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RNA Synthetic Activity of Glutamate Dehydrogenase

Determination of Enzyme Purity, RNA Characteristics, and Deamination/Amination Ratio

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Abstract

The activity of glutamate dehydrogenase (GDH), an important enzyme in carbon and nitrogen metabolism, is routinely assayed by photometry. The RNA synthetic activity of the enzyme provides new technologies for assaying its activity. The enzyme was made to synthesize RNAs in the absence of DNA and total RNA but with different mixes of the four nucleoside triphosphates (NTPs) in order to investigate the RNA characteristics. RNase VI (hydrolyzes base-paired residues) digested the poly (U,A) RNA completely because the U and A residues were evenly distributed to produce many base-paired regions. Therefore, the synthesis of RNA by GDH was by random addition of NTPs. The RNA synthetic activity of the enzyme was at least 50-fold more active in the deamination than in the amination direction, thus providing a robust technology for assay of the enzyme's activity. cDNAs prepared from the RNAs were subjected to restriction fragment differential display polymerase chain reaction analyses. Sequencing of the cDNA fragments showed that some of the RNA synthesized by GDH shared sequence homology with total RNA. Database searches showed that the RNA fragments shared sequence homologies with the G proteins, adenosine triphosphatase, calmodulin, phosphoenol pyruvate (PEP) carboxylase, and PEP carboxykinase, thus explaining the molecular mode of their functions in signal transduction.

Index Entries: Glutamate dehydrogenase; α-ketoglutarate; differential display polymerase chain reaction; nucleotide chromatography; deamination; amination.

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Introduction

Glutamate dehydrogenase (GDH) (EC 1.4.1.2) is a multi-isoenzymic oxidoreductase. It has been purified from microbes as well as plant and animal tissues *(1–5)*. Photometry is routinely used to assay GDH's activities as signaling indexes in the response of tissues and cells to redox changes in the environment *(6–8)*. To improve the interpretation of the amination and deamination activities, the redox changes in the environment have been correlated to the changes in the GDH isoenzyme distribution patterns *(9–13)*. The correlation was dependent on the fact that the GDH multiple molecular isoenzymes were partly due to the ability of the enzyme to polymerize in the presence of nucleotides *(14,15)* and other nucleophiles, and to the nonproteolytic cleavage of the subunits *(16)*. However, GDH isoenzymes stained with formazan in polyacrylamide gels were not always visible for those tissues whose GDH amination and deamination activities were very low *(17)*. The recent demonstration of the RNA synthetic activity of GDH *(18)* provides a new technology for assaying the amination/deamination activity of the enzyme. However, the RNAs synthesized by the enzyme have not been characterized. In addition, the effects of the oxidative and reductive environments on the RNA synthetic activity have not been studied. Characterization of the structures of the RNAs will broaden the technological applications of the enzyme.

In the present study, we used peanut as the experimental organism. We report herein that the enzyme synthesized more RNA in the deamination than amination direction, and that some of the RNAs synthesized encoded short sequences of several signaling proteins.

Materials and Methods

Purification of GDH Isoenzymes

GDH isoenzymes were extracted from 30 g of control or treated peanut (*Arachis hypogaea* L) seedlings *(18)* by homogenizing in a blender at maximum speed with 100 mL of ice-cold extraction buffer *(19)* at 3°C. RNase A and DNase 1 were added to the extraction buffer solution to bring each to 5 U/mL prior to tissue homogenization. The homogenate was left at room temperature for 15 to 20 min to allow further degradation of nucleic acids. Tissue debris was pelleted by centrifugation (4000*g*, 4°C, 30 min), and the supernatant was frozen at –80°C, thawed at 4°C, and recentrifuged (10,000*g*, 4°C, 30 min) to remove cellular debris. The resulting supernatant was saturated to 50% with solid $(NH_4)_2SO_4$. The precipitated protein was pelleted by centrifugation (10,000*g*, 4°C, 30 min); dissolved in a minimum volume of 0.1 *M* Tris-HCl buffer (pH 8.0); and dialyzed exhaustively at 4°C for 48 h against four changes of 10 m*M* Tris-HCl buffer (pH 8.0), each change being 5 L of the buffer solution. The volume of the extract after dialysis was ~50 mL. The dialyzed extract (crude enzyme) was subjected to Rotofor (Bio-Rad, Hercules, CA) isoelectric focusing *(10)*. After dialyzing

the Rotofor fractions exhaustively at 4°C to remove urea and ampholyte, aliquots of the fractions were subjected to native 7.5% polyacrylamide gel electrophoresis (PAGE) (100 V, 20 h, 4°C) to remove other protein and nucleic acid contaminations. GDH isoenzymes were eluted from the electrophoresed gel at subzero temperature using whole gel eluter (Bio-Rad). Control extractions of the enzyme were performed as just described, but without the DNase and RNase treatment.

To test for DNA contamination of GDH with or without DNase treatment of the tissue homogenate, 0.5-mL aliquots of the Rotofor fractions and whole gel elution fractions were extracted with phenol-chloroform (pH 8.0) followed by alcohol precipitation of the DNA. After pelleting and air-drying the DNA, 30 µL of TE buffer was added to the tube. The contaminating DNA was evaluated by agarose gel electrophoresis. It was further analyzed by Taq 1 restriction enzyme digestion, ligation of DisplayProfile adapters to the ends of the restriction fragments, followed by polymerase chain reaction (PCR) amplification using DisplayProfile control primers as described by the manufacturer (Display Systems Biotech, Vista, CA). Control amplification without DNA was also set up.

To evaluate the extent of the persistence of total RNA in the purification of GDH isoenzymes, peanut total RNA (~5 µg) dephosphorylated with calf intestinal alkaline phosphatase and labeled with 1 µL of [γ^{32}P] ATP (7000 Ci/mmol) (ICN, Aurora, OH) using polynucleotide kinase (KinaseMax kit from Ambion, Austin, TX) was added to each Rotofor fraction and electrophoresed (100 V, 20 h, 4°C) through native 7.5% polyacrylamide gel (PAG). The gels were electrophoresed in duplicate. One gel was removed as soon as the bromophenol blue dye had reached the bottom of the gel (~10-h run). Electrophoresis with the second gel was continued for an additional 10 h. After staining the two gels for GDH activity, they were dried and autoradiographed.

GDH (~0.1 mg) purified from DNase- and RNase-treated homogenate was hydrolyzed with proteinase K (0.1 mg) at 35°C overnight. Nucleotides that were bound to the GDH were isolated by adding ethanol to the hydrolysate to 70% (v/v) saturation followed by centrifugation at top speed to remove the precipitate. Controls without proteinase K digestion were also set up. After vacuum concentration of the supernatant to about 1 mL, the nucleotide contents were fractionated by chromatography through a 5-mL Econo-Pac Q cartridge (Bio-Rad) strongly basic anion exchanger. The automated Econo System was programmed at a flow rate of 5 mL/min with a multistep of discontinuously increased NaCl concentrations using distilled water as the initial eluent. Elution peaks were collected by time windows, and the absorbance at 260 nm of each peak was measured. Nucleotides present in each peak were identified by chromatography of authentic samples.

