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METHOD

Development of a tRNA-dependent in vitro translation system

RICHARD J. JACKSON,[1] SAWSAN NAPTHINE,[2] and IAN BRIERLEY[2]

[1]Department of Biochemistry, University of Cambridge, Old Addenbrookes Site, Cambridge CB2 1GA, United Kingdom
[2]Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom

ABSTRACT

A method is described for depleting rabbit reticulocyte lysates and wheat germ extracts of endogenous tRNAs by affinity chromatography using a matrix generated by coupling ethanolamine to epoxy-activated Sepharose 6B. Greater than 90% depletion of tRNA is achieved with the result that translation becomes in effect absolutely dependent on added tRNA. This depletion procedure should prove very useful for studying the influence of tRNA concentration, and the spectrum of the tRNA population, on recoding events such as programmed frameshifting and readthrough of termination codons.

Keywords: leaky termination; programmed ribosomal frame-shifting; rabbit reticulocyte lysate; wheat germ system

INTRODUCTION

The accuracy of decoding mRNA and the efficiency of various "programmed errors" in translation, such as programmed frameshifting, are believed to be influenced by the relative abundance of different tRNA species in the total tRNA population. Attempts to test this proposition by manipulating the tRNA population distribution of cell-free mRNA translation extracts are somewhat limited by the pool of endogenous tRNAs in such extracts, which restricts the degree to which the population can be skewed simply by addition of supplementary tRNAs. The degree of freedom for manipulation of the tRNA population would obviously be much greater if such cell-free extracts could be depleted of their endogenous tRNAs. The current perception is that there is no simple method available for achieving such depletion. Methods for the depletion of Krebs II ascites cell extracts (Le Meur et al., 1976) and wheat germ extracts (Pfitzinger et al., 1989) have been described, but they involve rather cumbersome procedures that have not been shown to have general applicability.

We have fortuitously discovered a very simple one-step method of depleting eukaryotic cell-free mRNA translation systems of tRNA with such a high efficiency of depletion that the systems become effectively tRNA dependent. This serendipitous discovery arose out of attempts to define why sustained high rates of translation initiation in rabbit reticulocyte lysates required the presence of glucose 6-phosphate, not only as a means of generating NADPH, but also as a cofactor per se, as shown by the fact that glucose 6-phosphate was still required even if NADPH was generated by another route, such as via cytoplasmic isocitrate dehydrogenase (Hunt et al., 1983; Jackson et al., 1983a, 1983b). To explore this cofactor role we passed reticulocyte lysate through an affinity column prepared by coupling glucose 6-phosphate to epoxy-activated Sepharose, with the hope that the flow-through lysate would exhibit the same properties as a gel-filtered lysate lacking sugar phosphates but containing a suitable reducing system (either dithiothreitol or isocitrate/isocitrate dehydrogenase/NADP), namely a high rate of translation for some 15–20 min, followed by a shut off due to inefficient (re)initiation (Hunt et al., 1983; Jackson et al., 1983a, 1983b). In fact the flow-through lysate was highly restricted in translation from the very start of the incubation, and polysome analysis showed that this was due to inhibition of elongation (or termination) rather than to defective initiation. Elution of the column with a KCl gradient yielded fractions capable of rescuing this deficiency. The rescue activity eluted after most of the proteins and was coincident with a peak of material absorbing at 260 nm with a high $A_{260/280}$ ratio. The

Reprint requests to: Richard J. Jackson, Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Old Addenbrookes Site, Cambridge CB2 1GA, UK; e-mail: rjj@mole.bio.cam.ac.uk.

obvious conclusion was that the column might have depleted the lysate of tRNA, and this was confirmed when direct assay showed a depletion of greater than 90% of the tRNA present in the parent lysate, and supplementation of the flow-through lysate with bona fide tRNA was found to effect an almost complete rescue of translation activity.

Because there was no a priori reason why the glucose 6-phosphate moieties covalently bound to the resin should remove tRNA with such extraordinary efficiency, we reasoned that the depletion was more likely to be due to the chemical groups linked to the resin in the final blocking step, which involves reaction of the resin with ethanolamine. Accordingly we took fresh epoxy-activated Sepharose and incubated it directly with the blocking reagent, ethanolamine, and found, not surprisingly with hindsight, that this "affinity matrix" was just as effective in depleting reticulocyte lysates of tRNA. It is this ethanolamine-Sepharose that is the basis of our procedure for preparation of tRNA-dependent cell-free translation extracts.

RESULTS

Development of a protocol for the preparation of tRNA-depleted reticulocyte lysates

