

SHORT COMMUNICATION

A mRNA molecule encoding truncated excitatory amino acid carrier 1 (EAAC1) protein (EAAC2) is transcribed from an independent promoter but not an alternative splicing event

XIAO PING JIN, JI BIN PENG, FANG HUANG, YA NI ZHU, JIAN FEI*, LI HE GUO*

Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

ABSTRACT

Glutamate transporter EAAC1 removes excitatory neurotransmitter in central nervous system, and also absorbs glutamate in epithelia of intestine, kidney, liver and heart for normal cell growth. When a mouse cDNA was screened using EAAC1 cDNA fragment as probe in our lab, a transcript (GenBank U75214) encoding an EAAC1 protein with 148 residues truncated at N-terminal was cloned and named as EAAC2. Sequence analysis shows that EAAC2 has its own start code and unique 5' TR that is different from that of EAAC1. A mouse genomic library was screened and a positive clone including EAAC1 CDS was sequenced (GenBank AF 322393) and indicates that normal EAAC1 transcript (GenBank U73521) is transcribed from 10 exons in terms of exon I, II, III, IV, V, VI, VII, VIII, IX, X, and EAAC2 transcript is consisted by exons from IV to IX as same as that of EAAC1 and with its unique exon β upstream to exon IV and exon d downstream to IX. EAAC2 transcript has a cluster of transcriptional start sites not overlapping with the transcriptional start sites of EAAC1. These results indicate that EAAC2 is transcribed from an independent promoter but not an alternative splicing event.

Key words: *genomic organization, glutamate transporter, transcriptional start sites EAAC1.*

INTRODUCTION

Glutamate transporters are able to uptake neurotransmitter glutamate from synaptic cleft and maintain its concentration at low level ($< 1 \mu\text{M}$) thus to protect neurons from excitotoxicity caused by Glu [1]. Up to now, five subtypes of glutamate transporter GLAST (EAAT1)[2], GLT-1 (EAAT2)[3], EAAC1 (EAAT3)[4], EAAT4[5] and EAAT5[6] have been cloned. Excitatory amino acid carrier 1 (EAAC1) is a high-affinity Na^+ -dependent L-glutamate/D, L-aspartate cell-membrane transporter

protein. EAAC1 cDNA was originally cloned from rabbit small jejunum[4], followed by finding its counterparts in rat [7-9], mouse[10] and human[11-14]. Human EAAC1 gene was mapped to 9p24, which was linked to dicarboxylic aminaciduria and neurodegenerative disorders[15]. Loss of function studies by means of chronic antisense oligonucleotide administration[16] and gene knockout[17] revealed that EAAC1 deficiency did not lead to neurodegeneration, although EAAC1 was believed to be a neuronal glutamate transporter. Functional EAAC1 was assembled as pentamer in EAAC1 expressed *Xenopus* oocyte plasma membrane[18].

However, genomic organization of EAAC1 was not fully understood, only its partial genomic organization was documented[17]. During the experi-

* Corresponding author: To whom correspondence and reprint requests should be addressed.

E-mail: mhzhang@sunm.shenc.ac.cn

Tel: 86-21-64315030-2069

Received March-25-2002 Revised July-15-2002 Accepted July-25-2002

ment of screening of mouse EAAC1 cDNA in our lab, a truncated EAAC1 transcript was cloned and named as EAAC2. In order to address the genomic organization relationship between EAAC1 and EAAC2 transcripts, we cloned the mouse genomic fragment for EAAC1, and also identified transcriptional start sites for EAAC1 and EAAC2 by 5' RACE. We also detected their expressions at transcriptional level by RT-PCR.

MATERIALS AND METHODS

Mouse EAAC1 and EAAC2 cDNA cloning

The method for cloning EAAC1 cDNA and EAAC2 cDNA from the neonatal Balb/C mouse brain cDNA library (Stratagene Cat. No. 937301) was detailed in literature[19].

Mouse genomic organization analysis

The mouse genomic library (Stratagene Cat. No. 946309) was screened with EAAC1 full length cDNA as a probe. The positive genomic clones were mapped by suitable restriction endonucleases and subcloned. DNA sequence was analyzed by automatic fluorescent sequencer. The DNA sequence data were aligned and analyzed by GCG software package.

