We have investigated the mechanism of overproduction of the multifunctional protein catalyzing the first three steps of UMP biosynthesis in stable mutants of Syrian hamster cells in culture. The rate of degradation of this protein is unaltered in one mutant cell line which overproduces it by 118-fold. In all mutants tested, the increase in the rate of synthesis of this protein is equal to the increase in its steady state concentration. There is a similar correlation between steady state levels of this protein in vitro and the capacity of polysomal RNA isolated from these cells to direct the synthesis of the protein in vitro. The one mutant cell line studied contains large amounts of a polysomal poly(A)-containing RNA (Mr = 2.7 ± 0.2 × 10^6) that is not detected in wild type cells. This large RNA co-sediments in sucrose gradients with the capacity to direct the synthesis of the multifunctional protein in vitro.

The transition state analog N-(phosphonacetyl)-L-aspartate (PALA)\(^{1}\) is a specific inhibitor of aspartate transcarbamylase (ATCase, EC 2.1.3.2), the second enzyme of de novo pyrimidine nucleotide biosynthesis in eukaryotes (Collins and Stark, 1971; Swyryd et al., 1974). PALA kills mammalian cells in tissue culture by blocking this essential pathway specifically (Swyryd et al., 1974). However, mutant cells resistant to low concentrations of PALA arise spontaneously in the population (Kempe et al., 1976). The frequency of mutation to PALA resistance depends on the type of cell and is not affected by the base alteration mutagen ethyl methanesulfonic acid (Kempe et al., 1976).

Kempe et al. (1976) obtained many mutants of simian virus 40-transformed Syrian hamster cells resistant to high levels of PALA by stepwise selection. Some of these mutants are resistant to 5000 times the concentration of PALA required to inhibit growth of the wild type cell line by half. All resistant clones tested have the same kinetic constants for the ATCase reaction as the sensitive cells. The uptake and stability of PALA are the same in both PALA-sensitive and -resistant cells. However, all resistant clones have increased ATCase activity per cell, and the extent of the increase depends on the PALA concentration used for selection. In every cell line tested, the increase in specific activity of ATCase corresponds closely to the increase in concentration of ATCase active sites determined by titration of crude extracts with \(^3\)H-labeled PALA. In some mutants resistant to 25 mM PALA, the highest concentration used for selection, the specific activity of ATCase in crude extracts is more than 100 times greater than in wild type cells. With a few exceptions, the PALA-resistant phenotype was stable after growing the mutants without PALA for at least 60 generations.

The specific activities of carbamyl-phosphate synthetase (glutamine) (EC 2.7.2.9) and dihydro-orotase (EC 3.5.2.3), the first and third enzymes of the pathway, are increased coordinately with that of ATCase in PALA-resistant mutants. All three activities co-purify from a variety of eukaryotic sources (Mori and Tatibana, 1973 and 1975; Hoogenraad et al., 1971; Shoaf and Jones, 1973; Ito and Uchino, 1972). Recently, Coleman et al. (1977) have shown that all three activities co-purify as a high molecular weight protein from one clone of PALA-resistant Syrian hamster cells, and that all three activities are contained within a single polypeptide chain of molecular weight about 200,000. We will refer to this multifunctional protein as CAD (carbamyl-P synthetase, aspartate transcarbamylase, dihydro-orotase).

We now show that the increase in the amount of CAD in several PALA-resistant mutants is due to an increase in its rate of synthesis rather than to a decrease in its rate of degradation. The increased rate of synthesis is accompanied by an increase in the amount of a single poly(A)-containing RNA which directs the synthesis of CAD in vitro.

**METHODS**

**Cells and Cell Culture**—Procedures for growing cells and for preparing dialyzed serum are described by Swyryd et al. (1974). The origin and selection of PALA-resistant cell lines used in this study are described by Kempe et al. (1976). All cells are derivatives of the SV28 line of simian virus 40-transformed Syrian hamster cells (Wiblin and MacPherson, 1972). Cloned PALA resistant line 97-3 was selected in 1 mM PALA; lines 165-21 and 165-23 were selected in multiple steps to resist 25 mM PALA; and line 165-23R is a partial revertant of 165-23 which arose spontaneously after about 5 months in culture without PALA and which has about one-half the ATCase specific activity of 165-23.

**Aspartate Transcarbamylase Assays**—Enzyme activity was measured in extracts of subconfluent cells prepared by sonication and assayed as described by Kempe et al. (1976). Protein concentrations were determined according to Bradford (1976).

