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SELECTIVE INHIBITION OF PROTEINS SYNTHESIZED FROM DIFFERENT mRNA SPECIES IN RETICULOCYTE LYSATES CONTAINING L-PYRROLINE-5-CARBOXYLIC ACID

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SUMMARY: L-Pyrroline-5-carboxylic acid is a naturally occurring nonprotein amino acid present in human plasma that changes concentrations with diet. L-pyrroline-5-carboxylic acid inhibited net synthesis of globin in untreated reticulocyte lysates in a dose dependent manner. This inhibition was greater than that observed with equimolar GSSG or $NADP^+$ and was prevented by a NADPH generating system. L-pyrroline-5-carboxylic acid also inhibited net synthesis of proteins from brome mosaic and alfalfa mosaic virus mRNAs to different extents. However, no effect on the translation of the naturally uncapped encephalomyocarditis virus mRNA was observed. In general, mRNAs that are considered strongly competitive, such as alfalfa mosaic virus 2 and 4, were more resistant to this inhibitory process. These results indicate that pyrroline-5-carboxylic acid can initiate a differential effect on proteins synthesized from different mRNA species by an as yet unidentified mechanism.

Although it has been firmly established that optimal protein synthesis in rabbit reticulocyte lysates requires both sugar phosphates and a NADPH generating system coupled with a thioredoxin/thioredoxin reductase system the precise reasons for these requirements remain unclear (1-6). Changes in cellular $NADP^+$/NADPH ratios may effect enzymatic reactions dependent on either NADPH or $NADP^+$. Since NAD(H) exists primarily in the reduced state it appears that mechanisms capable of generating $NADP^+$ may have the greatest regulatory significance (7,8). The nonprotein amino acid L-pyrroline-5-carboxylic acid (P5C) has been indirectly shown to affect the $NADP^+$/NADPH couple using the activity of the oxidative limb of the pentose pathway as an end point (9-11). P5C is also a component of a proposed NADPH linked hydride ion shuttle that has been demonstrated in a reconstituted system (12,13). Additional evidence for such a NADPH linked shuttle has come from the kinetic characteristics of liver pyrroline-5-carboxylate reductase (EC 1.5.1.2) and reconstitution of shuttle activity in a cell-free liver system (14). L-P5C is present in human plasma and changes with diet, and in at least one species it is present in unusually high levels in aqueous humor as compared to plasma (15-17). More recent studies suggest that proline and P5C may have a regulatory role in the

Abbreviations: P5C, L-Δ^1-pyrroline-5-carboxylic acid; eIF-4F, eukaryotic initiation factor-4F; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TEMED, N,N,N^1,N^1- tetramethylethylenediamine; DTT, DL-dithiothreitol.

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symbiotic relationship between soybean nodules and bacteroids (18). In view of the studies suggesting a possible regulatory role for P5C, the effect of this nonprotein amino acid on protein synthesis in a cell-free system was tested.

We report studies demonstrating that P5C, or a compound produced during its catabolism, inhibits net protein synthesis in untreated rabbit reticulocyte lysates. The surprising findings were that the degree of inhibition of globin synthesis observed with P5C was greater than that seen with equimolar concentrations of GSSG and $NADP^+$ and that P5C inhibited proteins synthesized from several capped mRNAs to different degrees. Proteins synthesized from the naturally uncapped eIF-4F insensitive mRNA of encephalomyocarditis virus was not effected by the presence of P5C, however $NADP^+$ was inhibitory.

