Optimisation of growth hormone production by muscle cells using plasmid DNA

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Abstract

The production of peptide hormones by skeletal muscle tissue is a promising area of gene therapy. Skeletal muscle myogenesis can be induced *in vitro*, resulting in the fusion of mononucleate myoblasts to form multinucleate myotubes, and delivery vectors are first tested *in vitro*. C2C12 myoblasts transfected with pcDNA3-GH, which used the human cytomegalovirus (CMV) promoter, secreted immunoreactive GH with comparable biological activity to pituitary GH. Mouse myeloid leukaemia cells, which express the mouse GH receptor were used for the bioassay, and activation of these cells by GH was measured by a colorimetric microculture tetrazolium assay. Cells were incubated with a tetrazolium salt (MTS) and an intermediate electron acceptor (phenazine methosulphate, PMS), and formazan production was measured as optical density (O.D.) at 490 nm.

The efficiencies of several plasmid expression vectors were compared in differentiated and non-differentiated muscle cells, as a function of bioactive GH secreted by the transfected cells. Ten-day differentiated C2C12 myotubes transfected with pcDNA3E-GH, which used the CMV promoter and a rat myosin light chain enhancer element,

secreted significantly more biologically active GH than myotubes transfected with pcDNA3-GH (0.82 ± 0.06 vs 0.57 ± 0.05 respectively, $P < 0.001$). This was consistent with reduced CMV promoter activity in myotubes. Myoblasts transfected with pcDNA3-GH secreted more bioactive GH than 10-day transfected myotubes (1.1 ± 0.1 vs 0.77 ± 0.07 respectively). However, the responses were indistinguishable (both 1.0 ± 0.09) if both the myotubes and myoblasts had been transfected with pcDNA3E-GH. Substitution of the vector pMHLC-GH, which used a muscle-specific truncated rabbit myosin heavy chain promoter, and the myosin enhancer resulted in a marked decrease in the responses to the conditioned medium from fused myotubes compared with the vectors pcDNA3-GH and pcDNA3E-GH (0.24 ± 0.02 vs 0.57 ± 0.05 vs 0.82 ± 0.06 respectively). We concluded that the combination of CMV promoter and myosin light chain enhancer in pcDNA3E-GH had the greatest expression efficiency of the several plasmid vectors which we investigated.

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Introduction

Gene transfer into skeletal muscle is currently being developed as a method for producing proteins with potential therapeutic benefits, and a wide variety of applications have been envisaged, including treatment for muscular dystrophy (Floyd *et al.* 1997), motor neurone diseases (Haase *et al.* 1996) and peripheral vascular disease (Takeshita *et al.* 1994). There have been some promising results from clinical trials using muscle gene transfer as an instrument of therapeutic drug delivery. For instance, significant clinical improvements were observed in a recent phase 1 clinical trial, where plasmid DNA encoding

vascular endothelial growth factor had been injected into the limb muscles of patients with critical limb ischaemia. New collateral blood vessel growth, improved distal blood flow and healing of ischaemic ulcers were observed, resulting in successful limb salvage in 3 patients recommended for below-knee amputation (Baumgartner *et al.* 1998).

Recent studies have shown that skeletal muscle can be adapted for use as an artificial endocrine tissue (reviewed in MacColl *et al.* 1999), and this methodology has been used to produce physiologically active leptin (Murphy *et al.* 1997) and erythropoietin (Tripathy *et al.* 1994). Additionally, gene transfer into muscle has been used to

Figure 1 Plasmid vectors used to produce recombinant GH in C2C12 myoblasts and myotubes. 1, pcDNA3-GH; 2, pcDNA3E-GH; 3, pMHLC-GH. The construction of each vector is described in the Materials and Methods section and the relevant sequences are shown here (not to scale): human cytomegalovirus immediate early promoter (pCMV), rabbit myosin heavy chain promoter (pMHC), rat myosin light chain enhancer (MLCE), rat growth hormone complementary DNA (RGH cDNA), bovine growth hormone polyadenylation signal (pA), *E. coli* origin of replication (ColE1), ampicillin resistance gene (Amp).

produce the key hormones and growth factors which regulate growth. These include growth hormone releasing hormone (Draghia-Akli *et al.* 1998), growth hormone (Barr & Leiden 1991, Dahler *et al.* 1994, Anwer *et al.* 1998) and insulin-like growth factor-I (Barton-Davis *et al.* 1998).

