Glutamate dehydrogenase, an enzyme central to glutamate metabolism, is deficient in patients with heterogeneous neurological disorders characterized by multiple system atrophy. There is evidence for multiplicity of human glutamate dehydrogenase, which may account for the heterogeneity of the above disorders. However, only one mRNA that is encoded by an intron-containing gene (GLUD1) is presently known. Because blindness due to neuroretinal degeneration can occur in rare forms of multiple system atrophy, we searched for retina-specific GLUD mRNA(s) by screening a ρgt10 library derived from human retina. A novel cDNA encoded by an X chromosome-linked intronless gene, designated GLUD2, was isolated and characterized. Reverse transcription-polymerase chain reaction analysis of human tissues revealed that the novel cDNA is expressed in human retina, testis, and, at lower levels, brain. In vitro translation of mRNAs derived from GLUD1 and GLUD2 genes generated proteins with distinct electrophoretic characteristics. The retinal cDNA was expressed in the baculovirus heterologous system, producing a protein capable of catalyzing the oxidative deamination of glutamate. The mobility of the expressed protein on SDS-polyacrylamide gel electrophoresis and its catalytic properties were very similar to those of the naturally occurring human brain glutamate dehydrogenases. The novel gene will be useful for understanding the biology of human neural and testicular tissues and in the study of X-linked neurodegenerative disorders.

Glutamate dehydrogenase (EC 1.4.1.3) catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate using NAD and/or NADP as cofactors. Two structurally distinct forms of the enzyme showing specificity for either NAD or NADP are known to exist in lower organisms (1). In contrast, all mammalian glutamate dehydrogenases known are capable of using both cofactors (1). Although this is considered to be a mitochondrial enzyme, there is evidence for the cellular presence of nonmitochondrial glutamate dehydrogenase activity (2, 3).

Glutamate dehydrogenase is partially deficient in patients with heterogeneous neurological disorders characterized by the degeneration of multiple neuronal systems (4). Dysregulation of glutamate metabolism occurs in such patients with multiple system atrophy and is thought to cause neuroexcitotoxic nerve cell death (5). The brains of these patients show loss of glutamate receptors (6, 7) and a selective atrophy of regions that receive glutamatergic innervation (8) and are normally rich in glutamate dehydrogenase immunoreactivity (9). In these brain regions, the enzyme is localized in astrocytic processes associated with glutamatergic terminals (9) and is thought to be involved in the detoxification of transmitter glutamate (10). Given the extensive nature of the glutamatergic pathways in brain (11), glutamate dehydrogenase may play a role in a number of human neurodegenerations.

In human tissues and in rat brain, the enzyme is shown to exist in two catalytically active forms, designated as soluble and particulate glutamate dehydrogenases, which differ in their resistance to thermal inactivation and allosteric regulation characteristics (12, 13). In some patients with multiple system atrophy, the decrease in enzyme activity is limited to the particulate form (12, 14).

Work at the protein level (15) showed that glutamate dehydrogenase purified from human brain consists of four electrophoretically distinct isoforms. These are differentially distributed in the two catalytically active isofoms of the enzyme (16). Concurrent molecular biological studies showed the presence of four different sized mRNAs and multiple gene copies for glutamate dehydrogenase in 17, 18, thus suggesting a genetic basis for the multiplicity of this protein. To date, however, only one cDNA (17, 19, 20) encoding for human glutamate dehydrogenase is known. This derives from an intron-containing gene (21) that maps to human chromosome 10 (22, 23). Since blindness due to neuroretinal degeneration can occur in some rare types of multiple system atrophy (4, 15), we searched for retina-specific GLUD mRNA(s) by screening a cDNA library derived from human retina poly(A)+ RNA. A novel cDNA encoded by an X chromosome-linked processed (retroposon) intronless gene was cloned and characterized.

