

The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression

(transcription factors/*Kid-1*/ZNF2/kidney)

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ABSTRACT We have previously reported the cloning, sequencing, and partial characterization of *Kid-1*, a zinc finger-encoding cDNA from the rat kidney. The *Kid-1* protein and approximately one-third of all other zinc finger proteins contain a highly conserved region of ≈ 75 amino acids at their NH₂ terminus named Krüppel-associated box (KRAB), which is subdivided into A and B domains. The evolutionary conservation, wide distribution, and genomic organization of the KRAB domains suggest an important role of this region in the transcriptional regulatory function of zinc finger proteins. The functional significance of the KRAB domain was evaluated by studying transcriptional activities of yeast GAL4–rat *Kid-1* fusion proteins containing various regions of the non-zinc-finger domain of *Kid-1*. Transcriptional repressor activity of GAL4–*Kid-1* fusion proteins maps to the KRAB-A domain. The KRAB-A domain of another zinc finger protein, ZNF2, also has repressor activity. Site-directed mutagenesis of conserved amino acids in this motif results in decreased repressor activity. Thus, we have established a functional significance for the KRAB-A domain, a consensus sequence common in zinc finger proteins.

A limited number of structural motifs have been described for eukaryotic transcription factors. A common motif is the zinc finger structure, which is highly conserved and found in many different species (1). Though the crystal structure of a co-crystal between the three zinc fingers of *zif-268* (*Egr-1*) and their binding site has been elucidated (2), little is known about the interaction between zinc fingers and DNA and how these protein–DNA interactions are involved in the regulation of transcription.

Approximately one-third of all zinc finger proteins contain an evolutionarily conserved region of about 75 amino acids at their NH₂ terminus—the Krüppel-associated box (KRAB), a sequence motif of hitherto unknown function. Similar to regions found in a subset of homeodomain proteins (the paired box and POU domain), the KRAB domain is rich in charged amino acids (3). Because of the potential α -helical structure of KRAB domains, it has been proposed that this domain mediates protein–protein interactions and functions as a transcriptional regulatory domain (3), but this hypothesis has not been previously tested.

Kid-1 is a rat zinc finger gene that is expressed primarily in the kidney (4). *Kid-1* mRNA levels are regulated in renal ontogeny and during repair after ischemic and folic acid insults to the kidney (4). *Kid-1* encodes a 68-kDa protein with 13 zinc fingers. When COS or LLC-PK₁ cells are transfected with fusion constructs encoding the non-zinc-finger region of rat *Kid-1* and the yeast GAL4 DNA-binding domain, chloramphenicol acetyltransferase (CAT) activity from cotransfected reporter constructs containing GAL4 binding sites and

either a minimal promoter or a simian virus 40 (SV40) enhancer is strongly repressed (4). We designed experiments to test the hypothesis that the transcriptional repressor activity resided in the KRAB domain of *Kid-1* (Fig. 1). Our mutagenesis studies with the *Kid-1* protein define the KRAB-A domain as a strong transcriptional repressor motif.

METHODS

Expression Plasmids. Plasmids encoding chimeric proteins of the DNA-binding domain (amino acids 1–147) of GAL4 and various parts of the non-zinc-finger region of *Kid-1* (designated *Kid-1N*) were constructed from pBXG1/*Kid-1N* in sense orientation (4) (designated pBXG1/*Kid-1N,s*), encoding a GAL4–*Kid-1* fusion protein containing the entire non-zinc-finger region of *Kid-1* (Fig. 2). pBXG1/*Kid-1,AB* was made by vector self-ligation after cutting pBXG1/*Kid-1N,s* with *Bam*HI. To construct pBXG1/*Kid-1,A(-)* lacking the *Kid-1* KRAB-A domain, pBXG1/*Kid-1N,s* was digested with *Eco*RI and filled in by using the Klenow fragment. The insert was subsequently removed by digestion with *Xba* I, and the vector fragment was isolated. Insert was prepared by digesting pBXG1/*Kid-1N,s* with *Nco* I, filling in the overhang with the Klenow fragment, and releasing the insert with *Xba* I. To construct pBXG1/*Kid-1,A* and pBXG1/*Kid-1,B*, fragments containing the *Kid-1* KRAB-A- or B-encoding sequences (designated *Kid-1A* and *Kid-1B*) were PCR-amplified from pBXG1/*Kid-1,AB* with primers containing an *Eco*RI site or *Xba* I site. PCR products were digested with *Eco*RI and *Xba* I and cloned into the *Eco*RI/*Xba* I-cut pBXG1 vector. To construct pBXG1/ZNF2,A, the exon encoding the KRAB-A domain of ZNF2 (designated ZNF2,A) was PCR-amplified from human genomic DNA with primers containing *Eco*RI and *Xba* I sites and subcloned into *Eco*RI/*Xba* I-cut pBXG1. pCDM8/*Kid-1, ℓ* , in which *Kid-1, ℓ* signifies coding region for the full-length *Kid-1* protein, was engineered by ligating *Bst*XI-adapters to a *Kid-1* cDNA fragment extending from nucleotide 312 to 2123 [as numbered in the cDNA (4), where the putative start codon is at 312 and the stop codon is at 2040] and ligating it into *Bst*XI-cut pCDM8. PCR products were sequenced in their entirety by the chain-termination method. Cloning sites of all constructs were sequenced (5).

