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Functional and Conserved Domains of the *Drosophila* Transcription Factor Encoded by the Segmentation Gene *knirps*

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The *Drosophila* gap gene *knirps* (*kni*) is required for abdominal segmentation. It encodes a steroid/thyroid orphan receptor-type transcription factor which is distributed in a broad band of nuclei in the posterior region of the blastoderm. To identify essential domains of the *kni* protein (KNI), we cloned and sequenced the DNA encompassing the coding region of nine *kni* mutant alleles of different strength and *kni*-homologous genes of related insect species. We also examined in vitro-modified versions of KNI in various assay systems both in vitro and in tissue culture. The results show that KNI contains several functional domains which are arranged in a modular fashion. The N-terminal 185-amino-acid region which includes the DNA-binding domain and a functional nuclear location signal fails to provide *kni* activity to the embryo. However, a truncated KNI protein that contains additional 47 amino acids exerts rather strong *kni* activity which is functionally defined by a weak *kni* mutant phenotype of the embryo. The additional 47-amino-acid stretch includes a transcriptional repressor domain which acts in the context of a heterologous DNA-binding domain of the yeast transcriptional activator GAL4. The different domains of KNI as defined by functional studies are conserved during insect evolution.

Genetic analyses combined with molecular analyses have defined a cascade of segmentation gene activities in *Drosophila melanogaster*, which organizes the larval body pattern within a single-layer epithelium, the blastoderm (for reviews, see references 1 and 14). This cascade of hierarchical and cross-regulatory gene interactions is initiated in response to maternally derived, localized morphogens which define the spatially restricted domains of zygotic gap gene expression (29; for reviews, see references 28 and 46). The activities of gap genes are required for the establishment of a contiguous set of segments along the anterior-posterior axis of the embryo (9, 30; for a review, see reference 32). Among these first zygotically active segmentation genes, *knirps* (*kni*) is essential for the development of the abdominal region of the embryo. In the absence of *kni* activity in homozygous *kni* lack-of-function mutant embryos, the establishment of abdominal segments is abnormal. The head, the thorax, and the tail region of such embryos develop normally, but of the eight abdominal segments (A1 to A8), only A8 is formed properly. In place of the other abdominal segments, a single large denticle field that contains pattern elements of both A1 and A7 is found (21).

kni encodes a Cys_2/Cys_2 type of zinc finger protein. The *kni* protein (KNI) shares extensive sequence similarity in the DNA-binding domain with nuclear hormone receptors of vertebrates (27). KNI has recently been shown to function as a transcriptional regulator (13). It acts as a repressor that antagonizes transcriptional activation by the anterior morphogen bicoid (BCD) through competition for overlapping DNA-binding sites in the *cis*-acting region of the gap gene *Krüppel* (*Kr*) (30, 51), which is expressed and required in the region anterior to the *kni* expression domain in blastoderm embryos. Here we describe the identification and characterization of functional sequences of KNI by analyzing the biological consequences of the molecular lesions of various *kni* alleles and by cotransfection experiments in *Drosophila* tissue culture cells. The identified functional sequences such as the DNA-binding domain, the nuclear localization signal (NLS), and a repressor domain are conserved in *kni*-homologous genes of other insect species.

MATERIALS AND METHODS

Sequence analysis of *kni* alleles. Mutant *kni* genes were cloned by PCR amplification of genomic DNA of mutant *kni* embryos as the template, using two primers surrounding the translated part of the *kni* transcript: primer 1 (5′-CGCGGGATTCTTTACAATCTTCCAG-3′, positions 2553 to 2578 of the genomic *kni* sequence [27]) starting 5′ of the initiation codon ATG in exon 2 and primer 2 (5′-CTCTAGACTCCTC CCACTTGGT-3′, for the opposite strand at positions 4100 to 4114) located 3′ of the stop codon in exon 3. The underlined sequences indicate synthetic restriction sites (*Eco*RI and *Xba*I, respectively) that were used for subcloning of the resulting amplified 1.6-kb genomic fragments of the different mutant *kni* alleles into correspondingly digested pBluescript II vector (Stratagene). For each mutant, two independent clones were sequenced on both strands by using the dideoxynucleotide method (41).

Gel mobility shift assays. For production of in vitro-translated truncated *kni* proteins, the *kni* cDNA clone pcJ15 (27) was digested with either *Bam*HI (for KNI^{1-74}), *Pst*I (for KNI^{1-91}), *Bgl*I (for KNI^{1-99}), or *Nde*I (for KNI^{1-154}), resulting in defined interruptions of the *kni* transcription unit. The linearized plasmid DNAs were then transcribed and translated by using rabbit reticulocyte lysate as instructed by the manu-

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facturer (Promega). For bandshift assays, 5 μl of in vitro-translated truncated KNI was incubated (15 min, room temperature) in a total reaction volume of 20 μl containing binding buffer B (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 50 mM KCl, 7% glycerol, 0.1 mM $ZnSO_4$, 1 mg of bovine serum albumin [BSA] per ml), 1 to 5 fmol of ^{32}P-end-labeled *Kr*16 element (a 16-bp double-stranded oligonucleotide [5'-ACTGAACTAAATCCGG-3'] containing a KNI in vitro binding site [13]), 1 μg of poly(dI-dC) (Pharmacia), and 1 μg of salmon sperm competitor DNA. Samples were separated on a 10% native polyacrylamide gel (44).

Recombinant plasmids. The recombinant plasmids pPackniM42, pPackniΔ75-93, pPackniΔ159-164, and pPackniΔ187-199 were used for the expression of the Cys-42→Leu replacement mutant KNI^{Leu-42} and the protein deletions KNI$^{\Delta 75-93}$, KNI$^{\Delta 159-164}$, and KNI$^{\Delta 187-199}$, in which the *kni* box or other conserved sequence motifs have been deleted (see Results). For construction of the expression plasmids, corresponding genomic fragments of the *kni* gene were cloned into the pMc5-8 vector (45) and modified by site-directed mutagenesis using the following primers: kniM42 (5'-GCCCTCGTTCTTG AGCTCGCTGATGGTG-3', complementary to positions 2903 to 2930 [27]; altered nucleotides are underlined), kniΔ75-93 (5'-GCTGCGGCGGCCTGTTCGTG-Δ-CCCCTTCGACAT GCCCACG-3', complementary to positions 2996 to 3014 and 3072 to 3091, deleted *kni* box coding sequence indicated by -Δ-), kniΔ159-164 (5'-GCAGGGCGGCGGATGGATCCGA CAG-Δ-CAGAGGCATATGCTGGTGCG-3', complementary to positions 3247 to 3266 and 3285 to 3309, deleted region coding for LGYPSY marked by -Δ-), and kniΔ187-199 (5'-GCAGAAGCCGCTGCAGCGGCAGCACTTGC-Δ-GGGC GACTGGTGCGGTACACCGCCCATC-3', complementary to positions 3323 to 3350 and 3390 to 3418; -Δ- marks deleted region coding for FQLPPHLLFPGYH). Different subfragments of the genomic *kni* clone pEH5.4 (27) were then exchanged with the different mutated *kni* sequences. Finally, the complete modified *kni* coding sequences were isolated from these plasmids as 2.6-kb *Nru*I fragments and inserted into the expression vector pPac (18). The KNI-expressing plasmid pPackni was constructed analogously. For construction of the BCD effector plasmid DNA pPacTNbcd, see reference 6. Reporter plasmid pKr16AdhCAT and reference plasmid pPaclacZ have been described previously (13). The fusion protein GAL4$^{1-148}$/KNI$^{189-254}$ as well as GAL4$^{1-148}$ and KNI$^{189-254}$ were expressed from the following modified pPac expression plasmids. pPacGal4 1-148 was generated by inserting the 444-bp end-filled *Hin*dIII-*Bam*HI fragment from the *GAL4* cDNA plasmid pMA424 (25), which encodes the DNA-binding domain of GAL4 (amino acids 1 to 148), into the blunt-ended *Bam*HI restriction site of the expression plasmid pPac (18). pPackni189-254 was constructed by fusing the linker FUS5 (5'-CATCATTATGGATCCATAATG-3') to the end-filled 199-bp *Pvu*II-*Cla*I *kni* fragment (positions 3357 to 3555, coding for amino acids 189 to 254), generating a start codon (boldface) at amino acid position 188 and flanking the DNA fragment by two *Bam*HI restriction sites (underlined) that were used for the insertion into the corresponding site of pPac. pPacGal4 1-148/kni189-254 was created by inserting the end-filled 199-bp *Pvu*II-*Cla*I *kni* fragment into the end-filled *Bam*HI restriction site of pMA424. The resulting fusion gene was excised by using *Hin*dIII and *Sal*I, end filled, and inserted into the blunt-ended *Bam*HI restriction site of pPac.

