Vitamin H-regulated transgene expression in mammalian cells

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ABSTRACT

Although adjustable transgene expression systems are considered essential for future therapeutic and biopharmaceutical manufacturing applications, the currently available transcription control modalities all require side-effect-prone inducers such as immunosuppressants, hormones and antibiotics for fine-tuning. We have designed a novel mammalian transcription-control system, which is reversibly fine-tuned by non-toxic vitamin H (also referred to as biotin). Ligation of vitamin H, by engineered Escherichia coli biotin ligase (BirA), to a synthetic biotinylation signal fused to the tetracycline-dependent transactivator (tTA), enables heterodimerization of tTA to a streptavidin-linked transrepressor domain (KRAB), thereby abolishing tTA-mediated transactivation of specific target promoters. As heterodimerization of tTA to KRAB is ultimately conditional upon the presence of vitamin H, the system is vitamin H responsive. Transgenic Chinese hamster ovary cells, engineered for vitamin H-responsive gene expression, showed high-level, adjustable and reversible production of a human model glycoprotein in bench-scale culture systems, bioreactor-based biopharmaceutical manufacturing scenarios, and after implantation into mice. The vitamin H-responsive expression systems showed unique band pass filter-like regulation features characterized by high-level expression at low (0–2 nM biotin), maximum repression at intermediate (100–1000 nM biotin), and high-level expression at increased (>100 000 nM biotin) biotin concentrations. Sequential ON-to-OFF-to-ON, ON-to-OFF and OFF-to-ON expression profiles with graded expression transitions can all be achieved by simply increasing the level of a single inducer molecule without exchanging the culture medium. These novel expression characteristics mediated by an FDA-licensed inducer may foster advances in therapeutic cell engineering and manufacturing of difficult-to-produce protein therapeutics.

INTRODUCTION

Precise transgene expression dosing in mammalian cells has become a cornerstone for synthetic biology (1–4), (pre)clinical gene therapy studies (5–8), drug discovery (9,10), biopharmaceutical manufacturing (11–13) as well as for numerous applications in functional genomic research (14–16).

Research and development of inducible mammalian transgene expression systems has focused on clinically licensed small-molecule inducers to minimize pleiotropic side effects in cell culture, animal models or ultimately in clinical trials (17). Several antibiotics (18–22), steroid hormone analogs (23–25) and immunosuppressive substances (26,27) have been successfully used to modulate transgene expression in vitro and in vivo. Immunosuppressive drugs such as rapamycin can trigger transgene expression by conditional heterodimerization of FKBP, fused to a DNA-binding domain, and FRB, fused to a transactivation domain, which form a chimeric transactivator inducing transcription from specific promoters (26). Steroid hormone-based systems capitalize on the cytosolic sequestration of nuclear hormone receptors engineered to contain heterologous DNA binding and transactivation domains by endogenous heat-shock protein 90 [Hsp90; (28)]. Addition of steroid hormone analogs (for example, tamoxifen) displaces Hsp90 which results in nuclear translocation of the transactivator and induction of cognate promoters (29,30). Antibiotic-dependent expression systems [tetracyclines (19), macrolides (21), streptogramins (18)] take advantage of engineered prokaryotic repressors which activate or repress synthetic target promoters in an antibiotic-responsive manner (17).

The prototype tool for heterologous mammalian gene regulation is the tetracycline-responsive expression system
(known as the TET system) which consists of an Escherichia coli-derived tetracycline-dependent transactivator (tTA, a fusion of the TetR repressor and the Herpes simplex VP16 transactivation domain) that binds and activates tetracycline-responsive promoters (P_{hCMV-1}, heptameric TetR-specific operator sites [tetO] linked to a minimal version of the human cytomegalovirus immediate early promoter) (19). In the absence of tetracycline antibiotics, tTA binds tetO and triggers P_{hCMV-1}-driven transgene expression. In the presence of tetracycline, tTA-tetO binding is abolished and transcription remains shut down (19). Despite solid regulation characteristics, ongoing concerns about long-term side effects of antibiotics (31,32), as well as the rapid emergence of antibiotic resistance in bacteria exposed to subclinical environmental antibiotic concentrations (33), have prevented the use of the TET systems in gene therapy and biopharmaceutical manufacturing scenarios. Despite advantages of using clinically licensed drugs for therapeutic transgene expression fine-tuning, there are ongoing concerns about side effects associated with their prolonged administration in a clinical setting (28,32,34). For example, sustained administration of immunosuppressive drugs increases the susceptibility to bacterial infections (35), persistent intake administration of immunosuppressive drugs increases the risk for the emergence of multidrug-resistant pathogenic bacteria (33). Therefore, the design of novel transgene control modalities responsive to side-effect-free trigger molecules remains a current priority (17,39).

