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SPECIAL ISSUE PATTERNS & PHENOTYPES

Working Map of the Lampbrush Chromosomes of *Xenopus tropicalis*: A New Tool for Cytogenetic Analyses

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The amphibian *Xenopus tropicalis*, whose genome has been recently sequenced, has become an important model organism for vertebrate developmental genetics. The development of cytogenetic tools in this new model organism should contribute to an understanding of the organization of the amphibian genome and the mapping of a variety of loci of interest. In this respect, oocyte lampbrush chromosomes are particularly useful for the localization of genomic sequences expressed during oogenesis. We have constructed a working map of *X. tropicalis* lampbrush chromosomes, which allows the 10 bivalents of the oocyte karyotype to be readily identified by distinctive combinations of specific landmark structures composed of lateral loops, spheres, and granules. We have also established the patterns of RNA Pol III sites over the chromosomes by immunofluorescence using antibodies directed against two Pol III subunits. Specific staining patterns were found for each chromosome, which constitute a supplementary tool for their identification. *Developmental Dynamics 238:1492–1501, 2009.* © 2009 Wiley-Liss, Inc.

Key words: lampbrush chromosomes; map; *Xenopus tropicalis*

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INTRODUCTION

Due to their large size, amphibian lampbrush chromosomes (LBCs) have provided for several decades a system of considerable value for cytological and molecular analyses of transcriptional processes. LBCs are observed at the diplotene stage in the oocytes of many animals, but they are particularly developed in amphibians. The two homologues are associated in bivalents and several thousand pairs of lateral loops unfold along their chromosome axis, giving them a characteristic feathery aspect (Callan, 1986; Morgan, 2002; Gall et al., 2004). Light and electron microscopy investigations have shown that each lateral loop consists of one or a few extremely long transcription units. Transcriptional inhibition studies and immunostaining approaches have demonstrated that the vast majority of lateral loops are transcribed by RNA polymerase II, while few of them are transcribed by RNA polymerase III. Immunostaining studies using a large panel of specific antibodies have shown that components of the splicing machinery, 3′-processing, and RNA packaging proteins or some other proteins involved in general translational repression, are associated with these nascent transcripts, suggesting that the regulation of gene expression occurs at an early transcriptional stage in the amphibian oocyte (for a review, see Morgan, 2002).

Amphibian lampbrush chromosomes also constitute a suitable model for studying the molecular organization and function of genomic sequences, which are expressed during oogenesis. In situ hybridization of specific probes to nascent transcripts of lateral loops consistently yield strong signals because they bind to many closely packed RNA transcripts in these loops (Weber et al., 1989; Angelier et al., 1996). The location of these hybridizing loops can be precisely defined and LBC maps were established in many amphibian species thanks to the presence of lateral loop landmarks

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with specific morphology and invariant location along the chromosome axis (for a review, see Callan, 1986).

Although most sequences studied previously and used for mapping the landmarks were repetitive sequences, the recent accumulation of amphibian sequence data and the fast development of cytogenetic techniques have made possible the extension of these approaches to single copy genes expressed on LBCs.

In this context, the amphibian *Xenopus tropicalis*, whose genome has been recently sequenced (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html), has become an important model organism for vertebrate developmental genetics. Compared with the closely related species *X. laevis*, which is allotetraploid, *X. tropicalis* is diploid with a smaller genome size (1.7×10^9 bp vs. 3.1×10^9 bp) and 10 chromosomes instead of 18 in *X. laevis*. Thus, the development of cytogenetic tools in this new model organism should contribute to a better understanding of the organization and expression of the amphibian genome and the mapping of a variety of loci of interest. To this aim, we have constructed a working map of the *X. tropicalis* lampbrush chromosomes in which the 10 bivalents of the oocyte karyotype can be readily identified by distinctive landmarks.

RESULTS

General Characteristics of the *X. tropicalis* Lampbrush Chromosomes

In addition to the 10 LBCs, the germinal vesicle (GV) of *X. tropicalis* oocytes was found to contain approximately 1,000 nucleoli and 100 spherical bodies similar to the Cajal bodies (CBs) described in many amphibian GVs (for a review, see Gall et al., 2004). The vast majority of these bodies had a diameter of 2–3 μm and were free in the nucleoplasm, whereas a small fraction of them reached 8 μm in diameter and were in general attached to the chromosomes. The GV also contained approximately 1,000 smaller particles, whose size was less than 0.5 μm in diameter and could correspond to the B-snurposomes described in other amphibian GVs (for a review, see Gall et al., 2004).

Construction of the LBCs map (Fig. 1) resulted from observations carried out on 100 LBCs spreads prepared mainly from stage V–VI oocytes isolated from 10 different non–hormonally or hormonally stimulated *X. tropicalis* females. Despite their small size, the lampbrush chromosomes of the diploid *X. tropicalis* oocytes exhibited lateral loops, which were relatively well extended. As reported previously for different amphibian species, these loops were more developed in stage IV oocytes, in which transcription was very intense, than in stage VI oocytes, in which it was slowed down. However, similarly to what was described for *X. laevis* (Gall et al., 1991), lateral loops became less extended when oocytes at any stage were incubated in vitro for more than 4–5 hr, reflecting a slowing down of transcription. No recovery of transcription was observed after overnight incubation. LBCs axes became condensed and foreshortened, and only landmark loops remained visible (not shown). LBCs lateral loops in oocytes from hormonally stimulated females were also slightly more extended than those in oocytes from non–hormonally stimulated females, but the difference was not as clearly visible as reported for *X. laevis* (Callan et al., 1987). Although the lateral loops were more extended in stage IV than in stage VI oocytes, we decided to establish the *X. tropicalis* LBCs map with oocytes at the border between stages V and VI (0.6–0.8 mm in diameter) for the following practical reasons: (1) stage VI oocytes were more abundant in non–hormonally stimulated females while the LBCs exhibited the same features as those observed in stage IV oocytes; (2) the GV content of stage IV oocytes took twice as long to disperse as that of stage V–VI oocytes (see the Experimental Procedures section); (3) chromosomes were well separated from each other in larger oocytes greatly facilitating the preparation of GV spreads by eventual untrained investigators. In this study, we have used the relative length of the different LBCs, the specific landmarks that they exhibited and their Pol III-labeling pattern as identification criteria.

