Antidiphtheritic Activity of a Fragment of the Epidermal Growth Factor Precursor

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Expression of a DNA fragment coding for the 76-208 domain of protein molecule of the epidermal growth factor precursor, which contains a sequence of 133 N-terminal amino acid residues, was attained by gene engineering and molecular biology methods. The fusion protein was produced with very low efficacy in *E. coli* cells. It was purified by metal-affinity chromatography and its specific antitoxic activity evaluated in guinea pigs was at least 10^6 IU/mg protein.

Key Words: *epidermal growth factor precursor; fusion protein; metal-affinity chromatography; antitoxic activity*

Diphtheritic toxin (DT) actively adsorbs on the receptor site of a protein molecule of the epidermal growth factor precursor (EGFp) [2] which may be the N-terminal sequence of amino acid residues, starting from 76th to the 208th ($EGFp_{76\text{-}208}$).

We attempted to obtain a DNA fragment coding the $EGFp_{76\text{-}208}$, to express its genetic information in *E. coli* as a fusion protein, and to evaluate its antitoxic activity.

Biotechnological strategy of obtaining $EGFp_{76\text{-}208}$ as a fusion protein could become the basis for therapeutic and prophylactic antidiphtheritic preparations and for a DNA vaccine against diphtheria.

MATERIALS AND METHODS

Microbiological and gene engineering methods [1, 9,11] were used in the study; primary nucleotide sequence was determined automatically [8]. Total poly $(A)^+$-mRNA was isolated from newborn human kidney cells using magnetic oligo(dT)-adsorbent [3] (Promega Protocol No. 246), reverse transcription PCR was carried out [6], antitoxic polyclonal antibodies were used in blot-hybridization [5], affinity chromatography was performed on nickel-Sepharose according to the QIAGEN method, and N-terminal amino acids were analyzed [7]. Antidiphtheritic activities of the resultant preparations were measured at the L. A. Tarasevich Institute for Standardization and Control of Viral Preparations by skin tests on guinea pigs [4]. Primers for PCR were constructed using the OLIGO 4.0 software.

Components of media were used as described previously [1] for microorganisms, antibiotics, salts, phosphoramidite derivatives of deoxynucleotides, restriction endonucleases Xho I, EcoR I, BamH I, bacteriophage T4 ligase, Taq polymerase, AMV revertase, paper filters, and buffer solutions in appropriate concentrations from Difco, Sigma, Applied Biosysthems, Promega, and Whatman. Peroxidase conjugate with polyclonal antibodies to the "toxic" sequence of DT (a fragment including amino acid residues 1-386) was kindly provided by Dr. V. V. Sviridov from I. I. Metchnikov Institute of Vaccines and Sera, Russian Academy of Medical Sciences.

RESULTS

cDNA was obtained and amplified in the reverse transcription PCR using the following primers: $5'OP_4$-CTG

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TABLE 1. Comparison of Nucleotide Sequences

Insert	Number of replacements	Position of replacement in coding translation frame (*third position)
1	5	37, 77, 201*, 278, 443
2	10	23, 29, 85, 115, 127, 189*, 235, 277, 318*, 356
3	2	155, 395
4	4	94, 109, 191, 437
5	6	12*, 34, 57*, 257, 378*, 447
6	3	79, 236, 327
7	13	115, 119, 127, 221, 259, 299, 321, 335, 367, 387, 417, 421, 431, 439
8	7	163, 190, 244, 292, 339, 354, 396
9	3	45*, 141*, 297*
10	4	235, 249, 273*, 307
11	14	47, 89, 115, 133, 147*, 172*, 203, 225*, 258*, 271, 292, 318*, 345*, 414*

Note. Insert No. 9 contains: $T_{45}\rightarrow A(Val)$, $G_{141}\rightarrow T(Val)$, $G_{297}\rightarrow A(Ser)$.

