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Activation and repression by the C-terminal domain of Dorsal

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SUMMARY

In the *Drosophila* embryo, Dorsal, a maternally expressed Rel family transcription factor, regulates dorsoventral pattern formation by activating and repressing zygotically active fate-determining genes. Dorsal is distributed in a ventral-to-dorsal nuclear concentration gradient in the embryo, the formation of which depends upon the spatially regulated inhibition of Dorsal nuclear uptake by Cactus. Using maternally expressed Gal4/Dorsal fusion proteins, we have explored the mechanism of activation and repression by Dorsal. We find that a fusion protein containing the Gal4 DNA-binding domain fused to full-length Dorsal is distributed in a nuclear concentration gradient that is similar to that of endogenous Dorsal, despite the presence of a constitutively active nuclear localization signal in the Gal4 domain. Whether this fusion protein activates or represses reporter genes depends upon the context of the Gal4-binding sites in the reporter. A Gal4/Dorsal fusion protein lacking the conserved Rel homology domain of Dorsal, but containing the non-conserved C-terminal domain also mediates both activation and repression, depending upon Gal4-binding site context. A region close to the C-terminal end of the C-terminal domain has homology to a repression motif in Engrailed – the eh1 motif. Deletion analysis indicates that this region mediates transcriptional repression and binding to Groucho, a co-repressor known to be required for Dorsal-mediated repression. As has previously been shown for repression by Dorsal, we find that activation by Dorsal, in particular by the C-terminal domain, is modulated by the maternal terminal pattern-forming system.

Key words: Dorsal, Groucho, Activation, Repression, Transcription, *Drosophila*

INTRODUCTION

The dorsoventral (D/V) axis of the *Drosophila* embryo is specified by the Rel family transcription factor Dorsal, a maternal morphogen that directs the spatially restricted transcription of zygotically active D/V patterning genes (Morisato and Anderson, 1995). A signal transduction cascade that is specifically activated on the ventral side of the embryo results in the dissociation of Dorsal from its cytoplasmic inhibitor Cactus, allowing Dorsal nuclear uptake (Belvin and Anderson, 1996). As a result, a nuclear concentration gradient of Dorsal is established in the syncytial blastoderm embryo, with highest concentrations in the ventral nuclei and diminishing concentrations towards the dorsal side of the embryo (Gay and Keith, 1990). Once in the nucleus, Dorsal directs the ventral-specific activation of *twist* (*twi*) and snail (*sna*), and the ventral specific repression of *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*) (Huang et al., 1993; Ip et al., 1991; Ip et al., 1992; Jiang et al., 1991; Pan et al., 1991; Ray et al., 1991).

How does Dorsal both activate and repress transcription in the same cells? Numerous studies have established that the context of the Dorsal-binding sites in the regulatory regions of Dorsal target genes determines the regulatory outcome (Jiang et al., 1992; Pan and Courey, 1992). Genes that are activated by Dorsal contain enhancers termed ventral activation regions (VARs) (Ip et al., 1992; Jiang et al., 1991; Pan et al., 1991). The Dorsal sites in VARs are the only elements within these enhancers that are crucial for activation. In contrast, genes that are repressed by Dorsal contain silencers termed ventral repression regions (VRRs) (Huang et al., 1993; Ip et al., 1991; Kirov et al., 1994). Although the Dorsal sites in these silencers are required they are not sufficient for repression (Huang et al., 1995; Jiang et al., 1993; Kirov et al., 1993). VRRs contain additional elements close to the Dorsal-binding sites that are also required for repression, and mutagenesis of these sites converts the VRR into a VAR.

Activation by Dorsal may depend upon direct interactions with a number of factors. For example, the TBP-associated factors $TAF_{II}60$ and $TAF_{II}110$ have been found to interact with Dorsal and to be required for Dorsal-mediated activation in vitro. In addition, expression of the Dorsal target genes *twi* and *sna* is weakened by a simultaneous reduction in the concentrations of Dorsal and $TAF_{II}60$ or $TAF_{II}110$ in the early embryo (Pham et al., 1999; Zhou et al., 1998). The *Drosophila* CREB-binding protein (dCBP; Nej – FlyBase), a co-activator with histone acetyl transferase activity, has also been implicated in Dorsal-mediated activation (Akimaru et al., 1997a). Distinct domains in Dorsal mediate the interactions with these targets. dCBP binds to the conserved N-terminal Rel

homology domain (RHD) of Dorsal (Akimaru et al., 1997b), a domain that also mediates DNA binding and dimerization. In contrast, $TAF_{II}60$ and $TAF_{II}110$ interact with the non-conserved C-terminal domain (CTD) of Dorsal (Pham et al., 1999). In accordance with these findings, cell transfection assays indicate the presence of activation domains in both the RHD and CTD (Shirokawa and Courey, 1997).

Repression by Dorsal also requires multiple additional factors. As mentioned above, VRRs contain sites in addition to the Dorsal sites that are required for repression. These sites are thought to serve as binding sites for factors that we now term 'assistant repressors'. Biochemical analysis identified the products of the *grainyhead* (*grh*) or *dead ringer* (*dri*) and *cut* (*ct*) genes as factors that may serve as assistant repressors at the *dpp* or *zen* VRRs, respectively (Huang et al., 1995; Valentine et al., 1998). Although eliminating these factors from the early embryo does not result in major defects in the ventral repression of the endogenous Dorsal-target genes, Ct and Dri are nevertheless required for repression by a minimal *zen* VRR. Thus, multiple VRRs that interact with a variety of assistant repressors may control the overall pattern of *zen* and *dpp* transcription.

Dorsal-mediated repression of the endogenous *dpp* and *zen* genes, as well as Dorsal-mediated repression of a *lacZ* reporter under the control of the *zen* VRR is strongly dependent upon the product of the *groucho* (*gro*) gene (Dubnicoff et al., 1997). Gro is a WD repeat-containing protein that acts as a co-repressor in multiple developmental pathways (Chen and Courey, 2000; Fisher and Caudy, 1998; Parkhurst, 1998). Small peptide motifs mediate Gro recruitment to a variety of DNA-bound transcriptional repressors. For example, the homeodomain repressor Engrailed (En) recruits Gro via a ~10 amino acid motif termed the Engrailed homology 1 (eh1) motif (Jiménez et al., 1997; Jiménez et al., 1999; Smith and Jaynes, 1996), while the Hairy and Runt family repressors recruit Gro via C-terminal WRPW/Y motifs (Aronson et al., 1997; Fisher et al., 1996). In contrast to factors like En, Runt and Hairy, no discrete motifs have been identified in Dorsal that mediate Gro recruitment and transcriptional repression. The RHD of Dorsal is sufficient for binding to Gro (Dubnicoff et al., 1997), and analysis of a *dorsal* (*dl*) allele encoding just the RHD shows that this truncated form of Dorsal is able to repress transcription weakly (Isoda et al., 1992). Both Dorsal and the assistant repressor Dri have been found to bind Gro. Conversion of Dorsal from an activator to a repressor by assistant repressors may involve the cooperative recruitment of the Gro co-repressor by DNA-bound Dorsal and nearby DNA-bound assistant repressors, resulting in the formation of a DNA-bound repression complex (Valentine et al., 1998).

Here we show that, in addition to interacting with the Dorsal RHD, Gro also interacts with the CTD. A motif in the CTD with partial homology to the eh1 motif is largely responsible for the interaction between Gro and the CTD. When the CTD is targeted to a modified VRR in the form of a Gal4 fusion protein, it directs transcriptional repression. Deletion of the eh1 motif severely weakens repression by the CTD. We also find that, in addition to repression, the CTD directs activation when targeted to a promoter via the Gal4 DBD. Activation by the Dorsal CTD is down regulated by the *torso* (*tor*) receptor tyrosine kinase, suggesting that the CTD is a direct or indirect target of the terminal pattern-forming system.