**Preparation of Antibodies**—The multifunctional protein was purified from 165-23 cells according to Coleman et al. (1977). Rabbits were injected subdermally with 100 µg of purified protein in...
Freund's complete adjuvant and were boosted at 2-week intervals with 100 µg of purified protein in Freund's incomplete adjuvant. This regime yielded poor antibody titers, so the amount of protein in each injection was increased to 1 mg, which increased the titers substantially. Antibody titers were determined using Staphylococcus aureus protein A in the assay described by Crawford and Lane (1977) with to label 1 mg of protein to a specific activity of approximately 10³ cpm/mg. Antibody titers were determined using Staphylococcus aureus microcuries of [14C]formaldehyde (New England Nuclear) were used to label 1 mg of protein to a specific activity of approximately 10⁵ cpm/mg.

Preparation of Polyomavirus RNAs—Polyosomes were prepared after lysis of the cells with NP-40 (Gieken et al., 1971). Cells grown in roller bottles were fed with fresh medium 3 to 4 h before harvest, washed with ice cold isocitic buffer, scraped into ice cold buffer, collected by centrifugation at 750 x g for 5 min, and washed twice with ice cold isocitic buffer. The polysome pellets were resuspended in ice cold 10 mM KCl, 5 mM MgCl₂, and 40 µg/ml of heparin in a Beckman SW 41 tube and sedimented at 273,000 x g for 135 min at 4°C. The supernatant was removed and the polysome pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 1% SDS, and 100 µg/ml of Proteinase K (Merck) and incubated for 30 min at 37°C. Sodium acetate buffer, pH 5.0, was added to 50 mM and the RNA was then divided into two equal parts. To one part, 50 mM sodium acetate buffer, pH 5.0, followed by two extractions with chloroform. The RNA was then precipitated with 2.5 volumes of absolute ethanol at −20°C overnight (Palmiter, 1974). The precipitate was washed twice with 3 M sodium acetate, pH 6.0, followed by two washes with 75% ethanol. The RNA samples were dried at room temperature in a vacuum desiccator and dissolved in sterile, quartz-distilled water. The RNA could be stored as an ethanol precipitate for at least 6 weeks without any loss of translational activity.

Protein Synthesis in Vitro—The mRNAs-dependent rabbit reticulocyte lysate described by Pelham and Jackson (1976), modified as described above, was used. Lysates (100 to 200 µl) were made 1.0 mm MgCl₂, 40 units/ml of creatine phosphokinase (Calbiochem), and either 125 µCi/ml of [3H]leucine (58 Ci/mmol, Amersham/Searle) or 300 µCi/ml of [35S]methionine (1175 Ci/mmol, Amersham/Searle). The amino acid mixture used is the one described by Lingrel and Borns (1963) except that it contained 240 µM methionine when [35S]methionine was used. The [3H]leucine was dried in a vacuum desiccator to remove the ethanol in which it was stored. Each reaction mixture also contained 50 µg/ml of rabbit liver trichloroacetic acid-precipitable radioactivity, and the remainder was used. Lysates (100 to 200 µl) were made 1.0 mm MgCl₂, 40 units/ml of creatine phosphokinase (Calbiochem), and either 125 µCi/ml of [3H]leucine (58 Ci/mmol, Amersham/Searle) or 300 µCi/ml of [35S]methionine (1175 Ci/mmol, Amersham/Searle). The amino acid mixture used is the one described by Lingrel and Borns (1963) except that it contained 240 µM methionine when [35S]methionine was used. The [3H]leucine was dried in a vacuum desiccator to remove the ethanol in which it was stored. Each reaction mixture also contained 50 µg/ml of rabbit liver trichloroacetic acid-precipitable radioactivity, and the remainder was used.

Immunoprecipitation of CAD Synthesized in Vitro—Reaction mixtures were diluted with 3 volumes of TEN buffer (25 mM Tris-HCl (pH 7.4), 10 mM EDTA, 350 mM NaCl, 0.15% Triton X-100) containing 25 µCi/ml of [3H]leucine (58 Ci/mmol, Amersham/Searle) or 300 µCi/ml of [35S]methionine (1175 Ci/mmol, Amersham/Searle). After labeling, the medium was incubated with the cell-free extract described by Lingrel and Borns (1963) except that it contained 240 µM methionine when [35S]methionine was used. The [3H]leucine was dried in a vacuum desiccator to remove the ethanol in which it was stored. Each reaction mixture also contained 50 µg/ml of rabbit liver trichloroacetic acid-precipitable radioactivity, and the remainder was used.

