Journal of Cell Science 101, 759-772 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

A novel structure associated with a lampbrush chromosome in the chicken, *Gallus domesticus*

IRINA SOLOVEI, ELENA GAGINSKAYA

Biological Research Institute, University of St. Petersburg, Russia

TERRY ALLEN

Paterson Laboratory, Christie Hospital, Manchester, England

and HERBERT MACGREGOR*

Department of Zoology, University of Leicester, Leicester LE1 7RH, England

*Author for correspondence

Summary

At a site near the end of the short arms of lampbrush bivalent 2 in the chicken (*Gallus domesticus*) there is always a marker structure that appears in the phase-contrast light microscope as a solid object with diffuse edges measuring about 4 μm across. When examined by transmission electron microscopy in thin section, this object appears as a loose bundle of fibres. In some preparations individual fibres appear 15-16 nm thick, smooth in outline and solid in cross-section. In other preparations they are 32-38 nm thick, rougher in outline and ring-like in cross-section. High-resolution scanning electron micrographs of the chromosome 2 marker show it to be a loose bundle of spaghetti-like fibres that is quite unlike anything previously seen on a lampbrush chromosome of any organism. As with the sectioned material, fibres in some preparations were smooth and 15-16 nm in diameter, whereas those in others were more knobbly and about 35 nm thick. The fibres appear to branch and in some cases it is clear that the daughter strands of a branch have the same dimensions as the parent strand. Free ends are rare. Total length of fibre material present at one marker locus is estimated to be between 500 and 2000 μm. Similar structures are not present on the lampbrush chromosomes of quail, wood pigeon or chaffinch.

The nature of this fibrous marker, referred to in this paper as the "spaghetti marker", is discussed in relation to lampbrush chromosome function and to events that take place during the lampbrush phase of oogenesis in chicken. Evidence is discussed in relation to the possibility that the chromosome 2 marker represents a novel form of nuclear RNP or the specific association of some structural protein with one chromosome locus.

Key words: oocyte nucleus, lampbrush chromosome, scanning electron microscopy.

Introduction

Lampbrush chromosomes are well known as enormously elongated diplotene bivalents found in the growing oocytes of all animals except mammals, some insects and some reptiles. These chromosomes support a widespread transcription of RNA from many thousands of promoters distributed throughout the lengths of all the chromosomes. RNA polymerase transcribes past the end of structural gene sequences and continues on to form contiguous transcripts of whatever sequences lie downstream (Macgregor and Andrews, 1977; Varley et al., 1980; Diaz et al., 1981; Gall et al., 1983). The newly formed RNA transcripts remain associated with the chromosomal DNA template. The end result of this process is a large number of exceedingly long transcription units, each of them polarised in the sense of carrying RNA transcripts that are progressively longer from one end of the transcription unit to the other. These transcription units constitute the lateral loops that are the basis of the lampbrush form, and until quite recently RNA transcription and the arrangement and expression of DNA sequences have been the major foci for lampbrush chromosome research (Callan, 1986; Macgregor, 1987).

This paper is about a study of the lampbrush chromosomes of the domestic chicken (*Gallus domesticus*), and it focusses specifically on a novel structure

such as has never been seen in any previous studies of lampbrush chromosomes. The earliest studies of lampbrush chromosomes from birds, carried out with the techniques introduced by Gall (1954) and Callan and Lloyd (1960), were those of Koecke and Muller (1965) and Ahmad (1970). However, methods for preparing avian lampbrush chromosomes for modern molecular and cytogenetic investigations were worked out much later and independently by Hutchison (Hutchison and Weintraub, 1983; Hutchison, 1987) and by Gaginskaya and coworkers (Gaginskaya et al., 1984; Kropotova and Gaginskaya, 1984; Chelysheva et al., 1990; Solovei et al., 1990). These authors have confirmed that avian lampbrush chromosomes exhibit all the main features that have been described for those of amphibians.

At an early stage in their studies of fixed and stained isolated lampbrush chromosomes from chickens Chelysheva et al. (1990) noticed that the conspicuous object located near the end of the short arm of chromosome 2 was different from all other distinctive objects associated with the chromosomes in the sense that it never in any circumstances assumed the appearance of a loop. Experimental treatments, such as reducing the ionic strength of the chromosome isolation medium, have the effect of removing the RNP from all loops and often exposing the fundamental loop-like organisation of conspicuous structures that in normal circumstances have a solid, round or irregular appearance - the "lumpy loops" of Callan and Lloyd (1960). The objects located at the Chelysheva et al. locus LL22 never reacted in this manner.

Subsequent to the studies published in 1990 (Chelysheva et al., 1990; Solovei et al., 1990), it was decided to investigate certain aspects of the fine structure of chicken lampbrush chromosomes employing transmission electron microscopy (TEM) of sectioned material with special regard to the marker at LL22. Preparations of lampbrush chromosomes were made according to the method described by Mott and Callan (1975). The short arm of chromosome 2 was specifically selected for thin sectioning. Sections through most regions of the chromosomes produced no surprises. In general the structure of chromomeres and lateral loop ribonucleoprotein appeared more or less similar to that which had been described in electron micrographs of lampbrush chromosomes from other species (Mott and Callan, 1975; N'Da et al., 1986). The one outstanding exception was the marker at position LL22. Sections through the region of the LL22 marker showed evidence of a loose bundle of strands having an appearance that was quite different from that of neighbouring loop ribonucleoprotein. The area of section occupied by the stranded material was about 4 μm in diameter, which was consistent with the observed size of the LL22 marker as seen in unfixed lampbrush preparations examined with a phase-contrast microscope.

In view of the extraordinary appearance of the LL22 marker in light-microscope preparations and TEM sections, it was decided immediately that this region required further investigation and, taking account of the size of the whole marker and the sizes and packing of its individual strands, high-resolution scanning electron microscopy (SEM) coupled with light microscopy and cytochemistry seemed the most promising approaches. In this paper we describe the structure of the LL22 marker, we present the results of some cytochemical tests and we offer some suggestions as to the nature of this hitherto unknown class of object.

Materials and methods

Oocytes for lampbrush studies were obtained from commercial line Zarya 17 and Rhode Island Red crosses of between 21 weeks ("point of lay") and 31 weeks old. Chickens were killed by cervical dislocation and the whole ovary was removed and placed in a clean dry beaker, covered with foil and stored on ice. The oocytes remain in good condition for lampbrush studies for a maximum of 12 h after removal from the bird.

In the chicken, the lampbrush chromosomes are found in oocytes ranging from 0.13 mm to 2.5-3 mm in diameter (Gaginskaya, 1972a; Hutchison, 1987; Chelysheva et al., 1990). The best preparations of lampbrush chromosomes can be made from oocytes of between 1 mm and 2.5 mm diameter. The germinal vesicle of a 1.2 mm oocyte measures about 100 μm diameter and that of a 2.5 mm oocyte has a diameter of about 300 μm. For this study, oocytes of between 0.5 mm to 3.5 mm diameter were mainly used, although some preparations were made from post-lampbrush oocytes of up to 7 mm diameter. The chromosomes were isolated manually employing the standard lampbrush technique as described by Macgregor and Varley (1988) with some minor modifications. Individual oocytes or small groups of oocytes of the required size were dissected out and transferred to a separate dish containing "5:1 + phosphate" (Gall et al., 1981; 83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 7.2). Chicken ovary, unlike the ovaries of amphibians, is densely compacted with collagen and connective tissue and it is best to remove most of this material from around the oocyte before trying to isolate the germinal vesicle. The germinal vesicle was removed in 5:1 + phosphate by stabbing the oocyte with a dissecting needle and then carefully watching as the yolky cytoplasm streamed out of the hole. The germinal vesicle appears as a small clear interruption in the flow of yolk as it emerges from the hole. It is perfectly round, turgid, transparent and glistening in appearance. The germinal vesicle can be seen inside intact oocytes of less than 1.2 mm diameter if bright substage transmitted illumination is used on the dissecting binocular microscope. After isolation, the germinal vesicle is picked up immediately in a small-bore Pasteur pipette and transferred to a lampbrush observation chamber (Macgregor and Varley, 1988) containing 3/4 strength 5:1 plus phosphate with 0.1% formaldehyde. The nuclear envelope is removed manually, either with two pairs of very fine forceps or with tungsten needles, and the nuclear contents are allowed to disperse on the bottom of the observation chamber.

Some preparations were examined directly, without centrifugation or fixation, by phase-contrast microscopy. In order to examine the effects of nucleases and proteases, chromosomes were either dissected directly into 3/4 strength 5:1 plus phosphate to which the enzyme had been added to a concentration of 0.1 mg/ml, or they were isolated in 3/4 strength 5:1 plus phosphate and the enzyme was added later. With the latter technique, the observation chamber was covered with a coverslip, the chromosomes were allowed to

disperse and enzyme solution subsequently added to the edge of the coverslip so that it slowly mixed with the contents of the observation chamber. The final enzyme concentration in the region of the chromosomes was estimated to be about 0.01 mg/ml. The effects of deoxyribonuclease (DN-EP, Sigma), ribonuclease A (Sigma, pre-boiled in sodium acetate at pH 5 to eliminate DNase activity), Pronase and trypsin were examined in this manner and recorded by flash photomicrography of phase-contrast images. Magnesium chloride (6×10^{-4} M) was added to the isolation medium for studies of the effects of deoxyribonuclease. The effects of several restriction enzymes, *Hae*III, *Eco*RI and *Alu*I, on the lampbrush chromosomes of chickens and particularly on the LL22 marker were also examined in this study. For all restriction enzyme studies, chromosomes were isolated into Tris-buffered saline (TBS) plus magnesium (80 mM KCl, 20 mM NaCl, 20 mM Tris, 0.6 mM $MgCl_2$, pH 7.8).

