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EXPRESSION OF DNA SEQUENCES CONTAINING NEURON SPECIFIC ENOLASE GENE IN *ESCHERICHIA COLI*

S.K. Dutta, Nandan Bhattacharyya, R. Parui* and Mukesh Verma

Molecular Genetics Laboratories, Departments of Botany and Genetics & Human Genetics; and Cancer Research Center, Howard University, Washington, DC 20059

*Department of Medical Genetics,
Howard University Hospital, Washington, DC 20060

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SUMMARY: There is evidence that the gene for gamma-gamma enolase (neuron specific enolase, NSE) is regulated during cell differentiation and development, conserved in a variety of organisms and contains mRNA destabilizing sequences. In order to investigate further the mechanisms of these processes and to obtain large quantity of this protein, the NSE gene was isolated from neuroblastoma cells and cloned in *E.coli* using standard molecular biology techniques. The NSE gene expression was studied and the expressed protein (recombinant NSE) was characterized extensively. The recombinant NSE behaves like parental NSE in antisera specificity, resistance for chaotropic agents like urea, thermal stability at higher temperatures etc. The physical parameters like secondary structure, hydrophilicity, antigenic index and flexibility of the expressed protein were studied. The results of the present investigation collectively form the basis for initial investigations of how the expression of NSE gene is regulated. This is the first report where the recombinant NSE gene has been characterized so extensively.

Enolase (2-phospho-D-glycerate hydrolase) is the most extensively studied enzymes of the 10 that make up the glycolytic pathway (1-9). It catalyses the reversible conversion of 2-phosphoglycerate and phosphoenol pyruvate in the glycolytic pathway. Neuroblastoma cell line NG108 is reported to synthesize the gamma gamma form of enolase, called NSE. Being the vital enzyme of the glycolytic pathway, NSE has been the center of attraction for neurobiochemists. In neurons, it constitutes about 3% of total neuronal soluble protein and studies performed in different laboratories suggest that the appearance of NSE is correlated with neuronal maturation (3). Recent investigations (3, 8) showed the presence of five types of isoenzymes of the homodimer, viz., alpha-alpha, beta-beta, gamma-gamma, alpha-beta and alpha-gamma. As yet the remaining beta-gamma combination has not been found (8). The isoenzyme alpha-alpha is found in various tissues and in glial cells within adult nervous tissues; therefore, it is called non-neuronal enolase (NNE). The beta-beta isoenzyme is localized in the muscle tissue. Further, the isoenzyme gamma gamma is found only in the nervous tissue and is localized in the neurons and neuroendocrine cells; therefore it is designated as NSE. It has been demonstrated that during cell differentiation in immature neurons and muscle cells the switch phenomena to gamma-gamma (NSE) and alpha-gamma from alpha-alpha occurs (3). These findings suggest that NSE is a marker for mature neurons and that NNE is the marker for mature glial cells. In our laboratory we have purified NSE from neuroblastoma cells and characterized it extensively (1, 5, 10-12). NSE has been used as a diagnostic marker for cancer of different kinds like peripheral neuronal tumor (13), lung carcinoma (14), neuroblastoma (14), Markel cell tumor (15), retroperitoneal tumor (16), melanoma (17), medulloblastoma (18, 19) etc.

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To characterize NSE biochemically as well as immunologically, it was essential to obtain a large amount of highly purified enzyme; therefore, we have cloned the DNA fragment of NG108 which contains coding sequences for the NSE gene along with flanking sequences containing the regulatory sequences required for the expression of this gene. The expression of the NSE gene in *Escherichia coli* is evident from the experiments reported in these studies.

In this paper we provide evidence that the NSE clone pNGE7 constructed by us can express NSE in *E. coli* cells. The utilization of this clone to study differential expression of NSE genes in development, molecular evolution and stability of mRNAs has been elucidated.

MATERIALS AND METHODS

pNGE7 clone contains DNA sequences encoding NSE isolated from NG108, a hybrid cell line made by Marshall Nirenberg (NIH) from the mouse neuroblastoma and rabbit glial cells. The gene was cloned in phagemid bluescript expression vector and transformed in *E. coli* JM109. Details of molecular cloning procedures are being published separately. The clone pNGE7 is tetracyclin sensitive and ampicillin resistant and it can also be screened by blue/white color reaction with IPTG/XGal (20).

