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A novel, inducible, eukaryotic gene expression system based on the quorum-sensing transcription factor TraR

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Bacteria adapt their pattern of gene expression in response to a variety of external cues, including fluctuations in population density. This type of bacterial cell-to-cell communication is referred to as quorum-sensing. Quorum-sensing systems are present in many bacterial species and constitute a large collection of ligands and cognate receptors. The availability of such diversity offers interesting opportunities for biotechnological exploitation. We describe here the transformation of the quorum-sensing system of *Agrobacterium tumefaciens* into a transcription regulatory system that works in mammalian cells. The *A. tumefaciens* TraR protein was fused to the eukaryotic activation domain of NF-κB p65, generating a novel chimaeric transcriptional activator that stimulates gene transcription in different human cell lines from a minimal promoter containing the TraR DNA recognition sequence in the presence of the *Agrobacterium* quorum-sensing signal molecule *N*-(3-oxo-octanoyl)homoserine lactone (3-oxo-C_8-HSL). The basal level of transcription was low in the absence of 3-oxo-C_8-HSL, and gene expression was stimulated up to 1,000-fold at a saturating concentration of 3-oxo-C_8-HSL.

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INTRODUCTION

Many experiments, projects and programmes in developmental biology and gene therapy rely on the conditional regulation of the expression of a transgene introduced into cultured cells or living organisms. For this purpose, several artificial systems have been developed in which a transcription factor is activated by an exogenously administered small molecule (for reviews see Saez *et al.*, 1997; Harvey & Caskey 1998). Although the usefulness of such systems has been demonstrated, several fields of biology and medicine would benefit from improved or additional versions of artificial gene regulation systems that could complement and back up the existing ones. An interesting opportunity for the development of a novel and versatile system is offered by prokaryotic quorum-sensing systems (Miller & Bassler, 2001). Through quorum-sensing, many bacterial strains are capable of reprogramming gene expression in response to fluctuations of their population density. A prototypical Gram-negative bacterial circuit is accomplished by a simple mechanism consisting of a signal molecule, an acylated homoserine lactone (AHL), that accumulates in the immediate external environment, and a cognate transcription factor activated by it. Over 50 species of Gram-negative bacteria produce AHLs that differ in the acyl side chain. The most attractive feature of this two-component regulatory system is that there are several natural variants. Moreover, signals can act in parallel or in series in an intraspecific network. The bacterial quorum-sensing system thus represents a natural combinatorial library that could be exploited to generate reagents for the development of artificial eukaryotic gene regulation systems. To this end, it is necessary to re-engineer the basic prokaryotic module—that is, the signal and its cognate 'receptor'—such that it functions in eukaryotic cells.

As a prototype, we chose the TraR/*N*-(3-oxo-octanoyl) homoserine lactone (TraR/3-oxo-C_8-HSL) quorum-sensing system of the plant pathogen *Agrobacterium tumefaciens* (Hwang *et al.*, 1994; Fugua & Winans, 1994). TraR belongs to the LuxR family of transcriptional activators (Miller & Bassler, 2001). Binding of the small, diffusible, *Agrobacterium* quorum-sensing signal 3-oxo-C_8-HSL (Fig. 1A; Piper *et al.*, 1993; Zhang *et al.*, 1993) results in TraR activation, and subsequent interaction with promoters containing one or more copies of an 18-base pair (bp) inverted repeat called the *tra* box (Fuqua & Winans, 1996). The three-dimensional structure of TraR has been solved (Vannini *et al.*, 2002; Zhang *et al.*, 2002). The structure shows the presence of two separate domains: the amino-terminal domain contains the ligand-binding domain and the dimerization domain, whereas the carboxy-terminal domain contains the DNA-binding domain. This modular structure makes the LuxR family of transcriptional activators a promising candidate for genetic manipulations.

We describe here the construction of a eukaryotic transactivator consisting of the ligand-binding and DNA-binding domains of the quorum-sensing transcription factor, TraR, fused to a eukaryotic transactivation domain. We demonstrate that this chimaeric protein retains specific and inducible DNA binding activity *in vitro* and shows ligand-dependent induction of gene expression in cell culture.

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RESULTS

DNA-binding activity of TraR fusion proteins

The complementary DNA fragment corresponding to the TraR wild-type protein was cloned into a eukaryotic expression vector. Different eukaryotic activation domains were fused to the C terminus of TraR. However, none of them maintained the capacity to bind DNA *in vitro* and were not used for further studies (data not shown).

