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# Efficient Translation of Tobacco Mosaic Virus RNA and Rabbit Globin 9S RNA in a Cell-Free System from Commercial Wheat Germ

**(wheat germ extracts/protein synthesis/gel electrophoresis/peptide analysis)**

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**ABSTRACT Extracts prepared from commercially available wheat germ efficiently translate messenger RNAs from either viral or eukaryotic origin. The addition of tobacco mosaic virus RNA stimulated amino-acid incorporation more than 100-times and rabbit globin 9S RNA between 20- and 30-times. The *in vitro* product directed by globin 9S RNA comigrated precisely with authentic rabbit globin on sodium dodecyl sulfate–polyacrylamide gels. Furthermore, during high voltage ionophoresis two [$^{35}$S]methionine-labeled tryptic peptides synthesized *in vitro* comigrated in two dimensions with $\alpha$T5 and $\beta$T5 tryptic peptides from authentic [$^{35}$S]methionine-labeled rabbit globin.**

Numerous eukaryotic cell-free systems faithfully translate viral and eukaryotic messenger RNAs. These include Krebs II ascites tumor (1–7), rat and mouse liver (8, 9), reticulocyte lysate (10–13), HeLa cells, mouse L cells and CHO cells (5). However, many of these systems are limited by the efficiency of translation (mol of amino acid incorporated per mol of mRNA), and identification of products is hampered by high levels of endogenous protein synthesis occurring in the absence of added RNA.

We wish to report a simple method of preparing active extracts from commercial wheat germ that translate efficiently both viral and eukaryotic messenger RNAs.

## MATERIALS AND METHODS

Fresh commercial wheat germ (not toasted) was supplied by the "Bar-Rav" Mill, Tel Aviv, Israel and was stored under reduced pressure at 2°. Tobacco mosaic virus (TMV) variety vulgare was a gift of Dr. P. J. G. Butler (M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.). HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid), ATP (disodium salt), GTP (sodium salt), creatine phosphate (disodium salt), and creatine phosphokinase (EC, 2.7.3.2) were purchased from Sigma Biochemicals. Trypsin treated with TPCK (EC 3.4.4.4) and pancreatic ribonuclease A (EC 2.7.7.16) were from Worthington. All radioactive amino acids were obtained from the Radiochemical Centre, Amersham, U.K. with the following specific activities: $^{14}$C-labeled amino-acid mixture (>45 Ci/atom carbon), [$^{14}$C]leucine (342 Ci/mol), [$^{14}$C]-phenylalanine (480 Ci/mol), [$^{3}$H]leucine (38 Ci/mmol), and [$^{35}$S]methionine (the specific activity at the time of use as stated in the text).

*Preparation of Wheat-Germ Extracts.* The 30,000 $\times$ *g* supernatant (S-30) of wheat germ was prepared by modifications of published procedures (5, 14, 15). Wheat germ (6 g) was ground in a chilled mortar with an equal weight of sand and 28 ml of a solution containing 20 mM HEPES (pH 7.6) (adjusted with KOH), 100 mM KCl, 1 mM magnesium acetate, 2 mM $CaCl_2$, and 6 mM 2-mercaptoethanol. The homogenate was centrifuged at 30,000 $\times$ *g* for 10 min at 0–2°, and the supernatant was removed, avoiding both the surface layer of fat and the pellet. The S-30 fraction was made up to 3.5 mM magnesium acetate and preincubated with 1 mM ATP (neutralized), 20 $\mu$M GTP, 2 mM dithiothreitol, 8 mM creatine phosphate, and 40 $\mu$g of creatine phosphokinase per ml of homogenate for 12–15 min at 30°. Finally, 10–12 ml of the preincubated S-30 fraction was passed through a column (50 $\times$ 2-cm) of Sephadex G-25 (coarse), equilibrated with 20 mM HEPES (pH 7.6), 120 mM KCl, 5 mM magnesium acetate, and 6 mM 2-mercaptoethanol at a flow rate of 1.3–1.4 ml/min. The peak of the turbid fraction was pooled and dispensed through a sterile syringe into liquid nitrogen. The frozen spheres were stored thus for 6 months without any detectable loss of activity.

