Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 330–333, January 1985
Biochemistry

Isolation and characterization of factors from wheat germ that exhibit eukaryotic initiation factor 4B activity and overcome 7-methylguanosine 5′-triphosphate inhibition of polypeptide synthesis

(eukaryotic protein synthesis/capped and uncapped mRNAs)

SANDRA LAX, WILLIAM FRITZ, KAREN BROWNING, AND JOANNE RAVEL

Department of Chemistry and Clayton Foundation Biochemical Institute, The University of Texas, Austin, TX 78712

Communicated by Lester J. Reed, September 17, 1984

ABSTRACT **Three highly purified preparations (preparations I, II-1, and II-2) have been obtained from wheat germ and shown to support *in vitro* polypeptide synthesis directed by capped or uncapped mRNAs in a eukaryotic initiation factor 4B (eIF-4B)-deficient system. The three preparations differ, however, in polypeptide composition and in the ability to overcome the inhibitory effect of 7-methylguanosine 5′-triphosphate (m^7GTP) on *in vitro* polypeptide synthesis. Preparation I contains two polypeptides (M_r = 80,000 and 28,000), which are present in a 1:1 molar ratio and are associated in a high molecular weight complex. Preparation II-1 contains two major polypeptides (M_r = 220,000 and 26,000) and preparation II-2 also contains two major polypeptides (M_r = 110,000 and 26,000). Preparations II-1 and II-2 are high molecular weight complexes; neither contains detectable amounts of a M_r 80,000 or a M_r 50,000 component. Preparations II-1 and II-2 both overcome m^7GTP inhibition, whereas preparation I does not. These findings raise several questions with regard to the identity of eIF-4B and its relationship to cap recognition factors.**

Eukaryotic initiation factor 4B (eIF-4B) has been purified by several groups of investigators and identified as an 80,000-dalton polypeptide that is required for initiation of polypeptide synthesis (1–7). Some preparations of eIF-4B also contain a 24,000-dalton polypeptide that crosslinks to the 5′ end of capped mRNA (8). Preparations of the 24,000-dalton cap binding protein (CBP) free of the 80,000-dalton polypeptide have been obtained from rabbit reticulocytes (9, 10). This factor, designated CBP I, stimulates the translation of capped mRNAs (11) and overcomes 7-methylguanosine 5′-triphosphate (m^7GTP) inhibition of polypeptide synthesis *in vitro* (10). The 24,000-dalton CBP has also been isolated as part of a protein complex, designated CBP II (12) or eIF-4F (13), which contains two higher molecular weight polypeptides (M_r = 200,000 and 46,000) but no M_r 80,000 polypeptide. The 46,000-dalton polypeptide has been identified as eIF-4A (13, 14). CBP II or eIF-4F stimulates globin synthesis in an eIF-4B-deficient system (13) and restores translation of capped mRNAs in extracts of polio virus-infected cells (12, 13, 15).

In this investigation, we have obtained three highly purified preparations from wheat germ, all of which support polypeptide synthesis in an *in vitro* system deficient in eIF-4B. These preparations differ, however, in polypeptide composition and in the ability to overcome m^7GTP inhibition of polypeptide synthesis. Preparation I contains two polypeptides (M_r = 80,000 and 28,000) in close to a 1:1 molar ratio. Preparation II-1 contains two major polypeptides (M_r = 220,000 and 26,000) and preparation II-2 also contains two major polypeptides (M_r = 110,000 and 26,000). Neither preparation II-1 nor II-2 contains detectable amounts of a M_r 80,000 polypeptide. Preparations II-1 and II-2, but not preparation I, overcome m^7GTP inhibition of polypeptide synthesis in a system containing saturating levels of eIF-4B. A preliminary report of this work has been published (16).