The eukaryotic activation domain, F3, which consists of three 13-amino-acid repeats derived from the *Herpes simplex* virus protein VP16 (Baron *et al.*, 1997), or the transactivation domain of NF-κB p65 (Burcin *et al.*, 1999) were fused N-terminally to TraR. In both of these fusions, the activation domains are separated from TraR by an amino-acid linker. In addition, a nuclear localization signal (NLS) was added to the linker region of p65 to ensure nuclear localization of the chimaeric protein. Constructs are shown in Fig. 1B. Wild-type TraR and the chimaeric proteins were synthesized by *in vitro* translation in the presence or absence of 3-oxo-C_8-HSL using rabbit reticulocyte lysate, and were then tested for DNA-binding activity by gel retardation assays (Fig. 2A). The amount of protein produced was not influenced by the presence of the ligand (data not shown). An oligonucleotide containing the *tra* box sequence (Fig. 1B) was used as specific DNA probe. Translation of the wild-type TraR construct in the absence of 3-oxo-C_8-HSL produced a protein that did not bind to DNA, even if the ligand was added during the incubation with the DNA (lanes 1 and 2). If translation was performed in the presence of 3-oxo-C_8-HSL, the resulting protein was able to bind DNA, and no further addition of 3-oxo-C_8-HSL during DNA incubation was required (lanes 3 and 4). Specificity of binding was demonstrated using an excess of non-radiolabelled specific or non-specific competitor DNA (lanes 5 and 6). The chimaeric proteins were produced under the same conditions to assess whether they maintained the tight dependence on 3-oxo-C_8-HSL for functional protein expression and specificity of DNA binding. Fig. 2A shows that the chimaeric proteins behave similarly to wild-typeTraR. The continuous presence of 3-oxo-C_8-HSL during protein synthesis is necessary and sufficient for DNA-binding activity (lanes 7–18).

The ligand-binding domain of TraR is located at the N terminus of the protein. Addition of a transactivation domain to the N terminus of TraR might therefore disturb the correct folding of the protein, or reduce the affinity of the ligand-binding domain for 3-oxo-C_8-HSL. To address this question, we measured the DNA-binding activity of TraR and p65NTraR as a function of increasing concentration of 3-oxo-C_8-HSL (Fig. 2B). TraR and p65NTraR were translated *in vitro* at 3-oxo-C_8-HSL concentrations between 0 and 80 μM, and the level of DNA binding was measured as the fraction of retarded DNA probe. The concentration of 3-oxo-C_8-HSL resulting in half-maximal binding (EC_{50}) is comparable for the two constructs, and it can therefore be concluded that the affinity of the TraR ligand-binding domain for 3-oxo-C_8-HSL is not reduced significantly in the p65NTraR chimaeric protein.

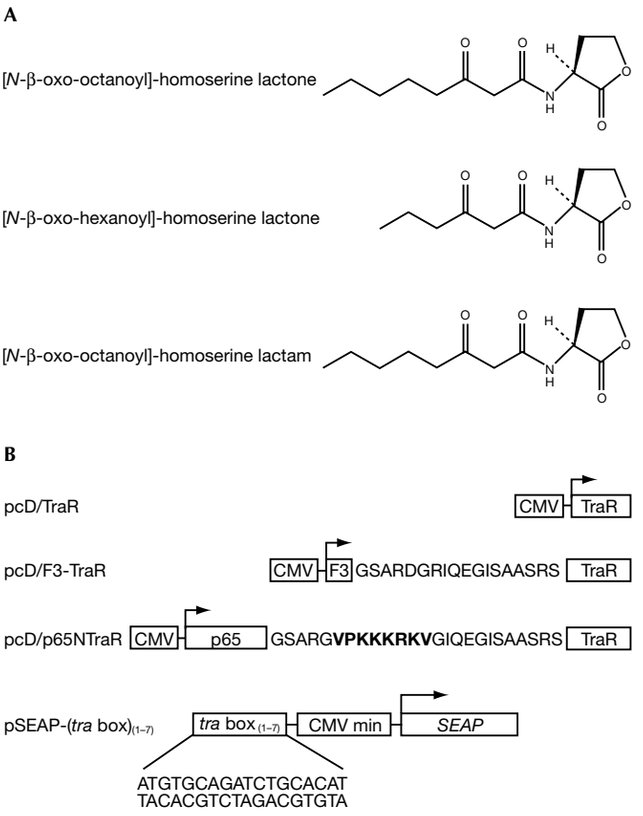

Fig. 1 | Schematic representation of DNA constructs, and structure of compounds used in this study. (**A**) The structure of the AHL compounds used in this study are shown. (**B**) The amino-acid sequences of the linker regions between the activation domains and TraR are shown. The nuclear localization signal used in pcD/p65NTraR is indicated by bold letters. The DNA sequence of the *tra* box is indicated. CMV, CMV promoter. CMV min, CMV minimal promoter.

