A Simple Procedure for the Isolation and Purification of Protamine Messenger Ribonucleic Acid from Trout Testis

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Preparation of milligram quantities of purified poly(A)⁺ (polyadenylated) protamine mRNA from trout testis tissue was accomplished by a simple procedure using gentle conditions. This involves chromatography of the total nucleic acids isolated by dissociation of polyribosomes with 25 mM-EDTA to release messenger ribonucleoprotein particles and deproteinization of the total postmitochondrial supernatant with 0.5% sodium dodecyl sulphate in 0.25 M-NaCl by binding it to a DEAE-cellulose column. Total RNA was bound under these conditions, and low-molecular-weight RNA, lacking 18S and 28S RNA, could be eluted with 0.5 M-NaCl and chromatographed on oligo(dT)-cellulose columns to select for poly(A)⁺ RNA. Further purification of both the unbound poly(A)⁻ RNA and the bound poly(A)⁺ mRNA on sucrose density gradients showed that both 18S and 28S rRNA were absent, being removed during the DEAE-cellulose chromatography step. Poly(A)⁻ RNA sedimented in the 4S region whereas the bound poly(A)⁺ RNA fraction showed a main peak at 6S [poly(A)⁺ protamine mRNA] and a shoulder in the 3–4S region. Analysis of the main peak and the shoulder on a second gradient showed that most of the main peak sedimented at 6S, whereas the shoulder sedimented slower than 4S. The identity of the poly(A)⁺ protamine mRNA was established by the following criteria: (1) purified protamine mRNA migrated as a set of four bands on urea/polyacrylamide-gel electrophoresis; (2) analysis of the polypeptides synthesized in the wheat-germ extract by starch-gel electrophoresis showed a single band of radioactivity which co-migrated exactly with the carrier trout testis protamine standard; and (3) chromatography of the polypeptide products on CM-cellulose (CM-52) showed the presence of three or four radioactively labelled protamine components that were co-eluted with the unlabelled trout testis protamine components added as carrier. The availability of large quantities of purified protamine mRNA should now permit a more thorough analysis of its physical and chemical properties.

Several methods for preparing eukaryotic mRNA, each involving multistep procedures, have been described previously. In a step common to most procedures, the RNA from polyribosomes and/or the postmitochondrial supernatant fraction is extracted with phenol at various pH values, ionic strengths and temperatures in the presence of chloroform and 3-methylbutan-1-ol (Penman, 1966; Lee *et al.*, 1971; Brawerman *et al.*, 1972; Aviv & Leder, 1972; Perry *et al.*, 1972). Since the discovery of poly(A) sequences at the 3'-terminus of most eukaryotic mRNA species (Lee *et al.*, 1971; Darnell

et al., 1971; Edmonds *et al.*, 1971), the extraction procedures have been modified to minimize losses of poly(A)-containing mRNA (Brawerman *et al.*, 1972). Eukaryotic mRNA species were further purified by specific adsorption of the poly(A)-containing mRNA on oligo(dT) (Aviv & Leder, 1972) and poly(U) (Sheldon *et al.*, 1972; Adesnik *et al.*, 1972) covalently bound to cellulose or Sepharose. However, the mRNA fractions are usually still contaminated with rRNA even after rechromatography on these affinity columns (Gielen *et al.*, 1974; Bantle *et al.*, 1976).

In some cases (Lockard & Lingrel, 1969; Jacobs-Lorena & Baglioni, 1972; Schimke *et al.*, 1974) mRNA can be deproteinized without phenol extraction, by sucrose-gradient sedimentation of polyribosomes after dissociation with buffer containing SDS. Specific mRNA molecules have also been extracted from messenger ribonucleoprotein particles (Sampson *et al.*, 1972; Ernst & Arnstein, 1975;

Abbreviations used: SDS, sodium dodecyl sulphate; poly(A)⁻ RNA, RNA lacking poly(A) tracts; poly(A)⁺ RNA, polyadenylated RNA; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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Chen *et al.*, 1976; Gedamu *et al.*, 1977a) isolated on sucrose gradients from polyribosomes dissociated with EDTA. All of these methods are time-consuming and result in variable losses of mRNA.

Krystosek *et al.* (1975) have modified these methods and have isolated globin mRNA from polyribosome fractions deproteinized with 0.5% SDS in 0.5M-NaCl by using oligo(dT)-cellulose chromatography. This method, although rapid and gentle, has the disadvantage that the poly(A)-containing RNA is still contaminated with rRNA, perhaps owing to specific base-pairing between an mRNA sequence in the initiation region and a complementary sequence in rRNA (Shine & Dalgarno, 1974; Steitz & Jakes, 1975; Sanger *et al.*, 1977; Ravetch *et al.*, 1977).

