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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 Tn*10* 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 transfected 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 used. To circumvent limitations due either to the leakiness of utilized promoters or the pleiotropic effects of inducing agents, chimeric transcription factors have been generated, for example, by fusing the strong transcriptional activator domain of VP16 to the Tn*10*-derived prokaryotic tetracycline repressor (TetR) protein (16). In general, chimeric transcription factors of this kind, TetR-VP16 (16), GAL4-VP16 (33), LexA-VP16 (7), and LacR-VP16 (22), are targeted to minimal promoters which are fused to multiple DNA binding sites specific 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 (30) or the estrogen receptor (38) 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 NH_2 terminus of Kox1 was constructed by PCR amplification. A PCR fragment was generated by using two synthetic oligonucleotides, 5′ATCAGGAATTC AACCATGGCTAGATTAGATAAAAG3′ and 5′GTCCTGTCGAC**CTTTCTC TTCTTTTTTGG**CGACCCACTTTCACATTT3′, and pUHD15-1 (16), introducing an *Nco*I site around the translational start of the TetR preceded by an *Eco*RI 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 *Sal*I restriction site (underlined). The resulting PCR fragment was digested with *Eco*RI and *Sal*I. A second PCR fragment was generated by using two oligonucleotides, 5′GACAGGTCGACGGCGGTGGT GCTTTGTC3′ and 5′GCTGCGGATCC**TTA**AACTGATGATTTGATTTC3′, and plasmid pKox1 (35), introducing a *Sal*I site at the NH_2-terminal end of the KRAB domain of Kox1 and a stop codon at position 121 of Kox1 followed by a *Bam*HI restriction site. After digestion with *Sal*I and *Bam*HI, both fragments were ligated into *Eco*RI- and *Bam*HI-digested pUHD10-1 (12), generating pCMV-tetR-KRAB. To generate pCMV-tetR-KRAB-hyg, a *Hin*dIII-*Bsm*I fragment of plasmid p220.3, a derivative of p220.1 (13) (kindly provided by M. Gossen and H. Bujard) containing the hygromycin resistance gene under control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter, was introduced into a *Pvu*II site downstream of the polyadenylation signal in pCMV-tetR-KRAB. To generate pCMV-tetR, pCMV-tetR-KRAB was digested with *Sal*I and *Bam*HI, 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-Stop).

Plasmid ptetO7-CMV-L was generated by ligating the 786-bp *Xho*I-*Bam*HI

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cytomegalovirus (CMV) promoter fragment of pUHD10-1 (12) into *Xho*I-*Bam*HI-digested pUHC13-4 (16). This places seven *tetO* sequences just upstream of the human CMV immediate-early promoter/enhancer (4). Plasmid ptetO7-TK-L was generated by ligating an *Xho*I-*Bgl*II fragment from pT109luc (29) comprising the HSV TK promoter (−109 to +52, both GC boxes and the CCAAT box present) into *Xho*I-*Bam*HI-digested pUHC13-4 (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% CO_2 environment at 37°C. Cells were plated at a density of 10^5 cells per 35-mm-diameter dish and transfected at 80% 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 *Asp* 700. Forty-eight hours after transfection, cells were trypsinized and split at a ratio of 1:10, and resistant colonies were selected in Iscove 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 *Hin*dIII and pNEO5′ (26). Colonies resistant to G418 (500 μg/ml) were grown in presence 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 500 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 in an Eppendorf centrifuge for 10 s. Aliquots (10 μl) of the supernatants were mixed with 350 μl of 25 mM glycylglycine–15 mM $MgSO_4$–5 mM ATP and assayed for luciferase activity (14) in a Lumat LB9501 (Berthold, Wildbad, Germany), using the integral mode (10 s). D-Luciferin (L 6882; Sigma) was used at 1 mM. Protein content of the lysates was determined by the method of Bradford (5).

Generation of Kox1 antiserum. Recombinant Kox1 protein was generated by using the T7 expression vector pAR3039 (31). Briefly, the *Bam*HI cDNA fragment of Kox1 (35) was cloned into the *Bam*HI site of pAR3039, expressed in the *Escherichia coli* host strain BL21(DE3), and the Kox1 protein was purified by Mono S fast protein liquid chromatography (36). Functionally active protein was obtained after renaturing Kox1 in a dialyzing procedure adapted for zinc finger proteins (28a). One rabbit was immunized with renatured Kox1 protein.

