AN INVESTIGATION OF RIBONUCLEOPROTEIN PROCESSING IN AMPHIBIAN OOCYTES

William Guy Faulkner Whitfield

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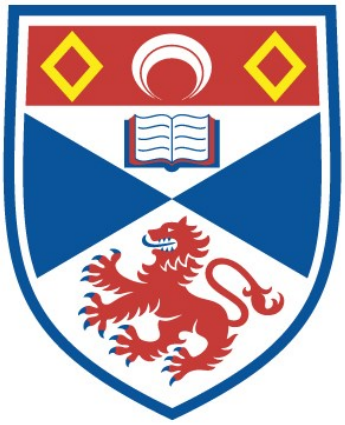
University of St Andrews



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by

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A Thesis submitted for the Degree of Doctor of Philosophy

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AN INVESTIGATION OF RIBONUCLEOPROTEIN PROCESSING IN AMPHIBIAN OOCYTES

SUMMARY

Two forms of ribonucleoprotein complex from oocytes of Triturus cristatus carnifex are investigated, heterogeneous nuclear ribonucleoprotein (hnRNP) and a free 40S cytoplasmic RNP particle that stores 5SRNA and transfer RNA in pre-vitellogenic oocytes.

HnRNP, isolated from homogenates of Triturus ovaries, has been characterized by sucrose gradient centrifugation, isopycnic centrifugation, electron microscopy and treatment with non-ionic detergent. The results largely confirm previous observations by Malcolm and Sommerville (1974) although electron microscopy of thin-sectioned hnRNP material revealed a considerable degree of cytoplasmic contamination of the preparation.

This finding was confirmed by characterization of the polypeptide components of the hnRNP fraction and by comparison with the polypeptide spectrum of manually isolated oocyte nuclei. These studies further revealed that not only were there very few major polypeptides common to both the hnRNP preparation and isolated oocyte nuclei but that the majority of the "hnRNP" polypeptides could be isolated from the oocyte cytoplasm.

Comparison of the polypeptide spectra of hnRNP, oocyte nuclei and rat-liver hnRNP "core particles" suggest that a "core protein" homologue may be present in oocyte nuclei though not in the "hnRNP" preparation. Immunostaining of SDS/polyacrylamide gel transfers with an antiserum to rat-

liver hnRNP "core protein" revealed the presence of antigenically related polypeptides in the "hnRNP".

It is suggested that a large proportion of the so-called hnRNP preparation from Triturus oocytes could represent partially processed messenger RNP in association with membranous supramolecular structures.

The 40S cytoplasmic RNP accumulated in previtellogenic Triturus oocytes contains 5S RNA and transfer RNA with two proteins of molecular mass 45,000 and 39,000 (P45 and P39). The particle has a buoyant density of 1.53 g cm^{-3} and consists of four identical subunits as shown by salt dissociation and isopycnic centrifugation experiments.

Treatment with SDS completely dissociates the RNP complex into its separate components. These can be reassociated into subunits and even intact 40S RNP particles upon removal of the SDS by dialysis. The stable RNA/protein interactions can be demonstrated by analysis of reformed RNP complexes using isopycnic centrifugation and are found to be: 5S RNA/P45, 3(tRNA)/P45, 5S RNA/P39 and 5S RNA/P45/P39.

Indirect immunostaining of frozen oocyte sections with antisera to P45 and P39 suggest a purely cytoplasmic location for P39 whilst P45 is also found in the nucleus.

The relationship between the 40S RNP particle proteins with transcription of 5S RNA and transfer RNA is discussed and the possibility that P39 is related to 5S RNA associated ribosomal proteins is also considered. A scheme for the formation and breakdown of 40S RNP storage particles is presented.

DECLARATION

I hereby declare that this thesis is of my own composition, and that the experimental work was performed by me alone apart from a proportion of that reported in Chapters 1 and 3 which was undertaken and published jointly with Dr. P.-M. Kloetzel and Dr. J. Sommerville in Nucleic Acids Research (1981), 9, No. 3 pp. 605-621 and International Cell Biology 1980-1981 pp. 66-71, (Edited by H.G. Schweiger), Springer-Verlag. None of the material in this thesis has been submitted for any other degree.

WILLIAM WHITFIELD.

C E R T I F I C A T E

I certify that Mr. William Whitfield has spent a minimum of nine terms engaged in research work on the processing of ribonucleoprotein in amphibian oocytes.

C O N T E N T S

Chapter 2 cont:

C H A P T E R 3.

Chapter 3 cont:

GENERAL INTRODUCTION

In recent years, the control of gene expression in eukaryotic cells has become a central field of study in experimental biology. Major advances in genetic manipulation have allowed a much clearer picture of both prokaryotic and eukaryotic genetic activity to emerge.

It has been known for some time that the rate of protein synthesis in bacteria is directly dependent on the messenger RNA supply (Jacob and Monod, 1961), moreover the mRNA supply is controlled by the promotion or inhibition of specific gene transcription (Englesberg et al., 1969; Greenblatt and Schlief, 1971; Zubay et al., 1970; Gilbert et al., 1973). Hence in prokaryotes, where the primary RNA transcript is the mRNA, gene expression would seem to be regulated mainly at the transcriptional level.

In contrast, the system of gene expression in eukaryotes is manifestly more complex. In general, eukaryotic primary transcripts are not directly equivalent to their messenger RNA counterparts as is the case in bacteria. Vertebrate transcription units have been demonstrated to be longer than the mature mRNA they produce (Weber et al., 1977; Goldberg et al., 1977; Evans et al., 1977; Darnell,1979; Ziff,1980). Before a mature messenger RNA is translated in the cytoplasm several processing steps have been shown to be necessary both during and after transcription. Firstly the 5' end of the transcript is "capped" by the addition of a methylated guanylate residue (Shatkin, 1976), a 3' polyadenylate segment or "tail" is then added after transcription (Jelinek et al. 1973; Nevins and Darnell, 1978; Ford et al., 1978; Nevins et al., 1980), any internal non-coding sequences (introns) are removed, and the remaining RNA pieces (exons) spliced together (Gilbert, 1978; Berget et al., 1977; Chow et al., 1977; Kessig, 1977).

These steps, together with both storage and transport of the mature mRNA from the nucleus to the polysomes, (all steps specific to eukaryotes), provide extra opportunities for the regulation of gene expression over and above transcriptional control (Darnell, 1979). In a simple transcription unit, encoding a single protein the only possible direct processing control takes the form of a "process versus discard" decision. This hypothetical possibility would involve little or no processing of a particular transcript in one cell type or cell in a particular condition but successful processing of the same primary transcript in another cell type or another physiological condition (Darnell, 1979 and 1982). Although no specific case of gene control through process versus discard decisions has yet been demonstrated, candidate transcripts of this type do exist in culture cells and some tissues (Darnell, 1982).

In a complex transcription unit whose primary transcript can yield two or more mRNAs encoding different polypeptides, differential processing may occur in one of two ways. The selection of one specific poly(A) site where several are available and the differential splicing of transcripts are both feasible methods for the generation of different mRNAs from single complex primary transcripts. Multiple poly(A) sites have been noted in the major late adenovirus transcription unit and in those of the heavy chains of the immunoglobins and in both cases there is evidence that some controlled selection of sites occurs (Reviewed by Darnell, 1982). Evidence for differential splicing is found once again in the adenovirus late promoter. In L1 poly (A^+) nuclear RNA molecules three products are generated during the late stages of infection but only one in the early stages (Shaw and Ziff, 1980). Finally, very convincing evidence for differential processing allied to developmental regulation is revealed in the expression of the peptide hormone calcitonin. Alternative processing of the calcitonin gene primary transcript results in the production of two differing mRNAS

and their products, calcitonin and calcitonin gene related peptide (CGRP). The calcitonin mRNA predominates in the thyroid gland whereas the CGRP message appears to predominate in the hypothalamus, suggesting a tissue specific control of gene expression by means of differential processing. (Amara et al., 1982)

Although very little is known of the involvement of ribonucleoprotein structure in the aforementioned processing events, both processing and the storage and transport of hnRNA must involve RNA/protein interaction of a highly specific nature. Thus it is very important to view all the events of RNA processing with consideration for the ribonucleoprotein organization involved, knowledge of the structure and function of ribonucleoproteins may well prove critical to a complete understanding of the post-transcriptional regulation of gene expression.

Since the earliest biochemical investigation of hnRNP by Georgiev and co-workers in 1965 (Samarina et al., 1965) there have been extensive investigations of all aspects of hnRNP structure and function (Reviewed by Martin et al., 1980). Though the quantity of descriptive data accumulated over the years has been immense there has been little progress in relating structure to function. In fact there is still considerable disagreement concerning the number and nature of the hnRNP proteins. Nevertheless there is a certain amount of common ground between investigators in this field and some tentative models of hnRNP structure have been put forward (Samarina et al., 1968; Martin et al., 1978; Sommerville 1979b; Stevenin and Jacob, 1979).

Most of the investigations of hnRNP have utilized somatic cells as their experimental medium however Sommerville and co-workers have used the amphibian oocyte as a convenient source of material sharing many of the characteristics expected of hnRNP. The amphibian oocyte has

been the chosen system of study in this work for several reasons. Amphibian species have high C-values ranging from 1-100 pg,with those of Xenopus laevis and Triturus cristatus at 3.1 pg and 23 pg respectively (Reviewed by Sommerville, 1977). Investigation of the morphology and composition of lampbrush chromosomes and of the associated nascent transcripts is greatly facilitated by their giant size and by the dimensions of the oocyte nucleus (Gall, 1954; Callan and Lloyd, 1960; Gall and Callan, 1962; Hill et al., 1974; Maundrell, 1975). Estimates of the transcriptional activity of amphibian oocytes indicate that it is at least 100 times that of most somatic cells (MacGillivray and Rickwood, 1974) and considerable accumulation of transcription products occurs in the oocyte nucleoplasm (up to 30 ng of RNA in Triturus mid-vitellogenic stages) (Edstrom and Gall, 1963; Gall, 1966). Hence the amphibian oocyte represents a rich source of ribonucleoprotein and furthermore is accessible to the addition of exogenous materials such as nucleic acids (Gurdon et al., 1976; De Robertis et al., 1982), protein (Gurdon, 1970; Bonner, 1975(a) and (b); De Robertis et al., 1978; Mills et al., 1980), ribonucleoprotein (De Robertis et al., 1982) or antibodies (Scheer et al., 1979), by microinjection. Using this system genetic activity and intracellular transport can be investigated.

In this thesis I have concentrated primarily on two species of ribonucleoprotein found in amphibian oocytes, representing opposite poles of complexity in terms of their organization and composition. The first two chapters have been devoted to an investigation of heterogeneous hnRNP in amphibian oocytes (Sommerville, 1973; Malcolm and Sommerville, 1974 and 1977) with reference to the more extensively characterized hnRNP of mammalian somatic cells (Martin et al., 1980). In Chapter 3 the structure of a cytoplasmic RNP particle that stores 5S RNA and transfer RNA is

investigated in detail and a tentative model for the RNP structure and function is proposed. In general, in this thesis, ribonucleoprotein structures are considered not merely as a convenient method of packaging RNA but as an essential condition for regulating the transduction of information between gene and gene product. It is believed that a greater understanding of the association of RNA sequences with specific proteins will prove vital to any model for the control of gene expression in eukaryotic cells.

C H A P T E R 1.

INTRODUCTION

Considerable importance is lent to the structures in which hnRNA is complexed from the observation that heterogeneous nuclear RNA (hnRNA) is the precursor of functional messenger RNA. The interactions between specific proteins and hnRNA must be of major significance to the events that occur during mRNA maturation.

The earliest biochemical evidence of hnRNA/protein associations in eukaryotic cells indicated that hnRNP has a subunit structure and a polydisperse distribution in sucrose gradients between 30S and 200S (Samarina et al., 1965 and 1968). Mild ribonuclease digestion causes a quantitative conversion of large polyparticles to 30S particle subunits; Georgiev and co-workers coined the term "informofers" for these 30S hnRNP subunits (Samarina et al., 1968).

Further investigations of the protein components of hnRNP have yielded conflicting results. Claims ranging from a single polypeptide species (Samarina et al., 1968) to a heterogeneous protein complement (Pederson, 1974; Augenlicht and Lipkin, 1976; Gallinaro-Matringe et al., 1975) have been made for hnRNP. These diverse results are largely due to the varying methods by which hnRNP is isolated. In general, those workers claiming a relatively simple composition for hnRNP subcomplexes (Martin et al., 1978 and 1979(a); Beyer et al., 1977; Karn et al., 1977; Billings and Martin, 1978) have utilized the method, or modifications of the method of Georgiev and co-workers (Samarina et al., 1965). This isolation procedure relies upon endogeneous nuclease activity at raised pH to release hnRNP "core particles" from higher order structures in intact nuclei. The 30S subcomplexes in the nuclear extract are then purified from any contamination by sucrose gradient centrifugation. All the alternative

methods of isolating hnRNP complexes involve the disruption of isolated nuclei by either sonication (Pederson, 1974; Augenlicht and Lipkin, 1976; Gallinaro-Matringe, 1975; Alonso et al., 1981) or cavitation (Faiferman et al., 1970). These preparations avoid the extensive degradation of the hnRNA that is inherent in the nuclear incubation methods and probably recover both structural proteins and more peripherally associated processing enzymes as well as contaminating nuclear material, thus accounting for the increased complexity of the polypeptide spectrum of hnRNP isolated in this manner.

The association of hnRNP with chromatin and possibly with the so-called nuclear matrix (Berezney and Coffey, 1974; Faiferman and Pogo, 1974; Herman et al., 1978; Miller et al., 1978) causes certain problems when trying to define the criteria of purity for hnRNP complexes. The relationship of hnRNP structure to function may well be inseparably linked to the associations of the extracted material with nuclear matrix or other nuclear components. Thus studies of functional processes in isolated "purified" material may prove disappointing.

In both of the aforementioned methods of hnRNP preparation the initial isolation step has involved the purification of nuclei in order to minimise the risk of cytoplasmic contamination. However it is possible to isolate a defined fraction from homogenates of amphibian oocytes by differential centrifugation and sucrose gradient purification, containing a high proportion of the rapidly labelled hnRNA in association with a broad spectrum of heterogeneously sized polypeptides. Recovery of both rapidly labelled hnRNA and protein in this fraction can be almost doubled by pretreatment of the labelled oocytes with concentrations of actinomycin-D known to cause retraction of the loops of lampbrush chromosomes and concommitant

stripping of the nascent transcripts (Sommerville, 1973). Analysis of spread preparations of this material in the electron microscope reveals large aggregates of about 25 nm diameter beaded particles (Malcolm and Sommerville, 1974) similar in appearance to that of nascent RNP on the transcriptionally active loops of lampbrush chromosomes (Mott and Callan, 1975; Malcolm and Sommerville, 1977). Protein components of this material have been used to generate antibodies in rabbits some of which give specific indirect immunofluorescent staining of the nascent transcripts on lampbrush chromosome loops (Scott and Sommerville, 1974; Sommerville et al., 1978).

In this chapter I have tried to determine to what extent hnRNP prepared from amphibian oocyte homogenates is a homogeneous preparation as suggested previously (Malcolm and Sommerville, 1974). To this end, the purified material has been analysed further by electron microscopy and subjected to various dissociative agents and treatments in order to select the specific RNA bound proteins. The relatively high percentage of protein ($>$ 95%) associated with the hnRNP isolated in this manner is considered with respect to cytoplasmic contamination and to any possible association of hnRNP with nuclear matrix.

MATERIALS AND METHODS

1. Radioactive labelling of amphibian ovaries

Ovaries containing either previtellogenic or early vitellogenic oocytes were excised from anaesthetized female Triturus cristatus carnifex. The ovaries were washed in modified Barth's solution (Gurdon, 1974) and incubated in the same solution containing 10 µci/ml ^{14}C-amino acid mixture (50 mCi/milliatom carbon, Radiochemical Centre, Amersham) or 0.4 mCi/ml ^{3}H-uridine (27 Ci/mmole, Radiochemical Centre, Amersham) together with 50 units/ml each of penicillin, streptomycin and kanamycin. After incubation at 20°C for 24 h the ovaries were rinsed extensively in modified Barth's medium before use.

2. Isolation of hnRNP from Triturus cristatus carnifex oocytes

Labelled or unlabelled ovaries from Triturus were washed and homogenized in a solution containing 0.1 M NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris/HCl pH 7.5 and 8.5% sucrose (Sommerville, 1973). The homogenate was centrifuged at 300 x g for 5 min to remove yolk, chromatin and connective tissue and the supernatant recentrifuged at 4000 x g for 15 min. The pellet of crude hnRNP was resuspended in homogenization buffer and 0.2 M Na_2 EDTA was added to a final concentration of 5 mM. This suspension was applied to a 25-55% sucrose gradient made up in 0.2 M NaCl, 5 mM Na_2 EDTA, 5 mM 2-mercaptoethanol and 10 mM Tris/HCl pH 7.5, and centrifuged at 180,000 x g for 1 h at 4°C in an M.S.E. 6 x 14 ml Ti swing-out rotor. The gradient was fractionated, monitoring at 254 nm and the hnRNP containing fractions retained. After dilution with three volumes of 0.2 M NaCl, 10 mM Tris/HCl pH 7.5, 5 mM 2-mercaptoethanol the hnRNP was pelleted by centrifugation at 12,000 x g for 30 min.

3. Fractionation of Triturus oocyte hnRNP components by homogenization and recentrifugation

Sucrose gradient fractions containing the labelled hnRNP zone were diluted and the hnRNP pelleted as described above. The hnRNP pellet was resuspended in the same buffer by homogenization in a Teflon/glass Dounce homogenizer and the suspension applied to a 5-60% sucrose gradient in 0.2 M NaCl, 5 mM Na_2EDTA, 5 mM 2-mercaptoethanol, and 10 mM Tris/HCl pH 7.5. Centrifugation was for 15 h at 110,000 x g in an M.S.E. 6 x 14 ml Ti swing-out rotor at 4°C. The gradient was fractionated, monitoring at 254 nm. Aliquots of each fraction were precipitated with cold 10% TCA and after 30 min at 4°C the precipitates were filtered through 2.5 cm Whatman GF/A filters, washed extensively with 5% TCA and finally with 70% ethanol, before drying and counting in a toluene based scintillation cocktail (NE 233). The remainder of each fraction was precipitated at -20°C after the addition of two volumes of ethanol. The polypeptide profile of each gradient fraction was analysed using SDS/polyacrylamide slab gel electrophoresis.

4. Sarkosyl treatment of Triturus oocyte hnRNP

The hnRNP pellet from the sucrose gradient fractions was raised in 2 ml of 0.5% Sarkosyl (Ciba Geigy) containing 10 mM Tris/HCl pH 7.5 and 5 mM 2-mercaptoethanol. After gentle homogenization in a Teflon/glass Dounce homogenizer the suspension was centrifuged at 100,000 x g for 1 h at 4°C in an M.S.E. 10 x 10 ml aluminium fixed-angle rotor. Both the pellet and the supernatant were retained for analysis on SDS/polyacrylamide slab gels.

5. Determination of the buoyant density of RNP particles by isopycnic centrifugation

Labelled RNP complexes were fixed by the addition of formaldehyde to a final concentration 3.4%. Formaldehyde was always made up freshly from paraformaldehyhde to a concentration of 17% and neutralized by the addition of 1M KOH. Formaldehyde was added to the RNP suspension in 10 µl aliquots with continuous vortex mixing in order to minimise precipitation. After a 3 h incubation at room temperature the fixed RNP was applied to 24 - 60% CsCl gradients containing 0.1% Brij, 10 mM potassium phosphate buffer pH 7.0 (Sommerville, 1974). Gradients were centrifuged at 75,000 x g for 12 h. at 20°C before being fractionated, monitoring at 254 nm. Aliquots of each fraction were TCA precipitated, filtered and counted as described previously. The density of each fraction was obtained by weighing 100 µl aliquots on a microbalance.

6. SDS/polyacrylamide gel electrophoresis

Both homogeneous and gradient SDS/polyacrylamide gels were run, utilizing a discontinuous buffer system modified from that of Laemmli (Laemmli, 1970). A 15 cm separating gel containing 0.375 M Tris-sulphate pH 8.3 and 0.15% SDS was overlaid with 1 cm of a 6% stacking gel containing 0.0625 M Tris-sulphate pH 6.9 and 0.15% SDS. The electrophoresis buffer contained 0.05 M Tris, 0.4 M glycine pH 8.3 and 0.15% SDS. Gels were always 1 mm thick, and 1 cm wide perspex well formers were usually employed. Samples were applied in a buffer containing 1% SDS, 30 mM Tris/sulphate, pH 6.9, 10% 2-mercaptoethanol and 25% glycerol. Prior to application samples were heated for 5 min at 95°C. A potential of 200 volts was applied for times that varied according to the acrylamide concentration of the gel used.

In general 12% homogeneous gels were employed for most purposes however gradient gels of either linear or exponential profile were used in certain circumstances, these were poured with the aid of an LKB ULTROGRAD 11300 gradient mixer.

7. Electron microscopy of Triturus oocyte hnRNP

(i) Droplet diffusion spreading of hnRNP

Ribonucleoprotein particles were adsorbed onto "Formvar" coated grids (Malcolm and Sommerville, 1974 and 1977) from 50 µl droplets of RNP suspension in 0.1 M phosphate buffer, pH 7.2. After 30 min the grids were transferred to droplets of 2.5% glutaraldehyde and fixed for 15 min. After dehydration through an ethanol series to 70% ethanol the grids were stained in either 0.2% uranyl acetate or phosphotungstic acid in 70% ethanol, in certain instances preparations were rotary shadowed with platinum-palladium. Grids were viewed in a Philips EM301 electron microscope.

(ii) Thin sectioning of fixed hnRNP

Ribonucleoprotein gradient fractions were fixed, stained and embedded for electron microscopy directly (Hurkman et al., 1981). The hnRNP peak fraction was fixed with 0.5% glutaraldehyde for 1 h at 4°C. Aqueous 25% glutaraldehyde was added to the gradient fractions in small aliquots with continuous vortex mixing. Fractions were diluted five-fold with PBS (0.9% NaCl, 10 mM phosphate buffer pH 7.0) and centrifuged at 12,000 x g 30 min. The pellets were rinsed with PBS and postfixed in the dark for 2 h in buffered 2% osmium tetroxide. Fixed pellets were washed in water and block-stained overnight in 0.5% uranyl acetate before being embedded in Araldite. Thin sections were viewed in a Philips EM301 electron microscope.

8. Isolation of mitochondria from Triturus ovary, liver and culture cells

(i) Triturus ovary

Ovaries were excised from anaesthetized, mature Triturus females. The crude hnRNP fraction was isolated as previously detailed, before recovering the oocyte mitochondria from the remaining supernatant by centrifugation at 24,000 x g for 20 min. The mitochondrial pellet was raised in a small quantity of 25% sucrose in 0.2 M NaCl, 5 mM Na_2 EDTA, 5 mM 2-mercaptoethanol and 10 mM Tris/HCl pH 7.5 and layered on a 25 to 55% sucrose gradient in the same buffer and centrifuged at 180,000 x g for 1 h at 4°C in an M.S.E. 6 x 14 ml Ti swing-out rotor. Gradients were fractionated, monitoring at either 254 nm or 280 nm and the peak of absorbing material mid-way down the gradient was retained. After dilution of the mitochondrial fraction with two volumes of buffer they were centrifuged at 12,000 x g for 30 min and the mitochondrial pellet retained.

(ii) Triturus liver

Livers were excised from anaesthetized, mature Triturus females, the gall bladder removed and any blood rinsed off in 0.9% NaCl 10 mM Tris/HCl pH 7.4 (TBS). After a final wash in 0.1 M NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 8.5% sucrose, 10 mM Tris/HCl, pH 7.5, the livers were finely chopped with scissors and homogenized gently with ten strokes of a Teflon/glass Dounce homogenizer. The homogenate was then centrifuged at 400 x g for 10 min, the pellet discarded, and the supernatant overlayed on a cushion of 55% sucrose in 0.2 M NaCl, 5 mM Na_2 EDTA, 5 mM 2-mercaptoethanol and 10 mM Tris/HCl, pH 7.5 before centrifuging at 12,000 x g for 20 min. The crude mitochondrial fraction was recovered from the

interphase, diluted with three volumes of 25% sucrose in the same buffer and pelleted by centrifugation at 12,000 x g for 20 min. Thereafter, the pellet of mitochondria was processed as for oocyte mitochondria.

(iii) Triturus culture cells

Triturus culture cells were maintained and harvested as described by Rudak (1976). After washing in TBS the cells were vigorously homogenized with ten strokes of a Teflon/glass Dounce homogenizer. Mitochondria were then purified from the cell homogenate in the same way as for oocyte mitochondria.

9. Fractionation of exogenous labelled oocyte nuclear components in an unlabelled Triturus hnRNP preparation

Triturus oocytes were labelled with H^3-uridine and ^{14}C-amino acid mixture as described earlier. After several washes in Barth's medium (Gurdon, 1974) nuclei were isolated from early vitellogenic oocytes of about 0.8 mm diameter into a buffer containing 70 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/HCl pH 7.4, and 2% PVP (see Materials and Methods, Chapter 2, for a more detailed description of this procedure). The isolated nuclei were cleaned and stockpiled in a small droplet of the same solution on a siliconized glass slide in a humidity chamber at 0°C.

Two hundred nuclei were accumulated before adding them to a homogenate of an unlabelled Triturus ovary, their homogenization was completed with a further five strokes of the Teflon/glass homogenizer. Thereafter the hnRNP preparation was continued as normal. The 25 - 55% sucrose gradient was fractionated and the fractions precipitated with 10% TCA, filtered through 2.5 cm diameter Whatman GF/A filters, and rinsed

with three changes of 5% TCA and one of 70% ethanol before drying and counting in a toluene based scintillation cocktail (NE 233).

Alternatively the total 300 x g supernatant was loaded onto a 10 - 60% sucrose gradient in 0.2 M NaCl, 5 mM Na_2 EDTA, 5 mM 2-mercaptoethanol and 10 mM Tris/HCl, pH 7.5, and centrifuged at 180,000 x g for 1 h at 4°C in an M.S.E. 6 x 14 ml Ti swing-out rotor. The gradient was fractionated and their radioactivity counted as outlined above.

RESULTS

1. Composition of hnRNP from Triturus oocytes

HnRNP was isolated from homogenates of early to mid-vitellogenic ovaries of Triturus cristatus carnifex by differential centrifugation and sedimentation on 25-55% sucrose gradients (Sommerville, 1973). The hnRNP sediments as a visible turbid zone to a density position at about 42% (w/v) sucrose (Fig. 1.1), equivalent to a density of 1.18 gcm^3 and a protein to RNA ratio in excess of 20:1. The hnRNP sediments very rapidly at a rate greater than 1500S suggesting that it is in a highly aggregated state.

SDS/polyacrylamide electrophoresis of the hnRNP proteins reveals a heterogeneous polypeptide distribution (Fig. 1.2) with molecular masses ranging from 10,000 to 200,000. The most prolific polypeptides lie in the 40,000 to 70,000 molecular mass range.

2. Electron microscopy of Triturus oocyte hnRNP

Material from the hnRNP density peak was prepared for electron microscopy in two different ways, either by droplet diffusion spreading (Malcolm and Sommerville, 1974) or by thin sectioning of fixed, pelleted material (Malcolm and Sommerville, 1974; Hurkman et al., 1981).

Droplet diffusion spreading of the hnRNP fraction reveals large aggregates of beaded material which are densely stained by both uranyl acetate and phosphotungstic acid (Fig. 1.3a). The beaded particles, though irregular in shape, have a mean diameter of approximately 20 nm. Occasionally in rotary shadowed preparations, fine fibrils are observed to emanate from the RNP aggregates. These fibrils are seen much more clearly after treatment of hnRNP aggregates with 4 M urea (Fig. 1.3b). a network of filamentous structures being revealed.

Fig. 1.1.

Absorbance and distribution of the radioactively labelled RNA and protein after centrifugation of the resuspended crude hnRNP in a 25-55% sucrose gradient.

- - - A_{254}; ●—● RNA; o—o protein.

Fig.1.1

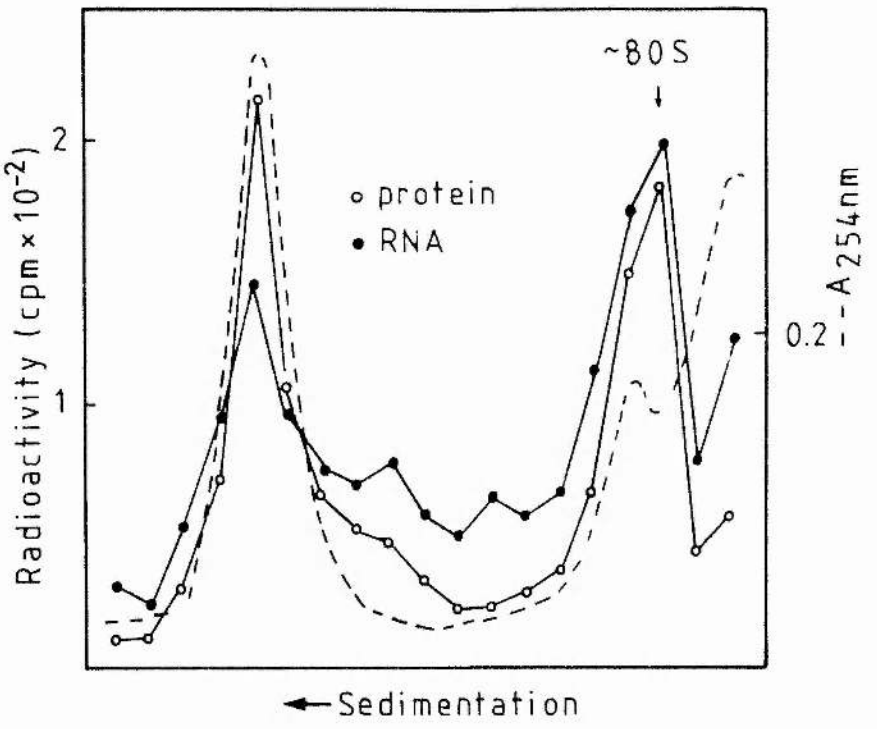

~80S
protein
RNA
Radioactivity (cpm × 10⁻²)
2
1
0.2
- - A254nm
Sedimentation

Fig. 1.2.