Fresh and centrifuged GV spread preparations showed 10 bivalents whose chromosome axial length ranged from 47 to 17 μm. As reported by Callan et al. (1987) for *X. laevis* LBCs, these values should not be considered as accurate because they were estimated from centrifuged preparations in which the chromosomes may have been submitted to some degree of stretching. *X. tropicalis* LBCs were numbered from I to X in decreasing length order according to the criterion followed in other species. Their relative length shown in the working map of Figure 1 corresponds to the ratio between the absolute length of each chromosome and the length of chromosome V to which an arbitrary value of 100 units was given. Because the centromeres were not visualized, chromosomes were oriented in such a way that landmark loops were on the right side to assign a right and a left end to each chromosome. Although the majority of lateral loops conformed to a "standard type" in which the RNP matrix showed a similar organization, several LBCs exhibited distinctive structures, which qualified them as landmarks. The most prominent landmarks were elongated giant structures, whose length could reach 14 μm. They were present on bivalents II and VI (Figs. 1–4) and fusion of sister loops was often observed. These loops appeared vacuolated and exhibited distinctive shapes such as "boxing gloves" or "buffalo horns" (Fig. 3). They may be compared with the "giant bodies" described in LBCs of *Triturus vulgaris meridionalis* (Barsacchi et al., 1970) and *Rana esculenta* (Giorgi and Galleni, 1972). We referred to these loops as mega fusing loops (MFL). A second class of landmarks was represented by giant formations of globular shape and homogeneous structure. They were observed on bivalents IV, V, and IX (Figs. 1–3). Because these structures appeared similar to the loops described in different amphibian species (for a review, see Callan, 1986) and referred to as giant fusing loops (GFLs) by Callan (1963), we also referred to these loops as "GFLs." Each homologue bore only one of these structures, indicating that GFL sisters were always fused. Spherical formations observed on bivalents VI (Figs. 1–4) and IX (Figs. 1, 6, 7) defined a third type of land-

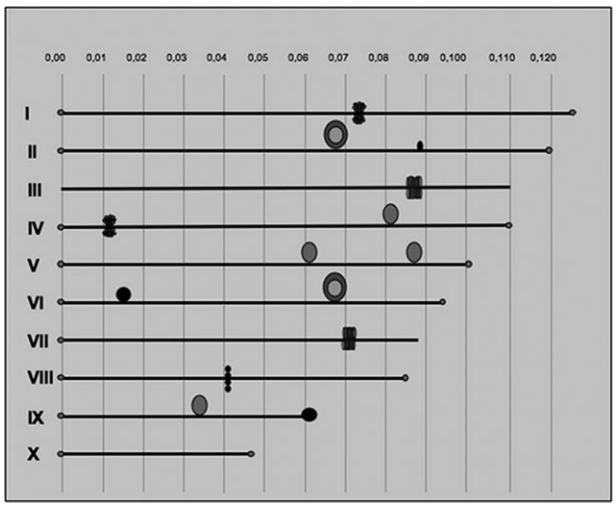

Fig. 1. Working map of the 10 lampbrush chromosomes of *Xenopus tropicalis* oocyte. The chromosomes were ordered and numbered according to their relative length expressed as the ratio between the length of each chromosome and that of chromosome V, whose length was taken as 100 units. Chromosomes were arbitrarily oriented so that their cytological landmarks were on the right side. A description of the landmarks is provided in the text.

mark. They were 2–8 μm in diameter, whereas those from smaller oocytes at the border between stages IV and V were larger (Fig. 6C). Spherical formations were also found sometimes associated with different chromosomes such as chromosome I (Fig. 5) or chromosome VII (not shown). Although they closely resembled similar structures found in many amphibian oocytes (for a review, see Callan, 1986) and which were initially called spheres and later identified as CBs (for a review, see Gall et al., 2004), we preferred to refer to these formations as "spheres" pending further molecular evidence of their exact identity. A fourth set of landmarks was composed of tightened lateral loops of fibrillar aspect (fibrillar loops, FLs) followed by a region marked by several long and thin loops, which could be compared with the region referred to as "exploded" by Callan et al. (1987) in LBCs III and XI of *X. laevis*. FLs were observed on bivalents III and VII (Figs. 1–3). A fifth type of lateral loops with a granular matrix (GrLs) was present on bivalents I and IV (Figs. 1–3). All of these different landmarks could be readily recognized in fixed preparations and allowed 8 of the 10 chromosomes of the oocyte karyotype to be easily identified (Fig. 3). As shown for bivalent VI (Fig. 4), these landmarks were better preserved in paraformaldehyde-fixed than in ethanol-fixed preparations.

As a complementary means of identifying all 10 chromosomes, even when their structure was damaged, we analyzed the distribution of RNA Pol III loci using antibodies against two subunits of pol III core polymerase: RPC53, which is unique to pol III; or RBP6, which is shared by the three polymerases pol I, pol II, and pol III (see the Experimental Procedures section). Using these antibodies Murphy et al. (2002) have demonstrated that the distribution of Pol III loci provided specific patterns, which allowed most of the 18 LBCs of *X. laevis* to be recognized. As exemplified for bivalents IX and X (Fig. 7), these two antibodies stained the same specific set of sites on the 10 *X. tropicalis* bivalents facilitating, therefore, their identification (Figs. 5, 7). These sites corresponded to some axial and terminal granules and were assumed to correspond to pol III loci because the anti-RBP6 antibody, although not specific for pol III, stained the same set of loci as did the anti-RPC53 antibody, which is itself specific for pol III. In contrast, these two antibodies stained differentially the other nuclear structures. The anti-RBP6 antibody strongly stained nucleoli, but very weakly the "spheres," the lateral loops and the multiple other CB-like structures in the nucleoplasm (Fig. 7C). The anti-RPC53 antibody strongly stained the "spheres" and the CB-like structures, as well as other landmarks such as MFLs, lumpy structures, and GFLs (Figs. 5, 7I), and the numerous granules associated to the double-loop bridges at one end of the chromosome X (Fig. 7L).

Individual Characterization of the *X. tropicalis* Lampbrush Chromosomes

The landmark type, the presence of terminal granules and the pattern of Pol III loci were taken into consideration for the following description of each chromosome (Figs. 2–7).

Chromosome 1.

