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Characterization of axolotl lampbrush chromosomes by fluorescence *in situ* hybridization and immunostaining

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Abstract

The lampbrush chromosomes (LBCs) in oocytes of the Mexican axolotl (*Ambystoma mexicanum*) were identified some time ago by their relative lengths and predicted centromeres, but they have never been associated completely with the mitotic karyotype, linkage maps or genome assembly. We identified 9 of the axolotl LBCs using RNAseq to identify actively transcribed genes and 13 BAC (bacterial artificial clone) probes containing pieces of active genes. Using read coverage analysis to find candidate centromere sequences, we developed a centromere probe that localizes to all 14 centromeres. Measurements of relative LBC arm lengths and polymerase III localization patterns enabled us to identify all LBCs. This study presents a relatively simple and reliable way to identify each axolotl LBC cytologically and to anchor chromosome-length sequences (from the axolotl genome assembly) to the physical LBCs by immunostaining and fluorescence *in situ* hybridization. Our data will facilitate a more detailed transcription analysis of individual LBC loops.

Keywords

Axolotl; *Ambystoma mexicanum*; Lampbrush chromosomes; Centromere; BAC FISH

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Declaration of competing interest

The authors have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2021.112523.

1. Introduction

The largest known chromosomes occur in the oocyte nucleus of the Mexican axolotl, *Ambystoma mexicanum*. First described by Walther Flemming in 1882 as “strange and delicate structures” [1], these chromosomes consisted of a long linear axis from which numerous loops extended laterally. A decade later, they were given the name lampbrush chromosomes (LBC) because of their resemblance to the brushes used to clean the chimneys of oil lamps, similar to today’s test tube brushes [2]. Little progress was made in understanding the structure and function of LBCs until the 1950s, when techniques were developed for isolating them from living oocytes of frogs and salamanders. Within a few years it was shown that the loops represent regions of intense transcriptional activity and are part of an enormously long, continuous strand of DNA. It is now known that only a fraction of the genome is being transcribed, but those genes that are transcribing do so at a rate far above that found in somatic cells. In this context, the “rate of transcription” refers to the number of polymerase II molecules per unit length of gene. In other words, individual genes of LBCs are visible in the light microscope because they are densely packed with polymerase molecules and the nascent transcripts that extend from them [3].

Throughout most of oogenesis LBCs are arrested in the diplotene stage (prophase) of the first meiotic division. Homologous chromosomes are held together by one or more chiasmata. The majority of the chromatin is transcriptionally inactive and is condensed in chromomeres that punctuate the axis of the chromosome. Hundreds of pairs of loops extend laterally from the chromomeres. It was predicted early on and later proven that loops are sites of active transcription [3,4]. Each loop contains one or more thin-to-thick regions corresponding to transcription units. Immunostaining shows that most loops are transcribed by polymerase II (Fig. 1) and only a minority by polymerase III [5,6].

Although LBCs are best known from large vertebrate oocytes, they actually occur in most, if not all large meiotic cells that lack nurse cells. Thus, in addition to fish, reptiles, birds, and amphibians they are found in many insects and even some plants (Callan, 1986). The generally accepted explanation for their high level of transcription is the need to accumulate a large amount of RNA in a limited time from a tetraploid (2 N/4C) set of chromosomes. Whereas large somatic cells rely on polyploidy to provide the templates for transcription, the oocyte must undergo meiosis, which is not compatible with polyploidy. As an alternative, the tetraploid (2 N/4C) LBCs in large oocytes transcribe a set of genes at a high rate (that is, at maximal packing of polymerase), which unfolds the DNA. This unfolding leads to the formation of loops of chromatin visible by conventional light microscopy.

Recent advances in sequencing make it possible to explore these chromosomes further. A full genome assembly was made available in 2018 [7], and a recent iteration has produced chromosome-scale scaffolds [8]. Smith et al. anchored these scaffolds to corresponding physical chromosomes using fluorescence *in situ* hybridization (FISH) with previously developed BACs [9]. In order to identify LBCs according to their corresponding mitotic chromosomes, we used a subset of these probes to assign numbers to the LBCs. We have used thirteen of the BAC clones from the 2019 study (8) as hybridization probes on LBCs,

effectively anchoring 9 of the chromosome-length sequences and associated linkage groups to specific LBCs.

