NEW METHODS AND TECHNOLOGIES

Retroviral vectors containing Tet-controlled bidirectional transcription units for simultaneous regulation of two gene activities

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ABSTRACT

In this study retroviral self-inactivating (SIN)-vectors were constructed, that allow simultaneous regulation of two genes by integration of bidirectional Tet controlled transcription units. Marker genes (luciferase and eGFP) were expressed under the control of various bidirectional promoters P_{tet}bis, in order to determine (i) the fraction of HtTA-1 cells exhibiting tight doxycycline (Dox) dependent control; (ii) possible effects of the vector backbone on the regulation of gene transcription; (iii) the possibility for crosstalk between different minimal promoters within P_{tet}bi. When HtTA-1 cells, constitutively expressing the Tet-Transactivator (tTA), were transduced by S2f-*l*MCg retroviral vector, a high percentage (40) of the cell population displayed tight regulation (5000 fold) of P_{tet}bi activity over a wide range of Dox concentrations. As a result of our comparative study on the activity of virus derived minimal promoters (from MMTV, HIV and CMV), a clear hierarchy of activity as well as a different sensitivity to external influences among the various promoters studied was observed. Furthermore, our results strongly support the idea, that viral elements such as part of the MuLV pol/env region significantly affect the regulation capacity of an integrate. Taking into account our observations as outlined above, we succeeded in generating significantly optimized Tet regulated retroviral vectors. The application of such a one-step transfer system for P_{tet} controlled genes would be of particular relevance to applications where cellular systems do not allow prolonged selection procedures as it is the case with primary cells considered for *ex vivo* gene therapy.

KEYWORDS: *Tet-System, bidirectional regulation, retroviral vector, cell populations, minimal promoters*

INTRODUCTION

Over the past decade, the study on eukaryotic gene function has greatly profited from methodological advances which have enabled scientists to create temporally defined and reversible changes in gene activity. Among the experimental systems that have emerged so far, the principle of tetracycline controlled transcription (Gossen and Bujard, 1992; Gossen et al, 1995) has found widespread application (Gossen and Bujard, 2002). Recent research focusing on tet-dependent transactivators as well as P_{tet}-promoter revealed the high flexibility of both components and let to its further application in basic research (Urlinger et al, 2000).

However, two major prerequisites have to be fulfilled, should a target gene be regulated within a desired activity window: (i) tTA, or its reverse counterpart rtTA have to be

present at a certain intracellular concentration; and (ii) the target gene has to be integrated in a genomic environment that does not interfere with P_{tet}-function.

In earlier studies Ptet was shown to be susceptible to activation from nearby sequences, resulting in a non-regulatable background activity. Furthermore, the level of tTA (rtTA) dependent activation was found to be largely defined by its (Ptet) integration site (Gossen and Bujard, 1992). Despite all this, further studies revealed the existence of loci, where Ptet shows no measurable activity in its un-induced state, though when induced initiates transcription at least as efficient as the strongest RNA polymerase II promoters (Baron and Bujard, 2000; Yin et al, 1996). These loci are referred to as „silent but activatable" (s/a) and they have been functionally characterized in many cellular systems as well as in transgenic mice (Grossen and Bujard, 1992; Baron and Bujard, 2000; Schönig et al, 2002). The fact that Ptet when integrated at an s/a loci allows for tight control makes it an favorable tool, especially under conditions when transduction can not be followed by rigorous selection, as it is the case in gene therapy. Here, a given transgene (therapeutic gene) has to be controlled in all target cells irrespective of its chromosomal integration site.

Among the various known gene transfer systems, the use of retroviral vectors offers a large number of advantages: (i) High transfer efficiency (100 % of all dividing cells); (ii) high probability for single copy integration, depending on the multiplicity of infection (MOI); (iii) broad transduction range, *e.g.*, envelope pseudotype broad host range viruses enable the transduction of cells recalcitrant to other transfer methods, whereas non-replicating cells are amenable to lentiviral transduction; and (iv) precise DNA integration, leading neither to rearrangements nor to multiple integrations at the insertion site.

So far, several attempts have been made to combine the useful properties of Tet-control and retroviral vector systems. In gene therapy vector development focused in particular on the following strategies, employing: (i) A two vector system, whereby Ptet controlled response and transactivator expression unit reside on independent vectors (Lindemann et al 1997; Vigna et al, 2002); (ii) the so called "self-contained" (SIN) one-vector system carrying both Ptet-controlled response as well as the tTA or rtTA encoding unit, driven by an independent promoter, in the same viral backbone (Paulus et al, 996; Hwang et al, 1997; Lida et al, 1996); and (iii) an autoregulatory one-vector system where tTA or rtTA are controlled via Tet dependent autoregulation (Hoffman et al, 1996; Unsinger et al, 2001). The latter system poses the following problems: (i) A functional system requires a low but defined transactivator level. Since transactivator as well as target gene are under Ptet control, the system can never be completely shut off and therefore does not enable tight regulation; (ii) transactivator expression cannot be controlled independently from target gene expression; and (iii) high overproduction of tTA or rtTA is detrimental to the cell due to squelching.

In the work described here, we have investigated: (i) The possibility to generate cell populations by retroviral transduction with high Tet-regulatory potential; and (ii) the effect of specific sequence elements of the retroviral backbone on the regulation of P_{tet}bi.

Our studies show that in HtTA-1 cell populations which are constitutively producing tTA, transduction with retroviral SIN-vectors results in a sizeable fraction of cells which contain proviral insertions in loci where Dox-controlled expression can be regulated over a wide range ($>10^3$ fold). Our data also indicate that there is crosstalk within P_{tet}bi. Moreover, it appears that even small sequence alterations within the viral backbone significantly affect the responsiveness of Tet-regulation. Furthermore, the regulatory properties of P_{tet}bi-3 found in MuLV based SIN-vectors were retained after transfer to a lentiviral SIN-vector, indicating a broad application for a bidirectional regulatory unit.

MATERIALS AND METHODS

Cell culture

293T cells were cultured in Dulbecco´s modified Eagles medium (DMEM, Invitrogen) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, Invitrogen), 1 mg/ml succrose and 2 mg/ml glutamine. HtTA-1 cells (1), constitutively providing the tTA tet-responsive transactivator, were cultured in Earl´s modified Eagles medium (EMEM, Invitrogen) supplemented with 10% (v/v) FCS and 2 mg/ml glutamine. Cultures were split at 70-80% confluency. Cells were harvested after a wash with PBS by incubation with PBS/EDTA and subsequently transferred into fresh medium or used for analysis.

