Stringent doxycycline-dependent control of gene activities using an episomal one-vector system

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INTRODUCTION

Conditional expression systems are of pivotal importance for the dissection of complex biological phenomena. Here, we describe a novel EBV-derived episomal replicating plasmid (pRTS-1) that carries all the elements for conditional expression of a gene of interest via Tet regulation. The vector is characterized by (i) low background activity, (ii) high inducibility in the presence of doxycycline (Dox) and (iii) graded response to increasing concentrations of the inducer.

The chicken beta actin promoter and an element of the murine immunoglobin heavy chain intron enhancer drive constitutive expression of a bicistronic expression cassette that encodes the highly Dox-sensitive reverse tetracycline controlled transcriptional activator rtTA2S-M2 and a Tet repressor-KRAB fusion protein (tTSKRAB) (silencer) placed downstream of an internal ribosomal entry site. The gene of interest is expressed from the bidirectional promoter Ptetbi-1 that allows simultaneous expression of two genes, of which one may be used as surrogate marker for the expression of the gene of interest. Tight down regulation is achieved through binding of the silencer tTSKRAB to Ptetbi-1 in the absence of Dox. Addition of Dox releases repression and via binding of rtTA2S-M2 activates Ptetbi-1.

ABSTRACT

Conditional expression systems are of pivotal importance for the dissection of complex biological processes. One of the most powerful approaches for the dissection of complex biological processes is the ability to individually manipulate the activity of a single gene or a few genes within the context of a cell or a living organism. The tetracycline-regulatable gene expression system (Tet system) has, for a number of reasons, gained wide acceptance. By adapting prokaryotic transcription control elements to the eukaryotic transcriptional machinery, a highly selective regulatory circuit was established (1), which greatly profited from the exceptional specificity of interaction between operator and repressor of the Tn10-encoded tetracycline resistance operon (2). The system is robust and works efficiently not only in a variety of cultured cells of different origin, but also in whole organisms including yeast, Dictyostelium, Drosophila, amphibia and mammals such as rodents and non-human primates (3,4).

Two complementary systems have been developed in which the addition of the tetracycline derivative doxycycline (Dox) either switches transcription of a target gene off (tTA or ‘Tet Off’ system) (1) or on (rtTA or ‘Tet On’ system) (5). An additional advantage of the system is that it also enables control of the level of gene expression by titrating the dose of Dox. Moreover, there is a wealth of information on the pharmacology and pharmacokinetics of tetracycline and its derivatives, which is of particular value for in vivo applications.

The Tet system, as initially designed, consists of two components: a tetracycline controlled transcriptional activator (tTA or rtTA) and a fTAtfTA responsive promoter Ptet (6).
directing the transcription of the gene of interest. Bidirectional tTA/rTTA responsive promoters, P\textsubscript{tetbi}-1 have been developed, allowing the coordinate expression of two genes, of which one may be used as a reporter for the expression of the gene of interest (6,7). For reasons discussed earlier (1,6), the best results are obtained when the genes encoding the two components of the Tet system including the genes of interest, are introduced into cell lines or transgenic animals separately from each other in a two-step procedure, whereby in a first step, cell lines or, for example, mouse lines are generated that produce tTA or rTTA appropriately. These lines are generally suitable for introducing a variety of genes of interest controlled by P\textsubscript{tet} or P\textsubscript{tetbi}-1. In the case of cell lines, this second step is accomplished by a second transfection and selection of appropriate clones, whereas for transgenic animals double transgenic individuals are obtained by breeding (4,8). This procedure avoids co-integration of the two transcriptional units and thus, crosstalk between the promoter driving the tTA/rTTA genes and P\textsubscript{tet}. An additional advantage of this procedure is that well-characterized transactivator-expressing cell lines are available suitable for the transfer of a P\textsubscript{tet} controlled target gene. At the mouse level, a ‘zoo’ of ~150 mouse lines has been described, transgenic for either the TTA or rTTA genes under the control of a cell type specific promoter, or for various genes of interest controlled by P\textsubscript{tet}, thus, allowing a multitude of combinations for \textit{in vivo} studies (4).