Mean relative length: 125 units. GrLs: 72 units. This chromosome was the longest of the oocyte complement. In good preparations, it could be identified by a loop with a granular matrix (GrL) at a locus lying two-thirds from its right extremity (Fig. 2). Spheres were sometimes associated with its left telomere. Because the chromosome axis was frequently coiled up the right and left ends were difficult to distinguish. The terminal granules were always fused. Both terminal and axial granules were intensely stained with the anti-RBP6 and anti-RPC53 antibodies. The anti-RPC53 antibody also stained spheres when present (Fig. 5).

Chromosome II.

Mean relative length: 119 units. MFLs: 70 units. LS: 89 units. Thanks to its MFLs (Figs. 2, 3) and lumpy structures (LS), this chromosome was one of the four bivalents of the oocyte complement, which were the easiest to be identified. Anti-RBP6 and anti-RPC53 antibodies immunostained axial and terminal granules. The anti-RPC53 antibody immunostained also the MFLs and lumpy structures (Fig. 5).

Chromosome III.

Mean relative length: 110. FLs: 89 units. It could be identified by several pairs of fibrillar matrix (FL) located near its right end (Figs. 2, 3). The bivalent also exhibited a particular configuration, which allowed its identification. On the right side, the extremities of the two homologues were largely spread out from the chiasma

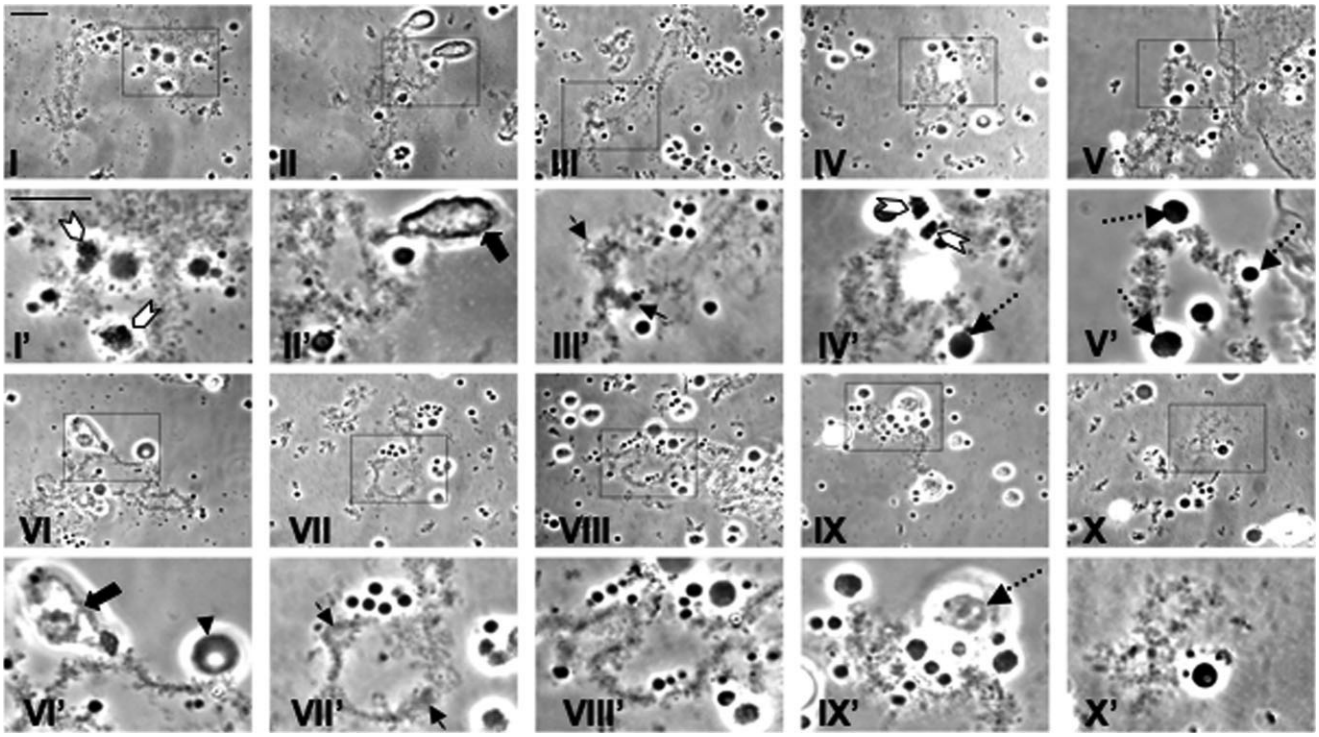

Fig. 2. The 10 bivalents of *Xenopus tropicalis* from fresh and centrifuged preparations. The 10 bivalents were numbered with roman numerals from I to X according to their relative length. I′ to X′: High magnifications of the squares in I to X showing the different landmarks which characterize each bivalent. White arrows point to granular loops (GrLs) on chromosomes I and IV; black arrows point to the mega fusing loops (MFLs) on chromosomes II and IV; small arrows point to the fibrillar loops (FLs) on chromosomes III and VII, dotted arrows point to giant fusing loops on chromosomes IV, V, and IX, arrowhead point to the "sphere" (S) on chromosome VI. Scale bars = 10 μm.

on both sides of the bivalent (Fig. 3). Anti-RBP6 and anti-RPC53 antibodies immunostained axial granules and the left end of the chromosome, although no terminal granules were observed (Fig. 5).

Chromosome IV.

Mean relative length: 109. GFL: 80 units. GrL: 72 units. It could be easily identified by the presence of a GFL near its right extremity and by the presence of a GrL on the opposite side (Figs. 2, 3). Both terminal and axial granules were intensely stained by the anti-RBP6 and anti-RPC53 antibodies. The latter stained also the GFLs (Fig. 5).

Chromosome V.

Mean relative length: 100. GFLs: 60 and 80 units. It was easily identified by two GFLs, which were always located on the right side (Figs. 2, 3). Its two extremities bore terminal granules, which were immunostained by the anti-RBP6 and anti-RPC53 antibodies. These two antibodies also labeled axial granules, while the anti-RPC53 antibody immunostained the GFLs (Fig. 5).

Chromosome VI.

Mean relative length: 93. S: 14 units. MFL: 68 units. Similarly to chromosome II, this chromosome bore MFLs on the right side, but it could be distinguished from it by its shorter length and the presence of "spheres" (Figs. 2–4). Its two extremities bore terminal granules. Anti-RPC53 antibody immunostained axial and terminal granules as well as the "spheres" and the MFLs (Fig. 5), while the anti-RBP6 antibody immunostained the axial and terminal granules only.

