# Translational fusion of heat labile enterotoxin chain B and β-subunit of human chorionic gonadotropin: periplasmic expression in *Escherichia coli* and its immunogenicity

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**Abstract** A fusion gene was constructed consisting of heat labile enterotoxin chain B (LTB) of *E. coli* genetically linked at its C-terminus to the β-subunit of human chorionic gonadotropin in translational fusion, under the control of tac promoter and LTB signal sequence. Expression of the fusion gene (about 5 μg/ml) in *E. coli* was confirmed by immunoblot analysis using both anti-LTB and anti-βhCG polyclonal antibodies. The fusion protein was efficiently processed and exported to the periplasmic space. LTB in the fusion protein retained its ability to bind to GM1 ganglioside receptor. Mice immunized with the fusion protein produce antibodies that recognize recombinant βhCG and the native hCG suggesting its potential use as a contraceptive vaccine.

*Key words:* Periplasmic expression; Fusion protein; βhCG; Signal sequence; Immunogenicity

## 1. Introduction

Human chorionic gonadotropin (hCG), a heterologous mammalian placental protein, has been the first choice as a target for immunocontraception [1]. A member of the family of glycoproteins consisting of two dissimilar subunits, α and β, it is produced by the trophoblast cells during the first trimester of pregnancy. The α-subunit is similar to that of other members of the family but the β-subunit has a distinctive amino acid sequence that confers the hormonal specificity. To avoid cross-reaction with other members of the glycoprotein family, the β-subunit has been used in contraceptive vaccine preparations. However, being a self protein, βhCG is not immunogenic by itself and is, therefore, conjugated to a non-self carrier protein. In all the hCG based vaccines that have entered clinical trials, conjugation has been achieved through chemical conjugation. Gene fusion offers a practical alternative to chemical conjugation as it allows the protein to be conjugated to a carrier protein through the recombinant route. The fusion protein thus obtained would have a consistent composition unlike that produced by chemical conjugation. Cholera toxin chain B (CTB) and heat labile enterotoxin chain B (LTB) of *E. coli* have gained interest as fusion partners in recent years. Oral administration of these proteins stimulates both systemic and mucosal immunity [2–6]. This property can be exploited to produce vaccines that can be

delivered orally, not only for infectious disease control but also to control mucosal physiological processes such as fertility [7].

In the present study, LTB was cloned at the N-terminus of βhCG to generate a translational fusion gene under the control of tac promoter and LTB signal sequence. We report here the expression, efficient processing and export of the fusion protein to the periplasm in *E. coli*. Our studies also indicate that the fusion protein could bind to GM1 ganglioside receptor, the receptor for LTB, in an in vitro receptor binding assay and was found to be immunogenic in mice.

## 2. Materials and methods

### 2.1. Reagents

Enzymes were purchased from New England Biolabs, USA. Bacto-tryptone, Bacto-agar and Bacto-yeast extracts were from Difco Laboratories, USA. Ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), Freund's complete (CFA) and incomplete adjuvant (IFA) were obtained from Sigma Chemicals, USA. Recombinant protein A was from Boehringer Mannheim, Germany. [$^{32}$P]α-dCTP was purchased from Amersham, UK. Goat anti-βhCG and anti-CTB polyclonal antibodies were provided by the reagent bank of the National Institute of Immunology, India and anti-goat IgG-HRP conjugate was obtained from Vector Laboratories Inc., USA. Purified hCG and monoclonal antibodies to hCG were provided by Dr. S.K. Gupta.

### 2.2. Bacterial strains and plasmids

*E. coli* DH5α and HB101 cells were used as bacterial hosts. The clone pKGβhCG [8], having βhCG structural gene under the control of tac promoter and LTB signal sequence, and plasmid pBSLTB (constructed in our laboratory) containing the LTB gene were used as the parent clones. It is to be noted that as a result of the cloning strategy of LTB in pBSLTB, the termination codon of LTB was deleted and a *Sac*I site was introduced at that position. In order to generate the fusion construct, the plasmid pBSLTB was digested with *Sac*I and the LTB structural gene fragment thus released was cloned into *Sac*I digested pKGβhCG to give an N-terminal translational fusion of LTB to βhCG. The resulting construct is designated pLTBβhCG. The termination codon for the fusion protein was provided by βhCG gene present in the pKGβhCG clone. Ligation and transformation with various plasmids as well as analysis of the transformants were carried out using standard techniques [9].

