High expression of human chorionic gonadotrophin β-subunit using a synthetic vaccinia virus promoter

A Gupta, S Chandrasekhar, R Pal, S Ahlawat and O Singh
National Institute of Immunology, New Delhi 110067, India

(Requests for offprints should be addressed to O Singh, Immunoendocrinology Laboratory, National Institute of Immunology, New Delhi 110067, India; Email: om@nii.res.in)

(A Gupta is now at Department of Pathology, Wayne State University School of Medicine, 540 E Canfield Avenue, Detroit, Michigan 48201, USA; S Chandrasekhar is now at Division of Surgical Oncology, Massachusetts General Hospital, 100 Blossom Street, Boston, Massachusetts 02114, USA)

ABSTRACT
We have constructed a recombinant vaccinia virus to express the β-subunit of human chorionic gonadotrophin (βhCG), a secretory glycoprotein that is used as an antigen for a contraceptive vaccine. The cDNA encoding the subunit was cloned under the control of a synthetic promoter that could be recognised by a vaccinia virus RNA polymerase to direct transcription. The peak expression level of βhCG directed by a late synthetic promoter (Psyn) was 11.5 µg/ml, a level that was at least sixfold higher than that directed by the p7.5 early/late promoter. The expressed protein was correctly processed post-translationally such that it attained a conformation with correctly folded discontinuous epitope(s) similar to that seen in native βhCG.

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INTRODUCTION
Human chorionic gonadotrophin (hCG) is a member of a family of heterodimeric glycoprotein hormones that also includes luteinizing hormone (LH), follicle-stimulating hormone and thyroid-stimulating hormone. Each hormone consists of a common α-subunit and a hormone-specific β-subunit, encoded by separate genes. hCG is produced by the peri-implantation embryo and stimulates the corpus luteum to continue production of progesterone until the placenta assumes the role of producing this steroid. Because of the crucial role played by hCG in the establishment and maintenance of early pregnancy, it has been considered to be a target for an anti-fertility vaccine. A prototype vaccine based on the entire β-subunit of hCG (βhCG) generating hCG-neutralising antibodies (Singh et al. 1989, Pal et al. 1990) has completed clinical testing in women (Talwar et al. 1994), demonstrating the feasibility of the approach in preventing pregnancy.

Conventionally, βhCG is obtained by dissociating native hCG, a procedure that is cumbersome and expensive. The isolated βhCG is invariably contaminated with trace amounts of α-subunit, an impurity that may lead to generation of antibodies with undesirable reactivity. With a view to expressing βhCG by a recombinant method, a vaccinia virus (VV) recombinant was previously constructed. This virus (vSS1) expressed only moderate levels of βhCG however (Srinivasan et al. 1995). The gene for βhCG was placed under the control of the widely used VV p7.5 early/late promoter (Cochran et al. 1985). In the present study, we report construction of a VV recombinant using a late synthetic promoter (Psyn) (Davison & Moss 1990), which can express high levels of βhCG that is immunologically similar to the native molecule.

MATERIALS AND METHODS
Plasmids, virus and primary cell cultures
The cloning vector pBluescript KS− was obtained from Stratagene, La Jolla, CA, USA. The VV (WR strain) and vaccinia transfer vector pMJ602 (Davison & Moss 1990) were gifts from Dr B Moss,
National Institutes of Health, Bethesda, MD, USA. The monkey CV1 cells and human TK~143B cells (obtained from the National Centre for Cell Science, Pune, India) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone Labs Inc., Logan, UT, USA). Restriction enzymes were procured from Boehringer Mannheim, Mannheim, Germany; other fine chemicals, media and antibiotics were from Sigma Chemical Co., St Louis, MO, USA or Gibco-BRL, Gaithersburg, MD, USA, and radioisotopes were from Amersham International plc, Amersham, Bucks, UK. All cloning steps were performed as described by Maniatis et al. (1982).

**Transfection and selection of recombinants**

CV1 cells (1 × 10^6) were seeded into a T-25 culture flask and incubated overnight at 37°C to obtain a confluent monolayer. A total of 3 × 10^6 cells were infected with VV (WR) at a multiplicity of infection (MOI) of 0.05 plaque-forming unit (pfu)/cell in medium containing 2.5% FBS, and incubated for 2 h with intermittent rocking. The inoculum mixture was aspirated and the cells, after washing with serum-free medium, were overlaid with 10 µg DNA complexed with lipofectin (Gibco-BRL) as per the manufacturer’s instructions. Following incubation for 5 h at 37°C in a humidified atmosphere containing 5% CO₂, the DNA-containing medium was replaced with 2 ml normal growth medium supplemented with serum. Two days later, the cells (containing the vaccinia recombinants) were scraped, centrifuged at 1800 g at 4°C for 5 min, suspended in 0.5 ml DMEM with 2.5% FBS and lysed by three cycles of freeze-thawing followed by sonication.