Transcriptional activity of p65NTraR in mammalian cells

We demonstrated that F3-TraR and p65NTraR bind to the *tra* box sequence in a ligand-dependent manner. To assess the transcriptional activity of the chimaeric fusion proteins, one, two, four or seven *tra* boxes were cloned upstream of the minimal cytomegalovirus (CMV) promoter, driving in turn the expression of a reporter gene encoding human secreted alkaline phosphatase (SEAP) (Fig. 1B). As a control for the specificity of DNA binding, a second construct was made in which seven repeats of the recognition sequence of LuxR (Devine *et al.*, 1989) were cloned in front of the CMV minimal promoter. The reporter gene constructs were individually cotransfected with the transactivators in HeLa cells, and cells were incubated in the presence or absence of 20 μM 3-oxo-C_8-HSL for 24 h (Fig. 3). 3-Oxo-C_8-HSL-dependent induction of SEAP became detectable in the presence of two *tra* boxes, and reached a maximum of activity with seven *tra* boxes. No SEAP activity was detectable with seven *lux* boxes in front of the minimal promoter, indicating that induction by p65NTra is DNA-sequence specific. The fusion protein F3-TraR, when co-transfected with a SEAP construct with seven *tra* boxes in front of the promoter—pSEAP-(*tra* box_7)—showed an activity that was ~7% of that obtained with p65NTraR. Conversely, expression of wild-type TraR protein, without a eukaryotic activation domain, was not able to induce transcription in eukaryotes (data not shown). These experiments show that the combination of TraR with eukaryotic activation domains results in a DNA-sequence-specific transcriptional activator, which depends on the presence of the ligand 3-oxo-C_8-HSL.

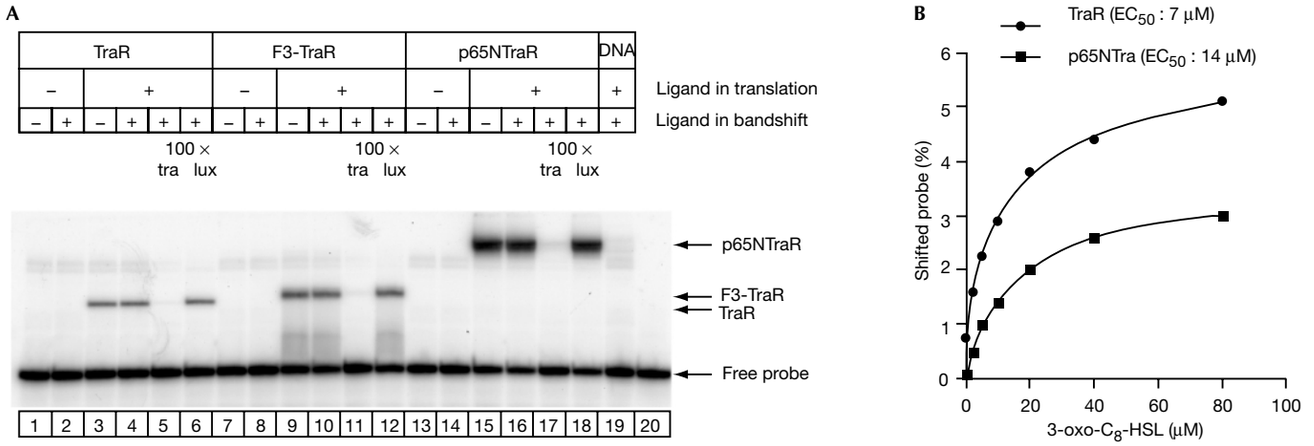

Fig. 2 | DNA-binding activity of *in vitro* translated TraR, p65NTraR and F3-TraR. (**A**) Gel-retardation assay. TraR, F3-TraR and p65NTraR were synthesized by *in vitro* translation either in the absence (−) or presence (+) of 20 μM 3-oxo-C_8-HSL. Proteins were incubated with the specific DNA probe in the absence (−) or presence (+) of 20 μM 3-oxo-C_8-HSL and loaded onto a 6% non-denaturing polyacrylamide gel. Competition was performed with a 100-fold excess (100×) of *tra* box (tra) or *lux* box (lux) oligonucleotides. (**B**) Titration curve of 3-oxo-C_8-HSL. TraR and p65NTraR were translated *in vitro* in the presence of 0, 5, 10, 20, 40 or 80 μM 3-oxo-C_8-HSL and the gel-retardation experiment was performed at the same 3-oxo-C_8-HSL concentrations. The percentage of shifted probe was plotted against the 3-oxo-C_8-HSL concentration.

