Efficient control of gene expression by a tetracycline-dependent transactivator in single Dictyostelium discoideum cells

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Abstract

We established a tetracycline-regulated gene expression system that tightly controls expression of genes in Dictyostelium discoideum. The control elements are contained in two plasmid vectors, one being an integrated plasmid encoding a chimeric tetracycline-controlled transcriptional activator protein (tTA<sup>s</sup>1). The second component is an extrachromosomal plasmid harboring the gene of interest preceded by an inducible promoter. This promoter contains a tetracycline-responsive element, which is the binding site for tTA<sup>s</sup>1. Tetracycline prevents tTA<sup>s</sup>1 from binding to the tetracycline-responsive element, rendering the promoter virtually silent. In the absence of tetracycline, tTA<sup>s</sup>1 binds to its target sequence and strongly induces gene expression. The kinetics of activation and repression of the system were monitored using luciferase as a reporter. The results reveal efficient inhibition of gene expression by low concentrations of tetracycline and an induction of gene expression by several orders of magnitude within a few hours after removal of tetracycline. Green fluorescent protein (GFP) provided information about the effects of modulation of the tetracycline concentration on gene expression, at the single cell level, using fluorescence activated cell sorting (FACS). We also report that not all cells in a clonal population express the reporter gene. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Green fluorescent protein; Fluorescence activated cell sorting; Inducible promoter; Luciferase

1. Introduction

Dictyostelium discoideum is a soil amoeba having signal transduction pathways similar to those of mammalian cells, leading to chemotaxis and cell differentiation. Furthermore, it shows mammalian-like glycosylation and architecture (Slade et al., 1997; Parent and Devreotes, 1999). Since D. discoideum cells can be easily transformed and cultured, and are haploid during the major part of their life-cycle, rapid screening of mutants can be performed for studying the role of endogenous genes in D. discoideum (Kim et al., 1997).

In addition, D. discoideum can be employed to express functional heterologous proteins (Slade et al., 1997) and for mutant screening purposes of complex recombinant human glycoproteins (Linskens et al., 1999). Modulation of the level of expression of endogenous genes in a time-controllable fashion is a valuable tool for analyzing the phenotypes associated with mutations in genes. Tight control of gene expression is especially advantageous for potential cytotoxic proteins. However, previous attempts to regulate gene expression in vegetatively growing cells of D. discoideum have met with limited success, due to the lack of tightness of control (Blusch et al., 1992; Dingermann et al., 1992; Wetterauer et al., 1993).

The tetracycline-controlled inducible expression system, originally described by Gossen and Bujard (1992), provides tight control of gene expression in mammalian cells by low, non-toxic levels of tetracycline (Tc). Briefly, this system employs a Tc-controlled transcriptional activator (tTA), that is negatively regulated by Tc. This transactivator interacts specifically with an inducible promoter, that controls the transcription of
the gene of interest. The transactivator (ITA) is a fusion of the DNA-binding Tet repressor (TetR) from *Escherichia coli* to the activation domain from herpes simplex virus protein 16 (VP16AD), thereby converting it from a repressor into a eukaryotic transcriptional activator protein (Gossen and Bujard, 1992). The inducible promoter consists of a minimal promoter, preceded by a Tc-responsive element (TRE) encompassing seven copies of a Tc-resistance operator (tetO) from *E. coli* (Gossen and Bujard, 1992).

In the absence of Tc, ITA binds specifically to TRE and strongly activates the minimal promoter, leading to transcription of the gene of interest. In the presence of Tc, however, a conformational change in the Tet repressor moiety prevents ITA from binding to the TRE and the promoter is silent (Gossen and Bujard, 1992; Shokett and Schatz, 1996). Gene expression can be gradually reduced by adding increasing amounts of Tc (Gossen and Bujard, 1992).

We have adapted the Tc-controlled expression system for application in *D. discoideum*. A modified transactivator (ITA) that is encoded on a vector randomly integrated in the genome is constitutively expressed under control of the complete *D. discoideum* actin15 promoter (Cohen et al., 1986; Hori and Firtel, 1994). An extra-chromosomal response plasmid contained a luciferase or GFP gene fused to a Tc-inducible promoter (TRE-P<sub>min</sub>) consisting of a minimal *D. discoideum* actin15 promoter preceded by seven copies of tetO. We report here our results from experiments expressing firefly luciferase (de Wet et al., 1987) and GFP (Cheng et al., 1996) using this inducible system. While luciferase expression provided information about the kinetics and induction in cells at the population level, FACS enabled us to investigate the regulation of GFP expression at the individual cell level.

