THE STRUCTURE OF NUCLEAR RIBONUCLEOPROTEIN OF AMPHIBIAN OOCYTES

DAVID B. MALCOLM AND JOHN SOMMERVILLE
Department of Zoology, University of St Andrews, St Andrews, Fife, KY16 9TS, Scotland

SUMMARY
Nuclear RNP from Triturus oocytes is organized as strings of beads which can be converted into 20-nm-diameter monoparticles with mild RNase treatment or into 5-nm-thick linear fibrils with low salt treatment. The protein component comprises a heterogeneous size-range of polypeptides which differ from the polypeptides of the other nucleoproteins of oocytes. The RNA is of high molecular weight, sediments mostly in excess of 50 s, and is capable of assuming considerable secondary structure. Duplex regions in the form of hairpin loops are present and may serve as focal points in the condensation of the RNP transcript fibres to generate the periodic beaded structure. The structure of the beads may be maintained by means of protein-protein interaction since at salt concentrations between 1 and 2 M NaCl all of the proteins are released in a cooperative manner as various sized aggregates which sediment at 15-30 s. There are no specific proteins obviously peculiar to either the beaded or the fibrillar RNP configuration. The various properties of nuclear RNP are compared with those of chromatin.

INTRODUCTION
It is generally observed that the various types of primary RNA transcript become associated with specific proteins. Those products of transcription which are not destined to become ribosomal components, or those which contain small RNA species such as tRNA, are collectively referred to as heterogeneous nuclear ribonucleoprotein (hnRNP).

Although there is a significant amount of variation in the composition of hnRNP found in different types of organism and in different tissues, many common features exist. (1) The hnRNP embodies rapidly labelled RNA which has a DNA-like base composition. This RNA comprises a population of molecules in the nucleus of heterogeneous size (hnRNA) which is generally of higher molecular weight than the mRNA population of which it is presumed, in part, to be the precursor (reviewed by Lewin, 1975a). (2) The hnRNA associates with a heterogeneous size range of non-basic proteins (Faiferman, Hamilton & Pogo, 1971; Niessing & Sekeris, 1971; Albrecht & Van Zyl, 1973; Gallinaro-Matringe & Jacob, 1973; Sommerville & Hill, 1973; Pederson, 1974) of which some are apparently enzymes involved in the cleavage and modification of the RNA (Niessing & Sekeris, 1970, 1973), others bind specific nucleotide sequences such as poly(A) (Kish & Pederson, 1975) but most have an unknown function. (3) The nuclear RNA-protein complexes exhibit a wide polydispersity in sedimentation coefficient (40-300 s) but are of uniform and specific
buoyant density, generally about 1.4 g cm⁻³, which is equivalent to a protein:RNA ratio of at least 4:1 (reviewed by Georgiev & Samarina, 1971). Under physiological conditions hnRNP has a particulate structure which is unaffected by EDTA but highly sensitive to RNase. Treatment with RNase or with 2 M NaCl results in the conversion of large RNP aggregates either to particles sedimenting as units of about 30 s (Samarina, Lukanidin, Molnar & Georgiev, 1968) or as differentially released protein complexes sedimenting at less than 15 s (Stevenin & Jacob, 1974).

Thus, where differences exist, they exist primarily in the interpretation of structural organization. Native hnRNP has been alternatively interpreted as consisting of stable 30-s protein aggregates (informofers) linked together by surface binding of RNA (Georgiev & Samarina, 1971) or as periodically folded RNP fibrils (Stevenin & Jacob, 1974; Gallinaro-Matringe, Stevenin & Jacob, 1975). The latter interpretation gains some support from the observation that RNP transcripts assume a fibrillar configuration when dispersed in very low salt at pH 8-9 (Miller & Hamkalo, 1972). In the present report we consider the possible molecular arrangement in the alternative particulate and fibrillar configurations of nuclear RNP.

In many respects the primary transcript RNP derived from the lampbrush chromosomes of amphibian oocytes is similar to the hnRNP extracted from other cell types. For instance it contains rapidly labelled RNA with a DNA-like base composition (Sommerville, 1973), a small percentage of which is homologous to oocyte mRNA nucleotide sequences (Sommerville & Malcolm, 1976). This RNA is associated with rapidly labelled protein (Sommerville, 1973) which consists of a large number of different polypeptides with considerable diversity of molecular weight (Sommerville & Hill, 1973; Scott & Sommerville, 1974; Maundrell, 1975). Also the RNA-protein complex is organized in the form of linear aggregates of 20-30 nm particles (Malcolm & Sommerville, 1974; Mott & Callan, 1975).

However, oocyte nuclear RNP tends to differ from most other hnRNP with respect to: the gigantic size of RNP aggregates (Sommerville, 1973; Malcolm & Sommerville, 1974) and the very high molecular weight RNA that they contain (Sommerville & Malcolm, 1976); its extremely low density which is equivalent to a protein:RNA ratio in excess of 20:1 (Sommerville, 1973); its long term stability during oogenesis when it possibly exists as an informational storage product (J. Sommerville, unpublished results). These various properties greatly facilitate the isolation of nuclear RNP and the subsequent direct visualization of its structure.

