Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1015–1020, February 1996
Biochemistry

Krüppel-associated box-mediated repression of RNA polymerase II promoters is influenced by the arrangement of basal promoter elements

(Krüppel-associated box domain/initiator element/TATA box)

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Communicated by Robert P. Perry, Fox Chase Cancer Center, Philadelphia, PA, October 16, 1995 (received for review August 7, 1995)

ABSTRACT **The evolutionarily conserved Krüppel-associated box (KRAB) is present in the N-terminal regions of more than one-third of all Krüppel-class zinc finger proteins. Recent experiments have demonstrated that the KRAB-A domain tethered to a promoter DNA by connecting to heterologous DNA-binding protein domain or targeted to a promoter-proximal RNA sequence acts as a transcriptional silencing of RNA polymerase II promoters. Here we show that expression of KRAB domain suppresses *in vivo* the activating function of various defined activating transcription factors, and we demonstrate that the KRAB domain specifically silences the activity of promoters whose initiation is dependent on the presence of a TATA box. Promoters whose accurate transcription initiation is directed by a pyrimidine-rich initiator element, however, are relatively unaffected. We also report *in vitro* transcription experiments indicating that the KRAB domain is able to repress both activated and basal promoter activity. Thus, the KRAB domain appears to repress the activity of certain promoters through direct communication with TATA box-dependent basal transcription machinery.**

While much attention has been focused on understanding how cellular transcription factors activate gene transcription, increasing evidence suggests that regulation of many genes is the result of a balance between positive and negative regulatory proteins. However, compared to activators, the number of transcriptional repressors that have been characterized is small. Analyses of the mechanisms of transcriptional repression by sequence-specific DNA-binding negative regulatory proteins have thus far revealed several distinct functional classes. (*i*) A repressor protein binds to DNA and may lead to a change in local "chromatin structure" and thus impair binding of other transcription factors to their cognate binding sites (1). (*ii*) Transcriptional repression can result from competition for the same DNA-binding sites or steric hindrance between repressors and positively acting transcription factors. Because this type of repression results from displacement of transcription factors from the DNA, such repressors might not necessarily possess an active repression function (2–5). (*iii*) Proteins that do not bind DNA directly but instead recognize their appropriate DNA-bound proteins (6, 7)—for example, the adenovirus E1B 55K protein represses p53-mediated activation upon binding to p53 without displacing it from its DNA-binding site (7). In yeast, $\alpha 2$ and MCM1 recruit the SSN6/TUP1 repressor (8, 9). (*iv*) Proteins that recognize a DNA element that can function in an orientation and distance-independent manner to block (silence) the formation of an active transcription complex. In this case, the repressor (silencer) could function in a promoter and distance-independent manner (10–12). (*v*) Transcription factors that activate in one circumstance and repress in another. The *Drosophila* Krüppel acts as such a dual-function regulator. *In vitro* monomeric Krüppel protein interacts with TFIIB to activate transcription, whereas an interaction of the Krüppel dimer with TFIIE results in transcription repression (13).

We and others have recently reported that the evolutionarily conserved protein domain, called Krüppel-associated box (KRAB), present in the N terminus of a large number of Krüppel-type zinc finger proteins is a strong repressor domain (11, 14, 15). Hundreds of genes encoding Krüppel-type zinc finger domains have been isolated, identifying them as a major class of eukaryotic DNA-binding proteins (16). The exon/intron organization of several KRAB zinc finger protein genes indicated that the two modules (KRAB-A and KRAB-B) of the KRAB domain are encoded by two separate exons (17–19). The repression property of the KRAB domain resides in the A module, whereas the B module does not appear to contribute significantly to repression of the KRAB domain (11, 14, 15). We have recently determined that the KRAB domain is functional when targeted to a promoter-proximal RNA sequence (20).

To begin to decipher how the KRAB domain transmits its repression signal to the promoter, we report a number of different but complementary *in vivo* and *in vitro* assays that have allowed us to delineate some properties of KRAB-mediated repression. *In vivo* transfections, in which the KRAB repressor and a defined activating domain were both targeted to a promoter, indicated that the presence of specific basal promoter elements can profoundly affect KRAB-mediated repression. The *in vitro* transcription assays indicated that the KRAB domain is able to repress both activated and basal promoter transcriptional activity and this repression requires that a TATA box be located on the promoter. Thus, the arrangement of basal promoter elements may provide a mechanism for differential response to KRAB-mediated repression.