MATERIALS AND METHODS

Materials. Wheat germ was kindly supplied by J. M. deRosier (International Multifoods, Minneapolis, MN). Wheat germ extracts were prepared as described (17) except that 0.5 mM phenylmethylsulfonyl fluoride and 0.1 mg of soybean trypsin inhibitor (STI) per ml were added to the extraction buffer. Salt-washed ribosomes and 0–40% and 40–60% ammonium sulfate fractions from the 120 mM KCl postribosomal supernatant were prepared as described (4). m^7GTP was obtained from Pharmacia P-L Biochemicals and m^7GTP linked to Sepharose through the γ-phosphate (18) was kindly supplied by Robert Rhoads (University of Kentucky, Lexington). Yeast polysomal RNA was prepared by a modification of a previously described procedure (4) and satellite tobacco necrosis virus RNA (STNV RNA) was obtained as described (19).

Assays for eIF-4B and m^7GTP Reversal Activities. The standard reaction mixture contained in 100 μl: 24 mM Hepes·KOH, pH 7.6/2.4 mM dithiothreitol/0.1 mM spermine/35 mM KCl/95 mM KOAc/3 mM $Mg(OAc)_2$/34 μM [^{14}C]leucine (200 cpm/pmol)/50 μM of the other 19 amino acids/1 mM ATP/0.2 mM GTP/7.8 mM creatine phosphate/3 μg of creatine phosphokinase/0.75 A_{260} unit of wheat germ tRNA/1–2 A_{260} units of yeast polysomal RNA/2.5–3.0 A_{260} units of salt-washed wheat germ ribosomes and additional supplements as indicated. The reaction mixture was incubated for 30 min at 25°C and the amount of [^{14}C]leucine incorporated into hot trichloroacetic acid-insoluble material was determined as described (4). To measure eIF-4B activity, the standard reaction mixture was supplemented with 600 μg of the 40–60% ammonium sulfate fraction and 8 μg of eIF-3 prepared by a modification of a previously described procedure (20). A unit of eIF-4B activity is defined as a 1 pmol increase in the amount of leucine incorporated into polypeptide. To assay for m^7GTP reversal activity the standard reaction mixture was supplemented with 300 μg of the 0–40% ammonium sulfate fraction, 600 μg of the 40–60% ammonium sulfate fraction, and 25 μM m^7GTP. A unit of m^7GTP reversal activity is defined as a 1 pmol increase in the amount of leucine incorporated into polypeptide in the presence of 25 μM m^7GTP. Protein concentrations were determined with Bio-Rad reagent by the method of Bradford (21).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: eIF, eukaryotic initiation factor; m^7GTP, 7-methylguanosine 5′-triphosphate; CBP, cap binding protein; STI, soybean trypsin inhibitor; STNV, satellite tobacco necrosis virus.

Chromatography of the 0–40% Ammonium Sulfate Fraction on DEAE-Cellulose. The 0–40% ammonium sulfate fraction of the 120 mM KCl postribosomal supernatant derived from 200 g of wheat germ was dialyzed against buffer B (20 mM Hepes·KOH, pH 7.6/0.1 mM EDTA/1 mM dithiothreitol/10% glycerol) containing 40 mM KCl (buffer B-40). These and all subsequent procedures were carried out at 4°C and all samples were stored at −70°C. The sample (75 ml containing 2.2 g of protein) was applied to a 200-ml DEAE-cellulose column (Whatman DE52) equilibrated in buffer B-40. After washing with buffer B-40, the column was developed with a 600-ml linear gradient from 40 to 150 mM KCl in buffer B. Fractions of 10 ml were collected and aliquots of the fractions were assayed for eIF-4B and m^7GTP reversal activities. A typical elution profile is shown in Fig. 1. The column was subsequently developed with a second linear gradient from 150 to 300 mM in buffer B to remove eIF-3 from the column.

Chromatography of DEAE Peak I and Peak II Fractions on Phosphocellulose. The peak I fractions from two DEAE-cellulose columns were pooled (designated DE I) and the sample (200 ml containing 120 mg of protein) was applied to a 12-ml phosphocellulose column (Whatman P11) equilibrated in buffer B-80. The column was washed with buffer B-80 and then developed with a 60-ml linear gradient from 80 to 300 mM KCl in buffer B. Fractions of 1.2 ml were collected. The eIF-4B activity eluted from the column between 170 and 210 mM KCl. Fractions containing high amounts of eIF-4B activity were pooled and designated PC I.