The reporter plasmid pAdh86-5UAS$_G$ was described previously (42).

For expression of the fusion proteins KNI$^{71-93}$/β-galactosidase (β-Gal), KNI$^{64-81}$/β-Gal, and KNI$^{52-93}$/β-Gal, the *lacZ* gene of *Escherichia coli* was fused with different parts of the *kni* coding sequence. In addition to the *lacZ* gene, the expression plasmid pkni71-93/lacZ contains a 76-bp *Sal*I-*Sma*I fragment, coding for an ATG start codon and the *kni* box. It was obtained by PCR amplification of a genomic *kni* fragment by using primers kni-box367 (5'-GCGTCGACATGTCGAAGGGGG GATCC-3') and kni-box369 (5'-GCCCCGGGCTCCTGCAG CAGACAATGG-3') surrounding the *kni* box (positions 3015 to 3071) and introducing an ATG codon (boldface) and additional synthetic restriction sites for *Sal*I and *Sma*I (underlined). This fragment was fused into pBluescript II (Stratagene) together with a 3.1-kb *Sma*I-*Pst*I fragment derived from the fusion vector pMC1871 (Pharmacia) encoding amino acid residues 6 and onwards of *E. coli* β-Gal. The *kni* box/*lacZ* fusion gene was then isolated as a 3-kb *Sal*I fragment, end filled, and ligated into the end-filled *Bgl*II site of pRmHa-3glob to generate pkni71-93/lacZ. pRmHa-3glob was constructed by inserting a 235-bp *Pst*I-*Hin*dIII fragment from pSP64T (19) which contains the globin mRNA leader and 3'-flanking region from *Xenopus laevis* into pRmHa3 (1a), which differs from the vector pRmHa1 (2) in the polylinker sequence.

pkni64-81/lacZ and pkni52-93/lacZ were constructed analogously. For pkni64-81/lacZ, a synthetic 69-bp *Sal*I-*Sma*I oligonucleotide containing an ATG start codon and *kni* sequences (positions 2982 to 3035) was fused to the 3.1-kb *Sma*I-*Pst*I fragment from pMC1871 (see above). pkni52-93/lacZ was constructed by using a 126-bp *Sal*I-*Sma*I fragment obtained by PCR amplification of the region from positions 2946 to 3071 with primer 5 (5'-GTCGACATGAAGAAGAACCACACCA CC-3') and primer 6 (5'-GCCCCGGGCTCCTGCAGCAGA CAATGG-3'), introducing an ATG codon (boldface) and a *Sal*I or *Sma*I restriction site (underlined).

Cotransfection experiments. *Drosophila* Schneider line S2/M3 cells were grown (4) and then plated and transfected (18) as described previously. For transfection, a total of 20 μg of DNA containing various amounts of effector plasmid DNA, 1 μg of reporter plasmid DNA, 3 μg of pPaclacZ reference plasmid DNA, and pBluescript carrier DNA (Stratagene) was used (see Fig. 3). Cotransfection experiments with *GAL4/kni* fusion gene constructs were performed with 6 μg of the reporter plasmid pAdh86-5UAS$_G$ and 4 μg of the different *GAL4/kni* expression plasmids (see Fig. 4). Transfected cells were grown for 80 h, washed once with phosphate-buffered saline (PBS), and lysed by repeated freeze-thaw cycles. Cell debris was removed by centrifugation. The supernatant was assayed for β-Gal activity to account for variations of transfection efficiency. Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (10) or by using a commercially available CAT enzyme-linked immunosorbent assay (5 Prime → 3 Prime, Inc., Boulder, Colo.).

Immunofluorescence microscopy. *Drosophila* Schneider cells were plated at a density of 4×10^5/ml on coverslips in 80-mm-diameter tissue culture dishes and transfected with 10 μg of expression plasmid DNA and 10 μg of pBluescript carrier DNA. At 48 h after transfection, the cells on the coverslips were washed in PBS, fixed in 50% acetone–methanol for 2 min, and washed again in PBS. They were incubated for 1 h at room temperature with primary antibodies, either anti-KNI antibody (diluted 1:50 in PBS–3% BSA [33]) or anti-β-Gal antibody (ABCR GmbH) diluted 1:4,000. After washing with PBS, the secondary Texas red-labeled antibody [F(ab')$_2$ fragment of anti-rabbit/mouse immunoglobulin G; Jackson ImmunoResearch Laboratories, Inc.] diluted 1:200 in PBS–3% BSA was added. The DNA in the nuclei of the cells was visualized with Hoechst dye (H23858; 0.5 μg/ml of PBS).

TABLE 1. Genetic, phenotypic, and molecular properties of different *kni* alleles

kni allele	Phenotype of homozygous embryos[a]	Mutation induced in marker chromosome[b]	Molecular lesion (position of nucleotide exchange and of altered amino acid)[c]	Reference(s)
FC13	Loss of function	*ru st e ca*	Deletion of transcription unit	21, 22, 27
7G	Loss of function	*ru h th st cu e^s sr ca*	T→A (2934); Cys→Ser (48)	16, 48
IIE	Loss of function	Wild type	T→A (2934); Cys→Ser (48)	16, 48
19	Loss of function	*st e*	G→A (2977); Arg→His (62)	21, 48
301	Loss of function	*th st cp in ri p*	C→T (3225); stop (145)	21
357	Loss of function	*th st cp in ri p*	C→A (3347); stop (185)	21
T	Strong	*TM1*	G→A (2884); Arg→His (31)	21, 27, 48
IIV	Intermediate	Wild type	C→T (2640); Thr→Ile (21)	16, 48
5F	Intermediate	*ru h th st cu e^s sr ca*	G→A (3001); Gly→Asp (70)	16, 27, 48
14F	Weak	*st e*	245-bp deletion (3489–3733) frameshift; 105 additional amino acids after position 232	16, 22, 27, 48

