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In vitro synthesis of a putative precursor of mitochondrial ornithine transcarbamoylase

(mitochondrial enzymes/protein biosynthesis/immunoprecipitation/precursor proteins/ornithine carbamoyltransferase)

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ABSTRACT **Ornithine transcarbamoylase (OTCase; ornithine carbamoyltransferase; carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3), a major mitochondrial matrix enzyme in ureotelic animals, is synthesized on cytoplasmic ribosomes and translocated across both mitochondrial membranes to the matrix. In an attempt to identify the primary translation product (or an early intermediate) that is the substrate for this transport process, we translated rat liver polysomal RNA *in vitro* by using the rabbit reticulocyte lysate system. Immunoprecipitation of the [^{35}S]methionine-labeled translation mixture was performed by using monospecific OTCase antiserum and the immunoadsorbent *Staphylococcus aureus*. Approximately 0.3% of total trichloroacetic acid-insoluble ^{35}S-labeled material was specifically precipitated. Analysis of the precipitate by fluorography of a dried sodium dodecyl sulfate/polyacrylamide gel showed a single major translation product whose mobility corresponded to a polypeptide of 43,000 daltons, a value ≈4000 daltons greater than that noted for the "mature" OTCase subunit isolated from rat liver. This translation product was not precipitated by preimmune rabbit serum, and excess unlabeled mature OTCase competed with it for interaction with OTCase antiserum. These results suggest that rat liver OTCase, like a number of other cytoplasmically synthesized organellar proteins, is initially made as a larger precursor that contains an amino acid sequence necessary to confer on OTCase its transport properties. The potential application of these findings to the study of inherited complete OTCase deficiency in humans is discussed.**

Ornithine transcarbamoylase (OTCase; ornithine carbamoyltransferase; carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) is a major enzyme located in the mitochondrial matrix of ureotelic animals (1). It constitutes 3–4% of the mitochondrial protein (2), catalyzes the second step of the urea cycle, and is composed of a trimer of identical polypeptide subunits (2–4). The "mature" enzyme from bovine, rat, and human liver has a molecular weight of 108,000–114,000; each subunit has a molecular weight of 36,000–38,000 (2–4). Because the OTCase subunit is encoded by a nuclear gene on the X chromosome (5, 6), it is almost certainly synthesized on cytoplasmic ribosomes (reviewed in ref. 7). The newly synthesized subunit (or, conceivably, the completed trimer) must then traverse both the outer and inner mitochondrial membranes to achieve its final location in the mitochondrial matrix. The present study is aimed at defining the mechanism of this translocation process.

The transfer of polypeptides into or across membrane bilayers has been most extensively studied for various secretory proteins and has given rise to the so-called "signal hypothesis" (8). It appears that these proteins are made as larger precursors that are translocated across intracellular membranes *while* they are being synthesized. Information is now beginning to accumulate regarding the distribution mechanisms for cytoplasmically synthesized proteins ultimately located in various intracellular organelles. Once again, larger precursors have been demonstrated for proteins destined for localization in watermelon glyoxysomes (9), in the chloroplast stroma of *Chlamydomonas* (10) and of various higher plants (11, 12), in the inner mitochondrial membrane of yeast (13, 14), and in the yeast mitochondrial matrix (15). However, in these instances, additional posttranslational events appear to be required for the transfer of the precursor into the appropriate organelle and, finally, for the processing of the precursor into the "mature" protein.

To our knowledge, only a single mitochondrial matrix protein from mammalian sources has been examined in this way. Shore *et al.* (16) used an *in vitro* cell-free translation approach to identify in rat liver a putative precursor of the first enzyme of the urea cycle, carbamoyl-phosphate synthetase I [carbamoyl-phosphate synthetase (ammonia), EC 6.3.4.16]. This precursor had a molecular weight ≈5500 larger than the mature mitochondrial form of the enzyme. Initial experiments aimed at demonstrating mitochondrial transport or processing of this putative precursor were, however, unsuccessful. In the present experiments we report the *in vitro* synthesis of a putative precursor for the OTCase subunit from rat liver that is ≈4000 daltons larger than its mature counterpart.