Assay of the Enzymes: Routinely the activity of the enzyme was measured by determining the velocity measurement at 240 nm as described by us previously (1).

Ammonium Sulfate Precipitation: Overnight grown cells of pNGE7 were collected (as described under materials and methods) and homogenized with 3 volumes of 10 mM Tris HCl, pH 7.0, 1 mM MgSO4, 1.4 mM beta-mercaptoethanol and acid washed sea sand. The extract was centrifuged and the supernatant was brought to 40% ammonium sulfate saturation. The resulting suspension was then centrifuged at 20,000 g for 30 min at 4°C. The 40% supernatant fraction was then adjusted to 80% ammonium sulfate saturation and stirred for 45 min at 4°C. This suspension was then centrifuged at 20,000 g for 20 min at 4°C and the protein pellet so obtained was dissolved in 10 mM Tris HCl, pH 7.0, 1 mM MgSO4. This protein fraction was then dialysed extensively against the Tris Mg^{++} buffer overnight at 4°C.

DEAE Cellulose Chromatography: Preswollen DEAE cellulose was passed through acid (0.5 N HCl) and base (0.5 N NaOH) cycles and equilibrated in 10 mM Tris HCl, pH 7.0, 1 mM MgSO4 as described (1). About 200 mg of protein was applied on the DEAE cellulose column (2.5 x 20 cm) in a volume of 70 ml. All the operations were done at 4°C unless otherwise stated, and the flow rate was maintained 0.5 ml /min. The column was washed twice with 10 mM Tris-HCl, pH 7.0, 1 mM MgSO4 and a linear gradient of NaCl (0-0.5 M) prepared in column buffer was then run through the column. Basically two major peaks were obtained, first for NNE and the second for NSE. NSE was characterized further.

Sephadex G-150 Chromatography: Sephadex G-150 was used in the column (1.5 x 50 cm), and the pooled fractions (after ion-exchange chromatography) were passed through this column. The active fractions after Sephadex G-150 chromatography were concentrated and used for the characterization of the enzyme. The protein isolated by this method seems to be homogeneous as it migrates as a single band (monomer mol. Wt. 45,000) on denaturing acrylamide gels. Sedimentation analysis of enolase with GM (guanidine-HCl containing 0.02% glutathionine) yields a molecular weight of 80,000 indicating the dimeric nature of the protein.

Amino Acid Analysis: The amino acid composition of enolase was determined according to Benson and Here (21) and for this purpose Glenco Amino Acid Analyzer, Model MM70, Glenco Scientific Instruments Company, was used. Initially a 1 mg/ml stock solution of protein (enzyme enolase) was made in 0.2 M lithium citrate buffer (pH 2.2% $\pm$ 0.1). This solution was hydrolysed with 6N HCl containing 0.02% phenol at 110°C for 24 hours. Then the solution was further diluted with the citrate buffer to make a final concentration 50 ng/0.1 ml. To estimate the amino acids present in the sample, 0.1 ml of the diluted solution was injected. The composition of amino acids in enolase was calculated on the basis of standard amino acid runs. Norleucine was used as the internal standard. Nomenclature for different enolases was as follows: EE for *E. coli* enolase (which is NNE), CE for enolase of NG108 and CE for enolase of clone pNGE7.

RESULTS AND DISCUSSION

After cloning the NSE gene from neuroblastoma, its expression in *E. coli* was studied and the results are presented in Figures 1-3 and Tables 1 and 2. As a first step the enzyme was purified by a series of chromatographic steps from *E. coli* cells transformed with vector only (Fig. 1A) and then from pNGE7 transformed cells (Fig. 1B). From *E. coli* cells containing vector only, single peak was observed whereas from pNGE7 transformed cells two peaks, first one eluted at no or very low salt concentration (NaCl) and second at high salt concentration, were observed (Fig. 1B). The activity of enolase in those peaks was determined and the enzyme was subjected to biochemical analysis like antisera neutralization, resistance to heat, urea etc. and results are presented in Fig. 2. Enolase from *E. coli* transformed with vector (i.e. with NSE gene) reacts with NNE antisera. The first peak of the pNGE7 (Fig. 1B) reacts with NNE antisera and the second peak reacts with NSE antisera. This suggests that pNGE7 cells express enolase protein which is neutralized by NSE antisera (Fig. 2C).