SDS/polyacrylamide gradient gel electrophoresis of Triturus hnRNP protein. Molecular mass is indicated at x 10^{-3}.

Fig.1.2

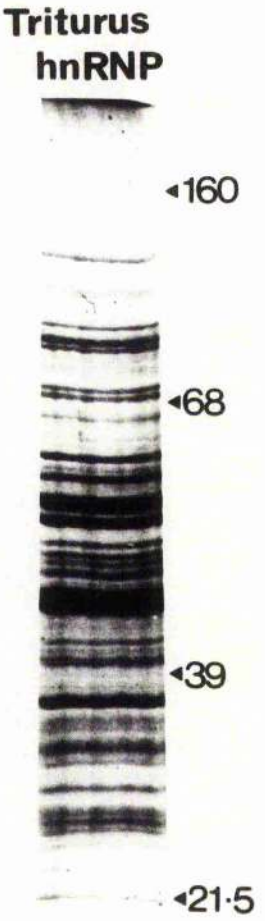


Fig. 1.3 a - f.

Electron micrographs of material derived from sucrose gradient fractionation of Triturus oocyte hnRNP.

(a) Nuclear RNP aggregate obtained from the first sucrose gradient separation (See Fig. 1.1). This preparation has been rotary-shadowed and fine fibrils can be seen (arrows) emanating from the dense RNP structures.

(b) The same material as shown in (a) but after treatment with 4 M urea. Here the RNP is more dispersed and many filamentous structures can be seen with RNP attached to them.

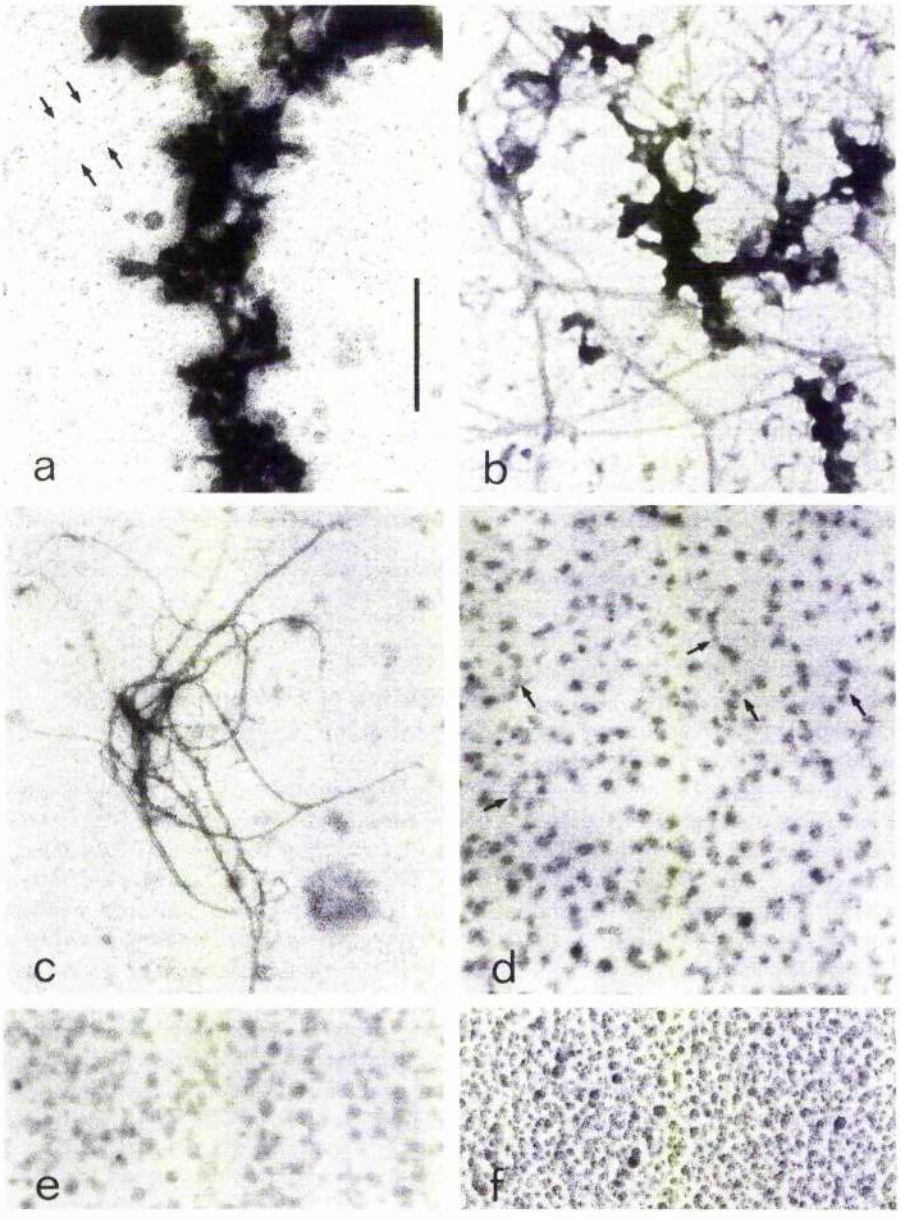
(c) Isolated filamentous network derived from the A_{254} peak near the bottom of the second sucrose gradient fraction (see Fig. 1.7).

(d) RNP particles derived from the gradient regions containing the peaks of radioactivity shown in Fig. 1.7. The 20 nm particles are often interconnected as linear strings (arrows).

(e) Protein aggregates derived from the unlabelled, absorbing peak of material near the top of the gradient shown in Fig. 1.7.

(f) The same material as seen in (e) but after rotary shadowing. The preparations are stained with phosphotungstic acid as described by Malcolm and sommerville, (1977).

All micrographs are x 90,000 (the bar represents 0.2 μm).


a
b
c
d
e
f

However, when the hnRNP-containing gradient fraction is fixed, and embedded for electron microscopy quite different structures are revealed (Fig. 1.4a). A high percentage of the material is seen to be mitochondrial in origin, their characteristic double membrane and infolding cristae being very obvious. Comparison of this material with sections taken from a higher speed pellet of ovary homogenates (Fig. 1.4b), expected to contain the bulk of the oocyte mitochondria, shows very few differences between the two. However regions of amorphous, aggregated material are much more prevalent in the hnRNP containing peak (Fig. 1.4c and d).

3. Sarkosyl treatment of Triturus oocyte hnRNP aggregates

If the hnRNP pellet is resuspended in a buffer containing 0.5% sarkosyl an enrichment of certain polypeptide species is found associated with the bulk of the labelled RNA in the 100,000 x g pellet (Fig. 1.5a). Several RNA associated polypeptides of molecular masses between 20,000 and 84,000 are detected. The polypeptide profile of the 100,000 g supernatant is very similar to that of the untreated material (Fig. 1.5b).

4. Buoyant density determination of Triturus oocyte hnRNP

Density equilibrium centrifugation of the formaldehyde-fixed hnRNP fraction on 24-60% CsCl gradients yields a density peak containing the bulk of the labelled RNA at a density of 1.39 gcm^3. In these gradients a considerable quantity of precipitated proteinaceous material bands near the top of the gradient and does not appear to be associated with any labelled RNA.

Fig. 1.4 a- d.

Electron micrograph of thin sectioned material derived from sucrose gradient fractionation of Triturus oocyte hnRNP and mitochondrial preparations.

(a) Material purified from the 4000 x g pellet of ovary homogenate showing characteristic cross-sections of mitochondria, the infolding cristae are clearly visible.

(b) Material purified from the 12,000 x g pellet of ovary homogenate showing the expected mitochondrial cross-sections.

(c and d) Regions of amorphous, aggregated material peculiar to the hnRNP containing 4000 x g pellet. The preparations were prepared and stained according to the method of Hurkman et al., (1981). All micrographs are x 45,000 (the bar represents 0.4 μm).

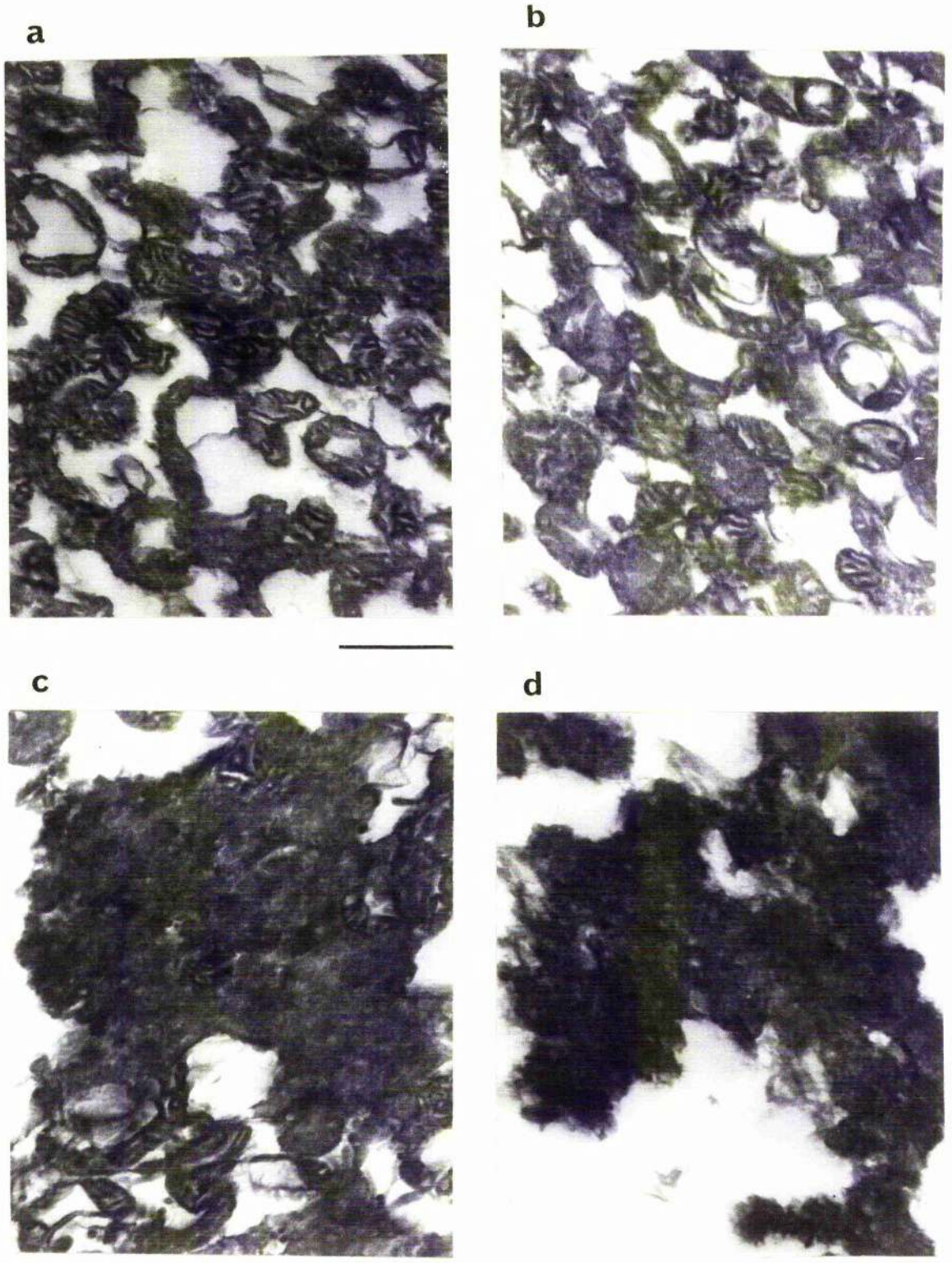

a
b
c
d

Fig. 1.5 a - b.

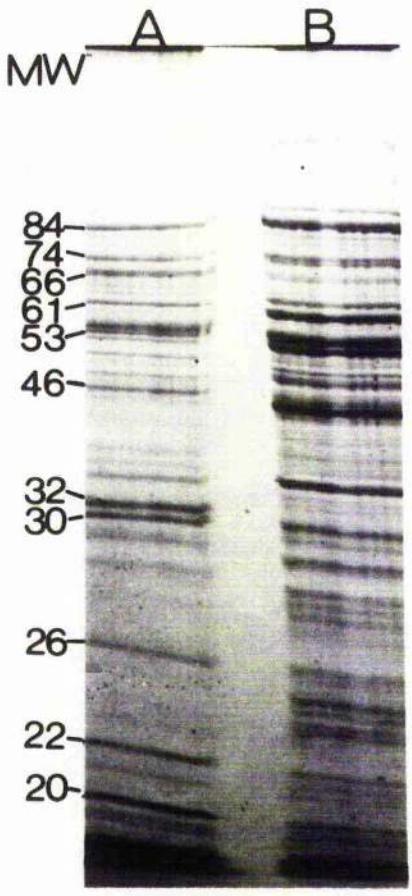
SDS/polyacrylamide gradient gel of Triturus oocyte hnRNP proteins.

(a) Proteins remaining associated with the labelled RNA in the 100,000 x g pellet after treatment with 0.5% - sarkosyl.

(b) Proteins remaining in the 100,000 x g supernatant after treatment of the hnRNP with 0.5% sarkosyl.

Molecular mass is indicated at x 10^{-3}.

Fig.1.5



After treatment with 0.5% sarkosyl (see above) prior to fixation, a density peak at 1.43 gcm^3 is detected (Fig. 1.6). The density of the native hnRNP at 1.39 gcm^3 and the sarkosyl-treated hnRNP at 1.43 gcm^3 correspond to protein to RNA ratios between 4:1 and 3:1 which is fairly typical for hnRNP (Preobrazhensky and Spirin, 1978).

5. Separation of the components of Triturus oocyte hnRNP aggregates by rehemogenization and recentrifugation

When labelled hnRNP from the initial sucrose gradient fractionation is pelleted, raised in the same gradient buffer, applied to a 5-60% sucrose gradient and recentrifuged at 70,000 x g for 15 h then the profile of absorbance and the distribution of radioactivity is very different from that observed during the first run. Instead of a single turbid zone containing the labelled RNP, the absorbing material and the radioactivity are heterogeneously distributed throughout the gradient. Three distinct regions can be determined (Fig. 1.7); though considerably reduced from the first run, there remains some turbidity at a density position of 1.18 gcm^3 but the bulk of the absorbance at both 254 nm and 280 nm is found at the top of the tube with a peak of sedimentation at 10-20S. Neither of these fractions contain a signicant level of ^3H-uridine or ^{14}C-amino acid incorporation. Most of the labelled RNA and protein is detected across a heterogeneous sedimentation range between 30S and 120S, though the relative distribution of counts between the three fractions varies between preparations.

Density equilibrium centrifugation shows that the labelled material in the 30-120S region has a density of 1.39 gcm^3 (Fig. 1.8) identical to that of the native hnRNP preparation. The density of the unlabelled material sedimenting at 10-20S is much lower at about 1.25 gcm^3 (data not shown).

Fig. 1.6 a - b.

Density equilibrium centrifugation of formaldehyde fixed hnRNP material. RNP was fixed with neutralized 17% formaldehyde to a final concentration of 3.4% and centrifuged through a preformed 24 - 60% CsCl gradient.

(a) Native Triturus oocyte hnRNP.

(b) Sarkosyl treated Triturus oocyte hnRNP.

■——□ RNA; —X——X— density.

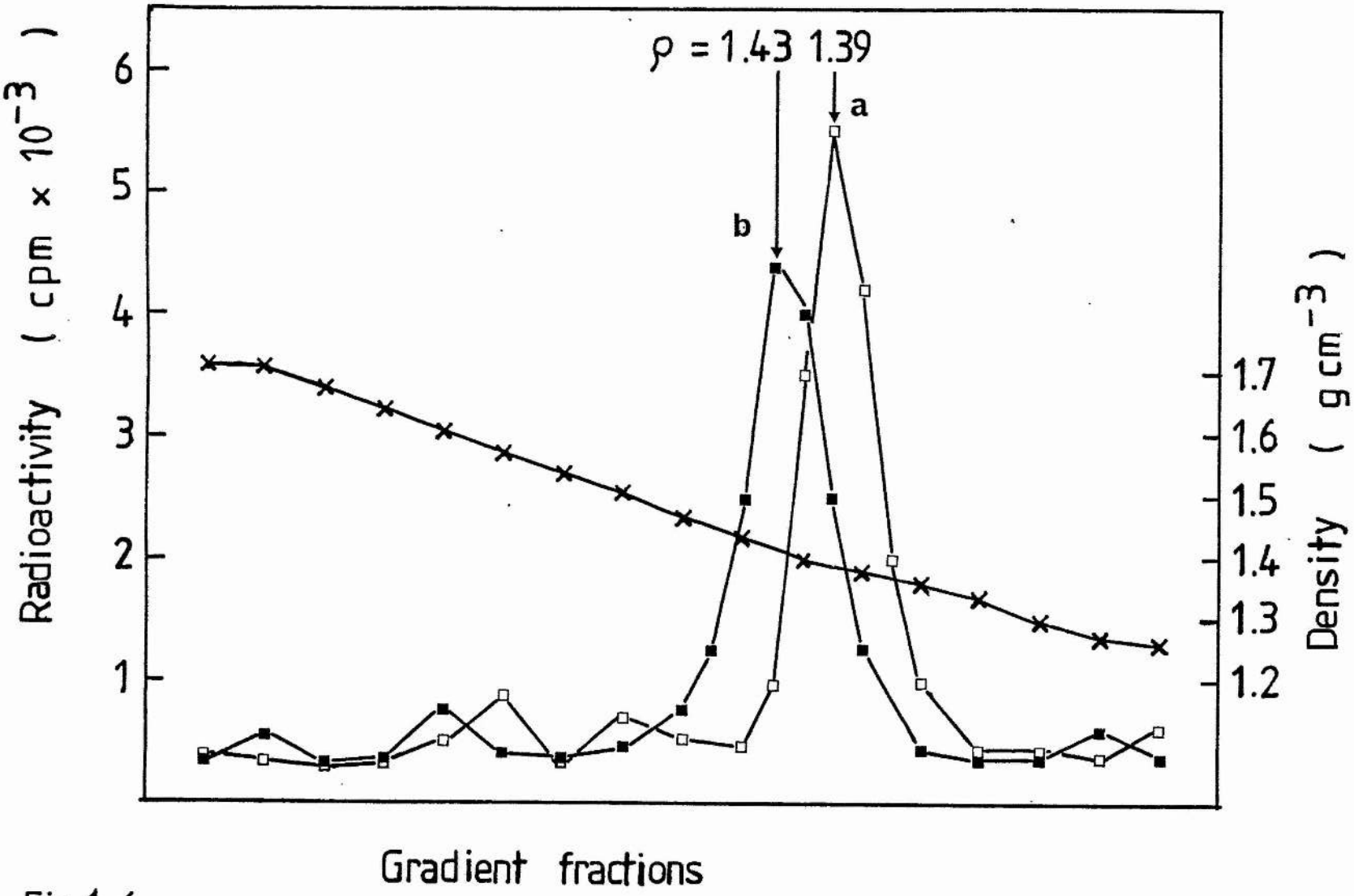

ρ = 1.43 1.39
a
b
Radioactivity (cpm × 10⁻³)
Density (g cm⁻³)
Gradient fractions
1
2
3
4
5
6
1.2
1.3
1.4
1.5
1.6
1.7

Fig.1.6

Fig. 1.7.

Absorbance and distribution of radioactively labelled RNA and protein after rehomogenization and recentrifugation of the turbid hnRNP fraction in a 5-60% sucrose gradient. Three main fractions are obtained:

(1) A rapidly sedimenting matrix with a density of 1.18 g cm^{-3} in sucrose.

(2 and 3) Heterogeneously sedimenting RNP which contains most of the rapidly labelled RNA. This RNP fraction has a density of 1.39 gcm^{-3} in CsCl after formaldehyde fixation and a density of 1.43 gcm^{-3} after treatment with 0.5% sarkosyl prior to fixation.

(4) Slowly sedimenting protein aggregates.

A_{254} - - - ; ●——● RNA.

Fig.1.7

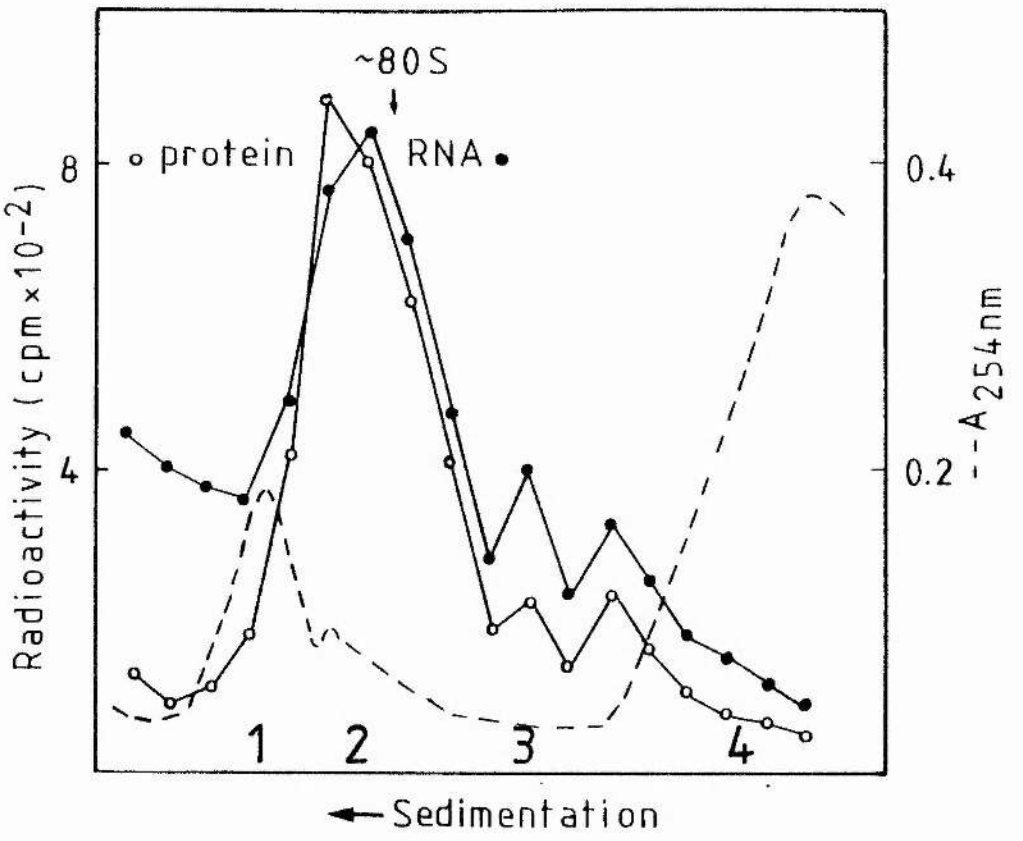
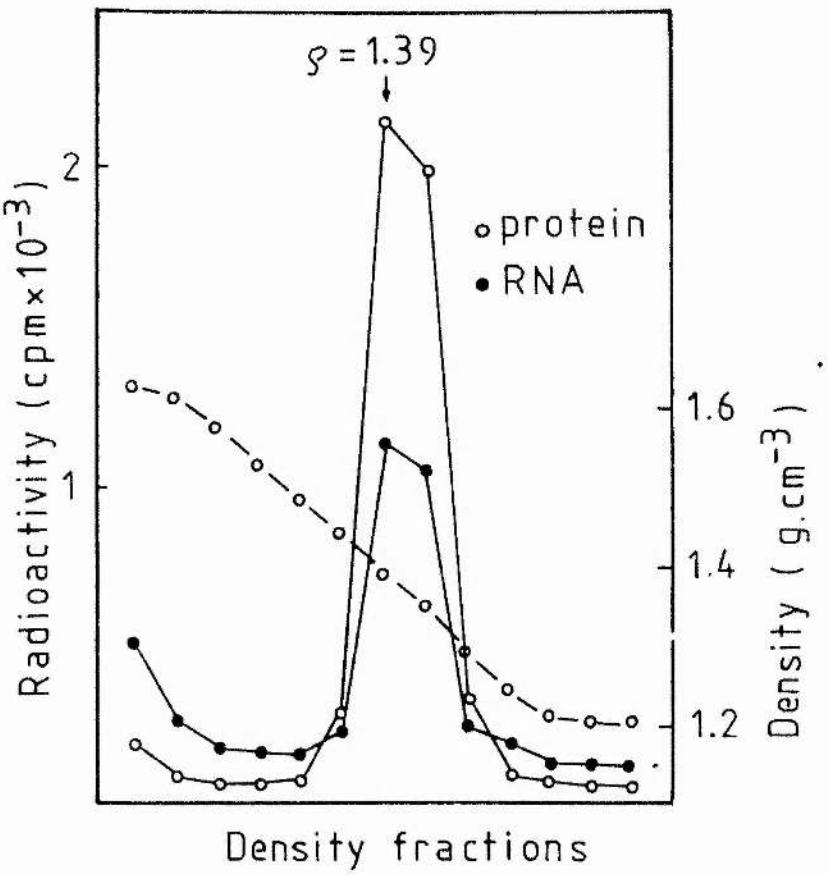

~80S
o protein
RNA •
8
4
0.4
0.2
Radioactivity (cpm×10⁻²)
- - A 254nm
1
2
3
4
← Sedimentation

Fig. 1.8.

Density equilibrium centrifugation of formaldehyde-fixed hnRNP material. The rehomogenized hnRNP preparation was fixed with neutralized 17% formaldehyde to a final concentration of 3.4% and centrifuged through a preformed 24-60% CsCl gradient.

Fig.1.8


ρ = 1.39
protein
RNA
Radioactivity (cpm×10⁻³)
Density (g.cm⁻³)
Density fractions
2
1
1.6
1.4
1.2

Examination of the recentrifuged fractions by electron microscopy reveals that the material continuing to sediment to a density of 1.18 gcm^3 consists largely of a fibrous network (Fig. 1.3c) the main filaments having a width of 10 nm with finer filaments branching from them.

The unlabelled material sedimenting at 10-20S consists of roughly spherical particles with diameters in the range of 5-20 nm (Fig. 1.3e and f). However the beaded nature of this fraction is not evident at low concentration.

The labelled RNP fractions appear as beaded particles of approximately 20 nm diameter, most of which are connected in linear arrays of 2-6 units (Fig. 1.3d).

The polypeptide profile of the recentrifuged material is quite distinctive (Fig. 1.9). Most of the polypeptides present in the original hnRNP preparation are represented in the top fractions sedimenting at 10 to 20S, very little labelled RNA or protein associates with this fraction. The labelled protein is largely associated with the labelled RNA as RNP with a modal sedimentation velocity of about 80S, and consists of a set of six major polypeptides within the molecular mass range 52,000 to 68,000 with minor bands at 72,000, 74,000 and 84,000.

The fibrous network is associated with a heterogeneous assortment of polypeptides over a similar range of molecular mass as the RNA associated fraction and it is not certain, as yet, which of the bands correspond to the fibrous material.

Fig. 1.9.

SDS/polyacrylamide gradient gel electrophoresis of recentrifuged Triturus hnRNP proteins, showing the polypeptide constituents of the gradient fractions (1 to 4) described in Fig. 1.7; track (T) contains the native hnRNP preparation. Molecular mass is indicated at x 10^{-3}.

Fig.1.9

6. Fractionation of exogenous, labelled oocyte nuclear components in an unlabelled Triturus hnRNP preparation

If manually isolated nuclei from ^{3}H-uridine labelled oocytes are included in an otherwise standard hnRNP preparation from unlabelled ovary homogenate then about 10% of their labelled RNA is detected co-sedimenting with the hnRNP peak (Fig. 1.10). Approximately 80% of the label sediments with a sedimentation coefficient in the range of 40S to 200S.

If however the entire 300 x g supernatant, rather than just the 4000 x g pellet of crude hnRNP derived from it, is fractionated on a 10% to 60% sucrose gradient then both the radioactive RNA and protein (from double labelled oocyte nuclei) are detected co-sedimenting to an approximate density of 1.18 gcm^{-3}, some radioactivity is also detected sedimenting between 40S and 200S but the bulk of the counts are recorded in the top fractions of the gradient (Fig. 1.11). An accurate assessment of the relative quantities of radioactivity in these gradients is complicated by the rather high levels of quenching produced by the heavy precipitates in some fractions and also by the possibility that unincorporated radioactive precursors might be retained in association with precipitated material.

Fig. 1.10.

Absorbance and distribution of radioactively labelled RNA from exogenous Triturus oocyte nuclei in a normal preparation of hnRNP from an unlabelled Triturus ovary homogenate in a 25-55% sucrose gradient.

●——● RNA; ——— A_{254}.

