

Contextual interactions determine whether the *Drosophila* homeodomain protein, Vnd, acts as a repressor or activator

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ABSTRACT

At the molecular level, members of the NKx2.2 family of transcription factors establish neural compartment boundaries by repressing the expression of homeobox genes specific for adjacent domains [Muhr *et al.* (2001) *Cell*, 104, 861–873; Weiss *et al.* (1998) *Genes Dev.*, 12, 3591–3602]. The *Drosophila* homologue, *vnd*, interacts genetically with the high-mobility group protein, *Dichaete*, in a manner suggesting co-operative activation [Zhao and Skeath (2002) *Development*, 129, 1165–1174]. However, evidence for direct interactions and transcriptional activation is lacking. Here, we present molecular evidence for the interaction of Vnd and Dichaete that leads to the activation of target gene expression. Two-hybrid interaction assays indicate that Dichaete binds the Vnd homeodomain, and additional Vnd sequences stabilize this interaction. In addition, Vnd has two activation domains that are typically masked in the intact protein. Whether *vnd* can activate or repress transcription is context-dependent. Full-length Vnd, when expressed as a Gal4 fusion protein, acts as a repressor containing multiple repression domains. A divergent domain in the N-terminus, not found in vertebrate Vnd-like proteins, causes the strongest repression. The co-repressor, Groucho, enhances Vnd repression, and these two proteins physically interact. The data presented indicate that the activation and repression domains of Vnd are complex, and whether Vnd functions as a transcriptional repressor or activator depends on both intra- and inter-molecular interactions.

INTRODUCTION

Homeobox genes play essential instructional roles in many developmental processes, including patterning the early embryo and specifying embryonic cell lineages. The NK-type homeobox genes encode transcription factors with a divergent homeodomain and two additional highly conserved domains, the candidate Groucho interaction domain referred to as the NK decapeptide or Engrailed homology (EH) domain, and the NK-2 box or the NK-2 specific domain (1–3). The *Drosophila* NK-2 homeobox gene, *vnd*, specifies ventral neuroblast identity (4,5). This dorsal–ventral patterning gene represses the expression of the homeobox gene, *intermediate neuroblast identity* (*ind*), which is expressed in neuroectodermal cells adjacent to the bilateral columns of *vnd* expressing ventral cells. Three *vnd* binding sites in the *ind* enhancer mediate this repression (6).

The question of whether Vnd can directly activate transcription has not been addressed, despite the fact that various potential target genes are not activated in *vnd* mutants. These include the proneural genes, *achaete* and *scute*, the homeobox gene, *Nk6* and the zinc finger transcription factor, Escargot. *vnd* also autoactivates its own expression (7–9). In addition, epistasis tests and double mutant analyses suggest that Vnd and the high-mobility group (HMG) proteins, Dichaete and Sox Neuro, co-operate to regulate patterning and cell fate decisions in the *Drosophila* neuroectoderm (10,11). *NKx2.2*, the closest vertebrate homologue of *vnd*, is also required for dorsal–ventral patterning of the ventral neural tube, as well as pancreatic specification (12–14). Within the neural tube, NKx2.2 interacts with the co-repressor, Groucho, to repress inappropriate expression of target genes (1). Groucho lacks a DNA-binding domain (DBD), but regulates transcription by interacting with DNA-binding transcription factors to form a repression complex [for review see (15)].

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Vnd is a 723 amino acids protein (3,16) that is significantly larger than its vertebrate counterparts, such as NKx2.2. This *Drosophila* protein is likely to be complex, as it may perform functions that are executed by the multiple vertebrate NKx2 family members. In this report, we dissected the domains of the Vnd protein using a heterologous system in transient transfection assays. If Vnd regulatory domains can function in a heterologous context with a DBD from another transcription factor, that domain contains sufficient sequence information to execute a specific regulatory function. These domain swap experiments identified multiple repression domains in Vnd. We found that the conserved homeodomain and NK-2 box have significant repressor activity when expressed in conjunction with other Vnd sequences. We also uncovered two activation domains that are masked by the repressor activity of the intact Vnd protein in cell culture. In immunoprecipitation and mammalian two-hybrid interaction assays, we found that Vnd physically interacts with the high-mobility Sox activator, Dichaete, through the homeodomain. While Vnd had little effect on its own enhancer, co-expression with Dichaete enhances its activation capacity co-operatively in transient transfections. Likewise, under similar assay conditions Vnd requires the co-repressor, Groucho, to repress the *ind* enhancer, through which Vnd represses *ind* expression in embryos. These data are the first biochemical evidence for Vnd-dependent activation, and suggest that Vnd-mediated activation or repression depends to a large extent on the availability of co-factors.

MATERIALS AND METHODS

Tissue culture expression and reporter vectors

A 2.44 kb fragment corresponding to the Vnd open reading frame (ORF), plus 10 bp 5' and 257 bp 3' of the ORF, was cloned into the EcoR1 site of the pCMV-flag vector (Sigma) for expression in mammalian cells. CMV-flag-*groucho 4* (*CMV-grg4*) was kindly provided by Greg Dressler (Department of Pathology, University of Michigan, Ann Arbor, MI). A 2.5 kb EcoR1–BamH1 fragment corresponding to one of the *vnd* enhancers was isolated from a P1 library and subcloned into the Kpn1–BglII site of the pGL3 promoter vector (Promega), following the addition of linkers. A 600 bp fragment corresponding to a dimer of the *ind* enhancer element, to which Vnd binds (6), was also cloned into pGL3. The pFox Luc1-7xNK2 vector that contains seven NKx2.2 consensus-binding sites upstream of the luciferase gene was provided by Michael German (Department of Medicine, University of California at San Francisco, CA). A 1 kb Pvu11–XbaI fragment, lacking sequences encoding the first 30 amino acids of Dichaete, was cloned into the Not1 site of pACT (Promega) and of pCMV-flag, following fill in with Klenow. John Nambu (Biology Department, University of Massachusetts at Amherst, Amherst, MA) kindly gave us the *Dichaete* cDNA. The pACT vector encodes the VP16 activation domain.

Tissue culture Gal4 fusion constructs

For the generation of Gal4–Vnd fusion constructs, relevant *vnd* cDNA fragments were subcloned into pBind vector

Table 1. Restriction sites used to generate pBind-constructs

Construct name	Encodes amino acids	Restriction sites
A1	3–717	Asp1–Bgl1
A2	3–613	Asp1–Acc1
A3	3–519	Asp1–EcoRV
A4	3–406	Asp1–Kpn1
A5	3–217	Asp1–Pvu11
A6	3–197	Asp1–BstX1
A7	217–717	Pvu11–Bgl1
A8	406–717	Kpn1–Bgl1
A9	519–717	EcoRV–Bgl1
A10	613–717	Acc1–Bgl1
A11	657–717	Asp1–Bgl1
A13	217–519	Pvu11–EcoRV
A14	217–406	Pvu11–Kpn1
A15	406–519	Kpn1–EcoRV
A16	535–613	BamH1–Acc1
A17	535–616	BamH1–Acl1
A18	566–613	Acl1–Acc1

(Promega), which encodes the Gal4 DBD (amino acids 1–147) driven by the cytomegalovirus (CMV) promoter and enhancer, using appropriate restriction enzymes and Klenow fragments (see Table 1). pBind also encodes *Renilla* luciferase under the control of the SV40 early enhancer and promoter. To express the NK decapeptide and the NK-2 box as Gal4 chimeras, complimentary oligonucleotides were used. The sequence of the positive-strand oligonucleotide for the NK decapeptide is: 5'-GGCTTCCATATATCGGACATCTTGA-ATTTGGAGGGCTCTGAGCTGAAGAATGCAGCAGC-3'. The sequence of the NK-2 box oligonucleotide is: 5'-CATCGCCCCGTCGGGTAGCCGTTCCAGTTCTGGTGA-GGAACGGAAAGCCCTGCTTGGGCGATAGTTCCAAA-CTGGGAGCCG-3'. Complimentary oligonucleotides were kinase-treated, denatured, annealed and cloned into pBind. Each construct was sequenced using vector-specific primers.

