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ISOLATION OF MURINE NEURON-SPECIFIC AND NON-NEURONAL ENOLASE cDNA CLONES

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SUMMARY : cDNA clones corresponding to subunits of neuron-specific (γγ and αγ) and non-neuronal (αα) enolase isozymes were characterized from two mouse brain cDNA libraries. Our hybridization data revealed a partial homology of the coding sequences of mouse α, mouse γ and rat γ mRNAs. The noncoding sequences, however, appear to be specific for each mouse mRNA. Although coding for two polypeptides of the same molecular weight, the mRNA for the γ subunit (2600 bases) is larger than that for the α subunit (1900 bases). The noncoding sequences for neuron-specific γ mRNA (about 1300 bases) are therefore longer than those of the non-nervous tissue specific α mRNA (about 600 bases).

Three dimeric forms of the glycolytic enzyme enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) are found in extracts of adult mammalian brain : an αα form, which is also present in other tissues, and γγ and αγ forms which are specific to nervous tissue (1).

In fetal brain αα enolase is the predominant isozyme (2, 3, 4). Immunohistochemical analysis has shown that a switch from αα to γγ enolase occurs specifically in post-mitotic neurons when they have reached their final location (5). Low levels of γγ and αγ enolases have been observed in early embryos which contain no recognizable neurons (3) and some evidence for γ gene expression in neuro-glial precursor cells which are still dividing has also been obtained (6, 7). However, the net increase in the γ subunit level is a postnatal event (2, 3, 4) and appears correlated with the functional maturation of neurons (5). Thus, in mature brain, α is the only subunit present

Abbreviation : bp, base pairs.

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in glial cells whereas γ subunit is expressed only in neurons (5). However, in Purkinge cells both γ and α subunits have been detected as γγ, αγ and αα dimers, γγ remaining the predominant form (8). The differential expression of α and γ genes during development is therefore a marker event in the maturation of glial and neuronal lineages and studies of molecular mechanisms involved should provide important data on the differentiation of nervous cells.

Our previous studies have shown that the expression of α and γ gene products, during brain development (4) and in differentiating neuron-like cells in culture (9, 10), is controlled at the level of their rate of synthesis (11, 12) which appears to depend primarily on the relative amounts of corresponding translatable mRNAs (11, 13). We also observed, by denaturing sucrose gradient analysis, that the translatable γ mRNA is much longer than the α mRNA (13). cDNA probes for mouse neuron-specific and non-neuronal enolases were necessary to further characterize mouse α and γ mRNA sequences and continue our studies on the developmental control of cerebral enolases expression. In this report we describe the isolation of these clones and present a comparison of the structural features of mouse α and γ mRNAs based on colony hybridization, hybrid-selected translation and Northern blot analysis data.

MATERIAL AND METHODS

Preparation of poly (A^+)RNA from adult mouse brain and liver : Poly(A^+) RNA used for cloning was prepared and enriched for α and γ sequences as previously described (13). For Northern blot analysis and hybrid-selected translation assays, total poly (A^+)RNA was obtained according to Auffray (14).

Construction of cDNA libraries and colony screening : A first cDNA library was prepared using the enriched mRNA fraction. Double stranded-cDNA was synthesized according to Wickens (15). cDNAs of more than 400 bp in length were inserted into the Pst1 site of pBR322 vector after dG-dC tailing and used to transform *E. coli* 1106. A second library was constructed in *E. coli* HB 101 using the Gubler technique (16) to prepare the double-stranded cDNA. The initial screening was performed with a rat γ cDNA probe of 510 bp from pUC8-3-1 clone. This clone was isolated from a brain cDNAs library, constructed in the expression vector lambda gt11 and screened with affinity-purified antibodies to rat γγ enolase. After subcloning into pUC8, the γ cDNA insert was further characterized by hybrid-selected translation and sequencing, and showed to correspond to the C-terminal sequence of the rat γ subunit (Bishop and Zomzely-Neurath, manuscript in preparation). All hydridizations and washings were performed under low stringency conditions.

Hybrid-selected translation : 5 µg of recombinant plasmid DNA prepared by alkaline lysis method were denatured and bound to a nitrocellulose membrane (17). After hybrid-selected translation, labelled α and γ polypeptides were immunoprecipitated from the translation products using specific αα or γγ antisera tested previously (4, 18). Unambiguous identification of *in vitro* synthesized α and γ subunits was obtained as previously described (13).

