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Identification of an upstream regulatory element reveals a novel requirement for Ind activity in maintaining *ind* expression

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Abstract

A maternally established gradient of nuclear Dorsal protein is the first step in subdivision of the *Drosophila* neurectoderm into stripes of homeodomain gene expression. Dorsal in combination with the EGF and TGFβ signaling pathways are key regulators of the expression of the genes *ventral nervous system defective (vnd), intermediate neuroblasts defective (*ind), and *muscle segment homeobox* (*msh*) in the developing neurectoderm. These three genes encode homeodomain transcription factors that can repress each other, which ensures adjacent, non-overlapping expression domains. Expression of *vnd*, *ind*, and *msh* is maintained after decline in EGF and TGFβ signaling, but the relevant positive transcriptional regulators have not yet been defined. Here we show that Ind can bind DNA with the same sequence specificity as its murine ortholog Gsh1. We have identified a novel upstream regulatory element at the *ind* locus containing predicted Ind binding sites, and we show that Ind activity is both necessary and sufficient for reporter gene expression from this element. We conclude that Ind can act as a transcriptional activator, and that positive autoregulation of Ind is a mechanism for persistent *ind* expression within the developing embryonic nervous system.

Keywords

Drosophila; Intermedate neuroblast defective; CNS; Enhancers

1. Introduction

Development of the embryonic central nervous system (CNS) in *Drosophila* initiates with the formation of the neural stem cells, called neuroblasts. Formation of the neuroblasts is a precise process that is repeated in each segment of the embryo (Broadus et al., 1995; Campos-Ortega and Hartenstein, 1985; Doe, 1992). Initially, these neuroblasts form in three columns on either site of the ventral midline. These columns are defined by the expression of three homeodomain transcription factors encoded by the *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*) and *muscle segment homeobox* (*msh*) genes.

Subdivision of the CNS across the Dorsoventral axis appears to be a two step process. Initially the ventral neuroectoderm in *Drosophila* is subdivided into three domains, established by signaling pathways that pattern the embryo on a global basis (Von Ohlen and Doe, 2000). Specifically, the Dorsal morphogen gradient is required for Vnd expression (Mellerick and Nirenberg, 1995; Von Ohlen and Doe, 2000). Both the Dorsal and Epidermal Growth Factor Receptor (Egfr) signaling pathways are required for initiation of Ind (Von Ohlen and Doe,

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2000). In the Msh domain, Dorsal is required to keep Decapentaplegic (Dpp) signaling low (Von Ohlen and Doe, 2000). The second step involves an interaction between these three proteins and a transcriptional repression hierarchy (Cowden and Levine, 2003; Von Ohlen and Doe, 2000). In this hierarchy, Vnd represses Ind in the ventral column and Ind represses Msh in the intermediate column (Mc Donald et al., 1998; Von Ohlen and Doe, 2000; Weiss et al., 1998).

The product of the Ind gene is exclusively expressed in the intermediate column during the early stages of CNS development. This homeodomain protein is required for proper formation and specification of the neuroblasts that derive from this domain (Weiss et al., 1998). As described above initiation of the stripes of Vnd, Ind and Msh expression is driven by the maternal Dorsal gradient. However, how these stripes are maintained is not as well understood. Vnd has been shown to respond to the Dorsal gradient via a neurectodermal specific enhancer, located in the first intron of the gene (Markstein et al., 2004; Stathopolous et al., 2002). In addition, it has been suggested that Vnd is capable of activating its own expression (Yu et al., 2005). Although there is no *in vivo* evidence of Vnd auto-regulation, in cultured cells Vnd is capable of binding to and activating transcription from an enhancer located upstream of its coding sequences (Wang et al., 2005; Yu et al., 2005). This suggests that regulation of Vnd expression may involve modular activity of multiple enhancer elements. Specifically, both an initiator element, that is capable of responding to the Dorsal gradient and other global patterning signals, and a maintenance element that is regulated by Vnd itself are required for complete Vnd expression.

Initial expression of Ind is controlled by an enhancer element located downstream of the *ind* coding sequence. This element contains known Vnd, Ets and Dorsal binding sites (TVO unpublished; (Cowden and Levine, 2003; Stathopolous and Levine, 2005; Weiss et al., 1998). While direct mutagenesis of these sites has not been performed, expression from this construct does respond to mutations in components of these pathways in a manner consistent with their regulation of Ind (TVO unpublished observation). Here we have identified a novel regulatory element located upstream of the *ind* coding sequence that is capable of directing *ind* dependent reporter gene expression. We also present data suggesting that in addition to its role as a transcriptional repressor, Ind also plays a key role in maintaining its own expression. Specifically, we show that Ind is not only required for but is also sufficient to activate expression from this upstream element.

2. Results

2.1 The Ind homeodomain binds DNA in a sequence specific manner

Ind is a homeodomain-containing protein and we predicted that it is a sequence specific DNA binding protein. Therefore, we wanted to determine the DNA binding specificity for Ind. Previously, (Valerius et al., 1995) demonstrated that the mouse Ind ortholog, Gsh1, was a sequence specific DNA binding protein, with the sequence specificity (GC T/C A/C ATTA G/A). An alignment of Ind and Gsh1 revealed a high degree of sequence identity within the homeodomains, particularly within the domains known to make contact with the DNA (Fig 1A). Thus, we predicted that Ind would bind DNA with the same sequence specificity as Gsh1. To test this we designed dsDNA oligos with either the consensus Gsh1 binding sites or a mutagenized oligo with a four base pair substitution of GGGG in place of the ATTA core sequence. We tested whether Ind would bind to these oligonucleotides using electrophoretic mobility shift assays with a bacterially expressed his6 tagged Ind homeodomain (His6IndHD) protein. We found that the His6Ind HD protein was able to shift the fragment of DNA containing the Gsh1 like binding site. We also find that unlabeled wild type Gsh1 fragments were efficiently able to compete for binding in a concentration dependent manner whereas the

mutant Gsh1 fragment did not compete (Figure 1B). Therefore, we concluded that Ind can bind DNA with similar sequence specificity as its mouse counterpart Gsh1.

