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The Product of the Murine Homolog of the *Drosophila extra sex combs* Gene Displays Transcriptional Repressor Activity

OLEG N. DENISENKO AND KAROL BOMSZTYK*

Department of Medicine, University of Washington, Seattle, Washington 98195

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The heterogeneous nuclear ribonucleoprotein K protein represents a novel class of proteins that may act as docking platforms that orchestrate cross-talk among molecules involved in signal transduction and gene expression. Using a fragment of K protein as bait in the yeast two-hybrid screen, we isolated a cDNA that encodes a protein whose primary structure has extensive similarity to the *Drosophila melanogaster extra sex combs* (*esc*) gene product, Esc, a putative silencer of homeotic genes. The cDNA that we isolated is identical to the cDNA of the recently positionally cloned mouse embryonic ectoderm development gene, *eed*. Like Esc, Eed contains six WD-40 repeats in the C-terminal half of the protein and is thought to repress homeotic gene expression during mouse embryogenesis. Eed binds to K protein through a domain in its N terminus, but interestingly, this domain is not found in the *Drosophila* Esc. Gal4-Eed fusion protein represses transcription of a reporter gene driven by a promoter that contains Gal4-binding DNA elements. Eed also represses transcription when recruited to a target promoter by Gal4-K protein. Point mutations within the *eed* gene that are responsible for severe embryonic development abnormalities abolished the transcriptional repressor activity of Eed. Results of this study suggest that Eed-restricted homeotic gene expression during embryogenesis reflects the action of Eed as a transcriptional repressor. The Eed-mediated transcriptional effects are likely to reflect the interaction of Eed with multiple molecular partners, including K protein.

It is becoming increasingly apparent that many interactions between molecular partners involved in signal transduction and gene expression occur in the context of a novel class of proteins that act as docking platforms or scaffolds. These proteins have the ability to assemble multiple factors, allowing multilateral cross-talk and integration of inputs from different signal transduction and gene expression pathways. STE5 is an example of a scaffold protein in *Saccharomyces cerevisiae* that assembles MEKK, MEK, and MAPK, the three main components of the cascade that triggers the pheromone response (27). There are examples of such proteins in higher eukaryotic cells, including the heterogeneous nuclear ribonucleoprotein K protein (56), Sin (2), and Sam68 (41). All three of these proteins are made up of modules, each of which is able to recruit a unique set of molecular partners. For example, K protein, Sam68, and Sin all contain multiple SH3-binding domains that can engage protein tyrosine kinases, such as Src and Fyn. Sam68 and K protein, but not Sin, also contain the evolutionarily conserved KH domains that appear to mediate the ability of these proteins to bind RNA. Sam68 and K protein also bind single- and double-stranded DNA, but it is not known if the KH domains also mediate these protein-DNA interactions. The broad biochemical properties exhibited by K protein or Sam68 suggest that, in vivo, these proteins might be engaged in different types of molecular interactions. Recent studies revealed that K protein has a particularly diverse repertoire of molecular partners, which, in addition to kinases, the proto-oncoprotein Vav, and nucleic acids, also includes the TFIID TATA binding protein, TBP (33), and the transcriptional repressor, Zik1 (17). Identification of these factors as K protein molecular partners suggests that K protein might be involved in transcriptional control. This postulate is supported by the observation that in vitro addition of K protein has been reported to stimulate or repress transcription. These observations suggest that proteins that act as docking platforms might be involved in the regulation of transcriptional activation and repression (9).

Because K protein appears to function as a modular docking platform (9), it can be used as a tool to uncover novel factors involved in signal transduction and gene expression. Using a fragment of K protein in the yeast two-hybrid screen, we isolated a mouse cDNA that encoded a putative transcriptional repressor whose primary structure has similarity to the *Drosophila melanogaster extra sex combs* (*esc*) gene product (25). Using positional cloning, another group has recently shown that mutation of this gene is responsible for severe defects in anterior-posterior patterning during mouse embryogenesis and has denoted the gene *eed*, for *embryonic ectoderm development* (48).

MATERIALS AND METHODS

Cell lines. Glomerular epithelial cells, pre-B lymphocytes (70Z/3) (37), epidermoid cells (KB) (6), kidney cells (COS) (30), and human leukemia T cells (Jurkat) were grown as described before (40, 42).

Reagents. The bacterial expression vector pGEX-KT was provided by J. Dixon (University of Michigan). The mammalian expression vector pM1 was kindly provided by I. Sadowsky (University of British Columbia, Vancouver, Canada). The pGL3 luciferase reporter vectors, pGL3-Enhancer, containing the SV40 enhancer, and pGL3-Control, containing both the SV40 promoter and the SV40 enhancer, were purchased from Promega (Madison, Wis.). Glutathione agarose beads were obtained from Sigma (St. Louis, Mo.).

RNA extraction and Northern blot analysis. Total RNA was extracted essentially as previously described (12). Cells or animal tissues were washed with phosphate-buffered saline (PBS). Two milliliters of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol) was added to each plate or to 100 mg of animal tissue to lyse the cells. The final RNA was dissolved in 25 μl of water and used for Northern blot analysis. RNA was analyzed essentially as described previously (28). After first being denatured in formaldehyde and formamide at 65°C for 15 min, the RNA samples were cooled on ice. Ten micrograms of total RNA per lane was resolved by electrophoresis for 4 h at 70 V with a 1.2% agarose gel containing 2.2 M formaldehyde. RNA was transferred overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.05 M sodium citrate) to a Hybond N+ membrane (Amersham, Arlington Heights,

* Corresponding author. Mailing address: Department of Medicine, 356521, University of Washington, Seattle, WA 98195. Phone: (206) 543-3792. Fax: (206) 685-8661. E-mail: karolb@u.washington.edu.

Ill.) and dried at 80°C for 45 min. The membranes were prehybridized for 2 h at 42°C in prehybridization buffer (50% formamide, 5× Denhardt solution, 5× SSC, 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of denatured salmon sperm DNA/ml, and 0.1 mg of yeast tRNA/ml). After prehybridization, ^{32}P-labeled cDNA probe (2×10^6 cpm/ml) was added, and hybridization was carried out overnight at 42°C. Following hybridization, the membranes were washed two times in 2× SSC with 0.1% SDS at 22°C for 10 min and then twice in 0.1× SSC with 0.1% SDS at 50°C for 20 min and autoradiographed.

Yeast two-hybrid system library screen. The procedure used here was based on methods previously described (21, 57). Briefly, for the screen, the L40 strain of yeast (generated by Stanley Hollenberg), which is auxotrophic for His, Trp, Leu, and adenine, was used. The L40 yeast strain contains both *lacZ* and *HIS3* marker genes under the control of minimal GAL1 promoters fused with multimers of LexA DNA-binding sites. Yeast strains were grown at 30°C in rich medium containing 1% yeast extract, 2% Bacto Peptone, 2% glucose, and 0.1 mg of adenine/ml.

To construct the bait, the open reading frame of K protein was used as a template for PCR. The PCR primers were programmed to contain a *Bam*HI site on the 5′ end and a stop codon and a *Sal*I site on the 3′ end. After digestion, the PCR-generated fragment was subcloned in frame with LexA into the pBTM116 vector, a DNA-binding domain plasmid (pBTM116 was constructed by Paul Bartel and Stan Fields). The cDNA library for the yeast two-hybrid screen was generated by random-primed cDNA synthesis from the epidermoid KB cells (John Sims, Immunex Corporation). This library was inserted into the transcription activation domain vector, pGADH, to generate a Gal4 activation domain-cDNA hybrid protein.

Yeast cells were first transformed with the bait plasmid, and then the L40 LexA-K protein strains were transformed with the cDNA library. Screening for positive clones containing the LexA-K fusion protein plasmid and the activation domain-library plasmid was done by growth on His$^-$ selective medium and by testing the colonies for β-galactosidase activity (57). To eliminate false-positive clones, we mated clones containing the primary positive cDNA library plasmids with an opposite mating type strain transformed with a LexA-Lamin fusion protein plasmid, pLAM5 (generated by R. Sternglanz), as a nonspecific bait (4).

Screening of the murine cDNA library and DNA sequencing. A partial cDNA labeled by random priming with [α-^{32}P]dCTP (Amersham) was used to screen approximately 2×10^6 PFU of a λgt10 cDNA library prepared from 22D6 pre-B cells (provided by A. Bothwell, Yale University) as previously described (35, 36). Several clones were plaque purified, and the cDNA inserts were subcloned into pBluescript vector (Stratagene, La Jolla, Calif.) for sequencing. Sequencing was performed with the DyeDeoxy terminator cycle-sequencing kit (Perkin-Elmer).

In vitro transcription and translation. Partial or complete cDNAs were used as templates for in vitro transcription by SP6 or T7 DNA-dependent RNA polymerases to generate mRNAs for in vitro translation as previously described (24). In vitro translation in a rabbit reticulocyte lysate cell-free system (Promega) was performed according to the manufacturer's protocol.

In vitro binding studies. Five microliters of the in vitro translation reaction system containing ^{35}S-labeled products was added to a suspension of 10 μl of glutathione beads bearing either full-length K protein or a fragment of K protein fused to glutathione *S*-transferase (GST) in 100 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1.0 mM $MgCl_2$, and 0.1% Nonidet P-40). After being mixed for 60 min (4°C), the beads were washed three times with 400 μl of binding buffer and boiled with 30 μl of SDS sample buffer. Released proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Plasmid constructs. The *Stu*I-*Sma*I fragment, pG5bCAT (29), containing five Gal4-binding DNA elements and a TATA box, was introduced into the *Sma*I site of pGL3 enh plasmid (Promega) to create a reporter plasmid, pG5GL3 enh. The expression plasmid pM1Eed was constructed by inserting the full-length coding region of Eed, which was modified by PCR to create appropriate cloning sites, into the *Bam*HI site of the pM1 plasmid (45). The final construct contained an insert encoding Gal4 DNA-binding domain-Eed fusion protein under the control of the SV40 promoter. The plasmid pM1EedΔ1 was derived from pM1Eed by cutting at the *Bam*HI-*Sal*I fragment and blunt ligating the linearized plasmid, creating a Gal4-EedΔ1 fusion protein. pM1EedΔ2 was derived from pM1Eed by cutting at the *Sal*I-*Hin*dIII fragment and blunt ligating the linearized plasmid, creating a Gal4-EedΔ2 fusion protein. pM1EedΔ3 was derived from pM1EedΔ1 by cutting at the *Msc*I-*Hin*dIII fragment and blunt ligating the linearized plasmid, creating a Gal4-EedΔ3 fusion protein. For the full-length Eed expression, the Eed cDNA (nucleotides 133 to 1790) was inserted into pM1 vector linearized with *Bgl*II and *Pst*I. The *eed* point mutants, $T^{1040} \rightarrow C$ and $T^{1031} \rightarrow A$, were generated using a QuikChange site-directed mutagenesis kit (Stratagene).

