

## Cloning and In vitro Expression of the Human Alpha Fetoprotein Gene

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Alpha-fetoprotein is known as a tumor marker. Strong conjugating features of the alpha-fetoprotein and its ability for specific binding with receptors of the tumor cells offers opportunity for the targeted delivery of cytostatic and cytotoxic drugs into malignant cells. Human AFP are able to induce apoptosis and to inhibit growth of the tumor cells. Technology of the recombinant DNA is the way to simplify and cheapen the production of the human AFP. Complementary DNA encoding human alpha-fetoprotein was cloned into bacterial vectors under the control of the T7 bacteriophage promoter. Various 5'- and 3'- untranslated regions (5'- and 3'-UTRs) were used as a translational enhancers. Transcription of the recombinant constructs by the T7 RNA polymerase and subsequent in vitro translation of the resulted transcripts in the wheat germ cell-free system were performed. It was shown that the human fetal blood protein could be correctly synthesized in plant cell-free system.

**Key words:** AFP, translational enhancers, in vitro translation, wheat germ cell-free system.

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Alpha-fetoprotein (AFP) is a mammalian embryo-specific protein, also known as a tumor marker and could be found in some cases of liver and reproductive system cancers<sup>1</sup>.

Human AFP (AFPh) is a 70 kDa glycoprotein containing up to 4 % of carbohydrates. It is a transport protein possessing strong conjugating features. It is known that some receptors of tumor cells are AFP ligands<sup>2</sup>. This feature is used for the targeted delivery of cytostatic and cytotoxic drugs into malignant cells<sup>3,4,5,6,7</sup>. Development of the drugs inducing apoptosis in the tumor cells is one of the perspective directions in oncology. The ability of the AFPh to induce apoptosis and to inhibit growth of some tumors by expression of the p53 gene were shown in several researches<sup>8,9,10,11,12,13,14</sup>.

Alternatively, AFP conjugation with antisense oligonucleotides to the mRNAs of the genes participating in cell proliferation and apoptosis could make sense. AFP conjugate with an antisense oligonucleotide for the mRNA of the antiapoptotic *bcl-2* gene induced apoptosis and subsequent death of the tumor cells<sup>15</sup>.

Thus, biological features of the AFPh offers great opportunities to develop various anticancer drugs that could be highly selective to the tumor cells. However, this promising perspective is limited to the complexity and the cost of the AFPh purification from the abortion serum. Recombinant systems producing AFPh could be the solution.

The aims of this work were AFPh gene cloning and optimization of its expression in a plant in vitro system.

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## MATERIALS AND METHODS

### Human hepatocellular carcinoma (HepG2) cell line was used as a DNA source

RNA was purified by commercial "RNAqueous®" kit using manufacturer's guide (Ambion).

The reverse transcription reaction was driven by the use of the *moloney murine leukemia virus reverse transcriptase (M-MLV RT)* (Thermo Scientific). The subsequent polymerase chain reaction was directed by the PL polymerase (Syntol).

### DNA cloning was performed according to<sup>16</sup>

DNA sequencing by Sanger was made in "ABI PRISM 3101 Genetic Analyzer" (Applied Biosystems) using "BigDye® Terminator v3.1 Cycle Sequencing Kit" according to manufacturer's recommendations (Applied Biosystems).

### Sequence of the 5'-UTRs with underlined *HindIII* and *NcoI* restriction sites

[p]: AAGCTTAGTCGACCATGG

[2xARC]: AAGCTTAC AAATACT  
CCCCACAACAGCTTACA AATACTCCCC  
ACAACAGCTTGTCGACCATGG

[3xARC]: AA GCTTACAAATACTCCCCACA  
C A G C T T A C A A A T A C T C  
CCCCACAACAGCTTGTCGACCATGG

[PVY]: AAGCTTAATTAATAA CAACTCAAT  
ACAACATAAGAAAA ACAACGCAAAAACA  
CTCATAAACGCTC ATTCTC ACTCAA  
GCAACTTGCTAAG TTTCAGTTTAAATCAT  
TTCCTTGCAATTCTC TAGAACA ATATTG  
GAAACCATTTCAACTCAACAAGCAATTC  
ATCACTT CCAACCAATTTCA GATCCTCÑ  
ATGG

