Volume 13 Number 12 1985 Nucleic Acids Research

Molecular cloning and the nucleotide sequence of cDNA to mRNA for non-neuronal enolase (αα enolase) of rat brain and liver

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Received 12 March 1985; Revised and Accepted 21 May 1985

ABSTRACT

The nucleotide sequence for αα enolase (non-neuronal enolase: NNE) of rat brain and liver was determined from recombinant cDNA clones. The sequence was composed of 1722 bp which included the 1299 bp of the complete coding region, the 108 bp of the 5'-noncoding region and the 312 bp of the 3'-noncoding region containing a polyadenylation signal. In addition, the poly(A) tail was also found. A potential ribosome-binding site was located 30 nucleotides upstream to the initiation codon in the 5'-noncoding region. The amino acid sequence deduced from the nucleotide sequence was 433 amino acids in length and showed very high homology (82%) to the amino acid sequence of γγ enolase (neuron-specific enolase: NSE), although the nucleotide sequence showed slightly lower homology (75%). The size of NNE mRNA was approximately 1800 bases by Northern transfer analysis and much shorter than that of NSE mRNA (2400 bases) indicating a short 3'-noncoding region. A dot-blot hybridization and Northern transfer analysis of cytoplasmic RNA from the developing rat brains using a labeled 3'-noncoding region of cDNA (no homology between NSE and NNE) showed a decrease of NNE mRNA at around 10 postnatal days and then a gradual increase to adult age without changes of mRNA size. Liver mRNA did not show any significant change during development.

INTRODUCTION

Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is a glycolytic enzyme which was discovered a long time ago. Recent investigations (1-3) showed the presence of three types of isozymes composed of homodimer 1) αα, 2) ββ, and 3) γγ in the mammalian tissues. The isozyme αα is found in various tissues and in glial cells within adult nervous tissues, therefore it is called non-neuronal enolase (NNE). The ββ isozyme is localized in the muscle tissue. Further, the isozyme γγ is found only in the nervous tissue and localized in the neurons and neuroendocrine cells; therefore, it is designated neuron-specific enolase (NSE). Immature neurons and muscle cells predominantly contain αα (NNE), but the switch phenomena to γγ (NSE) and ββ from αα (NNE) occur during cell differentiation. These findings indicate that NSE is a marker for mature neurons and NNE is that for mature glial cells. The studies on the molecular mechanism that controls the expression

of these enolase isozyme genes would provide important information about cell-specific or developmental stage-specific expression as in the genes of other isozymes like aldolase (4, 5), amylase (6, 7), alcohol dehydrogenase (8) and pyruvate kinase (9, 10), etc. Such studies would also give valuable information about the process of neuronal and glial differentiation in the central nervous tissue.

We already studied the cell-free translation of NNE- and NSE mRNA in the liver and brain (11-13). Further, recently we succeeded in cloning of cDNA for NSE mRNA and determined its nucleotide sequence (14, 15). In this report, we describe the cloning of cDNA for NNE mRNA. The nucleotide sequence of this cDNA is also described. From the viewpoint of evolutionary implication, the homology of nucleotide and amino acid sequences between NNE and NSE is compared. Further, the examination of NNE mRNA in the rat developmental brain and liver was carried out using a dot-blot hybridization and Northern transfer analysis. These developmental changes are compared with that of NSE mRNA.

MATERIALS AND METHODS

Enzymes and reagents

Reverse transcriptase (RNA-dependent DNA polymerase, EC 2.7.7.49) was purchased from J.W. Beard (Life Sciences, St. Petersberg, FL). Restriction endonucleases were obtained from Takara Shuzo Co. (Kyoto, Japan) and New England Biolabs Inc. (Beverly, MA). T4 polynucleotide kinase (EC 2.7.1.78), DNA polymerase (EC 2.7.7.7) and terminal deoxynucleotidyl transferase (EC 2.7.7.31) were also purchased from Takara Shuzo Co. Oligo(dT)-cellulose (type 3), $(dG)_{12-18}$ and $(dT)_{12-18}$ were from Collaborative Research (Waltham, MA). [γ-^{32}P]-ATP (5000 Ci/mmol), [α-^{32}P]-ddATP (7500 Ci/mmol), [α-^{32}P]-dCTP (3000 Ci/mmol) and [^{35}S]-methionine (1200 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan).

