratio, or the length of β-structure DNA per μm of chromatin is therefore

$$\left(\bar{x} \times 200 \text{ bp/}\mu\text{m chromatin}\right) \times 3.4 \times 10^{-4} \ \mu\text{m/bp}.$$

(1 bp β-structure DNA measures 0.34 nm or 3.4×10^{-4} μm)

For determinations of packing ratio the number of nucleosomes on each of 10 randomly selected, straight, transcriptionally inactive regions of chromatin, 1 μm in length, was calculated. A preparation made under spreading which I found resulted in high contrast and good preservation of structural detail was used and give a DNA packing ratio of 2.1 (Table III, p57). The mean diameter of nucleosomes in this preparation was 14.7 nm ± 2.5 nm, slightly larger than the generally accepted size, and probably a result of rotary shadowing.

Earlier preparations showed variable degrees of disruption of the nucleosome structure so that, in my experience, packing ratio varied between 1.0 (apparent lack of nucleosomes, with assumed maximal stretching of the chromatin) and a maximum of 2.1. It is now known that the detergent Joy

TABLE III.

The DNA packing ratio (μm of β-structure DNA per μm of chromatin) of transcriptionally inactive regions of the chromatin of different cell types, mean nucleosome diameter, and dispersal conditions for each preparation.

Cell type	DNA packing ratio		Number of 1 μm regions of chromosomes analysed	Mean nucleosome diameter (nm)	Method of visualisation	Sample size	Dispersal time (min)	Joy concentrations (%)
X. laevis cultured cells	2.1		10	14.7 ± 2.5	stain and shadowing	10	15	0.1
T.c. carnifex cultured cells	1.88		10	11.9 ± 2.4	stain and shadowing	10	20	0.1
X. laevis liver	1.84		10	17.4 ± 5.8	stain and shadowing	10	60	0.1
T.c. carnifex liver	1.63		10	16.0 ± 4.0	stain only	10	75	0.2
N. maculosus liver	1.52	1.64 1.4	10	15.4 ± 5.5	stain and shadowing	10	15	0.1
T.c. carnifex neurulae	1.35		10	-	stain only	10	75	0.2
X. laevis cultured cells plus cortisol	1.87	1.98 1.77	10	- 13.8 ± 5	- stain and shadowing	- 10	15 44	0.1 0.1
T.c. carnifex oocyte ribosomal genes	1.35		10	-	-	-	75	0.2

removes chromatin proteins (Scheer, 1978), and the long dispersal times I used are probably responsible for this variation. Thus, unlike the case of chromatin spread in "pH 9 water" it is not clear how valid my data on packing ratio are; they probably represent only an approximation to the *in vivo* condition.

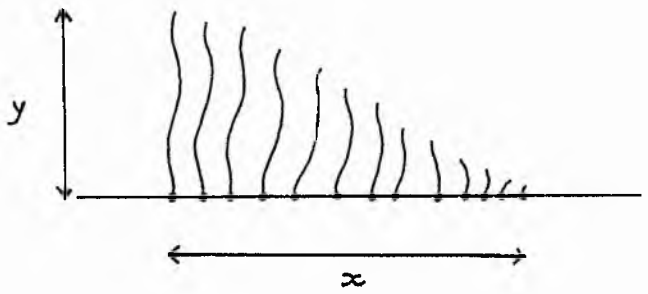
(2) DNA/RNA packing ratio (DNA/RNA P.R.)

The DNA/RNA packing ratio is a measurement of the amount of foreshortening undergone by the primary transcripts of a transcription unit, and is expressed as a ratio between the length of the terminal transcript of an array and the total β-structure DNA length of the transcription unit (Laird & Chooi, 1976; Laird *et al.*, 1976). DNA/RNA packing ratio was determined as described by Laird.

In favourable cases, for arrays consisting of three or more transcripts, a Laird Analysis was done to give an estimate of transcription unit length, and to determine whether or not the fibrils indeed belonged to the same transcription unit. DNA/RNA packing ratio was determined for arrays from preparations where the DNA packing ratio of transcriptionally active regions was known. Arrays whose DNP axis was beaded were considered. I assumed beads on the axis lacking attached transcripts, to be nucleosomes. However, without Sarkosyl-treatment, there is no reliable way of determining whether such beads are nucleosomes rather than polymerases. The contrast and size of the beads resembled nucleosomes rather than polymerases in my preparations, but as polymerases were only observed on ribosomal transcription units, the significance of this is debatable. The number of such beads per array was used to calculate DNA PR for transcriptionally active regions.

Length of array x DNA packing ratio = length of β-structure DNA
(of transcriptionally active regions) in T. unit.

$$\text{DNA/RNA packing ratio} = \frac{\text{length of array x DNA packing ratio (active regions)}}{\text{length of terminal fibril of array}} = \frac{x}{y}$$



In a few cases, DNA/RNA packing ratio was determined for transcription complexes on unbeaded chromatin, taking the DNA packing ratio of the transcription unit axis to be 1.0 (fully stretched). When values for the DNA packing ratio of transcriptionally active regions were not available, the value for inactive regions was employed, to calculate DNA/RNA packing ratio.

Using these criteria as defined by Laird I found that T.c. carnifex oocyte ribosomal genes (Appendix III) (Table VI, p59) had a DNA/RNA packing ratio of 13.3. This is rather high and may be due to insufficient spreading of the preparation, which was one of the earliest that I made. In X. laevis culture cells the only clear array was found in a preparation where little or no nucleosome structure was observed. Thus I assumed the DNA packing ratio to be 1.0. Table VI shows that this array (Fig. I-8a) had a DNA/RNA packing ratio of 10 - 12.

TABLE VI.

The DNA/RNA packing ratio (degree of foreshortening) of terminal (longest) RNP fibrils of transcription complexes from different cell types.

To determine the length of β-structure DNA per transcription complex, the DNA packing ratio of transcriptionally active regions of chromatin was used, where available (see Tables III and IV).

Cell type	Transcriptional complex length (μm)	DNA length (μm)	DNA packing ratio	Length of terminal RNA fibril of complex (μm)	DNA/RNA packing ratio
X. laevis cultured cells	3.48	3.48	1.0	0.29-0.35	10-12
T.c. carnifex cultured cells	-	-	-	-	-
X. laevis liver	2.59 1.28	4.2 2.1	1.62 1.62	3.42 1.70	1.23 1.23
T.c. carnifex liver	-	-	-	-	-
N. maculosus liver	3.79 1.52	8.4 2.16	1.42 1.42	0.22,0.32 1.49	17-25 1.45
T.c. carnifex neurulae	> 2.86	> 2.86	1.0	0.72	⩾ 4.0
X. laevis cultured cells + cortisol	13.8 3.45 2.77	24.0 6.0 4.5	1.74 1.74 1.63	4.2 1.54 0.22	5.71 3.9 20.5
T.c. carnifex oocyte ribosomal genes	2.15 2.32 3.12 1.94 2.2 1.99 2.13 1.99 2.2 2.3	2.9 3.13 4.21 2.62 2.97 2.68 2.87 2.68 2.97 3.1	1.35	0.21 0.24 0.24 0.32 0.19 0.19 0.27 0.24 0.24 0.19	13.8 13.04 17.5 8.2 mean 15.6 13.3 14.1 10.6 11.2 12.4 16.3

(b) Mitochondrial DNA

(1) Replication

During my investigation of transcription in X. laevis cultured cells, I noticed, in whole cell preparations, rare circular DNA molecules. About one third of these bore structures resembling the "bubbles" characteristic of replicating DNA (Fig. I-10a,-c). These DNA circles were always "smooth" with a width, in rotary shadowed preparations of 12 - 16 nm. Although rotary shadowing increases dimensions by metal deposition, a comparison with nuclear chromatin on the same preparation suggests that these rings were covered with protein, rather than being naked DNA.

The putative "fork" structures always showed a much greater degree of contrast that the rest of the circle, and in this were comparable to RNP from the same preparation.

It is not clear why this should be so, for it is not likely that newly replicated DNA should be associated with twice as much protein. That the proteins might be qualitatively different and thus vary in their staining properties is also hard to understand, and this casts doubt on the idea that the "bubbles" are indeed forks.

The circles (Table VII, p61) were all roughly the same size, around 4.4 μm in contour length, which is similar to values previously obtained for mitochondrial DNA (Dawid & Wolstenholme, 1968; Wolstenholme & Dawid, 1968; Wolstenholme et al., 1973b; Koike & Wolstenholme, 1974; Borst & Grivell, 1978; Pinon et al., 1978). That they are mitochondrial in origin is supported by the fact that they were only seen in whole cell preparations. Their size is such that they could not be mycoplasma genomes, and there was little or no bacterial contamination on my preparations. Finally, their uniform length argues against an interpretation that they are randomly broken pieces of DNA that have recircularised.

The circles I observed always lacked a beaded structure, unlike those observed by Pinon et al. (1978) who observed large 20 nm beads in X. laevis mitochondrial circles. To my knowledge, my findings are the first such obtained with the Miller technique.

When the putative "fork" regions of the circles were measured it was seen (Table VII, p61) that they all fell between 0.15 and 0.29 μm in length. When the measurements were expressed as percentages of the total circle length, in order to compensate for variation in spreading conditions they all fell between 4.7 and 6.7%. Tandem arrays of forks were never observed. If these structures are indeed forks, this would imply that replication of these circles is synchronous. However, my pictures were obtained from several preparations made at different times, from different cultures of randomly growing cells, which argues against this. The results may have been artefactual due to the small sample size, but nonetheless it is reasonable to assume that circles with many different lengths of "fork" would have been found.

TABLE VII.

Characteristics of putative mitochondrial circles.

Replicating forms

Treatment	Circle length (μm)	Mean μ	Fork length (μm)	Mean μ	Percentage of total contour length comrised by replication fork	Mean %
- cortisol	-	4.39	0.15	0.21	-	5.84 ± 0.84
	4.34		0.29		6.68	
	4.44		0.19		5.0	
+ cortisol	4.97	4.64	0.28	0.24	5.6	5.15 ± 0.45
	4.31		0.20		4.7	
Mean of total sample		4.52		0.22		5.69 ± 0.99

TABLE VIII.

Transcribing forms

Treatment	Circle length (μm)	Mean (μm)	Transcript length (μm)	Position on circle (μm)	Length of "transcription unit" (μm)
- cortisol	5.02	4.65	-	-	-
	4.98		0.2	-	-
	4.37		1.3	0	3.15
	-		1.13	0.38	
	4.24		0.23	-	-
+ cortisol	3.39	4.43	-	-	-
	5.47		0.21	-	-
	-		1.01	-	-
	-		0.67	-	-
Mean of total sample	-	4.58	-	-	-

In Novokoff rat ascites hepatoma cells (Koike & Wolstenholme, 1974) replication of mitochondrial DNA circles is discontinuous. As with Drosophila mitochondrial DNA (Goddard & Wolstenholme, 1978) replication is unidirectional from a specific origin. When fork lengths are expressed as a percentage of total circle length, it is found that over the first 2% - 44% of the circle, fork lengths fall into 7 or 8 discrete size classes. This suggests that over the length of the mitochondrial circle there are several points at which DNA replication can be blocked. Although the replication intermediates found in these cells are also characteristic of normal chick and rat liver cells (Koike & Wolstenholme, 1974) it is not clear whether this type of "discontinuous" replication occurs in other mitochondrial DNAs. There is no direct evidence in Drosophila, X. laevis or mouse L-cells, that mitochondrial DNA synthesis is discontinuous and this also applies to Crithidia kinetoplast DNA (Berk & Clayton, 1976; Manning & Wolstenholme, 1976; Barat et al., 1977; Goddard & Wolstenholme, 1978). The nature of mitochondrial DNA replication in yeast (Borst & Grivell, 1978) is still not clear.

If my pictures do indeed represent replicating mitochondrial DNA molecules the presence of only one size class of "blister" is certainly consistent with such a discontinuous mode of replication. However, the Miller spreading methodology, like biochemical extraction procedures, (Koike & Wolstenholme, 1974) may result in the removal or dilution of factors necessary for replication to proceed over "stop points", thus giving rise to discrete size classes of replication fork that would be absent in vivo. If this is so, it is hard to understand why "stop points" should be necessary in the first place, if replication over them is continuous in vivo. The length distribution of the forks is thus unlikely to be artefactual. In qualification, however, it must be considered that isolation

and spreading procedures need not necessarily terminate replication at the same place as that which it had reached when preparation was made.

All the rings that I found were fairly well spread. None had the supercoiled structure reported by other authors (Dawid & Wolstenholme, 1968; Koike & Wolstenholme 1974). Oligomeric molecules consisting of series of linked circular molecules were not seen (Wolstenholme et al., 1973b; Berk & Clayton, 1976).

(2) Transcription

Among the DNA circles that I found, there were some that appeared to be in the process of transcribing RNA. These were commoner than the putative replicating circles. The size of these circles fell around a mean of 4.7 μm, which suggests that they were mitochondrial genomes (Table VIII). I consistently obtained a lower value for circle length than the previously determined value of 5.35 μm for X. laevis mitochondrial DNA (Dawid & Wolstenholme, 1968) but this may be due to differences in spreading conditions.

The presumptive RNA (Figs I-11a-d and I-12a) of these circles varied in length from 0.2 to 1.13 μm (Table VIII), a size distribution overlapping that for X. laevis primary transcripts from nuclear chromatin. In appearance it exhibited a degree of contrast greater than that of the circles to which it was attached and similar to that of the putative "forks" seen on other circles. It did not have any apparent subunit structure. This may be more artefactual than real, as RNP from nuclear chromatin had the same appearance on this preparation, whereas it had a distinctly particulate structure in other preparations.

In one instance it was possible to measure the separation between two putative transcripts attached to one ring, and to make a Laird Analysis (Fig. I-12a-c). The two molecules were about 0.4 μm apart, and attached to a circle 4.4 μm in length. If the separation between them is indeed 0.4 μm (Fig. I-12b) then a Laird Analysis (see above for definition) suggests that they might form part of a transcription unit at least 3.2 μm long, i.e. 73% of the entire contour length of the circle. If, on the other hand, they are separated by 4.0 μm of DNA, then to obtain the observed conformation, transcription must proceed without termination, more than once around the circle. It is not possible to differentiate between these two alternatives here. The second alternative cannot be eliminated because small DNA circles bearing transcripts longer than the contour length of the ring to which they were attached and therefore indicative of transcription moving several times round the ring, have been observed in Dytiscus oocytes (U. Scheer, pers. comm.).

Although I did not succeed in my efforts to isolate X. laevis mitochondria from cultured cells, and so lyse them and visualise their DNA, the size distribution of those circles that I did find does suggest that they are mitochondrial. To my knowledge, the only pictures of transcribing mitochondrial circles that have so far been published, are those of Chooi & Laird (1976), which indicated a coupling of transcription and translation.

It is known that the mitochondrial genomes of yeast and Neurospora crassa (Dawid, 1970; Borst & Grivell, 1978; Hahn et al., 1979) contain genes for the mitochondrial ribosomal RNAs, and about 12 - 15 transfer RNA genes used in mitochondrial protein synthesis. The mitochondrial genome also codes for an unknown number of proteins of the mitochondrial inner membrane (Bos et al., 1978). If my pictures do represent mitochondrial DNA circles

in the process of transcription, then a transcription unit size of 3.2 μm would imply the production of polycistronic RNA, which would also be the case, if transcription proceeded around the ring more than once. Unfortunately I know of no studies on mitochondrial primary transcripts. The presence or absence of processing would modify this conclusion. The results of Dawid (1970) deal only with bulk RNA extracted from X. laevis mitochondria, and demonstrate that three main size classes of RNA exist, 21S, 13S, and 4S RNA, of which the first two are structural components of the mitochondrial ribosome.

(c) Rings attached to X. laevis chromatin

In one preparation of X. laevis culture cell chromatin, small rings were seen to be attached to the DNP (Fig. I-13a-d). That these were not merely accidental twists in the chromatin was suggested by their great numbers and by the fact that many occurred on lengths of DNP that had been stretched by centrifugation (Fig. I-13a). Finally, they were found only on this one preparation, which was characterized by a dispersal time of only 15 min. A short dispersal might be expected to preserve details of chromatin secondary structure that might otherwise be disrupted.

The small circles seemed to be of various types, some resembling twists in the chromatin (Fig. I-13b), and others the putative excision intermediates seen on lampbrush chromosome RNA (Fig. I-13c) (Scheer et al., 1976; Hill, 1979 in press). A third class of circle resembled primary transcript molecules whose free end was somehow associated with the chromatin axis (Fig. I-13d).

I decided to measure the sizes of these circles, and the contour lengths of a random sample fell around a median of 0.4 μm, with a range of

2.3 μm (Fig. I-14a). The distribution was skewed slightly towards the right, and for this reason the median value is more meaningful than the mean. No discrete size classes were observed.

This distribution overlaps the one obtained for X. laevis culture cell primary transcripts from nuclear chromatin so that it is conceivable that the rings might be RNP.. However, they were more frequent than RNP, which may rule this out. Moreover their contrast was similar if not identical to that of the chromatin, and, although having a beaded structure, it resembled DNA rather than RNA. These facts argue against such an interpretation, but it would be informative to spread the chromatin of these cells in the presence of Sarkosyl, which removes nucleosomes, but not RNA polymerases, from the chromatin (Scheer et al., 1977).

The possibility exists that the rings are "supernucleosome" breakdown products. "Supernucleosomes" in the sense of Hozier et al. (1977) are higher order chromatin packing structures containing 8 - 9 nucleosomes. Dispersal removes most of the protein from chromatin, but enough might be left to hold the remnants of the supernucleosome together. This could be resolved by counting the beads on the rings, but as it is not clear how heterogeneous supernucleosomes are, in their nucleosome content, this would not be very illuminating. Unfortunately, the nucleosomes on the preparation where the rings were found were so closely packed that it was not possible to count them accurately. Some of thelarger rings could represent structures of totally different origin.

A third possibility is that the rings could represent putative excision intermediates. This is supported by the structure of some of the loops (Fig. I-13c). However, even these might represent supernucleosomes less dissociated than the open rings. Excision of DNA sequences implies that

amplification of some sort occurs if information is not to be lost from the genome. Ribosomal amplification is the commonest type of DNA sequence amplification, but cannot be occurring here as most of the rings are much less than the necessary 2 μm in length. Evidence exists that in Daucus carota, nonribosomal DNA amplification occurs. In explants of carrot tissue, a heavy satellite occurs that is not detectable in the DNA of the whole carrot plant, and metabolic turnover of the DNA occurs (Schäfer et al., 1978). Treatment of carrot plants with gibberellic acid results in the unique sequence fraction of untreated carrot DNA hybridising to the middle-repetitive fraction of the DNA of treated plants (Schäfer & Neumann, 1978). Thus the unique sequence class is labile and apparently amplified. These results do not rule out the amplification of nongenic sequences because the unique sequence fraction includes a large number of noncoding sequences. The results of Strom (Strom & Dorfman, 1976) suggest that during differentiation in the chick limb bud, cartilage specific sequences are amplified. However, it is not yet known definitely whether very small, noncoding sequences are amplified and excised.

(d) Coincident transcription and replication

Electron microscope evidence for the coupling of transcription and replication has been obtained from Drosophila melanogaster embryos (McKnight & Miller, 1977; McKnight et al., 1977). Here, homologous RNP fibre arrays occur on the two arms of replicating regions of DNA.

Only in two instances were putative replicating regions bearing RNP, found (Fig. I-15a-b). One of these (Fig. I-15a) was equivocal, perhaps consisting of two strands of DNP crossing each other, the end of one of which had broken and then become more condensed in structure. However, the contrast and width of the putative RNA molecule were much greater than that

of the DNA, which does have a few nucleosomes on it. Such forms, when found, were not included in transcript length distributions.

(e) Higher order packing structures

One complete partially spread nucleus was found where the chromatin appeared as thick "cables", considerably thicker than the usual form of chromatin from these cells (Fig. I-16). At high magnifications the cables were seen to have a segmented structure which in some places had broken down to release spirals perhaps consisting of linked beads (Fig. I-17a-b). One odd characteristic of the cables was that in places they appeared double (Fig. I-18a). The cables were, on average, about 29 nm wide, whereas the segments were about 16 nm wide. The beads in the loose regions had a diameter of about 15 nm (Fig. I-19), and the double regions each had a width of 15 nm.

This cable structure may be analogous to the 20 - 30 nm chromatin fibres described in chicken erythrocyte chromatin and other systems (Olins, 1977; Renz et al., 1977), and which consist of superpacked nucleosomes. The structures I found are probably more similar to the 30 - 50 nm solenoid structure derived from a coiled 10 nm "nucleofilament" (Finch & Klug, 1976), as discontinuous clumps of nucleosomes were not seen (Olins, 1977). The do-called "supernucleosomes" were also absent from preparations of X. laevis culture cell chromatin.

The integrity of the 30 nm knobby chromatin fibre is dependent on the presence of histone H1 and at low ionic strength, as is the case for Miller spreads, the fibre is disrupted (Renz et al., 1977). This is probably why I only observed this type of structure once. Organic drying agents such as ethanol and metylbutane also distort chromatin structure to some extent,

so that the structure I saw may bear little relation to chromatin structure *in vivo*. Differences in ionic strength and isolation procedures are perhaps responsible for the coiled appearance of the chromatin fibres, which appear as linked clumps or large beads after mild digestion and lysis of nuclei. Biochemical evidence suggests that the 30 nm fibre unfolds to produce nucleosome chains. My observations are in agreement with this. The large size (15 nm) of the beads may be due to metal shadowing of the preparation.

(f) Contrast

The contrast of many of my preparations was poor, and evidence from various sources shows that dispersal of chromatin in Joy results in the removed of some chromatin proteins, thus reducing its stainability. Nucleosome structure is also disrupted and it was for this reason that I did not do any studies on nucleosome distribution. Any distributions that occur are likely to be largely artefactual.

(ii) *Triturus cristatus carnifex* cultured cells. Characteristics of transcription

As with *X. laevis* cultured cells, during my investigation of transcription, I used a variety of methods to visualise RNA synthesis. I obtained most of my results with the modification involving the lysis of Nonidet P-40 extracted nuclei, but a number of data were obtained with later modifications of the Miller technique which resulted in greater contrast and preservation of structural detail.

Most of the preparations were obtained from cells inoculated at twice the usual subculture density (8.6×10^5 - 1.8×10^6 cells/large vessel),

and grown for two days at 25°C, until they had attained log phase. Growth phase was checked by determining growth rate of the cells at various inoculation densities (Appendix II). I had technical difficulties with isolation and dispersal of the cells at lower cell concentrations, which necessitated this. Triturus cells are 3 - 6 times larger than those of X. laevis, and so a culture vessel of the same size as those used for X. laevis will contain fewer cells. Many of these are broken due to their fragility, and the Nonidet pellet is often difficult to see, and therefore easy to disrupt and lose. Later on I found that adequate preparations could be obtained from cells grown at lower inoculation densities, and the most effective method was to grow cells in Petri dishes, scrape them off in "Joy", and disperse them immediately. However, transcription figures were always extremely rare in this cell line and little or no difference in their frequency occurred in different types of preparation.

Dispersal of Nonidet P-40 extracted nuclei resulted in the presence of large nucleus-sized clumps of chromatin on the grids. These were very compact (Fig. I-20a), although a few fragments occurred. Nearly all had a clearly beaded structure (Fig. I-20a-b) and the beads were approximately 29 nm in diameter. Thus they appear to be analogous to the "supernucleosomes" described by other authors (Olins, 1977; Renz et al., 1977). Structures resembling the "cables" observed in X. laevis culture cell chromatin were not observed.

The clumps of beaded chromatin were usually surrounded by halos of well-spread filaments, almost always entirely smooth and devoid of nucleosomes. These chromatin filaments were usually of low contrast (Fig. I-20b) and measured about 13 nm in stained preparations, thus demonstrating that in spite of the absence of nucleosomes, the DNA was associated with proteins.

TABLE IX.

Lengths of clearly measurable transcripts from a long transcription complex found in a preparation of *T.c. carnifex* cultured cells

Cell type	Transcript length (μm)
T.c. carnifex cultured cells	2.7) adjacent 10.8) 5.9 4.5 1.9

In all these preparations, transcription occurred on "smooth" DNA filaments of this type, but this was thought to be artefactual, due to extended dispersal in 0.2% Joy. Later preparations showed many nucleosomes, with which transcripts could be associated.

Transcription figures were rarer in these preparations than in X. laevis culture cell chromatin. Of 34 preparations, only 4 showed clear instances of transcription. The great majority of these consisted of single isolated RNP fibrils (Fig. I-21a-b), although two putative arrays were seen. The criteria used for selection and measurement were the same as for X. laevis preparations.

Isolated RNP fibrils had a mean length of 0.33 μm (Fig. I-22) but the sample size (Table I, p54) was small, so that it is debatable whether these results have any statistical significance. One group of two fibrils was found, and here the terminal RNP fibril measured about 0.4 μm.

Only two arrays of RNP fibrils were found in these cells (Figs I 23a-b and I-26). Both consisted of long stretches (9 - 15 μm) bearing long, rather sparse RNP transcripts, and reminiscent of transcription on T.c. carnifex lampbrush chromosomes (Scheer et al., 1976); in one case the array stretched over most of one grid square. Neither array showed a clear length gradient of RNP fibrils and thus were clearly of nonribosomal origin. It was not possible to make a Laird Analysis of either of these arrays as, for a large part, they were obscured by chromatin. I cannot say how many transcription units were involved. A third array consisting of four very long transcripts was found but the ends of the fibrils were not traceable.

Several of the transcripts on one array (Figs I-23a, I-24 and I-25) were measurable. The longest (Fig. I-23b. Table IX, p90) was 10.8 μm, and as such compares well with the longest transcripts found in T.c. carnifex

lampbrush chromosome arrays. The others measured were between 2 and 6 μm in length. It is interesting to note that the 10.8 μm transcript is adjacent to one of only 2.7 μm, and may indicate the occurrence of processing. Some parts of this array show short gradients of fibrils which may rule out random breakage as the origin of the size difference of these two transcripts. In certain transcripts, secondary structure was extensive (Fig. I-24).

The second array bore very long fibrils but it was not possible to photograph the ends of many of these (Fig. I-26), so that further analysis was not possible. Long arrays of this type were never observed in X. laevis. To my knowledge, this is the first time that long fibrils of this type have been found in chromatin from cultured cells.

The DNP axes of the two arrays lacked nucleosomes, but their width (over 10 nm) implied some association of the DNA with chromatin proteins. The transcripts had a diffuse subunit structure, and in this resembled those found in X. laevis chromatin. Where clearly visible, subunits were up to 20 nm in diameter. In more recent preparations, under better conditions of both cell culture and chromatin spreading, rare isolated RNP fibrils occurred (Fig. I-21a-b) but arrays of any sort were either absent of undetectable. A major drawback of the Miller technique is that only small amounts of chromatin can be conveniently scanned. Spreading of a population of randomly growing cells or nuclei means that chromatin at all stages of the cell cycle is present on the grid, and these may vary in transcriptional activity. For cultured cells, which express relatively few genes it is necessary to perform biochemical experiments to supplement the data from spreads.

As with X. laevis, RNA polymerases were not detectable at the point of attachment of the transcript to its DNP axis. In the first long array

I found, RNP fibrils were distributed fairly uniformly along the DNP axis. In the second one, on the other hand, putative transcripts tended to be arranged in blocks (Figs I-26 and I-27). Although RNP ends were not visible, so that the number of separate transcription units cannot be determined, this may imply that here transcription proceeded in short bursts.

In T.c. carnifex cultured cells (Table II, p56) isolated fibrils comprised 73% of the total transcription, whereas troups of two fibrils made up only 9%. Arrays comprised about 18%. In this T.c. carnifex shows a somewhat greater percentage of arrays than does X. laevis. Because of the small sample size, however, these distributions may be artefactual. They could imply, nonetheless, that although less transcription seems to occur in T.c. carnifex culture cells, a higher percentage of certain sequences are transcribed. On the other hand, the fact that most tissue culture lines are thought to express similar numbers of genes, would seem to militate against this conclusion.

Ribosomal transcription units were never seen. Neither small circles attached to chromatin, nor putative mitochondrial circles were observed. This is unfortunate as the work of Wolstenholme & Dawid (1968) suggests that there is a size difference between the mitochondrial DNAs of anurans and urodelans. Transcription units of opposite polarity were apparently absent (Angelier & Lacroix, 1975; Grainger & Ogle, 1978).

(a) Packing ratios

It was not possible to determine DNA packing ratios for early preparations of T.c. carnifex culture cell chromatin due to the absence of nucleosomes. Because these were the only preparations to show arrays of RNP fibrils, it was impossible to calculate the degree of foreshortening of the RNP.

In a later preparation, the DNA packing ratio of transcriptionally inactive regions was found to be 1.88 (Table III, p57). Nucleosomes were 11.9 nm ± 2.4 nm (rotary shadowed preparation). No arrays were seen.

(b) Coincident transcription and replication

In one of the two long arrays that I found (Fig. I-26) part of the DNA axis appeared double for about 8.5 μm of its length, and had the form of a typical replication "bubble". This region was quite heavily covered in putative transcripts. Both arms of the "fork" appeared to have transcripts attached. As both ends of the "bubble" had attached fibrils, it is possible that the "bubble" really consists of a long piece of chromatin that had looped around the DNP axis. The fact that both arms of the "bubble" apparently bore RNP does not necessarily rule this out, as a piece of transcribing chromatin could have got looped around. However, the fact that the RNP was of similar length on both sides of the "bubble" does make this interpretation less likely.

Although the ends of many of the putative transcripts in the bubble region could not be traced, they were visible at other places along the array, which strengthens the interpretation that this is in fact a transcription figure. Many long transcripts were visible of the size order found in the first long array.

If the fibrils on the bubble are indeed RNP, this structure resembles those already observed in Drosophila embryo chromatin (McKnight & Miller, 1977; McKnight et al., 1977), where homologous arrays of fibrils occurred on each arm of the presumptive replication fork. Replication of DNA is bidirectional in Triturus (Callan, 1972), whereas transcription in this particular array is probably unidirectional, although this cannot be stated

with certainty. If progress of the "bubble" is also bidirectional, replication must be proceeding in a direction opposite to that of transcription.

The results of McKnight et al. (1977) suggest that in the case of Drosophila embryo ribosomal genes, a replication fork cannot enter an active ribosomal gene opposite to the polarity of RNA polymerase movement. In my pictures, one cannot distinguish whether replication started before transcription or vice versa. Transcripts were probably present on both sides of the putative fork (Fig. I-26) which may imply that RNA synthesis was in progress before replication started. Although many pictures have been published of transcription units through which replication has progressed, I know of none where a fork has apparently initiated in the centre of an array, and progressed outwards. However, my pictures should be treated with caution, as the identification of DNP and RNP is not absolutely proven and the number of transcription units comprising this array is unknown.

(4) Higher order packing structures

In many stained preparations of T.c. carnifex culture cell chromatin, clumps roughly the size of individual nuclei, and consisting of spherical subunits about 29 nm in diameter, were found (Fig. I-28). Many of these appeared to be associated with networks of collagen (Fig. I-20a).

In favourable cases the strings of beads, which may be equivalent to the "superbeads" of Renz (Renz et al., 1977; Pinon et al., 1978) and other authors, appeared stretched (Fig. I-29a). Stretched beads were less discrete than when unstretched, but were nonetheless clearly visible. The chromatin fibre between the superbead remnants was some 16 - 19 nm wide, and had no resolvable structure. I did not find any clear instances of a transition between the superbead structure and the unbeaded spread chromatin (Fig. I-29b).

In a few cases, structures resembling partially unwound spirals of nucleosomes were seen (Fig. I-29c). The beads in these structures were indistinct but were about 13 - 19 nm in size, and so may represent nucleosomes. They did not, however have a clumped structure like the superbeads, and it is not clear what relation they bear to the latter.

In preparations of spread whole cells, superbeads were almost entirely absent, as were chromatin clumps of any size. Superbeads were found in greatest numbers in spreads of NP40 extracted nuclei. This suggests that Nonidet P-40 in some way facilitates their visualisation. That they were not an artefact is indicated by the presence of short lengths of superbeads in later preparations, as well as their occurrence in spread chromatin of X. laevis liver cells (Chapter II, this study).

If the beads I found are analogous to the superbeads described by other authors, they differ inasmuch as the nucleosome subunit structure could not be seen (Renz et al., 1977; Rattner & Hamkalo, 1979). This could, however, have been due to my method of visualisation. These putative higher order structures were much more frequent than the cables found in X. laevis culture cell chromatin and may imply a greater degree of metabolic activity in the latter cell line.

The possibility does exist that the superbeads are in some way a preparative artefact, because T.c. carnifex nuclei, at this stage of my work, were routinely spread in 0.2% Joy, whereas X. laevis chromatin was spread in 0.1% Joy. A preparation of X. laevis nuclei dispersed in 0.2% Joy was made, but was unsuccessful, and I have not yet been able to repeat this experiment.

Much evidence suggests that the superbead structure is an universal unit of chromatin packaging (Olins, 1977; Renz et al., 1977). If so it is not clear why "cables" rather than superbeads were found in X. laevis chromatin. Differences in spreading conditions may have caused a distortion and compaction of the superbead structure but it is still difficult to account for the double aspect of the cables. Such double regions were entirely absent from T.c. carnifex culture cell superbeads.

(iii) Incorporation of ^{3}H-uridine by cultured cells

The Miller technique is relatively insensitive, and only a small proportion of the total transcription in a cell or population of cells can be examined. In order to look at the total RNA population, I decided to carry out some biochemical experiments. To determine the rate of incorporation of ^{3}H-uridine by my cultures, cells of each line were labelled with low specific activity ^{3}H-uridine. For each sample (see Materials and Methods) both cell number and total incorporation were determined. The results were expressed as incorporation per cell per hour, and incorporation per cell per 1% cell cycle time. Cell counts allowed me to determine Tau, the mean cell cycle time, for each culture.

I found the mean cell cycle time to be 30.6 hr for X. laevis and 94.2 hr for T.c. carnifex cultured cells (Fig. I-30a-b). The rate of incorporation per cell per hour (Fig. I-31) was similar for both cell lines. When incorporation was expressed as cpm per cell per 1% cell cycle time, T.c. carnifex was seen to incorporate label about 4 times faster than Xenopus (Fig. I-32). As the cell cycle time was about three times greater for T.c. carnifex, this implies that, per cell cycle, the total RNA synthesised per cell was about 4 times greater in T.c. carnifex than in X. laevis.

If one now assumes that pool sizes are the same for the two cell lines, that the rate of addition of nucleotides to RNA is similar, that the two cell types have an equivalent number of sites synthesising RNA, and that the polymerase density of both cell types is similar, this result implies that in T.c. carnifex the sites synthesising RNA are on average about 4 times as long as in X. laevis.

Established cell lines do not express many genes, merely those that are concerned with the "housekeeping" functions required by every cell. It is therefore likely that both my cultures were expressing rather similar numbers of genes.

To further test the above assumptions I attempted to measure polymerase density in these cells, but without success. On the other hand the similarity of thedistributions of different types of transcription figure in spreads of chromatin from these cells do suggest that polymerase density does not differ greatly between the two cell lines.

I was not able to determine pool sizes. The rate of uptake of isotope into a pool affects the incorporation pattern seen. An appreciable amount of TCA-precipitable incorporation had occurred after 15 min of labelling of both cultures, which may suggest that there was little or no lag between the addition of label and its incorporation into RNA. Finally, eukaryotic RNA polymerase rates are very unifrom, with a maximum rate, at 25°C, of less than 2×10^3 nucleotides per minute per polymerase (Kafatos & Gelinas, 1976).

The mostserious objection to my interpretation of these data is that I did not determine how much of the observed incorporation was due to ribosomal RNA synthesis. Thus my rate values may be spurious, and it would

be desirable to try to eliminate ribosomal RNA synthesis. Another objection to my interpretation is the possibility of mycoplasma contamination of my cultures, which was not determined. Uptake of ^{3}H-uridine by mycoplasma would greatly affect any rate differences that might exist.

Triturus cells are about 3 - 6 times larger than X. laevis cells. Thus the concentration of RNA is likely to be similar in both cell types, if one considers the amount of RNA synthesised per cell cycle.

(iv) Size determination of RNA from X. laevis and T.c. carnifex cultured cells

In order to supplement the data from Miller spreads, I decided to carry out a conventional RNA extraction and sizing experiment. Figure I-33 shows that a greater proportion of labelled total cellular RNA is of higher molecular weight in T.c. carnifex than in X. laevis. X. laevis, on the other hand, appears to synthesise more small RNA. T.c. carnifex RNA shows slight peaks at about 58S, 68S and 92S, and there are suggestions of peaks at these positions in the X. laevis distribution. On the other hand the T.c. carnifex distribution is still level when the X. laevis distribution is beginning to tail off.

These data suggest that T.c. carnifex culture cells do indeed make larger RNA than cells of X. laevis. Unfortunately, due to technical problems I was not able to similarly extract and size nuclear RNA from these cells. The distributions discussed here therefore include a large proportion of small cytoplasmic RNA. This, together with a necessary DNAse step in the extraction procedure may be responsible for a less striking difference in size distribution than that observed by Lengyel & Penman (1975). If their data are valid, one might expect a slightly greater mean size difference than 2 - 2.5 in the case of organisms where the C-value difference is greater than five to sixfold, as in the case of Aedes and Drosophila.

Due to the high C-values of these organisms, I found it necessary both to treat whole cells with DNAse, to reduce aggregation due to DNA released by broken cells, and also to treat the nucleic acids with DNAse at various points during extraction. If this was not done, labelled RNA became trapped in large aggregates of DNA which sedimented in the high molecular weight region of the gradients, giving spuriously high count levels in these regions. Although extremely pure enzyme was used one cannot be absolutely certain that contaminating RNAse was absent. Thus any size difference that exists might be further diminished by this procedure.

The phenol extraction method I used increases the likelihood of aggregation of the RNA (Federoff et al., 1977), even after denaturation. I was unsuccessful in my attempt to measure size differences in RNA centrifuged through formamide, where denaturing conditions are present in the gradient itself.

As with the incorporation rate experiment, a large proportion of the total RNA sample consists of ribosomal RNA. In an attempt to eliminate this component, and so accentuate the differences between the nonribosomal populations, I labelled cells in the presence of low concentrations (0.04 μg/ml) of actinomycin D, for a total of 1 hr 25 min. In HeLa cells (Penman et al., 1968) such low concentrations of actinomycin D cause a preferential reduction in the synthesis of 45S ribosomal RNA. However, my cell cultures appear to be sensitive even to low concentrations of this drug, as RNA synthesis was eliminated completely. In this experiment RNA was isolated by a process of enzymic digestion and centrifugation through caesium chloride (Glisin et al., 1974). This technique reduces the degree of breakage of high molecular weight transcripts, as does isolation of RNA from preparative agarose gels (Case & Daneholt, 1976).

Although the RNAs of both cell cultures were coextracted, it would be informative to extract RNAs after mixing the two cell types together following labelling with either ^{3}H-uridine of ^{14}C-uridine, which would give some measure of the degree of degradation that occurred (Lengyel & Penman, 1975).

A final qualification is the possibility of mycoplasma contamination of my cultures, which might affect the size distributions seen, although, if one assumes that increase in C-value results in an increase in RNA length, high molecular weight RNA on the gradients would not be masked.

(v) Gel electrophoresis of bleomycin-treated chromatin

Very few of my Miller spreads showed contrast approaching that of typical oocyte ribosomal gene preparations, and I suspected that this was because Joy in the spreading solution was removing proteins from the chromatin and thus reducing its stainability. To test this hypothesis chromatin extracted with spermidine, which preserves nucleosome structure, and chromatin from a Miller spread were electrophoresed through agarose gels. Both chromatin samples were treated with bleomycin (Kuo & Hsu, 1978) which attacks chromatin preferentially at internucleosome linker regions.

Both Xenopus and Triturus culture cell chromatin behaved in the same way. Bleomycin treatment resulted in the spermidine extracted DNA showing a banding pattern typical of different sized aggregates of nucleosomes (Fig. I-34), whereas in the absence of the antibiotic, all the fluorescence was at the high molecular weight end of the gel, showing that little or no autodigestion had occurred.

When Miller spread chromatin was treated with bleomycin no fluorescence was observed. The untreated control showed fluorescence only at the high molecular weight end of the gel (Fig. I-35).

These results indicate that the Miller technique as I used it in some way disrupts nucleosome structure. This allows bleomycin to break the DNA into very small fragments that run off the gel, rather than cutting between nucleosomes. It must therefore be borne in mind that observations made on nucleosomes from chromatin dispersed in detergent-containing spreading solutions may be artefactual.

In many cases, the smooth DNA of my early preparations where protein removal was likely to be maximal was about 10 nm wide, suggesting that the chromatin proteins, rather than being removed, had adopted a non-nucleosomal conformation.

The Miller technique used to prepare chromatin for this experiment was an early modification. Later versions resulted in higher contrast, and an improved preservation of nucleosome structure. However, I was not able to repeat this experiment using later modifications of my spreading method.

These data are in accord with the findings of Scheer (1978) that 0.3% Joy removes 75% of the chromatin proteins. The preparative method I used results in large clumps of "superbeads" being visible in electron microscope preparations. Thus one might expect some residual subunit structure to persist in gels of bleomycin treated spread chromatin. That none was observed may perhaps be due to bleomycin cleaving the chromatin into separate superbeads, which later unravel and lose their nucleosome structure.

(vi) Cellular and nuclear dimensions of cultured cells of X. laevis and T.c. carnifex

I wished, for a number of reasons, to know the dimensions of cells and nuclei in my tissue-culture lines. It is relatively easy to measure fixed and stained material, but even the best fixatives e.g. Sanfelice, result in considerable shrinkage of cells and nuclei. It was therefore necessary to take measurements from living cells.

All the size distributions were slightly skewed to the right so that a minimum cell or nuclear size must exist. For this reason, I determined median rather than mean values. Although the distributions are not normal I calculated the variance for each histogram, in order to assess the degree of spread of the size distributions.

Figs I-36a and I-37a show that at high inoculation densities, the median cell volumes of X. laevis and T.c. carnifex are 4492 μ^3 and 26914 μ^3 respectively, T.c. carnifex having cells some six-fold greater in volume. The variances of these distributions were 8% and 14% of the medians, respectively, showing that there was little spread in the distributions. Nuclear areas are 205 μ^2 and 1386 μ^2 respectively (Figs I-38a and I-39a) and show that T.c. carnifex has nuclei about 6.8 times greater in cross-sectional area than X. laevis. The variances were 6% and 9% of the median values, respectively. If the cell layers of both species are similar in thickness, then this reflects a real difference in the size of the nuclei.

At lower cell densities the size difference was less marked; it is not clear why, unless the selection procedure or sample size I used was in some way responsible. Cell volumes (Figs I-36-c and I-37c) fell around 7500 μ^3 for Xenopus and 19900 μ^3 for T.c. carnifex. Variances were 19% and 11% of

the median values. I obtained values of about 300 μ^2 and 1100 μ^2 (Figs I-38c and I-39c) for nuclear areas of the two cultures. Variances were 4% and 6% of the median values. In these cultures, T.c. carnifex has a cell volume only 2.7 times greater than that of X. laevis and a nuclear area greater by a factor of 3.5.

Both the absolute values and their relationship to each other vary with inoculation density. However, these data do agree with previous findings (Van t'Hof & Sparrow, 1963; Szarski, 1976; Olmo & Morescalchi, 1978) that nuclear size and generally also cell size correlate positively with C-value.

DISCUSSION

The experiments described in this chapter indicate that in the case of tissue-culture cells, T.c. carnifex synthesizes both longer primary transcripts and hnRNA than does X. laevis. Although the mean values for isolated RNP fibril length are very similar, 0.33 μm and 0.44 μm respectively, cells of T.c. carnifex can synthesize primary transcripts of up to 11 μm in length. RNP of this size order was never seen in Xenopus chromatin, where the longest RNP fibril observed was 2 μm long.

A similar relationship between C-value and hnRNA size has been noted in a variety of species (Lodish et al., 1973; Kung, 1974; Lengyel & Penman, 1975; Hereford & Rosbash, 1977; Williamson & Tobin, 1977; Busby & Bakken, 1979). However, none of these experiments demonstrate as great a range of primary transcript size as the five-fold difference I found. This difference though great, is still somewhat less than the 7 - 10-fold difference in the actual C-value. As much evidence points to a similarity in gene number amongst eukaryotes (Rosbash et al., 1975; Edstrom & Lambert, 1975) this would imply that, assuming coding sequences to be approximately the same length in both Triturus and Xenopus, not all the extra DNA is transcribed.

In a study of the DNA sequence organization of anurans and urodelans Baldari and her colleagues (Baldari & Amaldi, 1976, 1977) found that within the same taxonomic group, differences in C-value were largely due to differences in the amount of repetitive sequences. Within each taxonomic group, species of different C-value had similar amounts of unique DNA, although higher C-value species within each group had shorter unique sequences. The actual lengths of unique sequences were found to be similar in the Anura and Urodela. The differences between the two groups, involved all sequence classes. Furthermore, between the two groups differences in

C-value due to variations in the amount of repetitive sequences did not involve variation in length of the repetitive sequences (380 bp long). Thus, within the same subclass, increase in C-value is due partly to increase in the number of repetitive sequences and partly to changes in interspersion period (unique sequence length). Between subclasses, changes occur over the whole genome, which nonetheless maintains the same general organization.

HnRNA consists largely of interspersed repetitive and unique sequences (Jelinek et al., 1973; Herman et al., 1976; Davidson et al., 1977). This, and the data of Baldari & Amaldi (1976, 1977) certainly do not exclude the idea that between Xenopus and Triturus there exists a pronounced difference in hnRNA length. However, it is not clear why a high C-value species should transcribe a greater proportion of the interspersed noncoding sequences in its genome. For increase in C-value to result in the synthesis of longer primary transcript molecules, interspersed noncoding sequences would have to be evenly distributed between coding regions.

The significance of apparent C-value dependent differences in primary transcript size is not yet clear. The recent discovery of DNA sequence classes of different degrees of repetition in the genome, and their relative spatial organization (Crain et al., 1975; Davidson et al., 1975a, b; Goldberg et al., 1975; Manning et al., 1975) has led to the idea that these sequences might constitute regulatory elements of some sort. The most comprehensive model to account for eukaryote sequence organization is that of Britten and Davidson (Davidson, et al., 1977).

Briefly, it is assumed that the coordinated activity of many different genes is required to establish a differentiated state. The structural genes, coding for proteins, are conceived of as belonging to batteries,

defined as those groups of strutural genes which share a given receptor sequence, to which an activator RNA or protein can bind. Binding results in transcription of the contiguous structural gene. The activator RNA or protein molecules are encoded in the so-called integrator genes. Patterns of activation of sets of batteries are determined by the coordinate transcription of sets of integrator genes, leading to the production of sets of regulatory molecules governing functionally related gene batteries. Finally, to ensure the coordinate activation of each set of structural gene batteries, it is suggested that the integrator genes of each integrator gene set are transcribed as a single hnRNA molecule.

This model differs fundamentally from those that postulate a precursor-product relationship between the primary transcript and mRNA (Bastos & Aviv, 1977; Egyházi, 1978), in that the great bulk of the hnRNA is considered to be regulatory in function. The precursors to polysomal messenger RNA are assumed to be transcribed in a process separate from the transcription of giant hnRNA. hnRNA, which is known to be very heterogeneous in its sequence organization, would thus consist of repetitive regulatory sequences interspersed with single copy spacer sequences.

A number of observations do, however, conflict with this model, primarily the data on Drosophila sequence organization and primary transcript length. The interspersion pattern of the Drosophila genome consists almost entirely of repetitive sequences from 0.5 - 1.3 kb in length interspersed with single copy sequences of about 13 kb in length (Manning et al., 1975). Although some clusters of middle repetitive DNA may exist, there is little evidence (Crain et al., 1975) to suggest that a significant proportion of the Drosophila genome has the short period interspersion pattern. The evidence of Lengyel and Penman (1975) demonstrates that the mean length of Drosophila culture-cell hnRNA is about 4700 bp. Although

The relationship of the coding sequences to the single copy DNA is obscure, if Davidson's suggestion that hnRNA is a coordinate regulatory transcript of great sequence length and heterogeneity is valid, it is hard to reconcile these data with the model. Drosophila hnRNA is not long enough to include both unique and repetitive sequences of the length found in Drosophila DNA. However, primary transcripts of at least 10 μm (30,000 bp) long are synthesized in Drosophila spermatocytes (Glätzer, 1975) and band-sized primary transcripts have been detected in other Drosophila tissues (Chooi, 1976). Nonetheless, this does not dispose of the lack of correlation between interspersion pattern period and culture cell hnRNA size. However, it must be remembered that hnRNA is not necessarily equivalent to the primary transcript, which may be longer (Old et al., 1977; Scheer et al., 1979). If so, the Britten and Davidson model may apply, i.e. the 4700 bp long Drosophila culture cell hnRNA may be a processing product.

The two ideas are compatible if some portion of the Drosophila genome shows a short-period interspersion pattern, which does not appear to be the case. If it were, it would easily account for the suggestion of Wold (Wold et al., 1978) that there are two classes of primary transcript, long hnRNA molecules of unknown function, and premessenger RNA. It must be remembered that the Drosophila interspersion pattern is characteristic of small genomes, and suggests reduction of the genome by deletion, resulting in long repetitive DNA clusters instead of small separate sequences. The complexity of single copy sequences in Drosophila hnRNA represents a fraction of the genome similar to that found in vertebrate and echinoderm cells (Davidson et al., 1977), so that the long interspersed unique DNA may have the same complexity as several short interspersed sequences of the short-period interspersion pattern. This might also be true of the

repetitive sequences, and in this case, the Drosophila data would agree fairly well with Davidson's model.

On the other hand, the results of Wold (Wold et al., 1978) show that in the sea urchin virtually all blastula messenger RNA sequences are present in the nuclear RNAs of adult tissues, although most of these messenger RNA sequences are absent from the cytoplasmic and polysomal RNAs of adult cells. Blastula messenger RNA sequences are present in hnRNA at the same concentration as the nuclear transcripts of most unique DNA sequences. Calculations suggest that a majority of these molecules are not message precursors. They suggest that nuclear RNA consists of both message precursor and a second component of unknown function. These data are in accord with the hypothesis that hnRNA is mainly regulatory in function, and the presence of extensive overlap in sequence between different cell types does not require an extreme RNA processing model, or exclude transcriptional control.

The two main branches of the Britten and Davidson model involve either RNA or protein activators. Both give rise to testable predictions. If integrator genes produce activator RNAs, and if receptor sequences adjacent to structural genes are repetitive, the activators would also be transcribed from repetitive sequences. This would permit overlap between gene batteries. Thus hnRNA is envisaged as a sequence of repetitive integrator gene transcripts, each about 300 base pairs long, and linked by single copy spacer DNA. Preliminary calculations (Davidson et al., 1977) show that repetitive sequence transcripts are present in the nucleus at concentrations high enough to saturate putative binding sites within a few minutes of their synthesis, which certainly lends credibility to this idea. High hnRNA turnover rates are interpreted as a means of regulating the steady state concentration of activator sequences, and to change the state of differentiation, so that regulation is a dynamic process.

The major flaw of this theory is that it fails to account satisfactorily for C-value dependent size differences in hnRNA. Given DNA sequence organization and hnRNA sequence structure, there is no reason to assume that hnRNA size should change with C-value. Davidson (Davidson et al., 1977) suggests that because free hnRNA length differs from that of hnRNA still attached to its DNA template, hnRNA size could be arbitrary, and perhaps due to almost random cutting at spacer sequences. Thus it would be desirable to look at the hnRNA of a specific gene. A good candidate would be the silk fibroin gene, whose putative transcription unit has been visualised (McKnight et al., 1976). Although it might be difficult to eliminate isolation artefacts, I think it might be possible to determine whether the size of the free hnRNA varied in this way. Electron microscopic evidence exists for the excision of short lengths of RNA from lampbrush chromosome nascent RNP (Scheer et al., 1976), but it is not clear how this fits in with the above observations, unless short regulatory sequences are being clipped out, rather than the processing of a large to a small molecule.

If activator molecules are proteins there is no need for most of the main features of hnRNA organization that have been proposed. If a battery of structural genes is defined as that set of genes sharing receptor sequences belonging to one repetitive sequence family, with each battery activated by the binding of one species of activator protein, only one activator per battery is required for any differentiated state. Consideration of gene number, gene number per battery, and activator number per battery suggests that the complexity of integrator gene sequences coding for protein activators is less than 10% of that of the structural genes expressed at any time, i.e. less than 1% of the hnRNA complexity which is generally ten times greater than that of the mRNA. This is far less than would be expected from the known size and sequence organization of hnRNA, and implies that integrator genes must be repetitive if the transcription

unit is indeed a device for coordinate regulation. This alternative is also unsatisfying as it now fails to explain the presence of short interspersed sequences in the hnRNA.

If activator molecules are indeed RNA, larger nuclei would require a larger number of hnRNA molecules than smaller nuclei, in order to maintain activator concentration. My data represent incorporation into total RNA rather than exclusively hnRNA, and it is not clear how much they are affected by possible mycoplasma contamination (see Results above). I found that per cell cycle, cells of T.c. carnifex were synthesizing nearly four times as much RNA as X. laevis. The difference in nuclear size between these species is large but the significance of my result is debatable, for the reason outlined above. However, a study of hnRNA synthesis rate would be a desirable experiment to do, as this is a field in which little work has been done. Unfortunately, in the case of cultured cells my Miller spread data were too sparse to check rate of synthesis by measuring polymerase density or percentage of total transcription comprised by arrays.

The second major model proposed to explain hnRNA structure is that of Cavalier-Smith (1978). This model is fundamentally quite different from the one outlined above. Much experimental work shows DNA content to correlate strongly with cell and nuclear volume, cell cycle length and mean generation time (van t'Hof & Sparrow, 1963; Nagl & Ehrendorfer, 1974; Olmo & Morescalchi, 1978), and although it is difficult to prove a causal relationship, these observations can best be explained by postulating that DNA has two functions unrelated to its coding capacity. The first is the control of cell volume by the control of replication origins, as is known to occur in bacteria (Lewin, 1974), and the second, which will be discussed here, is the determination of nuclear volume by bulk DNA (Morescalchi,

1977a, b). This latter function is referred to as "nucleotypic" (Bennett, 1972; Bennett & Smith, 1972) rather than "genic".

Cell growth rates depend upon cell volume and the area of nuclear envelope available for the transport of metabolites. This is itself dependent upon nuclear volume and hence on DNA content, especially as the nuclear membrane is attached to some of the chromatin. Nuclear pore number per unit area is roughly constant in most species (Cavalier-Smith, 1978) and thus it has been suggested that eukaryote DNA consists of G-DNA, which codes for protein, and S-DNA, which plays a major role in nuclear volume determination. It may also share some of the function of G-DNA by coding for nucleoskeletal RNA.

Eukaryotes are subject to widely differing forms of natural selection, of which the most important are the two opposing forces of r- and k-selection. r-selection is characteristic of species that need to colonise ephemeral environments and favours rapid development and reproduction, small body size and short lifetime. k-selection on the other hand is most important for those species that compete in stabler but crowded environments. k-selection favours slower development, delayed reproduction, large body size and longer lifetime (Bachmann, 1972a), and for any species there is a particular compromise between these two forms of selection. Cavalier-Smith suggests that organisms adapt to varying r- and k-selection by evolving particular cell volumes and cell growth rates. Among unicellular organisms such as bacteria, selection is for small size and high growth rate, and C-values are therefore small. On the other hand, slow-growing algae with large cells have high C-values. This relationship is true of both pro- and eukaryotes, though in the latter case it is somewhat obscured by three factors. First, the indirect relationship between cell and organismic

growth rates, secondly, independent variation in cell and body size, and thirdly, variation in cell size and growth rates in different cells in the body. For instance, among plants, r-selected annuals have low C-values, whereas k-selected perennials have high C-values. Slow sluggish amphibians such as Amphiuma and Triturus have the highest C-values, whereas small-celled rapidly metabolising species such as birds and teleost fish, the lowest.

As developmental processes are sensitive to cell size and growth rate, it follows that different cell sizes and growth rates are likely to be optimal in different tissues. Thus a means of modulation of cell and nuclear volume is required. Cavalier-Smith suggests that this modulation could be achieved by polyteny or endopolyploidy, which result in changes in nuclear DNA content, or by the synthesis of nucleoskeletal RNA. It is suggested, therefore, that the major function of hnRNA sequences is to change nuclear volume, and that only a small fraction functions as a messenger RNA precursor. These hnRNAs, whose sequences might be irrelevant, could combine with swelling proteins to achieve changes in nuclear volume (Cavalier-Smith, 1978). Differences in the amount of primary transcript might exist between different tissues of the same organism. However a second means of varying hnRNA content is that of varying the length of hnRNA. In some ways, this mechanism is more economical than changing polymerase density, and is a way of exploiting the extra DNA sequences already in the genome. During the course of my research I found that hnRNA length varied between different cell types of an organism, and also in the cells of different species whereas the percentage of arrays in any sample of transcription figures was more or less constant. This may mean that hnRNA length modification rather than copy number modulation is the means of control actually used by the cell. It is conceivable that in some situations, both methods of control would be used (Chapter IV this study).

This model then accommodates the observed correlation of hnRNA size with C-value far more effectively than Davidson's hypothesis. It also accounts for the fact that in most cases, differences in hnRNA length are not as great as the difference in C-value. S-DNA is postulated to be largely interspersed throughout the genome, but some is also present as blocks of largely untranscribed sequences, which may be identical to constitutive heterochromatin.

The results of Wold (Wold et al., 1978), outlined earlier, fit in quite well, and the model predicts that different sets of genes would be used in different tissues according to their nuclear volume requirements. An intriguing possibility, suggested by the considerable degree of sequence overlap in hnRNA between different tissues, is that under certain conditions, hnRNAs of different lengths might be transcribed from the same functional unit.

A number of testable predictions result from this hypothesis. Firstly, increase in the amount or length of hnRNA synthesized should result in an increase in nuclear volume (Chapter IV this study). Different tissue types should also show differences in transcription patterns analysable by the Miller spreading procedure. Polymerase density, the percentage of the total transcription represented by arrays, and RNA length might all be expected to vary. Finally, I predict that in certain situations, a small C-value species could synthesise RNA of a size order generally found only in a species of much higher C-value (Chapter IV this study, Chooi, 1976). This is reinforced by the accumulation of evidence demonstrating the lability of all components of the eukaryote genome (Stanfield & Helinski, 1976; Strom & Dorfman, 1976; Schäfer & Neumann, 1978; Schäfer et al., 1978). All these propositions are testable using the Miller technique, and the rest of my thesis describes my attempts to do so.

This model clarifies the situation in Drosophila, whose small hnRNA is now seen to be an answer to a requirement for small cell and nuclear size. Drosophila, with its small body and cell size, and fast developmental rate, is an extremely r-selected organism (Cavalier-Smith, 1978) adapted to an ephemeral and unstable environment. Small genome size is necessary for the maintenance of these characteristics. Modulations in nuclear size over and above that due to the small DNA content are perhaps at a premium, in order to preserve the fast developmental rate. It would therefore be very interesting to investigate transcription in different tissues of Drosophila, as opposed to amphibians, which, together with the difference in the sequence interspersion pattern of their genomes, are extremely k-selected organisms with large nuclei and cells, and long developmental times.

The major flaw of this model is the fact that it is hard to reconcile the nucleoskeletal function of hnRNA with its short half-life of 30 min - 1 hr (Attardi et al., 1966; Soeiro et al., 1968). However, short half-life could conceivably be a means of effecting rapid modulation of differentiated states. Small changes in the rate or type of RNA being made would be quickly manifested in the population of molecules present in the nucleus. Hitherto unsuspected components of the genome might also synthesize longer-lived nucleoskeletal RNA. For instance, there is some evidence that satellite sequences are transcribed on occasion (H.C. Macgregor, pers. Comm.)

A second consideration is that although it is true to say that the nuclear membrane is attached to the chromatin of some nuclei, it is not true of all. Obvious exceptions are dipteran salivary gland nuclei, and oocyte nuclei. Thus the relationship between DNA content and nuclear volume is not necessarily direct. Furthermore, although nuclear pore number

per unit area of the nucleus is constant in many species, this parameter can vary between the nuclei of different tissues in a single organism.

The most important requirement, at present, is for more information on hnRNA complexity and sequence organization. Modifications of the Miller spreading technique allowing in situ hybridisation would be invaluable, as would a means of scanning preparations for RNP at low magnifications. Although autoradiography is now possible, the technique is very time consuming and tedious, and not suitable for all systems.

C H A P T E R II.

TRANSCRIPTION PATTERNS IN LIVER CELLS OF *Xenopus laevis*, *Triturus cristatus carnifex* AND *Necturus maculosus*

INTRODUCTION

The results of my investigation into primary transcript length in cultured cells of *Triturus cristatus carnifex* and *Xenopus laevis* suggest that with increase in C-value there is a concomitant increase in the length of the hnRNA. However, tissue culture cells, although an excellent system for biochemical and labelling experiments, are not ideally suited to the Miller spreading technique. Their low transcriptional activity makes it difficult to detect enough transcription figures to form a statistically significant sample for analysis.

In order to supplement the data presented in Chapter I, I decided that it would be necessary to examine the effects of C-value on primary transcript length in the somatic tissues of *X. laevis* and *T.c. carnifex*. It was also possible, during the course of my research, to study transcription in tissues of *Necturus maculosus*, although a cell culture of this species was not available. As the C-value of *N. maculosus* is about 52 pg, this extended the scope of my work.

For this investigation I decided that it would be most informative to look at liver cell transcription in these animals. The liver, due to its role in detoxification and other metabolic processes, is of high metabolic activity. Thus it is likely that in the electron microscope a large number of transcription figures should be seen, allowing more reliable conclusions to be made. The easy availability of liver was also an advantage. For several reasons, I wished to make a study of tissue- and

stage-specific transcription in the Amphibia, and liver cells were a natural choice for this. Furthermore, their use would extend the applicability of the Miller technique.

One possible disadvantage of liver cells, as a system for the study of transcription, is that in many vertebrates they show a high degree of polyploidy, which might be expected to affect the patterns seen, and confuse any differences due to variation in C-value. On the other hand, adult Axolotl and possibly other amphibian livers contain very few if any polyploid cells, up to 1% at the most. (H.G. Callan, pers. Comm.) The results of Collins (1978) demonstrate that in rat liver cells of different ploidy, there is little or no difference in the amount of RNA synthesized per ploidy level. Although it is obscure whether the length of the primary transcript would be affected, these data do imply that there are an approximately constant number of actively transcribing sites, regardless of DNA content, in rat liver cells. If valid for amphibian liver cells, this result makes it all the more desirable to study transcription in this tissue.

Finally, as mentioned in the Discussion to Chapter I, I wished to further investigate Cavalier-Smith's proposal (1978) that variation in C-value and the amount of RNA synthesis in some way modulates nuclear and cell size to meet environmental requirements.

MATERIALS AND METHODS

As with tissue culture spreads, I used different modifications of the spreading technique that I devised for liver cells. In nearly all cases, the last described variation was used, and gave the most consistent results. However, in the case of T.c. carnifex liver, which was studied some time before the others, a different technique was used, which is described at length below. All solutions and glassware were treated as for culture-cell preparations.

(i) Removal of liver

Females of X. laevis, T.c. carnifex (Gerrard & Haig; Xenopus Ltd.), and of N. maculosus (Xenopus Ltd.) were anaesthetised in a 1% solution of MS222 (Sandoz). The animals were opened and a piece of liver 3 - 4 mm cube was removed from the edge of the lobe nearest the opening. In the case of X. laevis, chromatin from such a piece of tissue was largely inactive (see Results), so that a larger piece proved to be necessary. This allowed cells from the body of the tissue to be spread. The piece of tissue was placed in a sterile plastic Petri dish (Falcon), and kept on ice until required. In practice it was used at once.

(ii) Lysis and dispersal of liver cells

Preparations of T.c. carnifex liver were made using the first "Joy" solution (0.2%) described below. All other preparations involved the use of the second solution (0.1%) which gave better results.

(1) A 0.2% solution of Joy in distilled water was adjusted to pH 8.7 with stock borate buffer.

(2) A 0.1% solution of Joy in distilled water, containing 0.05 M sucrose (RNase Free. Serva) and 100 µg/ml yeast transfer RNA (final vol. 20 ml) was adjusted to pH 8.7 with stock borate buffer.

The Petri dish containing the liver was placed on the stage of a dissecting microscope. Using two pairs of sharpened No. 4 watchmakers' forceps, and avoiding blood vessels, a piece of tissue about 0.3 - 0.5 mm cube was teased away. This was washed briefly in pH 9 water to remove blood and surface débris. The tissue was then placed in a 100 µl droplet of Joy, on Parafilm, in a sterile plastic Petri dish (Falcon). After about 1 min, to allow slight disaggregation of the tissue, the piece of liver was carefully macerated, using watchmakers' forceps, and the chromatin allowed to disperse. Unlike Harper and Puvion-Dutilleul (1979, in press) I did not find it neceessary to homogenise the liver, or isolate nuclei. The advantage of my method is its rapidity; the time from opening of the animal to dispersal of the tissue is only 5 - 10 min.

Dispersal times varied from 75 min for T.c. carnifex liver to 15 - 30 min for X. laevis and N. maculosus liver. I found that for tissues from all these species, 15 min was an adequate dispersal time, resulting in optimum spread, and structural preservation, of the specimen.

(iii) Further processing of preparations

The first few preparations of X. laevis liver that I made were extremely dirty, and this was thought to be due to the high melanin content of this tissue. Melanin was less of a problem in spreads of the other two species.

To overcome this, dispersal of X. laevis liver cells was allowed to proceed for slightly longer than usual (20 - 30 min), which resulted in most of the melanin sinking to the bottom of the Joy droplet as a greyish deposit. The supernatant containing the chromatin was then carefully pipetted off, disturbing the layer of pigment as little as possible. This protocol gave fairly clean preparations.

Centrifugation, washing, drying and staining of these preparations was as described in Chapter I. 8 grids were made per preparation.

RESULTS

(i) Transcription patterns in liver cells of Xenopus laevis

Three preparations were made of X. laevis liver cells, of tissue taken from the edges of lobes of liver. Smooth or nucleosomal DNP was absent from all these preparations, although the chromatin was otherwise well-spread and contrasted. All the chromatin took the form of lengths of "superbeads", and was either well spread or appeared as oval clumps, probably unspread nuclei. The superbeads were from 26 - 45 nm in diameter, roughly spherical, with no observable substructure (Fig. II-1a). In this they resembled those found in T.c. carnifex culture-cell chromatin. In a few cases (Fig. II-1b) they were stretched, consisting of dark elongated masses, connected by a fibril 8 nm in width. These measurements were made on stained preparations, and compare well with those obtained from T.c. carnifex culture-cells.

In a few cases, clumps of chromatin were covered by long strands showing a repeating structure (Fig. II-1a), and presumed to be collagen. These preparations were very dirty, probably due to the large amounts of melanin in the cells.

Later preparations were made from tissue derived from deeper within the liver, and here the chromatin was entirely different in its characteristics. It was well spread, although a few clumps occurred, and largely nucleosomal (Fig. II-2). Superbeads were absent. The nucleosomes measured 16 nm $\pm$ 3 nm in diameter, a relatively high value, probably due to rotary shadowing of the preparation. Nucleosomes occurred in both transcriptionally inactive and active regions (Figs II-2 and II-5a, b). The beads observed in transcription complexes are presumed to be nucleosomes because at no

time were clearly discernible RNA polymerase molecules ever seen at the point of attachment of the transcript to its DNP axis. Their size was also similar to that of nucleosomes in the same preparation.

In the case of transcription, the selection and measurement criteria that I used were the same as those discussed in Chapter I. Single fibrils formed a distribution falling around a median of 0.66 μm (Figs II-3a and II-4a, b) and with a range of 6.3 μm (Table I, p54). It was possible to measure the lengths of the terminal fibrils of arrays of two or more fibrils (Figs II-3b and II-4c), and these fell around a median of 0.4 μm, with a range of 3.6 μm (Table I, p54). These values are, surprisingly, lower than those for single fibrils, probably because of the small sample size available for analysis. All these fibrils and arrays are presumed to be of nonribosomal origin, because of their characteristic morphology. Arrays were sparsely covered with transcripts, which had a diffusely beaded appearance.. No clear fibril length gradients were apparent. Tandem repetition was not observed (Foe et al., 1976; Laird & Chooi, 1976; Laird et al., 1976). I did not observe any ribosomal transcription in these preparations.

Only two analysable nonribosomal arrays were seen (Fig. II-5a, b) and of these, the RNP of one (Fig. II-5a) had the diffusely beaded appearance previously observed in culture-cell RNP. The beads were 14 - 18.7 nm in size. The contrast of the RNP was very similar to that of the DNP. The RNP of the second array (Fig. II-5b) was very much more contrasted than the DNP. Unlike that of the first array it was not clearly beaded, and had extensive secondary structure, in this resembling primary transcripts in D. melanogaster embryos (Laird & Chooi, 1976). High contrasted RNP of this latter type, was, however, rare in preparations of X. laevis

liver cell chromatin. Laird Analysis showed that these arrays measured 1.3 μm and 2.6 μm respectively. Detached RNP was also seen in these preparations, the result of either processing or endogenous nuclease action (Fig. II-4a). There was little evidence of processing, except in the case of one array (Fig. II-5b).

Transcription figures in liver cells were more frequent than in cultured cells, so that the sample available for analysis was larger (Table I, p54). This allowed me to estimate the percentages of the total transcription made up by different types of transcription figure. Isolated RNP fibrils made up 86% of the total, whereas groups of two fibrils separated by less than 1.0 μm of DNP, made up 9.2%. Arrays of three or more fibrils comprised a further 4.6% of the total (Table II, p56). The great predominance of isolated fibrils prevented an analysis of the percentage transcriptional activity of the chromatin (McKnight & Miller, 1976) as well as the fact that although transcription was more frequently seen than in cultured cell chromatin, it was still not so frequent as to make this type of analysis possible.

The DNA/RNA packing ratio represents the degree to which the RNP fibrils of a transcription unit are foreshortened. For each of the two analysable arrays that I found, the DNA/RNA packing ratio was 1.23 (Table VI, p59). As no other arrays were analysed it is not clear whether this value of 1.23 is universal for X. laevis liver, or whether a broad distribution, or size classes of packing ratio might occur. My results differ from those of McKnight and Miller (1976) who show that in D. melanogaster embryos, nonnucleolar arrays fall into two classes. These are characterized by a length of up to 2 μm, and a high RNA polymerase density (nonnucleolar Type I), or a length of 3.6 μm, and a lower polymerase density (nonnucleolar Type II). However, the small sample size could again account for my results.

The preservation of nucleosomes was such as to allow analysis of their distribution. Dispersal of the chromatin was in Joy, which is known to remove chromatin proteins and disrupt nucleosome structure. In spite of this I decided to measure DNA packing ratios to see whether this removal was reflected in the chromatin structure observable in the electron microscope. Assuming Joy removes protein equally from chromatin of different origins, under the same dispersal conditions, then the packing ratios of preparations dispersed in similar conditions should be comparable. The DNA packing ratio for transcriptionally inactive regions (mean of 10 randomly selected 1 μm regions) of the chromatin was 1.84 (Table III, p57).

Although preparations made under different conditions, and from different chromatin, will vary, it should be possible to obtain a valid estimate of the DNA packing ratios of transcriptionally active and inactive chromatin from the same preparation, assuming that Joy removes proteins equally from transcribing and inactive regions of the DNP. Only a few nonribosomal transcription complexes were available for analysis in this cell type. All had DNP axes with a beaded structure. The mean number of nucleosomes per micrometer of chromatin was determined for the arrays (Table IV, p105) and this gave a value for DNA packing ratio of 1.62, which is lower than the value of 1.84 for transcriptionally inactive regions. If the assumptions I made are valid, this suggests that although transcriptionally active chromatin is nucleosomal, its structure is more extended than the rest of the DNP. However, it is possible that transcripts on an array may conceal nucleosomes. If the number of transcripts is added to the number of beads per array, DNA packing ratio is now 2.0, higher than for inactive regions (Table IV, p105). This result is in agreement with those of Busby & Bakken (1979) for Strongylocentrotus purpuratus gastrulae.

TABLE IV.

The DNA packing ratio (μm of β-structure DNA per μm of chromatin) of transcriptionally active regions of the chromatin of different cell types. The values obtained when it is assumed that each RNP fibril of a transcription complex conceals a nucleosome are also given.

Cell type	DNA packing ratio	Sample size (transcription complexes)	DNA packing ratio, assuming transcripts conceal nucleosomes
X. laevis cultured cells	-	-	-
T.c. carnifex cultured cells	-	-	-
X. laevis liver	1.62	3	2.0
T.c. carnifex liver	-	-	-
N. maculosus liver	1.42	2	1.69
	-	-	-
T.c. carnifex neurulae	-	-	-
X. laevis cultured cells	1.74	2	2.23
plus cortisol	1.63	6	2.5

In these cells, isolated RNP fibrils formed the commonest type of transcriptional structure. I therefore decided to look at nucleosome distribution near single fibrils to see if it bore out the results presented above. If chromatin structure is indeed less condensed in regions of transcription, the number of nucleosomes in a 0.5 μm interval should increase with distance away from the transcribing region. It was only possible to measure the nucleosome density for about 2.0 μm on either side of the RNP fibril.

The mean number of nucleosomes in 0.5 μm intervals to either side of an isolated RNP fibril were determined. It is not possible to determine the direction of transcription, in the case of isolated RNP fibrils, so the values for 0.5 μm intervals on either side of the fibril were pooled.

For X. laevis liver cells there is a slight increase in mean nucleosome number (Table V, p106) with distance away from the RNP fibril, which seems to support my hypothesis. The ranges of values for each interval do not overlap. However, the increase is very small (1 nucleosome/μm DNP) and may be a chance effect.

(a) Anomalous putative RNP

In a few cases, putative RNP of an anomalous structure was observed. These structures resembled groups of two RNP fibrils, whose free ends had in some way become associated to form a ring-like structure (Fig. II-6a, b). In some cases, the attachment points of the two presumptive transcripts to the DNP were so closely apposed as to suggest that they were in fact loops of DNP (Fig. II-6a). The lack of differentiation of contrast between axis and loop, and the similarity of their beading, support this interpretation. In some cases, RNP and DNP had similar contrast. However, in other cases (Fig. II-6b) the attachment points were sufficiently well separated to suggest

TABLE V.

Nucleosome number (per 0.5 μm of chromatin) for intervals increasingly distal to an isolated RNP fibril attached to the DNA. Values for regions on either side of the transcription event were pooled.

Cell type		Intervals of chromatin distal from transcript (at position 0 μm)				Sample size
		0 - 0.5 μm	0.5 - 1 μm	1.0 - 1.5 μm	1.5 - 2.0 μm	
X. laevis cultured cells		-	-	-	-	-
		-	-	-	-	
T.c. carnifex cultured cells		-	-	-	-	-
		-	-	-	-	
X. laevis liver	mean	13.2	13.3	14.3	14.3	10
	range	12.5 ± 4.8	12.5 ± 5.5	14 ± 4	14 ± 4	
T.c. carnifex liver		-	-	-	-	-
N. maculosus liver	mean	12.2	11.5	11.7	11.2	10
	range	13.5 ± 6.5	10.5 ± 5.5	14.5 ± 6.5	11.5 ± 3.5	
T.c. carnifex neurulae	mean	6.5	6.27	6.2	5.7	10
	range	-	-	-	-	
X. laevis cultured cells + cortisol	mean	14.3	15.6	15.3	16.0	10
	range	14.5 ± 4.5	15.5 ± 4.5	14.5 ± 3.5	16 ± 3	

that the structures were RNP, as does the fact that most of these structures occurred on well-stretched chromatin axes, which may rule out their being random twists. Unlike X. laevis culture cell chromatin, no rings of any type were seen.

(ii) Transcription patterns in liver cells of Triturus cristatus carnifex.

Melanin was not a problem with spreads of T.c. carnifex liver, unlike X. laevis, so that the preparations I obtained were cleaner than those of the latter species. On the other hand, the T.c. carnifex liver was less easy to macerate, so that chromatin occurred as large viscous clumps in the Joy droplet. This meant that loading of the grids was more difficult to control. The problem was partly overcome by using small pieces of tissue and relatively large Joy droplets.

Preparations of T.c. carnifex liver were made quite early in the course of my research, and involved long (75 min) dispersal times, in Joy lacking RNA or sucrose. Nonetheless, contrast and preservation of structural detail was good. A few preparations made later, involving Joy containing small RNA from yeast, sucrose, and shorter dispersal times, show little difference save in the occurrence and distribution of nucleosomes. There was no significant difference in the frequency of transcription complexes. Their frequency did vary a little between preparations, perhaps due to differences in transcriptional activity between different regions of the liver, as appears to be the case in X. laevis; however, these differences were not so marked in T.c. carnifex.

