

Expression of *GAD67* and Novel *GAD67* Splice Variants During Human Fetal Pancreas Development

GAD67 Expression in the Fetal Pancreas

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Abstract Glutamic acid decarboxylase (*GAD*) is a major inhibitory neurotransmitter in the brain, which catalyses the reaction of L-glutamate to γ -aminobutyric acid. There are two isoforms of *GAD*, a 65-kDa form and a 67-kDa form, which are encoded by two different genes. As previous studies suggested a role for *GAD67* splice variants during fetal pancreas development, we have investigated the mRNA expression of *GAD67* and *GAD67* splice variants in a series of 14 human fetal pancreases between 14 weeks gestation and term and in adult control pancreases by RT-PCR. In this study, we demonstrate mRNA expression of *GAD67* and four *GAD67* splice variants, including *GAD25*, in human fetal and adult specimens. Some of the splice variants, including various proportions of exon 7 or a new exon between exons 6 and 7, have not been described

before in the human pancreas. We speculate that the expression of these *GAD67* splice variants might be related to human fetal pancreas development.

Keywords fetal development · *GAD* · pancreas
alternative splicing · human development

Introduction

Two forms of glutamic acid decarboxylase (*GAD*) occur in mammalian tissues: a 65 kDa form (*GAD65*) and a 67-kDa form (*GAD67*). *GAD65* and *GAD67* are enzymes that catalyze the reaction of L-glutamate to γ -aminobutyric acid, a major inhibitory neurotransmitter in the central nervous system. The two *GAD* isoforms are highly homologous but are encoded by separate genes [1]. *GAD65* is localized on chromosome 10, and *GAD67* on chromosome 2 [2]. Alternative splicing of the *GAD67* gene can result in two additional isoforms of the protein [3, 4].

In humans, autoreactivity against *GAD* is often found in type 1 diabetes mellitus. Some 10 to 30% of the newly diagnosed patients have *GAD67* antibodies, and up to 80% have *GAD65* antibodies. Because the prevalence of *GAD67* autoreactivity is less than *GAD65*, and is usually detected in patients with *GAD65* antibodies, the *GAD67* antibodies are assumed to play a less prominent role in the pathogenesis of β -cell destruction [3]. However, there are studies where *GAD67* reactivity was found in patients without reactivity against *GAD65* [5–7].

GAD65 and *GAD67* are both highly expressed in the brain, but the two forms appear to be differentially expressed in pancreatic islets. In human islets, *GAD65* is

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abundant, but *GAD67* could not be detected with immunohistochemistry, in situ hybridization, immunoprecipitation, or Western blotting [3, 8–10]. In one study, limited *GAD67* expression was demonstrated in human islets by RNase protection analysis [11]. In contrast, rat islets express both forms, whereas mouse islets express predominantly *GAD67* [4, 8, 10, 12, 13].

In addition to the native form of *GAD67*, of which exon 7 is spliced out of the mRNA, other forms of alternative splicing of the *GAD67* gene have been described in the rodent brain. This alternative splicing resulted in two additional *GAD* transcripts in which either the first 80 or the first 86 bp of exon 7 were inserted into the full length *GAD67* message, respectively [4, 14]. Transcripts with the 80-bp insertion result in two overlapping open reading frames, encoding an enzymatically inactive 25-kDa protein (*GAD25*) and an enzymatically active 44-kDa protein (*GAD44*). A second stop codon at the end of the 86-bp insertion abolishes the translation of *GAD44* (Fig. 1A). This alternative splicing was only observed in embryonic and fetal mice, but not in adult animals [4, 14, 15], leading to the conclusion that this alternative splice variant could have a developmental function. Alternative splicing of the *GAD67* gene has also been demonstrated in cells of adult human pancreatic islets. Exon 7 was inserted after exon 6 of

GAD67, resulting in a shorter transcript of *GAD67* mRNA, encoding *GAD25* [3].

