Regulated Transport of Messenger Ribonucleic Acid from Isolated Liver Nuclei by Nucleic Acid Binding Proteins†

R. Bruce Moffett* and Thomas E. Webb

ABSTRACT: Rat liver nucleocytosolic messenger ribonucleic acid (mRNA) transport is shown to be regulated by proteins with a high affinity for nucleic acids. In the cell-free system described, the energy-dependent transport of all RNA classes [transfer RNA (tRNA), mRNA, and ribosomal RNA (rRNA)] exhibited a dependence upon the availability of discrete minor sets of cytosol proteins. In addition to having a different level of saturation, only the mRNA "transport protein" activities are increased by adenosine cyclic 3′,5′-phosphate (cAMP), an effect most likely mediated by a cAMP-dependent protein kinase. The mRNA transport proteins were isolated from cytosol by precipitation with streptomycin sulfate followed by deoxyribonucleic acid (DNA)–cellulose affinity chromatography, or from oligo(thymidylate)–cellulose bound cytoplasmic messenger ribonucleoprotein (mRNP) particles by high-salt extraction. Either method yielded a protein fraction which exhibited a 1000-fold increase in mRNA transport activity as compared to cytosol. Over one-half of the mRNA transport activity is associated with the mRNP of the cell. A partial homology between the cytosol and mRNP-derived proteins was demonstrated by polyacrylamide gel electrophoresis. One major (20 000 daltons) and several minor proteins (23 000, 52 000, 54 000, and 72 000 daltons) were in common. Nuclear 4–5S RNA, mRNA, and rRNA pulse labeled in vivo for 20 min exited from in vitro incubated nuclei in three phases, according to their differential in vivo rates of labeling and intranuclear pool sizes. The amount of nuclear RNA transported in vitro as mRNA (about 1.0%) agrees with the in vivo estimates. Additional evidence for in vivo equivalence was provided by the physicochemical characterization and bioassay of the RNA. The transported mRNA sedimented in urea–sucrose gradients as an 8–18S heterodisperse product. This RNA initiated cell-free translation with the synthesis of precursor peptides as diverse in size as those for albumin and α_{2U}-globulin. The relative abundancies of various transported mRNAs were different than the corresponding abundancies of liver cytoplasmic mRNAs.

Evidence from diverse sources indicates that significant regulation of genetic expression occurs at the posttranscriptional nuclear level. The complexity of single-copy nuclear ribonucleic acid (RNA) is 4–10-fold greater than cytoplasmic RNA even in systems where the two classes do not differ greatly in size (Bantle & Hahn, 1976; Mansson & Harris, 1979; Harpold et al., 1979; Samal & Bekhor, 1979). This apparent nuclear RNA restriction, i.e., wherein all nuclear RNA is not transported to the cytoplasm, applies to repetitive sequences (Garrett et al., 1973) as well as to single-copy RNA. Dramatic changes in the proportion of single-copy sequences restricted to the nucleus occur during early development (Kleene & Humphreys, 1977; Shiokawa et al., 1979; Wold et al., 1978) and during specific stages of differentiation (Chan, 1976; Mauron & Spohr, 1978). Furthermore, the efficiency of messenger RNA (mRNA)[1] processing and transport, i.e., nuclear restriction, responds to growth and nutritional stimuli (Johnson et al., 1976; Murty et al., 1977) and appears to be modified by carcinogenesis (Shearer, 1979; Patel et al., 1979).

The mechanisms regulating nuclear processing and nucleocytoplasmic transport of mRNA are unknown. The enzymes involved in these processes and the specific proteins associated with both the transcripts and the mature products, however, probably (Samarina et al., 1968; Schweiger & Kostka, 1977; Jeffrey, 1977) contribute to the regulatory system. Components of the nuclear matrix associated with hnRNA (Faiferman & Pogo, 1975) may contribute toward the movement of the RNA to the nuclear pore complex and thus exert feedback control. Indeed, any mRNA- or hnRNA-associated protein, if concentration limited, could constitute an element in a nucleocytoplasmic feedback system.

One approach to elucidate the mechanisms of nuclear processing and nucleocytoplasmic transport of messenger RNA is to study these processes and quantitatively evaluate alternate models [cf. Tobin (1979)] in a defined cell-free system. In the present paper, the kinetics of a nucleocytosolic RNA transport system are shown to parallel those observed in vivo, and a specific cytosol protein fraction essential to the transport of functional messenger RNA is isolated.

Experimental Procedures

Preparation of Nuclei. Male (250 g) Sprague-Dawley rats were fasted overnight and injected with either 30 μCi of [6-^{14}C]orotic acid or 1.0 mCi of [5-^{3}H]orotic acid (see text). Following 20 or 30 min of in vivo labeling, livers were perfused with 0.25 M sucrose and 3.3 mM calcium acetate (0 °C), minced, and homogenized with a loose Teflon pestle (4 strokes; 1100 rpm) in 15 volumes of 2.3 M sucrose and 3.3 mM calcium acetate (Marzluff et al., 1973). A 30-mL glass homogenizing vessel with a size B nonserrated Teflon pestle (A.H. Thomas Co., Philadelphia, PA), machined to give a clearance of 0.28 mm, was used for homogenization. These mild conditions assured both the integrity of the nuclei and the nuclear RNA precursors, e.g., 45S rRNA. After filtration

† From the Department of Physiological Chemistry, The Ohio State University College of Medicine, Columbus, Ohio 43210. *Received August 20, 1980; revised manuscript received December 29, 1980.* This research was supported by Grant GM/CA 29221 from the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; cAMP, adenosine cyclic 3′,5′-phosphate; mRNA, messenger ribonucleic acid; tRNA, transfer RNA; rRNA, ribosomal RNA; poly(A), poly(adenylic acid); oligo(dT), oligo(thymidylate); EDTA, ethylenediaminetetraacetate; TMK buffer, 50 mM Tris-HCl, pH 7.9 at 20 °C, 2.5 mM $MgCl_2$, and 25 mM KCl; RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein; ATA, aurintricarboxylic acid; hnRNA, high molecular weight nuclear RNA.

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through cheesecloth, the nuclei were sedimented at 35000*g* for 60 min at 3 °C, washed by resuspension in 1.0 M sucrose–1.0 mM calcium acetate, collected at 2000*g* for 4 min, and finally resuspended in the same buffer.

Cytosol Preparation and Fractionation. Livers from rats fasted overnight were perfused with 0.25 M sucrose in TMK buffer (TMK buffer: 50 mM Tris-HCl, 2.5 mM $MgCl_2$, and 25 mM KCl, pH 7.9 at 20 °C) and then homogenized (size B Teflon pestle, 0.15-mm clearance) in 2 volumes of 0.25 M sucrose–TMK buffer. Crude cytosol was prepared by centrifuging the 20000*g* for 10 min supernatant at 145000*g* for 120 min. This cytosol was then either fractionated further by streptomycin sulfate treatment or used to produce "normal" cytosol containing all of the transport activity. "Normal" cytosol was prepared by precipitation of the crude cytosol proteins with 65% ammonium sulfate followed by dialysis of the proteins against TMK buffer overnight. Thus, to insure consistency, the cytosol and all partially purified fractions (except the mRNP-derived protein fraction) were precipitated with 65% ammonium sulfate and dialyzed before use, as described below. Where indicated, fractionation of the crude cytosol was achieved by addition of 1 volume of 4% streptomycin sulfate in H_2O. After the solution was stirred on ice for 20 min, the resultant precipitate was collected at 20000*g* for 10 min; the supernatant was then diluted with 2 volumes of TMK buffer, precipitated with 65% ammonium sulfate, and dialyzed against TMK buffer overnight. Residual mRNA, tRNA, and rRNA transport activity in the streptomycin-treated cytosol could be inactivated by allowing the supernatant to stand at 4 °C in excess of 60 min prior to ammonium sulfate treatment. Stirring longer than the indicated 20 min progressively and irreversibly inactivated the transport factors. Almost complete inactivation of residual activity in the treated cytosol also occurred if the ammonium sulfate treatment was omitted. The precipitated transport factors were reactivated by immediately dissolving the precipitate in 3 mL of 20% ammonium sulfate, reprecipitating with 30 mL of 70% ammonium sulfate (65% final concentration), and, after resuspension, dialyzing against 75 mM NaCl–TMK buffer overnight.

