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Original article

Structure of lampbrush chromosome loops during different states of transcriptional activity as visualized in the presence of physiological salt concentrations

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Lampbrush chromosomes of amphibian oocytes were isolated in the presence of near-physiological salt concentrations, to preserve their native state, and studied by electron microscopy of ultrathin sections. The transcriptional state of the lampbrush chromosomes was experimentally modulated by incubating the oocytes for various time periods in medium containing actinomycin D. The observations show that the structure of the lateral loops changes rapidly in response to alterations in transcriptional activity. During decreasing transcriptional activity and reduced packing density of transcripts, the chromatin axis first condensed into nucleosomes and then into an approximately 30 nm thick higher order chromatin fiber. Packaging of the loop axis into supranucleosomal structures may contribute to the foreshortening and retraction of the loops observed during inhibition of transcription and in later stages of meiotic prophase. The increasing packing density of the DNA during the retraction process of the loops could also be visualized by immunofluorescence microscopy using antibodies to DNA. The dependence of the loop chromatin structure on transcriptional activity is discussed in relation to current views of mechanisms involved in gene activation.

lampbrush chromosomes — chromatin structure — electron microscopy — immunofluorescence microscopy — DNA antibodies

INTRODUCTION

Several lines of evidence suggest that changes in chromatin structure are involved in the transcriptional regulation of gene expression (for details see refs. [23, 31, 42]). Nevertheless, the relationship between chromatin structure and transcription is still poorly understood. Differences between transcriptionally active and inactive chromatin have been mainly established at the primary level of chromatin packaging, the nucleosomes. Combined structural and biochemical observations indicate that nucleosomes are highly dynamic structures which undergo reversible conformational changes during transcription. This is particularly evident with nucleolar chromatin, *i.e.* pre-rRNA genes [12, 13, 20, 34, 45], but is not as clear for RNA polymerase II-transcribed genes.

Based on observations made with electron microscopic spread preparations of highly active non-nucleolar chromatin, such as lampbrush chromosome loops, we came to the conclusion that in stages of maximum packing density of transcribing RNA polymerases the chromatin fiber is largely extended and nucleosomes are absent [13]. However, we also noted that in stages of reduced transcriptional activity, when transcriptional complexes are more widely spaced on the chromatin axis, nucleosomal particles do appear in the immediate vicinity of the RNA polymerase. This suggested that nucleosomes unfold anterior to transcribing polymerases and rapidly reform after each transcriptional event [34]. The rapid reformation of nucleosomes after the passage of a polymerase seems to be a general feature of non-nucleolar chromatin [*e.g.*, 12, 21, 24, 34; for further refs. see 23, 30, 31]. Correspondingly, an altered nucleosomal organization of chromatin, lacking the canonical nucleosomal repeat, has been found in biochemical experiments, for example, by nuclease digestion of transcriptionally active protein-coding genes such as heat shock genes of *Drosophila* [22, 44], chicken ovalbumin genes [4, 5] and Balbiani ring genes of *Chironomus* [43] and by cross-linking of histones to specific DNA sequences [19].

Much less is known about the next higher level of chromatin folding, *i.e.*, the chick chromatin fiber with a diameter of usually about 30 nm [for reviews see 10, 17], and its relation to transcription. At

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present it is not clear, for example, whether the gene activation involves the unfolding of the thick chromatin fiber into a more extended nucleofilament and whether structural changes accompanying activation precede or are the consequence of transcription. Unfortunately, the higher order organization of chromatin cannot be readily studied in electron microscopic spread preparations since the methodology involves the unravelling of chromatin by exposure to very low ionic strength solutions that are known to destabilize higher order packaging [32, 41, 45]. However, in a few cases chromatin can be isolated at physiological salt concentrations thereby allowing the identification of transcribed chromatin regions with the attached transcripts by electron microscopy. Lampbrush chromosome loops of amphibian oocytes represent such an example. As originally shown by Mott and Callan [26], lampbrush chromosomes can be isolated under near physiological conditions and studied by electron microscopy of ultrathin sections ('end-embedding procedure'). Using this method, Spring and Franke [40] concluded that the intensely transcribed chromatin of lampbrush chromosome loops is not compacted into nucleosomal and supranucleosomal particles. A similar absence of higher order structures has also been found in the actively transcribed regions of Balbiani ring genes of *Chironomus* salivary glands prepared *in situ* by conventional electron microscopy [1, 29]. In addition, it was observed by Andersson *et al.* [2, 3] that the thick chromatin fiber rapidly forms after experimental inhibition of transcription of the Balbiani ring genes with the drug DRB.