5' RACE

Poly A+ mRNA was isolated from tissues of neonatal mice and adult mice by OligotexTM mRNA Mini Kit (Qiagen Cat. No. 70022). The first-strand cDNA synthesis and 5' RACE PCR amplification were performed according to manufacturer's instructions (Clontech, Cat. No. K1811-1). For 5' RACE of EAAC1 transcript, P1 primer (All the primers sequence information were listed in Tab 1) was used as EAAC1 gene specific primer in the first round PCR, and P2 primer was used as EAAC1 nest primer in the second round PCR. For 5' RACE of EAAC2 transcript, P3 primer was used as EAAC2 gene specific primer in the first round PCR, and P4 primer was used as EAAC2 nest primer in the second

round PCR. Sequences of 5' RACE PCR products were analysed directly or after T-Vector cloning by automatic fluorescent sequencer.

RT-PCR

Total RNA was extracted from mice tissues using Trizol reagent (Gibcol BRL), then treated with RNase-free DNase I to eliminate the contaminant of chromosomal DNA. Approximately 5 μ g total RNA was converted into the first-strand cDNA using Oligo dT15 as primer. The E. coli RNase H (TaKaRa) was used to remove the RNA templet. The cDNA was used as template in PCR amplification. P5 and P6 primers were used for the specific region of EAAC1 transcript and P7 and P8 primers for PCR amplification of the specific region of EAAC2 transcript. PCR amplification of GAPDH was used as reference that generated by P9 and P10 primers.

RESULTS AND DISCUSSION

Characters of EAAC1 and EAAC2 transcripts

The EAAC1 cDNA that we cloned in this paper is totally in length of 3727 bp. The sequence was deposited in GenBank (U73521). Its open reading frame from nucleotide 76 to 1648 encoded a protein of 523 residues, which was identical to the sequence of EAAC1 protein reported previously[10], except amino acid substitution of methinine at site of 141 for isoleucine. Both polyadenylation signal and translational initiation context of EAAC1 transcript were consistent with the consensus sequences. During our cloning EAAC1 cDNA, we obtained a novel cDNA clone with the insertion sequence of 2176 bp. The sequence was deposited in GenBank (U75214) and named as EAAC2. With comparison to EAAC1, EAAC2 had 288 bp specific sequence in 5' untranslated region, but its rest sequence was identical to EAAC1 except its 3' untranslated region

Tab 1. PCR primers list

Primer	Nucleotide Sequence	GenBank and Position
P1	5' AAT TCC TAG TAC CAC GGC GGC CAC G 3'	EAAC1: U73521: 172-148.
P2	5' CAT GGT GAG CAC TGA CGT TTG 3'	EAAC1: U73521: 79-59.
P3	5' GTT CTG TTT CTC ATC TGG CGT CCG 3'	EAAC2: U75214: 196-173.
P4	5' CAG AAG TCT TCC TCA TCA AC 3'	EAAC2: U75214: 100-81.
P5	5' GCT CGA ATT CAC CAT GGG GAA GCC CAC 3'	EAAC1: U73521: 72-90.
EcoR I		
P6	5' GGT CCA ACA TGG CAT CCA TGG 3'	EAAC1: U73521 509-489.
P7	5' GCA GGA CTT CCC TGA GAA CAG 3'	EAAC2: U75214: 27-47.
P8	5' TTG GAG ACA CGG AAA ACA G 3'	EAAC2: U75214: 288- 270.
P9	5' ACG ACC CCT TCA TTG ACC 3'	GAPDH: M32599: 141-159.
P10	5' AGA CAC CAG TAG ACT CCA CG 3'	GAPDH: M32599: 345-325.

was shortened for 1341 bp. Its open reading frame from nucleotide 296 to 1423 encoded a protein of 375 residues, which was identical to EAAC1 protein from residue 149 to 523 with 148 residues truncated at N-terminal. However, both polyadenylation signal and translational initiation context of EAAC2 transcript were not typical but potential.