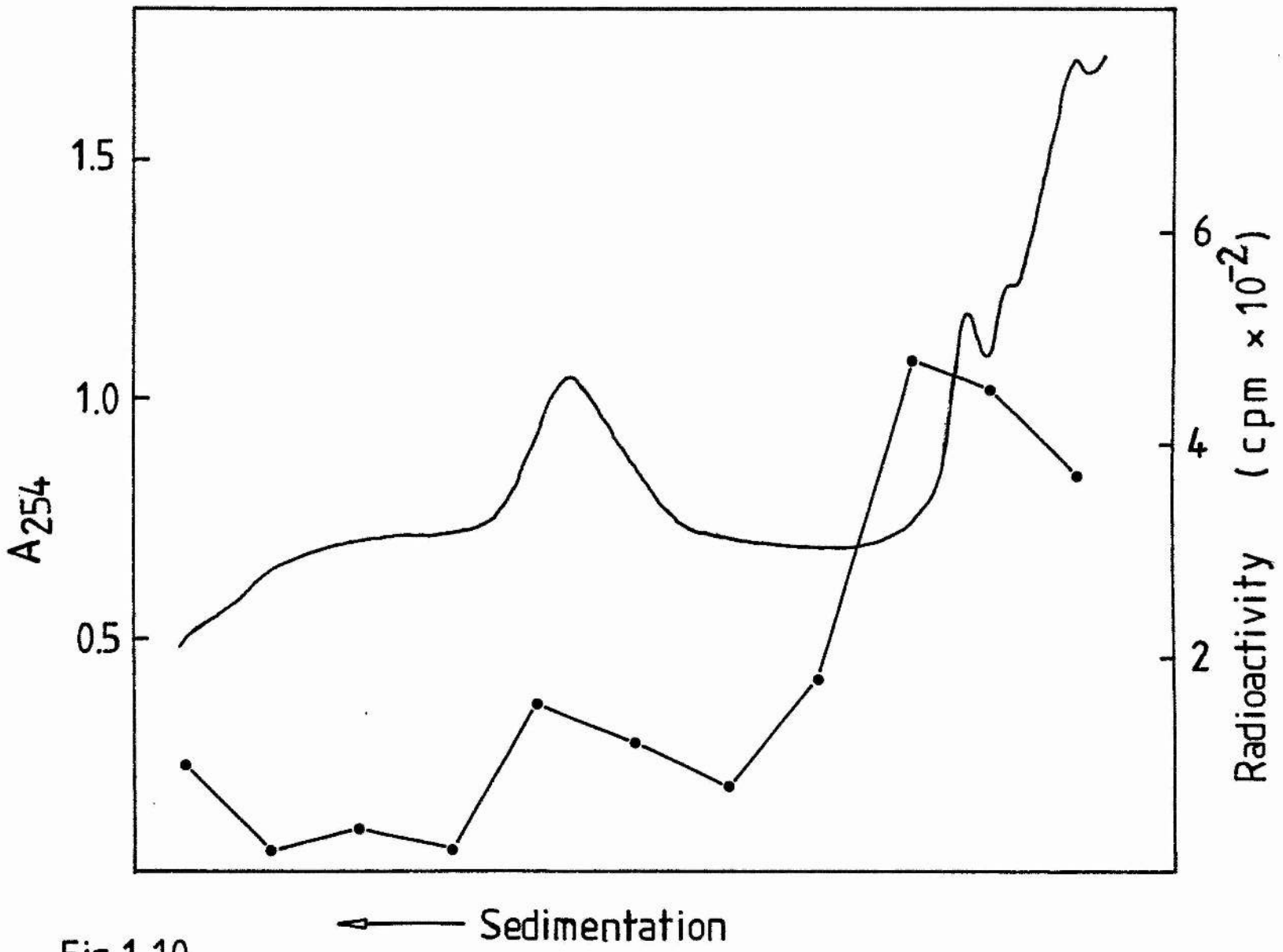

1.5
1.0
0.5
A_{254}
6
4
2
Radioactivity (cpm $\times 10^{-2}$)
Sedimentation

Fig.1.10

Fig. 1.11.

Absorbance and distribution of radioactively labelled RNA from exogenous Triturus oocyte nuclei in a preparation of hnRNP from the 300 x g pellet of an unlabelled Triturus ovary homogenate in a 20-60% sucrose gradient.

●——● RNA; ——— A_{254}.

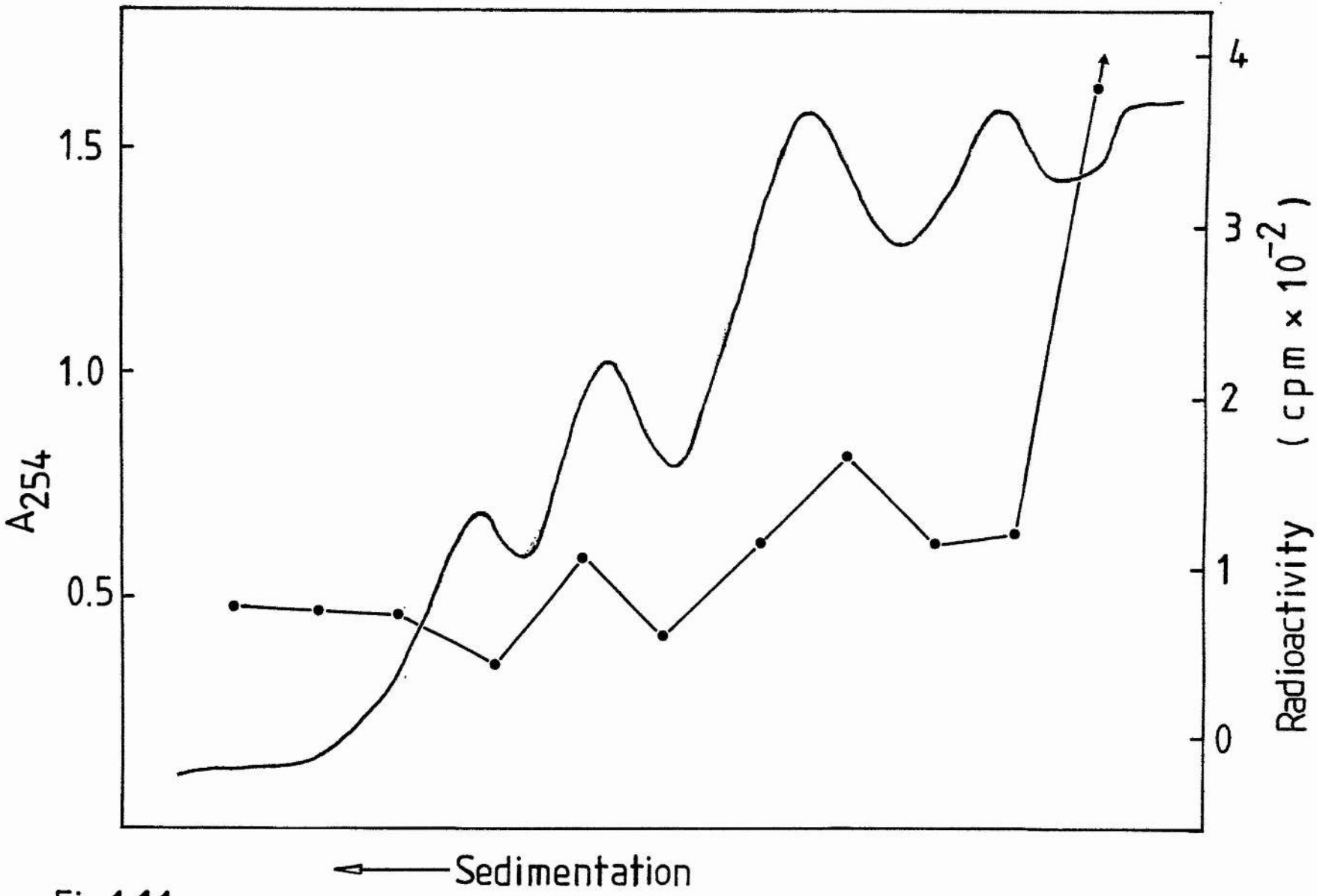
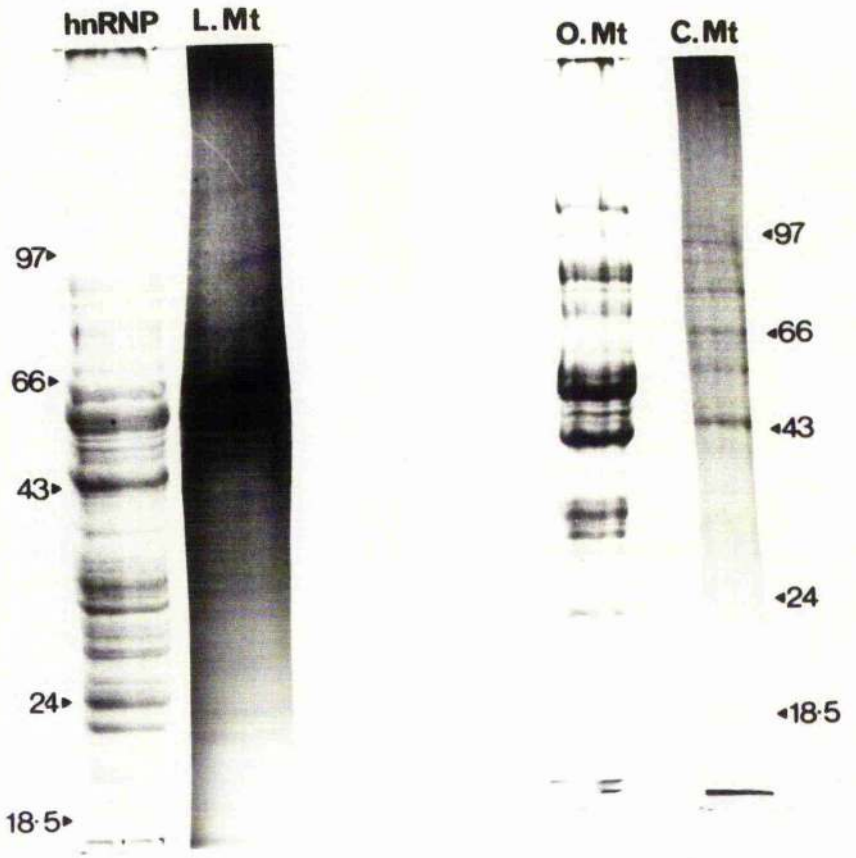


Fig.1.11

Fig. 1.12.

SDS/polyacrylamide gels comparing Triturus oocyte "hnRNP" proteins with Triturus liver mitochondrial proteins (L. Mt.), Triturus oocyte mitochondrial proteins (O. Mt.), and Triturus culture cell mitochondrial proteins. Molecular mass is indicated at x 10^{-3}.

Fig.1.12



DISCUSSION

It is a general feature of oogenesis that macromolecules and organelles are accumulated to prepare the oocyte for the rapid cycles of cell division that accompany embryogenesis (see Davidson, 1976 for review). Amphibian oogenesis entails intensive transcription of both ribosomal and chromosomal RNA (Gall, 1966; Davidson and Hough, 1969; Davidson et al., 1966). This high synthetic activity results in a mature oocyte of Xenopus laevis containing about 2×10^{11} polyadenylated RNA molecules (Cabada et al., 1977) in the form of either hnRNP or messenger RNP complexes. Thus, in amphibian oocytes, the storage role of RNP complexes is of particular significance, perhaps more so than in somatic cells.

In view of this functional difference and the morphological diversity of lampbrush chromosome lateral loops evident from both light microscopy Callan and Lloyd, 1960) and electron microscopy (Mott and Callan, 1975), it seems reasonable to expect differences in the organization and polypeptide composition of germ line hnRNP in comparison to its somatic equivalent. With these considerations in mind, the heterogeneous polypeptide constitution of the Triturus hnRNP and its lack of homology with hnRNP from other sources (such as rat liver), is not particularly surprising. The status of this fraction as a homogeneous hnRNP preparation is supported by various lines of evidence catalogued below (as summarised by Sommerville, 1977).

(a) It is rapidly labelled when oocytes are incubated in the presence of radioactive precursors of both RNA and protein (Sommerville, 1973).

(b) The protein to RNA ratio is greater than 30:1, a ratio similar to that for chromosome bound transcripts (Sommerville, 1973).

(c) A substantial increase in the amount of labelled RNA and protein in this material results from treating the oocytes with 25µg/ml of actinomycin D, a treatment known to release RNP transcripts from the chromosomes (Sommerville, 1973).

(d) It consists of linear arrays of 20 nm particles which are similar in appearance to chromosomal RNP (Malcolm and Sommerville, 1974; Sommerville and Hill, 1973). These linear strings of beads are reduced to monoparticles by low concentration of ribonuclease but not deoxyribonuclease (Malcolm and Sommerville 1974 and 1977).

(e) The beaded RNP can be converted into long fibrils by treatment with dissociative agents and in this state resembles the RNP fibrils of spread chromosomes in structure and dimension (Sommerville and Malcolm, 1976; Malcolm and Sommerville, 1977).

(f) The RNA extracted from this material has a base composition which is significantly different from ribosomal RNA (Sommerville, 1973); it has a heterogeneous range of sedimentation coefficients (Sommerville and Malcolm, 1976; Malcolm and Sommerville 1977; Sommerville, 1973), and it contains some nucleotide sequences that are homologous to oocyte polyadenylated mRNA sequences (Sommerville and Malcolm, 1976).

(g) The protein derived from this material consists of large numbers of different polypeptides with a considerable diversity of molecular mass (Sommerville and Malcolm, 1976; Scott and Sommerville, 1974; Sommerville et al., 1978; Sommerville and Hill, 1973). The polypeptide profile on SDS/polyacrylamide gels covers the molecular mass range of 10,000 to 150,000 and has little or no coincidence with the polypeptide profile of any other cell fraction, including nucleolar proteins (Sommerville

and Hill, 1973), ribosomal proteins, 42S RNP proteins, and DNP histones (Malcolm and Sommerville, 1977), but is similar to the polypeptide profile of proteins extracted from the nucleoplasm and the chromosomes of manually isolated oocyte nuclei (Maundrell, 1975).

(h) Antiserum prepared against the RNP proteins reacts specifically with the lateral loops of lampbrush chromosomes and not with the condensed DNP of the chromomeres or with nucleoli (Scott and Sommerville, 1974; Sommerville et al., 1978).

The homogeneous status of the hnRNP preparation must now be reconsidered in the light of the evidence revealed in this chapter.

The fate of exogenous, labelled, oocyte nuclear components when added to a normal unlabelled hnRNP preparation confirm that some of the labelled RNA in the hnRNP peak is nuclear in origin however the high proportion of label at the top of the gradient indicates either that there is considerable breakdown of hnRNA during the process of nuclear isolation and hnRNP preparation or that the bulk of the label is incorporated into smaller RNP structures. The latter possibility is accounted for in part by the likelihood of considerable incorporation of ^{3}H-uridine into ribosomal RNA, but smaller hnRNP complexes cannot be excluded. Unfortunately this experiment has not been repeated with the use of low concentrations of actinomycin D to inhibit polymerase I activity, which might well reveal the precise location of the nascent RNP by causing an alteration in the radioactive profile of the gradient in comparison with an untreated preparation.

The contradictory nature of the electron microscopic data displayed here is somewhat confusing. Although the droplet diffusion spreading of hnRNP largely confirms the findings of Malcolm and Sommerville (1974 and 1977) and further suggests the presence of some sort of fibrous network,

possibly nuclear matrix, in the material, the thin sections of fixed hnRNP quite clearly indicate a high level of mitochondrial contamination. This contamination is confirmed both by the presence of mitochondrial ribosomal RNA on agarose gels of the total RNA from the hnRNP peak, and, by the coincidence of latent malic dehydrogenase activity with that peak, released by concentrations of Triton X 100 known to cause mitochondrial lysis (R.M. Johnson, personal communication).

Previous high voltage electron microscopy of 5 μm sections of fixed hnRNP material revealed irregularly shaped particulate structures between 50 and 100 nm in diameter (Malcolm and Sommerville, 1974). The "completeness" of the individual particles, commented upon at the time was considered to be due to glutaraldehyde fixation rounding off the less coherent structures of the native state. It is tempting to reinterpret this result as being due to mitochondrial contamination, however the size of even the largest aggregate is considerably less than that shown by the thin sectioned mitochondrial material and unless some artifact of fixation is again invoked then it is as difficult to reconcile these particles with mitochondria as it is with hnRNP.

The obvious beaded nature of the hnRNP material seen in droplet diffusion spreads, its linear "beads-on-a-string" appearance when treated with formamide and its reduction to monomer particles upon mild ribonuclease treatment (Malcolm and Sommerville, 1977) would all seem to be incontrovertible proof that, whatever the degree of cytoplasmic contamination, some hnRNP complexes must be present. Why it is that mitochondrial structures are not evident in droplet diffusion spreads of this material is unclear, perhaps the mitochondria for reasons of size or otherwise do not adsorb to the 'formvar' coated grids, allowing a misrepresentation of the structures present in the gradient fraction.

Although the specific reaction of antisera with lampbrush chromosome lateral loops is suggestive of a ribonucleoprotein antigen, the manner in which these antisera were raised (Scott and Sommerville, 1974; Sommerville et al., 1978) against selected size classes or pI ranges does not precisely define the antigenic polypeptides involved. These antigens may or may not be major constituents of the material in the density peak and as such only qualitative rather than quantitative conclusions can be made; hnRNP complexes must be present in this gradient fraction but whether they represent a major contribution to the polypeptide complement is open to doubt.

The claim (Sommerville, 1977) that there is similarity between the polypeptide profile of proteins in the hnRNP peak and those of manually isolated oocyte nuclei (Maundrell, 1975) is considered in more detail in Chapter 2 but the homologies that do exist would appear to be minor (as might be expected since the preponderance of oocyte nuclear proteins are found in the soluble fraction and are thus unlikely to be of heterogeneous ribonucleoprotein origins (Maundrell, 1975).

Further complications in the characterization of this material stem from the unknown extent to which the stage of oocyte development influences the partition of sub-cellular components. In early experiments (Sommerville, 1973; Malcolm and Sommerville, 1974 and 1977; Sommerville and Malcolm, 1976) exclusively previtellogenic oocytes were used however subsequent preparations have been rather less rigorous in the selection of oocyte stages. Furthermore, seemingly minor variations in isolation procedure (e.g. an increase of the duration of the 4000 x g centrifugation) may also contribute to some of the conflicting evidence. It would therefore seem that the hnRNP preparation from homogenates of Triturus oocytes is contaminated to an uncertain degree with mitochondria and in addition may well be associated with some form of nuclear matrix.

In order to investigate this further the hnRNP peak material was treated with a number of dissociative agents and the polypeptides that sedimented in association with the radioactive RNA during high speed centrifugation were characterized by SDS/polyacrylamide gel electrophoresis. Previous results of salt dissociation experiments show that in Triturus hnRNP the forces involved in protein/protein interaction are greater than those involved in binding the bulk of the protein to the RNA (Malcolm and Sommerville, 1977). As the salt concentration is increased there is a progressive and proportionate removal of proteins in the form of aggregates sedimenting at 15 to 30S. This result is contrary to the usual behavior of somatic cell RNP where differential removal of proteins occurs under high salt conditions (Pederson, 1974; Gallinaro-Matringe et al., 1975).

Treatment of Triturus hnRNP with varying concentrations of urea and ribonuclease gave only minor changes in the RNA associated polypeptide spectrum (data not shown) however the use of 0.5% sarkosyl (a non-ionic detergent) resulted in considerable enrichment of certain polypeptide species sedimenting in conjunction with the RNA. Since it is known that treatment with 0.5% sarkosyl, whilst removing most of the chromatin associated proteins, does not disrupt the integrity of nascent RNP fibrils in spread preparations (Scheer, 1978) it seems likely that these enriched polypeptide species represent RNP proteins. However antisera raised against selected, sarkosyl resistant hnRNP polypeptides not only showed very low affinity but also gave only weak unlocalized fluorescence with both cryostat sectioned ovary and Triturus culture cells (data not shown). This is a rather disappointing result especially since the low molecular mass enriched polypeptide (32,000, 30,000, 26,000, 22,000 and 20,000) are in a similar molecular mass range to some of the putative snRNA binding proteins (Lerner and Steitz, 1979). Antisera to two of these polypeptides,

P22 and P26 have yielded a positive immunostaining reaction with lampbrush chromosome loops (J. Sommerville and P.-M. Kloetzel, unpublished).

As an alternative approach to further separating the components of the hnRNP containing sucrose gradient fraction, the material was pelleted, homogenized and recentrifuged. The resulting gradient subfractions can be divided into three major zones, the residual turbid region, a strongly absorbing proteinaceous peak at 10 to 20S and a heterogeneous distribution of labelled RNP sedimenting between 30 to 120S. The sedimentation, density value and appearance of this material is characteristic of hnRNP though the restricted group of associated polypeptides, mostly in the mass range of 50,000 to 68,000 are different from those described for hnRNP from mammalian cells (Beyer et al., 1977; Martin et al., 1977; Karn et al., 1977). Despite these differences the two groups do have several properties in common. For instance, they are comprised of both acidic and basic polypeptides (cf.Beyer et al., 1977 and Chapter 2) and readily precipitate in the absence of the RNA (cf. Martin et al., 1977; Karn et al., 1977), and they are not labelled as rapidly as the RNA or certain other nuclear proteins (cf. Martin et al., 1979; Dixon and Ford, 1980b) (data not shown). The correlation, if any, between the hnRNP associated polypeptides revealed by sarkosyl treatment and by recentrifugation is confined to the 50,000 to 80,000 molecular mass range.

The validity of most approaches to hnRNP purification are called into doubt by evidence that suggests that certain biochemical treatments result in the artifactual rearrangement of proteins associated with the RNA (Stevenin and Jacob, 1979). Stevenin et al., also observed that during the course of ribonuclease hydrolysis of pre-messenger ribonucleoproteins unexpected increases in monoparticle sedimentation rate occurred, once again due to an artifactual aggregation of proteins (Stevenin et al., 1979). Hence any prolonged isolation procedure or biochemical treatment

runs a considerable risk of implicating proteins as hnRNA associated which are not normally so *in vivo*. It is therefore desirable to verify the status of hnRNP proteins by several different procedures of isolation and analysis wherever feasible. On this basis, the correlation of the putative hnRNP proteins from the sarkosyl treatment and the recentrifugation is inadequate for the definite implication of any particular polypeptide as an hnRNP constituent although the molecular mass range between 50,000 and 80,000 seems similarly represented in both methods.

As an alternative approach to the positive identification of hnRNP proteins in this fraction the process of eliminating the more obvious contaminating polypeptide species was also adopted. To this end mitochondria from *Triturus* oocytes, liver and culture cells were prepared and their polypeptide profiles compared to that of the hnRNP preparation. Preliminary results of this comparison indicate considerable similarity between oocyte "mitochondrial" and hnRNP preparations but far less homology with mitochondria from other sources. Since the purity of these mitochondrial preparations is as yet unproven, (and doubtful in view of the variation in polypeptide pattern evident [Fig. 1.12]. Further investigation is needed before any meaningful conclusions are possible.

From the evidence described in this chapter hnRNP prepared from homogenates of amphibian ovary is not a homogeneous preparation as first considered by Malcolm and Sommerville (1974). Contamination of the preparation is, in part, mitochondrial in origin despite the relatively low centrifugation speeds used in the preliminary isolation step. The extent of this mitochondrial contamination, though apparently considerable as judged by electron microscopy of thin sections of the pelleted material, has not been unequivocally demonstrated and may be related to the oocyte stages employed. The presence of RNP in the preparation is confirmed

by isopycnic centrifugation, though the typical hnRNP density peak at 1.39 gcm^{-3} (representing a protein to RNA ratio of 4:1) indicates that a considerable proportion of the protein in the original material (protein to RNA ratio of $>$ 20:1) is not closely associated with the RNA and is lost during fixation. Sarkosyl treatment of the hnRNP also dissociates a high proportion of the protein from the hnRNP, the residual RNP is stable to formaldehyde fixation and isopycnic centrifugation, yielding a peak at a slightly higher density. Whether or not the RNA associated polypeptides of the native and sarkosyl treated RNP particles separated by isopycnic centrifugation are the same, cannot be demonstrated after formaldehyde fixation. The utilization of ultraviolet light cross-linking (Greenberg, 1979) or reversible cross-linking reagents (Karn et al., 1977) might resolve this difficulty.

Recentrifugation of hnRNP provides a method by which the RNA associated proteins can be recovered without recourse to dissociative agents. However the results are rather variable and the mechanisms by which RNP is released from associated material or contaminants are not obvious, (they may be dependent on endogenous nuclease activities or perhaps osmotic effects).

In view of the unknown extent of cytoplasmic contamination it is difficult to determine whether the fibrous network revealed by electron microscopical examination of the hnRNP in droplet diffusion spreads represents a form of nuclear matrix or extranuclear cytoskeletal elements.

If the data accumulated here is to be satisfactorily interpreted it is necessary to know which fractions of the hnRNP preparation are predominantly nuclear in origin and which are cytoplasmic. The approach adopted in the following chapter endeavours to clarify this issue and to demonstrate further the possibility that germ-line hnRNP differs in organization and composition to that found in somatic cells.

C H A P T E R 2.

AMPHIBIAN OOCYTE HnRNP PROTEINS

INTRODUCTION

Ever since the earliest biochemical investigations of the structure of hnRNP (Samarina et al., 1965) not only has there been considerable controversy regarding the nature and number of proteins associated with such material but there has also been very little progress made in relating hnRNP structure to its function. To a certain extent the poor understanding of the functional aspects of hnRNP is directly related to the confusion that exists over its composition. In addition, the considerable structural heterogeneity and complexity of hnRNP does not readily lend itself to any form of in vitro analysis, and opportunities for tracing processing pathways in vivo are few. In order to relate the specific hnRNP components to particular processing events it is desirable to investigate a system, analagous to that of the cytoplasmic storage RNP of Triturus oocytes (reported here in a later chapter), where both the RNA species and the processing steps are reasonably well defined. An example of such a defined approach towards an understanding of RNP function is the way in which antisera from patients with the autoimmune disease systemic lupus erythematosus (S.L.E.) have been utilized to probe the function of small nuclear RNP (snRNP). Antibodies to snRNP's have been shown to be produced by SLE sufferers (Lerner and Steitz, 1979), these antibodies have been introduced into both HeLa cell nuclei and HeLa cells that have been infected with adenovirus (Yang et al., 1981; Lenk et al., 1982), causing inhibition of splicing and an alteration of the normal expression of two late adenovirus genes, thus implicating snRNP in splicing events.

An alternative, more general approach to identifying the role of particular RNP proteins in hnRNA processing is to prepare specific antibodies against them and study their chromosomal distribution on the nascent transcripts of lampbrush chromosomes using indirect immunostaining techniques (Scott and Sommerville, 1974; Sommerville et al., 1978 ; Jones et al., 1980). In agreement with the cytological evidence of lampbrush loop heterogeneity (Callan and Lloyd, 1960; Varley and Morgan, 1978) the immunostaining of specific loops with antibodies to RNP proteins suggests that sequence specific protein binding does occur. It is to be expected that sequence specific RNP proteins will prove to be of greater interest than the hnRNP "core proteins" characterized in somatic tissues (Reviewed by Martin et al., 1980) which probably have a predominantly structural role in accordance with their lack of sequence specificity.

If any functional interpretation of amphibian oocyte RNP is to be successful it is necessary that its protein components are identified unambiguously. Previous investigations of nuclear RNP extracted from homogenates of amphibian ovarian tissue have suggested that the nuclear RNP fraction is a structurally homogeneous preparation (Malcolm and Sommerville, 1974; 1977). Evidence presented in the preceeding chapter would argue that this is not the case and that there is considerable cytoplasmic contamination in such preparations. Nevertheless the evidence supporting a significant concentration of nuclear RNP in this fraction cannot be ignored (see Chapter 1 for discussion) and a closer investigation of the origins of the protein components of this fraction is clearly required.

Most methods of hnRNP isolation involve the initial preparation of purified cell nuclei from which hnRNP can then be extracted. In this respect, as a source of hnRNP, amphibian ovarian tissue is at a disadvantage

since the only methods by which the large and fragile oocyte nuclei can be isolated en masse involve prolonged incubations that are detrimental to the integrity of the hnRNP (Scalenghe et al., 1978; Darnborough and Ford, 1981). However it is possible to prepare small numbers of highly purified oocyte nuclei by manual dissection (Gall, 1954; Callan and Lloyd, 1960; Gall and Callan, 1962). Not only can extremely clean preparations of nuclear material be made in this manner but specific oocyte stages can be selected for study, and the various nuclear components can be manually fractionated for further investigation (Hill et al., 1974; Maundrell, 1975; Krohne and Franke, 1980a and b). The total mass of protein in a 0.8 mm diameter oocyte of Triturus cristatus carnifex is about 1.2 µg of which about 100 ng are nucleolar and 30 ng are chromosomal protein (Maundrell, 1975). Hence if SDS polyacrylamide gels of oocyte nuclear protein are silver stained (Switzer et al., 1979; Oakley et al., 1980; Merril et al., 1981) (a method at least fifty times more sensitive than conventional gel staining techniques), then not only can the major nuclear proteins be revealed but many minor species also, without requiring the tedious isolation of large numbers of nuclei. In this way it is possible to verify the nuclear origin of putative hnRNP proteins from total ovary homogenates and to characterize directly the nuclear RNA associated proteins.

Where comparisons are made between putative hnRNP proteins and proteins of confirmed nuclear origin it is necessary that they be chacterized in an unambiguous manner. In preparations where several polypeptides of very similar molecular mass are present then one-dimensional electrophoretic techniques are inadequate in their resolution of the components and they do not rigorously define the identity of specific polypeptides. These problems are largely overcome by the adoption of two-dimensional

electrophoretic methods where polypeptides can be defined by both molecular mass and charge. HnRNP proteins have been fractionated by two dimensional electrophoresis (Beyer et al., 1977; Brunel and Lelay, 1979; Wilks and Knowler, 1980 and 1981) utilizing either isofocussing or non-equilibrium pH-gradient electrophoresis (NEPHGE) in the first dimension. The use of NEPHGE overcomes the problems encountered in electrofocussing the highly basic hnRNP core proteins which tend to be lost from the cathodic end of the gel when focussed to equilibrium (Wilks and Knowler, 1980). An alternative method of two-dimensional electrophoresis, specifically designed to fractionate basic proteins has been utilized by Darnborough and Ford (1981) to characterize oocyte specific messenger RNP proteins in Xenopus laevis. In this system separation in the first dimension is achieved on a Triton/acid/urea gel system and thereafter by SDS/polyacrylamide gradient electrophoresis.

