Pattern of expression of glutamic acid decarboxylase mRNA in the developing rat brain

(gene expression/neuron development)

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ABSTRACT The time and pattern of appearance of glutamic acid decarboxylase (glutamate decarboxylase; EC 4.1.1.15) (GAD) mRNA during the development of the rat brain were analyzed. RNA transfer blot analysis of poly(A)+ RNA from whole brain shows that a 3.7-kilobase transcript is the most abundant form of the message from embryonic day 15 (E15) through adulthood. By E15 this form is present at about 50% of its adult abundance relative to other poly(A)+ mRNA species. At birth the abundance is approximately the same as in the adult. In contrast, the enzyme activity level is only 8% of the adult level at birth and increases 3 weeks to reach adult levels. There are qualitative changes in GAD mRNA during development. Several large (7-9 kilobases) transcripts with strong homology to GAD are enriched in early developmental stages but are barely detectable in the adult. A nuclease protection assay shows a developmentally regulated heterogeneity in a coding portion of the mRNA.

Glutamic acid decarboxylase (glutamate decarboxylase; EC 4.1.1.15) (GAD) serves as a favorable model for the study of gene expression in the vertebrate brain. GAD catalyzes the formation of the neurotransmitter γ-aminobutyric acid (GABA) from L-glutamic acid and thus plays a crucial role in a functionally and numerically important class of neurons, GABAergic inhibitory neurons. The expression of GAD is highly tissue and cell specific (1-3). In the adult, with a few minor exceptions, GAD is found only in neurons. Inhibitory neurons high concentrations of the enzyme, whereas neighboring excitatory neurons appear to lack it entirely (4). In situ localization studies (5) show that GAD mRNA is enriched in the same set of neurons that contain the enzyme. This suggests that transcriptional activation and/or message stabilization play important roles in regulating GAD levels. Thus, study of the regulation of GAD gene expression should shed light on the mechanisms by which central nervous system neurons become different from one another.

The availability of cloned cDNAs corresponding to GAD mRNA now allows levels of the mRNA present in the developing brain to be analyzed directly. By using a cDNA to a feline GAD (3), we have selected cDNA clones for the rat mRNA. These have been used to characterize the mRNA for GAD from the whole brain and the striatum of a developmental series of fetal and postnatal rats. The results show that GAD mRNA is present in the brain as early as embryonic day 15 (E15). By the first postnatal day (P1), levels of GAD mRNA in the whole brain are almost at their adult levels relative to other poly(A)+ RNAs. This contrasts strongly with the level of enzyme activity that, at birth, is only 8% of its adult specific activity. There are also qualitative developmental changes in the sequences hybridizing to the GAD probes that may play an important role in the development of GABAergic neurons.

METHODS

Library Screening. A cDNA library from adult rat hypothalamus in λgt11 was provided by R. Goodman (Tufts University, Medford, MA). This library was screened by using the feline GAD cDNA as a probe. The feline GAD cDNA was labeled with 32P by nick-translation. Nitrocellulose filters with lifts of λgt11 were incubated at 65°C in hybridization solution [5 × Denhardt's solution (0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone), 750 mM NaCl/1.25 M NaHPO4/5 mM EDTA, pH 7.4, 0.5% NaDodSO4, 0.5 mg of salmon sperm DNA per ml, and 20% formamide] for 2 hr without probe and then for 15 hr after the addition of probe. Positive plaques were visualized by autoradiography and purified by replaquing.

RNA Preparation. RNA was purified by the method of Chirgwin et al. (6). In brief, tissues were homogenized in guanidine hydrochloride, and the RNA was isolated by two rounds of ethanol precipitation. Poly(A)+ RNA was selected by the method of Aviv and Leder (7).

RNA Transfer Blots. Samples of poly(A)+ RNA were loaded onto formaldehyde/agarose gels and electrophoresed to separate according to size. Equal amounts (as determined by O.D.260) of RNA were loaded onto each lane. Control experiments in which [3H]mRNA was included in the samples loaded onto the oligo(dt) columns showed that recovery of poly(A)+ RNA did not vary in samples from different aged tissues.

