PROTEINS OF THE NEWT OOCYTE NUCLEUS: 
ANALYSIS OF THE NONHISTONE PROTEINS 
FROM LAMPBRUSH CHROMOSOMES, 
NUCLEOLI AND NUCLEAR SAP 

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SUMMARY
An electrophoretic analysis has been carried out on the total protein of lampbrush chromosomes, nucleoli and nuclear sap, obtained from newt oocyte nuclei. In each case, distinctive and heterogeneous banding patterns are observed. The absence of detectable quantities of histones in oocyte chromatin is noted. In the case of the lampbrush chromosome preparation, it is concluded that all protein species are derived from the ribonucleoprotein matrix of the lateral loops.

INTRODUCTION
The proteins of isolated chromatin fall into 2 classes: the histones and the acidic proteins or non-histones. Whilst the histones from a wide variety of sources have been well characterized (see Elgin, Froehner, Smart & Bonner, 1971) the precise definition of the acidic proteins of chromatin has remained somewhat elusive. The difficulties involved in analysing complex mixtures of non-histones have been repeatedly discussed (Johns & Forrester, 1969; Comings & Tack, 1973). One recurrent problem is that in cell homogenates, acidic proteins show a tendency to aggregate non-specifically with both nucleoplasmic and cytoplasmic contaminants, which are thus adsorbed to and extracted with the chromatin. Johns & Forrester showed that much of what had formerly been regarded as chromosomal non-histone protein was in fact loosely bound contaminating protein which could be washed off in 0·35 M saline.

Amphibian oocytes provide a rare opportunity for overcoming many of the difficulties encountered in the study of nuclear proteins. This material offers 2 major advantages. First, oocytes are sufficiently large to allow manual dissection of individual nuclei, which subsequently can be washed to remove all traces of adhering cytoplasm. The isolation procedure takes about one minute. Macgregor (1962) has shown by interference microscopy that no exchange of protein takes place across the membrane of an oocyte germinal vesicle in 0·25 M KCl, 0·05 M NaCl within the first 2 min following isolation. This allows ample time for isolation, washing and collection. In preparing nuclei by this method, the only possible source of cytoplasmic contamination is through protein adhering to the outside of the nuclear membrane. However,
as was described earlier (Hill, Maundrell & Callan, 1974), analysis of an appropriate quantity of nuclear membrane alone yields no detectable protein of either cytoplasmic or membrane origin. Thus the possibility of cytoplasmic contamination of nuclear preparations in the experiments described below can be confidently eliminated.

A second feature of amphibian oocytes which is a considerable advantage in the present context, is that the nucleus is organized into 2 distinct zones, with a peripheral region of nucleoli surrounding a central mass of lampbrush chromosomes and nucleoplasm. In this work, as in our earlier report (Hill et al. 1974), I have taken advantage of the fact that in immature oocytes the nucleoli are firmly attached to the inner surface of the nuclear membrane. Thus simply by removing the nuclear membrane, the nucleus is divided into a chromosomal fraction and a nucleolar fraction. This is in marked contrast to the difficulties involved in fractionating most interphase nuclei, in which the nucleoli and the chromosomes are closely associated (Hnilica, 1967).

The total mass of nuclear protein in a 0.8-mm oocyte of Triturus cristatus carnifex is about 1.2 μg, of which about 100 ng are nucleolar protein, and about 30 ng are chromosomal protein (author's unpublished observations). Thus it is necessary to dissect relatively few oocytes to provide sufficient protein for analysis on polyacrylamide gels. In the present report I compare, by SDS-gel electrophoresis, the total protein from 3 cytologically defined nuclear fractions: chromosomes, nucleoli, and nuclear sap.

MATERIALS AND METHODS

Females of the crested newt Triturus cristatus carnifex (Laurenti) were obtained from Gerrard & Haig, Newdigate, Surrey, and kept in the laboratory at 18 °C. Ovaries were removed and stored in sealed embryo cups on ice for use later the same day.

