Cloning and Sequencing of a Deoxyribonucleic Acid Copy of Glyceraldehyde-3-phosphate Dehydrogenase Messenger Ribonucleic Acid Isolated from Chicken Muscle†

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ABSTRACT: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purified from the breast muscles of 3-week-old chickens and used to raise a specific antiserum in rabbits. This antiserum was coupled to an in vitro translation assay to monitor the purification of GAPDH mRNA. RNA was isolated from identical breast muscles and consecutively fractionated with several techniques to yield a preparation of GAPDH mRNA which was at least 50% pure. Double-stranded cDNA was made against this purified RNA, inserted into pBR322, and used to transform *Escherichia coli*. Recombinants were screened by colony filter hybridization with a cDNA probe made against the purified RNA. The hybridization-positive clone with the largest insert, pGAD-28, was then characterized by using pGAD-28–cellulose to select complementary RNA from total poly(A) RNA and then translating the hybridization-selected RNA in vitro. The single translation product was shown to be GAPDH by (1) comigration with pure GAPDH on sodium dodecyl sulfate–polyacrylamide gels, (2) precipitation with specific anti-GAPDH antiserum, (3) cyanylation fingerprinting, and (4) AMP–agarose affinity chromatography. pGAD-28 was mapped with several restriction enzymes and then sequenced by the method of Maxam and Gilbert [Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560]. The 1261-nucleotide insert was found to contain 29 nucleotides of noncoding sequence at the 5′ end, the entire coding region, and 230 nucleotides of the 3′-noncoding region including a poly(A) addition signal (AATAAA) and the first five residues of the poly(A) tail.

As our understanding of muscle development improves, it is becoming increasingly apparent that the regulation of the glycolytic enzymes is central to myogenesis. Different types of mature muscle fibers (e.g., white or red) differ in their concentration of glycolytic enzymes (Bass et al., 1970; Lebherz et al., 1978). Moreover, the enzyme profiles of the different fiber phenotypes are linked to other observable parameters such as twitch duration, innervation patterns, membrane properties (Buller et al., 1960; Henneman & Olson, 1965), and contractile protein isoforms (Sarkar et al., 1971; Perry, 1973; Salmons & Sreter, 1976). Whether these differentiated functions are regulated by a common signal, or if indeed one of them is the regulator of the others, remains to be seen.

Although we are studying the regulation of glycolysis in muscle, these enzymes are extremely interesting in the general context of cell biology. That is, unlike some differentiated products such as hemoglobin, which are maximally produced in one tissue and absent in others, glycolytic enzymes are ubiquitous at constitutive levels but can still be highly induced in certain tissues, for example, in fast twitch muscle (Lebherz, 1975). Moreover, several glycolytic enzymes are coordinately regulated (Shackelford & Lebherz, 1981; Stone & Schwartz, 1982) and may provide an excellent system for unraveling such regulation in eukaryotes. Even more fundamental, several glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] and aldolase are known to bind to actin and tropomyosin to a large extent (Arnold & Pette, 1970; Knull et al., 1980). Thus, these enzymes may play a role in the assembly of the cytoskeleton of all cells, or conversely, the cytoskeleton may provide a framework for precise physical arrangement of the glycolytic enzymes permitting efficient passage of metabolites from enzyme to enzyme (Ottaway & Mowbray, 1977).

Our intent is to study the regulation of glycolytic enzymes in chicken muscle at the nucleic acid level which involves the structure of the genes as well as their transcriptional activity. The first step in such a study is to procure a pure nucleic acid probe which contains sequences from a chicken glycolytic enzyme gene. Holland & Holland (1980) have previously reported the cloning of two glucose-inducible glycolytic enzyme genes from yeast: GAPDH and enolase (Holland et al., 1981). In this report, we describe the synthesis, recombinant cloning, and sequencing of a DNA molecule bearing the entire coding region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from the chicken. GAPDH was chosen for the synthesis of our first glycolytic enzyme gene probe because (1) it is the most abundant glycolytic enzyme in fast twitch chicken muscle and consequently has an abundant RNA which can be substantially purified, and (2) only one GAPDH isozyme exists in the chicken which reduces the ambiguity involved in correlating gene activity with enzyme concentrations in developmental studies (Lebherz & Rutter, 1967).

Materials and Methods

Materials. Three-week-old White Leghorn chicks were purchased from Animal Specialties, Houston, TX. Breast muscles were removed, rinsed in cold phosphate-buffered saline, frozen immediately in liquid nitrogen, and stored at −80 °C until used. Liquified phenol was purchased from Fisher Scientific Co. and distilled immediately before use. Oligo-

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[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tris-HCl, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

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(dT)–cellulose (6–18-mer average) was purchased from Collaborative Research. Restriction endonucleases, calf thymus terminal deoxynucleotidyltransferase, alkaline phosphatase, and T_4 DNA polynucleotide kinase were all purchased from Bethesda Research Laboratories. Nonlabeled deoxynucleotides and N^6-(6-aminohexyl)adenosine monophosphate–agarose were purchased from P-L Biochemicals. S_1 nuclease, poly(dT), and poly(rA) were purchased from Miles Laboratories. [^{3}H]dCTP (25 Ci/mmol) was purchased from New England Nuclear, while [^{35}S]methionine (755 Ci/mmol), [α-^{32}P]dATP (400 Ci/mmol), and [γ-^{32}P]dATP (2000 Ci/mmol) were purchased from Amersham Corp. Avian myeloblastosis virus (AMV) reverse transcriptase was supplied by Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, FL. Dithiodinitrocyanobenzoic acid (TNB-CN) was purchased from Eastman.

Purification of Glyceraldehyde-3-phosphate Dehydrogenase. Chicken muscle GAPDH was isolated by using a modification of the procedure developed by Allison & Kaplan (1964). Breast muscles (100 g) from 3-week-old chickens were minced with scissors and then homogenized with a Polytron (Brinkmann) in 400 mL of ice-cold 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and 1 mM β-mercaptoethanol. The homogenate was centrifuged at 20000*g* for 20 min, and the pellet was discarded. The supernatant was brought to 60% $(NH_4)_2SO_4$, clarified by centrifugation, and then brought to 80% $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation, and the pellet was resuspended in 25 mL of homogenization buffer and dialyzed extensively against this same buffer. This protein solution, enriched in GAPDH, was further purified by affinity chromatography on a 1 × 5 cm N^6-(6-aminohexyl)adenosine monophosphate–agarose column (Ohlsson et al., 1972). After the sample was applied, the resin was washed with homogenization buffer followed by 0.1 M sodium phosphate (pH 7.5). GAPDH greater than 95% pure was then eluted from the column with 1 mM NAD. The eluted enzyme was then assayed by the procedure of Velick (1955).

Raising Anti-GAPDH Antibody. Each of two New Zealand White rabbits were injected with 1 mg of purified chicken glyceraldehyde-3-phosphate dehydrogenase in complete Freund's adjuvant (multiple sites over a period of 6 weeks). Rabbits were bled every 2 weeks, and the serum was assayed for anti-GAPDH activity in immunodiffusion gels composed of 1% Nobel Agar (Difco) and phosphate-buffered saline.