MATERIALS AND METHODS

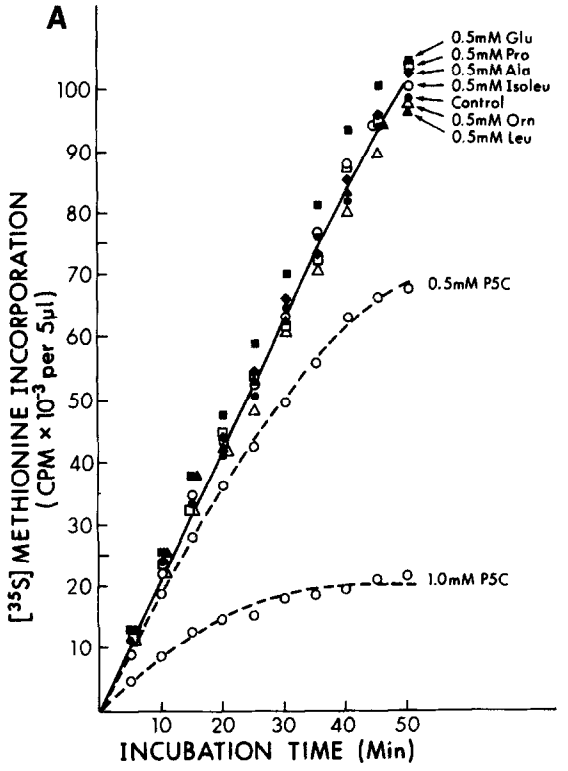
Reagents: All reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Brome mosaic virus mRNA was from Promega Biotec (Madison, WI) and alfalfa mosiac virus mRNA was a gift from Dr. Therese Godefroy-Colburn. Encephalomyocarditis virus mRNA was prepared as previously described (19).

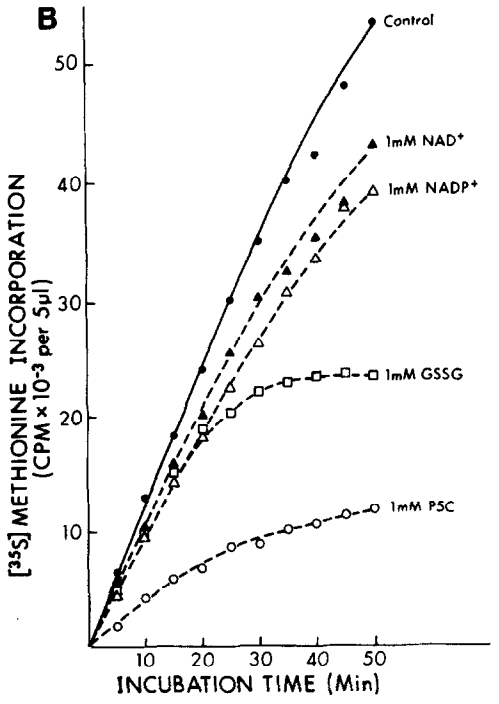
Enzymatic synthesis and purification of L-P5C: L-P5C was synthesized from L-ornithine using rat liver ornithine amniotransferase as described previously (20). Stock solutions of 5-10 mM were stored in 1N HCl at 4^o C. Immediately prior to its use in incubations small quantities of stock were lyophilized, extracted with 100 µl of HPLC grade methanol (Fisher Scientific) to remove salt, dried and then re-dissolved in 20-50 µl of 0.01 N HCl. The concentrated solution was assayed and appropriate quantities diluted with 20 mM Hepes-pH 7.4 and pH adjusted with 2 N KOH immediately before it was added to incubations (21).

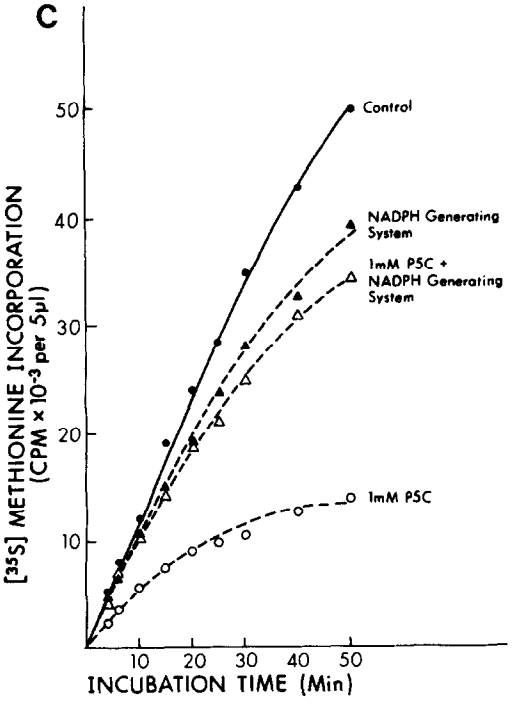
Kinetic studies of endogenous globin mRNA translation in reticulocyte lysates: Rabbit reticulocyte lysate was prepared as described elsewhere except that hemin was added at the time of lysis (22). Creatine phosphokinase and creatine phosphate were from Calbiochem (San Diego, CA). Incubations were at 30^o C with a final volume of 100 µl and contained: 80 µl lysate, 20 mM Hepes-pH 7.4, 0.5 mM ATP, 0.1 mM GTP, 15 mM creatine phosphate, 1.5 units creatine phosphokinase, 100 mM KCl, 0.5 mM $MgCl_2$, 40 or 20 µM hemin as specified, 25 µM each of 19 amino acids (minus methionine), and 10 µCi [^{35}S] methionine (1000 Ci/mmol, New England Nuclear). All other additions were pH adjusted immediately prior to their addition (particularly P5C, GSSG or nicotinamide coenzymes) and incubations were started by adding lysate. Samples (5 µl) were removed with a fixed volume pipet, spotted on Whatman 3MM filters and processed as described elsewhere (1). Cell-free translations of viral mRNA were in incubations as described above using untreated reticulocyte lysate. Samples were taken at the times specified in legends, analyzed by 10% SDS-PAGE and fluorography using Enlightening (New England Nuclear) with the recommended procedure of the manufacturer (23).