To study in greater detail how the formation of the higher order structures is related to the specific transcriptional activity of a given chromatin strand, I have examined the structural organization of loop chromatin of 'native' lampbrush chromosomes in states of maximal and reduced transcriptional activities. The activity of amphibian lampbrush chromosomes can be modulated by varying the exposure of oocytes *in vitro* to the drug actinomycin D (AMD) and monitored by light microscopy, using the extent of loop retraction as a measure of transcription [6, 18]. Earlier studies based on electron microscopic spread preparations have shown that AMD induces a premature release of the RNP transcripts from the loop axes, thus leading to a progressive decrease in their packing density along the loops [34].

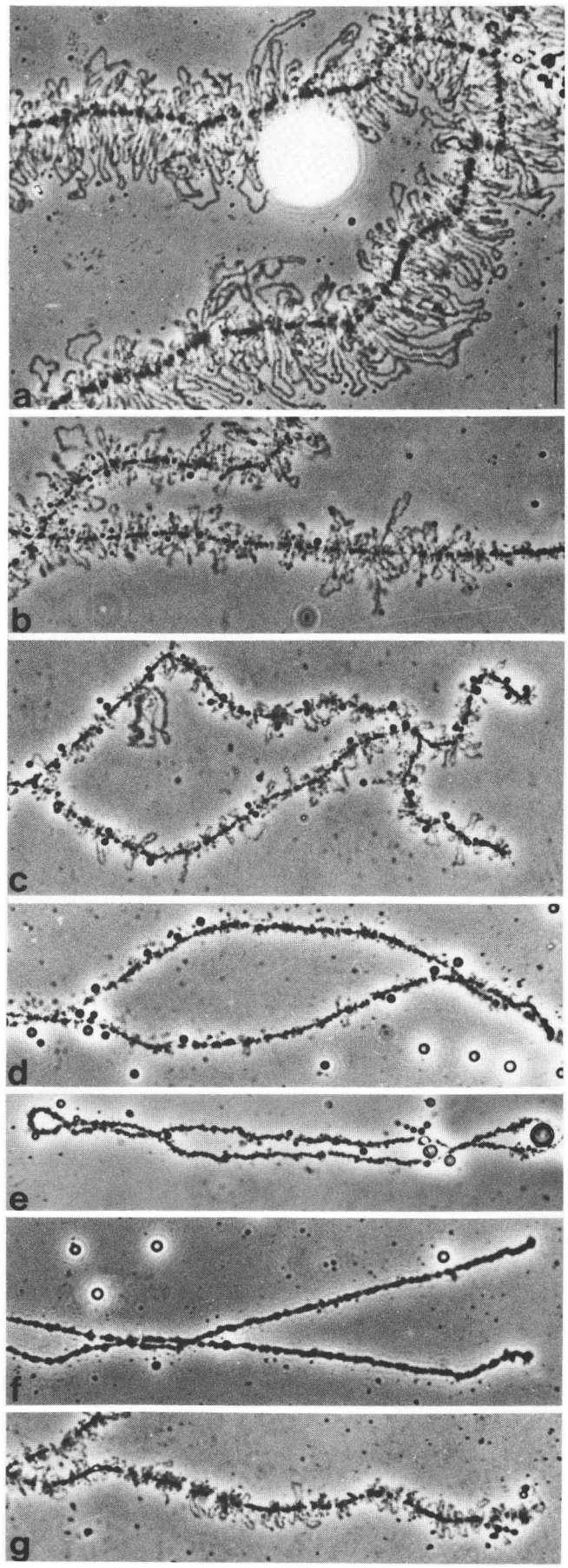


FIGURE 1. — Progressive retraction of the lateral loops of *Triturus* lampbrush chromosomes after increasing exposure times of oocytes to AMD. The lampbrush chromosomes were isolated in the presence of physiological salt concentrations and photographed with an inverted microscope using phase contrast optics. **a,** control; **b,** 1 hr; **c,** 2 hr; **d,** 3 hr; **e,** 4 hr; **f,** 5 hr after AMD treatment (50 μg/ml). When oocytes are transferred after AMD treatment (5 hr) to medium without the drug, loops reappear within 20 hr **(g).** All micrographs are magnified to the same scale. $\times$ 600; bar = 20 μm.

The observations made in this study support the concept of a highly dynamic nature of the chromatin fiber and suggest that it is the transcriptional process as such that transforms the higher order thick fiber into a more extended conformation. This property of the chromatin fiber may also provide an explanation for the apparent retraction of lateral loops which accompany decreasing transcriptional activities.

