

Chip interacts with diverse homeodomain proteins and potentiates Bicoid activity *in vivo*

Emi Torigoi*, Idriss M. Bennani-Baiti†, Christina Rosen†, Katie Gonzalez*, Patrick Morcillo†, Mark Ptashne*†, and Dale Dorsett*††

*Molecular Biology Program, Sloan-Kettering Division of the Weill Graduate School of Medical Sciences of Cornell University, and †Molecular Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

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The *Drosophila* protein Chip potentiates activation by several enhancers and is required for embryonic segmentation. Chip and its mammalian homologs interact with and promote dimerization of nuclear LIM proteins. No known *Drosophila* LIM proteins, however, are required for segmentation, nor for expression of most genes known to be regulated by Chip. Here we show that Chip also interacts with diverse homeodomain proteins using residues distinct from those that interact with LIM proteins, and that Chip potentiates activity of one of these homeodomain proteins in *Drosophila* embryos and in yeast. These and other observations help explain the roles of Chip in segmentation and suggest a model to explain how Chip potentiates activation by diverse enhancers.

The *Drosophila* Chip protein is provided maternally and is also expressed zygotically, and both supplies are required for normal development (1). Embryos lacking maternal Chip die without forming segments, and those lacking zygotic Chip form segments and hatch but die as larvae (2). It has been proposed that Chip facilitates enhancer–promoter communication at many genes (1–3). That idea was suggested by genetic studies indicating that the Su(Hw) insulator protein counteracts Chip activity at the *cut* locus (2). Thus, when the gypsy transposon is inserted at virtually any place between a wing-specific enhancer and the promoter of *cut*, Su(Hw) binds to gypsy and blocks communication between the enhancer and promoter, resulting in a *cut* wing phenotype (4, 5, and reviewed in refs. 3 and 6). Chip was identified as a mutation that further reduced expression of a *cut* gene bearing a gypsy insertion (2). These studies also indicated that the Su(Hw) bound to gypsy DNA in a *cut* gene on one chromosome works, to some extent, to block activation by an enhancer in a wild-type *cut* gene on the homologous chromosome. Under this situation of inter-chromosomal enhancer-blocking, expression of the wild-type *cut* gene is much more sensitive to reduction of Chip activity than to reductions in the activities of all other known *cut* regulators, including the enhancer-binding protein Scalloped (2).

Several recent studies have described interactions of Chip and homologous proteins with other proteins *in vitro*. Vertebrate Chip homologs were isolated as proteins that bind zinc-chelating structures called LIM domains that mediate diverse protein interactions (7–9). Nuclear LIM proteins include those that consist solely of a few LIM domains, and certain DNA-binding proteins that also contain homeodomains (LIM-HD proteins, reviewed in ref. 10). The Chip mammalian homologs promote formation of homotypic and heterotypic dimers of LIM-HD proteins *in vitro* (11). *Drosophila* Chip also interacts with LIM domains and supports activity of the Apterous LIM-HD protein in wings (1, 12–16). It also has been reported that a mammalian Chip homolog interacts with a mammalian HD protein (9).

Interactions between Chip and LIM proteins do not explain the full range of Chip's *in vivo* functions. None of the known *Drosophila* LIM proteins is needed for segmentation, and none of the known LIM proteins is required for activation of most genes, e.g., *cut* and *even-skipped* (*eve*), known to be regulated by Chip. In contrast, many segmentation genes, such as *eve*, are

activated by HD proteins. Here we show that *Drosophila* Chip interacts with diverse HD proteins and with the Su(Hw) insulator protein through a domain distinct from the region that interacts with LIM proteins. We also present two experiments showing that Chip potentiates the activity of the Bicoid (Bcd) HD protein in both *Drosophila* embryos and in yeast.

Materials and Methods

Affinity Chromatography. Glutathione-S-transferase (Gst)-Chip and mutant Gst-Chip fusion proteins (see below) were expressed in *Escherichia coli* (1). Glutathione-agarose beads (Sigma) were incubated with bacterial extracts overnight at 4°C and washed with 4 vol of extraction buffer (20 mM Hepes, pH 7.5/0.1 M NaCl/10% glycerol/0.1% Nonidet P-40/1 mM 2-mercaptoethanol), 2 vol of binding buffer (10 mM Pipes, pH 6.8/0.45 M NaCl/5% glycerol/0.1% Nonidet P-40/1 mg/ml BSA/1 mM DTT), and 8 vol of phosphate-buffered saline (1 mM KH₂PO₄/10 mM Na₂HPO₄/137 mM NaCl/2.7 mM KCl, pH 7.0) containing 1 mM DTT. Washes contained protease inhibitors (1 mM phenylmethylsulfonyl fluoride/1 μg/ml each of leupeptin, aprotinin, and pepstatin). The beads were equilibrated in PBS containing 1% Triton X-100, 1 mM DTT, and 25 mg/ml BSA and protease inhibitors and stored at 4°C. Before binding reactions, beads were incubated overnight at 4°C with 1 ml of blocking solution (PBS with 2% nonfat milk/1% Triton X-100/1 mM DTT/protease inhibitors) per 100 μl of beads.

[³⁵S]proteins for binding to Gst-Chip beads were produced by *in vitro* translation using the TNT kit (Promega), [³⁵S]methionine (NEN, 1,000 Ci/mmol), and transcription templates described below. ZnCl₂ (1 mM) was added to Apterous and Su(Hw) translations.