Preparation of RNA

The rat brain free polysomes and liver total polysomes were isolated as described previously (11). Polysomal RNA was isolated by phenol-chloroform-isoamyl alcohol extraction procedure. Poly(A) RNA was isolated from the polysomal RNA by oligo(dT)-cellulose chromatography (12, 13). Cytoplasmic RNAs from the rat brain and liver were prepared as described previously (12).

Construction and cloning of double-stranded cDNA

Double-stranded cDNAs were prepared from brain and liver polysomal poly(A) RNA according to the procedure of Land et al. (16, 17); first-strand

synthesis was accomplished using reverse transcriptase with oligo(dT)$_{12-16}$ as primer and the cDNA was tailed with dCTP. For second-strand synthesis, oligo(dG)$_{12-16}$ was used as primer for a large fragment of DNA polymerase I. The cDNAs were inserted into the PstI site of pBR322 using the dG-dC tailing technique. Transformation of Escherichia coli DH1 was carried out according to the procedure of Dagert et al. (18).

Colony hybridization

A cDNA insert (pRSE-116) containing the coding region of NSE cDNA was labeled by nick-translation. In order to carry out colony screening, colony hybridization with [^{32}P]-labeled pRSE-116 was performed at 55°C for 16 hr in 6 x SSC (1 x SSC = 0.15 M NaCl, and 0.015 M sodium citrate, pH 7.2), 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.5% SDS and 0.5 mg/ml denatured salmon sperm DNA and then washed at 55°C with 6 x SSC and 0.2% SDS according to the modified procedure of Hanahan and Meselson (19). [^{32}P]-labeled pRSE-116 cross-hybridized with NNE mRNA and NNE cDNA under these conditions.

Isolation of plasmid DNA

Plasmid DNA was isolated from cloned bacteria by the methods of Holmes and Quigley (20) or Birnboin and Doly (21).

Hybrid-selected translation assay

Hybrid-selected translation assay was carried out according to the modified method of Parnes et al. (22). Each 5 µg of plasmid DNA containing cDNA isolated as described above was denatured by boiling and addition of alkaline solution (final 0.5 M NaQH), then neutralized and immediately bound to a nitrocellulose filter (Millipore HAWP025). The filters were cut into a 7 mm square and washed with the solution containing 65% (v/v) formamide, 20 mM PIPES, pH 6.4, 0.2% SDS, 0.4 M NaCl and 100 µg/ml yeast tRNA for 10 min at 27°C, and then hybridized with 50 µg/ml of brain free polysomal poly(A) RNA under the above condition at 50°C for 4 hr. The hybridized RNA was eluted from the filters by heating for 1 min at 100°C in 0.2 ml of H_2O containing yeast tRNA and quickly frozen. The eluted mRNA was translated in a rabbit reticulocyte lysate system and the products were analyzed by two-dimensional gel electrophoresis before and after immunoprecipitation as previously described (12).

Restriction nuclease mapping of plasmid DNA

Conditions for restriction endonuclease cleavage of plasmid DNA were essentially as indicated by the supplier. Fragments were electrophoresed on 1% agarose gels or 8% acrylamide gels. These gels were stained with

ethidium bromide and visualized by ultraviolet irradiation.

DNA sequence determination

Appropriate restriction fragments were end-labeled at the 3'-termini with [^{32}P]-ddATP using deoxynucleotidyl terminal transferase or at the 5'-termini with [γ-^{32}P]-ATP using polynucleotide kinase. Nucleotide sequences were determined by the method of Maxam and Gilbert (23). Sometimes the [^{32}P]-labeled single strand was isolated and its sequence was determined.

Northern transfer analysis of brain and liver poly(A) RNA

Rat brain and liver polysomal poly(A) RNAs (2 µg/lane) after formamide (60%) and formaldehyde (2.2 M) treatment were electrophoresed on a 1.3% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose filter and hybridized with the nick-translated [^{32}P]-labeled NNE cDNA insert (pRNE-11) in 50% formamide, 5 x Denhardt's solution, 5 x SSC, 20 mM Na-phosphate (pH 7.0) and 10% dextran sulfate at 42°C for 16 hr. After washing in 2 x SSC and 0.1% SDS at 42°C, the RNA blot was fluorographed at -80°C using an intensifying screen (24). Similar analyses with cytoplasmic RNA from the developing rat brain and liver were also carried out.