The protocol for depleting reticulocytes of tRNA was worked out using native lysate (not treated with micrococcal nuclease) prepared in-house. Lysates prepared in this way are typically 25–40 mM in K^+, 0.5 mM in ATP, and 0.1 mM in GTP (Jackson et al., 1983a). They are routinely supplemented with 20 μM hemin before use. If the assay mix is 60–80% (by volume) lysate, it is not necessary to add additional ATP and GTP; it suffices to add 10 mM creatine phosphate plus some creatine kinase to regenerate ATP (and indirectly regenerate GTP). It is also necessary to supplement the assay with 100 mM KCl and 0.5 mM $MgCl_2$ (Jackson & Hunt, 1983). For the depletion trials, a portion of this additional 100 mM KCl and 0.5 mM $MgCl_2$ was added to the lysate, which had already been supplemented with hemin. The columns were equilibrated with Buffer A (25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM HEPES-KOH, pH 7.4) supplemented with the same additional KCl and $MgCl_2$; for example, if the lysate had been supplemented with 50 mM KCl and 0.25 mM $MgCl_2$, then the equilibration was with Buffer A containing 50 mM additional KCl and 0.5 mM additional $MgCl_2$. (The ionic composition of Buffer A is designed to mimic that of the lysate itself.) In the trial runs, a column of bed volume 1.2–1.5 mL was loaded with 2.7 mL of lysate at a flow rate of 10 mL per h, with all operations carried out at 2–4 °C. The column was subsequently washed with equilibration buffer, and then, before the next use, with Buffer A supplemented with 0.5 M KCl. Fractions of about 0.42 mL were collected, and after addition of any missing KCl and $MgCl_2$, the fractions were assayed for translation activity in the presence or absence of added calf-liver tRNA (50 μg/mL).

When a trial of this type was conducted with lysate supplemented with 100 mM KCl and 0.5 mM $MgCl_2$, the activity of the flow-through lysate exhibited fairly high dependence on tRNA, as judged by the ratio of incorporation with and without added tRNA after 60 min incubation, and if tRNA was added, the flow-through lysate recovered 80% of the activity (again assayed over 60 min) of the parent lysate (Fig. 1A). On the other hand, if no KCl or $MgCl_2$ was added to the lysate prior to loading on the column, then the activity of the flow-through lysate in the absence of added tRNA was even lower, but the recovery on tRNA addition was also lowered, typically to 25–33% of the parent lysate activity (data not shown). It seems that at 100 mM KCl, the column is somewhat leaky in retention of tRNA, while at very low salt, tRNA retention is very efficient but other components essential for protein synthesis must also be absorbed to a certain extent.

A good compromise seemed to be supplementation of the lysate with 50 mM KCl and 0.25 mM $MgCl_2$ prior to loading on the column (Fig. 1B). This gave up to 75% recovery of activity (provided tRNA was added), and the tRNA dependence was greater than in Figure 1A (100 mM KCl). Figure 2 shows the concentration dependence with respect to tRNA of lysate depleted under these conditions: half-maximal recovery of activity was seen at a concentration of added tRNA of less than 10 μg/mL, and maximal recovery at 20–25 μg/mL, with no significant difference between commercial calf-liver tRNA and reticulocyte tRNA prepared in-house.

For routine purposes we used a 1.5 mL column, loaded with 5 mL of lysate supplemented with 50 mM KCl and 0.5 mM $MgCl_2$, and operated at the same flow rate and temperature as in the trials. The first ~1.2 mL of lysate to emerge from the column and the last ~0.8 mL were discarded, because these fractions often have low activity. The problem with the trailing edge fractions is likely to be one of dilution, while at the leading edge, in addition to dilution, there may be complications due to gel-filtration effects of the column, or to the fact that Sepharose and Sephadex matrices tend to absorb ribosomes (Jackson & Hunt, 1983; Jackson et al., 1983a). The central ~3 mL of flow-through were routinely pooled, aliquotted and snap-frozen in liquid nitrogen. These tRNA-depleted lysates do seem rather more fragile than the parent lysate, and so frequent thawing and refreezing of these aliquots is not recommended. We do not know the capacity limit of such columns. We have applied 10 mL of lysate to a 1.5-mL bed volume column and found no indication that the capacity had been saturated: the later flow-through frac-