The peak II fractions from two DEAE-cellulose columns were pooled (designated DE-II) and the sample (200 ml containing 200 mg of protein) was applied to a 20-ml phosphocellulose column equilibrated in buffer B-120. The column was washed with buffer B-120, followed by buffers B-170, B-250, and B-400. Fractions of 2 ml were collected. The 250 mM KCl fractions containing eIF-4B and m^7GTP reversal activities were pooled and designated PC-II-1; the 400 mM KCl fractions containing eIF-4B and m^7GTP reversal activities were pooled and designated PC II-2.

Chromatography of Fractions PC I, PC II-1, and PC II-2 on m^7GTP-Sepharose. A m^7GTP-Sepharose column (0.8 ml) was equilibrated in buffer B-120. The KCl concentration of fraction PC I was lowered to 120 mM by addition of buffer B containing no KCl and the sample (12 ml containing 3 mg of protein) was applied to the column. After washing with 40 column volumes of buffer B-120, the column was washed with buffer B-120 containing 70 μM m^7GTP. Fractions of 0.7 ml were collected and assayed for eIF-4B and m^7GTP reversal activities. The fractions containing protein that was eluted from the column with m^7GTP were pooled and designated mGS I. The m^7GTP-Sepharose column was washed with buffer B-1000 and then with 20 mM Tris·HCl, pH 7.6/0.2% $NaDodSO_4$ prior to reuse (18). Fractions PC II-1 and II-2 were chromatographed on m^7GTP-Sepharose as described above. The fractions containing protein that was eluted from the column with m^7GTP were pooled and designated mGS II-1 and mGS II-2, respectively.

RESULTS

eIF-4B obtained from extracts of wheat germ as described (4, 17) contained, in addition to a M_r 80,000 polypeptide, a number of other polypeptides. These partially purified preparations supported polypeptide synthesis in an eIF-4B-deficient assay system (4) and also overcame the inhibitory effect of m^7GTP on polypeptide synthesis (unpublished data). In an effort to obtain more highly purified preparations of eIF-4B and to separate the eIF-4B and m^7GTP reversal activities, the 0–40% ammonium sulfate fraction from the 120 mM KCl postribosomal supernatant was applied to a DEAE-cellulose column in 40 mM KCl and the column was developed with a linear gradient from 40 to 150 mM KCl. As shown in Fig. 1, eIF-4B activity eluted from the column in two distinct peaks, one between 60 and 90 mM KCl (peak I) and the other between 110 and 130 mM KCl (peak II). m^7GTP reversal activity was observed in both peaks; however, the distribution of the two activities between the peaks was not the same. Close to equal amounts of eIF-4B activity were found in the peaks I and II, whereas more than twice as much m^7GTP reversal activity was found in peak II than in peak I (Table 1).

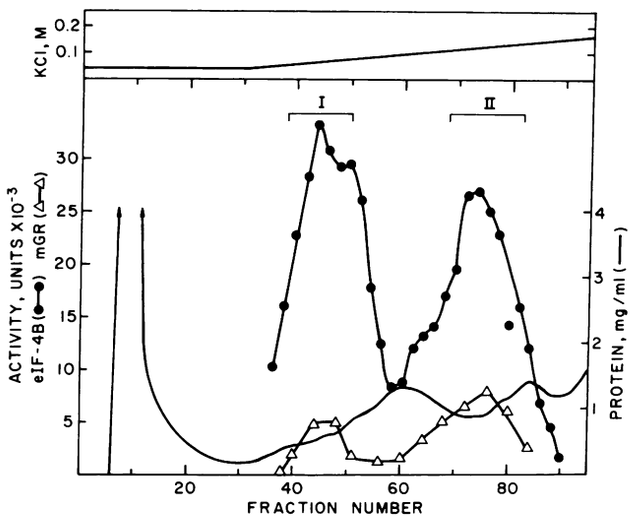

FIG. 1. Chromatography of 0–40% ammonium sulfate fraction on DEAE-cellulose. The 0–40% ammonium sulfate fraction was chromatographed on DEAE-cellulose, and aliquots of the fractions were assayed for the ability to support polypeptide synthesis (●) and to overcome m^7GTP inhibition of polypeptide synthesis (△). mGR, m^7GTP reversal.