Basal and induced transcriptional activity of p65NTraR

An inducible gene-expression system should fulfil the requirements of giving a low basal expression level in the uninduced state and a high level of induction on addition of the inducing agent. We investigated these two criteria using the TraR expression system (Fig. 4). The plasmids, as indicated in the figure legend, were transfected into HeLa cells (Fig. 4A), and SEAP activity was measured as described for Fig. 3. Maximum SEAP activity for the pSEAP-(*tra* box)$_7$ reporter plasmid alone was about 2×10^4 arbitrary light units (ALU). Cotransfection of 0.5 μg of the eukaryotic transactivator plasmid pCD/p65NTraR resulted in a sharp increase of SEAP activity (up to 1.2×10^7 units) that was strictly dependent on the presence of 3-oxo-C_8-HSL. Without exposure of the transfected cells to 3-oxo-C_8-HSL, the transcriptional level was low and, most importantly, the presence of the p65NtraR transactivator did not affect the basal level of SEAP activity (Fig. 4; compare columns 5, 7 and 9 with 11, 13 and 15). Moreover, increasing the amount of the p65NTraR transactivator plasmid to 1 μg did not significantly increase basal SEAP activity in the absence of 3-oxo-C_8-HSL (column 21). This indicates that the TraR expression system is tightly regulated in HeLa cells, and that activity of the transactivator strictly depends on the presence of 3-oxo-C_8-HSL. In parallel, up to 5 μg of a construct containing the SEAP reporter gene placed downstream of the non-inducible strong CMV promoter (pVIJN-SEAP; Chastain *et al.*, 2001) were transfected into HeLa cells (columns 1–4). The expression level of the induced TraR expression system was comparable to the gene expression driven by the strong eukaryotic promoter (compare columns 16 and 22 with column 4). The same experiment was repeated using the three human cell lines, Huh7 (hepatoma), 293 (transformed kidney) and RD (rhabdomyosarcoma) (Fig. 4B). In all three cell lines, a robust induction of SEAP was detectable only when the transactivator p65NTraR together with 3-oxo-C_8-HSL was present. Basal levels of SEAP expression were higher in these cell lines than in HeLa cells, which might be explained in part by higher transfection efficiencies.

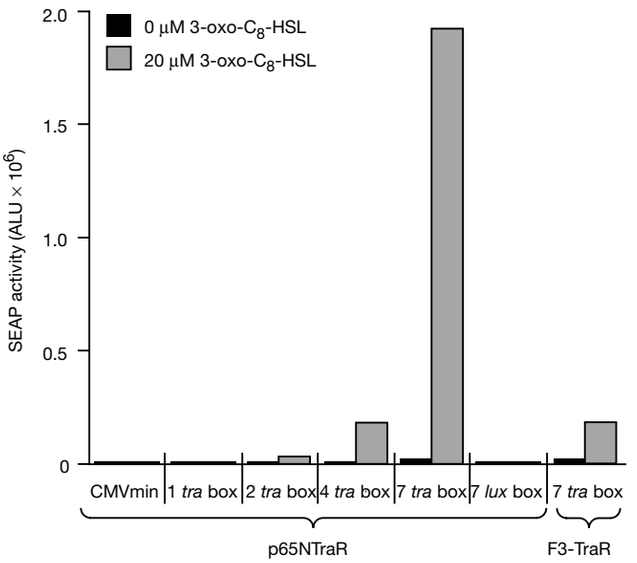

Fig. 3 | SEAP activity in transfected HeLa cells. HeLa cells were transfected with pcD/p65NTraR, pcD/F3-TraR and the SEAP reporter gene containing 0, 1, 2, 4 or 7 *tra* box sequences upstream of the minimal CMV promoter, as indicated in the figure, in the presence or absence of 20 μM 3-oxo-C_8-HSL. SEAP activity was measured 24 h after addition of 3-oxo-C_8-HSL. ALU, arbitrary light units.

3-Oxo-C_8-HSL is an *N*-acyl homoserine lactone, and is a member of a family of common microbial quorum-sensing molecules. A TraR-dependent gene expression system should strictly depend on 3-oxo-C_8-HSL and show variable responsiveness to

