Regulated expression of the *diphtheria toxin* A gene in human glioma cells using prokaryotic transcriptional control elements

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Because accurate regulation of toxin gene expression is critical for safe and effective gene therapy applications, the authors have examined the regulation of diphtheria toxin A (*DTA*) fragment expression in human glioma cell lines using two transcriptional control systems derived from *Escherichia coli*: the tetracycline (Tet) system and the lactose (Lac) system. The Tet system includes a tetracycline-controlled transactivator (tTA), a tTA-responsive minimum human cytomegalovirus (hCMV) promoter controlling the expression of the *DTA* gene, and tetracycline as an allosteric inhibitor. The Lac system includes the lac repressor (lacR), a lacR-regulated Rous sarcoma virus–long terminal repeat (RSV-LTR) promoter controlling the expression of the *DTA* gene, and isopropyl-thio-β-D-galactoside (IPTG) as an allosteric inducer. Expression plasmids encoding either tTA or lacR were transfected into U-87MG and U-343MG glioma cells along with the responsive *DTA* plasmid. Cell killing was monitored by the ability of the toxin to abolish protein synthesis and was quantitated using a luciferase reporter gene. In the Tet system, tumor cell killing could be regulated by tetracycline up to 120-fold. In contrast, only a twofold IPTG-dependent regulation was obtained using the Lac system because of an incomplete repression of *DTA* expression in the uninduced state. Replacement of the RSV-LTR promoter with the heavy metal–inducible mouse metallothionein-I promoter in the lacR-responsive unit, as well as the generation of a clonal glioma cell line expressing lacR, did not significantly enhance regulation of *DTA* in the Lac system. In conclusion, this study demonstrates that the Tet system is of potential use in gene therapy applications in which regulated expression of a therapeutic gene is an important issue.

**KEY WORDS** • diphtheria toxin • gene therapy • glioblastoma multiforme • Lac system • Tet system • tetracycline

Transfer of toxin genes into tumor cells is a promising gene therapy strategy against cancer. Two classes of genes exist that encode enzymes that are either indirectly or directly cytotoxic to mammalian cells. Cytosine deaminase and herpes simplex virus thymidine kinase (HSV-tk) belong to the class of enzymes that are indirectly cytotoxic to cells. These enzymes are also referred to as drug-conditional toxins, because they kill cells by activating a nontoxic prodrug (such as ganciclovir or 5-fluorocytosine) to an active form. On the other hand, prokaryotic toxins, such as diphtheria toxin and pseudomonas A endotoxin, are examples of enzymes that are directly toxic to cells because of their ability to abolish cellular protein synthesis.

Diphtheria toxin is a polypeptide of 535 residues produced and secreted by *Corynebacterium diphtheriae*. Receptors for this toxin are present on most mammalian cells; once inside the cell, the polypeptide is extremely toxic because it inhibits host cell protein synthesis through adenosine diphosphate (ADP) ribosylation of elongation factor–2. As little as one molecule of fully active diphtheria toxin may be sufficient to abolish protein synthesis and provoke cell death. The diphtheria toxin A fragment (DTA; amino acids 1–193) is the catalytic component of the toxin and is responsible for its toxicity; the B fragment (amino acids 194–535) mediates entry into the host cell by binding specific cellular receptors that facilitate translocation of the toxin into the cytoplasm. Because of the widespread distribution of diphtheria toxin receptors on most cells, therapeutic approaches have been devised that deliver DTA selectively to neoplastic cells, avoiding binding to, and killing of, normal cells. For example, DTA and other protein toxins have been conjugated to monoclonal antibodies that recognize cell surface antigens or to ligands for cellular receptors expressed selectively on neoplastic cells. Effective cell killing has also been accomplished using direct transfection of the *DTA* gene into tumor cells.
In contrast to most drug-conditional cytotoxic therapies, bacterial toxins can kill cells at extremely low concentrations, do not require cell replication for killing, and do not depend on exogenously applied compounds that may display general toxicity, such as ganciclovir. However, because bacterial toxins can act directly on cellular metabolism, tissue specificity and the timing and level of expression must be stringently regulated in a clinical setting. Therefore, in devising approaches that involve the delivery of toxin genes to tumor cells, accurate and efficient mechanisms to control toxin gene expression must be developed.

