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PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES XXXI: PURIFICATION OF Co-eIF-2C AND STUDIES OF ITS ROLES IN PEPTIDE CHAIN INITIATION

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Co-eIF-2C activity has been purified from high salt wash of reticulocyte ribosomes. The purified preparation is free from detectable levels of eIF-2, Co-eIF-2A, Co-eIF-2B and RF activities. The final step in the purification process involves use of a phosphocellulose chromatography and elution of Co-eIF-2C activity from the column with a buffer containing 2 M urea. This step results in complete removal of the contaminating Co-eIF-2B activity from the purified Co-eIF-2C preparation.

Upon polyacrylamide gel electrophoresis, the final Co-eIF-2C preparation shows a single protein band under non-denaturing conditions. In the presence of SDS, the gel picture shows five prominent polypeptide bands (approximate mol. wt: 100,000; 67,000; 53,000; 45,000; and 40,000) and several faint bands.

Purified Co-eIF-2C preparation strongly stimulates Met-tRNA$_f$·40S·AUG complex formation in the presence of eIF-2 and such stimulation is almost completely inhibited by HRI plus ATP. This study thus delineates the minimum factor requirements, namely, eIF-2 and Co-eIF-2C for formation of a stable Met-tRNA$_f$·40S·AUG complex.

Recent work done in our laboratory and elsewhere indicates that several ancillary protein factors such as Co-eIF-2A (2-10), Co-eIF-2B (11-16), Co-eIF-2C (15-20) and RF (21-23) are required for efficient ternary complex formation by eIF-2 and its proper functioning during peptide chain

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Abbreviations: eIF-2, eukaryotic peptide chain initiation factor 2, forms Met-tRNA$_f$·eIF-2·GTP complex; Co-eIF-2A, stimulates Met-tRNA binding to eIF-2; Co-eIF-2B, promotes dissociation of the ternary complex at high Mg^{2+} concentrations; Co-eIF-2C, relieves Mg^{2+} inhibition of ternary complex formation; HRI, the heme-regulated protein synthesis inhibitor; RF, reverses protein synthesis inhibition in heme-deficient reticulocyte lysates.

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initiation (for reviews, see Refs. 24,25). Co-eIF-2C promotes ternary complex formation by eIF-2 in the presence of Mg^{2+} (15,20,22). Co-eIF-2A binds to preformed ternary complex and forms a stable quaternary complex, Met-$tRNA_f$·eIF-2·GTP Co-eIF-2A (5,6). The precise function of Co-eIF-2B in peptide chain initiation is not known. In partial reactions, Co-eIF-2B promotes dissociation of the ternary complex in the presence of high Mg^{2+} (5 mM) and low temperature ($0^{o}C$) (11,12).

Protein synthesis inhibitors such as HRI (heme-regulated protein synthesis inhibitor) and dsI (double-stranded RNA activated inhibitor) inhibit protein synthesis presumably by phosphorylating the smallest subunit (α-subunit) of eIF-2 (24,25); eIF-2α(P) thus formed is not recognized by two eIF-2-ancillary factors, Co-eIF-2B (13-16) and Co-eIF-2C (15-20).

RF, a protein complex isolated from reticulocyte cell-supernatant, reverses protein synthesis inhibition in heme-deficient reticulocyte lysates (21-23,26,27). The same RF preparation also reverses ternary complex inhibition by HRI and ATP as RF can recognize eIF-2α(P) and stimulate Met-$tRNA_f$ binding to eIF-2α(P) in the presence of Mg^{2+} (21,22). Recent work done in our laboratory indicates that an active RF preparation contains both Co-eIF-2B and Co-eIF-2C activities, and the Co-eIF-2B and Co-eIF-2C activities in this preparation are not inhibited by HRI plus ATP (23). Presumably, the Co-eIF-2B and Co-eIF-2C activities in active RF preparation can recognize eIF-2α(P) and thus promote protein synthesis in heme-deficient reticulocyte lysates. The mechanism of such altered recognition of Co-eIF-2B and Co-eIF-2C activities, as they exist in RF protein complex, is not known.