Immunofluorescence. HeLa A12 cells were grown in tissue culture chamber slides (Miles Scientific), washed with PBS, fixed in 3% paraformaldehyde in PBS for 10 min, washed three times with PBS, and permeabilized with 0.6% Nonidet P-40 in PBS for 5 min. After washing with PBS, the fixed monolayer was blocked by incubation with 3% bovine serum albumin (BSA) in PBS for 20 min to reduce nonspecific antibody adsorption. Cells were then washed with PBS and incubated for 40 min with the rabbit anti-human Kox1 immune serum diluted 1/500 in 3% BSA in PBS. After four washes with PBS, cells were incubated for 20 min in the dark with a 1/100 dilution of a fluorescein-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim) in 3% PBS. Finally, cells were washed five times with PBS and covered with 4 μl of 90% glycerol–1 mM KI–0.2 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml (for staining of the DNA). Coverslips were applied and sealed in place with clear nail polish. Cells were visualized by fluorescence microscopy with a Zeiss microscope at a magnification of 1:200.

Electrophoretic mobility shift assay. A12 cells were grown in 35-mm-diameter dishes to 80% confluence, washed with PBS, and scraped off the plate, and the sedimented cells were resuspended in 200 μl of a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 25% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM Na_3VaO_4, 25 mM β-glycerophosphate, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. After freeze-thawing and incubation for 10 min on ice, the cellular debris was sedimented (10 min, 13,000 × *g*). Aliquots of total cell extract (2 μg of total protein) were mixed with 2 fmol of ^{32}P-labeled $tetO_2$ DNA (16) in 25 μl of binding buffer containing 10 mM HEPES-KOH (pH 7.9), 1 mM EDTA, 4% Ficoll 400, and 4 μg of sonicated calf thymus DNA. After 15 min at room temperature, the binding reaction mixture was loaded onto a 4% polyacrylamide–0.2% bisacrylamide gel with 0.25× Tris-borate-EDTA (1) as the running buffer. Polyacrylamide gel electrophoresis (PAGE) was performed at room temperature at 7.5 V/cm. For assays in which antibodies were included, binding was allowed for 10 min before addition of 2.5 μl of a 1:10 dilution of serum in binding buffer, and incubation was continued for an additional 5 min at room temperature. When deoxycholate (DOC) was used, appropriate dilutions of the DOC in binding buffer were added after 10 min incubation in absence of DOC. Dried gels were visualized by autoradiography using Kodak AR-5 film.

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 L-methionine-free modified Eagle's medium (GIBCO) supplemented with 5% dialyzed FCS and 500 μCi of L-[^{35}S]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 Na_3VaO_4, 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 precleared 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.

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 NH_2-terminal 121 amino acid residues of Kox1, containing the KRAB domain (35), to the C terminus of the prokaryotic TetR encoded by Tn*10* from *E. coli* (16) 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 TetR-KRAB (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 pUHC13-4 (16). In these constructs, the CMV or HSV TK promoter was inserted downstream of seven *tetO* sequences and upstream of the luciferase as a reporter gene. The right boundary of the fragment containing the seven *tetO* sites 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 *tetO* 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 *tetO* 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 *tetO* sequences and should thereby 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