Cell-Free Translation in Reticulocyte Lysate. Selected RNAs were translated in the presence of [^{35}S]methionine in an mRNA-dependent rabbit reticulocyte lysate which had been digested with micrococcal nuclease [Pelham & Jackson, 1976; described by Schwartz & Rothblum (1980)].

Immunoprecipitation of Cell-Free Translation Products. Antiserum (25 μL) was mixed with 20 μL of reticulocyte lysate, 20 μL of 5% Triton X-100, and 35 μL of phosphate-buffered saline. Mixture was incubated at room temperature for 30 min and then at 4 °C overnight. Protein A–Sepharose (Pharmacia) was then used to isolate the immunoprecipitates as described by Moss & Schwartz (1981). Precipitates were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis of Proteins. Samples were electrophoresed for 5–6 h at 40 mA on 10% or 10–16% gradient polyacrylamide slab gels containing 1% $NaDodSO_4$ (Laemmli, 1970) with a 3% polyacrylamide stacking gel. Gels were fixed, stained in 1% Coomassie blue–50% Cl_3AcOH, and destained in 7% acetic acid–30% methanol. Gels containing ^{35}S-labeled peptides from translation assays were autoradiographed with Kodak royal blue X-O-Mat film.

Purification of GAPDH mRNA. Nucleic acids were isolated from frozen 3-week-old breast muscles by successive $NaDodSO_4$–phenol extractions as previously described (Schwartz & Rothblum, 1980). The DNA was removed from the RNA by a series of 3 M NaOAc (pH 6.0) washes (Palmiter, 1974). Poly(A)-containing RNA was obtained by passing total RNA through an oligo(dT)–cellulose column twice, as described by Aviv & Leder (1972). Poly(A)-containing RNA was then heat denatured and further fractionated by centrifugation on 5–20% linear sucrose gradients in 0.02 M NaOAc (pH 5.0) and 0.001 M Na_2EDTA. RNA migrating between 15 S and 18 S was further purified by electrophoresis on 3.3% polyacrylamide gels containing the disulfide cross-linker *N'*,*N'*-cystamine bis(acrylamide) (Hanson, 1976). Gels were stained with methylene blue, and selected RNA bands were sliced out of the gel and dissolved in β-mercaptoethanol as previously described (Schwartz & Rothblum, 1980). Acrylamide polymers were removed from the RNA by passage over a cellulose column in the presence of buffered ethanol (Franklin, 1966). Aliquots of fractions isolated from gels and sucrose gradients were analyzed for translational activity and then used for cDNA synthesis.

cDNA Synthesis. Single- and double-stranded complementary DNA (cDNA) molecules were transcribed from gel-purified GAPDH mRNA by using avian myeloblastosis virus reverse transcriptase according to Schwartz et al. (1980).

Construction and Cloning of Chimeric DNA. Double-stranded cDNA was nicked with S_1 nuclease (Monahan et al., 1976), and approximately 15 dC residues were then added to the 3'-OH termini by using terminal deoxynucleotidyltransferase in the presence of $CoCl_2$ (Roychoudhury et al., 1976). pBR322 was linearized with restriction endonuclease *Pst*I (which inactivates the ampicillin resistance gene on the plasmid), and 15 dG residues were added to the 3'-OH termini (Dugaiczyk et al., 1980). Equimolar amounts of the "tailed" cDNA and pBR322 were mixed in 0.2 M NaCl, 10 mM Tris (pH 7.6), and 1 mM EDTA, such that the final DNA concentration was 8 μg/mL. This mixture was heated to 60 °C and allowed to cool to room temperature over a period of 4 h. The resultant chimeric plasmids were used to transform *E. coli* K12 strain RR1 (McReynolds et al., 1977). Bacterial colonies containing recombinant DNA molecules were detected by antibiotic sensitivity. These were screened for possession of GAPDH sequences by the Grunstein–Hogness (1975) colony filter hybridization procedure by using ^{32}P-labeled single-stranded cDNA (made against gel purified GAPDH mRNA) as a probe. Finally, plasmid DNA was isolated from cultures of hybridization-positive colonies (Katz et al., 1977). All experiments conformed to the National Institutes of Health guidelines for recombinant DNA research.

Identification of Cloned DNAs by Translation of RNA Selected by Hybridization to Plasmid–DNA–Cellulose. A 1-mg sample of plasmid DNA from each positive clone was covalently linked to 40 mg of diazobenzyloxymethylcellulose according to the protocol of Noyes & Stark (1975). Aliquots of plasmid–cellulose (200 μL packed volume) were placed in 1.5-mL Eppendorf centrifuge tubes with 50% formamide, 0.05 M Tris (pH 7.5), 1 mM EDTA, 0.4 M NaCl, 0.5% $NaDodSO_4$, 200 μg of poly(A)-containing RNA, 50 μg of poly(A), and 50 μg of *E. coli* tRNA in a final volume of 600 μL (Schwartz et al., 1980). The tubes were heated to 80 °C for 2 min, and the contents were then allowed to hybridize for 18 h in a shaking water bath at 37 °C. The DNA–cellulose was washed with 600 μL of hybridization buffer twice at 37 °C,

twice at 55 °C, and finally twice with 1 mL of ice-cold 2 × SSC. Specifically bound RNA was then removed from the DNA–cellulose by resuspending it in 200 μL of chelexed formamide, heating it to 80 °C for 2 min, and centrifuging. The RNA was precipitated from the supernatant by making it contain 50% formamide and 0.5 M NaCl and then by adding 2.5 volumes of ethanol and incubating at −20 °C overnight. Precipitated RNAs were then translated in a cell-free reticulocyte lysate as described above.

Cyanylation Fingerprinting of Native and Lysate-Translated GAPDH. Proteins were cleaved at cysteinyl residues under conditions similar to those of Jacobson et al. (1973). Purified chicken GAPDH (100 μg) and GAPDH synthesized in vitro were denatured in 6 M guanidine hydrochloride containing 0.5 mM DTT, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.0). This mixture was treated with 10 mM dithiodinitrocyanobenzoic acid (TNB-CN) at 25 °C for 30 min. The preparation was then extensively dialyzed against 50% acetic acid and dried under a stream of nitrogen gas. The GAPDH powder was resuspended in 8 M urea containing 1 M borate buffer (pH 9.2) and incubated at 37 °C for 12 h. The reaction was terminated with excess DTT. The GAPDH fragments were dialyzed against 1% $NaDodSO_4$ containing 50 mM Tris-HCl (pH 7.5) in tubing with a M_r 3000 limit and analyzed on $NaDodSO_4$–polyacrylamide gels as described above.