As an adjunct to the electrophoretic characterization and comparison of hnRNP proteins from different sources, the development of "Western blotting" techniques, whereby proteins are transferred by diffusion or electrophoretically from acrylamide gels to nitrocellulose sheets or other media, has proved invaluable for the immunolocalization of specific polypeptide antigens in complex protein mixtures (Renart et al., 1979; Towbin et al., 1979; Bowen et al., 1980). Using antibodies raised against proteins from the 30S ribonucleoprotein complexes of mouse Taper hepatoma ascites cell nuclei, homologous antigens are detected in extracts of total nuclear protein fractionated by SDS/polyacrylamide gel electrophoresis (Jones et al., 1980). The conserved nature of the hnRNP "core proteins" from somatic cells should enable their homologues in amphibian oocyte nuclei to be recognized using specific antisera. Martin et al., (1980)

show that antisera against 30S hnRNP core proteins react specifically with Triturus oocyte lampbrush chromosomes. Although the loops are highly stained and the polarity of width of the staining around the loop core reflects the nascent RNP polarity in some cases, caution must be exercised in the interpretation of such results since serum concentrations above 1:250 react non-specifically with lampbrush loop transcripts (Sommerville et al., 1978).

The objectives of the work reported in this chapter are as follows:

(a) To characterize the nuclear components present in the crude hnRNP preparations from ovary homogenates by comparison with proteins extracted from manually isolated and fractionated oocyte nuclei.

(b) To utilize two-dimensional electrophoretic techniques to identify the hnRNP "core protein" homologues in manually isolated oocyte nuclei.

(c) To identify proteins, antigenically related to the hnRNP "core proteins", in both the crude hnRNP preparation and the manually isolated oocyte nuclei.

The crude hnRNP preparation is reconsidered as a significant source of nuclear ribonucleoprotein and the possibilities of contamination by stored cytoplasmic messenger RNP are discussed. It is proposed that amphibian oocyte hnRNP and perhaps germ-line hnRNP in general may differ in several respects from that isolated from somatic cells.

MATERIALS AND METHODS

1. Manual isolation of oocyte nuclei

Ovaries were excised from anaesthetized female Triturus cristatus carnifex of varying stages of maturity and washed and stored in modified Barth's medium (Gurdon, 1974) at room temperature. Small portions of ovary were transferred to a black glass embryo-cup containing nuclear isolation buffer (70 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM Tris/HCl pH 7.4, 2% Polyvinylpyrolidone). The oocytes were punctured using a sharp steel dissecting needle and the nuclei expelled by gently squeezing with watchmakers forceps. The nuclei were picked up with the aid of a flame polished, narrow-bore pipette (Callan and Lloyd, 1960) and transferred to a second embryo-cup containing clean buffer. All visible traces of yolk and cytoplasm were removed by repeatedly drawing the nuclei up and down a narrow-bore pipette. The clean nuclei were transferred, for storage, to a small droplet of nuclear isolation buffer on a siliconized glass slide in a humidity chamber at 0°C.

2. Fractionation of oocyte nuclear homogenates on small sucrose gradients

Isolated oocyte nuclei were homogenized in 50 ul of the appropriate buffer by drawing them in and out of an Eppendorf pipette until all the nuclei were obviously dispersed. The homogenate was applied to a 1 ml 25% to 70% sucrose gradient in the same buffer. Gradients were formed as single steps and allowed to diffuse overnight at 4°C. After centrifugation in an M.S.E. 3x 5 ml aluminium rotor (with 1 ml adaptors) at 80,000 x g for 4 h at 4°C, gradients were rapidly frozen in liquid nitrogen and fractionated by cutting the tube into several equal lengths whilst still frozen. Fractions were defrosted in Eppendorf tubes and aliquots precipitated for radioactive counting and gel electrophoresis as described earlier.

3. Microdissection of oocyte nuclei

Oocyte nuclei were dissected manually into fractions containing soluble nucleoplasm, nuclear membranes and nucleoli, and chromatin by an adaptation of the method of Maundrell, (1975). The nuclear membranes of isolated oocyte nuclei (from immature oocytes of approximately 0.8 mm diameter, at which stage the bulk of the nucleoli adhere to the nuclear membrane) were torn open with a sharp tungsten needle, and the nuclei incubated for 15 min at 0°C in a buffer containing 0.1 M NaCl,0.1mM $CaCl_2$ and 10 mM Tris/HCl pH 7.5. In this buffer the nucleoplasm becomes fluid (Callan and Lloyd, 1960) and can be separated from the nuclear membrane and its adherent nucleoli by gentle pipetting with a narrow-bore, flame polished pipette. Nuclear membranes and nucleoli were then stockpiled in a small droplet of nuclear isolation buffer on a siliconized slide in a humidity chamber at 0°C. Chromatin, free nucleoli, and any other insoluble nuclear constituents were separated from the soluble nucleoplasmic proteins by centrifugation of the nuclear membrane free residue in an M.S.E. benchtop Eppendorf centrifuge at high speed for 10 min. After precipitation overnight at -20°C with two volumes of ethanol, the soluble nuclear fraction, the insoluble nuclear fraction and the nucleolar and membrane fraction were all raised in electrophoresis sample buffer for analysis on SDS/polyacrylamide gels.

4. Extraction of hnRNP from rat liver

(i) Isolation of hepatocyte nuclei

Monomer 40S hnRNP particles were extracted from rat liver as described by Karn et al., (1977).

Livers were excised rapidly from four to six-month old male or female rats (freshly killed by concussion followed by cervical section), and placed into ice-cold 0.32 M sucrose, 3 mM $MgCl_2$. The livers were minced finely with scissors and suspended in fresh 0.32 M sucrose, 3 mM $MgCl_2$ ($\sim$ 50ml per 10g of tissue). Aliquots were homogenized using 20 strokes of a motor driven Teflon pestle at 1600 r.p.m. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000 x g for 5 min. The supernatant was aspirated off the crude red nuclear pellet which was then resuspended in 2.2 M sucrose, 5 mM $MgCl_2$ ($\sim$ 30 ml per 10 g of tissue), 30 ml aliquots of the suspension were layered onto 10 ml cushions of 2.2 M sucrose, 5 mM $MgCl_2$ and centrifuged in an M.S.E. 6 x 38 ml Ti rotor at 80,000 x g for 1 hr. A clean white pellet of nuclei was obtained.

(ii) Extraction of 40S hnRNP monomers from rat hepatocyte nuclei

The purified nuclear pellet was suspended in Buffer 'A' (0.1 M NaCl, 10 mM triethanolamine, pH 7.0, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 6 ml per 10 g of tissue). The suspension was centrifuged at 800 x g for 5 min to pellet the nuclei, and this washing process was repeated three times before resuspending the nuclei in Buffer 'B' (0.1 M NaCl, 10 mM triethanolamine pH 8.0, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 6 ml per 10 g of tissue). After a single, rapid wash in Buffer 'B' the nuclei were pelleted at 800 x g for

5 min and the pellet resuspended in 1 ml of Buffer 'B'. The nuclear suspension was stirred gently (by means of a small magnetic stirrer) at 4°C for 30 min, it was then centrifuged at 800 x g for 5 min and the supernatant (containing released 40S hnRNP monomers) retained. This extraction was repeated a further three times, and the four pH 8.0 extracts combined. Nuclear debris was removed by centrifugation at 2000 x g for 10 min and the 2 ml aliquots of the supernatant were applied to 38 ml 15 to 30% sucrose gradients in Buffer 'B'. These were centrifuged in an M.S.E. 6 x 38 ml Ti rotor at 80,000 x g for 15 h. The gradients were then fractionated and the 40S peak of material absorbing at 254 nm was retained.

5. Two dimensional resolution of proteins on polyacrylamide gels

(i) Isoelectric focussion (first dimension)

Isoelectric focussing was carried out in 15 cm x 2.5 mm (i.d.) glass tubes according to a modification of the method of O'Farrell (1975). Using an acrylamide stock solution containing 28.38% acrylamide (BIO RAD) and 1.62% bis acrylamide (BIO RAD) isofocussing gels were made containing 4% acrylamide, 9 M urea, 2% Nonidet P40 and 2% of the appropriate ampholines (LKB). After degassing the mixture under vacuum for 1 min, 10 µl of 10% ammonium persulphate and 7 µl of TEMED were added per 10 ml of gel mixture. Gel tubes, sealed at one end with a double thickness of Parafilm were filled to the 12 cm level with gel mixture, overlayed with isobutanol (saturated with water) and left to polymerize at room temperature in a vertical position. After two hours the isobutanol was displaced with water and the gels left for a further hour to complete polymerization. The Parafilm was then removed from the base of the gel

tubes, the gels overlayed with 25 µl of sample buffer (9M urea, 2% NP40, 2% Ampholines, 5% 2-mercaptoethanol) and the tubes filled up with upper electrode buffer (0.02 M NaOH degassed). The lower and upper electrode reservoirs of the electrophoresis chambers were filled with 0.01 M H_3PO_4, and 0.02 M NaOH (degassed) respectively and the gels pre-run negative (top) to positive (bottom) at 200 v for 15 min, 300 v for30 min and 400 v for 30 min. The upper reservoir was then emptied and all the overlay flushed off the gels with fresh 0.02 M NaOH. Samples were then loaded and overlayed with 25 µl of sample buffer diluted 1:1 with water. The tubes and upper reservoir were carefully filled with 0.02 M NaOH and the samples run at 400 v for 12 h and finally 800 v for 1 h.

After electrophoresis the gels were gently forced from the tubes using a 10 ml syringe bearing a short adaptor made from P.V.C. tubing. The gels were then either equilibrated and loaded onto the second dimensions immediately or, more usually, frozen and stored at -70°C and equilibrated just before use.

(ii) SDS/polyacrylamide gel electrophoresis (second dimension)

Second dimension SDS/polyacrylamide gel electrophoresis was carried out as described in Chapter 1, utilizing a 15 cm, linear 10 to 20% acrylamide gradient, overlayed with a 1 cm stacking gel. Molecular weight markers (Sigma Chemical Co.) were run in parallel with the second dimension, loaded into a shallow 1 cm wide sample well at one side of the stacking gel.

First dimension gels were equilibrated for 30 min at room temperature in 5 ml of protein sample buffer, before being secured in place along the top of the second dimension with the aid of a 1% solution of agarose

made up in protein sample buffer. Gels were run at 200 v overnight. Where direct comparisons were made between samples, several second dimension gels were run sandwiched together for greater reproducibility of electrophoresis conditions.

6. Silver staining of SDS/polyacrylamide gels

Where appropriate gels were stained using a modification of the method of Oakley et al., (1980).

After fixation for at least 1 h in a solution containing 50% methanol and 10% acetic acid gels were given two 20 min washes in distilled water and were then placed in a solution containing 0.05 M di-sodium tetraborate and 1% glutaraldehyde for 15 min with continuous gentle agitation. The gels were then washed extensively in several changes of distilled water for at least 1 h before transfer to ammoniacal silver solution made up freshly as follows; to 50 ml of distilled water containing 2.3 ml of 1 M, NaOH and 1.4 ml of concentrated (35%) ammonium hydroxide solution, were added 10ml of 10% (w/v) silver nitrate. During the addition the solution was stirred constantly such that the brown precipitate forming initially was completely dispersed before further additions were made. The resulting clear solution was made up to 250 ml with distilled water before immediate use.

After 15 min in the ammoniacal silver solution the gels were washed for a further 15 min in distilled water before the stain was developed in 500ml of a solution containing 10% ethanol, 3 ml of freshly made 1% citric acid and 125 µl of 37% (w/v) formaldehyde. When the staining reached the desired intensity gels were washed in several changes of distilled water, and stored in sealed polythene bags.

All manipulations were carried out in clean plastic staining trays and, where handling of the gels proved unavoidable, vinyl gloves were always employed to prevent marking the gels with fingerprints. Overstained gels or gels with a high background were destained according to the method of Switzer et al., (1979).

7. Immunostaining of gel transfers

"Western" transfers were performed according to the method of Towbin et al., (1979), using a commercially produced electrophoretic transfer apparatus (BIO RAD Laboratories).

Immediately after running, gels were rinsed briefly in transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3), a pre-wetted nitrocellulose sheet (Millipore Corporation) was placed on top of the gel and excess liquid and air bubbles displaced by rolling gently with a 25 ml glass pipette. Gel and filter were then firmly sandwiched between two Scotch-Brite^{tm} pads and placed into the transfer apparatus such that the nitrocellulose filter was anodal to the gel. The apparatus was then filled with transfer buffer and the transfer run overnight at 30v and 0.1 A.

To visualize the transferred protein bands, the nitrocellulose filter was stained in a solution of 0.1% Amido Black in 40% methanol and 5% acetic acid for 5 min and rapidly destained in a solution of 50% methanol, 5% acetic acid.

In order to immunostain the filters the untreated blot was first incubated in a solution of 1% BSA, 10% Calf serum in TBS (0.9% NaCl, 10 mM Tris/HCl, pH 7.4) at 37°C for1 h, to saturate any further binding capacity of the nitrocellulose. The filter was then heat sealed in a close fitting polythene bag into which was injected the primary rabbit antiserum diluted 1:50 with 1% BSA in TBS. The filter was incubated in this way for 4 h

at room temperature with occasional agitation, after which it was washed with three changes of TBS over a period of 30 min. The incubation was repeated with horseradish peroxidase conjugated sheep anti-rabbit IgG (MILES YEDA) at 1:500 dilution for 2 h at room temperature. After washing for 30 min in five changes of TBS the blots were soaked in a solution containing 25 μg ml^{-1} o-dianisidine (FLUKA), 0.01% H_2O_2, 10 mM Tris/HCl, pH 7.4, prepared freshly from stock solutions of 1% o-dianisidine in methanol and 0.30% H_2O_2. The colour reaction was stopped after 20 min by washing with distilled water and the blots dried between filter paper. Dried blots were stored protected from the light and photographed on Kodak Ektachrome 64.

8. Production of antisera to rat liver hnRNP core proteins

Rat liver core proteins were purified by preparative SDS/polyacrylamide electrophoresis. The 32,000 molecular mass polypeptide band was located by the staining of a test strip, and eluted from the homogenized gel by dialysis against 1% SDS, 0.1 M NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris/HCl, pH 7.5 for 24 h. After dialysis against PBS (0.9% NaCl, 10 mM phosphate buffer pH 7.0) the protein antigen was incorporated into a multiple emulsion (Herbert, 1967) for subcutaneous injection into male or female Dutch rabbits. About 500 μg of protein was emulsified with an equal volume of a mixture of 9 parts of the mineral oil Drakeol 6VR (Pennsylvania Refining Co.) and one part of the emulsifier Arlacel A (Atlas Powder Co.). The water-in-oil emulsion was dispersed in an equal volume of 2% Tween 80 in 0.14 M NaCl and administered as a single subcutaneous injection. Booster doses were given after a period of 50 days and the rabbit was bled from the marginal vein of the ear

10 days later. The blood was allowed to clot at 4°C and the serum decanted off the clot, any remaining cells were removed by centrifugation at 2000 x g for 10 min. Serum was stored in 0.5 ml aliquots at -70°C.

Antisera were tested for specificity on Ouchterlony double diffusion plates made from 1% agar in 1.5 M NaCl, 4 mM EDTA, 10 mM Tris HCl, pH 8.4.

RESULTS

1. Manual isolation and fractionation of oocyte nuclei

Nuclei can be isolated manually with relative ease from oocytes of Triturus cristatus carnifex utilizing a buffer containing 70 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM Tris/HCl, pH 7.4 and 2% polyvinylpyrolidone. In this buffer the presence of Mg^{2+} ions maintains the nucleoplasm in a gelled state allowing the nuclei to be easily manipulated and avoiding extensive leaching of soluble nuclear protein over short periods of time. The presence of 2% polyvinylpyrolidone renders the buffer approximately iso-osmotic to the nucleoplasm, avoiding excessive swelling or shrinkage of the nuclei which is liable to cause rupture of the nuclear membrane and loss of soluble nuclear components.

When nuclei are stored in this buffer at 0°C, no morphological changes are evident even after 6 h.

Comparison of total nuclear protein between pre-vitellogenic, mid-vitellogenic and late-vitellogenic oocytes by one-dimensional SDS/polyacrylamide gel electrophoresis (Fig. 2.1) reveals a surprising qualitative similarity in the polypeptide species present. The pre-vitellogenic track represents twice as many nuclei as both the mid-vitellogenic and late-vitellogenic tracks, reflecting the considerable size difference between these three oocyte stages (Pre-vitellogenic oocyte nuclei from Triturus are generally less than 100 µm in diameter, early to mid-vitellogenic between 100 µm and 150 µm and late-vitellogenic about 200 µm in diameter.). It should be noted however that silver staining techniques are not quantitative so that the intensity of staining is not necessarily related to the amount of a particular protein present.

Fig. 2.1.

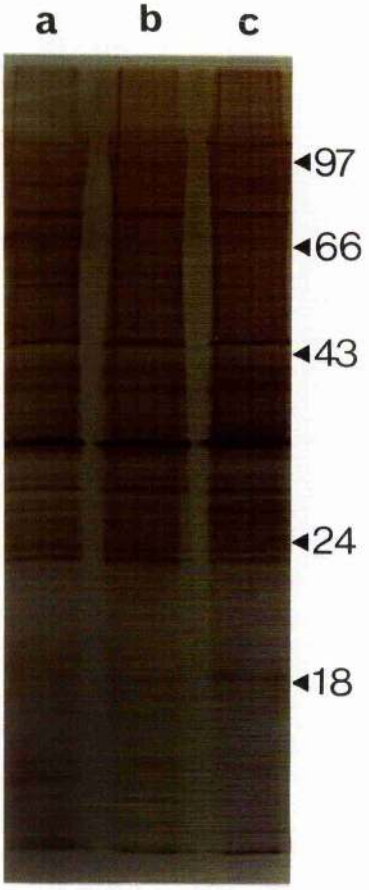
SDS/polyacrylamide gel electrophoresis on a homogeneous 15% acrylamide gel of total protein from manually isolated Triturus oocyte nuclei.

(a) Total protein from 20 pre-vitellogenic oocyte nuclei.

(b) Total protein from 10 mid-vitellogenic oocyte nuclei.

(c) Total protein from 10 late-vitellogenic oocyte nuclei.

The gel was silver stained by a modification of the method of Oakley et al., (1980). Molecular mass is indicated at x 10^{-3}.


a
b
c
97
66
43
24
18

In fact certain nuclear proteins in Xenopus have been noted to be strongly agyrophillic in nature, staining much more intensely than other more prolific species (Williams et al., 1982)

Although difficult to estimate accurately there are probably in excess of 200 polypeptide bands resolved on these gels indicating how complex the polypeptide composition of the oocyte nucleus must be.

When the polypeptide profiles of the manually separated nuclear components are examined by gel electrophoresis (Fig. 2.2) it becomes evident that by far the greatest proportion of the nuclear proteins are to be found in the "soluble" nucleoplasmic fraction. This has been noted previously (Maundrell, 1975) though the gels used in this analysis were inadequate to reveal the full extent of the similarity. Likewise, it can be seen that the chromatin fraction contains many proteins in common with the nucleolar fraction in which the enrichment of two polypeptide species with molecular masses of 97,000 and 38,000 is particularly striking. The likelihood that the chromatin fraction is significantly contaminated with nucleoli that were either free of, or dislodged from, the nuclear membrane would explain the presence of nucleolar proteins with the residual chromatin proteins, though it cannot be ruled out that both nucleoli and chromatin may have some proteins in common.

An alternative approach to the fractionation of oocyte nuclear contents is to adopt normal sucrose gradient centrifugation techniques. Since the quantities of material being fractionated are very small it is advantageous to utilize 1 ml sucrose gradients, over a wide range of sucrose concentration, upon which the total nuclear homogenate can be fractionated. The omission of any prior centrifugation steps avoids the loss of material frequently incurred when dealing with very small volumes of sample.

Fig. 2.2.

Silver stained SDS/polyacrylamide gradient gel of proteins from manually isolated Triturus oocyte nuclei.

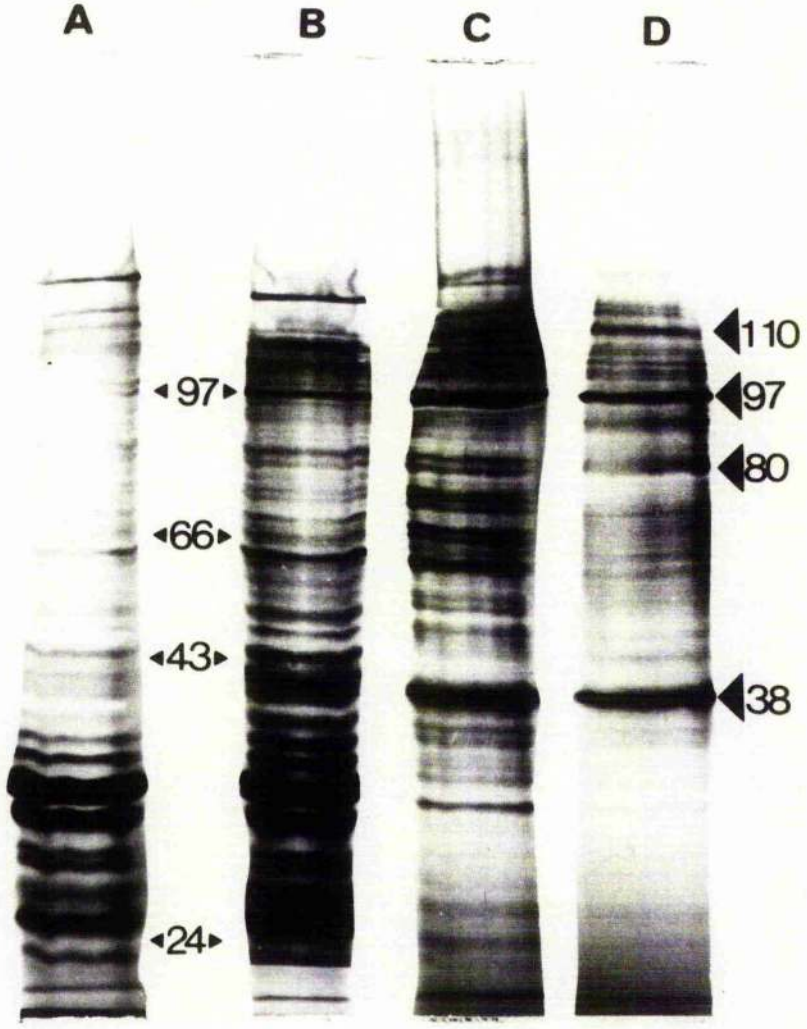
(a) Proteins of the "soluble" nucleoplasmic fraction obtained from the supernatant after centrifugation of nuclei homogenized in a saline containing 0.1 mM $CaCl_2$. The protein from approximately 20 nuclei was loaded onto this track.

(b) Total nuclear protein from about 20 nuclei, showing the similarity between the "soluble" protein fraction and the total protein.

(c) Proteins of the insoluble pelleted material obtained by centrifugation of the nuclear residue after the manual removal of the nuclear membrane and attached nucleoli. Protein from approximately 50 nuclei was loaded onto this track.

(d) Protein from approximately 50 nuclear membranes and associated nucleoli manually isolated from oocyte nuclei. Early vitellogenic oocytes were used for all isolations. Molecular mass is indicated at x 10^{-3}.

Fig. 2.2



Using these methods it was discovered that the efficiency of the fractionation obtained was highly dependent on the prevailing ionic milieu and particularly sensitive to divalent cation concentration. Fig. 2.3 shows the polypeptide profile obtained when the nuclei were homogenized in nuclear isolation buffer (70 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM Tris/HCl, pH 7.4, 2% polyvinylpyrolidone) and the homogenate fractionated on 25% to 70% sucrose gradients containing the same buffer. It can be seen that there are only very minor qualitative differences in the polypeptide profile between fractions all the way down the gradient, merely a steady decrease in the quantity of material towards the bottom of the tube. The pelleted material does show a significant enrichment of polypeptides of molecular mass of 39,000, 65,000, 67,000, 69,000 and 114,000.

The lack of any obvious resolution of nuclear components under these conditions is most probably related to the presence of 5 mM $MgCl_2$ in the homogenization buffer. As already observed the presence of Mg^{2+} ions results in the gelling of the nucleoplasm, a property exploited in the production of lampbrush chromosome preparations (Callan and Lloyd, 1960). Gentle homogenization of nuclear gels may well result in the generalized aggregation of nuclear contents associated with gelled material, centrifugation probably only serves to fractionate aggregates according to their size, and does not result in any useful separation of nuclear material.

When conditions under which the nuclear sap is fluid are adopted during the fractionation procedure a completely different result is obtained. In a saline containing no Mg^{2+} and 0.1 mM $CaCl_2$ a peak of labelled RNA (Fig. 2.4) can be isolated in conjunction with a specific set of

Fig. 2.3.

Silver stained SDS/polyacrylamide gradient gel of the polypeptide profile across a 25 - 70% sucrose gradient fractionation of total oocyte nuclear homogenate. Three hundred nuclei were homogenized in a saline containing 5 mM $MgCl_2$. Track (P) represents the material recovered from the pellet and fractions (1) to (7) are taken from the bottom to the top of the gradient respectively. A native Triturus hnRNP preparation was run in parallel for comparative purposes. Molecular mass is indicated at x 10^{-3}.

Fig.2.3

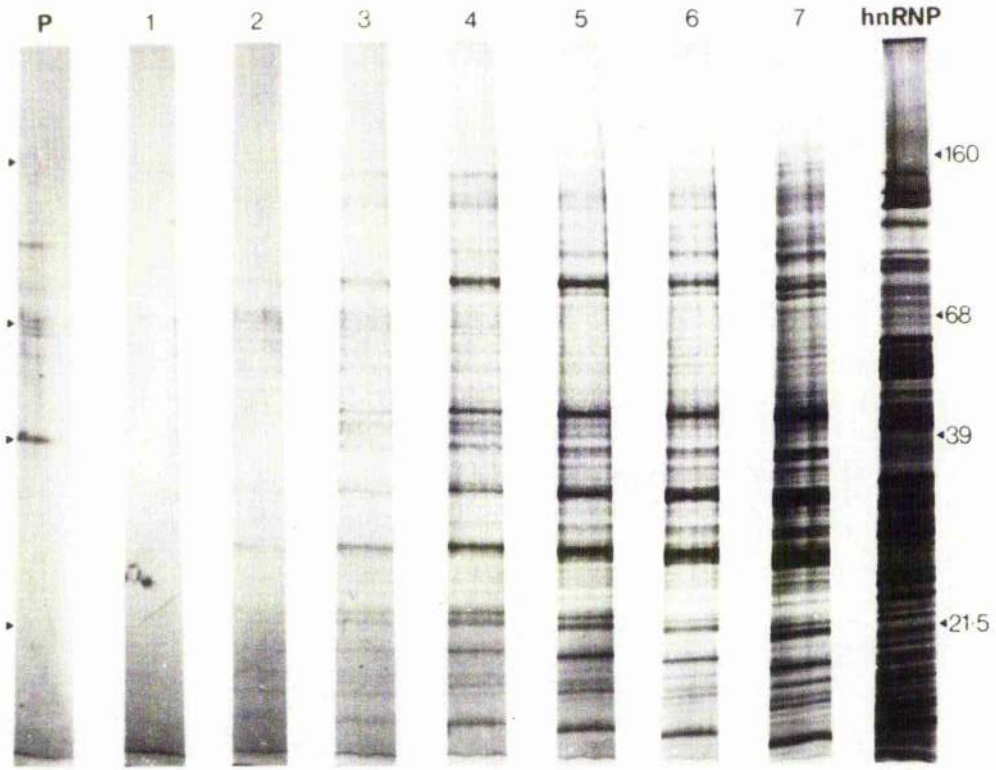
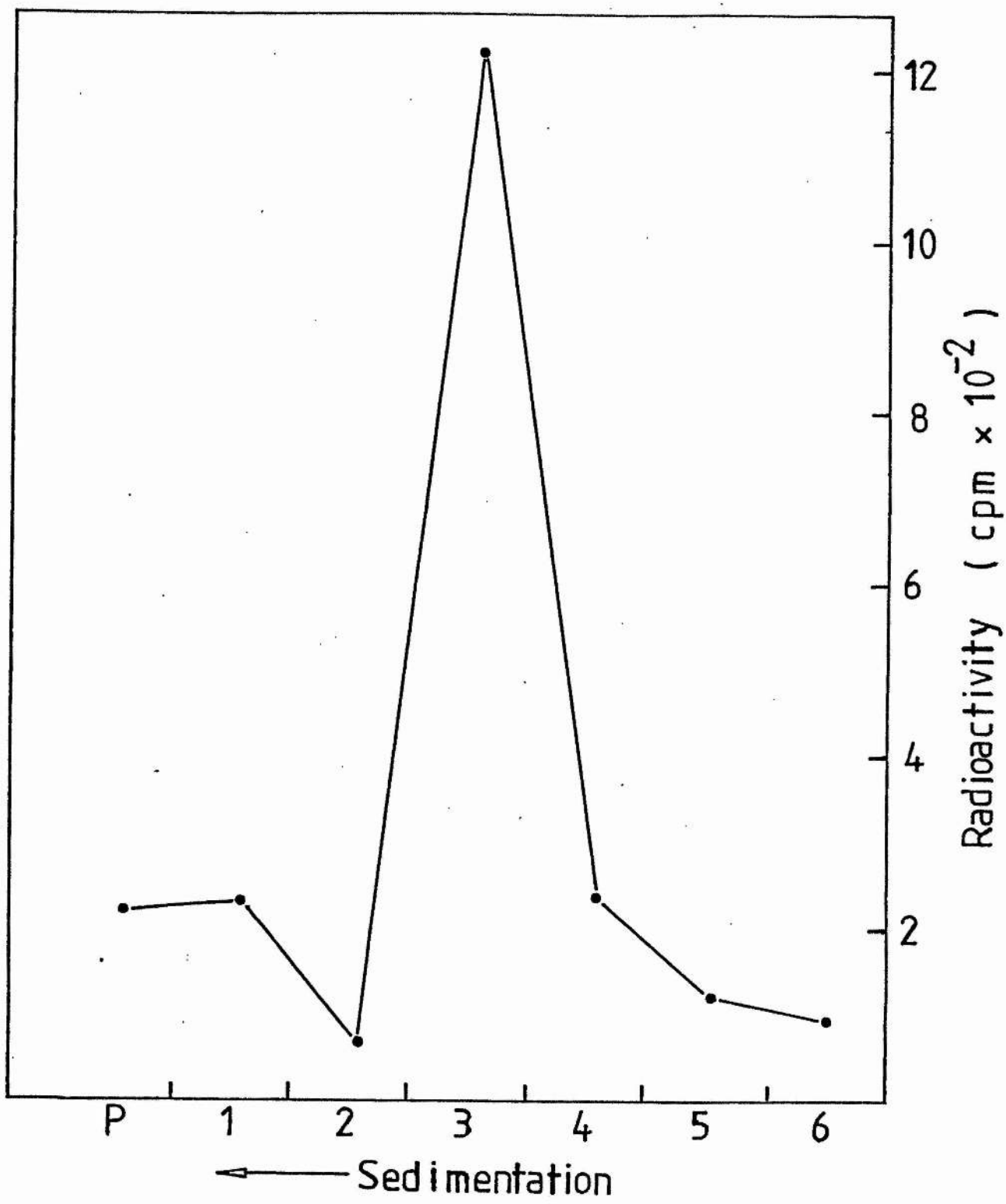


Fig. 2.4.

