Streptomyces-derived quorum-sensing systems engineered for adjustable transgene expression in mammalian cells and mice

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ABSTRACT

Prokaryotic transcriptional regulatory elements have been adopted for controlled expression of cloned genes in mammalian cells and animals, the cornerstone for gene-function correlations, drug discovery, biopharmaceutical manufacturing as well as advanced gene therapy and tissue engineering. Many prokaryotes have evolved specific molecular communication systems known as quorum-sensing to coordinate population-wide responses to physiological and/or physicochemical signals. A generic bacterial quorum-sensing system is based on a diffusible signal molecule that prevents binding of a repressor to corresponding operator sites thus resulting in derepression of a target regulon. In *Streptomyces*, a family of butyrolactones and their corresponding receptor proteins, serve as quorum-sensing systems that control morphological development and antibiotic biosynthesis. Fusion of the *Streptomyces coelicolor* quorum-sensing receptor (ScbR) to a eukaryotic transactivation domain (VP16) created a mammalian transactivator (SCA) which binds and adjusts transcription from chimeric promoters containing an SCA-specific operator module (P_{SPA}). Expression of erythropoietin or the human secreted alkaline phosphatase (SEAP) by this quorum-sensor-regulated gene expression system (QuoRex) could be fine-tuned by non-toxic butyrolactones in a variety of mammalian cells including human primary and mouse embryonic stem cells. Following intraperitoneal implantation of microencapsulated Chinese hamster ovary cells transgenic for QuoRex-controlled SEAP expression into mice, the serum levels of this model glycoprotein could be adjusted to desired concentrations using different butyrolactone dosing regimes.

INTRODUCTION

Integrated molecular interventions in mammalian cells and animals which require precisely adjusted expression of desired transgenes (1) have fostered recent advances in prototype gene therapy scenarios (2), drug discovery (3), biopharmaceutical manufacturing (4,5), gene-function analysis (6–8) and the design of animal models mimicking prominent human disease phenotypes (7).

In recent years, a variety of heterologous transgene control systems have been designed for use in mammalian cells and transgenic animals (1,9). Most modulate target gene transcription in response to antibiotics (10–12), immunosuppressive agents (13) or steroid hormones and their analogs (14,15). Although well established in basic research, ongoing concerns about low or leaky expression, promotion of antibiotic resistance, difficulties in managing the levels of regulating chemicals and interference of inducers with host regulatory networks may jeopardize long-term use of current gene control arrangements in human therapy and substantiate the need for gene regulation systems responsive to a new class of molecules (16–18).

Quorum-sensing systems regulate prokaryotic gene expression in response to fluctuations in cell-population density. Quorum-sensing bacteria produce and release chemical signal molecules and communicate within and across species to regulate a diverse array of activities including symbiosis, virulence, competence, conjugation, motility, sporulation, biofilm formation and antibiotic production (19,20). In *Streptomyces*, a family of related butyrolactones and their

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corresponding receptor proteins serve as quorum-sensing systems to coordinate morphological development and antibiotic biosynthesis (20,21).

Here we report the use of *Streptomyces coelicolor* and *Streptomyces pristinaespiralis* butyrolactone-responsive quorum-sensing systems to adjust transgene expression in mammalian cells and mice. The use of quorum-sensing pheromones *in vivo* did not elicit prominent side effects suggesting that butyrolactones are safe inducers. Since most bacterial species are endowed with a specific quorum-sensing system (19,22), *Streptomyces*-derived QuoRex technology is a promising prototype of a new mammalian gene control system using an alternative class of inducing molecules.

MATERIALS AND METHODS

Synthesis and formulation of SCB1 and MP133

Racemic 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide (SCB1) was synthesized as depicted in Figure 1A. The butanolide (**2**) was formed by the reduction of diethyl formylsuccinate (**1**) (23) with $NaBH_4$, and the hydroxyl group of this butanolide was subsequently protected with a trimethylsilyl ether (24). The alkyl chain of SCB1 was synthesized starting from diethyl malonate and 1-bromo-4-methylpentane (25), which resulted in a 42% (w/w) yield of 6-methylheptanoic acid (**4**) over three steps. The acid was converted into its acid chloride with thionyl chloride and attached to 3-(trimetylsilyloxymethyl)-butanolide (**3**) using lithium diisopropylamine in dry tetrahydrofuran. After removal of the trimethylsilyl group, racemic 2-(6-methyl-heptanoyl)-3-(hydroxymethyl)-butanolide (**5**) or A-factor was obtained. The reduction of the ketone carbonyl group of **5** with $NaBH_4$ and separation by preparative C18 reversed-phase HPLC resulted in racemic mixtures of the diastereoisomers of SCB1 (**6**). Diastereomers were over 99% pure as assessed by HPLC, and NMR-based analysis revealed no cross contamination by other diastereomers. A detailed protocol for the synthesis of SCB1 is provided at http://www.biotech.biol.ethz.ch/martinf/download/QuoRex.zip.

Synthesis of 4-methyl-3-pentyl-2(5*H*)-furanone (MP133) is shown in Figure 1B. Two synthetic pathways were designed for MP133. Whereas the first strategy follows Demnitz's approach to furanones (26), the second protocol is analogous to the preparation of some furanones which naturally occur in *Streptomyces antibioticus* (27). According to the synthesis by Grossmann and Séquin (27), the readily available methylated furanone (**7**) was condensed with pentanal. Subsequent deprotection with BBr_3 followed by $NaBH_4$-reduction resulted in a racemate (**8**). This racemate was then tosylated at the side chain hydroxy group, and the tosylate obtained reduced with $NaBH_4$ to give MP133 (**9**). Supplementary information on the synthesis of MP133 is available at http://www.biotech.biol.ethz.ch/martinf/download/QuoRex.zip.

SCB1 and MP133 were dissolved in DMSO at 100 mg/ml and diluted to reach a final DMSO concentration of 0.1% (v/v) in all cell culture experiments. Both butyrolactones were used at a concentration of 10 μg/ml unless stated otherwise.