The genomic organizations of EAAC1 and EAAC2 transcripts

To further study whether the EAAC2 is a spliced variation of EAAC1 or an independent transcript, we screened the genomic library of mice. Three posi-

tive phage clones were fished out by the probe of EAAC1 cDNA. With the overlapping insertion genomic sequence of 18.2 kb, 15.2 kb and 16.2 kb respectively in these three clones, a physical map of 33 kb was constructed. Approximately 25 kb of genomic sequence from Sal I to BamH I was sequenced in order to address the genomic organization relationship between EAAC1 and EAAC2 transcripts, the sequence data of 25407 bp was deposited in Genbank (GenBank AF322393). The genomic organizations of EAAC1 and EAAC2 transcripts were showed in Fig 1 and Tab 2.

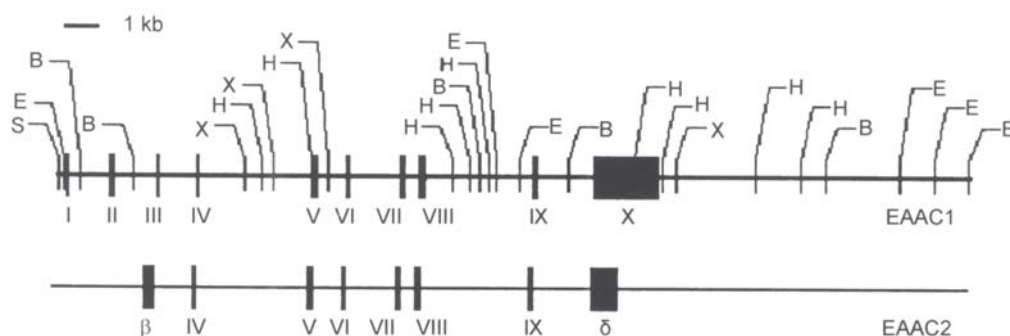


Fig 1. The genomic organizations of EAAC1 and EAAC2 transcripts

The upper line showed the physical map of mouse genomic DNA (33 kb) derived from three overlapping positive clones screened from mice genomic library. Restriction enzymes used: B: BamH I, E: EcoR I, H: Hind III, S: Sal I and X: Xba I; Solid bar: exon. EAAC1 is transcribed from exon I, II, III, IV, V, VI, VII, VIII, IX, X (the region from nucleotide 1 to nucleotide 308 of EAAC1 transcript is not included in this genomic sequence). EAAC2 is transcribed from exon β , IV, V, VI, VII, VIII, IX, δ and showed in the line below.

Tab 2. The genomic organizations of EAAC1 and EAAC2 transcripts

Exon	Size bp	EAAC1 U73521	EAAC2 U75214	Genomic AF322393	Intron Size bp	5' Splice site	3' Splice site
I	92	309-401		277-369	1531	CTAG↓gt	cag↓GTGT
II	114	402-516		1896-2010	1517	TCAG↓gt	cag↓GTAT
b	334		1-334	3236-3569			cag↓GAAC
III	42	517-559		3527-3569	1380	GCAG↓gt	cag↓CTG
IV	95	560-655	335-430	4950-5045	4061	TGAG↓gt	cag↓TACA
V	184	656-840	431-615	9106-9290	1060	TGTG↓gt	cag↓AACA
VI	107	841-948	616-723	10351-10458	1827	GCGG↓gt	tag↓CTAC
VII	122	949-1071	724-846	12285-12407	598	CCAG↓gt	cag↓GCTT
VIII	194	1072-1266	847-1041	13005-13199	3907	TCAG↓gt	cag↓TTCG
IX	134	1267-1401	1042-1176	17106-17240	2100	TCCT↓gt	tag↓CATT
d	969		1177-2146	19340-20309		AATGAA↓	cag↓GGAC
X	2282	1402-3684		19340-21619		AATAAA↓	

The region from nucleotide 309 to 3727 of EAAC1 transcript was distributed into 10 exons, in terms of exon I, II, III, IV, V, VI, VII, VIII, IX, X (Fig 1 and Tab 2). The corresponding genomic sequence from nucleotide 1 to 308 of EAAC1 transcript and its upstream regulation and control sequence were not included in this genomic sequence. All exon and intron junctions were followed the "GT-AG" rule (Tab 2)[20]. Two polyadenylation signals were located at nucleotide 21614 and nucleotide 21174.