Distribution of radioactively labelled RNA from a homogenate of 300 nuclei fractionated in a 1 ml 25-70% sucrose gradient. Nuclei were homogenized in a saline containing 0.1 mM $CaCl_2$. Centrifugation was at 80,000 x g for 4 h at 4°C. Aliquots from each fraction were precipitated for radioactive counting as described in the text.

Fig. 2.4



polypeptides (Fig. 2.5), (distinct from the bulk of the nuclear protein remaining near the top of the gradient). Although examination of this gradient fraction by phase contrast microscopy showed no evidence of the presence of nucleoli, comparison of the associated polypeptide spectrum with that of the manually isolated nucleolar fraction (Fig. 2.2) would indicate that some of the major polypeptides are common to both preparations, in particular prominent bands at molecular masses of 38,000, 80,000 and 97,000. Thus it would appear that perhaps the bulk of the material located in the peak is nucleolar in origin, having sedimented to a density position at about 55% (w/v) sucrose.

2. Comparison of HnRNP preparations from nucleated and enucleated oocytes

One hundred mid- to late-vitellogenic oocytes were selected from a Triturus ovary, manually defolliculated (with the aid of two pairs of No. 5 watchmakers forceps), and the nuclei isolated from fifty of them as previously described. The residual oocyte cytoplasm from this process was pooled and used in a parallel hnRNP preparation with the remaining intact oocytes. The polypeptide constitution of both preparations was compared by SDS/polyacrylamide gel electrophoresis (Fig. 2.6). The striking similarity between the preparations indicates that nearly all the major polypeptides of the "hnRNP" can be located in the cytoplasm. Although this result does not exclude a nuclear role for any of these proteins, and although the silver stained gel cannot give an accurate quantitative assessment of the relative contribution of cytoplasm and nucleoplasm to these preparations, it would certainly appear that the majority of the proteins are derived from the cytoplasm.

Fig. 2.5.

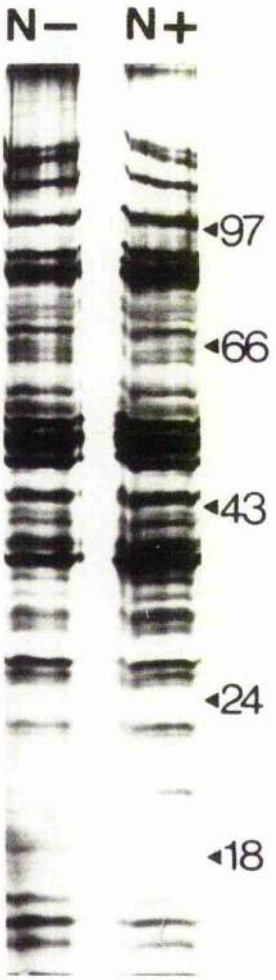
Silver stained SDS/polyacrylamide gradient gel of the polypeptide profile from the sucrose gradient fractionation described in Fig. 2.4. Track (P) represents the material recovered from the pellet and fractions (1) to (6) are taken from the bottom to the top of the gradient respectively. A native Triturus hnRNP preparation was run in parallel for comparative purposes. Molecular mass is indicated at x 10^{-3}.

Fig.2.5

Fig. 2.6.

Silver stained SDS/polyacrylamide gel comparing the proteins of "hnRNP" preparations from 50 normal mid-vitellogenic oocytes (N+) and 50 enucleated mid-vitellogenic oocytes (N-). HnRNP was prepared as usual, with both preparations treated in an identical manner. Molecular mass is indicated at x 10^{-3}.

Fig. 2.6



3. Comparison of "hnRNP" proteins with protein from Triturus oocyte nuclei by two-dimensional electrophoresis

Fig. 2.7 shows the two-dimensional separations of Triturus "hnRNP" proteins and total oocyte nuclear protein. The juxtaposition of the gel patterns reveals very little homology; only one of the major "hnRNP" polypeptide species corresponds with any certainty to a nuclear polypeptide, having a molecular mass of 95,000 and an approximate pI of 8.0. None of the characteristic set of "hnRNP" proteins in the molecular mass ranged between 40,000 and 66,000 are anything other than partially coincident with polypeptide spots of the nuclear protein pattern.

Although the full multiplicity of the "hnRNP" fraction is possibly not fully represented due to slight underloading or understaining of the gel, and recognition of homology between minor nuclear proteins and enriched "hnRNP" proteins might be difficult, it must be concluded that apart from P95 already mentioned, none of the other constituent proteins of the "hnRNP" fraction are present in the nucleus to any significant concentration.

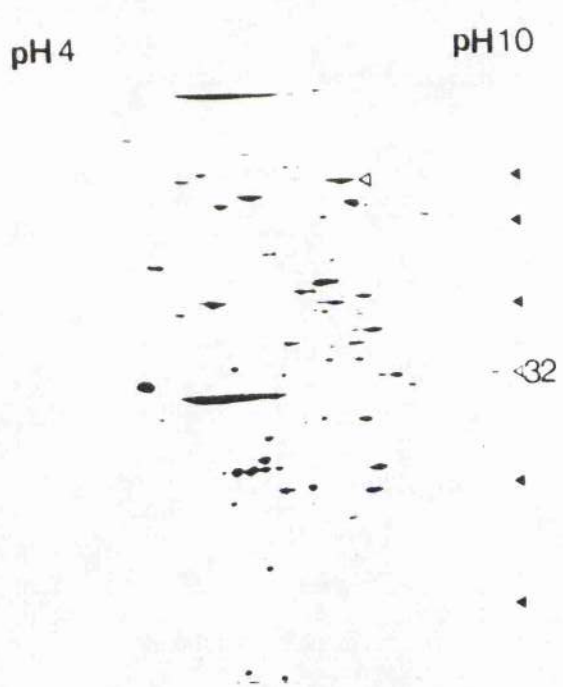
4. Isolation and characterization of rat liver hnRNP "core proteins": comparison with oocyte "hnRNP" and nuclear proteins

Incubation of isolated rat hepatocyte nuclei in an approximately isotonic buffer at pH 8.0 releases hnRNP: monomer particles by endogenous nuclease activity (Samarina et al., 1965; Karn et al., 1977). Purification of the nuclear extract by sucrose gradient centrifugation yields a peak of material sedimenting at approximately 40S and absorbing strongly at 254 nm (Fig. 2.8). Examination of the polypeptides associated with this 40S peak reveals a relatively simple polypeptide pattern (Fig. 2.9).

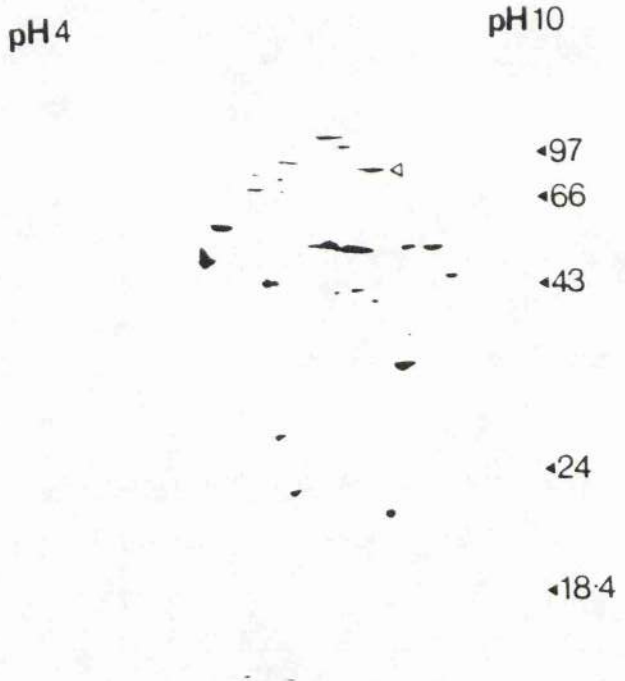
Fig. 2.7.

Two-dimensional polyacrylamide gel electrophoresis of Triturus" hnRNP" proteins and total oocyte nuclear protein. Separation in the first dimension was by isoelectric focussing according to the method of O'Farrell et al., (1977), separation in the second dimension was by SDS/polyacrylamide gradient gel electrophoresis. First dimension gels contained 1.2% of pH 3.5-10 Ampholines, 0.4% of pH 5-8 Ampholines and 0.4% of pH 9-11 Ampholines (LKB). The approximate pH range represented by the gels shown is indicated at the top of each photograph. Gels were silver stained. Molecular mass is indicated at $\times 10^{-3}$.

Fig. 2.7



Triturus Nuclear Protein



Triturus hnRNP

Fig. 2.8.

Absorbance of rat liver hnRNP "core particles" in a 15 to 30% sucrose gradient. Centrifugation was for 80,000 x g for 15 h at 4°C. The approximate sedimentation coefficient was estimated by comparison with the sedimentation of the small ribosomal subunit on a similar gradient.

——— A_{254}.

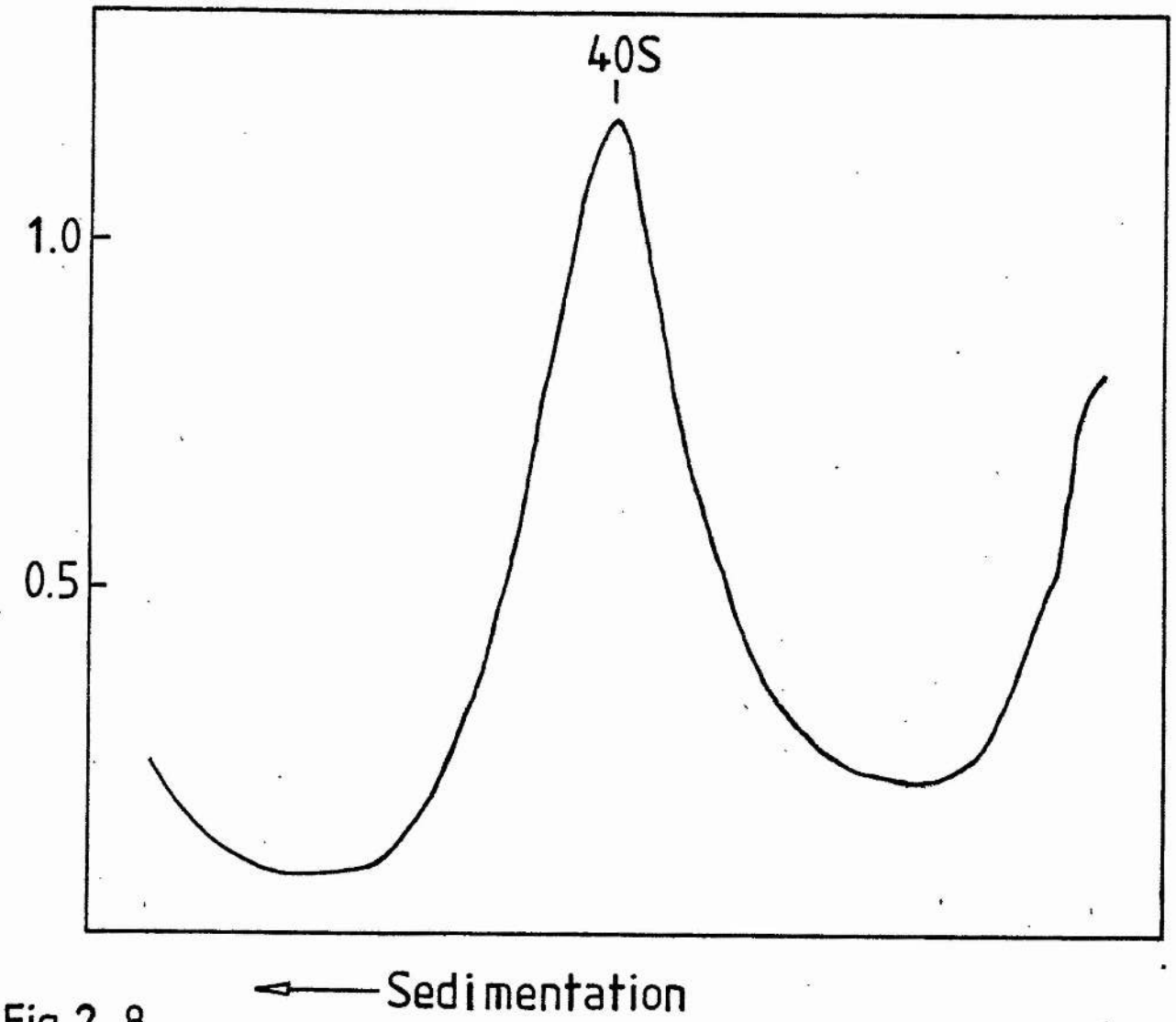


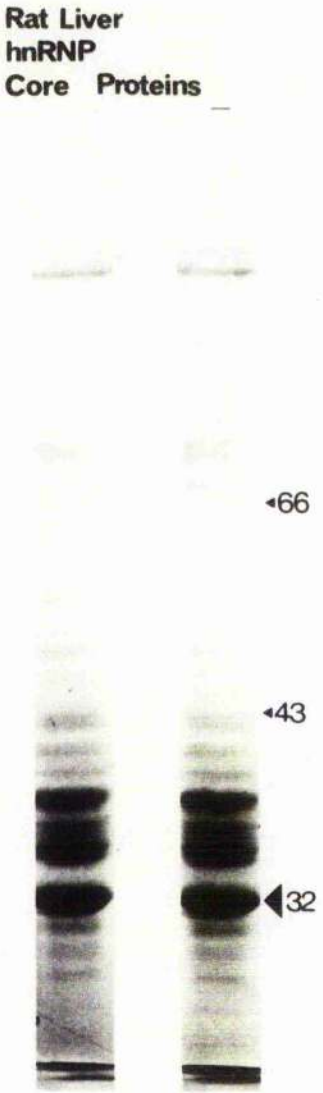
Fig. 2. 8

Fig. 2.9.

SDS/polyacrylamide gel electrophoresis of rat liver hnRNP "core proteins". The two tracks represent different preparations.

Molecular mass is indicated at x 10^{-3}.

Fig.2.9



Six major bands are obvious, having molecular masses of 32,000, 34,000, 34,500, 35,000, 36,000 and 38,000. The number and migration of hnRNP "core proteins" detected on SDS/polyacrylamide gels has been shown to be critically dependent on the nature of the detergent used and the electrophoretic conditions (Billings and Martin, 1978) and also on the maturity of the rats used in the preparation (W.G.F. Whitfield, unpublished observation). However provided the livers used were from rats between four to six months old, a consistent polypeptide pattern was obtained (the two tracks in Fig. 2.9 represent different preparations).

Two-dimensional electrophoretic comparison between rat liver hnRNP "core proteins" and Triturus oocyte nuclear protein (Fig. 2.10) shows only a small, minor nuclear protein spot corresponding to the major hnRNP "core" protein of molecular mass 32,000 (P32). None of the other core proteins in that range appear to be represented. A similar situation exists in the oocyte nuclei of Xenopus laevis (Fig. 2.11) where two spots are found with similar position and morphology to the major P32 polypeptide of the rat liver hnRNP "core proteins". In both Triturus and Xenopus the putative P32 equivalent is a minor nuclear protein in a region of the gel with few, if any, surrounding polypeptide spots.

5. Immunostaining of gel transfers

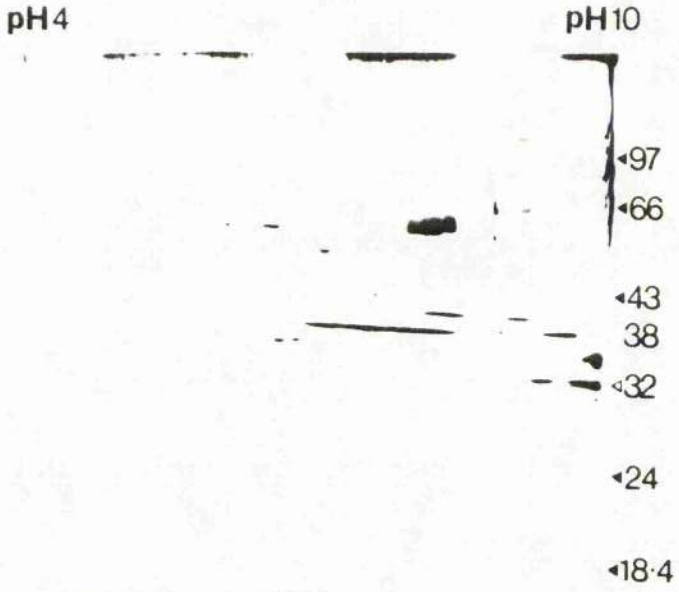
Antiserum to the major rat liver core protein P32 was tested for specificity against homologous purified protein antigen and native 40S hnRNP monomer particles by Ouchterlony double diffusion. Precipitin lines were never obtained against the purified protein antigen since although a variety of agents were used, adequate solubilization of the antigen other than in high concentrations of SDS, proved difficult. Similar

Fig. 2.10.

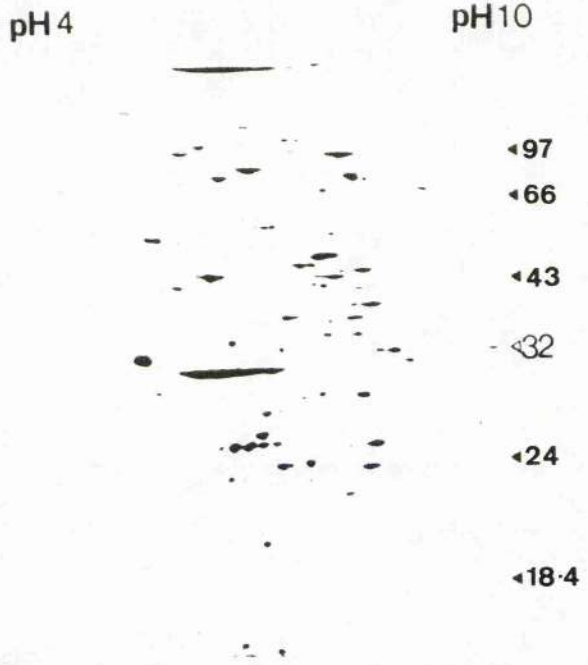
Two-dimensional polyacrylamide gel electrophoresis of rat liver hnRNP "core proteins" and Triturus oocyte nuclear protein. ▷ Symbol indicates the 32,000 molecular mass "core protein" and its putative oocyte homologue.

Molecular mass is indicated at x 10^{-3}.

Fig.2.10



Rat Liver hnRNP



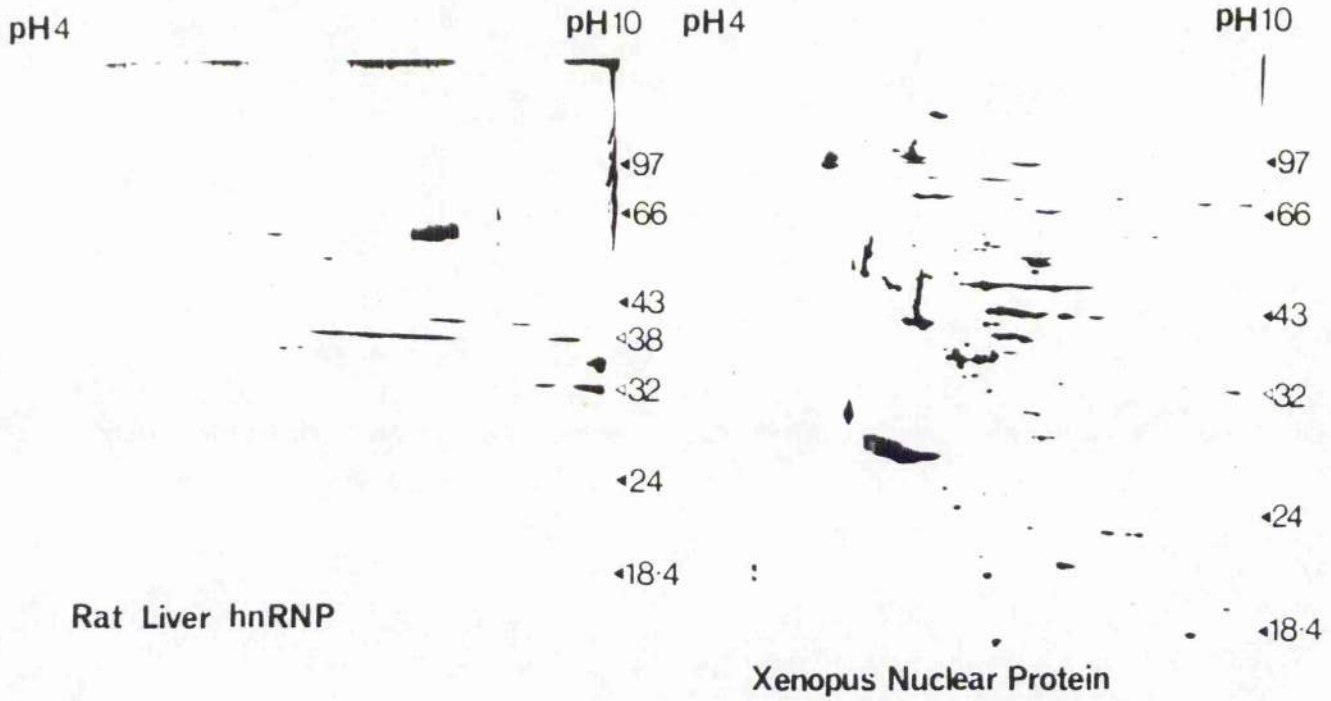
Triturus Nuclear Protein

Fig. 2.11.

Two-dimensional polyacrylamide gel electrophoresis of rat liver hnRNP "core proteins" and Xenopus oocyte nuclear protein.

▷ Symbol indicates the 32,000 molecular mass "core protein" and it putative oocyte homologue. Molecular mass is indicated at x 10^{-3}.

Fig.2.11


pH4
pH10
97
66
43
38
32
24
18·4
Rat Liver hnRNP
pH4
pH10
97
66
43
32
24
18·4
Xenopus Nuclear Protein

problems were encountered with the native 40S hnRNP particle which was prone to non-specific precipitation before it had even diffused out of the sample well. This difficulty was offset in part by the incorporation of 1.5 M NaCl in the Ouchterlony plate (Jones et al., 1980) observed that 1.5 M NaCl gave optimal immunoprecipitation of "core protein" antigens), under which condition faint precipitin lines were obtained (data not shown)

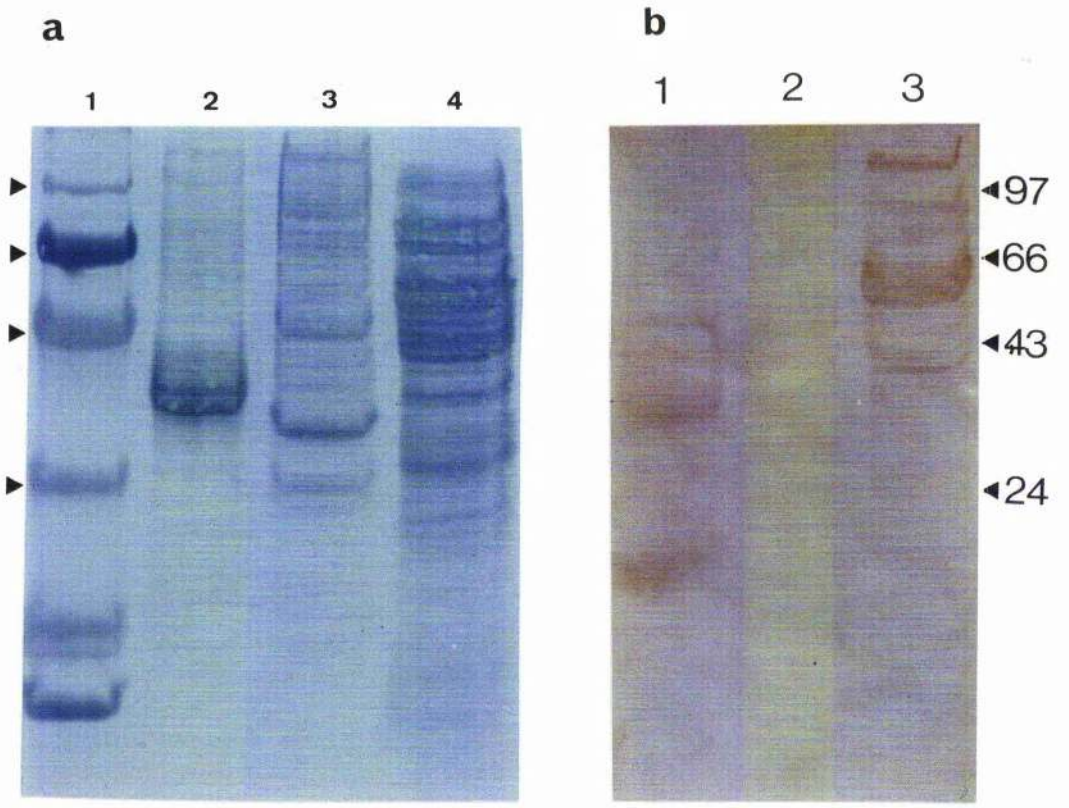
When anti-P32 is used to immunostain electrophoretic gel transfers of Triturus hnRNP and oocyte nuclear protein some rather interesting results are obtained (Fig. 2.12). The homologous reaction of anti-P32 with rat liver hnRNP "core proteins" gives a distinct reaction with the 32,000 molecular mass band and with at least two bands of higher molecular mass in the "core protein" range. This probably indicates shared antigenic determinants between different "core proteins". Disappointingly the nuclear protein track has no obviously stained polypeptide bands suggesting that either the concentration of any oocyte "core protein" homologue is too low to detect by this method or that the antiserum does not react with it. In contrast, the Triturus hnRNP track contains at least four polypeptide bands that give a reaction of greater intensity than the homologous one. Tight bands, at molecular masses of 110,000 and 37,000 give noticably strong colour reactions, and do not seem to reflect a non-specific binding of the antibody to overloaded protein bands since neither stain particularly strongly with amido-black on the comparative track (Fig. 2.12a). Even the two reactive bands at 52,000 and 56,000 although probably overloaded with protein, seem to be giving specific reaction with anti-P32 since adjacent, bands of equivalent loading remain relatively unstained. Pre-immune serum on a control transfer gave no reaction with either the total Triturus oocyte nuclear protein or the hnRNP track.

Fig. 2.12 a - b.

Electrophoretic SDS/polyacrylamide gel transfer onto nitrocellulose sheet.

(a) Gel tracks have been stained with 0.1% amido black for comparative purposes. Gel tracks in order are (1) molecular mass markers (2) rat liver hnRNP "core proteins" (3) Triturus oocyte nuclear protein (50 mid-vitellogenic nuclei) (4) Triturus "hnRNP" proteins.