MATERIALS AND METHODS

General. Sprague–Dawley rats weighing 100–250 g were used for purification of both polysomal RNA and mitochondrial OTCase. The reticulocyte lysate was prepared from New Zealand white rabbits (≈2.3–2.7 kg). [^{35}S]Methionine (600–1400 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and sodium boro[^{3}H]-hydride (5 Ci/mmol) were purchased from Amersham; electrophoresis reagents, from Eastman; micrococcal nuclease, from Worthington; creatine kinase, from Sigma. Formalin-fixed *Staphylococcus aureus* cells were a gift of W. Summers, and bentonite was provided by J. Pawelek.

Purification and Labeling of OTCase. OTCase was purified from rat liver mitochondria as described by Clarke (2). The final enzyme preparation had a specific activity of ≈225 μmol of citrulline formed/min per mg of protein when assayed by a slight modification of the method of Nuzum and Snodgrass (17) and was homogeneous on sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gel electrophoresis.

In some experiments purified OTCase was ^{3}H-labeled by using the reductive methylation procedure described by Means (18). To 200 μl of a solution containing pure OTCase at 0.125 mg/ml were added 4 μl of 200 mM formaldehyde and approximately 5 mCi (10 mM final concentration) of sodium boro[^{3}H]hydride. The reaction was stopped after 1 min by addition of 6 μl of 1.0 M Tris·HCl (pH 7.5); the reaction mixture was then dialyzed for 4 hr against 1000 vol of 10 mM potassium phosphate buffer (pH 7.0) to remove label not associated with protein.

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Abbreviations: OTCase, ornithine transcarbamoylase; pOTCase, putative precursor of OTCase; NaDodSO$_4$, sodium dodecyl sulfate.

Isolation of RNA. Rat liver polysomes were isolated as described by Taylor and Schimke (19), with minor modifications. The liver homogenate was centrifuged at 27,000 × *g* for 10 min, using bentonite rather than heparin as an RNase inhibitor. Discontinuous sucrose gradient centrifugation was performed in a Beckman SW 27 rotor (112,000 × *g* for 2.5 hr) or a Sorvall SS90 vertical rotor (26,500 × *g* for 100 min) by overlaying ≈15-ml samples of homogenate supernatant on gradients of 6 ml of 2.5 M sucrose, 12 ml of 1.0 M sucrose, and 2 ml of 0.5 M sucrose. Heparin was also omitted from this step. Polysomes were collected as described (20) and total RNA was extracted according to the method of Palmiter (21). After being precipitated twice with ethanol, the RNA was dissolved in sterile distilled water at 10 mg/ml and frozen at −15°C for use without further purification.

Cell-Free Translation. Micrococcal nuclease-treated rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (22), and was used for translation exactly as described by Cremer *et al.* (23). Aliquots (13 μl) were added to tubes containing 1 μl of rat liver polysomal RNA and 2–3 μl (≈15 μCi) of [^{35}S]methionine; the reaction could be scaled up at least 10- to 20-fold with no loss of translation efficiency. Reaction mixtures were incubated at 30°C for 75 min, then transferred to an ice bath for the start of the immunoprecipitation procedure. Incorporation of [^{35}S]methionine into protein was determined by precipitating 3-μl aliquots of translation mixture on Whatman 3 MM filter discs for 15 min in ice-cold 5% trichloroacetic acid and washing as follows: 15 min in boiling 5% trichloroacetic acid, 15 min in cold 5% trichloroacetic acid, and two times for 15 min each in absolute ethanol. After drying, the filters were placed in scintillation vials and their radioactivities were measured in 4.5 ml of Aquasol.

Immunological Procedures. Anti-OTCase antiserum was raised in rabbits immunized with homogeneous bovine OTCase, prepared as described by Marshall and Cohen (3) except that preparative isoelectric focussing was added as the final purification step. The antiserum, which inhibited OTCase activity in homogenates of rat liver almost as effectively as it did in homogenates of bovine liver, was monospecific when analyzed by immunoelectrophoresis and Ouchterlony immunodiffusion (data not shown). The IgG fraction of this antiserum, used in some experiments, was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (24).