In E. coli synthesis of activated vitamin H (biotin) as well as biotinylation of target proteins is catalyzed by the dual-function biotin ligase BirA: (i) BirA activates biotin (biotinyl-5'-adenylate) and transfers the biotin moiety to an acceptor lysine within a specific signal sequence (40). (ii) Furthermore, intracellular biotin concentrations are feedback-controlled by biotinyl-5'-adenylate which triggers binding of BirA to a specific 40 bp operator site, thereby inhibiting transcription of the divergently oriented bioA-BFCD operon and preventing de novo synthesis of biotin (40). Several synthetic biotinylation signals have been recently described [Avitag signal, GLNDFIEAQKIEWHE, target lysine in bold print, (41)] and used in combination with E. coli BirA for in vivo and in vitro purification of biotinylated target proteins (42), chromatin immunoprecipitation, as well as for detection of protein–DNA complexes (43,44).

Capitalizing on the high affinity of biotin to (strept)avidin [10^{-11} M; (45)], we have designed a chimeric transrepressor, which is assembled from tTA-Avitag and streptavidin-KRAB (kruelppel-associated box protein of the human kox-1 gene) components by biotin-triggered heterodimerization, and capable of tTA-mediated induction of P_{hCMV-1} in a vitamin H-adjustable manner. Precise transgene expression fine-tuning using a non-toxic vitamin may represent an important advancement for the gene therapy and biopharmaceutical manufacturing communities.

**MATERIAL AND METHODS**

**Vector construction**

pWW938 (PSV40-tTA-AT-pA) encoding the tetracycline-dependent transactivator [tTA; (19)]-Avitag (AT) fusion (tTA-AT) under the control of the simian virus 40 promoter (PSV40) was constructed by PCR-mediated amplification of the Herpes simplex VP16 transactivation domain from pWW35 (21) using oligonucleotides OWW18 (5'-GTACGAATTCCTCCACGATCGTTGCA TCGAATTCCTGAGCCGAAGATGTCGTTCAGAC-3', annealing sequence in lower case) and OWW248 (5'-GGATCAAGCTTTGGCGCCGCCTATTTTGGCA TCGAATTCCTGAGCCGAAGATGTCGTTCAGAC-3', annealing sequence in lower case) and subsequent ligation of the VP16-AT module (BssHII/HindIII restriction site in italics) and subsequent ligation of the VP16-AT module (BssHII/HindIII) into pSAM200 (18). pWW944 (PSV40-SA-KRAB-pA) encoding streptavidin (SA) fused to the kruelppel-associated box protein of the human kox-1 gene (KRAB) was constructed by PCR-mediated amplification of streptavidin from pWW801 [PSV40-TetR-SA-pA, PSV40-simian virus 40 promoter; TetR, tetracycline-dependent repressor; (39)] using oligonucleotides OWW434 (5'-GGATCGAATTCCTCCACGATCGTTGCA TCGAATTCCTGAGCCGAAGATGTCGTTCAGAC-3', annealing sequence in lower case, EcoRI restriction site in italics) and in italics) and OWW433 (5'-GGATCAAGCTTTGGCGCCGCCTATTTTGGCA TCGAATTCCTGAGCCGAAGATGTCGTTCAGAC-3', annealing sequence in lower case, BssHII restriction site in italics) and subsequent ligation of the SA module (EcoRI/BssHII) into pWW43 (21). pWW732 (P_{hCMV-1}-IFN-β-pA) harboring beta interferon (IFN-β) under the control of the tetracycline-responsive promoter (P_{hCMV-1}) was constructed by excising IFN-β (EcoRI/HindIII) from pWW430 (46) and ligating it into pMF11 [P_{hCMV-1}-SEAP-pA, (18)], thereby replacing SEAP (human placental secreted alkaline phosphatase), pWW804 (P_{hCMV-1}-BirA-pA-PSV40-neo^R pA, P_{hCMV}, human cytomegalovirus immediate early promoter; PSV40, simian virus 40 promoter; neo^R, neomycin resistance gene) encoding the E. coli biotin ligase BirA has been described previously (39), in brief, BirA was excised (EcoRI/SpeI) from pGEM-SD2 [Strouboulis,J., unpublished data, (42)] and ligated (EcoRI/XbaI) into pMF150 [P_{hCMV}-PIP-pA-PSV40-neo^R-pA (18)]. BirA harbors an N-terminal hemaglutinin (HA) tag. The triple-transcript vector pTT-Bio [pWW1091, PSV40-SAKRAB-P_{GTX}-tTA-AT-P_{GTX}-BirA-pA, P_{GTX}, P_{GTX}, synthetic promoter (47)] was constructed in a three-step procedure: (i) SA-KRAB excised from pWW944 (EcoRI/HindIII) was ligated into the corresponding sites of the first multiple cloning site (MCS) of pCF263 [PSV40-MCS1 P_{GTX}-MCS2-P_{GTX}-MCS3-pA, (47,48)] resulting in plasmid pWW1089. (ii) BirA excised from pWW804 (SpeI/SmaI) was ligated (SpeI/SmaI) into the third MCS of pWW1089 resulting in plasmid pWW1090. (iii) Finally, tTA-AT excised from pWW938 (NotI) was ligated in...
sense orientation (NotI) into the second MCS of pWW1090 thereby resulting in pTT-Bio.

