Assay of Protamine Messenger RNA from Rainbow Trout Testis*

LASHIT EW GEDAMU$ AND GORDON H. DIXON

From the Biochemistry Group, University of Sussex, Brighton, England, and Division of Medical Biochemistry,§ Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 1N4, Canada

A low molecular weight RNA fraction possessing protamine mRNA activity was prepared from rainbow trout testis polysomes. Addition of low molecular weight RNA to a Krebs II ascites S-30 cell-free protein synthesis system strongly stimulated [14C]arginine incorporation into acid-insoluble material. This stimulation was completely abolished by 10−4 M aurintricarboxylic acid, an inhibitor of eukaryotic protein synthesis at the level of initiation. Starch gel electrophoresis showed that labeled arginine was incorporated in vitro into products identical with both authentic protamine and histones as found previously (Gilmour, R. S., and Dixon, G. H. (1972) J. Biol. Chem. 247, 4621-4627).

The 4 to 6 S RNA fraction, isolated from the polysomal low molecular weight RNA by sucrose gradient fractionation, enhanced the incorporation of [14C]arginine into acid-insoluble material and when this product was examined by starch gel electrophoresis, it co-migrated with authentic rainbow trout protamine.

A 4 to 6 S RNA fraction was also prepared from the postribosomal supernatant of trout testis cells which, when assayed in the ascites cell-free system, stimulated [14C]arginine incorporation into acid-insoluble material. The polypeptide product synthesized in response to this RNA fraction was analyzed by starch gel electrophoresis and carboxymethylcellulose (CM52) chromatography. Chromatography on CM52, which allows the separation of the three components of trout testis protamine, C₁, C₂, and C₃ (Ling, V., Jergil, B., and Dixon, G. H. (1971) J. Biol. Chem. 246, 1168-1176) as individual peaks, showed that [14C]arginine was incorporated into all three components. A comparison of the incorporation of [14C]arginine and [14C]leucine in the presence of either trout testis sRNA or rabbit globin mRNA showed that while the incorporation of both labeled amino acids was stimulated by rabbit globin mRNA, there was no [14C]leucine incorporation in the presence of trout testis sRNA, although [14C]arginine was extensively incorporated. Since leucine is absent from trout testis protamine (Ando, T., and Watanabe, S. (1969) Int. J. Protein Res. 1, 221-224), this finding suggests that the major mRNA activity in trout testis sRNA is that for protamine. The involvement of methionyl-tRNA in the initiation of protamine synthesis (Wigle, D. T., and Dixon, G. H. (1970) Nature 227, 676-680) was confirmed by the observation that the sRNA fraction containing protamine mRNA was able to form an 80 S initiation complex in a rabbit reticulocyte lysate system (Darnbrough, C., Legon, S., Hunt, T., and Jackson, R. J. (1973) J. Mol. Biol. 76, 379-403). In addition, polypeptide profiles of the fractionated Krebs II ascites S-30 in the presence of sRNA showed that 70% of the nascent protamine was associated with the monoribosome peak and 30% with the disome peak.

Protamines are a group of highly basic nuclear proteins bound to the DNA of sperm cells of most higher organisms. Protamine from rainbow trout (Salmo gairdnerii) has a molecular weight of close to 5000 and it has been shown that total protamine can be resolved by chromatography on CM-cellulose columns (1) into three components with different amino acid compositions. From the amino acid sequences of these components (2) it was evident that of the total of 31 to 33 amino acids, 21 to 23 are arginine together with a limited range of neutral amino acids; proline, serine, valine, alanine, and isoleucine but no leucine. There is strong evidence that this unique sperm-specific nuclear protein is synthesized by the conventional mechanisms of protein synthesis in the cytoplasm (3) of early and middle spermatids (4). The synthesis seems to occur mainly on the disomes (1) and is inhibited (5) by puromycin and cycloheximide, but not by actinomycin D, over 6 to 10 hours suggesting its synthesis is in response to a stable mRNA. The involvement of methionine as the initiating amino acid both in vivo (6) and in vitro (7) also strongly supports a

*This work was generously supported by the Medical Research Council of the United Kingdom.
† British Council Predoctoral Fellow.
§ Address to which correspondence concerning this paper and requests for reprints should be sent.

1446
classical route of synthesis involving a messenger RNA for protamine.

In the present paper we report the existence of protamine mRNA activity in two distinct cell compartments, namely, bound to polyribosomes and free in the cell sap. We have used a cell-free system derived from Krebs II ascites cells to assay the ability of this mRNA to direct the synthesis of protamine.

The advantage of this system is that it possesses a low level of endogenous messenger activity and using it a number of eukaryotic mRNAs have been shown to be translated faithfully and efficiently (8 13).

**MATERIALS AND METHODS**

**Preparation of Low Molecular Weight RNA from Trout Testis Polysomes**—Low molecular weight RNA was prepared from frozen rainbow trout testis at the protamine stage, as described by Gilmour and Dixon (7) with the following modifications. The postmitochondrial supernatant was treated with 1% Nonidet P-40 so that polysomes rather than microsomes were sedimented by centrifugation using a 50 Ti rotor for 3 hours at 140,000 g in a Beckman L-2-65B centrifuge at 4°. As an additional deproteinizing step the low molecular weight RNA fraction was made 1% in sodium dodecyl sulfate, heated at 37° for 3 min, and was further purified on a 15 to 30% sucrose gradient prepared in 10 mm Tris-HCl (pH 7.6)/50 mm NaCl and centrifuged at 38,000 rpm for 24 hours in an SW 40 rotor. The region of the gradient containing RNA sedimenting at greater than 18 S was discarded and the remainder was divided into two fractions 6 to 18 S and 4 to 6 S, made 0.2 M in NaCl and precipitated overnight with 3 volumes of ethanol. The precipitates were collected, washed once more with 0.2 M NaCl/ethanol (1/2), and dried in vacuo. Each fraction of RNA was resuspended in the minimum amount of water and stored frozen in small aliquots at -40°.

