A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins

(transcription factors/Kid-1/ZNF2/coiled-coil motif/TIF1/PHD domain)

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ABSTRACT The Krüppel-associated box A (KRAB-A) domain is an evolutionarily conserved transcriptional repressor domain present in approximately one-third of zinc finger proteins of the Cys₂-His₂ type. Using the yeast two-hybrid system, we report the isolation of a cDNA encoding a novel murine protein, KRAB-A interacting protein 1 (KRIP-1) that physically interacts with the KRAB-A region. KRIP-1 is a member of the RBCC subfamily of the RING finger, or Cys₃HisCys₄, family of zinc binding proteins whose other members are known to play important roles in differentiation, oncogenesis, and signal transduction. The KRIP-1 protein has high homology to TIF1, a putative modulator of liganddependent activation function of nuclear receptors. A 3.5-kb mRNA for KRIP-1 is ubiquitously expressed among all adult mouse tissues studied. When a GAL4-KRIP-1 fusion protein is expressed in COS cells with a chloramphenicol acetyltransferase reporter construct with five GAL4 binding sites, there is dose-dependent repression of transcription. Thus, KRIP-1 interacts with the KRAB-A region of C2H2 zinc finger proteins and may mediate or modulate KRAB-A transcriptional repressor activity.

Limited numbers of structural motifs have been described for eukaryotic transcription factors. A common motif is the Cys₂-His₂ zinc finger structure that is highly conserved and found in many different species (1). Approximately one-third of all Cys₂-His₂ zinc finger proteins contain evolutionarily conserved regions at their amino terminus, which have been termed Krüppel-associated boxes (KRAB-A and -B), containing a total of about 75 amino acids (2). 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 (2).

Our studies with Kid-1, a KRAB-containing protein with 13 zinc fingers, revealed that its amino terminus, which contains the KRAB-A and -B domains, when fused to GAL4, confers transcriptional repression on promoter constructs with GAL4 binding sites (3). We subsequently established that the KRAB-A regions of Kid-1 and other zinc finger proteins are responsible for the transcriptional repression (4). Similar results were reported by Rauscher and colleagues (5). Site-directed mutagenesis of conserved amino acids in the KRAB-A motif results in decreased repressor activity (4).

Others have reported that the KRAB-A domain, tethered to RNA polymerase II promoters via a GAL4-binding domain, represses transcription in a distance-independent manner (6). The KRAB-A domain, when tethered to the transactivating response element, has also been shown to repress both basal and Tat-mediated human immunodeficiency virus 1 (HIV-1) long terminal repeat-driven gene expression (7). This observation led to the proposal that control of HIV-1 gene expression might be achieved with the KRAB-A domain fused to Tat to generate transdominant negative mutants. Fusion of the KRAB-A domain of human Kox1 to the Tet repressor derived from Tn10 of Escherichia coli results in the production of a tetracycline-controlled hybrid protein that, when bound to the tetO sequences upstream of a cytomegalovirus-driven luciferase reporter construct, represses luciferase expression (8). This repression was found with different promoters and from tetO sequences placed as far as 3 kb from the transcriptional start site. Thiesen and colleagues also found that a 110-kDa protein coimmunoprecipitates with the TetR-KRAB protein, suggesting the presence of an interacting protein. It was the purpose of our studies to identify proteins that interact with the KRAB-A domain to gain insight into potential mechanisms of KRAB-A-mediated repression.

METHODS

Two-Hybrid Screen. A two-hybrid screen was performed to isolate genes encoding proteins that associate with the KRAB-A domain. The two-hybrid screen was carried out in the yeast strain MaV103 using a system devised by Vidal and coworkers (9, 10). The *GAL4–GAL80*-deleted MaV103 strain is auxotrophic for uracil, leucine, and tryptophan and carries three chromosomally integrated reporter genes whose expression is regulated by different GAL4 responsive promoters: *GAL1::HIS3, SPAL10::URA3, GAL1::lacZ.*

To construct the KRAB-A bait, the nucleotides encoding the first 54 amino acids of Kid-1 (3), beginning with the initiation codon and containing the KRAB-A domain, were PCR-amplified from pMFH2-GAL4-Kid1A (11). The PCR primers were constructed to incorporate a *Sal*I site in the 5' end and a *Bam*HI site in the 3' end. After restriction enzyme digestion, the PCR product was subcloned in-frame with GAL4 into pPC97 to create pPC97/KRAB-A. The pPC97 plasmid has stop codons 3' from the multiple cloning site in all reading frames and carries the *LEU2* yeast selectable marker.

The cDNA library for the yeast two-hybrid screen was constructed in the pPC86 (12) vector modified by adding a 600-bp stuffer into the *Bgl*II site to facilitate the recovery of *Eco*RI- and *Spe*I-digested clones (a kind gift of Joshua La Baer of the Massachusetts General Hospital Cancer Center). The pPC86 vector carries the *TRP1* yeast selectable marker. cDNA

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Abbreviations: KRAB, Krüppel-associated box; KRIP, KRAB-A interacting protein; 3-AT, 3-aminotriazole; CAT, chloramphenicol acetyltransferase; HA, hemagglutini; DB, GAL4 DNA binding domain; AD, GAL4 transactivation domain; FOA, 5-fluoroorotic acid. Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U67303).

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was derived from poly(A) RNA isolated from 12- to 13-day whole murine fetuses and cloned directionally into the EcoRI(5') and SpeI (3') sites of the vector. Fusion proteins are encoded containing the simian virus 40 large tumor antigen nuclear localization sequence and the transactivation domain (AD) of GAL4 at the amino terminus (AD library).

MaV103 yeast cells containing pPC97/KRAB-A were transformed with the AD library by the lithium acetate method (13). Approximately one million transformants were plated onto leucine- and tryptophan-deficient synthetic complete [(SC)-Leu-Trp] medium, replica-plated after 2 days onto (SC)-Leu-Trp-His medium containing 50 mM 3-aminotriazole (3-AT; Sigma) to select for GAL1::HIS3-dependent His/AT prototrophy, and subsequently replica-plated again onto SC-Leu-Trp-His medium containing 50 mM 3-AT. Twelve positive clones were picked after an additional 3 days of incubation. pPC86 plasmids that conferred resistance to 50 mM 3-AT were rescued from yeast by electroporation of E. coli XL1 Blue bacteria with total yeast DNA. Fresh MaV103 yeast cells containing pPC97/KRAB-A were transformed with these plasmids, encoding putative KRAB-A interactors, and activation of all three reporters was tested as follows (10): (i) His prototrophy on SC-His plus 30 or 50 mM 3-AT; (ii) β-galactosidase activity, using a filter lift assay; (iii) URA3 activation on SC-Ura and SC+FOA (5-fluoroorotic acid). Two clones [15-2, named KRIP-1.2 (KRAB-A interacting protein 1.2); and 15-3, named KRIP-1.3] scored positively for the four phenotypic assays, suggesting that they were true interactors. Both of the clones were closely related structurally (see Results).

Screening of Murine cDNA Libraries and Sequencing. A cDNA library from embryonic day 13 to 14 kidney constructed in λ ZAP-II was provided by K. Hession of Biogen. A 500-bp 5' restriction fragment of the partial length clone obtained by the two-hybrid screen, KRIP-1.2, was used to screen approximately 1.5×10^6 plaques using standard techniques with duplicate filter lifts. Two clones approximately 3 and 3.5 kb long were plaque-purified, subcloned into pBluescript (Strategene), and sequenced on both strands by the chain-termination method (14). Computer analysis was carried out with the sequence analysis software package of the Genetics Computer Group at the University of Wisconsin, Madison (15).