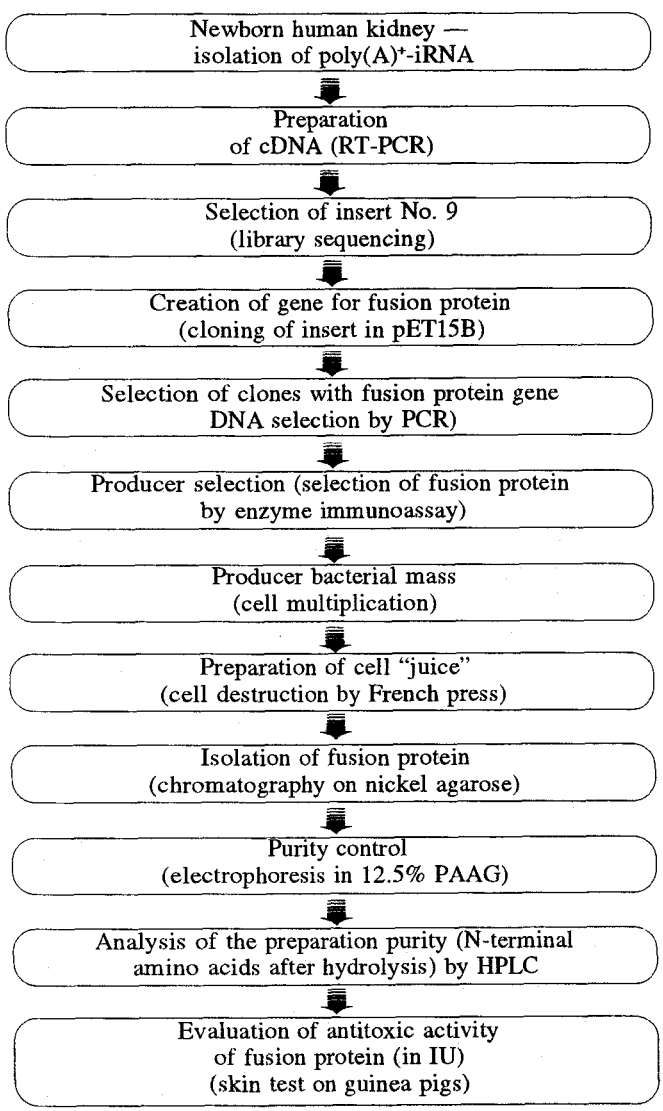

Fig. 1. Laboratory protocol for obtaining fusion protein.

AACCTCGAGATTGACACAGAAGGAACC-3'OH and 5'OP_4-CTGAACCTCGAGTATTTTCTCTGATG TCTCCA-3'OH. In comparison with the mobility of control fragments of DNA, the resultant product had about 450 b.p. (estimated size 462 b.p.). It had Xho I sites on its terminals (CTC GAG), which are absent from the coding cDNA [2]. After cloning of the amplification product, nucleotide sequence was determined in pGEM-T plasmid in 11 preparations of plasmid DNA isolated from Lac^--Amp^R transformants (Table 1). Comparison of the results with the translation frame [10] of sequence No. X04571 (ENTREZ database), coding for EGFp, showed that in only one insert (No. 9) the replacements or noncoincidences did not alter the coding sense of the frame. DNA from insert No. 9 was reclined into Xho I site of plasmid pET15B so that a gene was formed coding for the fusion protein with $EGFp_{76-208}$ structure.

The expression of the resultant plasmid was studied in BL31 cells. Cells of 37 Amp^R clones, whose DNA contains an insert of about 450 b.p., was lyzed on filters and then blot hybridization was carried out using DT and antibodies to DT_{1-386} (a peroxidase conjugate). About 12 clones were detected, which were positive in enzyme immunoassay; clone A211 cells contained the greatest amount of the material: 0.05%.

Fusion protein was isolated and purified from 14 g bacterial mass (Table 2). At the final stage 70 μg protein purified to 98% was obtained.

The specific antidiphtheritic activity of the resultant fusion protein was about 10^6 IU/mg.

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TABLE 2. Isolation and Purification of $EGFp_{76-208}$

Stages	Methods	Amount of protein, mg	Level of $EGFp_{76-208}$, mg	Concentration	Method for purity control (result)
Bacterial mass	—	2100	120000	1	—
Cellular "juice"	French press	870	46500	2.5	Phoresis in 12.5% PAAG (26 kD strip)
Purified "juice"	Sedimentation (25,000g/min)	14.2	6800	18.75	Phoresis in 12.5% PAAG (26 kD strip)
Affinity chromatography	Ni-liganding of His_6	0.0715 (final eluate: 0.15 M imidasole in 50 mM Tris-HCl buffer, pH 4.3)	70	1800	Determination of N-terminal L amino acids (*)

Note. *The structure of 46% N-terminals is MSSHHHHHHSSGLVPRGSLVDAG, of 46% SSHHHHHHSSGLVPRGSLVDAG, and 2% are other N-terminals.

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