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Sequence-Specific Transcriptional Repression by KS1, a Multiple-Zinc-Finger–Krüppel-Associated Box Protein

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The vertebrate genome contains a large number of Krüppel-associated box–zinc finger genes that encode 10 or more C_2-H_2 zinc finger motifs. Members of this gene family have been proposed to function as transcription factors by binding DNA through their zinc finger region and repressing gene expression via the KRAB domain. To date, however, no Krüppel-associated box–zinc finger protein (KRAB-ZFP) and few proteins with 10 or more zinc finger motifs have been shown to bind DNA in a sequence-specific manner. Our laboratory has recently identified KS1, a member of the KRAB-ZFP family that contains 10 different C_2-H_2 zinc finger motifs, 9 clustered at the C terminus with an additional zinc finger separated by a short linker region. In this study, we used a random oligonucleotide binding assay to identify a 27-bp KS1 binding element (KBE). Reporter assays demonstrate that KS1 represses the expression of promoters containing this DNA sequence. Deletion and site-directed mutagenesis reveal that KS1 requires nine C-terminal zinc fingers and the KRAB domain for transcriptional repression through the KBE site, whereas the isolated zinc finger and linker region are dispensable for this function. Additional biochemical assays demonstrate that the KS1 KRAB domain interacts with the KAP-1 corepressor, and mutations that abolish this interaction alleviate KS1-mediated transcriptional repression. Thus, this study provides the first direct evidence that a KRAB-ZFP binds DNA to regulate gene expression and provides insight into the mechanisms used by multiple-zinc-finger proteins to recognize DNA sequences.

One of the largest families of potential transcriptional regulators is the Krüppel-associated box–zinc finger proteins (KRAB-ZFPs) (2). In the human genome, for example, approximately 300 to 700 different genes encode C_2-H_2 zinc fingers, one-third of which also contain a KRAB domain (1, 2, 6). These KRAB-ZFPs share a common modular structure with an approximately 70-amino-acid KRAB domain at the N terminus and multiple C_2-H_2 zinc finger motifs at the C terminus (2). Because the C_2-H_2 zinc fingers have been shown to bind DNA in a sequence-specific manner in several other proteins, the KRAB-ZFPs have long been proposed to function as transcription factor proteins (2). Further support for this idea has been based on the findings that the KRAB domain represses transcription when fused to the heterologous GAL4 DNA binding domain (20, 26, 33). Interestingly, this transcriptional repression appears to be mediated through a corepressor protein, KRAB-associated protein 1 (KAP-1; also called KRAB-interacting protein 1 [KRIP-1] and transcriptional intermediary protein 1β [TIF1β]) (12, 18, 21). This 100-kDa protein has been shown to bind to the KRAB domains of several different KRAB-ZFPs and contributes to the ability of this domain to repress gene expression in GAL4-based transcriptional regulatory assays. Therefore, KRAB-ZFPs have been proposed to regulate transcription by binding to a specific DNA sequence via their zinc finger motifs, interacting with a KAP-1 complex through their KRAB domains and thereby repressing gene expression.

Despite the strong evidence that the KRAB domain functions as a potent transcriptional repressor motif, the ability of the KRAB-ZFPs to regulate transcription by binding DNA in a sequence-specific manner has remained unclear. For example, the zinc finger region of the KRAB-ZFP Kid-1 binds to heteroduplex DNA structures but is unable to selectively bind either single- or double-stranded DNA (9). In contrast, the ZNF74 KRAB-ZFP has been shown to specifically interact with poly(G) and poly(U) RNA but not to poly(A) RNA, poly(C) RNA, or DNA (14). However, this protein also interacts with the nuclear matrix and the large subunit of RNA polymerase, suggesting a role for ZNF74 in RNA metabolism as well as transcriptional regulation (15). Finally, another member of this protein family, ZNF85, was recently shown to bind both DNA and RNA, but in a non-sequence-specific manner (28). Indeed, no KRAB-ZFP has yet been shown to bind DNA via its zinc finger motifs to repress gene expression. Therefore, the ability of KRAB-ZFPs to regulate transcription in a site-specific manner remains to be established.

The C_2-H_2 zinc finger motifs, like those in the KRAB-ZFPs, represent one of the most common types of DNA binding domains found within eukaryotic transcription factors (1, 19). This motif frequently occurs in tandem repeats and is defined by the presence of the consensus sequence ϕ-X-Cys-X_{2-4}-Cys-X_3-ϕ-X_5-ϕ-X_2-His-$X_{(3,4)}$-His, where X represents any amino acid and ϕ represents a hydrophobic residue (19). The two cysteine and histidine residues coordinate a zinc ion and fold this domain into a finger-like projection that can interact with DNA (19). Structural studies of C_2-H_2 zinc finger domains

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from several different proteins, such as Zif268, Tramtrack, and Gli1, reveal that each motif is capable of contacting three to four nucleotides (10, 11, 23, 24). These findings led to the hypothesis that members of the C_2-H_2 ZFP family bind DNA and function as transcriptional regulators. In support of this prediction, a large number of proteins, most of which contain four or fewer zinc finger motifs, are among the best-characterized transcription factors that specifically bind DNA (5, 19, 27, 32). Interestingly, however, the vertebrate genome encodes a large number of multiple ZFPs that contain 10 or more C_2-H_2 zinc fingers, few of which have been shown to either bind DNA in a sequence-specific manner or function in transcriptional regulation.

In this paper, we have used KS1 (KRAB suppressor of transformation 1), a KRAB-ZFP containing 10 C_2-H_2 zinc fingers, to test the hypothesis that a KRAB-ZFP utilizes its multiple zinc finger domains to bind DNA and function as a transcriptional repressor. Towards this end, we have used a random oligonucleotide binding assay to identify a 27-bp KS1 binding element (KBE) that is specifically recognized by KS1. Moreover, we demonstrate that KS1 represses both basal and activated expression of KBE-containing reporter constructs. Deletion analysis reveals that the KS1 protein requires 9 of its 10 C-terminal zinc finger motifs as well as the N-terminal KRAB domain for this transcriptionally repressive function. Furthermore, biochemical assays show that the KRAB domain of KS1 interacts with the KAP-1 corepressor protein, and mutations that abolish this interaction alleviate KS1-mediated transcriptional repression. Together, these results demonstrate for the first time that a KRAB-ZFP can repress transcription via its own DNA binding domain. The contribution of these findings toward our current understanding of multiple ZFPs and their ability to function as sequence-specific transcription factors is discussed.

MATERIALS AND METHODS

Plasmid constructs. The GST-KS1 construct used for the random oligonucleotide binding (ROB) assay was generated by PCR amplifying the 10 zinc fingers of KS1 (amino acids 213 to 566; GenBank accession no. U56732) and cloning this fragment in frame with the glutathione *S*-transferase (GST) coding sequence in pGEX4T-3 (Pharmacia, Piscataway, N.J.). The wild-type KRAB domain of KS1 (amino acids 1 to 104) was PCR amplified and cloned into either pGEX5X-1 (Pharmacia) or the mammalian expression vector pcDNA3.1 HisA (Invitrogen, Carlsbad, Calif.). Site-directed mutagenesis of the KS1 KRAB domain (DV to AA [see Fig. 5A]) was done by standard overlapping PCR mutagenesis. This PCR product was subsequently cloned into pGEX5X-1 or pcDNA3.1 HisA vector or used to replace the wild-type KRAB domain within the full-length KS1 expression vector. The KS1 (1, 2, 8–10) zinc finger construct was generated by digesting the full-length pcDNA 3.1 HisA KS1 construct with *Bam*HI (releasing zinc fingers 3 to 7) followed by religation. All of the other KS1 zinc finger deletion and site-directed mutants were generated by PCR amplifying the indicated zinc fingers and cloning these fragments in frame with the N terminus of the KS1 protein. The KS1 expression constructs containing the N-terminal deletions were previously described (13). Firefly luciferase reporter constructs were assembled by annealing oligonucleotides containing three copies of either the ROB1, KBE, or Del1 sequence (see Fig. 1 and 2) flanked by overhanging *Asp*718-I/*Bgl*II restriction enzyme sites for subsequent cloning into either pGL3 promoter or pGL3 control vectors (Promega, Madison, Wis.). The *Renilla* luciferase reporter vector used to normalize for transfection efficiency was created by cloning the Rous sarcoma virus long terminal repeat promoter from pREP7 (Invitrogen) into the promoterless pRL null vector (Promega). Hemagglutinin (HA)-tagged KAP-1 cDNA was generously provided by Joseph Bonventre (Harvard, Cambridge Mass.), and the HA-tagged Kid-1 expression plasmid was kindly supplied by Ralph Witzgall (Ruprecht-Karls-Universität, Heidelberg, Germany). For coimmunofluorescence studies with HA-tagged Kid-1, the KAP-1 gene was cloned in frame with the Xpress-tagged pcDNA3.1 HisA vector. The sequences of all of the constructs were verified by direct DNA sequencing.