Site-Directed Mutagenesis. A PCR-based strategy (6) was used to construct mutants with single amino acid changes in the KRAB-A domain of *Kid-1* (*Kid-1,A*). Two PCR reactions were run with pBXG1/*Kid-1,A* as a template. In the first reaction, one primer, “F,” complementary to the 3'-end of GAL4, was paired with a primer containing the desired

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Abbreviations: KRAB, Krüppel-associated box; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase. PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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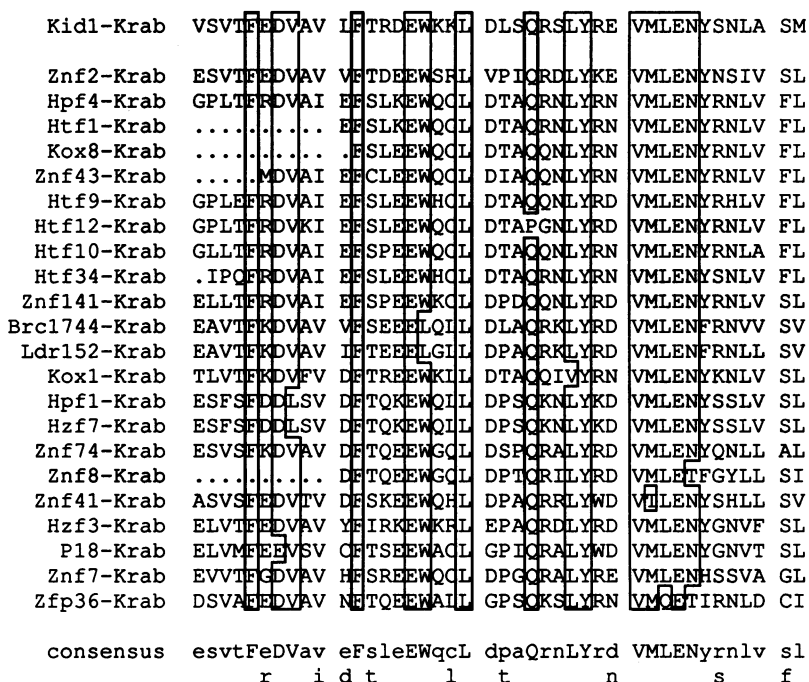


FIG. 1. Comparisons of KRAB-A regions of various zinc finger proteins. The second exon of Kid-1 encodes amino acids 12 through 53. A consensus sequence is presented at the bottom of the alignment with capital letters indicating at least 90% identity among the various proteins and with small letters indicating at least 25% identity but less than 90%. When more than one amino acid is common, the less frequent one is listed on the second line of the consensus sequence.

mutation. For the second reaction, the primer "R," complementary to the 3' end of Kid-1,A was paired with a primer overlapping the region of the mutation. The products from these two PCR reactions overlapped over a stretch of at least nine nucleotides and were used in a third PCR reaction with primers "F" and "R," which in ~50% of the cases yielded a full-length Kid-1,A fragment with the desired nucleotide change(s). PCR products were digested with *EcoRI* and *Xba* I and cloned into the *EcoRI/Xba* I-cut pBXG1 vector. The absence of additional mutations was verified by sequencing the entire PCR product. Mutations are named by listing the wild-type amino acid first, followed by the position in Kid-1 (Fig. 1) and the amino acid to which it is changed.