MATERIALS AND METHODS

Light- and electron microscopy

The newts *Triturus cristatus carnifex* were reared in our laboratory. A piece of an ovary was removed from an anesthetized female and placed in modified Barth's medium [14]. Actinomycin D (Serva, Heidelberg) was added at a final concentration of 50 μg/ml. Lampbrush chromosomes were manually isolated from mid-sized oocytes (0.8-1.0 mm in diameter) in a medium containing 83 mM KCl, 17 mM NaCl, 0.01 mM $CaCl_2$, buffered with 10 mM Tris-HCl to pH 7.2. The nuclear envelope was manually removed, the nuclear contents were allowed to disperse in a small observation chamber [8] and after about 15 min the preparation was centrifuged (800 × g, 10 min) to attach the chromosomes to a coverslip forming the bottom of the chamber. Photographs were taken with phase contrast optics (40 ×) using an inverted Zeiss microscope IM 35 (Carl Zeiss, Oberkochen, F.R.G.). For electron microscopy, specimens were fixed with glutaraldehyde followed by osmium tetroxide and flat-embedded in Epon essentially as described [26, 40]. Electron micrographs were taken with a Zeiss EM 10.

Immunofluorescence microscopy

The hybridoma clone AK 30-10 producing monoclonal antibodies (IgM) to DNA was the result of a fusion of cells of the mouse myeloma line Ag 8.653 with the spleen cells of a BALB/c mouse that was previously immunized with the high salt and detergent-resistant material of a rat hepatoma. Immunoglobulins were purified from ascites fluid by gel filtration on Sephacryl S300 columns (Pharmacia, Uppsala, Sweden) as described [16]. The antibody has been shown to bind to double- and single-stranded DNA (from prokaryotes and eukaryotic nuclei and mitochondria) but not to RNA [25]. A detailed account on this antibody, including binding studies by competitive ELISA-tests with a variety of synthetic oligonucleotides and natural nucleic acids, will be presented elsewhere [38].

For immunofluorescence microscopy, chromosomes attached to coverslips were transferred to 70% ethanol (10 min) and then into phosphate-buffered saline (PBS). Antibodies to DNA were added at a concentration of 10 μg/ml for 20 min at room temperature. Following several washes in PBS, FITC-labeled goat anti-mouse IgG/IgM (Medac, Hamburg; diluted 1:500) was added for another 20 min. After several washes in PBS, the coverslip was mounted in Elvanol. Photographs were taken with a Zeiss photomicroscope III equipped with epifluorescence illumination.