2.2 Ind activity is required to maintain ind expression

While initiation of *ind* transcription is known to be controlled by the early patterning signals, it is less clear what proteins are required for maintenance of expression. One candidate for a gene product controlling maintenance is Ind itself. In order to determine if maintenance of *ind* expression is dependent on Ind activity, we examined the expression of *ind* mRNA in *ind* mutant embryos. Of the collection of *ind* alleles available, most were generated by P-element excision and delete most, if not all, the coding sequence of the gene (Weiss et al., 1998). However, one allele, ind^{RR108}, was generated by EMS mutagenesis and thus had the potential to produce transcript. When we examined ind^{RR108} homozygous mutant embryos for presence of *ind* transcripts, we found that they did indeed express transcript (Figure 2). *ind* mRNA expression is initiated normally in these embryos (Figure 2A–B). However, expression of *ind* mRNA is not maintained properly in later stage ind^{RR108} mutant embryos. Specifically, as early as stage 7 we saw reduced expression of *ind* mRNA in the neurectoderm (Figure 2D). By stage 11, expression of *ind* transcripts is completely gone from the presumptive central nervous system (Figure 2G&H). However, expression is maintained in the head, demonstrating that the loss of expression is not due to failed staining. We concluded from this that *ind* expression in the head region is regulated independently of expression in the trunk regions. These data are consistent with the hypothesis that maintenance of *ind* transcription during CNS formation is dependent on the ability of Ind to positively regulate its own expression.

2.3 Putative ind binding sites are located upstream of the Ind coding sequence in a novel regulatory element

If Ind is capable of positively regulating its own expression, there should be an enhancer element that Ind is acting through. In addition to other groups, we have independently identified an *ind* regulatory element located 3' to the *ind* coding sequence. This regulatory element drives expression of the *lacZ* reporter gene in a pattern consistent with the *ind* expression pattern (Figure 3A). Expression from this regulatory element responds to the DV signaling pathways in a manner consistent with regulation of *ind* (Stathopolous and Levine, 2005; Weiss et al., 1998) TVO unpublished results). However, we failed to see expression of the *lacZ* transcript from this element in the neuroblasts derived from the *ind* domain (Figure 3). This, in combination with our observation that Ind activity is required to maintain expression of ind transcripts (Figure 2), led us to speculate that an additional enhancer element might be required for expression of *ind* at later stages of development. Expression from this element should be dependent on the activity of Ind. We predicted that such an enhancer element should contain sequences to which Ind will bind. Sequence analysis failed to reveal Gsh1/Ind binding sites located within the previously described downstream regulatory element. We used the cis-analyst search program available at the Berkeley *Drosophila* Genome Project as well as the seqseek program developed by Eric Johnson, (University of Oregon) to search genomic sequences around the *ind* gene for Gsh1/Ind-like binding sites (see Materials and Methods).

Interestingly, both search programs identified four potential Gsh1/Ind binding sites (Figure 4A). Whether they identified the same sites was unclear, because only the seekseq program provided detailed sequence of the region. Based on the seekseq program these sites were all located within approximately 2.8 kb upstream of the *ind* coding sequence. Therefore, we generated new reporter constructs with the upstream genomic fragment driving expression of the *lacZ* reporter gene. An approximately 4.2 kb fragment of upstream genomic sequences, designated GN4 (Genomic N-terminal 4 kb), drives expression in a pattern in the embryo that replicated the expression of endogenous *ind* (Fig 4). Specifically, *lacZ* transcription was initiated as early as stage 6 (data not shown), shortly after endogenous *ind* mRNA was initiated

and was maintained until after stage 11. Multiple independent insertions gave identical expression patterns. The observation that *lacZ* message initiated from this element at stages earlier than the *ind*^{RR108} mutants predict, is not entirely surprising; because Ind may be sufficient to activate its own expression before it is necessary. These results do not exclude the possibility that the upstream regulatory element, like the downstream element, is capable of responding to the early DV signaling pathways that initiate *ind* expression. However, this element does not contain consensus-binding sites for the known transcription factor components of the Dorsal, EGFR or Dpp pathways (data not shown).

Because the identified fragment was so large, 4.2 kb, we wanted to further narrow down the region responsible for maintenance of *ind* expression. To do this we built two additional reporter constructs. *GN1.2LacZ* contains the proximal 1.2 kb of genomic DNA upstream of *ind* coding sequences (Figure 5C). This element does not contain any of the potential Ind binding sites. *GN1.6lacZ* contains the next 1.6 kb of genomic DNA upstream of Ind and includes all four putative binding sites. When we examined expression from these reporter constructs in transgenic embryos we found that *GN1.2 lacZ* transgene always failed to express in an *ind*-like pattern (Figure 5C). However, the *GN1.6lacZ* transgene did drive *lacZ* expression in an *ind*-like pattern (Figure 5D). This provided further evidence that a region of genomic DNA containing Ind-like binding sites is important for conferring *ind*-like reporter gene expression. Finally, we performed site directed mutagenesis of the putative Ind binding sites within the GN1.6 element. We find that expression from the mutant *GN1.6LacZ* reporter construct is reduced relative to wild type but not entirely absent (Figure 5E). These results suggest that there are either additional Ind binding sites not identified by computational means or that other factors in addition to Ind are capable of regulating expression from this element. It is also possible that Ind binds to sites different from that of the Gsh consensus sequence. Nevertheless, the result that expression is reduced when the putative binding sites are mutated further supports our hypothesis that Ind binding to this enhancer is important for expression.

