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Mapping of transcription units on Xenopus laevis lampbrush chromosomes by in situ hybridization with biotin-labeled cDNA probes

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A non-radioactive in situ hybridization method is described for the localization of transcription units of defined genes to lateral loops of Xenopus laevis lampbrush chromosomes. Two Xenopus cDNA probes were used encoding the nucleolar protein NO38/B23 and cytokeratin 1(8). Both proteins are known to be synthesized in Xenopus oocytes, and Northern blot analysis revealed the presence of the corresponding mRNAs in different oogenic stages. The probes were enzymatically labeled with biotin-dCTP and hybridized to lampbrush chromosomes. The sites of hybridization were detected either by indirect immunofluorescence microscopy using rabbit antibodies against biotin and fluorescein-conjugated anti-rabbit IgG or enzymatically using peroxidase-conjugated streptavidin. The probe encoding the nucleolar protein hybridized to two sets of lateral loops on different bivalents, the cytokeratin probe to at least four. Our finding that each probe hybridized to more than one chromosomal locus may reflect the tetraploid nature of the Xenopus laevis genome or results from cross-hybridization to other transcriptionally active members of the NO38/B23-nucleoplasmin or the cytokeratin-lamin gene families. The method described should facilitate further in situ hybridization studies with appropriate genomic clones in order to map specific DNA sequences to defined loop regions and to come to a better understanding of the relationship between loop organization and gene transcription unit.

Introduction

Lampbrush chromosomes provide the unique opportunity to portray transcriptionally active genes directly in cytological preparations. These chromosomes are subdivided into distinct structural domains reflecting regions of differential transcriptional activities, notably the lateral loops and the compact chromomeres which are arranged in linear arrays to form the chromosomal axes (for reviews see [10, 14, 54]). The numerous lateral loops projecting from the chromosomal axes are the manifestation of ongoing transcription mediated by the type II RNA polymerase [7, 59]. Each lateral loop contains a single chromatin fiber with almost fully extended DNA [50] which carries nascent transcripts in form of closely spaced ribonucleoprotein (RNP) fibrils (for structural data derived from electron microscopic spread preparations see [5, 30, 42, 43, 49, 54]). In light microscopic preparations the multitude of nascent RNP transcripts appear to form a coat or matrix around the loop axis, often arranged in a thickness gradient reflecting the increasing transcript length as the polymerases move along the DNA template. According to the specific arrangement of the matrix material, loops containing a single transcription unit (TU) or multiple TUs of different lengths and polarities have been distinguished [15, 16, 53, 54].

The fact that TUs can be defined at the light microscopic level makes lampbrush chromosomes an attractive model system to define the genetic content and transcriptional pattern of individual loops by in situ hybridization and to study their protein composition by immunofluorescence microscopy (for refs. see [10, 39]). Since each TU of a chromosomal loop contains numerous transcripts, usually in nearly maximal loading density, hybridization of single-stranded DNA or RNA probes to the multiple nascent RNA chains as opposed to the DNA coding sequence provides a means to amplify considerably the detection signal [47]. Using this approach, histone genes have been mapped to specific lampbrush chromosome loops of newt oocytes and their transcription analyzed in detail [8, 10, 15, 16, 26]. Based on these studies, Gall and coworkers proposed a "readthrough transcription" model which essentially implies that transcription starts at or near the promoter of a histone gene and runs without interruption into satellite DNA sequences located downstream thereof [15, 16, 27]. However, the histone genes might be an exceptional case, so that the general value of the "readthrough" model for lampbrush loops has to be tested for other genes as well.

Although the use of the large lampbrush chromosomes from newts and salamanders facilitates cytological studies, the enormous genome size and the high proportion of repetitive DNA sequences renders it difficult to isolate genomic DNA clones of defined genes. In contrast, Xenopus

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laevis has a moderate genome size of about 3 pg, and a large number of cloned DNA sequences are available. Unfortunately, the chromosomes are shorter and the lateral loops smaller than in newts, a feature which hitherto complicated the cytological analysis of Xenopus lampbrush chromosomes considerably (for correlation between nuclear DNA content and average size of lampbrush chromosome loops see [52]). However, with a recently developed spreading technique it is now feasible to prepare Xenopus lampbrush chromosomes in a quality that is sufficient for cytological in situ hybridization studies [11, 12]; see also [32].

For the present study we have used biotinylated cDNA probes to map structural genes on specific loops of Xenopus lampbrush chromosomes by "RNA transcript hybridization" in situ. We hope that this approach will allow us in the near future using the appropriate genomic DNA clones, to map the distribution of coding and non-coding flanking sequences along individual chromosomal loops and to define those sequences involved in anchoring the loop bases into the chromomeric axis.

Materials and methods

Lampbrush chromosomes

Chromosomes were prepared from Xenopus laevis oocytes as described in detail by Callan, Gall and Berg [11] with some modifications. Thus, a freshly isolated nucleus was immediately transferred into the preparation chamber containing the dispersing medium [11] but lacking $CaCl_2$. After mechanical removal of the nuclear envelope, the nuclear content dispersed readily even in the absence of calcium ions. If exposure of the isolated nuclei to the phosphate-buffered isolation medium was short (less than 30 s), extensive gelification of the nuclear content could be avoided and so we succeeded in obtaining useful lampbrush chromosome preparations also from early oogenic stages (around the onset of vitellogenesis). After centrifugation, the preparations were processed as described by Gall et al. [26] and could be stored up to several weeks.