[a] The segmentation phenotype of *kni* mutant embryos is characterized by a reduced number of abdominal segments compared with the eight segments (A1 to A8) observed in wild-type embryos (30). In all *kni* mutant embryos, abdominal segment A8 develops normally. *kni* loss-of-function alleles show a fusion of abdominal segments A1 to A7 (16), indicating that segmentation of the abdomen is heavily disrupted. Strong, weak, and intermediate *kni* phenotypes develop a total number of two to three, three to four, and four to five abdominal segments, respectively (22, 27). With the exception of the X-ray-induced allele kni^{FC13}, all alleles listed were induced by ethyl methanesulfonate (EMS). While homozygous kni^{FC13} embryos lack *kni* transcripts as a result of a deletion of the transcription unit (27), the EMS-induced alleles express mutated forms of KNI. The spatial pattern of *kni* protein expression in blastoderm embryos of EMS-induced alleles was analyzed by anti-KNI antibodies but showed no detectable differences from the staining pattern in wild-type embryos.

[b] Genotypes of the marker chromosomes used in the different mutagenesis experiments are described in reference 24.

[c] For positions of nucleotides and amino acids, see reference 27.

Nucleotide sequence accession number. The DNA sequence of the *Drosophila virilis kni* gene will appear in the EMBL/GenBank sequence database under accession number L36177.

RESULTS

kni alleles can be ordered into a phenotypic series reflecting different levels of residual *kni* activities. The weakest *kni* mutant phenotype is characterized by four to five abdominal denticle belts (kni^{14F}); the next-stronger alleles develop three to four abdominal segments (kni^{IIV} and kni^{5F}) and two to three abdominal segments (kni^{T}), respectively. The *kni* lack-of-function mutants (kni^{7G}, kni^{IIE}, kni^{19}, kni^{301}, and kni^{357}) develop a single large abdominal denticle field in addition to the normal A8 (Table 1). To initiate a functional analysis of the *kni* gene product, we examined whether these mutants express significant portions of the KNI primary sequence that are recognized by specific anti-KNI antibodies. Embryos homozygous for the above-described *kni* alleles showed blastoderm staining patterns indistinguishable from the antibody staining patterns in the wild type (data not shown).

Mutational changes in the *kni* coding sequence. To identify functionally important domains within the primary sequence of KNI (Fig. 1), we cloned and sequenced the DNA of nine *kni* alleles. In each of them, missense mutations leading to single amino acid replacements or truncations of the KNI primary sequence have been identified (Table 1). Three of the five ethyl methanesulfonate-induced *kni* lack-of-function mutations were caused by amino acid replacements in the conserved DNA-binding domain of KNI (Fig. 1; Table 1). In the kni^{7G} and kni^{IIE} alleles, which originated from different mutagenesis experiments, Cys-48 is replaced by Ser. In kni^{19}, Arg-62 is replaced by His. The two lack-of-function alleles kni^{301} and kni^{357} contain stop codons which interrupt the open reading frame in positions 145 and 185, respectively (Fig. 1; Table 1). The resulting truncated mutant *kni* proteins carry no biological *kni* function, as deduced from the strong mutant phenotype of the homozygous larvae. Of the four hypomorphic *kni* mutants that carry residual *kni* activity, three are single amino acid replacements in different positions of the KNI DNA-binding domain (Fig. 1; Table 1). The fourth and weakest mutant, kni^{14F}, represents a truncated version of KNI. kni^{14F} carries a 245-bp deletion within the coding region that results in a frameshift. KNI^{14F} is therefore a 337-amino-acid hybrid protein that contains the N-terminal 232 amino acids of KNI (Fig. 1; Table 1). These results show that an intact N-terminal half of KNI is essential for KNI function and that the 185–232 amino acid interval, defined by the lack-of-function phenotype of kni^{357} and the weakest mutant phenotype of kni^{14F}, carries important sequences for the function of KNI in vivo.

Evolutionary conservation of KNI sequences. Assuming that the evolutionary conservation of protein regions reflects the necessity of those domains for the function of the protein, we cloned and sequenced the *kni*-homologous gene of *D. virilis*, a *Drosophila* species separated from *D. melanogaster* by some 40 to 80 million years of evolution. As shown in Fig. 1, the overall sequence identity between the two proteins is 78%. Only one conservative amino acid replacement has been found within the N-terminal 100 amino acids, the region that includes the Cys_2/Cys_2 zinc finger domain and the *kni* box, a sequence motif (positions 75 to 93) which is shared by the gene products of the *D. melanogaster* genes *kni*, *knirps-related* (*knrl*) and *embryonic gonad* (*egon*) (39). The rest of the proteins contains only small stretches of common sequences.

The 185–232 sequence interval of KNI (see above) contains a conserved stretch of alanines which is flanked by short conserved sequence motifs on each side: FQLPPHLLFPGYH in positions 187 to 199 and YRQEMYKHRQSVDS in positions 214 to 227 (Fig. 1). The putative DNA-binding domain, the *kni* box, the FQLPPHLLFPGYH sequence motif, and the stretch of alanines are also conserved in the KNI-like proteins of *D. virilis* and of the more distantly related dipteran *Musca domestica* (43). In addition, these protein motifs are also found in the protein encoded by the *D. melanogaster* gene *knrl* (31) (Fig. 1).

In the wild-type embryo, *knrl* is unable to substitute the segmentation function of *kni*, since its primary transcript of 23 kb is too large to be transcribed in full length during the short nuclear division cycles prior to cellular blastoderm (40). However, the *knrl* protein had been shown to bind to the KNI target sequences and to repress BCD-dependent gene activation in a manner indistinguishable from KNI. Furthermore, *knrl* activity can partially replace KNI function in *kni* lack-of-function mutant embryos when expressed from an intronless transgene

