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Characterization of a highly efficient protein synthesizing system derived from commercial wheat germ*

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ABSTRACT

A crude extract of commercial wheat germ is capable of translating mRNAs from widely different sources with high efficiencies. Of six wheat germs analyzed only one was found capable of a high level or incorporation with natural mRNAs. Under optimum conditions at a saturating level of Tobacco Mosaic Virus (TMV) RNA (4.5 μg) and labeled amino acid, 68% of all the available ^{14}C leucine is incorporated in 70 min. at 30°C with a stimulation of 425 fold above background (with an efficiency of 252 moles leucine/mole TMV RNA). Thus this system which is 30 fold more efficient for TMV translation than previous reported wheat germ cell free systems is capable of yielding 568 pmoles of ^{14}C leucine incorporated into protein in a 50 μl assay. 80% of the proteins produced have a molecular weight greater than TMV coat protein (17,400). This level of incorporation requires optimization of extract concentration, pH, Mg^{+2}, K^{+} and spermine concentration as well as the method of extract preparation.

Samples of crude polysomal RNA from hen oviducts (3% mRNA) and chorionating moth follicular cells (1% mRNA) are also translated in the wheat germ cell free system with high efficiency.

INTRODUCTION

Crude extracts of commercial wheat germ have recently been shown capable of translating mRNAs from several different sources. These consist of TMV RNA (1), SV40 (2), rabbit globin RNA (1), collagen mRNA (3), BMV RNA (4) and QB RNA (4). Studies of the products made in vitro directed by various mRNAs in the cell free wheat germ (1-6) and wheat embryo systems (7-10) indicate faithful translation. However, although many mRNAs are capable of directing the synthesis of their various proteins in the wheat system, the relative efficiencies of these mRNAs are apparently quite different and further study will require their individual optimization.

In order to use the wheat germ cell free system to study the translation of various mRNAs and to analyze translational control processes, a readily available commercial source of wheat germ is needed. In this communication we report the analysis of six commercially available wheat germs only one of which was found capable of yielding a highly efficient protein synthesizing system (Old Stone Mill raw wheat germ). The other wheat germs tested gave either no stimulation whatever or a stimulation two orders of magnitude lower than the highly active germ. The reaction parameters for optimum translational efficiency for TMV RNA were determined and for the most part appear to be similar to the optimum conditions for oviduct and moth follicular cell polysomal RNA translation. A wheat germ protein synthesizing system has distinct advantages over other cell free systems. These advantages involve the ease of preparation (75 min), the ready availability in large quantities, low cost and the high translational efficiency obtainable.

MATERIALS AND METHODS

Preparation of Wheat Germ Extract. A crude wheat germ extract was prepared by modification of the procedures of Roberts et al. (1) and Marcus et al. (11). All steps were performed at 4°C. Generally 2 grams of raw wheat germ were ground dry for approximately 60 seconds in a cooled mortar with an equal weight of powdered glass (obtained by crushing pasteur pipettes). Four mls of extraction buffer containing 20 mM Hepes (pH 7.6) (adjusted with KOH), 100 mM KCl, 1 mM MgAc, 2 mM $CaCl_2$ and 1 mM DTT was then added followed by gentle swirling for 15-30 seconds. The resultant thick paste was scraped out of the mortar with a spatula and spun at 16,000 rpm for 12 min. in a Sorvall SS34 rotor. After centrifugation, the supernatant was removed with a pasteur pipette taking care to avoid the thick upper fatty layer. At this point in the procedure, the A_{260} was found to be 400 with an A_{260}/A_{280} ratio of 1.33. One ml of supernatant was then

applied to a Sephadex G-25 column (medium) (21 x 1 cm) which was equilibrated and eluted with a buffer containing 20 mM Hepes (pH 7.6), 120 mM KCl, 5 mM MgAc and 1 mM DTT. The sample was eluted at a flow rate of 3 ml/min. and 0.5 ml fractions were collected. The total running time including sample addition was 5 min. All fractions higher than 90 OD/ml A_{260} were pooled (A_{260}/A_{280} = 1.5) and centrifuged for 20 min. at 16,000 rpm in an SS34 rotor. This entire procedure was generally completed in 75 min. The A_{260} of the resultant supernatant was 75 to 90 OD/ml with an A_{260}/A_{280} of 1.6-1.65 and 15 μl was used to obtain the optimum level of amino acid incorporation in a 50 μl assay.