DNA probes

Xenopus cDNA probes used in the present study encoded the nucleolar protein NO38 (probe NO38-185 [58]) which is homologous to mammalian nucleolar protein B23 [13, 57] and a cytoskeletal protein equivalent to cytokeratin No. 8 of mammals (probe pKXL 1/8 [23]). The cloned probes were generously provided by Marion Schmidt-Zachmann, Jürgen Franz and Werner W. Franke of the Institute of Cell and Tumor Biology at the German Cancer Research Center in Heidelberg.

The DNA (30–60 ng per assay) was biotinylated with biotin-16-dCTP (Enzo Biochem., Ortho Diagnostic Systems, Neckargemünd/FRG) using the random-primed oligonucleotide labeling protocol originally described by Feinberg and Vogelstein [20]. The reaction was terminated by precipitating the DNA with ethanol. After washing the DNA pellet with 70% ethanol, the probe was resuspended in 5 × sodium salt citrate (SSC) to a final concentration of approximately 10 to 50 ng/µl and denatured in a boiling waterbath.

In situ hybridization

Hybridization was performed with 3 µl of the heat-denatured probe for 2 h at 60 °C. In order to avoid evaporation of the hybridization solution, a coverslip was added and sealed with rubber cement. After hybridization, the coverslip was removed under 2 × SSC and the specimen washed twice in 2 × SSC and once in phosphate-buffered saline (PBS) for several minutes each.

The hybridized biotinylated DNA was routinely detected by indirect immunofluorescence microscopy. Rabbit anti-biotin antibodies (Enzo Biochem.; diluted 1:50 in PBS) were added to the preparation for 1 to 2 h at 37 °C. Then the slides were washed several times in PBS and incubated with fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG (Dianova, Hamburg/FRG; diluted 1:20 in PBS) for 30 min at room temperature. After a final PBS wash, the preparations were mounted in a glycerol/PBS (9:1) mixture containing p-phenylenediamine to reduce the fading [33]. In order to facilitate identification of the chromosomes, the DNA-specific dye Hoechst 33258 (5 µg/ml in PBS) was added to the preparation for a few minutes prior to mounting.

In some experiments, the hybridized probe was detected histochemically. The preparation was incubated with peroxidase-conjugated streptavidin (Enzo Biochem., DETEK I-hrp, dilution 1:200 in PBS containing 1% bovine serum albumin) for 30 min at room temperature. After rinsing once in PBS, freshly prepared hrp-reaction mixture (500 µg aminoethylcarbazole in 1 ml 100 mM Na-acetate, *p*H 4.5, containing 0.025% H_2O_2) was added for 1 to 2 h, washed in PBS and mounted as described above.

For controls, lampbrush chromosome preparations were digested with RNase A (Boehringer Mannheim; 100 µg/ml in 2 × SSC) for 60 min at 37 °C prior to hybridization. Micrographs were taken with a Zeiss Axiophot microscope equipped with epifluorescence phase-contrast optics.

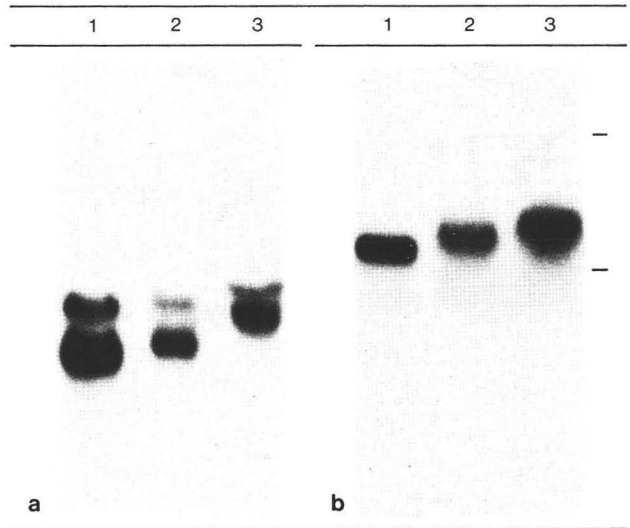


Fig. 1. RNA blot analysis of electrophoretically separated RNA from different oogenic stages of Xenopus laevis (*lane 1*, previtellogenic; *lane 2*, mid-sized; *lane 3*, mature oocytes). Total RNA corresponding to 10 oocytes of each stage was separated on agarose gels, blotted on nitrocellulose filters and probed with clone NO38-185 (**a**) and clone pKXL 1/8 (**b**). Horizontal bars indicate positions of 18S and 28S Xenopus rRNAs, run for reference on the same gels as size markers. The apparent decrease in gel electrophoretic mobility of the RNA bands from later oogenic stages is caused by the presence of increasing amounts of rRNA.