Dispersal of chromatin was excellent in all preparations, whether made with 0.2% or 0.1% Joy. In the latter case, dispersal time was only 30 min. Of nine preparations made (8 grids each), seven yielded analysable data.

This high proportion seems to be characteristic of liver cells and is greater than for tissue-culture preparations, although it is impossible to judge accurately the amount of chromatin on any grid.

The appearance of the chromatin varied between preparations. In many, a nucleosomal structure was apparent (Fig. II-7) and in others the DNP was relatively smooth, as for culture-cell chromatin (Fig. II-8a). This variation is probably a preparative artefact, as noted in Chapter I, as T.c. carnifex liver chromatin spread under optimal conditions showed a high frequency of nucleosomes. Nucleosomes were 16 nm ± 4 nm in diameter, and were present in both transcriptionally active and inactive regions (Figs II-7 and II-10b). As with other tissue types, clear RNA polymerases were rarely visible at the attachment points of RNP to DNP. In one case (Fig. II-8a), a putative RNA polymerase molecule, measuring 28.5 nm in diameter, was observed. This value is greater than previous estimates of 11.5 - 14.5 nm (Miller & Hamkalo, 1972; Franke et al., 1976a; Morgan, 1978). "Smooth" DNP measured 8.5 - 20 nm in width, indicating that the DNA was associated with protein.

Superbeads were scarce in these preparations, although a few short lengths were observed. None occurred as large clumps, and this may be connected with the high metabolic activity of liver cells.

Ribonucleoprotein was found in the form of transcription complexes of various types, but also occurred as clumps (Fig. II-7). These may have been superimposed but tangled transcription complexes, or detached transcripts, perhaps the result of nuclease action or processing. Detached transcripts also occurred in preparations of X. laevis liver (Fig. II-4a) where there was scant evidence for processing.

Transcription predominantly took the form of isolated RNP fibrils, which formed a distribution skewed rightward around a median of 0.6 μm, and with a range of 6.5 μm (Figs II-8a, b; II-9a) (Table I, p54). Terminal fibrils of arrays of two or more fibrils formed a similar distribution around a median of 0.95 μm, and with a range of 9.6 μm (Figs II-9b and II-10a) (Table I, p54). The longest fibril seen was 9.8 μm in length (Fig. II-11).

Several long transcription units were seen and in one of these transcription extended over at least 4 -5 μm (Fig. II-12), although only a few fibrils were clear. Laird Analysis was not possible. The lengths of some of the transcripts that I found are of the same size order as those observed in lampbrush chromosome transcription complexes (Scheer et al., 1976). A value for array length of 3.21 μm was obtained for another array.

Of the arrays found, several (Figs II-10a and II-11) included long transcripts adjacent to short transcripts, where the separation between the two fibrils was less than the difference in length between them. Such configurations could indicate the existence of proessing, although the possibility of random breakage of RNP cannot be eliminated. If they do represent processing, they were more frequent in T.c. carnifex than in X. laevis.

RNP had a beaded appearance, and was more contrasted than the DNP in the same preparation (Fig. II-7). Some variation in these characteristics did occur, however (Figs II-8b and II-10a). The beads, as for culture cell transcripts were less discrete than nucleosomes, and the RNP had a more "fuzzy" appearance. Beads had a mean size of 26 nm. Most of the RNP from newt liver showed little or no secondary coiling, except in the case of the long lampbrush-complex-like array (Fig. II-12), and in this differed from the primary transcripts of D. melanogaster embryos as visualized by Laird and Chooi (1976).

The relatively large sample size of transcription complexes allowed me to determine, with reasonable assurance, the percentage frequency of different types of complex. Isolated fibrils comprised 66%, groups of two fibrils closer together than 1 μm, 25%, and arrays of three or more fibrils 8.5% of the total transcription (Table II, p56).

In one preparation, where chromatin superstructure had been well preserved, it was possible to calculate the DNA packing ratio of transcriptionally inactive regions (Table III, p57), which was 1.63. I was not, however, able to compare the nucleosome distribution in transcribed and untranscribed regions of chromatin. Unfortunately no arrays were observed in the preparation for which the DNA packing ratio was known, so that I was unable to calculate the DNA/RNA packing ratio. Due to the variability of my preparations, and dispersal of the chromatin in Joy, which removes some protein, DNA/RNA packing ratio was only measured in preparations where the DNA packing ratio was known.

Ribosomal genes, with their characteristic "Christmas-tree" structure, tandemly repetitive arrangement and RNP fibril length gradient, were never observed (Trendelenburg et al., 1973). Rings, whether of mitochondrial or other origin, were also absent.

(a) Anomalous RNP-like structures

In several preparations of T.c. carnifex liver cells, long structures resembling RNP fibrils were observed (Fig. II-13a, b). These were of higher contrast than the neighbouring DNP, and either free or present as clumps. They were at least 2 μm long, and occasionally over 14 μm in length. None were seen to be attached to a presumptive DNP axis.

The fibrils were anomalous in that they appeared to bear short lateral fibrils along their length. Laird Analysis however, gave completely meaningless results for several of these long fibrils, which at first I thought to be sparsely transcribing, tandemly reiterated ribosomal genes. This, together with the fact that the presumed "RNP" lateral fibrils were always much shorter than either ribosomal or nonribosomal transcripts observed in preparations from any cell type, persuaded me that they were not in fact primary transcripts. Many fibrils were only 24 nm in length, and so may be supercoiled regions rather than true lateral fibrils. The fact that the fibrils were often more contrasted than the axis supports this idea.

If these structures are free transcripts their high degree of secondary structure is baffling, for it was not present in RNP still attached to the chromatin. Their great length is compatible with the data from lampbrush chromosome spreads, where transcripts of up to 10 μm in length have been seen. In most cases, the ends of the fibrils disappeared into dense clumps (Fig. II-13a), which argued for a much greater length. The fact that I never saw any attached to DNP also argued against the interpretation that they are primary transcript molecules. That the fibrils are DNP is not supported by visual evidence, for in all cases their contrast was greater than that of the rest of the chromatin. The results of the Laird Analysis also imply that the fibrils are not DNP. Few clear beads occurred along the length of the fibrils, and those that were present did not resemble nucleosomes on the same preparation (Fig. II-13a), being irregular in size and distribution, and more electron-dense than nucleosomes.

(iii) Transcription patterns in liver cells of Necturus maculosus

To complete my study of C-value dependent differences in transcription I decided to examine the chromatin of N. maculosus liver cells. The results presented here are incomplete as I was only able to make two preparations; however both of these yielded data.

As with preparations of the other two species, the chromatin of N. maculosus cells appeared as well spread, beaded fibres. Partially dispersed nuclei were occasionally seen, but clumps were in general small. Dispersal was maximal after 15 min of spreading, and both contrast and the preservation of structural detail were optimal at this spreading time. In my experience 0.1% Joy will lyse even the very large cells of N. maculosus. This is in contrast to the case of plethodontid spermatocytes, which require 0.365% Joy (Morgan, 1978) for lysis to occur.

Little or no "smooth" DNP was seen in these preparations, and super-beads were also apparently absent. Nucleosomes measured 16.5 nm ± 4 nm in diameter, approximately the same as those found in spreads of other livers (Fig. II-14a-c). Both actively transcribing and inactive regions of the chromatin were characterised by a nucleosomal structure (Fig. II-14a, b and II-18a, b).

As with all the cell types so far described, nearly all the transcription observed was in the form of single RNP fibrils attached to the DNP (Fig II-14a-c). In a few cases (Fig. II-18b) a putative RNA polymerase molecule was observed at the base of a transcript, and this measured 16.5 nm ± 4 nm in diameter. All the transcription that I observed was nonribosomal according to the criteria discussed in Chapter I. Selection and measurement were also according to these criteria.

Isolated RNP fibrils formed a distribution falling around a median of 1.1 μm, and with a range of 9.13 μm (Fig. II-15a) (Table I, p54). Terminal fibrils of arrays of two or more fibrils had a median of 1.96 μm and a size range of 7.08 μm (Figs II-15b and II-16) (Table I, p54). Transcription complexes of three or more fibrils showed low polymerase densities, and long complexes of lampbrush chromosome array-like morphology were not detected. As with T.c. carnifex liver, but unlike X. laevis liver, long transcripts sometimes occurred next to short ones (Fig. II-17) incidating the occurrence of procelling or of random breakage. Putative detached transcripts were also found.

RNP was beaded in structure and never showed the degree of secondary structure occasionally seen in X. laevis liver cell primary transcripts. Beads were less discrete than on the DNP, and measured 13 - 15 nm in diameter. This value is less than the 20 - 30 nm measured for compacted lampbrush chromosome RNP (Mott & Callan, 1975).

I was able to calculate the frequency of different types of array and found that single RNP ribrils formed 65.2% of the total transcription (Table II, p51). Arrays of two fibrils formed 23.9%, and arrays of three or more fibrils 10.9%. In all the cell types, irrespective of origin that I investigated this relative frequency was always the same, i.e. single fibrils were in excess of groups of two fibrils, which were commoner than arrays of three or more fibrils. This differs from Busby & Bakken's (1979) observation that in Strongylocentrotus purpuratus multiple-fibril arrays were more frequent than groups of two fibrils, isolated fibrils being in excess of either of the other types of array.

The values for DNA packing ratio of transcriptionally inactive regions that I obtained for the two preparations that I made were 1.4 and 1.64

respectively (Table III p57). When the DNA packing ratio of transcription complexes was measured for the preparation where the DNA packing ratio of inactive regions was 1.64 it was found to be 1.42 (Table IV p105). If RNP fibrils were assumed to conceal a nucleosome, and their number added to the total number of beads per array, the packing ratio obtained was 1.69 (Table IV p105). Thus it would seem that, as with X. laevis, transcribing chromatin in N. maculosus has a more extended structure than non-transcribing chromatin.

Determination of nucleosome number per 0.5 μm interval away from isolated fibrils (Table V p106) gave an inconclusive result, with little difference between the values for each interval. This is probably due to the limitations of the method of analysis that I used. However my result may imply that differences in nucleosome distribution are very small for such sparsely transcribing units as the ones I studied.

Laird Analysis was possible for most of the arrays that I found, so that DNA/RNA packing ratio could be determined (Table VI p59). In the first preparation DNA/RNA packing ratios were 1.45 for one array (Fig. II-18a) and between 17.0 and 25.0 for a second array (Fig. II-19 a-c). This second array resembled a typical ribosomal gene in morphology but its length (3.8 μm) was considerably greater than for ribosomal genes of either X. laevis or T.c. carnifex (~ 2.0 μm). Although it may be that ribosomal genes in Necturus are larger than in the other species, this is not likely, and this array may therefore represent a second class of ribosomal gene-like transcription unit.

In a second preparation, three arrays were found. Laird Analysis for these gave no clear length value, as the lateral fibrils did not form a definite length gradient. The DNA/RNA packing ratios are 1.4, 0.44 and 0.92.

The last two values, being less than 1.0, indicate that either no foreshortening of the primary transcript had occurred, or that Laird Analysis gave an inaccurate value for array length. The latter is the most likely explanation of my result. Circular DNA was not observed.

DISCUSSION

The three species X. laevis, T.c. carnifex and N. maculosus have haploid DNA contents of about 3 pg, 23 pg and 52 pg respectively. Mean single RNP fibril lengths are 0.66 μm for X. laevis, 0.6 μm for T.c. carnifex, and 1.1 μm for N. maculosus respectively. At first sight it seems that T.c. carnifex liver primary transcripts are smaller than those of X. laevis. However the size range (although this is only a minimum value) of the transcripts shows some increase with C-value, from 6.3 μm, through 6.5 μm, to 9.13 μm for Necturus, and further, long transcription units of lampbrush-chromosome type were seen on the chromatin of T.c. carnifex, but not on that of X. laevis. It is not clear how much the small size of the sample that I analysed affects these conclusions. None of the size distributions that I obtained have any large gaps so that it is likely that my values are a valid measure of differences in primary transcript length, and therefore transcription unit length. None of the histograms showed obvious size classes; all formed a normal distribution slightly skewed to the right.

Further corroboration comes from a consideration of the lengths of the longest or terminal RNP fibrils of transcription units, although here, the sample size was only about half of that of the single fibrils. All the length distributions show gaps, so that more data are necessary before a firm conclusion can be drawn. This is all the more necessary, as arrays of lateral fibrils can be more reliably identified as RNP than single

fibrils, which, in some cases, might be broken replication forks. Terminal fibril lengths range from 0.4 μm in X. laevis, through 0.95 μm in T.c. carnifex, to 1.96 μm in N. maculosus. The size ranges also increase from 3.6 μm in X. laevis, through 9.6 μm in T.c. carnifex, with 7.08 μm for Necturus. The values for X. laevis are probably lower than they should be, as one would expect the mean of terminal fibril length to be greater than that of single fibrils, the former consisting of RNP nearer the end of transcription than the latter. If it is valid to assume, first, that liver cells of all these species are expressing similar numbers and types of genes, and secondly, that the degree of foreshortening of the RNP is the same for all species, then this result suggests that with increase in C-value, transcription unit length also increases.

The C-values of the organisms that I used are in the ratio 1:7.6:17.3. Primary transcript length, on the other hand, does not show so great a range in size. The difference in length of RNP between X. laevis, with the smallest genome, and N. maculosus, with the largest, is about two-fold, except in the case of terminal fibrils of arrays, which are in the ratio 1:2.4:4.9. As mentioned above, this last is probably an overestimate of the true relationship. The results are similar to those that I obtained from culture-cells, where the difference in primary transcript length between Xenopus and Triturus is less than the difference in their C-values. The results presented in this chapter are, however, more reliable than those from cultured cells.

In liver cells of T.c. carnifex and N. maculosus, I found RNP molecules of a size order up to 10 μm, previously only observed in lampbrush chromosomes (Angelier & Lacroix, 1975; Scheer et al., 1976) or spermatocytes

(Kierszenbaum & Tres, 1974, 1975; Glätzer, 1975; Amabis & Nair, 1976; Morgan, 1978). In one or two cases (Fig. II-12) these large molecules had the "bushy" structure reported for spermatocyte transcripts, but for the most part they were long and extended, resembling shorter transcripts in appearance (Figs II-10a and II-11). This strengthens their identification as RNP, unlike the case of spermatocytes (Morgan, 1978), and in this they resemble the transcripts of lampbrush chromosomes. In no case, however, were the putative circular excision products characteristic of lampbrush chromosome RNP ever seen (Angelier & Lacroix, 1975; Scheer et al., 1976).

In an attempt to further characterize stage-specific transcription I looked at the percentage of total transcription encompassed by different types of transcriptionaly event (Table II). It is clear that the values I obtained are very similar for T.c. carnifex and N. maculosus. X. laevis, on the other hand, apparently shows a higher percentage of isolated RNP fibrils. That this may be artefactual is indicated by the presence of anomalous ring-like structures attached to the DNP (Fig. II-6a, b). If these are indeed, as suggested, groups of two fibrils whose free ends are in some way associated, then the value for arrays of two fibrils in X. laevis (9.2%) should be higher, and bring the values for this species much closer to those for the other two. Bearing this in mind, together with the probable similarity in gene number in these species, I think it is possible to conclude that liver cells from these three organisms are of a similar degree of transcriptional activity. However, it would be desirable to know the number of different genes being expressed in these tissues, for instance by determining polysomal mRNA complexity. A determination of the percentage of the chromatin being transcribed would also throw some

light on this problem. However, I was not able to perform this experiment for liver cells.

My figures differ from those of Busby & Bakken (1979) who found that for gastrulae of the sea-urchin Strongylocentrotus purpuratus, arrays of three or more fibrils were more frequent than arrays of two RNP fibrils. On the other hand, isolated fibrils were the commonest type of transcriptional event. This indicates that embryos of S. purpuratus are transcribing certain gene sequences more rapidly than are liver cells, and this phenomenon may be connected to the specific needs of embryonic cells.

Because of the good preservation of structural details in preparations of liver cells, especially of Xenopus and Necturus, I was able to examine DNA and DNA/RNA packing ratios, together with nucleosome distribution with respect to transcription. If it is assumed that, under similar conditions of dispersal, Joy removes proteins from chromatin from differenc sources, to the same extent, then chromatin of different cell types can be compared (Table III, p57). For liver cells, the DNA packing ratios of transcriptionally inactive regions were 1.84 for X. laevis, 1.63 for T.c. carnifex and 1.4 - 1.64 for Necturus maculosus. However, spreading conditions, i.e. dispersal time and Joy concentration, varied between 20 - 75 min, and 0.1 - 0.2% respectively. These factors must be considered in the interpretation of my data.

The values for DNA packing ratio are quite similar but it would be dangerous to use them to conclude anything about differential gene expression. The similarity of transcription pattern that I observed in the three types of liver cell investigated suggests that DNA packing ratio ought to be similar in each cell type, assuming that a more extended chromatin structure is concomitant with increased transcriptional activity. The values I determined vary more than might be expected on this assumption and may be more preparative artefacts than reflections of the in vivo situation.

It is valid, however, to compare nucleosome distributions for transcribing and nontranscribing regions of the chromatin within the same preparation, regardless of dispersal conditions, and assuming that Joy removes proteins from these regions to the same extent. Where nucleosome distribution in transcription complexes could be determined (Table IV, p105), for Xenopus and Necturus, it was found that the DNA packing ratio was lower than for transcriptionally inactive regions, thus implying a more extended structure. This conclusion is qualified by the fact that I interpreted beads along the arrays, and lacking attached RNP, as nucleosomes rather than RNA polymerase molecules (Laird & Chooi, 1976; Laird et al., 1976; Scheer, 1978). In my preparations, polymerases could rarely be distinguished at the base of nonribosomal transcripts, which strengthens this interpretation. On the other hand, in the few instances where they could be identified, they were almost exactly the same size as nucleosomes. Furthermore, RNP molecules of less than 0.15×10^6 Daltons molecular weight cannot be resolved by the Miller Technique, and will appear as "beads" on the DNP, hardly distinguishable from nucleosomes (Scheer, 1978). A more rigorous investigation might involve the use of the anionic detergent "Sarkosyl" which removes all chromatin superstructure but leaves transcription complexes

intact (Scheer et al., 1977). These considerations perhaps imply that the packing ratio of transcriptionally active DNP is lower than the values I obtained. The difficulty lies in demonstrating whether, in spreads, transcription complexes have a nucleosomal or nonnucleosomal structure.

When nucleosome distribution in intervals increasingly distal to an isolated RNP fibril is considered, it is apparent (Table V, p106) that for X. laevis liver cells, the mean number increases with distance from the transcript, albeit only slightly. For N. maculosus, on the other hand, it remains constant or even declines slightly. I was not able to make such an analysis for T.c. carnifex liver cells. These apparently contradictory results are probably due to the method of analysis and the nature of the transcription complexes themselves. However, it does seem that the structure of transcriptionally active chromatin in amphibian liver cells is more extended than that of inactive chromatin.

In recent years, a number of lines of evidence, both biochemical and morphological, have led to the tentative conclusion that nucleosome structure is preserved in transcriptionally active regions of the genome. The results of digestion with staphylococcal or micrococcal nuclease have been interpreted as showing that both transcriptionally active and inactive regions of the genome have a nucleosomal structure. Avian reticulocyte globin genes, ovalbumin genes (Felsenfeld, 1978), Tetrahymena pyriformis ribosomal genes (Mathis & Gorovsky, 1976) rat liver (Lacy & Axel, 1975), mouse, X. laevis and Physarum polycephalum ribosomal genes (Reeves, 1976; Butler et al., 1978; Gottesfeld & Melton, 1978; Grainger & Ogle, 1978) have been investigated. However, the major drawback of these experiments is that only in a few cases (Reeves, 1976; Butler et al., 1978) is it known that an appreciable number of genes is transcriptionally active at the time of isolation.

It is known that DNAse I preferentially degrades transcriptionally active chromatin (Felsenfeld, 1978). This is the case for both maximally and sparsely transcribed genes so that the transcription complex itself does not confer sensitivity. DNAse I-sensitive regions are staphylococcal nuclease resistant in a way that indicates they must be protein-associated. McKnight et al. (1977) have shown that transcriptionally active regions of the genome react with antibodies to the histones H2B and H3. The DNAse-I-sensitive regions are packaged with proteins in a way that mimics nucleosomes but such that only DNAse I attack is permitted. These observations indicate that there is no simple relationship between chromatin accessibility to nuclease and its transcriptional activity (Reeves & Jones, 1976). They point to a modification of nucleosome structure, rather than its absence or unmodified presence in transcriptionally active regions (Weintraub et al., 1976; Foe, 1977; Gottesfeld, 1978; Scheer, 1978).

The fractionation experiments of Gottesfeld (1978) demonstrate that both transcriptionally active and inactive components of rat liver chromatin have a beaded structure. The chemical composition, sedimentation properties, differential sensitivity to DNAse I and nuclease S_1, together with optical melting behaviour of the active fraction, all confirm the idea that this fraction has a more open configuration. In the past, fractionation experiments have been dogged by the difficulty of eliminating cross-contamination of active with inactive fractions, but the procedure used here reduces this.

The biochemical work done to date implies that inactive and transcriptionally active chromatin, whether the latter is of ribosomal or nonribosomal origin, and including satellite DNA (Lipchitz & Axel, 1976; Lee, 1978), has a beaded structure (Brown et al., 1977). This is somewhat at variance

with the morphological data obtained from Miller spreads, even though the results of Reeves (1976) show that with increasing transcriptional activity, the bead number of ribosomal genes decreases.

In a number of systems, Miller spreads show that maximally transcribing ribosomal genes lack a nucleosomal structure (Foe et al., 1976; Franke et al., 1976a; Laird & Chooi, 1976; Laird et al., 1976; Woodcock et al., 1978; Foe, 1978; Franke & Scheer, 1978; Scheer, 1978; Villard & Fakan, 1978). On the other hand, nonnucleolar transcription units tend to be beaded in morphology (Foe et al., 1977; Franke & Scheer, 1978), although it is not yet absolutely proven that the "nucleosomes" of these arrays are not in fact polymerases (Laird & Chooi, 1976; Laird et al., 1976; Franke & Scheer, 1978; Busby & Bakken, 1979). Nonetheless the DNA packing ratios of nonnucleolar arrays are less than that of inactive chromatin, so that transcription seems to be accompanied by an opening out of chromatin structure (Laird & Chooi, 1976; Laird et al., 1976; Busby & Bakken, 1979).

It is debatable whether the "subunits" visualised in many digestion experiments are indeed analogous to the nucleosomes seen in the electron microscope (Brown et al., 1977), and evidence has recently been produced that DNP in vitro, although appearing "smooth" in the electron microscope, can have a subunit structure with respect to nuclease attack (Moudrianakis et al., 1977; Woodcock & Frado, 1977; Foe, 1978). The nucleosome itself may be a transient structure within the transcription unit (Scheer, 1978) implying that chromatin structure is dynamic rather than static.

Bearing in mind the fact that my preparations were of chromatin dispersed in solutions containing Joy, my results are basically in accord with what is so far known about nucleosome organization and distribution.

I found greater differences in DNA packing ratio between transcribing and nontranscribing chromatin than did Busby & Bakken (1979), and although this might be an effect of sample size, these data are also compatible with the observations of Foe (1977) who found that in O. fasciatus chromatin, DNA packing ratios for inactive, ribosomal and nonribosomal chromatin were 2.3; 1.6 - 1.9, and 1.0 - 1.2 respectively. Unfortunately, ribosomal genes were not evident in any preparation of liver cells, so that I was unable to calculate DNA packing ratio for purposes of comparison with those for transcriptionally inactive DNP and nonribosomal arrays.

I was also able to determine the DNA/RNA packing ratio for a number of arrays from X. laevis and N. maculosus. Compared to the value of 13.3 I obtained for T.c. carnifex oocyte ribosomal genes (Appendix III), the values for nonribosomal arrays from these cells fell around 1.23 - 1.45 or between 17 - 25. This sharp demarcation into classes may be significant. The high value was that for an array (Fig. II-19 a-c) resembling a classical ribosomal gene in morphology, but longer than is usual for amphibian ribosomal genes. Its sparse coverage by RNP, and lack of tandem repetition suggest that it is nonribosomal. However, in its DNA/RNA packing ratio it resembles ribosomal genes. All the arrays of low DNA/RNA packing ratio were of nonribosomal morphology so that in my hands, DNA/RNA packing ratio could be used as a criterion for distinguishing between these two types of array. This is in contrast to the situation in Oncopeltus fasciatus (Foe et al., 1976) where the DNA/RNA packing ratios for all arrays, irrespective of origin, were between 6 and 7. This may be due to differences in spreading conditions and it is necessary to study more arrays before this question can be resolved.

Although it is conceivable that the array of ribosomal morphology is really ribosomal, its length (3.8 μm) is so much greater than that of ribosomal genes in Amphibia, that stretching may not be responsible for it. If it is nonribosomal then DNA/RNA packing ratio cannot be used as a criterion to distinguish between these two types of array. It is intriguing, however, to speculate that a high DNA/RNA packing ratio on the other hand, is characteristic of those genes whose products, like the 45S preribosomal RNA precursor, transfer RNAs, and 5S RNAs, are never translated, and so need not maintain an extended conformation for polyribosome attachment. It is still unknown whether other classes of nontranslated RNA exist, besides the ribosomal and transfer RNAs, and it would be desirable to investigate this.

There was some variation in the size of the subparticles visible in primary transcripts. These were determined to be about 20 nm in diameter, in amphibian oocyte transcripts (Sommerville, 1973; Malcolm & Sommerville, 1974; 1977; Mott & Callan, 1975) whereas, in stained preparations of liver chromatin I found their dimensions to be 14 - 19 nm for X. laevis, about 26 nm for T.c. carnifex, and 13 - 15 nm for N. maculosus. This range of values is probably due to the diffuse appearance of RNP in my preparations, which made accurate measurement difficult.

I did not observe any circular DNA molecules in my liver cell preparations; it is not clear why, for the conditions of spreading were similar to those used for culture cells, and in both cases, whole cells were used. In the latter case, putative mitochondrial DNA was seen. It might be possible to extract and spread the contents of mitochondria both to examine replication and transcription of these genomes and to see if there were any C-value dependent difference in mitochondrial DNA contour length.

Such a difference, of 20%, has been shown to exist between mitochondrial circles of the urodelans Necturus maculosus, and Siredon mexicanum, and the anurans Xenopus laevis and Rana pipiens (Wolstenholme & Dawid, 1968).

C H A P T E R III.

TISSUE-DEPENDENT TRANSCRIPTION PATTERNS IN *Xenopus laevis* AND *Triturus cristatus carnifex*

INTRODUCTION

During the course of my investigations, I experienced some difficulty in obtaining transcription figures from the chromatin of *X. laevis* and *T.c. carnifex* tissue-culture cells, due to their low RNA synthetic activity. In order to obtain more data on C-value dependent differences in transcription patterns I decided to make spreads of tissues of these organisms that might show a higher degree of RNA synthetic activity than cultured cells. The results I obtained also allowed me to compare transcription patterns in different tissues of the same species.

A number of investigations suggest that, in general, embryonic cells show a relatively high rate of RNA synthesis, so I decided to use newt and frog embryos fur further study. Miller spreading demonstrates that embryonic cells produce large amounts of hnRNA, and transcription patterns have been characterized in embryos of *Oncopeltus fasciatus* (Foe *et al.*, 1976; Laird *et al.*, 1976; Foe, 1978), *Drosophila melanogaster* (Laird & Chooi, 1976; McKnight & Miller, 1976; McKnight & Miller, 1977; McKnight *et al.*, 1977). and *Strongylocentrotus purpuratus* (Busby & Bakken, 1979). Determinations of the percentage of the genome that is transcriptionally active suggest that in embryos the level of hnRNA synthesis is high (McKnight & Miller, 1976). A number of biochemical investigations have also been performed (for review see Davidson, 1976), for both the sea urchin and Amphibia, where embryonic RNA synthesis has been extensively characterized (Brown & Gurdon, 1965; Lerner *et al.*, 1965; Denis, 1974; Kung, 1974; Hough-Evans *et al.*, 1977; Kleene & Humphreys, 1977). In the embryo of *X. laevis*,

transcription levels are negligible, and protein synthesis is actinomycin-insensitive until the gastrula stage (Denis, 1974) so I decided to use neurulae, a slightly later developmental stage, for my investigation of embryonic RNA synthesis.

An advantage is the easy availability, in great number, of amphibian embryos. Injection of animals with chorionic gonadotrophin allows one to obtain embryos all the year round for X. laevis, and during the breeding season (duration about 1 month) for T.c. carnifex.

To date, the Miller spreading technique has not been used systematically to study tissue-specific transcription patterns. Embryogenesis in D. melanogaster is characterized by a progressive activation of nucleolar and nonnucleolar genes, and there is an increase in the amount of chromatin transcribed, (McKnight & Miller, 1976). In few other cases where information on different tissues of one organism is available, for instance Drosophila hydei spermatocytes and polytene chromosomes (Derksen, 1975; Glätzer, 1975) the difficulty of obtaining good spreads in the latter case precludes all but a descriptive approach. A large variety of cell types from the rat have been investigated, but in only one instance, the cortisol stimulation of primary cell cultures, has a quantitative analysis been possible (Puvion-Dutilleul et al., 1978). The other cell types studied have shown only low levels of transcriptional activity, so that again, only a qualitative analysis was possible (Puvion-Dutilleul & Bernadac, 1976; Puvion-Duteileul et al., 1977; Harper & Puvion-Dutilleul, 1979, in press). Thus a study of transcription as a function of cell type is clearly necessary if gene regulation is to be understood.

The results of biochemical analysis are sparse. In Rana pipiens it has been shown that hnRNA size increases during development, and size

differences in the hnRNA exist between axial and belly regions of tailbud embryos (Shepherd & Flickinger, 1979). However, although precautions were taken to minimise nuclease activity in the RNA preparation, and to ensure that aggregation of the RNA did not occur, it has been suggested (Federoff et al., 1977) that the preparative technique used by these authors results in some degree of aggregation of hnRNA. Aggregates of RNA can be eliminated during analysis of a Miller spread (see Chapter I this study), for only clear RNP fibrils or transcription complexes need be considered. Thus the type of analysis described in this chapter is all the more necessary in order to supplement the biochemical data.

MATERIALS AND METHODS

(i) Injection of T.c. carnifex with chorionic gonadotrophin

The breeding season of T.c. carnifex in captivity in Scotland occurs in March and lasts for about one month. To obtain embryos for Miller spreads, newts were injected with chorionic gonadotrophin several days before these were needed. 10 female T.c. carnifex in breeding condition (Naples, 1977) were injected with 100 international units (i.u.) each of horse chorionic gonadotrophin (400 iu/mg. CIBA) dissolved in distilled water. Each female newt was placed in a tank containing Elodea, together with a male T.c. carnifex (uninjected) also in breeding condition (Day 1). Newts were fed on Day 1 with live Tubifex worms. Neurulae were obtained on the eighth day after injection, as described in Hamburger (1966). Newts and embryos were maintained at 16°C in a room specially designed for this purpose.

Day 1	Inject newts. Place in tanks with weed.	Feed.
2		Feed.
3	Remove weed. Add pesticide-free grass.	
4		
5		
6	Late blastulae	
7	Gastrulae	
8	Neurulae	

On Day 3, the Elodea was removed, and pesticide-free grass added in its place. Eggs are laid on the grass and are much easier to find than when laid on Elodea. Eggs laid on the grass were collected daily and placed in small plastic tanks containing 1/10 strength Steinberg saline. They were sorted into stages according to the stage series for X. laevis

(Nieuwkoop & Faber, 1956; Hamburger, 1966; Rudak, 1976), and checked daily. Any mouldy embryos were discarded.

Stage (Nieuwkoop & Faber, 1956)	
6 - 8	blastulae
9 - 12	gastrulae
13 - 20	neurulae

Neurulae between stages 16 and 17 were used.

(ii) Solutions

(a) Full strength Steinberg solution (FSS). (S. Hennen, pers. Comm. to H.G.C.).

	per litre
17% NaCl	20 ml
0.5% KCl	10 ml
0.8% $Ca(NO_3)_2.4H_2O$	10 ml
2.05% $MgSO_4.7H_2O$	10 ml
1.00 N HCl	4 ml
Tris buffer	560 mg
Streptomycin sulphate	50 mg
Pencillin-G-sodium	30 mg
Glass-distilled water	946 ml

Stock components were stored at 0 - 4°C.

The complete solution was Millipore filtered into sterile glass bottles as for tissue-culture solutions. Dilution was with non-sterile distilled water because 1/10 Steinberg solution was not required sterile. All solutions were stored at 0 - 4°C.

(b) Joy

Chromatin was spread in a 0.2% solution of Joy in distilled water, adjusted to pH 8.7 with stock borate buffer.

(c) Sucrose-formalin fixative

0.1 M sucrose (Analar) in distilled water containing 10% formalin (Analar) was adjusted to pH 8.5 with 0.1 M NaOH.

(iii) Decapsulation of embryos (Rudak 1976)

To remove the outer membranes of the embryos, they were placed in FSS in a small glass Petri-dish with a layer of black wax at the bottom. The outer gelatinous membrane was removed by piercing it at the top, going through the gelatinous layer, and coming out at the bottom and into the wax, with one point of a pair of sharpened stainless steel No. 5 watchmakers' forceps. Keeping the forceps still, the point of a tungsten needle was brought close to the embedded forceps point and both were quickly drawn across each other in a scissor-like motion. When this is done successfully, the embryo in its vitelline membrane is released from its gelatinous capsule.

The embryo was then transferred using a bent, wide-bore, siliconised glass pipette, to a small plastic Petri dish containing FSS over 2% Agar (Noble; Difco) in FSS.

The vitelline membrane was removed from the embryo by grasping the membrane at the top with a pair of sharpened stainless steel No. 5 watchmakers' forceps, then inserting the tip of a tungsten needle at this point and making a tear in a downwards direction, thus tipping out the ball of cells on to the agar surface.

(iv) Dispersal of chromatin

Using a pair of sharpened number 5 watchmakers' forceps and a tungsten needle, small pieces of tissue (1 mm^3) were cut out of the neural fold region of a decapsulated embryo, and rinsed briefly in pH 9 water to remove Steinberg solution, whose high salt concentration would be likely to prevent dispersal of the chromatin. A few minutes previous to this operation 100 µl droplets of Joy were placed on clean squares of Parafilm in sterile plastic Petri dishes (Falcon). A piece of washed tissue was placed in a droplet and carefully macerated with two pairs of sharpened no. 4 watchmakers' forceps. The Petri-dishes were covered and the chromatin allowed to disperse for 75 min at room temperature. For each experiment, eight small Petri dishes, each containing 1 droplet of Joy, were set up. Chromatin from 10 - 20 µl of each droplet was centrifuged on to one grid (8 grids prepreparation).

Fixation and staining of chromatin were as described in Chapter I for preparations of culture-cell chromatin. Preparations were not rotary shadowed.

(v) Injection of X. laevis with chorionic gonadotrophin.

On Day 1 three male X. laevis were anaesthetised in 0.1% MS222 (Sandoz) and injected in the dorsal lymph sac (Brown, 1970) using a long needle, with 300 iu (0.5 ml vol) horse chorionic gonadotrophin in distilled water (CIBA). The frogs were then kept in glass tanks, at 18°C for 1 day.

On Day 2 the three male X. laevis injected on day 1, and three female X. laevis, were anaesthetised as before. The males were each injected with 300 iu (0.3 ml vol) of Horse chorionic gonadotrophin and the females were given 500 iu (0.5 ml)of chorionic gonadotrophin.

The frogs were then divided into mating pairs, and placed in glass tanks which had been aerated, and provided with a plastic neeeting framework to prevent damage to any eggs that might be laid. The mating pairs were maintained at 18°C.

Although I ran two experiments, in one case giving the females rather than the males a primer injection of hormone, the frogs failed to go into amplexus and no eggs were laid, although it should be possible to obtain embryos all the year round in this species.

RESULTS

(i) Transcription patterns in embryonic cells of Triturus cristatus carnifex

Under the dissecting microscope T.c. carnifex embryonic tissue appeared to consist of large cells filled with yolk. In the case of DNA fibre autoradiographs of T.c. carnifex blastulae and gastrulae, a network of some material, possibly glycogen, prevented the DNA from free extension (H.G. Callan, personal communication). This was not the case for Miller spreads of neurulae, probably because any yolk particles or glycogen sank to the bottom of the Joy droplet in the same way as melanin from X. laevis liver cells (Chapter II, this study). In addition, the sucrose in the fixative prevents cell debris from pelleting on top of the chromatin.

Preparations from T.c. carnifex neurulae consisted of dense networks of chromatin. It was difficult to adjust the loading of the grids, even though small pieces of tissue were used, due to the high C-value of this species, and in general the amount of chromatin on the grids made scanning for RNP difficult. Ideally, pieces of tissue less than 1 mm^3 should have been used, or a larger droplet of Joy (200 µl).

Much of the DNP in these preparations was of a "smooth" appearance and nucleosomes were sparse (Fig. III-1a). Superbeads were never observed. Nucleosomes measured 14.8 nm ± 3 nm in stained preparations. The lack of extensive nucleosomal structure is probably due to the lengthy dispersal of the chromatin in the presence of a relatively high concentration of Joy. "Smooth" fibrils were about 14 nm wide, indicating that they consisted of DNA associated with protein. Earlier investigations (see Chapter I) show that their presence is an artefact of the spreading conditions that I employed (nearly all the DNP of preparations spread under conditions of minimum detergent concentration and dispersal time had a nucleosomal configuration.)

Transcription figures gave the impression of being commoner than in either of the cell-lines that I studied, or in liver cells of T.c. carnifex. However, this is an impression difficult to substantiate, because there is no way of determining how much chromatin is present on a grid. Transcription figures were not frequent enough to allow me to determine the percentage of the genome being transcribed.

Transcription was again predominantly in the form of isolated RNP fibrils. Isolated fibrils (Fig. III-1a-c) formed a skewed distribution (Fig. III-2a) around a median value of 0.82 μm, and with a range of 2.3 μm (Table I, p54). Few arrays were found so that only a small sample was available for analysis of the length of terminal fibrils of transcription complexes. These formed a distribution falling around a median of 1.3 μm, and with a range of 1.4 μm (Figs III-2b and III-3b-c). (Table I, p54).

As with other cell types, I was able to determine the percentage of different types of array. Isolated fibrils formed 82.7% of the total transcription observed whereas groups of two fibrils made up 9.6% of the whole. Arrays of three or more fibrils (Fig. III-3c) formed 7.7% of the total transcription (Table II, p51). These figures are very similar to those determined by Busby & Bakken (1979) for embryos of Strongylocentrotus purpuratus, where single fibrils made up 82% of the total, groups of two fibrils 7.0%. and arrays of three or more fibrils 11.0%.

The RNP of these preparations showed a higher degree of contrast than did the DNP (Figs III-1b-c and III-3a-b). It was greater in width than the DNP, at 15.7 nm ± 4.3 nm but a beaded structure was not clearly definable. In a few cases (Fig. III-3a and c) configurations indicative of RNA processing of breakage were found.

Although much of the DNP was "smooth" in appearance, I was able to determine the DNA packing ratio for transcriptionally inactive regions of the chromatin. I obtained a value of 1.35 (Table III, p57) for regions of DNP having a beaded structure. None of the smooth DNA (packing ratio assumed to be 1.0) was included, so it is more reasonable to express the DNA packing ratio as being 1.0 - 1.35. Clearly analysable transcription complexes were not available so that I was unable to determine the DNA packing ratio for transcriptionally active regions of embryo chromatin.

However, I was able to measure the nucleosome density in regions of chromatin progressively distal to an isolated RNP fibril (Table V, p106). The mean number of nucleosomes per 0.5 μm interval were very similar for all the intervals, as was the range of values per interval. This result is inconclusive. One drawback of the method of analysis that I used is that because it is not possible to determine the direction of transcription for a transcription complex carrying only one RNP molecule, the fact that identical intervals on either side of the fibril are summed means that any differences in nucleosome distribution between either side of the fibril may be obscured.

In arrays of T.c. carnifex embryo chromatin there was no apparent nucleosome structure. Thus I assumed the DNA packing ratio of these regions to be 1.0, and the DNP to be maximally extended. In one case an array was found, having a DNP length of about 2.86 μm (Fig. III-3c). The length of its terminal fibril was 0.72μm, giving a DNA/RNA packing ratio of about 4.0 (Table VI, p59). This value is only a rough approximation, for it was not possible to determine the position of the initiation point of transcription for this array with certainty. Thus the true DNP length is likely to exceed 2.86 μm and the DNA/RNA packing ratio will be greater than 4.0.

Ribosomal transcription was never observed in these preparations and this confirms the report of Busby & Bakken (1979) on transcription in S. purpuratus embryos. DNP rings, of mitochondrial or other origin, were also absent.

(a) Putative polyribosome-like structures

In several preparations of embryo chromatin, structures resembling polyribosomes were found (Fig. III-4). These consisted of varying lengths (up to 4.0 μm long) of darkly staining oval bodies connected by a fine fibril. The oval bodies measured 26 nm ± 1.7 μm by 34.7 nm ± 7 μm, and thus are probably too large to be supernucleosomes. Their oval shape and arrangement on the interconnecting fibril also precludes this interpretation.

These structures bear a superficial resemblance to beaded high molecular weight RNA extracted from amphibian oocytes (Sommerville, 1973; Malcolm & Sommerville, 1974, 1977). The double structure of some of the beads seen in my preparations resembles ribosomes rather than RNP particles. Also, it is not clear why RNP attached to DNP should not also have this structure, if the "polysomes" are in fact RNP.

None of the polysome-like structures were seen to be attached to a DNP axis and there is certainly no evidence for a coupling of transcription and translation, unlike the situation in bacteria (Miller et al., 1970). Similar polysomes were observed by McKnight and his colleagues (McKnight et al., 1976) in spreads of Bombyx mori silk-gland cells, and by Daneholt (Daneholt et al., 1976) in Chironomus tentans salivary glands.

Unfortunately I was not able to make spreads of Xenopus embryonic tissue because, despite two attempts, I was not able to obtain embryos.

COMPARISON OF TRANSCRIPTION PATTERNS IN DIFFERENT TISSUES OF Xenopus laevis

I observed a considerable difference in the overall characteristics of transcription between X. laevis cultured cells and liver cells, the two tissues I was able to study (see Chapters I and II of this study for details).

Isolated RNP fibrils in X. laevis liver cells have a median value 1.5 times as great (0.44μ and 0.66μ) and a range three times as great (2.1μ and 6.3μ) as in cultured cells (Table I, p54). Unfortunately, I was not able to measure terminal fibril lengths in preparations of X. laevis cultured cells, so that a strict comparison cannot be made. These differences are significant and probably stage-specific, reflecting those sets of genes expressed in each tissue.

There is also a marked difference between the two cell types, in the distribution of different types of transcription complex. In cultured cells, 94.4% of the transcription takes the form of isolated RNP fibrils, whereas only 5.6% consists of arrays (of 3 or more fibrils). Groups of two fibrils were absent. On the other hand, only 86.1% of the total transcription in liver cells, consisted of isolated fibrils, and arrays of two or more fibrils comprised 13.8% (9.2% + 4.6%. See Table II, p56). This suggests that liver cells are transcribing proportionately greater amounts of certain DNA sequences than are cultured cells, and that these sequences are part of the more numerous transcription complexes seen in chromatin from this cell type.

DNA packing ratio for transcriptionally inactive regions of X. laevis cultured cells was 2.1, whereas for liver cells I obtained a value of 1.84 (Table III, p57). This suggests that in the more transcriptionally active tissue, liver, even transcriptionally inactive regions of chromatin have a more extended structure. However, the conditions of dispersal varied between

the two preparations. In the case of cultured cells, dispersal was for 15 min in a solution containing 0.1% Joy, whereas in the case of liver cells, dispersal was for 1 hr in 0.1% Joy. This means that more protein is likely to have been removed from the liver cell chromatin, perhaps resulting in a lower DNA packing ratio. If the packing ratio for liver cells is in reality greater than 1.84, then it approaches that for cultured cells. As this value describes the foreshortening of transcriptionally inactive regions of the DNP, it is not really incompatible with the observed differences in transcriptional activity between the two tissues. Loosening of the chromatin structure might occur only in potentially active regions (see Chapter II of this study).

I was only able to determine the DNA packing ratio for transcriptionally active regions in X. laevis liver (Table IV, p105) so that comparison is not possible. In the same way, analysis of nucleosome number at intervals increasingly distal to an isolated RNP fibril was only possible in the case of liver cells.

The small sample of arrays available for analysis affects my determinations of DNA/RNA packing ratio (Table VI, p59), but I found that this parameter apparently fell into classes. For X. laevis liver, the values I obtained were 1.23 and 1.23, whereas for cultured cells a value (1 array) of 10 - 12 was obtained. Although these values might define the limits of a normal distribution, as has already been noted for Oncopeltus fasciatus (Foe et al.. 1976), the differences in morphology between transcription complexes in my preparations may also support the first interpretation. The results for other cell types also support the conclusion that DNA/RNA packing ratio shows size classes. Ideally such comparisons are best made between preparations spread under the same conditions, although in practice the morphology of arrays in general did not vary under different spreading conditions.

Analysis of transcription patterns in Necturus maculosus liver cells indicates that DNA/RNA packing ratio may be a criterion as to whether a primary transcript is translated, rather than a means of distinguishing between ribosomal and nonribosomal transcription complexes, and if this is so, DNA/RNA packing ratio would be expected to show size classes.

Ribosomal transcription was observed in X. laevis cultured cells but was apparently absent in liver cells. This may be a tissue-specific characteristic, but it is difficult to believe that ribosomal RNA synthesis is entirely lacking in a somatic tissue. Early embryonic stages rely on maternally synthesized ribosomes (Foe, 1977; Busby & Bakken, 1979) and are characterized, both biochemically and morphologically, by an absence of ribosomal RNA synthesis. However, all somatic tissues, especially such metabolically active ones as liver, must support some degree of ribosomal RNA synthesis. This might be expected to be intense in liver cells. The absence of such transcription complexes in X. laevis liver cells may be due to chance inasmuch as I made fewer preparations than for X. laevis culture cells. Furthermore, these genes are probably present exclusively as a block of tandem repeats integrated into the genome, so that a relatively large amount of chromatin would have to be scanned before ribosomal transcription units would be noted. In cultured cells, I observed that the ribosomal genes tended to occur in large clumps of tandemly repeated complexes, and were rare.

Circular DNA molecules were a characteristic of Xenopus cultured cells, but were not seen in preparations of liver cell chromatin. As far as the putative mitochondrial circles are concerned, this is probably a chance effect, as it is likely that liver cells contain many active mitochondria. Rings of other types may have been visualised in X. laevis cultured cells because the preparation in which they were found was characterized by a

very short (15 min) dispersal time. This may have resulted in the preservation of structural details lost from the liver cell chromatin. Thus it is probably not true that the presence of the types of circle noted here (Chapters I and II) is a tissue-specific characteristic. My results do not eliminate their occurrence in other cell types.

COMPARISON OF TRANSCRIPTION PATTERNS IN DIFFERENT TISSUES OF *Triturus cristatus carnifex*

I was able to examine three different cell types from *T.c. carnifex*, tissue-culture cells, liver cells and embryonic cells. The general trends in transcription pattern between different tissues are similar for both *T.c. carnifex* and *X. laevis*.

The median length of isolated RNP fibrils increases from 0.33 μm in cultured cells, through 0.6 μm in liver to a maximum of 0.82 μm in embryonic cell chromatin in a ratio of about 1:1.8:2.5 (Table I, p54). There is also a difference in the total range of length between these three cell types. The range for cultured cells is 1.03 μm, and for liver cells 6.5 μm, a difference greater than for the median values. This may be partly because I was only able to analyse a small sample from culture cells. The range for embryonic cells is 2.3 μm (Table I, p54), and implies that the distribution of RNP lengths is narrower than for the other cell types. The sample sizes for liver and embryonic cell RNP were similar and no gaps occurred in the histograms. Thus the calculated values are probably a valid index of transcriptional events in these cells. If so, the difference in range between liver and embryonic cells (6.5 μm and 2.3 μm respectively) is probably a tissue-specific characteristic, and does not follow the difference in median value. In both *T.c. carnifex* and *X. laevis*, liver cells apparently make longer primary transcripts than do cultured cells. However, long molecules up to 10 μm (30,000 bp, Scheer *et al.*, 1979) in length were observed in *T.c. carnifex* cultured cell chromatin. Nonetheless, the difference is likely to be real, as the frequency of long RNP molecules was greater in liver cell chromatin. Such molecules were never seen in embryonic cells.

A similar trend is observable if the terminal RNP fibrils of arrays of two or more fibrils are considered. Median terminal fibril length increases from 0.4 μm in culture cells to 0.95 μm in liver respectively, and to 1.3 μm in embryonic cells, i.e. a ratio of 1:2.4:3.2 (Table I, p54). This relationship is probably a better approximation to real differences in transcription unit length between these tissues than is single fibril length. Similarly the range of values also varies, being 0.2 μm in cultured cells 9.6 μm in liver cells, and 1.4 μm in embryonic cells (Table I, p54). Although these data are incomplete, due to the small number of samples available for analysis, the range of values is narrower in embryonic cells than in liver and the three values are related to each other as are the median values for isolated fibril lengths. This suggests that embryonic cells are expressing genes whose transcription unit lengths are more similar than those in liver cells, although their mean length is greater than in either cultured cells or liver.

One qualification must be borne in mind when interpreting the size distributions of terminal fibrils of arrays. In all cases of groups of two fibrils, the spacing bewween the molecules was less than 1 μm (see Chapter I). However such configurations fell into two classes, both of which were included in my analyses. the first class consists of two fibrils the difference in whose length is about the same as their spacing on the DNP axis. Here, the longer fibril was interpreted as being the terminal fibril. The second class consists of two fibrils the difference in whose length is greater than their spacing on the DNP. These latter configurations could be the result of either processing or breakage. If the former has occurred, then the shorter of the two fibrils is the terminal fibril. If the latter, then either fibril could be the terminal fibril. In fact, the longer fibril was consistently included as the terminal fibril. The

fact that terminal fibril distributions nearly always gave a median value greater than for the single fibril distributions suggests that this is probably not a significant source of error.

I was able to assess the percentage of different types of array for all the tissues I used. Due to the small sample size for arrays from cultured cells, the values for these are probably less reliable than for the other two tissues. I obtained values for single fibrils, for groups of two RNP fibrils, and for arrays of three or more fibrils, respectively, of 72.7%, 9.1%, 18.2% for cultured cells, 66.2%, 25.3% and 8.5% for liver, and 82.7%, 9.6% and 7.7% for embryonic cells (Table II, p56). These values differ markedly, more so than did the values I obtained for liver cells of species differing in C-value (Chapter II. This study).

In liver cells, arrays of all types comprise nearly 34% of the total transcription, whereas they form only 17% of the total in embryos (Table II, p56). This suggests that greater amounts of certain gene sequences are being transcribed in liver cells than in embryonic cells. It is not clear how this is related to the observation that the range of RNP fibril length is greater in liver than in embryos. However, determinations of the percentage of different types of transcription complex take no account of the absolute number of transcription complexes per nucleus, which cannot be determined in my preparations. Only in cultured cells is the percentage of groups of two fibrils less than the percentage of arrays of three or more fibrils (Busby & Bakken, 1979).

It may be that the metabolic requirements of embryos dictate the expression of more different genes than in liver cells. Thus the percentage of isolated fibrils would be higher, as it is, in embryonic cells, which show the highest percentage of single fibrils of the three tissues. On the

other hand, that small subset of liver cells spread in my preparations may have had a need for a large number of transcripts of a few sequences, i.e. of key enzymes.

The DNA packing ratio of inactive regions of the chromatin was 1.88 for T.c. carnifex cultured cells dispersed for 20 min. in the presence of 0.1% Joy. Values for the other two tissues were 1.63 for liver and 1.35 for embryos, both for chromatin dispersed for 75 min in the presence of 0.2% Joy, and which are therefore comparable (Table III, p57). Slight differences in the protocol for making up the sucrose-formalin fixative may affect my interpretation, however. This result implies that transcriptionally inactive regions of the T.c. carnifex neurula genome are more extended than similar regions of the liver genome. This observation agrees well with the observed greater transcriptional activity of embryo chromatin. In reality (see section (i) of Results, this chapter) the DNA packing ratio of embryonic cells is likely to be less than 1.35, as DNP lacking nucleosomes was not included in my analysis. It would be interesting to compare the DNA packing ratios for these two tissues after dispersal of the chromatin in more favourable conditions (i.e. for 15 min in the presence of 0.1% Joy) to see if this relationship still held true.

Unfortunately, analysable arrays were not found in preparations of any of these tissues so that I was not able to determine the DNA packing ratios for transcriptionally active regions of the genome. The mean nucleosome number for regions increasingly distal from a single RNP fibril remains roughly constant for embryonic cells but I was not able to define this parameter for either cultured cells or liver. Similarly, a DNA/RNA packing ratio was only determined for one array (Table VI, p59) in embryonic cells; I obtained a value of at least 4.

Ribosomal genes were absent from preparations of all these tissues. Low transcriptional activity probably accounts for the absence of "Christmas trees" from cultured cell chromatin but it is not clear why they were absent from liver cells of T.c. carnifex, and the other two species that I investigated (Chapter II, this study).

Ribosomal RNA synthesis was apparently absent from embryonic cells. Although early embryonic stages synthesise little ribosomal RNA, in X. laevis and probably also in T.c. carnifex, ribosomal RNA synthesis has already begun by the neurula stage (Brown & Gurdon, 1965). My result may be due to chance and the fact that I was not able to make many preparations. Furthermore, individual cells in the neurula may differ in the extent to which they are transcribing ribosomal RNA. I spread pieces of tissue cut out of the neural fold region, and there is no guarantee that the same cells were spread in any two preparations.

Rings were absent in preparations of all these tissues. Putative polysomes appeared in preparations of embryo chromatin alone; it is not clear why. Differences in polysome length and therefore the degree to which they pellet down should not be responsible, for most mRNAs are of similar length. Polysomes would not however occur in most of my culture cell preparations as they were spreads of nuclei rather than cells, and lacked cytoplasm. However, this still does not account for their absence from liver cells, where whole cells were dispersed, and which are transcriptionally, and therefore probably also translationally, active.

DISCUSSION

The results presented and discussed in this chapter show that primary transcript length can vary between different tissues of an organism. Both T.c. carnifex and X. laevis tissue-culture cells, the least transcriptionally active of the cell types that I studied, apparently transcribe the shortest RNP, whereas embryonic cells (in T.c. carnifex), transcriptionally most active, synthesise the longest. In both species the RNP of liver cells is longer than that of culture cells. However T.c. carnifex cultured cells synthesise occasional (10 - 11 μm) long transcripts. Parameters such as the distribution of transcription events amongst different types of transcription complexes, also vary as a function of cell type, and demonstrate that tissues differ in the relative amount as well as in the length of the RNA transcribed. Evidence also exists (Shepherd & Flickinger, 1979) for tissue-specific variation in hnRNA length in Rana pipiens.

My data are of limited significance on their own, and merely imply that at different times, different populations of genes coding for primary transcripts of various lengths, are activated. In recent years, however, an appreciable body of evidence has accumulated, showing that many differentiated states share a large proportion of both their hnRNA and mRNA sequences. Assuming that there is some direct relationship between the primary transcript and the biochemically defined hnRNA, these data have a direct bearing on the interpretation of my results.

In man, liver and leukaemic cells show a 75 - 85% homology of their messenger RNAs, a result similar to that obtained for chicken and mouse cell mRNAs (Ostrow et al., 1979). An exhaustive analysis of Strongylocentrotus purpuratus mRNAs from seven different tissues (Galau et al., 1976) demonstrates clearly that cells from these tissues share a small subset of their mRNAs, the complexity of which is about 2.1×10^6 nucleotides, and coding for no

more than 1000 - 1500 genes. It is possible to estimate gene number from messenger RNA complexity data because in most eukaryotes messenger RNAs are of similar size and complexity (Galau et al., 1974; Anderson et al., 1976). A straightforward explanation for this observation is that all cell types express a small number of genes coding for the "household functions" required by every cell, regardless of its state of differentiation. The situation becomes more complex when the nuclear RNA is considered, and it is clear that there is no simple relationship involved.

In tissues of the rat (Chikaraishi et al., 1978) extensive overlap was noted between the nuclear RNAs of different tissues, when single copy sequences were considered. Estimates of the gene number expected for the single copy sequence complexity of each hnRNA sample were also made. However, I do not think this latter procedure justified. The average length of rat brain poly A-containing RNA is 4500 base pairs (Chikaraishi et al., 1978), but this is the value for the abundant low-complexity fraction of the hnRNA, made up largely of the transcripts of repetitive genes. If this length value is valid for the complex RNAs, considered here, which form the smaller fraction by mass, but the greater fraction of the complexity, then gene number can be calculated from complexity values. However, there is no compelling reason for this assumption, as it is known that hnRNA varies greatly in length. Its sequence organization is also such that in some cases greater sequence complexity will correlate with transcript length. Finally, only a small proportion of the unique sequences expressed in the hnRNA consists of protein coding sequences.

A contradictory result was obtained for Tripneustes gratilla by Kleene & Humphreys (1977) who showed that between the blastula and pluteus stages there was no change in hnRNA complexity. This might be because these

stages are more similar in their patterns of gene expression than other groups of tissues. It is possible that hnRNA length could vary in the absence of changes in complexity, but the facts known about hnRNA sequence organization argue against this.

Conversely, in S. purpuratus (Ernst et al., 1979) some single copy sequences present in intestine hnRNA are absent from gastrula hnRNA. A more complex situation emerges from the experiments of Wold (Wold et al., 1978) who showed that in the same organism, blastula mRNA sequences were present in the nuclear RNA of adult tissues, but absent from the messenger RNAs of these tissues.

When the repetitive sequences of S. purpuratus hnRNA are considered (Scheller et al., 1978) it is seen that different families of repetitive sequences are highly represented in the hnRNA of different cell types, although a few copies of each family are present in all cell types (adjacent to housekeeping genes?). Most of the 3'-proximal sequences of large poly A-containing hnRNA are homologous to mRNA (Hahn et al., 1978).

At the most, these data imply that, between different developmental stages and cell types, message sequences can show a large degree of overlap. The single copy sequence fraction of the hnRNA, whether coding or noncoding, can show either slight or extreme overlap between differentiated states. On the other hand, the hnRNAs of different tissues seem to have a smaller fraction of their repetitive sequences in common. Between synthesis of hnRNA and the appearance in the cytoplasm of mRNA, there appears to be a selection step that removes many sequences, assuming a precursor-product relationship between hnRNA and mRNA (Giorno & Sauerbier, 1976; Bastos & Aviv, 1977; Egyházi, 1978). Differences in hnRNA complexity could, to some extent, imply differences in the mean length of primary transcripts, which is

consistent with the sparse data on hnRNA size differences among tissues. Although overlap in expressed sequences exists (Galau et al., 1977), different tissues also express non-overlapping subsets of their DNA sequences. Unfortunately it is not known exactly which genes are included in these two subsets, and it is clear that much more work must be done before the true significance of these results emerges.

Neither in the hnRNA nor in the primary transcript distribution of any cell type is it known which molecules represent which gene, whether "housekeeping" or coding for some differentiated product. Although it is generally assumed that hnRNA and the primary transcript are homologous, in defence of which assumption is cited the parallel between the molecular weight distributions of the two classes of RNA (Scheer et al., 1979), there is evidence, morphological and biochemical, for the processing of primary transcripts (Derman et al., 1976; Giorno & Sauerbier, 1976; Old et al., 1977). Thus the primary transcript populations of diverse tissues might conceivably have even more sequences in common than does the hnRNA. If so, it is hard to understand the significance of the observed marked differences in primary transcript length between different cell types.

Recently, various models have been proposed to explain the function of hnRNA and the regulation of gene expression in eukaryotes. As outlined in the Discussion to Chapter I of this study, the two main models are those of Britten and Davidson (Davidson et al., 1977) and Cavalier-Smith (Cavalier-Smith, 1978). The former model exploits what is already known about DNA sequence organization in eukaryotes, and defines hnRNA as a coordinate regulatory transcript. The structural genes are postulated to belong to gene batteries, defined as those groups of structural genes which share a given receptor sequence, to which an activator RNA or protein can bind. Binding results in transcription of the contiguous structural gene. The

activator RNA or protein molecules are encoded in the so-called integrator genes. Patterns of activation of sets of batteries are determined by the coordinate transcription of sets of integrator genes, leading to the production of sets of regulatory molecules governing functionally related gene batteries. Finally, to ensure the coordinate activation of each set of structural gene bztteries, it is suggested that the integrator genes of each integrator gene set are transcribed as a single hnRNA molecule. Thus the great bulk of the hnRNA is visualised as being regulatory in function.

This model predicts that in various states of differentiation overlapping but partially distinct regions of the genome would be transcribed, which appears to be the case. To relate this to observations on primary transcript length, it is vital to determine which genes are being transcribed in any two differentiated states whose degree of RNA sequence overlap is known, as well as the relationship this bears to the distribution of primary transcript lengths. Only in this way can the observed differences in primary transcript length be rationalised. Tissue-specific differences in primary transcript length might be explained on this theory by postulating that larger gene batteries are being used by those cells making longer primary transcripts (regulatory hnRNA), and this is worth investigating.

The activator RNA branch of the model (see Chapter I, this study) predicts that repetitive sequence families should not show a great deal of overlap between hnRNA populations, which seems to have been experimentally substantiated for the sea-urchin (Scheller, et al., 1978). If repetitive sequences are distributed at random with respect to the location of particular transcription units, this result would be impossible, because of the large fraction of the genome represented in the hnRNA of each cell.

The other main prediction arising from this branch of the model is that large nuclei, such as early embryo or oocyte nuclei, require a larger number of hnRNA molecules than small nuclei in order to maintain activator RNA sequence concentration. Thus one would expect to find correlations between nuclear size and hnRNA transcription rate, turnover rate or both.

A higher percentage of multifibril arrays in any random sample of transcription units (assuming one transcription unit is equivalent to one functional unit, and that the number of transcription units per cell is known) implies that a larger number of primary transcripts are being made. Both embryonic and liver cells are large (Szarski, 1976) and amphibian livers do not contain many polyploid cells. The percentage of multifibril arrays in different tissues of T.c. carnifex is 17.3% in embryos, and 34% in liver. However, as I was not able to determine the number of transcription units per cell in either of these tissues, my results are not sufficient to prove or disprove this aspect of the Britten and Davidson model. It might be possible to estimate transcription unit number per cell by autoradiographic methods and it is definitely worth attempting to do this.

The model of Cavalier-Smith (Cavalier-Smith, 1978) postulates that most of the DNA content of a nucleus plays a major role in nuclear and hence cell volume determination. It is suggested that the major function of hnRNA sequences is to change nuclear volume independently of changes in DNA content, and that only a small fraction functions as message precursor.

As with Britten and Davidson's model, this theory makes a variety of testable predictions. Its main weaknesses are that in postulating increase in the amount of nucleoskeletal DNA to be the main means of increasing nuclear and cell size it fails to account for the fact that many nuclei attain a large size and increase their surface-area to volume ratio by

invagination. Similarly, many anurans of low C-value have oocytes of a size comparable to those of species with a high C-value. A further problem is that all anuran oocytes have sacculated nuclear membranes, whereas those or urodeles are essentially smooth. Finally, hnRNA generally has a very short half life, although this is not true of the hnRNA of Drosophila hydei spermatocytes (Söderstrom and Parvinen, 1976; Geremia et al., 1977).

If hnRNA functions as means of modulating nuclear size, then its amount or length (see Chapter I of this study) should correlate positively with this parameter. Unfortunately, due to technical difficulties I was unable directly to determine nuclear and cell size for any of the tissues discussed in this chapter. The best way of determining nuclear and cell size in liver might be to set up primary cultures (Puvion et al., 1974) of liver. Such cultures still retain enough differentiated characteristics to be comparable to liver cells in situ, but allow nuclear and cell size to be measured more easily.

I found that liver cells synthesised longer primary transcripts than culture cells, in both T.c. carnifex and X. laevis. It has been shown (Szarski, 1976) that in mammals liver cells are among the largest of all somatic cells. The great size of liver cells, however, is partly due in the above cases, to polyploidy, and it is necessary to measure size differences between diploid liver cells of different species. This is also the case among birds, although the tendency is less marked than in mammals.

The data of Szarski & Czopek (1965) demonstrate that the liver cells of the Urodela are in general larger than those of anurans, which reflects the size differences that I found among liver cell primary transcripts of these two groups. The proportion of polyploid cells in amphibian livers is probably very small, and ploidy classes were not apparent in the

histograms of Szarski & Czopek (1965). If, therefore, in amphibians as well as in mammals, liver cells are among the largest of all somatic cells, it would be reasonable to state that increase in cell and nuclear size is accompanied by an increase in primary transcript length and amount (transcriptional activity). If this is true, it would imply that Triturus neurula cells must be larger than liver cells, as primary transcript length is greater in this cell type. The degree of transcriptional activity could not be determined.

The problem of nuclear size and hnRNA synthesis could be investigated in other systems, for instance Vicia faba meristematic cells, where there is a positive linear correlation between nuclear RNA content and chromosome volume. This confirms previous work showing that a large natural variation in chromosome size exists between cells in plant meristems of different ages (Bennett, 1970; Bennett et al., 1972). It has been suggested (Bennett, 1970) that this increase in chromosome volume is due to a phenomenon similar to the puffing of polytene chromosomes, so that in the light of my results, it would be illuminating to examine nuclear and cell size, the amount and length of hnRNA or primary transcript etc. This would extend the scope of the Miller technique, which among plant cells has been applied to few species (Berger & Schweiger, 1975a, b, c; Woodcock et al., 1975; Franke et al., 1976a).

As yet, insufficient data exist to allow either regulatory model to be confidently eliminated. My data on transcript length and amount in different cells could support either of the models outlined above. It is not yet possible to distinguish between the possibilities that first, cells using large batteries of genes and hence making long hnRNA, synthesise large

quantities of primary transcripts in order to maintain activator concentration in a large cell, or secondly, that requirement for a large cell size results in the production of large amounts of long primary transcripts.

One prediction unique to Cavalier-Smith's theory is that depending on the state of differentiation in which it is expressed, a gene will code for transcripts of different lengths. The length variation will depend on the distance between different genes. The pattern of this variation might depend on the sequence interspersion pattern of the genome. To eliminate this possibility it is necessary not only to know the difference in length of the primary transcripts of different cell types, but also which subsets of genes are expressed separately or shared. Finally the relationship between primary transcript length and gene expressed must be defined.

Differentiation will only be understood when the questions and problems mentioned above are clarified. To date, little is known with certainty about the mechanisms of gene regulation. I think it would be interesting to extract and size the hnRNAs (biochemically defined) of the various tissues that I studied, to see if their size distribution reflects that of the primary transcripts. To date, I know of no study of this type. Sequence distributions in the hnRNAs of these tissues, determinations of hnRNA and mRNA complexity would all yield information that might help towards a resolution of this problem.

C H A P T E R IV.

THE EFFECT OF CORTISOL ADMINISTRATION ON TRANSCRIPTION PATTERNS IN CULTURED CELLS OF *Xenopus laevis* AND *Triturus cristatus carnifex*

INTRODUCTION

As my attempts to characterize C-value-dependent transcription patterns progressed, it became increasingly clear that the transcriptional activity of cultured cells was low, and that few transcription complexes would be available for analysis. It was primarily for this reason that I decided to attempt hormone treatment of cultured cells, i.e. in order to obtain more transcriptional figures. This step was suggested by the work of Puvion-Dutilleul and her colleagues (Puvion-Duttilleul *et al.*, 1978) who demonstrated that rat hepatocyte primary cultures respond to cortisol treatment with a spectacular increase in the amount of transcription observable in the electron microscope.

The main difference between this work and mine was that in the former, primary cultures derived from a mammalian tissue known to be responsive to cortisol were used, whereas in mine, established cell lines of amphibian origin constituted the experimental system. Primary cultures retain many characteristics of the differentiated state from which they are derived, and liver cells are known to respond to cortisol (Puvion-Dutilleul *et al.*, 1978). Thus it was to be expected that a marked response to hormone treatment would occur. On the other hand, the *X. laevis* line that I used had been in continuous culture for at least eleven years and is derived from kidney rather than liver. The newt line was derived from abdominal skin cells and had been in culture for three years. Both cell lines are likely to have lost all traces of any differentiated characteristics that they might have possessed. Nonetheless, steroid hormones are present in amphibians

Barrington & Jørgensen, 1968), have a substantial effect on all aspects of metabolism, and are known to affect gene expression at the transcriptional level (lewin, 1974). Thus I decided to investigate any possible effects of cortisol on my cultured cells. In the eventuality that cortisol might have no effect, I decided also to study the effects of thyroxine treatment. Thyroxine was chosen because it is chemically unrelated to the steroid hormones. Thus if the cells failed to respond to cortisol, they might respond to thyroxine.

Apart from the work of Puvion-Dutilleul et al., (1978) no studies upon the response of transcriptional processes to hormones, and using the Miller technique, have yet been done. They would, if performed, supplement the biochemical evidence, which concentrates on aspects such as RNA and DNA sequence organization (Mascheck et al., 1977; Parker & Mainwaring, 1977; Konstantinove et al., 1978; Schäfer & Neumann, 1978), conformational changes in receptor molecules (Senior & Frankel, 1978) and the general induction of RNA synthesis (Gvozdev et al., 1975; De Groot et al., 1977; Jain, 1977; Burns et al., 1978; Thomas et al., 1978). For this reason I felt that my study would be a valuable addition to what is currently known about the control of gene expression (Lewin, 1974).

The second main reason for this study was that I wished to test directly one of the main predictions of Cavalier-Smith's (1978) model for the function of DNA sequences in the eukaryote genome. Briefly, this model postulates that the primary function of the bulk of hnRNA is as a mechanism for controlling nuclear and hence cellular dimensions. An increase in the amount of hnRNA synthesised is conceived of as resulting in an increase in the size of the nucleus. Cultured cells, if responsive to a stimulus such as hormone adminstration, form an ideal system in which to test this hypothesis.

Monolayer cultures such as the cells that I used, can be exposed to hormones much more conveniently and reproducibly than tissue cells. Both biochemical and morphological studies can be performed. Finally, the measurement of nuclear and cellular dimensions is easier than with somatic tissues, where the cells are generally growing in a solid mass rather than in a monolayer or in suspension.

Increase in the amount of RNA synthesised in response to hormone treatment, if such occurs, can be assessed by determining the percentage of total transcription made up of multifibril transcription complexes. Direct measurement of RNA fibril or transcription complex length will reveal any changes that might occur in this parameter. Finally, Miller spreading of hormone-treated cells is the most direct way of demonstrating the transcriptional control postulated to be the result of hormone action (Lewin, 1974; Puvion-Dutilleul et al., 1978). None of the studies done to date say anything about the effects of hormone treatment on the primary transcript (as opposed to hnRNA) or its length, a gap which the Miller technique is admirably suited to filling.

MATERIALS AND METHODS

(i) Culturing of cells

(a) X. laevis

For hormone treatment, cells of X. laevis were grown at a concentration known to allow a high rate of RNA synthesis, as measured by the incroporation of tritiated uridine (see Chapter I. this study). This was lower than the inoculation density usually employed for routine subcultures. Details of all processes are as described in the Materials and Methods section of Chapter I.

A. Cells were inoculated routinely into sterile plastic culture vessels (75 cm^2 growth area) at the same cell density as the uridine incorporation experiment (1.95 x 10^6 cells per flask). The cultures, two per experiment, test and control, were grown at 25°C for 2 days to ensure the attainment of log phase. A few cultures were set up at normal subculture density but the results from these were not included in my analysis.

B. This second method of setting up cultures for hormone treatment was the one that I found gave the most successful spread preparations, and the one that I ahered to. I employed a modification of the method used by Puvion-Dutilleul et al., (1978). A confluent culture was trypsinized routinely and cells inoculated into small (60 x 15 mm) sterile plastic Petri dishes (Falcon). The growth area of these Petri dishes was 22 cm^2 ao that 5.7 x 10^5 cells were inoculated per dish, to give the same final concentration as (A). The cells were grown, without gassing, for 2 days at 25°C, until they had reached log phase. Two dishes, test and control, were set up for each experimental run.

(b) T.c. carnifex

Both of the culture methods used for X. laevis cells were employed for T.c. carnifex. Cultures were inoculated at the same density as the incorporation experiment (Chapter I, this study) to ensure a high level of transcription, or at the same density as X. laevis. In one case, cultures were set up at 3×10^5 cells per Petri dish. Two cultures, test and control, were set up for each experimental run.

(ii) Cortisol treatment. X. laevis and T.c. carnifex

Following the suggestion of Puvion-Dutilleul et al. (1978) I treated cells with 20 µg/ml of cortisol for 5 hr, at 25°C. 5 hr was chosen as a treatment time because it allowed treatment and spreading to be carried out on one day. I thought that shorter treatment times, although adequate for hepatocyte primary cultures (Puvion-Dutilleul et al., 1978) might not elicit a response in X. laevis or T.c. carnifex cultured cells.

Cortisol (hydrocortisone-21-phosphate, Sigma) was dissolved in culture medium to a final concentration of 20 µg/ml. Carrier was not required as the hormone is soluble in aqueous media. To sterilize, medium containing hormone was Millipore-filtered into sterile bottles. All media were brought to 25°C before use. Old medium was decanted or pipetted from cultures in log growth, and 15 ml of the hormone-containing medium (for 75 cm^2 flasks) or 5 ml (for Petri dishes) added in its place. Control cells were given a similar medium-change, but with medium lacking cortisol. In the case of culture vessels, all media were added to the side of the flask opposite the monolayer. The flask was then securely capped, making sure that no medium had touched the cells, and the cultures turned over at zero hours. Thus the time of exposure to cortisol could be controlled precisely.

The cultures were placed in the incubator, and allowed to grow undisturbed for 5 hr, at 25°C. In a few cases, T.c. carnifex cells were treated for 5 hr with 40 µg/ml cortisol.

(iii) Thyroxine treatment (T.c. carnifex cells only)

Thyroxine(L-form, sodium salt, Sigma) was suspended, at a final concentration of 20 µg/ml of medium, in 1 ml distilled water. 1 drop of 0.1 N NaOH was added to dissolve the hormone, and the solution made up to the required volume with prewarmed culture medium. 1 drop of 0.1 N HCl was added to restore pH to its original value and the solution filter-sterized as above.

Cells were treated with thyroxine for 5 hr at 25°C, as above.

(iv) Solutions

As with preparations of other cell types, different modifications of the solutions required were used. They were made up as described in Chapter I.

(a) Sucrose-formalin fixative

0.6846 g RNAse-free sucrose (Serva), 10 ml 20% paraformaldehyde, 10 ml distilled water, adjusted to pH 8.5 with 20 µl of 0.1 N NaOH. Final solution 0.1 M sucrose; 10% formaldehyde.

(b) Sucrose-Joy (Spreading solution 1)

0.3423 g RNAse-free sucrose in 20 ml 0.1 - 0.2% Joy, adjusted to pH 8.7 with stock borate buffer.

(c) Sucrose-Joy + yeast tRNA (spreading solution 2)

0.3423 g RNAse-free sucrose in 20 ml 0.1% Joy containing 100 µg/ml yeast tRNA (Sigma), and adjusted to pH 8.7 with stock borate buffer. This modification gave the best results. About half the preparations were made with Joy containing RNA.

(v) Miller-spreads of hormone-treated cells

Cells grown in culture vessels were trypsinized routinely and the chromatin spread as described in detail in Chapter I. Whole cells were lysed and dispersed in 0.1% ~ 0.2% Joy in a clean Eppendorf tube. Dispersal was for 60 - 75 min.

The cells grown in Petri dishes were spread by a modification of the method of Puvion-Dutilleul et al. (1978) described in Chapter I. Cells in Petri dishes were washed with pH 9.0 water, which was decanted and replaced by 0.1% Joy. The cells were then scraped off with a rubber policeman, and 5 µl of this suspension added to 95 µl droplets of Joy (0.1%) on clean Parafilm. The cells were allowed to spread for 15 min - 1 hour. Test and control preparations were made at the same time and 4 grids of each were prepared per experiment.

Centrifugation and staining of the chromatin were as described in Chapter I of this study. As with all the preparations that I made, dispersed chromatin was layered on to sucrose-formalin fixative in the centrifugation champber, using a pulled Pasteur pipette. This procedure did not affect the morphology of oocyte ribosomal genes, so I assumed that the RNP of other chromatin would also be unaffected.