GST fusion protein purification. Each GST construct was transformed into BL21 bacteria (Stratagene, La Jolla, Calif.), and fusion protein expression was induced by the addition of 2 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. The GST fusion proteins were subsequently purified by glutathione-Sepharose 4B affinity chromatography according to manufacturer's suggestions (Pharmacia) in a buffer containing 20 mM HEPES (pH 7.9), 150 mM KCl, 50 μM $ZnCl_2$, 0.1% Nonidet P-40 (NP-40), 20% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride.

ROB assay and gel shift assays. The ROB assay was performed essentially as described by Blackwell and Weintraub (3). In brief, a random library of DNA sequences was generated by synthesizing an oligonucleotide containing a 35-bp random core sequence flanked on each side by 20 bp (5′-TACAAGATCCGG AATTCCTACN$_{35}$GACGGATCCGGCGATAAGACA-3′). A forward primer (5′-TACAAGATCCGGAATTCC-3′) and a reverse primer (5′-TGTCTTATCG CCGGATCC-3′) were synthesized in order to amplify the library. The double-stranded oligonucleotides used in the first round of DNA binding were generated by one cycle of PCR (3 min at 94°C, 2 min at 55°C, and 10 min at 72°C) using *Pwo* polymerase (Boehringer Mannheim, Indianapolis, Ind.) with a 10 M excess of reverse primer to random oligonucleotide. The PCR product was purified on a 3% low-melting-point agarose gel and end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase according to the manufacturer's suggestions (Promega). Gel shift assays were performed using 200 ng of purified GST or GST-KS1 fusion proteins incubated in a buffer containing 20 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 6% glycerol, 200 μg of bovine serum albumin per ml, and 50 μg of poly(dI-dC)·poly(dI-dC) per ml for 10 min at room temperature. Approximately 0.3 ng of the end-labeled probe was then added to each reaction for an additional 20 min and subsequently loaded onto a 4% polyacrylamide gel. Samples were run for 3 h at 120 V at room temperature, vacuum dried, and exposed to X-Omat film (Eastman Kodak Co., New Haven, Conn.). The DNA sequences bound by GST-KS1 were purified by excising specific protein-probe complexes from the gel and incubating the gel slice in a mixture of 0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS) for 3 h at 37°C. The samples were then briefly centrifuged, transferred to a fresh tube, extracted twice with phenol and twice with chloroform, and ethanol precipitated with 10 μg of glycogen. The DNA was subsequently reprecipitated with 0.3 M sodium acetate and ethanol and resuspended in 50 μl of water, and 10 μl was used in a standard 100-μl PCR. Control reactions with no oligonucleotide template were always used and did not yield a product. The PCR sample was then purified on a 3% agarose gel, end labeled, and used in the next round of gel shift analysis. After the seventh round of purification, the PCR product was digested with *Eco*RI and *Bam*HI, cloned, and sequenced. The sequences were analyzed with MacVector (Eastman Kodak Co.) and Genetics Computer Group (Madison, Wis.) DNA software. To test the ability of the GST-KS1 fusion protein to bind the purified sequences, the eight different 35-bp ROB sequences shown in capital letters in Fig. 1 were synthesized, annealed, and used for gel shift analysis as was performed for the ROB assay. The deletion and site-directed mutant primers shown in Fig. 4A were also synthesized and annealed by standard molecular techniques. In addition, a control GC box (5′-A TTCGATCGGGGCGGGGCGAGC-3′), mutant GC box (5′-TTCGATCGGT TCGGGGCGAGC-3′), and GT box (5′-ATTCGATCGGGGT-GGGGCGAG C-3′) were used as indicated in Fig. 2 (4).

Cell culture, transcriptional reporter assays, and Western blot analysis. The Chinese hamster ovary (CHO) cell line was obtained from the American Type Culture Collection (Manassas, Va.) and cultured under an atmosphere containing 5% CO_2 in F-12 medium plus 5% fetal bovine serum, 5% newborn calf serum, 100 U of streptomycin per ml, and 100 U of penicillin (Life Technologies, Rockville, Md.) per ml. For transcriptional regulatory assays, approximately 3×10^5 CHO cells in 35-mm-diameter tissue culture wells were transfected by using Lipofectamine (Life Technologies) with 2 μg of effector plasmid, 0.4 μg of pGL3 firefly reporter plasmid, and 0.1 μg of *Renilla* luciferase control plasmid, unless otherwise indicated. Twenty-four hours after transfection, proteins were isolated and the relative luciferase expression was assayed using the Dual Luciferase Assay and a Turner 20/20 luminometer (Promega). In all experiments, luciferase activity was determined using equal amounts of protein, and firefly luciferase values were normalized to *Renilla* luciferase activity. Each experiment was performed at least three different times in duplicate, and the mean and standard deviations were calculated. The expression of each KS1 deletion protein was determined by Western blot analysis. In brief, 24 h after transfection, cells were washed in cold phosphate-buffered saline and lysates were harvested in 1 ml of lysis buffer (8 M urea, 100 mM sodium phosphate [pH 8.0], 10 mM Tris-HCl [pH 8.0], 0.5 mM phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, and 10%

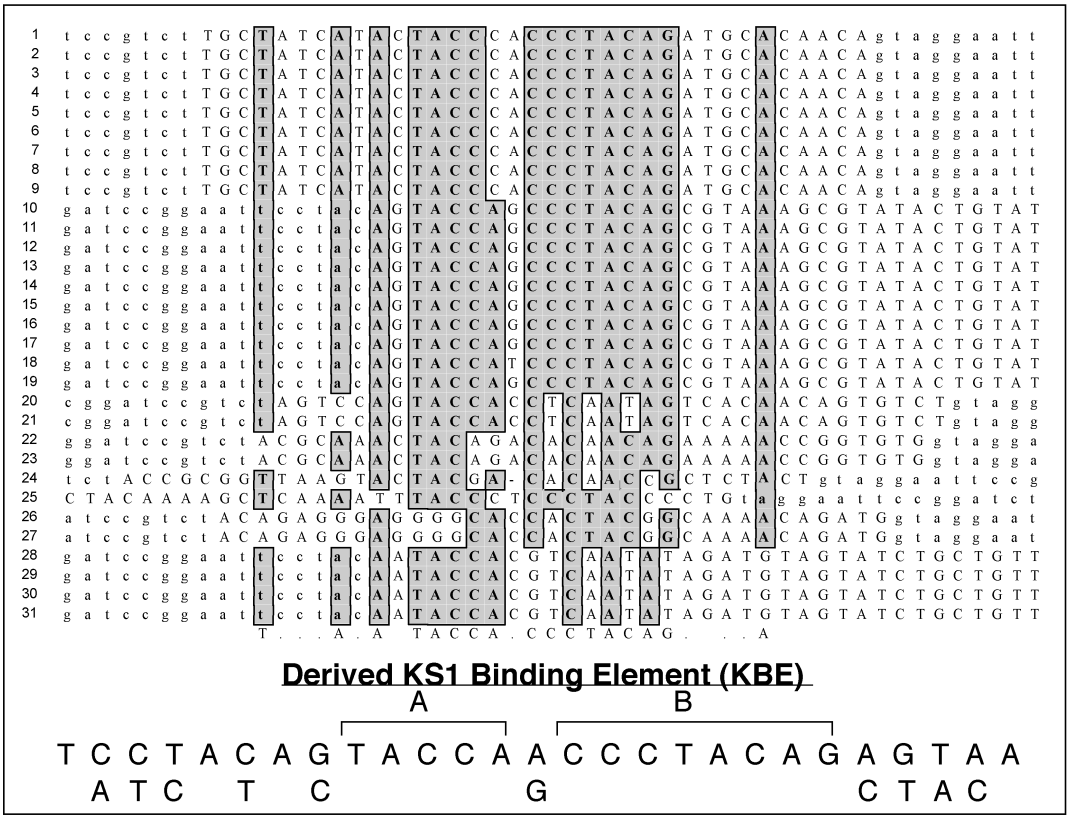

FIG. 1. ROB assay for KS1. ROB oligonucleotides with a 35-bp degenerate core were end labeled with ^{32}P, incubated with GST alone or with GST-KS1, and separated by nondenaturing polyacrylamide gel electrophoresis. After seven rounds of ROB purification, 31 oligonucleotides were cloned, sequenced, and aligned. Uppercase letters represent the 35-bp random sequence from each clone, whereas lowercase letters represent the flanking sequence. Gray residues indicate greater than 60% identity, and a derived consensus KBE is shown.

glycerol) for 20 min at room temperature with gentle rocking. Lysates were cleared by centrifugation at 10,000 × *g* for 10 min, NP-40 was added to the supernatant to 0.5% (vol/vol) together with 30 μl of washed Ni-nitrilotriacetic acid–agarose beads (Qiagen, Valencia, Calif.), and samples were incubated for 2 h at room temperature. Bead-bound proteins were collected by centrifuging, separated by SDS-polyacrylamide gel electrophoresis, and detected by Western blot analysis using the anti-Xpress D-8 Omni-probe antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, Calif.), a goat anti-mouse peroxidase-conjugated secondary antibody (Sigma, St. Louis, Mo.), and chemiluminescence (Boehringer Mannheim).