2. Materials and methods

2.1. Culture conditions and transformation of *D. discoideum* cells

Cells of strain AX3 were grown in axenic medium essentially as described in Watts and Ashworth (1970). with 10 g/l of glucose instead of 15.4 g/l. *D. discoideum* cells were transformed with circular plasmid DNA by electroporation as previously described (Heikoop et al., 1998; Howard et al., 1988). Depending on the antibiotic-resistance cassette present in the plasmids, the medium was supplemented with 10 mg/l of blasticidin S (ICN) and/or 20 mg/l of G418 (Gibco). The cell lines obtained after transformation of the transactivator plasmid (see Section 2.2.2) served as host cell lines for the transformation of response plasmid (see Section 2.2.1), containing either a luciferase or a GFP encoding reporter gene (see Sections 2.2.3 and 2.2.4, respectively). After this second transformation the cells were maintained in the presence of both blasticidin and G418. Tetracycline (Roche) was present at the concentrations indicated in the experiments.

2.2. Construction of plasmids

Most of the plasmids were constructed as described below by multiple cloning steps according to standard methods (Sambrook et al., 1989). Codon preference and induction initiation signals are different in *D. discoideum* (Fey et al., 1995; Firtel and Chapman, 1990; Sharp and Devine, 1989; Vervoort et al., 2000) as compared with those of other organisms. During cloning we adapted sequences to match the preferred usage in *D. discoideum*. *E. coli* XL1-Blue (Stratagene) was used as a host for the construction of all the plasmids mentioned. PCR was performed with Taq DNA polymerase (Roche) or the Expand High Fidelity System (Roche). Amplified DNA sequences were analyzed by dideoxy sequencing.

2.2.1. Construction of the response plasmid

Except for the Tc-inducible promoter, the response plasmid MB38 (Fig. 1) contains essentially the same elements as the previously described extrachromosomal plasmid MB12N (Heikoop et al., 1998). In short, the Tc-inducible promoter (TRE-P<sub>min</sub>) was created by multiple cloning steps using pGEMNB, a pGEM7Zf(+) (Promega)-derived plasmid in which we introduced an NdEl- and BglII-site in between the BamHI- and HindIII-site of the multiple cloning region. First, the TRE from plasmid pTRE (Clontech), containing seven copies of tetO, was cloned in pGEMNB after digestion with Smal and XhoI (plasmid MB24). Second, the XhoI/BglII fragment containing the complete A15P region of plasmid BS19.2H (gift from R. Firtel) was subcloned in pGEMNB (plasmid MB26). The minimal *D. discoideum* actin15 promoter (A15P<sub>min</sub>) consisting of region −127 to −14 of A15P (Cohen et al., 1986; Hori and Firtel, 1994), was isolated from MB26 after digestion with Accl, filling in the overhanging ends using Klenow, and cutting with BglII. Subsequently, the A15P<sub>min</sub> fragment was fused downstream of TRE in MB24 cut with Smal and BglII, to give the Tc-inducible promoter (Fig. 1b). To complete the construction of MB38, the A15P of the expression...
Fig. 1. Response plasmid. (a) Map of the extrachromosomal response plasmid MB38. The plasmid contains sequences for propagation (ColEl ori) and ampicillin resistance (ampR) in E. coli (from pBluescript SK), a blasticidin resistance cassette (bsr) consisting of the blasticidin deaminase gene under control of the complete D. discoideum actin15 promoter and the D. discoideum actin8 terminator, sequences for extrachromosomal maintenance in D. discoideum (G4/D5, G5/D6), a D. discoideum origin of replication (OriDd), and the inducible promoter (TRE-Pmin) upstream of the D. discoideum 2H3 terminator (2H3T). Four unique restriction sites are present for insertion of the gene to be expressed. (b) Sequence of the inducible promoter and the multiple cloning region of MB38. Bases 8086–8392 correspond to the Tc-responsive element (TRE) containing seven copies of the tet operator sequence (shown in bold letters). Bases 8393–8506 contain the D. discoideum actin15 minimal promoter (Pmin), corresponding to the −127 to −14 region of the D. discoideum actin15 promoter. The position of the D. discoideum TATA box is indicated, as well as the unique restriction sites for insertion of the gene to be expressed. Note that the cloned insert must have an initiation codon.

