Tetracycline-Reversible Silencing of Eukaryotic Promoters

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A tetracycline-controlled transrepressor protein has been engineered to silence transcriptional activities of eukaryotic promoters that are stably integrated into the chromatin of human cells. By fusing the KRAB domain of human Kox1 to the Tet repressor derived from Tn10 of Escherichia coli, a tetracycline-controlled hybrid protein (TetR-KRAB) was generated and constitutively expressed in HeLa cells. The TetR-KRAB protein binds to tet operator (tetO) sequences in the absence but not in the presence of tetracycline. When TetR-KRAB bound to tetO sequences upstream of the immediate-early promoter-enhancer of human cytomegalovirus (CMV), the expression of a CMV-driven luciferase reporter construct (ptetO7-CMV-L) was repressed in transient transfection experiments. This silencing was found to operate on different promoters and from tetO sequences placed more than 3 kb from the transcriptional start site. We constructed a stable, doubly transected cell line (TIS-10) carrying a chromosomally integrated ptetO7-CMV-L reporter construct and expressing the TetR-KRAB protein. Upon addition of tetracycline, luciferase expression was induced more than 50-fold above the baseline level, with half-maximal induction by 2 days. Furthermore, a protein of around 110 kDa was found to coimmunoprecipitate with the TetR-KRAB fusion protein. This protein might play a role as an adaptor protein mediating the silencing exerted by the TetR-KRAB protein. The TetR-KRAB silencing system should be useful as a genetic switch for regulating the expression of chromosomally integrated heterologous and endogenous genes present in mammalian genomes.

Inducible gene expression has been a valuable tool for the study of gene function in bacteria, yeasts, and Drosophila melanogaster. In mammalian cells, eukaryotic promoter systems that respond to inducing agents such as glucocorticoid hormone (19, 23), heat shock (40), heavy metal ions (28), or interferon (32) have been fused to sequence-acting protein domains originating from the glucocorticoid receptor (30) or the estrogen receptor (38) have been fused to sequence-specific DNA binding domains for the factor in question. This results in marked transcriptional stimulation of the respective minimal promoter. In order to modulate the transcriptional activity of chimeric transcription factors, movable steroid binding domains originating from the glucocorticoid receptor or the estrogen receptor have been fused to sequence-specific DNA binding domains. In the case of a GAL4-VP16-estrogen receptor fusion, estrogen administration has been shown to produce a functionally active chimeric transcription factor that activates a target promoter (6).

In the tetracycline system, the TetR protein fused to the transactivating domain of VP16 (called tTA) has been shown to bind to and strongly activate minimal promoter systems containing seven tetracycline operator (tetO) sequences (15, 16). The binding of tTA to the tetO sequences is blocked by tetracycline, preventing the activation of the target promoter which generates a very tight genetic switch (16).

In this report, we present a novel system for tetracycline-controlled silencing of eukaryotic promoters. Here, the KRAB repressor domain of the human Kox1 zinc finger protein (27) has been fused to the TetR protein. In the absence of tetracycline, this chimeric DNA-binding protein (TetR-KRAB) exerts its silencing activity by binding to several cis-acting tetO sites placed at a distance from the transcriptional initiation site of a eukaryotic promoter. Promoter activity is restored upon administration of tetracycline, which prevents binding of TetR-KRAB to the tetO sequences. We also present evidence that the TetR-KRAB forms a complex with a protein of around 110 kDa. This protein, provisionally called SMP1 (silencing-mediating protein 1), might constitute a putative corepressor protein that mediates the repression activity employed by the KRAB domain of Kox1.