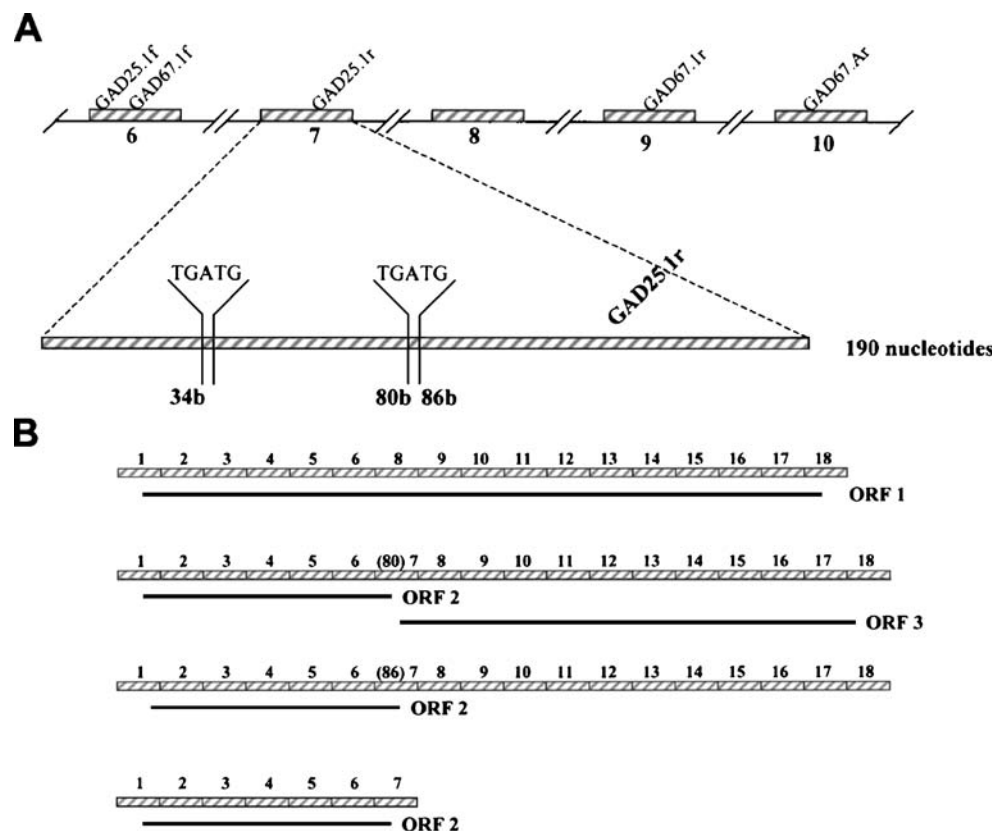
The potential role of *GAD* in pancreas development [4, 16] and the lack of knowledge of the normal expression of different *GAD* isoforms during normal fetal pancreas development led us to investigate *GAD* expression at the mRNA level in a series of human fetal pancreas specimens of 14 weeks gestation until term. In this paper, we demonstrate *GAD67* and *GAD67* splice variants, including *GAD25* mRNA expression by RT-PCR, between 14 weeks gestation and term and in the adult pancreas.

Materials and Methods

Tissue Specimens

Human fetal ($n=14$) and adult ($n=4$) pancreatic specimens were retrieved from the Erasmus MC Tissue Bank, Department of Pathology, Rotterdam, The Netherlands, following approval of the experimental design and protocols by the Medical Ethical Committee. The fetuses had died of causes not related to the pancreas. Autopsy was performed within 24 h after death. The adult pancreatic tissue was obtained from normal tissue in pancreatic

Fig. 1 **A** Schematic representation of exon 6 to exon 10 of *GAD67*, including the localization of the primers used for the RT reaction (*GAD67.Ar*) and PCR (*GAD25.1f*, *GAD25.1r*, *GAD67.1f*, and *GAD67.1r*). In the adult form, exon 7 is spliced out of the mRNA, but in embryonic mouse brain, a part of exon 7 is spliced out in two different ways. Either the first 80 bp (I-80) or the first 86 bp (I-86) is retained in the mRNA, and because an overlapping translation stop–start sequence occurs after 34 nucleotides in exon 7, the I-80 and I-86 inserts result in two “open reading frames” (ORFs) and one ORF, respectively, as displayed in subpanel (B). **B** Overview of alternative mRNA splice variants, including the predicted ORFs; ORF1 encodes *GAD67*, ORF2 encodes *GAD25*, whereas ORF3 encodes *GAD44*. The additional 6 bp in the I-86 message contains an in-frame stop codon which abolishes the translation of *GAD44*



resection specimens. Histological analyses confirmed the presence of normal tissue in these adult pancreas samples.

