

# Tetracycline-regulated Gene Expression Mediated by a Novel Chimeric Repressor That Recruits Histone Deacetylases in Mammalian Cells\*

Received for publication, July 23, 2001, and in revised form, September 24, 2001  
Published, JBC Papers in Press, October 1, 2001, DOI 10.1074/jbc.M106924200

Wei Jiang<sup>‡</sup>, Lan Zhou<sup>§</sup>, Benjamin Breyer<sup>‡</sup>, Tao Feng<sup>§</sup>, Hongwei Cheng, Rex Haydon, Akira Ishikawa<sup>¶</sup>, and Tong-Chuan He<sup>||</sup>

From the Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, Illinois 60637, the <sup>§</sup>Department of Biochemistry and Molecular Biology, Chongqing University of Medical Sciences, Chongqing 400046, China, and the <sup>¶</sup>Department of Orthopaedic Surgery, Yamagata University School of Medicine, 2-2-2, Iida-Nishi, Yamagata 990-9585, Japan

Regulated gene expression will provide important platforms from which gene functions can be investigated and safer means of gene therapy may be developed. Histone deacetylases have recently been shown to play an important role in regulating gene expression. Here we investigated whether a more tightly controlled expression could be achieved by using a novel chimeric repressor that recruits histone deacetylases to a tetracycline-responsive promoter. This chimeric repressor was engineered by fusing the tetracycline repressor (TetR) with an mSin3-interacting domain of human Mad1 and was shown to bind the *tetO*<sub>2</sub> element with high affinity, and its binding was efficiently abrogated by doxycycline. The chimeric repressor was shown to directly interact with mSin3 of the histone deacetylase complex. This inducible system was further simplified by using a single vector that contained both a chimeric repressor expression cassette and a tetracycline-responsive promoter. When transiently introduced into mammalian cells, the chimeric repressor system exhibited a significantly lower basal level of luciferase activity (up to 25-fold) than that of the TetR control. When stably transfected into HEK 293 cells, the chimeric repressor system was shown to exert a tight control of green fluorescent protein expression in a doxycycline dose- and time-dependent fashion. Therefore, this novel chimeric repressor provides an effective means for more tightly regulated gene expression, and the simplified inducible system may be used for a broad range of basic and clinical studies.

The ability to regulate the expression of a gene of interest in mammalian cells possesses unlimited potential in both basic science and clinical medicine (1, 2). Earlier inducible expression systems were primarily based on endogenous elements that responded to exogenous signals or stresses, such as heat shock (3, 4), hormones (5–8), or metal ions (9–11). A common problem associated with these systems was their low specificity caused by the pleiotropic effects of their inducers. Higher spec-

ificity of gene induction was achieved by using nonmammalian or mutated endogenous control elements in several inducible systems, including the *lac* repressor/operator, FK506/rapamycin, ecdysone-inducible, RU486/mifepristone, and tetracycline (Tc)<sup>1</sup>-inducible systems (12–26). The Tc-inducible system is derived from the gene regulation features of Tc resistance determinants in Gram-negative bacteria (27). Each Tc determinant consists of a resistance protein (*e.g.* TetB) and a regulatory/repressor protein (*i.e.* TetR). The TetR protein regulates the expression of seven Tc resistance proteins. In the absence of Tc, TetR is bound to the *tet* operon (*tetO*<sub>2</sub>). In the presence of Tc, TetR binds to Tc allosterically and rapidly dissociates from *tetO*<sub>2</sub>, allowing expression of Tc resistance proteins (28–35). The best characterized TetR protein is a repressor for the resistance protein TetB, or TetR(B) (or TetR, thereafter) (36). Gene expression in eukaryotes regulated by TetR was first demonstrated in plant cells (37). Subsequently, Tc-responsive promoters were developed for regulated gene expression in mammalian cells (19, 38), which have been proven suitable for both *in vitro* and *in vivo* gene expression (39–41). Despite significant improvements in the Tc-inducible systems (2, 36, 42), two significant shortcomings have limited their use in many circumstances. First, they usually exhibit a significant level of basal expression. This “leakiness” makes it difficult to establish inducible clones expressing toxic genes, tumor suppressor genes, or genes involved in apoptosis. Second, they require two-stage transfections to establish stable inducible lines. This can be problematic because clonal variations may affect functional outcomes of a given gene.

Recently, significant progress has been made in understanding eukaryotic gene regulation in the context of chromatin. Activation and repression of gene expression correlates with the acetylation state of histones (43–46). Acetylated histones are correlated with active gene expression, whereas deacetylated histones are correlated with repressed gene expression. Recently, enzymes that carry out histone acetylation (histone acetyltransferases) and deacetylation (histone deacetylases, or HDACs) have been identified. Transcription activators are often associated with histone acetyltransferases, and repressors can interact with HDACs, leading to local chromatin modifica-

\* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>‡</sup> These authors contributed equally to this work.

<sup>||</sup> To whom correspondence should be addressed: Molecular Oncology Laboratory, The University of Chicago Medical Center, 5841 S. Maryland Ave., MC 3079, Rm. J-611, Chicago, IL 60637. Tel: 773-702-7169; Fax: 773-834-4598; E-mail: tche@surgery.bsd.uchicago.edu.

<sup>1</sup> The abbreviations used are: Tc, tetracycline; GFP, green fluorescent protein; HDAC, histone deacetylase; SID, mSin3 interaction domain; TetR, Tc repressor; *tetO*<sub>2</sub>, Tc operator site; THE, Tc-controlled heterologous expression; TRSID, chimeric TetR composed of TetR and SID; IRES, internal ribosome entry site; CMV, cytomegalovirus; GST, glutathione S-transferase; tTA, Tc-controlled transactivator; rtTA, reverse tTA; PA, polyadenylation signal.

tion of specific genes (47–50). One of the most important HDACs is conferred by the mSin3 complex, which is comprised of at least seven subunits (51, 52). The Mad-Max complex has been shown to repress gene expression via an association with the mSin3 complex (53). The Myc/Max/Mad network comprises a group of transcription factors whose distinct interactions result in gene-specific transcriptional activation or repression (54). By forming heterodimers, the Myc-Mad complex recruits co-activators and activates E-box-containing promoters, whereas the Mad-Max heterodimer functions as a transcriptional repressor (54, 55). Recently, Mad1-mediated repression has been shown to require the formation of a ternary complex with Max and mSin3. The mSin3 interaction domain (SID) has been mapped to the N-terminal 35 amino acid residues of human Mad1 protein (56).