Immunoprecipitation of CAD Synthesized in Vitro—Reaction mixtures were diluted with 3 volumes of TEN buffer (25 mM Tris-HCl (pH 7.4), 10 mM EDTA, 350 mM NaCl, 0.15% Triton X-100) containing 25 µCi/ml of [3H]leucine (58 Ci/mmol, Amersham/Searle) or 300 µCi/ml of [35S]methionine (1175 Ci/mmol, Amersham/Searle). After labeling, the medium was incubated with the cell-free extract described by Lingrel and Borns (1963) except that it contained 240 µM methionine when [35S]methionine was used. The [3H]leucine was dried in a vacuum desiccator to remove the ethanol in which it was stored. Each reaction mixture also contained 50 µg/ml of rabbit liver trichloroacetic acid-precipitable radioactivity, and the remainder was used.
sample as an internal standard for immunoprecipitation and recovery. The mixture was incubated at room temperature for 15 min after which the staphs were removed by centrifugation at 8000 x g for 15 min at 4°C. This incubation in the absence of antibody was essential for obtaining low backgrounds. The supernatant solutions were transferred to clean tubes and 5 µl of immune or nonimmune serum were added. Unfractionated serum gave lower backgrounds than did purified IgG and did not degrade purified CAD. Reaction mixtures were incubated overnight at 4°C, 25 µl of staphs were added and incubation was continued for 10 min at 0°C. The staphs were collected by centrifugation at 8000 x g for 2 min at 25°C. The pellets were washed twice by resuspending them vigorously in a Vortex mixer to 700 µl of TENT containing 700 mM NaCl and either 2 mM leucine or 2 mM methionine followed by one wash with 700 µl of 10 mM Tris-HCl (pH 7.4). The staphs were pelleted after each wash by centrifuging them for 30 s at 8000 x g. Immune complexes were eluted from the staphs with 8 M urea, 1% SDS, 10 mM Tris-HCl (pH 7.4) for 60 min at 25°C. The staphs were removed by centrifugation at 8000 x g for 1 min and the supernatant solutions were added to 25 µl of SDS sample buffer for electrophoresis (Webber and Osborn, 1969), heated to 85°C for 15 min, centrifuged at 8000 x g for 1 min, and loaded onto a 4.5% polyacrylamide slab gel in SDS, prepared and run as described by Weber and Osborn (1969). Six 2 mm slices were cut from each gel track after staining, starting 2 mm above the visible band of CAD. The slices were dissolved in 400 µl of Soluene 350 overnight at 37°C, 4 ml of toluene scintillation fluid were added, and the samples were counted. Backgrounds were determined by counting corresponding gel slices taken from blank tracks and from tracks containing material precipitated by nonimmune serum. In quantitative experiments, 32P-labeled protein. Immunoprecipitates of reactions programmed by RNA isolated from sucrose gradient fractions were analyzed on slab gels by fluorography according to Laskey and Mills (1975).

Labeling of RNA with [32P]Phosphate—Two roller bottles each of SV28 and 165-23 cells were grown to approximately 50% confluency in DME medium containing 10% calf serum. The medium was removed and replaced with 15 ml of DME medium containing 12.4 mg/liter of NaH2PO4, H2O (10% of the original phosphate concentration), 10% calf serum, and 2.5 mCi of [32P]phosphate (New England Nuclear). Cells were labeled for 2 h at 37°C and then 50 ml of DME medium containing the standard phosphate concentration and 10% calf serum were added. After 30 to 45 min at 37°C, the cells were harvested and RNA was prepared as described above.

Electrophoresis of RNA in Glycerol Gels—Samples were run in horizontal slab gels (14 x 23 cm) containing 1% agarose and 10 mM CH3HgOH according to Bailey and Davidson (1976). The RNA was precipitated with ethanol in the presence of 15 µg/ml of rabbit liver tRNA, washed with 75% ethanol, and resuspended in electrophoresis buffer containing 10 mM CH3HgOH, 5% glycerol, and 0.025% bromphenol blue. The gels were run at 20 to 25 V (measured across the gel) for 12 to 14 h, washed with 0.5 M acetate followed by water, dried, and autoradiographed with an intensifying screen (Kodak X-Omataric regular) at ~80°C.

Electrophoresis of RNA in Glycerol Gels—Samples were precipitated, washed and dried as above, and resuspended in the denaturing buffer containing 1 M glyoxal purified over mixed bed resin (Amberlite MB-3), 50% (v/v) dimethyl sulfoxide, and 10 mM NaH2PO4/Na2HPO4 buffer, pH 7.0. The samples were heated at 50°C for 1 h and were run on 1% agarose horizontal slab gels at 50 V for 3 to 4 h. RNA was visualized with acridine orange and photographed by short wavelength UV light.