When the DE peak I material was chromatographed on phosphocellulose as described in *Materials and Methods*, most of the eIF-4B activity eluted from the column between 170 and 210 mM KCl. Very little m^7GTP reversal activity (≤10%) was found in the fractions containing the eIF-4B activity (PC I). Subsequent adsorption of the PC I material on m^7GTP-Sepharose and elution with m^7GTP yielded a preparation (mGS I) that had high eIF-4B activity and very little, if any, m^7GTP reversal activity. When the DE peak II material was chromatographed on phosphocellulose, eIF-4B and m^7GTP reversal activities were found in both the 250 mM KCl wash (PC II-1) and the 400 mM KCl wash (PC II-2). Subsequent chromatography of these two fractions on m^7GTP-Sepharose yielded two preparations (mGS II-1 and

Table 1. Separation and purification

Fraction	Protein, mg	Units × 10^{-3}		Units/mg of protein × 10^{-3}	
		eIF-4B	m^7GTP reversal activity	eIF-4B	m^7GTP reversal activity
0–40% $(NH_4)_2SO_4$*	4400	—	—	—	—
DE peak I	120	340	54	2.8	0.5
PC I	6	160	—	27	—
mGS I	1	80	—	80	—
DE peak II	200	340	120	1.7	0.6
PC II-1	8	72	27	9	3.4
mGS II-1	0.5	40	15	80	30
PC II-2	5.5	22	13	4	2.4
mGS II-2	0.25	9	6	36	24

*Derived from 400 g of wheat germ.

mGS II-2) that exhibited high eIF-4B activity and high m^{7}GTP reversal activity (Table 1).

Analysis of the phosphocellulose and m^{7}GTP-Sepharose fractions by NaDodSO$_4$/polyacrylamide gel electrophoresis is shown in Fig. 2. The PC I fraction (lane 3) contained large amounts of two polypeptides (M_r = 80,000 and 28,000) and no detectable polypeptides with M_rs greater than 80,000. In contrast, the PC II-1 (lane 4) and PC II-2 (lane 5) fractions contained a large number of polypeptides, several with M_rs greater than 80,000. Subsequent chromatography of the phosphocellulose fractions on m^{7}GTP-Sepharose yielded preparations with the following compositions: mGS I (lane 6), two polypeptides (M_r = 80,000 and 28,000); mGS II-1 (lane 7), two major polypeptides (M_r = 220,000 and 26,000); and mGS II-2 (lane 8), two polypeptides (M_r = 110,000 and 26,000). Estimates of the molar ratios of the polypeptides obtained by scanning the Coomassie blue-stained gel indicated that the molar ratio of the M_r 28,000 polypeptide to the M_r 80,000 polypeptide present in mGS I was ≈1:1. The ratio of the M_r 26,000 polypeptide to the M_r 220,000 polypeptide present in mGS II-1 was ≈4:1, and the ratio of the M_r 26,000 polypeptide to the M_r 110,000 polypeptide present in mGS II-2 was ≈2:1. Attempts to separate the high and low molecular weight polypeptides present in these preparations by chromatography on Sephacryl S-400 in the presence of 0.5 M KCl were unsuccessful. With all three preparations, both the low and high molecular weight polypeptides eluted from Sephacryl S-400 at a position corresponding to a M_r of 400,000 or greater, and the ratios of the two polypeptides remained the same.

