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A Direct Contact between the Dorsal rel Homology Domain and Twist May Mediate Transcriptional Synergy

JILL M. SHIROKAWA AND ALBERT J. COUREY*

Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90095-1569

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The establishment of mesoderm and neuroectoderm in the early *Drosophila* embryo relies on interactions between the Dorsal morphogen and basic–helix-loop-helix (bHLH) activators. Here we show that Dorsal and the bHLH activator Twist synergistically activate transcription in cell culture and in vitro from a promoter containing binding sites for both factors. Somewhat surprisingly, a region of Twist outside the conserved bHLH domain is required for the synergy. In Dorsal, the rel homology domain appears to be sufficient for synergy. Protein-protein interaction assays show that Twist and Dorsal bind to one another in vitro. However, this interaction does not appear to be of sufficient strength to yield cooperative binding to DNA. Nonetheless, the regions of Twist and Dorsal required for the binding interaction are also required for synergistic transcriptional activation.

The maternally encoded morphogen Dorsal functions to specify cell fate as a function of position along the dorsal-ventral axis of the *Drosophila* embryo. During oogenesis and the early stages of embryogenesis, multiple cascades of interactions between maternally expressed gene products establish a nuclear concentration gradient of the Dorsal morphogen, with peak levels in ventral nuclei and progressively lower levels in lateral and dorsal nuclei (32, 36, 42). Once established, this gradient of the Dorsal morphogen directs cell fate by regulating the expression of zygotic genes in a region-specific manner. These genes then direct the differentiation and subdivision of the germ layers.

The Dorsal morphogen is a member of the rel family of transcription factors, a family that includes the vertebrate factors NF-κB (39) and the product of the c-*rel* proto-oncogene (42). Dorsal directs the formation of the mesoderm from the ventral-most cells of the blastoderm embryo, in part, by transcriptionally activating the zygotic genes *twist* (*twi*) and *snail* (*sna*) (17, 19, 30, 46). The Dorsal morphogen directs the establishment of dorsal ectoderm and amnioserosa, in part, by repressing the zygotic genes *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*) in the ventral cells of the embryo, thereby confining their expression to dorsal and dorsal-lateral regions of the embryo (15, 34, 43). Finally, Dorsal initiates differentiation of the ventral neurogenic ectoderm, in part, by activating the zygotic genes *rhomboid* (*rho*) and *single-minded* (*sim*) in ventral-lateral cells (16, 22).

What determines the borders of a Dorsal-mediated domain of activation or repression? Part of the answer to this question appears to lie in the intrinsic affinity of Dorsal for its binding sites within a given target gene. For example, an enhancer in the *twi* gene termed the proximal ventral activator region contains Dorsal binding sites that are poor matches for the consensus Dorsal binding site (19, 30, 45). These low-affinity sites are apparently occupied only at the high Dorsal concentrations found in the ventral-most cells of the embryo, and thus this enhancer directs expression in a narrow ventral domain that occupies about 15 to 20% of the circumference of the embryo. Mutagenesis of these sites to convert them into perfect matches for the consensus binding site results in a broadening of the expression domain to include about 25 to 30% of the circumference of the embryo (20).

While intrinsic binding-site affinity is of some importance, it is not the sole or perhaps even the main determinant of the borders of target gene expression. In particular, it is thought that cooperative or synergistic interactions with other sequence-specific transcription factors bound to the target genes can somehow potentiate Dorsal action, thereby broadening domains of Dorsal-mediated activation or repression (14, 20). Synergistic transcriptional activation occurs when two or more activators give a transcriptional response that is greater than the sum of the response of each of the activators working alone (8). By permitting different combinations of activators to elicit different levels of transcription, synergy may be critical for the generation of spatially regulated patterns of gene expression during development. Transcriptional synergy may also provide a means by which smooth morphogen gradients can be interpreted to result in sharp borders of target gene activity (6). Cooperative DNA binding of two or more transcription factors can generate synergistic transcriptional activation. Alternatively, there is evidence that transcriptional synergy can occur when two factors simultaneously interact with different components of the general transcription machinery (3, 27, 37). In this case, the activators could elicit a synergistic response because their combined energies of interaction with the general transcription machinery would result in an exponential increase in the stability of the initiation complex.

A number of studies suggest that interactions between Dorsal and factors containing basic–helix-loop-helix (bHLH) domains are important for the development of the mesoderm. For example, embryos that are heterozygous for *twi* (which encodes a bHLH factor) and that are laid by *dorsal* (*dl*) heterozygous females show severe disruptions in mesoderm development (24, 40, 47). Similar genetic interactions are also observed between *dl* and other genes encoding bHLH factors, including *daughterless* (*da*) and the genes of the *Achaete Scute* complex (AS-C) (12). The disruption of the mesoderm appears

* Corresponding author. Mailing address: Department of Chemistry and Biochemistry, UCLA, 405 Hilgard Ave., Los Angeles, CA 90095. Phone: (310) 825-2530, Fax: (310) 206-4038. E-mail: courey@chem.ucla.edu.

to result from the significantly reduced levels of *twi* and *sna* promoter activity exhibited by these embryos. In the case of *sna*, both Dorsal sites and E boxes have been identified in the control region of the gene that mediate the regulation by Dorsal and bHLH factors (17). Paradoxically, however, while the *twi* regulatory region contains essential Dorsal binding sites, it does not contain any obvious E boxes (bHLH domain recognition elements) that could mediate the regulation of this gene by bHLH factors.

The interaction of Dorsal with bHLH activators is also important for initiating the differentiation of the neurogenic ectoderm. For example, in the ventral-lateral regions of the embryo where there is a low concentration of Dorsal protein, bHLH proteins appear to interact with Dorsal to activate *rho* (12). The *rho* regulatory region contains closely linked Dorsal binding sites and E boxes. Mutation of either the Dorsal sites or the E boxes causes a severe reduction in *rho* promoter activity, implying that factors bound to these two types of sites work together to synergistically activate *rho* transcription (16).

While it seems clear that Dorsal-bHLH factor synergy is critical for dorsal-ventral pattern formation, the mechanism behind this synergy is unknown. Coimmunoprecipitation assays have shown that Dorsal can directly bind to bHLH factors such as T4 (one of the AS-C gene products) and Twist (12), although the functional significance of this binding interaction has not been determined. Finally, recent evidence suggests that the Dorsal-bHLH interactions mediating mesoderm and neuroectoderm differentiation are fundamentally different (44), since in one case (neuroectoderm) the Dorsal and bHLH sites must be closely linked to one another to allow synergy, while in the other case (mesoderm) there is no such requirement.

In this report, we present the results of experiments investigating the mechanism of synergistic activation by Dorsal and Twist. We present direct biochemical evidence that Dorsal and the Twist bind to one another and that they synergistically activate transcription in cultured cells and in a cell-free transcription system. Structure-function studies of both proteins suggest that the same domains that mediate the protein-protein binding interaction also mediate synergistic activation.

MATERIALS AND METHODS

Cotransfections. Expression vectors pPacTwi, pPacTwi-N85 pPacTwi-N173, pPacTwi-N273, pPacTwi-N346, pPacTwi354, pPacDl, pPacDl-C590, pPacDl-C470, and pPacDl-C380 were constructed by PCR subcloning as follows. Full-length Twist cDNA, Twist N-terminal deletion cDNAs, Twist 354 cDNA, and full-length Dorsal cDNA were PCR amplified to contain *Sal*I ends and ligated into the *Xho*I site in the pPacU vector containing a Ubx leader sequence (7). Dorsal C-terminal deletion cDNAs were amplified to contain *Bcl*I (5′) and *Sal*I (3′) ends and ligated into the pPacU vector (containing a Ubx leader sequence) between the *Bam*HI and *Xho*I sites. The Twist internal deletion (Δ142-346) was created by ligating the N-terminal *Bam*HI fragment (amino acids 1 to 141) removed from pPacTwist into the *Bam*HI site of pPacTwi-N346 (amino acids 347 to 490). p65 and p50 cDNA were obtained from Sankar Ghosh and blunt-end cloned into the blunted *Bam*HI site of a pPacU vector containing no Ubx leader sequence.