(vi) Determination of cell and nuclear dimensions

For X. laevis and T.c. carnifex measurements were made on cells grown at the cell densities described in Chapter I. All other details were as described in Chapter I. For each experiment four small vessels were set up, two for nuclear cross-sectional area (test and control) and two for cell size determinations (test and control).

Hormone treatment. X. laevis and T.c. carnifex

The effect of cortisol on nuclear and cell size was investigated for both cell lines, but that of thyroxine was not studied (see Results, this chapter). Cells in log phase were treated with cortisol as described above.

The criteria for selection of cells or nuclei, and their measurement, were as described in Chapter I (this study).

RESULTS

(i) The effect of cortisol treatment on transcription patterns in X. laevis cultured cells.

Preparations of chromatin from cortisol-treated cells were made from cells grown at two different cell densities. These were the normal subculture density (5 x 10^6 cells per large vessel) and a lower inoculation density of about 1.95 x 10^6 cells per large vessel. The latter cell concentration was that used for the determination of ^{3}H-uridine incorporation rate, and resulted in a higher rate of transcription than the former concentration, as measured by this criterion. For quantitative analysis, preparations made exclusively from cells grown at this lower density were used, although the results of hormone treatment were similar for both types of preparation.

The appearance of the chromatin varied between preparations, depending on the spreading time (15 min - 1 hr), but it always had a beaded structure (Figs IV-1 and IV-2 b-c). These beads, by their size (14 nm $\pm$ 5 nm in a rotary shadowed preparation) and ubiquitous distribution on the chromatin, are presumed to be nucleosomes, and considered as such in my analysis. In preparations made under optimal conditions (15 min spreading in 0.1% Joy) they appeared to be more closely spaced than in other preparations (Fig. IV-1).

The use of cultured cells for making spread preparations allows one to control precisely the amount of material on the grid. This was not so easy for somatic tissues, where large clumps or even superimposed layers of chromatin were noted. In preparations of culture cell chromatin, the nuclear material appeared either as small clumps, usually less than the size of a nucleus, with long fibres radiating from them in all directions

or as large masses of parallel strands (Fig. IV-1). The contrast and degree of spreading of these preparations was good.

All the chromatin appeared as beaded fibres, although bead density varied. Fibres similar to the thin, low contrast, smooth DNP observed in plethodontid spermatocyte chromatin (Morgan, 1978) and in my own preparations of amphibian somatic cell chromatin (Chapters I, II, and III, this study) were rare. This may be due to the more favourable preparative conditions used for the spreads discussed here. Supernucleosomes appeared to be absent, as did structures resembling the cables of X. laevis culture cells grown in the absence of hormone.

Cortisol treatment had a marked effect on nonribosomal transcription in these cells. On scanning a grid, it was seen that transcription figures were more frequent than in untreated cells. Of 10 preparations, 8 yielded analysable data, a frequency greater than for unstimulated cells (see Chapter I, this study).

The criteria that I used for the selection and measurement of RNP molecules or transcription complexes are those that are outlined in Chapter I. As with the other tissues that I studied, transcription primarily took the form of isolated RNP fibrils. These formed a skewed distribution around a median of 0.74 μm, and with a range of 7.5 μm (Figs IV-2a-c and IV-3a) (Table I(ρ54)). The distribution was smooth and size classes were not apparent. A smaller sample (Table I(ρ54)) was available of the terminal RNP fibrils of arrays of two or more fibrils (Figs IV-3b anc IV-4 a-b). These formed a similar distribution around a median of 0.65 μm, and had a size range of 7.1 μm.

Multifibril arrays were rare even in the chromatin of stimulated cells, although more common than in the control preparations. Six clear arrays were observed (Figs IV-5 a-b) and of these, the point of initiation of transcription could be determined for three. Values for total transcription unit length of 2.77 μm, 3.45 μm and 13.8 μm were obtained, which would suggest an increase in transcription unit length after hormone treatment. However, in the case of the 13.8 μm array (Fig. IV-6) the complex consisted of only two RNP fibrils separated by a length of chromatin greater than 1 μm, and thus was considered as two separate transcription events. The attached RNP fibrils were 3.2 μm and 4.2 μm in length. If this putative long array is omitted, the array lengths obtained do not differ greatly from those for unstimulated cells (3.5 μm and 2.8 μm. See Chapter I, this study). However, I was only able to analyse a few arrays, and more must be done before any firm conclusions can be drawn. Dur to the low number of arrays, I was unable to determine the RNA polymerase density per μm of DNP (Puvion-Dutilleul et al., 1978). Nonribosomal arrays did not fall into classes (McKnight & Miller, 1976).

In hormone treated X. laevis culture cell chromatin isolated RNP fibrils comprised 71.8% of the total transcription, whereas groups of two fibrils made up 18.3%, and arrays of three or more fibrils 9.8% (Table II, p56). The total percentage of multifibril arrays of all types was therefore 28.1%.

In nearly all the preparations that I made, the RNP showed higher contrast than the DNP, and had the diffusely beaded structure noted before. It was linear in configuration, about 170 nm wide, and showed little secondary structure (Fig. IV-6), unlike spermatocyte RNA (Glätzer, 1975; Amabis & Nair, 1976, Morgan, 1978) or lampbrush chromosome RNA (Scheer et al., 1976; Hill in press 1979). In a few cases the contrast of the RNP was similar to that of the DNP. This RNP (Fig. IV-2b) had a very

extended structure, with beads similar to nucleosomes. It is possible that such "transcripts" are broken replication forks, which latter, unbroken,were frequent in these preparations. However, if this were so, it is hard to see why they should all be broken at one end i.e.

or why tandem arrays of forks (Fig. I-4) never showed breaks. Further, pseudomultifibril arrays derived from forks broken in other ways should perhaps have been commoner i.e.

In one preparation, putative "bushy" RNP was observed. This RNP was of high contrast, associated with DNP, and showed extensive secondary structure (Fig. IV-7a). In many cases, lengths of DNP bearing such "RNP", in excess of tens of microns in length occurred. Only in a few cases was the "RNP"sufficiently extended to allow its identification as such. These arrays never showed a clear fibril length gradient and the density

of fibrils was low. If they are in fact transcription complexes their configuration, which was reminiscent of lampbrush chromosome transcription units, argues a more extensive activation of transcription by cortisol than had hitherto been seen. Large detached clumps of "RNP" also occurred. In a second preparation tandemly arranged (Fig. IV-7b-c) long, "bushy" RNP fibrils were seen, having less secondary structure than the fibrils discussed above. They resembled spermatocyte transcripts in appearance (Glätzer 1975; Amabis & Nair, 1976; Morgan, 1978).

Although transcriptional activity was more evident in these preparations than in others that I made, it was still not so frequent as to allow a determination of the percentage of the chromatin that was transcriptionally active.

In cells grown at a low inoculation density, ribosomal genes were identified by their tandem repetition, and where evident, RNP fibril gradients, as well as by their length (matrix units 2.7 μm, spacer units 2.0 μm (Table X, Fig. IV, 8a-b), which is somewhat greater than the values determined by Scheer for X. laevis oocyte ribosomal genes (2.22 μm and 1.24 μm respectively, Scheer et al., 1977). In most cases, however, few or no lateral fibrils were present, perhaps due to endogenous nuclease activity, and arrays of RNA polymerase molecules alone identified the transcriptionally active regions of ribosomal repeats. Matrix length was measured from the first to the last polymerase. The polymerases were identified as such by their high contrast and large size, which was consistently greater than that of nucleosomes (Fig. IV-8a-b) at 17.4 nm x 23.7 ± 3 nm (rotary shadowed preparation). Ribosomal genes were the only transcription complexes where clear polymerases were evident.

Table X (p 55) shows that ribosomal matrix units are more similar to each other in length than spacer units. The difference between my values and those of Scheer et al. (1977) is probably due to the small number of units available to me for analysis, their poor preservation, and possibly some degree of stretching of the DNP axis.

In one case (Fig. IV-9a) a short length (0.4 μm) of polymerases was seen. This may represent a sparsely transcribing gene, or transcription within a spacer unit. Transcription of spacers was not otherwise observed.

In a few cases it was possible to look at the nucleosome distribution of transcribing ribosomal genes. Nucleosomes were identifiable in a metal shadowed preparation by their size, 15.4 nm ± 5 nm and by their contrast, which was consistently less than that of RNA polymerase molecules. Nucleosomes were visible in spacer regions (Fig. IV-8b) but absent, or nearly so, in matrix units, even when polymerase density was low.

Ribosomal genes were also seen in preparations of cells grown at high density before hormone treatment. As before, they occurred in blocks of tandem repeats. Their preservation was better than those found in later preparations, and Laird Analysis could be done, giving a mean matrix unit length of 1.45 μm (N = 5. see Table XI (p 55) and Fig. IV-9b). Spacer regions were not clearly spread. The RNP was short, up to 0.23 μm in length, and suggesting a high degree of foreshortening. The mean matrix unit length is less than for cells grown at lower densities, perhaps because the arrays in the high density cell preparations often showed an irregular (Fig. IV-9b) distribution of fibrils, suggestive of low transcriptional activity (Scheer, 1978). This made accurate determination of initiation point difficult.

(a) Packing ratios

(1) DNA packing ratio

The DNA packing ratio (DNA, P.R) of transcriptionally inactive regions of chromatin was determined for two preparations, and found to be 1.98 and 1.77 (mean 1.87) (Table III p57).

In both preparations of hormone treated cells for which I was able to calculate the DNA packing ratios of transcriptionally quiescent regions, I was also able to determine the DNA packing ratio of transcription complexes. In all these preparations, arrays had a beaded structure (Fig. IV-5 a-b), and I assumed that beads lacking attached RNP were nucleosomes. Where P.R. (inactive) was 1.98, the DNA P.R. (transcription-complexes) was 1.74 (Table IV p105). Where DNA P.R. (inactive) was 1.77, the DNA P.R. (transcription-complexes) was 1.63.

However, in any array, an RNP molecule could conceal a nucleosome. If the number of transcripts per complex is added on to the total bead number for each complex, and the final figure used to calculate DNA packing ratio, the DNA P.R. (± complexes) rises to 2.23 from 1.74 - 1.92, and to 2.5 from 1.63 (Table IV p105). These values are higher than the packing ratio for transcriptionally inactive regions of the same preparation. If, however, nucleosomes are transient structures, as suggested by Scheer (1978) and Hill (1979 in press) this calculation is meaningless, as the nucleosomes are postulated to dissociate prior to the passage of an RNA polymerase molecule, and reassociate in its wake. Experiments involving Sarkosyl would be useful in distinguishing between nucleosomes and polymerases in transcription complexes.

I was able to measure the number of nucleosomes at intervals increasingly distant from an isolated RNP fibril, for one preparation (Table V, p 106). The values show a slight increase for intervals up to 2.0 μm on either side of the lateral fibril, but the data as a whole are inconclusive. The method of analysis I used is subject to various limitations (for discussion see Chapters II and III, this study) which reduce the significance of these data.

(2) DNA/RNA packing ratios

The DNA/RNA packing ratio of the terminal RNP fibril for two arrays for which the DNA packing ratio of transcriptionally active regions was known, and of which the position of the initiation point of transcription could be determined, was calculated (Table VI, p 54). I obtained values of 3.9 and 20.5. For the long array of 13.8 μm (considered in the analysis as two isolated single fibrils) a value of 5.7 was obtained.

(b) Coincident transcription and replication

In two cases (Figs IV-4a and IV-10a) transcription figures were seen of such a conformation as to suggest coincident DNA and RNA synthesis. In both cases, a lateral fibril occurred near a putative replication fork. The lateral fibrils were identified as RNP by their structure and contrast. The DNP was thinner and of lower contrast than the RNP which in one instance (Fig. IV-10a) was far enough away (0.9 μm) from the fork to eliminate the possibility of it being a piece of DNP that was running parallel to another strand of DNP for some of its length. In the second case (Fig. IV-4a) the transcript was nearer the fork, but the presence of a second transcript, of the same morphology as the first and on a nonreplicating region of the DNP, supported the interpretation of the transcription figure as constituting

simultaneous RNA and DNA synthesis. Figs IV-10b-c show structures that may be putative transcription complexes in the region of replication forks. Figures IV-10a and IV-4a are less equivocal than ones previously seen and support observations (McKnight & Miller, 1977; McKnight et al., 1977) made on Drosophila melanogaster.

Unlike the case of D. melanogaster embryos I never observed long transcriptional complexes associated with replication forks. This may be due to the relatively low transcriptional activity of my material.

(c) Putative mitochondrial DNA circles

(1) Replicative forms

As in the case of X. laevis cultured cells untreated with cortisol, in preparations from treated cells I observed circular molecules which I interpreted as mitochondrial genomes. Preparations were made from whole cells, and consequently mitochondria would be lysed together with nuclei.

About half the structures I found had what appeared to be a replication fork (Fig. IV-11a). Table VII(p61) shows that the rings themselves measured about 4.64 μm (N = 2) in contour length, which is of the same order as mitochondrial genomes from other systems (Dawid & Wolstenholme, 1968a; Wolstenholme & Dawid, 1968; Polan et al., 1973; Borst & Grivell, 1978; Pinon et al., 1978) including X. laevis cultured cells untreated with cortisol. The "fork" regions were of higher contrast than the rest of the circle, the latter being similar in contrast to the nuclear chromatin and therefore not likely to be single stranded. It is by no means clear why the fork regions should be of higher contrast. Table VII(p61) also shows that fork length, and thus the percentage of the total contour length made up by the fork, were similar for all the circles (5.69% ± 0.99% N = 4).

Ths is an unexpected result, for the lengths of a random sample of replication forks should form a normal distribution. The observations presented in Chapter I suggest that the replication of mitochondrial DNA in Novikoff rat ascites hepatoma cells (Wolstenholme et al., 1973a; Koike & Wolstenholme, 1974) may be discontinuous, and this might account for my results. However, there is no direct evidence for discontinuity of replication of the mitochondrial DNA of D. melanogaster (Goddard & Wolstenholme, 1978), Tetrahymena pyriformis (Goldbach et al., 1979), Crithidia acanthocephali (Manning & Wolstenholme, 1978) or mouse L-cells (Berk & Clayton, 1976).

Table VII (p 61) shows the pooled results from both stimulated and unstimulated cells. The results were essentially similar for both types of preparation (see Chapter I for further discussion).

(2) Transcribing circles

The majority of the circles that I found appeared to have attached RNP transcripts (Fig. IV-11b). These lateral fibrils were identified as such by their structure and contrast, which resembled the transcripts of nuclear chromatin. The measurable rings (Table VIII (p 61) N = 2) were about 4.4 μm in contour length. Thus both replicative forms and transcribing circles are likely to be of mitochondrial origin. The contrast of the RNP resembled that of the fork regions of the replicative forms whereas the rest of the DNP circle of both types of ring was of similar contrast i.e.

In one case (Fig. IV-11b) three putative transcripts were found attached to one circle. I was unable to determine their spacing, however, and so could not make a Laird Analysis, unlike the case of one circle from a control preparation (Fig. I-12a-c). This latter demonstrated that a transcription unit comprising up to 73% of the total mitochondrial circle might exist.

RNP of a length greater than 1.01 μm was seen attached to circles from stimulated cells. This is less than the maximum length of such fibrils in control preparations. Thus cortisol cannot be proven to stimulate transcription in these circles. However, more circles should be analysed before firm conclusions can be drawn. I was able only to analyse 11 circles *in toto* (Tables VII & VIII(61)).

Interlocked oligomers of mitochondrial circles and circles of a size order suggesting concatenation were not observed. Such structures have been observed among both mouse and rat mitochondrial circles, in human granulocytic leukaemic leukocytes, hamster kidney cells and L-cells (Wolstenholme *et al.*, 1973b; Berk & Clayton, 1976). However, my data are not sufficient to exclude the possitility of their occurrence.

(d) Free circles

In one preparation of chromatin from hormone treated cells, free rings were observed (Fig. IV-12a-c). These were unlike the putative mitochondrial circles described above, and also apparently absent from control preparations, although this is hard to prove on morphological evidence alone. Most of the circles had an irregularly beaded morphology, the beads being somewhat larger than supernucleosomes (24 nm ± 4 nm diameter). Mitochondrial circles from the same preparation were unbeaded.

Fig. IV-13 shows the length distribution of a random sample of these circles. The median value was 1.26 μm, and the range at least 14.8 μm. There is a suggestion of size classes at 0.5 μm and 1.6 μm, but the distribution is otherwise approximately normal. The smallest circle observed was less than 0.2 μm in contour length, and the longest over 14.0 μm. The distribution is unlike that of the putative mitochondrial circles.

It is not clear why these circles are of an angular rather than curved shape. It may be because certain points became attached to the carbon film first, with the rest of the circle sliding over the support film when the grid was dipped in photoflo after centrifugation.

The circles were only found in one preparation, made from whole cells, so that their source could be either nuclear or cytoplasmic. I never observed any in preparations from unstimulated cells, so they may result from cortisol treatment. However, morphological investigations alone are insufficient to rule out their occurrence, albeit at a very low level, in unstimulated cells. All the rings were transcriptionally inactive and none had replication forks.

The nature of these circles remains obscure. A possibility exists that they are amplified ribosomal DNA circles, but this is not borne out by observations. In Dytiscus oocytes (Scheer & Zentgraf, 1978) transcriptionally inactive ribosomal circles, containing varying numbers of rDNA repeats occur, and they have a superbeaded structure. The smallest of these is about 0.7 μm long, containing an rDNA monomer of 8.6 μm extended DNA length. The smallest circle that I found was less than 0.2 μm in length. Thus, although size classes may exist among these circles, it is unlikely that they are amplified ribosomal genes. In the preparations where the rings were found ribosomal RNA synthesis did occur, but such transcription

units were apparently on linear DNA. Thus it is unlikely that the rings consist of ribosomal DNA. It is conceivable that if the cells were exposed to cortisol for more than 5 hours, RNA synthesis might occur on the rings, but this possibility was not checked.

The difference in morphology and size distribution between these circles and those identified as mitochondrial genomes makes it unlikely that the circles are mitochondrial in origin. The lack of clear subclasses excludes the possibility that they are mitochondrial concatemers. Absence of clear size classes may also be attributable to the differences in bead size and degree of stretching that I observed between different circles. The real size of the circles will therefore be greater than the values given here, but still different from those for either ribosomal DNA circles or mitochondrial genomes.

Similar rings have been observed in eggs and in cultured cells of Drosophila melanogaster (Stanfield & Helinski, 1976). In cultured cells the circles range in contour length from 0.09 to 7.3 μm, with a mean of 1.1 μm, and the means and length distributions differ for each source. Both logarithmic and stationary phase cells contained approximately 3 - 40 circular molecules per cell, and exposure to various drugs was seen to cause changes in the number of small circles per cell. Such rings occur in Xenopus, trypanosomes, yeast, and other systems, including a variety of tissue-culture lines (Stanfield & Helinski, 1976). In the majority of these cases the rings are heterogeneous in size, with buoyant densities either like that of the nuclear and/or mitochondrial DNA, or unlike any other DNA component in the cell. Furthermore, the intracellular location of these circles varied with the organism and both cytoplasmic and mitochondrial locations have been reported. Pulse-chase experiments indicate that in some cases at least, the circles arise from pre-existing nuclear DNA.

The size distribution I obtained resembles that for the Drosophila rings. In the latter case, biochemically isolated circles were measured, and a beaded structure was not evident. The rings I found may be homologous to the Drosophila rings. Statistical analysis of the rings from Drosophila cultured cells indicates the presence of at least eleven distinct size classes (Stanfield & Helinski, 1976).

A variety of origins have been suggested for these circles, for instance that they arise from recombination events between homologous regions on the chromosomal DNA. Other possibilities are the induction of integrated viral genomes or reassociation of the single-stranded ends of linear DNA fragments. A microorganisomal or plasmid origin cannot be eliminated. To date, there is insufficient evidence positively to rule out any of these alternatives. However, their presence does underline the lability of the eukaryote genome.

(e) Rings attached to the chromatin

Small rings (Fig. IV-14 a-c) were observed to be attached to spread chromatin from stimulated as well as unstimulated cells (for extended discussion see Chapter I, this study). In untreated cells (Fig. I-14a) the circles had a median length of 0.4 μm, and a range of 2.2 μm, whereas in hormone-treated cultures they had (Fig. I-14b) a median length of 0.48 μm and a range of 4.95 μm. This difference may be hormone-dependent.

The size distribution of these small attached circles differed from both that of the putative mitochondrial DNP (Tables VII and VIII (p61)) and the free circles discussed above (Fig. IV-12a-c). Thus it is unlikely that they are precursors of the free circles.

(ii) The effect of cortisol treatment on cellular and nuclear dimensions of X. laevis cultured cells.

(a) Cell volume

In cells grown at high density, cell volume showed a 9% increase after cortisol treatment, from 4492 μm^3 to 4919 μm^3 (Fig. I-36a-b). The variances of these distributions were 8% and 5.3% of the medians respectively. However, in low density cultures, cortisol treatment resulted in a decrease of about 4% in cell volume (Fig. I-36 c-d). Further, the values themselves were greater than in the former cultures, it is not clear why. Figs. I-36 c-d show that the value for the control is 7468 μm^3 whereas that for the hormone treated culture is 7184 μm^3. The variances were 19% and 18% of the median values respectively. As with nuclear area these values are similar for both treated and untreated cultures, and confirm the pattern formed by the median values.

(b) Nuclear cross-sectional area

The effects of cortisol on nuclear optical cross-sectional area were determined for cells grown at two different densities. The higher inoculation density was the one at which cells were grown for Miller spreads. The lower cell density should allow a more accurate determination of nuclear area, as the separation between cells is greater. Consequently, the cells are well stretched, allowing nuclei to be more easily measured (See Chapter I, this study, for full details of measurement procedure). Size distributions were skewed rightwards, showing that a minimum size probably exists for both cells and nuclei. Although the distributions were not normal, variances were determined for each sample, in order to assess the spread of the distributions.

At high density, the median nuclear cross-sectional area of the control cells (Fig. I-38a) was 205 μm^2, whereas the value for the cortisol treated culture was 216 μm^2 (Fig. I-38b) an increase of about 5%. The variances were 6% and 5% of the medians respectively, showing that there was little difference in the spread of the distributions. On the other hand, at low cell density I observed a decrease of 4% in median nuclear cross-sectional area after hormone treatment (Fig. I-38 c-d) i.e. from 299.3 μm^2 to 286.9 μm^2. The variances of this distributions were 4.2% and 5.3% of the median values, respectively, suggesting that cortisol did not affect the spread of values significantly.

The difference in nuclear size after cortisol treatment, if any, is slight. It is not clear why the two sets of values should differ so much, from 220 μm^2 to 290 μm^2, unless the sample size (N = 50) was too small. Cells grown at lower density should, in theory, give the more accurate value. If so, the latter value implies that cortisol treatment may actually result in a reduction of nuclear area.

However, certain qualifications must be borne in mind. First, the nuclei were elliptical in cross-section. Any irregularities in shape may have obscured real differences resuling from hormone administration. Secondly, the low cell density cultures were grown at an inoculation density little higher than the minimum cell density (about 1×10^5 cells per small vessel) required for growth to continue in these cells. Thus, if they were synthesising less RNA than the cultures grown at high density, changes in nuclear area might be less evident.

Finally, small differences in nuclear area resulting from cortisol stimulation of RNA synthesis might not be detectable by the relatively insensitive technique that I used. Xenopus nuclei are small and difficult

to measure with a micrometer scale, and small changes might be missed. These results are inconclusive. Distortion of the cells by the coverslip may be in part responsible for the wide variation in cell volume between the two experiments. The size, after trypsinisation, of cells grown at different densities may vary. The data, although they do not demonstrate a cortisol-induced change in cell size, do not eliminate the possibility. It has to be considered that Cavalier-Smith (1978) suggests that changes in nuclear size effect changes in cell size. Thus the former might be more marked than the latter which is dependent on it, being a stage further removed from the process of transcription, the original modulation mechanism.

(iii) The effect of cortisol administration on transcription patterns in T.c. carnifex cultured cells.

Three preparations (24 grids) were made of T.c. carnifex cells treated for 5 hr with 20 µg/ml of cortisol, at 25°C. None of these showed any stimulation of transcription, which was sparse in control preparations (See Chapter I, this study). I therefore concluded that either T.c. carnifex culture cells were insensitive to the hormone or that stimulation was so slight as to be negligible. The fact that this cell line originated from abdominal skin rather than kidney (Rudak, 1976) may be the reason for its insensitivity to cortisol stimulation. It was for the reason that I decided to examine the effects of thyroxine treatment on these cells. When the cells were treated for 5 hr with 40 µg/ml of cortisol (1 preparation) again no change in the frequency of transcription was observed. It is likely therefore, that the cells are insensitive to this hormone, in contrast to the considerable stimulation that occurs in X. laevis.

(iv) The effect of thyroxine treatment on transcription patterns in T.c. carnifex cultured cells

Two preparations of chromatin from thyroxine treated cultures were made. Neither showed any observable stimulation of transcription. This may be partly due to the poor quality of the preparations at this time and so cannot be taken as conclusively showing that T.c. carnifex cells are insensitive to thyroxine. Unforunately I was not able to make preparations under more favourable conditions.

(v) The effect of cortisol treatment on cellular and nuclear dimensions in T.c. carnifex cultured cells

(a) Cell volume

At high cell density I obtained a median value of 26914 μm^3 for the control, and 22741 μm^3 for the test, a decrease of 15.5% for the cortisol treated culture (Fig. I-27 a-b). The variance of the distribution of cortisol treated cells was less (8% of the median) than for the control (14.3% of the median), which reflects the median values. At lower densities I observed a 10% increase in cell volume after hormone treatment, from 19863 μm^3 to 21978 μm^3 (Fig. I-37 c-d). The variances of these two distributions were similar, at 11.5% and 11.8% of the median values respectively.

(b) Nuclear cross-sectional area

At high cell density median nuclear cross-sectional area (Figs I-39 a-b) is 1385.6 μm^2 in the control, and 1273.8 μm^2 in the treated cells, a decrease of about 8%. Variances were 9% and 4% of the median values respectively, which suggests a slight narrowing of the distribution of treated cells. In the case of cells inoculated at a lower density nuclear area

was about 1060.3 μm^2 for the control and 1243.6 μm^2 for the test, an increase of nearly 15% (Fig. I-39 c-d). The variances were, however, very similar, at 6% and 4.3% respectively, and do not reflect the increase in the median values. These values are likely to be the more accurate because cells were better spread and nuclei easier to measure.

Measurement was facilitiated, at least for nuclear area, in cells grown at low density, and it suggests that there is a substantial increase in nuclear area in T.c. carnifex cultured cells in the presence of cortisol. However if measurements made at high cell density are inaccurate it is hard to understand why cortisol treatment resulted in an apparent reduction in the size of both cells and nuclei. This may be a chance effect. The percentage changes are similar for all cultures so that these results might be due to chance variation. The qualifications mentioned above also apply to the results presented here. At this stage all that can be said is that if cortisol stimulation of transcription results in changes in nuclear and cell size (which cannot be eliminated by my experiments) the changes are too small to be detectable by the techniques I used.

DISCUSSION

The data presented in this chapter demonstrate that exposure of X. laevis tissue culture cells to cortisol results in the transcription of RNA nearly twice as long as in unstimulated cells. The range of transcript lengths increases 3.6 fold between unstimulated and stimulated cells (Table I, p54). Finally, the number of multifibril transcription complexes increases by nearly 23% (Table II, p56) from 5.6% to 28%, suggesting a real augmentation in the amount of transcription as well as in the lengths of the transcripts themselves. However, though high, the level is never as high as in liver cells, where the percentage of multifibril arrays could be as high as 35%.

Hormone treatment resulted in an apparent stimulation of ribosomal RNA synthesis; ribosomal genes were more frequently seen in preparations of cells grown at both high and low cell densities.

In accord with current concepts about nucleosome distribution are my observations on the morphology of X. laevis culture cell ribosomal genes. Nucleosomes were present in spacer regions, but not within the matrix units themselves. Most of the matrix units that I found were sparsely transcribing so that if nucleosomes were present they would have been easily seen. That a few nucleosomes were present in spacer regions conforms with the observation that sub-maximally active ribosomal cistrons have more nucleosomes than maximmaly active ones (Reeves, 1976; Scheer, 1978). I did not find densely fibril-covered ribosomal transcription units like those in oocyte preparations, in which both matrix and spacer regions have a "smooth" non-nucleosomal structure (Franke & Scheer, 1978; Scheer, 1978).

Analysis of nucleosome distribution in transcriptionally inactive regions of chromatin suggests that the DNP of a relatively transcriptionally

active cell type, has a more extended structure than that of a relatively inactive cell type. The DNA P.R. of transcriptionally inactive regions of chromatin from hormone-treated cells was lower than that of similar regions of chromatin from untreated cells, when identical dispersal conditions were employed. This is in accord with the experimental evidence discussed more fully in Chapter II of this investigation. I also found that the chromatin of transcriptionally active regions has a more extended, though not "smooth" structure, than the rest of the DNP. The differences I found are more pronounced than those reported for Strongylocentrotus purpuratus embryo chromatin (Busby & Bakken, 1979).

Table VI (p59) shows the values for DNA/RNA P.R. that I obtained for non-ribosomal transcription complexes from different sources. Although the total sample is small (N = 10), there is an indication that DNA/RNA P.R. falls into classes of 1.2 - 1.4, 3 - 12, and 30 - 25. If real, this result is at variance with the observations of Foe et al. (1976) in Oncopeltus fasciatus, who showed that DNA/RNA P.R. forms a normal distribution.

Cortisol stimulation of X. laevis cells had no detectable effect upon the size of nuclei or cells. The small size of X. laevis culture cell nuclei and cells may have prevented the detection of such an effect by the method of analysis that I used or the increase in the amount of RNA synthesis may have been insufficient to cause detectable changes in nuclear or cell size. This was also the case for T.c. carnifex, which appeared to be insensitive to both cortisol and thyroxine. Thus I was unable to confirm Cavalier-Smith's suggestion that increase in the amount of transcription results in an increase in cell size.

Puvion-Dutilleul and her colleagues (1978) did not report the occurrence of rings of DNA, which I observed in cortisol-treated cultures of X. laevis. My results do not prove that unstimulated cells lack these rings but rather that hormone stimulation results in an increase in their number, as is

known to be the case with drugs such as cycloheximide and puromycin (Stanfield & Helinski, 1976).

The differences in the transcription patterns that I observed are greater than those noted by Puvion-Dutilleul et al. (1978) for cortisol treated rat primary culture cells. Furthermore, in the case of the rat culture cells no determination of the percentages of different types of transcription complex was made. However, a slight increase in polymerase density between untreated and treated cells, together with the differences in sample size for each type of preparation, imply that here also cortisol treatment resulted in an increase in the amount of transcription.

Transcriptional control of gene expression is known to be the case for the chicken ovalbumin gene (Swaneck et al., 1979). It would be illuminating, therefore, to determine the percentages of different types of array in cells known to be expressing genes for certain differentiated products, and compare them with the values for cells not synthesising those products. If transcriptional control is the case, increase in the amount of a gene product will be accompanied by increase in the percentage of arrays as compared with isolated fibrils.

Changes in the percentage of arrays could also be used to distinguish between trasncriptional and translational control in cells already expressing a gene, i.e. in the case of a fine tuning rather than an on/off switch mechanism. This is an easy though perhaps indirect way of distinguishing between transcriptional and translational control mechanisms. In the absence fo criteria for distinguishing specific genes from each other morphologically, in Miller spreads, (McKnight et al. 1976), and of modifications of the technique allowing in situ hybridization of DNA or RNA sequences to transcription complexes, such an investigation would be useful. A system

to which it could be applied is the chicken oviduct, where on hormone stimulation coordinate synthesis of ovalbumin and related proteins occurs. Recently, two genes, X and Y, of unknown function, and contiguous with the ovalbumin gene, have been discovered (Carey, 1979). All are controlled by steroid hormones. Genes X and Y do not code for any of the abundant oviduct proteins, and expression of the ovalbumin gene is "dominant" over expression of X and Y, in hormone treated oviduct. It is not yet clear whether regulation of the amount of ovalbumin as opposed to the products of X and Y is exerted at the transcriptional or the translational level.

The suggestion that changes in the percentage of transcription complexes could be used to demonstrate transcriptional control is valid as long as the sample of transcription complexes selected is random. Furthermore, it is probable that only large differences in the percentages of different types of array would be detectable. Any conclusion drawn, at least for such systems as cultured cells, would have to be from an average of several experiments, in order to compensate for the fact that chromatin from cells at all stages of the cell cycle would be present on the grids. The transcriptional activity of such cells may vary.

A considerable body of biochemical investigations have been performed, with which my findings are in general agreement, and these data imply that hormones in general effect changes in the pattern of gene expression. The fact that a substantial change occurs in the length of primary transcripts in cortisol-treated cells, as well as in the amount of transcription bears this out (Puvion-Dutilleul et al., 1978. This study). Hormone treatment stimulates mRNA synthesis in the mouse (Toole et al., 1979) and nuclear RNA synthesis in chick oviduct (Swaneck et al., 1979) and rat (Degroot et al.,

1977; Aziz & Knowler, 1978; Thomas et al. 1978). Ecdysterone treatment prompts changes in gene expression in Calliphora vicina (Mascheck et al., 1977) and hormones specifically induce the expression of the vitellogenin gene in the rooster (Burns et al., 1978). General increases in the amount of RNA synthesis in response to hormones have been reported for the rat uterus (Jain, 1977), prostate (Loor et al., 1977; Parker & Mainwaring, 1977) and liver (Konstantinova et al., 1978). The role of hormones in effecting changes in gene expression is consolidated by the reported decrease in total RNA synthesis in Drosophila cell lines caused by ecdysterone and 2-deoxy-α-ecdysone (Gvosdev et al., 1975). Senior and Frankel (1978) have suggested that steroid hormones exert their effect by regulating the abundance of certain RNA sequences (Parker & Mainwaring, 1977) and similar effects have been noted in other systems (Lewin, 1974).

In the case of steroid hormones, it is generally accepted that upon entering the cell the hormone binds to cytoplasmic receptor proteins. These hormone-receptor complexes may act as gene regulators (Lewin, 1974; Senior & Frankel, 1978). In a few cases hormone-mediated changed in transcription pattern have been specifically linked to changes in the DNA sequences transcribed. In Calliphora vicina ecdysterone induces the transcription of new unique sequences (Mascheck et al., 1977) whereas in rat liver nuclei, cortisone induces both in vivo and in vitro, a preferential activation of RNA synthesis on repetitive DNA sequences (Konstatinova et al., 1978). It is not clear in this case, however, which class of repetitive sequence is involved.

Considerable evidence exists (discussion see Chapter III of this study) to show that different tissues express both similar and diverse subsets of genes. However, in no case where this has been observed have

specific genes been identified. This consideration is of importance in discussing the possible role of either the primary transcript or hnRNA.

It has been suggested (Davidson et al., 1977) that hnRNA (here considered to be homologous to the primary transcript) is a coordinate regulatory transcript. That is, some signal causes the transcription of an integrator gene set whose product is an hnRNA molecule whose interspersed repetitive sequence elements consitutue the signals whereby structural gene transcription is switched on. Activator RNA regions of the hnRNA molecule (the repetitive sequences), are postulated to bind to receptor sequences adjacent to structural genes, causing transcription to occur. Coordinate transcription of integrator gene sequences is only necessary, however, if structural genes have only one adjacent receptor sequence. Thus the inclusion of any structural gene in more than one gene battery requires that integrator genes be repetitive. This appears to be the case, inasmuch as a smaller number of distinct repetitive sequence families is contiguous with the structural genes expressed at any stage (Davidson et al., 1977). Thus expression of a large gene battery necessitates the synthesis of a long hnRNA, which will probably be processed, so that individual activator RNAs can bind to their appropriate receptor sequences. Primary transcripts are thought to be processed during their synthesis (Scheer et al., 1979), which seems to bear this out.

In both the rat and X. laevis, cortisol treatment results in the synthesis of longer primary transcripts. Whether this is a general effect of hormones is still uncertain although the proportion of high molecular weight hnRNA seems to increase in the rat after oestradiol treatment (Aziz & Knowler, 1978). Although it is not known, it is reasonable to assume that cortisol stimulates transcription of the same subsets of genes in both these cell

types. If hnRNA is indeed a regulatory molecule of the type envisaged by Britten & Davidson (Davidson et al., 1977) then this suggests that cortisol treatment results in the activation of a large number of genes. Unfortunately, I was only able to measure RNP fibril length rather than transcription unit length so that in many cases transcripts from the same gene but at different stages of synthesis would have been included in my sample. However, in the case of rat hepatocyte primary culture cells (Puvion-Dutilleul et al., 1978) mean transcription unit length showed an increase in the presence of hormone so that it may be true to say that more different genes were being expressed after hormone treatment i.e. a larger battery. So far, the results are in accord with the Britten and Davidson model.

Although I attempted to measure both nuclear and cell size in my cultures I did not obtain any conclusive results. Although T.c. carnifex cell cultures are a more favourable material for determinations of nuclear size, they were apparently insensitive to both cortisol and thyroxine. It is worth pursuing this line of research with more favourable systems, however, as it constitutes an eaxy way of distinguishing between the two main models for eukaryote gene regulation (Davidson et al., 1977; Cavalier-Smith, 1978).

An interesting observation is that after cortisol treatment, X. laevis cells are transcribing RNA of a length previously only observed in cells of T.c. carnifex. Thus it appears that the postulated C-value dependent differences in primary transcript length are not absolute, but that in certain situations, overlap can occur. This is in accord with Cavalier-Smith's suggestions (for a discussion see Chapter I of this study) and implies that transcription unit lengths overlap between species of widely differing C-value. This is demonstrated by the observations of Glätzer (1975) that

in Drosophila spermatocytes, primary transcripts of a length approaching those previously only observed on amphibian lampbrush chromosomes, are found. These spermatocyte transcripts are larger than the hnRNA of Drosophila culture cells (Lengyel & Penman, 1975), which is about 4500 bp long, equivalent to a primary transcript of 1.5 μm in a Miller spread. Although it is conceivable that the small culture cell RNA is a processing product, it is unlikely that these two populations of RNA molecules are of similar length. At this stage it is impossible to distinguish between the Britten and Davidson and the Cavalier-Smith model for gene regulation, because although the different sizes of the Drosophila RNAs could be reflected in differences in nuclear and cell size between spermatocytes and culture cells, it could equally well be argued that Drosophila culture cells synthesise small hnRNA because fewer genes, or relatively small gene batteries are being expressed, as would be the case in culture cells, where only "housekeeping genes" are being used.

The results presented here are incomplete and other experiments that could be done are the sizing of hnRNA from control and hormone-treated cells, on sucrose gradients, to see if the size difference between the primary transcripts is carried over into the hnRNA. Finally it would be illuminating to investigate the rate of incorporation of ^{3}H-uridine before and after cortisol treatment. Sequence analysis of the hnRNA could also be done.

The Miller technique can be applied to a wide variety of systems, and be used to investigate a number of different problems. For instance, large cells such as brain cells should be synthesising a great deal of RNA (Cavalier-Smith, 1978). Thus it would be fruitful to characterize transcription in these cells, as also in nurse cells. Cavalier-Smith suggests that the function of lampbrush chromosomes is to increase oocyte size, and this type of chromosome is absent in systems where the oocyte is associated

with nurse cells. It might be expected, therefore, that the nurse cell has taken over the function of the lampbrush chromosomes, and transcription in these cells might be extensive.

Another interesting system is the oocyte of the tailed frog Ascaphus truei. This species, the most primitive of living Anurans, has a C-value of about 3.8 pg, somewhat higher than that of X. laevis. At the start of meiotic prophase each oocyte contains eight germinal vesicles, each with its full complement of lampbrush chromosomes. In Rana the total volume of all the nuclei in a polynucleate oocyte is the same as for the nucleus of a uninucleate oocyte, and this is also the case for A. truei, all of whose germinal vesicles contain active lampbrush chromosomes (Macgregor & Kezer, 1970). If Cavalier-Smith's suggestions are correct then either the polymerase density on A. truei lampbrush chromosomes should be one eighth that on the lampbrush chromosomes of uninucleate oocytes such as those of X. laevis, or the overall length of loops transcribed per chromosome set should be one eighth as long in A. truei as in X. laevis.

The Miller technique could also be used to further investigate the transcription of mitochondrial genomes. Unlike the isolation techniques used in most of the studies done to date (Wolstenholme et al., 1973b), the Miller technique is gentle and less likely to cause disruption of such structures as transcription complexes.

There is also a need for some modification of the Miller technique that would allow electron microscope scanning at low magnifications for transcription figures. The applicability of the technique would be greatly extended if some sort of in situ hybridization of nucleic acids to transcription complexes were possible. Finally, the technique could also be used to investigate the question of gene dosage in S-phase.

Evidence both biochemical and morphological exists for the simultaneity of transcription and DNA replication during S-phase (Mittermayer et al., 1964; Klevecz & Stubblefield, 1967; de la Torre et al., 1975; McKnight & Miller, 1977; McKnight et al., 1977). Differences in DNase resistance during the cell cycle imply a cycle of chromatin condensation. Highly condensed chromatin binds less actinomycin D than does more extended chromatin. The binding capacity of chromatin for actinomycin D decreases progressively throughout S-phase, although it is high during G_1 and early S-phase (Pederson & Robbins, 1972). This argues for a less condensed structure of the chromatin during early S-phase, which would facilitate transcription.

When the rate of RNA synthesis during various stages of the cell cycle is investigated, it is seen to be constant in G_1, rises in S and continues in G_2 at a greater level than in G_1 (Pfeiffer, 1968; Pfeiffer & Tolmach, 1968; Lewin, 1974). That this is a general effect and not due to changes in polymerase activity is demostrated by the fact that all classes of RNA are affected equally.

The increase in the rate of RNA synthesis during S-phase is, however, too great to be accounted for entirely by gene dosage effects due to DNA replication, which may imply that in early S-phase at least, transcriptionally active regions of the chromatin might be preferentially replicated. The rate of DNA synthesis in S-phase is greatest later in S, which lends credence to this view (Dendy & Cleaver, 1964).

The results of McKnight et al. (1977) suggest that at least for ribosomal genes a replication fork cannot progress into a transcriptionally active ribosomal cistron. The synchronization of a population of HeLa cells, the system in which the RNA synthesis rate results were obtained, might allow one to determine whether during early S-phase, active regions

of the genome were preferentially replicated. The technique might also be used to investigate the timing of replication of genes, although as far as the ribosomal genes are concerned, replication occurs throughout S-phase (Balazs et al., 1973).

SUMMARY

CHAPTER I.

The ultrastructure of transcription in cultured cells of T.c. carnifex was compared with that of cultured cells from X. laevis, using the "Miller spreading technique". Primary RNA transcripts of nonribosomal origin, up to 10 μm in length, and arranged in long transcription complexes were observed in T.c. carnifex, whereas the maximum length of primary transcripts in X. laevis was about 2 μm. In both cell lines transcription was sparse and predominantly visualised as isolated RNP fibrils. Ribosomal transcription complexes were observed in X. laevis but not in T.c. carnifex.

T.c. carnifex chromatin had a nucleosomal structure under favourable spreading conditions. The DNA packing ratio of transcriptionally inactive regions was 1.88. X. laevis chromatin also had a nucleosomal conformation and a DNA packing ratio of 2.1.

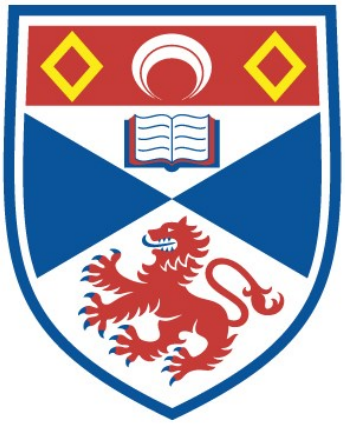
A large proportion of the T.c. carnifex culture cell chromatin consisted of linearly arranged beads approximately 29 nm in diameter, and considered to be homologous to the "supernucleosomes" described by other authors. In X. laevis, "cables" about 29 nm wide, consisting of segments 16 nm wide, were seen. These cables may represent a higher order packing structure.

In X. laevis chromatin unbeaded rings of DNA, identified by their size as mitochondrial genomes, were found. Some of these had putative replication forks whose uniform length suggested "discontinuous" replication. A number of the circles were associated with lateral fibrils whose appearance and contrast suggested that they might be RNP. These are considered to be mitochondrial genomes in the process of transcription. Smaller beaded rings of variable size and unknown significance, and attached to the DNP of X. laevis, were also found.

TRANSCRIPTION IN AMPHIBIA IN RELATION TO THE C-VALUE PARADOX : AN ELECTRON MICROSCOPIC STUDY

Sandya Narayanswami

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1979

T.c. carnifex cells in culture incorporated ^{3}H-uridine into total cellular RNA at a rate nearly four times as great as X. laevis when computed per cell per 1% cell cycle time. The rate per cell per hour was similar for both cultures. The labelled total cellular RNA of T.c. carnifex cells in culture showed a broader size distribution on sucrose gradients than did that of X. laevis.

The banding patterns on agarose gels of chromatin prepared by the Miller technique after bleomycin treatment and of chromatin extracted by a procedure that preserves nucleosome structure, were compared. Chromatin spread in the presence of Joy undergoes a dissociation of its nucleosome structure.

CHAPTER II.

Patterns of transcription in liver cells of X. laevis, T.c. carnifex and N. maculosus were compared, using the "Miller" technique. Chromatin had a nucleosomal structure and transcription complexes occurred on beaded chromatin. N. maculosus, with the largest C-value (52 pg) synthesised the longest primary transcripts. Transcription complexes of two or more RNP fibrils were rare in the chromatin of these species. The percentages of different types of transcription complex (1 RNP fibril, 2 RNP fibrils, 3 or more RNP fibrils) were similar for the three species.

The DNA packing ratios of transcriptionally inactive regions of chromatin were compared to those of transcriptionally active regions. For X. laevis and N. maculosus, where this parameter was measured, the DNA packing ratio was lower for transcriptionally active regions than for the inactive regions.

The DNA/RNA packing ratios, i.e. the degree of foreshortening of primary transcripts, of four transcription complexes analysed were 1.23 - 1.5 and 17 - 25.

Ribosomal transcription was not observed in any of these cell types. DNA rings were also apparently absent. Finally, supernucleosomes were rare or absent in chromatin prepared from the liver cells of all three species.

CHAPTER III.

The ultrastructure of transcription in T.c. carnifex neurula cells was investigated, using the Miller technique. Transcription occurred predominantly in the form of isolated RNP fibrils whose median length was greater than for T.c. carnifex liver cells or cultured cells, in that order. The percentage of transcription complexes of two or more RNP fibrils was greater in T.c. carnifex liver (33.8%) than in T.c. carnifex embryos (17.3%) Ribosomal transcription was not observed in any of these chromatins.

The DNA packing ratio of transcriptionally inactive regions of the chromatin was less in T.c. carnifex embryo chromatin ($\leqslant$ 1.35) than in liver chromatin (1.63). The embryo cells were the more transcriptionally active of the two cell types.

Transcription patterns in different tissues of X. laevis were compared. In both cultured cells and liver, transcription occurred predominantly as isolated RNP fibrils. Their median length was greater in liver than in tissue-culture cells. The percentage of multifibril transcription complexes was greater in liver (13.8%) than in cultured cells (5.6%). Ribosomal arrays occurred in culture cell chromatin but were absent in the chromatin of liver cells.

Higher order packing structures (supernucleosomes) were present in liver chromatin (transcriptionally inactive) from regions near the edges of liver lobes. Chromatin from cells deeper within the tissue (transcriptionally active) lacked supernucleosomes.

CHAPTER IV.

The Miller technique was used to investigate the ultrastructure of transcription in X. laevis and T.c. carnifex culture cells treated with cortisol or thyroxine. After cortisol treatment of X. laevis the median lengths of isolated nonribosomal RNA fibrils, and the terminal fibrils of multifibril complexes, increased twofold, as did the percentage of multifibril transcription complexes (from 5.6% to 28.1%). The DNA packing ratio of transcriptionally inactive regions of the chromatin was slightly less than that of untreated cells spread under the same conditions. There was also a decrease in the DNA packing ratio of transcriptionally active regions of the chromatin of treated cells as compared to the value for inactive regions. The DNA/RNA packing ratios of three transcription complexes were 4, 5.7, and 20.5.

Supernucleosomes and other higher order packing structures were not observed in preparations of chromatin from hormone-treated cells. Cortisol treatment resulted in a stimulation of ribosomal transcription. Ribosomal transcription complexes were sparsely covered with lateral fibrils. The matrix units lacked nucleosomes, which were present only in spacer regions.

Rings of three types were seen in preparations of hormone treated cells. One class was identified on the basis of contour length as being of mitochondrial origin. Unbe aded rings with both replication forks or attached RNP fibrils were found, as in untreated cells. Free circles of various sizes, and with a beaded structure resembling supernucleosomes were seen. Finally, small rings attached to the DNP occurred. These were of various sizes, and the range in their controur length was greater than that of similar rings occurring in control preparations. The distribution was not, however, the same as that of the free circles of either class.

T.c. carnifex cells failed to respond to either cortisol or thyroxine. I did not treat X. laevis cells with thyroxine.

Nuclear and cellular dimensions were determined for X. laevis culture cells before and after cortisol treatment, in cultures grown at two different concentrations. The results were inconclusive but were not sufficient to eliminate the possibility that an increase in the amount of transcription causes an increase in the size of the nucleus. The results were similarly inconclusive for T.c. carnifex cultured cells treated with cortisol.

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APPENDIX I.

Calibration of electron microscope magnifications

During the course of my research it transpired that the electron microscope (Philips EM 301) had not been calibrated. After calibration of the magnifications it was seen that a substantial difference existed between the real and the apparent values. To correct measurements taken from negatives made before calibration and to determine whether, over this period of time, the magnification values had drifted, I re-examined old preparations. These had all been made before calibration, but at intervals of several months. RNP molecules that had been photographed at an apparent magnification of x 19000 were re-photographed as the same apparent magnification (real magnification x 15500). The two negatives of each molecule were compared. I found that the difference, if any, was slight (1 - 5%) and not great enough to affect the conclusions I had made from my data. It is unlikely, furthermore, that the real magnification had changed much over the course of time that the electron microscope had remained uncalibrated.

APPENDIX II.

Growth rates of tissue-culture lines. Xenopus laevis and Triturus cristatus carnifex.

Several times during the course of my research it became necessary to determine whether my culture lines were in logarithmic growth. This allowed me to determine when rates of transcription were likely to be maximal. Growth rate was checked during the course of the uridine incorporation experiment but at other times cells of a known concentration were inoculated into flasks and the cell number determined at intervals of 12 hr or 24 hr. Subsequent experiments were either conducted at these concentrations, or concentrations less than or very close to those known to result in the attainment of log phase after about 2 days growth at 25°C.

MATERIALS AND METHODS

Using the routine subculturing methods described more fully in Chapter I of this study, confluent cultures of cells were trypsinized and the cells counted in a haemocytomer. Cells were inoculated at the required density into small (25 cm^2 growth area) sterile plastic culture vessels (Falcon) each containing a total volume of 5 ml medium. Two vessels were set up for each point of the growth curve. The cells were incubated for 24 hr at 25°C to ensure attachment to the substrate.

X. laevis cells were sampled at 12 hr intervals whereas the more slowly growing T.c. carnifex line (Rudak, 1976) was sampled at intervals of 24 hr. At each sampling time, cells were removed from the incubator and trypsinized routinely, using siliconized pipettes. The cell suspensions were placed in siliconized glass centrifuge tubes and centrifuged for 5 - 10 min at 900 rpm in a bench centrifuge (MSE). The supernatant was decanted and a known

volume of AWS or Versene added to the cells. Using a siliconized Pasteur pipette, the cells were gently resuspended, prior to counting in a haemocytometer (Paul, 1975). Four independent determinations were made for each sample, so that each point on the growth curve was the mean of eight values. Growth curves were used to determine the onset and duration of various phases of growth, and to determine Tau, the mean generation time of the culture.

(ii) Cell concentrations at which growth curves were made

(a) Growth rates were determined for cells grown at the normal subculture density (Fig. 1a,b). This was done for one of the T.c. carnifex cultures that I used, interchangeably, in experiments. Both lines exhibited similar kinetics of growth.

Inoculation densities

X. laevis	1.6×10^6 cells per small culture vessel.
T.c. carnifex	$1.7 - 3.0 \times 10^5$ cells per small culture vessel.

(b) Cells of each T.c. carnifex line were inoculated at twice the usual subculture density. This was necessary for RNA extraction experiments where the yield of highly labelled RNA from cells grown at the normal density was insufficient. At higher densities it was thought that the T.c. carnifex line, which showed contact inhibition, would have moved into stationary phase after a few days. However the growth rates (Fig. 1ab) show that this is not the case, and that log phase lasts for at least four days after attachment. Experiments were conducted with cells of 2 - 3 days in age.

Inoculation densities

T.c. carnifex "black" 5.4×10^5 cells per small vessel.

" "red" 6.0×10^5 cells per small vessel.

This was not done for X. laevis, as even at this concentration the cells were at a lower density than that normally employed for routine subculture. This is because X. laevis cells are 4 - 7 times smaller than those of T.c. carnifex.

(c) During the course of the uridine incorporation experiment it was necessary to know the cell number at the various sampling times. The inoculation densities that I used were different from those above and this allowed me to determine that cells grown at about 1.95×10^6 cells per parge (75 cm^2 growth area) culture vessel had attained logarithmic growth after two days in culture. This inoculation density was used for Miller spreads of cultured cells.

APPENDIX III.

Visualisation of nucleolar transcription in Xenopus laevis and Triturus cristatus carnifex oocytes

INTRODUCTION

During the early stages of my research I decided that it would be useful to have some test of the effectiveness of the Miller spreading technique in my hands. Oocyte ribosomal genes are a useful system because they have been extensively characterized and are of a known morphology. Their presence in oocytes at high frequency together with their tandem repetition and high transcriptive activity allow easy isolation and visualisation. Changes in their morphology from the standard one described in much of the literature (Trendelenburg et al., 1973) would be attributable to the isolation method that I used.

MATERIALS AND METHODS

(i) Solutions

(a) Nuclear isolation medium ("3:1")

A solution containing 3 parts 0.1 M KCl to 1 part 0.1 M NaCl was diluted from stock solutions and stored at +4°C.

(b) Dispersal medium "pH 9 water"

Freshly boiled water was adjusted to pH 9.0 with stock borate buffer (BDH. pH 9.2) using a plastic 1.0 ml tissue-culture pipette.

(ii) Procedure for spreading T.c. carnifex and X. laevis oocyte ribosomal genes

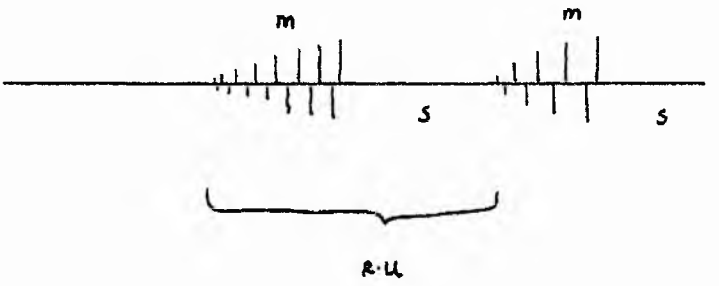
A female T.c. carnifex (Gerrard & Haig) was anaesthetised in a 1% solution of MS222 (Sandoz) and a piece of ovary removed. The ovary was stored at +4°C in a dry, sealed, embryo glass. To spread chromatin, a few oocytes were placed in an embryo glass containing "3:1", on the stage of a dissecting microscope. Oocytes of from between 0.98 - 1.00 mm in diameter were broken open using two pairs of sharpened No. 4 watchmakers' forceps. This caused the yolk to be released together with the oocyte nucleus (Callan & Lloyd, 1960), which was cleaned of yolk by repeated sucking in and out of a finely pulled Pasteur pipette (diam. 0.5mm). As quickly as possible, the cleaned nucleus was transferred to a droplet of pH 9 water in a sterile plastic Petri dish, and washed to remove saline. This step was repeated as chromatin disperses best under conditions of low ionic strength. The nucleus was then transfrred to a third droplet of pH 9 water. Using sharpened No. 5 watchmakers' forceps, and a fine tungsten wire (Callan & Lloyd, 1960) the nucleus was broken open and the nuclear membrane removed. The nuclear contents were allowed to disperse undisturbed, at room temperature, for from 45 - 80 min.

The procedure for X. laevis oocytes was exactly the same, save that oocytes of from 1.1 - 1.2 mm were used. Further processing of preparations was as for other cell types.

(iii) Measurements

For measurement a random sample of transcription units and spacers was considered. Only sufficiently stretched repeating units with well identified regions were included. Lengths were measured with a calibrated

map-measurer and for each value, the mean of two determinations was taken. For determination of repeating unit length the length of the spacer to the right of each matrix unit was added on to the matrix unit length i.e.



However, there is no way of telling which spacer of the two on either side of a matrix unit actually "belongs" to it. Ideally, in determinations of repeating unit length, both spacers should be included. The results of spreading several nuclei were pooled for analysis.

RESULTS

(i) T.c. carnifex ribosomal genes

Nucleolar chromatin appeared as clumps of material, at the periphery of which well spread DNP was seen, often containing tanscriptionally active ribosomal genes (Fig. 3). These had the classical "Christmas-tree" morphology (Fig. 3) and were tandemly repetitious in arrangement, being separated by transcriptionally inactive spacer regions of variable length (Fig. 4). Size distributions of matrix unit (Fig. 5a), spacer (Fig. 5b)

and repeating unit length (Fig. 5c) yielded values of 2.37 μm, 1.46 μm and 3.82 μm respectively. Spacer regions exhibited a greater heterogeneity in lengththan matrix units. These values are similar to those already determined for Triturus cristatus oocyte ribosomal genes (for review see Trendelenburg et al., 1973), 2.4 μm, 2.2 μm and 4.6 μm respectively. My values are somewhat smaller but this may be attributable to differences in the degree of dispersal of the nucleolar genes.

Ribosomal genes showed clear RNP fibril length gradients, and in a few cases, the distal region of the matrix units carried RNP with the terminal "knobs" indicating packaging of the ribosomal precursor (Fig. 4). However, the heavy shadowing of my preparations tended to obscure structural detail. I observed very little spacer transcription (Figs. 3 and 6 a-c) although in two cases (Figs 3 and 6a) a short matrix similar to the"preclude complexes" observed by other authors (Scheer et al., 1977) was seen. Matrix units of opposite polarity were apparently absent.

Nucleolar chromatin was exclusively beaded in appearance (Fig. 3 and 6 a-c). Beads occurred in spacer units but in my preparations it was not possible to say whether they were polymerases or nucleosomes. They were 29 nm ± 9.7 nm in diameter (N = 10) in a shadowed preparation. In most cases, the matrix units were so densely covered in lateral fibrils that it was not possible to determine whether beads lacking attached transcripts occurred in these regions. In a few cases (Fig. 7)the presence of a well stretched matrix unit allowed me to determine that such beads were rare or absent.

This is in accord with the observations of Scheer (1978) who has suggested that highly active nucleolar chromatin lacks nucleosomes, which do, however, occur in the spacer regions of submaximally active ribosomal genes.

I assumed that the beads on spacer units were nucleosomes, and obtained a value for the DNA packing ratio of these regions of 1.35 (Table III, p57). This low value is in accord with the idea that the chromatin of transcriptionally active regions of the genome is in a relatively extended conformation.

I was also able to calculate the DNA/RNA packing ratio of matrix units (Table VI, p59) using the above value for DNA packing ratio. I obtained a mean value of 13.3, which is high and implies a considerable foreshortening of the primary transcripts. This may be an artefact of speading conditions. Furthermore, if it is true that the matrix units themselves lack nucleosomes, unlike the spacers with their beaded structure, then this value is an overestimate. In this case, the DNA packing ratio of the matrix units would be in the region of 1.0, rather than 1.35.

These results conform closely to previous observations (Trendelenburg et al., 1973; Scheer, 1978), and suggest that in my hands the Miller spreading technique yields preparations that give a valid idea of transcriptional events in the chromatin.

(ii) X. laevis ribosomal genes

Oocytes of a diameter of 1.1 mm were used to make spread preparations of X. laevis nucleolar genes. Unfortunately, I had no success with these preparations. Lampbrush chromosomes were in a poor state of preservation. A certain proportion of the chromatin took the form of circular clumps, that on occasion had beaded and transcriptionally inactive fibrils radiating from their peripheries (Fig. 8). These may be transcriptionally quiescent nucleoli. Scheer et al. (1977) were successful in obtaining spread preparations of X. laevis ribosomal genes from oocytes of 0.5 mm diameter, spread for 10 - 20 min in a variety of solutions. They observed that the nucleoli are tightly

adherent to the nuclear membrane, which is included in the spreading solution together with the nuclear contents. I did not include the nuclear membrane in the spreading solution, which may be the reason for my lack of success. Even with long spreading times (30 - 105 min) chromatin clumps remained relatively condensed. This may have been due to the nature of the sucrose-formalin fixative, which was made up with stock formalin rather than freshly made formalin from paraformaldehyde which I used later in this study. Thus the sucrose-formalin would have been too acid and this might have affected the quality of my preparations.

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Transcription in Amphibia in relation to the C-value Paradox. An electron microscopic study.

Volume II. Illustrations

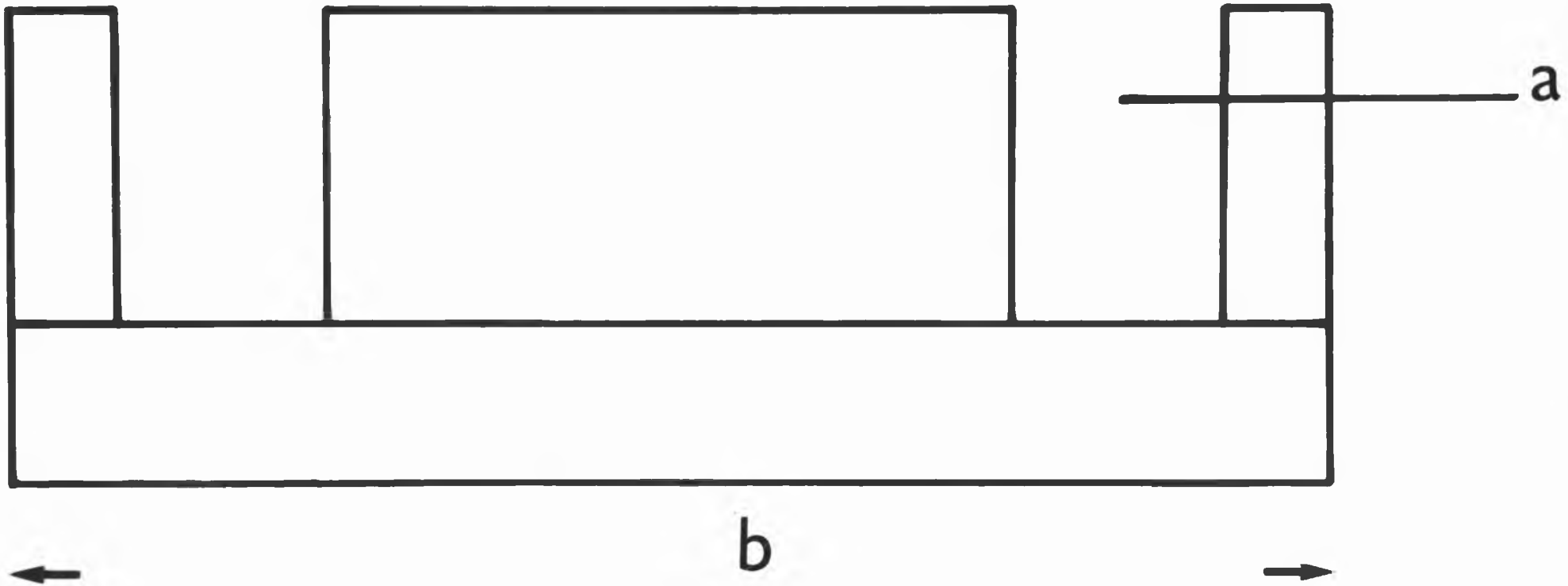
by

SANDYA NARAYANSWAMI

I - 1 A diagrammatic representation of a cross-section through a microcentrifugation chamber.

(a) microcentrifugation well 4 mm in diameter and 6 mm deep.

(b) diameter of chamber 2.5 cm.

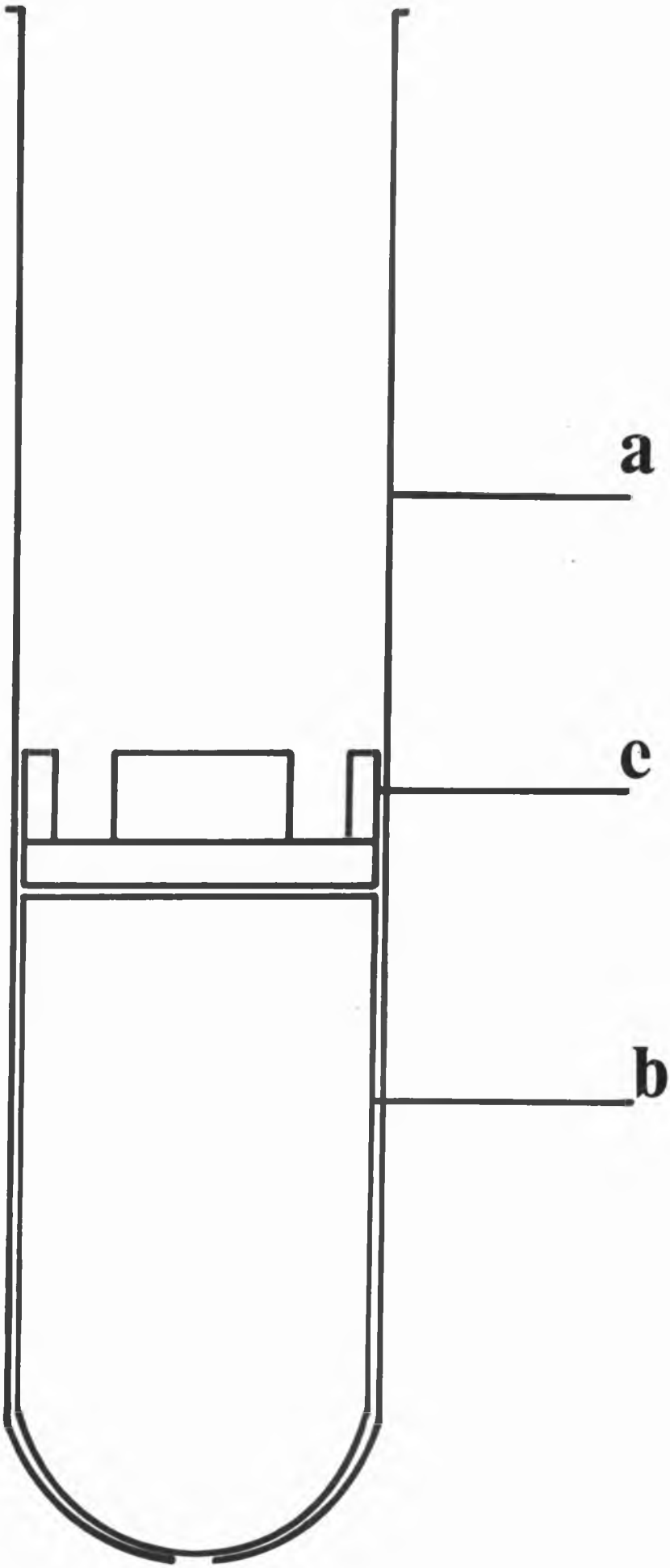

a
b

I - 2 A diagrammatic representation of the centrifuge tube and Araldite plug used during centrifugation of Miller spreads.

(a) centrifuge tube.

(b) Araldite plug.

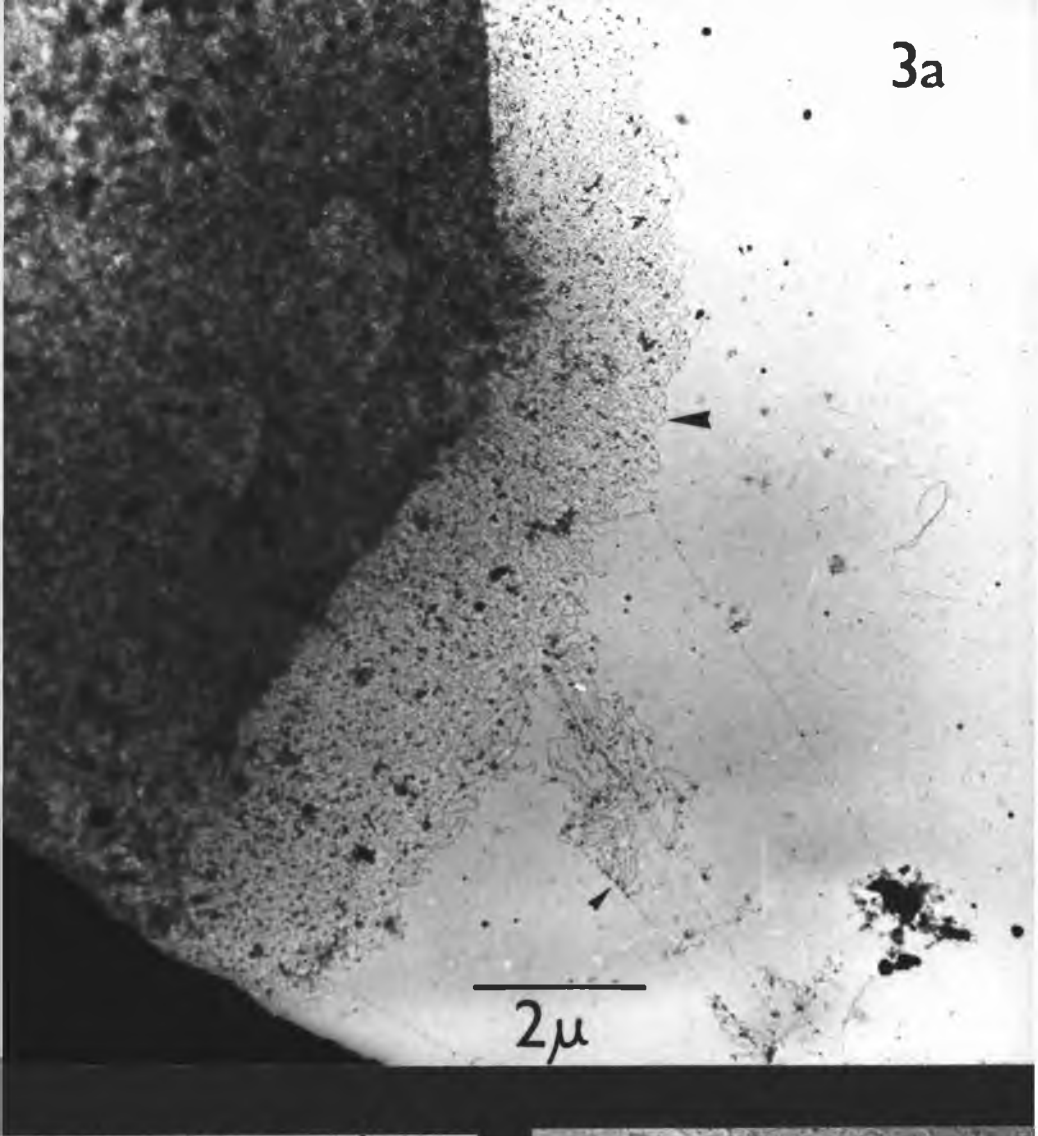
(c) microcentrifugation chamber.

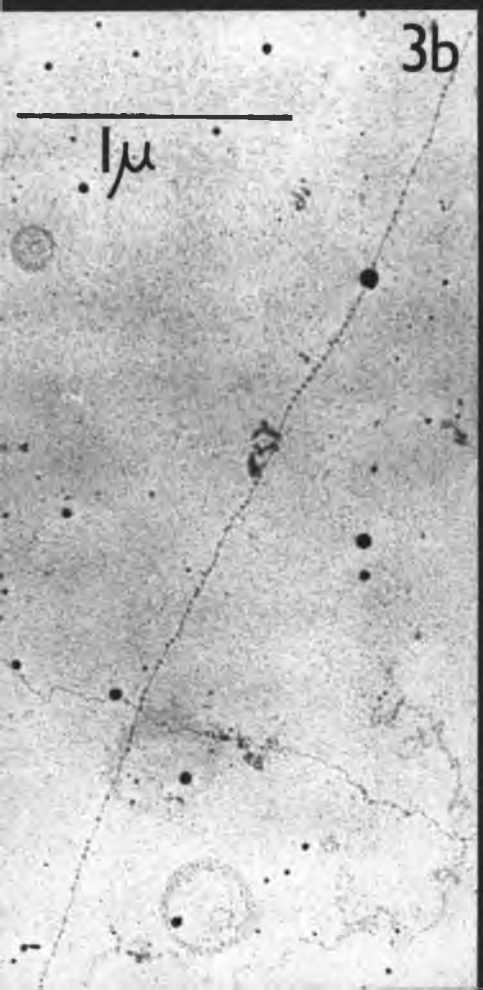

a
c
b

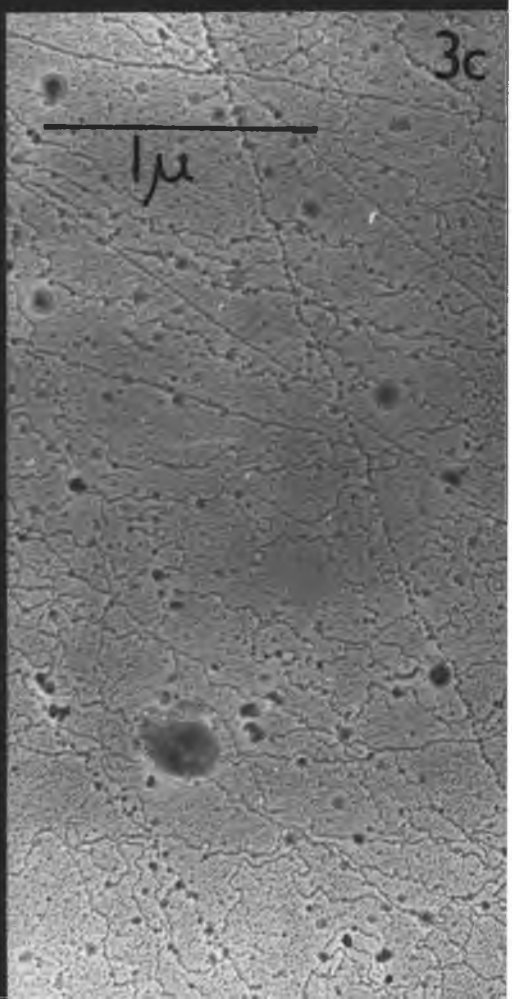
I - 3a Partially spread Nonidet-P40 extracted X. laevis cultured cell nucleus. Large arrow denotes halo of chromatin fibres around the central undispersed mass of DNP. Small arrow indicates fibres radiating from the halo and bearing putative RNP.

I - 3b Nucleosomal chromatin showing the regular arrangement of beads along the DNP.

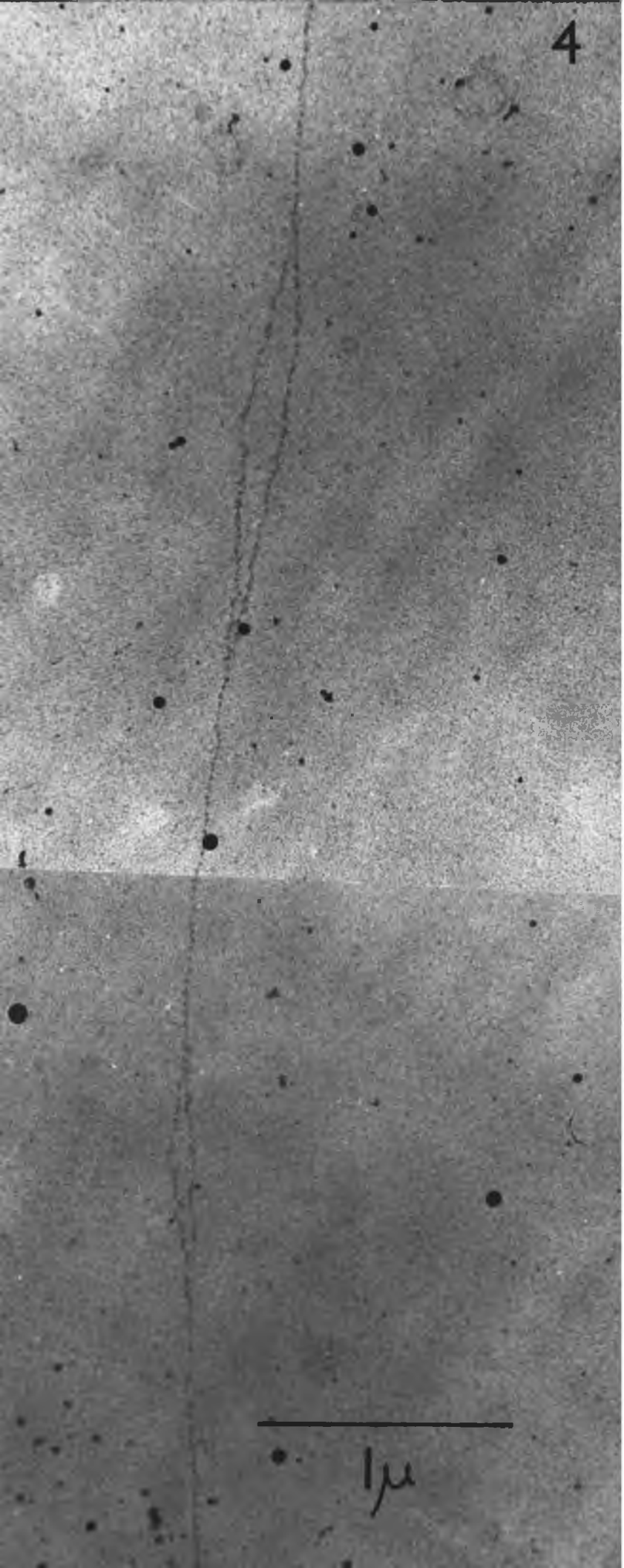
I - 3c Well spread chromatin showing both beaded and "smooth" DNP lacking nucleosomes.