Isolation and microdissection of nuclei

A portion of ovary was placed in an embryo cup containing 0.1 M NaCl, and examined under a binocular dissecting microscope at x 16 magnification. Oocytes of 0.7–0.8 mm diameter were selected for dissection. The oocyte was punctured using a sharp needle, and gentle pressure was applied until the nucleus emerged. The nucleus was then picked up in a flame-polished fine pipette (see Callan & Lloyd, 1960) and transferred to a second embryo cup containing clean 0.1 M NaCl, where it was pipetted to and fro until all visible traces of cytoplasm and yolk were removed. Fractionation of the nucleus from this stage onwards depends on the property of immature oocytes discussed above, namely that the nucleoli of an isolated nucleus are firmly attached to the nuclear membrane.

To obtain chromosomes, the nuclear membrane plus attached nucleoli was removed with a needle and forceps as described by Callan & Lloyd (1960), and the nuclear contents containing chromosomes and sap allowed to sink to the bottom of the embryo cup. In 0.1 M NaCl without divalent cations, the nucleoplasm remains gelled for some minutes, and with practice can be readily transferred to a 1-ml centrifuge tube by pipetting. The centrifuge tube containing 0.1 M NaCl, with 0.5 x 10⁻⁴ M CaCl₂ included to ensure sap dispersal, was kept on melting ice throughout the period of collection. As a rule, 100 chromosome sets were collected; this operation takes approximately 4 h. The massed chromosomes were then centrifuged at 750 g for 5 min and the supernatant containing the nuclear sap was removed. The chromosomal pellet was washed twice in 0.1 M NaCl, 0.5 x 10⁻⁴ M CaCl₂ and the final washing supernatant withdrawn with a fine-bore pipette, leaving at most 1–2 μl of fluid covering the pellet. Proteins were solubilized as described below.
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To obtain nucleoli from a washed nucleus, the nuclear membrane was slashed with a sharp tungsten needle, and the nuclear contents removed by further pipetting. This procedure leaves the nuclear membrane as a flaccid sac, with most of the nucleoli attached to its inner surface. In this condition it can be handled easily. Sixty-five nuclear membranes plus attached nucleoli were collected in 0.1 M NaCl, 0.5 x 10^{-4} M CaCl_2, and centrifuged and washed as above.

To prepare nuclear sap for electrophoretic analysis 7 washed nuclei were collected in a minimal volume of 0.1 M NaCl, 0.5 x 10^{-4} M CaCl_2. The nuclei were ruptured by passing through a fine pipette, and centrifuged at 750 g for 5 min to pellet the chromosomes, nucleoli and nuclear membranes. The supernatant containing the nuclear sap was then withdrawn using a fine pipette, and the final volume made up to 12 μl. Preparations of total nuclear protein were made in a similar manner except that the centrifugation step was omitted. Proteins were solubilized as described below.

The cytological appearance of the isolated nuclear fractions is recorded in an earlier publication (Hill et al. 1974).

Solubilization of nuclear preparations

Nuclear proteins were solubilized using 1% (w/v) sodium dodecylsulphate (SDS), 0.14 M 2-mercaptoethanol. The effect of this treatment on cytological preparations which had been allowed to stand for several hours was observed by phase-contrast microscopy. All nuclear structures, chromosomes, nucleoli and nucleoplasmic granules were completely dispersed within several minutes of addition of the reagents.

Pelleted samples, that is chromosomal and nucleolar preparations, were solubilized by addition of 15 μl of 1% SDS, 0.14 M 2-mercaptoethanol, 0.01 M Tris pH 8.6. Supernatant proteins, in a final volume of 12 μl, were solubilized by addition of 3 μl of 5% (w/v) SDS, 0.70 M 2-mercaptoethanol, 0.05 M Tris pH 8.6. All samples were kept under nitrogen for 3 h.

S-carboxymethylation of the reduced sulphhydryl groups of solubilized proteins had no detectable effect on the resulting electropherogram. This procedure was generally omitted. However, in experiments in which radioactive iodoacetic acid was used to assay cysteine residues, the protein-SDS samples were first held in a 95 °C waterbath for 1 min to ensure that all sulphhydryl groups were exposed, and then S-carboxymethylated following the procedure of Crestfield, Moore & Stein (1963).