There are, however, numerous applications where a reliable one-step transfer of P\textsubscript{tet} controlled genes along with the tTA/rTTA encoding transcription unit would be of great advantage or even essential. They include the transfer of Tet-regulated genes into cell lines or primary cells that are difficult to transfect and/or subject to subsequent selection processes. Moreover, various approaches in gene therapy rely on the controlled expression of therapeutic genes, which should be transmitted \textit{ex vivo} or \textit{in vivo} in a simple one-step procedure. Last but not least, it would often be convenient to quickly generate cells that allow the control of a gene function for a given period of time. Accordingly, various vectors have been described carrying the entire Tet-regulatory circuit of which some establish themselves as epiphenons and, thus, avoid the influence of chromosomal integration sites on P\textsubscript{tet} as well as on the promoter driving the tTA/rTTA gene (9–13). However, episomally existing transcription units are not subject to the same chromatin suppression seen when integration in an appropriate chromosomal locus has occurred. As a consequence, a significant tTA/rTTA-independent activity of P\textsubscript{tet} may be observed, as also experienced in transient expression studies. P\textsubscript{tet} can, however, be shielded from such unspecific activation by tetracycline-controlled transcriptional silencers (tTS) (14), and tightly regulated adenoviral vectors making use of rTA in combination with tTS\textsuperscript{kid} have been described (15,16).

Here, we report on the development of a broadly applicable EBV-based vector that contains all the elements for controlling the activity of a gene of interest via Dox. We show that the vector stably replicates in EBV-positive and -negative cells and demonstrates tight regulation by controlling growth inhibitory genes. The design of the vector facilitates the exchange of individual elements, particularly of target genes and, thus, makes the system useful for a variety of experimental applications, both in human and, contrary to expectation (17), also in rodent cell systems.

**MATERIALS AND METHODS**

**Cloning of pRT-1 and pRTS-1**

Details of the construction and the DNA sequence of pRT-1 and pRTS-1 are available upon request.

**Cell culture**

Cells were cultivated in growth media consisting of RPMI1640 supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO\textsubscript{2}. For growth in 96-well plates, 100 μM α-thioglycoler, 3 mM sodium pyruvate and 20 nM bathocuprine-disulfonate were additionally added (18,19). TGR-1 rat fibroblasts were cultured at 8% CO\textsubscript{2}.

**Transient transfection and quantification of eGFP, NGF receptor and luciferase expression**

DNA (10–20 μg) was transfected into 10⁷ cells by electroporation in 400 μl Optimem (Invitrogen) at 230 V and 960 μF using a Biorad electroporation apparatus leaving ~40–60% of the cells alive. Immediately after electroporation, cells were resuspended in 1 ml FCS and 9 ml growth medium without FCS was added. Cells were incubated in tissue culture flask at 37°C and 5% CO\textsubscript{2}. After two days in culture, cells were counted, an aliquot treated with Dox, and eGFP and NGF receptor expression quantified by flow cytometry using FACScan. NGF receptor staining was performed using HB8737 mouse monoclonal antibody at a dilution of 1:50 as primary antibody and phycoerythrin (PE)-labelled secondary goat anti-mouse IgG at 1:200 (Jackson labs). Luciferase activity was determined using a Berthold luminometer as described previously (20).

**Stable transfection and establishment of cell lines by limited dilution**

Electroporation was carried out as described above and the cells were allowed to recover in 10 ml growth medium for two days at 37°C and 5% CO\textsubscript{2}. Cells were counted and plated into 96-well plates in 100 μl at a density of 500, 50 and 5 viable cells per well. Cells were cultivated in growth medium supplemented additionally with 100 μM α-thioglycoler, 3 mM sodium pyruvate and 20 nM bathocuprine-disulfonate. After 5–6 days, 100 μl supplemented growth medium was added containing 250 μg/ml or 300 μg/ml hygromycin B for BJAB or Raji cells, respectively. Replacing 50 μl of the cell supernatant with supplemented fresh selection medium weekly, the hygromycin B concentration was slowly increased to a final concentration of 250 μg/ml hygromycin B for BJAB and 300 μg/ml for Raji cells. Within the next 2–4 weeks, hygromycin B resistant cells grew out. If cell lines grew out in <30% of the wells, the outgrowing cells were regarded as single cell clones. When an appropriate cell density was reached, cells were expanded successively into 48 and 24 well plates, and finally into 75 ml tissue culture flasks and maintained in culture under hygromycin B selection in the absence of Dox.