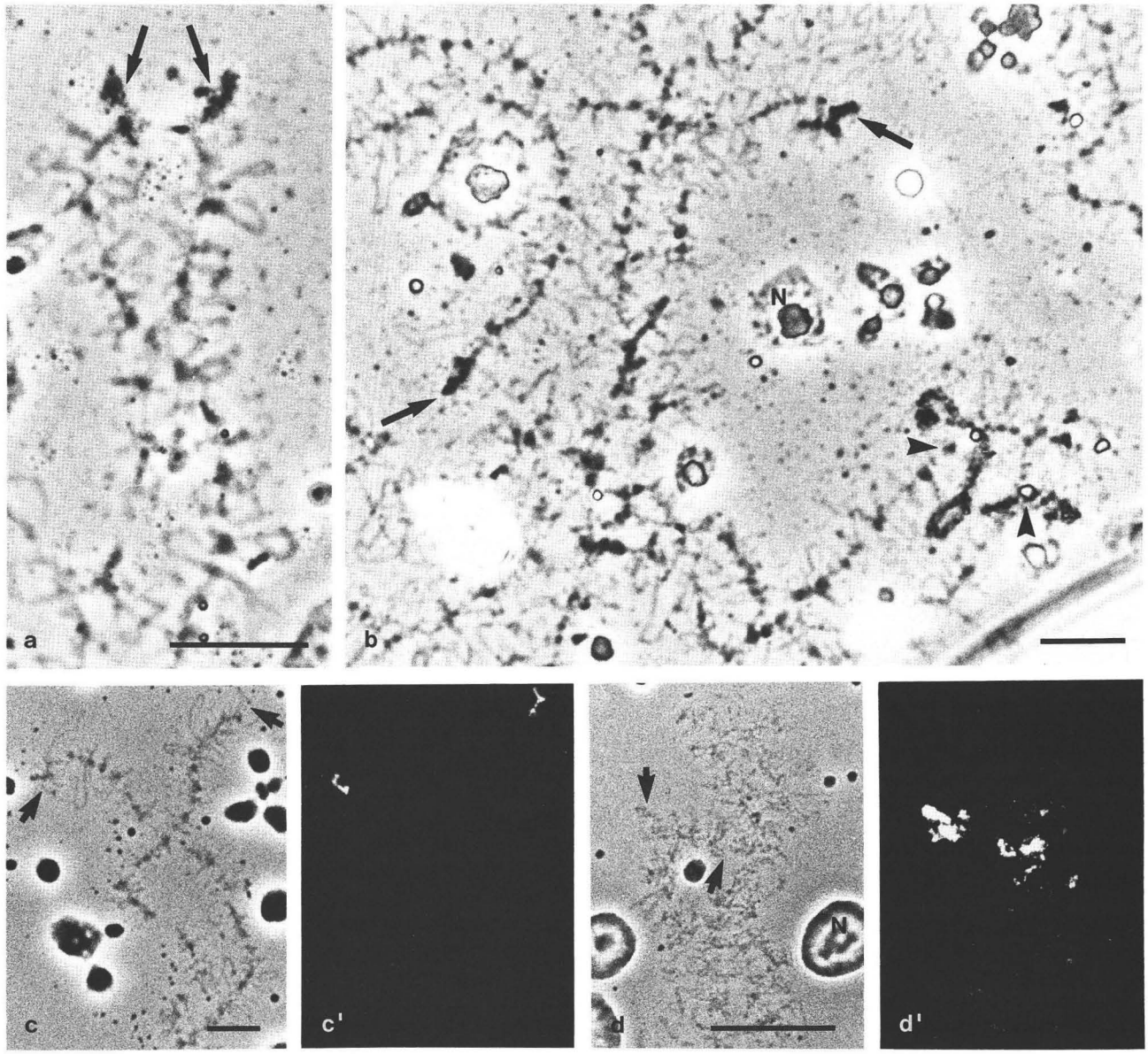


Fig. 2. In situ hybridization of biotinylated cDNA probe NO38-185 to Xenopus lampbrush chromosomes. Hybrid detection by the streptavidin-peroxidase reaction (**a, b**) and by indirect immunofluorescence microscopy (**c', d'**; the corresponding phase-contrast images are shown in **c, d**). Labeled loops are located at the ends of the homologous chromosomes of a bivalent (*arrows* in **a–c**). A second pair of loops is situated at a subterminal position (*arrowheads* in **b** and *arrows* in **d**). — N Nucleolus. — Bars 10 μm.

RNA blot analysis

Xenopus oocytes were manually divided into three size classes, i.e., previtellogenic (ca. 0.2 mm in diameter, Dumont stage I [18]), mid-sized (ca. 0.6 mm, Dumont stage IV) and fully mature (ca. 1.2 mm, Dumont stage VI). Total RNA was extracted with SDS-phenol [55], separated on 1% agarose gels containing formaldehyde and transferred to nitrocellulose paper according to standard procedures [40]. The hybridization probes were radiolabeled with [^{32}P]-ATP (3000 Ci/mmol) by the oligonucleotide labeling method [20].

