Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 304 (2003) 167–175
BBRC
www.elsevier.com/locate/ybbrc

Tetracycline-regulated secretion of human insulin in transfected primary myoblasts

Kathleen T. Scougall and James A.M. Shaw*

Diabetes Research Group, School of Clinical Medical Sciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

Received 27 February 2003

Abstract

A mechanism for safely regulating transgene expression will be necessary for gene therapy approaches to endocrine disorders. In this study, a two-plasmid tetracycline-inducible system was used to regulate expression of human proinsulin (hppI1) and a mutated proinsulin construct (hppI4, allowing cleavage by furin) in primary rat soleus myoblasts. In hppI1 and hppI4 transient transfections, the presence of 0.01 and 0.1 μg/ml tetracycline for 48 h inhibited pro/insulin secretion to 19–27% and 7–12%, respectively, compared to tetracycline untreated myoblasts. Following a 48 h tetracycline incubation (1.0 μg/ml), pro/insulin secretion in hppI1 and hppI4 transfected myoblasts was reduced to <4% of that in cells incubated without tetracycline. Pro/insulin secretion equivalent to that of untreated cells was restored following tetracycline withdrawal and incubation for a further 72 h. Conversion of proinsulin to insulin in transfected myoblasts was <1% for hppI1 and >45% for hppI4. In conclusion, regulated insulin secretion has been achieved in a dose-dependent and reversible manner in primary myoblasts.

Keywords: Muscle; Myoblast; Tetracycline; Proinsulin; Insulin; Plasmid DNA; Regulated secretion; Primary culture; Diabetes; Gene therapy

Skeletal muscle is an attractive candidate tissue for in situ or ex vivo gene delivery. It is abundant, easily accessible, and can mediate transgene expression for up to 19 months following plasmid injection [1]. The potential of muscle tissue for gene therapy has been demonstrated for erythropoietin [2], growth hormone [3], leptin [4], factor IX [5] and insulin replacement. Constitutive secretion of processed human insulin has been demonstrated in rodents following simple intramuscular injection with a preproinsulin plasmid modified for furin recognition [6–8]. A mechanism for safely regulating secretion following plasmid-mediated transfection would greatly enhance the suitability of this approach for therapeutic use. This will be particularly important for insulin replacement in diabetes providing a mechanism for preventing dangerous hypoglycaemia.

Gene expression can be regulated at the level of transcription. Several small molecule transcriptional regulatory systems have been developed including those based on ecdysone [9], FK506/rapamycin [10], RU486/mifepristone [11], and tetracycline [12]. The small molecules reversibly bind to modified transcription factors inducing or repressing transgene transcription. The tetracycline system has several advantages: (1) tetracycline, an antibiotic, has been well characterised for clinical use; (2) the doses required to regulate transcription are not toxic; (3) the integral regulatory elements employed are prokaryotic and will not interfere with host mammalian transcription machinery [13]; and (4) efficient regulation of reporter gene expression has been demonstrated following plasmid-mediated gene transfer to muscle in vivo [14].

In this study we have used a tetracycline-responsive promoter to demonstrate that insulin secretion can be controlled in a dose-responsive and reversible manner in transfected primary myoblasts. This approach offers a safe and robust mechanism for regulating therapeutic protein levels in muscle-targeted gene therapy.

Materials and methods

Cell culture reagents were purchased from Invitrogen (Paisley, UK) and chemicals were purchased from Sigma–Aldrich (Poole, UK) and

* Corresponding author. Fax: +44-191-222-0723.
E-mail address: jim.shaw@ncl.ac.uk (J.A.M. Shaw).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00553-9

BDH (Poole, UK) unless otherwise stated. Restriction and DNA modification enzymes were purchased from Promega (Southampton, UK). Plasmids pTRE, pTet-off, and CMV-β were obtained from Clontech (Basingstoke, UK) and pCR3 from Invitrogen.

Plasmid construction. Wild-type (hppI1) and mutant (hppI4, in which the PC2 and PC3 dibasic cleavage sites have been mutated to furin consensus cleavage sites) preproinsulin inserts have been described previously [8]. HppI1 and hppI4 cDNAs were ligated into pTRE using *Eco*RI sites to give pTRE-hppI1 and pTRE-hppI4, respectively. The β-galactosidase gene was excised from construct CMV-β by *Not*I digestion and sub-cloned as a *Not*I fragment into pCR3. Following pCR3-β digestion with *Eco*RI/*Xba*I, the β-galactosidase insert was sub-cloned into equivalent digested pTRE (pTRE-β). All constructs were characterised by restriction digests and purified by DNA columns (Qiagen, Crawley, UK).

Cell cultures. Rat hind limb soleus muscles were removed from adult male Sprague–Dawley rats. Connective tissue was removed and the muscle was dissociated mechanically by scalpel blade and then enzymatically by incubation with collagenase (Type-I, 2.5 mg/ml, Sigma) for 40–60 min at 37 °C. The muscle slurry was pipetted several times in Hams-F10 nutrient mixture supplemented with 20% fetal bovine serum (FBS), 2.5 ng/ml basic fibroblast growth factor (bFGF, Promega), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Fungizone was used to supplement initial medium at a concentration of 2.5 μg/ml and then reduced to 0.5 μg/ml through subsequent feedings. The resuspended muscle solution was dispensed into 6-well plates (1 muscle/plate), pre-coated with Matrigel solution (diluted 1:25, Matrigel:DMEM, Becton–Dickinson, Oxford, UK). Cells were grown in a humidified incubator at 37 °C in 5% CO_2 and fed every 2–3 days.

Transfection and tetracycline addition. Myoblasts were transfected using 1 μg of each cDNA with Transfast (Promega) according to manufacturer's guidelines and a DNA:lipid ratio of 1:1. Stock tetracycline (10 mg/ml, Sigma–Aldrich) was made up in 70% ethanol. Further dilutions were performed in growth medium to give final concentrations ranging from 0.0001 to 1 μg/ml. After transfection, cells were rested for 24 h before addition of tetracycline.