**HLA class II restricted T cell recognition**

BL30-B95.8 cells were co-transfected with 10 μg pRTS-M1 (encoding the matrix protein M1 gene of human influenza virus) and 10 μg pCMV-NGFR. After 48 h of transfection,
live cells were incubated with the α-NGF receptor mouse monoclonal antibody HB8737 (ATCC, Manassas, VA, USA) for 15 min on ice. After removal of unbound antibody by washing, cells were incubated for 10 min with a MicroBeads coupled goat-anti-mouse antibody (Miltenyi Biotec), and MACS-purified. After expansion of the cells, M1 protein expression was induced by addition of 1 μg/ml Dox to the culture medium. Two days later, the transfected cells were co-cultured with the M1-specific CD4+ T cell clone E5 and T cell stimulation was monitored by measuring GM-CSF release into the medium (21).

Transfection of TGR-1 rat fibroblasts

In general, 6 × 10^5 TGR-1 cells were transfected with 6 μg of pRTS-1 DNA using PolyFect (Quiagen) and expanded in 200 μg/ml hygromycin B for at least 2 weeks. Selection was performed either in the presence or in the absence of Dox. Cells expanded in the absence of Dox were treated with 1 μg/ml Dox for 24 h to activate conditional gene expression. For proliferation assays, TGR-1 cells were transfected with 6 μg of either the vector expressing luciferase (pRTS-1), or, instead of luciferase, the gene encoding the nucleolar protein Bop1 wt (pRTS-Bop1), or its N-terminal deletion mutant Bop1Δ (pRTS-Bop1Δ). Plasmids encoding for Bop1 wt and Bop1Δ were kindly provided by D. Pestov, and used for further cloning into pRTS-1 constructs. Transfected cells were expanded under 200 μg/ml hygromycin B selection for 2 weeks in the absence of Dox, and eGFP expression subsequently determined by flow cytometry 24 h after the addition of Dox. Episomal replication of the plasmids was visualized by gel electrophoresis in 1% agarose after lysis of the cells in the gel (22). To evaluate the copy number, different number of Raji cells were included as well as TGR-1 cells to which different amounts of pRTS-1 DNA had been added.

RESULTS

Design of the episomal expression vector pRTS-1

All functional elements required for tetracycline regulation were placed on one contiguous AscI–NotI restriction fragment of 9.7 kb (Figure 1). This fragment comprises two expression cassettes, one that responds to Dox, and a second conferring Dox responsiveness. The latter is composed of the chicken-beta-actin promoter (CAG) flanked by the mouse immunoglobulin heavy chain intron enhancer (Eμ) and drives expression of two genes encoding rtTA2S-M2 (23) and tTSKRAB (24) separated by an internal ribosomal entry site (IRES). The rtTA2S-M2 is an optimized version of the original rtTA, exhibiting significantly reduced affinity to tetO in absence of Dox and a highly increased sensitivity to Dox (23). Conversely, tTSKRAB binds to tetO only in the absence of Dox (or tetracycline) and dissociates when an effector is added. The use of natural sequence variants of TetR ensures that the reverse Tet-transactivator and the Tet-silencer can only homo- and not heterodimerize (24,25). Simultaneous expression of both Tet-controlled transcriptional regulators from the same bicistronic expression cassette should confer active repression of Ptet in the absence of Dox, whereas repression is relieved and activity of Ptet is induced by the addition of Dox (14,16). The chicken-beta-actin promoter was selected because it is active in many cell types including ES cells, and also in transgenic mice. Furthermore, it appears to be less sensitive to epigenetic silencing than many widely used viral promoters (26,27). As the research of some of us is focused on Epstein–Barr virus and B cell lymphomagenesis, the Eμ intron enhancer was included for efficient expression of Dox-controlled regulators in B cells.