GST pulldown assays. For GST pulldown assays, 3×10^6 CHO cells were labeled with [^{35}S]methionine for 4 h at 37°C, lysed at 4°C for 20 min in RIPA buffer (150 mM NaCl, 0.5% NP-40, 50 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$, 10 μg of aprotinin per ml, and 0.5 mM phenylmethylsulfonyl fluoride), and incubated with 2 μg of GST, GST–KRAB wild-type domain (wt), or GST-KRAB mutant domain (mt) for 2 h at 4°C. Glutathione-conjugated Sepharose beads were added for an additional hour, and complexes were pelleted by centrifugation at 500 × *g* for 5 min, washed five times with RIPA buffer, and separated by SDS-polyacrylamide gel electrophoresis. The gel was subsequently treated with AutoFluor (National Diagnostics, Atlanta, Ga.), dried, and exposed for autoradiography at −80°C. Pulldown assays using either GST, GST-KRAB wt, or GST-KRAB mt were also performed with approximately 3×10^6 CHO cells transfected with 10 μg of an HA-tagged KAP-1 expression vector. Forty-eight hours after transfection, nonradioactive pulldown assays were performed as described above followed by Western blot analysis using a rat anti-HA monoclonal antibody (Boehringer Mannheim), a goat anti-rat peroxidase-conjugated secondary antibody (Sigma), and chemiluminescence (Boehringer Mannheim).

Immunofluorescence and confocal microscopy. Immunofluorescence and confocal microscopy were performed essentially as previously described (4). Briefly, 4×10^5 CHO cells were transfected using Lipofectamine with either 2.5 μg of HA-tagged KAP-1 and Xpress-tagged KS1 or 2.5 μg of Xpress-tagged KAP-1 and 2.5 μg of HA-tagged Kid-1 expression vectors as indicated in Fig. 8. Twenty-four hours after transfection, the cells were harvested by trypsinization and plated onto coverslips coated with poly(L-lysine) (Sigma). After an additional 24 h, the cells were washed with phosphate-buffered saline and fixed with −20°C methanol for 10 min and immunofluorescence was performed. Xpress-tagged KS1 was localized with a mouse anti-Xpress monoclonal antibody (1:1,000; Invitrogen) and a tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100; Boehringer Mannheim), whereas Xpress-tagged KAP-1 was detected with the same primary antibody and a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:500; Molecular Probes, Eugene, Oreg.). HA-tagged KAP-1 and Kid-1 were subsequently detected by direct immunofluorescence using a fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated 12CA5 mouse anti-HA monoclonal antibody (1:30 dilution; Boehringer Mannheim) for KAP-1 and Kid-1, respectively. Cellular DNA was then stained for 10 min at 37°C using 0.5 μg of Hoechst 33342 (Molecular Probes) per ml in phosphate-buffered saline. Fluorescein and rhodamine fluorescence were observed with 488- and 568-nm excitation wavelengths from an argon krypton laser on a Zeiss LSM-510 confocal laser scanning microscope. Hoechst staining was observed with an emission wavelength of 385 to 470 nm by using an Enterprise laser (Coherent Laser Group, Santa Clara, Calif.).

RESULTS

Identification of a KBE. KS1 encodes a recently identified KRAB-ZFP that suppresses neoplastic transformation induced by several oncogenes (13). In this study, we wanted to investigate the role of KS1 as a sequence-specific transcription factor. To address this question, we performed a ROB assay using a GST fusion protein containing the 10 C_2-H_2 zinc finger motifs of KS1 (GST-KS1). This fusion protein or the GST protein

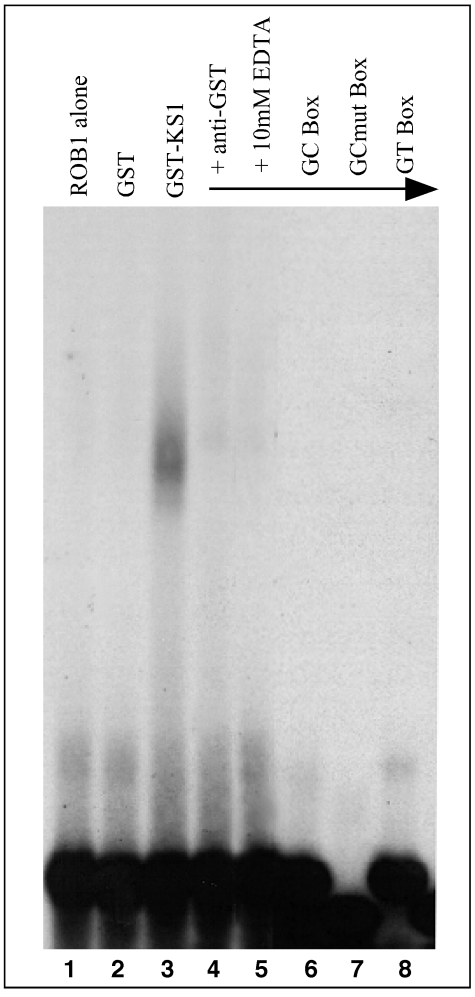

FIG. 2. Electromobility shift assay of KS1 with ROB-purified sequences. An electromobility shift assay was performed using either the GST protein alone or the GST-KS1 fusion protein. Lanes 1 to 5, ROB1 probe; lane 6, GC box probe; lane 7, GCmut probe; lane 8, GT box probe; lane 1, no proteins; lane 2, 200 ng of GST; lanes 3 to 8, 200 ng of GST-KS1; lane 4, 5 μg of anti-GST antibody; lane 5, 10 mM EDTA.

alone was incubated with radiolabeled double-stranded oligonucleotides containing a random core of 35 bp flanked on each side by 20 bp of known DNA sequence. Specific KS1-DNA complexes were then detected by gel shift analysis, and the bound oligonucleotides were purified, PCR amplified, radiolabeled, and used for another round of gel shift analysis. After seven rounds of purification, 31 different oligonucleotides were cloned and sequenced. As shown in Fig. 1, alignment of these sites revealed the presence of eight unique sequences that share a highly conserved region, which we have called the KBE. This element spans 27 nucleotides, is approximately 50% GC rich, and contains two central core sequences (A and B) that are almost identical among the clones. Database comparisons reveal that the KBE sequence does not contain binding sites for other previously characterized transcription factors and thus represents a potentially novel DNA target sequence (29).

To test the specificity of KS1 binding to the purified ROB sequences, we performed gel shift analyses using individually radiolabeled ROB probes. As shown in Fig. 2, the GST-KS1 fusion protein binds to the ROB1 sequence (sequences 1 to 9 in Fig. 1), while no binding is detected with the GST protein alone (Fig. 2, compare lanes 2 and 3). Similar studies using this method revealed that KS1 is also able to bind to the seven other purified ROB sequences (data not shown). Further characterization of the KS1-ROB1 interaction demonstrated that this binding is abolished by incubation with an antibody directed against GST (lane 4) or with the addition of the zinc chelating agent EDTA (lane 5). These results indicate that the GST-KS1 fusion protein interacts with this site and that correct folding of the zinc finger region of KS1 is necessary for this interaction, respectively. Finally, failure of the GST-KS1 protein to interact with probes containing well-characterized binding sites for other zinc finger transcription factors reveals that this protein binds to DNA in a sequence-specific manner (Fig. 2, lanes 6 to 8) (5, 27, 32).

