Human high affinity, Na^+-dependent L-glutamate/L-aspartate transporter GLAST-1 (EAAT-1): gene structure and localization to chromosome 5p11-p12

Wilhelm Stoffel[a,*], Jürgen Sasse[a], Maria Düker[a], Rolf Müller[a], Kay Hofmann[a], Thomas Fink[b], Peter Lichter[b]

[a]*Institut für Biochemie, Universität zu Köln, Joseph-Stelzmann-Str. 52, D-50931 Köln, Germany*
[b]*Institut für Virusforschung, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany*

Received 11 March 1996; revised version received 11 April 1996

Abstract The gene of the human L-glutamate transporter hGLAST-1 (EAAT-1) has been isolated and characterized. The 1626 bp cDNA open reading frame (542 aa) is distributed over ten exons and at least 85 kb on chromosome 5p11-p12. The gene is unrelated to any other previously described neurotransmitter transporter gene family, but its exon/intron structure corresponds largely to that of the Na^+-dependent neutral amino acid transporter ASCT-1. GLAST-1, ASCT-1 and the glutamate transporters GLT-1 and EAAC-1 have strongly similar amino acid sequences. The L-glutamate transporter gene structures might help to understand the correlation of L-glutamate reuptake in neurodegenerative disorders.

Key words: Glutamate; Aspartate; Neurotransmitter transporter; hGLAST-1

1. Introduction

Neurotransmitter transporters have important functions in the clearance of the synaptic cleft from small neurotransmitter molecules following their interaction with specific receptors at the postsynaptic membrane. The concentration of the neurotransmitter L-glutamate in excitatory synapses of CNS is regulated by Na^+-coupled L-glutamate/L-aspartate transporters, GLAST-1 (EAAT-1) [1], GLT-1 (EAAT-2) [2], EAAC-1 [3] and EAAT-4 [4] which form a new family of membrane proteins. Sequence analysis of these four eukaryotic glutamate transporters, of the neutral amino acid transporter ASCT-1 and the bacterial H^+-dependent glutamate transporter GltP [5], Na^+- dependent GLS [6] and the C4-dicarboxylate carrier DCTA [6] suggests a topology common to all members of this family of polytopic membrane proteins, although the similarity of their amino acid sequences ranges only between 25 and 50%. This is largely due to the different ions. Na^+ ions drive the transport in eukaryotic and H^+ ions in prokaryotic cells against the gradient of the transported compounds. They cotransport Na^+ and countertransport K^+ ions [7-9].

The hydropathy plot of all transporters of this family is almost identical. Unlike the 12 transmembrane helix (TMH) topology of the Na^+/Cl^--coupled neurotransmitter transporters (dopamine, serotonine, glycine, GABA, adrenaline) [10] in which transmembrane domains largely correspond to single exons the topology of all GLAST-1-related transporters convincingly reveals only a six TMH motif in the N-terminal half but an undefined C-terminal membrane topology [1-3,11].

Here we report the cloning and characterization of the first gene structure of the excitatory amino acid (Glu/Asp) transporter family, hGlast-1 (EAAT-1). The coding sequence is distributed over nine exons with one untranslated exon I preceding.

We determined the hGlast-1 locus on chromosome 5p11-p12 by fluorescence in situ hybridization. In a previous communication the mouse and human gene EAAT-1 was assigned to mouse chromosome 15 and a region of syntenic homology on human chromosome 5p13 [12].

2. Materials and methods

2.1. Vectors, chemicals and enzymes

The human brain cDNA and the human placenta genomic library in λ-EMBL3-Sp6/T7 were obtained from Clontech (Palo Alto, CA). Oligonucleotides were prepared using an Applied Biosystems 380A oligonucleotide synthesizer. [γ-^{32}P]ATP, [α-^{32}P]dATP and [^{35}S]dATP were purchased from Amersham-Buchler (Braunschweig, Germany), Taq DNA polymerase, restriction endonucleases and other enzymes from Boehringer Mannheim (Germany). DNA sequencing was performed using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Digoxigenin-11-dUTP and anti-digoxigenin conjugated to rhodamine were purchased from Boehringer Mannheim (Germany). Biotin-11-dUTP was purchased from Sigma, avidin conjugated to FITC was obtained from Vector Laboratories.