MATERIALS AND METHODS

Plasmids

An *hsp83* promoter based P-element expression vector was constructed as follows. A ~1 kb PCR fragment was generated that contains 873 bp of 5′ flanking sequences, the first non-coding exon, and the translational start signal of *hsp83*. The forward primer was AATTGGTACCGGGTCCTAACGGGAACTTGAAGAAGTGC; the reverse primer was AATTGGATCC**CTCGGGCAT**GTTGTATGT-ATGTTTTTCGTTCTATC. Underlined bases indicate restriction enzyme sites; boldface indicates the first three codons of *hsp83*. This was cloned between the *Kpn*I and *Bam*HI sites of PHWZ128 (Liaw et al., 1995), to generate P-*hsp83*. Insertion of the *hsp83* sequences into PHWZ128 in this way removes the *lacZ*-coding sequences and leaves the *hsp70* polyA signal intact. A 900 bp PCR fragment containing the 3′*bcd* UTR was cloned in the *Bam*HI site of P-*hsp83* to generate P-*hsp83*-3′*bcd*UTR.

Plasmids encoding Gal4/Dorsal fusion proteins were made by PCR amplification of *dorsal* sequences followed by cloning between the *Hpa*I and *Xba*I sites of pJL2, which includes sequences encoding amino acids 1-94 of Gal4 (Chasman et al., 1989). The Δeh1 derivative was generated by oligonucleotide directed mutagenesis of the *dorsal* sequences, deleting the sequences encoding Dorsal amino acids 628 to 645. The resulting plasmids were used for bacterial expression of the Gal4/Dorsal variants and as templates to generate PCR fragments for cloning into the P-element expression vectors. PCR fragments encoding Dorsal and Gal4/Dorsal fusion proteins were inserted into the *Bam*HI site of P-*hsp83* or P-*hsp83*-3′*bcd*UTR site using *Bcl*I restriction sites incorporated into the PCR primers. Sequences encoding the nt1 epitope followed by a stop codon were added to the 5′ end of each reverse primer. The nt1 epitope is TSPTKKSAPLRITKPQPTS.

The reporters containing the wild-type VRR were described previously (Huang et al., 1993). The modVRR was created by oligonucleotide-directed mutagenesis of the S3 and S4 Dorsal sites in the wild-type *dpp* VRR. The reporter containing four Dorsal-binding sites upstream of the *hsp70* core promoter was described previously (Pan and Courey, 1992). A line carrying the reporter containing four Gal4-binding sites upstream of the *hsp70* core promoter was obtained from the Bloomington Stock Center.

The plasmid encoding GST/$Dl_{357-678}$ was made by *Eco*RI and *Mfe*I digestion of GST-Dorsal (Dubnicoff et al., 1997) followed by re-ligation. The plasmids encoding GST-CTD and GST-CTD-Δeh1 were made by PCR amplification of the appropriate Dorsal coding sequences followed by insertion into the *Eco*RI site of pGEX-4T1 (Amersham-Pharmacia). All constructs were verified by DNA sequencing.

Protein-protein interaction assays

GST/Dorsal derivatives were produced and purified from *Escherichia coli* BL21 cells as described previously (Dubnicoff et al., 1997). Binding assays were carried out as described previously (Dubnicoff et al., 1997).

Generation of transgenic flies and mutant alleles used

P-elements were introduced into w^{1118} flies as described previously (Rubin and Spradling, 1983). Lines carrying third chromosome insertions of the P-element encoding Dorsal/nt1 were used to produce the following stock: dl^1/SM6; P[Dorsal/nt1]/TM3. dl^1 is a protein null. Homozygous dl^1 females from this stock could then be used to generate embryos that lacked endogenous Dorsal. The *tor* gain-of-function allele used was tor^{4021}, which contains a single histidine to lysine amino acid replacement in the extracellular domain. The loss-of-function allele used was tor^{PM51}, which contains a single alanine to threonine amino acid replacement in the tyrosine kinase domain. Flies carrying a P-element transposon coding for an activated form of *Toll* ($Toll^{10B}$) fused to the *bcd3′*UTR and have been previously described (Huang et al., 1997).

Antibody staining and in situ hybridization

Females carrying a P-element expression vector were crossed with males carrying the appropriate *lacZ* reporter genes. Embryos (0-3 hours) were collected and fixed as described previously and stored in ethanol at –20^{O}C. In situ hybridization was carried out as described previously (Tautz and Pfeifle, 1989) using antisense RNA probes. The expression patterns of the reporters and the maternally expressed fusion proteins were verified using multiple independent transgenic fly lines. Whole-mount antibody staining with α-nt1 monoclonal antibody (Cagan et al., 1992) was carried out using the Vectastain ABC kit (Vector laboratories).

RESULTS

Transcriptional regulation by recombinant Dorsal

For the maternal expression of transgenes encoding Dorsal variants, we used an *hsp83* promoter/enhancer based expression vector (Fig. 1A) similar to one that has been used previously (Govind et al., 1993). The promoter/enhancer region in this vector directs constitutive expression in the female germline, i.e., heat shock is not required to activate the promoter (Xiao and Lis, 1989). To provide a means for monitoring expression, we appended sequences encoding a 19 amino acid epitope (nt1) for which a monoclonal antibody is available (Cagan et al., 1992) to the 3′ end of each transgene. The transgenes were found to be expressed in late oogenesis in the ovaries of transgenic females (data not shown) and the maternal gene products could be detected in the embryos laid by such females (Fig. 2A). As previously reported for wild-type Dorsal (Drier et al., 1999), the recombinant Dorsal/nt1 protein is produced in multiple electrophoretically distinct isoforms that most likely reflect different phosphorylation states (Fig. 2A, lane 2).

We introduced the transgene encoding Dorsal/nt1 into embryos devoid of endogenous Dorsal. Dorsal/nt1 rescues the maternal effect lethality to varying degrees (data not shown) – the extent of rescue is very similar to that which has been previously observed for an *hsp83/dorsal* transgene (Govind et al., 1993) and depends upon the level of Dorsal expression in any given transgenic line. Anti-nt1 staining of embryos laid by mothers carrying the transgene indicates that the recombinant protein is present in a ventral-to-dorsal nuclear concentration gradient (Fig. 3D,L). To test the ability of Dorsal/nt1 to repress transcription, we used a previously characterized reporter (Fig. 1B, VRR/*Kr*/*lacZ*) that contains a VRR from the *dpp* gene upstream of the *Krüppel* (*Kr*) stripe enhancer, which directs *lacZ* expression (Huang et al., 1993). Analysis of the paternally contributed reporter indicates that Dorsal/nt1 is able to repress transcription via the *dpp* VRR (Fig. 3, compare panels B and E). Thus, Dorsal/nt1 is largely able to substitute for endogenous Dorsal.