Independent of FISH with BAC clones, we developed cytological maps for LBCs by immunostaining with antibodies against polymerase III and a new hybridization probe for the centromere, AmexCen. This probe was named using the species name, *A. mexicanum* and the cytogenetic nomenclature for centromere, cen. Whereas most loops stain with antibodies against polymerase II, far fewer stain with polymerase III, and the patterns made by these stains help identify individual LBCs. The AmexCen probe is the first to localize specifically to the centromeres of axolotl LBCs.

2. Methods

2.1. Oocyte isolation

An adult female axolotl was placed in 2 L of anesthetic solution (0.2% MS222) for about 20 min, until the animal stopped moving or righting itself when flipped over [10]. The animal was removed from the water and placed belly-up on a wet paper towel in a bed of ice chips. A 2–3 cm incision was made with surgical scissors in the lower abdomen and forceps were used to move organs aside and pull some ovarian tissue outside of the body. A piece of ovary (1–3 cm in length) was placed in a Petri dish with OR2 saline, and the axolotl was sutured with surgical silk. Vetbond (3M™ Vetbond™ Tissue Adhesive no. 1469SB) was added to the sutured wound and left until dry, and then the animal was placed back in a bowl of clean water. The axolotl was watched carefully until her wound healed (~6–8 weeks). Then the sutures were removed, and she could rejoin the colony.

2.2. Lampbrush chromosome (LBC) preparation

We isolated germinal vesicles (GVs) by hand from nearly mature oocytes, removed the nuclear envelope, and spread the contents on a microscope slide (for detailed methods of LBC preparation, see Ref. [10]). The entire slide was then centrifuged at 5000 rpm to attach the chromosomes, after which it was fixed for up to 1 h in 4% paraformaldehyde and held in PBS at 4 °C [10]. The best preparations came from oocytes with diameters of 1.6–1.8 mm. Many of these displayed 14 pairs of unbroken chromosomes with well-expanded lateral loops. Selected slides were then used for BAC FISH.

2.3. Centromere probe development

Using coverage data from another project [8], we identified a candidate centromere repeat. *Ambystoma mexicanum* shotgun sequence data from SRX800915 were mapped to the genome assembly PGSH00000000.1 using BWA-MEM with option -a [11] and filtered by SAMtools view with option -F 2308 [12]. Depth of coverage calculated across the entire genome, using genomecov -bga from bedtools v2.23.0 [13], revealed that scaffold PGSH01000799.1 has intervals with the highest coverage values, some reaching 35,000 times the normal read coverage across a randomly chosen scaffold. Note that GenBank scaffold PGSH01000799.1 is the same as AMEXG_0030079802, which can be viewed using the UCSC interface [14] at the Sal-Site [15] genome browser https://ambystoma.uky.edu/genome-resources. The centromere candidate sequence, which is 55

bases in length, appears in the scaffold as a tandem repeat. We found similar sequences in a large number of relatively short scaffolds as well as in each chromosome-scale scaffold. This sequence appears in the genome with many variations, and blast alignments with identities >80% and lengths >45 bases show hits to each chromosome, with most chromosomes showing a cluster in 1–2 places (Supplementary Fig. 1). Due to the general difficulty of assembling highly repetitive regions like centromeres, we anticipated a pattern where fewer alignments occurred throughout the chromosomal scaffolds and more occurred near a particular locus. Other repetitive sequences show random alignments throughout the assembly without clustering. These data gave us some confidence that there would be limited regions in which the probe would hybridize. Using BLAST [16] on the non-redundant NCBI database, we found a hit with 90% identity to an *Ambystoma bishopi* microsatellite Amb29 sequence (GenBank: KP289108). A 55 base DNA oligo with the following sequence, including a 5' Cy3 modification, was designed and obtained from Integrated DNA Technologies: TATCACATCTCATGTTATAGGAAGGATCTCAGGTTTGGTATTAGAAGCATTGAGA.

This probe hybridizes to a single site on each LBC (Fig. 4), corresponding to the positions of the centromeres as originally determined by Callan [17].