The Dox-responsive expression cassette is composed of a bidirectional promoter, driving expression of two genes in a coordinated fashion, one of which encoding the firefly luciferase, the other eGFP. The luciferase gene is flanked by StfI sites that generate non-cohesive ends that can be used as universal cloning sites for genes of interest. Generation of non-cohesive sites by digestion with StfI guarantees that neither the vector nor the insert can self-ligate, thereby ensuring high cloning efficiency (28). The luciferase gene is followed by the β-globin intron and polyadenylation site. The eGFP gene is flanked by unique SwiI and BglII sites and can be replaced by other genes, for example, the truncated version of the NGF receptor leaving the SV40 polyadenylation signal in place (Figure 1).

The vector backbone comprises the ampicillin-resistance gene, the bacterial colE1 origin of replication, an optimized
hygromycin B resistance gene driven by the SV40 early enhancer-promoter, the EBV episomal origin of replication (oriP) and the EBNA1 gene (17). The SV40 enhancer-promoter is flanked by unique BstI107I and SnaBI sites, and the hygromycin B resistance gene by SnaBI and NotI so that the promoter, the gene encoding the selectable marker or both can be replaced. The EBNA1 gene is transcribed from a cryptic promoter in pBR322, and is derived from the original episomal EBV vector described by Yates et al. (17). The modified hygromycin B resistance gene is derived from pAT76 (29). In pAT76, the Kozak sequence has been optimized and cryptic splice sites have been removed. The vector has been designated pRTS-1 (reverse transactivator silencer-1). A vector lacking IRES-tTSKRAB but otherwise identical was generated for comparison and has been designated pRT-1.

BJAB cells were transiently transfected by electroporation with pRTS-1 or the corresponding vector lacking the Tet-silencer tTSKRAB (pRT-1), treated with 1 μg/ml Dox or left untreated. Luciferase activity was measured in cell lysates after 48 h. As shown in Figure 2A, luciferase activity was slightly higher in cells transfected with the control vector lacking tTSKRAB and treated with Dox. There was, however, a dramatic difference in the luciferase activity of cells that had not been treated with Dox. The difference in luciferase activity +/- Dox was ~30-fold for pRT-1, and due to the low background, ~1000-fold for pRTS-1. Dox-dependent co-expression of the two genes driven by PoriBi-1 was verified by FACS analysis of Raji cells transfected with a construct in which the luciferase gene had been replaced by a bicistronic expression cassette encoding activation-induced deaminase (AID) and the truncated NGF receptor separated by an IRES (Figure 3).

Low luciferase and eGFP background of human B lymphoma cells transfected with pRTS-1

To study inducibility and background of expression, BJAB and Raji cells were transfected with pRT-1 and pRTS-1: 72 h after transfection, cells were seeded in 96-well plates at 5, 50, and 500 cells per well, and hygromycin B selection was started after another three days. Cell lines growing out at the lowest cell density were regarded as single cell clones, and cultured in duplicates: 48 h after addition of Dox to one or both, luciferase and eGFP expression were quantified (Figure 2B and C). This experiment led to three important observations. First, not all hygromycin B-resistant cell clones express the gene of interest upon Dox treatment. Second, even though we are dealing with episomally replicating vectors, there is variation among different cell clones in terms of inducibility by Dox and the basal level of expression in the absence of Dox. Third, and most importantly, simultaneous expression of tTSKRAB along with rTA2S-M2 reduced the background by 1–2 orders of magnitude without significantly affecting inducibility by Dox: 9 out of 10 pRTS-1 transfected clones exhibited a barely measurable background activity in the absence of Dox (Figure 2C). In about one-fifth of pRTS-1 transfected clones, the low background was associated with response of >80% of the cells to Dox. Whether the variation in expression observed within one clone is due to rapidly evolving heterogeneity in expression or due to outgrowth of more than one clone within one well, cannot be discriminated at present. Due to the low background and varying inducibility, the factor of induction varied between 1000- and 140 000-fold in cells transfected with pRTS-1, whereas it was typically in the range of 30- to 100-fold in cells transfected with pRT-1. The vector replicated episomally and the copy numbers in BJAB cells varied between 3 and 10 (data not shown). Three representative clones of each group are depicted in Figure 2B and C.