Transcriptional activation and repression by a Gal4/Dorsal fusion protein

We next tested the possibility of using Gal4 DNA-binding

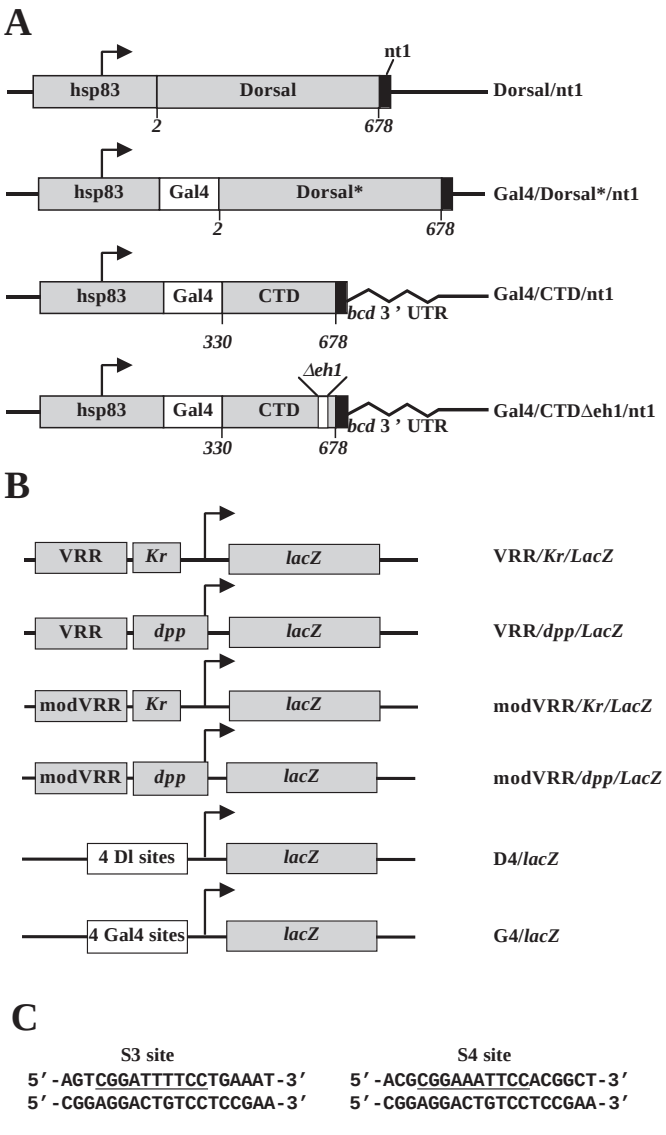

Fig. 1. P-element constructs used in this study. (A) Expression vectors. The names of the encoded proteins are shown to the right of each construct. A 1 kb region from the *hsp83* gene controls the expression of the indicated Dorsal derivatives. Gal4 sequences include amino acids 1-94 (the DNA-binding domain). The numbers indicate the range of Dorsal amino acid residues in each construct. Sequences coding for a 19 amino acid epitope derived from the N-terminal region of the Bride of Sevenless protein were appended to each construct. The asterisk indicates a triple point mutation in the Dorsal RHD that prevents DNA binding. The Gal4/CTD/nt1 and Gal4/CTDΔeh1/nt1-encoding constructs include a 900 bp region from the *bcd* 3′ untranslated region (3′UTR) that directs anterior localization of the mRNA. The transgene encoding Gal4/CTDΔeh1/nt1 contains a deletion that removes sequences encoding Dorsal amino acids 628-645, including the eh1 motif. Diagrams are not to scale. (B) Reporter constructs. An 800 bp VRR from the *dpp* gene was placed upstream of either the *Kr* enhancer or the *dpp* 5′ flanking region and promoter. Conversion of two Dorsal-binding sites within the *dpp* VRR to consensus Gal4-binding sites (see C) results in the modified VRR (modVRR). '4 Dl sites' indicates four tandemly repeated Dorsal binding sites, whereas '4 Gal4 sites' indicates four tandemly repeated Gal4-binding sites. The *Kr* enhancer-containing constructs, the D4/*lacZ* construct and the G4/*lacZ* construct contain core promoter sequences from the *hsp70* gene. Diagrams are not to scale. (C) Sequences of wild-type and mutated S3 and S4 Dorsal-binding sites. The wild-type sites are shown on top (with Dorsal-binding sites underlined), whereas the mutated sites, which constitute consensus Gal4 recognition elements, are shown beneath.

domain (DBD) fusion proteins to map regulatory domains in Dorsal. The advantages of this approach are twofold. First, the use of Gal4 DBD fusion proteins should allow us to examine the function of recombinant proteins in an otherwise wild type (i.e., dl^+) background by using the appropriate reporters. Second, the use of Gal4 fusion proteins should allow us to dissect the Dorsal protein without being concerned about maintaining the Dorsal RHD.

The fusion proteins generated for this analysis contain the Gal4 DBD fused to the N-terminal end of full-length Dorsal (Gal4/Dorsal*/nt1), or two different forms of the Dorsal CTD (Gal4/CTD/nt1 and Gal/CTDΔeh1/nt1) (Fig. 1A). When a Gal4/full-length Dorsal fusion protein was expressed in *E. coli* and tested for DNA binding, it was found to bind with normal specificity and affinity to both Dorsal and Gal4 sites (data not shown). This indicates that the fusion of the Gal4 DBD to the N terminus of Dorsal adjacent to the RHD does not interfere with folding of the RHD. To ensure that the Gal4/Dorsal fusion proteins expressed in embryos would bind only to Gal4 sites and thus be unable to function through any Dorsal sites present in the reporters, we introduced a triple point mutation in the RHD known to abrogate DNA binding (Kumar et al., 1992; Xu and Gélinas, 1997). The asterisk in the name of the Gal4/Dorsal*/nt1 fusion protein indicates the presence of this triple point mutation. Whole-mount antibody staining of embryos expressing Gal4/Dorsal*/nt1 reveals that there is a ventral-to-dorsal concentration gradient of the transgenic protein (Fig. 3G). Given that the Gal4 DBD includes its own nuclear localization signal (NLS), this finding implies that Dorsal may be actively retained in the cytoplasm of the syncytial embryo, and that the function of Cactus is not solely to mask the Dorsal NLS (see Discussion).

To assay activation by Gal4/Dorsal*/nt1, we used a reporter in which *lacZ* is under the control of the *hsp70* core promoter and four upstream Gal4-binding sites (G4/*lacZ*, Fig. 1B). Just as endogenous Dorsal activates transcription of a reporter containing four tandem Dorsal sites upstream of the *hsp70* core promoter (Pan and Courey, 1992), we might expect Gal4/Dorsal*/nt1 to activate G4/*lacZ*. In accordance with this expectation, we detected weak ventral specific *lacZ* expression (Fig. 3H) in embryos containing maternally expressed Gal4/Dorsal*/nt1 and the G4/*lacZ* reporter. For reasons that are not clear, activation is not uniform along the anteroposterior axis and so gaps are often observed in the expression pattern. A similar patchy ventral expression pattern is sometimes detected with a D4/*lacZ* reporter activated by endogenous Dorsal (not shown).

To assay repression by Gal4/Dorsal*/nt1, we used a reporter based on the *dpp* VRR (Fig. 1B). Mutagenesis of the two highest affinity Dorsal-binding sites (the S3 and S4 sites) in the VRR was previously shown to result in a marked decrease in repression activity (Huang et al., 1993). To create reporters that would be responsive to Gal4/Dorsal fusion proteins, we replaced the S3 and S4 Dorsal-binding sites with consensus Gal4-binding sites to create a modified form of the VRR (modVRR) (Fig. 1C). As expected, this modification results in a significant reduction in the ventral repression directed by the VRR. This is seen using the reporter modVRR/*dpp*/*lacZ* in which *lacZ* is under the control of the –980 bp *dpp* 5′ flanking region (Fig. 3, compare J with K) as well as the reporter modVRR/*Kr*/*lacZ* in which *lacZ* is under control of the *Kr* stripe enhancer (Fig. 3, compare C with F). Thus, efficient repression of both *Kr* enhancer-driven and –980 *dpp* promoter-driven transcription is dependent upon the S3 and S4 sites. The residual dorsal/ventral asymmetry of the expression patterns in the presence of the modVRR is due to remaining unmutated Dorsal-binding sites in the modVRR, which are able to interact with the endogenous Dorsal protein in these embryos (Huang et al., 1993).