To create K protein plasmids for expression in mammalian cells, the PCR-generated full-length K protein open reading frame was ligated into either *Bgl*II-*Bam*HI (for K protein expression) or *Bam*HI (for Gal4-K protein expression) of linearized pM1. All constructs were verified by sequencing and cell-free translation.

Transient transfection and luciferase reporter gene assay. COS cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum to approximately 60 to 75% confluency in 100-mm-diameter dishes and were transfected by the DEAE-dextran method (30). Briefly, cells were treated with a total of 5 to 10 μg of plasmid DNA premixed with 5 ml of Dulbecco's minimal essential medium and 0.16 mg of DEAE-dextran/ml. After a 4-h incubation, cells were shocked for 1 min at room temperature with 10% dimethyl sulfoxide in 1× PBS and washed twice with PBS and fresh medium was added. After 48 h, transfected cells were washed twice with PBS, scraped with a rubber policeman, and spun down in a microcentrifuge. The cell pellet was lysed in 150 μl of ice-cold 1× lysis buffer (Promega). The supernatant was assayed for luciferase activity by the standard Promega protocol with a luminometer.

Jurkat cells (23) were grown in suspension at 37°C in complete RPMI 1640 medium supplemented with 5% Fetal Clone (40). They were transfected with SuperFect transfection reagent (Qiagen Inc., Santa Clarita, Calif.) according to the manufacturer's protocol. After 48 h, transfected cells were spun down in a microcentrifuge and were washed twice with PBS. The cell pellet was lysed in 150 μl of ice-cold 1× lysis buffer (Promega). The supernatant was assayed for luciferase activity by the standard Promega protocol with a luminometer.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank and EMBL Data Bank under accession no. U97675.

RESULTS

Isolation of the murine homolog of the *Drosophila extra sex combs* protein with K protein as a bait in a yeast two-hybrid screen. A fragment of K protein (amino acids [aa] 318 to 337) (56) was used as a bait in a two-hybrid screen of an epidermoid KB cell cDNA library to identify K protein partners. Several true-positive colonies were obtained in which analysis showed that the partners were identical. A cDNA fragment of ~1.7 kb was isolated and partially sequenced.

The partial cDNA was used to screen a λgt10 cDNA library (46) derived from the murine pre-B cell line, 22D6. From this screen, three clones were isolated and subcloned into a pBluescript vector. The longest clone was used for sequencing both strands. The complete nucleic acid sequence of the 2,031-bp-long cDNA clone is shown in Fig. 1. The first ATG of the longest open reading frame is located at position 459, but it is not the translation initiation start site (see below). The deduced amino acid sequence of the longest open reading frame showed extensive similarities with the protein encoded by the *Drosophila extra sex combs* gene, Esc (Fig. 2) (25), indicating that we had cloned the mouse homolog of this gene. During the course of our work, Schumacher et al., using positional cloning, also cloned the mouse *esc* gene and demonstrated that mutations in this gene are responsible for the mouse defect of body axis formation, embryonic ectoderm development (48). We will therefore refer to this gene as *eed* and to the protein it encodes as Eed.

Tissue distribution and expression of *eed* transcripts. The full-length cDNA was used as a probe to define the tissue distribution of *eed* transcripts. Total RNA, isolated from a variety of mouse tissues and cells grown in culture, was separated by size on an agarose-formaldehyde gel and blotted onto a nylon membrane. Figure 3A shows the autoradiogram of the Northern blot. There were two transcripts, with apparent sizes of 2.3 and 1.7 kb. In the mouse tissues that were examined, *eed* transcripts were detected in ovaries (Fig. 3A, lane 2) but very little or no transcript was found in the brain (Fig. 3A, lane 1), muscle (Fig. 3A, lane 3), or liver (Fig. 3A, lane 4) samples. Among the cell lines tested, *eed* mRNA was most abundant in the murine pre-B lymphocyte 70Z/3 cell line (37); the message was also present, but at much lower levels, in the human epidermoid carcinoma KB cell line (5) (Fig. 3A, lane 5) and the rat primary glomerular epithelial cell line (42) (Fig. 3A, lane 7). The sizes of the *eed* transcripts were indistinguishable, suggesting that the processing of the *eed* message in the different tissues is similar. In the Northern blot the size of the 2.3-kb band is larger, while the 1.7-kb band is smaller, than those of the 2.03-kb-long *eed* cDNA that we cloned. However, gel electrophoresis of the mRNA synthesized in vitro with the *eed* cDNA clone as the template showed one RNA band whose apparent size was 2.2 kb (Fig. 3B). Taking into account that the

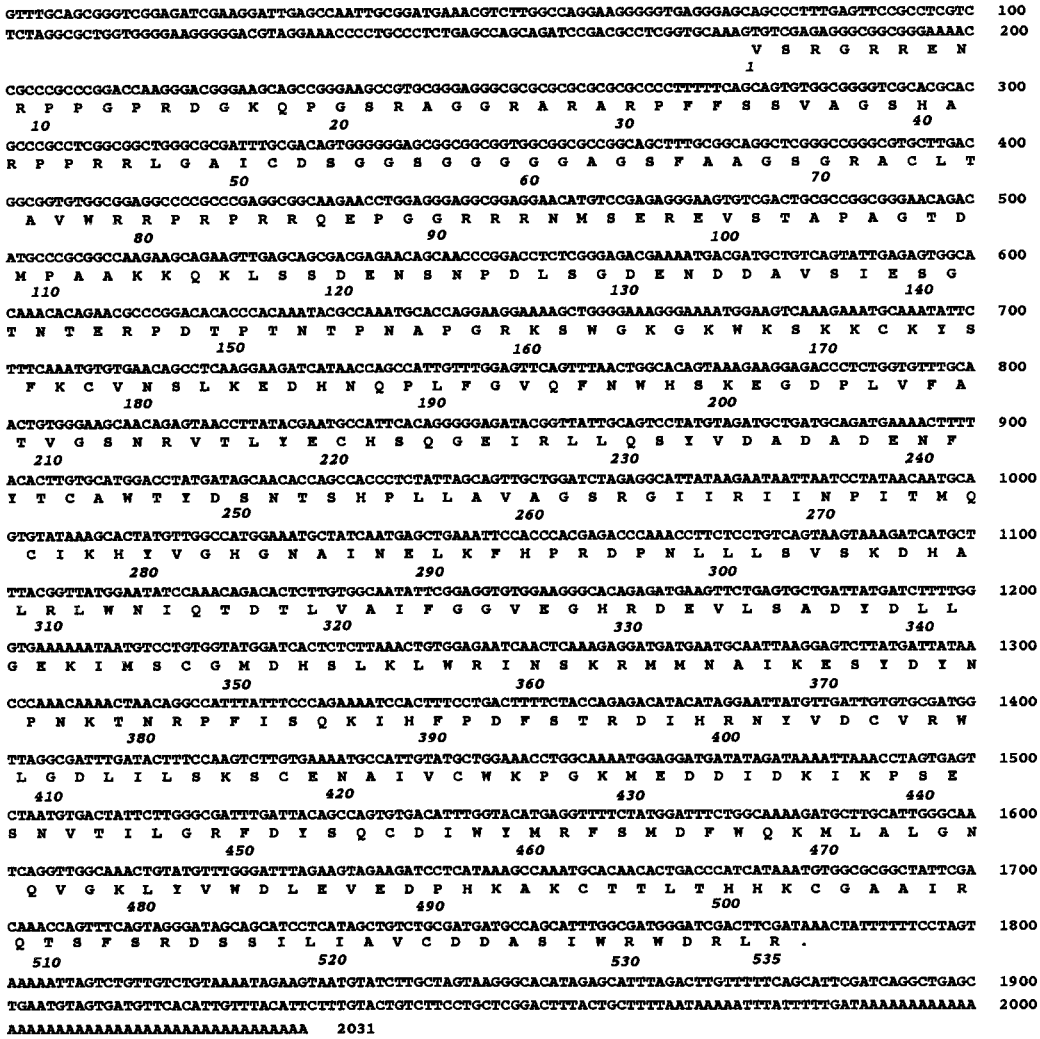

FIG. 1. The complete nucleotide and deduced amino acid sequences of an *eed* clone isolated from a murine pre-B lymphocyte cDNA library. Numbering of the amino acids deduced from the longest open reading frame begins with the putative start codon GTG (see text for explanation).

eed mRNA should contain a 100- to 200-bp-long poly(A) tail in cells, the *eed* cDNA that we cloned corresponds to the upper (2.3-kb) band seen in the Northern blot (Fig. 3A). The lower (1.7-kb) band may represent a product of alternative splicing.

A Western blot probed with antiserum raised in rabbits against GST-Eed fusion protein (Fig. 4) revealed a major immunoreactive band of approximately 69 kDa in extracts from 70Z/3 cells (Fig. 4, lane 3), a cell line that expressed the highest levels of the *eed* message (Fig. 3A). This band was not seen with a preimmune serum obtained from the same rabbit (Fig. 4, lane 1). The 69-kDa band was not detected in the brain (Fig. 4, lane 2), KB (Fig. 4, lane 4), or GE (Fig. 4, lane 5) cells. The major band at 60 kDa seen in the brain sample was also seen with the preimmune serum (data not shown), and therefore it is not specific.