The RNA was blotted onto nitrocellulose with 1.5 M NaCl/0.15 M sodium citrate for 15 hr. After baking, the nitrocellulose was hybridized with hybridization solution (see above) for 2 hr at 65°C. 32P-labeled antisense RNA from pRGB4 (about 0.5 μg of 1 × 107 cpm/μg) was then added to the hybridization solution and incubation was continued at 65°C for 15 hr. The unbound probe was removed by washing three times with 0.15 M NaCl/15 mM sodium citrate for 10 min at room temperature; this was followed by two washes with 15 mM NaCl/1.5 mM sodium citrate for 1 hr at 58°C. Positive bands were visualized by autoradiography.

Solution Hybridization. An excess of 32P-labeled antisense RNA (≈2 × 105 cpm per sample) made from pRGB5 was hybridized to 25 μg of whole brain RNA from the indicated tissue in 80% formamide for 15 hr at 45°C. Unbound probe was degraded by the addition of RNases A and T1. After proteinase K treatment and phenol/chloroform extraction, the samples were precipitated with ethanol and run on a 6% polyacrylamide sequencing gel. The protected probe was visualized by autoradiography.

Abbreviations: GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; E, embryonic day; P, postnatal day.

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RESULTS

Selection and Characterization of Rat GAD cDNAs. The feline GAD cDNA characterized by Kaufman et al. (3) was used to select homologous cDNAs from the rat. A cDNA library from rat hypothalamus in Agt11 was obtained from R. Goodman (Tufts University) and screened for clones cross-hybridizing with the feline GAD probe. Four positive clones were selected and shown to be stable upon recloning. One was selected for further study and was used to construct the plasmid pRGB2 shown in Fig. 1. The insert size is 2.1 kilobases (kb). It was judged to be homologous to feline GAD by the following criteria. (i) On RNA transfer blot analysis of rat brain RNA, it gave a pattern indistinguishable from the feline GAD. (ii) A 260-base-pair (bp) segment was subcloned to give plasmid pRGB5 (Fig. 1). The cDNA in this fragment was sequenced. The sequenced segment was highly homologous to the feline GAD from nucleotides 704 to 964 of the map of Kobayashi et al. (8); 235 of 260 bases were exact matches. Eighty-four of 86 deduced amino acids were exact matches. (iii) In situ hybridization localization in the cerebellum showed a pattern strikingly similar to the results for the feline probe reported by Wuschell et al. (5) (G. Szebenyi and D.I.G., unpublished observations). The cDNA in pRGB2 was subcloned directionally to give pRGB4 (Fig. 1). This plasmid was used to generate antisense probe for RNA transfer blot analysis and in situ hybridization. The plasmid pRGB5 was also constructed by subcloning pRGB2.

RNA Transfer Analysis of GAD mRNA During Rat Brain Development. Total RNA was prepared from a series of developmental stages of the rat brain. Poly(A)$^+$ RNA was then selected by chromatography on oligo(dT)-cellulose. Control experiments were done in which trace amounts of $[^3]H$mRNA from myeloma cells were included with the brain RNA. Recoveries of this tracer did not vary with mRNA from different aged tissues. Equal amounts of poly(A)$^+$ RNA were loaded on each lane of the gel in the experiment illustrated in Fig. 2. The results show that at each age tested the major form of RNA that hybridized with the rat GAD probe is a band of 3.7 kb. In other experiments (data not shown), samples of RNA from rat liver, gut, and muscle were analyzed. These did not show any specific hybridization. These results are in accord with the findings of Kaufman et al. (3) as to the size and the tissue distribution of mRNA recognized by the feline GAD probe. The results in Fig. 2 show that there are substantial quantities of GAD mRNA at E15 and that the amounts present by P1 are at about the same level as in samples from the adult brain. Densitometric measurements show that the bands at E15 and P1 have 55% and 94% of the density of the adult samples, respectively. At early developmental stages there are several additional bands above the major 3.7-kb band. These bands disappear later in development.

Nuclease Protection Assay of RNA from Whole Brain and Striatum. To extend the results with RNA transfer blot analysis to smaller samples and avoid the necessity of poly(A)$^+$ selection, a protection assay was developed using a segment of the GAD cDNA 260 bp in length. This segment is the one found in pRGB5 and shown by direct sequence analysis to be highly homologous to a segment in the feline GAD probe (see above). In samples from the adult brain (Fig. 3, lane 2) a 230-bp segment of the probe is protected. Lane 1 shows a control using liver RNA. In this control there are no protected pieces as expected from the known distribution of the mRNA for GAD. Experiments were also done with RNA from E15, gut, limb, and liver. There were no protected bands in any of these samples (data not shown). Fig. 3 also makes two additional points. (i) It shows that there is a low but detectable level of GAD mRNA at E15 and that the levels rise rapidly so that by P1 there is an amount

![Fig. 1](image-url) Recombinant plasmids containing GAD cDNA. All plasmids are derived from the BlueScribe plasmid. GAD inserts are indicated by the heavy lines. The restriction maps made for each plasmid are indicated. Numbers are map positions in kilobases. The positions of the T3 and T7 polymerase promoters are indicated.
similar to that in the adult. In this assay there seems to be a slight increase between P1 and the adult. (ii) There is a smaller protected piece at early stages (lanes 3–5) with a size of 200 bp.

