The role of lampbrush chromosomes in the formation of nucleoli in amphibian oocytes

By H. C. Macgregor

(From the Department of Natural History, The University, St. Andrews, Fife)

With 3 plates

Summary

Theories concerning the mode of origin of peripheral nucleoli in amphibian oocytes have been examined and tested.

In Triturus cristatus the giant fusing loops of the 3 shortest lampbrush bivalents resemble nucleoli when viewed in phase contrast and may be considered as possible sites of production of nucleoli. Giant fusing loops, however, differ from peripheral nucleoli in certain important respects, and animals lacking giant fusing loops on their lampbrush chromosomes nevertheless have normal peripheral nucleoli. Therefore, similarity in appearance between objects attached to lampbrush chromosomes and free peripheral nucleoli may not be significant.

In oocytes of T. c. carnifex, T. c. karelinii, and T. c. damubalis, peripheral nucleoli do not increase in number during the lampbrush phase of oogenesis, except by division of pre-existing nucleoli towards the end of oogenesis. There are about 1,000 nucleoli per oocyte nucleus in each of these sub-species.

In T. c. cristatus there are more nucleoli in large oocytes than in small ones, and it seems likely that in this sub-species the giant fusing loops add to the existing population of nucleoli in an oocyte by successively growing and shedding new nucleoli. A similar situation probably holds in Plethodon cinereus.

Hexaploid oocytes from triploid females of Ambystoma jeffersonianum have 3 times as many nucleoli as diploid oocytes from diploid females of the same species. The number of nucleoli in an amphibian oocyte nucleus is therefore related to the number of sets of chromosomes in the cell.

In yolky oocytes from hypophysectomized newts most peripheral nucleoli are firmly attached to the inner surface of the nuclear membrane; whereas in similar oocytes from unoperated or gonadotrophin-treated animals none of the nucleoli is so attached.

On the basis of these observations 2 mechanisms are suggested for the formation of amphibian oocyte nucleoli. The first of these mechanisms probably operates in T. c. carnifex, where all peripheral nucleoli are formed before or soon after the chromosomes assume the lampbrush form, and no part of a lampbrush chromosome is involved in a process which adds to the existing population of nucleoli. The second mechanism probably operates in T. c. cristatus, where most of the peripheral nucleoli are formed before the lampbrush phase of oogenesis but a nucleolar organizer on the lampbrush chromosomes continues to grow and detach nucleoli throughout oogenesis. Both these mechanisms are discussed in terms of what is known of the chemical composition and function of peripheral nucleoli.

Introduction

The 'peripheral nucleoli' in yolky oocytes of Triturus cristatus are large bodies 5 to 10 μ in diameter. They are irregularly rounded, often vacuolated, and appear bright in phase contrast. In a newt oocyte of about 1 mm diameter there may be as many as 1,500 peripheral nucleoli, not attached to the
Macgregor—Nucleoli in amphibian oocytes

chromosomes, but lying near or closely appressed to the nuclear membrane. Whilst something is known of the chemistry of amphibian oocyte nucleoli from the studies of Gersch (1940), Guyénot and Danon (1953), Gall (1954), and Brown and Ris (1959), remarkably little can be said with surety concerning their origin.

The studies of Duryee (1941; 1950), Gall (1954), and Miller (1961) have led to a general acceptance of the notion that the nucleoli of an amphibian oocyte are produced in association with the lampbrush chromosomes and shed by these chromosomes. Such a conclusion is by no means fully justified. The studies of Gersch (1940), Guyénot and Danon (1953), and Miller (1962) on amphibian oocytes, and those of Chouinard (1963) on fish oocytes, form a basis for quite a different scheme. Their observations suggest that the lampbrush chromosomes may not be involved in the formation of new nucleoli, and that the multi-nucleolar condition may be established before the lampbrush phase of oogenesis. The question remaining unsettled, therefore, concerns the role of lampbrush chromosomes in the formation of peripheral nucleoli. Are all peripheral nucleoli in an oocyte formed early in oogenesis before the chromosomes assume the lampbrush form, or are individual nucleoli produced one after another at certain loci on the lampbrush chromosomes? This paper describes an attempt to answer this question.

In this laboratory we are familiar with the lampbrush chromosomes of 4 sub-species of T. cristatus (Callan and Lloyd, 1960). In each of these newts the lampbrush-chromosome loci which might be considered as possible sites of production of oocyte nucleoli can be identified with ease. Of all the objects attached to the lampbrush chromosomes, the giant fusing loops (see Callan and Lloyd, 1960) of the 3 shortest chromosomes are most similar in appearance to oocyte nucleoli (figs. 1, 2, and 3). Giant fusing loops are characteristically present on chromosomes X, XI, and XII of T. c. carnifex, on chromosomes X and XII of T. c. karelinii, and on chromosome XII of T. c. cristatus and T. c. danubialis. The giant fusing loops of chromosome XII in T. c. cristatus, T. c. karelinii, and T. c. danubialis are homologous with the multiple giant fusing loops of chromosome XII in T. c. carnifex. Callan and Lloyd (1960) have shown that within a given sub-species the presence or absence of giant fusing loops is an individual-specific character, and that if giant fusing loops are present at certain loci in one oocyte of an animal then they will be present at the same loci in all oocytes of that animal which are between 0.6 and 1.7 mm in diameter.