In this paper we report further studies on the structural organization of the nuclear RNP of amphibian oocytes and we consider analogies with the other major nucleoprotein complexes of the cell, particularly the chromosomal DNP.

MATERIALS AND METHODS

Preparation of chromosomes and RNP for electron microscopy

Lampbrush chromosomes and nuclear RNP were isolated from early vitellogenic oocytes of Triturus cristatus carnifex and were prepared for electron microscopy as described by Malcolm & Sommerville (1974). Dispersed chromosomes were isolated under conditions of low salt and high pH (0.1 mM borate buffer, pH 8.5-9.0) by a technique similar to that described by
Oocyte nuclear RNP

Spring, Scheer, Franke & Trendelenburg, (1975). Chromosomes and RNP, or the material resulting from prior treatment with NaCl, SDS-guanidine, formamide and RNase, were stained with uranyl acetate or phosphotungstic acid and in certain instances rotary-shadowed with platinum-palladium.

Extraction of chromatin and histone

Blood was collected from decapitated *Triturus* by allowing them to bleed directly into a solution containing 1% citrate. The blood cells were then pelleted by centrifugation at 500 g for 5 min. Chromatin was isolated from *Triturus* erythrocyte nuclei and the histones were extracted as described by Destrée, d’Adehart-Toorop & Charles (1972). The final chromatin suspension, cleared of particulate material and pigment by centrifugation at 10,000 g for 10 min, was dialysed against 10 mM NaCl prior to spectrophotometric analysis. The histones were precipitated from H₂SO₄ extracts with 4 volumes of ethanol, pelleted and thoroughly washed with ethanol before lyophilization and subsequent resuspension in electrophoresis sample buffer.

Isolation of nuclear RNP

Nuclear RNP from *Triturus* ovaries was extracted by the procedure described by Sommerville & Malcolm (1976). After separation in a 30-55% sucrose gradient containing 0.1 M NaCl, 2 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5, the compact turbid zone of RNP was retained, diluted with an equal volume of ice-cold H₂O and pelleted at 6000 g for 20 min. Pellets were rinsed and resuspended in the appropriate treatment solution.

Extraction of RNA and iodination

RNA was extracted from nuclear RNP particles, *Triturus* oocyte ribosomes and the 40 s oocyte RNP which contains 4.8 and 5.8 RNA as described previously (Sommerville & Malcolm, 1976). After digestion with proteinase K (Boehringer, 10 μg/ml in 0.1% SDS, 5 mM EDTA, 10 mM Tris-HCl, pH 7.8) for 1 h at 37 °C, the RNA was again extracted with phenol, precipitated with 2.5% sodium acetate, pH 5.0 and 2 volumes of ice-cold ethanol. The pelleted RNA was lyophilized, resuspended in 0.1 M ammonium acetate pH 4.8 and labelled with ¹¹¹I-sodium iodide as described previously (Sommerville & Malcolm, 1976).

Gel electrophoresis

RNP particles, in the native state or after the various treatments described in the text, were suspended in 1% SDS, 5% glycerol, 5 x 10⁻⁴% bromophenol blue, 50 mM dithiothreitol, 30 mM Tris-HCl pH 6.8. Fifteen-microlitre samples were boiled for 3.5 min and applied to an SDS-acrylamide slab gel. The stacking layer contained 3.5% acrylamide and the running gel 15% acrylamide. The dimensions of the gel were approximately 20 x 20 cm x 1.5 mm thick and electrophoresis was performed at 100 V for 16 h. The gels were stained with 0.05% Coomassie brilliant blue in 50% methanol, 10% acetic acid and destained with 10% methanol, 10% acetic acid. Dried gels were scanned using a Joyce–Loebl densitometer.

RNA samples were separated in 3% acrylamide disk gels (Loening, 1969) containing 60% formamide. After electrophoresis the gels were sectioned into 1-mm-thick slices and radioactivity counted in a Nuclear Enterprises 8312 β/γ Automatic Spectrometer.

Hydroxyapatite chromatography

Hydroxyapatite crystals suspended in 10 mM phosphate buffer (PB: an equimolar mixture of NaH₂PO₄ and Na₂HPO₄, pH 6.8) were packed in columns made from Pasteur pipettes to give a bed volume of about 0.5 ml. The columns were equilibrated with 50 mM PB and the sample was applied in 5-20 μl of this same buffer. Elution was carried out with a linear gradient of 50 mM to 0.5 M PB of 80 ml total volume; 1-8-ml fractions were collected and radioactivity was counted in the β/γ spectrometer.
Fig. 1. Different configurations of chromosomal RNP transcripts. A, lampbrush loop showing beaded configuration of transcripts on chromosomes isolated in isotonic solution (75 mM KCl, 25 mM NaCl). B, C, matrix of transcripts on a loop-equivalent which has been allowed to disperse in 0.1 mM borate, pH 8.5. Note the high density of transcripts and the increase in transcript length from left to right. Arrows indicate branching structures on distal region of transcript. A, × 15,500; B, × 7,750; C, × 22,000.