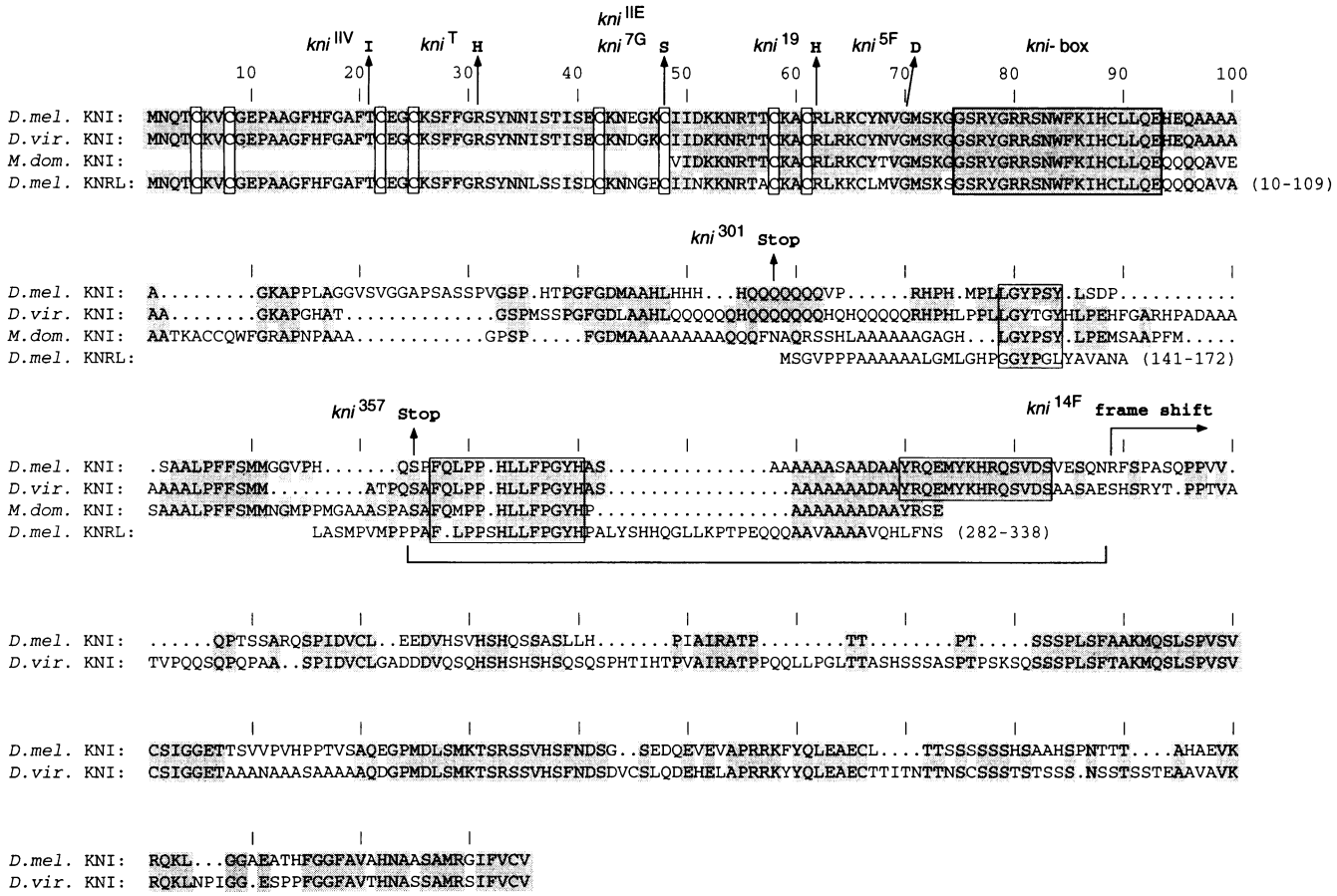

FIG. 1. Alignment of KNI-homologous proteins from different insect species and localization of mutations found in different *kni* alleles of *D. melanogaster*. The complete amino acid sequences of *kni* proteins from *D. melanogaster* (*D.mel.* KNI [27]) and *D. virilis* (*D.vir.* KNI) are compared with the partial KNI sequence of *M. domestica* (*M.dom.* KNI [43]) and three homologous stretches of the *knrl* protein of *D. melanogaster* (*D.mel.* KNRL [31]). Numbers in parentheses refer to the amino acid positions shown for KNRL. Conservation of amino acids between the different proteins are indicated by a grey background. The functional cysteine residues of the N-terminal DNA-binding domains are marked by boxes. Adjacent to this Cys_2/Cys_2 zinc finger domain, the absolutely conserved *kni* box (positions 75 to 93 [39]) is located. Other conserved protein motifs mentioned in the text (LGYPSY, positions 159 to 164; FQLPPHLLFPGYH, positions 187 to 199; YRQEMYKHRQSVDS, positions 214 to 227) are marked by boxes. KNI of *D. virilis* (481 amino acids) differs in size by additional 52 amino acids from KNI of *D. melanogaster* (429 amino acids). Both proteins are highly conserved and have a sequence identity of 78%. While the N-terminal 100 amino acids are absolutely conserved (besides a conservative Glu→Asp exchange), the remainders of the proteins are characterized by several stretches of identity interrupted by gaps or divergent amino acid regions. A similar situation is found for KNI of *M. domestica*. KNRL shows a high degree of sequence similarity to KNI in the N-terminal region including the DNA-binding domain and the adjacent *kni* box (39). In addition, two other homologous protein regions of KNRL discussed in the text are aligned. Mutational changes found in nine different *kni* alleles (see Table 1) are indicated by arrows above the sequences. Six *kni* alleles revealed single amino acid exchanges within the DNA-binding domain. The two independent alleles kni^{IIE} and kni^{7G} show an identical Cys-48→Ser exchange. The amino acid sequences of kni^{301} and kni^{357} are terminated by stop codons in positions 145 and 185, respectively. A deletion of 245 bp in the coding sequence of kni^{14F} causes a frameshift. Consequently, the KNI^{14F} sequence is interrupted after amino acid 232 and followed by 105 additional amino acids. While kni^{357} represents a loss-of-function mutation, homozygous kni^{14F} embryos show only a weak mutant phenotype. Thus, both mutations define a protein region of KNI (positions 185 to 232, indicated by a horizontal bracket) that adds an important contribution to the KNI function, considering the striking differences in the phenotypes of kni^{357} and kni^{14F}. In this region, a functional repressor domain was identified (see text).

(40). The conservation of different distinct stretches of amino acids of the functionally related *knrl* protein suggested that they are necessary for KNI to function as a transcription factor in the embryo. We therefore examined the functional requirement of the conserved sequences by deletion analysis, using in vitro assay systems and transfection experiments with tissue culture cells.

Sequence requirement for the DNA-binding of KNI. To define the minimal sequence requirement for the DNA binding of KNI, we examined truncated forms of KNI for the ability to form DNA-protein complexes with a 16-bp KNI target DNA sequence (Fig. 2A). Gel mobility shift assays with in vitro-translated KNI showed that the 91 N-terminal amino acids of KNI are both necessary and sufficient for DNA binding. This amino acid stretch includes the proposed Cys_2/Cys_2-type zinc finger DNA-binding domain and the conserved *kni* box (Fig. 2). A smaller KNI deletion mutant (KNI^{1-74}) lacking the *kni* box failed to bind to DNA. Furthermore, KNI is capable to bind as a monomer to its target DNA (Fig. 2B), suggesting that dimerization of KNI is not required for the recognition of and binding to DNA sequences. This finding is consistent with the lack of palindromic or tandemly repeated KNI-binding sites in