Results

RNA gel blot hybridization

To show that the genes for nucleolar protein NO38/B23 and cytokeratin are in fact transcribed during Xenopus oogenesis, total RNA was examined by gel electrophoresis and Northern blot hybridization. The presence of mRNA for NO38/B23 and cytokeratin could be readily detected (Fig. 1). The NO38/B23 DNA hybridized to a major band

corresponding to an mRNA of approximately 1.25 kb and a weaker band of 1.5 kb (Fig. 1a; see also [58]) and the cytokeratin clone to a single band corresponding to 2.2 kb (Fig. 1b; see also [23]). Furthermore, the Northern blots demonstrated that the mRNAs were already present in the early previtellogenic oocytes at levels comparable to or even slightly higher to those found at the termination of oogenesis (Fig. 1; total RNA from 10 oocytes each was applied per gel lane). The same pattern of accumulation has been found for a number of other mRNA species [9, 19, 28, 29, 46, 61–63].

In situ hybridization with biotinylated cDNA probes

In a first set of experiments we compared the detection signals of the hybridized probe NO38-185 as obtained by indirect immunofluorescence microscopy and the streptavidin-peroxidase reaction. The probe was hybridized to the nascent RNA transcripts of Xenopus laevis lampbrush chromosomes using a modification of the method originally introduced by Pukkila [47]. Both detection protocols gave essentially identical results (Fig. 2). For NO38-185 DNA, the hybridization was confined to two major sites on two different chromosome bivalents. One chromosomal locus was located terminally (Figs. 2a–c), the other at a subterminal position of a different chromosome bivalent (Figs. 2b, d). Although apparently the same loci were recognized by the different detection methods, the clarity and resolution of the hybridization signals differed considerably. Immunofluorescence microscopy produced a much more distinct and precise signal with a superior resolution and probably also higher sensitivity as compared to the peroxidase method. In particular, the discrimination between the brown precipitate of the peroxidase reaction and phase-dark structures such as the chromomeres and the numerous amplified nucleoli or fragments therefrom often proved to be difficult (e.g., Fig. 2b). Furthermore, the enzyme reaction product tended to obscure underlying structures such that the correlation with a specific loop was difficult or even impossible (Fig. 2a). These problems did not arise when immunofluorescence microscopy was employed since the brilliantly fluorescing loops stood out clearly against the dark background (Figs. 2c′, d′). The numerous amplified nucleoli present in the chromosome preparations served as an internal control; in "good" preparations they were not labeled above background levels.

For reasons discussed above we routinely used immunofluorescence microscopy for detection of the hybridized DNA probes. Hybridization times of 2 h turned out to be sufficient; prolonged incubation times did not generate substantially brighter signals. Hybridization in the presence of 40% formamide and at lower temperatures [26] resulted in comparable hybrid detection signals. However, the optical contrast of the lampbrush chromosomes was considerably reduced which made structural analyses of labeled loops difficult.

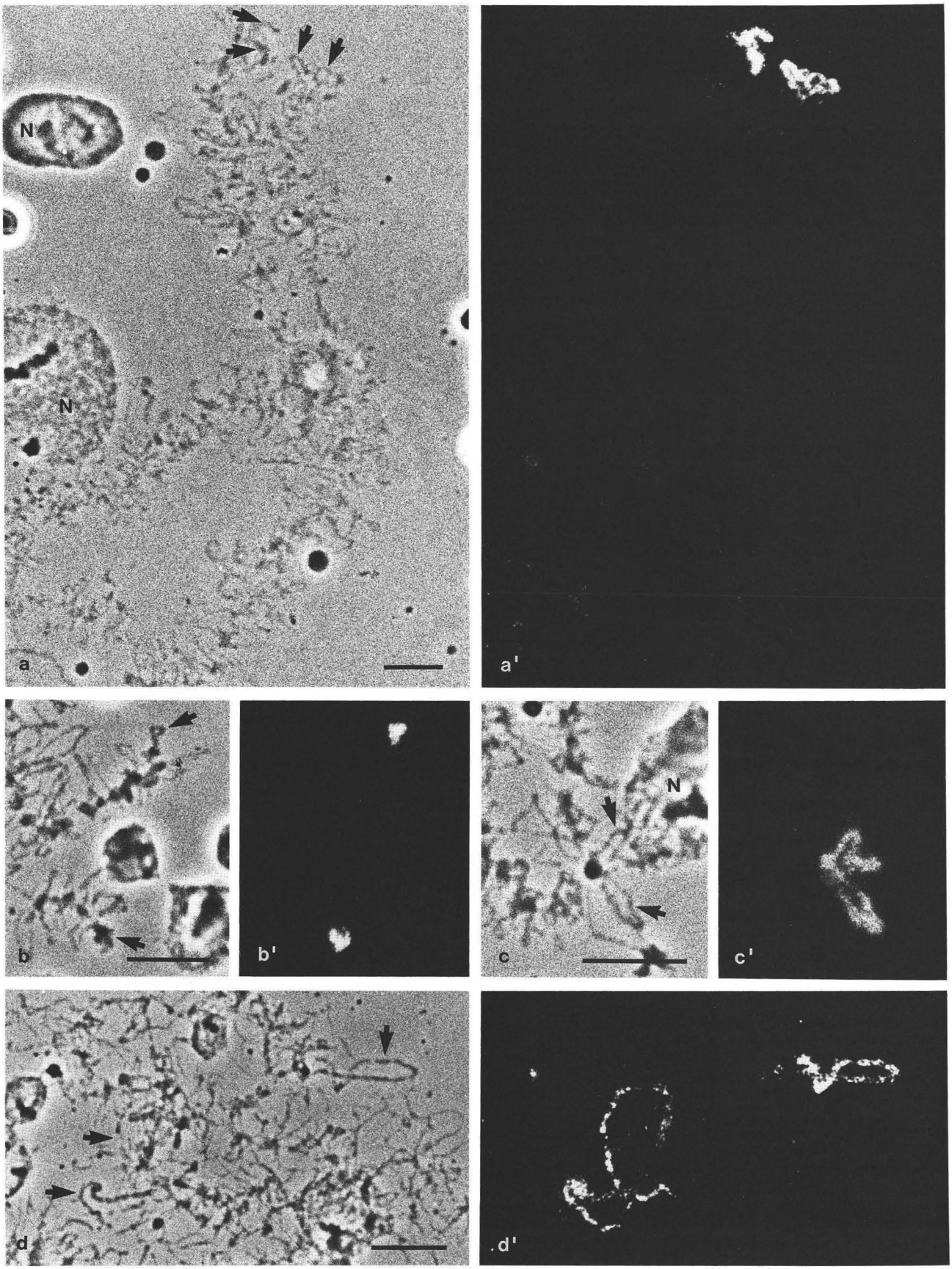
For controls, lampbrush chromosomes were hybridized with biotinylated plasmid DNA alone and processed for immunofluorescence microscopy. No signal could be detected in such preparations (not shown). Extensive digestion of the isolated chromosomes with RNase (which substantially decreased the optical contrast of the lateral loops) prior to addition of the biotinylated cDNA probes prevented hybridization (data not shown). This result confirms that under the conditions chosen (i.e., no denaturation of the DNA contained in the cytological preparation) the DNA probes hybridized to the loop-associated nascent RNA transcripts.

