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Analysis of intron-exon positioning on glutamate decarboxylase and its relation with evolution

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Glutamate decarboxylase (GAD) is an enzyme that catalyzes a production of GABA. The positions of intron-exon boundaries are located on mammalian/fruit fly GAD genes and their relations with the evolution, function, and structure of the protein are considered. The intron-exon boundaries were assigned on the primary structures of GAD65 of human, rat, and mouse, GAD67 of human, rat, mouse and chicken, and fruit fly GAD. GAD65 and 67 share an identical positions for intron-insertion and similar amino acid sequences except highly heterogeneous exons 1-3 coding region. When fruit fly GAD was compared with mammalian GADs, N-terminal region was constructed with only 20 amino acids for exon 1 in fruit fly GAD whereas 100 residues for exon 1-3 in mammalian GADs. The total number of introns in fruit fly GAD is significantly lower than that in mammalian GADs as if fruit fly has lost its introns during the evolution. The presence of common ancestral gene for fruit fly and mammalian GADs is assumed. The intron-exon boarders are assigned on the predicted structures of the examined GADs. Most of the intron insertion positions are not located directly on the defined secondary structural units or in the hydrophobic core. We have examined the relationship between the intron-exon boundary and a module, a most compact unit in protein structure proposed by Go [1], and have confirmed the boundary exhibits close correlation with the module boundary.

Keywords: bioinformatics, evolution, exon, GAD, intron, module

Introduction

Glutamate decarboxylase (GAD, EC 4.1.1.15) is an enzyme to synthesize γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in higher animals [2]. GAD is widely distributed among living cells of various organisms from mammalians to single cell organisms [2, 3]. Recently, it has shown that mammals have two distinct genes for GAD; for instance, human has one on chromosome 2 and the other on chromosome 10, where each produces GAD67 and GAD65, respectively [4-6]. The two GADs respond differently to their common cofactor pyridoxal 5'-phosphate (PLP) and differ in subcellular location [7, 8], which suggests that GAD67 and GAD65 may have different physiological roles. Therefore, the functional roles of GAD isoforms have been investigated [3].

Obata and others have created knock-out mice to demonstrate different functionality of GAD isoforms. For example, GAD67-/- mice born with cleft palate are dead within a day after birth

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Abbreviations: 3D, three dimensional; DDC, dihydroxyphenylalanine decarboxylase or L-aromatic amino acid decarboxylase; GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; PDB, Protein Data Bank; PLP, pyridoxal 5'-phosphate

while GAD65-/- mice are survived with a slightly increased tendency in having seizures [9]. GAD67 protein is a hydrophilic and soluble polypeptide which is found mainly in the cell bodies of neurons and in the cytosol of other cells particularly pancreatic β-cells. GAD65 is more hydrophobic and less soluble than GAD67 and reversibly anchored to the membrane of synaptic vesicles of neurons and synaptic-like microvesicles in pancreatic β-cells [7]. It has been suggested that GAD67 is responsible for most of the metabolic GABA synthesis in the brain [8, 9].

Pancreatic GAD65 was identified as a target antigen for autoantibody found in the blood circulation of patients with insulin-dependent (type I) diabetes [10, 11]. Accumulated evidences have suggested that GAD isoforms play different roles within a single cell. Moreover, it is probable to assume that the differences of functionality of the isoforms could be attributed to the differences in their structures. However, for GADs from most of the species, direct correlation of the exon boarders and the tertiary or secondary structure, has not been established and needs to be elucidated. Recently, an X-ray structure of GAD is reported for *E. coli* GAD_B by Capitani *et al.* who performed a homology modeling of GAD65 based on reported structures of pig DOPA decarboxylase (DDC) and *E. coli* GAD_B [PDB No. 1JS6 and 1PMM] [12, 13].

Eukaryotic genes encoding proteins are split by introns, whereas prokaryotic genes are not split by introns except for some cases such as plasmid of *Agrobacterium rhizogenes* [14]. The origin of those spliceosomal introns and their functional roles are still under investigation. It is still controversial that whether or not 1) introns existed in the ancestral genome of prokaryotes, eukaryotes, or both, or 2) introns were inserted into eukaryotic genomes in later stage during the evolution [15]. If it would be the former case, prokaryotes should have lost the introns from their genomes and slimmed down the size of genomic sequences. If the latter case is assumed, the introns could be inserted randomly into the eukaryotic genes, where the introns might have originated from the insertion elements such as transposable elements. Doolittle and Gilbert have suggested that the resulting introns would not contribute to the creation or modification of the protein function [16, 17].

A term "domain" is referred as a critical structural unit when the three-dimensional (3D) structure of protein is discussed. In 1981, Go introduced a concept of "module" which is a part of domain structure but more compact than domain [1]. A module is a peptide segment of about 15 residues long in an average and constructs a compact structural unit in the protein structure. The term "module" is also referred to as the most compact functional unit of protein [1, 18, 19].

Recent studies have demonstrated that the location of introns in a gene exhibits close correlation with module boundaries in a protein structure, which has drawn an attention as the understanding of intron positions is crucial for considering the protein structure/function relationships [1, 18, 20]. A protein evolutional study allows the origin of intron to be traced back into the ancestral gene; however, the roles of intron as the mediator of exon shuffling have not been fully realized. In this study, GAD genomes of fruit fly, mouse, rat, chicken and human are analyzed for the intron-exon borders as well as their structural and functional relationships, particularly linked to the evolution of GAD.

Materials and Methods

Multiple alignments - Primary amino acid sequences and intron-exon information on GADs were obtained from the Ensemble genome browser (http://www.ensembl.org) for mammals and GTOP (http://spock.genes. nig.ac.jp/ genome/gtop-j.html) for fruit fly. The multiple alignments were carried out using the CLUSTALW program (http://www.ebi.ac.uk/clustalw/). The access numbers in Ensemble for human, rat, mouse and chicken, and GTOP for fruit fly are as follows: human GAD65: ENST00000259271, human GAD67: ENST00000249471, rat GAD65: ENSRNOT0000000000824901, rat GAD67: ENSRNOT00000000008, mouse GAD65: ENSMUST00000028123, mouse GAD67: ENSMUST00000028507, chicken GAD67: ENSGALT00000015628 , fruit fly: CG14994.