**Preparation of Trout Testis Supernatant RNA (sRNA)**—Rainbow trout testis at the protamine stage was collected in October 1972 at the Sun Valley Trout Farm, Mission, British Columbia, and stored at -40°. The tissue was homogenized in cold Buffer A containing 50 mm Tris-HCl (pH 7.6)/25 mm KCl/5 mm magnesium acetate in a Waring Blendor at full speed for 3 min at 4°. The homogenate was centrifuged at 13,000 rpm in the MSE 50 superspeed 8 x 50 ml angle rotor for 30 min to remove nuclei and mitochondria. The postmitochondrial supernatant after filtration through cheesecloth was centrifuged further for 4 to 4.5 hours at 27,000 rpm. The postribosomal supernatant was applied to a DIFACE-cellulose column (2.5 x 30 cm) which had been equilibrated with 50 mm Tris-HCl (pH 7.6) at 4° and the column was then washed with 200 ml of 50 mm Tris-HCl (pH 7.6). Most proteins were removed by washing the column with 0.35 to 0.9 M NaCl in 50 mm Tris-HCl (pH 7.6). sRNA and other acidic material bound to the column was eluted with 0.75 M NaCl/50 mm Tris-HCl (pH 7.6) as a single peak with A260/A230 ratio of 1.7 to 1.8. The fractions comprising this peak were pooled and were either precipitated directly from 3 volumes of ethanol or first extracted with phenol (14) and the RNA in the aqueous phase precipitated with 5 volumes of ethanol. It was found that RNA could be satisfactorily extracted from the material precipitated by ethanol directly from the column fractions. The resulting RNA was either lyophilized or dried in vacuo, resuspended in water, and stored frozen in aliquots at -40°.

**Protein Synthesis in Krebs II Ascites Cell-free System**—The standard assay conditions consisted of a volume of 30 ml: 15 ml of preincubated ascites S-30 (9); 25 mm Tris-HCl (pH 7.6), 75 mm KC1, 2.25 mm magnesium acetate, 1 mm ATP, 0.1 mm GTP, 0.3 mg/ml of creatine kinase (EC 2.7.3.2), 2.4 mg/ml of creatine phosphate, a mixture of 19 amino acids (40 pm each) excluding unlabeled arginine but to which 0.05 pCi [14C]arginine (336 mCi/mmol) was added. The components required to produce these standard total concentrations were stored separately in concentrated solutions and appropriate dilutions were made. This complete mixture will be referred to below as the protein synthesis "master mix." Bound conditions were used for the ascites S-30 that had been separated into ribosomes and cell sap by a further centrifugation except that under these conditions the ribosomes were preincubated with cell sap and master mix without label at 37° in a final volume of 50 ml containing 25 ml of cell sap and 5 ml of ribosomes, to reduce endogenous incorporation. The reactions were terminated by spotting aliquots on to filter paper discs (2.4 Whatman No. 3MM) which were immediately immersed in 5% trichloroacetic acid/0.25% sodium tungstate, pH 2, at room temperature. The trichloroacetic acid-tungstate was then heated to boiling for 10 min, the filter papers washed in trichloroacetic acid-tungstate, ethanol, and ether, and then dried. The precipitates were immersed in 4 ml of toluene scintillation fluid and counted.

**Preparation of Krebs II Ascites Cell-free System (Ascites S-30)**—Krebs II ascites S-30 was prepared as described by Mathews and Korner (15) with slight modifications. Washed cells were homogenized in 1 volume of buffer containing 1.5 mm magnesium acetate, 10 mm Tris-HCl pH 7.6, and 6 mm 2-mercaptoethanol. After homogenization, tonicity was restored by the addition of one-third of the original cell volume of 10-fold concentrated buffer Medium K, containing 2.5 mm MgCl2, 75 mm KC1, and 25 mm Tris-HCI pH 7.6. The homogenate was centrifuged at 30,000 x g for 10 min and preincubated for 30 to 35 min as described by Mathews and Korner (15) and passed through a Sephadex G-25 column equilibrated with Medium K. The fractions comprising the breakthrough peak were pooled, divided into small aliquots, and frozen in liquid nitrogen. This cell-free extract remained active for several months.

**Ribosomes from Cell Sap**—Ribosomes and cell sap were prepared from a nonpreincubated S-30. For ribosome preparation, the S-30 was treated with 1% Nonidet P-40 and layered over 2 ml of 1 M sucrose in Medium K which was itself layered over 5 ml of 2 M sucrose in Medium K. Cell sap was prepared from untreated S-30. Centrifugation was carried out in the 50 Ti rotor for 3 hours at 125,000 rpm in either the Beckman L-2-65 or L-2-65B. The ribosome pellet was resuspended in Medium K and aggregates removed by low speed centrifugation. Cell sap was passed through a Sephadex G-25 column equilibrated with Medium K. Both cell sap and resuspended ribosomes were stored frozen in small aliquots in liquid nitrogen and retained activity for a minimum of 3 to 4 months.

**Rabbit Reticulocyte Lysates**—Anemia was induced in rabbits weighing 2.5 to 3 kg by four daily injections of 0.7 to 1.0 ml of 2.5% phenylhydrazine solution. No injection was given on the 5th day. Blood was collected on the 6th day by heart puncture. The cells were washed three times with reticulocyte saline (0.13 m NaCl/5 mm KCI/7.5 mm MgCl2) by centrifuging for 5 min at 2,000 x g and resuspending the pellet. A concentrated lysate (1:1) for use in incorporation studies was prepared (16) by lysing the cells with addition of an equal volume of distilled water, and the lysate was freed of debris by centrifugation at 15,000 x g for 10 min and the supernatant rapidly frozen in small portions and stored in liquid nitrogen.