Gedamu & Dixon (1976b) have shown that protamine mRNA can be purified by affinity chromatography of total cellular RNA on oligo(dT)-cellulose followed by sucrose-density-gradient centrifugation and more recently the method has been applied to messenger ribonucleoprotein particles (Gedamu *et al.*, 1977a). However, the procedure yielded only limited quantities of purified protamine mRNA with variable contamination with rRNA. Since milligram quantities of purified protamine mRNA are necessary for work involving extensive structural and functional studies, a faster and more efficient method for the isolation and purification of this mRNA was desirable. In the present paper a very rapid and gentle procedure is described, involving a one-step fractionation of the RNA, which yields large quantities of active protamine mRNA.

Materials and Methods

Cell fractionation

Rainbow-trout (*Salmo gairdnerii*) testes at the protamine stage were collected in September 1974 from Dantrout, Brande, Denmark, and stored at -70°C . The tissue was homogenized in 2 vol. of buffer containing 75 mM-NaCl, 5 mM-Tris/HCl, pH 7.6, and 25 mM-EDTA (disodium salt) in a Waring Blendor at full speed for 3 min at 4°C . The homogenate was centrifuged at 30000g to remove nuclei and mitochondria. The postmitochondrial supernatant after filtration through cheesecloth was used as a source for protamine mRNA preparation. However, if mRNA was to be extracted from either polyribosomal or postribosomal supernatant, the tissue was homogenized in buffer containing 50 mM-Tris/HCl, pH 7.6, 25 mM-KCl, 5 mM-magnesium acetate (Gedamu & Dixon, 1976b), and the 30000g postmitochondrial supernatant fractionated as described previously (Gedamu *et al.*, 1977a).

DEAE-cellulose chromatography

The procedure was performed at room temperature (20°C). The postmitochondrial supernatant

and/or the postribosomal supernatant was adjusted to 0.25M-NaCl/10 mM-Tris/HCl (pH 7.6)/0.5% SDS at room temperature and applied to a DEAE-cellulose column (3.5 cm $\times$ 25 cm). It is important that this column should be equilibrated in the application buffer without SDS. The column was then washed with 400–500 ml of application buffer to remove most of the cellular basic proteins. RNA and acidic proteins bound to the column were eluted with 0.5M-NaCl/10 mM-Tris/HCl (pH 7.6)/0.5% SDS as a single peak. It normally takes about 48 h to process material obtained from 1–3 kg of tissue (this corresponds to approx. 25000 A_{260} units of total RNA/kg of tissue) up to this stage.

Oligo(dT)-cellulose chromatography

The material eluted from the DEAE-cellulose column with the buffer containing 0.5M-NaCl was chromatographed on an oligo(dT)-cellulose column containing 10 g of oligo(dT) (type T3; Collaborative Research, Waltham, MA, U.S.A.) as described previously (Aviv & Leder, 1972; Gedamu & Dixon, 1976b). The poly(A)⁺ RNA which passed unretarded through the column in 0.5M-NaCl/10 mM-Tris/HCl (pH 7.6) buffer was recycled once or twice on oligo(dT)-cellulose. The poly(A)⁺ RNA was eluted with water. Each RNA fraction was made 0.24M in ammonium acetate (Osterburg *et al.*, 1975) precipitated with 2 vol. of ethanol, dried, dissolved in water and stored at -40°C .

Sucrose-density-gradient centrifugation

The RNA samples from the oligo(dT)-cellulose column were layered on a 12 ml 10–30% or 15–35% (w/v) linear sucrose density gradient made in 10 mM-Tris/HCl (pH 7.6)/30 mM-KCl/1 mM-EDTA (disodium salt) buffer for further purification and centrifuged in a SW 41 rotor as indicated in the legends to the Figures and Tables. The desired RNA fractions were pooled and concentrated by ethanol precipitation for further purification.

Polyacrylamide-gel electrophoresis of protamine mRNA

Electrophoresis of RNA samples was performed in 6% (w/v) polyacrylamide/8M-urea slab gels as described previously (Sanger & Coulson, 1975; Iatrou & Dixon, 1977; Gedamu *et al.*, 1977b). RNA zones were detected by staining the gel with Stains-All (Dahlberg *et al.*, 1969) overnight and destaining with water.

Assay in vitro for synthesis and product analysis

A cell-free protein-synthesizing system (S-30) was prepared from wheat germ (Canasoy, Vancouver,

B.C., Canada) by the procedure of Roberts & Paterson (1973). Protamine mRNA activity was assayed in a 50 μ l reaction mixture that contained 25 μ l of preincubated wheat-germ S-30 fraction, 20 mM-Hepes, pH 7.6, 75 mM-KCl, 2.5 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, creatine kinase (0.3 mg/ml; 44.6 units/mg; Worthington Biochemical Corp., Freehold, NJ, U.S.A.), 8 mM-phosphocreatine, 40 μ M (each) of 19 non-radioactive amino acids except arginine, 2 mM-dithioerythritol and [14 C]-arginine (specific radioactivity, 336 mCi/mmol) or [3 H]arginine (specific radioactivity 23 Ci/mmol). Radiochemicals were from New England Nuclear (Boston, MA, U.S.A.). Incubation was at 30°C for 45 min and mRNA was present as indicated. The reaction was terminated and hot-trichloroacetic acid/tungstate-precipitable material was measured as previously described (Gedamu & Dixon, 1976a).

