Binding sites for transcription factor NTF-1/Elf-1 contribute to the ventral repression of *decapentaplegic*

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The Dorsal morphogen is a transcription factor that activates some genes and represses others to establish multiple domains of gene expression along the dorsal/ventral axis of the early *Drosophila* embryo. Repression by Dorsal appears to require accessory proteins that bind to corepression elements in Dorsal-dependent regulatory modules called ventral repression regions (VRRs). We have identified a corepression element in *decapentaplegic* (*dpp*), a zygotically active gene that is repressed by the Dorsal morphogen. This *dpp* repression element (DRE) is located within a previously identified VRR and close to essential Dorsal-binding sites. We have purified a factor from *Drosophila* embryo extracts that binds to the DRE but not to mutant forms of the DRE that fail to support efficient repression. This protein also binds to an apparently essential region in a VRR associated with the *zerknüllt* (*zen*) gene. One of the DREs in the *dpp* VRR overlaps the binding site for a potential activator protein suggesting that one mechanism of ventral repression may be the mutually exclusive binding of repressor and activator proteins. We have found the DRE-binding protein to be identical to NTF-1 (equivalent to Elf-1, the product of the *grainyhead* gene), a factor originally identified as an activator of the *Ultrabithorax* and *Dopa decarboxylase* promoters. NTF-1 mRNA is synthesized during oogenesis and deposited in the developing oocyte where it is available to contribute to ventral repression during early embryogenesis. Previous studies have shown that overexpression of NTF-1 in the postblastoderm embryo results in a phenotype that is consistent with a role for this factor in the repression of *dpp* later in embryogenesis.

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Dorsal/ventral pattern formation in the *Drosophila* embryo is mediated by the morphogen encoded by the maternally active *dorsal* (*dl*) gene. Dorsal protein forms a nuclear concentration gradient along the dorsal/ventral axis of the blastoderm embryo (Steward et al. 1988; Roth et al. 1989; Rushlow et al. 1989; Steward 1989). At high concentrations, Dorsal activates *twist* and *snail* in the most ventral region of the embryo, which gives rise to the mesoderm (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991; Ip et al. 1992a). Intermediate concentrations of Dorsal allow the expression of *rhomboid* and *single-minded* in ventrolateral regions, which become neurogenic ectoderm (Ip et al. 1992b; Kasai et al. 1992). Low concentrations of Dorsal (or no Dorsal) result in the expression of *decapentaplegic* (*dpp*), *zerknüllt* (*zen*), and *tolloid* (*tld*) in dorsal and dorsolateral regions, which dif-

ferentiate into amnioserosa and dorsal ectoderm (Ip et al. 1991; Huang et al. 1993; Kirov et al. 1994). Transcriptional activation and repression of the genes mentioned above are mediated by Dorsal-binding sites in their regulatory regions. In particular, the ventral-specific repression of *dpp* is mediated by multiple Dorsal-binding sites in *cis*-regulatory modules that have been termed ventral repression regions (VRRs) (Huang et al. 1993). Mutations in these Dorsal-binding sites result in ventral derepression. The *dpp* VRRs, which are located in the second intron of the gene ~2 kb downstream of the transcriptional start site, are long-range silencers that can repress transcription from a distance.

The effect of Dorsal protein on gene expression depends on the context of the Dorsal-binding sites. Isolated Dorsal-binding sites mediate only weak activation; for Dorsal binding to give rise to high levels of expression, interactions between Dorsal and other activator proteins, such as Zeste and certain basic-helix-loop-helix transcription factors are required (Pan et al. 1991; Jiang and Levine 1993). For transcriptional repression, the cor-

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rect positioning of Dorsal-binding sites relative to the binding sites for other proteins in the VRR is critical. This is apparently because interactions between Dorsal and other DNA-binding proteins are required for repression. Proteins that assist Dorsal in the repression of transcription are termed corepressor proteins and the elements within VRRs to which they bind are termed corepression elements (for review, see Courey and Huang 1995). A VRR in the *zen* gene contains two putative corepression elements termed AT-1 and AT-2. Mutagenesis of AT-1 results in a dramatic reduction in ventral repression, whereas mutagenesis of AT-2 converts the *zen* VRR into a ventral-specific transcriptional enhancer (Jiang et al. 1993; Kirov et al. 1993). In addition, the *zen* VRR contains binding sites for a high mobility group (HMG)-like *Drosophila* factor (DSP1) that blocks Dorsal-mediated activation in yeast cells and converts Dorsal from an activator to a repressor in HeLa cells (Lehming et al. 1994).

In this paper we report the identification of a corepression element in the major *dpp* VRR. This element (the *dpp* repression element or DRE) constitutes a novel corepression element, as it does not bear homology to the previously identified corepression elements in the *zen* VRR. Because a region of the *zen* VRR essential for repression contains a DRE sequence, the DRE may mediate not only *dpp* but also *zen* ventral repression. A DRE-binding protein has been purified from *Drosophila* embryo extracts and found to be identical to the *Drosophila* transcription factor NTF-1 (equivalent to Elf-1), a factor that activates the *Drosophila Ultrabithorax* (*Ubx*) and *Dopa decarboxylase* (*Ddc*) promoters in vitro (Bray et al. 1989; Dynlacht et al. 1989). Overexpression of NTF-1 in the postblastoderm *Drosophila* embryo (Attardi et al. 1993) was shown previously to result in a phenotype that is consistent with a role for NTF-1 in the repression of *dpp* during later embryogenesis.