KS1 represses transcription of promoters containing the ROB1 site. We next wanted to determine if KS1 regulates basal and/or activated transcription. For this purpose, we cloned three copies of the ROB1 sequence upstream of a luciferase reporter gene under the control of either the simian virus 40 promoter alone (basal transcription) or the simian virus 40 promoter and enhancer elements (activated transcription). These assays show that KS1 can repress transcription of both of these reporters approximately 75% compared to control reporter vectors that lack the ROB1 sequence, demonstrating that KS1 represses transcription in a ROB1-dependent manner (Fig. 3A and B). Furthermore, by titrating the amount of KS1 transfected with the promoter-enhancer 3× ROB1 reporter vector, we show that KS1 represses transcription in a dose-dependent manner (Fig. 3C). Transcriptional regulatory assays were also used to determine if another KRAB-ZFP can regulate the expression of the 3× ROB1 reporter. To address this question, we performed transcriptional regulatory assays using *Kid-1*, a KRAB-encoding gene containing 13 C_2-H_2 zinc finger motifs (34). Importantly, while the KRAB domain of this protein has been previously shown to repress transcription in GAL4 assays, the full-length Kid-1 protein is unable to repress the expression of the 3× ROB1 reporter vector (Fig. 3D) (33). Taken together, these results demonstrate that KS1 can regulate transcription by binding DNA in a sequence-specific manner.

Defining the KBE. To better define a minimal KS1 binding sequence, we used deletion and site-directed mutagenesis in conjunction with gel shift analysis and transcriptional reporter assays. The results shown in Fig. 4A demonstrate that KS1 is able to bind the derived 27-bp consensus KBE from Fig. 1. In addition, deletion mutations that remove 5 bp from either end of the KBE sequence completely abolished KS1 binding (lanes 4 and 5). Interestingly, site-directed mutagenesis used to replace the two highly conserved regions within the KBE sequence (A and B) with guanine nucleotides significantly decreased the affinity of KS1 binding but did not completely abolish this interaction (lanes 6 to 8). Together, these in vitro data suggest that while the A and B boxes within the KBE sequence contribute to KS1 binding, DNA sequences outside of these boxes are necessary for this interaction. In agreement with these findings, in vivo reporter assays demonstrate that

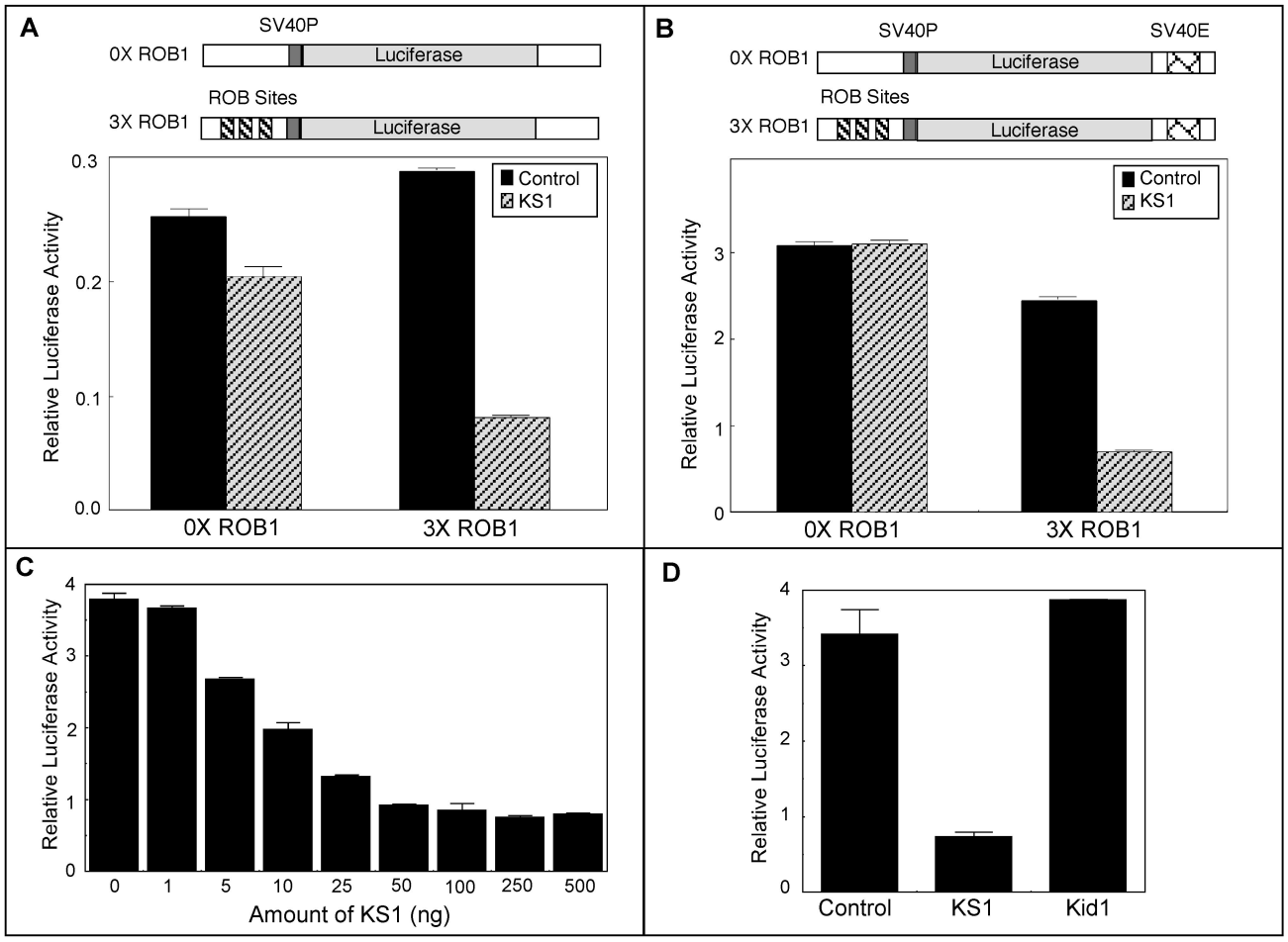

FIG. 3. KS1 represses the transcription of promoters containing the KS1 binding sites. CHO cells were cotransfected with various reporter vectors and a KS1 or Kid-1 expression vector as indicated. Firefly luciferase activity was measured and normalized to *Renilla* luciferase activity by a dual luciferase reporter system, and the mean and standard deviation were determined for each experimental condition. Histogram of relative luciferase activity shows that KS1 represses both basal (A) and activated (B) transcription of reporters containing three copies of the ROB1 sequence. Note that KS1 has no effect on a reporter vector lacking these sites. SV40, simian virus 40. (C) Increasing amounts of KS1 expression vector (0 to 500 ng) were cotransfected into CHO cells with the 3× ROB1 reporter vector as indicated. As shown in the histogram, KS1 is able to repress the expression of this reporter in a dose-dependent manner. (D) The 3× ROB1 reporter vector was cotransfected into CHO cells with either a KS1 or Kid-1 expression vector. The histogram shows that KS1 and not the Kid-1 KRAB-ZFP represses the expression of this reporter.

KS1 represses transcription through the consensus KBE sequence but not a deletion mutant (Del1) that lacks these flanking sequences (Fig. 4B).

KS1 requires zinc fingers 2 to 10 and the KRAB domain to repress gene expression. KS1 contains 10 C_2-H_2 zinc finger motifs, nine of which are clustered at the C terminus with a 56-amino-acid spacer separating an additional N-terminal zinc finger domain (Fig. 5A). To determine which of the zinc finger motifs within KS1 are required for repressing the expression of promoters containing the KBE site, we generated KS1 deletion constructs that lacked various zinc finger motifs (Fig. 5A). Each of these constructs was expressed and properly localized to the nucleus of CHO cells as determined by Western blot analysis and immunofluorescence, respectively (Fig. 5C and data not shown). As shown in Fig. 5B, the N-terminal zinc finger motif and linker region were dispensable for KS1-mediated transcriptional repression as a construct containing zinc fingers 2 to 10 (ZF2-10 [lane 2]) fused to the N terminus of KS1 maintains strong transcriptional repression. In contrast, deletion of either zinc finger 2 (ZF3-10 [lane 6]) or zinc finger 10 (ZF2-9 [lane 3]) abolished the ability of KS1 to repress transcription. Furthermore, several other KS1 proteins containing zinc finger mutations, including an internal deletion of zinc fingers 3 to 7 (ZFΔ3-7 [lane 9]) and point mutations within zinc finger 2, 3, or 7 (lanes 11 to 13) that change one of the required cysteine or histidine residues to either alanine or leucine, are unable to repress gene expression. Thus, these data indicate that KS1 requires its nine clustered C-terminal zinc finger motifs to repress the expression of promoters containing the KBE site.