Restriction Endonuclease Mapping of Plasmid DNA. Reaction conditions for restriction endonuclease cleavage of plasmid DNA varied among the several enzymes used and were essentially as suggested by the supplier. One unit of enzyme was added per μg of DNA substrate and incubated for 1 h at 37 °C (except for *Pst*I which was incubated at 30 °C). *Hae*III-digested pBR322 and *Hin*dIII-digested SV40 were used as molecular weight standards. Digested DNAs were electrophoresed at 70 V on agarose slab gels in TEA buffer containing 50 mM Tris (pH 8.4), 20 mM NaOAc, 18 mM NaCl, and 2 mM EDTA. Restriction sites within the largest *Hha*I fragment of plasmid pGAD-28 were determined by first labeling the 5′ termini with [γ-^{32}P]ATP (2000 Ci/mmol) by using T_4 DNA polynucleotide kinase (Maxam & Gilbert, 1977) and then digesting the labeled fragment with one or two restriction enzymes.

Nucleic Acid Sequencing. Various restriction fragments were labeled at either their 5′ termini (see above) or their 3′ termini (AMV reverse transcriptase and [α-^{32}P]dNTP). Each labeled fragment was cut with a restriction enzyme, and the resulting fragments were separated on polyacrylamide or agarose gels. These single-end-labeled fragments were then sequenced by the method of Maxam & Gilbert (1977).

Results and Discussion

Breast muscles from 3-week-old chickens were homogenized, and the postmitochondrial supernatant proteins were electrophoresed on a $NaDodSO_4$–10% polyacrylamide gel and stained with Coomassie blue (Figure 1, lane A). Triosephosphate isomerase (M_r 28 500), phosphoglycerate mutase (M_r 30 000), lactate dehydrogenase (M_r 34 500), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), aldolase (M_r 40 000), creatine kinase (M_r 43 000), enolase (M_r 50 000), pyruvate kinase (M_r 57 000), and phosphorylase (M_r 92 000) were all identified as major components of the fast twitch muscle cytosol by comparing the electrophoretic mobilities of the major cytosol components with those of purified enzymes (Petell et al., 1981; N. Reiss and R. J. Schwartz, unpublished results).

Immunoprecipitation of Translated Glyceraldehyde-3-phosphate Dehydrogenase. We chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for further study because (1) it is the most abundant cytosolic protein in fast twitch muscle and (2) the enzyme is composed of identical subunits, which allows unambiguous measurements of the protein in developmental studies. GAPDH was purified as described under Materials and Methods, and the eluted fraction with the highest GAPDH specific activity (90 units/mg) was analyzed by gel electrophoresis (Figure 1, lane B). The gel was overloaded in an attempt to detect minor contaminants. In the absence of detectable contaminants, we estimated this preparation of GAPDH to be at least 95% pure and used it for the generation of a specific antiserum in rabbits as described under Materials and Methods. When the serum was analyzed with an Ouchterlony double immunodiffusion test (Figure 2), a single precipitin line was formed between the serum in the center well and purified GAPDH in well 1. The identical result was obtained with a crude soluble extract of chicken muscle in well 4. The serum did not precipitate GAPDH from yeast (well 2) or rabbit (well 3), nor did it react with other glycolytic enzymes from the chicken such as lactate dehydrogenase (well 5) and aldolase (well 6).

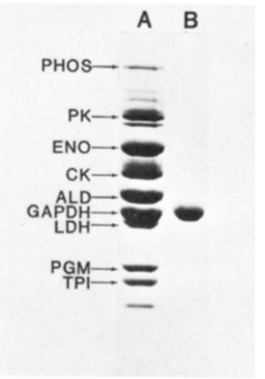

FIGURE 1: Purification of glyceraldehyde-3-phosphate dehydrogenase from chicken breast muscle. Frozen chicken breast muscle was homogenized in 4 volumes of 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and 1 mM β-mercaptoethanol. The postmitochondrial supernatant (20000*g*) was fractionated by ammonium sulfate precipitation, and the GAPDH-enriched material was purified by affinity chromatography on AMP–agarose as described under Materials and Methods. The crude postmitochondrial supernatant (lane A) and the purified GAPDH (lane B) were electrophoresed on 10% polyacrylamide slab gels containing 1% $NaDodSO_4$ which were stained with Coomassie blue. Identities of the protein bands were determined by comparison of their electrophoretic mobilities with those of known proteins (Petell et al., 1981; N. Reiss and R. Schwartz, unpublished results). Abbreviations are the following: PHOS, phosphorylase; PK, pyruvate kinase; ENO, enolase, CK, creatine kinase; ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PGM, phosphoglycerate mutase; TPI, triose-*p*-isomerase.

To further test the specificity of the anti-GAPDH antiserum, and to develop the conditions for assaying GAPDH mRNA translation activity by immunoprecipitation of cell-free translation products, we performed the following experiments. RNA was isolated from the breast muscles of 3-week-old chicks and then enriched for poly(A)-containing mRNA by hybridization to oligo(dT)–cellulose. This RNA was translated in a nuclease-treated reticulocyte lysate in the presence of [^{35}S]methionine, and the products were analyzed by polyacrylamide gel electrophoresis and autoradiography (Figure 2B, lane 1). Incubation of the translation products with GAPDH antiserum resulted in the precipitation of 15% of the radioactivity incorporated into protein in the translation mixture (Table I). This precipitated product was analyzed by gel electrophoresis and autoradiography (Figure 2B, lane

Table I: Purification of Glyceraldehyde-3-phosphate Dehydrogenase mRNA Measured by Translational Activity

stage of purifcn	total [^{35}S]Met incorpn (cpm/μg of RNA)	radioact. bound to anti-GAPDH serum (cpm/μg of RNA)	% incorpd into GAPDH	x-fold purifcn
total RNA[a]	37 000	3 500	10.1	
poly(A) RNA	458 400	68 700	14.9	22
sucrose gradient	1 736 000	537 900	30.9	176
preparative polyacrylamide gels	1 682 000	943 400	56.1	309
pGAD-28–cellulose	1 675 000	1 541 000	92.0	505

[a] RNA isolated from 3-week-old chick breast muscle.

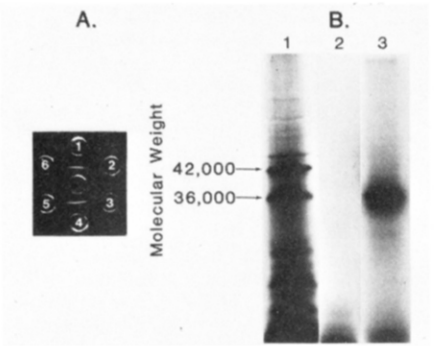

FIGURE 2: Specificity of immunoprecipitation with glyceraldehyde-3-phosphate dehydrogenase antiserum. In panel A, undiluted GAPDH antiserum was placed in the center well of an immunodiffusion gel composed of 1% Nobel agar in phosphate-buffered saline. Purified chicken muscle GAPDH (1 mg/mL; well 1), yeast GAPDH (1 mg/mL; well 2), rabbit GAPDH (1 mg/mL; well 3), chicken breast postmitochondrial supernatant (2 mg/mL; well 4), chicken muscle lactate dehydrogenase (1 mg/mL; well 5), and chicken muscle aldolase (1 mg/mL; well 6) were tested for immunoprecipitation. ^{35}S-Labeled proteins were translated in a reticulocyte lysate by using poly(A)-containing RNA from 3-week-old breast muscle as a template and were electrophoresed as described under Materials and Methods (lane 1). Equal amounts of this total translation product were treated with 50 μL of preimmune rabbit serum (lane 2) and 50 μL of rabbit anti-GAPDH serum (lane 3) as described in the text. An arrow points to the migration of GAPDH (M_r 36 000).