Cell culture, transfections, one- and two-hybrid cell culture assays, and luciferase assays

293 cells were plated at 500 000 cells/ml into 6-well plates, cultured in DMEM + 10% fetal calf serum (FCS) containing 1% penicillin/streptomycin at 37°C, and transfected the following day with Fugene-6 (Roche), according to the manufacturer's instructions. The volume of Fugene-6 used was twice that of the DNA. pBind (Promega) encodes *Renilla* luciferase, which was used to monitor transfection efficiency. The pG5luc reporter (Promega) encodes firefly luciferase downstream of five Gal4-binding sites. For the chimeric one-hybrid assays, 1.5 µg of DNA was transfected and the molar ratio of the expression vector to the reporter vector was 2:1. For the co-transfection of pCMV-flag-*groucho 4* and pCMV-flag-*dichaete* with the pBind-*vnd* and pCMV-*vnd* constructs, the ratio of pBind to pG5luc was 2:1. pGem-3Z was added to maintain the amount of DNA transfected constant. For the two-hybrid assay, the molar ratio of the pACT to pBind to pG5Luc constructs was 1:1:1, and 2.5 µg of DNA was used per transfection. The Checkmate Two Hybrid kit (Promega) was used according to the manufacturer's instructions.

Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS), scraped into passive lysis buffer (Promega) and rocked for 15 min gently at

room temperature (RT). Debris was pelleted and 20 μ l of lysate was assayed for *Renilla* and firefly luciferase activity using the Dual Luciferase Kit (Promega), according to the manufacturer's instructions. Note that the pBind vector encodes the *Renilla* luciferase, while the pG5luc vector encodes the firefly luciferase. Each transfection was performed at least three times. *Drosophila* S2 cells were maintained at RT in M3 media containing 10% FCS and 1% penicillin/streptomycin, and were otherwise handled similar to the 293 cells.

Co-immunoprecipitation and western analyses from tissue culture cells

4.5 μ g of pBind-*vnd* or empty pBind, 1.5 μ g of pCMV-flag-*grg4* or pCMV-flag-*dichaete* or empty vector, and 1.6 μ g of pGL3-*vnd* or pGL3-*ind* were transfected into 100 mm dishes containing 293 cells. Forty-eight hours after transfection, cells were washed with PBS, scraped and the supernatant removed following centrifugation. Cells were resuspended in immunoprecipitation (IP) buffer [20 mM Tris-HCl, 100 mM NaCl, 10 mM NaF, 1 mM Na₃VPO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche) containing 0.5% Triton X-100] and briefly vortexed. Non-specific binding proteins were removed by pre-incubating the supernatant with protein A/G PLUS agarose (Santa Cruz Biotechnology) for 30 min at 4°C, and then removing them by centrifugation. Cell lysates were then incubated with M2 anti-Flag antibody (Sigma), and rotated overnight at 4°C, following incubation with protein A/G PLUS agarose, and rotation at 4°C for 2 h. Beads were precipitated by centrifugation, and washed three times with IP buffer containing 0.1% Triton X-100. Then, beads were resuspended in SDS-PAGE sample buffer. Immunoprecipitates were separated by SDS-PAGE electrophoresis, transferred onto Immobilon-P (Millipore) membrane, and western blotted. Duplicate blots were incubated with anti-Flag antibody (Sigma) to detect the Flag-tagged Groucho 4 and Dichaete proteins, or a Gal4-specific antibody (Santa Cruz Biotechnology) to detect Gal4-Vnd chimeric protein. Binding of peroxidase-conjugated secondary antibodies was detected by chemiluminescence, using the Lightning kit (Perkin Elmer).

Yeast constructs, *in vitro* transcription and translation, and related immunoprecipitations

Asp1-Bgl1, Asp1-EcoRV and Kpn1-Bgl1 *vnd* cDNA fragments (Table 1) were PCR-amplified using a 5' primer with an EcoRI site added, and a 3' primer with a SalI site added, and cloned into the bait vector, pGBKT7 (Clontech). PCR products encoding the ORFs of *Dichaete* and fly *groucho*, containing synthetic 5' EcoRI and 3' XhoI sites, were also cloned into the pGADT7 prey vector (Clontech). Sequencing was used to confirm the identity of the PCR products, and that the individual fragments had been cloned in frame. Using the T7 promoter, located upstream of the myc tag in pGBKT7, and the hemagglutinin (HA) tag in pGADT7, individual prey and bait proteins were *in vitro* transcribed and translated using the TNT T7 Coupled Reticulocyte Lysate kit (Promega) with ³⁵S-labeled methionine, according to the manufacturer's instructions. Immunoprecipitations were performed using the HA antibody from the Matchmaker Co-Immunoprecipitation kit (Clontech), according to the manufacturer's instructions.

Following separation by SDS-PAGE, labeled proteins in the *in vitro* transcription-translation reactions and the co-immunoprecipitations were identified using autoradiography.

RESULTS

Transient expression of Vnd in *Drosophila* S2 or vertebrate 293 cells had minimal effects on reporter gene activation or repression, using three different candidate targets, despite strong Vnd expression in both cell types. The candidate target sequences tested were as follows: the *vnd* enhancer that directs *vnd*-specific expression from stage 6 onwards (17), the repression element previously identified in the *ind* enhancer (6) and a concatenate of seven binding sites corresponding to the consensus NKx2.2 recognition motif (18) (data not shown). Watada *et al.* (18) similarly found that intact Nkx2.2 had minimal effects on reporter expression driven by seven consensus Nkx2.2 binding sites. To facilitate the identification of the functionally important domains in Vnd, we used a one-hybrid assay in which Vnd was fused to the DBD of Gal4. Using this assay, we were able to delineate different Vnd domains and test their efficacy on reporter gene activation or repression using a Gal4-specific reporter construct (Figure 1).

Identification of activation and repression domains in Vnd

Full-length Vnd fused to the Gal4 DBD represses firefly luciferase reporter expression 7- to 8-fold (construct A1, Figure 1C), consistent with its potential role as a repressor in the developing nervous system. To delineate the repression domain more precisely, we made C- and N-terminal deletions of Vnd and fused these to the Gal4-DBD. Deletion of 100 amino acids at the C-terminal of Vnd, including the conserved NK-2 box (construct A2, Figure 1C), leads to a slight, but reproducible, 10% increase in repression activity, relative to A1. Further deletions encompassing the homeodomain (construct A3, Figure 1C) cause a 75% decrease in repressor activity relative to full-length Vnd. However, repressor activity is restored to the level of full-length Vnd if additional sequences are deleted to amino acid 217 (constructs A4 and A5, Figure 1C). These results suggest that sequences spanning amino acids 217-406 likely have de-repression or activator function. The first 217 amino acids of Vnd (construct A5, Figure 1C) include the NK decapeptide or the EH domain (1,2). Surprisingly, when the NK decapeptide is removed (construct A6, Figure 1C), the repression activity of the chimera is not greatly affected. Thus, C-terminal deletions of Gal4-Vnd chimeras uncover multiple redundant repression domains.