Assay of RNA Synthetic Activity of GDH

RNA synthesis by GDH isoenzymes was assayed *(18)* in solutions of 0.1 *M* Tris-HCl buffer (pH 8.0) containing 0.06–1.0 m*M* each of uridine

5'-triphosphate (UTP), adenosine triphosphate, guanosine 5'-triphosphate (GTP), and cytidine 5'-triphosphate (CTP); 0.07–100 m*M* NH_4Cl; 0.01–50 m*M* α-ketoglutarate (α-KG); 0.1–20 m*M* $CaCl_2$; 0.1–1 m*M* NADH; 5 U of RNase inhibitor; 5 U of DNase 1; and actinomycin D. The reaction was started by the addition of 0.1 mL of GDH charge isomers containing ~6 µg of protein/mL. The final volume of the reaction was brought to 0.4 mL with 0.1 *M* Tris-HCl buffer (pH 8.0). Control reactions with the enzyme but without the substrates (NTPs) and/or the effectors (NADH, NAD^+, NH_4Cl, $CaCl_2$, and α-KG) were also set up.

For determination of the GDH deamination/amination ratio, RNA synthesis was assayed with cocktails in 0.1 *M* Tris-HCl buffer (pH 8.0) containing the four NTPs (0.6 m*M* each), $CaCl_2$ (3.5 m*M*), 5 U of RNase inhibitor, 5 U of DNase 1, and 5 µg of actinomycin D in the amination and deamination directions. Reactions in the deamination direction were conducted by adding the synthetic cocktail to different micro-test tubes each containing a fixed concentration of NAD^+ (0.23 m*M*) and different concentrations of L-glu (2–250 m*M*). Reactions in the amination direction were conducted by adding the synthetic cocktail to different micro-test tubes each containing fixed concentrations of NADH (0.23 m*M*) and NH_4Cl (0.9 m*M*), and different concentrations of α-KG (2–250 m*M*). The reaction was started by the addition of 0.1 mL of pooled GDH charge isomers containing ~6 µg of protein/mL. The GDH used was purified from control peanut seedlings. The final volume of the reaction was brought to 0.4 mL with 0.1 *M* Tris-HCl buffer (pH 8.0). A control reaction with the enzyme but without the substrates and the effectors (NTPs, NADH, NAD^+, NH_4Cl, $CaCl_2$, and α-KG) was also set up.

Reactions were incubated at 16°C overnight and stopped by phenol-chloroform (pH 5.5) extraction of the enzyme. The RNA was precipitated with ethanol. RNA yield and quality were determined by photometry and by electrophoresing through 1.5% Seakem GTG agarose (FMC, Rockland, ME). Assays were carried out in duplicate to verify the reproducibility of the results.

Characteristics of RNA

RNAs were synthesized with mixes of 3 µL of each of two [α-^{32}P] NTPs (10 mCi/mL) (ICN), mixes of 2 µL of each of three [α-^{32}P] NTPs, and mixes of 1.5 µL of each of four [α-^{32}P] NTPs. NADH was not added, but NH_4Cl (0.8 m*M*), $CaCl_2$ (3.0 m*M*), α-KG (10.0 m*M*), DNase 1 (5 U), RNase inhibitor (5 U), and actinomycin D (5 µg) in 0.1 *M* Tris-HCl buffer (pH 8.0) were added. The reaction was started by the addition of ~0.6 µg of GDH to bring the final volume per reaction to 0.4 mL. Reactions and precipitation of the product RNA were carried out as described above. Each radioactive RNA (10 µL in each microcentrifuge tube) was digested with 0.01 U each of RNase A, RNase VI, or RNase T_1 (Ambion) at room temperature for 15 min according to Ambion's protocol. There were also controls without RNase. The RNase digestion products were electrophoresed through 1.5% agar-

ose, electroblotted (Bio-Rad's semidry trans-blot cell) onto nylon membrane, followed by autoradiography of the membrane.

Total RNA was isolated from 5 g of peanut seedlings *(20)*. The yield and purity of the RNA were determined by photometry and by agarose gel electrophoresis.

Identification of RNA

RNAs were synthesized using the whole-gel fractions 4 to 15 of GDH isoenzymes of chitosan-treated peanut seedlings in the presence of equal concentrations of the four NTPs. cDNAs were then synthesized with 2 µg of each product RNA, or with total RNA of peanut using random hexamer primer. The standard differential display PCR procedure *(21)* was followed, but the buffer solutions, enzymes, and restriction fragment differential display (RF-DD) PCR method of Display Systems Biotech were used without any modification. To verify reproducibility of the fractionation patterns, the reverse transcriptase-PCR and restriction fragment amplification were repeated with the RNA synthesized by a second preparation of GDH from the same batch of chitosan-treated peanut seedlings.

Selected cDNA fragments were subcloned into pCR4-TOPO vector and transformed into TOP10 One Shot Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA), followed by overnight growth on selective plates. About five positive transformant colonies were picked per plate and cultured overnight in Luria-Bertani medium containing 50 µg/mL of ampicillin. Plasmid DNA was purified with a plasmid miniprep kit (CPG, Lincoln Park, NJ), and the insert cDNA fragment was sequenced with M13 forward and reverse primers by MWG Biotech (High Point, NC).

Functional Characterization of RNAs

The nucleotide sequences of the cDNA fragments were used to search the GenBank database with the BLAST algorithm *(22)*. Putative functions were assigned to the highest BLASTN scores.

Results and Discussion

RNA- and DNA-Free GDH Isoenzymes

The isoenzyme distribution pattern (Fig. 1A) of the GDH extracted from chitosan-treated peanuts was obtained by GDH activity staining of the electrophoresed native polyacrylamide gel. After cryoelution, whole-gel fractions 3 to 18 were active in the synthesis of RNA (Fig. 1B). Silver staining and immunochemical assay with anti-GDH antibody showed that the GDH isoenzymes electroeluted from native polyacrylamide gel were free from contaminating proteins *(18)*. The GDH isoenzyme distribution pattern when the homogenate was not treated *(23)* with RNase was similar to that of the RNase-treated tissue homogenate, thus confirming that total RNA was not bound to the enzyme.

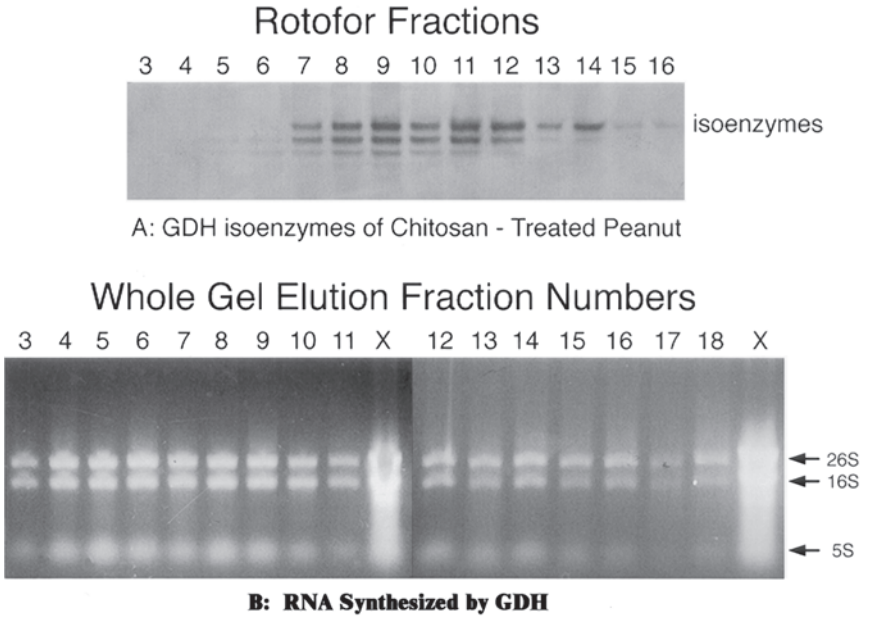

Fig. 1. Activity of GDH isoenzymes. **(A)** GDH isoenzyme pattern of chitosan-treated peanut. The enzyme extract was purified by $(NH_4)_2SO_4$ precipitation, isoelectric focusing, and native PAGE. Gel was stained for GDH activity with tetrazolium blue reagent. **(B)** RNA synthesis by GDH charge isomers. The GDH charge isomers were eluted from native gel by cryoelectrophoresis. The whole-gel fractions were used for RNA synthesis in cocktails containing the four NTP mixes, modulators, and DNase 1. The RNA products were electrophoresed through agarose gel. Total RNA (lane X, left = 6 µg; lane X, right = 12 µg) was used as the marker.