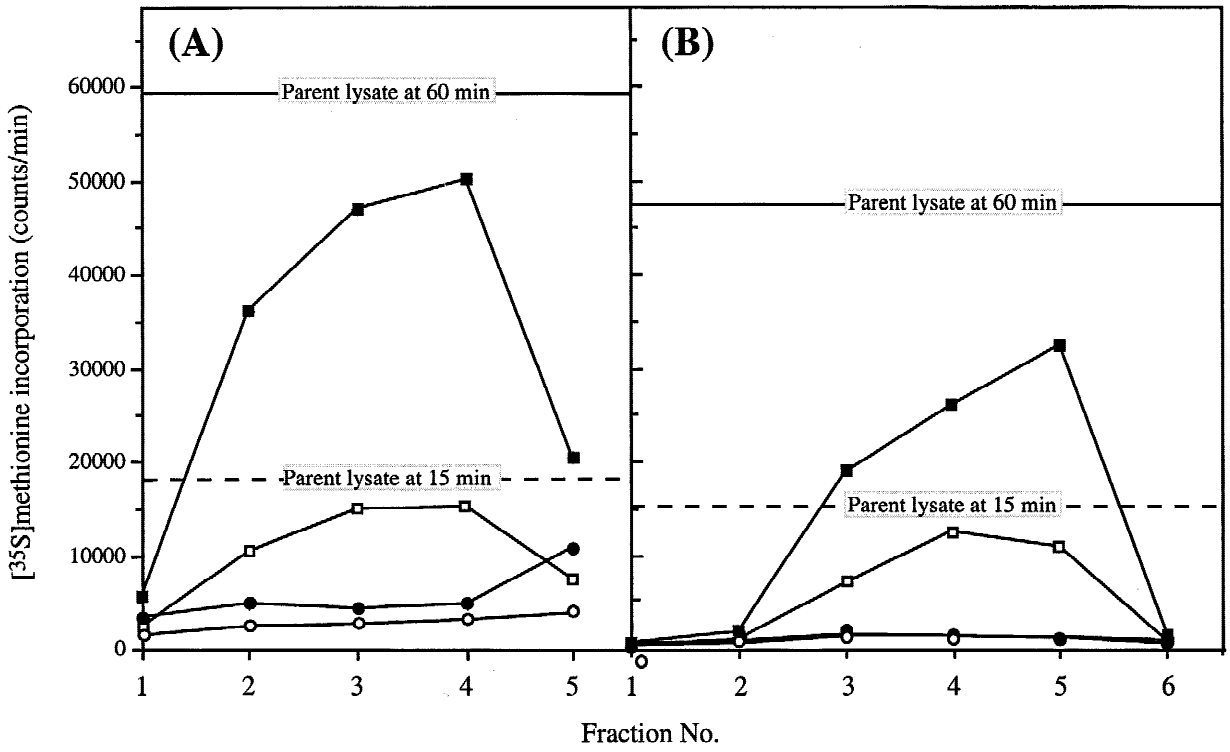

FIGURE 1. Effect of salt concentration on the efficiency of tRNA depletion and recovery of activity. **A:** Rabbit reticulocyte lysate (already supplemented with 20 μM hemin) was made 100 mM in added KCl and 0.5 mM in added $MgCl_2$; 2.7 mL were passed through a 1.2-mL affinity column that had been equilibrated with Buffer A containing 100 mM additional KCl and 0.5 mM additional $MgCl_2$. Fractions of approximately 0.42 mL were collected and assayed for [^{35}S]methionine incorporation in the presence (squares) or absence (circles) of 50 μg/mL calf-liver tRNA. Samples (5 μL) were taken for determination of acid-precipitable radioactivity after either 15 min (open symbols), or 60 min (filled symbols) incubation at 30 °C. **B:** The same procedure was followed except that lysate was made 50 mM in added KCl and 0.25 mM in added $MgCl_2$ before loading on the affinity column, which had been equilibrated in Buffer A containing 50 mM additional KCl and 0.25 mM additional $MgCl_2$. Symbols are as for **A**. In both panels the horizontal dashed line shows the incorporation observed in 15-min assays of the parent lysate, and the unbroken line the incorporation after 60 min incubation. (Addition of 50 μg/mL tRNA to the parent lysate did not have any significant effect on incorporation.)

tions were no less dependent on added tRNA than the earlier flow-through material.

Application of the tRNA-depletion method to mRNA-dependent reticulocyte lysates and wheat germs systems

We next examined whether the tRNA depletion was comparably successful when nuclease-treated cell-free extracts were used as starting material, with the aim of generating an in vitro translation system dependent on both mRNA and tRNA. These experiments were carried out with reticulocyte lysate (RRL) and wheat germ extracts (WG) purchased from Promega. In comparison to "home-made" lysates, the basic commercial translation systems are simple to prepare in that they require only the addition of amino acids and mRNA for functionality. However, they are less flexible in terms of ease of manipulation of their ionic composition, and this was an important consideration given the bearing that ionic strength had on the extent of tRNA depletion and subsequent activity of native lysates in the trial experiments detailed above. For this reason, the ethanolamine-Sepharose columns were loaded with RRL/WG whose endogenous ionic composition had been manipulated by dilution with water and/or addition of KOAc/$Mg(OAc)_2$. Although a number of conditions were tried, the best results were obtained when the KOAc concentration of the load material matched that of column Buffer A supplemented with 80 mM KOAc and 0.5 mM $Mg(OAc)_2$. For RRL, 1 mL was supplemented with amino acids (each to 20 μM; no methionine), diluted with water to 70% relative concentration (i.e., 0.42 mL water added per mL RRL) and passed over a 1-mL column of ethanolamine-Sepharose. Prior to loading, the column was washed with 10 vol of Buffer A followed by a further 10 vol of Buffer A supplemented with 80 mM KOAc and 0.5 mM $Mg(OAc)_2$. Based on the manufacturer's product information and estimated endogenous ion concentrations (Jackson & Hunt, 1983), the K^+ and Mg^{2+} concentrations in the column equilibration buffer (105 mM and 1.5 mM, respectively) were

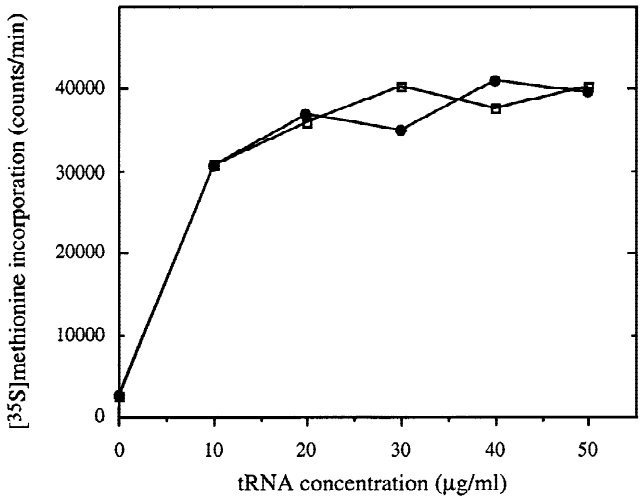

FIGURE 2. Effect of tRNA concentration and origin on the recovery of translation activity. The affinity depletion was carried out as in Figure 1B. Flow-through fractions 3, 4, and 5 were pooled, and the pool assayed for incorporation of [^{35}S]methionine in the presence of increasing concentrations of commercial (Boehringer Mannheim) calf-liver tRNA (open squares), or rabbit reticulocyte lysate tRNA prepared in-house and purified by gel filtration using Sephacryl S-300 (filled circles). Samples (5 μL) were taken for determination of acid-precipitable radioactivity after 60 min incubation at 30 °C.

