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EXPRESSION OF A BIOLOGICALLY ACTIVE OVINE TROPHOBLASTIC INTERFERON USING A BACULOVIRUS EXPRESSION SYSTEM

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SUMMARY: Ovine trophoblast protein (oTP) an embryonic interferon, which plays a key role in maternal recognition of pregnancy, has been expressed in insect cells using a baculovirus expression system. A cDNA coding for oTP was inserted downstream of the strong polyhedrin promoter. Cells infected with recombinant virus produced biologically active oTP and greater than 90% was secreted into the culture medium during infection. High amount of antiviral activity were produced (up to 5 x 10^5 IU per ml of culture medium). Recombinant oTP (roTP) was purified by immunoaffinity chromatography and found to be identical to authentic oTP with respect to molecular mass and N-terminal amino acid sequence.

In ruminants the trophoblast of developping conceptuses produces a major protein, which has been initially characterized in the sheep (1,2) and subsequently in the bovine species (3). The two proteins, ovine trophoblastin (oTP) and bovine trophoblast protein-1 (bTP-1), which are synthetized and secreted between days 11 and 21 in the sheep and between days 12 and 25 in the cow, play a central role in the establishment of pregnancy by blocking into uterus the pulsatile secretion of prostaglandins

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ABBREVIATIONS
oTP, ovine trophoblast protein; roTP, recombinant ovine trophoblast protein; bTP-1, bovine trophoblast protein-1; IFN-a, alpha interferon; IFN-aII, class II alpha interferon; PTH, phenyl thiohydantoins; MDBK, Madin-Darby bovine kidney; VSV, vesicular stomatitis virus.

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and thereby ensuring the maintenance of the corpus luteum function (reviewed in 4).

The amino terminal sequence of purified oTP (5,6) and sequencing of cDNA for both sheep (7, 8, 9) and cattle (10, 11) revealed a high degree of similarity with alpha-interferons (IFN-α) and more precisely with class II α-interferon (IFN-αII) subfamily. The antiviral and antiproliferative properties of these trophoblast proteins have confirm that they belong to the interferon family (12).

Purification of oTP from cultured embryos in the quantities required for physiological and pharmacological studies would be impractical in cost and effort. The ability to produce oTP throught recombinant DNA technology should provide alternate means for generating sufficient for studies the physiological function of this molecule.

The baculovirus expression system utilizing *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) in *Spodoptera frugiperda* cells has shown great potential for synthesis of a variety of proteins (13).

In the present study, we report the isolation and characterization of a biologically active roTP from a baculovirus expression system. The purified roTP was correctly matured as indicated by N-terminal sequence analysis.

MATERIALS AND METHODS

Restriction enzymes and other DNA modifying enzymes were purchased from Boehringer Mannheim and used according to the supplier's instructions. Reagents for Edman sequencing were provided by Applied Biosystem. Other reagents were of analytical grade for Biochemical use.

Cell and virus: *Spodoptera frugiperda* (Sf21) cells were maintained at 28°C as monolayer cultures in TC100 modified medium supplemented with 10% foetal calf serum (unpublished data). For serum-free cultures SF900 medium (GIBCO) was used. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant viruses were propagated in Sf21 cells (14).

Construction of recombinant baculovirus: The recombinant plasmid pUC18 tropho (9) containing oTP cDNA was used to construct recombinant baculovirus transfer vector. The plasmid was cleaved with NcoI and SspI and the 833bp fragment containing the entire coding region, one additional base before the ATG translational start signal, and the 246bp of the 3' untranslated sequence was blunt-ended with the Klenow fragment of DNA polymerase I. The resulting DNA fragment was cloned into the unique SmaI site of the transfer vector pGmAc3 (15) and used to transform *E. coli* strain Hb101. Clones harboring plasmid with the insert in the correct orientation with respect to polyhedrin promoter were identified by restriction endonuclease mapping. Recombinant transfer vector DNA was cotransfected into Sf21 cells with wild-type AcNPV DNA by calcium phosphate transfection procedure (16). In vivo homologous recombination between wild type baculovirus sequences and recombinant plasmids resulted in the generation of recombinant viruses, which were then plaque purified by screening for the occlusion-negative phenotype (17). Restriction analysis of

DNA from several of these occlusion-negative viruses confirmed that oTP had been transferred to AcNPV genome.

Immunoblot analysis: Sf21 cells were seeded at 1.5 x 10^7 cells per plate and infected with recombinant virus at a multiplicity of infection of 10 pfu/cell. Mock-infected and wild type-infected cells were used as controls. At 18, 24, 36, 48 and 72 hours after infection the medium was collected and clarified by centrifugation for 5 min. at 3000 g. Thirty µl aliquots of the medium were analysed by SDS-PAGE on 12% polyacrylamide gels (18), transferred to nitrocellulose and probed with a rabbit antitrophoblastin serum (provided by P. Reinaud). The antigen-antibody complexes were detected by reaction with anti-rabbit IgG conjugated with horseradish peroxydase and visualized with 4-chloro-naphtol.