MATERIALS AND METHODS

Reporter Plasmids. The human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR)-based reporters 83-HIV-CAT, G5-83-HIV-CAT, and G5-38-HIV-CAT have been described (CAT, chloramphenicol acetyltransferase) (12, 20). T7G5TATA was constructed by cloning the 7X tetracycline operator (tetO) sequences, obtained by digestion of the plasmid pHUC13-3 (21) with *Xho* I and *Sma* I, upstream of the GAL4 DNA-binding domain of G5E1b (22) digested with *Xho* I/*Hin*dIII. The plasmid T7G5-I was constructed in two steps. (*i*) The double-stranded oligonucleotide (upper strand, 5′-

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Abbreviations: KRAB, Krüppel-associated box; TetR, tetracycline repressor; tetO, tetracycline operator; Inr, initiator element; HIV, human immunodeficiency virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; AdMLP, adenovirus major late promoter.

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CTAGAGCGTTCGTCCTCACTCTCTTCCGCGCGGTT-AC-3′) containing the adenovirus major late promoter (AdMLP) initiator element (Inr) sequence flanked by the *Xba* I and *Kpn* I sites was cloned into *Xba* I/*Kpn* I sites of the plasmid G5E1b. Then the 7X tetO sequences, recovered by digestion with *Xho* I/*Pst* I from the plasmid T7G5TATA, were inserted into *Xho* I/*Pst* I upstream of the GAL4 DNA-binding sites. In a similar manner, the plasmid T7G5TATA-I was constructed by first cloning the double-stranded oligonucleotide (upper strand, 5′-GATCCGCGTTCGTCCTCACTCTC-TTCCGCGGTAC-3′) containing the AdMLP Inr sequence flanked by the *Bam*HI and *Kpn* I sites into G5EIb digested with *Bam*HI/*Kpn* I. Successively, the 7X tetO sequences, recovered by digestion with *Xho* I/*Pst* I from the plasmid T7G5TATA, was inserted in the *Xho* I/*Pst* I sites upstream of the GAL4 sites. The G5-Sp-Inr was constructed by inserting in the *Xba* I site of T7G5-I upstream of the Inr sequences an oligonucleotide containing two Sp1 DNA-binding sites derived from the early simian virus 40 promoter. The plasmid constructions were analyzed by DNA sequencing.

Effector Plasmids. GAL4-VP16, GAL4-SP1, GAL4-E1A, GAL4-p65rel(A), and GAL4-E2F have been described (22–24). GAL4–KRAB (previously named GAL4-18 KRAB) has been described (11) and it encodes a GAL4 fusion protein consisting of the GAL4 DNA-binding domain fused to 55 amino acids of the KRAB-A domain present in the p18 cDNA (25). To construct the tetracycline repressor (TetR)-KRAB effector plasmid, the TetR DNA-binding coding region (aa 1–206) was PCR amplified from pUHD15-1 (21) with 5′ *Hin*dIII and 3′ *Eco*RI adapter primers (5′ primer, 5′-CCCG-CAAGCTTGCCGCGATTTCA-3′; 3′ primer, 5′-CGGGAA-TTCGGACCCACTTTC-3′). The PCR product was subcloned into plasmid pSG424 and GAL4–KRAB was digested with *Hin*dIII and *Eco*RI to substitute the GAL4 coding region, resulting in pTetR and TetR-KRAB, respectively. To construct the TetR-KRABmut, PCR-mediated site-directed mutagenesis was used. The mutant plasmid harbors M → K, L → K, and E → K mutations in the KRAB domain. All plasmids were analyzed by DNA sequencing to confirm correct construction. Full details of each construction are available upon request.

Transfection and CAT Assay. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed by calcium phosphate precipitation with subconfluent cell cultures using different amounts of reporter and effector plasmids. For normalization of transfection efficiencies a β-galactosidase expression plasmid was included in the cotransfections (pSV-β-Gal expression plasmid; Promega). CAT assays were performed with different amounts of extract to ensure linear conversion of the chloramphenicol with each extract and results are presented as means ± SD of at least four duplicated independent transfection experiments. CAT activity was quantified by using the Molecular Dynamics PhosphorImager system.