EAAC2 transcript was distributed into 8 exons, in terms of exon β , IV, V, VI, VII, VIII, IX, δ (Fig 1 and Tab 2). Among them, exon IV, V, VI, VII, VIII, IX were identical to exons for EAAC1 transcript. Exon β contained the 288 bp specific sequence at 5' untranslated region of EAAC2 transcript and the sequence overlapped with exon III at the 3' end. Exon δ was identical to exon X but with 1310 bp shortened at 3' end. All exon and intron junctions were consistent with consensus sequence (Tab 2)[20]. There was no TATA box, CAAT box, CpG Island and octamer consensus sequences upstream from exon β . Its polyadenylation signal AATGAA at nucleotide 20309 was not typical but potential.

Transcriptional start sites of EAAC1 and EAAC2 transcripts

We employed 5' RACE method to test whether the 5' sequences of EAAC1 and EAAC2 transcripts were complete, and to identify their transcription start sites. EAAC1 transcript 5' RACE product with the length of about 150bp was amplified from

mRNA of 24 to 36 hours postnatal mice (D1) cortex, D1 kidney, and D1 intestine, of 72 to 96 h postnatal mice (D3) intestine and of one year old mouse whole brain tissue (Fig 2). These sequences fully matched to EAAC1 sequence from nucleotide 1 to 79 and extended 33 bp in 5' upstream direction as following: 5' G A A T G G C A G C G C T G T A C A G C G G C C G G T G C G C G T 3' . Since the genomic sequence corresponding the region from nucleotide 1

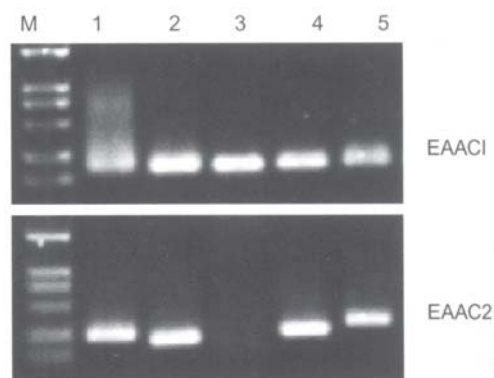


Fig 2. 5' RACE of EAAC1 and EAAC2 transcripts

5' RACE of EAAC1 transcript was done using P1 as gene specific primer for the first round PCR amplification and using P2 as nest primer for the second round PCR. 5' RACE of EAAC2 transcript was done using P3 as gene specific primer for the first round PCR amplification and using P4 as nest primer for the second round PCR. Lane M: DNA Marker (TaKaRa), 50 ng for each band at sizes of 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp and 100 bp; Lane 1: D1 cerebral cortex including hippocampus; Lane 2: D1 kidney; Lane 3: D1 intestine; Lane 4: D3 intestine; Lane 5: One year old mouse whole brain.

Tab 3. 5' RACE of EAAC1 and EAAC2 transcripts

Tissue	Transcript	Additional Sequence	Genomic Site	5' Most Genomic	Nucleotide Context
D1 Cortex	EAAC1	+33 bp	n/a	n/a	
	EAAC2	+89 bp	3147-3236	3147:	ttt六GGG
D1 Kidney	EAAC1	+33 bp	n/a	n/a	
	EAAC2	+45 bp	3191-3236	3191:	gac六AGA
D3 Intestine	EAAC1	+33 bp	n/a	n/a	
	EAAC2	+65 bp	3172-3236	3172:	act六GGA
Mature Mouse Brain	EAAC1	+33 bp	n/a	n/a	
	EAAC2	+120 bp	3116-3236	3116:	aca

Note: n/a=not available

to 308 of EAAC1 transcript did not locate in our genomic sequence, we were not able to analyze the transcriptional start sites of EAAC1 transcript. The fact that both additional sequence and additional length (Tab 3) were identical in all products from different source indicated that EAAC1 transcript was transcribed from a unique transcriptional start site.