Reporter vectors TKCAT5X(dl-Ebox), TKCAT5X(dl-mEbox), and TKCAT5X (mdl-Ebox) were constructed by ligating fivefold multimers of dl-Ebox, mdl-Ebox, and dl-mEbox into the −37TKCAT (7) vector between the *Bam*HI and *Sph*I sites. *Bam*HI/*Sph*I multimer fragments were obtained from pE4T-5X(dl-Ebox), pE4T-5X(dl-Ebox), and pE4T-5X(mdl-Ebox) vectors (see below). The reporter vector TKCAT4dl was constructed by inserting an oligonucleotide with the sequence 5′ TCGACATTGGGTTTCTCCCAGTATTGGGTTTCTCCCAG TATTGGGTTTCTCCCAGTATTGGGTTTCTCCCAGTG 3′ into the *Sal*I site.

Calcium phosphate cotransfections into *Drosophila* Schneider line 2 (SL2) cells and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (7). A Molecular Dynamics PhosphorImager was used to quantitate data from the thin-layer chromatography plates.

HeLa nuclear extract in vitro transcription assays. The in vitro transcription templates pE4T-5X(dl-Ebox), pE4T-5X(dl-mEbox), and pE4T-5X(mdl-Ebox) were constructed by ligating the *Kpn*I (blunted)/*Xba*I fragments from the pAEA vectors containing these multimers into the pE4T vector (4) between the *Hin*cII and *Xba*I sites.

The pAEA vector containing multimerized DNA fragments were created by oligomerizing oligonucleotides containing the desired transcription factor binding sites (26). The sequence of one strand of each oligonucleotide binding site, with the Twist and Dorsal binding sites underlined, is as follows: dl-Ebox, 5′ CCGAGAGCATATGTTTTGGGGGATTTTCCCAAC 3′; dl-mEbox, 5′ CCGAGAGACTAGCTTTTGGGGGATTTTCCCAAC 3′; and mdl-Ebox, 5′ CCGAGAGCATATGTTTTGTTAAAGCTTAACAAC 3′.

In vitro transcription assays using HeLa nuclear extracts were performed as described previously (4). HeLa nuclear extracts were provided by the lab of Stephen T. Smale, while the purified Gal4+AH (amphipathic helix) protein and the pG5E4T template were provided by Michael Carey.

Baculovirus-expressed Dorsal protein. The recombinant Dorsal baculovirus was created and selected as a non-occlusion body virus (2). Dorsal protein was DNA expressed and affinity purified as described previously (14).

Expression and purification of GST fusion proteins. For bacterial expression of glutathione *S*-transferase (GST) fusion proteins, pGex expression vectors (Pharmacia) were used. The pGexKGTwist vector has been previously described (16). Expression vectors pGexTwi-N28, pGexTwi-N159, pGexTwi-N234, and pGexTwi-N332 were constructed by PCR cloning as follows. Twi-N28, Twi-N159, and Twi-N234 cDNAs were PCR amplified to contain *Sma*I (5′) and *Eco*RI (3′) ends and ligated into the *Sma*I and *Eco*RI sites in the pGex2T vector. Twi-N332 cDNA was PCR amplified to contain the *Bam*HI (5′) and *Eco*RI (3′) sites and cloned into the pGex2T vector between the *Bam*HI and *Eco*RI sites.

GST-Twist, GST–Twi-N28, GST–Twi-N159, GST–Twi-N234, GST–Twi-N332, and GST proteins were prepared from bacterial extracts and purified by glutathione affinity chromatography (16).

Thrombin cleavage of GST-Twist and DNA affinity purification of Twist. GST-Twist was expressed in bacteria as described previously (16) except left bound to the glutathione-agarose beads. To 0.5 ml of the bead-fusion protein complex, 25 U of human thrombin (Sigma) was added in a total volume of 1.0 ml of phosphate-buffered saline (pH 7.4), and the mixture was incubated at room temperature for 2 h with rotation. Cleaved GST was removed by centrifugation (500 × *g*). The supernatant containing Twist and thrombin was removed and loaded onto a 0.5-ml Twist E-box DNA affinity column.

The oligomerized oligonucleotide Twist E-box binding sites were coupled to cyanogen bromide-activated Sepharose (29). The sequence of one strand of the oligonucleotide binding site is 5′GATCGAGAGCATATGTTTTGGCGCC3′. The Twist E-box column was washed with 10 column volumes of buffer H (25 mM HEPES [pH 7.6], 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol [DTT], 0.05% Nonidet P-40 [NP-40]) containing 0.1 M KCl. A step gradient was performed at 0.2, 0.5, and 1 M KCl in buffer H. Twist eluted from the DNA affinity column at 0.5 M KCl and was dialyzed into buffer D′ (20 mM HEPES [pH 7.9], 50 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT).

Expression and purification of His_6-TwistΔN protein. The *Nae*I (blunted)/*Eco*RI fragment obtained from the pGexKGTwist vector was cloned into the ptrcHisB (Invitrogen) histidine fusion expression vector between the *Bam*HI (blunted) and *Eco*RI sites.

The His_6-TwistΔN fusion was expressed in *Escherichia coli* as follows. A saturated culture was diluted 1:100 into 500 ml of LB containing 50 μg of ampicillin per ml and grown with shaking at 37°C until the optical density at 550 nm reached 0.2. Isopropylthio-β-D-galactoside (IPTG) was added to a concentration of 0.4 mM, and the bacteria were grown for 2 h at 37°C. Cells were harvested by centrifuging at 6,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 25 ml of ice-cold HEMG (25 mM HEPES [pH 7.6], 100 mM KCl, 12.5 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA, 1 mM DTT) and repelleted in an SS-34 rotor at 5,000 × *g* for 10 min at 4°C. The cell pellet was then resuspended in 5 ml of ice-cold HEMG containing 0.1% NP-40. Lysozyme was added to a concentration of 0.5 mg/ml, and the mixture was incubated on ice for 15-min. The mixture was sonicated at 0.6 relative output (35%) for two 15-s intervals on ice. The lysate was centrifuged at 85,000 × *g* for 30 min at 4°C. The supernatant containing the soluble fusion protein was frozen in liquid nitrogen and stored at −80°C.

The soluble extract obtained was dialyzed into column binding buffer (20 mM phosphate buffer [pH 7.8], 0.5 M NaCl, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite) and loaded onto a 1-ml Ni-Sepharose column (Invitrogen). The column was extensively washed with column binding buffer followed by extensive washing with column wash buffer (20 mM phosphate buffer [pH 6.3], 0.5 M NaCl, 0.1% NP-40). The fusion protein was eluted with a step gradient of 50 mM, 300 mM, 500 mM, and 1 M imidazole. The purest fraction of the fusion was dialyzed into HEMG containing 0.1% NP-40 and stored at −80°C.

In vitro transcription and translation. In vitro-transcribed Dorsal (dlE) and Dorsal 379 (dl379) mRNAs were prepared from pAR plasmids containing the Dorsal cDNAs under control of the T7 promoter. In vitro transcription reactions were performed with linearized pARdlE and pARdl379 templates at a concentration of 0.1 μg/μl in a mixture containing 80 mM HEPES (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 5 U of yeast inorganic pyrophosphatase per ml, 0.8 U of RNase block per μl, 1.8 U of T7 RNA polymerase per μl, and 3 mM each ATP, GTP, CTP, and UTP for 2 to 6 h at 37°C. Following the

transcription reaction, the DNA template was removed by digesting the reaction for 15 min at 37°C with RNase-free DNase (0.1 U/μl). The reaction was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) twice and with chloroform-isoamyl alcohol (24:1) once and then ethanol precipitated.

The Dorsal mRNA (5 μg), Dorsal 379 mRNA (5 μg), and luciferase mRNA (1 μg) were translated for 1 h at 30°C, using rabbit reticulocyte lysate (35 μl) with 4 μl of [^{35}S]methionine (1,000 Ci/mmol, 10 μCi/μl), 0.02 mM minus-methionine amino acid mixture, and 40 U of RNase block in a total volume of 50 μl. The translation products were analyzed on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel.