Preparations of the lampbrush chromosomes from a newt's oocytes, when made in the way described by Gall (1954) or Callan and Lloyd (1960), usually include many free peripheral nucleoli. A direct comparison between the properties of giant fusing loops and the free nucleoli of a single oocyte is therefore possible. The staining of these objects with toluidine blue at pH 5.4 has been examined before and after digestion with ribonuclease, and the actions of proteolytic enzymes and ribonuclease on unfixed giant fusing loops and nucleoli have been observed.
If peripheral nucleoli are produced one by one at certain loci on the lampbrush chromosomes, and if they detach from these loci and migrate across the nuclear sap to occupy their characteristic position near to the nuclear membrane, then large oocytes ought to have more nucleoli than smaller ones, unless a compensatory loss of nucleoli is occasioned in one way or another. This possibility has been tested by counting the nucleoli in oocytes of different sizes.

In the somatic cells of many animals, including *Ambystoma tigrinum* (Dearing, 1934) and *Ambystoma mexicanum* (Fankhauser and Humphrey, 1943), there is a clear relationship between the number of nucleoli and the number of sets of chromosomes present in a nucleus. There is no comparable record of a relationship between the number of peripheral nucleoli and the number of sets of chromosomes in an amphibian oocyte nucleus. Peripheral nucleoli have been counted in the hexaploid oocytes of triploid *A. jeffersonianum* (Macgregor and Uzzell, 1964) and in diploid oocytes from normal females of the same species to see whether nucleolar number and chromosome number are related in these cells.

If a mature female newt is hypophysectomized or injected with mammalian gonadotrophin, certain changes take place in its oocyte nuclei and lampbrush chromosomes. The changes which involve peripheral nucleoli are described here since they help towards an understanding of the mode of origin of these structures.

**Material and methods**

The animals used in this study were: *Triturus cristatus carnifex* (Laurenti), collected near Naples, Italy, and supplied by Dr. P. Dohrn; *T. c. cristatus* (Laurenti), collected in the south of England and supplied by the dealer L. Haig of Newdigate, Surrey; *T. c. karelinii* (Strauch), collected near Istanbul and supplied by Dr. A. Sengün; *T. c. danubialis* (Wolterstorff), collected near Vienna, Austria, and supplied by Professor E. Mainx; and *Ambystoma jeffersonianum* (Green), diploid and triploid females collected in Dodge County, Wisconsin, and Washtenaw County, Michigan, U.S.A., respectively, and supplied by Dr. T. M. Uzzell, Jr.

The techniques for obtaining oocytes, isolating oocyte nuclei, and removing nuclear membranes were those described by Callan and Lloyd (1960). Oocyte nuclei were isolated in a 5:1 mixture of 0.1 M potassium and sodium chlorides. Isolated nuclei were transferred to Callan and Lloyd's 'C' medium in an observation chamber (see Callan and Lloyd, 1960) for removal of their membranes. Medium C consists of 7 parts of the 5:1 KCl/NaCl mixture together with 3 parts of 0.001 M KH₂PO₄. Since nuclei burst within a minute of isolation in medium C, whole nuclei were examined in observation chambers containing 0.2 M 5:1 KCl/NaCl.

The techniques for fixing, staining, and digesting lampbrush chromosomes and nucleoli were the same as those described by Macgregor and Callan (1962), as were the techniques for digesting unfixed chromosomes and nucleoli.
Preparations were fixed by exposure to commercial formaldehyde vapour for 1 h, followed by immersion in 4% formaldehyde solution for 12 h or more. Some fixed preparations were stained for 40 min in a 0.2% solution of toluidine blue (G. T. Gurr, London) in 0.01 M phosphate buffer at pH 5.4, washed in a similar buffer, dehydrated, cleared, and mounted. Other preparations, prior to staining, were incubated for 4 h at 37°C in ribonuclease (0.2 mg per ml dissolved in distilled water), with controls incubated in water only.

Unfixed chromosomes and nucleoli were digested with pepsin at pH 6.2 and pH 2.0, trypsin at pH 6.2 and pH 7.8, or ribonuclease at pH 6.2. All enzymes were used at an effective concentration of 0.25 mg per ml in modified C medium of the appropriate pH.

All staining and digestion experiments were carried out on the chromosomes and nucleoli of oocytes 0.9 mm in diameter from *T. c. carnifex* and *T. c. cristatus*.

Preparations of lampbrush chromosomes and nucleoli in their observation chambers were observed with an inverted phase-contrast microscope (IPCM) (Cooke, Troughton, and Simms Ltd., York, England) giving dark contrast (alternatively known as bright field). Photographs of the primary image formed by the objective lens were taken with a single exposure camera loaded with Ilford Micro-neg Pan Film. A conventional light source with a ‘compur’ shutter of 35 mm aperture, interposed between the light source and the condenser, was used.