Structure analysis – Secondary structure prediction was performed with the program

Table 1 Comparison of amino acid sequences at the site where intron being allocated among mammalian and fruit fly GADs. The amino acid at the intron site is indicated in red (majority) or blue (minority) with its residue number beneath.

boundary number	human67	rat67	mouse67	chicken67	human65	rat655	mouse6	boundary number	fly
1	TTY 28	TTY 27	TTY 27	TTY 24	TAR 26	TAR 26	TAR 26		
2	CGF 49	CGF 48	CGF 48	CGF 45	CAL 46	CAL 46	CAL 46		
3	RDL 102	QDL 101	QDL 101	RDL 99	TDL 96	TDL 96	TDL 96	1	YDL 21
4	TGH 183	TGH 182	TGH 182	TGH 179	TGH 174	TGH 174	TGH 174	2	TGH 100
5	NMF 213	NMF 212	NMF 213	NMF 212	NMF 204	NMF 204	NMF 204		
6	PGG 251	PGG 250	PGG 250	PGG 247	PGG 242	PGG 244	PGG 242		
7	QSH 290	HSH 289	HSH 289	HSH 286	HSH 281	HSH 281	HSH 281		
8	ERG 316	ERG 315	ERG 315	ERG 312	ERG 307	ERG 307	ERG 307		
9	KGY 335	KGF 334	KGY 334	KGY 331	KGF 326	KGF 326	KGF 326		
10	DAA 374	DAA 373	DAA 373	DAA 370	DAA 365	DAA 365	DAA 365	3	IDA 289
11	ERA 395	ERA 394	ERA 394	ERA 391	ERA 386	ERA 386	ERA 386	4	ERA 312
12	KGI 422	KGI 421	KGI 421	KGI 418	EGL 413	EGL 413	EGL 413	5	EDG 338
13	KGT 472	KGT 471	KGT 471	KGT 468	KGT 463	KGT 463	KGT 463		
14	EPE 508	EPE 507	EPE 507	EPE 504	KPQ 499	KPQ 499	KPQ 499		
15	KVA 538	RVA 537	RVA 537	RVA 534	KVA 529	KVA 529	KVA 529	6	GKI 453

JPRED (http://www.compbio.dundee.ac.uk/~www-jpred/). Relative solvent accessibility of each amino acid of pig DDC and *E. coli* GAD_B was evaluated by the program DSSP (http://swift.cmbi.ru.nl/gv/dssp/) based on their X-ray structures.

X-ray structures of pig DDC and *E. coli* GAD_B - The crystal structures of DDC from *Sus scrofa* (Protein Data Bank (PDB) entry no. 1JS3) and GAD_B from *E. coli* at low pH (active form, PDB entry no. 1PMM) were obtained from PDB (http://www.rcsb.org/pdb/Welcome.do). Structural alignments among *E. coli* GAD_B, pig DDC and human GAD65 sequences were carried out based upon Capitani's study [12].

Evolutional tree - Evolutional analysis for amino acid sequences of mammalian and fruit fly GADs was performed by the method of neighbor-joining using CLASTALW. The evolutional tree was generated by the software Tree View (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

Multiple alignments - The coding regions of human, rat and mouse GAD65 genomes are composed with 16 exons (Fig. 1a). Exon 1 contains the 5' untranslated region of GAD65 mRNA, and exon 16 contains the protein's carboxyl terminal region and a small portion of untranslated region. Similarly, the coding region of human GAD67 genomes consists of 16 exons. Rat, mouse, and chicken GAD67 genomes contain 17 exons, where an additional exon, exon 0, together with a part of exon 1 contains the 5' untranslated region of GAD67 mRNA (Table 1). Exon 16 of GAD67 covers the entire 3' untranslated region of GAD67 mRNA. The genomes of both GAD isoforms from mammalian species that we have examined exhibit high identity in the intron insertion positions. The comparison of the primary structures GAD65 from mammalian species showed over 90% identity (Fig.

(a)

```
                                         ①                   ②
humanGAD65      MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYGDAEKPAESGG 60
ratGAD65        MASPGSGFWSFGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYGDSEKPAESGG 60
mouseGAD65      MASPGSGFWSFGSEDGSADPENPGTARAWCQVAQKFTGGIGNKLCALLYGDSGKPAEGGG 60
                *****************.*.*******************************: ****.**

                                                   ③
humanGAD65      SQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPACDGERPTLAFLQDVMNILLQ 120
ratGAD65        SVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPACEGERPTLAFLQDVMNILLQ 120
mouseGAD65      SVTSRAATGKVACTCDQKPCNCPKGDVNYAFLHATDLLPACDGERPTLAFLQDVMNILLQ 120
                * ..***: *.**:******.*.* *****:**********:******************

                                                                     ④
humanGAD65      YVVKSFDRSTKVIDFHYPNELLQEYNWELADQPQNLEEILMHCQTTLKYAIKTGHPRYFN 180
ratGAD65        YVVKSFDRSTKVIDFHYPNELLQEYNWELADQPQNLEEILTHCQTTLKYAIKTGHPRYFN 180
mouseGAD65      YVVKSFDRSTKVIDFHYPNELLQEYNWELADQPQNLEEILTHCQTTLKYAIKTGHPRYFN 180
                **************************************** *******************

                                       ⑤
humanGAD65      QLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFS 240
ratGAD65        QLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFS 240
mouseGAD65      QLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFS 240
                ************************************************************

                 ⑥                                      ⑦
humanGAD65      PGGAISNMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
ratGAD65        PGGAISNMYAMLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
mouseGAD65      PGGAISNMYAMLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
                ***********:***:*************:******************************

                      ⑧                  ⑨
humanGAD65      LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAVADICKKYKIW 360
ratGAD65        LIKCDERGKMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAVADICKKYKIW 360
mouseGAD65      LIKCDERGKMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAVADICKKYKIW 360
                *********************.**************************************

                    ⑩                    ⑪                          ⑫
humanGAD65      MHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGVPLQCSALLVREEGLMQNCNQ 420
ratGAD65        MHVDAAWGGGLLMSRKHKWKLNGVERANSVTWNPHKMMGVPLQCSALLVREEGLMQSCNQ 420
mouseGAD65      MHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGVPLQCSALLVREEGLMQSCNQ 420
                *********************.**********************************.***

                                                          ⑬
humanGAD65      MHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHVDKCLELAEY 480
ratGAD65        MHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHIDKCLELAEY 480
mouseGAD65      MHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHIDKCLELAEY 480
                **************************************************:*********

                                  ⑭                             ⑮
humanGAD65      LYNIIKNREGYEMVFDGKPQHTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEY 540
ratGAD65        LYNIIKNREGYEMVFDGKPQHTNVCFWFVPPSLRVLEDNEERMSRLSKVAPVIKARMMEY 540
mouseGAD65      LYTIIKNREGYEMVFDGKPQHTNVCFWFVPPSLRTLEDNEERMSRLSKVAPVIKARMMEY 540
                **.************************::*****.*************************

humanGAD65      GTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
ratGAD65        GTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
mouseGAD65      GTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
                *********************************************
```