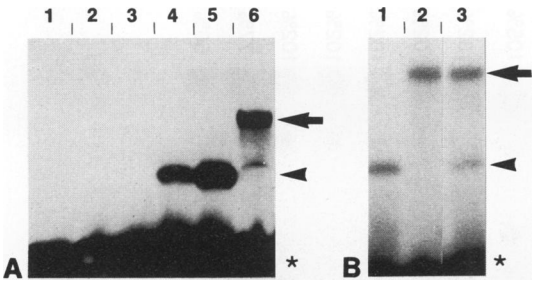

FIG. 2. Minimal sequence requirement for the binding of KNI to target DNA. In vitro-translated truncated KNI and ^{32}P-labelled *Kr*16 element DNA were used in gel shift mobility experiments to localize KNI protein domains essential for DNA binding. (A) The DNA was incubated without protein (lane 1), with unprogrammed reticulocyte lysate (lane 2), or with in vitro-translated KNI^{1-74} (lane 3), KNI^{1-91} (lane 4), KNI^{1-99} (lane 5), or KNI^{1-154} (lane 6). The positions of complexes formed between *Kr*16 element DNA and KNI^{1-91} or KNI^{1-99} are marked with arrowheads; the KNI^{1-154}-DNA complexes are marked with arrows. Free probe is marked with asterisks. While the C-terminal amino acids 92 to 429 are dispensable, the presence of the *kni* box (amino acids 75 to 93; lanes 4 to 6) is a prerequisite for DNA binding. (B) To test whether DNA binding requires dimerization of KNI and whether the N-terminal 100 amino acids could be involved, KNI^{1-99} (lane 1), KNI^{1-154} (lane 2), or a mixture of KNI^{1-99} and KNI^{1-154} (through cotranslation in vitro; lane 3) was incubated with the *Kr*16 element DNA. In lane 3, only KNI^{1-99} (arrowhead) and KNI^{1-154}-DNA complexes (arrow) are detectable. Note that an intermediate complex which would be indicative of heterodimers between KNI^{1-99} and KNI^{1-154} was not observed even after overexposure of the autoradiograph. This indicates that dimerization is not required for the DNA binding of KNI.

the control regions of KNI target genes (12, 13). We note that KNI also does not bind to the *ultraspiracle* gene product, the retinoid X receptor homolog of *Drosophila* species, which acts as heterodimeric partner for a number of nuclear receptor proteins (unpublished results). Thus, the KNI monomer is able to bind DNA, and therefore its DNA-binding properties can be distinguished from those of the known Cys_2/Cys_2-type nuclear receptors, which require homodimer or heterodimer formation for the binding to target DNA (20, 26, 34, 49, 50).

Suppression of BCD-dependent transcriptional activation. The one molecularly known KNI function in the embryo is repression of the activation of *Kr* gene expression by the anterior morphogen BCD, a homeodomain-containing transcription factor (13). In this context, KNI function is part of the regulatory interactions which set the posterior limit of the *Kr* expression domain in the blastoderm embryo (8, 15, 17). This aspect of KNI function is also experimentally accessible through cotransfection experiments in tissue culture cells. In this assay system, KNI-dependent repression of BCD-dependent activation is mediated by a 16-bp *cis*-acting element (*Kr*16 element) containing overlapping binding sites for KNI and BCD (13). We used this assay system to evaluate the function of the conserved DNA-binding domain, the *kni* box, and sequences within the 185–232 amino acid interval of KNI which are functionally defined by the *kni* mutant alleles (see above).

We cotransfected *Drosophila* Schneider cells (18) with BCD-expressing plasmid DNA and with increasing amounts of plasmids allowing the expression of either wild-type KNI or KNI mutants generated by site-directed mutagenesis (Fig. 3). In response to BCD expression alone, CAT gene expression mediated by the *Kr*16 element is activated. Cotransfection of BCD- and wild-type KNI-expressing plasmids resulted in KNI-dependent repression (Fig. 3a). Cotransfection of KNI^{Leu-42}, in which the Cys-42 residue of the DNA-binding domain was replaced by Leu, had no effect on the BCD-dependent CAT gene activation (Fig. 3b), indicating that an intact DNA-binding domain is necessary for KNI function in vivo. $KNI^{\Delta 75-93}$, $KNI^{\Delta 159-164}$, and $KNI^{\Delta 187-199}$ (Fig. 3c to e), which lack the *kni* box or one of the conserved sequence motifs in positions 159 to 164 and 187 to 199 (Fig. 1), were able to suppress BCD-dependent CAT gene activation, but all deletions cause a loss of repressor activity compared with wild-type KNI activity (Fig. 3a). KNI, the Cys→Leu replacement mutant KNI^{Leu-42}, as well as the deletion proteins $KNI^{\Delta 159-164}$ and $KNI^{\Delta 187-199}$ are efficiently imported into the nuclei of transfected Schneider cells (Fig. 3f, g, and i), while the deletion protein $KNI^{\Delta 75-93}$ is localized in both the nucleus and the cytoplasm of the cells (Fig. 3h). This finding suggests that the KNI sequence interval between amino acids 75 and 93 (Fig. 1) is involved in the nuclear translocation of the KNI protein (see below) and that the loss of suppression of BCD-dependent activation might therefore be due to a lowered nuclear protein concentration rather than to the loss of repressor activity per se. The results obtained with the other KNI deletions and the replacement mutant indicate that the DNA-binding domain and the *kni* box, as well as an intact 185–232 amino acid region, are essential for KNI-dependent suppression of BCD-dependent activation in the assay system applied.

KNI contains an alanine-rich transcriptional repressor domain. Molecular analysis of the *kni* mutant alleles showed that the 185–232 sequence interval of KNI is essential for *kni* function (see above). It contains the FQLPPHLLFPGYH motif in front of an alanine-rich region which is conserved among *D. melanogaster*, *D. virilis*, and *M. domestica* (Fig. 1). To determine whether this region of KNI contributes to its function as a transcription factor, we fused it with the DNA-binding domain of the yeast transcription factor GAL4. The resulting hybrid protein was composed of the N-terminal 148 amino acids containing the DNA-binding domain of GAL4 linked to the 189–254 amino acid interval of KNI (see Materials and Methods). We examined the regulatory abilities of the $GAL4^{1-148}/KNI^{189-254}$ fusion protein on gene expression mediated by multiple GAL4 target sequences (UAS_G) in front of the *ADH86* promoter in *Drosophila* Schneider cells (Fig. 4). Expression of neither the GAL4 DNA-binding domain nor the $KNI^{189-254}$ protein caused repression of UAS_G-mediated reporter gene expression, while expression of the $GAL4^{1-148}/KNI^{189-254}$ fusion protein decreased the level of reporter gene expression about 20-fold (Fig. 4). These results show that the $KNI^{189-254}$ sequences can provide repressor activity to a heterologous DNA-binding domain, indicating that the 189–254 region of KNI contains a functional repressor domain.

KNI nuclear localization signal. KNI, as well as KNI^{Leu-42}, $KNI^{\Delta 159-164}$, and $KNI^{\Delta 187-199}$, accumulated in the nuclei of Schneider cells which were transfected with the corresponding expression plasmid DNA (Fig. 3f, g, and i). $KNI^{\Delta 75-93}$ was observed in both the cytoplasm and the nucleus of the transfected cells (Fig. 3h), suggesting that the conserved *kni* box itself or overlapping neighboring sequences could represent the NLS of KNI. The overlapping 52–93 amino acid region of KNI (Fig. 5) covers sequences similar to the NLS of nucleoplasmin (38), which shows a bipartite structure and consists of two clusters of basic amino acids separated by 10 other amino acids. To functionally localize the NLS of KNI within the 52–93 amino acid interval, we constructed hybrid genes encoding the bacterial enzyme β-Gal fused either to the 19 amino acids of the *kni* box (positions 75 to 93) or to sequences extending from within the Cys_2/Cys_2 domain of KNI into the