3a
2μ


3b
1μ


3c
1μ

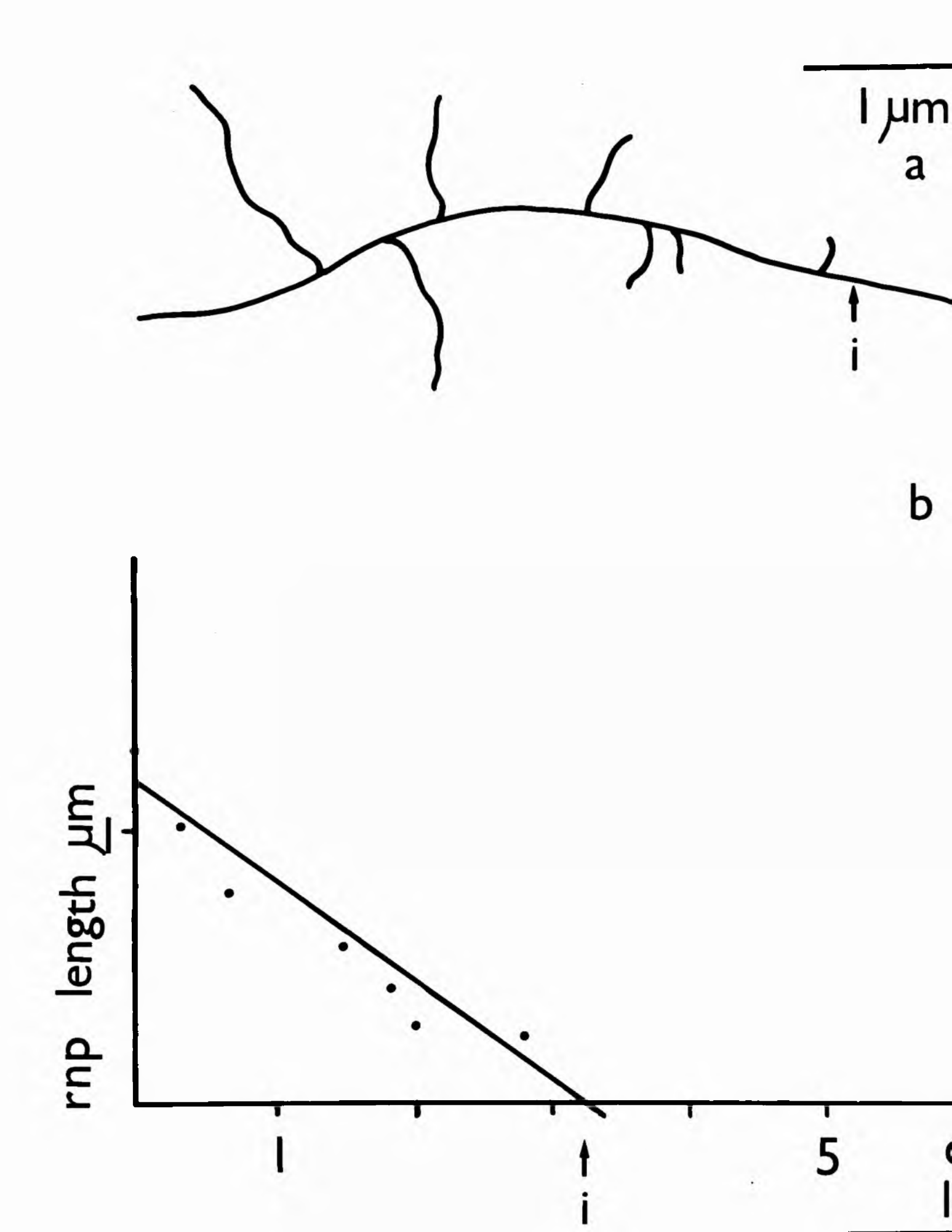
I - 4 Tandemly arranged replication forks found in a preparation of chromatin from X. laevis culture cells grown at low cell density.


4
1μ

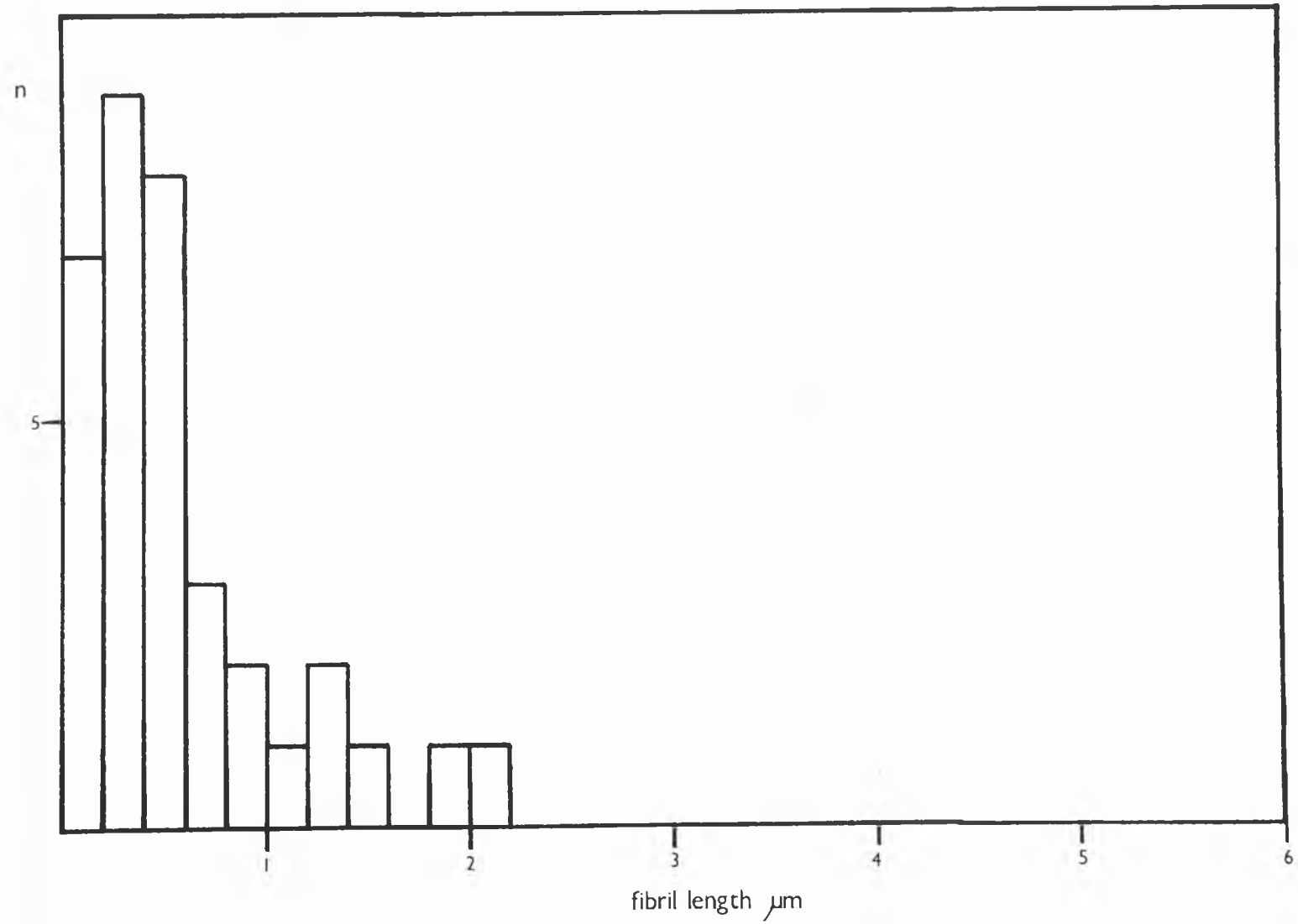
I - 5 Diagrammatic representation of the determination of initiation point of a transcription complex by Laird Analysis.

(a) Tracing of the transcription complex showing RNP fibril distribution on the DNP axis.

(b) Plot of RNP fibril length against position of each fibril on the DNP axis. A line drawn through the resulting slope cuts the x-axis at the presumptive initiation point of the complex.


1 µm
a
i
b
rnp length µm
1
i
5

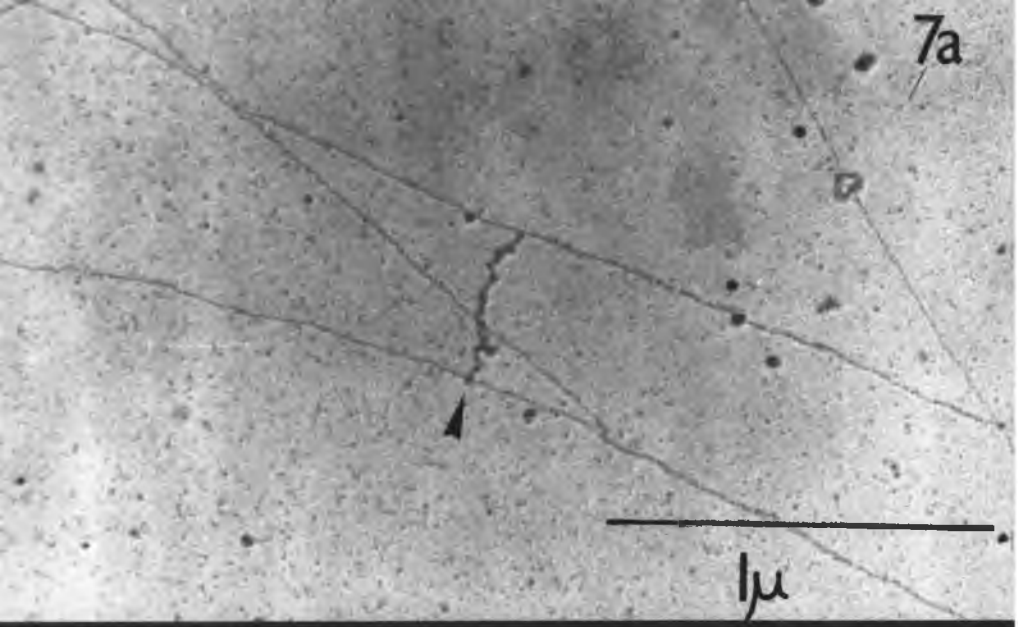
I - 6a Histogram showing the length distribution of isolated RNP fibrils from X. laevis cultured cell chromatin. Median value 0.44 μm. Range 2.1 μm.


n
5
1
2
3
4
5
6
fibril length μm

I - 7a Arrow indicates an isolated RNP fibril attached to X. laevis cultured cell chromatin, and showing the higher contrast generally characteristic of RNP molecules visualised by the Miller spreading technique.

I - 7b Arrow indicates an isolated RNP fibril of some 2 μm in length, the longest that was found attached to X. laevis cultured cell chromatin. This structure may consist of two fibrils entangled, and exhibits clearly the diffusely beaded structure characteristic of primary transcript molecules.

I - 7c Putative transcription complex found on X. laevis cultured-cell chromatin. RNP fibrils were not clearly traceable so that Laird Analysis was impossible.

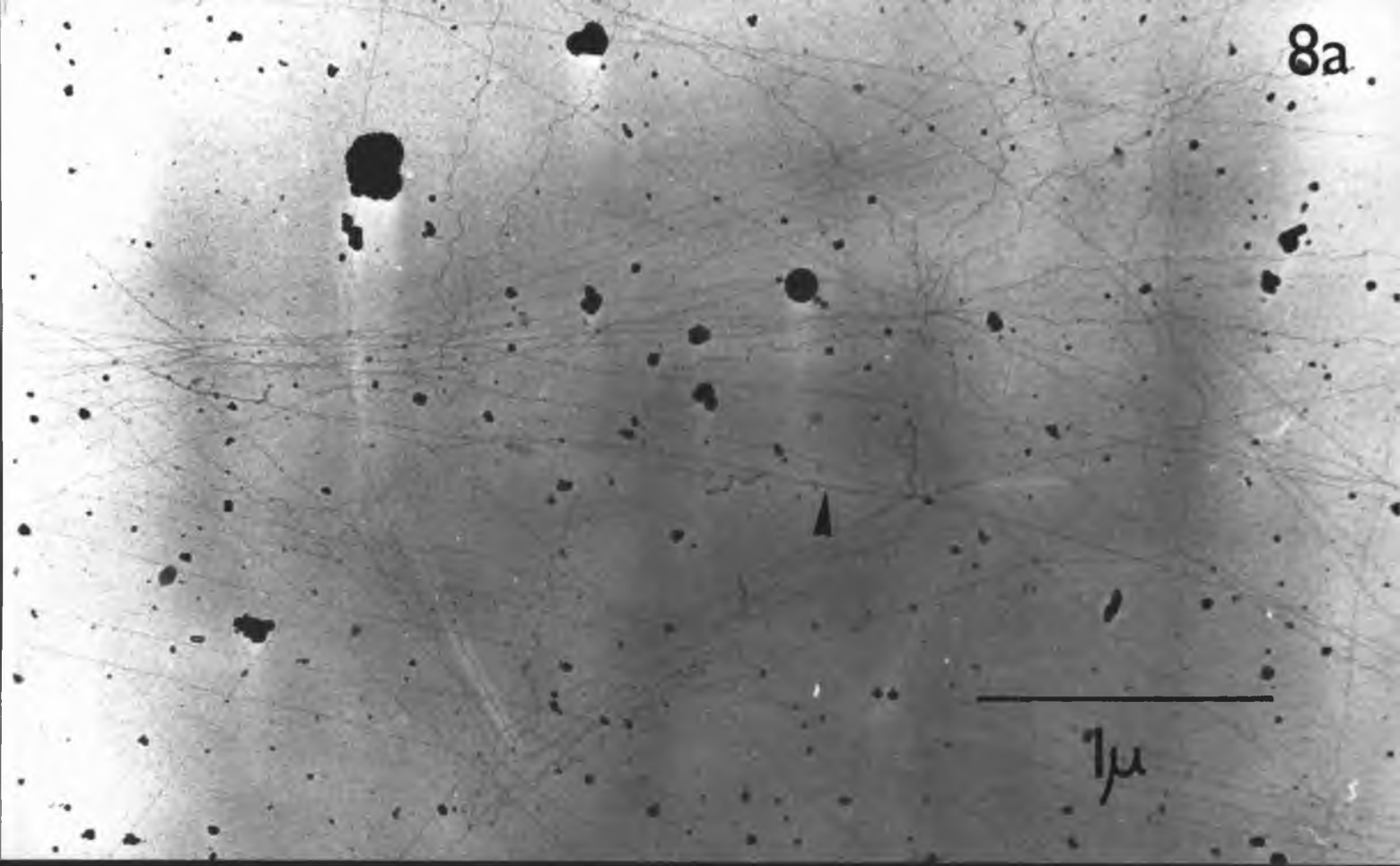

7a
1μ

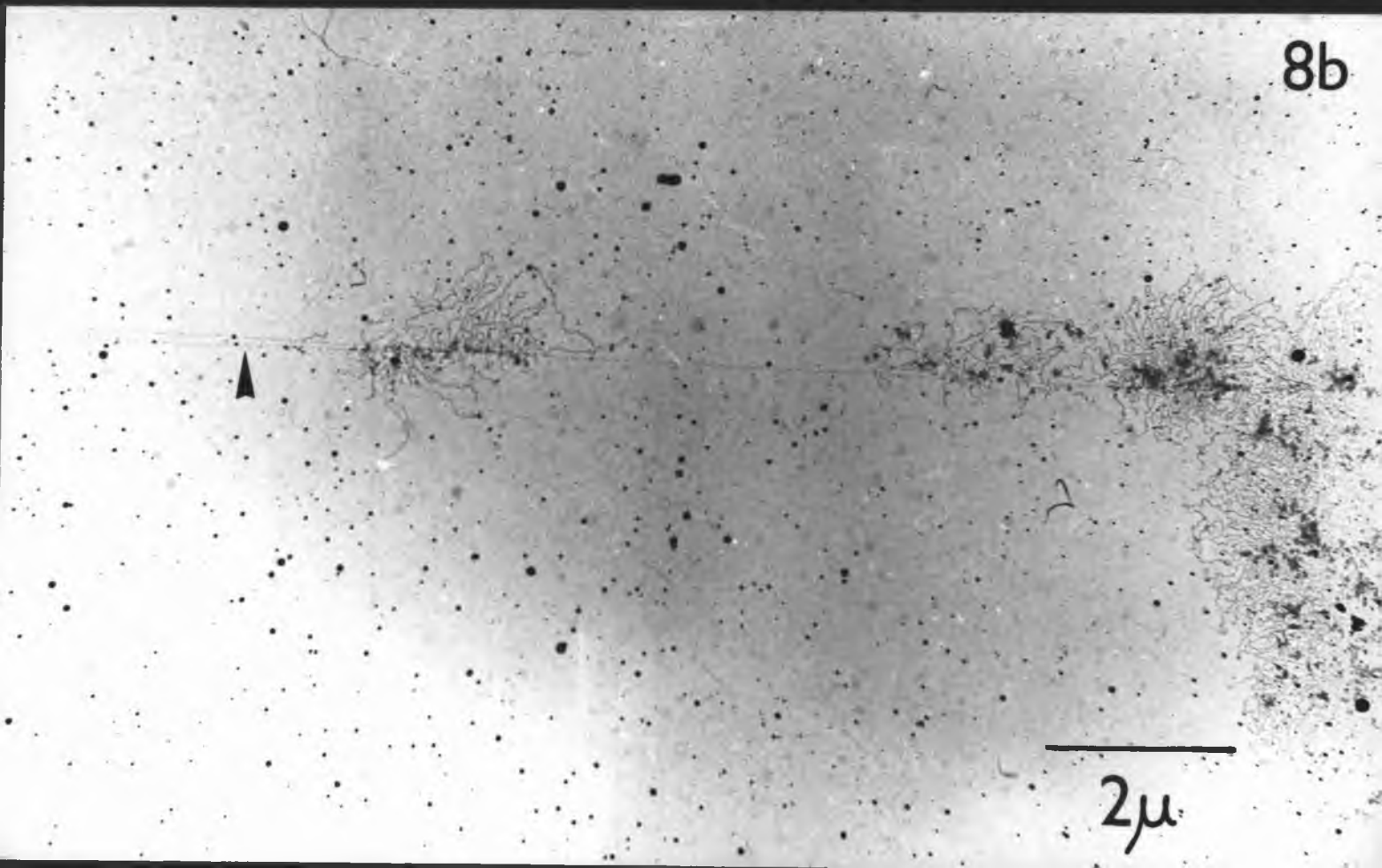

7b


7c

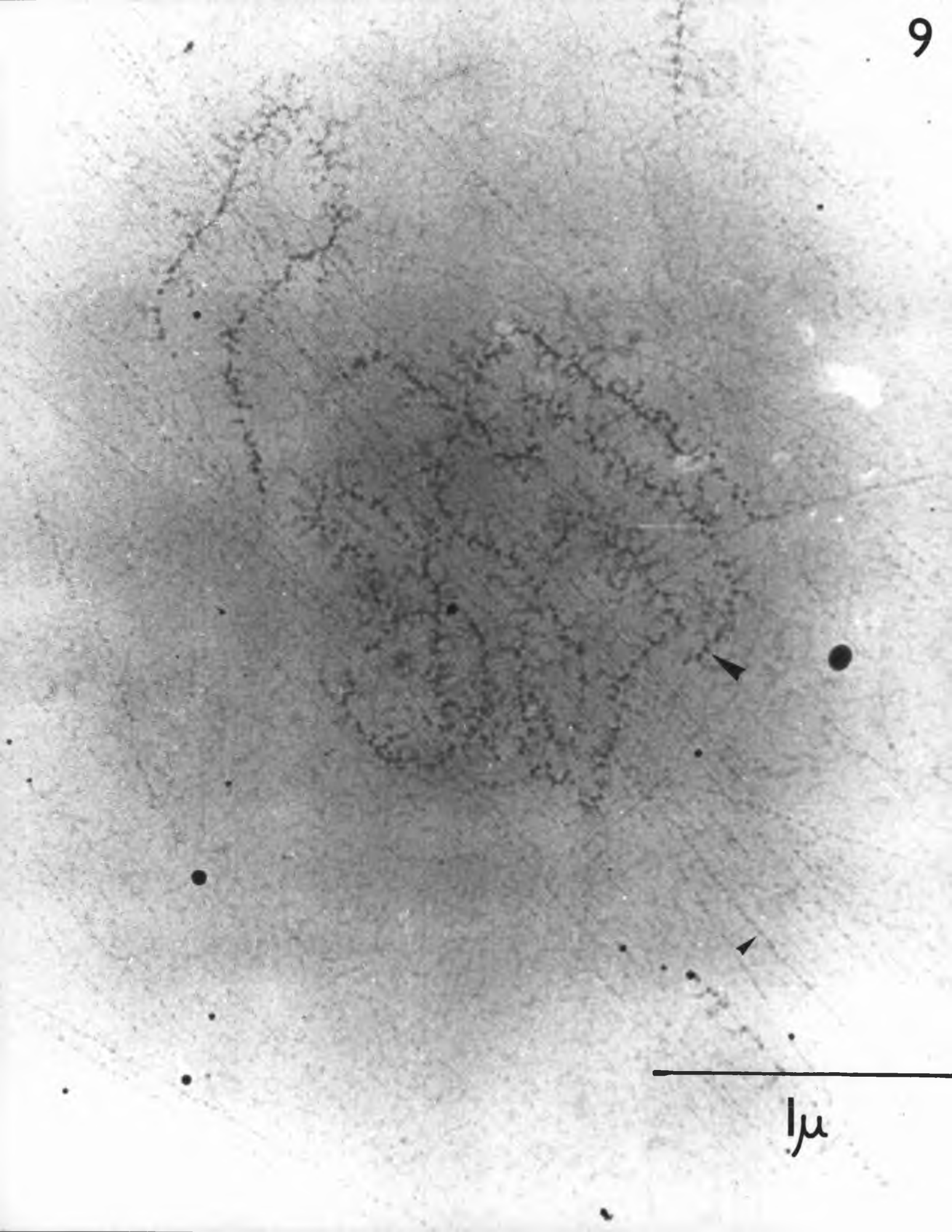
I - 8a Arrow indicates a transcription complex consisting of three to four RNP fibrils, and found on chromatin of X. laevis cultured cells grown at high cell density. Laird Analysis gave a value for transcription complex length of 3.5 μm.

I - 8b As Fig. I - 8a. This transcription complex was approximately 2.8 μm long. Arrow denotes region of putative DNA replication.


8a
1μ


8b
2μ

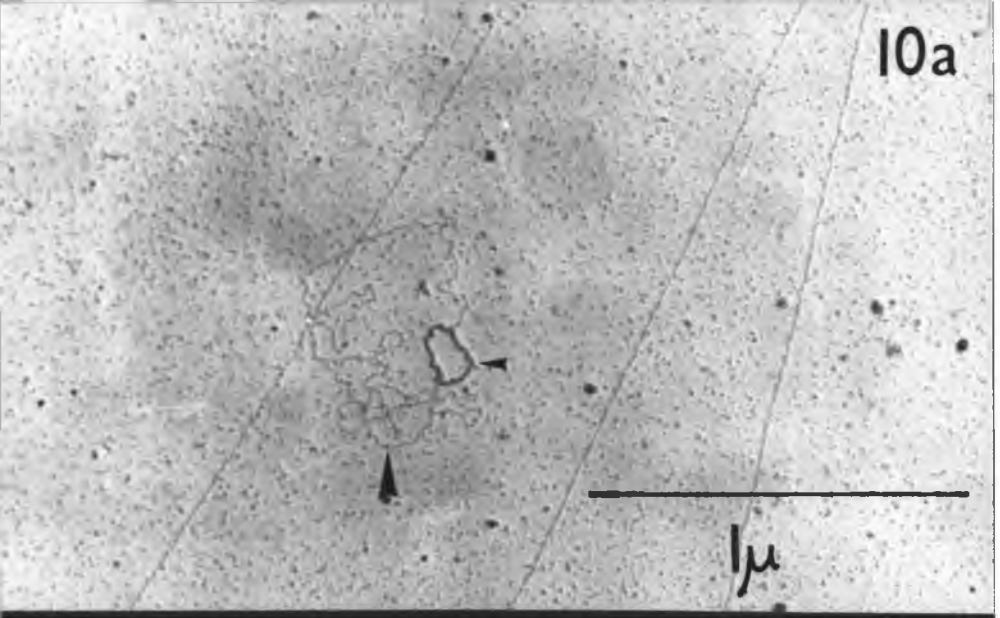
I - 9. Clump of moderately transcriptionally active ribosomal transcription complexes from X. laevis cultured cells grown at low cell density. Arrow indicates RNA polymerase molecules, which were of higher contrast than nucleosomes (small arrow) on this preparation.

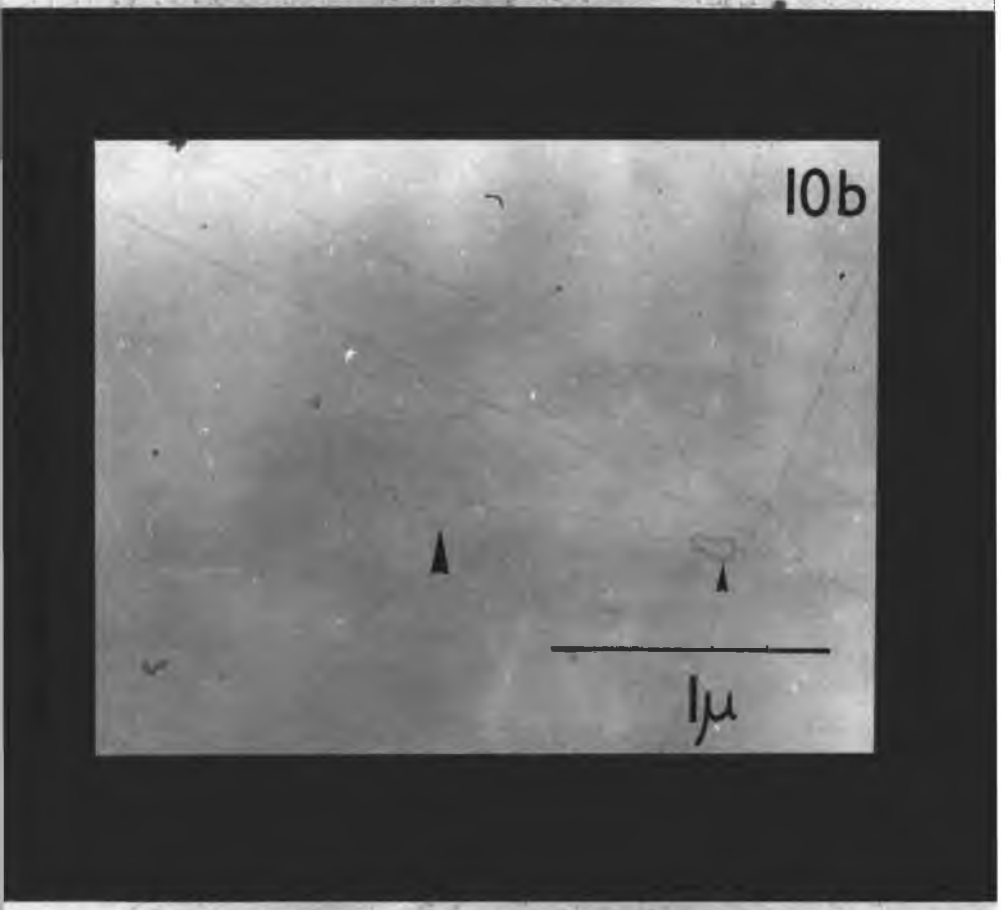

1μ

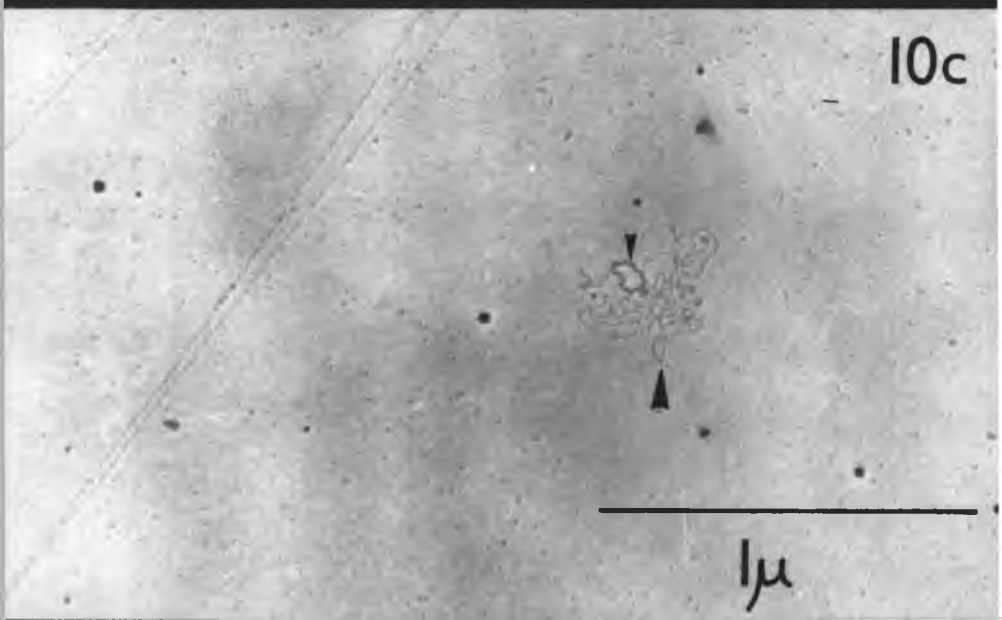
I - 10a Arrow denotes putative mitochondrial DNA circle found in chromatin from X. laevis cultured cells grown at low cell density. Small arrow denotes putative replicating region, of higher contrast than the rest of the circle.

I - 10b As Fig. I - 10a.

I - 10c As Fig. I - 10a.


10a
1μ


10b
1μ

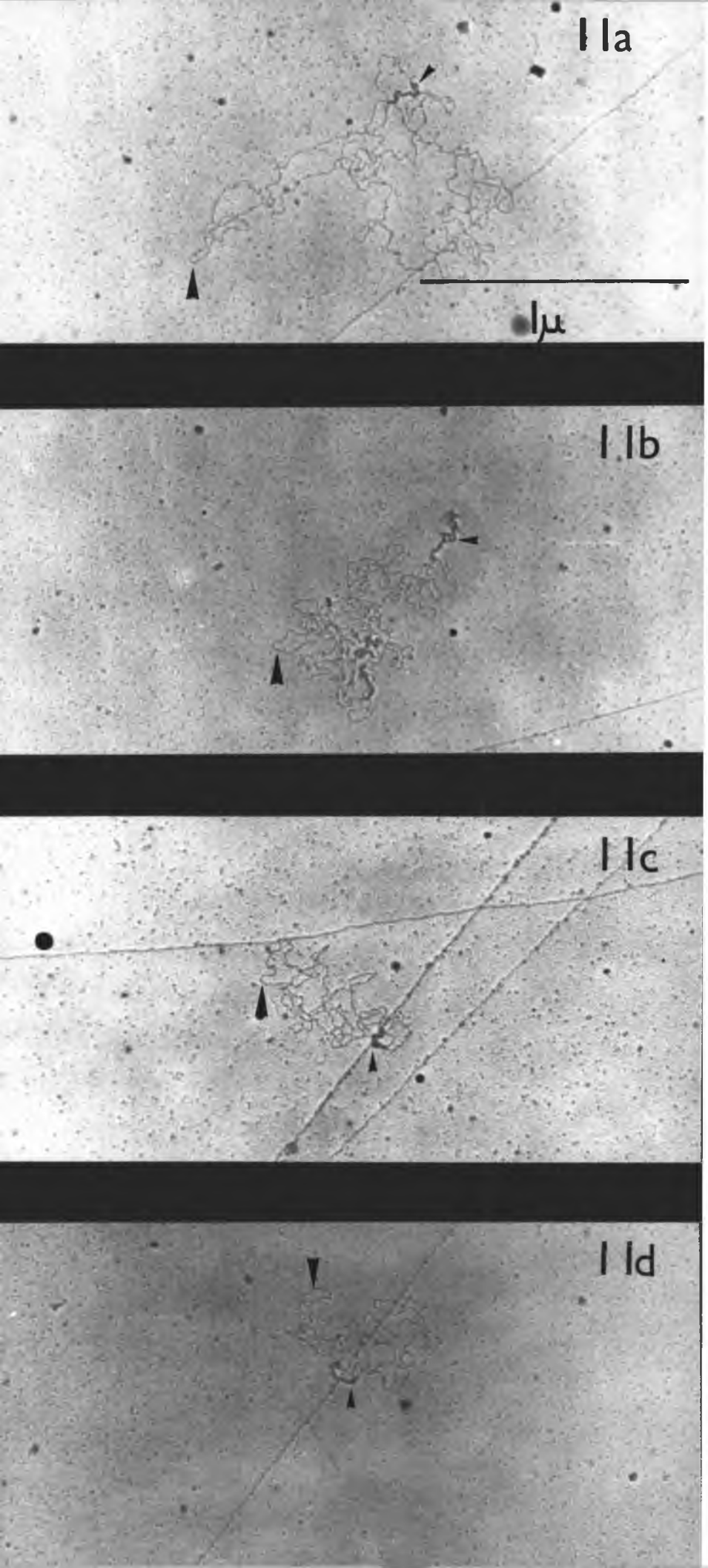

10c
1μ

I - 11a Arrow denotes putative mitochondrial DNA circle found in chromatin from X. laevis cultured cells grown at low cell density. Small arrow denotes putative attached RNP molecule, of higher contrast than the rest of the circle.

I - 11b As Fig. I - 11a.

I - 11c As Fig. I - 11a.

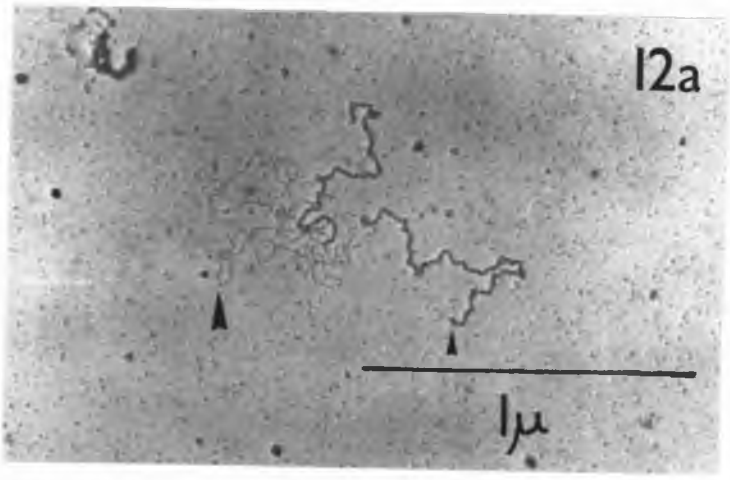
I - 11d As Fig. I - 11a.


I Ia
I Ib
I Ic
I Id

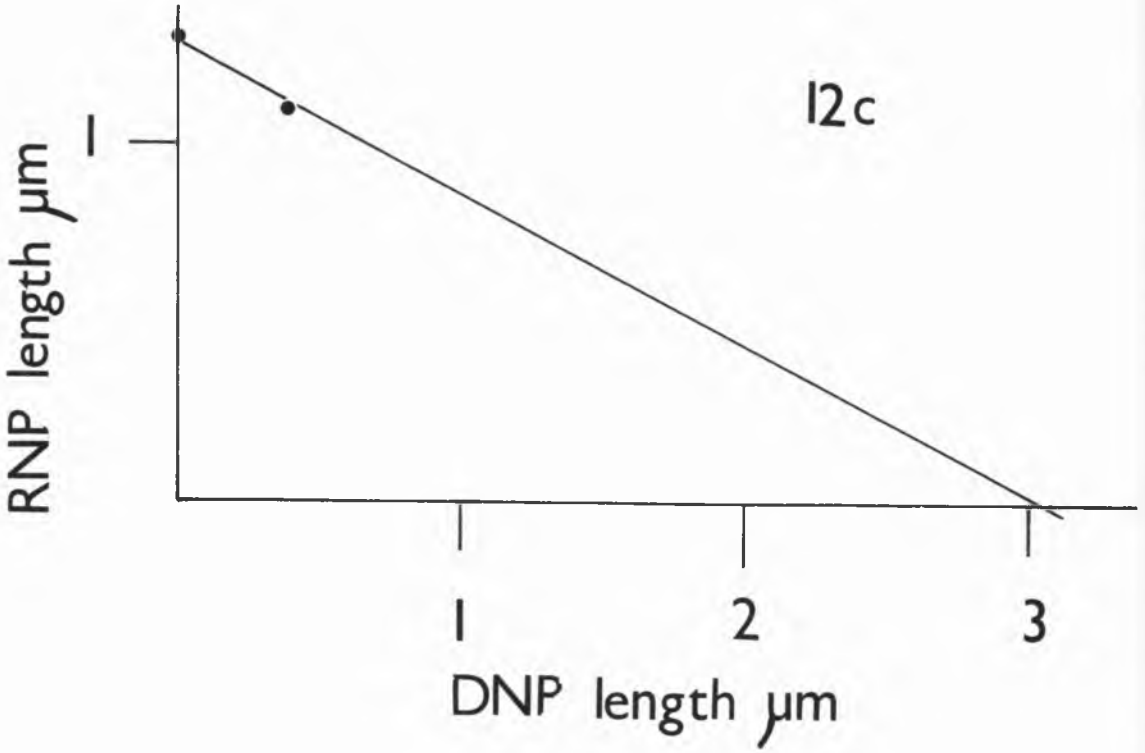
I - 12a Putative mitochondrial DNA circle (large arrow) bearing two attached transcript molecules (small arrow), and found in chromatin from X. laevis cultured cells grown at low cell density.

I - 12b Tracing of I - 12a.

I - 12c Plot of RNP fibril length against position of transcript on the DNP circle, assuming the spacing of the two fibrils to be 0.4 μm. A line drawn through the resulting slope gives a transcription complex length of up to 3.2 μm.


12a
1μ


12b

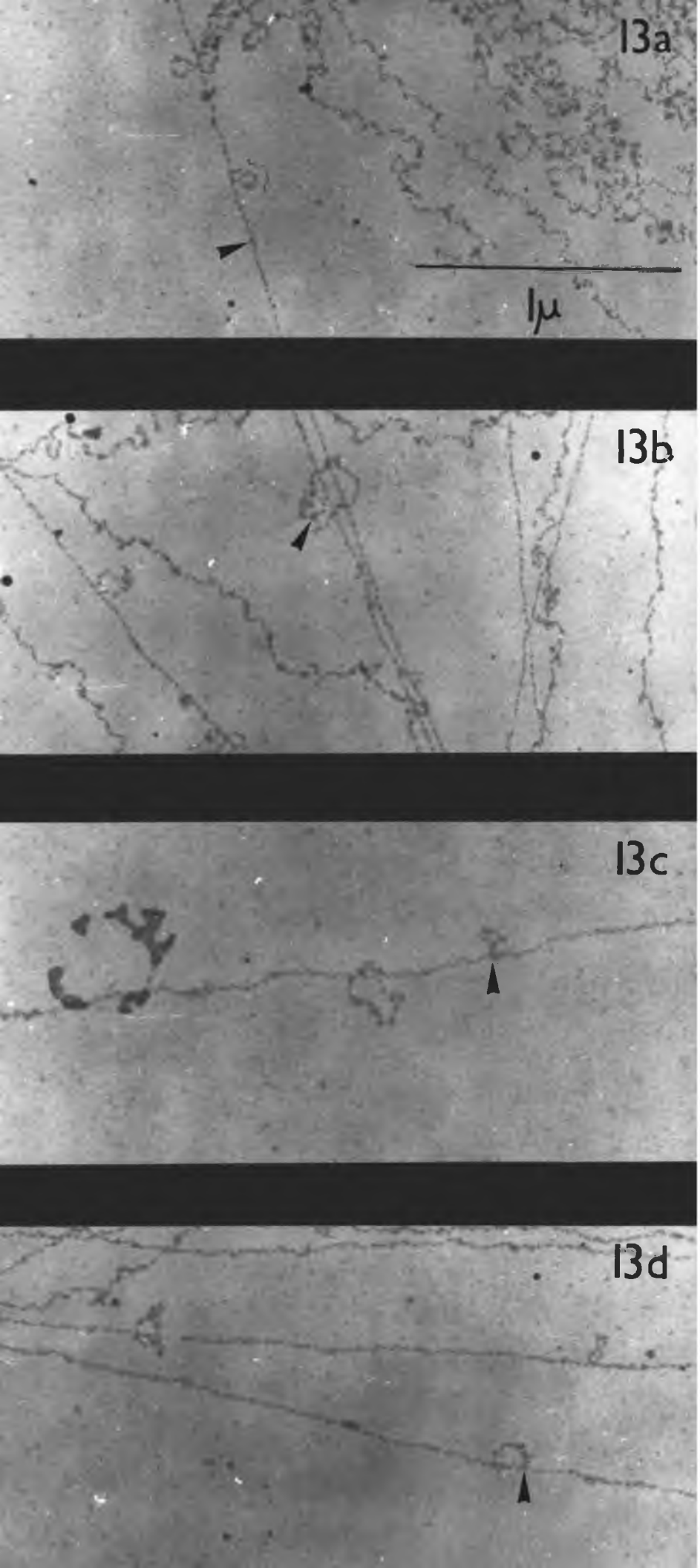

12c
RNP length μm
1
1
2
3
DNP length μm

I - 13a Small rings attached to chromatin of X. laevis cultured cells grown at low cell density and spread for 15 min in 0.1% Joy. Arrow denotes chromatin axis, stretched by centrifugation. Unstretched chromatin lies adjacent to the stretched region.

I - 13b As Fig. I - 13a. Arrow denotes circle resembling a twist in the chromatin.

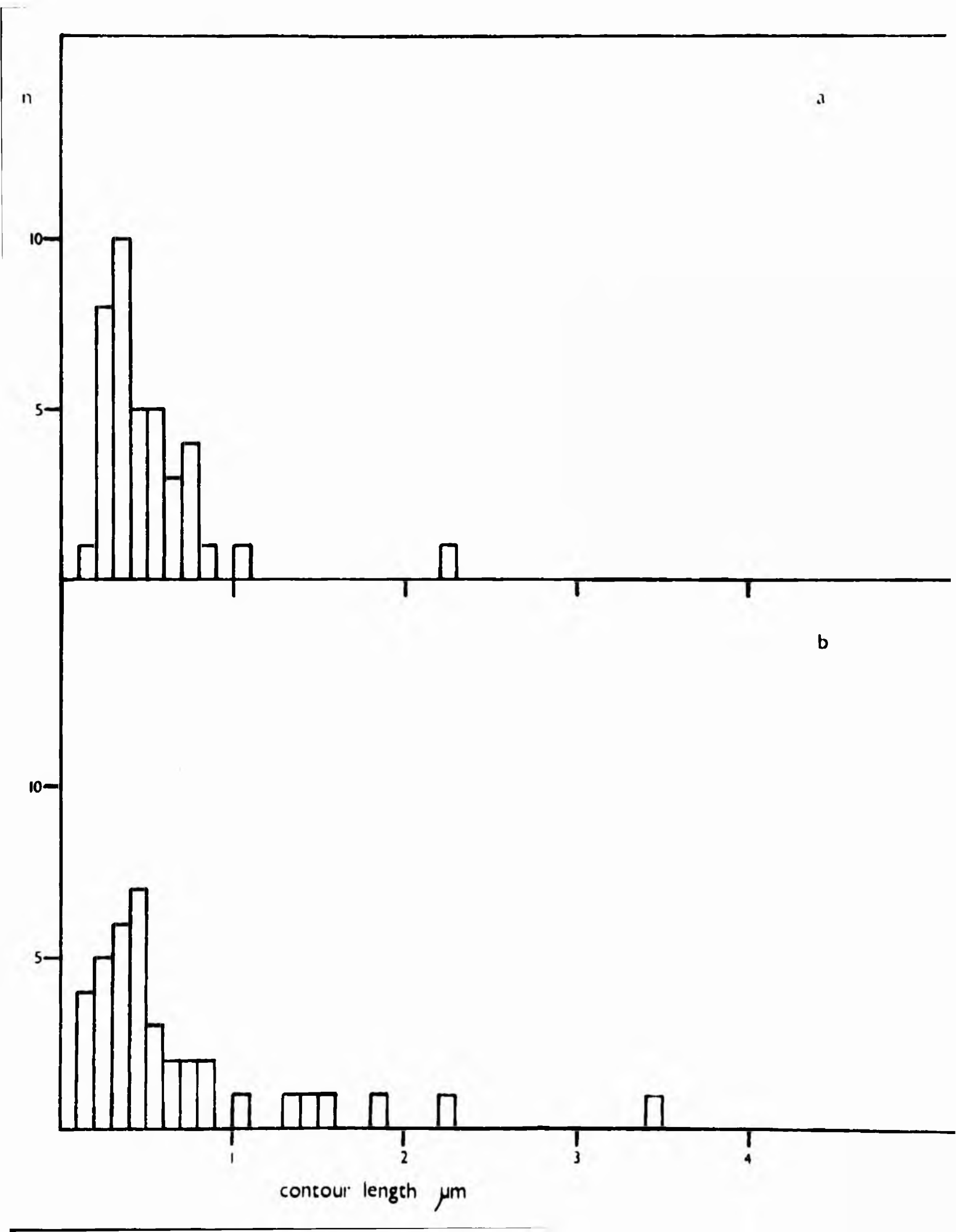
I - 13c As Fig. I - 13a. Arrow denotes a circle resembling the excision intermediates seen on lampbrush chromosome RNP.

I - 13d As Fig. I - 13a. Arrow denotes a circle resembling a primary transcript molecule whose free end has become associated with the chromatin axis.


13a
1μ
13b
13c
13d

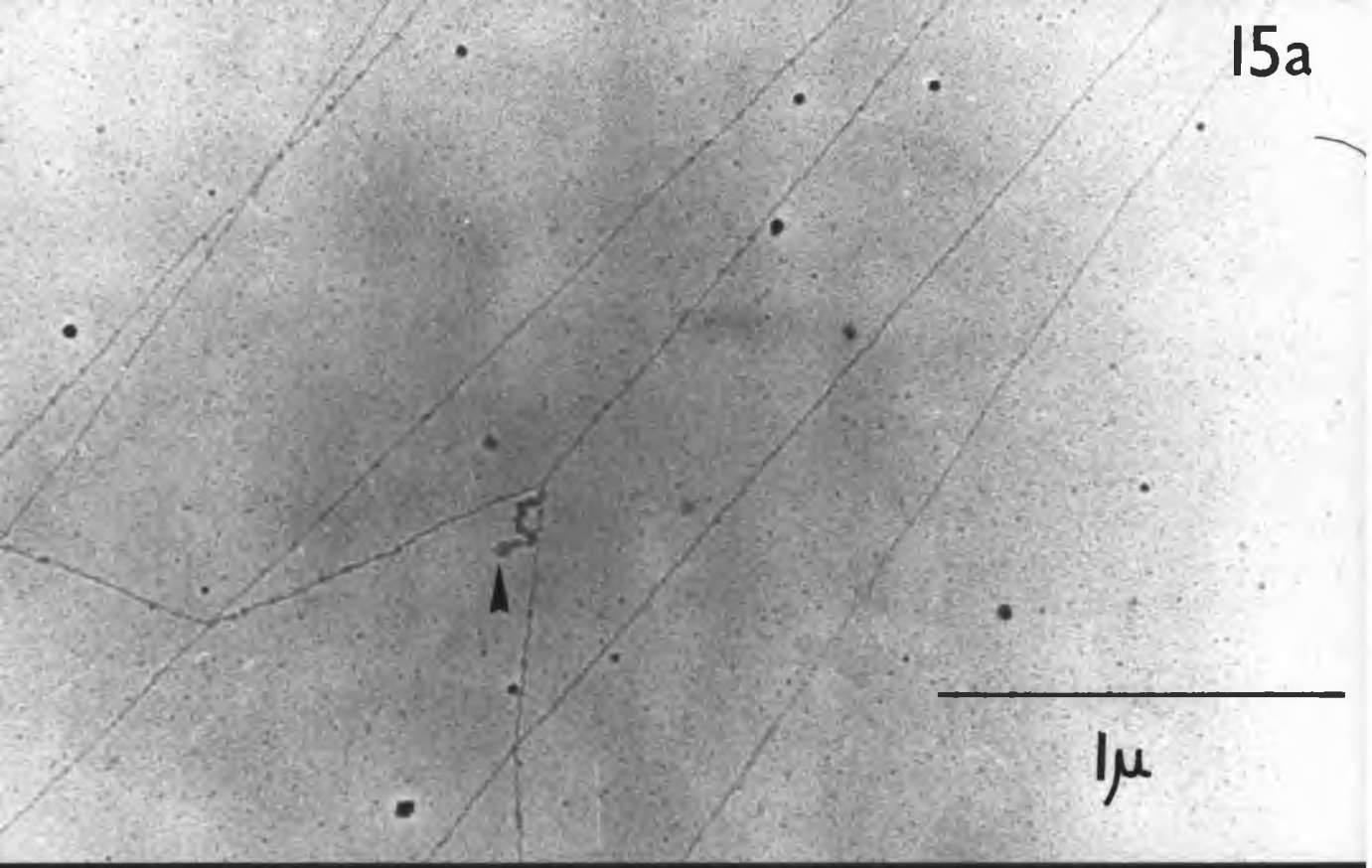
I - 14a Histogram showing the distribution of the contour lengths of a random sample of the small rings attached to X. laevis cultured cell chromatin (Figs I - 13 a-d). Median value 0.4 μm. Range 2.3 μm.

I - 14b Histogram showing the distribution of the contour lengths of a random sample of the small rings attached to X. laevis cultured cell chromatin, after 5 hr treatment with 20 μg/ml cortisol, at 25°C (see Chapter IV). Median value 0.47 μm. Range 4.95 μm.


n
a
10
5
b
10
5
1
2
3
4
contour length μm

I - 15a Arrow denotes putative primary transcript attached to a replicating region of the DNP. Due to the proximity of the "fork" to the "RNP" this structure may represent two strands of DNP crossing each other, one of which has broken off and become more condensed in structure.

I - 15b As Fig. I - 15a. The distance of the putative primary transcript from the region of ongoing DNA replication (arrow) suggests that this structure really represents coincident transcription and replication.


15a
1μ

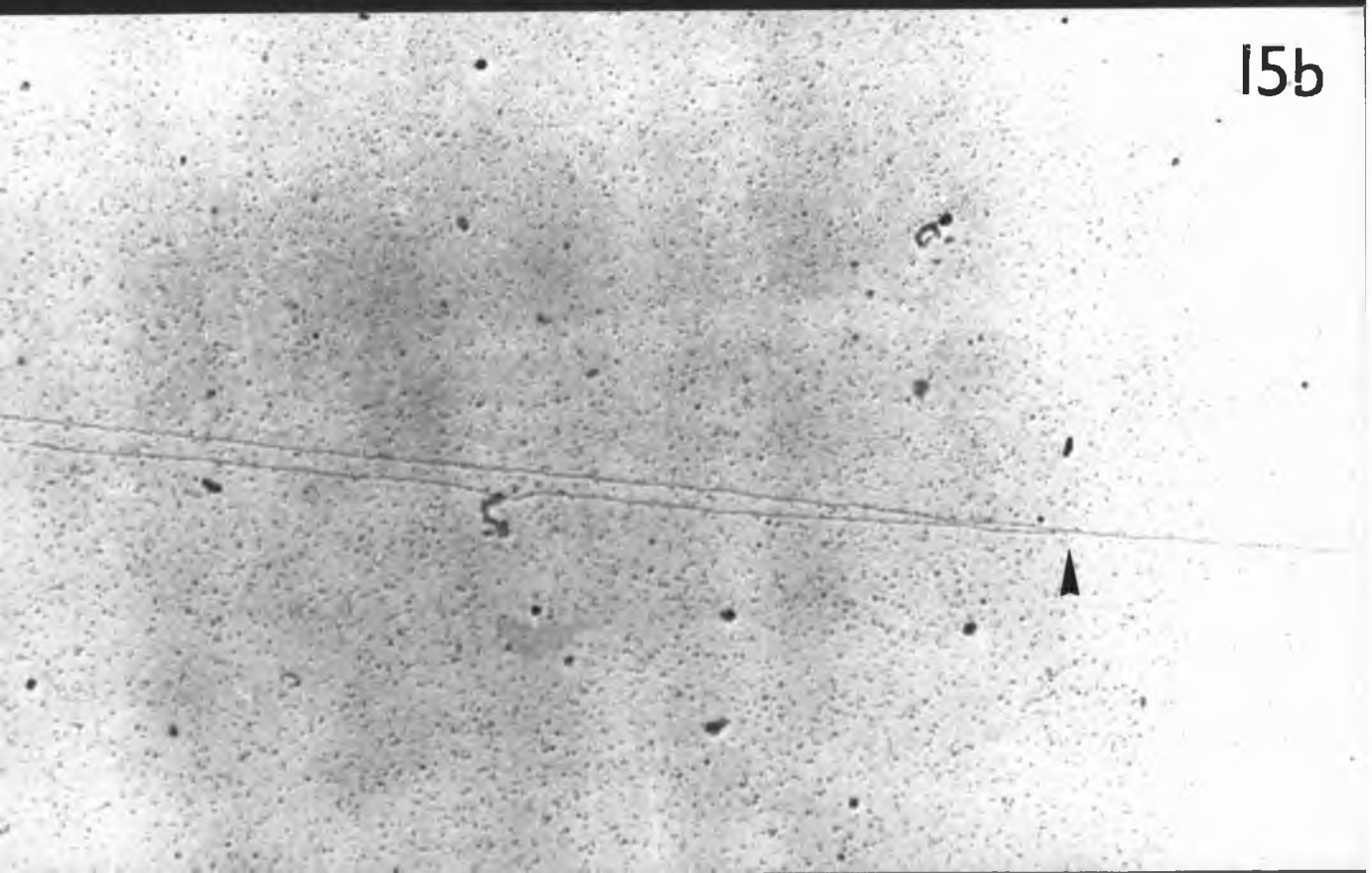
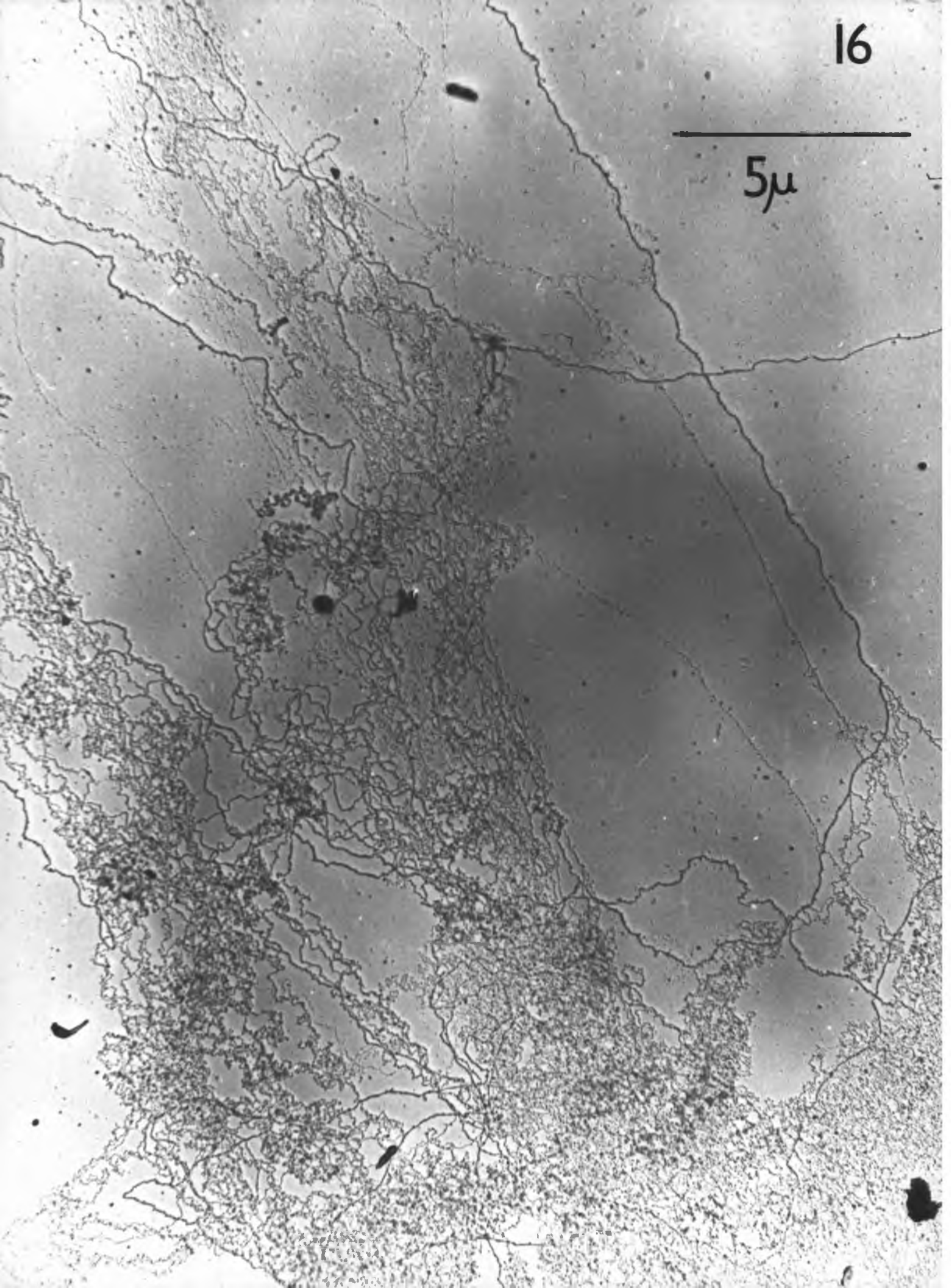
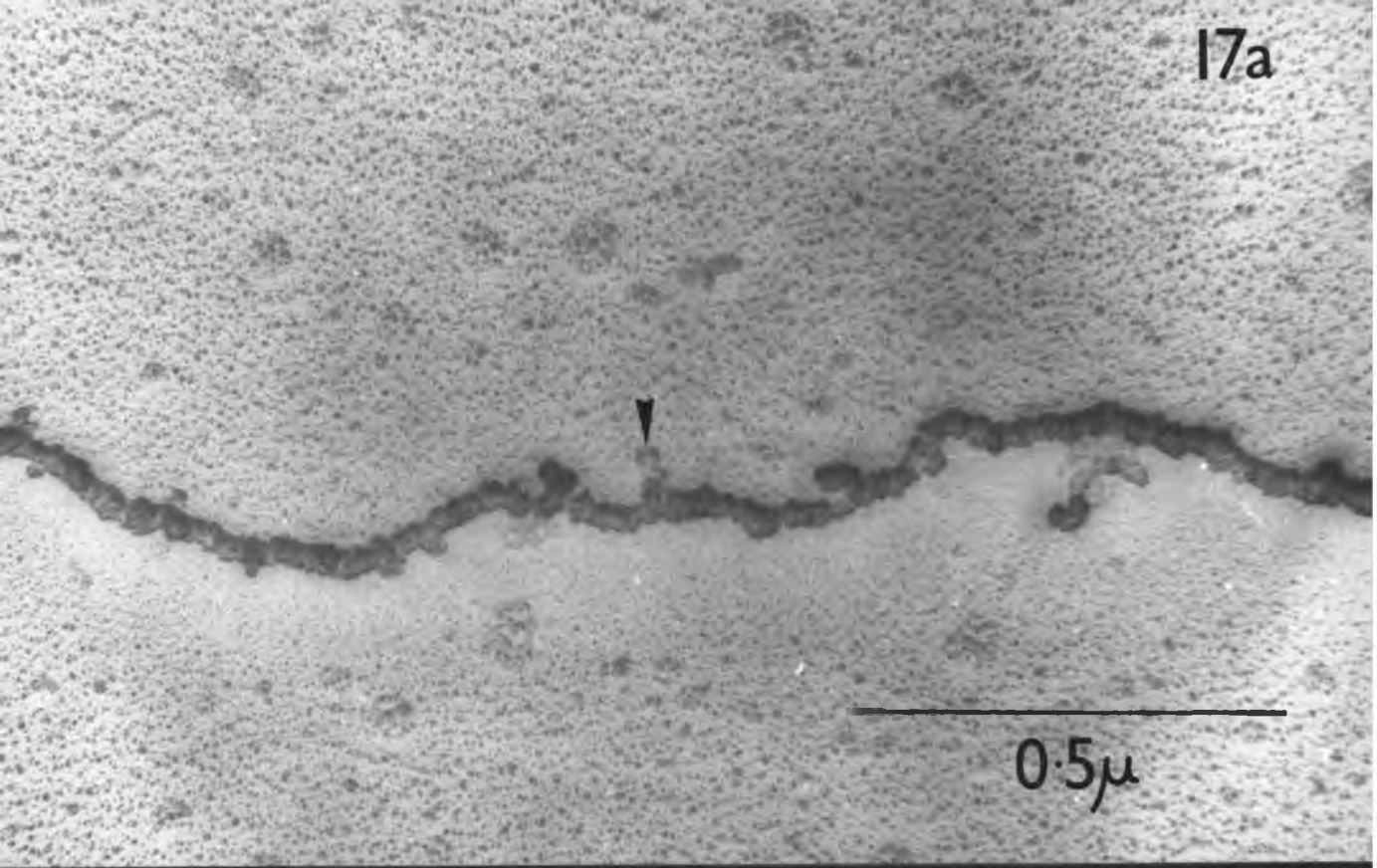

15b

Fig. I - 16 Part of a partially spread nucleus of an X. laevis cultured cell grown at high cell density, and showing the "cable" structure of the chromatin.


16
5μ

I - 17a High power photographs of the "cable" structures presented in Fig. I - 16. The segmented structure of the cables is clearly visible. Arrow denotes a region of the cable which may consist of a length of nucleosomal DNP that has unravelled.

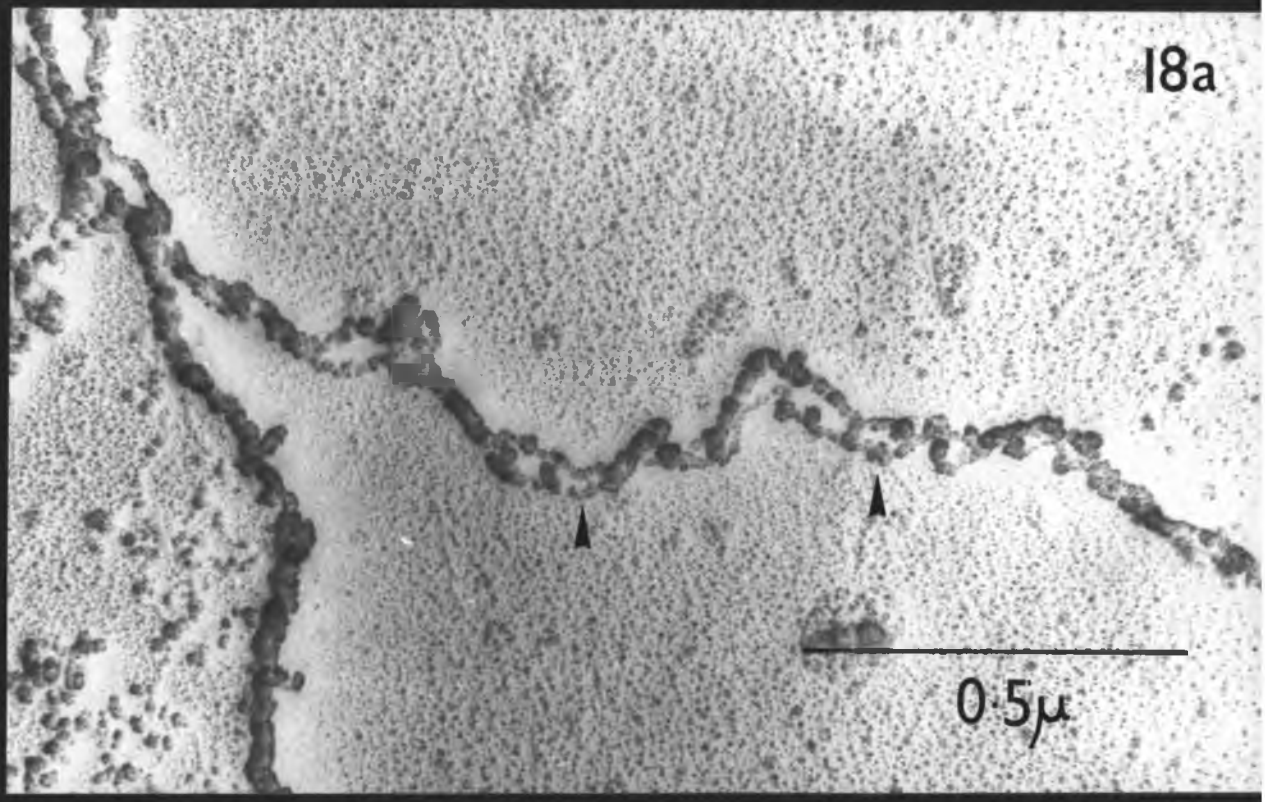
I - 17b As Fig. I - 17a.

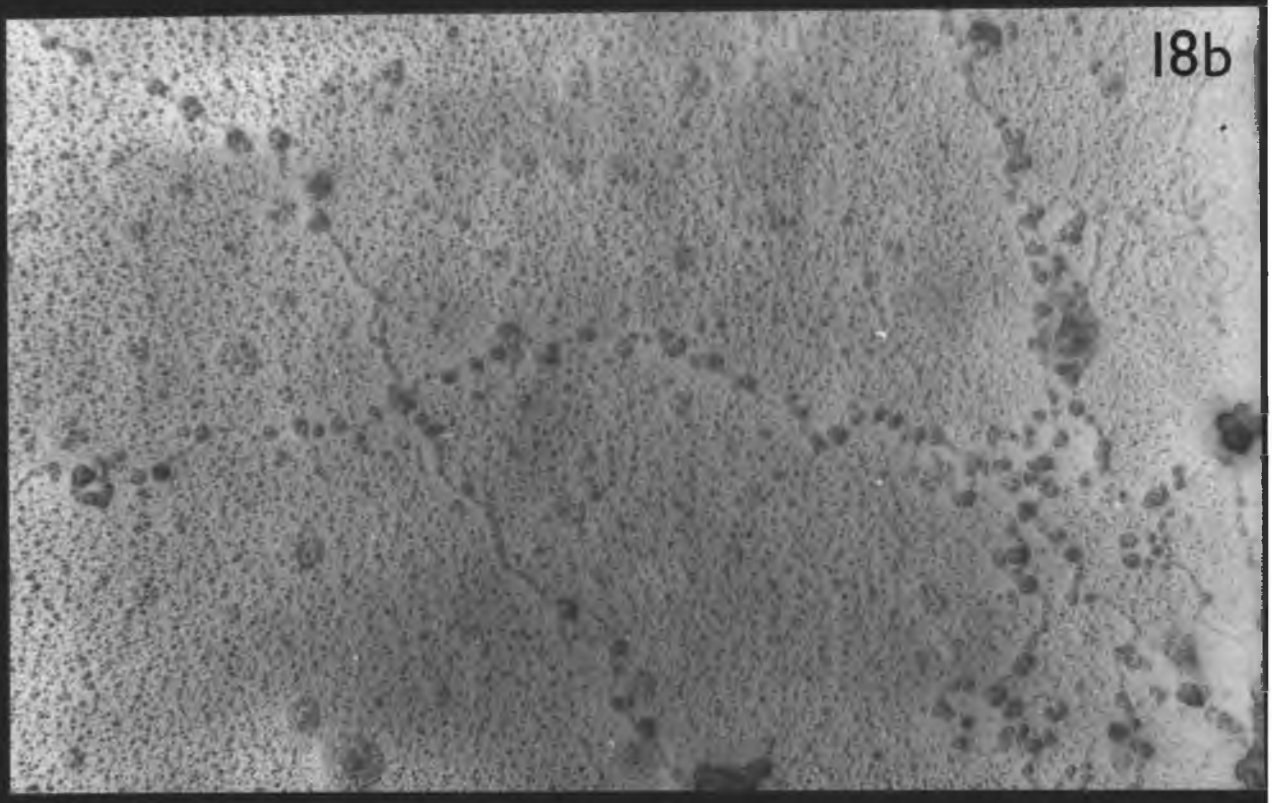

17a
0·5μ


17b

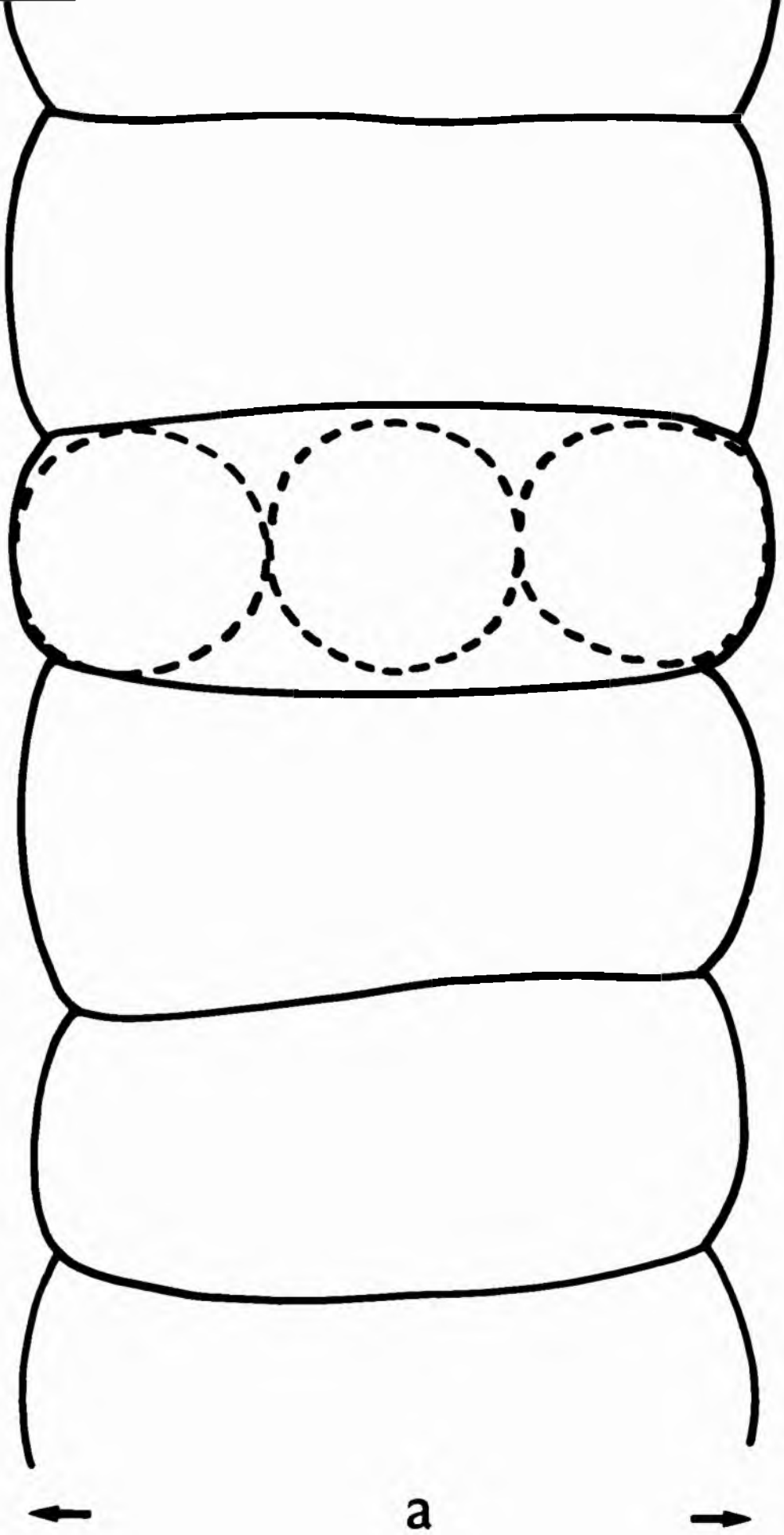
I - 18a As Figs I - 17 a-b. Arrows denote regions of the "cables" having a double structure. These double regions are connected by chromatin having a segmented structure of similar appearance and dimensions to those presented in Figs I - 17 a-b.

I - 18b Nucleosomal DNP from the same preparation as the one in which the "cables" were found.


18a
0·5μ

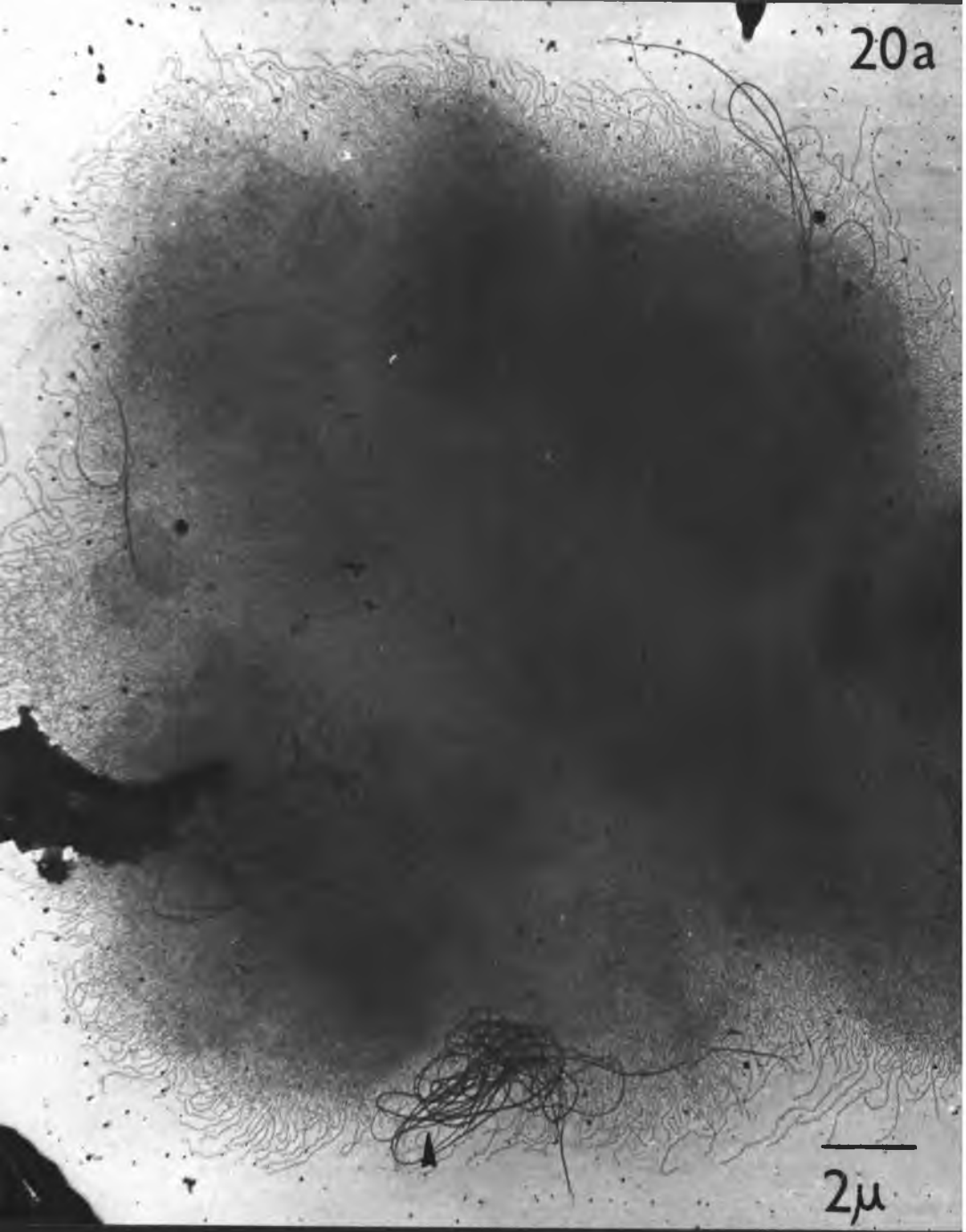

18b

I - 19 A diagrammatic representation of the "cables" found in one preparation of X. laevis cultured cells. The cables were approximately 29 nm wide (a) and consisted of segments 16 nm wide (b). The segments may consist of coiled nucleosomes, which were about 16 nm in diameter in this preparation.


a

I - 20a Compact chromatin clump probably consisting of an unspread Nonidet-P40 extracted T.c. carnifex cultured cell nucleus. The beaded structure of the chromatin is most evident at the periphery of the clump. Arrow denotes superimposed clumps probably consisting of collagen fibres. These were commonly associated with the chromatin.