Plasmid construction

The *S.coelicolor*-derived butyrolactone-dependent transactivator SCA (ScbR-VP16) was constructed by PCR-mediated amplification of *scbR* from *S.coelicolor* A3(2) *scbR* using oligonucleotides OWW36 (5′-gtacgaattcccaccatggccaagcag-gaccgggcg-3′) and OWW37 (5′-gcgcgcggctgtacgcg-gagtccttcccggtcggtgccagtt-3′) followed by cloning (EcoRI/BssHII) into pWW35 (10), thereby resulting in pWW122. Similarly, the *S.pristinaespiralis*-derived transactivator SPA (SpbR-VP16) was designed by cloning PCR-amplified [oligonucleotides OWW34 (5′-gtacgaattcccaccatggcgcgcggcag-gagcgg-3′) and OWW35 (5′- gcgcgcggctgtacgcggagtggg-cggcgggctggg-3′)] *spbR* from *S.pristinaespiralis* (strain NRRL2958) EcoRI/BssHII into pWW35 to give pWW123. SCA- and SPA-responsive promoters [P_{SCA} (O_{scbR}-$P_{hCMVmin}$); P_{SPA} (O_{papRI}-$P_{hCMVmin}$)] were assembled by amplifying $P_{hCMVmin}$-encoding fragments from pRevTre (Clontech, Palo Alto, CA) with OWW39 (5′-gatcgacgtcgccattgaaaaccgaccgtg-ccgttttttttcctgcaggtcgagctcggtacccgggtc-3′; O_{papRI} site underlined) and OWW22 (10) as well as OWW49 (5′-gatcgacg-tctaagatacagactgagcggtttttttttcctgcaggtcgagctcggtacccgggtc-3′; O_{scbR} site underlined) and OWW22 (10), respectively. The P_{SCA} and P_{SPA}-encoding fragments were cloned (AatII/EcoRI) into pWW36 (10) to result in plasmids pWW124 (P_{SCA}-SEAP-pA) and pWW135 (P_{SPA}-SEAP-pA), respectively. The EPO expression vector pWW156 (P_{SPA}-EPO-pA) was constructed by EcoRI/XhoI-mediated swapping of SEAP (pWW135) with EPO encoded by pMF242 (10–12). The SCA-encoding retroviral vector pWW158 was constructed by excising SCA (EcoRI/BamHI) from pWW122 and cloning it (EcoRI/BglII) into pMSCVneo (Clontech). pWW122-derived retroviruses were produced following Clontech's protocol and used for transduction of CHO-K1. The bacterial *scbR* expression vector pWW314 (P_n-*scbR*-*his6*-*pA*) was constructed by PCR-mediated amplification of *scbR* from pWW122 using primers OWW304 (5′- cggaattcccaccatgca-tatggccaagcagg accgggc-3′) and OWW305 (5′-gctctagag-caagcttttaatggtgatggtgatgatgggatccacgcggaaccagaccgtccttccc-ggtcggtgcca-3′) followed by cloning (NdeI/HindIII) into pRSETmod (Weber,C.C., Link,N., Fux,C., Zisch,A.H., Weber,W. and Fussenegger,M., submitted for publication). Plasmid pWW56 has been described previously (10).

Gel retardation assays

P_{SCA}- and P_{SPA}-encoding probes were generated by PCR using plasmids pWW124 and pWW135 as templates, [^{32}P]dATP and primers OWWF (5′-ggttccgccacatttcccc-3′) and OWWR (5′-aagctgactctagaggatcc-3′). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) were transformed with pWW314 and grown in LB to an OD_{600} of 0.6 prior to induction of *scbR* with 1 mM IPTG for 3 h. Transgenic *E.coli* were collected by centrifugation and disrupted in TA buffer [10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) Triton X-100, 50 mM NaCl]. Crude protein extract (10 μg) was incubated with SCB1 for 10 min in the presence of 2 μg of competitor DNA [poly(dI–dC)·(dI–dC)] and then mixed with the probe. Protein–DNA complexes were resolved on 5% (w/v) polyacrylamide-TBE [90 mM Tris, 90 mM borate, 2 mM EDTA (pH 8)] gels, run at room
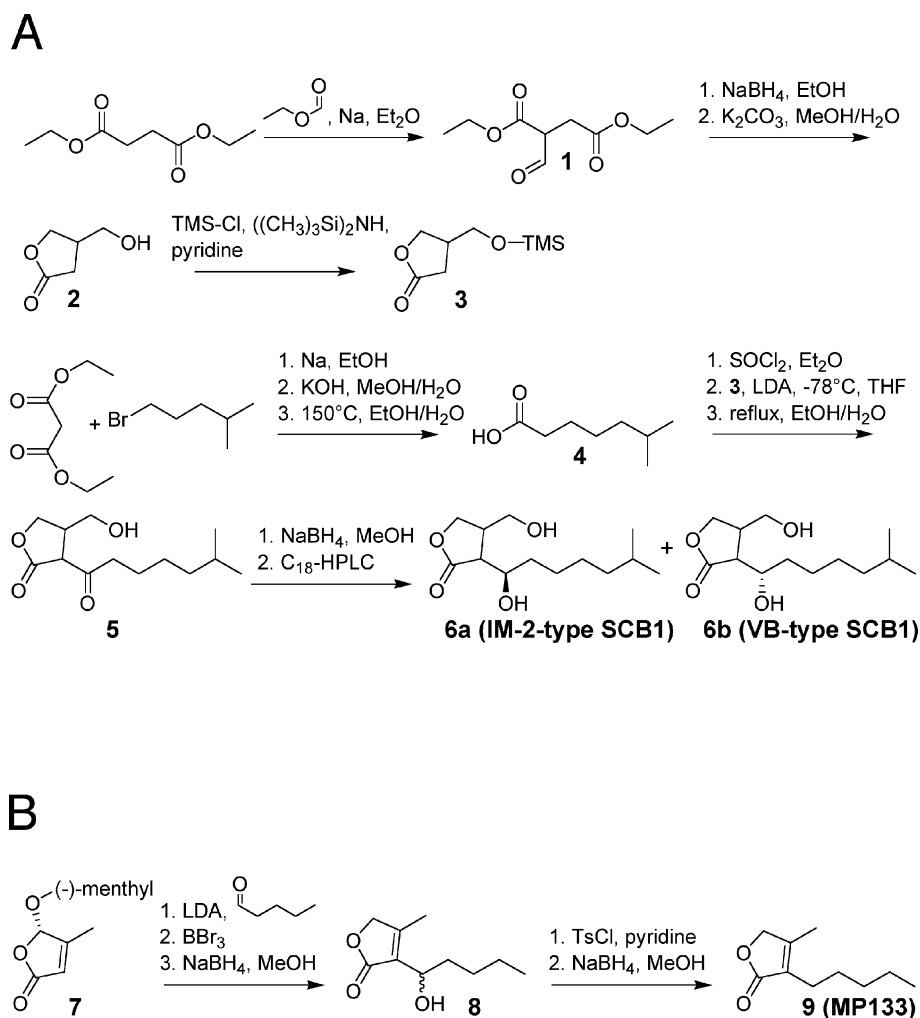
A

