Cell-type specificity of short-range transcriptional repressors

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Transcriptional repressors can be classified as short- or long-range, according to their range of activity. Functional analysis of identified short-range repressors has been carried out largely in transgenic *Drosophila*, but it is not known whether general properties of short-range repressors are evident in other types of assays. To study short-range transcriptional repressors in cultured cells, we created chimeric tetracycline repressors based on *Drosophila* transcriptional repressors Giant, *Drosophila* C-terminal-binding protein (dCtBP), and Knirps. We find that Giant and dCtBP are efficient repressors in *Drosophila* and mammalian cells, whereas Knirps is active only in insect cells. The restricted activity of Knirps, in contrast to that of Giant, suggests that not all short-range repressors possess identical activities, consistent with recent findings showing that short-range repressors act through multiple pathways. The mammalian repressor Kid is more effective than either Giant or dCtBP in mammalian cells but is inactive in *Drosophila* cells. These results indicate that species-specific factors are important for the function of the Knirps and Kid repressors. Giant and dCtBP repress reporter genes in a variety of contexts, including genes that were introduced by transient transfection, carried on episomal elements, or stably integrated. This broad activity indicates that the context of the target gene is not critical for the ability of short-range repressors to block transcription, in contrast to other repressors that act only on stably integrated genes.

Transcriptional repressors play critical roles in a variety of cellular and developmental processes, but the activities of these repressors are not well understood. These proteins can be classified as short- or long-range repressors, according to their range and selectivity of action (1, 2). As defined in *Drosophila* embryo assays, short-range repressors such as Snail, Giant, Krüppel, and Knirps must bind within 100 bp of activators to inhibit their activity. As a result, this type of repressor can specifically block individual enhancer elements, leaving other enhancer elements within a complex regulatory region free to engage the transcription apparatus (3–6). In contrast, long-range repressors such as Hairy can inhibit when bound anywhere within several kilobases of the transcriptional start site, resulting in simultaneous inhibition of multiple regulatory elements (2, 7). The mechanistic differences between long- and short-range repressions have not been elucidated, but different types of corepressors may underlie these two distinct modes of repression (8, 9).

Critical questions remain concerning the nature of short-range repression. Interaction with the C-terminal-binding protein corepressor is important for function of several short-range repressors (8), but it is not known whether all short-range repressors exhibit the same spectrum of activities or cofactor requirements. The action of short-range repressors has been studied both on chromosomally integrated reporter genes in the embryo and on transiently transfected reporters in cell culture systems (10–13), but no comparative study has determined whether these proteins exhibit different activities depending on the chromosomal state of the target gene.

Because of their local action, short-range transcriptional repressors may be useful for controlled regulation of gene expression. The regulation of inducible promoters via chimeric tetracycline repressor (TetR) proteins has attracted considerable interest for use in ectopic expression systems in cell culture (14–16), microbes (17, 18),

plants (19), and whole animals (20–24). In these systems, a chimeric protein consisting of the *Escherichia coli* TetR protein fused to an activation domain binds to promoters containing Tet response elements (TREs) (14, 25). On addition of tetracycline or doxycycline, the chimeric protein is released from the promoter and the gene is inactivated. TetR DNA-binding domains with reverse specificity have been developed to permit activation of target genes on addition of the drug (26–28). Although this system can be highly regulated, low-level basal expression can be a problem in the case of potentially toxic gene products (27, 29). To overcome this problem, higher specificity Tet DNA-binding domains have been recently developed (30, 31). Many endogenous genes accomplished tight regulation by the coordinated action of repressors and activators. To mimic such composite systems, a Tet repressor can be combined with a Tet activator to give repression and activation in the absence and presence of doxycycline, respectively. Such combined Tet-based activation/repression systems were recently developed for yeast (18) and mammalian systems (32–34). Most of these systems use the KRAB repressor domain (32–36). Whether KRAB repressors can work in nonvertebrate cell types has not been reported, however.

To investigate the properties of short-range repressors within the context of the Tet regulatory system, and to provide the basis for their use in a system of tight regulation of Tet-mediated gene expression, we created a panel of transcriptional repressors based on well characterized short-range repressors from *Drosophila*. The chimeric proteins show reproducible repression activity in the Tet system in a variety of cell types and on stably integrated or transiently introduced reporter genes. Compared with the mammalian Kid repressor, these repressors may be the preferred alternative for regulation of expression in some cell types and with certain transgene configurations.