2.2.2. Construction of the transactivator plasmid

The transactivator plasmid MB35 (Fig. 2) is based on the integrating D. discoideum vector BS18 2H3, conferring G418-resistance to D. discoideum host cells. It contains the TA0 encoding gene (tTA0) under control of A15P and the 2H3 terminator. tTA0 was adapted for use in D. discoideum as follows. First, the 621 bp TetR part of tTA0 was obtained by PCR with plasmid pTet-O (Clontech) as a template, using ATTCC-3 as the 3'-primer. Five As were introduced upstream of the initiation codon (underlined), and BglII- and BamHI-sites were included for cloning purposes. Second, the 255 bp 3' part of VP16AD in plasmid pTet-Off was amplified by PCR, concomitantly, seven codons in the 5' region from the resulting short VP16AD as well as the stop codon (underlined) were replaced by codons preferred by D. discoideum (table from Vervoort et al., 2000), using 5'-GGGTTTGATGACACACTCGT-AGACTCTCAA of A15P and the 2H3 terminator (2H3T). Sequences analysis revealed the presence of one silent mutation (nucleotide 768, GGT to GGC) in VP16AD'. Via multiple cloning steps using pGEMNB, the modified activation domain
Fig. 2. Transactivator plasmid. (a) Map of the integrating transactivator plasmid MB35. The plasmid contains sequences for propagation (ColEI ori) and ampicillin resistance (ampR) in E. coli (from pUC18), a D. discoideum actin6 promoter-driven G418 resistance cassette (neoR) and the gene encoding the transactivator (tTA) driven by the complete D. discoideum actin15 promoter (A15P) and followed by the D. discoideum 2H3 terminator (2H3T). (b) Schematic representation of the gene encoding the transactivator (tTA*). Bases 1–621 encode the original 207 amino acids of TetR (terR), bases 622–876 the original 85 C-terminal amino acid residues of the VP16 activation domain (VP16AD). Numbers indicate nucleotide positions in tTA*, amino acid residues are presented below the nucleotide sequence. Letters in bold designate bases and amino acid residues of VP16AD. Nucleotides mutated to create codons adapted to the preferred codon usage in D. discoideum are indicated by an asterisk. The gene contains one silent mutation (nucleotide 768) introduced by PCR (not shown).

(VP16AD) was cloned downstream the modified TerR moiety. The resulting tTA* was inserted into the BglII-site of BS18.2H3.

2.2.3. Construction of luciferase reporter plasmids

The firefly luciferase gene was used as reporter gene (de Wet et al., 1987). Four As were introduced in front of the ATG start codon and six of the first 10 codons were adapted to the preferred codon usage of D. discoideum (Sharp and Devine, 1989; Vervoort et al. 2000). For this purpose, a double-stranded synthetic oligonucleotide containing BamHI- and NarI-compatible overhanging ends, sense oligonucleotide: 5'-GATCCAAAAATGGAAGATGCTAAAAATATTAAGAAAGGTFCCAG-3' (start codon underlined), antisense oligonucleotide: 5'-CGCTGGACCTTCTTAATATTCTTAAAGGTTATGGAAGATGCTAAAAATATTAAGAAAGGTFCCAG-3' (start codon underlined), was inserted in BamHI/NarI-digested plasmid pGEM-huc (Promega). This modified luc* was subcloned as a BamHI/NarI-fragment downstream of TRE-Pmax or A15P in the Midl-(rendered blunt by Klenow) and BglII-sites of MB38 and MB33, respectively.

2.2.4. Construction of GFP reporter plasmids

The GFP-S65T gene (Cheng et al., 1996) was adapted for usage in D. discoideum. In the modified gene (gfp"") a TAA stop codon was introduced, as well as four As in front of the ATG start codon. Plasmid p865T-C1 (Clontech) served as template for the amplification of GFP-S65T. The following primers were used: 5'-AGCTTGGATCCTATCAACGGACCTTCTAAATATTCTTAAAGGTTATGGAAGATGCTAAAAATATTAAGAAAGGTFCCAG-3' (start codon underlined) as 5'-primer
and 5′-TGTTAGATCG TTACTGTAT AGTTCCAT-CC-3′ as 3′-primer (stop codon underlined), containing a BamHI- and a RsrII-site, respectively, for insertion of gfp in the RsrII-sites of MB38 and MB33.

2.3. RNA preparation and hybridization

Cells (1 × 10⁷) were grown on a 9 cm Petri dish and washed once with EB [10 mM sodium phosphate buffer (pH 6.7), 50 mM sucrose] at 22°C. Cells were collected by centrifugation and resuspended in 0.6 ml RLT lysis buffer (Qiagen). The lysed cells were homogenized by passing them several times through a 0.5 × 16 mm needle fitted to a syringe. Total cellular RNA was prepared using the RNeasy kit (Qiagen) following the manufacturer’s protocol. For Northern blot analysis, 10 ng of RNA was diluted in RNA gel loading buffer and resolved on a formaldehyde gel. After electrophoresis, RNA was transferred onto a Nitran membrane (Schleicher & Schuell) and cross-linked by UV. Radioactive probes were generated by end-labeling with [γ-32P]ATP (Amersham). The synthetic antisense oligonucleotides 5′-GGGAAAACAG CACGTGGAGC ATTCGTACCA GCAAAAACGG C-3′ and 5′-CGCCTGGACCT TTCTTAATAT TTTAGCATC TTCCATTTTGT-3′ served as actin15 and luc probe, respectively. Hybridization was first performed at 50°C with the luc′ probe in 0.5 M Na₂HPO₄, H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 0.2 mg/ml BSA, 7% SDS. After hybridization, filters were washed 2 × 15 min in 40 mM Na₂HPO₄, H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 5% SDS and 4 × 15 min in 40 mM Na₂HPO₄, H₃PO₄ (pH 7.2), 1 mM Na₂EDTA, 1% SDS at 65°C (actin15 probe) or 50°C (luc′ probe) quantified using a PhosphoImager TRE-P (Molecular Dynamics, ImageQuant software). The blot was stripped and re-probed with the actin15 probe at 65°C. The luc′ hybridization signal was normalized relative to the actin15 hybridization signal in each sample.