MATERIALS AND METHODS

Library Screening and Characterization of cDNA and Genomic DNA Clones—cDNA libraries were constructed in ρgt10 using poly(A)+ RNAs isolated from human retina (gift of Dr. J. Nathans) and testis (CLONTECH); they were screened using the GLUD1 cDNA (17). The genomic DNA library was constructed in AEMBL4 phage (21) and was also screened using the GLUD1 cDNA. Genomic clones were mapped by single and double digestions using BamHI, BglII, HindIII, and EcoRI and subcloned into pUC19. The cDNA clones were subcloned into pBlueScript SKI+ (Stratagene) and used for sequence analysis. Double-stranded DNA sequencing was performed using the United States Biochemical Corp. Sequenase kit. For sequence verification, both strands of
the DNA were sequenced. Analysis of the sequence data was done using Genetics Computer Group software (J. Devereux, Genetics Computer Group, Inc., Madison, WI).

Identification of Human Chromosomal Region Containing GLUD2 Gene—This was accomplished by Southern blot analysis of DNA derived from CHO/human hybrid cell lines containing single human chromosomes (Coriell Institute, Camden, NJ). A 486-bp DNA fragment corresponding to the 5'-end of the GLUD1 and GLUD2 genes (bp -50 to 436; see Fig. 1B) was PCR-amplified. The PCR mixture (50 μl) contained 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl, pH 8.8, 6.7 mM MgCl₂, 10 μM each dNTP, 100 μM primers, 500 μM dNTPs, 50 μg of hybrid line DNA, and 200 ng of each primer (5'-GCGGAGTGCGCCGCA-3' and 5'-GAATTCGAGAACGTTTATCGGCT-3'; and GLUD2-specific probe, 5'-GGCCGAGTCGGCGGACGCCGAGCCACCCAG-3' (GLUD2 residues that differ from those of GLUD1 are underlined). The translational initiation site is indicated by +1. The two decanucleotide direct repeats are indicated at each end. The first polyadenylation signal (last) used by the GLUD1 cDNA (17) is shown by a dashed underline. The unique SalI site nucleotide sequence is shown in boldface. The abbreviations used are: CHO, Chinese hamster ovary; bp, base pairs; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TEMED, N,N,N',N'-tetramethylethylenediamine; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

Fig. 1. Structure of novel GLUD2 gene. A, genomic organization of the GLUD2 gene locus. Three genomic clones (AL9, AL10, and AL101) spanning the GLUD2 gene are depicted schematically at the top. Below is a map of the genomic GLUD2 locus containing the sites of four restriction endonucleases. An expanded map of a 5.2-kilobase (kb) segment containing the coding area and the flanking 5'- and 3'-untranslated regions (UTR) of the GLUD2 gene is shown at the bottom. The solid bar represents the coding region, and the dotted areas represent the nontranslated sequences. The nucleotides and deduced amino acids corresponding to the GLUD1 gene are depicted schematically at the top. Below is a map of the genomic GLUD2 locus containing the sites of four restriction endonucleases. An expanded map of a 5.2-kilobase (kb) segment containing the coding area and the flanking 5'- and 3'-untranslated regions (UTR) of the GLUD2 gene is shown at the bottom. The solid bar represents the coding region, and the dotted areas represent the nontranslated sequences.

The GLUD2 gene contains a 14-kilobase (kb) segment that spans the GLUD2 gene and is depicted schematically at the top. Below is a map of the genomic GLUD2 locus containing the sites of four restriction endonucleases. An expanded map of a 5.2-kilobase (kb) segment containing the coding area and the flanking 5'- and 3'-untranslated regions (UTR) of the GLUD2 gene is shown at the bottom. The solid bar represents the coding region, and the dotted areas represent the nontranslated sequences. The nucleotides and deduced amino acids corresponding to the GLUD1 gene are depicted schematically at the top. Below is a map of the genomic GLUD2 locus containing the sites of four restriction endonucleases. An expanded map of a 5.2-kilobase (kb) segment containing the coding area and the flanking 5'- and 3'-untranslated regions (UTR) of the GLUD2 gene is shown at the bottom. The solid bar represents the coding region, and the dotted areas represent the nontranslated sequences.
ATGATCCATC-3') containing EcoRI restriction sites. The PCR products were ligated to the baculovirus transfer vector (pVL1392, Invitrogen) and were used to transform *Escherichia coli* (INVaP5R). The proper orientation of the insert was verified by DNA sequencing. Cells of the insect Spodoptera frugiperda (Sf9) were cotransfected with the plasmid DNA and modified baculovirus DNA (Baculo Gold, Pharmingen) and were maintained at 27 °C for 4 days. The recombinant virus was amplified with two additional rounds of infection.