similar to those of the diluted lysate (calculated to be 97 mM K^+, 1.27 mM Mg^{2+}). For depletion of WG extracts, 1 mL was supplemented with amino acids (each to 60 μM; no methionine) and KOAc (to 40 mM), the extract diluted with water to 60% relative concentration (i.e., 0.66 mL water added per mL WG extract), and then loaded onto a 1-mL column of ethanolamine-Sepharose and processed as for RRL. In the case of the WG system, the diluted extract had a K^+ concentration calculated to be identical to that of the column equilibration buffer (105 mM), but the Mg^{2+} concentration was considerably higher (2.5 mM in the diluted extract as opposed to 1.5 mM in the column equilibration buffer).

Column profiles from RRL and WG depletion experiments are shown in Figure 3. In each case, 13 fractions of approximately 0.2 mL were collected, the first three fractions discarded (as they largely comprised column buffer), and 50-μL aliquots taken from the remaining fractions for assessment of translational activity and tRNA content (the remainder was stored at −70 °C). Translational activity was monitored using a capped, synthetic mRNA transcript derived from plasmid pFScass 5 (Brierley et al., 1992). This transcript contains the ribosomal frameshift signal of the coronavirus infectious bronchitis virus (IBV) cloned within the influenza A/PR8/34 PB2 gene (Young et al., 1983), and specifies an 18-kDa nonframeshift product and a 22-kDa −1 frameshift product. Aliquots (10 μL) of each column fraction were supplemented with [^{35}S]methionine (7.5 μCi), the pFScass 5 reporter mRNA added (to 25 μg/mL), and translation carried out in the presence or absence of calf-liver tRNA (50 μg/mL) at 25 °C (WG) or 30 °C (RRL) for 60 min. Products were separated by SDS-PAGE and detected by autoradiography. Nucleic acids were isolated from the remaining 30 μL of each fraction by phenol/chloroform extraction and ethanol precipitation, and analyzed by agarose gel electrophoresis. As can be seen in Figure 3A, the translational activity of the RRL was entirely dependent upon the addition of exogenous calf-liver tRNA. About 95% of the tRNA had been removed from the active fractions, whereas most of the other nucleic acid species were retained. Taking into account load (L) and column fraction volumes, and the intensity of the nonframeshifted and frameshifted translation products, we estimate that about 40% of the total translation activity loaded onto the column was recovered under these conditions. The WG column profile (Fig. 3B) was different in that all of the activity loaded was recovered, but the tRNA depletion was not quite as good (~90%), and most of the unsupplemented fractions retained a trace of activity (fractions 7, 8, 9, 10, and 11). This was found to be a consequence of the KOAc concentration of the WG loaded in this particular experiment (calculated as 133 mM); subsequent reduction (to 103 mM) improved the tRNA dependence of the active fractions without greatly compromising activity (data not shown). For either translation system, the activity in each fraction did not correlate precisely with total nucleic acid content. This was especially evident with the WG system; although fractions 4, 5, and 6 had no detectable activity, their nucleic acid contents were very similar to that of the active fractions. We do not know why these early fractions are inactive; it may be that an important component of the wheat germ extract is initially depleted following binding to the column, but that the column is saturated rapidly and subsequent fractions are active. Alternatively, gel filtration of an essential component may be occurring. Consistent with either hypothesis, increasing the bed volume of ethanolamine-Sepharose beyond 2 mL has yielded lysates with reduced activity. We have also found that it is not possible to identify the active WG column fractions on the basis of eluate opacity. In Figure 3B for example, the later column fractions (11, 12, and 13) retained significant mRNA- and tRNA-dependent translational activity, but were clear.

In the experiments described above, the tRNA dependency of the flow-through fractions was tested using a reporter mRNA that directed the synthesis of relatively small proteins. To assess the ability of the tRNA-dependent lysates (td-RRL, td-WG) to synthesize substantially longer proteins, we programmed translation reactions with commercially available RNAs (Promega) derived from brome mosaic virus (BMV) or firefly luciferase (luc). The BMV RNAs specify polypeptides of 104 kDa (RNA 1a), 94 kDa (2a), 32 kDa (3a), and 20 kDa (4) (Ahlquist et al., 1984), and the luc mRNA,