The amount of extract made available by this procedure is ample for several hundred assays and is stored in polypropylene microfuge tubes (Brinkman Inc.) in liquid N_2 to maintain stability for long periods of time. It was found that prolonged storage at -70°C led to a decrease in extract activity.

Preparation of TMV RNA. TMV RNA was isolated by the procedure of Marcus, Efron and Weeks (11). It was stored in small aliquots at -70°C to maintain stability.

Protein Synthesis Assay. The complete system in a final volume of 50μl contained: 15 μl of wheat germ extract following the second S30 spin, 20 mM Hepes (pH 7.4) (adjusted with KOH), 2 mM DTT, 1 mM ATP (neutralized with KOH), 20 μM GTP, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase, 2.5 mM MgAc, 100 mM KCl, .25 μc of ^{14}C leucine (312 μc/μmole) and 25 μM of all 19 unlabeled amino acids.

Assays were incubated for 60 min. at 30°C. The reactions were stopped by addition of 1 ml of 5% trichloracetic acid (TCA) and were then taken up to 90°C for 15 min. and finally cooled in ice water for 1 to 2 min. The resultant protein precipitates were mixed thoroughly and collected

onto Whatman GF/A filters (previously moistened with cold 2% TCA). Assay tubes were rinsed four times with 2% TCA and all filters were rinsed an additional four times. Filters were dried at 150°C for 15 min. and counted in omnifluor-toluene in a Nuclear Chicago scintillation counter with a ^{14}C efficiency of 95%.

Crude polysomal RNA from the chorionating follicular cells of Antheraea polyphemus and crude polysomal RNA of hen oviducts were the generous gifts of Dr. Richard Gelinas and Dr. Richard Palmiter respectively of Harvard University, Cambridge, Mass. TMV (variety vulgare) infected leaves was a generous gift of Dr. Barbara Filner of Columbia University. The variety of wheat germ found to yeild a highly efficient cell free system was purchased from Niblach's Inc. of Rochester, New York (Brand name; Old Stone Mill Wheat Germ) and was stored at 4°C. Other wheat germs tested were purchased from Sigma Biochemicals, Shiloh Farms and several local health food suppliers (Brand names: Crown, Elam's and Golden Harvest raw wheat germs), Creatine phosphate (disodium salt), creatine phosphokinase (powder), spermine tetrahydrochloride, Hepes, ATP (disodium salt), GTP (sodium salt) and cycloheximide were all purchased from Sigma Biochemicals. ^{14}C leucine (312 μc/μmole) was purchased from Schwarz Mann.

RESULTS AND DISCUSSION

Under the conditions described in this communication, high levels of efficiency were obtained for the translation of TMV RNA, polysomal RNA of chorionating moth follicular cells and polysomal RNA of hen oviducts in a cell free system derived from commercially available wheat germ (Niblach's Inc. of Rochester, N.Y.,; Brand name: Old Stone Mill). This activity was shown to be highly dependent on an energy generating system, very sensitive to inhibition by cycloheximide and stimulated an additional 60% in the presence of 30 μg/ml of spermine tetrahydrochloride as shown in Table I. The additional stimulation due to sper-

mine was only found with TMV RNA and not with oviduct or moth follicular cell mRNAs. Interestingly, a polyamine, similar to spermine, has been found in TMV virions (12). The level of efficiency (mole amino acid/mole TMV RNA) is approximately 30 fold higher than previously reported for the incorporation of ^{14}C leucine in a wheat germ system (1) and approximately 13 fold higher for the incorporation of leucine directed by TMV in a wheat embryo cell free system (11). However, recently the incorporation with ^{35}S methionine has been enhanced by approximately one order of magnitude over the level previously reported (Bryan Roberts, personal communication). In addition, the total amount of leucine incorporated, directed by a saturating level of TMV RNA, is quite comparable to the level of leucine obtained using BMV RNA in a wheat germ cell free system as described by Davies and Kaesberg (4). Using the wheat germ described in this report, the optimum levels of amino acid incorporation directed by TMV RNA occurred at 2.5 mM $Mg^{+}2$ (Fig. Ia), 95-110 mM K^{+} (Fig. Ib), pH 7.4 (Hepes) (Fig. Ic) and in the presence of 30 μg/ml of spermine (Fig. Id).