(b) Gel tracks have been subject to indirect immunostaining by the peroxidase/o-dianisidine colour reaction (Towbin et al., 1979). Primary antiserum (anti P32 rat liver "core protein") was used at a 1:50 dilution. Gel tracks in order are (1) rat liver hnRNP "core proteins" (2) Triturus oocyte nuclear protein (3) Triturus "hnRNP" proteins. Stained blots were photographed using Kodak Ektachrome 64. Molecular mass is indicated at x 10^{-3}.


a
1
2
3
4
b
1
2
3
97
66
43
24

DISCUSSION

The evidence described in the previous chapter casts considerable doubt on the suggested nuclear location of a high proportion of the polypeptides found in Triturus oocyte hnRNP preparations. The polypeptide spectrum of manually isolated Triturus oocyte nuclei is very complex (Fig. 2.1) so that the recognition of similarities with that of "hnRNP" preparations is extremely difficult. The lack of any qualitative differences between oocyte nuclear protein preparations from early, mid, and late vitellogenic stages, when considered in the light of the stage specific variation in the classes of RNA synthesised, indicates that hnRNP proteins may not be present in sufficient concentration in oocyte nuclei to be easily detectable in a complex, one-dimensional protein spectrum. Furthermore, when manually isolated nuclei are manually separated into three different fractions containing "soluble" components, nucleoli and nuclear membranes, and chromatin, the great majority of the proteins are detected in the "soluble" fraction which has a protein spectrum almost identical to that seen on electrophoretic separations of total oocyte nuclear protein (Fig. 2.2). The considerable size of hnRNP polyparticles and the association of perhaps half of the hnRNP with the lampbrush chromosome loops as nascent transcripts (Sommerville, 1973), suggests that hnRNP should fractionate with the chromatin under the conditions of mild homogenization and low speed centrifugation applied. Enrichment of certain proteins in the chromatin pellet is certainly evident, though many would appear to be common to the nucleolar fraction. This may be related to an observation of Christensen et al. (1981) who, with the aid of an antiserum prepared against a nuclear protein

from the slime mould Physarum, showed that an antigenically similar protein may be associated with both rRNA and hnRNA in Drosophila. These authors speculated that this protein may have a general RNA packaging function in lower eukaryotes whilst having evolved a more specialized role exclusive to pre-messenger RNA packaging in higher eukaryotes. Hence the possibility that some Triturus oocyte nuclear proteins have a similar dual role in both hnRNP and nucleoli cannot be dismissed.

The employment of sucrose gradient fractionation of oocyte nuclear homogenates in an attempt to enrich the hnRNP fraction for comparison with "hnRNP" isolated from total ovary homogenates was complicated by the effects of divalent cation concentration. The marked variations in consistency of oocyte nucleoplasm between different species and different physiological states in any one species has been noted with regard to the problems this creates during the preparation of spread lampbrush chromosomes (Callan, 1963). The finding that the nuclear sap of Axolotl could be dispersed in saline containing low concentrations of $CaCl_2$ (Callan, 1966) has led to the routine incorporation of 10^{-5}-5 x 10^{-5} M $CaCl_2$ in media for the preparation of lampbrush chromosomes from many species. Gounon and Karsenti (1981) have shown that actin is present in considerable quantities in the oocyte nucleus of the newt Pleurodeles waltlii. They found that chelation of Ca^{2+} results in the formation of a nucleoplasmic gel composed of individual microfilaments. The formation of actin cables was induced by the addition of phalloidin, a drug known to stabilize F-actin. Electron microscopic examination of these nuclear actin cables shows that they consist of tightly associated microfilaments

to which RNP-like particles are bound. It seems probable that a similar mechanism, of Ca^{2+} mediated actin de-polymerization underlies the gel/sol transition in Triturus oocytes. The association of nuclear components, and especially RNP, with the actin gel explains the failure of sucrose gradient fractionation of nuclear homogenates in the absence of Ca^{2+} ions. This is consistent with the more effective fractionation of nuclear contents obtained when 0.1 mM $CaCl_2$ is included in the saline used for nuclear homogenization and the sucrose gradient solutions (Figs. 2.4 and 2.5). Nevertheless, virtually all the radioactively labelled RNA is detected in a single peak at about 55% sucrose, presumably both hnRNA and rRNA are represented in this peak but it would be necessary to inhibit RNA polymerase I activity with low levels of actinomycin D to reveal the relative contribution of each RNA class. Without an adequate separation of hnRNP and nucleolar material it is difficult to ascribe any particular protein to one or other source.

The enrichment of a similar pattern of polypeptides in both the sucrose gradient peak (Fig. 2.5 track 3) and the manually isolated nucleolar and chromosomal nucleoplasmic fractions (Fig. 2.2 C and D) can be interpreted in various ways. The obviously enriched bands might represent nucleolar material alone if the chromosomal preparation is contaminated to a considerable extent with nucleoli. Conversely, hnRNP may be a contaminant of the nucleolar preparation or as already postulated hnRNP and nucleoli may have some polypeptides in common. It can be stated with rather more certainty that there is no obvious similarity between the polypeptide pattern of the nuclear RNA associated gradient fraction (Fig. 2.5 track 3) and that of the oocyte "hnRNP" fraction shown on the same gel.

As an alternative to searching for the proteins from the oocyte "hnRNP" fraction amongst the multitude of nuclear proteins, the problem was approached from the reverse standpoint, namely an "hnRNP" preparation was made from a known number of selected oocytes and compared with an "hnRNP" preparation from an equivalent set of manually enucleated oocytes. The two gel tracks in Fig. 2.6 show little, if any, qualitative differences implying that most of the visible polypeptide bands are largely of cytoplasmic rather than nuclear origin. Two alternative conclusions can be drawn from this discovery regarding the nature of the RNP found in this fraction. On the one hand if we consider the hnRNP to be a minor component, co-isolating with a considerably greater mass of cytoplasmic material then specific hnRNP proteins might well remain undetected in a one dimensional electrophoretic comparison. If on the other hand we still consider the bulk of the proteins to be RNP proteins then it must be concluded that either the same proteins that bind hnRNA in the nucleus are present in some similar form in the cytoplasm, perhaps as stored messenger RNP, or that the whole fraction represents cytoplasmic RNP containing hnRNA-like sequences. This final possibility derives some support from the observations of Anderson et al. (1982). They showed that in Xenopus, incompletely processed mRNA precursors are accumulated in the oocyte cytoplasm, and that egg poly $(A)^+$RNA is physically similar to the nuclear RNA of somatic cells. These transcripts represent a majority of the mass of the oocyte's poly$(A)^+$RNA. Their sequence complexity, though less than that of oocyte hnRNA is greater than that of the oocyte mRNA. Thus it is feasible that these partially processed poly$(A)^+$RNA sequences are represented in the Triturus "hnRNP" preparations. The poly$(A)^+$ mRNA of Xenopus

oocytes is found in 40-200S particles containing polypeptides of molecular mass coinciding quite closely with those of the 50-60,000 mass range in Triturus "hnRNP" (Darnborough and Ford, 1982). These oocyte specific mRNP proteins are reported to be highly basic, unlike their Triturus "hnRNP" counterparts, some of which are slightly acidic in nature. Despite these differences some of the Triturus "hnRNP" proteins could be related to these oocyte-specific mRNP proteins found in Xenopus, further reinforcing the possibility that cytoplasmic RNP is present in the Triturus "hnRNP" preparation.

Comparison of Triturus "hnRNP" proteins with total nuclear protein with one dimensional electrophoresis is rendered inadequate by the multiplicity of polypeptide bands found in both sources. This prevents the resolution of any minor similarities in polypeptide composition that might be present. The superior resolution of two-dimensional electrophoresis, coupled with its more rigorous characterization (charge as well as molecular mass), provide a more suitable technique for comparative purposes. Using this method to compare Triturus "hnRNP" with Triturus total nuclear protein a single polypeptide of molecular mass 95,000 and pI of about 8.0 appears to be the only major species common to the two sources. Whether or not it is this polypeptide (alone or with other undetected minor components) that is associated with the hnRNA present in the "hnRNP" prep is a matter for speculation. It is clear from the one-dimensional gels (Fig. 2.6) that P95 is definitely a cytoplasmic protein in addition to being present in the nucleus. It is also possible that it is related to the RNA associated polypeptide (P97) enriched during sucrose gradient centrifugation of oocyte nuclear homogenate (Fig. 2.5) although there is a discrepancy in molecular mass and no two-dimensional electrophoretic comparison has been made.

Nuclear ribonucleoprotein from rat liver has been extensively characterized by many different groups (Beyer et al. 1977; Martin et al., 1980). Jones et al., (1980) report considerable conservation of the hnRNP "core proteins" between vertebrate species. Using antisera to rat liver hnRNP the presence of antigenically similar material on the loops of Xenopus lampbrush chromosomes was shown by indirect immunofluorescence (Martin et al., 1980). Thus it would appear that analogous "hnRNP" core proteins might be present in amphibian oocyte nuclei, and might therefore be detectable by two-dimensional electrophoretic comparison. This is in fact the case, polypeptide spots, corresponding in both size and charge to the major rat liver hnRNP "core proteins" can be detected in both Xenopus and Triturus. (Figs 2.10 and 2.11). The scarcity of any other polypeptide species in the same region of the gel makes it unlikely that these polypeptides are unrelated and only fortuitously share the same molecular mass and charge. Cross comparison between Figs. 2.7 and 2.10 shows no comparable hnRNP "core protein" analogues in the Triturus "hnRNP" preparation suggesting that perhaps there are different "families" of hnRNP to be found in the amphibian oocyte.

Some support for this possibility is derived from the use of an antibody raised against the major rat liver "core protein" P32. In addition to immunostaining the homologous rat liver hnRNP "core proteins" on "Western Blot" transfers, four cross reacting polypeptide bands are detected in the Triturus hnRNP preparation only one of which corresponds in molecular mass to any of the rat liver hnRNP "core proteins" (Fig. 2.12). Neither the "core proteins" nor the cross-reacting Triturus "hnRNP" bands are detected by immunostaining

of the total oocyte nuclear protein transfer. This is possibly due to an inadequate level of protein renaturation, in combination with the relatively low band loadings found in the nuclear protein track, such that the quantity of antigen presented in a recognizable state is too low to be detected.

Before considering the cross-reacting bands in the Triturus "hnRNP" preparation it is instructive to note that although the antiserum used was raised against a specific rat liver hnRNP "core protein" antigen (P32), it also stained the higher molecular mass "core proteins" on the transfer. (Fig. 2.12). This antigenic relatedness within the family of rat liver hnRNP "core proteins" has been noted previously (Martin et al., 1979). It seems quite likely that the antigenic sites recognized involve the RNA binding regions of the molecules which may well have been highly conserved structures during evolution. With this in mind the cross-reactivity of the polypeptide bands detected with anti-P32 in Triturus "hnRNP" may well be a result of a functional relationship, namely that they bind RNA in a similar fashion to the"core proteins". P37, the only cross reacting Triturus hnRNP protein that corresponds in size to the "core proteins", might also be compared with the 38,000 molecular mass polypeptide enriched in the manually isolated Triturus oocyte chromatin fraction (Fig. 2.2) and the RNA associated polypeptide (also 38,000 molecular mass) enriched by sucrose gradient centrifugation of oocyte nuclear homogenates (Fig. 2.5). It is tempting to consider these three polypeptides to be one and the same although the evidence to suggest this is sparse and largely circumstantial.

Of the remaining cross-reactive Triturus "hnRNP" polypeptides, the 110,000 molecular mass band is perhaps the most interesting since a band of similarly high molecular mass is occasionally detected with anti-"core protein" antisera on transfers of rat liver hnRNP "core protein" preparations. (Data not shown). This suggests that it may be an hnRNP protein common to both amphibian oocytes and mammalian somatic cells, although its low concentration in both Triturus hnRNP and rat liver "core protein" preparations probably means that it is not a "core protein" itself but is either associated with a specific subclass of hnRNP or transiently associated with hnRNP in general.

The major objective of the varied investigative methods described in this chapter was to identify which of the polypeptide components of the Triturus "hnRNP" preparation are associated with the hnRNA known to be present in that source. Since other more extensive investigations of hnRNP in somatic cells (Reviewed Martin et al., 1980) have suggested a solely nuclear location for the hnRNP "core proteins", comparison of Triturus "hnRNP" with total oocyte nuclear protein seemed a sensible approach. The revelation that only one major nuclear polypeptide (P95) was evident in the "hnRNP" preparation suggested that an enrichment step might be necessary in order to see hnRNP proteins amongst the considerable quantity of oocyte nuclear material. The use of sucrose gradients and manual dissection was helpful to this end with both methods implicating similar polypeptides as RNP associated. However without the inhibition of rRNA synthesis it was not possible to say with certainty whether these polypeptides were associated with hnRNA or rRNA. The polypeptide profile of "hnRNP"

prepared from enucleated oocytes confirmed the suspected extensive cytoplasmic contamination of the isolated material, it seems likely that many of the major proteins present are mitochondrial in origin, and that no exclusively nuclear polypeptides can be detected in one-dimensional gels of this material, though since probably less than 3% of the "hnRNP" peak material is RNA (Sommerville, 1973) this latter observation is not altogether surprising. The detection of polypeptides antigenically related to rat liver hnRNP "core proteins" in oocyte "hnRNP" is difficult to interpret but at the very least suggests that some form of RNP is present in this fraction. This is contradicted by the absence of any "core protein" homologue on 2D gels of oocyte "hnRNP". Polypeptides of closely related size and charge to the rat liver hnRNP "core proteins" are detected in the oocyte nuclei of both Xenopus and Triturus although they do not represent the considerable proportion of total nuclear protein that these polypeptide species do in somatic cell nuclei. The evidence would therefore suggest that although oocyte nuclei may contain hnRNP with a "core protein" structure as found in somatic cells, other candidate RNP proteins are present either associated with hnRNP "cores" or involved in completely different types of hnRNP complex, perhaps specific to the oocyte and related to processes unique to oogenesis.

CHAPTER 3.

ANALYSIS AND RECONSTRUCTION OF A CYTOPLASMIC RNP PARTICLE FROM AMPHIBIAN OOCYTES.

INTRODUCTION

During the first meiotic prophase of amphibian oogenesis a high proportion of the oocytes' synthetic activity is directed towards the production of components required subsequently in early embryogenesis. In particular, both ribosomes and transfer RNA are accumulated in considerable quantities (Brown and Littna; 1964a and b: Davidson et al., 1964; Ford, 1971; Mairy and Denis, 1971), for instance, mature vitellogenic oocytes of Xenopus laevis accumulate about 1.1×10^{12} ribosomes (Perkowska et al., 1968).

Considerable reiteration of both 5S and tRNA genes is found in amphibian genomes, there being over 2×10^4 5S RNA genes (Brown and Weber, 1968; Brown et al., 1971) and about 8×10^3 transfer RNA genes in Xenopus (Birnstiel et al., 1972; Clarkson et al., 1973). The synthesis of 5S RNA and tRNA is at a maximum during early diplotene (Ford, 1971; Mairy and Denis, 1971) and these two RNA species represent more than 80% of the total amount of RNA accumulated in previtellogenic oocytes.

Xenopus 5S RNA genes can be divided into two distinct groups according to the specificity of their transcription. One group is expressed in both oocytes and somatic cells whilst the other is transcribed exclusively in the oocyte (Wegnez et al., 1972; Ford et al., 1973). Oocyte 5S RNA is known to be more stable that somatic 5S RNA (Mairy and Denis, 1972; Denis and Mairy, 1972), this increased stability may be due to several different factors (Denis, 1974). Firstly there would seem to be a lower concentration of nucleases in oocytes than in somatic cells; secondly

the primary and secondary structures of the two 5S RNA species differ (Ford and Southern, 1972; Wegnez and Denis, 1973; Denis et al., 1972) which is perhaps of significance to their intrinsic stability; thirdly some protection from nuclease digestion must be derived from the nucleoprotein structures in which the oocyte 5S RNA is stored (the same almost certainly applying to transfer RNA).

In the previtellogenic oocytes of Xenopus nearly 50% of the 5S RNA and 90% of the tRNA is complexed in ribonucleoprotein particles which sediment at 42S in sucrose gradients (Ford, 1971; Denis and Mairy, 1972; Wegnez and Denis, 1973). Excepting a small percentage of ribosome associated 5S RNA, in previtellogenic oocytes the remaining 50% of the 5S RNA is found in smaller cytoplasmic RNP particles sedimenting at 7S (Picard and Wegnez, 1979) or 10S (Dixon and Ford, 1980b). Similar 42S particles have been described for the urodelan amphibian Triturus cristatus (Sommerville, 1977; Malcolm and Sommerville, 1977) and for the teleost fish Tinca tinca (Denis et al., 1980).

As oogenesis proceeds the level of 42S particles gradually declines in parallel with the incorporation of 5S RNA into the ribosomes. Although no 42S particles can be detected in mature oocytes, 20% of the 5S RNA still remains in a 7S/10S particle (Dixon and Ford, 1980b), however the protein associated with the 7S/10S particle of previtellogenic stages cannot be detected in mature vitellogenic oocytes (Dixon and Ford, 1980b).

An in vitro transcription assay using unfertilized Xenopus egg extracts (which are normally incompetent for 5S gene transcription) has been utilized to characterize a factor necessary for 5S gene transcription. The factor, derived from Xenopus ovary homogenates, has been shown to bind specifically to an intragenic region (Engelke et al., 1980) that corresponds, to a close approximation, with the 5S gene control region described by Sakonju

Bogenhagen et al., 1980

et al. (Sakonju et al., 1980;). A specific transcription factor that can bind either the 5S gene or 5S RNA has also been described in previtellogenic Xenopus oocytes (Pelham and Brown, 1980). Chymotryptic digests of the purified transcription factor of Engelke et al. (1980) are identical with those of the smaller of two closely related proteins isolated from the 7S/10S RNP particle (Pelham and Brown, 1980). The co-identity of the transcripion factor and the 7S/10S RNP protein suggests a mechanism by which 5S RNA synthesis might be controlled in vivo. The activity of 5S RNA gene transcription might gradually be inhibited as the 5S RNA produced sequesters the transcription factor into the 7S/10S RNP particle, hence 5S RNA synthesis would ultimately be limited by the size of the available pool of transcription factor.

The role of the 42S particle proteins with regard to 5S RNA transcriptions is still unclear. Although there is evidence that one of the 42S particle proteins is similar to the 7S/10S particle protein (transcription factor) (Picard et al., 1980; Dixon and Ford 1980b) this is contradicted by chymotryptic digestion analysis (Pelham and Brown, 1980) and by cyanogen bromide cleavage (P. Barrett, 1982 personal communication) and also by the failure of ribonuclease-treated 42S particles to protect the 5S gene control region from nuclease digestion in "footprinting" experiments (Pelham and Brown, 1980). This confusion may well result from inadequate resolution of 42S particle proteins in Xenopus laevis, where two minor protein species as well as two major protein constituents can be resolved (more clearly in Xenopus borealis) (Dixon and Ford, 1980b) introducing uncertainty over which protein digests, from this heterogeneous mixture, have been compared.

In contrast the composition of the 40S particle of Triturus is particularly clear. As in Xenopus the ratio of 5S RNA to transfer RNA

is 1:3. The two RNA species are complexed with only two proteins with molecular masses of 45,000 and 39,000 (P45 and P39), previously reported as being 49,000 and 38,000 respectively (Sommerville, 1977; Sommerville et al., 1978).

The nature and sequence of the RNA/protein interactions have not previously been well defined although immunofluorescence analysis has indicated that the larger of the two particle proteins reacts specifically with only one loop pair situated on chromosome X and the smaller of the two proteins reacts with several loop pairs of atypical morphology on different chromosomes (Sommerville et al., 1978). Gall and co-workers have shown that polymerase III transcription in oocytes of the newt Notophthalmus viridescens occurs at a small number of discrete sites in condensed chromatin and not on the lampbrush loops at all. These sites include the centromere bars of three or four chromosomes which are probably involved in 5S RNA transcription (Schultz et al., 1981). Hence association of the 40S particle proteins of Triturus with specific lampbrush loops does not necessarily represent any binding to 5S RNA or tRNA at their sites of transcription, though it should be noted that Triturus does not exhibit any chromosomal structures analogous to the centromeric bars of Notophthalmus. Whatever the relationship between the 40S particle proteins and polymerase III transcription of 5S RNA and tRNA may be it is clearly necessary for a more extensive investigation of the RNA/protein associations before any further speculation on a regulatory function is developed.

In order to understand the general principles governing the formation and stability of RNP complexes and their role in the modulation of gene expression it is convenient to utilize the relatively simple cytoplasmic 40S RNP particle of Triturus oocytes. The small number of well characterized constituents facilitate a comprehensive analysis of the nature

of their interactions. In this Chapter the native 40S particles of Triturus oocytes have been dissociated, their RNA and protein components isolated and a method established for the complete reconstruction of 40S particles from the individual components. Furthermore, the in vitro formation of stable RNA/protein complexes is related to the observations on the location of the protein components in cellular structures.

It is hoped that these studies will serve not only as a framework for the mechanisms involved in the generation and utilization of the 40S RNP storage particle, which after all may be peculiar to oogenesis, but will also provide a general approach for the reconstruction of more complex RNP particles.

MATERIALS AND METHODS

1. Isolation of 40S RNP storage particles

Ovaries, containing only previtellogenic or early vitellogenic oocytes, were dissected from anaesthetized, immature, female Triturus cristatus carnifex. The ovaries were washed in modified Barth's solution (Gurdon, 1974) and incubated in a small sterile petri dish (placed in a humidity chamber), in this same solution containing 0.4 mCi/ml ^{3}H-uridine (27 Ci/mMole, Radiochemical Centre, Amersham) or 10 μCi/ml ^{14}C-amino acid mixture (50 mCi/milliatom carbon, Radiochemical Centre, Amersham) together with 50 units/ml each of penicillin, streptomycin (BDH Chemicals Ltd.) and kanamycin (GIBCO). After incubation at 20°C for 24 h the ovaries were washed and homogenized in a solution containing 0.1 M NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 8.5% sucrose and 10 mM Tris/HCl, pH 7.5 as described previously (Sommerville et al., 1978). Occasionally, 30 mM NaCl was substituted for 0.1 M NaCl. The homogenate was clarified by centrifugation at 24,000 x g for 15 minutes and the supernatant was layered on 15 to 30% sucrose gradients containing 0.1 M (or 30 mM) NaCl, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.5. After centrifugation at 70,000 x g in an M.S.E. 6 x 14 ml Ti swing-out rotor for 18 h at 4°C (or at 200,000 x g for 5 h) the 40S peak of material absorbing at 254 nm was collected, as was the ribosome peak.

2. Isolation of the 40S RNP proteins

The 40S RNP containing fraction was centrifuged in an M.S.E. 10 x 10 ml aluminium fixed angle rotor at 110,000 x g for 4 h at 4°C and the pellet resuspended in protein sample buffer (1% SDS, 35 mM Tris/sulphate, pH 6.9, 10% 2-mercaptoethanol, 25% glycerol) and heated for 5 min at 95°C. Both preparative and analytical gel electrophoresis was carried out in 1 mm thick, 15 cm long, 8-20% SDS/polyacrylamide slab-gel gradients, using a discontinuous Tris/sulphate, Tris/glycine pH 8.3 buffer system, modified from that of Laemmli (Laemmli, 1970). A potential of 200 v was applied for 20 h at 4°C and the gels were fixed and stained in 0.05% Coomassie Brilliant Blue R250 in 40% methanol, 10% acetic acid and destained in 10% methanol, 10% acetic acid. Proteins were located on preparative gels by staining and destaining a test strip and cutting out the corresponding regions from the unstained gel. The proteins were eluted by dialysis of homogenized gel slices against 1% SDS, 0.1M NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris/HCl, pH 7.5 for 24 h. Molecular weights of the proteins were determined using protein standard mixture (Boehringer and Sigma).

3. Isolation of 5S RNA and transfer RNA

Pelleted 40S RNP was resuspended in a preincubated solution containing 100 µg/ml proteinase K (Boehringer), 1% sarkosyl (Ciba-Geigy), 20 mM EDTA, 50 mM Tris/HCl, pH 8.4. After incubation at 18°C overnight, NaCl was added to 0.2 M and the RNA was precipitated at -20°C with two volumes of ethanol. The two RNA species were electrophoretically separated on 10 cm long, 5 mm diameter, 7.5% polyacrylamide tube gels using a Tris/phosphate buffer system (Loening, 1967). Electrophoresis was continued

at 8 mA per tube until the bromophenol blue marker had reached the end of the gel (usually after about 4 h). The gels were then scanned at 260 nm in a Joyce Loebl U.V. Scanner, and the positions of the 5S RNA and tRNA bands noted. RNA was eluted from the appropriate regions by dialysis of the homogenized gel slices against 1% SDS, 0.6 M sodium acetate, pH 7.5.

4. Isolation of the 5S RNA/protein complex from ribosomes

Ribosomes were isolated from homogenized ovaries in parallel with the 40S preparation previously described. After homogenization of the ribosomal pellet in a buffer containing 0.5 M KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris/HCl pH 7.5 and centrifugation of the homogenate on 15 to 30% sucrose gradients in the same buffer, under similar centrifugation conditions to those for the 40S preparation, the gradients were fractionated monitoring absorbance at 254 nm and the fractions containing the 60S ribosomal subunit retained. The large ribosomal subunits were pelleted by centrifugation at 110,000 x g for 4 h in an MSE 10 x 10 ml aluminium fixed angle rotor. The 60S pellet was raised in buffer containing 25 mM EDTA to release the 5S RNA/protein complex (Grummt et al., 1974) and purified by centrifugation on 5 to 20% sucrose gradients at 200,000 x g for 60 h in an M.S.E. 6 x 14 ml Ti swing-out rotor.

5. Reconstruction of RNP particles

Prior to mixing, the isolated, labelled RNA and protein fractions were extensively dialysed against RNP reconstitution buffer containing 0.2 M NaCl, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 mM Tris/HCl, pH 7.5 plus 1% SDS. For reconstitution, the two isolated RNA species and the two isolated proteins were mixed in various combinations and the mixtures

were dialysed against RNP reconstitution buffer until the SDS was removed. In general, mixing and dialysis were performed at 18°C over 24 h in a volume of 2-5 ml containing the appropriate molecular ratio of components found in the 40S RNP particle and in the range of 2-10 μg of each component. After this, samples were used directly for analysis of the samples formed, or were first dialysed against reconstitution buffer containing 30 mM NaCl in place of 0.2M NaCl.

6. Determination of the buoyant density of reconstituted RNP particles

Reconstituted RNP particles were fixed with neutralized formaldehyde as detailed in chapter 1, applied to preformed 24-60% CsCl gradients and centrifuged at 75,000 x g for 12 h at 20°C in an M.S.E. 3 x 5 ml aluminium swing-out rotor. The labelled material from the gradient fractions was precipitated with trichloroacetic acid and collected on nitrocellulose filters (Millipore). Radioactivity was determined by counting the washed and dried filters in a toluene based scintillation cocktail (NE 233), the RNA/protein ratio of individual peaks was determined using an empirical formula (Percentage protein = $(1.85 - \rho)/0.006$) (Spirin, 1969).

7. Immunological procedures

The production of antibodies against isolated RNP proteins and indirect immunofluorescent staining were as described in Sommerville et al., (1978).

Preparative SDS/polyacrylamide gels were run, protein bands excised and the proteins eluted as described earlier. After dialysing against PBS, to remove the bulk of the SDS, protein fractions were incorporated into multiple emulsions for subcutaneous injection into male or female Dutch rabbits. Between 100-500 μg protein was emulsified with an equal volume of a mixture of 9 parts of the oil Drakeol 6VR (Pennsylvania Refining

Co.) and one part of the emulsifier Arlacel A (Atlas Powder Co.). This water-in-oil emulsion was dispersed in an equal volume of 2% Tween 80 in 0.14 M NaCl.

The multiple emulsion was administered as a single subcutaneous injection, a booster dose was given after a period of 50 days and the rabbit was bled from the marginal vein of the ear 10 days later. The blood was allowed to clot at 4°C and the serum decanted off the clot, any remaining cells were removed by centrifugation at 2000 x g for 10 min. Serum was stored in 1 ml aliquots at -70°C.

Antisera were tested for specificity on Ouchterlony double diffusion plates made from 1% agar in 0.9% NaCl, 0.2% sarkosyl, 4 mM EDTA 10 mM Tris/HCl, pH 8.4.

Cryostat sections were cut from frozen ovary at -20°C and fixed in 100% ethanol at -20°C prior to immunostaining. After 10 min rehydration in 0.9% NaCl, 10 mM Tris/HCl pH 8.0 (TBS). The sections were incubated for 30 min at 18°C in a humidity chamber, under a droplet of 1:100 dilution of antiserum in TBS. The sections were then thoroughly washed with several changes of TBS over a period of 30 min before the application of 1:100 diluted fluorescein conjugated sheep anti-rabbit immunoglobulin (Miles-Yeda Ltd.) after a further 30 min incubation, sections were washed thoroughly in TBS and mounted in 9/1 glycerol/TBS. Sections were examined and photographed using a Wilde epifluorescence microsope with excitor and barrier filters optimized for the vizualization of fluorescein isothiocyanate. Photomicrographs were taken on Kodak Tri-X Pan film.

RESULTS

1. Composition of Triturus oocyte 40S RNP

The 40S RNP particles were isolated from homogenates of previtellogenic ovaries of Triturus cristatus carnifex by sedimentation in 15-30% sucrose gradients [Fig. 3.1] The yield of 40S particles was generally 2-3 times the mass of ribosomes although this ratio was greatly reduced if more mature ovaries were used, as observed for Xenopus laevis (Ford, 1971). Earlier investigations have shown that the 40S particles contain tRNA and 5S RNA in the ratio of 3:1 [Fig. 3.2a] (Sommerville et al., 1978), complexed with just two polypeptide species. The use of SDS polyacrylamide gradient gels has allowed more precise calibration of their molecular mass, previously quoted as 38,500 and 49,000 they are now considered to be 39,000 and 45,000 respectively, (subsequently referred to as P39 and P45) [Fig. 3.2b].

