Tetracycline-regulated secretion of human insulin in a transfected non-endocrine cell line

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Abstract

Long-term constitutive secretion of insulin by implantation of *ex vivo* transfected cells such as fibroblasts or myoblasts or *in situ* by intramuscular injection of naked plasmid DNA provides a potential approach to gene therapy for diabetes mellitus. A mechanism for regulating insulin secretion will be necessary to realize the therapeutic potential of this approach. A second obstacle is the inability of non-endocrine host cells to fully process proinsulin. Therefore, alteration of the wild-type cDNA will be necessary to achieve processing of proinsulin by endogenous endoproteases within these cells. The cDNAs for β-galactosidase (β), human wild-type proinsulin (hppI1) and a mutated construct (hppI4), in which the dibasic PC2 and PC3 cleavage sites had been altered to form furin cleavage sites, were sub-cloned into four vectors (pCR3, pVR1012, pIRES, pTRE), including a tetracycline responsive plasmid (pTRE) that requires co-transfection with another plasmid encoding a transactivator (pTet-off) for transgene expression. Transient transfection of the COS-7 fibroblast cell line with these constructs was performed using DEAE-dextran and liposomes. Analysis of vector efficiencies revealed that pTRE/pTet-off>pIRES>pCR3>pVR1012. Further analysis demonstrated total pro/insulin secretion of 2·33 ng/10^6 cells/24 h with ≥25% processed to insulin in hppI-1.pTRE/pTet-off-transfected cells compared with 0·39 ng/10^6 cells/24 h and >70% processing in hppI-4.pTRE/pTet-off-transfected cells. In co-transfection studies with pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off constructs, pro/insulin secretion was inhibited to 65–66% and 36–38% of control (100%) in the presence of 0·01 and 0·1 μg/ml tetracycline respectively over a 24-h incubation period. Furthermore, reversal of tetracycline inhibition was demonstrated for pTRE-hppI1/pTet-off- and pTRE-hppI4/pTet-off-transfected cells. After a 48-h incubation with 1·0 μg/ml tetracycline, total pro/insulin levels were 10 and 14% compared with untreated cells respectively. On tetracycline removal, total proinsulin levels increased and were equivalent to untreated groups 72 h later. In conclusion, regulation of fully processed human insulin secretion has been achieved in a transiently transfected non-endocrine cell line.

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Introduction

Transfer of genes encoding therapeutic proteins into accessible somatic cell may lead to new potential treatments for a broad range of diseases (MacColl *et al.* 1999, Spirito *et al.* 2001). Host fibroblasts or myoblasts can be derived from minimally invasive biopsies, transfected *ex vivo* and implanted, thereby avoiding the need for immunosuppression. In addition, transgenes can be expressed in host skeletal muscle *in situ* following intramuscular injection of viral vectors or plasmid DNA (Wells 1995, van Deutekom *et al.* 1998).

Host-derived fibroblasts have been considered for growth hormone delivery (Heartlein *et al.* 1994) and insulin replacement in diabetes (Kawakami *et al.* 1992, Taniguchi *et al.* 1997, Falqui *et al.* 1999). Constitutive secretion of fully processed human insulin was attained in transfected fibroblasts using a preproinsulin construct in which PC2 and PC3 cleavage sites were altered to allow furin

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recognition (Falqui *et al.* 1999). *In vivo* studies in rodents were, however, complicated by hypoglycaemia due to unregulated cell proliferation, leading to increased insulin secretion (Falqui *et al.* 1999). Prolonged systemic secretion at therapeutically active concentrations of proteins including growth hormone (Dhawan *et al.* 1991), factor IX (Hortelano *et al.* 2001) and α1 antitrypsin (Bou-Gharios *et al.* 1999) has been demonstrated in transfected myoblasts following implantation in rodent muscle.

In vivo gene transfer to muscle can mediate transgene expression for up to 19 months (Wolff *et al.* 1992). This approach has been evaluated for DNA vaccination (Brower 1998), cytokine delivery (Prud'homme 2000) and treatment of heart disease (Teiger *et al.* 2001), in addition to erythropoietin (Tripathy *et al.* 1996), growth hormone (Rivera *et al.* 1999) and leptin replacement (Murphy *et al.* 1997). Constitutive secretion of processed human insulin has been demonstrated for up to 5 weeks in rodents following simple intramuscular injection with a preproinsulin plasmid modified for furin recognition (Abai *et al.* 1999, Kon *et al.* 1999, Shaw *et al.* 2002). Sustained glucose lowering was demonstrated without dangerous hypoglycaemia (Shaw *et al.* 2002). In individuals with haemophilia B, factor IX has been successfully expressed in muscle following injection with an adeno-associated viral vector containing the transgene (High 2001).

Plasmid DNA for *ex vivo* or *in vivo* therapeutic gene transfer has several advantages in comparison with viral vectors. These include relative ease of production on a large scale, cost-efficiency, lower toxicity and decreased immunogenicity. Although transgene expression remains comparatively low, there is considerable ongoing research addressing novel delivery methods to maximise plasmid uptake and retention together with plasmid modifications to optimise mRNA expression and stability (Xu *et al.* 2001, Liu & Huang 2002).

A mechanism for safely regulating constitutive secretion following plasmid-mediated transfection, and more specifically for 'turning down' transgene protein levels, would greatly enhance the applicability of this approach for therapeutic use. This will be particularly important for insulin replacement in diabetes, allowing titration of a constant background level of insulin between meals and a mechanism for averting dangerous hypoglycaemia.

Several small molecule transcriptional regulatory systems have been developed including those based on tetracycline (Gossen & Bujard 1992), FK506/rapamycin (Rivera *et al.* 1996), RU486/progesterone (Wang *et al.* 1994) and ecdysone (No *et al.* 1996). These small molecules reversibly bind to modified transcription factors inducing or repressing transcription of a transgene. 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 (Blau & Rossi 1999) and (4) efficient regulation of reporter gene expression has been demonstrated following plasmid-mediated gene transfer to muscle *in vivo* (Dhawan *et al.* 1995).

The objective of these studies was to evaluate the potential for tetracycline-regulated secretion of fully processed human insulin, following transient transfection of the non-endocrine COS-7 fibroblast cell line. Initially, efficiency of transgene uptake and expression employing a range of plasmids under the control of a constitutive cytomegalovirus or tetracycline repressible promoter were compared using a β-galactosidase reporter gene and a wild-type human preproinsulin gene. Thereafter, proinsulin synthesis, processing and secretion together with the potential for tetracycline transcriptional regulation were evaluated using a wild-type preproinsulin plasmid and a mutant plasmid modified to enable cleavage by furin.

Materials and methods

Cell culture reagents were purchased from Invitrogen Ltd (Paisley, UK) and chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) and BDH (Poole, Dorset, UK) unless otherwise stated. Restriction and DNA modification enzymes were purchased from Promega (Southampton, UK). Plasmids pIRES, pTRE, pTet-off and CMV-β were obtained from CLONTECH Laboratories Ltd (Basingstoke, Hants, UK), pCR3 from Invitrogen Ltd and pVR1012 from Vical Inc (San Diego, CA, USA). The monkey kidney fibroblast, COS-7 cell line was a gift from Prof. A Rees, University of Aberdeen, UK.