Fourteen snap-frozen pancreatic samples between 14 weeks gestation and term (gestational ages: 14, 16, 18, 19, 21, 26, 27, 29, 32 ($n=2$), 33, 36, and 40 ($n=2$) weeks, respectively) and four adult snap-frozen pancreatic samples were stored in liquid nitrogen and were used to isolate RNA. Paraffin samples were fixed by immersion in 4% buffered formaldehyde and embedded in paraffin according to the standard procedures.

RNA Isolation

Total RNA was extracted using Trizol reagent (Life Technologies, Rockville, MD, USA) following the manufacturer's instructions. After RNA isolation, the quality of 5 μ g RNA was checked with an MOPS gel (0.2 M 3-*N*-morpholinepropanesulfonic acid, 0.05 M sodium acetate, and 0.01 M Na₂EDTA).

cDNA Synthesis and PCR Amplification

One microgram of total RNA from 18 pancreases was reverse-transcribed with a mix of specific reverse primers for *GAD67* (5'-AGTGACTCTGTTCTGAGGTG-3'), *POLR2A* (5'-TTGTTAGAGTCCACAAGCAG-3'), and oligo-dT (T12). cDNA synthesis was performed in a final volume of 20 μ l containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 3 pM specific primer mix, 1 mM dNTPs, 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, San Diego, CA, USA), and 30 U RNase inhibitor (Promega, Madison, WI, USA). The RT mix was incubated for 1 h at 37°C. Polymerase chain reaction was performed with 1 μ l cDNA in a final volume of 15 μ l containing 1.5 mM MgCl₂; 10 mM Tris-HCl; 50 mM KCl; 0.02 mM dATP; 0.2 mM dGTP, dTTP, and dCTP; 0.8 μ Ci α^{32} P-dATP (Amersham, Buckinghamshire, United Kingdom), 15 pmol of each forward and reverse primer for *GAD67* and *POLR2A* (Table 1), and 1 U *Taq* polymerase (Promega, Madison, WI, USA). PCR was performed for 35 cycles at 95°C for 30 s, 58°C for 45 s, and 1 min at 72°C, followed by 1 cycle at 72°C for 10 min. PCR products were run on a denaturing 6% polyacrylamide gel (Fluka, Neu-Ulm, Germany). After electrophoresis, the gel was dried and

exposed to an x-ray film. Negative controls for the RT reaction were performed by omission of either M-MLV reverse transcriptase or RNA, and for the PCR by omission of cDNA.

DNA Sequencing

To confirm that the additional bands represented both splicing forms, two 50 μ l PCRs were performed with two cDNA samples, respectively, at 14 and 19 weeks. Polymerase chain reaction was performed under the identical conditions as the previous PCR except that this mix contained 0.2 mM dNTPs instead of 0.02 mM dATPs and 0.8 μ Ci α^{32} P-dATP. The PCR products were run on a 2% agarose gel, and the additional bands were excised out of the gel and purified using a QIAquick Gel Extraction Kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. The purified PCR products were commercially sequenced (Baseclear, Leiden, The Netherlands).