Transient transfection and harvest of viral particles

Transient virus production was carried out by the Ca-phosphate coprecipitation method (Southern and Berg, 1982) of a three plasmid system (Lindemann et al 1997). $1x10^6$ 293T cells were transferred to 60 mm dishes the day before transfection and maintained in DMEM, supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen) and 100U/ml of Pen/Strep. A total amount of 15 µg plasmid DNA was transfected including 5 µg pHIT60 (gag/pol expression plasmid; Soneoka et al, 1995), 5 µg pczVSV-G (VSV-G envelope expression plasmid; Pietschmann et al, 1999) and 5 µg of the respective transfer vector. 16-18 hours after transfection the medium was replaced by 3 ml DMEM, supplemented with 5 mM Na-butyrate which was replaced by DMEM after 6-8 hr. After another 16 hr, the culture supernatant was harvested, purified from cell debris by filtration (0,45µm), supplemented with polybrene (5 µg/ml), aliquoted and either used directly or stored for later use at –20°C. Lentiviral vectors were produced as described (Dull et al, 1998). Viral supernatants were titrated on HtTA-1 cells following a freeze/thaw cycle. Three to five dilutions were used to determine viral titers based on eGFP-positive cells resulting in $5.1\text{-}5.6x10^5$ infectious particles for all vectors.

Infection and enrichment of HtTA-1 cell populations

Infection of HtTA-1 cells was performed on 6-well plates. The day before transduction $2x10^5$ cells were transferred to each well. After 24 hr cells were washed twice with

DMEM and incubated with infectious particles (IPs) at a MOI of 0,03-0,05 to ensure single copy integration of the vectors in the majority of transduced cells. Cultures were incubated with IPs for 4 hr followed by its replacement with EMEM cell culture medium. For analysis of transduction efficiency cells were cultured for 48 hr in the presence or absence of 100 ng/ml of Dox, harvested and monitored for eGFP expression on a FACS-calibur (Beckton Dickinson). The cell suspension was supplemented with 1 µg/ml propidium iodide in order to exclude dead cells from measurements. Populations achieved consisted of 1.8-5.6x10^3 individual eGFP-positive clones. For enrichment of eGFP-positive populations, a fraction of the cells cultured in the absence of Dox were transferred to 9 cm dishes and cultivated for another 48 hours under the same conditions. The "total" pools containing all eGFP-positive cells were established by two rounds of enrichment (FACS-vantage, Becton Dickinson). The sorted cells were kept for six days in the presence of 100 ng/ml Dox and than split into culture medium +/- Dox. After another four days the eGFP/Luciferase expression was determined or the cells were harvested for RNA/DNA-analysis.

Determination of luciferase activity

An aliquot of cells (70-80% confluent) prepared for FACS-analysis was transferred to an Eppendorf tube (around 1x10^5 cells) and centrifuged for 3 min at 850x *g* in a Heraeus centrifuge at room temperature. Cell pellets were lysed in 100 µl of lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM Dithiothreitol, 2 mM diaminocyclohexane tetraacetic acid, 10% (v/v) glycerol, 1% (v/v) Triton X-100). Cell debris was removed by centrifugation (13000x *g* at 4°C for 2 min) and 2-10 µl of the supernatant were analyzed for luciferase activity as described (Gossen and Bujard, 1992). Protein concentration was determined by the method of Bradford (Bradford, 1976). Regulation factors were calculated as quotient of the luciferase activity in the "on"-state, devided by its activity in the "off"-state of the Tet-system.

Northern analysis of total RNA

For RNA analysis the enriched cell populations, cultured in the presence of Dox (100 ng/ml), were split to 9 cm dishes. After an 96 hr incubation +/- Dox, the cells were harvested as described above. Total RNA was isolated by the acidic phenol method (Chomczynski and Sacchi, 1987) and subjected to northern hybridization analysis according to previously published method (Löw and Rausch, 1994), except that CPD-Star (Tropix) was used as substrate for chemiluminescent detection. Rat GAPDH served as internal standard. All probes used were biotin labelled during PCR amplification (Löw and Rausch, 1994). Detection of the relative mRNA steady states was achieved via exposure to X-Ray film (Kodak Bio-Max light, Sigma). RNA size markers were from Promega. Oligonucleotides used for probe synthesis:

luc:
Sense: 5´-TTACAGATGCACATATCGAGG
Antisense: 5´-CCTCTGG ATCTACTGGGTTA

gapdh (rat):
Sense: 5´-AATGCATCCTGCACCACCAAC
Antisense: 5´-GCCATATTCATTGTCATACCAGG

egfp:
Sense: 5´- TTCAGCGTGTCCGGCGAG
Antisense: 5´- TCCTCGATGTTGTGGCGG

Southern hybridization analysis of genomic DNA

Genomic DNA was isolated according to standard procedures (Ausubel et al, 2001). The high molecular weight DNA was CsCl- purified. For Southern blot analysis 10 µg of the genomic DNA was digested with Eco RI, separated on non-denaturing agarose gel (1x TAE, 0.8% (w/v) agarose), blotted according to standard procedure (Löw and Rausch, 1996). The DNA was hybridized with a probe directed against luciferase (as above). As control the S2f-*l*MC*g* plasmid DNA was digested with *Bam*HI and *Eco*RI to release the *luc* orf together with the regulatory unit (2.2 kb), and 4x10^6 molecules were loaded on the gel, supplemented with 10 µg of shared salmon sperm DNA. HtTA-1 genomic DNA served as negative control.

For PCR analysis of the S2f-*l*MC*g* proviral regulatory unit about 100 ng genomic DNA was used as template. The primers employed to amplify the proviral regulatory unit were originally used to adapt the 3´-restriction sites of the MMTV-minimal promoter (MPM-4: 5´-GGGGATATCTG GCCATGGTGTCGACCCGACCTGAGGGTGACCGGG), and, CMV-minimal promoter (MPC-3: 5´-GGGGAT ATCTGGCCATGGTGAATTCCGCGGAGGCTGGATCG. The amplified proviral region spans the whole Tet-regulatory unit, including both minimal promoters and the *tetO* heptamer. The PCR-reaction was carried out in a total volume of 50 µl, with 1 U of Taq-polymerase (MRC-Holland, Amsterdam, Netherlands) and 1x reaction buffer (MRC-Holland), 10 pmol each primer, 100 µM each dNTP and 2.5% (v/v) formamide. Cycles were: (1x)-94°C, 4:30 min; (30x) 94°C, 0:30 min – 72°C, 1:30 min; (1x) 72°C, 5 min. The PCR products were ethanol precipitated (0.3M NaAc, 2.5 vol EtOH, over night at –20°C) and dissolved in 20 µl 1/10 TE (1 mM Tris pH 8.0, 0.1 mM EDTA). Five µl aliquots were digested with either *Hin*dIII or *Xho*I (1 hr, 37°C). The digestion products were separated on a 6% (w/v) native polyacrylamide gel and stained with ethidium bromide. DNA size markers were from Invitrogen (1kb ladder).