To test whether a chimeric TetR repressor that recruits HDACs can confer a tight regulation of gene expression, we engineered a hybrid repressor, namely TRSID, by fusing the TetR with a SID derived from human Mad1 protein (56). TRSID was shown to retain *tetO<sub>2</sub>* binding ability and Tc inducibility and was also shown to directly interact with the mSin3-HDAC complex. By using a single vector system that contained both a chimeric repressor expression cassette and a Tc-responsive promoter, we have demonstrated that the chimeric repressor exhibited significantly tighter regulation of gene expression, and its repression was efficiently relieved by doxycycline. This tightly controlled gene expression system should be valuable for a wide range of gene function studies and clinical applications.

#### EXPERIMENTAL PROCEDURES

**Cell Culture, Medium, and Chemicals**—Human embryonic kidney cell line HEK 293, human colon cancer line HCT116, and human osteosarcoma line 143B were purchased from the American Type Culture Collection (Manassas, VA). 293 cells were maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Mediatech), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO<sub>2</sub>. HCT116 cells were maintained in McCoy's 5A (Mediatech) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO<sub>2</sub>. 143B cells were maintained in Eagle's minimal essential medium (Mediatech) supplemented with 10% fetal bovine serum, 2.0 mM L-glutamine, 1× nonessential amino acids (Mediatech), 1.0 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (Mediatech) at 37 °C in 5% CO<sub>2</sub>. Unless otherwise indicated, all chemicals were purchased from Sigma.

**Construction of the Geneticin-selectable Expression Cassettes for TetR and the Chimeric Repressor TRSID**—Both TetR and TRSID expression cassettes were constructed by multiple subcloning steps. Details of cloning procedures are available upon request. Briefly, the TetR coding sequence (underlined) was amplified by polymerase chain reaction with the following pair of oligonucleotides: 5'-CCGCTCGAGATGTCTAGATAGATAAAAAGTAAAG-3' and 5'-CATGCATGCTTAGGACCACTTTCACATTTAAGTTG-3'. The amplified fragment was cloned into pSL301 (Invitrogen) at *Xho*I and *Sph*I sites, resulting in pSL-TetR. The SID was amplified with the following pair of oligonucleotides: 5'-GATCCATATCGCGATTAGAAAAACAACCTAAATGTGAAA GTGGGTCCGGACCGGTATGGCGGCGCGGTTTCGGATG-3' and 5'-CGCGGATCCCTTAGTCCTTGTATTGTATGGTAAAC-3' (the TetR sequence is underlined, and the SID sequence is italicized). To construct the hybrid repressor TRSID, the amplified SID fragment was subcloned into pSL-TetR at the *Nde*I and *Bam*HI sites to replace the 3'-end of TetR sequence, resulting in pSL-TRSID. Subsequently, an internal ribosome entry site (IRES)-neomycin resistance cassette was added to the 3'-end of both TetR and TRSID to enable a bicistronic expression of TetR or TRSID, and a geneticin resistance selection marker in mammalian cells. The final expression cassettes for both TetR (CMV-TetR-IRES-Neo-SV40 PA) and TRSID (CMV-TRSID-IRES-Neo-SV40 PA) were flanked with *Mlu*I sites and were readily subcloned into the pCMV-TO4 vector (see below). All polymerase chain reaction-amplified DNA fragments were sequenced to verify their nucleotide sequence authenticity.

**Construction of a *tetO<sub>2</sub>*-containing CMV Promoter and pTRE and pTHE Vectors**—To engineer a *tetO<sub>2</sub>*-containing CMV promoter that is

responsive to TetR or the chimeric repressor TRSID, we designed two overlapping oligonucleotides that contained four copies of the *tet* operator site *tetO<sub>2</sub>* (underlined) and multiple cloning sites (italicized): 5'-G-CAGAGCTCTCCCTATCAGT GATAGAGATCTCCCTATCAGTGATA-GAGATCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAG-ATCGAGCTGTTTGTAGTGAACCGTCAG-3' and 5'-CGAGCGCGCCGTCGAGTTAACCGCGGACCGGTCGACAGGCGCTGAATTCGGTGTCT-TCTATGGAGGTCAAACAGCGTGGATGGCGTCTCCAGGCGATCT-GACGGTTCACTAAACAGC-3'. Two oligonucleotides were denatured, annealed, and extended with Platinum *Taq* (Life Technologies, Inc.) for one cycle to generate a cassette that was subsequently cloned downstream to a CMV promoter, resulting in pCMV-TO4. In this vector, the first *tetO<sub>2</sub>* site was precisely positioned 10 nucleotides downstream from the TATA box of the CMV promoter (Fig. 1B). The cloned fragment was verified by DNA sequencing. Finally, the *Mlu*I fragment containing the TetR or TRSID expression cassette (see above) was subcloned into the pCMV-TO4 vector, generating pTRE and pTHE, respectively. Other vectors were also constructed, including pTRE-GFP, pTHE-GFP, pTRE-Luc, and pTHE-Luc. DNA sequences and maps for pTRE and pTHE vectors are available at the following website: mywebpage.netscape.com/ucmolab/home.html.