***In Vitro* Transcription.** The glutathione *S*-transferase (GST)–GAL4 and GST–GAL4-KRAB fusion proteins were generated by PCR amplification, and the PCR products were inserted into pGEX2T (Pharmacia). The plasmids were analyzed by DNA sequencing. The GST fusion proteins were expressed in *Escherichia coli* by induction with 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested 3 hr after induction. After sonication and centrifugation, the proteins were purified using a glutathione-Sepharose column

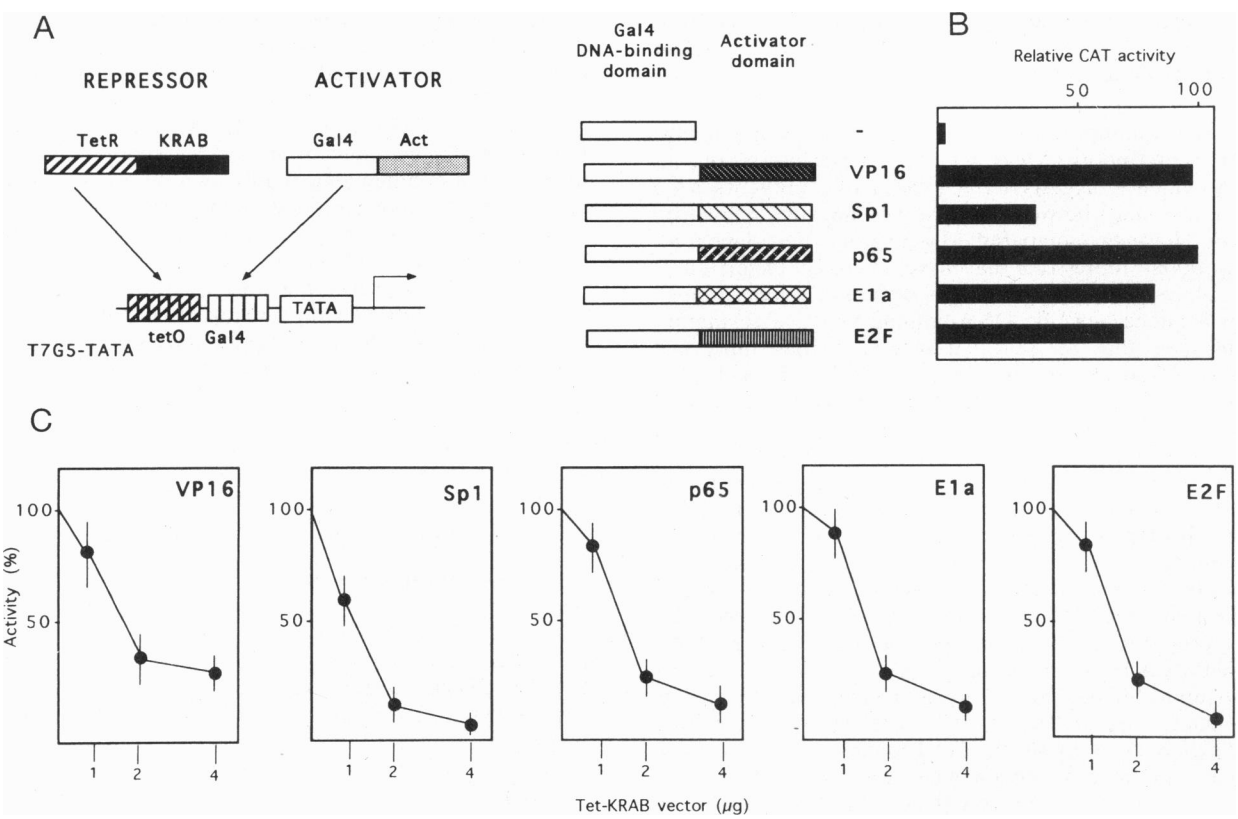

FIG. 1. KRAB represses transcription in the presence of defined activator. (*A*) Schematic diagram of experimental planning to determine KRAB-mediated repression of defined activators. (*B*) T7G5-TATA reporter (2 μg) was cotransfected into HeLa cells with the indicated GAL4 activator expression vector (1 μg), and CAT activities relative to the sample without activator are diagrammed. (*C*) TetR–KRAB represses expression activated by VP16, SP1, p65(relA), E1A, and E2F GAL4 fusions. T7G5-TATA (2 μg) was cotransfected with 1 μg of each effector plasmid as indicated in the presence of increasing amounts of TetR–KRAB in a total of 20 μg adjusted with the parental plasmid pTetR. The ordinate is the percentage change in CAT activity due to cotransfection with the TetR–KRAB plasmid at the amount indicated on the abscissa. Values represent averages of five independent transfections after normalization for the internal control β-galactosidase activity.