Insulin and proinsulin ELISA. ELISA for human intact proinsulin was purchased from DAKO (DakoCytomation, Ely, UK) and human proinsulin and specific insulin kits were purchased from Mercodia (Uppsala, Sweden). Assays were performed according to manufacturer's instructions. Cross-reactivities for the ultrasensitive human insulin ELISA were <0.01% for C-peptide, <0.01% for proinsulin, 98% for proinsulin des (64–65), 56% for proinsulin split (65–66), <0.5 for proinsulin des (31–32), and <0.5% for proinsulin split (32–33). Cross-reactivities for the human proinsulin ELISA were <0.03% for insulin, <0.006% for C-peptide, 84% for proinsulin des (64–65), 90% for proinsulin split (65–66), 95% for proinsulin des (31–32), and 95% for proinsulin split (32–33). DAKO human intact proinsulin ELISA cross-reacts 66% with 65–66 split proinsulin but not with insulin or 32–33 split proinsulin [15]. Cross-reactivities are for human peptides.

Immunocytochemistry. Muscle slurry was plated onto Matrigel-coated coverslips in 6-well plates. Cells were fixed using 5% (v/v) acetic acid/ethanol for 10 min at room temperature and then at −20 °C for 20 min. All further procedures were carried out at room temperature. Non-specific antibody binding was blocked using blocking buffer (2% (w/v) BSA, 0.2% (v/v) Tween 20, and 6.7% (v/v) glycerol in PBS) for 1 h. Monoclonal desmin primary antibody (Sigma–Aldrich) was diluted in blocking buffer (1/50) and applied to cells for 1 h. Cells were then washed four times in washing buffer (2% (w/v) BSA, 0.4% (v/v) Tween 20, and 6.7% (v/v) glycerol in PBS). Rabbit anti-mouse TRITC (tetramethylrhodamine isothiocyanate, Sigma–Aldrich) conjugate was diluted in blocking buffer (1/250) and added to cells for 1 h in darkness. After a further four washes, coverslips were mounted in Vectashield aqueous mounting medium containing DAPI (4,6-diamidino-2-phenylindole) nuclear stain (Vector Laboratories, Peterborough, UK) and analysed by digital fluorescence microscopy. Fluorescence microscopy was carried out using a Zeiss Axiolan II transmission microscope with images captured using a cooled coupled device camera and processed using Vysis/Quip software.

β-Galactosidase staining. Cells were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS for 5 min at 4 °C. Cells were washed in PBS and stained in X-gal solution (1.3 mM $MgCl_2$, 3 mM K_3 $Fe(CN)_6$, 3 mM K_4 $Fe(CN)_6$, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase, Melford Laboratories, Chelsworth, UK) in PBS) for 2–4 h at 37 °C. To visualise nuclei, Hoechst nuclear stain (Sigma–Aldrich) diluted in PBS (10 ng/ml) was applied to cells.

Protein quantification. Intracellular protein was extracted in PBS from cell pellets by freeze–thaw (3 cycles: liquid nitrogen; 37 °C water bath; vortex suspension). Following the final cycle the extracts were centrifuged at 4 °C for 10 min, 12,000*g*. Protein concentration in the supernatants was quantified by the Bradford method following manufacturer's guidelines (Bio-Rad Laboratories, Hemel Hempsteasd, UK).

RNA isolation and Northern blotting. Preproinsulin mRNA levels were analysed by Northern blotting. Total RNA was isolated from cultured cells in 1 ml of TRIZOL reagent (Invitrogen), loaded onto a 1.2% agarose gel containing formaldehyde (0.7%), and electrophoresed for 2–3 h at 65 V. The electrophoresed RNA was transferred onto a nitrocellulose N^+ membrane. Hybridisation was performed with a digoxigenin (DIG) labelled antisense RNA encoding the preproinsulin or GAPDH genes. Procedures were carried out according to manufacturer's guidelines (Roche, Lewes, UK).

Statistical analysis. Values are reported as means ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA) and Student's *t* test. A p value of <0.05 was accepted as statistically significant.

Results

Characterisation and transfection efficiency of primary myoblasts

Isolated primary rat myoblasts were characterised using immunocytochemical staining for desmin, a muscle specific marker, and through the ability of myoblasts to form multinucleated myotubes. A desmin-positive (red) myoblast surrounded by desmin-negative fibroblasts (nuclei, blue) confirms antibody specificity (Fig. 1A). Fusion of five myoblasts is illustrated, resulting in myotube formation (Fig. 1B). After optimisation, greater than 90% of cells demonstrated desmin staining, confirming the purity of myoblast cultures.

The tetracycline transcriptional regulatory system [12] requires co-transfection with pTRE and pTet-off plasmids. Plasmid pTet-off encodes a transactivator (VP16/TetR) that binds reversibly to the tetracycline-responsive element (7 copies of a 42 bp sequence of the tet operator (tetO) fused upstream of a minimal CMV promoter) within pTRE, initiating transcription.

To determine the efficiency of transient liposomal transfections in primary rat myoblasts, initial studies with β-galactosidase reporter constructs were performed. The β-galactosidase gene was sub-cloned downstream of the minimal CMV promoter in pTRE to generate plasmid pTRE-β. Co-transfections with pTRE-β/pTet-off were compared with single transfections using CMV-β, a plasmid containing the full length, constitutively active