Fig. 1 Comparison of the amino acid sequences at the sites of intron intervention in the coding region of mammalian GADs and fruit fly GAD cDNAs. (a) mammalian GAD65, (b) mammalian GAD67, (c) human GAD67 and GAD65, (d) human GAD67, GAD65 and fruit fly GAD, respectively. Numbers on the right are residue positions. The intron insertion sites are shown in gray highlight. The numbers of intron insertion sites are shown above with circle or square for mammalian or fruit fly, respectively. Deduced protein sequences are aligned by using the program CLUSTALW. Amino acid identity is indicated by asterisk for the match, colon for strong similarity and dot for weak similarity. Absence of the compatible residues at the position is indicated by blank space.

(b)

```
                                             ①                    ②
humanGAD67        MASSTPSSSATSSNAGADPNTTNLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEK 60
ratGAD67          MASSTPSP-ATSSNAGADPNTTNLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEK 59
mouseGAD67        MASSTPSP-ATSSNAGADPNTTNLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEK 59
chickenGAD67      MASSAPSS----SNDAPDPAPTNLRPTTYDSWCGVAHGCTRKIGLKICGFLQRTNSLEDK 56
                  ****:**.    ** ..** .*********:***********:***************:*

                                                           ③
humanGAD67        SRLVSAFKERQSSKNLLSCENSDRDARFRRTETDFSNLFARDLLPAKNGEEQTVQFLLEV 120
ratGAD67          SRLVSAFRERQASKNLLSCENSDPGARFRRTETDFSNLFAQDLLPAKNGEEQTVQFLLEV 119
mouseGAD67        SRLVSAFRERQSSKNLLSCENSDQGARFRRTETDFSNLFAQDLLPAKNGEEQTAQFLLEV 119
chickenGAD67      GRIVGSLKERQSSKNLLACENSEREARFRRTETDFSNLYARDLLPAKNGEEQTMQFLLEV 116
                  .*:*.:::***:*****:****:  *************:*:************ ******

humanGAD67        VDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDHPESLEQILVDCRDTLKYGV 180
ratGAD67          VDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDHPESLEQILVDCRDTLKYGV 179
mouseGAD67        VDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDHPESLEQILVDCRDTLKYGV 179
chickenGAD67      VDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDNPESLEQILVDCRDTLKYGV 176
                  ****************************************:*******************

                    ④                             ⑤
humanGAD67        RTGHPRFFNQLSTGLDIIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLKKMREIVGWS 240
ratGAD67          RTGHPRFFNQLSTGLDIIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLKKMREIIGWS 239
mouseGAD67        RTGHPRFFNQLSTGLDIIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLKKMREIVGWS 239
chickenGAD67      RTGHPRFFNQLSTGLDMIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLRKMREIIGWS 236
                  ****************:*********************************:*****:***

                            ⑥                                      ⑦
humanGAD67        SKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVPKLVLFTSEQSHYSIKKAGAA 300
ratGAD67          NKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVPKLVLFTSEHSHYSIKKAGAA 299
mouseGAD67        NKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVPKLVLFTSEHSHYSIKKAGAA 299
chickenGAD67      NKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVPKLVLFTSEHSHYSIKKAGAA 296
                  .***********************************************:***********

                                 ⑧                  ⑨
humanGAD67        LGFGTDNVILIKCNERGKIIPADFEAKILEAKQKGYVPFYVNATAGTTVYGAFDPIQEIA 360
ratGAD67          LGFGTDNVILIKCNERGKIIPADLEAKILDAKQKGFVPLYVNATAGTTVYGAFDPIQEIA 359
mouseGAD67        LGFGTDNVILIKCNERGKIIPADLEAKILDAKQKGYVPLYVNATAGTTVYGAFDPIQEIA 359
chickenGAD67      LGFGTDNVILIKCNERGKIIPADLEAKILEAKQKGYVPLFVNATAGTTVYGAFDPIQEIA 356
                  ***********************:*****:*****:**::********************

                               ⑩                    ⑪
humanGAD67        DICEKYNLWLHVDAAWGGGLLMSRKHRHKLNGIERANSVTWNPHKMMGVLLQCSAILVKE 420
ratGAD67          DICEKYNLWLHVDAAWGGGLLMSRKHRHKLSGIERANSVTWNPHKMMGVLLQCSAILVKE 419
mouseGAD67        DICEKYNLWLHVDAAWGGGLLMSRKHRHKLSGIERANSVTWNPHKMMGVLLQCSAILVKE 419
chickenGAD67      DICEKYNLWLHVDAAWGGGLLMSRKHRHKLNGIERANSVTWNPHKMMGVLLQCSAILVRE 416
                  ******************************.***************************:*

                   ⑫                                                 ⑬
humanGAD67        KGILQGCNQMCAGYLFQPDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFENQI 480
ratGAD67          KGILQGCNQMCAGYLFQPDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFENQI 479
mouseGAD67        KGILQGCNQMCAGYLFQPDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFENQI 479
chickenGAD67      KGILQGCNQMCAGYLFQQDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFENQI 476
                  ***************** ******************************************

                                             ⑭                             ⑮
humanGAD67        NKCLELAEYLYAKIKNREEFEMVFNGEPEHTNVCFWYIPQSLRGVPDSPQRREKLHKVAP 540
ratGAD67          NKCLELAEYLYAKIKNREEFEMVFNGEPEHTNVCFWYIPQSLRGVPDSPERREKLHRVAP 539
mouseGAD67        NKCLELADYLYAKIKNREEFEMVFDGEPEHTNVCFWYIPQSLRGVPDSPERREKLHRVAP 539
chickenGAD67      NKCLELAEYLYTKIKNREEFEMVFEGEPEHTNVCFWYIPPSLRGMPDCDERREKLHRVAP 536
                  *******:***:************:************** ****:**. :******:***

humanGAD67        KIKALMMESGTTMVGYQPQGDKANFFRMVISNPAATQSDIDFLIEEIERLGQDL 594
ratGAD67          KIKALMMESGTTMVGYQPQGDKANFFRMVISNPAATQSDIDFLIEEIERLGQDL 593
mouseGAD67        KIKALMMESGTTMVGYQPQGDKANFFRMVISNPAATQSDIDFLIEEIERLGQDL 593
chickenGAD67      KIKALMMESGTTMVGYQPQGDKVNFFRMVISNPAATKSDIDFLIEEIERLGQEL 590
                  **********************.*************:***************:*
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Fig. 1b. (continued)