MATERIALS AND METHODS

Plasmid constructions. The fusion between TetR and 121 amino acids of the NH₂ terminus of Kox1 was constructed by PCR amplification. A PCR fragment was generated by using two synthetic oligonucleotides, 5'-ATCAGGATTC AACCATGCTAGTATAGATAAAAAG3' and 5'-GTCGTCGACACTTTGCTTCTTTCTC TTCCTTTTTTGCCGAGCCACTTTTCACATT3', and pUHD15-1 (16), introducing an Ncol site around the translational start of the TetR preceded by an EcoRI restriction site (sites are underlined). In addition, six amino acids comprising the simian virus 40 (SV40) large-T-antigen nuclear localization sequence (20) (nucleotides in boldface) were fused in frame with the last amino acid residue of TetR followed by a SalI restriction site (underlined). The resulting PCR fragment was digested with EcoRI and SalI. A second PCR fragment was generated by using two oligonucleotides, 5'-GACACTGTCGACGGCGGTGTTGCCTTCTG3' and 5'-GTCGTCGGATCCATTAAACCTGATGATTTGATTTC3', and plasmid pKox1 (35), introducing a SalI site at the NH₂-terminal end of the KRAB domain of Kox1 and a stop codon at position 121 of Kox1 followed by a BamHI restriction site. After digestion with SalI and BamHI, both fragments were ligated into EcoRI- and BamHI-digested pUHD10-1 (12), generating pCMV-tetR-KRAB. To generate pCMV-tetR-pCMV-tetR-KRAB was digested with SalI and BamHI, and the ends were filled in and religated. This retains the fusion of the complete TetR to the nuclear localization signal of SV40 large T antigen (Pro-Lys-Lys-Lys-Arg-Lys) followed by five amino acids originating from the vector backbone (Val-Glu-Ile-Gln-Thr-Ser).

Plasmid ptetO7-CMV-L was generated by ligating the 786-bp Xho1-BamHI

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cytochrome c (CMV) promoter fragment of pUHD10-1 (12) into XhoI-BamHI-digested pUC13-4 (16). This places seven tetr sequences just upstream of the human CMV immediate-early promoter/enhancer (4). Plasmid pTetO7-TK-L was generated by ligating an XhoI-BglII fragment from pT109ulc (29) comprising the HSV TK promoter (−109 to +52), both GC boxes and the CAT site of XhoI-BamHI-digested pUC13-4 (16).

Cell culture and transfections. HeLa cells were grown in Iscove’s modified Dulbecco medium (GIBCO) supplemented with 5% fetal calf serum (FCS); Boehringer Mannheim) in a 5% CO2 environment at 37°C. Cells were plated at a density of 105 cells per 35-mm-diameter dish and transfected at 90% confluence with 5 μg of plasmid DNA (4.5 μg of the expression and 0.5 μg of the reporter plasmid) as a calcium phosphate precipitate (1). After the transfection, cells were further incubated in Iscove’s modified Dulbecco medium supplemented with 5% FCS and no or 0.5 μg of tetracycline (Sigma) per ml for 24 h before determination of luciferase activities from cell extracts. Determination of β-galactosidase activities (1) from cotransfected pCH110 (2 μg per dish; Pharmacia) was used to control for transfection efficiencies.

To establish stable cell lines expressing the TetR-KRAB fusion protein, HeLa cells were transfected with 2.5 μg of pCMV-tetR-KRAB-hyg DNA linearized with Aps 700. Forty-eight hours after transfection, cells were trypsinized and split at a ratio of 1:10, and resistant colonies were selected in Iscove’s modified Dulbecco medium supplemented with 5% FCS (Boehringer) and 250 μg of hygromycin B (Sigma) per ml. Resistant colonies were subcloned by limiting dilution in nonselective medium and tested for expression of functional TetR-KRAB protein, resulting in the A12 cell line. To establish a cell line containing the pTetO7-CMV-L construct together with pCMV-tetR-KRAB, A12 cells were transfected with 5 μg of a 15:1 mixture of pTetO7-CMV-L linearized with HindIII and pNeo5 (26). Twenty colony units (CUs) were plated on G418 (500 μg/ml) to obtain colonies of 0.5 μg of tetracycline per ml, and luciferase activities were determined from extracts. One positive clone, called TIS-10, was chosen for further characterization.