Polypeptides synthesized *in vitro* were extracted and analysed by starch-gel electrophoresis and by CM-cellulose chromatography as previously described (Ling *et al.*, 1971; Gilmour & Dixon, 1972; Gedamu & Dixon, 1976a).

Results

Gedamu & Dixon (1976b) showed that poly(A)⁺ protamine mRNA can be isolated from both polyribosomal and postribosomal supernatant fractions of trout testis tissue by extraction of total cellular RNA with phenol/chloroform/3-methylbutan-1-ol and purified by chromatography on an oligo(dT)-cellulose column followed by sucrose-density-gradient centrifugation. There was some evidence, however, that this procedure often led to aggregation of rRNA with mRNA species.

We have used the method described by Krystosek *et al.* (1975), which involves deproteinization of total cellular RNA from polyribosomes by 0.5% SDS in the presence of 0.5 M-NaCl followed by chromatography on oligo(dT)-cellulose columns to prepare poly(A)⁺ protamine mRNA from both polyribosomal and postribosomal supernatant fractions of trout testis. Fig. 1(a) shows a sucrose-gradient analysis of the poly(A)⁺ protamine mRNA isolated from postribosomal supernatant, sedimenting, as expected, at approx. 6S with a shoulder in the 4S region in relation to the 4S and 18S trout testis RNA markers analysed in parallel gradients. The RNA fraction which did not bind to oligo(dT)-cellulose [poly(A)⁻] was mainly 4S RNA, with a small amount of 5S RNA (results not shown). When the poly(A)⁺ mRNA isolated from the polyribosomal fraction was analysed in a similar way, it was found to be contaminated with other cellular RNA species. This contaminated RNA was heated at 60°C for 5 min to release the poly(A)⁺ mRNA species from any complexes with contaminating RNA species (Bantle *et al.*, 1976) and

Fig. 1. Sucrose-gradient analysis of the poly(A)⁺ protamine mRNA isolated from (a) the postribosomal supernatant and (b) the polyribosomal fraction of trout testis

(a) The postribosomal supernatant from 150g of testis at a late stage of development was made in 0.5 M-NaCl/0.5% SDS/10 mM-Tris/HCl, pH 7.6, and applied to an oligo(dT)-cellulose column pre-equilibrated in the same buffer at room temperature. The RNA fraction eluted from the column with water [poly(A)⁺ mRNA] was precipitated with ethanol, pelleted (30000g for 20 min) and dissolved in 500 μ l of water. Then 250 μ l (9 A_{260} units) of the poly(A)⁺ mRNA was applied to 11.6 ml 10–30% linear sucrose gradients in buffer containing 10 mM-Tris/HCl, pH 7.6, 30 mM-KCl and 1 mM-EDTA. Gradients were centrifuged in a Beckman SW41 rotor at 39000 rev./min for 24 h at 4°C and A_{260} was then monitored. Trout testis RNA containing 4S, 18S and 28S RNA was also centrifuged in parallel gradients. (b) Polyribosomes from 150g of tissue were dissolved in buffer containing 10 mM-Tris/HCl (pH 7.6)/0.5% SDS; NaCl was added to a final concentration of 0.5 M and the sample was then applied to an oligo(dT)-cellulose column. The RNA eluted from the column with water was heated at 60°C for 5 min, cooled and was made 0.5 M with respect to NaCl. It was rechromatographed on oligo(dT)-cellulose column and the RNA fractions bound (—) and unbound (----) to the column in the 0.5 M-NaCl were analysed by sucrose-density-gradient centrifugation as described in Fig. 1(a) for 21 h.

Table 1. *Activities of RNA fractions in a wheat-germ cell-free system*

Poly(A)⁺ mRNA was isolated from the polyribosomal and postribosomal supernatants of trout testis by the method of Krystosek *et al.* (1975). RNA was determined (Gedamu & Dixon, 1976*b*) and fractions were assayed in the wheat-germ cell-free system at various stages during purification as described in the Materials and Methods section. [³H]Arginine incorporated into hot trichloroacetic acid/tungstate-precipitable polypeptides was determined in a 40 µl sample. Radioactivity incorporated into the endogenous proteins (8536 c.p.m.) was subtracted. The standard poly(A)⁺ protamine mRNA was purified, after phenol extraction of trout testis postribosomal supernatant, by the method of Gedamu & Dixon (1976*b*), and showed the typical set of four closely spaced sub-bands described by Gedamu *et al.* (1977*b*) as in Fig. 6, slot 4.