Results

Identification of *dpp* repression elements

To identify possible DNA sequences serving as binding sites for transcription factors controlling *dpp* expression, we carried out DNase I footprinting and sequence analysis of the region of the *dpp* gene spanning from +1608 to +2148. This region, which lies within the second intron of the gene, was shown previously to contain a VRR as well as a general activation region (Huang et al. 1993). We refer to the VRR in this region as the major *dpp* VRR. This VRR contains two Dorsal-binding sites (dl-A and dl-B) that are critical for the ventral repression of *dpp* (Huang et al. 1993). DNase I footprinting assays show that the major *dpp* VRR is protected extensively by *Drosophila* embryo nuclear extracts (Fig. 1A,B).

The region was also searched for previously identified transcription factor-binding sites, matches for the *zen* AT-rich corepression elements (Jiang et al. 1993; Kirov et al. 1993), as well as for repetitive DNA sequences (Fig. 1B). The rationale behind searching for repetitive sequences is that regulatory proteins frequently interact

with repeated elements in an important regulatory region. Other than the Dorsal-binding sites, no significant matches to binding sites for previously known factors were revealed by this search. Matches for the AT-rich sites were found only when two mismatches were allowed. However, several repetitive sequences were identified. Two repeated TACCTGC elements were of particular interest, (1) because they coincide with regions that were protected from DNase I digestion by nuclear extracts, and (2) because they are each within 50 bp of one of the two critical Dorsal-binding sites (dl-A and dl-B). For reasons that will be made apparent below, we refer to these two elements as *dpp* repression element A (DRE-A) and *dpp* repression element B (DRE-B).

To test the role of DRE-A and DRE-B in ventral repression, clustered point mutations were introduced into these elements and the effects of these mutations were assayed by germ-line transformation. Specifically, four substitutions were introduced into each of the two TACCTGC elements by site-directed mutagenesis of a fragment of *dpp* extending from +1608 to +2400, which includes the major *dpp* VRR. The mutated DNA was linked to the *lacZ* reporter gene under control of the *dpp* promoter and introduced into *Drosophila* by P-element-mediated germ-line transformation. In this experiment, and in all subsequent experiments involving P-element-mediated transformation, the results reported for each construct were reproducibly observed in multiple independent transformant lines.

The expression pattern of the *lacZ* reporter gene, as revealed by mRNA in situ hybridization, demonstrates that simultaneously mutating DRE-A and DRE-B results in partial ventral derepression (Fig. 2). Embryos containing the wild-type DREs always exhibit completely dorsal-specific *lacZ* expression with a relatively sharp border between the expressing and nonexpressing domains (Fig. 2A,C). Mutation of the DREs results in embryos exhibiting a gradient of *lacZ* expression that extends all the way to the ventral midline (Fig. 2B). Thus, one or both DREs function as corepression elements. Once cellularization begins (nuclear cycle 14), ventral repression is reestablished in embryos containing mutant DREs. In the late cellular blastoderm embryo, there is no difference between embryos bearing wild-type and mutant DREs, suggesting that additional unidentified corepression elements cooperate with Dorsal-binding sites to bring about complete ventral repression at this stage (data not shown). Although the DRE mutant embryos exhibit ventral expression in the syncytial blastoderm embryo, mutagenesis of other footprinted elements within the VRR outside the DREs and Dorsal-binding sites had very little effect on the pattern of reporter gene expression (Fig. 2C; data not shown).

Purification of a DRE-binding protein

To further investigate the mechanism of *dpp* ventral repression, we purified a DRE-binding protein based on its sequence-specific interaction with DRE-B. As noted above (see Fig. 1), 0- to 12-hr *Drosophila* embryo extracts

Figure 1. Analysis of the major *dpp* VRR reveals multiple potential regulatory sites. (A) DNase I footprinting with 0- to 12-hr *Drosophila* embryo nuclear extracts. Footprinting probes were 5' end-labeled on the coding strand at position +1605 (left) or at position +1835 (right). The coordinates shown to the left of each panel are in base pairs and are relative to the transcriptional start site at +1. (Lanes 1) G+A chemical sequencing ladder; (lanes 2) G chemical sequencing ladder; (lanes 3,8) no protein controls; (lanes 4,5) 4 μ l of nuclear extract; (lanes 6,7) 8 μ l of nuclear extract. (B) Analysis of the *dpp* VRR sequence. Shading indicates regions protected from DNase I digestion by 0- to 12-hr nuclear extracts. Two Dorsal binding sites (dl-A and dl-B, enclosed in rectangles) were shown previously to be required for ventral repression (Huang et al. 1993). Each of these Dorsal-binding sites falls within 50 bp of a repeated TACCTGC element (DRE-A and DRE-B, enclosed in ovals). In addition, a third repeat of this element, whose function has not been analyzed, is found farther downstream (DRE-C) on the noncoding strand. A short palindromic sequence consisting of two inverted repeats of the sequence TAAAAGGAC (PLS, indicated by two converging arrows) is found adjacent to DRE-B.

protect the DREs in the *dpp* VRR from DNase I digestion. In our initial efforts to purify the activity responsible for this footprint, 0- to 12-hr *Drosophila* embryo nuclear extracts were subjected to heparin-Sepharose chromatography. Footprinting activities protecting DRE-B eluted at 0.3–0.35 M KCl. However, in addition to protecting wild-type DRE-B, this activity also protected probes containing a mutated form of DRE-B that fails to support efficient repression (Fig. 3A). Thus, a specific DRE-B-binding activity is undetectable in the heparin-Sepharose fractions.