To confirm and extend our observations, we also analyzed N-terminal deletions (Figure 1D). Deletion of amino acids 3-217 significantly reduces repressor activity (construct A7, Figure 1D), consistent with the activity of constructs A5 and A6. Further N-terminal deletion to amino acid 519 increases repressor activity (constructs A8 and A9, Figure 1D), although not quite to the full-length Vnd levels. Again, these results are consistent with the homeodomain having repressor function. The C-terminal 60 amino acids are able to activate reporter gene expression, consistent with the slight increase in repression observed upon deletion of this sequence in A2

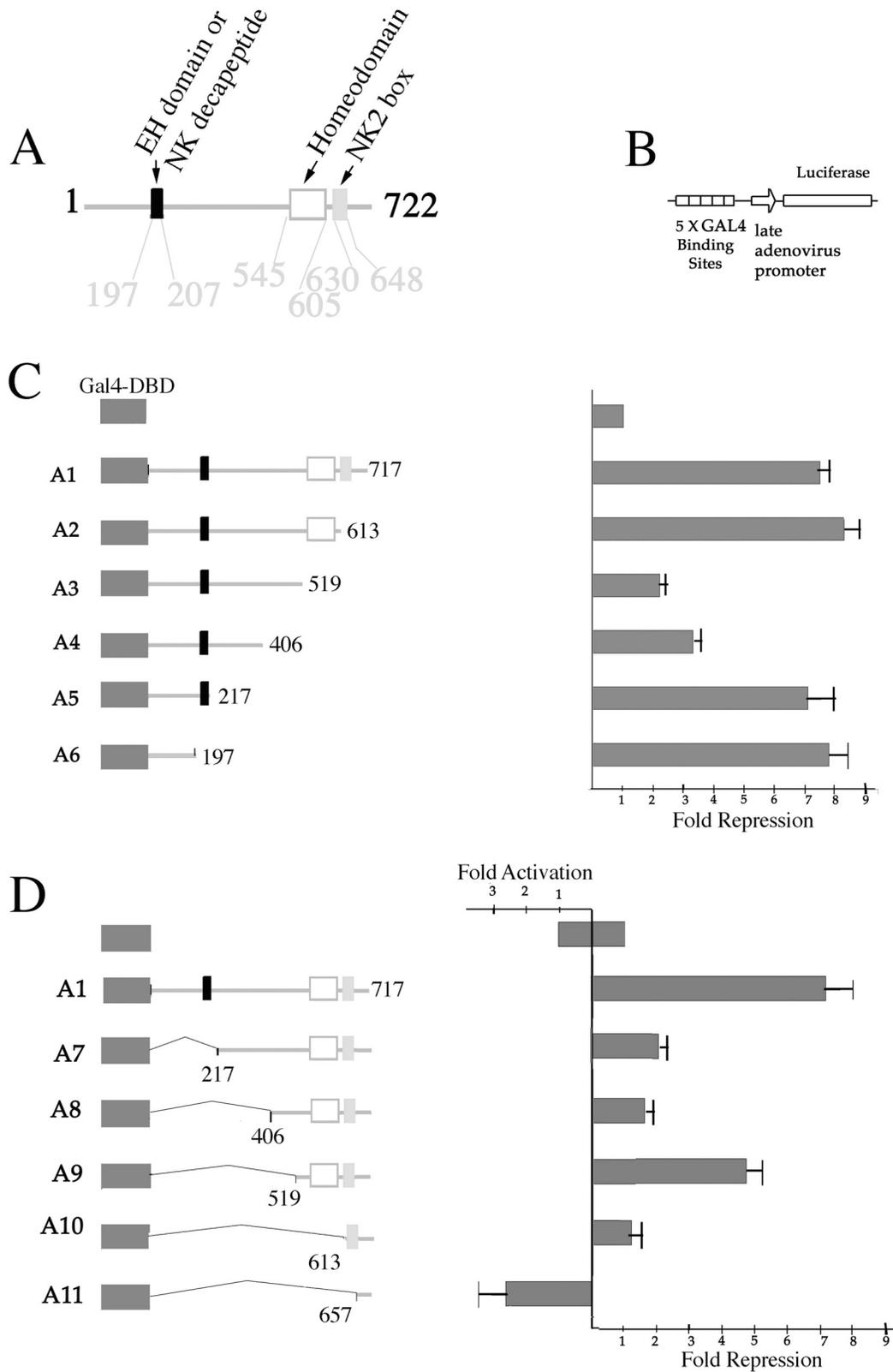


Figure 1. Structure–function analysis of Vnd–Gal4 fusion proteins. **(A)** Schematic representation of the Vnd ORF from amino acids 1 to 722. Salient features depicted are: the NK decapeptide, black box; the homeodomain, open box; and the NK-2 box, solid gray box, as well as the relevant amino acids spanning the different domains. **(B)** Schematic representation of the luciferase reporter gene driven by five Gal4-binding sites upstream of the adenoviral late promoter. **(C)** C-terminal deletions of Vnd fused to the Gal4-DBD are shown schematically. The levels of luciferase activation or repression are shown normalized. A typical baseline reading for *Renilla* luciferase (R) was 6200, and firefly luciferase (F) was 12.9. The F/R value was set at 1-fold. All constructs begin at amino acid 3 and extend to the indicated C-terminal residues. **(D)** N-terminal deletions of Vnd fused to the Gal4-DBD are shown with relative luciferase values as described in (C). Transfections were done in triplicate with average levels of activation or repression shown. The error bars are 1 SD from the mean.

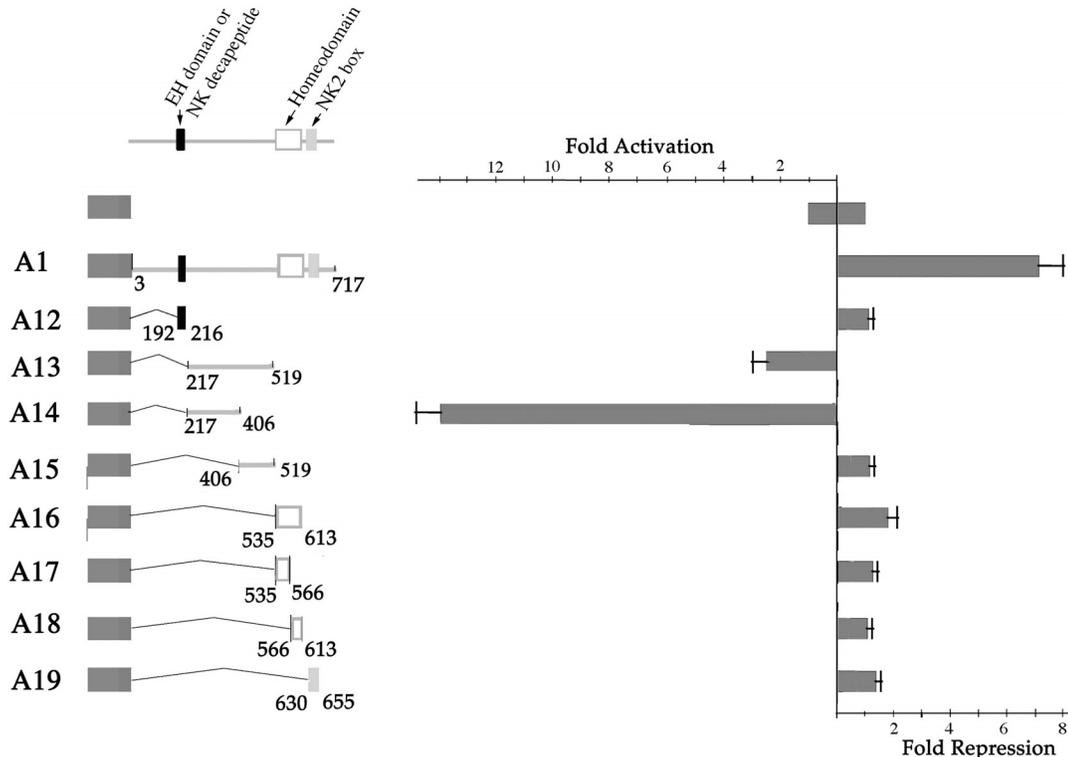


Figure 2. Analysis of separate Vnd domains fused to the Gal4-DBD. Fusion proteins containing the indicated Vnd amino acids fused to Gal4-DBD are shown schematically. The level of activation or repression is shown relative to the Gal4-DBD alone, which has an assigned value of 1. Note the delineation of a strong activation domain spanning amino acids 217–406 with construct A14.

(Figure 1C). Taken together, the C- and N-terminal deletions indicate that the sequences spanning the homeodomain and NK-2 box, and the sequences spanning 3–217, are able to independently repress activation. However, the region spanning 217–519 can function to de-repress these domains individually. In the presence of both repressor domains, the effect of the depression region is minimal.

