Assembly of Lampbrush Chromosomes from Sperm Chromatin

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We have examined the behavior of demembranated sperm heads when injected into the germinal vesicle (GV) of amphibian oocytes. *Xenopus* sperm heads injected into *Xenopus* GVs swelled immediately and within hours began to stain with an antibody against RNA polymerase II (Pol II). Over time each sperm head became a loose mass of chromosome-like threads, which by 24–48 h resolved into individually recognizable lampbrush chromosomes (LBCs). Although LBCs derived from sperm are unreplicated single chromatids, their morphology and immunofluorescent staining properties were strikingly similar to those of the endogenous lampbrush bivalents. They displayed typical transcriptionally active loops extending from an axis of condensed chromomeres, as well as locus-specific "landmarks." Experiments with [^{3}H]GTP and actinomycin D demonstrated that transcription was not necessary for the initial swelling of the sperm heads and acquisition of Pol II but was required for maintenance of the lampbrush loops. Splicing was not required at any stage during formation of sperm LBCs. When *Xenopus* sperm heads were injected into GVs of the newt *Notophthalmus*, the resulting sperm LBCs displayed very long loops with pronounced Pol II axes, like those of the endogenous newt LBCs; as expected, they stained with antibodies against newt-specific proteins. Other heterologous injections, including sperm heads of the frog *Rana pipiens* and the zebrafish *Danio rerio* in *Xenopus* GVs, confirm that LBCs can be derived from taxonomically distant organisms. The GV system should help identify both cis- and trans-acting factors needed to convert condensed chromatin into transcriptionally active LBCs. It may also be useful in producing cytologically analyzable chromosomes from organisms whose oocytes do not go through a typical lampbrush phase or cannot be manipulated by current techniques.

INTRODUCTION

More than 100 years ago Flemming (1882) described giant chromosomes in the oocyte nucleus or germinal vesicle (GV)[1] of the salamander *Ambystoma mexicanum*. His initial brief observations were soon followed by detailed investigations that established the existence of giant chromosomes in GVs of many animals, both vertebrate and invertebrate (Rückert, 1892; Car-

noy and Lebrun, 1897; Maréchal, 1907). Rückert named them lampbrush chromosomes (LBCs) because of their fancied resemblance to the brushes used for cleaning kerosene lamp chimneys. Their overall organization and functional significance have been the subject of extensive experimentation and speculation (reviewed in Callan, 1986; Davidson, 1986). Because LBCs occur in oocytes during the growth period, they are in an arrested diplotene phase of meiosis I with homologous chromosomes held together at one or more chiasmata. Each homologue has a 4′,6-diamidino-2-phenylindole (DAPI)-positive axis of chromomeres, which correspond to transcriptionally inactive regions where sisters are closely associated. Numerous pairs of loops extend laterally from the

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[1] Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; GV, germinal vesicle; LBC, lampbrush chromosome; mAb, monoclonal antibody.

chromomere axis; these consist of transcriptionally active regions where sisters are completely independent of one another. At the light optical level loops are not of uniform thickness but consist of one or more thin-to-thick regions, which correspond to transcription units. The electron microscope shows that the thin-to-thick morphology reflects the increasing length of nascent ribonucleoprotein (RNP) fibrils along the transcription unit in the direction of transcription.

Despite a wealth of detailed morphological and molecular information on LBCs, fundamental questions remain about their structure and especially about the significance of their highly active transcription. At one time their structure was regarded as unusual, but the discovery of looped chromatin domains in somatic nuclei (Paulson and Laemmli, 1977; Saitoh and Laemmli, 1993; Yokota et al., 1995) suggests that the lampbrush condition may be a good model for chromosomes in general. What factors cause a condensed chromosome to assume the lampbrush condition remain obscure, however. Questions about LBC structure and activity would be easier to address in a system in which the lampbrush state could be experimentally induced and altered. Recently, we have discovered that sperm chromatin injected into an amphibian GV gives rise within hours to typical transcriptionally active LBCs. This system should be useful in assessing the relative importance of cis and trans factors in establishing the morphological and molecular attributes of LBCs. It may also permit analysis of transcriptionally active chromosomes from organisms whose oocytes cannot be handled by current techniques or do not go through a typical lampbrush phase.

MATERIALS AND METHODS

Oocytes

A female *Xenopus laevis* was anesthetized in 0.1% methanesulfonate salt of 3-aminobenzoic acid ethyl ether (tricaine methanesulfonate or MS222; product A5040, Sigma, St. Louis, MO). A sample of ovary was removed surgically and held at 18–20°C in a small Petri dish of OR2 saline (Wallace *et al.*, 1973). In some experiments the ovary sample was treated with crude collagenase from *Clostridium histolyticum*, which removes the outer layers of follicle cells and causes individual oocytes to come apart. Treatment was for 2 to 6 h at room temperature in 0.2% collagenase (type II, Sigma, product C6885) made up in Ca^{2+}-free OR2. In other cases, individual oocytes or small clumps of oocytes with their follicle layers intact were separated with jeweler's forceps.

GV Spreads

Detailed instructions for studying LBCs of *Xenopus* were published earlier (Gall *et al.*, 1991), but a few critical modifications now dramatically improve the quality of GV spreads (Gall, 1998). Injected oocytes were transferred one at a time to isolation medium (83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0). The GV was removed with forceps and transferred within 20 s to spreading medium (21 mM KCl, 4 mM NaCl, 1.6 mM Na_2HPO_4, 0.9 mM KH_2PO_4, 1 mM $MgCl_2$,

1 mM dithiothreitol, 0.1% paraformaldehyde, pH 7.0). The nuclear envelope was removed with jeweler's forceps, and the nuclear gel was transferred with a pipette to a spreading chamber. If the procedure was carried out quickly, dispersal of the nuclear gel occurred within minutes and centrifugation could begin almost immediately. Slides were centrifuged in a special holder in the Sorvall HS4 rotor (DuPont, Wilmington, DE) at 5000 rpm (4800 $\times$ *g*), 4°C for 30 min. GV spreads were fixed with 2% paraformaldehyde in phosphate-buffered saline for 1 h or longer.

Immunofluorescence

GV spreads were blocked with 10% horse serum in phosphate-buffered saline for 15–30 min and then incubated with primary antibody for 1 h. After a brief rinse in 3% horse serum, they were treated with a secondary antibody for another hour. Rabbit serum was diluted 1:200 in 10% horse serum, whereas monoclonal antibodies (mAbs) were used as undiluted culture supernatant. Secondary antibodies were Cy3- or fluorescein-conjugated goat anti-mouse or goat anti-rabbit sera (Cappel/Organon Teknika, Durham, NC). Preparations were mounted in 50% glycerol containing 1 mg/ml phenylenediamine, pH 9, and 1 μg/ml DAPI.

Sperm Heads

Sperm heads were demembranated with lysolecithin as originally described by Gurdon (1976). Sperm heads of *X. laevis* and *Silurana (Xenopus) tropicalis* were prepared from testes as described (Newmeyer and Wilson, 1991). Sperm of the mouse *Mus musculus* and the zebrafish *Danio rerio* were prepared in the same way; mature sperm from the mouse were obtained from the epididymis, whereas mature sperm from the zebrafish were squeezed from adult males. Sperm of the cricket *Acheta domesticus* were obtained by homogenizing six to eight pairs of testes from adult insects in distilled water, which effectively destroys most cells but leaves mature and nearly mature sperm intact. The sperm were concentrated by centrifugation and then treated with lysolecithin as for the other species. In each case sperm counts were made with a hemacytometer.

Oocyte Injections

Some oocytes were partially defolliculated with collagenase before injection, whereas others were used without enzyme treatment. Defolliculated oocytes are easier to penetrate with the injection needle, but this advantage is offset by the time required for defolliculation and the somewhat greater fragility of treated oocytes. Before injection, oocytes were centrifuged at 600 $\times$ *g* for 20 to 30 min to bring the GV to the surface, where its position can be detected as a depigmented area. Glass needles were made from capillary tubing (0.5 mm i.d. and 1.2 mm o.d.) by using a Vertical Pipette Puller (David Kopf Instruments, Tujunga, CA). Before use, the needle was siliconized and the tip was broken off with forceps to give an internal diameter of 10–20 μm. The needle was filled from the back with 4–5 μl of sperm suspension and then with mineral oil. Injections of 4.8 nl or 9.6 nl were made under a dissecting microscope with a Drummond Nanoject microinjection apparatus (Drummond Scientific, Broomall, PA), which employs a plunger to displace liquid in the needle. We found it advantageous to make injections soon after filling the needle with the sperm suspension. Test spottings onto a microscope slide showed that the number of ejected sperm decreased with time after filling, presumably due to settling of sperm heads and adherence to the side of the needle.