**Cell-free Synthesis in Reticulocyte Lysates**—The protein-synthesizing system contained 2 volumes of 1:1 lysate, 1 volume of master mix and 2 volumes of water or other additions. The master mix solution contained 1 mm ATP, 0.2 mm GTP, 15 mm creatine phosphate, 0.3 mg/ml of creatine kinase (EC 2.7.3.2), 2 mm magnesium acetate, 20 mm Tris-HCl, pH 7.6, an amino acid mixture containing 18 unlabeled amino acids minus arginine, and [14C]arginine. Hemin was added to 25 to 30 μM, a level found to be optimal for the protein-synthesizing system.

**Preparation of Rabbit Globin mRNA**—Rabbit globin mRNA was prepared by the procedure of Evans and Lingrel (17) with the following modifications. Polyribosomes were obtained from the lysate by centrifugation in reticulocyte saline buffer (Buffer A) (10 mm Tris-HCl (pH 7.6)/30 mm KCl/2 mm magnesium acetate) for 3 hours at 50,000 rpm in the Spinco SW 60 Ti rotor through 9 ml of 1 M sucrose in Buffer A layered over 2 ml of 2 M sucrose in Buffer A. The 14 S rRNA complex was obtained by dissociating polyribosomes with EDTA (18) and purified by sucrose gradient centrifugation. The 9 S messenger RNA was then prepared by treatment of the 14 S rRNA with 1% sodium dodecyl sulfate and purified on a sucrose gradient. The RNA was ethanol-precipitated, pelleted by centrifugation, dried in vacuo, and redissolved in a small amount of distilled water and stored at -20°.

**Preparation of [35S]Met-tRNA<sup>Met</sup>—** Rabbit reticulocyte tRNA was prepared by phenol extraction of rabbit reticulocyte cell sap followed by addition of 0.1 volume of 0.2 m potassium acetate, pH 5.2, and ethanol (3 volumes) to precipitate the RNA in the aqueous phase. Any aminoacylated tRNA was removed by incubating the tRNA with 15 mm Tris-HCl for 2 hours at 37° and was subsequently charged with...
RNA was added at a concentration of 125 μg/ml. Endogenous synthesis; M, in the presence of supernatant RNA. PGlml. Endogenous synthesis; 04, with added low molecular weight RNA was present at a concentration of 150 to 155.7 μg/ml. 0.4 ml of cold buffer (30 μM Hepes (pH 7.0)/50 mM KCl/3 mM MgCl₂) was added, and the samples applied to 5 ml of 10 to 30% sucrose gradients made up in the diluting solution. Successive fractions were collected and analyzed as described by Darnbrough et al. (20). Preparation and Analysis of Ribosome-bound Protamine Peptides—The preincubated fractionated ascites cell-free system has very few polysomes and is very suitable for the detection of those polysomes associated with protamine synthesis following the addition of protamine mRNA. Incubation was carried out as described in the figure legends and each reaction mixture was applied to a 5-ml 10 to 30% sucrose gradient made in 30 mM Hepes/30 mM KCl/3 mM MgCl₂ (pH 7.0) and centrifuged under the conditions of the shift experiment except that the running time was 80 min. Isolation of Incubation Products—The products from the ascites S-30 incubations with RNA fractions from trout testis were isolated as described previously (1). Characterization of Synthetic Products by Starch Gel Electrophoresis—Urea-aluminum lactate gels were prepared as described by Sung and Smithies (22) except that 45 g of Connaught starch (Connaught Laboratories, Toronto) were mixed and the gel prepared and poured into a Smithies' gel tray. The surface of the gel surrounding the slots was outlined with petroleum wax; the samples (incubation products) were dissolved in 0.1 N HCl and applied to the slots which were then sealed with wax. Electrophoresis was carried out in the cold room for 15 to 17 hours at 4 volts/cm. After electrophoresis the gels were trisected horizontally. The bottom slab was stained with covalt-Amid black 10B and destained with 1 N sulfuric acid. Radioactivity was determined (4) by cutting the middle slab into 3-mm slices and incubating them in scintillation vials with 0.5 ml of NCS reagent (Amersham/ Searle) overnight at room temperature. Five milliliters of Triton-toluene (3:7) scintillation fluid was added and the capped vials incubated at 40-50°C for a further 4 hours, cooled down, and counted. Characterization of Synthetic Product by CM-cellulose Chromatography—The acid-soluble material synthesized in the Krebs II ascites S-30 system in the presence of trout testis mRNA was dissolved in 20 mM sodium acetate, pH 5.5, and applied to a CM-cellulose (Whatman CM52) column (1 x 50 cm) equilibrated in the same buffer. The column was washed with 40 ml of 20 mM sodium acetate, pH 5.5, to remove most of the free [³⁵S]arginine and then with 40 ml of 0.66 M LiCl-aceate, pH 5.5, to wash out the histones which were added as carriers with unlabeled protamine. An acid extract of immature trout testis chromatin was the carrier material and contains approximately equal amounts of histones and protamine. The column was then eluted with a linear gradient of LiCl generated from 100 ml of each of 0.66 and 1.1 M LiCl in sodium acetate buffer, pH 5.5. The flow rate was about 15 ml/hour and 1.5 ml fractions were collected and their absorbance at 230 and 225 nm determined. The salt concentration in every fifth fraction was measured with a Radiometer conductivity meter making reference to standard solution of lithium chloride. Alternate fractions of 0.6 ml were withdrawn, and 4 ml of Triton-toluene scintillation fluid was added and the samples counted.