GST protein-protein interaction assays. GST fusion proteins were expressed as stated previously. One microgram of each GST fusion protein was bound to 15 to 30 μl of glutathione-agarose beads in 40 mM HEPES (pH 7.5)–50 mM KCl, 5 mM $MgCl_2$–0.2 mM EDTA–1 mM DTT, 0.5% NP-40 (HEMNK). Approximately 10 to 25 μl of the translation products was added to the beads in a total volume of 600 μl of HEMNK, and binding was allowed to proceed for 1 h at 4°C on a rocking platform. The beads were washed with 1 ml of HEMNK five times, and the bound proteins were eluted with 25 to 50 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and were resolved by SDS-PAGE. For experiments using ^{35}S-labeled proteins, the gel was dried and exposed to a phosphorimaging screen. The scanned image was visualized and bands were quantitated using a PhosphorImager. For experiments using biotin-labeled proteins, bound proteins were transferred to nitrocellulose and probed with streptavidin-horseradish peroxidase, blocked in 5% nonfat milk, probed with streptavidin-horseradish peroxidase, and visualized by enhanced chemiluminescence.

RESULTS

Dorsal and Twist proteins synergistically activate transcription in *Drosophila* SL2 cells. To determine if the synergy between Dorsal and Twist as revealed by genetic analysis could be due to a direct interaction between these factors, we performed cotransfection experiments with *Drosophila* SL2 cells. These assays used expression vectors in which the Twist and Dorsal coding regions have been placed under the control of the *Drosophila* actin 5C promoter, as well as a CAT reporter vector. The reporter vector bears five copies of a regulatory module containing Dorsal and Twist binding sites inserted upstream of the herpes simplex virus thymidine kinase core promoter [TKCAT5X(dl-Ebox) (Fig. 1A)]. This regulatory module is identical to that used previously to demonstrate a genetic interaction between Dorsal and Twist. It was designed to mimic the orientation and spacing of Dorsal and bHLH factor binding sites found in the *rho* promoter (16).

When introduced into SL2 cells alone, the reporter vector directs levels of CAT expression not significantly higher than the level directed by a similar CAT vector lacking the Dorsal and Twist binding sites (data not shown). This finding indicates that these cells lack endogenous factors capable of productively interacting with the Dorsal and Twist binding sites. Cotransfection of low levels (20 to 60 ng) of either the Twist or Dorsal expression vector resulted in a modest (two- to threefold) increase in reporter gene activity, indicating that each factor has some intrinsic ability to activate the reporter promoter. Simultaneous addition of both expression vectors resulted in an approximately 30-fold increase in reporter gene activity (Fig. 1B). Thus, the combined factors function in a synergistic (greater than additive) manner to activate the reporter promoter (Fig. 1C).

Activation by both Dorsal and Twist in this cotransfection system is, with a minor exception, binding site dependent. When we mutagenize the Dorsal binding sites in the reporter promoter {leaving the Twist binding sites intact [Fig. 2A, TKCAT5X(mdl-Ebox)]}, we observe no activation by Dorsal alone over the basal level and modest (threefold) activation by Twist alone (Fig. 2B). The introduction of Dorsal in addition to Twist results in no further transcriptional activation.

When we mutagenize the Twist binding sites in the reporter promoter, leaving the Dorsal binding sites intact [TKCAT5X(dl-mEbox) (Fig. 2A)], we observe no activation by Twist alone and modest (10-fold) activation by Dorsal alone (Fig. 2C, right).

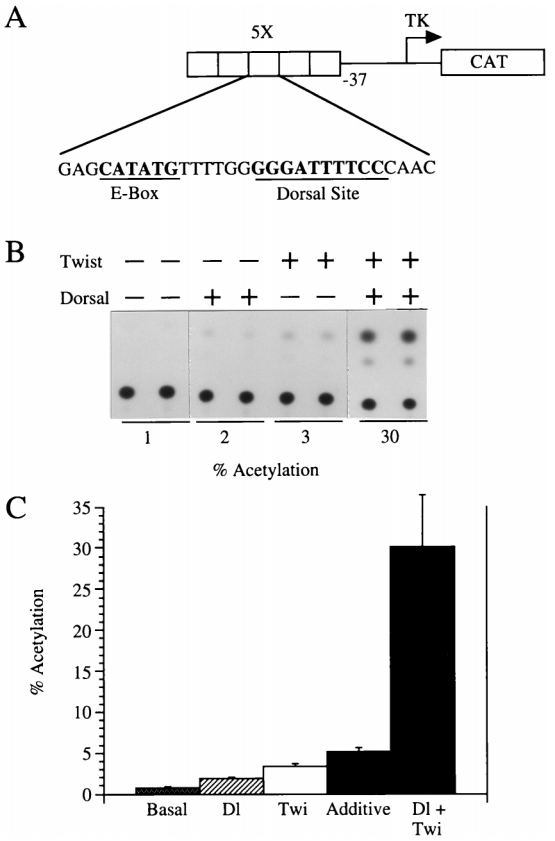

FIG. 1. Dorsal and Twist synergistically activate transcription in *Drosophila* SL2 cells. (A) Schematic diagram of the reporter construct, TKCAT5X(dl-Ebox), which contains five copies of an oligonucleotide containing Dorsal and Twist binding sites (5X) upstream of the herpes simplex virus thymidine kinase core promoter (TK) driving expression of the CAT reporter gene. (B) The TKCAT5X(dl-Ebox) reporter (5 μg) was introduced into SL2 cells with and without Dorsal (60 ng) and Twist (20 ng) expression vectors. Activity is from an average of duplicates and is presented in percent acetylation. (C) Bar graph summarizing the data from panel B. The bar labeled "Additive" indicates the level of activity that would be expected if Dorsal and Twist acted in an additive way to activate transcription.

Somewhat surprisingly, the introduction of Twist in addition to Dorsal results in a small but reproducible twofold stimulation of transcription over that observed with Dorsal alone. Apparently, the elimination of the Twist binding sites from the reporter greatly reduces, but does not completely eliminate, the ability of Twist to stimulate transcription from a promoter containing Dorsal protein-occupied Dorsal binding sites. This apparent Twist binding site independent stimulation of transcription by Twist could potentially result from cryptic Twist binding sites somewhere in the vector or from nonspecific DNA binding by Twist. However, this is unlikely since deletion of the Twist DNA binding domain did not eliminate this weak binding site-independent activity (Fig. 2D). In contrast to the result observed with Twist, binding site-independent activation of a promoter containing occupied Dorsal binding sites was not observed with transcription factor Sp1 (data not shown). This result suggests that the ability to interact with Dorsal in a binding site-independent manner is specific for Twist.

Mapping the functional domains in Dorsal and Twist required for simple and synergistic activation. As demonstrated above, Dorsal and Twist are each able to direct modest levels of transcriptional activation on their own (simple activation),