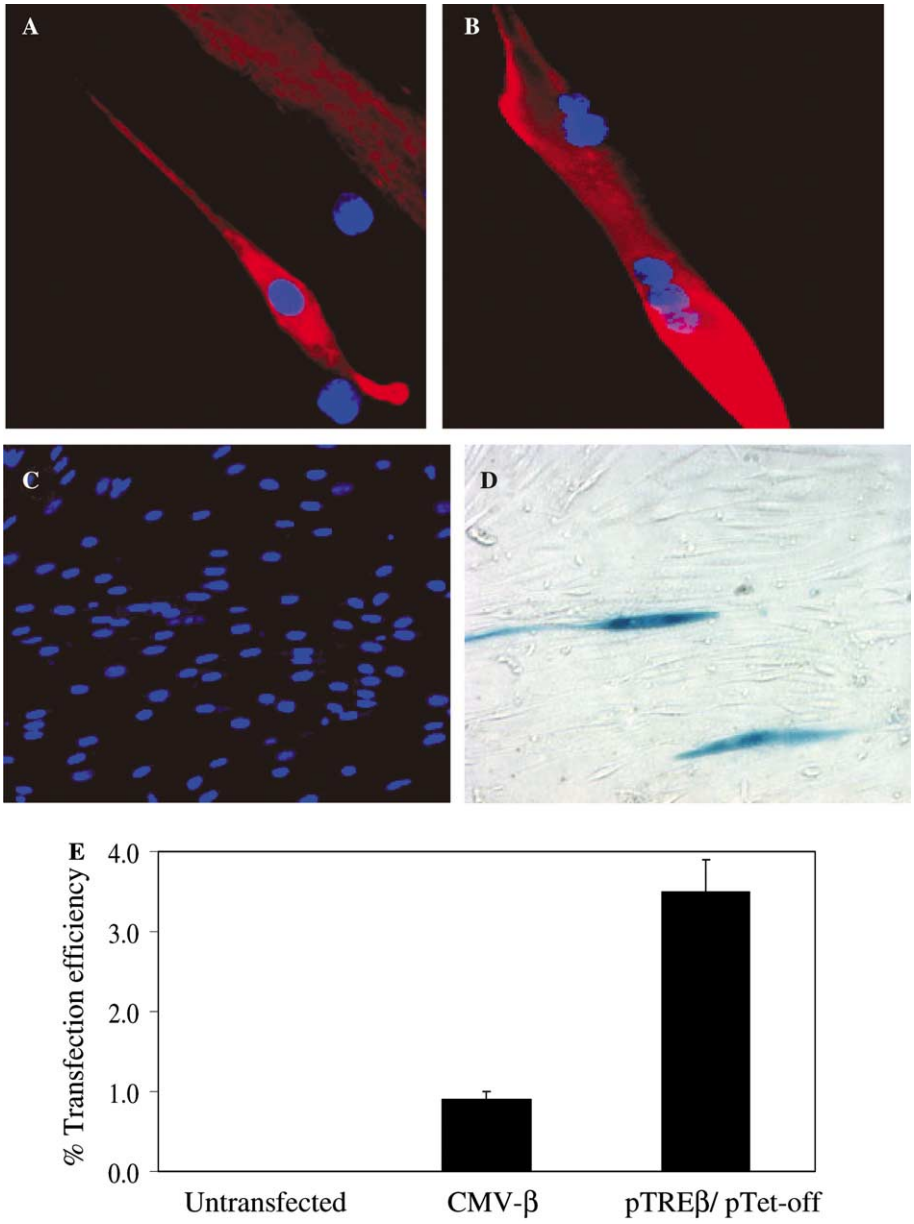

Fig. 1. Characterisation and transfection of primary rat myoblasts. Isolated myoblast cultures were positively identified using mouse anti-desmin antibodies (A) and through demonstration of cell fusion (B). Desmin (red) and DAPI nuclear (blue) staining is demonstrated in figures A and B (magnification 650×). Myoblasts were transfected with plasmids encoding transgenes for β-galactosidase (pCMV-β and pTRE-β/pTet-off). Transfected cells were fixed and stained with (C) Hoechst nuclear stain and (D) X-gal, 48 h post-transfection (magnification 200×). (E) The number of X-gal and nuclear stained cells in 10 fields of view (5 central and 5 peripheral, magnification 200×) was counted for each well and the mean determined (percentage of positively transfected cells = (mean X-gal positive cells per field/mean total number of cells (measured by nuclear staining) per field) × 100 ($n = 3$, mean ± SD).

CMV promoter, and water (control). The total number of cells visualised by Hoechst nuclear stain (Fig. 1C) and the number of blue, X-gal stained cells (Fig. 1D) were counted. Transfected cells were expressed as a percentage of the total cell count in a field of view. Transfection efficiencies were 3.5% for pTREβ/pTet-off, 0.9% for CMVβ, and 0.0% for control cells. Therefore, in primary myoblasts, transfection with the two-plasmid system does not reduce the percentage of cells successfully expressing transgene in comparison to a single plasmid.

Tetracycline-regulated proinsulin secretion in transiently transfected primary myoblasts

The potential for regulated pro/insulin secretion by tetracycline administration was evaluated in myoblasts in vitro. Tetracycline reversibly binds to the transactivator preventing binding to the tetO promoter, thereby inhibiting transgene transcription. Tetracycline-responsive insulin constructs (pTRE-hppI1 and pTRE-hppI4) were studied. In cells transfected with pTRE-hppI1 or

pTRE-hppI4 alone, no significant pro/insulin (proinsulin and insulin) secretion was detected in comparison to negative control transfections (pTet-off).

Following co-transfection with pTRE-hppI1 and pTet-off in the absence of tetracycline, peak total pro/insulin secretion was 251 pmol/l/24 h (Fig. 2A). Over the first 24 h period post-transfection there was no significant difference between groups in the total amount of pro/insulin secreted (ANOVA, $p = 0.98$). Addition of 0.01, 0.1, and 1.0 μg/ml tetracycline to medium and incubation for a further 24 h (24–48 h, post-transfection) significantly reduced levels of total pro/insulin secretion to 53%, 50%, and 51% of untreated cells (100%), respectively ($p < 0.05$). This was reduced further by an additional 24 h tetracycline incubation (48–72 h, post-transfection) to 19%, 11%, and 12% of control in 0.01, 0.1, and 1.0 μg/ml tetracycline, respectively ($p < 0.05$). Incubation at 0.0001 and 0.001 μg/ml tetracycline reduced pro/insulin secretion in comparison to untreated cells (87% and 68%, respectively, at 24 h and 93% and 51%, respectively, at 48 h), although decrements were not statistically significant.