It is known from the reports of Guénot and Danon (1953) and Gall (1954) that in small oocytes of *T. cristatus* and *Triturus viridescens* the peripheral nucleoli are attached to the nuclear membrane and evenly distributed over its surface. The peripheral nucleoli of larger oocytes lie near to the nuclear membrane but are usually not attached to it; they are held in place by gelatinous nuclear sap. Because of this difference, 2 methods were used for counting nucleoli, one for oocytes of less than 0.8 mm diameter in which most of the nucleoli were attached to the nuclear membrane, and another for those of more than 0.8 mm diameter in which most of the nucleoli lay free in the peripheral nuclear sap.

Each nucleus from a small oocyte was placed in an observation chamber containing 0.2 M saline, and the chamber was covered with a coverslip. The

![Fig. 1. Bivalent X of *T. c. carnifex* homozygous for the presence of giant fusing loops; on both homologues the matrices of sister giant fusing loops have fused. In C medium.](image1)

![Fig. 2. Bivalent XI of *T. c. carnifex* homozygous for the presence of giant fusing loops; there is no fusion between sister giant fusing loops. In C medium.](image2)

![Fig. 3. Bivalent XII of *T. c. carnifex* homozygous for the presence of giant fusing loops; ‘double bridge’ breaks have occurred at the giant fusing loop loci on both homologues. In C medium.](image3)

![Fig. 4. Part of bivalent X of *T. c. carnifex* stained with toluidine blue at pH 5.4.](image4)

![Fig. 5. Part of bivalent X of *T. c. carnifex* stained with toluidine blue at pH 5.4 after incubation in a solution of ribonuclease at a concentration of 0.25 mg per ml at 37°C for 4 h.](image5)

I, giant fusing loops; n, free nucleolus.
FIGS. 1–5
H. C. MACGREGOR
observation chamber was then transferred to the stage of an IPCM and the nucleus brought into view. The nucleoli in contact with that part of the nuclear membrane furthest from the objective lens were then drawn to show their distribution over an area of approximately 3,000 \( \mu^2 \). Drawings were made using a 95\( \times \) oil immersion objective, 10\( \times \) eyepieces, and a camera lucida. Immediately after completion of a drawing, the diameter of the nucleus was measured using a micrometer eyepiece. The observation chamber was then removed and tilted on its side so as to roll the nucleus into a new position. A second drawing was made and the nuclear diameter was again measured. If nucleoli were not attached to the nuclear membrane then they fell downwards out of the plane of focus as the nuclear sap became hydrated. All nuclei in which this happened were rejected. Given 2 values for the diameter of a nucleus and 2 values for the number of nucleoli per unit of area of its surface, it is possible to estimate the total number of nucleoli in that nucleus. Two values for each factor were deemed necessary since the nucleoli may not be distributed evenly around the periphery of the nucleus.

For a larger oocyte the nucleus was isolated and its membrane removed and spread out alongside the chromosomes and nucleoli on the bottom of the observation chamber. All the nucleoli in the nucleus could then be counted using the IPCM, 10\( \times \) objective, and camera lucida. As each nucleolus was counted its position was marked on a piece of paper pinned to the drawing-board of the camera lucida. In this way all the nucleoli in a nucleus were counted.

Details of the methods and timing involved in hypophysectomizing newts and injecting gonadotrophins have been given in a previous paper (Macgregor, 1963). The observations described here were made upon oocytes from certain \( T. c. carnifex \) females before hypophysectomy and 30 days after hypophysectomy, and from \( T. c. carnifex \) females before treatment with gonadotrophin and 2 days after the second of 2 injections each consisting of 200 i.u. of a mixture of chorionic and serum gonadotrophin.

**Observations**

Nucleoli and giant fusing loops stain intensely with toluidine blue at pH 5.4 (fig. 4). Pretreatment with ribonuclease has no effect upon the stainability of the spheres on chromosomes V and VIII (Macgregor and Callan, 1962); it reduces but does not eliminate the staining of all other objects. However, nucleoli and giant fusing loops from the same nucleus, stained with toluidine blue, match one another precisely whether pretreated with ribonuclease or not (fig. 5). Nothing stains with toluidine blue in preparations which have been treated with boiling trichloroacetic acid for 15 min prior to staining.

Nucleoli isolated in 0.2 % veronal acetate or ammonium acetate at pH 6.5 swell, burst, and exude their contents in a sequence of events which I have called ‘decapsulation’ (fig. 6). A freshly decapsulated nucleolus can be seen to consist of a blob of refractile material, probably corresponding to the
nucleolar ‘core’, lying beside an empty ‘hull’ (Miller, 1961). Giant fusing loops isolated in veronal or ammonium acetate disintegrate into a mass of tiny granules.

The effect of trypsin at pH 6·2 is similar in some respects to that of veronal or ammonium acetate. Within 5 min of application of the enzyme solution all nucleoli become less refractile, nucleolar vacuoles become larger, and where there are 2 or more vacuoles in one nucleolus these merge to form a single large vacuole (figs. 7 and 8). All vacuoles seem to be full of minute particles which show violent brownian movement. About 10 min after the start of digestion each nucleolus begins to swell and its vacuole grows. After 12 to 15 min of enzyme action the nucleoli burst and their contents flow out and disperse, leaving empty hulls (figs. 8 and 9). The latter are slowly digested and disappear after about 1 h. The action of trypsin at pH 6·2 on the giant fusing loops is quite different. They may be slowly reduced in size (figs. 10 and 11), or rapidly and completely stripped of their matrix (figs. 12 and 13).