Materials and Methods

Construction of Chimeric TetR Proteins. An *Xba*I Tet-controlled transcription activator (tTA) fragment from pRevTetoff with the Tet repressor fused to the VP16 activation domain was cloned into pBluescript SK (+) (Stratagene) to create pBStTA. DNA fragments encoding the transcriptional repressors were amplified by PCRs with the following primers, Knirps (amino acids 80–429): 5′GCGGCCGCGCG**CGT**CGCTCCAACTGGT-TCAAGATCCATTGTCTGC3′, 5′GCGGCCGCGCGCTTA-GACACACACGAATATTCCCCTCATGGCACT3′, Giant (a.a 2–389), 5′GCGGCCGCGCG**CTA**ATGCACGAGAAACT-CATGGCCGGGCAGTTCT3′, 5′GCGGCCGCGCGCTTA-CTCGTAGTATGCGGCATCCTTAACCTGGCT3′, dCtBP (a.a 5–353), 5′GCGGCCGCGCG**CTG**ATGATGCCGAAGCGT-TCGCGCATCGATGTCA3′, 5′GCGGCCGCGCGCTTACT-CACTTACTAGAAGTACTCCTTGTTGACGCAATT3′. Bold

letters indicate first residue of fusion protein, and underlined letters indicate C-terminal residues. PCR products were digested with *Bss*HII and ligated into pBStTA, yielding Tet-Knirps (SRA<u>RRS</u> to <u>VCV</u>), Tet-Giant (SRA<u>LMH</u> to <u>YYE</u>), and Tet-dCtBP (SRA<u>LMM</u> to <u>EYF</u>). Underlined residues indicate first three and last three amino acid residues from the repressor fused to the C terminus of Tet. To generate Tet-Stop, an array of four stop codons (5′<u>TAGTAAGTGAGTA</u>AAAGCTTACGAAAAA-CAATTACGGGTCT3′) was inserted after codon 213 of Tet by PCR-mediated mutagenesis (stop codon underlined). Tet-repressor fusions were inserted into pRevTet previously digested with *Xba*I to remove tTA-coding region, placing Tet-repressor sequences under the control of the human cytomegalovirus immediate early promoter. To generate *Drosophila* expression constructs, pActin5C-HisA (Invitrogen) was digested with *Kpn*I and *Xba*I and oligonucleotides 5′GAAGCTTGAATTC<u>CAACCAAAAT</u>GT3′ and 5′CTAGA<u>CATTTTGGTT</u>GGAATTCAAGCTTCGTAC3′ with a consensus *Drosophila* Kozak sequence and translation start codon sequence (underlined), were inserted yielding pAX. Tet chimeric genes were inserted into the *Xba*I site of pAX, placing them under *Drosophila* actin 5C promoter. pRevTetoff, pRevTRE-Luc, ptTS-Kid, and pCMVTRE-Luc were from CLONTECH. Plasmids were purified by using Qiagen (Chatsworth, CA) columns.

Cell Culture Conditions. HeLa cells and HeLa X1/6 (H. Bujard, Heidelberg University) were grown in DMEM containing 10% FBS (GIBCO/BRL). HeLa pCEPTetPLuc cells (U. Rodeck, Thomas Jefferson University, Philadelphia) were grown in DMEM supplemented with 10% FBS and 100 μg/ml of Hygromycin B. INS-1, rat insulinoma β cells, were cultured in RPMI medium 1640 with 10% FBS, sodium pyruvate and β-mercaptoethnaol, as described (37). *Drosophila* S2 cells (American Type Culture Collection) were maintained at 22–24°C in Schneider *Drosophila* medium (GIBCO/BRL) supplemented with 10% FBS.

Transient Transfection and Luciferase Assays. HeLa cells were seeded at 2×10^5 cells, and INS-1 cells were seeded at 1.5×10^6 cells per well on six-well plates. Transfection mixtures contained 4 ml of serum-free media, 4 μg of reporter plasmid, 2 μg of Tet chimeric construct, and 12 μl of Lipofectamine (2 mg/ml, GIBCO/BRL) and were incubated for 30 min at room temperature to form DNA–liposome complexes. Cells were washed with 2 ml of serum-free media twice, and 1 ml of the transfection mixture was added to each well and incubated for 4 h. The medium was then replaced with 3 ml of fresh complete medium with or without 1 μg/ml of doxycycline (Sigma), cells were incubated for 24 h, medium was replaced and cells incubated an additional 24 h. Luciferase assays were performed with Luciferase Assay System from Promega according to the manufacturer's instructions and analyzed with a Turner (Palo Alto, CA) TD20e Luminometer. Luciferase activity was normalized to protein, as determined by the Lowry protein assay (38).

To assay repressor activity in the presence of Tet-VP16, transfections were carried out with 5 μg of DNA [2 μg of TRE luciferase reporter, 1 μg of Tet-VP16, Tet chimeric repressor (0–2 μg) and cytomegalovirus promoter (pCMV) carrier DNA] by using 10 μl of Lipofectamine. For HeLa pCEPTetPLuc and HeLa X1/6 cells with integrated luciferase reporters, the mixture had no reporter. Cells were incubated for 4 h after treatment with transfection mixture, medium was replaced with complete medium, and cells were incubated for 40 h. Transfection assays were carried out in duplicate, and each independent experiment was repeated three or more times.