Electrophoresis

Protein samples solubilized as above were made 10% (v/v) in glycerol, and 0.01% (w/v) in bromophenol blue. The total samples were analysed on SDS-acrylamide gels using the procedure of Shapiro, Viñuela & Maizel (1967). The gels of 5% acrylamide were polymerized in glass tubes of 2 mm internal diameter and 6 cm long. Electrophoresis was at 50 V (constant voltage) for 10 min, until the protein had entered the gel, and then at 150 V until each buffer front had moved a measured distance along the gel. Gels were stained in 0.25% Coomassie brilliant blue in 7% acetic acid, 25% methanol for 3 h, and were destained in the same solution without dye. For molecular weight determination, bovine serum albumin, phosphorylase a, ovalbumin, carbonic anhydrase and myoglobin were obtained from Sigma Chemical Co. and prepared for electrophoresis as described earlier (Hill et al. 1974). The calibration gels were electrophoresed alongside the experimental samples.

RESULTS

In an earlier report (Hill et al. 1974) we established that non-histone proteins are genuine constituents of newt oocyte chromatin, and showed that total oocyte chromatin, defined as nucleoli, nuclear membranes and lampbrush chromosomes, had 2 major protein components with estimated molecular weights of 110,000 and 43,000 Daltons. Preliminary attempts at chromatin fractionation showed that the 2 major proteins were components of the nucleoli. In the present communication, I have extended this approach but using SDS/2-mercaptoethanol for solubilizing proteins rather than
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guanidine hydrochloride/2-mercaptoethanol as used before. The results corroborate
those previously published regarding the nucleolar proteins and in addition reveal
the protein composition of lampbrush chromosomes. Analyses of total proteins from
(a) whole nuclei, (b) nuclear sap, (c) nucleoli and (d) lampbrush chromosomes are
presented in Fig. 1.

SDS is an extremely powerful agent for the molecular dispersal of proteins. In
SDS, all proteins become saturated with negative charge and during electrophoresis
at neutral pH, migrate towards the anode. Spectroscopic analysis of the stained gels
in Fig. 1 reveals that less than 1% of the protein in each sample remains trapped at
the origin. Fig. 1 therefore represents a complete display of the polypeptides in each
sample.

As noted previously, the similarity in protein composition between whole nuclei
and the low-speed nuclear supernatant (Fig. 1, gels a and b) indicates that the bulk
of the protein in an oocyte nucleus is in the nuclear sap. As would be expected, total
nuclear protein solubilized in SDS/2-mercaptoethanol produces an extremely com-
plex mixture of polypeptides, and while 25 or so major bands are discernible, there
are clearly a large number of minor species which contribute to the heavily staining back-
ground. To a lesser extent this is also true of the chromosomes and nucleoli. Both
the latter fractions are resolved into a similar number of polypeptides (ca. 20) in SDS/
2-mercaptoethanol; the banding pattern of each is distinctive, and shows no evidence
of cross-contamination. The question of proteins common to both the chromosomes
and the nucleoli is currently under investigation. From the gels in Fig. 1 it is evident
that the major nucleolar protein, which has an estimated molecular weight of 110000
Daltons, has a counterpart of similar electrophoretic mobility in the chromosomes.
However, preliminary experiments in which 3H-labelled iodoacetic acid was used to
assay the cysteine content of each protein indicate that, although of similar molecular
size, the 2 proteins differ in amino-acid composition. In view of the fact that both
nucleoli and chromosomes are actively engaged in RNA transcription, and also in view
of any possible involvement of the nucleolus, or its products, in the expression of
structural genes (Leavitt, Moldave & Nakada, 1970; Deák, Sidebottom & Harris,
1972), the possibility that other proteins are common to both chromosomes and
nucleoli remains an important subject for further investigation.