2.4. Luciferase assays

Cells were grown in 24-well multishes until 50–90% confluency was reached (0.5 × 10⁶ cells), and resuspended in 0.5 ml of EB. After centrifugation, the cells were resuspended in 30 μl of CCLR (Promega) supplemented with 1× Complete Mini EDTA-free (Roche) protease inhibitor cocktail and 5 mM of the phosphatase inhibitor sodium orthovanadate (ICN) (Rutherford et al., 1997). After incubation at 22°C for 20 min, the cell extracts were frozen at −80°C. Immediately after thawing, 5 μl of cell extracts were mixed with 50 μl of Luciferase Assay Reagent (Promega) in Fluoro Nunc opaque white 96-well plates (Nunc). Luminescence was detected using a FL600 Fluorescence microplate reader (Bio-Tek Instruments). The lamp was turned off and the emission filter was removed during measurements. Luminescence was quantified using the luminescence of recombinant luciferase (Promega) as a standard. The luciferase activity was normalized to the protein concentration of the cell extract as determined by the method of Bradford (1976).

2.5. Flow cytometry

Expression of gfp, displaying excitation and emission maxima at 490 and 511 nm, respectively (Cheng et al., 1996), was analyzed by FACS analysis using an Epics Elite flow cytometer (Coulter). Approximately 3000 or 5000 intact cells were illuminated at 488 nm and fluorescence was detected in the FITC (525/20 nm) channel. Non-specific fluorescence was detected using a 575/30 nm emission filter in the PE channel. The relative fluorescence signal is given in arbitrary units (AU).

Cells were grown on plates until 10–30% confluency, washed 3 × with EB at 22°C, and resuspended in EB at a concentration of 1–5 × 10⁶ cells/ml. When cells were cultured in the presence of Tc, the same concentration of Tc was added to EB. Cells were kept on ice until FACS analysis was carried out.

3. Results

3.1. Construction and characterization of the inducible promoter

TRE-Pmax, the promoter that can be negatively regulated by Tc, was harbored in the extrachromosomally maintained response plasmid MB38. To generate TRE-Pmax, the enhancer sequences upstream from position +127 of the complete D. discoideum A15P (Cohen et al., 1986; Hori and Firtel, 1994) were replaced by a DNA fragment containing seven tetr sequences (Gossen and Bujard, 1992) (Fig. 1b). The orientation of TRE-Pmax was opposite to that of the A15P of the blastocidin resistance cassette (Fig. 1), in order to avoid possible readthrough from the A15P on transcription from TRE-Pmax. In order to test the residual activity of TRE-Pmax, luciferase expression driven by TRE-Pmax was measured in AX3 cells, in the absence of transcriptional activator protein and Tc. Luciferase activity was indistinguishable from the signal measured in AX3 cells lacking a luciferase gene, which is undetectable (below 0.2 ng luciferase/mg protein) (Table 1), whereas the luciferase expression level driven by A15P ranged from 447 to 876 ng luciferase/mg total protein. Similar results were obtained for cells growing in the presence of 2 mg Tc/l (data not shown). These results demonstrate that the minimal promoter region of TRE-Pmax is silent.
Fig. 3. Time course of Tc action. (a) Northern blot analysis of luciferase mRNA (luc) produced by 8Dluc cells, AX3 cells transformed with the A15P/luc plasmid (AX3luc) or untransformed (AX3) and tet cells (tet). Total RNA was isolated from cells grown in the absence of Tc for 4 days (0 Tc), after removal of Tc (2/0 Tc) or addition of Tc (0/10 Tc) at different times (h) indicated. The act15 mRNA (act15) is shown as a loading control. (b) Cells of clone 8Dluc were grown on plates in HG5 medium in the absence (diamonds) or presence (2 mg/l) (squares) of Tc. Induction of luc mRNA (open circles, dotted line) and luciferase activity (circles) was monitored after removal of Tc at time 0. Reduction of luciferase mRNA (open squares, dotted line) and luciferase activity (×) was measured after the addition of Tc (10 mg/l) to Tc-free medium at time 0. A relative luciferase activity of 100% corresponds to 958 ng luciferase/mg protein.