Previously, we have reported (1) that NSE is more resistant to heat and higher concentrations of urea; therefore, we tested purified NNE (EE) and NSE (CE), isolated from pNGE7, for these treatments and the results are shown in Fig. 2A and 2B. The figure clearly illustrates that NNE is inactivated completely when heated at 50°C for 5 minutes, whereas NSE is inactivated to only 30%. NSE (Fig. 2B) is inactivated completely at 70°C. The results suggest that NSE is more resistant to heat than NNE.

When NNE (EE) and NSE (CE) isolated from pNGE7 were treated with different concentrations of urea (as shown in Fig. 2A), NSE was found to be much more resistant to urea than was NNE. For example, treatment of 1M (or 1000 mM as shown in Fig. 2A) urea at most completely inactivated the NNE whereas NSE showed about 78% activity. As a further step the amino acid composition of CE was determined and its comparison with rat NSE and NNE composition is presented in Table 1. As the results clearly show, CE is

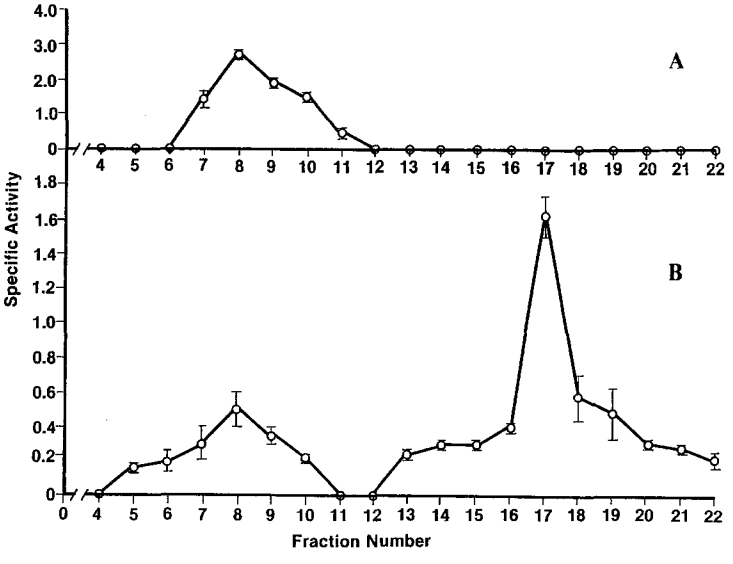

Fig. 1. DEAE cellulase fractionation profiles of purified enolase from *E. coli* cells transformed with vector without the insert (A) and pNGE7 containing the NSE gene insert (B). The details are discussed in the text.

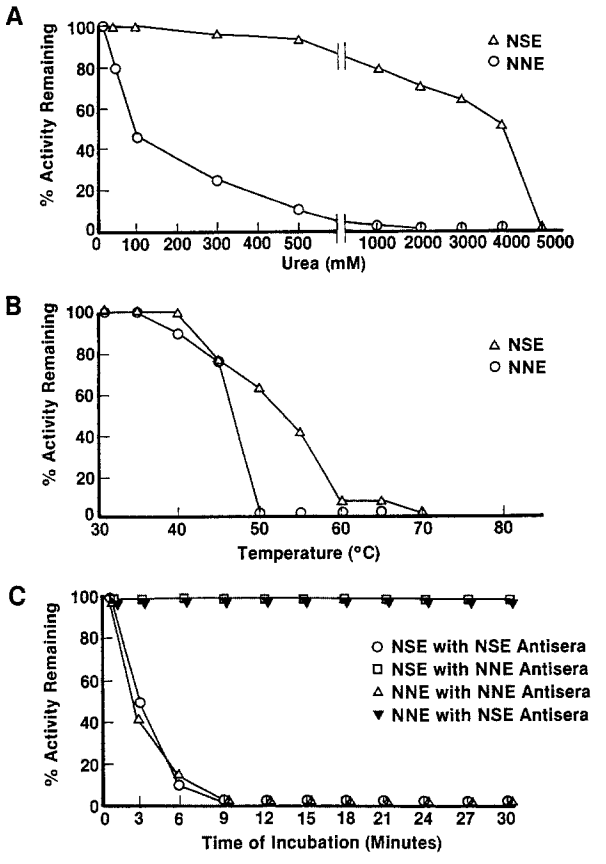

Fig. 2. Characterization of the NSE produced by pNGE7 by urea treatment (A), resistance to temperature (B) and neutralization with antisera (C). The methodology has been explained in previous publications from our laboratory (1, 10).