RESULTS

Organization of RNP on isolated chromosomes

To provide a basis for the structural analysis of isolated nuclear RNP it is worth considering first the organization of RNP prior to its release from the chromosomes. Depending upon the method of preparation, nuclear RNP in the process of transcrip-
Oocyte nuclear RNP can be displayed in either a beaded or a fibrillar configuration. These alternative configurations, each equated with a unit of transcription, are shown in Fig. 1. When the chromosomes of Triturus are isolated under conditions isotonic with the nuclear contents, the RNP can be seen to be deployed as strings of beads, each bead being about 20 nm in diameter (Fig. 1A). This is the general condition pertaining to the structure of the lampbrush chromosome loop (Malcolm & Sommerville, 1974; Mott & Callan, 1975). Alternatively, when the same material is isolated in low salt concentration at slightly elevated pH (0.1 mM sodium borate, pH 8.5) linear arrays of RNP fibrils, each about 5 nm across, are produced (Fig. 1B). These are the transcriptional matrices which have been described in detail not only for the oocytes of the amphibian genera Notophthalmus (Miller & Hamkalo, 1972) and Pleurodeles (Angelier & Lacroix, 1975), but also for a range of other cell types including Acetabularia (Spring et al. 1975), Drosophila spermatocytes (Glätzer, 1975), Schizosaccharomyces, Drosophila embryonic cells and HeLa cells (Hamkalo, Miller & Bakken, 1974). The general picture is one of RNP fibrils increasing in length as the RNA polymerases proceed along the DNP from the point of initiation to the point of termination of transcription.

The progressive unfolding of the RNP fibrils can be followed by examining chromosome preparations after various times in the dispersion medium. This process is most easily studied in transcription matrices containing a relatively small number of well spaced polymerases. Fig. 1B, C shows that the transcripts proximal to the DNP axis retain some of their beaded configuration while the more distal regions become increasingly fibrillar and even appear to form branching structures at the termini (arrows) (see also Glätzer, 1975). This effect may be related to RNA duplex formation which is described later.

In order to investigate the molecular basis of the alternative structural states of RNP, i.e. beaded and fibrillar, and to examine the nature of the bead to fibre transformation, further studies have been carried out using isolated chromosome-derived RNP.

Composition of isolated nuclear RNP

Large nuclear RNP complexes can be extracted from homogenates of oocytes by differential centrifugation and can be isolated subsequently as a narrow zone of rapidly sedimenting material in a 30–55 % sucrose gradient (Sommerville, 1973). Prior treatment with 5 mM EDTA and 0.1–0.5 M NaCl has little or no effect on the gradient profile, nor on the composition of the material recovered from the peak. Therefore, 0.3 M NaCl and 5 mM EDTA are incorporated routinely in the gradient buffer in order to minimize the binding of extraneous protein and to dissociate any polyribosomes which might cosediment with the nuclear RNP; 0.1 M NaCl was used in order to study in more detail the effect of increased salt concentration. Fig. 2 shows the gradient separation of nuclear RNP and also indicates that the level of rapidly-incorporated [3H]uridine which we can obtain by labelling newt ovaries is low in this fraction. It should be emphasized that the RNA content of nuclear RNP is small (< 3 % of the total mass), but despite poor yields the RNA has been extracted
and measurements of molecular size, base composition and sequence complexity have been reported previously (Sommerville, 1973; Sommerville & Malcolm, 1976). Solubilized protein from nuclear RNP produced a polypeptide profile on SDS-acrylamide gel electrophoresis which is characteristically heterogeneous in the size range of 10000–150000 Daltons. As Fig. 3 shows, this pattern is quite distinct from the protein profiles of the other major nucleoprotein fractions of the cell: histones, proteins associated with precursor 4s and 5s RNA, and ribosomal proteins appear to have a polypeptide size distribution in no way similar to that of nuclear RNP. In addition we have previously established that there is no homology between nuclear RNP proteins and nucleolar proteins (Sommerville & Hill, 1973). Therefore there is no apparent contamination of nuclear RNP with other protein-containing components.
Although adsorption of soluble proteins during RNP isolation is a possibility, this is unlikely, again because there is no obvious relationship between the electrophoresis profiles of RNP proteins and soluble proteins, but also because the protein constitution of the RNP particles is unchanged by high salt (0.5 M NaCl) treatment which would be expected to remove adventitiously bound protein. We propose that the profile shown in Fig. 3 represents the natural polypeptide constitution of nuclear RNP.
Linear organization of nuclear RNP in relation to the structure of primary transcript RNA

As can be seen in Fig. 4A, isolated nuclear RNP, when viewed by electron microscopy, has the appearance of a tangled string of bead-like particles, each being about 20 nm in diameter (Malcolm & Sommerville, 1974). These aggregates assume linear configurations when allowed to disperse in 85% formamide. The resulting periodically beaded structure is shown in Fig. 4B. Whereas DNase has no effect on the linear integrity of the aggregates, low concentrations of RNase A (< 1 μg/ml) rapidly cause random dispersal of single beads which gradually disintegrate. Released monoparticles in the process of breakdown are shown in Fig. 4C. Therefore it is likely that the fine fibres which can be seen to connect the beads have a backbone of covalently linked RNA. This is consistent with previous findings that the RNA extracted from nuclear RNP is of high molecular weight (Sommerville, 1973; Sommerville & Malcolm, 1976), mostly sedimenting at > 50S (Fig. 5, inset). Also the compact state of the beads when linked, in contrast to their disintegration in the presence of RNase, suggests that RNA in some way maintains the structure of the bead itself. Indeed, after RNase treatment some RNA remains associated with the individual beads. This is demonstrated by sedimentation analysis of [3H]uridine-labelled RNP, briefly treated with RNase then fixed with formaldehyde. As Fig. 10A (p. 158) shows, most of the RNase-resistant radioactivity remains associated with particles sedimenting at 20–30S.