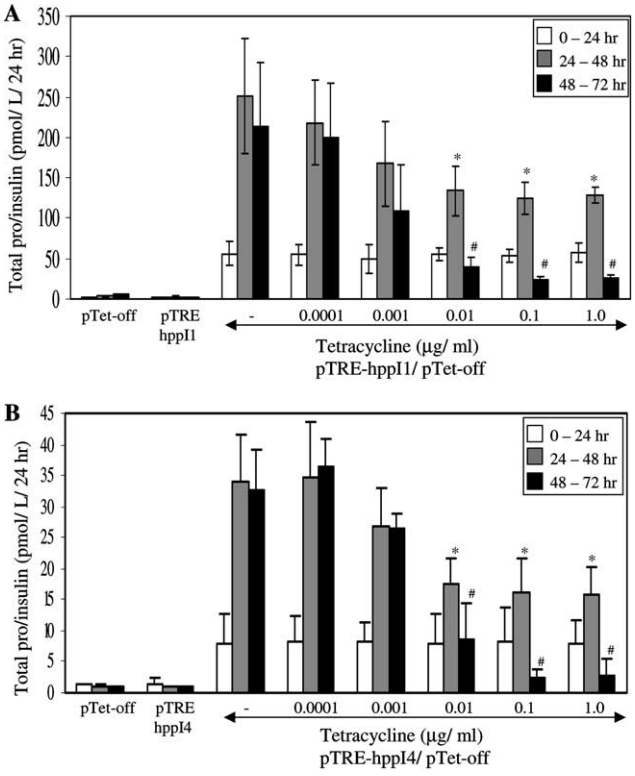

Fig. 2. Tetracycline-regulated pro/insulin secretion in primary myoblasts. Primary myoblasts were transfected with pTRE-hppI1 (A) or pTRE-hppI4 (B) alone or in conjunction with pTet-off. Medium was collected at 0–24, 24–48, and 48–72 h and tetracycline was added at 24 and 48 h. Total pro/insulin (proinsulin and insulin) results are expressed as pmol/l/24 h ($n = 3$, means ± SD). *$p < 0.05$ compared to tetracycline untreated, 24–48 h; #$p < 0.05$ compared to tetracycline untreated, 48–72 h.

A similar pattern was observed in pTRE-hppI4/pTet-off transfected cells (Fig. 2B). Following co-transfection with pTRE-hppI4 and pTet-off in the absence of tetracycline, peak total pro/insulin secretion was 34 pmol/l/ 24 h. As above, there was no significant difference between groups in the levels of total pro/insulin secreted during the first 24 h post-transfection (ANOVA, $p = 0.99$). Secreted total pro/insulin levels were reduced significantly by the addition of 0.01, 0.1, and 1.0 μg/ml tetracycline for 24 h to 52%, 48%, and 47%, respectively, of untreated control (100%, $p < 0.05$). Following maintenance in the same concentrations of tetracycline (0.01, 0.1, and 1.0 μg/ml) for an additional 24 h, levels were reduced further to 26%, 7%, and 8%, respectively ($p < 0.05$). Incubation with tetracycline for 24 or 48 h at 0.0001 μg/ml did not reduce pro/insulin secretion compared untreated cells. A tetracycline concentration of 0.001 μg/ml did reduce pro/insulin secretion (24 h, 79%; 48 h, 80%), although this was not significant.

Tetracycline addition was not associated with cell death, growth inhibition or proliferation as the intracellular protein concentration between wells of individual plates was not significantly different ($p > 0.05$, ANOVA). Intracellular proinsulin levels were also measured to determine whether tetracycline was preventing secretion. Tetracycline addition was not associated with intracellular accumulation of proinsulin and intracellular levels accounted for 3.0–8.8% and 0–5.9% of overall (secreted and intracellular) pro/insulin levels for pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off transfections, respectively. For pTRE-hppI1/pTet-off transfections intracellular proinsulin was 14.9 pmol/l in untreated cells compared to 1.0 pmol/l in tetracycline treated (1.0 μg/ml)) and for pTRE-hppI4/pTet-off co-transfections intracellular levels were 1.9 pmol/l (untreated) compared to 0.1 pmol/l (1.0 μg/ml).

Regulation of preproinsulin mRNA expression by tetracycline in transiently transfected primary rat myoblasts

To confirm that tetracycline was acting at the level of gene transcription, mRNA was analysed by Northern blotting. Myoblasts were transfected individually with plasmids pTRE-hppI1 and pTRE-hppI4 or co-transfected with pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off (Fig. 3). Transfected cells were incubated for 24 h, after which tetracycline was added in increasing amounts to give final concentrations of 0.001, 0.01, 0.1, and 1.0 μg/ml. Cells were incubated for a further 24 h after which the medium was harvested (24–48 h) and total RNA was extracted from the cell pellet. Total pro/insulin concentrations were measured by ELISA. Total RNA was analysed by Northern blotting using preproinsulin and GAPDH probes. Proinsulin mRNA was detected in pTRE-hppI1/pTet-