Sample	Amount of RNA added (µg)	[³ H]Arginine incorporated (c.p.m./40 µl)	Specific radioactivity (c.p.m./µg)
First oligo(dT)-cellulose-bound RNA fraction	2.5	22 500	9000
Second oligo(dT)-cellulose-bound RNA fraction	1.0	33 400	33 400
Unbound RNA after second oligo(dT)-cellulose column	10.0	6800	680
Poly(A) ⁺ protamine mRNA (main peak)	0.5	25 500	51 000
Poly(A) ⁺ protamine mRNA (shoulder)	0.5	7500	15 000
Poly(A) ⁺ RNA sedimenting faster than protamine mRNA	2.0	14 500	7250
Poly(A) ⁺ protamine mRNA from postribosomal supernatant	0.8	42 800	53 000
Poly(A) ⁺ protamine mRNA (standard)	0.4	24 900	62 250

Scheme 1. *Scheme for the isolation of protamine mRNA from trout testis*

cooled quickly in ice. The salt concentration was adjusted to 0.5M-NaCl/10mM-Tris/HCl, pH 7.6, and the RNA rechromatographed on oligo(dT)-cellulose. Fig. 1(b) shows a sedimentation analysis on a sucrose gradient of the RNA fractions bound and unbound in high salt after the rechromatographic step. After heat dissociation, the poly(A)⁺ mRNA species isolated from both the polyribosomal and post-ribosomal poly(A)⁺ protamine mRNA fractions now show similar profiles (compare Figs. 1a and 1b).

The incorporation of labelled arginine into hot-trichloroacetic acid/tungstate-precipitable material was used as a measure of protamine mRNA (translational) activity in the wheat-germ system of Roberts & Paterson (1973). As shown in Table 1, the specific activity of the protamine mRNA isolated from the polyribosomal fraction increased at each stage of purification, from 18% in the first oligo(dT)-cellulose step to about 80–90% in the sucrose-density-gradient step (Fig. 1b), compared with a standard of highly purified poly(A)⁺ protamine mRNA prepared by the phenol method described by Gedamu & Dixon (1976b). The poly(A)[−] RNA fraction removed by the second oligo(dT)-cellulose step was relatively inactive (Table 1). The protamine mRNA isolated from the postribosomal supernatant after one oligo(dT)-cellulose step followed by one sucrose-gradient centrifugation was, however, 85% as active as the standard protamine mRNA. These experiments indicate that this method can be used directly to isolate protamine mRNA from the post-ribosomal supernatant. However, additional steps are required to remove contaminating RNA if the source of the mRNA is the polyribosomes or the total postmitochondrial supernatant.

A simple procedure for the isolation of poly(A)⁺ protamine mRNA from the cytoplasm of trout testis, which is well-adapted to large scale preparations, is outlined in Scheme 1. The cytoplasmic fraction was adjusted to 0.25M in NaCl, 0.5% in SDS and applied to a DEAE-cellulose column. It is very important to equilibrate the DEAE-cellulose column with 0.25M-NaCl/10mM-Tris/HCl, pH 7.6, without any SDS. Under these conditions basic proteins pass through the column, but nucleic acids and acidic proteins bind to the DEAE-cellulose. When the salt concentration was increased to 0.5M, low-molecular-weight RNA species and acidic proteins were eluted from the DEAE-cellulose column. A typical absorption profile of this material eluted from the column is shown in Fig. 2. High-molecular-weight rRNA and other RNA species (Gedamu, 1974), which cause problems by binding unspecifically to oligo(dT)-cellulose or forming aggregates with poly(A)⁺ mRNA when applied in large amounts, remained bound to the column and were thus removed from the low-molecular-weight RNA in one step. The eluate was then applied directly to an

Fig. 2. *Chromatography of trout testis postnuclear supernatant on a DEAE-cellulose column*

Trout testis postnuclear supernatant in 0.25M-NaCl/0.5% SDS/10mM-Tris/HCl (pH 7.6) was applied to a DEAE-cellulose column (3.5cm × 25cm) equilibrated in 0.25M-NaCl/10mM-Tris/HCl, pH 7.6. After washing out the basic protein in the application buffer, the RNA and acidic proteins were eluted with 0.5M-NaCl/0.5% SDS/10mM-Tris/HCl, pH 7.6. Fractions (about 10ml) were collected and their A_{260} was measured. About 3500–4000 A_{260} units of low-molecular-weight RNA/kg of tissue was obtained.

oligo(dT)-cellulose column in tandem with the DEAE-cellulose column, and the poly(A)⁺ RNA species were bound to it, whereas both RNA species lacking poly(A) tracts and any acidic proteins were eluted from the column by washing with 0.5M-NaCl/10mM-Tris/HCl, pH 7.6, buffer. The bound RNA was finally eluted with water after the oligo(dT)-cellulose had been thoroughly washed with 0.1M-NaCl/10mM-Tris/HCl, pH 7.6 (Fig. 3). Only one peak of material absorbing at 260nm was eluted with water, but no material absorbing at 260nm was eluted when the column was washed with 0.1M-NaCl (Fig. 3). This is in marked contrast with the results obtained when total RNA was extracted from trout testis with phenol/chloroform/3-methylbutan-1-ol (Gedamu & Dixon, 1976b). When this RNA was applied to an oligo(dT)-cellulose column, the 0.1M-NaCl wash contained substantial amounts of species absorbing at 260nm, which were shown to be mainly 18S and 28S rRNA. This difference illustrates the major improvement in the present method. The material eluted with 0.5M-NaCl (not shown in Fig. 2) was mainly low-molecular-weight RNA (see Fig. 5a).