To assess whether the KRAB domain is necessary for KS1-mediated transcriptional repression, we created N-terminal deletion and site-directed mutations of KS1 (Fig. 6A). Each of these KS1 deletion proteins was expressed (Fig. 6C) and localized to the nucleus as determined by immunofluorescence (data not shown) (13). As shown in Fig. 6B, KS1 deletion

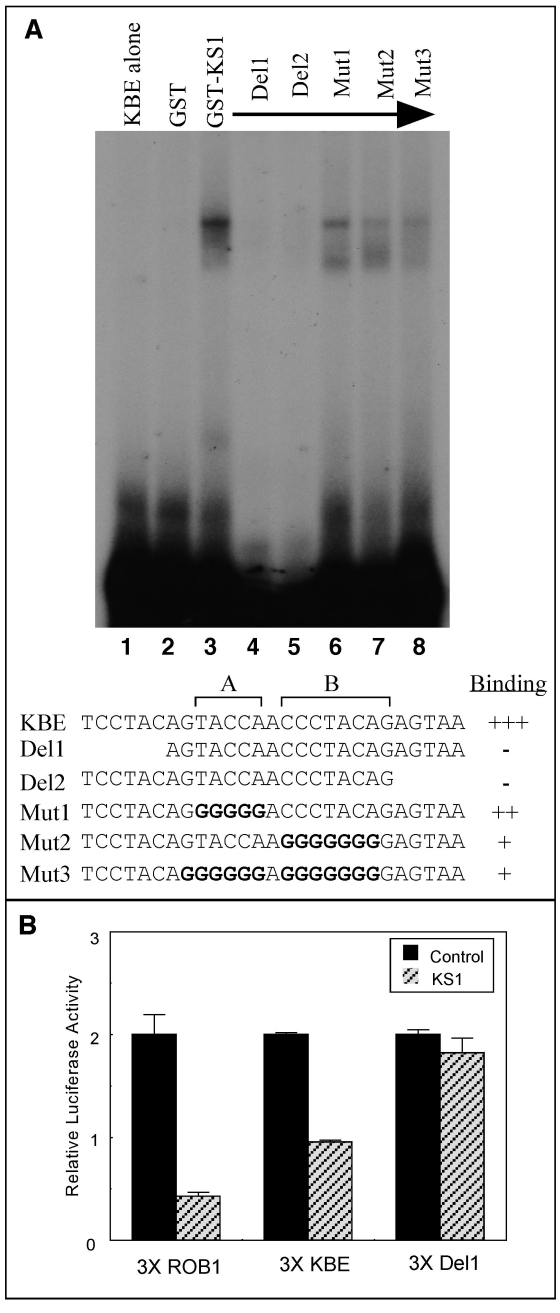

FIG. 4. Defining the KBE. (A) Electromobility shift assay was performed using the GST-KS1 fusion protein with deletion and site-directed mutated KBE probes. Lane 1, no proteins; lane 2, 200 ng of GST; lanes 3 to 8, 200 ng of GST-KS1 with the various probes as indicated. The relative binding affinity of KS1 for the different probes is shown. (B) Luciferase assays using a reporter vector containing three copies of the ROB1, KBE, or Del1 sequence were performed as described for Fig. 3. The histogram of luciferase activity shows that KS1 is able to repress expression of a reporter containing three copies of the KBE. Note that KS1 is unable to repress a reporter containing three copies of the Del1 mutant sequence.

mutants lacking the KRAB domain [KS1 (105-566) (lane 2) and KS1 (213-566) (lane 4)] were unable to repress transcription, whereas a construct lacking the region between the KRAB domain and the zinc finger region [KS1 (Δ105-212) (lane 3)] maintained strong transcriptional repression. Furthermore, a KS1 gene containing a mutation within the KRAB A domain (KS1 KRAB mt [lane 5]), a mutation previously shown to abolish KRAB-mediated transcriptional repression in GAL4-based assays, was unable to repress transcription (20). Therefore, these mutagenesis data demonstrate that a functional KRAB A domain is required for KS1-mediated transcriptional repression.

KS1 colocalizes and interacts with the KAP-1 corepressor protein. Recently, three independent groups have identified a KRAB A-interacting protein, which they individually named KAP-1, KRIP-1, or TIF1β (referred to as KAP-1 in this paper) (12, 18, 21). Sequence alignment of the KS1 KRAB A domain reveals high homology with several different KRAB domains, including that found in KOX1, the protein originally used to identify KAP-1 (Fig. 7A) (12, 21). These data support the hypothesis that KS1 will also interact with the KAP-1 corepressor protein. To test this possibility, we generated GST fusion proteins containing either the wild-type or a mutant KS1 KRAB domain (Fig. 7B). GST pulldown assays using [^{35}S]methionine-labeled cell lysates demonstrated that the wild-type KRAB domain of KS1 interacts with a single 100-kDa protein, a size that corresponds to that of KAP-1 (Fig. 7C) (12, 18, 21). Furthermore, pulldown assays from cells transfected with an HA-tagged KAP-1 expression vector followed by Western blot analysis reveal that this protein is indeed KAP-1 (Fig. 7D). In contrast, the mutant KS1 KRAB domain that was unable to direct KS1-mediated transcriptional repression (Fig. 6B) does not interact with KAP-1.

To determine if KS1 and KAP-1 are colocalized within the nucleus, we performed an immunofluorescence assay for these proteins in transfected CHO cells. As shown in Fig. 8, KS1 and KAP-1 are similarly distributed within discrete regions of the nucleus. This pattern of expression is not observed for all KRAB-ZFPs, as several members of this family have been localized to the nucleolus (17). The Kid-1 protein, for example, is found extensively within the nucleolus of transfected cells (Fig. 8I to L) (17). Together, these results demonstrate that KS1 and KAP-1 are localized within similar regions of the nucleus, a finding that is consistent with the ability of these two proteins to interact in GST pulldown assays. Furthermore, these data support a role for KS1 and KAP-1 to function together to regulate gene expression.

The KAP-1 corepressor protein enhances KS1-mediated transcriptional repression. Based on our studies indicating that KS1 binds to KAP-1 in vitro and that these proteins colocalize in transfected cells, we next determined if KS1-mediated transcriptional repression is sensitive to the presence of KAP-1. To address this question, we first determined if overexpression of the KS1 KRAB domain, which should compete with the full-length KS1 protein for any titratable factors involved in transcriptional repression (e.g., KAP-1), is sufficient to abolish KS1-mediated transcriptional repression. To perform this experiment, CHO cells were transfected with a limiting amount of full-length KS1 and increasing amounts of an expression vector containing the KS1 KRAB domain that lacks the DNA binding motif. As shown in Fig. 9A (lanes 3 to 6), transfection of the KRAB domain abolishes the ability of KS1 to repress transcription in a dose-dependent manner. In