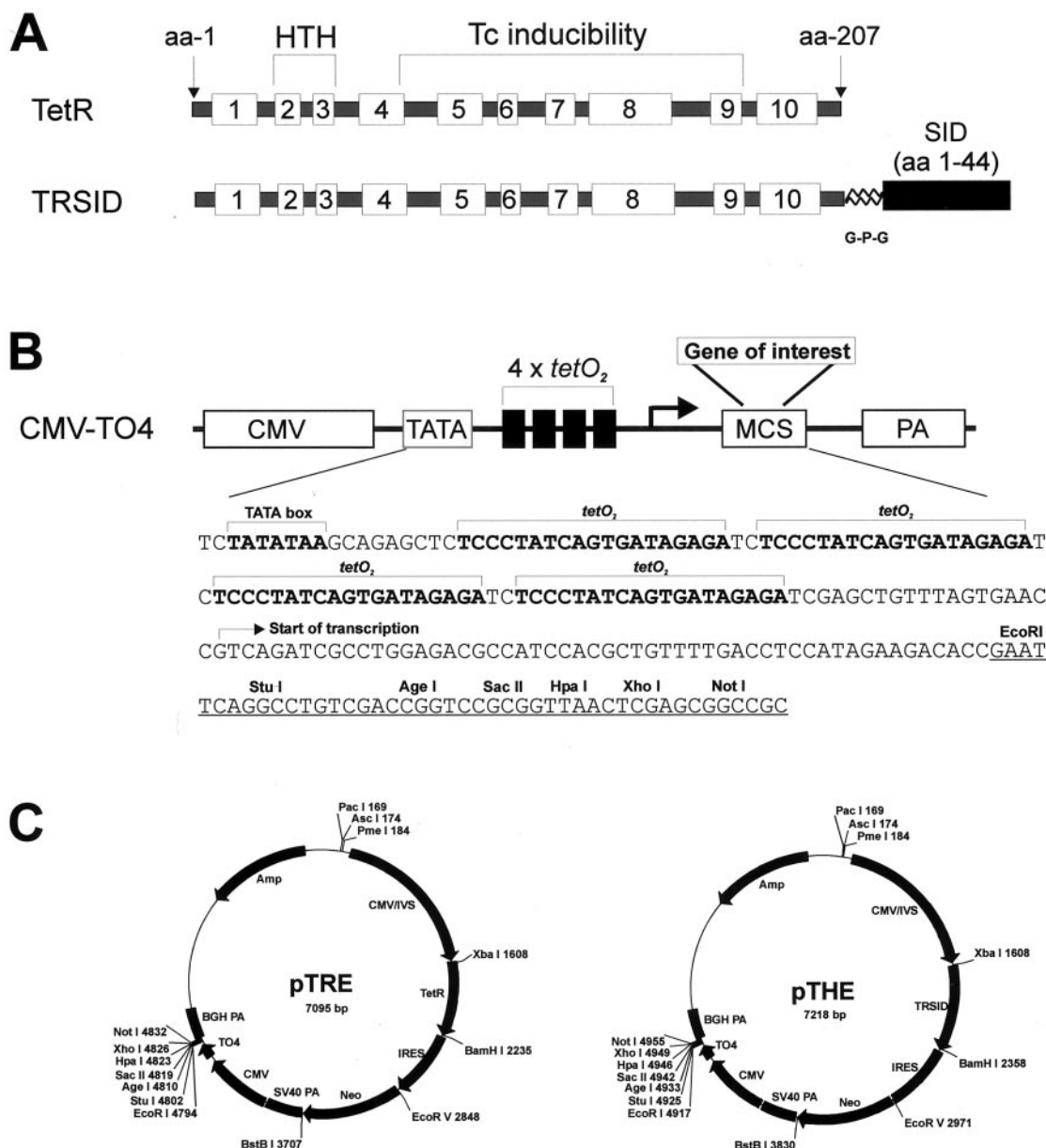
**Construction and Purification of GST Fusion Proteins**—For the construction of GST fusion proteins with TetR or the chimeric repressor TRSID, the coding sequences of TetR or TRSID were subcloned into pGEX-3X (Amersham Pharmacia Biotech) at a filled-in *Eco*RI site, resulting in pGST-TetR and pGST-TRSID, respectively. The cloning junctions were verified by DNA sequencing. Purification of GST fusion proteins was performed essentially as described previously (57). Briefly, exponentially growing BL21 bacterial cells that contained GST fusion vectors were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C. Cells were collected and lysed by sonication. GST fusion proteins were purified by using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and eluted with reduced glutathione (10 mM, Sigma). The purified proteins were quantified by using a BCA-200 Protein Assay kit (Pierce) and kept at -80 °C.

**Electrophoretic Mobility Shift Assays**—DNA binding assays were performed essentially as described previously (57). Briefly, each DNA binding assay was supplemented with 0.1–0.2 µg of GST fusion proteins; a nonspecific competitor, poly(dI-dC) (6 µg/ml); and 0.5 ng of <sup>32</sup>P-labeled probe (≈10<sup>6</sup> dpm). For competition assays, a 50-fold excess of unlabeled probe was added. The *tetO<sub>2</sub>* probe was formed by annealing 5'-CTCCCTATCAGTGATAGAGAT-3' and 5'-ATCTCTATCACTGATAGGGAG-3'. To test Tc inducibility, various amounts (ranging from 0.01 to 10.0 ng) of doxycycline were added to the DNA binding assays.

**GST Fusion Protein Pull-down Assay and Western Blotting Analysis**—Exponentially growing 293 cells were collected and lysed in a cell lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% Nonidet P-40) containing protease inhibitors (Roche Molecular Biochemicals). The cleared cell lysate was incubated with 0.5 mg of GST-TetR or GST-TRSID proteins at 4 °C for 60 min. Next 100 µl of a 50% slurry glutathione-Sepharose 4B (Amersham Pharmacia Biotech) was added to the GST fusion protein mixtures. As a control, an equal volume of cleared lysate was incubated with 100 µl of a 50% slurry of glutathione-Sepharose 4B. After 30 min of incubation at 4 °C, the protein-bound glutathione-Sepharose 4B was washed with phosphate-buffered saline three times. Bound proteins were eluted by boiling the samples in Laemmli sample buffer and were loaded onto a 4–20% gradient SDS-polyacrylamide gel. After being resolved by electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore) via electroblotting. The membrane was blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) at room temperature for 1 h and probed with a rabbit anti-mSin3A antibody (Santa Cruz Biotechnology) for 60 min followed by a 30-min incubation with anti-rabbit IgG conjugated with horseradish peroxidase (Pierce). The presence of mSin3 protein was detected by using the SuperSignal West Pico chemiluminescent substrate kit (Pierce).

**Luciferase Assays**—Exponentially growing cells were seeded in 12-well cell culture plates for 4 h and transfected with 0.2 µg of a luciferase reporter plasmid and 0.2 µg of pCMV-β per well using LipofectAMINE (Life Technologies, Inc.). At 15 h after transfection, doxycycline (100 ng/ml) was added to the transfected cells. At 48 h after induction, cells were lysed and collected for assays of luciferase activity using the Luciferase Assay kit (Promega). Each assay condition was done in triplicate, and transfection efficiency was normalized by β-galactosidase assays.