Luciferase assays. Cells were grown in absence or presence of tetracycline (0.5 μg/ml) in 35-mm-diameter dishes, washed with 2 ml of phosphate-buffered saline (PBS), and then lysed in 50 μl of 20 mM Tris-HCl (pH 7.8)–150 mM NaCl–1 mM EDTA–1 mM dithiothreitol–0.6% Nonidet P-40. The lysate was collected and centrifuged at 10,000 × g for 10 min. Aliquots (10 μl) of the supernatants were mixed with 350 μl of a buffer containing 20 mM Hepes–150 mM NaCl–200 mM glycyglycine–15 mM MgSO4–5 mM thiourea, and the labeled proteins were visualized by autoradiography.

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RESULTS

Construction of expression plasmids. It was recently shown that a fusion protein of the yeast GAL4 DNA binding domain and the KRAB domain of Kox1 expressed in mammalian cells strongly repressed a cotransfected chloramphenicol acetyltransferase reporter construct containing GAL4 binding sites upstream of the TK promoter (27). We decided to take advantage of this finding. We fused the first NH2-terminal 121 amino acid residues of Kox1, containing the KRAB domain (35), to the C terminus of the prokaryotic TetR encoded by Tn10 from E. coli (20) to create a tetracycline-responsive transcriptional silencer protein, TetR-KRAB. As a linker between the two domains, six amino acids comprising the nuclear localization sequence of SV40 large T antigen (20) were added to favor nuclear localization of the TetR-KRAB fusion protein in mammalian cells. In plasmids used for expression of TetRKRAB (pCMV-tetR-KRAB and pCMV-tetR-KRAB-hyg) in mammalian cells, the sequence encoding TetR-KRAB is flanked upstream by the human CMV immediate-early promoter and downstream by the SV40 poly(A) site. As a control, the KRAB domain was removed, creating TetR fused to the nuclear localization signal followed by five additional amino acids in pCMV-tetR. The reporter plasmids (pTetO7-CMV-L and pTetO7-TK-L) are based on pUC13-4 (16). In these constructs, the CMV or HSV TK promoter was inserted downstream of seven tetr sequences and upstream of the luciferase as a reporter gene. The right boundary of the fragment containing the seven tetr sequences is located more than 685 and 109 bp upstream of the transcriptional initiation site of the CMV and TK promoters, respectively. Relevant features of plasmids pCMV-tetR-KRAB, pCMV-tetR, and pTetO7-CMV-L are outlined in Fig. 1. The rationale is to generate a fusion protein TetR-KRAB that binds in cis to tetr sequences upstream of the transcriptional initiation site of the CMV promoter (Fig. 1) or to any other promoter. Binding of the KRAB domain to the tetr sites should result in active repression of productive transcription measured by luciferase activity. Addition of low levels of tetracycline to the growth medium should prevent TetR-KRAB from binding to the tetr sequences and should therefore restore full productive transcriptional initiation from the respective promoter.

Repression of transcription mediated by the TetR-KRAB fusion protein. HeLa cells were cotransfected with the reporter plasmid pTetO7-CMV-L and one of the expression plasmids pCMV, pCMV-tetR-KRAB, and pCMV-tetR and grown with binding buffer were added after 10 min incubation in absence of DOC. Dried gels were visualized by autoradiography using Kodak AR-5 film.