Further purification of the transport factors was achieved by affinity chromatography on DNA–cellulose. Following 6–8 h of dialysis against 75 mM NaCl–TMK buffer, the streptomycin precipitate was clarified, and nonspecific cellulose binding proteins were removed by centrifugation at 20000*g* for 10 min and elution through a cellulose column equilibrated with 75 mM NaCl–TMK. Calf thymus DNA–cellulose columns were prepared according to Alberts & Herrick (1971). The column flow-through (unbound) and wash (75 mM NaCl–TMK buffer) fractions were collected. The bound material was subsequently eluted with 2.0 M NaCl–TMK buffer (Johnson et al., 1975). Both fractions were dialyzed for 12 h against 75 mM NaCl–TMK buffer and then reduced in volume by dialysis against Ficoll. Prior to use, all cytosol and fractionated materials were clarified at 20000*g* for 10 min.

The mRNA-derived proteins were isolated from cytoplasmic polysomal RNP particles. Polysomes were isolated from a 20000*g* for 10 min supernatant fraction of a rat liver homogenate prepared in 0.25 M sucrose–TMK. The supernatant fraction was brought to 1% Nonidet P40 and layered over 2.0 M sucrose–TMK buffer, and the polysomes were sedimented at 145000*g* for 4 h. Isolated polysomes were suspended in 0.02 M EDTA and 0.225 M NaCl–TK buffer, passed over a blank cellulose column, and then bound to oligo(dT)–cellulose at 4 °C (Kumar & Pederson, 1975; Barrieux et al., 1976; van Venrooij et al., 1977; Cardelli & Pitot, 1977). After being extensively washed with 0.225 M NaCl–TMK buffer (0.25 M total salt) to remove residual ribosomal subunits and loosely associated proteins, the remaining proteins were eluted with 2.0 M NaCl–TMK buffer (Bearden & Chandra, 1976). The eluted fractions were dialyzed overnight against 75 mM NaCl–TMK buffer and then concentrated by dialysis against Ficoll.

The protein content of the various fractions was determined by standard Lowry procedures [cf. Schumm & Webb (1975)]. Comparisons of the population of peptides present in the various fractions were made by one-dimensional NaDodSO_4–polyacrylamide slab gel electrophoresis (Laemmli, 1970). Electrophoresis was performed in 11% acrylamide and 0.1% NaDodSO_4 gels by using a Pharmacia slab gel apparatus. Gels were fixed in 10% acetic acid and 50% methanol and stained with Coomassie blue. Albumin as well as the light and heavy chains of immunoglobulin was used as a molecular weight standard along with a standard series of molecular weight markers (Bio-Rad), including phosphorylase (94000), BSA (68000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21000), and lysozyme (14300).

Cell-Free Nuclear RNA Transport System. The standard assay system contained 40 mM Tris-HCl, pH 7.85 at 20 °C (final assay pH is 7.45 at 30 °C), 20 mM KCl, 2.0 mM $MgCl_2$, 0.545 mM $CaCl_2$, 0.27 mM $MnCl_2$, 4.5 mM NaCl, 4.5 mM spermidine, 1.8 mM dithiothreitol, 2.3 mM Na_2HPO_4, 500 mg/mL yeast RNA [oligo(dT) purified], 170 mM sucrose, 2.0 mM ATP, 3.5 mM phosphoenolpyruvate, 0.3 mM GTP, 0.41 mg/mL methionine, 1.0 μM cAMP (except as indicated), 4.4 mM $(NH_4)_2SO_4$ added with 35 units of pyruvate kinase, 4×10^6 prelabeled nuclei/mL, and cytosol protein as indicated. Unless otherwise noted, incubations were conducted at 30 °C for 40 min. Additional phosphoenolpyruvate was added at 12-min intervals where indicated. Following the incubation, nuclei were removed at 10000*g* for 8 min, and the RNA either was precipitated with 10% trichloroacetic acid for direct radioassay or was purified as described below.

The yeast RNA used to complement the natural ribonuclease inhibitor was converted to uniformly low molecular weight species which were easily separated from the larger mRNA species transported in the assay by guanidine-HCl extraction or by banding in CsCl or sucrose gradients. Specifically, the yeast RNA was hydrolyzed with 0.1 M NaOH (25 °C for 1 h) and then neutralized. High molecular weight RNA was precipitated from 8.0 M guanidine-HCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0 [cf. Cox (1968)]; after being dialyzed against 0.1 M NaCl, the low molecular weight RNA was recovered from the supernatant by ethanol precipitation, dried to remove ethanol, and dissolved in TMK buffer. In some assays (see text), the endogenous ribonuclease activity in the assay was further suppressed with 0.3 mM aurintricarboxylic acid (ATA) (Zaug & Cech, 1980). At this concentration, the ATA does not significantly alter the in vitro release of RNA over a 3-h period, and the transported RNA exhibits higher translational activity.

RNA Extraction and Analysis. Total liver RNA, isolated by homogenization of rat liver in 10 volumes of 4.0 M guanidine thiocyanate, 1.0 M β-mercaptoethanol, and 20 mM sodium acetate, pH 5.0 (Ullrich et al., 1977), was layered over 5.7 M CsCl–50 mM EDTA and centrifuged (131000*g*) at 25 °C for 36 h (Glisin et al., 1974). The RNA precipitate was resuspended in H_2O, then 2 volumes of 8.0 M guanidine-HCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0, were added, and the RNA was reprecipitated with 0.5 volume of ethanol

at −20 °C for 4 h (Cox, 1968). The RNA was reprecipitated twice with ethanol.

In vitro transported RNA was isolated from the postnuclear (10000*g* for 8 min) supernatant as follows. Protein was removed by the pH 9.0 $NaDodSO_4$–phenol extraction procedure (Brawerman, 1974). Contaminating yeast RNA, ATA (when present), and transported 4–5S RNA were removed by precipitation of the larger mRNA and rRNA with 0.5 volume of ethanol (−20 °C) after adjusting the extract to 4.0 M guanidine-HCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.5 (Cox, 1968). Finally, the high molecular weight RNA fraction was further purified to remove a translation inhibitor on urea–sucrose gradients as follows. The ethanol-precipitated RNA, dissolved in 6.0 M urea, 0.5% $NaDodSO_4$, 5.0 mM EDTA, and 10.0 mM Tris-HCl, pH 7.5, was heat denatured (70 °C for 5 min) and then layered over linear 7.5–30% sucrose gradients prepared in 6.0 M urea, 1.0 mM EDTA, 0.5% $NaDodSO_4$, and 10.0 mM Tris-HCl, pH 7.5. The gradients were formed over a 40% sucrose cushion in the same buffer medium and were centrifuged for 24 h at 280000*g* in a Beckman SW41 rotor. Fractions were collected for radioassay in liquid scintillant.