2.4 Expression of GN4lacZ requires Ind activity

If this novel upstream regulatory element responds to positive regulation by Ind, we predicted it would not be expressed in *ind* mutant embryos. Conversely, the downstream element that responds to the early DV patterning signals should be initiated normally in *ind* mutant embryos. To test this hypothesis we built recombinant fly lines in which the upstream regulatory element or the downstream regulatory elements were recombined onto the *ind* mutant chromosomes. For these experiments we used two different *ind* alleles; *ind* $^{16.2}$ and *ind* ^{RR108}(Weiss et al., 1998). We found that in both mutant backgrounds, the absence of *ind* activity resulted in a loss *lacZ* mRNA expression from the upstream regulatory element (*GN4lacZ*; Figure 6B&C). However, we were able to detect normal initiation of expression from the downstream element (*g3.3lacZ*; Figure 6E&F). These results were consistent with our hypothesis that the downstream element responds to the early dorsoventral patterning cues and the upstream element responds directly to Ind activity. In addition, when we examined expression from the *GN4lacZ* construct in a Paired Gal4-UAS Ind gain of function background we were able to detect ectopic *lacZ* expression in the Prd pattern (Figure 6G). This suggests that not only is Ind activity necessary for reporter expression from this element but Ind is also sufficient to activate expression of *lacZ* when Ind is ectopically expressed outside the endogenous Ind domain. These results further support our hypothesis that Ind positively regulates its own expression via this newly identified enhancer element located upstream of the coding region.

3. Discussion

Patterning of the CNS in *Drosophila* involves regulation of gene expression by three DV restricted homeodomain containing proteins. Previous data suggest that this is accomplished

by a cascade of transcriptional repression (Cowden and Levine, 2003; Stathopolous and Levine, 2005). It is in fact well documented that the DV restricted homeodomain proteins act as transcriptional repressors. Recent papers have suggested that at least Vnd can act as a transcriptional activator (Yu et al., 2005) (Uhler et al., 2002). Here we present in vivo data suggesting that the Ind homeodomain protein can also act as a transcriptional activator. Specifically, the data show that Ind activity is required to maintain *ind* expression and that this autoregulation takes place through a previously uncharacterized regulatory element located upstream of the *ind* coding sequence. It is entirely possible that an additional as yet unidentified positive regulator is required for the maintenance of Ind expression.

Our results suggest that Ind can act as a transcriptional activator. However, we cannot rule out the possibility that loss of *ind* function causes derepression of a repressor. One possible repressor of *ind* could be Msh. We do not think this is the case for three reasons; first I have previously demonstrated that expression of Msh is expanded ventrally in *ind* mutant embryos (Weiss et al., 1998). In *ind* mutant embryos Msh expression is expanded ventrally into the Ind domain at the time of initiation, around stage 6. However, *ind* mRNA in the *RR108* mutant appears largely normal until stage seven and eight. Second, Cowden and Levine, (2003) state that Msh over-expression does not repress *ind* expression (Cowden and Levine, 2003). It is possible there could be another as yet unidentified repressor as suggested in (Stathopolous and Levine, 2005). Third, expression of *ind* does not expand dorsally in *msh* mutant embryos (data not shown).

Regulation of *ind* expression appears to involve to separable regulatory elements. The previously described element that is located downstream of the *ind* coding sequence and is required for initiation (Cowden and Levine, 2003; Stathopolous and Levine, 2005; Weiss et al., 1998). An additional element located upstream of the coding sequence appears to be dependent on Ind activity. A parallel regulation might also be possible for Vnd where the early neurectodermal enhancer is located within the first intron (Markstein et al., 2004; Stathopolous et al., 2002). However, elements controlling expression in neuroblasts are located upstream of the coding sequence (Shao et al., 2002). Moreover, Vnd can bind to the upstream element and regulate reporter gene expression from it (Saunders et al., 1998; Wang et al., 2005; Yu et al., 2005). It should be noted, that these results are based on tissue culture reporter assays and not in vivo results. Nevertheless, these data do support the idea that similar to *ind, vnd* expression might be regulated by separable enhancer elements which control initiation and maintenance independently.

The ability of Vnd to act as a transcriptional activator appears to be in part regulated by interaction with the HMG domain-containing protein Dichaete (Yu et al., 2005). Genetic data suggest that Ind and Vnd interact with Dichaete in a similar manner (Buescher et al., 2002; Zhao and Skeath, 2002). Ind expression is normal in *dichaete* mutant embryos (Zhao and Skeath, 2002). Thus, we hypothesized that expression of Ind is most likely initiated normally in *dichaete* mutants and despite the apparently normal expression of Ind protein in *dichaete* mutants we might see an effect on expression from our upstream regulatory element. However, following recombination of the GN4lacZ transgene onto the *dichaete* chromosome we failed to see a loss of lacZ expression (data not shown). Therefore we are not convinced that Dichaete is involved in this aspect of Ind function.