Treatment of nuclear RNP with 0.1% SDS and 4 M guanidine removes most of the protein but does not generate linear structures. Instead, complex folded molecules are formed, one of which is shown in Fig. 4D. These nuclear RNA molecules have a secondary structure which is apparently more extensive than that contained in native RNP, nevertheless they are reminiscent of the terminal complexes seen in some chromosomal RNP fibres which have been spread in low salt (see Fig. 1C). The considerable secondary structure of nuclear RNA, as revealed by electron microscopy, is not easily resolved into single-strand and double-strand regions. It is also difficult at this stage to decide how much homology, in terms of size and spacing of presumed duplex regions, there is between different molecules. As stated above, it is unlikely that such complex interaction is normally present in the RNP transcript. A possible explanation is that extensive duplex formation results from the removal of protein. On the other hand we have argued previously (Malcolm & Sommerville, 1974) that regions of double-stranded RNA exist in the RNP complex, specifically in the beaded regions. This view derives some support from the finding that RNase-resistant RNA remains associated with released monoparticles (see Fig. 10A).

The secondary structure of nuclear RNA is almost completely removed when the SDS/guanidine-treated molecules are spread on a hypophase of 60% formamide. The resulting molecular configurations represent the linear forms of the primary transcripts, which vary in length from 2 to 20 μm and which have been described previously (Sommerville & Malcolm, 1976). A feature of these RNA molecules is occasional thickenings along their length. These we have attributed to the presence of
Fig. 4. Effect of various treatments on the structure of nuclear RNP as seen by electron microscopy. A, untreated nuclear RNP. B, nuclear RNP treated with 85 % formamide showing individual beads and thin connecting fibre (f). C, nuclear RNP treated with 1 μm/ml RNase A showing dispersed beads. D, nuclear RNP treated with 0.1 % SDS, 4 M guanidine. Most of the protein has been removed and considerable RNA secondary structure in the form of duplexed regions is apparent. E, F, nuclear RNP treated as in D, then spread on a hypophase of 60 % formamide. Loop-back structures (lb) are often seen at one end of the linear molecules while terminal blobs (tb) are seen at the other end of the same molecules.

Preparations A–C were made as described by Malcolm & Sommerville (1974) while (D–F) were made as described by Sommerville & Malcolm (1976). A–F, × 58,000.
tenaciously bound proteins, for we have found no equivalent structures in phenol-
extracted molecules. Especially interesting are the characteristic structures found
at the 2 termini of each molecule and shown in Fig. 4E, F. The larger of the two can be
seen, where deproteinization is sufficient, to consist of a stable ‘loop-back’ region
which is 0.2–0.3 μm in length irrespective of the total length of the molecule. Although
this structure is partially duplexed in 60% formamide, we have not as yet studied

Fig. 5. Sedimentation of nuclear RNP-derived RNA before and after iodination.
Inset shows sedimentation profile of [3H]uridine-labelled RNA. RNA was extracted
from nuclear RNP, as isolated in Fig. 2, by phenol extraction and proteinase K diges-
tion. Sedimentation of RNA after iodination at 2 days (○) and after incubation at 60°C
for 17 days (●) is shown. All samples were centrifuged through 15–30% sucrose gradi-
ets containing: 0.1 M NaCl, 1 mM EDTA, 0.5% SDS, 10 mM Tris-HCl, pH 7.5.
the stability of duplexed regions under more stringent denaturing conditions. The other terminal structure is roughly oval, measuring about 30 x 60 nm, but its molecular composition is not known. In considering the orientation of the terminal structures, the loop-back region is analogous to the thickening seen at the tips of growing RNP fibrils, at the presumptive 5' terminus.

Fig. 6. Ribonuclease digestion of iodinated RNA. Purified RNA extracted from nuclear RNP and *Triturus* ribosomes was digested at 37 °C for 1 h with various concentrations of RNase A plus RNase T1 (at 10 units/μg RNase A). Iodinated nuclear RNA (○) and ribosomal RNA (●) samples were adjusted to 0.12 M PB, 5 mM EDTA prior to incubation.

These preliminary observations on the structure of nuclear RNA molecules by electron microscopy are consistent with earlier findings using labelled nuclear RNA which we shall briefly report here. Because of the difficulty in pulse-labelling newt oocyte RNA with radioactive precursors and because of the low yields of purified nuclear RNA which can be obtained, we have had to resort to *in vitro* labelling with 125I in order to study basic aspects of nuclear RNA structure. Iodination of RNA molecules is subject to several serious restrictions: labelling is exclusive to the C-5 positions of cytosine; there is probably preferential single-strand to double-strand labelling; any tenaciously bound protein will be labelled also; the labelled RNA tends to be more unstable and subject to breakdown.
The size distribution of oocyte nuclear RNA before iodination and 2 days and 17 days after iodination is shown in Fig. 5. Considerable reduction in sedimentation coefficient due to the iodination procedure, from over 50s to about 6s, and subsequent breakdown with time to 4s and less is apparent. All studies on the labelled RNA were carried out as soon as possible after iodination.