RNA dot-blot hybridization

Cytoplasmic RNAs isolated from the whole brains and livers of the developing rats at various ages were denatured with formaldehyde, spotted on the nitrocellulose filter presoaked in the 20 x SSC and hybridized with [^{32}P]-labeled NNE cDNA (pRNE-11) according to the procedure of White and Bancroft (24, 25).

In vitro translation

mRNA-dependent reticulocyte cell-free reactions and analysis of the translation products were carried out essentially according to the procedure of Yoshida et al. (12).

RESULTS AND DISCUSSION

Cloning of NNE cDNA

Poly(A) RNAs isolated from the rat brain free polysomes and liver total polysomes were used to construct rat brain and liver cDNA libraries. These procedures were performed essentially according to the method of Land et al. The [^{32}P]-labeled NSE cDNA insert (pRSE-116) was used to screen the clones containing NNE cDNA inserts. The conditions for colony hybridization were slightly modified to weak stringency. The strongly positive clones contained NSE cDNA. After several weakly hybridization-positive clones were selected, the size of these inserted cDNAs was determined by digestion of the plasmid DNA with several endonucleases and electrophoresis of the DNA fragments on an

agarose or polyacrylamide gel. One clone (pRNE-11) containing the longest insert was isolated. In order to identify this candidate as NNE cDNA clone, we used the hybrid-selected translation assay to examine the recombinant plasmid prepared from this clone as described in the methods. The translation products were analyzed on two-dimensional gel electrophoresis. One of the products was observed to comigrate with an authentic NNE spot which showed Mr. 47000 and pI 6.5 (Fig. 1A). In addition to this spot, an endogenous spot and the product from the carrier yeast tRNA were also observed.

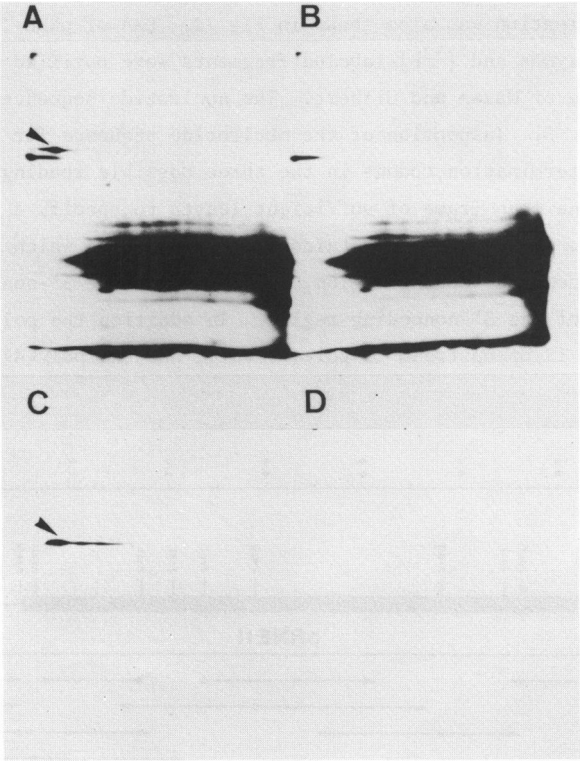


Figure 1. Identification of rat NNE clone by hybrid-selected translation. Rat brain free polysomal poly(A) RNA was hybridized to pRNE-11 or pBR322 which had been bound to a nitrocellulose filter. The hybridized mRNA was eluted and translated in a reticulocyte lysate system containing [^{35}S]-methionine. The products were either directly fractionated by two-dimensional electrophoresis or electrophoresed after immunoprecipitation. A, hybrid-selected with pRNE-11; B, hybrid-selected with pBR322; C, immunoprecipitation of products hybrid-selected with pRNE-11; B, immunoprecipitation of products hybrid-selected with pBR322. The arrowheads indicate the presence of rat NNE.

When the labeled polypeptides were purified by immunoprecipitation and run on a two-dimensional gel (Fig. 1C), a single labeled spot was found to react with the antisera against NNE. However, control experiment did not show any synthesized NNE spot (Fig. 1B and 1D). Thus a clone contained the plasmid containing NNE cDNA insert.