Transcription and Splicing

Transcription was monitored by injecting [^{3}H]GTP into the cytoplasm of oocytes. [^{3}H]GTP (250 μCi; 5.7 Ci/mmol; Amersham, Arlington Heights, IL) in 50% ethanol was evaporated to dryness and redissolved in 1.25 μl of H_2O. Approximately 1 μCi (5 nl) was injected into each oocyte. At various times after injection, GV

spreads were made and fixed in 2% paraformaldehyde. They were dehydrated in an ethanol series and air dried from acetone. Unincorporated label was removed with 5% trichloroacetic acid at 4°C for 5 min. After a second dehydration and drying from acetone, the slides were dipped in NTB2 autoradiographic emulsion (Eastman Kodak, Rochester, NY). Autoradiographic exposure was for 1.5 d, followed by development in D19 developer for 2 min. GV contents were stained through the emulsion with Coomassie blue (Gall *et al.,* 1991). To inhibit splicing, 10 ng of an oligodeoxynucleotide complementary to nucleotides 28–42 of *Xenopus* U2 small nuclear RNA (snRNA; 5′-CAGATACTACACTTG-3′) was injected with sperm into the GV (Pan and Prives, 1988; Tsvetkov *et al.,* 1992). The oligonucleotide hybridizes with U2 snRNA in the GV, which is then destroyed by endogenous RNase H activity within 1–4 h. Preparations of LBCs made 24 h after injection show unusually large loops that lack all detectable U2 snRNA (Tsvetkov *et al.,* 1992).

RESULTS

Injection of Sperm Heads

Initial experiments involved injection of demembranated *X. laevis* sperm heads into *Xenopus* GVs of stage IV–V oocytes (1.0–1.2 mm in diameter). In various experiments, the injected volume was 5–10 nl, nominally containing 1–5 sperm heads/nl, but the number of injected heads was often lower than calculated, probably due to adherence of the heads to the inside of the needle. At various times after injection, GVs were manually dissected from the oocytes, their contents were allowed to disperse in specially prepared well slides, and the slides were centrifuged to insure attachment of the GV contents to the glass substrate. We have now carried out several dozen injection experiments, each involving 20–60 oocytes. In a typical experiment, 90–95% of the injected oocytes appear normal after 24 h, and of these, 60–90% received sperm heads in the GV, the remaining ones presumably representing cases where the needle missed the GV or sperm heads were not ejected from the needle.

Initial Swelling of Sperm Heads

Demembranated *Xenopus* sperm heads as used for injection are worm-like structures roughly 20 μm in length, tapered toward both ends with a maximal diameter near the middle of about 1 μm (Figure 1A). After injection into the GV, they were easily recognized by their morphology and their intense staining with the DNA-specific dye DAPI. The first sign of change in the sperm heads was swelling (Figure 1, B and C), which took place without formation of a membrane or envelope detectable by phase contrast or differential interference (DIC) microscopy (Figure 2B). The timing of subsequent events was variable from experiment to experiment, possibly related to differences in oocytes from different females. In many
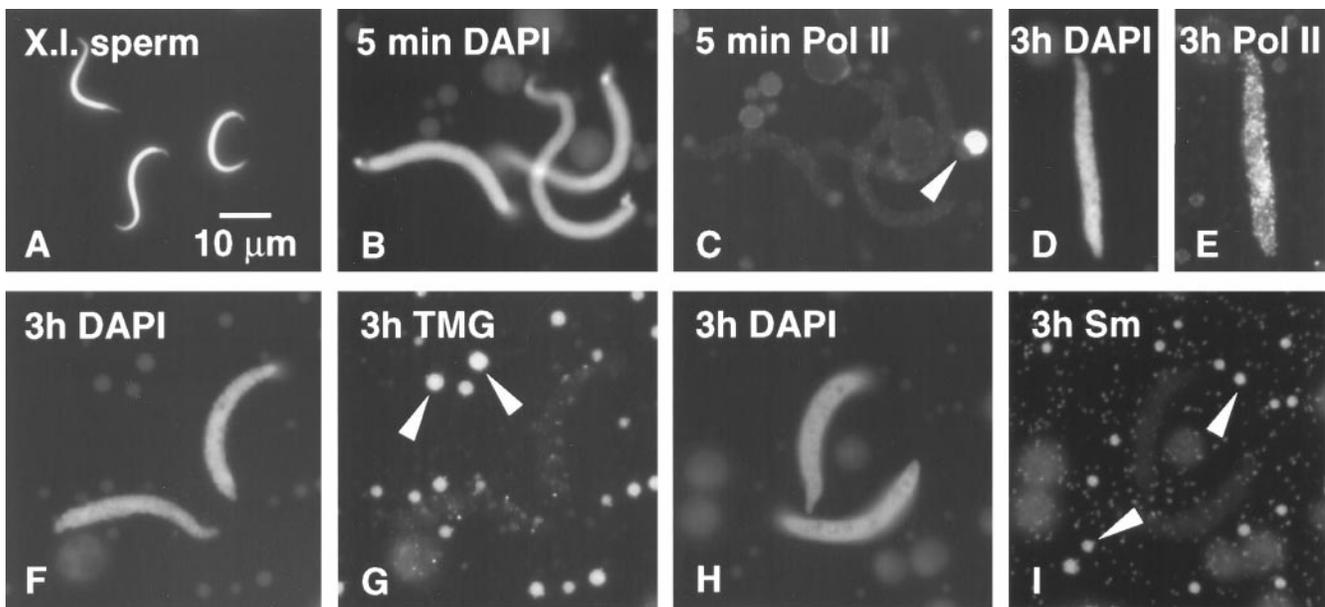
Figure 1. (A) Demembranated sperm heads of *X. laevis* as injected (DAPI stain for DNA). (B) Swollen sperm heads 5 min after injection into a *Xenopus* GV (DAPI stain). (C) Same field stained with mAb H14 against RNA Pol II; sperm heads are negative, whereas a coiled body (sphere) stains brightly (arrowhead). (D) Sperm head 3 h after injection into a GV (DAPI stain). (E) Same sperm head shows strong staining with mAb H14 against RNA Pol II. (F) Sperm heads 3 h after injection into a GV (DAPI stain). (G) Same field stained with mAb K121 against the trimethylguanosine (TMG) cap of snRNAs. Sperm heads are negative; B-snurposomes are stained (arrowheads). (H) Sperm heads 3 h after injection into a GV (DAPI stain). (I) Same field stained with mAb Y12 against the Sm epitope of snRNPs. Sperm heads are negative; B-snurposomes are stained (arrowheads).
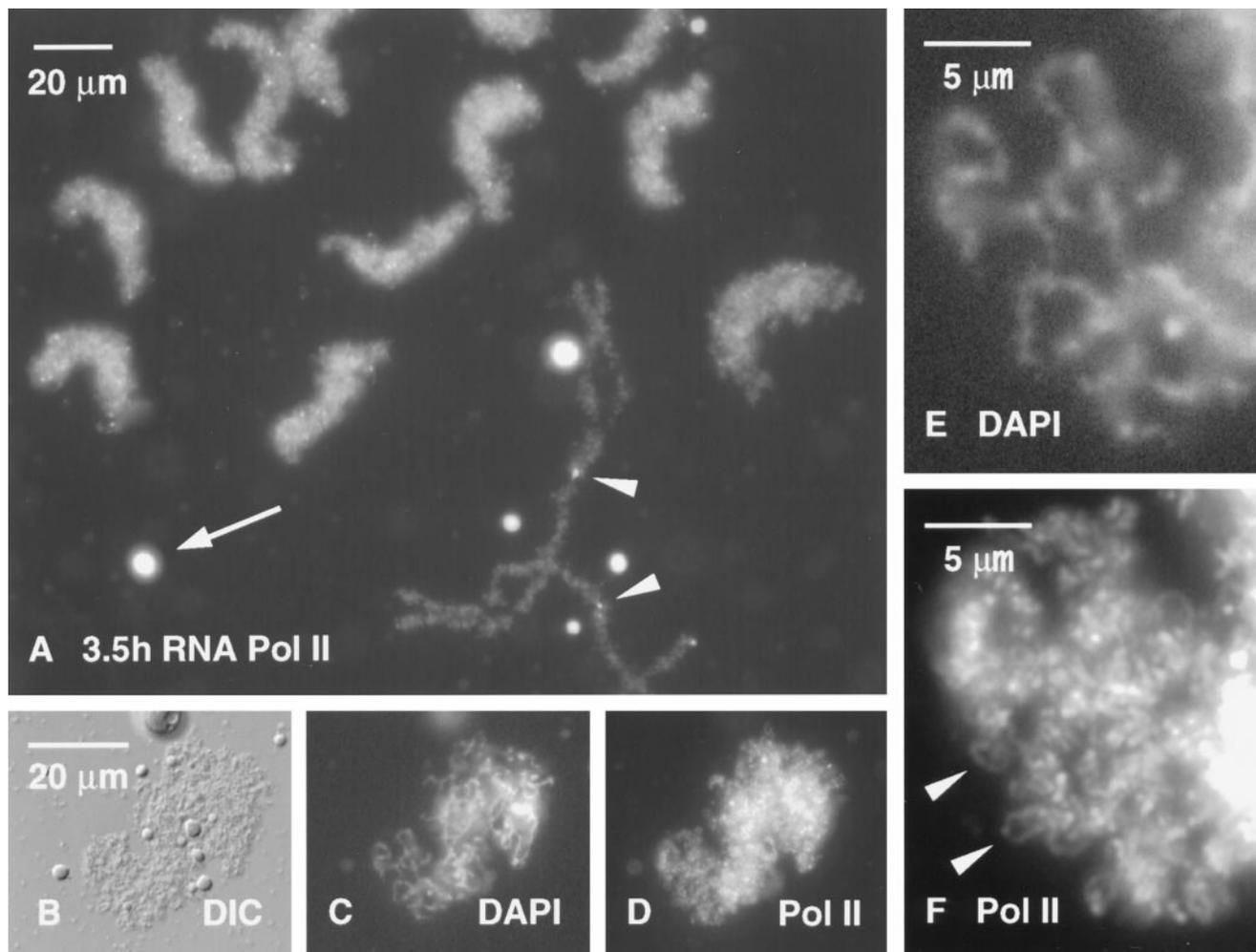
Figure 2. (A) Group of *Xenopus* sperm heads 3.5 h after injection into a *Xenopus* GV have swollen extensively and stain strongly with mAb H14 against RNA Pol II. An endogenous lampbrush chromosome is fused at its terminal granules to two other chromosomes (arrowheads). Several brightly staining coiled bodies (spheres) are also present (arrow). (B) DIC image of a single sperm head from the same GV. (C) DAPI stain reveals individual chromatids within the sperm head. (D) Staining with mAb H14 against RNA Pol II gives a strong reaction in the sperm head. (E) Higher magnification of C showing DAPI-positive chromatids. (F) Individual Pol II-positive loops (arrowheads) can be seen in this enlargement of the same region from D.