Plasmid constructs

All retroviral constructs were based on a modified SFG-ECT2-luc SIN-vector (Lindemann et al, 1997). Modifications of this backbone include: (i) Removal of the MuLV pol/env region harboring the native splice acceptor (SA); (ii) introduction of FLP-recombinase recognition sites (FRT) into the *Bgl* II (Fn) or *Bsp*EI (F5) site of SFG-ECT2-Luc vector as double stranded oligonucleotides, that will allow retargeting of the tagged locus (Seibler and Bode, 1997). Given here is only the sense-strand for Fn: 5'-GATCCGAAGTTCCTATTCCGAAGTTCCTATTCT TCAAAAGGTATAGGAACTTCAGATC, and F5: 5'-GGATCCGAAGTTCCTATTCCGAAGTTCCTATTCTT CAAAAGGTATAGGAACTTCAGATCTAAGCTTTTC CATGG; (iii) introduction of a modified SV40 late

polyadenylation signal (pA) to precisely terminate the antisense orientated transcription. The modifications belong to the SV40 pA-early signals on the opposite strand that were eliminated by point mutations; (iv) the constitutive transport element (cte) of simian retro virus-1 (SRV-1) for optimization of the nucleo/cytoplasmic transport process of unspliced transcripts (Saavedra et al, 1997). The resulting vector was termed S2f-(*l*MCg), we occasionally refer to as the "standard vector" (Figure 1C).

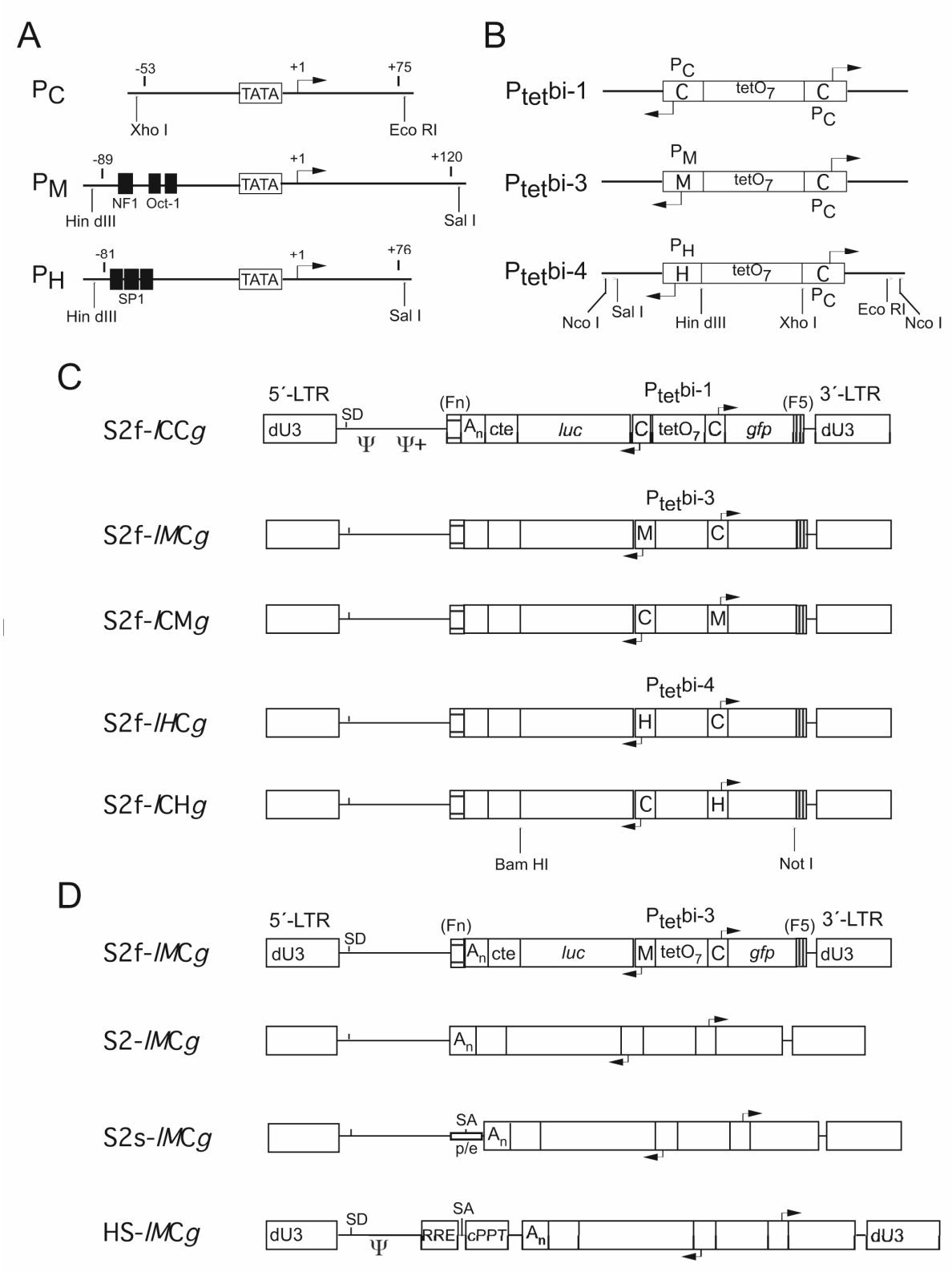


Figure 1. Transcription units and viral backbones. **(A)** Minimal promoters used for the generation of bidirectional promoters. P_C, P_M and P_H are derivatives of the hCMV IE promoter, the 5' LTR of MMTV and HIV-1, respectively. The nucleotide sequences used are delineated by the upstream (-83, -89 and –81) and by downstream positions (+75, +120 and +76,) respectively, +1 being the transcriptional start site. Unique endonuclease cleavage sites flanking the minimal promoter sequences are indicated as well as the transition to the initiating codon and AP-1, SP-1 and NFκB recognition motifs located within P_M and P_H respectively. **(B)** Outline of the bidirectional promoters (not drawn to scale). Endonuclease cleavage sites, that may allow to exchange minimal promoters and those for the entire P_{tet}bi are valid for all units but were indicated only for Ptet-bi-4. The minimal promoters were fused to a tet-operator heptamer ($tetO_7$), NcoI cleavage sites may be used for inverting P_{tet}bi within the transcription unit, while *Sal*I/*Eco*RI restriction sites can be used for the exchange of the complete regulatory units. **(C)** Outline of the standard proviral MuLV-based S2f-vector containing a bidirectional transcription unit controlled by P_{tet}bi-1, P_{tet}bi-3 and P_{tet}bi-4, respectively. The vector encodes a self-inactivating SIN 5'-LTR and 3'-LTR (dU3) and respective packaging signals indicated as "Ψ" and "Ψ+", where psi+ indicates the first 385 bp of the *gag* open reading frame, the start codon of which is deleted. The splice donor (SD) is shown. It is followed by a wildtype FRT site (Fn), a mutated polyadenylation signal from SV40 (An) and a constitutive transport element (cte) of SRV-1. The latter two sequences define the end of the luciferase transcript (*luc*), which is initiated at P_{tet}bi. The transcript of the *egfp* gene initiated in opposite direction is terminated at the viral poly A site within the 3' LTR. A mutant FRT site (F5) adjacent to the *egfp* gene is indicated. The entire bidirectional transcription unit can be removed or replaced via *Not*I/*Bam*HI cleavage as indicated only for S2f-*l*CHg. **(D)** Viral backbone variants. The MuLV based vector S2 was obtained by removal of the entire sequence of the SFG-ECT2-luc backbone via *Bgl*II/*Bsp*EI restriction digest and insertion of a similar digested, antisense orientated SV40-pA-signal 3´ to the Ψ/Ψ+ region together with a suitable polylinker. Introduction of the splice acceptor (SA) signal and surrounding sequences of the MuLV pol/env sequence (p/e) into the S2 backbone results in S2s-vector. Finally, the HIV-2 based vector HS-*l*MC*g* also contains deleted U3 region and packaging signals, both HIV-specific. In addition, a Rev response element (RRE) and the central polypurin tract (cPPT) between the packaging signal and the terminus of the *luc* gene. The wild type splice donor (SD) and splice acceptor (SA) pair is indicated.