B

Figure 1. Synthesis of SCB1 (**A**) and MP133 (**B**). More detailed information is provided at http://www.biotech.biol.ethz.ch/martinf/download/QuoRex.zip.

temperature and constant voltage (7 V cm^{-1}) for 2 h, dried and visualized by autoradiography.

Cell culture, transfection, stable cell lines and reporter gene assays

Chinese hamster ovary cells (CHO-K1, ATTC CCL-61) were cultured in FMX-8 complete medium (FMX-8; Cell Culture Technology, Zürich, Switzerland) supplemented with 10% (v/v) FCS and 1% (v/v) of a penicillin/streptomycin solution (cat. no. P4458; Sigma, St Louis, MO), African green monkey kidney cells (COS-7, ATCC CRL-1651), human embryonic kidney cells [HEK293-T (28)], baby hamster kidney cells (BHK-21, ATCC CCL-10) and human hepatocellular carcinoma cells (HepG2, ATCC HB-8065) were cultivated in DMEM-complete medium [DMEM supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin solution]. Human umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany) and mouse embryonic stem cells (StemCell Technologies, Meylan, France) were cultivated according to the suppliers' protocols.

Twelve hours prior to transfection, 60 000 CHO-K1 cells were seeded per well of a 24-well plate. Plasmid DNA (1.2 µg per well; if a cotransfection was made, equal amounts of each plasmid were used) in 0.5 M CaCl$_2$ (final volume: 12 µl) was mixed with 12 µl of phosphate solution (50 mM HEPES, pH 7.05, 280 mM NaCl, 1.5 mM Na$_2$HPO$_4$), vortexed for 5 s and incubated for 25 s prior to addition of 0.4 ml of FMX-8 medium containing 2% (v/v) FCS. The culture medium was replaced by the transfection mixture and cells were incubated for 5 h prior to performing a 30 s glycerol shock [0.5 ml of 5% (v/v) glycerol in FMX-8 medium containing 2% (v/v) FCS] and one wash step (1 ml of FMX-8 complete medium). The cells were cultivated in 0.5 ml of FMX-8-complete medium in the presence and absence of the regulating butyrolactone until reporter gene expression was quantified.

For transfection of HEK293-T, COS-7, BHK-21 and HepG2 cells, 120 000 cells were seed per well of a 12-well plate containing 1 ml of DMEM complete medium and grown for 12 h prior to transfection. Total plasmid DNA (0.6 µg) in 50 µl of 0.25 M CaCl$_2$ was added dropwise to 50 µl of 2× HBS (100 mM HEPES, pH 7.1; 280 mM NaCl, 1.5 mM

Na_2HPO_4) while vortexing, followed by addition to the cells. After a 4 h incubation time, the supernatant was removed and the cells were washed once with pre-warmed DMEM complete medium. Subsequently, the cells were cultured in 1 ml of DMEM complete medium per well in the presence or absence of regulating butyrolactone until assessment of reporter gene expression. Mouse ES cells (60 000 cells/well of a 12-well plate) were transfected using FuGENE 6 (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the suppliers instructions. The regulating butyrolactone was added 4 h post-FuGENE 6-mediated transfection.

For HUVEC transfection, 300 000 cells were seeded per well of a 6-well plate 12 h prior to transfection. Total plasmid DNA (20 µg) in 150 µl of PBS (10 mM NaH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl) were mixed with 150 µl of DEAE Dextran solution (0.83 µg/µl in PBS; cat. no. D9885; Sigma). The cells were washed twice with PBS and incubated with the transfection mix for 30 min at 37°C in a humidified atmosphere containing 5% (v/v) CO_2-containing atmosphere at 37°C. The plate was gently shaken every 10 min. After 30 min, 2 ml of medium (EC medium; Promocell, Heidelberg, Germany) supplemented with 100 µM chloroquine (cat. no. C6628; Sigma) was added to the cells followed by incubation for another 2.5 h. The medium was removed and the cells were treated with 1 ml of 9% (v/v) DMSO in EC medium for 90 s followed by a washing step and incubation in the presence and absence of the regulating butyrolactone for expression profiling of the reporter gene.

The stable SCA production cell line CHO-SCA was constructed by transducing CHO-K1 with pWW158-derived retroviruses. For construction of the double transgenic cell line CHO-SCA-SEAP, $CHO-SCA_1$ was cotransfected with pWW124 and pZeoSV2 (Invitrogen, Carlsbad, CA). Stable cell lines were selected and maintained in culture medium supplemented with appropriate antibiotics (400 µg/ml G418, 100 µg/ml zeocin). Mixed stable cell populations were cloned using FACS-mediated single cell sorting (FACStar^{Plus}; Beckton Dickinson, San Jose, CA). For all analyses CHO-SCA-SEAP was used at a density of 66 000 cells/ml. SEAP and EPO expression were quantified as described previously (12). All data represent average values of three independent transfections/cultures.

In vivo methods

Cells were encapsulated in alginate-poly-(L-lysine)-alginate beads (alginate-PLL-alginate; 200 cells/per capsule, 2×10^6 cells/animal) as described previously and injected intra-peritoneally into female OF1 mice (oncins france souche 1; Iffa-Credo, Lyon, France) (10,29) obtained from Iffa-Credo. One hour after capsule implantation, mice were injected with different IM-2-SCB1 doses, diluted in 0.9% (w/v) NaCl from a stock solution of 100 mg/ml in ethanol. Serum SEAP levels were quantified from at least eight mice per dose as described (29, Fux,C., Weber,W. and Fussenegger,M., submitted for publication). All experiments involving animals were approved by the French Ministry of Agriculture and Fishery (Paris, France) and performed by M.D.E. at the Institut Universitaire de Technologie, IUTA, F-69622 Villeurbanne Cedex, France.