Drosophila S2 cells were plated at 1×10^6 cells per well on 12-well plate and grown for 24 h at 22–24°C. Each transfection mixture contained of 54 μl of 2 M CaCl$_2$, 3 μg of TRE-Luc, 1.5 μg of Tet-VP16, Tet-repressor construct (0–3 μg), and pAX carrier DNA (total 7.5 μg) in 450 μl of water. Solution was dropped slowly into
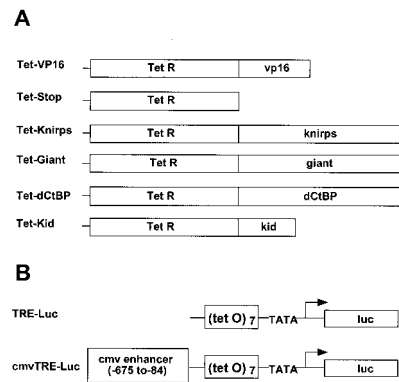

Fig. 1. Tet chimeric proteins and reporter constructs. (*A*) Tet-VP16 consists of TetR fused to the transactivator domain of the herpes simplex virus VP16 (amino acids 363–490). Tet-Stop contains only TetR protein. Tet-repressors contain residues from Knirps (amino acids 80–429), Giant (amino acids 2–389), dCtBP (amino acids 5–353), or Kid (amino acids 12–74). (*B*) Reporters. TRE-Luc contains seven TREs upstream of the human CMV promoter sequences. cmvTRE-Luc contains the complete CMV enhancer region (−675 to −84) 5′ of the TRE sequences.

450 μl of 2× HeBS [50 mM Hepes (pH 7.1)/1.5 mM NaHPO$_4$/280 mM NaCl] and incubated for 30 min at room temperature to form DNA–Ca phosphate complex (39). Transfection mixture was evenly dropped into the wells containing cells, and cells were incubated for 2 days.

Gel Mobility-Shift Assays. HeLa cells were grown in 10-cm dishes to 80% confluency and transiently transfected with 20 μg of plasmid DNA by using Lipofectamine. Forty hours later, transfected cells were washed with PBS, lysed with 400 μl of lysis buffer [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/1 mM PMSF/0.2 μg/ml of leupeptin] and incubated for 15 min on ice. Twenty-five microliters of 10% Nonidet P-40 was added, samples were cleared by centrifugation, and supernatant was removed. The pellet was resuspended in 50 μl of nuclear extract buffer [20 mM Hepes (pH 7.9)/400 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM DTT/2 mM PMSF/0.2 μg/ml leupeptin] and incubated for 15 min on ice. Nuclear extracts were collected by centrifugation. Five micrograms of nuclear extract was mixed with binding buffer containing 15 mM Hepes (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 12% glycerol, 0.25 mg/ml sonicated salmon sperm DNA, 0.25 mg/ml BSA, and 4 fmol of double-stranded ^{32}P-end-labeled (10,000 cpm) synthetic 1× TRE oligonucleotides (wild type 5′TCGAGTTTACCAC**TCCCTATCAGT-GAT**AGAGAAAAGTGAAAG3′ (TRE in bold) and mutant 5′TCGAGTTTACCAC<u>CTTTCGCTGACAGCG</u>AGAGAAA-GTGAAAG3′ (altered residues underlined) and incubated for 15 min on ice. Ten- and 100-fold molar excess unlabeled specific or nonspecific probes were added before nuclear extract. DNA–protein complexes were resolved on 6% nondenaturing polyacrylamide gels in 40 mM Tris/glycine (pH 8.3)/2 mM EDTA at 25°C (6–7 cm/V). Gels were analyzed with a PhosphorImager (Molecular Dynamics).

Results

Tetracycline-Repressor Chimeras. To assay the effectiveness of short-range repressors in the Tet system, chimeric proteins were created by fusing repression domains from the *Drosophila* Giant, Knirps, and dCtBP proteins to the Tet DNA-binding domain. Also tested were the Tet DNA-binding domain alone (Tet-Stop) and the Tet-Kid protein derived from the vertebrate Kid-1 KRAB repressor (33). Expressed from the CMV promoter, these genes produce chimeric proteins that bind to the TREs in the absence of doxycycline (Fig. 1*A*). Parallel constructs for insect cells use the actin 5C
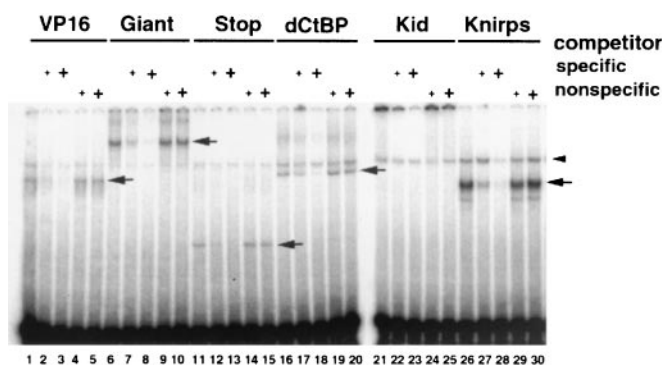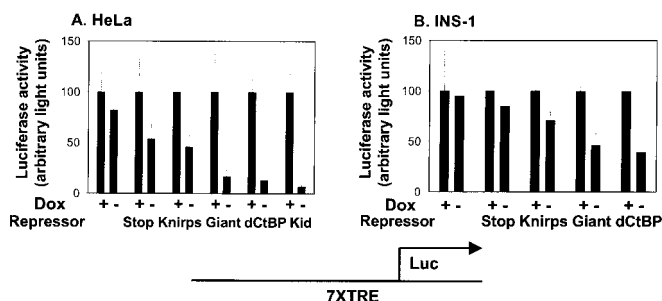
Fig. 2. Expression of Tet-repressor proteins. HeLa cells were transfected with indicated constructs and nuclear extracts analyzed by gel shift assay. Arrows indicate specific DNA and Tet chimeric protein complexes; arrowhead indicates a nonspecific band present in all samples. Specificity of binding was tested by addition of 10- or 100-fold molar excess unlabeled oligonucleotide. As quantitated by PhosphorImager analysis, Tet-Giant levels were >3-fold higher than those of Tet-dCtBP and Tet-Stop, and Tet-Knirps levels were ≈3-fold higher than those of Tet-Giant. Similar results were obtained in three independent transfection experiments.