cases the swollen sperm heads remained more or less unchanged during the first 3–6 h (Figure 1, D–I), whereas in other experiments there was marked decondensation and evidence of LBC structure (Figure 2F).

For monitoring changes in the composition of sperm heads, we used three antibodies that detect transcription and splicing components of the nucleus: mAb H14 against RNA polymerase II (Pol II; Bregman *et al.*, 1995), mAb Y12 against the Sm proteins associated with splicing small nuclear RNPs (snRNPs) (Lerner *et al.*, 1981), and mAb K121 against the TMG cap of snRNAs (Krainer, 1988). mAb H14 is particularly useful for the study of LBCs because it stains only the axis of the lateral loops, leaving the RNP matrix unstained.

In this way one can follow the course of individual loops even in regions where there is considerable overlap. Among the extrachromosomal organelles in the GV, mAb H14 is highly specific for the coiled bodies (spheres), leaving essentially everything else unstained (Figures 1C and 2A). It is not known whether the staining of coiled bodies is due to Pol II or to a cross-reacting epitope. Antibodies Y12 and K121 allow the identification of snRNPs; both stain the matrix of the loops (Figure 3) and the snRNP-containing bodies in the GV referred to as B-snurposomes (Wu *et al.*, 1991; Figure 1, G and I).

Sperm heads begin to stain with mAb H14 within the first few hours after injection (Figure 1, D and E), presumably due to uptake of Pol II from stored re-
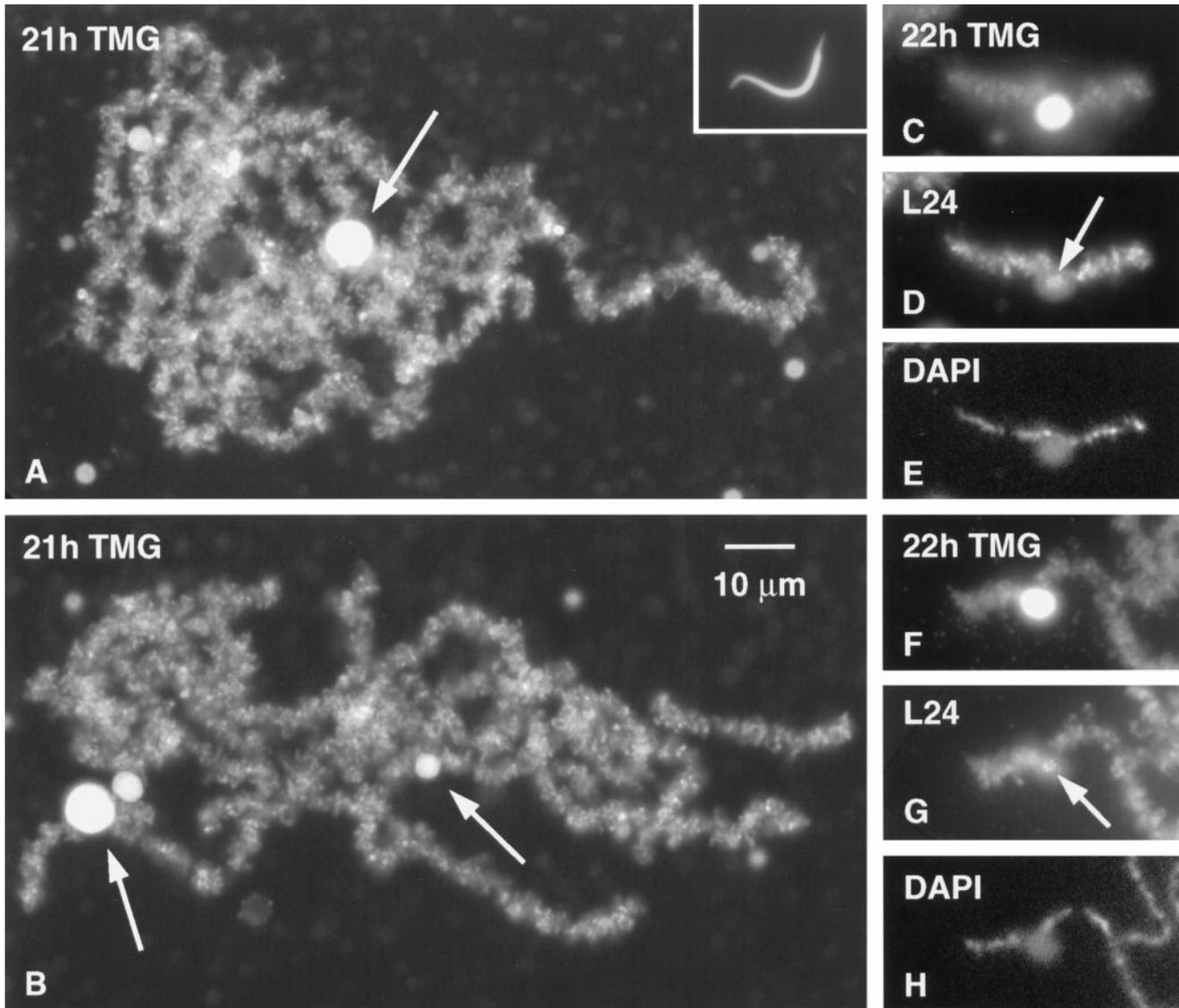
Figure 3. (A and B) Two *Xenopus* sperm heads 21 h after injection into a *Xenopus* GV. Each has resolved into a loose cluster of chromosomes with most of the features of the endogenous LBCs. Stained with mAb K121 against the TMG cap of the splicing snRNAs. The loops of the sperm LBCs stain strongly for snRNAs. Each cluster of chromosomes also displays one very large K121 (+) mass and several smaller masses (arrows). These almost certainly correspond to the similar masses that occur on chromosomes 6, 8, and 14. Inset in A shows sperm head at same magnification. (C) Single sperm LBC with a large K121 (+) mass attached near its center. The position of the mass and the relative size of this chromosome identify it as chromosome 14. (D) Same chromosome was double stained with serum L24 against protein xnf7 (Reddy *et al.*, 1991). This antibody stains small inclusions in the mass (arrow) and most of the lampbrush loops, as it does on endogenous LBCs. (E) DAPI staining of the same chromosome to show the axis of condensed chromomeres. (F–H) K121, L24, and DAPI staining of another example of what is probably sperm LBC 14, attached at one end to another chromosome. Arrow in G points to L24 (+) granules inside the mass.

serves in the GV. As we discuss below, this uptake is not accompanied by transcription and occurs even when transcription is inhibited by actinomycin D. Splicing factors are not taken up initially, as demonstrated by the absence of Y12 and K121 staining (Figure 1, F–I). As the sperm heads continue to expand, they stain more and more intensely for Pol II (Figure