(Pharmacia). *In vitro* transcription experiments were performed using the HeLa cell extract transcription kit from Promega and 100 ng of template as indicated in the text. The template was incubated with purified GST–GAL4–KRAB or GST–GAL4 proteins for 10 min at 4°C. The amount of GAL4 protein used to supplement the extract was equalized on the basis of a gel mobility-shift assay with an oligonucleotide containing a GAL4 DNA-binding site, and the total amount of protein concentration was equalized with bovine serum albumin. HeLa cell extract was added and incubated for 15 min at 30°C. Transcription was initiated by addition of nucleotides and allowed to proceed for 45 min at 30°C. Transcription products were analyzed by primer extension using a CAT primer as described (12). Reaction products were resolved on a 6% polyacrylamide/8 M urea gel and the Molecular Dynamics PhosphorImager system was used to quantitate the extended products.

RESULTS

Transcriptional Activation by Different Positive Regulators Is Suppressed by KRAB. We have previously shown that the KRAB domain tethered to the HIV-1 promoter by a GAL4 DNA-binding domain actively repressed transcription in a distance-independent manner (11). Repression at distance supports a mechanism requiring protein–protein interactions between distantly bound KRAB domain and proximal factors looping out intervening DNA. Alternatively, the KRAB domain tethered to DNA may alter (bending) promoter topology, such as described for the δ/YY1 transcription factor (26) and more recently for the YB-1 protein, a repressor of the human major histocompatibility complex class II genes (27). It has been suggested that YB-1 binds to specific DNA sequences and induces local unwinding of the DNA duplex. Hence, the distortion would prevent binding of the nearby activators and would thereby result in gene repression. This possibility is unlikely because we have recently demonstrated that the KRAB domain can efficiently repress transcription from nascent RNA targets (20). These data suggest that KRAB-mediated repression may not require a stable interaction with the promoter DNA but rather a mechanism using protein–protein interaction with components of the general transcription machinery.

To determine whether the KRAB domain may affect the activating function of a defined activator by interference with a common component of basal preinitiation multiprotein complex, we developed an *in vivo* transcription assay in which various well characterized transcription activation domains were fused to the yeast GAL4 DNA-binding domain, and their activity was assayed on a reporter plasmid. To evaluate the extent of repression, the KRAB domain was connected in frame to the C terminus of the prokaryotic TetR encoded by Tn*10* from *E. coli*. Thus, the TetR–KRAB chimeric protein was able to bind to the tetO sequences. As template we constructed the T7G5-TATA reporter, which contains the CAT gene under the control of the E1b TATA box with 5 GAL4 DNA-binding and 7 tetO sequences. Relevant features of the effectors and reporter plasmids are outlined in Fig. 1*A*. HeLa cells were transfected with the reporter T7G5-TATA in the presence of the various GAL4 activators. As expected, the GAL4 chimeric activators stimulated transcription when allowed to bind next to the TATA box (Fig. 1*B*). However, GAL4-dependent transcriptional activation was repressed by coexpression of the TetR–KRAB chimeric protein in a dose-dependent manner (Fig. 1*C*). The specificity of KRAB-mediated repression was demonstrated by the results reported in Fig. 2*A*, showing that neither the Tet–KRABmut protein bearing a mutation that has been previously shown to abolish repression (14) nor the KRAB domain lacking the TetR DNA-binding domain influenced significantly promoter activity. To ensure that comparable amounts of Tet–KRAB and Tet–KRABmut proteins were made in each transfection,