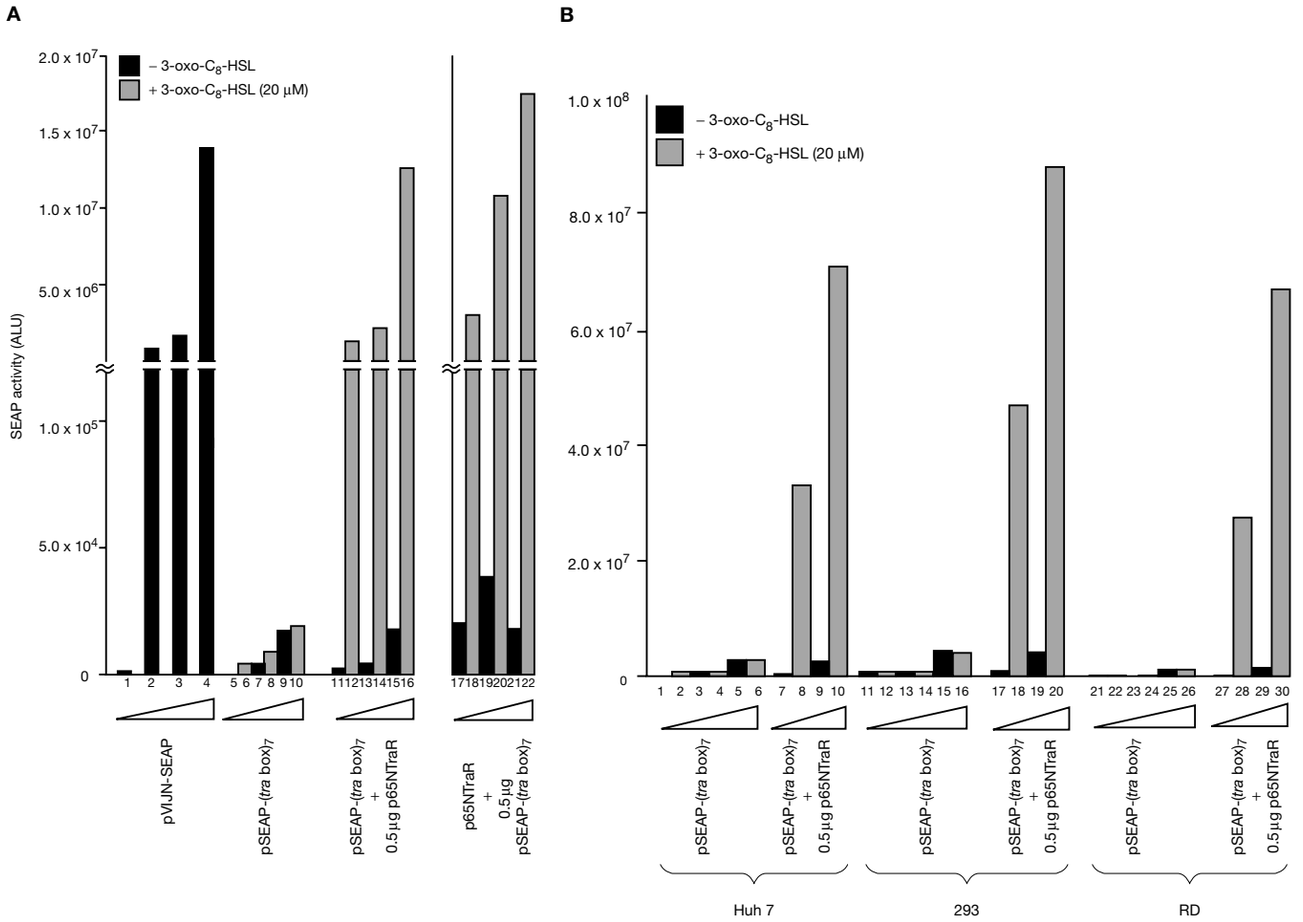

Fig. 4 | Basal and induced levels of the p65NTraR expression system. (**A**) HeLa cells were transfected with the indicated plasmids in the presence or absence of 20 μM 3-oxo-C_8-HSL. SEAP activity was measured after 24 h. The following amounts of the indicated SEAP reporter plasmid were transfected: 0 μg (column 1), 0.05 μg (columns 2, 5, 6, 11 and 12), 0.1 μg (columns 3, 7, 8, 13 and 14) or 0.5 μg (columns 4, 9, 10 and 15–22). The following amounts of plasmid pcD/p65TraR were cotransfected with the reporter plasmid: 0.1 μg (columns 17 and 18), 0.5 μg (columns 11–16 and 19 and 20) or 1 μg (columns 21 and 22). (**B**) Transfection of Huh7, 293 and RD cells in the absence (black bars) or presence (gray bars) of 3-oxo-C_8-HSL. The following amounts of pSEAP-(*tra* box)$_7$ plasmid were transfected: 0.05 μg (columns 1, 2, 7, 8, 11, 12, 17–, 8, 21, 22, 27 and 28), 0.1 μg (columns 3, 4, 13, 14, 23 and 24) or 0.5 μg (columns 5, 6, 9, 10, 15, 16, 19, 20, 25, 26, 29 and 30). Columns 1–10, Huh7; columns 11–20, 293; columns 21–30, RD.