Our data provides evidence that following initiation by global patterning signals, expression of the Ind homeodomain is maintained by the activity of Ind itself. This occurs through a newly identified regulatory element that is positioned upstream of the coding sequence and away from the element controlling initiation. A parallel type of regulation might occur for the Vnd homeodomain protein. However, additional work is required to confirm this hypothesis.

4. Materials and Methods

4.1 Fly stocks and In situ hybridizations

All fly stocks were maintained on standard media, *yw* flies were used for wildtype. The ind mutant fly lines used were *ind*^{RR108}*/TM3 ftz lacZ; ind* $^{16.2}$*/TM3 ftz lacZ* (Weiss et al., 1998). Recombination crosses were done as described in; Greenspan, 1997). The Prd Gal4 line was obtained from Chris Q. Doe (Oregon). All other fly lines are transgenics of constructs described in sections 4.2.

In situ hybridizations were done according to standard procedures (Jiang et al., 1991; Tautz and Pfeiffle, 1989)

4.2 Constructs

To build the UASind construct, PCR primers were designed to amplify the coding sequence from *ind* cDNA in NB40 were as follows: IndF cccgctcgagcggggaaataccccagaaacccaagatg and IndR ggggtaccccacgcctcaaccttcaattcgtg (Weiss et al., 1998). This product was cloned into pUAST (Brand and Perrimon, 1993) at the *Xho*I/*Kpn*I sites.

GN4 lacZ—Phage DNA containing the genomic sequences surrounding Ind coding region were kindly provided by Joe Weiss (Stanford). A 4.5 kb *Eco* RI to *Xho* I fragment was cloned into the CaSper Hs43 lacZ reporter vector. Restriction mapping of phage clones was done via Southern blotting using the Chemiluminscent Dig labeling kit (Roche). Multiple single and double digests were performed, and blots were probed with Dig-labeled probes to either the *ind* cDNA or the previously described domain containing Vnd binding sites (Weiss et al., 1998). The *g3.3 lacZ* construct was built with a 2.5 kb *Sal*I/*Eco*RI fragment located approximately 1.5 kb downstream of the stop codon of *ind*. This fragment was also ligated into the CaSper Hs43 LacZ reporter vector.

GN1.2 lacZ—The 1.2 kb PCR product was amplified from GN4 using the following primers: GN1.2 F tggatgatccttgccgc and GN1.2 R cgatcgctgactgtgcg. GN1.2 PCR product was cloned in to the TOPO TA cloning vector (Invitrogen) and subsequently moved into the pCaSpeR HS43 lacZ reporter vector at the *Eco*RI site.

GN1.6 LacZ—1.6 Kb ClaI fragment from GN4 was cloned into Bluescript KS at the *Cla*I site, then moved in to the pCaSpeR HS43 lacZ reporter vector with the *Not*I and *Xba*I sites. The Non-Mammalian Model Systems unit at Duke University injected all transgenes.

4.3 His Tagged ind homeodomain and DNA binding experiments

The coding region for the C-terminal of the Ind protein was cloned into the pET28c vector (Novagen) between the *Not* I and *Hind*III sites. His6IndHD was expressed and purified by Abgent Corporation (San Diego CA). Oligomers containing the Gsh1 binding site are tgaccagctaattagagacacatt and aatgtgtctctaattagctggtca. Oligos containing the mutant site are tgaccagctaggggagacacatt and aatgtgtctccccctagctggtca these serve as a control for non-specific binding. WT and mutant oligos were annealed to form dsDNA molecules. EMSA assay was performed using the Dig non-radioactive Gel shift kit (second generation, Roche). The DNA/protein complexes were resolved on a 5% non-denaturing polyacrylamide gel.

4.4 Sequence analysis by Cis analyst and seq seek searches

We used two separate search programs to search for Gsh1/Ind-like binding sites in the genomic regions around Ind. We used the cis-analyst search program available at the Berkeley Drosophila Genome Project (BDGP; http://rana.lbl.gov/cis-analyst/cgi/viewer.php; (Berman

et al., 2001) website as well as the seqseek program developed by Eric Johnson, (University of Oregon; http://flycompute.uoregon.edu/cgi-bin/indiv_gene.pl; (Freeman et al., 2003) to search genomic sequences around the *ind* gene for Gsh1 like binding sites. In the cis-analyst program we entered up to 10 of the sites identified in (Valerius et al., 1995). In the seekseq program we use the consensus sequence gcymattaa and degeneracy model of gcnnattan to identify potential sites. Both programs identified multiple potential sites within approximately the same upstream region. Whether they identified the same sites in unclear, as only the seqseek program gave a detailed output.

4.5 Site directed mutagenesis

Putative Ind binding sites in the GN4 enhancer element we mutagenized using the Quick Change Multi-site directed mutagenesis kit (Stratagene). The following mutagenic primers were used to convert the core ATTA sequence to GGGG or CCCC.