With the DNA probes employed, hybridization resulted in a precisely coincident labeling pattern of the two homologues of a chromosome bivalent which illustrates the specificity of the hybridization reaction (Figs. 2–5). The hybridization sites were consistently the lateral loops of the lampbrush chromosomes and not the chromosome axes. This could be directly observed in preparations stained with the peroxidase reaction (Figs. 2a, b) and by a comparison of the immunofluorescence pattern with the corresponding phase-contrast image (Figs. 2c, c′; 2d, d′; 3–5). Labeled loops occurred in pairs at each of the two homologous chromosomes of a bivalent (Figs. 2b; 3c, d; 5b), in agreement with the general organization of these meiotic chromosomes and their tetraploid nature (see [10]). Thus, each labeled loop was present in four separate copies per nucleus as could be seen in favorable preparations (Fig. 2b; frequently, however, sister loops were closely entangled with each other so that they could hardly be resolved as two individual loops).

The sizes of the chromosomal loops varied considerably not only in different oogenic stages but also in equally sized oocytes taken from different animals. In agreement with Callan, Gall and Berg [11] we noted that oocytes of 1 mm diameter generally exhibited larger loops upon isolation from freshly ovulated females as compared to unstimulated animals in which oocyte growth is presumably slowed down. The size differences of chromosomal loops might be related to differential spacing of the RNA polymerases as indicated by experiments in which the transcriptional state of lampbrush chromosomes was modulated [50]; see also [11, 31, 44]. According to these results, the apparent foreshortening of lateral loops is brought about by a condensation into nucleosomes and higher order structure of those chromatin stretches which are transiently free of transcripts [50].

Size differences of loops carrying the NO38/B23 TUs are illustrated in Figure 3. A pair of labeled loops was located near the telomeric regions of each of the two homologous chromosomes of the bivalent shown (Figs. 3a, a′; b, b′). These loops were either large and contorted (Fig. 3a) or, when oocytes from unstimulated females were used, relatively small (Fig. 3b). We also noted size differences of the other labeled loop pair which was located at a subterminal position of the other bivalent (Figs. 3c, c′; d, d′). The length of individual loops ranged from 12.5 μm (Fig. 3c) to 27 μm (Fig. 3d).