After 10 h of native PAGE, activity staining of the gel for GDH activity showed that the GDH isoenzymes had migrated only 1 cm into the top of the 14-cm-long separating gel (Fig. 2A), whereas autoradiography showed that total RNA had migrated rapidly through, completely ahead and away from the GDH zone of the gel (Fig. 2B). Alignment of the autoradiograph and the GDH-stained gel confirmed that the total RNA was clearly separated from the GDH isoenzymes. In addition, the total RNA profile (Fig. 2B) was different from the GDH isoenzyme pattern (Fig. 2A), thus further confirming that GDH did not bind any RNA.

After 20 h of electrophoresis, the GDH isoenzymes had migrated only 2.3 cm into the gel (Fig. 2C), and at that time, autoradiography did not detect any radioactivity in the gel. Therefore, GDH so prepared was free from RNA contamination. The high molecular mass (>350 kDa) of the GDH hexamers and possibly their reversible polymerization *(14,15)* retarded their migration in the native gel, so that after 20 h of electrophoresis at 100 V, the hexamers just entered the separating gel, while other proteins and total RNA migrated out of the GDH zone of the gel. Therefore, GDH eluted from

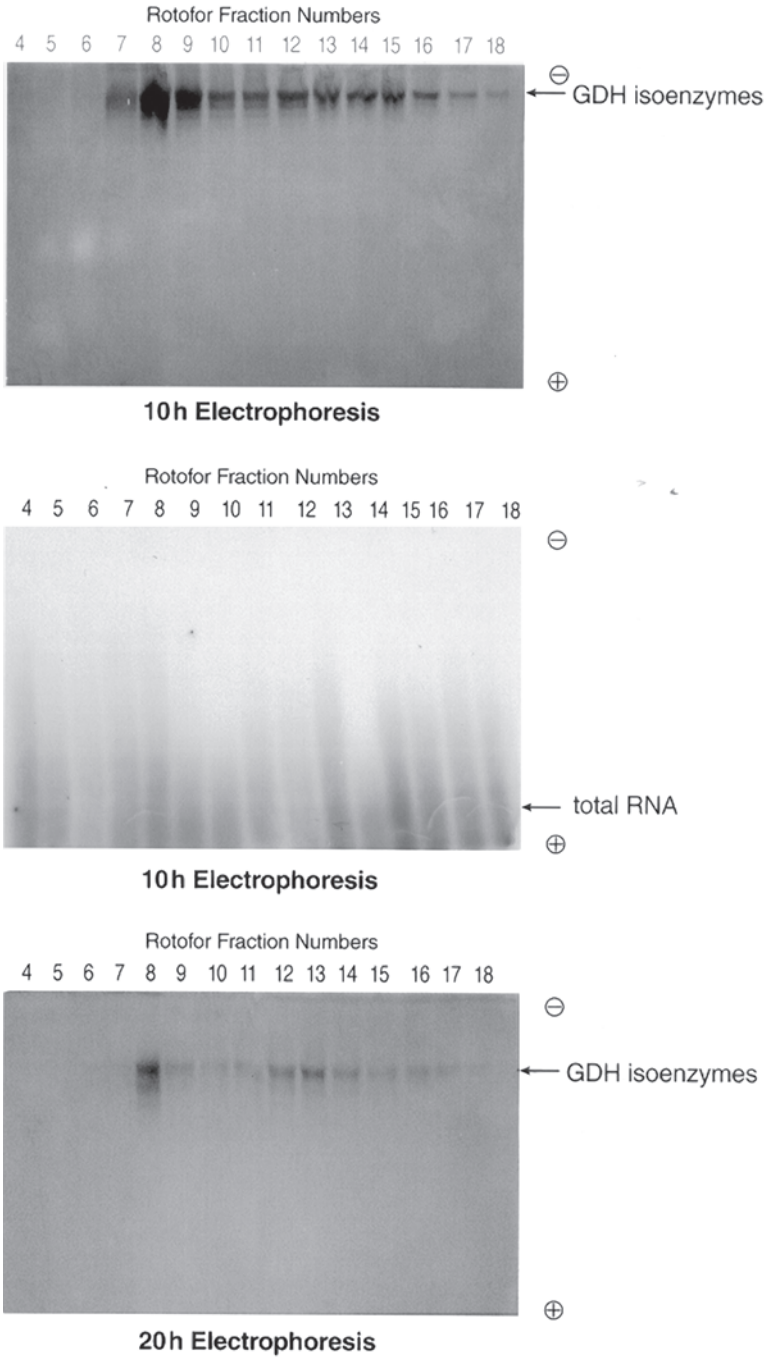

Fig. 2. Electrophoretic separation of GDH isoenzymes from total RNA. Labeled total RNA (5 μg) was added to each Rotofor fraction and subjected to native 7.5% PAGE. Gels were run in duplicate. After 10 h, one gel was removed and stained for GDH activity **(A)** followed by autoradiography **(B)** to detect RNA. After an additional 10 h of electrophoresis, the second gel was removed and stained for GDH activity **(C)** followed by autoradiography.

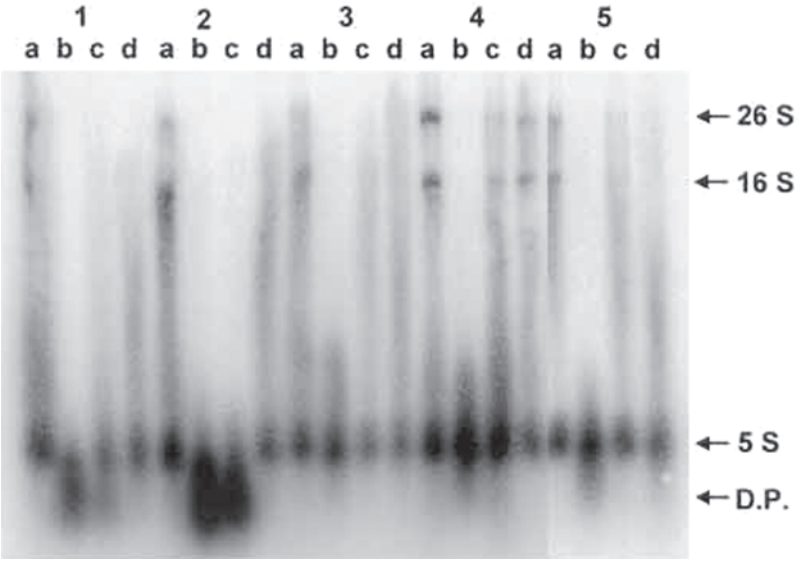

Fig. 3. RNase digestion of RNAs synthesized by GDH. Equal volumes (0.2 mL) of GDH isoenzymes were added to cocktails containing 3 µL of each of two [α-^{32}P] NTPs, DNase 1, and modulators. The two NTP mixes were (1) C + A, (2) U + A, (3) C + G, (4) U + C, and (5) U + G. The product RNAs were ethanol precipitated, dissolved in distilled water, and aliquots were subjected to (a) control (no RNase), (b) RNase A, (c) RNase VI, and (d) RNase T_1 digestion. The digestion products were electrophoresed through agarose gel and transblotted to nylon membrane, and the membrane was autoradiographed. D.P., digestion product.

the gel was free not only from other proteins, but also from total RNA contamination.