Results

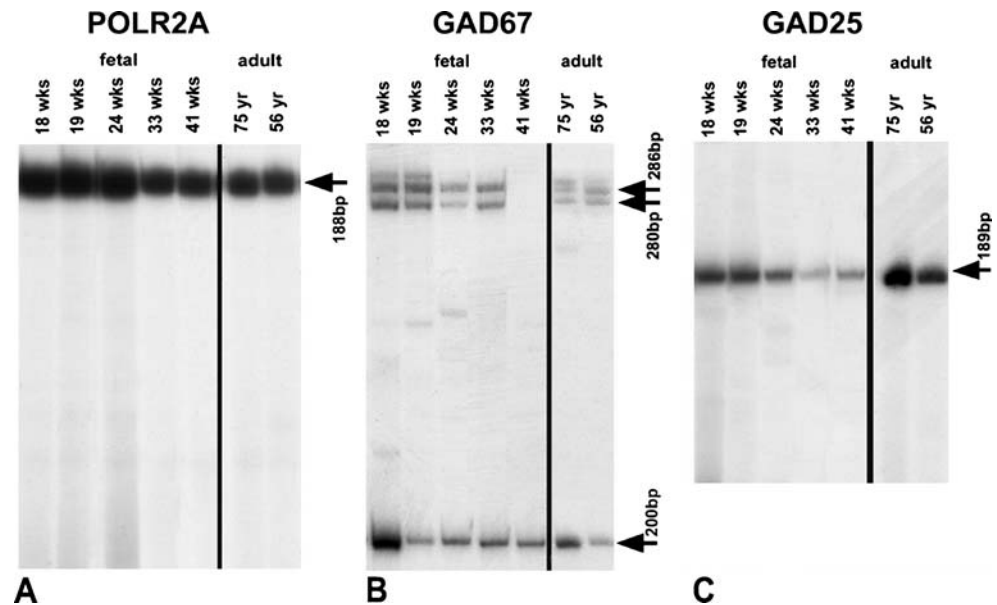
RT-PCR

The housekeeping gene *POLR2A* was used as a positive control for the RT reaction and showed positive results for all samples (Fig. 2A). Using the *GAD67* primers, four distinct PCR products per sample were observed. In all samples, a 200-bp amplified product was present, which represents the adult *GAD67* isoform, without insertion of exon 7. The 280-bp product and the 286-bp product were highly reproducible and detected in most fetal and all adult samples (Fig. 2C). Three fetal pancreatic samples showed only the 286-bp additional band. An additional PCR product of about 330-bp appeared reproducibly in the majority of samples. This product was sequenced, as well as the 280-bp and the 286-bp PCR products (see below). To detect the *GAD25* mRNA, we performed PCR with specific primers for *GAD25*, i.e., a forward primer in exon 6 and a reverse primer in the last part of exon 7 (Fig. 1). The results show an expression of this *GAD25* form in all fetal and adult pancreas specimens (Fig. 2C). In general, there appeared to be no differences in expression levels by RT-

Table 1 Primers used for PCR

Specificity	Forward (5'-3')	Reverse (5'-3')	Expected product size
<i>GAD67</i>	gagaatggctgacatcaacg	gaagtactttagcgcgagcag	200 bp
<i>GAD25</i>	tttaaccagctctcca	tcagttaccatcacctctg	189 bp
<i>POLR2A</i>	cggatgaactgaagcgaatg	ttgtaggtccacaagcag	188 bp

Fig. 2 A–C Results of RT-PCR experiments of seven representative samples, with primers against *POLR2A* (A), *GAD67* (B), and *GAD25* (C). The arrows indicate the sizes of the PCR products, which are 188 bp for *POLR2A* (A); 200, 280, and 286 bp for *GAD67*, respectively (B); and 189 bp for *GAD25* (C)



PCR between the different gestational ages or the adult samples for all of the primer combinations used.

Sequence Analysis

Sequence analysis with specific forward and reverse primers revealed that the two additional PCR products of 280 and 286 bp consisted of the adult form with an insertion of either the first 80 or 86 bp of exon 7. The additional product of 330 bp (Fig. 3), consisting of exons 6 to 9, with an insertion of 68 bp between exons 6 and 7 (Fig. 4), appeared to be a new splice product. This new exon was flanked by authentic splice junctions: 5'-AG/GT-3'. The additional sequence was homologous to a part of intron 6 of the human *GAD67* gene (51.128–51.195 of GenBank™ accession number AC007405). The first 213 amino acids of the predicted protein are homologous to *GAD25*,

followed by another 26 amino acids. The predicted molecular weight would approximately be 29.3 kDa.

Discussion

The potential role for *GAD* in pancreas development and the lack of knowledge about the distribution of the isoforms and splice variants of *GAD* led us to investigate *GAD67* and *GAD25* expression in the human fetal pancreas. We performed RT-PCR, on human fetal and adult pancreases, and found mRNA expression of *GAD67* and *GAD25* from 14 weeks gestation onward until term, as well as in the adult pancreas specimens. Apart from the adult form of *GAD67*, the RT-PCR experiments revealed three additional splice variants, which have not been described previously in the human pancreas.