An explanation for this lack of DRE specificity is provided by the results obtained when the nuclear extract is applied to SP-Sepharose and eluted with a series of salt steps. DNase I footprint analysis of the fractions from this column reveal two chromatographically distinct activities covering DRE-B, one eluting at 0.21 M KCl and the other eluting at 0.3 M KCl (Fig. 3B). The weak foot-

print found in the 0.21 M KCl fractions is centered well around DRE-B, whereas the stronger footprinting activity in the 0.3 M salt fractions includes the DRE but is centered over a palindromic element (PLS) adjacent to the DRE. It is this PLS-binding activity that appears to be primarily responsible for the footprints observed in the heparin-Sepharose fractions.

To explore the possibility that the weak footprinting activity eluting from the SP-Sepharose column at 0.21 M salt was DRE-specific, this activity was further purified and concentrated by DEAE-Sephacel chromatography and then footprinted using probes containing wild-type and mutant versions of DRE-A. As shown in Figure 3C, the activity binds to a probe containing the wild-type DRE but not to a probe containing the same quadruple point mutation that resulted in ventral derepression. Similar results are obtained with wild-type and mutant versions of DRE-B (see below).

Figure 2. DRE-A and DRE-B are required for efficient ventral repression in the blastoderm embryo. Transgenic embryos bearing the *lacZ*-coding region under the control of the *dpp* promoter and 5'-flanking region plus wild-type and mutant versions of the major *dpp* VRR were collected and stained to reveal the pattern of *lacZ* mRNA expression. *lacZ* mRNA was detected by whole-mount in situ hybridization with digoxigenin-labeled RNA probes. The embryos are shown in sagittal view and are oriented so that dorsal is up and anterior is to the left. (A) The expression pattern directed by the wild-type VRR. (B) The expression pattern directed by a VRR in which DRE-A and DRE-B have been mutagenized as shown. (C) The expression pattern directed by a mutant *dpp* VRR in which footprinted elements adjacent to each of the DREs have been mutagenized. In A and C, expression is specific for the dorsal side of the embryo; in B ventral derepression attributable to the inactivation of DRE-A and DRE-B results in a gradient of expression that extends to the ventral midline.

Further purification of the DRE-binding protein was achieved by DNA affinity chromatography and velocity sedimentation through a glycerol gradient (Fig. 4A). The footprinting activity was found to copurify with three polypeptide species of ~130, 120, and 77 kD in molecular mass (Fig. 4B). The finding that these three species cosediment with one another in the absence of DNA indicates that they most likely form a complex in solution.

We also carried out UV cross-linking studies utilizing a ^{32}P -labeled oligonucleotide probe in which the 5-methyl groups on thymine residues were replaced with iodine atoms. The iodine atoms render the probe extremely photoreactive without significantly altering the affinity of the probe for the DRE-binding protein as assessed by electrophoretic gel mobility shift assays (data not shown). DRE-binding protein purified as shown in Figure 4, A and B, was incubated with this probe and irradiated with UV light. The reaction products were then analyzed by SDS-PAGE and autoradiography. All three species became covalently attached to the radioactive probe (Fig. 4C), giving rise to three species with ap-

parent molecular masses of ~140, 130, and 90 kD. The increased apparent molecular mass after cross-linking is attributable to the mass of the covalently attached probe. The signal was competed away by the cold DRE-containing oligonucleotide, and ~10- to 20-fold less efficiently by an oligonucleotide containing a mutated (nonfunctional) DRE. Additional radiolabeled species visible in the autoradiograph of the cross-linking gel may be attributable to occasional multiple cross-linking events, photolysis of the protein, or to the probe cross-linking to itself (see legend to Fig. 4). These UV cross-linking results indicate that all three polypeptide species of the DRE-binding protein are in contact with the DRE.

As noted above, SP-Sepharose chromatography separates an activity that protects a palindromic sequence (the PLS, +1907 to +1930) adjacent to DRE-B from the DRE-binding protein (Fig. 3B). To explore the possibility of an interaction between these two factors, footprinting reactions were carried out in which increasing amounts of the DRE-binding protein were mixed with the SP-Sepharose fraction containing the PLS-binding activity (Fig. 5A). The probe was the region from the *dpp* VRR containing DRE-B and the adjacent PLS. The results show that the binding of the two proteins is mutually exclusive. In the presence of the PLS-binding protein, higher concentrations of DRE-binding protein are required before a footprint is detected over DRE-B (cf. lanes 5–8 with lanes 9–12). Furthermore, at concentrations of DRE-binding protein sufficient to saturate DRE-B (lane 12), the PLS-binding protein is unable to occupy the adjacent element.