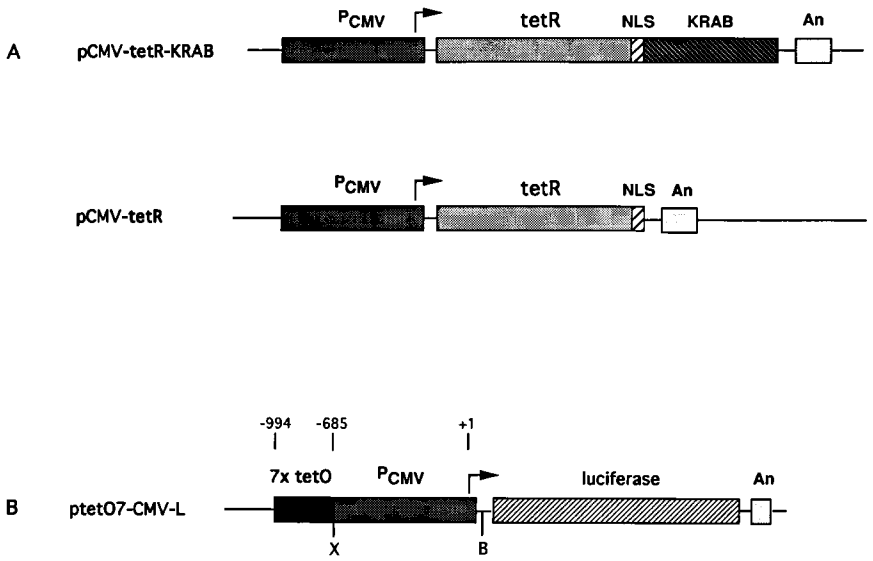

FIG. 1. Schematic representation of main features of expression and reporter constructs. (A) In the expression plasmid pCMV-tetR-KRAB, the TetR-KRAB sequence is placed downstream of the strong immediate-early human CMV promoter (P_{CMV}) and is followed by an SV40 polyadenylation signal (An). The TetR-KRAB 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 NH_2 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 transcriptional 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 transcriptional start site of the CMV promoter and the sites for insertion of the promoter(s), X (*Xho*I) and B (*Bam*HI), are indicated.

or without tetracycline for 1 day (Fig. 2). In transfections using the control vector pCMV lacking TetR sequences, similar luciferase activities were obtained from cells grown with or without 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 μg of tetracycline per ml to the medium. No repression was observed when TetR fused to the nuclear localization 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 construct 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 interfered 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 control of the TK promoter inserted into pCMV-tetR-KRAB (see Materials 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 immunofluorescence 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).

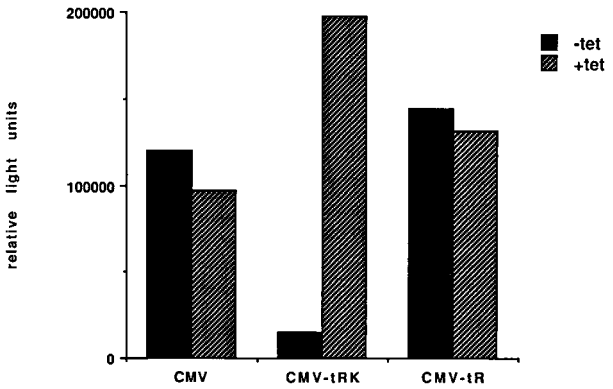

FIG. 2. Tetracycline-reversible silencing of the CMV promoter by TetR-KRAB in transiently transfected HeLa cells. HeLa cells (70 to 80% confluent) grown in 35-mm-diameter dishes without tetracycline (tet) were transiently transfected at a molar ration of 1:5 with the reporter plasmid ptetO7-CMV-L (0.5 μg) and 2.5 μg 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 μg of tetracycline per ml before luciferase activities were determined from aliquots (1/50) of total cell extracts.

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 tetracycline 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