For immunoprecipitation of [^{35}S]methionine-labeled proteins synthesized *in vitro*, a procedure very similar to that described by Cremer *et al.* was adopted (25). Aliquots (35 μl) of the cell-free translation mixture were diluted with 55 μl of 0.15 M NaCl/0.01 M EDTA/0.5% Triton X-100 and centrifuged for 2 min at full speed in a Fisher tabletop microcentrifuge. In some experiments, 7.5 μg of unlabeled OTCase was added at this point. Then 45 μl of a 1:50 dilution of anti-OTCase antiserum or control rabbit serum was added. After incubation for 2 hr at 4°C, 90 μl of 2% (wt/vol) unlabeled methionine was added, followed by 20 μl of a 10% (wt/vol) suspension of formalin-fixed *S. aureus* cells (26). The mixture was incubated at room temperature for 5 min and the bacteria–antigen–antibody complex was pelleted by centrifugation for 2 min at full speed in a Fisher tabletop microcentrifuge. The pellet was washed three times by resuspending in 50 μl of 150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$/10 mM Tris·HCl, pH 7.2; the last wash was accompanied by transfer to a clean tube. The final pellet was resuspended in 15 μl of gel sample buffer. Immediately prior to electrophoresis, the sample was heated in a boiling-water bath for 3 min, *S. aureus* cells were pelleted as above, and the supernatant was loaded on the gel.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out according to the method of Laemmli (27) on 12.5% polyacrylamide slab gels, using a 3.5% stacking gel. Gels were fixed and stained in 10% acetic acid/20% 2-propanol/0.1% Coomassie brilliant blue (vol/vol/wt) for 1 hr and destained overnight in 10% acetic acid/10% methanol (vol/vol). Radioactive bands were visualized by fluorography of dried gels on Kodak XR-5 x-ray film (28).

RESULTS

When total polysomal RNA isolated from rat liver was used to direct protein synthesis in the nuclease-treated rabbit reticulocyte lysate system, incorporation of [^{35}S]methionine into protein was stimulated 5- to 10-fold. A large number of different proteins were synthesized under these conditions, as demonstrated by fluorography of a dried $NaDodSO_4$/polyacrylamide gel (Fig. 1, lane b). To determine which of these bands was OTCase, immunoprecipitation of the translation mixture was performed with partially purified monospecific anti-OTCase antiserum, followed by addition of the immunoadsorbent *S. aureus* (26). A single major translation product