2A). DAPI staining for DNA provides the first indication of individual chromatids within these swollen heads (Figure 2, C and E). Even at this early stage, careful examination reveals the presence of Pol II (+) loops extending from the DNA (Figure 2F, arrowheads). These loops signal the onset of RNA synthesis and the accumulation of nascent transcripts.
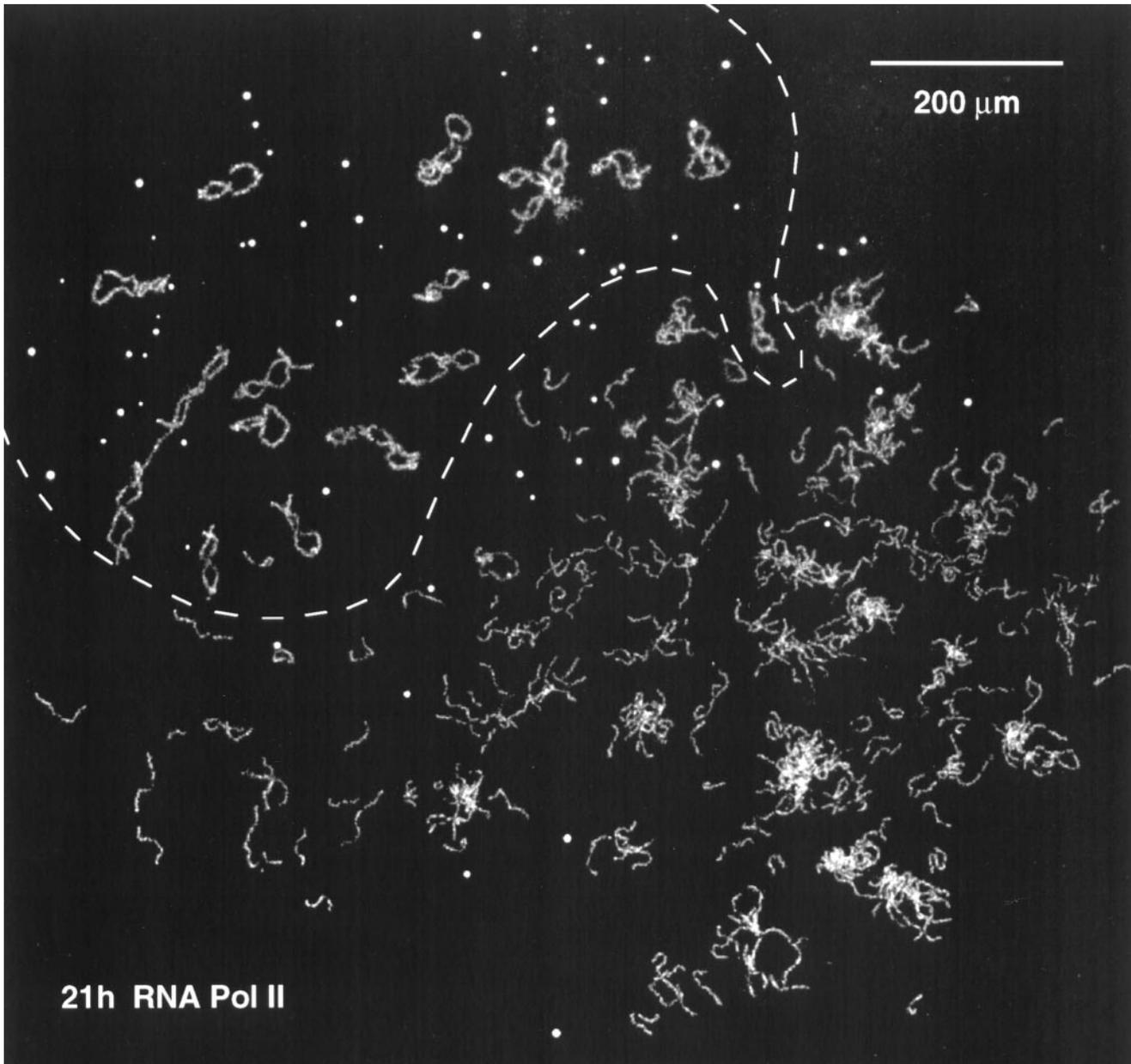
Figure 4. Overview of the contents of a single *Xenopus* GV 21 h after injection of *Xenopus* sperm heads. The sperm heads have been converted into loose clusters of chromatids or single chromatids that have the characteristic features of LBCs. This GV probably received 15–20 sperm heads. The 18 endogenous LBCs and most of the coiled bodies (spheres) occupy the area above the dashed line. This nucleus was stained with mAb H14 against RNA Pol II.

Appearance of Definitive Lampbrush Structure

The sperm heads eventually resolved into loose clusters of fuzzy threads that closely resembled the endogenous LBCs (Figure 3). Over time these clusters fell apart into individual chromosomes or small groups of chromosomes, which we refer to as "sperm LBCs." The sperm LBCs were easily recognizable, because they were unpaired and slightly shorter than the 18

endogenous bivalents in the same GV (Figure 4). Sperm LBCs did not disperse evenly throughout the volume of the GV but remained together, presumably moving only slowly from the site of injection. In various experiments, from 1 to about 20 sperm heads were injected into a single GV. Within these limits, the system was not saturable, in the sense that sperm LBCs appeared similar in morphology and staining
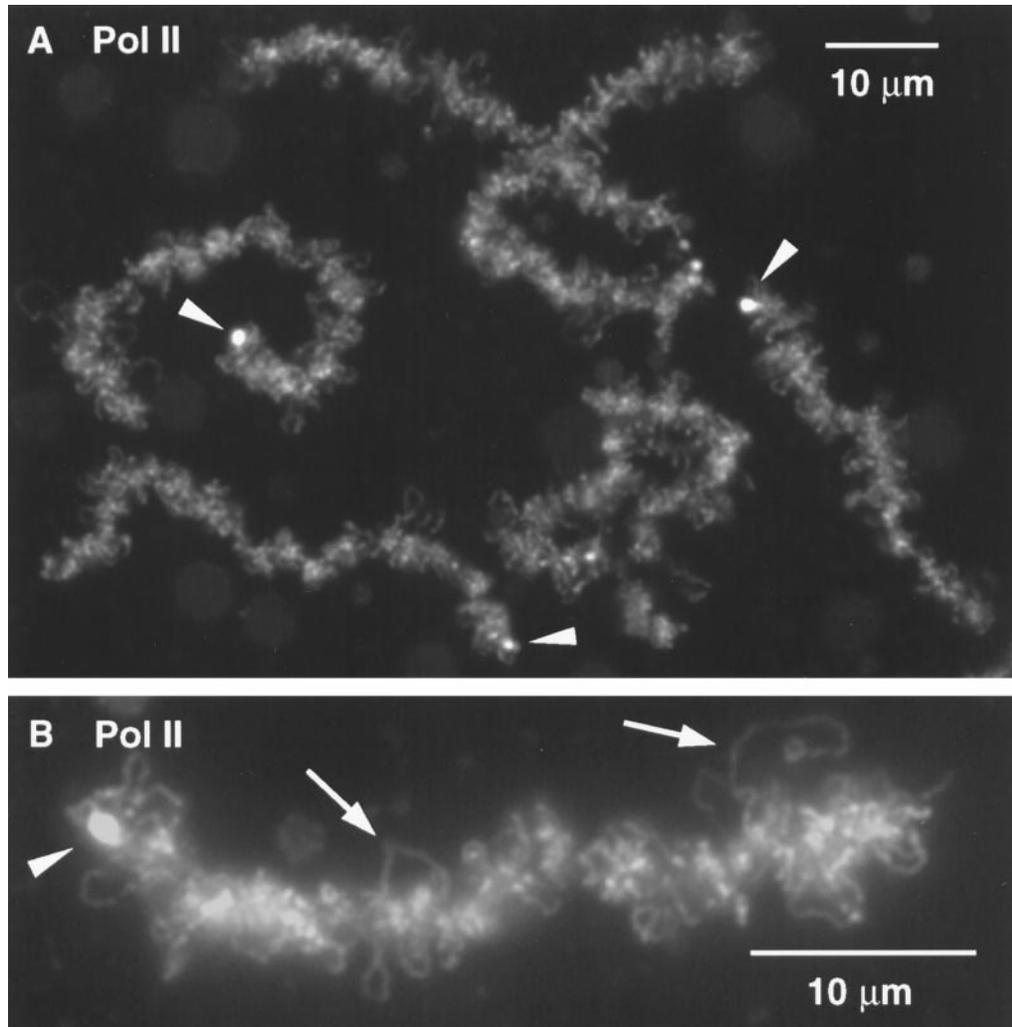
Figure 5. (A) Higher magnification of several sperm LBCs from the GV shown in Figure 4, stained with mAb H14 against RNA Pol II. The overall fuzzy appearance of the chromosomes is due to staining of the axes of the lateral loops. In addition, mAb H14 stains the terminal granules (arrowheads) found at the end of the long arm of 15 of the 18 *Xenopus* LBCs. (B) Single sperm LBC at still higher magnification, stained with mAb H14. The arrowhead points to the terminal granule, and arrows point to loop axes (DNA axes covered with Pol II).

properties whether derived from one or many sperm heads.