Expression plasmids

The mammalian expression vectors used in this study are described in Table 1. For eukaryotic transgene expression, all plasmids consisted of the human cytomegalovirus (CMV) enhancer/promoter, with the exception of tetracycline responsive plasmid (pTRE). For transgene polyadenylation and transcriptional termination, bovine growth hormone (BGH) or simian virus 40 (SV40) sequences were employed. Genes encoding either ampicillin or kanamycin resistance were used for bacterial selection. All plasmids contained the neomycin phosphotransferase gene for mammalian selection, with the exception of pTRE, which has no selectable marker. Plasmid pCR3 has been optimised to remove potential RNA secondary structure sequences (Mizushima & Nagata 1990). Plasmid pIRES has been modified to possess an internal ribosomal entry sequence which permits translation of two open reading frames from one messenger RNA and a synthetic intron known to enhance the stability of mRNA (Rees *et al.* 1996). Plasmid pVR1012 has been optimised for enhanced transgene expression (Hartikka *et al.* 1996). The tetracycline transcriptional regulatory system (Gossen & Bujard 1992) 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. In the presence of tetracycline, binding of the transactivator protein is inhibited, blocking gene transcription. Transgene expression can be regulated, as binding of tetracycline is both reversible and dose-responsive.

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) human preproinsulin cDNAs have been described previously (Shaw *et al.* 2002) and were available as inserts in pCR3 (pCR3-hppI1 and pCR3-hppI4) and pIRES (pIRES-hppI1 and pIRES-hppI4). Wild-type and mutant insulin cDNAs were ligated into pTRE using EcoRI sites to give pTRE-hppI1 and pTRE-hppI4 respectively. Inserts were sub-cloned as EcoRI fragments into pCR3. Inserts incorrectly orientated were cleaved using BamHI and XbaI and sub-cloned into BamHI/XbaI digested pVR1012 to give correctly orientated inserts pVR1012-hppI1 and pVR1012-hppI4. The β-galactosidase gene was excised from construct CMV-β by NotI digestion and sub-cloned as a NotI fragment into pCR3 and pIRES (pCR3-β, pIRES-β). Following pCR3-β digestion with EcoRI/XbaI and Pst/XbaI, the β-galactosidase insert was sub-cloned into equivalent digested pTRE and pVR1012 respectively (pTRE-β, pVR1012-β). All constructs were characterised by restriction digests and purified by QIAGEN DNA columns (QIAGEN Ltd, Crawley, West Sussex, UK).

Cell cultures

COS-7 cells were maintained in growth medium consisting of Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), penicillin G (100 U/ml), streptomycin (100 μg/ml), and cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

Transfections

Transient transfections were performed with DEAE-dextran (Amersham-Pharmacia plc, Little Chalfont, Bucks, UK) using previously described protocols (Luthman & Magnusson 1983, Lopata *et al.* 1984) or with cationic lipsomes, TransFast, purchased from Promega. COS-7 cells were transfected at 70-80% confluence in 6-well plates (diameter 3·5 cm/well). Each well was transfected using 2 μg of each cDNA, 1 mg/ml DEAE-dextran/PBS in a final volume of 200 μl Hank's balanced salt solution and incubated on cells for 20 min at 37 °C/5% CO_2, with gentle agitation every 10 min. Growth medium (1 ml) supplemented with chloroquine (100 μM) was added and the cells incubated at 37 °C/5% CO_2 for a further 2·5 h. Cells were shocked with 10% (v/v) DMSO in growth medium for 2·5 min, which was then replaced with standard growth medium. Transfection using Transfast was performed using 1 μg of each DNA and using a charge ratio of lipid reagent to DNA of 1:3. The manufacturer's

Table 1 Comparison of mammalian expression plasmids

Plasmid (supplier)	Size (kb)	Promoter	Poly-A tail	Mammalian selection	Bacterial selection	Additional elements	Reference
pCR3 (Invitrogen)	5·1	Human CMV	BGH	Neomycin/kanamycin	Ampicillin	Potential RNA secondary structure sequences have been removed	Mizushima & Nagata (1990)
pIRES (Clontech)	5·3	Human CMV	BGH	Neomycin/kanamycin	Ampicillin	pIRES contains a synthetic intron that enhances mRNA stability and an internal ribosomal entry sequence, allowing translation of two proteins from one mRNA	Rees *et al.* (1996)
pVR1012 (Vical Inc.)	4·9	Human CMV	BGH	Neomycin/kanamycin	Kanamycin	pVR1012 has been optimised for gene delivery and unnecessary viral backbone elements have been removed	Hartikka *et al.* (1996)
pTRE (Clontech)	3·1	7 copies of tetO upstream of minimal CMV promoter (lacks enhancer)	SV40	None	Ampicillin	pTRE/pTet-off co-transfection is required for transgene expression. Regulated transcription can be achieved by tetracycline addition	Gossen & Bujard (1992)
pTet-off (Clontech)	7·4	Human CMV	SV40	Neomycin/kanamycin	Ampicillin		

instructions were followed for transfection. Medium was collected and replaced at 24-h intervals and cells were harvested following completion of the experiment.

Tetracycline addition

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 μg/ml to 100 μg/ml. After transfection, cells were rested for 24 h before the addition of tetracycline. Tetracycline supplemented medium was replaced at 24-h intervals.

1011 and 1014 pro/insulin radioimmunoassay (RIA)

Cell medium and extracts (freeze-thawed in PBS) were assayed in duplicate by in-house RIA as described previously (Shaw *et al.* 2002). Briefly, human recombinant insulin (Sigma-Aldrich) was used as a standard for RIA (0–5 ng/ml). Non-specific (1011) and specific (1014) antibodies (Linco Research Inc, St. Charles, MO, USA) were used for the detection of total insulin-like immunoreactivity (ILI) and mature insulin respectively. Tyrosine-14-labelled insulin (^{125}I, Amersham-Pharmacia plc), medium/cell extracts and anti-insulin serum were incubated overnight at 4 °C. Precipitating complex (γ-globulins and PEG-16000) was added, vortexed, centrifuged and the supernatant discarded. The precipitates were counted for 60 s using a gamma scintillation counter. All reagents used in the initial incubation mixture were diluted in RIA buffer (50 mM PBS, 25 mM EDTA, 138 mM NaCl, 1% (w/v) BSA, 0·1% (w/v) NaN_3, pH 7·4).

Proinsulin and insulin ELISA

Commercially available human proinsulin and ultrasensitive insulin ELISA kits were purchased from Mercodia, Uppsala, Sweden. Samples were vortexed and centrifuged prior to analysis and the manufacturer's protocol was followed. Completed plates were measured at absorbance A_{450} on a Dynatech MR 7000 plate reader.

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 $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase (Melford Laboratories Ltd, Chelsworth, Suffolk, 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.