The restriction sites of all Tet system components were adjusted via PCR, cloned into pBluescript SK II+ vector (Stratagene) and verified by sequencing. *Tet* operators, minimal promoters (Figure 1A and 1B) and reporter genes were sub-cloned into pUHR plasmid backbones providing a suitable multiple cloning site for the construction of bidirectional expression cassettes prior to their transfer to the retroviral vectors.

The MuLV backbone variants S2 and S2s (Figure 1D) were obtained by insertion of a suitable polylinker into the *Bgl*II and *Bam*HI sites of the SFG-ECT2 Luc vector, thereby removing the viral pol/env fragment together with the luciferase open reading frame. For construction of the S2-backbone the pA/cte-element was inserted into the *Bgl*II site of the SFG-intermediate. The S2s backbone was constructed by re-insertion of the MuLV pol/env fragment as 5´-*Bam*HI/*Bgl*II-3´ fragment into the *Bgl*II digested SFG-intermediate, thereby retaining the *Bgl*II restriction site into which the pA/cte-element was inserted as 5´-*Bcl*I/*Bgl*II-3´ fragment. The final vectors S2-*l*MC*g* and S2s-*l*MC*g* were obtained after insertion of the bi-directional response unit containing P_{tet}bi-3, released as *Bam*HI/*Not*I fragment from the standard vector.

For construction of the lentiviral SIN-vector the WPRE-element was removed from the parental plasmid (pRRL.sin.ppt.hPGK.EGFP.pre; Follenzi et al, 2000). The remaining vector obtained the pA/cte-element as *Xho*I/*Bam*HI fragment and subsequently the bi-directional response unit as 5´-*Bam*HI/blunt-3´ fragment, resulting in the HS-(*l*MC*g*) lentiviral SIN-vector. Detailed sequence information of the constructs shown in Figure 1 will be supplied upon request.

RESULTS

Experimental strategy

Throughout the entire study HtTA-1 cells were used, which constitutively expressed tTA at a concentration level which allowed systematic comparison of various inducible vectors (Gossen and Bujard, 1992). For quantitative analysis of cell populations showing P_{tet} controlled transcription the reporter genes firefly luciferase (*luc*) and the enhanced green fluorecent protein (eGFP) were placed under the control of different bidirectional promoters P_{tet}bis (Baron et al, 1995). The obtained constructs allowed detection and enrichment of P_{tet}bi active cells by fluorescence activated cell sorting (FACS) based on GFP expression, while the effect of regulation factors on P_{tet}bi were studied by measuring luciferase activity in the induced and uninduced state. However, when interpreting the data, it has to be kept in mind that the two indicators differ significantly with respect to quantitative parameters. Whereas luciferase and its mRNA are short-lived (combined halflife in HtTA-1 cells is around 3 hr) and therefore reach steady state level rapidly, eGFP is long-lived and accumulates over time reaching equilibrium point only after a couple of days. Moreover, luciferase assays are extremely sensitive picking up ≤ 7 molecules per cell (Kistner, 1996). By contrast, considerable intracellular eGFP levels have to be reached before a signal is detected by FACS, therefore, monitoring of eGFP does not enable the analysis of tightly controlled gene expression.

At the level of transcription, one has a certain degree of freedom in order to obtain an appropriate amount of a gene product, by using promoters of different strength. We have examined several minimal promoters, which were fused to an array of seven *tet* operators following the symmetric design of P_{tet}bi-1 (Baron et al, 1995). In general, constructs consisted of two minimal promoter sequences derived from the human cytomegalovirus (CMV) IE promoter, P_C, flanking the *tet* operators (Figure 1A and 1B). For generation of asymmetric regulatory units, minimal promoters of the LTRs of mouse mammary tumor virus (MMTV) P_M (Hoffmann et al, 1997), and of human immunodeficiency virus type I (HIV-1), P_H, were fused to one end of a seven *tetO* sequence, while at the other end

the CMV derived minimal promoter P_C was retained. The symmetric as well as the asymmetric bidirectional promoters were inserted into the S2f backbone in the context of *luc/egfp* transcription units.

To account for possible effects caused by the arrangement of genes and signals within the vector, the various P_{tet}bi elements were examined upon integration in both orientations (Figure 1B and 1C). Moreover, we modified the S2f-*l*MC*g* standard backbone by either removal or addition of individual elements (FRT sites, pol/env region) in order to explore possible context dependent influences (Figure 1D).

The P_{tet}bi-3 transcription unit was also inserted into a lentiviral SIN backbone (Follenzi et al, 2000) yielding HS-*l*MC*g* lentiviral vector, with the aim to investigate whether its properties were transferable or specific for the MuLV-based retroviral vector.

Symmetric bidirectional regulatory unit is not tolerated in the retroviral backbone

In an initial experiment, we attempted to generate HtTA-1 cell populations containing the S2f-*l*CC*g* or S2f-*l*MC*g* provirus (Figure 1C). Titration of the viral supernatants on HtTA-1 cells revealed titers of >5 x 10^5/ml for S2f-*l*MC*g*. By contrast, only very low titers (<1 x 10^2/ml) were obtained with S2f-*l*CC*g*. FACS analysis of the respective HtTA-1 cells confirmed that the inefficient virus production was restricted to S2f-*l*CC*g*. This led us to the conclusion that the extended complementarity of sequences within the symmetric P_{tet}bi-1 and the stable secondary structure resulting from it might interfere with packaging and/or the reverse transcription during viral replication. Therefore, symmetric regulatory units were excluded from further studies.