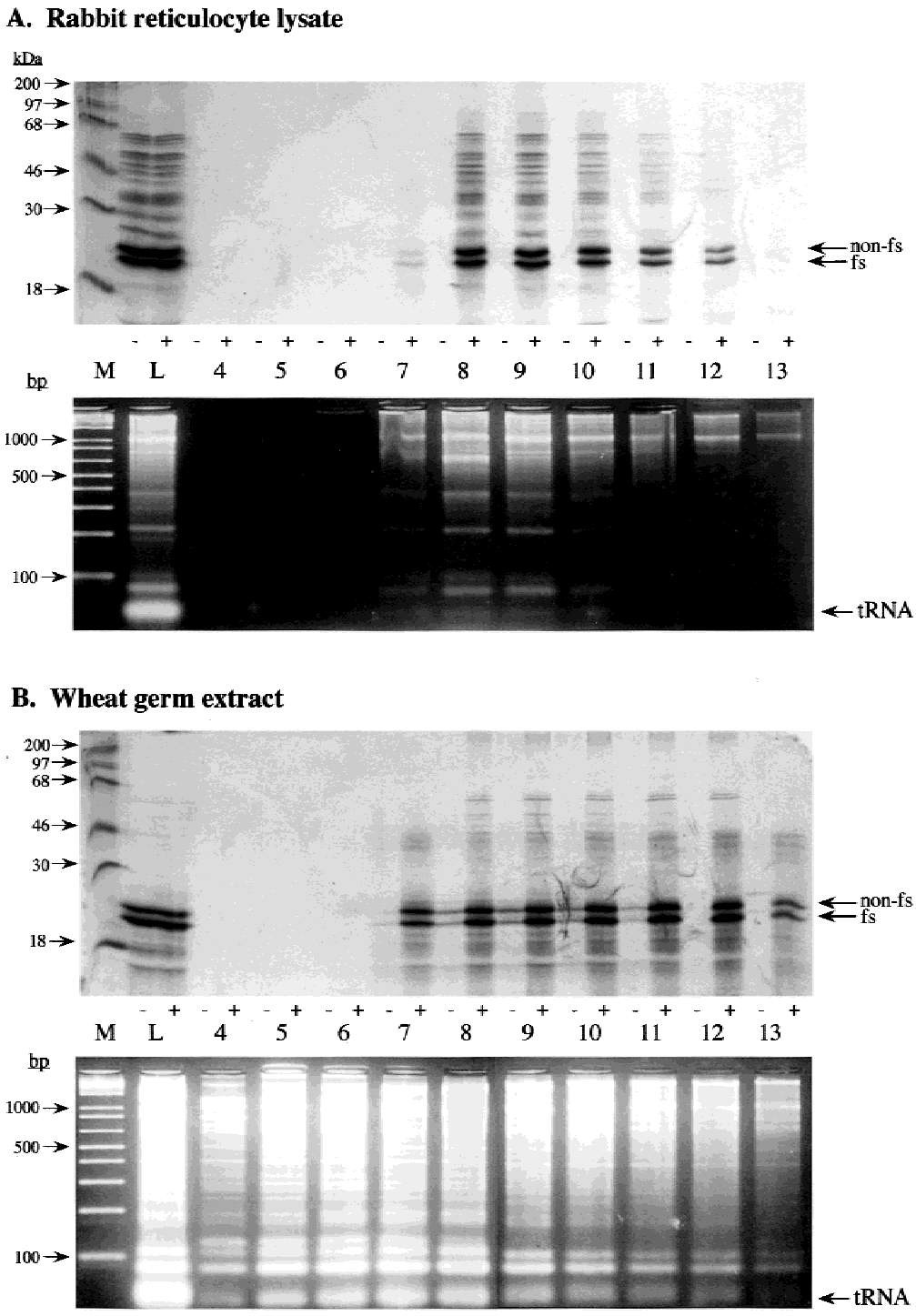

FIGURE 3. Ethanolamine-Sepharose column profiles. Commercial (Promega) rabbit reticulocyte lysate (**A**) or wheat germ extracts (**B**) were depleted of tRNAs by ethanolamine-Sepharose chromatography. The columns were equilibrated and the load material prepared as described in the text. Fractions (lanes 4–13) were collected and tested for their ability to translate a synthetic mRNA derived from *Bam*HI-digested plasmid pFScass 5 in the absence (−) or presence (+) of added calf-liver tRNA (to 50 μg/mL) (upper gel of each panel) and for nucleic acid content (lower gel of each panel). Translation products were labeled with [^{35}S]methionine, separated on SDS-15% polyacrylamide gels and detected by autoradiography. The pFScass 5 mRNA directs the synthesis of a 19-kDa nonframeshift (non-fs) and a 22-kDa −1 frameshift (fs) product (Brierley et al., 1992). L lanes represent translations of the column load material. Nucleic acids were separated on 3% TBE-agarose gels, stained with ethidium bromide and the gel photographed under UV illumination. Markers (M) used were ^{14}C-labeled protein size standards (Amersham Pharmacia Biotech) and kb+ ladder (Gibco-BRL).