Chromosome VII.

Mean relative length: 88. FLs: 70 units. Like chromosome III, this chromosome exhibited several FLs (Figs. 2, 3) but it could be distinguished from it by its shorter length and the presence of terminal granules at its left extremity. The immunostaining pattern with the anti-RBP6 and anti-RPC53 antibodies facilitated its identification because 3 stained loci were observed at its right extremity, while only one locus was found on bivalent III (Fig. 5).

Chromosome VIII.

Mean relative length: 84. This bivalent lacked regular identifying landmarks. However, nucleoli-like structures were often observed at the level of one loop, located in its middle. Terminal granules were observed at its two extremities. The anti-RBP6 and anti-RPC53 antibodies stained the terminal and axial granules. The anti-RBP6 antibody stained also these nucleoli-like structures (Fig. 5).

Chromosome IX and chromosome X.

Mean relative lengths: 60 and 46 units. These two small bivalents could not always be easily identified in the oocyte karyotype because they exhibited a fragile and complex structural organization, which appeared split in many instances (Fig. 6A,B). As shown in Figure 5C, bivalent IX appeared often broken at the level of its GFLs, leading to the formation of circular

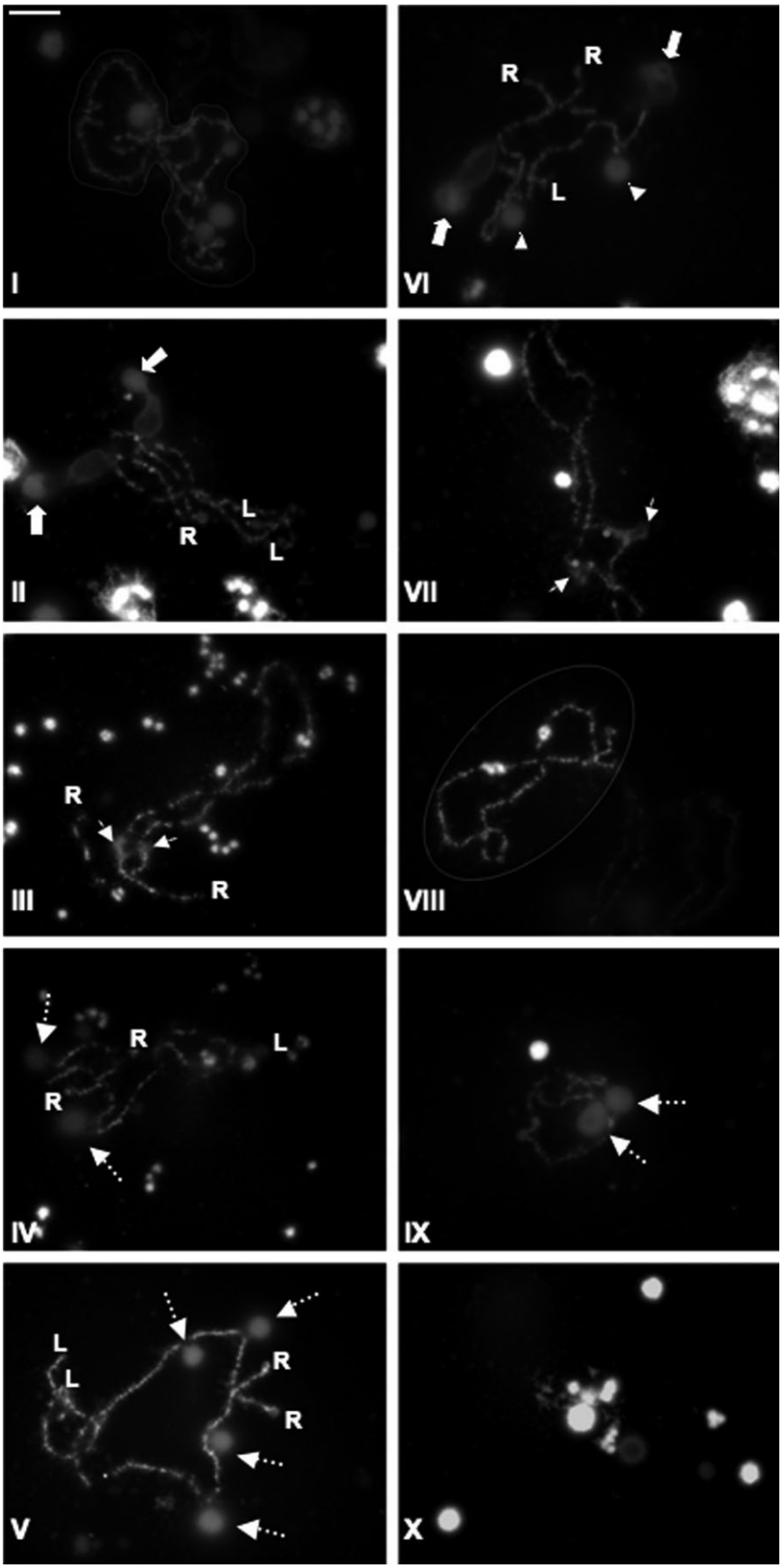

Fig. 3.

mini-chromosomes (Fig. 6D,E). The chiasma in the middle of chromosome X separated two regions (Fig. 6F–I). Lateral loops conformed to the "standard type" in one, while in the other they corresponded to several bunches of small loops. Numerous granules of different sizes were associated with these loops. The fusion between the granules of adjacent or more distant lateral loops created an important stretching, which led to their separation from the chromosomal axis and the formation of "double-loop bridge" structures. As shown in Figure 7, a specific pattern of immunostaining with anti-RBP6 and anti-RPC53 was found for these chromosomes.

DISCUSSION

We have identified the 10 lampbrush chromosomes making up the karyotype of the *X. tropicalis* oocyte and produced a comprehensive cytological map. In previous studies the establishment of LBCs maps in different amphibian species has been based on different criteria including the relative length of the chromosomes, the position of the centromere or nucleolar organizer, the presence of loops with specific morphology, and the presence of a variety of other chromosomal structures, which can be considered as landmarks. Because centromeres could not be directly identified on the *X. tropicalis* chromosomes, we have ordered and numbered them according to their relative length, and arbitrarily fixed their orientation relative to the cytological landmarks they bore. It should be noted that our ordering differs from that proposed by Tymowska and Kobel (1972) for metaphase chromosomes according to chromosome size and centromere position. Because a new order of metaphase chromosomes is currently being established, it becomes important to corre-

Fig. 3. The 10 bivalents of *Xenopus tropicalis* from paraformaldehyde-fixed and propidium iodine–stained preparations. The 10 bivalents were numbered with roman numerals from I to X. Large arrows point to the mega fusing loops (MFLs), small arrows point to the fibrillar loops (FLs), dotted arrows to the giant fusing loops (GFLs), arrowheads point to the spheres. L, left end; R, right end. Scale bar = 10 μm.