RESULTS AND DISCUSSION

Initial studies showed that P5C markedly inhibited the translation of endogenous globin mRNA in rabbit reticulocyte lysates. The inhibition of globin synthesis by the presence of P5C was dose dependent (Fig. 1, Panel A) and was observed in at least 5 laboratory prepared and 2 commercial (Promega Biotec, Madison, WI) lysates. The effect varied from a 70% to 40% inhibition of control levels depending on which lysate preparation was studied. In Figure 1, Panel B the inhibition observed with the P5C at a final concentration of 1 mM is compared with that of GSSG, NAD^+, and $NADP^+$. Addition of glucose plus NADPH at the start of incubations prevented the inhibition of globin synthesis in lysates containing P5C (Fig. 1, Panel C). These results indicated that the inhibition observed in lysates containing P5C could at least


A
0.5mM Glu
0.5mM Pro
0.5mM Ala
0.5mM Isoleu
Control
0.5mM Orn
0.5mM Leu
0.5mM P5C
1.0mM P5C
[35S] METHIONINE INCORPORATION (CPM × 10-3 per 5µl)
INCUBATION TIME (Min)


B
Control
1mM NAD+
1mM NADP+
1mM GSSG
1mM P5C
[35S] METHIONINE INCORPORATION (CPM × 10-3 per 5µl)
INCUBATION TIME (Min)


C
Control
NADPH Generating System
1mM P5C + NADPH Generating System
1mM P5C
[35S] METHIONINE INCORPORATION (CPM × 10-3 per 5µl)
INCUBATION TIME (Min)

be prevented by a NADPH generating system. However, the reason for different extents of inhibition with equimolar GSSG, $NADP^+$, and P5C remain unclear at this time. The observation that 1 mM P5C produced a greater inhibition than 1 mM $NADP^+$ suggests that P5C was not acting solely by its ability to generate $NADP^+$ during its catabolism to proline.

To further study this inhibitory phenomenon the effect of P5C on the translation of several well studied viral mRNAs (brome mosaic virus, BMV; alfalfa mosaic virus, AMV; and encephalomyocarditis virus, EMC) was determined. Addition of P5C to lysates containing both endogenous globin mRNAs and exogenous BMV mRNAs produced a quantitatively different degree of inhibition of proteins synthesized from individual viral mRNA species (Fig. 2, Panels A, B, and C). The apparent degree of inhibition was enhanced if lysates were preincubated with P5C prior to the addition of [^{35}S]methionine (Fig. 2, Panel B and C). Untreated lysates were still active after this 60 min preincubation as demonstrated by a continued increase in proteins synthesized up to 120 min of incubation (Table I). Quantitation of proteins synthesized from different mRNA species in incubations containing 1 mM P5C indicated that BMV 1a+2a and BMV 3a were inhibited to a greater extent than globin or BMV 4 mRNA (Fig. 2, Panel C). Similarly, studies with alfalfa mosaic virus (AMV) mRNAs indicated that proteins synthesized from messages considered more competitive were relatively resistant to the inhibition caused by the addition of P5C (Fig. 2, Panel D). Globin synthesis was reduced by up to 35% but no effect on the synthesis of proteins from the more competitive AMV 2 and AMV 4 mRNAs was seen.