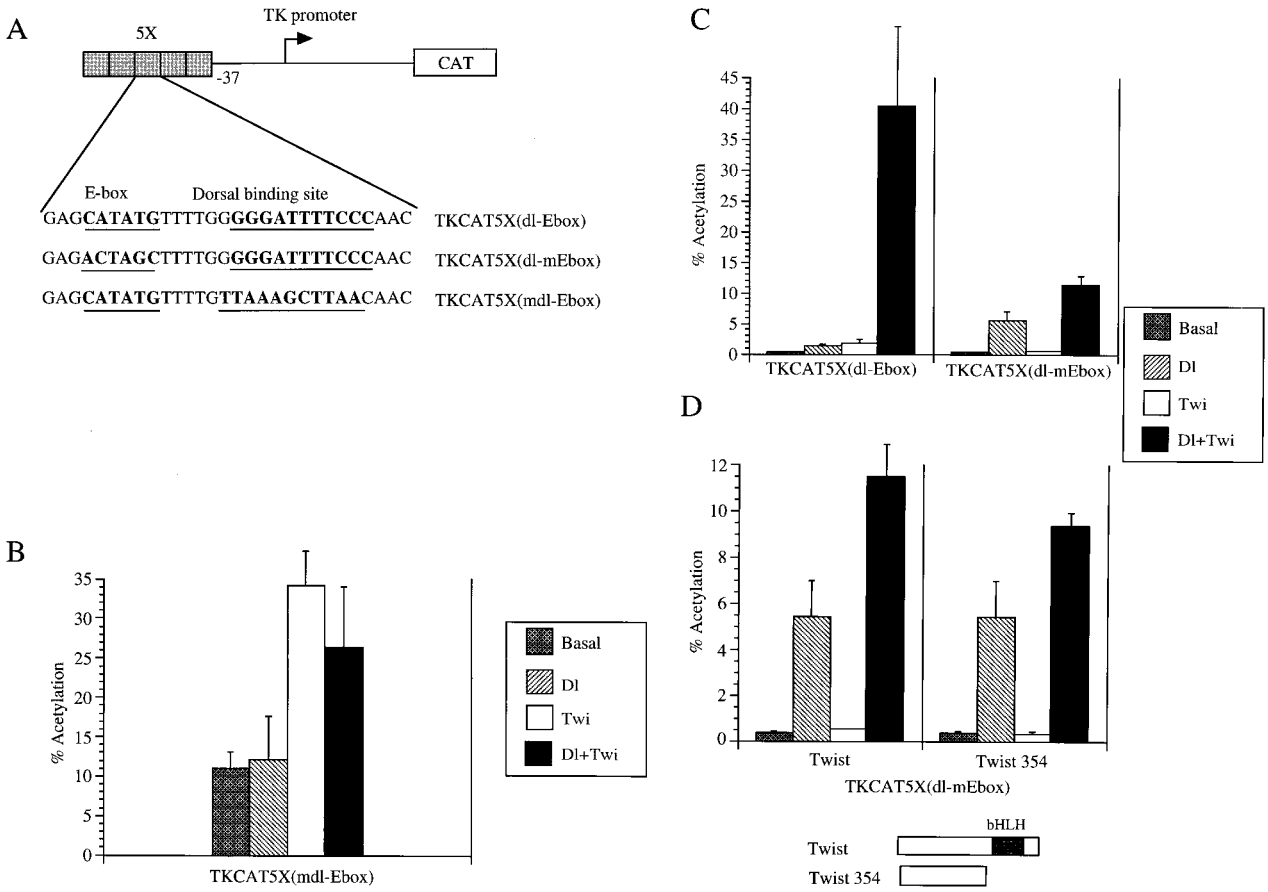

FIG. 2. Dorsal can recruit Twist to a promoter lacking Twist binding sites. (A) Schematic diagrams of reporter constructs (notation is as in Fig. 1A). The TKCAT5X (dl-Ebox) reporter is depicted in Fig. 1A. The TKCAT5X(dl-mEbox) reporter is similar to the TKCAT5X(dl-Ebox) reporter except that the Twist E-box binding site contains a 4-bp substitution mutation. The TKCAT5X(mdl-Ebox) reporter is also similar to the TKCAT5X(dl-Ebox) reporter except that the Dorsal binding site is abolished. (B) Transient transfection assays in SL2 cells were performed with the TKCAT5X(mdl-Ebox) reporter. This reporter (5 μg) was transfected with Dorsal (60 ng), Twist (20 ng), or Dorsal (60 ng) and Twist (20 ng) expression vectors. Percent acetylation values represent averages of duplicate assays. (C) Transient transfection assays in SL2 cells were performed with the two different reporter constructs, TKCAT5X(dl-Ebox) and TKCAT5X(dl-mEbox). Reporters (5 μg) were cotransfected with the Dorsal (60 ng), Twist (20 ng), or Dorsal (60 ng) and Twist (20 ng) expression vectors. Percent acetylation values represent averages of duplicate assays. (D) Transient transfection assays in SL2 cells were performed with the TKCAT5X(dl-mEbox) reporter. The reporter (5 μg) was transfected with Dorsal (60 ng), Twist (20 ng), Twist 354 (20 ng), Dorsal (60 ng) and Twist (20 ng), or Dorsal (60 ng) and Twist 354 (20 ng) expression vectors. Percent acetylation values represent averages of duplicate assays.

while the combination of the two factors results in a greater than additive effect on reporter gene activity (synergistic activation). To determine if the same regions of these proteins mediate these two types of activation, we carried out a deletion analysis of these factors.

Twist is a 490-amino-acid protein with a bHLH motif near its C terminus. N terminal to the bHLH domain are two Gln-rich regions (Gln-1 and Gln-2). Since Gln-rich domains frequently play roles in transcriptional activation, it seemed possible that the Gln-rich domains of Twist play a role in simple or synergistic activation. To test this possibility, we constructed expression vectors encoding a series of deletion variants of Twist (Fig. 3). To examine the effects of the deletions on simple activation, the vectors encoding each variant were cotransfected into SL2 cells along with the CAT reporter vector. To examine the effects of these deletions on synergistic activation, the vectors encoding each variant were cotransfected into SL2 cells along with the CAT reporter vector and the full-length Dorsal expression vector. For the experiments examining simple activation (Fig. 3, −Dorsal), it was necessary to use 100-fold-higher concentrations of the Twist expression constructs than were used in the experiments examining synergistic activation (Fig. 3, +Dorsal). This is because simple activation is intrinsically weak in this assay—the higher concentrations of Twist expression vectors are therefore required to obtain simple activation levels large enough to be accurately quantified. As a result, the level of synergy between full-length Dorsal and full-length Twist appears artificially low in this experiment. The data in Fig. 1B and 2C (left) much more accurately reflect the true magnitude of the synergy, since in these experiments the same concentrations of the Twist expression vector were tested in the presence and absence of the Dorsal expression vector.

Assays of reporter gene activity suggest that the two Gln-rich regions each make independent contributions to synergistic activation (Fig. 3). An 85-amino-acid N-terminal deletion that removes Gln-1 reduces reporter gene activity threefold. Further deletion of an additional 88 amino acids does not result in any further reduction in reporter gene activity. Deletion of the next 100 residues to remove Gln-2 results in an additional twofold drop in reporter gene activity. The bHLH domain alone does not direct levels of acti-

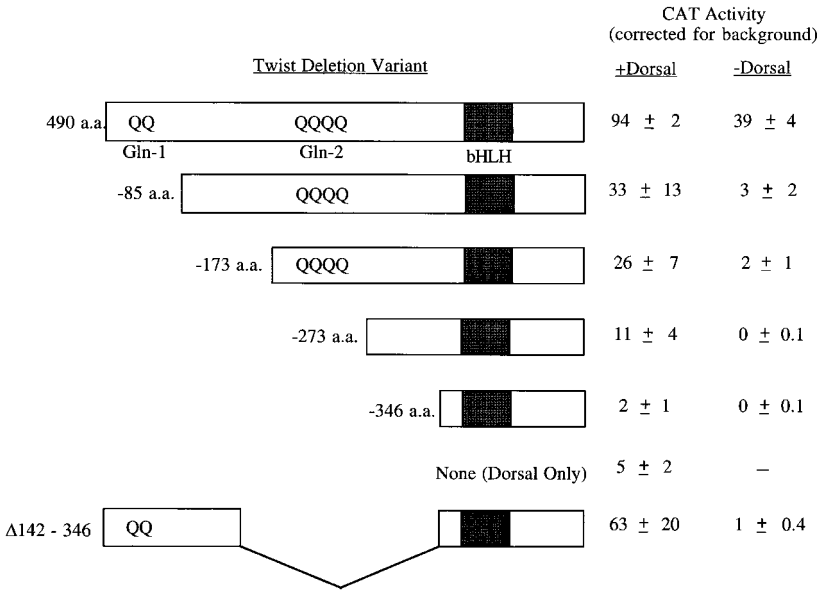

FIG. 3. The Twist N terminus contains two domains that independently mediate synergistic transcriptional activation with Dorsal. Transient transfection assays similar to those shown in Fig. 1 were performed for a series of deletion variants of the 490-amino-acid (a.a.)-long Twist protein in the presence and absence of Dorsal. For the experiments reported in the +Dorsal column, 60 ng of the Dorsal expression vector and 20 ng of a vector expressing one of the Twist variants were transfected with the TKCAT5X(dl-Ebox) reporter (5 μg) shown in Fig. 1A. For the experiments reported in the −Dorsal column, 2 μg of each Twist variant was transfected with the TKCAT5X(dl-Ebox) reporter. Results represent CAT activity as percent acetylation and are averages of triplicate assays. All values have been corrected by subtraction of the background CAT activity observed in the absence of activators. For the series of experiments shown in the +Dorsal column, the background CAT activity was 1.8% acetylation, while for the series of experiments shown in the −Dorsal column, the background CAT activity was 1.1% acetylation.

vation that are higher than those directed by Dorsal protein alone.

To determine if Gln-1 can synergize with Dorsal in the absence of Gln-2, we tested an internal deletion of Twist lacking Gln-2 but containing Gln-1 (Δ142-346). When cotransfected with the Dorsal expression vector and the reporter, this vector directs activation levels about two-thirds as high as that directed by full-length Twist (Fig. 3). Thus, Gln-1 and Gln-2 appear to function in an additive manner to direct synergistic activation.

