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Distinct Protein Forms Are Produced from Alternatively Spliced Bicistronic Glutamic Acid Decarboxylase mRNAs during Development

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It has been shown that the enzyme glutamic acid decarboxylase (GAD; EC 4.1.1.15), which catalyzes the conversion of L-glutamate to γ-aminobutyric acid in the central nervous system of vertebrates, can be first detected in rodents at late embryonic stages. In contrast, we have found that the gene coding for the 67-kDa form of GAD is already transcriptionally active at embryonic day E10.5 in the mouse. In addition to the 3.5-kb adult-type mRNA, we have detected two 2-kb embryonic messages that contain alternatively spliced exons of 80 (I-80) and 86 (I-86) bp, respectively. The overlapping stop-start codon TGATG, found in the embryonic exons, converts the monocistronic adult-type transcript into a bicistronic one, coding for a 25-kDa leader peptide and a 44-kDa enzymatically active truncated GAD. A second stop codon at the 3′ end of the 86-bp exon abolishes the expression of truncated GAD. The products of the two embryonic mRNAs were identified in a rabbit reticulocyte in vitro translation system, COS cells, and mouse embryos. The two GAD embryonic forms represent distinct functional domains and display characteristic developmental patterns, consistent with a possible role in the formation of the γ-aminobutyric acid-ergic inhibitory synapses.

Eukaryotic cells use a variety of posttranscriptional mechanisms to expand the coding capacity of their genomes and to provide additional levels for regulation of gene expression. By the constitutive, developmentally regulated, and tissue-specific alternative splicing of precursor mRNAs (reviewed in references 9, 37, and 38), multiple mRNA species can be produced from a single primary transcript, as for instance in the case of Ubx complex of *Drosophila melanogaster* (28), neural cell adhesion molecules (49), or the muscle-specific troponin T mRNA which can be spliced in 128 different ways (10, 39).

Translation of bicistronic mRNAs could additionally contribute to the heterogeneity of protein forms synthesized from the same mRNA. The majority of eukaryotic mRNAs, which are structurally and functionally monocistronic, are thought to be translated according to the scanning model (29), which states that the 40S ribosomal subunits bind initially to the 5′ end of the mRNA and then migrate linearly in the 3′ direction and initiate translation at the first AUG codon of the open reading frame (ORF). The sequence around functional AUGs is nonrandom; thus, they could be classified as strong or weak depending on how well the flanking nucleotides fit the consensus for initiation 5′-CCPuCCAUGG-3′ (31). However, some eukaryotic, mainly viral, mRNAs are functionally bicistronic because of the mechanisms of leaky scanning or reinitiation of translation (reviewed in references 32, 33, and 35). Briefly, if the consensus sequence for translation initiation around the 5′ proximal AUG is weak, or if a strong AUG is followed by an in-frame stop codon, the ribosomes can migrate further on the mRNA and initiate translation at a downstream AUG, provided that the latter is in a context favorable for initiation. An upstream AUG, if used, has an inhibitory effect on the downstream initiation. Initiation at a downstream in-frame AUG by leaky scanning results in the synthesis of truncated protein forms in addition to the long form, in most cases with distinct biological functions (16, 34, 45, 47). The scanning ribosomes can also initiate translation at an out-of-frame start codon, in which case functionally distinct proteins are synthesized from different overlapping ORFs of the same message, a strategy widely used by animal and plant viruses (32, 58). The termination-reinitiation mechanism is usually applied in the translation of variety of cellular eukaryotic messages, in which the major ORF is preceded by one or more short upstream ORFs. The products of these minicistrons are nonfunctional peptides; thus, their translation is considered to have solely a regulatory role with regard to the translation of the downstream cistron (1, 21, 33, 50).

The translational coupling at an overlapping stop-start codon often found at the intercistronic boundaries of bacterial polycistronic mRNAs is another mechanism by which structurally distinct polypeptides can be produced coordinately from adjacent ORFs (42, 57, 63). In bacteria, the extent of coupling of two ORFs depends on the distance between their corresponding stop and start signals and the strength of the intercistronic Shine-Dalgarno sequence (15, 51). In addition to bacteria, this type of strategy has been shown to be utilized by some eukaryotic viruses (12, 20, 22, 24) and artificially constructed operons (43).

In this paper, we provide evidence that the eukaryotic cellular mRNA coding for the 67-kDa form of the neurotransmitter synthesizing enzyme glutamic acid decarboxylase (GAD; reviewed in reference 18) is rendered structurally and functionally bicistronic through the developmentally regulated alternative splicing of a single exon. The embryonic exon-derived TGATG overlapping stop-start codon splits the ORF of GAD67 into two tandemly arranged ORFs, coding for a 25-kDa leader peptide and a 44-kDa enzymatically active truncated GAD, respectively. A second stop codon interrupting the reading frame of truncated GAD is found on a

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developmentally earlier transcript, in which a 6-bp-longer embryonic exon is spliced.

MATERIALS AND METHODS

Isolation of DNA, library screening, and sequencing. Plasmid DNA was purified essentially as described previously (48). For transfection in COS cells, plasmid preparations were banded twice in CsCl gradient. Genomic clones were isolated from a Charon 4A mouse genomic library (kindly provided by Shirley Tilghman, Princeton University) by screening with [^{32}P]dCTP-labelled mouse GAD cDNA at high stringency. DNA fragments were subcloned into M13 and sequenced by the dideoxy chain-termination method (U.S. Biochemical Sequenase kit).

RNA purification and Northern (RNA) blot analysis. For matings, C57BL/6 females and CBA males were used. The day of the plug was considered day 0.5 postcoitum. Total RNA from mouse embryo heads (embryonic days E10.5, E11.5, and E12.5) or embryonic (E13.5, E15.5, and E18.5), newborn (P0), and adult (A) brains was extracted by the guanidine isothiocyanate method (13). For Northern blot analysis, 40 μg of total RNA samples was run on a 1.2% formaldehyde agarose gel and transferred to nitrocellulose. Filters were hybridized either with [^{32}P]dCTP-labelled mouse GAD67 cDNA or with 3′ [^{32}P]dATP-tailed 42-nucleotide (nt) embryonic exon-specific oligonucleotide 5′-CAACCAACACTCCCTCATGTCTGATGGC ATATTGGTATTGGC as probe.

First-strand cDNA synthesis-PCR amplification. A total of 10 μg of total RNA from adult brain and embryo heads or brains was reverse transcribed with random primer with Moloney murine leukemia virus reverse transcriptase or SuperScript Preamplification System (Bethesda Research Laboratories). An aliquot of the first-strand cDNA (1/10) was PCR amplified with the following primers: +E2, 5′-CCGGAATTCCG/CACA CCAGTTGCTGGAGG (from position 428; the *Eco*RI site within the extension is underlined); −E3, 5′-CAGGTCGACC TG/ACAAACACGGGTGCAATT (from position 665; the *Sal*I site is underlined); −E4, 5′-TTGGGCACAGCCGCCAT GCC (from position 839); +P1, 5′-GACGCCCCCTGGGAA CTTG (from position −46); and −P4, 5′-GGGCACAAGCAT GGCCGATG (from position 1824). The positions on the cDNAs are calculated relative to their distance from the A of the first ATG codon of GAD67 cDNA (27). +E2 and −E3 primer pairs were used to amplify the embryonic insertion. The full coding regions of the embryonic mRNAs were amplified in two overlapping pieces with two primer pairs, +P1/−E4 and +E2/−P4, respectively, as follows: 1 cycle of 95°C for 5 min, 60°C for 2 min (for all primer pairs), and 72°C for 5 min; and 35 cycles of 94°C for 1.2 min, 60°C for 1.5 min, and 2 min at 72°C for primers +E2/−E3 or 4 min for the other primers.