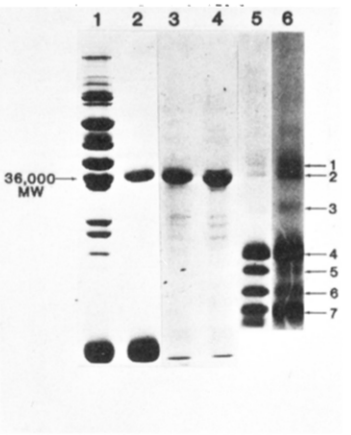

FIGURE 3: Electrophoresis of translation products of RNA selected by hybridization to pGAD-28–DNA–cellulose. Total poly(A)-containing muscle RNA was hybridized to pGAD-28–DNA–cellulose as described under Materials and Methods. Hybridized RNA was eluted and translated, and the synthesized polypeptides were then electrophoresed either on a NaDodSO$_4$–10% polyacrylamide slab gel (lanes 1–4) or on a 10–16% polyacrylamide gradient slab gel (lanes 5 and 6). In lanes 1 and 4, the postmitochondrial supernatant and a reticulocyte lysate were mixed together, while in lanes 2 and 3 pure GAPDH and a reticulocyte lysate were combined and electrophoresed. Lanes 1 and 2 were stained with Coomassie blue, and lanes 3 and 4 are the autoradiograms of these two lanes. Lane 5 is a stained cyanylation fingerprint of chick muscle GAPDH. Lane 6 is the autoradiograph of the cyanylation fingerprint of the translation product. Note that the molecular weight indication applies only to lanes 1–4.

3) and found to consist of a single species of M_r 36 000, which comigrated with purified GAPDH. The specificity of the antiserum was further confirmed by incubating the translation products with preimmune rabbit serum. In this experiment, less than 1% of the radioactive protein was precipitated from the mixture; gel electrophoresis and autoradiography showed that no translated species were selectively precipitated (Figure 2B, lane 2).

Amplification of Recombinant GAPDH cDNA Plasmids. Having established the specificity of the GAPDH antiserum, we used the cell-free translation and immunoprecipitation assay to monitor the purification of GAPDH mRNA. Poly(A)-containing RNA was isolated from breast muscles of 3-week-old chicks as described under Materials and Methods. Cell-free translation and selective immunoprecipitation revealed that poly(A)-containing RNA had 22-fold more GAPDH translational activity than total RNA (Table I). A 9-fold increase in GAPDH translational activity was achieved when RNAs greater than 18 S were removed by fractionation on a sucrose gradient. When the sized RNA was electrophoresed on a disulfide cross-linked polyacrylamide gel, it was found to consist predominantly of two species. The upper band (band I) migrated at 1600 nucleotides and was found to contain α-actin translational activity (Schwartz & Rothblum, 1980). The lower band (band II) migrated at 1390 nucleotides and contained a GAPDH translational activity 300-fold greater than that in total RNA (Table I). Band II translation products migrated as a broad single band on a NaDodSO$_4$–polyacrylamide gel (Schwartz & Rothblum, 1980), but only 56% of the products were precipitated by GAPDH antiserum (Table I). This probably reflects the presence of other mRNAs in band II, possibly including those which encode lactate dehydrogenase (M_r 34 000), aldolase (M_r 38 000), and α-tropomyosin (M_r 36 000). When conventional methods of nucleic acid purification failed to completely purify GAPDH mRNA, we decided to complete the purification by cloning GAPDH cDNA in *E. coli*. cDNA was made against gel purified band II RNA and inserted into the *Pst*I site of pBR322 as described under Materials and Methods. These chimeric molecules were used to transform *E. coli* K12 strain RR1 in the presence of $CaCl_2$ (McReynolds et al., 1977). Growth of these bacteria on antibiotic plates yielded 400 clones that were ampicillin sensitive and tetracycline resistant. These were replica plated, transferred to nitrocellulose, and hybridized in situ (Grunstein & Hogness, 1975) with [^{32}P]cDNA made against band II RNA. Autoradiograms of these filters showed that 30 of the 400 clones contained sequences complementary to the band II cDNA. Plasmid DNA was isolated from each of these hybridization-positive clones and digested with the restriction enzyme *Hha*I. Agarose gel electrophoresis of these digests revealed that clone pGAD-28 had the largest cDNA insert, approximately 1300 base pairs, and thus this clone was chosen for further study.

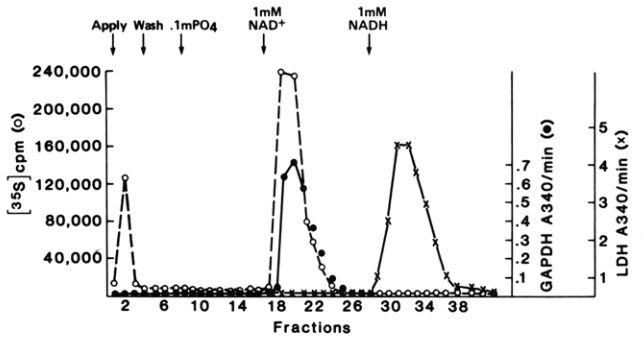

FIGURE 4: Affinity chromatography of translation products of pGAD-28-selected RNA. Translation products described in Figure 3 were chromatographed onto a 1 × 3 cm N^6-(6-aminohexyl)adenosine monophosphate–agarose column. The reticulocyte lysate, GAPDH, and lactate dehydrogenase were applied in 10 mM Tris-HCl (pH7.5), 1 mM Na_2EDTA, and 1 mM β-mercaptoethanol. The resin was washed with 0.1 M sodium phosphate (pH 7.5) and then with 1 mM NADH. Individual fractions were assayed for GAPDH activity (●), lactate dehydrogenase activity (×), and acid precipitable (10% trichloroacetic acid) radioactivity (○).

Positive Confirmation of the GAPDH Insert by Hybridization to DNA–Cellulose. To verify that the cDNA insert of pGAD-28 encodes GAPDH, we performed the following experiments. pGAD-28 DNA was covalently linked to granulated diazobenzyloxymethylcellulose (Noyes & Stark, 1975), and this material was used to select complementary RNA from total poly(A) RNA as described under Materials and Methods. The hybridization-selected RNA was eluted from the DNA–cellulose with chelexed formamide and translated in the cell-free reticulocyte lysate. The radioactive products of this translation were coelectrophoresed on a Na-DodSO_4–polyacrylamide gel with (1) unlabeled postmitochondrial supernatant proteins from chicken breast muscle and (2) unlabeled purified GAPDH (Figure 3, lanes 1 and 2). Autoradiography of this gel revealed a single major translation product which comigrated with purified GAPDH (lanes 3 and 4).