I Immunoprecipitation. HeLa and HeLa A12 cells were grown in 100-mm-diameter dishes to 80% confluence, washed, and grown for 2 h in 4 ml of tetracycline-free modified Eagle’s medium (GIBCO) supplemented with 5% dialyzed FCS and 500 μg of [35S]methionine (Amersham). Following the addition of 4 ml of Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 5% FCS, cells were grown for an additional 30 min, washed with PBS, harvested, and lysed by freeze-thawing in 500 μl of immunoprecipitation buffer (IP buffer; 50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 0.05% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol) followed by centrifugation for 10 min at 10,000 × g. The crude supernatant was adjusted to 1% BSA in IP buffer and incubated with 50 μl of a 50% slurry of protein G-agarose beads (Boehringer). The supernatant was divided in two aliquots; to each, 2 μl of the anti-Kox1 rabbit immune serum was added. After 2 h of incubation at 4°C, a slurry of 50 μl of protein G-agarose was added, and the mixture was incubated for an additional 30 min. The beads were collected and washed three times in IP buffer. To one aliquot of the beads, 50 μl of IP buffer containing 0.1% DOC was added; after 5 min at room temperature, the beads were separated from the supernatant. Fifty microliters of IP buffer was added to the beads, and the samples were adjusted to SDS-sample buffer and separated on an SDS–12% polyacrylamide gel (1). The gel was soaked in Amplify (Amersham), and the labeled proteins were visualized by autoradiography.

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or without tetracycline for 1 day (Fig. 2). In transfections using
the control vector pCMV lacking TetR sequences, similar lu-
ciferase activities were obtained from cells grown with or with-
out tetracycline. In contrast, luciferase activity was reduced
about 10-fold in transfections with pCMV-tetR-KRAB (Fig. 2,
CMV-tRK). In these cells, luciferase expression was restored
by addition of 0.5 \( \mu g \) of tetracycline per ml to the medium.
No repression was observed when TetR fused to the nuclear lo-
calization sequence was expressed alone (Fig. 2, pCMV-tR),
demonstrating that the repression is mediated by the KRAB
domain. Similar results were obtained for the reporter con-
struct ptetO7-TK-L carrying the HSV TK promoter (data not
shown). No differences in luciferase activities were observed
for reporter constructs lacking tetO sequences (data not
shown). This observation suggested that TetR-KRAB inter-
fere with productive initiation at the CMV or TK promoter
only when bound to tetO sequences. We next made stable
transfectants expressing TetR-KRAB under the control of
the CMV promoter. The construct used (pCMV-tetR-KRAB-hyg)
contained a hygromycin resistance gene under the contro-
lof the TK promoter inserted into pCMV-tetR-KRAB (see Ma-
terials and Methods). Clones resistant to hygromycin B were
assayed for expression of the TetR-KRAB protein, and the
intracellular localization of the TetR-KRAB hybrid protein
was visualized by indirect immunofluorescence. In one of the
stable cell lines examined, A12, the TetR-KRAB protein was
localized predominantly to the nucleus by indirect immuno-
fluorescence using an anti-Kox1 immune serum (Fig. 3d and h).
The staining was specific since the preimmune serum showed
very little staining (Fig. 3b and f). A12 cells (Fig. 3d) showed a
much stronger nuclear staining than the parental HeLa cells
(Fig. 3h), as a result of the expression of the TetR-KRAB
protein. The staining of HeLa nuclei (Fig. 3h) suggests that
nuclear proteins that are immunologically related or identical
to Kox1 are expressed in HeLa cells. Kox1 mRNA has been
detected in HeLa cells (35).

The luciferase activities from HeLa A12 cells transiently
transfected with plasmids pCMV-L and pTK-L, which lack
tetO sequences, were not altered by the presence of tetracy-
cline in the medium (Fig. 4). However, tetO sequences inserted
in ptetO7-CMV-L and ptetO7-TK-L resulted in a significant
reduction of luciferase activities in the absence of tetracycline
of more than 1 order of magnitude (Fig. 4). Addition of
tetracycline fully restored the expression of luciferase (Fig. 4).
Luciferase activities of these plasmids transfected into parental
HeLa cells that lack stably expressed TetR-KRAB protein
were not affected by tetracycline (data not shown). Increasing