Analysis of proviral transcription

In order to examine the function of bidirectional transcription units, HtTA-1 cells were transduced with S2f-*l*MCg at a MOI of <0.1, thereby ensuring single copy integrations in the majority of transduced cells. eGFP-positive cells, obtained upon induction, were enriched by FACS and designated "total pool". As described below (see Figure 5), this pool was sub-divided further on. In detail, whereas a highly expressing population (high pool) could be isolated in the induced state (*i.e.*, in absence of Dox), and a eGFP-negative population (low pool) was isolated in the un-induced state (*i.e.*, in presence of Dox). From the latter 47, individual clones were derived, out of which clone 29 was chosen for further analysis, as it displayed regulatory properties comparable to the low pool.

Cells originating from the total, the low and the high pool were divided into two equal aliquots and were cultured in the presence or absence of Dox. After four days, cells were harvested and mRNA was analyzed by northern blotting. The results depicted in Figure 2A show that: (i) When P_{tet}bi-3 is induced, the mRNA steady state of *egfp* as well as *luc* correlates with the found enzymatic data (see Figure 5); (ii) the size of *luc* mRNA confirmed that transcriptional termination occurred at the internal SV40 poly A site.

The background activity as was detected in the analysis of the total pool and the high pool is in agreement with the respective FACS pattern in the un-induced state, where a minor fraction of cells gives a signal up to the third decade of fluorescence intensity (see Figure 5).

The regulatory properties of transcription units transferred to a genome are known to vary due to locus dependent effects. Accordingly, we assumed to find a rather broad distribution of expression levels in the induced as well as uninduced state within our pools of transduced cells. Yet, the observed variability could, at least in part, also be due to rearrangements within the (pro)viral genomes. In particular, the presence of two P_C promoters within P_{tet}bi-1 gave rise to a significant instability of the viral genome, most likely due to structural peculiarities introduced by long inverted repeats. We therefore analyzed, whether an array of seven *tet* operators which themselves are arranged as inverted repeats may also cause rearrangements in the asymmetric bi-directional promoters. In order to investigate the integrity of the promoter sequence, DNA was extracted from cells of the low and total pool as well as from clone c29 and the proviral Tet-control region was analyzed by PCR followed by endonuclease cleavage. The results from analyses of high and low pool derived DNA (Figure 2C) confirmed that the vast majority of proviral inserts carried intact bidirectional promoters, independent of their origin (pool). Furthermore, only a minor fraction of cells were shown to have lost one or two tet-operators during virus production. Our findings confirm that P_{tet}bi-3 is indeed stably transferred and maintained. This observation is supported by the result obtained with DNA from clone 29, which, according to southern blot analysis (Figure 2B), contains only a single proviral insert. Moreover, even after numerous cell divisions (>30 passages) the provirus did not show any trace of rearrangements within P_{tet}bi-3.

Properties of bidirectional promoters in retroviral constructs

Asymmetric bidirectional promoters underlie a number of influences that may affect their function. Thus, it is not clear whether two different minimal promoters, when combined in a P_{tet}bi arrangement, will affect each others function, nor can we exclude influences from the vector backbone itself.

In order to study the possibility for promoter crosstalk, we examined three minimal promoters, P_C, P_M and P_H, in two P_{tet}bi combinations (CM and CH). In detail, promoters were integrated in two orientations in the standard transcription unit encoding luciferase and eGFP, respectively. The four resulting retroviral vectors S2f-*l*MC*g*, S2f-*l*CMg, S2f-*l*CH*g* and S2f-*l*HC*g* as outlined in Fig. 1C were used to transduce HtTA-1 cells at low MOI (<0,1) and eGFP-positive populations were enriched by FACS. The various pools obtained were finally analyzed in the induced and uninduced state as shown in Figure 3.

Comparison of the mean fluorescence of uninduced cells containing either the S2f-*l*CMg or S2f-*l*MC*g* proviral genome revealed that P_M produces a much lower background than P_C (mean fluorescence 3 vs 38; Figure 3).

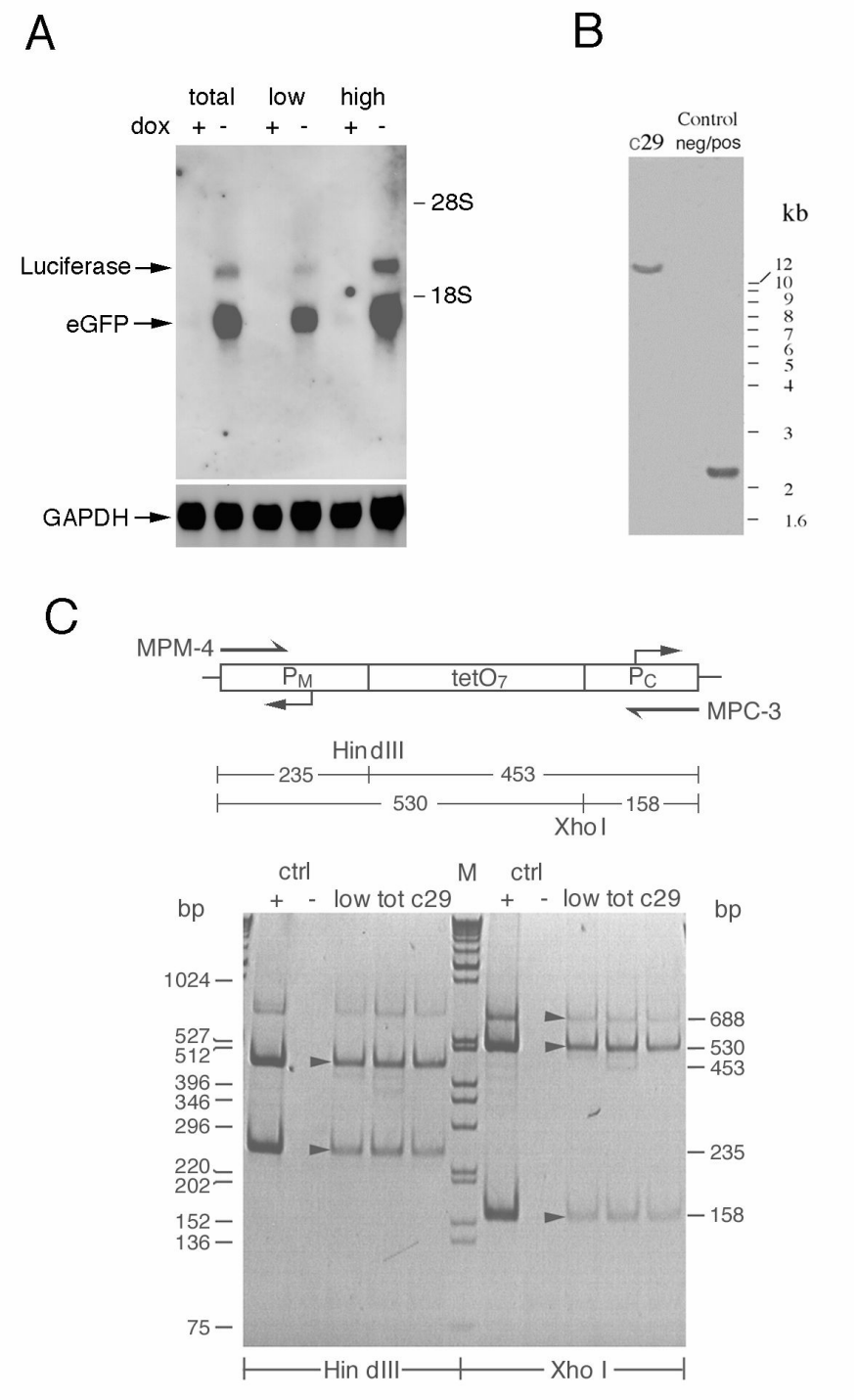
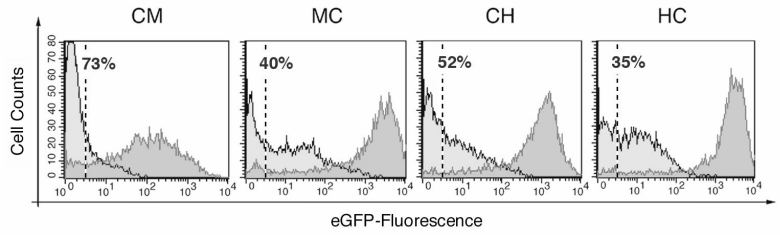