Embryos bearing maternally expressed Gal4/Dorsal*/nt1 and the modVRR/*Kr*/*lacZ* reporter were generated and subjected to in situ hybridization. Examination of the *lacZ* expression pattern indicates that Gal4/Dorsal*/nt1 can mediate ventral specific repression of the modVRR/*Kr*/*lacZ* reporter (Fig. 3, compare F with I). Thus, just as we observed for activation, the Dorsal RHD need not bind DNA directly for Dorsal to carry out its repression function. Rather the recruitment of Dorsal to the DNA template via a heterologous DNA-binding domain is sufficient.

Groucho interacts with a region in the Dorsal CTD that contains an eh1-like motif

Deletion analysis of Dorsal indicates that the RHD interacts directly with Gro (Dubnicoff et al., 1997). In agreement with these data, previous analysis of mutant Dorsal alleles indicated that the RHD was sufficient for transcriptional repression.

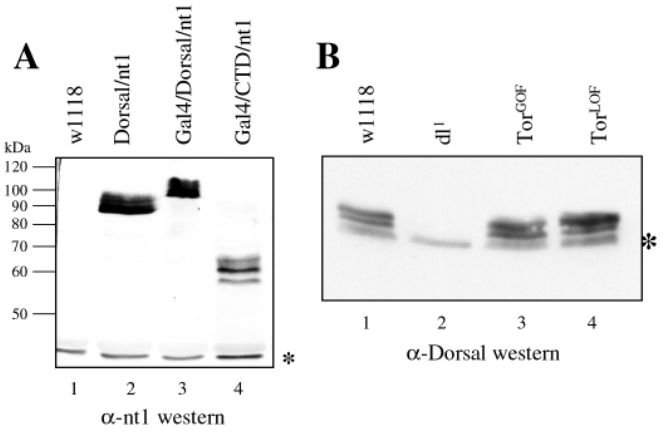

Fig. 2. Immunoblots of embryo lysates. (A) Analysis of recombinant Dorsal expression in *Drosophila* embryos. Approximately 80 0-3-hour-old embryos laid by mothers bearing one of the expression vectors were homogenized in SDS-PAGE loading buffer. The lysate was resolved by 8% SDS-PAGE and then transferred to PVDF membranes, which were probed with the α-nt1 monoclonal antibody. Lane 1, w^{1118} embryos used as a negative control; lane 2, lysate of embryos containing maternally expressed Dorsal/nt1; lane 3, lysate of embryos containing maternally expressed Gal4/Dorsal*/nt1; lane 4, Lysate of embryos containing maternally expressed Gal4/CTD/nt1. The asterisk indicates the position of a cross-reacting protein detected even in embryos not expressing an nt1 fusion protein, which serves as an internal control for loading. (B) Effects of *tor* mutations on Dorsal mobility. Embryo extracts were prepared as in (A) and probed with an α-Dorsal antibody; lane 1, lysate of w^{1118} embryos; lane 2, lysate of embryos laid by females homozygous for a null *dorsal* mutation (dl^1); lane 3, lysate of embryos laid by females heterozygous for a *tor* gain-of-function mutation; lane 4, Lysate of embryos laid by females homozygous for a *tor* loss-of-function mutation. The asterisk indicates the position of a cross-reacting band that is detected even in embryos that lack Dorsal.

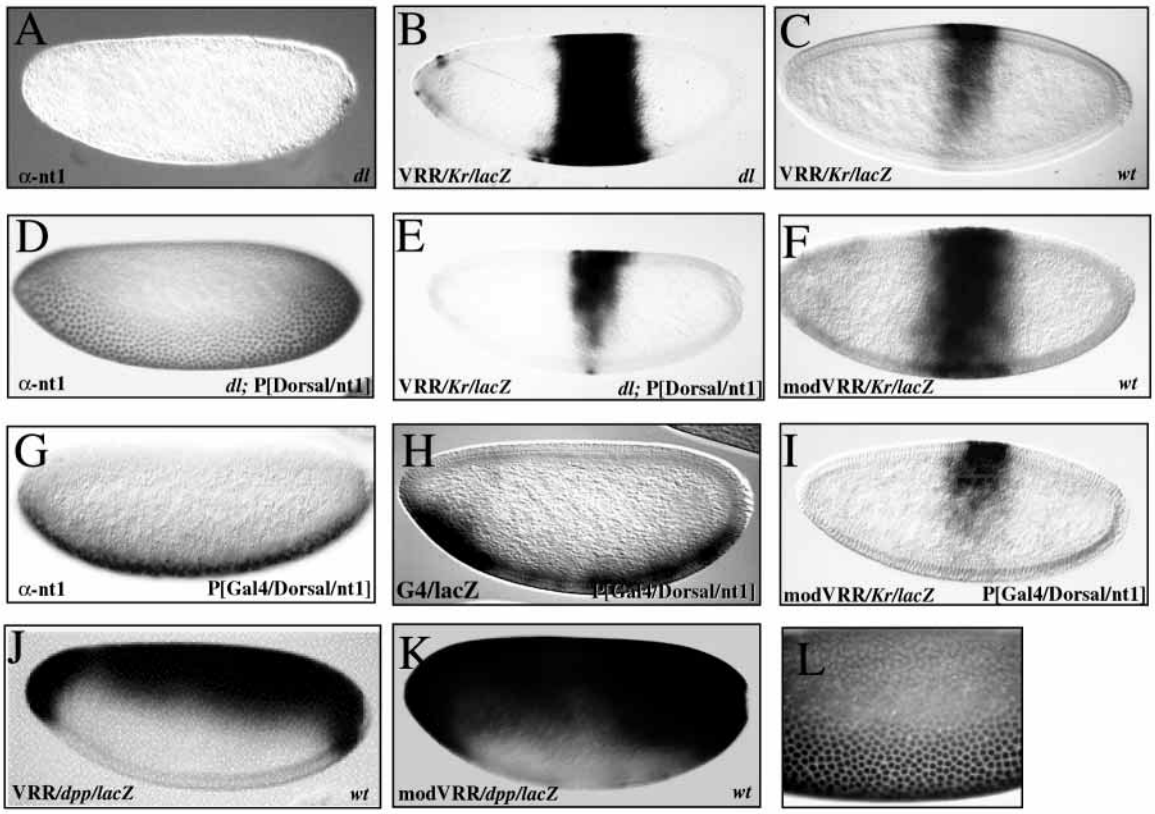

Fig. 3. Transcriptional regulation by Dorsal/nt1 and Gal4/Dorsal*/nt1. (A,D,G,L) α-nt1 whole-mount antibody staining of embryos laid by mothers that lack endogenous Dorsal protein (A), lack endogenous Dorsal and carry one copy of the Dorsal/nt1-encoding transgene (D,L), or carry one copy of the Gal4/Dorsal*/nt1-encoding transgene in an otherwise wild-type background (G).The image shown in L, which is a magnified view of the same embryo shown in G, clearly demonstrates the Dorsal/nt1 nuclear concentration gradient – nuclear staining is observed ventrally, uniform staining is observed laterally, and cytoplasmic staining is observed dorsally. (B,C,E,F,H-K) In situ hybridization with an antisense *lacZ* RNA riboprobe. The maternal genotype is indicated in the bottom right hand corner of each panel. The reporter gene is indicated in the bottom left hand corner of each panel.

However, the repression mediated by the RHD alone is very weak compared to that mediated by full-length Dorsal (Isoda et al., 1992; R. D. F.-S., S. J. and A. J. C., unpublished), implying that regions outside the RHD contribute to transcriptional repression.