Immunocytochemistry

COS-7 cells were seeded onto coverslips (washed in a solution of 1% DECON, rinsed thoroughly and autoclaved) in 6-well plates. Cells were fixed using 5% (v/v) acetic acid/ethanol for 10 min at room temperature 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, 6·7% (v/v) glycerol in PBS) for 1 h. Primary antibodies: guinea pig anti-human insulin (provided by Prof. A Bone, University of Brighton, UK) and mouse anti-human vimentin (Sigma-Aldrich) were diluted in blocking buffer (1/500 and 1/50 respectively) 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, 6·7% (v/v) glycerol in PBS). Secondary antibodies (Sigma-Aldrich): rabbit anti-mouse TRITC (tetramethylrhodamine isothiocyanate) conjugate and mouse anti-guinea pig FITC (fluorescein isothiocyanate) conjugate were diluted in blocking buffer to 1/250 and added to cells for 1 h in darkness. After a further four washes in wash buffer, coverslips were mounted in Vectashield aqueous mounting medium containing DAPI (4,6-diamidino-2-phenylindole) nuclear stain (Vector Laboratories Ltd, Peterborough, UK) and analysed by digital fluorescence microscopy. Fluorescence microscopy was carried out using a Zeiss Axioplan II transmission microscope with images captured using a Photometrics Sensys camera and recorded using VijsisQuips software (Vysis, Surrey, UK).

Cell counts

Trypan Blue (0·4%, Invitrogen Ltd.) exclusion was used to count viable cells on a haemocytometer.

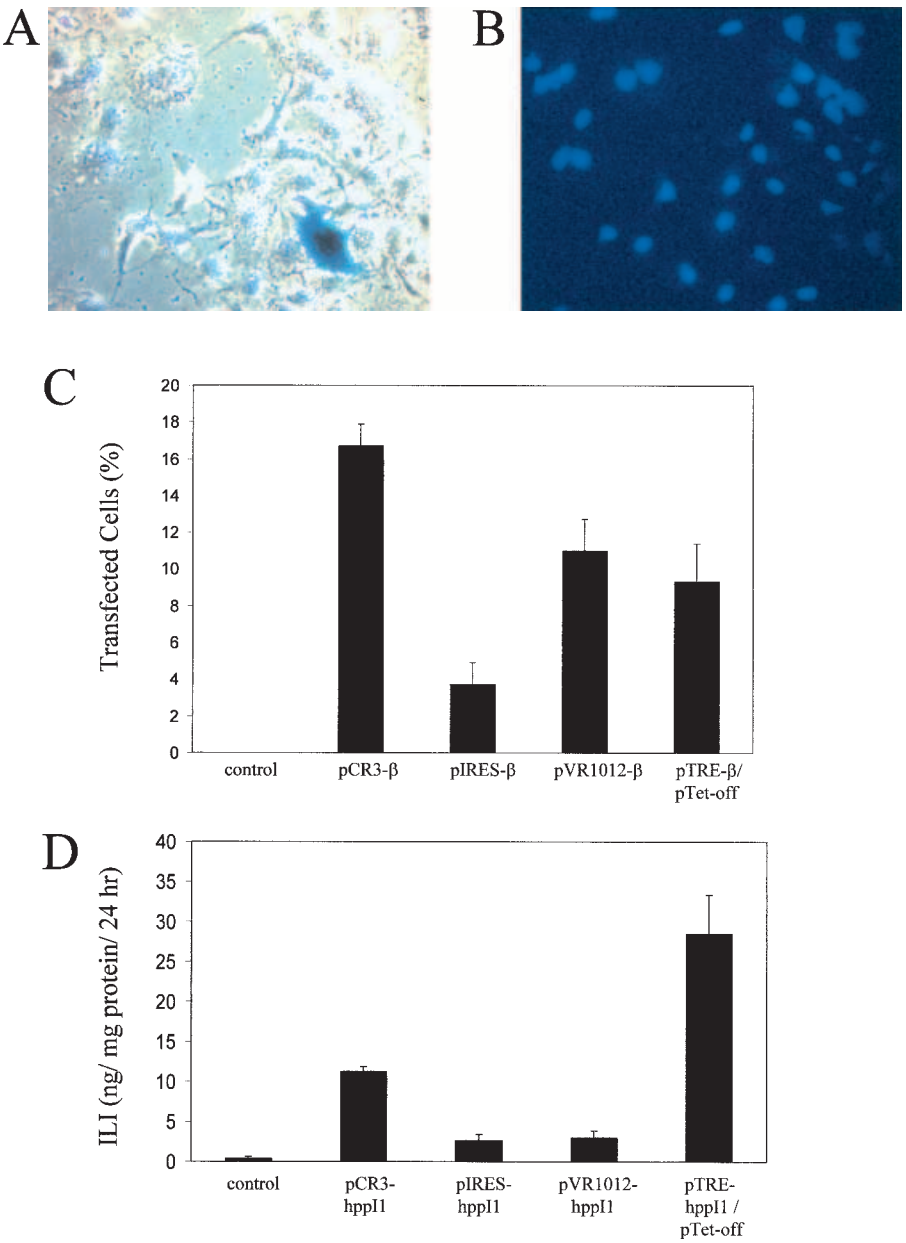
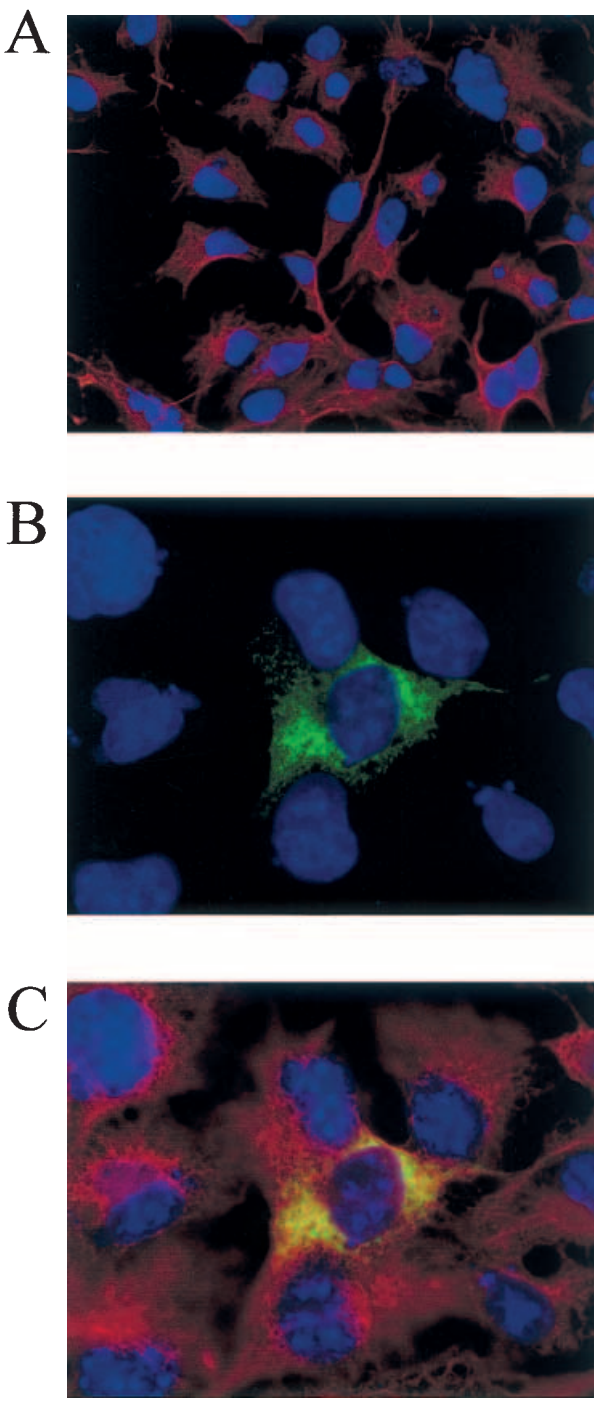