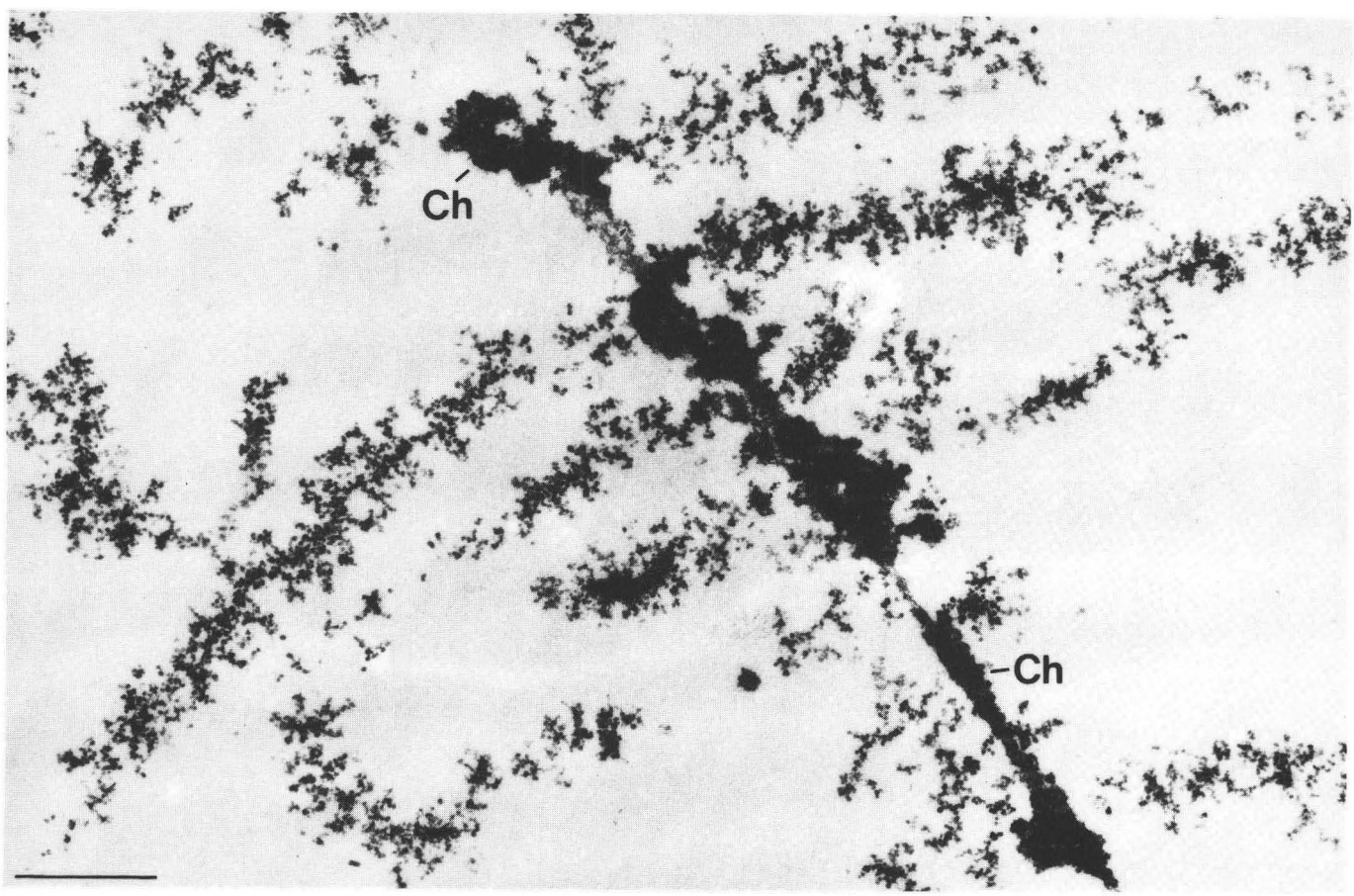


FIGURE 2. — Survey electron micrograph of an ultrathin section of a flat-embedded lampbrush chromosome from a growing oocyte. Numerous lateral loops with attached RNP transcripts extend from the dense central chromosome axis which is subdivided into structural units, the chromomeres **(Ch).** × 19 000; bar = 1 μm.