Northern Blot Analysis. A human multiple tissue Northern blot (CLONTECH) was hybridized at 65°C with the 2582-bp KRIP-1.2 cDNA, labeled with [³²P]dCTP by random priming, and washed at 65°C according to the procedure described by CLONTECH.

Transfection Protocols and Chloramphenicol Acetyltransferase (CAT) Assays. COS cells were plated 2 days prior to transfection at a density of 2.5×10^5 cells per 100 mm-dish. For transfections, cells were generally exposed to 20 µg of total DNA in 5 ml of DMEM/10% NuSerum (Collaborative Biomedical Products, Bedford, MA)/DEAE-dextran (400 µg/ ml)/0.1 mM chloroquine. One microgram of a luciferaseexpressing plasmid, poLucSV/T1 (provided by C. S. Shelley, Massachusetts General Hospital) was included for normalization of transfection efficiencies. Three to 4 h 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). Cells were then 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·HCl (pH 7.8) and subsequently broken up by freezing-thawing three times in a dry-ice/ethanol bath and 37°C water bath. The supernatant was assayed for CAT and luciferase activities as described (3). CAT activity is expressed as the ratio [monoacetylated [¹⁴C]chloramphenicol/ (monoacetylated plus nonacetylated) [¹⁴C]chloramphenicol] and is normalized to luciferase activity.

Coimmunoprecipitation Studies. To confirm the direct interaction of KRIP-1 with the KRAB-A region of Kid-1 (Kid-1A) or ZNF2 or the entire non-zinc-finger region of Kid-1 (Kid-1N), the 2.6-kb KRIP-1.2 cDNA from pPC86-KRIP-1.2 was subcloned into the expression vector pMT3, which encodes fusion proteins in-frame with the 9-amino acid hemagglutinin (HA) epitope tag. COS cells were cotransfected with pMT3-KRIP-1.2 and with the expression vector pBXG1 containing an insert encoding GAL4 fusion proteins with Kid-1A, Kid-1N, the KRAB-A region of ZNF2, or the GAL4 binding protein alone. In other studies the vector pMFH was used (11) to encode a GAL4 Kid-1A or Kid-1N fusion protein preceded in-frame by a Flag epitope, from bacteriophage T7. Transfections were done using DEAE-dextran, as described. Forty-eight hours later cells were lysed and cellular proteins were immunoprecipitated for 4 h with anti-HA antibody using protein G-agarose (Boehringer, Mannheim) (16). Proteins were separated on a 10-12% SDS/PAGE gel and electrophoretically transferred to an Immobilon-P membrane (Millipore). Western blot analysis was then performed using a polyclonal antibody to GAL4 or monoclonal antibody to T7 Flag.

Protein Gel and Western Blot Analysis. SDS/PAGE gel and Western blot analyses were performed according to standard protocols (14). An anti-GAL4 antibody (obtained from S. A. Johnston and K. Melcher, University of Texas Southwestern Medical Center) was used at a 1:3000 dilution. A commercial monoclonal T7⁻Tag antibody (IgG2b, κ) directed against the Flag epitope was obtained from Novagen and used at 1:3000 dilution. Immune complexes were detected with the Renaissance light detection kit from DuPont.

RESULTS

Isolation of a cDNA That Encodes a KRAB-A-Interacting Protein. Despite the highly reproducible and well-character-

Yeast Two-Hybrid Selection



FIG. 1. Selection of a KRAB-A interacting protein using a yeast two-hybrid screen. The second through the fifth panels demonstrate phenotyping on four media. Growth or LacZ expression reflects the expression levels of three GAL4-responsive reporter genes. Yeast transformants were patched in duplicate on SC-Leu-Trp (Leu-Tryp-) medium in the panel on the left. The yeast cells were then replica plated onto the other four plates. In each panel are yeast cells transformed with pPC97/GAL4 encoding only the DNA binding (DB) region of GAL4 or pPC97/KRAB-A, which includes DB and KRAB-A (DB/KRAB-A), with the pPC86 vectors encoding one of the following: the activation domain (AD), AD and KRIP-1.3 (AD/ 15-3), or AD and KRIP-1.2 (AD/15.2). The arrows designate (from left to right on each panel) one negative control (DB + AD), one intermediate positive control (DB-retinoblastoma + AD-E2F1), and two positive controls (DB-Fos + AD-Jun, and full-length GAL4containing binding domain and activation domain) (9). Proteinprotein interaction confers ability to grow on His-Leu-Tryp- medium in the presence of 50 mM 3-AT (second panel) and growth in Ura⁻Tryp⁻Leu⁻ medium (fourth panel). In the third, panel β -galactosidase activity induced by protein-protein interaction, is demonstrated. The fifth panel reveals FOA sensitivity resulting from the protein-protein interaction.