2.2. cDNA cloning

Screening of the human brain cDNA library with the randomly labeled rat GLAST-1 cDNA yielded the clone λ-hGlast-1 with an insert size of 3 kb [13]. Its product is active in heterologously expressing HEK-293 cells. The cDNA insert was released by *Eco*RI digestion, subcloned into pBluescriptII SK+ and mapped by double digestion with the restriction enzymes *Eco*RI, *Bam*HI, *Sac*I, *Xba*I and *Xho*I and sequenced using the dideoxy chain termination method [14].

2.3. Genomic cloning

Four independent and partially overlapping genomic clones λ-g4, λ-g3, λ-g14, λ-g3/9c were obtained by screening a human placenta genomic library in λ-EMBL3-Sp6/T7 with several hGlast-1 cDNA fragments suitable as hybridization probes. For further characterization the four independent genomic clones were mapped using the enzymes *Eco*RI, *Bam*HI, *Sac*I and *Xho*I. Genomic fragments containing coding sequences were identified by Southern blot analysis with cDNA fragments and cDNA-derived oligonucleotides as hybridization probes. Positive genomic fragments were isolated, if necessary sub-mapped with *Pst*I, *Hin*dIII, *Kpn*I and *Pvu*II and subsequently sequenced. Mapping, hybridization analysis and cloning were carried out according to standard procedures [15].

*Corresponding author. Fax (49) (221) 478-6979; e-mail akc03@rs1.rrz.uni-koeln.de

The nucleotide sequences reported in this paper have been submitted to the EMBL/GeneBank data repository and assigned the accession numbers Z 31703-31710 and Z 31713.

PII S0014-5793(96)00424-3

2.4. Chromosomal mapping

Phytohemagglutinin-stimulated human lymphocytes were cultured for 72 chromosome preparations. After denaturation at 70°C for 2 min in 70% deionized formamide/2×SSC the slides were dehydrated in ice-cold ethanol. Hybridization with labeled DNA fragments at 37°C and posthybridization washes were performed as described previously [16]. Biotin- or digoxigenin-labeled probes were detected by incubation with avidin conjugated to fluorescein isothiocyanate (FITC) or anti-digoxigenin conjugated to rhodamine, respectively. For chromosomal banding counterstaining with 4,5-diamino-2-phenylindol-dihydrochloride (DAPI) was applied.

Genomic clone λ-g4 was labeled by nick translation using digoxigenin-11-dUTP [17] or biotin-11-dUTP [18]. This probe was used for chromosomal in situ suppression (CISS) hybridization to elongated metaphase chromosomes as described previously [16,19]. Briefly, 80–160 ng labeled probe were combined with 10 mg Cot1 DNA, precipitated by ethanol and resuspended in 10 ml hybridization cocktail (50% formamide, 10% dextran sulfate, 2×SSC). After heat denaturation at 75°C for 5 min preannealing of probe and competitor DNA was done at 37°C for 10 min. Hybridization signals were evaluated using a Zeiss Axioplan microscope equipped for epifluorescence microscopy and recorded with a CCD camera (Photometrics). Photographs of an electronical superposition of FITC and DAPI images were taken directly from the video screen.

2.5. Computational sequence analysis

Database searching and protein comparisons were performed using the software package of the Wisconsin Genetics Computer Group [20]. For hydrophobicity analysis the program PROFILEGRAPH [21] was used. Restriction maps were verified and refined by the program COMAP (K. Hofmann, unpublished).