Although the mechanism of this differential effect on net protein synthesis from viral mRNAs has not been identified, one possible explanation would be a defect in a message discriminatory initiation factor (24). Evaluation of the reported relative competitive efficiencies of BMV, AMV, and globin mRNAs indicate that the general pattern of inhibition observed in lysates containing P5C was consistent with greater inhibition of the less competitive mRNAs (24,25). Proteins synthesized from the naturally uncapped EMC mRNA were not decreased in lysates containing P5C (Fig. 3, Panel A and B). However, $NADP^+$ did inhibit synthesis of EMC mRNA encoded proteins (Fig. 3, Panel A). This observation is of interest and suggests that EMC mRNA translation may be particularly sensitive to $NADP^+$. The lack of a P5C effect on protein synthesized from EMC mRNA suggests that the 5' cap or other structural features absent in EMC mRNA may in some way be important in the inhibitory process observed with globin, BMV and AMV mRNAs. In addition, the possibility of altered thiol/disulfide status of initiation factors being

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Fig. 1. Inhibitory effect of L-P5C on protein synthesis, comparison of the effect of other oxidizing agents, and prevention of the effect by a NADPH generating system. Incubations (100 µl) containing 80 µl of untreated reticulocyte lysate were as described in Methods. [^{35}S]Methionine incorporation into trichloroacetic acid insoluble protein was determined in 5 µl samples at the indicated times. Panel A: As indicated either 50 nmol of P5C or 50 nmol of designated amino acids, 100 nmol P5C or an equal volume of buffer (control) was added to separate incubations immediately before adding lysate. The final hemin concentration in all incubations was 20 µM. Panel B: Incubations were identical to those in Panel A except 40 µM hemin was present and, as indicated 100 nmol NAD^+, 100 nmol GSSG or 100 nmol P5C was added immediately prior to starting incubations. Panel C: Incubations were identical to those in Panel A except that either 100 nmol P5C and/or a NADPH generating system (500 nmol glucose and 50 nmol NADPH) was present as indicated.