Pepsin at pH 6·2 causes a slow reduction in size of the giant fusing loops but has no evident effect upon the nucleoli. Ribonuclease at pH 6·2 first causes the giant loops to become increasingly vacuolated; later they slowly shrink to about half their original volumes. Ribonuclease, like pepsin at this pH, has no visible effect upon the nucleoli. Both nucleoli and giant fusing loops are rapidly and completely dissolved by trypsin at pH 7·8 and by pepsin at pH 2·0.

Nucleolar counts were not obtained from oocytes of less than 0·15 mm in diameter since the nuclei of oocytes smaller than this are too difficult to isolate. No counts were obtained from oocytes larger than 1·5 mm in diameter since in the largest oocytes the nucleoli clump tightly around the chromosomes in the centre of the nucleus and some fusion of nucleoli may take place. Counts of the number of nucleoli per oocyte nucleus were taken from oocytes within this size range from 2 females of *T. c. carnifex*, one female of *T. c. karelinii*, 2 females of *T. c. cristatus*, and 2 females of *T. c. danubialis*. Counts from

---

**Fig. 6.** Oocyte nucleoli decapsulated after being isolated into 0·2% ammonium acetate; *c*, nucleolar 'core'; *h*, nucleolar 'hull'.

**Fig. 7.** Oocyte nucleoli isolated in C medium.

**Fig. 8.** Same as fig. 7, 12 min after addition of trypsin at an effective concentration of 0·25 mg per ml and pH 6·2. Arrows point to places where the nucleolar hull has broken down, releasing the contents of the nucleolus.

**Fig. 9.** Same as fig. 7, 18 min later.

**Fig. 10.** Parts of the left arms of bivalent XI of *T. c. carnifex* showing the giant fusing loops; sister loops have fused on the upper homologue but not on the lower one. In C medium.

**Fig. 11.** Same as fig. 10, 45 min after addition of trypsin at an effective concentration of 0·25 mg per ml and pH 6·2. Arrows point to the remains of the giant fusing loops.

**Fig. 12.** Part of bivalent XI of *T. c. carnifex*, including 2 homologous pairs of giant fusing loops. The arrow points to a pair of giant fusing loops which have formed a ‘double bridge’. In C medium.

**Fig. 13.** Same as fig. 12, 5 min after application of a solution of trypsin at an effective concentration of 0·25 mg per ml and pH 6·2. Arrow points to a ‘double bridge’ formed by the unbroken axes of the giant loops exposed as matrix has dissolved.
Figs. 14-23
H. C. MACGREGOR
larger oocytes in *T. c. carnifex* were often complicated by the fact that the nucleoli were clumped together in groups of 2 to 5. Each member of a group was smaller than any of the solitary nucleoli in the same preparation. In some cases members of groups were attached to one another by thin strands (fig. 20). I have assumed that each group results from the division of a single large nucleolus. Accordingly, some counts from larger oocytes are counts of solitary nucleoli and clusters of smaller nucleoli.

Nucleolar counts from *T. c. carnifex* are shown in the third and fourth columns of table 1. They show that in a nucleus of 90 μ diameter, in which one cannot even guarantee the presence of lambrush chromosomes, there are as many objects distributed over the inner surface of the nuclear membrane as there are free nucleoli in a nucleus of 570 μ diameter, in which the lambrush chromosomes are past the peak of their activity.

Counts from *T. c. karelinii* are shown in the fifth column of table 1. The situation in this sub-species is not significantly different from that in *T. c. carnifex*.

A glance at columns 8 and 9 of table 1 will show that in *T. c. danubialis* the situation is more complex. In this sub-species nucleoli are consistently more numerous during the first half of oogenesis. In nuclei of 120 to 130 μ diameter there are small peripheral nucleoli, firmly attached to the nuclear membrane, and arranged in groups of 2 to 5. In all larger nuclei there are typical peripheral nucleoli which are more or less uniform in size and evenly distributed over the inner surface of the nuclear membrane. In table 1 the first 7 counts in column 8 and the first 5 counts in column 9 are of groups of nucleoli per small oocyte nucleus; the remainder of the counts in columns 8 and 9 are of solitary nucleoli.

In *T. c. carnifex* there are objects which might be described as peripheral nucleoli in oocytes of 0.08 to 0.1 mm diameter. In oocytes of 0.15 mm diameter...
nucleoli are recognizable as small (1 to 5 μ across), dense, roughly hemispherical bodies which I shall call 'primary' nucleoli (fig. 17). These are evenly distributed over the inner surface of the nuclear membrane. In isolated nuclei they are firmly attached to the nuclear membrane. They are not vacuolated, and they do not produce the bright haloes which are so characteristic of the nucleoli of larger oocytes when viewed in phase contrast.