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1261 1321 1381 1441 1501	H CTC L AAJ K ACC T GA' D GTC V	IGA E CAA N ACT L CAA K TGT V CAA N	GCC P CGC A CTT GGA E CAA K TGA D	CCT L TCA H GGC A GGT M GAT M TGC A	CGT V CAA K TTC. S TCG R GGC A CCA Q	GCT L GGAA D AACT L AAAG S CAT (Î) GAA K	GTT F CCA GGT V CTC S TCT L GGT V	CTG C TCA Q GAA K GAT I GAC T	TGA E GTA Y ACG R CCG R GAT I CGA E	GAG S CCA' Q TCT (1) CCA Q CAT M GGG G	GTT GTT G GGT G GGT K G GAA K TCA Q	FGA D TTT L GGA D GGA E GGA Q	CACI T GGAI E CAAI K D GGAI E GGAI E	ACT L AGA D ACA H TGI V GAA N ACC R	TTGC: A TTGC: A TGCA Q TTAA K TTAA K	CTG	CCG R GAG R ACT C GCG R GCG R GCG R GCG R	CGA D GAA N TCA Q AGT TCG R TCG R CCA Q	CTG CCA Q GAA K GCA Q AGT V GCA H	CCAG Q ACGT R AAAC N GGTT V TCTG (L) CTGG W	2 C O I L E D	234 254 274 294 314 334
1261 1321 1381 1441 1501	H CTC L AAA K ACC T GA U GTC V	IGA E CAA N ACT L CAA K TGT V CAA N	GCC P CGC A CTT C C C C C C C C C C C C C C C C	CCT L TCAA H GGC A GGT M GAT M TGC A	CGT V CAA K FTC. S FTCG R GGC A CCA Q	GCT L GGAA D ACT L AAAG S CAT (Î) GAA K	GTT F CCA GGT V CTC S TCT L GGT V	CTG C TCA Q GAA K GAT I GCA Q GAC T	TGA E GTA Y ACG R CCG R GAT I CGA E	GAG S CCA' Q TCT D CCA Q CAT M GGG G	GGTT GGTT GGGT GGGT K CAA CAA	FGA D TTT L GGA D GGA S GGA E GGA E GGA	GGAL GGAL E CAAA K D GGAL E GGAL E	ACT L AGA D ACA H TGT V GAA N ACC R	TGCACC T TTGC. A TGCAC Q A TAAAK K TCT L	CTG	CCG R GAG R ACT C GCG R GCG G GCG R GCG R	CGA D GAA N TCA Q AGT V TCG R CCA Q	CTG CCA Q GAA K Q GCA Q AGT V GCA H	CCAG Q ACGT R AAAC N GGTT V TCTG (_) CTGG W	2 C O I L E D C	234 254 274 294 314 334
1261 1321 1381 1441 1501 1561	H CTC L AAA K ACC T GAC V V ACC	IGA E CAA N ACT L CAA K TGT V CAA N CAT	GCC P CGC A CTT CTT C C CTT C C C C C C C C C C	GGCT GGCC A GGCT M GGAT M TGCC A CAAA	CGT V CAA K FTC R GGC R GGC A CCA Q AAT	GCT	GTT F CCA GGT V CTCC S TCT L GGT L GGT	CTG C TCA Q GAA K GAT I GCA Q GAC T GCA	TGA E GTA Y ACCG R CCCG R GAT I CCGA E CCCA	GAG S CCA' Q TCT L CCA Q CAT M GGG G GGA	GGTT GGTT GGGT GGGT GGAA K CAA QAAA	FGAA D TTTV L GGAA CAT	CACI T GGAI E CAAI K TGA' D GGCT GGAI E TTTT	ACT L AGA D ACA H TGI V GAA N ACC R GCC	TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: C TTGC:	CTG	CCG R GAG R GCG R GCG R GCG R GCG R GCG R GCG R GCG C CTC	CGA D GAA N TCA Q AGT TCG R CCA Q TTG	CTG CCA Q GAA K GCA Q AGT V GCA H GCA	CCAG Q ACGT R AAAC N GGTT V TCTG CTGG W TCTG	2 C O I L E D C O	234 254 274 294 314 334
1261 1321 1381 1441 1501 1561	H CTC L AAA K ACC T GAC U V ACC T	IGA E CAA N ACT L CAA K TGT V CAA N CAA	GCC P CGC A CTT CTT C C CAA K CAA K CAA K T GAC T	GGCT GGCT M GGCT M TGCC A CAAA K	CGT V CAAA K FTCC R GGCC A CCA Q AAAT I	GCT L GGAA D ACT L AAAG S CAT (Î) GAA K Q	GTT F CCA GGT V CTC S TCT L GGT K	GAA GAA K GAA K GAA K GAA T GCA H	TGA E GTA Y ACCG R CCCG R I CCGA E CCCA Q	GAG S CCA Q TCT (L) CCA Q CAT M GGGG GGA E	CTG GTT F TGG GGT GGT K C Q ACA H	FGAA D TTT L GGAA CAT I	GGAL GGAL E CAAL K D GGAL E GGAL E TTTT L	ACT L AGA D ACA H TGI V GAA N ACC R GCC R	TTGC: A TTGC: A TGCA Q TTGCA K TGCA K TTAA K TTTT F	CTG CAC: T GAAA K GGAA CAC: T GGAA CAC: T GGAA CAC: T GGAA CAC: T GGAA CAC: T GGAA C C C C C C C C C C C C C	CCG R GAG R GCG R GCG R GCG R GCG R GCG R CTC S	CGA D GAA N TCA Q AGT TCG R CCA Q CCA Q TTG W	CTG CCA Q GAA K GCA Q AGT V GCA H GGC A	CCAG Q ACGT R AAAC N GGTT V TCTG CTGG W TCTG L	2 C O I L E D C O I	234 254 274 294 314 334
1261 1321 1381 1441 1501 1561	H CTC L AAA K ACC T GAA V ACC T C C C	IGA E CAA N ACT L CAA K TGT V CAA N CAA	GCCC P CGCC A CTTT C C CTTT C C C CTT C C C C C T C	CCT L TCAA GGCC A GGCT M TGCC A CAAA K TAA	CGT V CAA K TTC S TTCG R GGC A CCA Q AAAT I	GCT L GGAA D AAAG S CAT L AAAG S CAT L GAA K TCA Q	GTT F CCAA GGT V CTCC S TCT L GGT L GGT K AGC	CTG C TCA Q GAA K I GCA Q GAC T GCA H TCT	TGA E GTA Y ACG R CCG R GAT I CGA E CCA Q	GAG S CCA Q TCT (L) CCA Q CAT M GGG G GGA CAT	GGTT GGTT F GGGT GGGT K CTCA Q ACA H CTCC	FGA D TTT L GGA D GGA E GGA Q CAT I	CACJ T GGAJ E CAAL K TGA D GGAJ E CAAL E CAAL	ACT L AGA D ACA H TGT V GAA N ACC R R GCC R	TTAAA K TTAAA K TTAAA K TTTT F	$\begin{array}{c} \text{CTG} \\ \bigcirc \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	GAG R GAG R ACT C C C C C C C C C C C C C C C C C C	CGAA D GAA N TCA Q AGT TCG R CCA Q TCG R CCA	CTG CCA Q GAA K GCA Q AGT V GCA H GGC A	CCAG Q ACGT R AAAAC N GGTT V TCTG CTGG W TCTG L	2 COLLED COLL	234 254 274 294 314 334 354
1261 1321 1381 1441 1501 1561 1621	H CT(L AAJ K AC(T GA' V AC(T C GA' E	IGA E CAA N ACT L CAA K TGT V CAA N CAA S GAG	GCCC P CGCC A CTTT CTT C CAA K TGAA D GACC T TGAA D	CCT L TCA GGC A GGC A GGT M TGC A CAA K K N	CGT V CAA K TTC S TCCG R GGCC A CCA I I CCAA N	GCT L GGAA D AAAG S CAT L AAAG S CAT L GAA K TCA Q TTAC T	GTT F GGT V CTC S TCT L GGT K GGAA K AGC	GAA GAA GAA GAA GAA GAA GAA GAA GAA H TCT	TGA E GTA Y ACG R CCG R GAT I CCGA E CCA Q CTT L	GAG S CCA ^I Q TCT Q CCA M GGG G GGA E GGA E CAT	GGTT F GGTT G GGT G GGT K TCA K C C C C C C C C C C C C C C C C C C	FGAA D TTT L GGAA D GGAA E GGAA E GGAA L CAT I I TAA	CACJ T GGAJ E CAAJ K TGA' D GGAJ E CAAJ K CAAJ CAAJ CAAJ CAAJ CAAJ CAAJ CA	ACT L AGA D ACA H TGI V GAA N ACC R GCC R GCC R	TAAA T TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: T T T TGC: T T TGC: A T T TGC: A T T T T T T T T T T T T T T T T T T	CTG AGT AGT CAC. T GAAA K GGAA CTA Y	CCG R GAG R GCG R GCG R GCG R GCG R CTC S TF	CGAA D GAA N TCA Q AGT TCG R CCA Q TTG W CCA	CTG CCA Q GAA K Q GCA Q AGT V GCA H GGC A C T	CCAG Q ACGT R AAAAC N GGTT V TCTG U CTGG W TCTG L GCAT H	2 COILED COIL	234 254 274 294 314 334 354
1261 1321 1381 1441 1501 1561 1621	H CTC L AAJ K ACC T GA V ACC T C ACC T C GA C C T C GA C	IGA E CAA N ACT L CAA K TGT V CAA N CAA S GAG	GCCC P CGCC A CTTT C CAA K CAA K CAA K D GGAC T TGA	CCTUL TCAU GGCCA GGCT M TGCCA A CAAA K N	CGT V CAA K TTC S TCG R GGC A CCA Q AAT I CAA N	GCT L GGAA D AACT L AAAG S CAT C C CAT C C C C	GTT F CCA GGT V CTC S TCT L GGT K GGAA K AGC A	GAA GAA GAA GAA GAA GAA GAA GAA T C C	TGA E GTA Y ACGG R GAT I CGA E CCA Q CTT L	GAG S CCA Q TCT Q CCA M GGG G GGA E CAT M GGG G GGA CAT M	GTTGG GTTF G GGT K GGT K C C C C C C C C C C C C C C C C C C	FGAA D GGAA D GGCA S GGAA E GGAA E CAT I I TAA K	CACI T GGAI E CAAI K TGA' D GGAI E TTT' L GAAA K	ACT L AGA D ACA H TGT V GAA R GCG R GCG R GCG L	TTGC: TTGC: A TGCA A TGCA A TGCA A TTGC: A TGCA A TGCA A TGCA A TGCA A A TGCA A A TGCA A A TGCA A A A A A A A A A A A A A	$\begin{array}{c} \text{CTG}\\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	CCG R GAG R GGG R GGG G GGG R GGG R GGG R GGG R GGG R GGG R TTT F	CGAA D GAA N TCA Q AGT TCG R CCA Q TTCG R CCA Q CCA Q CCA	CTG CCA Q GAA K Q GCA V GCA H GCC A GCT L	CCAG Q AACGT R AAAAC V TCTG CTGG U CTGG U CTGG U CTGG CAT H	2 COILED COIL	234 254 274 294 314 334 354 374
1261 1321 1381 1441 1501 1561 1621 1681	H CTC L AAJ K ACC T GAC T GAC T GAC C GAC C GAC	IGA E CAA N ACT L CAA K TGT V CAA N CAT GAG S GGC	GCC P CGC A CTTT GGA E CAA K TGA C T GAC T TGA	$\begin{array}{c} CCT^{i}\\ L\\ TCA^{i}\\ \hline \\ \hline$	CGT V CAA K TTCC S CCA Q CCA CCA CCA CCA N AAT	GCT L GGAA D AACT L AAAG S CAT C C CAT C CAT C CAT C CAT C CAT C C CAT C C CAT C C CAT C C CAT C CAT C C CAT C CAT C C CAT C C C C	GTT F CCA GGT V CTC S TCT L GGT K AGC A TGT	$\begin{array}{c} CTG^{\circ}\\ \hline \\ C \\ \hline \\ C \\ \hline \\ TCA^{\circ}\\ \hline \\ \\ TCA^{\circ}\\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	TGA E GTA Y ACG R CCG R GAT I CGA E CCA Q CTT L TCC	GAGG S CCA Q TCT C CCA Q CAT M GGG G G G G G G CAT M TGT	GGTT GGTT GGTT GGTT GGTT GGTT GGTA K TCA Q ACA H CTC S GGA	TGA D TTT L GGA D GGA S GGA S GGA CAT I TAA K GCC	CACJ T GGAJ E CAAA K D GGAJ E CAAA K CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C C CAAA C C CAAA C C CAAA C C CAAA C C CAAA C C CAAA C C CAAA C C C CAAA C C C CAAA C	ACT L AGA D ACA H TGI V GAA N GCG R GCG R GCG R GCG R GCG R	TTGC: A TTTT F TTGC: A TTGC: A TTTTT T TTGC: A TTGC: A TTGC: A TTTTT T TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC:	$\begin{array}{c} \text{CTGC}\\ \hline \\ \text{AGT}\\ \hline \\ \text{CAC}\\ \text{T}\\ \text{CAC}\\ \text{T}\\ \text{CAC}\\ \text{T}\\ \text{GAA}\\ \text{CAC}\\ CA$	$\begin{array}{c} CCG \\ R \\ \hline \\ GGAG \\ R \\ \hline \\ \\ GCG \\ R \\ GGGG \\ R \\ GGGG \\ R \\ GGGG \\ R \\ CTC \\ S \\ TTT \\ \hline \\ GAA \\ GAA \\ \end{array}$	CGAA D GAAA N TCA Q AGT TCG R CCA Q TTG W CCA Q GTT	$\begin{array}{c} CTG\\ \hline \\ CCA\\ Q\\ GAA\\ K\\ GCA\\ Q\\ AGT\\ V\\ H\\ GGCA\\ GCT\\ L\\ \hline \\ TCA\\ \end{array}$	CCAG Q AACGT R AAAAC V TCTGG U CTGG CTGG GCAT H GCGGT	2 C O I L E D C O I L	234 254 274 294 314 334 354 374