The use of inducible eukaryotic promoters, such as the metallothionein and mouse mammary tumor virus promoters, for the control of gene expression has been hindered by their limited increases on induction and the pleiotropic effects of the inducers, heavy metals, and steroids. In contrast, prokaryotes harbor several transcription systems that can stringently and specifically regulate gene expression through the actions of physiological inducers and inhibitors. In the *Escherichia coli* lac operon (lac) operon, the lac repressor protein (lacR, encoded by the *lacI* gene) binds as a homotetramer to lac operator sequences (lacO) downstream of the lac promoter, leading to inhibition of transcription of the *lacZ, lacY*, and *lacA* genes. Physiological inducers (allolactose) or synthetic inducers (IPTG) bind to lacR and cause a conformational change and decrease in affinity for lacO, resulting in dissociation of the lacO/lacR complex and induction of transcription. Similarly, in the tetracycline (tet) resistance operon of *E. coli*, the tet repressor (tetR) inhibits transcription of tetracycline resistance–mediating genes by binding to the tet operator (tetO), whereas tetracycline as the physiological inducer reestablishes transcription by removing tetR from tetO.

Both the Lac and Tet systems have been adapted for use in mammalian cells. In the Lac system, a modified form of lacR, containing a nuclear localization signal, was shown to downregulate the expression of a cotransfected gene under the control of a promoter containing lacO sequences. Furthermore, expression of the cotransfected gene was inducible with isopropyl-thio-β-D-galactoside (IPTG). An interesting modification was introduced into the Tet system for its use in mammalian cells by Gossen and Bujard. By fusing tetR with the transcriptional activating domain of virion protein 16 (VP16) of the herpes simplex virus, a tetracycline-controlled transactivator (tTA) was generated. This tTA still efficiently binds tetO sequences and tetracycline; however, rather than repressing transcription, tTA now activates transcription because positively acting cellular transcription factors are recruited by the VP16 activating domain. The tTA has been shown to stimulate transcription of a minimum promoter containing tetO sequences by up to five orders of magnitude in cultured mammalian cells and transgenic mice. Furthermore, in the presence of tetracycline, which removes tTA from tetO, transcription from the minimum promoter can be repressed.

Here we show that prokaryotic transcription control systems are able to modulate the expression of *DTA* in human glioma cells and, consequently, the rate of tumor cell killing by up to 120-fold. We found that the Tet system was superior to the Lac system in controlling toxin gene expression. More generally, our data indicate that prokaryotic transcription control elements are promising tools for achieving regulated expression of therapeutic genes.

**Materials and Methods**

**The Plasmids**

A 635-bp *DTA* fragment encoding the *DTA* subunit was generated using polymerase chain reaction (PCR) from pTH-1.8 included in the 5’ PCR primer was an optimal Kozak sequence (CCACCATG) for efficient translation initiation. The PCR-generated *DTA* fragment was inserted into the cloning vector pCRRI and designated pCR-DTA.

The Tet system expression vectors include the regulator plasmid pHUD15-1 encoding tTA, which is under the transcriptional control of a human cytomegalovirus (hCMV) promoter/enhancer, and the response plasmid pHUD10-3, which includes a minimum hCMV promoter that is tTA dependent because of heptamerized upstream tetracycline operators.13 The *DTA* was excised from pCR-DTA by Eco RI digestion and ligated into the unique Eco RI site of pHUD10-3 to construct the response plasmid pHUD-DTA.