In overall structure sperm LBCs closely resemble the endogenous chromosomes in the same nucleus. They each have a DAPI-positive chromomere axis (Figure 3, E and H), which is less intensely stained than that of the endogenous LBCs, presumably reflecting the difference between a single chromatid and two closely paired sister chromatids. Loops on the sperm LBCs stained with antibodies against various proteins associated with nascent transcripts, such as serum L24 against xnf7 (Reddy *et al.*, 1991; Figure 3, D and G), and they also contained splicing factors, as demonstrated by staining with mAbs Y12 and K121 (Figure 3, A–C and F). The axis of each loop was well-delineated with mAb H14 against Pol II (Figure 5B).

Sperm LBCs Are Complete Chromosomes

The longest of the 18 lampbrush bivalents of *Xenopus* is just twice the length of the shortest, and the remaining chromosomes form a closely graded series (Müller, 1974; Callan *et al.*, 1987). The sperm LBCs are similarly graded in size with no particularly long or short members (Figures 4 and 5). Superficially, therefore, they look as if they might be derived from undefined fragments of sperm chromatin. However, closer inspection and comparison with the endogenous bivalents show that most of them are whole unbroken chromosomes.

Of the 18 bivalents, 15 possess a conspicuous terminal granule at the end of the long arm, referred to in earlier articles as a telomere (Callan *et al.*, 1987). This granule contains the genes coding for 5S rRNA (Callan *et al.*, 1988) and stains intensely with mAb H14, although there is no direct proof that it contains RNA Pol II. These granules tend to fuse with one another in various combinations. Nearly every GV has at least one bivalent in which the terminal granules of the two homologues are fused into a single granule (Figure 6A), and fusions between the termini of nonhomolo-
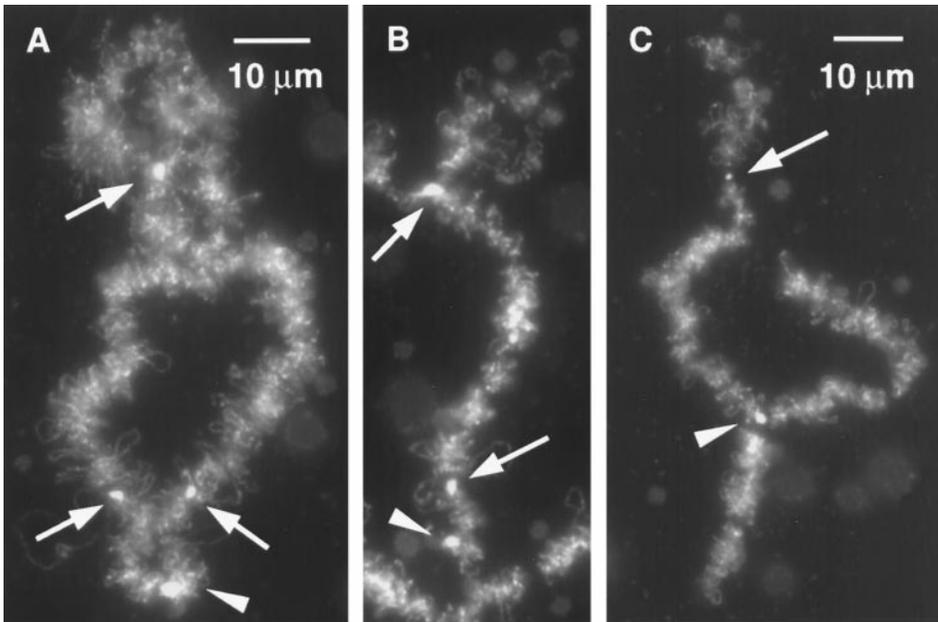
Figure 6. (A) Bivalent 11 is identifiable by a terminal granule at the end of its long arm (arrowhead) and axial granules at positions 0.16 and 0.80 (arrows). In this example there is a single fused granule at position 0.80. The centromere lies immediately beyond the fused granule but is not visible with this stain. The short arm has a characteristic diffuse structure. Sample is stained with mAb H14 against RNA Pol II. (B) Chromosome 11 derived from a sperm. The terminal granule (arrowhead), two axial granules (arrows), and the diffuse short arm are recognizable. (C) Another example of chromosome 11 derived from a sperm. The axial granule at 0.16 is not evident in this image, but the chromosome is reliably identified by the axial granule at 0.80 (arrow), the diffuse short arm, and the terminal granule (arrowhead), which is fused to the terminal granules of two other chromosomes to give a triradiate figure.

gous chromosomes are equally frequent (Figure 2A, arrowheads). Casual inspection of sperm LBCs shows that many also have one end capped by a terminal granule that stains with mAb H14 (Figure 5, A and B). Nonhomologous fusions between sperm LBCs are especially common, giving rise to multiarmed configurations that look like branched chromosomes (Figure 6C).

The strongest proof that sperm LBCs are whole chromosomes comes from identification of specific chromosomes. Among the easiest LBCs to identify is chromosome 14. Near the middle of this chromosome is a short region to which are attached oval or spherical masses that stain intensely with mAbs Y12 and K121. Such a mass is a conspicuous feature in each cluster of sperm LBCs (Figure 3, A and B), and in many cases chromosome 14 can be individually recognized (Figure 3, C–H). Chromosome 11 is also easy to identify. In addition to a prominent terminal granule, it has two axial granules that stain brightly with mAb H14 (Figure 6A). The axial granule nearest the end of the long arm is the locus of the U2 snRNA genes and is often associated with a prominent pair of loops; the second axial granule lies immediately adjacent to the centromere. Highly characteristic of chromosome 11 is the short arm, in which sister chromatids are not paired, giving the whole region a loose and unstructured appearance. Figure 6, B and C, shows two examples of sperm LBCs that display the characteristics of chromosome 11. In Figure 6B all of the features just mentioned are recognizable; in Figure 6C the axial granule at the U2 locus is not obvious, but

the chromosome is otherwise identifiable as chromosome 11.

Sperm LBCs Contain a Single Unreplicated Chromatid

Each sperm contains a haploid number of unreplicated chromatids, 18 in the case of *Xenopus*. Consequently, each sperm LBC should consist of an unreplicated chromatid, unless DNA synthesis occurs after the sperm is injected. When sperm heads are incubated in *Xenopus* egg extract, they enter the S phase and carry out DNA synthesis, as shown originally by Lohka and Masui (1983). However, the S phase of an oocyte occurs before the onset of meiosis, and there is no evidence for DNA synthesis in GVs at the lampbrush stage. Nevertheless, some caution is necessary in evaluating the DNA synthetic capacity of the GV, because an unusual amplification of rDNA does take place at pachytene in amphibian oocytes (Brown and Dawid, 1968; Gall, 1968).

The existence of two chromatids in each homologue of a lampbrush bivalent was originally inferred from the paired nature of the lateral loops (discussed in Callan, 1986). If sperm LBCs consist of unreplicated chromatids, they should have unpaired loops. Although the majority of sperm LBC loops are too short and similar in appearance for evaluation, their unpaired condition is revealed under several circumstances. First, when a specific loop is recognizable because of size or unusual morphology, it is always single, never paired, as it
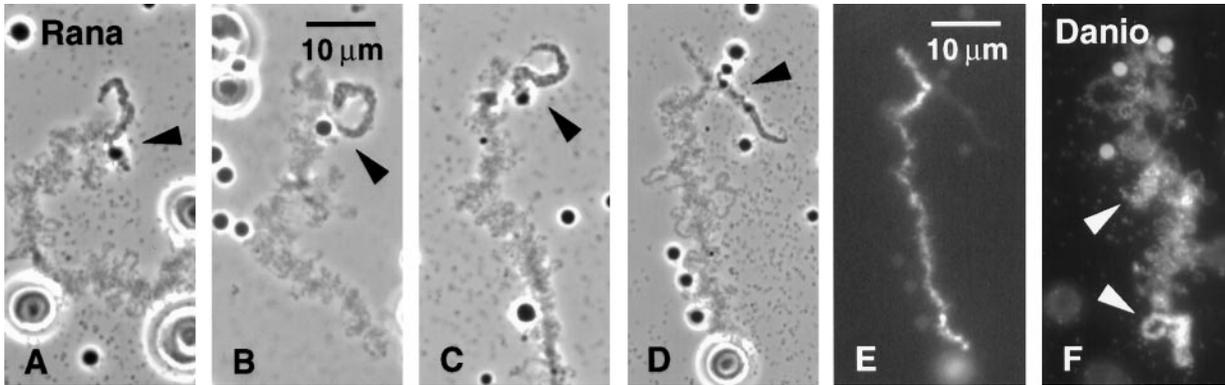
Figure 7. (A–D) Phase-contrast images of four examples of the same sperm LBC derived from *R. pipiens* sperm heads injected 2 d previously into a *Xenopus* GV. Near one end of this chromosome is an unusually large loop with a prominent granule at its base (arrowheads). The loop is single, as expected for an unreplicated chromatid. (E) Same field as D, DAPI stain to show the prominent DNA axis. (F) Sperm LBC 2 d after injection of zebrafish *D. rerio* sperm heads into a *Xenopus* GV. Sample is stained with mAb K121 against TMG. Arrowheads point to two loops with unusually heavy matrix.