Cloning procedures and plasmid constructs. Fragments generated with primers +E2 and −E3 were digested with *Eco*RI and *Sal*I restriction enzymes, respectively, and cloned into Bluescript (Stratagene). PCR products generated with the other two primer sets were phosphorylated with polynucleotide kinase, and the ends were repaired with Klenow DNA polymerase. The +P1/−E4 amplified fragment containing the 5′ portion of the embryonic GAD cDNA and the +E2/−P4 amplified one, corresponding to the 3′ end, were inserted into the *Hin*dII and *Eco*RV sites of Bluescript. Clones containing the embryonic insertion were identified by colony hybridization and PCR. The two portions of the embryonic GAD cDNAs were spliced together at the *Eco*RV site as follows: the *Eco*RV-*Hin*dIII fragment of the 5′ clone was inserted into the *Eco*RV-*Hin*dIII sites of Bluescript carrying the 3′ portion of the embryonic GAD cDNA. The final products were sequenced.

For bacterial expression, the *Bam*HI fragment from the adult and embryonic I-80 cDNAs, containing the entire coding region with the exception of the N-terminal 15 amino acids (aa), was cloned into the *Bam*HI site of plasmid pAR3039 (54), resulting in a fusion protein between the N-terminal 13 A of gene *10* protein and GAD, under the control of the T7 polymerase promoter. GAD proteins were expressed in DE3 (Promega), carrying the inducible T7 polymerase gene. Growth of bacteria and Western blot (immunoblot) analysis of the expressed proteins were performed as described previously (27).

For transfections in COS cells, the *Hin*dIII-*Asp* 718 fragment of the adult GAD67 cDNA was filled in with Klenow DNA polymerase and inserted into the blunt-ended *Hin*dIII-*Bam*HI sites of the eukaryotic expression vector pBC12B (14). The embryonic versions were generated by switching the *Eco*RV-*Cla*I fragments (partial *Eco*RV-*Cla*I digestion). The truncated versions of all plasmids were generated by deletion of the coding region upstream of the *Eco*RV site.

In vitro translation. Capped mRNAs were synthesized in vitro according to the Stratagene protocol (mCAP mRNA capping kit) on linearized plasmid templates carrying the adult (A) or embryonic cDNAs (I-80 and I-86) and their truncated versions. A total of 2 μg of capped mRNA samples was translated in vitro in rabbit reticulocyte lysate or wheat germ extract (Promega), supplemented with 40 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham). The average incorporation was 3×10^6 to 6×10^6 cpm per reaction. A total of 2.5 to 10% of the labelled protein product was applied on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) or used for immunoprecipitation.

Tissue culture, transformation, and immunoprecipitation. COS cells were maintained and transformed essentially as described in reference 14. Special precautions were taken to avoid possible degradation. The cells were quickly lysed in 2% SDS–5% β-mercaptoethanol, and the lysates were immediately boiled for 5 min. Immunoprecipitations were performed as described in reference 14 in radioimmunoprecipitation assay buffer containing 1 mg of trypsin inhibitor per ml. The integrity of the RNA was checked on Northern blots. Also, after a 12-h chase we did not observe changes in the overall pattern of the immunoprecipitated products on SDS gels. This is in agreement with the computer studies, which predicted a more than 20-h half-life for each of the products translated from the multiple ORFs on GAD mRNAs.

All primary sera and their preimmune sera were used at a dilution of 1:500. Incubation was for 2 h at room temperature with gentle mixing. A 1/20 volume of protein A-Sepharose (Pharmacia; 1:1 slurry in radioimmunoprecipitation assay buffer) was added, and incubation was continued for 30 min. The immunoprecipitates were washed three times with radioimmunoprecipitation assay buffer and twice with 50 mM Tris-Cl (pH 7.5)–100 mM NaCl–1 mM EDTA (TBSE), dissociated by being heated for 2 min at 100°C in 25 μl of 2× SDS-PAGE sample buffer, and run on SDS–12% PAGE (36). Rainbow molecular weight protein markers (Amersham) were used as standards. The gels were dipped in Amplify (Amersham) and fluorographed. In some cases, the gels were blotted (see below), and the nitrocellulose membrane was exposed for autoradiography.

Immunoprecipitation of in vitro-translated proteins was performed overnight under native conditions (0.1 M phosphate-buffered saline [PBS], pH 7.3, 4°C) in the presence of eight different protease inhibitors. Antibodies were used at a

dilution of 1:500. Incubation with protein A-Sepharose was for 1 h at 4°C.

GAD-specific antibodies. The rabbit serum no. 6799 has been described in detail previously (27). Serum no. 8876 was raised in a rabbit against the C-terminal exon-derived peptide of the leader peptide H-Met-Pro-Ser-Asp-Met-Arg-Glu-Ser-Trp-Leu-Leu-Arg-OH, conjugated to keyhole limpet hemocyanin. The rabbit serum no. 8877 was raised against the N-terminal peptide of the 44-kDa truncated GAD form H-Met-Gly-Leu-Arg-Ala-Glu-Pro-Lys-His-Glu-Ser-Asp-Leu-Gln-Arg-Cys-NH2, conjugated to keyhole limpet hemocyanin. Both peptides were synthesized and conjugated in the Research Division of Hoffmann-La Roche by Danho Walheed. The anti-peptide antibodies were affinity purified on membrane strips carrying bacterially expressed immobilized antigen as described previously (48).

Immunoblotting. Tissue homogenates were prepared, subjected to SDS-PAGE, and blotted essentially as described previously (27). To visualize smaller proteins, blotting was initially performed at 60 V; the voltage was subsequently increased to 80 V to ensure blotting of larger proteins also. For staining of immunoblots, all rabbit sera were used in a dilution of 1×10^3 to 2×10^3. The affinity-purified sera no. 8877 and 8876 were used in 1:250 and 1:500 dilutions, respectively. The sheep anti-rat GAD serum no. 1440 (41) was diluted 2×10^4. The blots treated with this serum were subsequently reacted with rabbit anti-sheep immunoglobulin G (Boehringer; dilution, 10^4). Anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega; dilution, 7.5×10^3) was applied as secondary antibody, and the color reaction was developed as recommended by the manufacturer.