### Table 1

Counts of the number of nucleoli per oocyte nucleus in 2 females of *T. c. carnifex*, 1 of *T. c. karelinii*, 2 of *T. c. cristatus*, and 2 of *T. c. danubialis*. Counts marked with an asterisk are of groups of nucleoli rather than solitary nucleoli.

<table>
<thead>
<tr>
<th>Oocyte diam., mm</th>
<th>Nuclear diam., mm</th>
<th>Nucleoli counted in</th>
<th>Carnifex 1</th>
<th>Carnifex 2</th>
<th>Karelinii 1</th>
<th>Karelinii 2</th>
<th>Cristatus 1</th>
<th>Cristatus 2</th>
<th>Danubialis 1</th>
<th>Danubialis 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.09</td>
<td>981</td>
<td>—</td>
<td>—</td>
<td>480</td>
<td>355</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.20</td>
<td>0.11</td>
<td>866</td>
<td>—</td>
<td>—</td>
<td>432</td>
<td>326</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.25</td>
<td>0.15</td>
<td>836</td>
<td>—</td>
<td>—</td>
<td>977</td>
<td>1170*</td>
<td>1042*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.30</td>
<td>0.18</td>
<td>907</td>
<td>—</td>
<td>—</td>
<td>1000</td>
<td>919</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.35</td>
<td>0.20</td>
<td>1000</td>
<td>919</td>
<td>—</td>
<td>513</td>
<td>491</td>
<td>1104*</td>
<td>1148*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.40</td>
<td>0.24</td>
<td>715</td>
<td>—</td>
<td>—</td>
<td>368</td>
<td>490</td>
<td>1176*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.45</td>
<td>0.26</td>
<td>1014</td>
<td>824</td>
<td>1247</td>
<td>741</td>
<td>630</td>
<td>1392*</td>
<td>1054*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.50</td>
<td>0.28</td>
<td>948</td>
<td>1112</td>
<td>1130</td>
<td>724</td>
<td>994</td>
<td>1324*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.55</td>
<td>0.30</td>
<td>803</td>
<td>—</td>
<td>—</td>
<td>1253</td>
<td>724</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.60</td>
<td>0.32</td>
<td>1240</td>
<td>—</td>
<td>—</td>
<td>1096</td>
<td>444</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.65</td>
<td>0.34</td>
<td>1102</td>
<td>—</td>
<td>—</td>
<td>1012</td>
<td>900</td>
<td>624</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.70</td>
<td>0.37</td>
<td>976</td>
<td>—</td>
<td>—</td>
<td>365</td>
<td>511</td>
<td>800</td>
<td>844</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.75</td>
<td>0.39</td>
<td>811</td>
<td>1102</td>
<td>—</td>
<td>614</td>
<td>732</td>
<td>776</td>
<td>781</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.80</td>
<td>0.40</td>
<td>859</td>
<td>874</td>
<td>972</td>
<td>—</td>
<td>659</td>
<td>672</td>
<td>790</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.85</td>
<td>0.41</td>
<td>979</td>
<td>1215</td>
<td>—</td>
<td>—</td>
<td>613</td>
<td>781</td>
<td>885</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.90</td>
<td>0.43</td>
<td>959</td>
<td>—</td>
<td>—</td>
<td>806</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.95</td>
<td>0.45</td>
<td>1040</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>714</td>
<td>781</td>
<td>751</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.00</td>
<td>0.46</td>
<td>1020</td>
<td>—</td>
<td>—</td>
<td>967</td>
<td>952</td>
<td>947</td>
<td>890</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.05</td>
<td>0.47</td>
<td>1052</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>758</td>
<td>808</td>
<td>790</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.10</td>
<td>0.48</td>
<td>1155</td>
<td>—</td>
<td>—</td>
<td>847</td>
<td>768</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.15</td>
<td>0.49</td>
<td>1109</td>
<td>941</td>
<td>1141</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.20</td>
<td>0.50</td>
<td>1160</td>
<td>990</td>
<td>896</td>
<td>920</td>
<td>737</td>
<td>804</td>
<td>793</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.25</td>
<td>0.51</td>
<td>858</td>
<td>—</td>
<td>—</td>
<td>608</td>
<td>757</td>
<td>855</td>
<td>901</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.30</td>
<td>0.52</td>
<td>1023*</td>
<td>—</td>
<td>—</td>
<td>700</td>
<td>679</td>
<td>911</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.35</td>
<td>0.53</td>
<td>740*</td>
<td>—</td>
<td>—</td>
<td>919</td>
<td>926</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.40</td>
<td>0.54</td>
<td>1003*</td>
<td>1008*</td>
<td>—</td>
<td>790</td>
<td>709</td>
<td>802</td>
<td>858</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.45</td>
<td>0.57</td>
<td>936*</td>
<td>—</td>
<td>—</td>
<td>983</td>
<td>823</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.50</td>
<td>0.60</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>763</td>
</tr>
</tbody>
</table>

Primary nucleoli are often attached to one another by threads, and in some cases a thread emerges from a primary nucleolus and ends blindly against the nuclear membrane. Each primary nucleolus grows, spreads itself out over the surface of the nuclear membrane, and gives rise to a refractile, non-vacuolated plate of material, a 'secondary' nucleolus (fig. 18). Secondary nucleoli are evenly distributed over the inner surface of the nuclear membrane. The largest of them may be 10 to 15 μ wide. Some are connected to their neighbours by threads. Secondary nucleoli are characteristic of oocytes ranging from 0.2 to 0.6 mm diameter in *T. c. carnifex*.