3. Results and discussion

3.1. Cloning of the human GLAST-1 (hGlast-1) gene

Fragments of the GLAST-1-encoding cDNA clone were used to screen a human placenta genomic DNA library. In two rounds of screening the four partially overlapping phage clones λ-g4, λ-g3, λ-g14, and λ-g3/9c were obtained (Fig. 1). These four clones span a minimum of 85 kb of the human genome and harbor the complete coding region of the GLAST-1 gene. The gene is organized in ten exons interrupted by nine introns (Table 1). The exon sizes vary considerably. While exon I contains the noncoding region the translation start codon ATG is preceded in exon II by a 5′ untranslated region of additional 104 bp. The smallest exon V consists of only 43 base pairs coding for 14 amino acids. The largest intron sizes are those of introns 2 and 3 of about 30 and 33 kb, respectively. The sizes of the gap between λ-g4 and λ-g3 could not be determined by Southern blot analysis and no contig-forming phage clone was obtained for that region; the exact size of intron 1 is therefore arbitrary.

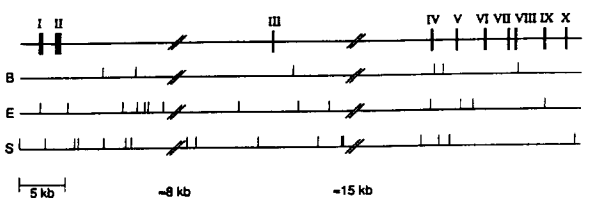

Fig. 1. Organization of the human GLAST-1 gene. The upper trace depicts the arrangement of the ten exons (filled boxes with Roman numbers). Protein-coding regions are represented by filled boxes, 5′ and 3′ untranslated regions by open boxes. Restriction sizes of three enzymes B (*Bam*HI), E (*Eco*RI) and S (*Sac*I) used for the restriction mapping are shown in the linearized hGlast-1 gene locus which is expanded over more than 85 kb.

Table 1
Splice junction sequences and exon sizes of the human GLAST-1 gene

Exon no.	Size (bp)	Donor sequence	Acceptor sequence
I*	104		AAAAGgtaagtgtca
II	76	cccattctagTTGTG	TGTGGgtgagtcatt
III	138	tttccttcagGTACA	CACAGgtacc
IV	205	cctccaccagGAATG	ATCAGgtatgtcctt
V	43	ttctttgcagGAACA	AACAGgtaaacactc
VI	293	tatttttaagTTTAA	ATGTGgtatgtattt
VII	234	cgtgtggagGTATG	TCAAGgtagtatgta
VIII	195	ctgttctcagTTCTG	ATCAGgtacaaggaa
IX	135	ctgttaacagCATCA	TTCCTgtgagtatgc
X	205	ctgcctgcagGGATC	

*Non-coding.

3.2. Nucleotide sequence of hGlast-1

All coding portions of the GLAST-1 gene together with some of their flanking regions were characterized by DNA sequencing (Table 1). The sequence data are retrievable under the accession numbers Z 31703-31710 and Z 31713 in the GeneBank.

The nucleotide sequence of the ten exons encoding the human GLAST-1 protein was identical to the cDNA sequence (confirmed by the original hGlast-1 sequence [22]).

3.3. Characterization of the 5′ end of the human GLAST-1 gene

We tried to determine the site of transcription initiation by primer extension analysis using poly(A)$^+$ RNA obtained from human fetal brain tissue samples. However no transcription start signal was observed. Although Glast-1 is expressed exclusively in brain [1] no well-defined cis-elements were observed in the 5′ nucleotide sequence upstream of the translation start.

3.4. Chromosomal localization of hGlast-1

In order to localize the gene for the human GLAST-1 we applied the technique of chromosomal in situ suppression (CISS) hybridization. The genomic clone λ-g3/9c spanning exons 7 to 10 was hybridized to elongated metaphase chromosomes. Detection of the biotinylated or digoxigenin-labeled probes with avidin or antibodies coupled to fluorescent dyes resulted in specific signals in the short arm of chromosome 5 (Fig. 2). For quantitative evaluation 17 metaphase spreads were analyzed. In all cases specific hybridization signals were observed on both homologs of chromosome 5 (see Fig. 2). Comparison of the hybridization signal with chromosomal bands obtained by DAPI staining allowed the region of the human GLAST-1 locus to be narrowed to chromosome 5p11-p12.