**Establishment of Stable Inducible Lines Using pTHE-GFP**—HEK 293 cells were plated in a 25-cm<sup>2</sup> flask and transfected with 2 µg of *Pac*I-linearized pTHE-GFP plasmid DNA by using LipofectAMINE



**FIG. 1. Schematic representation of TetR, TRSID, a Tc-responsive expression cassette, and the chimeric repressor-containing pTHE vector.** A, structures of TetR and chimeric repressor TRSID. Ten  $\alpha$ -helices are numbered. Helices 2 and 3 are responsible for  $tetO_2$  DNA binding activity, and helices 4–9 are required for Tc binding. aa, amino acid; HTH, helix-turn-helix domain. B, the  $tetO_2$ -containing CMV-driven expression cassette (see text for detail). MCS, multiple cloning sites. C, construction of the chimeric repressor-containing pTHE inducible system. The single vector pTRE was constructed (pTRE was used as a control). See “Experimental Procedures” for details. Full-length sequences and vector maps are available at [mywebpage.netscape.com/ucmolab/home.html](http://mywebpage.netscape.com/ucmolab/home.html).

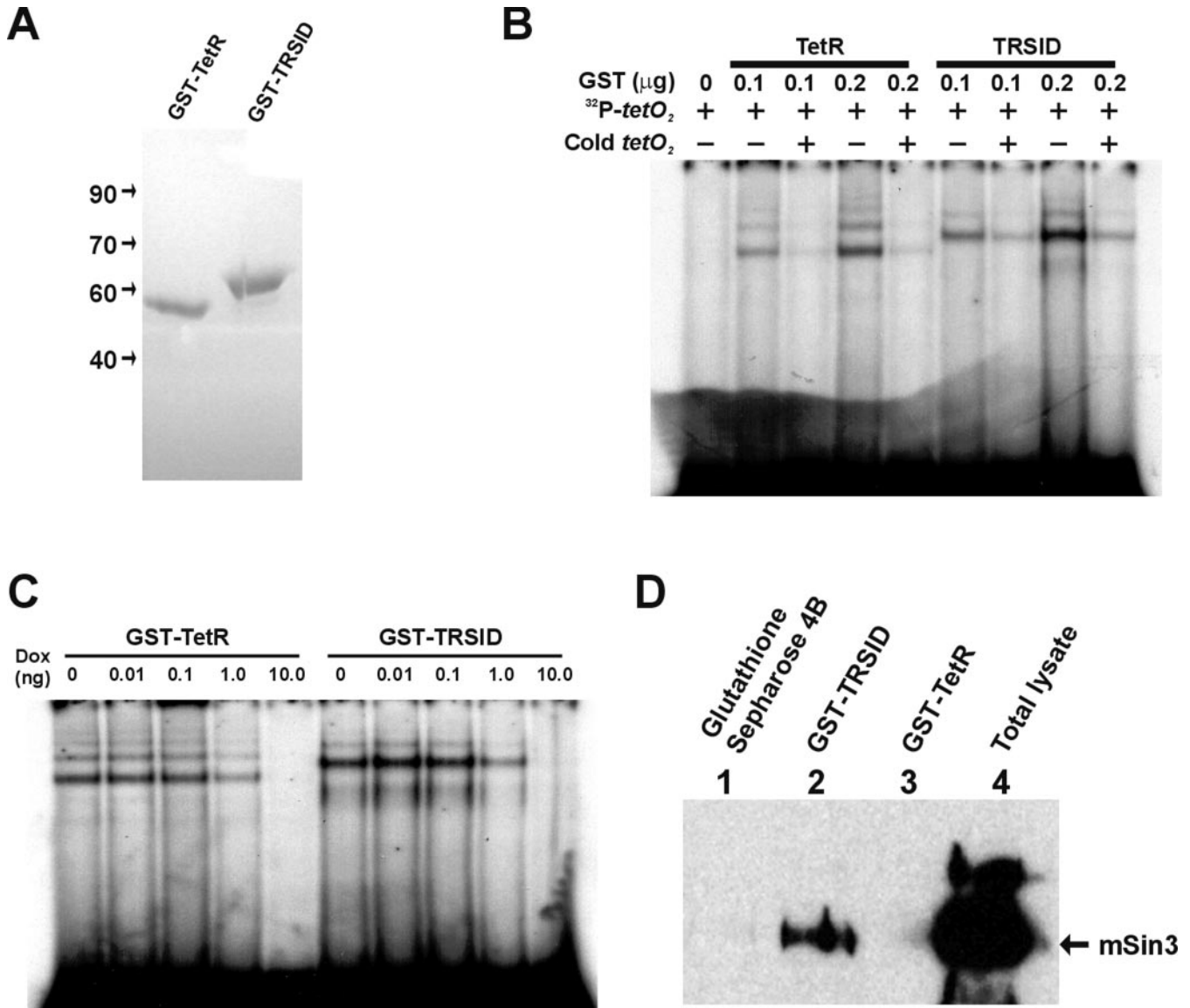
(Life Technologies, Inc.). At 24 h after transfection, cells were replated into 96-well plates with various densities and selected in G418 (0.3 mg/ml) for 2 weeks. Clones derived from single cells were grown up for further analysis.

## RESULTS

**Construction of the Chimeric Repressor TRSID**—Controlled gene expression is important for gene function studies and therapeutic applications. Despite improvements, current Tc-inducible systems continue to be limited by two factors: high basal level of expression and inconvenient two-stage establishment of inducible lines (2, 36). In an attempt to reduce the basal level of expression, we constructed a hybrid repressor, TRSID, which contained the full-length TetR fused with a well characterized SID (amino acids 1–44) derived from human Mad1 protein (Fig. 1A). A small linker sequence, Gly-Pro-Gly, was introduced between the two moieties. It has been demon-

strated that the SID is necessary and sufficient to recruit histone deacetylases to the vicinity of the transcription start site and to repress transcription activity (56). Proper configuration of the chimeric repressor TRSID was verified by DNA sequencing. The prototypic TetR was included as a control for our studies (Fig. 1A). To engineer a TRSID-mediated repression system, we introduced four copies of the canonical  $tetO_2$  site in the context of a CMV promoter. To maximize the potential suppressive effects of the repressor proteins, we placed the first copy of the  $tetO_2$  site precisely 10 base pairs downstream of the TATA box (Fig. 1B).