Characterization of mouse brain enolase mRNAs by Northern blot analysis : 5 µg of poly (A^+)RNA were treated with formaldehyde (19) before agarose gel electrophoresis. All subsequent steps were performed as described by Thomas (20).

RESULTS

I - Isolation and characterization of cDNA clones for α and γ mRNAs : Poly (A^+)RNA from mouse brain was enriched for α and γ mRNAs from 0.2 to 1 % and 0.06 to 0.2 % respectively and used to construct a first cDNA library as described in Methods. 5400 recombinants clones were ordered and screened using the insert of pUC8-3-1 as a probe. Two positive clones were obtained containing 1450 bp and 950 bp inserts respectively with an internal Pst1 site as determined by electrophoresis of Pst1 digests on agarose gel. These presumptive α or γ clones were identified as α clones by hybrid-selected translation and designated pBE α1 and pBE α2 (see fig. 1 and below). No γ clone was detected with the pUC8-3-1 probe.

During a second screening of the library with pBE α1 insert, 3 other clones containing short inserts (about 300 bp) were detected, confirmed to be α clones by Northern blot analysis (see following section) and designated pBE α3, pBE α4 and pBE α5.

To improve the yield of longer inserts a second library was prepared using the Gubler technique for cDNA synthesis and 9200 recombinants were screened with pBE α1 insert as the probe. Three highly positive clones were selected as putative α clones. The clone with the largest insert (1800 bp) showing an internal Pst1 site was characterized as an α clone by hybrid-selected translation and designated pBE α6. The two other clones with short inserts (about 300 bp) were identified as α clones by Northern blot analysis and designated pBE α7, pBE α8. Three weakly positive clones were also selected as presumptive γ clones with pBE α1 probe and contain respectively 2000, 1600 and 1200 bp inserts without an internal Pst1 site. These clones were identified as γ clones, the first by hybrid-selected translation, the other two by Northern blot analysis (see following section). They were designated pBE γ1, pBE γ2 and pBE γ3 respectively. As shown in fig. 1, the translation products immunoprecipitated with γγ antiserum gave a single strongly labelled band comigrating with the carrier for pBE γ1 and PUC8-3-1 clones. A less intense radioactive band was also obtained with αα antiserum. The reverse was observed for pBE α6 with αα and γγ antiserum (see Discussion).

When the first library was rescreened with the pBE γ 1 probe, a strongly positive clone, with a short insert (about 300 bp) designated pBE γ4, previously

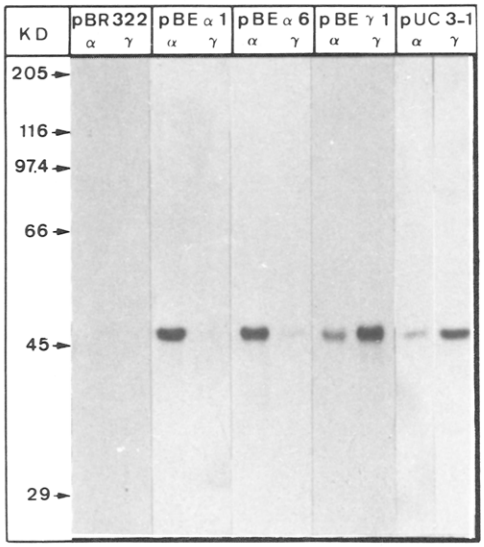

Fig. 1. Identification of brain enolase cDNA clones by hybrid-selected translation. Gel electrophoresis of the translation products obtained with mRNA eluted from plasmid DNAs and immunoprecipitated with αα or γγ antiserum. pBE α 2 gave the same results as pBE α 1 and pBE α 6 (data not shown).

undetected with the pUC8-3-1 and pBE α1 probes, was revealed and confirmed to be a γ clone by Northern blot analysis. In addition, no cross hybridization was observed between the pBE γ1 probe and the α clones from both libraries containing short inserts (pBE α 3, 4, 5, 7, 8), whereas weak cross reactions were obtained with clones including long inserts (pBE α 1, 2, 6).

II. Northern blot analysis : Northern blot analysis using pBE α6 DNA as the probe has permitted identification in total poly (A^+)RNA from brain and liver of a unique sequence of 1900 bases corresponding to α mRNA (fig. 2A). With the pBE γ1 DNA probe in total poly(A^+)RNA of brain but not liver, a single sequence of 2600 bases was observed and corresponds to the γ mRNA (fig. 2B).