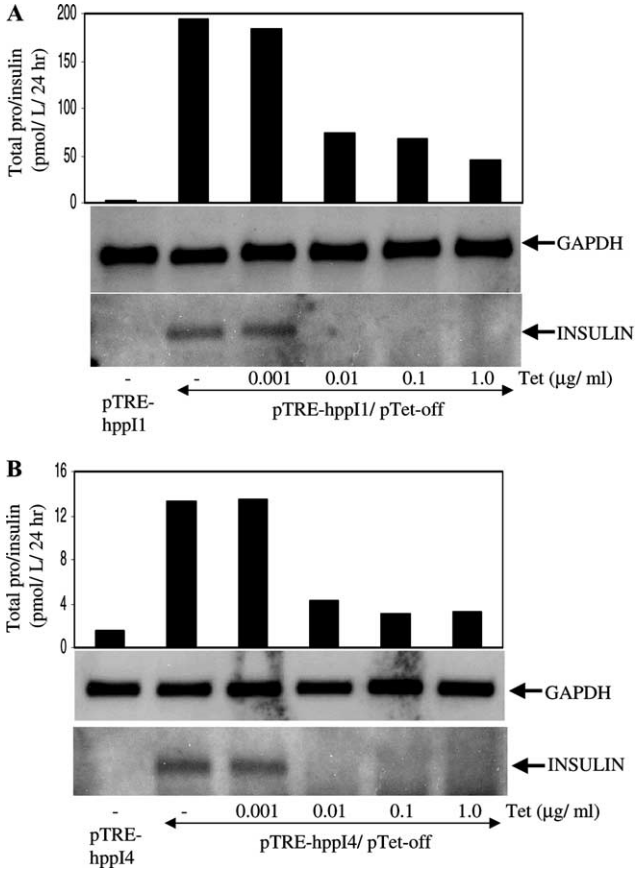

Fig. 3. Tetracycline-regulated preproinsulin transcription in primary myoblasts. Myoblasts were transfected with pTRE-hppI1 (A) or pTRE-hppI4 (B) alone and in conjunction with pTet-off. Tetracycline was added to fresh medium at final concentrations of 0.001, 0.01, 0.1, and 1.0 μg/ml, 24 h after transfection. Medium was harvested 24 h later (24–48 h), assayed by ELISA, and is expressed as total pro/insulin (proinsulin and insulin), pmol/l/24 h. Cells were lysed in TRIZOL and total RNA was analysed by Northern blotting using insulin and GAPDH probes.

off and pTRE-hppI4/pTet-off co-transfections in the absence and presence of 0.001 μg/ml of tetracycline, but not at higher concentrations (0.01–1 μg/ml) confirming transcriptional regulation.

Reversal of tetracycline inhibition in primary myoblasts

To assess reversal of tetracycline inhibition, tetracycline was added to and then removed from transfected cells. Medium was collected every 24 h and analysed for secreted proinsulin and insulin. Primary myoblasts were transfected with pTRE-hppI1/pTet-off or pTRE-hppI4/pTet-off. Transfected wells were separated, 1 day after transfection, into three groups: (1) tetracycline untreated, (2) 48 h tetracycline (1.0 μg/ml) treated, with subsequent removal or (3) continually treated with tetracycline (1.0 μg/ml). In the absence of tetracycline (group 1), total pro/insulin secretion peaked at day 3 post-transfection for both hppI1 (3167 pmol/l) and hppI4 (230 pmol/l) transfections. Thereafter total pro/insulin secretion declined daily and, at day 7, levels were reduced to 35% and 28% of peak levels at day 3 for pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off, respectively. For comparison, total pro/insulin secretion in tetracycline treated groups is expressed as a percentage of the level in untreated groups at the same time point (Figs. 4A and B). Continuous addition of 1 μg/ml tetracycline (group 3) to pTRE-hppI1/pTet-off transfected cells reduced the total pro/insulin secreted to 24, and 1% over days 2 and 3, respectively, after which a level of $<1\%$ was maintained in comparison to untreated cells. For pTRE-hppI4/pTet-off transfected cells, addition of tetracycline continuously (group 3) reduced the total pro/insulin secreted to 23% and 3% over days 2 and 3, respectively, after which a level of $<1\%$ was maintained. To group 2, tetracycline was added for 2 days before being withdrawn. Upon tetracycline withdrawal proinsulin secretion increased to 3%, 29%, 101%, and 118% for pTRE-hppI1/pTet-off transfected cells and 8%, 51%, 102%, and 101% for pTRE-hppI4/pTet-off transfected cells over days 4, 5, 6, and 7, respectively, compared to untreated cells (group 1). At the end of the study intracellular protein concentrations were quantified. There was no significant difference between groups ($p = 0.45$, ANOVA).

Analysis of proinsulin processing in transfected myoblasts

Processing of proinsulin to insulin was assessed in pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off transfected myoblasts. Levels of secreted mature insulin were compared to intact proinsulin and proinsulin (total, including split forms) over 72 h. In pTRE-hppI1/pTet-off co-transfected cells, $>75\%$ of total pro/insulin secreted remained intact with $<1\%$ processing to mature insulin (Fig. 5A). On transfection with pTRE-hppI4/pTet-off, $<1\%$ of total pro/insulin secreted remained intact proinsulin with 40–54% processed to proinsulin split forms and 46–60% processed to mature insulin (Fig. 5B).

Comparison of hppI1 and hppI4 expression levels

Levels of total pro/insulin measured in the medium of pTRE-hppI4/pTet-off co-transfected myoblasts were consistently lower than in pTRE-hppI1/pTet-off co-transfected cells. Comparison of pTRE-hppI4/pTet-off and pTRE-hppI1/pTet-off revealed no difference in proinsulin mRNA levels, despite an average 12-fold decrease in total pro/insulin secreted from pTRE-hppI4/pTet-off transfected cells (Fig. 6A). Processing to insulin is greater in pTRE-hppI4/pTet-off transfected cells. This raises the possibility that insulin in preference to proinsulin is metabolised by myoblasts or degraded by serum within the medium. Insulin degradation or metabolism would result in an underestimation of the