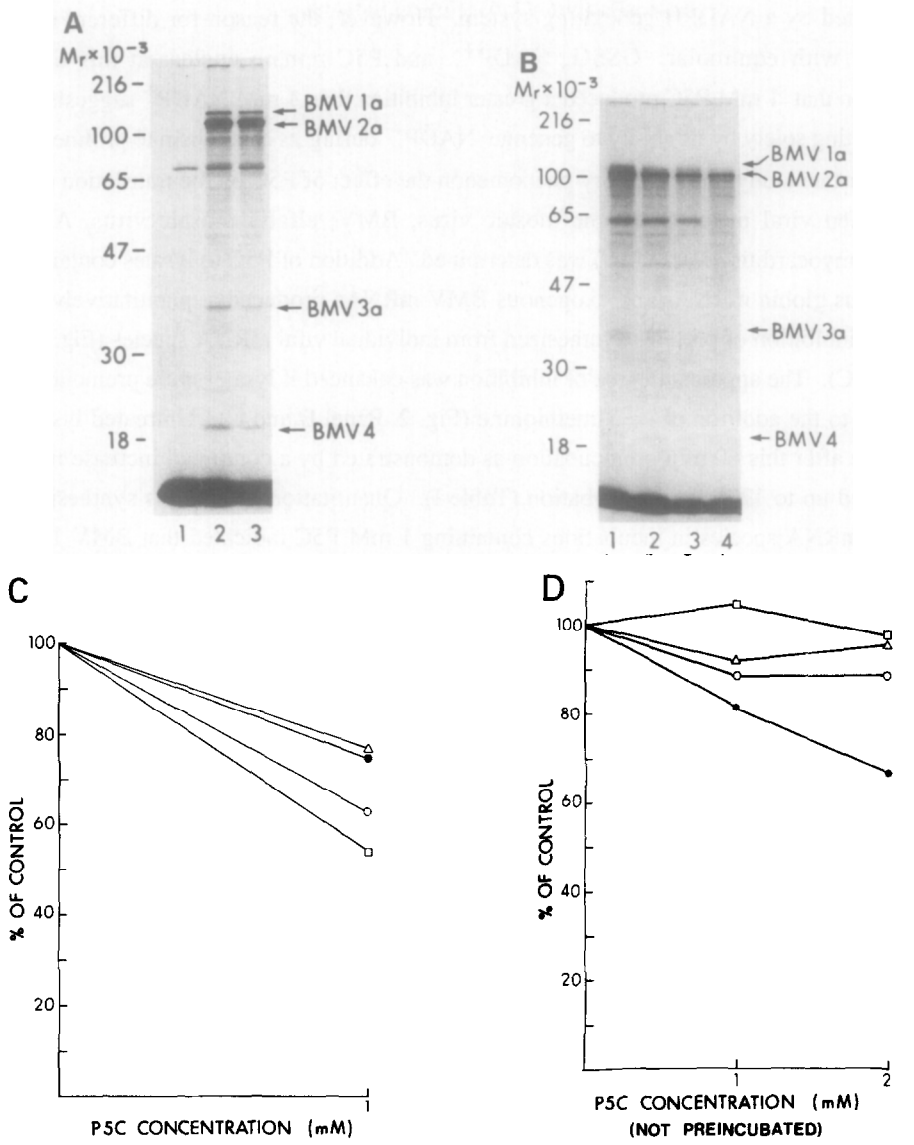

Fig. 2. Differential Inhibition of Proteins synthesized from Globin, BMV and AMV mRNAs in Lysates Treated with L-P5C. Incubations were identical to those in Fig. 1 except they contained 50 μCi of [^{35}S]methionine (1330 Ci/mmol), purified viral mRNA, and P5C or $NADP^+$ were present as indicated. Panel A: Purified brome mosaic virus (BMV) mRNA (1.0 μg) (Lanes 2 and 3) and 2 mM P5C (Lane 3) were present at the start of incubations. Samples (5 μg) were taken at 100 min and one third analyzed by SDS-PAGE as described in Methods. Gels were treated for fluorography and autoradiograms prepared as described in Methods. The autoradiogram shown was obtained following a 24 h exposure. Panel B: Incubations were identical to those in Panel A except that three separate incubations containing BMV had final concentrations of 1 mM P5C (Lane 2), 2 mM P5C (Lane 3), and 2 mM $NADP^+$ (Lane 4), respectively. The incubation represented in Lane 1 had only purified BMV mRNA added. In addition [^{35}S]methionine was not added until 60 min of incubation and samples were taken at 100 min and analyzed as in Panel A. The autoradiogram shown was obtained following a 48 h exposure. Panel C: Incubations were identical to those in Panel B where [^{35}S]Methionine was not added until 60 min and samples were taken for analysis at 100 min. Quantitation of globin and viral proteins synthesized was done by densitometric scanning of autoradiograms using a Joyce Loebl laser densitometer. Values are expressed as percent of control (P5C absent) incubations. Quantitation of BMV 1a+2a (o), BMV 3a (□), BMV 4 (Δ) encoded proteins and globin (●) synthesized are shown here. Panel D: Incubations were identical to those in Panel A (no preincubation) except they contained 0.5 μg of purified alfalfa mosaic virus (AMV) mRNA. Quantitation of protein synthesized from AMV 1 (o) AMV 2 (□) , AMV 4 (Δ) and globin (●) mRNA following 120 min of incubation are shown.

Table 1. Quantitation of Proteins Synthesized in Preincubated Reticulocyte Lysates

	Incubation time (min)		
	80	100	120
Control	61,285	114,371	136,149
+ BMV mRNA	87,721	145,687	168,675

To determine the effect of a 60 min preincubation on lysate activity total protein synthesis was quantitated in the control incubations for the experiments described in the legend for Fig. 2, Panel C. [^{35}S]Methionine was added to incubations after 60 min. Samples (5 µl) were taken at indicated times and [^{35}S]methionine incorporation into total protein was determined as described in Methods. The absence (control) or presence of BMV mRNA (1 µg) is indicated. The data represent acid insoluble radioactivity recovered and are expressed in CPM per 5 µl samples.

involved in this mechanism is worth noting. Particularly in view of the evidence for thiol and disulfide groups in the 25 kDa mRNA cap binding protein, the existence of a 24 kDa protein in reticulocyte lysates that changes its thiol /disulfide status depending on incubation conditions, the indirect evidence that P5C can alter $NADP^+$/NADPH ratios in intact cells and lysates, the evidence