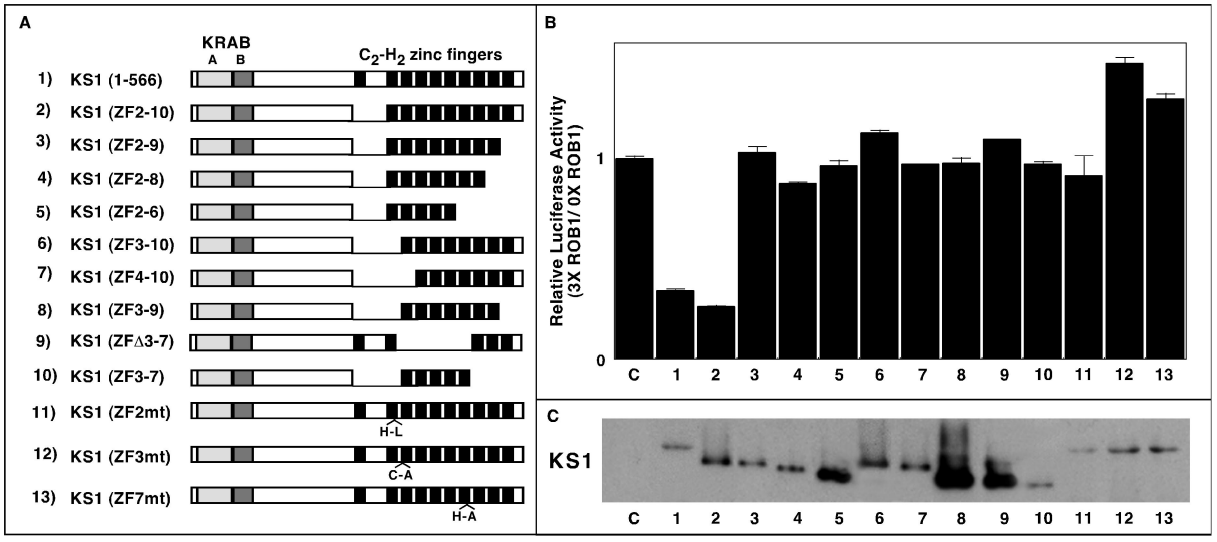

FIG. 5. Mapping of the zinc finger motifs required for KS1-mediated transcriptional repression. (A) Physical maps of the KS1 expression vectors transfected into CHO cells and tested in transcriptional regulatory assays. (B) Cells were transfected with either the 0× ROB1 or the 3× ROB1 reporter vector and the various deletion and site-directed mutants as indicated. Luciferase activities were determined as described for Fig. 3, and the 3×/0× values with standard deviations were graphed in the histogram. Note that the construct containing the nine clustered zinc fingers of KS1 was able to repress transcription [KS1(ZF2-10)], whereas all other zinc finger mutations abolished KS1-mediated transcriptional repression. (C) The various KS1 deletion proteins were detected by Western blot analysis as described in Materials and Methods. Note that all of the KS1 deletion constructs are expressed at similar or higher levels than the full-length KS1 protein.

contrast, expression of the mutant KS1 KRAB domain, which does not interact with the KAP-1 corepressor protein in vitro (Fig. 7), has no effect on KS1-mediated transcriptional repression (Fig. 9A, lanes 7 to 10). Western blot analysis reveals that both the wt and mt KRAB domains are expressed at similar levels in the presence of full-length KS1 (Fig. 9B). Furthermore, control experiments using KRAB wt in the absence of the full-length KS1 protein show no effect on reporter gene expression (Fig. 9A, lanes 11 to 14).

Because a defining feature of corepressor proteins is their ability to stimulate the repression activity of their target transcription factor, we next tested the ability of KAP-1 to enhance KS1-mediated transcriptional repression. To perform this experiment, we first inhibited the transcriptional repression in-

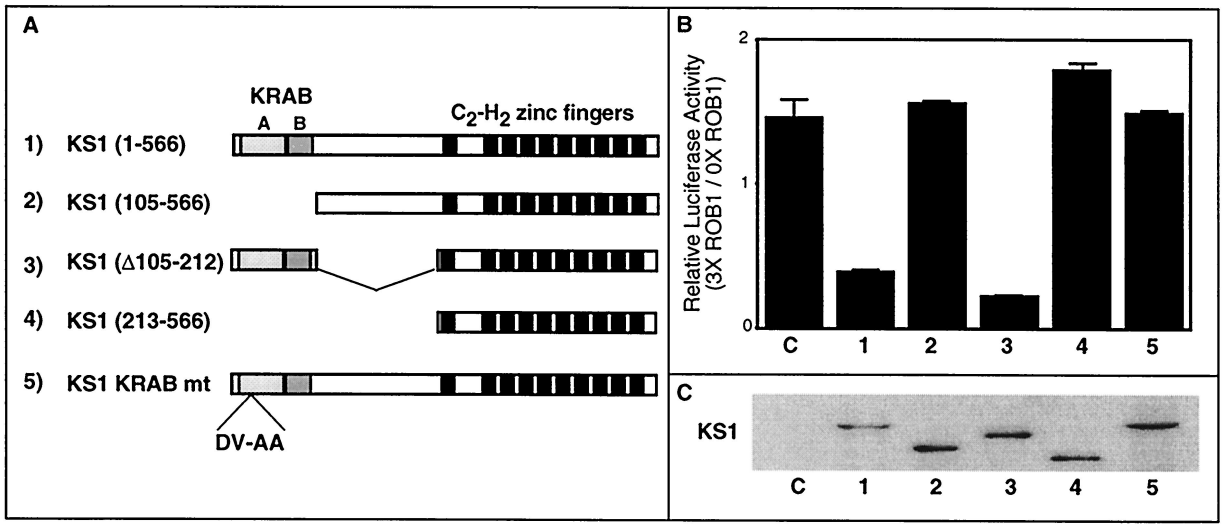

FIG. 6. Mapping of the transcriptional repressor activity within KS1. (A) Physical maps of the KS1 expression vectors transfected into CHO cells and tested in transcriptional regulatory assays. (B) Cells were transfected with either the 0× ROB1 or the 3× ROB1 reporter vector and the various deletion and site-directed mutants as indicated. Luciferase activities were determined as described for Fig. 3, and the 3×/0× values were graphed in the histogram. Note that both constructs containing KRAB wt were able to repress transcription, whereas deletion or mutation of this region abolished KS1-mediated transcriptional repression. (C) The KS1 deletion proteins were detected by Western blot analysis as described in Materials and Methods. Note that all of the KS1 deletion constructs are expressed at similar or higher levels compared with the full-length KS1 protein.

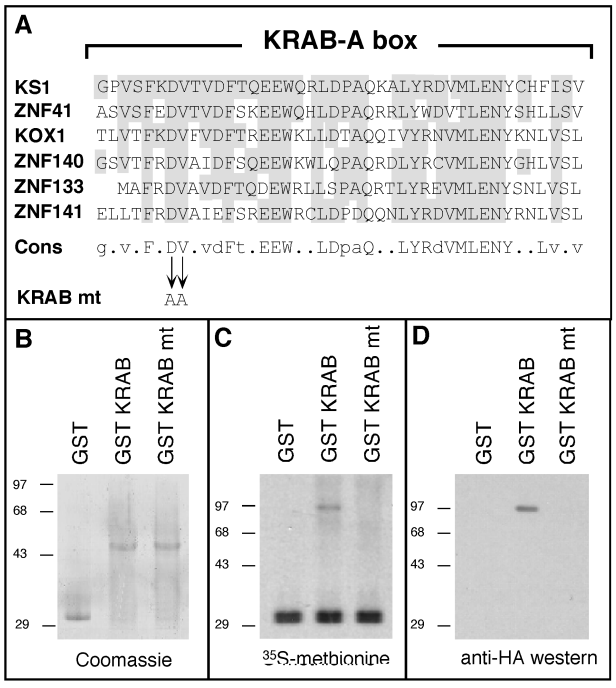

FIG. 7. The KRAB domains of KS1 interact with KAP-1. (A) Comparison of the KS1 KRAB-A motif with the KRAB-A domain from other KRAB-ZFPs. Identical residues are shaded. The KRAB-A consensus sequence (Cons) is derived from the work of Bellefroid et al.; uppercase letters represent highly conserved residues, lowercase letters represent moderately conserved residues, and dots represent unconserved residues (2). The KRAB mutation that abolishes KS1-mediated transcriptional repression is indicated. (B) Coomassie blue gel analysis of the GST, GST-KRAB, and GST-KRAB mt fusion proteins used for the GST pulldown assays. (C) To determine the proteins with which the KRAB domain interacts, CHO cells were labeled with [^{35}S]methionine, lysed in RIPA buffer, and incubated with either the GST, GST-KRAB, or GST-KRAB mt protein. GST pulldown assays were performed as described in Materials and Methods. Note that KRAB wt interacts with a protein of approximately 100 kDa, whereas the mutant KRAB domain does not. The doublet of ≈30 kDa corresponds to endogenous GST. (D) To determine whether the 100-kDa KRAB-interacting protein was KAP-1, CHO cells were transfected with an HA-tagged form of KAP-1. Western blot analysis using an anti-HA antibody demonstrates that the KRAB wt of KS1 interacts with the KAP-1 protein.

duced by KS1 through the addition of the KRAB domain alone. We then added increasing amounts of KAP-1 to determine if this protein could then restore KS1-mediated repression. Figure 9C demonstrates that increased expression of KAP-1 strengthens KS1-mediated transcriptional repression in a dose-dependent manner (lanes 4 to 7), whereas expression of KAP-1 in the absence of KS1 has no effect on reporter activity (lanes 8 to 11). This effect is not due to loss of KS1 expression, as Western blot analysis demonstrates that both proteins are expressed at equivalent levels when either transfected alone or cotransfected (Fig. 9D). Therefore, these data suggest that KS1 can function as a DNA binding protein that tethers KAP-1 to specific sequences in order to repress transcription.

