Cloning and Expression of Rat GFAP cDNA in *Escherichia coli*

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 1, pp. 74-77, January, 2007
Original article submitted March 17, 2006

A cDNA fragment encoding GFAP was amplified by reverse transcription PCR from total mRNA isolated from primary culture of rat astrocytes and cloned for expression in *Escherichia coli* using pET-28a vector. High level of GFAP expression was confirmed by SDS-PAGE, while immunochemical identity was verified by immunoblotting. The constructed producer strain is a cheap source of GFAP and can be used for diagnostic purposes.

Key Words: *glial fibrillary acidic protein (GFAP); GFAP recombinant protein; Escherichia coli; central nervous system*

Glial fibrillary acidic protein (GFAP) [4] is the main component of intermediate filaments in astrocytes; it participates in the formation of cell skeleton of differentiated forms of these glial cells and forms the abluminal constituent of the blood-brain barrier. Active clinical and laboratory studies of GFAP are explained by the possibility of using this natural astrocyte marker in CNS diseases characterized by direct or indirect involvement of these cells into the pathological process [5,6,13,14] and in diseases associated with dysfunction of the blood-brain barrier [9,10,15].

Appreciable amounts of standard preparations of the test antigen are needed for the development of quantitative immunochemical methods of GFAP assay (enzyme immunoassay, immunochemical, immunoblotting, *etc.*) [1]. High level of the expression of target cDNA can be attained by using *E. coli* BL21[DE3] strain and pET-28a cloning vector after induction of the promotor. Cloning in the described vector makes is possible to obtain a fusion protein containing an extra N-terminal fragment consisting of a hexahistidine motive and a thrombin-recognizable site. This property gives new opportunities for rapid and effective single-step purification of the target protein by metal chelating affinity chromatography.

The aim of our study was to obtain an effective producer of rat recombinant GFAP on the basis of *E. coli* BL21[DE3] strain and pET-28a cloning vector (Novagen).

MATERIALS AND METHODS

Brain cortex of 3-4-day-old rat pups was isolated for preparing primary culture of normal astrocytes. The tissue was treated with trypsin and dissociated to homogeneous cell suspension. Dissociated cells were cultured in nutrient medium (90% DMEM, 10% PBS, 2 mM glutamine, 0.8% glucose, 25 mM HEPES; all reagents from Gibco) until the monolayer formation.

Bacterial strains *E. coli* XL-1Blue (Stratagene) and BL21[DE3] [9] were cultured in Luria—Bertani medium with 30 mg/ml kanamycin. Transformation was carried out by electroporation or "calcium method" [3,12].

Poly-A^+ mRNA was isolated and synthesis of cDNA first strand was carried out. The cells (~1 mln.) were treated with trypsin and washed in Hanks

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0007-4888/07/1431-0068

solution. Polyadenylated fraction of mRNA was isolated using a Kit for Isolation of Poly(A) mRNA on Magnetic Particles (Sileks-M) according to manufacturer's instruction. Reverse transcription reactions were carried out using M-MLV reverse transcriptase (SibEnzim) at 37°C for 1 h under conditions recommended by the manufacturer.

PCR, reverse transcription PCR, and construction of the plasmid were carried out. PCR amplification of GFAP cDNA fragment was carried out using primers rN-GFAP-f (5'-GCAAGATGGAGC GGAGACGT-3') and rN-GFAP-r (5'-ACACTGTA TGGCAAGGGCCG-3') and Taq DNA polymerase (SibEnzim). A total of 25 cycles were carried out: 30 sec at 94°C, 30 sec at 61°C, and 2 min at 72°C. Amplification was carried out using "hot start" PCR. The resultant product was reamplified in the next round using primers rGFAP-f (GCATCATAT GGAGCGGAGACGTAT) and rGFAP-r (GCGAAT TCTCACATCACATCCTTGT). All gene engineering manipulations were carried out in accordance with standard protocols [11]. The resultant PCR product was treated with FauNDI and EcoRI restriction endonucleases (SibEnzim). Vector pET-28a was treated with the same enzymes and then dephosphorylated using CIAP alkaline phosphatase (SibEnzim). The reaction products were separated by preparative electrophoresis in 1.5% agarose gel and isolated using silica-spin microcolumns (Helicon) in accordance with manufacturer's instructions. Eluted fragments were ligated using bacteriophage T4 DNA-ligase (SibEnzim). The resultant preparation was used for electrotransformation of competent *E. coli* XL-1Blue cells. Clones containing recombinant plasmids were selected by PCR with primers gfap350-s (5'-ACATCGAGATCGCC ACCTA-3') and T7term (5'-GCTAGTTATTGCTC AGCGG-3') and a program of 30 cycles: 15 sec at 94°C, 15 sec at 56°C, and 30 sec at 72°C.