Figure 1 Comparison of vector efficiency in the COS-7 cell line. COS-7 cells were transfected with empty plasmid (control) or plasmids encoding transgenes for β-galactosidase (pCR3-β, pIRES-β, pVR1012-β and pTRE-β/pTet-off) and hppI1 (pCR3-hppI1, pIRES-hppI1, pVR1012-hppI1 and pTRE-hppI1/pTet-off). β-Galactosidase-transfected cells were fixed and stained with (A) X-gal and (B) Hoechst nuclear stain 48 h post-transfection (magnification ×200). (C) The number of X-gal- and nuclear-stained cells in 8 fields of view (4 central and 4 peripheral) were 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). The results are from three separate experiments (n=3, mean±S.D). (D) COS-7 cells were transfected with plasmids encoding hppI1. Following transfection, medium was changed after 24 and 48 h, cells were incubated for a further 24 h, after which the medium was harvested, representing 48–72 h. Intracellular protein concentrations were measured and pro/insulin levels adjusted accordingly. ILI (insulin-like immunoreactivity) was measured by 1011 RIA and is expressed as ng/mg protein/24 h (n=3, mean±S.D).

RNA isolation and Northern blotting

Preproinsulin mRNA levels were analysed by Northern blotting. Total RNA was isolated from cultured cells in 1 ml TRIZOL reagent (Invitrogen Ltd.) following the manufacturer's instructions. To determine equal loading, RNA was loaded onto a 1% agarose gel and stained with ethidium bromide. Approximately 10 µg total RNA were loaded onto a 1·2% agarose gel containing formaldehyde, morpholinopropane-sulphonic acid (MOPS), sodium acetate, EDTA pH 7·0 and transferred onto a nitrocellulose N^+ membrane (Hybond, Amersham-Pharmacia plc.) using the capillary blotting technique. Hybridisation was performed with a digoxigenin (DIG)-labelled antisense RNA encoding the preproinsulin gene. Procedures were carried out according to the manufacturer's guidelines (Roche Diagnostics Ltd, Lewes, East Sussex, UK). Plasmid pCR3-hppI1 was linearised (BamHI) and used as a template for *in vitro* RNA transcription (SP6 RNA polymerase) in the presence of DIG-11-UTP to create the preproinsulin probe.



Statistical analysis

Values are reported as means ± standard deviation (S.D.). Data were analysed by one-way analysis of variance (ANOVA) and Student's *t*-test. A P value of $<0{\cdot}05$ was accepted as statistically significant.

Results

Transgene expression in transiently transfected COS-7 cells

COS-7 cells were transiently transfected with plasmids (pCR3, pIRES, pVR1012 and pTRE/

Figure 2 Immunocytochemical staining of transfected COS-7 cells. Cells were fixed, then incubated with guinea pig anti-insulin (1/500) and mouse anti-vimentin (1/50) primary antibodies as described in the methods section. Anti-guinea pig FITC (1/250) conjugate and anti-mouse TRITC conjugate (1/250) secondary antibodies were used to visualise insulin (green) and vimentin (red) staining. Nuclei were stained with DAPI blue. (A) Triple stain of COS-7 cells co-transfected with pTRE (no insert)/pTet-off. Vimentin (red) and nuclear (blue) staining are demonstrated. Insulin staining (green) is absent (magnification ×400). (B and C) COS-7 cells co-transfected with pTRE-hppI1/pTet-off. Double nuclear and insulin staining demonstrates a single, successfully co-transfected cell (B, magnification ×650). Triple staining of the same field confirms vimentin expression in a cell expressing the insulin transgene in addition to DAPI blue nuclear staining (C, magnification ×650).

Table 2 Percentage inhibition of pro/insulin secretion by tetracycline. COS-7 cells were co-transfected with either pTRE-hppI1/pTet-off or pTRE-hppI4/pTet-off. Tetracycline was added after 24 h and again at 48 h post-transfection to fresh medium at final concentrations of 0·01, 0·1 and 1·0 μg/ml. In separate experiments medium was harvested after 24 (n=3) and 48 (n=2) h tetracycline incubations (48 and 72 h post-transfection respectively) and total pro/insulin levels were measured by 1011 RIA. Pro/insulin levels from tetracycline-treated cells are expressed as a percentage over untreated cells (100%)

	pTRE-hppI1/pTet-off		pTRE-hppI4/pTet-off	
	24 h	48 h	24 h	48 h
Tetracycline concentration (μg/ml)				
0·000	100%	100%	100%	100%
0·01	66%	50%	65%	29%
0·1	38%	7%	36%	5%
1·0	40%	9%	46%	6%

pTet-off) encoding transgenes for β-galactosidase (β) and wild-type human preproinsulin (hppI1). Transfection efficiency was determined by two methods. First, by determination of percentage transfection efficiency by microscopic quantification, whereby the number of blue, X-gal stained cells (Fig. 1A) and the total number of cells visualised by Hoechst nuclear stain (Fig. 1B) were counted. Transfected cells were expressed as a percentage over the total cell count in a field of view (Fig. 1C). Secondly, the total amount of pro/insulin secreted from transfected cells was measured using RIA (Fig. 1D).

There was no background X-gal staining visualised in cells transfected with plasmids containing no transgene inserts. The highest percentage of X-gal-positive cells was seen following transfection with pCR3-β (16·7%), followed by pVR1012-β (11·0%), pTRE-β/pTet-off (9·3%) and pIRES-β (3·7%). In contrast, co-transfection with pTRE-hppI1 and pTet-off resulted in maximal secretion of total pro/insulin into the surrounding culture medium (28·48 ng/mg protein/24 h) compared with pCR3-hppI1 (11·28 ng/mg protein/24 h), pVR1012-hppI1 (3·03 ng/mg protein/24 h) and pIRES-hppI1 (2·68 ng/mg protein/24 h). Secreted pro/insulin levels were adjusted for intracellular protein concentration.

If the levels of secreted pro/insulin are divided by the percentage of β-galactosidase-positive cells, a comparison of relative promoter strength, mRNA stability and efficiency of translation for each plasmid can be made. Co-transfection of pTRE and pTet-off (3·06 ng/μg protein/24 h/percent of transgene expressing cells) resulted in an 11-fold higher amount of pro/insulin secreted by each cell expressing both transgenes in comparison with pVR1012 transfection (0·28). By this method, expression was 4·25 times greater in cells transfected with pTRE/pTet-off in comparison with pIRES (0·72) and 4·5 times greater than with pCR3 (0·68). These results indicate that even if basic promoter/poly A structures are similar, apparently minor variations can lead to considerable differences in transgene expression by cells which have taken up and retained the plasmid.