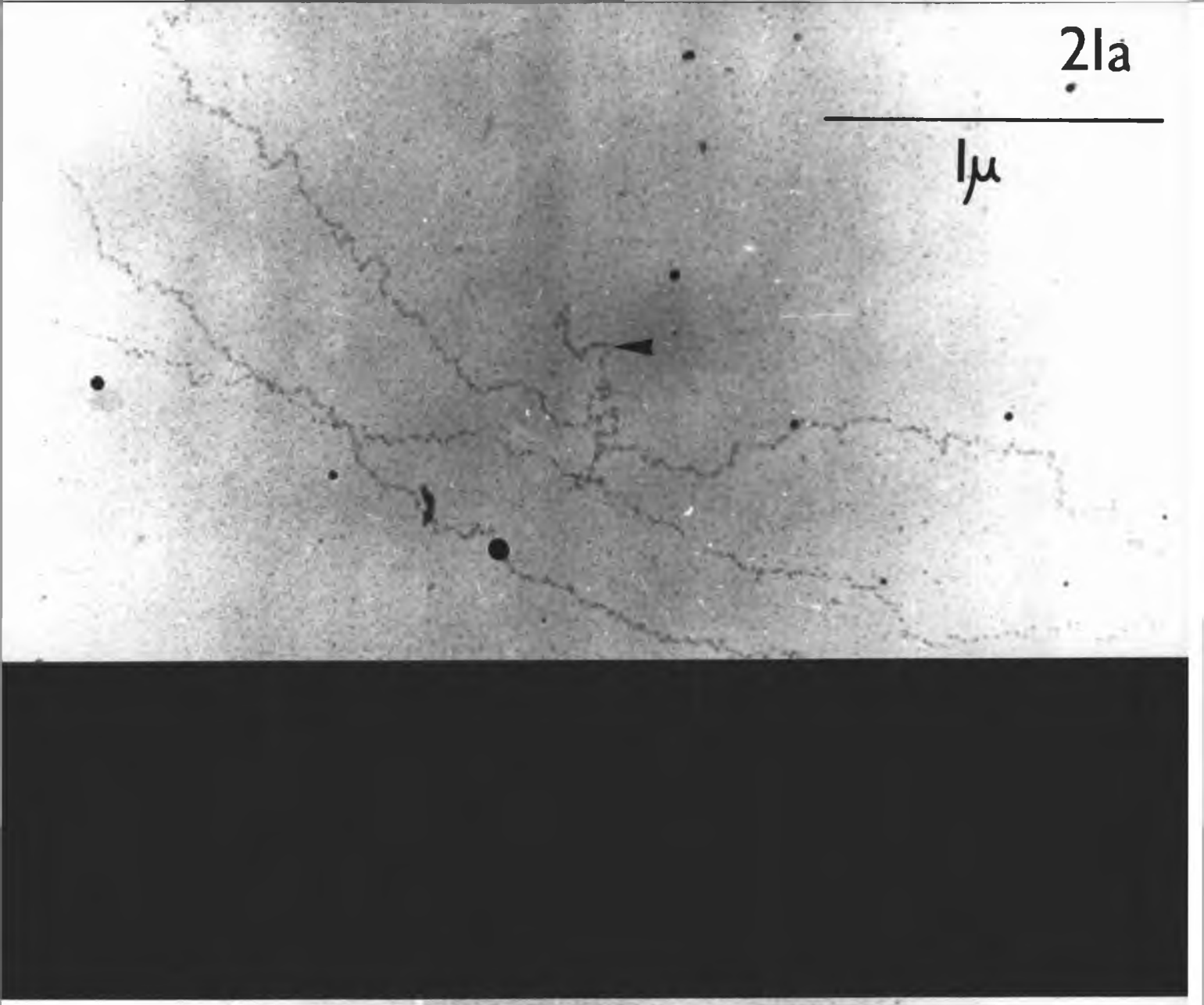
I - 20b High power photograph of the periphery of a chromatin clump similar to Fig. I - 20a. Arrow denotes halo consisting of low-contrast, non-nucleosomal DNP fibres radiating from the main mass of the chromatin.


20a
2μ


20b
2μ

I - 21a Arrow denotes an isolated primary transcript molecule found in a preparation of T.c. carnifex cultured cell chromatin. The transcript has a beaded structure, as does the DNP axis to which it is attached.

I - 21b As Fig. I - 21a


21a
1μ

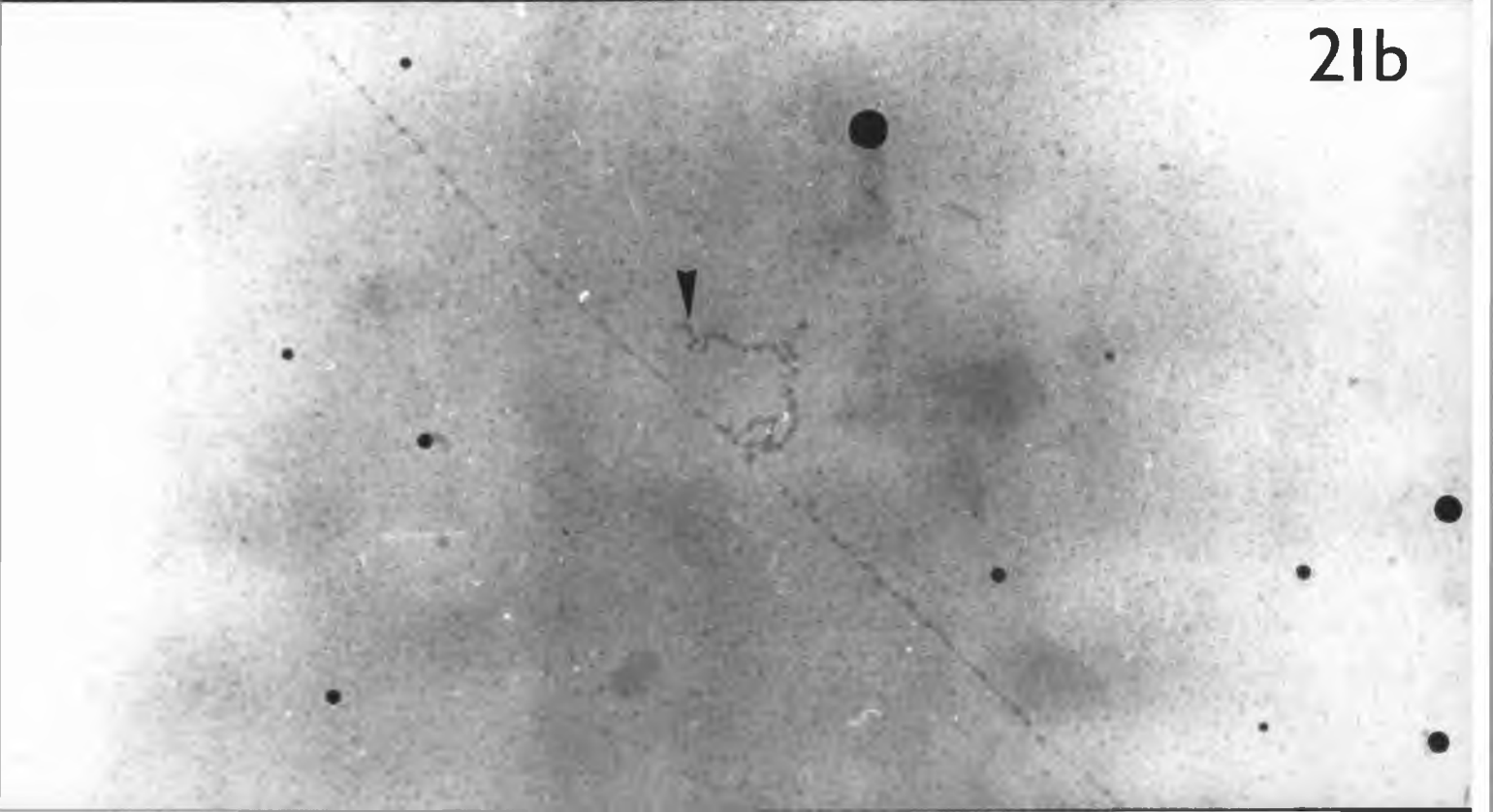
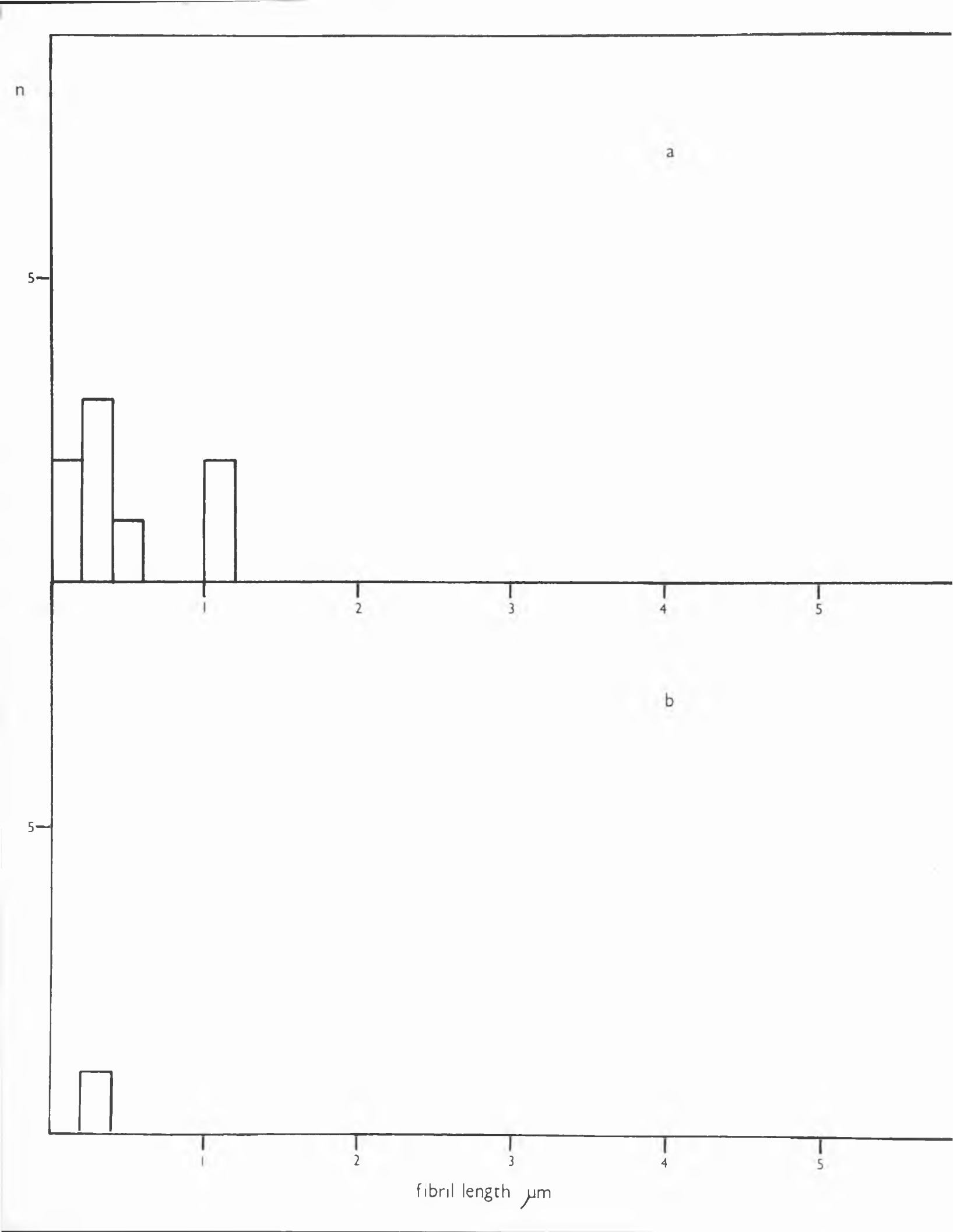

21b

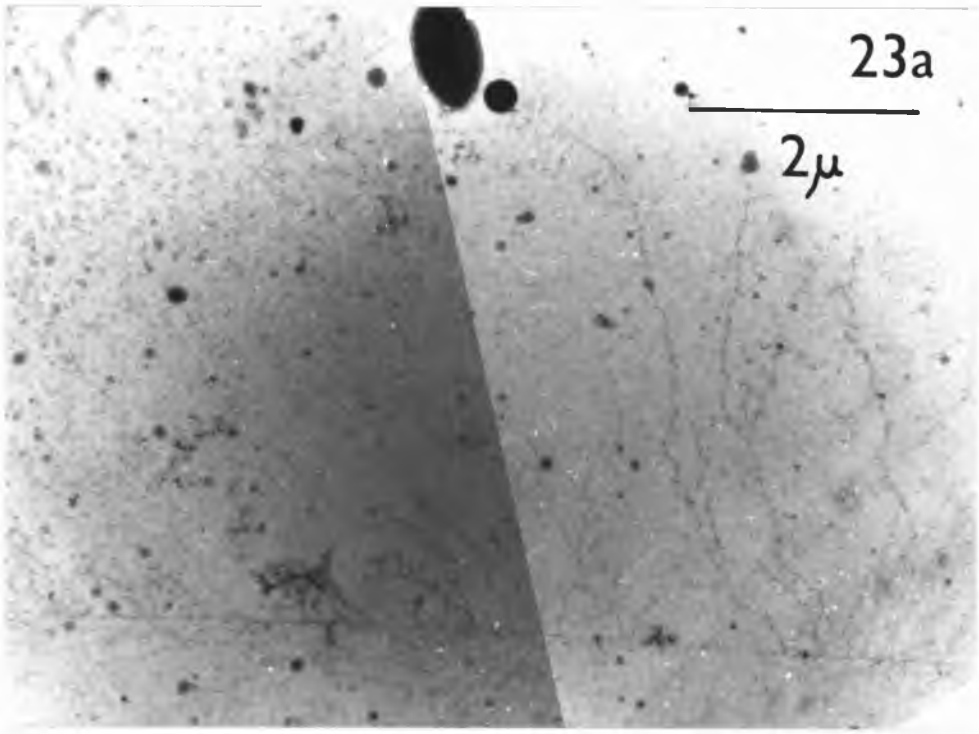
Fig. I - 22a Histogram showing the distribution of contour lengths of isolated RNP fibrils from T.c. carnifex cultured cell chromatin. Median value 0.33 μm. Range 1.03 μm.

Fig. I - 22b Histogram showing the distribution of contour lengths of the terminal RNP fibrils of transcription complexes consisting of two more adjacent transcripts from T.c. carnifex cultured cell chromatin. Median value 0.4 μm. Range 0.2 μm.

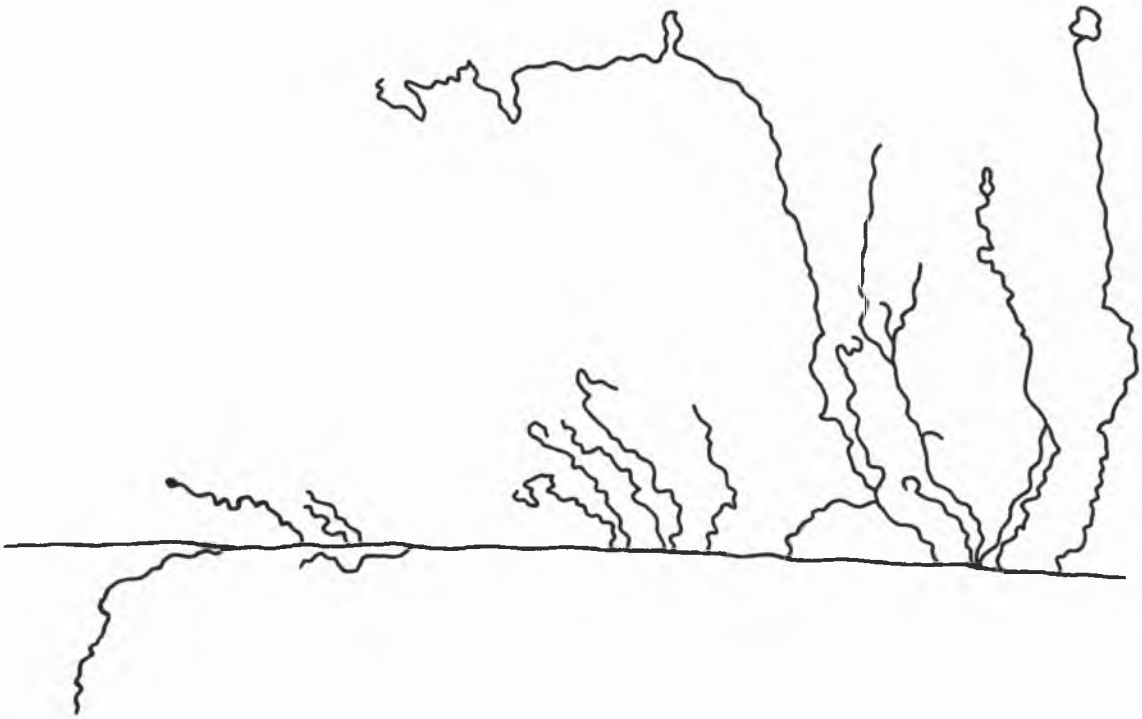

n
a
5
1
2
3
4
5
b
5
1
2
3
4
5
fibril length μm

I - 23a Low power photograph of a long transcription complex found in T.c. carnifex cultured cell chromatin. Most of the complex was obscured by chromatin and only the portion presented here was clear. The longest fibril measured 10.8 μm. Laird Analysis of this array was not possible.

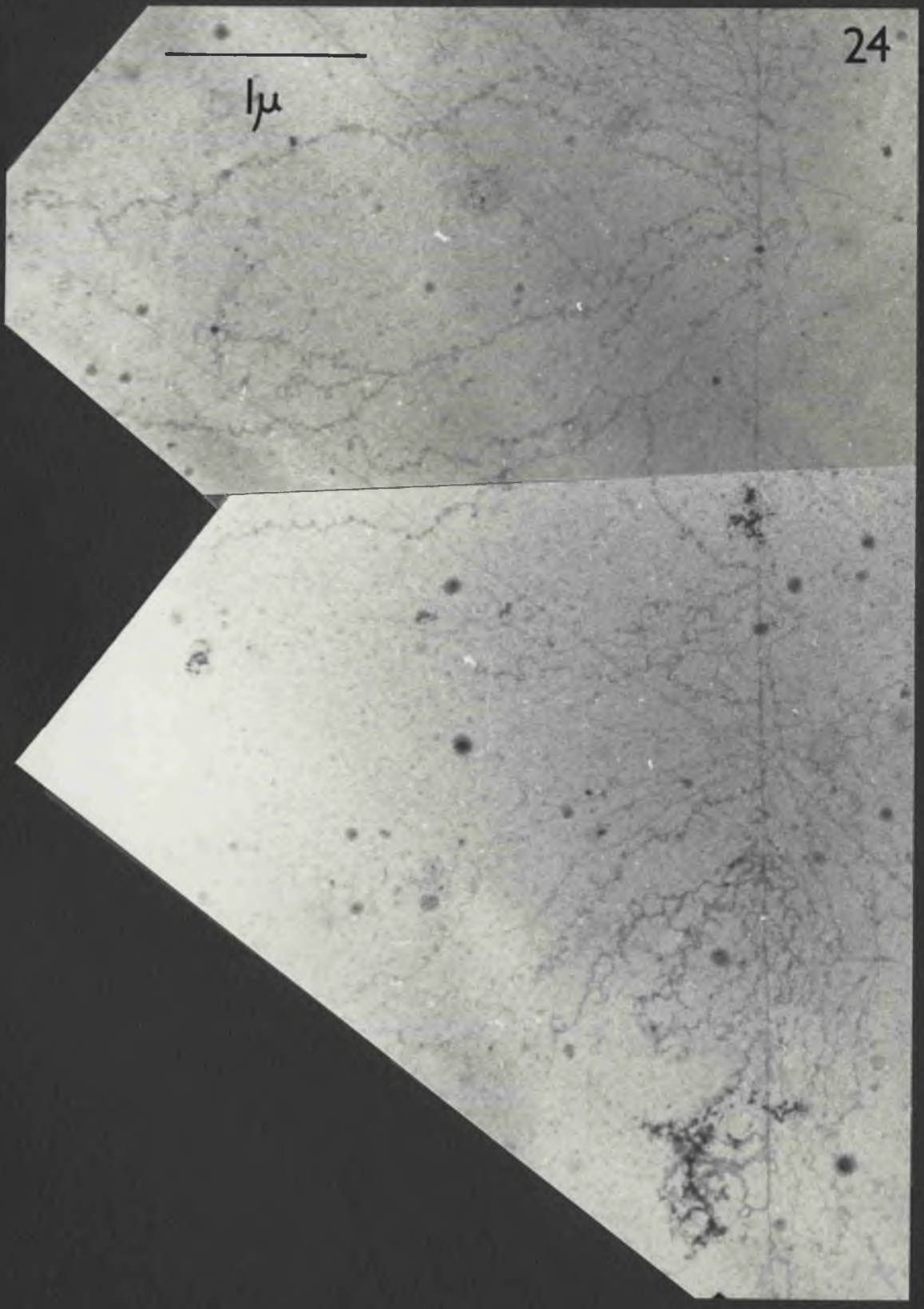
I - 23b Tracing of Fig I - 23a.



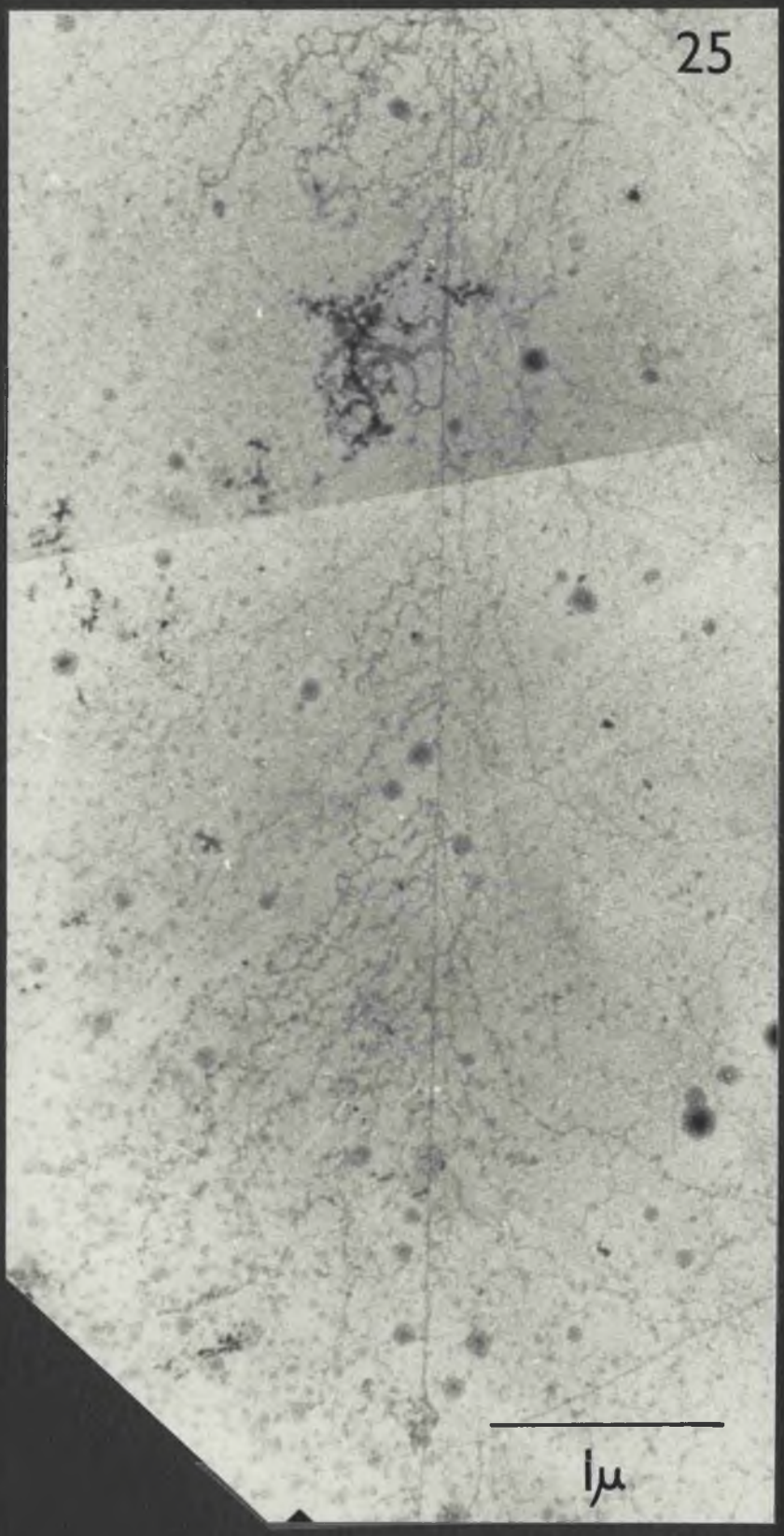
23b



I - 24 High power photograph of part of the transcription complex in Figs I - 23 a-b. The primary transcript 10.8 μm long (Fig. I - 23b) is seen to be adjacent to one only 2.7 μm long, which may indicate processing or breakage. The DNP axis of this complex is smooth, although its dimensions suggest association of the DNA with protein. This is in contrast to the attached RNP, which exhibits a diffusely beaded appearance.


1μ
24

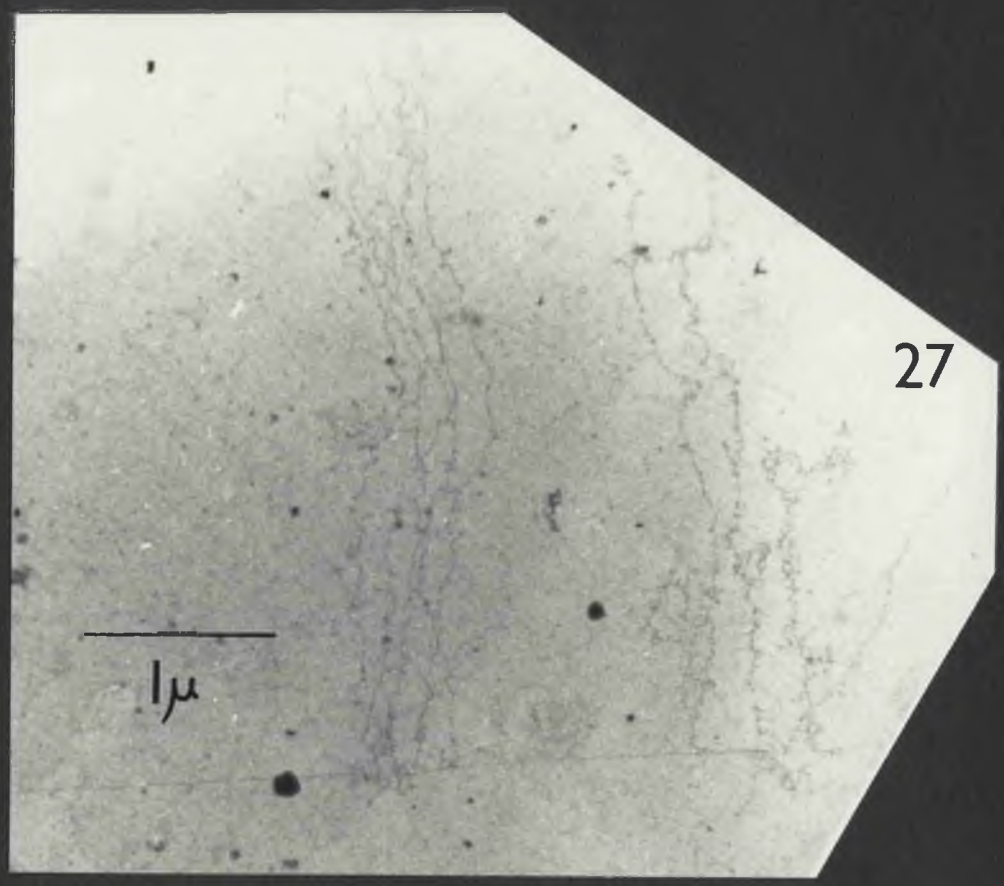
I - 25 Part of the array in Fig. I - 24. Groups of relatively short lateral fibrils separated by lengths of DNP lacking attached transcripts, are evident.


25
1μ

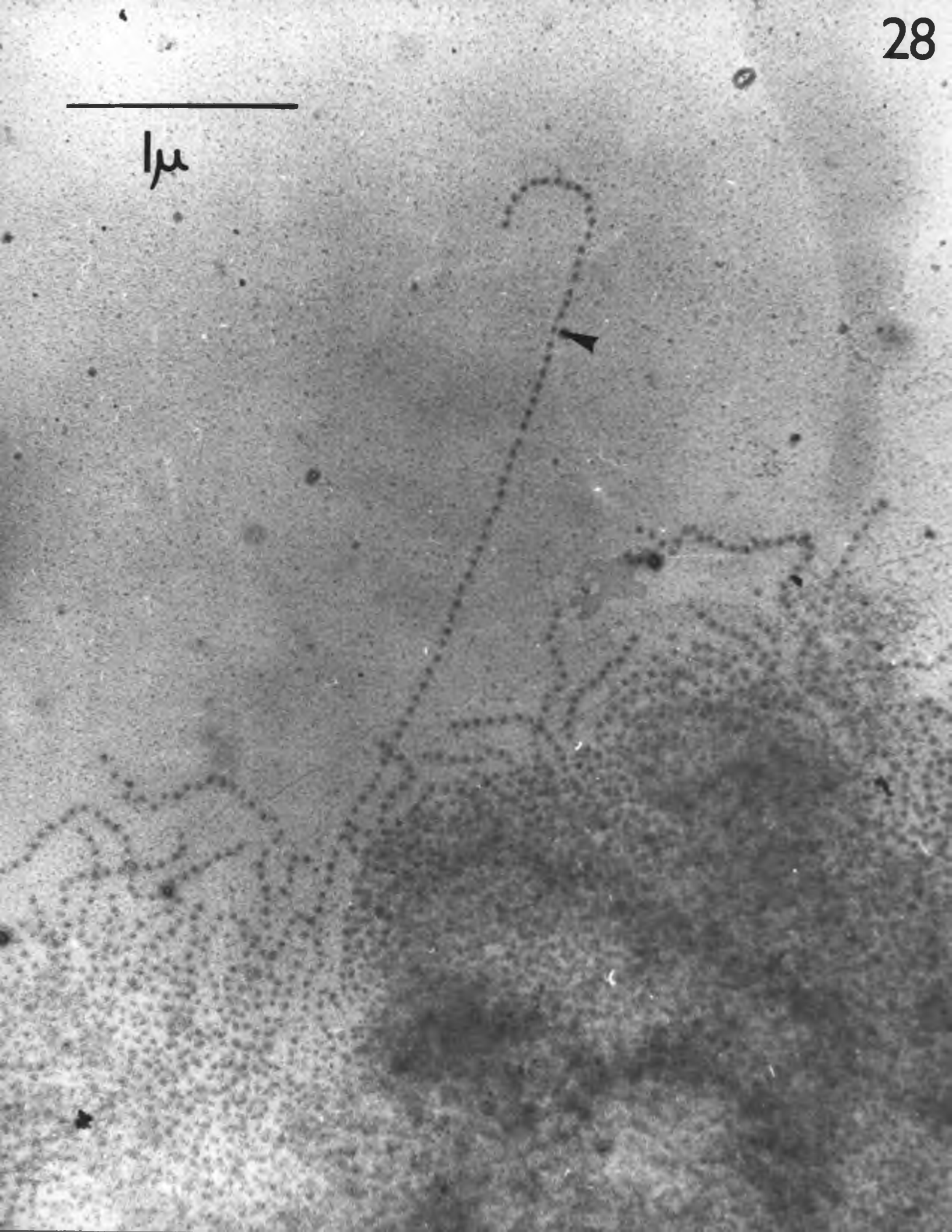
I - 26 A second long transcription complex found in chromatin of T.c. carnifex cultured cells. The ends of the lateral fibrils were not traceable so that their identity as RNP is not proven. However, fibrils (large arrow) of a size order seen in Figs I - 23 a-b, are present. Transcripts were arranged in blocks. The small arrow indicates a putative replicating region of this transcription complex. The DNP of this complex had a smooth appearance, though the dimensions of the axis suggested association of the DNA with protein.

1μ

I - 27 Part of the transcription complex shown in Fig. I - 26, showing the arrangement of the lateral fibrils in blocks. The ends of these RNP molecules were not traceable.


27
1μ

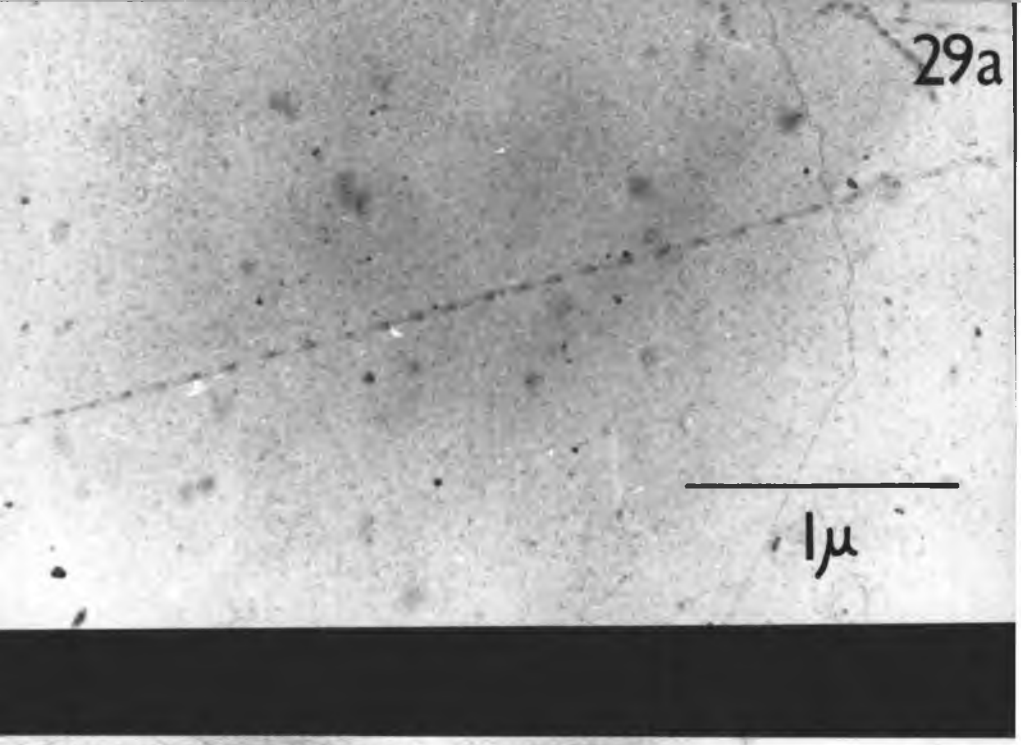
I - 28 Part of a chromatin clump consisting of a partially spread T.c. carnifex cultured cell nucleus, and consisting of beads 29 nm in diameter and probably analogous to the "superbeads" described in other systems. The arrow denotes a well displayed length of beads. A few low-contrast unbeaded RNP fibrils are also ivisble at the periphery of the clump.

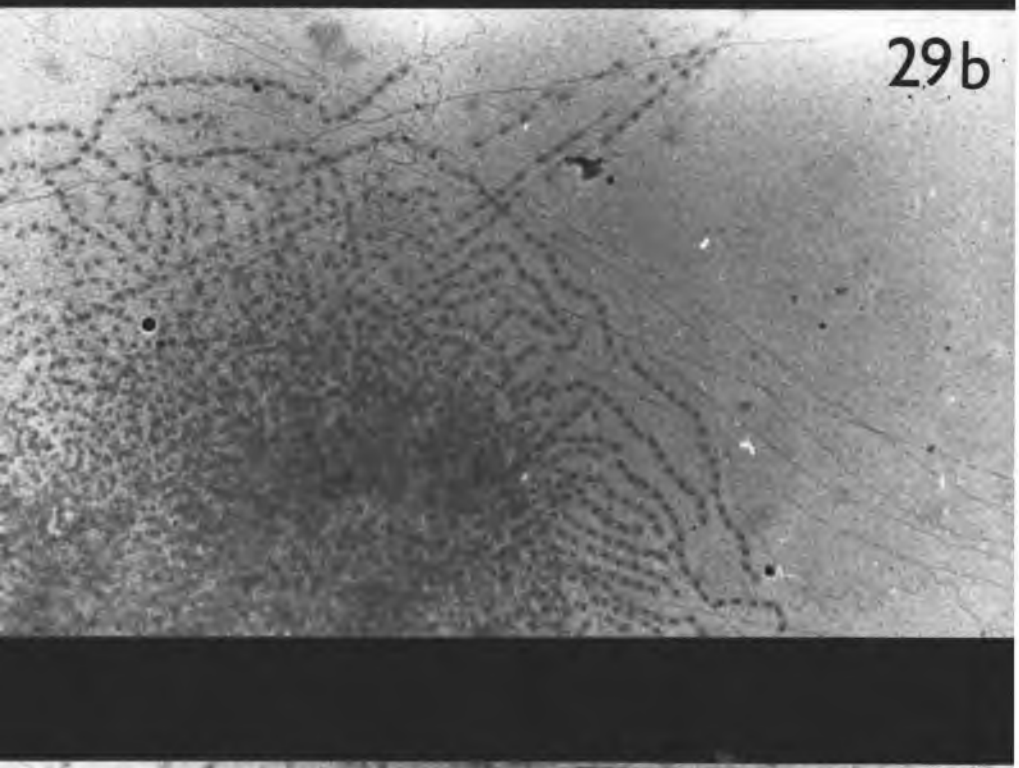

1μ

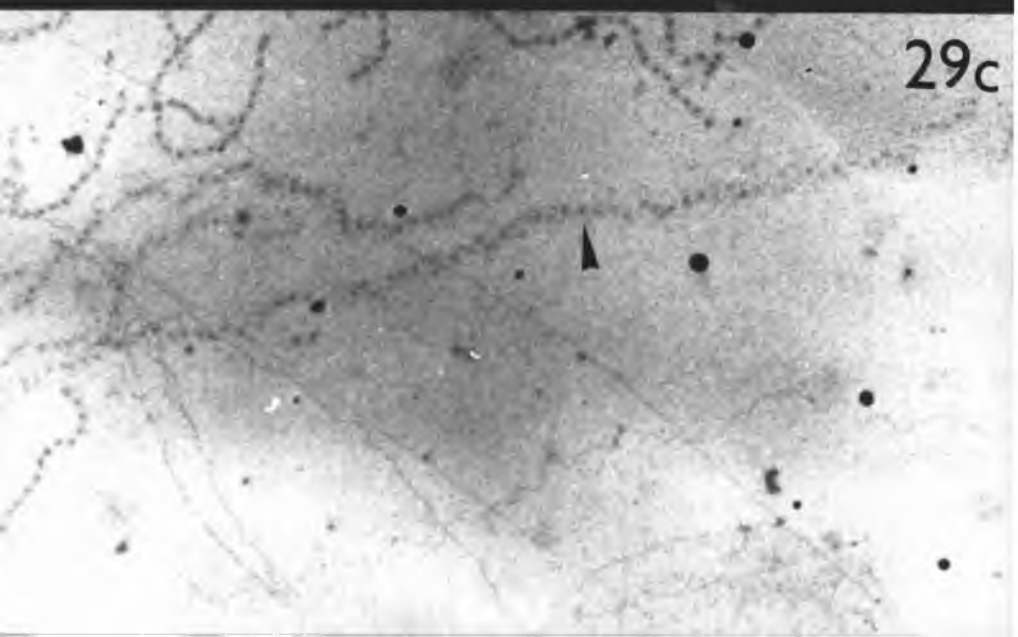
I - 29a A length of "stretched" superbeads from T.c. carnifex cultured cells, showing the diffuse appearance of these structures when stretched. No resolvable sub-structure was apparent.

I - 29b As Fig. I - 29a. Both beaded and smooth chromatin are present but there is no evidence of a clear transition from one conformation to the other.

I - 29c Arrow denotes a structure resembling a partially unwound spiral of nucleosomes. The size of the beads (13 - 19 nm) suggests that they may be nucleosomes but there is no evidence of their association to form clumps perhaps analogous to the superbeads in Figs I - 28 and I - 29 a-b.

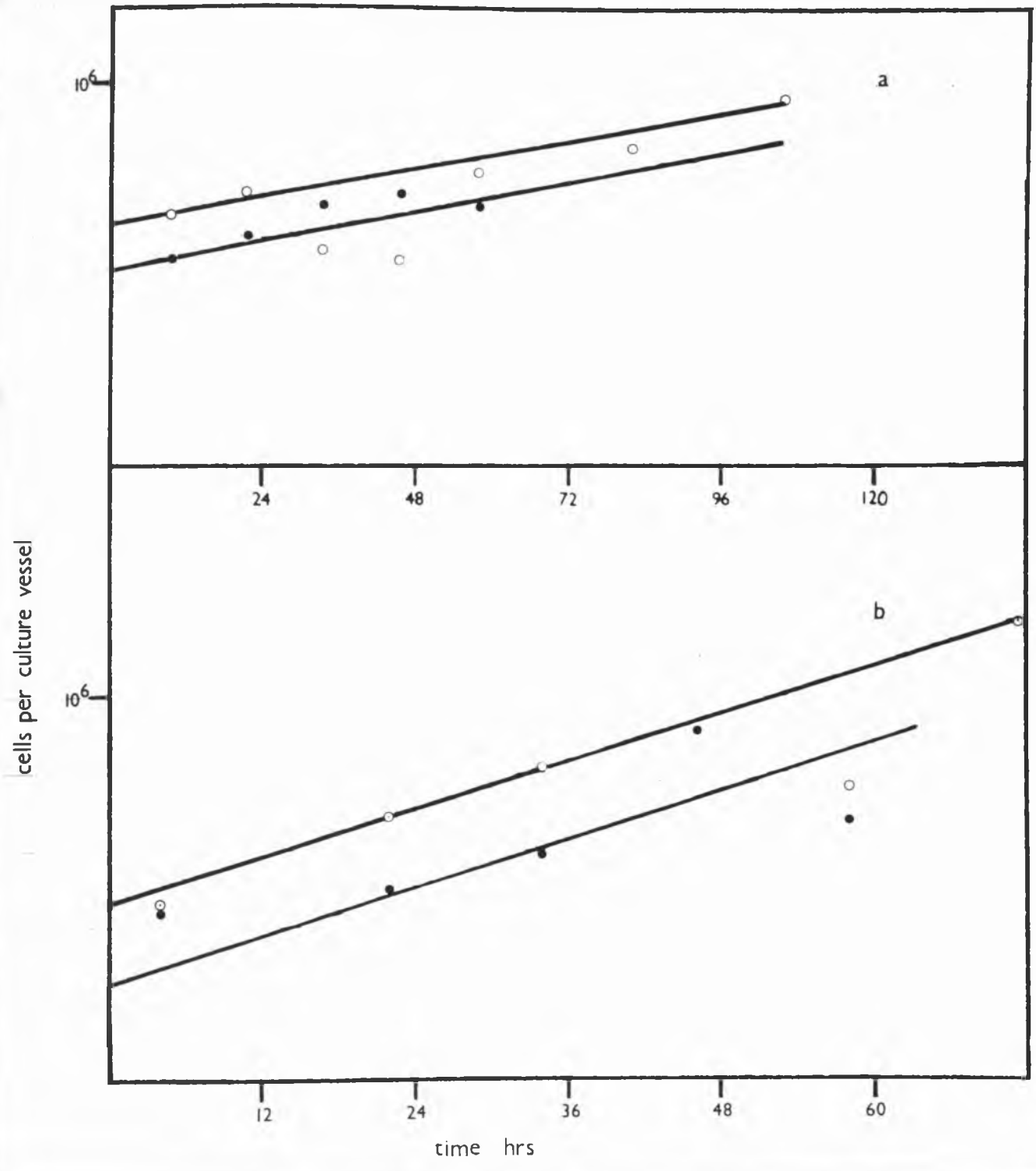

29a
1μ


29b

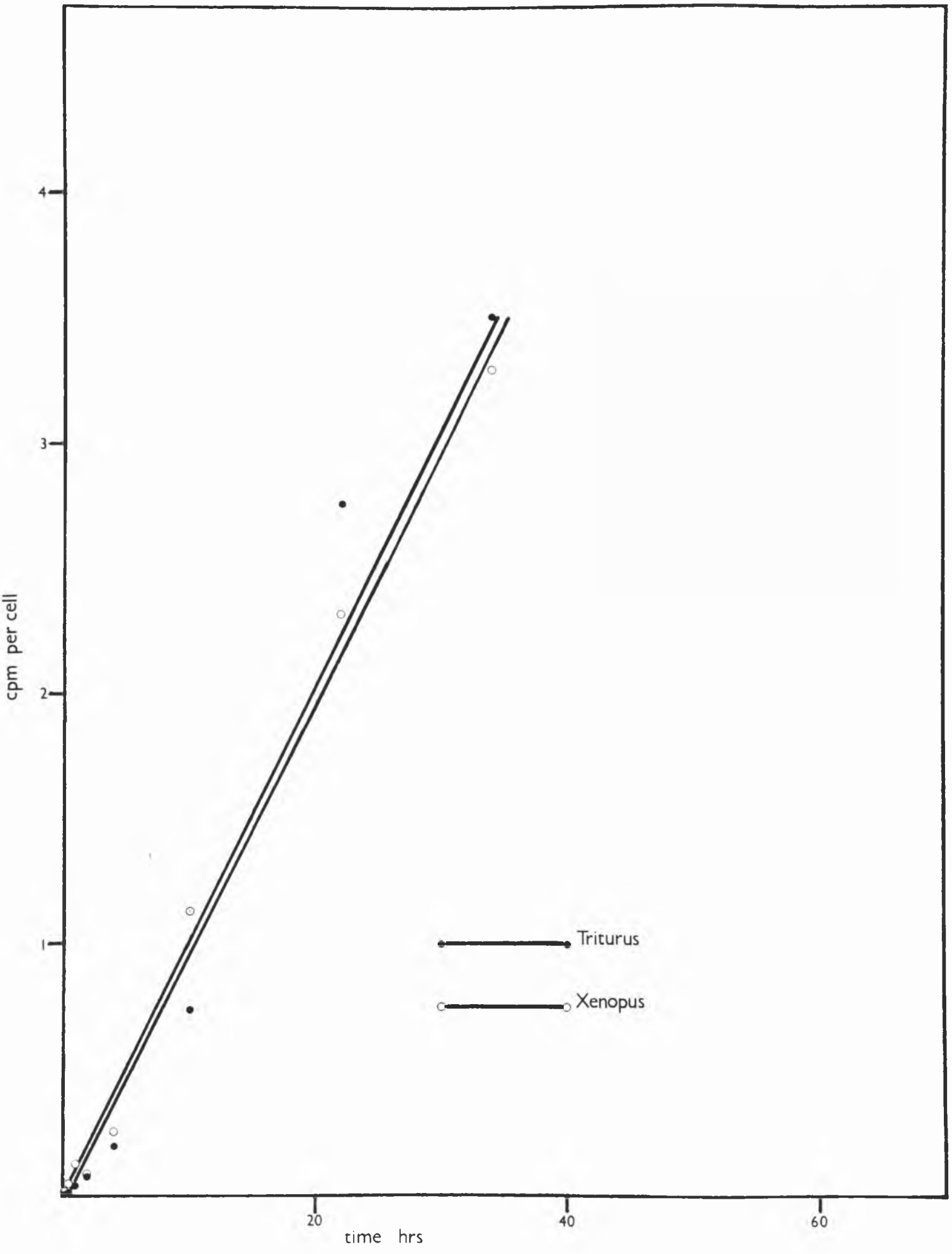

29c

I - 30a Growth rates of T.c. carnifex cultured cells. The upper slope represents the rate of increase in cell number of the unlabelled (control) culture. Tau is approximately 94.2 hr.

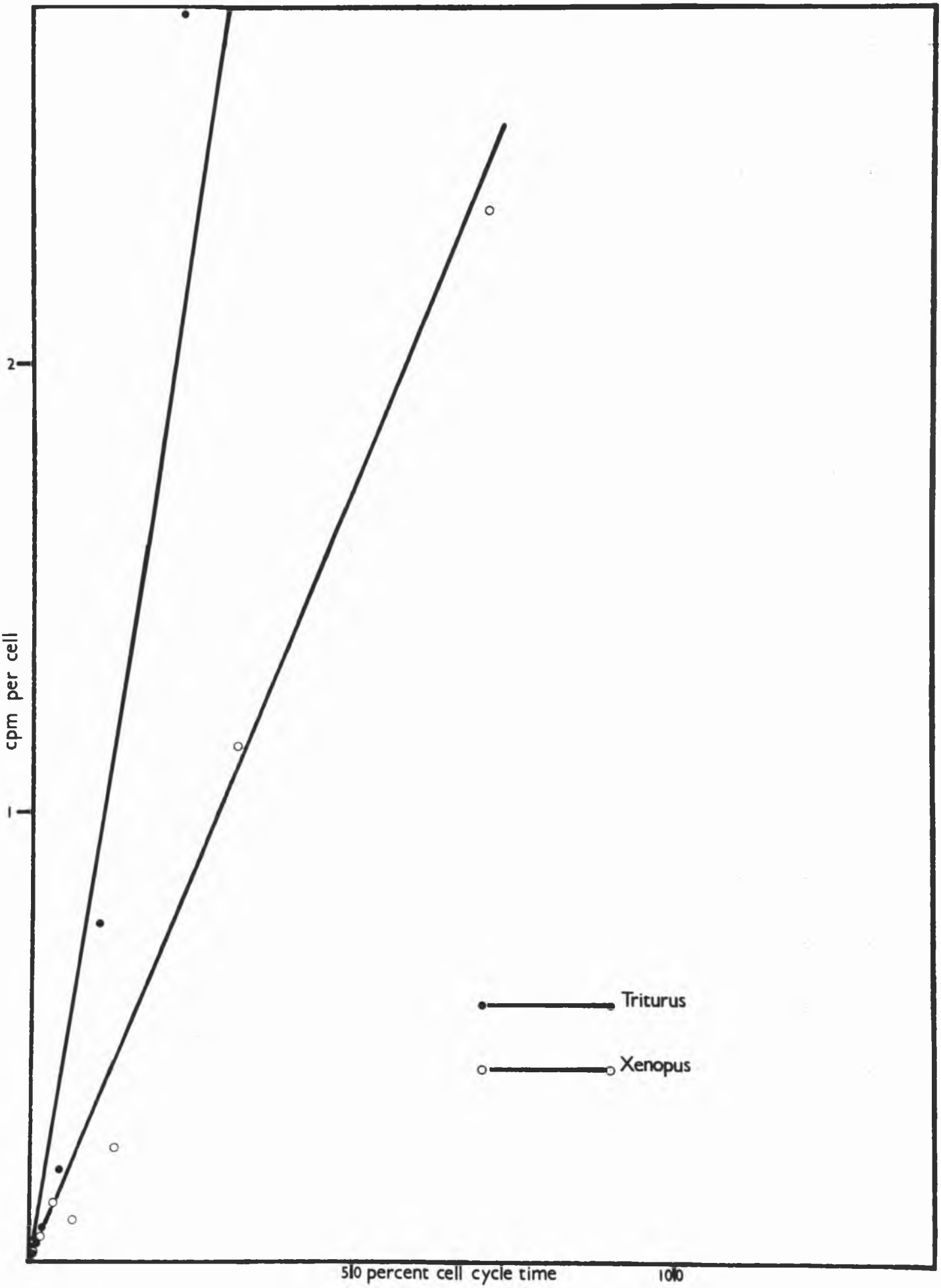
I - 30b Growth rates of X. laevis cultured cells. The upper slope represents the rate of increase in cell number of the unlabelled (control) culture. Tau is approximately 30.6 hr.


a
10^6
24
48
72
96
120
b
10^6
12
24
36
48
60
cells per culture vessel
time hrs

I - 31 Graph representing the rate of incorporation of ^{3}H-uridine per cell with time of cultured cells of T.c. carnifex and X. laevis. The rates of incorporation are similar for both cultures. The graph represents incorporation into total cellular RNA.

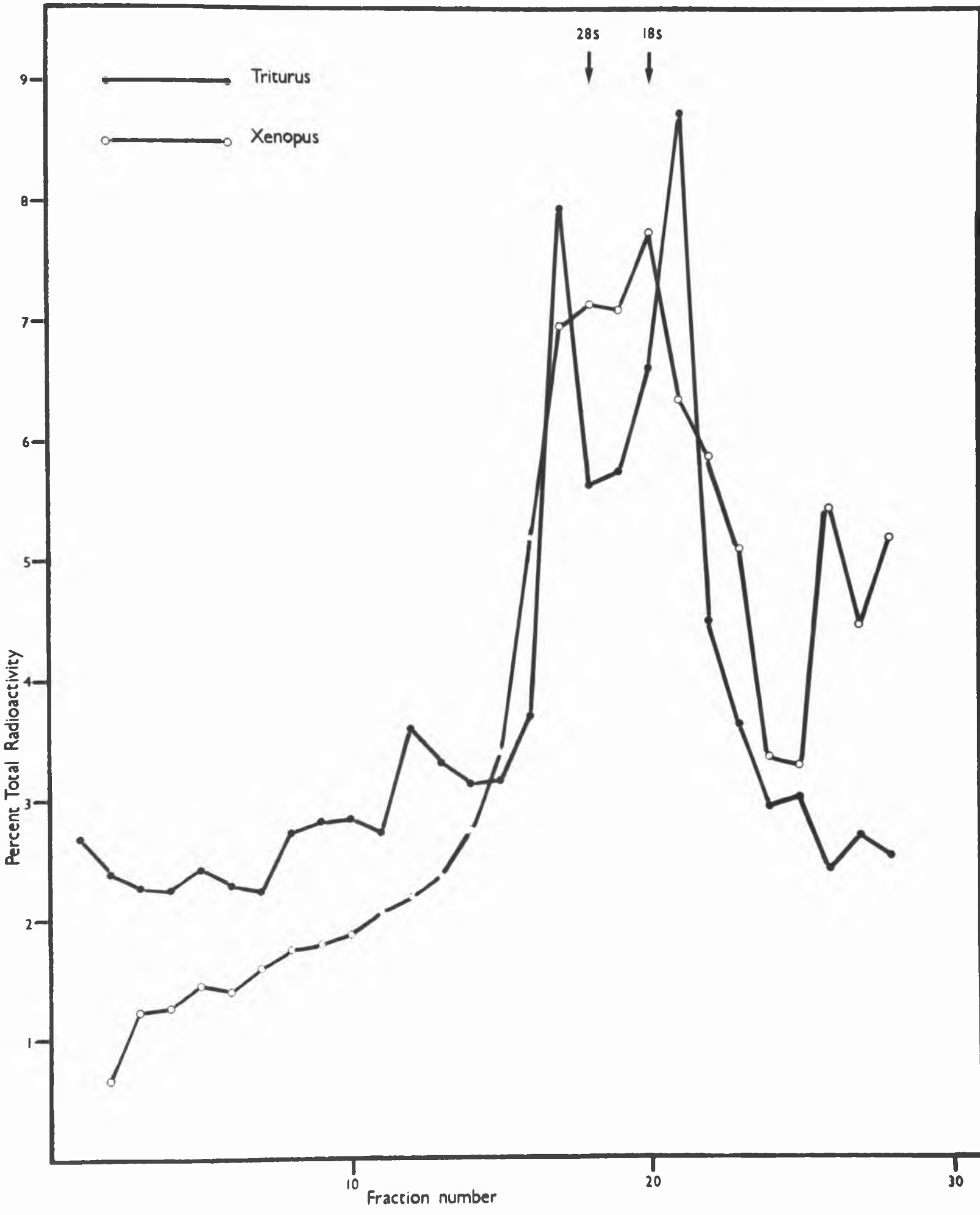

4
3
2
1
cpm per cell
20
40
60
time hrs
Triturus
Xenopus

I - 32 Graph representing the rate of incorporation of ^{3}H-uridine per cell per 1% cell cycle time of cultured cells of T.c. carnifex and X. laevis. T.c. carnifex incorporates label nearly four times faster than X. laevis. The graph represents incorporation into total cellular RNA.

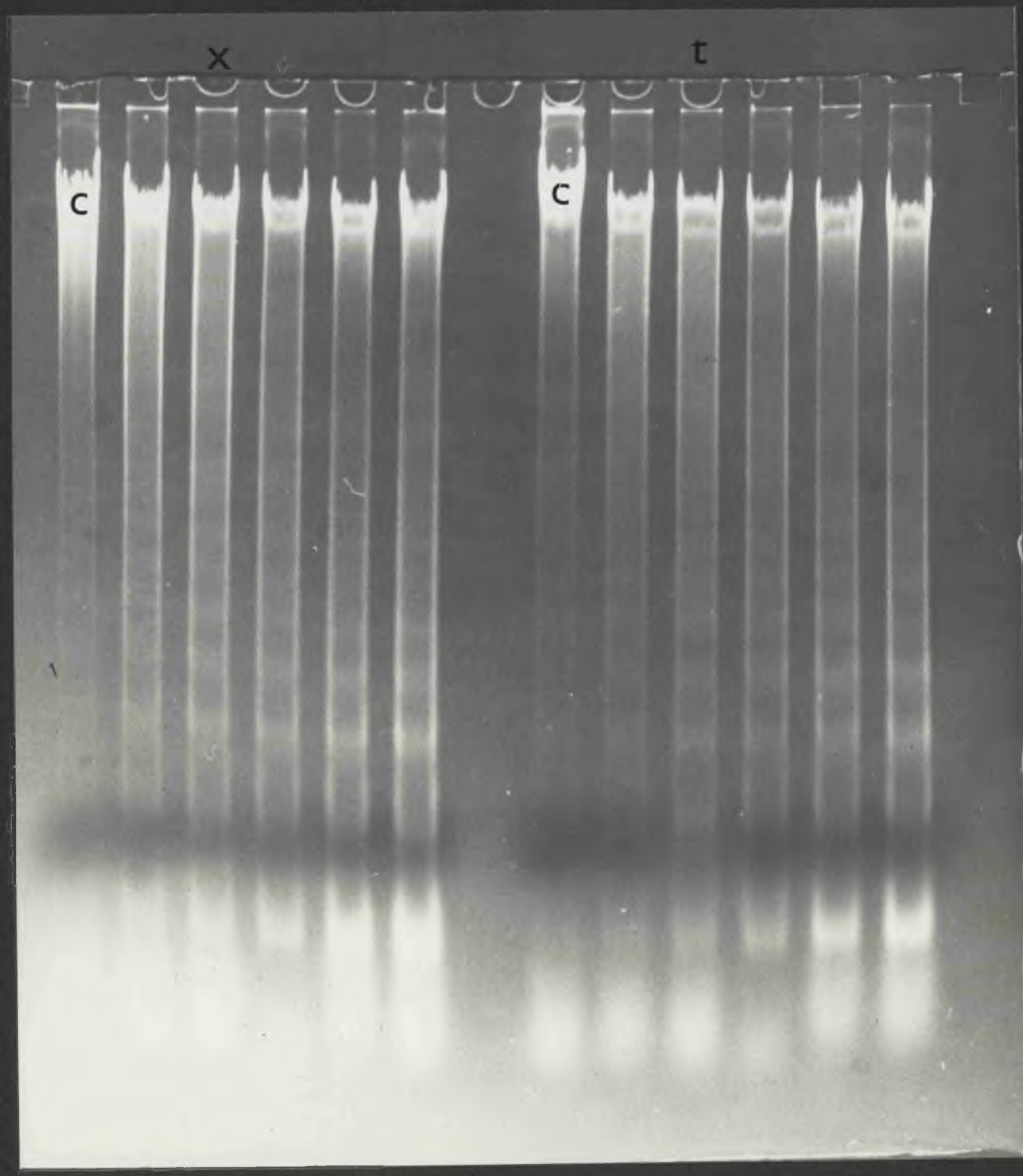

2
1
cpm per cell
Triturus
Xenopus
5|0 percent cell cycle time
10|0

I - 33

Figure demonstrating the distribution of ^{3}H-uridine-labelled whole-cell RNA from T.c. carnifex and X. laevis cultured cells on a sucrose gradient. A greater proportion of labelled RNA is of higher molecular weight in T.c. carnifex than in X. laevis. The arrows denote the approximate position of the 28S and 18S pre-ribosomal RNAs, which were used as molecular weight markers. Their position was determined by making a trace of ultraviolet absorbance.


28s
18s
Triturus
Xenopus
9
8
7
6
5
4
3
2
1
Percent Total Radioactivity
10
20
30
Fraction number

I - 34 The banding pattern on an agarose gel of spermidine extracted DNA from X. laevis (X) and T.c. carnifex (T) cultured cells, after treatment with bleomycin. The banding pattern is typical of the different sized aggregates of nucleosomes resulting from bleomycin treatment of chromatin. In the absence of antibiotic (channel XC or TC) all the fluorescence is at the high molecular weight end of the gel, showing that little or no autodigestion of the chromatin occurs.


X
t
C
C

I - 35 The banding pattern on an agarose gel of Miller spread chromatin extracted from X. laevis (X) and T.c. carnifex (T) cultured cells, after treatment with bleomycin. Miller spreading disrupts nucleosome structure such that bleomycin can now break the DNA into small fragments that run off the gel. Thus no fluorescence is visible on the test channels. The untreated control (XC and TC) showed fluorescence at the high molecular weight end of the gel.

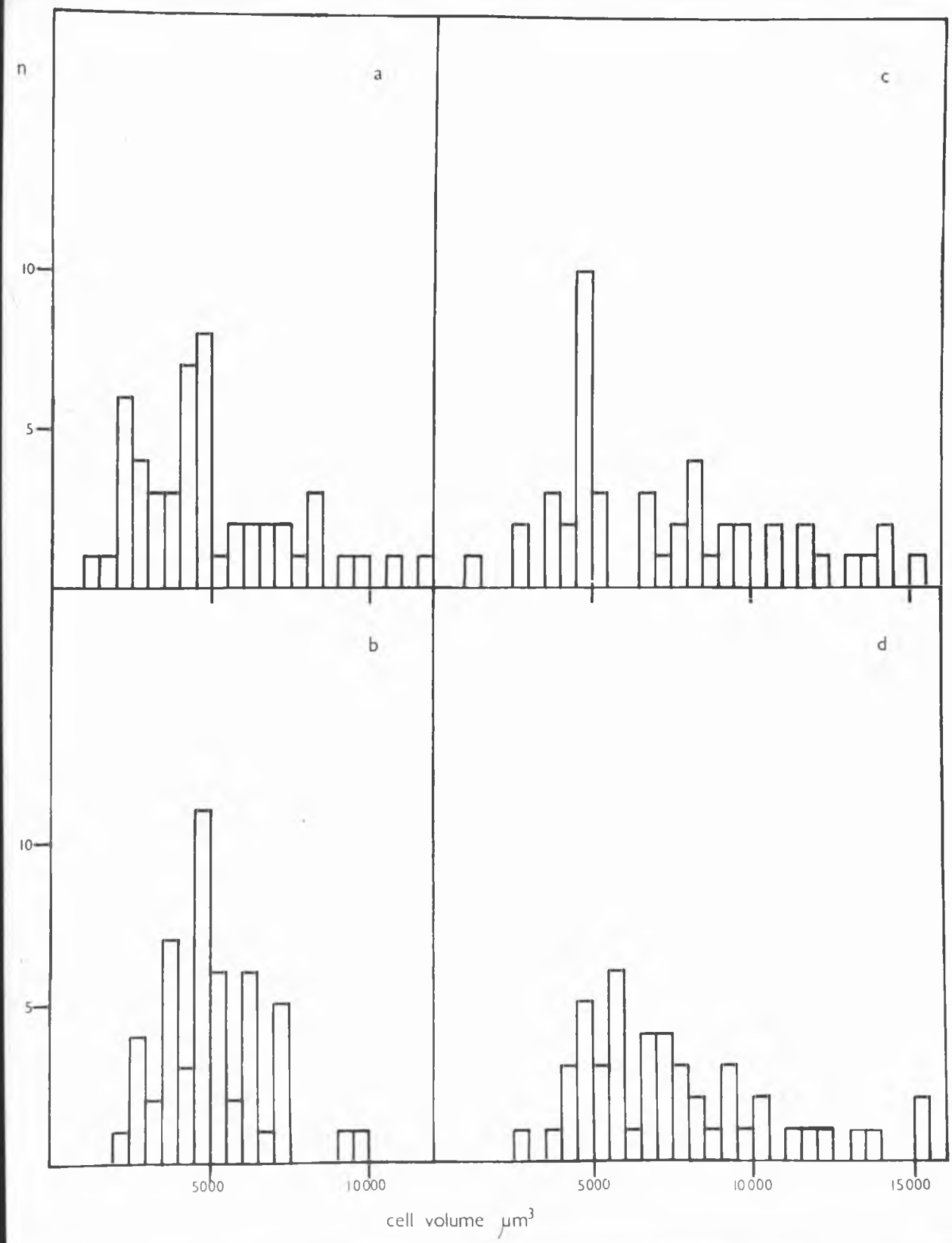

X
t
c
c

I - 36a Histogram to show the distribution of cell volumes in X. laevis cultured cells grown at high inoculation density, in the absence of cortisol. Median value 4491.7μ^3. Variance 8%

I - 36b As Fig. I - 36a. Cells grown in the presence of 20 μg/ml cortisol. Median value 4919.2μ^3. Variance 5.3%.

I - 36c Histogram to show the distribution of cell volumes, in X. laevis cultured cells grown at low inoculation density, in the absence of cortisol. Median value 7467.6μ^3. Variance 19%.

I - 36d As Fig. I - 36c. Cells grown in the presence of 20 μg/ml cortisol. Mean value 7184.4μ^3. Variance 18%.

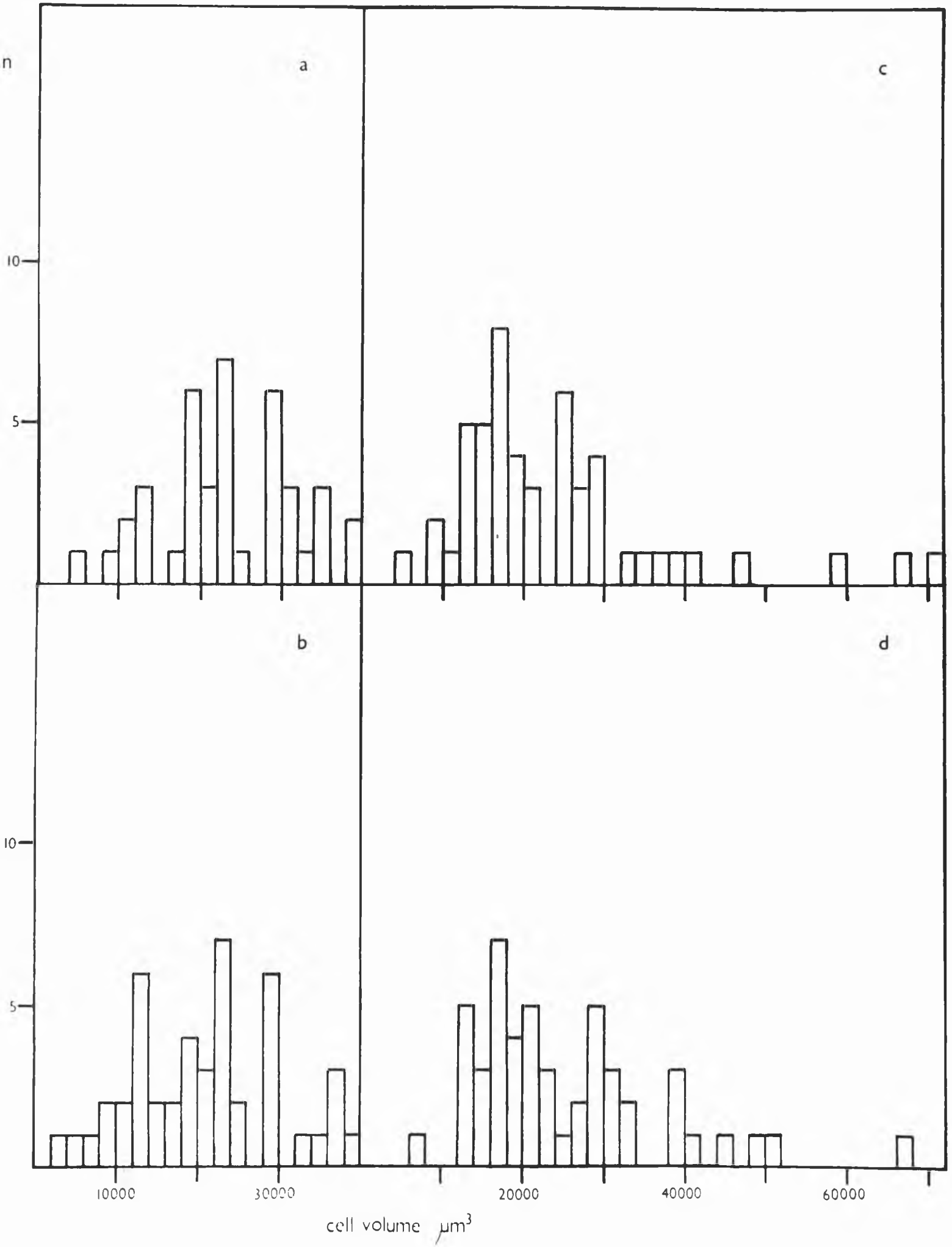

n
a
c
10
5
b
d
10
5
5000
10000
5000
10000
15000
cell volume µm3

I - 37a Histogram to show the distribution of cell volumes in T.c. carnifex cultured cells grown at high inoculation density in the absence of cortisol. Median value 26914μ^3. Variance 14%.

I - 37b As Fig. I - 37a. Cells grown in the presence of 20 µg/ml cortisol. Median value 22741μ^3. Variance 8%.

I - 37c Histogram to show the distribution of cell volumes in T.c. carnifex cultured cells grown at low inoculation density in the absence of cortisol. Median value 19862.7μ^3. Variance 11%.

I - 37d As Fig. I - 37c. Cells grown in the presence of 20 µg/ml cortisol. Median value 21978μ^3. Variance 11.8%.

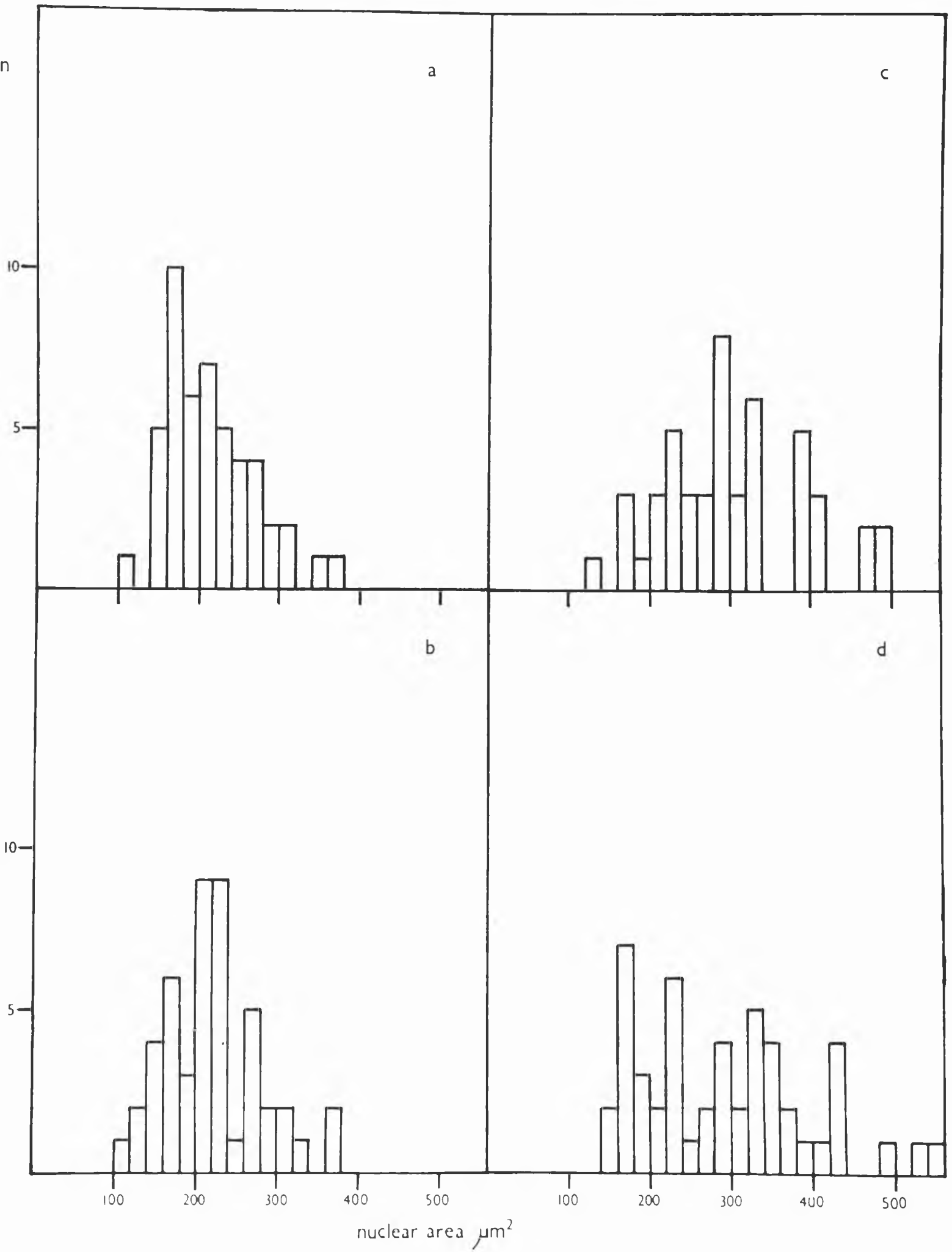

n
a
c
10
5
b
d
10
5
10000
30000
20000
40000
60000
cell volume μm³

I - 38a Histogram to show the distribution of nuclear cross-sectional areas in X. laevis cultured cells grown at high inoculation density, in the absence of cortisol. Median value 205.1μ^2. Variance 6%.

I - 38b As Fig. I - 38a. Cells grown in the presence of 20 µg/ml cortisol. Median value 215.9μ^2. Variance 4.7%.

I - 38c Histogram to show the distribution of nuclear cross-sectional areas in X. laevis cultured cells grown at low inoculation density, in the absence of cortisol. Median value 299.3μ^2. Variance 4.2%.

I - 38d As Fig. I - 38c. Cells grown in the presence of 20 µg/ml cortisol. Median value 286.9μ^2. Variance 5.3%.