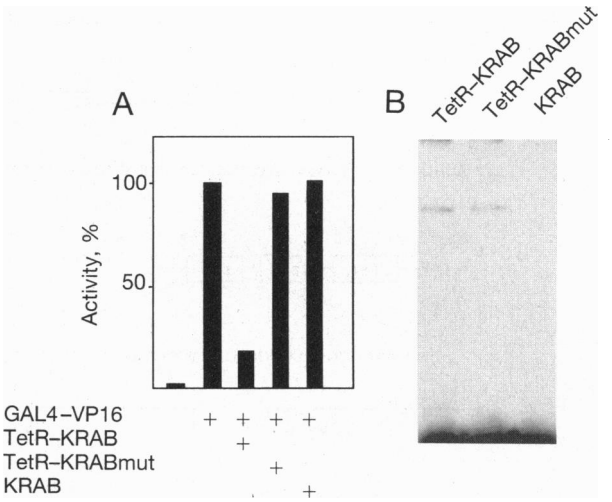

FIG. 2. TetR–KRABmut and KRAB domain unable to bind DNA are defective for repression. TetR–KRABmut and KRAB domain were tested for their ability to repress transcription activated by GAL4-VP16. (*A*) T7G5-TATA (2 μg) was cotransfected into HeLa cells with GAL4-VP16 (1 μg) together with 4 μg of each effector plasmid as indicated. CAT activities were determined and presented as described in Fig. 1. Values are representative of three independent duplicated experiments. (*B*) Gel mobility-shift assay with a DNA fragment containing the tetO DNA-binding sites and protein extracts from transfected cells with TetR–KRAB, Ter–KRABmut, and KRAB expression plasmids as indicated.

electrophoretic mobility retardation analysis was carried out as reported in Fig. 2*B*.

Basal Promoter Elements Influence the Response to KRAB. A large number of basal promoters are devoid of a TATA box and in many cases the presence of the Inr has been demonstrated. We sought to determine the KRAB repression on AdMLP Inr-bearing promoters. To this end, we constructed two reporters: T7G5-TATA-Inr and T7G5-Inr. The two reporter plasmids are isogenic except for the presence of the TATA element, and their structure is depicted in Fig. 3*A*. First, the effects of the various activator domains present in the GAL4 chimeric proteins were determined. Consistent with previous results we found that both TATA and Inr promoters responded to the GAL4-based activators, with the exception of the E1a activating domain, which as previously reported is strictly dependent on the presence of the TATA box (22). Therefore, the GAL4–E1a fusion protein is unable to transactivate the T7G5-Inr reporter, whereas the T7G5-TATA-Inr was fully responsive. Furthermore, we noted that activation of Inr promoters is always weaker than that observed in TATA-containing promoters (Fig. 3*A*).

To determine the repressive effects of the KRAB domain, the reporter plasmids described in Fig. 3*A* were cotransfected into HeLa cells with the indicated GAL4 fusion proteins in the presence of increasing amounts of the TetR–KRAB expression vector. Coexpression of TetR–KRAB protein was found to repress in a dose-dependent manner GAL4 chimeric protein-mediated activation of the TATA box-containing promoter, whereas Inr-mediated expression was unaffected (Fig. 3*B*). A relatively low level of repression of Inr-dependent promoter was observed only with a high dose of TetR–KRAB repressor in the context of the Sp1 activating domain. It thus appeared that inhibition of expression by KRAB was directed through the particular sequence motif responsible for accurate initiation of transcription.

We noticed that the repression activity observed using the T7G5-TATA or the T7G5-TATA-I was very similar (compare KRAB-mediated repression reported in Figs. 1*C* and 3*B*) perhaps because the TATA box dominates the Inr in estab-