Immunocytochemical confirmation of insulin biosynthesis and vimentin expression in transiently transfected COS-7 cells

Pro/insulin biosynthesis following pTRE-hppI1/pTet-off co-transfection, together with expression of the mesenchymal marker vimentin in COS-7 cells was confirmed by immunocytochemical staining (Fig. 2).

Regulation of pro/insulin secretion by tetracycline in transiently transfected COS-7 cells

The potential for regulated pro/insulin secretion by tetracycline administration was evaluated in COS-7 cells transiently transfected *in vitro*. Pro/insulin

secretion detected by 1011 RIA following transfection with pTRE-hppI1 and pTRE-hppI4 was not significantly higher than background (pTet-off). In the absence of tetracycline, total pro/insulin secretion was 2·33 ng/10^6 cells/24 h following co-transfection with pTRE-hppI1 and pTet-off (Fig. 3A). Addition of 0·01, 0·1 and 1·0 μg/ml tetracycline to medium for 24 h (24–48 h post transfection) reduced the levels of total pro/insulin secreted into the medium to 66, 38 and 40% of untreated cells (100%) respectively (Table 2). A similar pattern was observed in pTRE-hppI4/pTet-off-transfected cells. Total pro/insulin secretion (determined by 1011 RIA) of 0·39 ng/10^6 cells/24 h was attained after co-transfection in the absence of tetracycline (Fig. 3B). Secreted levels were reduced by the addition of 0·01, 0·1 and 1·0 μg/ml tetracycline to 65, 36 and 46% of control (100%) respectively (Table 2).

In a separate study, medium was harvested after a further 24-h incubation with tetracycline (Table 2). This resulted in a further percentage reduction in pro/insulin secretion to a minimum of 7% following pTRE-hppI1/pTet-off co-transfection and 5% after pTRE-hppI4/pTet-off transfection in the presence of 0·1 μg/ml tetracycline.

Processing of proinsulin to insulin was assessed in hppI1 and hppI4 transfected cells. Levels of secreted mature insulin (determined by 1014 RIA) were compared with total pro/insulin secretion (determined by 1011 RIA). In hppI1-transfected cells, processing of proinsulin to insulin varied from 8–25% (Fig. 3A). In hppI4-transfected cells, 72–100% of total ILI was processed to mature insulin (Fig. 3B).

To determine if tetracycline was preventing pro/insulin secretion, intracellular concentrations were measured; they were 2–4% and 10–16% of the total amount secreted for pTRE-hppI1/pTet-off- and pTRE-hppI4/pTet-off-transfected cells respectively. There was no observed intracellular accumulation of pro/insulin related to increasing tetracycline concentration in co-transfections with pTRE-hppI1/pTet-off or pTRE-hppI4/pTet-off (absence of tetracycline-mediated intracellular accumulation confirmed by ANOVA).

Viable cell numbers were also compared to determine if reduced pro/insulin secretion was due to tetracycline-induced cell death. There were no significant differences observed with increasing concentrations of tetracycline up to 1 μg/ml in either wild-type or mutant co-transfections. Numbers of viable cells per well at 48 h following *in vitro* transfection and after exposure to tetracycline for 24 h were in the range 1·2–1·4 × 10^6 following pTRE-hppI1/pTet-off transfection and 2·0–2·6 × 10^6 after pTRE-hppI4/pTet transfection.

To determine potential interference of tetracycline with 1011 and 1014 RIAs, tetracycline was added to pCR3-hppI1 (a tetracycline unresponsive plasmid)-transfected cells at final concentrations of 0, 0·01, 0·1, 1, 10 and 100 μg/ml, 24 h post-transfection. Medium was harvested a further 24 h later and assayed by non-specific (1011) and specific (1014) RIA with pro/insulin expressed as ng/10^6 viable cells/24 h. At tetracycline concentrations of 0–10 μg/ml there were no significant differences between treated and untreated groups with either 1011 or 1014. Only at a tetracycline concentration of 100 μg/ml, higher than that used in any of the other studies reported here, were levels of secreted pro/insulin and insulin significantly lower, relating to lower cell numbers at this concentration (data not shown).

Regulation of preproinsulin mRNA expression by tetracycline in transiently transfected COS-7 cells

To confirm the effect of tetracycline on gene transcription, preproinsulin mRNA levels were analysed by Northern blot. COS-7 cells were transfected individually with plasmids pTet-off, pTRE-hppI1 and pTRE-hppI4 or co-transfected with pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off. Transfected cells were incubated for 24 h, after which tetracycline was added in increasing amounts to give final concentrations of 0·01, 0·1 and 1·0 μg/ml. Cells were incubated for a further two 24-h periods in medium containing tetracycline, then medium was harvested (48–72 h) and total RNA extracted from the cell pellet. Total secreted pro/insulin concentrations were measured by 1011 RIA (Fig. 4A). Samples containing 10 μg total RNA were run on a formaldehyde/1·2% agarose gel. RNA was also run on a 1% agarose gel and stained with ethidium bromide to confirm equal loading (Fig. 4B). After separation, the Northern blot was probed with a digoxigenin-labelled preproinsulin antisense RNA (Fig. 4C).