**FIG. 1. Schematic representation of main features of expression and reporter constructs.**

A) In the expression plasmid pCMV-tetR-KRAB, the TetR-KRAB
sequence is placed downstream of the strong immediate-early human CMV promoter (PCMV) and is followed by an SV40 polyadenylation signal (An). The TetR-KRAB
fusion protein is composed of the complete 207-amino-acid sequence of TetR fused to 6 amino acids representing the SV40 large-T-antigen nuclear localization
sequence (NLS) and a 121-amino-acid KRAB domain originating from the NH2 terminus of human Kox1 containing the KRAB A and B domains (27). The KRAB
domain has been removed in the control expression plasmid pCMV-tetR. (B) The reporter plasmid ptetO7-CMV-L consists of seven tetO sequences placed upstream
of the promoter/enhancer sequence (positions −675 to +75, +1 being the transcriptions start site) of human CMV followed by sequences encoding firefly luciferase
and an SV40 poly(A) site (An). The distances of the boundaries of the tetO-containing fragment (−994 and −685) with respect to the transcriptions start site of the
CMV promoter and the sites for insertion of the promoter(s), X (XhoI) and B (BamHI), are indicated.

**FIG. 2. Tetracycline-reversible silencing of the CMV promoter by TetR-
KRAB in transiently transfected HeLa cells.** HeLa cells (70 to 80% confluent)
grown in 35-mm-diameter dishes without tetracycline (tet) were transiently trans-
fected at a molar ratio of 1:5 with the reporter plasmid ptetO7-CMV-L (0.5 \( \mu g \))
and 2.5 \( \mu g \) of one of the expression plasmids pCMV (CMV), pCMV-tetR-KRAB
(CMV-tRK), and pCMV-tetR (CMV-tR). After transfection, cells were grown
for an additional 24 h with or without 0.5 \( \mu g \) of tetracycline per ml before
luciferase activities were determined from aliquots (1/50) of total cell extracts.
the distance of the tetO sequences to the transcriptional start site to about 3,600 bp still resulted in significant tetracycline-reversible repression of luciferase expression by TetR-KRAB which was just threefold lower than that at a distance of 685 bp (13a). Furthermore, the orientation of the tetO-containing fragment with regard to the promoter had no influence on the observed repression (13a). All of the many different promoters tested so far in this system were found to respond to TetR-KRAB-mediated silencing equally well (data not shown). We conclude that TetR-KRAB stably produced in HeLa cells is able to actively repress the productive expression from both the CMV and TK promoters by binding to cis-acting tetO sequences placed at a distance from the respective transcriptional initiation sites.

The time course of tetracycline action. The time course of tetracycline action is shown in Fig. 5. A. Luciferase activities were determined in extracts from TIS-10 cells after growth in medium containing or lacking tetracycline. The presence of tetracycline for 24 h in the growth medium of TIS-10 cells resulted in more than 10-fold increases in luciferase activity. No obvious change in growth behavior was detected in the presence of tetracycline, which demonstrates that the TetR-KRAB fusion protein can locate the tetO sequences in the chromatin of mammalian cells and repress the activity of the ptetO7-CMV-L construct within the genomic environment in a reversible fashion. A tetracycline concentration of 0.1 μg/ml was sufficient to release repression (data not shown). This concentration is well below the concentration of 10 μg/ml above which inhibition of growth had been observed (reference 16 and data not shown).

**Kinetics of tetracycline action.** The time course of tetracycline action is shown in Fig. 5. B. Luciferase activities were determined in extracts from TIS-10 cells after growth in medium containing or lacking tetracycline. The presence of tetracycline for 24 h in the growth medium of TIS-10 cells resulted in more than 10-fold increases in luciferase activity. No obvious change in growth behavior was detected in the presence of tetracycline, which demonstrates that the TetR-KRAB fusion protein can locate the tetO sequences in the chromatin of mammalian cells and repress the activity of the ptetO7-CMV-L construct within the genomic environment in a reversible fashion. A tetracycline concentration of 0.1 μg/ml was sufficient to release repression (data not shown). This concentration is well below the concentration of 10 μg/ml above which inhibition of growth had been observed (reference 16 and data not shown).
Luciferase activities were determined from aliquots of extracts (1/50) of cells. Tetracycline per ml in the growth medium. After the indicated times, relative luciferase activities were determined from 1/50 of extracts from cells grown with or without tetracycline. Data from three individual dishes (TIS-10-1 to TIS-10-3) are shown.