To assess the potential of the candidate regulatory domains to function independently, we assayed the activity of constructs encoding specific internal Vnd regions that were delineated by the N- and C-terminal deletions. Surprisingly, we found that the capacity of the EH domain, the homeodomain and the NK-2 box to repress transcription is minimal, when expressed independent of other Vnd sequences (Figure 2, constructs A12, A16–A19, respectively). Assuming that steric hindrance does not account for these observations, these results suggest that these domains interact with one another, or other Vnd sequences, in the intact protein to repress transcription. More significantly, a strong activation domain was found that maps onto the amino acids 217–406 (construct A14, Figure 2). Again, this is consistent with the de-repression activity observed in the previous deletions. No difference in luciferase levels was seen when Vnd was expressed independent of the Gal4-DBD with the empty pBind vector, which indicates that the Vnd-dependent effects seen are not due to Vnd activity in a target DNA-independent manner. Thus, Vnd has two activation domains, spanning 217–406 and 657–717, which are typically masked in the intact protein, suggesting that Vnd can function as an activator under the appropriate circumstances. For each of the constructs generated in

Figures 1 and 2, western blotting with an antibody against the Gal4-DBD generated the expected sized protein at levels consistent with the efficiency of transfection (data not shown). Furthermore, all of the Vnd chimeric proteins were found in the nucleus of transfected cells when the same antibody was used for immunostaining (data not shown).

Interaction of Vnd with co-factors

For transcriptional activation, co-activators are typically required in addition to the general transcription factors needed for basal transcription. Recent genetic analyses suggest that the HMG proteins, Dichaete and Sox neuro interact with Vnd (10,11). Zhao and Skeath (12) found that the frequency of loss of marker expression in two ventral neuroblasts was increased in *vnd*, *Dichaete* double mutants relative to that seen in either *vnd* or *Dichaete* single mutants. Because HMG proteins bend DNA to align non-contiguous DBDs for interaction with other transcription regulators and physically interact with other regulatory proteins to activate transcription (19–23), we asked whether Vnd physically interacts with Dichaete by doing a two-hybrid interaction assay in mammalian cells using the Checkmate Kit (Promega). We introduced a chimeric plasmid, pACT-*Dichaete*, which encodes the VP16 activation domain in frame with the *Dichaete* ORF into the one-hybrid assay. We found that when increasing concentrations of VP16–Dichaete are co-expressed with Gal4–Vnd, the repression of baseline luciferase activity caused by the full-length Vnd–Gal4 chimera was significantly reduced. When Dichaete was expressed at a 4-fold molar excess relative to Vnd, repression was reduced ~6-fold (Figure 3, A1).

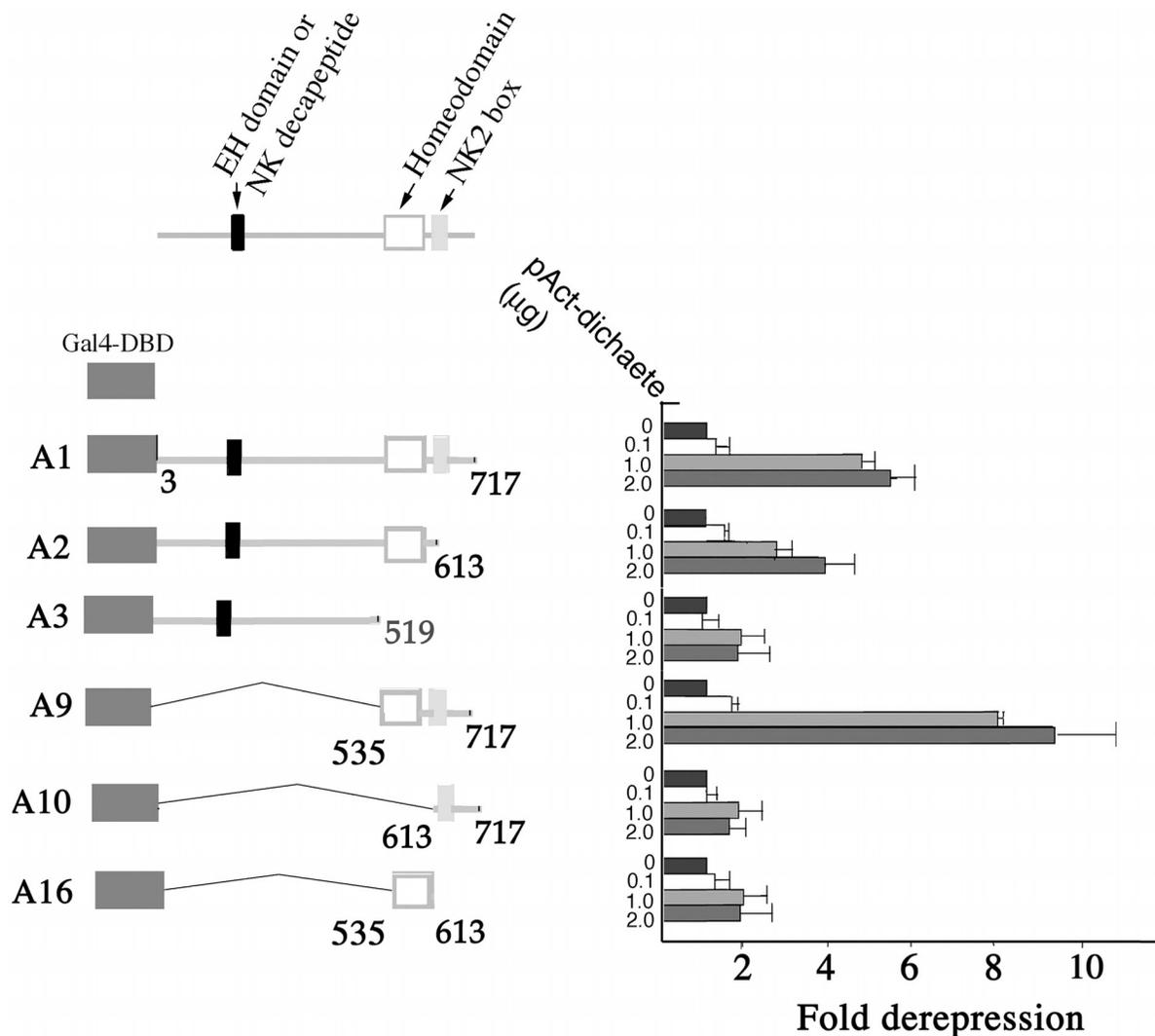


Figure 3. Interaction of Vnd with the high-mobility protein, Dichaete, relieves Vnd-Gal4-mediated repression. The indicated Vnd-Gal4 fusion proteins were expressed transiently with increasing amounts of a pACT-*Dichaete* expression plasmid. Expression of the VP16 activation domain fused to Dichaete from this construct relieves Vnd-Gal4-mediated repression of the luciferase reporter because of Vnd-Dichaete interactions. The baseline for each experimental series was set using pACT-*Dichaete* with empty pBind. Because the Vnd domains have regulatory activity in the one-hybrid assay, the data are expressed as fold de-repression. Note that full de-repression of Vnd-Gal4 by Dichaete requires the presence of the homeodomain and sequences C-terminal to this domain.

Figure 3 shows the results of mapping the region of Vnd that interact with Dichaete, using our series of Gal4-*vnd* deletion constructs. There was a 3- to 3.5-fold alleviation of repression when a Gal4-Vnd chimera encoding the N-terminal of Vnd, including the homeodomain (Figure 3, amino acids 3-613, A2), was co-expressed with VP16-Dichaete, relative to empty pBind plus pACT-Dichaete. Excluding the homeodomain in the N-terminal construct (Figure 3, amino acids 3-519, A3) leads to only very slight alleviation of repression, which suggests that the Vnd homeodomain is important for Dichaete interaction; whereas, expression of the C-terminal amino acids 519-717, including the homeodomain (Figure 3, A9), resulted in an 8- to 9-fold alleviation of repression. This indicates that Vnd sequences in this region must interact with Dichaete strongly. To further define the Dichaete interaction domain in the C-terminal region of *vnd*, we expressed the Vnd homeodomain and the downstream region, independent of each other, as Gal4 chimeras. When expressed in conjunction

with VP16-Dichaete, neither peptide activated luciferase activity significantly, compared to when the domains were expressed together (Figure 3, A10 and A16). Although we cannot rule out steric hindrance accounting for the inability of the smaller Vnd sequences to interact with Dichaete, these results suggest that the homeodomain is necessary, but not sufficient, for binding of Vnd to Dichaete. The C-terminal sequences downstream of the homeodomain may facilitate Dichaete binding. In agreement with these observations, the homeodomains of Bicoid, and Oct-type homeodomain proteins has been implicated in the interaction of these transcription factors with other HMG proteins (24-26).