Mapping of the translation initiation site. If the ATG at position 459 is the initiation codon (Fig. 1), then the protein encoded by this cDNA would be 441 aa long and would have a predicted molecular mass of 50.2 kDa. However, the Western blot with anti-Eed serum revealed a protein of 69 kDa (Fig. 4, lane 3), a nearly 20 kDa difference. Since this discrepancy can be accounted for by a number of factors, we first examined the electrophoretic mobility of proteins synthesized in a cell-free system with the cloned *eed* cDNA as a template (24). SDS-PAGE of the translational products revealed one major protein of 69 kDa and several less intense bands (Fig. 5A, lane 2,

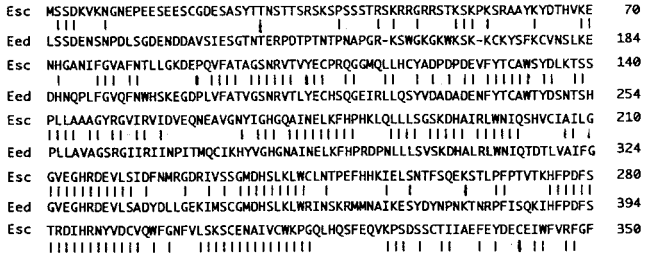

FIG. 2. Similarity of the deduced amino acid sequence of the cloned murine *eed* cDNA to the primary structure of the *Drosophila* Esc. Only the region of similarity is shown, i.e., for Eed, aa 114 to 533, and for Esc, aa 1 to 419.

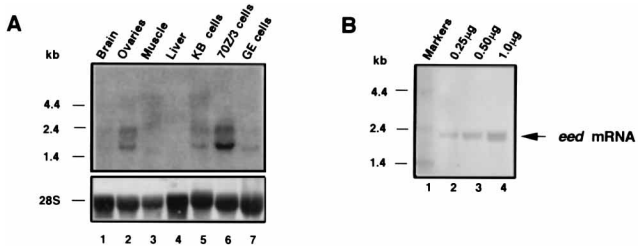

FIG. 3. Northern blot analysis of *eed* mRNA in mouse tissues and cultured cells. Total RNA was isolated, electrophoresed on a formaldehyde-agarose gel, and blotted to a nylon membrane. (A) The blot was hybridized with ^{32}P-labeled *eed* cDNA and autoradiographed (upper panel). The same membrane was stripped and reprobed with ^{32}P-labeled 28S probe as a loading control and autoradiographed (lower panel). (B) Electrophoretic mobility of in vitro-synthesized *eed* mRNA. The *eed* cDNA cloned into pBluescript was transcribed by T7 polymerase. Given amounts of the transcript were loaded on a formaldehyde agarose gel (lanes 2 to 4). Molecular size markers were run in lane 1. The gel was stained with ethidium bromide and was photographed.

and Fig. 5B, lane 1). Therefore, the size of the major in vitro-synthesized protein is similar to that of the major 69-kDa band seen in the Western blot with the anti-Eed serum (Fig. 4, lane 3), indicating that the cloned cDNA contains the full-length open reading frame of the Eed protein.

To test if the ATG at position 459 was the start codon in the *eed* mRNA, we used PCR and appropriate-sense primer to delete the entire region 5′ to the first ATG from the *eed* cDNA clone and then used the PCR product as a template for cell-free transcription and translation. To make the ATG a stronger translation start site, we designed the sense primer so that the ATG is followed by G rather than T, i.e., ATGT→ATGG (Fig. 5). SDS-PAGE and autoradiography of the ^{35}S-labeled translation products synthesized from this template revealed one major protein with a molecular mass of around 55 kDa (Fig. 5A, lane 1, band d). The size of this band is considerably smaller than the major band seen on the Western blot of 70Z/3 extracts (Fig. 4, lane 3) or the major product of the cell-free translation of the full-length *eed* cDNA clone (Fig. 5A, bands b). These results indicate that the *eed* cDNA clone contains a translation initiation site upstream of the first ATG.

To identify the codon that initiates the in vitro synthesis of the major protein (Fig. 5, bands b), we designed a number of synthetic oligonucleotides and used the *eed* cDNA clone as a template to amplify different-length DNAs for cell-free transcription and translation. The autoradiograph of the ^{35}S-labeled in vitro-translated protein products from these DNA templates is illustrated in Fig. 5A (lanes 3 to 5). The template which starts at position 143 generated a major product that was indistinguishable from that synthesized with the full-length clone (Fig. 5A, compare lanes 2 and 3). Importantly, the mRNA containing a 210-bp deletion at its 5′ end (Fig. 5A, lane 4) failed to produce the major band at 69 kDa made from the full-length template (Fig. 5A, bands b), but two of the lower-molecular-mass minor bands were accentuated (Fig. 5A, bands c and d). Based on this analysis, it appears that the major initiation codon of the *eed* cDNA must be contained within the nucleotide region from positions 143 to 211. Inspection of this region revealed a GTG sequence at position 177 that is flanked by a good consensus sequence for initiation of translation and thus could be one potential initiation codon. There are many examples of proteins from diverse species where GTG is the initiation codon (7, 8, 16, 51). To test if this is indeed the first codon for the major translation product of the *eed* cDNA template (Fig. 5A, bands b), we designed a sense oligonucleotide primer for PCR to convert G at position 177 to A and T at position 181 to G (GTGT→ATGG) (Fig. 5) and used the PCR product as a template for in vitro transcription and translation. In this mutated template the putative initiation codon, GTG, was converted to ATG flanked by a strong consensus sequence for translational initiation (32). This altered template produced the same major 69-kDa band as did the wild-type cDNA (Fig. 5A, compare lane 5 to lanes 2 and 3), while the minor bands of lower molecular mass were synthesized at either lower (band a) or the same (bands c and d) levels. This set of experiments suggests that the GTG at position 177 is the initiation codon for the major protein synthesized in a cell-free system. Starting with this GTG, the open reading frame is 1,605 nucleotides long, encoding a protein containing 535 amino acids with a predicted molecular mass of 60.0 kDa and a pI of 8.9.

The cDNA clone that was used here for cell-free transcription and translation may represent an alternatively spliced mRNA with an intron present that, by chance, contains nucleotide sequences, such as the GTG, that can initiate translation upstream of the first ATG, which is at position 459. To determine if the protein pattern produced in in vitro translation was unique to the *eed* cDNA that was cloned from the pre-B cell library, we used mouse kidney cDNA and appropriate oligonucleotide primers in PCR to amplify another set of templates for cell-free translation. In vitro transcription and translation of three independently amplified DNAs from kidney cDNA produced one major band, which had a molecular size indistinguishable from the one generated from the *eed* cDNA clone isolated from the pre-B cell cDNA library (Fig. 5B, compare lane 1 to lanes 2 to 4). Moreover, the PCR-generated templates produced similar profiles of the minor protein bands, except that the weaker band of higher molecular mass seen with the cDNA clone (Fig. 5B, lane 1, band a) was not seen with any of the three independently generated kidney mRNAs. All PCR-generated templates were sequenced and were indistinguishable from the clone we isolated.

The Western blot analysis of extracts from the pre-B 70Z/3 cells (Fig. 4) and the cell-free translation experiments (Fig. 5) provide strong evidence that the ATG at position 459 (Fig. 1) is not the initiation codon for translation of *eed* in 70Z/3 cells. The cell-free translation of the wild-type template and the different mutated *eed* templates suggest that the GTG at po-

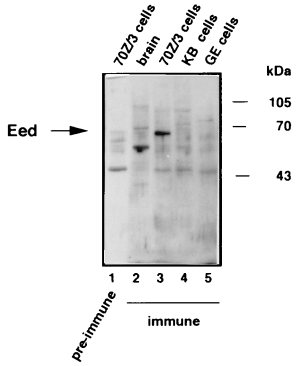

FIG. 4. Western blot analysis of Eed derived from brain cells and various cell lines. Protein extracts from tissue and cells grown in culture were prepared as described previously (56). Fifty micrograms of protein extract per lane was resolved by SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane for immunoblotting with either preimmune (lane 1) or immune (lanes 2 to 5) anti-Eed sera. The anti-Eed antibody was raised against a GST-Eed fusion protein in rabbits. Both the preimmune and immune sera were used at a 1:5,000 dilution. The blots were developed by chemiluminescence. The position of Eed is indicated by the arrow. Molecular mass markers are shown on the right.