```
(c)                                                 ①                    ②
humanGAD67      MASSTPSSSATSSNAGADPNTTNLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEK 60
humanGAD65      MAS--PGS-GFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYGD---AEK 54
                ***. *.* : .*: *:. . .    * *  :** **:  *  :* *:*.:*      **

                                                            ③
humanGAD67      SRLVSAFKERQSSKNLLSCENSDRDARFRRTETDFSNLFARDLLPAKNGEEQTVQFLLEV 120
humanGAD65      PAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPACDGERPTLAFLQDV 114
                .   .. :  ::: .  :*  .:: .   :.:.::: *.* ***** :**. *: ** :*

humanGAD67      VDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDHPESLEQILVDCRDTLKYGV 180
humanGAD65      MNILLQYVVKSFDRSTKVIDFHYPNELLQ---EYNWELADQPQNLEEILMHCQTTLKYAI 171
                ::***:** *:*******:***:*::**:    :* **:*:*:.**:**:.*: ****.:

                  ④                         ⑤
humanGAD67      RTGHPRFFNQLSTGLDIIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLKKMREIVGWS 240
humanGAD65      KTGHPRYFNQLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGWP 231
                :*****:*********::***.:*********************:* :********:**.

                      ⑥                                    ⑦
humanGAD67      SKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVPKLVLFTSEQSHYSIKKAGAA 300
humanGAD65      GGSGDGIFSPGGAISNMYAMMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAA 291
                . .***************::* **:* ***** *****:*:*: ****:**:*:**..**

                           ⑧                 ⑨
humanGAD67      LGFGTDNVILIKCNERGKIIPADFEAKILEAKQKGYVPFYVNATAGTTVYGAFDPIQEIA 360
humanGAD65      LGIGTDSVILIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAVA 351
                **:***.******:****:**:*:* :*********:*** *.*************:  :*

                         ⑩                 ⑪
humanGAD67      DICEKYNLWLHVDAAWGGGLLMSRKHRHKLNGIERANSVTWNPHKMMGVLLQCSAILVKE 420
humanGAD65      DICKKYKIWMHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGVPLQCSALLVRE 411
                ***:**::*:****************: **.*:*************** *****:**:*

                 ⑫                                               ⑬
humanGAD67      KGILQGCNQMCAGYLFQPDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFENQI 480
humanGAD65      EGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFEAHV 471
                :*::*.**** *.**** **:**:********:*******:**:****:****.*** ::

                                            ⑭                           ⑮
humanGAD67      NKCLELAEYLYAKIKNREEFEMVFNGEPEHTNVCFWYIPQSLRGVPDSPQRREKLHKVAP 540
humanGAD65      DKCLELAEYLYNIIKNREGYEMVFDGKPQHTNVCFWYIPPSLRTLEDNEERMSRLSKVAP 531
                :**********  ***** :****:*:*:********** ***  : *. :* .:* ****

humanGAD67      KIKALMMESGTTMVGYQPQGDKANFFRMVISNPAATQSDIDFLIEEIERLGQDL 594
humanGAD65      VIKARMMEYGTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
                 *** *** *****.*** ***.*************:.****************
```

Fig. 1c. (continued)

1a). Similar results were obtained for GAD67 (Fig. 1b). On the other hand, when the primary structures were compared between GAD67 and GAD65 from the same species, the sequence homology declined to 65% [21]. Despite of the sequence diversities among mammalian species, the positions of the exon-intron boundaries in all mammalian GADs were virtually identical (Table 1 and Fig. 1d). It is of interest to note that most of the heterogeneity was found within N-terminal 100 residues. The N-terminal regions of human as well as the other mammalian GADs have been coded by exons 1-3 (Fig. 1c). When details of exons 1-3 were compared, the level of heterogeneity appeared to be different for each of the exons. While there are about 30% residues conserved between GAD65 and GAD67 on exon 1 and exon 2, the frequency of the conservation becomes extremely low in case of exon3.

Fruit fly GAD genomes include seven exons, of which exon 0 and a part of exon 1 contain the 5' untranslated region of fruit fly GAD mRNA (Table

```
(d)                                           ①                   ②
humanGAD67   MASSTPSSSATSSNAGADPNTTNLRPTTYDTWCGVAHGCTRKLGLKICGFLQRTNSLEEK 60
humanGAD65   MASPGSGFWSFGSEDGSGDSEN---PGTARAWCQVAQKFTGGIGNKLCALLYGD---AEK 54
fruit fly    MSLNPNGYKLSERTG--------------------------------------------- 15
              *  ..    ...*

                                                          ③/[1]
humanGAD67   SRLVSAFKERQSSKNLLSCENSDRDARFRRTETDFSNLFARDLLP-AKNGEEQTVQFLLE 119
humanGAD65   PAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLP-ACDGERPTLAFLQD 113
fruit fly    ------------------------------------KLTAYDLMPTTVTAGPETREFLLK 39
                                                 * * **:* :  .   *  ** .

humanGAD67   VVDILLNYVRKTFDRSTKVLDFHHPHQLLEGMEGFNLELSDHPESLEQILVDCRDTLKYG 179
humanGAD65   VMNILLQYVVKSFDRSTKVIDFHYPNELLQ---EYNWELADQPQNLEEILMHCQTTLKYA 170
fruit fly    VIDVLLDFVKATNDRNEKVLDFHHPEDMKR---LLDLDVPDRALPLQQLIEDCATTLKYQ 96
             *:::**::* : **. **:***:*.:: .     : ::.*:.  *:::: .*  ****

   ④/[2]                          ⑤
humanGAD67   VRTGHPRFFNQLSTGLDIIGLAGEWLTSTANTNMFTYEIAPVFVLMEQITLKKMREIVGW 239
humanGAD65   IKTGHPRYFNQLSTGLDMVGLAADWLTSTANTNMFTYEIAPVFVLLEYVTLKKMREIIGW 230
dmel GAD     VKTGHPHFFNQLSNGLDLISMAGEWLTATANTNMFTYEIAPVFILMENVVLTKMREIIGW 156
             ::****::*****.***::.:*.:***:**************:*:* :.* *****:**

                   ⑥                                      ⑦
humanGAD67   SSKDGDGIFSPGGAISNMYSIMAARYKYFPEVKTKGMAAVP-KLVLFTSEQSHYSIKKAG 298
humanGAD65   PGGSGDGIFSPGGAISNMYAMMIARFKMFPEVKEKGMAALP-RLIAFTSEHSHFSLKKGA 289
fruit fly    SG--GDSILAPGGSISNLYAFLAARHKMFPNYKEHGSVGLPGTLVMFTSDQCHYSIKSCA 214
             ..  **.*::***:***:*::: **.* **: * :* ..:*  *: ***::.*:*:*. .