The identity of the other presumptive α and γ clones was similarly confirmed. The corresponding nick-translated plasmid DNA recognized either α or γ mRNA.

DISCUSSION

We have detected clones for the mouse α subunit with a rat γ cDNA probe as well as mouse γ clones with a mouse α cDNA probe.

Our hybrid-selected translation experiments permitted unambiguous identification of the putative α and γ clones. Indeed, the αα and γγ antisera used were previously shown to be strictly specific for the α or

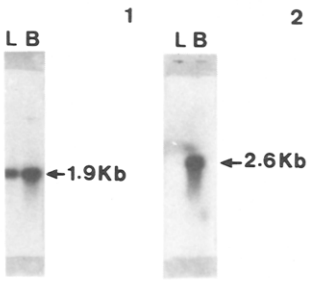

Fig. 2. Size determination of mouse enolase mRNAs by Northern blot analysis. Poly (A^+)RNA of brain (B) or liver (L) : 5 µg per lane. Hybridization with pBE α 6 (1) and pBE γ 1 (2) nick-translated plasmid DNAs. pBE α 1, 2, 3, 4, 5, 7, 8 gave the same results as pBE α 6 and pBE γ 2, 3, 4 as pBE γ 1 (data not shown).

γ subunits respectively (4, 18). The absence of the hybrid form αγ among the translation products of brain poly(A^+)RNAs in a cell free system was also demonstrated (13). Therefore, the presence of a significant radioactive band when translated products of mRNA eluted from mouse and rat γ clones were immunoprecipitated with αα antiserum and vice-versa shows cross hybridization between α and γ sequences. The less intense radioactive band obtained in the case of α clones and γγ antiserum probably reflects the lower proportion of *in vitro* translatable γ mRNA compared to that of α mRNA in mouse brain (13). A cross hybridization between both these sequences was also indicated by the pattern of colony hybridization and strongly suggest a partial homology between mouse α, mouse γ and rat γ sequences.

These results are in agreement with data demonstrating a high degree of homology between rat α and γ cDNA sequences (21) and suggest that the genes for αα and γγ isozymes probably evolued from the same ancestral gene. Indeed, a partial homology has been observed between the aminoacid sequence of yeast enolases and that of the rat γ subunit deduced from the pUC8-3-1 cDNA sequence (Bishop and Zomzely-Neurath, manuscript in preparation) and from a rat γ cDNA recently sequenced (22) indicating that enolase has been remarkably conserved during eucaryotic evolution.

Northern blot analysis of mouse brain poly(A^+)RNA revealed the existence of a single mRNA species for each enolase subunit. From the respective sizes of the α and γ mRNAs, 1900 and 2600 bases, it appears that the pBE α 6 insert (1800 bp) is probably a complete copy of α mRNA sequence and that the pBE γ 1 insert (2000 bp) corresponds to about 80 % of the γ mRNA sequence. As expected from the cellular specificity of αα and γγ isozymes, our γ cDNA

probes did not recognize any mRNA sequence in poly(A^+)RNAs from mouse liver, whereas α cDNA probes detected a single mRNA species of the same size as that of brain α mRNA. The difference in size between the α and γ mRNAs confirms our previous data obtained by gradient analysis (13). Since murine α and γ subunits have the same molecular weight (45 000 daltons) requiring about 1300 bases for the coding regions, it appears that the noncoding sequences for neuron-specific γ mRNA are longer than for ubiquitous α mRNA. In addition, from our colony hybridization data the noncoding sequences appear to be specific for each RNA. Indeed, taking into account the cloning technique used, short α and γ inserts (about 300 bp) should probably correspond to 3' noncoding regions, and we observed that α and γ clones with such inserts could only be detected with a homologous cDNA probe.

These data strongly suggest that mouse α and γ mRNAs possess specific structural features identical to those already observed for these mRNAs in rat (21, 23) and are consistent with those of Milner and Sutcliffe showing that mRNAs expressed specifically in rat brain tend to be considerably longer than mRNAs which are not specific to the central nervous system (24).

Our mouse α and γ cDNA probes now permit further analysis of the regulatory processes involved in the developmental and cell type specific expression of cerebral enolases. The sequence analysis of α and γ mRNAs has been undertaken and the developmental expression of each mRNA is under investigation.

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