A major difficulty in studies of the mechanism of eIF-2-ancillary factor activities is that the factors, Co-eIF-2B, Co-eIF-2C and RF are high molecular weight protein complexes and are heavily cross-contaminated. As mentioned above, RF preparation contains Co-eIF-2B and Co-eIF-2C activities. Purified Co-eIF-2B and Co-eIF-2C preparations do not reverse protein synthesis inhibition in heme-deficient reticulocyte lysates and presumably do not contain the full complements of RF factor. However, both Co-eIF-2B and Co-eIF-2C preparations are heavily cross-contaminated. Consequently, it has not yet been possible to precisely define the roles of these factor activities in specific step(s) in peptide chain initiation.

In this paper, we describe extensive purification of Co-eIF-2C from reticulocyte ribosomal salt wash and its role in Met-$tRNA_f$·40S·AUG initiation complex formation. The final step in the purification process involves use of a phosphocellulose chromatography and elution of Co-eIF-2C activity from the column with a buffer containing 2 M urea. This step

resulted in complete removal of the contaminating Co-eIF-2B activity from the purified Co-eIF-2C preparation. In partial reactions, homogeneous preparations of eIF-2 and Co-eIF-2C promoted Met-tRNA$_f$ binding to 40S ribosomes in the presence of AUG-codon and such binding was strongly inhibited by HRI plus ATP. This study thus establishes the minimum factor requirements, namely, eIF-2 and Co-eIF-2C, for formation of a stable Met-tRNA$_f$·40S·AUG initiation complex.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (> 1000 Ci/mmol) was purchased from Amersham/Searle and was diluted with unlabelled methionine to 10,000 to 20,000 cpm/pmol. Trinucleoside diphosphate A_pU_pG was purchased from Miles Laboratories.

Reticulocyte Ribosomes, Ribosomal Subunits and Initiation Factors.

Rabbit reticulocyte ribosomes, 40S ribosomal subunit and ribosomal 0.5 M KCl wash were prepared according to the procedure described previously (12). The peptide chain initiation factor eIF-2 and HRI were purified according to the procedure of Das et al. (1,15)

Purification of Co-eIF-2C.

Co-eIF-2C activity was purified from reticulocyte ribosomal 0.5 M KCl wash. The purification procedure up to the ammonium sulfate precipitation step was the same as described previously (15). Dialyzed Fraction II preparation (200 mg, from 15 rabbits) was applied onto a phosphocellulose column previously equilibrated with Buffer A (20 mM Tris-HCl, pH 7.8; 1 mM dithiothreitol, 50 µM EDTA and 10% (v/v) glycerol) containing 0.1 M KCl. The column was washed thoroughly with the same buffer. The adsorbed proteins were then eluted from the column using Buffer A containing 0.7 M KCl (Fraction III). Fraction III preparation was concentrated by ammonium sulfate (0 → 65%) precipitation. The precipitate was suspended in Buffer A containing 0.1 M KCl and dialyzed against the same buffer. The dialyzed suspension was applied onto a hydroxylapatite column (column vol., 20 ml) previously equilibrated with Buffer B (20 mM potassium phosphate, pH 7.8; 1 mM dithiothreitol, 50 µM EDTA and 10% (v/v) glycerol). The column was washed with Buffer B containing 0.2 M potassium phosphate (pH 7.8). Co-eIF-2C activity was then eluted by washing the column with Buffer B containing 0.5 M potassium phosphate (Fraction IV).

Fraction IV preparation was concentrated by ammonium sulfate (0 → 65%) precipitation and the precipitate was dissolved in Buffer A containing 0.1 M KCl and was dialyzed against the same buffer. The dialyzed suspension was then applied onto a CM-Sephadex C-50 column (column vol., 40 ml) previously equilibrated with Buffer A containing 0.18 M KCl. Co-eIF-2C activity was not adsorbed onto the column and was eluted in the column flow-through (Fraction V).