Purification and determination of N-terminal amino acid sequence of roTP: Immunopurification of roTP was performed with a Trisacryl GF200 coupled to monoclonal antibody (12Φ34) (19). The culture medium (50ml) was applied at a flow rate of 5ml/h on a chromatographic column containing 1ml gel (6.9 mg IgG.per ml). The column was washed with phosphate buffer-saline (PBS), then PBS containing NaCl 2M until A280 returned to base line. The oTP was eluted with 50 mM Na citrate buffer pH2.8, neutralized and titrated for antiviral activity.

Automated Edman degradation of the purified roTP was carried-out using an Applied Biosystems 470A sequenator coupled with a model 120 phenyl thiohydantoins (PTH) HPLC analyser with reagents and methods of the manufacturer. Before sequencing roTP was reduced and alkyled with 4-vinyl pyridine according to Henschen (20).

Antiviral assay: Cell-free culture supernatants were collected and antiviral titers were determined by standard assay in microtiter plates using MDBK (Madin-Darby bovine kidney) cells and Vesicular Stomatitis Virus (VSV) as a challenge virus (21).

RESULTS AND DISCUSSION

Expression of biologically active oTP in infected cells with recombinant AcNPV: The kinetic of oTP production by recombinant virus infected cells measured by titration of antiviral activity is shown on Fig. 1. Antiviral activity was detectable into the culture medium 18h post-infection. This activity continues to accumulate in the medium over the next 48-72h and reached a maximum of $5x10^5$ IU/ml at 72h post infection. Media samples from uninfected cells and cells infected with wild-type AcNPV had no detectable antiviral activity. In a separate experiment, the intracellular and extracellular levels of roTP was measured. At late times post-infection (72-96h) up to 90% of roTP was present in the medium.

Immunoblot analysis of roTP expressed in infected cells: The time course expression of roTP was also followed by immunoblotting. As observed by titration of antiviral activity amount of secreted roTP are low during the first 24h post-infection. These results were consistent with the late expression of the polyhedrin promoter (22). A single immunoreactive band with an apparent molecular mass (≈19 kDa) similar to the size of authentic oTP was detected in the medium 36h post infection (Fig. 2, lane 4). The concentration of roTP increase considerably at last times post-infection as

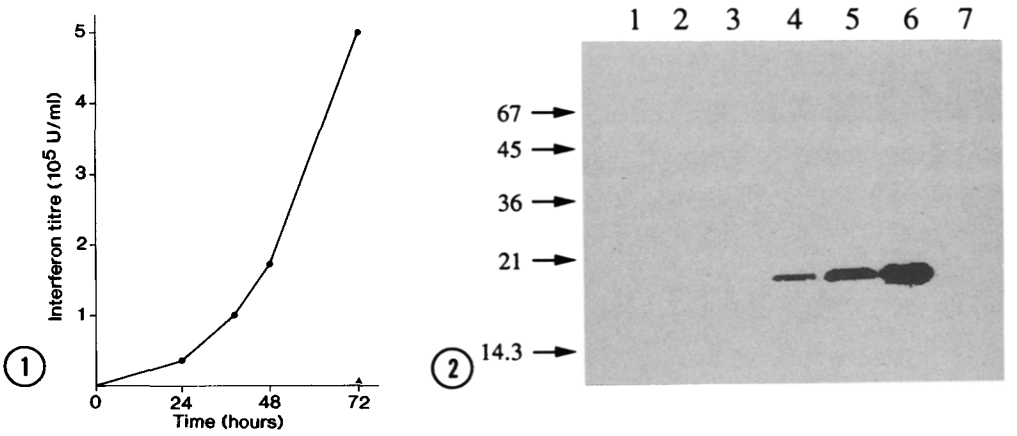

Fig. 1. Time course expression of oTP in recombinant virus infected cells.
Sf21 cells were infected with recombinant virus (10 pfu/cell) at a cell density of 1.5 x 10^6/ml. At the times indicated, the media were collected, centrifuged for 5 min at 3000 g and stored frozen until measured for antiviral activity.
● - Recombinant virus infected cells.
▲ - Wild-type virus infected cells.

Fig. 2. Immunoblot analysis of recombinant oTP secreted by Sf21 infected cells.
Thirty µl of media samples from infected and non-infected cells (see Fig. 1) were analyzed by SDS-PAGE on 12% polyacrylamide gel and transferred on to nitrocellulose as described in experimental procedure.
Lanes 1 and 7 medium from uninfected and wild-type virus infected cells respectively. Lanes 2 to 6 samples of recombinant virus infected cells at 18, 24, 36, 48 and 72 hours respectively. Molecular mass standards (kDa) are indicated on the left.

illustrated on Fig. 2 (lanes 5 and 6). No immunoreactive materiel was present in uninfected cells (Fig. 2, lane 1) or in cells infected with-wild type baculovirus (Fig. 2, lane 7).