Our study revealed *GAD67* mRNA expression in all fetal and adult pancreases. Whereas *GAD67* expression at the mRNA and protein level is well established in the mouse pancreas, only a few studies, using RNA protection assay analysis and RT-PCR, have demonstrated *GAD67* mRNA expression in the human pancreas [11, 17]. In contrast, *GAD67* was never detected in the human pancreas at the protein level, although some studies found *GAD67* autoantibodies in type 1 diabetes mellitus patients, in the absence of *GAD65* autoantibodies [5–7]. An explanation for the negative findings of previous studies could be that the *GAD67* protein has already degraded because the half-life of the *GAD67* protein is less than 4 h [18].

According to the literature, alternative splicing of the *GAD67* gene results in at least four mRNA products [3, 4].

Fig. 3 Four distinct RT-PCR products appear when *GAD67* primers are used in this human fetal pancreas of 16 weeks of gestation. In lane 1, the native *GAD67* PCR product of 200 bp is seen. Lane 2 represents the PCR products of the I-80 and I-86 splice variants, whereas lane 3 shows the newly found PCR product of 330 bp

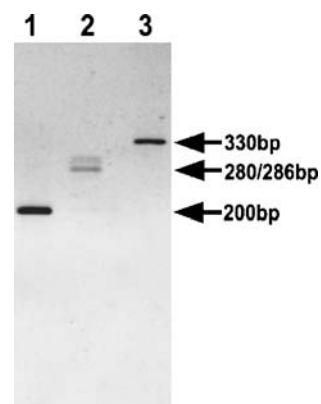


Fig. 4 Forward sequence of the additional PCR product of 330 bp

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exon 6                               new exon
ACCAACAT | GTCATGGTTAGCTTACAGACTGTCCATGTTGGGTCCTGAGATTTGGAAGA
                                                exon 7
GCCAAGAGGAATACAG | GCCATCAGACATGAGGGAGTGTGGTTGCTACGGTGATGGGGCT

CAGAGCAGAACCAAAGCATGATTGTGACCTCCAGAGGTGATG | GTTTACATATGAAATTGC
exon 8
ACCAGTGTGGTCTCCTCATGGAACAAATAACACTTAAGAAGATGAGAGAGATAGTTGGATGG
                                                exon 9
TCAAGTAAAGATGGTGATGGGATATTTTCTCCTGG | GGGCGCCATATCCAACATGTACAGC
ATCATGGCTGCTCGCTACAAGTACTTC

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In our study, we demonstrate four *GAD67* mRNA isoforms in the human fetal and adult pancreas samples. The first discovered transcript was an mRNA product, which lacks exon 7 and encodes for the native 67-kDa protein (*GAD67*) (see the “Results” section). A second transcript comprised the first seven exons of the *GAD67* gene, encoding a 25-kDa protein (*GAD25*). This variant was previously reported in human nonneural tissues by Chessler et al. [19]. A third and fourth transcript contained the first 80 or 86 bp of exon 7. So far, these mRNA variants have only been described in embryonic mouse brain [3, 4]. In contrast to the mouse brain, where the *GAD67* mRNA splice variants seem to have a developmentally regulated expression pattern [4], we did not see an increase of the 80-bp splice form, nor did we detect a decrease of the 86-bp during gestation, compared to each other in the same sample. In addition, all different mRNA isoforms described above were detected in most human fetal pancreases from 14 weeks of gestation until term even in the adult pancreases. It should be noted, though, that these experiments were not set up to yield quantitative data and therefore must be interpreted with caution.

The additional RT-PCR product of 330 bp represents a heretofore undescribed splice variant of *GAD67*, with an insertion of an unknown exon before the I-86 insert. The predicted protein would consist of 213 amino acids, of which the first part consists of the same amino acids as *GAD25*, followed by another 26 amino acids, and would have a molecular weight of approximately 29 kDa. Szabo et al. [4] observed two isoforms of approximately 31 and 32 kDa in embryonic mouse brain by Western blot. These products were assumed to be the result of posttranslational modifications. The predicted protein of our newly found splice variant might actually represent one or both of the proteins found by Szabo et al. [4].

In summary, we found *GAD67* mRNA expression during the entire gestational period investigated. In addition, *GAD67* splice variants were present in both fetal and adult pancreases. We also found a heretofore undescribed *GAD67* mRNA splice variant, which is also found in the fetal and

adult pancreases. These results suggest that *GAD67* and its splice variants might play a role in the developing human fetal pancreas and in the maintenance of the human adult pancreas.

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