                        ⑧                ⑨
humanGAD67   AALGFGTDNVILIKCNERGKIIPADFEAKILEAKQKGYVPFYVNATAGTTVYGAFDPIQE 358
humanGAD65   AALGIGTDSVILIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLA 349
fruit fly    AVCGLGTDHCIVVPSDEHGKMITSELERLILERKAKGDIPFFVNATAGTTVLGAFDDINT 274
             *. *:***  *:: .:*:**:*.:::*  **: * ** :*: *.******* **** :

                     ⑩/[3]              ⑪/[4]
humanGAD67   IADICEKYNLWLHVDAAWGGGLLMSRKHRH-KLNGIERANSVTWNPHKMMGVLLQCSAIL 417
humanGAD65   VADICKKYKIWMHVDAAWGGGLLMSRKHKW-KLSGVERANSVTWNPHKMMGVPLQCSALL 408
fruit fly    IADICQKYNCWMHIDAAWGGGLLMSRKHRHPRFTGVERADSVTWNPHKLMGALLQCSTIH 334
             :****:**: *:*:**************:  ::.*:***:********:**. ****::

   ⑫/[5]                                                  ⑬
humanGAD67   VKEKGILQGCNQMCAGYLFQPDKQYDVSYDTGDKAIQCGRHVDIFKFWLMWKAKGTVGFE 477
humanGAD65   VREEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHVDVFKLWLMWRAKGTTGFE 468
fruit fly    FKEDGLLISCNQMSAEYLFMTDKQYDISYDTGDKVIQCGRHNDIFKLWLQWRAKGTEGFE 394
             .:*.*:: .**** * ***  **:**:*******.:***** *:**:** *:**** ***

                                  ⑭
humanGAD67   NQINKCLELAEYLYAKIKNR-EEFEMVFNGEPEHTNVCFWYIPQSLRGVPDSPQRREKLH 536
humanGAD65   AHVDKCLELAEYLYNIIKNR-EGYEMVFDGKPQHTNVCFWYIPPSLRTLEDNEERMSRLS 527
fruit fly    QQQDRLMELVQYQLKRIREQSDRFHLIL--EPECVNVSFWYVPKRLRGVPHDAKKEVELG 452
              : :: :**.:*    *::: : :.:::  :*: .**.**::* ** : .   ::   .*

⑮/[6]
humanGAD67   KVAPKIKALMMESGTTMVGYQPQGDKANFFRMVISNPAATQSDIDFLIEEIERLGQDL 594
humanGAD65   KVAPVIKARMMEYGTTMVSYQPLGDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
fruit fly    KICPIIKGRMMQKGTLMVGYQPDDRRPNFFRSIISSAAVNEADVDFMLDEIHRLGDDL 510
             ::.* **. **: ** **.*** . : **** :**..*...*:**:::**.***::*
```

Fig. 1d. (continued)

1). The exons 2-7 of fruit fly GAD and exons 4-16 of mammalian GADs show high amino acid sequence homology. Also fruit fly and mammalian GADs share almost identical exon-intron boundaries within this region (Fig. 1d). The number of exons in fruit fly GAD is significantly smaller than that of mammalians. This is because exon 3 in the fruit fly is extended so that it covers exons 5–10 of the mammalians (Table 1, Fig. 1d). In other words, fruit fly lacks introns corresponding to introns 5–10 of mammalians. Similarly, exon 6 of the fruit fly GAD, matching to the exons 13–15 of the

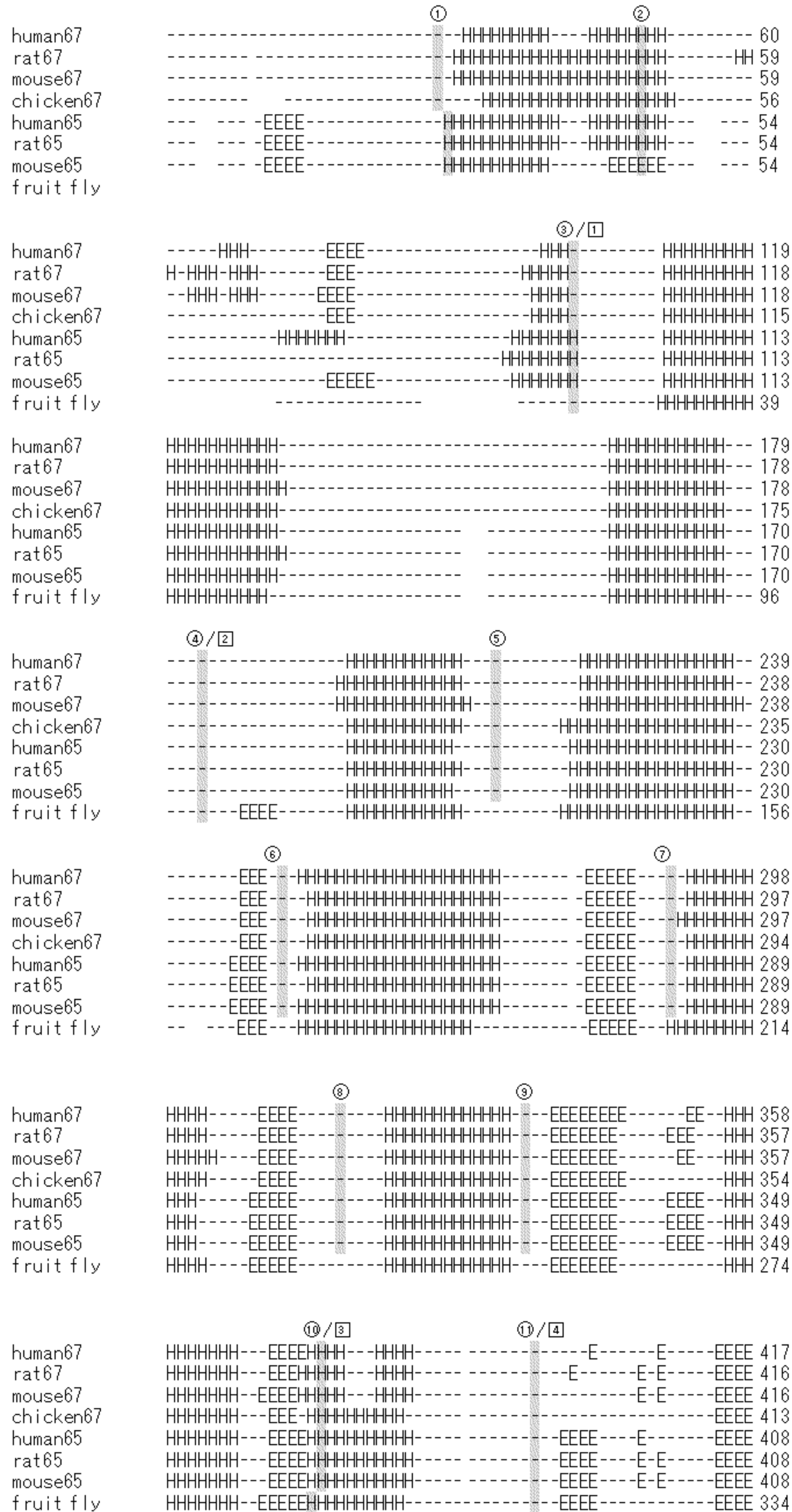

Fig. 2 Predicted secondary structures assigned on the aligned mammalian and fruit fly GAD amino acid sequences. Secondary structures predicted by JPRED: α-helix is indicated by H, β-structure by E and random structure by -. Numbers on the right are residue positions. The intron sites are shown in gray highlight. The numbers of intron insertion sites are shown above with circle or square for mammalian or fruit fly, respectively.