### In vitro transcription

In vitro transcription by the T7 RNA-polymerase according to<sup>17</sup>. 50 µL of the reaction mix contained 40 mM Tris-HCl (pH 7.9), 6 mM MgCl<sub>2</sub>, 10 mM DTT, 10 mM NaCl, 2 mM spermidin, 2 mM of each ribonucleoside triphosphate, 40 units of the ribonuclease inhibitor, 40 units of the T7 RNA-polymerase and 1 µg of DNA. The reaction lasted 2 hours at 37°C. Then the reaction mix was treated with chloroform. RNA precipitated in the presence of 3M LiCl. The pellet was washed with 70% ethanol after centrifugation at 14000 RPM.

Dried under vacuum the pellet was dissolved in H<sub>2</sub>O. RNA concentration was determined by spectrophotometry.

### In vitro translation

In vitro translation was carried out in the wheat germ cell-free system from the "Kazakhstanskaya 4" variety<sup>18</sup>. 25 µL of the reaction mix contained 20 mM Tris-Ac (pH 7.6), 90 mM KAc, 2 mM MgAc<sub>2</sub>, 10 mM phosphocreatine, 0.12 mg/mL *creatine phosphokinase*, 1 mM ATP, 0.1 mM GTP, 0.1 mM spermidin, 0.1 mM of each amino acid except methionine, 0.025 mM methionine, 0.075 mM [<sup>35</sup>S]-methionine, 1 µg of mRNA, 7 µL of the wheat germ extract. The reaction mix was incubated for 1 hour at 26°C.

### Autoradiography and western blot analysis

Protein electrophoresis was performed according to the classical Laemmli protocol<sup>19</sup>. The gel was dried and subsequent autoradiography using "Kodak" X-ray film was made. Western blot analysis was made according to<sup>20</sup>. Antibodies conjugated to horseradish peroxidase were used as a secondary to AFPh-specific monoclonal antibodies.

## RESULTS AND DISCUSSION

AFP gene belongs to the albumin gene family and located in the long arm of the fourth chromosome (4q11-q13)<sup>21</sup>. Like all secreted proteins, AFP is synthesized as a precursor, which becomes the mature protein after cleavage of the signal peptide and the process of glycosylation. The complete nucleotide sequence of the AFP gene is known. It consists of 20000 base pairs, contains 15 exons and 14 introns<sup>22</sup>.

Nucleotide sequence of the AFP mRNA consists of 2098 nucleotides and has three main parts: 44 noncoding nucleotides at the 5'-end, 1830 coding and 155 noncoding nucleotides at the 3'-end<sup>23</sup>. The signal peptide consists of 19 amino acids followed by 590 amino acids of the mature protein.

Thus, taking into account big size of the AFPh gene and many introns inside it seems impossible to express it neither in vitro nor in vivo in bacterial or plant systems. We decided to clone complementary DNA (cDNA) of the AFPh gene. mRNA sequence of the AFPh (GenBank:

NM\_001134) was analyzed with SnapGene® software (GSL Biotech) to build DNA restriction map. These data were used to design oligonucleotide primers. Total RNA was purified from the AFP<sub>h</sub> producing human hepatocellular carcinoma (HepG2) cell line (Fig. 1). It is seen from the Fig. 1 that 28S, 18S ribosomal RNAs and 4S transport RNA (about 100 nucleotides) are major components of the sample. The RNA sample was enriched by highly weight molecular components after precipitation by 3M LiCl, since low weight tRNA molecules was leaved in the supernatant fraction (lane 2).

Obtained RNA sample was used to synthesize cDNA of the AFP<sub>h</sub> by moloney murine leukemia virus reverse transcriptase with the use of antisense primer no.1 (5'GCATCTC GAGTTAACTCC CAAAGCAGCA CGAG TTTTGT) containing XhoI restriction site (underlined). Antisense oligonucleotide primer no.1 and sense oligonucleotide primer no.2 (5'GCAACCATGGAGTGGGTGGAATC), containing NcoI restriction site (underlined) were used to amplify the resulting cDNA. We performed 30 cycles of the following PCR program: 30 seconds at 94°C (initial denaturing lasted 4 minutes), 30 seconds at 48°C and 1 minute at 72°C (final elongation lasted 5 minutes). Results of the RT-PCR are represented in the Fig. 2.