**Functional Characterization of the Chimeric TRSID Repressor**—We next tested whether the hybrid repressor TRSID retained its ability to bind the  $tetO_2$  element. To facilitate these assays, we constructed a GST fusion protein, GST-TRSID, as well as GST-TetR for a control (Fig. 2A). As indicated in elec-



**FIG. 2. Functional characterization of the chimeric TRSID repressor.** *A*, expression and purification of GST fusion proteins, GST-TetR and GST-TRSID. About 2 μg of GST fusion proteins were resolved by 10% polyacrylamide gel electrophoresis and stained with Coomassie BR-250. *B*, electrophoretic mobility shift assays of TetR and chimeric TRSID. GST fusion proteins were incubated with <sup>32</sup>P-labeled *tetO*<sub>2</sub> probe formed by two complementary oligonucleotides in the presence or absence of a 50-fold excess of unlabeled *tetO*<sub>2</sub> cassette. *C*, effect of doxycycline on the DNA binding ability of TetR and TRSID. 0.2 μg of GST fusion proteins were incubated with a <sup>32</sup>P-labeled *tetO*<sub>2</sub> probe in the presence of the indicated amount of doxycycline (see text for details). *D*, interaction of the chimeric repressor with mSin3 of the HDACs. Total cell lysate was prepared from the exponentially growing 293 cells and was incubated with 0.5 mg of GST-TetR or GST-TRSID proteins at 4 °C for 60 min followed by incubation with glutathione-Sepharose 4B (Amersham Pharmacia Biotech). As a control, an equal volume of cleared lysate was incubated with glutathione-Sepharose 4B. The protein-bound glutathione-Sepharose 4B was eluted by boiling the samples in Laemmli sample buffer and resolved on a 4–20% gradient SDS-polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane (Millipore) via electroblotting and probed with a rabbit anti-mSin3A antibody (Santa Cruz Biotechnology). The presence of mSin3 protein was detected by using the SuperSignal West Pico chemiluminescent substrate kit (Pierce).

trophoretic mobility shift assays, the *tetO*<sub>2</sub> binding capability of the chimeric repressor TRSID was not affected by the fusion construction (Fig. 2*B*). Interestingly, the binding of TRSID to the *tetO*<sub>2</sub> element was reproducibly shown to be slightly stronger than that of the control TetR protein. Reasons for the enhanced binding ability of TRSID are not known.

Previous studies have indicated that although mutations in other regions also significantly affect its Tc-induced allosteric conformational changes, Tc inducibility is mostly determined by the carboxyl portion of the TetR protein (28–35). To assess whether the chimeric TRSID retained this inducibility, we included doxycycline, a water-soluble and more stable analogue of Tc, in the electrophoretic mobility shift assay reactions. As illustrated in Fig. 2*C*, addition of doxycycline at a concentration

as low as 1.0 ng/ml significantly reduced the *tetO*<sub>2</sub> binding ability of TRSID protein, which was completely abolished when 10 ng of doxycycline was used. There was no significant difference in the inducibility between control TetR and TRSID proteins (Fig. 2*C*). Taken together, these observations demonstrate that the chimeric TRSID repressor exhibited similar biochemical features to those of the control TetR.

We next tested whether the TRSID directly interacted with the mSin3 complex. Total cell lysate prepared from 293 cells was incubated with ~0.5 mg of GST-TetR or GST-TRSID followed by an incubation with glutathione-Sepharose 4B. As a control for nonspecific binding to the beads, total cell lysate was also incubated with glutathione-Sepharose 4B. Bound proteins were eluted and subjected to Western blotting analysis. The

TABLE I  
Comparison of doxycycline (Dox)-induced luciferase activity using pTRE and pTHE inducible vectors

Cell line	TRE-Luc		THE-Luc	
	-Dox	+Dox	-Dox	+Dox
143B				
Mean (S.D.)	36,265 (7,985)	159,997 (47,241)	1,391 (306)	83,288 (15,789)
-Fold induction		4.4		59.9
-Fold decrease in basal level				26.1
HCT116				
Mean (S.D.)	133,589 (34,448)	504,385 (57,917)	39,551 (6,229)	440,728 (29,188)
-Fold induction		3.8		11.1
-Fold decrease in basal level				3.4
HEK 293				
Mean (S.D.)	249,950 (31,907)	5,534,640 (1,570,912)	125,756 (13,385)	441,524 (65,350)
-Fold induction		22.1		3.5
-Fold decrease in basal level				2

presence of mSin3 was probed with an anti-mSin3A antibody. As shown in Fig. 2D, a specific interaction between mSin3 and TRSID was readily detected.

**Construction of pTHE Inducible Vector That Contained the Chimeric Repressor**—To circumvent the requirement of a two-stage transfection to establish Tc-inducible lines, we developed a single system, namely the Tc-controlled heterologous expression (THE) system (Fig. 1C). Specifically, in the pTHE system, a single vector contains two independent expression cassettes. The first one contains a TetR- or TRSID-responsive expression cassette, and the second cassette contains a CMV-driven bicistronic expression of a repressor protein (e.g. TetR or TRSID) and a G418 selection marker (Neo<sup>r</sup>), which was mediated by an IRES. The bicistronic configuration ensures a concordant expression of the repressor protein in G418-resistant cells. To further facilitate establishment of stable cells, octanucleotide-recognizing restriction endonuclease sites of *PacI*, *PmeI*, and *AscI* were engineered for construct linearization. It is expected that expression of a transgene would be suppressed by repressor TetR or TRSID and activated in the presence of Tc or doxycycline. Depending on the repressor, resultant vectors were designated as pTRE (i.e. TetR as a repressor) and pTHE (i.e. TRSID as a repressor), respectively (Fig. 1C).