2.4. Immunostaining of Pol II and Pol III

Immunostaining of LBC spreads was carried out according to published protocols [10]. The rat monoclonal antibody anti-phospho RNA Pol II (clone 3E7C7) was used at a dilution of 1:500. A rabbit polyclonal serum against the polymerase subunit RPB6 (a gift from Robert Roeder, Rockefeller University) was used at a dilution of 1:5000–1:10,000. Although RPB6 is a subunit common to all three RNA polymerases, this antibody is essentially specific for sites of Pol III transcription [18]. Slides were counterstained with DAPI and mounted in OR2 buffer [19]. The immunostained spreads were then analyzed visually and imaged with a Leica SP5 confocal laser scanning microscope in the case of Pol II and with an Olympus epifluorescence microscope in the case of Pol III. Because the pattern of Pol III sites is unique for each chromosome, all members of the genome can be easily distinguished.

2.5. BAC fluorescence in situ hybridization (FISH)

To select BAC clones that might hybridize to loops on the LBCs, we performed RNAseq on the cytoplasm of several axolotl oocytes to identify which genes were actively transcribed (PRJNA692843). BACs that contained parts of these genes were selected and used for fluorescence *in situ* hybridization (FISH). BAC probes were labeled with Cy3-and fluorescein-dUTP (Enzo Life Sciences) using nick-translation according to methods previously published [20]. FISH was carried out as described by Galkina et al. [21]. Images were captured on a Leica confocal microscope and pseudocolored using ImageJ (https://imagej.nih.gov/ij/).

2.6. FISH after immunostaining

Immediately following imaging, immunostained slides were placed in PBS to allow the coverslip to fall off. They were then dehydrated through an alcohol series and air-dried. A circle was drawn with a diamond pencil on the back of the slide to delineate the

chromosome area, and paraffin wax was removed with a razor blade. Slides were then placed in PBS, hybridized as described above, and re-imaged to visualize the centromeres. Two changes to the protocol were made for the centromere probe: 1) hybridization took place overnight at room temperature instead of 37 °C, and 2) post-hybridization washes were with 2X SSC at 37 °C.

2.7. Construction of LBC maps

Cytological maps of the 14 axolotl LBCs were constructed on the basis of relative lengths, centromere positions, and sites transcribed by RNA polymerase III. These maps are shown in Fig. 5; they are a composite of measurements made at different times and with different numbers of chromosomes. Centromere positions are based on 6 complete LBC preparations. An example of one LBC is shown in Fig. 4.

3. Results & discussion

3.1. BAC FISH

Table 1 shows the set of BAC clones provided by Jeramiah J. Smith and S. Randal Voss (University of Kentucky). These BAC clones were selected because they contain genes whose transcripts appear in oocyte RNAseq data and are presumably transcribed on LBC loops. DNA probes were hybridized to the RNA of the loops. We hybridized each probe a minimum of 4 times. Some probes gave a positive signal each time, whereas others were less consistent for unknown reasons. Some fluorescence can be visualized in extrachromosomal bodies on every spread, possibly due to autofluorescence. In all cases, however, localization on the LBCs was not difficult to determine.

The location of individual BAC clones on the LBCs correlated well with the reported locations of the genes on the linkage groups, on metaphase chromosomes and in the latest version of the assembly. Thus, we can confidently predict the position of genes that have not yet been assigned to positions on physical chromosomes.

Hybridization of BAC probes for NISCH and FGF9 are shown in Fig. 2. On the linkage map NISCH is located at 6.49 cM near the telomere of chromosome 2p; in an earlier study a NISCH probe hybridized to mitotic chromosome 2 [8]. Hybridization of the BAC probe for NISCH on a LBC preparation shows localization to a pair of loops near the tip of a long chromosome (Fig. 2A). It should be noted that here, as in other *in situ* hybridizations to LBC loops, the probe hybridizes to the nascent RNA transcripts on the loop, not to the (invisible) DNA axis of the loop.

The BAC probe for FGF9 hybridized near the middle of the q arm of chromosome 7 in mitotic spreads [8]. Hybridization of this probe on a LBC preparation shows localization to a pair of loops near one end of a medium length chromosome (Fig. 2B). Both the NISCH and FGF9 probes label the loops along their entire lengths, because the gene fragment in the BAC clone comes from the 5′ end of the gene.