**Glutamate Dehydrogenase Assay**—This was carried out in 50 mM triethanolamine buffer, pH 8.0, containing 100 mM ammonium acetate, 2.6 mM EDTA, 150 μM NADPH or 1.4 mM ADP, and 8 mM α-ketoglutarate or 25 mM glutamate (15). For determining GLUD2 activity, the cells were homogenized by glass-to-glass shear homogenization at about optimal levels (15). α-Ketoglutarate was varied between 0.6 and 8 mM, glutamate between 2.5 and 50 mM, and NADPH between 15 and 200 μM. For comparison, kinetic studies were done using readily solubilized and particulate-bound dehydrogenase purified from human brain.

**Purification of Human Brain Glutamate Dehydrogenase**—Brain tissue obtained at autopsy from patients without overt neurological anomalies was used for the purification of the readily solubilized and particulate-bound isoforms of human brain glutamate dehydrogenase. About 5–10 g of frozen tissue were homogenized by glass-to-glass shearing in 10 mM Tris-HCl, pH 7.4, containing 0.1 mM phenylmethanesulfonyl fluoride. A low speed supernatant, obtained by centrifugation at 480 x g for 10 min (4 °C), was further fractionated at 100,000 x g for 60 min (4 °C) into a high speed supernatant and a high speed pellet (13). The high speed supernatant was used for the purification of the soluble glutamate dehydrogenase, whereas the high speed pellet was further extracted (in 10 mM Tris-HCl, pH 7.4, containing 0.5 mM NaCl, 1% Triton X-100, and 0.1 mM phenylmethanesulfonyl fluoride) (15) and used for the purification of the particulate-bound enzyme. The purified enzymes were used for the kinetic comparisons described above. Also, glutamate dehydrogenase was purified from whole homogenates of human brain as previously described (15) and was used for raising polyclonal antibodies (see below). Purification of all three enzyme preparations was carried out as previously reported (15). Briefly, this involves ammonium sulfate fractionation (30–45% cut), hydrophobic interaction chromatography on a phenyl-Sepharose column, and affinity chromatography on a GTP-Sepharose column.

**SDS-PAGE**—This was performed at constant current (50 mA) with water cooling on vertical slab gels. The separating gel consisted of a 5–15% acrylamide linear gradient in 0.375 mM Tris-HCl, pH 8.8, containing 0.1% SDS, 0.05% ammonium persulfate, and 0.1% TEMED. The stacking gel comprised 4.5% acrylamide in 75 mM Tris-HCl, pH 6.8, containing 0.1% SDS, 0.05% ammonium persulfate, and 0.1% TEMED. 

**Immunoblotting**—SF9 cell extracts were analyzed using polyclonal antiserum against human brain glutamate dehydrogenase (100-fold diluted with the blocking buffer) and incubated for 1 h. The blot was washed several times with the diluent; the last wash did not contain Tween 80. The blot was incubated by washing thoroughly with the diluent, peroxidase-conjugated anti-rabbit immunoglobulins raised in swine (Dako Corp.) were added (200-fold diluted with the diluent) and incubated for 1 h. Then the blot was washed several times with the diluent; the last wash did not contain Tween 80. The blot was developed by incubation at room temperature in a mixture (20 ml) made of 5 parts 50 mM Tris-HCl, pH 7.6, 1.5 mM NaCl, and 0.1% Tween 80. Nonspecific binding was blocked with 2% bovine serum albumin in the diluent for 30 min. The blot was incubated for 1 h with polyclonal antisera against human brain glutamate dehydrogenase raised in rabbits (100-fold diluted with the blocking buffer). After washing thoroughly with the diluent, peroxidase-conjugated anti-rabbit immunoglobulins raised in swine (Dako Corp.) were added (200-fold diluted with the diluent) and incubated for 1 h. Then the blot was washed several times with the diluent; the last wash did not contain Tween 80. The blot was developed by incubation at room temperature in a mixture (20 ml) made of 50 mM Tris-HCl, pH 7.6, containing 0.5 mM NaCl and 1 part 4-chloro-1-naphthol solution (3 mg/ml freshy dissolved in ice-cold methanol). H2O2 (12 μl of 30% (v/v) solution) was added to the incubation mixture just before use. After the bands were visualized (10–15 min), the blot was washed with H2O and stored dry. For antibody production, glutamate dehydrogenase purified from whole homogenates of SF9 cells described above was subjected to SDS-PAGE. The protein band was cut from the gel and electroeluted into a dialysis tube. The purified protein was concentrated and used for raising polyclonal antibodies in rabbits employing standard procedures (2, 26). The specificity of the immune serum was confirmed by Western blotting of human brain homogenates (data not shown).