Fraction V Co-eIF-2C preparation was then diluted with equal volume of Buffer A minus KCl and was adsorbed onto a DEAE-cellulose column equilibrated with Buffer A containing 0.1 M KCl. The DEAE-cellulose column was then eluted using a potassisum chloride gradient (0.1 → 0.3 M) in Buffer A. Co-eIF-2C activity eluted from the column at approximately 0.18 → 0.22 M KCl concentration (Fraction VI).

Fraction VI Co-eIF-2C preparation was adjusted to 2 M in urea by addition of 8 M urea in Buffer C (20 mM Tris-HCl, pH 7.8; 10 mM dithiothreitol, 50 μM EDTA, 20% (v/v) glycerol) and incubated for 15 mins in ice. At the end of incubation, 4 M KCl was added to the solution to adjust the final KCl concentration to 0.25 M. The solution was applied onto a phosphocellulose column equilibrated with Buffer C containing 2 M urea and 0.25 M KCl. In the presence of urea, Co-eIF-2C activity was not adsorbed onto the column and was eluted with the column wash containing Buffer C, 0.25 M KCl and 2 M urea. The column wash was immediately concentrated by ammonium sulfate (0 →80%) precipitation. The precipitate was suspended in minimum volume of Buffer C containing 0.2 M KCl and was dialyzed for 18 hours against the same buffer with one change. The final preparation was dialyzed against Buffer A containing 0.1 M KCl for 3 hours (Fraction VII).

Fraction VII Co-eIF-2C preparation was stored in small aliquots in liquid nitrogen. Co-eIF-2C activity was stable in liquid nitrogen for several weeks.

Peptide Chain Initiation Assays.

eIF-2, Co-eIF-2B and Co-eIF-2C activities were assayed by the Millipore filtration methods as described previously (12,15). Met-tRNA binding to 40S ribosomes was assayed by a two-stage Millipore filtration assay method (1,28). Met-$tRNA_f$·40S·AUG complex was separated by sucrose density gradient centrifugation and the density gradient fractions were analyzed by Millipore filtration (1,28)

The detailed description of the assay procedure is also given in the legends of the figures.

RESULTS AND DISCUSSION

Purification of Co-eIF-2C.

Co-eIF-2C activity was purified starting from 0.5 M KCl wash of reticulocyte ribosomes. The results of a typical purification procedure is shown in Table I. Co-eIF-2C activity co-purified with eIF-2 and Co-eIF-2B activities up to the CM-Sephadex step and could not be assayed by the standard Millipore filtration method. Upon CM-Sephadex chromatography, Co-eIF-2B and Co-eIF-2C activities eluted with the column flow-through and was resolved from eIF-2 activity. eIF-2 Activity was adsorbed onto the CM-Sephadex column and was eluted with Buffer A containing 0.5 M KCl.

The DEAE-cellulose and phosphocellulose chromatographic procedures used in the final two steps of purification did not lead to significant enrichment of Co-eIF-2C activity but resulted in complete removal of Co-eIF-2B activity from the final Co-eIF-2C preparation. Use of urea during phosphocellulose chromatography apparently altered the chromatographic behavior of Co-eIF-2C. In the presence of 0.25 M KCl, Co-eIF-2C activity is ordinarily adsorbed onto the phosphocellulose column (see step 3) but in the presence of 2 M urea, Co-eIF-2C activity was not adsorbed onto the