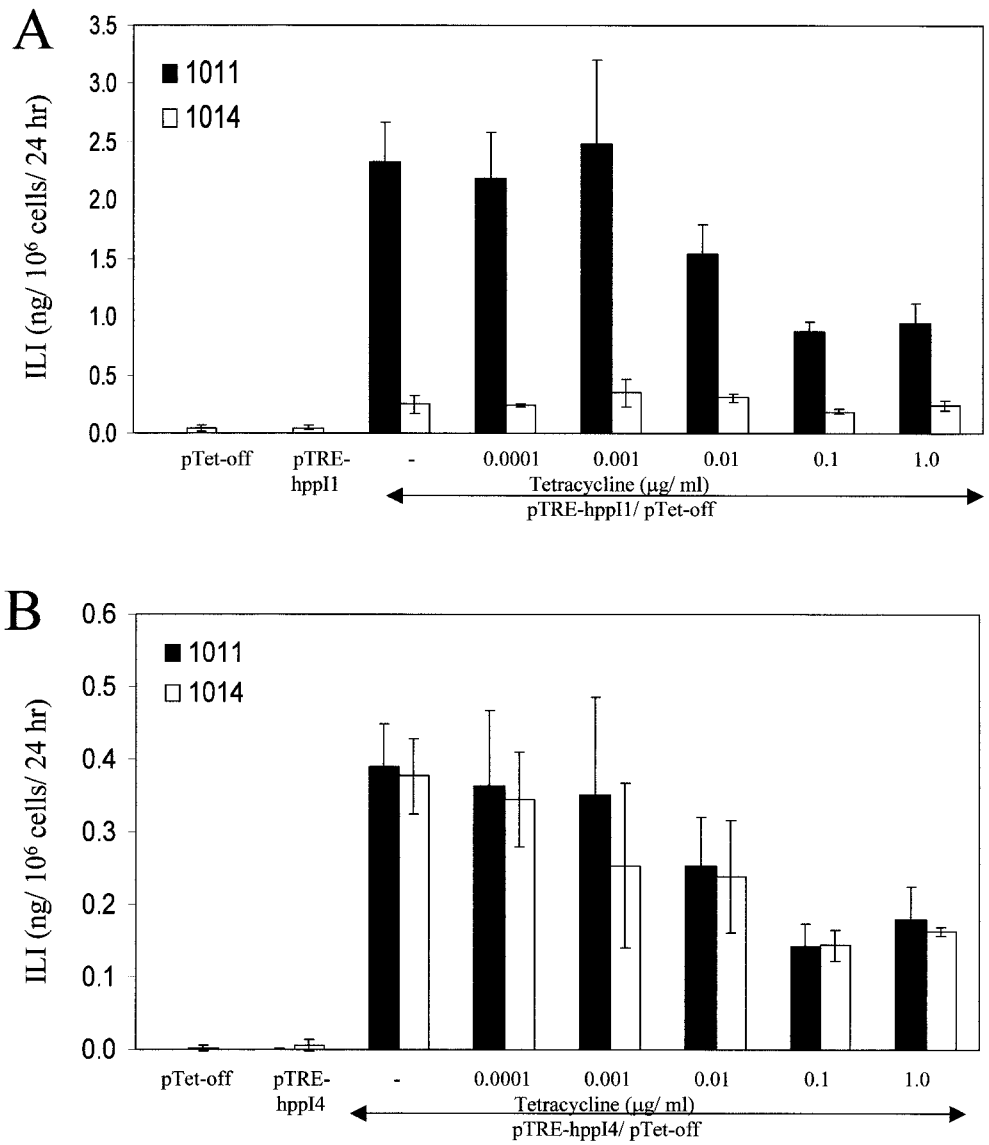


Figure 3 Tetracycline-regulated pro/insulin secretion in COS-7 cells. Cells were transfected with pTRE-hppI1 (A) or pTRE-hppI4 (B) alone and in conjunction with pTet-off. Tetracycline was added to fresh medium at final concentrations ranging from 0–1·0 µg/ml, 24 h after transfection. The medium was harvested 24 h later (24–48 h) and assayed by 1011 (solid bars) and 1014 (open bars) RIA. Cells were trypsinised and cell counts were taken for each well. ILI is expressed as ng/10^6 cells/24 h (n=3, mean±S.D).

Proinsulin mRNA was detected only in co-transfection of cells with pTRE-hppI1/pTet-off and pTRE-hppI4/pTet-off in the absence and presence of 0·01 µg/ml tetracycline. In the presence of 0·1 and 1·0 µg/ml tetracycline proinsulin mRNA expression was not detected in wild-type or mutant transfections.

Reversal of tetracycline inhibition in transiently transfected COS-7 cells

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 by

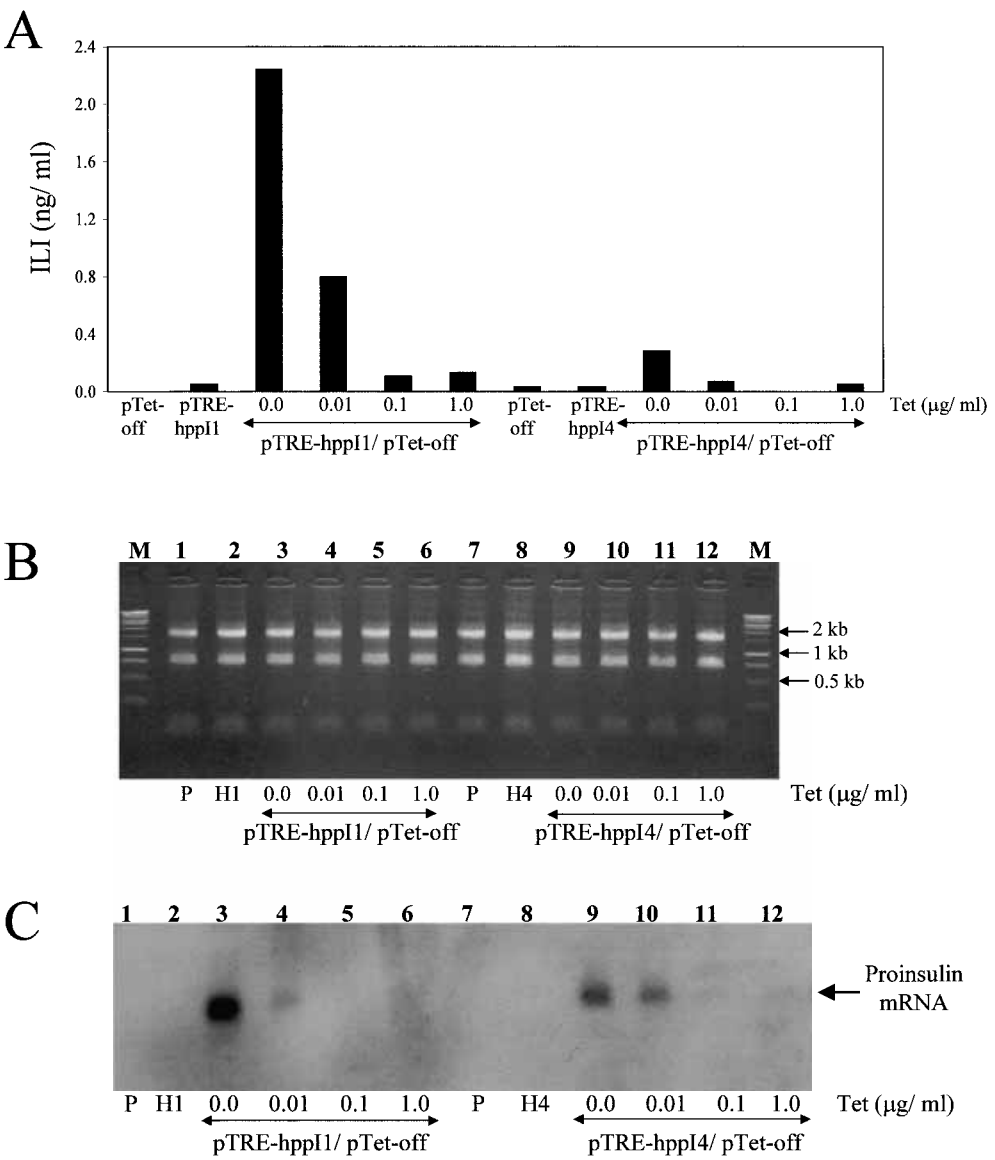


Figure 4 Tetracycline-regulated preproinsulin transcription in COS-7 cells. COS-7 cells were transfected with pTRE-hppI1 (H1) or pTRE-hppI4 (H4) alone and in conjunction with pTet-off (P). Tetracycline was added to fresh medium at final concentrations of 0·01, 0·1 and 1·0 μg/ml, 24 and 48 h after transfection. (A) The medium was harvested 48 h after initial tetracycline addition (48–72 h), assayed by 1011 RIA and ILI is expressed as ng/ml. Cells were lysed in TRIZOL. (B) Total RNA was extracted and analysed by 1% agarose gel electrophoresis to confirm equal loading. (C) Total RNA (10 μg) was analysed by Northern blot and probed with an antisense preproinsulin DIG-labelled RNA.

