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Short communication

High level expression of bioactive recombinant human growth hormone in the milk of a cloned transgenic cow

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Abstract

Transgenic farm animals have been proposed as an alternative to current bioreactors for large scale production of biopharmaceuticals. However, the efficiency of both methods in the production of the same protein has not yet been established. Here we report the production of recombinant human growth hormone (hGH) in the milk of a cloned transgenic cow at levels of up to 5 g l^{-1}. The hormone is identical to that currently produced by expression in *E. coli*. In addition, the hematological and somatometric parameters of the cloned transgenic cow are within the normal range for the breed and it is fertile and capable of producing normal offspring. These results demonstrate that transgenic cattle can be used as a cost-effective alternative for the production of this hormone.

Keywords: Recombinant hGH; Transgenic cloned cow; Animal biotechnology

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The expression of proteins with potential therapeutic applications in the milk of livestock species appears to be one of the most attractive commercial applications of animal transgenesis (Niemann and Kues, 2000). However, this method has been restricted to proteins required in large amounts that cannot be obtained cost-effectively in current bioreactors, such as alpha1 anti-trypsin (Wright et al., 1991) or serum albumin (Echelard et al., 2002). Levels of up to 30 g l^{-1} and 48 g l^{-1} have been reported for the production of these proteins in the milk of sheep and cows, respectively.

The present study was set up to analyze the feasibility of using the milk of transgenic cattle as an alternative for the commercial production of human growth hormone (hGH).

Recombinant hGH was produced in *E. coli* culture following the procedure routinely used for the commercial production of the hormone. A 650 bp cDNA encoding the hGH was cloned into a pBST plasmid under the control of a phage RNA polymerase promoter. The expression of the gene in the resultant construct is inducible with IPTG. One milliliters of bacterial suspension from the original culture was used to generate a subsequent culture in a New Brunswick Scientific IF 250 bioreactor. Bacteria were grown during 12 h and then induced to produce hGH for 3 h. They were subsequently collected by cross-flow filtration (Filtron equipment) and disrupted by high-pressure treatment (Rannie equipment). Material was centrifuged and the supernatant passed through an immunoaffinity column (Affigel-10 Bio-Rad Laboratories resin + anti-hGH monoclonal antibodies). Recombinant hGH was eluted and fully purified by RP-HPLC (C4) and further molecular exclusion chromatography.

For the production of cloned transgenic cows, fetal fibroblasts were obtained from a 75-day-old Jersey fetus and transfected with two separate plasmids, one containing the human growth hormone gene under the control of a bovine β-casein promoter, and the other bearing a neomycin resistance gene. After selection for 14 days with 800 μg ml^{-1} geneticin, several colonies were isolated. Three cell lines were obtained from those colonies: Leo 0, Leo 1 and Leo 2, which were used as donors.

Oocytes were aspirated from slaughterhouse ovaries and matured in TCM-199 + 5% FCS at 39 °C for 24 h. Mature oocytes were denuded by vortexing for 3 min in PBS with 1 mg ml^{-1} bovine testis hyaluronidase. Metaphases were assessed and oocytes were enucleated by visualization with Hoechst 33342 (5 μg ml^{-1}) under UV light (<6 s). Donor cells at G0/G1 stages were fused to enucleated oocytes by an electrical pulse. After 3 h, activation was induced by incubation in TL-HEPES with 5 μM ionomycin for 4 min and 2 mM 6-DMAP for 3 h. The oocytes were then washed with TL-HEPES and cultured in SOF medium with an atmosphere of 5% CO_2 + 5% O_2 + 90% N_2. Development to blastocysts (days 7–9) was recorded. One or two blastocysts per recipient cow were transferred non-surgically, and pregnancies at 30 days or 60 days were determined by ultrasonography.

Cleavage rates and blastocyst production were not significantly different between the different cell lines and control fibroblasts. A total of 15 transgenic calves were born (4 from cell line Leo 0 and 11 form line Leo 2, while line Leo 1 produced no pregnancies). The presence of the transgene in DNA isolated from the transgenic calves blood was verified by PCR amplification of DNA sections comprising part of the promoter transgene and the entire coding region. Only one calf from line Leo 0 showed the complete sequence for the hGH coding region and the β-casein promoter, whereas partial deletions in the 3′ end of the transgene were observed in the other animals. This is probably a consequence of the heterogeneity of the transgenic cell lines, since they were not subjected to clonal selection.

Lactation was induced in transgenic animals by a biphasic treatment. The first phase of the treatment involved the combined sc administration of estrogens (estradiol benzoate, Histeren®, Instituto Rosenbusch) and progestagens (medroxyprogesterone acetate, Pronal®, Aton), consisting of five successive applications of each drug, in a dose of 0.1 mg kg^{-1} BW and 0.25 mg kg^{-1} BW, respectively, every 48 h (i.e., on days 1, 3, 5, 7 and 9, assuming that the treatment commences on day 1). The second phase included the sc administration of dexamethasone (Decadron, Sidus) and oxytocin (Orasthin®, Hoechst Marion Roussel). A total amount of 20 mg of the former was injected over a period comprising days 18–20 (one-third of total mass being administrated each day), and three applications of 50 IU of the latter were given on days 21–23. Milking was started on day 24.