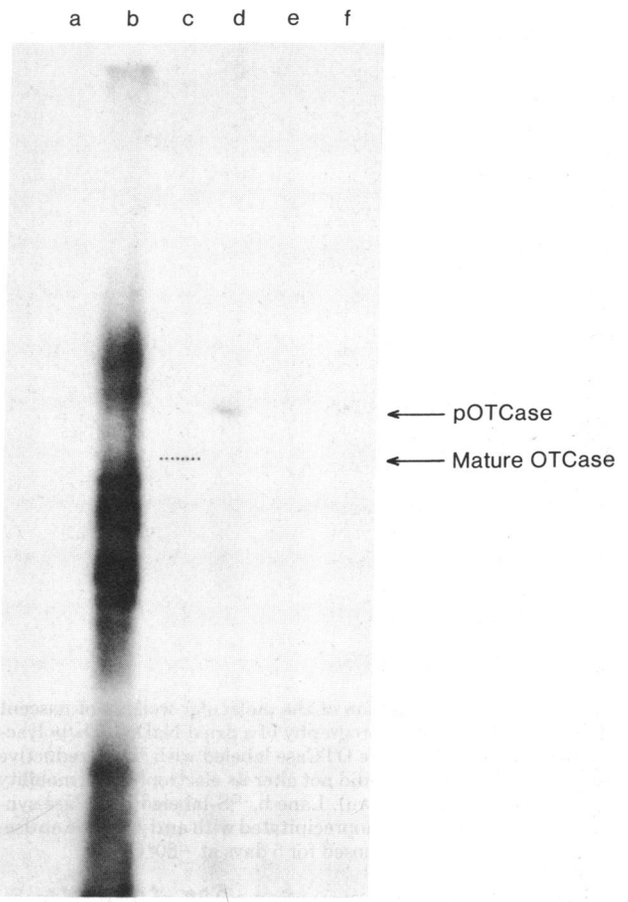

FIG. 1. Fluorography of a $NaDodSO_4$/polyacrylamide gel showing immunoprecipitation of a putative OTCase precursor (pOTCase) from the total cell-free translation products synthesized in the rabbit reticulocyte lysate system with rat liver polysomal RNA. The x-ray film was exposed for 32 hr at −80°C. Lane a, 3 μl of nuclease-treated lysate before addition of rat liver RNA; lane b, 3 μl of lysate after addition of rat liver polysomal RNA, showing synthesis of many different polypeptides; lane c, unlabeled mature OTCase subunit isolated from rat liver mitochondria (the dotted line indicates its position as determined by alignment of the Coomassie blue-stained gel with the fluorograph); lane d, pOTCase immunoprecipitated from 35 μl of translation mixture; lane e, immunoprecipitation performed as for lane d, except that 7.5 μg of unlabeled mature OTCase was present during incubation with antibody; lane f, immunoprecipitation of 35 μl of translation mixture performed with control rabbit serum.

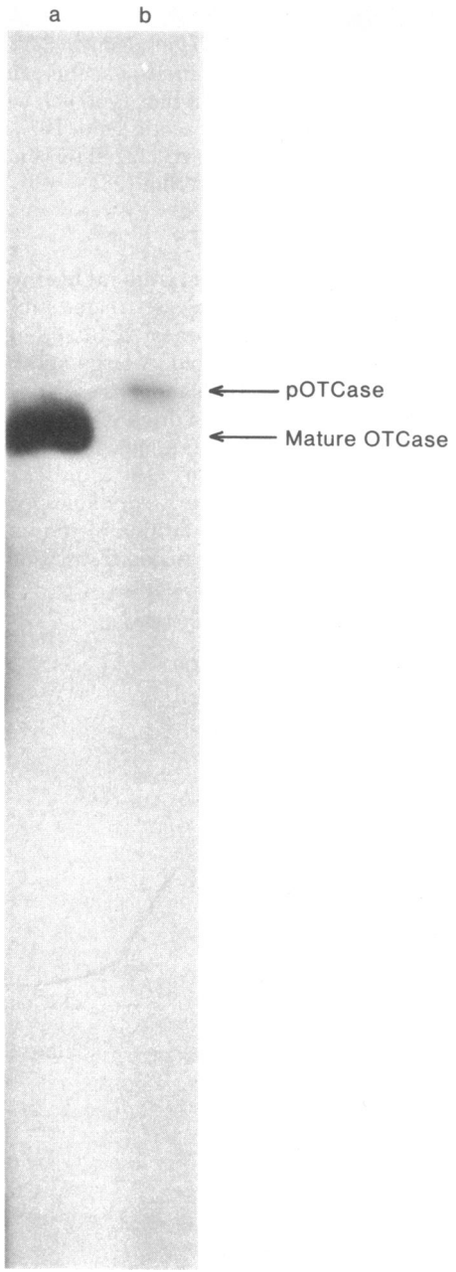

FIG. 2. Direct comparison of the molecular weights of nascent and mature OTCase by fluorography of a dried $NaDodSO_4$/polyacrylamide gel. Lane a, mature OTCase labeled with ^3H by reductive methylation; this procedure did not alter its electrophoretic mobility on these gels (data not shown). Lane b, ^{35}S-labeled pOTCase synthesized *in vitro* and immunoprecipitated with anti-OTCase antiserum. The x-ray film was exposed for 5 days at −80°C.

(designated pOTCase), representing ≈0.3% of total ^{35}S incorporated into trichloroacetic acid-insoluble material, was precipitated under these conditions (Fig. 1, lane d). This band was not precipitated by control rabbit serum (Fig. 1, lane f). Furthermore, excess unlabeled mature OTCase competed effectively with the *in vitro* product for interaction with the antibody (Fig. 1, lane e), thereby providing additional evidence for the identity of this band.