When an oocyte reaches about 0.5 mm diameter, its secondary nucleoli
start to thicken and become round or nearly so (fig. 19). As soon as a nucleolus has rounded off, it comes to lie free in the peripheral nuclear sap and shows no sign of attachment to the nuclear membrane under any circumstances. Not all nucleoli round off at once. A few nucleoli lie free in the nuclear sap in oocytes of about 0.6 mm diameter; in oocytes of 0.8 to 0.9 mm diameter, there are usually about as many nucleoli lying free in the nuclear sap as there are secondary nucleoli stuck to the nuclear membrane; and in a normal healthy animal all nucleoli are round and free in oocytes of more than 1 mm diameter.

### Table 2

<table>
<thead>
<tr>
<th>Oocyte diam., mm</th>
<th>Nuclear diam., mm</th>
<th>Nucleoli counted in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Triploid 1</td>
</tr>
<tr>
<td>0.35</td>
<td>0.26</td>
<td>—</td>
</tr>
<tr>
<td>0.40</td>
<td>0.29</td>
<td>—</td>
</tr>
<tr>
<td>0.45</td>
<td>0.31</td>
<td>—</td>
</tr>
<tr>
<td>0.50</td>
<td>0.33</td>
<td>—</td>
</tr>
<tr>
<td>0.60</td>
<td>0.38</td>
<td>—</td>
</tr>
<tr>
<td>0.65</td>
<td>0.40</td>
<td>—</td>
</tr>
<tr>
<td>0.70</td>
<td>0.42</td>
<td>—</td>
</tr>
<tr>
<td>0.80</td>
<td>0.44</td>
<td>—</td>
</tr>
<tr>
<td>0.85</td>
<td>0.45</td>
<td>—</td>
</tr>
<tr>
<td>0.90</td>
<td>0.45</td>
<td>—</td>
</tr>
<tr>
<td>0.95</td>
<td>0.47</td>
<td>—</td>
</tr>
<tr>
<td>1.00</td>
<td>0.49</td>
<td>—</td>
</tr>
<tr>
<td>1.10</td>
<td>0.51</td>
<td>—</td>
</tr>
<tr>
<td>1.20</td>
<td>0.53</td>
<td>—</td>
</tr>
<tr>
<td>1.30</td>
<td>0.54</td>
<td>—</td>
</tr>
<tr>
<td>1.40</td>
<td>0.55</td>
<td>—</td>
</tr>
<tr>
<td>1.50</td>
<td>0.58</td>
<td>—</td>
</tr>
<tr>
<td>Means of nucleolar counts</td>
<td></td>
<td>3823</td>
</tr>
</tbody>
</table>

Each free nucleolus grows, and when it reaches a certain maximum size it may break up into several smaller daughter nucleoli (fig. 20). Coincident with the start of contraction of the lampbrush chromosomes and retraction of their lateral loops, the nucleoli begin to leave the periphery of the nucleus and migrate inwards. In an oocyte of about 1.5 mm diameter a few nucleoli are clustered around the chromosome group: others are scattered throughout the nuclear sap. The ball of closely packed nucleoli in nuclei of mature oocytes measures some 50 to 80 µ in diameter.

In *T. c. cristatus* the number of nucleoli per oocyte nucleus varies between 350 and 1,000 (columns 6 and 7, table 1). This variability is not related to oocyte size but it is related to nucleolar size. The fewer the nucleoli in a nucleus, the larger they are. Nevertheless, small oocytes containing only 300
to 400 nucleoli are common enough in *T. c. cristatus*, whereas large oocytes with less than 600 nucleoli cannot be found. There is no evidence of nucleolar division in *T. c. cristatus* oocytes.

Nucleolar counts were obtained from oocytes of 0.35 to 1.5 mm diameter from 2 diploid females and 2 triploid females of *Ambystoma jeffersonianum*. Oocytes from diploid animals always contained 14 lampbrush bivalents. Oocytes from triploid animals always contained 42 lampbrush bivalents. Nucleolar counts are shown in table 2. There are no more nucleoli in large oocytes of *A. jeffersonianum* than in small ones; but there are about 3 times as many nucleoli in a hexaploid oocyte as in a diploid one.

There are many more nucleoli in lampbrush chromosome preparations made from oocytes of 1 mm diameter taken from gonadotrophin-treated *T. c. carnifex* than there are in comparable preparations made from the oocytes of untreated or hypophysectomized newts (figs. 14, 15, and 16). The lack of nucleoli in preparations from hypophysectomized newts is due to the fact that in yolky oocytes of these animals more than 80% of the nucleoli are firmly attached to the nuclear membrane, and when the membrane is dissected from a nucleus in an observation chamber the nucleoli come to be discarded with it. In yolky oocytes of gonadotrophin-treated newts, all nucleoli lie free in the peripheral nuclear sap. They therefore fall to the bottom of the observation chamber with the chromosomes when the nuclear membrane is removed and the nuclear sap disperses.