To test whether induction of gene expression from the pRTS-1 vector is reversible, eGFP was induced by addition of Dox to one of the pRTS-1 transfected BJAB cell clones followed by removal of Dox by three consecutive washes. Due to its long half-life, eGFP levels decreased only slowly over time: 19 days after removal of Dox, eGFP expression had reached almost the basal background level of uninduced cells. Re-addition of Dox revealed a very similar pattern of expression as observed in the first round of induction (data not shown). This shows that, as expected, the Dox-controlled activation repression system transferred by pRTS-1 works in a reversible fashion.

pRTS-1 driven antigen-specific T cell stimulation is strictly dependent on Dox

T cell receptor-dependent recognition of antigen by CD4+ and CD8+ T cells is a very sensitive process that is known to require only a few peptide molecules per cell (30). To assess the tightness of the Dox-regulated vector, we cloned the gene encoding the influenza matrix protein M1 into pRTS-1, and transfected the construct into the Burkitt lymphoma line BL30-B95.8 that expresses HLA-DR13, the restriction element for the M1-specific T cell clone E5 (21). Upon co-culture of untreated and Dox-treated BL30-B95.8 cells with the M1-specific CD4+ T cell clone, antigen-specific T cell stimulation was quantified by measuring GM-CSF release into the medium. As shown in Figure 4, GM-CSF release was strictly dependent on Dox treatment. We conclude that expression of influenza matrix protein M1 in BL30-B95.8 cells in the absence of Dox is below the critical threshold for eliciting GM-CSF release by the M1-specific CD4+ T cell clone.

Stable expression of an inhibitor of cell proliferation in rat TGR-1 fibroblasts

To test whether the vector also works in rodent fibroblasts, non-transformed rat TGR-1 cells were transfected with pRTS-1, and eGFP expression was monitored before and after selection with hygromycin B. If cells were selected with hygromycin B in the presence of Dox, large foci of green fluorescent cells grew out (Figure 5A). If selection was performed by hygromycin B in the absence of inducer and Dox added after 4 weeks, large foci of green cells were equally visible (Figure 5B), whereas no green fluorescent cells were apparent in the absence of Dox (Figure 5C). In TGR-1 cells, the majority of cells that were selected by hygromycin B also responded to Dox. Southern blot analyses of cells lysed in the agarose gel before gel electrophoresis (22) revealed that the vector was episomally replicated in TGR-1 cells (Figure 5G).

The highest challenge for a conditional vector system is the establishment of cell clones that express—upon addition of
the inducer—a toxic molecule or an inhibitor of cell proliferation. Low basal expression of the inhibitor in the absence of Dox may select for clones that do not express the inhibitor at all. To address the question whether cell lines can be established that express an inhibitor in a conditional fashion, an N-terminal deletion mutant of the nucleolar protein Bop1 (Bop1Δ) was cloned into pRTS-1. This mutant has been shown before to act as a dominant negative mutant of Bop1 and as an inhibitor of cell proliferation (31,32). TGR-1 cells were transfected by electroporation and selected for hygromycin B resistance. Dox was then added to the cultures, and eGFP expression and cell proliferation were monitored in the
As shown in Figure 5F, switching on expression of Bop1Δ reduced proliferation strongly (and of Bop1 wild type weakly). Yet, regardless of whether luciferase, Bop1-wt or Bop1Δ had been co-expressed with eGFP, there was no difference, neither in the number of cells expressing eGFP (Figure 5D) nor in the level of eGFP expression (Figure 5E) upon addition of Dox. We conclude that repression of Bop1Δ expression in the off-state was sufficiently efficient to establish stable cell lines conditional for this growth inhibitor.