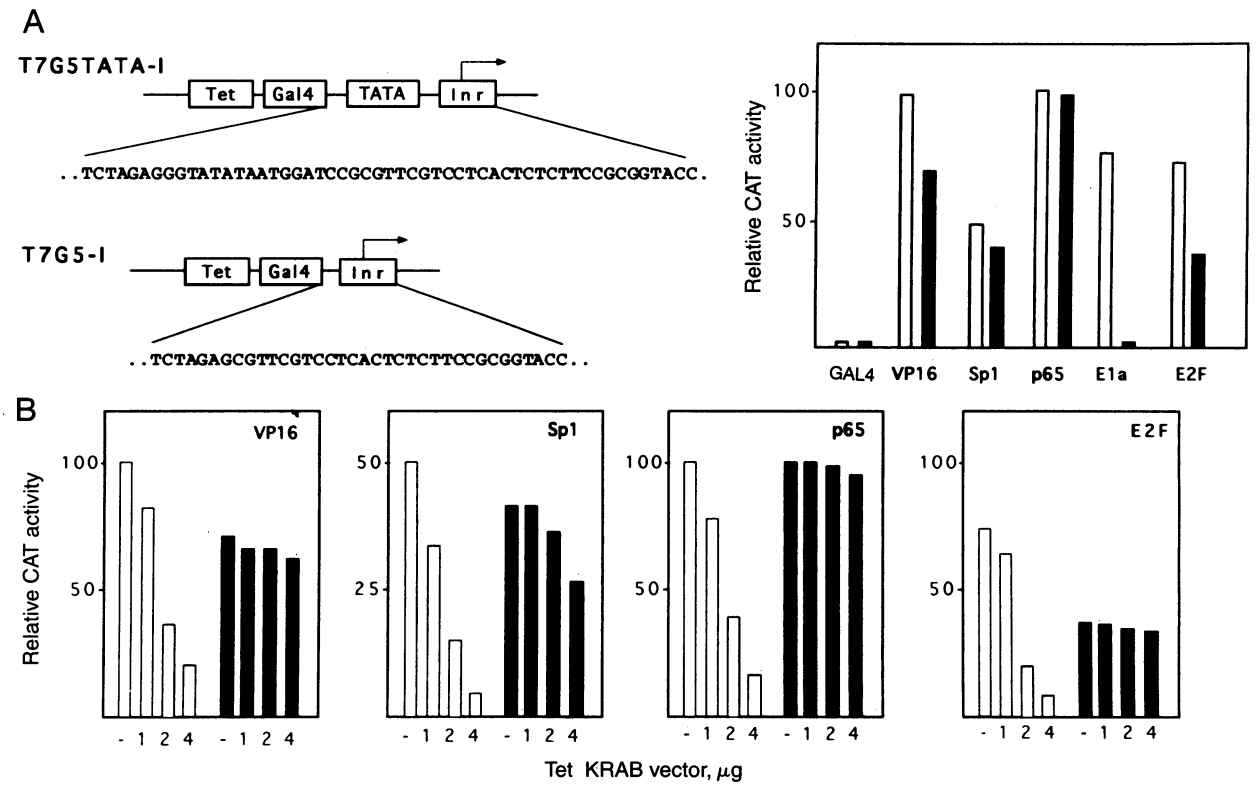

FIG. 3. TetR–KRAB-mediated repression is TATA dependent. (*A*) T7G5TATA-I and T7G5-I reporter plasmids containing the AdMLP Inr sequence with or without the TATA element were used to test for activity of the KRAB domain in the presence of different activators. Reporter plasmids T7G5TATA-I (2 μg; open bars) and T7G5-I (5 μg; solid bars) were cotransfected into HeLa cells with the GAL4 derivatives (2 μg) as indicated and the transcriptional activity of each GAL4 derivative is diagrammed at the right. (*B*) T7G5-TATA-I (2 μg; open bars) and T7G5-I (5 μg; solid bars) were cotransfected with 2 μg of each GAL4 effector plasmid as indicated in the presence of increasing amounts of TetR–KRAB in a total of 20 μg adjusted with the parental plasmid pTetR. CAT activities relative to the sample without activator are diagrammed. Values represent averages of five independent transfections.

lishing basal promoter activity (28, 29). A similar extent of KRAB-mediated repression was also noted for TATA and TATA-Inr reporters in the presence of GAL4–E1a (data not shown). These results suggested that the Inr element does not play a negative role in abrogating KRAB repression, but rather it is the presence of the TATA box that is strictly required for KRAB-mediated repression.

KRAB Domain Represses Transcription *in Vitro*. To further substantiate KRAB-mediated repression, we sought to determine whether the KRAB domain could repress transcription *in vitro*, and to this end we performed *in vitro* transcription reactions with a HeLa cell transcription system and a number of different templates. We produced recombinant proteins containing the GAL4 DNA-binding domain alone (GST–