ELISA. COS-7 cells were transfected with pTRE-hppI/pTet-off and pTRE-hppI4/pTet-off using Transfast. Transfast was used to improve transgene expression from transient transfections. 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 for both hppI1 (1623 pmol/l) and hppI4 (65 pmol/l) transfections. For comparison, tetracycline-treated

groups are expressed as a percentage over untreated (Fig. 5A and B). Continuous addition of 1 μg/ml tetracycline (group 3) to pTRE-hppI1/pTet-off-transfected cells reduced the total pro/insulin secreted with respect to untreated cells by 43, 10 and 7% over days 2, 3 and 4 respectively, after which a level of 7% was maintained. For pTRE-hppI4/pTet-off-transfected cells, addition of tetracycline continuously (group 3) reduced the total pro/insulin secreted by 40, 14 and 12% over days 2, 3 and 4 respectively, after which a level of 6–12% was maintained. In group 2, tetracycline was added for 2 days before being removed. During incubation with tetracycline secreted pro/insulin levels were similar to the tetracycline continually treated group. Upon removal of tetracycline, proinsulin secretion increased to 13, 55 and 92% for pTRE-hppI/pTet-off-transfected cells and 26, 61 and 95% for pTRE-hppI4/pTet-off-transfected cells over days 4, 5 and 6 respectively compared with untreated groups. At the end of the study intracellular protein concentrations were quantified. Although there was a marginal decrease in protein levels in tetracycline-treated (0·81–0·93 mg/ml) compared with untreated (1·0–1·1 mg/ml) groups this was not significant (P=0·819, ANOVA).

Discussion

In these studies, efficiency of transgene expression following transient transfection of the COS-7 fibroblast cell line, with a range of plasmids under the control of a constitutive CMV or a tetracycline repressible promoter has been compared employing a β-galactosidase reporter gene and a wild-type human preproinsulin gene. Highest pro/insulin secretion was attained on co-transfection with a tetracycline repressible transactivator plasmid and a construct in which preproinsulin was expressed under the control of a tetracycline responsive element. Regulation of pro/insulin secretion, mediated by changes in transgene transcription, by addition of tetracycline, was confirmed employing the tetracycline regulatable wild-type preproinsulin plasmid and an equivalent construct engineered to facilitate post-translational processing by furin. Moreover, in mutant transfections, greater than 70% of total pro/insulin secreted was fully processed to mature insulin.

The highest percentage of cells expressing the β-galactosidase transgene was achieved with the pCR3-β plasmid in COS-7 cells. X-gal staining of β-galactosidase-expressing cells was very clear allowing identification of positively transfected cells without ambiguity. It would be envisaged that, employing a single transfection method, the percentage of cells taking up each of the plasmids would be comparable. Efficiency of nuclear localisation of plasmid DNA following uptake is dependent on cell type and appears to be influenced by degree of degradation within the cytosol by nucleases (Coonrod *et al.* 1997, Pollard *et al.* 2001). We have previously reported enhanced preproinsulin transgene expression in primary fibroblasts in the presence of lysosomal nuclease inhibitors (Shaw *et al.* 2002). It may be that the plasmids employed in the present studies differed in their susceptibility to nuclease degradation.

Pro/insulin transgene expression was used to compare efficiencies of different plasmids, co-transfection with pTRE-hppI1 and pTet-off being most effective. An estimation of relative promoter strength, mRNA stability and translational efficiency was calculated by dividing the levels of pro/insulin secreted into the medium by the percentage of cells expressing the β-galactosidase reporter gene. Using this method, efficiency of transgene expression by positive transfected cells was higher with pTRE/pTet-off followed by pIRES, pCR3 and pVR1012. The plasmids pVR1012, pCR3 and pIRES all contain the CMV promoters and BGH termination sequences. The presence of a synthetic intron, reported to increase the stability of mRNA in pIRES and modification of pCR3 to remove potential RNA secondary structure sequences may account for higher relative transgene expression compared with pVR1012.

Tetracycline-responsive transcriptional regulation of preproinsulin transgene expression was evaluated to determine the feasibility of controlling therapeutic protein secretion in a transfected non-endocrine cell line. Pro/insulin secretion was reduced in a dose-dependent manner on 24-h incubation with tetracycline, with further reduction to less than 14% after a 48-h incubation; this was sustained over a period of 4 days. Reversibility of tetracycline repression was demonstrated, requiring 3 days of incubation without tetracycline before pro/insulin secretion levels were similar to untreated cells. Although preproinsulin mRNA was