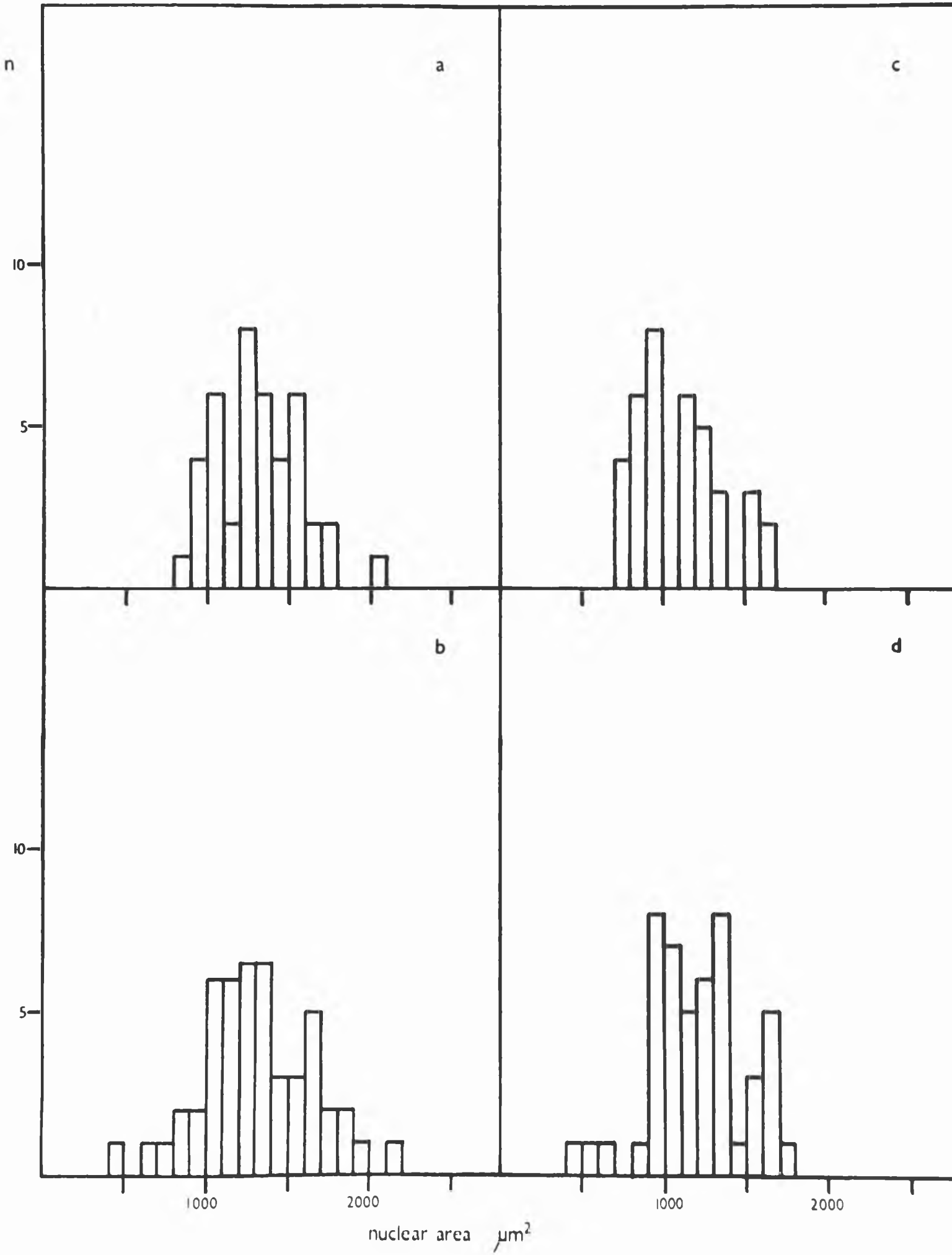

n
a
c
10
5
b
d
10
5
100
200
300
400
500
100
200
300
400
500
nuclear area μm²

I - 39a Histogram to show the distribution of nuclear cross-sectional areas in T.c. carnifex cultured cells grown at high inoculation density, in the absence of cortisol. Median value 1385.6μ^2. Variance 9%.

I - 39b As Fig. I - 39a. Cells grown in the presence of 20 μg/ml cortisol. Median value 1273.8μ^2. Variance 4%.

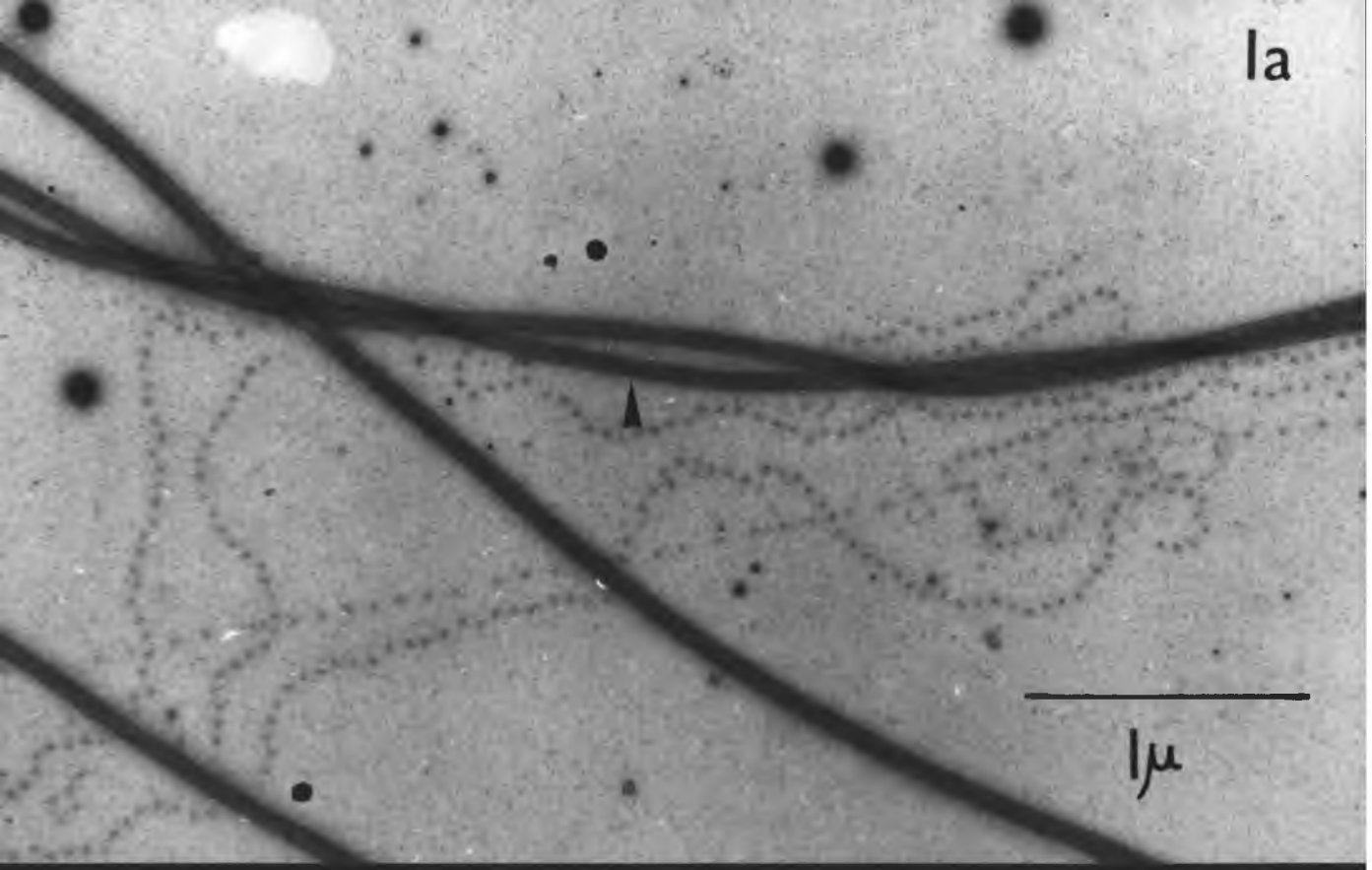
I - 39c Histogram, to show the distribution of nuclear cross-sectional areas in T.c. carnifex cultured cells grown at low inoculation density, in the absence of cortisol. Median value 1060.3μ^2. Variance 5.9%.

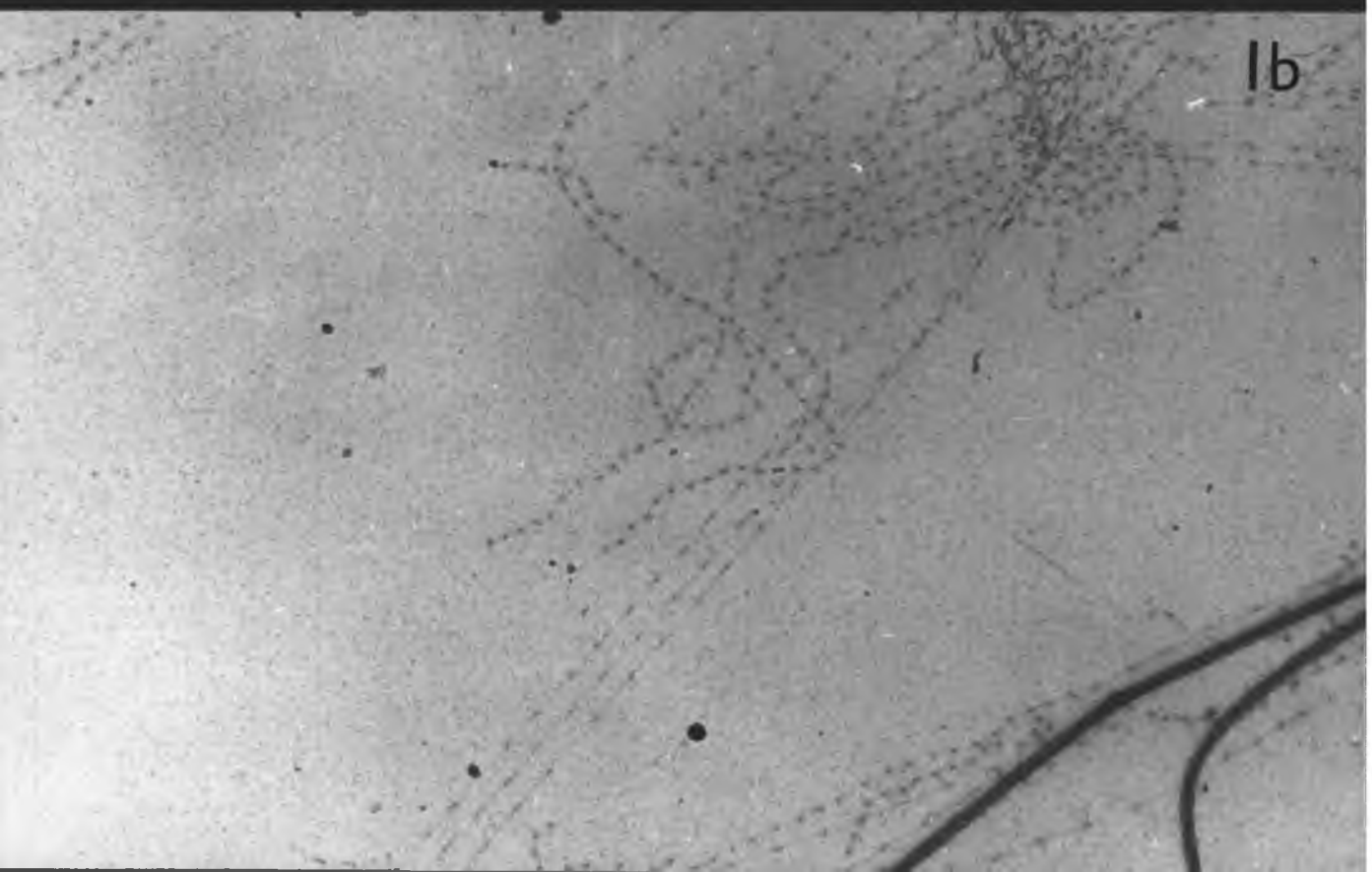
I - 39d As Fig. I - 39c. Cells grown in the presence of 20 μg/ml cortisol. Median value 1243.6μ^3. Variance 4.3%.


n
a
c
10
5
b
d
10
5
1000
2000
1000
2000
nuclear area μm²

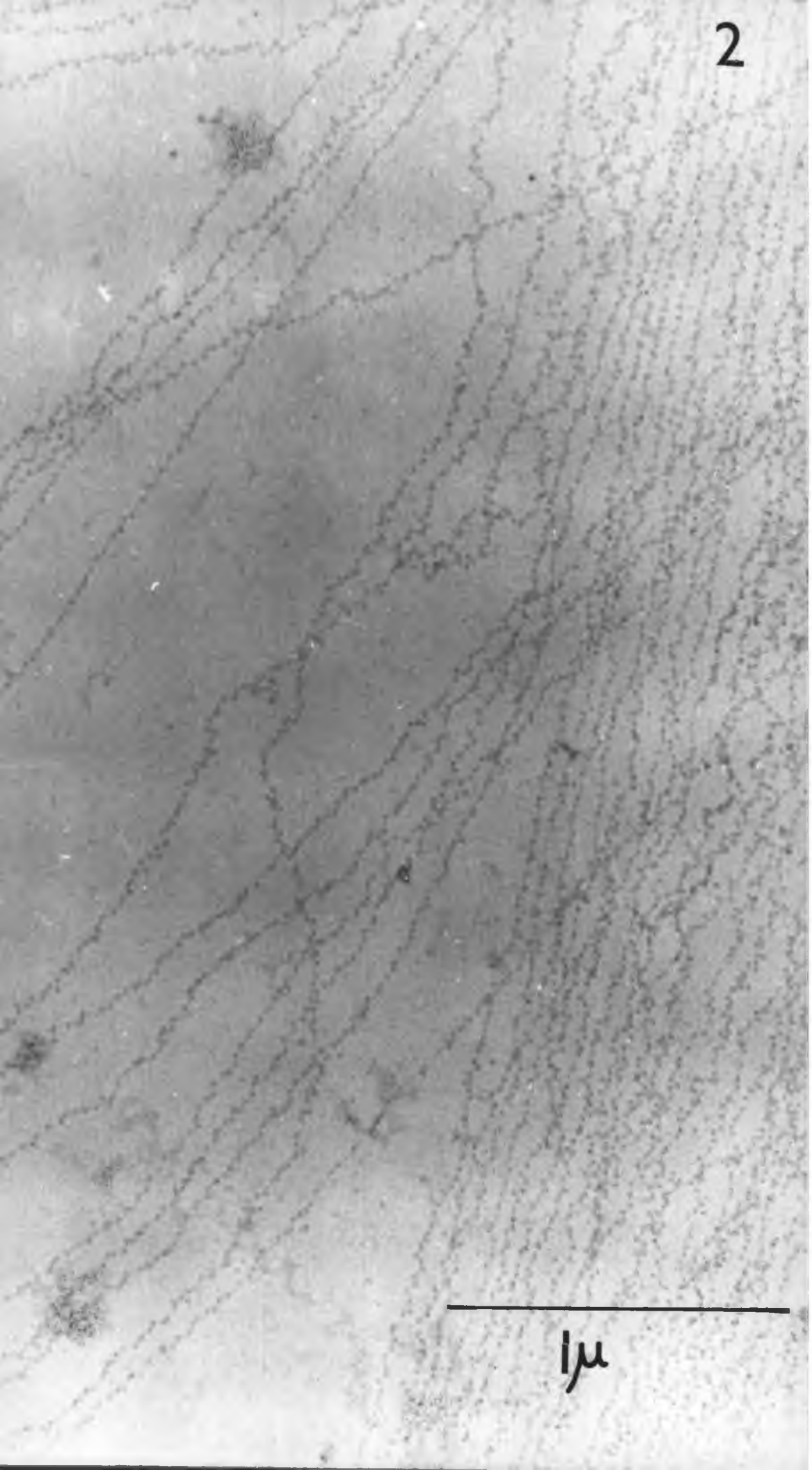
II - 1a Superbeads found in a preparation of X. laevis liver cell chromatin derived from cells at the periphery of the liver. Arrow denotes fibrils showing a repeating structure, and probably composed of collagen.

II - 1b As Fig. II - 1a. Stretched superbeads lacking a clearly observable substructure.


Ia
1μ

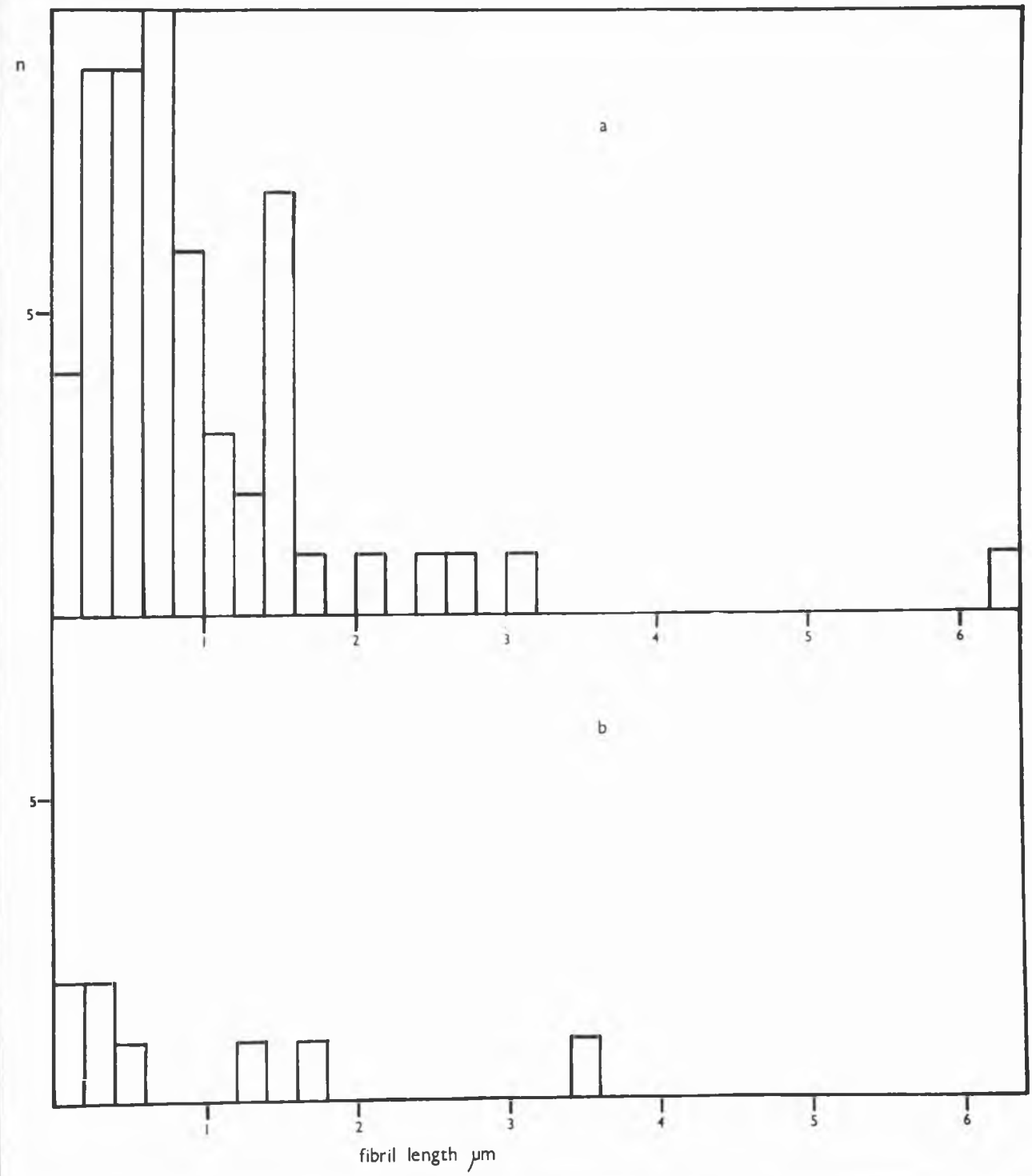

Ib

II - 2 Nucleosomal chromatin found in a preparation of *X. laevis* liver cells taken from deeper within the liver. The internucleosomal DNP is not clearly visible due to the close packing of the nucleosomes.


2
1μ

II - 3a Histogram showing the distribution of contour lengths of isolated RNP fibrils attached to X. laevis liver cell chromatin. Median value 0.66 µm. Range 6.3 µm.

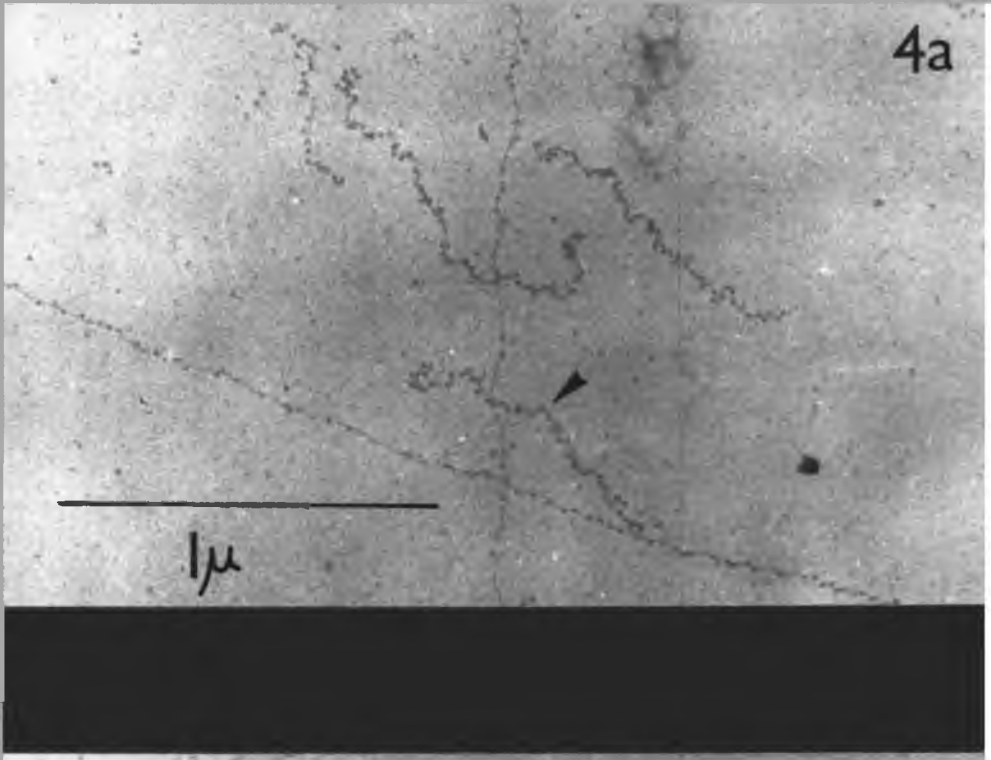
II - 3b Histogram showing the distribution of contour lengths of terminal RNP fibrils of transcription complexes comprising two or more RNP fibrils, from X. laevis liver cell chromatin. Median value 0.4 µm. Range 3.6 µm.

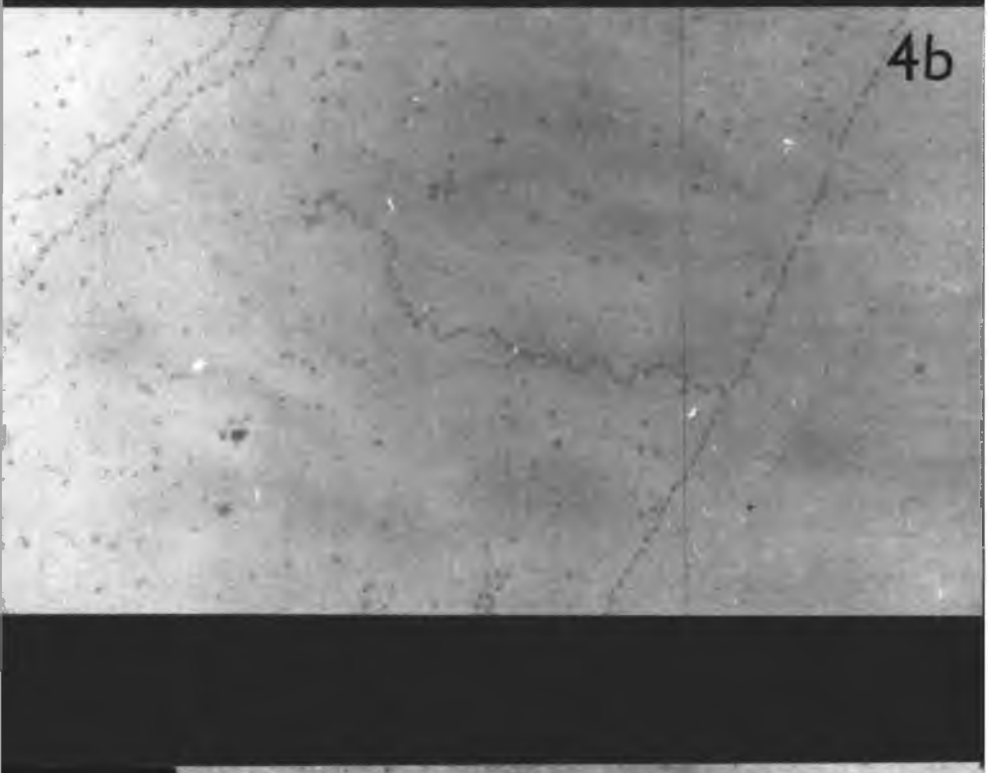

n
a
5
1
2
3
4
5
6
b
5
1
2
3
4
5
6
fibril length µm

II - 4a Arrow denotes an isolated RNP fibril attached to DNP from X. laevis liver. Putative detached primary transcripts are also present. The beaded structure and higher contrast of the RNP are apparent.

II - 4b As Fig. II - 4a.

II - 4c Group of two RNP fibrils from the same source as Figs II - 4 a-b. Arrow denotes a grid bar.

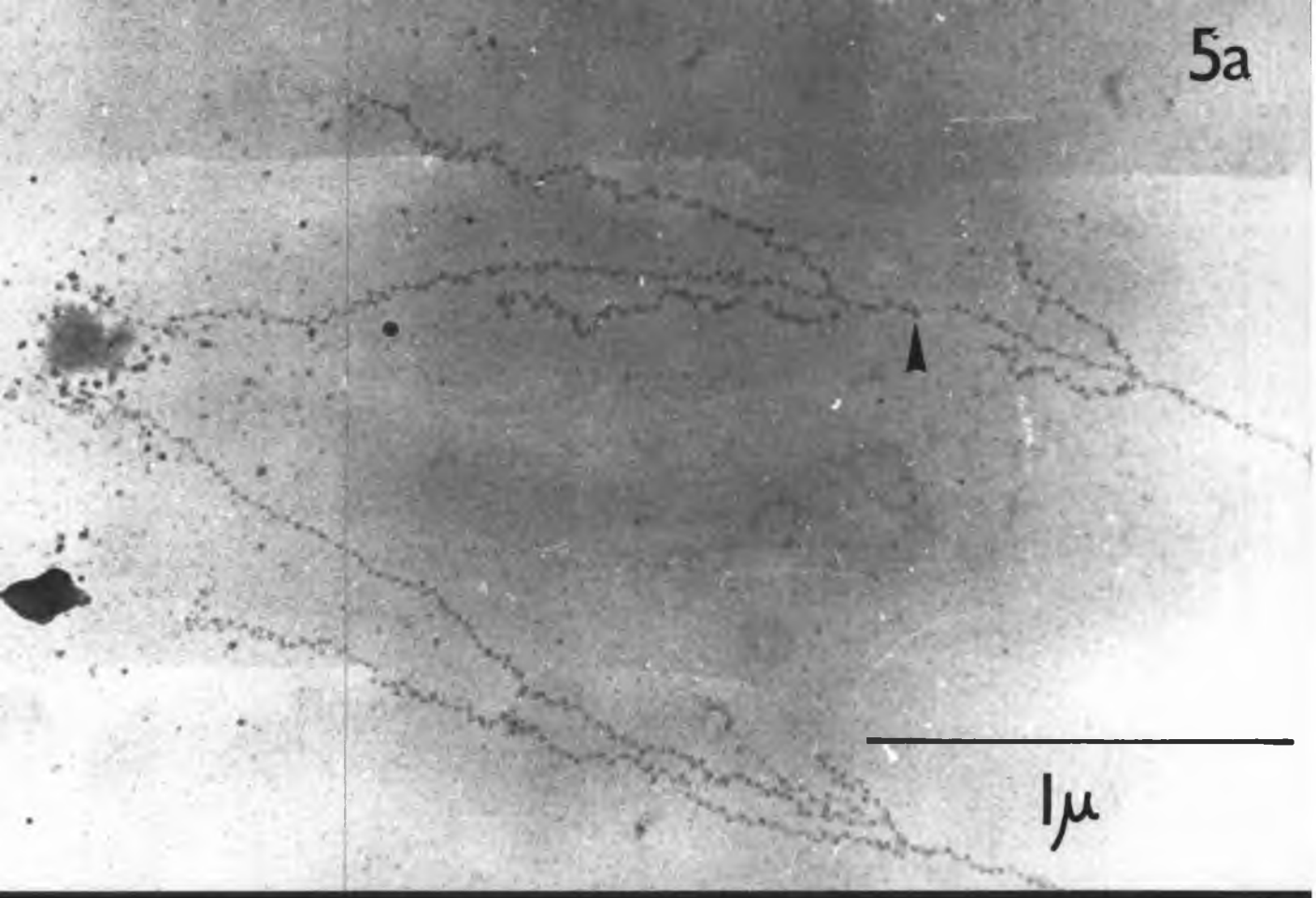

4a
1μ

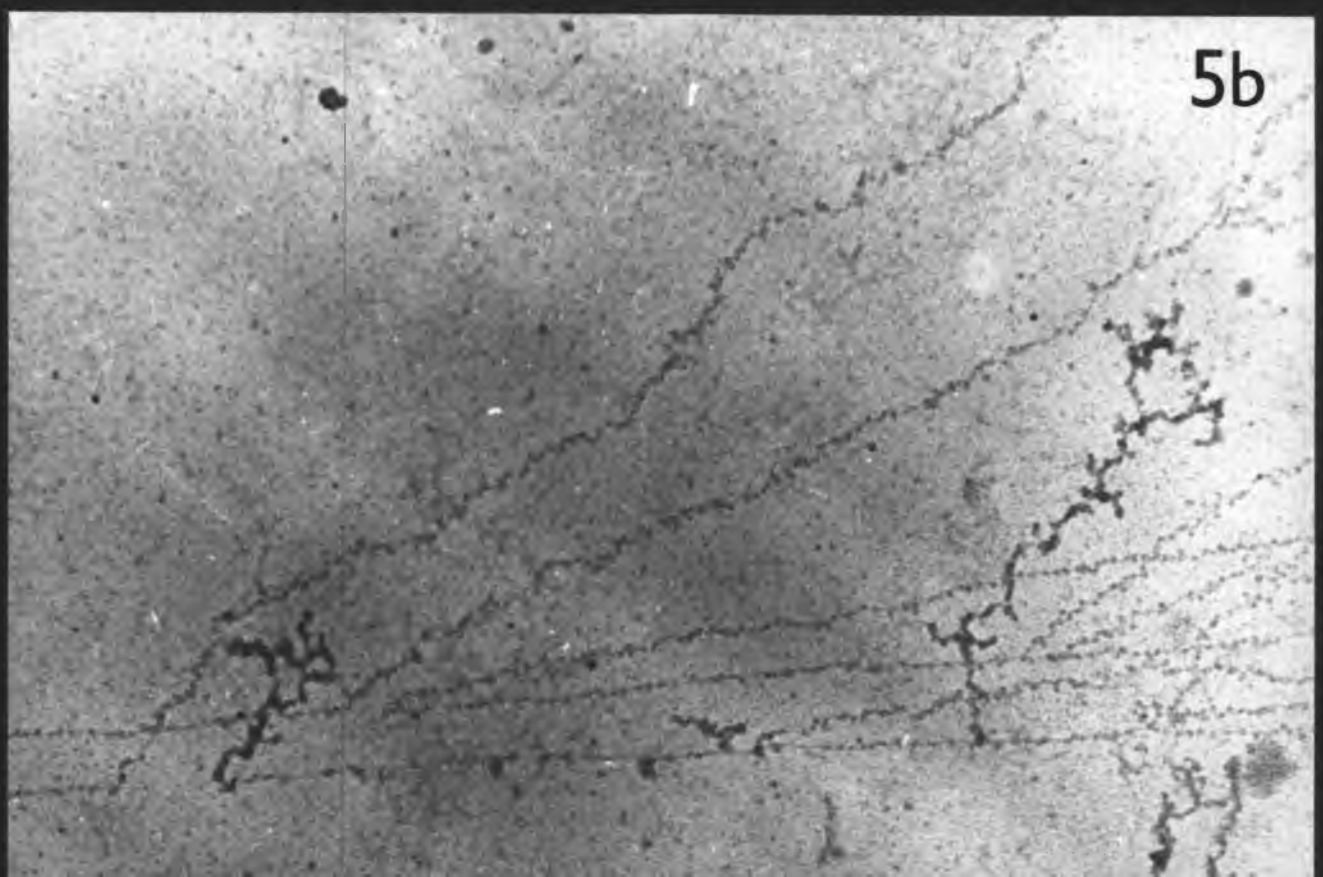

4b


4c

II - 5a Transcription complex from X. laevis liver chromatin, consisting of four RNP molecules. Both the RNP and the DNP axis of this complex have a beaded structure. Beads along the DNP (arrow) are presumed to be nucleosomes. Laird Analysis gave a value for transcription complex length of 1.3 μm. RNA polymerase molecules were not visible.

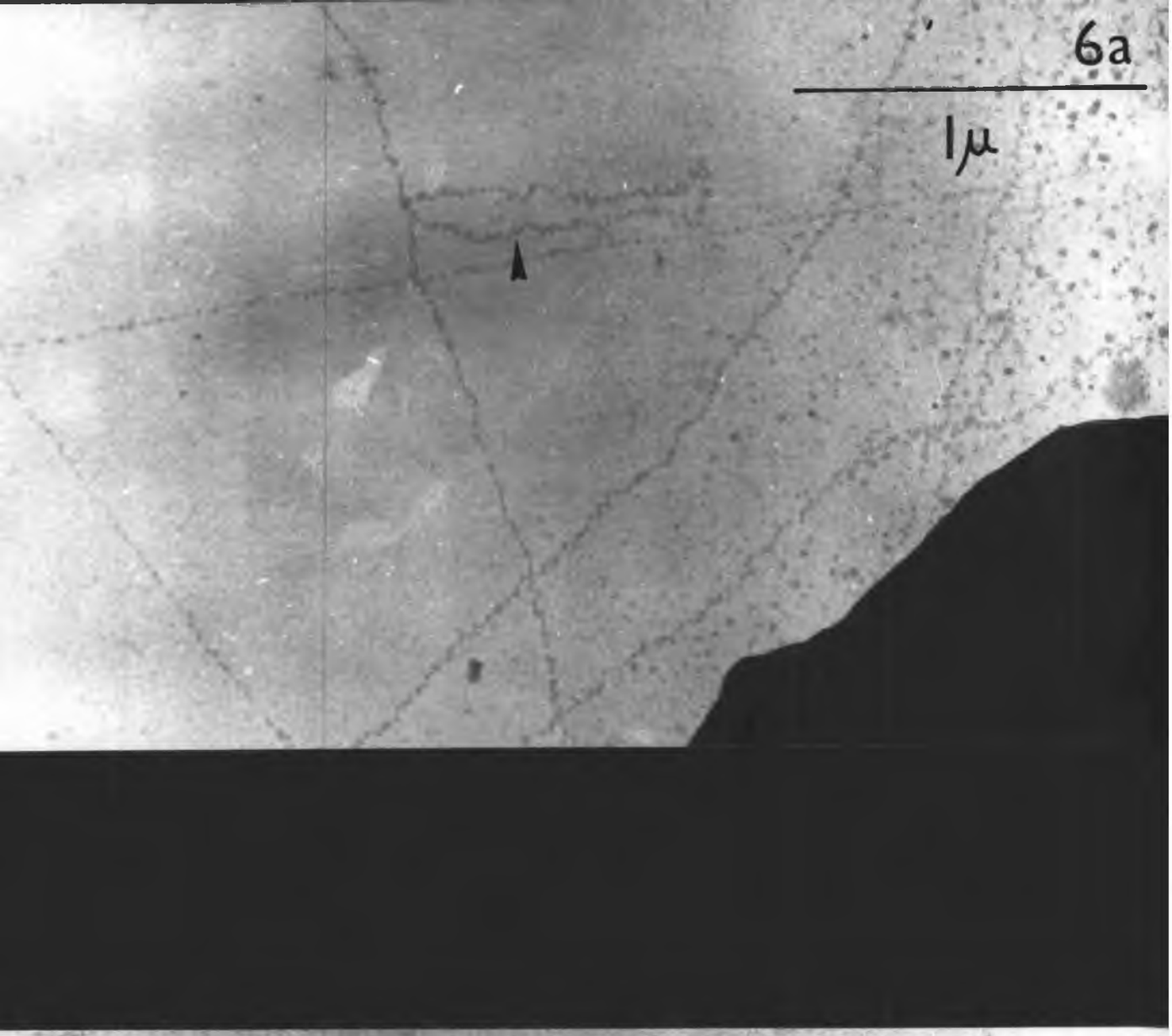
II - 5b As Fig. II - 5a. The DNP axis of this complex also has a beaded structure, but the RNP is more contrasted than the DNP, unlike Fig. II - 5a. The degree of secondary structure of the RNP is also greater than in Fig. II - 5a, and a beaded structure is not evident. Laird Analysis gave a value for transcription complex length of 2.6 μm. RNA polymerase molecules were not clearly visible.

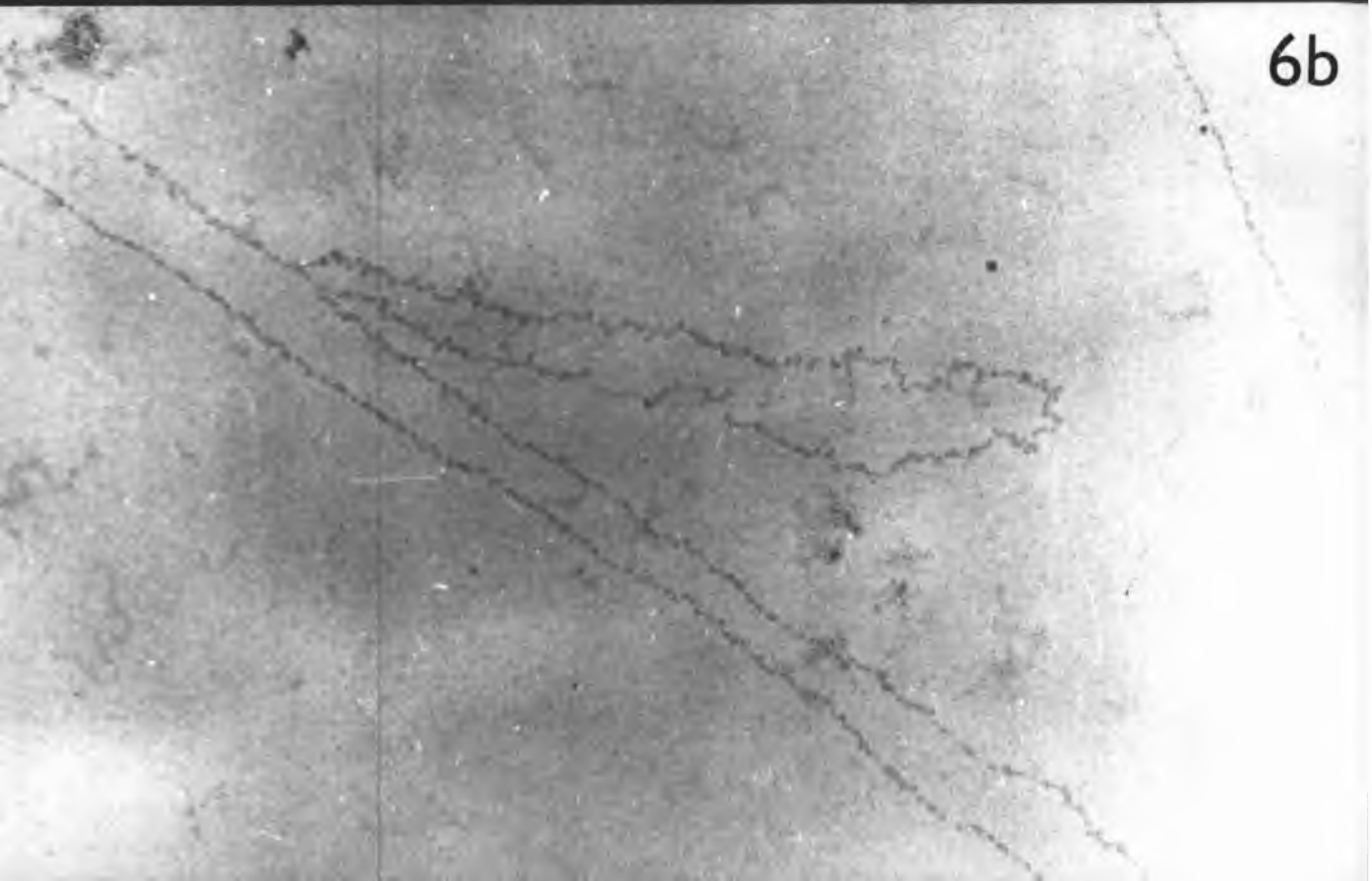

5a
1μ


5b

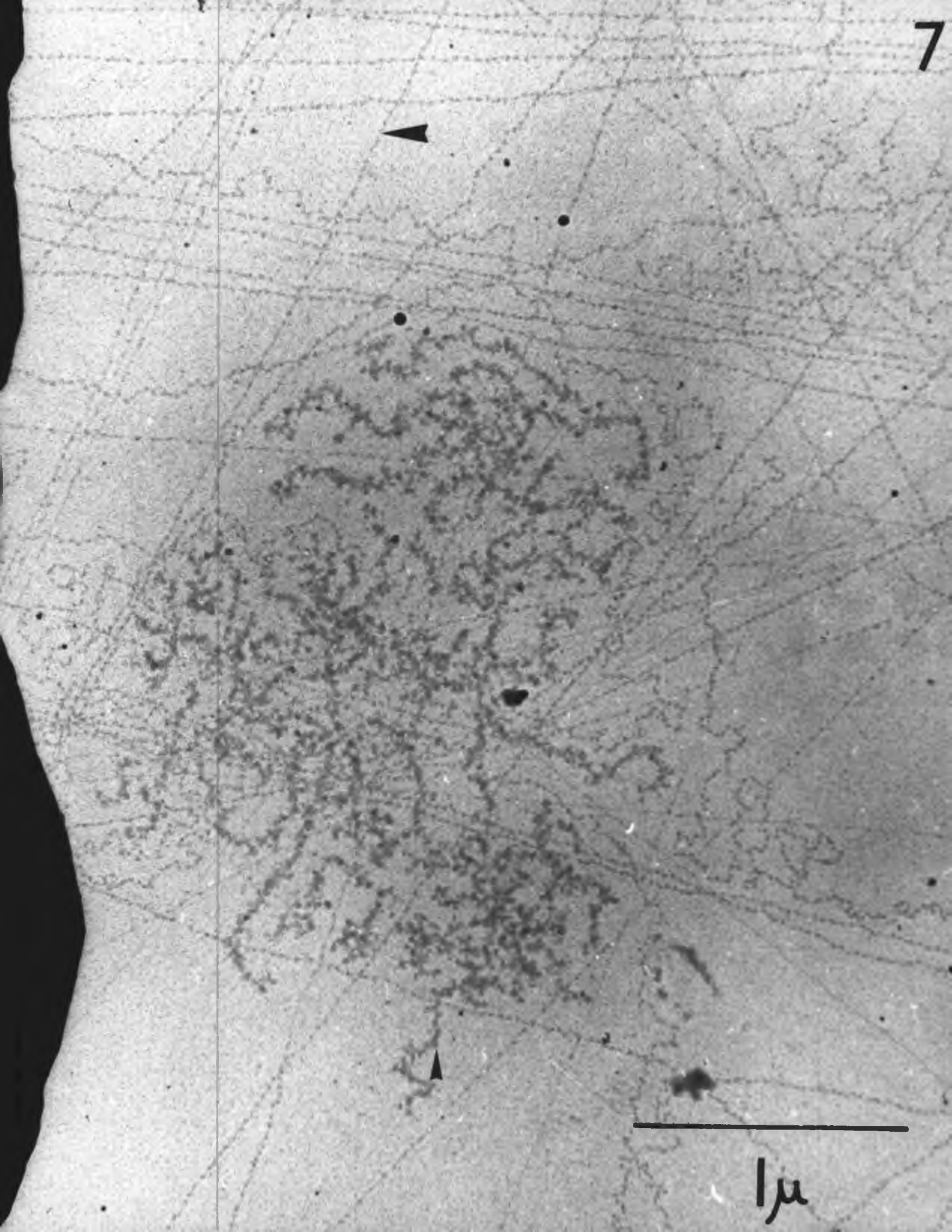
II - 6a Arrow denotes anomalous putative "RNP" from X. laevis liver cell chromatin. The close spacing of the two ends of the structure on the DNP may imply that it is a twist in the chromatin.

II - 6b As Fig. II - 6a. The relatively wide spacing of the attachment points (arrows) of this structure to the DNP may imply that it consists of two RNP molecules whose ends have in some way become associated.


6a
1μ

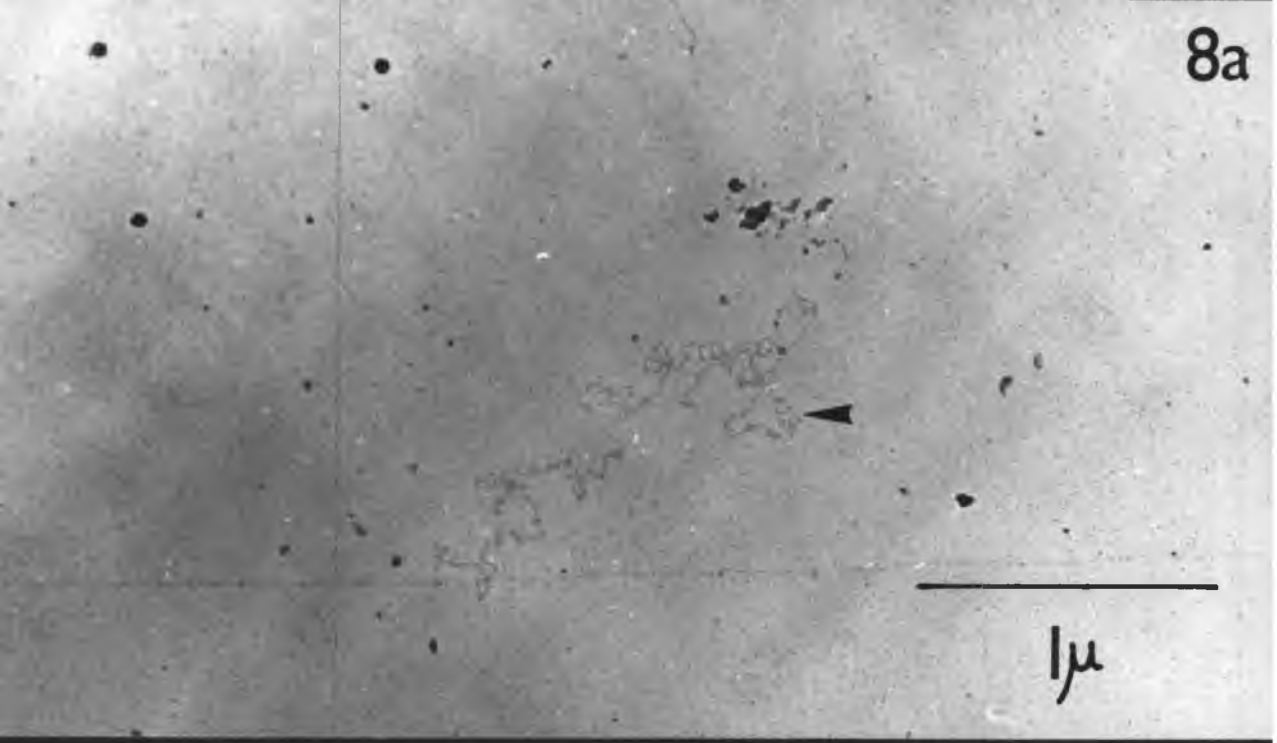

6b

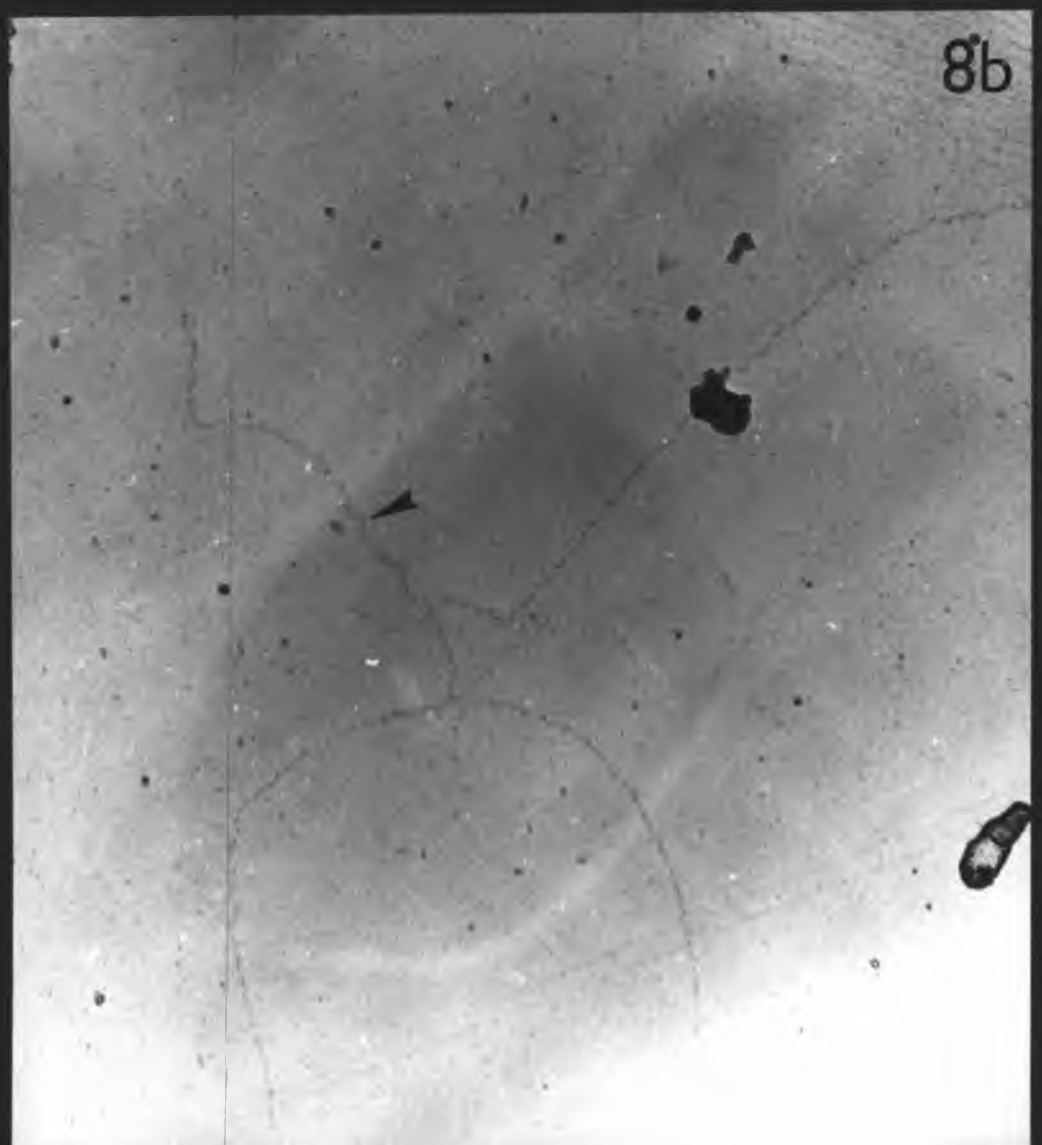
II - 7 Nucleosomal chromatin (large arrow) from T.c. carnifex liver cells. Small arrow denotes a clump of RNP molecules, which are, however, too tangled to be traced with certainty.


7
1μ

II - 8a Arrow denotes a long transcript attached to T.c. carnifex liver cell chromatin. An RNA polymerase molecule is visible at the point of attachment of the RNP to the DNP, which is relatively smooth in structure, without nucleosomes. The transcript has a beaded structure.

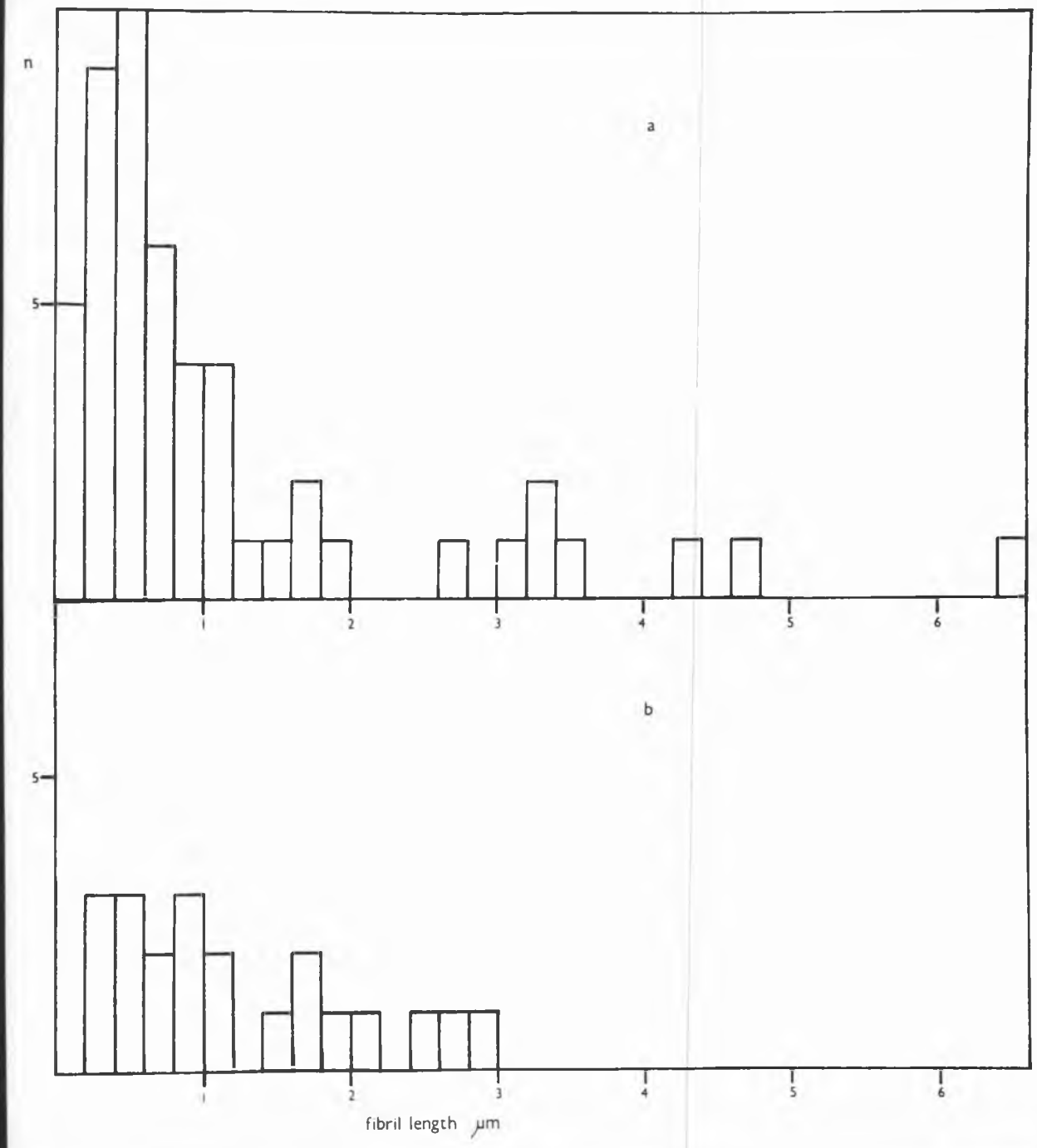
II - 8b As Fig. II - 8a. Both DNP and RNP have a beaded structure. The RNA polymerase at the attachment point of the RNP to the DNP, is not clearly visible.


8a
1μ


8b

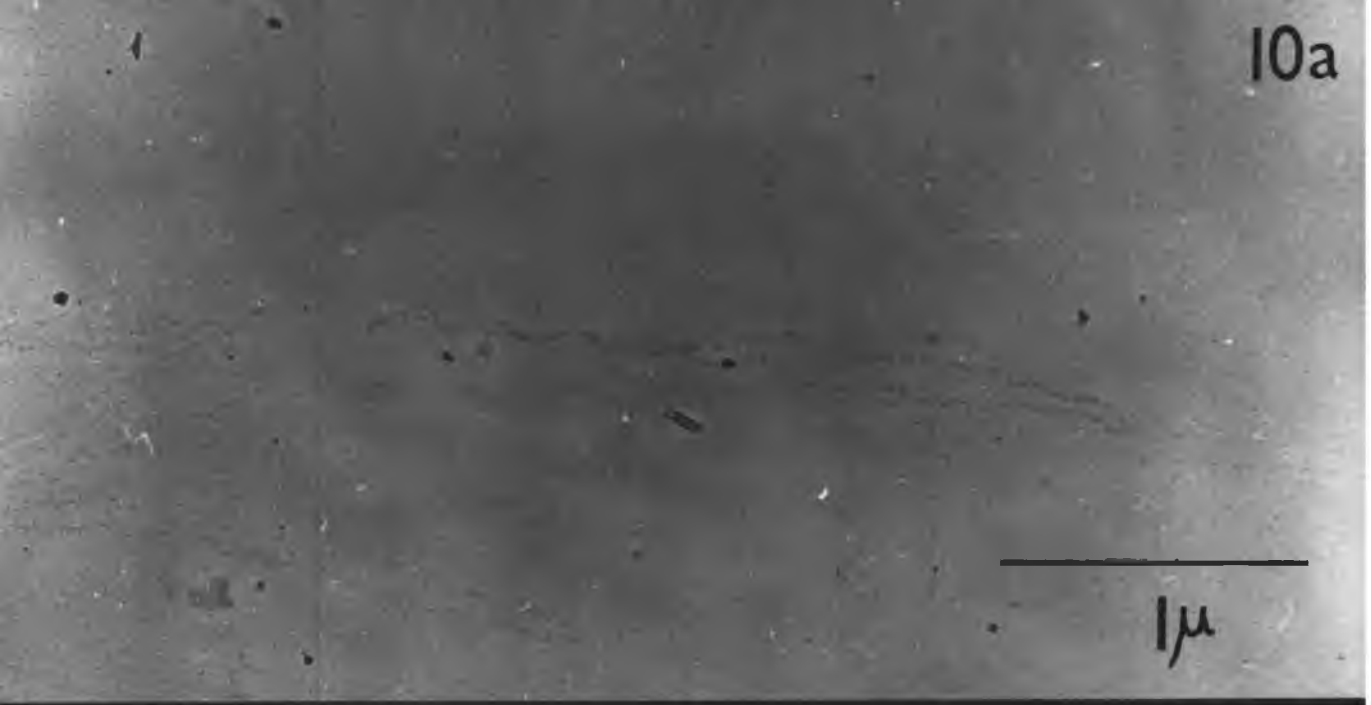
II - 9a Histogram showing the distribution of contour lengths of single RNP fibrils attached to T.c. carnifex liver cell chromatin. Median value 0.6 µm. Range 6.5 µm.

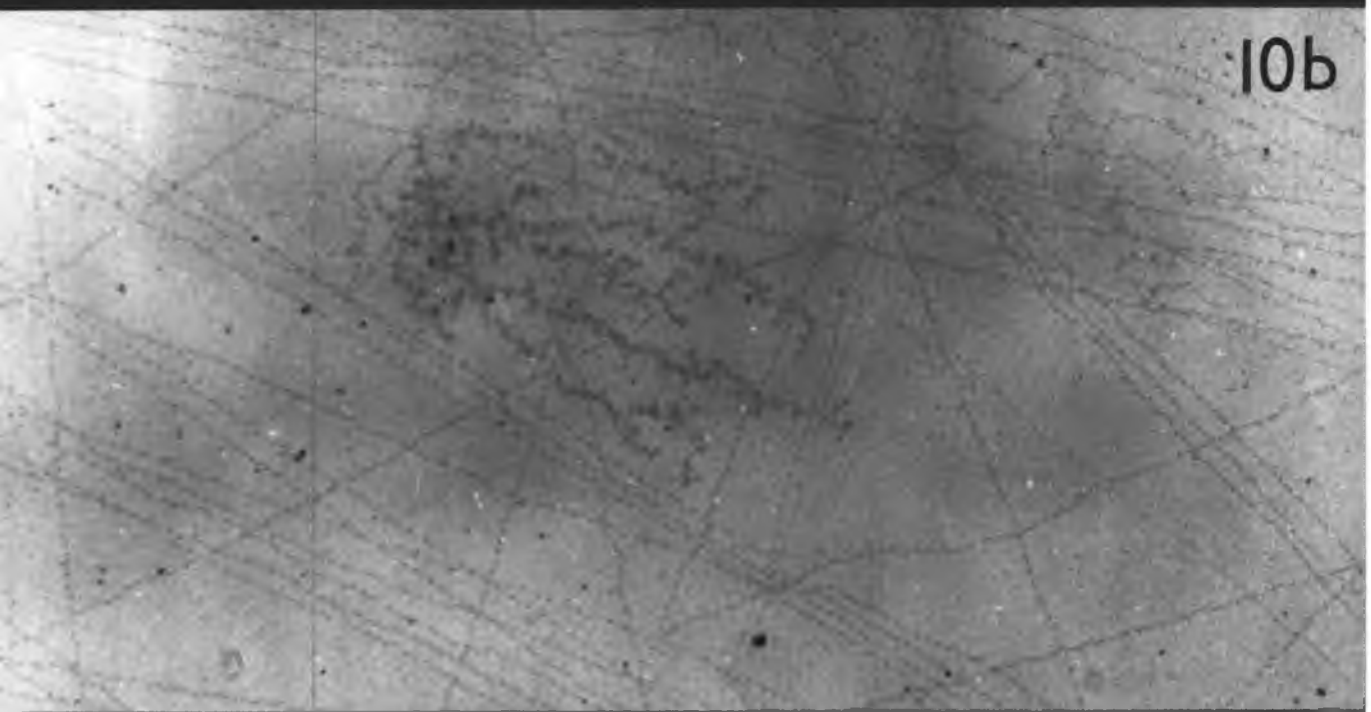
II - 9b Histogram showing the distribution of contour lengths of the terminal fibrils of transcription complexes consisting of two or more RNP fibrils, from T.c. carnifex liver cell chromatin. Median value 0.95 µm. Range 9.6 µm.


n
a
5
1
2
3
4
5
6
b
5
1
2
3
4
5
6
fibril length µm

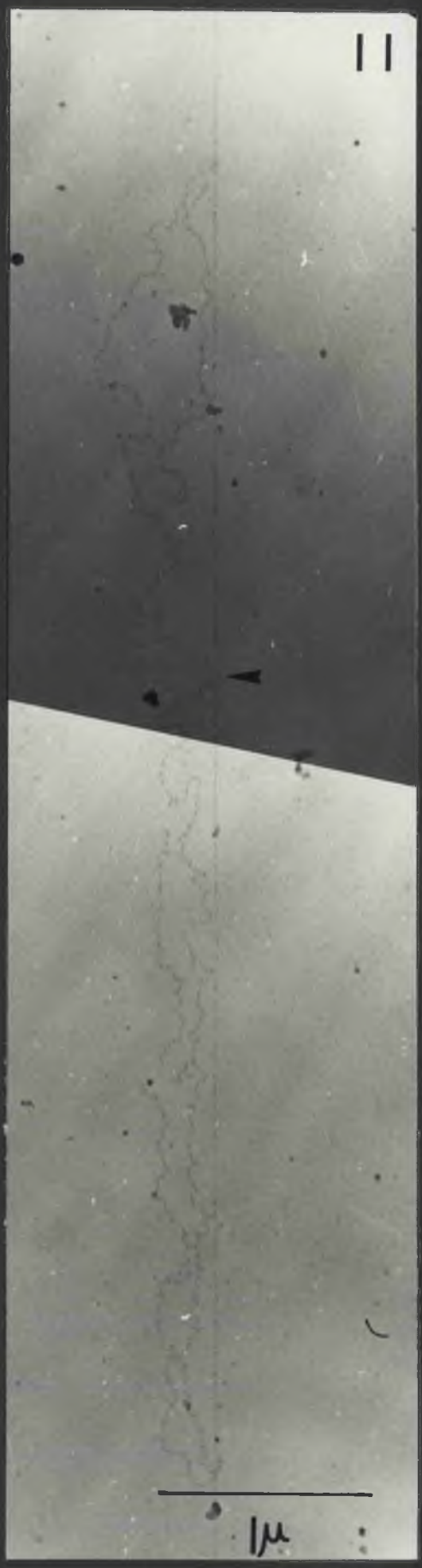
II - 10a Group of two RNP fibrils from T.c. carnifex liver cell chromatin. The beaded structure and relatively high contrast of the RNP are clear. The difference in length between the two RNP molecules is greater than their spacing on the DNP, which may imply processing.

II - 10b Transcription complex from the same source as Fig. II - 10a.


10a
1μ


10b

II - 11 Transcription complex from T.c. carnifex liver cells, consisting of three RNP fibrils, and of a configuration perhaps indicative of processing. Arrow denotes a transcript of 9.8 μm.

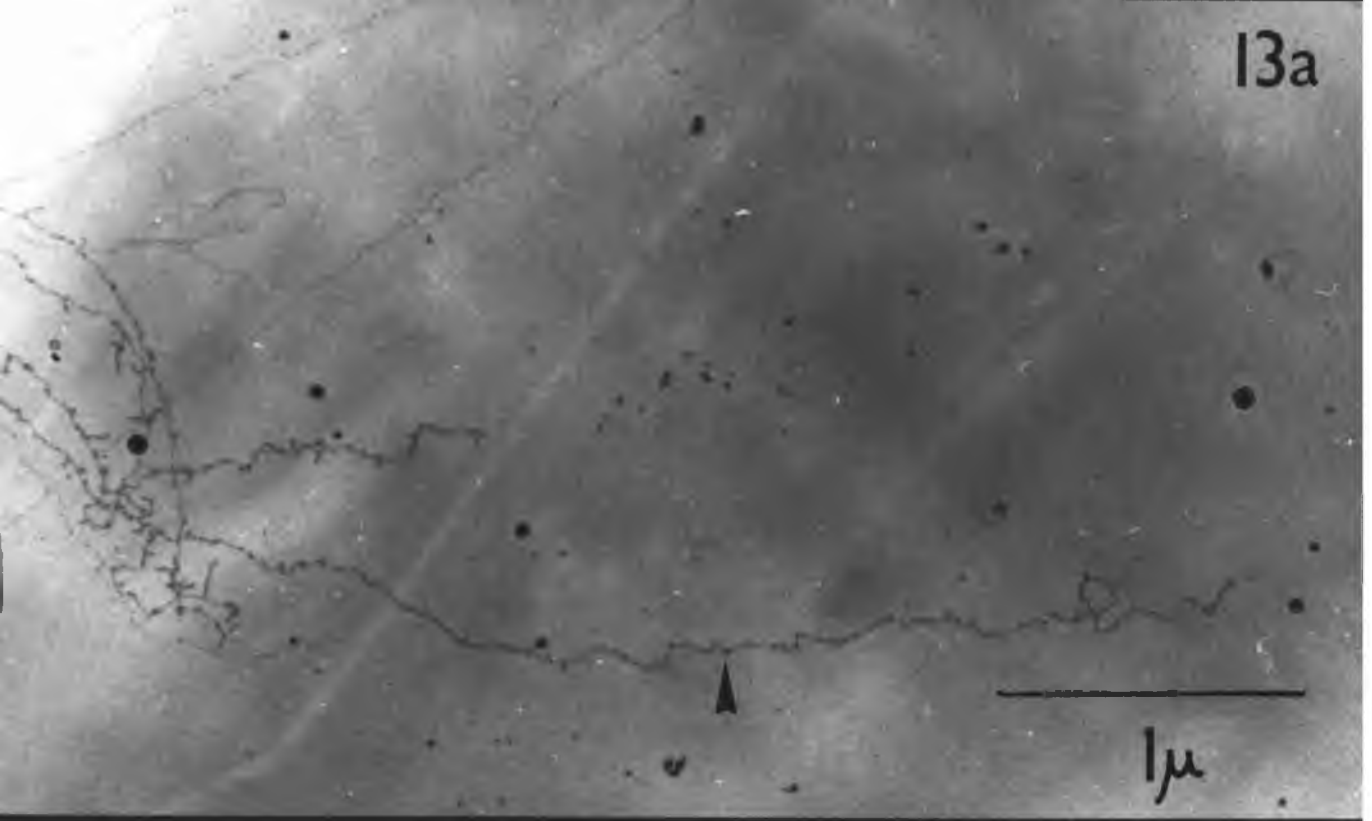

11
1μ

II - 12 Transcription complex from the same source as Fig. II - 11. Only a few fibrils were clear (arrows) but the length of this array is reminiscent of the transcription complexes seen on lampbrush chromosomes.


12
1μ

II - 13a Arrow denotes anomalous "RNP-like" structures from T.c. carnifex liver cell chromatin. DNP is present adjacent to the fibrils, and is seen to be of lower contrast, with less secondary structure. The fibril presented here disappeared into a clump, part of which is evident at the left of the picture.

II - 13b As Fig. II - 13a. Many of these fibrils have structures resembling the lateral fibrils seen on transcription complexes (arrow). Laird Analysis of these structures did not give a meaningful result, and a line could not be drawn through the distribution of fibril lengths.

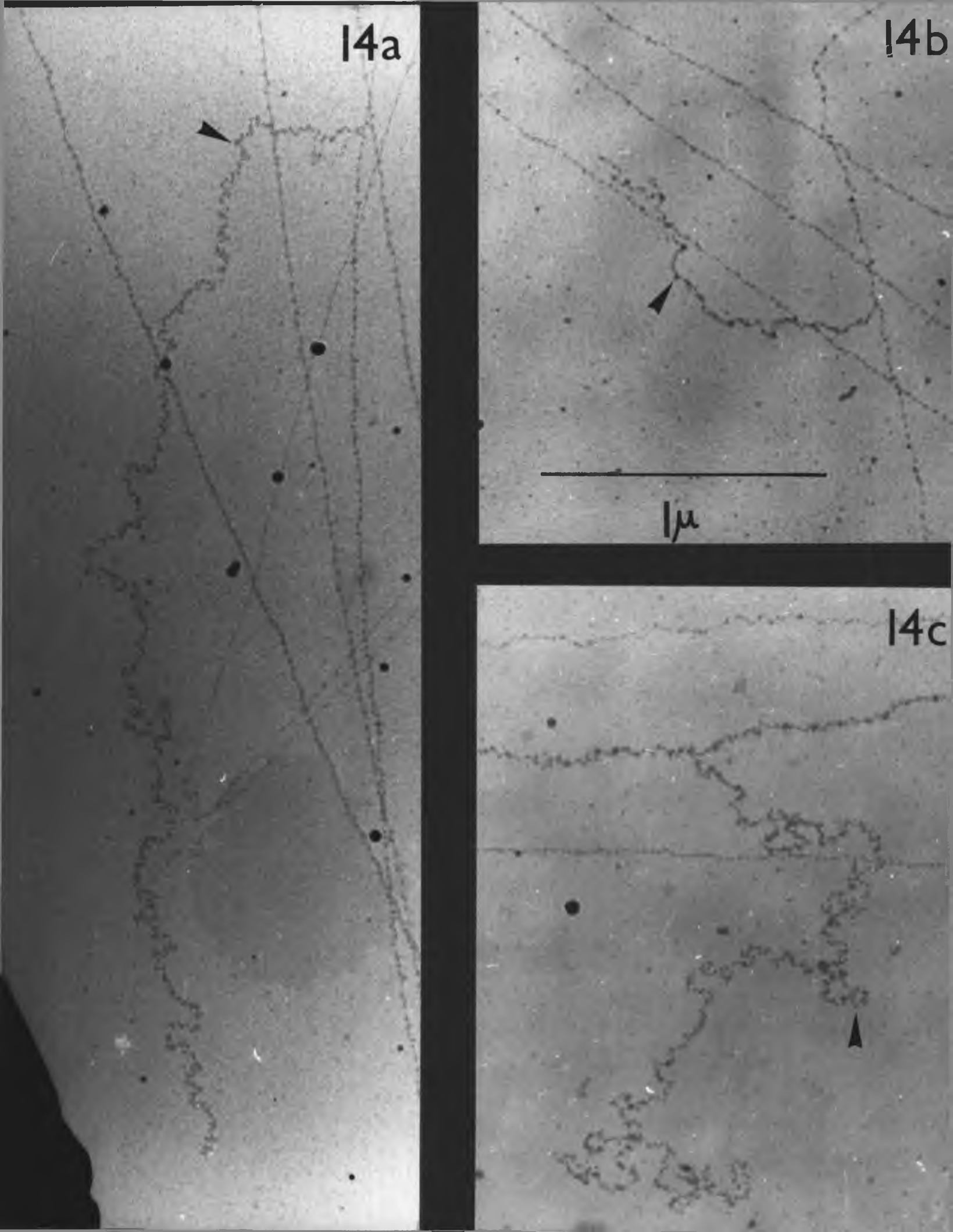

13a
1μ


13b

II - 14a Arrow denotes an isolated RNP fibril from N. maculosus liver cell chromatin. It is of extensive secondary structure and relatively high contrast. The nucleosomal structure of the DNP is apparent. RNA polymerases were not clearly visible.

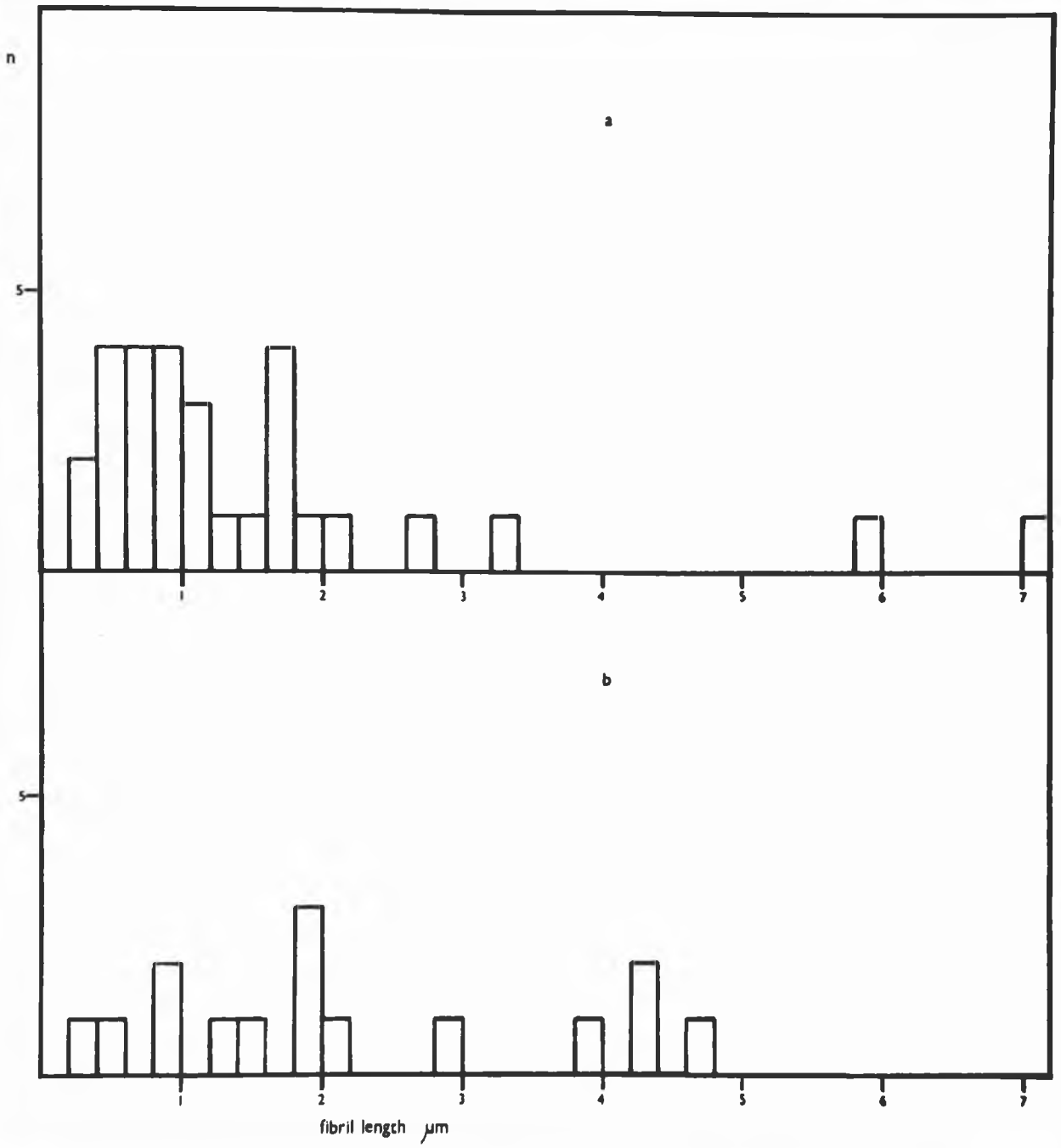
II - 14b As Fig. II - 14a.