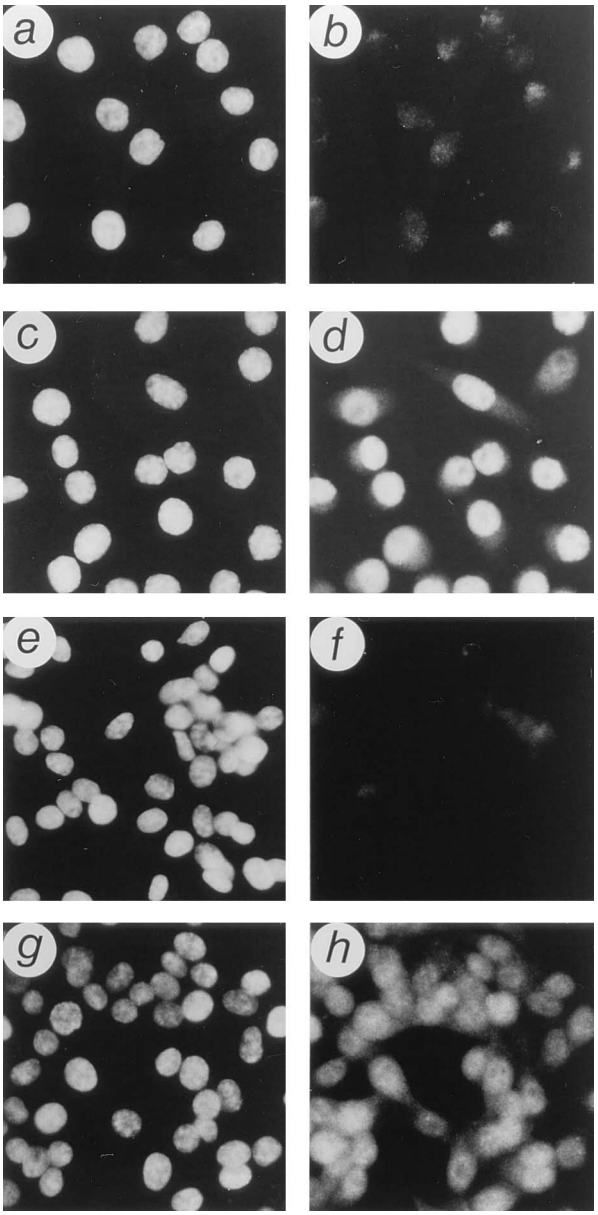

FIG. 3. Expression and nuclear localization of TetR-KRAB fusion protein in HeLa A12 cells. HeLa cells were stably transfected with pCMV-TetR-KRAB-hyg, and stable clones were selected in hygromycin B. The right-hand row of photomicrographs shows indirect immunofluorescence with an anti-Kox1 immune serum of stable transfected HeLa A12 (d) and HeLa (h) cells. A preimmune serum was used as a control for A12 (b) and HeLa (f) cells. Fluorescein-conjugated goat anti-rabbit antibodies were used as secondary antibodies. The DNAs of the respective cells were stained with DAPI to visualize the nuclear compartments and are shown in panels a, c, e, and g.

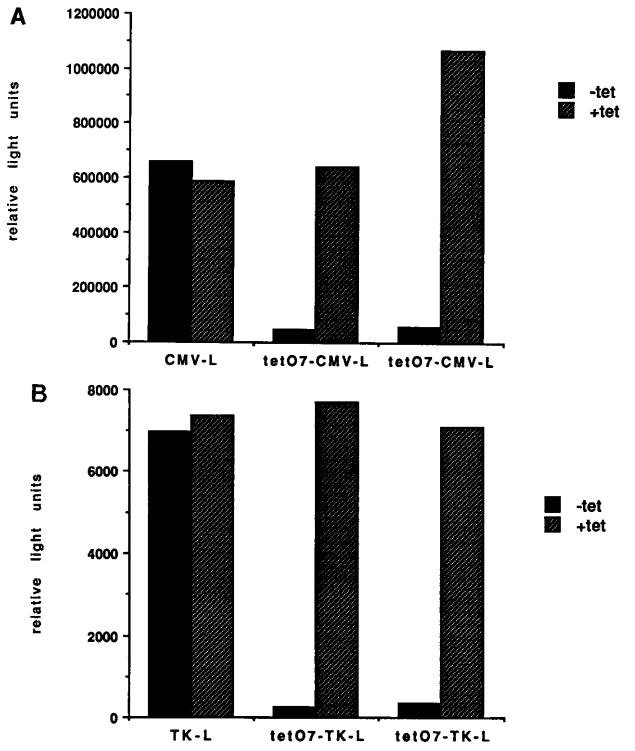

FIG. 4. Tetracycline-reversible silencing of the CMV and HSV TK promoters in HeLa A12 cells stably transfected with pCMV-tetR-KRAB. HeLa A12 cells (70 to 80% confluent) grown in 35-mm-diameter dishes without tetracycline (tet) were transiently transfected in duplicate with, per dish, 2.5 μg of pCMV-L or ptetO7-CMV-L (A) or 2.5 μg of pTK-L or ptetO7-TK-L (B). After transfection, the cells were grown in the presence or absence of 0.5 μg of tetracycline per ml in the medium for an additional 24 h before luciferase activities were determined from 1/50 of total extracts of transfected cells. Two independent experiments are shown for ptetO7-CMV-L (A) and ptetO7-TK-L (B).