DISCUSSION

KRAB-containing ZFPs have been proposed to function as sequence-specific transcription factors. This idea was derived from strong biochemical data demonstrating that the KRAB motif, a domain always associated with multiple C-terminal zinc fingers, represses transcription when fused to a heterologous DNA binding domain (20, 26, 33). However, rigorous support for this hypothesis requires the demonstration that KRAB-ZFPs use their zinc finger domains to recognize distinct DNA sequences and, more importantly, that they regulate promoters containing these elements. Unfortunately, thus far, this crucial piece of evidence has remained elusive. In the present study, however, we have identified a DNA sequence that is specifically recognized by the KRAB-ZFP, KS1. This finding has allowed us to test two different questions. First, can a full-length KRAB-ZFP function as a sequence-specific transcriptional repressor, and second, which of the 10 zinc finger motifs within this multi-zinc-finger protein are required to interact with this DNA binding site?

DNA binding activity of KS1. We initially attempted to deduce the DNA binding site for KS1 using information previously derived from a large number of biochemical and structural studies of non-KRAB C_2-H_2 ZFPs. These studies indicated that the DNA sequence bound by a zinc finger motif is determined by the identity of the −1, +3, and +6 amino acids within the α-helical region of each finger (8, 23, 24). However, as recently demonstrated, this a priori deductive approach can provide misleading information due to the fact that other residues within the α-helix can affect DNA binding specificity (31). Consequently, this phenomenon can complicate the predictions made for ZFPs, especially for proteins containing a relatively large number of zinc finger domains, such as KS1. Therefore, we instead used a random oligonucleotide selection approach to identify DNA sequences that specifically bind to this protein (3). The consensus KBE identified by this method is a 27-nucleotide sequence that does not contain binding sites for any previously identified transcription factor (Fig. 1) (29). Interestingly, however, an a posteriori analysis that compared the conserved A and B box sequences within the experimentally isolated KBE (TACCAACCCTAC AG) with a predicted binding sequence based upon the amino acid identities within the α-helical regions of zinc fingers 3 to 7 of KS1 (TACTACNNNTANTG) reveals highly similar sequences (35). However, in vitro gel shift assays demonstrated that this 14-bp sequence is not sufficient for KS1 to interact with through its zinc finger motifs (Fig. 4A). Furthermore, reporter assays revealed that zinc fingers 3 to 7 of KS1 were insufficient to direct transcriptional repression in vivo (Fig. 5B). Thus, the finding that KS1 binds to the 27-bp KBE site but not to shorter deletion mutants of this sequence suggests that most, if not all, of the zinc finger motifs of KS1 interact with nucleotides along the KBE site. Our finding that KS1 requires 9 of its 10 zinc fingers to repress gene expression in vivo (Fig. 5) correlates well with this hypothesis.

Transcriptional regulatory activity of KS1. The identification of a KBE allowed us to test the hypothesis that KS1 functions as a sequence-specific transcription factor using transcriptional regulatory assays. The results from these studies demonstrate that KS1 behaves as a transcriptional repressor of both basal and activated transcription through the KBE site and that the KRAB domain is required for this repressive function (Fig. 3 and 6). Moreover, the fact that the KRAB domain represses transcription within the context of the native

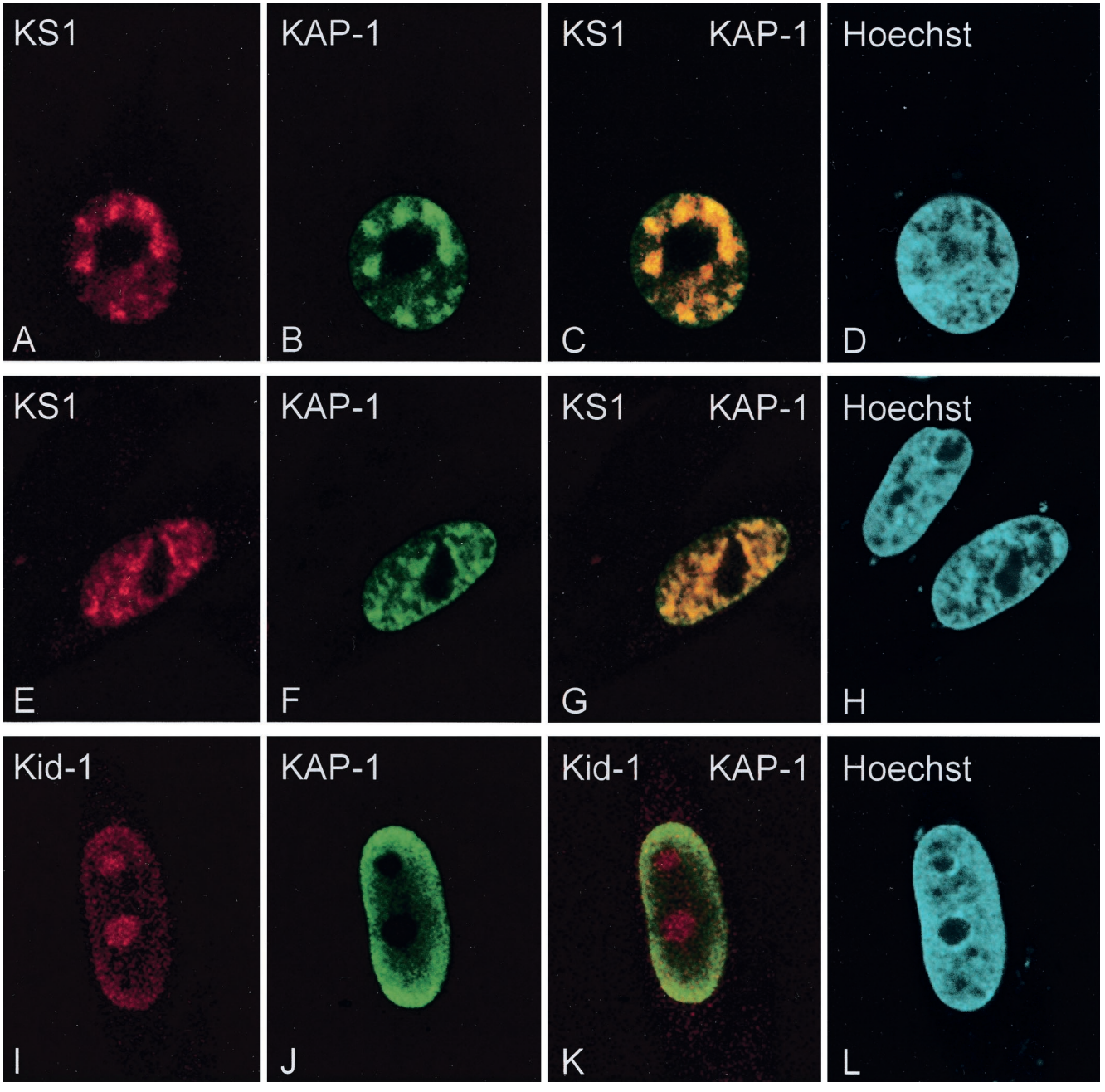

FIG. 8. KS1 colocalizes with KAP-1. For KS1 and KAP-1 colocalization, CHO cells were cotransfected with an Xpress-tagged KS1 construct and an HA-tagged KAP-1 vector (A through H). KS1 expression was detected with an anti-Xpress mouse monoclonal antibody and a rhodamine-conjugated anti-mouse secondary antibody (A and E), whereas the KAP-1 protein was detected with an anti-HA-umouse monoclonal antibody directly conjugated to fluorescein (B and F). Superimposing these images demonstrates that KS1 and KAP-1 are localized to the same regions of the nucleus, as shown in yellow (C and G). For Kid-1 and KAP-1 colocalization, CHO cells were transfected with an Xpress-tagged KAP-1 construct and an HA-tagged Kid-1 expression vector (I through L). KAP-1 was detected with an anti-Xpress mouse monoclonal antibody and a fluorescein-conjugated anti-mouse secondary antibody (J), Kid-1 was detected with an anti-HA-umouse monoclonal antibody directly conjugated to rhodamine (I), and the images were subsequently superimposed (K). Hoechst staining was used to stain cellular DNA (D, H, and L). Note that an untransfected cell shown in panel H shows no specific staining.

KS1 protein indicates that the zinc finger motifs do not interfere with the repressive function of this domain. This finding is important in light of the fact that the DNA binding motif of other transcription factors can mask the activity of certain transcriptional regulatory domains identified by GAL4-based reporter assays (7, 16, 36). Thus, the data derived from the in vitro selection protocol and the in vivo reporter assays indicate that the KBE is a bona fide binding site for KS1. Moreover, this functional analysis supports a model in which KRAB-ZFPs bind a cognate DNA binding site via their zinc finger motifs to repress transcription through their KRAB domains.