The Lac system vectors include the regulator plasmid p3’SS, which contains a modified lacI gene under control of a polyoma F9-1 promoter, and the plasmid pOPISCAT, which contains three ideal (Oid) 20-bp lacO sequences embedded within an SV40 intron downstream of the RSV-LTR promoter (LacSwitch system). The *DTA* fragment was excised from pCR-DTA with EagI and ligated into the NotI site of pHUD10-3 to construct the response plasmid pOP-RSV-DTA.

To replace the RSV-LTR promoter of pOP-DTA by the heavy metal–inducible mouse metallothionein-1 (MT-1) promoter, the RSV-LTR promoter was excised with SmaI and BglII. The remaining 6327-bp fragment was ligated to a 564-bp PuVII/BamHI-generated fragment containing the MT-1 promoter and an additional 25-bp O1 lacO immediately downstream of the promoter. The MT-1 promoter was generated by PCR using pCLH3A as the template and ligated into pCRII. The resulting construct containing four lac operators was named pOP-MT1-DTA.

Using p3’SS as the template, pMFGlac was constructed by PCR amplification of the entire lacI transcriptional unit. The 1990-bp PCR product included the F9-1 promoter at the 5’ end and the thymidine kinase polyadenylation signal at the 3’ end. The NcoI and BglII restriction sites were included in the PCR primers to facilitate cloning into the NcoI and BglII sites of the retroviral vector pMFG. Both pSVlac and pGl2 are expression plasmids that code for firefly luciferase under control of the SV40 promoter/enhancer.

**Cell Lines**

The human glioblastoma cell lines, U-87MG and U-343MG, were maintained in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% fetal calf serum and 100 U/ml penicillin G/100 μg/ml streptomycin sulfate.

**Establishment of lacR-Expressing Clones (U-343lac Cells)**

The U-343MG cells were plated at a density of 10^4 cells per 100-mm dish and incubated for 24 hours until a confluency of 60% was reached. One hour after addition of fresh medium, the cells were cotransfected with 12 μg pMFGlac and 3 μg pRSVneo (encoding the *neomycin resistance* gene) using the calcium phosphate technique. After 6 hours, cells were washed twice in phosphate-buffered saline. Forty-eight hours after transfection, the cells were split 1:5 into 100-mm dishes and selected in medium containing 400 μg/ml G418. The G418-resistant clones were expanded and screened for lacR expression using Western blotting analysis. From each clone, total cell lysates were prepared and protein concentration was determined using a DC protein assay kit, and sample buffer (50 mM Tris-HCl pH 6.8/2% glycerol, 5% 2-mercaptoethanol, 0.01 mg/ml bromophenol blue) was added to each sample (50 μg). Samples were
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then resolved by electrophoresis in 10% sodium dodecyl sulfate–polyacrylamide gels. Proteins were transferred to nitrocellulose, probed overnight with a rabbit anti-lacR antibody (diluted 1:1000), and developed using an indirect immunoperoxidase system and chemiluminescence. Proteins extracted from the E. coli strain DH5α served as a positive control for lacR.

**Transient Transfections With DTA Plasmids**

The U-87MG cells (10⁵, Lac and Tet systems) or the U-343lac cells (3 × 10⁵, Lac system) were plated onto 100-mm dishes. Transient transfections using the calcium phosphate technique were performed the following day 1 hour (Lac system) or 3 hours (Tet system) after washing. The U-87MG cells were preincubated with tetracycline at various concentrations 3 hours before transfection. The IPTG was added to U-87MG cells and to U-343lac cells at various concentrations 6 hours after transfection. Tetracycline and IPTG were present until harvesting. Luciferase assays were performed 24 hours posttransfection.

