

## MOLECULAR CLONING OF OVINE cDNA LEPTIN GENE

### CLONAREA MOLECULARĂ A ADNc AL GENEI LEPTINEI OVINE

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*An efficient bacterial transformation system suitable for cloning the coding sequence of the ovine leptin gene in E. coli DH5α host cells using the pGEMT easy vector it is described in this paper. The necessity of producing leptin is based on the fact that the role of this molecule in the animal and human organism is still unknown, leptin not existing as commercial product on the Romanian market. The results obtained in the bacterial transformation, cloning, recombinant clones selection, control of the insertion experiments and DNA computational analysis represent the first steps in further genetic engineering experiments such as production of DNA libraries, DNA sequencing, protein expression, etc., for a further contribution in elucidating the role of leptin in the animal and human organism.*

**Key words:** ligation reaction, bacterial transformation, recombinants selection

#### Introduction

The ability to manipulate living organisms at the genetic level is one of the main tools of modern biotechnology. Among the many systems available for heterologous protein production, the gram positive bacterium *Escherichia coli* remains one of the most attractive because of its availability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. The genetics of *E. coli* and the existence of a large number of compatible tools available for biotechnology, especially a wide number of vectors, recombinant fusion partners and mutant strains, recommend this system for all the DNA recombinant technology applications. Unlike small molecules typically synthesized by chemical means, most proteins are having such a molecular weight and are so complex, that necessitate their production in living systems, mostly by recombinant DNA technology. Cloning of any DNA fragment essentially involves four steps: fragmentation, ligation, transformation, and screening/selection. Although these steps are invariable among cloning procedures a number of alternative routes can be selected, all these being summarized in the cloning strategy. The recombinant proteins obtaining methods are presenting major advantages in order to avoid inconvenient as the risk of allergic and immune

reactions, virus contamination, usually present in the classic methods which are using hormones obtained from animal tissue.

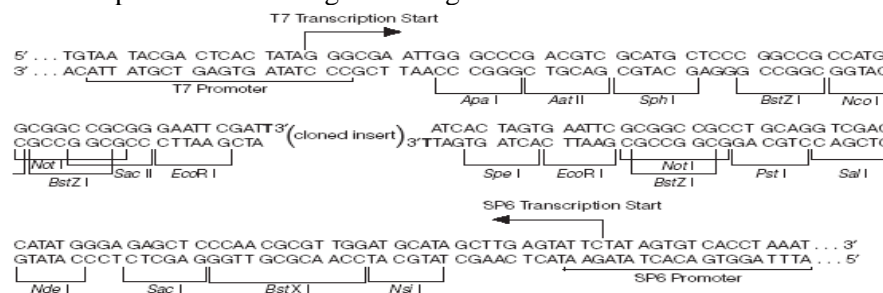
## Materials and Methods

### Biological material

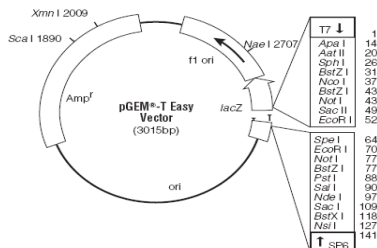
The ovine leptin gene isolated (from adipose subcutaneous tissue) by a specific RT-PCR reaction and a further purification process by cutting its band from the 1% agarose gel was used as starting material in the experiments; the pGEMT easy vector was achieved from Promega.

### Ligation reaction

Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it in vivo. The pGEMT easy vector is a convenient system for the cloning of PCR products. The leptin gene is composed of coding and non- coding sequences; the coding sequences and was obtained from the reverse transcription of messenger RNA that allow the production of a functional leptin molecule. The ovine leptin gene coding DNA segment has a suitable size (441 bp) to be inserted in the pGEMT easy vector. In the figure 1 is shown the place where the passenger will be inserted in the pGEMT easy vector, and in the figure 2 that the above sequence is inside of the lacZ region of the vector. A new plasmid containing the foreign DNA as insert was obtained.



**Figure 1.** The promoter and multiple cloning sequence of pGEMT easy vector.



**Figure 2.** pGEMT easy vector circle map and sequence reference points.

In the ligation reaction the DNA ligase reform phosphodiester linkages between adjacent 5'-phosphates and 3'-hydroxyls using an energy cofactor, for joining DNA molecules together. The insert and the linearized vector are incubated at 16°C for a couple of hours, in a reaction mix of 10 µl containing 3U/µl T4 DNA Ligase and the proportion insert: vector being equal to 1:5; the 2X Rapid Ligation Buffer contained ATP.

#### **CaCl<sub>2</sub> bacterial transformation**

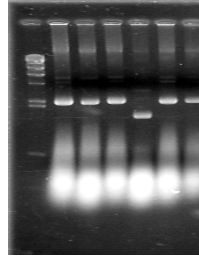
The recombinant plasmid containing the coding region of ovine leptin gene can be inserted into bacterial cell (*E. coli* DH5α). Competent cells for bacterial transformation were prepared by the calcium chloride method with an optimum concentration of 0,1M. *Escherichia coli* DH5α host cells were used for the transformation; the optimal optical density (OD<sub>600</sub>) range for competent cell preparation of the bacterial strain was 0.4-0.5. 25 ng/µl of the pGEMT easy vector containing the coding sequence of the ovine leptin gene are mixed with 10 µl *E. coli* DH5α competent cells, the heat shock being performed at 42°C for 45 seconds. The transformed cells were immediately placed in 1 ml LB medium and incubated at 37°C for 20 minutes.