other members of the family. We assessed signal specificity for p65NTraR-dependent induction of gene expression by comparing the concentration dependence of 3-oxo-C_8-HSL with that of the *Vibrio fischeri* signal 3-oxo-C_6-HSL (Fig. 1A). HeLa cells were cotransfected with pcD/p65NTraR and the SEAP reporter gene and incubated with different concentrations of 3-oxo-C_8-HSL or 3-oxo-C_6-HSL, respectively. Results are shown in Fig. 5. Gene induction mediated by p65NTraR in the presence of 3-oxo-C_8-HSL was half-maximal at a concentration of 6 μM. This value is comparable to the half-maximal concentration of 3-oxo-C_8-HSL required for DNA-binding *in vitro* (14 μM). Therefore, efficient diffusion of 3-oxo-C_8-HSL into the cell does not appear to be a limiting step in the activation process. The induction reached a plateau at 80 μM 3-oxo-C_8-HSL, which corresponds to a 1,000-fold induction. In contrast, SEAP activity in the presence of 3-oxo-C_6-HSL was low even at a concentration of 80 μM. This result indicates that TraR-mediated gene expression may respond to 3-oxo-C_6-HSL, but with a substantial quantitative difference between 3-oxo-C_8-HSL and 3-oxo-C_6-HSL.

We found that 3-oxo-C_8-HSL undergoes slow lactonolysis in aqueous solutions (Supplementary Table 1), which is in line with the findings of Yates *et al*. (2002). In addition, 3-oxo-C_8-HSL is not toxic up to 100 μM, and reaches 50% cytotoxicity at 500 μM (see Supplementary Table 2). To increase stability, we have synthesized (3-oxo-octanoyl)-homoserine lactam (see Supplementary Information) and tested its biological activity. This compound is completely stable under experimental conditions and non-toxic up to 500 μM (see Supplementary Tables 1 and 2). The lactam derivative activates TraR-dependent transcription with an EC_{50} of 70 μM and reaches around 75% of the maximal SEAP activity obtained with 3-oxo-C_8-HSL at a concentration of 80 μM (Fig. 5).

DISCUSSION

The aim of this work is the development of a new regulated gene-expression system in eukaryotes based on a quorum-sensing bacterial protein of the LuxR family. We achieved this by fusing a eukaryotic activation domain to the N terminus of the *Agrobacterium* TraR protein. As with wild-type TraR, the chimaeric transcription factors bind to DNA containing a *tra* box only in the presence of a cognate ligand, such as 3-oxo-C_8-HSL. 3-Oxo-C_8-HSL must be present during the protein synthesis reaction, which is in line with previous findings that the ligand is necessary for proper folding of TraR (Qin *et al.*, 2000; Zhu & Winans, 2001). In fact, the three-dimensional structure shows that the ligand-binding site is deeply embedded in the protein core, consistent with a key role of the quorum-sensing signal molecule in the correct folding of the nascent protein (Vannini *et al.*, 2002; Zhang *et al.*, 2002). This requirement poses limitations to the use of the system as there will always be a substantial lag in time between induction with the AHL and gene activation.

We observed that the affinity of 3-oxo-C_8-HSL for the ligand-binding domain of TraR is not changed significantly in the context of the chimaeric proteins (Fig. 2B). However, the apparent potency of 3-oxo-C_8-HSL for TraR in eukaryotic systems is lower than in bacterial strains (with induction of gene expression occurring at micromolar levels in eukaryotic systems as compared with nanomolar levels in bacteria). The reason for the observed diminished ligand potency is not understood at present, and further work will be needed to address this issue.

We investigated whether p65NTraR and F3-TraR were capable of inducing gene transcription in eukaryotic cells. The highest level of SEAP activity was obtained with seven *tra* boxes and p65NTraR as transactivator. The requirement for several operator sequences in front of a minimal promoter for maximal activity has been previously described (Gossen & Bujard, 1992). Even though DNA-binding activity is preserved in both chimaeric proteins, competence to activate transcription to high levels was obtained only for the p65NTraR fusion protein. Interestingly, the transcriptional activity of this chimaeric protein was not profoundly altered by deletion of the NLS in the linker region (data not shown). This observation is consistent with the finding that recombinant wild-type TraR localizes to the nuclei of eukaryotic cells (data not shown). Therefore, reduced activity of F3-TraR is unlikely to be due to less efficient translocation to the nucleus. In HeLa cells, the basal level of transcription in the absence of 3-oxo-C_8-HSL is low, indicating that there are no endogenous factors that activate the TraR expression system. Addition of 3-oxo-C_8-HSL to the cell culture medium leads to strong induction of gene expression, comparable to the activity of the strong, non-inducible CMV promoter. Robust induction of SEAP expression in the presence of p65NTraR and 3-oxo-C_8-HSL has also been shown in three additional human cell lines.