Dichaete interacts with the CNS-midline-specific proteins, Single-minded and Tango, to activate ventral midline Slit gene expression (23). A 2.5 kb region of the *vnd* enhancer directs ventral cord expression (17,27). We examined this region for the presence of consensus Dichaete-binding sites [ACAAT and ACAAAG (22)] and Vnd-binding

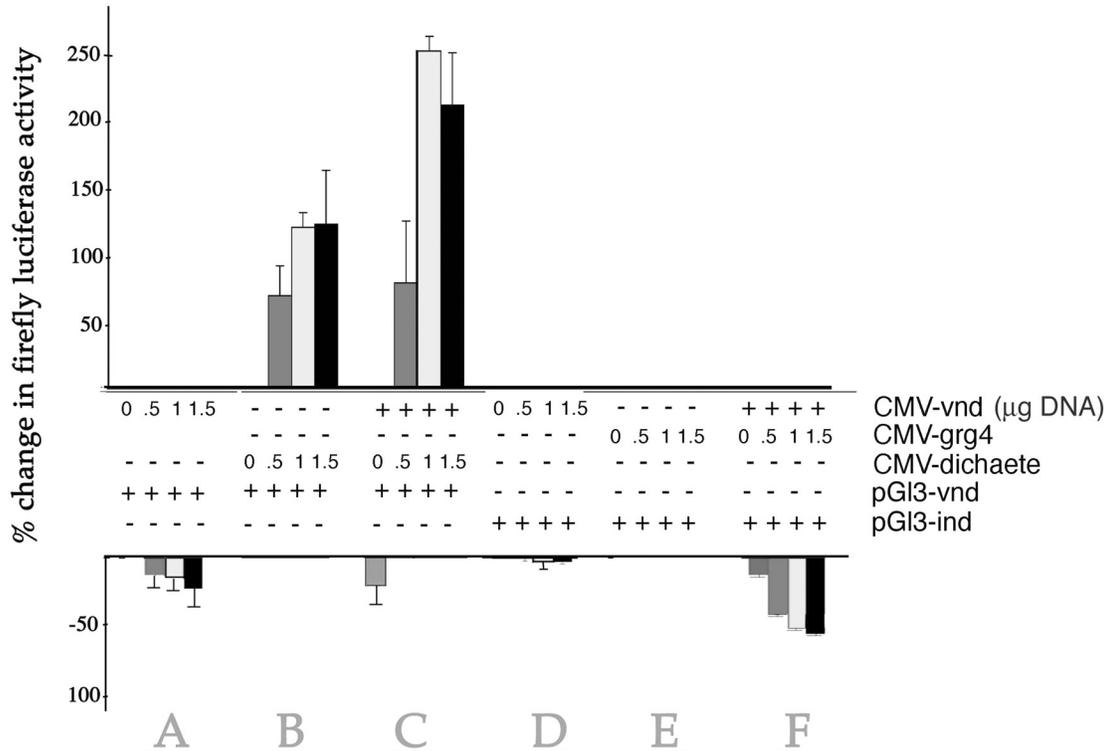


Figure 4. Effects of Dichaete and Groucho on Vnd regulatory activity. Cells were transiently transfected with the plasmids indicated. The reporter plasmid contained the *vnd* promoter fused to the luciferase reporter gene (pGL3-*vnd*) or an *ind* enhancer (pGL3-*ind*). The baseline firefly/*Renilla* luciferase reading was ~3. Vnd expression alone had negligible effects on both reporters (A and D). Expression of Dichaete alone was able to activate the *vnd* promoter ~1-fold, a 100% increase (B). Co-expression of Dichaete with Vnd increased the level of activation to >2.5-fold to 250% activation above background (C), suggesting co-operation. Expression of increasing amounts of the Groucho protein, Grg4, had no effect on reporter expression (E); whereas pGL3-*ind* reporter expression was suppressed by >50% when Grg4 and Vnd were co-expressed (F).

sites [GTGAACT (4)]. Two non-contiguous consensus Dichaete-binding sites are present at positions 350 and 1350, independent of three candidate Vnd-binding sites that have six out of seven of the consensus nucleotides at positions 50, 700 and 1020, respectively. We hypothesized that if Dichaete interacts with Vnd physically, this could cause bending of the *vnd* enhancer, so that the non-adjacent binding sites for Vnd and Dichaete are juxtaposed. This modulation of target DNA availability, combined with the likely conformational changes in Vnd resulting from its interaction with Dichaete, could potentially lead to the activation of reporter expression.

When we addressed this possibility initially in S2 cells, we found that although Vnd is strongly expressed in these cells under the control of the actin promoter, the effects on reporter expression driven by the *vnd* enhancer are negligible. Dichaete, when expressed alone caused weak activation of reporter expression, whereas co-expression of Dichaete and Vnd lead to enhancer activation at levels greater than when Dichaete was expressed on its own (data not shown). We repeated the experiment in 293 cells, using the CMV promoter to drive protein expression (Figure 4). With increasing concentrations of pCMV-*Dichaete*, luciferase expression driven by the *vnd* enhancer was increased to a maximum level of 150% [when the baseline was set at 0 (Figure 4, B)], whereas increasing the concentration of pCMV-*vnd* lead to a slight reduction in luciferase expression (Figure 4, A). However, when Vnd and Dichaete were co-expressed, reporter

expression was activated to a maximum level of 250% (Figure 4, C), relative to the baseline level of reporter expression. Because the sum of the effects of Vnd and Dichaete expressed together is significantly greater than when each transcription factor is expressed independently, our results suggest that these two regulators co-operate with the *vnd* enhancer to activate reporter expression. The capacity of Vnd to activate is target gene dependent. Dichaete did not affect Vnd activity when co-expressed in cells in the presence of a reporter driven by the *ind* enhancer, through which Vnd mediates repression in the embryo (6).

Groucho enhances Vnd repression

We also asked whether exogenous Groucho affects the capacity of intact Vnd to repress luciferase reporter expression, because Vnd has an NK decapeptide or EH domain. In addition, there are AT-rich domains in close proximity to the binding sites for Vnd in the *ind* enhancer (6). Groucho repression complexes typically bind these AT-rich domains when appropriately positioned (28). When we used the *ind* enhancer to direct luciferase reporter expression, luciferase activity was only very slightly repressed when Vnd was expressed alone. The repression was variable and the maximum level seen was a bare 15% decrease in luciferase activity (Figure 4, D). Likewise, when Grg4 was expressed alone, it had minimal effects on luciferase activity (Figure 4, E). However, when Vnd and Grg4 were co-expressed, repression of luciferase activity