Cell-Free Translation. Cell-free protein synthesis was carried out by using a rabbit reticulocyte lysate pretreated with the calcium-dependent micrococcal nuclease and fortified with mouse Ehrlich ascites pH 5 enzyme (Pelham & Jackson, 1976). Incorporation of [^{35}S]methionine into total acid-insoluble (hot trichloroacetic acid) material during a 1-h incubation at 30 °C was determined by liquid scintillation counting. Samples for $NaDodSO_4$–polyacrylamide gel electrophoresis were precipitated with 5% trichloroacetic acid and then dissolved in 2% $NaDodSO_4$, 25 mM Tris, pH 6.8, 5% glycerol, and 5% β-mercaptoethanol with bromophenol blue included as a marker. The labeled proteins were separated on one-dimensional 11.0% polyacrylamide slab gels (Laemmli, 1970). Gels were fixed with 10% acetic acid–50% methanol and prepared for fluorographic exposure on Kodak XR-2 X-Ray film following the procedure of Bonner & Laskey (1974).

The binding of labeled polyadenylated RNA to reticulocyte ribosomes was determined by analyzing the translation mix on 10–30% sucrose–TMK buffer gradients. For this purpose, labeled RNA was added to a 0.4-mL reticulocyte reaction mix to the optimum level for cell-free protein synthesis; samples were then incubated for 5 min at 30 °C. A control assay containing 5 mg/mL pactamycin, a potent inhibitor of translation initiation (Duncan et al., 1975), was incubated at 0 °C and analyzed. Gradients were centrifuged at 140000*g* for 8 h in a Beckman SW-28 rotor. After centrifugation, the gradients were monitored at 260 nm, and 0.3-mL fractions were collected for radioassay.

Results

Assay Protocol. Rat liver nuclei are prelabeled for either 20 or 30 min in vivo, as indicated. Subsequently, the release of the various types of labeled RNA is monitored in the cell-free transport system, which in addition to prelabeled nuclei contains homologous cytosol protein, spermidine, salts, sucrose, buffers, an RNase inhibitor, cAMP (as indicated), methionine, and GTP. Energy is supplied by ATP which is regenerated by phosphoenolpyruvate, the latter being added each 12 min of incubation at 30 °C. RNA transport is quantitated either directly by removing the nuclei and radioassaying the acid-insoluble RNA or indirectly by purifying the transported RNA before density gradient analysis or cell-free translation. Because high levels of cytosol are required for optimal RNA transport, unless otherwise indicated, the

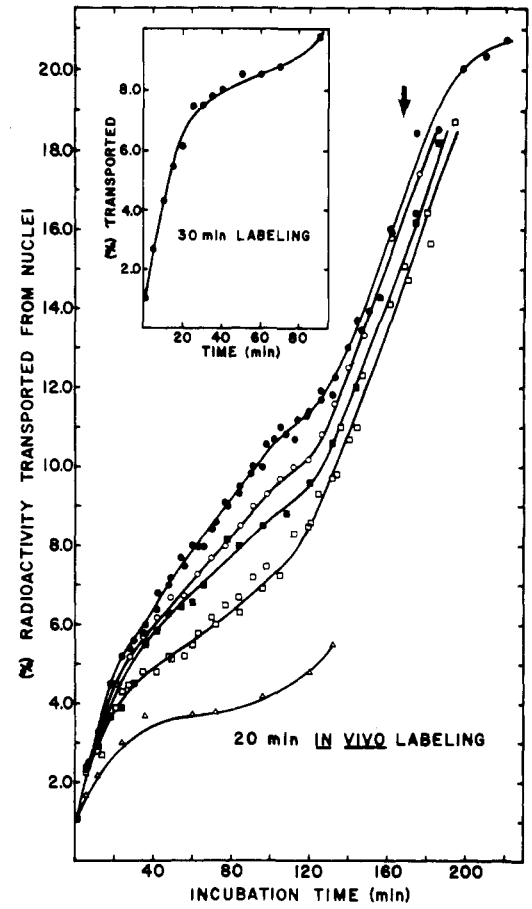

FIGURE 1: Time courses of RNA release from isolated nuclei. Rat liver nuclei were prelabeled for 20 min in vivo with [^{3}H]orotic acid. The in vitro RNA transport was performed at 30 °C. Phosphoenolpyruvate was added every 12 min with the last addition at 160 min (arrow). At each time point, the nuclei were removed from a 0.4-mL aliquot. The amount of transported labeled RNA was then determined and expressed as a percentage of the acid-insoluble radioactivity in nuclei at zero time. The curves do not extrapolate to zero since background counts (radioactivity released under non-transport conditions, 0 °C) were not subtracted. The cell-free assays varied only with respect to the cytosol proteins and the presence or absence of 1.0 μM cAMP as follows: (●) normal cytosol (15 mg of protein/mL) plus cAMP; (○) normal cytosol (15 mg of protein/mL) minus cAMP; (■) basal system reconstituted with streptomycin-precipitated cytosol proteins plus cAMP (the basal system contained 15 mg/mL streptomycin-treated cytosol protein and was reconstituted with 105 μg/mL streptomycin-precipitated proteins); (□) basal system of streptomycin-treated cytosol (15 mg of protein/mL) plus cAMP; (△) inactivated streptomycin-treated cytosol protein (15 mg/mL) plus cAMP (see text). Inset: (●) Labeled RNA transported from nuclei prelabeled in vivo for 30 min with [^{14}C]orotate to normal liver cytosol (15 mg of protein/mL) plus cAMP.

cytosol protein is normally held between 10 and 20 mg/mL assay mix. The nuclei are completely stable under these assay conditions, less than 1% nuclear lysis or DNA release occurring during even prolonged incubation at 30 °C (Schumm & Webb, 1975; Schumm et al., 1979). The amount of labeled RNA transported is expressed as the percentage of zero time acid-insoluble nuclear counts.

Temporal Release of Labeled RNA in Response to Total Cytosol. During a 20-min in vivo labeling with [^{3}H]orotic acid, only the intranuclear RNA precursors are labeled with little or no labeled RNA being transported in vivo from the liver nuclei. However, as shown in Figure 1, upon subsequent incubation of such nuclei in vitro, labeled transfer, messenger, and ribosomal RNAs are processed and released into the cell-free medium in a temporal sequence parallel to that observed for pulse-labeled RNA in vivo [cf. Levis & Penman

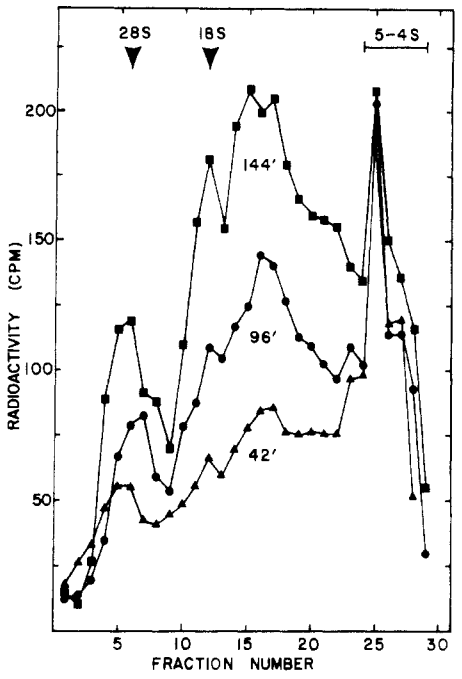

FIGURE 2: Denaturing gradient profiles of labeled RNA transported to normal cytosol plus cAMP. The nuclei were prelabeled in vivo for 20 min with [^{3}H]orotate, and the RNA was transported to normal cytosol (15 mg/mL) plus cAMP (1.0 μM, cf. Figure 1). The transported RNA was purified by phenol at pH 9.0 (see Experimental Procedures), dissolved at 70 °C for 5 min in 6.0 M urea–0.5% Na-DodSO$_4$–5 mM EDTA–10 mM Tris-HCl, pH 7.5, and then sedimented through denaturing gradients of 7.5–30% sucrose (with a 40% sucrose cushion) for 24 h at 280000g. After centrifugation, 0.35-mL gradient samples were radioassayed. The profiles shown are representative of the RNA transported over a period of (▲) 42, (●) 96, and (■) 144 min.