RESULTS

Two embryonic GAD67 mRNAs are detected in mouse embryos. One of the major issues that we addressed initially in this study was the transcriptional activation of the gene coding for the 67-kDa form of GAD. To this end, we performed PCR on first-strand cDNA (reverse transcription [RT]-PCR) from embryonic head RNA preparations with different sets of primers which span the entire coding region of mouse GAD67 cDNA. When primers +E2 (position 428) and −E3 (position 665) were used (A of the first ATG codon of GAD67 cDNA [27] is considered no. 1), two higher-molecular-weight bands were detected in addition to the 261-bp amplified product of the adult cDNA (Fig. 1). Although the PCR was not designed to be quantitative, a highly reproducible temporal profile of three amplified products was observed. While the low-molecular-weight band specific for the adult message increased with age, the embryo-specific bands showed the opposite tendency. We did not detect any other stage-specific bands with any other sets of primers from the coding region, which indicated that the GAD embryonic messages contained a single insert but their coding regions were otherwise colinear with the adult mRNA. To verify this hypothesis, we performed PCR on randomly primed first-strand cDNA of total RNA preparations from mouse embryonic brains (stage E13.5) with primers which amplify the whole coding region in two fragments, overlapping at the embryonic insert. The PCR products were subsequently cloned into Bluescript (Stratagene), and the minilibrary was screened by PCR with primers +E2 and −E3 for the presence of the embryonic insert. The sequence of independent clones revealed the existence of two distinct messages that contained inserts at position 634 on GAD cDNA of 80 and 86 bp, respectively (Fig. 2). The two inserts were identical with the exception of the 3′-most 6 bp at the end of the 86-bp insert. The rest of the coding region was identical to GAD67 cDNA. Most of the single base substitutions were silent and attributed to amplification and/or sequencing errors, since they did not match in different independent clones (56).

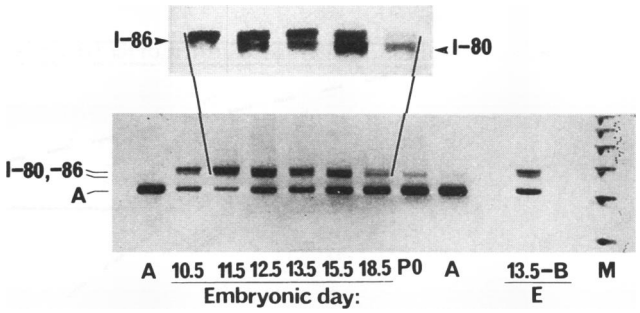

FIG. 1. RT-PCR analysis of GAD transcripts in mouse embryos. Total RNA from E10.5 to E13.5 mouse embryo heads or E15.5 to P0 and adult (A) mouse brains was reverse transcribed and amplified with primers +E2 and −E3. I-80 and I-86 are the 341- and 347-bp amplified products of the two embryonic mRNAs; A is the 261-bp product of the adult message; 13.5-B is RT-PCR of total RNA from E13.5 mouse trunks (bodies). M, molecular size markers.

The embryonic cDNAs were reconstructed from two independent PCR products representing the 5′ and the 3′ half of the messages, overlapping at the embryonic exon. The entire coding region of the embryonic GAD cDNAs is 1,860 and 1,866 bp, respectively (Fig. 2). For convenience, these messages and the corresponding cDNAs will be referred to as I-80 and I-86, respectively.

The structure of the embryonic insert suggested that it might be the product of a developmentally regulated alternative splicing. To verify this, we hybridized the embryonic exon-specific oligonucleotide probe to genomic clone 18A, which was known to contain the flanking exons 6 and 8 (Fig. 2). The single *Hin*dIII fragment that hybridized to the exon-specific probe was sequenced. An exact copy of the embryonic insert(s) was found on this fragment, flanked by authentic splice junctions: 5′-AG/GT (I-80), the rare 5′-TG/GT (I-86), and a common 3′ splice site, AG/G (Fig. 2). As a result of the utilization of a common 3′ and two different 5′ splice sites, 6 bp apart, the two exons differ by only 6 bp. The two 5′ splice sites are used unequally during development, which is reflected in the distinct developmental patterns of the two embryonic transcripts (Fig. 1): I-86 is prevalent at earlier developmental stages (E10.5 to E11.5) and is not detectable in the adult brain, whereas I-80 is more abundant at later embryonic stages (E12.5 to E15.5) and is also detectable at a very low level in the adult brain.

Northern blots of total mRNA extracted from mouse embryonic and neonatal brains and probed with the adult cDNA revealed the existence of a 2-kb transcript in addition to the 3.5-kb adult mRNA, which displayed a developmental pattern very similar to the one observed for the RT-PCR-generated embryo-specific bands (Fig. 3A and B and Fig. 1). When the same blot was rehybridized with an embryonic insert-specific 42-nt oligonucleotide probe, only the 2-kb, not the 3.5-kb, GAD mRNA was detected (Fig. 3C); thus, the 2-kb band represents both I-80 and I-86 transcripts. The difference in size between the adult and embryonic mRNAs is due primarily to the differential utilization of poly(A) addition sites (56).

GAD embryonic mRNAs are structurally bicistronic. The sequence of the embryonic exons revealed the existence of a stop codon in the main ORF, overlapped by an ATG codon in