Ethidium bromide-stained agarose gel electrophoresis showed that DNase 1 treatment of the peanut homogenate successfully removed all traces of DNA from the crude extract at the $(NH_4)_2SO_4$ precipitation and dialysis step. Extending the analysis through to PCR amplification showed that DNA was absent, thus confirming the agarose gel results. However, after the whole-gel elution step, there was no trace of DNA in the GDH isoenzyme preparation whether or not the tissue homogenate was treated with DNase 1. Therefore, treatment of the tissue homogenate with DNase doubly ensured that the electrophoretically purified enzyme was DNA free.

Characteristics of RNAs Synthesized by GDH

The RNAs synthesized by GDH in the presence of two NTPs, three NTPs, and four NTPs were in the 26S, 16S, and 5S rRNA molecular mass ranges (Fig. 3). All the RNAs containing at least a pyrimidine nucleotide residue were totally degraded by RNase A (Fig. 3). RNase VI (hydrolyzes base-paired residues) digested the poly (U,A) RNA completely, probably because the U and A residues were evenly distributed to produce many base-paired regions. Therefore, GDH synthesized RNA by random

addition of NTPs. The other mixes of two NTPs produced RNAs that were not readily degraded by RNase VI. In this class, the poly (U,C) RNA was most resistant, followed by the poly (U,G), poly (C,G), and poly (A,C) RNAs. The lack of base pairs in the structures of the poly (U,C), poly (U,G), and poly (A,C) RNAs is understandable because the U and C, the U and G, and the A and C residues did not form stable Watson-Crick base pairing. The lack of base pairs in the poly (C,G) RNA was surprising but may be owing to a higher frequency of addition of the C than the G by the GDH, thereby producing long stretches of C residues with few intermittent G residues in the RNA. The observed lower content of G residues in the poly (C,G) RNA was supported by the resistance of the RNA to RNase T_1 digestion (Fig. 3). The poly (C,G) RNA was as resistant to RNase T_1 digestion as those RNAs lacking G residues. Because it lacked any purine residue, the poly (U,C) RNA was the most resistant to RNase T_1 digestion (Fig. 3). Therefore, GDH synthesized different RNAs by random addition of NTPs depending on the prevalent pool of NTPs, the U + A mix being more reactive than the C + G mix. This is unlike the action mode of template-dependent RNA polymerase. RNA amplification or resynthesis by GDH of preexisting total RNA would have given RNase digestion patterns that were identical irrespective of the labeled NTPs present in the reaction cocktail.

All the RNAs that GDH synthesized with mixes of three NTPs and of four NTPs were totally degraded by RNase A (not shown) because of the presence of at least a pyrimidine nucleotide in the substrate mix. RNase VI encountered more resistance in the digestion of the three NTPs than in the four NTPs owing to less base pairing in the three NTPs' RNAs. The poly (U,A,G) RNA was the most susceptible to RNase T_1 digestion, thus confirming that the G residues were not involved in any base pairing. However, the poly (U,A,C), and poly (A,C,G) RNAs were totally resistant to RNase T_1 digestion. The poly (U,A,C) RNA lacked G residues. The resistance of the poly (A,C,G) RNA to RNase T_1 was probably owing to the G residues being base paired with the C residues.

Therefore, different RNAs were synthesized depending on the available NTP mix as different from the template-dependent RNA polymerases. The RNase digestions illuminated not only the unique primary structures but also the secondary structures of the RNAs. They also provided further evidence that the RNAs synthesized by GDH were not transcribed from DNA, not amplified from total RNAs, and not resyntheses of preexisting total RNAs.

Differential Display Patterns

The redox dependence of the RNA synthetic activity of GDH was clearly demonstrated by the resolution patterns (Fig. 4) in parallel comparison with the regular differential display of total RNA products. The PCR priming by display PROBE 16 (Fig. 4A) showed that the acidic isoenzymes (whole-gel fractions 4 to 9) were inactive in the synthesis of the oxidative