RESULTS

Response of Krebs II Ascites Cell-free System to Trout Testis Low Molecular Weight Polysomal RNA and Supernatant RNA

The addition of both low molecular weight polysomal RNA and supernatant RNA from trout testis to a preincubated Krebs II ascites S-30 caused a stimulation of the incorporation of [¹⁴C]arginine into hot trichloroacetic acid-tungstate-insoluble material (Fig. 1, a and b). The stimulation of [¹⁴C]arginine incorporation by both RNA preparations was routinely found to be between 2- and 3-fold. In a second experiment (Table I) it was observed that the addition of trout testis supernatant RNA to a preincubated Krebs II ascites S-30 stimulated [¹⁴C]arginine incorporation into hot trichloroacetic acid-tungstate-insoluble material but had no effect on the incorporation of [¹⁴C]leucine. On the other hand, rabbit globin mRNA stimulated both [¹⁴C]arginine and [¹⁴C]leucine incorporation. This result suggests that part of the stimulation observed in the presence of trout testis supernatant RNA is due to the synthesis of protamine since the amino acid sequence of trout testis protamine (2) shows that arginine comprises 2/3 of the

TABLE I

Comparison of leucine and arginine incorporation in response to rabbit globin mRNA and trout testis postribosomal supernatant RNA (sRNA) in Krebs II ascites S-30 system

A background which averaged 73 cpm was subtracted and the figures shown are averages of duplicate samples. Rabbit globin mRNA and total trout testis sRNA were present at concentrations of 13.3 and 100 μg/ml, respectively.

| Additions | [¹⁴C]Leucine incorporated/20 μl | Minus control | [¹⁴C]Arginine incorporated/20 μl | Minus control |
|-----------|-------------------------------|---------------|-------------------------------|---------------|
| Control   | 601                           | 427           |                               |               |
| + Globin mRNA | 2788                        | 2187          | 546                           | 119           |
| + sRNA    | 599                           | 0             | 1262                          | 835           |
| + Globin mRNA and sRNA | 2633                        | 2032          | 1091                          | 664           |
Assay of Protamine mRNA

FIG. 2. Analysis of the product of in vitro synthesis in the Krebs ascites S-30 directed by postribosomal supernatant RNA isolated from trout testis. Starch gel electrophoresis was carried out as described under "Materials and Methods." —— endogenous synthesis; —— in the presence of sRNA.

Characterization of Product Synthesized in Krebs II Ascites S-30 in Presence of Either Supernatant RNA or Low Molecular Weight Polysomal RNA from Trout Testis

In order to demonstrate the presence of protamine mRNA, the products synthesized in the ascites S-30 in the presence of the RNA fractions and [3H]arginine were studied. Two methods of analysis were reported here. First, the acid-soluble products were analyzed by electrophoresis on starch gels. After termination of the in vitro reaction, a mixture of unlabeled trout testis protamine and histones were added and the incubation mixture was extracted with 0.2 M sulfuric acid and the extracts run on starch gels as described under "Materials and Methods." In the absence of added RNA, one peak of radioactivity was observed which migrated on the starch gel in the same position as free arginine (Fig. 2). In the presence of supernatant RNA from trout testis, there were two peaks of radioactivity. The faster moving peak co-migrated with free arginine and the slower with the carrier protamine which was detected by staining one portion of the gel with Amido black prior to slicing the remainder for radioactive counting (Fig. 2). In the presence of low molecular weight polysomal RNA from trout testis, three peaks of radioactivity were observed (Fig. 3a). The slowest moving peak corresponded to the area of the gel containing marker histones. The major peak of radioactivity migrated in the protamine region and there was a third peak which migrated in the free arginine region. When only the 4 to 6 S region of the low molecular weight RNA, which had been separated on a sucrose density gradient, was added to a Krebs II ascites S-30, the synthesis of histones was abolished presumably due to the removal of the larger species of RNA containing histone mRNA (Fig. 3b).

Secondly, the cell-free products synthesized in the presence of supernatant RNA were analyzed by CM-cellulose chromatography using a lithium chloride/acetate gradient (1). The conditions of chromatography were such that three components of protamine from trout testis, Cα, Cβ, and Cγ, could be separated as individual peaks. It can be seen (Fig. 4) that the product synthesized in the cell-free system in the presence of trout testis supernatant RNA contained labeled components which co-chromatographed with Cα, Cβ, and Cγ. It can also be seen that these three components are not synthesized in equal amounts, there being more incorporation into Cβ than Cα, with very little into Cγ.

Partial Characterization of Protamine mRNA on Sucrose Gradients

Samples of both low molecular weight polysomal RNA and supernatant RNA from trout testis were analyzed by sucrose density centrifugation. After centrifugation under the conditions given in the legend of Fig. 5, RNA was extracted from various regions of the sucrose gradient of the low molecular weight polysomal RNA (Fig. 5a) and the 4 S region from the supernatant RNA (Fig. 5b). These RNA fractions were assayed for mRNA activity in the Krebs II ascites cell-free system. The

FIG. 3. Characterization of polypeptides synthesized in Krebs ascites cell-free system as directed by low molecular weight RNA and 4 to 6 S RNA. The acid-soluble polypeptides synthesized by the Krebs II ascites cell-free system (S-30) in vitro were extracted as described by Gilmour and Dixon (7) and separated by starch gel electrophoresis as described under "Materials and Methods." a, ——, endogenous [3H]arginine incorporation; ——, [3H]arginine incorporation in the presence of added low molecular weight RNA. By reference to a stained horizontal portion of the gel, it could be seen that the histones migrate 5 to 7 cm and protamines, 11 to 15 cm, from the starting slot. b, ——, [3H]arginine incorporation, in response to the RNA in the 4 to 6 S region of the low molecular weight RNA preparation after sucrose gradient analysis. The protamines migrated 12 to 14.6 cm from the top of the gel.
Assay of Protamine mRNA

Fig. 4. Characterization of protamine synthesized in vitro by chromatography on carboxymethylcellulose (CM52) as described previously (1). The [14C]arginine-labeled polypeptides synthesized in the Krebs II ascites S-30 in the presence of testis postribosomal supernatant RNA were extracted as described previously and applied to a CM52 column with 10 mg of cold carrier protamine. The column was eluted with a linear LiCl gradient. — —, conductivity; — —, radioactivity; bar graph, absorbance at 225 nm.