**Discussion**

In preparations of lampbrush chromosomes which have been stained with toluidine blue at pH 5.4, the only real distinction between giant fusing loops and free nucleoli is that the former are attached to the chromosomes, whereas the latter are free. Nevertheless, observations on unfixed material suggest that giant fusing loops and nucleoli differ in certain important respects. Therefore, unless the nucleoli change immediately after detachment from the chromosomes, the giant fusing loops cannot be considered as sites of production of nucleoli.

*T. c. danubialis* has giant fusing loops on the shortest lampbrush bivalent only. These loops may be present on both homologues, on one homologue only, or on neither (Callan and Lloyd, 1960). The oocyte nucleoli of animals which are homozygous for the absence of giant fusing loops have the usual appearance and are present in the usual numbers.

In *Triturus helveticus, Ambystoma jeffersonianum, Ambystoma macrodoxyllum, Desmognathus fuscus, and Ensatina eschscholtzii*, there are no objects attached to the lampbrush chromosomes which resemble peripheral nucleoli; yet all these animals have peripheral nucleoli (unpublished observations by J. Kezer and H. C. Macgregor).

S. Morton, in work not yet published, investigated whether there is a relationship between the number of recognizable giant fusing loops in oocytes and the number of nucleoli per somatic nucleus in the 4 sub-species...
of *T. cristatus* which I have named above. She showed that for a given individual the maximum number of nucleoli per somatic cell (fusion may result in fewer nucleoli) does not correspond with the number of giant fusing loops per oocyte nucleus, and that the maximum number of nucleoli varies between sub-species.

On the basis of these observations I suggest that similarity in appearance between attached objects and free nucleoli in the same oocyte nucleus is not necessarily significant. Somatic nucleolar organizer or oocyte nucleolar organizer, whether the same or different, may not be marked by the presence of conspicuous objects attached to the lampbrush chromosomes.

In *T. c. carnifex*, *T. c. karelinii*, and *T. c. danubialis* there are no more nucleoli in large oocytes than in small ones. It seems therefore that unless nucleoli are lost from a nucleus at the same rate as they are formed there can be no production of nucleoli during the lampbrush phase of oogenesis in these animals. Evidence for the extrusion of nucleoli from a nucleus is unsound. A turnover in the population of nucleoli in a nucleus is therefore improbable. I suggest that in *T. c. carnifex*, *karelinii*, and *danubialis* no locus on the lampbrush chromosomes is producing nucleoli, but that in these newt races all nucleoli are produced at an early stage before the chromosomes assume the lampbrush form.

The apparent correspondence in *A. jeffersonianum* between the number of oocyte nucleoli per nucleus and the number of sets of chromosomes is as one might expect. Nevertheless, this condition is remarkable in view of the large number of nucleoli present. Whether nucleoli form one after another on the lampbrush chromosomes or simultaneously by break-up of large nucleoli before the lampbrush phase, if hexaploid oocytes are to contain 3 times as many nucleoli as diploid ones, then the rate of production of nucleoli or the degree of fragmentation must be precisely regulated.

Why are all nucleoli attached to the nuclear membrane in nuclei isolated from yolky oocytes of hypophysectomized newts but free in nuclei of the same size from gonadotrophin-treated animals? It seems that gonadotrophin treatment, by stimulating oocyte metabolic activity, causes nucleolar material to accumulate more quickly, and hence the nucleoli round off at an unusually early stage. Hypophysectomy produces the reverse situation and indirectly inhibits the rounding-off and detachment of nucleoli from the nuclear membrane. Hypophysectomy affects nucleoli in other ways. The late secondary nucleoli of hypophysectomized newts are by no means normal. They are small half-round objects and are often attached to one another by threads in groups of 3 or 4. Why this should be so I cannot say.

The mode of formation of nucleoli in *T. c. cristatus* is evidently different from that in *T. c. carnifex*, *karelinii*, or *danubialis*. Yolky oocytes of *T. c. cristatus* always contain more than 600 nucleoli, whereas small yolkless oocytes may have only 300 to 400 nucleoli. It seems therefore that in this sub-species nucleoli are produced during the lampbrush phase of oogenesis. The most likely sites of production of nucleoli are the giant fusing loops. All females of
T. c. cristatus examined in this laboratory have been homozygous for the presence of giant fusing loops on bivalent XII. The similarity between free oocyte nucleoli and giant fusing loops in T. c. cristatus is more striking than in any of the other sub-species (fig. 21). Moreover, in preparations of lampbrush chromosomes from T. c. cristatus, the giant fusing loops are often surrounded by objects which are undoubtedly the shed products of this locus, but which are optically indistinguishable from nearby nucleoli.