Figure 5 shows that the TraR expression system is dose-dependent for 3-oxo-C_8-HSL. Increasing 3-oxo-C_8-HSL concentrations lead to increased SEAP activity, which reaches up to 1,000-fold induction at saturating levels of 3-oxo-C_8-HSL. As expected (Zhang *et al.*, 1993) 3-oxo-C_6-HSL, a closely related *N*-acyl homoserine lactone, elicits SEAP activity, but much larger amounts are required to produce SEAP levels comparable to those observed with 3-oxo-C_8-HSL. A lactone moiety, which undergoes spontaneous lactonolysis upon prolonged exposure to aqueous conditions (Yates *et al.*, 2002), is invariably present in all the quorum-sensing signal molecules of the LuxR family of

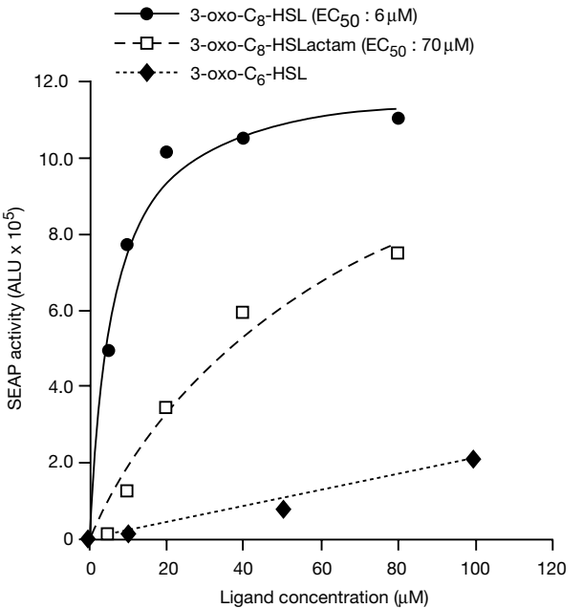

Fig. 5 | Titration curves of 3-oxo-C_8-HSL, 3-oxo-C_6-HSL and 3-oxo-C_8 HS-lactam in HeLa cell transfection experiments. HeLa cells were transfected with pcD/p65NTraR and pSEAP-(*tra* box)$_7$ and incubated with 0, 5, 10, 20, 40 or 80 μM of 3-oxo-C_8-HSL, 3-oxo-C_6-HSL or 3-oxo-C_8 HS-lactam. SEAP activity was measured after 12 h of incubation.

transcriptional regulators (Miller & Bassler, 2001). The limited chemical or enzymatic stability of lactones does, therefore, pose an obstacle to the future development of the system. In the attempt to design a ligand more suitable for experiments in eukaryotes, we have synthesized [*N*-β-oxo-octanoyl]-homoserine lactam (Fig. 1A). Notably, the [*N*-β-oxo-octanoyl]-homoserine lactam is stable under experimental conditions and is non-toxic up to a concentration of 500 μM. The potency of p65NTraR with this ligand is about ten times less than that of 3-oxo-C_8-HSL (an EC_{50} of 70 μM compared with 6 μM); however, similar biological activity could be reached by increasing the lactam concentration, permitted by the non-toxic nature of this compound. Thus, in spite of the striking conservation of the lactone ring in natural quorum-sensing signal molecules, it is possible to develop compounds that combine increased stability with biological activity.

In conclusion, we describe a novel eukaryotic gene-expression system based on engineered modules of bacterial quorum-sensing systems. Significant advantages of this system are the low basal transcriptional activity and a robust dose-dependent induction of target-gene transcription by the signalling molecule. This work demonstrates that it would be possible to engineer other LuxR proteins that respond to different AHLs and thus increase the repertoire of genes whose expression could be simultaneously regulated, provided that the cognate AHL for one LuxR protein does not activate the other. It is known that bacterial AHL signalling molecules can influence the behaviour of eukaryotic cells and tissues. In particular, certain AHLs have been shown to exhibit potent immune-modulatory and cardiovascular activity (Gardiner *et al.*, 2001; Smith *et al.*, 2001). Future work on the development of the system we describe will have to address these issues. Moreover, detailed

pharmacodynamic studies will have to be performed to test the utility of this system *in vivo*.