**Controlled Expression of Luciferase by the Chimeric Repressor**—To initially assess whether gene regulation was under a tight control in the pTHE vector, we constructed two reporter plasmids, pTHE-Luc and a control reporter, pTRE-Luc, both of which contained a gene encoding luciferase under the control of a *tetO*<sub>2</sub>-bearing CMV promoter. Upon introduction of reporter vectors into three mammalian cells (HEK 293, HCT116, and 143B), expression of luciferase was induced by doxycycline. Luciferase activity was assayed 48 h after induction. As summarized in Table I, the luciferase activity of both pTRE-Luc and pTHE-Luc-transfected cells was significantly induced by doxycycline in all three tested cell lines. Levels of induction were higher in 293 cells for pTRE-Luc (approximately 22-fold) and 143B cells for pTHE-Luc (approximately 60-fold). Moreover, a significant decrease of basal luciferase activity was documented in all the pTHE-Luc-transfected cell lines. The greatest reduction of basal activity was found in the 143B cells (approximately 26-fold decrease) when the luciferase activity of the uninduced state in pTRE-Luc- and pTHE-Luc-transfected cells was compared. It is noteworthy that the maximum activity induced by doxycycline seemed to be lower in the pTHE-Luc-transfected cells (especially in 143B and 293 cells). This result is consistent with that observed using the pTHE-GFP inducible vector (see below). Nevertheless, these quantitative data strongly suggest that the chimeric repressor drastically reduced the basal level of expression. Interestingly, the magnitude of induction and the fold of reduction in basal activity exhibited significant variations among the three cell lines

tested. Although the actual causes of these differences remain undefined, there may be two contributing factors. First, it seemed that a higher basal level of luciferase activity was directly related to higher transfection efficiency. We observed that among the three lines HEK 293 cells exhibited the highest efficiency of transfection and 143B cells exhibited the lowest. Second, the advantage of the chimeric repressor system may not be fully exploited in transient expression assays. Mechanistically, it is expected that the basal activity of pTHE system would be more significantly suppressed by the HDACs once the inducible vectors stably integrate into the host chromosomes.

**Regulated Expression of Green Fluorescent Protein by the Chimeric Repressor**—To further test the efficacy and kinetic features of the pTHE inducible system in mammalian cells, we constructed two GFP reporter vectors: pTHE-GFP and pTRE-GFP. After the vectors were transiently introduced into HEK 293 cells, expression of GFP was induced by doxycycline (at 100 ng/ml). As shown in Fig. 3A, GFP expression was readily detectable at 48 h after induction in both pTHE-GFP- and pTRE-GFP-transfected cells. However, the basal level of GFP expression was significantly higher in the pTRE-GFP-transfected cells than in the pTHE-GFP-transfected cells, strongly suggesting that the chimeric repressor TRSID indeed enhances the repression of basal expression on the *tetO*<sub>2</sub>-responsive promoter.

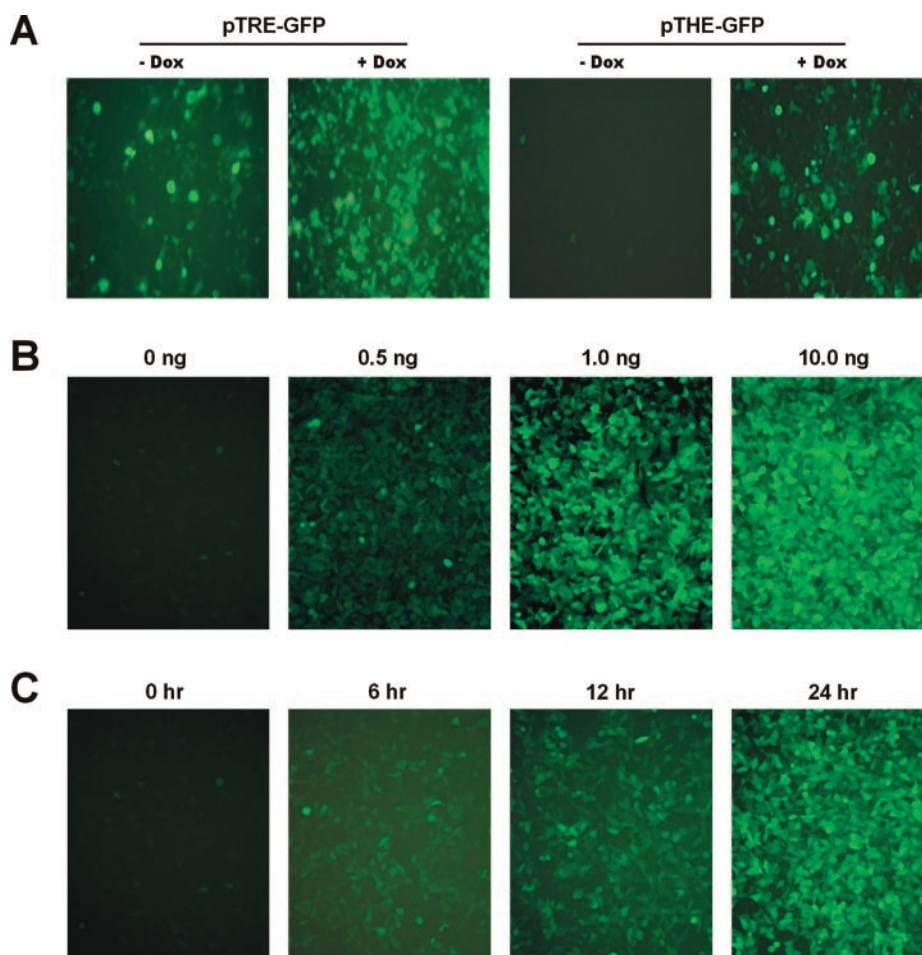
To further assess the kinetic characteristics of gene expression in the THE inducible system, we generated multiple HEK 293 clones that were stably transfected with the pTHE-GFP vector. We observed that on average about 10–20% of the stable clones derived from single cells were tightly regulated by doxycycline, and the tightly controlled expression was well retained in the cells recovered from cryogenic preservation. As illustrated in Fig. 3B, GFP expression was induced in a doxycycline dose-dependent manner. Apparent GFP expression was induced by doxycycline at a concentration as low as 0.5 ng/ml. Like most Tc-inducible systems, the dosage range of induction was fairly narrow (less than 10-fold) because GFP expression was induced close to the maximum level at 1.0 ng/ml doxycycline. In a time course study, we observed that appreciable GFP expression could be induced as early as 6 h but that the maximum level of induction was achieved at 24 h after induction (Fig. 3C). Thus, our collective data have demonstrated that the pTHE inducible system can tightly regulate gene expression in a Tc-dependent fashion and that the chimeric TRSID repressor significantly reduces the basal level of expression from a *tetO*<sub>2</sub>-containing CMV promoter.