Restriction endonuclease map and nucleotide sequence determination of plasmid DNA

Since pRNE-11 had the size of about 1700 - 1800 bp, the restriction endonuclease cleavage map of this cDNA was constructed by analysis of their single and double enzyme digests (Fig. 2). The strategy of the nucleotide sequence determination was also shown in Fig. 2. DNA of pRNE-11 was digested with several enzymes and [^{32}P]-labeled fragments were purified and sequenced by the procedure of Maxam and Gilbert. The nucleotide sequence of this cDNA is shown in Fig. 3. Inspection of the nucleotide sequence for location of initiation and termination codons in the three possible reading frames revealed only one open frame of sufficient length to specify a protein of the size of NNE. Thus pRNE-11 contained 1722 bp inserts which included the 1299 bp of the complete coding region, the 108 bp of the 5'-noncoding region and the 312 bp of the 3'-noncoding region. In addition the polyadenylation signal, AATAAA, 15 bp upstream to poly(A) tail, and the poly(A) tail were

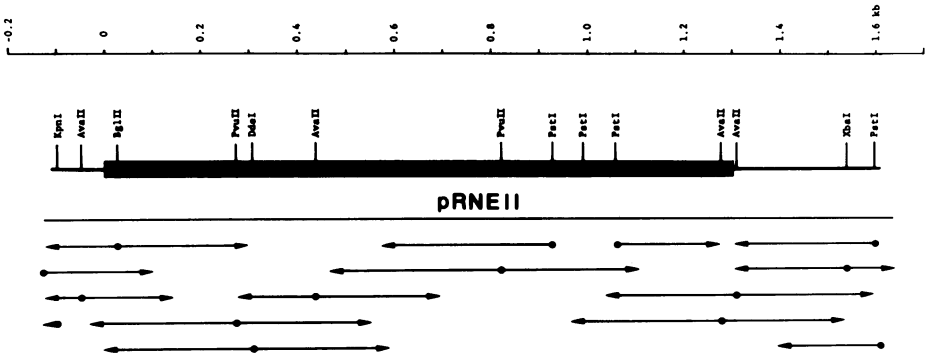


Figure 2. Restriction endonuclease map of rat NNE cDNA and sequence strategy. The top scale designates the nucleotide positions (in kb). The restriction endonuclease map is shown in the second line. The poly(dA)·poly(dT) tract and poly(dG)·poly(dC) tail are not included in the restriction map. The protein coding region is indicated by a closed box. The restriction endonuclease map shows only the relevant restriction endonuclease sites, and arrows under cDNA indicate the direction and extent of sequence determination.