would be in a typical bivalent (Figure 7, A–D). Second, some loops can be stained specifically with antibodies. Again, these loops are single on sperm LBCs (Figure 8, E and F). Third, when conventional LBCs are stretched, they display a phenomenon known as "double-loop bridges" (Callan, 1955; Callan and Lloyd, 1960). A double-loop bridge occurs when the bases of a pair of loops give way under tension; what were originally paired sister loops end up as parallel strands stretched out along the axis of the chromosome. Such double bridges have not been seen in sperm LBCs, but instead "single-loop bridges" are relatively common (Figure 8, G and H). These bridges confirm that a sperm LBC consists of a single chromatid.

Transcription and Splicing

It has been demonstrated many times that lampbrush loops are actively engaged in RNA synthesis and that inhibition of transcription with actinomycin D or α-amanitin leads to loss of loop RNP matrix and retraction of the loops (Izawa *et al.*, 1963; Schultz *et al.*, 1981; Callan, 1986; Figure 9G). To examine a possible relationship between transcription and early events in the formation of sperm LBCs, we injected [3H]GTP into the cytoplasm of oocytes, followed immediately by injection of sperm into the GV. Some oocytes were preincubated in actinomycin D for about 1 h before and were returned to actinomycin D after injection. In both sets of oocytes, sperm heads underwent initial swelling and by 1 h stained intensely with mAb H14 against RNA Pol II (Figure 9, A and B). At this early time splicing components were not demonstrable in the sperm heads (Figure 9, C–F). No further changes in the sperm heads were seen after longer incubation in actinomycin, whereas typical sperm LBCs eventually

appeared in oocytes held in OR2 saline. In oocytes held in OR2 saline for 7 h after injection of sperm and [3H]GTP, nucleoli and endogenous chromosomes were strongly labeled, whereas the swollen sperm heads were unlabeled (Figure 10A). Oocytes examined a day later displayed well-formed sperm LBCs, which by this time were actively transcribing (Figure 10B). [3H]GTP was not incorporated into any nuclear structures in actinomycin-treated oocytes (Figure 10C). Thus the initial stage of sperm enlargement and accumulation of RNA Pol II take place in the absence of transcription.

Earlier experiments with antisense oligodeoxynucleotides showed that transcription on LBCs can take place in the absence of U2 snRNA and thus does not require concomitant splicing (Tsvetkov *et al.*, 1992). We carried out a similar experiment that demonstrates assembly of sperm LBCs in oocytes in which the splicing machinery was disrupted. Figure 10, D and E, shows a sperm LBC from an oocyte injected 2 d earlier with sperm heads and with an oligodeoxyribonucleotide against U2 snRNA. This sperm LBC displays two features characteristic of U2-depleted GVs: the loops are unusually prominent and they show reduced staining with mAb K121. Because the targeted U2 snRNA was destroyed within the first 4 h (or less) after injection (Tsvetkov *et al.*, 1992), this experiment demonstrates that sperm LBCs can form in the absence of splicing.

In summary, the initial stage of sperm enlargement and accumulation of RNA Pol II normally occur without transcription and can take place even when transcription is inhibited by actinomycin D. Whether transcription is necessary for the emergence of cytologically recognizable chromosomes from the sperm head has not been tested, but the maintenance
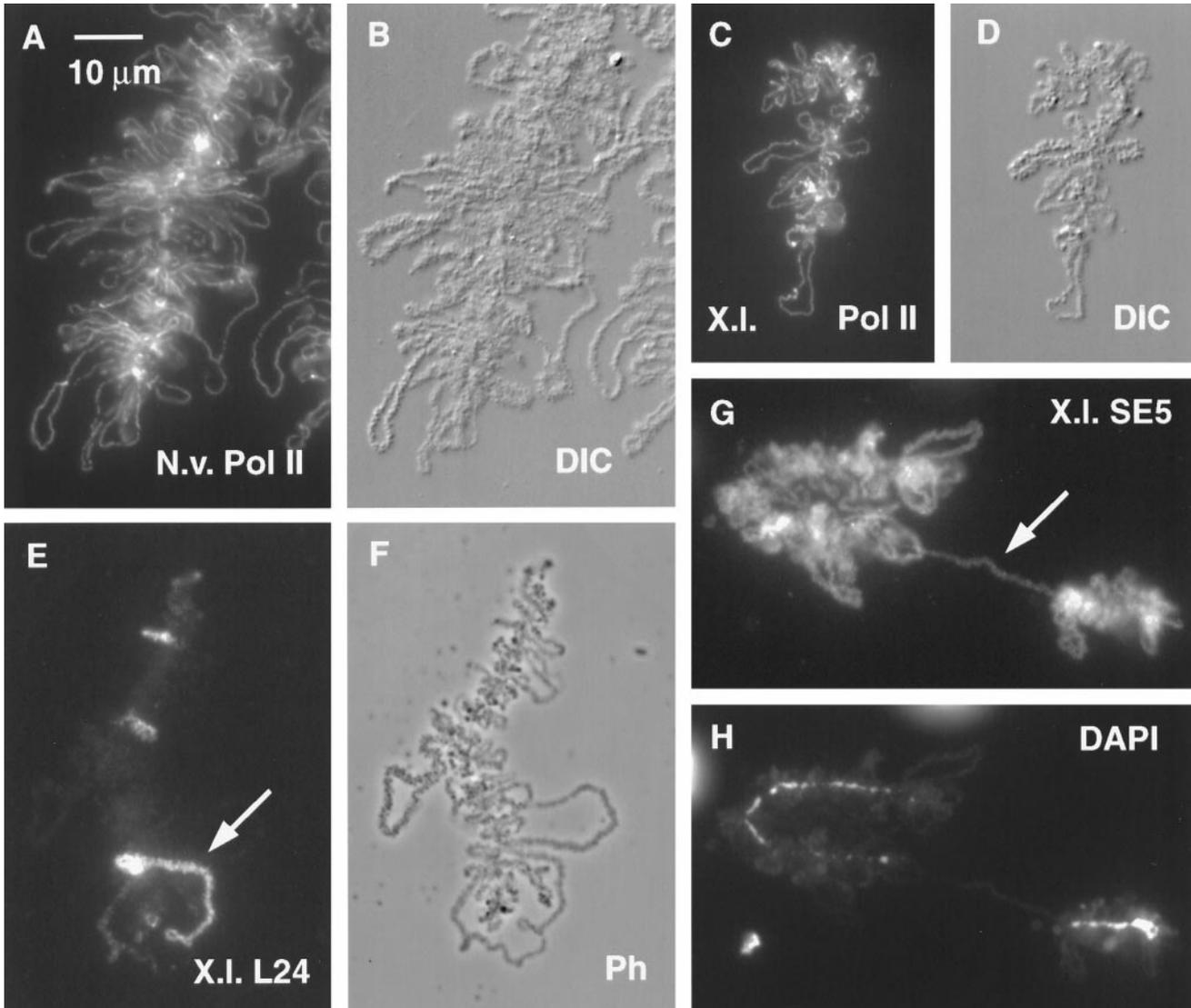
Figure 8. (A) Small portion of a typical LBC from a GV of the newt *N. viridescens*, stained with mAb H14 against RNA Pol II. (B) Same field by DIC. Compared with a *Xenopus* LBC, the newt LBC has longer loops, more heavily textured loop matrix, and unusually distinct Pol II (+) loop axes. In addition, newt LBCs are longer than *Xenopus* LBCs. (C) LBC derived from a *Xenopus* sperm head that had been injected 2 d previously into a newt GV. Sample was stained for Pol II. (D) Same field by DIC. Note how strongly this *Xenopus* LBC resembles a newt LBC except for length. (E) *Xenopus* sperm LBC 2 d after injection of *Xenopus* sperm into a newt GV. Sample was stained with serum L24 against xnf7 (Reddy *et al.*, 1991). Only a few loops react strongly with this antibody. The large loop (arrow) near the chromosome end is single, not paired like the loops of an oocyte LBC. This loop consists of two tandem transcription units, only one of which stains. (F) Same field by phase contrast. (G) *Xenopus* sperm LBC 2 d after injection into a newt GV. Sample was stained with mAb SE5 (Roth and Gall, 1987), which normally stains newt but not *Xenopus* LBC loops. Staining demonstrates that newt proteins were used to assemble the *Xenopus* LBC. Arrow indicates a single-loop bridge described in the text. (H) Same field (DAPI stain) showing that the loop bridge spans a gap in the chromomere axis.

of typical lampbrush loops does depend on transcription. Neither formation nor maintenance of sperm LBCs requires concomitant splicing.