RESULTS

Design of a mammalian transcription control system based on *Streptomyces* quorum-sensing components

Exploiting molecular design concepts of the butyrolactone quorum-sensing system which evolved in *Streptomyces* to control antibiotic biosynthesis and/or coordinate morphological differentiation, we have constructed binary mammalian transcription control units called the quorum-sensor-regulated gene expression system (QuoRex). The receptors of the *S.coelicolor* [ScbR; *S.coelicolor* butyrolactone transcriptional repressor; (30)] and *S.pristinaespiralis* [SpbR; *S.pristinaespiralis* butyrolactone transcriptional repressor (21)] butyrolactone quorum-sensing systems were each fused to the Herpes simplex virus-1 (HSV-1)-derived mammalian transactivation domain VP16 (31) thus creating the artificial mammalian transactivators SCA (ScbR-VP16) and SPA (SpbR-VP16) (Fig. 2). The SCA/SPA-specific mammalian promoters P_{SCA}/P_{SPA} were assembled by cloning the ScbR-/SpbR-specific operator modules O_{scbR}/O_{papRI} 5′ of a minimal human cytomegalovirus immediate early promoter ($P_{hCMVmin}$, Fig. 2). Cotransfection of the SCA/SPA expression vectors with plasmids encoding P_{SCA}- and P_{SPA}-driven human secreted alkaline phosphatase (SEAP) into CHO-K1 cells resulted in high SEAP expression levels (21.7 ± 2.7 U/l for SCA/P_{SCA}-SEAP-pA and 29.6 ± 3.5 U/l for SPA/P_{SPA}-SEAP-pA) which were comparable to those of a P_{SV40}-driven SEAP control construct (pWW56; P_{SV40}-SEAP-pA) (46.8 ± 6.7 U/l). Since the *S.coelicolor* and *S.pristinaespiralis* butyrolactone quorum-sensing systems are highly homologous, their derived mammalian transcription control units recognize the same operator sites in CHO-K1 cells: SCA binds and activates transcription from P_{SPA} (85.8 ± 5.8 U/l for SCA/P_{SPA}-SEAP-pA) and SPA promotes P_{SCA}-driven expression (36.0 ± 2.2 U/l for SPA/P_{SCA}-SEAP-pA). These findings demonstrate that the *Streptomyces* quorum-sensing components ScbR/O_{scbR} and SpbR/O_{papRI} retain their specific (cross)interaction and form a potent heterologous transcription unit in mammalian cells.

Butyrolactone responsiveness of QuoRex-controlled transgene expression in mammalian cells

Expecting that SCA-P_{SCA}, SCA-P_{SPA}, SPA-P_{SCA} and SPA-P_{SPA} interactions could be engineered for adjustable transgene expression in mammalian cells by specific butyrolactone derivatives, we have designed novel/refined protocols for the synthesis of racemic SCB1 which elicits antibiotic production in *S.coelicolor* (25,30) and MP133, a butenolide first isolated from *S.antibioticus* (32) (Fig. 1). The enantiomers of SCB1 were obtained as two racemic mixtures, IM-2 type SCB1 and VB type SCB1 (IM-2-SCB1 and VB-SCB1).

In order to qualify as molecules for controlling transgene expression in mammalian cells, SCB1 and MP133 should not have any undesirable physiological impact on host cells. An indicator for possible toxicity of SCB1 and MP133 is their influence on constitutive reporter gene expression. We have therefore added increasing SCB1 and MP133 concentrations to a CHO-K1 derivative engineered for constitutive SEAP expression. Both butyrolactones showed no effect on SEAP expression at concentrations up to 10 µg/ml. At a
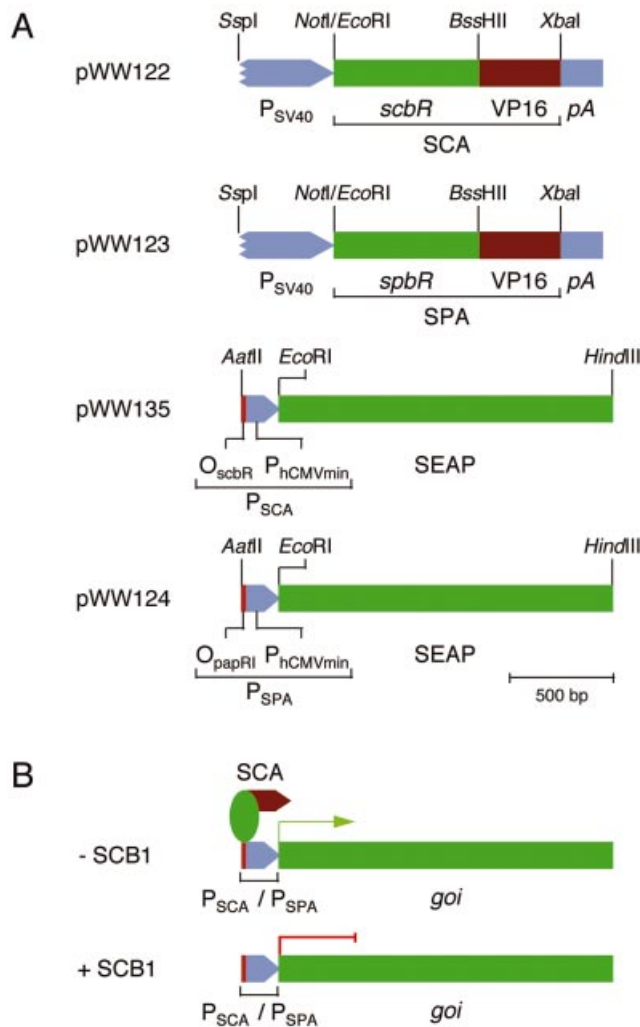
Figure 2. Design and function of the quorum-sensor-derived mammalian gene regulation system (QuoRex). (**A**) Diagram of QuoRex' genetic elements: the butyrolactone-dependent transactivators SCA and SPA were assembled by fusing the *S.coelicolor/S.pristinaespiralis* quorum-sensing receptors ScbR/SpbR 3′ to the Herpes simplex virus 1 (HSV-1)-derived mammalian transactivation domain. SCA and SPA were cloned into a constitutive mammalian expression configuration (pWW122/pWW123) driven by the simian virus 40 promoter (P_{SV40}) and terminated by an SV40-derived polyadenylation site (pA). SCA/SPA-specific promoters were constructed by cloning the ScbR-/SpbR-specific operator modules (O_{scbR}/O_{papRI}) adjacent to a minimal version of the human cytomegalovirus immediate early promoter ($P_{hCMVmin}$). (**B**) In the absence of regulating butyrolactones (–SCB1), the transactivator (SCA) binds to its cognate P_{SCA}/P_{SPA} promoters and initiates transcription of the cloned gene of interest (*goi*). However, addition of SCB1 (+SCB1) results in disruption of the SCA-P_{SCA}/P_{SPA} interactions and repression of *goi*.