```
                 ⑫/[5]                                    ⑬
human67      E---------------------------------------HHHHHHHHHHHHHHHHHHHH 477
rat67        E-----EE--------------------------------HHHHHHHHHHHHHHHHHHHH 476
mouse67      E---------------------------------------HHHHHHHHHHHHHHHHHHHH 476
chicken67    E-----E---------------------------------HHHHHHHHHHHHHHHHHHHH 473
human65      E------HHH------------------------------HHHHHHHHHHHHHHHHHHHH 468
rat65        E---------------------------------------HHHHHHHHHHHHHHHHHHHH 468
mouse65      E---------------------------------------HHHHHHHHHHHHHHHHHHHH 468
fruit fly    E-------HHH-----------------------------HHHHHHHHHHHHHHHHHHHH 394

                                            ⑭
human67      HHHHHHHHHHHHHHHHHHH- ---EEEE------EEEEEEE-----------HHHHHHHH 536
rat67        HHHHHHHHHHHHHHHHHHH- --EEEEE-----EEEEEEEE-----------HHHHHHHH 535
mouse67      HHHHHHHHHHHHHHHHHHH- --EEEE------EEEEEEEE------------HHHHHHH 535
chicken67    HHHHHHHHHHHHHHHHHH-- ---EEE------EEEEEEEE-----------HHHHHHHH 532
human65      HHHHHHHHHHHHHHHHHHH- --EEEEE-----EEEEEEEE----------HHHHHHHHH 527
rat65        HHHHHHHHHHHHHHHHHHH- --EEEEE-----EEEEEEEE----------HHHHHHHHH 527
mouse65      HHHHHHHHHHHHHHHHHHH- --EEEEE-----EEEEEEEE----------HHHHHHHHH 527
fruit fly    HHHHHHHHHHHHHHHHHHH---EEEE-   ----EEEEEE---------------HHHHH 452