*Preparation of RNA.* TMV RNA was isolated by the EDTA–phenol method of Marcus, Efron, and Weeks (*Methods in Enzymology*, in preparation). Preincubated Krebs S-30 fraction and rabbit globin 9S RNA was a generous gift of Uri Nudel, Department of Biochemistry, The Weizmann Institute of Science, Rehovot, Israel. [$^{35}$S]Methionine-labeled rabbit globin was prepared by the method of Schulman (16).

*Standard Protein Synthesis Assays* contained in a final volume of 50 $\mu$l, 10–20 $\mu$l of preincubated wheat-germ S-30 fraction, 20 mM HEPES (pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 20 $\mu$M GTP, 8 mM creatine phosphate, 40 $\mu$g/ml of creatine phosphokinase, 20–30 $\mu$M of the appropriate unlabeled amino acids, KCl, magnesium acetate, and radioactive amino acids as indicated. Each reaction was supplemented with 75 $\mu$g/ml of deacylated tRNA extracted from wheat germ by the method described (17).

Reactions were incubated for 90 min at 25°. Aliquots of 5 $\mu$l were pipetted immediately onto 2.5-cm discs of Whatman 3 MM paper, dried, placed into 10% trichloroacetic acid containing the appropriate unlabeled amino acids for 10 min, transferred to 5% trichloroacetic acid, boiled at 90° for 10 min, rinsed with cold 5% trichloroacetic acid, and dried by sequential rinsing in ethanol, ethanol–ether (3:1 v/v), and ether. Radioactivity was determined by liquid scintillation, with 10 ml of toluene scintillant. The efficiency of counting of $^{14}$C and $^{35}$S was 85%, and of $^{3}$H 25%. The remainder of the reaction mixture was made 10 mM with EDTA (pH 7.3) and 20$\mu$g/ml with pancreatic ribonuclease A. This was incubated at 37° for 15 min.

*Product Identification.* The *in vitro* products were analyzed by Na dodecyl sulfate–polyacrylamide gels as described by

Abbreviations: TMV, tobacco mosaic virus; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE 1. *Incorporation of radioactive amino acids directed by TMV RNA*

| Exp. | Amino acid | Amino-acid incorporation (cpm) Control* | TMV/RNA | Incorporation ratio (TMV RNA/control)* | Efficiency (mol amino acid per mol TMV RNA) |
|---|---|---|---|---|---|
| I | [14C]Phenylalanine | 1,360 | 9,700 | 7.1 | 3.9 |
| | [14C]Leucine | 1,400 | 13,840 | 9.9 | 8.3 |
| | [3H]Leucine | 4,372 | 203,200 | 46.5 | 4.0 |
| | [14C]Aminoacid mixture | 3,211 | 66,980 | 20.9 | — |
| | [35S]Methionine | 10,810 | 606,300 | 56.1 | 1.3 |
| II | [35S]Methionine | 16,200 | 1,666,000 | 102.9 | 3.0 |

50-μl Reactions were incubated at 25° for 90 min, with [35S]methionine (102 Ci/mmol). The reactions in experiment I contained 3.5 mM magnesium, 100 mM potassium, and 5 μg of TMV RNA; in experiment II, 3 mM magnesium, 100 mM potassium, and 6 μg of TMV RNA.

* Reactions were incubated for 90 min in the absence of added RNA.

Laemmli (18). Gels were stained with Coomassie Brilliant Blue, sliced longitudinally, dried, and autoradiographed. The method of tryptic digestion of the [35S]methionine-labeled products was as described by Mathews (1). The sample was subjected to high-voltage two-dimensional paper ionophoresis on Whatman 3 MM at pH 6.5 for 90 min at 3 kv in the first dimension. The dried strip was sewn into another piece of paper and subjected to electrophoresis in the second dimension at pH 3.5 for 60–90 min at 3 kv. The dried paper was autoradiographed. The peptides were eluted and hydrolyzed with 6 N HCl for analysis on a Beckman amino-acid analyzer.