The production of nucleoli at a particular locus on a lampbrush chromosome is probably not peculiar to T. c. cristatus. In work not yet published on Plethodon cinereus Kezer and Macgregor found that the nucleoli of yolky oocytes, if isolated in 0.05 M saline, often take the form of rings of large granules (fig. 22). There can be no doubt that these rings are the homologues of the round, refractile, peripheral nucleoli of Triturus oocytes. There are no other structures in P. cinereus oocytes which could be classed as nucleoli. In oocytes which have ring nucleoli, one locus near the middle of the seventh longest chromosome carries clusters of rings and loops which closely resemble the free nucleoli (fig. 22). In smaller oocytes neither the nucleoli nor the objects attached to the middle of chromosome VII are ring-like; nevertheless they resemble one another closely (fig. 23). Nucleoli from P. cinereus have not been counted but the peculiar and striking resemblance between objects attached to a lampbrush chromosome and free nucleoli is compelling evidence of the fact that in this species nucleoli are produced and shed by a nucleolar organizer on the lampbrush chromosomes.

Two general mechanisms appear to exist for establishing the multinucleolate condition in oocytes of Amphibia and some other vertebrates. Variations of the first of these mechanisms have been outlined by Gersch (1940), Guyénot and Danon (1953), Miller (1962), and Chouinard (1963). Their observations, together with my own, show that in some Amphibia all peripheral nucleoli are formed before or soon after the chromosomes assume the lampbrush form. No part of a lampbrush chromosome is involved in a process which adds to the existing population of nucleoli.

Variations of the second mechanism have been outlined by Gall (1954) and Duryee (1941; 1950). According to these authors, many of the peripheral nucleoli of amphibian oocytes are produced and shed by one or more nucleolar organizers on the lampbrush chromosomes, and these organizers are characterized by attached objects which resemble free nucleoli. This mechanism probably operates in T. c. cristatus and P. cinereus. Accordingly, the loci which produce oocyte nucleoli in T. c. cristatus do so before and throughout the lampbrush phase of oogenesis, whereas those in T. c. carnifex produce no new nucleoli after the chromosomes assume the lampbrush form.

Miller (1961; 1962) distinguishes 2 components, a fibrous ‘core’ and a granular ‘hull’ in oocyte nucleoli from Rana and Triturus. He states that the nucleoli of small oocytes have relatively more core than hull, and that some nucleoli from small Triturus oocytes consist entirely of core material. My observations on T. c. carnifex fit into Miller’s scheme. Primary nucleoli,
consisting mostly of core, appear before the lambrush phase and thereafter
grow by accumulation of hull material. The mode of formation of nucleoli
in *T. c. cristatus* is less certain. The objects which are shed by the giant fusing
loops are often as large as the free nucleoli of the same nucleus. To judge
from their size it is most unlikely that they consist entirely or even mostly of
core material; yet if both core and hull are present in a newly formed *cristatus*
nucleolus then it would seem that the synthesis of hull material may begin
before the nucleolus detaches from the chromosome.

The precise nature of core and hull components is not known, although
something can be inferred from histochemical and autoradiographic studies
and from electron micrographs. Both components contain ribonucleic acid
(RNA) and protein (Miller, 1962). Tritiated RNA precursors appear in the
hull but not in the core (Miller, 1962). It is not yet known whether RNA is
synthesized in the nucleoli or merely accumulates there. From unpublished
observations by H. G. Callan it seems likely, however, that at least some of
the nucleolar RNA is synthesized in the nucleolus since there is no lag between
labelling of the chromosomes and the nucleoli with tritiated uridine, and the
nucleoli become labelled before the nuclear sap. Izawa, Allfrey and Mirsky
(1963) and Callan (unpublished) found that actinomycin D inhibited the
incorporation of tritiated uridine into oocyte nucleoli. From this they
concluded that the synthesis of nucleolar RNA is dependent on deoxyribo-
nucleic acid (DNA). Therefore, if RNA is synthesized in the nucleolus
rather than accumulating there, then each nucleolus must contain some
DNA, and since the base ratios of nucleolar RNA correspond to those of
cytoplasmic ribosomal RNA (Edstrom and Gall, 1963), the DNA of all
nucleoli in an oocyte nucleus is likely to be similar.

Kezer, in work not yet published, and Miller (1964) have independently
provided conclusive evidence of the presence of DNA in amphibian oocyte
nucleoli. Kezer found that freshly isolated ring nucleoli from *P. cinereus*
disintegrated into their component granules when treated with deoxyribo-
nuclease, and Miller obtained similar results when he isolated nucleoli from
*T. viridescens* in dilute media and treated them with deoxyribonuclease. It
follows from these observations that the nucleolar organizer must be the site
of synthesis of 2 materials. The first of these is DNA; the second is RNA.
DNA may be synthesized during a brief, intense multi-replication of the
nucleolar organizer early in oogenesis, producing the full complement of
primary nucleoli, or by an extended multi-replication of the nucleolar organ-
izer throughout oogenesis. RNA may be synthesized in association with a
replica of the nucleolar organizer in a free nucleolus, or in association with the
nucleolar organizer itself on the lambrush chromosomes.

I wish to thank Professor H. G. Callan and Dr. James Kezer for helpful advice and
comments.

**References**

Macgregor—Nucleoli in amphibian oocytes