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.

Reversible repression of the ptetO7-CMV-L construct stably integrated into the genome of HeLa A12 cells. To quantify the tetracycline-inducible repression of the CMV promoter, the construct ptetO7-CMV-L was stably integrated into HeLa A12 cells that express the TetR-KRAB fusion protein. HeLa A12 cells were cotransfected with ptetO7-CMV-L linearized at the unique *Asp* 700 site and pNeo 5′ (26). One clone resistant to G418 that showed significant luciferase levels when grown in the presence of 0.5 μg of tetracycline per ml was selected and named TIS-10. 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 (Fig. 5). 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 reduce 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 tetracy-

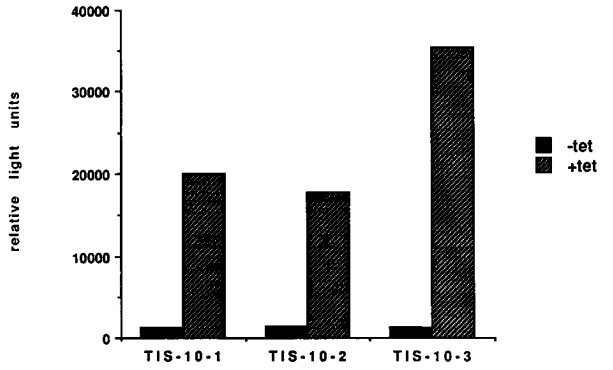

FIG. 5. Tetracycline-inducible silencing of a chromosomally integrated CMV promoter fused to *tetO* sequences. The ptetO7-CMV-L construct was stably integrated into HeLa A12 cells, resulting in the TIS-10 cell line. TIS-10 cells were grown in 35-mm-diameter dishes (70% confluence), and tetracycline (tet; 0.5 μg/ml) was either omitted or added to the growth medium. After 24 h, 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.

cline action on luciferase expression in TIS-10 cells was analyzed in two different ways. First, tetracycline was added to cultures grown in the absence of tetracycline (Fig. 6). By 24 h, the luciferase activity was stimulated 10-fold over the noninduced values. This stimulation had increased to more than 30-fold by 2 days and more than 50-fold after 3 days. In a second experiment, we measured the decrease of luciferase activities after removal of tetracycline. Within 3 days, these levels dropped more than 50-fold (data not 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).

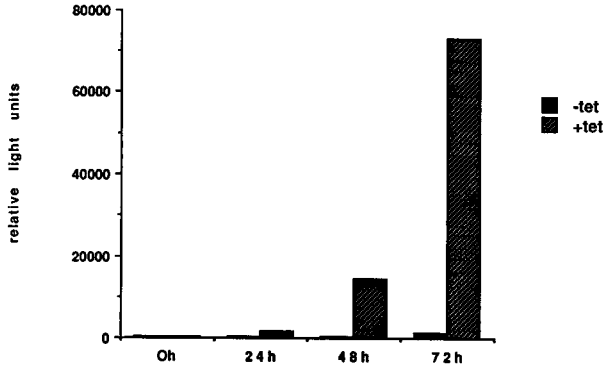

FIG. 6. Time course of tetracycline action in TIS-10 cells. TIS-10 cells were grown in the absence of tetracycline (tet), trypsinized, seeded in 35-mm-diameter dishes at low density (15% confluency), and further grown without or with 0.5 mg of tetracycline per ml in the growth medium. After the indicated times, relative luciferase activities were determined from aliquots of extracts (1/50) of cells.

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-[^{35}S]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 coimmunoprecipitated together with TetR-KRAB (Fig. 8, SMP1, 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 coimmunoprecipitation 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 SMP1.