## RESULTS

*Protein Synthesis Directed by TMV RNA.* In a preincubated S-30 fraction of wheat germ, incorporation of [35S]methionine is greatly enhanced on addition of TMV RNA, and is saturated at about 200 μg of RNA per ml (Fig. 1*a*). Stimulation of incorporation is optimal at a magnesium concentration of 2.5 mM (Fig. 1*b*) and at a potassium concentration of 100 mM (Fig. 1*c*). Time-course studies of reactions at 25° indicate a lag period of between 5 and 7 min, followed by linear incorporation for about 60 min and termination at 90 min (Fig. 1*d*). Incorporation of different radioactive amino acids directed by TMV RNA, their stimulation above endogenous protein synthesis, and the number of moles of each incorporated per mole of TMV RNA are summarized in Table 1, experiment I. Under optimal conditions, incorporation of [35S]methionine directed by TMV RNA was more than 100-times that of reactions incubated for the same time in the absence of added

TABLE 2. *Incorporation of radioactive amino acids by rabbit globin 9S RNA*

| Amino acid | Amino-acid incorporation (cpm) Control* | Rabbit globin 9S RNA | Efficiency (mol amino acid per mol 9S RNA)† |
|---|---|---|---|
| [14C]Aminoacid mixture | 5,120 | 171,900 | — |
| [35S]Methionine | 28,200 | 639,000 | 0.58 |

Reactions were done in 50 μl containing 1.25 μg of rabbit globin 9S RNA, 3 mM magnesium, 80 mM potassium, and 100 μCi/ml of [35S]methionine (89 Ci/mmol). They were incubated at 25° for 90 min.

* Reactions incubated in the absence of added RNA.

† Assumed a molecular weight of 200,000 for rabbit globin 9S RNA.

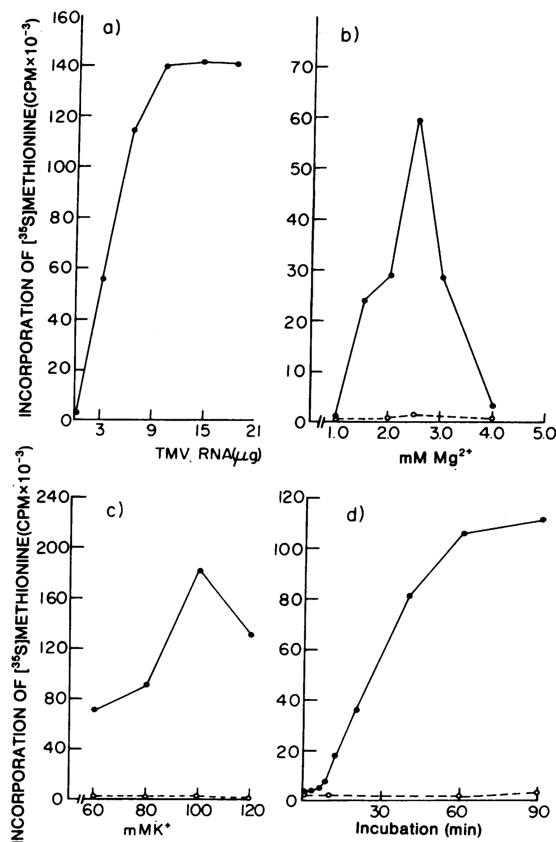


FIG. 1. Characteristics of protein synthesis directed by TMV RNA in wheat-germ extracts. (*a*) Response to added TMV RNA at 3 mM $Mg^{2+}$ and 100 mM $K^+$. (*b*) Effect of magnesium concentration on protein synthesis at 100 mM $K^+$ with 11 μg of TMV RNA. (*c*) Effect of potassium concentration on protein synthesis at 3 mM $Mg^{2+}$ with 11 μg of TMV RNA. (*d*) Time-course of protein synthesis at 3 mM $Mg^{2+}$, 100 mM $K^+$ with 11 μg of TMV RNA. Samples (50 μl) were incubated at 25° for 90 min (●——●) with TMV RNA or (O- - -O) without TMV RNA. Specific activity of the [35S]methionine was 89 Ci/mmol.