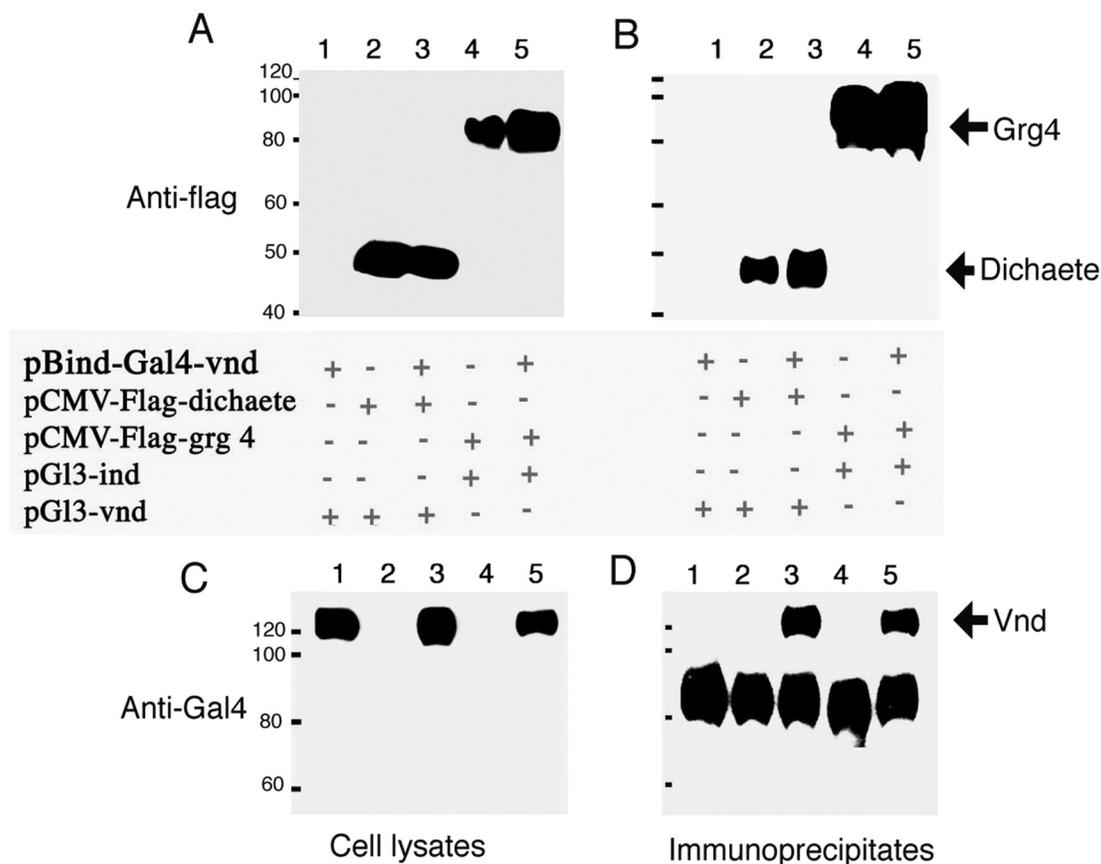


Figure 5. Vnd physically interacts with the co-activator, Dichaete, and the co-repressor, Groucho. Western blots of cell lysates (A and C) following transient transfections, and corresponding immunoprecipitates (B and D) incubated with an anti-Flag antibody (A and B) and anti-Gal4 antibody (C and D). Lanes correspond to transfections: Lane 1, pBind-*vnd*, pGL3-*vnd* and pCMV-flag; lane 2, pCMV-flag-*Dichaete*, pGL3-*vnd* and pBind; lane 3, pBind-*vnd*, pCMV-flag-*Dichaete* and pGL3-*vnd*; lane 4, pCMV-flag-*grg4*, pGL3-*ind* and pBind; and lane 5, pBind-*vnd*, pCMV-flag-*grg4* and pGL3-*ind*. In the cell lysates shown in (A), bands corresponding to Flag-tagged Dichaete and Groucho 4 proteins, with predicted molecular weights of 45 and 88 kDa are seen. In (C), the Gal4 antibody recognizes the Gal4-Vnd chimera (black arrowhead), with a predicted molecular weight of 90 kDa. (B) Western blot of immunoprecipitates generated using the anti-Flag antibody, which immunoprecipitates Dichaete and Grg4. (D) Vnd (black arrowhead) is pulled down when co-expressed with Dichaete, or Groucho 4. Thus, Vnd physically interacts with these co-regulators.

was significantly enhanced, and there was less variability (Figure 4, F). Again, Vnd's capacity to co-operate with Groucho in repressing gene expression was target-dependent, and required the *ind* enhancer. Co-expression of Vnd and Groucho in the presence of the *vnd* enhancer had no effect (data not shown). These combined results indicate that both co-factor availability and target DNA accessibility influence Vnd's capacity to activate and repress transcription.

Vnd can co-precipitate with both Dichaete and Groucho

Our data suggest that Vnd interacts directly with the co-activator, Dichaete, and the co-repressor, Groucho, and this interaction leads to either target activation or repression. To confirm this, we performed co-precipitation analyses. Because our Vnd antibody was relatively ineffective for western-blot analyses, we confirmed that the expression of Vnd fused to the Gal4-DBD with Flag-tagged Groucho or Dichaete resulted in enhanced repression and activation of *vnd* targets, similar to intact Vnd without an additional tag (see Figure 4; data not shown). When we immunoprecipitated Flag-tagged Dichaete

or Grg4 from cell lysates using a Flag antibody, Gal4-tagged Vnd was pulled down with both co-regulators. These results indicate that Dichaete and Grg4 physically interact with Vnd (Figure 5).

We further examined the physical interactions of Groucho and Dichaete with Vnd by addressing whether these co-factors interact in a yeast two-hybrid assay using the Matchmaker Gal 4 Two Hybrid System 3 kit (Clontech). We cloned full-length *vnd* into the bait vector, pGBKT7, as well as two smaller *vnd* domains. Because of concerns about steric interference that arose in the Vnd-Gal4 chimeric assays, we selected the A3 and A8 Vnd constructs for the yeast assays. A3 lacks the homeodomain and its C-terminal sequences, but includes the EH domain, whereas A8 extends from amino acid 406, and includes the homeodomain and Nk-2 box. Full-length fly *groucho* and *Dichaete* were also cloned into the pGADT7 prey vector. AH109 cells were transformed with the various constructs. Because all the three bait proteins drove alpha gal expression without an interacting protein, we monitored prey-bait interactions by measuring growth on *HIS*⁻ medium. We found that both Dichaete and Groucho interacted with full-length Vnd very poorly in this assay. When the

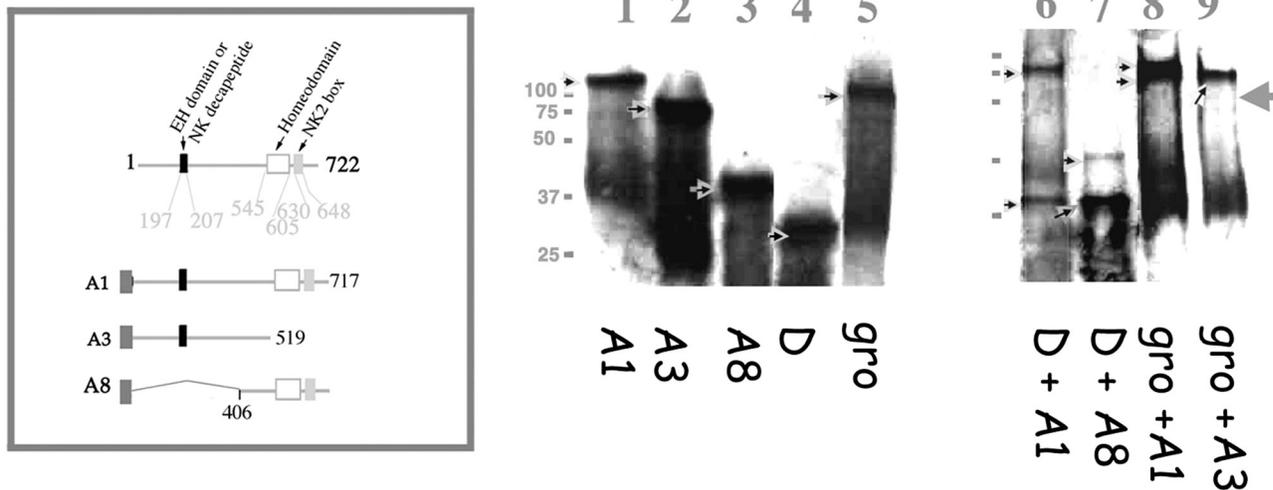


Figure 6. Groucho does not bind a Vnd deletion that includes the EH domain, but lacks the C-terminal region of the protein, which includes the homeodomain and the NK-2 box. Left top: Schematic diagram showing the Vnd protein containing the NK decapeptide (black box), the homeodomain (open box) and the NK-2 box (solid light gray box), as well as the relevant amino acids spanning the different domains. Left bottom: Constructs A1, A3 and A8 that were used for immunoprecipitation analyses. The dark gray box at the N-terminus depicts the myc tag. Lanes 1–5: S-35 distribution in rabbit reticulocyte lysates following *in vitro* transcription–translation of A1, A3 and A8, as well as Dichaete (D) and groucho (gro). Arrows indicate bands corresponding to the individual proteins. Lanes 6–9: S-35 distribution in immunoprecipitates from a mixture of rabbit reticulocyte lysates of HA-tagged D or gro with the Vnd peptides, following immunoprecipitation with a HA antibody. Lane 6: D and A1. Bands of ~100 and 35 kDa (arrows), corresponding to A1 and D, respectively. Lane 7: D and A3. Bands of ~45 and 35 kDa (arrows), corresponding to A4 and D, respectively. Lane 8: gro and A1. A doublet at ~90–100 kDa is seen (arrows), corresponding to A1 and gro, respectively. Lane 9: a single band at ~90 kDa is seen (arrow) corresponding to HA-tagged gro. However, A3 is not pulled down by gro. The gray arrowhead indicates the expected position of the Vnd peptide.