Figure 3. Impact of butyrolactone ligands on constitutive and QuoRex-controlled gene expression in CHO-K1. (**A**) Effect of the SCB1 diastereomers IM-2-SCB1 and VB-SCB1 as well as MP133 on constitutive SEAP expression in CHO-K1. Triplicate CHO-K1 cultures were transfected with a P_{SV40}-driven SEAP expression vector (pWW56) and grown for 48 h at the indicated ligand concentrations prior to quantification of SEAP in the culture medium. (**B**) Modulation of P_{SPA}-driven SEAP expression in CHO-K1 cells transiently transfected with pWW122 (P_{SV40}-SCA-pA) and pWW124 (P_{SPA}-SEAP-pA) following addition of different IM-2-SCB1, VB-SCB1 and MP133 concentrations. SEAP assays were performed 48 h post-transfection (all assays in triplicate).

concentration of 100 µg/ml, both SCB1 and MP133 were toxic/growth-retarding for mammalian cells as indicated by a depression in SEAP expression levels which was more prominent for both stereoisomers of SCB1 than for MP133 (Fig. 3A).

When used to adjust transient P_{SPA}-driven SEAP expression in CHO-K1 cells transfected with an SCA expression vector, addition of increasing concentrations of SCB1 correlated with decreasing SEAP production profiles indicating that this butyrola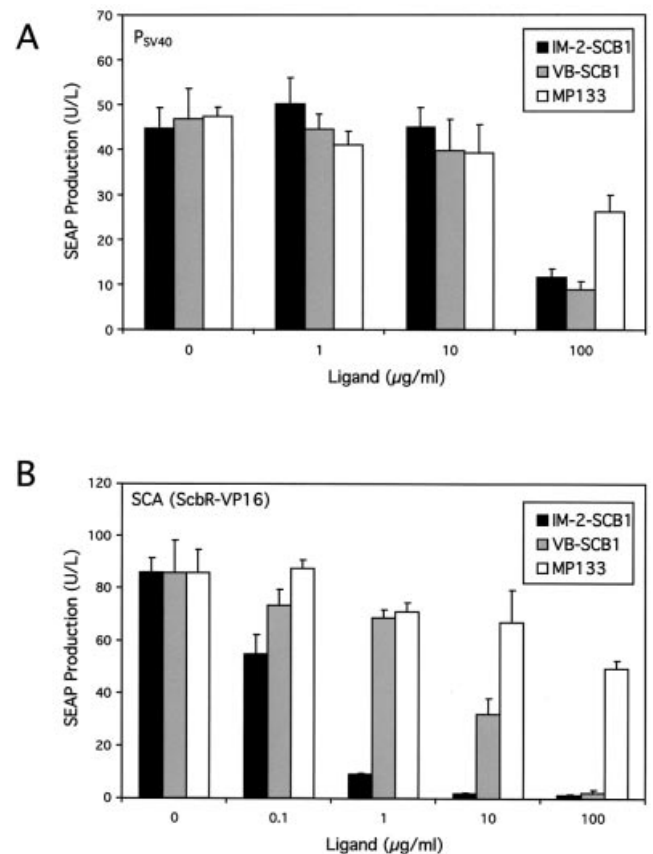ctone releases SCA from P_{SPA} which terminated transcriptional activation of SEAP expression (Fig. 3B). However, the structurally related MP133 was unable to modulate SEAP production significantly within its non-toxic concentration range. Interestingly, IM-2-SCB1 was more efficient than VB-SCB1 in changing SCA's allostery to prevent P_{SPA} binding. This was illustrated by a progressive IM-2-SCB1-mediated decrease of SEAP activity reaching basal expression at 10 µg/ml corresponding to 1.9% of fully induced expression. In contrast, addition of VB-SCB1 reduced expression of this human glycoprotein only to half-maximum levels at this concentration (Fig. 3B). The remarkable stereoselective discrimination of SCA between the two SCB1 diastereomers emphasizes the specificity of quorum-sensing phenomena. IM-2-SCB1 retained its excellent regulation performance in an isogenic QuoRex-controlled erythropoietin expression setting (P_{SPA}-EPO-pA; –IM-2-SCB1: 690 ± 28 mIU/ml; +IM-2-SCB1: 23.7 ± 5.2 mIU/ml).