To determine if regions outside the RHD can interact with Gro, we performed in vitro protein-protein interaction assays. GST fusion proteins containing full-length Dorsal, the RHD (amino acids 1-379) or the CTD (amino acids 357-678) were immobilized on glutathione beads and then tested for their ability to co-immobilize radiolabeled Gro produced in an in vitro translation system (Fig. 4A). In accordance with previous findings, Gro bound to full-length Dorsal (lane 3) and to the RHD (lane 4). In addition, we found that Gro was able to bind the CTD (lane 5).

Previous sequence analysis (Steward, 1987) has demonstrated extensive homology between En and the CTD of Dorsal, in a region of En that includes eh1, a motif responsible for Gro recruitment (Jimenez et al., 1997). Careful alignment of Dorsal and En sequences reveals a sequence within the Dorsal CTD that has significant homology to the En eh1 motif (Fig. 4B). To determine whether this divergent eh1 motif in Dorsal might contribute to Gro recruitment, we created a derivative of the GST-CTD fusion protein in which the last 40 amino acids of the CTD, including the eh1 motif have been removed. Analysis of the binding of Gro to this derivative indicates that removing the eh1 motif results in an 80% reduction in the affinity of Gro for the Dorsal CTD (Fig. 4C, compare lanes 5 and 6). Similar results were previously observed when the eh1 motif was removed from En (Jimenez et al., 1997).

Transcriptional repression by the eh1 motif

Having shown that the CTD can bind Gro, we were interested in examining the ability of this domain to repress transcription in the embryo. We therefore constructed a P-element vector for the maternal expression of the CTD fused to the Gal4 DBD (Gal4/CTD/nt1) (Fig. 1A). The RHD is essential for the regulated nuclear import of Dorsal (Govind et al., 1996) and, thus, we would not expect Gal4/CTD/nt1 to be localized in a dorsoventral concentration gradient in the embryo. To facilitate the analysis of the transcriptional activity of this fusion protein, we therefore targeted it to the anterior end of the embryo by adding a segment from the 3′ untranslated region (UTR) of the *bicoid* (*bcd*) gene to the 3′ UTR of the transgene. This region of *bcd* is sufficient for anterior mRNA localization (Huang et al., 1997). Whole-mount antibody staining of transgenic embryos shows that when expressed in this way, Gal4/CTD/nt1

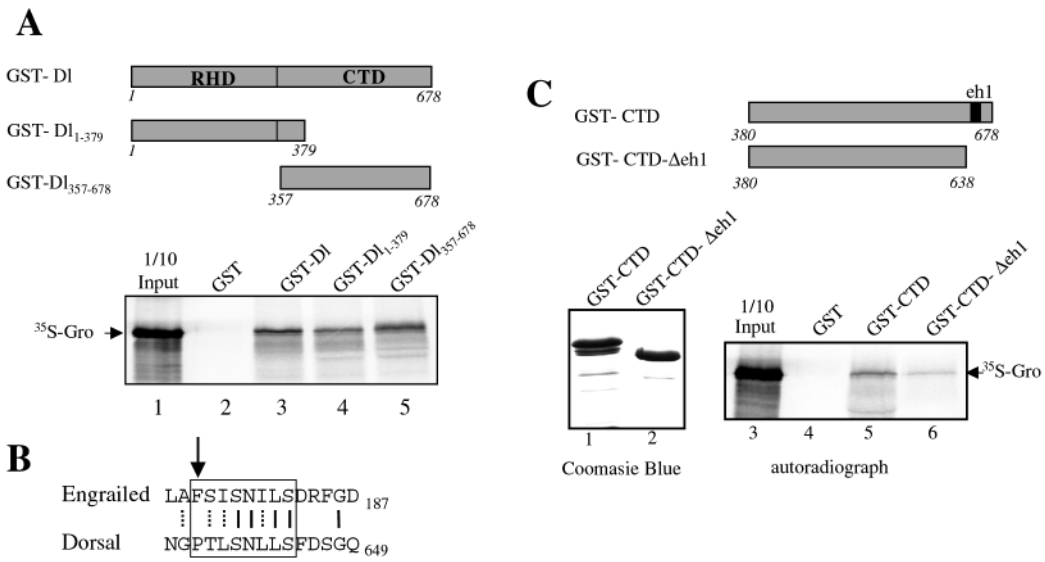

Fig. 4. An eh1-like domain mediates the interaction of Gro with the CTD of Dorsal. (A) Gro interacts with both the RHD and the CTD of Dorsal. The diagrammed regions of Dorsal were expressed with GST fused to the N-terminus. These proteins were immobilized on glutathione beads, which were then incubated with [^{35}S]-labeled Gro. The beads were extensively washed and immobilized proteins were displayed by SDS-PAGE. The resulting gel was visualized by autoradiography. Lane 1: one-tenth of the input Gro protein used for the assays shown in the remaining lanes; lanes 2-5, GST pulldown assays to examine the interaction between Gro and GST (Lane 2), GST-Dl (Lane 3), GST-Dl$_{1-379}$ (Lane 4), or GST-Dl$_{357-678}$ (Lane 5). (B) Sequence alignment of the En eh1 motif and with a similar motif in Dorsal. The box encloses the conserved eh1 core sequence. Unbroken lines represent sequence identity. Broken lines represent conserved substitutions. A phenylalanine residue that is absolutely conserved in En family proteins is indicated with an arrow. (C) The diagrammed regions of Dorsal fused to GST were tested for binding to Gro as in (A). Lanes 1 and 2, Coomassie Blue stained gel showing amounts of GST-CTD (Lane 1) and GST-CTDΔeh1 (Lane 2) fusion proteins used in the assays shown in this panel. Lane 3, one-tenth of the input Gro protein used for the assays shown in the remaining lanes; lanes 4-6, GST pulldown assays to examine the interaction between Gro and GST (Lane 4), GST-CTD (Lane 5) or GST-CTDΔeh1 (Lane 6). Quantitation of the bands in lanes 3-6 reveals the following percentages of the labeled Gro in the bound fractions – 0.083% (lane 4), 0.51% (lane 5) and 0.19% (lane 6).

is present in an anterior-to-posterior gradient in the blastoderm embryo (Fig. 5A). In over-stained embryos, Gal4/CTD/nt1 can be detected along the length of the embryo (not shown). Western blot analysis of embryos expressing Gal4/CTD/nt1 indicates the presence of multiple isoforms of the fusion protein (Fig. 2A), suggesting that the CTD could be a target for post-translational modification in the *Drosophila* embryo.

To test the ability of the CTD to mediate repression, we examined embryos containing maternal Gal4/CTD/nt1 and the reporter containing *lacZ* under the control of the *dpp* 5′ flanking region and the modified *dpp* VRR (Fig. 1B, modVRR/-980*dpp*/*lacZ*). We observed significant anterior repression of the reporter resulting in a gap, observed in about two-thirds of the blastoderm stage embryos, in the expression domain extending from 55% to 80% egg length (Fig. 5B). The failure of this gap to extend to the anterior pole of the embryo is expected, owing to the well-established role of the terminal system in alleviating Dorsal-mediated repression (Rusch and Levine, 1994).

To determine if the eh1 motif is required for repression by the CTD, we generated transgenic flies expressing a variant fusion protein with an internal deletion that removes 18 amino acids, including the eh1-like sequences (Fig. 1A, Gal4/CTDΔeh1/nt1). Western analysis indicates that this deletion variant is equal in stability to the Gal4 fusion protein containing the intact CTD and is also produced as multiple isoforms (data not shown). Whole-mount embryos stained with the α-nt1 antibody show a gradient of Gal4/CTD(Δeh1)/nt1 essentially identical to that observed for the fusion protein containing the intact CTD (Fig. 5, compare A with C). In contrast to embryos containing the intact Gal4/CTD/nt1, embryos containing similar amounts of the deletion variant never exhibit the anterior gap in the expression of the modVRR/*dpp*/*lacZ* reporter gene (Fig. 5, compare B with D), indicating that the eh1 motif makes an important contribution to repression by the CTD.