The factor that binds to the PLS may function as a transcriptional activator. This is demonstrated by an experiment in which four copies of the isolated palindromic element were inserted upstream of the *dpp* promoter and 5'-flanking region fused to the *lacZ* reporter gene. The *dpp* 5'-flanking region by itself directs little or no expression in the blastoderm embryo (Fig. 5B). However, addition of the palindromic element results in near uniform expression throughout the early embryo (Fig. 5C). The pattern includes almost the entire embryo, although there is usually a gap in the expression at the posterior end. This expression is first apparent in some embryos at nuclear cycle 13 and is always observed by nuclear cycle 14. The expression persists throughout gastrulation and germ band elongation. Thus, a short-range repression mechanism may contribute to ventral repression, i.e., the DRE-binding protein may reduce the level of transcription of *dpp* by preventing the binding of an activator protein.

A single point mutation that reduces the affinity of DRE-B for the DRE-binding protein results in ventral derepression

To further analyze the DNA-binding specificity of the DRE-binding protein, we generated a series of footprinting probes containing single and double mutations in DRE-B. The mutations affected DNA-binding activity to varying extents (Fig. 6A). Two of the three double muta-

Figure 3. Nuclear extracts contain a DNA-binding activity specific for the DRE, as well as an activity that binds to the palindromic sequence adjacent to DRE-B. (A) Nuclear extracts (0- to 12-hr) were applied to heparin-Sepharose column at 0.1 M KCl. The column was eluted with the indicated concentrations of KCl. The protein peaks observed at each salt step were pooled and assayed for DNase I footprinting activity using probes bearing either the wild-type DRE-B or a mutant DRE-B that fails to support ventral repression in *Drosophila* embryos (Fig. 2). The binding activity detected in the 0.3 and 0.35 M KCl steps binds equally well to the wild-type and mutant probes. (Lanes 1,5,6,10) No protein controls. (B) Nuclear extracts (0- to 12-hr) were applied to an SP-Sepharose column at 0.1 M KCl. The column was eluted with the indicated concentrations of KCl, and fractions were assayed for DNase I footprinting activity using a probe bearing the wild-type DRE-B. (Lane 1) No protein control. (Lane 2) Starting material. An activity generating a footprint centered over DRE-B elutes at 0.21 M KCl (lanes 3,4), whereas an activity generating a footprint centered over the adjacent PLS elutes at 0.3 M KCl (lanes 7,8). The 0.21 M salt step was diluted to 0.05 M KCl and fractionated on a DEAE-Sepharose column. The concentrated DRE-binding protein elutes in a 0.15 M salt step (lanes 9-12). The DRE-specific footprinting activity is very weak in the SP-Sepharose fractions (lanes 3,4) but is readily apparent after concentration on DEAE-Sepharose as shown in lanes 9-12. See Materials and methods for further details of the purification. (C) DEAE-Sepharose-purified DRE-binding activity was assayed using footprinting probes containing either a wild-type or mutant DRE-A. The activity binds to the wild-type probe (lanes 1,2), but not to the mutant probe (lanes 4,5). (Lanes 3,6) No protein controls. Similar results are observed with wild-type and mutant versions of DRE-B (Fig. 6; data not shown).

tions tested (lanes 7-15) had dramatic effects on DNA binding. Of the four single mutations (lanes 16-27), three had very minor effects on DNA binding. However, a single point mutation that altered G6 to C dramatically reduced binding (lanes 25-27). Thus, we decided to engineer this point mutation into the *dpp* VRR to test its effects on ventral repression. Specifically, we generated two VRR variants: one containing a quadruple point mutation in DRE-A and a wild-type DRE-B, and the other containing the quadruple point mutation in DRE-A and the single G6 to C point mutation in DRE-B. Both constructs were linked to the *lacZ* reporter gene under control of the *dpp* 5'-promoter region and introduced into the *Drosophila* germ-line by P-element mediated transformation. In situ hybridization reveals that mutagenesis of DRE-A alone (Fig. 6C) has very little effect on the ventral expression of the reporter gene relative to the wild-type VRR (Fig. 6B). However, the addition of the single G6 to C point mutation in DRE-B (Fig. 6D-G)

resulted in partial ventral derepression. Although the derepression is somewhat less dramatic than that observed in embryos containing quadruple point mutations in both DREs (see Fig. 2B), the embryos still exhibit a gradient of expression that extends to the ventral midline of the syncytial blastoderm embryo. Thus, a single point mutation that strongly reduces binding of the DRE-binding protein to DRE-B results in a reduction in Dorsal-mediated ventral repression.