**Cell culture**

Wild-type Chinese hamster ovary cells (CHO-K1; ATCC CCL-61) were cultivated either in biotin-containing or biotin-free ChoMaster® HTS medium (Cell Culture Technologies, Gränesano, Switzerland) supplemented with 5% fetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany, Cat. No. 3302, Lot. No. P231902) or 10% biotin-free serum replacement (KOSR, Invitrogen, Carlsbad, CA, USA, cat. no. 10828-028), respectively, and 1% of a penicillin/streptomycin solution (Sigma, St. Louis, MO, USA, Cat. No. 4458). Human embryonic kidney cells [HEK293-T (49)] were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 10% FCS or 10% serum replacement and 1% penicillin/streptomycin solution.

**Transfection**

All transfection protocols were optimized for a well of a 24-well plate. CHO-K1: 30 000 CHO-K1 were seeded 12 h before transfection (0.5 ml biotin-free HTS medium supplemented with 5% FCS). An aliquot of 12 µl 0.5 M CaCl2 solution containing 1.2 µg total plasmid DNA (for co-transfections, equal amounts of individual plasmids were used) were mixed with 12 µl phosphate solution (50 mM HEPES, pH 7,05, 280 mM NaCl, 1.5 mM Na2HPO4), vortexed for 5 s, incubated for 25 s at room temperature to allow formation of the transfection precipitate and transferred to 0.4 ml ChoMaster® HTS medium containing 2% FCS. The culture medium was replaced by the transfection mixture and the plates were centrifuged for 5 min at 1200 x g prior to a 90 min incubation period and a 30 s glycerol shock (0.5 ml 15% NaCl, 1.5 mM Na2HPO4, pH 7.1), incubated for 20 min at 37 °C before transfection (0.5 ml biotin-free HTS medium containing 2% FCS). The culture medium was replaced by the transfection mixture and the plates were centrifuged for 5 min at 1200 x g prior to a 90 min incubation period and a 30 s glycerol shock (0.5 ml 15% glycerol in ChoMaster® HTS medium containing 2% FCS). After washing once in 0.5 ml biotin-free ChoMaster® HTS medium, the cells were cultivated in biotin-free or biotin-containing ChoMaster® HTS medium supplemented with 10% KOSR serum replacement or 5% FCS as indicated. HEK293-T: 40 000 HEK293-T were seeded 12 h before transfection (0.5 ml DMEM, 10% FCS). Twenty microliter of 0.25 M CaCl2 containing 0.6 µg total DNA (for co-transfections, equal amounts of individual plasmids were used) were mixed with 20 µl 2× HBS solution (100 mM HEPES, 280 mM NaCl, 1.5 mM Na2HPO4, pH 7.1), incubated for 20 min at room temperature to allow formation of the transfection precipitate, which was transferred dropwise to the cell culture and centrifuged onto the cells (5 min at 1200 x g). Transfected cells were incubated for 90 min at 37 °C before they were washed once in FCS-free DMEM and then cultivated in DMEM containing 10% biotin-free KOSR.