Transfection Protocols and CAT Assays. COS cells were plated 2 days prior to transfection at a density of $\approx 2.5 \times 10^5$ cells per 100-mm dish. For transfections, cells were exposed to 20 μ g of total DNA in 5 ml of DMEM/10% NuSerum (Collaborative Biomedical Products, Bedford, MA)/400 μ g of DEAE-dextran per ml/0.1 mM chloroquine. One microgram of a luciferase-expressing plasmid, poLucSV/T1 (7), was included in all cotransfection experiments to normalize transfection efficiencies. Three to 4 hr after the addition of DNA, medium was removed, and cells were shocked for 2 min at room temperature with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS). After shock treatment, cells were washed once with PBS, and new medium was added.

Forty-eight hours after transfection, cells were washed twice with PBS, scraped with a rubber policeman into a microcentrifuge tube, and pelleted. The cell pellet was resuspended in 200 μ l of 0.25 M Tris chloride (pH 7.8) and subsequently broken up by subjecting it to a freeze/thaw cycle three times in a dry ice/ethanol bath and 37°C water bath. The supernatant was assayed for CAT and luciferase activities according to standard protocols (4). CAT activity is expressed as the ratio of monoacetylated [¹⁴C]chloramphenicol/(monoacetylated plus nonacetylated) [¹⁴C]chloramphenicol and normalized to luciferase activity.

Protein Gels and Western Blots. SDS/PAGE gels and Western immunoblots were performed according to standard protocols (8). An anti-GAL4 antibody (obtained from S. A. Johnston and K. Melcher, University of Texas Southwestern Medical Center) was used at a 1:1000 dilution. Immune complexes were detected with the Renaissance light-detection kit from DuPont.

RESULTS

To define the region in the Kid-1 protein that is able to mediate its repressor effect on transcription, we generated a set of expression plasmids encoding chimeras of the DNA-binding region of GAL4 with various regions of the NH₂ terminus of Kid-1 (Fig. 2). A plasmid that encoded GAL4-(1-147) and only the KRAB-A and -B domains (pBXG1/Kid-1,AB) induced a very similar level of transcriptional repression of CAT activity of the cotransfected pG5SV-BCAT reporter plasmid as the sense construct encoding the full-length non-zinc-finger region of Kid-1 (Kid-1N,s) (Fig. 3). The mean residual CAT activities in cells expressing Kid-1N,s or Kid-1,AB, were $16.7 \pm 1.9\%$ and $6.6 \pm 1.1\%$, respectively, when compared to cells transfected with an antisense construct of Kid-1N (pBXG1/Kid-1N,as), which served as a negative control (Fig. 3). CAT activity in cells cotransfected with the antisense expression plasmid pBXG1/Kid-1N,as was equal to the activity obtained from cells transfected with pBXG1, which encoded only the DNA binding region of GAL4 (data not shown). Transfection of pBXG1/Kid-1,A resulted in repression of transcription to $15 \pm 1.5\%$ of antisense CAT activity, a degree of repression almost identical to that seen with the plasmid encoding Kid-1N,s. Conversely, when the KRAB-A region was deleted from the NH₂ terminus of Kid-1 [pBXG1/Kid-1,A(-)] or when the construct encoded only the KRAB-B domain (pBXG1/Kid-1,B), transcriptional repression was almost completely absent. To demonstrate that a lack of repressor