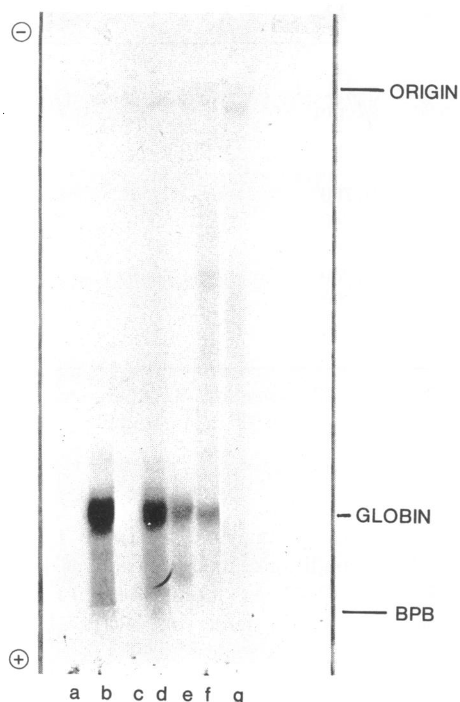


FIG. 2. Sodium dodecyl sulfate–gel electrophoresis of *in vitro* products of amino-acid incorporation directed by rabbit globin 9S RNA. Dried gels were autoradiographed for 16 hr; the *origin* marks the top of the separating gel and *BPB* the bromophenol blue front. Wheat-germ extracts incubated with [$^{14}$C]aminoacid mixture (*a*) without added RNA and (*b*) with 1.25 $\mu$g of 9S RNA; those incubated with [$^{35}$S]methionine were (*c*) without added RNA (*d*) with 1.25 $\mu$g of 9S RNA, and (*e*) with 0.5 $\mu$g of 9S RNA. Krebs ascites extracts incubated with [$^{35}$S]methionine were (*f*) with 0.5 $\mu$g of 9S RNA and (*g*) without added RNA. Specific activity of the methionine was as stated in Table 2.

RNA (Table 1, experiment II). The disparity in the estimated efficiency for [$^{14}$C]leucine and [$^{3}$H]leucine incorporation could result from the dilution of the tritiated amino acid of high-specific activity by residual unlabeled amino acid in the wheat-germ extract. Furthermore, it should be noted that these reactions were not at the optimum concentration for either TMV RNA (Fig. 1*a*) or magnesium (Fig. 1*b*).

The proteins synthesized *in vitro* in response to TMV RNA consist of a large number of polypeptides ranging in molecular weights from 10,000 to 140,000. The use of a nitrous-acid mutant of TMV (Ni 568) has shown that a specific peptide from the coat protein was faithfully translated in a wheat-germ S-30 fraction (Roberts, B. E., Mathews, M. B. & Bruton, C. J., *J. Mol. Biol.*, in press).

*Translation of Rabbit Globin 9S RNA.* Rabbit globin 9S RNA stimulated incorporation of both a mixture of $^{14}$C-labeled amino acids (containing 14 naturally occurring amino acids) and of [$^{35}$S]methionine into hot trichloroacetic acid-precipitable material (Table 2). On a molar basis, TMV RNA is more active than 9S RNA in directing incorporation of [$^{35}$S]-methionine; however, on a weight basis the reverse is true. The optimum ionic conditions for translation of 9S RNA were at 3.0 mM magnesium and 80 mM potassium. The *in vitro* products were separated in discontinuous Na dodecyl sulfate–14% polyacrylamide gels. Reactions incubated with either a mixture of [$^{14}$C]aminoacids (Fig. 2*a*) or [$^{35}$S]methionine (Fig. 2*c*), without added RNA, contained no labeled proteins discernable on autoradiographs of dried gels exposed for 18 hr. However, the *in vitro* product of incorporation directed by globin 9S RNA labeled with either a mixture of [$^{14}$C]aminoacids (Fig. 2*b*) or [$^{35}$S]methionine (Fig. 2*d*) comigrated precisely with unlabeled rabbit globin. Furthermore, the product synthesized under the direction of 9S RNA in the wheat-germ S-30 fraction comigrated with that synthesized in a preincubated Krebs ascites S-30 fraction (Fig. 2*f*). However, the ascites S-30 fraction synthesized numerous radioactive proteins in the absence of added RNA (Fig. 2*g*).