The pUHD-DTA and pOP-DTA plasmid DNAs were cotransfected with the appropriate regulator plasmids, together constituting 20 μg, at different ratios into U-87MG cells. In addition, 10 μg of luciferase plasmid DNA was used in all transfections for quantification of cell killing. For the Lac system, pGL2 was used instead of pSVLac as the reporter plasmid because the latter contains lacO sequences responsive to lacR/IPTG. To investigate the effects of the regulator-containing plasmids and the basal activity of the promotors responsible for expression of DTA, pUHD15-1 and p3'SS were replaced by equal amounts of pRK5, which lacks tetO and lacO regulatory sequences. The U-343lac cells were transfected with different amounts of pOP-DTA plasmid DNA, and the total amount of transferred DNA was equalized by addition of the parent vector, pOP3CAT. Identical precipitates were used for the transfections with or without addition of tetracycline or IPTG. All transfections were done in duplicate and performed at least twice.

**Luciferase Assay**

Cells were washed three times with PBS before they were lysed in 1 mM dithiothreitol/1% Triton X-100/luciferase buffer (25 mM glycéryl glycérol, 15 mM MgSO₄, 4 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid, pH 7.8) at 4°C. Cell lysates were scraped off the dishes and centrifuged for 4 minutes at 7800 g. Aliquots (100 μl) of the supernatant were mixed with 370 μl luciferase buffer containing 2 mM adenosine triphosphate, 1 mM dithiothreitol, and 15 mM potassium phosphate, and assayed for luciferase activity in a 1251 luminometer using the integral mode. The D- Luciferin was used at a saturating substrate concentration (0.2 mM in luciferase buffer). Luciferase activity was adjusted to the protein content of the lysates, as determined using a DC protein assay kit.

**Sources of Supplies and Equipment**

The U-87MG and U-343MG cell lines were obtained from the American Type Culture Collection (Rockville, MD) and from Dr. Mark Israel (University of California at San Francisco, San Francisco, CA), respectively.

The plasmids pUHD10-3 and pUHD15-1 were kindly provided by Dr. H. Bujard (University of Heidelberg, Heidelberg, Germany); pTH-1 by Dr. I. H. Maxwell (University of Colorado, Denver, CO); pRK5 and pRSVneo by Dr. R. Derynck (University of California at San Francisco); pSVLac by Dr. J. W. Henson (Massachusetts General Hospital, Boston, MA); pMPG by Dr. R. C. Mulligan (Massachusetts Institute of Technology, Cambridge, MA); and pCLH3A by Dr. V. Ramesh (Massachusetts General Hospital).

The cloning vector pCRII was purchased from Invitrogen (San Diego, CA). The LacSwitch System was obtained from Stratagene (La Jolla, CA), which also provided the anti-lacR antibody and the IPTG. The control vector pGL2 was obtained from Promega (Madison, WI). Vector (Burlingame, CA) provided the indirect immunoperoxidase system. The enhanced chemiluminescence (ECL) was provided by Amersham (Little Chalfon, England). The G418 and the DH5α were obtained from Life Technologies (Basel, Switzerland). The DC protein assay kit was obtained from Bio-Rad (Hercules, CA).

In the luciferase assay, we used a 1251 luminometer provided by Wallac (Gaithersburg, MD). The D- Luciferin was purchased from Sigma Chemical Co. (St. Louis, MO).

**Results**

**Regulation of DTA Gene Expression Using the Tet System**

We generated plasmid-based expression vectors containing the DTA gene under the control of the Tet system (Fig. 1 upper). Regulation and induction of the DTA gene in this system were examined in transient transfection assays involving cotransfection of a “regulator” plasmid (encoding tTA), a “response” plasmid (tTA-dependent promoter in front of the DTA gene), and an “indicator” plasmid (encoding luciferase under the control of the SV40 promoter) (Fig. 1 upper). The rationale for the indicator plasmid is that the degree of expression of the luciferase gene provides a highly sensitive means of measuring expression of the DTA gene as well as the effectiveness of the DTA protein to abolish protein synthesis by ADP ribosylation of elongation factor-2.