Preparations for light-microscopic cytochemistry or for electron microscopy were centrifuged at 1500 *g* for 20 min in a Sorvall RT-6000 bench top refrigerated centrifuge (see Macgregor and Varley, 1988) in order to stick the chromosomes firmly to the base of the observation chamber. They were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7. Preparations for fluorescence cytochemistry (Hoechst, Chromomycin A3, DAPI) were fixed in 2% formaldehyde for one hour and then in 70% ethanol. They were then returned to phosphate buffered saline (PBS: 0.02 M phosphate buffer, 0.15 M NaCl) and incubated at room temperature with the primary and secondary antibodies. Antibodies to chicken nuclear lamins A and B2 (kindly provided by Dr. E. A. Nigg) and to mammalian intermediate filaments (kindly provided by Dr. C. Ockleford) were selected for testing on the grounds of the finely fibrous appearance of the LL22 marker. Preparations were preincubated in 10% horse serum in PBS and then with the first antibody, employing ranges of concentrations recommended by Drs. Nigg and Ockleford. The preparations were then washed in 10% horse serum, incubated with the appropriate FITC-labelled secondary antibody, washed again with 10% horse serum, mounted and examined with a Carl Zeiss epifluorescence microscope.

All preparations for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7. Preparations for subsequent embedding and thin-sectioning for transmission electron microscopy and some preparations for scanning electron microscopy were post-fixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7, for 30 minutes. Subsequent treatment of chromosomes for thin sectioning was as described by Mott and Callan (1975). Regions of the chromosomes that carried distinctive markers were specifically selected for thin sectioning. Sections were cut at either 100 nm or 150-250 nm thickness and subsequently examined with a Tesla BS 500 electron microscope using accelerating voltages of either 60 or 90 kV. Preparations for scanning electron microscopy were dehydrated in an ethanol series, rinsed twice in amyl acetate and then critical-point dried from liquid CO_2. They were then coated with gold/palladium (estimated 2-3 nm thickness) or chromium (estimated 1-2 nm thickness) and examined in a high-resolution, field emission, in-lens scanning electron microscope (ISI/ABT DS 130F) at a variety of accelerating voltages within the range 3-25 kV.

For electron microscopy of negatively stained preparations, chromosomes were isolated directly onto a grid with a Formvar supporting film situated at the bottom of a lampbrush observation chamber. The preparations were centrifuged, fixed in 2.5% glutaraldehyde, washed and air dried. They were then covered for 30 s with a few drops of saturated uranyl acetate diluted 1:1 with water, washed in running distilled water, air dried and examined in a JEM-100CX electron microscope.

Results

Light microscopy

Altogether, lampbrushes from about 90 chickens (commercial cross Zarya 17) in the USSR and 15 chickens (Rhode Island crosses) in the UK have been examined in the current programme. All were homozygous for the presence of the LL22 marker. The marker has a distinctive appearance in phase-contrast (Fig. 1A). It sometimes consists of two components, a small compact dark structure about 1 μm in diameter situated in line with the chromosome axis, and a lighter and more diffuse area partially or wholly surrounding the dark object. The entire structure usually measures between 3 and 5 μm across. It is of a similar size in all oocytes of all sizes from a particular bird. LL22 is present in oocytes ranging from 0.4 mm to 3.5 mm diameter, the smallest and largest from which lampbrushes can be successfully isolated. It is absent from post-lampbrush chromosomes isolated from oocytes of 7 mm diameter. In general, its behaviour parallels that of all the other conspicuous markers in the lampbrush set, the majority of which are typically loop-like in organisation.

In some preparations the two homologous LL22 markers were fused together to form a single structure that joined the two half-bivalents at that locus. In all such cases, there were no chiasmata distal to LL22. In all of the many preparations examined in which there was a chiasma distal to LL22 in the short arm of chromosome 2, the LL22 markers were separate from one another.

Simple cytochemical tests in which the chromosomes were stained with Hoechst 33258, DAPI, chromomycin A3, gallocyanin-chrome alum and Coomassie blue all indicated that LL22 was not noticeably different from other marker structures on the chromosomes. In DAPI and Hoechst preparations the LL22 marker itself was unstained or very weakly stained, but a structure that appeared to be a large chromomere lying immediately under or beside LL22 regularly stained as brightly as neighbouring chromomeres in the chromosome axis. Typical lampbrush chromomeres are known to consist almost entirely of compacted chromatin (Callan, 1986).

In studies of the effects of nucleases and proteases on lampbrush chromosomes two matters are particularly important. First, conditions must be used that allow the enzyme to gain access to freshly isolated and unfixed chromosomes. Secondly, some kind of internal standard must be available to provide evidence that the enzyme is, in fact, doing what is expected of it. In our experiments, the actions of DNase I and of restriction endonucleases were proved by fragmentation of lampbrush loops and breakage of chromosome axes. The action of RNase was proved by the destruction of the RNP matrix of lampbrush loops and the consequent disappearance of the loops. These effects were particularly evident on the large landmark loops, TBL21 and

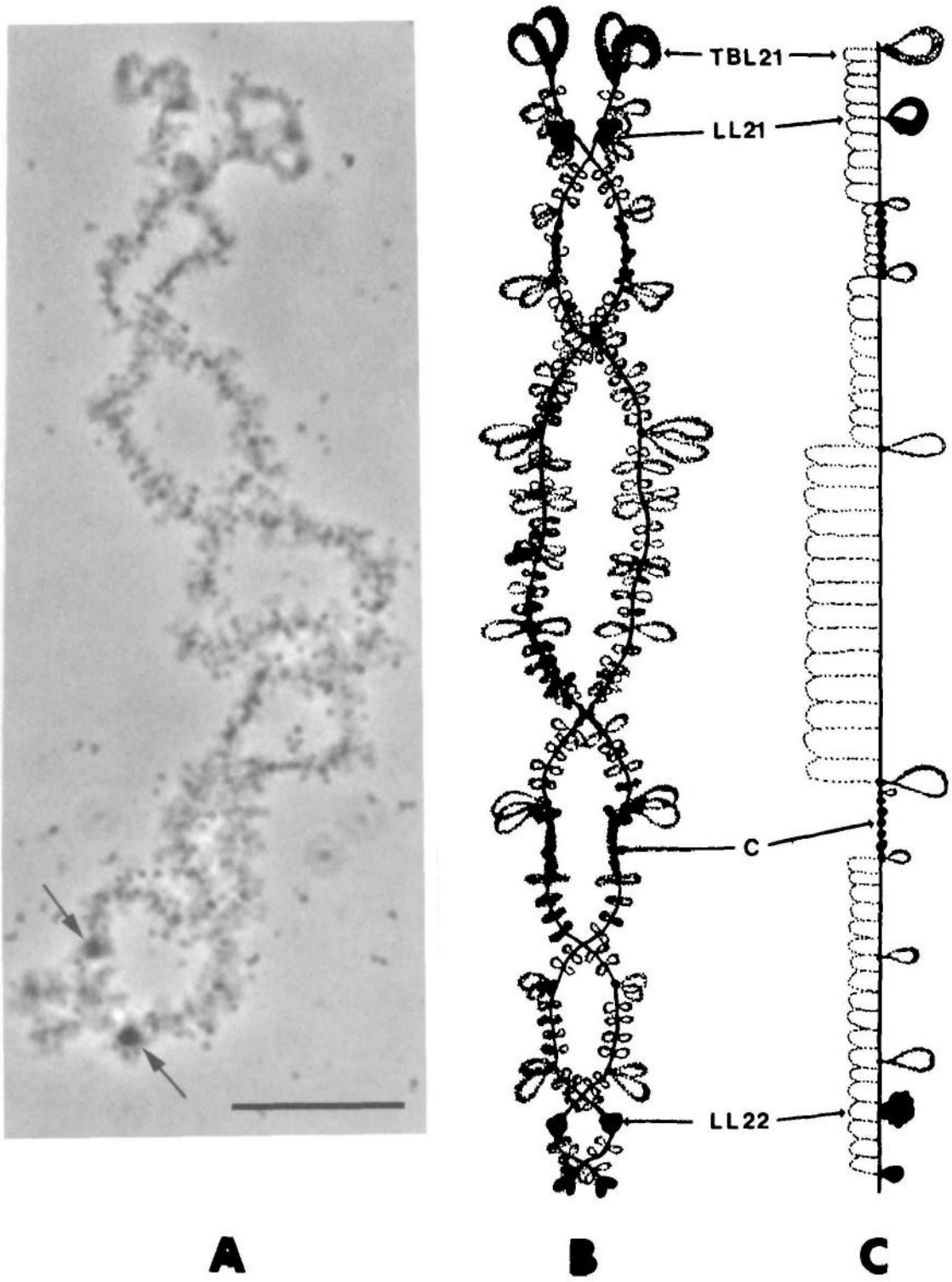
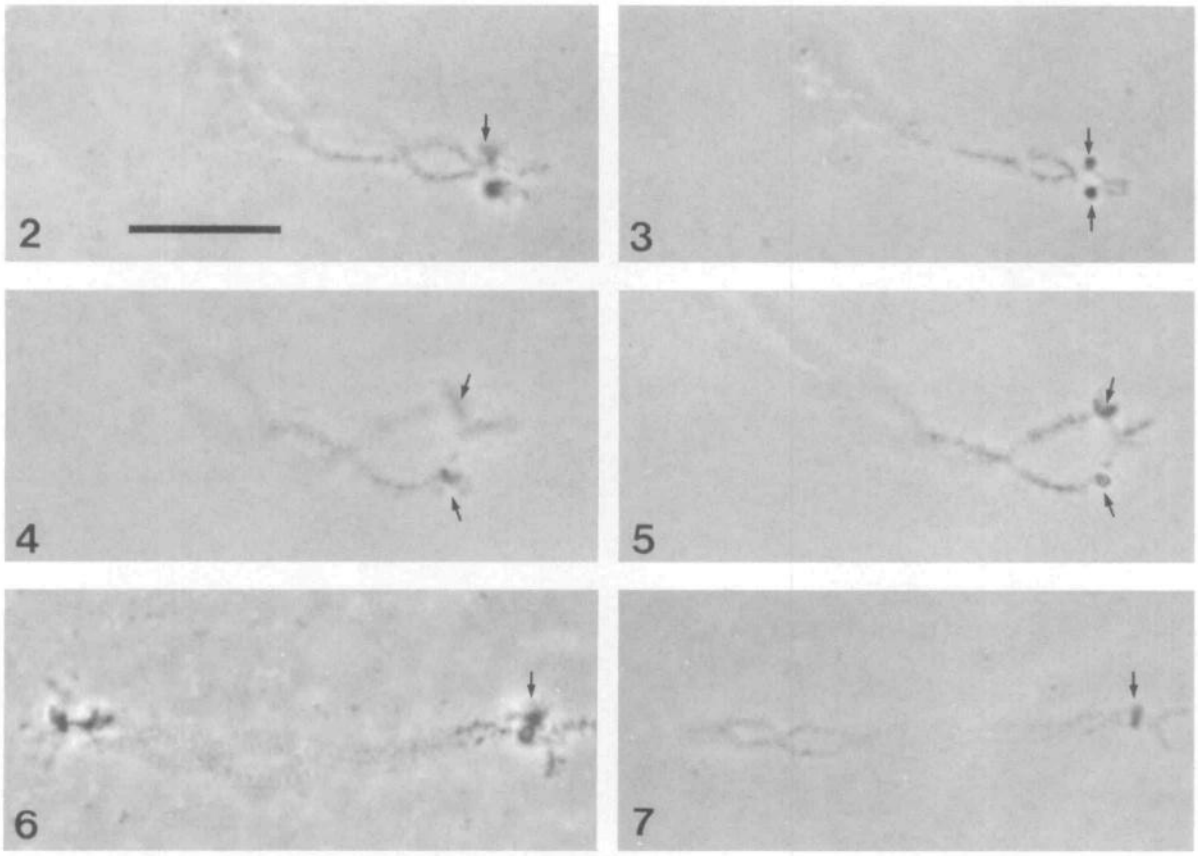