1261 1321 1381 1441 1501 1561 1621 1681	H CTC L AAJ K ACC T GAA T GAA T GAC T CGA R	IGA E CAA N ACT L CAA K TGT V CAA N CAT M GAG S GGC	GCC P CGC A CTT C C C C C C C C C C C C C C C C	CCTV L TCAU GGCC A GGCT M TGCC A CAAA K TAAAN CAAA K	CGT V CAAN K TTCC S TCG R GGC A CCA Q AAT I CAA N M	GCT L GGAA D AAAG S CAT L AAAG S CAT L GAA K CAT L GAA T CA T T CA T I GAT I	GTT F CCA GGT V CTC S TCT L GGT K GGAA K AGC A TGT V	GAA GAA K GAT GAA K GAA K GAA K GAA H TCT GGA D	TGA E GTA Y ACG R CCG R I CCA Q CTT L TCC P	GAGG S CCA Q TCT Q CCA Q CAT M GGGG G GGA E GGGA E TGT V	$\begin{array}{c} CTG^{G}\\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	TGAA D TTTY L GGAA D GGCA CAT I TAA K GCCC P	CACJ T GGAJ E CAAJ K TGA' CAAJ GGAJ CAAJ CAAJ CAAJ CAAJ CAAJ CAAJ	ACT L AGA D ACA H TGI V GAA N ACC R GCC R GCC C C G GCC G	TTGC: A TTGC A TTTGC A TTT	$\begin{array}{c} \text{CTG} \\ \hline \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $	CCG R GAG R GGG R GGG G GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GGG R GAGAR R CTC	CGA D GAA N TCA Q AGT CCA Q TCG R CCA Q TTG W CCA Q GTT F	CTG CCA Q GAA K Q GCA K Q GCA H GCCA C CCA Q GCA C CCA Q GCA C CCA Q GCA C CCA Q GAA K Q CCA Q GAA K CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA Q GAA K Q CCA C Q GAA K Q CCA Q CCA C Q GAA K C C C C C C C C C C C C C C C C C C	CCAG Q ACGT R AAAAC N TCTG GGTT V TCTG CTGG GCAT H GCGG W	2 COIL ED COIL	234 254 274 294 314 334 354 374
1261 1321 1381 1441 1501 1561 1621 1681	H CTC L AAA K ACC T GAA V ACC T CGA E CGA R	IGA E CAA N CAA K CAA K CAA N CAT GAG S GGC	GCCC P CGCCA CTTT GGAA E CCAA K TGA GACC T TGA D CCTT L	CCT L TCAN GGC A GGC M GGT M CAAA K CAAA K	CGT V CAAA K TTCCG R CCA CCA CCA AAAT I CAAA N AAAT M	GCT L GGAA D AAAG S CAT L AAAG S CAT L GAA K TCA Q TAC T TAC T I GAT	GTT F CCA GGT V CTC S TCT L GGT K AGC A TGT V	$\begin{array}{c} CTG^{\circ}\\ \hline \\ \hline$	TGA E GTA Y ACG R CCG R GAT I CCA Q CTT L TCC P	GAGG S CCA Q TCT (L) CCA M GGG G G G G G G G CA T G T G T G T G T G T G T	CTG ^C GTT ^G F GGTT GGTT GGAA K TCA Q ACA H CTC S GGA CTC S GGA	IGAA D TTT L GGAA D GGCA CAT I ITAA K GCCC P	GGAJ GGAJ CAAA K TGA' D GGT GGAJ K CAAA CAAA CAAA CAAA CAAA CAAA CAAA	ACT L AGA D ACA H TGI V GAA N ACC R GCC R GCC R GCC C C L GCC	TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A TTGC: A T T T T T T T T T T T T T T T T T T	$\begin{array}{c} \text{CTG} \\ \hline \\ \hline \\ \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ $	CCG R GAG R ACT GCG R GCG R GCG R GCG R CTC S TTT F GAA K	CGA D GAA N TCA Q AGT TCG R CCA Q TTG W CCA Q GTT F	CTG CCA Q GAA K GCA Q GCA H GGCC A GCT L TCA Q	CCAG Q ACGT R AAAC N GGTT V TCTG L CTGG W GCAT H GTGG W	2 C O I L E D C O I L	234 254 274 314 334 354 374 394
1261 1321 1381 1441 1501 1561 1621 1681 1741	H CTC L AAA K ACC T GAA D GAC T CGA R CGA R GAA	IGA E CAA N ACT L CAA K IGT V CAA N CAA S GGC S GGC	GCCC P CGCCA CTTT GGA E CAAA C TGA CCTT TGA D CCTT L CCAA	CCT ^C L TCAA GGC ^C A GGT ^C A GGT ^C A A CAAA K TGC ^C A CAAA K TGC ^C A CAAA	CGT V CAAA K TTCC R CCA CCA CCA AAAT I CCAA N AAAT M CTG	GCT L GGAA D ACT L AAAG S CAT L GAA K TCA Q GAA K TCA T GAT I GAC	GTT F CCA GGT V CTC S TCT L GGT V CTC S TCT L GGT V CTC S TCT L GGT V CTC C S TCT L C CA A C C C C C C C C C C C C C	$\begin{array}{c} CTG^{*}\\ \hline \\ \hline$	TGA E GTA Y ACG R CCG R CCG E CCA C CTT L TCC P	GAGG S CCA Q TCT D CCA M GGG G G G G G G G G G G G G G C T G T G	$\begin{array}{c} \text{CTG} \\ \hline \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $	IGAA D ITTT L GGAA D GGCA CAT I IAA K GCCC P	GGAJ GGAJ CAAA K TGA' D GGAJ GGAJ K TTT' L GGAA K TTCA' H	ACT L AGA D ACA H TGT V GAA N GCC R GCC R GCC CAA	TTGC. A TTGC.	$\begin{array}{c} TGG\\ \hline \\ G\\ \hline \\ G\\ G$	CCG R GGGG R GCG GCG GCG GCG GCG GCG GCG	CGAA D GAA Q AGT CCA Q TCG R CCA Q TTGA GTT F TGA	CTG CCA Q GAA K GCA Q GCA H GCCA L TCA Q GCG	CCAG Q ACGT R AAAC N GGTT V TCTG L CTGG U CTGG GCAT H GTGG W TCCT	2 C O I L E D C O I L	234 254 274 314 334 354 374 394
1261 1321 1381 1441 1501 1561 1621 1681 1741	H CTC L AAA K ACC T GA V ACC T GA GA C GA R CGA R D	IGA E CAA N ACT L CAA K TGT V CAA N CAA N GAG S GGC TCT L	GCC P CGC A CTT C C C C C C C C C C C C C C C C	CCT ^C L TCAA GGC ^C A GGT ^C A GGT ^C A CAAA K TGC ^C A CAAA K TGC ^C A	CGT V CAAA K TTCC S CCAA CCAA N CCAA N M CTG W	GCT L GGAA D ACT L AAG S CAT C CAT C GAA K TCA Q GAA K TAC T GAT I GAC T	GTT F GGT V CTC S TCT L GGT K AGC A A CAA K	$\begin{array}{c} \text{CTG}^{\text{CTG}} \\ \hline \\ \hline \\ \hline \\ \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\$	TGA E GTA Y CCGG R GAT I CGA E CCA Q CTT L TCC P TGC	GAGG S CCA ^I Q TCT Q CAT M GGG G G G G G CAT M TCT M TCT TCT TCT TCT TCT TCT TCT TC	$\begin{array}{c} \text{CTG} \\ \hline \\ \hline \\ \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $	IGAA D ITTT L GGA D GGA CAT I ITAA K GCCC F	CACJ T GGAJ E CAAA K D GGAJ CAAA C GGAJ K CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C C CAAA C C CAAA C C CAAA C C CAAA C	ACT L AGA D ACA H TGT V GAA N ACC R GCC R GCC R GCC R GCC R GCC R GCC R GCC R CAA K	TTGC. A TTGC. TTGC. A	$\begin{array}{c} \text{CTG}\\ \hline \\ \hline$	CCG R GAG R ACT L GCG R GCG R GCG R GCG R CTC S TTT F GAA K GGC A	CGAA D GAAA N CCA Q TCG R CCA Q TTG CCA Q TTGA F TGA	CTG CCA Q GAA K GCA CCA Q GCA K GCA CCA C Q GCA C CCA Q CCA Q CCA Q CCA Q CCA Q CCA Q Q AGT C C Q Q A A C C Q Q A A C Q Q A A C Q Q Q A A C Q Q Q A A C Q Q A A C Q Q A A C Q Q A A C Q Q A A C Q Q A A C Q Q A A C Q Q A A C A C	CCAG Q ACGT R AAAC N GGTT V TCTG G CTGG U GCAT H GTGG W TCCT P	C O I L E D C O I L	234 254 274 314 334 354 374 394
1261 1321 1381 1441 1501 1561 1621 1681 1741	H CTC L AAA K ACC T GA' U GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C GA' C C C C C C C C C C C C C C C C C C C	IGA E CAA N ACT L CAA K IGT V CAA N CAT M GAG S GGC A TCT L	GCC P CGC A CTT C C C C C C C C C C C C C C C C	CCT ^C L TCAA GGC ^C A GGC ^C A GGC ^C A GGC ^C A CAA K CAA K CAA K CAA K CAA	CGT V CAAA K TTCC S CCAA CCAA N CCAA N CCTG W CAC	GCT L GGGA D ACT L AAG S CAT L GAA K TCA Q GAA K TCA T GAA T I GAA T	GTT F CCA GGT V CTC S TCT L GGT C CTC S TCT L GGT K CAA K TCT TCT TCT C CAA	$\begin{array}{c} \text{CTG}^{\text{CTG}} \\ \hline \\ \hline \\ \hline \\ \hline \\ \hline \\ \\ \\ \hline \\ \\ \\ \\ \hline \\ \\ \\ \hline \\ \\ \\ \\ \hline \\ \\ \\ \hline \\ \\ \\ \\ \hline \\ \\ \\ \\ \\ \hline \\$	TGA E GTA Y ACG R CCG R CCG R CCA E CCA Q CTT L TCC P TGC A	GAGG S CCA ^I Q TCT Q CCA ^I CCA Q CAT M GGGG G GGA E CAT T TGT TGA E CAT	$\begin{array}{c} \text{CTG} \\ \hline \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $	IGAA D ITTT L GGA D GGA CAT I ITAA K GCCC F CTT	CACI T GGAI E CAAA E TGA' CAAA C GGAI C GGAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C CAAA C C CAAA C C CAAA C C CAAA C	ACT L AGA D ACA H TGT V GAA R GCC R GCC R GCC R GCC R GCC R GCC R GCC R GCC R	TTGC T TGC: A TGC	$\begin{array}{c} CTG \\ \hline \\ CAGT \\ \hline \\ CAGT \\ \hline \\ CAGT \\ \hline \\ \\ GAA \\ \hline \\ \\ \\ \\ GAA \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\$	CCG R GAG R GCG R C C C C	CGAA D GAAA N CCA Q TCG R CCA Q TTG R CCA Q GTT F TGA E	CTG CCA Q GAA K GCA CCA Q GCA K GCA CCA C Q GCA C CCA Q GCA C C C Q C C Q C Q C Q C Q C Q C Q C Q	CCAG Q ACGT R AAAC N GGTT V TCTG CTGG CTGG W TCTG GCAT H GCAT P AACC	C O I L E D C O I L	234 254 274 314 334 354 374 394 414
1261 1321 1381 1441 1501 1561 1621 1681 1741 1801	H CTC L AAJ K ACC T GA' CGA R CGA R GGA GGG G	IGA E CAA N ACT L CAA K IGT V CAA N CAT M GAG S GGC TCT L TAC T	GCC P CGC A CTTT GGAA C CCTT CAA C CCT CCAA C CCT CCAA N GAA N	CCT ^Q L TCAA GGC ^C A GGC ^C A GGC ^C A CAA K CAA K CAA K CAA K CCAA CCC S	CGT V CAAK K FTCC S CCA CCA CCA CCA CCA CCA CCA CCA CCA	GCT L GGGA D AACT L AAAG S CAT L AAAG S CAT T CA T CAT T GAA T CAT T GAA T CAT T GAA C T CAT C C CAT C C C C	GTT F CCA GGT V CTC S TCT L GGT V CTC S TCT L GGT K CAA K TCT V CAA K TCC	CTG CTG CTG CTG CTG CTG CTG CTG	TGA E GTA Y ACG R CCG R I CGA E CCA CTT L TCC P TCC A GCC P	GAGG S CCA Q TCT C C C C C C C C C C C C C C C C C	$\begin{array}{c} \text{CTG} \\ \text{CTG} \\ \text{G} $	IGAA D ITTT L GGAA D GGAC S GGAA CAT I IAAA K GCCC F F F	GGAJ GGAJ E GGAJ E GGAJ E GGAJ E TTT GGAJ GGAJ CAAL K GGAJ E TTT H TGG ⁴ CAAL CAA	ACT L AGA D ACA H TGT V GAA N C GCC R GCC R GCC R GCC R GCC R GCC R GCC R C AA C A C A C A C A C A C A C A C	TGCAC T TTGC: A TGCCAC Q TTGCA K TGCAC K TTTT F TGAT TGAT I SAGCA A	$\begin{array}{c} CTG \\ \hline \\ CAGT \\ \hline \\ CAGT \\ \hline \\ CAGT \\ \hline \\ GAA \\ \hline \\ \\ GGA \\ \hline \\ \\ GGA \\ \hline \\ \\ GAT \\ \hline \\ \\ \\ GAT \\ \hline \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $	$\begin{array}{c} CCG \\ R \\ \hline GGAG \\ R \\ \hline \\ GCG \\ R \\ \hline \\ GCG \\ R \\ GGG \\ G $	CGAA D GAAA Q AGT TCG R CCA Q GTT F TGA E CCP	$\begin{array}{c} CTG\\ CC\\ CC\\ Q\\ GAA\\ K\\ GCA\\ CC\\ A\\ GCA\\ CC\\ A\\ CC\\ CC\\ A\\ CC\\ CC\\ CC\\ A\\ CC\\ CC$	CCAG Q ACGT R AAAAC N GGTT V TCTG CTGG W TCTG L GCAT H GTGG W TCCT P AAAGC	C O I L E D C O I L	234 254 274 294 314 334 354 354 374 394 414