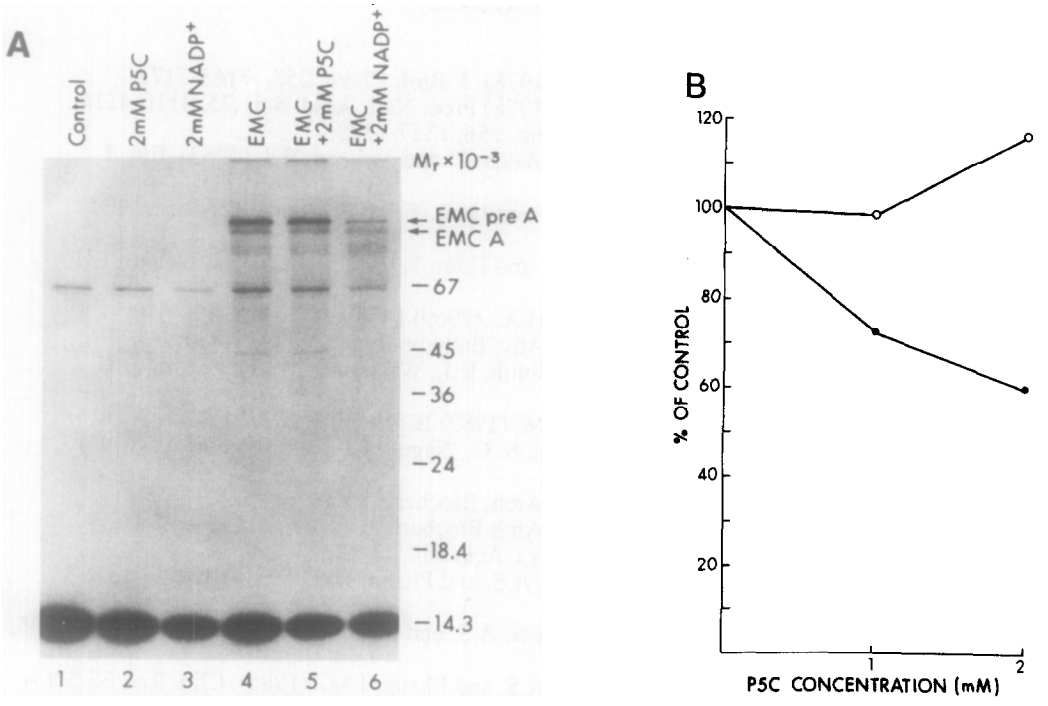

Fig. 3 Addition of P5C to Lysates does not Inhibit Translation of the Naturally Uncapped Encephalomyocarditis mRNA. Panel A: Incubations were identical to those in Fig. 2, Panel A (no preincubation) except 0.3 µg of purified encephalomyocarditis (EMC) mRNA (Lanes 4-6) was present and either 2 mM P5C (lanes 2 and 5) or 2 mM $NADP^+$ (Lanes 3 and 6) was present at the start of incubations. Samples were taken after 100 min of incubation, analyzed by SDS-PAGE and fluorograms prepared following a 24 h exposure. Panel B: Incubations were done under the same conditions as those in Panel A. Quantitation of EMC A+B proteins (o) and globin (•) synthesized in the presence of 1 mM or 2 mM P5C after 100 min are shown relative to control incubations (No P5C).

that specific cysteine residues are essential for the activity of at least some RNA binding proteins, and the report that NADPH is required for eIF-2B activity (6,9,10,27-29).

It is not yet clear if P5C itself, a carbon atom metabolite, or an indirect by-product generated during the catabolism of P5C is responsible for the effects reported here. Although the physiologic significance of these findings remain uncertain at this time, they may have implications for understanding the mechanism of in vivo translational control of a liver enzyme, ornithine aminotransferase (EC 2.6.1.13), that catalyzes the synthesis of P5C from ornithine (2,30,31). Furthermore, the possibility of pharmacologically manipulating the expression of either endogenous or transfected genes in animal systems or different cDNAs in a multicomponent BMV vector in plant systems is raised (31).