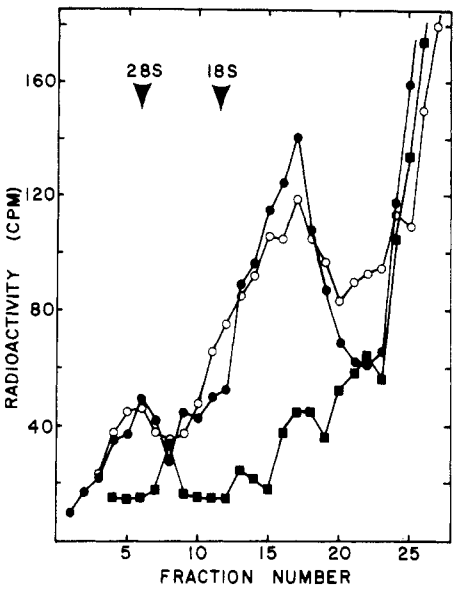

FIGURE 3: Denaturing gradient profiles of labeled RNA transported from 20-min prelabeled nuclei in normal basal and reconstituted systems. Assay conditions were as for Figures 1 and 2 with a uniform sampling time of 96 min from assays comprised as follows: (■) basal system (streptomycin-treated cytosol protein) plus cAMP; (●) basal system reconstituted with precipitated cytosol proteins plus cAMP; (○) normal cytosol (15 mg of protein/mL) without cAMP.

(1977) and Piper et al. (1979)]. The release of labeled RNA to normal, "unfractionated", cytosol (open circles) occurs in three phases over a 180-min period. In parallel to the in vivo situation, labeled tRNA, the nuclear pool of which is quickly saturated with labeled products, is transported predominantly during phase I (0–30 min). During phase II (30–110 min) and phase III (over 110 min), labeled mRNA and then rRNA, the nuclear pools of which become saturated with label more slowly, are transported sequentially in the in vitro system (see below). cAMP stimulates only phase II transport (closed circles).

Figure 2 indicates that by 42 min most of the 4–5S RNA is transported. The heterodisperse material in this 42-min preparation, while derived in part from phase II transport, is also comprised of the 1% zero time 0 °C released RNA. By 96 min and increasingly by 144 min, substantial 8–18S heterodisperse RNA ("putative mRNA") is released from the isolated nuclei without any accompanying release of hnRNA or of rRNA precursors. By 144 min, 18S and 28S rRNA begin accumulating among the transport products, with additional release occurring as long as energy is supplied. In addition to the increased radioactivity appearing in the 8–18S range during phase II transport, an increase in the proportion of polyadenylated RNA occurs between 42 and 96 min, as measured by affinity to oligo(dT)–cellulose. For example, 20% of the total transported RNA is polyadenylated at 42 min, while 35% is polyadenylated at 96 min.

Due to the delayed in vivo saturation of the large nuclear ribosomal pool with labeled products, only after depletion of the "putative mRNA" pool are labeled 18S and 28S rRNAs released in vitro. This rRNA release continues as long as a constant supply of energy is maintained and until the 45S ribosomal precursor pool is depleted (Schumm et al., 1979). However, if the energy supply is terminated at *any* time during the assay by cessation of phosphoenolpyruvate additions (arrow, Figure 1), then transport rapidly ceases. The cessation of transport upon the depletion of energy emphasizes the active-transport nature of continued RNA release and indicates the exceptional stability of the nuclei during these prolonged incubations.

The rate of cell-free RNA transport at 30 °C is approximately 25% of the in vivo 37 °C rate. However, the rate of transport is doubled when the cell-free assay temperature is raised to 35 °C (R. B. Moffett, unpublished experiments).

Temporal Release of Labeled RNA in Response to Isolated Transport Factors. The protein factors essential for phase II mRNA transport are selectively precipitated from the cytosol with 2% streptomycin sulfate. After removal of streptomycin sulfate from the supernatant by precipitation of the residual proteins with ammonium sulfate and dialysis, the streptomycin-treated cytosol supports 4–5S and ribosomal RNA transport, but not messenger RNA transport. Thus, a preferential reduction of phase II mRNA transport (Figure 1, open squares) and the absence of 8–18S heterodisperse material in the transported RNA preparation (Figure 3, solid squares) are observed. Guanidine-HCl–ethanol fractionation (Cox, 1968) of the pH 9.0 phenol-purified RNA indicates that less than one-third of the RNA transported from 30-min prelabeled nuclei during a 40-min incubation in normal cytosol is tRNA; if, however, the nuclei are incubated in streptomycin-treated cytosol, over two-thirds is tRNA. Since the streptomycin-treated cytosol appears to be deficient only in "mRNA" transport factors, assays containing this cytosol at a saturation level of 10 mg/mL (see below) are used subsequently as a basal system for assaying the mRNA transport activity of the various partially purified fractions.

Under appropriate conditions of ammonium sulfate treatment and dialysis, the streptomycin-precipitated transport factors obligatory for phase II transport are recovered in active form. When the reactivated streptomycin-precipitated fraction is added to a basal streptomycin-treated cytosol system, a

Table I: Cell-Free Nucleocytosolic RNA Transport: Reconstitution of Transport Factor Depleted Cytosol and Calculation of the Specific Activity of Purified Protein Fractions

	control systems				reconstituted basal systems[b]							
	basal system[a] (streptomycin-treated cytosol)		standard system (normal cytosol)		cytosol protein, streptomycin precipitated[c]		streptomycin-precipitated protein, DNA affinity chromatography[d]				polysomal mRNP particle proteins[e]	
							unbound		bound			
cAMP (μM)	0.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0
active fraction added[b] (mg of protein/mL)	10.0	10.0	10.0	10.0	0.070	0.070	0.056	0.056	0.014	0.014	0.017	0.017
net RNA transport[f] (units)	2.8 ± 0.4	3.0 ± 0.3	3.6 ± 0.4	5.0 ± 0.5	3.5 ± 0.3	4.2 ± 0.5	3.2	3.6	3.9	4.3	4.0 ± 0.5	4.2
enhancement over basal system (Δ units)		0.2	0.8	2.2	0.7	1.4	0.4	0.8	1.1	1.5	1.2	1.4
sp act. (Δ units/mg of protein)		0.020	0.080	0.22	10	20	7.1	14	77	110	71	83
total enhanced act.[g] (Δ units/g of liver)		0.96	3.8	11	3.4	6.7	1.9	3.8	5.2	7.4	6.8	7.4
sp act. rel to standard system[h]			1.0	2.7	125	250	89	179	962	1340	893	1040