3.2. Construction and transactivation potential of tTA

The transactivators tTA and tTA', originally described by Gossen and Bujard (1992), contain a C-terminal portion of the VP16 activation domain of 128 amino acids (VP16 codons 363–490) or 85 amino acids (VP16 codons 406–490), respectively. When fused to the E.coli TetR, both transcriptional activator proteins exhibited essentially the same transactivation potential (Gossen and Bujard, 1992). During cloning we adapted sequences to match the preferred usage in D. discoideum, since codon preference and translation initiation signals are different in D. discoideum (Fey et al., 1995; Firtel and Chapman, 1990; Sharp and Devine, 1989; Vervoort et al., 2000) as compared with other organisms. For instance, 17 of VP16 codons 363–405 are used at a frequency lower than 0.6% in D. discoideum, which may negatively influence expression.
The TetR region. The resulting coding sequence of tet coding sequence of AX3 TRE-P

3.3. Kinetics of activation and inactivation

We therefore decided to fuse a short activation domain (VP16AD), containing codons 406–490 of VP16, to the TetR region. The resulting coding sequence of TetR was expressed constitutively under control of the complete D. discoideum A15P (plasmid MB35, Fig. 2).

The capacity of TetR to activate luciferase expression and of Tc to reduce expression was assessed in different A15P expressing tet cell lines. A slight reduction in growth rate was observed in most of the tet cell lines (approximately one doubling per 16 h) as compared with AX3 cells (one doubling per 12 h). Upon transformation with the TRE-P\textsubscript{mec} luc\textsuperscript{−} response plasmid, cells were grown either in the absence or in the presence of 10 mg Tc/l. As shown in Table 1, in the presence of Tc luciferase activity was not or hardly detectable. In contrast, in the absence of Tc, TetR strongly activates the inducible promoter. The luciferase expression level ranged from 5% to 130% as compared with the expression driven by the D. discoideum complete A15P (Table 1). This variation is likely due to the different integration sites of the transactivator plasmid in the different tet cell lines. A variation in expression levels was also observed in individual subclones of the same tet cell line after transformation of the TRE-P\textsubscript{mec} luc\textsuperscript{−} response plasmid. The highest induction factor observed was approx. 3000-fold in tetr\textsuperscript{R}+TRE-P\textsubscript{mec} luc\textsuperscript{−} cells, clone D (8Dluc cells), resulting from an induced expression level of 110% and a basal expression level of 0.04% (Table 1). In other clones, the expression level under maximally inducing conditions was at least 500-fold above the basal expression level, which was below 0.03% (0.2 ng luciferase/mg protein).

3.3. Kinetics of activation and inactivation

The 8Dluc cell line, that expressed the highest level of luciferase in the induced state and shows a basal expression level just above the detection limit, was selected for further analysis. 8Dluc cells were grown for several days on Petri dishes in HG5, both in the absence and the presence of Tc. As shown in Fig. 3, removal of Tc (2 mg/l) by rinsing and replacing Tc-containing medium with Tc-free medium resulted in an increase (approximately one doubling per 16 h) as compared with AX3 cells (one doubling per 12 h). Upon transformation with the TRE-P\textsubscript{mec} luc\textsuperscript{−} response plasmid, cells were grown either in the absence or in the presence of 10 mg Tc/l. As shown in Table 1, in the presence of Tc luciferase activity was not or hardly detectable. In contrast, in the absence of Tc, TetR strongly activates the inducible promoter. The luciferase expression level ranged from 5% to 130% as compared with the expression driven by the D. discoideum complete A15P (Table 1). This variation is likely due to the different integration sites of the transactivator plasmid in the different tet cell lines. A variation in expression levels was also observed in individual subclones of the same tet cell line after transformation of the TRE-P\textsubscript{mec} luc\textsuperscript{−} response plasmid. The highest induction factor observed was approx. 3000-fold in tetr\textsuperscript{R}+TRE-P\textsubscript{mec} luc\textsuperscript{−} cells, clone D (8Dluc cells), resulting from an induced expression level of 110% and a basal expression level of 0.04% (Table 1). In other clones, the expression level under maximally inducing conditions was at least 500-fold above the basal expression level, which was below 0.03% (0.2 ng luciferase/mg protein).

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Maximal luciferase activity in the absence of tetracycline*</th>
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<tr>
<td>Host cell line</td>
<td>Reporter construct</td>
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<tr>
<td>AX3</td>
<td>A15P−</td>
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<td>AX3</td>
<td>TRE-P\textsubscript{mec} luc\textsuperscript{−}</td>
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<tr>
<td>iso2</td>
<td>TRE-P\textsubscript{mec} luc\textsuperscript{−}</td>
</tr>
<tr>
<td>iso3</td>
<td>TRE-P\textsubscript{mec} luc\textsuperscript{−}</td>
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<tr>
<td>iso4</td>
<td>TRE-P\textsubscript{mec} luc\textsuperscript{−}</td>
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<tr>
<td>iso5</td>
<td>TRE-P\textsubscript{mec} luc\textsuperscript{−}</td>
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* Cell lines expressing TetR from iso2 to iso15 were cloned with respect to TetR expression. After transformation of luc\textsuperscript{+} reporter plasmids, cells from different subclones were grown on plates in HG5 medium with or without Tc (10 mg/l) (+ Tc and − Tc, respectively). Presented are average values and standard deviations of two to three independent experiments n.d., not determined.