### 2.3. Induction of recombinant clones and immunoblot analysis of fusion protein

Cells harboring the recombinant plasmid pLTBβhCG, having the LTB insert either in the right or in the reverse orientation, were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml). Freshly subcultured 0.5 $A_{600}$/ml unit cells were induced with 1 mM IPTG for 8 h. Uninduced cells served as controls. Cells were harvested by centrifugation and total cell extract was prepared by resuspending the cell pellet of 1 ml culture in 50 μl reducing sample buffer. Extracts thus prepared were analyzed by 12% SDS-PAGE and immunoblot analysis [10] was carried out using both anti-LTB and anti-βhCG polyclonal antibodies to check for expression of the fusion

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protein. To determine the localization of the fusion protein in *E. coli*, total cell extract, periplasmic fraction [11] and culture supernatant of induced and uninduced cells were analyzed by Western immunoblot using anti-βhCG antibodies.

## 2.4. Receptor binding activity

The receptor binding capacity of LTB in the fusion protein was determined in vitro using a sandwich ELISA. 100 μl of GM1 ganglioside (10 μg/ml) was coated onto ELISA plates and incubated at 37°C for 1 h followed by overnight incubation at 4°C. Non-specific sites were blocked with 1% lactogen in PBS-Tween followed by successive incubations with 100 μl each of fusion protein, anti-CTB goat antiserum (1:1000) and anti-goat IgG-HRP conjugate (1:2000), respectively. All incubations were carried out at 37°C for 1 h each followed by thorough washes with 50 mM PBS containing 0.5% Tween-20. Subsequent to the final incubation and washes, 0.05% *ortho*-phenylene diamine (OPD) in citrate phosphate buffer (pH 5.0) and 1 μl/ml of hydrogen peroxide were added to develop color. The reaction was terminated using 50 μl of 5 N $H_2SO_4$ and the absorbance was read at 490 nm.

## 2.5. Immunization

To determine whether the recombinant fusion protein is immunogenic, 100 μl of emulsion of the periplasmic fraction containing the fusion protein with CFA was administered to 6–8 week old BALB/c inbred mice (*n*=10) at fortnightly intervals. Primary immunization with CFA was given subcutaneously and the subsequent boosters with IFA were given intramuscularly. The mice were bled retro-orbitally a week after the third booster. Antibody titers were assayed by ELISA and were expressed as the last dilution at which the absorbance at 490 nm was above the background level.