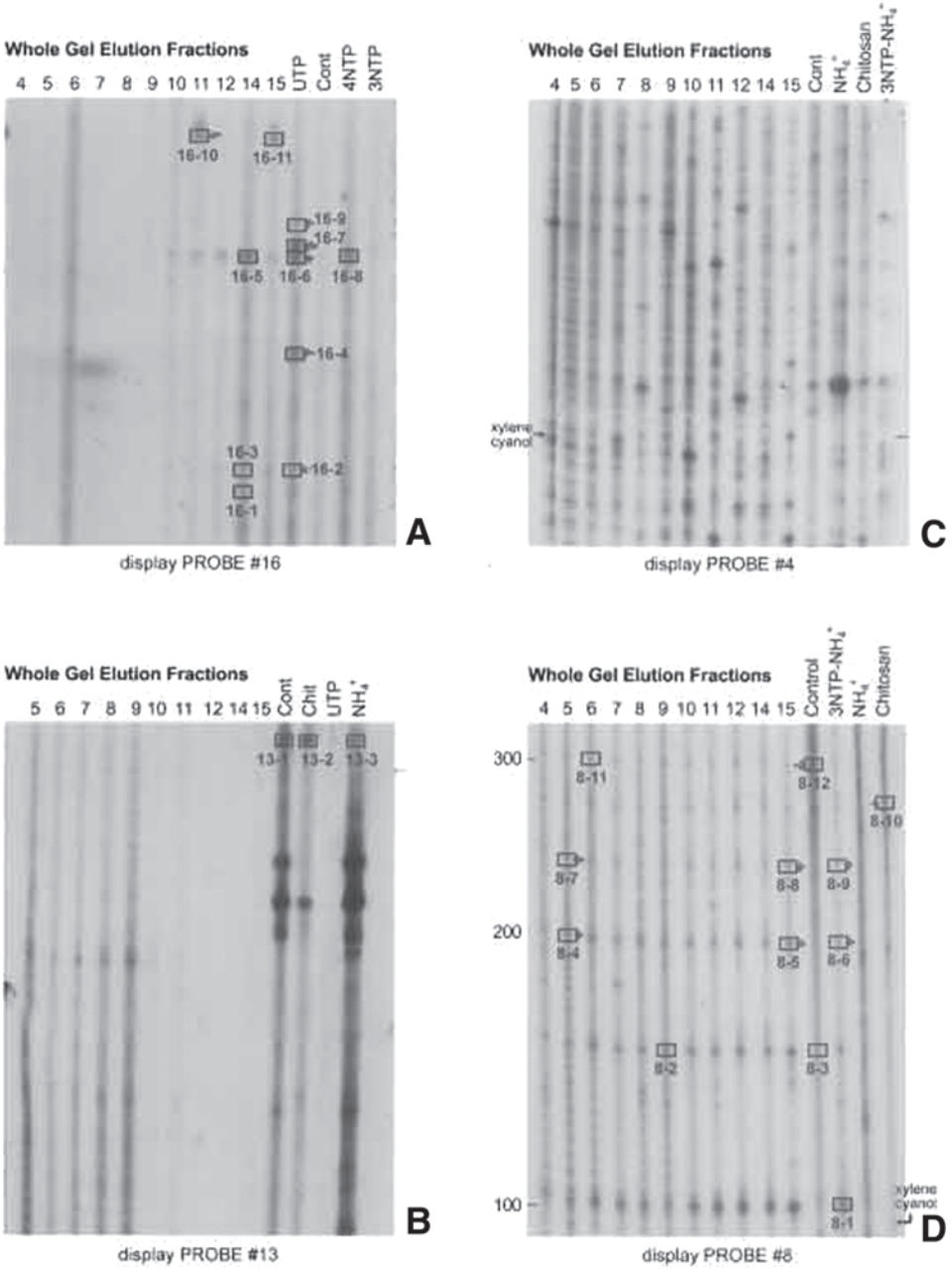

Fig. 4. Differential display for RNAs synthesized by GDH compared with differential display for total RNA. The RNA synthesized by each whole-gel eluted GDH fraction was used. After cDNA preparation (random hexamer primers) and Taq restriction enzyme digestion, the restriction fragments were amplified by displaySystem's DD-PCR method and fractionated on sequencing polyacrylamide gel. Total RNAs of peanut were similarly treated. **(A–D)** Differential display patterns when display PROBES 16, 13, 4, and 8, respectively, were used as PCR primers. The indicated fragments were sequenced.

subset of the RNA population, but that the basic isoenzymes (whole-gel fractions 10 to 16) were active in the synthesis of the reductive subset of the RNA population. The converse was the case in the PCR priming by displayPROBE 13 (Fig. 4B) because the acidic isoenzymes (whole-gel fractions 4 to 9) were very active in the synthesis of the oxidative subset of RNA, but the basic isoenzymes (whole-gel fractions 10-16) were inactive in the synthesis of the reductive subset of RNA. The display patterns obtained from the PCR priming by displayPROBE 4 (Fig. 4C) and displayPROBE 8 (Fig. 4D) were intermediate between those of displayPROBEs 16 and 13. Therefore, the RNA synthetic activity of GDH was dependent on the electrode potentials of the GDH isoenzymes. Some total RNA lanes did not possess any cDNA bands (Fig. 4). This is part of the nonreproducible nature of the differential display of total RNA products *(24)*. The display patterns obtained in the replicate RF-PCR experiments of the RNAs synthesized by GDH showed complete identity per displayPROBE. Therefore, the synthesis of RNA by GDH was a reproducible reaction.

Sequence Comparison

All the cDNA fragments resulting from a display pattern contained the same pair of displayPROBE sequences at their ends (Table 1). Nucleotide sequences of 26 cDNA fragments were determined from both ends of 15 fragments, from the 5' ends of 3 fragments, and from the 3' ends of 8 fragments. The nucleotide sequences are presented in Table 1.

Fragment 16-5 (RNA by GDH) and fragments 16-6 and 16-8 (total RNA) were completely homologous, thus confirming the accuracy of the sequence of the fragment. Fragment 16-6 was different from fragment 16-7 (total RNA) owing to a C deletion. Fragment 16-7 was not synthesized by GDH. Fragment 8-1 (total RNA) had only limited sequence homology with fragment 8-2 (RNA by GDH), but all of fragment 8-1 constituted the 5' section of fragment 8-3. Fragments 8-5 (RNA by GDH) and 8-6 (total RNA) had sequence homology except an insert of a block of 76 bases at the 5' end of fragment 8-5. Furthermore, fragments 8-8 (RNA by GDH) and 8-9 (total RNA) had complete sequence homology. Fragment 8-10 (total RNA) also shared extensive sequence homology with fragments 8-5 (RNA by GDH), 8-6 (total RNA), 8-8 (RNA by GDH), and 8-9 (total RNA), showing that many total RNAs and RNAs synthesized by GDH shared extensive sequence homologies. The other sequenced fragments of RNAs synthesized by GDH did not share homology with any of the sequenced total RNAs.

Functions of Sequenced RNAs

To assign functions to the cDNA fragments, their sequences were used to search the GenBank database with the BLASTN algorithm *(22)*. Putative functions were assigned to fragments based on the highest BLASTN scores. All the sequenced RNAs were assigned putative functions (Table 2).

Table 1
Nucleotide Sequences of Differential Fragments[a]

1. cDNA Fragment 16-1

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGCGCCAATGCGTTGAGTACCTTCAACGCCAAGAACGTCAACTACCAG
CGCACGCCGCACTTCAAGAACAAACCCGGCACGCGGCATAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGCGG

2. cDNA Fragment 16-2

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGGAGATCAAGGCACCCCATGTCTTGAGGGTGGGACGGTTATTTGCTCA
GGATAATAAAGGGCGGTTTCAGTTCAAAGTGCCTGAGCTTAGTATAGCGATGAGTCCTGACTGGGT**ACGCAGTCTACGAGACCAGTA**AGG
GCGAATTCGCGG

3. cDNA Fragment 16-3

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGTGAATAGGTCGTTGTGTTTCATGAGGCCTCCTTGATACTCATG
AACTACAGATATTTGACGTCAAAAATAATTCAAATAAGTTGTCCGACAATGCTGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**AG
GGCGAATTCGCGG

4. cDNA Fragment 16-4

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGCGGCCTGCATGCTCATGTTGCCAGTCTTGCCACCAGTACCCGTTCC
AGTGTCAGGAGCCGGGAACTGACCTACGCCATTTTTGTAAACACCGGTAGAAGAGGAATAAGGACTCCCGGAAGTGTGCCAAGTCACCAAGGTT
TCAACTTCGGGT**ACCAGTCTACGAGACCAGTA**AGGGCGAATTCGCGG

5. cDNA Fragment 16-5

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGGGCGTTGAACTGTAAGGCAAAGGCTTGTAAAGTGCGCCCCGCAGCAACGTCAGCCC
ACGGGTTTCTTCTCCCCAAGAAGAAATCAGAAAGAAGCGCAAATCATTGTCTTAAAAGCAATTTAAAGGGTTGACAGGGGTTTTTAAGATTGTA
GAATAGCGCGCCTCAGAGACGCTAACGCAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACCAAAACCATA**AGGGCGAATTCGCGG

6. cDNA Fragment 16-6

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGGGCGTTGAACTGTAAGGCAAAGGCTTGTAAAGTGCGCCCCGCAGCAACGTCAGCCCACG
GGTTTCTTCTCCCCAAGAAGAAATCAGAAAGAAGCGCAAATCATTGTCTTAAAAGCAATTTAAAGGGTTGACAGGGGTTTTTAAGATTGTAGAA
TAGCGCGCCTCAGAGACGCTAACGTAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGTTTAAACCTGCAGGACT

7. cDNA Fragment 16-7

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGGGCGTTGAACTGTAAGGCAAAGGCTTGTAAAGTGCGCCCCGCAGCAACGTCAGCCCACGGG
TTTCTTCTCCCCAAGAAGAAATCAGAAAGAAGCGCAAATCATTGTCTTAAAAGCAATTTAAAGGGTTGACAGGGGTTTTTAAGATTGTAGAATAGC
CGCGCCTCAGAGACGCTAACGTAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGTTTAAACCTGCAGGACTAG

8. cDNA Fragment 16-8

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGGGCGTTGAACTGTAAGGCAAAGGCTTGTAAAGTGCGCCCCGCAGCAACGTCAGCCC
ACGGGTTTCTTCTCCCCAAGAAGAAATCAGAAAGAAGCGCAAATCATTGTCTTAAAAGCAATTTAAAGGGTTGACAGGGGTTTTTAAGATTGTAG
AATAGCGCGCCTCAGAGACGCTAACGTAGCGATGAGTCCTGACCCGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGTTTAAACCTGCA
GGACTAG

9. cDNA Fragment 16-9

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGCCTGCCTAAACCTTCTTGAAGTAGTGGCGGCGGTCGTTTTCGGTGA
CTGTCTGCTGGAAAATGTCCGTCCAGAAATCCCGCTCCATTACGTCCTGGTGAAACATCACCCCGCAGATAACCTCCATCGGGTTGCACTTCAAAAG
CTCGGCAACCTTCACGGCCTGCTTGACGCTCATTTCATGTTTTCCCGCCTTCTGTAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**
AGGGCGAATTCGCGG

10. cDNA Fragment 16-10

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGCTATTTAGGTAGCGCCTCATGTATCACTGTAGGGGGTAGAGCACTGTTTCGGCTAGGGGGTC
ATCCCGACTTACCAAACCGATGCAAACTCCGAATACCTACAAGTGCCGAGCATGGGAGACACACGGCGGGTGCTAACGTCCGTCGTG
AAAAGGGAAACAACCCAGACCGTCAGCTAAGGTCCCAAAGTTATGGTTAAGTGGGAAACGATGTGGGAAGGCTTATACAGCTAGGAGGTTGGCT
TAGAAGCAGCCACCCTTTAAAGAAAGCGTAATAGCCTCACTAGTCGGGT**ACGCAGTTTACGAGACCAGTA**AGGGCGAATTCGTTTAAACCT
GCAGGACTAG

11. cDNA Fragment 16-11

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**TAGCTATTTAGGTAGCGCCTCATGTATCACTGTAGGGGGTAGAGCACTGTTTCGGCTAGGGGGTCA
TCCCGACTTACCAAACCGATGCAAACTCCGAATACCTACAAGTGCCGAGCATGGGAGACACACNGGCGGGTGCTAACGTCCG
TCGTGAAAAGGGAAACAACCCAGACCGTCAGCTAAGGTCCCAAAGTTATGGTTAAGTGGGAAACGATGTGGGAAGGCTTAGACAGCTAGG
AGGTTGGCTTAGAAGCAGCCACCCTTTAAAGAAAGCGTAATAGCTCACTAGTCGGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTC
GTTTAAACCTGCAGGACTAG

12. cDNA Fragment 13-1

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ACTGGTCTCGTAGACTGCG**TACCCGACTAACCCATGTGCAAGTGCC
GTTCACATGGAACCTTTCCCCTCTTCGGCCTTCAAAGTTCTCATTTGAATATTTGCTACTACCACCAAGATCTGCACCGACGGCCGCTCCG
CCCGGGCTCGCGCCCCAGGTTTTGCAGCGACCGCCGCGCCCTCCTACTCATCGCGGCATAGCCCTTGCCCCGACGGCCGGGTATAGGTCACGCGCT
TCAGCGCCATCCATTTTCGGGGCTAGTTGATTCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATG**TCGGTCAGGACTCATA**
AGGGCGAATTCGCGG

13. cDNA Fragment 13-2

CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**CATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATCAACTAGCCCCGAAAATGG
ATGGCGCTGAAGCGCGTGACCTATACCCGGCCGTCGGGGCAAGGGCTATGCCGCGATGAGTAGGAGGGCNGCGGCGGTCGCTGCAAAA
CCTGGGGCGCGAGCCCGGGCGGAGCGGCCGTCGGTGCAGATCTTGGTGGTAGTAGCAAATATTCAAATGAGAACTTTGAAGGCCGAAG
AGGGGAAAGGTTCCATGTGAACGGCACTTGCACATGGGTTAGTCGGG**TACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGTTTAA
ACCTGCAGGACTAG

14. cDNA Fragment 13-3

CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ACTGGTCTCGTAGACTGCG**TACCCGACTAACCCATGTGCAAGTGCCGTTCACATGGA
ACCTTTCCCCTCTTCGGCCTTCAAAGTTCTCATTTGAATATTTGCTACTACCACCAAGATCTGCACCGACGGCCGCTCCGCCCGGGCTCGC
GCCCCAGGTTTTGCAGCGACCGCCGCGCCCTCCTACTCATCGCGGCATAGCCCTTGCCCCGACGGCCGGGTATAGGTCACGCGCTTCAGCG
CCATCCATTTTCGGGGCTAGTTGATTCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATG**TCGGTCAGGACTCATA**AGGGCGAATTCGCGG

(continued)

Table 1 *(continued)*

15. cDNA Fragment 8-1
CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**GAACGGCATTGATAGCGATGAGTCCTGACCGACAACGGC
ATTGATAGCGATGAGTCCTGACCGGGT**ACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGCGG

16. cDNA Fragment 8-2
CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**GAACAACGGAACACGTGTGGCAGGGAACATTTTCTCTCTGGGCTTGGGTGTGACTGC
TTCCGATCTTTGACGGTGGAGCAATACAAGCGGGAATCGGG**TACGCAGTCTACGAGACCAGA**AGGGCGAATTCGTTTAAACCTGCAGGACTAG

17. cDNA Fragment 8-3
CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**GAACGGCATTGATAGCGATGAGTCCTGACCGACAACGGCATT
GATAGCGATGAGTCCTGACCGACAACGGCATTGATAGCGATGAGTCCTGACCGGG**TACGCAGTCTACGAGACCAGTTA**AGGGCGAATTCGCGG

18. cDNA Fragment 8-4
CCGCGAATTCGCCCTT**ATGAGTCCTGACCGA**GAAGGTTGCGCCCGACGATTGTGACGACGCCGAGCGGCGCGGCATCCTCCAGCCGCGT
CGCGATCCGATCGGGTACGCAGTCTACGAGACCAGTACGCAGTCTACGAGACCAGTACGCAGTCTACGAGACCAG**TACGCAGTCTACGA**
GACCAGTAAGGGCGAATTCGTTTAAACCTGCAGGACTAG

19. cDNA Fragment 8-5
CCGCGAATTCGCCCTT**ACTGGTCTCGTAGACTGCG**CACTGGTCTCGTAGACTGCGCACTGGTCTCGTAGACTGCGTTCTCGGTCAGGACT
CATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCAGCTATCAATGCCGTTGTCGGTCAGGACTCATCAGCTATCAATGTCGTTGTCGGT
CAGGACTCATCAGCTATCAATGCCGTTGTCGATCAGGACTCATCGCTATCAATGCCGTT**CTCGGTCAGGACTCATA**AGGGCGAATTCGTT
AACCTGCAGGACTAG

20. cDNA Fragment 8-6
CCGCGAATTCGCCCTT**ACTGGTCTCGTAGACTGCG**TACCCGGTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGCTA
TCAATGCCGTTGTCGGTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGCTATCAATGCCGTT**CTCGGTCAGGACTCATA**
AGGGCGAATTCGTTTAAACCTGCAGGACTAGTC