This M_r 36 000 translation product was then analyzed by cyanylation fingerprinting. The translation product and purified chicken GAPDH were each cleaved at cysteine residues with TNB-CN as described under Materials and Methods. The resulting fragments were electrophoresed on a NaDod-SO_4–10–16% polyacrylamide gradient gel, stained with Coomassie blue, and autoradiographed (Figure 3, lanes 5 and 6). Eight fragments were observed on the Coomassie stained gel while seven were observed on the autoradiogram, indicating that the smallest Coomassie fragment was devoid of methionine. The molecular weights of the five smallest bands were 500, 3900, 5400, 9700, and 17 000. These total 36 500 (very near the molecular weight of the uncut samples, M_r 36 000), suggesting that they result from total cleavage of the protein. The nucleotide sequence given below confirms the existence of four cysteine residues in GAPDH as well as the absence of methionine from the smallest fragment. Three heavier bands are also observed on both the gel and the autoradiogram

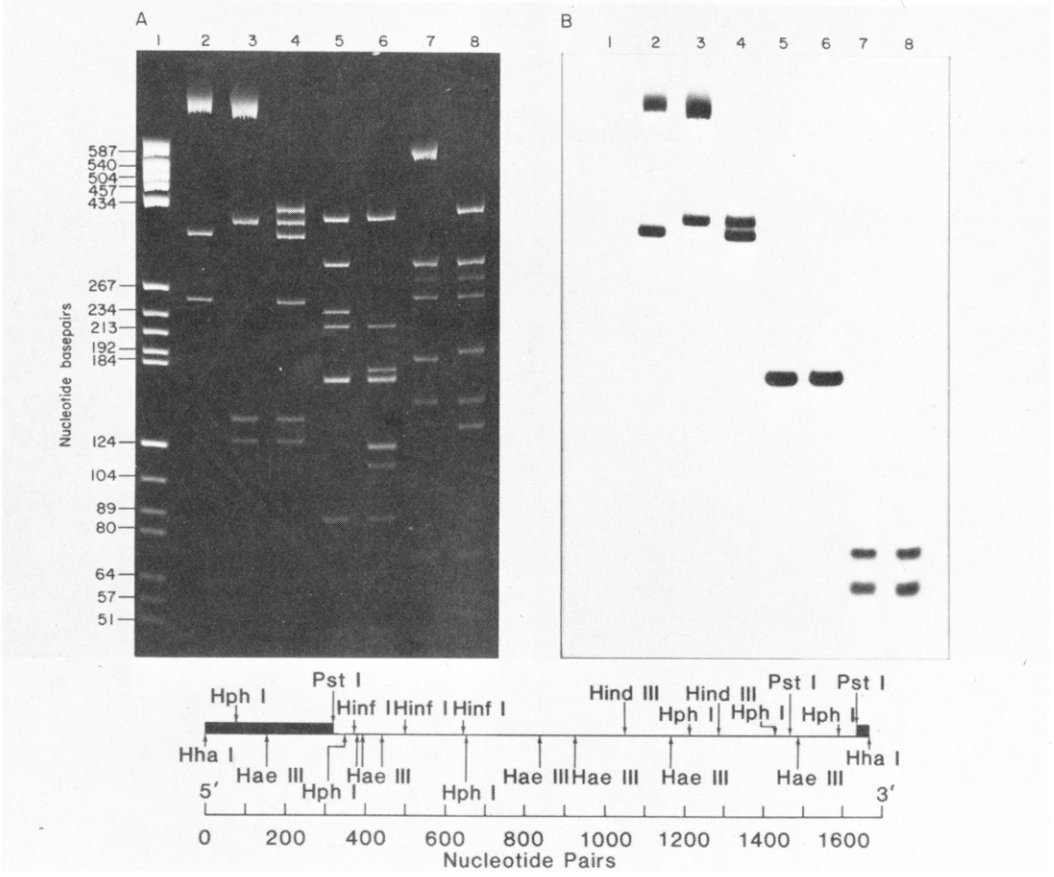

FIGURE 5: Restriction endonuclease map of pGAD-28. Digestion of pGAD-28 with *Hha*I released a 1650-nucleotide pair insert which was purified by preparative gel electrophoresis and then labeled at the 5′ termini with γ-^{32}P as described under Materials and Methods. The labeled fragment was digested with restriction endonucleases *Hin*dIII (lane 2), *Hin*fI (lane 3), *Hin*dIII plus *Hin*fI (lane 4), *Hae*III (lane 5), *Hae*III plus *Hin*dIII (lane 6), *Hph*I (lane 7), and *Hph*I plus *Hin*dIII (lane 8). Digests were electrophoresed on 5% polyacrylamide gels in TEA buffer. The gel was stained with ethidium bromide (5 μg/mL; panel A) and autoradiographed (panel B). pBR322 digested with *Hae*III served as a molecular weight standard in lane 1. A map was constructed from ordered fragments as well as from sequenced DNA. The darkened areas denote pBR322 DNA. The 5′- and 3′-transcriptional orientation was determined by nucleic acid sequencing (see Results and Discussion).