3.5. Polytopic transmembrane domain structure of GLAST-1

More than 15 different transport proteins belonging to the superfamily of Na^+/Cl^--coupled transporters were successfully cloned by homology screening facilitated by sequence similarity of previously cloned transporters such as the GABA transporter [23] and the noradrenaline transporter [10,24,25]. Among the 15 new members of this protein family obtained by homology screening none is able to transport the excitatory neurotransmitter L-glutamate [10,25]. This was not

Fig. 2. Localization of GLAST-1 gene to human chromosome 5p11-p12. The genomic clone λ3 was randomly labeled with dioxigenin and used for the hybridization to human metaphase chromosomes and detected by anti-digoxigenin antibodies conjugated to rhodamine. For chromosome banding counterstaining with DAPI was performed. The hybridization signal was assigned to chromosome 5p11-p12 marked with arrowheads.

surprising when the first three mammalian glutamate/aspartate transporters were cloned. GLAST-1 (EAAT-1) [1], GLT-1 (EAAT-2) [2], EAAC-1 (EAAT-3) [3] and EAAT-4 [4] form a novel family of integral membrane proteins completely different in their structure from the neurotransmitter transporters described before.

The hydropathy plot of the Na^+/Cl^--coupled transporters suggests twelve clearly defined hydrophobic domains most likely spanning the lipid bilayer as transmembrane helices. For the GLAST-related family of transporters a clear prediction of the transmembrane topology is not possible based on sequence considerations alone. GLAST-1, GLT-1, EAAC-1, ASCT-1 and the prokaryotic H^+-dependent glutamate symporters have six distinct hydrophobic domains in the N-terminal half of the molecule, all of them large enough to span the lipid bilayer as α-helices (Fig. 3). In the eukaryotic transporters GLAST-1, GLT-1, EAAC-1, ASCT-1 there is a seventh extended hydrophobic region located shortly after the proposed transmembrane helix VI of GLAST-1 (Cys^{185}–Ser^{237}), GLT-1 (Cys^{184}–Phe^{235}) and EAAC-1 (Cys^{158}–Tyr^{206}), which is absent in the prokaryotic transporters. The most intriguing feature with regard to the prediction of the transmembrane topology is an extended region of intermediate hydrophobicity within the C-terminal part of all members of the glutamate transporter family. A hydrophobicity analysis of this region with a window width of 9 reveals the presence of up to six short hydrophobic stretches with almost equal spacing. This feature, which is strictly conserved in both mammalian and prokaryotic GLAST-related transporters, had prompted us to speculate about alternatives to the classic α-helical transmembrane organization [1].

In a recent study on the topology of the prokaryotic glutamate transporter dctA from *Rhizobium meliloti*, truncated DCTA polypeptides ending at different positions between the six short hydrophobic stretches were fused to reporter enzymes to yield DCTA/lacZ and DCTA/phoA fusion proteins. The protein exhibited an alternating outside-inside pattern of the reporter enzyme activity [11]. This finding suggests that the short hydrophobic stretches are indeed membrane-spanning. Whether they form the core of relatively short transmembrane α-helices or span the membrane in some different way remains subject to speculation. The hydropathy plots of all excitatory amino acid neurotransmitter transporters including ASCT-1 make a transmembrane topology likely which is common to the whole protein family.

3.6. Position of the intron sites of hGlast-1 and Asct-1 in relation to transmembrane organization

Hydrophobic amino acid sequences sufficiently long to span the lipid bilayer are assumed to form transmembrane helices as autonomously folding domains [26]. Single transmembrane segments of many polytopic membrane proteins are encoded by individual exons with the intron positions located within the connecting hydrophilic loops. Exon modules that code for structural and functional elements recombine to form the gene. From this observation the exon-shuffling hypothesis has been deduced [27]. The gene family of the Na^+/Cl^--coupled neurotransmitter transporters, which also includes members transporting non-neurotransmitters, is an additional example for the common relation between transmembrane organization and intron site position. The mouse gene for the GABA transporter, which is relatively compact (12 kb), contains eleven introns, none of which dissects a putative transmembrane domain [10]. With only one exception each of the exons contains only a single transmembrane domain.