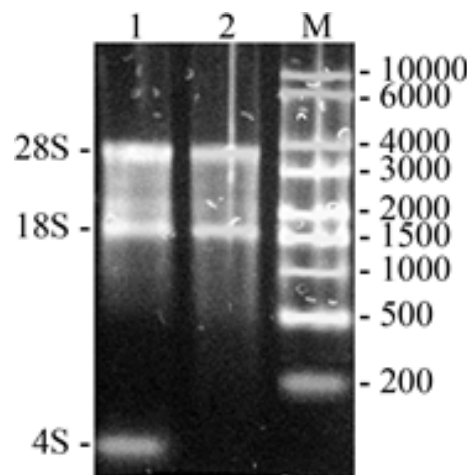
[Figure 2]

#### Electrophoregram shows resulting 2000 bp DNA amplification products presumably coding AFP<sub>h</sub> protein

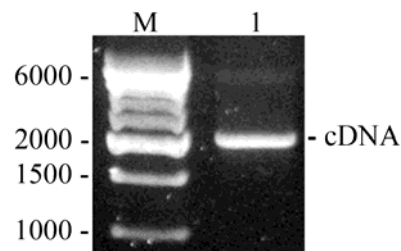
2000 bp fragments obtained were eluted from the gel and cloned into series of bacterial pIVEX-WG-1.3 vectors between NcoI-XhoI restriction sites under the control of the T7 bacteriophage promoter. The plasmids of this series differ from the analogous vectors constructs by the presence of the translational enhancers described earlier<sup>24</sup>. In addition, it is possible to insert various 5'-untranslating regions between HindIII-NcoI restriction sites to test if it increases efficiency of the mRNA translation.

The resulting recombinant plasmids, containing AFP<sub>h</sub> gene are schematically represented in the Fig. 3.

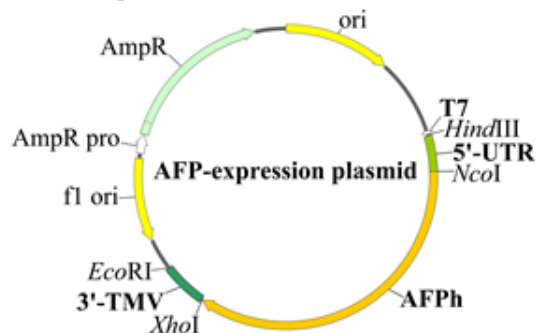
We performed DNA sequencing of the cloned AFP<sub>h</sub> gene from the 3'-end (about 500 nucleotides). Comparative Blast-analysis identified



**Fig. 1.** Nucleic acids purified from the human hepatocellular carcinoma (HepG2) cell line and separated by electrophoresis in 1% agarose gel. 1 – total nucleic acid sample; 2 – nucleic acid sample after precipitation by 3M LiCl; M – RNA marker (size marked in nucleotides on the right). Positions of the 28S rRNA, 18S RNA and 4S tRNA are marked on the left.



**Fig. 2.** RT-PCR products separated by electrophoresis in 1% agarose gel. M – DNA marker (size marked in nucleotide pairs on the left); 1 – cDNA of the AFP<sub>h</sub>.

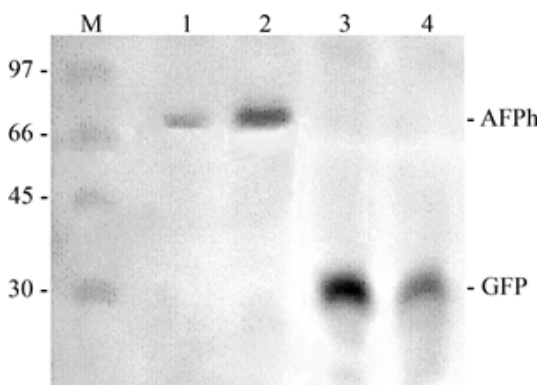


**Fig. 3.** Schematic representation of the plasmids for *in vitro* expression of the AFP<sub>h</sub> gene. Vectors with the molecular size about 5000 bp contain promoter of the bacteriophage T7, one of the 5'-UTR, AFP<sub>h</sub> gene and the 3'-UTR of the tobacco mosaic virus (3'-TMV).