Because individual chromosomes and genes are much more extended in the LBC stage than in mitosis, they permit precise localization of FISH signals. Fig. 3 shows hybridization to

mitotic and LBC spreads of BAC clones that contain portions of CHD4 and ETV4. CHD4 gives a strong signal near the end of the p arm of chromosome 3, close to the nucleolus organizer region, which appears as a secondary constriction on mitotic chromosomes (Fig. 3A) or as round nucleoli attached to the LBC (directly above the CHD4 signal in Fig. 3B). The sequence of this probe is known; it aligns to 109 bases in the first 5400 bases of the predicted CHD4 gene for the box turtle *Terrapene carolina*, suggesting that only a small piece of the CHD4 gene is present in the BAC probe (Fig. 3A). ETV4 gives a strong signal along the entire length of a loop on the q arm of LBC 13, in agreement with the hybridization on mitotic chromosomes. The portion of the gene contained within the BAC is near the 5′ end of the gene, and for that reason hybridization spans the length of the loop.

3.2. Centromeres

The positions of the centromeres on axolotl chromosomes were first described by Callan from mitotic preparations of larval tail fin, hepatocytes and brain cells [17]. He also identified the centromere positions in LBCs as “short lengths (10 μ or a little less) of chromosome axis devoid of lateral loops.” In our studies of LBCs, we have found it very difficult to identify the centromeres unambiguously on the basis of morphology alone, even though we know approximately where to look. Fortunately, one of our cloned sequences (AmexCen) from scaffold PGSH01000799.1, a highly repetitive sequence of 55 bases, hybridizes strongly to one short region on each LBC. This region is clearly the centromere or pericentromeric region, based on a comparison with the centromere positions described by Callan. An example of centromere hybridization on chromosome #5 is shown in Fig. 4B. This chromosome was also immunostained with an antibody against pol III (Fig. 4A).

3.3. LBC maps

In Fig. 5 we present diagrams of the 14 axolotl chromosomes based on relative lengths and centromere positions, as determined by *in situ* hybridization with the AmexCen probe. Also included are the positions of approximately 60 major sites of Pol III transcription, determined by antibody staining. As shown in an earlier study of the LBCs of *Xenopus laevis*, Pol III transcription takes place at a few loci on each chromosome [5], and the same is true for the axolotl. Along with relative lengths and centromere positions, the Pol III sites provide useful landmarks for rapid identification of each LBC. Taken together with the BAC probe FISH data, which have numbered the axolotl LBCs, Pol III immunostaining can now be used to identify each chromosome number without the hybridization of chromosome-specific probes. Future LBC studies can benefit from this efficient way of determining chromosome number.

The relative lengths of axolotl chromosomes were originally determined by Callan [17] based on both LBCs and mitotic chromosomes. Our LBC maps agree with his with one minor exception: we find that Callan’s LBC 6, which carries a pair of histone locus bodies near its middle, is the 5th longest by our measurements. Given the shallow gradient in relative lengths of the LBCs, we believe this difference reflects the difficulty in determining relative lengths, not a real biological difference between animals.

The axolotl mitotic chromosomes were most recently studied by Smith et al. [8]. Their Fig. 2 compares the cytological banding pattern of each mitotic chromosome with its linkage group. A partial correspondence between the mitotic and LBCs can be made based on relative lengths and centromere positions (chromosomes 1–3, 13, 14), but a full correspondence will require further study.

4. Concluding remarks

The enormous size of urodele LBCs opens the possibility for detailed cytogenetic analysis, but the full potential of these chromosomes has not been exploited in the past, primarily due to the scarcity of molecular probes. In addition, LBCs help reveal the complex organization of genes in the karyotype of species where standard cytogenetic analysis on mitotic chromosomes is unclear. In species where banding patterns are difficult, such as those enriched with repetitive elements, LBCs may help decipher differences among chromosomes for proper identification [22–24]. Many karyotypes, such as those of birds and reptiles, include a few large macrochromosomes and many tiny indistinguishable microchromosomes [24–26]. LBCs provide high resolution of these cytological systems, helping to decipher the order of cytological markers. These cytogenetic studies can elucidate evolutionary chromosomal rearrangements, including sex chromosomes [24].