21. cDNA Fragment 8-7
CTAGTCCTGCAGGTTTAAACGAATTCGCCCTT**ATGAGTCCTGACCGA**GAACGGCATTGATAGCGATGAGTCCTGACCGACAACGGCATTGA
TAGCGATGAGTCCTGACCGANAACGGCATTGATAGCGATGAGTCCTGACCGACAACGGCATTGATAGCGATGAGTCCTGACCGACAACGGC
ATTGATAGCGATGAGTCCTGACCGGG**TACGCAGTCTACGAGACCAGTA**AGGGCGAATTCGCGG

22. cDNA Fragment 8-8
CCGCGAATTCGCCCTT**ACTGGTCTCGTAGACTGCGTACCCG**GTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGCTAT
CAATGCCGTTGTCGGTCAGGACTCATCGCTATCAATGCCGTTCTCGGTCAGGACTCATCGCTATCAATGCCGTCGTCGGTCAGGACTCATCG
CTATCAATGCCGTT**CTCGGTCAGGACTCATA**AGGGCGAATTCGTTTAAACCTGCAGGACTAG

23. cDNA Fragment 8-9
CCGCGAATTCGCCCTT**ACTGGTCTCGTAGACTGCGTACCCG**GTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGCTA
TCAATGCCGTTGTCGGTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGCTATCAATGCCGTTGTCGGTCAGGACTCATCGC
TATCAATGCCGTT**CTCGGTCAGGACTCATA**AGGGCGAATTCGTTTAAACCTGCAGGACTAG

[a]Each fragment sequence is flanked by the vector's multiple restriction enzyme cloning sites (underlined), and the displaySystem's o-extension primer sequence (bold underlined). The fragment numbering system is the same as in Fig. 4.

Table 2
Characterization of Sequenced RNAs[a]

Fragment no.	Source of fragments	Putative function
16-1	RNA synthesized by GDH	PEP carboxylase
16-2	RNA synthesized by GDH	Sucrose synthase
16-4	Total RNA	Cellulose synthase
16-5	RNA synthesized by GDH	CaM
16-6	Total RNA	CaM
16-7	Total RNA	CaM
16-8	Total RNA	CaM
16-10	RNA synthesized by GDH	4.5S and 23S rRNA
16-11	RNA synthesized by GDH	4.5S and 23S rRNA
8-2	RNA synthesized by GDH	4S and 23S rRNA
8-4	RNA synthesized by GDH	PEP carboxykinase
8-5	RNA synthesized by GDH	G protein
8-7	RNA synthesized by GDH	Plasma membrane H^+ ATPase
8-8	RNA synthesized by GDH	G protein
8-9	Total RNA	G protein
8-10	Total RNA	5.8S rRNA
8-11	RNA synthesized by GDH	Exoglucanase
8-12	Total RNA	Plastid 26S rRNA
13-1	Total RNA	25S rRNA
13-2	Total RNA	25S rRNA
13-3	Total RNA	25S rRNA

[a]The cDNA fragments were sequenced and the nearest match was identified using BLASTN.

Fragments 8-5 and 8-8 (RNAs by GDH) and 8-9 (total RNA) showed homology to the Rac GTP-binding protein RNA. Elucidation of the mechanisms of the guanosine 5'-triphosphatases *(25)* and the G protein–coupled receptors *(26)* has made a great contribution to the pharmaceutical industry because it permitted the discovery and design of drugs that act on receptors *(27)*.

Fragments 16-5 (RNA by GDH) and 16-6, 16-7, and 16-8 (total RNA) demonstrated homology to calmodulin (CaM) mRNA. The CaM-binding proteins represent a mechanism for communication between Ca^{2+} and the nucleotide-mediated signal transduction pathway *(28)*. Peanuts were treated with nucleotides and chitosan in this project in order to induce the G proteins, the CaM, and the kinase cascade signaling pathways.

RNA fragments 16-10 and 16-11 (from GDH) showed structural homology to 4.5S and 23S rRNAs, respectively. RNA fragments 8-12 and 13-1 (total RNA of control peanut) showed similarities to 25S rRNA. RNA fragments 8-10 and 13-2 (total RNA) from chitosan treatment showed sequence similarities to 5.8S and 25S rRNAs respectively. Fragment 13-3 (total RNA) of the NH_4Cl-treated peanut also showed similarity to 25S rRNA. The homology of fragments 16-10 and 16-11 (RNAs by GDH) with

the 23S and 4.5S rRNAs is further evidence for the randomness of the addition of nucleotides by the GDH.

Fragment 8-11 (RNA by GDH) demonstrated sequence homology to exoglucanase mRNA. Host organisms produce glucanases, chitinases, chitosanases, and other hydrolases as part of the mechanism by which they transduce signals from fungal elicitors *(29)*, the strongest among which are chitosan derivatives *(9)*.

Fragments 16-1 and 16-3 (RNA by GDH) had structural homology to the RNAs encoding PEP carboxylase and sucrose synthase, respectively. In addition, fragment 8-4 (RNA by GDH) had structural homology to PEP carboxykinase. The three enzymes are involved in carbohydrate synthesis and storage *(30)*. GDH regulates biomass accumulation in plants *(17,31)*.

Fragment 16-4 (total RNA of the UTP-treated peanut) had sequence homology with the RNA that encodes cellulose synthase. The fragment was downregulated in the control, suggesting that its transcription was induced by oxidative stress. Fragments 8-2 and 8-7 (RNA by GDH) had sequence similarities to rRNA and plasma membrane adenosine triphosphatase (ATPase) RNA, respectively. The GDH isoenzyme pattern is regulated by cellular energy charge and membrane redox state *(9,32,33)*. Therefore, the synthesis of RNA encoding ATPase by GDH illuminates the relationship between the two enzymes. RNA fragments 8-1, 8-3, and 8-6 (total RNA) had structural homology to the mRNA of alcohol dehydrogenase.

The agreement of the structural chemistry of the RNAs synthesized by GDH with that of total RNA is confirmation of the chemistry of nucleic acids. However, although the RNAs synthesized by GDH were not as excellently translated as total RNA in vitro (unpublished result), the synthesis of RNAs by GDH in vitro does not confirm that they are translated in vivo, because translation and transcription are temporally coupled in the cell such that any mRNA not protected by ribosomes is rapidly degraded *(34)*. Nontranscriptional RNA synthesis by GDH has not been demonstrated to be coupled with translation. Therefore, this suggests that the metabolic role of the RNAs synthesized by GDH may be in part as signaling/regulatory molecules. GDH is a signaling enzyme *(35)*, but the signaling pathways have not been deciphered. The RNAs synthesized by GDH showed strong hybridization with several total RNA bands (unpublished results), thus suggesting that some might possess antisense silencing properties. It is therefore possible that many fragments of the RNAs synthesized by the enzyme interfere with gene expression. GDH isomerization interfered with total RNA contents *(23)*. The homology of many RNA fragments synthesized by the enzyme with total RNA sequences therefore supports the interference suggested. The role of RNA molecules in the regulation of gene expression is a rapidly growing field of biochemical research *(36,37)* with many potential technological applications. The nontranscriptional synthesis by GDH of RNAs that encode different signaling proteins and enzymes may be the mechanism by which GDH differentially transduces signals and communicates the messages to the diverse targets, thereby