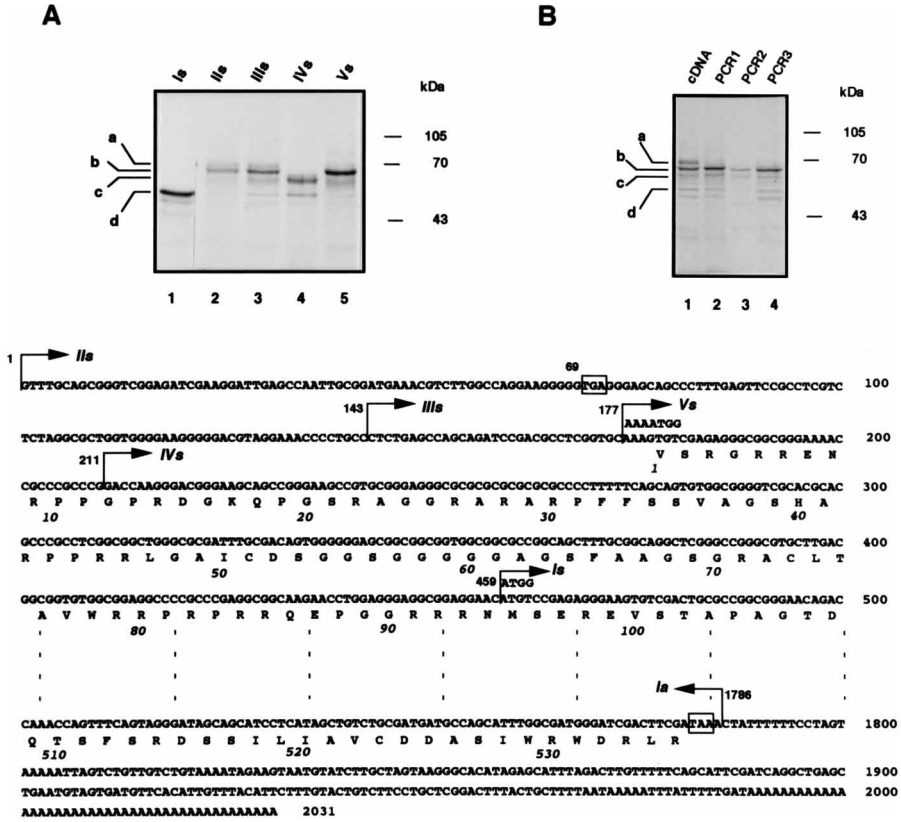

FIG. 5. Mapping of the translation initiation sites of the *eed* cDNA clone with a cell-free system. All mRNAs were translated in rabbit reticulocyte lysates with either the cloned cDNA or PCR-generated DNAs as templates for transcription. ^{35}S-methionine-cysteine was used in translation reactions to label proteins. Translation products were analyzed by SDS-PAGE and autoradiography. Major ^{35}S-labeled protein bands are designated a, b, c, and d. The nucleic acid sequences of the 5′ and 3′ ends of the *eed* cDNA clone are shown in the panel below the autoradiographs. The deduced N- and C-terminal amino acid sequences of the longest open reading frame are also shown. The relative positions of the deduced amino acid sequences from the putative initiation codon are shown in italics below the protein sequence. The stop codons are boxed. The 5′ position of the sense (*Is*, *IIs*, *IIIs*, *IVs*, and *Vs*) and anti-sense (*Ia*) oligonucleotide primers used in PCR to generate templates for transcription and translation are indicated by the arrows. The positions of the 5′ ends of the primers in reference to the cloned full-length cDNA are shown by the numbers adjacent to the arrows. (A) Analysis of translational products with DNA amplified with the indicated sense primers and with the cloned cDNA as a template for PCR. With the exception of the oligonucleotides IVs and Vs, primers had sequences identical to the corresponding nucleotide stretch of the cloned cDNA. In reference to the *eed* clone, the 5′ end of the primer IVs was changed, as shown, from ATGT to ATGG, and that of primer Vs was changed from AAAGTGT to AAAATGG, as shown. The same antisense primer, Ia, was used in all the PCR reactions. (B) The ^{35}S-labeled translation product synthesized from the wild-type full-length cDNA is shown in lane 1. Lanes 2 to 4 represent translation products from three (PCR1, PCR2, and PCR3) independently amplified DNAs from kidney cDNA with oligonucleotide primers that correspond to the 5′ and 3′ ends of the cloned cDNA.

sition 177 might be an initiation codon in vivo. Although it is conceivable that *eed* has a very short first exon that contains an initiating ATG that was not included in our cDNA clone, the observation that several independently generated templates from kidney and 70Z/3 cells produced similar products in cell-free translation makes that possibility less likely. This postulate is further supported by the fact that the cDNA sequence isolated by positional cloning is identical to our sequence (48).

Mapping protein domains responsible for the K protein-Eed interactions. To define the domain of K protein responsible for the engagement of Eed, we tested binding of the full-length in vitro-translated ^{35}S-labeled Eed to glutathione beads bearing GST fused to either full-length K protein or a fragment of K protein. After incubation with ^{35}S-labeled Eed, beads were washed and boiled in buffer containing SDS, and proteins were resolved by SDS-PAGE. The Coomassie blue-stained and autoradiographed gel is illustrated in Fig. 6. In agreement with the yeast two-hybrid screen, these results showed that Eed binds to the full-length GST-K protein (Fig. 6, lanes 1). Eed also bound to GST-K13 (aa 1 to 337) and GST-K3 (aa 171 to 337) but not to the GST-K12 (aa 1 to 209) deletion mutant (56). There was also very weak binding to the GST-K7 (aa 337 to 464) deletion mutant, but this binding was not seen consistently. This experiment shows that the Eed-binding site is contained within the aa 171 to 337 domain of the K protein. This domain may overlap with the K protein site that binds the Src and Vav SH3 domains (56) or the transcriptional repressor, Zik1 (17).

To approximate the Eed domain that interacts with K protein, we tested the binding of ^{35}S-labeled full-length Eed (aa 1 to 535) and the Eed deletion mutants EedΔN (aa 100 to 553) and EedΔC (aa 1 to 283) to glutathione beads bearing either full-length K protein or deletion fragments of K protein. The autoradiograph of the SDS-PAGE gel of protein eluted from the glutathione beads is illustrated in Fig. 7. As before, full-length Eed bound to GST-K (aa 1 to 464) (Fig. 7, lane 2) and GST-K3 (aa 171 to 337) (Fig. 7, lane 3) but no binding was detected to GST-K7 (aa 337 to 464) (Fig. 7, lane 4). A pattern identical to that seen with the full-length Eed was seen with the Eed mutant lacking the C terminus, EedΔC (aa 1 to 283), but in contrast, no binding to any of the GST-K fusion constructs was seen with the Eed deletion mutant lacking the N terminus, EedΔN (aa 100 to 553). These binding studies indicate that the first 100-aa stretch of Eed is critical for K protein binding.

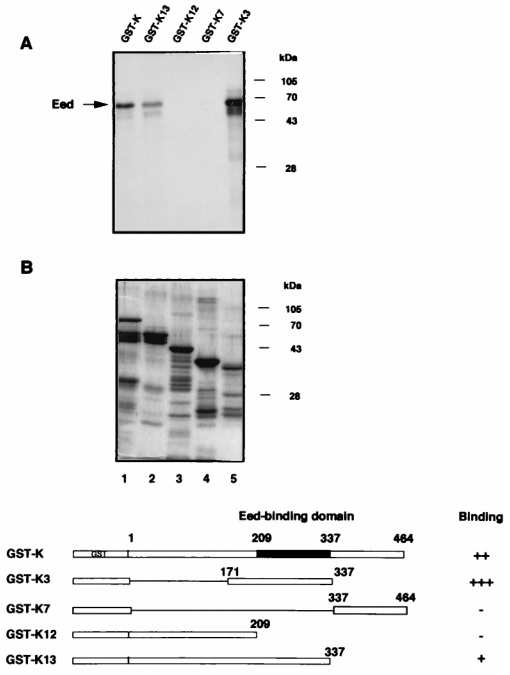

FIG. 6. Binding of Eed to K protein in vitro. *eed* mRNA was translated in a rabbit reticulocyte cell-free system. The ^{35}S-labeled translation products were incubated with glutathione beads bearing either the full-length K protein or deletion fragments of K protein mutants fused to GST (see text). After binding, the beads were washed and boiled in SDS buffer and eluted proteins were analyzed by SDS-PAGE. The Coomassie blue-stained gel (B) and its autoradiograph (A) are shown. The diagram below the gels depicts the GST-K protein constructs that were conjugated to the glutathione beads and used in the binding assays. Positions of the amino acid residues on the full-length K protein that correspond to the N- and C-terminal ends of the K protein fragments fused to GST are shown by the numbers above the bars. The approximate location of the Eed-binding domain in the K protein is shown by the shaded box.

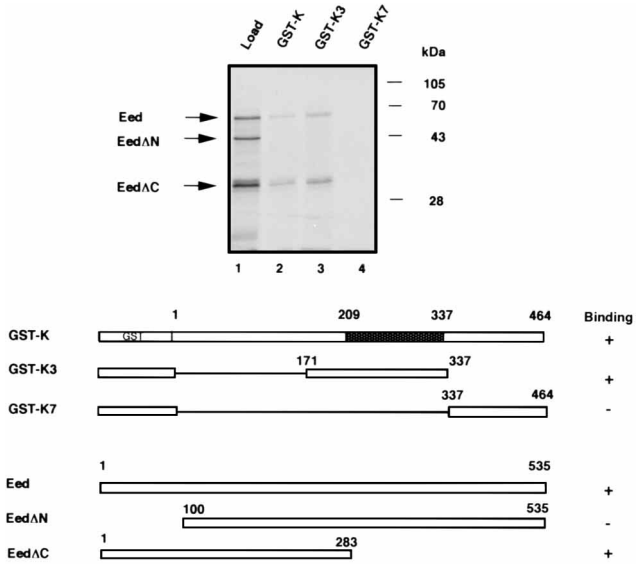

FIG. 7. Mapping of the K protein-binding domain to the Eed N terminus. Full-length DNA and two PCR-amplified DNA deletion constructs of the cloned cDNA were used to synthesize mRNA for cell-free translation of the full-length Eed (aa 1 to 535) and of the mutants lacking either the N (EedΔN, aa 100 to 535) or C (EedΔC, aa 1 to 283) terminus. The ^{35}S-labeled translation products (Load) were mixed together (lane 1), and an aliquot of the mixture was incubated with glutathione beads bearing either GST-K, GST-K3, or GST-K7 fusion protein. After binding, beads were washed and boiled in SDS buffer and eluted proteins were analyzed by SDS-PAGE and autoradiographed.

To narrow down the K protein-binding domain in Eed, we tested binding of various Eed N terminus deletion mutants to glutathione beads bearing full-length GST-K protein. The autoradiogram of an SDS-PAGE gel of the ^{35}S-labeled full-length and deletion-mutant proteins eluted from the GST-K beads is illustrated in Fig. 8. These results demonstrate that the full-length Eed (aa 1 to 553) and the deletion fragments EedΔN2 (aa 47 to 553) and EedΔN3 (aa 36 to 553) bound to K protein but the deletion mutant EedΔN1 (aa 95 to 553) did not. This experiment suggests that the K protein-binding domain includes the aa 47 to 95 region of Eed.