## 2.6. Colloidal immunogold assay

The serum samples were pooled and analyzed for the presence of



Fig. 2. Analysis of fusion protein expression. Total cell extract from cells grown in the presence or absence of IPTG were prepared by suspending 0.5 $A_{600}$/ml unit cells in reducing sample buffer and were analyzed by Western blotting. (A) Samples were immunoblotted with anti-βhCG polyclonal antibodies. Lanes 6 and 7 represent uninduced and induced samples of the clones containing the plasmid in the right orientation while lanes 8 and 9 correspond to the uninduced and induced cells of the reverse orientation clones, respectively. Lanes 1 and 2 are the uninduced and induced cells of the parent clone pKGβhCG expressing βhCG, lanes 3 and 4 are the induced and uninduced control cells which lack the recombinant plasmid. Lane 5 corresponds to the molecular weight markers. The arrowhead on the right points to the fusion protein and the arrowhead on the left points to βhCG. (B) Samples were immunoblotted with anti-LTB polyclonal antibodies. Lane 4 is the positive control corresponding to pure LTB. Lanes 5 and 6 correspond to uninduced and induced cells harboring the pLTBβhCG plasmid in right orientation, respectively. Lanes 7 and 8 correspond to uninduced and induced cells harboring the pLTBβhCG plasmid in reverse orientation. Lanes 9 and 10 are the negative controls consisting of uninduced and induced cells which lack the recombinant plasmid whereas lanes 1 and 2 correspond to uninduced and induced cells harboring pMMB68 plasmid expressing LTB. Lane 3 is the molecular weight marker showing a band at 30 kDa. The arrowhead on the right points to the fusion protein and the arrowhead on the left points to LTB.
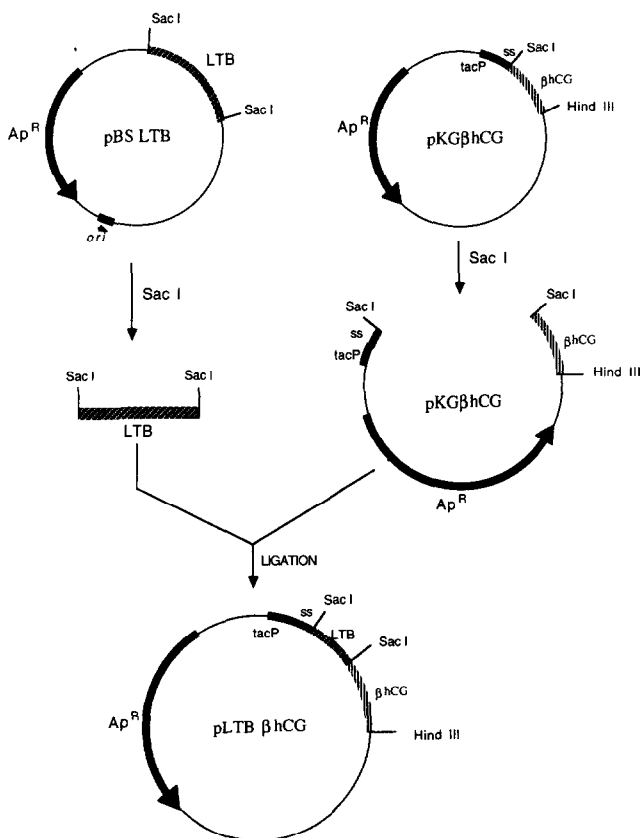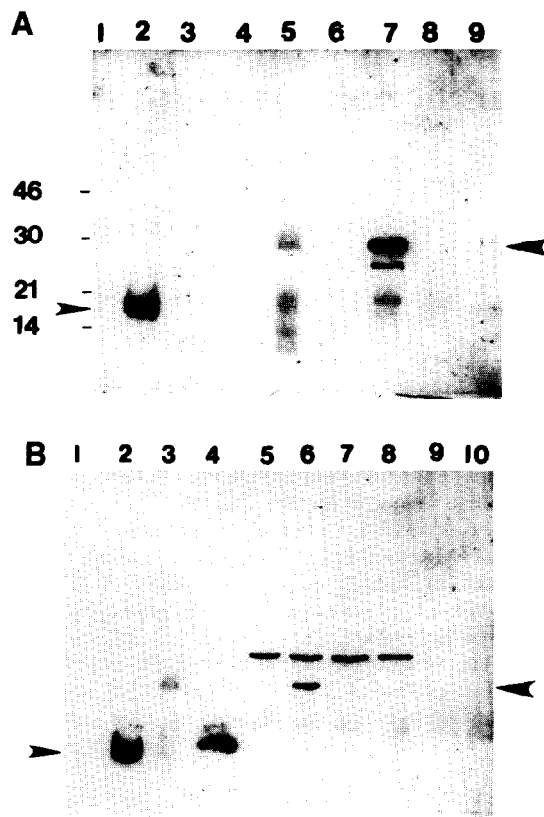


Fig. 1. Construction of the fusion plasmid pLTBβhCG. The 309 bp LTB fragment lacking the termination codon released by *Sac*I digestion of pBSLTB was cloned into *Sac*I linearized pKGβhCG. Fusion gene LTBβhCG is thus under the control of LTB signal sequence and tac promoter.