Gsh site 1:tc aaa atg caa atg tca caa cca ccc cat gtc aat aaa tga tta acc caa tc

Gsh site 2:gaa ttt ttt ttg aaa cac ccc ttg ccc cgg caa atc cgg ttt gat gtt ctc

Gsh site 3: gtt ttt ttt tgc aca gcc cgc cac ccc agc aga agc caa ata ctt aaa aac

Gsh site 4:cta cgt aga agt cgg tcg aca ccc cac aat taa cgt cag caa ttg tga tcg g

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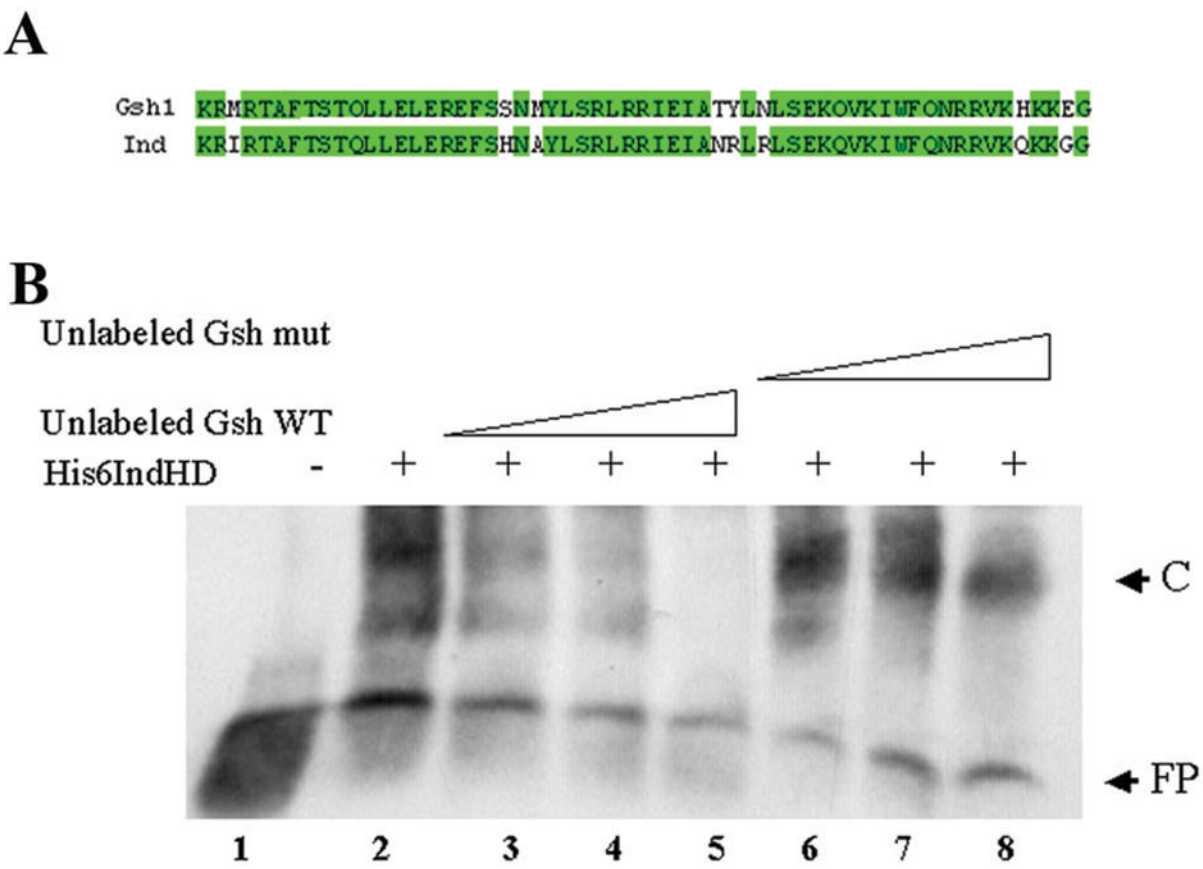

Figure 1.

A) Aligment of homeodomain sequences for Ind and Gsh1. Areas shaded green indicate regions of amino acid identity. Overall sequence identity for the homeodomains is 85%. B) Electrophoretic mobility shift assay demonstrating Ind binding to Gsh1 binding site. FP indicates the position of the free probe. C indicates position of the complex formed in the presence of His6-Ind homeodomain. Lane1) Free Gsh1 oligo. Lanes 2–8) + 0.5 mg His6IndHD, Lane 2) no competitor. Lane 3) 5ng unlabeled Gsh1. Lane 4) 10 ng unlabeled Gsh1. Lane5) 20 ng unlabeled Gsh1. Lane 6) 5ng unlabeled mutant Gsh1. Lane 7) 10 ng unlabeled mutant Gsh1. Lane 8) 20 ng unlabeled mutant Gsh1.