Transfections of human glioma U-87MG cells with the luciferase-indicator plasmid and different ratios of the DTA-response plasmid and the tTA-regulator plasmid (which should activate DTA gene transcription) resulted in
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We also generated plasmid-based expression vectors containing DTA under the control of the RSV-LTR promoter to which lacO sequences have been added (Fig. 1 lower). In contrast to the Tet system in which expression of DTA is stimulated when the tTA protein binds tetO sequences, expression of DTA in the Lac system should be abolished when lacR binds lacO sequences and restored when IPTG is added because of the dissociation of lacR from lacO sequences.

Transfections of U-87MG cells with the luciferase-indicator plasmid and different ratios of the DTA-response plasmid pOP-RSV-DTA and the regulator-plasmid p3’SS (which should repress DTA gene transcription in the absence of IPTG) resulted in luciferase activity ranging from 35 to 2% of the values obtained in transfections with luciferase-indicator plasmid alone (Fig. 2 right). Addition of IPTG (5 mM) had only a small effect on induction of DTA gene expression. The fold induction, determined by dividing the luciferase activity in the repressed state (without IPTG) by the luciferase activity in the induced state (with IPTG), did not exceed twofold and was much lower than those obtained using the Tet system (Fig. 2 right and Table 1). Concentrations of IPTG at 1 mM and 10 mM did not increase the fold induction, whereas toxic effects were observed at an IPTG concentration of 25 mM (data not shown).

Two additional strategies were used to improve the low induction ratios of the Lac system. First, a glioma cell line that constitutively overexpresses lacR was generated to prevent possible toxic effects of DTA that occur prior to the synthesis of sufficient amounts of lacR; this potentially could be occurring in the transient transfection experiments. Following cotransfection of U-343MG cells with pMFGlac (Fig. 1) and pRSVneo, several G418-resistant clones were tested for stable lacR expression. The immunoblotting results of three of these clones are shown in Fig. 3. A strong band of approximately 38 kD was seen in one of the clones (designated U-343lac), was absent in the parental cell line (U-343MG), and corresponded to the 38-kD band of monomeric lacR present in protein extract from the E. coli strain DH5α. When U-343lac cells were transfected with increasing amounts of the DTA-response pOP-RSV-DTA plasmid, IPTG induction increased only moderately to at best threefold (Fig. 4 and Table 1).
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**TABLE 1**

<table>
<thead>
<tr>
<th>System/Cells</th>
<th>Fold Induction</th>
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<tr>
<td><strong>Tet system</strong></td>
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<tr>
<td>U-87MG cells</td>
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<td>DTA/tTA (1:1)</td>
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<tr>
<td>DTA/tTA (1:20)</td>
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<tr>
<td><strong>Lac system</strong></td>
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<tr>
<td>U-87MG cells</td>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>pOP-RSV-DTA (1 µg)</td>
<td>2.9</td>
</tr>
<tr>
<td>pOP-RSV-DTA (10 µg)</td>
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</tr>
<tr>
<td>pOP-MT1-DTA (0.2 µg)</td>
<td>2.3</td>
</tr>
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</table>

*In the Tet system, optimal regulation is dependent on the ratio between the amounts of cotransfected tTA and DTA plasmids, whereas the low regulation of the Lac system cannot be markedly increased by different DTA/lacl plasmid ratios. Transfection of U-343lac cells showing stable expression of lacR, or use of a heavy metal/IPTG-inducible MT-1 promoter controlling expression of the DTA gene did not significantly increase regulation. Fold induction is determined by dividing the luciferase activity in the repressed state of DTA gene expression (plus tetracycline or minus IPTG) by the luciferase activity in the induced state of DTA gene expression (minus tetracycline or plus IPTG).