The striatum was chosen for analysis because a high proportion of its neurons are GABAergic (9). Fig. 4 shows that there is a substantial amount of message by E15 and that the amounts rise quickly so that by P1 the levels are equal to that of the P40 animal. The lower molecular weight protected piece is very prominent at early stages but disappears by P40.

**DISCUSSION**

In the adult brain, GAD is restricted to a diverse and numerically important subset of neurons, the GABAergic inhibitory neurons. In this report we have determined some of the characteristics of the GAD mRNA in a developmental series by using isolated cDNA probes from the rat. Our results are relevant to several issues involving gene expression in developing tissues.

The first is the time of first appearance of GAD mRNA. The E15 brain already contains substantial quantities of GAD mRNA. Given the relatively high levels found at this age, the first appearance of the message must have preceded E15 by some time. Unfortunately, direct measurements on younger ages by RNA transfer blot analysis are not practicable. By E15, the brain consists of a complex mixture of dividing precursor cells and young neurons and glia that have withdrawn from the cell cycle. A major question, left unresolved in this study, is the cellular localization of the GAD mRNA found at early stages of development. One possibility is that it only occurs in young neurons destined to become GABAergic. Alternatively, some of the GAD mRNA might also be found in dividing precursor cells or even in postmitotic neurons destined to be nonGABAergic. Perhaps in situ hybridization studies of early brain cell populations will allow identification of the cell type(s) that first transcribe the GAD gene.

In this study the relative abundance of GAD mRNA was first determined by analyzing equal amounts of poly(A)+ RNA from brains of different stages of development. This measures GAD mRNA as a percentage of the total poly(A)+ RNA. By this measure GAD mRNA has achieved ≈50% of its adult level by E15 and almost its full adult level by P1. In contrast, enzymatic activity at E15 is only 3% of the adult specific activity; at P1 the corresponding figure is 8% (10).

Several explanations can be advanced for the precocious appearance of GAD mRNA relative to enzyme activity. The most simple is that the rate of accumulation of protein is slow and that it takes several weeks to accumulate adult levels of the enzyme. It is also possible that the efficiency of translation of GAD mRNA increases with developmental time. Alternatively, the rate of degradation of GAD might be high early in development and decline with age. Additional studies in more simplified preparations such as cultured neurons are needed to decide among these different alternatives.

Finally, our results show that there are qualitative changes in GAD mRNA during development. In RNA transfer blots the major form of the mRNA throughout development is a 3.7-kb species. This is in agreement with published values for the GAD mRNA in adult rat brain (3, 11). However, at early developmental stages there are several much larger bands that are always lower in intensity than the 3.7-kb band. At E15 the most prominent is about 9 kb; at P1 a band of about 7 kb dominates. These bands are barely visible in RNA samples from the adult. Three interpretations of the larger bands seem plausible. They could be fully processed transcripts of a gene with regions of homology to the GAD gene. They might also be fully processed transcripts of the same gene that gives rise to the major 3.7-kb transcript, but including additional exons sequences of that gene. Finally, they might represent precursor transcripts from which all introns have not yet been removed.

Nuclease protection assays also reveal a developmentally regulated heterogeneity in GAD mRNA. Early in development two fragments of a 260-bp probe are protected. Later in development the smaller piece is no longer protected. The most likely interpretation of these results is that this region is transcribed from two exons separated by an intron and that the splicing pattern is changed during development. Alternatively, the protected pieces might come from transcripts of two related genes. If so, both of these genes are brain specific since protected fragments are not seen with embryonic gut, limb, or liver RNA. The transition in mRNA
pattern occurs at a time when GABAergic neurons are becoming connected with their targets and with afferent neurons. A reasonable hypothesis is that the formation of connections modulates the expression of the GAD gene.

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