[a] Rat liver cytosol was treated for 20 min with 2.0% streptomycin sulfate. The resultant precipitate was removed, and the supernatant proteins were precipitated with 65% ammonium sulfate and then dialyzed against TMK buffer to remove streptomycin sulfate. [b] The reconstituted system contained 10 mg/mL streptomycin-treated cytosol plus the active (purified) fraction derived from an equivalent amount of normal cytosol. Purified informosomal proteins were added to approximately the same concentration as the DNA-bound proteins. [c] Streptomycin-precipitated proteins (see *a* above) were reactivated by resuspending the precipitate in 65% ammonium sulfate and dialyzing against 0.075 M NaCl-TMK buffer to remove streptomycin sulfate. [d] Soluble, dialyzed, streptomycin-precipitated proteins were bound to DNA-cellulose in 0.075 M NaCl-TMK buffer and eluted with 2.0 M NaCl-TMK buffer. Both the flow-through and bound fractions were dialyzed against 0.075 M NaCl-TMK buffer. [e] EDTA-dissociated polysomes (0.02 M EDTA) were bound to oligo(dT)-cellulose in 0.25 M NaCl-TMK buffer. Informosomal proteins were eluted with 2.0 M NaCl-TMK buffer, and dialyzed against 0.075 M NaCl-TMK buffer. [f] Net transport = percentage of nuclear radioactivity released minus background (0 °C, zero-time contamination was approximately 1%). One unit = 1% net transport from a standard 30 °C, 40-min cell-free incubation, with nuclei prelabeled for 30 min in vivo. Data presented are based upon two or more independent experiments, sampled in triplicate. [g] Δ units of transport activity recovered from 1.0 g of fresh liver. Approximately 48 mg of cytosol protein and approximately 89 μg of mRNP particle protein [from 31 μg of polysomal poly(A^+) mRNA] are recovered from 1.0 g of fresh liver. [h] Specific activity relative to the standard (normal) assay without cAMP.

"normal" phase II transport is restored (Figure 1, solid squares), and 8–18S heterodisperse RNA (Figure 3, open circles) is once again transported. Both the temporal release (Figure 1) and the size distribution (Figure 3) of the RNA released in a reconstituted system compare favorably with those in a normal assay.

It should be emphasized that a three-phase transport is observed only with the brief (20 min) in vivo labeling, which does not allow saturation of nuclear mRNA and rRNA pools with labeled precursors. In this instance, unlabeled mRNA and rRNA are released during the incubations prior to their appearance as radioactive products. Indeed, if the in vivo labeling period is lengthened to 30 min, then tRNA and mRNA appear in the single combined phase of transport, with rRNA appearing after 60–70 min (Figure 1, inset). The percentage of radioactivity released as mRNA from 30-min prelabeled nuclei in vitro is consistent with the in vivo levels measured by Samal & Bekhor (1979). Ribosomal RNA transport is observed from the onset of incubation, if nuclei are prelabeled in vivo for 120 min (Schumm et al., 1979). A short in vivo labeling time of 20 min or less thus provides a means of differentiating between the transport of unlabeled, previously processed mRNAs during the initial phases of a cell-free transport and the transport of labeled mRNA precursors undergoing both processing and transport.

While the transported RNA appears relatively stable, i.e., little visible reduction in size occurs following transport (Figure 2, 96 and 144 min) for as long as 60 min, it is unlikely that all of the mRNA molecules transported near the beginning of an assay remain completely unmodified during an entire 180-min incubation. Therefore, to obtain translationally active mRNA, only that fraction of mRNA transported during the initial 40 min of a cell-free assay is isolated and studied in subsequent relevant experiments.

cAMP Increases the Effective Concentration of the Transport Factors. In assays which use 30-min prelabeled nuclei, a substantial fraction of labeled "mRNA" is released together with labeled tRNA to normal cytosol during the first 40 min of assay (Figure 1, inset); neither labeled rRNA, labeled hnRNA, or rRNA precursors, however, are released (Schumm & Webb, 1978). Figure 4 demonstrates that the dependency for cytosol protein of labeled RNA transport from 30-min prelabeled nuclei is *reduced* 50% by cAMP without altering the maximum level of transport. Thus, a doubling in activity of the mRNA transport factors is achieved, presumably via cAMP-dependent protein kinase induced phosphorylation. The increased level of transport in optimum (high) cytosol protein or with cAMP, as compared to suboptimum (low) cytosol protein or without cAMP, is due to differences in the rate and not the duration of transport. This holds for both 20- and 30-min prelabeled nuclei; in both cases, there is a linear mRNA transport with a fairly abrupt termination (Figure 1; solid circles vs. open circles). As may be seen from the concentration-dependency curve (Figure 4) of streptomycin-treated cytosol, both the mRNA transport factors as well as the cAMP stimulatory response are effectively removed by streptomycin treatment. In addition, the biphasic nature of the streptomycin-treated cytosol dependency curve indicates that, at least under this assay condition, transport of "tRNA" is also cytosol protein concentration dependent below 6–8 mg/mL. The rRNA transport factors have been described previously (Schumm et al., 1979).

Messenger RNA Transport Factors Are a Unique Class of Cytosol Proteins. Table I summarizes the purification of the transport factors and the response of each fraction to the addition of cAMP. With streptomycin-treated cytosol as a basal system, sufficient purified fraction is added to this transport factor depleted system to reconstitute a 10 mg/mL normal assay. Increasing the streptomycin-treated cytosol to 20 or 30 mg/mL does not increase the level of labeled RNA

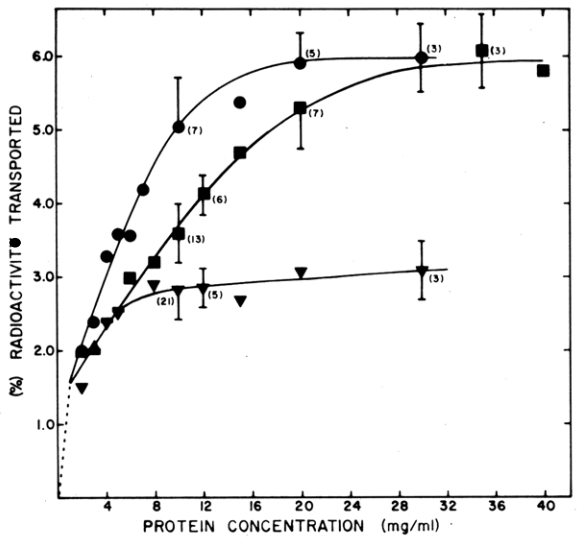

FIGURE 4: Transport protein dependency of labeled RNA transported from 30-min prelabeled nuclei. The labeling conditions were the same as those for Figure 1, inset. The data represent the average of two or more independent experiments, sampled in triplicate, with the percent nuclear counts transported as RNA to (▼) streptomycin-treated cytosol without cAMP, (■) normal cytosol without cAMP, and (●) normal cytosol with 1.0 μM cAMP.