Fig. 3. Tet-repressor proteins repress basal TRE-Luc promoter in mammalian cells. HeLa and INS-1 cells were transfected with 2 μg of TRE-Luc reporter plasmid and 1 μg of a Tet chimeric repressor construct. Cells were cultured with or without doxycycline (±Dox) before luciferase activity measurement. Tet-Kid was not tested in INS-1 cells. Data are the means ± SD for three independent experiments, each with duplicate measurements. Luciferase activity in the presence of the doxycycline was set at 100.

promoter. Luciferase reporter genes include a minimal TRE-Luc gene with seven TREs 5′ of the CMV basal promoter (−53 to +75) and cmvTRE-Luc, a reporter that contains the 590-bp CMV enhancer (−675 to −84) linked 5′ of the TRE elements of TRE-Luc (Fig. 1*B*).

Quantitation of TetR Proteins. To quantitate expression levels of functional Tet chimeric proteins, gel-shift assays were performed with HeLa nuclear extracts from cells transiently transfected with Tet constructs (Fig. 2). Specificity of binding was determined by competition with specific and nonspecific oligonucleotides. The expression levels varied between constructs; Tet-dCtBP and Tet-Stop were expressed at comparable levels, whereas Tet-Giant levels were ≈3-fold higher (see legend to Fig. 2). Tet-Knirps, which was inactive in HeLa cells (see below), was more abundant than the active Tet-Giant and Tet-dCtBP, indicating that the differences in repression effectiveness are not merely a reflection of relative protein expression levels. Tet-Kid formed complexes that did not migrate into the gel, precluding quantitation. This complex retained the probe in the well and was specifically competed by a TRE probe but not a nonspecific probe (Fig. 2, lanes 21–23). Previous studies have indicated that Gal4-Kid forms high molecular weight complexes that require detergent treatment to resolve on nondenaturing gels (40).

Repression of Basal Promoter Activity. TRE-regulated promoters may be transcribed under "nonactivating" conditions, because small amounts of Tet-activator can still bind and activate, or alternatively, the general transcription machinery may directly access the gene ("basal" transcription). We designed assays to simulate each of these possibilities by using both transiently transfected reporter genes and stably integrated reporters. To determine whether the Tet-repressor can act in the absence of added exogenous activators, the TRE-Luc reporter was transfected into human HeLa or rat INS-1 cells together with constructs encoding Tet-Giant, Tet-Knirps, Tet-dCtBP, Tet-Stop, or Tet-Kid. The repression levels measured as the ratio of repressed to not repressed luciferase expression of Tet-Giant and Tet-dCtBP chimeras were ≈5- to 10-fold in HeLa cells, whereas Tet-Knirps was less effective, repressing about 2-fold, indistinguishable from that of Tet-Stop, which lacks a repression domain. Tet-Kid was most effective, reducing expression 10- to 20-fold (Fig. 3*A* and Table 1). Consistent with doxycycline's ability to inhibit the Tet DNA-binding domain,

repression was relieved by treatment of the cells with the drug, indicating that the repression was specific for the Tet-chimeras. Similar, although weaker, repression effects were seen in the rat INS-1 pancreatic β cell line (Fig. 3*B* and Table 1). Again, Tet-Giant and Tet-CtBP were more active than Tet-Stop and Tet-Knirps, but overall levels of repression were reduced, a characteristic of all assays in this cell type (see below).