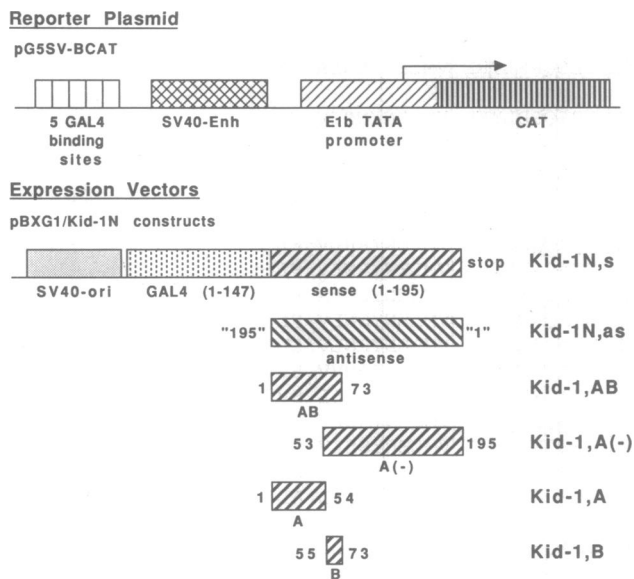


FIG. 2. Reporter and expression plasmids. pG5SV-BCAT contains five GAL4 binding sites upstream of the SV40 enhancer and the TATA box from the *E1B* promoter, which drive a CAT gene. In the expression vectors, transcription is driven by the SV40 enhancer. The DNA binding domain of GAL4 (amino acids 1–147) lies at the NH₂ terminus of fusion proteins with various portions of Kid-1. “Sense (1–195)” contains the first 195 amino acids of Kid-1 (including the first pair of cysteines of the zinc finger domain). In the antisense “as” construct, the orientation of the Kid-1 fragment is reversed. In the “AB” chimera, the KRAB-A and -B domains (amino acids 1–73) are fused to GAL4. In “A(–),” amino acids 53–195, lacking the KRAB-A domain, are fused to the GAL-4 DNA-binding domain. Mutants “A” and “B” code for fusion proteins of the GAL4 DNA-binding domain and the KRAB-A and -B domains of Kid-1, respectively.

activity of pBXG1/Kid-1N,as or pBXG1 was not simply due to lack of expression of the protein, we transfected COS cells with 3 μ g of either pBXG1/Kid-1N,s or pBXG1/Kid-1,A or 15 μ g of pBXG1 or pBXG1/Kid-1N,as. CAT activities were 21% (pBXG1/Kid-1N,s), 31% (pBXG1/Kid-1,A), and 108% (pBXG1) of the CAT activity in cells transfected with pBXG1/Kid-1N,as. Expression of proteins encoded by these plasmids was confirmed by Western blot analysis with an anti-GAL4 antibody (Fig. 4).

Similar to our previous observations with the pBXG1/Kid-1N,s plasmid (4), the pBXG1/Kid-1,A construct repressed transcription in a dose-dependent fashion (Fig. 5) with virtually identical efficiency as the full-length non-zinc-finger region construct (4). The plasmid, pBXG1/ZNF2,A, encoding a fusion protein of GAL4(1–147) with the KRAB-A region of ZNF2, was as effective a repressor as pBXG1/Kid-1N,s (Fig. 6). This indicates that the repressor activity of the KRAB-A domain is not specific to Kid-1.

To establish that binding to the GAL4 site on the reporter was necessary for transcriptional inhibition, the cDNA encoding the full-length Kid-1 protein (i.e., including all 13 zinc fingers) was subcloned into the expression vector pCDM8, yielding the construct pCDM8/Kid-1, ℓ . The binding site for the Kid-1 protein is undefined, but likely distinct from the GAL4 binding site. Cotransfection of pCDM8/Kid-1, ℓ with pG5SV-BCAT did not result in a reduction of CAT activity (Fig. 7).

Point mutations in the KRAB-A region of Kid-1 were generated by PCR to evaluate the importance of individual amino acids, which were highly conserved in all KRAB-A domains for which sequence information was available (Fig. 8). Each of the mutants repressed transcription from the CAT gene to a lesser extent than the wild-type KRAB-A domain. In the case of the