Plasmid DNA was isolated from 5 ml overnight culture using a Kit for Isolation of Plasmid DNA from *E. coli* (Helicon) according to the instruction or by the standard alkaline method with phenol-chloroform extraction. The isolated plasmids were cleaved with FauNDI and EcoRI restriction endonucleases (SibEnzim).

Sequencing was carried out with primers T7prom (5'-TAATACGACTCACTATAGGC-3') and T7term, "BigDye Terminator Kit" (Amersham), and ABI 3100 Genetic Analyzer automated sequencer (PINNI).

Strain *E. coli* BL21[DE3] was transformed by pT7rGFAP-1 plasmid and inoculated in 50 ml LB medium with 30 μg/ml kanamycin. After attaining optical density of 1 at λ=550 nm, IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 1 mM. After the start of induction, the cells were incubated for 3 h at 37°C with constant shaking (200 rpm) and aliquots were collected after 30 min, 1, 2, and 3 h. The aliquots were centrifuged for 3 min at 13,000*g* in a microcentrifuge and the precipitates were resuspended in 100 μl lyzing buffer containing 50 mM Tris-hydrochloride (pH 6.8), 100 mM β-mercaptoethanol, 1% SDS, 0.0025% bromophenol blue, and 10% glycerol. After 3-min heating at 100°C the samples were centrifuged and 15 μl supernatant was applied onto 12.5% SDS-PAAG. Transfer of protein bands onto nitrocellulose membrane was carried out in a blotter (Sigma) in 0.05 M Tris-glycine buffer with 20% methanol at a current of 0.8-1.0 mA/cm^2 for 2-3 h. After transfer the nitrocellulose was washed with distilled water and stained with 0.1% Ponso C solution in order to evaluate the quality of transfer. After satisfactory transfer, immunochemical development was carried out using murine monoclonal antibodies to GFAP (Laboratory of Immunochemistry, V. P. Serbskii Institute). After incubation with first antibodies, (1 h) the membranes were washed 3 times in PBS and biotinilated horse antimurine IgG (1:200; Vector Labs.) in 1% normal equine serum were added. The membranes were then again washed in PBS and GFAP band was visualized in a substrate

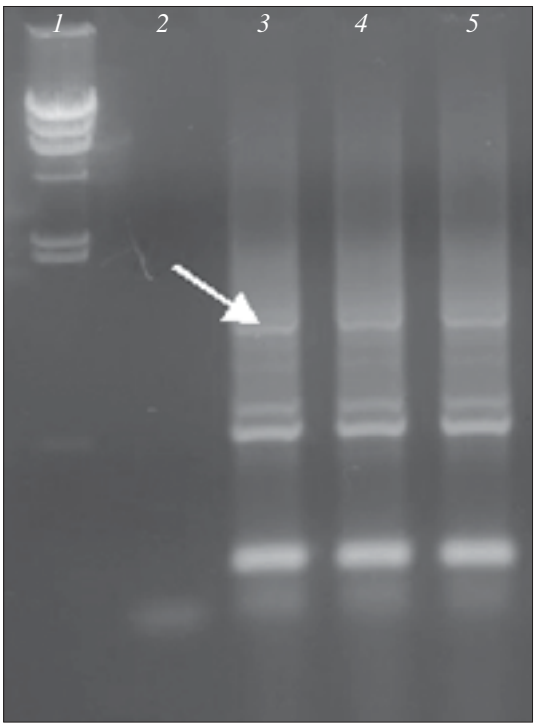

Fig. 1. Reverse transcription PCR of GFAP cDNA. *1*) molecular weight marker λ/HindIII: 564, 2027, 2322, 4361, 6557, 9416, 23,130 b.p.; *2*) negative control; *3-5*) reamplification with a pair of internal primers. Arrow shows GFAP amplicon.