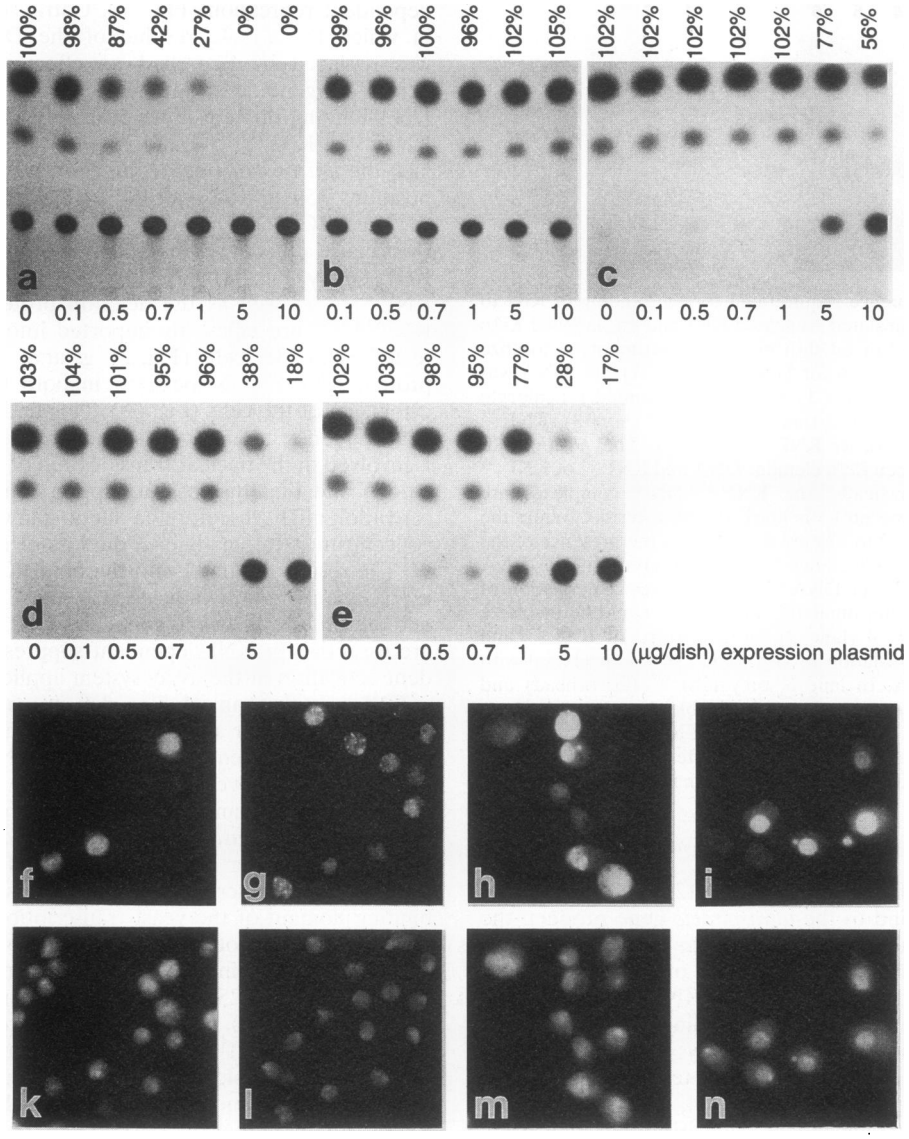

FIG. 3. Suppression of BCD-dependent activation by mutated *kni* proteins. (a to e) *Drosophila* Schneider cells were cotransfected with constant amounts of the BCD effector plasmid DNA pPacTNbcd (5 μg per dish), the pKr16AdhCAT reporter DNA (1 μg per dish), and increasing amounts of KNI effector plasmids pPackni (a), pPackniM42 (b), pPackniΔ75-93 (c), pPackniΔ159-164 (d), and pPackniΔ187-199 (e). To obtain the same repression of BCD-dependent activation of *Kr*16-CAT gene expression as observed for KNI, larger amounts of $KNI^{\Delta 187\text{-}199}$ and $KNI^{\Delta 159\text{-}164}$ are required. $KNI^{Leu\text{-}42}$ (encoded by pPackniM42; b) is unable to bind to DNA because of an amino acid replacement of Cys-42 by Leu (37). Note that although it should contain all other features of KNI, this protein does not repress at high concentrations (b), indicating that squelching is not the mechanism by which repression is achieved. The reduced repression of CAT gene activation observed for $KNI^{\Delta 75\text{-}93}$ might in part be due to a reduced efficiency of protein translocation into the nucleus. (f to n) Subcellular localization of in vitro-mutated *kni* proteins. The efficient translocation of KNI (f) and the mutated proteins $KNI^{Leu\text{-}42}$ (g), $KNI^{\Delta 75\text{-}93}$ (h), and $KNI^{\Delta 187\text{-}199}$ (i) into the nuclei of *Drosophila* tissue culture cells was examined by immunofluorescence microscopy. (f to i) Staining of the transfected cells with anti-KNI antibody (33); (k to n) the corresponding 4′,6-diamidino-2-phenylindole staining visualizing the nuclei of the same cells. All of the proteins are efficiently translocated into the nuclei except $KNI^{\Delta 75\text{-}93}$ (h), which is observed in both the cytoplasm and the nucleus. $KNI^{\Delta 159\text{-}164}$, which is not shown, reveals the same distribution as $KNI^{\Delta 187\text{-}199}$ (i).

kni box (positions 64 to 81 and 52 to 93, respectively; Fig. 5). While the *kni* box/β-Gal hybrid protein and the one containing the KNI 64–81 sequence interval was found in the cytoplasm, the hybrid protein containing the KNI 52–93 sequence interval accumulated in the nucleus (Fig. 6). Thus, the region adjacent to the zinc finger domain contains a functional NLS for KNI.

DISCUSSION

The results presented here define a minimal portion of the gap protein KNI which carries residual *kni* activity. This portion of KNI encompasses slightly more than the N-terminal half of KNI, and it contains several different functional domains as defined by their action in both in vivo and in vitro assay systems. The mutant protein KNI^{14F} containing only the

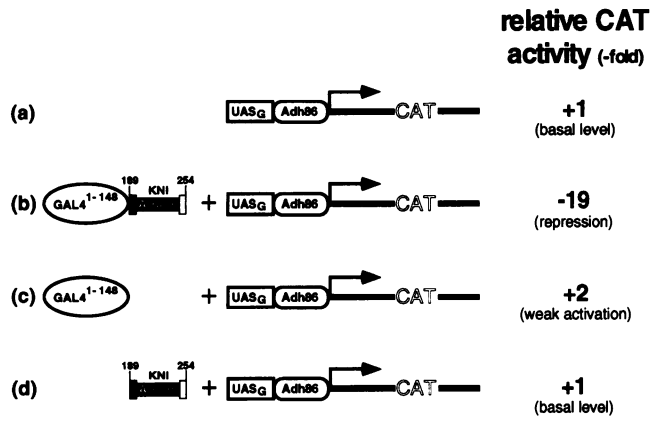

FIG. 4. Functional test for the KNI repressor domain by fusion to the DNA-binding domain of the yeast transcription factor GAL4. A schematic representation of the transfection experiments and the quantitative results (mean values of CAT activities) obtained from three independent series of measurements are shown. The CAT reporter plasmid pAdh86-5UAS$_G$ was transfected into *Drosophila* tissue culture cells (see Materials and Methods). The basal level of CAT reporter gene expression driven by the UAS$_G$ sequences in front of the ADH*86* promoter was monitored in the absence of any other DNA construct (a). Cotransfection of the N-terminal 148 amino acids of GAL4 containing the DNA-binding domain (GAL4$^{1-148}$) leads only to a weak increase of CAT activity (c). The basal level was also not changed significantly by the coexpression of a polypeptide composed of KNI amino acids 189 to 254 (d) including an alanine-rich region surrounded by other conserved sequences (see Fig. 1). However coexpression of the fusion protein GAL4$^{1-148}$/KNI$^{189-254}$ (b) decreased the basal level of CAT gene expression 19-fold, indicating that the KNI$^{189-254}$ sequences act as a repressor domain in conjunction with the DNA-binding region of a heterologous transcription factor. For a number of control experiments involving the GAL4 system as described here, see reference 42.