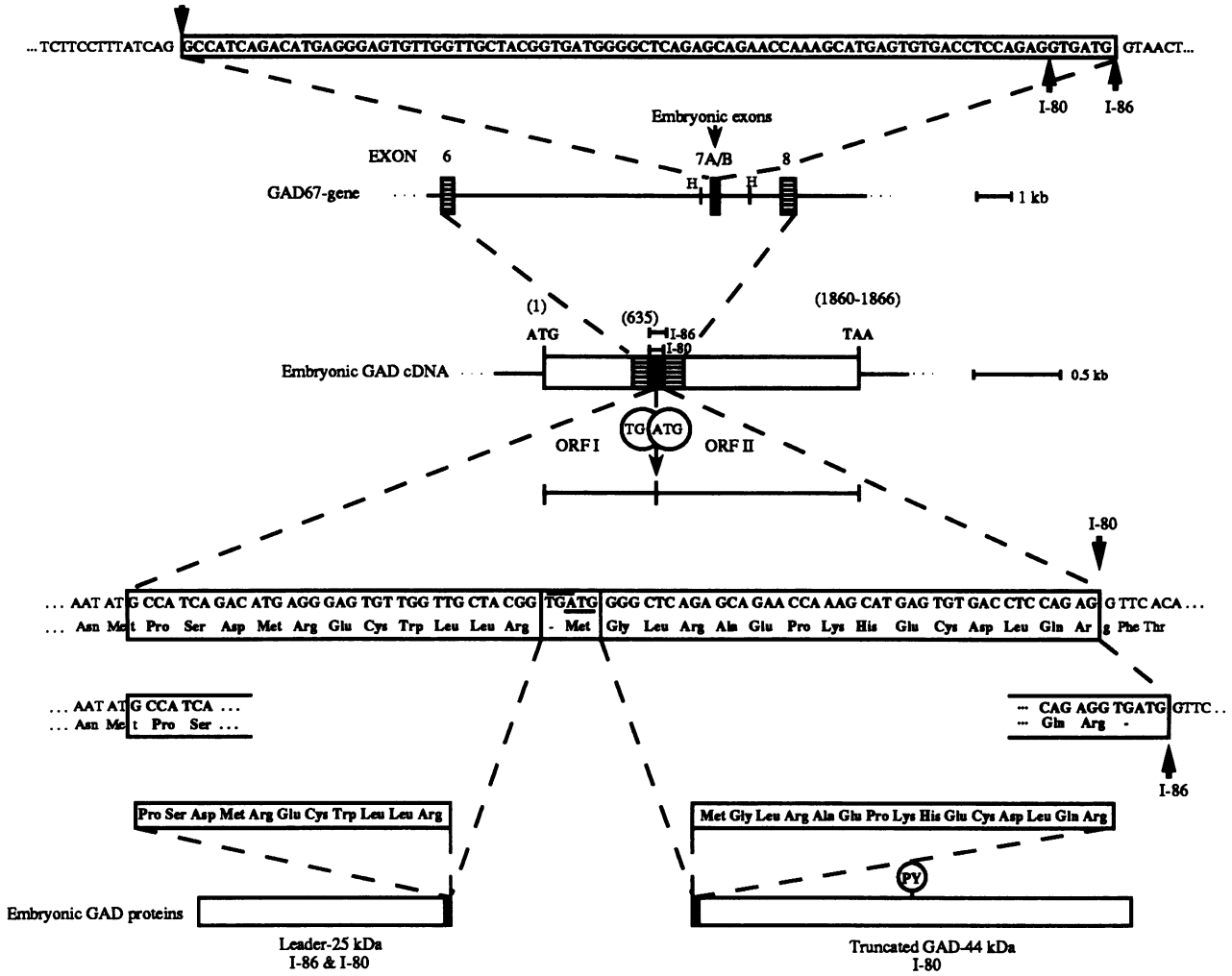

FIG. 2. Schematic presentation of the embryonic exon on GAD67 genomic and cDNA clones and the resulting putative protein products. Exons 6 and 8 are represented by striped boxes; embryonic exons 7A and B are represented by a filled box. The overlapping stop-start codon is circled. The corresponding amino acid sequences are given in a three-letter code. The leader peptide and the truncated GAD, encoded by ORF1 and ORF2, are identical to the adult cDNA in the regions indicated by empty boxes and contain exon-derived C (leader peptide) or N (truncated GAD)-terminal oligopeptides, indicated by filled boxes. PY is the pyridoxal phosphate binding site.

a context favorable for initiation, 5′-GGTGATGG-3′ (Fig. 2). In I-80, this ATG codon is in frame with the rest of the protein. In I-86, an additional in-frame stop codon interrupts the ORF initiating at the overlapping stop-start codon. Schematic presentation of the polypeptide products predicted to be synthesized from the two mRNAs based on analysis of their sequences is shown in Fig. 2. Both I-86 and I-80 could direct synthesis of a 25-kDa leader peptide (GAD25) from the upstream ORF (ORF1), which is identical to the corresponding part of the adult GAD form, but contains an additional 11 amino acids at its C terminus derived from the embryonic exon. In contrast, a termination-reinitiation at the stop-start codon TGATG could produce the 44-kDa truncated GAD (GAD44) only from I-80, since in I-86 the downstream ORF (ORF2) is interrupted by a stop codon. As indicated in Fig. 2, the truncated form retains the pyridoxal phosphate binding site, which is needed for enzymatic activity and has 15 additional aa at its N terminus, contributed by the embryonic exon.

Some important observations emerged upon detailed examination of the 80/86-bp exon that might be relevant to the

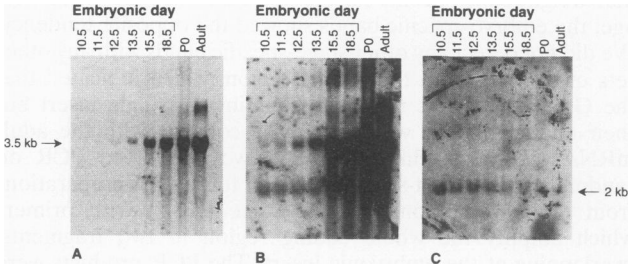

FIG. 3. Northern blot analysis of GAD transcripts during mouse embryonic development. A total of 40 μg of total RNA from embryonic, neonatal, and adult brains was run on a 1.2% formaldehyde-agarose gel, blotted, and hybridized with [^{32}P]dCTP-labelled GAD67 cDNA (A) or a ^{32}P-labelled 42-nt oligonucleotide probe derived from the embryonic exon (C). Panel B is a longer exposure of panel A. The sizes of the adult (3.5-kb) and embryonic (2.0-kb) mRNAs are indicated.

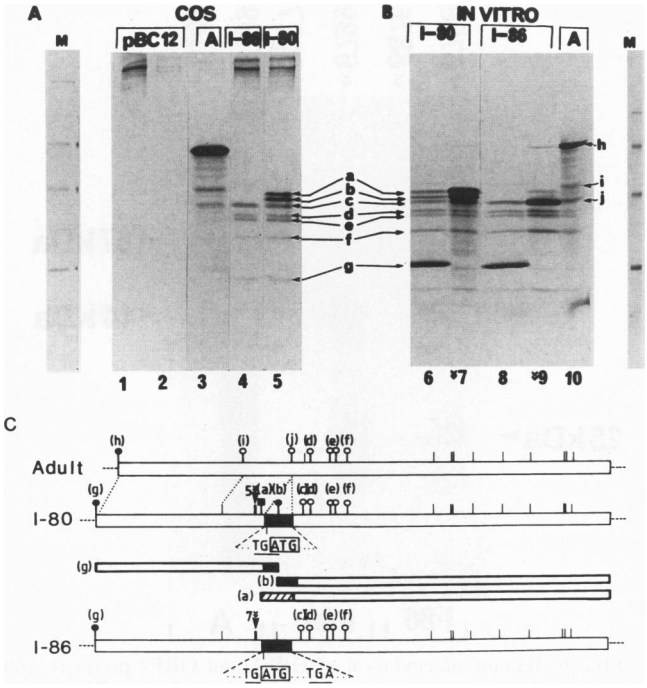

FIG. 4. Translation of GAD mRNAs in rabbit reticulocyte lysates and COS cells. (A) Embryonic (I-80, I-86) and adult (A) cDNAs and vector pBC12 were introduced in COS cells. At 65 h posttransfection, the cells were labelled with [^{35}S]methionine for 3 h and lysed. GAD protein products were immunoprecipitated with anti-GAD antibody 6799 (1:500). (B) I-80, I-86, and adult (A) synthetic mRNAs were translated in vitro in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham). The labelled products were run on SDS–10% PAGE and fluorographed. M, ^{14}C-labelled molecular size marker (from top to bottom, 200, 100 to 92.5, 69, 46, and 30 kDa). In lanes 7 and 9, the ORF1 in I-80 and I-86 was truncated up to position 585 (*Eco*RV site), 20 bp upstream of the first methionine of (a). (C) Schematic presentation of the ORFs derived from the three mRNAs and the protein products detected in COS cells and rabbit reticulocyte lysates. The vertical bars represent the internal methionines in the main ORF. The embryonic exons are given in filled boxes. The N-terminal part of product (a) is hatched. Filled circles indicate the start sites of ORF1 [band (g) of I-80 and I-86], of GAD adult mRNA [band (h)], and ORF2 [band (b)]. The translational start site of (a), which is upstream of the overlapping stop-start codon in I-80, is indicated by a filled square. Initiation codons of bands (c) to (f) are marked by empty circles. The *Eco*RV site which was the endpoint of the deletion of ORF1 is indicated by an arrowhead. The additional stop codon on I-86 is in frame with the ATG from the overlapping stop-start codon.

translation of ORF2. First, the sequence 5′-CCATCAG-3′ found 26 nt upstream of the overlapping stop-start codon shows a patchy complementarity to a conserved region at the 3′ end of eukaryotic 18S rRNA, 3′-GGAAGGC-5′, that may facilitate ribosome binding (30). Second, except in the middle of the embryonic exons, the pentanucleotide TGATG, an overlapping stop-start codon, is found at the very end of I-86 and at the initiation codon of ORF1 and adult GAD.