The second strategy involved replacement of the RSV-LTR promoter responsible for DTA gene expression with the MT-1 promoter. Lower basal DTA expression due to the less active MT-1 promoter (in the absence of heavy metals), as well as an additional lac operator, might result in better regulation. After transfection of pOP-MT1-DTA into U-343lac cells, luciferase activity remained relatively high (73% of the activity obtained with luciferase plasmid alone), indicating lower toxin expression by the MT-1 promoter in the repressed state. However, induction of DTA gene expression was still low (two- to threefold using 5 mM IPTG and 0.5 µM ZnSO4; Table 1).

**Discussion**

To regulate the expression of transfected toxin genes in neoplastic cells effectively, inducible systems require several features. Ideally, in the induced state, the concentration of the toxin should be high enough to guarantee killing of all transfected cells and the basal activity in the repressed or noninduced state should be low enough to prevent cell damage. Our data show that these requirements are potentially fulfilled by the Tet system, but not by the Lac system.

In the induced state, both systems were highly effective at abolishing protein synthesis. Using the Lac system, luciferase activity was decreased to less than 2%, but only after transfection of the highest amount of DTA plasmid. In contrast, the Tet system showed reduced luciferase activity to less than 1%, even after transfecting the lowest amount of DTA plasmid. The almost complete inhibition of protein synthesis in transfected cells 24 hours post-transfection reveals the extreme toxicity of DTA and its rapid action, which is independent of cellular replication.

When DTA expression was repressed in the two regulated systems (with tetracycline or in the absence of IPTG), luciferase activity was reduced by only 29% in the Tet system but by more than 65% in the Lac system. The reduction in luciferase activity indicates that low-level basal expression from the RSV-LTR and minimum hCMV promoters does occur in both systems. In accordance with these findings, low basal luciferase expression has also been observed in some tissues of mice that are transgenic for the luciferase gene under control of the tTA-responsive minimal hCMV promoter.10 The more pronounced degree of basal expression in the repressed state of the Lac system is explained by the relatively high basal activity of MT-1/lacO and RSV/lacO promoters used. The low basal or “leaky” DTA expression we observed in our studies with the Tet system can be circumvented in two different ways. 1) We have recently constructed a novel retroviral vector in which the Tet system components were incorporated in a manner that decreases basal or leaky expres-

**Fig. 3.** Results of immunoblot analysis of G418-resistant U-343MG clones cotransfected with a plasmid encoding lacR (pMFGlac) and a plasmid encoding neomycin resistance (pRSVneo) using an anti-lacR antibody. Protein extracts from *E. coli* (DH5α) were included as a positive control for lacR and protein extracts from U-343MG cells (parent) as a negative control. Lanes 1 to 3: three G418-resistant clones. Monomeric lacR (38 kDa) is detectable in one transfected clone (lane 3). This clone was designated U-343lac.

**Fig. 4.** Bar graph showing luciferase activity using the Lac system with U-343lac cells. Luciferase activity (rLU/mg protein) was assayed after transient cotransfection of U-343lac cells with the indicated amounts of pOP-RSV-DTA plasmid and 10 µg luciferase plasmid (pSVLac). Decreased luciferase activity in transfections with DTA plasmid compared to transfections with pSVLac alone is an indicator of cell killing. Ratios of luciferase activity in the absence and presence of IPTG indicate regulation (Fold Induction).
Using this vector, we have achieved controlled induction of apoptotic cell death of glioma cells in vitro and in vivo by transferring and tightly regulating the interleukin-1β-converting enzyme gene. Similarly, the retroviral Tet system may be effective at delivering and more stringently controlling the expression of DTA in tumor cells. 2) In the studies described here we used a fully functional DTA gene; however, a spectrum of attenuated DTA mutants have recently become available that demonstrate 10 to 10,000 times decreased elongation factor–2 ADP ribosylation activities.28 If a small amount of basal expression in the Tet system is unavoidable, a mutant and less active DTA coupled with the Tet system could be used rather than the fully active toxin in a tumor-killing paradigm.