The Mg^{++} and K^{+} optimums for TMV RNA (Fig. Ia and Ib) are essentially the same as previously described by Roberts and Paterson (1). However, both the shapes and slopes of the curves are substantially different in both cases. The Mg^{++} optimum for oviduct polysomal RNA and moth follicular cell polysomal RNA appear to be similar to the TMV optimum (2.5 mM). While the K^{+} optimum for TMV and moth polysomal RNA appear the same (90-110 mM), the K^{+} optimum for oviduct polysomal RNA is somewhat lower (80-95 mM).

The pH optimum of the wheat germ system tested is 7.4 (Fig. Ic). The protein synthetic activity drops to about 30% of the maximum stimulation directed by TMV at $\pm$ 0.3 pH units around the optimum. The pH of the extract after the second centrifugation was found to be 6.7. In order

Table I

Amino acid incorporation directed by TMV RNA, hen oviduct polysomal RNA and moth follicular cell polysomal RNA

	CPM[b]	Stimulation Above Background (+mRNA/-mRNA)	Efficiency moles ^{14}C leucine/ mole mRNA
Complete System[a] (+4.5 μg TMV RNA)	215,000	268	160[c]
+ spermine tetrahydrochloride (30 μg/ml)	340,000	425	252[c]
- mRNA	800	-	
+ cycloheximide (10 μg/50λ	1,400	1.7	
- (creatine phosphate and creatine phosphokinase)	4,000	5.0	
- ^{12}C amino acid mixture	100,000	125	
Complete System (+4.2 μg oviduct polysomal RNA)[f]	6,000	7.6	70[d]
Complete System (+10 μg moth follicular cell polysomal RNA)	56,200	70.2	140[e]

a. The complete system is described in materials and methods
b. Incubation at 30°C for 60 min with ^{14}C leucine (312 μc/μmole)
c. With the approximate molecular weight of TMV RNA as 2×10^6 (15)
d. 3% of crude hen oviduct polysomal RNA consists of mRNA and 1 μg of mRNA constitutes approximately 1 pmole of mRNA (16)
e. 1% of crude polysomal RNA of the chorionating follicular cells of Antheraea polyphemus consists of mRNA and assuming the average molecular weight is 1.5×10^5 (17)
f. At a K^+ of 90 mM with all other components as described in materials and methods.