Fig. 1. Phase-contrast micrograph (A) and accompanying diagrams (B and C) of lampbrush bivalent 2 from *Gallus domesticus*. The diagrams are redrawn from Chelysheva et al. (1990). (C) Centromeric region of the chromosome. TBL 21, the "telomeric bow loops" at the ends of the long arms. LL21, the "lumpy loops" near the ends of the long arms. LL22, the marker structure, referred to by Chelysheva et al. as "lumpy loops", that is the focus of the present study. The terminology applied to these structures by Chelysheva et al. follows that introduced by Callan and Lloyd (1960). Note that LL22 on both half-bivalents (arrows on Fig. 1A) appears as a darker blob surrounded by a halo of lighter material with indistinct boundaries. Bar in A, 20 μm.



Figs 2-7. Phase-contrast micrographs of freshly isolated and unfixed 2nd lampbrush bivalents before or after treatment with DNase, RNase or trypsin. Bar (20 μm) on Fig. 2 applies to all members of this series.
Fig. 2. Before treatment with DNase I. Arrow indicates the paired LL22 markers.
Fig. 3. The same bivalent as in Fig. 2, 2 h 15 min after addition of DNase I at a concentration of 0.1 mg/ml. The bivalent has been broken in several places by the enzyme. The LL22 markers (arrows) are reduced in size but are more compact and contrasty than in Fig. 2.
Fig. 4. Before treatment with RNase A. The LL22 markers in this preparation (arrows) were quite widely diffuse and of correspondingly low phase-contrast.
Fig. 5. The same preparation as in Fig. 4, 14 min after addition of RNase A (pre-boiled to eliminate DNase contamination). As with DNase I, the LL22 markers (arrows) are smaller but more compact after RNase treatment. There was no further change in the appearance of the markers over a period of several hours.
Fig. 6. Before treatment with trypsin. Arrow indicates LL22 markers. The LL21 and TBL markers at the other end of bivalent 2 are also in focus in this micrograph.
Fig. 7. The same preparation as in Fig. 6, 35 min after addition of trypsin. All lateral loop material has disappeared. The markers at LL21 and TBL have disappeared. LL22 markers are greatly reduced in size *and* contrast.

LL21 (see Fig. 1), at the end of chromosome 2 opposite to that bearing LL22. The actions of Pronase and trypsin are characterised by a rapid disappearance of all loops and landmark structures, leaving bare chromosome axes of low phase refractility. DNase I, *Hae*III, *Alu*I, *Eco*RI and RNase A all had the expected effects on loops and chromosomes and all had the same effect on the LL22 marker. In no case was the marker completely destroyed. After 2 h in DNase I several axial breaks were apparent in bivalent 2 and the LL22 marker had changed from a relatively large object with a loose fluffy appearance to a small, compact and contrasty granule (Figs 2 and 3). The same effect was produced by RNase after about 20 min, except that all loops, including the large ones at TBL21, disappeared, and there were no breaks in the chromosomes' axes (Figs 4 and 5). Trypsin and Pronase destroyed all loops, including the markers at LL21 and TBL21, and reduced the chromosome axis to a faint and slightly swollen, but unbroken, strand. LL22 remained distinct even after 50 min in trypsin, when all other markers had long since disappeared, although it was much reduced in both size *and* contrast (Figs 6 and 7).

Immunofluorescence

No evidence was found for binding of either chick anti-lamins (anti-A and anti-B2) or anti-vimentin to any structure on chicken lampbrush chromosomes, including the LL22 marker. All three antibodies that were used were shown by immunofluorescence to bind specifically to nuclear envelope (anti-lamins) or cytoplasm (anti-vimentin) of chicken erythrocytes under exactly the same conditions as used in the preparation

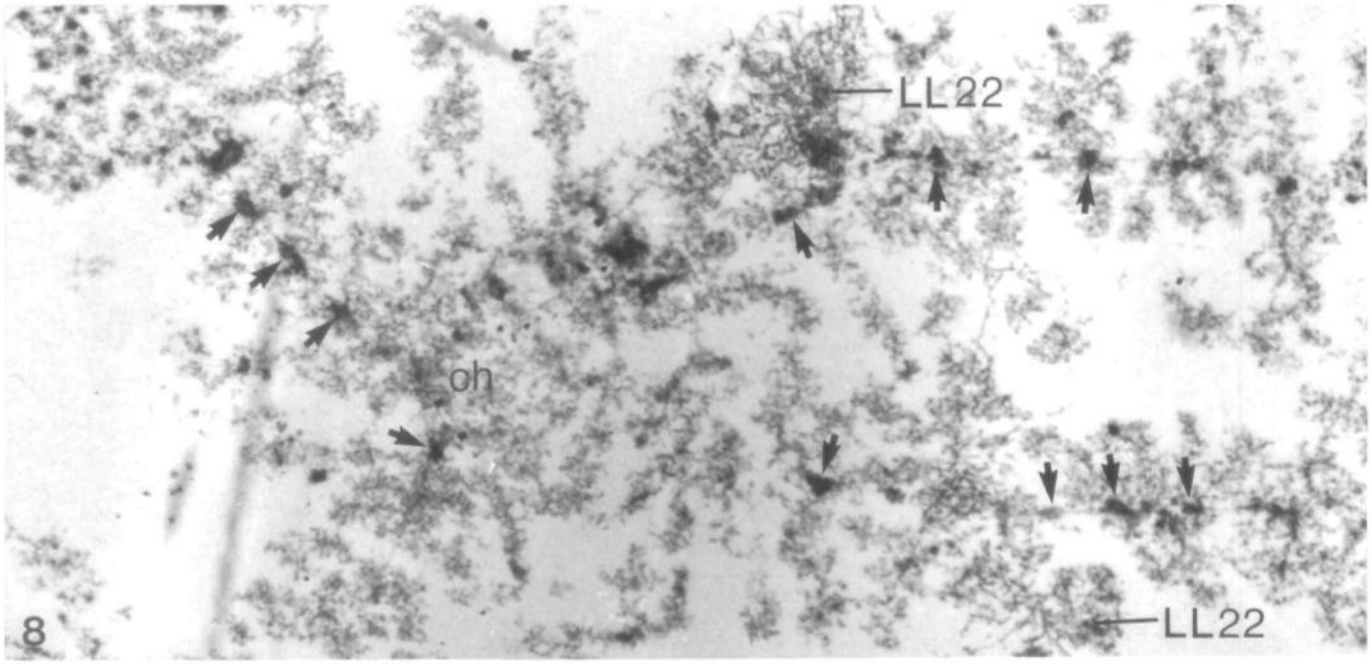


Fig. 8. Transmission electron micrograph (TEM) of a longitudinal section through the region of lampbrush bivalent 2 that includes LL22. The small unlabelled arrows indicate axial chromomeres. ch, position of a chiasma. The material of LL22 lies on either side of the chromosome axis and has a finely fibrous appearance that is quite different from the loop RNP of the remainder of the chromosome. ×7000.

of lampbrush chromosomes. Anti-lamins also bound specifically to pieces of oocyte nuclear envelope that lay alongside the chromosomes in lampbrush preparations. Control preparations, in which only the second (FITC-labelled) antibody was used, were entirely negative.

Transmission electron microscopy of thin sections

Electron micrographs have been made from sections in two ranges of thickness. The thicker ones, 150-250 nm, sacrifice some of the details of fine structure but give an overview of the relative positions of objects along the length of a lampbrush chromosome. Thin sections, about 100 nm, allow interpretation of fine structure. Altogether 22 chromosomes 2 were embedded and sectioned for transmission electron microscopy.