**Construction of the stable cell line pBioCHO-SEAP**

CHO-K1 were first co-transfected with plasmids pWW938 (Psv40-TA-AT-pA), pWW944 (Psv40-SA-KRAB-pA) and pWW804 (PbcMV-BirA-pA), also carrying a constitutive expression cassette conferring resistance to G418) at a ratio of 15:15:1, selected for 12 days in ChoMaster® HTS medium containing 5% FCS and 400 µg/ml G418 and subjected to single-cell cloning. Individual clones, which functionally express pWW938- and pWW944-encoded genes, were then co-transfected with pMF111 (PphCMV-1-SEAP-pA), pWW804 (PbcMV-BirA-pA) and pPUR (confering constitutive resistance to puromycin; Clontech, Palo Alto, CA, USA) at a ratio of 15:15:1 followed by selection for 10 days in ChoMaster® HTS medium containing 5% FCS, 400 µg/ml G418 and 15 µg/ml puromycin and single-cell cloning. Individual cell clones were assessed for biotin-triggered SEAP production and BioCHO-SEAP1 was chosen for further studies.

**Analytic assays**

BirA (HA-tagged) and tTA-AT (TetR-VP16-Avitag) were quantified by resolving cleared cell lysates on a 10% SDS–polyacrylamide gel followed by western blot analysis using anti-HA-tag (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat. No. sc-805) or anti-VP16 (Santa Cruz, Cat. No. sc-7545) as primary antibodies for detection and horseradish peroxidase-coupled anti-IgG antibodies for chemiluminescence-based visualization (ECL plus, GE Healthcare, Piscataway, NJ, USA, Cat. No. RPN2132) with a Chemilux CCD camera (Intas, Göttingen, Germany). Chemiluminescence signals were indicated as integrated optical density (IOD). Streptavidin-KRAB was quantified by incubating cleared cell lysate (40 µl) with 1 µg/ml FITC-biotin (60 µl) (Fluka, Buchs, Switzerland, Cat. No. 53608) for 30 min before measuring fluorescence intensity. Fluorescence of FITC-Biotin bound to streptavidin is quenched and can be used to quantify streptavidin (50). Fluorescence quenching is indicated in relative fluorescence units (RFU). SEAP production was quantified as detailed by Schlatter and coworkers (51) In brief, the cell culture supernatant, collected 48 h after seeding/transfection, was heated to 65 °C for 30 min to inactivate endogenous phosphatases and then centrifuged for 2 min at 14 000 x g to remove cell debris. An aliquot of 80 µl supernatant were mixed with 100 µl SEAP buffer (20 mM homoarginine, 1 mM MgCl2, 21% (v/v) diethanolamine, pH 9.8) and the reaction was started by addition of 20 µl nPP (120 mM p-nitrophenylphosphate in SEAP buffer) and the light absorbance timecourse scored at 405 nm. SEAP levels were calculated according to Lambert-Beer’s law using the slope of the timecourse and the specific absorption coefficient of the reaction product p-nitrophenolate (εpNP = 18 600 cm⁻¹ M⁻¹). IFN-β production was profiled by ELISA (PBL Biomedical Laboratories, Piscataway, NJ, USA, Cat. No. 41400-1A) and the cell number was determined either by a Casy™ Counter TTC (Schärfe System GmbH, Reutlingen, Germany) or by using a colorimetric WST-1-based assay (Roche Applied Science, Rotkreuz, Switzerland, Cat. No. 11644807001). All experimental
data represent average values derived from three independent experiments with SDs indicated by the error bars.

**Bioreactor operation**

Cells were cultivated in a BioWave® 50SPS bioreactor (Wave Biotech AG®, Tagelswangen, Switzerland) equipped with Wave Bag® 2LOpt for optical pH and DO control of the 11 culture. The bioreactor was operated at a rocking rate of 16 min\(^{-1}\), a rocking angle of 5°, and an aeration rate of 50 ml/min with inlet gas humidification to prevent evaporation of the medium (HumiCare® 200, Gruender Medical, Freudenstadt, Germany). Two milliliter samples were withdrawn at the indicated points in time. Samples for assessment of SEAP production were centrifuged (2 min, 14,000 \( \times \) g) and the supernatant was stored at \(-20^\circ\text{C}\) until analysis. Cell density was quantified using the colorimetric WST-1 kit (Roche Applied Science, Rotkreuz, Switzerland, Cat. No. 11644807001).