For species with small oocytes that normally lack LBCs, LBCs may be induced [27] and subsequently used for cytogenetic studies. Previous studies have demonstrated that demembranated sperm heads from several species, including zebrafish (*Danio rerio*), *Xenopus*, *Rana*, human and mouse, form LBCs when injected into the oocyte nucleus of the frog or newt [27,28].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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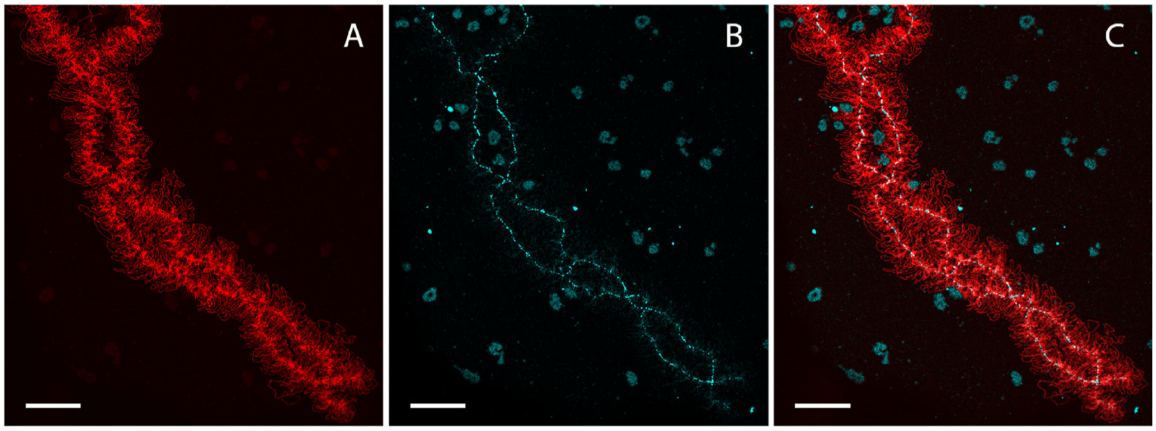

Fig. 1.

An axolotl lampbrush chromosome stained with DAPI and an antibody against RNA polymerase II. A) Pol II immunostaining highlights the loops of the LBC. B) DAPI stains the condensed chromomeres along the LBC axis. C) Overlay of DAPI (cyan) and Pol II (red). Bars = 50 μm.

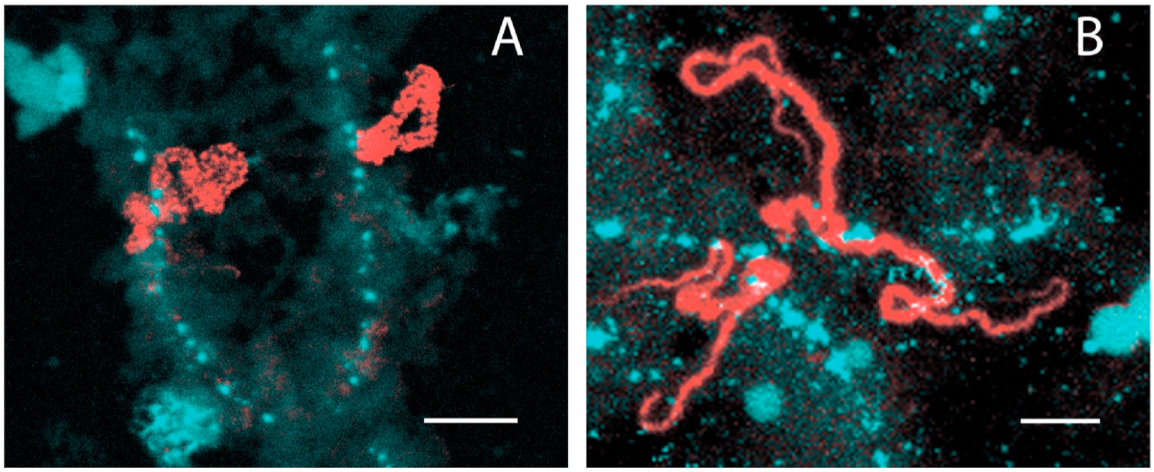

Fig. 2.