co-transformants were plated on HIS^{-} , LEU^{-} , TRP^{-} medium with concentrations of 3-AT ranging from 0 to 15 mM, it took 5 days at 30°C for small colonies to grow, relative to unrelated controls that grew well after 3 days. Transformants encoding Groucho and the A3 Vnd deletion mutant failed to activate HIS^{-} expression, while transformants encoding the A8 Vnd deletion mutant and Dichaete grew well on HIS^{-} , LEU^{-} , TRP^{-} medium containing 10 mM 3-AT after 3 days at 30°C (data not shown).

Since these observations question our finding that Vnd can co-precipitate with both Dichaete and Grg 4, we tested whether the bait and prey proteins interact in co-precipitation assays. Using the Matchmaker Co-IP kit (Clontech) and the TNT T7 Coupled Reticulocyte Lysate System (Promega), we made ^{35}S -labeled A1, A3 and A8 Vnd from the bait plasmids *in vitro*, with N-terminal Myc-tags (but lack the Gal4-DBD; Figure 6, lanes 1–3). Similarly, we made ^{35}S -labeled Groucho and Dichaete from the T7 prey plasmids, which have a HA tag (but lack the Gal4 activation domain; Figure 6, lanes 4 and 5). The HA antibody proved more effective than the Myc antibody in immunoprecipitations (data not shown). Figure 6 (lanes 6–9) shows the results of pull downs with the HA antibody. Confirming our results from the co-immunoprecipitations from tissue culture cells, we found that either HA-tagged Dichaete or Groucho can pull down full-length Vnd (Figure 6, lanes 6 and 8, respectively). Vnd lacking the first 408 amino acids (A8) interacted with Dichaete (Figure 6, lane 7), but the amount precipitated was less than when full-length Vnd was co-precipitated (Figure 6, lane 6). We were surprised to find that the Vnd deletion mutant, A3, which contains the EH domain but lacks N-terminal sequences including the homeodomain and the NK-2 box, failed to interact with Groucho (Figure 6, lane 9) in three independent

experiments. These results indicate that sequences in the C-terminal region of Vnd are required for Groucho binding to the EH domain, and potentially explain why we were unable to assign repressor function to the EH domain in the Gal4–Vnd chimera dissection assays. In agreement with these findings, Winnier *et al.* (29) reported that in *Caenorhabditis elegans* mutant Unc-4 failed to interact with Unc-37, the worm Groucho, in two-hybrid interaction assays, and did not exhibit *in vivo* repressor activity when the EH1 domain was intact, but sequences N-terminal to the domain are missing.

DISCUSSION

Our dissection of the Vnd homeodomain protein in cell culture suggests that this transcription factor is a complex combination of repression and activation domains. Both intra- and inter-molecular interactions are apparently involved in the DNA binding, activation and repression activity of Vnd. Chimeric analyses of Vnd subdomains indicate that there are at least three repression domains and two activation domains that can contribute to the regulatory capacity of this protein. These activation and repression domains likely interact in the intact protein to generate different patterns of regulation. Our analyses indicate that the co-repressor, Groucho, promotes the repressional activity of Vnd, whereas interaction with the transcriptional activator and DNA bending protein, Dichaete, leads to target activation. Figure 7 summarizes our current model for Vnd regulation of target gene expression that is based on the data presented.

The Vnd homeodomain plays a complex role in the function of this protein. Although capable of binding its target *in vitro* (3,6,30), the homeodomain apparently does so very