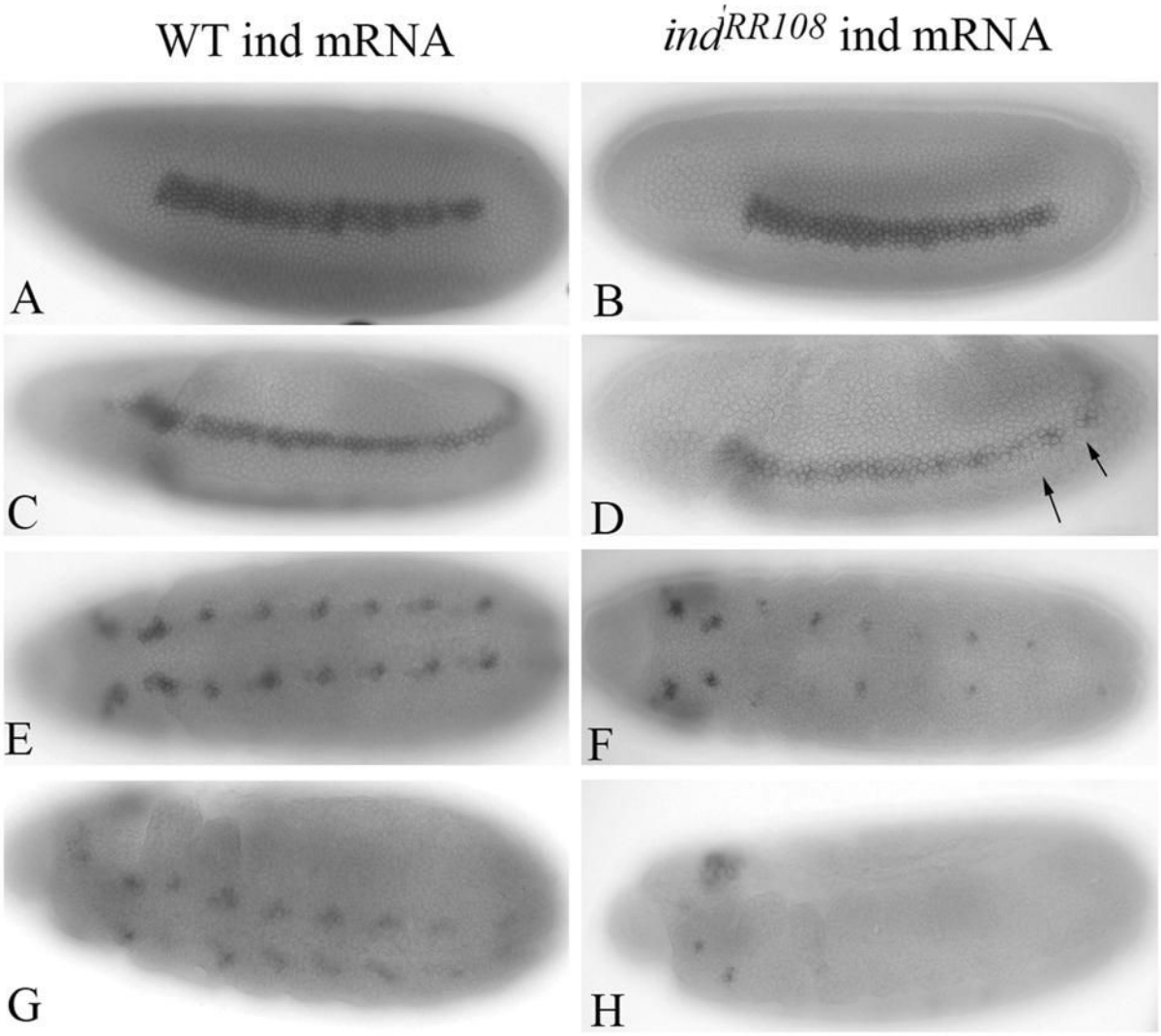

Figure 2.
Ind activity is required to maintain expression of *ind* mRNA. A, C, E, G) Wild type *ind* mRNA expression. (B, D, F, H) expression of *ind* mRNA in ind^{RR108} homozygous mutant embryos. Expression is initiated normally (compare A and B). At stage 8 expression of *ind* mRNA begins to deteriorate (compare C and D) Arrow indicates a gap in *ind* pattern beginning to form at stage 8. *ind* message is gone from the trunk regions by late stage 11.