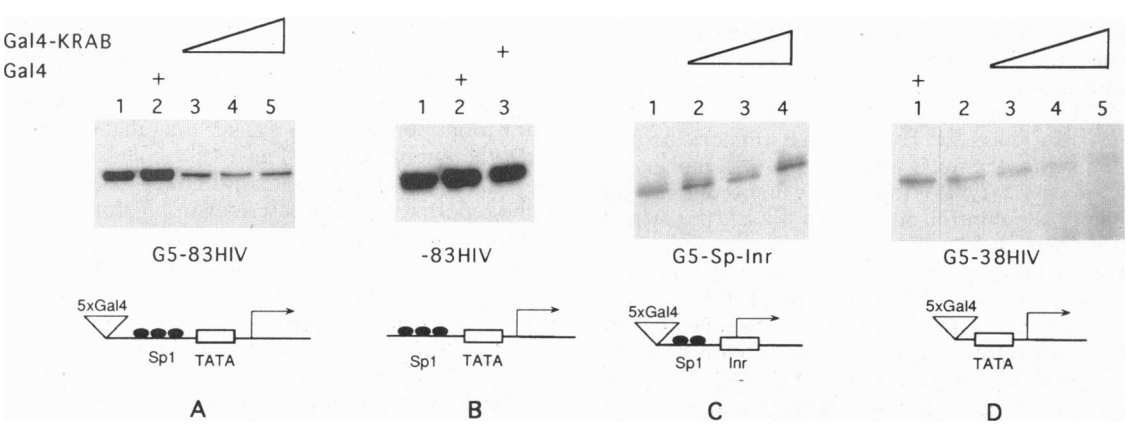

FIG. 4. KRAB domain represses transcription *in vitro*. RNA synthesized *in vitro* was detected by primer-extension analysis using a primer complementary to the CAT coding region. Purified GAL4–KRAB proteins (50, 150, and 300 ng) were used in the *in vitro* reactions reported in *A* and *D* (lanes 3–5) and *C* (lanes 2–4). In *B* (lane 3) 250 ng of GAL4–KRAB was used; 100 ng of purified GAL4 protein was used in *A* and *B* (lane 2) and in *D* (lane 1). The presence of GAL4 fusion proteins in the nuclear extract is indicated above each lane. The G5-83HIV, -83HIV, G5Sp-Inr, and G5-38HIV templates were used in *A, B, C*, and *D*, respectively. Quantitation by densitometry of primer-extension products (value of templates in the absence of GAL4 proteins set as 100) is as follows. *A*: lane 1, 100; lane 2, 120; lane 3, 55; lane 4, 38; lane 5, 29. *D*: lane 1, 120; lane 2, 100; lane 3, 52; lane 4, 31; lane 5, 15. In *B* and *C* no significant differences were determined by densitometry of the primer-extension products. Results shown are from a single experiment. Although the degree of change varied slightly between experiments, the relative effects of the GAL4 fusion proteins are consistent in five independent experiments using different preparations of nuclear extracts.

GAL4) or fused to the KRAB (GST–GAL4–KRAB). Different templates, whose relevant features are outlined in Fig. 4, were used and the *in vitro* transcriptions were performed in the presence or absence of the GAL4 proteins. The amount of GAL4 protein used to supplement the extracts was equalized on the basis of a gel mobility-shift assay with an oligonucleotide containing a single GAL4 DNA-binding site, and the total amount of protein concentration was equalized with bovine serum albumin. As reported in Fig. 4*A*, *in vitro* transcription of the G5-83HIV template was specifically repressed by the GAL4–KRAB protein, whereas no significant effects were observed with the GAL4 protein and with the same template (-83HIV) lacking the GAL4 DNA-binding sites (Fig. 4*B*). Using as template the plasmid G5-Sp-Inr, whose transcription initiation is governed by Inr, no significant effect was observed in the presence of the GAL4–KRAB fusion protein (Fig. 4*C*). Lack of repression of Inr-driven promoter substantiates the *in vivo* results reported in Fig. 3. Since the G5-83HIV template contains three Sp1 DNA-binding sites, KRAB-mediated repression may be due to either a masking effect on the positively acting Sp1 protein or a direct repression of the basal promoter. Therefore, we sought to determine whether the KRAB domain may affect transcription of a template in the absence of transactivators. To this end, the G5-38HIV plasmid (12), which is devoid of Sp1 DNA-binding sites, was used as template. As reported in Fig. 4*C*, repression of the relatively low level of basal transcription was specifically observed in the presence of the GAL4–KRAB protein.

DISCUSSION

The experiments described here demonstrate that KRAB-mediated repression is influenced by the arrangement of basal promoter elements. *In vitro* transcription assays indicated that the KRAB domain is able to repress both activated and basal promoter activity. These data suggest an interaction of the KRAB domain with components of the basal promoter complex. The presence of a charged, amphipathic helix with potential leucine zipper-like structure is suggestive of protein–protein interaction ability. Accordingly, amino acid substitutions in the predicted KRAB amphipathic helix abolished repression function (14).