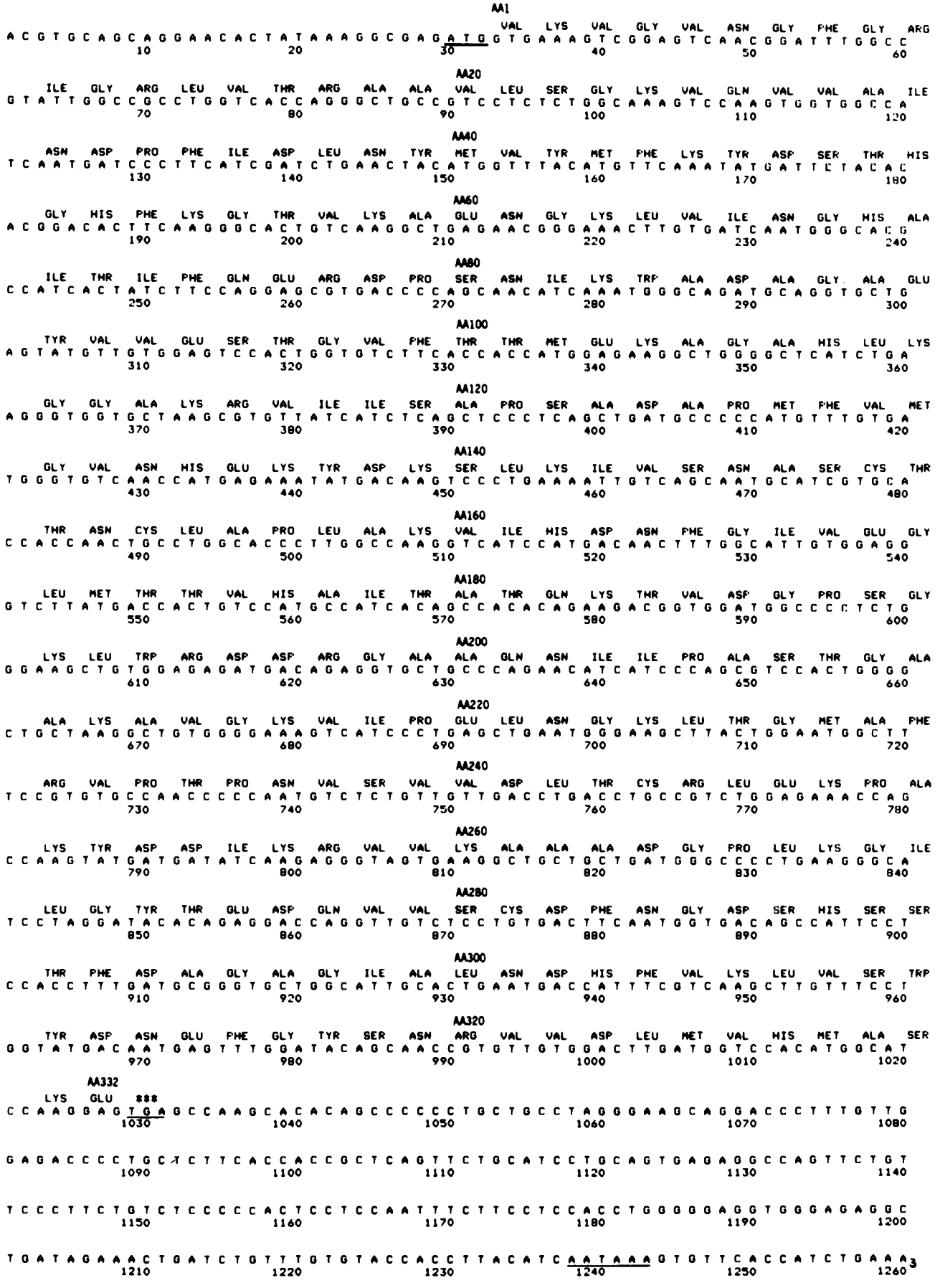

FIGURE 6: Complete nucleotide sequence of the pGAD-28 insert. Initiation codon (ATG), termination codon (TGA), and putative poly(A) addition signal (AATAAA) are underlined.

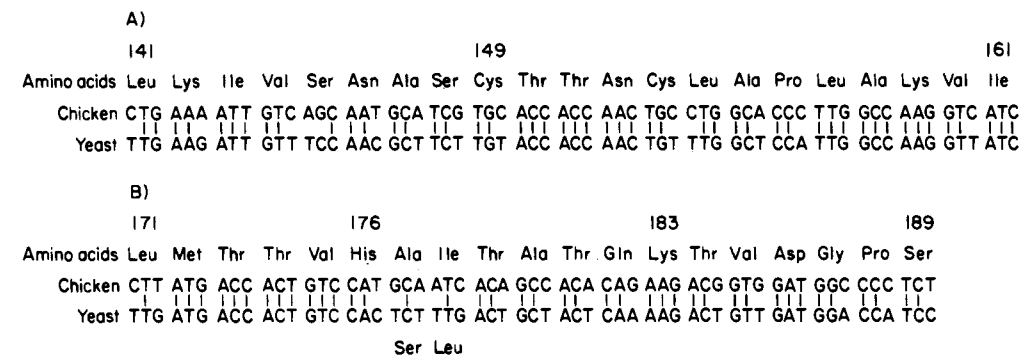

FIGURE 7: Comparison of chicken and yeast glyceraldehyde-3-phosphate dehydrogenase coding sequences in the regions which specify catalytically important residues. Data for chicken are from Figure 6 and for yeast are from Holland & Holland (1979).

and undoubtedly result from partial digestion of the protein. In any case, the digestion patterns of the pGAD-28-directed translation product and purified chicken GAPDH match exactly, except of course for the unlabeled fragment. This can be seen by comparing lanes 5 and 6 of Figure 3. In another experiment, the product translated from pGAD-28-selected RNA was incubated with GAPDH antiserum, which precipitated 92% of the radioactivity incorporated into protein (Table I). Again, the precipitated protein was eluted from the protein A–agarose and found to comigrate with purified GAPDH on a $NaDodSO_4$–polyacrylamide gel (data not shown).

Dehydrogenases which require pyridine nucleotides as well as some other related enzymes bind tightly to AMP–agarose (Kaplan et al., 1974). As a result this material has been used for affinity purification of lactate dehydrogenase and GAPDH (Ohlsson et al., 1972). For final identification, the translation product directed by pGAD-28-selected RNA was mixed with pure chicken GAPDH and lactate dehydrogenase and then applied to a 1 × 3 cm column of AMP–agarose. Approximately 10% of the total radioactive translation products did not bind to the column and was removed by several washes of application buffer. However, a stringent wash with 0.1 M sodium phosphate (pH 7.5) failed to remove any additional radioactivity. When 1 mM NAD was added, 90% of the applied radioactivity coeluted with GAPDH enzyme activity (Figure 4). NADH (1 mM) was then added which eluted the lactate dehydrogenase enzyme activity without liberating any additional radioactivity. The unlabeled GAPDH and the radioactive translation product which coeluted were electrophoresed on a $NaDodSO_4$–10% polyacrylamide gel and were found to have identical electrophoretic mobilities, as previously shown in Figure 3. Binding of the translation product to AMP–agarose suggests that the nascent protein is able to fold into its native conformation in the reticulocyte lysate.

Nucleotide Sequence of the pGAD28 Insert. pGAD-28 was digested with several restriction endonucleases and the resulting map (Figure 5) established the size of the cDNA insert and guided our preparation of various labeled fragments needed for sequencing. The sequence of the entire insert of pGAD28 was determined by the method of Maxam and Gilbert and is shown in Figure 6. The insert contains 29 noncoding nucleotides at the 5′ end, the initiation codon (ATG), 996 coding nucleotides, a termination codon (TGA), and 230 noncoding nucleotides at the 3′ end. The putative poly(A) addition signal AATAAA (Proudfoot & Brownlee, 1976) is present in this 3′-noncoding region and is 14 nucleotides away from the beginning of the poly(A) tail. Thus the insert of pGAD28 has all the features of a typical eukaryotic mRNA.

The amino acid sequence of chicken GAPDH has not been previously determined, and thus the nucleotide sequence of pGAD28 gives us the first opportunity to compare chicken GAPDH with those from yeast (Holland & Holland, 1979), lobster (Davidson et al., 1967), and pig (Harris & Perham, 1968). The chicken GAPDH amino acid sequence agrees with that of yeast at 210 of 332 positions, and with lobster at 262 positions. The pig sequence has the greatest homology with chicken GAPDH, agreeing at all but 26 positions. Moreover, 19 of these 26 differences can be explained by single nucleotide substitutions.