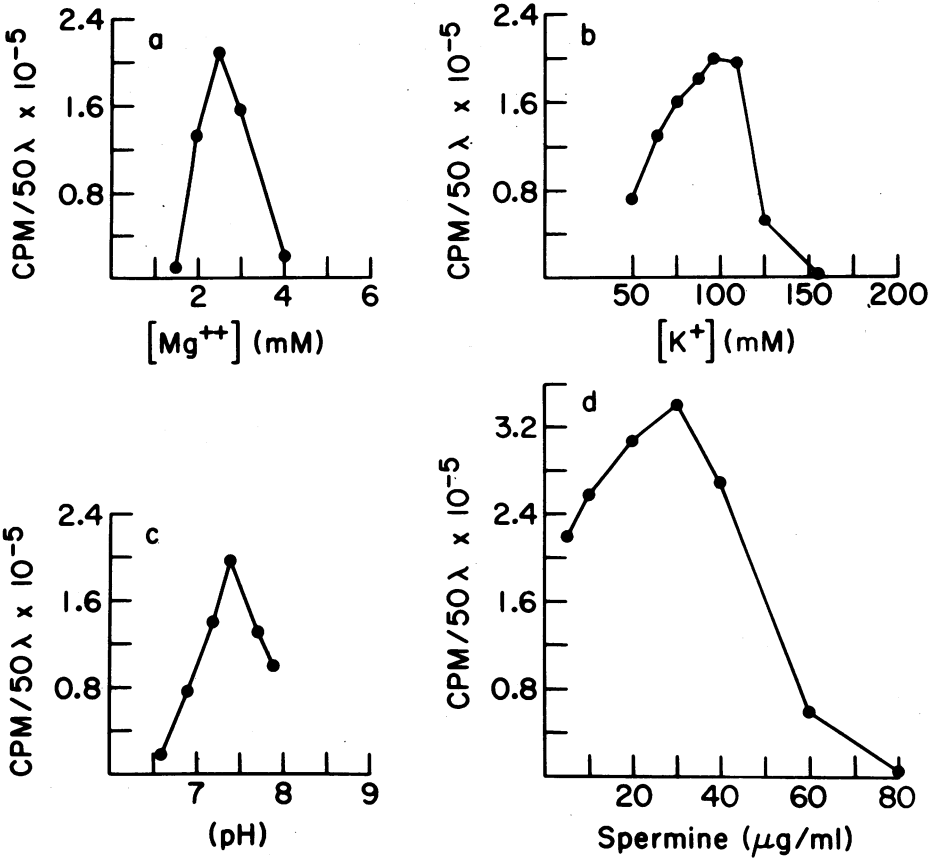

Figure I

All incubations were carried out for 60 min. at 30°C with 4.5 μg of TMV RNA per 50 μl assay. (a) Magnesium concentration scan at 110 mM K^+ and pH 7.4. (b) Potassium concentration curve with 2.5 mM MgAc at pH 7.4. (c) pH curve with 2.5 mM MgAc and 110 mM K^+. (d) Spermine tetrahydrochloride concentration scan with 110 mM K^+, 2.5 mM MgAc at pH 7.4.

to obtain a final pH of 7.4 in the reaction, Hepes buffer at pH 8.1 was added to the assay to yield a final concentration of 20 mM Hepes.

Spermine stimulates the incorporation directed by TMV at a range of concentrations (i.e., 10-45 μg/ml) (Fig. Id). The addition of spermine (20 μg/ml) has no effect upon moth follicular cell polysomal RNA and appears to have an inhibitory effect on oviduct polysomal RNA translation in the wheat germ cell free system.

The protein synthetic activity with TMV RNA saturates at an RNA concentration of 90 μg/ml (Fig. IIc) and reaches termination in 70 min. at 30°C (Fig. IId). The time course of incorporation directed by TMV (in the absence of spermine) appears to indicate a lag period of 3 min. followed by linear incorporation for 60 min. until termination at 70 min. (the degree of incorporation between 60 and 70 min. generally amounts to an additional 3%). ^{14}C leucine at .25 μc/50 μl (16.7 μM leucine) is required to saturate the system. Isotopic dilution experiments indicate no apparent level of unlabeled leucine in the extract. At limiting concentrations of leucine (i.e., .05 μc/50 μl, 3.3 μM leucine), essentially 100% of the ^{14}C leucine added to the reaction is incorporated into radioactive protein in 60 min. (Fig. IIa). The effect of extract concentration on amino acid incorporation indicates that the activity does not rise in a linear fashion with the extract (Fig. IIb). The incorporation into radioactive protein saturates at an extract concentration of 22 OD/ml A_{260} with no additional requirement for tRNA. The slight inhibition noted at a concentration of 26 OD/ml (A_{260}) is due to a 10% rise in the Mg^{+2} concentration. At a Mg^{+2} concentration of 2.75 mM, the activity (in the absence of spermine) drops by approximately 10% (Fig. Ia). Analysis of the TMV proteins produced on 14% polyacrylamide slab gels indicate a large and complex distribution of proteins of molecular weights 9,000 to 80,000 with 20% of the proteins of molecular weight lower than TMV coat