**Animal studies**

CHO-K1 transgenic for pWW938, pWW944, pWW804 and pMF111 were encapsulated in 400 \( \mu \)m alginate-PLL (poly-1-lysine)-alginate beads using the Inotec Encapsulator Research IE-50R (Inotech Biotechnologies Ltd., Basel, Switzerland) according to the manufacturer’s protocol and at the following specific settings: 0.2 mm nozzle, 20 ml syringe at a flow rate of 315 units, nozzle vibration frequency 1108 s\(^{-1}\), voltage for bead dispersion 800 V. Female OF1 (oncins france souche 1) mice were obtained from Iffa-Credo and kept on a biotin-free diet (Société SAFE, Augy, France) for two weeks, whereas the control mice were fed the same diet supplemented with 4 mg/kg biotin. Biotin deficiency was monitored using the forced swim test (52). 700 microliter of ChoMaster® HTS medium containing 50% capsules (2 \( \times \) 10\(^6\) cells, 200 cells/capsule) were injected intraperitoneally into the mice. When indicated, biotin was dissolved in 0.9% NaCl and administered by intraperitoneal injection 1 h after capsule implantation. Blood samples were collected retroorbital and serum was produced using microtainer SST tubes (Beckton Dickinson, Franklin Lakes, NJ, USA, Cat. No. 365968). Serum of control mice (implanted with capsules and kept with or without biotin supplementation) did not show any detectable SEAP levels. All experiments involving animals were conducted according to European Community legislation (86/609/EEC), and have been approved by the French Republic (No. 69266310) and the Institut Universitaire de Technologie, IUTA, F-69622 Villeurbanne Cedex, France.

**Inducers**

D (+)-biotin (Acros Organics, Geel, Belgium, Cat. No. 23009) was prepared as a 10 \( \mu \)M stock solution in \( \text{H}_2\text{O} \) and was typically used at a final concentration of 100 nM unless stated otherwise. At concentrations above 10 \( \mu \)M, biotin was dissolved as stock solution in DMSO. Avidin (Pierce, Rockford, IL, USA, Cat. No. 21121) was prepared as a 100 \( \times \) stock solution in phosphate-buffered saline (PBS; 20 mM NaH\(_2\)PO\(_4\), 150 mM NaCl, pH 7.2). The concentration of avidin was expressed as biotin-binding capacity. Tetracycline (Sigma, Cat. No. T7660) was prepared as a 1 mg/ml stock solution in \( \text{H}_2\text{O} \) and used at a final concentration of 2 \( \mu \)g/ml. For determination of biotin stability, ChoMaster® containing 100 \( \mu \)M biotin and 5% FCS was incubated for one week at 37°C and biotin samples were quantified daily using a HABA (2-(4-hydroxyazobenzene) benzoic acid)-based biotin quantification kit (EZ biotin quantification kit, Pierce, Rockford, IL, USA Cat. No. 28005). No significant decrease in biotin concentration could be observed. In pigs, biotin shows a half-life time of up to 22 h (53).

**RESULTS**

**Design of a vitamin H-responsive mammalian expression system**

In order to enable precise expression dosing by vitamin H, we have converted proven tetracycline-responsive expression system into a vitamin H-adjustable transcription control modality (Figure 1A). In the presence of biotin (+ Biotin) constitutive expression of the *Escherichia coli* biotin ligase BirA [pWW804; \( \text{P}_{\text{Bcmv}} \text{-BirA-pA}; (42,54) \)] biotinylates tetracycline- [ITA, TetR-VP16; (19)] dependent transactivators, which have been fused to the synthetic biotinylation signal (Avitag, AT; (41); pWW938, \( \text{P}_{\text{SV40}} \text{-TATA-AT-pA}, \) Figure 1C-i) thereby promoting biotin (B)-dependent heterodimerization with the krueppel-associated box protein of the human kox-1 gene (KRAB; SA-KRAB; pWW944, \( \text{P}_{\text{SV40}} \text{-SA-KRAB-pA} \) which has been fused to streptavidin (SA) (Figures 1A and C-ii). Since KRAB-mediated silencing overrides the VP16-based transactivation capacity of TATA-AT, the chimeric transrepressor ([TATA-BSA-KRAB] binds and represses the established tetracyline- (\( \text{P}_{\text{Bcmv}} \text{-1; pMF111, P}_{\text{Bcmv}} \text{-1-SEAP-pA} \) responsive promoter (Figure 1C-iii). At high biotin concentrations (Figure 1C-iv), all avitag and streptavidin sites are expected to be saturated by free biotin thereby competitively inhibiting SA-AT heterodimerization and de-repressing target gene expression.

Biotin deprivation (Figure 1C, −Biotin) prevents or abolishes heterodimerization of chimeric transrepressors thereby enabling classic tTA-mediated transgene expression which can be repressed by addition of regulating tetracycline antibiotics (Figure 1C, +TET) independent of biotin availability. Expression of all biotin-responsive regulatory genes has been validated by western blot analysis or fluorescence quenching (Figure 1B).