To better understand KS1-mediated transcriptional repres-

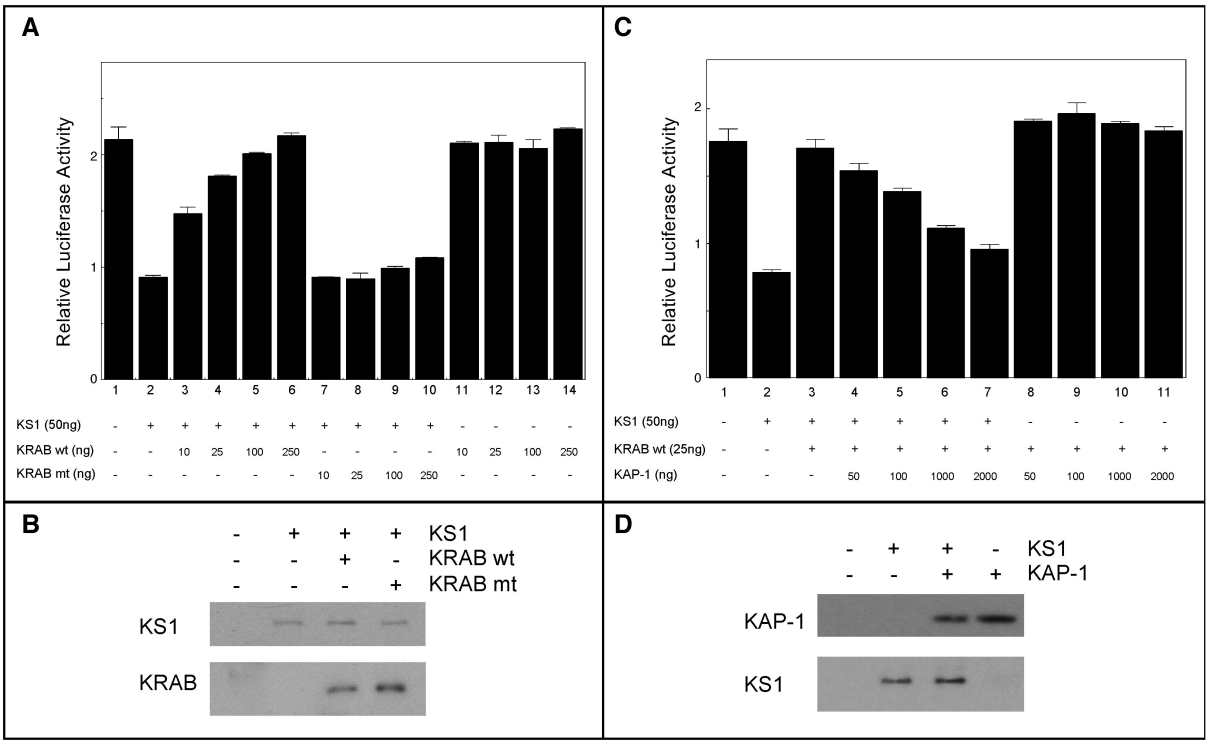

FIG. 9. KAP-1 enhances KS1-mediated transcriptional repression. CHO cells were transfected with various constructs as indicated, and luciferase assays were performed as described for Fig. 3. (A) Histogram of luciferase activity from an experiment using either KRAB wt or KRAB mt, both of which lack the zinc finger motifs of the full-length KS1 protein. Note that expression of the KRAB domain alleviates the ability of KS1 to repress luciferase expression, whereas expression of the mutant KRAB domain has no effect on KS1-mediated repression. (B) Western blot analysis was used to detect the Xpress-tagged KS1 KRAB proteins (wt or mt) from CHO cells cotransfected with the Xpress-tagged full-length KS1 protein as indicated. (C) Histogram of luciferase activity from CHO cells transfected with increasing amounts of a KAP-1 expression vector. Note that addition of KAP-1 increases the transcriptional repression activity of KS1. (D) Western blot analysis was used to detect the Xpress-tagged full-length KS1 protein and the HA-tagged KAP-1 protein from transfected CHO cells as indicated.

sion, we tested whether this activity is modulated by the KAP-1 nuclear protein. To begin to address this question, we utilized several different biochemical, cellular, and transcriptional regulatory assays to provide evidence that KAP-1 can function as a corepressor for KS1. First, we demonstrated that the KRAB domain of KS1 interacts with the KAP-1 protein from cell lysates in pulldown assays (Fig. 7). Second, mutations within the KRAB domain that abolish the interaction with KAP-1 in vitro result in a loss of transcriptional repression activity by KS1 (Fig. 6 and 7). Third, we demonstrated that KS1 and KAP-1 are colocalized in similar regions of the nucleus (Fig. 8). Finally, we show that the expression of the KAP-1 protein enhances the transcriptional repression activity of KS1 through the KBE site (Fig. 9). While these results do not demonstrate that KS1 directly interacts with KAP-1, the GST pulldown assays performed under high-stringency conditions are consistent with this hypothesis, as the KS1 KRAB domain interacts with only one protein from metabolically labeled cell lysates (Fig. 7). Furthermore, the fact that the KRAB domain from other proteins does directly bind to KAP-1 also supports that the KS1-KAP1 interaction is direct (25). Taken together, these results are consistent with data derived from studies using KRAB-containing GAL4 chimeric proteins and support the hypothesis that KAP-1 functions as a corepressor protein for a full-length KRAB-ZFP (12, 18, 21).

Potential cellular roles for KRAB-ZFPs. In light of our finding that KS1 can function as a sequence-specific transcription factor, it is useful to review the proposed cellular functions for other members of the KRAB-ZFP family. The Kid-1 ZFP, for example, can bind to heteroduplex DNA structures and is localized to the nucleolus (9, 17). Once localized to this region of the nucleus, Kid-1 leads to the disintegration of the nucleolus, and nucleolar run-on assays demonstrated that rRNA synthesis was greatly reduced in cells transfected with this protein (17). Moreover, the KRAB domain of Kid-1 was necessary for both of these cellular phenomena, suggesting that this protein may repress RNA polymerase I transcription (17). Interestingly, however, it has been recently reported that the KRAB domain of KOX1 cannot repress transcription of RNA polymerase I in GAL4-based assays (22). One explanation for this finding is that the KRAB domain may function differently in the full-length Kid-1 protein than in a chimeric fusion protein. Another possibility is that the KRAB domains of KOX1 and Kid-1 behave differently at RNA polymerase I promoters. Thus, future studies are needed to delineate between these possibilities and to demonstrate that the full-length Kid-1 protein can specifically repress gene expression within the nucleolus.

In contrast to Kid-1, the ZNF74 protein is found within discrete granular nuclear structures, is tightly associated with

the nuclear matrix, binds to RNA, and interacts with RNA polymerase II (14, 15). This KRAB-ZFP contains a truncated KRAB A domain and 12 different C_2-H_2 zinc finger motifs that are sufficient for targeting this protein to the nuclear matrix as well as for RNA binding (14). In addition, ZNF74 interacts with the hyperphosphorylated form of RNA polymerase IIo and colocalizes with this protein in nuclear domains enriched in splicing factors (15). These findings suggest that ZNF74 may regulate gene expression through both transcriptional and posttranscriptional mechanisms. However, since ZNF74 contains a truncated KRAB A domain, the role of this protein in transcriptional repression remains to be established.

Our findings demonstrate that KS1, like ZNF74, is not expressed within the nucleolus but is localized to other regions of the nucleus. Moreover, we have shown that KS1 binds to DNA in a sequence-specific manner and not only interacts with the KAP-1 corepressor protein but also is colocalized with this protein within the nucleus. Furthermore, we have shown that KS1 functions as a transcriptional repressor of both basal and activated RNA polymerase II promoters using a reporter system, a function stimulated by the expression of KAP-1. These KS1 data, together with results obtained from studies using other KRAB-ZFPs, suggest that members of this large family of proteins may be divided into subgroups based upon their expression patterns within the nucleus and on their potentially distinct cellular functions.

In conclusion, here we provide direct experimental evidence that KS1 fulfills the criteria for a sequence-specific transcriptional repressor. To our knowledge, this is the first member of the KRAB-containing family of proteins that in its native form has been demonstrated to have this activity. Furthermore, the identification of the KBE site in conjunction with deletion analysis of KS1 provided us with an assay for mapping the domains required for KS1-mediated DNA binding and transcriptional repression. Therefore, these data are important as part of the theoretical framework for understanding the role of multiple ZFPs and the control of gene expression.

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