Fig. 5. a, sucrose gradient analysis of low molecular weight RNA. The low molecular weight RNA prepared as described under "Materials and Methods" was applied to a 12-ml 15 to 30% linear sucrose gradient in buffer containing 100 mM Tris-HCl (pH 7.6)/300 mM KCl. Gradients were centrifuged at 36,000 rmp for 24 hours at 4°C in an SW 40 rotor and analyzed at 260 nm by a Unicam SP800 continuously recording spectrophotometer. b, sucrose gradient centrifugation of trout testis supernatant RNA. Approximately 10,000 units of postribosomal supernatant RNA from trout testis were applied to a 15 to 30% linear sucrose gradient made up in buffer containing 1.0 mM Tris-HCl (pH 7.6)/300 mM KCl. Centrifugation was carried out in an SW 40 rotor at 36,000 rpm for 20 hours at 4°C.

bulk of the protamine mRNA activity was found at the top of the gradient in the 4 to 6 S region. A small amount of activity was seen in the 6 to 18 S region as well, however, there was no protamine mRNA activity in the 18 S region of the gradient (data not shown).

Characterization of Protamine mRNA Translation in Krebs II Ascites Cell-free System

Time Course and mRNA Concentration—Fig. 6 shows the effect of adding increasing amounts of sRNA to an ascites S-30 system. The stimulation of [14C]arginine incorporation reaches a plateau at 4 µg/30 µl reaction mixture (120 µg/ml) of sRNA. Similar amounts of sRNA could also saturate the fractionated Krebs II ascites S-30 (Fig. 7a). Addition of 100 µg/ml of low molecular weight testis polysomal RNA saturates the Krebs II ascites S-30.* In the presence of sRNA, incorporation of [14C]arginine in the Krebs II ascites S-30 remains linear for the first 30 min and then the rate slows down (Fig. 1b). Fig. 7b shows the rate of incorporation of [14C]arginine in a fractionated Krebs II ascites S-30 as directed by sRNA. The rate of incorporation was linear for the first 10 min and then declined. In the presence of trout testis low molecular weight polysomal RNA, incorporation of [14C]arginine by the ascites S-30 remains linear for the first 30 min (Fig. 1a).

Effect of Adding Transfer RNA—It has been reported recently that the translation of a number of messenger RNAs including EMU RNA (23) and rabbit globin mRNA (24) in the ascites S-30 is dependent on, or can be stimulated by, the addition of tRNA. It was therefore worthwhile to test the effect of the addition of tRNA on protamine mRNA translation. The addition of either ascites tRNA, reticulocyte tRNA, or trout testis tRNA (purified on BD-cellulose) to a preincubated ascites S-30 had no effect on the incorporation of [14C]arginine.
In the presence of sRNA or low molecular weight polysomal RNA, the addition of tRNA caused a 25% increase in the incorporation of \[^{14}C\]arginine when compared to the value obtained in the presence of the RNA alone (Table II). It appears therefore that the translation of protamine mRNA in the ascites S-30 used here is only slightly stimulated by the addition of tRNA so that none of the tRNAs required for the insertion of the limited range of amino acids present in protamine are limiting in the Krebs II ascites S-30 system.

**Effect of Aurintricarboxylic Acid**—Aurintricarboxylic acid is an inhibitor of protein synthesis which acts at the level of initiation in eukaryotic systems. Addition of this compound in optimal amounts to a cell-free system should therefore inhibit the translation of exogenous mRNA. In general, a concentration of aurintricarboxylic acid of 10^{-4} M has been shown to be optimal in inhibiting the initiation of protein synthesis in mammalian systems (25, 26). Fig. 8 shows that the stimulation of incorporation of \[^{14}C\]arginine incorporation by low molecular weight polysomal RNA is completely abolished by the addition of 10^{-4} M aurintricarboxylic acid, an observation which further supports the idea that the observed stimulation is due to the synthesis of protamine directed by protamine mRNA. Similar observations have been made with testis sRNA containing protamine mRNA activity.

**“Shift” Experiment**—It is now generally accepted that the initiation of polypeptide synthesis in both prokaryotic and eukaryotic cells involves the incorporation of a methionyl residue by a special initiating tRNA which binds to the initiation AUG codon in the messenger RNA. The synthesis of protamine both in vivo (6) and in vitro (7) has been shown to involve the incorporation of an unblocked methionyl residue at the NH\(_2\)-terminal position of the nascent protamine chain.

Recently Darnbrough et al. (20) have developed a system in which they can study the mechanism of initiation of protein synthesis in a rabbit reticulocyte lysate. This system can be used to detect the presence of a messenger RNA by following the “shift” in methionine label (in \[^{35}S\]Met-tRNA\(^{Met}\)) from the 40 S ribosomal subunits in the absence of mRNA to the 80 S ribosome upon addition of mRNA. The experiment is performed in the presence of a saturating amount of sparsomycin to prevent movement of the 80 S ribosome along the messenger, thus, there is no translation and initiation stops at the formation of this 80 S complex. In Fig. 9, it can be seen that in the absence of any exogenous mRNA, a peak of radioactivity is associated with the small 40 S ribosomal subunit of the sucrose gradient (Fig. 9a). The addition of either rabbit globin mRNA (Fig. 9b) or trout testis sRNA (Fig. 9c) causes a change in the radioactivity distribution leading to an increase in label in the 80 S region at the expense of label associated with the 40 S ribosomal subunit. There is a larger shift of labeled [\(^{35}S\)Met-tRNA\(^{Met}\) in the presence of rabbit globin mRNA than in the case of trout testis sRNA so that it is likely that the concentration of trout testis sRNA used did not saturate the system. Since this assay depends on the presence of an AUG codon at the initiation site of mRNA, this observation of the “shift” of [\(^{35}S\)Met-tRNA\(^{Met}\) from the 40 S to the 80 S complex in the presence of trout testis sRNA indicates the presence of an mRNA containing an AUG-initiating codon.