61 kDa. We tested these RNAs in combination with a variety of tRNAs including wheat germ (WG) and calf-liver (CL) tRNAs from a commercial source (Sigma), baby hamster kidney cell tRNAs (BHK) prepared in-house (Marczinke et al., 2000), and *Escherichia coli* JM101 tRNAs (*E. coli*) purified under acidic conditions to preserve their aminoacyl groups (see Materials and Methods). The results are shown in Figure 4A,B alongside a translation series performed using nondepleted (yet appropriately diluted) RRL and WG for comparison (Fig. 4D). A number of features emerged from this study. First, the translation efficiency of the depleted lysates was highly dependent on the kind of tRNA employed. Elongation was most efficient with CL and BHK tRNAs (Fig. 4A,B, lanes 2, 3, 6, and 7), but less so with WG tRNAs (Fig. 4A,B, lanes 1 and 5). The precharged *E. coli* tRNAs were capable of supporting protein synthesis to a certain degree in the td-WG system (Fig. 4A,B, lane 4), but not in td-RRL (Fig. 4A,B, lane 8), at least with the mRNAs used here. The td-WG preparation used in this experiment was well depleted, and control translations without added mRNA did not produce detectable translation products (data not shown).

To some extent, the synthesis of longer proteins was also dependent on the mRNA used. For example, in td-RRL programmed with BMV RNAs and CL tRNA (Fig. 4A, lane 6), the intensity of the 32-kDa, 94-kDa, and 105-kDa proteins, in comparison to that of the 20-kDa protein, was not as great as that seen in non-depleted RRL (Fig. 4D, lane 5). However, with the optimal tRNA supplement (CL), synthesis of the 61-kDa luciferase protein proceeded as well in td-RRL (Fig. 4B, lane 6) as it did in nondepleted lysates (Fig. 4D, lane 6). Similarly, translation of the pT7CGL mRNA (described