antibodies by colloidal immunogold assay [12]. Colloidal gold solution was prepared as described [12] and 1 ml of this solution was mixed with 17 μl of 1% $K_2CO_3$. To this mixture, 6 μg of purified native hCG was added and vortexed for a few seconds. After incubation at room temperature (RT) for 5 min, 6 μl of 20% BSA was added and the contents were centrifuged at 5000 rpm for 5 min at RT. The pellet was resuspended in 50 μl of the supernatant containing the colloidal gold mixture, discarding the remainder. Nitrocellulose strips (BA 85) were dotted with 5 μg of recombinant protein A and dried at RT. 40 μl of the pooled serum, 10 μl of 50 mM PBS pH 7.2, 10 μl of hCG-colloidal gold conjugate and finally, 10 μl of 10% alkali-treated casein were added in the wells of a microtiter plate. The contents were thoroughly mixed and nitrocellulose strips (NCS) were placed in the

wells. For positive control, the NCS was dotted with $P_3W_{80}$, a monoclonal antibody to hCG. Sera from unimmunized control mice were also tested. To determine whether the antibodies could bind to recombinant βhCG, the latter was conjugated to colloidal gold and tested as described above.

## 3. Results and discussion

### 3.1. Analysis of the expression of pLTBβhCG

The strategy for the construction of the fusion gene plasmid pLTBβhCG is shown in Fig. 1. The fusion gene pLTBβhCG is thus under the control of tac promoter and LTB signal sequence. E. coli cells harboring the recombinant plasmid pLTBβhCG with the insert in either the right or the reverse orientation were induced with IPTG and an expression level of 3–5 μg/ml of fusion protein was observed. Authenticity of the fusion protein was confirmed by Western blot analysis using both anti-βhCG (Fig. 2A) and anti-LTB (Fig. 2B) polyclonal antibodies. A 30 kDa band corresponding to the expected size of fusion protein was detected with both antibodies in induced cells harboring pLTBβhCG only (Fig. 2A, lane 7; Fig. 2B, lane 6). A band at a slightly higher molecular weight was also observed when the samples were blotted with anti-LTB antibody (Fig. 2B, lanes 5–8). This is probably a non-specific band as it could be observed in the uninduced and induced cells harboring the plasmid in reverse orientation. However, this band was not picked up when these samples were blotted with anti-βhCG antibody (Fig. 2A). The fusion protein could not be detected in uninduced cells suggesting a stringent control on inducibility.

In order to check the localization of the expressed product, different cellular fractions of induced and uninduced cells harboring the plasmid pLTBβhCG were analyzed. The fusion protein band could be detected as a single band in the total cell extract and periplasmic fraction of induced cells (Fig. 3, lanes 2 and 3) but not in the culture supernatant (lane 4). The fusion protein could not be detected in the corresponding fractions of uninduced cells. The detection of the fusion protein in the periplasmic fraction of induced cells indicated that the fusion protein was exported across the cytoplasm, into the periplasmic space but not secreted further into the extracellu-
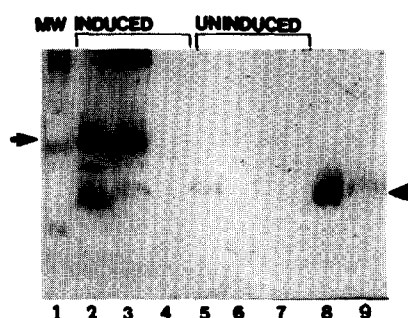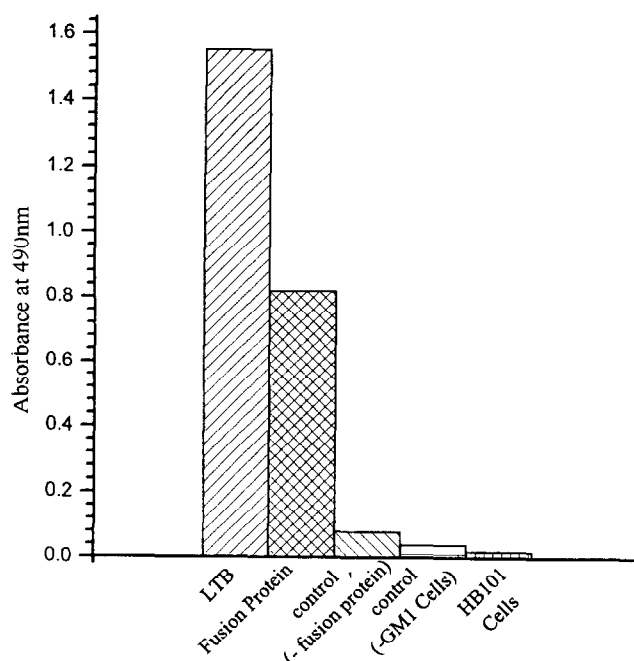