The hybrid protein (TetR-KRAB) produced in HeLa A12 cells is associated with a cellular protein of around 110 kDa. To investigate the mechanism of TetR-KRAB-mediated repression, we performed electrophoretic mobility shift analysis with labeled synthetic tetO DNA and crude extracts from A12 cells stably producing the TetR-KRAB protein. Incubation of the labeled tetO sequences with crude extract from A12 cells led to the formation of a very slowly migrating specific complex C1 (Fig. 7A, lane 3). The migration of this complex cannot be explained simply by a dimer of the 38-kDa TetR-KRAB protein bound to a 21-bp tetO DNA fragment. In several experiments, a significant fraction of the specific complexes did not even enter the gel, suggesting that large complexes might have been formed. Such complexes were not observed with extracts from parental HeLa cells (Fig. 7A, lane 2). Complex formation was sensitive to tetracycline (1 μg/ml) and specifically competed for by an excess of unlabeled tetO DNA, while DNA fragments with unrelated sequences had no effect (data not shown).

To investigate further the nature of these large complexes, the ionic detergent DOC was added to the binding reactions in order to dissociate weak protein-protein interactions (2). The presence of 0.05 to 0.2% DOC resulted in the disappearance of the large complex C1 and the formation of a faster-migrating complex, C2 (Fig. 7A, lanes 4 to 6). The nature of complex C2 was further investigated by adding a Kox1-specific immune serum (28a). In the presence of a 1:200 dilution of the polyclonal anti-Kox1 immune serum together with 0.1% DOC, a retarded complex, C3, was formed (supershift; Fig. 7B, lane 5). No supershift was observed with the preimmune serum (Fig. 7B, lane 4), which suggested that the faster-migrating complex C2 was probably composed of a TetR-KRAB dimer bound to the tetO sequence. The large complex C1 was also detected in extracts from HeLa cells transiently transfected with pCMV-tetR-KRAB (C1; Fig. 7C, lane 3). In contrast, a much smaller complex, C4, was formed with extracts from HeLa cells transfected with pCMV-tetR expressing the 25-kDa TetR protein fused to the nuclear localization sequence but lacking the KRAB domain (C4; Fig. 7C, lane 4; see also Fig. 1 and 2).

Complex C4, migrating just slightly above a nonspecific complex (asterisk in Fig. 7C), is not formed in the presence of 1 μg of tetracycline per ml and is insensitive to low levels of DOC (not shown).

The very slowly migrating complex C1 could be the result either of TetR-KRAB dimers associating with each other to form a larger complex or of a complex in which the KRAB domain of Kox1 is associated with a cellular factor(s). As a first step toward distinguishing between these possibilities, we metabolically labeled HeLa A12 cells and HeLa cells with [L-35S]methionine and prepared extracts. Extracts were immunoprecipitated with the rabbit anti-Kox1 immune serum and protein G-Sepharose beads and separated by SDS-PAGE (Fig. 8). Immunoprecipitates of extracts from HeLa A12 cells showed two bands corresponding to the predicted size of the TetR-KRAB fusion protein of 38 kDa (Fig. 8, lane 2). This doublet was specifically missing in extracts from HeLa cells (Fig. 8, lane 5). The occurrence of a doublet may indicate posttranslational modification, such as phosphorylation, of the TetR-KRAB fusion protein synthesized in HeLa cells. In addition, a band corresponding to a protein of around 110 kDa was specifically communoprecipitated together with TetR-KRAB (Fig. 8, SMPL, lane 2). This band was not visible in extracts from HeLa cells (Fig. 8, lane 5). Most significantly, if the protein G-Sepharose beads were extracted once with 50 μl of IP buffer containing 0.1% DOC, the 110-kDa protein was released from the beads (Fig. 8, lane 3) and appeared in the DOC supernatant (Fig. 8, lane 4). The DOC sensitivity of the association of the 110-kDa protein to TetR-KRAB in the communoprecipitation is strikingly reminiscent of the DOC sensitivity of the large C1 complexes formed in extracts from HeLa A12 cells with labeled tetO sequences in the electrophoretic mobility shift analysis (Fig. 7A, lanes 3 to 5). In addition, the large complexes are not formed with the TetR protein lacking the KRAB domain. We therefore propose that a protein of around 110-kDa interacts with the KRAB domain of the TetR-KRAB fusion protein. This protein might be involved in the mechanism of repression of promoters from a distance by TetR-KRAB. We have tentatively named this protein SMPL.
DISCUSSION