Testing of Eed transcriptional activity. Based on the observations that Esc silences homeotic genes in *Drosophila*, it has previously been suggested that Esc might be a transcriptional corepressor (25). Like Esc, Eed is thought to regulate embryonic development by repression of homeotic genes in mice (20, 48), an effect which might be transcriptionally mediated. To determine if Eed can affect transcription, full-length Eed (aa 1 to 535) was fused in frame to the Gal4 DNA-binding domain (aa 1 to 147) in the mammalian expression vector pM1 (44). pM1 containing only the Gal4 DNA-binding domain was used as a control. These plasmids were transiently coexpressed with the reporter luciferase gene plasmid in COS cells. The results of the luciferase activity assays are shown in Fig. 9. Compared to the expression of Gal4 DNA-binding domain that was used as a control (Fig. 9A, Gal4), expression of the full-length Gal4-Eed protein (Fig. 9A, Gal4-Eed) resulted in a fourfold reduction in the activity of the luciferase gene driven by the minimal promoter containing the Gal4 DNA elements (Fig. 9B, compare shaded bars Gal4-Eed to Gal4). The level of repression of the reporter gene increased with increasing amounts of Gal4-Eed plasmid. In contrast, expression of the full-length Gal4-

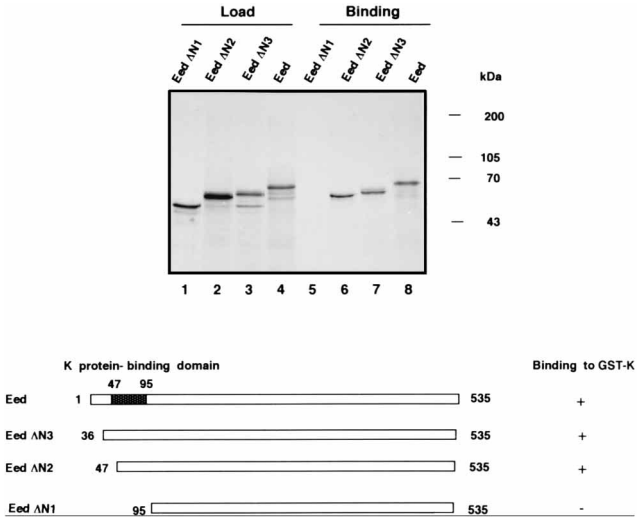

FIG. 8. Narrowing down the location of the K protein-binding domain in the N terminus of Eed. Synthesis and binding of ^{35}S-labeled full-length Eed and Eed deletion mutants was done as described in the legend to Fig. 7. The ^{35}S-labeled products from the in vitro translation (Load) are shown in lanes 1 to 4, and ^{35}S-labeled proteins eluted from glutathione beads bearing the full-length GST-K fusion protein (Binding) are shown in lanes 5 to 8. Lanes 1 and 5, EedΔN1 (aa 95 to 535); lanes 2 and 6, EedΔN2 (aa 47 to 535); lanes 3 and 7, EedΔN3 (aa 36 to 535); and lanes 4 and 8, full-length Eed (aa 1 to 535). The bars shown below the autoradiograph depict the ^{35}S-labeled Eed used. The shaded area defines the region that contains the K protein-binding domain. +, binding; −, no binding.

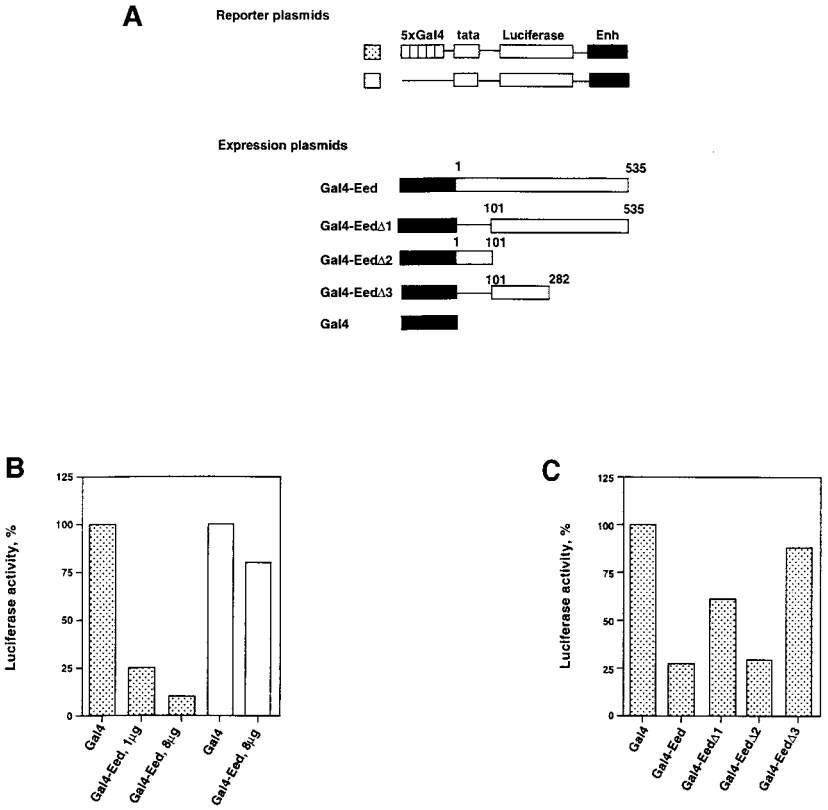

FIG. 9. Effects of Gal4-Eed protein on transcription of the luciferase reporter gene in COS cells. (A) Plasmids used in transient cell transfections. Reporter plasmids: luciferase reporter gene pGL3-Enhancer vector containing an SV40 enhancer (Enh) and a minimal promoter (tata) either with (shaded boxes) or without (clear boxes) five Gal4-binding elements (5×Gal4). Expression plasmids: mammalian vector, pM1, was used for expression of either Gal4 DNA-binding domain alone (Gal4) or a fusion of Gal4 DNA-binding domain with either the full-length Eed (Gal4-Eed, aa 1 to 535) or Eed deletion mutants (Gal4-EedΔ1, aa 101 to 535; Gal4-EedΔ2, aa 1 to 101; and Gal4-EedΔ3, aa 101 to 282). (B) Effect of expression of Gal4 DNA-binding domain (Gal4) and Gal4 DNA-binding domain-Eed fusion constructs (Gal4-Eed) on expression of the luciferase reporter gene in COS cells. Expression plasmids (1 and 8 μg) with 0.5 μg of luciferase reporter plasmid were used for cotransfections. The amount of DNA was adjusted with pM1 so that the total amount of DNA used in each transfection was the same. Forty-eight hours after transfection, cells were pelleted and lysed in reporter lysis buffer. Luciferase activity was measured with a luminometer (10) and normalized for protein concentration, and the final values were calculated relative to the activity measured for the Gal4 DNA-binding domain (Gal4). The data shown are representative of one out of five experiments. (C) Mapping of the transcriptional repression domains by comparing transcriptional activity of the full-length Gal4-Eed and that of a panel of Gal4-Eed deletion mutants expressed in COS cells. The data shown represent one out of three experiments that were done as described for panel B.

Eed in these cells had little or no effect on the same minimal promoter, in which the five Gal4 DNA sites were not included (Fig. 9B, compare clear bars Gal4-Eed and Gal4). These results show that Gal4-Eed inhibits transcriptional activity of a target promoter that contains the cognate Gal4-binding DNA motifs.

To map the domain within the Eed protein that mediates this transcriptional repression, we constructed a number of Eed deletion mutants fused to the Gal4 DNA-binding domain and tested the effects of these constructs on the transcriptional activity of the promoter containing Gal4 elements (Fig. 9C). As in the previous experiment (Fig. 9B), full-length Gal4-Eed inhibited the expression of the luciferase reporter gene (Fig. 9C, compare Gal4-Eed to Gal4). The same degree of repression was seen with Gal4-EedΔ2, a deletion mutant that contains only the first 101 aa of the Eed protein (Fig. 9C, compare Gal4-EedΔ2 to Gal4). Some transcriptional suppression was also seen with the Eed mutant that lacks the N terminus, Gal4-EedΔ1 (aa 101 to 535) (Fig. 9C, compare Gal4-EedΔ1 to Gal4), while the deletion mutant missing both the N and C termini of Eed, Gal4-EedΔ3 (aa 101 to 282), did not repress the activity of this promoter (Fig. 9C, compare Gal4-EedΔ3 to Gal4). This deletion analysis suggests that Eed may contain two transcriptional repression domains, one in the N terminus and the other in the C terminus, but in the COS cells the Eed N terminus was consistently a more potent transcriptional repressor than the C terminus of this molecule. The location of the two Eed repressive domains is similar to the position of transcription repression domains found in the other *Drosophila* WD-40 repeat protein, Groucho (22, 39).

We next tested the transcriptional activity of the Gal4-Eed protein in the human T-cell leukemia Jurkat cell line (23). The reporter gene construct, Gal4-pGL3-Enhancer (Fig. 9A), yielded low luciferase activity in Jurkat cells, and therefore, in these cells we used instead the pGL3-Control plasmid, which contains an SV40 promoter and an SV40 enhancer, into which we inserted five Gal4 DNA elements upstream of the promoter (Fig. 10A). In Jurkat cells there was a 17-fold repression of the reporter gene activity with the expression of the full-length Gal4-Eed fusion protein compared to that of the control (Fig. 10B, compare Gal4-Eed to Gal4). We found that Gal4-Eed was consistently a more potent repressor of the Gal4-SV40 promoter-driven luciferase gene in Jurkat cells than it was a repressor of the reporter gene driven by the Gal4-minimal promoter in COS cells (Fig. 9 and 10, compare Gal4 to Gal4-Eed). These considerable discrepancies may reflect differences that exist between the two cell types.