In contrast to SCA-dependent transactivation of target promoters, neither SCB1 nor MP133 were able to disrupt SPA-P_{SPA} or SPA-P_{SCA} interactions suggesting that SCB1 is
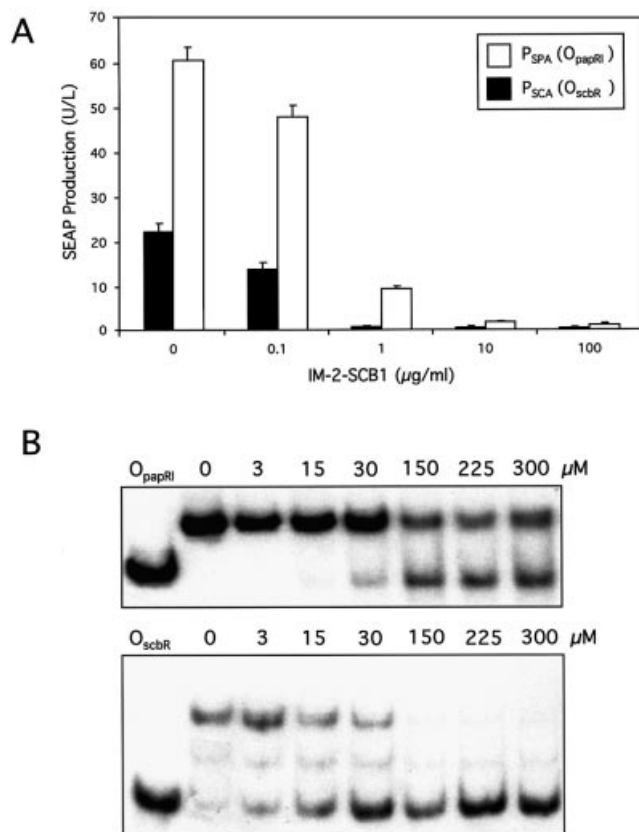
Figure 4. Specificity and affinity of SCA for *S.coelicolor*/*S.pristinaespiralis* O_{scbR}/O_{papRI} operator modules. (**A**) SCA-dependent SEAP expression levels driven by P_{SPA}/P_{SCA} promoters in CHO-K1 cells in the presence of different IM-2-SCB1 concentrations. CHO-K1 cells were cotransfected with the SCA expression plasmid pWW122 (P_{SV40}-SCA-pA) and the SCA-dependent SEAP expression vector pWW124/pWW135 (P_{SPA}-SEAP-pA/P_{SCA}-SEAP-pA). Transfected cell lines were grown for 48 h in medium containing the indicated IM-2-SCB1 concentrations prior to quantification of SEAP expression levels. (**B**) Gel retardation assays showing interactions of ScbR with O_{scbR} and ScbR with O_{papRI}. The ScbR and radioactively labeled O_{scbR} and O_{papRI} protein–DNA complexes were incubated with various IM-2-SCB1 concentrations and resolved on a polyacrylamide gel. ScbR is released from O_{scbR} at lower IM-2-SCB1 concentrations compared to O_{papRI} indicating higher affinity of this *S.coelicolor* quorum-sensing receptor for *S.pristinaespiralis* O_{papRI} than for its autologous O_{scbR}.

Table 1. Expression characteristics of SCA/P_{SPA}-driven secreted alkaline phosphatase in different cell lines in the presence and absence of IM-2-SCB1

Cell line	–IM-2-SCB1	+IM-2-SCB1
CHO-K1	60.4 ± 8.3	1.81 ± 0.17
BHK-21	5.0 ± 0.4	0.23 ± 0.04
COS-7	7.5 ± 0.5	0.14 ± 0.04
HEK 293-T	228.0 ± 7.6	33.5 ± 0.11
Hep G2	2.1 ± 0.1	0.096 ± 0.035
HUVEC	0.5 ± 0.08	0.0001 ± 0.0001
Mouse ES	1.0 ± 0.02	0.027 ± 0.007

Cells were transfected with plasmids pWW122 (P_{SV40}-SCA-pA) and pWW124 (P_{SPA}-SEAP-pA) and grown for 48 h in the presence (10 µg/ml) and absence of IM-2-SCB1 before scoring of SEAP production (in U/l).

In order to investigate whether variations in expression levels were associated with different affinities of SCA for P_{SCA} and P_{SPA}, *in vitro*-formed SCA-P_{SCA} and SCA-P_{SPA} complexes incubated at increasing IM-2-SCB1 concentrations were resolved on polyacrylamide gels. These gel retardation assays provided biochemical evidence that SCA did indeed have higher affinity for the heterologous P_{SPA} than for P_{SCA} which apparently renders the SCA-P_{SPA} configuration more effective for controlling transgene expression in mammalian cells (Fig. 4B).

Detailed expression analysis of the SCA-PSPA-based QuoRex system

The performance of the SCA-P_{SPA}-based QuoRex system was evaluated in a variety of mammalian cell types including human primary cells and mouse embryonic stem cells. To this end, we cotransfected the SCA expression vector pWW122 (P_{SV40}-SCA-pA) and the SCA-responsive SEAP expression vector pWW124 (P_{SPA}-SEAP-pA) into different cell lines and assayed the SEAP levels in their culture supernatants following cultivation for 48 h in the presence and absence of IM-2-SCB1. In all cell lines tested, high SEAP expression could be monitored in the absence of IM-2-SCB1 whereas complete repression was achieved following addition of this butyrolactone (Table 1).

We also evaluated QuoRex's key regulation characteristics (dose-dependence, expression kinetics and reversibility) in a stable expression configuration. QuoRex was engineered into CHO-K1 cells following a two step procedure. The SCA expression unit was transduced using a transgenic retrovirus ($5'$LTR-ψ^+-SCA-P_{PGK}-neo^R-$3'$LTR) and individual CHO-K1-derived cell clones were assessed for QuoRex performance after transient transfection with the P_{SPA}-driven SEAP expression construct pWW124. The regulation performance of four independent CHO-SCA clones in the presence and absence of IM-2-SCB1 is shown in Table 2. Because of its balanced expression profiles combining high maximum SEAP expression levels as well as tight repression, we chose CHO-SCA$_{35}$ for further studies. The P_{SPA}-driven SEAP expression unit was stably integrated into CHO-SCA$_{35}$ and individual clones were scored for their regulation performance in the presence and absence of IM-2-SCB1. Table 2 shows four different double transgenic clones of which CHO-SCA$_{35}$-SEAP$_{16}$ was chosen for further analysis.

specific for SCA (*in vitro* and *in vivo*; data not shown). Thus, SPA awaits isolation and characterization of SpbR-specific butyrolactones before implementation of the *S.pristinaespiralis*-derived mammalian gene regulation system can proceed (20).

DNA-binding specificity of SCA

The regulatory activity of SCA for promoters P_{SCA} and P_{SPA} was analyzed following cotransfection of pWW122 (P_{SV40}-SCA-pA) with either pWW135 (P_{SCA}-SEAP-pA) or pWW124 (P_{SPA}-SEAP-pA) into CHO-K1. Surprisingly, SCA activated the promoter containing the heterologous operator sequence (O_{papRI}) more efficiently than the same promoter with its cognate operator (O_{scbR}). At all IM-2-SCB1 concentrations tested (0–100 µg/ml), the SCA-P_{SCA} configuration only reached approximately one-third of the SEAP expression levels compared to the heterologous SCA-P_{SPA} combination (Fig. 4A).

