ABSTRACT

Lymphoma is the fifth most common cancer in the USA. (American Cancer Society) Hes1 is a significant signal effector in the signalling pathway allowing the proliferation of lymphocytes. It regulates the transcription of other genes through various novel mechanisms some known, others being researched upon. Being a consequential molecule, its own activities are regulated by other proteins being investigated. This project targeted to analyse the Hes1 protein structure through X-ray crystallography and perhaps progress towards a possible drug for cancer. The research methodology involved isolating the gene through the amplification; forming a recombinant plasmid vector; transforming bacterial cells with the recombinant vector; expressing the protein and optimising the conditions for expression. Several other steps for the purpose of verification of the gene sequence as well as protein were also carried out. Difficulties in amplifying and expressing the entire gene lead to the eventual amplification the truncated DNA-binding domain of the Hes1 gene. Despite a frame-shift mutation in the amplified gene sequence, the protein expressed seemed to be of an appropriate size. Further confirmation could be carried out in the form of His-tag binding or mass spectrometry.

INTRODUCTION

Hes 1 is a regulator that is involved in the control of several pathways which are important in the proliferation of the cell. Thus the mechanisms of control adopted by Hes1 are well-studied. Being an important multi-functional transcriptional controller, the regulation of its own transcription has been well-researched. However, the protein structure of Hes 1 has the protein structure of Hes1 has not been profoundly analysed. This research project aimed to clone the Hes1 gene into a modified vector and express the protein; eventually examining the protein structure by X-ray crystallography. In the future, an antibody blocking Hes 1 protein can be designed allowing it to serve as a cancer drug.

MATERIALS AND METHODS

3 types of primers were constructed for isolating the gene coding whole protein (WP), the DNA binding domain(region of protein that binds to DNA to control transcription) and Orange domain (structural feature that has been conserved among its family of transcriptional
regulators). In all 3 types of primers, the forward contained the EcoR1 restriction site and the reverse contained the Xho1 site. PCR was performed 5 times to achieve good clones made visible through gel electrophoresis. 2 different cell lines (SUPM-2 & SUDHL-2) were used, altering annealing temperatures. These clones were then extracted from the gel using the protocol of a PCR purification kit. The purity and concentration of the clones was checked using a spectrophotometer.

These GOI clones and pET-M vector were then double digested using EcoR1 and Xho1 enzymes. The double digested vector was run on gel and extracted from the gel; its purity and concentration checked. The 2 double digested GOI and vector are then ligated. These ligated samples were transformed into DHP-α cells by inducing competency through a heat shock. These cells were plated on ampicillin (Amp) medium and allowed to grow. Colonies were picked at random to undergo colony PCR. T7 promoter and terminator primers were used. Comparative analysis between the negative control and samples allowed the colonies with recombinant plasmids to be identified.

Sequencing PCR was then carried out. The colony with the recombinant plasmid was first inoculated in a culture medium. After which the curing of the plasmids was conducted by following the protocol of a Plasmid Quick kit. These plasmid samples then underwent PCR using either the T7 promoter or terminator as primers. They were then sent for sequencing.

Small-scale protein expression was then conducted by first transferring the transformed plasmids to BL-21 cells. These cells are inoculated in culture tubes containing Luria-Bertani broth and Amp. After the optical density has reached 0.6, these cells are induced with different concentrations of Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells are then sonicated and centrifuged separating soluble proteins and insoluble proteins. These protein samples are subjected to SDS-PAGE. A faint band at 10kD is observed. Western blotting is used to transfer the proteins to a membrane and an antibody that binds to the His-tag present in the POI is used to probe and identify the presence of the POI.

RESULTS AND DISCUSSION

Eventually clones of the DNA-binding domain and Orange domain were obtained from the SUPM-2 cell line at annealing temperatures between 55–65°C. Their purity and concentration were checked to ensure they were good enough to be used for the further experiments.

Double digestion is done to ensure that the GOI was not inserted into the vector in an inverted manner. This would prevent protein expression. It also helped to ensure that the vector did not re-anneal after being digested and different sticky ends would be present. DHP-α cells are bacterial cells specialised for taking up the recombinant plasmids due to more porous cell walls, absence of plasmids and it also has the benefit of being non-pathogenic thus, facilitating its use in the laboratory. The polylinker sites in the vector are situated within the T7 site which
can be transcriptionally activated by gratuitous inducer IPTG. Hence in a recombinant plasmid the GOI is located within the T7 site. Thus the T7 primers used in colony PCR should amplify the GOI in the recombinant plasmid as opposed to just the T7 site in the negative control. This leads to a difference in size.

The sequencing results showed a frame-shift mutation which resulted in a premature termination codon preventing the last 19 amino acids from being translated. However, the sequencing results arrived after protein expression had been performed.

BL-21 cells are specialised for protein expression as they are protease-deficient. IPTG acts as an inducer removing repression of the Lac operon in which the GOI is present allowing the Protein of Interest (POI) to be over-expressed. Despite the mutation a 10kD protein is detected in the soluble protein samples through SDS-PAGE which is not present in the negative control. The mutation should have caused the protein to be smaller whereas the actual size of the protein is around 10.3kD. Western blotting allows the proteins to be transferred to a membrane which binds proteins. Milk protein binds indiscriminately to the rest of the membrane (excluding the areas where the POI has already binded) and prevents the antibody from binding to the membrane. Hence the primary-antibody from the mouse, binds to the tag of 6 histidines in the protein. Being anti-mouse, the secondary-antibody binds to the primary-antibody. The reporter enzyme attached to secondary-antibody that catalyses a reaction product that is chemiluminescent and hence darkens sensitive X-ray film enabling concentration-dependant detection. The Western blot was unsuccessful. The positive control did not show a band; perhaps the antibody was defective. Further tests (his-tag purification, mass spectrometry) can be conducted to confirm the identity of the protein. After the protein has been identified X-ray crystallography can elucidate protein structure and eventually be used to design an antibody.

REFERENCES