Single nucleotide mutations within *eed* are responsible for

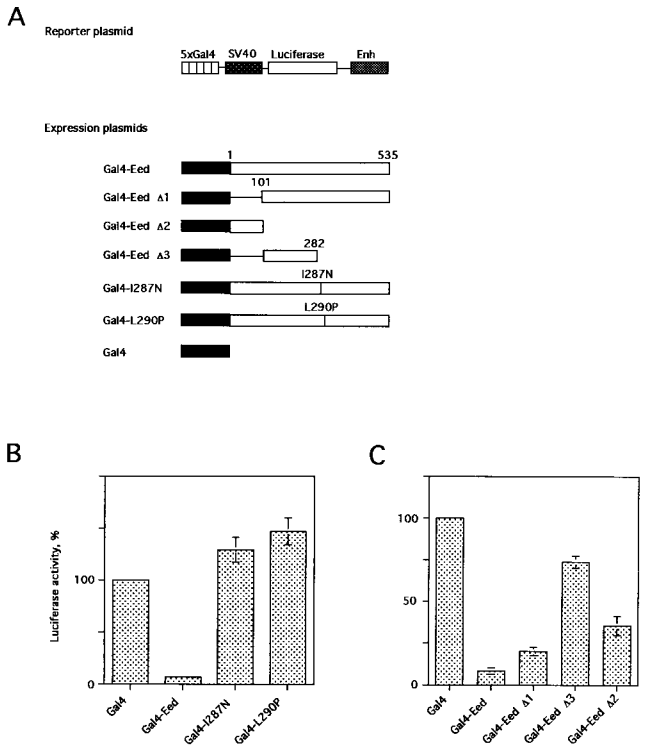

FIG. 10. Effects of Gal4-Eed protein on transcription of the luciferase reporter gene in Jurkat cells. (A) Plasmids used in transient transfections. Reporter plasmid: luciferase reporter gene pGL3-Control vector containing an SV40 promoter (SV40) and an SV40 enhancer (Enh) with five Gal4-binding elements (5×Gal4). Expression plasmids: mammalian vector, pM1, was used for expression of either Gal4 DNA-binding domain alone (Gal4) or a fusion of Gal4 DNA-binding domain with either the full-length Eed (Gal4-Eed, aa 1 to 535) or deletion mutants (Gal4-EedΔ1, aa 101 to 535; Gal4-EedΔ2, aa 1 to 101; and Gal4-EedΔ3, aa 101 to 282). Fusion of Gal4 DNA-binding domain with the full-length Eed containing either the Leu^{290}→Pro (Gal4-L290P) or Ile^{287}→Asn (Gal4-I287N) mutation was also used. (B and C) Effect of expression of Gal4-Eed fusion constructs on expression of the luciferase reporter gene (B) and mapping of the Eed transcriptional repression domains (C) in Jurkat cells. Expression plasmids (4 μg) with 1 μg of luciferase reporter plasmid were used for cotransfections. Forty-eight hours after transfection, cells were analyzed for luciferase activity. The data shown represent the means ± standard errors of the means of three independent experiments.

severe developmental abnormalities in mouse embryogenesis (48). A mutation in the coding region, T^{1040}→C, which leads to the substitution Leu^{290}→Pro (L290P), is lethal to the embryo, while mutation T^{1031}→A, which leads to the substitution Ile^{287}→Asn (I287N), is hypomorphic (48). Both mutations are localized within the second WD-40 repeat (Fig. 11). To gain insight into molecular mechanisms that may be responsible for these Eed developmental phenotypes, we generated Gal4-Eed fusion proteins containing these mutations and tested their transcriptional repression activity in Jurkat cells (Fig. 10A). In a sharp contrast to the wild-type Gal4-Eed fusion protein (Fig. 10A, Gal4-Eed), expression of the Gal4-Eed fusion protein containing either the hypomorphic mutation (Fig. 10A, Gal4-I287N) or the lethal mutation (Fig. 10A, Gal4-I290P) enhanced (by 30 to 50%) rather than repressed the Gal4-SV40 promoter (Fig. 10B, compare Gal4-Eed, Gal4-I287N, and Gal4-I290P to Gal4). These results suggest that the lethal and the hypomorphic phenotypes that result from these *eed* gene mutations may reflect abolition of the transcriptional repression exhibited by the wild-type Eed and that, as a result, these Eed mutants can no longer act as suppressors of homeotic genes.

The coexpression of Gal4-Eed deletion mutants with (5×Gal4)-pGL3-reporter gene constructs in COS cells suggested that Eed contains two transcription repression domains (Fig. 9C). In COS cells, the N-terminal domain appeared to be a more potent transcriptional repressor (fourfold repression) than the domain in the C terminus (30 to 35% repression), which includes the WD-40 repeats. Since Eed exhibited stronger transcriptional repression activity in Jurkat cells (Fig. 10) than in COS cells (Fig. 9), we repeated mapping of the Eed transcriptional repression domains in Jurkat cells (Fig. 10C). The Gal4-EedΔ1 fusion protein, which contains the WD-40 repeats but lacks the N terminus, repressed the activity of the Gal4-SV40 promoter fivefold (Fig. 10C, compare Gal4-EedΔ1 to Gal4), while the expression of the Gal4-EedΔ2 fusion protein, which contains only the first 101 aa of Eed, inhibited reporter gene expression threefold (Fig. 10C, compare Gal4-EedΔ2 to Gal4). In contrast, the Gal4-EedΔ3 fusion protein, a construct that contains the middle portion of the Eed molecule, inhibited luciferase expression by only 25% (Fig. 10C, compare Gal4-EedΔ3 to Gal4). Thus, as in the COS cells transfections (Fig. 9C), the mapping series of experiments done in Jurkat cells confirmed that Eed contains two separable transcription repression domains (Fig. 10C), but in contrast to the COS cell experiments, these results show that the C-terminal part of Eed, which contains the WD-40 repeats, may possess higher transcriptional repression activity in Jurkat cells than the Eed N-terminal part, which binds K protein. These differences may reflect differential expression of Eed protein partners in the two cell types.

Eed exhibits transcriptional repression activity when tethered to a promoter by the Gal4-K fusion protein. The yeast two-hybrid screen (Fig. 1) and the in vitro binding experiments (Fig. 6 to 8) revealed that Eed interacts with K protein. Moreover, the in vitro binding experiments allowed us to map the K protein-binding domain to the Eed N terminus (Fig. 8), a region that contains one of the two Eed transcriptional repression domains (Fig. 11). To explore the possibility that K protein participates in Eed-mediated transcriptional repression activity, we tested luciferase reporter gene expression in Jurkat cells driven by the (5×Gal4)-SV40 promoter in cotransfection experiments with Eed and K protein (Fig. 12A). Coexpression of Gal4-K protein with Eed inhibited the level of the luciferase reporter gene activity 2.5-fold (Fig. 12B, compare Gal4-K + Eed to Gal4-K). These results suggest that Eed can display transcriptional repression activity when recruited to a target promoter by K protein. Cotransfection of the Eed deletion mutant that lacks the K protein-binding domain (EedΔ1) did

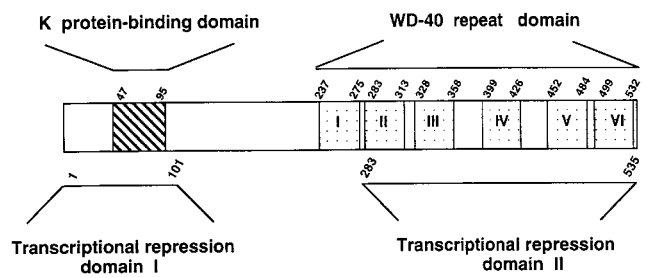

FIG. 11. Modular structure of Eed. Six (I to VI) WD-40 repeats (34) are located in the C-terminal half (aa 237 to 532). The K protein-binding domain is located in the N terminus (aa 47 to 95). Eed contains two transcriptional repression domains, one in the N terminus (aa 1 to 101) and one in the C terminus (aa 283 to 535).

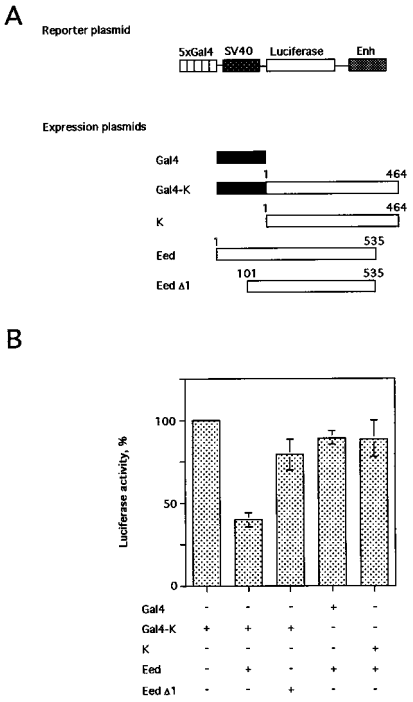

FIG. 12. Effects of Eed on transcription of the luciferase reporter gene when recruited to the target promoter by Gal4-K protein. (A) Plasmids used in transient transfections in Jurkat cells. Reporter plasmid: same as in Fig. 10A. Expression plasmids: mammalian vector, pM1, was used for expression of either Gal4 DNA-binding domain alone (Gal4) or a fusion of Gal4 DNA-binding domain with the full-length K protein (Gal4-K, aa 1 to 464). The pM1 vector with the Gal4 DNA-binding domain deleted was also used to express the full-length K protein (K, aa 1 to 464), full-length Eed (Eed, aa 1 to 535), and an Eed deletion mutant lacking the K protein-binding domain (EedΔ1, aa 101 to 535). (B) Effect of coexpression of Eed variants with K protein constructs on luciferase reporter gene activity. The symbols below each column designate which plasmid was (+) or was not (−) used in the cotransfection experiments with the luciferase reporter gene. K protein plasmid constructs (1 μg) and 3 μg of Eed plasmid constructs with 1 μg of luciferase reporter plasmid were used for cotransfections. The total amount of DNA was adjusted with pM1 plasmid. Forty-eight hours after transfection, cells were pelleted and analyzed for luciferase activity. The final values were normalized for protein concentration. The data shown represent means ± standard errors of the means of three independent experiments.

not significantly affect the luciferase reporter gene activity (Fig. 12B, compare Gal4-K + EedΔ1 to Gal4-K). Similarly, there was no effect on the reporter gene expression when Gal4 DNA-binding domain (Fig. 12B, Gal4) was co-transfected with the full-length Eed or when the full-length K protein without Gal4 DNA-binding domain (Fig. 12B, K) was co-transfected with the full-length Eed (Fig. 12B, compare Gal4 + Eed and K + Eed to Gal4-K). These experiments provide further evidence that Eed and K protein interact in vivo and that recruitment of Eed to a target promoter by K protein can allow Eed to repress gene transcription. Thus, results from these series of cotransfection experiments may reflect involvement of K protein in Eed-mediated transcriptional repression.