II - 14c As Fig. II - 14a.

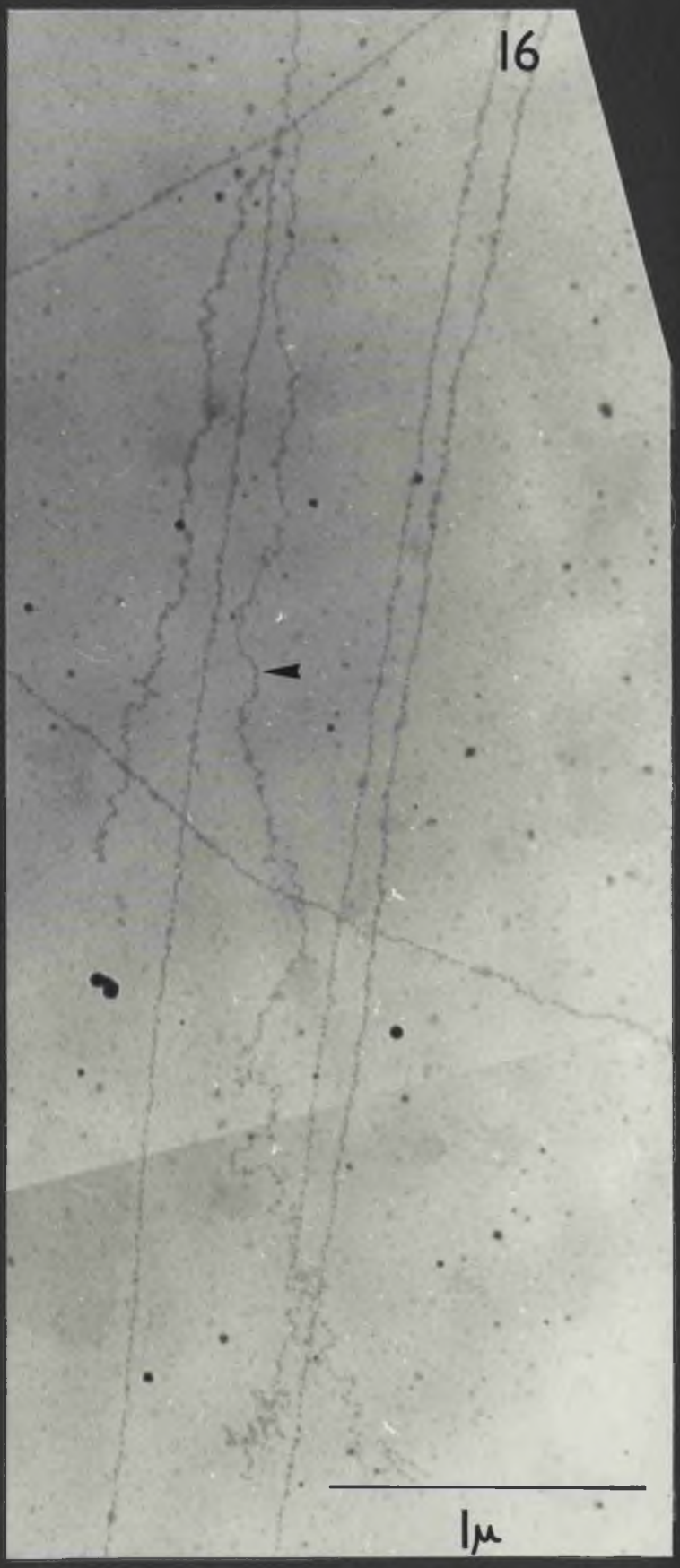

14a
14b
1μ
14c

II - 15a Histogram showing the distribution of contour lengths of isolated RNP fibrils attached to N. maculosus liver cell chromatin. Median value 1.1 μm. Range 9.13 μm.

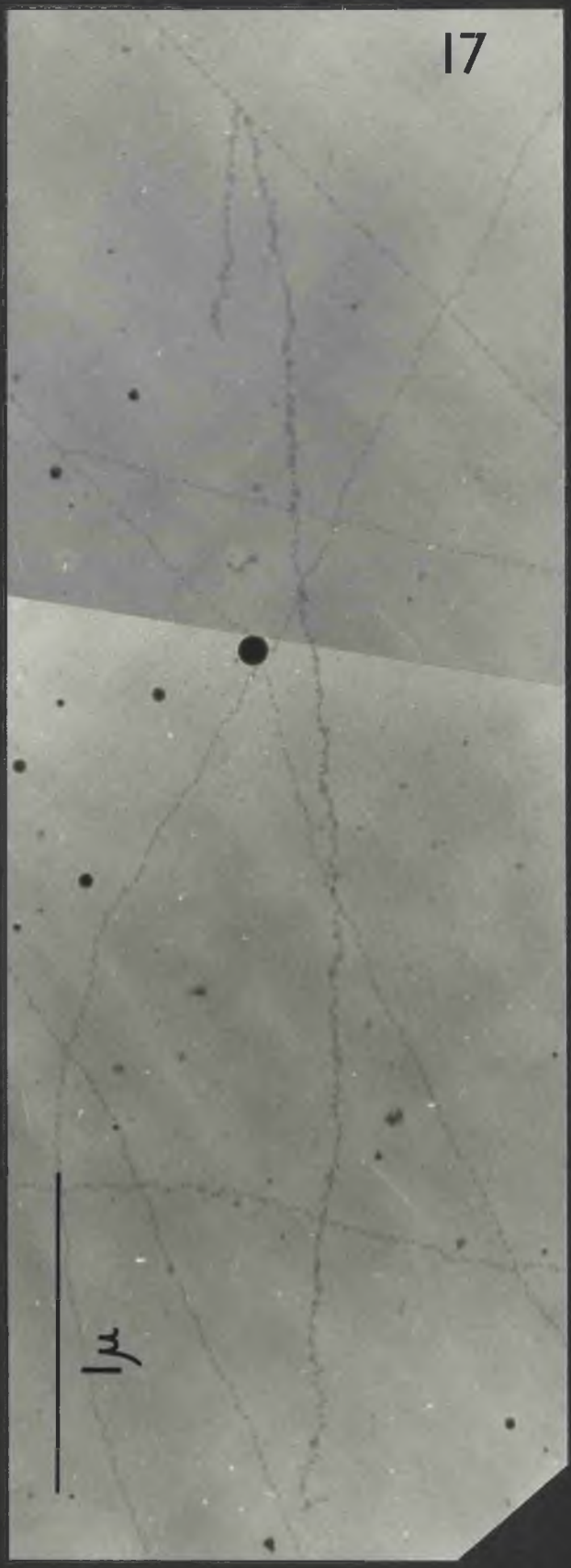
II - 15b Histogram showing the distribution of contour lengths of terminal RNP fibrils of transcription complexes consisting of two or more RNP fibrils, from N. maculosus liver cells. Median value 1.96 μm. Range 7.08 μm.


n
a
5
1
2
3
4
5
6
7
b
5
1
2
3
4
5
6
7
fibril length µm

II - 16 Group of two RNP fibrils from N. maculosus liver cell chromatin. The configuration of these fibrils, where the difference in their length is greater than their spacing on the DNP, may be indicative of processing. The longer of the two fibrils (arrow) was considered as the terminal fibril.

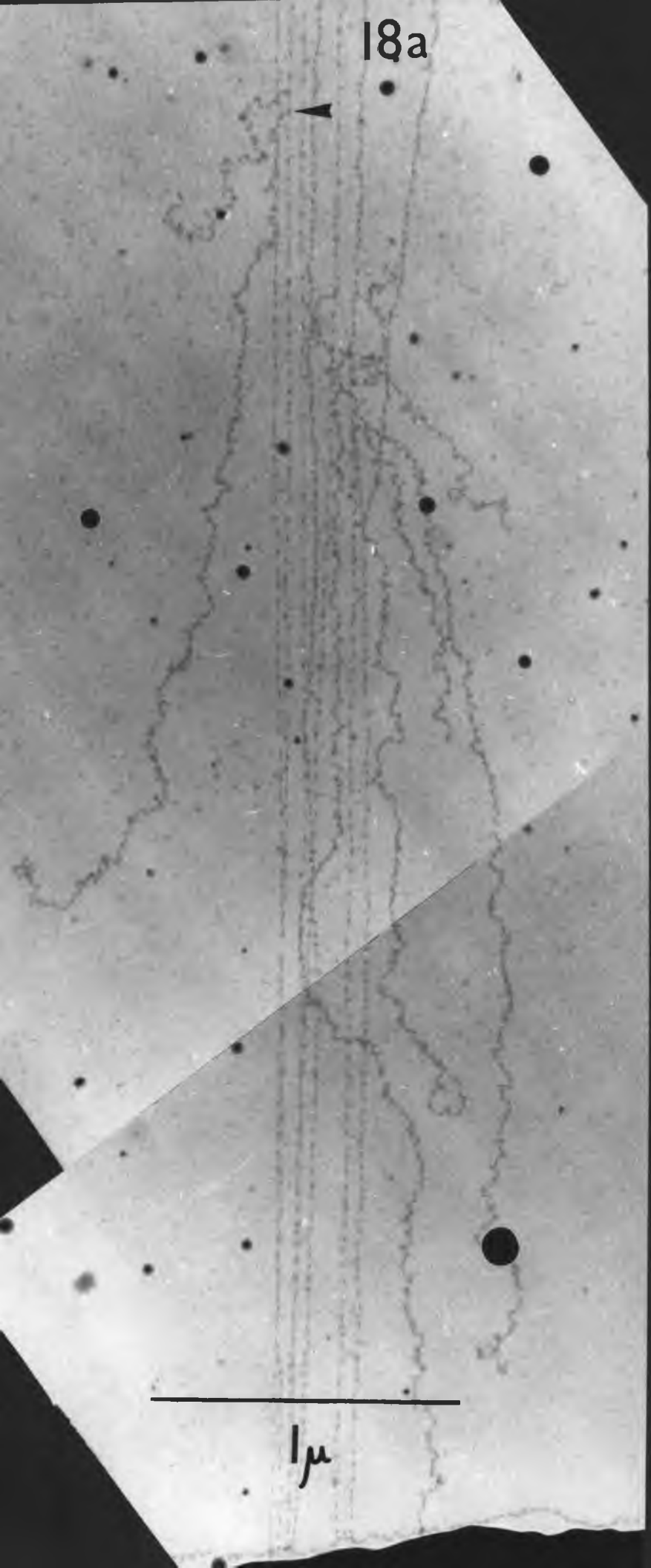

16
1μ

II - 17 As Fig. II - 16.


17
1μ

II - 18a Group of transcription complexes from N. maculosus liver cells. The large arrow denotes a putative RNA polymerase molecule. Laird Analysis was done, but did not give a reliable value for transcription complex length. The nucleosomal structure of the DNP is clearly visible.

II - 18b Transcription complex from the same source as II - 18a. The DNP axis of this complex is beaded, and these beads were assumed to be nucleosomes.

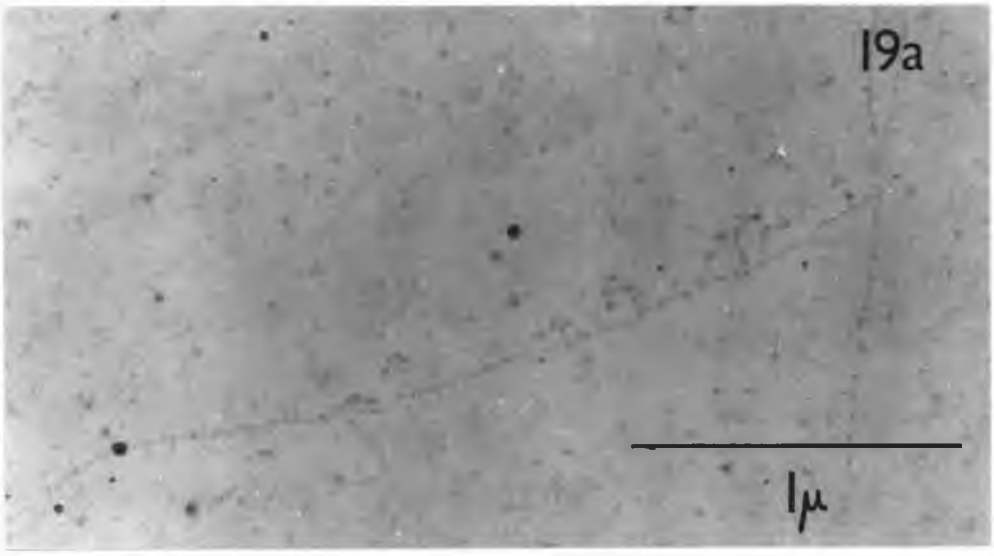

18a
1μ


18b

II - 19a Transcription complex from N. maculosus liver cell chromatin.

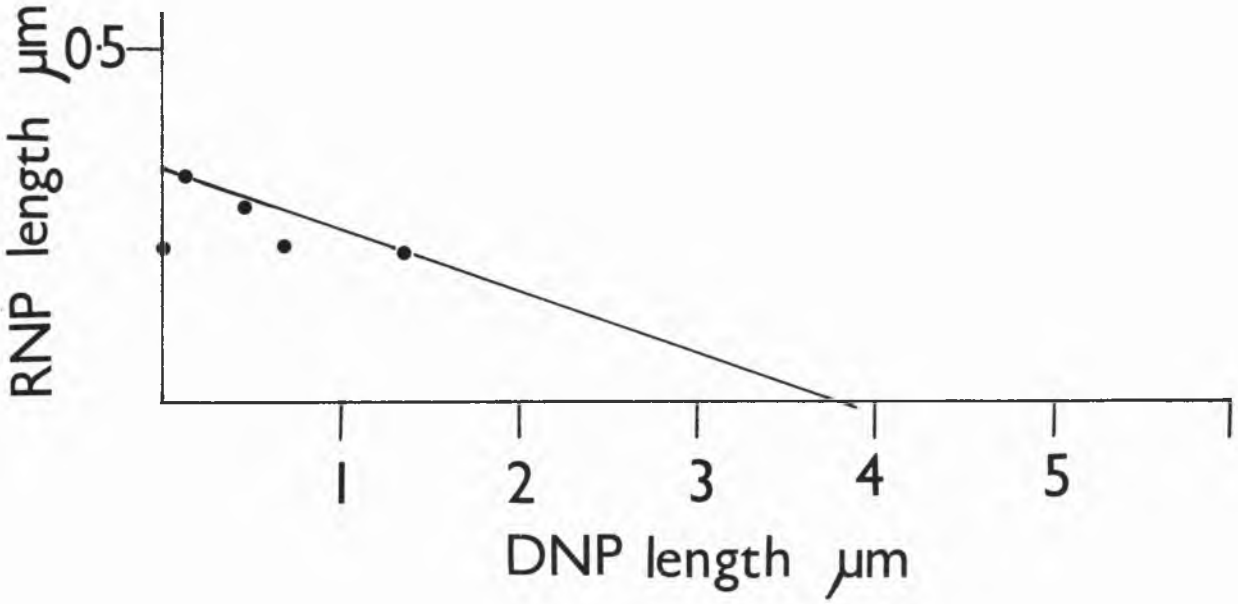
II - 19b Tracing of Fig. II - 19a.

II - 19c Laird Analysis of transcription complex presented in Fig. II - 19a. A value of 3.8 μm was obtained for array length, when RNP fibril length is plotted against position of the fibril in the array.



19b

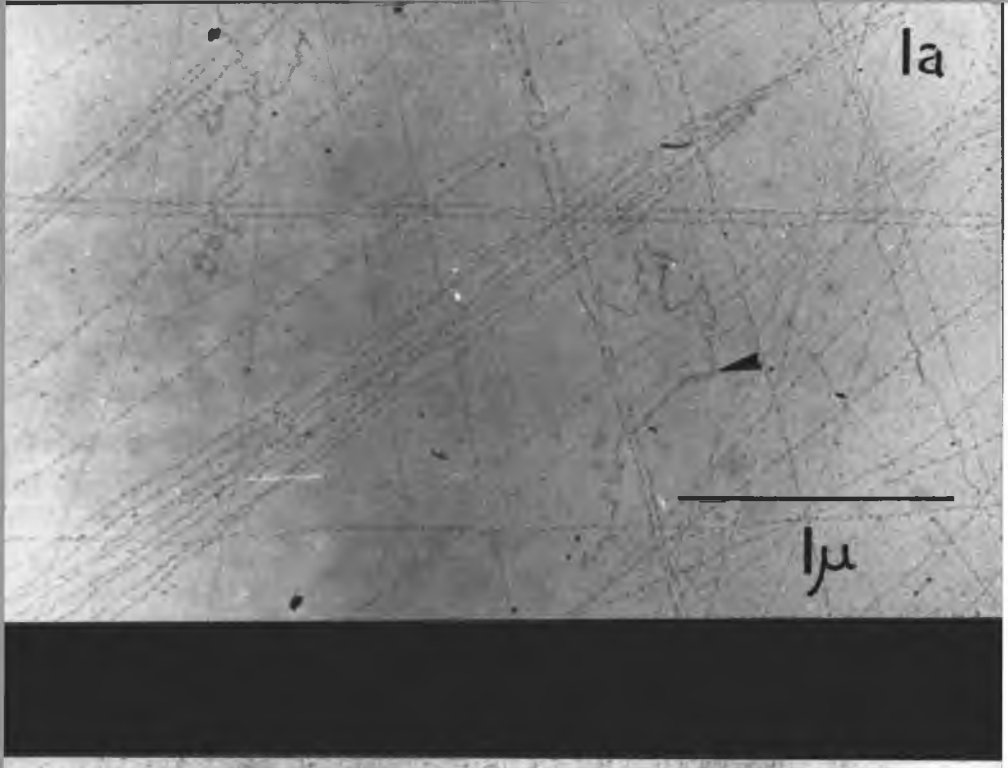
19c

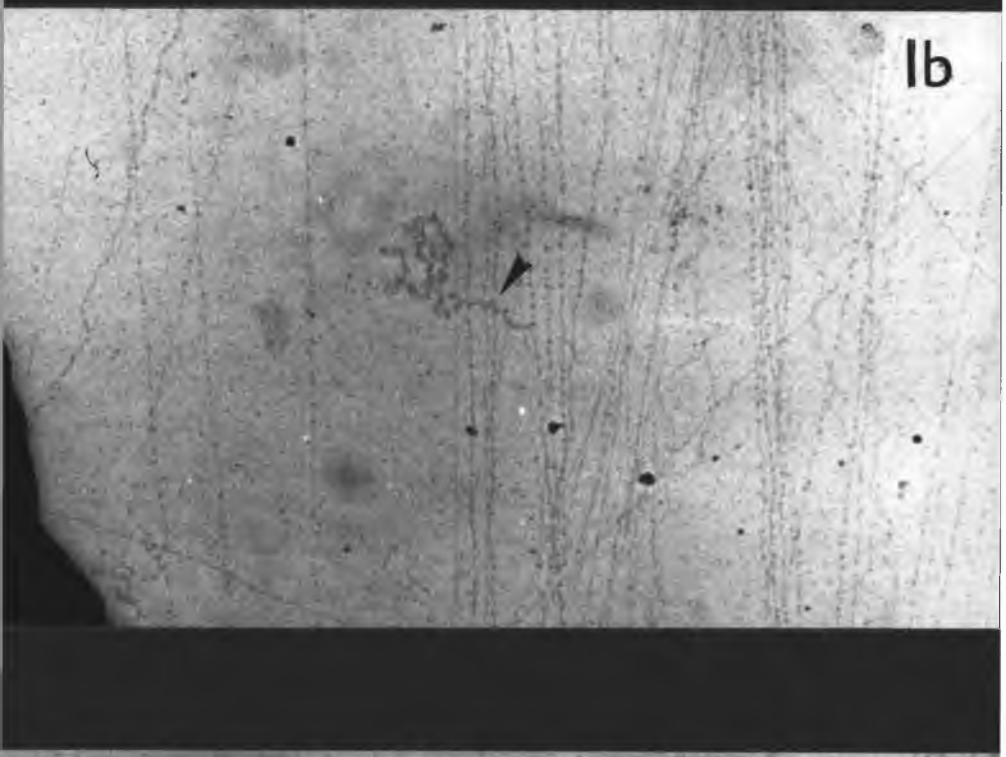


III - 1a Arrow denotes an isolated RNP fibril attached to chromatin from T.c. carnifex neurula cells. The DNP is partly of nucleosomal structure, but a certain proportion is smooth and lacks beads.

III - 1b As Fig. III - 1a.

III - 1c As Fig. III - 1a.

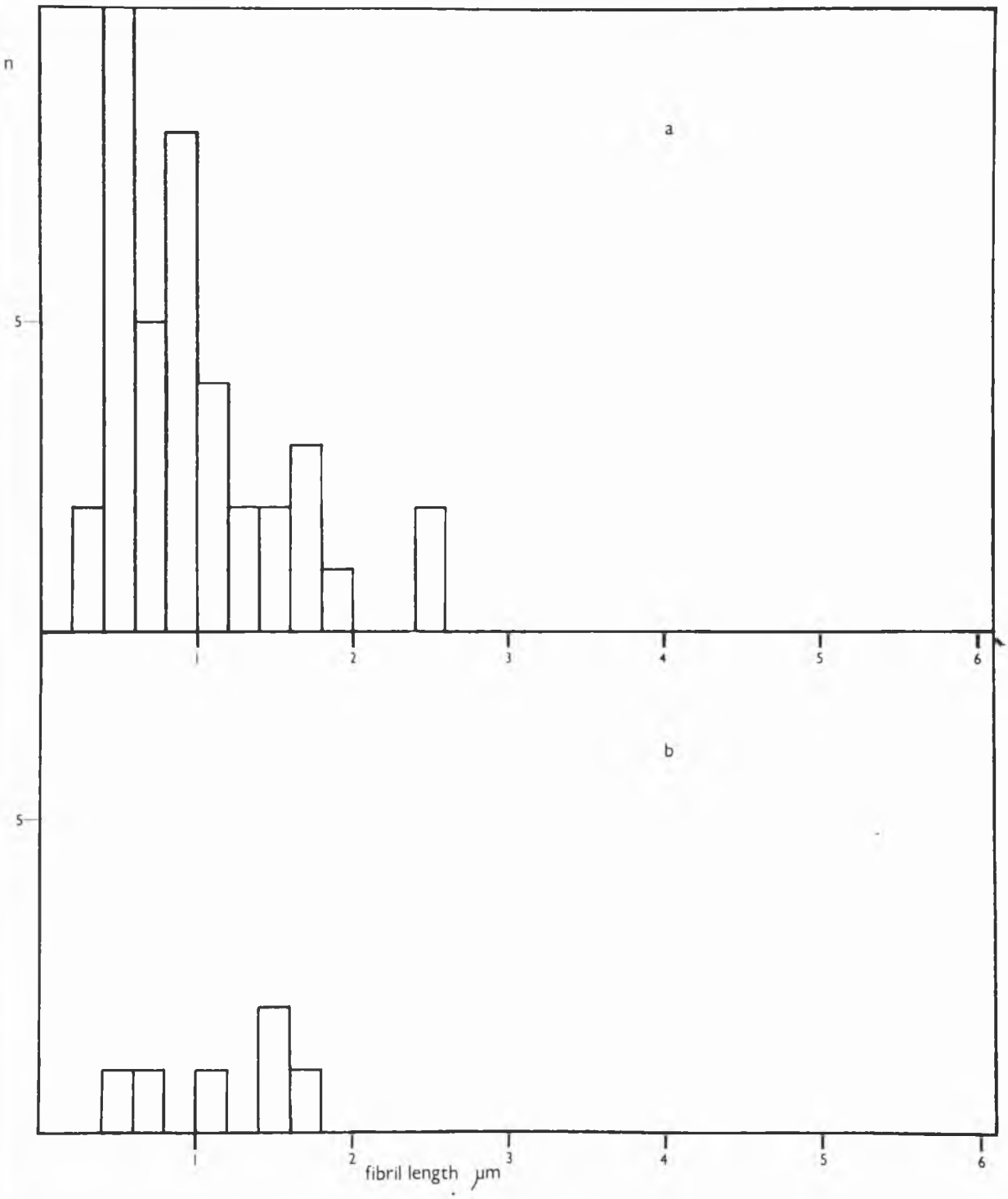

Ia
Iμ


Ib


Ic

III - 2a Histogram showing the distribution of contour lengths of isolated RNP fibrils attached to the chromatin of T.c. carnifex neurula cells. Median value 0.82 μm. Range 2.3 μm.

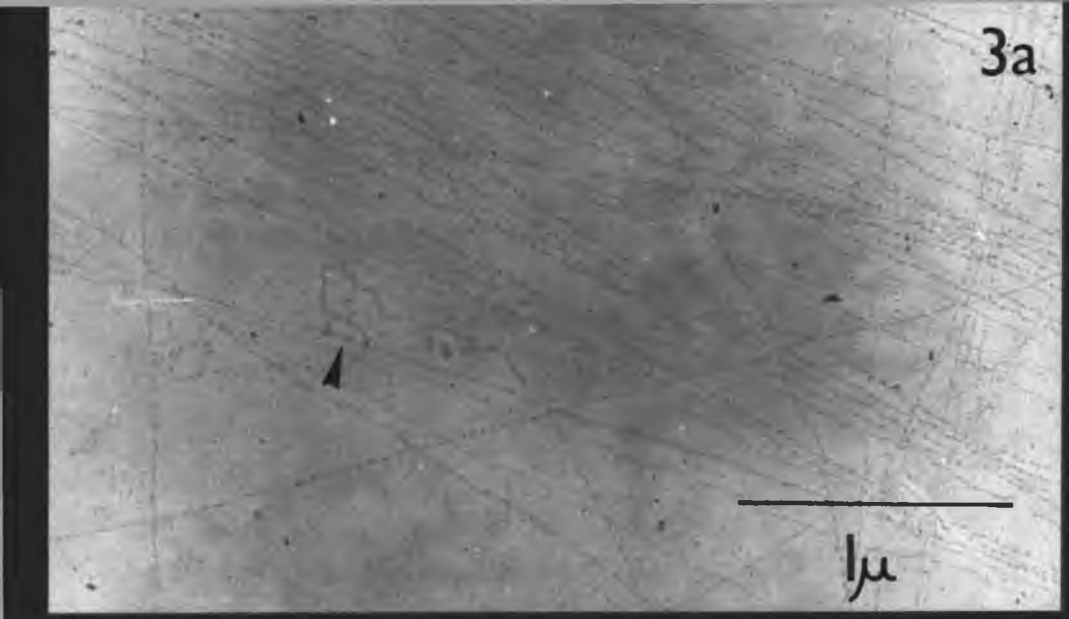
III - 2b Histogram showing the distribution of contour lengths of the terminal RNP fibrils of transcription complexes consisting of two or more RNP fibrils, from T.c. carnifex neurula cells. Median value 1.3 μm. Range 1.4 μm.

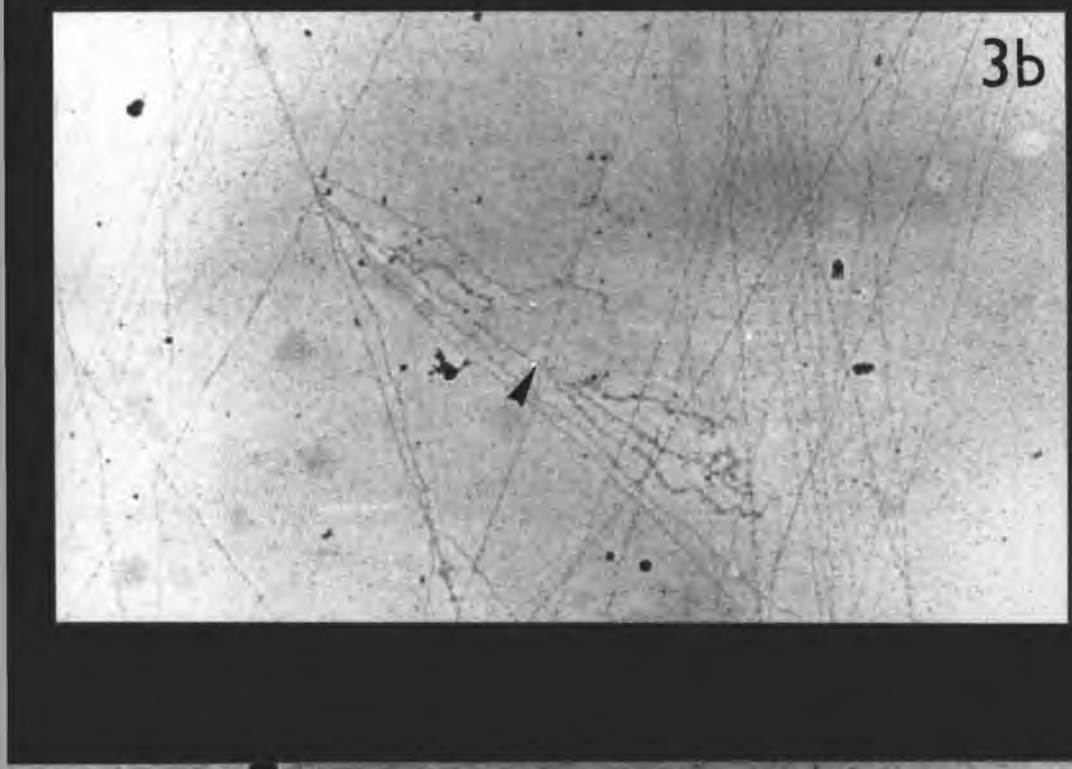

n
a
5
1
2
3
4
5
6
b
5
1
2
3
4
5
6
fibril length µm

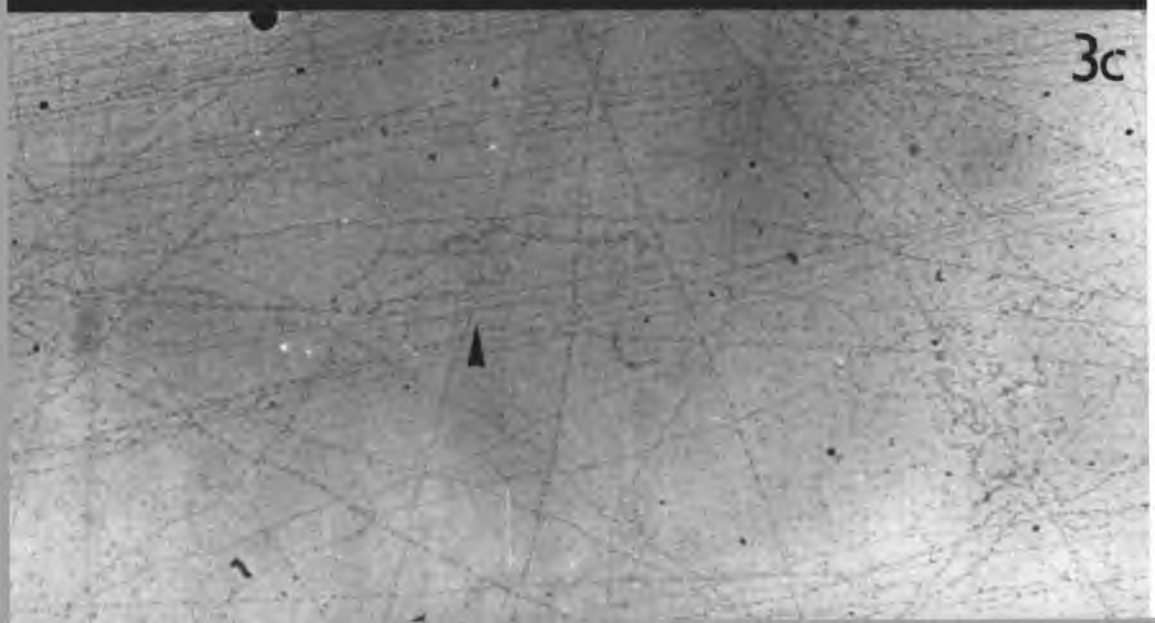
III - 3a Arrow denotes a group of two RNP fibrils from T.c. carnifex neurula chromatin, whose configuration may be indicative of processing.

III - 3b Arrow denotes a transcription complex from the same source as Fig. III - 3a. Laird Analysis was not possible.

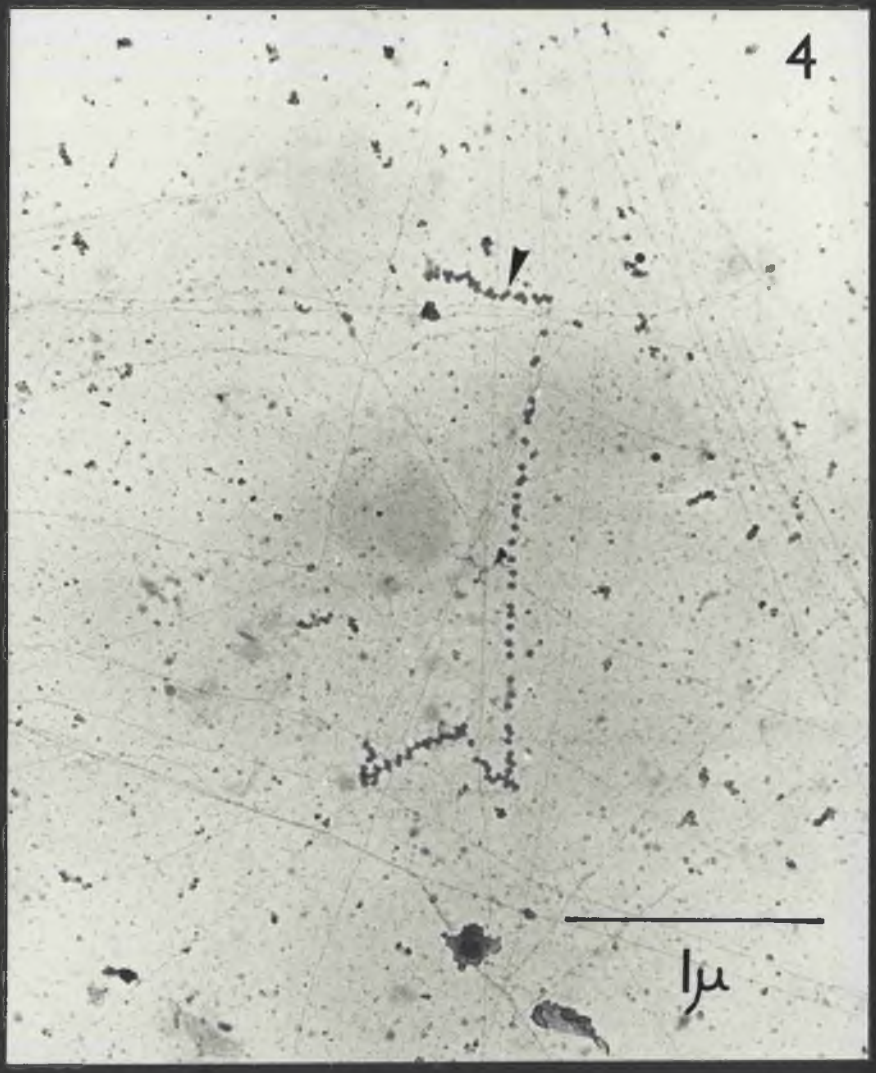
III - 3c As Fig. III - 3b. Laird Analysis gave a value for transcription complex length of 2.86 μm.


3a
1μ

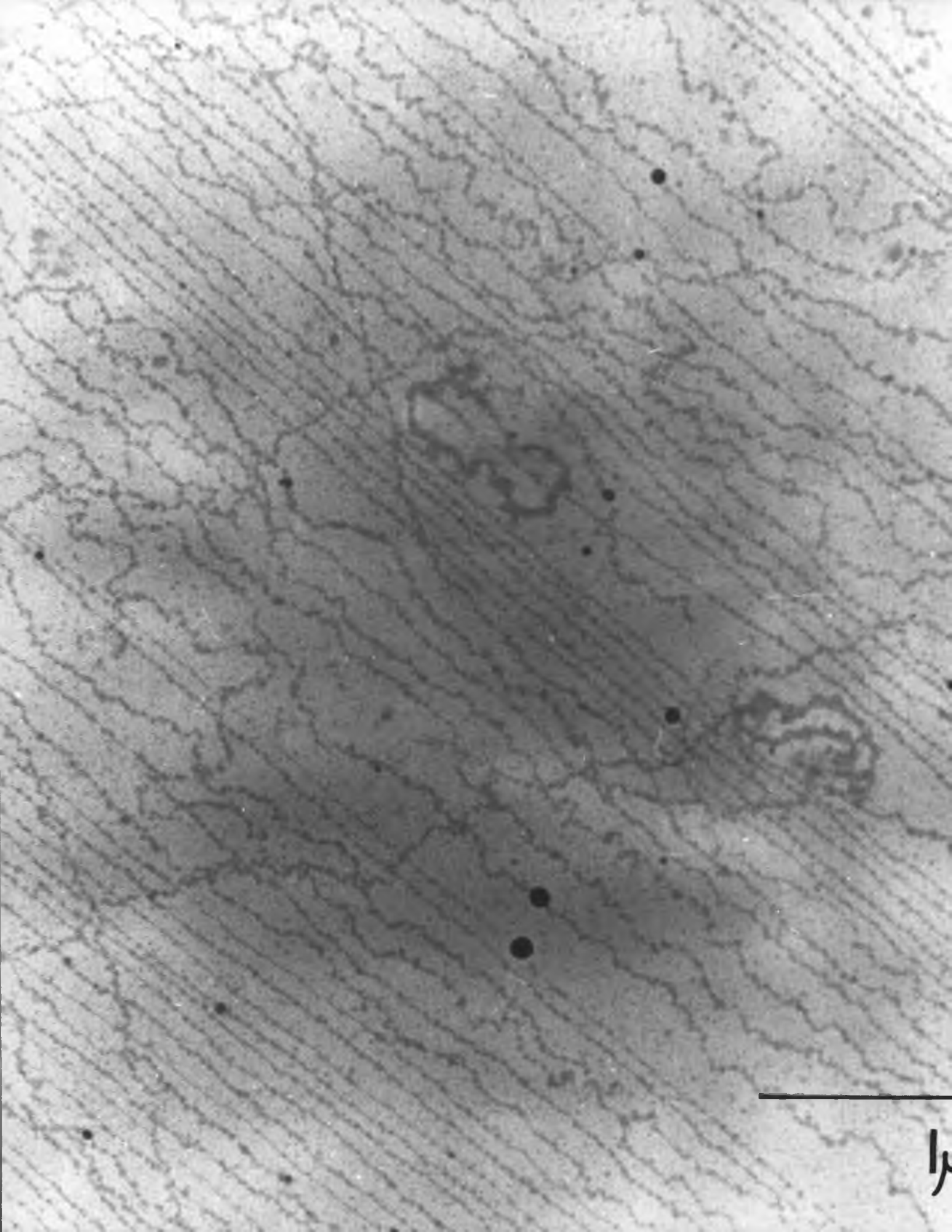

3b


3c

III - 4 Arrow denotes a putative "polysome"-like structure found in preparations of T.c. carnifex neurula cell chromatin. A fine fibril is visible connecting the spherical darkly staining bodies, and this may be RNP.


4
1μ

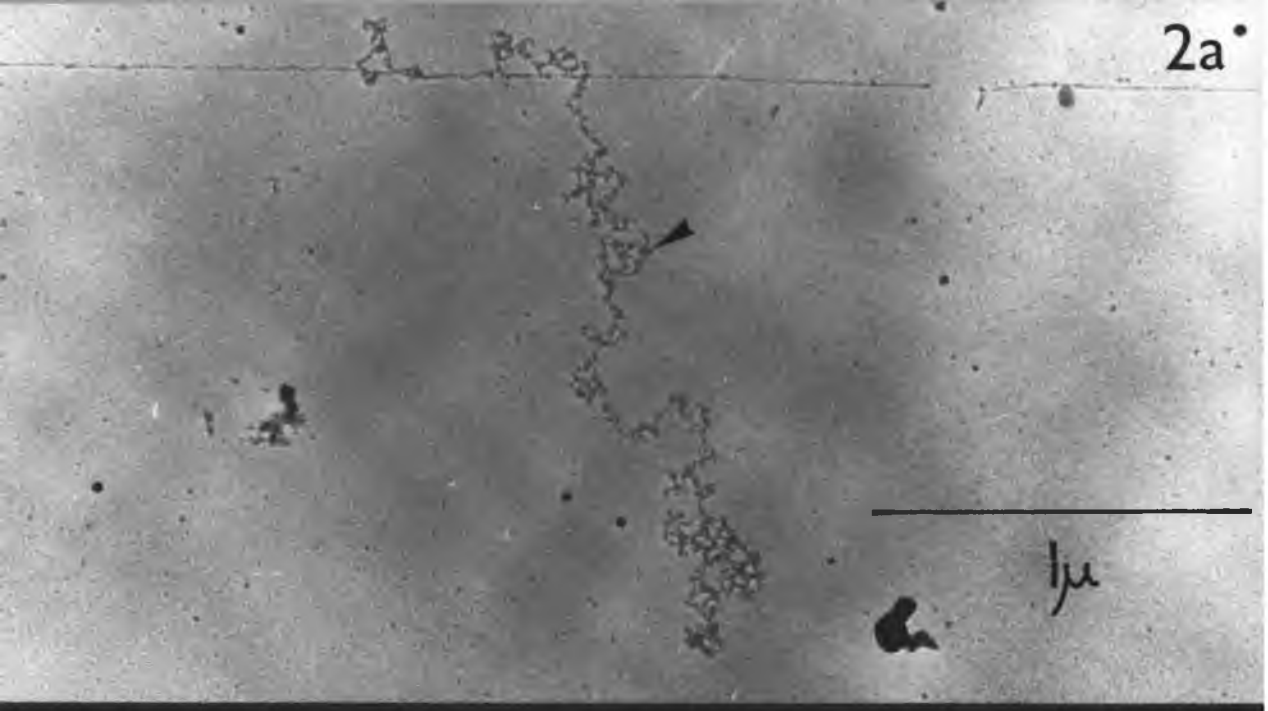
IV - 1 Spread chromatin from a preparation of X. laevis cultured cells treated with 20 µg/ml cortisol. DNP often occurred as large masses of parallel strands, probably as a result of centrifugation. Nucleosomes were closely spaced so that the interconnecting DNP was seldom visible.



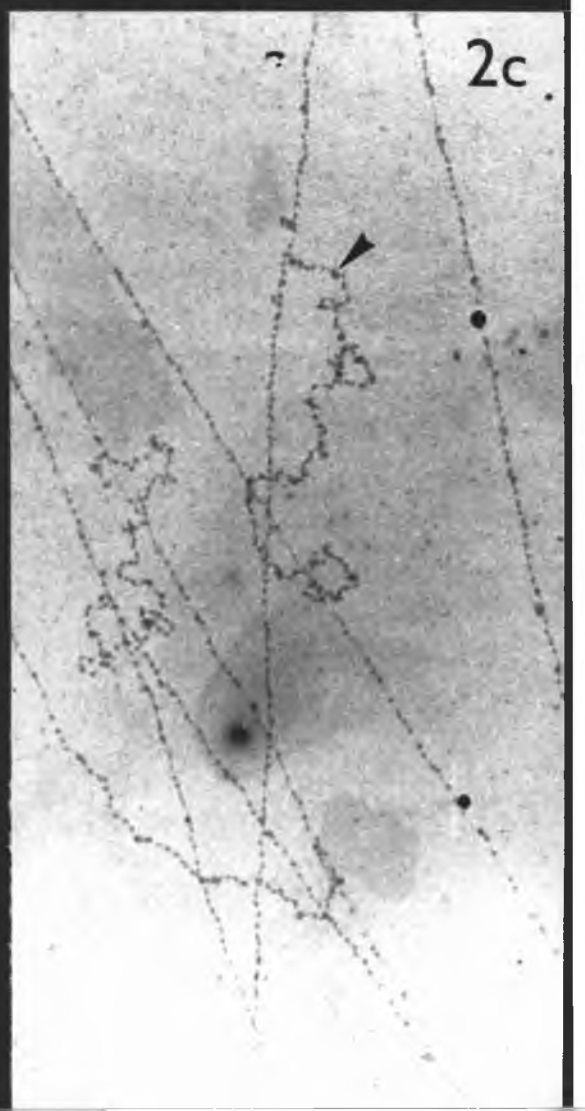
IV - 2a Arrow denotes an isolated RNP fibril attached to chromatin from cortisol-treated X. laevis cultured cells. The great length and high contrast of such primary transcripts is particularly clear. Secondary structure is extensive in this lateral fibril, but varied considerably between different fibrils.

IV - 2b As Fig. IV - 2a. The primary transcript is more extended than in Fig. IV - 2a, and the difference in contrast between RNP and DNP is not so great. The RNP has a clearly beaded structure.

IV - 2c As Fig. IV - 2a.

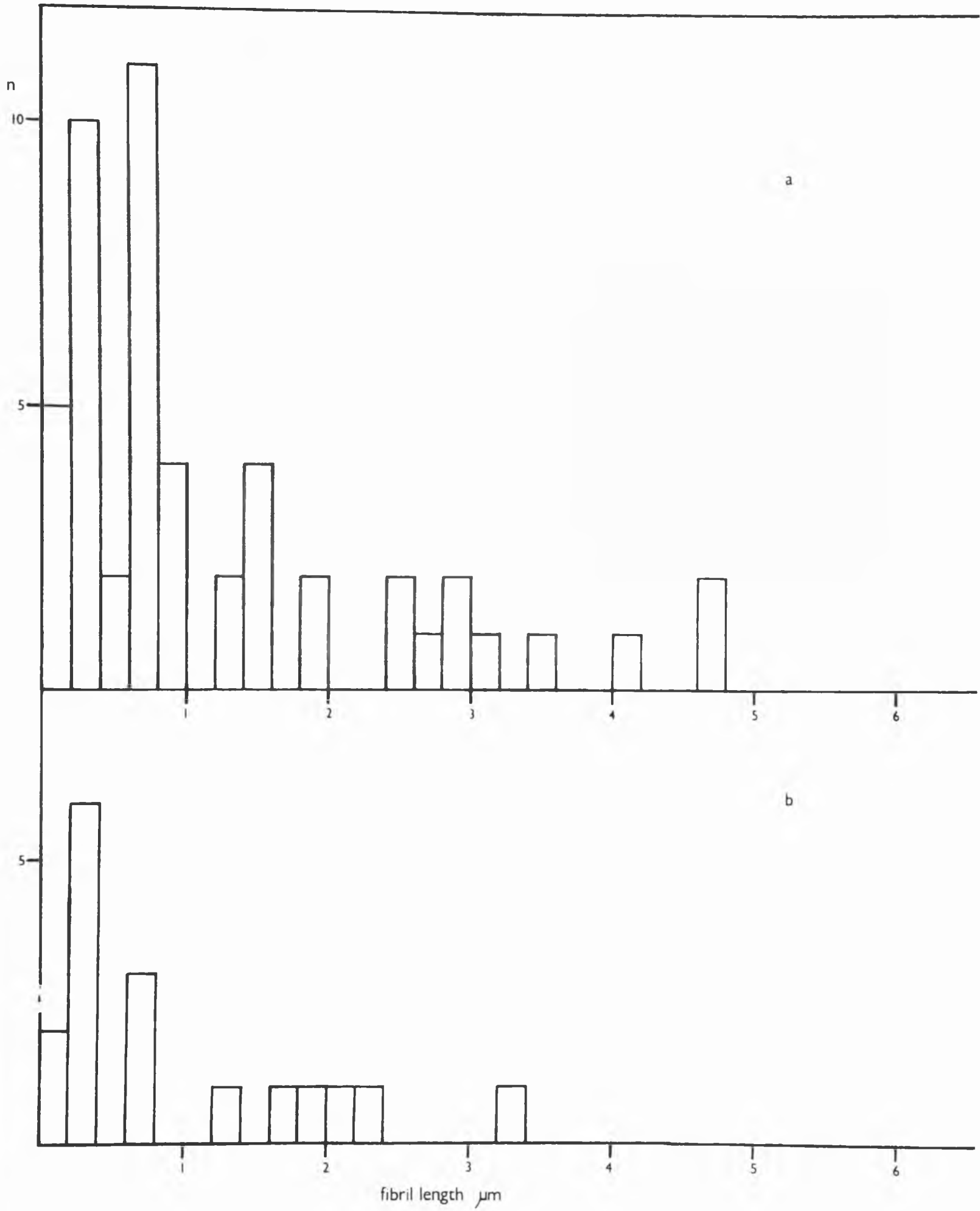

2a
1μ


2b


2c

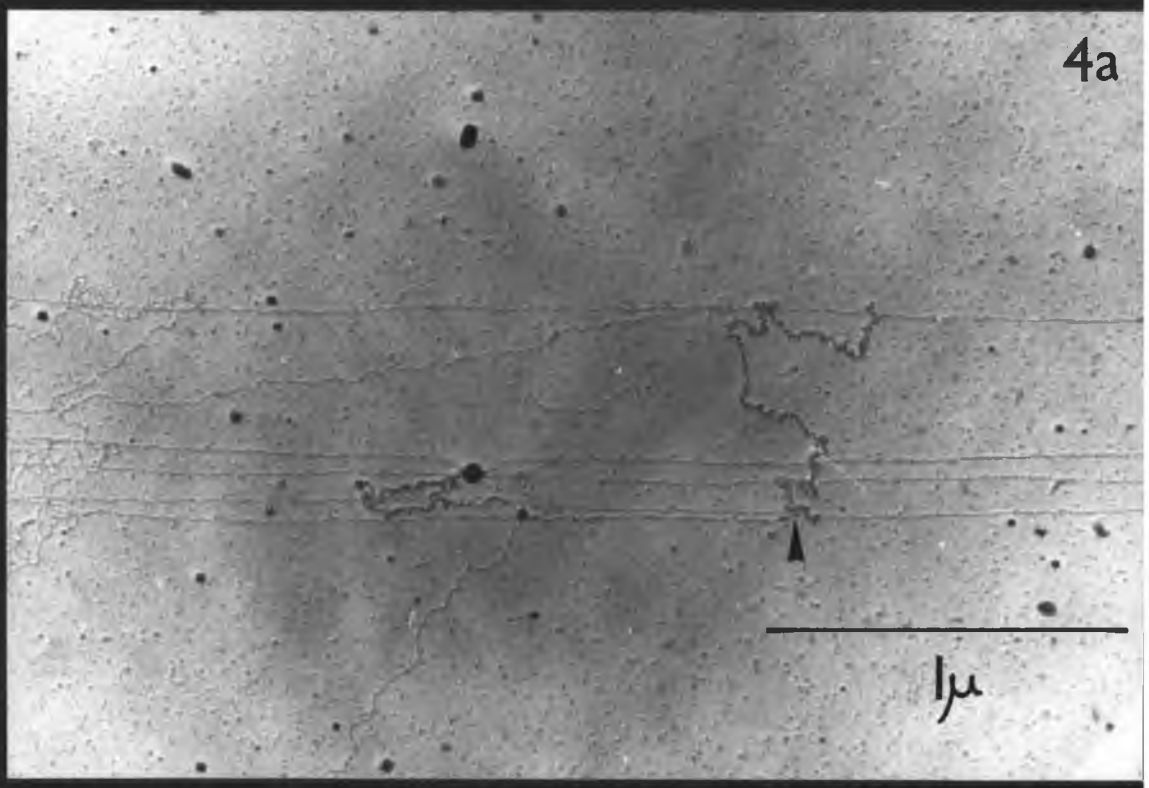
IV - 3a Histogram showing the distribution of contour lengths of isolated RNP molecules attached to chromatin of X. laevis cultured cells treated with cortisol. Median value 0.74 µm. Range 7.5 µm.

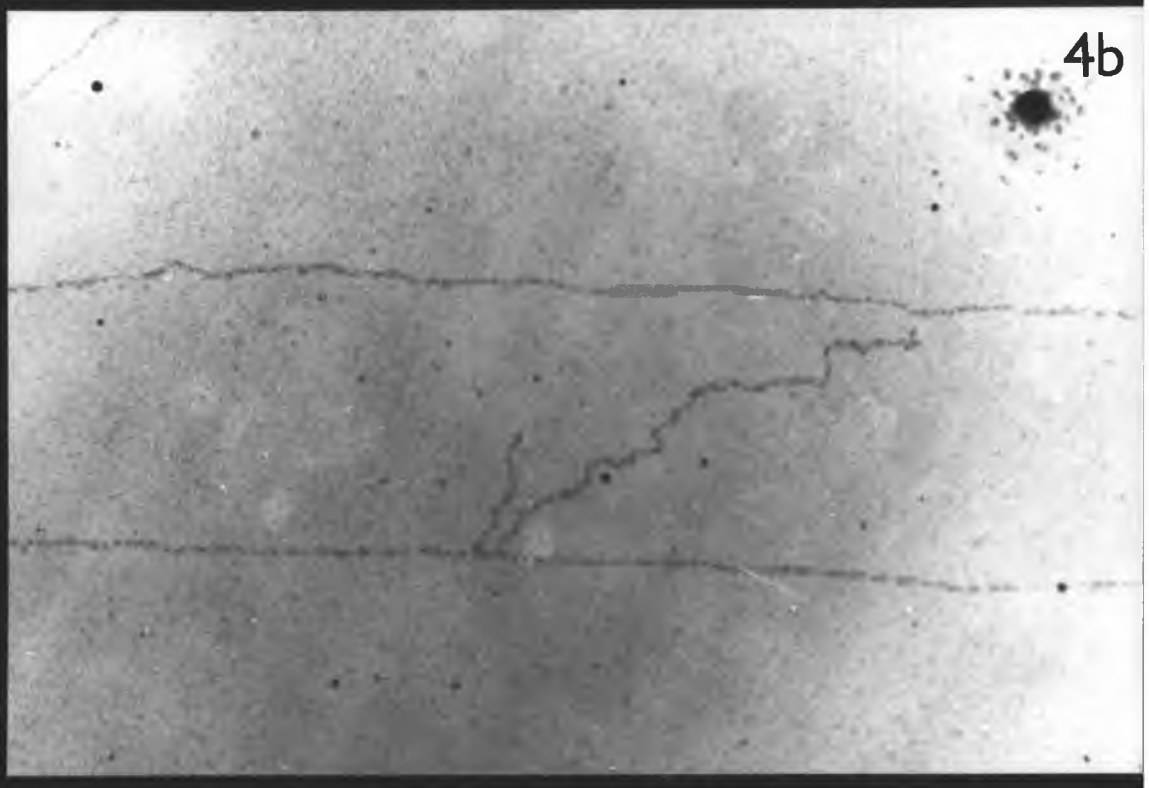
IV - 3b Histogram showing the distribution of contour lengths of terminal fibrils of transcription complexes consisting of two or more $R^{N}P$ fibrils, from X. laevis cultured cells treated with cortisol. Median value 0.65 µm. Range 7.1 µm.


n
10
5
a
1
2
3
4
5
6
b
5
1
2
3
4
5
6
fibril length μm

IV - 4a Group of two RNP fibrils attached to chromatin of cortisol-treated X. laevis cultured cells. RNP is identified by its high contrast and condensed structure. Arrow denotes a putative replicating region of the DNP.

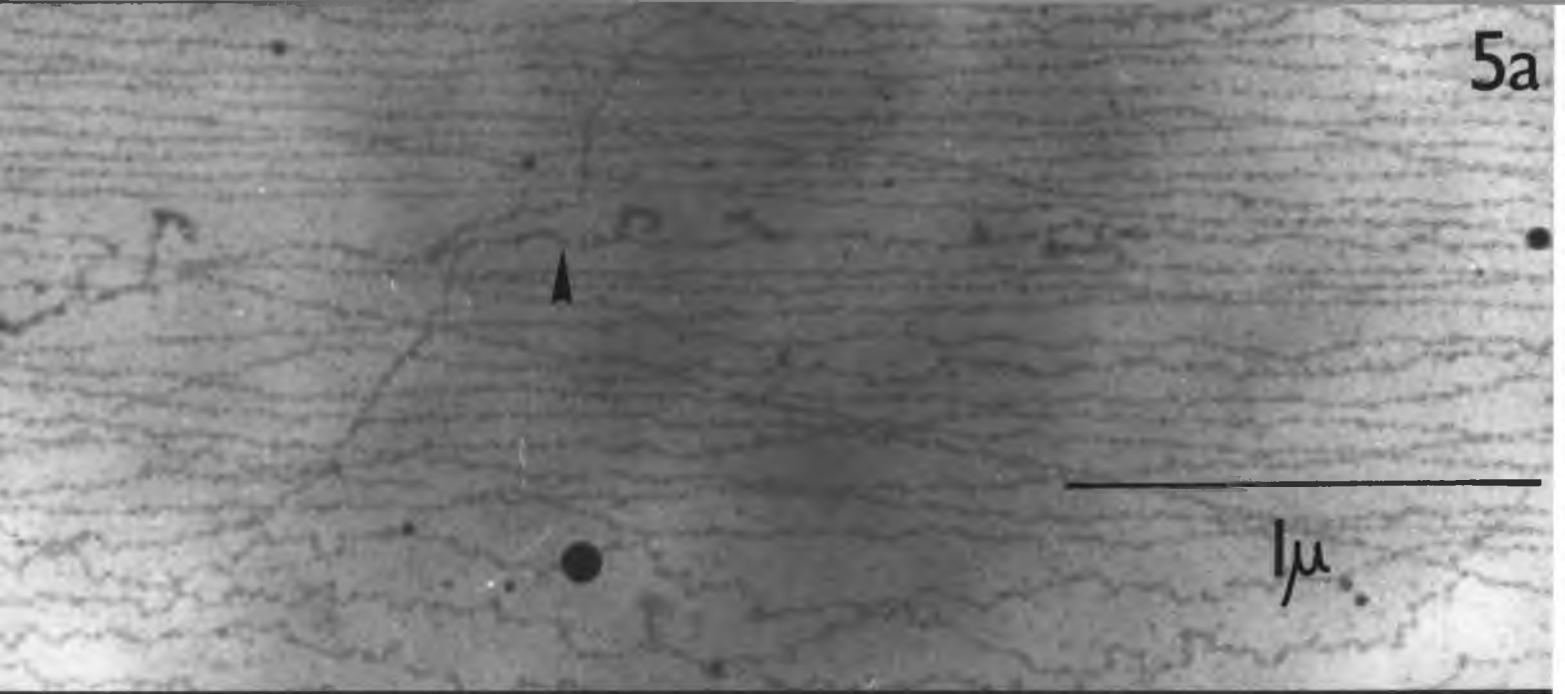
IV - 4b As Fig. IV - 4a.

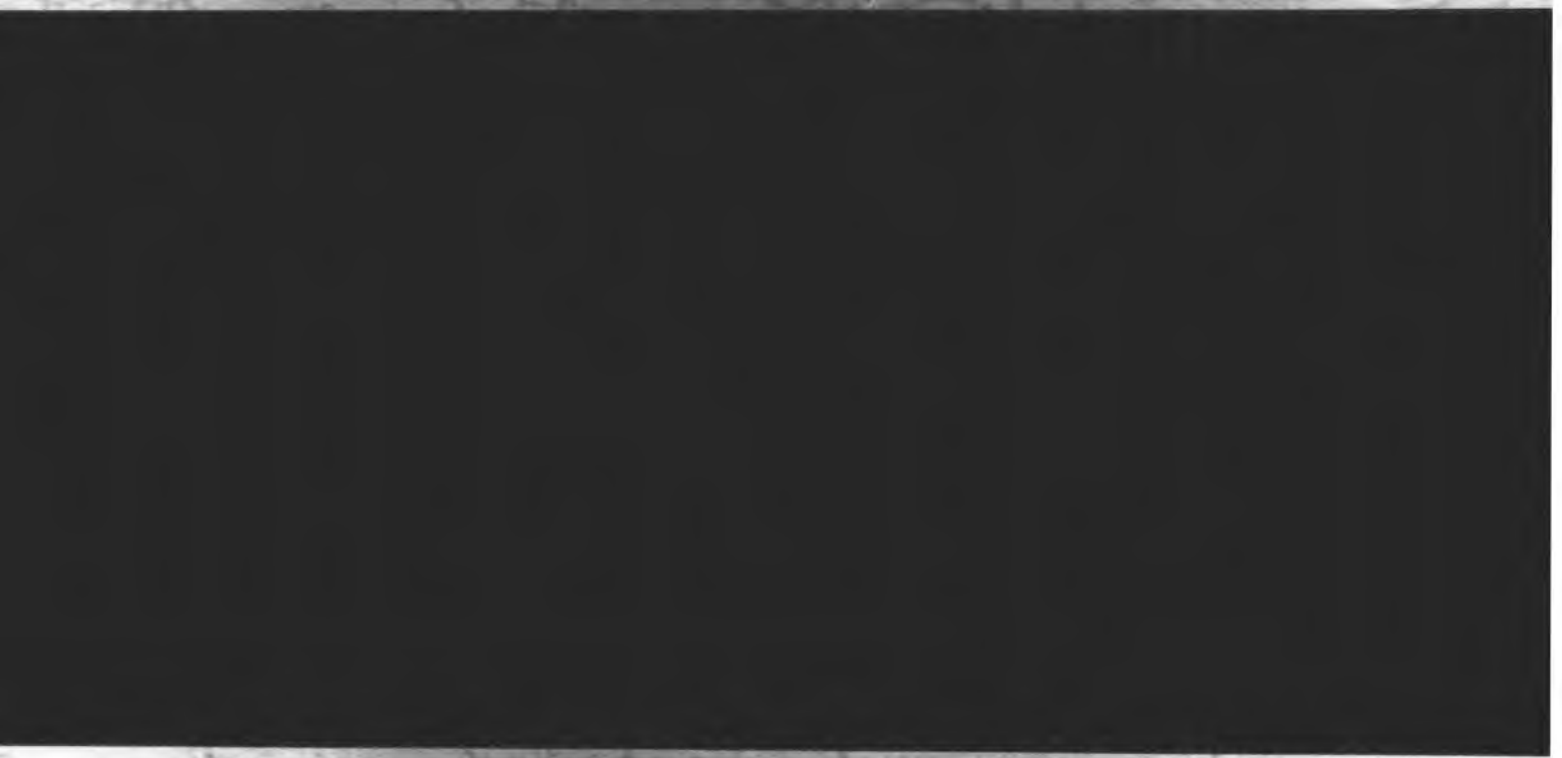

4a
1μ

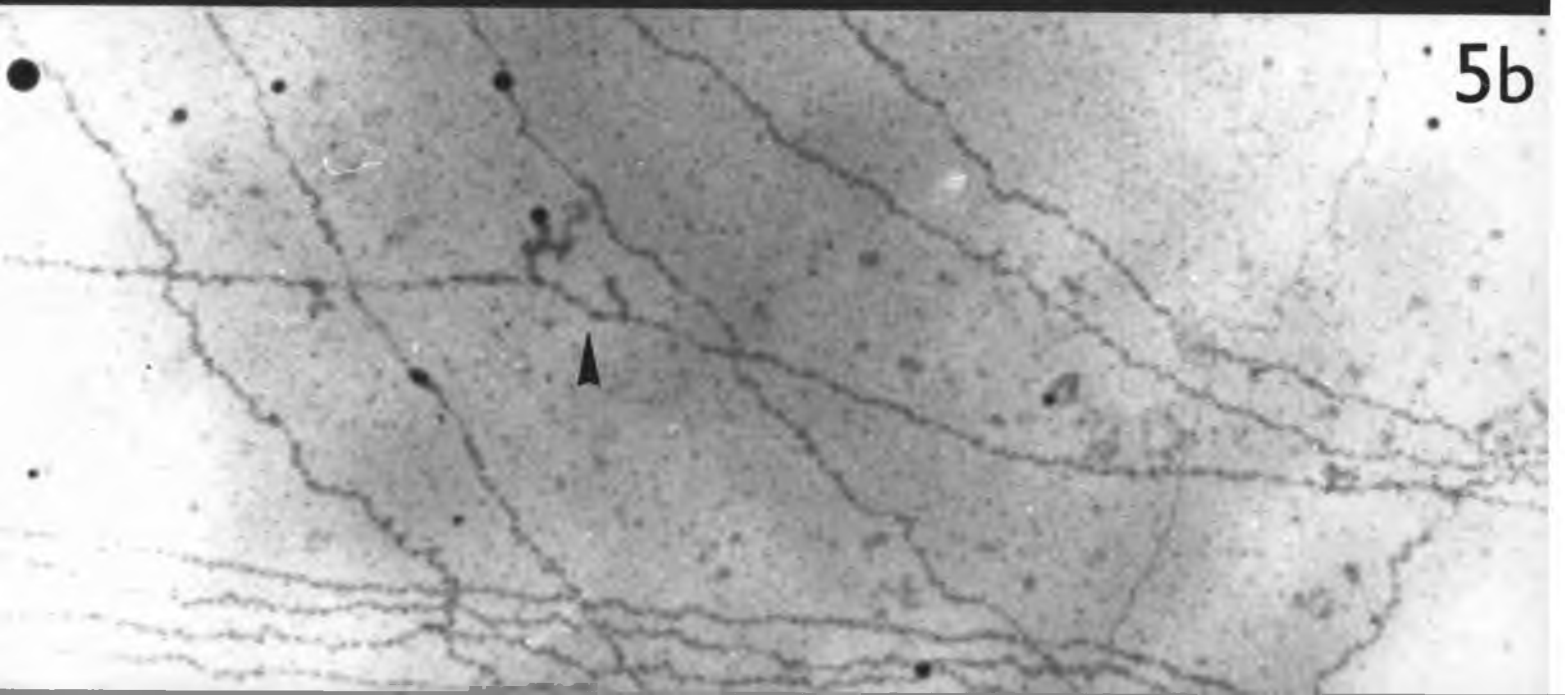

4b

IV - 5a Arrow denotes a transcription complex found in chromatin of cortisol-treated X. laevis cultured cells. Laird Analysis gave a value for transcription complex length, of 3.45 μm.

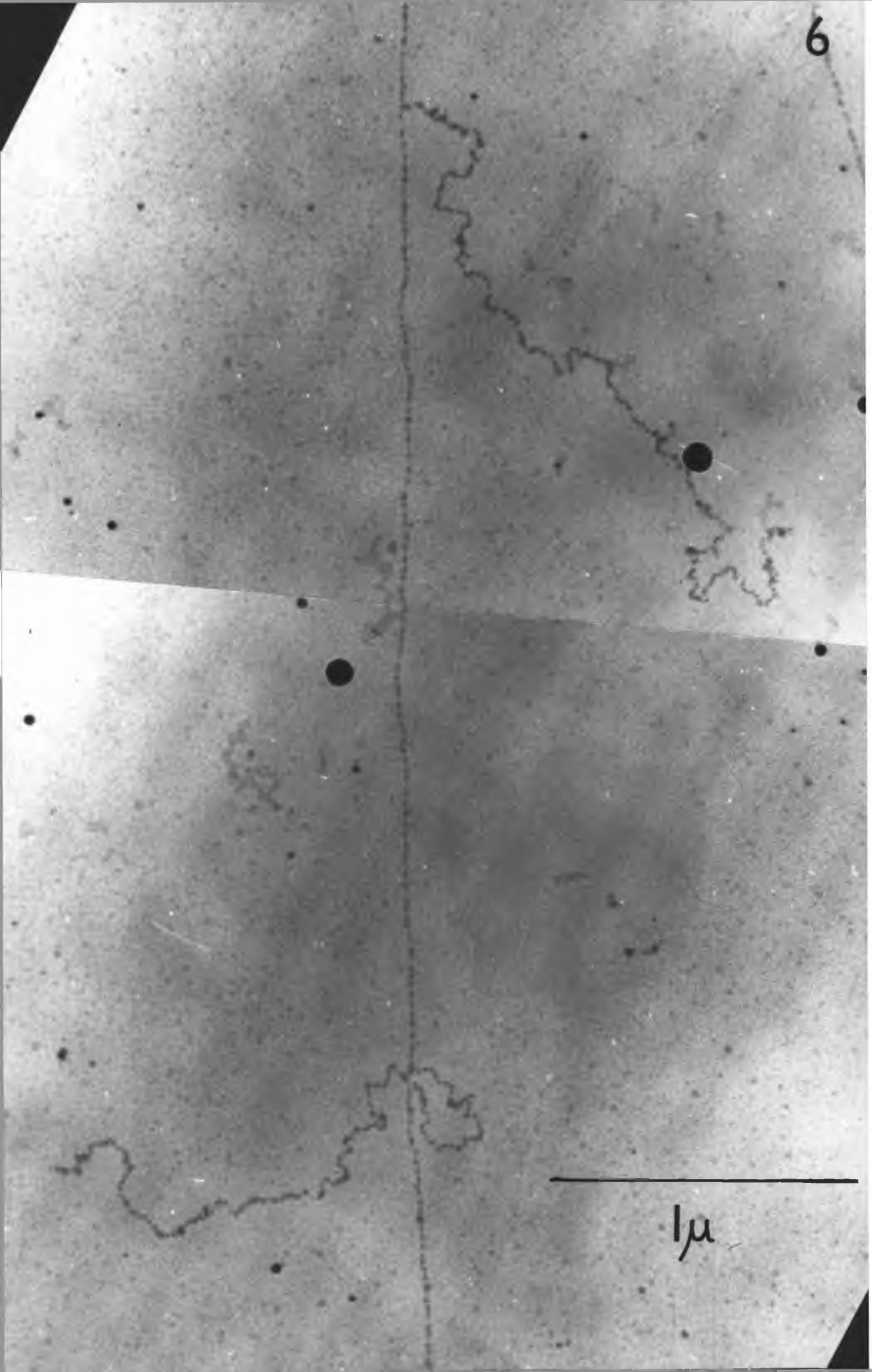
IV - 5b As Fig. IV - 5a. Laird Analysis gave a value for transcription complex length of 2.77 μm.


5a
1μ




5b

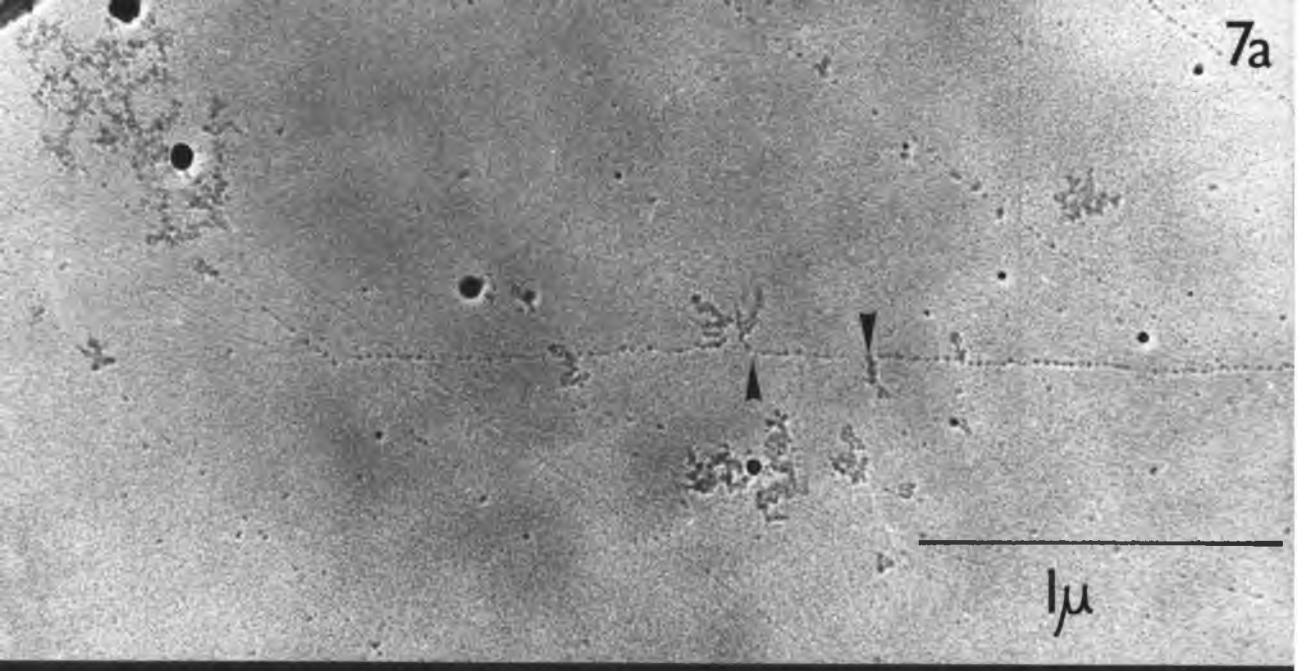
IV - 6. Group of two RNP fibrils from the same source as Figs IV - 5a - b. As the separation of these two fibrils is greater than 1 μm, they were counted as two separate transcription complexes in my analysis. However, if they are counted as one transcription complex, Laird Analysis gives a value for complex length of 13.8 μm. The DNP axis is clearly of a beaded structure.

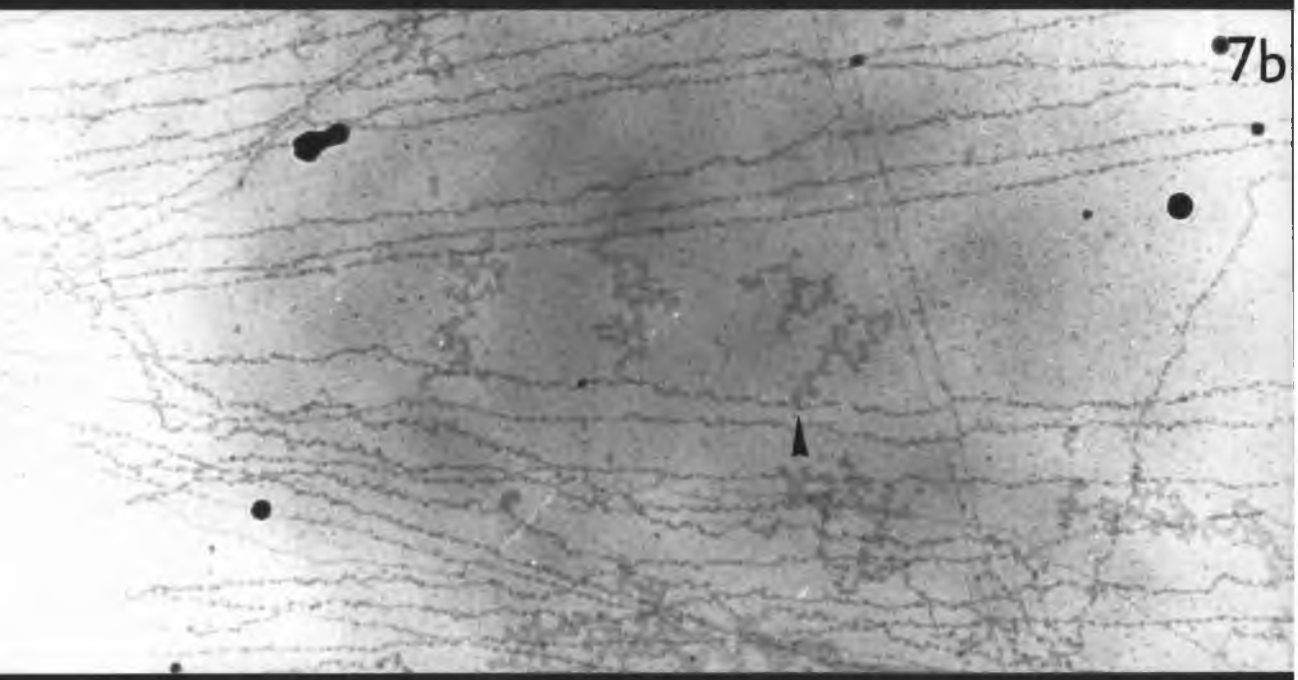

6
1μ

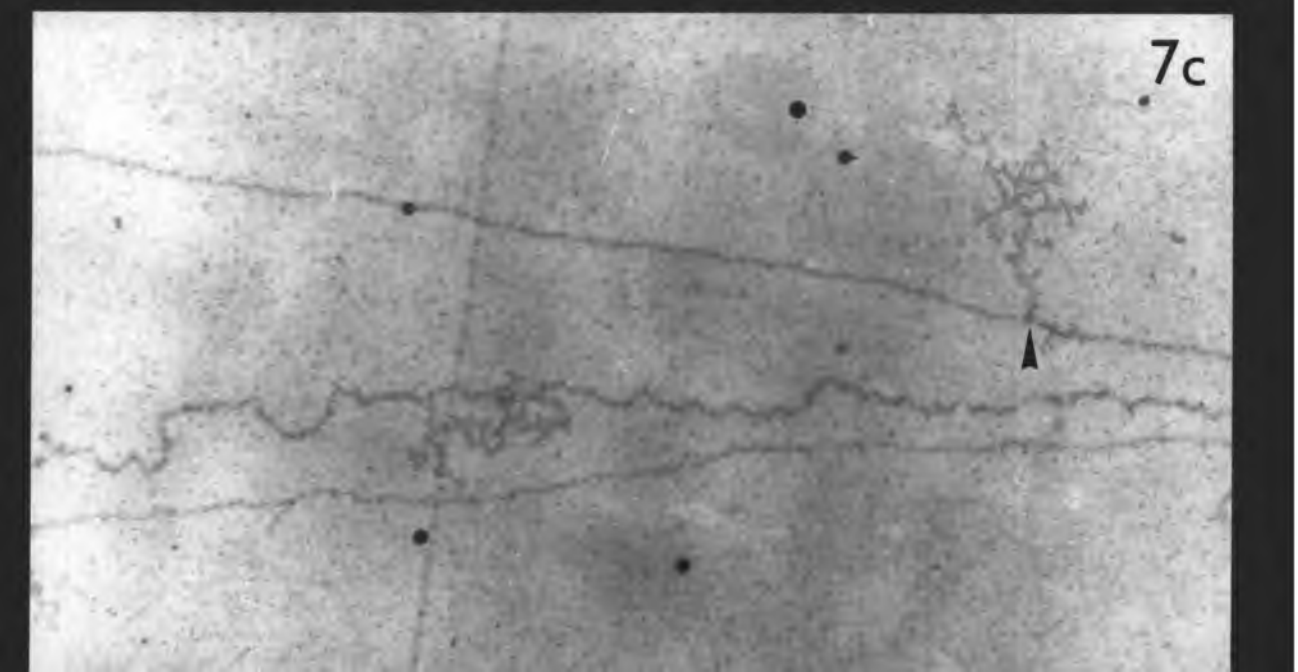
IV - 7a "Bushy" RNP from X. laevis cultured cells treated with cortisol (arrows).