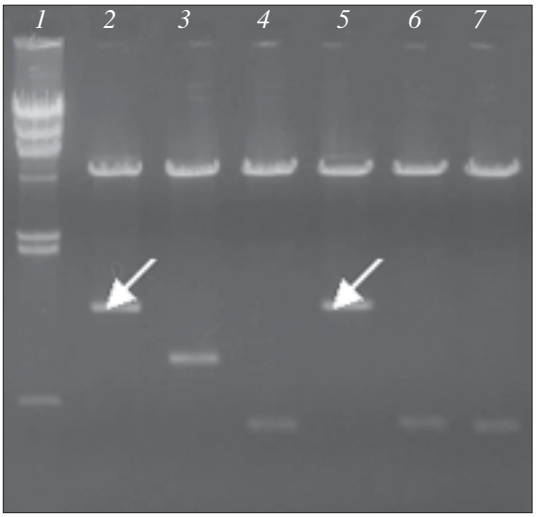

Fig. 2. Restriction analysis of recombinant plasmids. *1*) molecular weight marker λ/HindIII: 564, 2027, 2322, 4361, 6557, 9416, 23,130 b.p.; *2-7*) plasmid DNA from recombinant clones. Arrows show inserts of expected molecular weights.

mixture (0.025% diaminobenzidine in 0.01% H_2O_2). Membranes were washed in distilled water, dried in air, and scanned.

RESULTS

Primary culture of normal rat astrocytes intensively expressing GFAP served as the source of the target mRNA. Primer sequences were developed on the basis of previously determined rat GFAP mRNA sequence [7]. GFAP cDNA fragment was amplified in two PCR rounds using internal primers containing synthetic recognition sites for NdeI and EcoRI restriction endonucleases. Visualization of the reaction products in 1.5% agarose gel showed a product with expected molecular weight of 1308 kb and some abortive and nonspecific products (Fig. 1).

The resultant PCR product was cloned in pET-28a vector carrying bacteriophage T7 promotor and terminator and an effective translation initiation site. Twenty randomly selected transformant colonies were tested for the presence of the insert. After amplification, a product with expected molecular weight (350 kb; Fig. 2) was obtained from all tested colonies. From these, 6 clones were selected at random for isolation of plasmid DNA for subsequent restriction analysis and determination of the nucleotide sequence of inserts.

Plasmid from the clone containing the insert with intact nucleotide sequence was used for transformation of BL21[DE3] producer strain carrying bacteriophage T7 RNA polymerase gene under lac-promotor control. The resultant transformant was cultured in broth culture until the mean logarithmic phase and induced by adding IPTG. Aliquots taken after 30 min, 1, 2, and 3 h were analyzed by electrophoresis in SDS-PAAG. Accumulation of protein with expected molecular weight (50 kDa) was observed in aliquots after addition of IPTG (Fig. 3, *a*).