Skeletal muscle consists of different types of multi-nucleated fibres, originating from myoblast stem cells which fuse during the embryonic development of muscle tissue (myogenesis). In postnatal life, the self-renewal and growth of differentiated muscle tissue is further mediated by a resident population of mononuclear myogenic precursors, the satellite cells. In response to muscle injury, satellite cells divide and fuse to repair or replace damaged fibres (Ferrari *et al.* 1998). The bulk of muscle cells in developed muscle tissue are, however, non dividing and gene delivery vectors are stably maintained in post-mitotic muscle fibres following intramuscular injection. This provides muscle with a considerable advantage over other tissues used for the production and secretion of therapeutic proteins, as a long-term release of protein can be achieved following gene transfer *in vivo* (Tripathy *et al.* 1996).

Myogenic differentiation can also be manipulated in culture, as myoblasts can be induced to differentiate *in vitro*. By replacing the culture medium with serum-deficient fusion media, a clear morphological change from dividing myoblasts to quiescent multinucleated myotubes occurs after several days. This allows gene transfer vectors such as plasmid DNA molecules to be tested in differentiated muscle cells *in vitro*, before advancing to *in vivo* studies (Dahler *et al.* 1994, Novo *et al.* 1997).

Here, we have tested several plasmid DNA vectors encoding rat growth hormone, in transfected, cultured rodent muscle cells. The plasmid vectors used for these studies contained viral or muscle-specific promoters, or a combination of both types (Fig. 1), and murine C2C12

myoblasts (Blau *et al.* 1985) were used to analyse each plasmid transfect *in vitro*. Initially, the bioactivity of recombinant rat growth hormone produced by transfected C2C12 myoblasts was compared with pituitary-derived rat growth hormone (GH), using a somatogenic bioassay. The target cells used for this bioassay were mouse myeloid leukaemia cells, which were producing the murine growth hormone receptor (Rowlinson *et al.* 1996). A microculture tetrazolium assay (MTA) was used to measure activation of myeloid cells by growth hormone (described by Goodwin *et al.* 1996). The bioassay was used to compare the amounts of recombinant rat GH produced by undifferentiated myoblasts and by differentiated myotubes, as a function of each plasmid transfection (MacColl *et al.* 1998).

Materials and Methods

Synthesis of rat GH cDNA

Materials Total pituitary tumour RNA was provided by Dr D Flavell, Division of Neurophysiology, National Institute of Medical Research, London, UK. The first strand cDNA synthesis kit was supplied by Clontech Laboratories UK Ltd (Basingstoke, UK) and Taq DNA polymerase by Life Technologies Ltd (Paisley, UK). Polymerase chain reaction (PCR) oligonucleotides were synthesised by Oswell DNA Service, Southampton, UK. Electrophoresis grade agarose and ethidium bromide were supplied by Sigma-Aldrich Company Ltd (Gillingham, UK).

Reverse transcription (RT)-PCR

A rat growth hormone complementary DNA (GH cDNA) was made from total rat pituitary RNA by RT-PCR. First strand cDNA was primed with an oligomeric dT primer and synthesised using avian myeloblastosis virus (AMV) reverse transcriptase, according to the manufacturer's instructions. PCR oligonucleotides were based on the published GH cDNA sequence (Seeburg *et al.* 1977) and each PCR ampimer contained an *SalI* restriction endonuclease site (underlined) to facilitate cloning of the PCR product in each of the three expression vectors used in these studies. The PCR oligonucleotides were designed to make a product containing the entire open reading frame and the 3' untranslated region (UTR) of the GH cDNA. A 5' UTR was provided by each of the GH expression vectors. The primers used were:

forward 5': CCGTCGACATGGCTGCAGACTCTCA GACTCCC and
reverse 3': CCGTCGACATTAGGACAAAGTGTAGG GGTGGC.