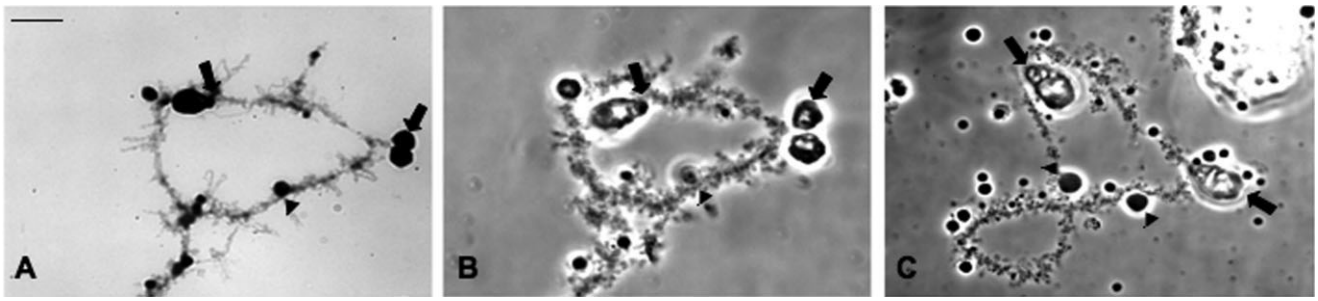

Fig. 4. The bivalent VI from fresh, alcohol-fixed and paraformaldehyde-fixed preparations. **A:** Bivalent VI from alcohol-fixed and Coomassie blue stained preparation. **B:** The same bivalent from the same preparation before fixation. **C:** The bivalent VI from another preparation fixed with paraformaldehyde and observed under phase contrast. Large arrows point to the mega fusing loops (MFLs), arrowheads point to the "spheres." Note that all the characteristic features of this bivalent were conserved regardless of the fixative. However, chromosome structure was best conserved with paraformaldehyde fixative. Scale bar = 10 μm.

late the lampbrush chromosomes numbering with that of the mitotic chromosomes. Such a correlation could be achieved using two complementary approaches. One would be the comparison of the position of centromeres on these two types of chromosomes. Position of the centromeres on LBCs of *X. tropicalis* could now be determined by in situ hybridization of centromeric repeat one (Fcr1) probe as for *X. laevis* (Edwards and Murray, 2005) or by injecting transcripts of the myc-tagged CENP-C protein in the *X. tropicalis* oocyte (Joseph Gall, Carnegie Institution, personal communication). The other would be to carry out in situ hybridization of specific probes for single copy genes on both sets of chromosomes, a technique which has been applied successfully to *X. tropicalis* mitotic chromosomes (Krylov et al., 2007).

The most striking feature of *X. tropicalis* GV spreads was that each of the 10 LBCs exhibited one or several specific morphological characteristics which made their identification unambiguous. In contrast, only 10 of the 18 LBCs in the closely related species *X. laevis* could be readily identified on the sole basis of their morphology. It has been admitted for a long time that the relative length of lateral loops in one species can be directly correlated to the C-value of its genome (Macgregor, 1980). Because the size of the *X. tropicalis* genome is nearly two-thirds of that of *X. laevis*, we assumed that the lateral loops would be of a very small size. Remarkably and contrary to our expectation, many landmarks were of a large size as compared to the relatively small size of the corresponding chromosomes. It was the case for the MFLs of bivalent VI, the GFL of bivalent IX, the GLs of bivalent X and the lateral loops in the "exploded" region of bivalent VII. In this context, it should be noted that Gall and Murphy (1998) have demonstrated that the degree of development of lateral loops can be dependent on the physiology of the oocyte rather than on the genome C-value (see for review, Morgan, 2002).

We have also studied the distribution of RNA Pol III subunits by immunofluorescence using the anti-RPB6 and anti-RPC53 antibodies. The use of the anti-RBP6 antibody for detecting Pol III loci may have seemed inappropriate because the RPB6 subunit was also shared by RNA pol I and pol II, but Murphy et al. (2002) showed that it gave a brilliant staining of pol III loci on *X. laevis* LBCs, which allowed their unambiguous identification. Similarly, we found a specific staining pattern for each *X. tropicalis* lampbrush chromosome, which provided an additional tool for their identification. Because these two antibodies recognized Pol III, we could assume that the sites labeled by both antibodies, such as axial and terminal granules, corresponded to pol III loci. The significance of the staining of the MFLS, GFLs, and lumpy structures by the anti-RPC53 antibody remained to be elucidated. This antibody also stained strongly the spheres and the CB-like structures in agreement with their putative role. The CBs, which correspond to the coiled bodies found in mammalian somatic cells, were previously designated as sphere organelles or coiled bodies in amphibians GVs. Their sites of attachment to the LBCs were identified as the histone genes loci in several amphibian species (Gall et al., 1981, Callan et al., 1991). From the seminal studies of Gall and coworkers, it is now well admitted that CBs are the sites of preassembly of the nuclear transcription machinery and assembly or modification of the nuclear RNA processing machinery (for review, see Gall et al., 2004) and, therefore, contain the core units of all three RNA polymerases. In this context, it was surprising to observe that the anti-RPB6 antibody, which is directed against the RBP6 subunit shared by pol III and pol II, stained neither the spheres and CB-like structure nor the axes of lateral loops, while it strongly stained the nucleoli as expected. This lack of staining of lateral loops transcribed by pol II was in accordance with the observation reported by Murphey et al. (2002) that this antibody failed to detect the RPB6 subunit when part of the pol II on LBCs of *X. laevis*. An investigation of the distribution and targeting of other pol III subunits and pol III transcription factors would be required to explain these results. In the context of our study, we considered that these antibodies provided a complementary means of identification of the LBCs only.