Luciferase activity was related to the A15P-driven luciferase expression level in AX3 cells (100% = 675 ng luciferase/mg total protein).

We therefore decided to fuse a short activation domain (VP16AD), containing codons 406–490 of VP16, to the TetR region. The resulting coding sequence of TetR was expressed constitutively under control of the complete D. discoideum A15P (plasmid MB35, Fig. 2).

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Fig. 4. Dose–response analysis of Tc-regulated luc\textsuperscript{+} expression. Relative luciferase activity in 8Dluc cells grown on plates in HG5 medium containing different concentrations of Tc as indicated. After 4 days, the cells were assayed for luciferase activity. Average values of two independent experiments are given. A relative activity of 100% corresponds to 958 ng luciferase/mg total protein.
Table 2
Mean GFP fluorescence in GFP-positive cells

<table>
<thead>
<tr>
<th>Host cell</th>
<th>Reporter construct</th>
<th>Mean fluorescence in GFP-positive cells (AU)</th>
<th>% of GFP positive cells (of total cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX3</td>
<td>A15P/gfp*</td>
<td>119 (32%)</td>
<td>136 (28%)</td>
</tr>
<tr>
<td>tet</td>
<td>TRE-Pmsn/gfp*</td>
<td>108 (32%)</td>
<td>109 (38%)</td>
</tr>
<tr>
<td>tet</td>
<td>TRE-Pmsn/gfp*</td>
<td>101 (39%)</td>
<td>136 (39%)</td>
</tr>
<tr>
<td>tet</td>
<td>TRE-Pmsn/gfp*</td>
<td>120 (32%)</td>
<td>92 (21%)</td>
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<tr>
<td>gfp</td>
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</table>

* Cell lines tet, tre and tch were clonal with respect to tTA expression. Cells of subclones obtained after transformation of gfp* reporter plasmids were grown on plates in Tc-free HG3 during 4 days. GFP expression in individual cells was analyzed using FACS. GFP fluorescence was the mean fluorescence signal in GFP positive cells in arbitrary units (AU), determined using excitation at 488 nm and a 525 nm emission filter.

within 30 min. This provided us with an estimation of the biological half-life of luc mRNA of less than 6 min. However, after 48 h (about three doubling times) approx. 6% of the maximal luciferase activity was still present per cell. Presumably this luciferase was synthesized prior to the addition of Tc, and diluted in the biomass by cell divisions. Studies with cycloheximide confirmed that luciferase was relatively stable in 8Dhec cells with a half-life of at least 30 h (data not shown).

3.4. Quantitative control of luciferase expression

Dose-response analysis of luciferase expression levels was examined in cell extracts of 8Dhec cells exposed to Tc concentrations ranging from 100 ng/l to 30 mg/l during 4 days. The Tc concentrations used had no effect on growth rate or cell morphology. Fig. 4 shows that maximal induction was achieved at Tc concentrations of 0.01 mg/l or below. Increasing Tc concentrations resulted in a decrease of the luciferase expression level. Approximately half of the maximal luciferase expression level was measured at a Tc concentration of 0.1 mg/l, while at concentrations of 1 mg/l or higher tTA was totally inactivated and luciferase activity was undetectable.

3.5. GFP expression under inducing and non-inducing conditions

FACS analysis of GFP expressing cells enabled us to study the activity of the inducible promoter in individual cells. Three of the tet cell lines were transformed with the TRE-Pmsn/gfp* response plasmid. The GFP fluorescence patterns of fully induced cells (in the absence of Tc) and cells grown under repressing conditions (3 mg Tc/l) were compared to the fluorescence patterns obtained with various cell lines not expressing GFP (non-transformed tet cells and AX3 cells, as well as AX3 cells transformed with the TRE-Pmsn-plasmid MB38). In addition, we examined expression of GFP under the control of A15P in AX3 cells. We determined that the autofluorescence was similar in all cell lines that were not transformed with a gfp reporter plasmid, and not influenced by the presence of Tc (data not shown). All non-induced tet+ TRE-Pmsn/gfp* cell lines exhibited fluorescence patterns indistinguishable from autofluorescence.

As shown in Table 2, GFP expression under control of A15P in AX3 cells was quite similar in different subclones, with a mean fluorescence of 109-136 AU in only 28-38% of the cells. Apparently, in 62-72% of the cells, GFP expression is not detectable. When tet+ TRE-Pmsn/gfp* cells were maximally induced, we also observed that only a portion of the cells showed GFP fluorescence. Among different tet+ TRE-Pmsn/gfp* cell lines, the mean GFP fluorescence and the fraction of positive cells ranged from 44 AU in 11% of the cells to 229 AU in 58% of the cells. In this experiment, the strongest (tTA-regulatable cell line (tet5+ TRE-Pmsn/gfp*, clone 5Bgfpl) expressed about 3-fold more GFP than cells expressing GFP under the control of A15P.