The coding portion of the protamine message would be expected to contain 96 to 99 nucleotides, a region approximately one-fifth the size of the coding portion of the globin mRNA. The average number of ribosomes associated with globin message in reticulocyte polysomes is five (16) and since protamine mRNA is one-fifth the size, it would be predicted that with the same packing, only a single ribosome at a time would be associated with a monocistronic protamine mRNA. Lang et al. (3) discussed the apparent discrepancy between this extrapolation from globin-synthesizing polysomes and the observed appearance of nascent protamine chains on diribosomes in intact trout testis spermatid cells in terms of whether or not the presence of a dicistronic messenger for protamine but could not distinguish between these possibilities. This question was, therefore, re-examined in the heterologous translation system described above of the Krebs II ascites S-30 programmed with trout testis sRNA containing messenger activity for protamine.

### Table II

| Assay of Protamine mRNA | 1451 |

**Comparison of stimulation of \[^{14}C\]arginine incorporation into acid-precipitable polypeptides by various RNA fractions and effect of tRNA using fractionated Krebs II ascites system**

\[^{14}C\]Arginine incorporation into acid-precipitable polypeptides was determined as under "Materials and Methods." Incorporation in the absence of ribosomes (which averaged 320 cpm) was subtracted.

| RNA added | \[^{14}C\]Arginine incorporated/40 \(\mu\)l | Minus control | Specific activity |
|-----------|------------------------------------------|--------------|------------------|
| Control   | 296                                      | 834          | 167              |
| Low molecular weight polysomal RNA (total) | 1130 | 1216 | 1484 |
| 4 to 6S region from ouroside gradient | 0.84 | 1542 | 1216 |
| sRNA (total) | 3.6 | 1817 | 1521 | 424 |
| Total RNA prepared from ascites cells | 3.0 | 374 | 78 | 26 |
| sRNA (3.6 \(\mu\)g) plus ascites tRNA (3.0 \(\mu\)g) | 2267 | 1951 |

In the presence of sRNA or low molecular weight polysomal RNA, addition of tRNA caused a 25% increase in the incorporation of \[^{14}C\]arginine when compared to the value obtained in the presence of the RNA alone (Table II). It appears therefore that the translation of protamine mRNA in the ascites S-30 used here is only slightly stimulated by the addition of tRNA so that none of the tRNAs required for the insertion of the limited range of amino acids present in protamine are limiting in the Krebs II ascites S-30 system.
Assay of Protamine mRNA

activity and after 5- and 10-min incubation, the polysomes were fractionated on sucrose gradients and the distribution of [14C]arginine in the nascent protamine associated with the polysomes was determined.

Fig. 10, A and D, show that there was no [14C]arginine label associated with ribosomes after either 5 and 10 min of incubation in the absence of added mRNA. In the presence of 7.5 μg of sRNA, a small amount of label was observed in the monosome region (Fig. 10B) after 5 min of incubation and there was a significant increase at 10 min (Fig. 10C).

At 15 μg of sRNA, there was much more label associated with the monosome peak at 5 min and this increased at 10 min (Fig. 10, E and F). In contrast to the results of Ling et al. (3) the major peak of the newly synthesized protamine is found associated with the monosomes and a minor peak in the disome region (70% of the radioactivity on the monosome and 30% on the disome).

DISCUSSION

An important consideration in studies of the translation of an isolated messenger RNA is the selection of an appropriate translation system. Gilmour and Dixon (7) used a preincubated trout liver ribosome system to detect the presence of the messenger RNA for protamine in a low molecular weight RNA fraction prepared from trout testis polysomes. This system had the advantage of being derived from an easily available tissue in the same animal but one which does not synthesize protamine endogenously and hence has a very low background incorporation. However, the activity of the preincubated trout liver ribosomes was low and somewhat variable. Mathews and Korner (15) found that ribosomes prepared from Krebs II ascites cells after preincubation under the conditions of protein synthesis could faithfully translate heterologous messenger RNAs such as that for globin. Since then a number of messenger RNAs including those for lens crystallins, immunoglobulin light chain and histones as well as the RNAs of EMC virus, bacteriophage Qβ, and reovirus have been translated in the Krebs II system. This cell-free system, which has a low background incorporation of [14C]arginine has proved both sensitive and convenient for detecting and assaying protamine mRNA. Addition of low molecular weight RNA prepared both from trout testis polysomes (Fig. 1a) and postribosomal supernatant (Fig. 1b) to the Krebs II ascites S-30 leads to marked stimulation of incorporation of [14C]arginine into hot trichloroacetic acid tungstate-precipitable material. At low concentrations of added low molecular weight RNA, the increase in [14C]arginine incorporation was linearly related to RNA concentration but the system became saturated as the RNA concentration was increased (Figs. 6 and 7a). Gilmour and Dixon (7) showed with the trout liver ribosome cell-free system that methionine was incorporated into the NH₂-terminal position of nascent protamine chains, an observation consistent with the NH₂-terminal methionine incorporation in vitro indicated that de novo initiation rather than chain elongation was taking place in the trout liver cell-free system. A sensitive assay for initiation is the effect of ariurincarboxylic acid on in vitro incorporation of [14C]arginine into protamine. As seen in Fig. 8, ariurincarboxylic acid at 1 × 10⁻⁴ M completely inhibits protamine synthesis by the Krebs II ascites S-30 and since it is known to affect the initiation step specifically, this total inhibition indicates that substantial de novo initiation must be taking place in the ascites S-30. The [14C]arginine-labeled product of synthesis in the presence of added low molecular weight RNA was further characterized by starch gel electrophoresis as described by Gilmour and Dixon (7) and Fig. 2a shows both the resolution of [14C]arginine-labeled protamine from free [14C]arginine and the strong dependence of the incorporation upon added protamine mRNA. As shown by Gilmour and Dixon (7), crude low molecular weight RNA from testis polysomes supports the incorporation of [14C]arginine into
Fig. 10. Polysome profiles of a fractionated Krebs II ascites cell-free system in the presence and absence of postribosomal supernatant RNA containing protamine messenger RNA (sRNA). The reaction mixtures (135 µl) contained Krebs II ascites ribosomes, cell sap, and the "master mix" without label and were preincubated for 10 min at 37° and then chilled in ice. Potassium and magnesium ion concentrations were 83 and 2.8 mM, respectively. The reaction was started by the addition of [14C]arginine plus water or an sRNA solution to bring the final volume to 150 µl. At the end of the incubation periods indicated below, the reaction mixtures were diluted with 2 volumes of a buffer containing 30 mM Hepes/30 mM KCl/3 mM MgCl₂, (pH 7.0) and layered onto a 5-ml 10 to 30° sucrose gradient made up in the same buffer. Gradients were centrifuged for 80 min at 45,000 rpm and 4° in an SW 50 rotor. The collection and analysis of fractions is as described in the "shift" experiment. A, control incubated for 5 min; B, 7.5 µg of sRNA incubated for 5 min; C, 15 µg of sRNA incubated for 5 min; D, control incubated for 10 min; E, 7.5 µg of sRNA incubated for 10 min; F, 15 µg of sRNA incubated for 10 min. Optical density and radioactivity are shown by continuous and dotted lines, respectively.