To compare the evolutionary conservation of GAPDH with that of other proteins, we estimated the absolute rate of divergence between chicken and pig GAPDH by the method of Dayhoff (1969). The 26 amino acid differences indicate an evolutionary distance of 7.8 PAMs (accepted point mutations per 100 residues). If one assumes that the pig and chicken diverged 300 million years ago (Dayhoff, 1969), the vertebrate GAPDHs are diverging at a rate of 1.3 PAMs per 100 million years. Comparison of this value with 0.1–0.9 for histones, 3.0 for cytochrome *c*, and 40 for the rapidly diverging K C region of mammalian immunoglobulins (Dayhoff, 1969) reveals that GAPDH is among the most highly conserved proteins known. Using the same type of calculation, yeast and chicken GAPDH are found to differ by 38 PAMs. This suggests that these two organisms diverged more than one billion years ago.

Olsen et al. (1975) showed that GAPDH could be divided into two functional domains, an external NAD binding region (amino acids 1–147) and an internal catalytic region (amino acids 148–332). The differences between the chicken and pig sequences were distributed equally between these two domains; but, when chicken GAPDH was compared to yeast, greater homology was found within the catalytic domain (74%) than within the NAD binding domain (49%). The regions that are conserved between chicken and yeast consist of short strings of 5–10 amino acids except for the 21 amino acid string (141–161) which contains the catalytically active Cys-149 (Figure 7). This "essential thiol polypeptide" (Perham, 1969) is totally conserved among *E. coli*, yeast, lobster, chicken, pig, and human (Allison, 1968). Comparison of the nucleotide sequences in chicken and yeast which encode this region reveals a 78% homology. In addition, this region contains two strings of continuous nucleotide homology (11 nucleotides each) between the two species and emphasizes the functional importance of this portion of the protein. Other residues thought to be important for catalysis such as His-176 and Lys-183 are situated in short strings of amino acids which are also completely conserved among the eukaryotic species noted above (Figure 7).

The overall nucleotide homology between the mRNAs of yeast and chicken GAPDH is relatively low. The first 300 nucleotides, which encode the NAD binding region, have only a 36% homology. In the next 300 nucleotides, which include the catalytic residues, the homology improves to 57%, but, in

Table II: Codon Usage in Chicken[a] and Yeast[b] GAPDH Genes

		chick	yeast			chick	yeast
Asp	GAT	11	9	Val	GTT	8	22
	GAC	11	16		GTC	16	15
					GTA	1	0
Asn	AAT	8	0		GTG	12	0
	AAC	9	12				
				Met	ATG	9	6
Thr	ACT	6	12				
	ACC	9	12	Ile	ATT	4	9
	ACA	4	0		ATC	15	11
	ACG	1	0		ATA	0	0
Ser	TCT	4	13	Leu	TTA	0	0
	TCC	8	12		TTG	2	21
	TCA	2	0		CTT	4	0
	TCG	1	0		CTC	1	0
	AGT	0	0		CTA	1	0
	AGC	4	0		CTG	11	0
Glu	GAA	0	12	Tyr	TAT	5	0
	GAG	12	2		TAC	4	11
Gln	CAA	1	5	Phe	TTT	5	0
	CAG	4	0		TTC	8	10
Pro	CCT	2	0	Lys	AAA	9	1
	CCC	7	0		AAG	17	25
	CCA	3	12				
	CCG	0	0	His	CAT	6	0
					CAC	4	8
Gly	GGT	9	25				
	GGC	8	0	Arg	CGT	6	0
	GGA	6	0		CGC	1	0
	GGG	8	0		CGA	0	0
					CGG	0	0
Ala	GCT	17	25		AGA	2	11
	GCC	9	7		AGG	2	0
	GCA	6	0				
	GCG	2	0	Trp	TGG	3	3
Cys	TGT	1	2	stop	TAA	0	1
	TGC	3	0		TAG	0	0
					TGA	1	0

[a] Data from Figure 6. [b] Data from Holland & Holland (1979).

the final 392 nucleotides of coding sequence, the homology falls to 45%. The discrepancy between the amino acid (63%) and nucleotide (46%) homologies of chicken and yeast GAPDH reflects an evolutionary relaxation of the biased codon usage noted by Holland & Holland (1979). As seen in Table II, there is essentially random utilization of the codons specifying a given amino acid in chicken GAPDH with the exception being the exclusive use of GAG for glutamic acid. In marked contrast, 10 amino acids in yeast GAPDH are encoded by only one of the possible codons, and 5 others are specified by only two of the possible codons. The need for this codon bias either has been lost during evolution or has been satisfied by the codon bias of a single amino acid (Glu). The nucleotide sequence of GAPDH mRNAs from other species will be interesting in this regard.

We have recently screened a λ Charon 4A chicken genomic library (Dodgson et al., 1979) with pGAD28 and obtained several natural gene clones. These clones are currently being analyzed to determine the exact number of GAPDH genes in the haploid chicken genome, as well as to investigate whether the placement of intervening sequences correlates in any meaningful way with the highly conserved domains discussed above.

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Registry No. GAPDH, 9001-50-7; glyceraldehyde-phosphate dehydrogenase (chicken reduced), 84582-70-7; deoxyribonucleic acid (chicken glyceraldehyde-phosphate dehydrogenase messenger RNA-complementary), 84582-74-1.

References

Allison, W. S. (1968) *Ann. N.Y. Acad. Sci. 151*, 180–189.
Allison, W. S., & Kaplan, N. O. (1964) *J. Biol. Chem. 239*, 2140.
Arnold, H., & Pette, D. (1970) *Eur. J. Biochem. 15*, 360.
Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408.
Bass, A., Lusch, G., & Pette, D. (1970) *Eur. J. Biochem. 13*, 289.
Buller, A. J., Eccles, J. C., & Eccles, R. M. (1960) *J. Physiol. 150*, 417.
Davidson, B. E., Sajgo, M., Noller, H. F., & Harris, J. I. (1967) *Nature (London) 216*, 1181.
Dayhoff, M. O., Ed. (1969) *Atlas of Protein Sequence and Structure*, Vol. 4, pp 39–60, National Biomedical Research Foundation, Washington, DC.
Dodgson, J. B., Strommer, J., & Engel, J. D. (1979) *Cell (Cambridge, Mass.) 17*, 879.
Dugaiczyk, A., Robberson, D. L., & Ullrich, A. (1980) *Biochemistry 19*, 5869.
Franklin, R. M. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 1304.
Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961.
Hanson, J. N. (1976) *Anal. Biochem. 76*, 37.
Harris, J. I., & Perham, R. N. (1968) *Nature (London) 219*, 1025.
Henneman, E., & Olson, C. B. (1965) *J. Neurophysiol. 28*, 581.
Holland, J. P., & Holland, M. J. (1979) *J. Biol. Chem. 254*, 9839.
Holland, J. P., & Holland, M. J. (1980) *J. Biol. Chem. 255*, 2596.
Holland, M. J., Holland, J. P., Thill, G. P., & Jackson, K. A. (1981) *J. Biol. Chem. 256*, 1385.
Jacobson, G. R., Schaffer, H. M., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583.
Kaplan, N. O., Everse, J., Dixon, J. E., Stolzenback, F. E., Lee, C. Y., Lee, C. L. T., Taylor, S. S., & Mossbach, K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3450.
Katz, L., Williams, P. H., Sato, S., Leavitt, R. W., & Helinski, D. R. (1977) *Biochemistry 16*, 1677.
Knull, H. R., Bronstein, W. W., Besjardins, P., & Niehaus, W. G. (1980) *J. Neurochem. 34*, 222.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lebherz, H. G. (1975) *J. Biol. Chem. 250*, 5976.
Lebherz, H. G., & Rutter, W. J. (1967) *Science (Washington, D.C.) 157*, 1198.
Lebherz, H. G., Sardo, M. J., Pettell, J. K., & Shackelford, J. E. (1978) in *Protein Turnover and Lysosome Function* (Segal, H., & Doyle, D., Eds.) pp 655–685, Academic Press, New York.
Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.
McReynolds, L. A., Catterall, J. F., & O'Malley, B. W. (1977) *Gene 2*, 217.
Monahan, J. M., Harris, S. E., Woo, S. L. C., Robberson, D. L., & O'Malley, B. W. (1976) *Biochemistry 15*, 223.
Moss, M., & Schwartz, R. J. (1981) *Mol. Cell. Biol. 1*, 289.
Noyes, B. E., & Stark, G. R. (1975) *Cell (Cambridge, Mass.) 5*, 301.
Ohlsson, R., Brodelius, P., & Mossbach, K. (1972) *FEBS Lett. 25*, 234.