Repression of Tet-VP16-Activated Transcription. To determine whether the chimeric Tet-repressors can repress activated transcription, assays were performed by using transgenes activated by Tet-VP16. Increasing amounts of the Tet-repressor constructs were cotransfected into HeLa and INS-1 cells, together with a constant amount Tet-VP16 (Fig. 4). In both cell types, Tet-VP16 alone stimulated luciferase expression greater than 100-fold (data not shown). To counteract this activation, Tet-repressor proteins could block DNA binding by the activators ("passive" repression) and by interaction between the repression domain and the transcriptional machinery ("active" repression). In HeLa cells, the repression by Tet-Giant showed a dose-dependent response with increasing amounts of transfected gene, with 20- to 40-fold repression at higher levels of the gene (Fig. 4*A* and Table 1). Coexpression of the Tet-dCtBP protein resulted in up to a 7- to 8-fold reduction in VP16 mediated activation. Tet-Kid was most effective, with 50- to 140-fold repression. Neither Tet-Stop nor Tet-Knirps mediated effective repression in this assay (Fig. 4*A* and data not shown). In this and subsequent assays, repression by passive blocking of Tet-binding sites by Tet-Stop and Tet-Knirps is weak, probably because competition for all seven TRE-binding sites in the promoter would require a large molar excess of the Tet competitor. A similar pattern of repression was observed with the Tet-repressors in transfections of INS-1 cells, but with lower repression levels. Tet-Giant mediated ≈5-fold repression, whereas repression by Tet-dCtBP was only ≈3-fold. Tet-Stop and Tet-Knirps had less than 2-fold effects (Fig. 4*B*, Table 1, and data not shown). Tet-Kid was the most effective repressor, with 7- to 28-fold repression. In cases where repression was observed, a dose response was seen with increasing amounts of transfected repressor gene. Because they showed significantly higher activity than that of the DNA-binding portion alone encoded by Tet-Stop, Tet-Giant, Tet-dCtBP, and Tet-Kid appear to repress via "active repression," possibly by contacting specific targets in the transcription machinery.

Repression of CMV-Enhancer-Activated Expression. Integrated transgenes may be inappropriately activated by a combination of endogenous enhancer elements acting on the transgene (via "enhancer trapping") and low-level binding of the Tet-activator protein

Table 1. Activity of chimeric Tet-repressors

Cells	Reporter	Tet-Repressors				
		Stop	Knirps	Giant	dCtBP	Kid
Repression in the absence of Tet-VP16*						
HeLa	TRE-Luc	1.8 ± 0.2 (3)	2.2 ± 0.0 (3)	5.9 ± 1.3 (4)	7.5 ± 1.2 (4)	14 ± 2.8 (4)
	cmvTRE-Luc	0.9 ± 0.1 (3)	1.1 ± 0.2 (3)	5.5 ± 1.6 (5)	5.3 ± 2.9 (5)	107 ± 20 (3)
INS-1	TRE-Luc	1.2 (1)	1.4 ± 0.2 (3)	2.1 ± 0.3 (3)	2.5 (1)	—
	cmvTRE-Luc	2.6 ± 0.5 (2)	1.1 ± 0.1 (2)	3.5 ± 0.7 (2)	3.4 ± 1.3 (2)	24 ± 8.6 (2)
Repression in the presence of Tet-VP16†						
HeLa	TRE-Luc	1.4 ± 0.3 (3)	1.1 ± 0.3 (3)	26 ± 17 (3)	7.3 ± 1.1 (3)	127 ± 81 (3)
	cmvTRE-Luc	2.2 ± 0.5 (3)	1.6 ± 0.1 (3)	6.6 ± 1.3 (3)	4.3 ± 1.6 (3)	141 ± 83 (3)
INS-1	TRE-Luc	1.4 ± 0.5 (4)	1.1 (1)	4.7 ± 1.2 (5)	2.7 ± 0.9 (4)	14 ± 12 (3)
	cmvTRE-Luc	2.6 ± 2.0 (2)	—	1.9 ± 0.3 (2)	4.4 ± 2.5 (2)	42 ± 36 (2)
S2‡	TRE-Luc	1.5 ± 0.6 (3)	21 ± 2.3 (3)	105 ± 22 (3)	33 ± 5.6 (3)	1.0 ± 0.1 (3)
HeLa	pCEPTetPLuc§	0.9 ± 0.2 (6)	1.0 ± 0.3 (2)	6.7 ± 1.0 (6)	5.6 ± 1.8 (6)	161 ± 88 (4)
	HeLa X1/6¶	0.8 ± 0.1 (3)	1.2 ± 0.4 (3)	4.5 ± 1.1 (3)	2.9 ± 0.9 (3)	1,165 ± 600 (3)