BAC fluorescence *in situ* hybridization (FISH) on axolotl lampbrush chromosomes. A) BAC clone BbMex_4E17 (red) localizes to transcripts on a pair of loops near the telomere on the p arm of LBC 2. B) BAC clone AMMCBa_426N21 (red) localizes to transcripts on a pair of long loops on the q arm of a medium-sized chromosome. Chromosomes are counterstained with DAPI (cyan). Bars = 10 μm.

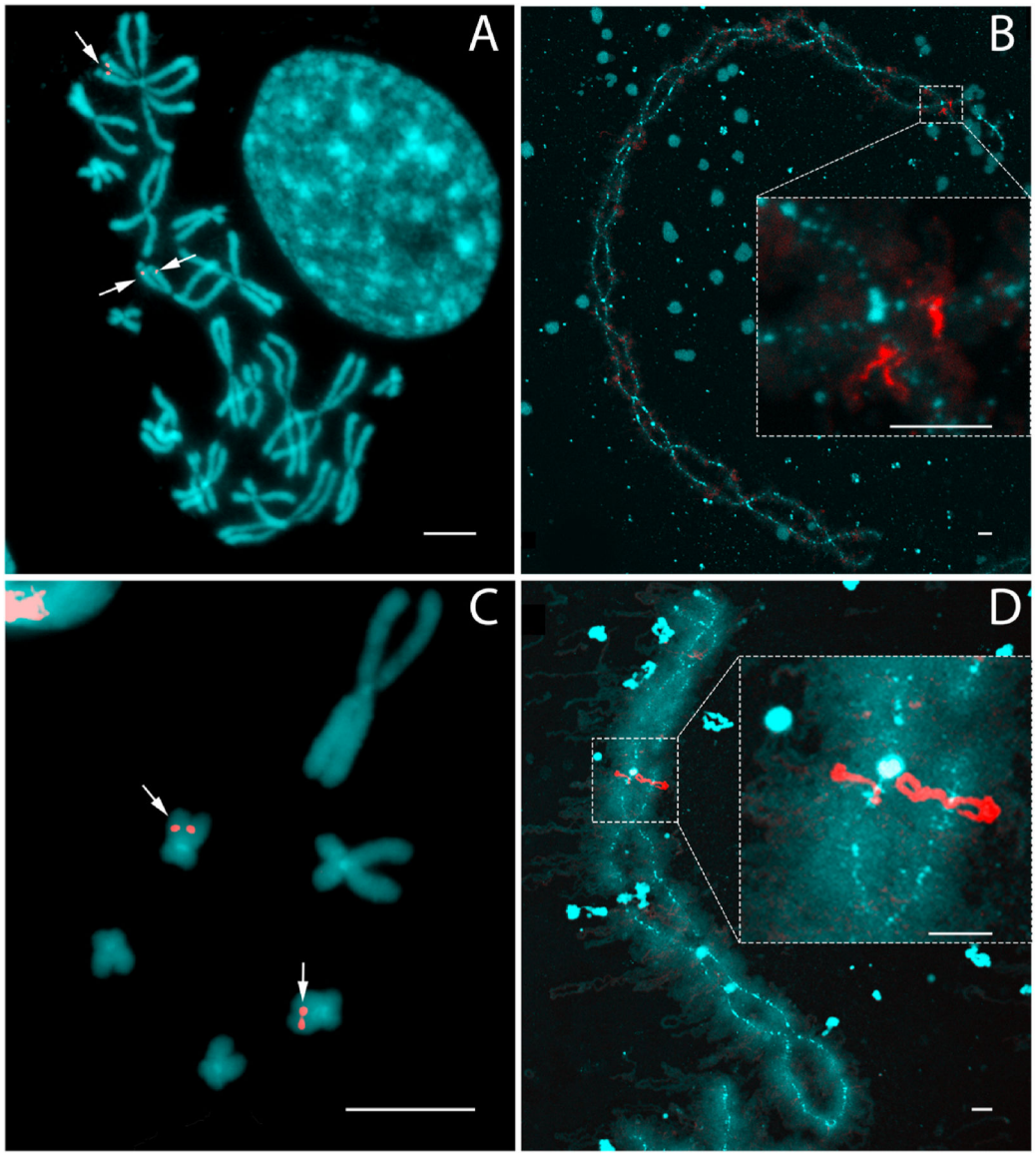

Fig. 3.