#### DISCUSSION

The arrival of the postgenomic era mandates the development of tightly regulated expression technologies for gene function studies as well as gene therapy. As one of the most com-

**FIG. 3. Tightly controlled GFP expression by the chimeric repressor.**

**A**, comparison of induced GFP expression in pTHE and pTRE vectors. HEK 293 cells were transiently transfected with pTHE-GFP or pTRE-GFP vectors and induced with doxycycline (100 ng/ml). GFP expression was recorded at 48 h after induction. **B**, dose-dependent induction of GFP expression with the pTHE-GFP vector. A stable pTHE-GFP line derived from 293 cells was induced with doxycycline at the indicated concentrations. GFP expression was recorded at 24 h after induction. **C**, time course induction of GFP expression with the pTHE-GFP vector. A stable THE-GFP line derived from 293 cells was induced with doxycycline (5 ng/ml). GFP expression was recorded at the indicated hours after induction.



monly used methods, conventional Tc-inducible systems usually exhibit a significant basal level of expression and require a two-stage procedure to establish stable inducible lines (2, 58). To overcome these shortcomings, we engineered a tightly controlled gene expression (pTHE) system mediated by a chimeric repressor that recruits histone deacetylases. The development of the pTHE inducible vector has conceptually benefited from recent progress in our understanding of chromatin remodeling and transcriptional regulation. Recent studies have demonstrated that the acetylation status of histones is a key determinant of transcriptional activity. Transcriptional activators are often associated with histone acetyltransferases and repressors interacting with HDACs. Recruitment of HDACs to specific promoter regions plays a major role in silencing gene expression (43–46). To exploit the gene silencing function of HDACs, we created a hybrid repressor by fusing the TetR with an mSin3 interaction domain of human Mad1 repressor (56). As assessed by electrophoretic mobility shift assays, the chimeric repressor TRSID was shown to retain its ability to specifically bind the *tetO*<sub>2</sub> element with high affinity. Furthermore, the TRSID binding to *tetO*<sub>2</sub> was dramatically reduced or abolished in a doxycycline dose-dependent fashion, suggesting that the chimeric repressor retained Tc inducibility. To avoid the conventional two-step establishment of stable lines, we constructed a single vector system, namely the pTHE vector, as well as a control vector, pTRE. Quantitative analyses using luciferase as a reporter demonstrated that the chimeric TRSID repressor significantly suppressed the basal level of expression. Accordingly, GFP expression in pTHE-GFP system is tightly regulated by doxycycline in a time course- and dosage-dependent manner. It is noteworthy that the maximum level of

gene expression was slightly lower in pTHE-GFP-transfected cells than that in pTRE-GFP-transfected cells. For reasons yet to be understood, this may reflect the fact that the chimeric repressor may bind *tetO*<sub>2</sub> with a higher affinity as suggested by the *in vitro* electrophoretic mobility shift assay results. Nevertheless, the Tc-induced expression of transgenes should remain high enough for most *in vitro* or *in vivo* studies.

Since Bujard *et al.* (19, 38) developed the prototypic Tc-inducible system for mammalian cells by converting TetR to a Tc-controlled transactivator (tTA), several modifications have been made. For instance, Shockett *et al.* (59) developed an autoregulatory tTA production for a Tc-inducible system that functioned in cultured cells and transgenic mice. In a series of studies, Blau and colleagues (40, 42, 60) demonstrated that a fine-tuned transcriptional regulation could be achieved by a binary Tc-regulatable retroviral system containing both transactivator tTA and transrepressor rtTA. Urlinger *et al.* (61) conducted a comprehensive mutagenic analysis and optimized the rtTA protein with a broader range of regulation and higher sensitivity. To avoid the potential toxicity associated with the VP16 transactivation domain, Akagi *et al.* (62) have recently constructed a novel Tc-dependent transactivator with an E2F4 transcriptional activation domain. On the contrary, several groups have explored the silencing potential of the TetR in mammalian cells (63–66).

The development of the THE inducible system has further exploited the potent silencing function of gene expression mediated by histone deacetylases. Thus, our THE inducible system should be superior to the current Tc-inducible system in the following areas. 1) It does not alter the intrinsic biochemical function of the prototypic TetR, which is to suppress gene

expression. 2) Strong exogenous transactivators (e.g. VP16), which may cause cellular toxicity due to overexpression, are not required for gene regulation. 3) It is more feasible to construct a single vector for a repressor-mediated inducible system because the close proximity of repressor and its responsive element would less likely interfere with each other and hence should not compromise the tight regulation of gene expression. Our findings have demonstrated that the THE inducible system is tightly regulated by doxycycline and exhibits significantly lower leakiness. This feature is particularly important for studies in which controlled expression of toxic genes, tumor suppressor genes, and apoptosis-inducing genes is desired. Moreover, the single vector system facilitates the establishment of inducible lines and minimizes the possible phenotypic aberrations caused by clonal variations. Thus, this inducible system should be highly valuable for a broad range of basic and clinical studies.

**Acknowledgments**—We thank Zita Dubauskas, Donald Vander Griend, Daniel Cahill, and Andrea Deyrup for critical comments on this manuscript. We also thank Dr. Nicole Purcell for expert assistance on fluorescence microscopy. We are grateful to Drs. Michael A. Simon, Terrance Peabody, and Carrie Rinker-Schaeffer of The University of Chicago for encouragement and support.