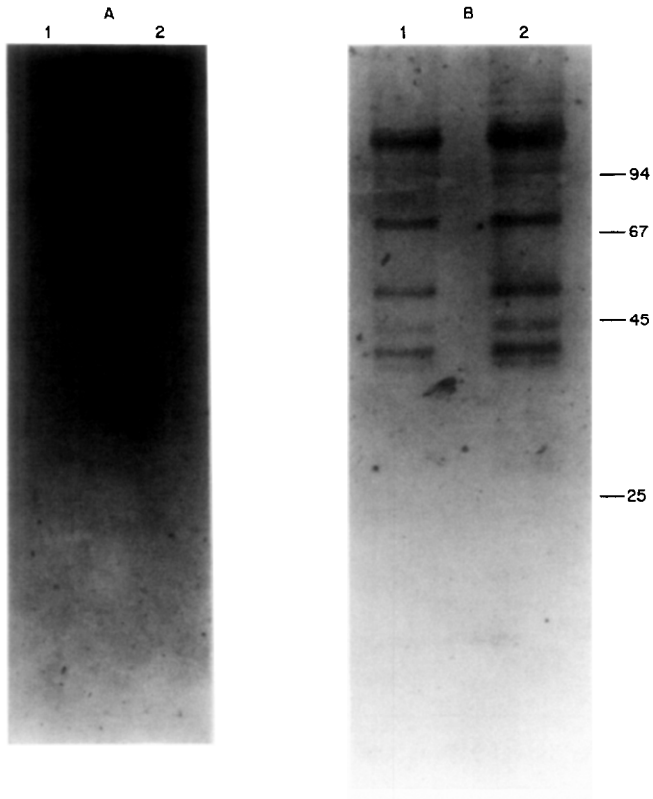

Fig. 1: Panel A: Polyacrylamide gel electrophoresis (in absence of sodium dodecyl sulphate) of purified Co-eIF-2C (Fraction VII). The electrophoresis was carried out in a 3.3→ 10% gradient gel and run at constant voltage of 75 volts for 8 hours. The concentrations of proteins used were; lane 1, 3 μgm; lane 2, 10 μgm.

Panel B: Polyacrylamide gel electrophoresis (in presence of sodium dodecyl sulphate) of purified Co-eIF-2C (Fraction VII). The samples were denatured by adding 1% SDS and heating at 100 C for 2 minutes and were analyzed in a 15% polyacrylamide and 0.09% bis-acrylamide gel in Tris-glycine buffer (pH 8.3). The electrophoresis was done at 140 volts for 2.5 hours. The gels were stained with Coomassie brilliant blue. Concentrations of proteins used were; lane 1, Co-eIF-2C, 10 μgms; lane 2, Co-eIF-2C, 15 μgm.

phosphocellulose column and was eluted from the column with eluting buffer containing 0.25 M KCl. This step also freed Co-eIF-2C preparation from detectable levels of Co-eIF-2B.

Upon polyacrylamide gel electrophoresis, the final Co-eIF-2C preparation showed a single protein band under non-denaturing conditions. In the presence of SDS, the gel picture showed several prominent polypeptide bands (approximate mol. wt. 100,000; 67,000; 53,000; 45,000 and 40,000) and numerous faint bands. Some of the prominent polypeptide components (such as 67,000; 53,000; 45,000; and 40,000 dalton molecular weight bands)

TABLE I

Purification of Co-eIF-2C from Reticulocyte Lysates

Step		Total Protein (mg)	Total Activities (Units) Co-eIF-2C[a]	Co-eIF-2B[b]	Specific Activities (Units/mg) Co-eIF-2C	Co-eIF-2B
1.	Ribosomal 0.5 M KCl wash					
2.	DEAE-cellulose	200				
3.	Phospho-cellulose	70				
4.	Hydroxyl-apatite	40				
5.	CM-Sephadex	22	4,400	1,100	200	50
6.	DEAE-cellulose gradient	8	2,000	320	250	40
7.	Phospho-cellulose (urea)	4	1,332	0	333	0

[a] One unit of activity is defined as the amount of protein required to stimulate the formation of 1 pmol of ternary complex using 0.5μ g (Fraction VII (1)) eIF-2 in the presence of 1 mM Mg^{2+} in 5 min at 37°C (15).