Fig. 3. Chromatography of low-molecular-weight RNA species from DEAE-cellulose on oligo(dT)-cellulose column

The low-molecular-weight RNA fraction eluted from the DEAE-cellulose with 0.5M-NaCl/0.5% SDS/10mm-Tris/HCl, pH7.6, (Fig. 2) was applied directly to an oligo(dT)-cellulose column at room temperature. The column was washed with 300–400 ml of the above buffer lacking SDS at 4°C, to remove SDS, followed by 0.1M-NaCl/10mm-Tris/HCl, pH7.6. The poly(A)-containing RNA was finally eluted with water.

Fig. 4. Spectra of poly(A)-containing RNA (—) and low-molecular-weight RNA (----)

The spectra were determined in the ACTA MVI Beckman spectrophotometer.

The poly(A)⁺ RNA bound to the column was not contaminated with protein, as evidenced by its u.v.-absorption spectra. As shown in Fig. 4, the spectrum of the poly(A)⁺ RNA fraction (continuous line) differs markedly from that of the low-molecular-weight RNA, applied to the oligo(dT)-cellulose column (broken line). In the latter, proteins absorb relatively strongly at 230–240nm and at 280nm. The poly(A)⁺ RNA spectrum is identical with that of purified RNA prepared by phenol extraction ($A_{260}/A_{280} = 2.10$).

Fig. 5. Sucrose-gradient analysis of RNA fractions after oligo(dT)-cellulose chromatography of low-molecular-weight RNA from trout testis postmitochondrial supernatant

(a) The poly(A)⁺ and poly(A)⁻ RNA fractions obtained from oligo(dT)-cellulose chromatography were concentrated by precipitating with ethanol as described in the Materials and Methods section. About 15 A_{260} units of the poly(A)⁺ RNA (—) and 12 A_{260} units of poly(A)⁻ RNA (----) were applied to 15–35% linear sucrose density gradients and centrifuged for 24h as in Fig. 1(a). The poly(A)⁺ protamine mRNA shoulder and the main peak are represented by A and B respectively. Positions of the RNA markers are indicated by 4S and 18S. (b) The poly(A)⁺ protamine mRNA main peak (B) and shoulder (A) from the previous gradient were pooled, concentrated and applied on a second sucrose density gradient. Analysis was performed under the same conditions as described in Fig. 5(a). The broken and continuous lines show the profiles of the poly(A)⁺ protamine mRNA shoulder and main peak respectively.

Both the RNA fraction bound to the oligo(dT)-cellulose column and that passing unretarded through the column in 0.5M-NaCl/10mm-Tris/HCl, pH7.6,

Table 2. Activities of poly(A)⁺ protamine mRNA fraction isolated from trout testis homogenate in wheat-germ cell-free system

Poly(A)⁺ protamine mRNA was isolated from trout testis postmitochondrial supernatant by the DEAE-cellulose oligo(dT)-cellulose method outlined in Scheme 1. The activities of the RNA fractions in supporting [³H]arginine incorporation into hot trichloroacetic acid/tungstate-precipitable material was determined as in Table 1.

Sample	Amount of RNA added (μg)	[³ H]Arginine incorporated (c.p.m./40 μl)	Specific radioactivity (c.p.m./μg)
First oligo(dT)-cellulose bound RNA fraction	0.6	19539	32565
Poly(A) ⁺ protamine mRNA (main 6S peak)	0.4	24929	62323
Poly(A) ⁺ protamine mRNA (3-4S shoulder)	0.5	8943	17886
Poly(A) ⁺ protamine mRNA (standard)	0.35	22006	62874

were further characterized by sucrose-density centrifugation (Fig. 5a). The bound fraction (continuous line) sedimented with the main peak at 6S and a shoulder at 3-4S, a behaviour seen in previous preparations of poly(A)⁺ protamine mRNA (Gedamu & Dixon, 1976b). The unbound fraction, however, sedimented at 4S (broken line). Both fractions appear to be free of rRNA, suggesting that the removal of rRNA from the low-molecular-weight RNA at the DEAE-cellulose step is complete.