Multiple protein products are synthesized from the embryonic GAD mRNAs in vitro and in COS cells. To check the validity of our assumption that the two ORFs on I-80 could direct expression of two distinct full-size proteins, we performed in vitro translation of synthetic GAD mRNAs in rabbit reticulocyte lysates as well as transient expression in COS cells (Fig. 4). Unexpectedly, a set of bands was detected [labelled (a) to (j)], products of translation of both adult (A) and embryonic (I-80 and I-86) mRNAs in COS cells and in the rabbit reticulocyte system (Fig. 4A and B). These bands were absent from immunoprecipitates performed with preimmune serum or immune serum from COS cells, transfected with the vector pBC12 (panel A, lanes 1 and 2).

TABLE 1. Molecular masses and sequences around the initiator codons of the translation products of the adult, I-80, and I-86 GAD mRNAs

Band[a]	Molecular mass (kDa)[b]	Position of ATG[c]	Sequence around ATG[d]	Transcript[e]
			GCC**Pu**CC ATG**G**	Consensus
h	66.5	1	GAG**C**TG ATG**G**	Adult
i	49.9	448	GAG**A**GC ATG**G**	Adult
j	43.0	634	ACC**A**TA ATG**G**	Adult
a	47.1	606	TGG**T**GA ATG**G**	I-80, I-86
b	44.6	672	ACG**G**TG ATG**G**	I-80
c	41.6	750	GTT**C**TC ATG**G**	I-80
d	38.1	766[f]	TCC**A**AT ATG**T**	Adult, I-80, I-86
e	37.6	778[f]	ACG**A**TC ATG**G**	Adult, I-80, I-86
f	35.8	823[f]	AAA**G**GC ATG**G**	Adult, I-80, I-86
g	25.0	1	GAG**C**TG ATG**G**	I-80, I-86

[a] Corresponding to protein bands in Fig. 3.
[b] Estimated molecular mass of the protein, initiated at the corresponding methionine.
[c] Position of initiator ATG on the corresponding cDNA, utilized for initiation. Numbering starts at A of the first ATG of adult cDNA (see text for details).
[d] Boldface type indicates the most conserved nucleotides.
[e] Specifies the transcript in which the particular ATG serves as initiator codon.
[f] Indicates only the position on the adult cDNA; for I-80 and I-86, 80 and 86 bp, respectively, should be added.

The observed bands correspond by molecular size to the putative translational products, initiated at internal methionines (Fig. 4C). The locations of the initiator ATGs for bands (a) to (j) on the three cDNAs, the sequences around these ATGs, and the estimated molecular sizes of the corresponding polypeptides are shown in Table 1. It should be noted that all methionines between positions 1 and 823 of the main ORF are utilized for translation initiation, the only exception being the methionine found at the end of exon I-86 as part of the stop-start signal TGATG (Fig. 2), which is shortly followed by a stop codon. The alternative reading frames of the adult GAD cDNA do not have a coding capacity for peptides larger than 6 kDa, and their possible translation was not studied.

The 25-kDa band (g) is produced by in vitro translation from I-80 and I-86 (Fig. 4, lanes 4, 5, 6, and 8), but not from the adult message (lanes 3 and 10); therefore, it is the candidate product of ORF1. Since it is translated from the first ATG codon, its intensity is much stronger than that of the other bands, in good agreement with the scanning mechanism. This polypeptide is less prominent in immunoprecipitates from COS cells, which could be explained by its tendency to form aggregates (26).

Instead of the expected one band, there are two additional products translated from I-80 that are missing in the I-86 lane [bands (a) and (b), respectively]. Our original assumption, based on the precise estimation of their molecular sizes, was that (i) the 44.6-kDa band (b) is the bona fide product of the ORF2 and (ii) the 47.1-kDa band (a) is translated from an upstream ATG, in frame with the initiation codon of ORF2 (+2 frame relative to ORF1). Thus, (a) could be initiated by ribosomes that reach back 63 bases after pausing at the overlapping stop-start codon. In addition to bacteria (45, 51), this type of translational coupling has been shown to occur in eukaryotic cells as well (59).

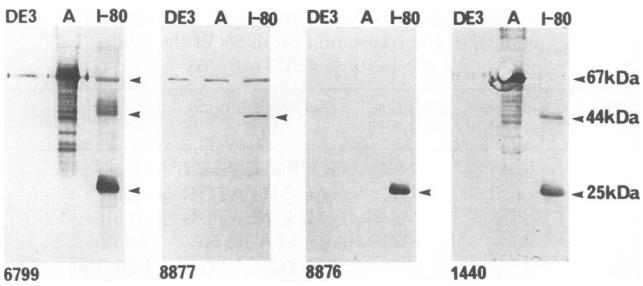

FIG. 5. Immunoblots of bacterially expressed GAD forms, stained with anti-adult GAD and anti-embryonic GAD antibodies. A total of 1 ml of IPTG (isopropyl-β-D-thiogalactopyranoside)-induced bacterial culture was centrifuged, the pellet was suspended in 100 μl of sample buffer, and 10 μl was run on a 10% SDS gel (36). The gel was blotted and reacted with the following antibodies: 6799, raised against the recombinant 67-kDa form of GAD; 8876, raised against the embryonic exon-derived COOH-terminal oligopeptide of the leader peptide; 8877, raised against the NH_2-terminal, exon-derived oligopeptide of the truncated GAD; and 1440, anti-rat GAD antibody (41). DE3, IPTG-induced strain DE3, carrying plasmid pAR3039 (54); A and I-80, IPTG-induced DE3, carrying the adult and I-80 cDNAs, respectively, in plasmid pAR3039. The molecular sizes of the stained GAD proteins are indicated.

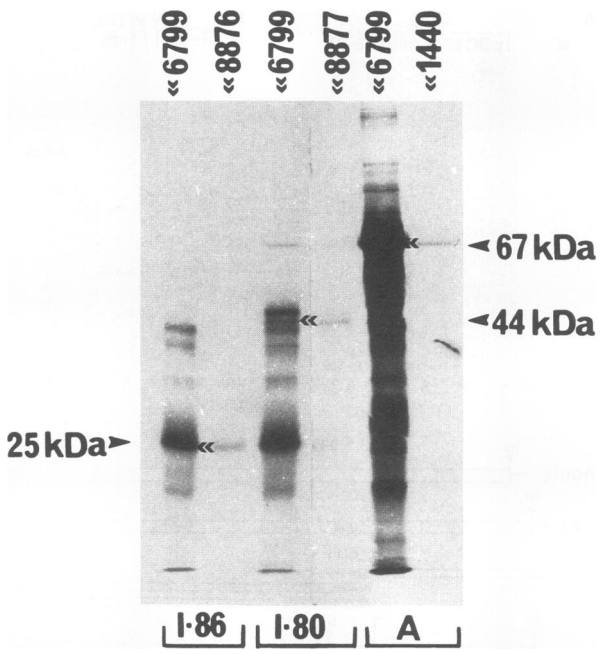

FIG. 6. Immunoprecipitation of ORF1 and ORF2 products with specific antibodies. GAD embryonic and adult synthetic mRNAs were translated in vitro, and the protein products were immunoprecipitated in PBS with antibodies 6799, 8876, 8877, and 1440 (1:500). The immunoprecipitates were run on SDS gels (36), blotted, and exposed for autoradiography.