PCR amplification of the cDNA was carried out with a Techne thermal cyler, using Taq DNA polymerase

according to the manufacturer's instructions. The conditions used for the PCR were 95 °C for 1 min, followed by 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and then 72 °C for 5 min. Five percent of the PCR reaction was run on a 0.7% w/v agarose gel, and a product of approximately 730 base pairs was visualised by staining the gel with ethidium bromide, as described in Sambrook *et al.* (1989). The remainder of the solution containing the PCR product was purified through a silica-gel membrane (Qiagen Ltd, UK, Crawley, UK).

GH vector construction

Materials Sall, XhoI, BamHI and BglII restriction endonucleases and T4 DNA ligase were supplied from Boehringer Mannheim UK Ltd (Lewes, UK). The plasmid pcDNA3 was supplied from Invitrogen (Groningen, Netherlands). Subcloning efficiency competent DH5 α cells were supplied by Life Technologies Ltd.

Methods Five micrograms of the PCR product were enzymatically digested for 24 h with 100 units Sall restriction endonuclease, and purified as described above. To make pMHLC-GH (Fig. 1), the 729 base pair product containing the GH cDNA was ligated into p β PASe9 (Miller *et al.* 1995), which had previously been digested with Sall. This vector contains a truncated rabbit myosin heavy chain beta cardiac promoter (Cribbs *et al.* 1989), and a rat myosin light chain enhancer (Donoghue *et al.* 1988), both of which have specific activity in differentiated muscle cells. The second vector, pcDNA3-GH, used a human cytomegalovirus (CMV) immediate early promoter to drive GH expression in muscle cells (Fig. 1). pcDNA3-GH was made by removing the GH cDNA from pMHLC-GH by Sall restriction digest, and by ligating to pcDNA3 digested with XhoI. The third vector, pcDNA3E-GH (Fig. 1), was made in two steps: first, the 920 base pair sequence containing the myosin light chain enhancer was removed from p β PASe9, using BamHI restriction endonuclease, and this was ligated into pcDNA3 digested with BglII to make pcDNA3E. Secondly, the 729 bp Sall DNA fragment containing the GH cDNA was ligated to pcDNA3E, which had been digested with XhoI, to make pcDNA3E-GH.

T4 DNA ligase was used for all DNA ligations, according to the manufacturer's instructions. Competent DH5 α *E. coli* cells were used to screen ligation reactions for recombinant DNA vectors, as described by Sambrook *et al.* (1989). Plasmid DNA, used for sequencing and muscle cell transfections, was prepared from transformed DH5 α cells by alkaline lysis and anion exchange chromatography, using a Maxi prep kit (Qiagen Ltd UK) and following the manufacturer's instructions. The PCR product was sequenced for insert orientation and for open reading frame fidelity using the method described by Sanger

et al. (1977), with a Sequenase version 2.0 kit (Amersham-Pharmacia Ltd).

In vitro muscle cell culture and transfections

Materials C2C12 myoblasts were obtained from the American Tissue Culture Collection (ATCC-CRL-1772; ATCC, Rockville, MD, USA); lipofectamine, fetal bovine serum (FBS), horse serum (HS), phosphate buffered saline (PBS), Optimem, and Dulbecco's modified Eagle's medium (DMEM) with glutamax were obtained from Life Technologies Ltd (Paisley, Strathclyde, UK). Corning 75 cm² vented flasks, 6 well tissue culture plates, Gentamycin and Ampicillin from Sigma Aldrich Ltd.

C2C12 myoblast and myotube cultures

Frozen C2C12 mouse myoblasts (Blau *et al.* 1985), were thawed rapidly at 37 °C, transferred into 15 ml growth medium (DMEM+glutamax containing 10% FBS and 0.5% gentamycin/ampicillin) in a 75-cm² vented flask, and medium was changed once the cells had adhered. Myoblasts were routinely grown to between 70–80% confluence, and split 1:20 every 2 days. Myoblasts were induced to fuse into myotubes by replacing growth medium with fusion medium, where 5% HS was used as the serum supplement, and fresh medium was added every 2 days. Differentiation of cells was established visually, as myoblast fusion into myotubule-like syncytia (Barnhart *et al.* 1998). Cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