**Vitamin H-controlled transgene expression in mammalian cell lines**

Vitamin H-controlled transgene expression was validated by co-transfection of CHO-K1 with pWW804 (\( \text{P}_{\text{Bcmv}} \text{-BirA-pA}, \) pWW944 (\( \text{P}_{\text{SV40}} \text{-SA-KRAB-pA}, \) pWW938 (\( \text{P}_{\text{SV40}} \text{-TATA-AT-pA}, \) and pMF111 (\( \text{P}_{\text{Bcmv}} \text{-1-SEAP-pA} \) followed by SEAP quantification 48 h after cultivation in the presence or absence of biotin and the regulating antibotic tetracycline (Figure 2A). Cells exhibited high-level SEAP expression in the absence of regulating agents and repressed transgene expression in the presence of biotin or tetracycline. The biotin-regulated gene expression
system also mediated conditional SEAP expression in human embryonic kidney cells (HEK293-T) (Figure 2B) and both vitamin H and tetracycline could be used to control the production of the multiple sclerosis therapeutic beta interferon (IFN-β; pWW732, P_hCMV/C3/C0-1-IFN-β-pA) (Figure 2C).

Specificity of the biotin-controlled expression system was assessed by selective omission individual components including (i) the biotin ligase BirA (pWW804), (ii) SA-KRAB (pWW944), (iii) BirA and SA-KRAB (pWW804, pWW944), (iv) BirA, SA-KRAB, tTA-AT (pWW804, pWW944, pWW938) or (v) by replacing the avitag-containing tTA-AT (pWW938) by the original tTA (pSAM200, PSV40-tTA-pA). Exclusion of either BirA, SA-KRAB or Avitag abolished biotin-responsive transgene repression while
tetracycline-responsive expression control remained intact (Figure 2D). In the absence of either tTA-AT or tTA no SEAP expression could be observed indicating that all components are essential and sufficient for biotin-controlled gene expression (Figure 2D). In all of these experiments, SEAP production reached identical maximum levels in the absence of biotin and tetracycline, indicating that the AVITAG fusion does not impact tTA function (tTA-AT, pWW938, 2.9 ± 0.3 U/l; tTA, pSAM200, 2.7 ± 0.1 U/l).

To provide biotin-controlled gene regulation in an easy-to-handle two-vector format (19), we have cloned
all components (SA-KRAB, tTA-AT, BirA) into a triple-transcript expression format, in which the first cistron (SA-KRAB) is transcribed from a simian virus 40 promoter (P_{SV40}) while subsequent cistrons two (tTA-AT) and three (BirA) are under control of compact synthetic promoters (P_{GTX}) (47,48) (pTT-Bio; P_{SV40}-SA-KRAB-P_{GTX}-tTA-AT-P_{GTX}-BirA-pA). Co-transfection of pTT-Bio with the response vector pMF111 into CHO-K1 cells resulted in high-level SEAP expression in the absence of biotin while addition of biotin and/or tetracycline shut off SEAP production (Figure 2E).

**Adjustability and induction kinetics of the biotin control system**

Exposure of CHO-K1 containing components mediating biotin-controlled SEAP expression (pWW938, pWW944, pWW804 and pMF111) to increasing vitamin H concentrations followed by scoring of SEAP production 48 h after transfection revealed adjustability over 2 logs of biotin concentrations. Maximum SEAP production occurred below 1 nM of biotin and SEAP repression to background levels occurred at 100 nM of biotin (Figure 3A). Biotin-dependent expression kinetics were assessed by cultivating CHO-K1 cells transfected with plasmids pWW938, pWW944, pWW804 and pMF111 for 68 h in the presence (100 nM) or absence of biotin which resulted in linear induction profiles between 5 and 53 h after transfection (Figure 3B).

**Validation of biotin-controlled product protein secretion in mice**

In order to validate biotin-responsive expression technology in a prototype gene therapy scenario, we micro-encapsulated CHO-K1 engineered for biotin-controlled production of the human model glycoprotein SEAP into coherent alginate-poly-l-lysine-alginate capsules and injected them intraperitoneally into mice kept on a biotin-free diet. After implantation, the mice were injected with either biotin (100 µg/ml) or doxycycline (100 mg/kg) and the serum levels of SEAP were profiled after 48 h. SEAP profiles confirmed maximum product protein production in the absence of biotin and repressed protein production in the presence of biotin or doxycycline (Figure 4A). Relative *in vivo* regulation profiles were similar compared to identical subcultures maintained *in vitro* suggesting that the biotin control system retains its regulation characteristics *in vivo* (Figure 4B). The higher basal expression in the presence of doxycycline or biotin *in vivo* (Figure 4A) compared to *in vitro* (Figure 4B) may result from lower bioavailability of the inducer *in vivo*. However, since biotin- and doxycycline-mediated *in vitro* and *in vivo* repression profiles are comparable, the regulation performance of both systems may be considered equivalent.