However, the organization in the human GLAST-1 and ASCT-1 genes is quite different, Fig. 3. Amino acid sequences encoded by the first three exons of the two genes have very little similarity. In hAsct-1 intron 1 dissects the nucleotide sequence encoding the amino acid sequence of the extracellular loop joining transmembrane helices III and IV. The coding sequence within the primary transcript of hGlast-1 up to this position is proceded by exon I of the untranslated 5′ region and is split by three more intron sequences. Introns 2, 3 and 4 dissect transmembrane helices I, II and III, respectively. The nucleotide sequence of intron 7 dissects the transmembrane helix VI of hGlast-1, but introns 8 and 9 are at 'conventional' positions within the hydrophilic loops following putative hydrophobic transmembrane domains. Closely related modules with exons VIII, IX and X of hGlast-1 and exons VI, VII and VIII of hAsct-1 are assembled in the highly conserved C-terminal part of both transporters with an almost identical hydropathy plot, Fig. 3. Exon sizes are very similar and introns dissect at almost identical positions in this part of the genes which encodes the very similar C-terminal part of both transporters. Their membrane topology and likewise that of GLT-1 and EAAC-1, the other members of this new family of plasma membrane transporters, is structurally ill-defined. Only topochemical studies will decide whether the exon-intron structure or the hydrophobicity profile on the amino acid sequence level has a higher predictive value for the theoretical prediction of the transmembrane organization of the GLAST-1.

3.7. Assignment of hGlast-1 locus to chromosome 5p11-p12

We assigned the hGlast-1 locus to chromosome 5p11-p12. The gene loci of mouse and human Glast-1 have previously been assigned to chromosome 15 of the mouse and slightly