The marker at LL22 is relatively easy to locate in longitudinal sections of end-embedded chromosomes on account of the distinctive appearance of its constituent strands. Sections through the region of the marker show it as a loose mass of interwoven fibres lying around or to one side of the main chromosome axis (Fig. 8). The fibres are 15-16 nm wide. They appear straight or curved and of uniform electron contrast across their width in longitudinal and in transverse view (Figs 9 and 10). The entire mass of fibres as seen in a section through the widest part of LL22 occupies a region 4-5 μm in diameter and it usually lies around or very near to one of the chromomeres of the chromosome axis (Figs 8 and 9). The centre portion of the mass of fibres is much more compact than the outer regions (Fig. 9). Where a section passes through the chromomere it is evident that LL22 fibres are closely associated with or penetrate right into the substance of the chromomere (Fig. 10). All of these dimensions and characteristics were seen in the great majority of preparations that we used for thin-sectioning and transmission electron microscopy.

In an earlier series of sections from which we obtained electron micrographs, the general organisation of the LL22 marker was exactly as described above, apart from the fact that the individual fibres were about twice as thick (32 nm), they were much rougher in appearance, sometimes showing evidence of twisting (Fig. 11), and in transverse section the individual fibres showed a less dense central core 3-5 nm wide (Fig. 12). We suspect that in this first set of preparations some aspect of the fixation or dehydration produced either a shortening and thickening of individual fibres or a coiling of individual fibres to make them appear shorter and thicker. Whichever state is artifactual, we consider it essential that both go on record, particularly since two sets of material prepared for scanning electron microscopy have also given LL22 fibres with correspondingly different properties.

Scanning electron microscopy

The first set of scanning electron micrographs (SEMs) that we obtained were from chromosomes that had been lightly centrifuged to flatten them and stick them to a glass coverslip, fixed in glutaraldehyde/osmium, critical-point dried and coated with chromium to produce an overall increase in thickness of about 2 nm. In these preparations the critical-point drying was good and individual chromosomes and their marker structures could be identified with confidence. To judge from our experience with SEMs of amphibian lampbrush

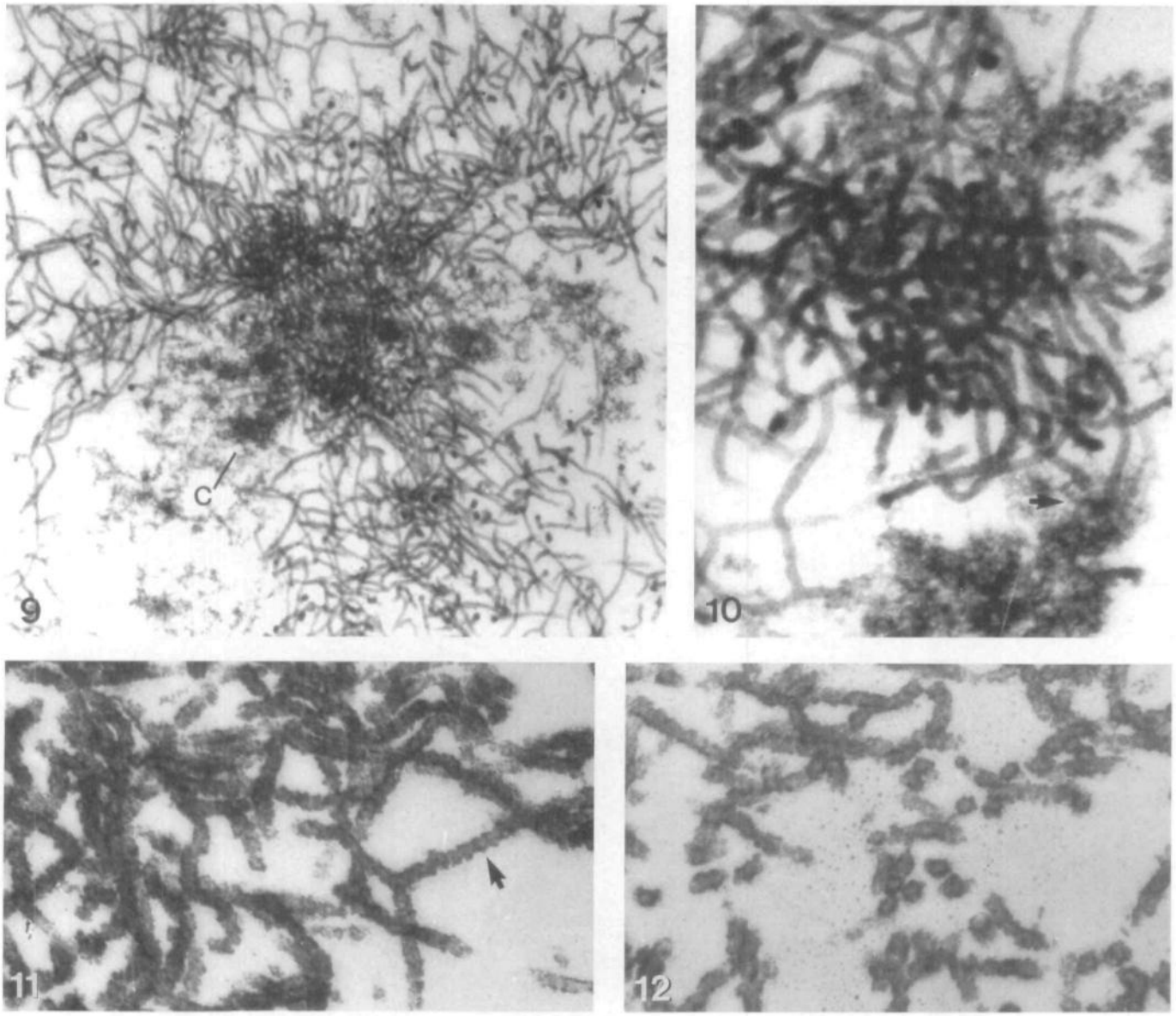


Fig. 9. TEM of a section through the greater part of an LL22 marker showing the relatively compact central portion with axial chromomeric material (c) close by. ×30,000.
Fig. 10. TEM of a section through part of the same LL22 marker region as shown in Fig. 2. The arrow indicates a close association between some of the LL22 fibres and a chromomere (arrow). This electron micrograph, as well as those shown in Figs 2 and 3, is from the second batch of material used for TEM studies in which the average fibre diameter was in the region of 15-16 nm, the fibres appearing smooth in outline and solid in cross-section. ×80,000.
Fig. 11. TEM of a section through part of an LL22 marker in the first batch of material used for TEM studies. Here the individual fibres are thicker, 32 nm, rougher in outline and sometimes show signs of a helical substructure (arrow). ×80,000.
Fig. 12. Same as Fig. 5 but showing the ring-like appearance of these larger (32 nm) fibres in cross-section. ×80,000.

chromosomes (HM and TA), the chromosomal morphology and fine structure were well preserved. Our studies were largely confined to chromosome 2 and more specifically to the LL22 marker that had shown a distinctive fibrous structure in TEM sections. An example of a typical chromosome 2 is shown in Fig. 13. The giant loops at the end of the long arm of chromosome 2 have retained their form and the LL22 marker is conspicuous and loosely fibrous in appearance (Figs 13 and 14). At higher SEM magnifications (×18,000 upwards) LL22 varies from a rough and compact texture in one region to a more open loose network of fibres in surrounding parts (Figs 14 and 15). There are few obvious free ends to the LL22 fibres. There is some evidence of branching of the fibres, but it is usually difficult to determine whether a fibre truly branches into two, or two closely apposed fibres simply separate into two single ones. The fibres are all between 32 and 38 nm wide and in many places they show some evidence of a tightly twisted sub-structure (Figs 15 and