A comparison of the abilities of the three m^{7}GTP-Sepharose preparations to support polypeptide synthesis in an eIF-4B-deficient *in vitro* system is shown in Fig. 3. With yeast polysomal RNA (a heterogeneous population of capped mRNAs) the highest amount of polypeptide synthesis was obtained with the mGS II-1 fraction (Fig. 3 *Left*). The mGS I fraction was as active as the mGS II-1 fraction at low levels; however, the maximal amount of polypeptide synthesis obtained was consistently lower. When low levels of the mGS I and the mGS II-1 fractions were both added (0.1 μg of each), the increase in the amount of leucine incorporated into polypeptide was equal to the sum of that obtained with each of the fractions alone, indicating that the effects were additive. When high amounts of the two fractions were both added (0.5 μg of each), the amount of polymerization obtained was no greater than that obtained with 0.5 μg of mGS II-1 alone, possibly because some other factor(s) became limiting. The total amount of polypeptide synthesis obtained with saturating levels of mGS II-1 or a mixture of mGS II-1 and mGS I was ≈70% of that obtained with saturating amounts of the 0–40% ammonium sulfate fraction (see Fig. 4). The mGS II-2 fraction also supported polypeptide synthesis; however, higher amounts of this fraction were required and the maxi-

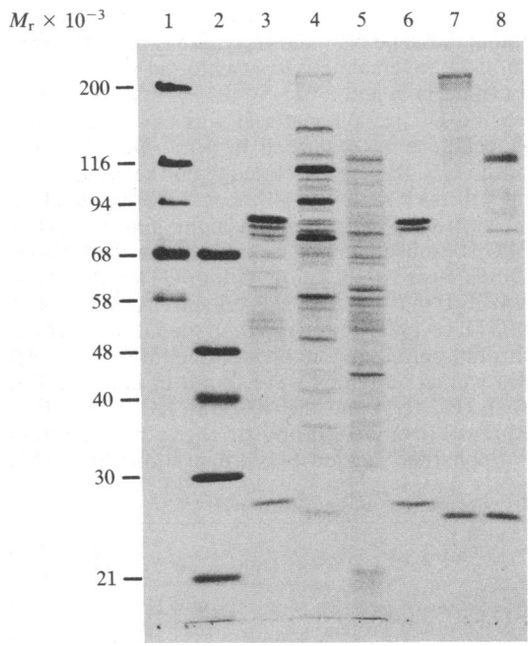

FIG. 2. Electrophoretic analysis of the phosphocellulose and m^{7}GTP-Sepharose fractions. Gel electrophoresis was carried out in the presence of NaDodSO$_4$ by a modification of the procedure of Laemmli (22). The gel contained 12.5% acrylamide and 0.07% bisacrylamide and was stained with Coomassie brilliant blue. Lane 1, myosin (M_r 200,000), β-galactosidase (M_r 116,000), bovine serum albumin (M_r 68,000), and catalase (M_r 58,000); lane 2, bovine serum albumin (M_r 68,000), fumarase (M_r 48,000), aldolase (M_r 40,000), carbonic anhydrase (M_r 30,000), and soybean trypsin inhibitor (M_r 21,000); lane 3, 7.5 μg of PC I; lane 4, 16 μg of PC II-1; lane 5, 15 μg of PC II-2; lane 6, 3.6 μg of mGS I; lane 7, 5.2 μg of mGS II-1; and lane 8, 6.3 μg of mGS II-2.