While the two Gln-rich regions function as independent modules in synergistic activation, the same is not true for simple activation (activation by Twist in the absence of Dorsal). For the analysis of simple activation, the Dorsal expression vector was omitted from the transfections, and, as mentioned above, we included relatively high concentrations of the vectors directing expression of the various Twist variants. Under these conditions, we observed approximately 40-fold activation with full-length Twist but no significant activation with any of the Twist deletion variants (Fig. 3).

Dorsal is a 678-amino-acid protein containing a rel homology domain (RHD) within its 341 N-terminal residues. To map the regions of Dorsal required for synergistic and simple activation, we generated a series of expression vectors encoding a number of C-terminal deletions of Dorsal. To assess the ability of each deletion variant to direct synergistic or simple activation, each Dorsal variant was introduced into SL2 cells either with or without full-length Twist (Fig. 4A). As with the assays of the Twist deletion variants, in our assays of the Dorsal deletion variants, the transfections examining simple activation (Fig. 4A, −Twist) used considerably higher concentrations of each of the Dorsal variant expression vectors than did the experiments examining synergistic activation (Fig. 4A, +Twist). Once again, this was necessary to obtain levels of simple activation that could be accurately quantified.

Progressive deletion of sequences from the C-terminal end of the protein first results in a dramatic decrease in the level of synergistic activation. Thus, removal of the C-terminal 88 amino acids decreases reporter gene activity about fivefold, while removal of the next 120 amino acids results in a further threefold reduction in activity. Surprisingly, however, removal of the next 90 amino acids to leave a 380-amino-acid protein that contains little more than the RHD results in a dramatic increase in transcriptional synergy. This truncated protein cooperates with Twist to synergistically activate transcription as well as full-length Dorsal. Thus, it appears that Dorsal contains two domains contributing to synergistic activation. The first maps to the C terminus, while the second localizes to the RHD. In the absence of the C-terminal domain, sequences residing between 380 and 590 amino acids from the N terminus appear to interfere with the ability of the RHD to direct synergistic activation.

While the RHD is sufficient for synergistic activation, it is incapable of directing simple activation (Fig. 4A). Deletion of the C-terminal 88 amino acids of Dorsal reduces simple activation by a factor of 2 to 3, while deletion of a further 120 residues abolishes simple activation altogether. Further deletion of the protein does not restore its ability to mediate simple activation.

Because the RHD of Dorsal is sufficient for transcriptional synergy, we decided to determine if other rel family members could cooperate with Twist to synergistically activate transcription. We therefore constructed expression vectors encoding the RHDs of p50 and p65, two mammalian rel family polypeptides. These two factors constitute the two subunits of the heterodimeric transcription factor NF-κB. In addition, each subunit can also bind DNA as a homodimer (23, 32, 33, 47, 48). The homodimers have consensus binding sites very similar to that of Dorsal (36) and would be expected to bind to the consensus Dorsal binding sites in our reporter vector. Cotrans-

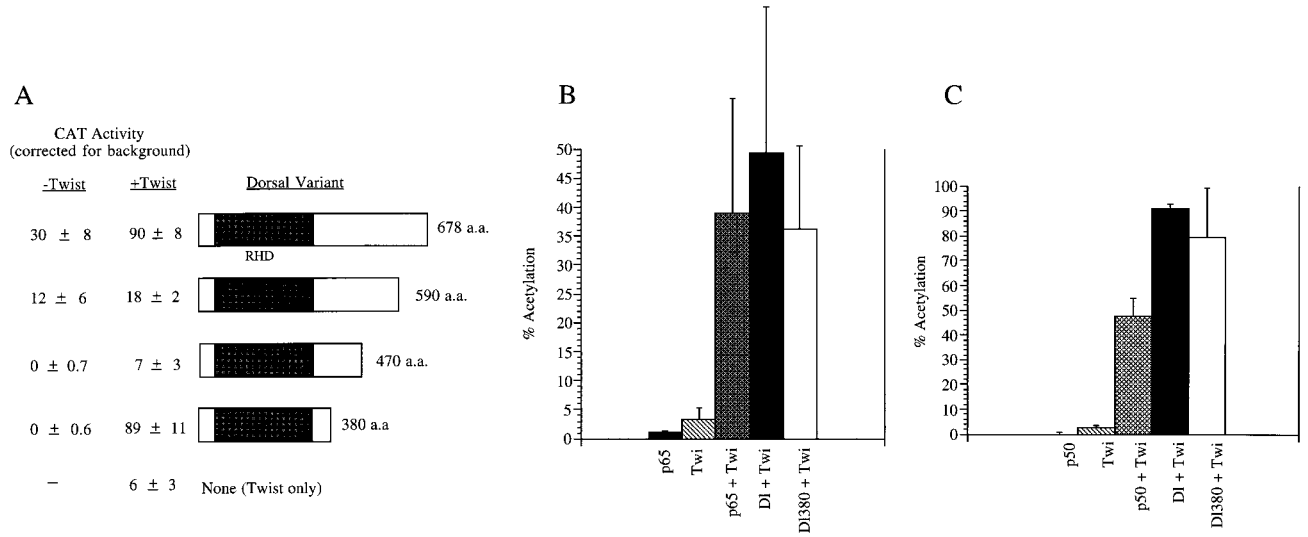

FIG. 4. The RHDs of Dorsal, p65, and p50 mediate synergistic transcriptional activation with Twist. (A) Transient transfection assays in SL2 cells were performed with a series of C-terminal deletion variants of the 678-amino-acid (a.a.)-long Dorsal protein in the presence and absence of Twist protein. For the experiments reported in the +Twist column, 20 ng of Twist and 60 ng of Dorsal variant expression vectors were transfected with the TKCAT5X(dl-Ebox) reporter (5 μg). Results are averages of duplicate assays. For the experiments reported in the −Twist column, 1.8 μg of each Dorsal variant was transfected with the TKCAT5X(dl-Ebox) reporter (5 μg). Results are averages of triplicate assays and represent CAT activity as percent acetylation. All values have been corrected by subtraction of the background CAT activity observed in the absence of activators, which was 1.5% acetylation. (B) Transient transfection assays were performed with a vector expressing the RHD of the mouse p65 protein (amino acids 1 to 399). The p65 expression vector (180 ng), the Twist expression vector (20 ng), or the p65 (180 ng) and Twist (20 ng) expression vectors were cotransfected with the TKCAT5X(dl-Ebox) reporter into SL2 cells. For comparison, Dorsal (60 ng) and Twist (20 ng) or Dorsal 380 (60 ng) and Twist (20 ng) expression vectors were cotransfected with the same reporter. Results represent CAT activity as percent acetylation and are averages of triplicate assays. All values have been corrected by the subtraction of the background CAT activity observed in the absence of activators (0.4% acetylation in this experiment). (C) Transient transfection assays were performed with a vector expressing the mouse p50 (amino acids 1 to 433) protein. The p50 expression vector (60 ng), the Twist expression vector (20 ng) or the p50 (60 ng) and Twist (20 ng) expression vectors were transfected with the TKCAT5X(dl-Ebox) reporter into SL2 cells. For comparison, Dorsal (60 ng) and Twist (20 ng) expression vectors or Dorsal 380 (60 ng) and Twist (20 ng) expression vectors were cotransfected with the same reporter. Results represent CAT activity as percent acetylation and are averages of triplicate assays. All values have been corrected by the subtraction of the background CAT activity observed in the absence of activators (1.3% acetylation in this experiment).

fection experiments demonstrate that these factors are both capable of synergizing with Twist. When p65 and Twist expression vectors were cotransfected with the reporter, we observed levels of CAT activity comparable to those observed with the combination of the Dorsal RHD and Twist (Fig. 4B). The combination of p50 and Twist gave levels of CAT activity that were about twofold lower than those observed with the combination of the Dorsal RHD and Twist (Fig. 4C). Thus, it appears that the interaction between Twist and Dorsal is mediated by some conserved feature of the RHD.