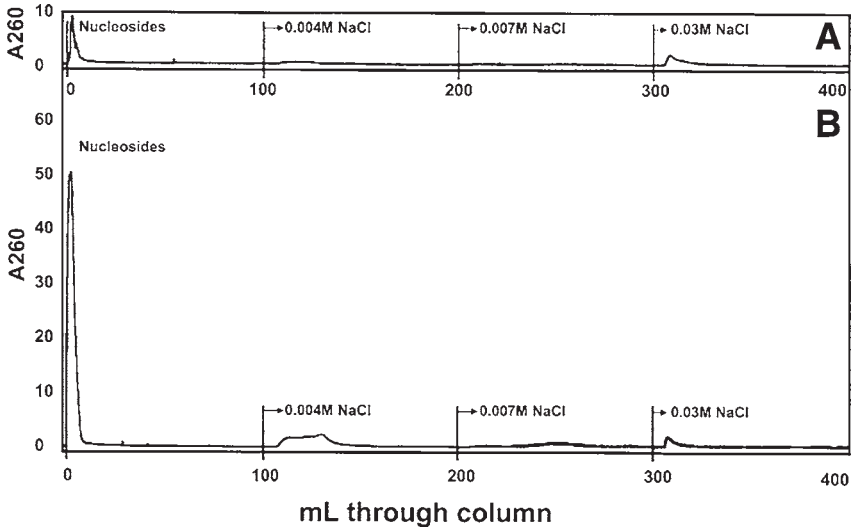

Fig. 5. Nucleosides recovered from GDH isoenzymes. Cryoelectrophoretically purified GDH isoenzymes of peanut were treated with **(A)** 70% ethanol and **(B)** proteinase K followed by 70% ethanol. The supernatants were concentrated by freeze-drying and were chromatographed on Econo-Pac Q strongly basic anion exchanger using increased concentrations of NaCl as the eluent, and the ultraviolet absorbance of eluate was measured with the automated Econo System. Eluent: 0.004 *M* NaCl elutes NADH, 0.007 *M* NaCl elutes mononucleotides, 0.03 *M* NaCl eluted di- and trinucleotides.

coordinately regulating gene expression, metabolism, and biomass accumulation, as has been reported in alfalfa cultivated on boron-amended acid soils *(38)*.

Mechanism of RNA Synthesis by GDH

Only nucleosides were recovered from GDH isoenzymes purified from RNase A-treated homogenate (Fig. 5). Since RNase A hydrolyzes single-stranded RNA to nucleoside 3'-monophosphates by cleaving after cytidine and uridine residues, only the 3'-terminal residue of the RNA is liberated as a nucleoside. Therefore, the GDH-binding site on the RNA is the 3'-growing terminus. Digestion of the GDH with proteinase K increased the recovery of nucleosides from ~0.7 to ~4.0 nmol/μg of GDH without any corresponding increases in the nucleoside mono-, di-, and triphosphates, thus confirming that the enzyme did not protect any total RNA sequences from RNase digestion, the 3'-terminus of RNA being the GDH-binding site. The concentration of nucleosides recovered suggested that one molecule of RNA was bound per hexameric charge isomer rather than per subunit polypeptide, thus confirming that the hexamers, but not the dissociated subunit polypeptides, were responsible for the RNA synthetic activity. These results showed that GDH did not use any template for the synthesis of RNA, that the growing end of the RNA was the 3'-terminus, and that the

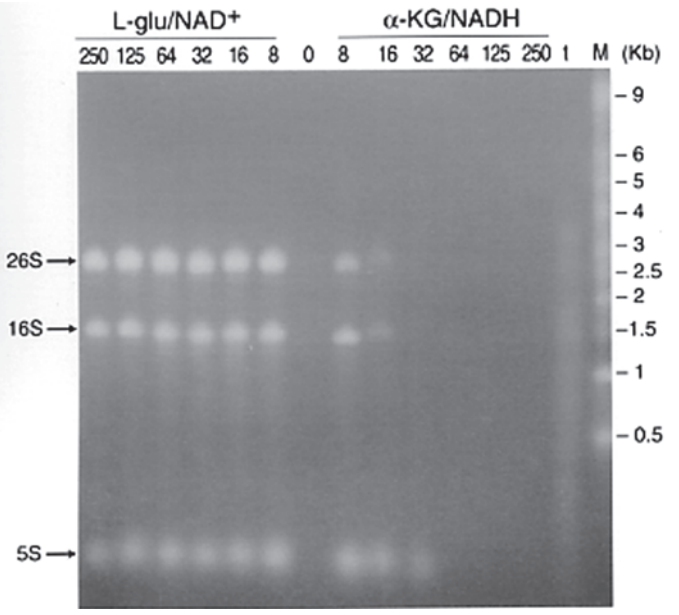

Fig. 6. Dependence of RNA synthetic activity of GDH on L-glu and α-KG. In the deamination direction, fixed concentrations of NAD^+ and $CaCl_2$ were added to varying concentrations of L-glu. In the amination direction, fixed concentrations of NADH, $CaCl_2$, and NH_4Cl were added to varying concentrations of α-KG. All reactions were at pH 8.0. Equal concentrations of the four NTPs were added followed by GDH (0.7 µg) to each reaction, incubated at 16°C overnight, and the RNA product was precipitated with ethanol. A control with GDH but without substrates and modulators was set up. The RNA products were electrophoresed through 1.5% agarose gel. RNA molecular weight markers (M) and peanut total RNA (t) were used as markers in the electrophoresis.

enzyme selected the incoming nucleotide that would be added to the growing end. The template-independent synthesis of RNA by GDH also illuminates the discussion on the origins of RNA and of life *(39)*.

Deamination/Amination Ratio

GDH isoenzymes synthesized RNA in the presence of a wider concentration range of L-glu (deamination) than of α-KG (amination) because α-KG concentrations >65 m*M* inhibited the enzyme whereas at least 250 m*M* L-glu did not cause any inhibition (Fig. 6). RNA synthesis by GDH in the deamination direction similar to that for the amination direction was conducted at pH 8.0 instead of 9.0 in order to ensure the stability of the RNA. Therefore, the determinants were limited to L-glu/NAD^+ for deamination and α-KG/NADH/NH_4^+ for amination. The results showed that the deamination and amination were quantitatively interdependent *(23)*, being integrated by the enzyme based on the concentrations of L-glu and α-KG. At a concentration of 32 m*M* L-glu, the RNA synthetic activity was about 50-fold more active than at a concentration of 32 m*M* α-KG.

This overwhelming displacement of the GDH reaction equilibrium toward deamination (Fig. 6) supported the catabolic function *(4,6,40)* proposed for the enzyme. A review of the GDH literature showed that the NADH/NAD^+ photometric assay of the enzyme supported the amination function, whereas the RNA, amino acid, α-KG, or NH_4^+ assays of the enzyme activity supported the deamination function. Therefore, the two basic approaches for monitoring the enzyme appear to be responsible for the debate about the function of the enzyme. Based on the complexity of the substrates required by the enzyme, and that NADH, NAD^+, and NH_4^+ modulators may not be required in some of the reactions *(18)*, it becomes clear why NADH/NAD^+ photometric assays may be inappropriate for routine monitoring of the enzyme activity. The unequivocal results in Fig. 6 and the simplicity by which they were derived suggest that assay of RNA synthetic activity is the robust technology for monitoring the enzyme.

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