Several models can be envisaged to explain how the KRAB domain might repress transcription. For example, KRAB may be a component in a mechanism that causes global changes in chromatin structure, such as is found in control of expression of the yeast mating-type locus (1–3, 30). This possibility is unlikely because expression of the recombinant GAL4–KRAB protein is sufficient to repress *in vitro* transcription. Alternatively, KRAB may function by inactivating or squelching a protein that normally activates pol II expression (4, 30). This possibility also seems very unlikely because KRAB-mediated repression is strictly dependent on binding in cis to the promoter. Finally, KRAB-mediated repression is strongly influenced by the arrangement of the basal promoter elements, suggesting that KRAB's effect on transcription is not due to a general nonspecific shutdown of RNA polymerase II machinery.

In recent work, we have shown that the KRAB domain can efficiently repress transcription from the HIV-1 LTR when targeted to a nascent RNA sequence (20). These results pose significant constraints on the possible mechanisms governing KRAB repression. Formation of the target RNA is dependent on transcription from the LTR, and a functional preinitiation complex must, therefore, have already been formed. The observation that KRAB function can be dissociated from any requirement for stable interaction with a DNA target sequence appears inconsistent with scenarios for repressor function that require formation on the DNA template of stable, multiprotein complexes involving KRAB and other cellular transcription factors. Instead, it appears more likely that the RNA-bound KRAB repressor domain serves to recruit cellular "corepressor(s)" to the transcription initiation complex. These putative corepressors may then modify the HIV-1 LTR initiation complex so that the rate of transcription initiation is repressed. However, it remains to be demonstrated whether the mechanism of KRAB repression is the same when it is targeted to nascent RNA as it is when it is targeted to promoter DNA.

We have compared the sensitivity of the repressive effects of KRAB of promoters using TATA-containing elements with those that use Inr. The KRAB domain does not mediate repression of the Inr-dependent transcription initiation promoter very effectively, although it functions effectively on promoters whose transcription initiation is controlled by the TATA box element. Thus, these data demonstrate that the arrangement of basal promoter elements, even in the context of identical activator domains, can profoundly influence the response to the strong KRAB-repressor domain.

The specific repression we observe both *in vivo* and *in vitro* is consistent with a direct interaction of the KRAB domain with TATA-dependent basal transcriptional machinery, either directly or by recruitment of a corepressor. Interestingly, both TATA and initiator elements use the TATA-binding protein (TBP) subunit of the TFIID complex for their activities (31), but each relies on different components of TBP-associated factors (TAFs) for function (32). Hence, it is likely that repression by KRAB is mediated through an interaction with a basal transcription component or a specific TAF, which results in interference with the assembly of an essential TATA transcription complex. Recent *in vitro* experiments suggested a close relationship between organization of core promoter elements (TATA vs. Inr) and the subunit architecture of TFIID (33). These findings suggested that TAFs may play an important role, not only as coactivators that mediate enhancer-dependent activation but also as core promoter recognition factors. Finally, the role of basal promoter elements as a selective determinant of transcriptional activator and repressor function *in vivo* has been recently documented (34–38). For example, p53 appears to repress effectively the TATA box-containing promoters, whereas it does not affect Inr-dependent promoters (35). On the contrary, the c-Myc protein appears to repress transcription by a mechanism dependent on the Inr of the basal promoters of susceptible genes (36). REST, a mammalian silencer protein that restricts sodium channel gene expression to neurons, functions effectively on neuron-specific genes whose promoters lack a conventional TATA motif (37). Recently, it has been shown that the arrangement of basal promoter elements may provide a mechanism for differential regulation of transcription by showing that changing the arrangement of core promoter elements can alter the response to transcriptional activation domains (38). Further studies of KRAB-mediated repression should add more specific information to the limited but fast-growing knowledge concerning the molecular mechanisms governing transcription repression in eukaryotes.

We thank Drs. R. Della Favera, M. Gossen, H. Bujard, B. Majello, and P. Baeuerle for the gift of plasmids and Miss Rosaria Terracciano for technical assistance. This work was paid for by a grant from the Italian Association for Cancer Research (AIRC) and from the AIDS Program 1994, Istituto Superiore di Sanità, Ministero della Sanità, Rome.

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