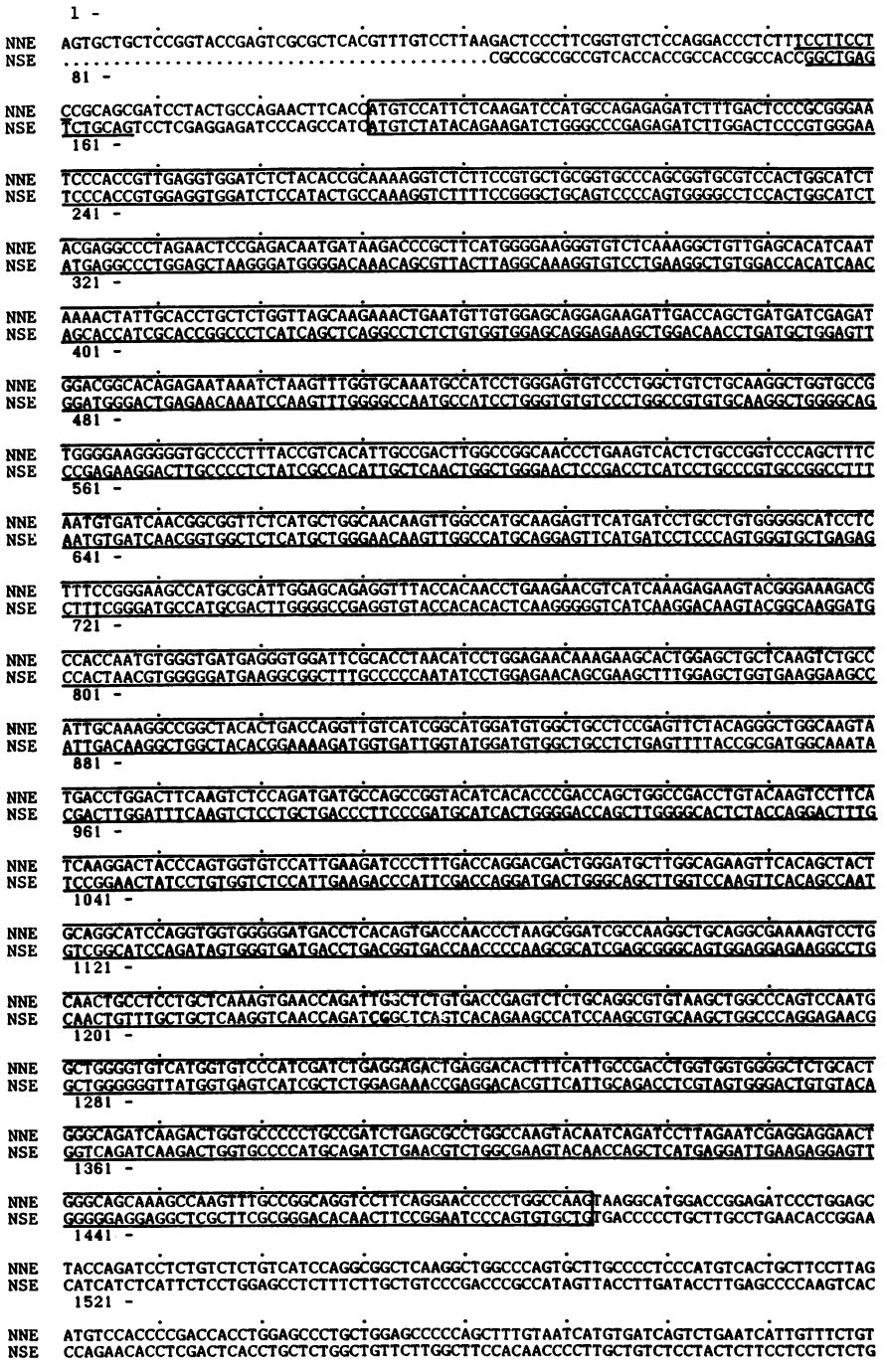
also found, indicating complete 3'-noncoding sequence. These data indicate evidence for the complete nucleotide sequence, considering the size of mRNA (1800 bp) and the size of poly(A) tail as described later.

The primary amino acid sequence of rat NNE was deduced from the nucleotide sequence of the coding region (Fig. 3 and 4). The single open frame encoded a polypeptide of 433 amino acids, indicating a molecular weight of 46984 daltons, while the molecular weight of the purified NNE has been calculated to be 47000 daltons by SDS polyacrylamide gel electrophoresis. The coding sequence starts at a methionine residue in position 109 and ends with a TAA termination codon in position 1411. It is possible that the amino terminus (serine) of mature NNE is generated directly by removal of the initiator methionine. This may be in agreement with the absence of precursor protein in the translation experiments. There is a codon ACC at positions 106-108 which is consistent with a functional initiation codon (Kozak) (26).

We succeeded in cloning of cDNA for rat NSE mRNA and determined its nucleotide sequence (14, 15). Its coding region was 1299 bp in length, coding a protein with 433 amino acid residues, the size of which was the same as that of NNE. The data on NSE cDNA will be published in a separate paper (15). The nucleotide sequences of the coding regions and the deduced amino acid sequences show a high homology between rat NNE and NSE (82% in amino acids and 75% in nucleotides) (Fig. 3 and 4). The slightly lower homology in the nucleotide sequences indicates the difference of the codon usage pattern between NNE and NSE. Especially, there are some different nucleotides in the third position of each codon. In the 5'- and 3'-noncoding regions, however, we could not find any homology between NNE and NSE (Fig. 3), although the 3'-noncoding region of NNE cDNA (312 bp) was much shorter than that of NSE (848 bp). Therefore, each 3'-noncoding region may be useful as a specific probe for examination of NNE- and NSE mRNAs.