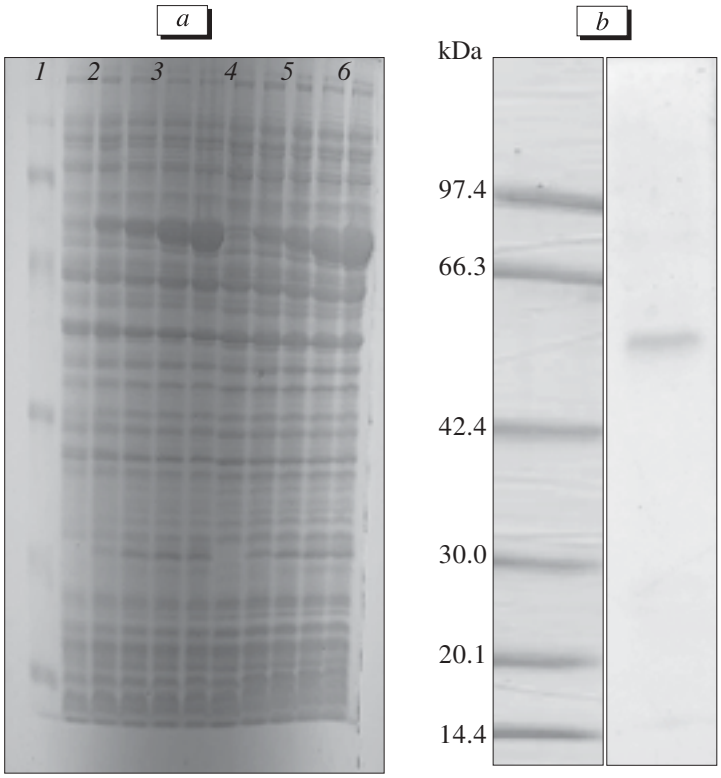

Fig. 3. Efficiency of GFAP expression in the producer strain. *a*) electrophoresis of total protein from GFAP producer clone: *1*) molecular weight markers; *2*) culture without induction; *3-6*) culture 0.5, 1, 2, and 3 h after induction, respectively; *b*) immunoblotting analysis of protein preparation from GFAP producer clone with monoclonal anti-GFAP antibodies.

Immunochemical identity of recombinant GFAP preparation was confirmed by immunoblotting with monoclonal anti-GFAP. GFAP-immunoreactivity was detected in the zone corresponding to the molecular weight of native GFAP (Fig. 3, *b*).

Hence, cloning of a reverse transcription-PCR product obtained on the matrix of polyadenylated mRNA from normal astrocyte culture in the pET-28a vector led to creation of a *E. coli* strain characterized by high level of expression of recombinant GFAP. This protein was immunochemically identical to GFAP preparation isolated from the brain tissue as described previously [1].

REFERENCES

1. V. P. Chekhonin, T. B. Dmitrieva, and Yu. A. Zhirkov, *Immunochemical Analysis of Neurospecific Antigens* [in Russian], Moscow (1999).
2. H. C. Birnboim and J. Doly, *Nucleic Acids Res.*, **7**, No. 6, 1513-1523 (1979).
3. S. N. Cohen, A. C. Chang, and L. Hsu, *Proc. Natl. Acad. Sci. USA*, **69**, No. 8, 2110-2114 (1972).
4. L. F. Eng and S. J. DeArmond, *Prog. Clin. Biol. Res.*, **59A**, 65-79 (1981).
5. L. F. Eng and R. S. Ghirnikar, *Brain Pathol.*, **4**, No. 3, 229-237 (1994).
6. L. F. Eng, R. S. Ghirnikar, and Y. L. Lee, *Neurochem. Res.*, **25**, Nos. 9-10, 1439-1451 (2000).
7. D. L. Feinstein, G. A. Weinmaster, and R. J. Milner, *J. Neurosci. Res.*, **32**, No. 1, 1-14 (1992).
8. J. Grodberg and J. J. Dunn, *J. Bacteriol.*, **170**, No. 3, 1245-1253 (1988).
9. F. Ikuta, Y. Yoshida, E. Ohama, *et al.*, *Acta Neuropathol.* Suppl. (Berl.), **8**, 103-110 (1983).
10. N. Marchi, M. Cavaglia, V. Fasio, *et al.*, *Clin. Chim. Acta*, **342**, Nos. 1-2, 1-2 (2004).
11. J. Sambrook and D. W. Russel, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. 2001.
12. K. Shigekawa and W. J. Dower, *Biotechniques*, **6**, No. 8, 742-751 (1988).
13. M. E. Smith, F. P. Somera, K. Swanson, and L. F. Eng, *Prog. Clin. Biol. Res.*, **146**, 139-144 (1984).
14. R. A. Sobel, S. J. DeArmond, L. S. Forno, and L. F. Eng, *J. Neuropathol. Exp. Neurol.*, **40**, No. 6, 625-632 (1981).
15. M. Trojano, C. Manzari, and P. Livrea, *Ital. J. Neurol. Sci.*, **13**, No. 9, Suppl. 14, 55-64 (1992).