#### **Recombinant clones selection and control of the insertion**

The *E. coli* DH5α transformed cells were plated on LB agar medium 50 mg/ml X-Gal, as the pGEMT easy vector contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the peptide coding region of the enzyme galactosidase, insertional inactivation of the peptide allows the directly identification of the recombinant clones by color screening on indicator plates. So the color selection marker of the cloning vector allows the blue/white screening (α-factor complementation) of the transformed colonies on X-gal medium. The pGEMT easy vector allows also the selective amplification of the recombinant clones, the transformed *E. coli* DH5α being protected by the ampicillin resistance gene (amp<sup>r</sup>) which can express an enzyme, which inactivate the specific antibiotic ampicillin. For a higher accuracy the control of the insertion was performed by extracting the plasmid DNA from 3 ml of a liquid culture of the transformed colonies using Wizzard Plus SV Minipreps DNA Purification System purchased from Promega according to the spin protocol of the manufacturer. The DNA extracted was controlled on 1% agarose gel and one colony was chosen for the enzymatic digestion (at 37°C). The reaction mix consists on 24 ng/µl plasmid DNA isolated from the colony number 1, 3U/µl EcoRI and 3U/µl BamHI (Promega) in a final reaction volume of 15 µl. DNA fragments generated by digestion with two restriction enzymes were then electrophoresed in 1% agarose.

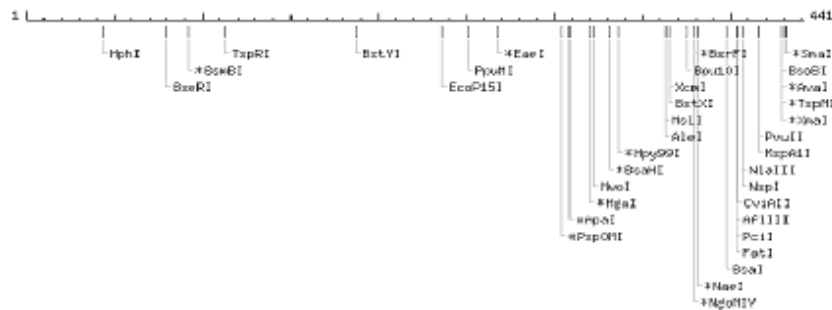
## Results and Discussions

The recombinant pGEMT easy vector containing the coding sequence of the ovine leptin gene was obtained by the ligation reaction performed, confirmed by the recombinant colonies grown on the LB 50 mg/ml Amp, 50 mg/ml X-Gal and the extraction of the plasmid DNA from the transformed colonies (3456bp).

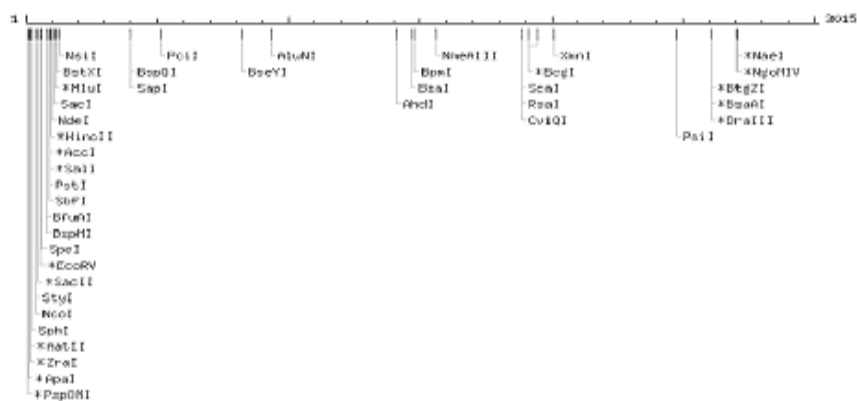


**Figure 3.** The transformed pGEMT easy vector (3456 bp)- migrated in agarose gel 1%: lane 1- Lambda DNA Ladder (Fermentas), lane 2-7:the transformed pGEMT easy vector extracted from the recombinant *E. coli* DH5α colonies (lane number 5 is showing a false recombinant).

The result of the computational digestion (performed using NEB tools) is showing that the enzymes *EcoRI* and *BamHI* are not cutting inside their nucleotide sequence, so two fragments are expected after the electrophoresis to be seen in the agarose gel.



**Figure 4.** Enzymes that cut in the 441 bp of the amplified coding sequence of the ovine leptin gene



The cleaved fragments of 3015 bp and 441 bp from the 1% agarose gel stained with ethidium bromide are the proof of the presence of the insert in the recombinant pGEMT easy vectors, confirming the success of the bacterial transformation process.

**Figure 6.** The transformed pGEMT easy vector digested with EcoRI and BamHI restriction enzymes- migrated in agarose gel 1%: lane 1- DNA Ladder 1 kb (Fermentas), lane 2: digested fragments of the transformed pGEMT easy vector- pGEMT easy vector (3015 bp), coding sequence of the ovine leptin gene (441 bp).

This paper describes an efficient bacterial transformation system suitable for the preparation of competent cells and recombinant plasmids. Using this method, a number of different plasmids can be amplified for further experiments, the recombinant DNA molecule can be amplified, purified, and perpetuated forever. This technique is one of the first stages of most of the genetic engineering experiments: production of DNA libraries, PCR, DNA sequencing, protein expression.

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