Both protamine and histones and we have confirmed this finding with the Krebs II ascites system (Fig. 3a). However, when the low molecular weight RNA is further purified by sucrose density gradient centrifugation (Fig. 5a) and the 4 to 6 S RNA peak assayed, the protamine mRNA activity is retained (Fig. 3b and Table II) but the histone mRNAs are removed (Fig. 3b). This finding is consistent with the larger size of the histone messenger RNAs (7 to 9 S) which would not be included in the 4 to 6 S peak of the gradient.

A rather surprising finding in the present study is the presence of a considerable amount of protamine messenger RNA in the postribosomal supernatant solution from trout testis (Fig. 1b and Table II). The mRNA from this postribosomal supernatant shows a peak sedimenting at 4 to 6 S in the sucrose density gradient (Fig. 5b) and produced a marked stimulation of incorporation of [14C]arginine into hot trichloroacetic acid-tungstate-precipitable material.

Control incubations containing ascites tRNA (Table II) or trout testis tRNA purified on BD-cellulose showed no such stimulatory effect.

In Fig. 2, the product of testis postribosomal sRNA translation in the ascites S-30 is shown to undergo co-electrophoresis with carrier protamine on starch gels while in Fig. 4 the product is further characterized by ion exchange chromatography on a carboxymethylcellulose column eluted with a gradient of lithium chloride in the presence of lithium acetate/acetate acid buffer, pH 5.0 (1). Three subcomponents of trout testis protamine, C₁, C₀, and C₃, are resolved by this procedure and it is clear that [14C]arginine is incorporated into each. However, C₃ shows the highest incorporation and possesses the greatest specific activity, thus the three components are not labeled equally or in proportion to the amounts of each present in the cold carrier protamines extracted from trout testis nuclei. There are at least two possible explanations for this result; either the sRNA fraction was isolated at a stage of testis development when the tissue is richest in the C₅₁₁ message or the Krebs II ascites S-30 is able to translate the protamine mRNA fractions for the three components with differing efficiencies. In the case of α- and β-globin mRNA, it is well known that there can be considerable variation in the rates of α to β chain synthesis in different cell-free translation systems which may be related to the presence or absence of initiation factors specific for one message or the other (28-30). We have observed wide variations in the distribution of incorporation into the three protamine components and those studies will be reported in a subsequent communication.

Protamine has a very limited range of amino acids in its structure and it is possible to compare the incorporation of an amino acid such as leucine which is not present in the known sequence, with one that is very frequent such as arginine. Such an experiment is described in Table 1 where the incorporation of [14C]leucine and [14C]arginine is compared when the ascites S-30 is programmed with either rabbit reticulocyte globin mRNA, trout testis sRNA, or a mixture of both. Leucine is a major constituent of both α and β chains and is extensively incorporated in response to globin mRNA but not at all in response to testis sRNA. This lack of stimulation of [14C]leucine incorporation by testis sRNA indicates that the amounts of mRNAs coding for leucine-containing proteins is undetectable in the total testis sRNA. In contrast, there is strong
stimulation of [14C]arginine incorporation by the testis sRNA but rather weak stimulation by globin mRNA; this is consistent with the presence of only 3 arginine residues out of 141 to 143 in both rabbit α- and β-globins as compared with 21 arginines out of 31 residues in the protamines. When both globin mRNA and testis sRNA are present simultaneously there is a slight decrease in the leucine and arginine incorporation, respectively, indicating a slight degree of competition between the two messages at the concentrations employed.

Another useful assay for the presence of messenger RNA is the "shift" assay described by Darnbrough et al. (30) which depends upon the formation of a complex between [32P]methyl-tRNA<sub>met</sub> and the small 40 S ribosome subunit in the presence of initiation factors. When mRNA containing the initiation codon, AUG, is present together with the large 60 S ribosomal subunit, then there is a shift of radioactivity from the 40 S region to the 80 S region. Since the "shift" assay is done in the presence of sparsomycin, a potent inhibitor of elongation, no polysomes are formed and the 32P label remains associated with the 80 S monoribosome peak. The occurrence of the expected shift in the presence of both globin mRNA and sRNA containing protamine mRNA shows that both possess the initiating codon characteristic of natural messenger RNA. In addition, the shift of [32P]methyl-tRNA<sub>met</sub> in the presence of protamine mRNA confirms the involvement of methionyl-tRNA in the initiation of protamine synthesis as previously reported in vivo by Wigle and Dixon (6) and in vitro by Gilmour and Dixon (7). When the exogenous testis sRNA is added to Krebs II ascites S-30 in the presence of [14C]arginine and the polysome profile examined at 5 min (Fig. 10, C and D) and 10 min (Fig. 10, E and F), it is seen that there is a stimulation of incorporation of [14C]arginine into nascent protamine associated largely (70%) with the monoribosome peak but with some label (30%) in the diribosome peak. This result is somewhat different to that in vivo in trout testis cells (3) where the predominant site of nascent protamine synthesis appeared to be the diribosome peak. The basis of this difference is not clear at the moment, but the efficiency of the heterologous in vitro protein synthesis system is so much reduced compared to the in vivo situation that the smaller polysome size in vitro may reflect the greatly reduced efficiency of mRNA translation under these artificial conditions. In an accompanying paper, Gedamu et al. (31) have shown that two editions of protamine mRNA exist in testis cells, one possessing a poly(A) region which binds tightly to oligodeoxythymidylate cellulose columns and one which does not. The postribosomal supernatant appears to be somewhat richer in the species of mRNA lacking poly(A) and since in the experiment depicted in Fig. 10, the source of the mRNA was postribosomal supernatant (sRNA), it is possible that the predominant location of nascent protamine on the monoribosome may be related to the presence of an appreciable amount of protamine mRNA lacking in poly(A) region. Further experiments are under way to examine this possibility.