These results may be very important when we consider the origin of enolase isozyme genes. In general the isozyme genes are believed to be originated from the same ancestral gene during the course of evolution. Our data which shows high homology may also support this idea. However, it is still unclarified what kind of changes in the gene structures and transcription factors have caused the cell-specific expression or development-specific expression of their coding protein.

Several isozymes were investigated in order to elucidate the molecular mechanism for similar tissue-specific or developmental stage-specific expression of each gene; for example, alcohol dehydrogenase (8, 27), amylase



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      1601 -
           .         .         .         .         .         .         .
NNE   CACCTGACTTTCCAGCTAGTGTCTGGAGCCCTCTGAACTCCAGCGTAATCTCTAGAGTGCCCACACCATCAAGACTCCCC
NSE   GGCCCCATTTTTGGGGGGATTCCAGTCTGCCCACTTTCCCTTCTATTCTCTCTAATCTTAAAAAAAAAAAAAAAATGACG
      1681 -
           .         .         .         .         .         .         .
NNE   CCAGTGGTTTACTTGCAAAAATAAAAGCTGCAGAAGCCCAAAAAA.....
NSE   ACTAGAAGAAGCGGTCCACAGAAGAACCGCCAGCGTCCGAGAGGAGCTTCAGGATTGGTGTGTTGGGGCGTTTAAAGTGG
      1761 -
                .         .         .         .         .         .         .
NSE   GGCCACGTGGCACGTGTGCTTCCCTGCCATCCATGGTGTGTTAAGCCTTGAACTATGCACAGAGCTGGTGTTTGGGGAGT
      1841 -
                .         .         .         .         .         .         .
NSE   GCTGGATGTGTGTGTGTTCACATTTGTTTGTTTGTTTATTTATTTATTCACTTATTTATTTCTCAGCCTGTCAGTCGTCT
      1921 -
                .         .         .         .         .         .         .
NSE   GCCATTACTCTTACAGTCTGAAAGCATCAGTATTTTCACGTGGTTCCATTTCAAGATGACCTAGGATGGGAGGTTTTGTT
      2001 -
                .         .         .         .         .         .         .
NSE   AGCATGGGAAAGGTCACAGAAAGGTTAGCAATGGTTTTTCATTTGGTGCACTAACTGAAGCTCGGTACTTTACAGAATGG
      2081 -
                .         .         .         .         .         .         .
NSE   GGCTGTGTACCCGGGGACTTTTCTCCTATAACTCTCTCCCCCAGCCCTAGGTTCCTCAGTCTTTTTCTCCGGCTGCACCA
      2161 -
                .         .         .         .         .         .         .
NSE   GAGCGCTGCCTCATTCCCCCGTGCCATGTCCCACAGTTGCCACTGTCTCTGTGGCTTTGAAATGACCAACCACTATTAAA
      2241 -
                .         .         .         .         .         .         .
NSE   GTCTGAACCACAGTGCACACCAAAAAA.....
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Figure 3. Nucleotide sequences of rat NNE cDNA (upper line) and rat NSE cDNA (lower line). Nucleotides are numbered in the 5' to 3' direction. The coding regions are boxed. The underlined sequences toward 5' end indicate the predicted binding site for rat 18S rRNA. The polyadenylation signals in the 3'-untranslated region are also underlined.

(6, 7, 28) and aldolase (4, 5). In the Drosophila alcohol dehydrogenase and α-amylase 1, the usage of two promotors was regulated not only developmentally but tissue-specifically, although there are different genes for pancreatic and salivary amylase isozymes. Tsutsumi et al. (29) suggested that the genes for aldolase subunits (A, B and C) are evolved from the same ancestral gene and the heterogeneity of the aldolase A mRNA in the fetal liver and muscle could be due to the difference of either the 5'- or 3'-untranslated regions.