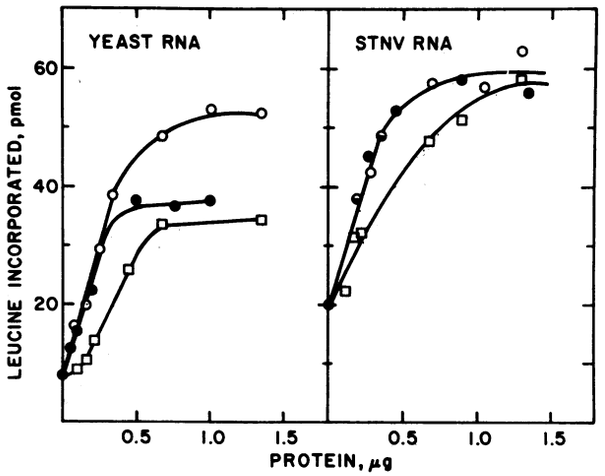

FIG. 3. Comparison of the abilities of mGS I, mGS II-1, and mGS II-2 to support polypeptide synthesis directed by yeast mRNA and STNV RNA in an eIF-4B-deficient system. The eIF-4B-deficient system contained either 1.3 A_{260} units of yeast polysomal RNA (*Left*) or 0.15 A_{260} unit of STNV RNA (*Right*) and either mGS I (●), mGS II-1 (○), or mGS II-2 (□) in the amounts indicated.

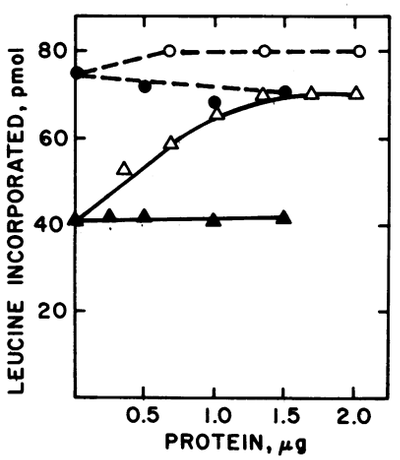

FIG. 4. Comparison of the ability of mGS I and mGS II-1 to overcome m^{7}GTP inhibition. The reaction mixture for measuring m^{7}GTP reversal activity was supplemented with mGS I (●, ▲) or mGS II-1 (○, △), as indicated, without m^{7}GTP (●, ○) or with 25 μM m^{7}GTP (▲, △).

mal amount of polypeptide synthesized was lower than that obtained with the mGS II-1 fraction. Results similar to those described above were obtained when globin mRNA was used (data not shown). With STNV RNA (an uncapped viral RNA), the mGS I and mGS II-1 fractions supported polypeptide synthesis equally well, and the total amount of polypeptide synthesized was the same (Fig. 3 *Right*). Higher concentrations of mGS II-2 were required but the total amount of polypeptide synthesized was the same as that obtained with the other two preparations.

The abilities of the mGS I and mGS II-1 fractions to reverse the inhibitory effect of m^7GTP on polypeptide synthesis directed by yeast mRNA are shown in Fig. 4. At 25 μM m^7GTP, polypeptide synthesis was inhibited about 50%, and the inhibition was overcome completely by addition of the mGS II-1 fraction. The inhibition was also overcome by the mGS II-2 fraction but higher concentrations of this fraction were required (data not shown). In contrast, very little, if any, reversal of m^7GTP inhibition was obtained with the mGS I fraction. Polypeptide synthesis directed by STNV RNA was not inhibited by 25 μM m^7GTP.

DISCUSSION

In this investigation three highly purified preparations have been obtained from wheat germ, all of which support polypeptide synthesis in an eIF-4B-deficient *in vitro* system. These preparations differ, however, in polypeptide composition and the ability to overcome m^7GTP inhibition of protein synthesis. Preparation I (mGS I) appears to be similar to eIF-4B isolated from rabbit reticulocytes and ascites and HeLa cells (2, 6, 7) in that one of the major polypeptides present in this preparation is a M_r 80,000 polypeptide. Preparation I differs, however, from rabbit reticulocyte eIF-4B in that it also contains a M_r 28,000 polypeptide that appears to be present in a 1:1 molar ratio to the M_r 80,000 polypeptide, and the two polypeptides appear to be associated in a high molecular weight complex. Preparation I does not overcome m^7GTP inhibition of polypeptide synthesis.

Preparation II-1 (mGS II-1) appears to be similar to CBP II (12) or eIF-4F (13) in that it is a high molecular weight complex, contains two polypeptides (M_r = 220,000 and 26,000), and reverses m^7GTP inhibition. Preparation II-1 differs from CBP II or eIF-4F in that it does not contain a M_r 48,000–50,000 polypeptide (presumably eIF-4A). Preparation II-2 (mGS II-2) also appears to be similar to CBP II and eIF-4F in that it is a high molecular weight complex, contains a M_r 26,000 polypeptide, and reverses m^7GTP inhibition.