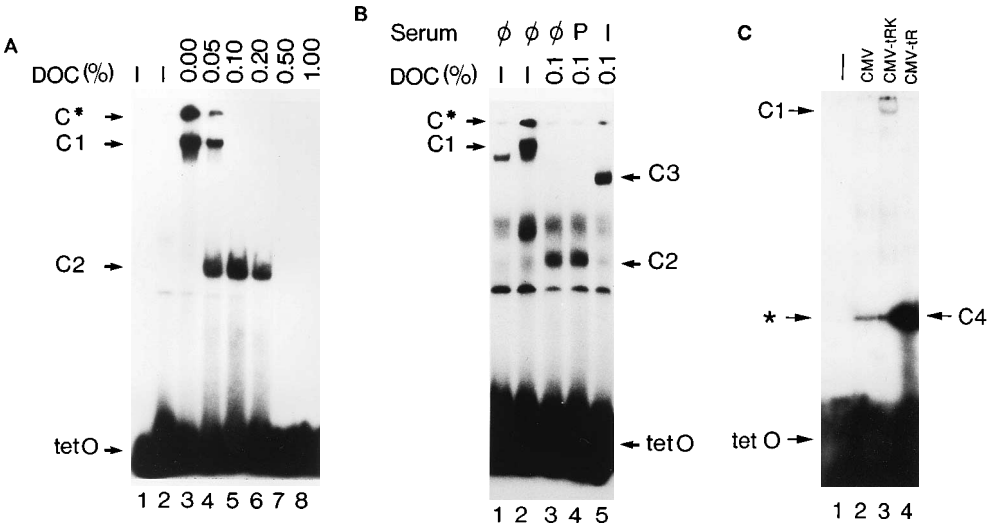

FIG. 7. DNA binding activity of TetR-KRAB from HeLa A12 cells. (A) Crude extracts from HeLa cells (lane 2) or HeLa A12 cells (lanes 3 to 8) were prepared and incubated with ^{32}P-end-labeled *tetO* DNA for 10 min before DOC was added to the indicated final concentration. After further incubation for 5 min, the samples were subjected to electrophoresis in a 4% polyacrylamide gel. As a control, no extract was added (lane 1). The positions of the free *tetO* DNA and complexes C*, C1, and C2 are indicated. (B) Crude extracts from HeLa (lane 1) or HeLa A12 (lanes 2 to 5) cells were prepared and incubated with ^{32}P-end-labeled *tetO* DNA for 10 min at room temperature before DOC was added to 0.1% (final concentration) where indicated (lanes 3 to 5). No serum (lane 3) or either a preimmune serum (P) or an anti-Kox1 immune serum (I) was added afterwards to 1% (final concentration) (lanes 4 and 5), and the samples were subjected to electrophoresis in a 4% polyacrylamide gel. The positions of the free *tetO* DNA and the different complexes (C*, C1, C2, and C3) that were formed are indicated. (C) Crude extracts from HeLa cells, transiently transfected with pCMV (lane 2), pCMV-tetR-KRAB (lane 3), or pCMV-tetR (lane 4), were prepared, and aliquots were incubated with ^{32}P-end-labeled *tetO* DNA for 15 min at room temperature and subjected to electrophoresis in a 5% polyacrylamide gel. The positions of the free *tetO* DNA, the specific complexes C1 and C4, and a nonspecific complex (*) are indicated.

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 NH_2 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 Tn*10*-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-

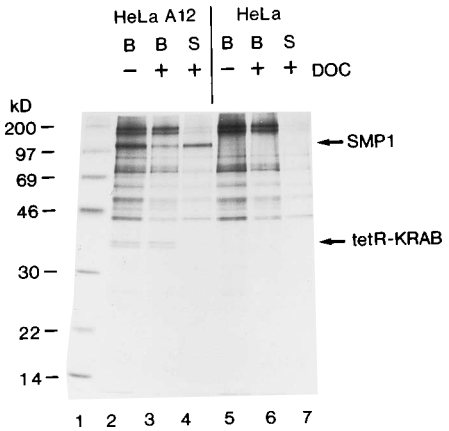

FIG. 8. A protein of around 110 kDa is coimmunoprecipitated with the TetR-KRAB from HeLa A12 cells. Whole cellular extracts of HeLa (lanes 5 to 7) and HeLa A12 (lanes 2 to 4) cells, labeled with L-[^{35}S]methionine, were incubated with an anti-Kox1 immune serum, and the immune complexes were collected with protein G-agarose beads (B; lanes 2 and 5). Half of the beads were incubated with a buffer containing 0.1% DOC, and the supernatants (S, DOC; lanes 4 and 7) were separated from the beads (B, DOC; lanes 3 and 6). The proteins on the beads and in the supernatants were analyzed by SDS-PAGE (12% gel) and fluorography. The positions of the 38-kDa TetR-KRAB fusion protein and a 110- to 120-kDa protein named SMP1 are indicated. The sizes of ^{14}C-labeled protein standards (lane 1) are indicated on the left.