1861	AAGC	AAGG	TTC	TGG	CAG	TAG	CCA	GCC	CAT	GGA	AGT	ACA	AGA	GGG	ATA	TGG	CTT	TGG	GTCA		
	KQ	G	s	G	s	s	Q	Р	М	Е	v	Q	Е	G	Y	G	F	G	s	4	54
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1981	GAGG	GAGA	GGT	AAG	TGG	CCT	CTT	AAG	GAA	GGI	GCC	ACG	TGI	GAG	CCI	TGA	ACG	CCI	GGAT		
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2041	Cmcc	2000	0.00	CmC	- m C 3	C 2C	~~~	~~~	100		2000	~ ~ ~			moo	mee					
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2101	GAGG	ACTA	CAA	TCI	GAT	TGT	TAT	TGA	GCG	TGG	TGC	TGC	TGC	AGC	CAC	TGC	TGG	TCA	GGCT		
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2221	GAAG	AAGA	GAC	AGA	AGC	TGC	TAT	TGG	AGC	TCC	ccc	GGC	TGC	ccc	CGF	GGG	TCC	TGA	AACC		
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2341	CCTA	GTGG	CAG	TAC	CAG	CTC	AGG	CTT	GGA	GGI	GGI	GGC	TCC	TGA	GGI	TAC	СТС	AGC	CCCA		
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2401	CTAN	CTCC	ccci	ACC	መልጥ	CCT0	CA	PC M	C 3 C	TCC	CAC	דמיד	CTTC	ccc	TCT	CTTC		222	ACCA	_	
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2461	GGTG	ACCT	GGT	CAT	GTG	TAA	CCA	TG	CGA	ATT	TTG	CTT	CCA	ССТ	GGA	TTG	CCA	CCT	ссст	D.	
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2521	GCCC	тсса	GGA	ጥርጥ	ጥሮሮ	100	GGA	363	מיינ	GAG	ጥጥር	CTTC	ልሮሞ	стс		CGT	GCT	ccc	TGAC		
2321	A L	Q	D	v	P	G	E	E	W	s	Ô	s	L	Õ	H	v	L	P	D	6	74
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2581	CTAA	AGGA	GGA	AGA	TGG.	AAG	CCT	CAG	CCT	GGA	TGG.	AGC	AGA	TAG	CAC	TGG	TGT	GGT	AGCT		
	ΓK	Е	Е	D	G	s	г	s	г	D	G	А	D	s	т	G	v	v	A	6	94
2641	AAAC	тстс	ACC	AGC	CAA	CCA	GCG	GAA	ATG	TGA	GCG	TGT	TCT	сст	GGC	сст	GTT	CTG	CCAT		
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2701	GAAC	CATG	CCG	TCC	CTT	GCA	TCA	GCT	GGC	TAC	CGA	СТС	TAC	ATT	CTC	CAT	GGA	GCA	GCCT	-	24
	E P	С	R	P	г	н	Q	г	A	т	D	S	т	r	S	м	Е	Q	P		34
2761	GGTG	GTAC	CCT.	AGA	CCT	GAC	CTT	GAT	TCG	TGC	TCG	сст	CCA	AGA	GAA	GCT	GTC	ACC	тсст		
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2821	TATA	GCTC	CCC	CCA	GGA	GTT	TGC	TCA	AGA	TGT	GGG	CCG	CAT	GTT	CAA	ACA	GTT	CAA	CAAG	-	- 4
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2881	CTGA	CTGA	GGA	CAA	GGC	AGA	TGT	TCA	GTC	CAT	CAT	CGG	СТІ	GCA	GCG	CTT	стт	TGA	GACA		
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2941	CGCA	TGAA	TGA	TGC	CTT	TGG	TGA	CAC	CAA	GTI	TTC	TGC	TGT	GCT	GGI	AGA	ACC	ACC	ACCA		1.4
	км	N	D	А	r	G	D	T	r	r	5	А	v	Ц	v	Е	P	P	P	0	14
3001	TTGA	ACCT	тсс	CAG	TGC	TGG	CCT	AAG	TTC	TCA	GGA	GCT	стс	TGG	ccc	TGG	TGA	TGG	cccc		
			P	s	A	G	L	s	s	Q	Е	L	s	G	Ρ	G	D	G	Р	8	34
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	L N	L	-																		
3061	L N Tgaa	L gctg	ggg	ctc	ttg	tgg	tca	gcc	cag	tcc	agc	tct	ggt	ctc	tgt	att	tto	acc	ccat		
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3061 3121 3181 3241	L N Tgaa accc cttc aaaa	L gctg tgtc acaa aaaa	ggg ctt aat	ctc tgg ggt	ttg tgg ttt	tgg cct tac	tca gac ttc	gcc tcc tgt	cag tgt gga	tcc tct ttt	agc tgc aat	tct tgg aaa	ggt ccc aac	ctc cat	tgt cgt act	att ccc gag	ttc ctc aaa	acc agt laaa	ccat ccct aaaa		
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3061 3121 3181 3241	L N Tgaa accc cttc aaaa	L gctg tgtc acaa aaaa	ggg ctt aat aaa	ctc tgg ggt	ttg tgg ttt	tgg cct tac	tca gac ttc	gcc tcc tgt	cag tgt gga	tcc tct ttt	agc tgc aat	tct tgg aaa	ggt ccc aac	ctc cat	tgt cgt act	att ccc gag	ttc ctc aaa	acc agt aaa	ccat ccct aaaa		