To help define the origin of the individual bands, we deleted most of the upstream ORF in I-80 and I-86 (a) (up to the *Eco*RV site, position 585 on the cDNA map, indicated by a double arrowhead in Fig. 4C), 20 bp upstream of the initiation codon of product (a). The results from the in vitro translation of the truncated mRNAs (Fig. 4B, lanes 7 and 9) were fully consistent with our original assumptions and could be summarized as follows. (i) Band (g), the leader peptide, disappeared. (ii) Bands (a) and (b) in lane 7 are the largest polypeptides synthesized from I-80, which is consistent with the first and second positions of their initiator ATGs. (iii) The second ATG on the truncated I-80 is the overlapping stop-start codon; therefore, (b) is the most likely candidate for the product of ORF2. (iv) (a) and (b) are not found among the products of the truncated I-86, in which an in-frame stop codon shortly follows the initiator codons of these polypeptides.

The products of ORF1 and ORF2 can be identified with specific antibodies. The translation of the embryonic exon results in the addition of 11 and 15 aa to the C terminus of the leader peptide and N terminus of truncated GAD, respectively (Fig. 2). We raised rabbit polyclonal antibodies against the exon-derived oligopeptides as follows. Serum no. 8876 was raised against the C-terminal 11 aa of the leader peptide. No. 8877 was raised against the 15-aa exon-derived N-terminal peptide of the truncated GAD. These antibodies were used in parallel with the previously characterized sera no. 6799 (27) and no. 1440 (41) to immunoprecipitate the GAD-related proteins from in vitro-translated embryonic and adult mRNAs. The specificity of all antibodies was tested on Western blots of bacterial extracts from strains overexpressing GAD67, GAD44, and GAD25 in the T7 polymerase expression system (54) (Fig. 5). The strain overproducing GAD67 was already described (27). GAD44 and GAD25 were produced coordinately from the bicistronic I-80 by translational coupling (26). As demonstrated in Fig. 5, no. 8876 bound specifically to GAD25, but not to GAD44. Similarly, no. 8877 did not cross-react with GAD25. No. 1440 and no. 6799 recognized both embryonic and adult GAD forms. No. 8877 and no. 6799 exhibited some nonspecific binding to a 60-kDa bacterial protein.

The [^{35}S]methionine-labelled immunoprecipitates of in vitro-translated GAD forms were run on protein gels that were electroblotted and autoradiographed (Fig. 6). In good agreement with our predictions, antibody no. 8876 specifically precipitates the 25-kDa polypeptide, product of ORF1. Serum no. 8877 precipitates predominantly the protein with a molecular size of 44 kDa, translated from ORF2. This serum also precipitates extremely inefficiently the 47-kDa protein, in which the antigen is at an internal, compared with a free, terminal position in the 44-kDa protein.

The protein forms synthesized from ORF1 and ORF2 of the embryonic GAD mRNAs are detected in mouse embryos. To identify the in vivo protein products of GAD embryonic mRNAs, we stained Western blots of mouse brain extracts from different embryonic stages with the anti-GAD serum no. 6799. In adult brain, this antibody stains predominantly the 67-kDa form but reacts also with the 65-kDa molecular form of GAD (Fig. 7a) (27). On Western blots, the 67-kDa form is usually first seen around E12.5, but it could be demonstrated as early as E11.5 by immunoprecipitation (26). In addition to the 67-kDa GAD form, a smaller 44-kDa band is specifically stained in homogenates from mouse and rat embryonic brains and trunks of mouse embryos. At higher protein concentrations, this band is seen until P14 to P21 (Fig. 7c). The developmental pattern of the 44-kDa GAD form is identical to that of I-80, which makes it a good candidate for the truncated GAD encoded by ORF2. When blotting was performed at low voltage, two additional intensely stained, closely spaced 31- to 32-kDa bands were detected on the developmental Western blots (Fig. 7a and d). This doublet is quite prominent at embryonic stages E10.5 to E11.5, when I-86 predominates; shows a maximum expression at E12.5 to E15.5, a period of most intensive synthesis of both embryonic mRNAs; and is

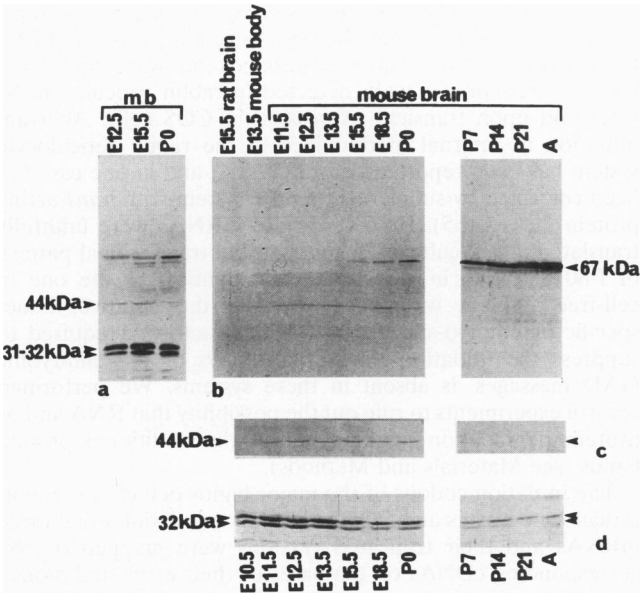

FIG. 7. Western blot of mouse embryonic and postnatal brain (or trunk) total homogenates. A total of 20 μg of protein per lane was run on SDS-PAGE and blotted at low intensity (a and d) and high intensity (b and c) of the field. The blots were stained with rabbit anti-GAD antibody 6799 (1:2,000). The 67-kDa adult GAD form, the 31- to 32-kDa doublet, and the 44-kDa truncated GAD are indicated.

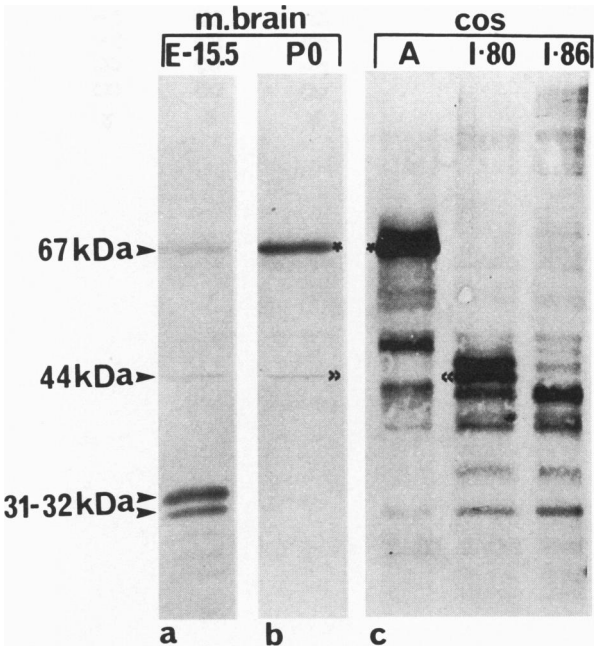

FIG. 8. Size comparison between GAD protein forms detected in mouse brains from stages E15.5 and P0 and the protein products synthesized in COS cells from the adult and two embryonic GAD mRNAs. Mouse (m) brain homogenates and immunoprecipitates from metabolically labelled COS cells with antibody 6799 were run on the same gel, which was subsequently blotted. The part shown in panel c was exposed for autoradiography. The part shown in panel b was stained by antibody 6799. Also shown for comparison is a blot from E15.5 brain homogenate that was run in parallel, blotted at low voltage, and reacted with 6799. The truncated 44-kDa GAD form shows a mobility identical to that of the second band from lane I-80 [corresponding to band (b), Fig. 4].

hardly detectable after P14, which suggests that it could be translated from ORF1.