**RESULTS**

**Screening of cDNA and Genomic DNA Libraries**—Screening of the retinal cDNA library led to the isolation of five clones specifying a novel cDNA, designated GLUD2. Using oligonucleotides specific for the new cDNA, we also screened a human testis agt10 cDNA library and isolated three additional clones specifying the same cDNA. Investigations using a human genomic library were carried out concurrently and led to the isolation of several genomic clones specific for glutamate dehydrogenase (21). Sequencing of these clones showed that the GLUD2 cDNA is encoded by a gene, the coding region of which is not interrupted by introns (Fig. 1). This gene was accordingly designated "intronsless GLUD," although the possibility of the presence of introns in its 5'-untranslated region cannot be excluded. The deduced amino acid sequence of the GLUD2 gene is 96% homologous to that of the GLUD1 gene (Fig. 1B). The GLUD2 gene has a 3'-AT-rich region and features direct repeat sequences that flank the coding sequence (Fig. 1).

**Linkage of GLUD2 Gene to Human Chromosome X**—PCR amplification of DNA derived from CHO/human hybrid lines gave positive products for cell lines that contained human chromosome 10 or X (Fig. 2). Both cell lines produced the same amplification product (~500 bp) as the cloned GLUD1 and GLUD2 cDNAs (Fig. 2). Southern blot analysis of the amplified products, using oligonucleotide probes capable of discriminating between GLUD1 and GLUD2 genes, revealed that the GLUD2 gene was linked to the human X chromosome, whereas the GLUD1 gene is linked to human chromosome 10 (Fig. 2).

**Expression of GLUD2 mRNA in Human Tissues**—The novel cDNA contains a restriction site for SalI in its coding region.
that is not present in GLUD1 cDNA (Fig. 1B). This made it possible to detect the expression of GLUD2 mRNA in human tissues using reverse transcription-PCR and restriction analysis. SalI digestion of amplified DNA, derived from various human tissues, showed that a novel mRNA is expressed in human retina and testis (Fig. 3A and B), while lower levels of expression are also detected in human brain (data not shown). Liver (Fig. 3, A and B) and the other tissues tested gave nondetectable expression products for GLUD2.

**Production of Glutamate Dehydrogenase Proteins—In vitro translation of mRNA corresponding to the GLUD2 gene generated a protein with a slightly lower electrophoretic mobility than that derived from the GLUD1 gene (Fig. 4A). In addition, expression of the GLUD2 cDNA in Sf9 cells, using the baculovirus expression system, produced a protein capable of catalyzing the reversible interconversion of glutamate to \( \alpha \)-ketoglutarate in the presence of either NAD(H) or NADP(H) (Table I). Since the host insect cells (Sf9) contained glutamate dehydrogenase activity exhibiting an absolute specificity for NAD(H), enzymatic assays were performed in the presence of NADP(H), thus eliminating all background activity. Kinetic analyses showed that the \( K_v \) values for \( \alpha \)-ketoglutarate and glutamate of the expressed protein were comparable to those of particulate and readily solubilized human brain glutamate dehydrogenases (Table I). Moreover, the glutamate dehydrogenase immunoreactivity seen on Western blots at various post-infection days correlated with the amounts of NADP(H)-dependent enzyme activity (Fig. 4B). On SDS-PAGE, the expressed protein migrated to just above the bovine liver glutamate dehydrogenase (used as a marker), as does the mature human brain enzyme (Fig. 4C).