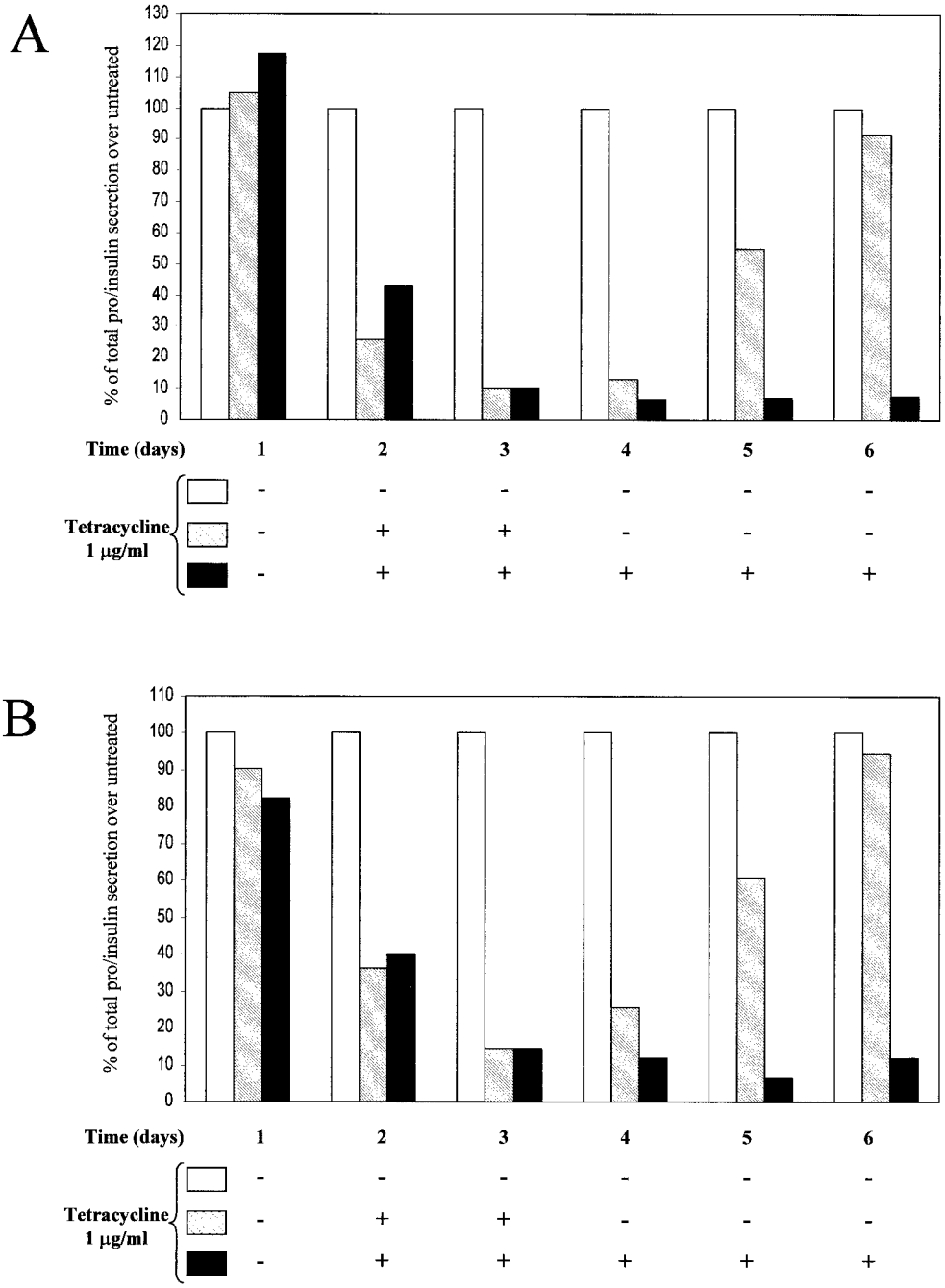


Figure 5 Reversal of tetracycline inhibition of pro/insulin secretion in transfected COS-7 cells. COS-7 cells were transfected with pTRE-hppI1/pTet-off (A) or pTRE-hppI4/pTet-off (B). Tetracycline was omitted (open bars), added during days 2 and 3 only (hatched bars) or added continuously after day 1 (solid bars) to culture medium post-transfection. Tetracycline was added at a final concentration of 1·0 μg/ml. The medium was harvested every 24 h and assayed using ELISAs. The total pro/insulin levels (proinsulin and insulin) are expressed as a percentage over the tetracycline-untreated group.

undetectable after 48-h exposure to a tetracycline concentration of 0·1 μg/ml or above, secreted pro/insulin remained measurable at 0·1 μg/ml tetracycline and could not be reduced by increasing the concentration to 1·0 μg/ml. This finding is in contrast to another study, where there was no detectable transgenic protein secretion at 0·1 μg/ml tetracycline (Sturtz *et al.* 1998). Tetracycline derivatives such as doxycycline demonstrate increased affinity for the transactivator and may enable complete inhibition of pro/insulin secretion at comparable concentrations. This transcriptional system has also been refined as a single plasmid containing both tetracycline repressible transactivator and tetracycline responsive element (O'Brien *et al.* 1997), and for the expression of two genes in a mutually exclusive manner (Baron *et al.* 1999).

Transient transfection of the COS-7 cell line with the mutant insulin construct (hppI4) resulted in approximately 80% lower levels of total pro/insulin secretion in comparison with equivalent wild-type (hppI1) transfections. Levels of rat proinsulin secretion were 40–50% lower in COS-7, CHO, HepG2 and HIH3T3 cells transfected with a mutant plasmid engineered to facilitate furin cleavage compared with wild-type transfections (Yanagita *et al.* 1993). This appears to be a consistent finding in all reported studies of unmodified and modified insulin gene transfer to non-endocrine cells, although the underlying mechanism remains unclear. Rapid internalisation and metabolism of insulin following binding to the insulin receptor or increased proteolytic degradation within the medium in comparison with unprocessed proinsulin may provide at least a partial explanation (Vollenweider *et al.* 1992).

Greater than 70% of total pro/insulin secreted by COS-7 cells transfected with the mutant insulin construct represented mature unmodified human insulin. In previous COS-7 cell studies employing rat insulin constructs, <5% processing was reported with wild-type and 60% with mutant transfections (Yanagita *et al.* 1993). Processing of both wild-type and mutant propeptides may be limited by endogenous furin expression. Correlation between furin level and processing in a range of non-endocrine cell types has been described in addition to enhanced processing in both wild-type and mutant transfections on furin co-transfection (Yanagita *et al.* 1993, Shaw *et al.* 2002).

The application of this system as a sole treatment for diabetes mellitus would be limited for four main reasons. First, physiological insulin secretion is linked to glucose levels and there is no mechanism for glucose sensing within this system. Secondly, insulin is secreted in two phases, the first acute phase lasting minutes followed by a sustained secondary phase. The system described here requires days to efficiently switch off and switch on insulin secretion, so could not be used for a rapid first phase response. Thirdly, the system is regulated at the level of transcription and not at exocytosis; therefore if a quicker acting derivative were used there would still be a time delay of hours (to allow for transcription, translation, protein synthesis and processing) between antibiotic addition and insulin secretion. Fourthly, the cells used (COS-7) do not have the capacity to store peptides and, therefore, if tetracycline regulation were linked to exocytosis there would not be a sufficient build-up of insulin for an abundant release upon stimulation.

The requirements mentioned above are necessary for an efficient first phase insulin response. 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, however, 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 (Bolli *et al.* 1999). Tetracycline-mediated transcriptional regulation of insulin secretion by transfected non-endocrine cells 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. Despite the advent of novel long-acting insulin analogues, none of the available insulin formulations for subcutaneous injection have been able to mimic physiological basal insulin replacement leading to a continued risk of hypoglycaemia due to inappropriate peaks and troughs of insulin action over a 24-h period (Owens *et al.* 2001).

A further benefit of this system is the concomitant secretion of C-peptide with insulin. C-peptide was originally thought to have no biological function except to participate in insulin

synthesis; however recent studies have indicated that it has a biological role with important physiological effects. C-peptide has been demonstrated to improve renal function, nerve function and glucose utilisation in animal and human studies of diabetes mellitus (Wahren *et al.* 2000). C-peptide elicits its effects by binding to cell membrane via G protein-coupled receptors (Rigler *et al.* 1999). In this study, the wild-type proinsulin cDNA 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. Mutation into the C-peptide to allow processing by furin may alter receptor binding of C-peptide. A recent paper, however, demonstrated that Glu^{27} was crucial for binding and is unaltered in our mutant (Pramanik *et al.* 2001). However, further studies would be necessary to demonstrate effective C-peptide membrane binding.

In conclusion, these studies provide important proof of principle for the use of non-endocrine cells to restore basal secretion of fully processed unmodified human insulin that can be safely titrated and 'turned down' to prevent hypoglycaemia. Efficient transgene expression has been demonstrated *in vitro* despite the use of a two-plasmid system without viral vectors or selection of stably transfected clones. Potential future therapeutic applications with this approach include intramuscular reimplantation of *ex vivo* transfected host-derived dermal fibroblasts or myoblasts with subsequent uptake into muscle fibres mediating sustained background insulin secretion. Moreover, successful delivery of these plasmids 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 basal insulin treatment for individuals with Type 2 diabetes and may help those with Type 1 diabetes who are not complying with daily insulin injections. Truly constant basal insulin levels may facilitate more physiological glycaemic control with reduced hypoglycaemia in combination with preprandial injections of fast-acting insulin. Refinement of this technique also holds promise for the treatment of a broader range of endocrine and other disorders.

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