In recent years, domains of various proteins that, when fused to DNA binding domains, can actively repress the activity of promoters containing appropriate target sequences have been described (reviewed in references 10 and 11). Among these is the KRAB domain of Kox1, which functions very efficiently (13a, 27). The KRAB domain is an evolutionarily conserved domain of 75 amino acids comprising a heptad repeat of methionine and leucine residues in Kox1 (35). It has been estimated that approximately one-third of all zinc finger-type DNA-binding proteins contain a KRAB domain NH₂-terminal to the zinc finger region (3). We show here that a fusion of the KRAB repression domain derived from the human Kox1 protein to the TnI0-derived E. coli TetR generated the TetR-KRAB hybrid protein, which functions as a potent DNA-binding-site-dependent transrepressor of transcription. The TetR-KRAB protein was overproduced in HeLa cells to significant levels and seems to be localized to the nucleus. The nuclear localization is presumably favored because of the presence of a nuclear localization signal derived from the SV40 large T antigen (20) between TetR and KRAB domains (Fig. 1). The strong nuclear staining in HeLa A12 cells (Fig. 3) is accompanied by the presence of a strong DNA binding activity toward tetO sequences in crude extracts (Fig. 7A). Furthermore, the anti-Kox1 immune serum detects the native KRAB domain in the TetR-KRAB protein, as demonstrated by the supershift of the TetR-KRAB/tetO complex (C3; Fig. 7B, lane 5). The TetR-KRAB produced in HeLa cells binds to tetO sequences in vitro, and this association is prevented by tetracycline (not shown). When the TetR-KRAB protein is bound to tetO sequences upstream of the enhancer and the transcriptional initiation site of promoters like the immediate-early human CMV promoter or the HSV TK promoter, the productive transcriptional activity (determined by using the luciferase gene as a reporter gene) of the respective constructs is repressed in transient assays. This repression is released by tetracycline and strictly dependent on both the presence of the KRAB domain (Fig. 2) and cis-acting tetO sequences (Fig. 4a and b). Repression by the TetR-KRAB protein is observable when tetO sequences are placed more than 3 kb upstream or downstream of the transcriptional initiation site of a eukaryotic promoter and independent of the orientation of the tetO sequences, suggest-
ing that the TetR-KRAB protein functions as a tetracycline reversible silencing protein (13a). Tetracycline-reversible silencing is also observed with TIS-10 cells that carry the pTetO-CMV-L construct stably integrated into the genome of HeLa A12 cells expressing the TetR-KRAB protein (Fig. 5). Here the addition of tetracycline to the medium of TIS-10 cells results in a more than 50-fold activation of productive transcription from the CMV promoter (Fig. 6). Removal of tetracycline from the medium of cells grown in the presence of tetracycline restores repression (not shown). The kinetics of this process is rather slow, with half-maximal effects by 2 days. Induction or repression is observed in rapidly dividing and nondividing, i.e., confluent, cells, suggesting that replication of the DNA may not be essential for the underlying mechanism (13a).