An encouraging feature of the results in Fig. 1 is that there appears to be no
entrainment of unbound sap protein contributing to the nucleolar or chromosomal
patterns (see for example that the several proteins in the molecular weight range
20000-32000 are virtually absent from both pelleted preparations), which demonstra-
tes the efficacy of the washing procedure. The question of bound contaminants,
however, is not so easily dismissed. For the reasons outlined in the Introduction, the
possibility that oocyte cytoplasm is a significant source of non-specifically bound
protein is remote. The possibility of protein being adsorbed from the nucleoplasm is,
however, unavoidable. On the other hand, within the cell nucleus, there must be
constant exchange of molecules between the chromosomes and the surrounding sap,
and quite probably, there is no clear dividing line between the two. Thus inevitably
any definition of chromosomal protein based on analysis of isolated chromosomes is
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an arbitrary one. In spite of such considerations, it is reasonable to assume that legitimate chromosomal proteins are more tightly bound than contaminating ones. An attempt has therefore been made in the following experiment to remove loosely bound proteins by washing the chromosome pellet in 0.35 M NaCl as described by Johns & Forrester (1969), instead of in the 0.1 M NaCl used routinely. Fig. 2 shows the results of an experiment in which equal numbers of chromosome sets were isolated and pelleted in 2 separate vials as described in Materials and methods, and then resuspended twice in either 0.1 M NaCl or 0.35 M NaCl. The final pellet in each case was solubilized in SDS/2-mercaptoethanol, and electrophoresed as described in Materials and methods. From a comparison of the quantity and pattern of the non-histone proteins obtained after each treatment, it is evident that there is very little, if any, loss of protein from the chromosomal preparation as a result of the higher concentration salt wash. Thus it appears that all major species of protein visualized in these experiments are tightly bound to the chromatin and therefore probably represent authentic chromosomal components.

A feature which has emerged from the study of oocyte chromatin is the low proportion of histone relative to total protein. Alkaline fast green staining of fixed cytological preparations (Macgregor & Callan, 1962) shows slight staining of the chromomeres, but no reaction over the lateral loops which contain the bulk of the protein. Furthermore, in biochemical analysis of whole chromatin from oocytes (Hill et al. 1974) very little protein was detectable in the region where histones would be expected to run. In the following experiment, I have analysed directly the protein associated with chromomeres, to assess what contribution they alone make to the total chromosomal protein.

Lampbrush chromosomes isolated directly into 2 μg/ml RNase made up in 2 M urea show extensive loss of loop matrix (Fig. 3B). The cytological evidence indicates that virtually all material remaining on the chromosomes after such treatment is chromomeric. Nuclear contents — that is nuclei after removal of membranes and attached nucleoli — were prepared as described in Materials and methods, and collected in 2 μg/ml RNase, 2 M urea, 0.1 M NaCl, 0.01 M Tris, 10^{-3} M MgCl₂, 0.5 x 10^{-4} M CaCl₂ pH 7.2, in a 1-ml centrifuge tube. Unsolubilized material was collected by low-speed centrifugation, washed as before in 0.1 M NaCl, 0.5 x 10^{-4} M CaCl₂, and finally solubilized in 1% SDS, 0.14 M 2-mercaptoethanol pH 8.6. The resulting gel after electrophoresis is shown in Fig. 1e. The absence of protein detectable on this gel endorses the cytological impression that the great majority of material associated with lampbrush chromosomes and analysed in the above experiments is non-histone protein of the lateral loop matrix.

DISCUSSION

Numerous reports on the protein composition of eukaryotic chromatin have demonstrated the great complexity shown by the non-histone fraction. In view of the variety of function ascribed to this fraction, however (Paul & Gilmour, 1968; Shea & Kleinmith, 1973; Brasch & Setterfield, 1974; Thomopoulos, Dastugue & Defer, 1974), such complexity is readily understandable.
In the present analysis of proteins of newt oocyte chromatin, I have taken advantage of the facility with which nuclei can be isolated and washed free from cytoplasmic contaminants, and then further fractionated by simple manipulation into a nucleolar fraction and a chromosomal fraction. Moreover, the cytological structures of newt oocyte nuclei are readily distinguishable by phase-contrast microscopy, which thus provides a means of monitoring the progress of nuclear fractionation, and of establishing the purity of the final preparations. SDS gel electrophoresis allows a total display of the polypeptide composition of each fraction. In the case of nucleolar and chromosomal preparations distinctive and heterogeneous banding patterns are obtained (Fig. 1, gels c and d), each containing 20 or more major protein components. Furthermore, both fractions reveal a notable absence of detectable quantities of histones. This situation in oocyte chromatin is consistent with the observation made by several authors that cell nuclei actively engaged in RNA synthesis are particularly rich in non-histone proteins (Elgin & Bonner, 1970; Johns & Forrester, 1969).