While studying the hybridization properties of iodinated nuclear RNA (Sommer-
ville & Malcolm, 1976) we were puzzled by the large amount of RNase-resistant radioactivity present in the preparation. That this effect was not a direct consequence of the iodination procedure was demonstrated by treating ribosomal RNA in an identical manner. Fig. 6 shows that whereas the background resistance of iodinated ribosomal RNA is 2–3 %, the RNase-resistant component of nuclear RNA amounts to 12–15 %. This result was tentatively ascribed to a greater extent of double-stranded structure in nuclear RNA compared with ribosomal RNA. Although the incomplete removal of protein from nuclear RNA could not altogether be eliminated as a possible explanation, this seemed unlikely to account for all of the nuclease resistance because both nuclear RNP and ribosomes had been thoroughly phenol-extracted and treated with protease. In order to investigate further the nature of the apparent double-stranded RNA, analysis by hydroxyapatite chromatography was performed.

As can be seen in Fig. 7A iodinated ribosomal RNA and DNA elute from hydroxyapatite (with a linear gradient of sodium phosphate buffer, PB) at about 0.14 and 0.23 M PB respectively. These elution profiles are typical of single-stranded and double-stranded nucleic acids and are used as elution standards. Iodinated nuclear RNA has a complex elution profile as shown in Fig. 7B with peaks of radioactivity at 0.11 M, 0.16 M and in the broad range of 0.25–0.5 M PB. This profile is to some extent simplified by heating the sample to 105 °C for 4 min. Fig. 7C shows that on subsequent hydroxyapatite analysis the peak originally eluting at 0.16 M PB is no longer apparent but a corresponding increase in radioactivity occurs in the peak eluting at 0.11 M PB. This result implies that the radioactivity eluted with 0.16 M PB contains RNA fragments with some duplexed regions which can be readily denatured but do not rapidly renature, at least in the trace concentrations that are available to us. However, the broad peak of radioactivity eluting at high PB concentrations appears not to be affected by heating and could be accounted for, at least in part, as being due to iodinated protein. As can be seen in Fig. 7D, although most of this material is resistant to RNase, it is susceptible to alkaline hydrolysis as judged from its elution properties. In addition, when the RNase-resistant fraction is heated to 105 °C for 4 min, cooled to 60 °C and then analysed by hydroxyapatite chromatography, there is an increase in the percentage radioactivity eluting in the range 0.25–0.5 M PB with increased time of incubation at 60 °C. As shown in Fig. 7D (inset) 54, 78, and 84 % of the input radioactivity elute with high PB after prior incubation for 20 min, 4 h and 16 h, respectively. These results taken together indicate a rapid renaturation of RNA sequences which were native duplexes before heating and probably linked in the form of hairpin structures before RNase treatment.

The size distribution of the RNase-resistant RNA fragments was determined by electrophoresis using formamide-acrylamide gels where it is compared with total iodinated nuclear RNA and with 28, 18, 5 and 4S markers. As Fig. 8 shows, RNase-resistant RNA migrates at a rate similar to the smallest nuclear RNA fragments with a peak migration rate roughly between that of the 4-S and 5-S RNA markers. We conclude that RNA duplexes are an integral part of oocyte nuclear RNA molecules and that they are probably organized as small hairpin structures. These findings are similar to results obtained in the study of hnRNA structure in HeLa cells (Jelinek...
The renaturation kinetics, sequence complexity and estimates of size of the RNA duplexes in *Triturus* nuclear RNA will be reported in more detail in a later report.

![Figure 8](image)

**Fig. 8.** Size of RNase-resistant iodinated RNA. Iodinated nuclear RNA (○) and the RNase-resistant fraction (●) were analysed by electrophoresis in 3% acrylamide gels containing 60% formamide. The gels were cut into 1-mm-thick sections and assayed for radioactivity. Labelled 28-, 18-, 5- and 4-s RNA preparations were used as markers.

**Structure and constitution of proteins in the RNP beads**

We have shown in Fig. 3 that oocyte nuclear RNP contains a heterogeneous collection of proteins. An important consideration concerns the protein composition of the beaded particles which are a basic feature of the RNP transcripts. For instance, do the RNA transcripts from different loops associate with the same proteins? Are all of the individual beaded structures found within a transcriptional unit identical? Are all the proteins found to be associated with the beaded configuration of nuclear RNP also involved in its linear configuration? Certainly there appear to be differences in the type of proteins which bind to the RNA products of at least a few genetic loci. For instance, a polypeptide of about 30,000 Daltons is found to be associated with only several of thousands of lambrush loops (Scott & Sommerville, 1974). Also, transcripts from specific genetic loci, such as tRNA and 5-s RNA associate with only
Oocyte nuclear RNP

2 major proteins to form RNP complexes (see Fig. 3 C). However, these examples may represent the exceptions. As far as can be resolved by the use of labelled antibodies prepared against proteins of different molecular size, a vast majority of the transcriptional products appears to share most of the individual proteins (Scott & Sommerville, 1974; J. Sommerville, unpublished results).