[b] One unit of activity is defined as the amount of protein required to dissociate 4 pmol ternary complex (preformed at 37°C in the absence of Mg^{2+}) to 2 pmol during second stage incubation in the presence of 5 mM Mg^{2+} and at the ice bath temperature for 5 min (12).

are also present in partially purified RF and Co-eIF-2B preparations (data not shown). However, it has not yet been possible to identify one or a group of polypeptide component(s) with any specific factor activity. The SDS-gel pattern of Co-eIF-2C as shown in Fig. 2 closely resembles the gel pattern reported by Siekierka et al., (22) for a similar protein factor, termed ESP.

The final Co-eIF-2C preparation was free from detectable levels of eIF-2, Co-eIF-2A, Co-eIF-2B and RF activities. Also, as reported previously (15,20,22), homogeneous Co-eIF-2C preparation relieved Mg^{2+} in-

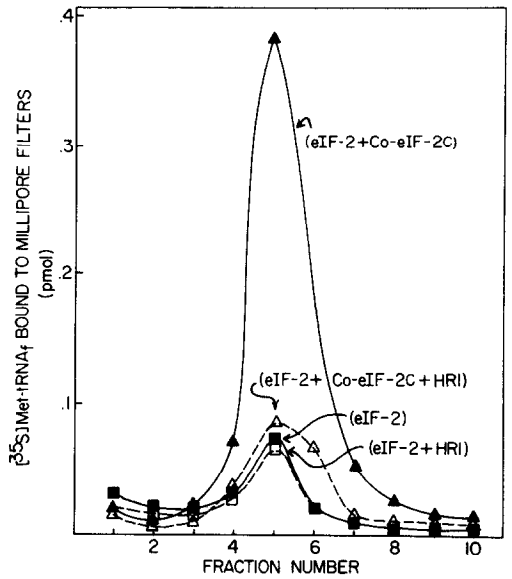

Fig. 2: Effect of addition of HRI plus ATP on [^{35}S]Met-tRNA$_f$·40S·AUG complex formation. A two-stage assay method was used. In Stage I, eIF-2 was preincubated for 5 minutes at 37 C in presence of 1 mM magnesium acetate, 0.2 mM ATP and, where indicated, HRI and Co-eIF-2C. In Stage II, more Mg^{2+} (to make the final concentration 2 mM), GTP, AUG, 40S ribosomes and [^{35}S]Met-tRNA$_f$ were added and the reaction mixtures were incubated for an additional 5 minutes at 37 C. The final reaction mixture in a total volume of 75µl contained: 20 mM Tris-HCl, pH 7.8, 100 mM KCl, 2 mM magnesium acetate, 0.15 mM ATP, 10µ gm bovine serum albumin, 2 mM dithiothreitol, 0.26 mM GTP, 0.08 A_{260} unit 40S ribosomes, 0.02 A_{260} unit AUG, 8-10 pmol [^{35}S]Met-tRNA$_f$, 1.2µ gm eIF-2, and, where indicated, 0.4µ gm HRI and 3µ gm Co-eIF-2C.

At the end of incubation, all reactions were terminated by addition of 50 µl potassium phosphate (0.1 M, pH 6.0). 100µl aliquots of the reaction mixtures were then layered on top of a sucrose gradient (14 → 27%) containing 20 mM potassium phosphate, pH 6.0, 100 mM potassium chloride, 2 mM magnesium acetate, 1 mM dithiothreitol and 50µ M EDTA. The gradients were centrifuged at 45,000 rpm for 105 minutes in a S.W. 50.1 rotor. The gradients were fractionated using an ISCO density gradient fractionator and 0.3 ml fractions from the top were collected into 3 ml wash buffer. The solutions were then filtered through Millipore filters. The filter papers were washed using the same wash buffer, dried and counted for radioactivity.

hibition of ternary complex formation by eIF-2 and such activity was almost completely inhibited by HRI and ATP (data not shown).

Role of Co-eIF-2C in Met-tRNA$_f$·40S·AUG Complex Formation.