Our results on the cDNA for NNE and NSE showed high homology of the coding regions and differences of 3'-noncoding regions (the size and sequences) and 5'-noncoding regions (sequences), but these data may not be sufficient to explain the cell-specific or development-specific expression of the isozyme genes. Cloning and characterization of genomic DNA for both isozymes may be important to elucidate the above mechanism. We are currently carrying out cloning of genomic DNA for NNE and NSE from a rat genomic DNA

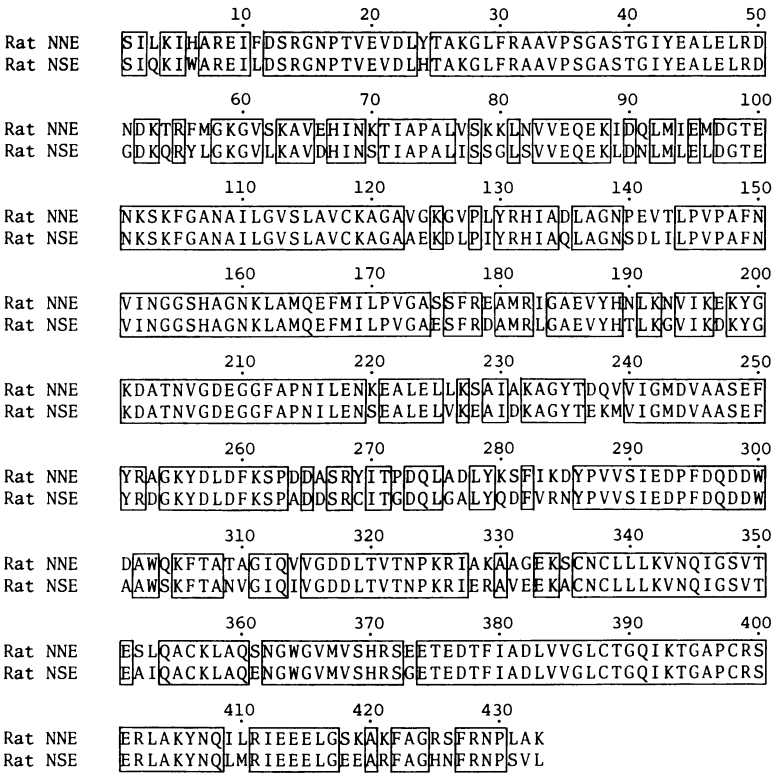

Figure 4. Homology of the amino acid sequences between rat NSE and NNE deduced from the nucleotide sequences in Fig. 3. The one letter amino acid notation is used. The homologous residues are boxed.

library using the cDNA probes. Our preliminary experiments on Southern transfer analysis with the rat total genomic DNA and the rat genomic DNA library showed evidence for different genes of NNE and NSE.

Size of NNE mRNA

The nick-translated [^{32}P] PstI-XbaI fragment (3'-noncoding region) of pRNE11 was used as a probe to establish the size of NNE mRNA in the rat brain. Fig. 5 shows that it is about 1800 bases in length by the Northern transfer technique. The size of mRNA from the liver was also similar to that of rat brain (Fig. 5). A protein of 47000 daltons requires 1299 bases for its coding region. Therefore, NNE mRNA must have about 500 noncoding bases including the 3'-poly(A) tail. Fig. 3 shows that the 5'-noncoding region is 108 bases long and has a GC content of 58%. There is a 8-base

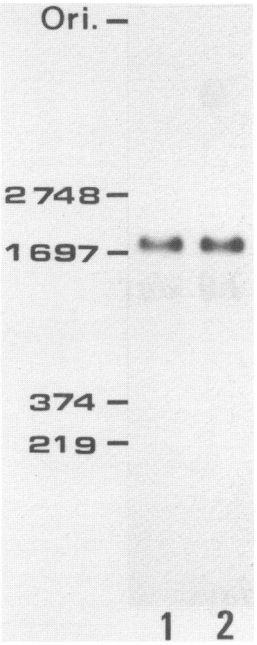

Figure 5. Northern transfer analysis. Two micrograms of poly(A) RNAs isolated from the rat brain free polysomes and liver total polysomes were fractionated by electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde. Resolved RNA was transferred to a nitrocellulose filter, and hybridized with nick-translated PstI-XbaI fragment of pRNE-11. ϕX174 DNA was digested with HapII and [^{32}P]-labeled for use as a molecular weight marker. Lanes: 1, RNA from liver; 2, RNA from brain.

pair stretch in this region (73-81) with partial homology to the 3'-end of 18S rRNA (30). This region that might hybridize to rat 18S rRNA (31) is as follows.