Olsen, K. W., Moras, D., Rossman, M. G., & Harris, J. I. (1975) *J. Biol. Chem. 250*, 9313.
Ottaway, J. H., & Mowbray, J. (1977) *Curr. Top. Cell. Regul. 12*, 107.
Palmiter, R. D. (1974) *Biochemistry 13*, 3606.
Pelham, H. R., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.
Perham, R. N. (1969) *Biochem. J. 111*, 17.
Perry, S. V. (1973) *Exploratory Concepts in Muscular Dystrophy* (Milhorat, A., Ed.) Vol. II, pp 319–330, Excerpta Medica, Amsterdam.
Petell, J. K., Sardo, M. J., & Lebherz, H. G. (1981) *Prep. Biochem. 11*, 69.
Proudfoot, N. J., & Brownlee, G. G. (1976) *Nature (London) 263*, 211.
Roychoudhury, R., Jay, E., & Wu, R. (1976) *Nucleic Acids Res. 3*, 101.
Salmons, S., & Sreter, F. A. (1976) *Nature (London) 263*, 30.
Sarkar, S., Sreter, F. A., & Gergely, J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 946.
Schwartz, R. J., & Rothblum, K. (1980) *Biochemistry 19*, 2506.
Schwartz, R. J., Haron, J. A., Rothblum, K. N., & Dugaiczyk, A. (1980) *Biochemistry 19*, 5883.
Shackelford, J. E., & Lebherz, H. G. (1981) *J. Biol. Chem. 256*, 6423.
Stone, E. M., & Schwartz, R. J. (1982) *Annu. Symp. Fundam. Cancer Res. [Proc.] 35*, 43.
Velick, S. F. (1955) *Methods Enzymol. 1*, 401.

Chemical Modification and Ligand Binding Studies with *Escherichia coli* Glutamate Synthase†

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ABSTRACT: The structure and function of *Escherichia coli* glutamate synthase were studied by ligand binding and chemical modification experiments. Binding of $NADP^+$ was to a single dinucleotide site per $\alpha\beta$ protomer with a K_d of approximately 5 μM. Phenylglyoxal modified an essential arginyl residue required for binding of $NADP^+$. *E. coli* glutamate synthase thus employs a single dinucleotide binding site which functions in the NADPH to flavin electron transfer for glutamine-dependent glutamate synthase and for direct reduction of 2-iminoglutarate by NADPH in the NH_3-dependent reaction. Binding of 2-oxoglutarate was complex. "Half of the sites" binding of 2-oxoglutarate ($K_d < 0.25$ μM) was obtained in the absence of glutamine. Binding to half of the sites was pH independent. In the presence of glutamine, the 2-oxoglutarate binding ratio was approximately 1 equiv per protomer (K_d = 2–3 μM) at pH 7.5. This binding was pH dependent and varied between 0.43 equiv per protomer at pH 6.7 and 2.3 equiv per protomer at pH 9.0. Correlation of half of the sites binding with negative cooperativity for 2-oxoglutarate saturation indicates the utilization of low-K_d 2-oxoglutarate sites for NH_3-dependent glutamate synthase. Binding of glutamine promotes a conformational change that exposes additional 2-oxoglutarate sites having a K_d of 2–3 μM which are utilized in the glutamine-dependent reaction. Chemical modification with pyridoxal 5′-phosphate caused inactivation of glutamine-dependent but not NH_3-dependent glutamate synthase. Inactivation was ascribed to modification of one to two lysyl residues per protomer by Schiff base formation. The essential lysyl residue has a role in the binding of glutamine.

Escherichia coli glutamate synthase [L-glutamine:2-oxoglutarate aminotransferase (reduced $NADP^+$ oxidizing), EC 2.6.1.53] is a non-heme iron–sulfur flavoprotein (Miller & Stadtman, 1972). The enzyme has an essential role in ammonia assimilation, particularly under conditions of low ammonia concentration (Tempest & Meers, 1970). Homogeneous enzyme has been isolated from *Escherichia coli* (Miller & Stadtman, 1972; Mäntsälä & Zalkin, 1976a), *Klebsiella aerogenes* (Trotta et al., 1974), and *Bacillus megaterium* (Hemmilä & Mäntsälä, 1978a,b). These enzymes appear similar and exhibit an $\alpha_4\beta_4$ subunit structure.

Glutamate synthase catalyzes a glutamine- and NH_3-dependent synthesis of glutamate as shown by eq 1 and 2.

$$\text{glutamine} + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightarrow 2\ \text{glutamate} + NADP^+ \quad (1)$$

$$NH_3 + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightarrow \text{glutamate} + NADP^+ \quad (2)$$

For the *E. coli* enzyme, the rate of NH_3-dependent glutamate synthesis is 5–7% of that of the amidotransferase reaction (Mäntsälä & Zalkin, 1976a). Treatments which release non-heme iron, acid-labile sulfur, and flavin completely inactivate the amidotransferase activity and concomitantly stimulate the NH_3-dependent activity approximately 5-fold (Mäntsälä & Zalkin, 1976b; Hemmilä & Mäntsälä, 1978a,b). Evidence has accumulated that the mechansim of reductive amination is different for amidotransferase and NH_3-dependent reactions (Mäntsälä & Zalkin, 1976b; Geary & Meister, 1977). Although the NH_3-dependent reaction is formally similar to that of glutamate dehydrogenase, the two enzymes are immunochemically unrelated, and their catalytic parameters are distinct (Mäntsälä & Zalkin, 1976b).

The present experiments were conducted to (a) determine if glutamine- and NH_3-dependent activities of glutamate synthase utilize the same substrate binding and catalytic sites and (b) determine the roles of residues essential for catalysis.

Experimental Procedures

Materials. Homogeneous glutamate synthase was prepared from *E. coli* as previously described (Mäntsälä & Zalkin, 1976a). Freshly prepared enzyme had a specific activity of

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