TABLE 1. Amino Acid Composition of Enolase Isoenzymes from Different Sources

	Molecular Percentage in		
Amino Acid	Rat NNE*	Rat NSE*	NG108 NSE
Aspartic acid	11.38	11.20	11.00
Threonine	4.30	4.00	4.01
Serine	6.53	5.88	5.75
Glutamate	9.85	11.22	10.56
Proline	4.07	3.73	3.95
Glycine	8.80	9.60	9.5
Alanine	11.04	10.56	10.75
Cysteine	1.38	1.28	1.30
Valine	7.99	7.85	7.7
Methionine	1.77	1.57	1.6
Isoleucine	6.06	5.00	5.10
Leucine	8.39	9.72	9.6
Tyrosine	2.04	1.97	2.0
Phenyl alanine	3.43	3.27	3.30
Lysine	8.72	6.85	6.50
Histidine	1.24	1.58	2.1
Arginine	3.95	4.69	5.0

* Data taken from (3).
The amino acid composition was determined as described (21).

TABLE 2. Characterization of Recombinant NSE Isolated from pNGE7 Clone

Properties	Rat NNE*	Rat NSE*	NSE of pNGE7+
Molecular weight	87,000	78,000	80,000
Subunit composition			
Subunit molecular weight	43,500	39,000	45,000
Isoelectric point	7.2	4.7	ND
Reactivity with anti NNE-serum	+++	---	---
Reactivity with anti NSE-serum	---	+++	+++
Substrate Km	1.3×10^{-4}	1.2×10^{-4}	1.1×10^{-4}
Chloride stability	No	Yes	Yes
Temperature stability	No	Yes	Yes
Urea stability	No	Yes	Yes

* These values were obtained from Marangos and Schmechel (3).
\+ From studies reported in this communication.
ND, not done.
Procedures of the determination of Km values, antisera treatment, stability to urea, heat and chloride have been described previously (1).

similar to the NSE of rat. Table 2 summarizes the physical characterization of EE and CE. The parameters like isoelectric point, molecular weight, subunit composition, Km of the substrate etc. were compared and it was concluded that CE is similar to NSE in its physical properties.

The secondary structure, hydrophilicity, surface probability, antigenic index and flexibility of the NSE were determined using IBI Pustell Protein Analysis Program (Ver 2.02). In the top part of the figure (Fig. 3) the information regarding the antigenic index is provided which combines the information from secondary structure predictions (based on the amino acid composition), flexibility predictions, surface probability and hydrophilicity predictions to produce a composite predicition of the surface contour of a protein, and thus the regions most likely to be antigenic. As seen in the figure the potential antigenic sites are present throughout. The protein secondary structure has been analyzed using the popular methods of Chou and Fasman (22) and Garnier et al. (23) because this analysis not only predicts the secondary structure of the protein, but also integrates data from both methods to provide a third display of regions of secondary structure. An "integrated" display of secondary structure is important because each method alone is only about 60% accurate. By showing the combined predictions, we are showing the regions in which one might feel more confident in the data. In the hydrophilicity curve (24), where the value of the graph is above the line (a positive number), the region is hydrophilic and vice-versa. The lower graph representing the surface probability of NSE uses solved crystallographic structures as its data set to derive properties of amino acids used for predictions. Each amino acid is assigned a probability of being a surface residue, or an amino acid with >20 argstorms of water-accessible surface in a solved structure (24). A value of 1.0 means the segment is definitely at the surface (although we realistically never achieve a perfect 1.0) and a value of 0.0 means the segment definitely is not at the surface (25). The flexibility analysis and pleated sheet structure analysis is based on the methods of Karplus and Schultz (26).