Rabbit globin contains two methionine residues, one in the $\alpha$ chain, tryptic peptide $\alpha$T5, and the other in the $\beta$ chain, $\beta$T5 (19). [$^{35}$S]Methionine-labeled tryptic peptides from a wheat-germ S-30 fraction were studied by two-dimensional high-voltage ionophoresis. In the absence of 9S RNA, two radioactive spots were obvious, one immobile at the origin and the other comigrating with methionine sulphone (Fig. 3*a*). On addition of 9S RNA, the *in vitro* products contained six well-defined tryptic peptides (Fig. 3*b*). The mobility of two peptides, I and II, in the first dimension at pH 6.5 compares with those of mouse $\alpha$T5 and $\beta$T5 synthesized in Krebs ascites (1) and in mouse and rat liver extracts (8). In the second dimension at pH 3.5, it compares with rabbit $\alpha$T5 and $\beta$T5 synthesized in extracts of reticulocytes (13). Recent experiments demonstrate that the tryptic peptides I and II synthesized in a S-30 fraction from wheat germ comigrate precisely with tryptic peptides synthesized in a Krebs ascites S-30 fraction (unpublished data).

Moreover, peptides I and II synthesized in a wheat-germ S-30 fraction comigrate in two dimensions with authentic [$^{35}$S]methionine-labeled rabbit globin $\alpha$T5 and $\beta$T5 tryptic peptides (Fig. 3*c*). We further characterized peptides I and II by mixing [$^{35}$S]methionine-labeled cell-free products from wheat germ with unlabeled globin (5 mg), digesting them with trypsin, and resolving the peptides in two dimensions. Peptides I and II were located by autoradiography, eluted, and hydrolyzed. The amino-acid composition was determined. Comparison with the published sequences for rabbit $\alpha$ and $\beta$ globin chains (19) identified peptide I as the tryptic peptide $\alpha$T5, residues 32–40 (Table 3). Peptide II had the same ratio and composition of amino acids as $\beta$T5, residues 41–59; however, the quantities were insufficient to permit accurate quantification. The peptides that comigrate during ionophoresis with authentic $\alpha$T5 and $\beta$T5 in two dimensions are synthesized in the wheat-germ system with an $\alpha$ to $\beta$ ratio between 1.4 and 1.9. The origin of the other peptides in the tryptic digests of the 9S RNA directed products (Fig. 3*b*) have not been investi-

TABLE 3. *Amino-acid composition of peptide I*

| Amino acid | No. residues observed* | No. residues calculated† |
|---|---|---|
| Lys | 0.95 | 1 |
| Thr | 1.70 | 2 |
| Pro | 0.92 | 1 |
| Gly | 1.00 | 1 |
| Met-$SO_2$ | 0.72 | 1 |
| Leu | 1.30 | 1 |
| Phe | 1.70 | 2 |

* Amino-acid analysis of peptide I.

† Calculated from the composition of tryptic peptide $\alpha$ T5 (residues 32–40) from rabbit globin (19).