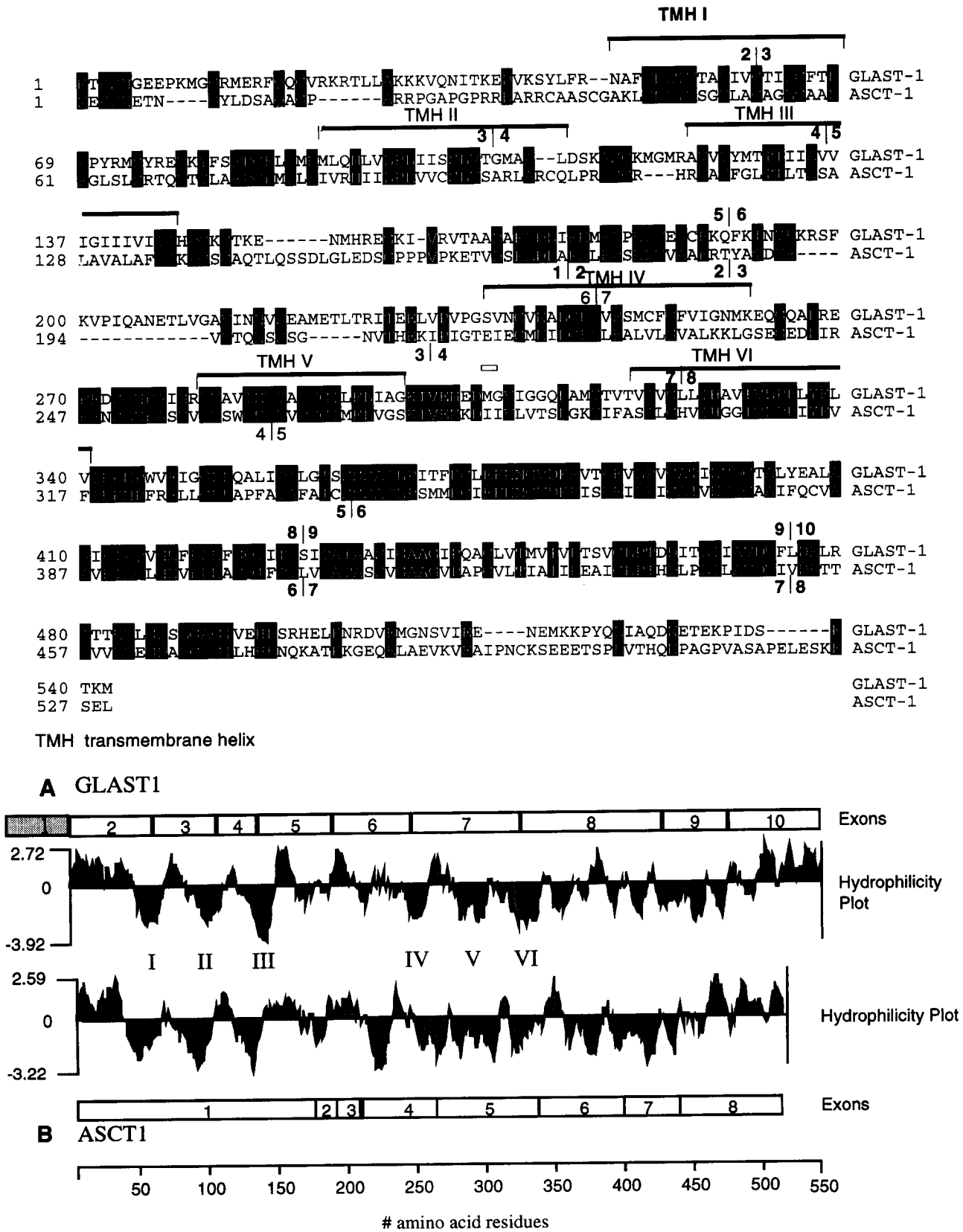

Fig. 3. (Above) Comparison of positions of introns in the aligned amino acid sequences of GLAST-1 and ASCT-1 (numbered vertical bars). The putative transmembrane helices are marked with horizontal bars (I–VI) above the sequences. (Below) Hydrophilicity plots and assignment of exons to amino acid sequences of (A) GLAST-1 and (B) ASCT-1. Roman numbers (I–VI) mark the putative transmembrane helices of these and the other members of the GLAST-1 family.

different from our localization to a region of synthenic homology on human chromosome 5p13. Eaac-1 was localized on chromosome 9q24 [28] and we have recently described the gene structure and chromosome locus of hAsct-1 on chromosome 2p13-p15 [29].

Although numerous speculations have been put forward which relate a disrupted glutamate transport and metabolism to neurodegenerative diseases, e.g. amyotrophic lateral sclerosis (ALS), Alzheimer's and Huntington's disease, no firm linkage analysis warrants any conclusion.

So far no association of a clinical phenotype to this GLAST-1 gene locus on chromosome 5p11-p12 has been found. This locus is too far distant from the critical region 5p15.3, a segment which is deleted in *cri du chat* syndrome [30,31]. However, the report that 5p interstitial deletions can cause a wide spectrum of clinical phenotypes requires an understanding of the role of the complete chromosomal region in 5p [32]. The hGlast-1 locus might therefore prove to be an additional valuable marker of chromosome 5p.

The Na^+-dependent, high affinity glutamate transporters known so far show complex expression patterns in neuronal and glial cells as well as in different regions of central nervous system [1,33,34]. Therefore an understanding of the association of neurodegenerative disorders of excitatory neurons with glutamate transporter mutations on the molecular level is pivotally associated with the knowledge of the gene structure and chromosome localization of all glutamate neurotransmitter transporters known so far.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (Sto 32/34-1).