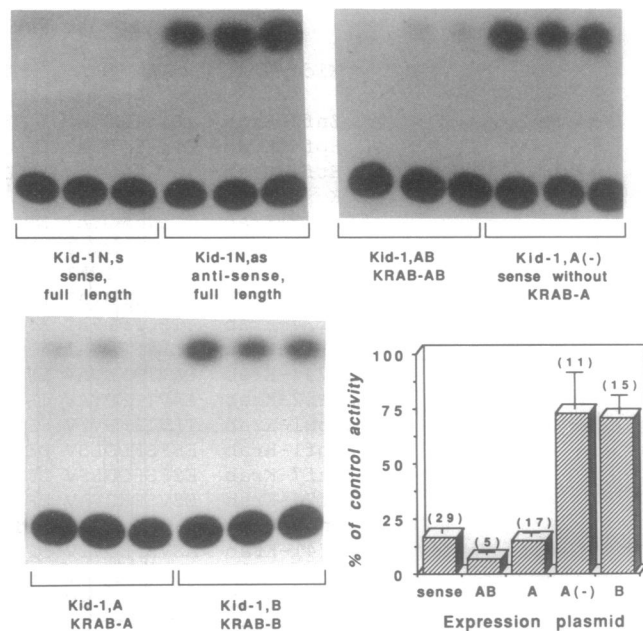


FIG. 3. Thin-layer chromatography assays of CAT activities in cells transfected with the pBXG1 expression constructs encoding fusion proteins of the DNA-binding region of GAL4 with Kid-1N,s, Kid-1N,as, Kid-1,AB, Kid-1,A(–), Kid-1,A, and Kid-1,B. Expression of fusion proteins containing Kid-1N,s, Kid-1,AB, or Kid-1,A results in marked repression of CAT activity, when compared with the control antisense construct (pBXG1/Kid-1N,as). In contrast, when the KRAB-A domain is missing from the NH₂ terminus of Kid-1 [Kid-1,A(–) or Kid-1,B], CAT activity is similar to that observed in the presence of the control antisense plasmid. (Lower Right) Graph providing quantitative CAT activity, with CAT activity obtained with the antisense pBXG1/Kid-1N,as plasmid taken as 100%. The numbers above the standard error bars indicate the number of independent experiments.

Phe-16, Val-19, and Leu-31 mutations, no difference was seen between a conservative (Phe \rightarrow Ala, Val \rightarrow Ala, or Leu \rightarrow Ala) and a nonconservative (Phe \rightarrow Asp, Val \rightarrow Glu, or Leu to Glu)

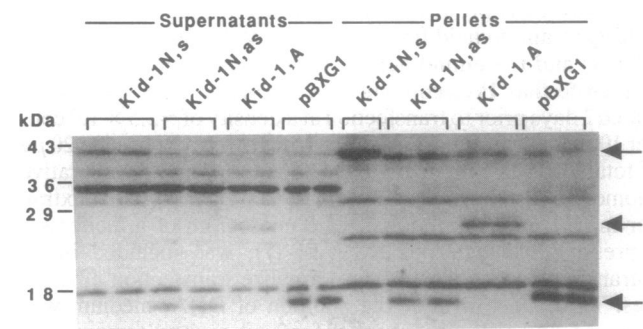


FIG. 4. Western blot with GAL4 antibodies. COS cells were transfected with 3 μ g of either pBXG1/Kid-1N,s or pBXG1/Kid-1,A or with 15 μ g of pBXG1 or pBXG1/Kid-1N,as. Pellets obtained after subjecting the transfected cells to freeze/thaw cycles were resuspended in 200 μ l of 1 \times PBS and sonicated; 50 μ l of 5 \times SDS sample buffer containing 625 mM Tris-HCl, 12.5% SDS, 50% glycerol, 12.5% 2-mercaptoethanol, and 0.05% bromophenol blue was added, and the samples were boiled for 5 min. Aliquots of the supernatants and pellets corresponding to equal amounts of luciferase activity were run on a 13% gel and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). GAL4 and GAL4 fusion proteins were detected with an anti-GAL4 antibody. Some of the smaller proteins leaked out into the supernatant during the freeze/thaw process. Arrows point to the fusion proteins. The GAL4/Kid-1N,s band is above an unrelated band similar in size.

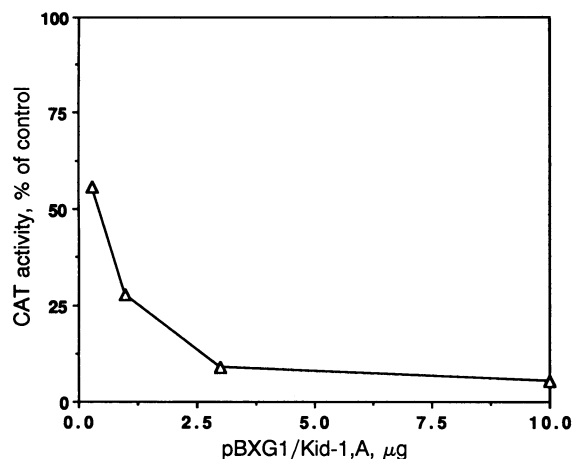


FIG. 5. Quantitation of CAT activities from thin-layer chromatography assays of cells transfected with various amounts of pBXG1/Kid-1,A and 3 µg of the pG5SV-BCAT reporter plasmid. There is a dose-dependent inhibition of CAT activity with increasing amounts of pBXG1/Kid-1,A transfected. CAT activities were unchanged with equivalent amounts of transfected plasmid encoding GAL4/Kid-1N antisense (pBXG1/Kid-1N,as; data not shown).