            ⑮/[6]
human67      HHHHHHHHHHHH---EEEEEE------EEEEEEE------HHHHHHHHHHHHHHHH-- 594
rat67        HHHHHHHHHHH----EEEE-------EEEEEEEE------HHHHHHHHHHHHHHHH-- 593
mouse67      HHHHHHHHHHHH---HHHH-------EEEEEEEE------HHHHHHHHHHHHHHHH-- 593
chicken67    HHHHHHHHHHHH----EE--------EEEEEEEE------HHHHHHHHHHHHHHH--- 590
human65      HHHHHHHHHHH----EEEEE------EEEEEEEE------HHHHHHHHHHHHHHH--- 585
rat65        HHHHHHHHHHHH---EEE--------EEEEEEEE------HHHHHHHHHHHHHHH--- 585
mouse65      HHHHHHHHHHHH---EEEEE------EEEEEEEE------HHHHHHHHHHHHHHH--- 585
fruit fly    HHHHHHHHHHHH---EEEEE------EEEEEEEE------HHHHHHHHHHHHHHH--- 510
```

Fig. 2. (continued)

mammalian GADs, does not have corresponding introns 13 and 14 in the mammalian GADs (Fig. 1d). The lengths of exons tend to be longer in fruit fly than those in mammals: The results are consistent with Go's description [22]. Exons 1-3 of mammalian GADs and exon 1 of fruit fly GAD were not homologous; however, a short segment at the end of exon 1 of fruit fly is conserved in both fruit fly and mammalian GADs (Fig. 1d).

Position of introns and its relationship with the secondary structure – The predicted secondary structure of the examined GADs are summarized in Fig. 2. In mammalian GADs, the position of introns in the secondary structures on the amino acid sequences and the intron-exon boundaries are highly conserved. The predicted secondary structure of fruit fly GAD was similar to exon 4 and thereafter of mammalian GADs. When the secondary structure assigned to N-terminal low homology region was examined, a segment covering entire exon 2 takes α-helical structure (Fig. 2). The secondary structure predicted for the remaining region exhibited close resemblance between the two isoforms. Therefore, the heterogeneous nature at the N-terminal region is considered.

The locations of introns are closely examined as they locate at the sites where no significant secondary structures being defined. Only two of fifteen mammalian GAD boundaries, #13 and #15, are located in the middle of their predicted long α-helical structure at the C-terminus region. In fruit fly, only intron #6 is equivalent to the two introns of mammals, locating at the middle of α-helix, and other five intron sites are outside of defined secondary structures.

Solvent accessibility of GAD and the positions of intron-exon boundaries – The solvent accessibility is one of good indicators that represent the spatial characteristics of the individual amino acid in the protein [22]. A three-dimensional model for human GAD65 was constructed by Capitani *et al.* based upon amino acid homology between human GAD65 and pig DDC and/or *E. coli* GAD_B, where X-ray structures of pig DDC and *E. coli* GAD_B were used as templates [12]. We have employed a similar approach as reported by Capitani *et al.* [12]. At first, an image of human GAD65 structure was superimposed onto pig DDC and *E. coli* GAD_B . Then, the spatial positioning of the intron-exon boundaries of GAD65 was assigned based on the

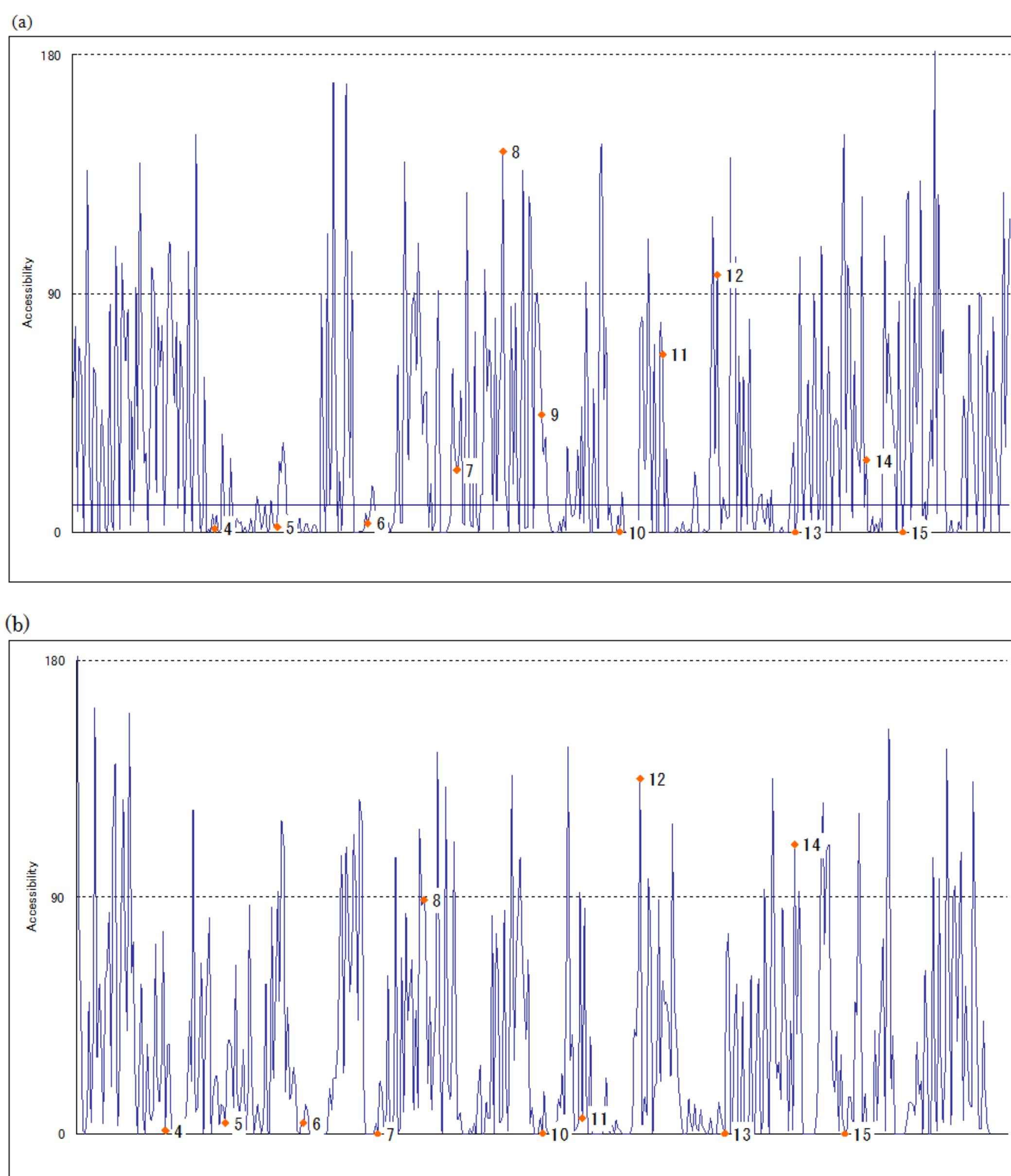

Fig. 3 Solvent accessibility profiles of (a) pig DDC and (b) *E. coli* GAD_B. Horizontal axis is amino acid residue numbers. Vertical axis is a relative solvent accessibility derived from crystal structure as calculated by DSSP program. Diamond marks show the amino acid compatible to the intron insertion sites of human GAD65.

solvent accessibility plots obtained from pig DDC and *E. coli* GAD_B as shown in Figs. 3a and 3b, respectively. The solvent accessibility plots for pig DDC and *E. coli* GAD_B were obtained by using DSSP program (Web site: http://swift.cmbi.ru.nl/gv/dssp/, Figs. 3a, 3b). In Figs. 3a and 3b, the sites corresponding to the intron-insertion positions on human GAD65 were indicated. As a result, out of twelve available intron sites in Fig. 3a, three introns, #8, 11, and 12, are on the surface and six intron sites, #4, 5, 6, 10, 13, and 15, are buried in the hydrophobic core regions. Remaining three intron sites, #7, 9, and 14 do not belong to either areas. In Fig. 3b, out of ten available intron sites, seven intron sites, #4-7, 11, 13 and 15, are positioned inside and the rest three introns, #8, 12, and 14 are found on the surface of the GAD molecule.

Discussion

Glutamate decarboxylase (GAD) are

distributed among the wide varieties of organisms including microorganisms, insects, plants, and animals [3]. Despite of the diverse distribution, GAD shows high amino acid sequence resemblances among species [3]. Although homology studies on GAD have been extensively carried out [23], little is understood how intron-exon shuffling has occurred in mammals and the relationship between the localization of intron-exon boarders and their positions in the secondary and tertiary structures. In this paper, we have assigned intron-exon boarders into the amino acid sequences of mammalian and fruit fly GADs and have tried to address the molecular evolution and predicted structure on mammalian GAD65.

Our comparative study on amino acid sequence homology between GAD65 and GAD67 has revealed a significant homology between two isoforms. Intron positions are almost identical between the isoforms. Since these facts can suggest that there was a common ancestral gene for mammalian GADs [21], we have looked into the evolutional process of GAD by examining the sequence homology and the intron positioning.

The heterogeneity between GAD65 and 67 lies mostly upon their N-terminal regions, where Bu and Tobin have noted the first 100 amino acid residues as a low homology region [21]. When

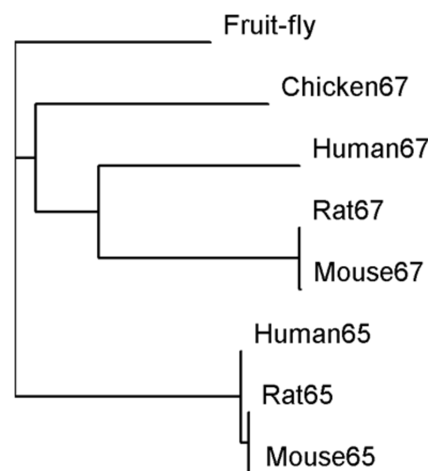

Fig. 4 Phylogenetic tree for mammalian GADs and fruit fly GAD. The presented tree was generated by the program of Tree View (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) based on the calculation by the method of neighbor-joining.