The domains of Dorsal and Twist that mediate transcriptional synergy also mediate a binding interaction between the two proteins. To determine if the synergistic activation by Dorsal and Twist observed in the transfection assay might reflect a protein-protein interaction between the two factors, we tested for a binding interaction between these two proteins. Twist was expressed in *E. coli* as a fusion to bacterial GST. The GST fusion protein was immobilized on glutathione-agarose beads, which were then incubated with in vitro-translated ^{35}S-labeled Dorsal protein. After extensive washing to remove nonspecifically bound proteins, the bound proteins were eluted with SDS, separated by SDS-PAGE, and visualized by autoradiography. While only 0.03% of the input Dorsal protein bound to a control resin containing unfused GST (Fig. 5A, lane 2), 11% of the input Dorsal protein bound to the immobilized GST-Twist fusion protein (lane 7). Dorsal and Twist are therefore capable of a specific protein-protein binding interaction.

To map regions of Twist responsible for Dorsal binding, we generated a series of fusion proteins in which GST was fused to a number of N-terminal deletion variants of Twist. In this way, we found that deletion of the first 28 amino acids of Twist to remove part of Gln-1 resulted in about a twofold drop in Dorsal binding (Fig. 5A, lane 6). Further deletion of the region up to the N-terminal border of Gln-2 had no additional effect on Dorsal binding (lane 5). However, N-terminal deletions that removed part of Gln-2 in addition to Gln-1 nearly eliminated Dorsal binding (lanes 3 and 4). Thus, the two Gln-rich regions that appear to mediate synergistic activation are also required for binding to Dorsal in vitro.

To determine if the RHD of Dorsal, which is sufficient for synergistic activation, is also sufficient for Twist binding, we examined the Twist binding activity of a C-terminal deletion variant of Dorsal containing only the RHD (Dorsal 379). For this experiment, full-length Dorsal and Dorsal 379 were labeled with [^{35}S]methionine and incubated with immobilized GST-Twist. Specifically bound Dorsal and Dorsal 379 were eluted and separated by SDS-PAGE. Both full-length Dorsal and Dorsal 379 were found to bind to the GST-Twist resin (Fig. 5B, lanes 1 and 2) but not to the control GST resin (data not shown). The luciferase control did not bind to the GST-Twist resin (Fig. 5B, lane 3). Apparently, the Dorsal RHD, which is sufficient for synergistic activation, is also sufficient for Twist binding.

The finding that Dorsal and Twist can bind to one another suggests that they may be able to bind to DNA in a cooperative manner. If this is so, then it could provide an explanation for the transcriptional synergy observed in the transfection assays. However, multiple attempts to detect cooperative binding using both gel shift and DNase I footprinting assays have yielded negative results. These experiments show that the affinity of the template for one factor is not altered in any detectable way by the presence of the other factor (data not shown).

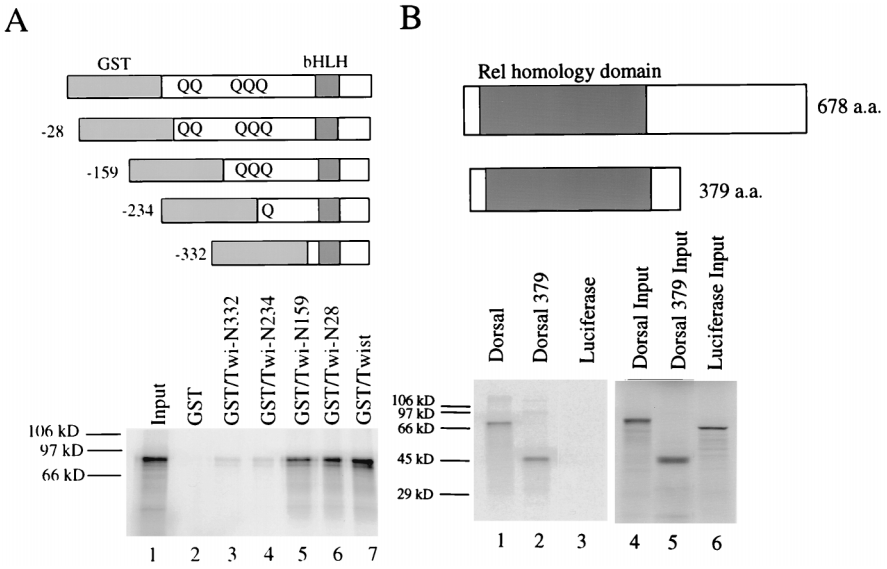

FIG. 5. Synergistic activation domains of Twist correspond to protein-protein interaction regions. (A) GST pull-down assay utilizing a series of GST-Twist N-terminal deletions incubated with ^{35}S-labeled Dorsal protein. Lane 1 contains an amount of input protein equal to 10% of the amount of Dorsal protein used in each of the protein-protein interaction assays shown in lanes 2 to 7 (B) GST-Twist was immobilized on glutathione-agarose beads, which were then incubated with two different ^{35}S-labeled forms of Dorsal (Dorsal and Dorsal 379) or ^{35}S-labeled luciferase. Lane 4 contains an amount of Dorsal input protein equal to 12% of the amount of Dorsal protein used for the protein-protein interaction assay shown in lane 1. Lane 5 contains an amount of Dorsal 379 input protein equal to 12% of the amount of Dorsal 379 protein used for the protein-protein interaction assay shown in lane 2. Lane 6 contains an amount of luciferase input equal to 12% of the amount of luciferase protein used for the protein:protein interaction assay shown in lane 3. a.a., amino acids.

Cell-free transcription assays confirm a role in transcriptional synergy for a region of Twist outside the conserved bHLH domain. Previous studies have shown that at least two bHLH factors in addition to Twist (Daughterless and T4) can genetically interact with Dorsal (12). Thus, our finding in cotransfection assays that a region of Twist N terminal to the conserved bHLH domain is required for transcriptional synergy was somewhat unexpected. Therefore, we used a cell-free assay system in an effort to confirm this finding. We used HeLa cell nuclear transcription extracts as opposed to *Drosophila* embryo extracts in these studies because the HeLa cell extracts are devoid of endogenous Twist or Dorsal. The use of the human system is appropriate because of the high degree of conservation of the basal transcriptional machinery in all eukaryotes (39). Purified factors used in the in vitro transcription assays include baculovirus-expressed Dorsal, *E. coli*-expressed Twist, and *E. coli*-expressed His_6-TwistΔN fusion variant (Fig. 6A).

The results of a typical experiment examining the interaction between full-length Dorsal and full-length Twist are shown in Fig. 6B, lanes 1 to 9. The quantitative results of this experiment are graphed in Fig. 6C. The template for the in vitro transcription assays consisted of the adenovirus E4 core promoter plus five upstream copies of the module containing Twist and Dorsal binding sites (Fig. 6A). When this promoter was used to program a HeLa cell extract, we observed low levels of basal transcription comparable to that observed for the E4 core promoter with no upstream elements (Fig. 6B, lane 1). Addition of 35 ng of Dorsal in the absence of Twist gave about a sixfold activation of the promoter (lane 2), while the addition of 100 ng of Twist in the absence of Dorsal resulted in a less than twofold stimulation of the promoter (lane 4). Higher concentrations of Twist did not result in any further stimulation (lanes 5 and 6). However, the combination of 35 ng of Dorsal and either 100 or 300 ng of Twist resulted in about 60-fold activation of the promoter (lanes 7 and 8). Thus, while Twist only weakly activates transcription on its own, addition of Twist to the in vitro transcription system containing Dorsal protein results in a further ~10-fold stimulation of transcription. Clearly, the factors function in a synergistic manner to activate the E4 promoter in vitro. At supersaturating Twist concentrations (lane 9, 35 ng of Dorsal and 500 ng of Twist), promoter activity begins to decrease, perhaps due to an ability of the Twist activation domains to squelch transcription by titrating away a critical coactivator (31).

In contrast to the results obtained with full-length Twist, a truncated form of Twist (His_6-TwistΔN) lacking sequences N-terminal to the bHLH domain (expressed as a His-tagged fusion protein) was incapable of synergistic activation in the in vitro transcription assay (Fig. 6B, lanes 15 and 16). Although only one reaction (at one protein concentration) is shown here, His_6-TwistΔN was assayed over a range of concentrations, all of which fail to support activated transcription with Dorsal (data not shown). Thus, in agreement with our transient transfection assays, synergy requires sequences N terminal to the bHLH domain.