Chromatography on Oligo(dT)-cellulose—RNA dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA was heated to 75°C for 5 min and quickly cooled in an ice/ethanol bath. NaCl was added to 0.5 M. We have not observed any significant degradation of the RNA due to heating under these conditions. The RNA was passed over a column of oligo(dT)-cellulose (200 mg, Collaborative Research, binding capacity = 71 A260/mg) which had been washed with 0.1 M KOH, neutralized with 1 M Tris-HCl (pH 7.4) and equilibrated with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. Bound RNA was eluted with 2 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. The RNA was then treated as described above and passed over the column again. Approximately 2% of the RNA was bound in the first pass and more than 90% in the second pass.

Sedimentation of RNA in Sucrose Gradients—The RNA was dissolved in 100 µl of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, heated to 75°C for 5 min and cooled quickly. Sodium lauryl sarcosinate was added to 1% and the sample was loaded onto a 10 to 30% (w/v) linear sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% sodium lauryl sarcosinate. The gradients were centrifuged in a Beckman SW 41 rotor at 272,000 x g at 4°C for 7 h. Fractions of approximately 300 µl were collected by puncturing the bottom of the tube.

RESULTS

Rates of Degradation and Synthesis of the Multifunctional Protein (CAD) in Vivo—Rates of degradation were determined by pulse-labeling PALA-sensitive or PALA-resistant cells with [3H]leucine, followed by a chase with unlabeled leucine for various times. Purified IgG directed against purified CAD was used to immunoprecipitate the protein from crude extracts. Purified CAD from PALA-resistant cells, labeled with 14C in vitro, was added to each precipitation as an internal standard. The immunoprecipitates were then fractionated by SDS-gel electrophoresis, and the radioactivity co-migrating with purified CAD was determined.

Three conditions must be met for this method to yield accurate results: 1) CAD from PALA-sensitive and -resistant cells must co-migrate on SDS gels; 2) since CAD represents only 0.05 to 0.1% of the total cellular protein in PALA-sensitive cells, accurate measurements of its abundance can only be made if it is separated from all other proteins; 3) there can be no selective loss of CAD from either PALA-sensitive or -resistant cells. As shown in Fig. 1, CAD from [3H]leucine labeled PALA-sensitive cells and [3H]leucine-labeled PALA-resistant cells co-migrate on SDS gels. Furthermore, CAD is the predominant labeled species in the immunoprecipitate. To demonstrate that the other two conditions are met, PALA-resistant and PALA-sensitive cells were labeled with [14C]- and [3H]leucine, respectively, for 3 days to achieve a steady state. Extracts from these cells were mixed and analyzed for the relative amounts of each label in CAD. As shown in the last column of Table I, the ratio of the two labels in CAD purified by this procedure is nearly the same as the ratio of the ATCase activities in the resistant and sensitive cells employed. Since the amount of ATCase activity is directly proportional to the amount of CAD protein (Kempe et al., 1976), the data in Table I indicate that the CAD is being recovered equally from extracts of PALA-sensitive and -resistant cells.
sensitive SV28 cells. The data of Table I also show that there is no contamination by other labeled proteins since only a small contamination would depress the ratios markedly. For example, a nonspecific contamination of as little as 0.05% of the total labeled protein would reduce the 165-23 to SV28 ratio from 110 to 74. In addition, while quantitative recovery of labeled CAD from 165-23; 0, CAD from SV28; W, total protein from 165-23; pulse-labeled with [3H]leucine and chased with unlabeled leucine. 0, CAD from SV28.

**TABLE I**

**Rates of synthesis of CAD in vivo**

<table>
<thead>
<tr>
<th>PALA-resistant line</th>
<th>Relative ATCase activity</th>
<th>Relative rate of CAD synthesis</th>
<th>Relative amount of label in CAD at steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td>165-23</td>
<td>118</td>
<td>101</td>
<td>110</td>
</tr>
<tr>
<td>165-23R</td>
<td>63</td>
<td>52</td>
<td>70</td>
</tr>
<tr>
<td>165-21</td>
<td>101</td>
<td>94</td>
<td>n.d.*</td>
</tr>
<tr>
<td>9-3</td>
<td>6.6</td>
<td>9.1</td>
<td>n.d.*</td>
</tr>
</tbody>
</table>

*a n.d., not determined.

...pale labeling PALA-sensitive SV28 cells and several PALA-resistant cell lines with [3H]- and [14C]leucine, respectively. The ratios of [3H] to [14C]-labeled CAD were measured from mixtures of sensitive and resistant cell extracts after immunoprecipitation and electrophoresis in SDS gels. The [3H] to [14C] ratios in total protein in the mixtures were calculated from the trichloroacetic acid-precipitable radioactivity of the extracts. As shown in Table I, Column 3, there is a good correlation between the relative rate of CAD synthesis and the relative amount of CAD in the various PALA-resistant cell lines.