Transfection of C2C12 myoblasts

A modified version of the method described by Trivedi and Dickson (1995) was used to transfect C2C12 myoblasts. Initially, a mixture of cationic liposome (lipofectamine) and a plasmid containing the chloramphenicol acetyl transferase (CAT) reporter gene (pcDNA3-CAT) was used to optimise the transfection of C2C12 myoblasts. Cells were lysed for 24 h after transfection and assayed for CAT activity following the method described by Gorman *et al.* (1982). This optimised method was used to transfect myoblasts with each of the GH expression plasmids. Dividing myoblasts were seeded in 6-well dishes (2 ml) at a final density of 7.5×10^4 cells/ml, and grown for 18 h. Lipofectamine (10 μ g) and plasmid DNA (2 μ g) were mixed together in 1 ml serum-free medium (Optimem), and incubated for 45 min at room temperature. Cultures were washed 3 times in PBS to remove serum, and overlaid with lipofectamine/DNA complexes. Each transfection was incubated in 5% CO₂/95% air at 37 °C for 5 h, and stopped by adding fusion medium. Transfected myoblasts were either grown for 48 h before analysis of rat

growth hormone production, or left over a 10-day period to reach confluence and to differentiate into myotubes.

Western blot for recombinant rat GH

Materials Affinity-purified rat GH and primary antibody to rat GH (rhesus anti-rat growth hormone, RGH-IC1) were obtained from the National Hormone and Pituitary Program (NHPP, Harbour-UCLA Medical Center, Torrance, CA, USA), USA. Microconcentrators with a 5 kDa molecular mass cutoff were obtained from Vivascience, Lincoln, UK Ltd and prestained molecular weight markers were obtained from New England Biolabs Inc., Hitchin, UK. All electrophoresis reagents, Tween-20, rabbit anti-monkey IgG alkaline phosphatase conjugate, EDTA, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) developing reagent were obtained from Sigma-Aldrich; Hybond-C nitrocellulose membrane was purchased from Amersham-Pharmacia Biotech Ltd, St Albans, UK.

Western transfer and detection of rat GH

Forty-eight hours after transfection, conditioned medium (500 µl) was removed from myoblasts, transferred to a microconcentrator and centrifuged at 13 000 g for 15 min. Cell lysates were prepared as described by Novo *et al.* (1997), and concentrated. Protein concentrates from each transfection were separated under reducing conditions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described in Sambrook *et al.* (1989). Protein molecular weight markers, rat growth hormone standard and protein concentrates were denatured at 80 °C for 10 min in 1 × sample loading buffer, and loaded onto a discontinuous polyacrylamide gel with a 4% acrylamide stacking gel and a 12% resolving gel. Each gel was run for 1 h with a constant current of 60 mA per gel.

Protein was transferred to Hybond-C membranes using the method described in Harlow and Lane (1988). Membranes were washed once in PBS and incubated overnight at 4 °C in blocking solution: PBS containing 5% w/v fat free milk powder (Marvel), and 0.01% v/v Tween 20. Membranes were incubated at 4 °C for 2 h with primary antibody (RGH-IC1) diluted 1 in 200 in blocking solution, and then washed 3 times in PBS containing 0.01% v/v Tween 20. Second antibody (rabbit anti-monkey IgG alkaline phosphatase conjugate) was diluted 1 in 2000 in blocking solution, and incubated with membranes at room temperature for 1 h. Membranes were then washed 3 times in 0.01% Tween 20 in Tris-buffered saline (pH 8). Alkaline phosphatase activity was detected by incubating nitrocellulose membranes with 5 ml developing reagent, containing BCIP and NBT. Reactions were stopped by adding 20 mM EDTA, pH 8.

Somatogenic bioassay for rat GH

Materials The creation of murine myeloid leukaemia cells that stably express the murine growth hormone receptor (FDCP1-MGHR) has been described previously (Rowlinson *et al.* 1996). RPMI-1640 was obtained from Life Technologies Inc. Penicillin, streptomycin, L-glutamine, Corning 25 cm² tissue culture flasks and 96 well microculture dishes were obtained from Sigma-Aldrich Company Ltd. Human pituitary growth hormone (hGH – IS 80/505) was provided by the National Institute for Biological Standards and Control (South Mimms, Herts, UK). 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulphophenyl) tetrazolium inner salt (MTS) and phenazine methosulphate (PMS) were obtained from Promega UK Ltd (Southampton, UK).