References

[1] Storck, T., Schulte, S., Hofmann, K. and Stoffel, W. (1992) Proc. Natl. Acad. Sci. USA 89, 10955–10959.
[2] Pines, G. et al. (1992) Nature 360, 464–467.
[3] Kanai, Y. and Hediger, M.A. (1992) Nature 360, 467–471.
[4] Arriza, J.L., Kavanaugh, M.P., Fairman, W.A., Wu, Y.-N., Murdoch, G.H., North, R.A. and Amara, S.G. (1993) J. Biol. Chem. 268, 15329–15332.
[5] Tolner, B., Poolman, B., Wallace, B. and Konings, W.N. (1992) J. Bacteriol. 174, 2391–2393.
[6] Engelke, T., Jording, D., Kapp, D. and Pühler, A. (1989) J. Bacteriol. 171, 5551–5560.
[7] Kanner, B.I. and Schuldiner, S. (1987) CRC Crit. Rev. Biochem. 22, 1–38.
[8] Bouvier, M., Szatkowski, M., Amato, A. and Attwell, D. (1992) Nature 360, 471–473.
[9] Klöckner, U., Storck, T., Conradt, M. and Stoffel, W. (1993) J. Biol. Chem. 268, 14594–14596.
[10] Liu, Q.R., Mandiyan, S., Nelson, H. and Nelson, N. (1992) Proc. Natl. Acad. Sci. USA 89, 6639–6643.
[11] Jording, D. and Pühler, A. (1993) Mol. Gen. Genet. 241, 106–114.
[12] Kirschner, M.A., Arriza, J.L., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Magenis, E. and Amara, S.G. (1994) Genomics 22, 631–633.
[13] Stoffel, W. and Blau, R. (1995) Biol. Chem. Hoppe-Seyler 376, 511–514.
[14] Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[16] Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D. (1988) Hum. Genet. 80, 224–234.
[17] Kessler, C., Höltke, H., Seibl, R., Burg, J. and Mühlegger, K. (1990) Biol. Chem. Hoppe-Seyler 371, 917–927.
[18] Langer, P., Waldrop, A. and Ward, D. (1981) Proc. Natl. Acad. Sci. USA 78, 6633–6637.
[19] Lichter, P., Tang, C., Call, K., Hermanson, G., Evans, G., Housman, D. and Ward, D. (1990) Science 247, 64–69.
[20] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucl. Acids. Res. 12, 387–394.
[21] Hofmann, K. and Stoffel, W. (1992) Comput. Applic. Biosci. 8, 331–337.
[22] Kawakami, H., Tanaka, K., Nakayama, T., Inoue, K. and Nakamura, S. (1994) Biochem. Biophys. Res. Commun. 199, 171–176.
[23] Guastella, J. et al. (1990) Science 249, 1303–1306.
[24] Pacholczyk, T., Blakely, R.D. and Amary, S.G. (1991) Nature 350, 350–353.
[25] Amara, S.G. and Kuhar, M.J. (1993) Annu. Rev. Neurosci. 16, 73–93.
[26] Popot, J.L. and de Vitry, C. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 369–403.
[27] Gilbert, W., Marchionni, M. and McKnight, G. (1986) Cell 46, 151–154.
[28] Smith, C.P., Weremowicz, S., Kanai, Y., Stelzner, M., Morton, C.C. and Hediger, M.A. (1994) Genomics 20, 335–336.
[29] Hofmann, K., Düker, M., Fink, T., Lichter, P. and Stoffel, W. (1994) Genomics 24, 20–26.
[30] Niebuhr, E. (1978) Hum. Genet. 44, 227–275.
[31] Overhauser, J., Beaudet, A.L. and Wasmuth, J.J. (1986) Am. J. Hum. Genet. 39, 562–572.
[32] Keppen, K.D., Gollin, S.M., Edwards, D., Sawyer, J., Wilson, W. and Overhauser, J. (1992) Am. J. Med. Genet. 44, 356–360.
[33] Rothstein, J.D., Martin, L.J. and Kungl, R.W. (1992) New Engl. J. Med. 326, 1464–1494.
[34] Danbolt, N.C., Pines, G. and Kanner, B.I. (1990) Biochem. 29, 6734–6740.