**Synthesis of CAD in Vitro**—The increased rate of synthesis of CAD in PALA-resistant cells could be due either to alterations in the protein synthetic machinery or to an altered structure or abundance of the mRNA(s) coding for CAD. To help distinguish among these alternatives, we have used cell-free protein synthesis to determine whether the increased rate of synthesis in vivo is due to an increase in the amount of specific mRNA(s). The message-dependent reticulocyte lysate protein synthesis system of Pelleman and Jackson (1976), supplemented with polyoma RNA isolated from PALA-sensitive and PALA-resistant cells. Reactions programmed with polyoma RNA isolated from PALA-sensitive cells (Fig. 3A) or PALA-resistant cell lines (Fig. 3C) stimulated synthesis of a protein which could be precipitated with CAD-specific antisera and which co-migrated with purified [14C]CAD on SDS gels. Identical reaction mixtures precipitated with nonimmune serum gave no such material (Fig. 3, B and D). Reaction mixtures without added RNA failed to direct the synthesis of any immunoreactive protein (Fig. 3E, open circles) or other product (Fig. 3F) which co-migrated with authentic CAD.

The amounts of CAD synthesized in vitro in response to RNA from different mutants are shown as a function of the concentration of added polyoma RNA in Fig. 4. RNA from PALA-resistant mutants stimulates the synthesis in vitro of far more CAD than does RNA isolated in an identical manner...
mRNA Overaccumulation in PALA-resistant Cells

**Fig. 4.** Synthesis of CAD in vitro. Incorporation of label into CAD during a 60-min reaction in the rabbit reticulocyte lysate system programmed with different amounts of total polysomal RNAs isolated from the indicated cell lines. The ordinate indicates the amount of radioactivity in CAD in a 50-μl reaction. Reactions programmed by RNA isolated from SV28 or 97-3 were 100-μl reactions.

**TABLE II**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Counts per min in total protein*</th>
<th>Counts per min in CAD</th>
<th>Fraction of counts per min in CAD</th>
<th>Relative synthesis of CAD</th>
<th>Relative ATCase activity in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV28</td>
<td>10,500</td>
<td>12</td>
<td>0.11</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>165-23</td>
<td>12,500</td>
<td>1,450</td>
<td>11.6</td>
<td>105</td>
<td>118</td>
</tr>
<tr>
<td>165-23R</td>
<td>12,500</td>
<td>750</td>
<td>6.0</td>
<td>55</td>
<td>63</td>
</tr>
<tr>
<td>165-21</td>
<td>12,000</td>
<td>1,600</td>
<td>13.3</td>
<td>121</td>
<td>101</td>
</tr>
<tr>
<td>97-3</td>
<td>15,000</td>
<td>100</td>
<td>0.67</td>
<td>6.6</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* From reactions containing 25 μg/ml of added RNA.

**Fig. 5.** Synthesis of CAD in vitro from sucrose gradient fractions of polysomal poly(A)-containing RNA. A and B, sucrose gradient profiles of 32P-labeled RNA bound to oligo(dT)-cellulose from PALA-sensitive SV28 or PALA-resistant 165-23 cells. Fractions within the dashed lines were pooled. Ribosomal RNA markers were run in parallel gradients. C and D, CH₃HgOH/agarose gels of RNA from the pooled fractions. Arrows indicate the position of the abundant large RNA found in 165-23 cells. Markers (MRK) are ribosomal RNAs from Syrian hamster cells. E and F, SDS-polyacrylamide gels of immunoprecipitated products of in vitro protein synthesis programmed by the same RNA fractions. Arrows indicate the position of pure CAD.

mRNA on a sucrose density gradient. Polysomal RNA labeled with [32P]phosphate was prepared from PALA-sensitive SV28 and PALA-resistant 165-23 cells. The fraction of polysomal RNA which bound to a column of oligo(dT)-cellulose was eluted and sedimented in a linear 10 to 30% (w/v) sucrose from PALA-sensitive cells. RNA from PALA-sensitive cells gives a low but clearly detectable synthesis of the protein, while RNA from the PALA-resistant lines gives levels of synthesis corresponding quantitatively to the amount of enzyme overproduction measured in vivo (see Table II). An equal mixture of RNAs from PALA-sensitive SV28 cells and from PALA-resistant 165-23 cells (118-fold overproduction) gives an intermediate level of synthesis, indicating the absence of any inhibitors or activators in the RNA preparations. In Table II are summarized the results obtained by comparing the slopes of the linear portions of RNA concentration profiles such as those shown in Fig. 4. For each PALA-resistant cell line, the capacity of the RNA to direct synthesis of CAD in vitro parallels almost exactly both the relative rate of CAD synthesis in vivo and the relative CAD concentration.