**Gradual induction of gene expression by tetracycline and Dox at the single cell level**

Addition of increasing amounts of Dox or tetracycline had revealed a dose-dependent increase in the amount of luciferase activity in previous experiments (data not shown). A gradual increase in luciferase activity with increasing Dox concentration is compatible with two interpretations. First, it might indicate that each single cell responds to an increase in Dox in a dose-dependent manner. Alternatively, it is also compatible with the model that each individual cell is able to discriminate between two states only, the off- and the on-state, and that an increase in Dox concentration would increase the likelihood that an individual cell is switching from the off- to the on-state. To discriminate between both models, eGFP expression was analysed with increasing Dox concentration. If the first model is correct, we would expect to see a gradual shift in the eGFP expression peak with increasing Dox concentration from absent, to low, over medium, to high expression. According to the second model, we would expect that the peak of eGFP-negative cells decreases, while a peak of highly eGFP-positive cells increases with increasing Dox concentration. The experiment revealed that gradual gene expression can indeed be achieved at a single cell level. Apparently, repression can be efficiently relieved at 1–2 ng/ml Dox (Figure 6A), presumably without affecting activation as has been shown previously (14). This conclusion is supported by
the notion that the mean fluorescence intensity reached at this low Dox concentration equals that obtained with vectors lacking tTSKRA in the absence of Dox (see also Figure 3B). At concentrations of 5–100 ng/ml Dox, the promoter is activated in a graded fashion resulting in a broad peak of eGFP-positive cells moving from the left to the right with increasing Dox concentration (Figure 6A). The reverse transactivator responded only poorly to tetracycline and was by about three orders of magnitude less sensitive to tetracycline than to Dox [(23); data not shown].
Inducibility is not stable over time

To study both background of expression and inducibility over time, in a long-term experiment, BJAB cells were transfected with pRTS-1 and several clones cultured in the absence of Dox for 5 and 9 months. Dox was added to aliquots of the cultures and eGFP expression monitored after 2, 5 and 9 months. Impaired inducibility was already evident after 5 months (Figure 7). After 9 months, clone H11 had retained its inducibility whereas clone B12 did not respond to Dox anymore (data not shown). Four additional clones cultured over 9 months had also lost their responsiveness to Dox. Remarkably, not only was inducibility lost or decreased over time, also the background of eGFP expression appeared to be considerably higher in cells that had lost their responsiveness to Dox (Figure 7 and data not shown). The latter observation argues for the fact that expression of the bicistronic rTA2\(^{S}\)-M2-rtSSKRAB transactivator-silencer cassette may not be stable over time in culture.

DISCUSSION

We describe here the prototype of a Dox-inducible episomal vector designated pRTS-1. This vector was designed to accommodate all the elements required for Dox-regulated gene expression on one episomally replicating plasmid including the gene of interest. The hallmark of pRTS-1 is its low background and its high inducibility in the absence and presence of Dox, respectively. Low background expression in the off-state was demonstrated by using an antigen-specific T cell stimulation assay and by establishing a cell line expressing a strong inhibitor of cell proliferation. To study antigen presentation in the off- and the on-state, BL30-B95.8, a Burkitt lymphoma line infected with EBV B95.8 \textit{in vitro}, was transfected with pRTS-1 encoding influenza matrix protein M1. These cells elicited an antigen-specific response in an M1-specific CD4\(^{+}\) T cell clone only if Dox was added, and not in the absence of Dox. As growth inhibitory protein, a dominant negative mutant of the nucleolar protein Bop1 that lacks 231 amino acids of the N-terminus and inhibits cell proliferation (31,32) was conditionally expressed from pRTS-1 in rat TGR-1 cells. Stably transfected cell lines could be established that exhibited pronounced cell cycle arrest upon addition of Dox. The tight regulation of the gene of interest observed in these experiments makes pRTS-1 a very useful experimental tool. Importantly, the vector enables the expression of a gene of interest in a graded fashion by titrating the Dox concentration. The vector carries the EBV plasmid origin of replication as well as EBNA1, the viral protein binding to oriP and maintaining episomal replication and segregation of the episome to daughter cells synchronously with the cell cycle. A bidirectional promoter is used to express the gene of interest. The second gene driven by the bidirectional promoter may encode a second protein to express a heterodimeric protein in an inducible fashion, or may encode a surrogate marker gene, like eGFP, to monitor expression of the gene of interest. The truncated NGF receptor gene may be used alternatively to sort those cells that respond to Dox. We have verified at the single cell level that the two genes transcribed from the bidirectional promoter are indeed expressed simultaneously (Figure 3). That both genes are also transcribed and expressed at an equivalent level has been carefully demonstrated by Hasan \textit{et al.} in transgenic mice (33).