BAC fluorescence *in situ* hybridization (FISH) on axolotl lampbrush and mitotic chromosomes. A) BAC AMMCBa_355L20 (red) localizes to the p arm of mitotic chromosome 3 and B) to a pair of loops near the telomere on the p arm of a long LBC. C) BAC clone AMMCBa_45F11 (red) localizes between the centromere and telomere on the q arm on mitotic chromosome 13 and D) to a pair of loops on the q arm of LBC 13. Chromosomes are counterstained with DAPI (cyan). Arrows point to signal from hybridization of the probe. Bars = 10 μm.

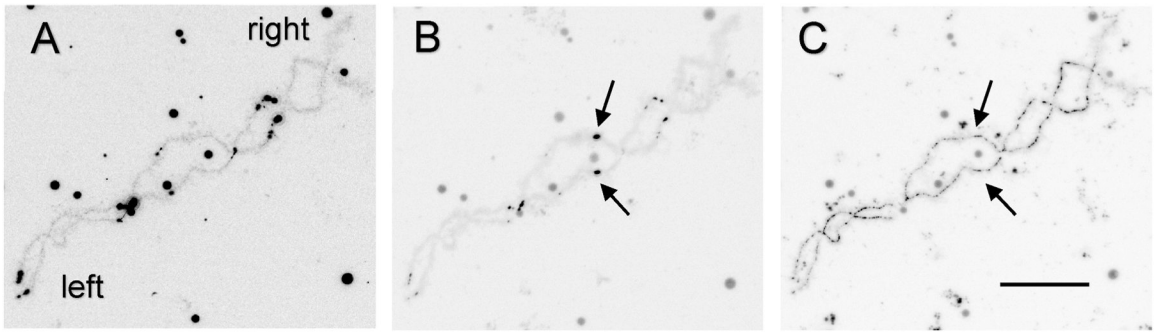

Fig. 4.

LBC #5 hybridized with a clone against the centromere and also immunostained with an antibody against pol III. A) Pol III sites detected by antibody staining. The pattern is shown diagrammatically in Fig. 5. B) The same preparation after *in situ* hybridization for the centromeres (arrows). The *in situ* procedure reduces but does not completely eliminate pol III staining. C) DAPI stain showing DAPI-positive chromomeres. The centromeres are also DAPI-positive. Arrows point to the centromeres. Bar = 100 μm.