FIG. 2. Nucleotide and protein sequence of KRIP-1. The RING finger, B1, B2, coiled coil domain, and PHD finger are boxed. In the case of the RING finger, B1, B2, and PHD finger region, the cysteines and histidines making up the consensus sequence are circled. In the case of the coiled coil domain, the amino acids that form the heptad repeat of hydrophobic amino acids are circled.

ized transcriptional repression conferred by KRAB-A in mammalian cells, the GAL4-KRAB-A fusion protein exhibited a strong transcriptional activation function in yeast cells when overexpressed from multicopy plasmids (17, 18) (data not shown). However, when GAL4-KRAB-A was expressed at more moderate levels from the centromeric two-hybrid plasmid, pPC97 (12), it exhibited a milder degree of activation of GAL1::HIS3 that could be counteracted by the addition of 50 mM 3-AT in the selective medium lacking histidine. Transformation of yeast cells with the bait pPC97/KRAB-A and the mouse embryonic library in pPC86 with subsequent replica plating of 1.5×10^6 transformants to selective medium resulted in the isolation of two yeast clones (containing the inserts KRIP-1.2 and KRIP-1.3). These yeast colonies grew, when glucose was the carbon source, on SC-Leu-His-Trp medium in the presence of 50 mM 3-AT, which selects for yeast cells that have higher levels of HIS3 gene expression, which is required to sustain growth. Each of the two interactor plasmids encoding fusion proteins of the GAL4 activation domain with KRIP-1.2 or KRIP-1.3 (AD/15-2 or AD/15-3 in Fig. 1) were purified and retransformed along with pPC97/KRAB-A plasmid encoding the GAL4 DNA binding domain/KRAB-A fusion protein (DB/KRAB-A in Fig. 1) or the vector containing only the GAL4 DNA binding domain (DB). Growth on SC-Leu-His-Trp medium occurred only when AD/15-2 or AD/15-3 were expressed with DB/KRAB-A. Expression of the GAL4 activation domain only with DB/KRAB-A or the GAL4 DNA binding domain only with AD/15-2 or AD/15-3 did not permit growth. Similar findings were observed when activity of the URA3 gene was evaluated on SC-Ura-Trp-Leu plates. An additional unique feature of the two-hybrid system used is that the expression of the URA3 gene can be counterselected on medium containing uracil and FOA where cotransformation of the interacting fusion proteins results in prevention of yeast growth (Fig. 1). Finally, the specificity of the interaction between KRIP-1.2 or KRIP-1.3 and KRAB-A