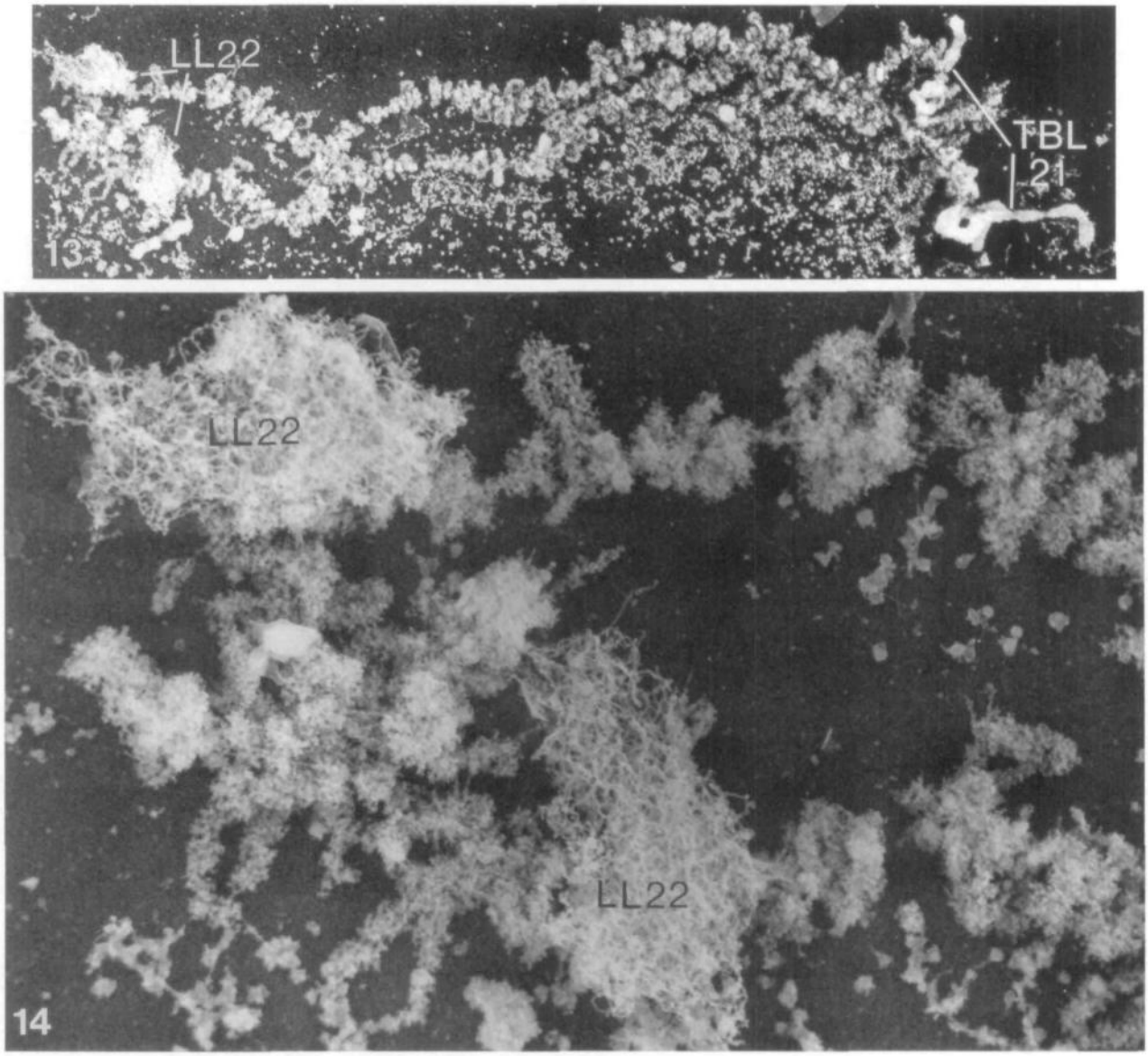


Fig. 13. Low-magnification scanning electron micrograph (SEM) of the whole of lampbrush bivalent 2 from the first set of material prepared for high-resolution SEM studies. The lateral loops and other structures associated with the chromosomes are well preserved. The LL22 markers are at the left end of the bivalent and the TBL21 (telomeric bow loops) show at the right-hand ends of the bivalent. ×6,250.

Fig. 14. Higher-magnification view of the LL22 regions on the bivalent shown in Fig. 13. The difference in appearances of the normal loop RNP and the spaghetti-like strands of the LL22 marker is particularly apparent in this picture. ×6,250.

16). Where the fibres are compacted together they frequently have a coarse knobbly appearance that could be indicative of some supercoiling (Fig. 16). At high magnification (>×100,000) these chromium-coated fibres have a smooth surface structure (Fig. 16).

The second set of SEMs that we obtained were from chromosomes that were isolated, fixed and dried in almost the same way except for the omission of osmium post-fixation. They were coated with gold/palladium to produce an overall increase in thickness of about 5 nm. In these preparations the lateral loops of the chromosomes had an appearance that suggested some loss of material during isolation and less than optimal fixation. Nevertheless, critical-point drying was good and individual chromosomes and their marker structures could be identified with confidence. In the first two preparations that we examined in this series the material at locus LL22 on both half-bivalents was in two forms. One of these was a smooth, irregularly shaped body about 1-2 μm across lying on the proximal side of the locus and over or alongside the main chromosome axis. The other material was a complex network of fibres extending over an area of several square micrometres (Fig. 17). The same general organisation was seen in several other preparations, although the smooth solid component was lacking and LL22 consisted entirely of a meshwork of fibres. Individual fibres in LL22 markers from this second series of preparations were between 15

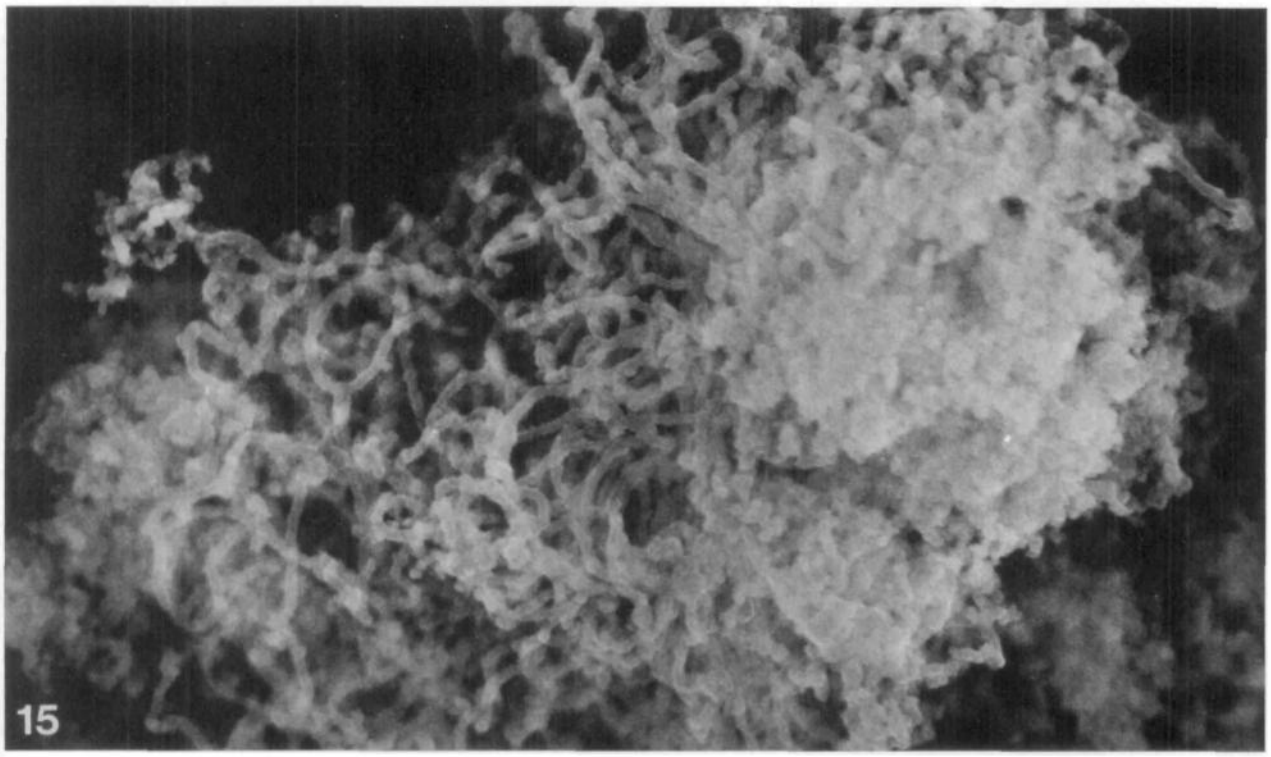


Fig. 15. Detail at ×33,500 magnification of an LL22 marker from the first series of preparations. In part of the marker, the strands are closely packed to give an almost solid structure, such as might explain the dense region seen in phase-contrast micrographs.

and 16 nm thick. This was best seen in preparations where the LL22 marker had been dispersed, probably mechanically, during preparation (Figs 18 and 19). Fibres often appear to be fused with one another, two or more together, to produce strands of up to 50 nm wide. In the region of the smooth, solid component the fibres show extensive fusion to produce strands up to 100 nm thick and they fuse with or adhere to the surface of the smooth body (Fig. 17). As in the first series of preparations, there are few, if any, visible free ends. High magnification (×100,000-×200,000) micrographs show the fibres with a coarse, granular surface structure that we consider to be resolution of the grain of the gold/palladium coating (Fig. 19). In both sets of preparations the fibrous component of LL22 has an appearance that is quite different from that of nearby lateral loop RNP.

Transmission electron microscopy of negative-stained chromosomes

The appearance of LL22 in negative-stained whole mounts was exactly as expected on the basis of our SEM studies. Three aspects of the fibre structure were particularly clear in negative-stained preparations (Fig. 20). The fibres are smooth in outline and structurally homogeneous. The fibre diameter is between 32 and 37 nm. Branching of the fibres is common but, where it occurs, the widths of the parent strand and the daughter branches are identical, which would not be expected if branching were a consequence of separation of two closely apposed strands.

Discussion

The phenomenon that we have described in this paper represents something entirely new, in the sense that nothing remotely comparable has ever been encountered in any previous study of lampbrush chromosomes. It has been demonstrated that in newt lampbrush chromosomes all loops, including the larger "landmark" structures, no matter what their appearance in the light microscope, are made up of the same basic 30 nm RNP particles (N'Da et al., 1986; Bonnanfant-Jais et al., 1991) and all loops exhibit the same basic organisation with nascent RNP transcripts attached by RNA polymerase molecules to the DNA loop axis from which they were transcribed (Scheer et al., 1976). The two notable exceptions to this rule are the "spheres", such as were characterised by Callan and Lloyd (1960) on the 5th and 8th chromosomes of crested newts and have since been shown to be present in germinal vesicles of a wide variety of other vertebrates (Gall and Callan, 1989) and the "protein bodies" described by Gaginskaya (1972b) and Khutinaeva et al. (1989) in the oocyte nuclei of certain species of bird. The LL22 marker is not made up of 30 nm RNP particles, and does not resemble a lampbrush loop. It seems highly unlikely that it is related to the "spheres" of amphibian