Cell culture FDCP1-MGHR cells were maintained in suspension culture, in 25-cm² flasks with growth medium consisting of RPMI-1640 medium without phenol red containing 10% FBS, 100 U penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine and 0.1 µg/ml human growth hormone. Myeloid cells were routinely passaged every 3 days, and cultures were incubated in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

Microculture tetrazolium assays

A microculture tetrazolium assay (MTA) was used to measure the metabolic activation of FDCP1-MGHR cells by growth hormone. Cells were resuspended in assay medium without hGH, with 10% HS used as the serum supplement. Cells were dispensed into 96-well microculture dishes (50 µl) at a final density of 8×10^5 cells/ml and activated with 50 µl aliquots of assay medium containing either recombinant rat GH derived from transfected muscle cells, or known amounts of pituitary-derived rat GH. For the comparison between recombinant rat growth hormone and pituitary standard, conditioned media from three separate transfections, shown by Western blot to contain immunoreactive GH, were pooled and serially diluted in assay medium before adding to FDCP1-MGHR cells. Assay plates were then incubated for 24 h in a humidified, gassed chamber at 37 °C.

MTS tetrazolium salt and PMS, an intermediate electron acceptor, were used for the colorimetric MTA (Goodwin *et al.* 1996). Twenty microlitres of a solution containing an optimised mixture of MTS+PMS (4.1 mM MTS+0.15 mM PMS) were added to assay wells and mixed. Each assay plate was then incubated in a dry, non-gassed incubator at 37 °C for 2 h. The quantity of soluble formazan produced by bio-reduction of MTS was determined by measuring optical density (O.D.) at 490 nm. Each sample was assayed in triplicate, and O.D. values expressed as the mean ± s.e.m. Significant

Figure 2 Western blot of recombinant growth hormone produced by transfected C2C12 myoblasts. Conditioned medium was removed from undifferentiated C2C12 myoblasts 2 days after transfection with either pcDNA3 or pcDNA3-GH. Three independent transfections were analysed and immunoreactive recombinant rat GH was detected using RGH-IC1 antisera, as described in the Materials and Methods section. Each replicate transfection is shown. Lane 1, 50 ng rat pituitary GH standard; lanes 2, 5, 8, conditioned media from C2C12 myoblasts transfected with pcDNA3; lanes 3, 6, 9, cell extracts from C2C12 myoblasts transfected with pcDNA3-GH; lanes 4, 7, 10, conditioned media from C2C12 myoblasts transfected with pcDNA3-GH.

differences between mean O.D. values were determined using a Student's *t*-test, with significance set as $P \leq 0.05$.

Results

Immunoreactivity of rat GH produced by C2C12 myoblasts

Undifferentiated C2C12 myoblasts produced immunoreactive recombinant rat growth hormone 2 days after transfection with pcDNA3-GH, and Fig. 2 shows a Western blot of conditioned media from 3 separate transfections. These contained recombinant protein which co-migrated at 22 kDa with pituitary-derived rat growth hormone. A more sensitive method of chemiluminescent

detection also demonstrated that rat GH was also present in cell extracts (data not shown). Rat GH immunoreactivity could not be detected using these methods, either in control C2C12 myoblasts or in cells transfected with plasmid without the GH cDNA (pcDNA3).