the exon boarders are marked upon the amino acid sequences, an interesting correlation between the low homology region and the exon boarder has become apparent; the low homology region is totally included within the exons 1-3. (Table 2, Fig. 1c). The remaining amino acid sequence that covers exons 4-16 is conserved between the isoforms (Fig. 1c). Table 2 summarizes the sequence alignment score for each exon component where exons 1-3 exhibits significantly low score. When the low homology region is examined further, the first 50 amino acids and the second 50 amino acids show significantly different homology patterns. Within the first 50 amino acid residues, the conserved amino acids are about 30%, as indicated * in Fig. 1c and this region originates from exons 1-2. On the other hand, within the second 50 amino acid residues that originated from exon 3, very little residues are conserved. Since the differences between mammalian GAD isoforms resides on the segment encoded by exons 1-3, this uneven distribution could lead us to explain how GAD65 and 67 would be evolved from the common mammalian ancestral gene.

The heterogeneity in exon 3 sequences for GAD65 and 67 provokes suspicion that this exon

Table 2. Comparison of alignment score between each exon. The score was yielded by alignment of each exon of human GAD65 and 67 using ClustalW program.

compared exon	score
1	16
2	40
3	10
4	57
5	83
6	78
7	71
8	73
9	73
10	71
11	80
12	85
13	76
14	69
15	60
16	85

could be inserted after the ancestral gene was evolved. However, the prediction of the secondary structure as shown in Fig. 2 strongly suggests that exons 1-3 have existed in the mammalian ancestral gene prior to the evolution. Therefore, it is reasonable to assume that the heterogeneity should be attributed to an accumulation of point mutations. It is still in question when and how the point mutations having been accumulated on exons 1-3. Nevertheless, the idea of comparing homology of the primary structures and exon positions would be a useful tool to study molecular evolution.

Fruit fly GAD and mammalian GADs exhibit high homology in their primary structures and good agreements on intron-insertion positions. It is strongly suggested that fruit fly GAD and the mammalian GAD isoforms share a common origin as shown in the evolutionary tree (Fig. 4) [21, 23]. However, there are several points that makes fruit fly GAD different from the mammalian isoforms. First, nine introns are missing in the fruit fly GAD: The number of introns in mammalian GADs is 15 whereas only 6 for fruit fly (Fig. 1). This could be explained by either fruit fly had lost nine introns or mammalians had gained them. It would be reasonable to assume that an ancestral gene already had introns prior to the evolution, so that mammalian GADs inherited those introns but fruit fly lost some of them during the evolution [18, 22]. Second point is that a large deletion at the N-terminal region of fruit fly GAD is observed and those deleted segments are corresponding to the entire exon 2 and most of exon 3 of mammalian GADs (Fig. 1d). Since there are few conserved residues near the first exon-intron boarder of fruit fly GAD, it could be assumed that the large deletion occurred after the evolution or extra sequences were gained in mammalian GADs after the evolution. By any means, the heterogeneity observed at the N-terminal regions of fruit fly and mammalian GADs couldn't be explained by mutations occurred at amino acid level. It is probable to assume that any divergence introduced in GADs could have taken place at DNA level, such as exon shuffling.

As discussed above, it is apparent that exons 1-3 of mammalian GADs are highly heterogeneous, probably due to their susceptibility to mutation. Exons 1-3 encode for the N-terminal 100 amino acid residues; the region is believed to act as the determining role for GAD protein localization in the cell. It has been shown that GAD67 localizes in the cytosol and GAD65 associates with vesicular membrane. GAD65 has three Cys and one Thr residues at the N-terminal region which are susceptible to palmitoylation and phosphorylation, respectively [24-26]. The entire N-terminal 100 residues may not be essential for the catalytic activity since the recombinant feline GAD67, that expressed with its N-terminal 80 residues truncated, exhibited almost full enzymatic activity [27]. Furthermore, other decarboxylases belong to the same vitamin B_6 family, such as DOPA decarboxylase, and histidine decarboxylase, do not have the N-terminal segments constructed with exons 1-3 of GADs [3]. It is reasonable to think that the role(s) of the N-terminal region of mammalian GADs would be the determining factor for cellular localization. The signal can be palmitoylation, phosphorylation, pI, hydrophobic, and/or hydrophilic nature built in the N-terminal region.

In addition to the unique primary structure at the N-terminal region of mammalian GADs, there are other characteristic differences between GAD isoforms. GAD67 seems to be tightly bind to pyridoxal 5'-phosphate (PLP), a cofactor of GAD, to form holo enzyme whereas GAD65 shows weaker binding to PLP in forming half-apo enzyme [4] [7, 8]. It could be assumed that the differences in N-terminal sequences would cause the altering effects on an affinity toward PLP on the mammalian GAD isoforms. Answer to this point may have to wait for the results of X-ray crystallographic analysis.

Go and her colleagues have reported that intron-exon boarders tend to appear right at the secondary structure and/or at the hydrophobic core region [22]. In our present analysis on GADs (Fig. 1b), two out of sixteen boundaries, #13 and #15, of mammals and one out of six, #6, of fruit fly were found at the middle of long helical structure. Most of the boundaries we have analyzed seem to be localized outside of the defined secondary structure. However, boundaries examined for the structures of pig DDC (Fig. 3a) and *E. coli* GAD_B (Fig.3 b) are found in the hydrophobic core, and

somewhat buried. This is consistent with Go's report [22], in which she has proposed a term "module", a minimum element of protein structure. The position of module in the protein structure is often correlated close to the intron-insertion positions [1, 18, 20]. Therefore, structural information on module can be provided by locating the intron-exon boundaries. Our current study has indicated that the intron-exon boundaries appear at the hydrophobic core regions.

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