3.6. Gradual regulation of GFP expression in individual cells

FACS analysis of GFP expression at a range of Tc concentrations was performed, in order to analyze whether partial induction in a cell population at intermediate Tc concentrations was either due to full induction
in an increasing number of cells, or to a gradual induction in all positive cells. Partial activation of GFP expression was studied by exposing tet2 and tet5 cells transformed with TRE-\textsc{pm} \textsc{c} (clones 2Agfp and 5Dgfp, respectively) to a wide range of Tc concentrations for 4 days.

Fig. 5a reveals that GFP expression was induced in a dose-dependent manner in individual cells. Only background fluorescence was observed in the presence of 3 or 10 mg/l Tc, while maximal GFP expression was reached at concentrations of 0.03 mg/l or lower. FACS analysis was not sensitive enough to distinguish between the background fluorescence or a low expression level of GFP, in cells grown at 1 mg/l of Tc. The FACS profiles for cells 3Dgfp (Fig 5b) clearly show that decreasing concentrations of Tc (0.3 or 0.1 mg/l Tc) resulted in a gradual increase of GFP fluorescence in the positive cells (in the region to the right of line R2 in the Fig. 5b). Apparently, Tc did not affect the number of positive cells, since the fraction of positive cells (approx. 60%) remained constant. Similar results were obtained with FACS analysis of cells of clone 2Agfp (data not shown).

4. Discussion

In this report we describe the construction and characterization of a tetracycline-regulated inducible gene expression system in \textit{D. discoideum}, based on a similar system for mammalian cells originally described by Gossen and Bujard (1992).

4.1. Inducible promoter

Transcription is driven by an inducible \textit{D. discoideum} promoter, TRE-\textsc{pm}, through the action of the transactivator protein \textsc{t}\textsc{ta}. The activity of \textsc{t}\textsc{ta} is repressed by Tc. In TRE-\textsc{pm}, the enhancer sequences of the \textit{D. discoideum} A15P (Cohen et al., 1986; Hori and Firtel, 1994) have been replaced by a DNA fragment containing seven \textsc{tet} operator sequences. TRE-\textsc{pm} was introduced in an extrachromosomal plasmid, which is stably extra-chromosomally maintained under selective conditions. This presents two advantages as compared to the use of an integrated vector. First, TRE-\textsc{pm} is not influenced by endogenous enhancing sequences, so is virtually silent under non-inducing conditions (Table 1). Second, recovery of the response plasmid from transformants is relatively straightforward, which may be useful in mutagenesis studies.

4.2. Analysis of \textsc{t}\textsc{ta}*

For efficient induction of TRE-\textsc{pm}, the intracellular concentration of \textsc{t}\textsc{ta} should be sufficiently high. On the other hand, a relatively high concentration of a VP16 fusion protein, like \textsc{t}\textsc{ta}*, is believed to be toxic due to transcriptional squelching effects (Gossen and Bujard, 1992; Shockett and Schatz, 1996). In order to obtain cell lines with optimal induction characteristics, we transformed cells with an integrating vector (Fig. 2) for constitutive expression of \textsc{t}\textsc{ta}*. Variation in both the copy number of this transactivator plasmid and the site of chromosomal integration will result in different \textsc{tet} cell lines which have varying expression levels of \textsc{t}\textsc{ta}*. Seven \textsc{tet} cell lines were analyzed. We observed a slight reduction in growth rate in most of the \textsc{tet} cell lines, suggesting that expression of \textsc{t}\textsc{ta} may have some negative effect on the transcriptional machinery or physiology of the cell.

The range of luciferase and GFP expression levels from reporter constructs in maximally induced cells varied widely among the various \textsc{tet} cell lines (Tables 1 and 2). Moreover, different subclones obtained from the same \textsc{tet} cell line displayed some variation in expression levels, which may be a result from variation in the copy number of the reporter plasmid. The maximum levels of luciferase and GFP expression were 1.3-fold and 3-fold higher, respectively, as compared with the levels obtained with A15P. The maximal induction factor of approx. 3000-fold (Table 1) is in the same range as observed for the \textsc{t}\textsc{ta}-based expression systems in mammalian cells (Gossen and Bujard, 1992; Kringlestein et al., 1998; Shockett and Schatz, 1996) and yeast (Gari et al., 1997). In summary, our results indicate that \textsc{t}\textsc{ta} is a potent transactivator in \textit{D. discoideum}.