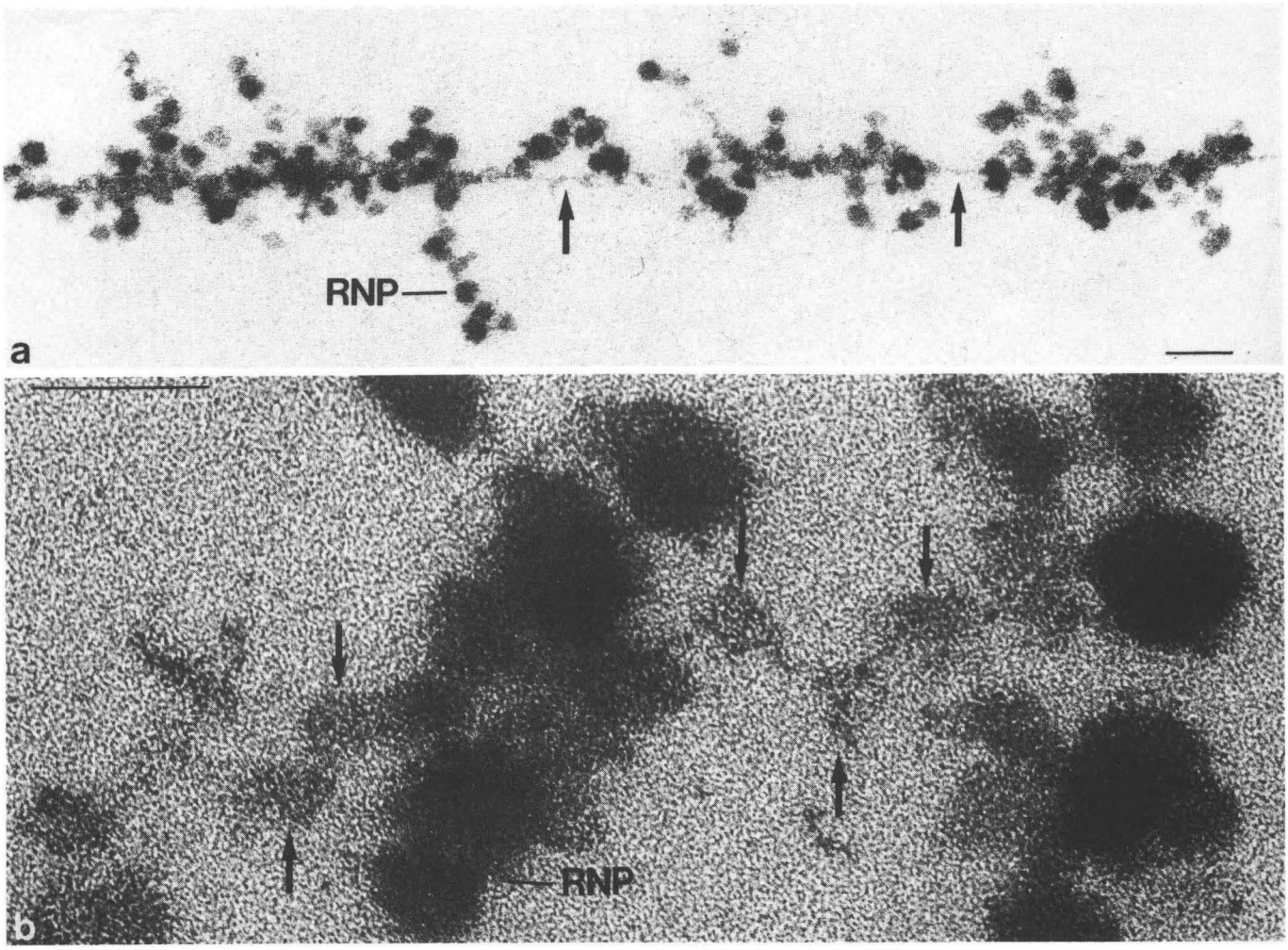


FIGURE 3. — Electron micrographs of flat-embedded lampbrush chromosomes isolated from maturing *Triturus* oocytes. Spacing of the nascent transcripts allow visualization of the underlying chromatin axis *(arrows* in **a**). At high magnification a beaded conformation of the chromatin axis between adjacent transcripts is recognized *(arrows* in **b**). Note the zig-zag arrangement of the nucleosome particles which are interconnected by thin filaments. RNP, nascent transcripts folded into linear arrays of RNP particles. **a,** × 95 000; bar = 0.1 μm; **b,** × 500 000; bar = 0.05 μm.

RESULTS

Light microscopic observations

Exposure of isolated *Triturus* oocytes to AMD induced a time-dependent foreshortening of the lateral loops of the lampbrush chromosomes as seen in light microscopic preparations (Fig. 1a-f). Approximately 4 hr after exposure to the drug the loops were barely recognizable (Fig. 1e); at 5 hr they were virtually absent and apparently amalgamated with the chromosome axis (Fig. 1f). This process of loop retraction was fully reversible. Upon removal of AMD from the medium the lateral loops re-appeared partially within 20-24 hr and almost completely after 48 hr indicating that transcription had been resumed (Fig. 1g). Thus, the experimental system used allowed to study chromosomes in various stages of transcriptional activities.

Electron microscopy of intensely transcribed chromosome loops

After flat-embedding of chromosomes isolated at near physiological salt concentrations, followed by ultrathin sectioning for electron microscopy, the lateral loops with numerous attached transcript fibrils could be clearly distinguished from the central chromosome axis containing the highly condensed, transcriptionally inactive chromatin (Fig. 2; for details see [26, 27, 40]). Each transcript fibril consisted of a linear array of densely stained RNP particles of rather regular size (25-40 nm; Fig. 3a). Usually the high number and packing density of the RNP transcripts obscured the chromatin axis. Occasionally, however, gaps between more distantly spaced transcripts allowed visualization of the underlying chromatin (Fig. 3a). At high magnification such transcript-free chromatin regions appeared as short chains of 10-15 nm particles