Bioactivity of recombinant rat GH

The conditioned media from C2C12 myoblasts transfected with pcDNA3-GH stimulated FDCP1-MGHR myeloid cells, which express the murine GH receptor, as measured by the MTA assay (Fig. 3). Hormonal specificity of these responses to the pooled media was demonstrated by the abolition of the bioactivity by preincubation with an

Figure 3 Stimulation of FDCP1-MGHR cells by rat GH produced by transfected C2C12 myoblasts. Conditioned media, containing immunoreactive rat GH from 3 separate transfections of C2C12 myoblasts, were pooled and assayed for somatogenic activity. Serial dilutions of pooled media, taken from undifferentiated myoblasts 48 h after transfection with pcDNA3-GH, were used to activate target cells in the colorimetric somatogenic bioassay, as described in the Materials and Methods section. Hormonal activation was quantified by the increased O.D. (490 nm) due to formazan production by FDCP1-MGHR target cells. Negative controls were run as described in the text. In addition, a dose-response curve to affinity purified rat pituitary GH standard was run in parallel in this bioassay. For both GH preparations, each dilution was tested in replicate wells ($n=3$) and all responses are expressed as mean O.D. $\pm$ S.E.M.

Figure 4 (a) Comparison between plasmid vector activities in undifferentiated C2C12 myoblasts, and in differentiated C2C12 myotubes. Conditioned media were harvested from cultures of undifferentiated C2C12 myoblasts 2 days after transfection, or from differentiated C2C12 myotubes 10 days after transfection, and tested for rat GH production using the somatogenic bioassay. The transfection variants were: 1, untransfected myotubes; 2, myotubes transfected with pcDNA3; 3, myoblasts transfected with pcDNA3-GH; 4, myotubes transfected with pcDNA3-GH; 5, myoblasts transfected with pcDNA3E-GH; 6, myotubes transfected with pcDNA3E-GH. Transfected myoblasts were induced to differentiate into myotubes, as described in the Materials and Methods section. Transfections were repeated three times and the conditioned medium from individual experiments were assayed for GH bioactivity in replicate wells ($n=3$). All responses are expressed as mean O.D. $\pm$ S.E.M., * $P<0.001$ for variant 4 compared with variant 3. (b) Comparison between 3 plasmid expression vectors in 10-day cultures of transfected C2C12 myotubes. The transfection variants were: 1, untransfected myotubes; 2, myotubes transfected with pcDNA3-GH; 3, myotubes transfected with pcDNA3E-GH; 4, myotubes transfected with pMHLG-GH. Transfected myoblasts were induced to differentiate into myotubes as described in the Materials and Methods section. For each plasmid, conditioned medium from 6 separate transfections was tested for rat GH bioactivity in replicate cultures of FDCP1-MGHR target cells ($n=3$) and all responses are expressed as mean O.D. $\pm$ S.E.M. * $P<0.001$ for variant 3 compared with variant 2.

antibody raised against the native hormone (results not shown). No stimulation was observed with the media harvested from negative controls of untransfected C2C12 myoblasts, or from cells that had been transfected with pcDNA3 only. A comparison between dose-response curves to the secreted recombinant rat GH and rat pituitary-derived GH standard, revealed essentially parallel response increments (Fig. 3).

Comparison of vector efficiency in muscle cells

The bioassay was used to compare the levels of recombinant rat GH secreted into conditioned medium by undifferentiated C2C12 myoblasts and fused myotubes, which had been transfected with plasmid GH expression vectors containing different combinations of viral and muscle promoter elements (Fig. 4a and b). Figure 4a shows the comparison of CMV promoter activity in both cell types. Ten days after transfection with pcDNA3-GH, the bioassay response to the rat GH secreted from differentiated myotubes was significantly less than that secreted from undifferentiated myoblasts (0.77 ± 0.07 vs 1.1 ± 0.1 respectively, $P<0.001$). These responses were indistinguishable (both 1.0 ± 0.09) if myotubes and myoblasts were instead transfected with pcDNA3E-GH, which included both the CMV promoter and the myosin light chain enhancer element (Fig. 4a).

In a separate series of transfections, we compared the efficiencies of different plasmids in 10-day cultures of transfected C2C12 myotubes. We confirmed that when myotubes had been transfected with pcDNA3E-GH they produced significantly more GH than myotubes transfected with pcDNA3-GH (0.82 ± 0.06 vs 0.57 ± 0.05 , $P<0.001$) (Fig. 4b). However, if the plasmid vector pMHLG-GH was substituted, there was a marked decrease in the responses to the conditioned medium from fused myotubes, compared with pcDNA3-GH and pcDNA3E-GH (0.24 ± 0.02 vs 0.57 ± 0.05 vs 0.82 ± 0.06 respectively) (Fig. 4b). This vector contained the myosin heavy chain promoter and myosin light chain enhancer element. We concluded that, since the responses observed were a direct function of the GH secreted, the combination of CMV promoter and myosin light chain enhancer in pcDNA3E-GH had the greatest expression efficiency of the vectors which we investigated.