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REFERENCES

1. Ernst, V., Levin, D.H. and London, I.M. (1978) J. Biol. Chem. 253, 7163-7172.
2. Ernst, V., Levin, D.H. and London, I.M. (1978) Proc. Natl. Acad. Sci. ,75, 4110-4114.
3. Jagus, R. and Safer, B. (1981) J. Biol. Chem. 256, 1317-1323.
4. Hunt, T., Hervert, P., Campbell, E.A., Deidakis, C. and Jackson, R.J. (1983) Eur. J. Biochem. 131, 303-311 .
5. Jackson, R.J., Campbell, E.A., Herbert, P. and Hunt, T. (1983) Eur. J Biochem. 131, 289-301.
6. Jackson, R.J., Herbert, P., Campbell, E.A. and Hunt, T. (1983) Eur. J. Biochem. 131, 313-324.
7. Veech, R.L., Eggleston, L.V. and Krebs, H.A. (1969) Biochem. J. 115, 609-619.
8. Krebs, H.A. and Eggleston, L.V. (1974) Adv. Enzyme Regul. 12 , 421-434.
9. Phang, J.M., Downing, S.T., Yeh, G.C., Smith, R.J., Williams, J.A., Hagedorn, C.H. (1982) J. Cell. Physiol. 110, 255-261.
10. Hagedorn, C.H., Yeh, G.C. and Phang, J.M. (1982) Biochem. J. 202, 31-39.
11. Yeh, G.C., Roth, E.F., Phang, J.M., Harris, S. C., Nagel, R.L., and Rinaldi, A. (1984) J. Biol. Chem. 259, 5454-5458.
12. Hagedorn, C.H. and Phang, J.M. (1983) Arch. Biochem. Biophys. 225, 95-101.
13. Hagedorn, C.H. and Phang, J.M. (1986) Arch Biochem. Biophys. 248, 166-174.
14. Hagedorn, C.H. (1986) Biochem. Biophys. Acta 884, 11-17.
15. Fleming, G.A., Hagedorn, C.H., Granger, A.S. and Phang, J.M. (1984) Metabolism 33, 739-742.
16. Fleming, G.A., Steel, G., Valle, D., Granger, A.S. and Phang, J.M. (1986) Metabolism 35, 933-937.
17. Fleming, G.A., Rodgers, Q.R., Granger, A.S. and Phang, J.M. (1985) Clin. Res. 33 526A.
18. Kohl, D.H., Schubert, K.R., Carter, M.B., Hagedorn, C.H. and Shearer, C. in press
19. Lawrence, C. and Thach, R.E. (1984) J. Virol. 14, 598-610.
20. Smith, R.J., Downing, S.J. and Phang, J.M. (1977) Anal. Biochem. 82, 170-176.
21. Johnson, A.B. and Strecker, H.J. (1962) J. Biol. Chem. 237, 1876-1881.
22. Safer, B., Jagus, R. and Kemper, W.M. (1979) Methods in Enzymology 60, 61-87.
23. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
24. Ray, B.K., Brendler, T.G., Adya, S., Daniels-McQueen, S., Miller, J.K., Hershey, J.W.B., Grifo, J.A., Merrick, W.C. and Thach, R.E. (1983) Proc. Natl. Acad. Sci. USA 80 663-667.

25. Godefroy-Colburn, T., Thivent, C. and Pinck, L. (1985) Eur. J. Biochem. 147, 541-548.
26. Herson, D., Schmidt, A., Seal, S., Marcus, A. and van Vloten-Doting, L. (1979) J. Biol. Chem. 254, 8245-8249.
27. Rychlik, W., Gardner, P.R., Vanaman, T.C. and Rhoads, R.E. (1986) J. Biol. Chem. 261, 71-75.
28. Romaniuk, P.J. and Uhlenbeck, O.C. (1985) Biochem. 24, 4239-4243.
29. Dholakia, J.N., Mueser, T.C., Woodley, C.L., Parkhurst, L.J. and Wahba, A.J. (1986) Proc. Natl. Acad. Sci. USA 83, 6746-6750.
30. Mueckler, M.M., Merrill, M.J. and Pitot, H.C. (1983) J. Biol. Chem. 258, 6109-6114.
31. Merrill, M.J. and Pitot, H.C. (1985) Arch. Biochem. Biophys. 237, 373-385.
32. Ahlquist, P., French, R., Janda, M. and Loesch-Fries, L.S. (1984) Proc. Natl. Acad. Sci. USA 81, 7066-7070.