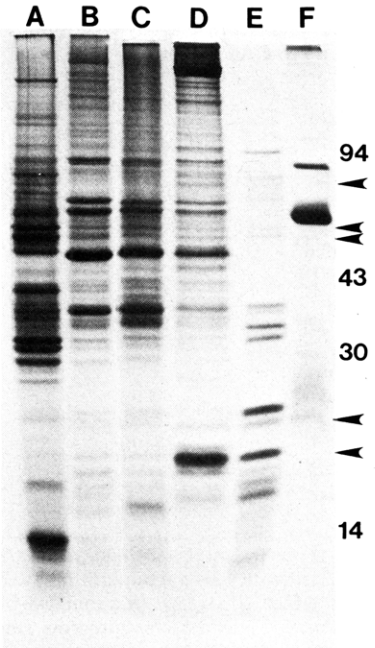

FIGURE 5: $NaDodSO_4$–polyacrylamide slab gel electrophoretogram of cytosol fractions exhibiting RNA transport activity. Approximately 75–100 μg of each protein fraction was separated in 11% acrylamide–0.1% $NaDodSO_4$ slab gels and then stained with Coomassie blue. (A) Total cytosol; (B) streptomycin precipitate fraction from total cytosol; (C) DNA column flow through of streptomycin precipitate (unbound in 75 mM NaCl–TMK buffer); (D) DNA column-bound fraction of streptomycin precipitate (bound in 75 mM NaCl–TMK buffer and eluted with 2.0 M NaCl–TMK buffer); (E) informosomal proteins derived from low salt isolated polysomal mRNP particles bound to oligo(dT)–cellulose in 0.225 M NaCl–EDTA–TMK buffer (proteins eluted with 2.0 M salt–TMK buffer); (F) standard proteins: albumin, 68 000; heavy chain IgG, 85 000; light chain IgG, 23 000–25 000 daltons. Additional molecular weight standards were run separately. Arrows indicate peptides found in both the (D) and (E) fractions.

transport when purified fractions are added. On the other hand, adding increasing amounts of the purified fraction in order to reconstitute a 15 mg/mL assay (by adding 21–24 μg/mL DNA-bound or informosome proteins) does proportionately increase mRNA transport. During purification, there is some loss of activity, particularly with regard to the cAMP-enhanced fraction, where a 30–40% reduction is observed. The enrichment for mRNA transport activity is indicated by a comparison of the specific activities of the reconstituted and standard systems. The streptomycin precipitate, representing only 0.7% of the total cytosol protein, exhibits a relative specific activity of 250 with and 125 without cAMP. Approximately 20% of the solubilized streptomycin-precipitated protein binds to DNA–cellulose in 75 mM NaCl–TMK buffer and is eluted with 2.0 M NaCl–TMK buffer. The DNA-bound fraction exhibits the highest specific activity, i.e., a 1340-fold enhancement with and a 962-fold enhancement without cAMP.

Polysomal mRNP particle proteins were found to be an alternative rich source of the transport factors. In order to effectively remove contaminating proteins, the liver polysomes pretreated with 1.0% Nonidet P40 were isolated by centrifugation through 2.0 M sucrose in low salt (TMK buffer). For the release of mRNP from the ribosomes and for the removal of loosely bound proteins, the polysome pellet was resuspended in 0.02 M EDTA and 0.25 M NaCl–TMK buffer, and the mRNP particles were bound to oligo(dT)–cellulose. After being extensively washed with 0.25 M NaCl–TMK buffer, proteins elutable with 2.0 M NaCl–TMK buffer were recovered. This protein fraction was tested for cell-free mRNA transport activity in a basal system containing streptomycin-treated cytosol (Table I). Upon addition of mRNP particle associated proteins, a net increase in transport of labeled RNA is observed, yielding final relative specific activities of 1040 and 893, with and without cAMP, respectively. Although there appears to be a loss of the cAMP enhancement with purification, the relative specific activities not only indicate that mRNA transport factors can be purified at least 1000-fold over normal cytosol but also emphasize that they represent only a very minor fraction of the total cytosol proteins. Finally, it is significant that over one-half (i.e., 6.8 units/g of liver) of the total mRNA transport activity in the liver is recovered in the mRNP particles (cf. Table I) despite significant losses of the polysomes at several steps in their preparation. Thus, both the cytosol and mRNP particles represent significant pools of the factors.

Electrophoretic Analysis of the Active Protein Fractions. The purified active fractions were analyzed by one-dimensional $NaDodSO_4$–polyacrylamide electrophoresis. The Coomassie blue stained profiles (Figure 5) show a substantial enrichment of a number of peptides over total cytosol. The patterns obtained exhibit several major bands as well as numerous minor components. Interestingly, both the DNA-bound cytosolic and mRNP particle fractions each contain a major 20 000 molecular weight protein (arrow, Figure 5). Further, several minor peptides, one of 23 000 daltons and others approximately the size of the poly(A)-binding proteins (Kumar & Pederson, 1975; Barrieux et al., 1976; Cardelli & Pitot, 1977; van Venrooij et al., 1977), are present in both fractions; these latter peptides are 52 000, 54 000, and 72 000 daltons. Proof of identity of common proteins in the cytosol and mRNP fractions will require testing of their cross-reactivity to specific antibodies.

Physiochemical Properties and Translatability of Transported Messenger RNA. Of critical importance to the demonstration of in vivo equivalence is proof that the transported products are functional. For determination of this, labeled messenger RNA, which had been transported into normal cytosol with cAMP during a 40-min incubation, was isolated

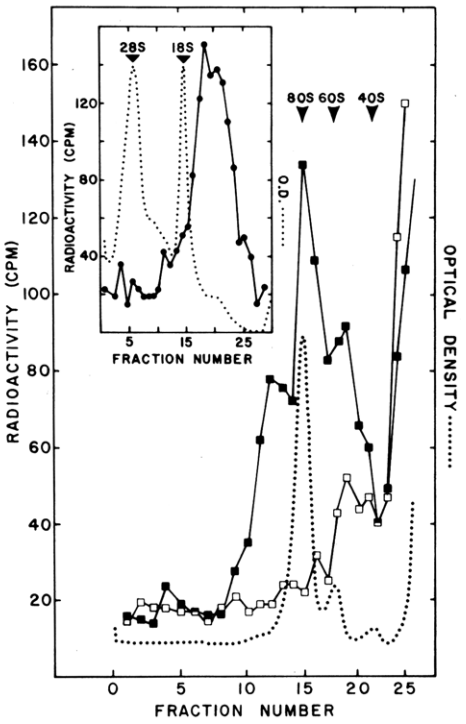

FIGURE 6: Incorporation of radioactive transported polyadenylated RNA into reticulocyte monosomes. Liver nuclei were prelabeled for 30 min in vivo with [^{3}H]orotic acid, and the RNA was transported in a cell-free assay containing 20 mg/mL normal cytosol plus 1.0 mM cAMP incubated at 30 °C for 40 min. Transported ^{3}H-labeled mRNA was purified by phenol–pH 9.0 extraction and oligo(dT)–cellulose affinity chromatography. The reticulocyte lysate cell-free translation system was incubated at either 0 or 30 °C for 7 min, and 0.4 mL was layered over a 37-mL 10–30% sucrose–TMK gradient; centrifugation was at 145000*g* for 8 h. Fractions were collected and counted directly. (□) Control assay (0 °C) containing 5 μg/mL pactamycin; (■) experimental assay incubated for 7 min at 30 °C; (···) optical density. Inset: Denaturing gradient profile of purified RNA (see Figure 2): (●) counts per minute; (···) optical density.