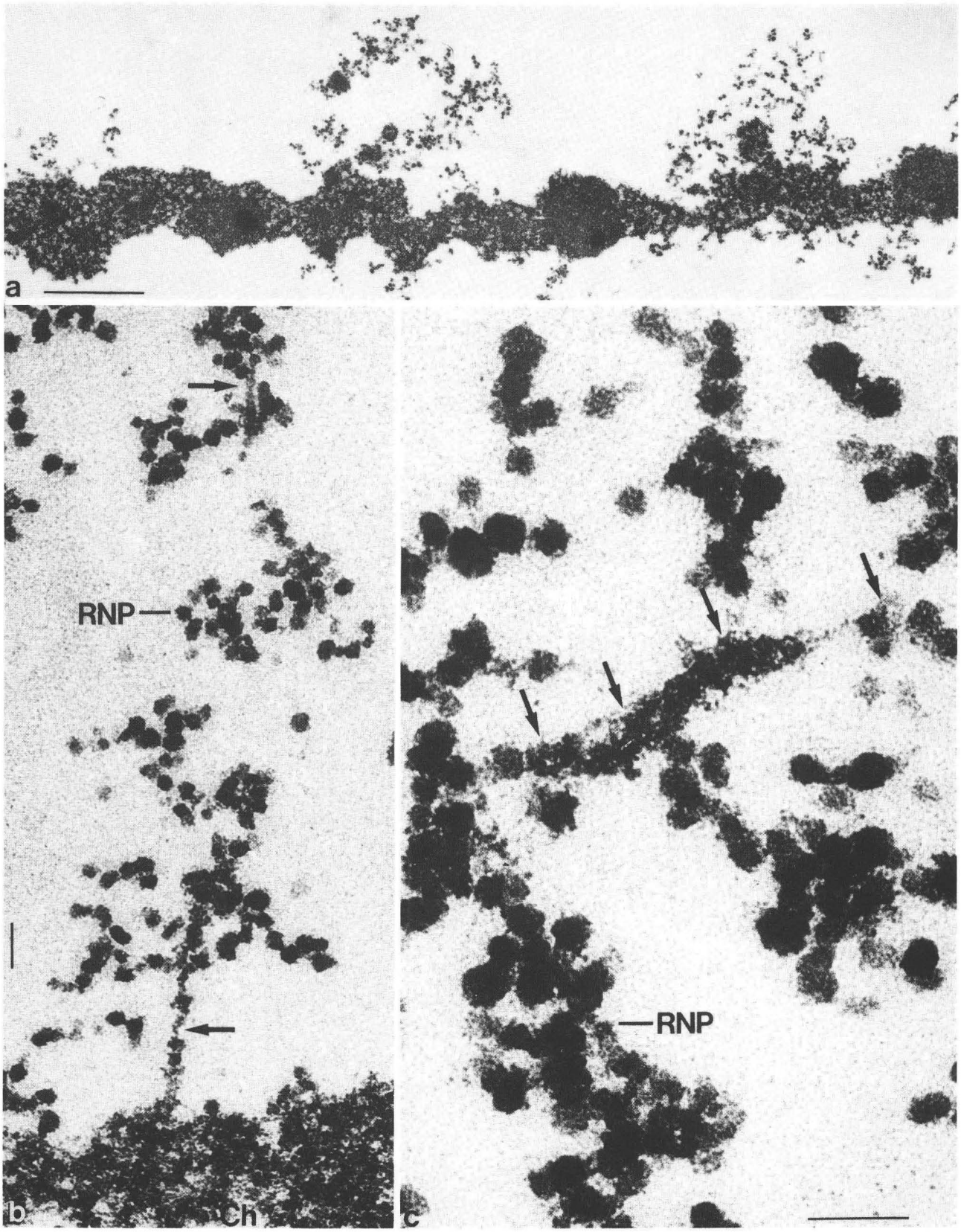


FIGURE 4. — Electron micrographs of *Triturus* lampbrush chromosomes with small retracting loops (4 hr treatment of oocytes with AMD). The survey micrograph shows a chromosome with very small transcript-covered lateral loops (**a;** the corresponding light microscopic situation is shown in Fig. 1**e**). The axis of such retracting loops is condensed into an approximately 30 nm thick fiber (*arrows* in **b** and **c;** the loop axis is only partially included in **b**). **Ch,** chromomere; **RNP,** nascent transcripts. **a,** × 18 000; bar = 1 μm; **b,** × 80 000; bar = 0.1 μm; **c,** × 180 000; bar = 0.1 μm.

interpreted as nucleosomes arranged in a loose zig-zag pattern (Fig. 3b; for identification of nucleosomes in ultrathin sections *c.f.* [9, 28, 45]). Further condensation of such short intragenic chromatin regions, free of transcripts, into larger, supranucleosomal arrangements was not observed in lampbrush chromosomes isolated from mid-sized oocytes.

Ultrastructure of moderately transcribed chromosome loops

When oocytes were exposed for 4 hr to AMD, the majority of the lateral loops were almost completely retracted as seen by light (Fig. 1e) and electron (Fig. 4a) microscopy. As shown in the survey electron micrograph of Fig. 4a, in some of the retracting loops numerous transcript fibrils remained associated with the loop axis and appeared to be organized in a rather close packing density. In contrast to the situation found in fully active transcription units, the chromatin axes of the small retracted loops were much thicker and consisted of an approximately 30 nm thick fiber with somewhat irregular contours (*arrows* in Fig. 4b and c). This thick chromatin fiber could be clearly described along the basal portions of the loops since lateral transcript fibrils were often lacking in these transition regions between the loops and the chromomeres (Fig. 4b). Whether the higher order structure of the loop chromatin was locally unfolded at transcript attachment sites could not be determined because of difficulties in identifying the small RNA polymerase particles (⁓12 nm) in such preparations.

In stages of increasing transcriptional activity, during recovery from AMD treatment (see Fig. 1g), the loop chromatin displayed essentially the same morphological features, *i.e.,* some early transcripts were found in association with thick chromatin fibers. Since the partially regenerated loops are known to be transcriptionally active [39], it can be concluded that higher order condensation of chromatin occurs next to active transcriptional complexes.

Binding of DNA antibodies to lampbrush loops in different states of activity

Lampbrush chromosomes isolated from growing oocytes were incubated with a monoclonal antibody against DNA and processed for immunofluorescence microscopy. The strong and selective fluorescence of the chromosome axes illustrated that the majority of the chromosomal DNA is transcriptionally inactive and compacted in the chromomeres (Fig. 5a; for a detailed model of lampbrush chromosome organization *c.f.* [7]) and that the local concentration of DNA in the lateral loop axes was below the detection limit of the immunofluorescence method used. The numerous small, individual fluorescing dots which surrounded the axes of the chromosomes were aligned along the lateral loops (compare Fig. 5a' with the corresponding phase contrast image of Fig. 5a). Therefore, each of these fluorescing entities represents a loop region with a high local DNA content. Such regions might contain non-transcribed spacers between adjacent transcription units [36] or intragenic chromatin regions of low transcriptional activity. Upon treatment of oocytes with AMD all the smaller retracting loops became positive (Fig. 5b', c'). Therefore, the specific packing density of DNA must be considerably higher in the small retracting loops of AMD-treated oocytes as compared with the fully extended state.