N-terminal half (amino acids 1 to 232; Fig. 1) of the 429 amino acids of KNI allows the development of up to five abdominal segments, compared with *kni* lack-of-function mutations (e.g., *kni*^{357}), which show only two segments. The N-terminal 100 amino acids of KNI^{14F} include evolutionary conserved sequence motifs such as the Cys_2/Cys_2 finger domain required for the DNA binding and an NLS followed by the *kni* box. The following 84 amino acids, which are also contained in the mutant protein KNI^{357}, which is interrupted after position 184, lack evolutionary conservation except for glutamine/alanine-rich stretches found in a number of transcription factors (3, 11, 47). Compared with KNI^{14F}, however, KNI^{357} does not provide any biological activity, as judged by the *kni* lack-of-function phenotype of the mutant embryo. Thus, the additional 47 amino acids (positions 185 to 232; Fig. 1) that distinguish KNI^{14F} from KNI^{357} must add significant biological activity to the remainder of the protein.

The 47-amino-acid sequence interval contains a stretch of alanines which is flanked by two short conserved sequence motifs on each side (see Results). Only the FQLPPHLLFP GYH motif followed by an alanine-rich region is conserved in the KNI functionally related *knrl* protein (31). The deletion of the FQLPPHLLFPGYH motif, in KNI$^{\Delta 187-199}$, resulted in a reduction of KNI repressor function as assessed by tissue culture cotransfection experiments (Fig. 3). This finding suggests that the competitive binding of KNI, which blocks the binding of the activator BCD, requires sequences of the KNI 187–199 amino acid region in addition to the DNA-binding domain. Thus, the FQLPPHLLFPGYH motif, which represents a short α helix, may be part of a protein domain which is involved in the competition of KNI and BCD for binding to the enhancer or in quenching that is part of KNI-dependent repression of BCD-dependent activation. Alternatively, it may represent a structural motif to expose the adjacent alanine-rich transcriptional repressor domain, which is able to function in conjunction with a heterologous DNA-binding domain of the yeast transcription factor GAL4 and thus represents a repressor domain. Although there is no direct sequence identity with other precisely characterized minimal repressor domains of the *Drosophila* transcription factor encoded by *even-skipped* (11) or the N-terminal transcriptional repressor domain encoded by *Kr* (23), the overall sequence similarity between the KNI repressor domain and the corresponding regions of these transcriptional repressors is obvious. Nevertheless, it may be that the alanine-rich region of KNI does not provide repression by itself but rather does so in combination with the FQLPPHLL FPGYH motif or other sequences contained within the KNI$^{189-254}$ region. Since basal transcription can be inhibited in the presence of this repressor domain, we suspect that it functions by negative interference with one or several of the basal transcription factors of the general transcription factor machinery which assemble at the promoter start site (for a review, see reference 5). We note that there is no direct evidence for whether this region of KNI functions as a repressor domain in the embryo and, if so, how it may work, but the biological importance of this region for *kni* function is most convincingly demonstrated by the differences in the embryonic phenotypes caused by the *kni*^{357} and *kni*^{14F} mutations, which differ by the sequences containing the repressor domain.

The sequences sufficient for the nuclear localization of KNI start within the second Cys_2/Cys_2 finger motif and extend into the adjacent *kni* box. This region (amino acids 52 to 93) contains several basic amino acids and has sequence similarities to the NLS motif that has been shown to be functional in nucleoplasmin (38) (Fig. 5). The *kni* box seemed to be a candidate for the NLS of KNI, since this sequence motif is absolutely conserved in the *kni* proteins of different insect species and in the proteins encoded by *knrl* and *egon* (39). In addition, it is located adjacent to the DNA-binding domain, a position typical for NLS of steroid/thyroid receptors of vertebrates, e.g., the glucocorticoid receptor or the estrogen receptor (35, 36). This hypothesis was supported by the fact that a deletion of the *kni* box (in KNI$^{\Delta 75-93}$) results in a less efficient nuclear localization of KNI (Fig. 5 and 6). However, the 71–93 amino acid interval including the *kni* box was not sufficient to localize a KNI$^{71-93}$/β-Gal hybrid protein in the nucleus by itself. The same observation was made with the overlapping KNI 64–81 sequences, which were chosen since they resemble the nucleoplasmin NLS. Only the KNI 52–93 sequences were sufficient to carry the β-Gal protein into the nucleus. This 42-amino-acid region is composed of an N-terminal sequence with homology to the bipartite structure of basic amino acids of the nucleoplasmin NLS and the C-terminal *kni* box (Fig. 5). Both protein motifs must be essential for the nuclear targeting of KNI, since KNI$^{\Delta 75-93}$, which lacks the *kni* box, and the fusion proteins KNI$^{71-93}$/β-Gal and KNI$^{64-81}$/β-Gal failed to become efficiently directed to the nucleus. Whether this sequence represents the minimal NLS of KNI remains to be shown.

Of the *kni* alleles analyzed by sequencing, three alleles represent *kni* lack-of-function mutations caused by single amino acid replacements in the conserved DNA-binding portion of the protein. The replacement of Cys-48 by Ser, as observed in the two independent alleles *kni*^{7G} and *kni*^{11E}, is