In a number of our preparations, in particular from young oocytes, the chromosomes were not well separated but remained entangled in form of larger aggregates (Fig. 4). In order to facilitate the assignment of labeled structures to specific loops and chromosomes, the preparations were additionally stained with Hoechst dye and examined by double-label fluorescence microscopy (Fig. 4). Under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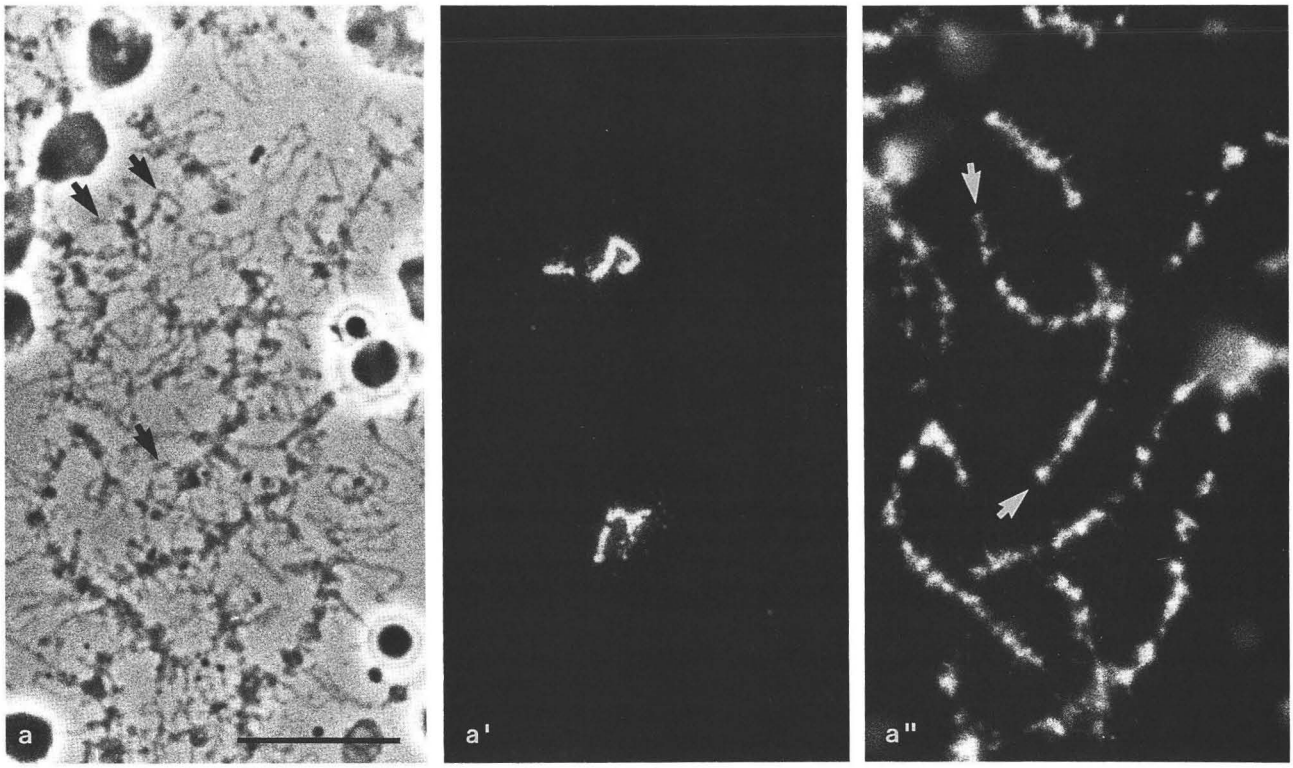

Fig. 4. Hybridization of probe NO38-185 to an aggregate of lampbrush chromosomes. The same field is shown in phase contrast (**a**), immunofluorescence (**a'**) and fluorescence microscopy after staining with Hoechst (**a"**). The Hoechst staining allows to trace individual chromosome axes (**a"**). Hybridization occurs on loop pairs situated at the ends of the homologous chromosomes of a bivalent (**a'**). Labeled loops are denoted by *arrows* in (**a**) and the chromosome ends to which they are attached by *arrows* in (**a"**). — Bar 20 μm.

these conditions the chromosome axes were brightly fluorescing and thus allowed to trace individual chromosomes (Fig. 4a″; the chromosome loops appear to be unstained due to their extremely low local DNA concentration, cf. [50]). In combination with phase-contrast microscopy, the labeled loops could thus be readily located to the ends of two homologous chromosomes (Fig. 4).

The hybridization pattern obtained with the cDNA clone pKXL 1/8 coding for a cytokeratin [23] was different from that of clone NO38-185. A larger number of chromosomal loci was labeled including some loops with a distinctly punctate hybridization pattern (Fig. 5). A major hybridization site could be located to a loop pair whose matrix was organized, at least in part, in form of numerous tiny granules which could be seen both by phase-contrast and immunofluorescence microscopy (Figs. 5a, a′). A pair of loops with comparable morphology has been described as the diagnostic feature of chromosome No. XVII [11]. The strongly fluorescing granules might indicate that defined subsets of adjacent RNP fibrils are organized into discrete structural entities. A different type of hybridization pattern is exemplified by another loop shown in Figure 5a′. Here, a region of strong fluorescence occurs next to an extended loop segment surrounded by a cloud of very small fluorescent entities. The significance of this hybridization pattern is as yet unclear. Finally, two pairs of large labeled loops with lengths ranging from 19 to 28 μm are shown in Figures 5b and b′. Again, in some regions of the labeled loops a spotty pattern of fluorescence is noticeable.

Fig. 3. Immunofluorescence detection of probe NO38-185 hybridized to lampbrush chromosomes (**a'-d'**). Corresponding phase-contrast images are shown for each case (**a-d**). The labeled terminal loops (*arrows* in **a, b**) can vary considerably in length depending on the size and metabolic state of the oocytes. Chromosomes were prepared from an early vitellogenic oocyte taken from a recently ovulated animal (**a, a'**) and from a larger oocyte of an unstimulated frog (**b, b'**). The subterminally located loop pair is denoted by *arrows* (**c, d**). — N Nucleolus. — Bars 10 μm.