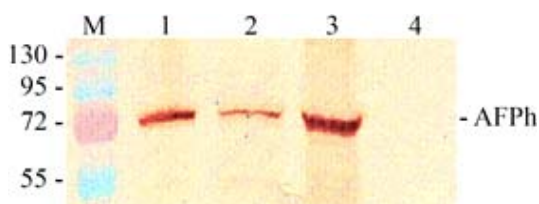
the sequence as AFP<sub>h</sub> mRNA from the NCBI “Nucleotide” database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

Thus, we developed three types of the recombinant plasmids that could be transcribed by the T7 RNA-polymerase and differing only in the structure of the 5'-UTR.

In subsequent experiments, the plasmids



**Fig. 4.** Autoradiography of the translation products from various mRNAs in the wheat germ cell-free system in the presence of the [<sup>35</sup>S]-methionine. M – protein marker (size marked in kDa on the left); 1 – AFP<sub>h</sub> translation products from the mRNA with the control 5'-UTR [pI]; 2 – AFP<sub>h</sub> translation products from the mRNA with the 5'-UTR [3xARC]; 3 – GFP translation products from the mRNA with the 5'-UTR [PVY]; 4 – GFP translation products from the mRNA with the 5'-UTR [2xARC].



**Fig. 5.** Western blot analysis. AFP<sub>h</sub> polypeptides synthesized in the wheat germ cell-free system. M – protein marker (size marked in kDa on the left); 1 – AFP<sub>h</sub> translation products from the mRNA with the 5'-UTR [3xARC]; 2 – AFP<sub>h</sub> translation product from the mRNA with the control 5'-UTR [pI]; 3 – AFP<sub>h</sub> translation products from the mRNA with the 5'-UTR [PVY]; 4 – control in vitro translation without exogenous mRNAs.

were linearized with EcoRI enzyme, which unique restriction site is situated after the 3'-UTR of the TMV genomic RNA. This linearized plasmids served as a template for in vitro transcription from T7 promoter. Equal amounts of uncapped mRNAs synthesized by the T7 RNA-polymerase were used as a templates for in vitro translation in the wheat germ cell-free system.

Proteins translated in vitro in the presence of the [<sup>35</sup>S]-methionine were separated in 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Then PAGE was dried and exposed with X-ray film. Results are represented in the Fig. 4.

As you can see from the Fig. 4, the presence of the 5'-UTRs [PVY] (lane 3) and [2xARC] (lane 4) resulted in high yield of the green fluorescent protein (GFP). Artificial translational enhancer [3xARC] provided high level of the AFP<sub>h</sub> synthesis (lane 2) compared to the AFP<sub>h</sub> translation when the [pI] was used as a 5'-UTR (lane 1).

Then, we analyzed products of translation of the [pI]-[AFP<sub>h</sub>]-[3'-TMV], [3xARC]-[AFP<sub>h</sub>]-[3'-TMV] and [PVY]-[AFP<sub>h</sub>]-[3'-TMV] mRNAs by western blot analysis (Fig. 5). Monoclonal antibodies to AFP<sub>h</sub> detected major polypeptides in the lanes 1-3 that match in size with AFP<sub>h</sub> protein (70 kDa).

## CONCLUSIONS

Obtained data shows the possibility of oncofetal AFP<sub>h</sub> protein synthesis in the wheat germ cell-free system.

It should be noted that the presence of the translational enhancers in mRNA significantly increased the yield of the synthesized protein. The highest yield was obtained using the 5'-UTR of the potato virus Y genomic RNA, although the artificial enhancer [3xARC] also significantly enhanced the translation of the AFP<sub>h</sub> mRNA.

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