The RNA sedimenting as the main peak (region B, Fig. 5a) and the shoulder (region A, Fig. 5a) was pooled from six 12ml gradients and further purified on sucrose gradients (Fig. 5b). The RNA in the main peak sedimented at 6S, with some evidence of contamination from the lower-molecular-weight RNA shoulder seen in the first gradient (Fig. 5a). The RNA originally sedimenting as the low-molecular-weight shoulder of the main peak, however, sedimented in the second gradient slower than the 4S trout testis RNA marker and showed a small shoulder on the heavy side co-sedimenting with the 6S RNA (Fig. 5b, broken line).

The protamine mRNA activities of the RNA fractions from both the main peak (Fig. 5b, region B) and the shoulder (Fig. 5b, region A) were assayed, and Table 2 shows that the RNA in the main peak (6S) is as active as poly(A)⁺ protamine mRNA purified by the phenol/chloroform/3-methylbutanol method. The RNA sedimenting at less than 4S was considerably less active, and the activity in the preparation is likely to be due only to contamination by 6S RNA (Fig. 5b). This suggests that the entire protamine mRNA activity sediments in the 6S region. Protamine mRNA activity was not found in the rRNA fraction bound to the DEAE-cellulose column.

Fig. 6 shows analysis of RNA fractions on a polyacrylamide slab gel under denaturing conditions. The unbound RNA fraction from the oligo(dT)-cellulose column is shown in slot 1. It consists of pre-

Fig. 6. Analysis of RNA fractions on a 6% polyacrylamide/8M-urea gel

The following RNA samples were analysed as described previously (Iatrou & Dixon, 1977; Gedamu *et al.*, 1977b). Slot 1: 90 μg of poly(A)⁻ RNA after oligo(dT)-cellulose chromatography of low-molecular-weight RNA (see profile in Fig. 5a). Slot 2: 15 μg of poly(A)⁺ protamine mRNA shoulder (Fig. 5b). Slot 3: 15 μg of poly(A)⁺ protamine mRNA main peak (Fig. 5b, B). Slot 4: 9 μg of poly(A)⁺ protamine mRNA standard. Positions of 4S and 5S RNA and of poly(A)⁺ protamine mRNA are indicated by arrows.

dominantly of 4S tRNA, with some 5S rRNA and other poly(A)⁻ RNA species with molecular weights greater than 5S rRNA. Some of these bands have been identified as protamine mRNA molecules lacking poly(A) tracts (Gedamu *et al.*, 1977b). The RNA fraction that was bound to oligo(dT)-cellulose in high salt and sedimented as the low-molecular-weight shoulder of the 6S-RNA peak during the first sucrose-gradient step is represented by slot 2. This material shows a diffuse distribution from less than 4S RNA to greater than 5S RNA. However, some very faint bands could be seen in the protamine mRNA region. Slot 3 shows poly(A)⁺ protamine mRNA prepared by the DEAE-cellulose/oligo(dT)-cellulose method after fractionation on two successive sucrose gradients. It shows four closely spaced bands, as reported previously (Iatrou & Dixon, 1977), and other faint bands in the low-molecular-weight region which could represent contamination with the low-molecular-weight RNA species from the shoulder. This is to be compared with a sample of poly(A)⁺ protamine mRNA (slot 4) prepared by the phenol/chloroform/3-methylbutan-1-ol method described previously (Gedamu & Dixon, 1976b) and which shows the same four closely spaced sub-bands (Iatrou & Dixon, 1977).

The polypeptide products synthesized in the wheat-germ translational system in response to the poly(A)⁺ protamine mRNA were analysed by starch-gel electrophoresis. In the presence of poly(A)⁺ protamine mRNA a major peak of radioactivity which co-migrated with carrier protamine shown in the stained gel (Fig. 7a) was observed, whereas in the control only the free [¹⁴C]arginine peak could be seen (Fig. 7b).

The individual protamine components synthesized in the wheat-germ cell-free system in response to this poly(A)⁺ protamine mRNA have been fractionated by CM-cellulose chromatography by using a LiCl gradient (Ling *et al.*, 1971; Gedamu & Dixon, 1976a,b). The conditions of chromatography were such that the three previously described protamine components from trout testis, C_I, C_{II} and C_{III}, could be separated as individual peaks. Fig. 8 shows that the product synthesized in the cell-free system in the presence of the poly(A)⁺ protamine mRNA contained label incorporated into all three protamine components, C_I, C_{II} and C_{III}. We have also observed the synthesis of an additional polypeptide which is eluted earlier than the three major components and which may be a fourth protamine component. These labelled protamine components, however, do not co-chromatograph precisely with unlabelled trout testis protamine components added as carrier during the extraction procedure, being eluted slightly earlier than their respective carrier protamine components. This behaviour may be due to the post-translational modification of the protamine com-

Fig. 7. Analysis by starch-gel electrophoresis of polypeptides synthesized in the wheat-germ cell-free system in the presence of protamine mRNA

Reaction mixtures (100 μ l) containing 50 μ l of wheat-germ extract were incubated with poly(A)⁺ protamine mRNA (Fig. 5b, B) under the conditions described in the Materials and Methods section. The reaction mixture was diluted to 500 μ l with water and 20 μ l samples were taken for measurement of trichloroacetic acid / tungstate-precipitable radioactivity. Polypeptides soluble in 0.2M-H₂SO₄ were extracted from the remainder (Gedamu & Dixon, 1976a). Dried samples were resuspended in 100 μ l of 0.1M-HCl and 50 μ l was applied to starch gel. Analysis was performed as described previously (Gedamu & Dixon, 1976a). Protamine mRNA was present in the reaction mixture at a final concentration of 15 μ g/ml. —, Product due to endogenous mRNA; ----, product obtained in the presence of protamine mRNA.