One Large mRNA Codes for CAD—Three mechanisms can account for the synthesis of one polypeptide chain with three enzymatic activities: 1) one large mRNA may be translated into one large polypeptide; 2) separate small mRNAs may be translated into separate small polypeptides which are joined subsequently to form the final protein; 3) a large mRNA may be translated into more than one protein with subsequent joining.

A single mRNA coding for CAD would have to be at least 6000 nucleotides long, far larger than the average mRNA in hamster cells. Therefore, we attempted to purify such an mRNA on a sucrose density gradient. Polysomal RNA labeled with [32P]phosphate was prepared from PALA-sensitive SV28 and PALA-resistant 165-23 cells. The fraction of polysomal RNA which bound to a column of oligo(dT)-cellulose was eluted and sedimented in a linear 10 to 30% (w/v) sucrose from PALA-sensitive cells. RNA from PALA-sensitive cells gives a low but clearly detectable synthesis of the protein, while RNA from the PALA-resistant lines gives levels of synthesis corresponding quantitatively to the amount of enzyme overproduction measured in vivo (see Table II). An equal mixture of RNAs from PALA-sensitive SV28 cells and from PALA-resistant 165-23 cells (118-fold overproduction) gives an intermediate level of synthesis, indicating the absence of any inhibitors or activators in the RNA preparations. In Table II are summarized the results obtained by comparing the slopes of the linear portions of RNA concentration profiles such as those shown in Fig. 4. For each PALA-resistant cell line, the capacity of the RNA to direct synthesis of CAD in vitro parallels almost exactly both the relative rate of CAD synthesis in vivo and the relative CAD concentration.

One Large mRNA Codes for CAD—Three mechanisms can account for the synthesis of one polypeptide chain with three enzymatic activities: 1) one large mRNA may be translated into one large polypeptide; 2) separate small mRNAs may be translated into separate small polypeptides which are joined subsequently to form the final protein; 3) a large mRNA may be translated into more than one protein with subsequent joining.

A single mRNA coding for CAD would have to be at least 6000 nucleotides long, far larger than the average mRNA in hamster cells. Therefore, we attempted to purify such an mRNA on a sucrose density gradient. Polysomal RNA labeled with [32P]phosphate was prepared from PALA-sensitive SV28 and PALA-resistant 165-23 cells. The fraction of polysomal RNA which bound to a column of oligo(dT)-cellulose was eluted and sedimented in a linear 10 to 30% (w/v) sucrose from PALA-sensitive cells. RNA from PALA-sensitive cells gives a low but clearly detectable synthesis of the protein, while RNA from the PALA-resistant lines gives levels of synthesis corresponding quantitatively to the amount of enzyme overproduction measured in vivo (see Table II). An equal mixture of RNAs from PALA-sensitive SV28 cells and from PALA-resistant 165-23 cells (118-fold overproduction) gives an intermediate level of synthesis, indicating the absence of any inhibitors or activators in the RNA preparations. In Table II are summarized the results obtained by comparing the slopes of the linear portions of RNA concentration profiles such as those shown in Fig. 4. For each PALA-resistant cell line, the capacity of the RNA to direct synthesis of CAD in vitro parallels almost exactly both the relative rate of CAD synthesis in vivo and the relative CAD concentration.

One Large mRNA Codes for CAD—Three mechanisms can account for the synthesis of one polypeptide chain with three enzymatic activities: 1) one large mRNA may be translated into one large polypeptide; 2) separate small mRNAs may be translated into separate small polypeptides which are joined subsequently to form the final protein; 3) a large mRNA may be translated into more than one protein with subsequent joining.

A single mRNA coding for CAD would have to be at least 6000 nucleotides long, far larger than the average mRNA in hamster cells. Therefore, we attempted to purify such an mRNA on a sucrose density gradient. Polysomal RNA labeled with [32P]phosphate was prepared from PALA-sensitive SV28 and PALA-resistant 165-23 cells. The fraction of polysomal RNA which bound to a column of oligo(dT)-cellulose was eluted and sedimented in a linear 10 to 30% (w/v) sucrose from PALA-sensitive cells. RNA from PALA-sensitive cells gives a low but clearly detectable synthesis of the protein, while RNA from the PALA-resistant lines gives levels of synthesis corresponding quantitatively to the amount of enzyme overproduction measured in vivo (see Table II). An equal mixture of RNAs from PALA-sensitive SV28 cells and from PALA-resistant 165-23 cells (118-fold overproduction) gives an intermediate level of synthesis, indicating the absence of any inhibitors or activators in the RNA preparations. In Table II are summarized the results obtained by comparing the slopes of the linear portions of RNA concentration profiles such as those shown in Fig. 4. For each PALA-resistant cell line, the capacity of the RNA to direct synthesis of CAD in vitro parallels almost exactly both the relative rate of CAD synthesis in vivo and the relative CAD concentration.