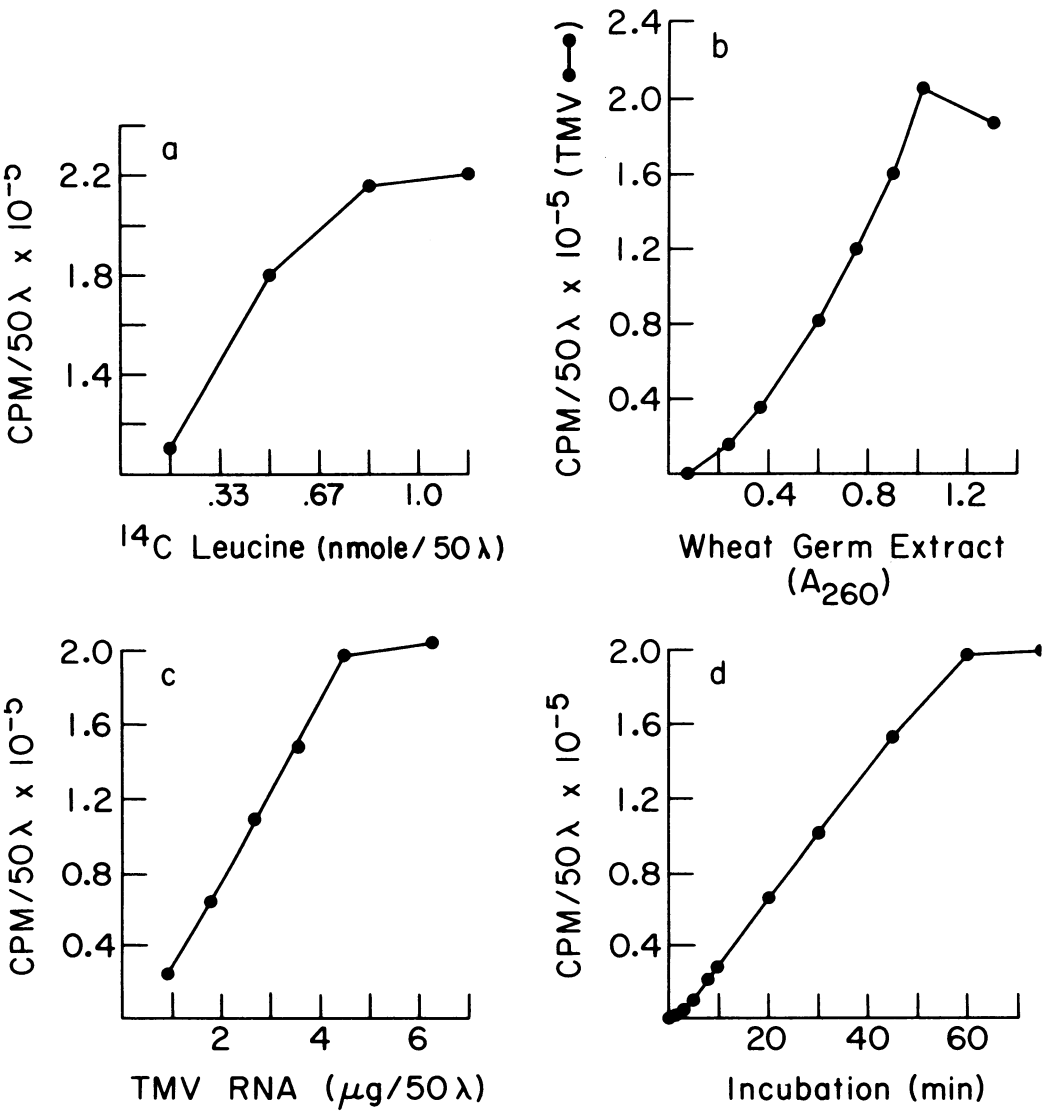

Figure II

All incubations were carried out for 60 min. at 30°C with 110 mM K^+, 2.5 mM MgAc at pH 7.4. (a) Level of protein synthetic activity at various ^{14}C leucine concentrations. (b) Effect of extract concentration on TMV translation (shown as amount of extract in A_{260} units added to a 50 μl assay). (c) Effect of TMV RNA concentration on protein synthetic activity. (d) Time course of protein synthesis in the absence of spermine was carried out in a 100 μl assay from which 5 μl aliquots were obtained at the times indicated and corrected for the level of radioactive protein in 50 μl.

protein (17,400). Similar complex distributions of proteins have been previously observed in TMV directed in-vitro protein synthesizing systems (5-7). Unfortunately, it is not known whether the translation of the high molecular weight proteins is correct at the present time.

Next, we investigated the relative abilities of different wheat germ extracts to support protein synthesis directed by TMV RNA and Poly U. Five other sources of wheat germ were tested and found to give protein synthetic activities far below that obtained with the Old Stone Mill variety (Table II). All these extracts were essentially as concentrated as the highly active one, and all were capable of stimulating protein synthesis directed by Poly U at relatively high levels of Mg^{++} (7-10 mM). Aminoacylation assays indicated active tRNA synthetases in all the germs tested. Mixing experiments (performed by combining aliquots of active

Table II

Protein Synthetic Activity of Various Wheat Germs directed by TMV RNA

	TMV (4.5 μg)	CPM+	Stimulation Above Background
Sample A	+	400	1.2
" "	-	330	-
Sample B	+	460	1.3
" "	-	360	-
Sample C	+	1740	2.5
" "	-	700	-
Sample D	+	1000	2.0
" "	-	500	-
Sample E	+	750	1.07
" "	-	700	-