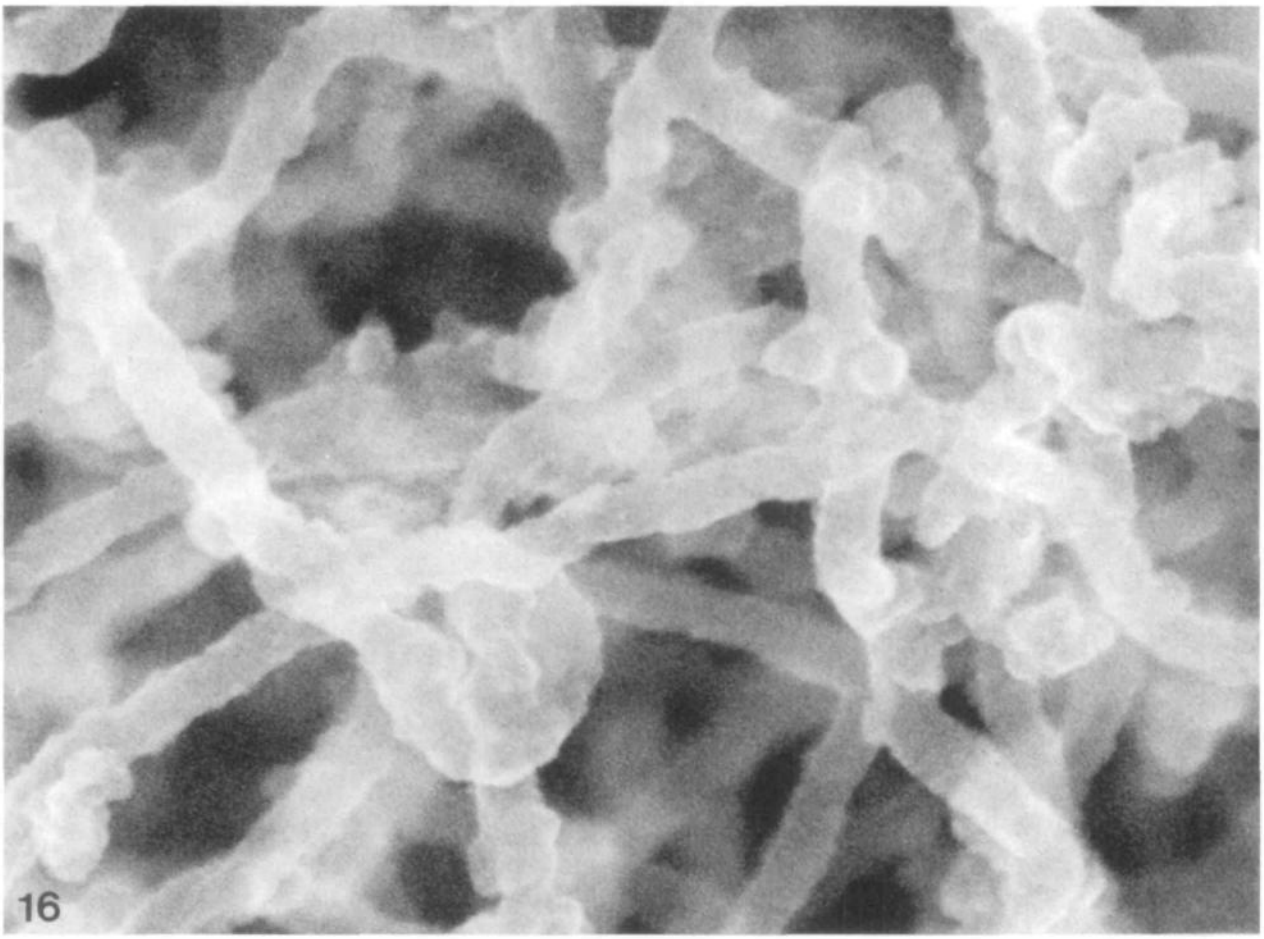


Fig. 16. High-resolution SEM photographed at an instrumental magnification of ×100,000 of part of an LL22 marker from the first series of preparations. Compare with Fig. 19. Here the fibres have received a high-resolution coating of chromium 1-2 nm in thickness. The average fibre width, allowing a total of 3 nm for coating thickness, is in the region of 35 nm. They have a smooth surface structure but an overall knobbly appearance, perhaps suggesting some degree of supercoiling. ×192,000.

oocytes and indeed it does not stain by indirect immunofluorescence with antibodies that are known to react with sphere proteins in *Triturus, Notophthalmus* and *Xenopus* (H. Macgregor, unpublished observations).

It has to be admitted immediately that even after quite a wide-ranging structural study and a range of experimental approaches we still do not know what LL22 is made of. Our objective at this stage must therefore be simply to publish the findings, integrate them into some reasonable hypotheses and then invite other investigators to help in discovering the nature and significance of this curious structure.

There are three main possibilities. First, the material at LL22 could represent a novel form of RNP with a linear superstructure rather than a particulate one; secondly, LL22 could be a novel form of chromatin; and thirdly, LL22 could represent an accumulation of large amounts of a specific protein or structural macromolecule at a single chromosomal locus. Whichever of these possibilities is nearest to the truth, one observation would seem to be of paramount importance. LL22 is a constant marker structure associated with a specific chromosomal locus, and it is present at the same position on both half-bivalents. Moreover, none of the many chickens that we have examined has been heterozygous for the presence of LL22. It is therefore difficult to avoid the conclusion that LL22 reflects some property of the chromosomal DNA at that locus.

Undoubtedly the simplest and most conservative hypothesis would be one involving a novel form of transcription and packaging of RNP. In this regard the following points are significant. Few free ends are evident. They might, of course, be obscured by fusion or just general tangling of the material, but they are probably not common. Careful modelling of the structures shown in Figs 14 and 17, with close attention to scale, has shown that the total strand length would be of the order of 500 to 2000 μm. Allowing a factor of 5:1 for normal foreshortening of RNP transcripts on lampbrush loops (Hill and Macgregor, 1980), these lengths rise to 2.5 to 10 mm. If we allow another factor of 5:1 for tight coiling of the 15 nm fibre such as can be shown, again by scale modelling, to produce the 35 nm fibres shown in Fig. 16, then that particular marker might be estimated to contain up to 50 mm of primary

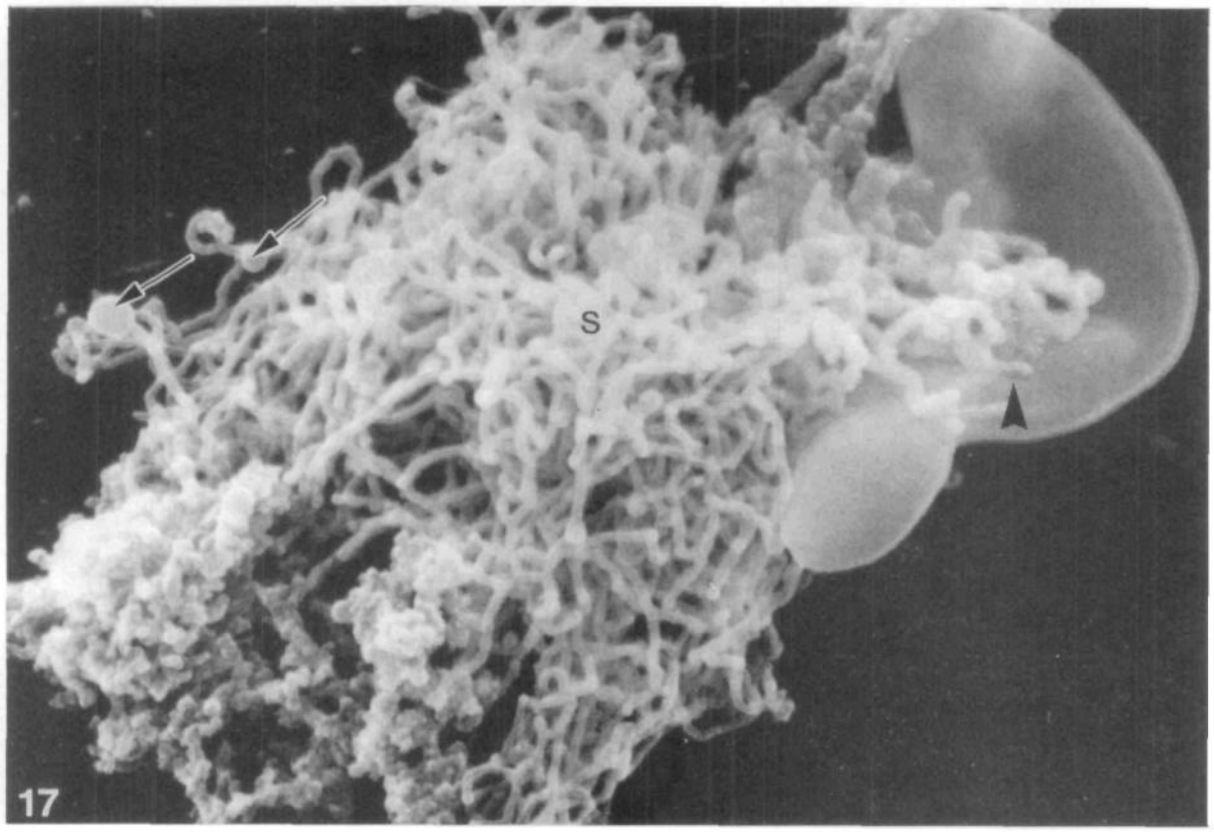


Fig. 17. SEM view of an LL22 marker from the second set of preparations made for scanning electron microscopy. The marker appears as a tangled mass of relatively smooth spaghetti-like fibres (s) lying alongside a smooth-surfaced solid mass of material. Note how the fibres become thicker in the region of the smooth solid structure and merge with its surface where they touch (arrowhead). Small spherical bodies with the same general appearance as the large solid structure lie amongst the spaghetti fibres (arrows). ×34,000.