Fig. 4. Receptor binding activity of the fusion protein. The ability of LTB in the fusion protein to bind to its receptor, GM1 ganglioside, was tested using an in vitro sandwich ELISA. Pure LTB was maintained as the positive control. The periplasmic fraction of the fusion protein was used for the test. E. coli HB101 cells and the reactants which were devoid of GM1 ganglioside and the fusion protein were used as negative controls.

lar medium as no protein band could be observed in the culture supernatant of induced cells. The reducing environment of E. coli cytoplasm is not conducive to the formation of disulfide bonds in cysteine rich proteins. It is, therefore, important that the protein be secreted to the outer oxidizing environment of periplasm or extracellular medium. By virtue of exporting the protein to periplasm, partial purification of the protein is achieved automatically since the E. coli periplasm contains only about 4% of the cellular proteins.

We have previously shown the efficient cleavage of the signal peptide and export of βhCG alone to the periplasm in E. coli using LTB signal sequence [8]. The detection of only a single band in total cell extract and periplasm (Fig. 3) suggests that the fusion protein consisting of two mature proteins, LTB and βhCG, in translational fusion, could be successfully expressed, efficiently processed and exported to the periplasm in E. coli using the LTB signal sequence.

The expression of the fusion protein improved with increasing concentration of IPTG until 0.5 mM beyond which no detectable increase in expression was observed (data not shown). The time course of fusion protein expression revealed that the level of expression increased with time attaining its maximum at 8 h and plateaued at later time points (data not shown).

### 3.2. Analysis of receptor binding activity

The periplasmic fraction of E. coli cells harboring pLTBβhCG or HB101 cells were tested for receptor binding activity of LTB in the fusion protein using GM1 sandwich ELISA. LTB in the fusion protein retained its ability to bind to GM1 ganglioside receptor as is evident from the high absorbance detected in the periplasmic fraction of E.



Fig. 3. Localization of the fusion protein in E. coli. Different cellular fractions of cells grown in the presence and absence of 1 mM IPTG were electrophoresed on a 12% SDS-PAGE and immunoblotted with anti-βhCG polyclonal antibodies. Lanes 2–4 represent the total cell extract, periplasmic fraction and supernatant of induced cells harboring pLTBβhCG. Lanes 5–7 represent the corresponding fractions of uninduced cells. Lanes 8 and 9 correspond to induced and uninduced E. coli cells expressing βhCG. MW denotes the molecular weight markers. The arrow points to the fusion protein and the arrowhead indicates βhCG.

*coli* cells containing pLTBβhCG (Fig. 4). However, its binding affinity for GM1 was reduced compared to standard LTB which was used as the positive control (Fig. 4). The binding affinity of the fusion protein to GM1 ganglioside compared to LTB, observed in the present study, supports the earlier finding [13] that fusion of CTB to a relatively large protein could alter its affinity for GM1. Background levels of absorbance observed in HB101 cells or control samples that lacked either fusion protein or GM1 demonstrate the specificity of the reaction. These data clearly suggest that fusion protein is biologically active in terms of GM1 receptor binding assay.

### 3.3. Immunogenicity of the fusion protein

To establish the immunogenicity of the recombinant fusion protein, mice were immunized with fusion protein as described in Section 2. Anti-LTB and anti-βhCG antibodies were assayed by ELISA in serum samples immunized with fusion protein. Antibody titers against each component of the fusion protein, i.e. LTB and βhCG, were found to be high, however, titers against LTB were 1:10000 and against βhCG were 1:5000. The serum samples from immunized mice were also tested for its reactivity to recombinant βhCG and native hCG by colloidal immunogold assay. The antisera raised against the fusion protein could bind to recombinant βhCG and native hCG (data not shown). These data suggest that the fusion protein is immunogenic and has a potential to be used as a contraceptive vaccine.

An expression cassette such as described here could be used to clone other genes of interest, using LTB as a fusion partner, thus bypassing the need for chemical conjugation of proteins, a process which is time consuming and cumbersome.

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