We further attempted to determine whether the embryonic GAD proteins correspond in size to any of the products of I-80 and I-86 expressed in COS cells or rabbit reticulocytes. To this end, we ran on the same gel homogenates of mouse embryonic brains next to immunoprecipitates from metabolically labelled COS cells, transfected with GAD cDNAs. The separated proteins were electroblotted onto nitrocellulose, which was autoradiographed (labelled part) or stained with the GAD-specific antibody 6799 (Fig. 8). We performed multiple experiments with extracts and immunoprecipitates from independent preparations to eliminate nonspecific factors that might contribute to altered electrophoretic mobility. On all gels, the 44-kDa brain protein (GAD44) comigrated with band (b), the second largest product translated from I-80, which we already showed to be the product of ORF2. The same approach could not be used to positively identify the leader peptide, since it was inefficiently immunoprecipitated from homogenates of transfected COS cells. Similarly, the 31- to 32-kDa doublet could not be related by size to the in vitro-translated leader peptide, which migrates faster on SDS gels.

The products of I-80 and I-86 in vivo were directly identified with the use of the embryonic GAD form-specific antibodies 8876 and 8877. Both antibodies were affinity purified on nitrocellulose strips (48) to eliminate nonspecific background and used to stain Western blots of E15.5 brain homogenates. Control staining with 6799, which was shown to react with both adult (GAD67) and embryonic (GAD25 and GAD44) forms, was also carried out (Fig. 9). Antibody 8876, specific for the leader peptide (GAD25), binds to the 31- to 32-kDa doublet, which is also recognized by the anti-GAD antibodies 6799 (Fig. 7 and 9) and 1440 (26). In addition to their retarded migration in SDS gels, the bands of the doublet display variable relative intensities at different developmental stages, which suggests that they might be modified posttranslationally. Serum 8877, specific for the N-terminal peptide of the truncated GAD, stained a 44-kDa protein, comigrating with the truncated GAD (GAD44), detected with serum 6799. In an independent experiment we also found that the electrophoretic mobility and staining properties of the bacterially expressed GAD44 are indistinguishable from those of embryonic GAD44 (26). These results argue that the 44-kDa embryonic GAD form is the product of ORF2 of I-80 mRNA, whereas the 31- to 32-kDa proteins are synthesized from ORF1 of both I-80 and I-86 mRNAs and are probably modified posttranslationally.

DISCUSSION

By developmentally regulated and tissue-specific alternative splicing, multiple protein forms can be produced from a single gene. As has been shown in numerous examples, usually one combination of exons is regulated in a tissue and/or developmental stage-specific way whereas the alternative combination(s) arises by default (4, 6, 11, 60). The developmentally regulated alternative splicing of GAD primary transcript described in this paper is an example of unusual complexity for the following reasons. First, the splicing of two almost identical exons is differentially regulated during development. Second, the overlapping stop-start codon on the embryonic exons converts the adult-type mRNA into structurally bicistronic mRNAs with tandemly arranged ORFs. Third, of two almost identical embryonic transcripts, only I-80 is both structurally

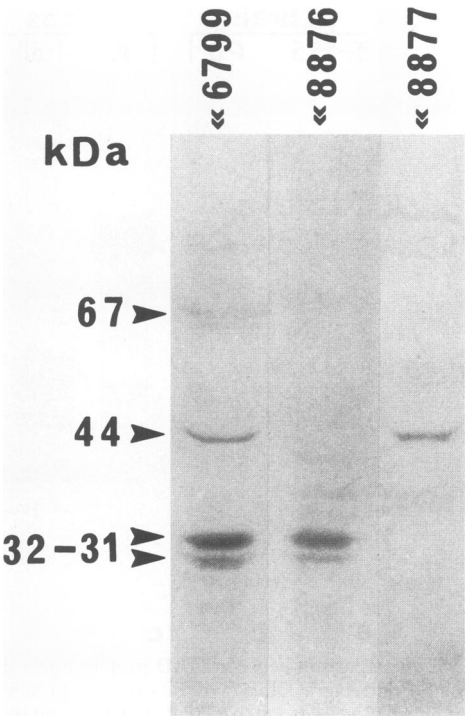

FIG. 9. Western blot of E15.5 mouse brain homogenates stained with anti-GAD and anti-GAD embryonic form-specific antibodies. A total of 20 μg of protein was run in each lane and blotted. The antibodies 8876 and 8877, specific for the leader peptide and truncated GAD, were affinity purified and used at a dilutions of 1:500 and 1:250, respectively. Anti-GAD antibody 6799 was diluted 1:2,000.

and functionally bicistronic. In I-86, an in-frame stop codon interrupts ORF2; therefore, I-86 is predicted to be monocistronic.

The splicing of GAD embryonic exons bears resemblance to the sex-specific splicing of mRNAs involved in *Drosophila melanogaster* sex determination (5, 38, 60). Similarly to the female-specific usage of the weaker 3′-splice site of *dsx* in the presence of the positive regulators *tra* and *tra*-2, the rare TG/GT 5′-splice site of I-86, which is preferentially utilized at earlier developmental stages, could be a target for positive regulators. In contrast, the conventional AG/GT 3′-splice junction of I-80 suggests that it is processed by default. The prevalence of I-80 over I-86 at later embryonic stages could be a result of the successful competition for splice factors at the two closely spaced 5′-splice sites. By analogy with the splicing of *Sxl* in the female mode, the down-regulation of I-80 in the adult mouse brain might occur by a blockage mechanism (exon skipping) (4, 23).

Because of the premature stop codon in the male-type exon of *Sxl* mRNA, a nonfunctional N-terminal peptide is synthesized (8). In contrast, the stop codon on the embryonic GAD exon is overlapped by an ATG codon in a favorable context for translation initiation, and the ORF initiated at this ATG (ORF2 of I-80) is in frame with the rest of the GAD protein. This tandem arrangement of the two ORFs is reminiscent of the structure of many bacterial polycistronic mRNAs (15, 25, 45, 51), which can direct the coordinate expression of functionally related, but structurally distinct, proteins by translational coupling. To our knowledge, GAD I-80 embryonic mRNA is the first known, naturally occurring, eukaryotic cellular bicistronic mRNA with tandemly arranged ORFs.

According to the predictions, based on the sequencing data, one and two proteins could be synthesized from I-86 and I-80, respectively. Instead, a number of bands that were initiated at internal methionines were detected in rabbit reticulocyte lysates and upon transient expression in COS cells. Aberrant initiation at internal methionines in the rabbit reticulocyte system has been reported before (17, 24) and in one case has been corrected by supplementing the system with *trans*-acting protein factors (55). However, these mRNAs were faithfully translated in cell culture. In contrast, the translational pattern of I-80 and I-86 in COS is almost identical to the one in cell-free systems, which suggests that the putative tissue-specific or embryo-specific *trans*-acting factor(s), required to suppress the initiation at internal ATGs of the embryonic GAD messages, is absent in these systems. We performed control experiments to rule out the possibility that RNA and/or protein degradation might generate the additional protein bands (see Materials and Methods).