RNA transcript. That would seem to be an inordinately large amount of RNA to accumulate at one lampbrush locus, but it is actually comparable to the amount that is associated with the axes of some of the large marker loops that regularly form on the lampbrush chromosomes of urodele amphibians. Could it be that LL22 represents transcription of a highly repeated DNA sequence at a level that, for purely physical reasons within the environment of the small chicken germinal vesicle, requires a system of packaging and processing that is different from that which operates on the normal lampbrush transcription unit? None of the evidence that is available conflicts with such a hypothesis. The marker is characteristic of the lampbrush phase. Like most conspicuous marker structures, it persists after the normal loops have retracted and then disappears right at the end of the lampbrush phase. The structure is reduced in size by both ribonuclease and proteases. The substance of LL22 has been reported to bind total DNA from chicken under conditions that would favour hybridisation of DNA to nascent loop RNP (Hutchison, 1987). However, recent experiments carried out in the Leicester laboratory and in Dr. Hutchison's laboratory in the USA lead us to the conclusion that the binding of labelled DNA to LL22 does not represent the formation of a hybrid nucleic acid complex. The details of these experiments will be reported elsewhere in the context of a study of the arrangement of DNA sequences on lampbrush chromosomes of chickens.

In a second hypothesis, the material at LL22 would consist of chromosomal DNA and associated proteins, essentially a modified form of chromatin. The evidence in support of this idea is minimal. The LL22 fibres seem to be closely associated with or perhaps continuous with the conventional chromatin of the chromosome axis. The general histochemistry of LL22 is not wholly inconsistent with loosely organised DNP, and the structure becomes smaller and more contrasty when exposed to DNase. Nevertheless, on all accounts we consider the modified chromatin hypothesis to be unlikely and we will not consider it further.

A third hypothesis would present LL22 as the result of a specific interaction between a chromomeric DNA sequence and a specific macromolecule that was abundant in the germinal vesicle. That hypothesis starts with the premise that LL22 does not consist of nucleoprotein and has nothing to do with transcription, and it takes special account of the fact that the strands of LL22 seem to cluster around what appears to be an axial chromomere and in some cases penetrate right into the substance of the chromomere. The chromomere itself is relatively large. Identification of the main

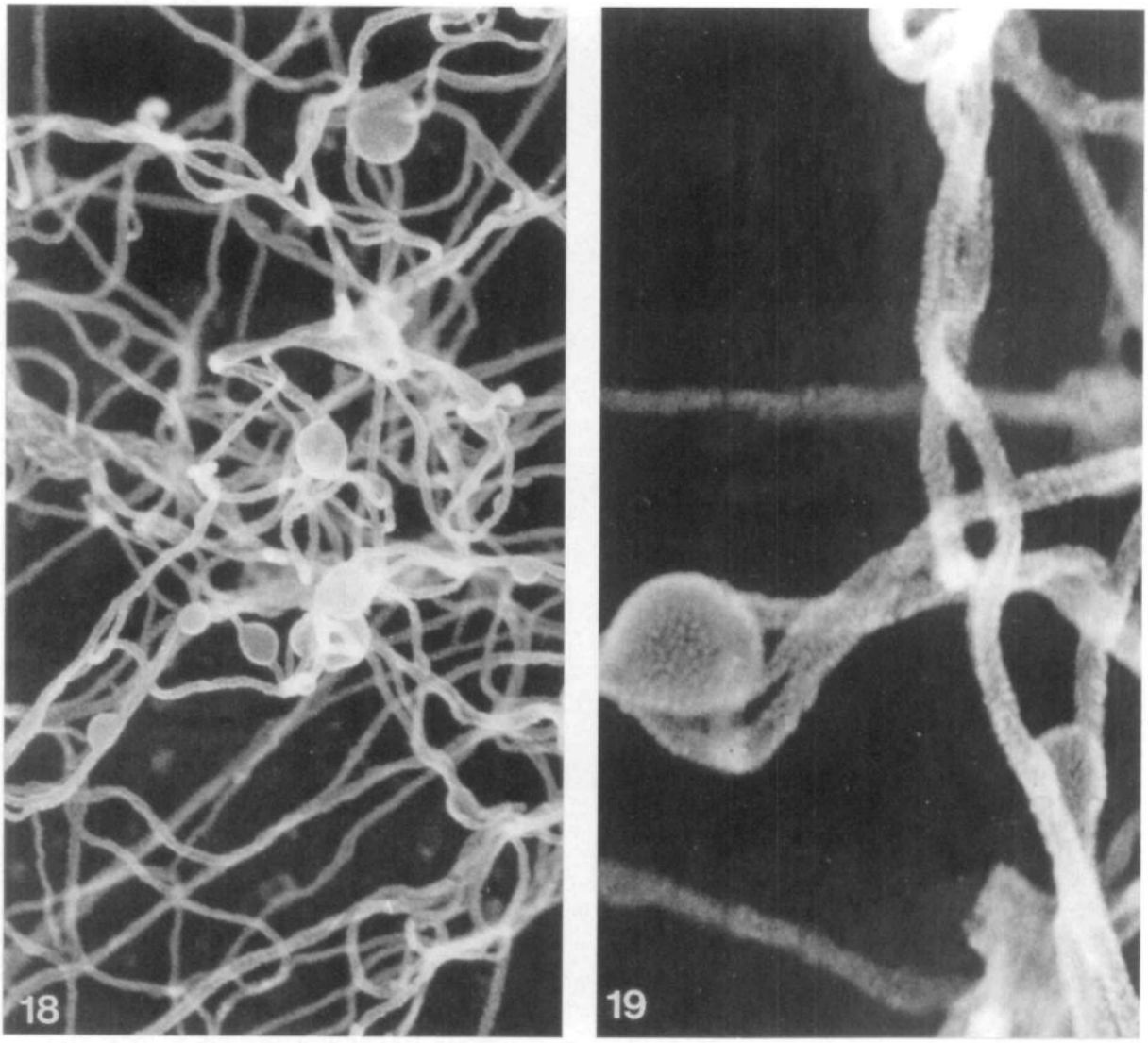


Fig. 18. SEM detail (×58,500) of LL22 fibres in a preparation where they were more dispersed, probably as a consequence of mechanical disturbance during manual isolation of the chromosomes. Fusion of fibres produces strands of up to 100 nm in thickness. Round objects with the same general surface properties and appearance as the material of the fibres give the impression of swellings along the lengths of some fibres.

Fig. 19. High-resolution SEM, photographed at ×100,000 instrumental magnification, in which the gold/palladium coating on the surface of the fibres has been resolved. The average single fibre width in micrographs such as this was estimated to be in the region of 15-16 nm. ×195,000. Compare with Fig. 16.

molecular components of LL22 strands is, of course, of paramount importance. So far this has proved difficult. Nucleases have effects that could be interpreted as indicating the presence of either DNA or RNA, and proteases fail to destroy the structure completely, although they do remove a substantial part of its substance. Immunofluorescence experiments with anti-lamins, anti-vimentin and anti-histone (H. Macgregor, unpublished) antibodies have all proved inconclusive or negative, although only the anti-lamin immunofluorescence tests employed antibodies to proteins from chickens.

In the search for clues to the molecular nature of LL22 several quite simple observations have to be kept in mind. The fibres are homogeneous and smooth, they are capable of branching, and two different forms of them (15 nm and 35 nm) have both been identified independently in two separate laboratories and employing different techniques, thin sectioning and SEM. Structurally, the nearest resemblance is between LL22 strands and the 'membranous tubules' identified in association with nucleoli and the nuclear envelope in germinal vesicles of amphibians (Leon et al., 1991). The latter have a diameter of about 30 nm (according to our measurements of Leon et al.'s micrographs). Leon et al. speculate that these "membranous tubules" may represent a store of prefabricated nuclear lamina components assembled in the germinal vesicle for later use in oogenesis. Our own experiments with monoclonal antibodies to chicken lamins would seem to rule out the lamin hypothesis, but we nevertheless consider that the structural resemblance between LL22 fibres and Leon

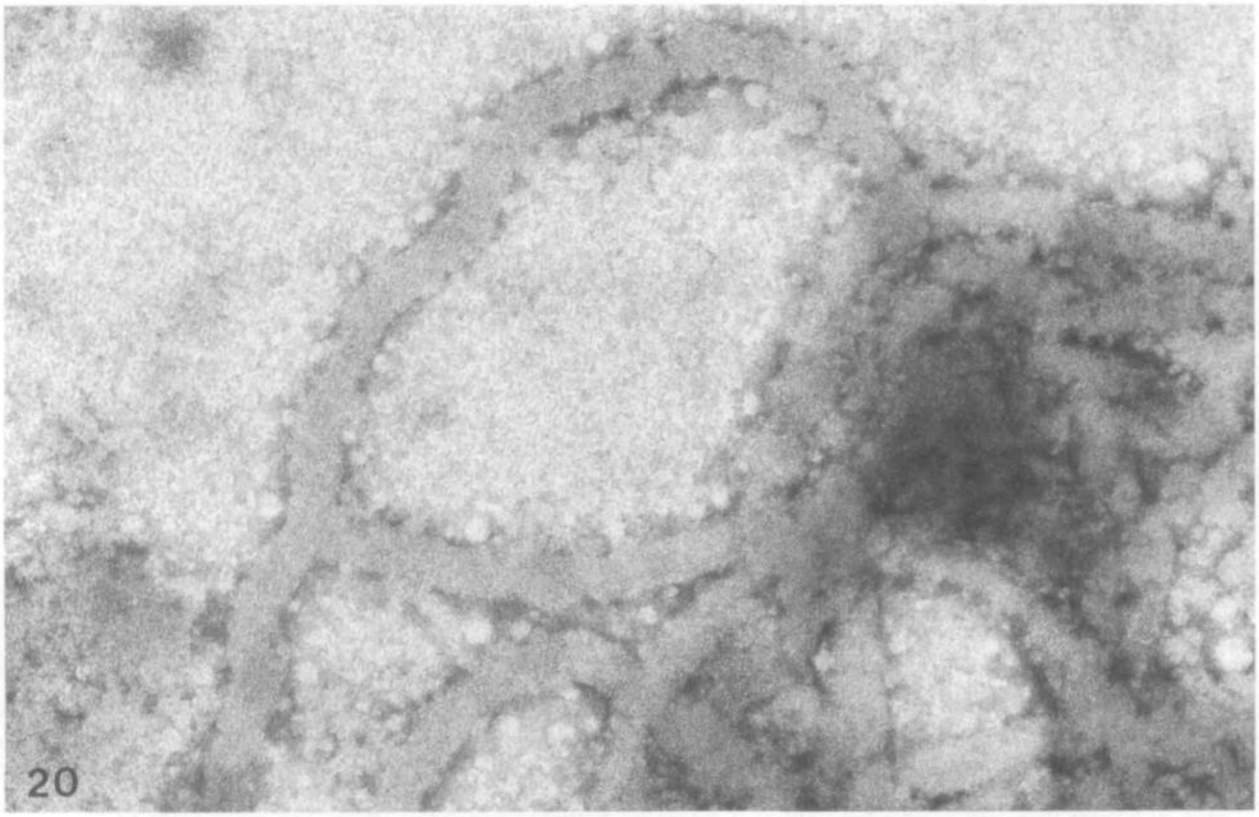


Fig. 20. Transmission electron micrograph of isolated LL22 fibres stained with uranyl acetate for negative contrast. Fibre thickness here is estimated to be in the region of 35 nm. Note the apparent branching of fibres on the left of the picture, with all three arms of the branch being of the same thickness. ×210,000.

et al.'s "membranous tubules" and the fact that both occur in the same cell type and at corresponding stages are likely to be significant.