IV - 7b As Fig. IV - 7a.

IV - 7c As Fig. IV - 7a.

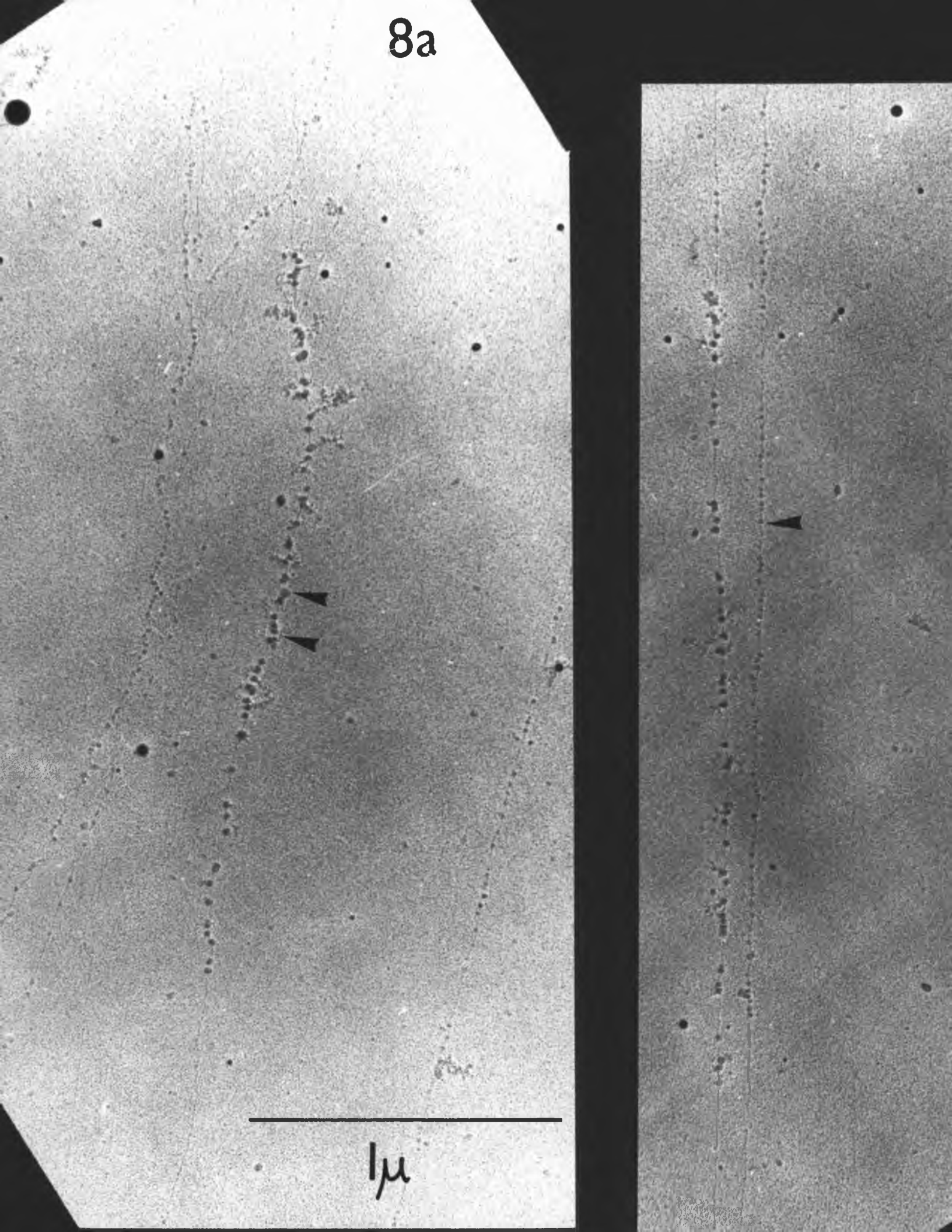

7a
1μ


7b


7c

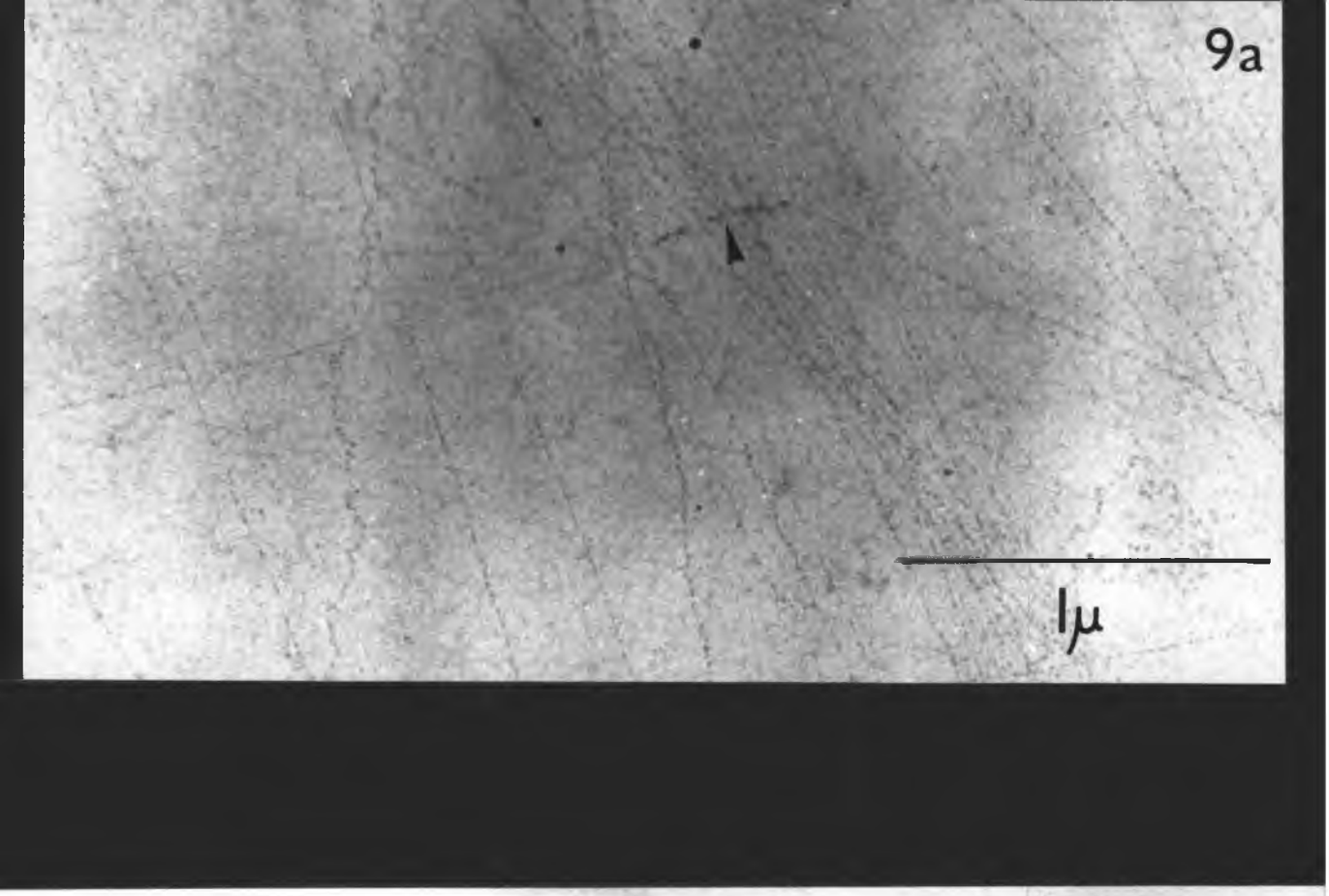
IV - 8a Ribosomal transcription complex from cortisol-treated X. laevis cultured cells grown at low inoculation density. Arrows denote RNA polymerase molecules. RNP was sparse, possibly due to the action of endogenous nucleases.

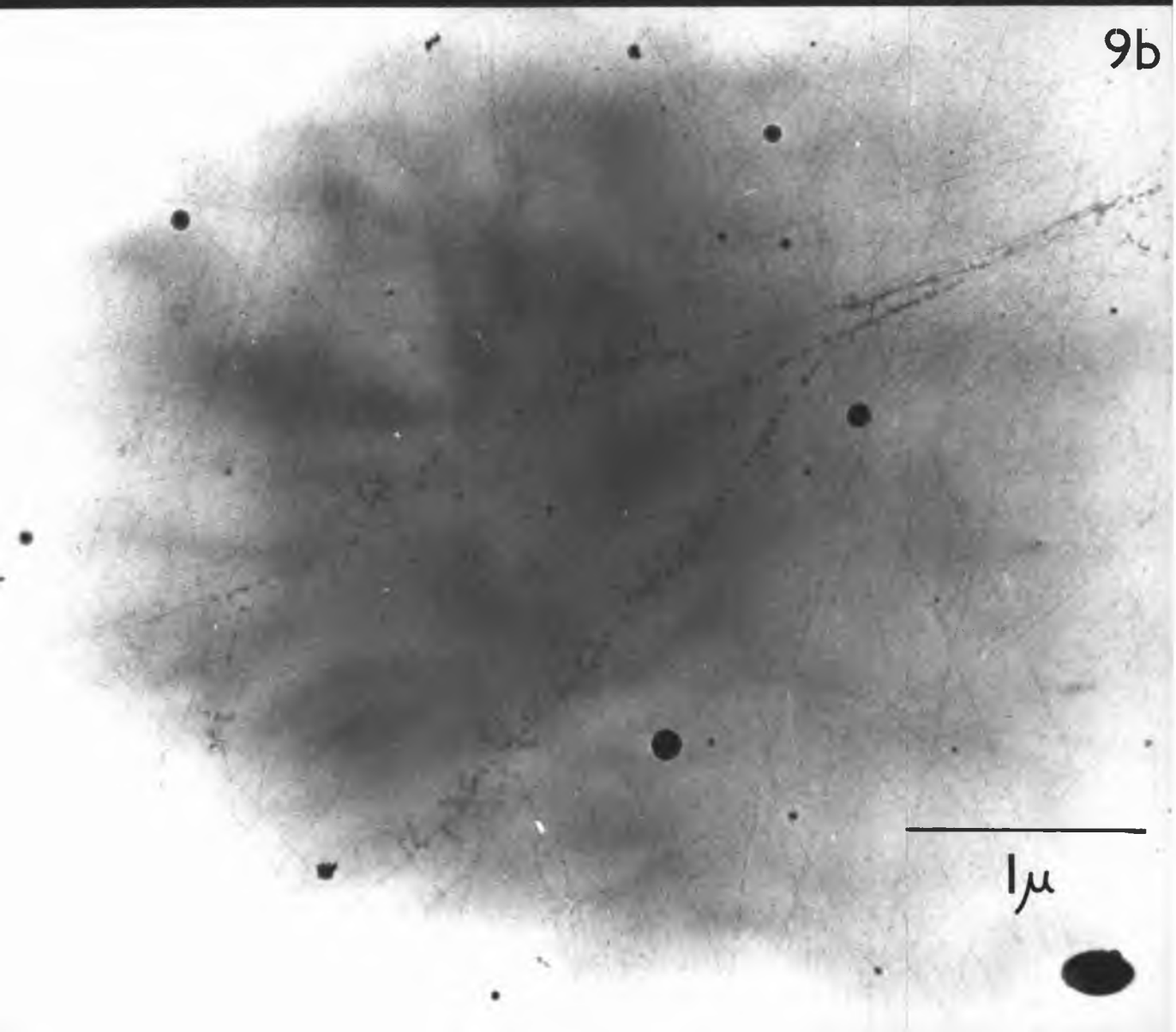
IV - 8b As Fig. IV - 8a. Arrow denotes a spacer region characterized by a nucleosomal structure which was absent in the matrix units.


8a
1μ

IV - 9a Arrow denotes a short length of RNA polymerases from chromatin of X. laevis cultured cells grown at low density before cortisol treatment. Such RNA polymerases may be indicative of spacer transcription, as clear polymerases were only seen on ribosomal transcription complexes.

IV - 9b Ribosomal transcription complexes from chromatin of X. laevis cultured cells grown at high inoculation density before cortisol treatment. Mean matrix unit length was 1.45 μm. Spacer regions were not clearly displayed.


9a
1μ

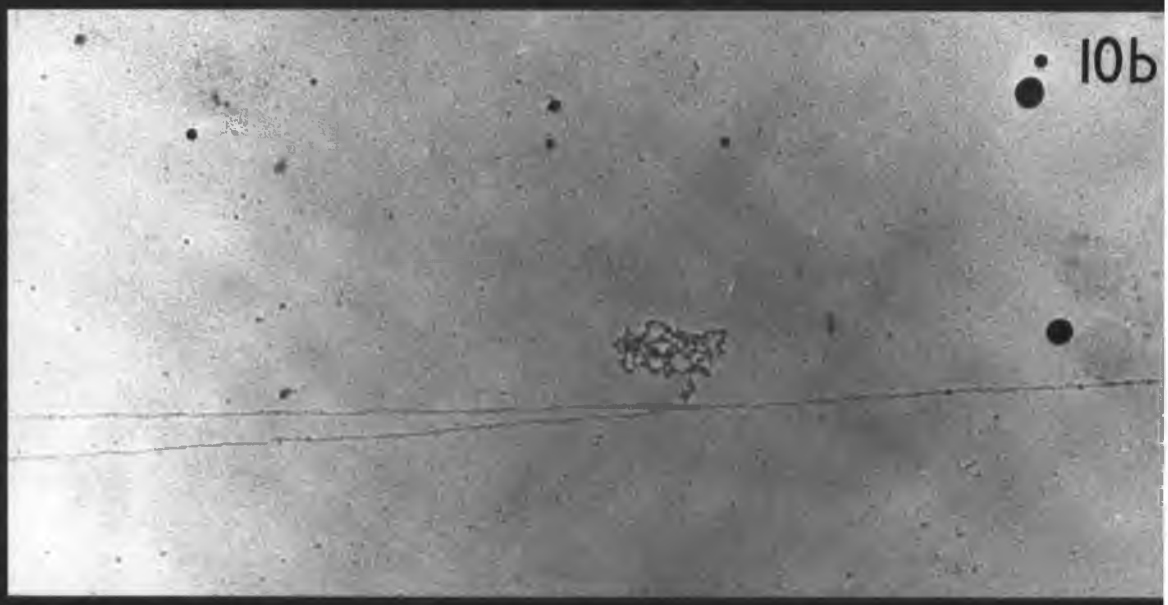

9b
1μ

IV - 10a Configuration indicative of simultaneous DNA replication and transcription. Arrow denotes replication fork. A long RNP fibril is present 0.9 μm away from the fork region.
p = polysaccharide

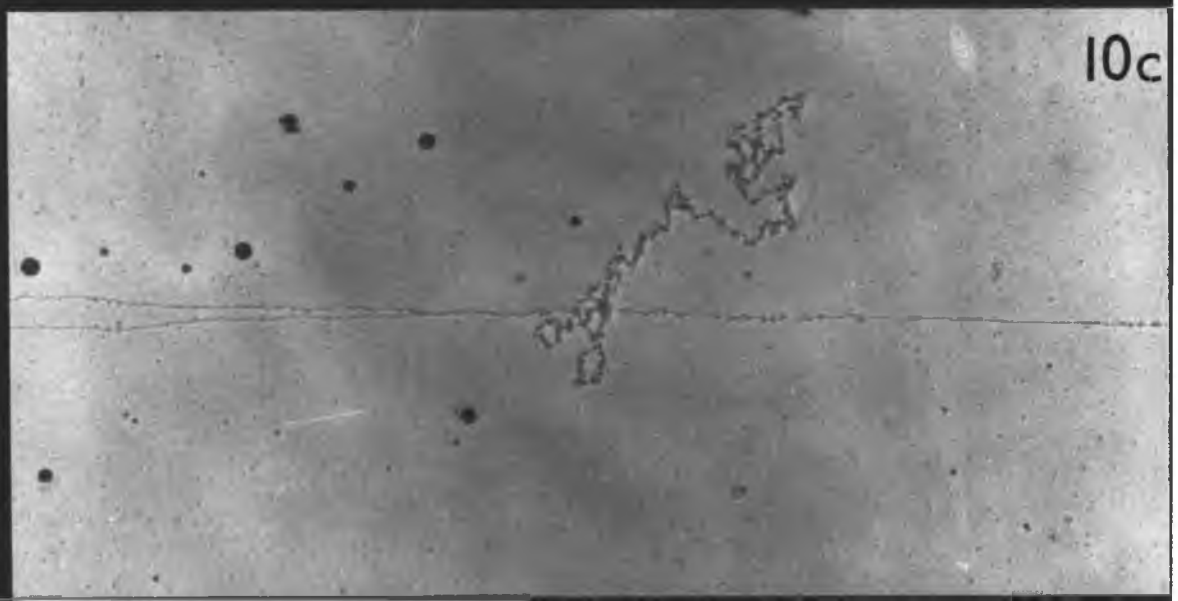
IV - 10b As Fig. IV - 10a. This structure is more equivocal than Fig. IV - 10a, and may represent two DNP molecules crossing each other, one of which has broken off and become more condensed.

IV - 10c As Fig. IV - 10b.

10a



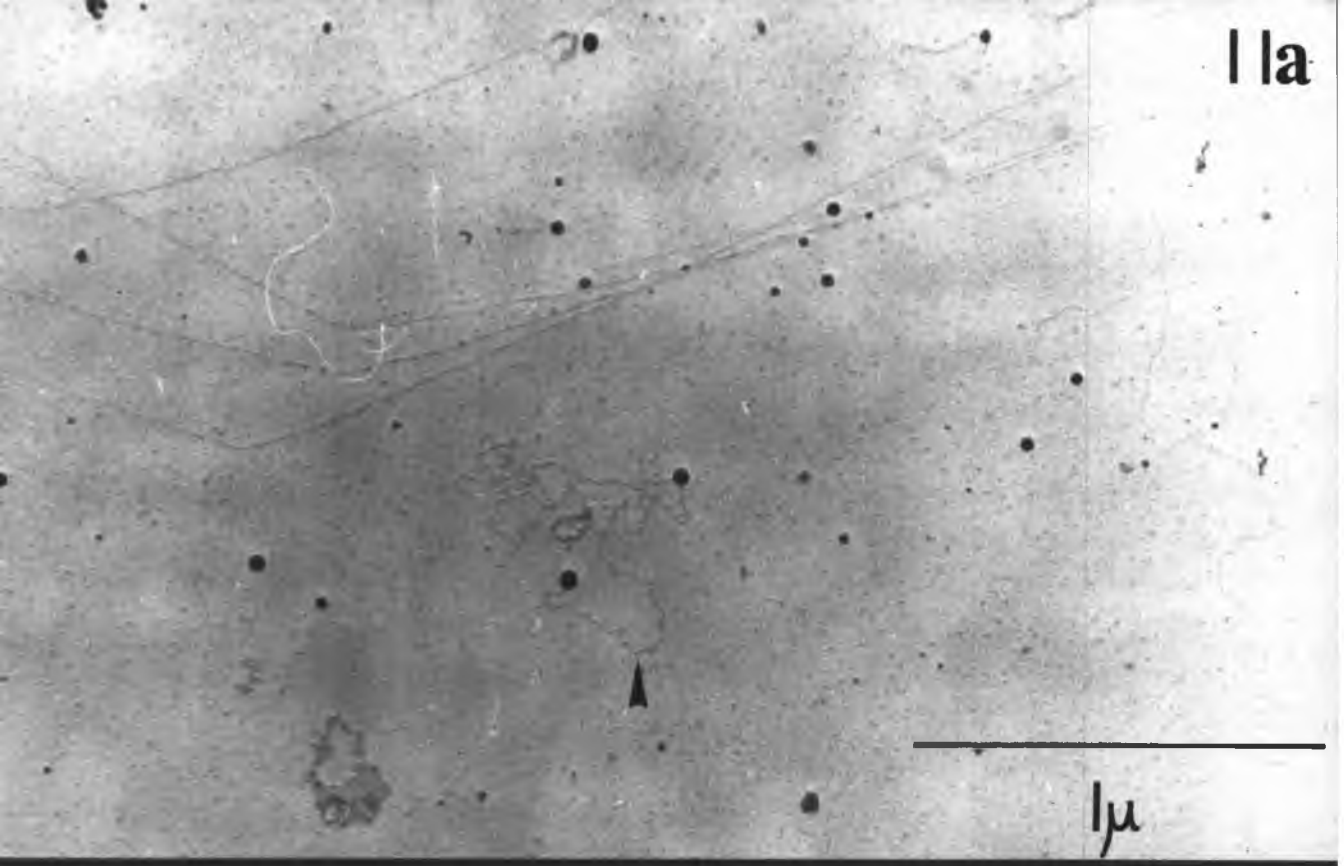
10b

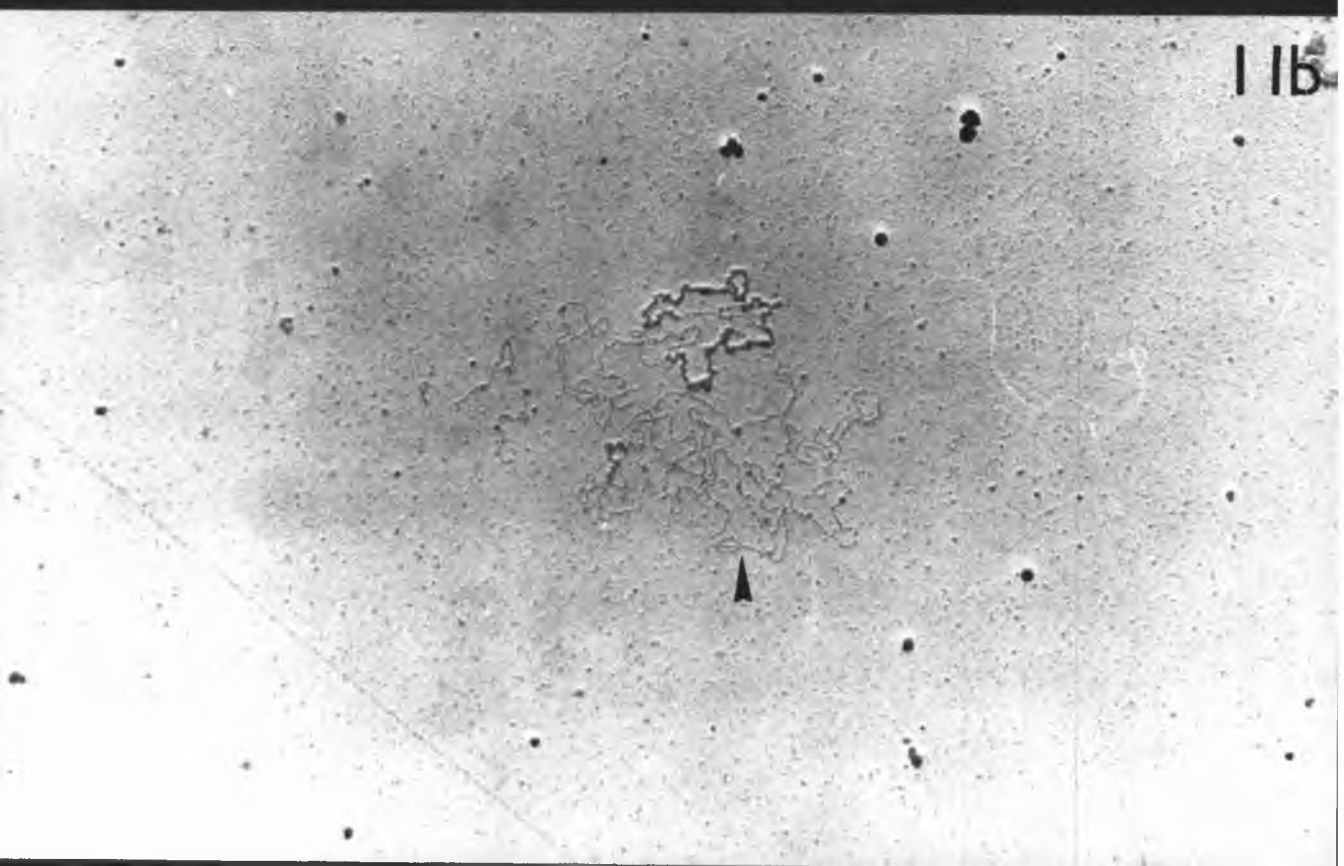


10c

IV - 11a Arrow denotes a putative mitchondrial DNA circle from X. laevis cultured cells treated with cortisol. This molecule has a "bubble" region perhaps comprising a replication fork.

IV - 11b Arrow denotes a putative mitochondrial DNA circle from the same source as Fig. IV - 11a. bearing three lateral RNP fibrils. These are identified as such by their high contrast and condensed structure. Laird Analysis was not possible.

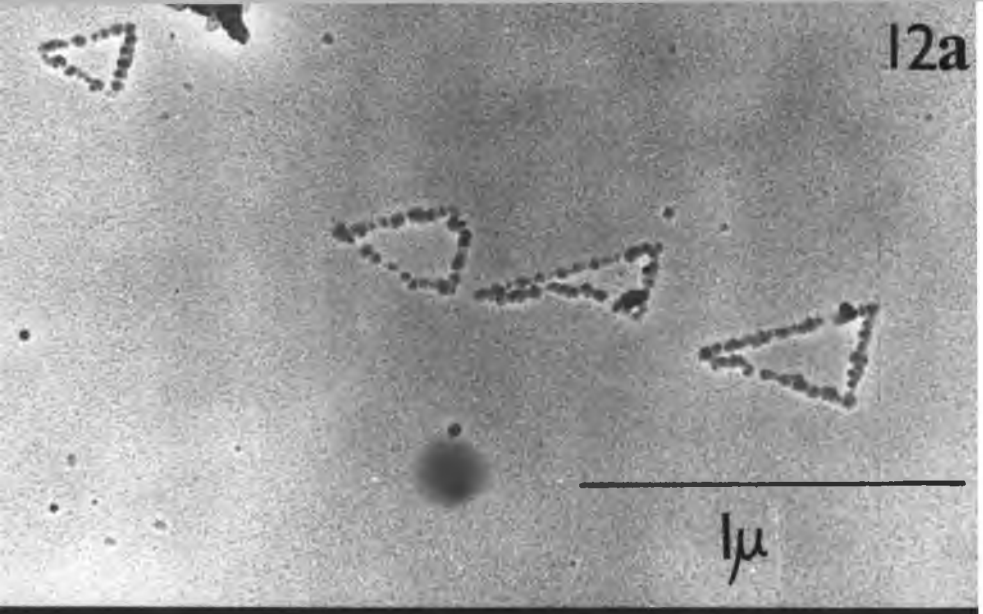

11a
1μ

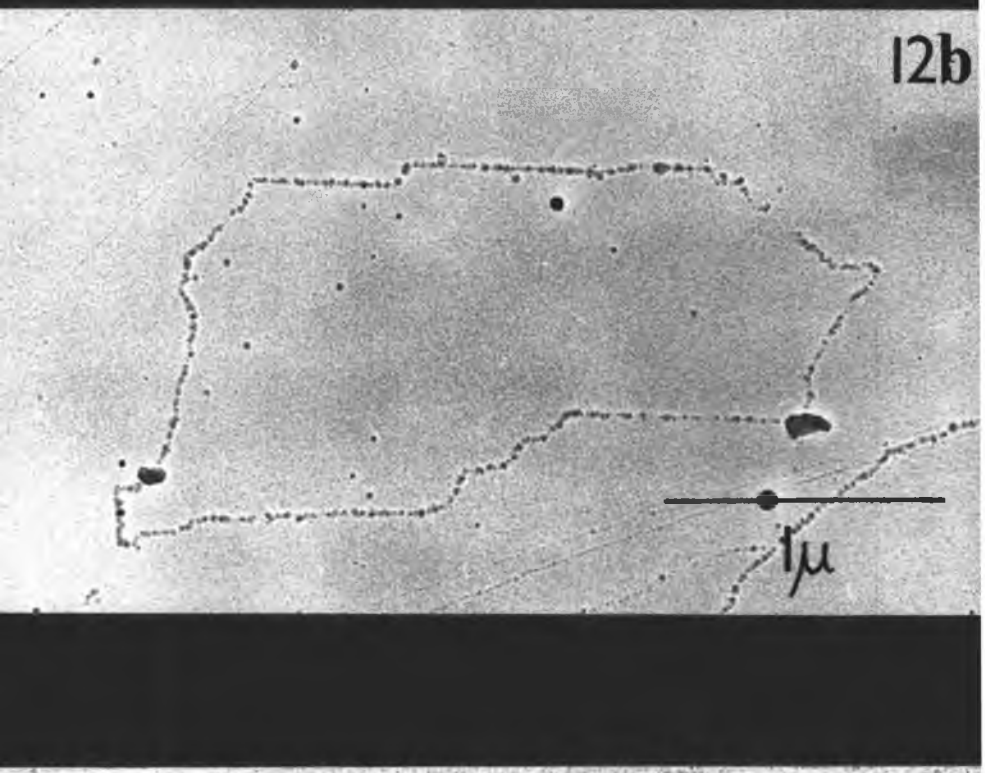

11b

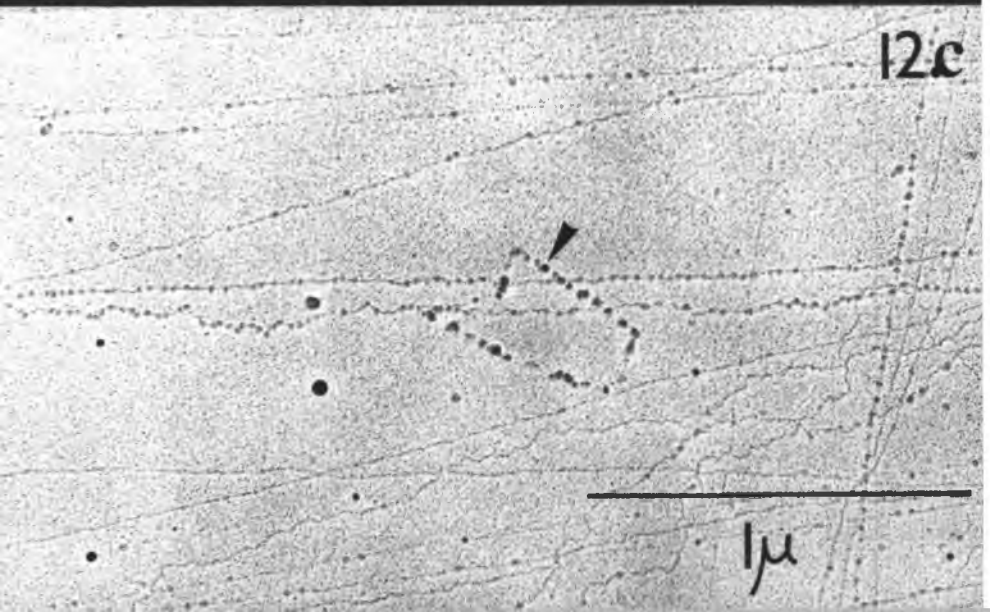
IV - 12a Free circles found in preparations from the same source as Fig. IV - 11a. The beaded structure of these rings is clear. The beads were slightly larger than supernucleosomes.

IV - 12b As Fig. IV - 12a.

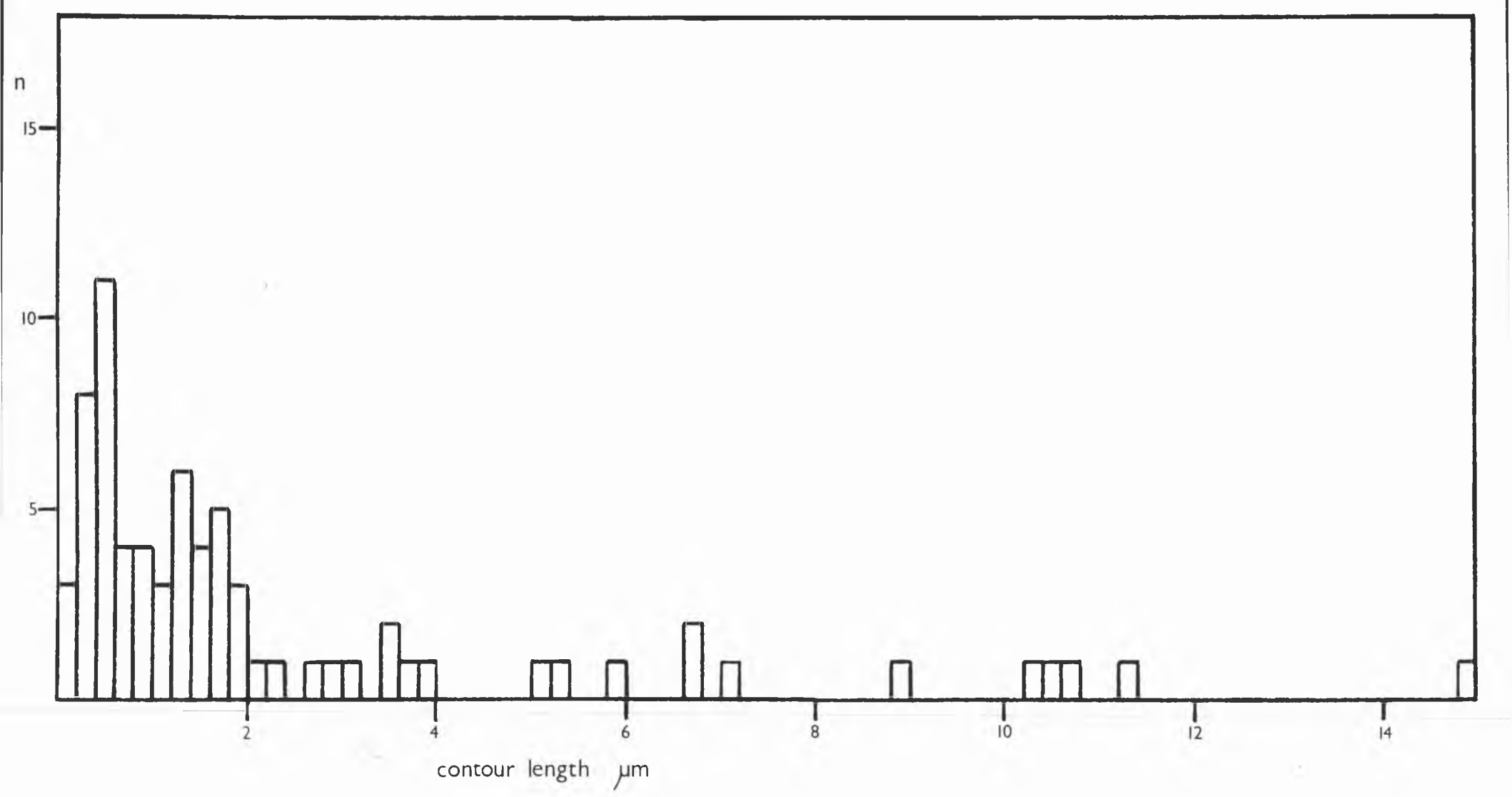
IV - 12c As Fig. IV - 12a (arrow). Nuclear chromatin, both nucleosomal and smooth, is also present.


12a
1μ


12b
1μ


12c
1μ

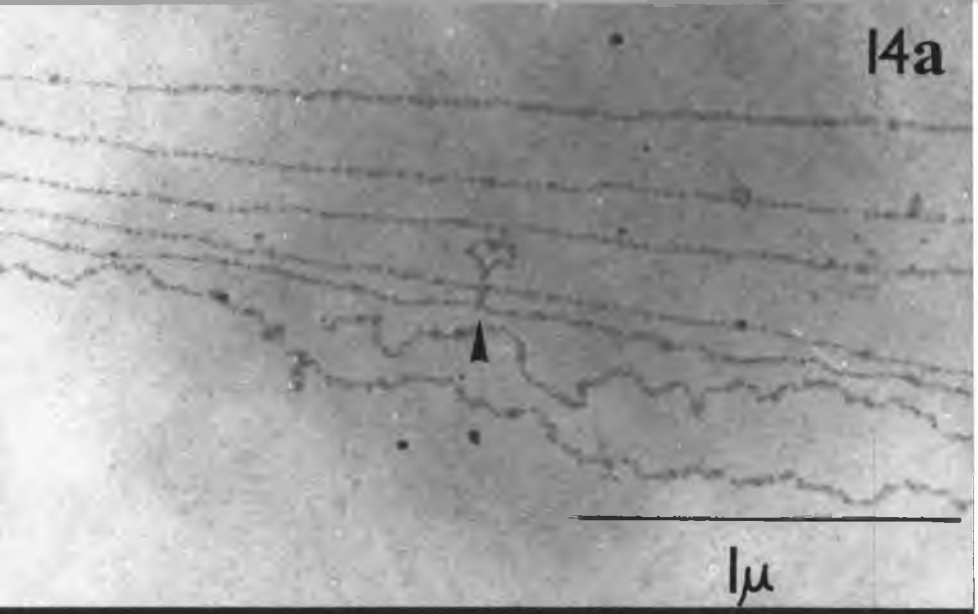
IV - 13 Histogram showing the distribution of the contour lengths of the free circles found in preparations of X. laevis cultured cells treated with cortisol. Median value 1.26 µm. Range 14.8 µm.

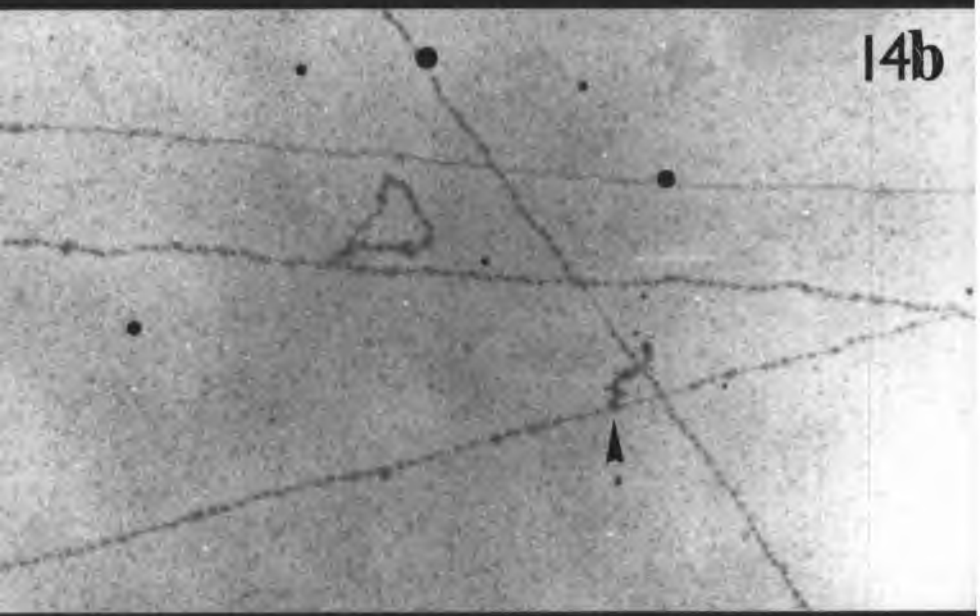

n
15
10
5
2
4
6
8
10
12
14
contour length µm

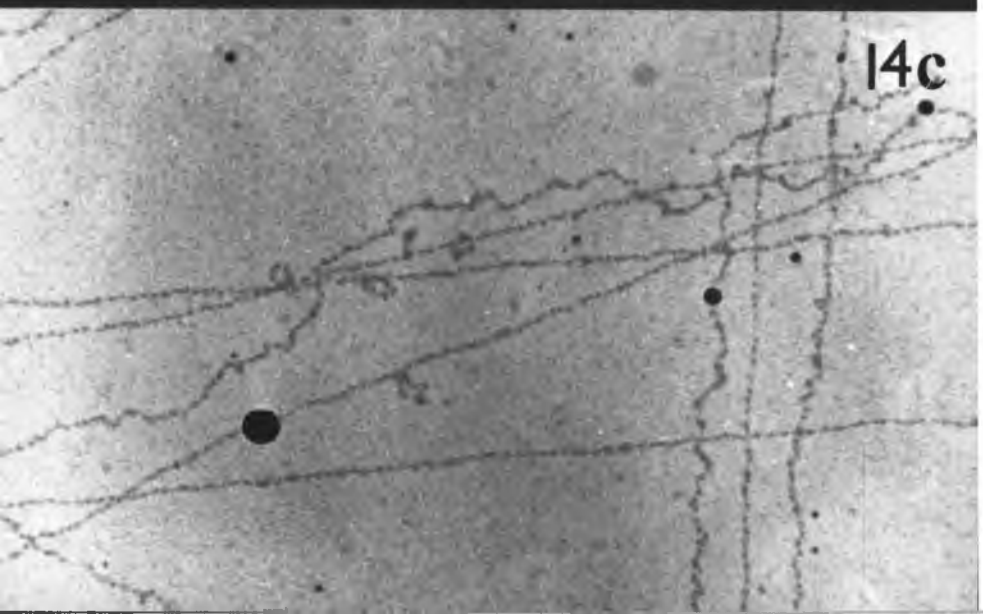
IV - 14a Arrow denotes a ring found on chromatin from X. laevis cultured cells treated with cortisol and resembling the excision intermediates of lampbrush chromosome RNP. Note the DNP axis stretched by centrifugation.

IV - 14b As Fig. IV - 14a. Arrow denotes an RNP fibril on the same preparation.

IV - 14c A group of small rings attached to chromatin of X. laevis cultured cells treated with cortisol.

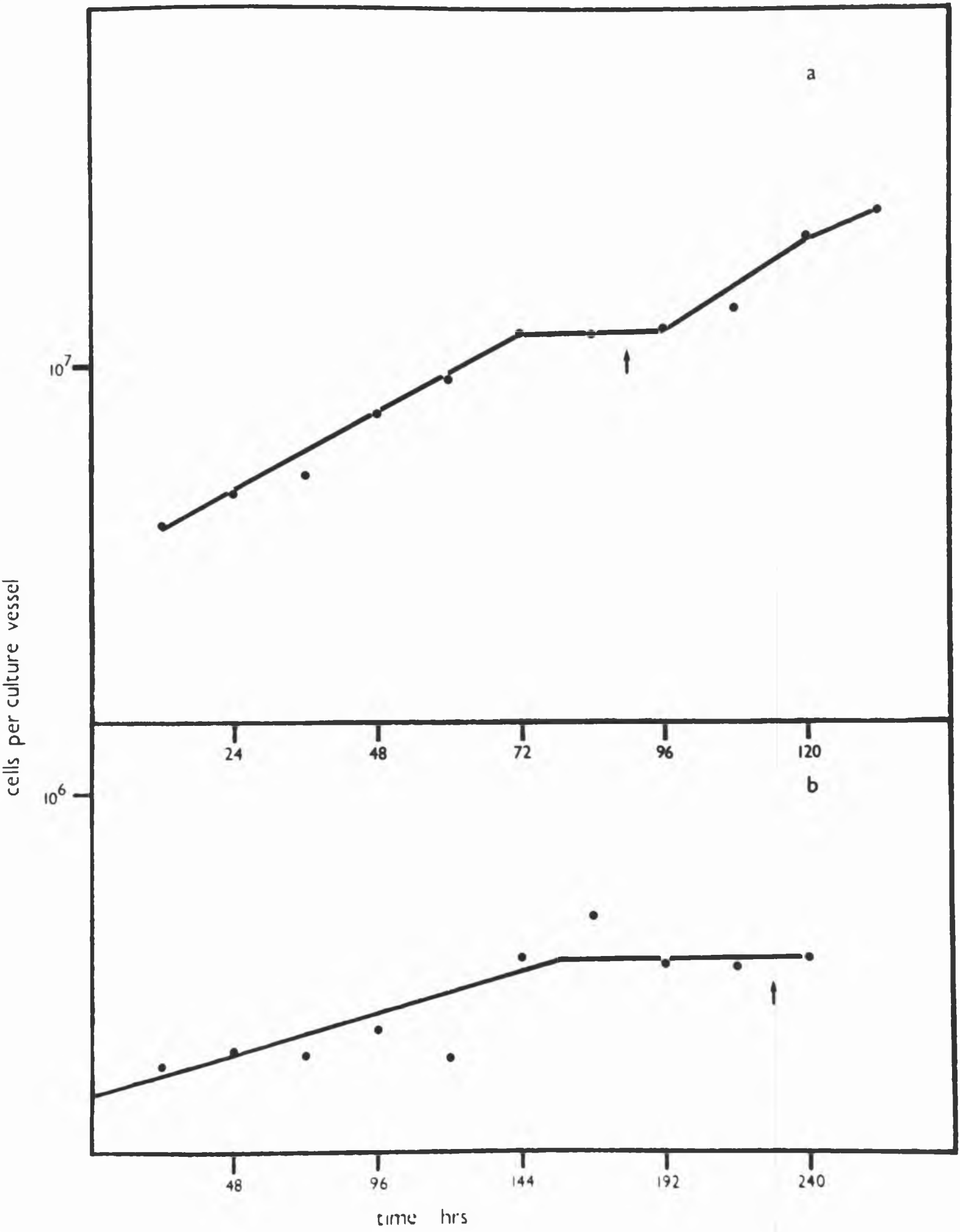

14a
1μ


14b


14c

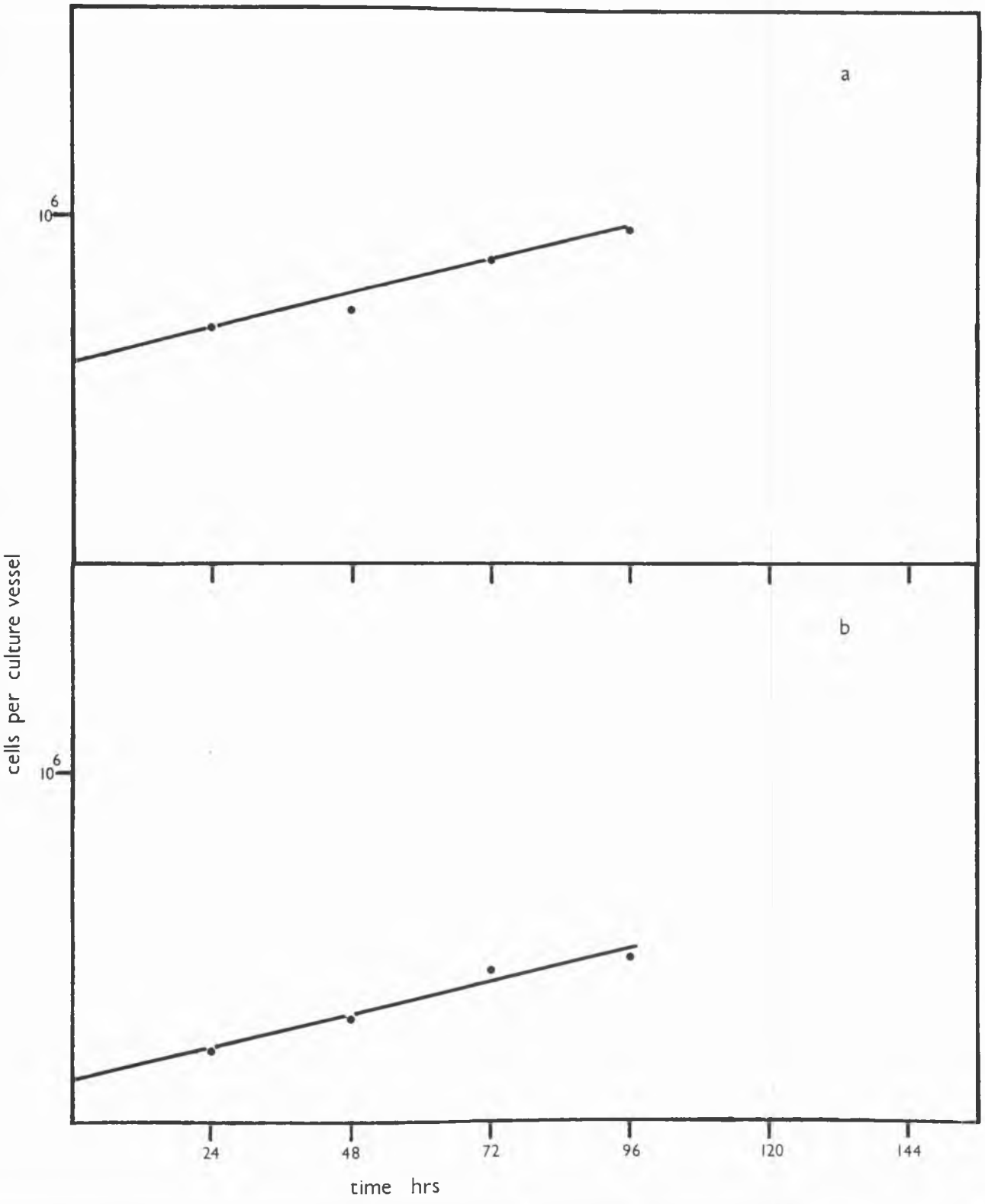
1a Graph showing the growth rate of X. laevis cultured cells at the cell density used for routine subculture purposes.

1b Graph showing the growth rate of T.c. carnifex cultured cells at the cell density used for routine subculture purposes.

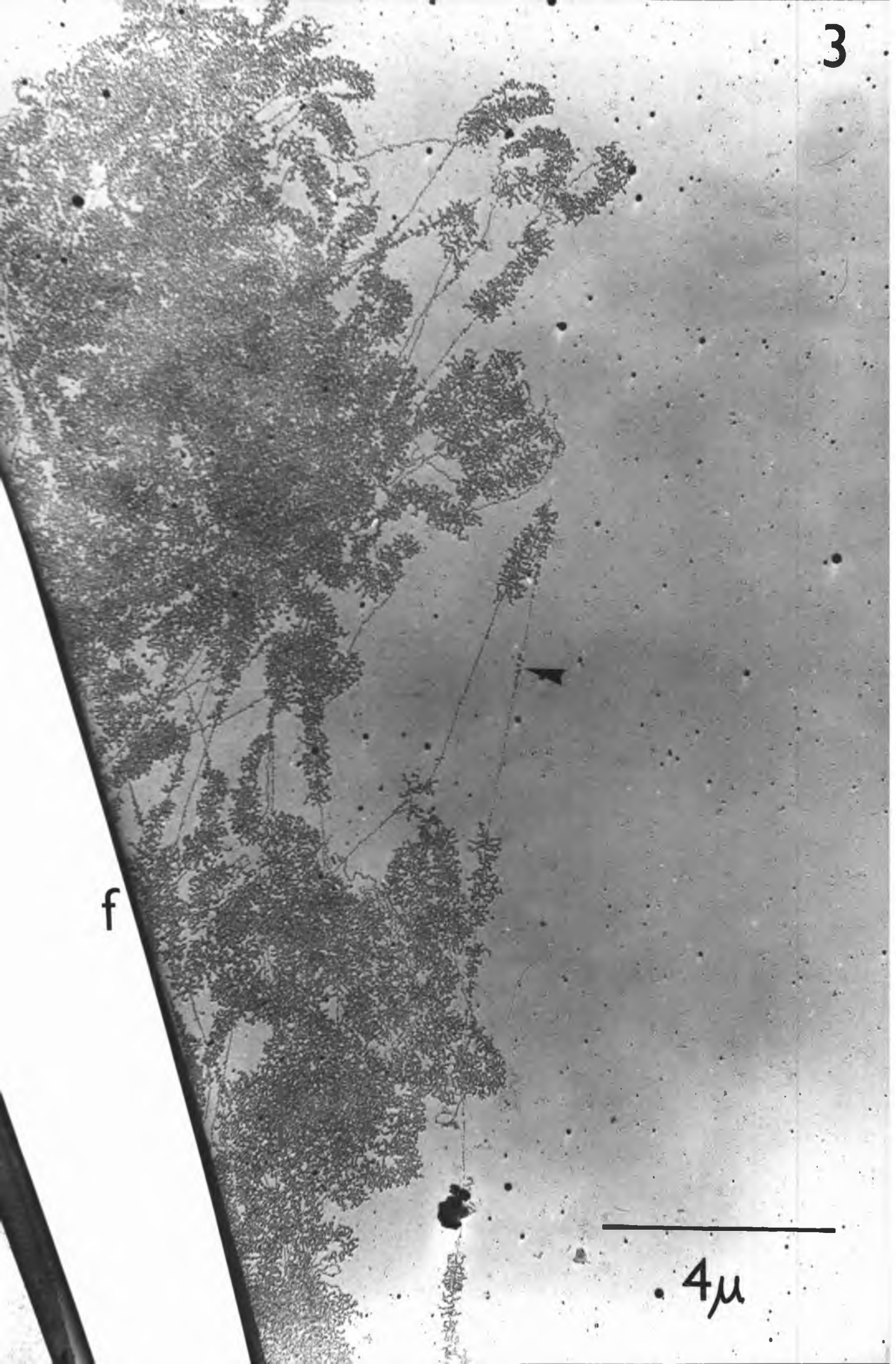

a
10^7
24
48
72
96
120
b
10^6
48
96
144
192
240
cells per culture vessel
time hrs

2a Graph showing the growth rate of the T.c. carnifex "red" line at twice the usual subculture density.

2b Graph showing the growth rate of the T.c. carnifex "black" line at twice the usual subculture density.


a
10^6
b
10^6
cells per culture vessel
24
48
72
96
120
144
time hrs

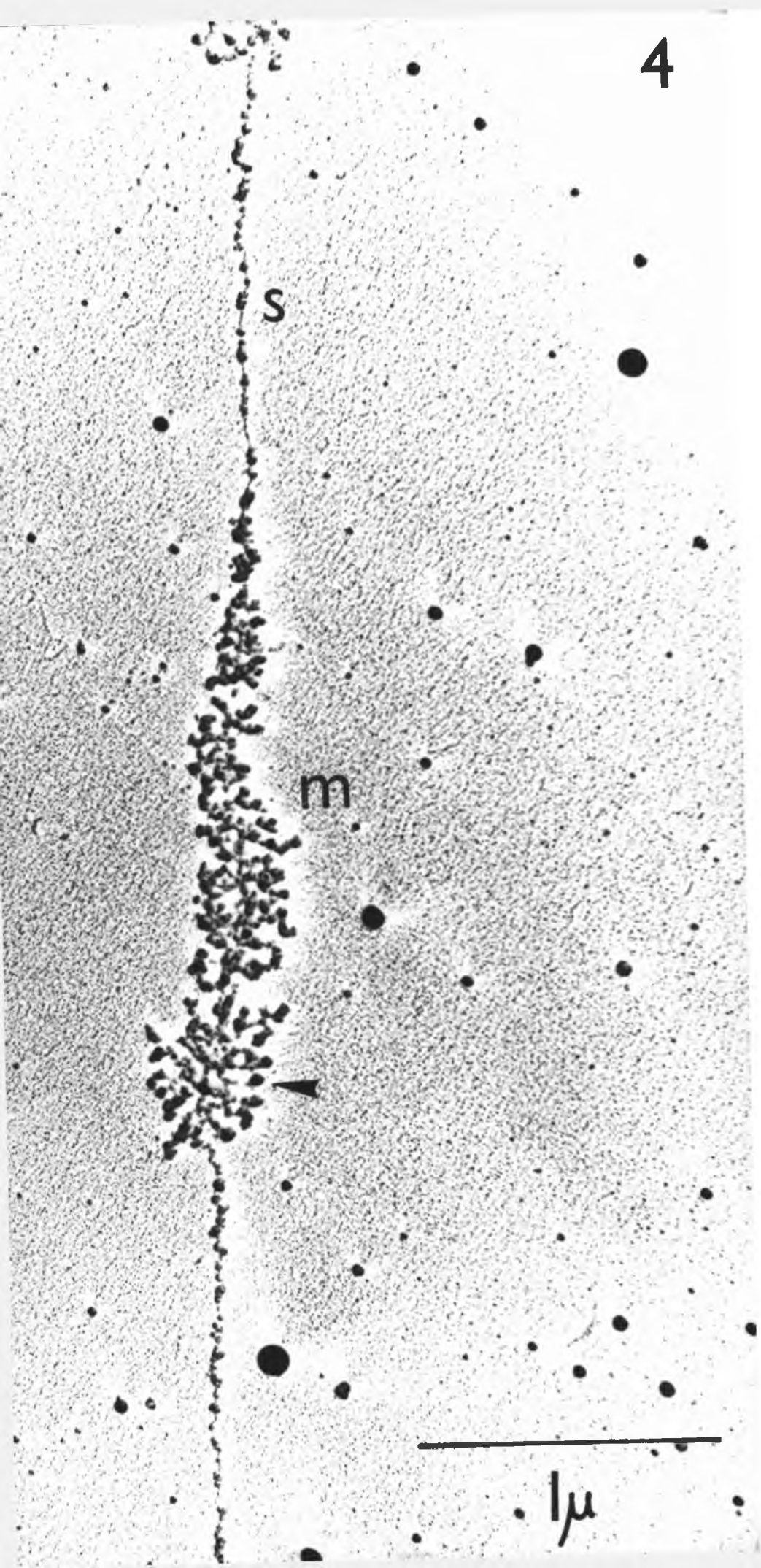
3 Clump of partially spread nucleolar chromatin from a T.c. carnifex oocyte. Its organization into alternating spacer and matrix units is clearly visible. Arrow denotes a "prelude complex" perhaps indicative of spacer transcription. f = break in the carbon film.


3
f
4μ

4. Repeating unit from T.c. carnifex oocyte nucleolar chromatin.

s = spacer unit. m = matrix unit.

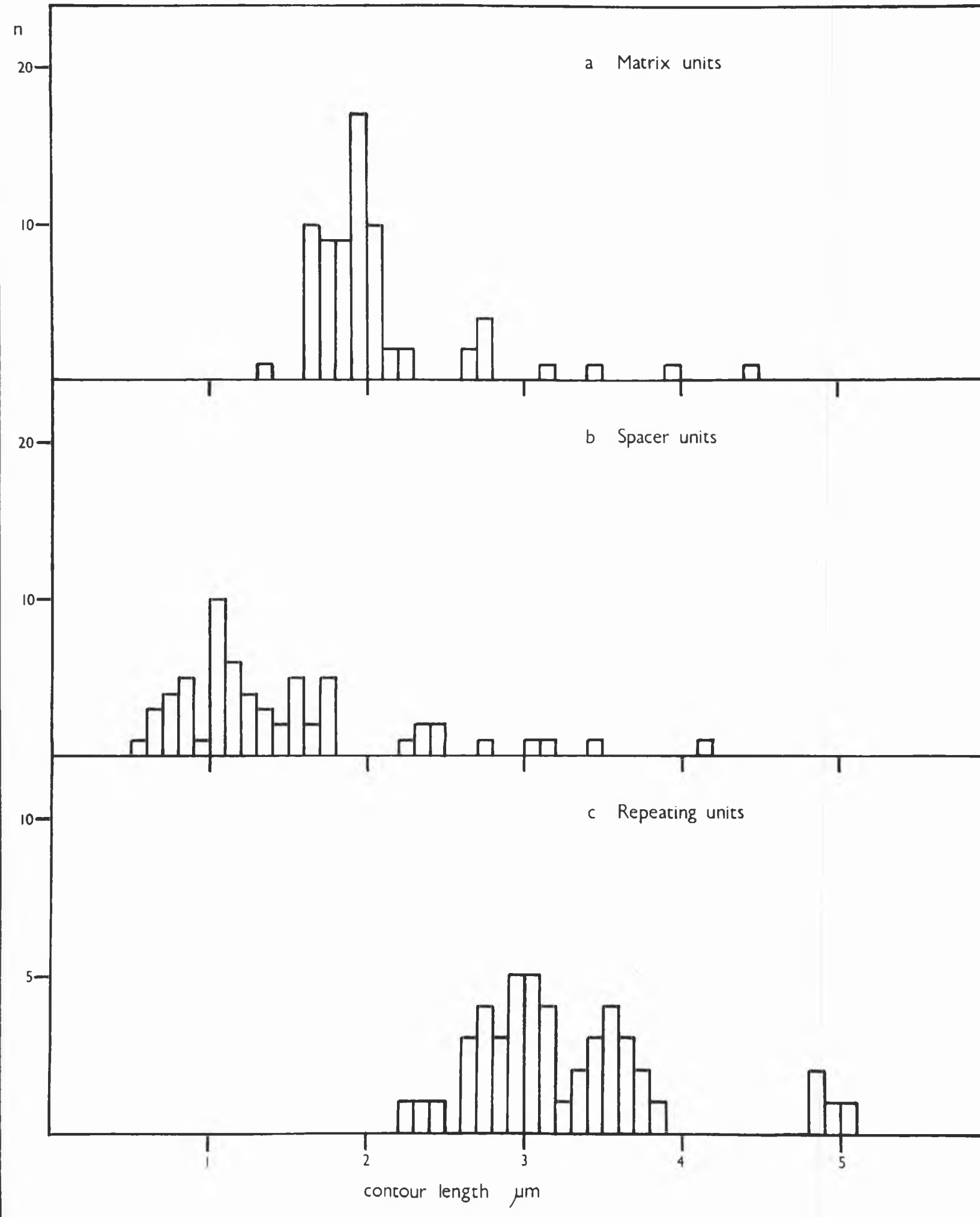
The beaded structure of the spacer unit is clear. Arrow denotes terminal "knobs" of the lateral fibrils of the matrix unit, indicative of packaging of the 45S preribosomal RNA.


4
s
m
1μ

5a Histogram showing the distribution of contour lengths of matrix units from T.c. carnifex oocyte nucleolar chromatin. Mean value 2.37 µm.

5b Histogram showing the distribution of contour lengths of spacer units from T.c. carnifex oocyte nucleolar chromatin. Mean value. 1.46 µm.

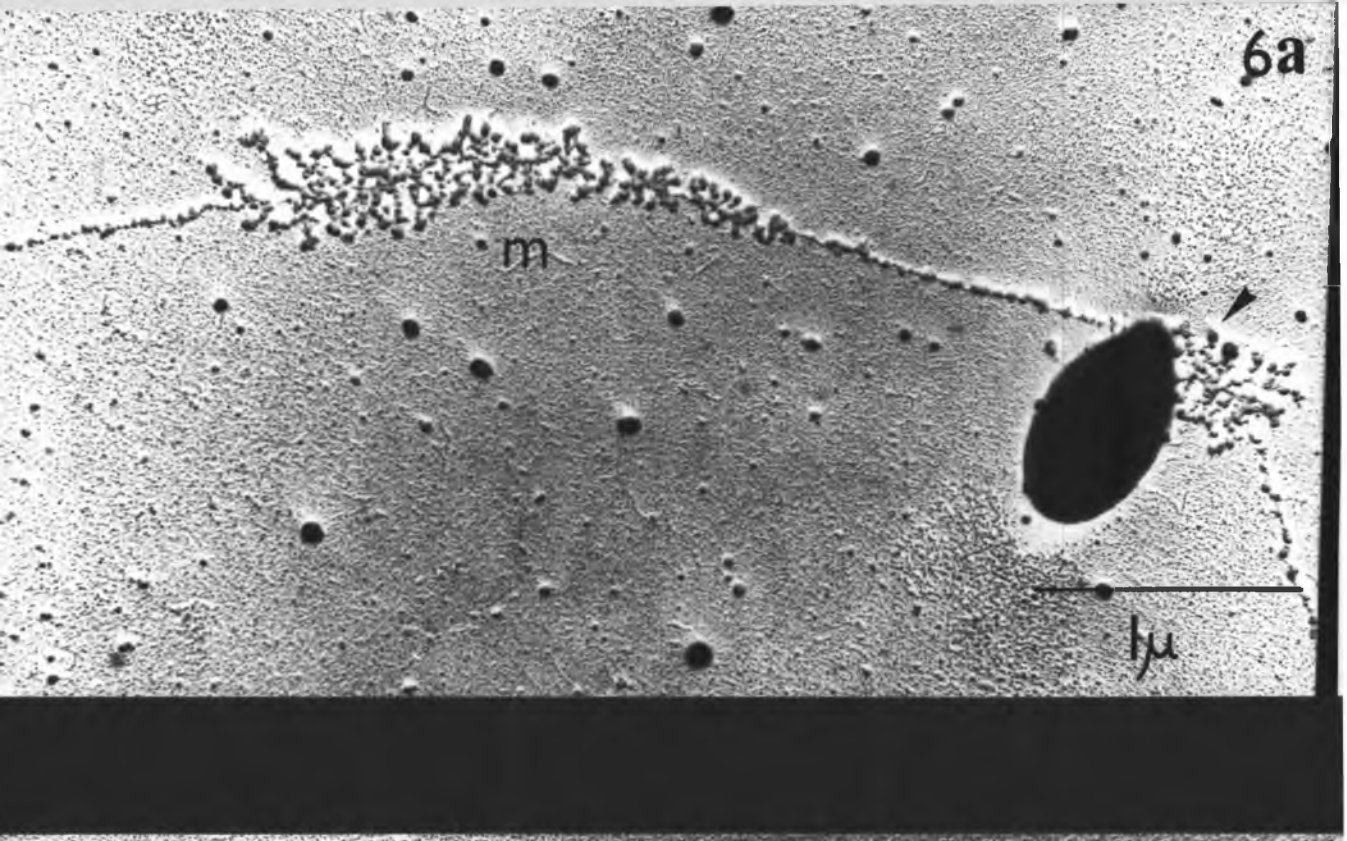
5c Histogram showing the distribution of contour lengths of repeating units from T.c. carnifex oocyte nucleolar chromatin. Mean value 3.82 µm.

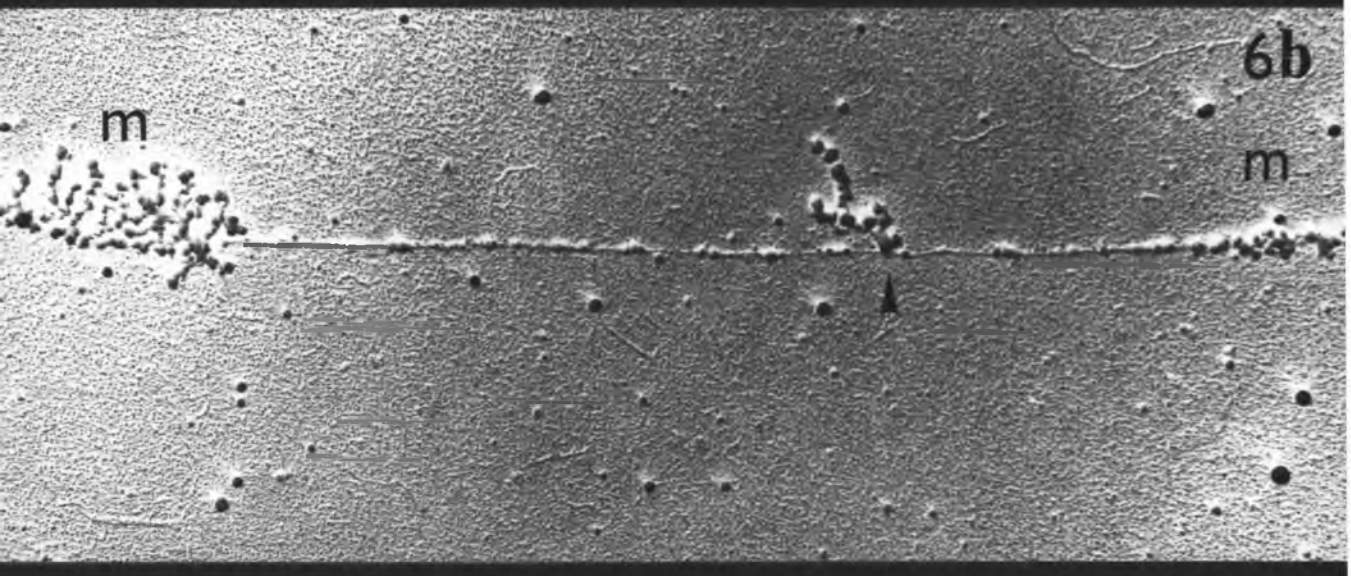

n
20
10
a Matrix units
20
10
b Spacer units
10
5
c Repeating units
1
2
3
4
5
contour length μm

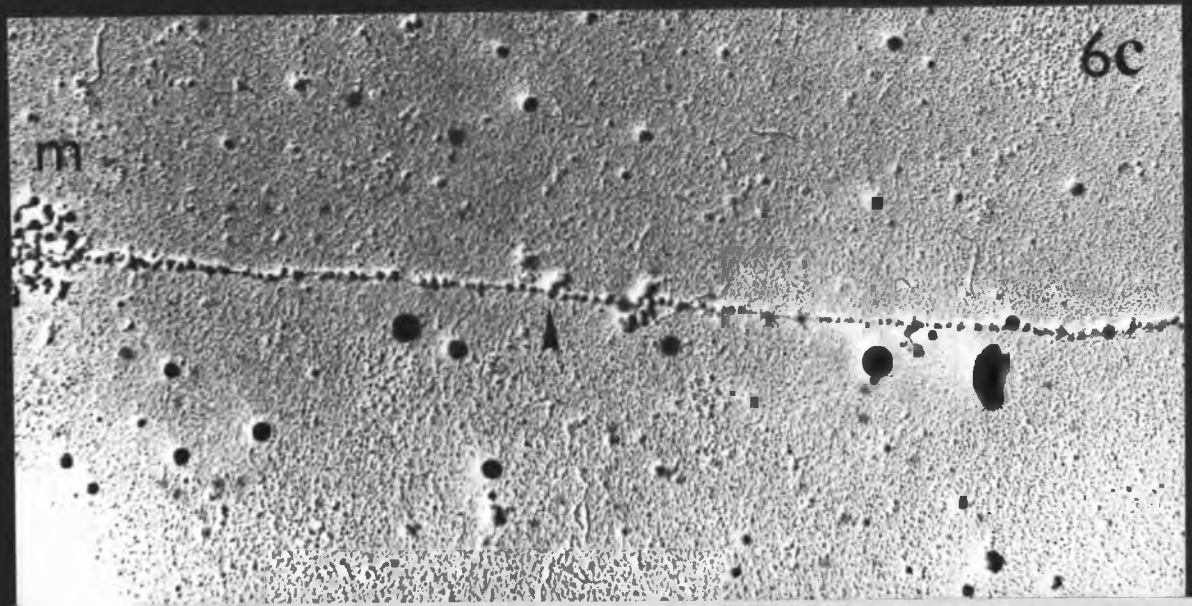
6a The arrow points to a region of a putative spacer unit, from T.c. carnifex oocyte nucleolar chromatin, and bearing RNP fibrils. A typical matrix unit (m) is included for comparison.

6b As Fig. 6a. m = matrix unit.

6c As Fig. 6a. m = matrix unit.


6a
m
1μ

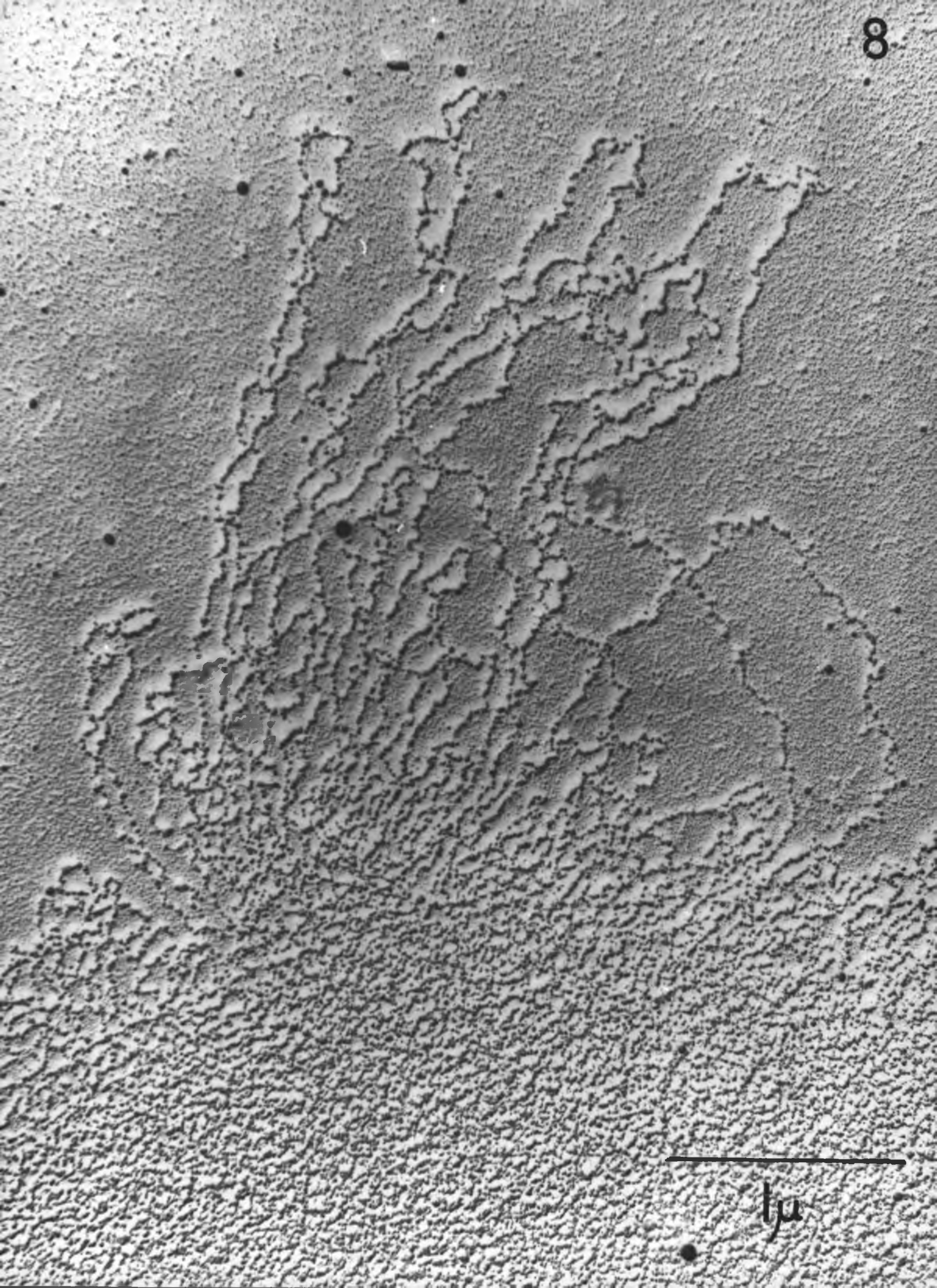

6b
m
m


6c
m

7. A stretched matrix unit (m) from T.c. carnifex oocyte nucleolar chromatin, demonstrating the probable absence of nucleosomes from the matrix units themselves. This is facilitated by the sparse coverage by RNP, of the matrix unit.

7
m
1μ

8. Clump of putative nucleolar chromatin from an oocyte of *X. laevis* (1.1 mm diameter) showing the beaded, transcriptionally inactive chromatin. These clumps were circular and roughly the size of nucleoli. All were transcriptionally inactive.


8
1μ