mutation. For the Tyr-39 mutations, a difference was seen between an alanine-substituted mutant and a mutant with an acidic residue. The loss of repressor activity was most pronounced for Tyr-39 → Asp. Tyr-39 → Ala and Glu-45 → Ala showed the strongest repressor effects among all mutants examined. While there was some variation in expression, each of the mutants was expressed at a level similar to that of the wild-type KRAB-A domain as determined by Western blots with anti-GAL4 antibodies (Fig. 8).

DISCUSSION

Our data provide functional evidence for the importance of the KRAB-A domain, a highly conserved motif found in many zinc finger proteins. It has been estimated that approximately one-third of the hundreds of zinc finger genes in the mammalian genome are members of the KRAB family (3). With this widespread conservation of the KRAB domain, it is possible that transcriptional repressor activity is associated with many members of the zinc finger family.

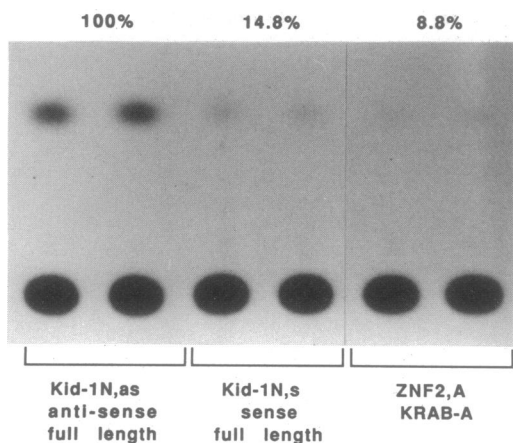


FIG. 6. Thin-layer chromatography assays of CAT activities in cells transfected with either pBXG1/Kid-1N,s or pBXG1/Kid-1N,as or an expression construct encoding the GAL4 DNA-binding domain and the KRAB-A domain of ZNF2. The KRAB-A domain from the human zinc finger protein ZNF2 represses transcription to an extent similar to that seen with Kid-1N,s.

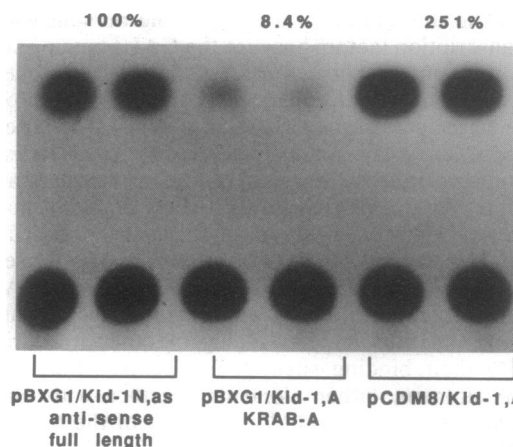


FIG. 7. Thin-layer chromatography assays of CAT activities in cells transfected with an expression construct encoding the full-length Kid-1 protein in pCDM8 (pCDM8/Kid-1,ℓ) or with Kid-1N,as or Kid-1,A in pBXG1. pCDM8/Kid-1,ℓ, which encodes Kid-1,ℓ without a GAL4 DNA-binding domain, has no effect on CAT activity when cotransfected with pG5SV-BCAT. The pCDM8 vector is very similar in design to the pBXG1 vector in that it can replicate extrachromosomally to very high copy numbers in COS cells because of its SV40 origin of replication.