*Cells were transiently transfected with 2 μg of reporter and 1 μg of Tet-repressor, ±1 μg/ml of doxycycline. Fold repression represents relative light units (RLU) in the presence of doxycycline divided by RLU value in the absence of doxycycline. Data represent means ± SD (n = number of independent experiments).
†Cells were transiently transfected with 2 μg of reporter, 1 μg of Tet-VP16, and 2 μg of Tet-repressors. Repression was measured relative to value of that Tet-VP16 alone.
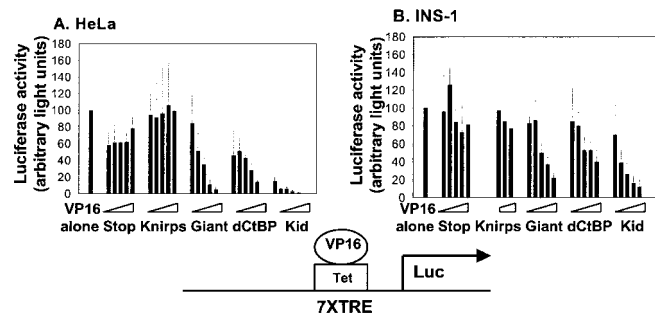‡*Drosophila* S2 cell line.
§HeLa pCEPTetPLuc has a stably replicating episome with TRE-Luc reporter.
¶HeLa X1/6 carries a chromosomally integrated TRE-Luc gene.

in the noninduced state. To assay the effectiveness of the Tet-repressor proteins in a context where both types of activities were present simultaneously, we used a TRE-luciferase reporter containing a strong CMV enhancer located 5′ of the seven TRE-binding sites. This enhancer, which binds Sp1 (41), increases luciferase expression ≈100-fold (not shown). We tested the ability of the Tet-repressors to interfere with expression driven by the CMV enhancer alone and found that the Tet-repressors that were active in our basal transcription assay also interfered with CMV enhancer-driven luciferase expression in HeLa cells (Fig. 5A). Tet-Giant and Tet-dCtBP exhibited 5-fold repression, whereas Tet-Knirps and Tet-Stop again had no discernible effect (Fig. 5A). Tet-Kid was much more active than the other repressors, showing over 100-fold repression of expression of the luciferase gene (Fig. 5A and Table 1).

Cotransfection of the Tet-VP16 gene with the CMV-TRE reporter further increased the expression of luciferase ≈10-fold,
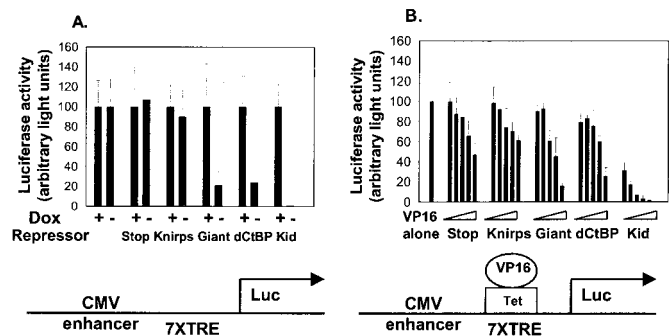
indicating that this promoter was still capable of additional induction (data not shown). Tet-Giant and Tet-dCtBP reduced the levels of reporter gene expression showing ≈7- and 4-fold repression, respectively, whereas Tet-Stop and Tet-Knirps showed comparable 2-fold effects (Fig. 5B and Table 1). Tet-Kid was much more effective, exhibiting over 100-fold repression. Similar effects were seen for cotransfections of INS-1 cells with the CMV-TRE reporter in the presence or absence of Tet-VP16, although the magnitude of repression was lower, as seen in experiments with the TRE-luciferase reporter (Table 1 and data not shown).

Repression of Activated Transcription in Stably Transformed Cells. The chromatin environment of a transiently transfected transgene is likely to be very different from that of a stably integrated gene, and it is known that transcriptional activators and repressors can, in

Fig. 4. Dose-dependent repression activity of Tet-repressors in the presence of the Tet-VP16. Two micrograms of TRE-Luc reporter, 1 μg of Tet-VP16 activator, and increasing amounts of Tet-repressor plasmid (0.1, 0.2, 0.5, 1, and 2 μg) were transfected into HeLa (A) and INS-1 (B) cells. Forty hours later, cells were harvested and analyzed for luciferase activity. Data are presented as the means ± SD for three or more independent experiments. In INS-1 cells, 0.5, 1, and 2 μg of Tet-Knirps were used in a single experiment. Luciferase activities are relative to that of Tet-VP16 alone, which was set at 100.

Fig. 5. Repression of CMV enhancer activity in transiently transfected HeLa cells. (A) Repression of CMV enhancer activity alone. Cells were transfected with 2 μg of cmvTRE-Luc and 1 μg of Tet-chimera constructs and cultured with or without doxycycline (±Dox). Data represent means ± SD for four or more independent experiments. Luciferase activity in the presence of doxycycline was set at 100. (B) Repression of CMV reporter in the presence of the Tet-VP16 activator. Two micrograms of cmvTRE-Luc, 1 μg of Tet-VP16, and 0.1, 0.2, 0.5, 1, or 2 μg of Tet-repressors were transfected. Data are the means ± SD for three independent experiments with duplicates with luciferase activity set at 100 for the value of Tet-VP16 alone.