A total of 5 × 10⁵ TK~143B cells, seeded in a six-well plate and grown to confluency in DMEM supplemented with 10% FBS and 5-bromodeoxyuridine (BrDU; Boehringer Mannheim; 25 µg/ml), were infected with the cell lysate obtained after transfection. Following incubation at 37°C for 2 h, the virus inoculum was aspirated and 1.5 ml selective agarose (low melting point agarose in DMEM with 2% FBS and 25 µg/ml BrDU) was added to each well. The agarose was allowed to solidify at 4°C and the cells were incubated for a further 48 h at 37°C. For visual selection of the recombinant virus, a second selective agarose overlay containing 5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside (X-gal; Amresco, Solon, OH, USA) (300 µg/ml) was added. Following overnight incubation, five to six blue plaques were collected by suction with a Pasteur pipette and transferred to 500 µl DMEM supplemented with 2.5% FBS. The virus particles were released by alternate cycles of freezing and thawing followed by indirect sonication for 30 s in an ice-water bath. After three to four rounds of purification, the recombinant plaque isolate was amplified by infection of successively larger number of cells. The selective drugs were added during the initial amplification steps after which no selection was required.

**Detection and characterisation of βhCG**

Amounts of βhCG expressed in culture supernatants were determined by competitive radioimmunoassay using a specific monoclonal antibody and purified native subunit as the standard (Singh & Capoor 1993). The affinity of βhCG for binding to antibodies (monoclonal and polyclonal) was determined by the cold displacement method essentially as described (Singh et al. 1989). Briefly, increasing concentrations of βhCG (recombinant or native) were incubated in a total volume of 0.5 ml with a limited amount of antibody in the presence of 125I-hCG for 24 h at 4°C. Antibody-bound fractions precipitated by polyethylene glycol molecular weight 800 (Amresco; 12.5% final concentration) were separated by centrifugation at 1500 g for 20 min, and counted for radioactivity in an LKB multi-γ-counter. The association constant (K_a) of βhCG-antibody interaction was calculated using a computer programme (Munson & Rodbard 1980). The ability of recombinant βhCG to associate with αhCG was tested in a mouse Leydig cell assay system as described (Rao et al. 1988). Culture supernatant containing expressed βhCG was incubated with a twofold excess of native αhCG in an end-to-end shaker for 24 h at room temperature. For Western blot, culture supernatant (harvested 72 h after infection) was fractionated on 12.5% denaturing SDS-PAGE (Laemmli 1970) and the resolved bands were transferred onto nitrocellulose membrane (Amersham International plc) in a transfer buffer containing 20 mM Tris–Cl, 150 mM glycine, pH 8.0 and 20% methanol. Non-specific sites were blocked by incubation with 2% bovine serum albumin (BSA) (in 50 mM phosphate-buffered saline, pH 7.4 and 0.05% Tween 20; PBST) for 1 h at room temperature. The filter was washed with PBST and then incubated for 2 h with the primary (βhCG-specific monoclonal) antibody. The membrane was washed again with PBST and incubated with goat anti-mouse IgG-horse radish peroxidase conjugate (Sigma) for 1 h. Following washings, reactive proteins were visualised using 3,3-diaminobenzidine.
RESULTS

Generation of recombinant VV expressing βhCG

A recombinant VV vAO1 containing the βhCG gene under the control of the synthetic promoter, Psyn, was constructed employing standard protocols (Mackett et al. 1984). To achieve this, a recombinant VV transfer vector pAO6 was generated by cloning a 600 bp βhCG gene fragment at the HindIII site of the VV vector pMJ602 downstream of Psyn (Fig. 1). The orientation of the gene with respect to the promoter was determined by restriction analysis followed by Southern blotting and probing with 32P-labelled βhCG (Fig. 2). The ability of the promoter to direct the expression of βhCG was checked in a transient expression system using CV1 cells. A significant amount (170 ng/ml) of βhCG was secreted into the culture supernatant but no activity was detected in the medium of cells infected with the wild-type virus. The expression cassette was inserted into the VV TK region by homologous recombination. The tk gene enabled a positive selection of the recombinant plaques in the presence of BrDU in a tk- cell line. The lacZ gene under the control of the VV p7·5 promoter resulted in blue recombinant plaques in the presence of the chromogenic substrate X-gal.