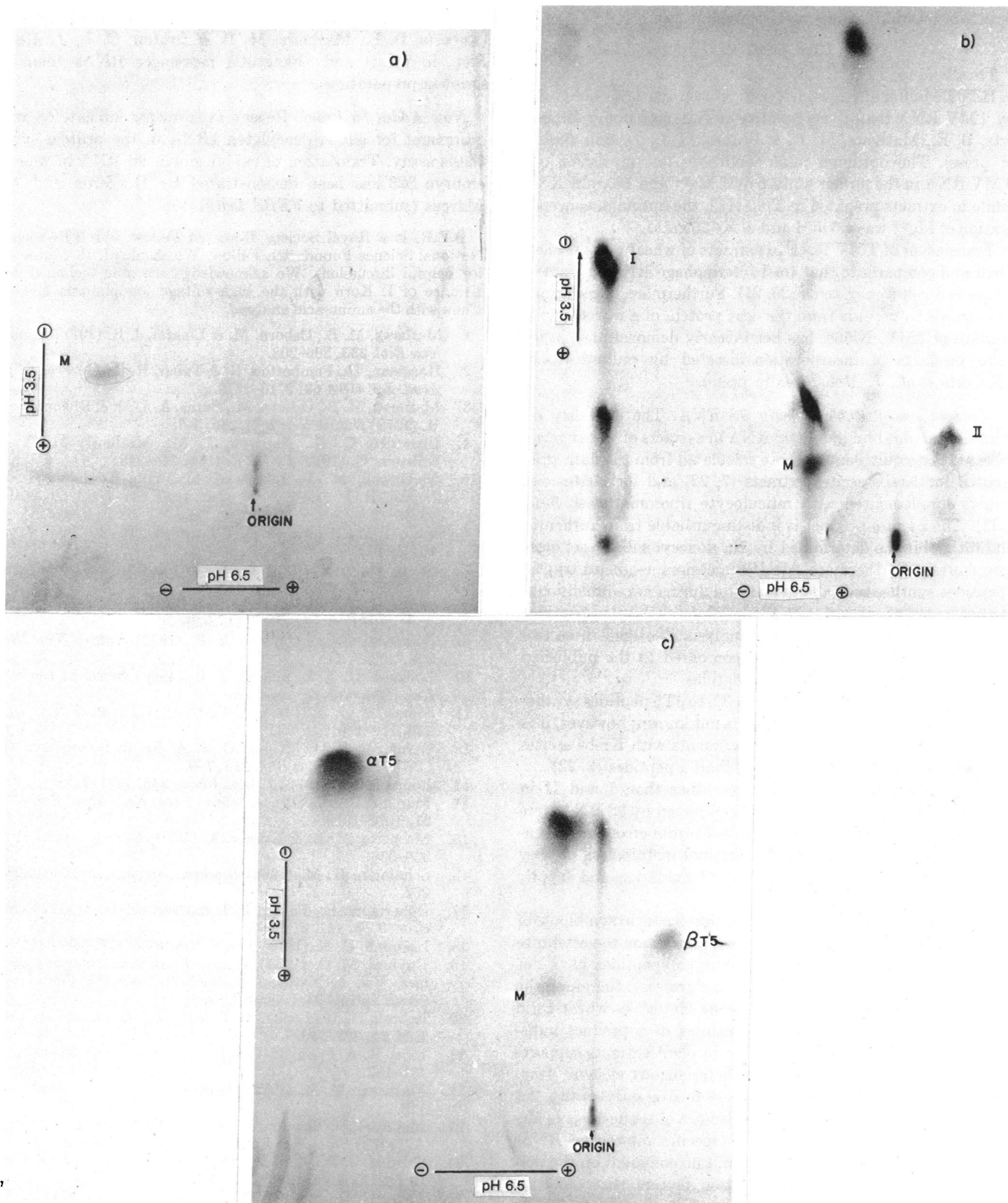


FIG. 3. Autoradiographs of electropherograms of tryptic peptides. Proteins labeled with [35S]methionine synthesized in reactions containing wheat-germ extracts (*a*) without added RNA (29,000 cpm) and (*b*) with 1.25 μg of globin 9S RNA (900,000 cpm), and also (*c*) [35S]methionine-labeled authentic rabbit globin (350,000 cpm) were digested with trypsin. The peptides were fractionated by ionophoresis on Whatman 3 MM paper at pH 6.5 at 3 kv for 60 min in the first dimension and at pH 3.5 at 3 kv for 60 min for (*a*) and (*b*) and 90 min for (*c*) in the second dimension. The spot indicated as *M* corresponds to methionine sulphone. The dried paper was autoradiographed for 6 days.

gated. Other eukaryotic messenger RNAs stimulate protein synthesis almost as efficiently as globin 9S RNA in the wheat-germ S-30 fraction (manuscript in preparation).