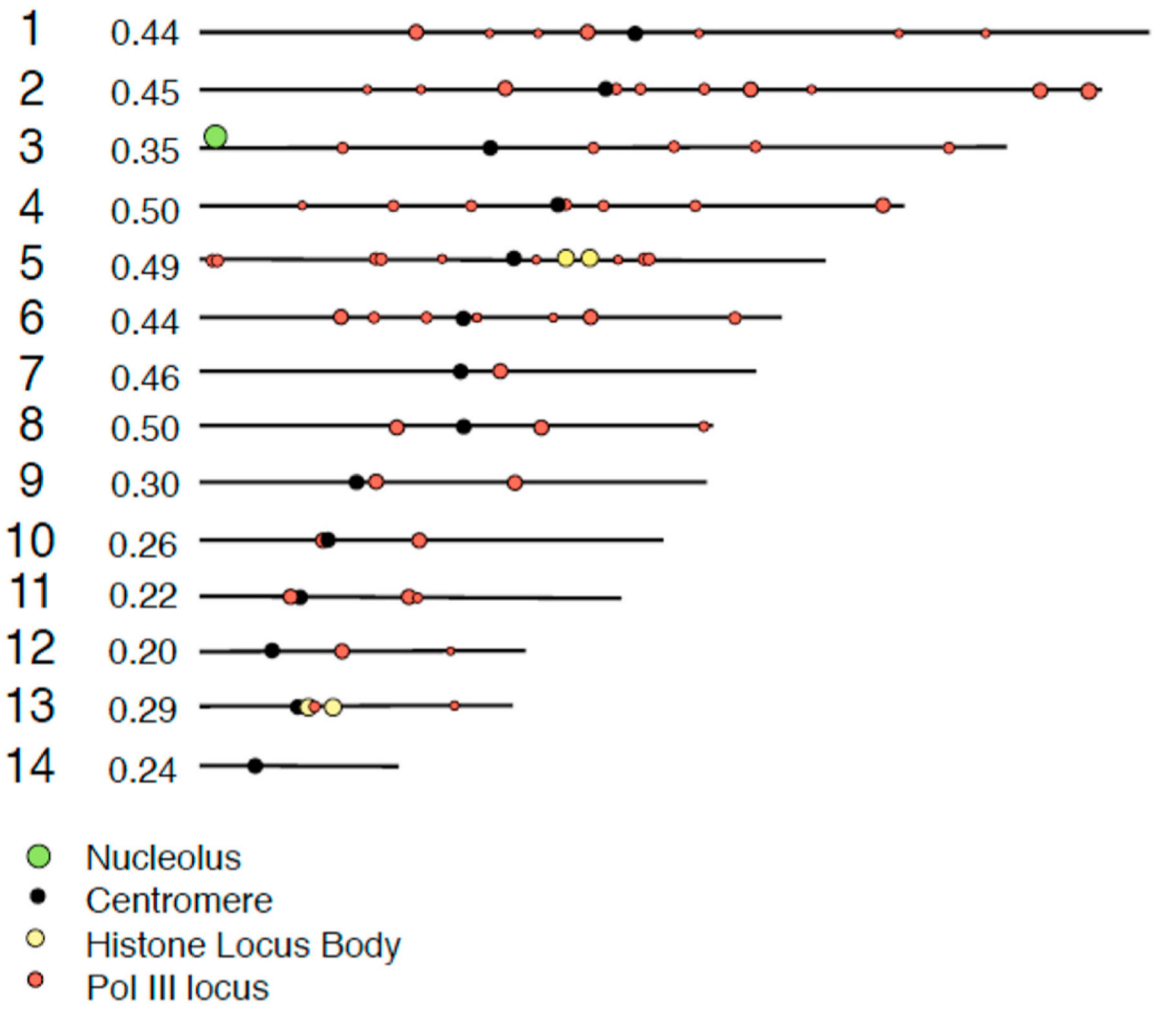

Fig. 5.

Ambystoma mexicanum LBC Maps. Maps of the 14 LBCs of *A. mexicanum* showing positions of the centromeres, the most prominent pol III loci, the four histone locus bodies, and the single nucleolus. The fraction preceding each chromosome is the position of the centromere, measured from the left end. The relative lengths and centromere positions are in approximate agreement with those originally determined by Callan (1966) with one minor exception: reversal of chromosomes 5 and 6.

Table 1

BAC probes used for FISH on LBCs.

BAC clone ID	Gene contained in BAC	Chromosome	Linkage map position (cM) [8]	Physical position (tel = telomere; cen = centromere; sat = satellite)
BbMex_134I3	CLDN7	1	13.507	1p-tel
BbMex_4E17	NISCH	2	6.479	2p-tel
AMMCBa_355L20	CHD4	3	44.253	3p sat
AMMCBa_97O23	TEX2	3	386.279	3q-4/5tel
BbMex_87I6	NANOG	3	39.033	3p sat
AMMCBb_66M18	JARID2	5	177.789	5p- 1/5 cen
AMMCBa_272K10	NIPBL	6	62.765	6p-close to tel
BbMex_75J4	KLF4	6	327.123	6q- 1/5 cen
AMMCBa_426N21	FGF9	7	257.352	7q 1/2 cen
BbMex_79E10	TMX2	12	141.384	12q 1/4 cen
AMMCBa_45F11	ETV4	13	97.224	13q 1/2
BbMex_22H14	TRMT5	14	79.687	14q 1/2
AMMCBa_508D24	DICER1	14	39.091	14q cen