Strikingly three types of the *X. tropicalis* landmarks appeared to be duplicated on two separate chromosomes. For example, a similar granular loop was observed on chromosomes I and IV; the MFLs were observed on chromosomes II and IV; the FLs were observed on chromosomes III and VII. We have previously shown that the

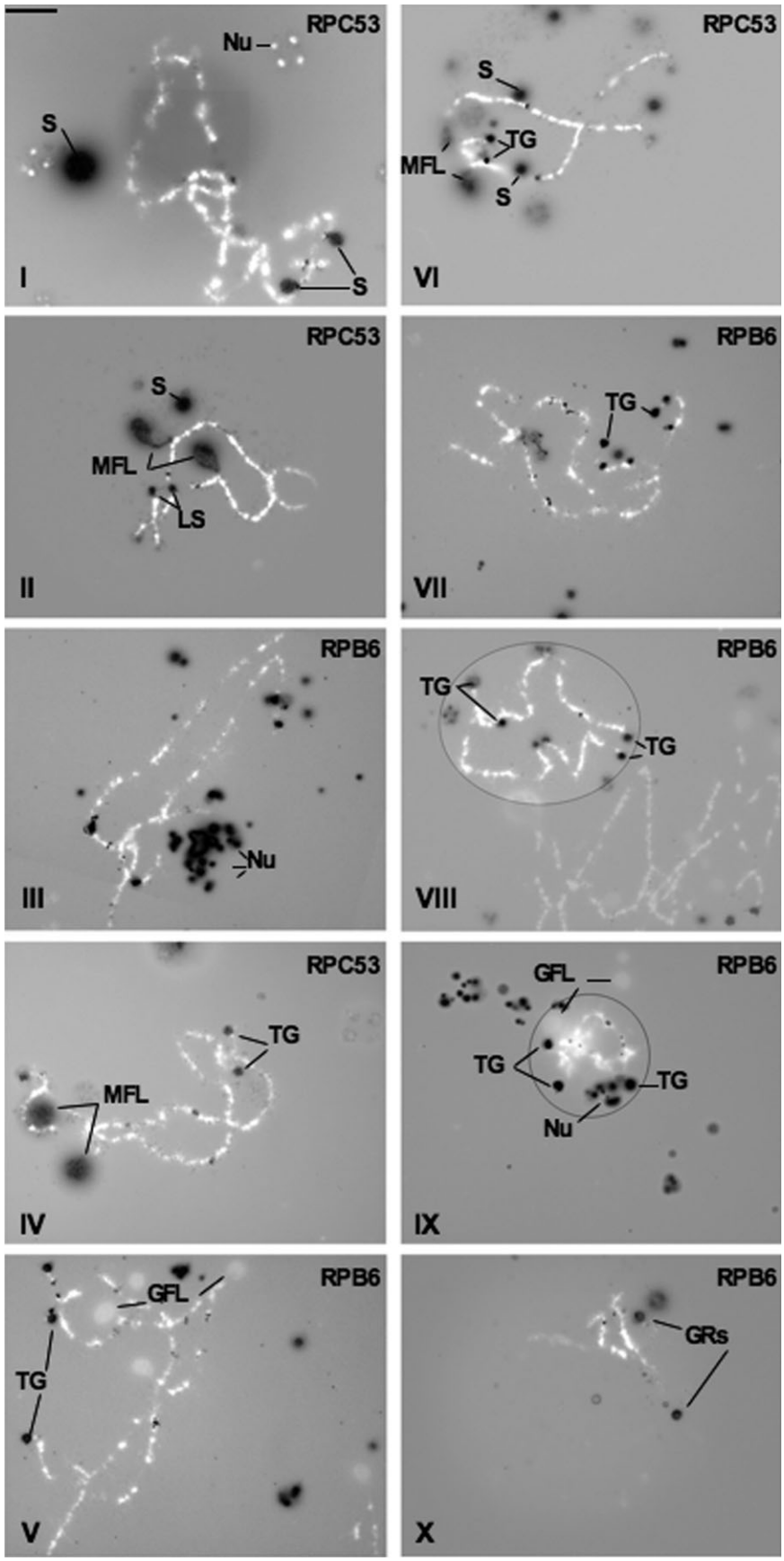

Fig. 5.

specific morphology of the RNP matrices of lampbrush chromosomes was dependent on the degree of compaction of the transcription products resulting from the packaging of RNP transcripts and the progressive coiling of the loop axis (Bonnanfant-Jais et al., 1986; N'Da et al., 1986; Penrad-Mobayed et al., 1986). We have also established that landmark loops of the same type share an ultrastructural similarity in different amphibian species (Bonnanfant-Jais et al., 1991). From these observations, we can assume that the *X. tropicalis* landmark loops, which belong to the same morphological type, have the same organization of their RNP matrix suggesting a possible duplication of the relevant genomic regions. Because the *X. tropicalis* genome was proven to be diploid, the significance of these apparent duplications remains to be assessed.

Our working map of the lampbrush chromosomes of *X. tropicalis*, can be considered as a new tool for cytogenetic analysis in this species. We are currently establishing the correspondence between LBCs and metaphase chromosomes by FISH analysis and positioning of the centromeres in LBCs.

EXPERIMENTAL PROCEDURES

LBCs spreads of *X. tropicalis* were prepared from seven non–hormonally or three hormonally stimulated females following a protocol modified from that initially described by Callan et al. (1987) for *X. laevis* LBCs and further detailed by Gall et al. (1991) in their general instructions for the

Fig. 5. Pol III sites in the lampbrush chromosomes of *Xenopus tropicalis*. The chromosomes were issued from two nuclei stained with anti-RPB6 or anti-RPC53 antibodies. Pol III loci are shown black against the white chromosome axis stained with Hoechst. Note that the two antibodies stained the axial and terminal granules and the numerous granules at one end of the chromosome X (Grs). Antibody against RPC53 also stained the mega fusing loops (MFLs), the spheres, and the giant fusing loops (GFLs), while the anti-RPB6 antibody stained the nucleoli. Grs, granules; N, nucleolus; S, sphere; TG, terminal granule. Scale bar = 10 μm.