Figure 2. Characterization of the S2f-*l*MC*g* proviral genome. **(A)** P_{tet}bi-3 controlled transcription in HtTA-1 cells transduced with S2f-*l*MC*g*. Cells of the total, the high and the low pool were analyzed. The Northern blot prepared with the respective purified RNAs was probed with labelled PCR-fragments specific for luciferase and eGFP coding sequences. A GAPDH probe served as internal control. Cells were grown in absence (-) or presence (+) of Dox (100 ng/ml). **(B)** Southern blot analysis of HtTA-1 cell clone, transduced with S2f-c(*l*MC*g*). Genomic DNA (10 µg) of a representative clone (c29) was analyzed by hybridization with luciferase probe after Eco RI digest of genomic DNA and electrophoretic separation on a 0.8% (w/v) agarose gel. As negative control serves HtTA-1 genomic DNA. As positive control about 4 x 10^6 copies of an *Eco*RI/*Bam*HI digest fragment of the pS2f-*l*MC*g* plasmid fragment was loaded. **(C)** Examination of the P_{tet}bi-3 integrity in proviral genomes. Genomic DNA from cells of the low and total pool as well as of c29 was subjected to PCR amplification using primers MPM-4 and MPC-3 as indicated. The DNA generated was cleaved with *Hin*dIII or *Xho*I resulting in fragments visualized by PAGE and ethidium bromide staining. Plasmid pS2f-*l*MC*g* was used as control (ctrl). Size markers (1 kb ladder, GIBCO) are given at the left, fragment size at the right. Arrowheads indicate the expected fragments outlined in the scheme.



	CM	MC	CH	HC
Mean Fluorescence				
uninduced	3	38	14	17
Luc Activity				
uninduced	$2.6x10^5$	$2.4x10^3$	$6.5x10^4$	$2.8x10^4$
induced	$8.3x10^6$	$1.1x10^6$	$9.3x10^6$	$6.4x10^6$
Reg. Factor	31	463	143	231

Figure 3. Expression of the *luc* and *egfp* gene in HtTA-1 cells transduced with S2f vectors. HtTA-1 cells were transduced with S2f viruses controlling *luc* and *egfp* via P_{tet}bi-3 or P_{tet}bi-4 integrated in both orientations (CM, MC and CH, HC). Cells positive for eGFP in absence of Dox were collected via FACS and analyzed in the induced (-Dox, 4days) and un-induced (+Dox, 10days) state, for eGFP via FACS and for luciferase activity (rlu/µg proteins). The upper part shows the FACS profiles in the induced (medium grey) and uninduced (light grey) state. The profile of plain HtTA-1 cells is indicated by vertical dotted lines (------). The lower part summarized the mean fluorescence (mfu) of the cells in the uninduced state as well as luciferase values (rlu/µg of protein) in the uninduced (+Dox) and induced (-Dox) state. Regulation factors were calculated as quotient of the two luciferase values. The data shown are representative and rely on one of two or three independently established cell populations. The percentage of cells that could be downregulated to HtTA-1 fluorescent background in this particular experiment is given in the histogram for each of the vectors. The data of all populations were summarized in Table 1.

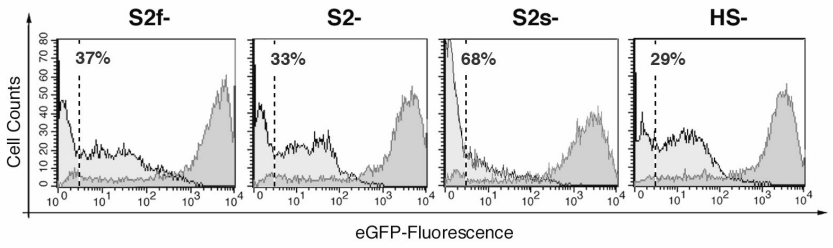
In agreement with this finding, in repressed populations containing S2f-*l*CM*g* about 73% of cells gave a fluorescence signal equivalent to the parental HtTA-1 cell line, whereas repressed pools containing the S2f-*l*MC*g* showed a much broader distribution. When induced, S2f-*l*CM*g* containing cell populations exhibit a broad distribution of fluorescence with a maximum at around 2 x 10^2, while S2f-*l*MC*g* cells appear highly activated moving out of the linear range of the FACS monitoring system. The luciferase activity of the two pools in the induced and un-induced state reveals that P_C can indeed be activated about 8 fold higher than P_M (Figure 3). However, since P_C causes an almost 100x higher background activity in the uninduced state, P_M exhibits a better regulation factor than P_C (463 vs 31; Figure 3).

When the two minimal promoters P_C and P_H, embedded in P_{tet}bi-4, were examined in the analogous way, a different picture emerged. Both cell pools (S2f-*l*CH*g* and S2f-*l*HC*g*) can be induced to similar high levels, as judged from respective luciferase values (9.3 x 10^6 and 6.4 x 10^6 rlu/µg protein respectively; Figure 3). Moreover, the two cell populations resemble each other also with respect to the mean fluorescence (14 vs. 17) and luciferase values (6.5 x 10^4 and 2.8 x 10^4 rlu/µg protein; Figure 3) in the uninduced state. The latter data contrast those obtained with P_{tet}bi-3 where the *luc* and the *egfp* gene are co-regulated by P_C and P_M. Indeed, in the context of P_{tet}bi-4, P_H lowers the background activity of P_C, as seen by the lower mean fluorescence (17 vs 38) and luciferase activity (6.5 x 10^4 vs 2.6 x 10^5 rlu/µg protein). The net result is an increase of the regulation factor of P_C in this proviral construct from 31 to 143. Thus, when organized in a P_{tet}bi arrangement, the various minimal promoters do not appear to influence each other's relative strength upon induction, but they apparently can affect the activity profile in the off-state (summarized in Table 1) as will be discussed below.