Like unfused Twist protein, the Twist-GST fusion protein (Fig. 6B, lanes 3, 12, 13, and 14; Fig. 6D, lanes 1 to 4) can also synergize with Dorsal protein to activate the E4 promoter, showing that the GST domain does not interfere with activation or transcriptional synergy in this assay. Both the simple and synergistic activation observed in vitro are almost completely binding site dependent, as shown by a series of experiments using full-length Dorsal protein and the Twist-GST fusion protein. When the extract is programmed with a promoter containing a mutant Twist binding site and an intact Dorsal binding site (Fig. 6D, lanes 7 to 12), we observe weak stimulation by Dorsal (lane 8) and almost no stimulation by Twist-GST in either the absence (lane 9) or presence (lane 10) of Dorsal protein. When the extract is programmed with a promoter containing a mutant Dorsal binding site and an intact Twist binding site (Fig. 6D, lanes 13 to 18), we observe weak stimulation by Twist-GST alone (lane 15) and no stimulation

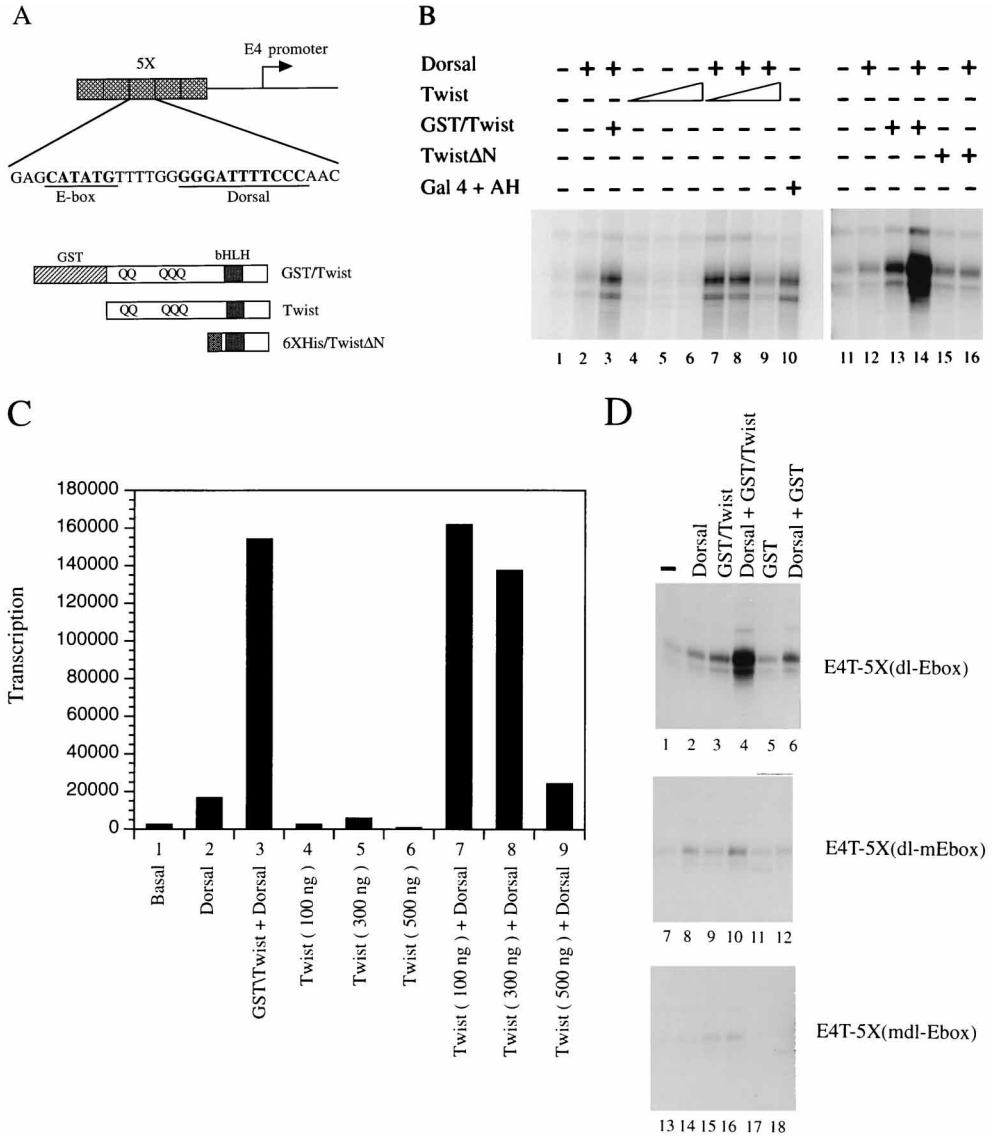

FIG. 6. In vitro transcription assays confirm a role in transcriptional synergy for the region of Twist N terminal to the bHLH domain. (A) Diagrams of the in vitro transcription template and of the forms of Twist used in the in vitro transcription assays. The template E4-5X(dl-Ebox) consists of five copies of an oligonucleotide containing Dorsal and Twist binding sites (5X) placed upstream of the adenovirus E4 core promoter. The template E4-5X(dl-mEbox) contains five copies of an oligonucleotide containing the Dorsal site and a mutated Twist E box site placed upstream of the adenovirus E4 core promoter. Likewise, the template E4-5X(mdl-Ebox) contains five copies of an oligonucleotide containing a mutated Dorsal site and an E-box site placed upstream of the adenovirus E4 core promoter. Factors used include baculovirus-expressed Dorsal protein, *E. coli*-expressed GST-Twist fusion protein, bacterially expressed Twist protein (generated by thrombin cleavage of the GST-Twist fusion protein and subsequent removal of the GST domain), and *E. coli*-expressed His_6-TwistΔN (a His-tagged truncated Twist protein lacking sequences N-terminal to the bHLH domain). (B) Dorsal and Twist synergistically activate transcription in vitro. A HeLa cell nuclear in vitro transcription system used 100 ng of the template described above. In the experiment displayed at the left, 35 ng of Dorsal (lane 2), 35 and 600 ng of GST-Twist (lane 3), 100, 300, and 500 ng of Twist (lanes 4 to 6), and 100, 300, and 500 ng of Twist plus 35 ng of Dorsal (lanes 7 to 9) were added to each HeLa transcription reaction; 30 ng Gal4+AH protein was used as a positive control with the template pG5E4T (200 ng) containing five Gal4 binding sites upstream of the core E4 promoter. The panel on the right represents a separate HeLa transcription experiment using the template used for lanes 1 to 10. Factors used include 35 ng of Dorsal (lane 12), 600 ng of GST-Twist (lane 13), 35 ng of Dorsal plus 600 ng of GST-Twist (lane 14), 50 ng of TwistΔN (lane 15), and 50 ng TwistΔN plus 35 ng of Dorsal (lane 16). The transcription extract used in the experiment on the right was more active than the one used for the experiment on the left. Thus, lane 14 shows a higher level of activity than lane 3. Note that the Twist protein derived from the thrombin-cleaved GST-Twist fusion protein was DNA affinity purified, while the GST-Twist fusion protein was not DNA affinity purified. Therefore, the GST-Twist preparation may contain some inactive forms of GST-Twist that do not bind DNA, explaining why higher concentrations of GST-Twist than of Twist are required to achieve optimal activation levels. (C) Quantitated data from panel B. (D) Dorsal-Twist synergistic activation is dependent on both the Dorsal binding site and the Twist E-box site in vitro. HeLa cell nuclear in vitro transcription reactions used 100 ng of each of the following templates: E4T-5X(dl-Ebox), E4T-5X(dl-mEbox), and E4T-5X(mdl-Ebox). Factors used include 35 ng of Dorsal (lanes 2, 8, and 14), 600 ng of GST-Twist (lanes 3, 9, and 15), 35 ng of Dorsal plus 600 ng of GST-Twist (lanes 4, 10, and 16), 600 ng of GST as a control (lanes 5, 11, and 17), and 35 ng of Dorsal plus 600 ng of GST as a control (lanes 6, 12, and 18). The three different template reactions were performed in one experiment but separated for ease of presentation.

by Dorsal in either the absence (lane 14) or presence (lane 16) of Twist-GST. As expected, the addition of the unfused GST protein to the transcription system has no effect on transcription levels in either the absence or presence of Dorsal protein (Fig. 6D, lanes 5, 6, 11, 12, 17, and 18).