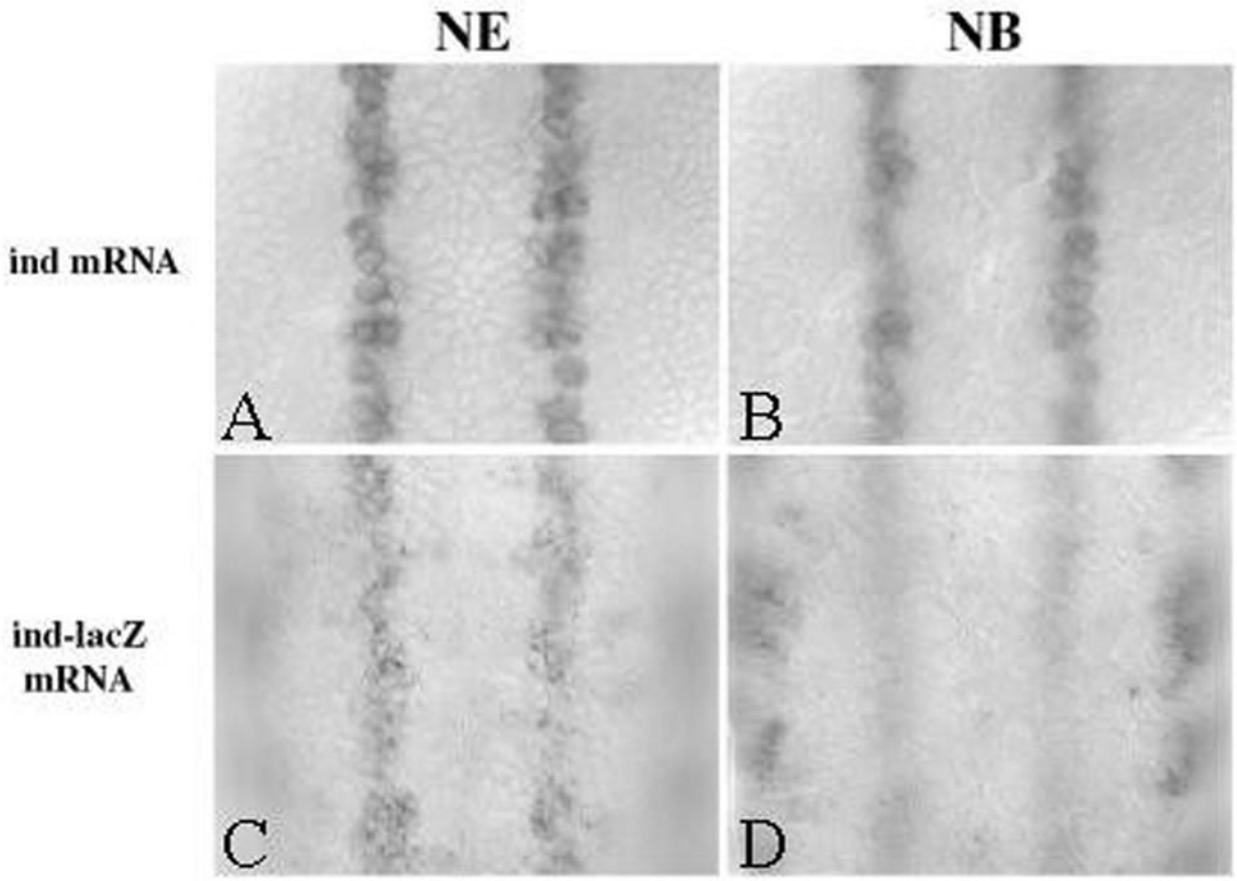

Figure 3.

Enhancer element located downstream of *ind* coding region drives expression in neurectoderm but not neuroblasts. Top panels show expression of endogenous *ind* mRNA in A) neurectoderm and B) neuroblasts. Bottom panels show expression of *ind* enhancer driven *lacZ* expression in C) neuroectoderm, but expression in D) neuroblasts is undetectable.

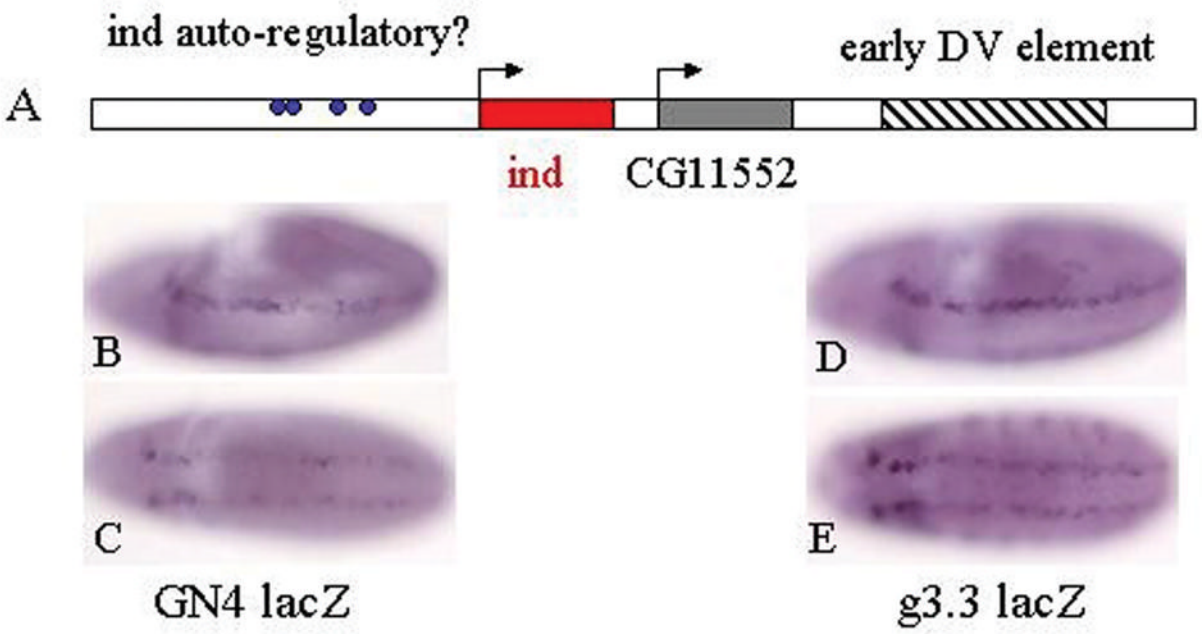

Figure 4.

Identification of a previously uncharacterized Ind regulatory element located upstream of *ind* coding sequences. A) Illustration of genomic region around *ind* gene. The hatched region encompasses the early DV enhancer. The *ind* gene is shown in red and the *CG11552* gene is gray. Blue dots indicate the relative positions of putative Gsh1/Ind binding sites. B) *lacZ* mRNA from GN4 upstream regulatory element in a stage 9 embryo. C) *GN4LacZ* mRNA expression in a stage 10/11 embryo. D) *lacZ* expression from downstream (*g3.3lacZ*) regulatory element in a stage 9 embryo. E) *lacZ* expression from downstream regulatory element in a stage 10/11 embryo.