A Tor-responsive activation domain in the CTD

Previous deletion analysis has suggested that the Dorsal CTD contains one or more activation domains (Isoda et al., 1992; Shirokawa and Courey, 1997). To test this possibility directly, we examined the ability of the Gal4/CTD/nt1 fusion protein to activate the G4/*lacZ* reporter. Embryos containing maternally provided fusion protein and the reporter exhibited specific *lacZ* expression at the anterior end of the embryo, indicating that the CTD can direct activation in the absence of the RHD (Fig. 6). Although Gal4/CTD/nt1 is present in a continuous gradient, we observe sharp borders of reporter gene expression, indicative of a threshold effect in the activation of the reporter. In syncytial blastoderm embryos, expression is absent from the anterior tip of the embryo (Fig. 6A), while this gap disappears after cellularization (Fig. 6B). This suggests that activation may be negatively regulated by the terminal system (see below).

Interestingly, in addition to abolishing CTD-dependent repression, the eh1 deletion in Gal4/CTD(Δeh1)/nt1 also

reduces the levels of activation. The overall extent of activation of the reporter is weaker than that observed with Gal4/CTD/nt1 (Fig. 6C,D) and activation is never established at the anterior tip of the embryo after cellularization (Fig. 6D). Furthermore, for reasons that are not clear, activation is consistently stronger on the dorsal than on the ventral side of the embryo. Thus, in addition to repression, the eh1 motif in Dorsal may be involved in transcriptional activation.

It is well established that repression by Dorsal is blocked by the terminal pattern-forming system. Although Dorsal is present in the nuclei at the poles of the embryo, it is unable to repress transcription at the poles unless the terminal pattern forming system is inactivated (Rusch and Levine, 1994). Our observation that the Gal4/CTD/nt1 fusion is unable to direct activation of the G4/*lacZ* reporter at the anterior tip of the syncytial blastoderm embryo suggests that the activation function of Dorsal may also be under the control of the terminal system. To examine this possibility further, we studied the transcriptional activation of a reporter gene containing four consensus Dorsal-binding sites upstream of the core *hsp70* promoter driving *lacZ* expression (D4/*lacZ*) (Pan and Courey, 1992). Consistent with the notion that activation by Dorsal is negatively modulated by the terminal system, the expression of this reporter is restricted to the ventral-most region of the embryo and is excluded from the poles (Fig. 7A). Deletion analysis of the *twi* gene has defined a promoter proximal and a promoter distal VAR (Jiang et al., 1991; Pan et al., 1991). In agreement with our findings for the D4/*lacZ* reporter, the *twi* proximal VAR, which consists of nothing but a series of Dorsal-binding sites, does not direct activation at the poles (Pan et al., 1991 and data not shown).

We also analyzed the expression pattern of the D4/*lacZ* reporter in embryos that have an ectopic anterior to posterior gradient of the Dorsal protein, owing to the presence of an activated form of Toll protein that has been targeted to the anterior of the embryo using the *bcd* 3′ UTR (Huang et al., 1997). In this case, we observed an additional anterior expression domain of the reporter (Fig. 7B). However, the domain does not extend to the anterior tip of the embryo, once again suggesting that Dorsal is inactivated by the terminal system.

We next examined the expression of the D4/*lacZ* reporter gene in embryos produced by mothers carrying *tor* loss- or gain-of-function mutations. In the absence of *tor* signaling, the D4/*lacZ* reporter was expressed at the poles of the embryo (Fig.7D), indicating that Tor negatively regulates Dorsal-mediated transcriptional activation. In *tor* gain-of-function embryos, we do not detect the expected further retraction of the ventral domain of expression of the reporter from the poles of the embryo. Rather the ventral domain is expanded dorsally (Fig. 7C and data not shown). Although this result seems surprising, it accords with previous observations regarding the role of the terminal system in modulating the function of other activators (Bellaïche et al., 1996; Janody et al., 2000; see Discussion).

To determine if the observed effects on Dorsal activation by the Tor system are mediated by the activation domain in the CTD, we introduced the transgene encoding Gal4/CTD/nt1 into mothers carrying the same gain- and loss-of-function *tor* mutations (Fig. 6E,F). As expected, if Tor inhibits CTD-mediated activation, we do not observe the anterior gap that is present in wild-type embryos. In contrast, in *tor* gain-of-function embryos, the posterior border of activation shifts posteriorly, whereas the anterior gap is still present (Fig. 6G,H).

Western blot analysis of whole embryo extracts from wild-type, or *tor* mutant embryos shows that in a *tor* loss-of-function mutant there is an increase in the lower mobility form of Dorsal relative to the faster mobility forms (Fig. 2B, arrow). As it is believed that the multiple forms of Dorsal are due to phosphorylation, these finding suggest that Dorsal could be a target for post-translational modification by the Tor system.

DISCUSSION

We have found that the Dorsal CTD contains both activation and repression domains. Repression by the CTD is largely dependent upon an eh1-like motif close to the C-terminal end of the region, which apparently functions to recruit the co-repressor Gro. Previous studies have shown that repression by Dorsal is blocked at the poles of the embryo by the action of the terminal pattern-forming system. Our findings demonstrate that activation by Dorsal may also be negatively modulated by the terminal system

Dorsal may be actively retained in the cytoplasm

It is not clear how the interaction between Dorsal and Cactus interferes with Dorsal nuclear uptake. The interaction may mask the Dorsal NLS from the nuclear import machinery. It is also possible that Cactus physically anchors Dorsal in the cytoplasm. Our analysis shows that a Gal4/Dorsal fusion protein is distributed in a ventral-to-dorsal nuclear concentration gradient, indicating that the NLS in the Gal4 DBD is not sufficient to drive constitutive nuclear import of the fusion protein. This result appears to be inconsistent with the idea that Cactus merely masks the Dorsal NLS, and instead, supports the idea that Dorsal is actively retained in the cytoplasm prior to Toll activation. However, our findings are at

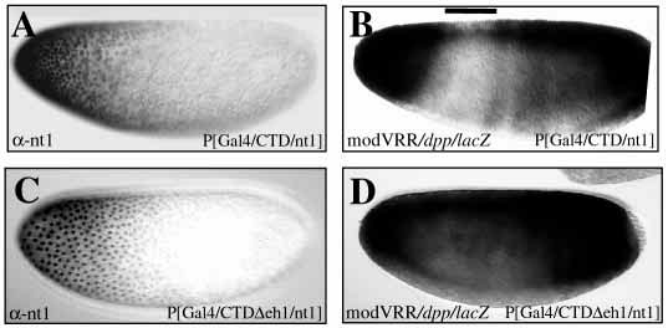

Fig. 5. Transcriptional repression by the CTD is dependent on the eh1 domain. (A,B) Embryos containing maternally expressed Gal4/CTD/nt1. (C,D) Embryos containing maternally expressed Gal4/CTDΔeh1/nt1. All embryos contain the modVRR/*dpp*/*lacZ* reporter. A and C are stained with α-nt1 antibodies. B and D are stained by in situ hybridization with antisense *lacZ* probe. The bar in B indicates the zone of repression that is consistently observed with the Gal4/CTD/nt1 fusion protein, which extends from about 55 to 80% of egg length at the dorsal midline. This zone of repression is absent from embryos containing the Gal4/CTDΔeh1/nt1 fusion protein (D).

odds with previous studies in which the attachment of the T-antigen NLS to NF-κB (the vertebrate homolog of Dorsal) was found to result in the constitutive nuclear uptake of the protein (Beg et al., 1992). This finding, as well as the finding that the binding of NF-κB to I-κB (the vertebrate homolog of Cactus) prevents recognition of the NF-κB NLS by an antibody (Henkel et al., 1992), suggests that I-κB acts by masking the NF-κB NLS. The apparent discrepancy between these earlier results and those reported here could reflect a real difference between the vertebrate and *Drosophila* systems. Alternatively, the observed differences could be due to differences between the Gal4 and T-antigen nuclear localization signals.