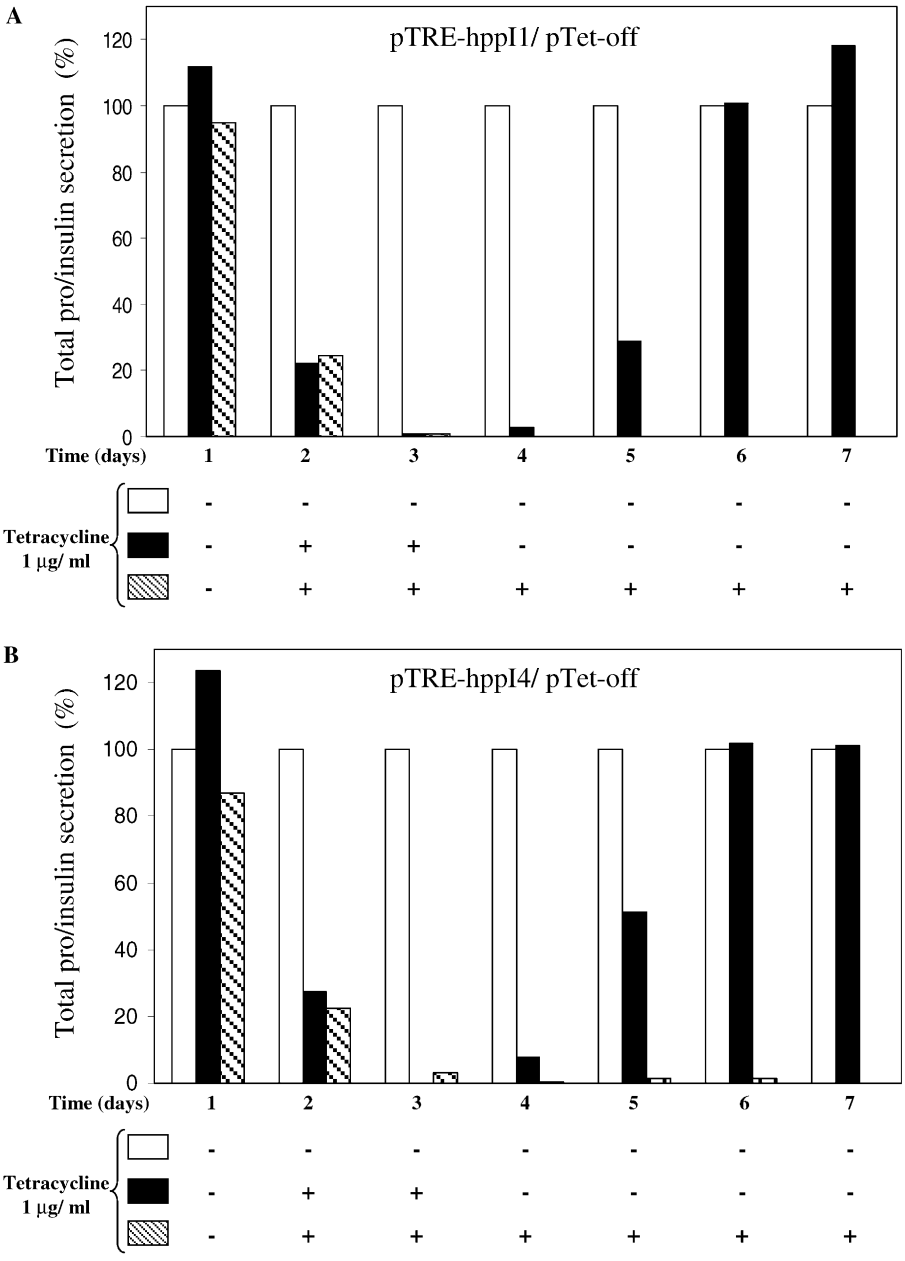

Fig. 4. Reversal of tetracycline inhibition in transfected myoblasts. Myoblasts were transfected with pTRE-hppI1/pTet-off (A) or pTRE-hppI4/pTet-off (B). Medium was replaced every 24 h following transfection. Tetracycline was omitted throughout (white bars), added during days 2 and 3 only (black bars) or added continuously after day 1 (hatched bars) to culture medium, at a final concentration of 1.0 μg/ml. Medium was assayed using ELISA. The total pro/insulin levels (proinsulin and insulin) are expressed as a percentage of those in the tetracycline untreated group ($n = 3$, means ± SD).

true insulin levels over a 24 h period, giving a possible explanation for the lower total pro/insulin levels.

Human recombinant proinsulin and insulin were diluted in complete medium and added to primary myoblasts or to an identical 6-well plate without cells. All plates were maintained in identical conditions (5% CO_2, 37 °C) for 24 h. An aliquot of spiked medium was frozen and unspiked medium without pro/insulin was analysed for background levels. Pro/insulin levels were determined by proinsulin and insulin ELISA (Figs. 6B and C). ELISA specificity was confirmed by the absence of immunoreactivity of proinsulin spiked media in the insulin ELISA and insulin spiked media in the proinsulin ELISA. Human insulin was significantly degraded by serum (32.6%, in comparison to that frozen at −20 °C) and metabolised by cells (64.3%, in comparison to that at 37 °C without cells). Overall insulin concentration had fallen to 24% of the initial level over a 24 h incubation with rat myoblasts. In contrast, there was no evidence of degradation or metabolism of human proinsulin. There

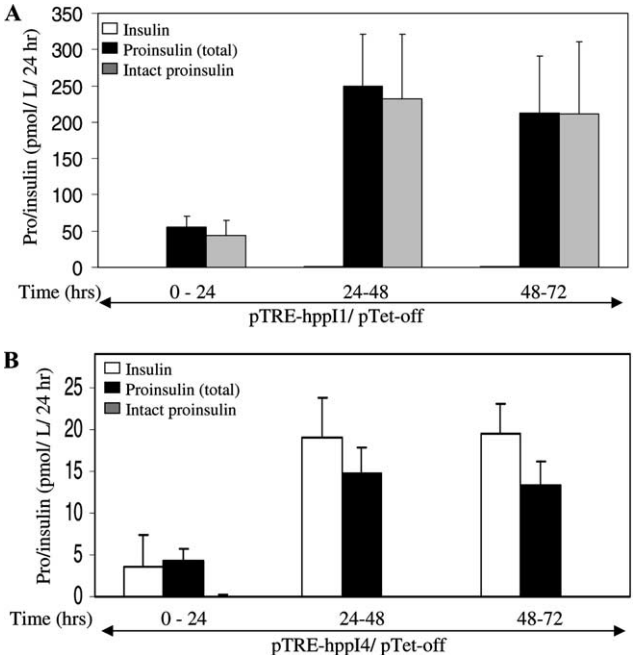

Fig. 5. Comparison of HPPI1 and HPPI4 processing. Using proinsulin, intact proinsulin, and insulin ELISA, pro/insulin secretion from pTRE-hppI1/ pTet-off (A) and pTRE-hppI4/ pTet-off (B) transfected myoblasts was compared at 0–24, 24–48, and 48–72 h post-transfection ($n = 3$, mean ± SD).