Despite these very useful properties, the vector also has its limitations: pRTS-1 was initially designed for use in human B lymphoma cell lines and EBV-immortalized cells. It soon became apparent that not all cells selected by the dominant marker indeed respond to Dox. To overcome this problem, we routinely use eGFP or truncated NGFR to trace those cells that respond to Dox. We have verified at the single cell level that the two genes transcribed from the bidirectional promoter are indeed expressed simultaneously (Figure 3). That both genes are also transcribed and expressed at an equivalent level has been carefully demonstrated by Hasan \textit{et al.} in transgenic mice (33).

Figure 6. Graded increase of eGFP expression of pRTS-1 transfected TGR-1 cells with increasing Dox concentration. TGR-1 cells transfected with pRTS-1 were plated in six-well plates and different concentrations of Dox added (A). The numbers depicted in the histograms represent ng/ml Dox. Mean fluorescence of eGFP expression was plotted versus the Dox concentration in a double logarithmic plot (B). Note that, although the peak of eGFP expressing cells is very broad at intermediate Dox concentrations (25–100 ng/ml), induction does not follow an ‘all or none’ pattern.
9 months, inducibility decreased significantly. It appears that the price to be paid for the efficiency savings using an episomal one-plasmid system is its limited stability over time. Additional work is required to define the reason(s) for this limited stability. There may be great variability between different cell lines. EBV-positive and -negative Burkitt lymphoma lines with so-called group I phenotype appear to be particularly difficult to work with in terms of transfection efficiency, heterogeneity and stability as compared with, for instance, TGR-1 rat fibroblasts (see Figure 5). The role of epigenetic factors regulating or limiting stability has to be defined. The fact that not only inducibility by Dox, but also repression was lost, argues for the fact that expression of the bicistronic rtTA2S-M2-tTSKRAB cassette has not been stable over time. To be able to select for expression of the bicistronic reverse transactivator-silencer cassette, we now are constructing a tricistronic expression cassette that expresses the puromycin or hygromycin B resistance gene from a second IRES.

Limited expression of transfected or transduced DNA over time is, however, not only a problem of this vector system. In cell culture, genes that are subject to negative transcriptional regulation are prone to silencing by epigenetic mechanisms over time (34). It is thus advisable to study the biological readout of the transfected gene of interest as soon as sufficient numbers of cells are available for the analysis. To speed up this process, we routinely co-transfect a non-replicating plasmid that encodes the truncated NGFR, a splice variant of CD34, or the extracellular domain of rat CD2 along with pRTS-1 or its derivative expressing the gene of interest and sort the transfected cells 48 h after electroporation using magnetic beads. The sorted cells are then cultivated in bulk in the presence of hygromycin B to expand the transfected cells. Depending on the initial transfection efficiency, sufficient cell numbers will be available after 2 to 4 weeks to study the phenotype of induced versus uninduced cells. For transient gene delivery, an adenovirus vector system expressing both, the reverse Tet-transactivator and the Tet-silencer, has been described (16).

The vector also replicates episomally in rat fibroblasts and is tightly regulated. Surprisingly, there was much less variation in the inducibility at the single cell level than in human B lymphoma lines. The vast majority of cells resistant to

Figure 7. Inducibility of eGFP expression is impaired when pRTS-1-transfected BJAB cells are cultivated in the absence of Dox over 5 months. Panels A and C represent two different pRTS-1-transfected BJAB cell lines analysed for eGFP expression in the absence and presence of Dox after 2 months in culture. Panels B and D show the pattern of eGFP expression in the same BJAB-transfected cell lines after 5 months in culture.
dominant marker selection also responded to Dox. This allowed us to establish cell lines that express dominant negative mutants of Bop1 inhibiting cell proliferation.

The vector has also been used successfully as an integrating vector in DT40 cells after linearization. As expected, there was marked clonal variation in the inducibility by Dox in various clones (Ertongur and Jungnickel, Personal communication).

In summary, pRTS-1 is the prototype of an improved Dox-regulated episomal ‘all-in-one’ vector characterized by low background, high inducibility and graded response to increasing Dox concentrations. It is particularly suited to study the biological impact of unknown genes in an isogenic system, for example, genes that have been identified by differential expression on microarrays.

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