**Stable CHO-K1 cell derivatives tetra-transgenic for biotin-responsive transcription-control components**

Extensive characterization of the biotin-responsive transcription control system for biopharmaceutical manufacturing requires availability of a stable cell line for long-term studies. We have therefore constructed a stable CHO-K1-derived cell line, which is tetratransgenic for pWW938 (P_{SV40}-tTA-AT-pA), pWW944 (P_{SV40}-SA-KRAB-pA), pWW804 (P_{hCMV}-BirA-pA) and pMF111 (P_{hCMV}-P_{−1}SEAP-pA) and produces SEAP in a biotin and tetracycline-responsive manner. Five out of 40 stable cell clones were cultivated for 48 h in biotin- and/or tetracycline-containing medium and profiled for SEAP production. All cell clones showed high SEAP production when grown in the absence of regulating molecules (−biotin, −tetracycline), while either addition of biotin or tetracycline abolished SEAP production (Figure 5A). The different expression levels observed for individual cell clones may result from random chromosomal integration and transcription-modulating impact of adjacent genetic elements. Likewise, transgene expression differences also exist between stable and transiently transfected populations, which produce the product protein from expression vector episomes (55,56). Since *Bio*CHO-SEAP clone no. 1 (BioCHO-SEAP1) showed the best regulation profile it was
chosen for further characterization. Expression stability of BioCHO-SEAP1 was confirmed by continuous cultivation of the cell line for 90 days, which did not reveal any statistically significant decrease in SEAP production (day 0, 0.67 ± 0.5 U/L; day 90, 0.65 ± 0.6 U/L). Biotin-responsive SEAP production kinetics for which BioCHO-SEAP1 was seeded in biotin-free or biotin-containing medium (t = 0) revealed a steady increase over time when cultured in the absence of an inducer, while biotin addition resulted in a gradual increase in SEAP levels only (Figure 5B). Reversibility of biotin-responsive gene expression induction was assessed by cultivating initial BioCHO-SEAP1 populations in either biotin-free or biotin-containing medium. After 31 and 100 h of cultivation, the cell population was split and the biotin status maintained or reversed as indicated. Sequential induction and repression of SEAP production was followed over a one-week period (Figure 5C). Biotin-controlled SEAP expression was reversible and the induction or repression kinetics were not influenced by the biotin cultivation history of the BioCHO-SEAP population (Figure 5C). The dose-response profiles recorded over 7 biotin concentration logs revealed unique expression characteristics: (i) gradual ON-to-OFF expression profiles between 1 and 100 nM, reflecting increasing heterodimerization of transsilencer and transsilencer-mediated repression of tTA-AT-driven Pϕ-AT transcription. (ii) Full OFF state at biotin concentrations ranging from 100 to 1000 nM, reflecting maximum transsilencing capacity. (iii) graded OFF-to-ON expression at biotin levels above 100 μM, suggesting that free biotin levels exceeding the ligation capacity of BirA may bind and sequester SA-KRAB thereby competitively inhibiting its heterodimerization with biotinylated tTA-AT (Figure 5D).

**Biotin-inducible expression in biotin-containing environments**

While all aforementioned experiments were conducted in biotin-free medium, standard cell culture media and serum additives contain biotin at an average level of 50 nM which fully represses the biotin control system (Figure 5D). Dose-response characteristics of BioCHO-SEAP1 cultivated in standard serum-containing cell culture medium and exposed to increasing biotin concentrations resulted in a gradual increase in SEAP production, confirming that the biotin control modality operates as an inducible expression system under standard culture conditions (Figure 6A). Furthermore, expression kinetics in the presence of 2000 μM biotin increased steadily, whereas the absence of additional biotin resulted in background expression only (Figure 6B).

A further strategy to induce the biotin control system is to sequester repressing biotin in standard medium by addition of avidin. Cultivation of BioCHO-SEAP1 in the presence of increasing avidin concentrations resulted in a gradual increase in SEAP production, thereby enabling adjustable transgene expression by administration of an exogenous protein (Figure 6C). Expression kinetics in the presence or absence of avidin increased steadily over time (Figure 6D). However, the overall regulation performance using avidin as an inducer was lower compared to biotin-triggered expression control (Figure 6A and C), suggesting that avidin is unable to fully compete with the thermodynamically favored enzymatic biotinylation of tTA-AT.