Influence of vector backbone and regulation efficiencies

To explore possible context dependencies of vector constructs on the regulation potential of P_{tet}bi-3, we compared the four vector backbones S2f-, S2-, S2s- and HS-containing the *luc*-P_{tet}bi-3-*egfp* transcription unit (Figure 1D). HtTA-1 populations were prepared as described above.

As summarized in Figure 4, a context dependency can indeed be shown. Thus, the removal of the FRT sites lowered the mean fluorescence and the luciferase value of the un-induced cells paralleled by an only moderate decrease of induction level, thus yielding an increased regulation factor. Reduction of the mean fluorescence and of the luciferase activity in un-induced cells is even more pronounced when the MuLV pol/env region harboring the splice acceptor site is introduced into the S2 vector resulting in the S2s backbone. The remarkable regulation factor of about 1300 fold seen with this pool of transduced cells is, however, primarily due to the reduced background activity of P_M in this context, as the maximal level of induction is lowered as well. Moreover, also unregulated background expression of P_C is significantly reduced leading to a total population in which about 68% of the cells can be downregulated to HtTA-1 level. Interestingly, a rather low background and moderate induction is also observed with cells harboring HS-*l*MC*g* provirus.



	S2f-	S2-	S2s-	HS-
Mean Fluorescence				
uninduced	40	25	12	22
Luc Activity				
uninduced	$4x10^3$	$1.8x10^3$	$7x10^2$	$1x10^3$
induced	$2x10^6$	$1.4x10^6$	$0.9x10^6$	$0.9x10^6$
Reg. Factor	509	797	1300	905

Figure 4. Effect of vector backbone modifications on P_{tet}bi-3 controlled expression. The bidirectional transcription unit lMC*g* incorporated in the vectors S2f, S2, S2s and HS was transferred into HtTA-1 cells. The profile of plain HtTA-1 cells is indicated by vertical dotted lines (------). Analysis of the resulting pools was carried out as described in Figure 3. The percentage of cells that could be downregulated to HtTA-1 fluorescent background is given in the histogram for each of the vectors. The data shown are representative and rely on one of two comparable measurements.

These results indicate a significant impact of elements within the viral backbone on the regulation potential of a Tet controlled transcription unit, which are apparently not specific for the employed minimal promoters. Furthermore, there is no functional restriction of the P_{tet}bi-3 regulatory unit to MuLV based vectors as could be demonstrated by its transfer to the lentiviral HS-backbone.

Isolation of cell populations with high regulation potential

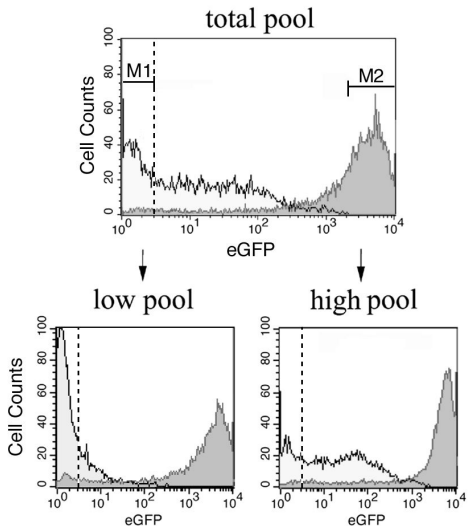
Based on the above data, it appeared obvious how to prepare cell populations, which would permit the stringent control of genes of interest in a P_{tet}bi context. We, therefore, prepared a "total pool" of HtTA-1 cells containing the S2f-*l*MC*g* provirus as described above. Samples of this pool in the induced and uninduced state were subjected to FACS and a "low pool" as well as a "high pool" was separated as shown in Figure 5. The FACS analysis of the two established pools (high and low) revealed that the low pool consisted of a rather homogenous population with a high regulation potential. This interpretation of the FACS pattern is supported by luciferase values of induced and un-induced cultures derived from the three pools. Accordingly, the regulation factor observed for the total pool was not improved in the high pool but it increased 8-fold to a value of 5300 in the low pool.

DISCUSION

Retroviral vectors offer distinct advantages for the transfer of genes into cells. In particular, vectors integrate into the genome of the target cells without causing rearrangements at the insertion site and allow stable expression of transgenes without the need for drug selection. Moreover, this process is efficient for a large spectrum of cells including primary cells. Transcription of inserted genes is, however, dependent on the chromatin environment surrounding the insertion site since RNA polymerase II promoters are generally susceptible to cis elements involved in transcription control which may act over considerable distance (Bulger and Groudine, 1999; Chambeyron and Bickmore, 2004; Lund and Lohuizen, 2004). This susceptibility to activation or inactivation from outside holds true also for P_{tet}, an artificial RNA polymerase II promoter responsive to the tetracycline controlled transactivators.

At this point, it is important to consider that MuLV-based retroviral vectors seem to preferably integrate into or close to transcriptional start sites of human cellular promoters (Wu et al, 2003; Nowrouzi et al, 2006), thus in many cases will expose the minimal promoters to unfavorable environment were enhancers but also silencers were active.

This in mind, we have explored the potential of Tet regulation in cell populations transduced with retroviral vectors that contain P_{tet} controlled transcription units in a background where the production of tTA was kept constant. The vectors contained two indicator genes, *luc* and *egfp*, whose transcription is coregulated by bidirectional promoters of the P_{tet}bi type. The two reporter functions enabled us to analyze populations of transduced cells at the single cell level via eGFP fluorescence and to quantify promoter activity by monitoring luciferase activity in cell populations. Our results, as summarized in Table 1, have yielded information relevant for the development of retroviral vectors as well as for the generation of cell populations containing Tet regulatable transcription units in a proviral context.



	total pool	low pool	high pool
Mean Fluorescence			
uninduced	38	3	40
Luc Activity			
uninduced	$3.2x10^3$	$0.35x10^3$	$4.2x10^3$
induced	$2.1x10^6$	$1.9x10^6$	$2.7x10^6$
Reg. Factor	643	5279	638

Figure 5. Generation of HtTA-1 cell populations with high regulation potential. HtTA-1 cells were transduced with S2f-*l*CM*g* viruses and eGFP-positive cells were obtained as described in Fig. 3. From these cells, a "low pool" was obtained by separating via FACS under un-induced conditions (+Dox) a population delineated by M1 in the most upper panel. Analogously, a "high pool" was obtained upon induction of the "total pool" as defined by M2. The lower panels show the induction profiles of the two pools. Induced (dark grey) and un-induced (light grey) states as well as the distribution of HtTA-1 cells (-----) are indicated. The lower part summarizes the mean fluorescence, the luciferase activities and regulation factors as in Figure 3. The data shown are representative and rely on one of two comparable measurements.