The DRE-binding protein binds a region of the zen VRR essential for repression

We suspect that the DRE-binding protein may also be required for the ventral repression directed by the *zen* VRR. Evidence for this conclusion comes from studies examining the regulatory sequences required for the activity of the *zen* VRR (Jiang et al. 1993; Kirov et al. 1993). These studies focused on the importance of the AT-rich

Figure 4. Purified DRE-binding protein consists of three polypeptides with apparent molecular masses of 130, 120, and 77 kD. (A) DRE-binding protein purification strategy (details are given in Materials and methods). (B) Velocity sedimentation of DRE-binding activity through a 10%–20% glycerol gradient. The indicated gradient fractions were analyzed by DNase I footprinting (*top*) using a DRE-B bearing probe and by SDS-PAGE (*bottom*). The footprinting activity cosediments with three polypeptides with apparent molecular masses of 130, 120, and 77 kD. (C) UV cross-linking analysis. A radiolabeled double-stranded DNA probe with iodine atoms substituted for the 5-methyl group on each thymine residue was incubated with purified DRE-binding protein in the presence of various competitors and irradiated with UV light. Products were subjected to SDS-PAGE and visualized by autoradiography. (Lane 1) Unirradiated control; (lane 2) no competitor; (lane 3) 10-fold molar excess of wild-type competitor *gs-1*; (lane 4) 100-fold molar excess of wild-type competitor *gs-1*; (lane 5) 10-fold molar excess of mutant competitor *gs-2*; (lane 6) 100-fold molar excess of mutant competitor *gs-2*. The arrows point to the three species thought to result from the covalent attachment of the three predominant polypeptides in the DRE-binding protein preparation (Fig. 4B) to the probe. Cross-linking to the probe increases the apparent molecular mass of each polypeptide by ~10 kD. The low molecular mass smear near the bottom of the gel, which is not subject to competition (and which is observed in the absence of DRE-binding protein; data not shown), probably results from the cross-linking of the probe to itself. The high molecular mass species near the top of the gel may result from the occasional cross-linking of one strand of probe to two polypeptide chains of the DRE-binding protein. Other faint bands may result from photolysis of the protein.

corepression elements in the *zen* VRR. Taken together, however, they also show that a region of the *zen* VRR containing an element with homology to the DRE is important for ventral repression. Specifically, Kirov et al. (1993) found that a 55-bp region from the *zen* VRR containing an AT-rich site and a Dorsal-binding site mediates ventral repression, whereas Jiang et al. (1993) found that a 37-bp region containing the same two elements failed to repress transcription (Fig. 7A). Our examination of the sequences present in the 55-bp fragment, but absent from the 37-bp fragment, reveals an element with homology to the *dpp* DREs (Fig. 7B). Purified DRE-binding protein binds to the DRE homologous element as shown by footprinting assays (Fig. 7C).

The DRE-binding protein is NTF-1

Shortly after purification of the DRE-binding protein to near homogeneity, we noticed that the molecular sizes of the polypeptides in our purified preparation were very similar to those of the polypeptides in purified NTF-1 (Dynlacht et al. 1989), equivalent to Elf-1, (Bray et al. 1989), the product of the *grainyhead* (*grh*) gene (Bray and Kafatos 1991). In addition, the DRE exhibits some sequence similarity to previously described consensus NTF-1-binding sites (Liaw et al., this issue). To determine whether the DRE-binding protein is NTF-1, we carried out DNase I footprinting assays using a probe from the *Ubx* proximal-promoter region containing NTF-1

Figure 5. Mutually exclusive-binding of the DRE-binding protein and a potential activator protein that binds to the PLS may contribute to ventral repression. (A) DNase I footprint analysis of the interactions between DRE-binding protein and a PLS-binding protein. The source of PLS-binding activity is a 0.3 M salt cut from an SP-Sepharose column similar to that shown in Fig. 3B, lanes 7 and 8. (Lanes 1,2) No protein controls. The remaining samples contained 1 μ l (lanes 3,4,9–12) of PLS-binding activity and 2 μ l (lanes 5,9), 5 μ l (lanes 6,10), 10 μ l (lanes 7,11), or 24 μ l (lanes 8,12) of DRE-binding protein. (B,C) Transgenic embryos bearing the *lacZ*-coding region under the control of the *dpp* promoter and 5'-flanking region alone (B) or the *dpp* promoter and 5'-flanking region plus four copies of an oligonucleotide containing the PLS (C) were collected and stained to reveal the pattern of *lacZ* mRNA expression. *lacZ* mRNA was detected by whole-mount in situ hybridization with digoxigenin-labeled RNA probes. The embryos are shown in sagittal view and are oriented so that dorsal is up and anterior is to the left.

binding sites. These experiments show that our purified DRE-binding protein binds specifically to the *Ubx* proximal-promoter generating footprints indistinguishable

from those published for NTF-1 [Fig. 8A, cf. Fig. 1 of Dynlacht et al. (1989)]. In addition, recombinant NTF-1 protein generates a footprint over DRE-B essentially indistinguishable from that generated by DRE-binding protein (Fig. 8B). Finally, antibodies raised against recombinant NTF-1 react with all three polypeptides in our NTF-1 preparation (Fig. 8C). In addition to verifying that the DRE-binding protein is NTF-1, the results of the immunoblot strongly suggest that the three polypeptide species that copurify as an apparent complex are all the products of a single gene. Their different apparent molecular masses may be attributable to posttranslational modification and/or proteolysis.

Maternal expression of NTF-1

Because *dpp* is one of the earliest genes to be expressed after fertilization (St. Johnston and Gelbart 1987), we would expect genes encoding potential regulators of *dpp* to be expressed maternally. As the maternal expression of *grh* (the gene encoding NTF-1) has not been reported previously, we examined egg chambers by in situ hybridization using a *grh* probe. By stage 6 of oogenesis, expression is clearly observed in the maternal germ line (Fig. 9A). Transcripts are most concentrated in the cytoplasm immediately surrounding the nucleus of the developing oocyte and are also observed in the nurse cells. By stage 10, expression in the nurse cells has increased. The apparent decrease in the amount of transcript in the oocyte may simply reflect its dilution by yolk. At this stage, the nurse cell transcripts appear to be streaming into the oocyte (Fig. 9B).