DISCUSSION

Using the yeast two-hybrid screen, we have identified a novel K protein molecular partner that contains a nearly 450-aa-long domain that is similar to the protein encoded by the *Drosophila extra sex combs* gene (*esc*) (Fig. 2) (25, 47). *esc* belongs to the *polycomb* group of genes that restrict expression of homeotic genes in domains along the anterior-posterior axis (50). During the course of our work Schumacher et al., using a positional-cloning method, isolated a murine cDNA (48) with a sequence identical to the cDNA that we cloned. They have demonstrated that mutation in this gene, previously denoted *eed* (for embryonic ectoderm development), is responsible for altered anterior-posterior patterning in mice (48). Remarkably, one of the known point mutations in *eed* is lethal to the embryo, underscoring the critical role of this gene in mouse embryonic development. Based on these observations, Eed is considered to be a global regulator of anterior-posterior patterning (20, 48). Thus, it appears that the structural similarity between Esc and Eed is reflected in their similar developmental roles in the respective species.

We found that the Eed mRNA is abundant in lymphocytes but present at low levels in fibroblasts and several mouse tissues (Fig. 3 and data not shown). This tissue distribution of Eed mRNA correlates with that of several other mammalian *Polycomb* group transcripts, such as Bmi-1 or EZH (1, 3, 11, 13). *Polycomb* group genes are likely to have similar tissue distributions because they form multimeric complexes (3) and cooperate to suppress gene expression (50). The role of Eed and other Polycomb group proteins in embryonic development has been extensively explored and is currently reasonably well defined (20, 48–50). In contrast, the role of these genes in the adult tissue is poorly understood. *bmi-1* knockout mice exhibit hematologic and immunologic abnormalities (3), suggesting that this Polycomb group protein may be involved in hematopoiesis and in lymphocyte development. The tissue distribution of Eed (Fig. 3 and data not shown) suggests that in adult organisms Eed might also be involved in these processes.

Both Esc and Eed contain six WD-40 repeats that span an over-300-aa stretch in the C terminus. WD-40 domains are highly conserved, usually 40 aa long, and end with Trp-Asp (WD-40). They were first identified in the β subunit of the heterotrimeric GTP-binding proteins (G proteins). Subsequently, WD-40 repeats were found to regulate a multitude of cellular processes and to be ubiquitously expressed in eukaryotic cells. On the molecular level, WD-40 repeats are thought to mediate both protein-protein and protein-nucleic acid interactions (34). Relevant to transcriptional processes, the WD-40 repeats were found in Tup1, a protein known to repress transcription of a number of genes in yeast (31, 54), and in *Drosophila* $TAF_{II}80$ and its human homolog, $TF_{II}100$ (18), which are components of the TFIID transcriptional complex (14). The protein Groucho, which is involved in the control of *Drosophila* neurogenesis, segmentation, and sex determination, also contains a WD-40 repeat domain in its C-terminal half (22, 39). Both the lethal and the hypomorphic mutations of Eed are present in the WD-40 repeats (48), indicating that these repeats play a key role in mediating the interactions of Eed with its molecular partners. A number of mutations of Esc that render it ineffective as a repressor of homeotic gene expression have likewise been mapped to the WD-40 repeats.

Although the C-terminal halves of Eed and Esc are remarkably similar, a domain that would resemble the N-terminal domain of Eed is absent in Esc. Notably, the Eed N terminus is crucial for K protein binding (Fig. 12). Inasmuch as their developmental function appears to be analogous, the functional significance of this major structural difference that exists between Eed and Esc structures is not clear. Nonetheless, it is conceivable that one of the Esc protein partners might provide the missing domain that allows the *Drosophila* K protein-like partner to interact with Esc.

Elegant studies done with *Drosophila* have provided evidence that Esc acts as a repressor of homeotic gene expression (47, 50), but until now no direct studies have been done to define Esc or Eed transcriptional activity. Using GAL4-Eed

fusion protein, we found that Eed can repress transcription of a reporter gene driven by a promoter that contains GAL4-binding motifs. Eed could be a very potent transcriptional repressor, because in some Jurkat cell experiments, Gal4-Eed inhibited the strong SV40 promoter as much as 30-fold. The potent Eed-mediated transcriptional repression of target promoters (Fig. 10) could therefore, at least in part, explain the Eed-restricted expression of homeotic genes in embryonic development.

Mapping experiments in COS and Jurkat cells (Fig. 9 and 10) suggest that Eed contains two transcriptional repression domains, one in the N terminus, where K protein binds, and the other in the C-terminal half of the protein, where the WD-40 repeats are located (Fig. 11). Although in both COS and Jurkat cells Eed displayed two transcriptional repression domains, the relative potency of each domain was different in the two cell lines. While in Jurkat cells the C terminus was a potent transcriptional repressor, the same domain in COS cells was a weak repressor of reporter gene expression (compare Fig. 9C and 10C). The different extent of Eed-mediated transcriptional repression in COS and Jurkat cells, and the different relative potencies the two transcriptional repression domains, may reflect differential expression of Eed protein partners in the two cell lines.

To gain more insight into the molecular mechanism that may be responsible for the Eed-mediated suppression of homeotic genes, we tested the effects of either the lethal mutation Leu^{290}→Pro or the hypomorphic mutation Ile^{287}→Asn (48) on the transcriptional activity of Eed. In a sharp contrast to the potent transcriptional repressor activity of the wild-type Gal4-Eed, both the Leu^{290}→Pro and the Ile^{287}→Asn Gal4-Eed mutants failed to inhibit the target Gal4-SV40 promoter (Fig. 10). These results suggest that a lack of the transcriptional repressor activity of these Eed mutants is responsible for the defects in the embryonic development described for these *eed* gene mutations. It seems that these mutations either dramatically alter the overall structure of Eed, thereby altering the tertiary structure of the WD-40 domain and masking the N-terminal transcriptional repression domain, and/or the altered WD-40 domains bind a protein or a protein complex that then masks both the C- and the N-terminal transcription repression domains.

The observation that Eed contains two transcriptional repression domains shows that it is similar to Groucho, which also contains N- and C-terminal transcription repression domains (22). Groucho is thought not to bind DNA by itself but rather to be recruited to target promoters through interaction with WRPW domains that are present in the Hairy-related basic helix-loop-helix proteins (22, 39). Once recruited to DNA by proteins, such as the *Drosophila* Hairy, Groucho is able to repress expression of target genes by mechanisms that are yet to be defined.

The structure and function of Eed are also reminiscent of yeast Tup1, which contains two discrete transcriptional repression domains and, like Eed, contains WD-40 repeats (31, 52–54). Tup1 is recruited to specific promoters by direct association with DNA-binding proteins like the α2 factor (31) or by an association with DNA-binding proteins, such as Mig1 or Rox1, through its close partner, Cyc8(Ssn6) (52, 53). The direct association between Tup1 and α2 is mediated by one of the seven WD-40 repeats (31). Cyc8 is made up of tandem TPR motifs, some of which serve to anchor Tup1 while others are used to recruit Tup1-Cyc8 complex to the cognate DNA proteins, such as Mig and Rox1 (53). Molecular partner(s) of Eed or Esc that may play a role similar to that of Cyc8 have not been identified, but it is conceivable that Cyc8-like function is provided by K protein. This postulate is supported by our observation that recruitment of Eed by Gal4-K protein inhibits activity of a target promoter (Fig. 12). This idea is further supported by the observation (17a) that, like Cyc8, which binds the zinc finger repressor Mig1 (52), K protein also interacts with the zinc finger transcriptional repressors, such as Zik1 (17) and Kid1 (58). Although results of the present study suggest that the K protein could be involved in the Eed-mediated transcriptional repression, Eed, like Tup1, is likely to interact with multiple factors, some of which may act as corepressors. This postulate is supported by the observation that Polycomb group proteins can exist in multimeric complexes (3). It is also conceivable that, depending on the target promoter, Eed-mediated transcriptional effects may be mediated or facilitated by a different set of protein partners, which may include K protein. K protein, through its action as a docking platform (9), could help to assemble a multimeric complex that would allow Eed to directly or indirectly interact with multiple factors.

Tup1-Cyc8 is thought to repress transcription both by interfering with the general transcriptional machinery (26) and by affecting chromatin structure (19, 43). A similar mechanism for repression of homeotic genes has been proposed for Esc (25). Given the observation that Eed appears to contain two separate transcription repression domains (Fig. 11), it is possible that, like Tup1-Cyc8, Eed may repress transcription by two mechanisms. (i) The Eed repression domain within the WD-40 repeat domain prevents a factor like $TF_{II}100$ ($TF_{II}80$), which contains WD-40 repeats (18), from recruiting a WD-40 ligand that might be necessary for the transcriptional activity of the TFIID complex. (ii) The repression domain in the Eed N terminus may maintain a repressive chromatin structure (15, 38) through interaction with histones or other chromatin proteins. This postulated mechanism is based on the observation that yeast Tup1, which appears to share some similarities with Eed, interacts with histones H3 and H4 (19). If repression by Eed is the result of chromatin nucleation and interference with the general transcription factors, it is conceivable that K protein might be involved in both of these repressive processes. Considering the observation that K protein is phosphorylated in response to growth factors (36, 55, 56), involvement of K protein would provide a way of regulating the Eed-mediated transcriptional repression by extracellular ligands.