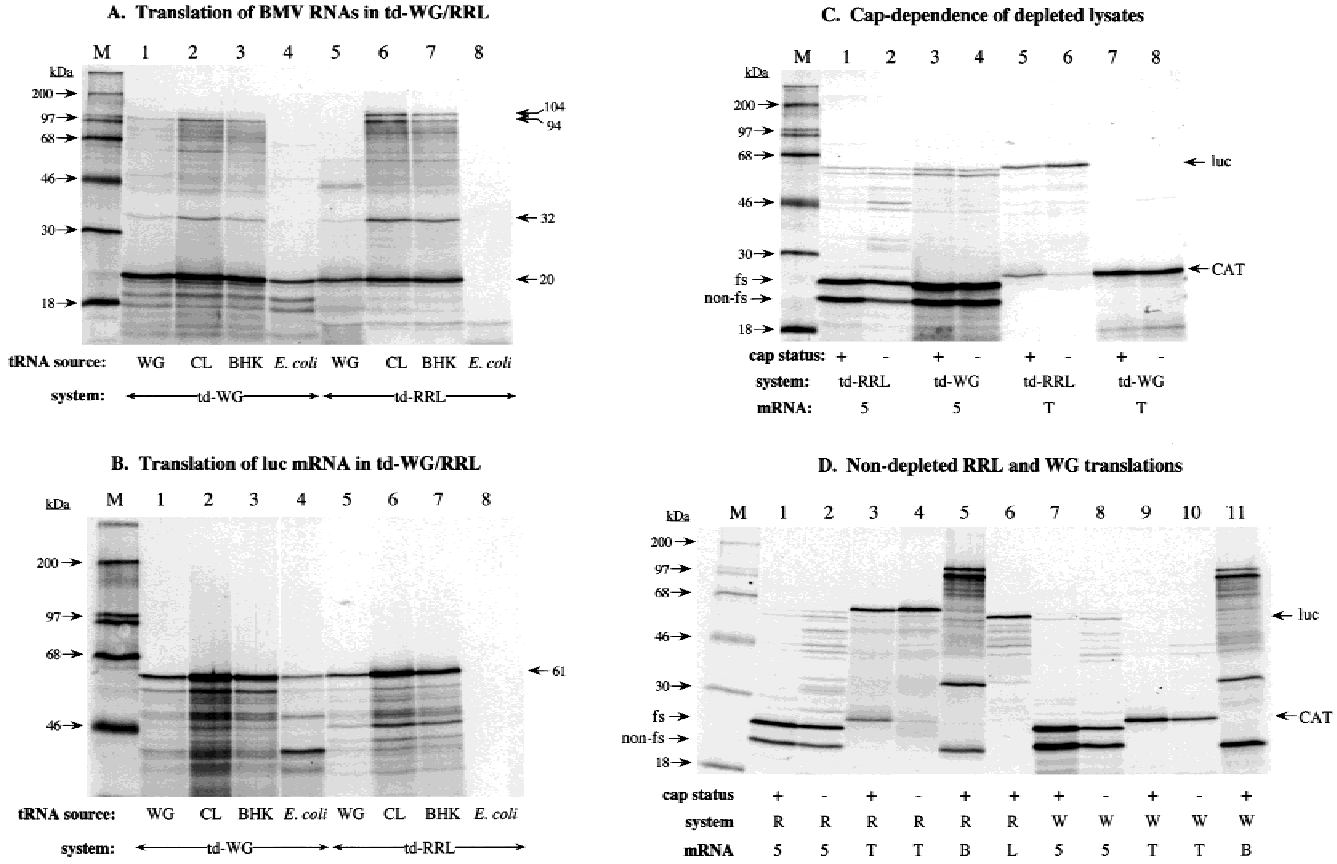

FIGURE 4. Functionality of the depleted lysates. **A**,**B**: The influence of the source of the tRNA on translation of BMV RNA (**A**) or luciferase (luc) mRNA (**B**) in tRNA-dependent wheat germ extracts (td-WG) or rabbit reticulocyte lysates (td-RRL). The tRNAs were added at 50 μg/mL and were from wheat germ (WG), calf liver (CL), BHK cells (BHK), or *E. coli* JM101 cells, as indicated. **C**: The cap-dependency of translation in tRNA-dependent wheat germ extracts (td-WG) or rabbit reticulocyte lysates (td-RRL). Capped (+) or uncapped (−) transcripts of pFScass 5 (designated "5"), or pT7CGL (designated "T"), were translated in td-WG or td-RRL (as indicated), in the presence of 50 μg/mL BHK cell tRNA. **D**: The cap dependency and efficiency of translation in the parent (nondepleted) extracts. Capped (+) or uncapped (−) transcripts of pFScass 5 (5) or pT7CGL (T), capped BMV RNAs (B), or capped luciferase mRNA (L) were translated in nondepleted wheat germ extract (W) or rabbit reticulocyte lysate (R). Both extracts were pretreated in exactly the same way as they would be if they were to be loaded on the affinity column, and thus the assays of **D** were done under the same conditions of salt concentration and extract dilution as those of **C**. Translation products were labeled with [^{35}S]methionine, separated on SDS-15% (**A**, **C**, **D**) or SDS-10% (**B**) polyacrylamide gels, and detected by autoradiography. Markers (M) were ^{14}C-labeled protein size standards (Amersham Pharmacia Biotech).

below) was qualitatively similar in the depleted (Fig. 4C, lanes 5 and 6) and nondepleted (Fig. 4D, lanes 3 and 4) systems.

The final question we addressed was the degree of cap dependence exhibited by the tRNA-dependent lysates (Fig. 4C) in comparison with undepleted lysates (Fig. 4D). We found that cap dependence was retained for td-RRL, but was noticeably reduced for td-WG. In td-RRL, translation of the frameshift reporter mRNA pFScass 5 was about threefold more efficient when the mRNA was capped (Fig. 4C, lanes 1 and 2) as also seen in undepleted RRL (Fig. 4D, lanes 1 and 2), but in td-WG, there was little difference between the capped and uncapped mRNAs, although protein synthesis was efficient (Fig. 4C, lanes 3 and 4). Undepleted WG extracts showed typical cap dependence (Fig. 4D, lanes 7 and 8). The same pattern was seen when lysates were programmed with capped or uncapped pT7CGL mRNA. This mRNA contains the internal ribosome entry sequence (IRES) of the GDVII strain of Theiler's murine encephalomyelitis virus (TMEV) flanked by chloramphenicol acetyl transferase (CAT, 5′) and luciferase (luc, 3′) coding sequences. In td-RRL, capped pT7CGL transcripts specified the synthesis of both CAT and luc, but uncapped RNA specified predominantly luc (Fig. 4C, lanes 5 and 6). This is a typical feature of the translation of capped and uncapped bicistronic mRNAs containing an intercistronic IRES of this kind (Hunt et al., 1993), where ribosomes have a choice of attachment sites. In td-WG, where the IRES is nonfunctional, only CAT was synthesized (Fig. 4C, lanes 7 and 8), and the yields of protein product were similar for capped or uncapped transcripts.

DISCUSSION

The ethanolamine-Sepharose column procedure described here clearly removes almost all of the tRNA from rabbit reticulocyte lysates and wheat germ extracts, without seriously depleting the system of other components necessary for protein biosynthesis. So effective is the depletion that it seems likely that, apart perhaps from tRNA associated with ribosomes, the whole tRNA population, including any associated with activating enzymes or elongation factor eEF1A, must be absorbed to the column. We do not know why this affinity matrix exhibits such a high selectivity towards tRNA. Although it obviously has anion exchange properties, this is unlikely to be the complete explanation. Passage of reticulocyte lysates through DEAE-Sepharose CL-6B results in a completely inactive translation system, perhaps not surprisingly in view of the fact that most of the ribosomes are bound to the column. The series of ω-aminoalkyl-Sepharoses are perhaps more analogous to ethanolamine-Sepharose, but we found that when lysates were passed through ω-aminododecyl-Sepharose or ω-aminohexyl-Sepharose, even in 0.1 M KCl, the flow-through lysate was inactive for reasons other than simply tRNA deficiency.

The method seems equally applicable whether the reticulocyte lysate has been pretreated with micrococcal nuclease or not, and works almost as well with wheat germ extracts. Although the depleted system (supplemented with tRNA) is efficient in the synthesis of short- to medium-length proteins, its ability to synthesize large proteins seems somewhat impaired. It is possible that this problem might be circumvented by using a higher salt concentration in the affinity column depletion procedure. As we have shown here, there is a certain trade-off between the degree of tRNA depletion and impairment of overall translation activity. Therefore, depending on whether the maximum possible depletion is required or not, it may be possible to increase the activity of the system at the price of a less complete depletion, simply by raising the salt concentration of the material loaded on to the affinity column. Alternatively, a lower capacity affinity matrix can be used. This is made by carrying out a "mock coupling," incubating the washed, epoxy-activated-Sepharose in 70 mM borate buffer, pH 8.5, at 34 °C for 60 h, followed by copious washing with water, before overnight incubation with the blocking reagent, 1 M ethanolamine. Other ways in which the system might be improved or adapted for specific purposes might be to use a medium other than Sepharose to avoid the gel-filtration effects that seem to prejudice the performance of the first few fractions of the flow-through lysate.