Discussion

The aim of this study was to compare the relative amounts of recombinant rat GH produced by three types of plasmid DNA vector in differentiated C2C12 myotubes and in undifferentiated C2C12 myoblasts, with a view to developing one or more of these for delivering GH *in vivo*.

Immunoreactive recombinant rat GH was secreted by C2C12 myoblasts transfected with pcDNA3-GH, and this had the same molecular weight as pituitary-derived rat GH. The somatogenic bioassay was then used to demonstrate that the immunoreactive protein had a comparable biological activity to native rat GH. Both recombinant and pituitary-derived GH generated similar dose-response curves over the same dilution range, when incubated with FDCP1-MGHR cells.

Using this bioassay to measure GH secretion as a function of promoter activity, the efficiency of the CMV promoter was then compared in both transfected C2C12 myoblasts and differentiated myotubes. We found that myotubes transfected with pcDNA3-GH produced significantly less biologically active GH when compared with myoblasts transfected with the same construct. However, when the CMV promoter was combined with a muscle-specific enhancer in the vector pcDNA3E-GH, equivalent levels of bioactive GH were secreted by transfected C2C12 cells irrespective of the state of differentiation of these muscle cells.

The decrease in the levels of GH secreted, following differentiation of transfected myoblasts into myotubes, and the subsequent attenuation of this effect when the CMV promoter was combined with the myosin light chain enhancer element in pcDNA3E-GH, was consistent with our current understanding of several of the cellular processes that control gene expression during myogenesis. First, the reduction in GH secretion observed in myotubes transfected with pcDNA3-GH may have been caused by the decrease in binding activity of the activator protein-1 (AP-1) transcriptional activator, which occurs following the induction of myogenesis *in vitro* (Lehtinen *et al.* 1996). AP-1 is a heterodimer consisting of Fos and Jun protein monomers, which specifically activates promoters that contain the binding site TGACTCA, including the CMV promoter used in pcDNA3-GH and pcDNA3E-GH (Lehtinen *et al.* 1996).

The difference between the levels of GH secreted by myotubes transfected with pcDNA3-GH, as opposed to pcDNA3E-GH must be due to the myosin light chain enhancer. This enhancer is activated specifically in differentiated C2C12 myotubes, but not in dividing myoblasts (Donoghue *et al.* 1988), as it contains several E box (CANNTG) and A/T rich (CTA)_n(T)_n DNA motifs. These sites bind to the myogenic differentiation factor (MDF) and myocyte enhancer families of transcription factors, which are expressed during muscle cell differentiation (Neville & Rosenthal 1996). The observations indicate that the myosin light chain enhancer compensates for the reduction in CMV promoter activity, which occurs following differentiation of transfected muscle cells.

In comparison with the plasmids using the CMV promoter, pMHLG-GH generated much lower levels of GH in transfected C2C12 myotubes. This vector used a truncated myosin heavy chain promoter in combination

with the myosin light chain enhancer element, and the results were consistent with previous observations where these vectors were used for the expression of human alpha-galactosidase (Novo *et al.* 1997). However, we have also shown that the promoter elements used in pMHLG-GH generate tissue-specific gene expression in muscle fibres (Skarli *et al.* 1998) and as other studies show that specific types of muscle promoters are effective at producing hormones in muscle cells (Barton-Davis *et al.* 1998, Li *et al.* 1999), this vector is being studied further.

In conclusion, the amount of biologically active growth hormone produced by transfected C2C12 myotubes was found to depend on the types of promoter element used in each plasmid vector, and a combination of viral and muscle-specific transcription elements proved to be the most effective. However, it is also recognised that plasmid vectors which are effective in cultured muscle cells, may not be as potent when used for gene transfer into skeletal muscle (Barnhart *et al.* 1998), and therefore these conclusions may not apply to *in vivo* situations.

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