We have previously reported that a high molecular weight factor preparation containing Co-eIF-2B activity and homogeneous eIF-2 were required for formation of stable Met-tRNA$_f$·40S·AUG complex (11,12). This Co-eIF-2B preparation also contained Co-eIF-2C activity. Recently, we have demonstrated that a factor preparation enriched in Co-eIF-2C activity but still

containing significant amounts of Co-eIF-2B activity stimulated Met-tRNA$_f$ binding to 40S ribosomes in the presence of AUG-codon and such Met-tRNA$_f$ binding was significantly inhibited by HRI plus ATP (1). The precise requirement of Co-eIF-2B and Co-eIF-2C in this Met-tRNA$_f$·40S·AUG complex formation could not be defined.

The availability of purified Co-eIF-2C preparation free from detectable levels of Co-eIF-2B activity prompted us to examine the precise factor requirements for Met-tRNA$_f$·40S·AUG complex formation. As shown in Fig. 2, this purified Co-eIF-2C preparation strongly stimulated Met-tRNA$_f$ binding to 40S ribosomes in the presence of eIF-2. Such Met-tRNA$_f$ binding to 40S ribosomes was entirely dependent on added AUG codon (data not shown). Also, Co-eIF-2C stimulated Met-tRNA$_f$ binding to 40S ribosomes was almost completely inhibited by HRI plus ATP.

In the experiment described in Fig. 2, we used 1.2 µg of eIF-2 (approximately 8 pmol). As reported previously (2), in the absence of Co-eIF-2A approximately 30% of input eIF-2 binds to Met-tRNA$_f$. Also, homogeneous eIF-2 preparations are unstable and lose considerable activity upon storage (29). The eIF-2 preparation (1.2 µg) used in Experiment 2 bound 1.5 pmol Met-tRNA$_f$ in the absence of Mg^{2+} and Co-eIF-2A and promoted formation of approximately 0.5 pmol Met-tRNA$_f$·40S initiation complex as measured using a glycerol density gradient centrifugation method.

The above results thus clearly demonstrate that eIF-2 and Co-eIF-2C alone can promote formation of a stable Met-tRNA$_f$·40S·AUG complex and such complex formation is strongly inhibited by a physiological protein synthesis inhibitor, namely HRI. The characteristics of requirements of other factors, such as Co-eIF-2B and Co-eIF-2A in Met-tRNA$_f$·40S initiation complex formation are not fully clear. We have not yet been able to obtain Co-eIF-2B preparations completely free from Co-eIF-2C activity. Addition of a factor preparation enriched in Co-eIF-2B activity consistently gave 30-50% stimulation of Met-tRNA$_f$·40S·AUG complex formation in the presence of saturating amounts of Co-eIF-2C (data not shown). The results of our preliminary experiments suggest that Co-eIF-2A may be required for physiological mRNA-directed Met-tRNA$_f$·40S initiation complex formation. This observation is consistent with our previous report that Co-eIF-2A is required for formation of a stable Met-tRNA$_f$·eIF-2·GTP complex in the presence of eukaryotic mRNAs (6).