In addition to being maternally expressed, *grh* is expressed in the blastoderm embryo. Zygotic transcripts are first visible at nuclear cycle 13 when they are faintly detected throughout the embryo (Fig. 9C). By nuclear cycle 14, *grh* expression intensifies and the pattern is refined in an unexpected way: The transcript is now excluded from a longitudinal domain along the ventral midline that appears to coincide with the mesodermal anlage (Fig. 9D,E). The expression patterns observed during later embryogenesis (data not shown) are in agreement with those published previously (Dynlacht et al. 1989).

Discussion

Multiple elements contribute to ventral repression

A variety of elements that contribute to ventral repression in the *Drosophila* embryo have been identified. These include Dorsal-binding sites (Huang et al. 1993; Ip et al. 1991; Kirov et al. 1994), the DRE, which we have shown to contribute to *dpp* repression and possibly *zen* repression, and AT-rich sequences (i.e., AT-1 and AT-2), which others have shown to contribute to *zen* repression (Jiang et al. 1993; Kirov et al. 1993). Although AT-1 and AT-2 may be two copies of the same element, we believe it is more likely that they are distinct elements, as the individual mutagenesis of these sites results in different

Figure 6. A single point mutation that blocks-binding activity also interferes with ventral repression. (A) DNase I footprinting assays on wild-type and mutant versions of DRE-B. DNase I footprinting assays were carried out to test the-binding of DRE-binding protein to a variety of DRE-B mutants. (Lanes 1–3) Wild-type DRE; (lanes 4–6) the indicated quadruple mutant DRE; (lanes 7–15) the indicated double-mutant DREs; (lanes 16–27) the indicated single-mutant DREs. (–) No protein controls; (+) each contains equal amounts of DNA-affinity purified DRE-binding protein. (B–G) Transgenic embryos bearing the *lacZ*-coding region under the control of the *dpp* promoter and 5'-flanking region plus wild-type and mutant versions of the major *dpp* VRR were collected and stained to reveal the pattern of *lacZ* mRNA expression. *lacZ* mRNA was detected by whole-mount in situ hybridization with digoxigenin-labeled RNA probes. The embryos are shown in sagittal view and are oriented so that dorsal is up and anterior is to the left. (B) The expression pattern directed by the wild-type major *dpp* VRR. (C) The expression pattern directed by a mutant *dpp* VRR containing a quadruple point mutation in DRE-A. (D–G) The expression pattern directed by a mutant *dpp* VRR containing a quadruple point mutation in DRE-A and a single point mutation in DRE-B. These four embryos are representative of the derepression observed prior to cellularization. Once cellularization begins, complete ventral repression is reestablished.

phenotypes (Jiang et al. 1993) and as they interact with different factors present in crude embryo extracts (Jiang et al. 1993; S.A. Valentine, unpubl.). In addition, a yeast assay system has been used to identify an HMG-like protein that binds to distinct sites in the *zen* VRR and converts Dorsal from an activator into a repressor in HeLa cells (Lehming et al. 1994).

We suspect that multiple corepressors, working through multiple corepression elements, are required to ensure full ventral repression in the early embryo. In accord with this idea, mutations in the DREs reduce but do not eliminate ventral repression, suggesting that the *dpp* VRR contains additional corepression elements. In addition, while mutations in the DREs result in partial

Figure 7. An element in the *zen* VRR that may be important for repression interacts with the DRE-binding protein. (A) Previous findings from other laboratories show that a 55-bp fragment from the *zen* VRR containing an element with homology to the DRE gives ventral repression (Kirov et al. 1993), whereas deletion of this DRE homologous element results in the loss of ventral repression (Jiang et al. 1993). (B) Alignment of two of the *dpp* DREs with the DRE in the essential region of the *zen* VRR. The DRE in *zen* overlaps a palindromic element. As a result, a portion of the DRE is repeated in an inverted orientation. (C) A DNase I footprinting assay using a probe from the *zen* VRR reveals that the DRE-binding protein binds specifically to the *zen* DRE.

ventral derepression prior to cellularization, this repression is restored during nuclear cycle 14. This suggests that other corepression elements play the dominant role after cellularization begins. Finally, although Dorsal sites and DREs are required for the repression of *dpp* (this paper), a short fragment from *dpp* containing only a Dorsal-binding site and a DRE is not sufficient for repression (J.-D. Huang, unpubl.).