**Biotin-inducible protein production in a prototype biopharmaceutical manufacturing scenario**

Despite intensive research no significant side effects have been discovered for biotin, neither following oral uptake nor after injection (57). Therefore, this FDA-licensed molecule represents an ideal inducer for any bioprocess application requiring precisely timed expression control (11,12,58). We have therefore evaluated the compatibility of vitamin H-responsive gene expression with standard bioreactor operation by cultivating BioCHO-SEAP1 for 53 h in a BioWave® bioreactor using 1 L of standard biotin-containing (non-supplemented) culture medium before adding 2 mM biotin to trigger SEAP production. While BioCHO-SEAP1 grew exponentially during the entire
period, SEAP expression remained silent until shortly after addition of biotin, thereby validating the suitability for timely controlled biotin-responsive gene expression in a prototype biopharmaceutical manufacturing scenario (Figure 7).

**DISCUSSION**

Adjustable transgene expression technology is fundamental to prototype gene therapy scenarios (6,7,59–61), functional genomic research (14,16), synthetic biology (1–3,62) and forefront biopharmaceutical manufacturing strategies (11,12,58,63). The novel vitamin H-responsive expression technology exhibits several advantages regarding standard operation and regulation performance compared to established systems which are typically triggered by side-effect-prone immunosuppressants, hormones or antibiotics (32,34,35,37,38): Vitamin H (biotin) is the first inducing agent with no or only low known side effects (e.g. to our knowledge, no LD₅₀ has ever been described). As biotin is a natural component of cell culture media, its removal or supplementation to native levels is not expected to require additional validation or licensing efforts for biopharmaceutical manufacturing as would be the case for pharmacologically active inducers such as hormones, immunosuppressors and antibiotics.

When comparing biotin-adjustable regulation characteristics to classic expression systems, biotin-adjustable transgene control exhibits unique band pass filter-like expression characteristics using a single inducer...
molecule: (i) within the low biotin concentration window (0–2 nM), transcription is fully induced, (ii) it is maximally repressed between 100 and 1000 nM biotin and (iii) again entirely turned on beyond 100 μM. This exclusive feature enables sequential induction, repression and re-induction using increasing doses of vitamin H, an expression pattern which has so far only been achieved in bacteria using a sophisticated gene network (64). The unique biotin-dependent band pass filter characteristic could be explained by a competition of tTA-AT and SA-KRAB for free biotin. At low biotin concentrations (0–100 nM), binding of biotin to tTA-AT [via the intermediate biotin–BirA complex, (65)] is thermodynamically favored [\( \Delta G_0 = -360 \text{ kJ/mol} \) for an amide bond (in trans) versus \( \Delta G_0 = -80 \text{ kJ/mol} \) for the non-covalent binding of biotin to SA-KRAB (66,67)], resulting in biotinylated tTA-AT and subsequent transcriptional shutdown after binding to SA-KRAB. At high biotin concentrations (>100 μM) free biotin is expected to saturate all tTA-AT and SA-KRAB.
biotin-binding sites thereby preventing dimerization with biotinylated tTA-AT. While basic research scientists may appreciate the extended regulation bandwidth emulating developmental expression patterns (64,68–70), the bio-pharmaceutical manufacturing could welcome the first opportunity to reverse the expression state of difficult-to-produce protein therapeutics without media exchanges and filtration operations.

Owing to the use of TetR as the DNA-binding component, the biotin-responsive gene expression technology is compatible with standard tetracycline-responsive promoters, established cell lines and mouse strains (15,19,20,59) and is unique in integrating two input signals: biotin and tetracycline. This dual-input sensitivity permits override of the biotin-controlled transgene expression by administration of tetracycline which may represent a useful safety feature in future cell engineering strategies. Since biotin is a physiological component the synthetic control modality may be used to convert endogenous biotin concentrations into a therapeutic transgene response. Scenarios to treat biotinidase-associated metabolic disorders by triggering therapeutic biotinidase expression at low pathologic biotin concentrations and shutting it off when physiological biotin concentrations are reached, could now become a reality (71–73). Despite the ubiquitous presence of biotin in living systems, interference-free transgene control can still be achieved by (i) addition of biotin-sequestrating avidin or by (ii) administration of excess biotin (>1000 nM) thereby taking advantage of the high-biotin regulation window of the aforementioned band-pass filter.

Considering all of the design and performance characteristics in mammalian cells, mice as well as during standard bioreactor operation, we are convinced that biotin-controlled transgene expression will foster advances in basic research, gene therapy scenarios as well as in biopharmaceutical manufacturing.

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