The DNA fragments of 5' RACE of EAAC2 transcript were in the length of 200 bp to 300 bp from the mRNA of D1 cerebral cortex, D1 kidney, D3 intestine and one year old mouse whole brain (Fig 2). Sequence analysis showed that they were perfectly identical to not only nucleotide 1 to 100 of EAAC2 transcript, but also the genomic sequence in consecutive. The additional length of EAAC2 5' RACE product in 5' upstream direction was 89 bp in the mRNA of D1 cerebral cortex, 45 bp in D1 kidney, 65 bp in D3 intestine and 120 bp in one-year-old mouse whole brain (Tab 3). The genomic contexts surrounding the 5' first nucleotides in EAAC2 5' RACE additional sequences were not consistent with splicing consensus sequences [20], which indicated that EAAC2 transcript was not the product of alternative splicing and supported that EAAC2 was initiated at different transcriptional start sites to that of EAAC1 in tested tissues. Combining of the result of genomic organization analysis, it does not seem that the transcript of EAAC1 and EAAC2 are created by the same promoter from different transcriptional start sites. We believe that the EAAC2 transcription is initiated from an independent promoter different from that of EAAC1.

Expressions of EAAC1 and EAAC2 transcripts at transcriptional level

RT-PCR with EAAC1 and EAAC2 5' specific primers indicated that both EAAC1 and EAAC2 were expressed in cerebral cortex, hippocampus, cerebellum, brain stem, lung and kidney tissues of D3 neonatal mouse (Fig 3).

In this paper we reported a new cDNA clone (EAAC2) encoding a N-terminal truncated EAAC1 protein was found. We also proved that EAAC2 transcription was initiated from its own promoter other than an alternative splicing of EAAC1. The expression pattern of EAAC2 is nearly identical in spatio-temporal manner to that of EAAC1. We expressed EAAC2 cDNA in *Xenopus* oocyte by infecting cDNA

of EAAC2 to measure if it could be Glu transporter, the answer was no (data not shown). We also co-expressed EAAC1 and EAAC2 in oocyte to see if EAAC2 had effect on the function of EAAC1, but no effect of EAAC2 was found on the function of EAAC1 (data not shown). To address the question that what is the biological function of EAAC2 is a big challenge to our further work.

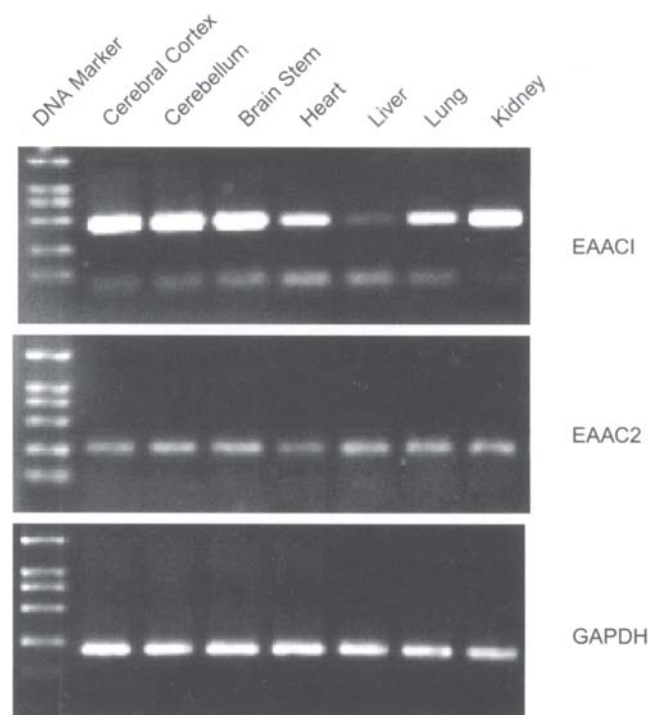


Fig 3. RT-PCR detections of EAAC1 and EAAC2 expression

EAAC1 5' specific sequence was amplified by PCR using P5 and P6 as primers. EAAC2 5' specific sequence was amplified by PCR using P7 and P8 as primers. House keeping gene GAPDH was amplified by PCR using P9 and P10 primers as positive control. The sample was collected from cerebral cortex including hippocampus, cerebellum, brain stem, heart, liver, lung and kidney of neonatal mice within 72 to 96 hours after postnatal (D3).