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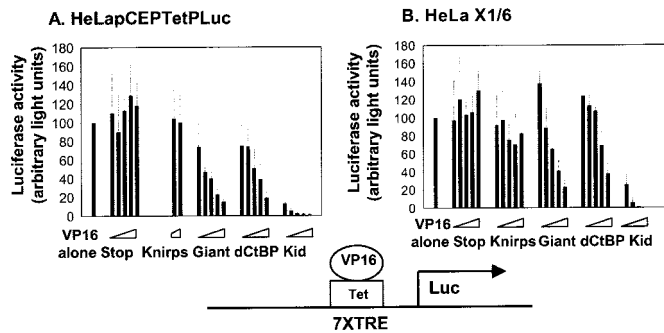
Fig. 6. Repression of activated gene expression in stably transformed cells. (*A*) HeLa pCEPTetPLuc and (*B*) HeLa X1/6 cells containing integrated TRE-Luc reporter genes were transfected with 1 µg of Tet-VP16 and 0.1, 0.2, 0.5, 1, or 2 µg of Tet-repressors. Data are means ± SD for three or more independent experiments. Tet-Knirps was tested in HeLa pCEPtetpLuc cells at the highest levels (1 and 2 µg) in two independent experiments. Luciferase activities are relative to that of Tet-VP16 alone (100).

some cases, act differently on transfected and stably integrated transgenes (42). To determine whether our panel of Tet-repressors would be effective on chromatinized genes, we tested their ability to repress transcription in two different cell lines that carry stable TRE-luciferase reporter genes. First, we used HeLa cells that were transfected with a stably replicating episomal element carrying the TRE-Luc reporter gene (pCEPTetPLuc) (43). Repression of basal expression by using transiently transfected Tet-repressors could not be measured effectively because of background expression from nontransfected cells (data not shown); therefore, we cotransfected the Tet-VP16 activator with the Tet-repressors. As previously shown (43), these cells show a robust >100-fold induction response to Tet-VP16 (not shown). In cotransfections with Tet-VP16 activator, Tet-Stop and Tet-Knirps had no significant repression (Fig. 6*A* and Table 1). In contrast, Tet-Giant and Tet-dCtBP were capable of repressing luciferase expression ≈5- to 7-fold in this cell line (Fig. 6*A*). Most effective in these assays was Tet-Kid, which was able to mediate >100-fold repression.

A similar but not identical pattern was seen with a TRE-luciferase reporter gene that is integrated into a chromosomal location in HeLa cells (HeLa X1/6), (Fig. 6*B*). This reporter gene is strongly induced by Tet-VP16 (≈10,000-fold; not shown). Tet-Giant mediated a 4- to 5-fold repression of luciferase expression in these cells, whereas Tet-Stop and Tet-Knirps had no significant activity. Tet-dCtBP was somewhat less effective than in other assays, mediating only a 3-fold reduction in luciferase activity. As seen with the episomally integrated transgenes, Tet-Kid was the most effective repressor, in this case providing up to 1,000-fold repression (Fig. 6*B*).

Repression Activity in *Drosophila* Schneider Cells. Giant, dCtBP, and Knirps are normally active in *Drosophila* and may interact with species-specific cofactors or targets; therefore, the chimeric Tet-repressors were transfected into *Drosophila* S2 cells. In competition assays with Tet-VP16, Tet-Giant and Tet-dCtBP showed strong 30- to 120-fold repression activities, whereas Tet-Stop had little effect (Fig. 7 and Table 1). In striking contrast to its activity in vertebrate cells, Tet-Kid was completely inactive in this assay, most likely because of the absence of a suitable corepressor (see *Discussion*). Tet-Knirps, inactive in the mammalian cells, was highly active in Schneider cells, mediating ≈20-fold repression (Fig. 7 and Table 1).

Discussion

Short-Range Repressors Possess Distinct Activities. In this study, we have characterized the activity of short-range transcriptional repressors from *Drosophila* in vertebrate and insect cell culture by
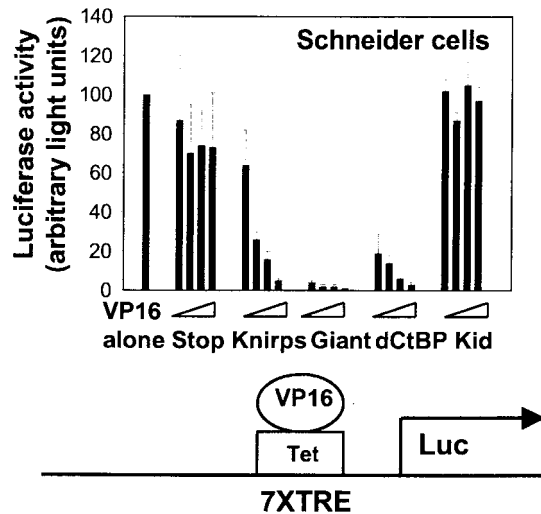
Fig. 7. Cell specificity of Tet-repressors measured in *Drosophila* S2 cells. TRE-Luc, Tet-VP16, and 0.25, 0.5, 1, or 2 µg of Tet-repressors were transfected into S2 cells as described. Data are means ± SD for three independent experiments. Luciferase activities are relative to that of VP16 alone (100).