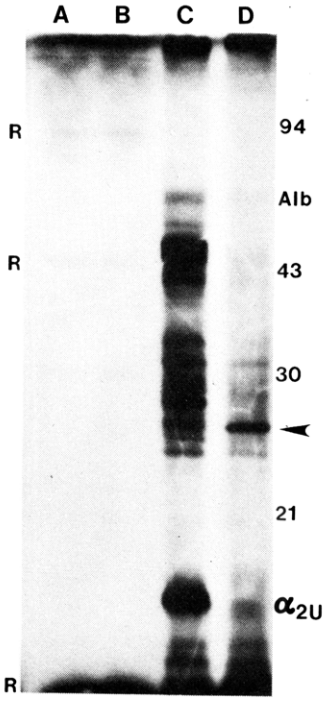

FIGURE 7: Fluorograms of translation products of transported mRNA separated by one-dimensional NaDodSO$_4$–polyacrylamide slab gel electrophoresis. Cell-free RNA transport was performed in normal cytosol (20 mg/mL) plus 1.0 μM cAMP or in streptomycin-treated cytosol (10 mg/mL) plus 1.0 μM cAMP at 30 °C for 40 min. Transported RNA was isolated by pH 9.0 NaDodSO$_4$–urea–sucrose gradient separation. Total rat liver RNA was isolated by guanidine thiocyanate–CsCl gradient centrifugation followed by guanidine-HCl extraction. Transported RNA at the optimal concentration of 18 μg/100 μL was added to a reticulocyte lysate cell-free translation system containing [^{35}S]methionine. Translation products were separated on 11% acrylamide gels containing 0.1% NaDodSO$_4$. Fluorographs were obtained upon a 3-week exposure of X-ray film. (A) Minus RNA control; (B) RNA transported to streptomycin-treated cytosol plus cAMP; (C) RNA isolated from total liver; (D) RNA transported to normal cytosol plus cAMP. R indicates endogenous reticulocytes background peptides. Arrow indicates peptide product of abundant transported mRNA. Standard proteins (LOW-SDS-PAGE, Bio-Rad), for determination of molecular weight, were separated in a run in parallel.

from liver nuclei prelabeled for 30 min, purified by pH 9.0 NaDodSO$_4$–phenol extraction, and then fractionated on oligo(dT)–cellulose. Approximately 40% of the labeled RNA bound to oligo(dT)–cellulose, i.e., was polyadenylated. As shown in the inset in Figure 6, the labeled polyadenylated transported mRNA is heterodisperse with a wide size distribution 8–18 S). Upon addition of this labeled polyadenylated RNA to a reticulocyte lysate cell-free translation system, the labeled RNA forms initiation complexes with ribosomes (Figure 6). Control incubations with pactamycin do not form initiation complexes (Figure 6).

Meaningful determinations of the mass of mRNA released in the cell-free system cannot be made directly due to the presence in the pH 9.0 NaDodSO$_4$–phenol guanidine-HCl purified RNA of transported rRNA and some contaminating yeast RNA. However, based upon the cell-free translation capacity of the transported RNA (Table II), 1.7 μg of mRNA is transported in a 10-mL transport assay during a 1-h incubation (i.e., after one-half of the phase II, presumptive mRNA, has been transported). This amount of transported mRNA (0.85% of the nuclear RNA) correlates well with the estimates on liver and HeLa cells (Sippel et al., 1977; Samal & Bekhor, 1979), which indicate that 1–2% of the total nuclear RNA is mRNA and mRNA precursors. As isolated by the phenol–guanidine-HCl procedures, the transported RNA has a contaminant which interferes somewhat with translation of the mRNA. Thus, before translation, the transported RNA is further purified by sedimentation through NaDodSO$_4$–urea–sucrose gradients. This procedure removes the inhibitor so that a linear increase occurs in the amount of [^{35}S]-methionine incorporated in response to the exogenous RNA up to 18 μg/100 μL of reticulocyte lysate. Messenger RNA preparations with good translational activity were recovered only from assays exhibiting substantial phase II transport (Table II). The RNA recovered from assays reconstituted with streptomycin-treated cytosol exhibited only 20% of the translational capacity of RNA transported in assays reconstituted with normal cytosol. The translational activity present in extracts of dialyzed cytosol or in the streptomycin precipitate of the cytosol was only 5–10% of the translational activity of the transported RNA.

Although as much as one-half of total liver mRNA is deficient in poly(A) (Grady et al., 1978; Moffett & Doyle, 1981), binding to oligo(dT)–cellulose was used to quantitate the release of poly(A$^+$) RNA (Table II). Transfer RNA was removed prior to oligo(dT)–cellulose chromatography by guanidine-HCl extraction. Under the transport conditions employed, i.e., 30-min prelabeling and 40-min transport, labeled rRNA is not released. Of the labeled RNA transported in the basal and standard systems, approximately 35% and 50% bound, respectively. As seen from the percentage of nuclear label which bound, poly(A$^+$) RNA is only transported from

Table II: Quantitation of mRNA Transported in Vitro

method of mRNA estimation	extracted RNA / total liver mRNA	RNA transported to cell-free media[a] cAMP (μM)	basal system (streptomycin-treated cytosol)	standard system (normal cytosol)	reconstituted system (basal + streptomycin precipitate)
labeled poly(A^+) RNA transported[b] (% nuclear cpm)		0.0 1.0	0.3 (0.12)[c]	1.0 1.4	1.0 1.3
stimulation of translation ([^{35}S]Met incorpn)[d]					
net stimulation (cpm/μL of assay)	36700	1.0	1400 (300)[c]	7400	5100
direct estimate of mRNA concn (ng of mRNA/μL of assay)[e]	8.0				
calcd mRNA transported (ng/mL of transport assay)[f]		1.0	332 (71)[c]	1750	1210
% total nuclear RNA (180 μg/10 mL) transported as mRNA		1.0	0.18 (0.04)[c]	0.97	0.67

[a] Final protein concentration in the cell-free system was 20 mg/mL (maximum transport in the presence of cAMP). Values are based upon a 45-min transport. [b] Nuclei were labeled in vivo for 30 min. Due to differences in the rates of nuclear pool saturation, variations in the labeling time result in variations in the percentage of label found in poly(A^+) RNA. [c] Values in parentheses represent the transport activity of streptomycin-treated cytosol after a 60-min exposure to streptomycin and indicate that much of the residual transport activity is lost. [d] Micrococcal nuclease treated reticulocyte lysate. [e] Based upon the absorbance of total liver RNA and the estimate that 5% of this RNA is mRNA. [f] Calculations were based on a stimulated incorporation of 4588 cpm/μg of total liver mRNA in 1.0-μL assay. Approximately 2.3% of the RNA recovered from a 10-mL transport was added to 25 μL of translation assay.

nuclei incubated in normal or reconstituted cytosol.

Fluorographic analysis of the translation products resolved by one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis confirms (Figure 7) that a number of normal liver mRNA species are released from nuclei to normal cytosol. No detectable liver products are visible in a comparable profile of the proteins synthesized in response to residual RNA released to the streptomycin-treated cytosol. Among the transported mRNAs are those coding for the precursors of albumin and α_{2U}-globulin, peptides which may be identified in total liver RNA preparations by their abundance and molecular weight. Although numerous liver mRNAs are present among the nuclear transport products, the apparent relative abundance of total liver and in vitro transported messengers is different; for example, there is an apparent underrepresentation of the abundant (albumin and α_{2U}-globulin) mRNAs. This discrepancy is consistent with the results of in vivo studies which indicate that abundant cytoplasmic mRNAs are not abundant in the nucleus (Herman et al., 1976; Sippel et al., 1977; Samal & Bekhor, 1979; Siegal et al., 1979).