4.3. Kinetics of induction and repression

Inactivation of \textsc{t}\textsc{ta}-regulated transcription is a rapid process. The data in Fig. 3 show that upon addition of Tc (10 mg/l), the luc RNA level decreased by 100-fold within 30 min. Since no new protein can be made, we conclude that the slow decrease of luciferase activity in the biomass is due to the relative stability of pre-existing luciferase in \textit{D. discoideum}. In contrast, switching the system on upon removal of Tc is a somewhat slower process than repression (Fig. 3). Half-maximal induction was reached by 8 h (luc m RNA) and 18 h (luciferase activity). This result is consistent with the observations in mammalian cells (Gossen and Bujard, 1992; Kringlestein et al., 1998), \textit{Drosophila} (Bello et al., 1998) and yeast (Gari et al., 1997). An alternative version of Tc-regulated systems employs reverse \textsc{t}\textsc{ta} (r\textsc{t}\textsc{ta}), a mutant of \textsc{t}\textsc{ta} requiring the addition of the Tc analog doxycycline to induce binding to the TRE (Gossen et al., 1995). Although it could be expected that the r\textsc{t}\textsc{ta} system would allow for faster induction kinetics, similar kinetics of activation and repression using either \textsc{t}\textsc{ta}- or r\textsc{t}\textsc{ta}-based systems were reported for mammalian cells (Gossen et al., 1995; Kringlestein et al., 1998).
Attempts to use rtTA in Drosophila (Bello et al., 1998) were unsuccessful, and in yeast repression of the rtTA-system was incomplete (Bello et al., 1998). A different approach to improve the kinetics of induction of the tTA<sub>i</sub>-based system in D. discoideum might be the use of a Tc-like compound with a short in vivo half-life, or an antagonist of Tc (Christ-Balz and Hooft van Huijsduijnen, 1996).

4.4 Quantitative regulation

The expression level of luciferase, driven by the inducible promoter TRE<sub>P</sub> in tTA<sub>i</sub> expressing cells, was quantitatively controlled by the concentration of Tc (Fig. 4). A dose-dependent response to Tc has also been reported for mammalian cells (Gossen and Bujard, 1992; Kringstein et al., 1998; Zeng et al., 1998) and yeast (Gari et al., 1997). The concentration range (0.01–1.0 mg/l) of Tc, allowing partial induction in D. discoideum, was in agreement with the range observed for yeast but differed markedly from the range typically reported for mammalian cells (0.008–0.1 mg/l). These different ranges are probably due to species-specific Tc metabolism.

4.5 Regulation at single cell level

FACS analysis allowed us to measure GFP expression in every single D. discoideum cell of a clonal population. As shown in Fig. 5, step-wise decreasing the concentration of Tc below 1 mg/l leads to a gradual increase of GFP expression at the individual cell level in GFP positive cells, rather than a gradual increase in the number of cells expressing GFP. This graded response in individual cells is in agreement with data obtained for mammalian cells (Kringstein et al., 1998; Zeng et al., 1998).

However, we noted a marked heterogeneity in GFP expression. Irrespective of whether GFP expression is controlled by A15P or regulated by tTA<sub>i</sub>, all clonal populations showed a bimodal distribution of cells, consisting of subpopulations of GFP-negative cells and GFP-positive cells (Table 2). Since the half-life of GFP in D. discoideum is relatively long (at least 30 h, our own observations and Deuschel et al., 1999), we conclude that GFP-negative D. discoideum cells did not express the reporter gene during several doubling times. Experiments using sorted cells of an AX3 cell line transformed with the A15P/gfp<sup>+</sup> plasmid showed that the GFP negative cells did not grow in the presence of blasticidin (data not shown), indicating that in these cells the reporter plasmid is lost. GFP expression in positive cells displayed a 100-fold range of fluorescence intensity. The mechanism for this heterogeneity is still unclear, but it is not specific for this expression system. It has been observed using integrating plasmids in D. discoideum for A15P-controlled expression of GFP (Pang et al., 1999), in Polysphondylium pallidum for actin<sup>+</sup> promoter (A6P)-controlled expression of GFP (Fey et al., 1995), in D. discoideum for A6P-controlled expression of beta-galactosidase (Dungermann et al., 1989) and it has been observed in mammalian cells expressing GFP (Cheng et al., 1996; Klucher et al., 1997).

4.6 Applications

The inducible expression system is particularly useful for several applications. Constructing gene knock-out strains may fail occasionally, which is often interpreted as that the gene is essential. Induced expression of the gene, in the absence of Tc, allows for isolation of a cell line with the disruption of the chromosomal gene; subsequently turning-off expression may provide proof that the gene is essential. Furthermore, the rapid decline of transcription upon addition of Tc allows for the analysis of mRNA and protein stability. An obvious application of the inducible system is the expression of toxic proteins. However, the protein may not be toxic, but merely reduce growth. During the initial isolation of transformants this may result in selection of epigenetic mutations that restore the growth rate. The isolation of stable transformants in the absence of expression prevents the selection of these unwanted mutations. The subsequently turned-on expression provides information on the function of the (toxic) protein.

In conclusion, we have established a tetracycline-regulated gene expression system allowing tight control of reporter gene expression in vegetatively growing D. discoideum cells. The system is robust, with no detectable expression in the ‘off’ state, and the expression can be modulated in a time- and level-controllable fashion. We expect this system to be a valuable tool for studying Dictyostelium development and signal transduction, as well as for expression of heterologous proteins.

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References