The molecular weight of nascent pOTCase was then compared with that of mature OTCase isolated from rat liver mitochondria. The two proteins were electrophoresed in adjacent slots of a $NaDodSO_4$/polyacrylamide gel and visualized either by Coomassie blue staining (for unlabeled mature OTCase) or by fluorography (for ^{35}S-labeled pOTCase and ^3H-labeled

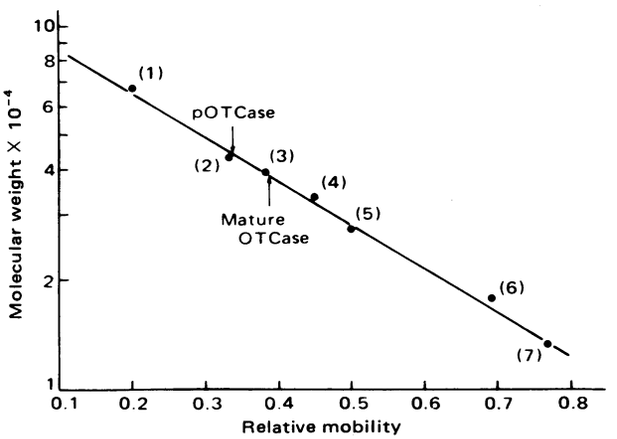

FIG. 3. Molecular weights of pOTCase and mature OTCase as estimated by their mobilities in $NaDodSO_4$/polyacrylamide gels. The numbers refer to the following standards: 1, bovine serum albumin (68,000); 2, ovalbumin (43,000); 3, aldolase (39,500); 4, aspartate transcarbamoylase catalytic subunit (34,000); 5, chymotrypsinogen (25,700); 6, aspartate transcarbamoylase regulatory subunit (17,000); 7, lysozyme (14,400).

mature OTCase). The data presented in Fig. 1 (lanes c and d) and Fig. 2 show clearly that pOTCase migrated more slowly than mature OTCase, indicating that pOTCase has a higher molecular weight. Quantitation of the molecular weight difference between pOTCase and mature OTCase was determined by comparing their electrophoretic mobilities with those of a number of pure standard proteins (Fig. 3). Mature OTCase had a mobility corresponding to a polypeptide with a molecular weight of ≈39,000. The pOTCase, on the other hand, was clearly larger, having an estimated molecular weight of ≈43,000. This molecular weight difference of ≈4000 was highly reproducible, being observed in ten consecutive experiments with material from two different rats.

DISCUSSION

The present results show that rat liver polysomal RNA directs the synthesis of a polypeptide (pOTCase) that specifically crossreacts with, but is slightly larger than, the mature subunit of mitochondrial OTCase. It could be argued that the size difference between pOTCase and mature OTCase reflects a *decrease* in size of the mature form produced during purification rather than an *increase* in the mass of the putative precursor. This seems most unlikely because the addition of protease inhibitors at all steps of the purification procedure used to prepare mature OTCase failed to change the size of the final product (data not shown).

We believe, on the other hand, that this *in vitro* product (pOTCase) represents a cytoplasmically synthesized precursor of the mature OTCase subunit. Several considerations support this conclusion. First, the larger size of pOTCase is not likely to be a translation artifact, because the reticulocyte lysate translates exogenous RNA with fidelity. In our system, immunoprecipitation of the same translation mixture with anti-rat serum albumin antiserum identified a specific product slightly larger than mature rat serum albumin, presumably representing intact preproalbumin (29). Second, the pOTCase band is not an artifact produced by the addition of rabbit serum, because it was not observed when preimmune rabbit serum was employed. Third, pOTCase is antigenically related to the mature OTCase subunit, because unlabeled OTCase effectively competed with the putative precursor for complexing by anti-OTCase antiserum. Fourth, similar results have been described for other cytoplasmically translated organellar proteins, including the mitochondrial matrix protein carbamoyl-phosphate

synthetase I mentioned earlier. Interestingly, pOTCase exceeds the mature OTCase subunit in size by an amount (≈4000 daltons) comparable to that observed for other putative precursors: ≈5500 for carbamoyl-phosphate synthetase (16); ≈6000 for the α subunit, ≈2000 for the β subunit, and ≈6000 for the γ subunit of F_1 ATPase from yeast mitochondria (15).

If pOTCase represents the form of the protein that is transported into mitochondria, and if the transport mechanism is similar to the posttranslational process described for other organellar proteins, it should be possible to demonstrate uptake by isolated mitochondria *in vitro*. One might predict that assembly of the enzymatically active trimer occurs only after transport and processing of individual subunits, but other models, including cotranslational transport, cannot be excluded *a priori*.