DISCUSSION

Previous studies have demonstrated that Dorsal and Twist, two transcription factors found in ventral and ventrolateral regions of the early *Drosophila* embryo, interact with one another to direct

cell fate in these regions of the embryo. Our studies using cell culture assays, in vitro protein-protein binding assays, and in vitro transcription assays strongly imply that this interaction is at least partly due to the ability of these two factors to synergistically activate target promoters. Furthermore, they suggest that a protein-protein interaction between the two factors may contribute to this synergy.

Dorsal and Twist synergistically activate promoters in a binding site-dependent manner. In cell transfection and in vitro transcription assays, we found that Dorsal and Twist each have some capacity to activate transcription individually (simple activation). However, together these factors were found to activate transcription synergistically; i.e., the combination of these factors results in much higher levels of promoter activity than would be expected if the effects of Dorsal and Twist were merely additive. For example, in cotransfection assays, levels of Dorsal and Twist that individually activate transcription 2- to 3-fold combine to give a 30-fold increase in the activity of a target promoter containing both Dorsal and Twist binding sites (Fig. 1C). The results of in vitro transcription assays (Fig. 6C) are in remarkably close quantitative agreement with the results of the cotransfection assays.

As expected, the observed simple and synergistic activation is largely dependent on binding sites in the promoter for both factors. Both in vitro and cell culture assays reveal that Dorsal-Twist synergistic activation is dependent on the Dorsal binding site (Fig. 2B and 6D). In addition to verifying that the Dorsal-Twist synergistic activation is Dorsal binding site dependent, the cell culture experiment depicted in Fig. 2B shows that mutation of the Dorsal binding site resulted in an increase in basal promoter activity. This higher than normal basal level may suggest that the altered promoter is now a target for the binding of an endogenous factor found in SL2 cells.

Somewhat surprisingly, our cotransfection assays show that Twist is able to weakly activate a promoter that lacks Twist binding sites, as long as that promoter contains occupied Dorsal binding sites. This weak binding site-independent activity is observed even after deletion of the Twist DNA binding domain. However, it is not observed when transcription factor Sp1 is used in place of Twist, nor is it observed in our in vitro transcription assays. We suspect that it results from a weak ability of Dorsal to recruit Twist to the DNA via a protein-protein binding interaction. Perhaps this interaction is stabilized by an accessory factor present in SL2 nuclei but lacking from the in vitro transcription system.

Numerous other examples are known of transcription factors that have the dual ability to regulate gene expression either by binding directly to the promoter or by binding to another promoter-bound factor (1, 5, 9–11, 29). The ability of Twist to regulate promoters lacking Twist binding sites may explain *twi* autoregulation. Twist is required for the maintenance of its own expression despite the lack of readily detectable Twist binding sites in the *twi* regulatory region (25). Perhaps Twist is recruited to its own promoter via protein-protein interactions with Dorsal, which binds to multiple sites within the *twi* regulatory region and which is responsible for the initiation of *twi* gene expression. Alternatively, it is possible that *twi* autoregulation is indirect.

Deletion analysis of Dorsal and Twist reveals different sequence requirements for synergistic and simple activation. Using deletion analysis, we have mapped regions of Dorsal and Twist that function in simple and/or synergistic activation. For both factors, we identified deletion variants that were adequate for synergistic activation but not for simple activation. In the case of Twist, we found that both of the Gln-rich regions were required for detectable simple activation, while either alone was sufficient for synergistic activation. In the case of Dorsal, we found that the RHD was sufficient for synergistic activation but that a region at the C-terminal end of the protein was additionally required for simple activation.

Available Twist antibodies are not sufficiently sensitive to detect the Twist variants in crude extracts of transfected cells. Thus, we have not been able to verify that all the Twist deletion variants are expressed at equivalent levels. However, results from in vitro transcription and GST pull-down assays suggest that the transfection experiments are valid. First, the in vitro transcription results confirm the transfection studies by showing that it is the regions outside of the bHLH domain that are required for transcriptional synergy. Second, the GST pull-down assays show that the regions important for protein-protein interaction coincide with the regions important for transcriptional synergy (as determined through the transfection deletion analysis).

The finding that the RHD of Dorsal is sufficient for synergistic activation led us to test the RHDs of the mouse factors p50 and p65 in our system. Transfection assays demonstrate that the RHDs from both of these proteins are able to synergize with Twist. This finding was not unexpected. A region of the mouse kappa light-chain enhancer contains rel family protein and bHLH family protein binding sites arranged in a manner similar to that observed for the *rho* neuroectoderm enhancer. Furthermore, when attached to an appropriate reporter gene and introduced into the *Drosophila* embryo, this region of the kappa enhancer can direct stripes of expression similar to those directed by the *rho* neuroectoderm enhancer (13). These findings suggest that the ability of rel family and bHLH family proteins to interact may have been conserved in evolution, in accord with our demonstration that both p50 and p65 can synergize with Twist.

The results of our deletion analysis of Dorsal are also in accord with studies analyzing the properties of mutant alleles of *dl*. As mentioned above, the C-terminal 208 amino acids of Dorsal are essential for simple activation. This domain also appears to play a nonessential role in synergistic activation. In agreement with these findings, embryos carrying a nonsense mutation in *dl* resulting in the deletion of the C-terminal 80 amino acids of the encoded protein fail to turn on genes such as *twi* that are normally activated by Dorsal (18).

Protein-protein interactions between Dorsal and Twist may be required for synergistic activation. Our GST pull-down assays show that Dorsal and Twist bind to one another in a specific manner, suggesting that a specific interaction between the two proteins could account for the observed transcriptional synergy. Indeed, when we examined deletion variants of Dorsal and Twist, we found that regions of the proteins required for the binding interaction colocalized with regions of the protein required for transcriptional synergy. For example, the two Gln-rich regions that contributed in an additive way to transcriptional synergy also contributed in an additive way to the binding interaction. The Twist bHLH domain, which is insufficient for synergistic activation, is also insufficient for Dorsal binding. Furthermore, the RHD of Dorsal, which is sufficient for transcriptional synergy, is also sufficient for Twist binding. These results are in accord with the findings of Gonzalez-Crespo and Levine (12), in which coimmunoprecipitation assays demonstrated binding between Dorsal and Twist. This earlier study did not include experiments to map regions of Twist required for the binding interaction. However, in agreement with our findings, it was found that the RHD of Dorsal was sufficient for binding.

Possible mechanisms for Dorsal-Twist synergy. One frequently suggested model for transcriptional synergy is cooperative binding to DNA. However, Dorsal and Twist do not appear to bind to the templates used in our assays in a cooperative manner. Apparently, the protein-protein interaction is not of sufficient affinity to yield cooperative binding in vitro. We cannot rule out the possibility that Dorsal-Twist binding is cooperative in vivo, in the context of a chromatinized template. However, we also observe transcriptional synergy in vitro, in the absence of a chromatin assembly system. Furthermore, the concentrations of Dorsal and Twist used in our in vitro transcription assays are sufficient to saturate the template in the absence of cooperative binding (data not shown), making it extremely unlikely that cooperative binding contributes to the observed transcriptional synergy.

In another model for transcriptional synergy, simultaneous interactions between the general machinery and different targets in the general transcription complex are proposed to account for transcriptional synergy (37). However, this model does not account for our finding that Dorsal-Twist synergy may involve a direct interaction between Dorsal and Twist. An alternative model, which does account for our findings, is that the interaction between Dorsal and Twist induces a conformational change in one of the factors that enables it to efficiently activate transcription.

Using coimmunoprecipitation assays, Gonzalez-Crespo and Levine (12) have previously examined binding of Dorsal to three bHLH factors, Twist, T4, and Daughterless. They found that whereas Twist and T4 bound to Dorsal, Daughterless did not. Intriguingly, we find that Dorsal and Da synergistically activate transcription in cell culture assays (unpublished data), despite the apparent lack of a direct interaction. Therefore, it is conceivable that different bHLH domain-containing transcription factors have evolved distinct mechanisms for synergizing with Dorsal. This conclusion is further supported by our finding that regions of Twist outside the conserved bHLH domain are required for transcriptional synergy both in a cell culture assay and in an in vitro transcription assay.

In conclusion, previous genetic studies strongly suggest that interactions between Dorsal and bHLH factors such as Twist are critical for establishing the border between the neurogenic ectoderm and the mesoderm in the blastoderm embryo. By illuminating the biochemical basis of these genetic interactions, the studies presented here strongly suggest that direct synergistic interactions between transcription factors help to set the borders of developmental domains.

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