ACKNOWLEDGEMENTS

This research was supported by foundations from Chinese Academy of Sciences and Special Funds for Major State Basic Research of China (G19990539).

REFERENCES

- [1] Kanai Y. Family of neutral and acidic amino acid transporters: molecular biology, physiology and medical implications. *Curr Opin Cell Biol* 1997; 9:565-72.

- [2] Storck T, Schulte S, Hofmann K, Stoffel W. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. *Proc Natl Acad Sci USA* 1992; **89**:10955-9.
- [3] Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, et al. Cloning and expression of a rat brain L-glutamate transporter [published erratum appears in *Nature* 1992 Dec 24-31;360(6406):768] [see comments]. *Nature* 1992; **360**:464-7.
- [4] Kanai Y, Hediger MA. Primary structure and functional characterization of a high-affinity glutamate transporter [see comments]. *Nature* 1992; **360**:467-71.
- [5] Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* 1995; **375**:599-603.
- [6] Arriza JL, Eliasof S, Kavanaugh MP, Amara SG. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci USA* 1997; **94**:4155-60.
- [7] Bjoras M, Gjesdal O, Erickson JD, Torp R, Levy LM, Ottersen OP, et al. Cloning and expression of a neuronal rat brain glutamate transporter. *Brain Res Mol Brain Res* 1996; **36**:163-8.
- [8] Kiryu S, Yao GL, Morita N, Kato H, Kiyama H. Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR. *J Neurosci* 1995; **15**:7872-8.
- [9] Velaz FM, McGraw TS, alandro MS, Fremeau-RT J, Kilberg MS, Anderson KJ. Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. *Am J Physiol* 1996; **270**:C67-C75.
- [10] Mukainaka Y, Tanaka K, Hagiwara T, Wada K. Molecular cloning of two glutamate transporter subtypes from mouse brain. *Biochim Biophys Acta* 1995; **1244**:233-37.
- [11] Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J Neurosci* 1994; **14**:5559-69.
- [12] Kanai Y, Stelzner M, Nussberger S, Khawaja S, Hebert SC, Smith CP, et al. The neuronal and epithelial human high affinity glutamate transporter. Insights into structure and mechanism of transport. *J Biol Chem* 1994; **269**:20599-606.
- [13] Kanai Y, Nussberger S, Romero MF, Boron WF, Hebert SC, Hediger MA. Electrogenic properties of the epithelial and neuronal high affinity glutamate transporter. *J Biol Chem* 1995; **270**:16561-68.
- [14] Shashidharan P, Huntley GW, Meyer T, Morrison JH, Plaitakis A. Neuron-specific human glutamate transporter: molecular cloning, characterization and expression in human brain. *Brain Res* 1994; **662**:245-50.
- [15] Smith CP, Weremowicz S, Kanai Y, Stelzner M, Morton CC, Hediger MA. Assignment of the gene coding for the human high-affinity glutamate transporter EAAC1 to 9p24: potential role in dicarboxylic aminoaciduria and neurodegenerative disorders. *Genomics* 1994; **20**:335-6.
- [16] Rothstein JD, Dykes HM, Pardo CA, Bristol LA, Jin L, Kuncl RW, et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 1996; **16**:675-86.
- [17] Peghini P, Janzen J, Stoffel W. Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. *EMBO J* 1997; **16**:3822-32.
- [18] Eskandari S, Kremann M, Kavanaugh MP, Wright EM, Zampighi GA. Pentameric assembly of a neuronal glutamate transporter. *Proc Natl Acad Sci USA* 2000; **97**:8641-46.
- [19] Peng Jibing, Fei Jian, Huang Fang, Jin Xiaoping, Gu Quanbao, Guo Lihe. Cloning and Analyzing of Members of Excitatory Amino Acid Transporter Family From Neonatal Mouse Brain. *Acta Biologicae Experimentalis Sinica* 2000; **33**(1):53-62.
- [20] Breathnach R, Chambon P. Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 1981; **50**:349-83.