was also shown using the LacZ reporter gene by transferring the colonies to a filter and incubating the filter in 5-bromo-4-chloro-3-indolyl β -D-galactoside.

The cDNA of clone KRIP-1.2 was 2582 bp long and that of KRIP-1.3 was 2453 bp long. We encountered some difficulty in obtaining a full-length clone likely due to the G+C-rich content of the 5' end of KRIP-1. A cDNA library from embryonic day 13–14 kidney constructed in λ ZAP-II was screened with a 500-bp 5' restriction fragment of clone KRIP-1.2. Two clones of approximately 3 and 3.3 kb long were plaque-purified and sequenced in pBluescript. Both the 3.0- and 3.3-kb clones were identical to KRIP-1.2 over their 2.6-kb 3' ends and extended 5' beyond KRIP-1.2. The 3.3-kb clone was designated KRIP-1. Its nucleotide and predicted protein sequences are presented in Fig. 2. KRIP-1 encodes an open reading frame of 834 amino acids with a calculated molecular weight of 88.9 kDa. The in vitro-translated protein migrated on SDS/PAGE gels with a molecular mass of approximately 105 kDa (data not shown). The sequence is alanine-rich (10.6% of amino acids), especially at the amino terminus. Upon search of GenBank, the KRIP-1 protein sequence was found to be distinct from but to have very high homology to that of TIF1 (transcriptional intermediary factor 1) (19), a putative modulator of the ligand-dependent activation function of nuclear receptors. Like TIF-1, KR IP-1 contains a RING finger domain at its amino terminus, followed by B1 and B2 domains and then a coiled coil domain (20). When KRIP-1.2 and KRIP-1.3 were compared with the KRIP-1 sequence, KRIP-1.3 was found to have a region of nt 695-823 (amino acids 47-89) deleted. This deletion disrupts the RING finger domain. The fact that the KRIP-1.3 peptide interacts with KRAB-A in the interactor screen indicates that the intact RING finger domain is not critical for this interaction. KRIP-1 contains a Cys₄-His-Cys₃ PHD finger domain (21) near the carboxyl terminus.

Tissue Distribution of KRIP-1. By Northern blot analysis, the KRIP-1 mRNA is approximately 3.5 kb and is expressed in each of the adult mouse tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, and kidney. The highest level of expression was found in testis (Fig. 3).

Direct Interaction Between KRIP-1 and KRAB-A in Mammalian Cells. To establish that the interaction identified by the two-hybrid system can occur in mammalian cells, KRIP-1.2 was coexpressed in COS cells with the KRAB-A regions of Kid-1 or ZNF2 or the entire non-zinc-finger region of Kid-1 (Kid-1N) fused to GAL4. Kid-1N and Kid-1A coimmunoprecipitated with KRIP-1.2 (Fig. 4). In addition the KRAB-A domain from ZNF2 also coimmunoprecipitated with KRIP-1.2, demonstrating that the interaction of KRIP-1.2 with the KRAB-A region of Kid-1 was not a unique property of the KRAB-A region of this protein.



FIG. 3. Multiple-organ Northern blot to detect KRIP-1 mRNA. Two micrograms of poly(A)-selected RNA from various organs was loaded onto each lane. The blot was hybridized at 65°C with the KRIP-1.2 cDNA and washed at 65°C in 0.1 × sodium chloride/sodium citrate (SSC)/0.1% SDS.