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REFERENCES

1. **Abel, K. J., L. C. Brody, J. M. Vlades, M. R. Erdos, D. R. McKinley, L. H. Castilla, S. D. Merajver, F. J. Couch, L. S. Friedman, E. A. Ostermeyer, E. D. Lynch, M.-C. King, P. L. Welcsh, S. Osborne-Lawrence, M. Spillman, A. M. Bowcock, F. S. Collins, and B. L. Weber.** 1996. Characterization of *EZH1*, a human homolog of *Drosophila Enhancer of zeste* near BRCA1. Genomics **37:**161–171.
2. **Alexandropoulos, K., and D. Baltimore.** 1996. Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, $p130^{Cas}$-related protein, Sin. Genes Dev. **10:**1341–1355.
3. **Alkema, M. J., M. Bronk, E. Verhoeven, A. Otte, L. J. van't Veer, A. Berns, and M. van Lohuizen.** 1997. Identification of Bmi-1 interacting proteins as constituents of a multimeric mammalian polycomb complex. Genes Dev. **11:**226–240.
4. **Bartel, P., C. Chien, R. Sternglanz, and S. Fields.** 1993. Elimination of false positives that arise in using the two-hybrid system. BioTechniques **14:**920–924.
5. **Bird, T. A., and J. Saklatvala.** 1989. IL-1 and TNF transmodulate epidermal growth factor receptors protein kinase C-independent mechanism. J. Immunol. **142:**126–133.
6. **Bird, T. A., H. Schule, P. B. Delaney, J. E. Sims, B. Thoma, and S. K. Dower.**

1992. Evidence that MAP (mitogen-activated protein) kinase activation may be a necessary but not sufficient signal for a restricted subset of responses in IL-1-treated epidermoid cells. Cytokine **4:**429–440.
7. **Bock, H., A. Brennicke, and W. Schuster.** 1994. Rps3 and rp116 genes do not overlap in Oenothera mitochondria: GTG as a potential translation initiation codon in plant mitochondria. Plant Mol. Biol. **24:**811–818.
8. **Boeck, R., J. Curran, Y. Matsuoka, R. Compans, and D. Kolakofsky.** 1992. The parainfluenza virus type 1 P/C gene uses a very efficient GUG codon to start its C′ protein. J. Virol. **66:**1765–1768.
9. **Bomsztyk, K., I. Van Seuningen, H. Suzuki, O. Denisenko, and J. Ostrowski.** 1997. Diverse molecular interactions of the hnRNP K protein. FEBS Lett. **403:**113–115.
10. **Brasier, A. R., J. E. Tate, and J. F. Habener.** 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques **7:**1116–1122.
11. **Chen, H., C. Rossier, and S. E. Antonarakis.** 1996. Cloning of a human homolog of the *Drosophila Enhancer of zeste* gene (EZH2) that maps to the chromosome 21q22.2. Genomics **38:**30–37.
12. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
13. **Cohen, K. J., J. S. Hanna, J. E. Prescott, and C. V. Dang.** 1996. Transformation by the Bmi-1 oncoprotein correlates with its subnuclear localization but not its transcriptional activity. Mol. Cell. Biol. **16:**5527–5535.
14. **Conaway, R. C., and J. W. Conaway.** 1993. General initiation factors for RNA polymerase II. Annu. Rev. Biochem. **62:**161–190.
15. **Croston, G. E., and J. T. Kadonaga.** 1993. Role of chromatin structure in regulation of transcription by RNA polymerase II. Curr. Opin. Cell Biol. **5:**417–423.
16. **Davidson, B. L., N. Golovoy, and B. J. Roessler.** 1994. A 13 base deletion in exon 1 of HPRTIllinois forms a functional GUG initiation codon. Hum. Genet. **93:**300–304.
17. **Denisenko, O. N., B. O'Neill, J. Ostrowski, I. Van Seuningen, and K. Bomsztyk.** 1996. Zik, a transcriptional repressor that interacts with the heterogenous nuclear ribonuclear particle K protein. J. Biol. Chem. **271:**27701–27706.
17a. **Denisenko, O. N., and K. Bomsztyk.** Unpublished data.
18. **Dubrovskaya, V., A.-C. Lavigne, I. Davidson, J. Acker, A. Staub, and L. Tora.** 1996. Distinct domains of $hTAF_{II}100$ are required for functional interaction with transcription factor TFIIFβ (RAP30) and incorporation into TFIID complex. EMBO J. **15:**3702–3712.
19. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
20. **Faust, C., A. Shumacher, B. Holdener, and T. Magnuson.** 1995. The *eed* mutation disrupts anterior mesoderm production in mice. Development **121:**273–285.
21. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
22. **Fisher, A. L., S. Ohsako, and M. Caudy.** 1996. The WRPW motif of the Hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Mol. Cell. Biol. **16:**2670–2677.
23. **Gillis, S., and J. Watson.** 1980. Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line. J. Exp. Med. **152:**1709–1719.
24. **Gurevich, V. V., I. D. Pokrovskaya, T. A. Obukhova, and S. A. Zozulya.** 1991. Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerase. Anal. Biochem. **195:**207–213.
25. **Gutjahr, T., E. Frei, C. Spicer, S. Baumgartner, R. A. H. White, and M. Noll.** 1995. The polycomb-group gene, *extra sex combs*, encodes a nuclear member of the WD-40 repeat family. EMBO J. **14:**4296–4306.
26. **Hershbach, B. M., M. B. Arnaud, and A. D. Johnson.** 1994. Transcriptional repression directed by the α2 protein *in vitro*. Nature **370:**309–311.
27. **Herskowitz, I.** 1995. MAP kinase pathways in yeast: for mating and more. Cell **80:**187–197.
28. **Johnson, R. J., H. Iida, C. E. Alpers, M. W. Majesky, S. M. Schwartz, P. Pritzl, K. Gordon, and A. M. Gwon.** 1991. Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis: α-smooth muscle actin is a marker of mesangial cell proliferation. J. Clin. Invest. **87:**847–858.
29. **Kakidani, H., and M. Ptashne.** 1988. Gal 4 activates gene expression in mammalian cells. Cell **52:**161–167.
30. **Kluxen, F. W., and H. Lubbert.** 1993. Maximal expression of recombinant cDNAs in COS cells for use in expression cloning. Anal. Biochem. **208:**352–356.
31. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeo domain protein α2. Genes Dev. **8:**2857–2867.
32. **Kozak, M.** 1996. Interpreting cDNA sequences: some insights from studies on translation. Mamm. Genome **7:**563–574.
33. **Michelotti, E. F., G. A. Michelotti, A. I. Aronsohn, and D. Levens.** 1996. Heterogeneous nuclear ribonucleoprotein K is a transcription factor. Mol. Cell. Biol. **16:**2350–2360.
34. **Neer, E. J., C. J. Schmidt, R. Nambudripad, and T. F. Smith.** 1994. The ancient regulatory-protein family of WD-repeat proteins. Nature **371:**297–300.
35. **Ostrowski, J., and K. Bomsztyk.** 1993. Purification of DNA-binding proteins using tandem DNA-affinity column. Nucleic Acids Res. **21:**1045–1046.
36. **Ostrowski, J., J. E. Sims, C. H. Sibley, M. A. Valentine, S. K. Dower, K. E. Meier, and K. Bomsztyk.** 1991. A serine/threonine kinase activity is closely associated with a 65-kDa phosphoprotein specifically recognized by the kappa B enhancer element. J. Biol. Chem. **266:**12722–12733.
37. **Paige, C. J., P. W. Kincaide, and P. Ralph.** 1978. Murine B cell leukemia line with inducible surface immunoglobulin expression. J. Immunol. **121:**641–647.
38. **Paranjape, S. M., R. T. Kamakaka, and J. T. Kadonaga.** 1994. Role of chromatin structure in regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63:**265–297.
39. **Paroush, Z., R. L. Finley, T. Kidd, S. M. Wainwright, P. W. Ingham, R. Brent, and D. Ish-Horowicz.** 1994. Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with Hairy-related bHLH proteins. Cell **79:**805–815.
40. **Rachie, N. A., R. Seger, M. A. Valentine, J. Ostrowski, and K. Bomsztyk.** 1993. Identification of an inducible 85-kDa nuclear protein kinase. J. Biol. Chem. **268:**22143–22149.
41. **Richard, S., D. Yu, K. J. Blumer, D. Hausladen, M. W. Olszowy, P. A. Connelly, and A. S. Shaw.** 1995. Association of p62, a multifunctional SH2- and SH3-domain-binding protein, with src family tyrosine kinases, Grb2, and phospholipase Cγ-1. Mol. Cell. Biol. **15:**186–197.
42. **Richardson, C. A., K. L. Gordon, W. G. Couser, and K. Bomsztyk.** 1995. IL-1β increases laminin B2 chain mRNA levels and activates NF-κB in rat glomerular epithelial cells. Am. J. Physiol. **268:**F273–F278.
43. **Roth, S. Y.** 1995. Chromatin-mediated transcriptional repression in yeast. Curr. Opin. Cell Biol. **5:**168–173.
44. **Sadowski, I., B. Bell, P. Broad, and M. Hollis.** 1992. Gal4 fusion vectors for expression in yeast or mammalian cells. Gene **118:**137–141.
45. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing GAL4 (1-147) fusions in mammalian cells. Nucleic Acids Res. **17:**7539.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sathe, S. S., and P. J. Harte.** 1995. The *Drosophila extra sex combs* protein contains WD motifs essential for its function as a repressor of homeotic genes. Mech. Dev. **52:**77–87.
48. **Schumacher, A., C. Faust, and T. Magnuson.** 1996. Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature **383:**250–253.
49. **Simon, J., A. Chiang, W. Bender, M. J. Shimell, and M. O'Connor.** 1993. Elements of the *Drosophila* bithorax complex that mediate repression by *Polycomb* group products. Development **158:**131–144.
50. **Struhl, G., and D. Brower.** 1992. Early role of the esc^+ gene product in the determination of segments in Drosophila. Cell **31:**285–292.
51. **Sugihara, H., V. Andrisani, and P. M. Salvaterra.** 1990. Drosophila choline acetyltransferase uses a non-AUG initiation codon and full length RNA is inefficiently translated. J. Biol. Chem. **265:**21714–21719.
52. **Treitel, M. A., and M. Carlson.** 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92:**3132–3136.
53. **Tzamarias, D., and K. Struhl.** 1995. Distinct TPR motifs of the Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. Genes Dev. **9:**821–831.
54. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cys8-Tup1 transcriptional co-repressor complex. Nature **369:**758–761.
55. **Van Seuningen, I., J. Ostrowski, and K. Bomsztyk.** 1995. Description of an IL-1-responsive kinase that phosphorylates the K protein. Enhancement of phosphorylation by sequence-selective DNA and RNA motifs. Biochemistry **34:**5644–5650.
56. **Van Seuningen, I., J. Ostrowski, X. Bustelo, P. Sleath, and K. Bomsztyk.** 1995. The K protein domain that recruits the IL-1-responsive K protein kinase lies adjacent to a cluster of Src- and Vav-SH3-binding sites. Implications that K protein acts as a docking platform. J. Biol. Chem. **270:**26976–26985.
57. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
58. **Witzgall, R., E. O'Leary, R. Gessner, A. J. Ouellette, and J. V. Bonventre.** 1993. Kid-1, a putative renal transcription factor: regulation during growth, ontogeny, and in response to ischemia and toxic injury. Mol. Cell. Biol. **13:**1933–1942.