## DISCUSSION

*Translation of TMV RNA.* Extracts of wheat germ prepared in HEPES buffer are more active in protein synthesis directed by TMV RNA than those isolated in Tris·HCl buffer (Roberts, B. E., Mathews, M. B. & Bruton, C. J., *J. Mol. Biol.*, in press). The optimum ionic conditions for translation of TMV RNA in the former was 2.5 mM $Mg^{2+}$ and 100 mM $K^+$, while in extracts prepared in Tris·HCl, the optimum concentration of $Mg^{2+}$ was 4.5 mM and of $K^+$, 55 mM.

Translation of TMV RNA in extracts of wheat germ is efficient and compares to that for bacteriophage RNA in *Escherichia coli* cell-free systems (20, 21). Furthermore, translation is accurate; a peptide from the coat protein of a nitrous-acid mutant of TMV, Ni568, has been clearly demonstrated in *in vitro* products of incorporation directed by mutant RNA (Roberts *et al.*, *J. Mol. Biol.*, in press).

*Translation of Rabbit Globin 9S RNA.* The efficiency of translation of rabbit globin 9S RNA in extracts of wheat germ was at least equivalent to those calculated from the data presented for Krebs ascites extracts (7, 22) and for ascites extracts supplemented with reticulocyte ribosomal wash fluid (23). The *in vitro* product is indistinguishable from authentic rabbit globin, as determined by Na dodecyl sulfate–gel electrophoresis. Furthermore, two [$^{35}$S]methionine-labeled tryptic peptides synthesized *in vitro* coincide during two-dimensional paper ionophoresis with two [$^{35}$S]methionine-labeled peptides from rabbit globin. Amino-acid analysis identified these two peptides as $\alpha$T5 and $\beta$T5 when compared to the published sequences for $\alpha$ and $\beta$ globin polypeptides.

The reason for the high ratio of $\alpha$T5 to $\beta$T5 peptides synthesized in wheat-germ S-30 fraction is not known; however, it is being studied in comparative experiments with Krebs ascites extract, which synthesizes more $\beta$ than $\alpha$ peptides (2, 22).

[$^{35}$S]Methionine-labeled peptides other than I and II in digests of products of incorporation directed by 9S RNA were not studied. However, they may result from erroneous initiation or failure to cleave the N-terminal methionine, or they may be an artifact of performic-acid oxidation and tryptic digestion.

Synthesis of a globin-like polypeptide occurs in symbionts of legumes and bacteria (30). However, its relation to vertebrate globin is questioned (19). Analogous polypeptides have not been reported for the Gramineae (the grasses); therefore, the presence of a "globin mRNA specific factor" in wheat-germ extracts is unlikely. Efficient synthesis of a product indistinguishable from authentic globin in plant extracts supports previous observations made in heterologous systems from animal origin (1–4, 7–9, 11, 12) and in frog oocytes (24, 25) and would appear to refute the existence of tissue-specific factors essential for translation of specific messenger RNAs (26–29). However, these data permit no comment on the possible existence of specific initiation factors that facilitate the more efficient translation of certain messenger RNAs. The advantages of using a preincubated wheat-germ extract as a heterologous cell-free system are as follows: (*a*) Rapid preparation of large quantities of extract from easily available starting material. (*b*) Low protein synthesis in extracts incubated in the absence of added RNA. (*c*) Marked stimulation of amino-acid incorporation by messenger RNAs of viral or animal origin, which, combined with (*b*), facilitates rapid and accurate autoradiographic location of the product and/or its tryptic peptides. (*d*) Ability to synthesize large polypeptides (>50,000 daltons) reported separately for TMV RNA (Roberts, B. E., Mathews, M. B. & Bruton, C. J., *J. Mol. Biol.*, in press) and eukaryotic messenger RNAs (manuscript in preparation).

*Note Added In Proof.* Recent experiments indicate no requirement for added deacylated tRNA in the protein synthesis assay. Translation of rabbit globin 9S RNA in wheat embryo S23 has been demonstrated by D. Efron and A. Marcus (submitted to *FEBS Lett.*).

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