The initiation codons of the major bands detected in rabbit reticulocyte lysates and COS cells upon translation of all three mRNAs and their truncated versions were mapped on the corresponding cDNAs on the bases of their estimated molecular sizes and relative intensities. The mechanisms of leaky scanning and termination-initiation (32, 35, 43, 44) could account for the initiation at most methionines. Band (b), initiated at the overlapping TGATG, and (a), initiated at an in-frame ATG codon, 63 bases upstream from the overlapping stop-start codon, could also be produced by a coupled termination-initiation. In favor of coupled translation is the observation that when full-length I-80 is translated in COS cells, (b), in spite of being initiated at a downstream ATG, is predominant to (a) (Fig. 4A, lane 5), whereas (a) is stronger than (b) when translated from the truncated I-80 by the leaky scanning mechanism (Fig. 4B, lane 7). The ratio of (b) to (a) in COS cells might be even higher given the inefficient immunoprecipitation of (b) by antibody 6799. The enhanced intensity of band (b) versus that of (a) in COS cells would be consistent with an enhanced translational coupling at the overlapping stop-start codon [band (b)] versus weaker coupling when the start codon is buried in the upstream ORF [band (a)] (15, 43, 52).

In contrast to the in vitro studies, two major protein forms are detected on immunoblots of mouse embryonic brain extracts, which correspond to the products of ORF1 and ORF2 by the following criteria. (i) Migration in SDS gels: the truncated GAD form always comigrates with band (b) from the in vitro-translated I-80 mRNA or COS cells transfected with I-80 cDNA; its molecular size, estimated from its migration relative to molecular size markers, exactly matches the size of the product of ORF2, calculated on the basis of its amino acid sequence. The presumed product of ORF1 appears usually as two closely spaced bands with molecular size greater than the size of the corresponding in vitro-synthesized band (31 to 32 compared with 25 kDa). We attribute this discrepancy to posttranslational modification(s), which plays an important role in its subcellular localization (in preparation). (ii) Developmental pattern: the pattern of distribution of the 44-kDa truncated GAD exactly matches the developmental pattern of I-80 with a low level of expression during stages E10.5 to E11.5 and a maximum of expression at E13.5 to E15.5. The 31- to 32-kDa bands are relatively more abundant at E12.5 to E15.5 when they are expressed from both embryonic messages. (iii) Staining with GAD-specific antibodies: the embryonic GAD protein forms are specifically detected on immunoblots and could be immunoprecipitated by anti-GAD antibody 6799, which recognizes multiple epitopes on both adult (GAD67) and embryonic (GAD25 and GAD44) forms, and the em-

bryonic form-specific antibodies 8876 (GAD25) and 8877 (GAD44) but not by the corresponding preimmune sera.

The apparent faithful initiation in vivo at the first ATG codons of ORF1 and ORF2 contrasts the impaired initiation in vitro and in COS cells and is probably a result of the combinatorial action of *trans*-acting factors and *cis* elements.

Among *cis* elements, mRNA secondary structure has been shown to be of primary importance in initiation (34). Surprisingly, computer search revealed no stable hairpin structures on either embryonic or adult mRNAs. Consistent with that, varying the concentrations of KCl in the in vitro translation assay (both reticulocyte lysates and wheat germ extracts from different manufacturers were tested) changes the overall efficiency of translation but not the relative intensities of the bands (56). A putative *cis*-acting element is the pentanucleotide TGATG, found in the beginning of ORF1 and ORF2 and in the end of exon I-86. This sequence is often used as a punctuation signal in translational coupling (42), although the exact mechanism is poorly understood. Finally, despite the existence of a ribosome binding site in eukaryotic mRNAs being disputed, homologies between eukaryotic mRNAs and 18S rRNA analogous to the prokaryotic Shine-Dalgarno sequence have been found (reviewed in reference 30) and in some cases proven to be indispensable for proper translation (46). Interestingly, we identified a sequence, complementary to the 3′ end of 18S rRNA in the vicinity of ATG of ORF2, that might act to selectively enhance initiation at this initiation codon.

Possible candidates for *trans*-acting factors could be the general translation initiation factors 4E, 4A, and 4F, which have been implicated in the selection of internal ATGs on bicistronic mRNAs (2, 58). Furthermore, the activity of the 4E subunit of eukaryotic initiation factor 2 has been shown to correlate with the differentiated state of PC12 cells (19), and the ratio of the two molecular forms of the 4A subunit of eukaryotic initiation factor 2 has been found to vary in a tissue-specific way (40), pointing to some tissue-specific and developmental stage-specific aspects of the translational process in eukaryotes. Alternatively, the putative factor could be specific for GAD, similar to the caulimoviral transactivator protein, which in combination with gene VII is required for the efficient translation of the tandemly arranged downstream ORFs of the polycistronic viral mRNA (20, 22). Undoubtedly, the identification of such a factor(s), which could enhance initiation at the first ATGs of ORF1 and ORF2 and suppress aberrant internal initiation on the embryonic GAD mRNAs, would provide us with important insights into the complex developmental and tissue-specific regulation of the gene.

What are the functional consequences of the complex posttranscriptional regulation of the GAD67 gene during development? According to previous reports, γ-aminobutyric acid (GABA) could be detected earlier (E13 in rats) than GAD (E16 in rats), and the levels of GABA in the embryonic and neonatal brain are as much as 20 to 50% of the adult level (reviewed in references 3, 61, and 62). Using much more sensitive methods, we could show that GAD mRNA coding for the 67-kDa form is already expressed in E10.5 mouse embryos (corresponding to E11.5 in rats), shortly after the onset of neuronal differentiation. The protein is detectable 1 day later, albeit at very low levels, and its concentration rises steadily with age. Two embryonic forms with a distinct temporal distribution are produced concomitantly from two embryo-specific mRNAs, a leader peptide (GAD25) and a truncated GAD (GAD44) that show a maximum of expression during the period of neuronal migration and differentiation (E12.5 to E15.5). We also found the 44-kDa truncated GAD in rat embryos, possibly synthesized from an embryonic rat brain mRNA with an almost identical alternatively spliced exon (7), pointing to the evolutionary conservation of this complex developmental regulation.

GAD25 and GAD44 represent different portions of GAD67 that are probably involved in different functions. Since GAD44 retains a larger portion of the protein including the pyridoxal phosphate binding site, we tested it for its ability to convert L-glutamate to GABA. Surprisingly, a β-galactosidase–GAD44 fusion protein exhibited activity equal to that of the fusion protein 0-20A (27), which includes almost the entire coding region of GAD (26). The leader peptide, which carries the signal mediating subcellular targeting of the enzyme (53), probably exerts regulatory functions. One of its primary roles could be related to the translation of GAD44 from ORF2. Rough estimation shows that the 44-kDa GAD represents 10 to 20% of the amount of GAD25 in both in vivo and in vitro assays. This translational suppression of the enzymatically active part of the protein could provide a quick control over the production of GABA in response to external factors.

Our preliminary results show that the adult (67-kDa) and truncated GAD, both capable of synthesizing GABA, are colocalized in a subpopulation of neuronal precursors in the embryonic central nervous system. Since the truncated GAD is predominantly detected during the period of neuronal differentiation and formation of inhibitory synapses, it might substitute for the synaptosomal GAD in the GABA-ergic neuronal precursors. The precise role of the embryonic GAD forms during development is currently being addressed by targeted homologous recombination.

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