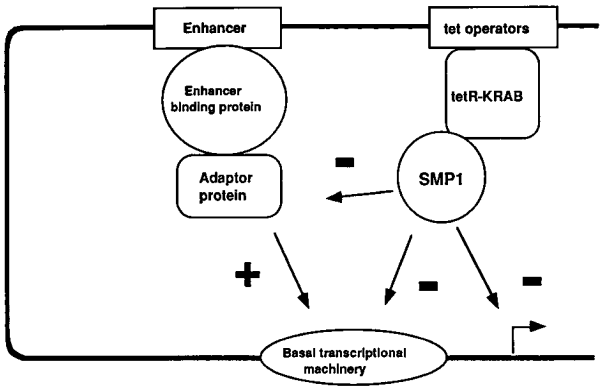

FIG. 9. Model of the silencing mechanism by TetR-KRAB and SMP1. An enhancer-binding protein exerts its stimulatory effect (+) on the basal transcriptional machinery through an adaptor protein. The TetR-KRAB protein binds to the *tetO* sequences, which can be regarded as a *cis*-acting silencer. The silencing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

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 ptetO7-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 in vitro (C1; Fig. 7A, lane 3). Low concentrations of DOC disrupt the large complexes and result in the formation of smaller complexes, presumably composed of TetR-KRAB dimers (C2; Fig. 7A, lanes 4 to 6). The TetR lacking the KRAB domain forms even smaller complexes (C4; Fig. 7C, lane 4). This finding suggests that at least one factor may be associated with TetR-KRAB and released upon treatment with DOC. The immunoprecipitations from extracts of HeLa A12 cells obtained by using the anti-Kox1 immune serum show that a protein of around 110 kDa is associated with the TetR-KRAB protein (Fig. 8). This protein dissociates from TetR-KRAB in the presence of DOC in a manner strikingly reminiscent of the DOC sensitivity of the large TetO/TetR-KRAB complex C1 formed with extracts from HeLa A12 cells (Fig. 7A and 8). The data suggest that the 110-kDa protein is part of the large complex C1 (Fig. 7A, lane 3). We tentatively named this protein SMP1 and propose that it is involved in mediating the silencing function of TetR-KRAB as outlined in Fig. 9. Transcriptional activator proteins bind to sites in the CMV enhancer and activate transcription from the basal transcriptional machinery in part by interacting with adaptor molecules that mediate the transactivation function; the function of SMP1 could be to mediate the silencing effect of the TetR-KRAB protein (Fig. 9). Further work will have to show whether the silencing exerted by the TetR-KRAB protein impinges directly or indirectly (i.e., via SMP1) on factors of the basal transcriptional machinery or if it works by influencing the chromatin structure of the target gene (Fig. 9). It is tempting to speculate that the KRAB-SMP1 interaction may share some similarity to protein-protein interactions that are involved in establishing repression or silencing in *Saccharomyces cerevisiae* as is the case for Ssn6-Tup1 and SIR1 (9, 21, 37).