At present we do not understand how the KRAB-A domain represses transcription. Our experiments with pCDM8/Kid-1,ℓ suggest that the KRAB-A domain has to be bound (indirectly through a DNA-binding domain) to DNA to be able to repress transcription. It does not suffice to overexpress a KRAB-A-containing protein like Kid-1 to repress transcription, arguing against a “squenching effect” by KRAB proteins. This lack of “squenching effect” infers specificity, since there are potentially large numbers of zinc finger proteins of the KRAB family expressed in a given cell with the specificity conferred by the DNA-binding characteristics of the specific zinc finger protein. In addition, the repressor effect of the GAL4/Kid-1,A construct cannot be attributed to “competition” with other factors for binding at the GAL4 binding sites because there are no known mammalian transcription factors that can bind to GAL4 binding sites, and a GAL4/Kid-1,A(-) mutant had only a minor repressor effect. We very likely can also rule out a purely “steric model” of repression in which the KRAB-A domain

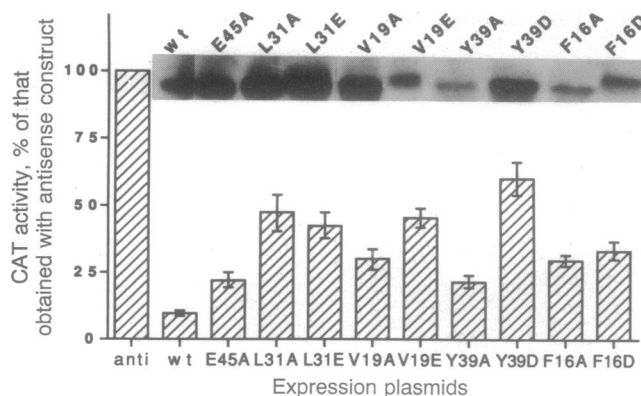


FIG. 8. CAT activities and Western blot of GAL4(1-147)-Kid-1,A fusion proteins with point mutations in the KRAB-A domain. CAT activities are shown as the percentage of the CAT activity from cells transfected with pBXG1/Kid-1N,as (“anti”); n = 5-10 for each construct. Western blots were performed as described in Fig. 4. Only the pellet fractions are shown. The mutants were all expressed, with most expressed at levels greater than or equal to levels of wild-type Kid-1N,s (wt).

prevents the interaction of a trans-acting domain with the basal transcription factors because the GAL4 binding sites in our reporter construct lie upstream of the SV40 enhancer and not between the SV40 enhancer and the TATA box and because larger proteins such as GAL4/Kid-1, A(-) are only weak repressors. The finding that GAL4/Kid-1N_s is also able to repress transcription from a basal promoter (4) argues against the hypothesis that the KRAB-A domain "masks" the activator region of a positively acting transcription factor. At present, we suggest the following two models for repression by the KRAB-A domain. Binding of a KRAB-A-containing protein may lead to a change in local "chromatin structure" and thus impair binding of other transcription factors to their binding sites. It is also possible that the KRAB-A domain, either alone or together with another interacting protein, prevents "assembly" of the basal transcription factors or "locks" them in an inactive state and therefore inhibits transcription.

Each of the point mutations we generated showed a loss of repressor activity although to various extents, emphasizing the importance of the absolutely conserved residues in the KRAB-A domain. Glu-45 → Ala and Tyr-39 → Ala were the mutants with the highest remaining repressor activity. In three of four residues examined (Phe-16, Val-19, and Leu-31), no difference was detected between a conservative and a nonconservative mutation, whereas in the other case (Tyr-39), a conservative mutation affected the repressor activity to a lesser extent than an acidic residue substitution. Among all the mutants, the Tyr-39 → Asp mutant repressed CAT activity least. The introduction of an acidic amino acid for Tyr-39 may result in the loss of interaction with the natural partner of the KRAB-A domain.

The KRAB-A domain is encoded by a single exon in the rat *Kid-1* gene (9) and the human *ZNF2* (10) and *ZNF45* (11) genes. A corresponding arrangement can be assumed for the human *ZNF43* gene, where one alternatively spliced mRNA species has been identified that lacks the KRAB-A region (12). Hence, by alternative splicing, cells may produce zinc finger proteins with or without the KRAB-A transcriptional repressor domain.

While there are at least four well-defined types of transcriptional activation domains, serine/threonine-rich, acidic, proline-rich, and glutamine-rich (13), to our knowledge the only other well-defined motif that has been postulated to mediate transcriptional repressor activity is an alanine-rich domain found in four transcriptional repressors from *Drosophila*: Krüppel (14), engrailed (15), even-skipped (16), and AEF-1 (14). In other cases where a repressor domain has

been delineated, such as Egr-1 (17), SRF (18), and E4BP4 (19), no obvious consensus sequence motifs have been identified. Our data indicate that the KRAB-A domain represents a widely distributed transcriptional repressor motif.

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