Heterologous Injections

We have carried out several experiments in which demembranated sperm heads of one species were in-

jected into the GV of another. Such experiments can begin to distinguish cis from trans factors in the assembly of LBCs; that is, to assess the relative contributions of sperm chromatin versus proteins and other factors provided by the host GV.

Sperm of *Silurana* (*Xenopus*) *tropicalis* were injected into *X. laevis* GVs. Sperm LBCs derived from *S. tropi-*
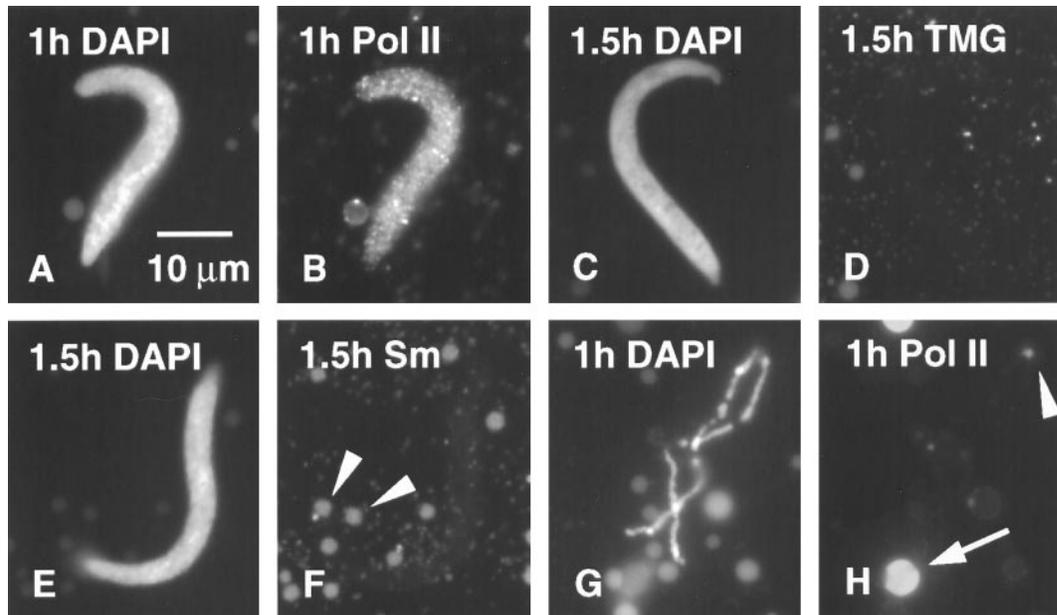
Figure 9. (A) *Xenopus* sperm head 1 h after injection into a *Xenopus* GV. The oocyte was incubated in actinomycin D (20 μg/ml) for 1 h before injection and was returned to the drug after injection (DAPI stain). (B) Same sperm head stained with mAb H14, showing uptake of Pol II by the sperm even though transcription was inhibited. (C) Sperm head 1.5 h after injection into an actinomycin-treated oocyte (DAPI stain). (D) Same sperm head stained with mAb K121 against TMG, showing the absence of splicing snRNAs. (E) Sperm head 1.5 h after injection into an actinomycin-treated oocyte (DAPI stain). (F) Same sperm head stained with mAb Y12, showing absence of Sm proteins. Arrowheads point to Y12 (+) B-snurposomes. (G) Highly contracted chromosome from the same GV that contained the sperm head shown in A and B (DAPI stain). (H) Same area showing absence of chromosomal stain with mAb H14, except for the terminal granule (arrowhead). A coiled body (sphere) shows typical staining (arrow).

calis were similar to those from homologous *Xenopus* injections. Because *S. tropicalis* has only 10 chromosomes in its haploid set, specific chromosomes were easier to identify, providing additional evidence that sperm LBCs are whole chromosomes. Sperm of the leopard frog *Rana pipiens* were also injected into *Xenopus* GVs. Again, the resulting sperm LBCs closely resembled the endogenous LBCs in both length and general morphology, and specific chromosomes could be recognized (Figure 7, A–D). When examined by DAPI staining (Figure 7E), the chromomere axes of the sperm LBCs were more prominent than those of the endogenous *Xenopus* bivalents, even though the comparison is between single and paired chromatids. The strong DAPI stain probably reflects the fact that *R. pipiens* has fewer chromosomes than *Xenopus* (n = 13 vs. n = 18) yet has a larger genome (6.8×10^9 bp vs. 2.8×10^9 bp [Dawid, 1965]), so that the "average" chromatid of *R. pipiens* contains about three times as much DNA as a chromatid from *Xenopus*. Perhaps less expected was the similarity of most loops on the *R. pipiens* sperm LBCs to those on the endogenous bivalents. LBCs of *R. pipiens* within their own oocytes display larger loops, and we were prepared to see more prominent loops on the sperm LBCs; that is, we had expected the origin of the sperm chromatin to have a greater effect on the morphology of the result-

ing LBCs. We have not yet carried out the reciprocal experiment of *Xenopus* sperm in *R. pipiens* GVs.

When sperm of the zebrafish *D. rerio* were injected into *Xenopus* GVs, the resulting LBCs resembled the endogenous *Xenopus* bivalents in general morphology, although they were much shorter. The *Danio* chromosome in Figure 7F was the longest one seen. Because *Danio* has a haploid number of 25 and a genomic DNA content of 1.7×10^9 bp (Postlethwait and Talbot, 1997), the "average" fish chromatid has about 0.44 times as much DNA as a *Xenopus* chromatid.

Unexpected results were obtained when we injected *Xenopus* sperm into GVs of the newt *Notophthalmus viridescens*. The difference in morphology between *Xenopus* and newt LBCs is considerable, the lampbrush loops of the newt being among the largest known (Figure 8, A and B). We were surprised to find that *Xenopus* sperm LBCs in GVs of *N. viridescens* resembled the endogenous newt chromosomes to a remarkable degree, except for their shorter overall length. The resemblance involved three features of the loops: length, morphology of the RNP matrix, and prominence of the Pol II axis. The most striking feature was the length of the loops, which was greater than that normally seen on *Xenopus* chromosomes (compare Figure 8, C–H, with Figures 5 and 6). Many loops were 30–40 μm in length, although on average they were
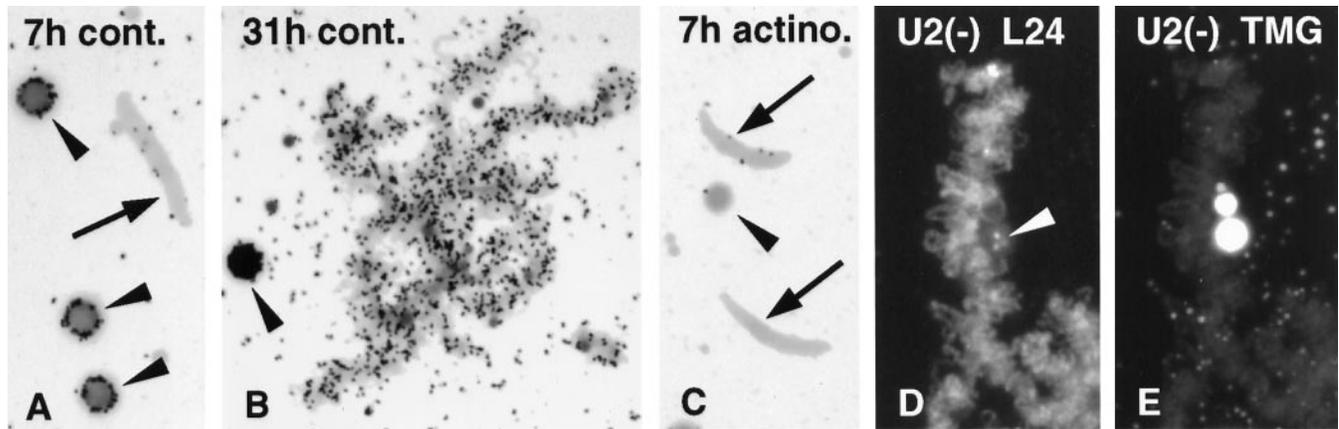
Figure 10. (A) [³H]GTP was injected into the cytoplasm of a *Xenopus* oocyte and 1 h later *Xenopus* sperm heads were injected into the GV. An autoradiograph of the GV contents made 7 h later shows no label in a sperm head (arrow) but strong label in three nucleoli (arrowheads), indicative of rRNA transcription (1.5-d exposure). (B) Autoradiograph of labeled sperm LBCs 31 h after injection of [³H]GTP and sperm heads as in A. Active transcription takes place on the loops of the sperm LBCs. A nucleolus (arrowhead) is blackened by silver grains (1.5-d exposure). (C) Oocytes were preincubated for 1 h in actinomycin D (20 μg/ml), injected with [³H]GTP and sperm heads as in A, and then returned to the drug. An autoradiograph of GV contents made 7 h later shows no label in the sperm heads (arrows) or in the endogenous nucleoli (arrowhead; 1.5-d exposure). (D) Sperm LBC from an oocyte depleted for U2 snRNA and stained with serum L24 against xnf7 (Reddy *et al.*, 1991). Loops are well stained, as are granules inside the large "mass" attached to the chromosome (arrowhead). (E) Same chromosome stained with mAb K121 against TMG. The loops show markedly reduced staining relative to untreated oocytes (compare Figure 3), but the K121 (+) masses are not affected.