We anticipate that this tRNA depletion procedure will prove very useful for analysis of the influence of the tRNA population spectrum on recoding events, such as frameshifting, readthrough of termination codons, and selenocysteine insertion at UGA codons. Although we have evaluated the viability of the protocol only in relation to reticulocyte lysates and wheat germ extracts, there is no reason to suppose that it will not be equally applicable to other systems. Given the (almost) universal conservation of tRNA structure among all known organisms, it seems almost a foregone conclusion that the procedure will certainly be just as effective in depleting tRNA from any other type of extract (with the possible exception of mammalian mitochondrial tRNAs). The only uncertainty is whether the column might not also absorb other components necessary for the in vitro translation activity of the particular cell-free extract concerned, but, as we have shown here, there is a good chance that any such problem can be overcome simply by manipulating the salt concentration at which the depletion is carried out.

MATERIALS AND METHODS

Materials

Epoxy-activated Sepharose 6B was purchased from Sigma. Calf-liver transfer RNA was from Sigma or Boehringer, wheat

germ tRNAs from Sigma. Reticulocyte tRNAs were prepared by phenol extraction of reticulocyte postribosomal supernatant, and were further purified by gel filtration on Sephacryl S-300 (Pharmacia). Charged *E. coli* tRNAs were prepared from *E. coli* JM101 by the procedure of Varshney et al. (1991). Inhibitory acidic polysaccharides were removed by DE52 column chromatography; 4-mL columns were equilibrated with 140 mM KOAc, pH 4.5, 100 μg of tRNA loaded, the column washed with 10 vol of equilibration buffer, and the tRNAs eluted with the same buffer containing 0.3 M NaCl. [^{35}S]methionine (SJ1515) was from Amersham International; it was diluted with unlabeled methionine to give a stock of 400 μM, 1 mCi/mL. Rabbit reticulocyte lysate was prepared as described previously (Jackson & Hunt, 1983). Nuclease treated RRL and wheat germ extracts were from Promega.

Column chromatography

Epoxy-activated Sepharose was swollen in water and washed copiously with water on a Buchner funnel to remove the preservative. For the preparation of high capacity ethanolamine-Sepharose, it was resuspended in 1 M ethanolamine (5 mL per mL swollen gel), incubated with gentle agitation overnight at room temperature, and finally washed with water until the pH of the washings fell to pH 7.

All column procedures were carried out at 4 °C using columns of 1.0–1.5 mL bed volume (0.4 × 5 cm), pumped at 10 mL/h. Buffers for column equilibration were based on Buffer A containing 1 mM dithiothreitol. Buffer A (25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM HEPES-KOH, pH 7.2) has a cation composition very similar to that of crude reticulocyte lysate (Jackson & Hunt, 1983). Columns were equilibrated with Buffer A plus additional KCl and $MgCl_2$ as detailed in the text. Columns were washed between runs with Buffer A containing 0.5 M KCl.

In vitro transcription

Plasmids were prepared for in vitro transcription as described previously (Brierley et al., 1989). In vitro transcription reactions were carried out essentially as described by Melton et al. (1984). For capped mRNAs, transcriptions included the synthetic cap structure m^{7}GpppG (New England Biolabs). Product RNA was recovered by a single extraction with phenol:chloroform:isoamyl alcohol (49:49:2) followed by ethanol precipitation in the presence of 2 M ammonium acetate. The RNA pellet was dissolved in water, and remaining unincorporated nucleoside triphosphates removed by Sephadex G-50 chromatography. RNA was recovered by ethanol precipitation, dissolved in water and checked for integrity by electrophoresis on 1.5% agarose gels containing 0.1% sodium dodecyl sulphate (SDS).

In vitro translation

The standard protein synthesis assay with native (non-nuclease-treated) lysate consisted of 0.8 vol reticulocyte lysate or column fraction (supplemented with hemin and containing 10 μg/mL creatine phosphokinase); 0.05 vol 2 M KCl, 10 mM $MgCl_2$; 0.05 vol 0.2 M creatine phosphate; 0.05 vol unlabeled amino acid mixture (lacking methionine); 0.05 vol [^{35}S]methionine (400 μM; 2.5 Ci/mMol). Incubation was at 30 °C for the times indicated and 5-μL samples were taken for assay of incorporation using trichloroacetic acid as described previously (Jackson & Hunt, 1983). In experiments in which the lysate was supplemented with KCl and $MgCl_2$ before passage through the column, the amounts of these salts added to the subsequent assays were reduced to achieve the same final concentrations as above.

In vitro translations with commercial nuclease-treated lysates (RRL and WG) were carried out as described (Brierley et al., 1987, 1989). Translation products were analyzed on SDS-10% or SDS-15% (w/v) polyacrylamide gels according to standard procedures (Hames, 1981). The relative abundance of products on the gels was estimated by measurement of [^{35}S]methionine incorporation using a Packard Instant Imager 2024 and adjusted to take into account the differential methionine content of the products.

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