The findings of this investigation raise several questions with regard to the identity of eIF-4B and its relationship to CBP II or eIF-4F. Is eIF-4B a M_r 80,000 polypeptide or is it a complex composed of M_r 80,000 and 28,000 polypeptides? Why does preparation I, which does not contain detectable amounts of M_r 220,000 or 26,000 polypeptides, support protein synthesis directed by capped mRNAs? Conversely, why do preparations II-1 and II-2, which do not contain detectable amounts of a M_r 80,000 polypeptide, support protein synthesis directed by capped or uncapped mRNAs? Is the M_r 110,000 polypeptide in preparation II-2 a degradation product of the M_r 220,000 polypeptide in preparation II-1? Further work is necessary to resolve some of these questions.

We thank Dr. John M. Clark, Jr., for the gift of STNV, Dr. R. E. Rhoads for the m^7GTP-Sepharose, and Glenna Donnell for careful preparation of the manuscript. This work was supported in part by National Institutes of Health Grant GM18775.

1. Schreier, M. H., Erni, B. & Staehelin, T. (1977) *J. Mol. Biol.* **116,** 727–753.
2. Benne, R., Luedi, M. & Hershey, J. W. B. (1977) *J. Biol. Chem.* **252,** 5798–5803.
3. Merrick, W. C. (1979) *Methods Enzymol.* **60,** 101–108.
4. Spremulli, L. L., Walthall, B. J., Lax, S. R. & Ravel, J. M. (1979) *J. Biol. Chem.* **254,** 143–148.
5. Seal, S. N., Schmidt, A. & Marcus, A. (1983) *J. Biol. Chem.* **258,** 859–865.
6. Trachsel, H., Erni, B., Schreier, M. H., Braun, L. & Staehelin, T. (1979) *Biochim. Biophys. Acta* **561,** 484–490.
7. Brown-Luedi, M., Meyer, L. J., Milburn, S. C., Yan, P. M.-P., Corbett, S. & Hershey, J. W. B. (1982) *Biochemistry* **21,** 4202–4206.
8. Sonenberg, N., Morgan, M. A., Merrick, W. C. & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4843–4847.
9. Sonenberg, N., Rupprecht, K. M., Hecht, S. M. & Shatkin, A. J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4345–4349.
10. Hellman, G. M., Chu, L.-Y. & Rhoades, R. E. (1982) *J. Biol. Chem.* **257,** 4056–4062.
11. Sonenberg, N., Trachsel, H., Hecht, S. & Shatkin, A. J. (1980) *Nature (London)* **285,** 331–333.
12. Tahara, S. M., Morgan, M. A. & Shatkin, A. J. (1981) *J. Biol. Chem.* **256,** 7691–7694.
13. Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J. & Merrick, W. C. (1983) *J. Biol. Chem.* **258,** 5804–5810.
14. Ederly, I., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H. & Sonenberg, N. (1983) *J. Biol. Chem.* **258,** 11398–11403.
15. Ederly, I., Lee, K. A. W. & Sonenberg, N. (1984) *Biochemistry* **23,** 2456–2462.
16. Lax, S., Fritz, W., Browning, K. & Ravel, J. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 1977 (abstr.).
17. Walthall, B. J., Spremulli, L. L., Lax, S. R. & Ravel, J. M. (1979) *Methods Enzymol.* **60,** 193–204.
18. Webb, N. R., Chari, R. V. J., DePillis, G., Kozarich, J. W. & Rhoads, R. E. (1984) *Biochemistry* **23,** 177–181.
19. Clark, J. M., Jr., & Klein, W. H. (1974) *Methods Enzymol.* **30,** 754–761.
20. Checkley, J. S., Cooley, L. & Ravel, J. M. (1981) *J. Biol. Chem.* **256,** 1582–1586.
21. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
22. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.