not so long as those on the endogenous newt chromosomes. The RNP matrix of the sperm LBC loops is both abundant and varied in morphology like that of newt LBCs (Figure 8, D and F). Similar variations in loop morphology occur on endogenous *Xenopus* LBCs but are less striking. A conspicuous feature is the staining of the loop axis with mAb H14 against RNA Pol II. In normal newt LBCs and in *Xenopus* sperm LBCs in the newt GV this axis is unusually prominent (Figure 8, A and C), whereas in normal *Xenopus* LBCs it is less conspicuous (Figure 6A).

We used two newt-specific antibodies to examine proteins associated with *Xenopus* sperm LBCs in the newt GV. mAb A33 detects a zinc-finger protein (A33) originally described from *Pleurodeles waltl* (Lacroix *et al.*, 1985; Bellini *et al.*, 1995), and mAb SE5 detects an unrelated protein (SE5) from *N. viridescens* (Roth and Gall, 1987). Both antibodies stain the RNP matrix of most but not all loops on LBCs of *N. viridescens*, whereas neither reacts with normal LBCs of *Xenopus*. In injection experiments, however, *Xenopus* sperm LBCs stained indistinguishably from the endogenous *N. viridescens* chromosomes in the same GV (Figure 8G). This observation demonstrates that newt proteins are used for the assembly of *Xenopus* sperm LBCs in newt GVs.

We have carried out other heterologous injection experiments with limited success. Lysolecithin-treated sperm heads from the mouse *M. musculus* and the cricket *A. domesticus* were injected into GVs of *Xenopus*. The sperm heads of both species underwent initial swelling and began to stain with mAb H14. In neither case, however, did individual chromosomes become visible, although a few short loops extended from some of the mouse nuclei. Samples of the same sperm heads formed typical in vitro nuclei after 30–60 min of incubation in *Xenopus* egg extract, suggesting that competence in the latter assay is not a sufficient criterion for competence in the GV system.

DISCUSSION

We have shown that typical transcriptionally active lampbrush chromosomes can be assembled from sperm chromatin when demembranated sperm heads of *Xenopus* are injected into the GV of intact *Xenopus* oocytes. About 15 years ago, Lohka and Masui (1983, 1984) found that such sperm heads transform into essentially normal pronuclei when incubated in an extract of *Xenopus* eggs, and the egg extract system has since been used to investigate many aspects of DNA replication and the cell cycle. The GV and egg extract systems are fundamentally different. In the egg extract, demembranated sperm chromatin becomes surrounded by a nuclear envelope and gives rise to a transcriptionally silent nucleus that may subsequently undergo DNA replication and enter mitosis. In the GV, however, the same demembranated sperm chromatin remains freely exposed to the surrounding milieu, does not undergo replication, and transforms into separate transcriptionally active giant chromosomes (Figure 11).

Behavior of demembranated sperm head in egg extract and in GV
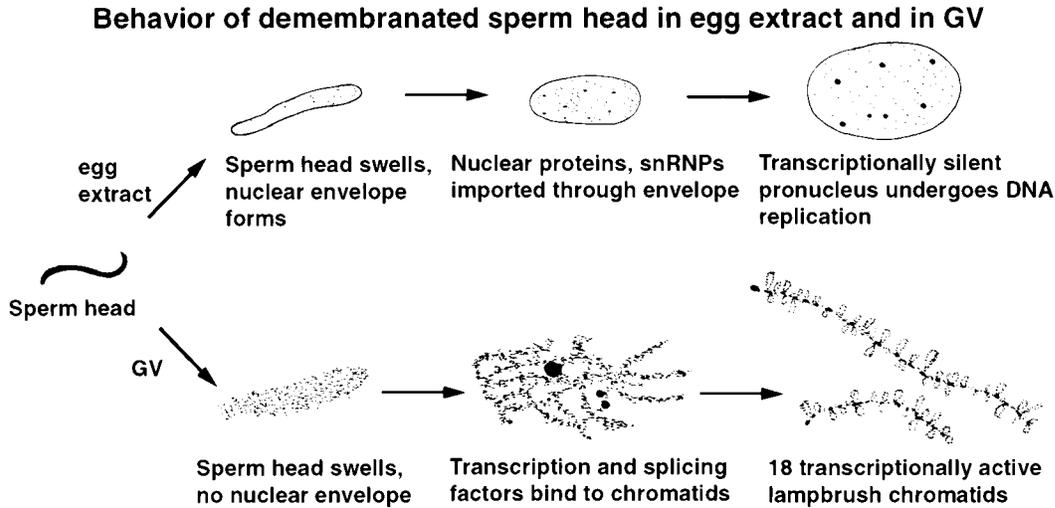
Figure 11. Comparison of the behavior of demembranated sperm heads after incubation in egg extract (top row) or injection into the GV (bottom row).

It has been documented many times that amphibian GVs contain large quantities of stored materials destined for the early embryo, including transcription and splicing factors. These factors allow the GV to transcribe injected DNA templates and to process the resulting RNA (reviewed in Gurdon and Melton, 1981; Davidson, 1986; Almouzni and Wolffe, 1993). GV extracts have been used to support Pol III transcription from DNA templates in vitro, as in the extensive studies of Brown and his colleagues (Birkenmeier *et al.*, 1978) on 5S RNA. On the other hand, aqueous GV extracts have been of limited use for the study of Pol II transcription, although GVs isolated under oil, and therefore retaining soluble cofactors, support efficient transcription from U1 snRNA templates (Lund and Dahlberg, 1989). In early experiments by Gurdon (1976), somatic nuclei were injected into both cytoplasm and GVs of *Xenopus* oocytes, and the subsequent morphological and transcriptional events examined in considerable detail. Nuclei injected into the GV swelled and became transcriptionally active but retained some type of investing material, presumably the nuclear lamina. LBCs may well have formed inside these nuclei without being detected as such.

The assembly of 18 giant LBCs from the chromatin of a minute sperm head within a matter of hours is a dramatic phenomenon, yet aspects of this transformation could have been anticipated from the studies just cited and from observations on LBCs themselves. As early as 1969, Snow and Callan (1969) studied the loss and reformation of lampbrush loops when newt oocytes were treated with actinomycin D. Within a few hours after oocytes were exposed to actinomycin D, the loops lost their RNP matrix and retracted into the chromomeres, which themselves coalesced to form shorter chromosomes. After removal of the drug, RNA synthesis resumed, and typical LBC morphology was regained over a period of 2–4 d. These experiments were carried out on ovaries of living animals. In a later study, Scheer (1987) carefully documented a similar course of events when excised newt oocytes were treated with actinomycin D in vitro. Even when oocytes are removed from a female *Xenopus* and placed immediately in a balanced salt solution, the LBCs exhibit similar signs of transcriptional shutdown during the first few hours (Gall *et al.*, 1991). After 18–24 h, however, the chromosomes increase in length and the loops return. Thus the behavior of endogenous LBCs should have suggested that exogenous chromatin might undergo a similar transformation.

The final form of LBCs derived from sperm chromatin must be influenced by features of the chromatin itself as well as by factors in the GV where the transformation takes place. It is not surprising that *Xenopus* sperm chromatin in a *Xenopus* GV gives rise to LBCs that are in many respects indistinguishable from the endogenous LBCs. On the other hand, the results of heterologous injections are not easy to predict. At the outset we were strongly influenced by an interpretation of LBC structure derived from comparative cytological studies. Some organisms, particularly salamanders, have enormous LBCs with very long loops, whereas others like *Xenopus* have intermediate sized chromosomes with modest loops, and still others have very short chromosomes with barely detectable loops. These differences correlate roughly with the genomic DNA content or C value of the various organisms (Macgregor, 1980). One expects overall chromosome length to correlate with C value, but why should loop length do so as well, if loops simply

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