One approach to the problem of differential binding of protein to RNA is the possibility of selective removal of certain proteins from nuclear RNP by increasing the concentration of NaCl. The precaution is taken in all salt-wash experiments to include

\[
\begin{array}{cccccc}
0.1 & 0.2 & 0.5 & 1.0 & 1.5 & 2.0 \\
0.1 & 0.2 & 0.5 & 1.0 & 1.5 & 2.0 \\
\end{array}
\]

\[
\begin{array}{c}
\text{0.1 mM borate} \\
P-1 \\
P-2 \\
\text{SN}
\end{array}
\]

![Fig. 9. Removal of proteins from nuclear RNP by high-salt and low-salt washes. Aliquots of nuclear RNP were pelleted, rinsed in the appropriate salt solution (0.1-2.0 M NaCl or 0.1 mM borate pH 8.5, each solution containing 5 mM 2-mercaptoethanol) and resuspended in that same solution. After shaking at 0 °C for 30 min the samples were centrifuged at 3000 g for 10 min. The resulting pellets were again rinsed and designated P-1. The supernatant was then centrifuged at 100000 g for 8 h and the pellets obtained this time were designated P-2. The supernatant was adjusted to 10% trichloroacetic acid, held at 0 °C for 6 h and centrifuged at 12000 g for 10 min. This was designated SN. Any precipitated material was rinsed with ethanol and air dried. The various pellets and precipitates were solubilized in 15 µl electrophoresis buffer and analysed on SDS-15% acrylamide gels. A, B, distribution of polypeptide bands after treatment with increasing NaCl concentrations. No polypeptide bands developed on staining the supernatant fractions from high-salt treated RNP, therefore this gel is not shown. C, distribution of polypeptide bands after treatment with 0.1 mM borate.](image-url)
a chemical reducing agent (5 mM 2-mercaptoethanol) to prevent the formation of disulphide linkage between proteins. After mixing the nuclear RNP with the appropriate concentration of NaCl and allowing it to disperse at 0 °C for 30 min, the material is then separated into 3 fractions: large particulate aggregates which sediment under those conditions used to pellet native nuclear RNP (3000 g for 10 min, P-1); smaller complexes which pellet after subsequent centrifugation at 100000 g for 8 h and which would include any 20-nm monoparticles (individual beads, P-2); and the remaining soluble material (SN) which precipitates in 5 % trichloroacetic acid. As can be seen from the SDS-acrylamide gels shown in Fig. 9 A, B, the effect of increasing

![Figure 10. Sedimentation of [3H]uridine-labelled nuclear RNP after treatment with ribonuclease, high salt and low salt. Labelled RNP, isolated as indicated in Fig. 2, was pelleted and resuspended in solutions containing: A, 1 µg/ml RNase A, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol; B, 2 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol; and C, 0.1 mM borate buffer, pH 8.5, 5 mM 2-mercaptoethanol. After shaking the samples at 0 °C for 30 min, they were centrifuged through 12 ml 15-30 % sucrose gradients, each incorporating a 1-ml cushion of 55 % sucrose. The RNase-treated sample was fixed with 3.5 % neutralized formaldehyde prior to centrifugation. Triturus 40S RNP was used as a sedimentation marker. The gradient effluents were monitored for absorption at 250 nm (solid lines) and then fractionated and assayed for radioactivity (circles).]

salt concentration, from 0.1 to 2 M NaCl, is to transform large aggregates to smaller complexes, there being an equivalent exchange of all protein components in the process. The effect is most apparent at salt concentrations between 1 and 2 M NaCl. No protein is detected as being soluble after any of the salt washes. Clearly all of the proteins associated with nuclear RNP are strongly bound and bound in an apparently cooperative manner. This is quite unlike the differential effect described in the removal of proteins from the nuclear RNP particles of rat brain (Gallinaro-Matringe et al. 1975) and from EDTA-dissociated ribosomal subunits of Xenopus (Ford, 1971).

In order to establish that protein-RNA disassociation genuinely occurs at high salt concentrations, [3H]uridine-labelled nuclear RNP was treated with 2 M NaCl
and subjected to sucrose gradient analysis. Fig. 10B shows that a broad protein peak sediments over the range 15–30S whereas the labelled RNA appears to sediment independently of the bulk of material absorbing at 280 nm, that is mostly in excess of 30S. That the protein released from RNP aggregates after salt treatment is in the form of molecular complexes is confirmed by electron microscopy. As Fig. 11A indicates, the RNP-derived material is seen as nothing more than a collection of various-sized particles. The RNA is not visible in these unshadowed preparations stained with uranyl acetate.

We conclude that treatment with high salt, in the range 1–2 M NaCl, results in the release of protein complexes which contain most, if not all, of the protein constituents

Fig. 11. Electron micrographs of RNP after treatment with high salt and low salt. Preparations were stained with phosphotungstic acid. A, particulate material produced by treating nuclear RNP with 2 M NaCl. × 36,000. B, RNP fibrils (f) formed by treating nuclear RNP with 0.1 mM borate, pH 8.5, 5 mM 2-mercaptoethanol. Some condensed RNP particles (p) are still present in this preparation. × 36,000. C, initial stage in the transformation of condensed particles (p) to fibrils (f) in borate buffer. × 36,000.
of untreated nuclear RNP. Thus there is no detectable selective effect in the removal of specific proteins from a population of nuclear RNP molecules, which implies that there is little heterogeneity in the protein constitution of nuclear RNP derived from different chromosomal loci.