One Large mRNA Codes for CAD—Three mechanisms can account for the synthesis of one polypeptide chain with three enzymatic activities: 1) one large mRNA may be translated into one large polypeptide; 2) separate small mRNAs may be translated into separate small polypeptides which are joined subsequently to form the final protein; 3) a large mRNA may be translated into more than one protein with subsequent joining.

A single mRNA coding for CAD would have to be at least 6000 nucleotides long, far larger than the average mRNA in hamster cells. Therefore, we attempted to purify such an
FIG. 6. Molecular weight determination of CAD mRNA. RNA samples were run on horizontal 1% agarose slab gels after denaturation with 1 M glyoxal (McMaster and Carmichael, 1977). VSV, vesicular stomatitis virus RNA, \( M_r = 3.6 \times 10^6 \) (Wagner, 1975); poliovirus RNA, \( M_r = 2.6 \times 10^5 \) (Granboulan and Girard, 1968); 28 S and 18 S rRNAs from HeLa cells, \( M_r = 1.75 \) and 0.67 \( \times 10^6 \) (McMaster and Carmichael, 1977).

gradient" (Fig. 5, A and B). The fractions indicated were precipitated with ethanol and one-half of each was analyzed in denaturing CH\(_3\)H\(_2\)OH gels (Bailey and Davidson, 1976) (Fig. 5, C and D). The other half of each was used to prepare protein synthesis in vitro (Fig. 5, E and F). A prominent band of large RNA is seen in the gradient fractions from PALA-resistant 165-23 cells (Fig. 5D), but no such band is apparent in the same fractions of the gradient from the PALA-sensitive cells (Fig. 5C). Strong stimulation of the synthesis of full length immunoreactive CAD is found in the same gradient fractions which contain the prominent large RNA species (Fig. 5F). Furthermore, as shown in Fig. 5F, the amount of CAD made in vitro is proportional to the abundance of the prominent RNA species in each of the fractions. Corresponding sucrose gradient fractions from PALA-sensitive cells did not produce enough CAD to be detected in this experiment (Fig. 5E).

Most of the immunoreactive material shorter than full length CAD appears to be CAD-specific since it is not seen in precipitations with nonimmune serum (Fig. 3, B, D, and E and other experiments not shown) and since its abundance is proportional to the amount of full length CAD (data not shown). This material may result from mRNA breakdown in the reactions, incomplete synthesis of some polypeptide chains, proteolytic activity in the reticulocyte lysate system, or a combination of these factors.

In Fig. 6 are shown data from several experiments in which the mobilities of RNAs of known molecular weight are compared with that of the RNA which codes for the multifunctional protein. In these experiments, the RNA was denatured with glyoxal in the presence of dimethyl sulfoxide, followed by separation of the denatured RNAs on agarose gels (McMaster and Carmichael, 1977). This method gives the same results as those obtained with RNA denatured with CH\(_3\)H\(_2\)OH, but is less dangerous and much more rapid. We estimate that the molecular weight of the CAD mRNA is 2.7 \( \pm 0.2 \times 10^6 \), corresponding to about 8000 nucleotides, 2000 nucleotides more than minimally required to code for a protein of \( M_r = 200,000 \).

**DISCUSSION**

Mutants resistant to high concentrations of PALA were selected in several steps, and in all mutates studied, the amount of CAD is increased roughly in proportion to the PALA concentration used for selection. Therefore, multiple mutations may account for the elevated levels of CAD in these cells. The increase in CAD steady state levels in PALA-resistant cells must be due to mutations affecting its rate of degradation or synthesis.

The half-life of CAD in one PALA-resistant mutant which overproduces CAD by 116-fold is about 75 h, the same as in the PALA-sensitive wild type. Since the doubling time of the wild type is 18 h, it is not possible to account for a significant increase in the amount of CAD in any PALA-resistant mutant by a decrease in its rate of degradation. It is interesting to note that CAD is an exception to the general correlations that in eukaryotes large proteins and enzymes at control points in metabolic pathways are degraded more rapidly than the average cellular protein (Goldberg and St. John, 1976).

Using an approach similar to that of Alt et al. (1976), we have shown that the relative rates of synthesis of CAD correlate very well with the relative steady state levels of CAD in several PALA-resistant cell lines. Therefore, an increase in the rate of synthesis is sufficient to explain the elevated levels of CAD in these cells.