Fig. 8. Characterization of protamine components synthesized in the wheat-germ cell-free system by chromatography on CM-cellulose column

A reaction mixture (500 μ l) containing 250 μ l of wheat-germ extract and 9.0 μ g of poly(A)⁺ protamine mRNA (Fig. 5b, B) was incubated in the complete assay system as described in the Materials and Methods section. The [³H]arginine-labelled acid-soluble polypeptides (Gedamu & Dixon, 1976a) synthesized in the presence of the protamine mRNA were applied to a CM-cellulose CM-52 column (0.8 cm $\times$ 60 cm) with 12 mg of a mixture of unlabelled carrier protamine and histones. The column was eluted with a 300 ml linear gradient of 0.66–1.10 M-LiCl/lithium acetate buffer, pH 5.1, (Ling *et al.*, 1971; Gedamu & Dixon, 1976a). Fractions (1.5 ml) were collected and radioactivity ($\circ$) was measured in 1.0 ml fractions. The protamine components C_I, C_{II} and C_{III} are assigned on the basis of the A₂₃₀ ($\bullet$).

ponents synthesized *in vitro* by phosphorylation of serine residues, present in the protamine sequence. The introduction of negatively charged phosphoryl groups into each of the labelled components has been shown previously (Marushige *et al.*, 1969) to cause them to be eluted earlier than the carrier components. Protein kinases, the enzymes responsible for basic protein phosphorylation, are widely distributed and are likely to exist in the wheat-germ system, and in the presence of the plentiful supply of ATP in the protein-synthetic system could readily phosphorylate the newly synthesized protamine polypeptides.

Discussion

Several methods have been described for the isolation of eukaryotic mRNA [for reviews see Mathews (1973) and Brawerman (1974)]. The most widely used procedure is that described by Aviv & Leder (1972), in which phenol-extracted RNA is adsorbed on oligo(dT)-cellulose columns to select for poly(A)-containing RNA. Phenol extraction is a complex,

poorly understood, procedure which can cause aggregation of RNA (Sedat & Sinsheimer, 1970; Schechter, 1973), removal of poly(A) regions from mRNA (Perry *et al.*, 1972) and selective loss of mRNA (Lee *et al.*, 1971; Brawerman *et al.*, 1972). Although these problems can be minimized by careful control of pH, ionic strength, temperature and use of a proportion of chloroform with the phenol, from our experience this type of mRNA extraction is time-consuming and awkward for large quantities of material in that large volumes of phenol must be shaken.

Krystosek *et al.* (1975) proposed an alternative method for the isolation of mRNA. In this procedure, polyribosomes were treated with SDS and applied directly to an oligo(dT)-cellulose column without deproteinization by phenol extraction. Application of this procedure to trout testis tissue revealed that the poly(A)-containing protamine mRNA bound to the oligo(dT) in high salt was contaminated with RNA species lacking poly(A) and sedimenting between 4S and 18S RNA (Fig. 1b, broken line). The specific activity of protamine mRNA at this step of purifi-

cation in the translational system *in vitro* was about 18% of that of pure standard. Further purification of this fraction led to mRNA of 80–90% purity. It is not possible to determine at the moment whether these contaminating RNA species are complexed with the poly(A)-containing mRNA species or bound non-specifically to oligo(dT)-cellulose. Apparently 18S rRNA can bind non-specifically to an oligo(dT)-cellulose column (Gielen *et al.*, 1974; Bantle *et al.*, 1976), and it has also been suggested that 18S rRNA may bind specifically to a region of the mRNA other than the poly(A) tail. The formation of such a complex has been proposed by Kabat (1975) for globin mRNA. Evidence that strong interaction might occur between rRNA and mRNA has also come from studies of mRNA primary structure. For example, in rabbit globin mRNA the sequence A-A-U-G-G-U around the initiation codon (Legon, 1976) is complementary to the sequence A-U-C-A-U-U at the 3'-terminus of rabbit reticulocyte 18S rRNA (Hunt, 1970).