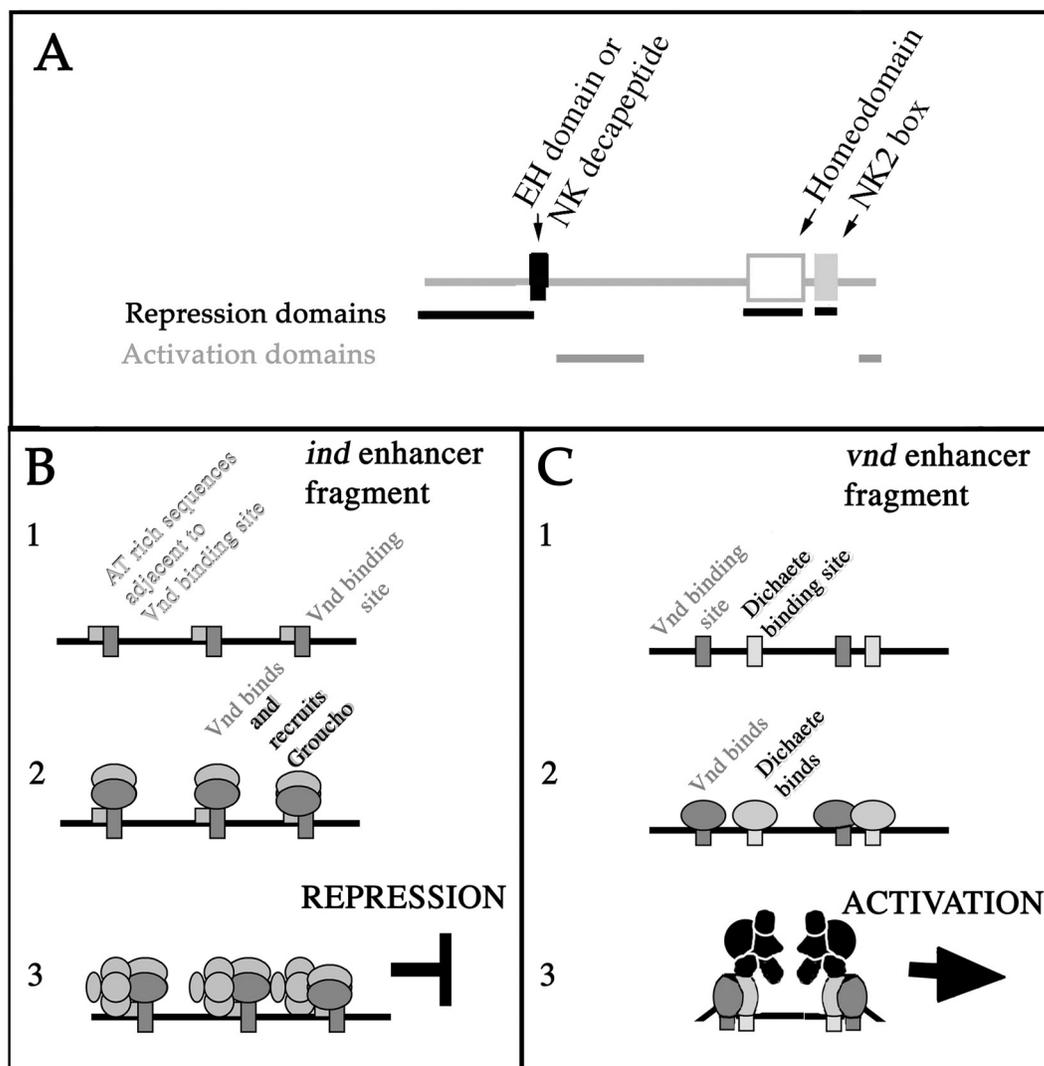


Figure 7. Summary of the regulatory domains in Vnd, and the mechanism of Vnd-mediated target gene regulation. (A) Schematic representation of the Vnd ORF showing the NK decapeptide (black box), the homeodomain (open box) and the NK-2 box (solid gray box). Below, the repression domains we identified are depicted in black, while the activation domains are depicted in gray. The homeodomain and the NK-2 box repress transcription, when expressed as Gal4–DBD chimeras in the context of other Vnd sequences, but have very little activity when expressed alone. The NK decapeptide has very little repressor activity in transient transfections. The first 197 amino acids strongly repress transcription. We identified a strong activation domain on the C-terminal side of the NK decapeptide (amino acids 217–406), and a weaker one at the C-terminal end of the protein (amino acids 657–717). (B) The 300 bp *ind* enhancer through which Vnd mediates repression is shown, and the three Vnd-binding sites within 50 bp [B1; (2)]. Adjacent to the Vnd-binding sites are AT-rich sequences, to which Groucho-interacting co-repressors bind in other enhancers (26). Vnd recruits Groucho, which does not directly bind DNA (B2). Groucho recruits its co-repressor complex, and this results in transcriptional repression (B3). (C) The 2.5 kb *vnd* enhancer is depicted (27). The exact binding sites of Vnd and Dichaete, although not functionally mapped, are schematized (C1). Once Vnd and Dichaete bind their respective sites (C2), and physically interact with each other (C3), this causes DNA bending because the sites are not directly adjacent to each other, and Dichaete has DNA bending activity. This combined effect of protein–protein interaction and DNA bending leads to reporter activation.

inefficiently within the context of the native protein in transient transfection assays. Watada *et al.* (18) showed that removal of the C-terminal of NKx2.2 downstream of the homeodomain leads to NKx2.2-activating reporter expression in tissue culture, although the intact protein was unable to do so. Co-factors are potentially required to stabilize the binding of the homeodomain to its target in the context of the complete protein. NMR analyses indicate that the conformation of the Vnd homeodomain is altered upon binding to target DNA (30). This potentially results in the protein surfaces available for contact with the transcriptional machinery, and with other regulatory proteins being altered. These intra-molecular

interactions would then provide a mechanism for transducing information from the DBD to the other regulatory domains within the protein. Our deletion analyses of Vnd–Gal4 chimeras in transient transfection assays suggest that the homeodomain interacts with repression domains at the N-terminal of the protein. In keeping with this speculation, we found that Groucho did not interact with a Vnd deletion, lacking the N-terminal 200 amino acids including the homeodomain and the NK-2 box, despite the presence of the EH domain (Figure 6). Paradoxically, the homeodomain and sequences on its C-terminal side are also required for the interaction of Vnd with the transcription activator and DNA bending protein,

Dichaete. Likewise, Bicoid and Oct 2A have been shown to interact with HMG proteins through their homeodomains (24–26). Taken together, these results suggest that conformational changes in the native protein, including local protein folding, play important roles in the capacity of Vnd to regulate gene expression.

Extending the findings of Xhao and Skeath (11), we show that Vnd's interaction with the high-mobility Sox protein, Dichaete, confers weak activation capacity on this homeodomain protein in transient transfections. Although Dichaete co-operated with Vnd to activate reporter expression driven by a *vnd* enhancer containing both Dichaete and Vnd binding sites, no effects were seen when both proteins were expressed in cells with a reporter driven by the *ind* enhancer (data not shown). Thus, Vnd's capacity to activate is dependent on both the availability of co-factors and the DNA target. In the context of the *vnd* enhancer, Vnd's interaction with Dichaete changes both the context of these proteins and also that of the target DNA. As an HMG protein, Dichaete's transcriptional co-activator ability is associated with its capacity to bend DNA to align non-contiguous sites for interaction with other transcription regulators [(20–22) and Figure 7]. The capacity of Dichaete to modulate target DNA architecture so that the contact DNA is altered potentially generates a new dimension to NKx2.2/Vnd regulation. To address the possibility that NKx2.2 regulates neural tube patterning by activating transcription, Muhr *et al.* (1) over-expressed the NKx2.2 homeodomain in frame with the VP16 activation domain in the chick neural tube. This substitution did not affect patterning of the neural tube, whereas over-expression of the NKx2.2 homeodomain fused to the Engrailed repression domain did. Consequently, Muhr *et al.* (1) concluded that NKx2.2's capacity to activate gene expression was not important in ventral neural tube patterning. However, these experiments modified the NKx2.2 protein but not its target DNA. Sox 1, 2 and 3, the vertebrate homologues of Dichaete, are expressed in overlapping patterns in the developing neural tube and Sox 2 function is necessary, but not sufficient, to direct cells to a neural fate (31,32). If NKx2.2 interacts with one, or all, of these proteins in the neural tube, this interaction could potentially confer transcriptional activation capacity on NKx2.2, by both reorganizing the target DNA and directly interacting with this transcription factor. This possibility has not yet been addressed. Interestingly, Watada *et al.* (17) also identified an activation domain at the C-terminal end of NKx2.2 that could be used in the presence of the appropriate transcriptional co-activators to activate target gene expression.

The intrinsic activity of Vnd's activation domains is suppressed in transient transfection assays in the context of the full-length protein, or when full-length Vnd is fused to Gal4. Both activation domains that we identified are predicted to have subdomains that are helical and enriched in homopolymeric alanine residues interspersed with bulky amino acids. The structure of the activation domain is as important as the amino acid sequence to its function (33,34). Further work will be required to define the limits of these activation domains and the critical amino acids involved.

We were surprised to find that a domain with no homology to the published regulatory domains at the N-terminus of Vnd had the strongest repression activity in our domain swap

experiments. This region is moderately conserved between *Drosophila virilis*, *Drosophila pseudoobscura* and *Drosophila melanogaster* (Z. Yu and D.M. Mellerick, unpublished data). However, the amino acid composition is not striking. One possibility is that the N-terminal extension of Vnd, lacking in vertebrate Vnd-type proteins, reflects greater functional complexity relative to the vertebrate counterparts. The alternative possibility that this domain is not functionally significant in the developing *Drosophila* embryo cannot be excluded at this time. Although, full-length Vnd co-precipitates with Groucho, a Vnd deletion lacking the N-terminal 200 amino acids, including the homeodomain and the Nk-2 box, failed to do so (Figures 5 and 6). Potentially, this explains why we were unable to assign repressor activity to the EH domain in the Gal4–Vnd chimera dissection analyses (Figures 1 and 2).

It should be kept in mind that how a transcription factor functions in a transient transfection assay does not always fully reflect its function in the context of the developing embryo, where the availability of co-activators, repressors and targets may be different. Tolkunova *et al.* (35) previously found that Engrailed uses two mechanisms to repress transcription—one that is predominant under normal transient transfection assay conditions, the other, which is predominant in an *in vivo* repression assay. The EH domain had only weak activity in transient transfection assays, despite the fact that this Engrailed domain mediates the *in vivo* repression activity and interacts specifically with Groucho, whereas two additional repression domains were more potent in transient transfections than *in vivo*. Koizumi *et al.* (36) recently found that over-expression of mutant Vnd lacking either the NK-2 box or the NK domain in transgenic embryos did not affect the early repression activity of the *vnd* transgene. Since both these domains are highly conserved, and associated with the repressor activity of the protein, these results were unexpected. One possible explanation for Koizumi's result is that deleting repression domains does not alter the activation capacity of Vnd. The mutant transgenes may be able to auto-activate expression of the endogenous *vnd* gene, resulting in its over-expression. Consequently, the effects of the mutations would be hidden by the over-expression of the endogenous gene. Determining whether the mutant *vnd* transgenes can rescue loss-of-function, *vnd* mutants will address this possibility. Thus, both tissue culture and transgenic *vnd* over-expression experiments have limitations in terms of their capacity to assign a specific function to an individual domain. Both these approaches highlight the context-dependence of Vnd activity.

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