Although further studies on the characterization of the strengths and weaknesses of this system will be necessary, we feel that our findings will open up new avenues towards the development of a highly versatile gene expression system suitable for many different applications.

METHODS

Plasmid construction. The wild-type *traR* gene was excised as an *Nde*I–*Eco*RI fragment from BJZ358 (a gift from S. Winans) and cloned into the vector pcDNA3 (Invitrogen), yielding plasmid pcD/TraR. For the construction of N-terminal fusion proteins, a DNA encoding an amino-acid linker (Fig. 1B) was inserted into an artificial *Bam*H1 site located at the N terminus of *TraR* (pcD/P-TraR) using conventional methods (Sambrook *et al.*, 1989). A DNA fragment corresponding to the transactivation domain of NF-κB p65 (amino acids 287–551) (Burcin *et al.*, 1999) was amplified by the polymerase chain reaction and the fragment was cloned in-frame at the N terminus of pcD/P-TraR to make pcD/p65-TraR. The nuclear localization signal VPKKKRKV was inserted into the linker region of pcD/p65-TraR (Fig. 1B) to give plasmid pcD/p65NTraR. The F3 fragment was constructed using overlapping synthetic oligonucleotides and cloned into pcD/P-TraR at the *Bam*HI site. The resulting plasmid is named pcD/F3-TraR. For the construction of the SEAP reporters, the CMV minimal promoter (Gossen & Bujard, 1992) was cloned into the *Bgl*II/*Eco*RI restriction sites of pSEAP2-basic (pSEAP-CMVmin; Clontech). *Tra*-box-containing oligonucleotides (3′-GTCGGCTGAAAGGGAATGTGCAGATCTGCA-CATCGGCAACGC-5′ and 3′-CGACGCGTTGCCGATGTGCAGATCT-GCACATTCCCTTTCAGC-5′) were annealed to each other and self-ligated. Ligation products were 5′-end-filled using the Klenow fragment of *Escherichia coli* DNA polymerase I and cloned into the 5′-end-filled *Xho*I restriction site of pSEAP-CMVmin, resulting in one *tra* box or multimers placed in front of the minimal CMV promoter. The CMV minimal promoter containing seven *lux* boxes was constructed in a similar way, using the *lux*-box-encoding oligonucleotides 5′-GTC-GAACATAAGTACCTGTAGGATCGTACAGGTTTACGCAAG-3′ and 5′-CGACCTTGCGTAAACCTGTACGATCCTACAGGTACTTATGTT-3′.

***In vitro* translation and gel retardation assays.** Proteins were translated *in vitro* and radioactively labelled with [^{35}S]methionine (Amersham) using the TNT T7 Coupled (Fig. 2A) or TNT T7 Quick Coupled (Fig. 2B) transcription/translation systems (Promega). The *tra* box and *lux* box probes were constructed by annealing the two 42-base oligonucleotides shown above.The *tra* box duplex was radioactively labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP with the Klenow enzyme. The binding reaction was performed with 10 µl *in vitro* translated protein in the presence of 50 fmol labelled oligonucleotide, 25 mM HEPES/NaOH pH 7.5, 2% glycerol, 5 mM EDTA, 100 ng poly[d(I-C)]·poly[d(I-C)], 0.1% Triton X-100 and 50 mM NaCl in a final volume of 20 µl. The incubation was performed for 30 min at 22 °C and 5 µl of 20% (w/v) Ficoll were added to the reaction mix at the end of incubation. 10 µl of the sample were loaded onto a 6% non-denaturing polyacrylamide gel. Electrophoresis was carried out at room temperature in 0.25 × TBE.

Transfection experiments and measurement of SEAP activity. 2×10^5 cells for HeLa and Huh7 and 5×10^5 cells for 293 and RD cells were placed in 35 mm culture dishes and transfected using the Fugene 6 transfection reagent (Roche). For each transfection, 0.5 µg of transactivator plasmid, 0.05 µg of SEAP reporter plasmid (unless shown otherwise in the figure legend), and 0.1 µg of plasmid expressing luciferase under the control of a CMV promoter were transfected. After 6 h, the medium was removed and fresh medium was added either without or with ligands, as indicated in the figure legend (Fig. 4). At 12 or 24 h after the addition of ligands, the medium was collected and SEAP activity was measured using the Phospha-Light Chemiluminescent Reporter Assay (Tropix). SEAP units (ALU) were measured using the TopCount NXT instrument (Packard) and correspond to 12.5 µl of medium. Experiments were performed in triplicate, and measurements varied within 5% of the indicated values. Luciferase activity was determined in cell extracts as a measure of transfection efficiency.

Supplementary data are available at EMBO reports Online (http://www.emboreports.org).

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