Dorsal-mediated activation and repression via Gal4-binding sites

The results presented here show that just as Dorsal sites function in a context-dependent manner in the presence of endogenous Dorsal, so too do Gal4 sites function in a context-dependent manner in the presence of a Gal4/Dorsal fusion protein. When Gal4/Dorsal*/nt1 binds to multiple tandemly repeated Gal4 sites upstream of a core promoter, the result is activation. In contrast, when Gal4/Dorsal*/nt1 binds a modified *dpp* VRR in which two critical Dorsal-binding sites have been replaced by Gal4-binding sites, the result is repression.

Thus, bringing Dorsal to its target sites is sufficient for both activation and repression – the RHD itself need not be directly engaged with the DNA. Similar phenomena have been observed for many regulatory factors, including factors that bind the human β-interferon enhancer. This enhancer contains binding sites for a number of factors, including NF-κB, and functions via the formation of a large cooperatively assembling multiprotein complex (an enhancesome) that includes DNA-binding proteins and co-activators (Carey, 1998; Merika et al., 1998; Thanos and Maniatis, 1995). When an NF-κB site in the β-interferon enhancer was replaced by a Gal4 site, transcriptional activity was restored by a Gal4/NF-κB fusion protein (Merika et al., 1998). By analogy with the β-interferon enhancesome, perhaps Dorsal, other DNA-bound repressors (the assistant repressors) and co-repressors such as Gro cooperatively assemble at the ventral silencer to form a 'silencesome'. As might be expected if silencer function required the assembly of such a complex, silencing by the *zen* VRR is crucially dependent upon the spacing between the sites for the DNA-binding proteins. Changing the spacing by a non-integral multiple of the DNA helical repeat distance severely abrogates silencing, presumably by rotating DNA-bound proteins onto opposite faces of the helix (Cai et al., 1996). Very similar spacing effects have been observed for enhancesomes (Thanos and Maniatis, 1995).

Repression and activation by the Dorsal CTD

Previous analysis revealed that the co-repressor Gro, which is required for Dorsal-mediated repression, interacts with the Dorsal RHD (Dubnicoff et al., 1997). This finding is consistent with the observation that truncated forms of Dorsal consisting of little more than the RHD are able to mediate partial repression of target genes such as *zen* and *dpp* (Isoda et al., 1992; R. D. F.-S., S. J. and A. J. C., unpublished). However, the repression directed by the RHD alone is weak relative to that directed by full-length Dorsal and it is therefore not surprising to discover, as reported here, an additional Gro-interaction domain in Dorsal, this one in the CTD. Although the CTD is not conserved between Rel family proteins, the Dorsal-related immunity factor (Dif) can partially substitute for Dorsal during embryogenesis (Stein et al., 1998). In addition, patterning of the chick limb may involve the regulation by NF-κB of the vertebrate orthologs of Dorsal-target genes (Bushdid et al., 1998; Kanegae et al., 1998). Given these similarities in function, how are we to explain the apparent absence of the eh1-like repression domain from Dorsal-homologues such as Dif and NF-κB? One possibility is that Rel family protein-mediated transcriptional repression is of relatively minor importance to pattern formation. This is possible because other redundant mechanisms involving Short gastrulation (Sog)-family inhibitors exist to ensure that Dpp-orthologs will not be active at inappropriate positions along the dorsal/ventral axis of the metazoan embryo. For example, in the *Drosophila* embryo, Sog is activated ventrally by Dorsal. The Sog protein is then secreted and serves to inhibit Dpp

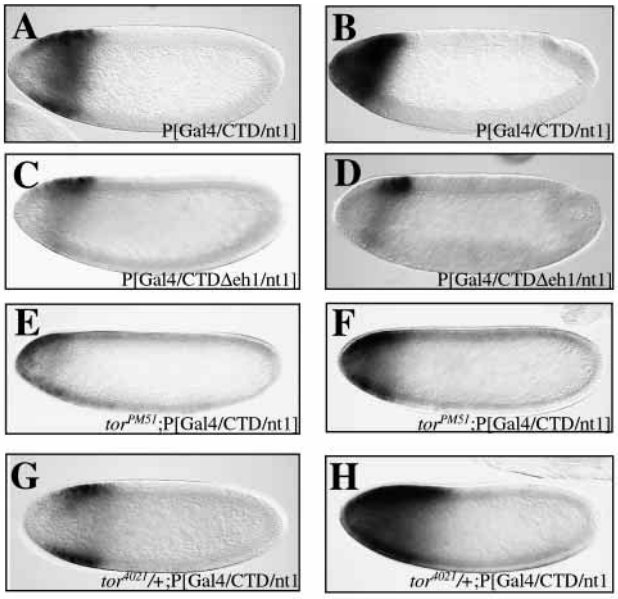

Fig. 6. The Tor RTK regulates transcriptional activation by the C-terminal domain of Dorsal. All embryos contain a copy of the reporter gene containing four Gal4-binding sites and a core hsp70 promoter (G4/*lacZ*), the expression of which was examined by RNA in situ hybridization with a *lacZ* antisense riboprobe. (A,C,E,G) Precellular embryos. (B,D,F,H) Embryos after the beginning of cellularization. (A,B) Embryo containing maternally-encoded Gal4/CTD/nt1. Prior to cellularization, activation is inhibited at the anterior pole of the embryo (A), whereas after cellularization the expression domain extends all the way to the pole (B). (C,D) Same as A and B, except the embryos contain Gal4/CTDΔeh1/nt1. Activation of the reporter never extends to the anterior end of the embryo, and there is a lower overall level of activation. (E,F) Same as A and B, except that the embryos are derived from females homozygous for a *tor* loss of function mutation (tor^{PM51}). Removal of Tor signaling results in the expression of the reporter all the way to the anterior tip of the early embryo and a reduction in the level of expression. (G,H) Same as A and B, except that the embryos are derived from females heterozygous for a *tor* gain-of-function mutation (tor^{4021}). The expression domain extends further towards the posterior than in a wild-type background.

signaling in a ventral-specific manner (Ashe and Levine, 1999; Biehs et al., 1996; Rusch and Levine, 1996). The additional Gro-interacting repression domain in the Dorsal CTD may have arisen relatively recently, perhaps as an evolutionary adaptation to allow more complete or more reliable repression of *dpp* and other genes that interact with *dpp* to pattern the dorsal ectoderm.

Transcriptional repressors that use the Gro co-repressor often recruit Gro using short peptide motifs. A prominent example of such a factor is Engrailed, which recruits Gro through a ~10 amino acid motif known as the eh1 motif. Previous analysis of Dorsal, which suggested that the determinants of Dorsal binding were spread broadly over the RHD (Dubnicoff et al., 1997), indicated that Dorsal might represent an exception to this rule. However, the studies presented here suggest that potent repression by Dorsal does require a region with homology to the eh1 motif. Thus, Engrailed and Dorsal may use a similar interface to recruit Gro. In this respect, it is interesting to note that Engrailed and Dorsal actually have a ~150 amino acid region of similarity, with the eh1 motif at the C-terminal end of this region (Steward, 1987). The similar region contains polyalanine stretches, which is a characteristic associated with other repression domains (Han and Manley, 1993). Perhaps this extended region of similarity plays some role in repression beyond that played by the eh1 motif (e.g. the recruitment of another co-repressor).