The reverse process, the treatment of nuclear RNP with increasingly low ionic strength buffer, is analogous to the treatment employed in generating linear RNP fibrils from the more-beaded structures seen on lampbrush chromosomes. We can question whether there is a difference in protein constitution of the alternative RNP configurations. Any differential removal of protein should be apparent from SDS-acrylamide gel analysis. In fact low concentrations of NaCl in the range 0.1 M to 1 mM have little effect in removing protein from the nuclear RNP aggregates. However, under the conditions of chromosome spreading, that is distilled water buffered with borate to pH 8.5 at a concentration not exceeding 0.1 mM, some of the protein is released from the RNP, this time partly in soluble form. As shown in Fig. 9c, there is no obvious selective removal of particular proteins. Therefore it would seem that the beaded structure of native RNP is not due to the presence of specific proteins which are lost in the conversion to more linear fibrils. Fig. 10c shows the gradient analysis of [H]uridine-labelled RNP after suspension in 0.1 mM borate buffer. It indicates that most of the material absorbing at 280 nm plus the radioactivity remain in the form of rapidly sedimenting material. The structure of this material, as seen by electron microscopy (Fig. 11B), is mainly in the form of long linear strands, presumably of RNP. The initial stages of the transformation of beaded nuclear RNP into more fibrillar material is shown in Fig. 11c. The extension of the RNP chain during this process has not been measured with any degree of precision but it would seem to be in excess of 10 times.

**Thermal dissociation of nuclear RNP**

Although complicated by several coincidental transformations such as development of hyperchromicity due to the melting of RNA duplexes, disruption of protein-protein interactions, RNA-protein dissociation and protein denaturation, an analysis of the thermal dissociation of RNP may provide some useful indications as to the forces involved in maintaining its native configuration. At present we can merely describe the overall optical effect of increasing temperature and the least we can show is that the thermal stability of nuclear RNP differs from other nucleoproteins such as chromatin.

As Fig. 12A indicates, nuclear RNP is characterized by a low 'hyperchromicity' of about 4% compared with the 22% of newt erythrocyte chromatin, and has a shallow and complex melting profile which differs from that of chromatin. The shape of the RNP melting profile can be compared directly with that of the chromatin when the values are normalized as a percentage of the final reading. Whereas chromatin increases its peak absorption at 260 nm with increasing temperature (Fig. 12c), nuclear RNP develops a peak of absorption at 280 nm (Fig. 12b) which is most likely due to the disassociation and solubilization of protein from particulate light-scattering material.
The 'melting' of nuclear RNP may be somewhat analogous to the effect observed on treating the RNP with SDS, where we observe an initial fusing of the protein beads prior to release of solubilized protein.

---

**Fig. 12.** Thermal stabilities and melting spectra of nuclear RNP and of erythrocyte chromatin. Nuclear RNP and *Triturus* erythrocyte chromatin were melted using a Unicam SP1800 UV-Spectrophotometer programmed to give a temperature increase of 0.25 °C/min and an absorption scan at 260 nm. A, melting profile of nuclear RNP (solid line), chromatin (broken line) and nuclear RNP normalized to the same percentage hyperchromicity as the chromatin (dotted line). B, wavelength scans of nuclear RNP at 30, 60, and 80 °C (lower, middle and upper curves, respectively). C, wavelength scans of erythrocyte chromatin at 30, 65, and 95 °C (lower, middle and upper curves, respectively).
DISCUSSION

We have presented here what is essentially a descriptive account of the structure of nuclear RNP in *Triturus* oocytes. A detailed biochemical analysis is prevented to a large extent by the difficulties in obtaining sufficient amounts of radioactively labelled material. However, there are similarities enough in the gross characterization of the RNP from *Xenopus*, *Rana*, *Notophthalmus* and *Ambystoma* (D. B. Malcolm & J. Sommerville, unpublished results) to suggest that we are considering general features of amphibian nuclear RNP. Further, there are obvious similarities between amphibian nuclear RNP and the nuclear RNP previously described in mammalian cells, particularly with respect to the heterogeneity and size range of RNP polypeptides (Gallinaro-Matringe et al. 1975), the beaded nature of isolated RNP (Georgiev & Samarina, 1971), and the fibrillar appearance of dispersed RNP transcripts (Miller & Hamkalo, 1972). It is worthwhile to consider the individual properties of nuclear RNP and in addition to see how they relate to the organization of a more extensively studied nucleoprotein, chromatin.