The main objective of the current investigation was to isolate large amount of NSE and characterize it using recombinant DNA techniques. The procedure adopted was to clone the DNA fragment that contains the complete sequences for the expression of NSE. This large amount of highly purified NSE enzyme will be useful for future in-depth biochemical studies in addition to those done in this report. Since one possible isoenzymic form of enolase beta-gamma has not been reported so far, we will attempt to determine if clone pNGE7 synthesizes any amount of that form. Furthermore, the clinical significance of NSE is enormous (1,

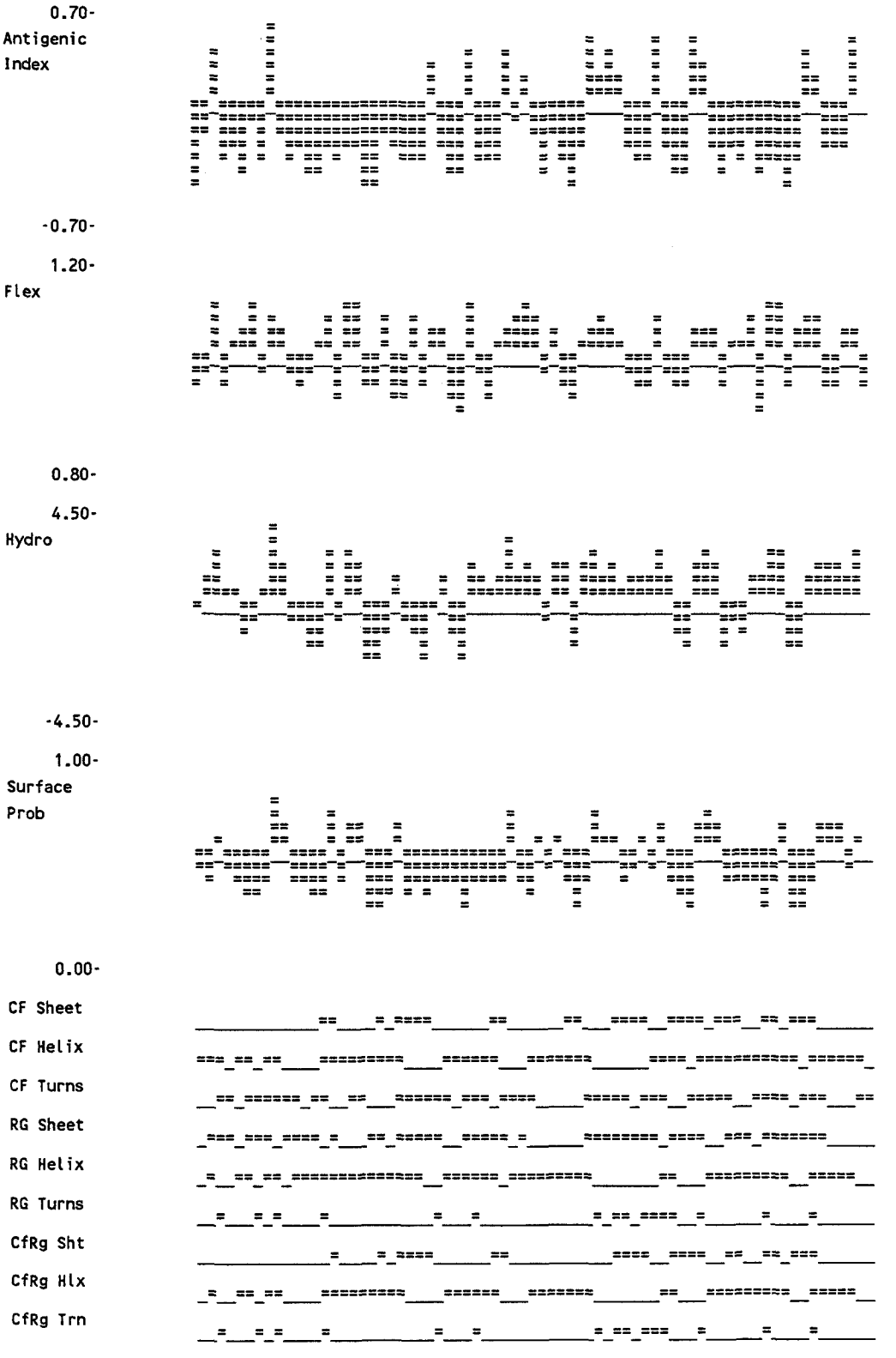

Fig. 3. Antigenic index, flexibility, hydrophobicity and surface probability of NSE. The description of the computer analysis is given in the text.