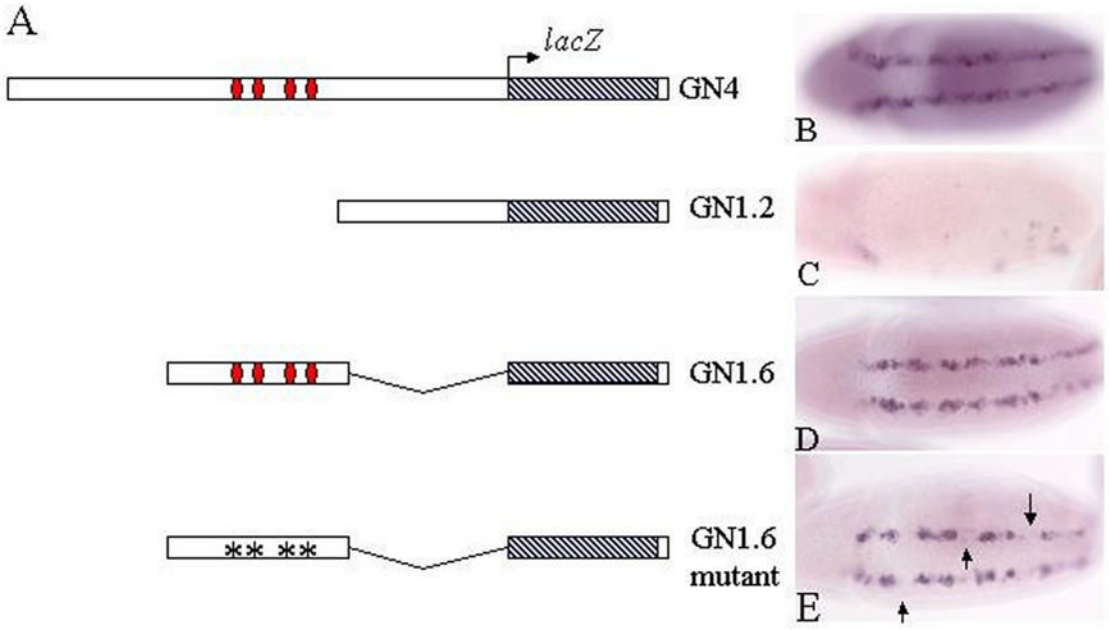

Figure 5.

Deletion analysis of GN4 enhancer reveals regulatory element is contained in 1.6 kb fragment containing potential Ind binding site. A) Schematic of reporter constructs built and tested in embryos. B–E) Expression of *lacZ* mRNA in stage 10 embryos from corresponding reporter constructs to the left of photo. B) *GN4lacZ*. C) *GN1.2 lacZ*. D) *GN1.6 lacZ*. E) Mutant *GN1.6 lacZ*, arrows indicate the presence of gaps in the pattern not observed with the wild type reporter.

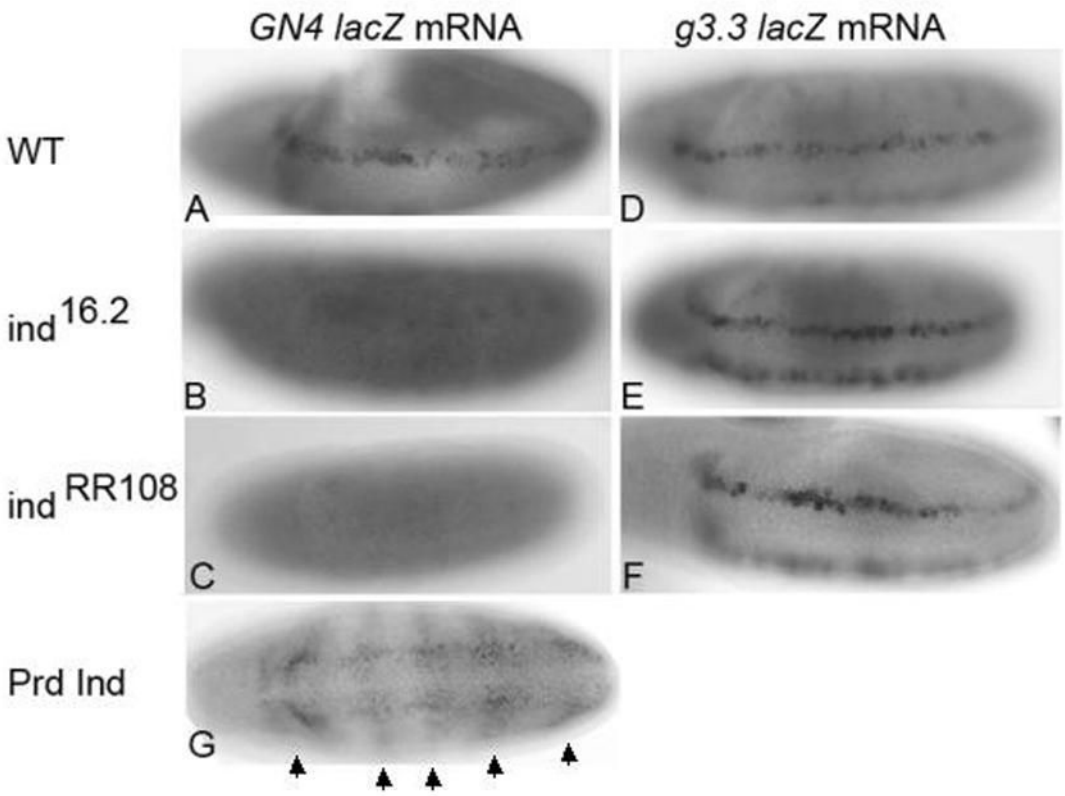

Figure 6.

Ind activity is necessary and sufficient for *GN4lacZ* expression. A) Expression of *GN4lacZ* in WT embryo. B–C) Expression of *GN4lacZ* is absent in *ind* mutant embryos either $ind^{16.2}$ (B) or ind^{RR108} mutants. D–F) Expression from the downstream regulatory element is unaffected in *ind* mutant embryos. G) Prd Gal4 UAS Ind embryo expressing *GN4lacZ* in Prd pattern. Arrows indicate the positions of pair rule-like stripes of *lacZ* expression observed in Prd Gal4 UAS Ind embryos. All embryos are stage 9–10 and anterior is to the left.