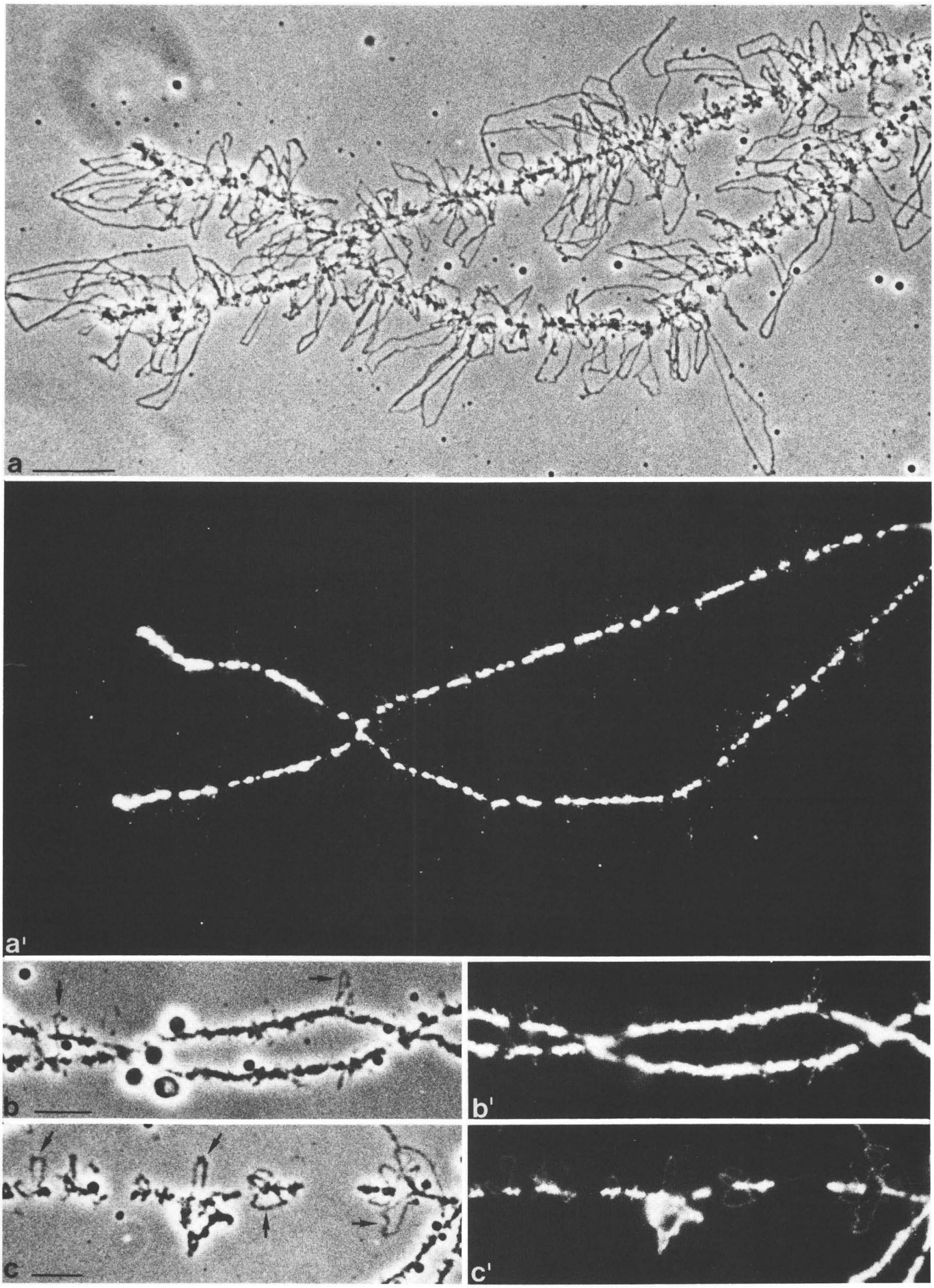
DISCUSSION

The present observations indicate that the chromatin of the lateral loops of amphibian lampbrush chromosomes is in a dynamic state and rapidly changes its specific compaction in response to changes of the transcriptional status. In earlier studies based on spread preparations at low ionic strength, the analysis of chromatin conformation in the lateral loops was restricted to the nucleosome level (for refs. see Introduction). The present study extends such analyses to higher order, supranucleosomal chromatin structures. The results demonstrate that the transcriptionally active chromatin of loops can be found in at least three different states of compaction.