Purification and N-terminal sequence analysis of roTP: roTP was purified from serum-free culture medium by immunoaffinity chromatography with an anti oTP monoclonal antibody (12Φ34) linked to glutaraldehyde actived Trisacryl GF 200. The elution profile of Fig. 3A shows that roTP was eluted using Na citrate buffer pH 2.8 with recoveries of antiviral activity of 70 to 80%. Analysis of various preparations by SDS PAGE shows that the elution of antiviral activity coincided with that of a highly purified 19 kDa protein (Fig. 3B, lanes 2 and 3), the size of authentic oTP. After passage through immunoaffinity column roTP preparations were estimated to be 95% homogeneous after electrophoresis by scanning densitometry of the stained gel. Immunopurified roTP was chromatographed on reverse phase HPLC and submitted to Edman degradation after reduction and alkylation.

The N-terminal sequence of the first 20 amino acids of roTP : Cys-Tyr-Leu-Ser-Gln-Arg-Leu-Met-Leu-Asp-Ala-Arg-Glu-Asn-Lys-Leu-Leu-

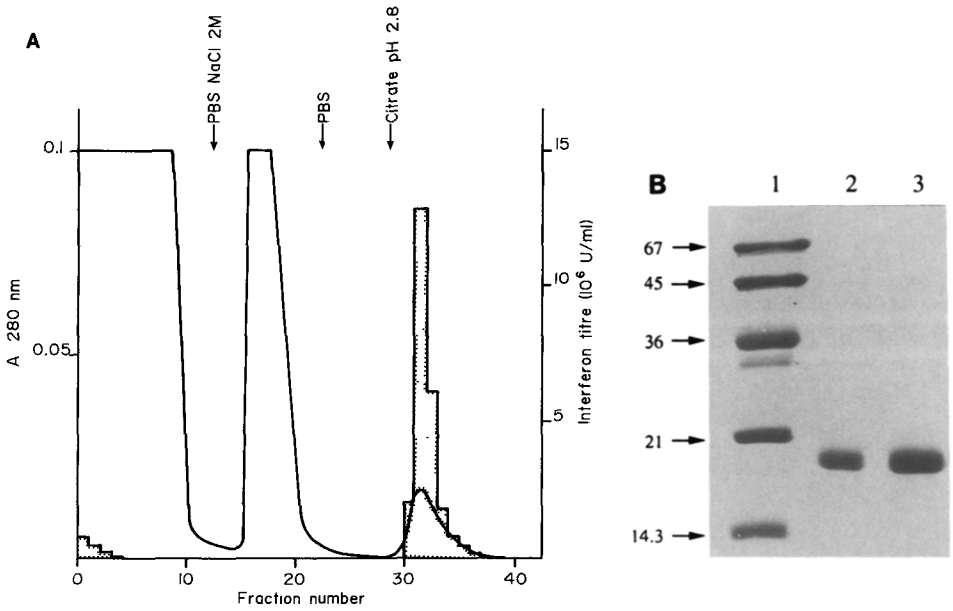

Fig. 3. Purification of recombinant oTP
A) Affinity chromatography of recombinant oTP
The medium from 50 ml culture infected Sf21 cells was applied on immunoabsorbant column. The column was washed with 5 vol. of PBS, then with 5 vol. of PBS containing 2M NaCl, before elution with Na citrate pH2.8 as described under "materials and methods". Fractions of 1ml were collected and analyzed for antiviral activity (bar graph) reported as the total activity of each fraction.
B) SDS PAGE analysis of immunopurified roTP
An aliquot of two separate preparations of immunopurified roTP was analyzed on 12% SDS polyacrylamide gel (lanes 2 and 3). Proteins were stained with Coomassie Brillant blue. Molecular mass markers are in kilodaltons (lane 1).

Asp-Arg matches perfectly the NH_2-terminal residues of natural oTP, showing that the signal peptide has been correctly processed.

In conclusion, we describe here, the expression of mature biologically active roTP in baculovirus expression systems. The highest level of roTP (2 to 3mg/l) were obtained from the baculovirus system in serum containing medium. Interestingly only a minor reduction in roTP expression ($\approx$20%) was observed in absence of serum. An efficient purification procedure was developed for roTP given good recoveries ($\approx$80%) of this protein with high biological activity. After this purification step this protein appear to be 95% pure and N-terminal sequence analysis revealed the expected sequence of authentic oTP.

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