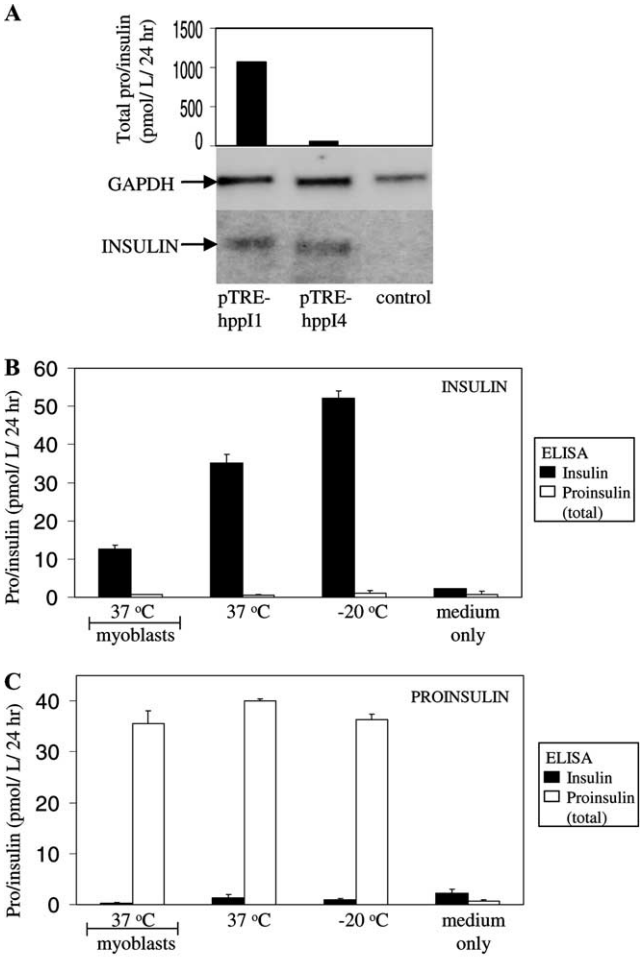

Fig. 6. Degradation/metabolism of insulin and proinsulin in myoblast cultures. Messenger RNA levels of pTRE-hppI1/ pTet-off, pTRE-hppI4/pTet-off transfected and untransfected (control) myoblasts were compared by Northern blotting using insulin and GAPDH probes (A is representative of three experiments). Human recombinant insulin (B) and proinsulin (C) were added to 6-well plates with or without myoblasts. Plates were incubated for 24 h in 5% CO_2/at 37 °C. Spiked medium was kept frozen at −20 °C and unspiked medium (medium only) was employed for comparison. Medium was assayed by proinsulin and insulin ELISA ($n = 3$, mean ± SD).

was no significant difference between the intracellular protein concentrations in the proinsulin and insulin treated myoblasts ($p > 0.05$), eliminating the possibility of fewer myoblasts in the proinsulin treated plates.

Discussion

In this study we have demonstrated regulated insulin secretion from skeletal muscle in a dose-responsive and reversible manner. There are several published reports on the secretion of mature insulin from myoblasts [8,16–19]. However, this is the first study addressing regulated human insulin secretion.

Regulation of transgene expression was studied using a two-plasmid tetracycline-inducible system. Primary myoblast transfections were initially compared using β-galactosidase constructs. Efficiency was low with <5% of cells staining positively using lipid vectors. However, co-transfection with the two-plasmid tetracycline system (pTRE-β/pTet-off) resulted in an increased number of positive transfected cells in comparison to a single-plasmid system (CMV-β). Following co-transfection with preproinsulin constructs and a tetracycline-repressible transcriptional activator, pro/insulin secretion was reduced in a dose-dependent manner following 24 and 48 h tetracycline incubations. Total pro/insulin secretion could be reduced to less than 1% with 1 μg/ml tetracycline and was sustained over a period of 4 days. This finding is similar to that of another study, where no transgenic protein (Granulocyte–Macrophage Colony-Stimulating Factor) secretion was detected following a 48 h incubation in 0.1 μg/ml tetracycline [20]. Reversibility of tetracycline repression was demonstrated, requiring 3 days incubation after tetracycline withdrawal before pro/insulin secretion levels were similar to untreated cells. Preproinsulin mRNA levels were analysed by Northern blotting, confirming tetracycline regulation at the level of transcription.

Transient transfection of primary myoblasts with the mutant insulin construct (hppI4) resulted in approximately 12-fold lower levels of detectable total pro/insulin secretion over a 24 h incubation in comparison to equivalent wild-type (hppI1) transfections. Levels of rat

proinsulin secretion from a similar furin-mutant were lower by 40–50% in COS-7, CHO, HepG2, and HIH3T3 cells compared to wild-type transfections [21]. In our study the differences were not related to mRNA levels. Partial explanation is provided by greater degradation by serum and metabolism by primary myoblasts of human insulin in comparison to proinsulin.

The application of this system as a sole treatment for diabetes mellitus is limited by the absence of a glucose sensing mechanism and the requirement of 2–3 days to efficiently switch off and on gene expression. This system could not, therefore, be used to restore rapid first phase insulin response. However, near physiological, post-prandial first phase insulin profiles can now be effectively replicated by injection of a short-acting insulin analogue at meal times. This has highlighted the need for restoration of basal insulin secretion, which is maintained at a constant low level between meals and through the night in those without diabetes [22]. Despite the advent of novel long-acting insulin analogues, none of the available insulin formulations are able to mimic physiological basal insulin leading to a continued risk of hypoglycaemia due to inappropriate peaks and troughs of insulin action over a 24 h period [23]. Tetracycline-mediated transcriptional regulation of insulin secretion offers the potential of long-term restoration of basal insulin secretion, together with a mechanism for titrating circulating background insulin levels according to fasting and late post-prandial glucose. A further advantage is that tetracycline could be used to "switch off" insulin expression in the event of dangerous hypoglycaemia.

A further benefit of this system is the concomitant secretion of C-peptide, which has been demonstrated to improve glucose utilisation, renal and nerve functions in mammalian models of diabetes mellitus [24]. To generate hppI4, the cDNA of hppI1 has been altered so that the 31-amino acid C-peptide has been mutated by two amino acids at both termini to produce basic residues to promote furin cleavage. This avoids alteration of the insulin A and B chains. Mutation into the C-peptide may alter receptor binding, although a recent paper demonstrated that Glu^{27}, which remains unaltered in our mutant, was crucial for binding [25]. However, further studies would be necessary to confirm effective C-peptide membrane binding.