A. Untreated wheat germ purchased from Sigma Biochemicals
B. Raw chunk wheat germ obtained from Shiloh Farms of Sulphur Springs, Arkansas
C. Crown natural untreated wheat germ distributed by Centennial Mills of Portland, Oregon
D. Golden Harvest raw wheat germ distributed by Natural Sale Co. of Pittsburgh, Pennsylvania
E. Elam's raw wheat germ distributed by Elam Mills of Broadview, Illinois
\+ Incubated for 60 min. at 30°C with ^{14}C leucine (312 μc/μmole) in the complete system as described in materials and methods.

and various inactive extracts) suggest the presence of inhibitors of protein synthesis directed by TMV RNA. Similar results with natural mRNAs and raw, untreated, food store wheat germ have been reported (4).

The reproducibility of the high level of efficiency described in this study requires several precautions with respect to the preparation of the wheat germ extract. Grinding with powdered glass in the absence of buffer (see Materials and Methods) was found to be the gentlest and most efficient manner of preparation. Grinding for longer periods of time was found to result in a variable level of efficiency in protein synthesis and in inhibition which became quite substantial if the grinding time was extended for 4 min. or more. After grinding, the extract is kept at a relatively high concentration (400 OD/ml A_{260}) in order to obtain a concentrated eluant following G-25 chromatography. In general, an additional centrifugation following the desalting step was found to yield an extract which gave more reproducibly high activity. Using this procedure, 15 μl of extract in a 50 μl assay is required to obtain the maximum level of activity. Of course, larger amounts of extract can be added if the concentration is lower and compensations can be made to avoid alterations in the Mg^{++}, K^{+}, DTT and Hepes concentrations. However, adding the extract in the smallest possible volume allows more freedom for the addition of other components, particularly mRNAs.

The high efficiencies of translation (moles amino acid incorporated/mole mRNA) reported in this paper for widely different mRNAs in a wheat germ extract further indicates the relative lack of specificity inherent in the wheat germ cell free system. Crude oviduct polysomal RNA is capable of directing the synthesis of ovalbumin in this wheat germ system as determined by immunoprecipitation (D. Lipsky and K. Marcu unpublished observation). Polysomal RNA of the chorionating follicular cells of Antheraea polyphemus directs the synthesis of completed protein products in a wheat

germ cell free system derived from Bar Rav Mill wheat germ. These products co-migrate with authentic egg shell proteins on polyacrylamide gels (R. Gelinas, personal communication). Similarly, analysis of the insect proteins synthesized in this study on 14% SDS polyacrylamide slab gels indicate proteins of molecular weights 7,000 to 20,000 which agrees with the size range of proteins analyzed by in vivo studies (13). 10% of the protein products are lower than 7,000 molecular weight. However, in comparing the relative efficiencies of moth polysomal RNA with these two varieties of active wheat germ, it appears that the assay described in this communication is capable of yielding at least a 30 fold greater efficiency of translation. A comparison of the levels of various insect proteins synthesized in these two wheat germ systems is currently under investigation.

Many studies involving cell free protein synthesis are more easily approachable with a highly efficient protein synthesizing system with a low endogenous level of incorporation. At present, our studies show essentially no endogenous mRNA activity. The level of incorporation without exogenous mRNA cannot be altered by adding high levels of cycloheximide or by omitting the energy generating system. However, the background level of hot TCA precipitable material is incubation time dependent and this may be due to aminoacyl-tRNA-protein transferase activities in the extract (14). This background level of incorporation remains unaffected following preincubation of the extract as previously described (1) and is tRNA dependent.

Due to the high level of efficiency of so many different mRNAs and the obvious ease of preparation of these extracts, the wheat germ cell free system can be utilized as a screening technique for the isolation and purification of mRNAs. The percentage of low molecular weight polypeptides produced appears to vary with the particular mRNA. As is apparent with hen oviduct and moth polysomal RNA, small quantities (several micro-

grams) of relatively crude preparations of messengers (1-3% mRNA) yield high levels of incorporation. However, it is not known whether all mRNAs translated in this particular wheat germ cell free system would exhibit similar efficiencies of translation. Studies with other mRNAs will require their individual optimization.

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