## REFERENCES

- Saez, E., No, D., West, A., and Evans, R. M. (1997) *Curr. Opin. Biotechnol.* **8**, 608–616
- Rossi, F. M., and Blau, H. M. (1998) *Curr. Opin. Biotechnol.* **9**, 451–456
- Evgen'ev, M., Levin, A., and Lozovskaya, E. (1979) *Mol. Gen. Genet.* **176**, 275–280
- Schweinfest, C. W., Jorczyk, C. L., Fujiwara, S., and Papas, T. S. (1988) *Gene (Amst.)* **71**, 207–210
- Lee, F., Mulligan, R., Berg, P., and Ringold, G. (1981) *Nature* **294**, 228–232
- Hynes, N. E., Kennedy, N., Rahmsdorf, U., and Groner, B. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2038–2042
- Klock, G., Strahle, U., and Schutz, G. (1987) *Nature* **329**, 734–736
- Israel, D. I., and Kaufman, R. J. (1989) *Nucleic Acids Res.* **17**, 4589–4604
- Mayo, K. E., Warren, R., and Palmiter, R. D. (1982) *Cell* **29**, 99–108
- Brinster, R. L., Chen, H. Y., Warren, R., Sarthy, A., and Palmiter, R. D. (1982) *Nature* **296**, 39–42
- Searle, P. F., Stuart, G. W., and Palmiter, R. D. (1985) *Mol. Cell. Biol.* **5**, 1480–1489
- Takekoshi, M., Maeda-Takekoshi, F., Ihara, S., Sakuma, S., and Watanabe, Y. (1993) *J. Gen. Virol.* **74**, 1649–1652
- Maniatis, T., Goodbourn, S., and Fischer, J. A. (1987) *Science* **236**, 1237–1245
- Ko, M. S., and Takano, T. (1989) *DNA* **8**, 127–133
- Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A., and Rossant, J. (1989) *Development* **105**, 707–714
- Hu, M. C., and Davidson, N. (1990) *Mol. Cell. Biol.* **10**, 6141–6151
- Zernik, J., Kream, B., and Twarog, K. (1991) *Biochem. Biophys. Res. Commun.* **176**, 1149–1156
- Fincato, G., Polentarutti, N., Sica, A., Mantovani, A., and Colotta, F. (1991) *Blood* **77**, 579–586
- Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5547–5551
- Natzle, J. E., Robertson, J. P., Majumdar, A., Vesenka, G. D., Enlow, B., and Clark, K. E. (1992) *Dev. Genet.* **13**, 331–344
- Gazit, G., Kane, S. E., Nichols, P., and Lee, A. S. (1995) *Cancer Res.* **55**, 1660–1663
- Mikulits, W., Chen, D., and Mullner, E. W. (1995) *Nucleic Acids Res.* **23**, 2342–2343
- No, D., Yao, T. P., and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3346–3351
- Lee, A. V., Weng, C. N., McGuire, S. E., Wolf, D. M., and Yee, D. (1997) *BioTechniques* **23**, 1062–1068
- Hoppe, U. C., Marban, E., and Johns, D. C. (2000) *Mol. Ther.* **1**, 159–164
- Wheeler, G. N., Hamilton, F. S., and Hoppler, S. (2000) *Curr. Biol.* **10**, 849–852
- Hillen, W., and Berens, C. (1994) *Annu. Rev. Microbiol.* **48**, 345–369
- Takahashi, M., Degenkolb, J., and Hillen, W. (1991) *Anal. Biochem.* **199**, 197–202
- Kaszycki, P., Guz, A., Drwiega, M., and Wasylewski, Z. (1996) *J. Protein Chem.* **15**, 607–619
- Smith, L. D., and Bertrand, K. P. (1988) *J. Mol. Biol.* **203**, 949–959
- Sizemore, C., Wissmann, A., Gulland, U., and Hillen, W. (1990) *Nucleic Acids Res.* **18**, 2875–2880
- Berens, C., Pfeleiderer, K., Helbl, V., and Hillen, W. (1995) *Mol. Microbiol.* **18**, 437–448
- Berens, C., Schnappinger, D., and Hillen, W. (1997) *J. Biol. Chem.* **272**, 6936–6942
- Orth, P., Cordes, F., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (1998) *J. Mol. Biol.* **279**, 439–447
- Orth, P., Schnappinger, D., Hillen, W., Saenger, W., and Hinrichs, W. (2000) *Nat. Struct. Biol.* **7**, 215–219
- Blau, H. M., and Rossi, F. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 797–799
- Gatz, C., and Quail, P. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1394–1397
- Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) *Science* **268**, 1766–1769
- Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10933–10938
- Kringstein, A. M., Rossi, F. M., Hofmann, A., and Blau, H. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13670–13675
- Bello, B., Resendez-Perez, D., and Gehring, W. J. (1998) *Development* **125**, 2193–2202
- Rossi, F. M., Kringstein, A. M., Spicher, A., Guicherit, O. M., and Blau, H. M. (2000) *Mol. Cell* **6**, 723–728
- Struhl, K. (1998) *Genes Dev.* **12**, 599–606
- Workman, J. L., and Kingston, R. E. (1998) *Annu. Rev. Biochem.* **67**, 545–579
- Luo, R. X., and Dean, D. C. (1999) *J. Natl. Cancer Inst.* **91**, 1288–1294
- Orphanides, G., and Reinberg, D. (2000) *Nature* **407**, 471–475
- Hassig, C. A., and Schreiber, S. L. (1997) *Curr. Opin. Chem. Biol.* **1**, 300–308
- Kuo, M. H., and Allis, C. D. (1998) *Bioessays* **20**, 615–626
- Johnson, C. A., and Turner, B. M. (1999) *Semin. Cell Dev. Biol.* **10**, 179–188
- Khochbin, S., Verdel, A., Lemerrier, C., and Seigneurin-Berny, D. (2001) *Curr. Opin. Genet. Dev.* **11**, 162–166
- Ng, H. H., and Bird, A. (2000) *Trends Biochem. Sci.* **25**, 121–126
- Ahringer, J. (2000) *Trends Genet.* **16**, 351–356
- Pazin, M. J., and Kadonaga, J. T. (1997) *Cell* **89**, 325–328
- Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000) *Annu. Rev. Cell Dev. Biol.* **16**, 653–699
- Schreiber-Agus, N., and DePinho, R. A. (1998) *Bioessays* **20**, 808–818
- Ayer, D. E., Laherty, C. D., Lawrence, Q. A., Armstrong, A. P., and Eisenman, R. N. (1996) *Mol. Cell. Biol.* **16**, 5772–5781
- He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) *Science* **281**, 1509–1512
- Gschwend, J. E., Fair, W. R., and Powell, C. T. (1997) *Prostate* **33**, 166–176
- Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6522–6526
- Rossi, F. M., Guicherit, O. M., Spicher, A., Kringstein, A. M., Fatyol, K., Blakely, B. T., and Blau, H. M. (1998) *Nat. Genet.* **20**, 389–393
- Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H., and Hillen, W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7963–7968
- Akagi, K., Kanai, M., Saya, H., Kozu, T., and Berns, A. (2001) *Nucleic Acids Res.* **29**, E23
- Kim, H. J., Gatz, C., Hillen, W., and Jones, T. R. (1995) *J. Virol.* **69**, 2565–2573
- Deuschle, U., Meyer, W. K., and Thiesen, H. J. (1995) *Mol. Cell. Biol.* **15**, 1907–1914
- Kim, H. J., and Kim, K. H. (1998) *Arch. Pharm. Res.* **21**, 320–325
- Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998) *Hum. Gene Ther.* **9**, 1939–1950.