using a range of assays. The restriction of Knirps repression activity to *Drosophila* cells, in contrast to the lack of species specificity seen with Giant, indicates that not all short-range repressors have identical biochemical activities, despite their common interactions with the CtBP cofactor corepressor (6, 8, 44). Knirps had not been previously tested in non-*Drosophila* cell lines, but Krüppel and CtBP, both short-range repressors, were demonstrated to mediate repression in mammalian cell culture (12, 13), and this study indicates that Giant, too, is active in mammalian cells. The inactivity of Knirps cannot be simply attributed to a lack of CtBP, as this protein is present in HeLa cells (51). Thus, the difference between Knirps and other short-range transcriptional repressors may indicate that the mechanism of repression is not identical between these proteins, even though Giant, Knirps, and Krüppel all interact with the CtBP. Indeed, increasing evidence suggests that these three proteins are able to mediate repression in both CtBP-dependent and -independent manners (44, 45, 50, 52). The functional differences we observe may be related to the CtBP-independent patterns of gene repression.

Cell-Type Specificity of Repressors. Tet-Giant and Tet-dCtBP were found to be active in mammalian cells on both integrated and transiently transfected reporter genes, but both are less effective than the mammalian repressor Tet-Kid. The lower level of repression is not simply because of an inability to inhibit the VP16 activator, because the Kid repressor was also more active on the CMV enhancer and in basal transcription assays (Figs. 3 and 5, Table 1). Rather, the difference in activities probably reflects differences in mechanisms of repression conferred by these proteins. Kid 1 is a member the KRAB family of zinc-finger-containing nuclear proteins that interact with the KAP-1 corepressor (8, 46, 47). This cofactor is thought to mediate repression by interactions with the heterochromatin protein HP-1 and histone deacetylases, perhaps inducing local heterochromatinization (46–48). KRAB repressors have been found to repress promoters over ranges of several kilobases in transient transfection assays and with integrated target genes (35, 48, 49). The inactivity of Tet-Kid in *Drosophila* cells indicates that this protein, and probably other repressors of its class, will not be suitable for regulation of the Tet system in this cell type and possibly other insect cells. The most likely reason for Tet-Kid inactivity is that KRAB and KAP-1 homologs do not exist

in *Drosophila*, rather than a fundamental difference in the transcriptional machinery of *Drosophila* and mammals, because the mammalian KAP-1 corepressor is capable of mediating repression in the *Drosophila* embryo (G. Attardo, D.N.A., and D. Schultz, unpublished results). Another more subtle example of cell-type specificity of repression may be represented in the uniformly higher repression levels in HeLa cells than in INS-1 cells, perhaps because of different levels of corepressors in the different cell lines. Cell-line variation of repressor effectiveness has been previously noted for a Gal4-CtBP chimera (12).

Optimizing a Repression/Activation System. Clearly, the selection of the repressor for use in a regulated gene expression system must take the cell type into account. Our study suggests that Tet-Knirps, Giant, and dCtBP fusion proteins should be effective in repression of Tet-regulated promoters especially in *Drosophila* cells. In mammalian cells, there may be circumstances in which short-range repression is preferable to long-range repression, such as when using stable cell lines that contain a Tet-regulatable transgene linked to the selectable marker gene. If regulation is achieved with a long-range repressor such as Tet-Kid, silencing of the promoter with Tet-binding sites may be accompanied by the inadvertent silencing of the nearby marker, thus interfering with drug selection of the transgene.

In our assays, the Tet-VP16 protein and the short-range Tet-repressor proteins both possess the same "B-type" dimerization domain (30); thus heterodimers may form between activator and repressor. Formation of these heterodimers may decrease VP16 activity somewhat, but previous studies in yeast indicated that it did

not preclude establishment of effective regulation (18). To develop this system for optimal *in vivo* regulation, however, it may be preferable to use the short-range Tet repressors in conjunction with a Tet activator possessing distinct nonoverlapping dimerization specificity (32).

Use of Transient Transfections to Study Repression. Transcriptional repression in the cell normally takes place on a chromatinized template, and many transcriptional regulators are known to act through cofactors that modify chromatin structure by acetylation and deacetylation. Thus, it is not surprising that some repressor proteins have been found to exhibit normal repression activity only when assayed on integrated transgenes, as opposed to transiently transfected genes, which may have a different chromatin structure (42). Our study is the first comprehensive comparison, to our knowledge, of the effectiveness of short-range repressors on transiently transfected genes versus a stably integrated gene. We find that these proteins are capable of mediating repression in both contexts, indicating that mechanistic analysis of short-range repressors by using transfection assays is likely to provide useful insights into the activities of these proteins.

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