In conclusion, efficient transgene expression has been demonstrated in vitro despite the use of a two-plasmid system. Future therapeutic applications with this approach include intramuscular reimplantation of ex vivo transfected host-derived myoblasts. Moreover, successful delivery of these plasmids in situ to host muscle offers the potential of long-term gene therapy mediated by simple intramuscular injection avoiding the need for cell culture, transplantation, immunosuppression or toxic small molecule transcriptional regulators. Although physiological minute-to-minute insulin secretion would not be attainable, this approach may be sufficient as a sole treatment for individuals with Type 2 diabetes and for restoration of basal insulin treatment in those with Type 1 diabetes. Refinement of this technique also holds promise for the treatment of a much broader range of endocrine, haematological, and other disorders characterised by protein deficiency.

Acknowledgments

Dr. K.T. Scougall was supported by University of Newcastle upon Tyne. Dr. J.A.M. Shaw was supported by a Glaxo Wellcome Senior Clinical Fellowship. We wish to thank Prof. K. Docherty for the provision of hppI1 and hppI4 cDNAs.

References

[1] J.A. Wolff, J.J. Ludtke, G. Acsadi, P. Williams, A. Jani, Hum. Mol. Genet. 1 (1992) 363–369.
[2] S.K. Tripathy, E.C. Svensson, H.B. Black, E. Goldwasser, M. Margalith, P.M. Hobart, J.M. Leiden, Proc. Natl. Acad. Sci. USA 93 (1996) 10876–10880.
[3] V.M. Rivera, X. Ye, N.L. Courage, J. Sachar, F. Cerasoli Jr., J.M. Wilson, M. Gilman, Proc. Natl. Acad. Sci. USA 96 (1999) 8657–8662.
[4] J.E. Murphy, S. Zhou, K. Giese, L.T. Williams, J.A. Escobedo, V.J. Dwarki, Proc. Natl. Acad. Sci. USA 94 (1997) 13921–13926.
[5] G. Hortelano, L. Wang, N. Xu, F.A. Ofosu, Haemophilia 7 (2001) 207–214.
[6] A.M. Abai, P.M. Hobart, K.M. Barnhart, Hum. Gene Ther. 10 (1999) 2637–2649.
[7] O.L. Kon, S. Sivakumar, K.L. Teoh, S.H. Lok, Y.C. Long, J. Gene Med. 1 (1999) 186–194.
[8] J.A. Shaw, M.I. Delday, A.W. Hart, H.M. Docherty, C.A. Maltin, K. Docherty, J. Endocrinol. 172 (2002) 653–672.
[9] D. No, T.P. Yao, R.M. Evans, Proc. Natl. Acad. Sci. USA 93 (1996) 3346–3351.
[10] V.M. Rivera, T. Clackson, S. Natesan, R. Pollock, J.F. Amara, T. Keenan, S.R. Magari, T. Phillips, N.L. Courage, F. Cerasoli Jr., D.A. Holt, M. Gilman, Nat. Med. 2 (1996) 1028–1032.
[11] Y. Wang, B.W. O'Malley Jr., S.Y. Tsai, B.W. O'Malley, Proc. Natl. Acad. Sci. USA 91 (1994) 8180–8184.
[12] M. Gossen, H. Bujard, Proc. Natl. Acad. Sci. USA 89 (1992) 5547–5551.
[13] H.M. Blau, F.M. Rossi, Proc. Natl. Acad. Sci. USA 96 (1999) 797–799.
[14] J. Dhawan, T.A. Rando, S.L. Elson, H. Bujard, H.M. Blau, Somat. Cell Mol. Genet. 21 (1995) 233–240.
[15] W.J. Sobey, S.F. Beer, C.A. Carrington, P.M. Clark, B.H. Frank, I.P. Gray, S.D. Luzio, D.R. Owens, A.E. Schneider, K. Siddle, Biochem. J. 260 (1989) 535–541.
[16] C. Arcelloni, L. Falqui, S. Martinenghi, A. Stabilini, A.E. Pontiroli, R. Paroni, J. Endocrinol. 166 (2000) 437–445.
[17] L. Gros, E. Riu, L. Montoliu, M. Ontiveros, L. Lebrigand, F. Bosch, Hum. Gene Ther. 10 (1999) 1207–1217.
[18] G.D. Simonson, D.J. Groskreutz, C.M. Gorman, M.J. MacDonald, Hum. Gene Ther. 7 (1996) 71–78.
[19] K. Yamasaki, T. Sasaki, M. Nemoto, Y. Eto, N. Tajima, Biochem. Biophys. Res. Commun. 265 (1999) 361–365.
[20] F.G. Sturtz, L. Cioffi, S. Wittmer, M.J. Sonk, A. Shafer, Y. Li, N.J. Leeper, J. Smith-Gbur, J. Shulok, D. Platika, Gene 221 (1998) 279–285.

[21] M. Yanagita, H. Hoshino, K. Nakayama, T. Takeuchi, Endocrinology 133 (1993) 639–644.
[22] G.B. Bolli, R.D. Di Marchi, G.D. Park, S. Pramming, V.A. Koivisto, Diabetologia 42 (1999) 1151–1167.
[23] D.R. Owens, B. Zinman, G.B. Bolli, Lancet 358 (2001) 739–746.
[24] J. Wahren, K. Ekberg, J. Johansson, M. Henriksson, A. Pramanik, B.L. Johansson, R. Rigler, H. Jornvall, Am. J. Physiol. Endocrinol. Metab. 278 (2000) E759–E768.
[25] A. Pramanik, K. Ekberg, Z. Zhong, J. Shafqat, M. Henriksson, O. Jansson, A. Tibell, M. Tally, J. Wahren, H. Jornvall, R. Rigler, J. Johansson, Biochem. Biophys. Res. Commun. 284 (2001) 94–98.