Whatever the mechanism, successful reconstitution of transport *in vitro* would give us the potential for examining this step in human livers characterized by inherited, complete OTCase deficiency (30). It is possible that an alteration in the amino acid sequence or conformation of a peptide unique to pOTCase could disturb the transport process and lead to phenotypic consequences indistinguishable from those caused by failure to synthesize the OTCase polypeptide or by the synthesis of a markedly unstable protein. Interestingly, recent studies of secreted prokaryotic proteins have established a precedent for such compartmentalization mutants. In *Escherichia coli*, a larger precursor of the maltose binding protein is normally synthesized in the cytoplasm and transported across the cytoplasmic membrane into the outer membrane. Mutants defective in this transport process have been identified and partially characterized (31). One of these shows cytoplasmic accumulation of the precursor form, caused apparently by a single amino acid substitution that may reside in the amino acid sequence of the peptide responsible for the enlarged size of the precursor, and therefore, likely to confer on the precursor its transport properties.

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1. Gamble, J. G. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248,** 610–618.
2. Clarke, S. (1976) *Biochem. Biophys. Res. Commun.* **71,** 1118–1124.
3. Marshall, M. & Cohen, P. P. (1972) *J. Biol. Chem.* **247,** 1641–1653.
4. Kalousek, F., Francois, B. & Rosenberg, L. E. (1978) *J. Biol. Chem.* **253,** 3939–3944.
5. Short, E. M., Conn, H. O., Snodgrass, P. J., Campbell, A. G. M. & Rosenberg, L. E. (1973) *N. Engl. J. Med.* **288,** 7–12.
6. Ricciuti, F. C., Gelehrter, T. D. & Rosenberg, L. E. (1976) *Am. J. Hum. Genet.* **28,** 332–338.
7. Schatz, G. & Mason, T. L. (1974) *Annu. Rev. Biochem.* **43,** 51–87.
8. Blobel, G. (1977) in *International Cell Biology, 1976–1977,* eds. Brinkley, B. R. & Porter, K. R. (Rockefeller Univ. Press, New York), pp. 318–325.
9. Walk, R.-A. & Hock, B. (1978) *Biochem. Biophys. Res. Commun.* **81,** 636–643.
10. Dobberstein, B., Blobel, G. & Chua, N.-H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1082–1085.
11. Chua, N.-H. & Schmidt, G. W. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6110–6114.
12. Highfield, P. E. & Ellis, R. J. (1978) *Nature (London)* **271,** 420–424.
13. Côté, C., Solioz, M. & Schatz, G. (1979) *J. Biol. Chem.* **254,** 1437–1439.
14. Michel, R., Wachter, E. & Sebald, W. (1979) *FEBS Lett.* **101,** 373–376.
15. Maccecchini, M.-L., Rudin, Y., Blobel, G. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 343–347.
16. Shore, G. C., Carignan, P. & Raymond, Y. (1979) *J. Biol. Chem.* **254,** 3141–3144.
17. Nuzum, C. T. & Snodgrass, P. J. (1976) in *The Urea Cycle,* eds. Grisolia, S., Baguena, R. & Mayor, F. (Wiley, New York), pp. 325–349.
18. Means, G. E. (1977) *Methods Enzymol.* **47,** 469–478.
19. Taylor, J. M. & Schimke, R. T. (1973) *J. Biol. Chem.* **248,** 7661–7668.
20. Palacios, R., Palmiter, R. D. & Schimke, R. T. (1972) *J. Biol. Chem.* **247,** 2316–2321.
21. Palmiter, R. D. (1974) *Biochemistry* **13,** 3606–3615.
22. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67,** 247–256.
23. Cremer, K., Bodemer, M. & Summers, W. C. (1978) *Nucleic Acid Res.* **5,** 2333–2344.
24. Livingston, D. M. (1974) *Methods Enzymol.* **34,** 723–731.
25. Cremer, K. J., Bodemer, M., Summers, W. P., Summers, W. C. & Gesteland, R. F. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 430–434.
26. Kessler, S. W. (1975) *J. Immunol.* **115,** 1617–1624.
27. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
28. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
29. Strauss, A. W., Donohue, A. M., Bennett, C. D., Rodkey, J. A. & Alberts, A. W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1358–1362.
30. Campbell, A. G. M., Rosenberg, L. E., Snodgrass, P. J. & Nuzum, C. T. (1973) *N. Engl. J. Med.* **288,** 1–6.
31. Bassford, P. & Beckwith, J. (1979) *Nature (London)* **277,** 538–541.