The density of the native 40S RNP particle was determined by isopycnic centrifugation in CsCl gradients after formaldehyde fixations. The single peak obtained banded at a density of 1.53 gcm $^{-3}$ [Fig. 3.3] corresponding to a composition of 46.7% RNA and 53.3% protein according to the empirical formulation of Spirin (1969).

Assuming that the molecular masses of 5S RNA and tRNA are 40,000 and 25,000 respectively the various data are consistent only if P45 and P39 are present in the 40S particle in the ratio of 2:1. The relative staining intensities of the particle proteins on SDS-polyacrylamide gels are somewhat variable, perhaps due to their highly basic nature effecting the quantitative binding of the Coomassie stain. Despite this anomalous behaviour, P45 is consistently present at higher concentration than P39.

Fig. 3.1.

Absorbance at 254 nm of Triturus high speed supernatant fractionated in a 15-30% sucrose gradient by centrifugation at 70,000 x g for 18 h at 4°C. The peak at the bottom of the gradient sedimenting at 80S is largely ribosomal material. Under these conditions the 40S RNP storage particles are found about mid-way down the gradient.

——— A_{254}.

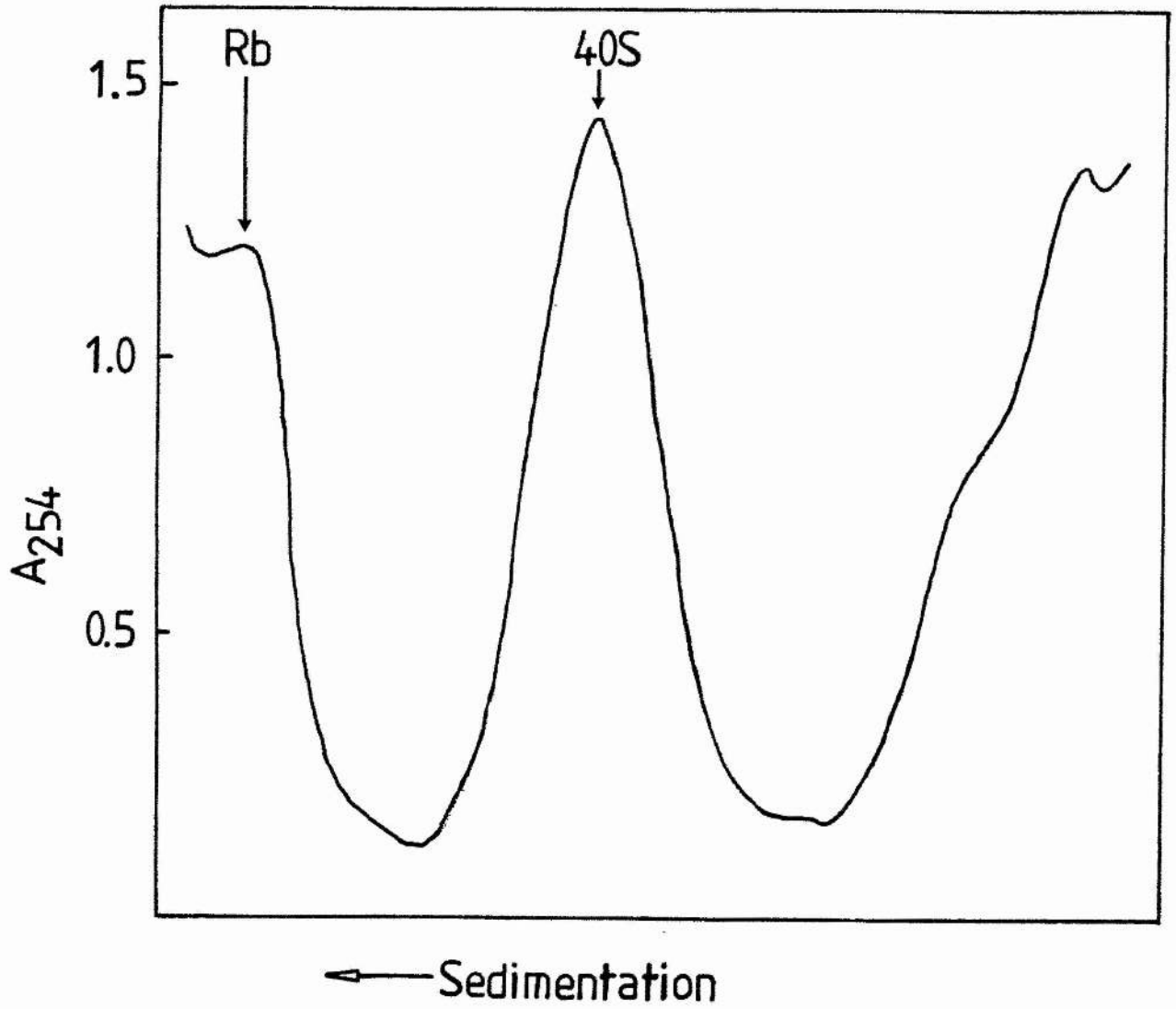


Fig.3.1

Fig. 3.2 a - b.

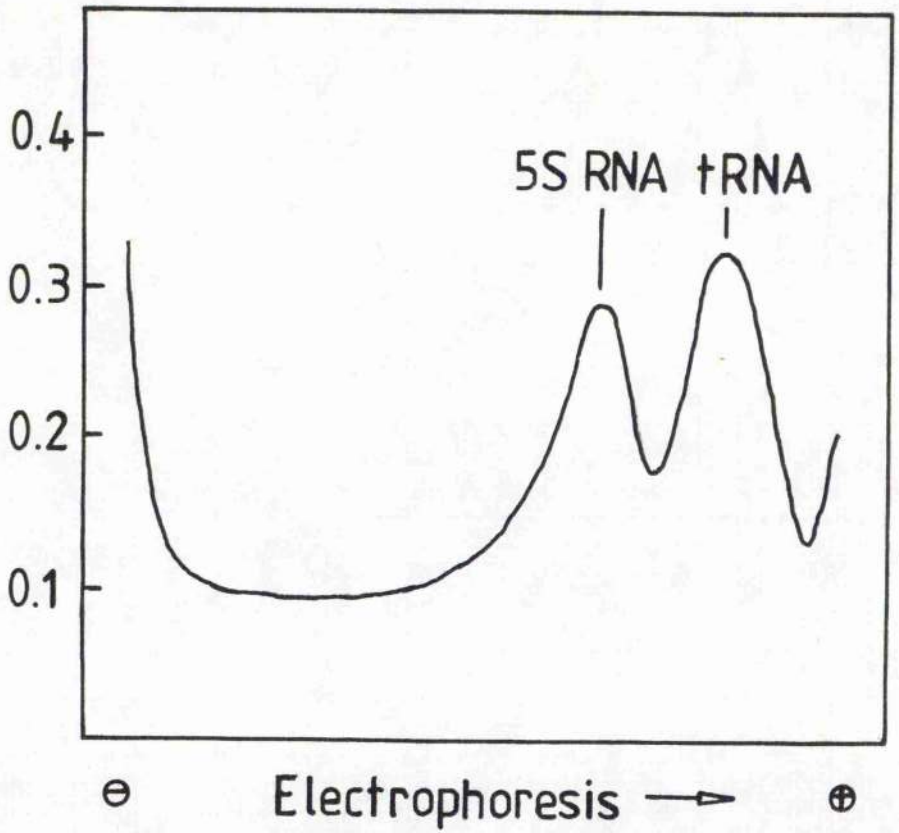
Triturus 40S RNP storage particle components.

(a) Triturus 40S RNP particle RNA electrophoresed in 7.5% polyacrylamide gels according to the method of Loening (1969). Only two peaks, migrating in positions identical to 5S and transfer RNA markers, are seen on scanning the gel using a Joyce-Loebl U.V. gel scanner.

——— A_{254}.

(b) Triturus 40S RNP storage particle proteins analysed by SDS/polyacrylamide gel electrophoresis. Two major proteins are visible with molecular masses of 39,000 and 45,000, a track containing Triturus oocyte ribosomal protein is shown adjacent to the 40S track for comparative purposes. Molecular mass is indicated at x 10^{-3}.

Fig. 3.2 a.



b.

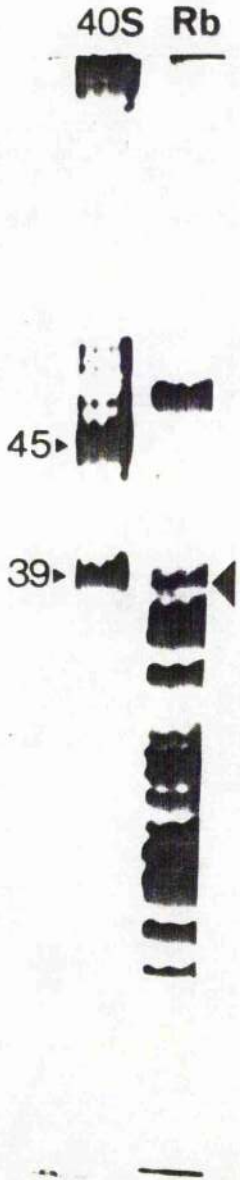
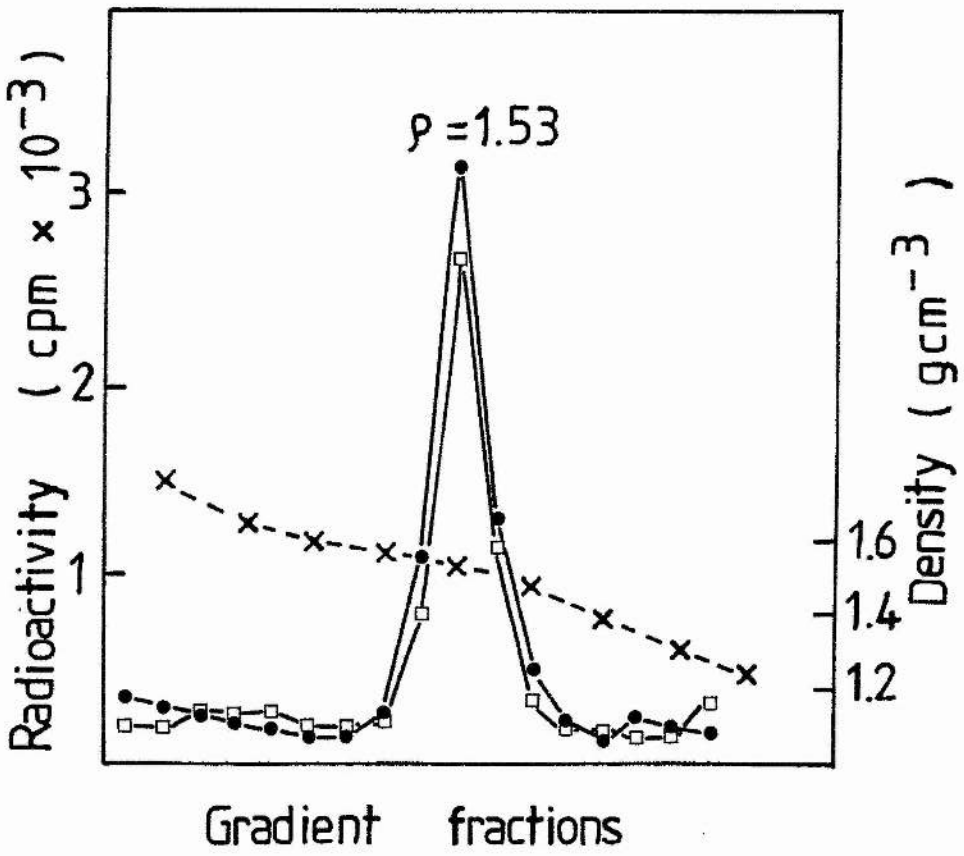


Fig. 3.3.

Density equilibrium centrifugation of formaldehyde fixed 40S RNP material. The 40S particles were fixed with neutralized 17% formaldehyde to a final concentration of 3.4% and centrifuged through a preformed 24-60% CsCl gradient.

●——● RNA;□——□ protein; ✕——✕ density.

Fig.3.3


ρ =1.53
Radioactivity (cpm × 10⁻³)
3
2
1
Density (gcm⁻³)
1.6
1.4
1.2
Gradient fractions

Treatment of the 40S particles with increasing salt concentrations results in a breakdown of the RNP complex and leads to the production of peaks of material previously reported as sedimented at 32S, 24S and 15S (Kloetzel et al., 1981) but now more rigorously assigned as 26S, 15S and 10S [Fig. 3.4]. Initially preparations of 40S particles utilized 0.1 M NaCl in which the RNP complex remained stable, however subsequent destabilization of the purified 40S particle in 0.1 M salt has led to the adoption of 30 mM NaCl concentration in all isolation buffers. Presumably the presence of certain cytoplasmic factors, lost during the purification process, helps to stabilize the 40S particle and maintain its integrity in 0.1 M salt.

Investigation of the 26S, 15S and 10S peaks, derived by salt dissociation from the 40S particle shows that they share the same protein and RNA components in the same relative proportions as the native particle (data not shown).

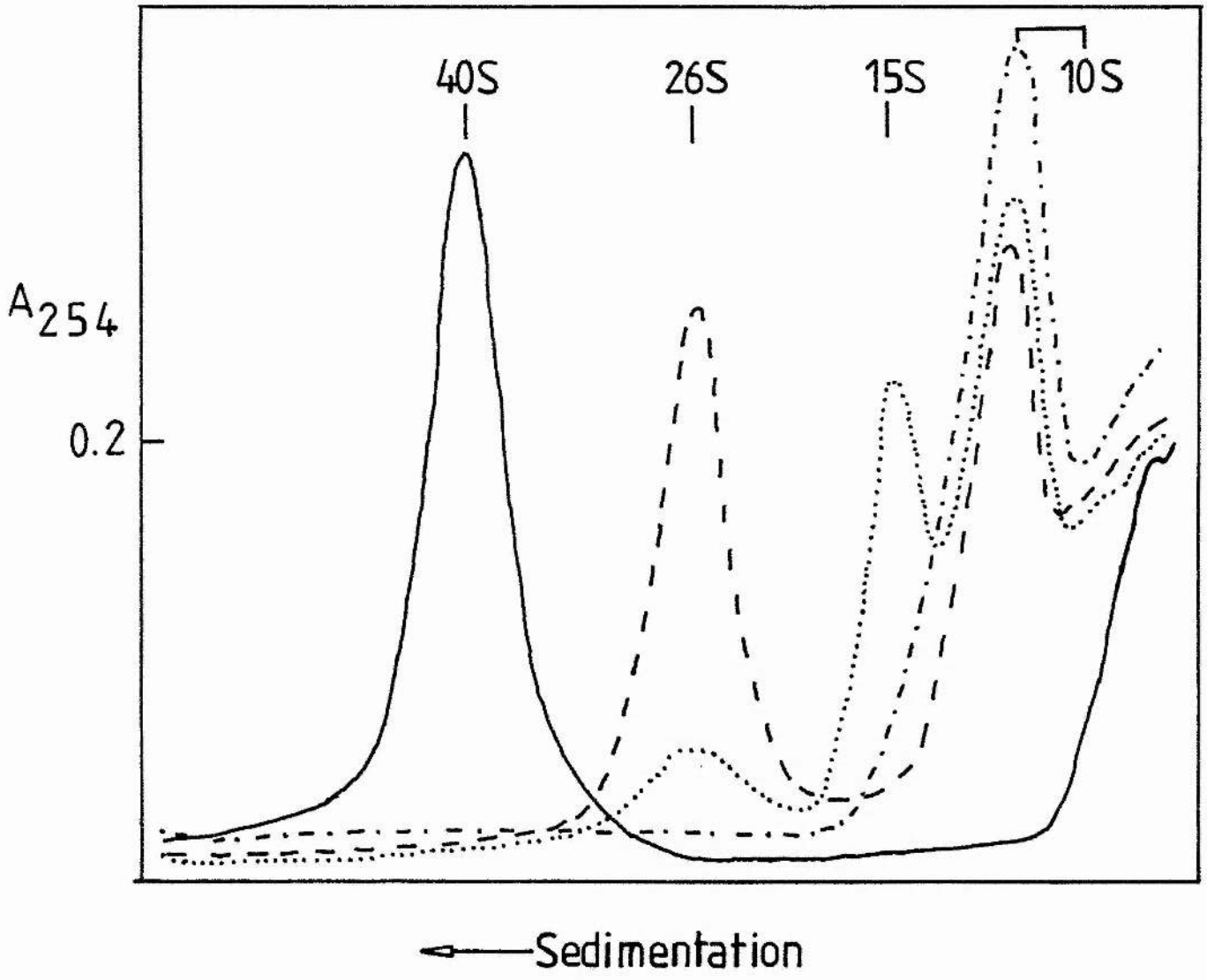
A possible interpretation of these observations is that the 40S particle consists of four monomeric subunits with a sedimentation rate of 15S, held together in the 40S particle by ionic interactions. The 24S peak would represent a dimer and the 10S peak a heterologous collection of the further degradation products of the 15S monomer. A simple stoichiometric solution to the arrangement of the 40S RNP components in the 15S subunit, compatible with the available data is as follows:

Each 15S particle must contain one molecule of 5S RNA, three molecules of tRNA, two molecules of P45 and one molecule of P39. The total molecular mass would thus be nearly 250,000 giving the 40S particle a mass of 10^6 which is near to that expected for its sedimentation rate. The RNA and protein content calculated for this model is 47.1% and 52.9% respectively, in close agreement with the experimentally-derived density.

Fig. 3.4.

Effect of increasing salt concentration on the sedimentation of 40S RNP particles. The 40S RNP peak from Triturus oocytes was pelleted, resuspended in solutions containing 0.1 M NaCl (– – –); 0.2M NaCl (.....); 0.5 M NaCl (-.-.-.), and sedimented in 15-30% sucrose gradients containing the same salt concentrations. The sedimentation profile of part of the original 40S RNP preparation (———) is superimposed for comparison.

Fig.3.4



2. Reconstruction of the 40S RNP particle

Complete dissociation of the 40S RNP complex into its separate components can be achieved by treatment with 8 M urea or 1% SDS. In concentrations of urea below about 6 M the particle proteins are observed to precipitate, even in the presence of RNA. Hence attempts to reform RNP complexes after disruption in 8 M urea or resuspension of gel purified component in 8 M urea fail, since in these conditions it would seem that the proteins are irreversibly denatured and precipitate as soon as the urea concentration is reduced below 6 M. Any RNA/protein interaction is thus forestalled. A similar phenomenon has been noted in attempts to reform a 5S RNA/protein complex from purified yeast ribosomal components (Nazar et al., 1979).

In contrast, when 1% SDS is used to dissociate the 40S RNP particle and is subsequently removed by dialysis against 0.2 M NaCl buffer, almost 100% of the labelled RNA can be found in an RNP complex having a buoyant density in CsCl of 1.53 gcm^{-3} [Fig. 3.5] identical to that of the native particle and its subunits. Analysis of the formed RNP by sedimentation in sucrose gradients shows a reconstitution efficiency of about 30% of the labelled RNA into 40S particles, most of the rest of the label being present in peaks corresponding to monomeric and dimeric subunits [Fig. 3.6].

The use of purified 5S RNA, tRNA, P45 and P39 to reform RNP complexes yields a similar result [Fig. 3.5] although the efficiency of reconstitution is relatively lower with only 55% of the labelled RNA being incorporated into RNP particles. The efficiency of reconstitution is partly dependent upon the molecular ratio of the RNA and protein components and it is difficult to achieve optimal conditions since although stringent precautions were taken against component degradation

Fig. 3.5.

Density equilibrium centrifugation of formaldehyde-fixed dissociated and reconstituted 40S RNP particles. ^{3}H-uridine labelled 40S RNP was dissociated in a solution containing 1% SDS, 0.2 M NaCl, 2mM $MgCl_2$, 5 mM 2-mercapto-ethanol, 10 mM Tris/HCl pH 7.5, fixed in 3.4% formaldehyde and analysed on a preformed 24-60% CsCl gradient (O——O). Part of the dissociated RNP was dialysed against the same solution minus the SDS prior to fixation and analysis. The density profile of reconstituted RNP (●——●) is indistinguishable from that of native 40S RNP. (see Fig. 3.3).

Fig.3.5

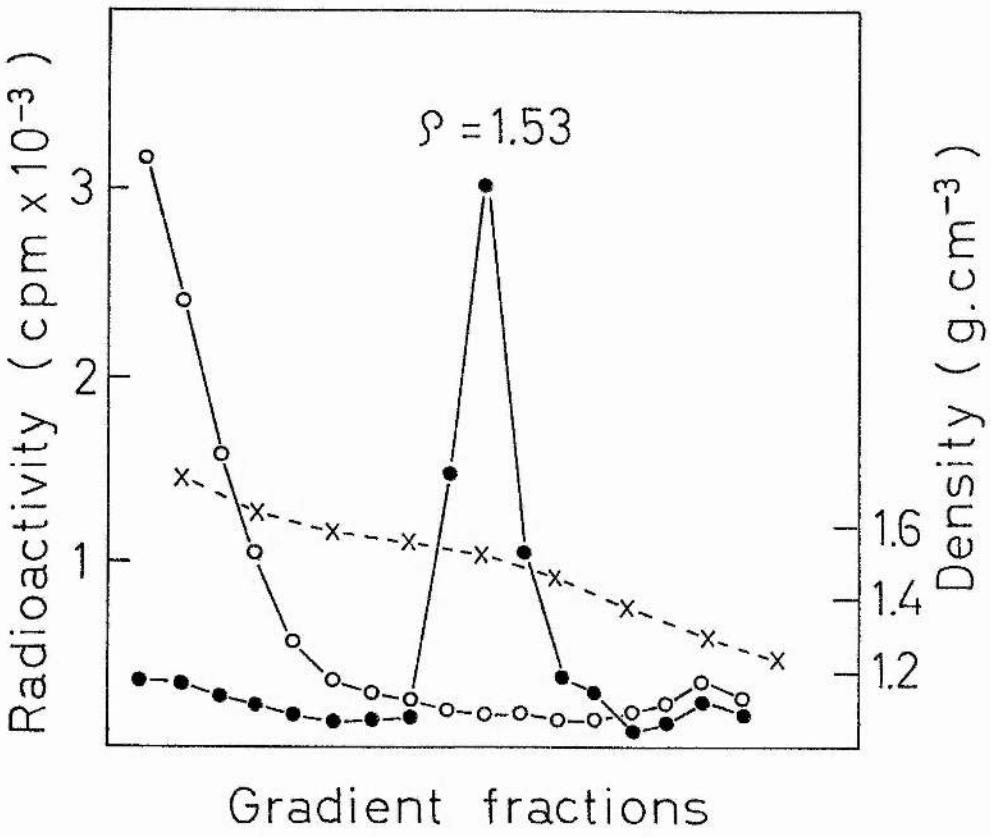

ρ = 1.53
Radioactivity (cpm x 10⁻³)
3
2
1
1.6
1.4
1.2
Density (g.cm⁻³)
Gradient fractions

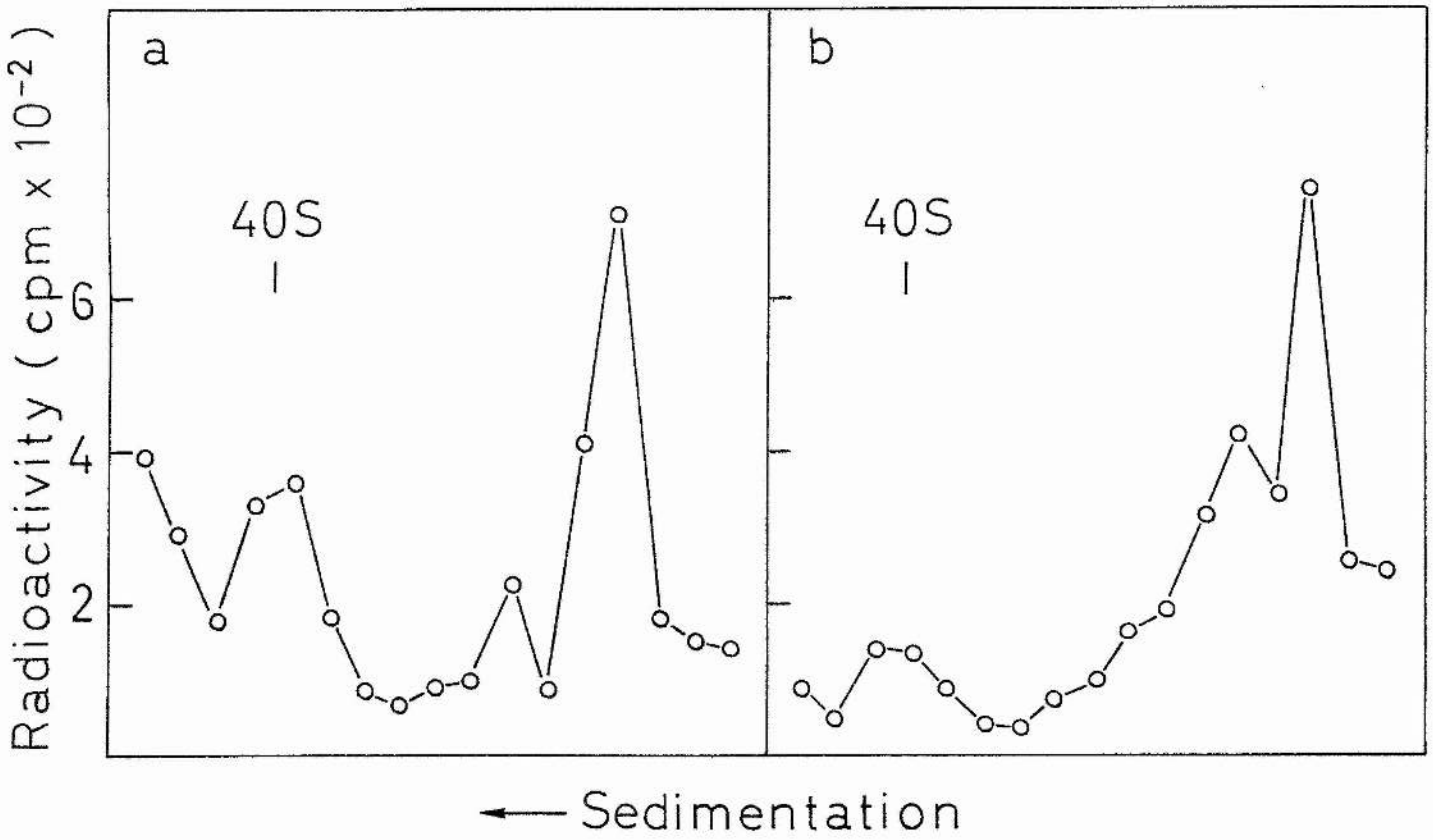
Fig. 3.6 a - b.

Sedimentation profiles on 15 - 30% sucrose gradients of reconstituted RNP.

(a) The reconstituted material shown in Fig. 3.5.

(b) RNP reconstituted from the four isolated components 5S RNA:tRNA:P45:P39 in the molecular ratio 1:3:2:1. RNA.

Fig. 3.6



(protein and RNA integrity was monitored wherever possible) proteolytic and ribonuclease inhibitors were never employed in case they interfered with the interactions of the particle.

3. RNA/protein binding experiments

In order to gain a deeper understanding of the binding specificities of the 40S RNP components and their arrangement within the particle, RNA/protein binding studies were performed using electrophoretically purified RNA and protein molecules. Conventional RNA/protein binding experiments frequently utilize membrane filter binding assays (Hsieh and Brutlag, 1979), however these methods do not disciminate between the formation of specific RNP complexes and the adventitious association of RNA with protein precipitates.

The basis of our approach is that radioactively labelled RNA is mixed with the appropriate protein solubilized in a solution containing 1% SDS, and any RNP complexes that form upon removal of the SDS by dialysis are fixed with formaldehyde and analysed by density gradient centrifugation. The detection of discrete density peaks is indicative of the formation of specific RNP complexes and the RNA/protein binding ratio can be calculated empirically using the formulation of Spirin, (1969). The results from most of the RNA/protein combinations investigated are shown in Fig. 3.7.

The reaction of 5S RNA with P45 results in the formation of a stable RNP complex with a density of 1.53 gcm^3 in CsCl [Fig. 3.7a]. The density of this sharp, single peak corresponds to that expected for a molecular ratio of 1:1, since no other peaks were ever detected it seems reasonable to conclude that P45 has a single, specific binding site for 5S RNA.

Combining P45 with tRNA gives rise to a much broader density distribution [Fig. 3.7c]. The peak density value of 1.58 gcm^{-3} corresponds closely to the theoretical density of 1.57 gcm^{-3} for an RNP complex containing one molecule of P45 and two tRNA molecules. The shoulders of the main peak, with densities of 1.45 gcm^{-3} and 1.62 gcm^{-3} suggest that complexes of P45 with one and three tRNA molecules could also be present. Thus P45 may have up to three binding sites for tRNA in addition to its single 5S RNA binding site.

No evidence was obtained of any interaction between P39 and tRNA [Fig. 3.7d] however P39 and 5S RNA combine to form an RNP complex with a density of 1.57 gcm^{-3} [Fig. 3.7b] corresponding to a simple 1:1 molecular ratio and indicating that P39, like P45, has a single, specific binding site for 5S RNA.