From the amino acid sequence of the rainbow trout protamines (2), it can be calculated that 3 × 31 to 33 = 93 to 96 nucleotides plus the initiating and terminating codons for a total of 99 to 102 nucleotides, would be required to code for protamine. This would represent the minimum size for the protamine mRNA. However, by analogy with other messenger RNAs (32) which are usually significantly larger than their predicted coding sizes, it would be expected that protamine mRNA would be at least 130 to 170 residues in length which would correspond to an S value of 5 to 6. In the accompanying paper (31), it is shown that poly(A) containing protamine mRNA which can be purified by oligo(dT)-cellulose chromatography followed by sucrose density gradient centrifugation has an S value of 5.7 corresponding to about 165 nucleotides. A significant proportion of the extra 63 to 66 nucleotides over the minimum coding size of 99 to 102 must be poly(A) but the exact size of this region is not yet known.

The sucrose density gradient centrifugation of sRNA (Fig. 5b) shows a single peak in the 4 to 6 S region; the great majority of the material must be tRNA but the total absence of 18 and 28 S ribosomal RNA indicates that the messenger activity present in the postribosomal supernatant cannot be due to contaminating polysomes containing bound mRNA but must represent a distinct postribosomal form of protamine mRNA.

REFERENCES
1. Ling, V., Jergil, B., and Dixon, G. H. (1971) J. Biol. Chem. 246, 1168-1176
2. Ando, T., and Watanabe, S. (1969) Int. J. Protein Res. 1, 221-224
3. Ling, V., Trowbridge, N. J. R., and Dixon, G. H. (1969) Can. J. Biochem. 47, 51-60
4. Louie, A. J., and Dixon, G. H. (1972) J. Biol. Chem. 247, 5498-5505
5. Ingles, C. J., Trowbridge, N. J. R., Smith, M., and Dixon, G. H. (1966) Biochem. Biophys. Res. Commun. 22, 627-634
6. Wigle, D. T., and Dixon, G. H. (1970) Nature 227, 676-680
7. Gilmour, R. S., and Dixon, G. H. (1972) J. Biol. Chem. 247, 4621-4627
8. Mathews, M. B., Osborn, M., Bens, A. J. M., and Bloemendal, H. (1972) Nature New Biol. 236, 5-7
9. Mathews, M. B., Fragelli, I. B., Osborn, M., and Arnstein, H. R. V. (1972) Biochem. Biophys. Acta 287, 113-123
10. Swan, D., Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1967-1971
11. Milstein, C., Brownee, G. G., Harrison, T. M., and Mathews, M. B. (1972) Nature New Biol. 239, 117-120
12. Jacobs-Lorena, M., Baglioni, C., and Borun, T. W. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2077-2080
13. Boime, I., and Leder, P. (1972) Arch. Biochem. Biophys. 153, 706-713
14. Kirby, K. S. (1967) in Techniques in Protein Biosynthesis (Campbell, P. N., and Sargent, J. K., eds) Vol. 1, pp. 263-285, Academic Press, New York.
15. Mathews, M. B., and Korner, A. (1970) Eur. J. Biochem. 17, 328-338
16. Lamfrom, H., and Knopf, P. M. (1964) J. Mol. Biol. 9, 578-575
17. Evans, M. J., and Lingrel, J. B. (1969) Biochemistry 8, 829-831
18. Hwa, G., Burney, L. A., Marbaix, G., and Lebelu, B. (1967) Biochim. Biophys. Acta 145, 629-636
19. Gupta, N. K., Chatterjee, N. K, Bose, K. K., Bhanduri, S., and Chung, A. (1970) J. Mol. Biol. 54, 145-154
20. Darnbrough, C., Legon, S., Hunt, T., and Jackson, R. J. (1973) J. Mol. Biol. 76, 379-403
21. Lingrel, J. B., and Bensox, H. (1963) Biochemistry 2, 309-314
22. Sung, M., and Smithies, O. (1969) Biopolymers 7, 39-58
23. Aviv, H., Boime, I., and Leder, P. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2303-2307
24. Metafora, S., Terada, M., Dow, L. W., Marks, P. A., and Bank, A. (1972) Proc. Natl Acad. Sci. U. S. A. 69, 1967-1980
25. Stewart, M. L., Grollman, A. P., and Huang, M. T. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 97-101
26. Webster, R., and Zindo, N. D. (1969) J. Biol. Chem. 42, 425-439
27. Rich, A., Warner, J. R., and Goodman, H. M. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 257-265
28. Howser, D. A., Pemberton, R., and Taber, R. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 2716-2719
29. Hall, N. D., and Arnstein, H. R. V. (1973) Biochem. Biophys. Res. Commun. 54, 1490-1497
30. Sampson, J., Mathews, M. B., Osborn, M., and Boghetti, A. F. (1972) Biochemistry 11, 3606-3610
31. Gedamu, L., and Dixon, G. H. (1970) J. Biol. Chem. 231, 1455-1463
32. Mathews, M. B. (1973) Essays Biochem. 9, 59-102
Assay of protamine messenger RNA from rainbow trout testis.
L Gedamu and G H Dixon

J. Biol. Chem. 1976, 251:1446-1454.

Access the most updated version of this article at http://www.jbc.org/content/251/5/1446

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/5/1446.full.html#ref-list-1