Discussion

Among the protein coding genes only the reiterated histone genes have been located so far by in situ hybridization to specific lampbrush chromosome loops of newt oocytes and their pattern of transcription analyzed [8, 10, 15, 16, 26, 27]. Although the large lampbrush chromosomes from newt and salamander oocytes favor cytological stud-

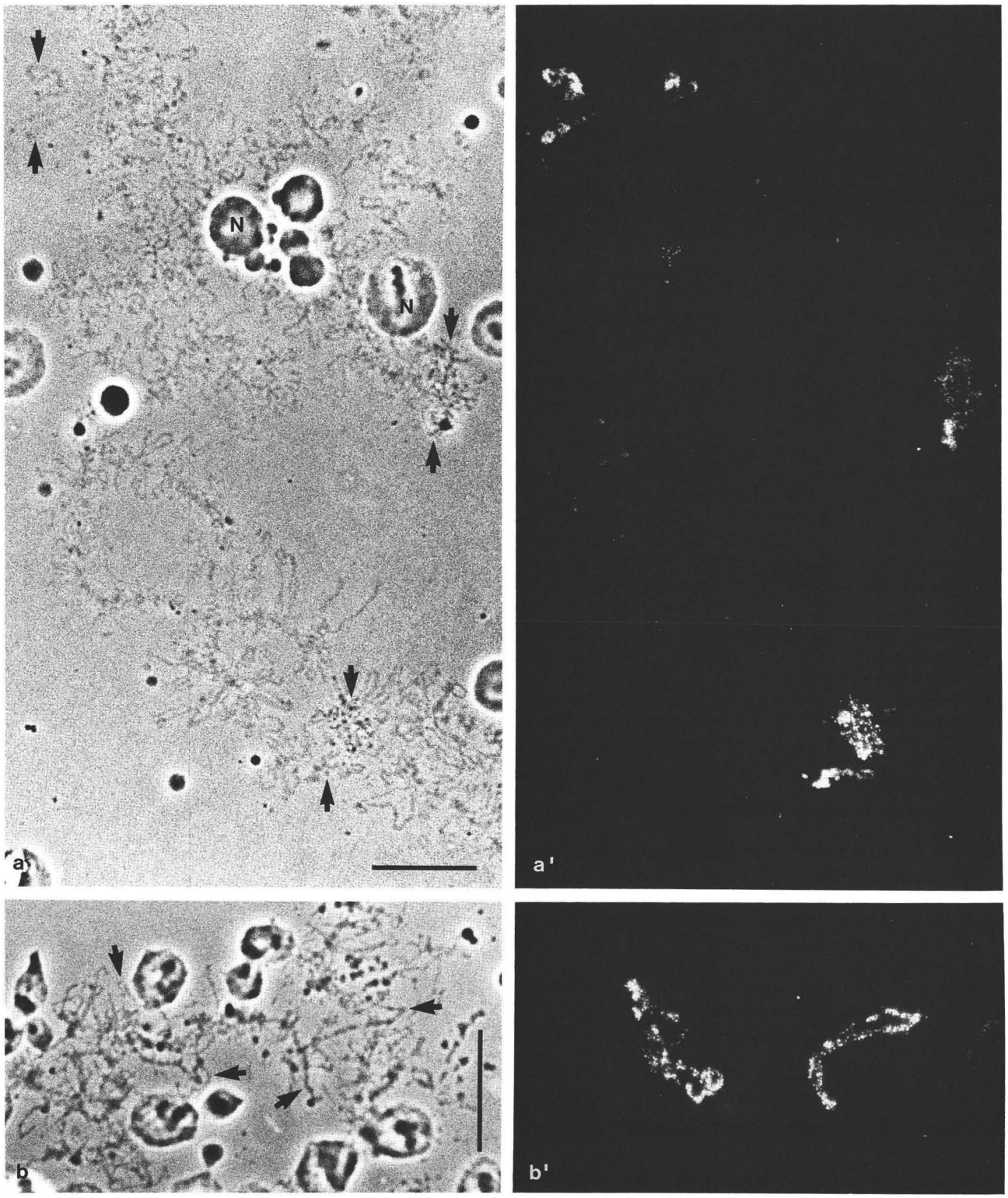

Fig. 5. Immunofluorescence detection of the cytokeratin probe pKXL 1/8 hybridized in situ to lampbrush chromosomes (**a', b'**). *Arrows* denote labeled loop pairs as seen in phase contrast (**a, b**). Note the different morphological aspects of the labeled loops. — N Nucleolus. — Bars 20 μm.

ies, we have decided to use the much smaller chromosomes of Xenopus laevis in order to take advantage of the broad spectrum of molecular probes available from this species. The small size of the lateral loops of the Xenopus lampbrush chromosomes made it necessary to apply a hybrid detection method with a resolution superior to that of hitherto employed approaches based on radiolabeled probes and autoradiography. It was found that immunofluorescence detection of hybridized biotinylated DNA probes gave the best results both in terms of sensitivity and resolution (for application of this non-radioactive methodology for localization of single-copy genes on metaphase chromosomes, polytene chromosomes and in interphase nuclei see, e.g., [1, 36, 37, 56, 64] and for visualization of specific primary RNA transcripts within interphase nuclei see [38]).