For many applications, it is of advantage to coregulate two genes (*e.g.*, α- and ß-chain of T-cell receptors) or to coregulate a gene of interest along with a gene encoding an easily assayable reporter function as a correlate for the activity of the target gene, without the constraints of bicistronic arrangements. This can be reliably achieved by using P_{tet}bi-type promoters. Interestingly, our data as well as a previous report (Unsinger et al, 2001) show that implementation of the symmetric P_{tet}bi-1 (Baron et al, 1995) in a retroviral context leads to extremely poor titers. The reason for this deleterious effect is, however, not the bidirectional transcription per se, but is most likely a structural constraint at the level of the viral RNA. By contrast, the asymmetric promoters P_{tet}bi-3 and P_{tet}bi-4 do not interfere with high titer virus production and are stably maintained in our retroviral backbones.

Transducing cells at low MOI by viral vectors such as S2f-*l*MC*g* or S2f-*l*CM*g* enabled us to isolate, via eGFP/FACS, cell populations consisting of 2-5 x 10^3 independent clones of which the majority contained a single integrate. Our results indicated that both, the luciferase and the eGFP gene were co-regulated. Moreover, the rate of transcription of the two divergently transcribed genes is dependent on the properties of the respective minimal promoters, but independent of the promoter orientation within the viral backbone. Thus, minimal promoters with different strength may be exploited to achieve a dosage effect for the genes expressed according to the hierarchy for the promoters examined here: $P_C \geq P_H > P_M$.

As the origin of the used minimal promoters differs, it could be expected that their susceptibility to outside activation varies accordingly. Examining our minimal promoters reveals that P_C (from human non-integrating DNA virus) is the most sensitive, giving rise to considerable eGFP and luciferase background levels followed by P_H (from integrating human lentivirus) and P_M (from integrating mouse retrovirus). However, the susceptibility depends on the combination of the minimal promoters within P_{tet}bi. Accordingly, P_C shows the highest background activity in P_{tet}bi-3 where it is combined with P_M, whereas considerably lower values are observed in the P_H/P_C combination of P_{tet}bi-4. As in both cases the

Table 1. Transcription control by P_{tet}bi –3 and P_{tet}-bi –4 in HtTA-1 cells transduced with various retroviral constructs This table summarizes data from populations obtained for the experiment described for Fig. 3. It compiles the P_{tet}-bi variants within the S2f backbone. For each of the vectors two or three independently established populations have been generated, each of which was measured twice.

		Fraction of transduced cells (%) showing Dox dependent GFP expr.[1)]		**Activity of Minimal Promoters in P_{tet}bi context**			
Provirus	Promoter	ON (- Dox)	OFF (+ Dox)	P_{min}	Fold luc induction[2)]	P_{min}	mfu in the OFF state (+Dox)
S2f	P_{tet}bi –3	96,5± 0,9	35 ± 2	P_M	538 ± 94	P_C	36,1 ± 2,10
		85,7± 7,6	61 ±11	P_C	22 ± 13	P_M	3,1 ± 0,01
	P_{tet}bi –4	98,0± 0,6	26 ± 3	P_H	160 ±101	P_C	18,5 ± 1,77
		95,8± 2,8	41 ± 5	P_C	130 ± 51	P_H	22,3 ± 0,46

[1)] All measurements are standardized using respective values of non-transduced HtTA-1 cells; the "OFF column" shows the fraction of cells, which are downregulated to HtTA-1 background in the presence of Dox, corrected for not inducible cells still present in the purified populations.

[2)] Values given were the mean of two or three independently established populations. The fold-regulation is expressed as the quotient of induced to uninduced luciferase activity.

maximal inducible levels did not change, these data indicate that P_H but not P_M is capable of shielding to some extent P_C from outside activation resulting in a considerable increase of the regulation factor (from 22 to 130; Table 1). It remains to be seen whether indeed P_H exerts some degree of locus control that can be exploited more generally.

On the other hand, it is surprising that while in the context of P_{tet}bi-4 P_H reduces the mean fluorescence of the whole cell population and, thus, the GFP background controlled by P_C to 19 (as compared to 36 in P_{tet}bi-3) the fraction of tightly regulated cells as seen in the FACS profile becomes smaller (Figure 4). This may be due to the cis elements still present in P_M (Oct-1 and NF-1) and P_H (3 SP-1 sites), which could have a differential effect on P_C.

The effect of lowered mean fluorescence and a concomitant decrease of tightly regulatable cells in purified HtTA-1 cell populations also becomes visible for the lentiviral backbone and, to a smaller extend, the MuLV-based S2 backbone, both expressing the two reporter genes under the control of P_{tet}bi-3 (Figure 4). This observation indicates that surrounding viral sequences can affect promoter dependent transcriptional control in a similar way as described above for the interference of the minimal promoters.

The influence of cis elements within the proviral genome is further underlined by the effects observed upon modification of the viral backbone. Both, the elimination of the FRT sites and the introduction of the MuLV pol/env fragment affect the regulation properties of the transcription unit in an unpredictable way (Figure 4), suggesting that probably no „neutral" sequences exist in retroviral backbones. On the other hand, these data show also that there is room for individual optimizations and that well functioning retroviral vectors first need to be optimized over several parameters as seen, *e.g.*, for S2s-*l*MC*g*. Remarkably, the strongest effect was exerted by introducing the pol/env fragment into the viral backbone: It reduced the activity of both P_C and P_M within P_{tet}bi-3 in the off state several fold, increasing the fraction of transduced cells showing the background fluorescence of the parent HtTA-1 cell line to 68%.

In the work described here, we analyzed bidirectional response units of the Tet system in cell populations upon transfer by retroviruses. As expected, many parameters influence the properties of the minimal promoters within P_{tet}. Some are vector-borne, others integration site-dependent. The phenotype we measure is the net result of complex combinations. In view of this complexity, it is surprising and encouraging that modifications in retroviral backbones can result in vectors with rather favorable properties. Thus, the finding that cell populations transduced with the S2s backbone show a 1300-fold range of regulation while in the off state more than 60% of the cells exhibit the background of the HtTA-1 parent cell line, suggests a remarkable degree of locus control by the proviral construct, especially when considering the preferential integration of MuLV-based retroviral vectors into promoter proximal regions. For many tasks, properties of cell populations as described here are already satisfying. They can, however, be considerably improved by a single sorting step resulting in cell populations with regulation windows exceeding three orders of magnitude.

Finally, as we learn more about the secrets of transcriptional insulation and locus control, gene transfer

via retroviral vectors may in the end develop into an approach with high predictability.

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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