The DRE, a corepression element in the dpp VRR, interacts with NTF-1

We have identified and purified a protein that interacts with the wild-type DRE, but not with mutant forms of the DRE that fail to support efficient repression. This protein was shown to be NTF-1 [equivalent to Elf-1 (Bray et al. 1989; Dynlacht et al. 1989), the product of the *grh* gene (Bray and Kafatos 1991)]. It has been shown previously that overexpression of NTF-1 between 4 and 10 hr of embryogenesis by heat-shocking embryos bearing *hsp70-grh* transgenes leads to a dorsal closure defect (Attardi et al. 1993). This phenotype is similar to the phenotype observed for mutations in two genes encoding members of the TGF- β receptor family (i.e., *thick veins* and *punt*) (Penton et al. 1994; Terracol and Lengyel 1994; Letsou et al. 1995; Ruberte et al. 1995). Because the *dpp* protein is known to signal through these receptors, it is likely that *dpp* also plays a role in dorsal closure. Therefore, overexpression of NTF-1, a putative repressor of *dpp*, could interfere with dorsal closure. The presumed function of *dpp* in dorsal closure is distinct from its early

function in dorsal/ventral pattern formation (Affolter et al. 1994; Terracol and Lengyel 1994). Nonetheless, the postulated repression of *dpp* by NTF-1 in the late embryo could still rely on interactions between NTF-1 and Dorsal as the Dorsal protein persists throughout embryogenesis (Steward 1989). Alternatively, in the presence of excess NTF-1, the repression of *dpp* may become Dorsal independent.

A possible link between the dorsal/ventral and terminal patterning systems

Rusch and Levine (1994) have shown that the Dorsal-mediated repression of both *dpp* and *zen* can also be alleviated by the action of the terminal maternal patterning system. Although this may be the result of the inactivation of Dorsal protein, the results reported here and in the accompanying paper (Liaw et al., this issue) suggest another possible explanation, that is, that the relief of Dorsal-mediated repression may be attributable, in part, to the inactivation of NTF-1. In addition to its potential role as a corepressor in the dorsal/ventral patterning system, NTF-1 appears to function as a repressor of *tailless* (*tll*), a major zygotic target of the terminal patterning system. Recent evidence indicates that activation of the terminal pathway, a receptor tyrosine kinase (RTK)-activated protein phosphorylation cascade, alleviates *tll* repression at the anterior and posterior termini of the embryo (Liaw et al., this issue). The finding that NTF-1 is a substrate for MAP kinase (one of the kinases in the RTK-activated phosphorylation cascade) (Liaw et

Figure 8. DRE-binding protein is equivalent to NTF-1. (A) DRE-binding protein protects the NTF-1-binding site in the Ubx 5'-flanking region from DNase I digestion. The footprinting probe used here is as described in Dynlacht et al. (1989). (Lane 1) No protein control; (lanes 2,3) 12.5 μ l of DRE-binding protein. (Left) The sequence of the protected region. The boxed portion of the sequence represents the NTF-1-binding site (Dynlacht et al. 1989). (B) Recombinant NTF-1 binds to DRE-B. (Lane 1) No protein control. Footprinting reactions contained 2 μ l (lane 2), 5 μ l (lane 3), or 10 μ l (lane 4) of DNA-affinity purified DRE-binding protein, or 25 μ l (lanes 5,6) of a recombinant NTF-1/GST fusion protein expressed in *Escherichia coli* and purified by chromatography on a glutathione affinity resin. (C) A Western blot probed with antiserum (kindly provided by R. Tjian, University of California, Berkeley) raised against vaccinia virus expressed recombinant NTF-1 protein. (Lane 1) The recombinant NTF-1 protein against which the antiserum was raised; (lane 2) DRE-binding protein. In addition to the three normal polypeptide components of DRE-binding protein with apparent molecular masses of 130, 120, and 77 kD, the Western blot reveals a series of lower molecular mass bands that we suspect to be degradation products of the larger species. Silver staining of a similar gel indicates that the amount of rNTF-1 in lane 1 is roughly equivalent to the amount of the 130- and 120-kD species in lane 2 (data not shown).

al., this issue) suggests that one of the repressors targeted by the terminal pathway may be NTF-1 itself.

Both because *dpp* and *tll* are expressed very early and because *grh* mutant embryos do not have detectable pattern defects [Bray and Kafatos 1991], any NTF-1 involved in regulating the initial expression of *dpp* and *tll* is most likely provided maternally. Consistent with this prediction, in situ hybridization to ovaries shows that NTF-1 mRNA is synthesized during oogenesis and deposited in the developing oocyte. One way to determine the requirement for maternal NTF-1 is to examine the phenotype of embryos resulting from females containing *grh*⁻ germ-line clones. We have recently made such females, and the resulting embryos exhibit a variable expansion of the *tll* expression domain [Liaw et al., this issue], confirming that NTF-1 functions as a repressor in the early embryo. We have not yet been able to draw definitive conclusions about the role of NTF-1 in *dpp* expression, and further analysis is under way.

Multiple mechanisms may contribute to ventral repression

We have shown previously that the *dpp* VRR can repress transcription over a long distance and thus has the char-

acteristics of a silencer [Huang et al. 1993]. The data presented here suggest that a short-range repression mechanism may also be important. Adjacent to DRE-B in the *dpp* VRR is a palindromic element that may serve as the binding site for a general activator. The NTF-1 and general activator footprints overlap one another, and binding to the two sites is mutually exclusive. Perhaps the VRR employs both long- and short-range repression mechanisms to ensure complete repression on the ventral side of the embryo.

How might Dorsal cooperate with NTF-1 and/or other corepressors to repress transcription? In the absence of corepression elements, Dorsal protein functions to activate transcription. Perhaps the corepressor proteins are the true repressors and the role of Dorsal is simply to facilitate-binding of these proteins to the DNA. Although we have been unable to demonstrate cooperative-binding of Dorsal and NTF-1 so far, it is possible that Dorsal facilitates NTF-1-binding in vivo by perturbing chromatin structure and thereby allowing NTF-1 access to the template.