Oogenesis in Amphibia is a period of intensive transcription of both ribosomal and chromosomal RNA (Gall, 1966; Davidson & Hough, 1969; Davidson, Crippa, Kramer & Mirsky, 1966). There is an increasing body of evidence that much of the messenger RNA transcribed during this time is stored in an inactive form in the oocyte for subsequent use by the developing embryo (Gross, 1967; Davidson, 1968). Rosbash & Ford (1974) estimate that less than 10% of the poly-A containing RNA in amphibian oocytes is associated with polysomes, while the majority of it is found complexed with protein, in free ribonucleoprotein particles having sedimentation coefficients ranging from 25 to 80 s. Furthermore, Rosbash & Ford show that virtually all poly-A containing RNA is synthesized during the early stages of vitellogenesis, during the period in which the lampbrush loops are maximally extended. Sommerville (1973) has shown that, in keeping with other eukaryotic cells, the DNA-like RNA molecules transcribed on the lateral loops are very large, with sedimentation coefficients of 50 s and greater. This finding is wholly consistent with the electron-microscopical observations of dispersed lampbrush chromosomes, in which nascent RNA molecules, complexed with protein, are seen as extended, high-molecular-weight transcripts of the DNA in the lateral loop axis (Miller & Bakken, 1972). In electron micrographs of sections through untreated lampbrush chromosomes (Mott & Callan, 1975) the RNP matrix is more compact, and has a particulate texture reminiscent of the 'informosome' organization of free nuclear ribonucleoprotein particles (Samarina, Lukandin, Molnar & Georgiev, 1968), many of which also contain very high-molecular-weight DNA-like RNA. However, the singular quality of the RNP matrix of lampbrush chromosomes is the high proportion of protein relative to DNA. Protein constitutes about 60% of most ribonucleoprotein complexes (Perry & Kelley, 1966), and about 80% of nuclear 'informosomes' (Samarina et al. 1967). On the lampbrush loops, the evidence from cytochemical (Izawa, Allfrey & Mirsky, 1963) and spectroscopic (Sommerville, 1973) measurement indicates that at least 97% of the ribonucleoprotein matrix is composed of proteins. It is the latter proteins which are displayed in Fig. 1d.

The assignment of localization and function to chromosomal non-histone proteins
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awaits clarification. In oocytes, in view of the morphological diversity of the lateral loops seen by both light microscopy (Callan & Lloyd, 1960) and electron microscopy (Mott & Callan, 1975), it seems reasonable to expect the heterogeneous protein composition shown in Fig. 1d. However, even the largest loops constitute no more than 1% of the total chromosomal protein, so it is improbable that any protein restricted to one or even a few loops would be detectable as a discrete band in the present analysis against the background of total chromosomal protein. Therefore, all major polypeptides visualized in Fig. 1d are most likely to be widely distributed throughout the genome, and involved in functions common to many or all loops, such as, for example, in the transcription and processing of RNA. Clearly this argument does not exclude the possibility of locus-specific proteins. Indeed in the situation in which some of the RNA is translated by the oocyte, and some of the RNA is stored until embryogenesis, it is tempting to speculate that the fate of newly transcribed RNA could be determined soon after transcription by association with particular proteins. As has been argued recently (Pederson, 1974), it may well be the case that the primary gene product is defined not only in terms of its nucleotide sequence, but also by the number and kinds of its associated proteins.

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REFERENCES

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Fig. 3. Phase-contrast microscopy showing the typical appearance of lampbrush chromosomes isolated (A) into Tris-buffered saline (0.1 M NaCl, 0.01 M Tris, 10^{-3} M MgCl_{2}, 0.5 \times 10^{-4} M CaCl_{2}, pH 7.3); and (B) into Tris-buffered saline containing 2 \mu g/ml RNase, 2 M urea. Both preparations were centrifuged at 1100 g for 5 min. Scale bar represents 50 \mu m.