1. Non-nucleosomal arrangement during intense transcriptional activity

Chromatin of lampbrush chromosome loops from growing oocytes is associated with numerous transcripts in close to maximal packing density and occurs in a non-nucleosomal form in which the DNA is probably extended to the length of its B-conformation [13, 34, 40]. Core histones remain associated with such heavily transcribed chromatin regions as shown by immunolabeling (unpublished observations). Furthermore, microinjected antibodies to histone H2B interfere with transcription of the chromosome loops [37].

FIGURE 5. — Immunofluorescence microscopy of *Triturus* lampbrush chromosomes after incubation with antibodies directed against DNA. A normal chromosome bivalent is shown in phase contrast **(a)** and epifluorescence optics **(a').** The chromosome axes are brightly fluorescent whereas the lateral loops are negative **(a').** After AMD-induced loop retraction (3 hr AMD-treatment) the small loops fluoresce **(b', c';** the corresponding phase contrast images are shown in **b** and **c;** *arrows* indicate the retracted loops). **a, a',** × 800; bar = 20 μm; **b, b',** × 1 100; bar = 10 μm; **c, c',** × 1 000; bar = 10 μm.


a
a'
b
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2. Nucleosomal arrangement during moderate transcriptional activity

In stages of moderate transcriptional activities which occur naturally in full-grown oocytes of several amphibian species or can be experimentally induced as a transitory state during AMD treatment of growing oocytes or during the recovery period from AMD, polymerases are more distantly spaced. Transcript-free stretches invariably assume a beaded, nucleosome-like conformation. This was shown earlier by the chromatin spreading technique [34] and was confirmed in the present study by analyzing sectioned lampbrush chromosomes isolated under near physiological salt conditions.

3. 30-nm thick chromatin fiber during low transcriptional activity

When individual transcripts are widely spaced, the nucleosomal chain condenses further into an approximately 30-nm thick fiber. It is important to emphasize that this higher order folding occurs between successive transcriptional events since transcripts are still attached to the thick chromatin fibers both in stages of decreasing (during AMD treatment) and increasing (recovery from AMD treatment) transcriptional activities and does not depend on the complete inactivation of the entire transcription unit. Since the partly retracted loops are transcriptionally active, as judged from light microscopic autoradiography (unpublished observations; see also [39]), condensation into a higher order structure appears to be an intrinsic property of the loop chromatin. Whether or not such supranucleosomal packaging structures are formed in a given loop seems to depend on the specific transcriptional status, *i.e.*, the time interval between successive transcriptional events. In this connection it is worth emphasizing that the thick chromatin fiber was observed in lampbrush loops in stages of decreasing and increasing transcriptional activity. Furthermore, selective induction of higher order compaction of loop chromatin by the action of the DNA-intercalating drug AMD can be excluded, since comparable structural chromatin transformations also occur in lampbrush chromosomes of full-grown oocytes, which are characterized by a natural transcriptional inactivation and loop retraction (data not shown).

The dynamic nature of lampbrush chromosome loops, together with its general tendency to form higher order structures as a response to reduced transcriptional activity, probably reflects a general behavior of active chromatin. Chromatin of the Balbiani ring genes of *Chironomus tentans* salivary glands is also rapidly transformed from an extended state into thick fibers upon reduction or completion of RNA synthesis [2, 3]. These and the present observation support a model for active chromatin in which the 30-nm chromatin fiber is unfolded only in the vicinity of the transcribing RNA polymerases. The generally held view of gene activation, *i.e.*, opening of the entire gene domain into an extended conformation, followed by transcriptional activation, seems to be at first sight incompatible with this model. However, DNAse I digestion experiments clearly indicate that conformational differences do exist between 'active' genes and the bulk of inactive chromatin which are independent of ongoing transcription (for refs. see [23, 42]). Thus, the primary steps in gene activation do not necessarily lead to a permanent unravelling of the higher order structure of the entire chromatin region containing the specific gene but involve more subtle changes in chromatin conformation and composition which, at this moment, are poorly understood.

The observation of a higher order compaction of loop chromatin in states of low transcriptional activity is consistent with the foreshortening of loops during experimental inhibition of transcription and in normal oocyte maturation. As shown in this study, the significantly higher compaction status of DNA in small loops, as compared to normal loops, can be directly demonstrated by immunofluorescence microscopy using antibodies to DNA. Apparently, the mechanism of loop retraction involves folding of the normally extended chromatin axis into nucleosomes and further into a 30-nm thick fiber.

In stages of complete cessation of transcription, the thick fiber may then be folded further and coiled (for possible models see [2, 3]). Because histone H1 seems to be responsible for the stabilization of higher order chromatin structures [10, 32, 41] one would expect to find H1 in the small retracting loops. Whether H1 is also present in the fully extended and maximally active chromosome loops remains unresolved.

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