When both 5S RNA and tRNA are combined with P45 two density peaks result [Fig. 3.7e]. The major peak, at a density of 1.53 gcm^{-3} corresponds to that already observed for the 5S RNA/P45 complex. The minor peak has a density of 1.61 gcm^{-3} and corresponds closely to the expected value for a complex of P45 and three tRNA molecules. It should be noted that the difference in peak height is probably not related to any differential stability of the two complexes but rather a consequence of the particular 5S/tRNA ratio employed in this experiment. Though only two density peaks are clearly visible in Fig. 3.7e it is uncertain whether other possible combinations can be excluded. For instance, P45 complexed with one molecule each of 5S RNA and tRNA (theoretical density 1.61 gcm^{-3}) or two molecules of P45 with one of 5S RNA and three of tRNA (theoretical density 1.59 gcm^{-3}) could all be represented in the smaller peak. Unfortunately no clear resolution of the different complexes is possible by sedimentation analysis and electrophoretic characterization of the formaldehyde fixed components is not adequate: this difficulty

Fig. 3.7 a - f,

Buoyant density profiles of RNP complexes formed on mixing the isolated RNA and protein components indicated. Conditions of reassociation and analysis were as described in Fig. 3.5. The appproximate molecular ratio (RNA:protein) used in (a) was 1:1; in (b - d) was 2:1; in (e) 5S RNA:tRNA:P45 was 3:1:6 and in (f) 5S RNA:P:45:P39 was 1:2:1.

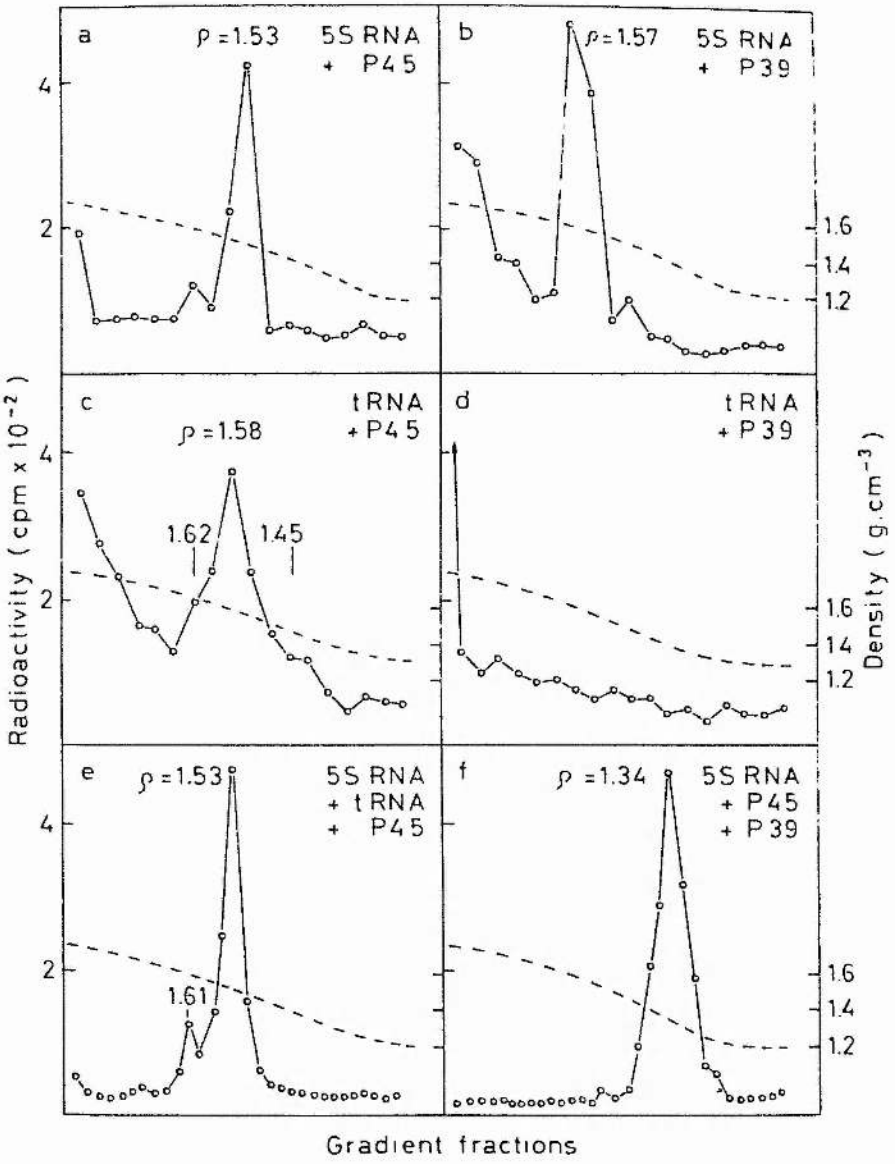

a
ρ =1.53
5S RNA
+ P45
b
ρ=1.57
5S RNA
+ P39
c
ρ =1.58
tRNA
+P45
1.62
1.45
d
tRNA
+P39
e
ρ =1.53
5S RNA
+ tRNA
+ P45
1.61
f
ρ =1.34
5S RNA
+ P45
+ P39
Radioactivity (cpm x 10⁻²)
Density (g.cm⁻³)
Gradient fractions

Fig. 3.7a-f

could probably be resolved by the use of a reversible cross-linking reagent such as 4-methyl mercaptobutyrimidate (Karn and Allfrey, 1977).

The addition of P39 to a preincubated mixture of 5S RNA and P45 results in the formation of a single peak with a density of 1.34 gcm^{-3} [Fig. 3.7f], a value much lower than that of the native particle or any of the previously described complexes. The increased percentage of protein can be accounted for if one molecule of P39 and one molecule of P45 bind to a P45/5S RNA complex although a small discrepancy exists betweem the theoretical density of 1.39 gcm^{-3} and the observed value. P39 would therefore seem to have a role in the formation of larger complexes by protein/protein interaction. This is confirmed by the results of another experiment where the addition of P39 to a preincubated mixture of P45, 5S RNA and tRNA (data not shown) results in a quantitative conversion to forms which have a density of 1.53 gcm^{-3} and sediment with a distribution similar to that in Fig. 3.6b It would therefore appear that P39 is necessary to facilitate the formation of complete monomeric subunits from P45/RNA complexes.

4. Immunological studies

The cellular locations of the two 40S RNP proteins are most easily determined by immunological analysis. Not only should it be possible to demonstrate the intracellular distribution of 40S RNP particles but the presence of P45 and P39 in precursors or products of the 40S particle should also be detectable.

Immunofluorescent staining of cryostat-sectioned ovary shows that whereas anti-P39 gives a predominantly cytoplasmic reaction [Fig. 3.8a] anti-P45 reacts somewhat more weakly, but with nucleus and cytoplasm to an almost equal extent, [Fig. 3.8b]

Fig. 3.8 a - b.

Indirect immunofluorescent staining of cryostat-sectioned *Triturus* ovary reacted with 1:100 dilutions of anti-P39 (a) and anti-P45 (B). Bars indicate 100 μm.

A

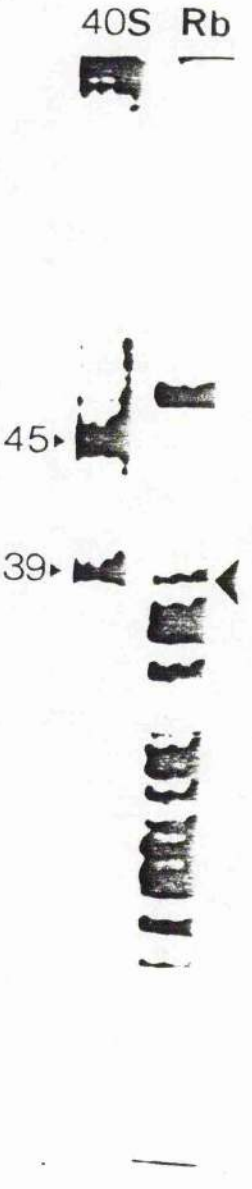
B

Fig. 3.9.

SDS/polyacrylamide gel electrophoresis of proteins from Triturus 40S RNP particles and from Triturus oocyte ribosomes. The positions of the 45,000 and 39,000 molecular mass marker proteins are indicated.

▶ Symbol indicates the ribosomal protein corresponding to P39.

Fig.3.9

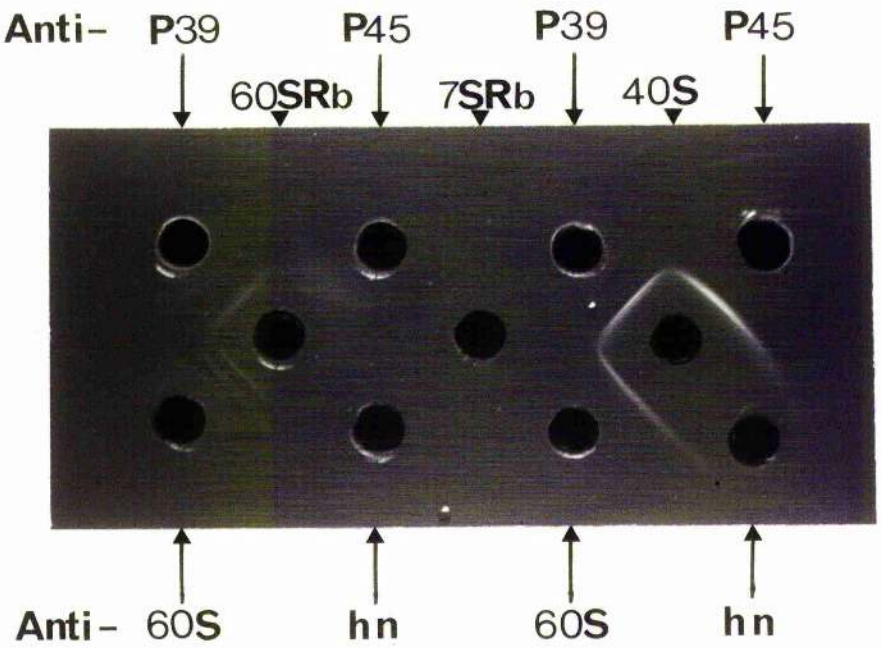


The relationship between 5S RNA/protein complexes in the 40S RNA particle and in the ribosome is also amenable to immunological investigation. It is apparent from SDS/polyacrylamide gradient gel analysis that P39 has an electrophoretic mobility very similar to one of the ribosomal proteins [Fig. 3.9], and it it possible that this ribosomal protein is associated with 5S RNA in the ribosomal 60S subunit (Picard and Wegnez, 1979; Nazar et al., 1979). However, when the 7S component is isolated from EDTA treated 60S subunits of Triturus ribosomes, this is found to contain 5S RNA plus a protein with a molecular mass of 33,000 (data not shown).

When antibodies raised against P45 and P39 are reacted in Ouchterlony double-diffusion tests with 40S particles, ribosomes and ribosomal subunits, anti-P45 precipitates 40S particles alone, whereas anti-P39 precipitates both 40S particles and protein from the ribosomal 60S subunit [Fig. 3.10] However anti-P39 forms no obvious precipitin line with the isolated ribosomal 7S complex, which contains only 5S RNA and the protein of molecular mass 33,000. Antibodies raised against the ribosomal 60S subunit precipitate 40S particles in addition to several of the subunit proteins. Therefore P39 appears to be antigenically related to at least one of the ribosomal proteins. (The fused precipitin lines formed by anti-P45, anti-P39 and anti-60S ribosomal with 40S RNP indicate that, unlike the ribosomal 60S subunit, the RNP storage particle is not dissociated in the presence of 0.2% sarkosyl. The possibility that P39, or a modified form of this protein accompanies 5S RNA into the ribosome is supported by experiments involving the immunostaining of protein gel-transfers (see chapter 1 for methodology). Although the immunoperoxidase staining was so weak in this instance as to be unrecordable, it was nevertheless evident that anti-P39

Fig. 3.10.

Ouchterlony double-diffusion plate showing reaction of anti-P45 (aP45), anti-P39 (aP39), antiserum to ribosomal 60S subunit proteins (a60s) and antiserum to hnRNP proteins (ahn) with 40S particles (40S), ribosomal 7S particles (7SRb) and ribosomal 60S subunits (60S Rb). The gel was made from 1% agar in 0.9% NaCl,0.2% sarkosyl, 4 mM EDTA, 10 mM Tris/HCl pH 8.4.


Anti- P39
P45
P39
P45
60SRb
7SRb
40S
Anti- 60S
hn
60S
hn

reacts with a ribosomal protein of equivalent molecular mass and also another protein with a molecular mass of 33,000. The low affinity demonstrated in the immunostaining of even the homologous antigen on protein gel-transfers may be a result of inadequate renaturation of these proteins in the transfer conditions used.

DISCUSSION

Analysis of the 40S RNP storage particle of Triturus indicates that, as in the 42S Xenopus equivalent, it contains only two RNA species, 5S RNA and transfer RNA. Unlike Xenopus, in Triturus there is no confusion about the number and molecular mass of the protein components, only two polypeptides are found in the 40S particle, having molecular masses of 45,000 and 39,000.

In common with its counterpart in Xenopus (Picard et al., 1980) the Triturus 40S RNP particle can be divided into four monomeric subunits by salt treatment, though it would seem to require higher salt concentrations to achieve this. Xenopus 40S particles are reduced to the monomeric state with just 0.125 M NaCl whereas 0.5 M NaCl is necessary to destabilize Triturus 40S.

Isopycnic density centrifugation in CsCl gradients shows the 40S particle to have a density of 1.53 gcm^{-3}, equivalent to a protein content of 53.3%. Taking into account the 3:1 ratio of tRNA to 5S RNA and a probable ratio of two molecules of P45 to every one of P39 in the 40S particle these data can be explained if the 15S monomeric subunit of the 40S RNP particle is considered to contain one molecule of 5S RNA, three of tRNA, two of P45 and one of P39. A comparable composition has been reported for the 42S RNP storage particle of the teleost Tinca tinca and the amphibian Xenopus laevis (Denis et al., 1980; Picard and Wegnez, 1980). Any other components would have to be present at levels of less than one molecule per 40S particle in order to conform with the data presented. It is quite likely that enzymes and other macromolecules are transiently associated 40S particles and that some minor heterogeneity can exist within the 40S RNP population.

The susceptibility of the 40S particle to dissociation at raised salt concentrations indicates that the monomeric subunits are bound to one another by ionic forces. From the equivalence of density and composition of monomer, dimer and the native particle it can be concluded that the increased salt concentration does not disrupt the subunits internal organization. The integrity of the 15S subunit must therefore be maintained by strong interactions, other than ionic, between its components. In urea concentrations above 6 M the complete disruption of subunit integrity it achieved suggesting that hydrogen bonds are critical in maintaining the native conformation of the particle. Any alteration in protein conformation would most probably reduce its RNA binding capacity and, in this case, denaturation would appear to be irreversible since the particle proteins precipitate when the urea concentration is reduced. The irreversible denaturation after urea treatment, particularly of P39, excludes this method of dissolution for binding studies. However 40S RNP particles can also be easily dissociated in the presence of 1% SDS. As the SDS concentration is gradually reduced by dialysis against 0.2 M NaCl the proteins seem to be able to renature sufficiently to interact with the RNA before the SDS concentration is so low that precipitation of the proteins occurs. This method offers a means of studying the specific interactions of proteins with RNA, a knowledge of which is crucial to our understanding of the mechanisms involved in the formation of 40S storage particles <u>in vivo</u>.

A stepwise analysis of the specific RNA/protein interactions is summarised in Table 3a, and explained below in greater detail.

The RNP complex formed by mixing P45 and 5S RNA appears to result from a specific one-to-one molecular interaction. In conditions of protein excess, nearly all of the RNA is bound in the form of an RNP which

Table 1. Density values of RNA/protein complexes formed in vitro.

Components	Observed density	Molecular ratio	Theoretical density*
5S RNA + P45	1.53	1:1	1.53
5S RNA + P39	1.57	1:1	1.57
tRNA + P45	1.45	1:1	1.46
	1.58	2:1	1.57
	1.62	3:1	1.62
tRNA + P39	-		
5S RNA + tRNA + P45	1.53	1:0:1	1.53
	1.62	0:3:1	1.62
		1:1:1	1.59
		1:3:2	1.59
5S RNA + P45 + P39	1.34	1:2:1	1.39
5S RNA + tRNA + P45 + P39	1.53	1:3:2:1	1.53

*Calculated from molecular weights of the components according to the formulation of Spirin (1969).

has a density very close to the expected theoretical value for a particle of that stoichiometry. This interaction may well be significant *in vivo*, not only in the 40S particle but also in association with 5S RNA in the nucleus, possibly at its site of transcription as previously postulated (Sommerville *et al.*, 1978). A nuclear role of P45 is supported by the immunostaining evidence of a nuclear as well as cytoplasmic location in previtellogenic oocytes.

In addition to binding 5S RNA, P45 can evidently form stable complexes with up to three tRNA molecules, indicating a surprising RNA binding capacity for this protein. However when both 5S RNA and tRNA are combined with P45 *in vitro* the formation of an RNP corresponding to one molecule of P45, one of 5S RNA and three of tRNA is not detected. Such a particle has a theoretical density of 1.68 gcm^{-3} but the highest density peak observed is only 1.61 gcm^{-3}. This can be explained if a complex of P45 with three tRNA molecules excludes 5S RNA binding or if a particle with a 1:1:1 molecular ratio of all three components is formed. Further analysis of the molecular composition of the density peaks was not attempted but the use of a reversible cross-linking reagent, to fix the RNP prior to isopycnic centrifugation, would facilitate the electrophoretic characterization of the components and hence resolve exactly which interactions occur. In the absence of this information it is only possible to speculate on a number of feasible alternatives (see Table 1). Whether or not P45 binds to 5S RNA at its site of transcription is still uncertain but if this were the case the nuclear role of P45 could be that of a carrier protein in the transport of 5S RNA and perhaps tRNA to the cytoplasm.

The binding studies also indicate that protein/protein interaction occurs between P45 and P39, especially when the former is already complexed with 5S RNA. Since 5S RNA can also produce a stable complex with P39

a mechanism whereby 5S RNA, complexed with P45 might transfer its binding affinity to P39 during the formation of a 40S particle in the cytoplasm seems feasible. Such a process would provide a means of confining 5S RNA to the cytoplasm, (since P39 would appear to be restricted from the nucleus according to the immunostaining results) which might be of significance if 5S transcription is controlled by a form of feedback inhibition as has been suggested (Sakonju et al., 1980; Bogenhagen et al., 1980).

In contrast, tRNA is never found to bind P39 and probably leaves the 40S complex as naked RNA. The significance of P39 would seem to be in its interaction with the small 5S/P45 and 3(tRNA)/P45 complexes to generate a 15S monomer, and under optimum salt concentrations to yield 26S dimers and 40S tetramers. Since the density of these particles is 1.53 gcm^{-3}, identical to that found for the native and salt dissociated particles it would seem that the 40S RNP is assembled by a very simple association of the smaller RNP complexes.

In addition to the 40S RNP particle, about 50% of the 5S RNA in previtellogenic oocytes of Xenopus is found in association with a second type of storage particle sedimenting at 7S (Picard and Wegnez, 1979). Recent reports indicate that the protein of the 7S particle may be identical to a transcription factor which can bind specifically, in an in vitro transcription system, to a control region located within the 5S gene, resulting in its activation (Engelke et al., 1980; Pelham and Brown, 1980).

The co-identity of the 7S particle protein and one of the 42S particle proteins has been reported (Picard et al., 1980; Dixon and Ford, 1980). however this has been contradicted by evidence that chymotryptic digests of the 7S particle protein (or "transcription factor") are different from those of the corresponding 42S particle protein. Dixon and Ford, (1980), have noted that the 7S/10S particle of previtellogenic oocytes contains a different protein to the 7S/10S particle they detect in mature

oocyte stages. The interrelationships of all these proteins are still not well characterized and a much more detailed analysis is required before any adequate explanation of function or comparison with the system in Triturus is possible.

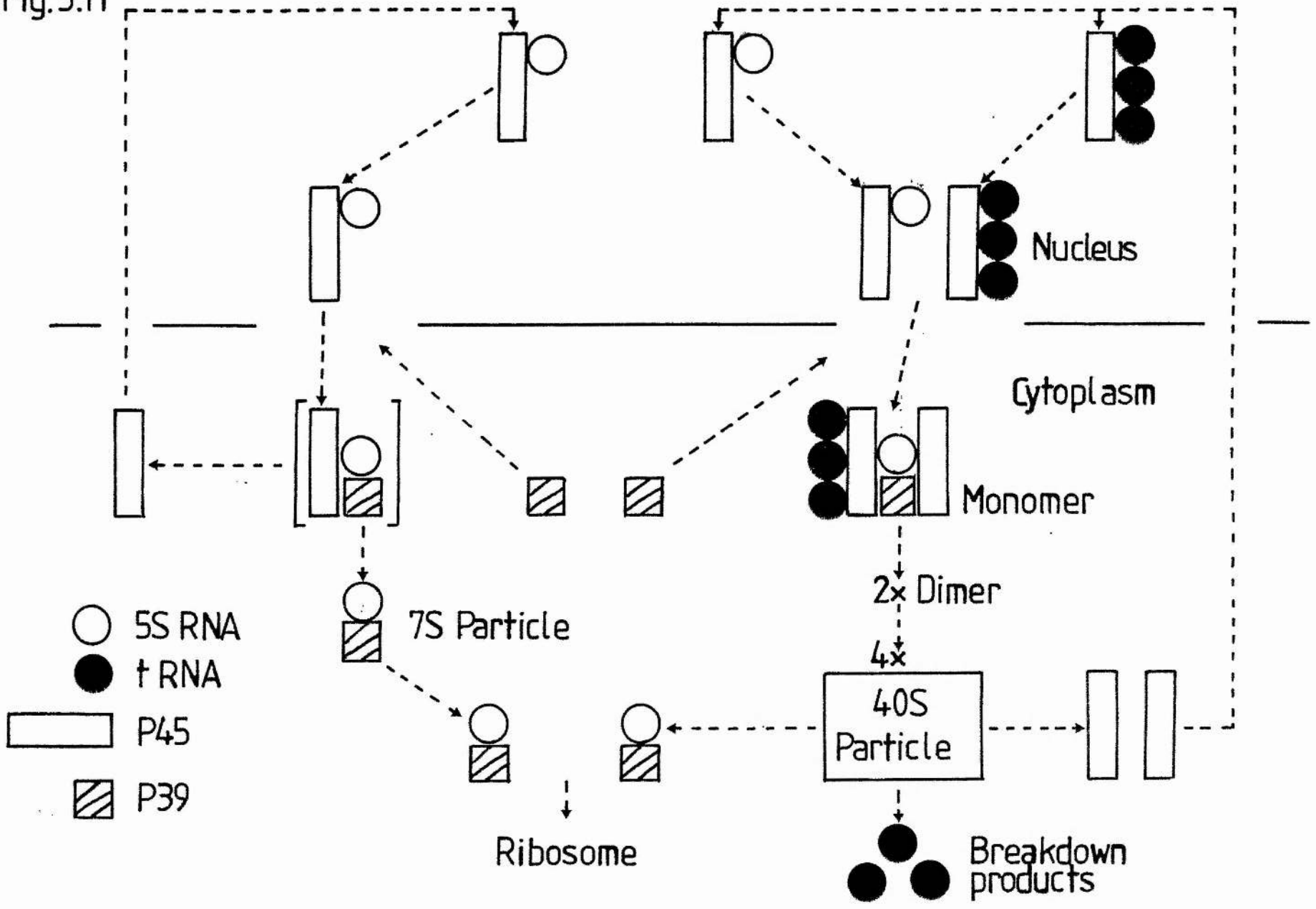
A similar situation exists regarding the relationship between the smaller of the two particle proteins and the 5S RNA associated ribosomal proteins. Preliminary immunostaining of gel transfers of ribosomal proteins with antibodies to P39 gave weak reactions with a protein of corresponding molecular mass and also with a protein of molecular mass of 33,000. Whether the latter protein is the same one that is co-released with 5S RNA from the 60S ribosomal subunit by EDTA treatment has not been established. The failure of anti-P39 to form a precipitin line on Ouchterlony double diffusion assays against the EDTA released 5S ribosomal complex would indicate that it is not the same protein and hence that some exchange of protein binding may occur either when 5S RNA enters the ribosome or artifactually when the subunit is released by EDTA treatment. In general, the immunostaining experiments must be interpreted with caution since it is quite likely that different proteins with similar binding functions will have some antigenic determinants in common.

A scheme for the formation and breakdown of 40S RNP storage particles is presented in Fig. 3.11. This is based on the observations made in the present study but does not exclude other interpretations, for instance that P39 may, to a limited extent, have some nuclear function, or that the various P39 proteins are translation products of different genes rather than modified forms of the translation product of one type of gene.

Fig. 3.11

Scheme for the formation and breakdown of 40S RNP storage particles. A pathway for the formation of 7S storage particles is suggested. As discussed in the text, the protein indicated as P39 may be different in the 40S storage, 7S storage and ribosomal complexes.

Fig. 3.11


Nucleus
Cytoplasm
Monomer
2x Dimer
4x
40S Particle
7S Particle
Ribosome
Breakdown products
5S RNA
t RNA
P45
P39

GENERAL DISCUSSION

In this thesis two widely differing types of ribonucleoprotein complex from amphibian oocytes have been investigated, heterogeneous ribonucleoprotein and a 40S RNP that stores 5S RNA and tRNA in the cytoplasm of previtellogenic oocytes.

The 40S RNP particle presents an ideal system for study. 5S RNA and transfer RNA are both transcribed by RNA polymerase III and though they are not destined to be translated, their vital functional roles in protein synthesis make the mechanisms by which their expression is controlled very important. The relationship between the structure of the 40S particle and the control of 5S RNA synthesis has already been discussed in Chapter III, however the analysis of the 40S particle reveals several features that are pertinent to RNP structure in general and some that are rather more specific to the 40S RNP complex.

The RNA/protein interactions in the 40S particle are not simply an electrostatic association of the basic proteins with nucleic acid but seem to be dependent on the specific hydrogen bonded configurations of P45 and P39. The sequence specificity of these two proteins is probably not typical of the hnRNP "structural" proteins where a wider variety of RNA sequences must be present. In addition to RNA/protein interactions P39 is important in protein/protein bonding within the 15S subunit of the 40S particle. The possibility of a protein "core" to hnRNP has been considered since the original "informofer" model suggested by Samarina et al., (1968). In contrast to the association of the RNA with a beaded core subunit, Stevenin and Jacob, (1974) suggested a "folded fibre" model

for hnRNP in which periodic foldings of an hnRNP fibril are stabilized by protein/protein interactions. The concept of a recycleable protein core complex (analagous to the nucleosome core in DNP) is probably an over simplification in view of the complex processes of hnRNA maturation and transport that occur (Malcolm and Sommerville, 1974; Martin et al.,) 1980). In either model of hnRNP structure protein/protein interaction would be a necessary factor in the architecture of the RNP complexes.

In Triturus oocytes there is considerable evidence to suggest that hnRNP particles are associated in vivo with a fibrillar protein matrix (Kloetzel, et al. 1981 and 1982). Unlike the 40S cytoplasmic particle in Triturus the hnRNP preparation seems to be far from pure. The material sediments at a much higher rate than somatic cell hnRNP, and bands at a density that indicates a protein/RNA ratio of more than 30:1. Considering the mitochondrial contamination evident from examination of thin sections of the pelleted material in the electron microscope (Fig. 1.4), and the cytoplasmic location of many of the polypeptides from the "hnRNP", it might be possible that the ribonucleoprotein moiety is associated with macromolecular structures in this preparation. Such associations have been described between hnRNP and nuclear matrix (Miller et al., 1978; van Eekelen and van Venrooij, 1981) and between hnRNA and mRNA with membranous structures during transport from nucleus to cytoplasm. (Shiokawa and Pogo, 1974). Shiokawa, (1983) discusses the possibility that partially processed mRNA is sequestered to supramolecular membranous structures that eventually associate with cytoskeletal elements in the oocyte. The nature and origin of these mRNP-binding structures has also been considered (Capco and Jeffery, 1982). These authors favoured annulate lamellae as

the carrier structures. Annulate lamellae were shown to originate at the periphery of the nuclear membrane and to separate into the cytoplasm (Franke et al., 1981) and are thus possible candidates for a role in transporting ribonucleoprotein from the nucleus to the cytoplasm. Although the mitochondria associated endoplasmic reticulum was suggested as a possible mRNA carrier structure, no labelling of the mitochondrial region is noted in autoradiographs of oocyte sections hybridized with [^{3}H] poly(U) (Capco and Jeffery, 1982). These authors show that poly(A)$^+$ RNA is localized just outside the nuclear membrane in stage III Xenopus oocytes but migrating to the subcortical region of the vegetal hemisphere in stage VI oocytes. The presence of a secondary accumulation of poly(A)$^+$ RNA in a sub-nuclear location in stage V oocytes is also shown. This is interesting with regard to the seasonal (stage) variation in the Triturus oocyte hnRNP preparation noted by Sommerville, 1979. A second peak of RNP banding at a slightly lower density appears during the summer months, correlated with the increased percentage of fully grown oocytes found in Triturus ovaries at this time. The base composition of this second peak is distinctive, with up to 60 mol. % uridylic acid in some fractions (Sommerville, 1973). The cellular localization of this uridylic acid rich RNP fraction might well be demonstrable using a labelled poly(A) probe for in situ hybridization on sectioned oocytes in the manner of Capco and Jeffery (1982).

The supramolecular associations of hnRNP in the nucleus, or of stored pre-mRNA in the cytoplasm of amphibian oocytes, are a manifestation of all the processes of modification and selection that are necessary to produce functional mRNA. Even the 40S cytoplasmic storage particle, with its restricted and well-defined components presents an interwoven relationship

between storage, nuclear/cytoplasmic transport and control of transcription that involves considerable problems of interpretation. The advent of techniques that allow the processing of hnRNP transcripts of known sequence to be followed through oogenesis will be helpful in furthering our knowledge of this field. However considerably more data on the structure and organization of hnRNP complexes and their association with supramolecular structures will be necessary before the role of RNP structure in gene expression is fully understood.

A C K N O W L E D G E M E N T S

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