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REFERENCES

1. Das, A., Bagchi, M.K., Ghosh-Dastidar, P. and Gupta, N.K. J. Biol. Chem., (in press).
2. Dasgupta, A., Majumdar, A., George, A.D. and Gupta, N.K. (1976) Biochem. Biophys. Res. Commun., **71**, 1234-1241.
3. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A. and Gupta, N.K. (1978) J. Biol. Chem., **253**, 6054-6059.
4. Ghosh-Dastidar, P., Yaghmai, B., Das, A., Das, H.K. and Gupta, N.K. (1980) J. Biol. Chem., **255**, 365-368.
5. Ghosh-Dastidar, P., Giblin, D.,, Yaghmai, B., Das, A., Das, H.K., Parkhurst, L.J. and Gupta, N.K. (1980) J. Biol. Chem., **255**, 3826-3829.
6. Roy, R., Ghosh-Dastidar, P., Das, A., Yaghmai, B. and Gupta, N.K. (1981) J. Biol. Chem., **256**, 4719-4722.
7. Malathi, V.G. and Mazumdar, R. (1978) FEBS Letters, **86**, 155-159.
8. Treadwell, B.V., Mauser, L. and Robinson, W.G. (1979) "Methods in Enzymology" (K. Moldave and L. Grossman, Eds.), Vol. 60, 181-193, Academic Press, New York.
9. MacRae, T., Houston K.J., Woodley, C.L. and Wahba, A.J. (1979) Eur. J. Biochem., **100**, 67-76.
10. Woodley, C.L., Roychowdhury, M., MacRae T.H., Olson, K.W. and Wahba, A.J. (1981) Eur. J. Biochem., **117**, 543-551.
11. Majumdar, A., Roy, R., Das, A., Dasgupta, A. and Gupta, N.K. (1977) Biochem. Biophys. Res. Commun., **78**, 161-169.
12. Majumdar, A., Dasgupta, A., Chatterjee, B., Das, H.K. and Gupta, N.K. (1979) "Methods in Enzymology" (K. Moldave and L. Grossman, Eds.), Vol. 60, 35-52, Academic Press, New York.
13. Das, A. and Gupta, N.K., (1977) Biochem. Biophys. Res. Commun., **78**, 1433-1441.
14. Ranu, R.S., London, I.M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R. and Gupta, N.K. (1978) Proc. Natl. Acad. Sci., U.S.A., **75**, 745-749.
15. Das, A., Ralston, R.O., Grace, M., Roy, R., Ghosh-Dastidar, P., Das, H.K., Yaghmai, B., Palmieri, S. and Gupta, N.K. (1979) Proc. Natl. Acad. Sci., U.S.A., **76**, 5076-5079.
16. Das, H.K., Das, A., Ghosh-Dastidar, P., Ralston, R.O., Yaghmai, B., Roy, R. and Gupta, N.K. (1981) J. Biol. Chem., **256**, 6491-6495.
17. deHaro, C., Datta, A. and Ochoa, S. (1978) Proc. Natl. Acad. Sci., U.S.A., **75**, 243-247.
18. deHaro, C. and Ochoa, S. (1978) Proc. Natl. Acad. Sci., U.S.A., **75**, 2713-2716.
19. deHaro, C. and Ochoa, S. (1979) Proc. Natl. Acad. Sci., U.S.A., **76**, 2163-2164.
20. Ranu, R.S. and London, I.M. (1979) Proc. Natl. Acad. Sci., U.S.A., **76**, 1079-1083.
21. Ralston, R.O., Das, A., Grace, M., Das, H.K. and Gupta, N.K. (1979) Proc. Natl. Acad. Sci., U.S.A., **76**, 5490-5494.
22. Siekierka, J., Mitsui, K. and Ochoa S. (1981) Proc. Natl. Acad. Sci., U.S.A., **78**, 220-223.
23. Ralston, R.O., Grace, M., Das, A. and Gupta, N.K. (1981) Fed. Proc., **40**, 1549.
24. Austin, S.A. and Clemens, M.J. (1980) FEBS Letters, **110**, 1-6.
25. Gupta, N.K. in "Current Topics in Cellular Regulation" (B.L. Horecker and E.R. Stadtman, Eds.), Academic Press, Inc., New York. (in press).
26. Ralston, R.O., Das, A., Dasgupta, A., Palmieri, S. and Gupta, N.K. (1978) Proc. Natl. Acad. Sci., U.S.A., **75**, 4858-4862.

27. Amesz, H., Gouman, S.H., Handrich-Morre, T., Voorma, H.O. and Benne, R. (1979) Eur. J. Biochem.., **98**, 513-520.
28. Chatterjee, B., Dasgupta, A., Majumdar, A., Palmieri, S. and Gupta, N.K. (1979) in "Methods in Enzymology" (K. Moldave and L. Grossman, Eds.) Vol. 60, 256-265, Academic Press Inc., New York.
29. Majumdar, A., Reynolds, S., Gupta, N.K. (1975) Biochem. Biophys. Res. Commun., **67**, 687-695.