Expression and characterisation of βhCG

Time kinetics of βhCG secretion were investigated in virus-infected cells. CV1 cells (2 × 10⁶) were infected at an MOI of 0·5 pfu/cell. Expression of βhCG by the recombinant virus vAO1 increased as a function of time (Fig. 3). βhCG could be detected within 6 h of VV infection and the saturation level (11·5 µg/ml) was attained at 48 h after infection. To determine the size of the expressed protein, culture supernatant of the cells infected with the recombinant virus vAO1 was subjected to Western blotting and the protein identified using a monoclonal antibody specific for βhCG. The migration pattern of recombinant βhCG was almost the same as that of the standard βhCG (Fig. 4), indicating that the size and overall extent of glycosylation of the vaccinia-expressed protein is similar to that of native βhCG. The expressed protein was evaluated for its reactivity with a specific monoclonal antibody as well as with antibodies generated by five women immunised with the βhCG vaccine during efficacy studies. These antibodies are bioneutralising in nature and recognise a discontinuous epitope on βhCG (Talwar et al. 1994, 1997). Analysis of cold displacement curves revealed that VV-expressed recombinant βhCG bound each antibody tested with the same affinity as did native βhCG (Kₐ=2·5 × 10¹⁰ M⁻¹ to 4·7 × 10¹⁰ M⁻¹) (Fig. 5). These results indicate that the expressed subunit folds to recreate the conformational integrity of the

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**FIGURE 1.** Construction of recombinant transfer vector pAO6 for the insertion of βhCG cDNA into the vaccinia genome. The gene for βhCG (β-hCG) was extracted from pAO1 by digestion with HindIII (right panel) and ligated to the VV transfer vector pMJ602 at its HindIII site (left panel). The recombinant clone pAO6 identified after colony hybridisation has the gene for βhCG under the control of Psyn (p-syn) and the lacZ gene under the control of an early portion of the p7·5 (p-7·5) promoter. TK-1 and TK-r refer to the segments of VV DNA that include the left and right portion of the thymidine kinase gene and pBR328 bacterial origin of DNA replication.
native molecule. To confirm this, binding of 
βhCG with LH/hCG receptors was also investigated.
Since isolated subunits cannot bind to receptor,
recombinant βhCG was associated with the native
αhCG and the potency of the resultant dimer tested
in an in vitro bioassay. As is evident from Fig. 6,
the dimer of recombinant βhCG and αhCG produced
a steroidogenic response parallel to that of native
dimer. On a weight basis, the recombinant dimer
was observed to be nearly as potent (94%) as the
native dimer, confirming that vaccinia-expressed
recombinant βhCG has maintained the integrity of
the molecule. As expected, medium containing
individual subunits did not produce any biological
activity.

DISCUSSION

We have here described the construction of a
recombinant VV vAO1 which can express high
levels of βhCG. The recombinant virus vAO1
expressed and secreted βhCG into culture medium
in a time-dependent manner until peak levels of
11.5 µg/ml were obtained 48 h post infection. The
levels of expression are at least sixfold higher than
those secreted by the VV recombinant (vSS1)
constructed earlier with p7.5 early/late promoter
(Chakrabarti et al. 1989). These results are in
contrast to the SV40 virus (Reddy et al. 1985)- and
yeast (Sen Gupta & Dighe 1999)-based systems
where co-expression of both α- and β-subunits was
required to obtain a reasonable yield. Since significantly high levels of expression could be achieved by VV-based system, low expression of βhCG by other expression systems may not be due to intrinsic instability of the molecule as suggested by Reddy et al. (1986).

βhCG consists of 145 amino acids, which include 12 cysteine residues that form six disulphide bonds. The three-dimensional structure of βhCG determined by X-ray crystallography (Lapthorn et al. 1994) has revealed a unique cystine knot motif (formed by three disulphide bonds) that largely determines the peptide folds of βhCG. βhCG has two N-linked and four O-linked glycosylation

**FIGURE 3.** Kinetics of βhCG secretion by cells infected with recombinant virus, vAO1. 2 × 10^6 CV1 cells were infected with 0.5 pfu/cell of recombinant VV vAO1 (with Psyn). Culture supernatants harvested at the time-points indicated were assayed for βhCG by competitive radioimmunoassay employing a specific monoclonal antibody.

**FIGURE 4.** Western blot analysis of total protein isolated from vAO1-infected CV1 cells. Culture supernatant of CV1 cells infected with the recombinant virus (lane 2) and purified native βhCG (lane 1) were fractionated in a 12.5% polyacrylamide gel under denaturing conditions. After blotting onto a nitrocellulose membrane, the proteins were identified by specific monoclonal antibody and HRPO-conjugated second antibody. mw, protein molecular weight marker lane.

**FIGURE 5.** Cold displacement analysis of βhCG–monoclonal antibody interaction. Slopes of the Scatchard curves with recombinant βhCG (●) and native βhCG (○) gave Kd values of 4.2 × 10^{10} M^{-1} and 4.5 × 10^{10} M^{-1} respectively.
required to be linked to protein carrier molecules to render it immunogenic. Protein carriers have limitations and can be substituted by a combination of helper T-cell peptides (Mandokhot et al. 2000). Fusion of peptides at the C-terminal of βhCG does not affect the folding or antigenicity of βhCG (Srinivasan et al. 1995, Gupta et al. 1998). It will be of interest to express βhCG fused with helper T-cell epitopes to construct a well-defined chimeric immunogen, obviating the need for chemical linkage that invariably results in batch-to-batch variation.

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