Alternatively, perhaps Dorsal protein and corepressors work together to recruit other proteins to the DNA that directly repress transcription. A precedent for this is pro-

Figure 9. Expression of NTF-1 during oogenesis and early embryogenesis. In situ hybridization to whole mount egg chambers and embryos was performed using a digoxigenin-labeled antisense *grh* RNA probe. [A] A stage 6 egg chamber oriented with anterior to the left. Strongest staining is observed around the oocyte nucleus (arrow), with weaker staining observed in the nurse cells. [B] A stage 10 egg chamber oriented with anterior to the left. By this stage, staining is strongly and specifically observed in the nurse cells. Initial deposition of nurse cell transcripts into the oocyte is inferred from the additional staining adjacent and posterior to the oocyte nucleus (arrow). [C] A late syncytial blastoderm embryo (nuclear cycle 13) in sagittal view. By this stage, zygotic transcripts are clearly visible throughout the embryo. Staining is consistently stronger on the dorsal side than on the ventral side of the embryo. [D] A cellular blastoderm embryo (early stage 5) in ventrolateral view. By this stage, transcripts have nearly disappeared from a longitudinal band ~15–20 cells wide straddling the ventral midline (roughly the mesodermal anlage). [E] A higher magnification view of the same embryo shown in D.

vided by the yeast silent mating type loci, which contain-binding sites for multiple transcription factors (Laurenson and Rine 1992). In isolation, several of these transcription factors have been shown to function as

activators. When combined with one another in the correct spatial orientation, these factors probably cooperate in the recruitment of a protein complex that includes products of some of the SIR loci (Moretti et al. 1994). The SIR proteins appear to function to organize the chromatin into a heterochromatic state that is inaccessible to the transcriptional machinery (Loo and Rine 1994). Perhaps silencing by the *Drosophila* VRRs also involves the formation of an inaccessible chromatin conformation.

Materials and methods

P-element constructs and whole mount in situ hybridization

Mutated versions of the *dpp* VRR were generated as described previously (Huang et al. 1993). The mutated DNA was inserted into the $-980/dpp/lacZ$ vector for introduction into the *Drosophila* germ line. To generate a P-element vector containing multiple copies of the PLS, an oligonucleotide containing the PLS was multimerized in the pAEA5 vector (Liaw 1994). DNA fragments containing tandem repeats of the oligonucleotide were amplified by PCR with primers that had an *EcoRI* site at their 5' ends. The PCR products were treated with *EcoRI* and cloned into the *EcoRI* site of the $-980/dpp/lacZ$ P-element vector. Whole mount in situ hybridization to embryos and ovaries was carried out as described in (Tautz and Pfeifle 1989). Staging of egg chambers was according to King (1970) and embryos according to Campos-Ortega and Hartenstein (1985).

Purification and assay of DRE-binding protein (NTF-1)

The DRE-binding protein (Fig. 4) was isolated as follows. Nuclear extracts were prepared according to the method of Soeller et al. (1988) and dialyzed to completion against HEMG [25 mM HEPES, K^+ at pH 7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 1 mM Sodium metabisulphite, 0.2 mM PMSF] containing 0.1 M KCl. The extract was applied to an SP-Sepharose column preequilibrated with the same buffer, washed extensively with this buffer, and then eluted with a series of salt steps (Fig. 4A). Elutions were monitored by UV absorbance and were continued until the absorbance of the eluate returned to baseline levels. The protein-containing fractions from the 0.21 M salt step were pooled together, diluted with HEMG to 0.05 M KCl and loaded onto a preequilibrated DEAE-Sepharose column. The column was washed with HEM2.5G (same as HEMG except that the $MgCl_2$ concentration is 2.5 mM) containing 0.05 M KCl and eluted with a series of salt steps (Fig. 4A). The DRE-binding activity eluted in the 0.15 M KCl step. The active fractions were pooled and applied to a DNA affinity column. DNA affinity chromatography was performed as described by Kadonaga and Tjian (1986). The experiment shown in Figure 4 used an affinity column containing the *dpp* VRR sequences from nucleotides 1914 to 1959. DNA affinity chromatography was carried out in HEM2.5GN buffer [same as HEM2.5G with the addition of 0.05% NP-40]. Active fractions were pooled and placed in dialysis tubing, which was first embedded in powdered polyethylene glycol to concentrate the activity and then dialyzed against HEM2.5GN containing a reduced concentration of glycerol (5%). The material was then layered onto a 10%–20% glycerol gradient for velocity sedimentation. Sedimentation was carried out in a Beckman SW-41 rotor at 36,000 rpm for 48 hrs. Fractions (0.5 ml) were dripped from the bottom of the tube before analysis by DNase I footprinting and SDS-PAGE.

Footprinting assays were carried out as described previously (Pan et al. 1991). For UV cross-linking studies, iododeoxyuridine substituted probes were synthesized on a Pharmacia Gene Assembler using an iodinated phosphoramidite (Glen Research).

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Binding sites for transcription factor NTF-1/Elf-1 contribute to the ventral repression of decapentaplegic.

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