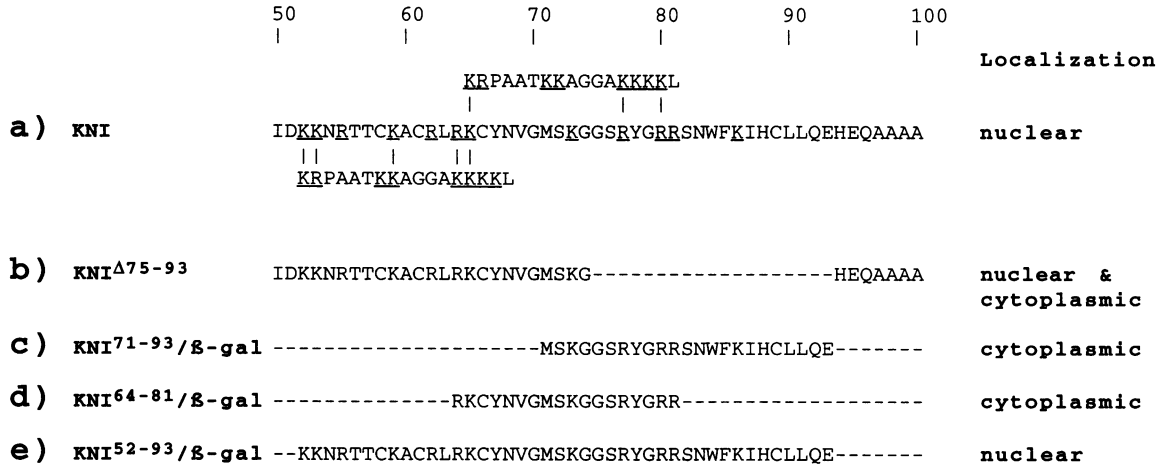

FIG. 5. Sequences required for the nuclear translocation of KNI. (a) Above and below the KNI sequence from amino acids 50 to 100, two possible alignments with the nuclear targeting sequence of nucleoplasmin (38) are shown. Basic amino acids are underlined. | indicates corresponding positions of basic amino acids in KNI and the NLS of nucleoplasmin. (b to d) Amino acids present in the in vitro-generated $KNI^{\Delta 75\text{-}93}$ and the KNI/β-Gal fusion proteins which have been tested for cellular localization by immunofluorescence (see Fig. 6). Note that the deletion of the *kni* box represented by $KNI^{\Delta 75\text{-}93}$ (b) inhibits efficient transport of the protein into the nucleus; however, a slightly larger isolated amino acid sequence ($KNI^{71\text{-}93}$/β-Gal) is unable to mediate the transport of β-Gal into the nucleus (c). Also, the isolated 64–81 amino acid interval of KNI (d) overlapping a region homologous with the NLS of nucleoplasmin does not act as a functional NLS in this assay. Only the sequence present in $KNI^{52\text{-}93}$ (e) mediates nuclear translocation of the fused β-Gal.

consistent with the argument that the DNA-binding zinc finger structure is impaired in the mutant protein. Similarly, the replacement of Arg-62 by His found in the kni^{19} allele may interfere with the formation of the finger structure. Of the four weak *kni* alleles, two contain single amino acid replacements in the DNA-binding region in positions that may interfere with the DNA-binding affinity of the mutant protein to the degree reflected in the mutant phenotype (Table 1). In one weak allele, kni^{5F}, Gly-70 is replaced by Asp. This amino acid replacement occurred in the NLS of KNI and could affect the nuclear localization of the mutated KNI^{5F}. From the *kni* mutant phenotype caused by this mutation (Table 1), the loss of KNI activity can be judged to be more than 50% of KNI wild-type activity. However, since antibody staining indicated that the mutant protein KNI^{5F} is localized in the nuclei of the embryos and a mutated $KNI^{52\text{-}93*}$/β-Gal fusion protein containing a Gly-70→Asp replacement also showed nuclear β-Gal staining in tissue culture (data not shown), we have to assume that this mutational change also affects other functions of KNI that are not yet known.

Concerning the DNA-binding properties, there are noticeable differences between KNI and the other members of the steroid/thyroid receptor superfamily. KNI is able to bind as a monomer to its target sequence, similarly to nerve growth factor I-B and steroidogenic factor 1 (52), and thus KNI is not part of a multifactor system which would offer the potential to generate many different combinations of receptors as suggested for most of the steroid and related receptors (reviewed in reference 34). Furthermore, KNI does not follow the general rule for target gene recognition, since it does not bind to response elements consisting of two distinct sequence motifs related to either AGAACA or AGGTCA (see reference 34 for a review) rather than to a single motif like ACTGAACTA AATCCGG (12). This KNI-binding motif shares four and five out of six nucleotides with this nuclear receptor consensus sequence, i.e., GAAC, and, on the opposite strand, AGxTCA. This may indicate that KNI and the known nuclear receptors originated from a common ancestor or few prototype DNA-binding factors.

The dissimilarities between KNI and the known members of the nuclear receptor superfamily makes one wonder whether KNI requires a ligand for its activity in the embryo. Although our present study does not exclude this possibility, it appears unlikely that a ligand is required for the KNI-dependent repression of BCD-dependent gene activation which is one of

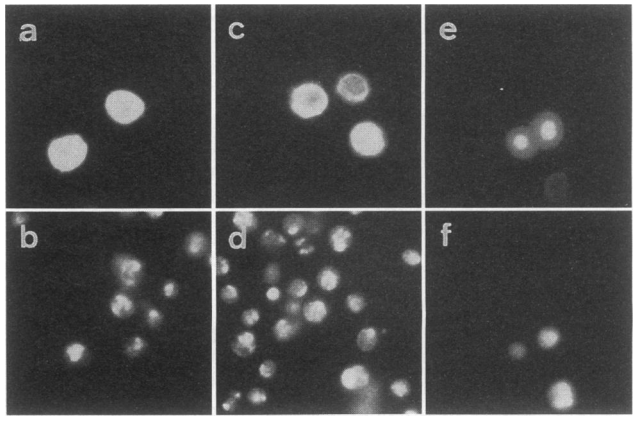

FIG. 6. Subcellular localization of KNI/β-Gal fusion proteins. After transfection of *Drosophila* Schneider cells, KNI/β-Gal fusion proteins ($KNI^{71\text{-}93}$/β-Gal [a], $KNI^{64\text{-}81}$/β-Gal [c], and $KNI^{52\text{-}93}$/β-Gal [e]; see Fig. 5) were detected by anti-β-Gal antibodies (ABCR GmbH) and visualized by immunofluorescence staining with Texas red-labeled antibody (Jackson ImmunoResearch Laboratories, Inc.). Panels b, d, and f show the 4′,6-diamidino-2-phenylindole staining of the nuclei of the same cells. While $KNI^{\Delta 75\text{-}93}$ lacking the *kni* box is expressed in the nucleus and the cytoplasm (see Fig. 3h), the fusion proteins $KNI^{71\text{-}93}$/β-Gal (a) and $KNI^{64\text{-}81}$/β-Gal (c) are found in the cytoplasm. Only $KNI^{52\text{-}93}$/β-Gal (e) is able to direct the β-Gal into the nucleus.

possibly several natural KNI functions in the *Drosophila* segmentation gene cascade acting at blastoderm (13; for a review, see reference 32). This conclusion is based on two arguments. First, KNI-dependent repression is mediated by a single nonpalindromic and nonrepeated DNA target site, unlike those which would be required for the DNA binding of nuclear hormone receptors (20, 26, 49, 50), and there is no indication that dimerization of KNI is essential for the DNA binding per se. Second, the *knrl* protein is able to replace KNI function although it shares only small stretches of sequence similarities aside from the N-terminal DNA-binding domain (40). In addition, the lack of sequences in the C-terminal region of mutated KNI did not cause an on/off state of activity but rather caused a gradual loss of KNI activity, as reflected in the allelic series of *kni* mutants lacking different C-terminal portions of the protein. If a ligand would act on KNI in a manner analogous to the ligands of the nuclear receptors, one would expect that KNI would immediately lose its activity when the corresponding ligand-binding domain is absent (for a review, see reference 7). Thus, we conclude that at least the aspect of KNI function that is involved in transcriptional repression is not dependent on a ligand molecule.

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