Blot: GAL4 Ab

FIG. 4. Coimmunoprecipitation of the KRAB-A domain from two distinct zinc finger proteins and KRIP-1.2. (A) COS cells were cotransfected with pMT3-KRIP-1.2, encoding the HA-tagged KRIP-1.2 with pMFH-Kid-1N, wild-type pMFH2, or pMFH2-Kid-1A, which encode T7-GAL4 fusion proteins with the non-zinc-finger region of Kid-1, no additional sequence, or the KRAB-A region of Kid-1 respectively. After cell lysis, preparation of soluble (S) and pellet (P) fractions, immunoprecipitation with the anti-HA antibodies, SDS/ PAGE resolution of the S, P, and immunoprecipitate fractions (IP), transfer to an Immobilon-P membrane, and reaction with anti-T7 flag antibody, it is apparent that Kid-1N and Kid-1A immunoprecipitate with KRIP-1.2. (B) pMT3-KRIP-1.2 was cotransfected with pBXG1 constructs encoding GAL4 fusion proteins of Kid-1N, Kid-1A, and the KRAB-A region of ZNF2. Kid-1N and the KRAB-A regions of Kid-1 and ZNF2 coimmunoprecipitate with KRIP-1.2 when the complexes are precipitated by anti-HA antibody. The blot is stained with anti-GAL4 antibody.

KRIP-1 Has Transcriptional Repression Activity. The pBXG1/KRIP-1.2 eukaryotic expression construct encoding a GAL4 DNA binding domain-KRIP-1.2 fusion protein was cotransfected with a CAT reporter plasmid containing five GAL4 binding sites (pG5SV-BCAT) into COS cells. When compared with cells cotransfected with the reporter construct and pBXG1/Kid-1N antisense, the CAT activity was lower in cells cotransfected with pBXG1/KRIP1.2 (Fig. 5). When a construct encoding only amino acids 382-834, excluding the RING finger, B1, B2, and coiled coil domain $(pBXG1/KRIP-1\Delta RBCC)$, was cotransfected with CAT reporter, the repression was equivalent to that observed with pBXG1/KRIP-1.2. A frameshift mutation in pBXG1/ KRIP-1 Δ RBCC, due to loss of a guanine nucleotide at 1702 that resulted in a stop codon at nt 1727-1729, eliminated the repressor activity of the protein. There was a dosedependent repression of CAT activity when the full-length



FIG. 5. Thin layer chromatography assays of CAT activities in COS cells cotransfected with a CAT reporter and with various KRIP-1 constructs. COS cells were cotransfected with 3 μ g of pG5SV-BCAT reporter construct, which contains five GAL4 binding sites, and with 20 μ g of the pBXG1 plasmid with inserts encoding a fusion protein of the DNA binding domain of GAL4 with Kid-1N antisense, full-length KRIP-1.2 encoding amino acids 43-834 of Krip-1, KRIP-1.2 in which the RING fingers, B1, B2, and coiled coil domains are deleted (GAL4-KRIP-1 Δ RBCC), or the latter with a frameshift mutation resulting in a stop codon at nt 1727. Full-length KRIP-1.2 and KRIP-1.2 without the RBCC domains exert equivalent transcriptional repression. Numbers at the bottom reflect relative CAT activity.

KRIP-1 was coexpressed as a GAL4 fusion protein with the pG5SV-BCAT reporter (Fig. 6).

DISCUSSION

While there are at least four well-defined types of transcriptional activation domains, serine/threonine-rich, acidic, proline-rich, and glutamine-rich (22), the only well-defined motif other than KRAB-A that has been postulated to mediate transcriptional repressor activity is an alanine-rich domain found in four transcriptional repressors from Drosophila: Krüppel (23), engrailed (24), even-skipped (25), and AEF-1 (23). In other cases where a repressor domain has been delineated, such as Egr-1 (26), SRF (27), and E4BP4 (28), no obvious consensus sequence motifs have been identified. The KRAB-A domain, which is present in approximately one-third of all Cys₂-His₂ zinc finger proteins, therefore, represents the first widely distributed transcriptional repressor motif. The potential α -helical structure of KRAB domains may mediate protein-protein interactions (2), but this hypothesis had not been previously tested. The fact that the repression afforded by the KRAB-A domain is distance-independent suggests that the mechanism of the repression is not related to steric hindrance and is consistent with a mechanism involving protein-protein interactions.

KRIP-1 belongs to the RBCC subfamily of RING finger proteins (20) that, in addition to the Cys₃-His-Cys₄ RING finger, contain a second cysteine-rich domain termed the B box, and a coiled coil motif carboxyl-terminal to the RING finger. Many members of the RING finger family have been implicated in the control of cell growth, differentiation, and development. The family includes many oncogenes, such as BRCA1, Mel18, and Bmi1 (20). The RBCC subfamily is particularly notable for the inclusion of a number of known oncoproteins, including Rfp/RET, the acute promyelocytic leukemia protooncogene PML, and T18 (20). Members of the RING finger family have been localized to the nucleus and proposed to interact with DNA. Mutation of cysteines in the RING finger domain of PML disrupts the folding of the protein and prevents PML nuclear body formation (20). Two of the germ-line mutations associated with predisposition to breast cancer occurs in the RING finger domain of the BRCA1 gene (29). Mutations in the B box or leucine coiled coil domain affect the normal interaction of PML with other nuclear proteins, interfering with the formation of nuclear bodies (30). The RBCC motif may be an integrated structural unit (20). The repressor function of KRIP-1 is localized to the carboxyl terminus of the molecule. While we have not identified the repressor domain, it is of interest that the PHD domain is located in this region of KRIP-1.

KRIP-1 is closely related to TIF1, a putative mediator of ligand-dependent activation function of nuclear receptors (19). TIF1, like KRIP-1 (data not shown), is localized in the nucleus of transfected cells. KRIP-1 may modulate or mediate the transcriptional repression of the KRAB-A motif. KRIP-1, like TIF1, may interact with one or more proteins important for the regulation of transcription. KRIP-1 is expressed ubiquitously. It is probable, therefore, that any tissue-specific repression is mediated by the KRAB-A-containing zinc finger protein.

The interaction of KRIP-1 with the KRAB-A domain of zinc finger proteins, if it carried over to other RBCC family members, might help to explain the importance of these proteins in control of proliferation and differentiation. If a KRAB-A-containing zinc finger transcription factor was important to repress proliferation and/or potentiate differentiation, the RBCC protein may mediate these effects. A mutation in the RBCC protein might serve to disrupt the ability of the KRAB-A-containing zinc finger protein to exert its repressor function. Uncontrolled proliferation, loss of differentiation and oncogenesis might be a consequence.



FIG. 6. Thin layer chromatography assays of CAT activities in COS cells cotransfected with a CAT reporter and with various amounts of full-length KRIP-1 constructs. The numbers at the top reflect the amount (in μ g) of pMFH₂/GAL4 plasmid encoding GAL4 binding domain only, pMFH₂/GAL4-KRIP-1 encoding a GAL4-KRIP-1 fusion protein, and pMT3-KRIP-1 encoding an HA–KRIP-1 fusion protein that cannot bind to the GAL4 binding sites of pG5SV-BCAT. Numbers at the bottom reflect relative CAT activity.

Note. After submitting the sequence of KRIP-1 to GenBank and while this manuscript was being prepared for submission, a study was published by Friedman *et al.* (31) in which the authors have used a different technique to clone and characterize KAP-1, the likely human homologue of KRIP-1.

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