Protein-nucleic acid association

RNA transcripts in amphibian oocytes associate with a large number of different polypeptides. Until we have a basis for separating different classes of nuclear RNP we are unable to say to what extent the polypeptide heterogeneity is due to the presence of different kinds of transcript. In HeLa cells it has been shown that only the nuclear RNP containing poly(A) sequences has a complex population of proteins associated with it (Kumar & Pederson, 1975). On the other hand, RNP lacking poly(A) sequences contains a single major protein of molecular weight 40,000 which seems to correspond to the ‘informoffer’ protein described by Lukanidin et al. (1972). It is not known if the 40,000 Dalton polypeptide with restricted specific localization in oocyte chromosomes (Scott & Sommerville, 1974) is associated also with poly(A)−RNA. In any event, it is obvious that the protein constitution of a major part of the nuclear RNP is far more complex, both in size and charge differences, than the limited number of small, basic proteins present in DNP.

Beads

The periodically beaded structure of nuclear RNP in amphibian oocytes is obvious both while the transcripts are associated with the chromosomes and after release and isolation (Malcolm & Sommerville, 1974). A beaded configuration for isolated nuclear RNP has been proposed for a variety of other organisms (Georgiev & Samarina, 1971) but the interpretation of RNA localized on the surface of a 30-s protein aggregate has been disputed and a periodically-folded RNP strand model has been proposed instead (Stevenin & Jacob, 1974). In the amphibian oocyte situation the beaded structure is broken down by 2 M NaCl and although protein aggregates are released, these are in the form of various sized smaller particles rather than discrete 30-s beads. Even after RNase treatment, when more uniform-sized particles are produced, these are obviously unstable. We conclude that the protein beads have no existence...
independent of the RNA and are formed by periodic condensates of linear RNP as a result of RNA secondary structure and/or protein-protein interaction. The size of nuclear RNP beads is about 20 nm in diameter, compared with the 7-nm beaded structures seen to be typical of chromatin preparations.

Sequence recognition

Regular periodicity of beaded structures along RNP transcripts and the generality of their occurrence on total RNP preparations implies that bead formation is not a consequence of specific nucleotide sequences, unless such sequences are regular periodic features of transcribed RNA. In general there is little evidence for sequence recognition by proteins and the generation of beaded structures in RNP can be considered as being analogous in this respect to the nucleosome-type organization of DNP (reviewed Lewin, 1975b). Notable exceptions to the postulate that proteins do not recognize RNA sequences are the specific proteins which appear to bind monotonous sequences such as poly(A) and oligo(U) (Kish & Pederson, 1975).

The significance of RNA secondary structure

The structural organization of RNA within the beads is still a subject for speculation. The general observations are that there is an apparent foreshortening of the RNP fibril relative to the length of DNA involved in individual transcriptional matrices and that on dissociating RNP in low salt or with detergents longer lengths of transcript, still associated with protein, are generated. It is likely then that the RNA is compacted in the region of the beaded structures.

It has been demonstrated in this report that isolated nuclear RNA has considerable secondary structure, particularly in the form of hairpin loops. The function of these RNA duplexes in the organization of the RNP is tentatively believed to be that of protein-binding sites. However, if this is so, regular spacing of duplexes is expected to be a feature of primary RNA transcripts. In fact the distribution of duplexes has not as yet been determined. Also, it is unlikely that duplex formation alone could account for the observed extent of RNP foreshortening. An alternative argument is that the extensive secondary structure seen in isolated nuclear RNA is a consequence of the removal of protein rather than a native feature. In other words the binding of protein to RNA immediately after transcription may serve to prevent extensive base sequence interaction. A compromise proposal is that intermittent hairpin loops along the length of the linear transcript may serve as focal points for the condensation of the RNP fibril, the resulting beaded structure being stabilized by means of protein-protein interaction.

Cooperative protein binding

The results of salt dissociation experiments show that in nuclear RNP the forces involved in protein-protein interaction are greater than those involved in binding the bulk of the protein to the RNA. Although there is progressive removal of protein at concentrations between 1 and 2 M NaCl, the various different-sized polypeptides are removed proportionately and in the form of large aggregates sedimenting at 15–30 s.
These protein aggregates are presumably derived from the beaded region of the RNP where protein-protein interaction occurs after localized condensations of the RNP fibrils. The forces responsible for the beaded configuration are fairly easily relaxed by treating the RNP with very low ionic strength buffer where again some of the protein is released which is representative of all the molecular weight classes. That is, we have been unable to show that particular proteins are present in the beaded structures which are not present in the linear RNP fibrils.

Our inability to separate different proteins on the basis of salt treatment of nuclear RNP is contrary to previous results which showed that there was differential removal of proteins from HeLa cell nuclear RNP (Pederson, 1974) and from rat brain nuclear RNP (Gallinaro-Matringe et al. 1975). We have no simple explanation as to why oocyte nuclear RNP should differ in this respect, although more extensive phosphorylation of oocyte proteins is a possibility. In fact the mode of release is more alike the cooperative phenomena observed in the release of histones from DNP (Ohlenbusch, Olivera, Tuan & Davidson, 1967).

In order to study more precisely the mode of protein-RNA interaction in oocyte RNP we hope that we shall soon be able to carry out reconstitution experiments involving an analysis of the binding of nuclear RNP proteins to the RNA.

We thank David Roche for assistance with the photography and Professor H. G. Callan for continued encouragement.

This work was supported by Grant no. B/SR/8841.8 from the Science Research Council of Great Britain.

REFERENCES
Oocyte nuclear RNP


(Received 23 September 1976)