The increased rate of CAD synthesis is due to an increase in the amount of translatable mRNA coding for this protein in every PALA-resistant cell line studied. The relative ability of polysomal RNA isolated from PALA-sensitive and PALA-resistant cells to program the synthesis of CAD in vitro correlates very well with the relative rates of CAD synthesis in vivo and also with the relative amounts of CAD protein in these cells. The increase in the amount of CAD specific translation could be due to an increase in the amount of specific mRNA in the cells, to activation of a pre-existing but translationally inactive population of specific mRNA molecules, or to modification of the structure of the specific mRNA to allow more efficient initiation or elongation. We believe the first possibility is the most likely, and in support of this, we find a prominent poly(A)-containing RNA of molecular weight 2.7 \( \pm 0.2 \times 10^6 \) that is present in one highly PALA-resistant cell line and is not detectable in the PALA-sensitive parental line. This RNA co-sediments in sucrose gradients with the capacity to stimulate the translation of the full length multifunctional protein in vitro. Experiments are currently in progress to quantitate the levels of CAD-specific mRNA in various PALA-resistant cell lines by other methods in order to show more conclusively that the CAD-specific mRNA itself is overaccumulated.

Our results also extend the understanding of the tight linkage of the first three enzymes of the pyrimidine biosynthetic pathway. Taking into account the observations that all PALA-resistant mutants examined overproduce the first three enzymes of de novo pyrimidine nucleotide biosynthesis coordinately (Kenpe et al., 1976), that all three enzymatic activities reside in one covalently linked polypeptide chain (Coleman et al., 1977), and that one large mRNA stimulates the translation of immunoreactive full length CAD in vitro, we propose that covalent association of these activities is the result of the continuous translation of the single mRNA. Although we have no direct evidence against the unlikely possibility that the three enzymatic activities are translated separately from a single mRNA and then joined subsequently, we have never observed significant amounts of discrete immunoreactive translation products which are less than full length, even though our immune serum does cross-react with partial synthesis products (see "Results"). Furthermore, there is very strong genetic and biochemical evidence in yeast that some proteins having more than one enzymatic activity in one polypeptide chain result from the continuous translation of a single mRNA (Manney, 1968; Shaffer et al., 1969; Tauro et
mRNA Overaccumulation in PALA-resistant Cells

Also consistent with this idea is the observation that fatty acid synthetase from rat liver, another multifunctional protein, is synthesized on large polysomes in vivo (Alberts et al., 1975) and can be made as a full length species in vitro as a product of runoff synthesis from polysomes (Strauss et al., 1975).

The ability to select for cells which overproduce a specific gene product by using a specific inhibitor is neither unique to PALA nor to eukaryotic cells. Roth and his collaborators (Anderson et al., 1975) have used aminopterin, a specific inhibitor of imidazole glycerol phosphate dehydrogenase, to select for Salmonella mutants which overproduce this enzyme by 2- to 4-fold. Several groups have used methotrexate, a tight-binding and specific inhibitor of dihydrofolate reductase, to select for mutants in several mammalian cell lines which overproduce this enzyme by at least 100-fold relative to the parental line. The only mechanism found for increasing the level of dihydrofolate reductase is an increase in the level of translatable mRNA (Kellem et al., 1976; Chang and Littlefield, 1976). In more recent studies, Alt et al. (1978) show that there is a one-to-one correlation between the degree of overproduction of dihydrofolate reductase, the level of specific mRNA, and the number of dihydrofolate reductase genes. The possibility of gene amplification, among others, is presently under investigation with PALA-resistant mutant lines.

Specific and potent inhibitors have been used in only a few instances to obtain mutants which overproduce the target enzymes. However, this approach may well be useful in obtaining different kinds of regulatory mutants in mammalian cells. The availability of large quantities of specific enzymes, specific mRNAs, and possibly the genes which encode them will facilitate the analysis at a molecular level of many of the processes involved in the control of gene expression in eukaryotes.

Acknowledgments—We thank Michael Long for valuable assistance with the rabbits and in the preparation and assay of the reticulocyte lysate. We are indebted to Dr. Lionel Crawford for generously providing us with Staphylococcus aureus and for his many helpful suggestions. We also thank Dr. Parker Suttle for his helpful suggestions and for providing us with purified CAD, Drs. Fred Alt, Rod Kellem, and Robert Schimke for valuable discussions and for making their data available to us prior to publication, and Dr. E. Ehrenfeld for supplying us with poliovirus and vesicular stomatitis virus.

REFERENCES


Shoaf, W. T., and Jones, M. E. (1973) Biochemistry 12, 4039-4051


N-(Phosphonacetyl)-L-aspartate-resistant hamster cells overaccumulate a single mRNA coding for the multifunctional protein that catalyzes the first steps of UMP synthesis.

R A Padgett, G M Wahl, P F Coleman and G R Stark


Access the most updated version of this article at http://www.jbc.org/content/254/3/974

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/3/974.full.html#ref-list-1