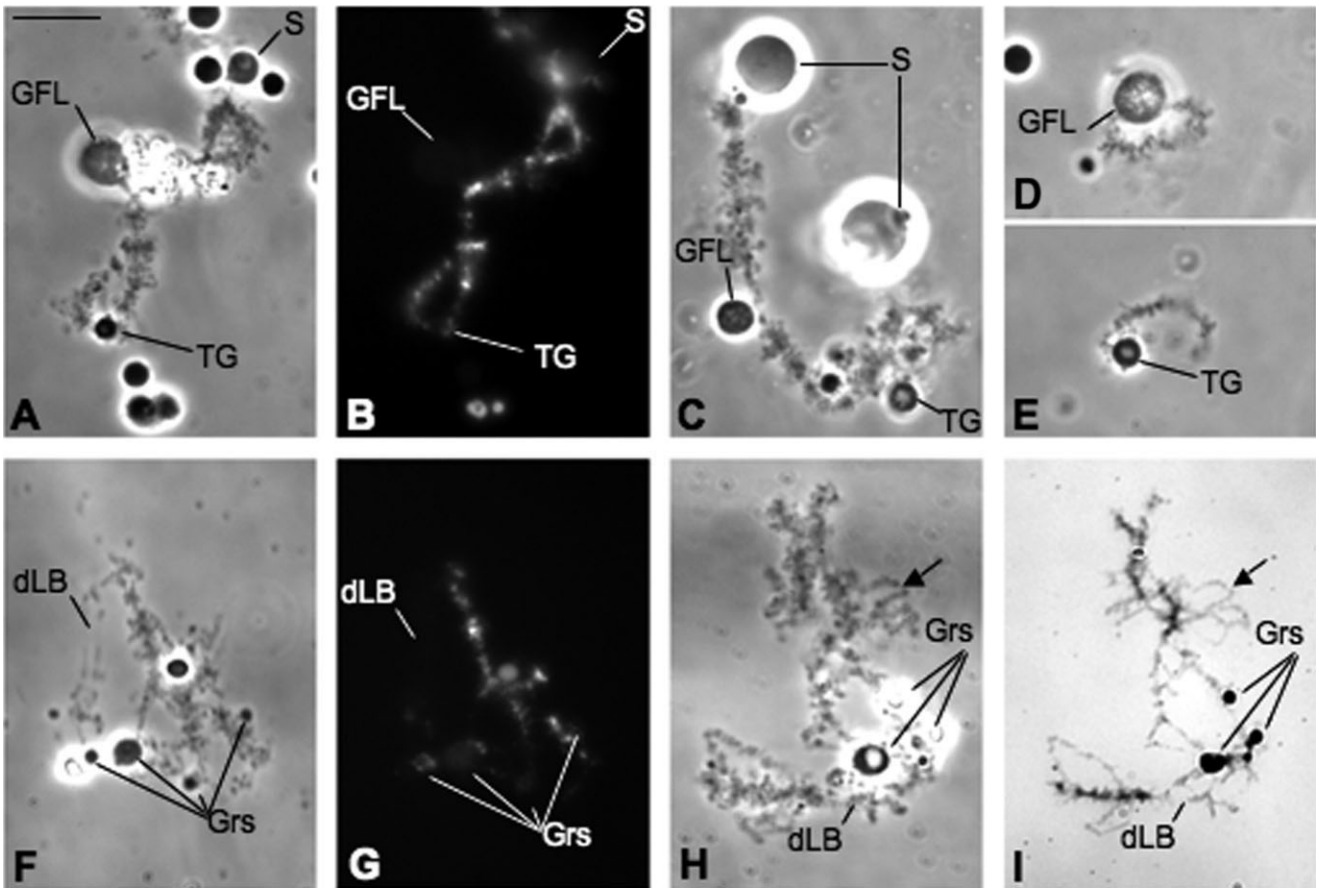

Fig. 6. The complex organization of bivalents IX and X. A–E: Bivalent IX. **A,B:** Phase-contrast and fluorescence images of the same bivalent showing its general characteristics. **C:** Phase-contrast from fresh preparation of stage IV–V oocyte, broken at the level of one giant fusing loop (GFL). Note the large size of the "spheres" at this stage. **D,E:** Phase-contrast showing two mini chromosomes which resulted from a break at the level of both GFLs. F–I: Bivalent X. **F,G:** Phase-contrast and fluorescence images of this bivalent showing its general characteristics. **H,I:** The same bivalent from the fresh (H) or ethanol-fixed preparation (I). Note the presence of "double-loop bridge" structures and the large size of loops of "standard" type (arrow). dLB, double-loop bridge; S, sphere; Grs, granules; TG, terminal granule. Scale bar = 10 μm.

preparation of GVs spreads of amphibian oocytes.

Six stages of oocyte development were distinguished in the ovary of non-hormonally mature females on the basis of the classification criteria introduced by Dumont (1972) for *X. laevis* oocyte development. Stages used for LBC spreads of different amphibian species ranged from IV to VI. In *X. tropicalis*, average sizes were 0.4–0.5 mm for stage 1V oocytes, 0.6–0.7 mm for stage V, and 0.7–0.8 mm for stage VI. After ovulation, the number of stage VI oocytes was dramatically decreased in the hormonally stimulated females.

The different steps of our protocol can be summarized as follows: (1) preparation of hormonally stimulated animals: mature females were stimulated by injection with human chorionic gonadotropin (100 IU/100 μl) to induce ovulation; (2) ovary biopsy: non–hormonally stimulated females or stimulated females 2 days after ovulation, were anesthetized by immersion in 0.1% MS222 (Amino-benzoic Acid Ethyl, Fluka). A sample of ovary was surgically removed, and oocytes 0.4–0.8 mm in diameter which are equivalent to IV–VI stages oocytes of *X. laevis* were dissociated and maintained in OR2 medium (Wallace et al., 1973) at 18°C for several hours. (3) Recovery of GVs: each oocyte was transferred to a Petri dish in the "5: $1+PO_4^{-3}$" solution (17 mM NaCl, 83 mM KCl, 6.5 mM NA_2HPO_4, 3.5 mM KH_2PO_4; Gall et al., 1991) and dissected using two pairs of forceps (Dumont no. 5). The isolated GVs were quickly transferred directly to the centrifugation chamber using a nuclear pipette and maintained on ice. (4) GV spread: the centrifugation chamber consisted of a glass slide with a central hole 5 mm in diameter which was sealed with a coverslip glued to the underside of the slide using paraffin and filled with dispersal medium (25% of $5:1+PO_4^{-3}$ solution, 10 μM $CaCl_2$, 0.1% w/v paraformaldehyde). The nuclear envelope was removed in the chamber with forceps, and the GV content was allowed to disperse at 4°C for 30 min (stage V–VI oocytes) or 1 hr (stage IV oocytes). Chambers were then centrifuged at 300 × *g* for 10 min, and at 1,500 × *g* for 30 min at 4°C. After centrifugation, preparations were observed under phase contrast with an inverted microscope (Carl Zeiss) and further post-fixed with 70% alcohol or 2% paraformaldehyde in phosphate-buffered saline (PBS).