NNE mRNA ..... ^{73}UCCUUCC$^{U}_{C}$^{81}.....

Rat 18S rRNA HO-AUUACUGAGGAAGG$_{C}$GU .....

The free energy of hybridization of a continuous 7-base pair stretch (73-79) is -17.8 kcal/mol/25°C (32). In the 3'-noncoding region, 312 nucleotides were found, including a polyadenylating signal, AATAAA as shown in many eukaryotic mRNAs (33). In addition, the poly(A) tail was also found, but this tail may be around 90 nucleotides in length. Thus these data indicate that our cDNA contains the nearly complete nucleotide sequence.

The developmental changes in the levels and size of NNE mRNA in rat brain and liver

Since the cDNA probe specific for NNE was available, the developmental changes of this mRNA were examined by a dot blot hybridization and Northern transfer hybridization analysis of cytoplasmic RNA from rat whole brain and liver using the labeled cDNA (3'-noncoding region fragment). Fig. 6 shows

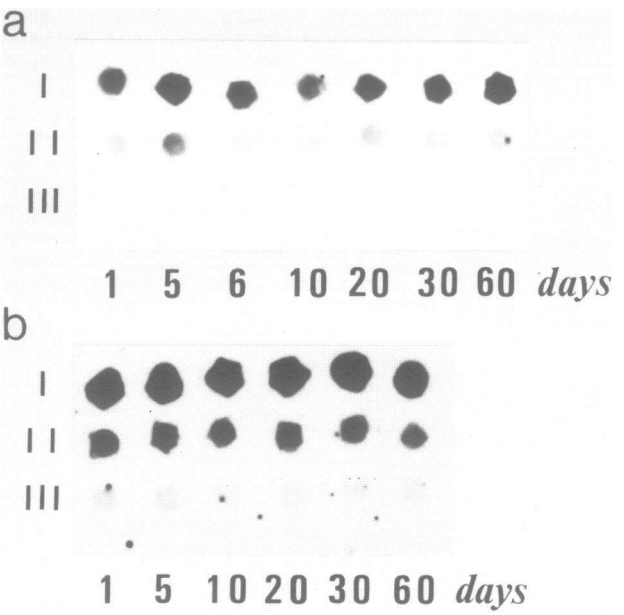

Figure 6. Estimation of NNE mRNA by dot blot hybridization. Serial 1 : 5 dilutions of cytoplasmic RNA isolated from brain (a) and liver (b) of rat of different developmental stages were spotted onto nitrocellulose filters and hybridized with [^{32}P]-labeled PstI-XbaI fragment of pRNA-11. Lanes: I - III, 6, 1.25, and 0.25 µg RNA, respectively.

a slight decrease at 10 postnatal days and then a gradual increase during 20-30 days in NNE mRNA concentration of the rat brain. These developmental changes of NNE mRNA were different from those of NSE mRNA. NSE mRNA concentration was gradually increased during 5-30 postnatal days (15). Fig. 6 shows no developmental changes in NNE mRNA concentration of the rat liver. The similar developmental changes of translatable NNE mRNA in a cell-free translational system were found, confirming a valley-like change of the NNE mRNA level in the brain and a minimal change in the liver (12). It is important, without the use of translation procedure, to get similar results using the direct quantitative analysis of mRNA for NNE.

The changes of the size of NNE mRNA during development of rat brain were examined using Northern transfer analysis. Any significant change of the size was not found as far as we examined (data not shown).

Since we can observe the specific bands of NNE and NSE mRNA in the rat liver and brain using 3'-noncoding sequences of NNE and NSE cDNA as the probes, we already observed the effects of some conditions on the mRNA of

both enolase isozymes using these cDNA (data not shown). These results in the adult rat brain may suggest the specific metabolic changes of neuron and glia-cells. Further the experiments in the fetal and young brains using dot blot hybridization and in situ hybridization may give information on the differentiation of neurons and glial cells. Furthermore, inducing of these cDNA combined with the specific promotor sequence into some cultured cells may also permit us another type of the studies on the cell-specific expression.

ACKNOWLEDGEMENTS

We thank Profs. S. Natori and M. Muramatsu of Tokyo University for their advice. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Public Health of Japan and from the Niigata Medical Foundation to Y.T.

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