27, 28 and references therein), because it is used as a diagnostic marker in several diseases including cancer (3) the clone pNGE7 should be very useful for studying the NSE gene and its regulation.

The comparative analysis of the primary structure of enolase indicate that the deduced amino acid sequence (433 residues) of human gamma enolase shows a 97% similarity with rat gamma enolase (6, 8). Both the 5'- and 3'-untranslated regions are similar (82% and 68%, respectively) to the analogous regions of the rat gamma enolase gene, suggesting that a strong selective pressure operates on noncoding segments of gamma-enolase mRNAs. The size of the gamma-enolase mRNA expressed in human and rat brain is 2.4 kb (6, 8). Rat NNE and NSE show high degree of homology (82% in amino acids and 75% in nucleotides) (29-32). However, no homology was found in the 5' and 3' noncoding regions, although the 3' noncoding region of NNE was much shorter than that of NSE. Each 3' noncoding region may be useful as the specific probe for examining NSE and NNE mRNA. These observations from different laboratories (including ours) may be significant when we consider the origin of enolase isozyme genes. In general, isozyme genes are believed to have originated from the same ancestral gene during the course of evolution (29-32). Results from Sakimura's group, Oliva's group and Lazar's group (2, 6, 8, 29-32) are in congruence with this idea. The above description favours NSE to be a model system to study molecular evolution as has been done by our group using the non-transcribed spacer region and coding sequences of the ribosomal RNA genes (33, 34).

The sequence conservation in the 3'-untranslated regions of NSE, lymphokine and protooncogene mRNAs has been reported (32). Two short conserved sequences in this region are worthy of comment. The first of these is a poly (A^+) stretch of 15 residues in the rat NSE mRNA which appears as a 12-nucleotides sequence with 8 A residues in the same position in the human NSE mRNA. This position corresponds to the poly (A^+) addition site in the mRNAs for the yeast enolase isozymes and for NNE (32) and suggests that during evolution the lengthening of the neuron-specific transcript may have involved an insertional event. The second feature is an ATTT sequence which is repeated 9 times in 40 residues in the rat NSE sequence and is seen in a similar position in the human NSE sequence where it is repeated 7 times in 28 residues. A similar sequence, which is unusual among eukaryotic mRNAs, has been reported in the 3'-untranslated regions of the numerous lymphokines, cytokine, and protooncogene mRNAs (32). It has been suggested that these gene products (which are not structurally related in protein-coding sequences) are transiently expressed and that this repeated sequence may be part of a mechanism for selective degradation of mRNAs containing it (35, 36). Insertion of this repeated sequence into the 3'-untranslated region of the stable beta-globin message resulted in dramatic increase in mRNA instability. pNGE7 can now be used for such studies. NSE, however, is a major (up to 3%) protein in adult neuronal cytoplasm and its mRNA appears to be abundant (28). Detailed study of the rates of NSE mRNA turnover may throw light on the function of this unusual repeated sequences in the 3'-untranslated region.

The prokaryotic bacterial cells like *E. coli* do not syntehsize splicing enzymes. These enzymes are essential for the expression of eukaryotic gene(s) which contain intron(s). It is not known yet whether NSE gene cloned by us has any intron. There are examples of eukaryotic genes, like histone genes studied in numerous organisms, which do not contain introns (37). Several organisms have shown intron variation within and between related species which has led to a new concept of the "optional intron" (38). Recently Huang (39) has made an extensive review of the literature on the possible regulatory role of introns and has provided examples of the possible functions of introns themselves as coding sequences. Using cDNA as a probe it has been reported that enolase mRNA size is of 1.8 kb (2). Due to lack of knowledge of the enolase gene, particularly those of NSE gene, it is not possible at this time to provide exact explanation

about the mechanism of NSE gene expression in *E. coli* cells, nevertheless, pNGE7 clone contains coding sequences for NSE as well as large extra DNA segments adjacent to NSE gene. It is not known whether those extra sequences might have any role in NSE gene expression in *E. coli.* Hence, extensive studies on the molecular structure of NSE gene are needed, which is the subject of our future investigations.

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