Using the commercially available Lac system, the induction ratio, calculated as the ratio of luciferase activity in the repressed versus induced states, did not exceed twofold in our transient transfection experiments, whereas a five- to 30-fold induction by IPTG has previously been reported using various other Lac systems.3,15,19 We therefore introduced two modifications in hopes of increasing IPTG-dependent regulation and decreasing high basal activity in the Lac system. First, we generated a U-343MG subclone that constitutively expresses lacR to account for possible DTA toxicity occurring prior to Lac synthesis and to increase intracellular levels of lacR to ensure that sufficient amounts were present for binding to lacO. However, in these U-343lac cells no significant increase in the induction ratio was observed. Second, when the IPTG-inducible RSV-LTR promoter was replaced by an IPTG-inducible MT-1 promoter, which should introduce additional regulation because of its heavy metal induction, again induction ratios could not be substantially increased and did not exceed twofold, although a lower level of noninduced cell death was achieved. The poor regulation of DTA toxicity using the Lac system is apparently related to its inability to repress DTA gene transcription sufficiently. We therefore conclude that the Lac system is not suitable for situations in which efficient repression of transgene expression is mandatory, such as in paradigms involving genes that encode highly potent toxins.

We have obtained regulation of up to 120-fold when using the Tet system, corresponding to data shown previously, in which the expression of reporter genes could be regulated between 15- and 100,000-fold in HeLa cells, and between two- and 5000-fold in various organs of mice that are double transgenic for tTA and a reporter gene.10,13 Besides its high regulation, controlled DTA expression by the Tet system offers additional advantages when used in vivo. First, tetracycline as an effector molecule has well-established pharmacological features and is broadly distributed in various tissues following ingestion. To enhance regulated gene expression in the brain, tetracycline derivatives, such as minocycline, with a higher ability to penetrate the blood-brain barrier can be used. Furthermore, although the toxicity of tetracycline is minimum at the concentration needed to regulate promoter activity, it can be replaced by derivatives showing less cytotoxicity and even higher affinity for tTA, such as anhydrotetracycline.12 Second, several reports have shown that inhibition of protein synthesis by proteins such as diphtheria toxin results in the activation of programmed cell death (apoptosis).6,21,28 Moreover, a significant “bystander” effect occurs in populations of cells induced to undergo programmed cell death.4,9,35 The bystander effect is defined as the eradication of nontransduced tumor cells by transduced tumor cells via mechanisms that have not been completely identified to date. These mechanisms may include exchange of toxic factors by way of gap junctions, uptake of apoptotic vesicles, release of toxic factors, and activation of the immune system.22 Thus, similar to the bystander effect observed in a population of cells expressing the thymidine kinase gene and exposed to ganciclovir (a prodrug that when incorporated into DNA is toxic to cells), the bystander effect is expected to occur in a population of tumor cells expressing the DTA gene, although in vivo experiments are required for definitively clarifying this point. In this regard, only a fraction of the tumor cells would need to be transduced with DTA for a significant tumoricidal effect. Third, the toxin is targeted to tumor cells in vivo because retroviral vectors transduce only replicating cells, that is, glioma cells and a few reactive glial cells and vascular cells, whereas quiescent parenchymal cells are not transduced. Moreover, possible leakage of DTA from transduced cells is not harmful to normal brain tissue, because the B fragment, which is required for uptake of the toxin, is absent. Fourth, the very low levels of basal gene expression of the retroviral Tet system33 should increase the number of packaging cells that release viral particles as well as the number of tumor cells transduced with a toxin gene. We therefore hypothesize that this retroviral vector that contains and regulates an adequate toxin gene will enable regulated cytotoxic gene therapy in vivo.

**References**

10. Furth PA, Onge LS, Böger H, et al: Temporal control of gene...
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35. Samejima Y, Meruelo D: Bystander killing induces apoptosis and is inhibited by forskolin. Gene Ther 2:50–58, 1995

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