**DISCUSSION**

Previous investigations using cDNA libraries derived from human liver, brainstem, and fibroblasts led to the isolation of 13 glutamate dehydrogenase-specific clones (17, 19, 20). All of these were shown to be identical by sequencing and restriction analysis. More recent studies on genomic clones (21) revealed that the cDNA specified by these clones is encoded by a 45-kilobase-long gene designated GLUD1, the coding region of which is interrupted by 12 introns. This gene maps to human chromosome 10 (22, 23). Four additional genes, designated GLUD2, GLUDP3, GLUDP4, and GLUDP5, are present in the human genome, but may represent truncated pseudogenes (21).

Here, in an effort to search for distinct mRNA species encoding human glutamate dehydrogenase, we screened cDNA libraries derived from human retina and testis. A novel cDNA, designated GLUD2, was isolated showing a 96% amino acid homology to the GLUD1 cDNA. Expression of GLUD2 in the baculovirus heterologous expression system produced a protein, the catalytic properties of which were very similar to those of human brain soluble and particulate glutamate dehydrogenases. The size of the expressed protein was also comparable to that of mature human brain enzymes. This indicates that the recombinant protein is processed within the host cells in a manner similar to that normally occurring in mammalian tissues and involves removal of the leader peptide (27).
to the GLUD1-derived polypeptide, the GLUD2-generated protein is more basic and shows a slightly lower electrophoretic mobility. These differences are consistent with the electrophoretic characteristics of glutamate dehydrogenase isoproteins purified from human brain (15).

The novel glutamate dehydrogenase is encoded by an X chromosome-linked gene, the coding region of which is not interrupted by introns (intronless). This gene contains direct de-canucleotide repeats that flank the region that is homologous to the GLUD1 gene. Such direct repeats are hallmarks of cDNA copies of processed transcripts acquired during insertion into the genome (28). Therefore, their presence indicates that this intronless gene might have evolved through retroposition of a spliced GLUD1 mRNA (retroposon) (28, 29).

Recently, several functional intronless genes generated by reverse transcription and insertion into the human genome (retroposons) have been identified (29-31). Some of these are expressed in neural tissues, encoding for G protein-linked receptors (29), or in testis, encoding for the metabolic enzymes pyruvate dehydrogenase and phosphoglycerate kinase (30, 31).

In contrast, the GLUD2 intronless gene is expressed in both neural and testicular tissues. The localization of the GLUD2 gene to human chromosome X and of the GLUD1 gene to human chromosome 10 represents a reversal of the chromosomal loci of pyruvate dehydrogenase and phosphoglycerate kinase; the intronless genes of pyruvate dehydrogenase and phosphoglycerate kinase map to human autosomal chromosomes, while their intron-containing genes map to the X chromosome (30, 31). Retroposition of the latter two genes to autosomal chromosomes might have occurred in order to serve the metabolic needs of sperm cells (31), half of which contain one X chromosome.

It is of interest that brain and testis show similarities with respect to their energy metabolism (31) and that glutamate, the substrate of glutamate dehydrogenase, is involved in both intermediary metabolism and neurotransmission processes. Hence, retroposition of the GLUD2 gene may be a mechanism that resulted in the multiplicity of glutamate dehydrogenase proteins expressed in human neural tissues. As such, it might have been selected out of necessity owing to the complex function of glutamate in these tissues.

Expression of the GLUD2 gene in human retina is of particular interest in view of data showing that glutamate is an important retinal excitatory transmitter (32). In addition, electroretinographic abnormalities have been detected in patients with spinocerebellar ataxia who have a selective deficiency of the heat-labile glutamate dehydrogenase (14). Cloning of the novel GLUD gene may thus be of importance for understanding the biology of the human nervous and male reproductive systems and the genetic analysis of X-linked neurodegenerations.

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REFERENCES