Many other proteins contain repression domains that function when fused to heterologous DNA binding domains (8, 9, 17, 21, 24, 25, 27, 34, 37, 39). The mechanisms by which such alanine-rich or proline-rich domains, as well as the KRAB domains, exert their repressing functions have not yet been identified (10, 11, 18). We show here that the TetR-KRAB protein produced in HeLa cells forms very large complexes with tetO sequences, which can be regarded as a cis-acting silencer. The silencing effect (−) of the TetR-KRAB fusion protein bound to its tetO sequences is mediated by the SMP1 protein and could act on different levels of transcriptional control as indicated.

![Diagram](image.png)

**FIG. 9.** Model of the silencing mechanism by TetR-KRAB and SMP1. An enhancer-binding protein exerts its stimulatory effect (+) on the basal transcriptional machinery through an adaptor protein. The TetR-KRAB protein binds to the tetO sequences, which can be regarded as a cis-acting silencer. The silencing effect (−) of the TetR-KRAB fusion protein bound to its tetO sequences is mediated by the SMP1 protein and could act on different levels of transcriptional control as indicated.

Additionally, any effect observed through the addition of tetracycline to the medium of TIS-10 cells expressing the TetR-KRAB protein (Fig. 5). Here the addition of tetracycline to the medium of TIS-10 cells results in a more than 50-fold activation of productive transcription from the CMV promoter (Fig. 6). Removal of tetracycline from the medium of cells grown in the presence of tetracycline restores repression (not shown). The kinetics of this process is rather slow, with half-maximal effects by 2 days. Induction or repression is observed in rapidly dividing and nondividing, i.e., confluent, cells, suggesting that replication of the DNA may not be essential for the underlying mechanism (13a).

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eukaryotes. Biochem. J. 296:521–541.
11. Cowell, I. G. 1994. Repression versus activation in the control of gene transcription. Trends Biochem. Sci. 19:38–42.
12. Deuschle, U., R. Pepperkok, F. Wang, T. J. Giordano, W. T. McAllister, W. Anorge, and H. Bujard. 1989. Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and lac repressor. Proc. Natl. Acad. Sci. USA 86:5400–5404.
13. Deiss, L. P., and A. Kimchi. 1991. A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. Science 252:117–120.
13a. Deuschle, U., and H.-J. Thiesen. Unpublished data.
14. DeWet, J. R., K. V. Wood, M. DeLuca, D. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7:725–737.
15. Gossen, M., A. Bonin, and H. Bujard. 1993. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. Trends Biochem. Sci. 18:471–475.
16. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89:5547–5551.
17. Han, K., and J. L. Manley. 1993. Functional domains of the Drosophila engrailed protein. EMBO J. 12:2723–2733.
18. Herschbach, B. M., and A. D. Johnson. 1993. Transcriptional repression in eukaryotes. Annu. Rev. Cell Biol. 9:479–509.
19. Heynes, N. E., N. Kennedy, U. Rahmsdorf, and B. Groner. 1981. Hormone-responsive expression of an endogenous proviral gene of mammary tumour virus after molecular cloning and gene transfer into cultured cells. Proc. Natl. Acad. Sci. USA 78:2038–2042.
20. Kaldner, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. Cell 39:499–509.
21. Keleher, C. A., M. J. Redd, J. Schultz, M. Carlsson, and A. D. Johnson. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell 68:709–719.
22. Labow, M. A., S. B. Baim, T. Shenk, and A. J. Levine. 1990. Conversion of the lac repressor into an allosterically regulated transcriptional activator for mammalian cells. Mol. Cell. Biol. 10:3342–3356.
23. Lee, F., R. Mulligan, P. Berg, and G. Ringold. 1991. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids. Nature (London) 351:226–232.
24. Licht, J. D., W. Hanna-Rose, J. C. Reddy, M. A. English, M. Ro, M. Grossel, R. Shakhnovich, and U. Hansen. 1994. Mapping and mutagenesis of the amino-terminal transcriptional repression domain of the Drosophila Krüppel protein. Mol. Cell. Biol. 14:4057–4066.
25. Licht, J. D., M. Ro, M. A. English, M. Grossel, and U. Hansen. 1993. Selective repression of transcriptional activators at a distance by the Dro-