Apart from the opportunity to understand the phenomenon of transcriptional repression exerted by the KRAB domain of Kox1, our data provide a basis for a novel way to regulate gene expression in higher mammalian cells. In contrast to a previously published system for highly efficient regulation of gene expression using a tTA-responsive promoter (15, 16), the tetracycline-reversible silencing of complex promoters by the TetR-KRAB protein offers the unique possibility of reversibly down-regulating the expression of cellular genes on top of their normal cellular regulation. Here, *tetO* sequences would be placed at a distance (0.1 to 3 kb) upstream or downstream from the transcriptional start site of the target gene such that the normal regulation is not disturbed. Binding of TetR-KRAB expressed in the very same cells results in transcriptional repression that can be released by adding tetracycline to the growth medium. Understanding the mechanism of the silencing exerted by the KRAB domain might allow improvement of the stringency of the system in order to render it applicable for the generation of cell lines or whole animals in which the expression of particular genes can be down-regulated in a reversible fashion.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
3. **Bellefroid, E. J., D. A. Poncelet, P. J. Lecoq, O. Revelant, and J. A. Martial.** 1991. The evolutionarily conserved Krüppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. Proc. Natl. Acad. Sci. USA **88:**3608–3612.
4. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Braselmann, S., P. Graninger, and M. Busslinger.** 1993. A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. Proc. Natl. Acad. Sci. USA **90:**1657–1661.
7. **Brent, R., and M. Ptashne.** 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell **40:**729–736.
8. **Bunker, C. A., and R. E. Kingston.** 1994. Transcriptional repression by *Drosophila* and mammalian polycomb group proteins in transfected mammalian cells. Mol. Cell. Biol. **14:**1721–1732.
9. **Chien, C-T., S. Buck, R. Sternglanz, and D. Shore.** 1993. Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. Cell **75:**531–541.
10. **Clark, A. R., and K. Docherty.** 1993. Negative regulation of transcription in

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. Ansorge, 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. **Kalderon, 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. Carlson, 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) **294:**228–232.
24. **Licht, J. D., W. Hanna-Rose, J. C. Reddy, M. A. English, M. Ro, M. Grossel, R. Shaknovich, 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 Drosophila Krüppel protein. Proc. Natl. Acad. Sci. USA **90:**11361–11365.
26. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequence. Cell **36:**391–401.
27. **Margolin, J., J. R. Friedman, W. K.-H. Meyer, H. Vissing, H.-J. Thiesen, and F. J. Rauscher III.** 1994. Krüppel-associated boxes are potent transcriptional repression domains. Proc. Natl. Acad. Sci. USA **91:**4509–4513.
28. **Mayo, K. E., R. Warren, and R. D. Palmiter.** 1982. The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells. Cell **29:**99–108.
28a. **Meyer, W. K.-H., and H.-J. Thiesen.** Unpublished data.
29. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
30. **Picard, D., S. J. Salser, and K. R. Yamamoto.** 1988. A movable and regulatable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell **54:**1073–1080.
31. **Rosenberg, A. H., B. N. Lade, D. S. Chui, S. W. Lin, J. L. Dunn, and F. W. Studier.** 1987. Vectors for selective expression of cloned DNA's by T7 RNA polymerase. Gene **56:**125–135.
32. **Ryals, J., P. Dierks, H. Ragg, and C. Weissmann.** 1985. A 46 nucleotide promoter segment from an IFN-α gene renders an unrelated promoter inducible by virus. Cell **41:**497–507.
33. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) **335:**563–564.
34. **TenHarmsel, A., R. Austin, N. Saenelli, and M. D. Biggin.** 1993. Cooperative binding at a distance by even-skipped protein correlates with repression and suggests a mechanism of silencing. Mol. Cell. Biol. **13:**2742–2752.
35. **Thiesen, H.-J.** 1990. Multiple genes encoding zinc finger domains are expressed in human T cells. New Biol. **2:**363–374.
36. **Thiesen, H.-J.** 1990. Target detection assay: a general method to determine DNA binding sites for putative DNA-binding proteins. Immunol. Methods **4:**61–74.
37. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature (London) **369:**758–761.
38. **Webster, N. J. G., S. Green, J. R. Jin, and P. Chambon.** 1988. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation domain. Cell **54:**199–207.
39. **Witzgall, R., E. O'Leary, A. Leaf, D. Önaldi, and J. V. Bonventre.** 1994. The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. Proc. Natl. Acad. Sci. USA **91:**4514–4518.
40. **Wurm, F. M., K. A. Gwinn, and R. E. Kingston.** 1986. Inducible overexpression of the mouse c-myc protein in mammalian cells. Proc. Natl. Acad. Sci. USA **83:**5414–5418.