Table 2. Expression characteristics of stable CHO-SCA and CHO-SCA-SEAP clones in the presence and absence of IM-2-SCB1

CHO-SCA				
Clone no.	15	16	35	38
–IM-2-SCB1	8.8 ± 2.9	6.1 ± 0.6	6.8 ± 0.3	6.7 ± 1.0
+IM-2-SCB1	0.13 ± 0.02	0.10 ± 0.12	0.15 ± 0.08	0.15 ± 0.03
CHO-SCA-SEAP				
Clone no.	10	16	63	109
–IM-2-SCB1	8.4 ± 0.4	5.2 ± 0.2	3.0 ± 0.1	13.7 ± 0.3
+IM-2-SCB1	0.67 ± 0.06	0.09 ± 0.02	0.17 ± 0.15	0.17 ± 0.17

CHO-SCA cell clones were transfected with pWW124 (P_{SPA}-SEAP-pA) and grown for 48 h in the presence and absence of IM-2-SCB1 before measuring SEAP activity (in U/l). CHO-SCA-SEAP cell clones were grown for 48 h with (10 µg/ml) and without IM-2-SCB1 before SEAP assays.

Upon exposure of CHO-SCA$_{35}$-SEAP$_{16}$ to increasing IM-2-SCB1 concentrations, SEAP expression could be adjusted to intermediate levels until it was completely repressed to detection limits when the butyrolactone concentration reached 0.5 µg/ml (Fig. 5A). Interestingly, the sensitivity of CHO-SCA$_{35}$-SEAP$_{16}$ to IM-2-SCB1 was one order of magnitude higher compared to transient transfections, a phenomenon which has previously been reported for double-transgenic cell lines harboring the tetracycline-response gene regulation system (33).

SEAP expression kinetics of CHO-SCA$_{35}$-SEAP$_{16}$ grown in the presence of 0.5 µg/ml IM-2-SCB1 or in the absence of this regulating butyrolactone were monitored for 48 h (Fig. 5B). Despite its excellent regulation profiles, high expression levels in the absence of IM-2-SCB1 and tight repression in its presence, the QuoRex system proved to be completely reversible following repeated cycles of addition and withdrawal of IM-2-SCB1 from the culture for a period of over 140 h (Fig. 5C).