The problem of complex-formation between mRNA and rRNA led us to develop an improved method for purification of poly(A)⁺ protamine mRNA from trout testis postmitochondrial supernatant. The method described here, which combines and modifies existing procedures, is very simple and rapid, and is easily carried out on a large scale. The use of 25mM-EDTA (disodium salt) in the homogenization step dissociates polyribosomes into ribosomal subunits and allows the release of messenger ribonucleoprotein particles, predominantly those for protamine, and a 7S complex consisting of 5S rRNA associated with proteins (Gedamu *et al.*, 1977a). The presence of SDS (0.5%) in the purification process causes the deproteinization of both the messenger ribonucleoprotein particles released from the polyribosomes and those free in the postribosomal supernatant, the 7S complex and the ribosomal subunits, and yields a mixture of free total cellular RNA and both basic and acidic proteins complexed with SDS. The application of this mixture to a DEAE-cellulose column in 0.25M-NaCl/0.5% SDS is important and critical. Under these conditions, basic proteins pass through the column and both total RNA and some acidic-protein-SDS complexes are bound to the column. By raising the salt concentration to 0.5M, low-molecular-weight RNA species and some acidic-protein-SDS complexes are eluted from the column. We have chosen 0.5M-NaCl as the most suitable eluent because previous experiments revealed that low-molecular-weight RNA species, including tRNA, 5S rRNA and protamine mRNA, were eluted from a DEAE-cellulose column with a linear salt gradient ranging from 0.25 to 0.50M (Gedamu, 1974). This has the advantage that the rRNA species, which are the major contaminants of poly(A)-containing RNA

and which cause problems during any mRNA purification, are left bound to the column (Fig. 5a). Moreover, the fraction eluted from the DEAE-cellulose column in 0.5M-NaCl can be applied directly to an oligo(dT)-cellulose column, allowing the binding of poly(A)-containing RNA species (Aviv & Leder, 1972). The procedure up to the sucrose-density-gradient centrifugation can be performed in one step, and large quantities of tissue (3–4kg) can be processed by this method. The yield of highly purified protamine mRNA from 1kg of tissue was approx. 1–1.5mg.

Protamine mRNA prepared in this way is as pure as that prepared either after deproteinization of polyribosomes by phenol extraction (Gedamu & Dixon, 1976b; and Fig. 6, slot 3) or from the protamine messenger ribonucleoprotein particles (Gedamu *et al.*, 1977a). The products synthesized in the wheat-germ translational system in the presence of protamine mRNA prepared by this method co-electrophorese with authentic protamine on starch gels (Fig. 7) and showed three or four protamine polypeptide components when analysed on a CM-cellulose column (Fig. 8).

The presence of RNA sedimenting as a shoulder of the main protamine mRNA peak (6S), with a peak at approx. 3–4S (Fig. 5b), has been also observed previously when protamine mRNA was isolated by the phenol extraction method (Gedamu & Dixon, 1976b). However, no detailed studies of the nature of this RNA were made at that time. In the present studies we found that this RNA migrates with a heterogeneous distribution as indicated in Fig. 6 (slot 2) and, after two successive sucrose-density-gradient centrifugations, application to an oligo(dT)-cellulose column showed that 67–70% of the RNA sedimenting as a shoulder could still be bound in high salt and eluted in water from the column (unpublished work). This suggests the presence of poly(A) sequences in the molecules, and is further supported by an analysis of its base composition, which showed over 90% A residues (results not shown). Moreover, determination of the poly(A) length revealed an average length of 60 residues, which is somewhat smaller than that found for the poly(A) length of the poly(A)⁺ protamine mRNA (K. Iatrou, L. Gedamu & G. H. Dixon, unpublished work). When assayed in the wheat-germ cell-free system, this poly(A)-rich shoulder RNA did not support [¹⁴C]-arginine incorporation into hot trichloroacetic acid/tungstate-precipitable polypeptides. We have observed also, when using either the phenol or the present method, that this material is found predominantly when mRNA is prepared from testis at an early stage of differentiation, where the amount of total cellular RNA is appreciably higher (Louie & Dixon, 1972) and decreased in quantity if the source of tissue is from the late stage (L. Gedamu, unpublished work). We

conclude that this material is probably a degradation product of the total cellular poly(A)⁺ mRNA in the trout testis. Since all poly(A)-containing RNA species have their poly(A) tail at the 3'-end of the molecule, this material might represent a collection of the 3'-ends of the poly(A)-containing mRNA molecules, and most probably a large proportion is derived from the poly(A)⁺ protamine mRNA, a major mRNA component. The method described here for the isolation of poly(A)⁺ protamine mRNA has some disadvantages as a general method for preparation of mRNA species in that it selects only low-molecular-weight size classes of mRNA under the conditions described. We have not tested its applicability, for example, in preparing specific messages such as globin and histone mRNA, but we think that it might be applicable to the isolation of other cellular mRNA species, of average and high molecular weight, by certain modifications such as increasing the salt concentration and also by adding 5–10% ethanol to facilitate the elution of these larger mRNA molecules from the DEAE-cellulose columns, although this would introduce the problem of possible contamination by 18S and 28S rRNA, which will probably be eluted under these higher-salt conditions.

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