While Dorsal can function as either an activator or repressor, Engrailed and all other previously characterized repressors containing eh1 motifs appear to be dedicated repressors (Han and Manley, 1993; Jiménez et al., 1997; Smith and Jaynes, 1996; Tolkunova et al., 1998). It was previously shown that the conserved phenylalanine in the eh1 domain is required for efficient Gro recruitment and transcriptional repression (Jiménez et al., 1999). The absence of this phenylalanine in the Dorsal motif could explain the ability of Dorsal to act as either an activator or a repressor depending upon binding site context. Perhaps this 'defect' in the Dorsal eh1 motif prevents Dorsal from recruiting Gro without help from other nearby DNA-bound repressor proteins (assistant repressors). In this respect, it is very interesting to note that Hairy family proteins, which are dedicated repressors, use a C-terminal WRPW motif to recruit Gro, while Runt family proteins, which can function as both activators and repressors, recruit Gro, at least in part, via a C-terminal WRPY motif (Aronson et al., 1997; Levanon et al., 1998; Westendorf and Hiebert, 1999). Perhaps the conversion of the C-terminal tryptophan to a tyrosine weakens Gro recruitment thereby allowing Runt family proteins to function as either activators or repressors depending upon binding site context.

Consistent with previous experiments showing that the CTD contributes to transcriptional activation in *Drosophila* S2 cells and in vitro (Shirokawa and Courey, 1997), we have found that this domain mediates activation in embryos. Transcriptional activation by the CTD may be mediated by the previously described interactions of this domain with $TAF_{II}110$ and $TAF_{II}60$ (Pham et al., 1999; Zhou et al., 1998).

Interestingly, the deletion that removes the eh1-like motif and prevents repression by the CTD also results in reduced transcriptional activation. There are multiple possible explanations for this observation. Perhaps Gro has some role in activation in addition to repression. This is reminiscent of studies suggesting that Tup1, a possible yeast ortholog of Gro, functions in both activation and repression (Conlan et al., 1999). Alternatively, it is possible that the activation and repression domains in the CTD overlap, but function via completely different co-regulators. If this is true, then one might expect the binding of the co-repressor and the co-activator to be mutually exclusive, thus ensuring that Dorsal cannot function at cross-purposes by simultaneously recruiting a co-activator and a co-repressor.

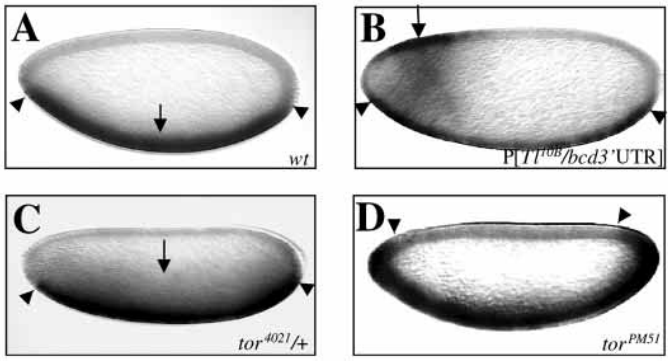

Fig. 7. Tor regulates Dorsal-mediated activation. All embryos bear the reporter gene containing four Dorsal-binding sites and a core hsp70 promoter (D4/*lacZ*), the expression of which was examined by RNA in situ hybridization with a *lacZ* antisense riboprobe. (A) Wild-type embryo demonstrating that expression of the reporter is restricted to the ventral-most region of the embryo (arrow), and it is absent from the poles of the embryo (arrowheads). (B) Embryo containing an ectopic anterior-to-posterior gradient of Dorsal protein, owing to the presence of maternally encoded activated form of the Toll receptor targeted to the anterior with the *bcd* 3′ UTR. An additional anterior stripe of activation is seen (arrow), but expression is still excluded from the poles (arrowheads). (C) Embryo laid by female bearing a *tor* gain-of-function mutation (tor^{4021}). Expression of the reporter is extends further dorsally (arrow) and is still excluded from the poles (arrowheads). (D) Embryo laid by female homozygous for a *tor* loss-of-function mutation (tor^{PM51}). The expression domain now wraps around the poles of the embryo (arrowheads).

Regulation of Dorsal function by the terminal system

When Gal4/CTD is targeted to the anterior end of the embryo, the resulting zone of repression does not include the anterior pole of the embryo. This lack of repression at the terminus of the embryo was expected, as it has been known for several years that the terminal pattern-forming system relieves repression by Dorsal (Rusch and Levine, 1994). A key finding in our understanding of this phenomenon came with the discovery and analysis of *capicua* (*cic*), a gene that encodes an HMG-box family transcription factor (Jiménez et al., 2000). In addition to being required for terminal pattern formation, Cic is also required for efficient Dorsal-mediated repression. Other HMG-box proteins (e.g. Lef1, HMG1 and HMG2) have been found to play architectural roles in enhancesome formation (Carey, 1998). Thus, as an HMG-box protein, perhaps Cic plays an architectural role in silencesome assembly. The finding that Cic appears to be degraded in response to Tor activation suggests that Cic may be a direct target of the terminal pattern forming system (Jiménez et al., 2000).

Previous evidence also hinted at a role of the terminal system in modulating Dorsal-mediated activation. When an artificial anterior-to-posterior gradient of Dorsal is established in the embryo, activation of a reporter gene under the control of the proximal *twi* VAR does not extend to the anterior pole of the embryo (Huang et al., 1997). This effect was attributed to the possible presence of Tor response elements in the *twi* VAR. However, as reported here, we find that even when activation is mediated by nothing but tandem Dorsal sites, this activation is still inhibited at the termini of the embryo by Tor. Likewise, Tor also blocks activation by Gal4/CTD through multiple Gal4 sites. As these artificial reporters are unlikely to contain Tor response elements distinct from the Dorsal or Gal4 sites, it is likely that the Tor pathway interferes directly with Dorsal-mediated activation, either by modifying Dorsal itself or by modifying a co-activator required for Dorsal activity. Consistent with the possibility that Dorsal itself is the direct target of the terminal system, we find that elimination of Tor activity results in an increase in the lower SDS-PAGE mobility form of Dorsal. As phosphorylation usually decreases SDS-PAGE mobility, this finding suggests that Tor activation might result in the dephosphorylation of Dorsal, either by inactivating a Dorsal kinase or by activating a Dorsal phosphatase.

In addition to blocking the activation of Dorsal target genes directly, the terminal system also blocks their activation indirectly, as *huckebein*, a zygotic target of the terminal system, clearly directs *sna* repression at the poles (Goldstein et al., 1999). Thus, there appear to be multiple perhaps partially redundant mechanisms to ensure that mesodermal determinants such as *twi* and *sna* will not be inappropriately expressed at the poles.

The effect of a *tor* gain-of-function mutation on activation by Dorsal and the Gal4/Dorsal fusion is not what would be predicted based upon the simple idea that Tor inhibits Dorsal-mediated activation. Instead of resulting in a further retraction of expression from the pole of the embryo, the gain-of-function mutation causes no obvious change in the size of the anterior gap. In addition, this mutation results in an expansion towards the posterior of Gal4/CTD-driven activation and a broadening in the D4/*lacZ* expression domain. These findings appear to be consistent with a model in which Tor has two completely different effects on Dorsal-mediated activation, inhibiting it at the poles and strengthening it away from the poles. This is precisely what has been observed for the for the interaction between Bcd and the terminal system (Bellaïche et al., 1996; Janody et al., 2000). Thus, the effects of Tor on activation may be very general. How Tor is able to function in these two opposite ways depending upon position in the embryo is not clear.

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