Alcohol-fixed preparations were dehydrated through an ethanol series,

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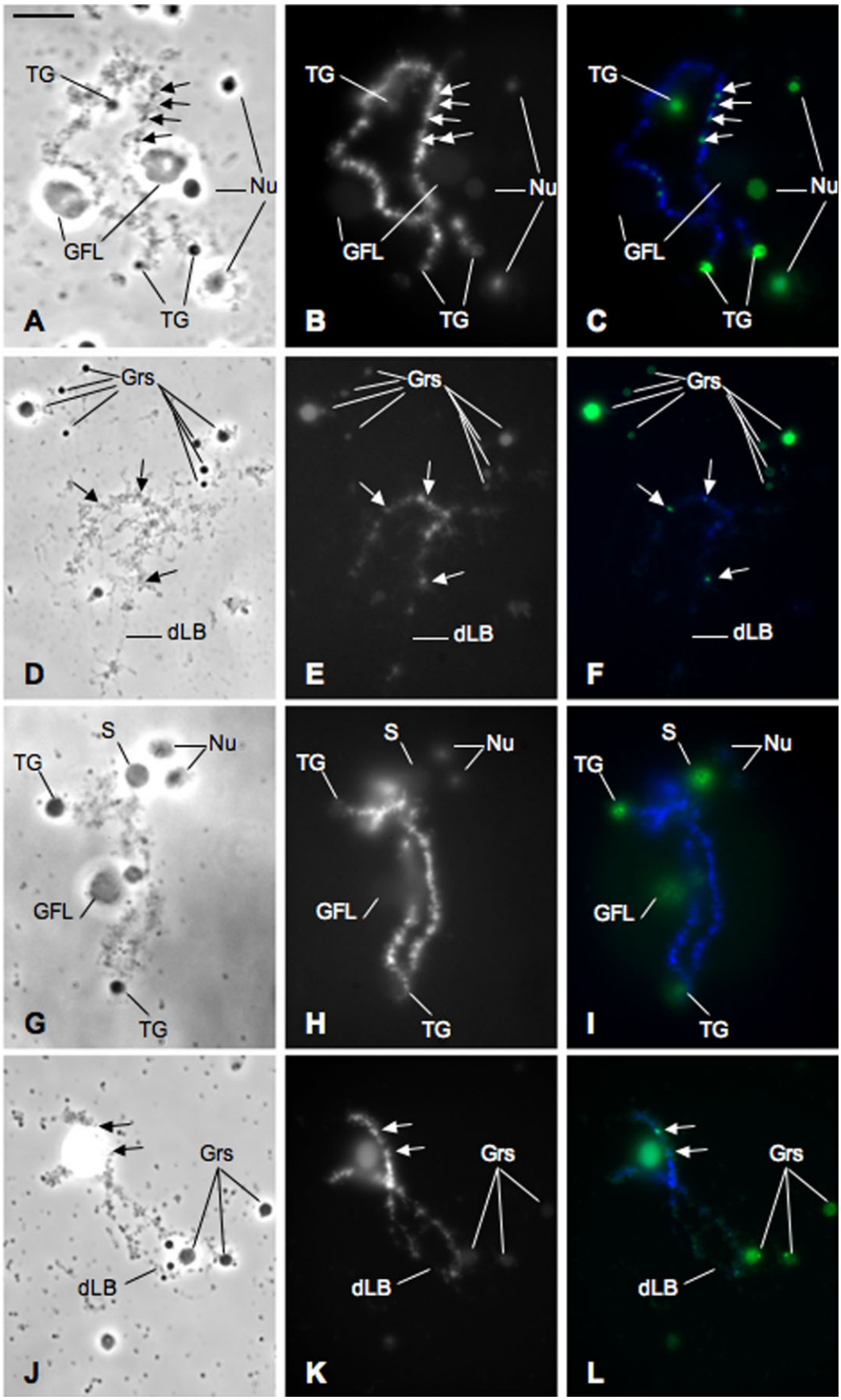

Fig. 7. The Pol III sites in the bivalents IX and X. **A–F:** Anti-RBP6 staining. **G–L:** Anti-RPC53 staining. Bivalent IX: A–C,G–I. Bivalent X: D–F, J–L. A,D,G,J: Phase contrast. B,E,H,K: Hoechst staining. C,F: Overlay of anti-RBP6 (green) and Hoechst (blue) staining. I,L: Overlay of anti-RPC53 (green) and Hoechst (blue) staining. Arrows point to the axial granules. Note that the antibody against RPB6 also stained nucleoli while the anti-RPC53 antibody stained the giant fusing loops (GFLs) and the spheres. dLB, double-loop bridge; Nu, nucleoli; S, sphere; TG, terminal granule. Scale bar = 10 μm.

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washed in xylene to remove paraffin wax, air-dried from acetone, and post-stained with Coomassie-blue R as described by Gall et al. (1981). They were observed at high magnification under a Leica microscope and photographed with a NIKON DXM-1200 camera. Paraformaldehyde preparations were post-stained either with Hoechst 33342 (1:1,000; Invitrogen) or with propidium iodide (10 μg/ml) (Sigma). Preparations with a coverslip laid on top using citifluor (Citifluor) as mounting medium were observed under a Leica microscope using a Leitz DMRB CDD camera.

Immunofluorescence assay for the identification of pol III loci on LBCs was carried out as described previously (Murphy et al., 2002) using either a rabbit polyclonal anti-RBP6 (RPC15) antibody directed against a subunit common to RNA Polymerases I, II, and III at a 1/250 dilution, or an anti-RPC53 directed against a subunit unique to Pol III at a 1/250 dilution (antisera kindly supplied by R.G. Roeder, Rockefeller University). After washing with PBS, slides were incubated with Alexa-488-rabbit IgG as a secondary antibody (Invitrogen) diluted to 1:1,000 and stained with Hoechst 33342. Fluorescence microscopy was performed using a Leitz DMRB CDD camera and images were assembled using Adobe Photoshop.

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