In the present study we demonstrate that biotinylated DNA probes in conjunction with immunofluorescence detection of the site of hybridization can be successfully used to locate the TUs of single-copy or near single-copy genes to specific loops of Xenopus lampbrush chromosomes. The approach described is very rapid and allows evaluation of the hybridization results within one day. From the first results obtained with this method it is noteworthy that the gene probes hybridized to more than one chromosomal locus, each locus being represented by two pairs of loops (each pair made up by sister loops originating from the same chromomere) located at corresponding positions of the two homologous chromosomes of a bivalent. Thus, transcripts homologous to the gene for nucleolar protein NO38/B23 are present at two major sites located on different chromosome bivalents, and cytokeratin-homologous transcripts are found at least at four major sites. A number of explanations might be envisaged to account for this situation.

(*i*) Both genes belong to larger multigene families whose members might also — dependent on the stringency of the hybridization conditions — be detected by the probes used. The nucleolar protein NO38/B23 is closely related to nucleoplasmin, an abundant nuclear protein of Xenopus oocytes [57, 58] whose genes are expressed also during oogenesis of Xenopus laevis [9]. The interpretation of our results is complicated by the fact that in addition several isoforms of both nucleoplasmin and protein NO38/B23 have been described (for refs. see [57]). In fact, the cross-hybridizing RNA band of approximately 1.5 kb (in addition to the major RNA band with 1.25 kb; Fig. 1a) indicates that there are two different transcripts which could be derived from two different loci.

Cytokeratins belong to the large "superfamily" of intermediate filament proteins including the nuclear lamina proteins which are differentially expressed in a cell-type specific pattern (for reviews see [22, 45, 60]). Xenopus oocytes synthesize three different cytokeratins [21, 24]. The probe pKXL1/8 codes for one of these cytokeratins, i.e., the equivalent to human cytokeratin No. 8 [23]. In addition, lamin L_{III} which is involved in formation of the lamina layer closely apposed to the inner nuclear membrane, is also expressed during Xenopus oogenesis ([6]; for review see [35]). Hence, it is not surprising that several different loci contain transcripts homologous to the cytokeratin gene used in our study.

(*ii*) Xenopus laevis is essentially a tetraploid species and it is thought that polyploidization had occurred in the course of its phylogenetic development, thus providing duplicate genetic information (for review see [34]). In fact, a number of single-copy genes including homeobox and c-myc genes have been shown to be duplicated in Xenopus laevis [25, 34, 62]. Two separate hybridization sites at different chromosome bivalents might therefore indicate the occurrence of duplicated genes, each one being transcriptionally active.

Our combined results based on Northern blots and in situ hybridization demonstrate that the genes for protein NO38/B23 and the cytokeratin 1/8 are transcriptionally active throughout most phases of Xenopus oogenesis. Although, for technical reasons, we have not examined oocytes larger than 1 mm in diameter, it is quite likely that the genes are continuously expressed until termination of the lampbrush chromosome phase just prior to maturation of the oocytes [41]. While the present data allow identification of defined transcriptionally active genes, they cannot establish a relationship between the TUs identified and the functional mRNA accumulated in the oocyte cytoplasm. Recently, however, we could definitely show by transferring individual nuclei from early vitellogenic Pleurodeles oocytes into Xenopus oocytes that lampbrush chromosomes do indeed synthesize functional mRNAs [51]. At first sight these findings appear to be in contradiction with the observed kinetics of mRNA accumulation during Xenopus oogenesis. As shown by the Northern blots, the final mRNA titers of full-grown oocytes are already established very early in oogenesis (comparable accumulation patterns have been measured for a number of other mRNAs and total poly(A) RNA, e.g., [9, 19, 28, 29, 46, 48, 61–63]). This apparent paradoxon was recently explained by measurements of the RNA metabolism and the flow of newly synthesized poly(A) RNA from the nucleus into the cytoplasm of variously sized vitellogenic Xenopus oocytes [2–4, 17]. According to these studies mRNA is continuously synthesized and exported into the cytoplasm throughout oogenesis, but net accumulation ceases — at about the onset of vitellogenesis — when synthesis is counterbalanced by degradation due to turnover of the stored pool of maternal mRNA (for detailed discussion see [14]). In a few cases, however, a continuous increase of mRNA levels has been measured throughout Xenopus oogenesis [65].

The aim of our studies is to understand the organization and transcriptional pattern of defined lateral loops in relation to specific single-copy or near single-copy genes. In the present report we have emphasized the methodological aspects of our in situ hybridization protocol which allows a rapid and sensitive assignment of cDNA probes to transcriptionally active loops of Xenopus lampbrush chromosomes. However, cDNA probes are restricted in their applicability in that they allow only the establishment of the chromosomal distribution of labeled loops and the determination of the approximate sizes of the constituent TUs. The TUs of the genes coding for protein NO38/B23 and cytokeratin 1/8 measured 27 and 28 μm, respectively, cor-

responding to 81 and 84 kb assuming full extension of the transcribed DNA. Experiments are now in progress to use appropriate genomic clones in order to correlate the distribution of coding and gene-flanking sequences to specific loop regions. In particular, we want to clarify whether the "readthrough" transcription model proposed by Gall and coworkers based on their studies of histone gene loops (for review see [27]) applies to lampbrush chromosome loops in general.

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