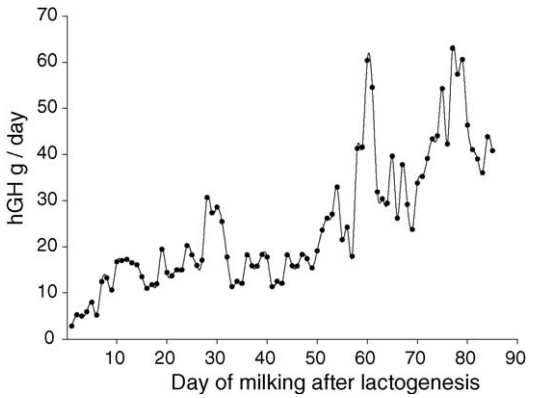

Fig. 1. Bioactive hGH levels in the milk of the transgenic cloned cow.

Bioactivity of hGH in the milk, measured by a Nb2 lymphoma cell bioassay (Tanaka et al., 1980), started to increase from values around 2 g l^{-1} at the onset of lactation and rose steadily reaching values of 5.0 g l^{-1} (Fig. 1). A highly significant correlation was observed between hGH bioactivity and its immunoactivity, as determined with a specific antibody. Milk hGH levels showed three peaks (30, 60 and 80 days after induction), which were associated with the onset of estrous cycles.

Human GH was also detected in the cow serum, reaching a maximum of 3000 ng ml^{-1} and then leveling at around 600 ng ml^{-1}. Since the presence of hGH in serum was observed 3 weeks before the onset of lactation, our interpretation is that rather than to an ectopic expression of the transgene, the circulating hGH may derive from leakage from the mammary gland due to a non-vectorial secretion before lactogenesis II. In fact, tight junctions between adjacent mammary secretory cells do not develop until just prior to parturition, and hence, proteins that are constitutively synthesized by the developing secretory epithelial cells are secreted into the interstitial fluid, and ultimately find their way into serum. This mechanism was described for the milk proteins α-lactalbumin (Akers et al., 1986) and β-lactoglobulin (Mao et al., 1991).

Analysis of the whey milk by SDS-PAGE and Coomassie blue staining showed a major band corresponding to recombinant hGH (Fig. 2A). This band represents about 10% of the total protein content. As

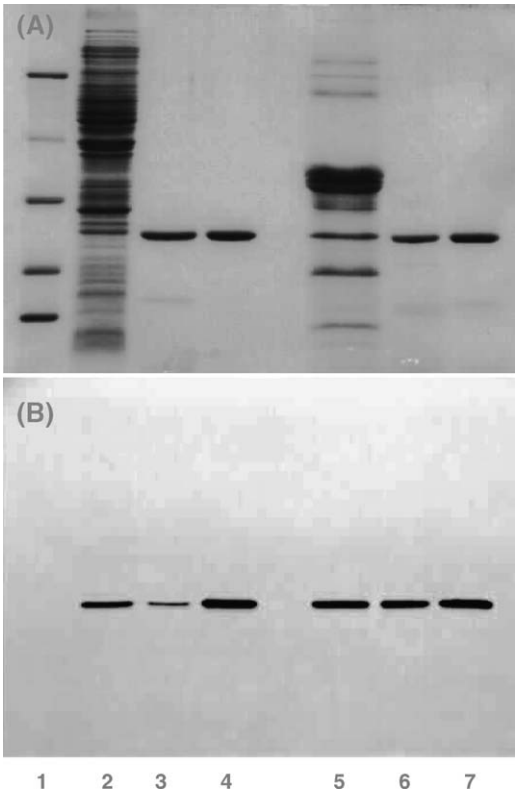

Fig. 2. Coomassie blue staining (panel A) and Western blot (panel B) of bacterial extracts and milk from the transgenic cow producing hGH. Lane 1, molecular weight marker; Lane 2, bacterial extract before immunoaffinity chromatography; Lane 3, bacterial extract after immunoaffinity chromatography; Lane 4, bacterial extract after final purification; Lane 5, whey milk before immunoaffinity chromatography; Lane 6, whey milk after immunoaffinity chromatography; Lane 7, whey milk after final purification.

comparison, in the cell extract from *E. coli* hGH represented less than 5%. Fig. 2B shows a Western blot of the same samples, which was carried out with a monoclonal antibody against hGH. A faint band corresponding to hGH cleavage products could be observed, being the proportion of cleaved products similar between bacterial cell extracts and bovine milk serum. Peptidic mapping of hGH from milk gave an identical result to that of the hormone produced by bacterial culture (data not shown).

In order to initiate a production herd, we have cloned two calves from ear fibroblasts of the founder cow. PCR and Southern blots analysis showed a genomic profile identical to that of the donor cow. In addition, after

standard procedures of superovulation and artificial insemination, we have recently obtained four calves from the founder cow (three males, one female), all of which bear the hGH transgene (Orellana, 2005). Although alterations in reproductive function have been reported in mice overexpressing hGH (Bartke et al., 1999), the production of viable pregnancies reported herein confirms the fertility of the transgenic cow.

We conclude that hGH can be produced at a large scale in the milk of transgenic cows. At present production rates, which are expected to increase when the cow reaches maturity, the annual amount of hGH produced from this single cow would be of about 24 kg, which represents a 545% increase over our yield when using conventional bacterial cultures (4.4 kg). Only about 15 animals would be required to meet the worldwide needs of this protein for the treatment of dwarfism in GH-deficient children.

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