In vivo quorum-sensing-responsive gene expression

Following successful validation of QuoRex-controlled gene expression in mammalian cells, we assessed its potential for adjustable transgene expression *in vivo*. Mimicking current gene therapy scenarios, we implanted CHO-SCA$_{35}$-SEAP$_{16}$ cells, encapsulated in biocompatible alginate-PLL-alginate beads, intraperitoneally into immunocompetent mice. Microencapsulation protects implanted cells from the mouse immune system and prevents any inflammatory response as well as the elimination of transgenic cells. Microencapsulated cells remained responsive to IM-2-SCB1 as this butyrolactone can diffuse through the alginate-PLL-alginate layers and SEAP, a model for therapeutic proteins, was secreted across the capsule (Fig. 6A). Following intraperitoneal implantation of the same *in vitro*-validated cell batch, the mice received different IM-2-SCB1 doses by injection (0–50 mg/kg). Serum SEAP levels of treated mice were quantified 72 h post-implantation. IM-2-SCB1-adjustable SEAP production including full repression to undetectable serum levels was reached in these animals at butyrolactone concentrations which showed no undesired effects on mouse physiology or behavior suggesting that quorum-sensing molecules were safe for *in vivo* applications. Control mice harboring non-engineered CHO-K1 cells did not contain elevated serum SEAP levels in the presence and absence of IM-2-SCB1. IM-2-SCB1-responsive SEAP expression profiles in mice resembled those observed *in vitro* with the same batch of
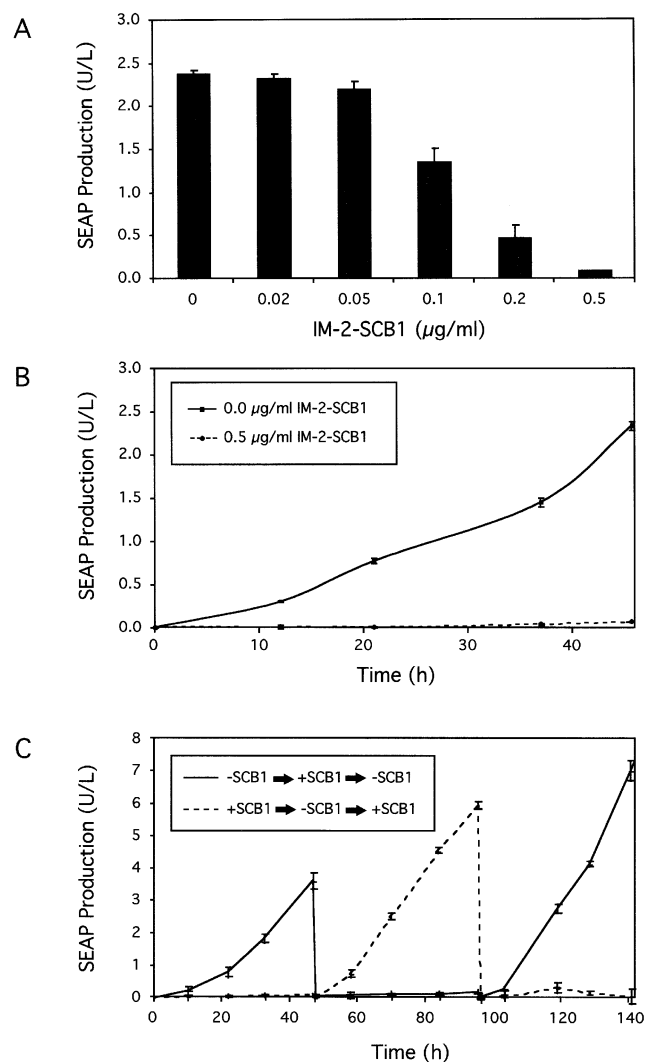
Figure 5. Regulation characteristics of CHO-SCA$_{35}$-SEAP$_{16}$ transgenic for QuoRex-controlled SEAP expression. (**A**) IM-2-SCB1-adjustable SEAP production. CHO-SCA$_{35}$-SEAP$_{16}$ cells (66 000 cells/ml) were cultivated for 48 h at indicated IM-2-SCB1 concentrations prior to quantification of SEAP production. (**B**) Expression kinetics of CHO-SCA$_{35}$-SEAP$_{16}$ cells cultivated in the presence and absence of IM-2-SCB1 for 48 h. (**C**) Reversibility of QuoRex-controlled transgene expression. Two cultures (66 × 10^6 CHO-SCA$_{35}$-SEAP$_{16}$ cells/ml) were grown initially in the presence and absence of IM-2-SCB1. At 48 h intervals the IM-2-SCB1 status was reversed (–IM-2-SCB1 → +IM-2-SCB1 → –IM-2-SCB1; +IM-2-SCB1 → –IM-2-SCB1 → +IM-2-SCB1). SEAP production was monitored during the entire cultivation.
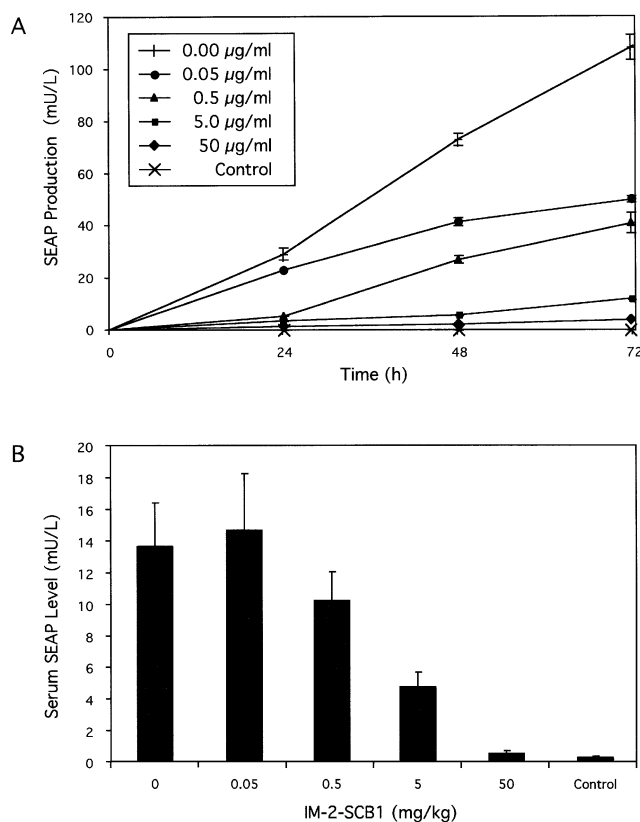
Figure 6. Butyrolactone-responsive SEAP expression from microencapsulated CHO-K1 cell derivatives in culture and in mice. (**A**) IM-2-SCB1-responsive SEAP expression from encapsulated cells transgenic for QuoRex-controlled SEAP expression. CHO-SCA$_{35}$-SEAP$_{16}$ or CHO-K1 control cells (2×10^6 cells per culture) were microencapsulated in alginate-poly-L-lysine-alginate microcapsules (400 µm in diameter, 200 cells/capsule) and cultivated in medium supplemented with different IM-2-SCB1 concentrations and analyzed for SEAP production after 72 h. (**B**) SCB1-adjustable SEAP expression in mice. The same microencapsulated batch of CHO-SCA$_{35}$-SEAP$_{16}$ and CHO-K1 control cells as used for *in vitro* cultivation [2×10^6 cells, 200 cells/capsule; see details in (A)] was implanted intraperitoneally into immunocompetent mice. At 1 h post-implantation the mice received IM-2-SCB1 injections at indicated doses. Blood samples were collected after 72 h and quantified for SEAP levels.

microencapsulated cells, thereby confirming the *in vivo* compatibility of the QuoRex system (Fig. 6).

DISCUSSION

Pharmacological control of transgene expression is a current priority in therapeutic engineering of mammalian cells to titrate levels of desired protein pharmaceuticals in gene therapy scenarios, or to reprogram preferred cell phenotypes for tissue engineering (6,10,34–38).

Quorum-sensing systems enabling coordination of gene expression within and between prokaryotic populations represent a vast reservoir of small-molecule response regulators, which could be adopted for use as heterologous mammalian gene regulation systems (19,22). As proof of principle we converted a butyrolactone quorum-sensing regulator, which controls antibiotics biosynthesis in *S.coelicolor* and *S.pristinaespiralis*, for use as gene control systems in mammalian cells and mice (21,25). ScbR and SpbR, as well as their

cognate operators O_{scbR} and O_{papRI}, are highly homologous and recognize similar DNA sequences (20). Interestingly, ScbR/SCA had stronger affinities for heterologous O_{papRI}/P_{SPA} than for their cognate O_{scbR}/P_{SCA} modules which is reflected in higher maximum expression levels of the SCA-P_{SPA} configuration compared to the sister SCA-P_{SCA} set-up. In contrast to the flexibility in sequence recognition, the interaction of these quorum-sensing receptors with regulating butyrolactones was highly specific. SCB1 exclusively interacts with ScbR and ScbR-derived mammalian transactivators but not with SpbR derivatives. Furthermore, SCA discriminates between the SCB1 diastereomers IM-2-SCB1 and VB-SCB1 since IM-2-SCB1 enables fine-tuning of transgene expression in mammalian cells with a lower concentration window than VB-SCB1.

An important characteristic of the QuoRex system is its *in vivo* compatibility showing negligible basal transcriptional activity and IM-2-SCB1-adjustable target gene expression in mice. Pioneering *in vivo* use of SCB1 suggested that this type of butyrolactone is well tolerated in animals when administered at regulating dosing regimes.

The prototype QuoRex system provides the first evidence that molecular interactions enabling bacteria to communicate as multicellular systems can be successfully transferred to a multicellular organism to fine-tune heterologous transgene expression to desired levels. There are many bacterial quorum-sensing systems, all consisting of protein receptors, their cognate butyrolactones and operator sequences. Thus, QuoRex represents a vast potential for development of a new generation of small molecule-adjustable gene control systems for clinical application (39).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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