A search has been made for LL22-like material associated with the lampbrush chromosomes of other bird species, Japanese quail (*Coturnix coturnix*), wood pigeon (*Columba palumbus*) and chaffinch (*Fringilla coelebs*). The first of these species belongs to the same family, the Phasianidae, as the chicken. Nothing resembling the LL22 marker could be found in Japanese quail. Likewise, nothing could be found in wood pigeon, although one of the longer chromosomes of this species does carry an extremely large and loosely organised lampbrush marker such as might represent a counterpart for LL22 in chicken. The marker to which we refer is not related in any way to the many conspicuous "protein bodies" described by Gaginskaya (1972b) and Khutinaeva et al. (1989) in pigeon (*Columba livia*) germinal vesicles. It should be added that the wood pigeon marker has a fine structure, as seen in scanning electron micrographs, that is quite different from that of LL22 and from anything previously seen on lampbrush chromosomes of birds or amphibians (Allen and Macgregor, unpublished observations). The Russian authors of this paper report that, on the basis of transmission EM studies of thin sections of oocyte nuclei, there is nothing comparable with the LL22 marker on chaffinch lampbrush chromosomes, although an exhaustive search by high-resolution scanning electron microscopy has not been made.

Whatever we may eventually discover about the LL22 marker one thing is certain: it is an entirely novel structure the like of which has never been seen before in association with a lampbrush chromosome. It is a structure that can only be seen in thin sections examined with a transmission electron microscope or in whole mounts examined with a *high-resolution* scanning electron microscope. We think it may offer opportunities for some interesting new insights into the role of the chromosomes and the germinal vesicle in oogenesis.

Throughout this paper we have referred to the object of study as LL22. The name was introduced by Chelysheva et al. (1990) on account of the superficial resemblance of the object to the "lumpy loops" described by Callan and Lloyd (1960) on the lampbrush chromosomes of crested newts. We now know that LL22 is not a lampbrush loop but has a peculiar identity of its own that has yet to be explained. Since the very start of our SEM study we have referred to Chelysheva et al.'s (1990) LL22 as the "spaghetti marker", and we think it may be useful to retain this morphologically descriptive name, just as Callan and Lloyd (1960) did with their "lumpy loops" "spheres" and "currant buns" on the lampbrush chromosomes of *Triturus*, until it is possible to define the molecular and functional nature of this bizarre structure.

We thank The British Council and The Wellcome Trust for their help and for funding visits to the UK by the two Russian authors of this paper, so making possible the collaborative effort that was needed to accomplish this research. We thank Lesley Barnett and Evie Roberts for their valuable technical assistance.

References

Ahmad, M. S. (1970). Development, structure and composition of lampbrush chromosomes in domestic fowl. *Can. J. Genet. Cytol.* **12**, 728-737.

Bonnanfant-Jais, M. L., Penrad-Mobayed, M. and Angelier, N. (1991). Ultrastructural similarity in landmark loops of amphibian lampbrush chromosomes. *Biol. Cell* **71**, 105-114.

Callan, H. G. (1986). *Lampbrush Chromosomes*. Springer Verlag, Berlin, Heidelberg, New York, Tokyo.

Callan, H. G. and Lloyd, L. (1960). Lampbrush chromosomes of crested newts *Triturus cristatus* (Laurenti). *Phil. Trans. Roy. Soc. Lond.* B, **243**, 135-219.

Chelysheva, L. A., Solovei, I. V., Rodionov, A. V., Yakovlev, A. F. and Gaginskaya, E. R. (1990). The lampbrush chromosomes of the chicken: the cytological maps of macrobivalents. *Cytology (Russ.)* **32**, 303-316.

Diaz, M. O., Barsacchi-Pilone, G., Mahon, K. A. and Gall, J. G. (1981). Transcripts from both strands of a satellite DNA occur on lampbrush chromosome loops of the newt *Notophthalmus*. *Cell* **24**, 649-659.

Gaginskaya, E. R. (1972a). The nuclear structures of oocytes in adult birds: chromosome behaviour during the oocyte growth. *Cytology (Russ.)* **14**, 426-432.

Gaginskaya, E. R. (1972b). The nuclear structures of oocytes in adult birds: protein bodies and karyosphere. *Cytology (Russ.)* **14**, 568-578.

Gaginskaya, E. R., Kropotova, E. V., Tsvetkov, A. G. and Vengerov, Yu. Yu. (1984). Morphology of transcription in lampbrush chromosomes from avian oocytes. *Cytology (Russ.)* **26**, 1064-1065.

Gall, J. G. (1954). Lampbrush chromosomes from oocyte nuclei of the newt. *J. Morphol.* **94**, 283-352.

Gall, J. G. and Callan, H. G. (1989). The sphere organelle contains small nuclear ribonucleoproteins. *Proc. Natl Acad. Sci. U.S.A.* **86**, 6635-6639.

Gall, J. G., Diaz, M. O., Stephenson, E. C. and Mahon, J. A. (1983). The transcription unit of lampbrush chromosomes. In *Gene Structure and Regulation in Development* (41st Symposium of the Society for Developmental Biology), pp. 137-146. Alan R. Liss, Inc., New York.

Gall, J. G., Stephenson, E. C., Erba, H. P., Diaz, M. O. and Barsacchi-Pilone, G. (1981). Histone genes are located at the sphere loci of newt lampbrush chromosomes. *Chromosoma* **84**, 159-171.

Hill, R. S. and Macgregor, H. C. (1980). The development of lampbrush chromosome-type transcription in the early diplotene oocytes of *Xenopus laevis*: an electron microscope analysis. *J. Cell Sci.* **44**, 87-101.

Hutchison, N. J. (1987). Lampbrush chromosomes of the chicken, *Gallus domesticus*. *J. Cell Biol.* **105**, 1493-1500.

Hutchison, N. J. and Weintraub, H. (1983). Lampbrush chromosome structure and expression in chicken oocytes. *J. Cell Biol.* **97**, 388a.

Khutinaeva, M. A., Kropotova, E. V. and Gaginskaya, E. R. (1989). Morphofunctional organisation peculiarities of the lampbrush chromosomes of oocytes in the pigeon, *Columbia livia*. *Cytology (Russ.)* **31**, 1185-1193.

Koecke, H. U. and Muller, M. (1965). Formweschsel und Anzahl der Chromosomen bei Huhn und Ente. *Naturwissenschaften* **52**, 483.

Kropotova, E. V. and Gaginskaya, E. R. (1984). Lampbrush chromosomes from the Japanese quail oocytes: light and electron microscopic study. *Cytology (Russ.)* **26**, 1008-1015.

Leon, P., Kezer, J. and Schabtach, E. (1991). Further observations on nucleolar tails in amphibian oocytes. *J. Cell Sci.* **99**, 515-522.

Macgregor, H. C. (1987). Lampbrush chromosomes. *J. Cell Sci.* **88**, 7-9.

Macgregor, H. C. and Andrews, C. (1977). The arrangement and transcription of "middle repetitive" DNA sequences on lampbrush chromosomes of *Triturus*. *Chromosoma* **63**, 109-126.

Macgregor, H. C. and Varley, J. M. (1988). *Working with Animal Chromosomes* (second edition). John Wiley and Sons, Chichester and New York.

Mott, M. R. and Callan, H. G. (1975). An electron microscope study of the lampbrush chromosomes of the newt *Triturus cristatus*. *J. Cell Sci.* **17**, 241-261.

N'Da, E., Bonnanfant-Jais, M. L., Penrad-Mobayed, M. and Angelier, N. (1986). Size uniformity of ribonucleoprotein matrix particles in loops of *Pleurodeles waltlii* lampbrush chromosomes visualized by electron microscopy. *J. Cell Sci.* **81**, 17-27.

Scheer, U., Franke, M. F., Trendelenburg, M. F. and Spring, H. (1976). Classification of loops of lampbrush chromosomes according to the arrangement of transcriptional complexes. *J. Cell Sci.* **22**, 503-519.

Solovei, I. V., Khutinaeva, M. A., Chelysheva, L. A., Rodionov, A. V. and Gaginskaya, E. R. (1990). Lampbrush sex bivalent of birds: identification and structure. *Cytology (Russ.)* **32**, 817-825.

Varley, J. M., Macgregor, H. C., Nardi, I., Andrews, C. and Erba, H. P. (1980). Cytological evidence of transcription of highly repeated DNA sequences during the lampbrush stage in *Triturus cristatus carnifex*. *Chromosoma* **80**, 289-307.

(Received 23 August 1991 - Accepted, in revised form, 9 January 1992)