Discussion

In view of the accumulating evidence for a secondary regulation of gene expression at the posttranscriptional nuclear level (Darnell, 1978; Darnell et al., 1973; Harpold et al., 1979; Lewin, 1975a, b), the development of a reliable cell-free nucleocytoplasmic mRNA processing and transport system is desirable. In modified form, the cytosol and energy-dependent cell-free system described in this report is the only system which supports the processing and transport of translationally active ribosomal subunits (Schumm et al., 1979). This system, as presently described, supports the transport of translationally active mRNA. Furthermore, this transport occurs in the absence of lytic release of DNA (Schumm & Webb, 1975), rRNA precursors (Yannarell et al., 1977), or, as demonstrated by its absence in transported populations (Figures 2 and 3), of hnRNA from the nuclei. "Nicking" of transported RNA occurs during extended incubations, resulting in mRNA isolated from lengthy (2–3 h) incubations being degraded (R. B. Moffett, unpublished experiments). However, background and quickly released mRNA are proportionately greater in short incubations. Therefore, the 40-min transports used for cell-free translation and poly(A^+) RNA determinations represent a compromise between transport and degradation, with the mRNA recovered probably representing only one-half of the total.

Three experiments demonstrate the in vivo equivalence of the cell-free system, including the intranuclear origin of the transported mRNA. First, translationally active mRNA is not transported to mRNA transport factor depleted cytosol. Second, labeled mRNA from pulse-labeled nuclei initiates protein synthesis in the reticulocyte cell-free system. Third, while many translation products synthesized in response to the transported mRNA are identical with those synthesized in response to total liver mRNA templates, for example, the precursors of albumin and α_{2U}-globulin, the relative abundancies of the transported mRNAs are different from those of total liver mRNAs. Although such differences are predicted from the results of comparable studies on the nuclear and cytoplasmic mRNA pools in rat liver (Sippel et al., 1977), quantitative analyses of the results from the cell-free system would be premature unless the influence of differential degradation and/or translation of the transported messenger is determined.

The estimate that as much as 50% of the labeled RNA larger than 4–5 S transported from 30-min prelabeled nuclei

during a 40-min incubation in vitro is poly(A^+) RNA is also consistent with in vivo data. For example, employing the single-copy DNA hybridization technique, Grady et al. (1978) determined that approximately 40% of mouse liver polysomal mRNA species are poly(A) deficient; i.e., the poly(A) tracts are not sufficiently long to bind to oligo(dT)–cellulose. Similarly, Moffett & Doyle (1981) showed by cell-free translation that only 50% of the translationally active mouse liver mRNA binds to oligo(dT)–cellulose. The cell-free system may be useful in evaluating the origin of the poly(A^+) mRNA and poly(A)-deficient mRNA.

The extent of mRNA transport in vitro depends upon the effective concentration of transport factors, which in turn partially depends upon the presence of cAMP. In fact, the concentration dependency provides evidence supporting the hypothesis of Yannarell et al. (1976) that a number of rate-limiting factors are involved, each reaching its saturation level at a different protein concentration. Further, the kinetics suggest that the factors interact with the mRNA in a quantitative, as opposed to a catalytic, manner, since at subsaturating concentrations less mRNA is transported even at prolonged incubation periods. This is further emphasized by the fact that the initiation and termination of phase II transport appear to be independent of the concentration of transport factors and the absence or presence of cAMP.

The reconstituted cell-free system will allow both the kinetic analysis and study of the mechanisms underlying both mRNA processing and transport. The differential rates of labeling of tRNA, mRNA, and rRNA observed in vivo (Brandhorst & McConkey, 1975; Levis & Penman, 1977), which are due to differences in pool sizes and rates of synthesis and processing, predict a phased release of labeled tRNA, mRNA, and rRNA from nuclei prelabeled in vivo for periods insufficient to saturate the mRNA and rRNA precursor pools. As predicted, a triphasic release of labeled RNA was observed.

The mRNA transport activity has been shown to reside in a minor "subset" of cytosol proteins. As little as 14 μg of purified mRNA transport factor added to a 1.0-mL assay (containing 10 mg of factor-depleted cytosol protein) is capable of eliciting normal mRNA transport. These mRNA transport proteins have been purified 1000-fold with respect to transport activity. Proteins with similar transport activity have been purified from the polysomal-derived mRNP particles. Without being corrected for polysome loss during purification, which may be as high as 50% (Blobel & Potter, 1967), at least 50% of the mRNA transport activity can be isolated in high purity from the 0.25 M salt-washed mRNP particles derived from the detergent-treated, step gradient purified polysomes. Some of the common proteins purified from the cytosol and from the mRNP particle fractions have molecular weights resembling the poly(A)-binding proteins (Kumar & Pederson, 1975; Barrieux et al., 1976; Cardelli & Pitot, 1977; van Venrooij et al., 1977). However, poly(A) polymerase is not concentrated in the active DNA bound fraction (R. B. Moffett, unpublished experiments), indicating that if it is involved in transport it is not rate limiting in the basal transport system. While the site(s) of action of these transport proteins as yet is (are) unknown, it is interesting to note that in the case of induced mRNA transport by tryptophan in rat liver (Murty et al., 1979) regulatory proteins specific for these RNA sequences are also induced.

Streptomycin precipitation not only provides a rapid effective first step in purification but also provides a mRNA transport factor depleted cytosol as a control. Although streptomycin (two guanidino groups) is more effective in precipitating transport proteins, other aminoglycosides, such as neomycin (six primary amino groups), kanamycin (four primary amino groups), and the basic peptide protamine (22 guanidino groups), also are effective agents (D. E. Schumm and T. E. Webb, unpublished experiments). Besides transport proteins, other regulatory proteins, notably the steroid receptor proteins, interact with protamine sulfate (Zava et al., 1976). As a class, such proteins must carry a relatively strong negative charge which provides an indication that they may be phosphoproteins. The increase in the effective concentration of the transport proteins by cAMP suggests that at least some are phosphoproteins under the regulation of a cAMP-dependent protein kinase and that their activity is governed by their level of phosphorylation. It is of interest that the affinity of proteins for nucleic acids is modified by phosphorylation (Gallinaro-Matringe et al., 1975). Also of relevance is the identification of cAMP-binding proteins and protein kinase activity in mRNA (Bag & Sells, 1979; Schweiger & Schmidt, 1974; Obrig et al., 1978; Quirin-Stricker & Schmitt, 1977) and in hnRNP (Blanchard et al., 1977, 1978) particles. It is thus evident that in addition to the nuclear pore nucleoside triphosphatase which has been implicated in the transport of ribonucleoproteins (Agutter et al., 1976) there appears to be additional levels at which ATP acts to stimulate mRNA transport. The transport proteins are most certainly synthesized in the cytoplasm and interact in some manner with the nucleus or with the nuclear RNA. The fact that the nuclei are isolated in hypertonic sucrose, a condition known to assure retention of certain nuclear proteins lost under hypotonic conditions [cf. Rose et al. (1976)] suggests, but does not prove, that the nuclear concentration of these proteins is limiting in vivo. Besides binding to DNA as reported in this study, the transport proteins also bind to mRNA–oligo(dT)–cellulose columns, but not to oligo(dT)–cellulose (R. B. Moffett, unpublished experiments). If future studies can establish unequivocal identity between the cytosolic proteins and the proteins strongly bound to the nucleic acids which exhibit mRNA transport activity, this will constitute strong evidence that they are an integral part of the mRNP particle.

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