Binding reactions were conducted for 1 hr at 4°C in blocking solution with a total volume of 200 μl, ≈10–20 μg of Gst fusion protein, and 10⁻¹³ mmol of [³⁵S]protein. The beads were washed three times with blocking solution for 10 min, twice with PBS containing 1% Triton X-100 for 10 min, and twice with PBS for 5 min. The beads were boiled in 40 μl of 2× SDS-PAGE sample buffer, and the retained [³⁵S]protein quantified with a phosphorimager after SDS-PAGE.

Gst-Chip Fusion Constructs. Gst-Chip was made from pGEX2T-Chip (5). Gst-Chip:Δ472–577 was made by PCR amplification (17) of the *Chip* cDNA sequence encoding residues 1–471 using a 5'-primer with a *Bgl*III site and a 3'-primer with a stop codon and an *Eco*RI site and cloning into the *Bam*HI and *Eco*RI sites

Abbreviations: Gst, glutathione-S-transferase; HD, homeodomain; Su(Hw), suppressor of hairy-wing; LID, LIM interaction domain; SID, self-interaction domain; Otd, Orthodenticle; OID, other interaction domain.

*To whom reprint requests should be addressed. e-mail: d-dorsett@ski.mskcc.org.

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of pGEX2T (Pharmacia). Gst-Chip:Δ441–454 was made by cloning a *Bgl*I to *Sma*I linker into the blunted *Bam*HI and *Ava*I sites of pGEX2T-Chip. Gst-Chip:Δ404–465 was made by substituting the *Mae*I (blunted) to *Eco*RI *Chip* cDNA fragment for the *Alw*NI (blunted) and *Eco*RI fragment of pGEX2T-Chip. Gst-Chip:Δ404–519 was made by deleting the *Alw*NI to *Bst*XI fragment of pGEX2T-Chip and blunt religation. Gst-Chip:Δ294–381 was made by deleting the *Sph*I to *Bss*HII fragment of pGEX2T-Chip. Gst-Chip:Δ384–438 was made by substituting a *Sma*I linker for the *Bss*HII to *Bam*HI fragment of pGEX2T-Chip. To make Gst-Chip:Δ457–471, the sequence encoding residues 471–577 was amplified by PCR with primers containing *Ava*I and *Afl*III sites and substituted for the 507-bp *Ava*I to *Afl*III fragment in the *Chip* cDNA. The resulting *Bam*HI to *Eco*RI *Chip* cDNA fragment was cloned into pGEX2T. Gst-Chip:Δ1–381 was made by cloning the blunted 856-bp *Bss*HII to *Eco*RI *Chip* cDNA fragment into the blunted *Xho*I site in pGEX1ZT (Pharmacia).

In Vitro Translation Vectors. The *apterous* cDNA was amplified by PCR with a 5'-primer containing the Kozak initiation sequence (18) and an *Nco*I site, and a 3'-primer containing an *Eco*RV site, and cloned into the *Nco*I and *Eco*RV sites of the pING14.1 expression vector (S. Ingles and I. Brierly, cited in ref. 19). pTN3-Bcd (20), pAR-Ftz (20), and pAR-Eve (20) were used to produce Bcd, Ftz, and Eve. The Orthodenticle (*Otd*) expression vector contains the *otd* cDNA in a pBluescript vector (Frieder Schöck, Max Planck Institute for Biophysical Chemistry, personal communication).

Bcd:1–255 was made by deleting the *Acc*I to *Eco*RI fragment of pTN3-Bcd. Bcd:1–190 was made by deleting the 1.6-kb *Bse*RI to *Eco*RI fragment of pTN3-Bcd. Bcd:1–166, Bcd:57–166, and Bcd:157–255 were made by PCR amplification of the appropriate coding sequences with a 5'-primer containing an *Nco*I site and initiation codon and a 3'-primer with an *Eco*RV site, and cloning the resulting DNA fragments into the *Nco*I and *Eco*RV sites of pING14.1.

The *Chip* cDNA-coding sequence was amplified by PCR using a 5'-primer with an *Nco*I site and a Kozak initiation site and a 3'-primer with an *Eco*RV site, and cloned into the *Nco*I and *Eco*RV sites of pING14.1. *Chip*:Δ404–519 was made by replacing the *Bss*HII to *Hind*III 668-bp fragment of pING14.1-Chip with the *Bss*HII to *Hind*III 322-bp fragment of pGEX2T-Chip:Δ404–519. *Chip*:Δ2–381 was made by cloning the blunted *Bss*HII to *Eco*RI *Chip* cDNA fragment into the blunted *Nco*I site in pING14.1.

The *Su(Hw)* cDNA was cloned as an *Xho*I to *Sma*I fragment (21) into the *Sal*I and *Sma*I sites of pGEM4Z. Circular pGEM4Z-Su(Hw) was used to produce full-length Su(Hw), and Su(Hw):ΔCTD (truncated at residue 780) was produced after linearization with *Bam*HI. Su(Hw):1–190 was made after cutting pGEM4Z-Su(Hw) with *Bsa*BI or *Xho*II. The Su(Hw) zinc finger domain (residues 204–672) was made by cloning the *Sal*I to *Nhe*I fragment from pSJ-*su(Hw)*^{Zn} (22) into the *Sal*I and *Nhe*I sites of pGEM4Z. Su(Hw):706–944 was made by cloning the *Pvu*II to *Hind*III 713-bp fragment of the *su(Hw)* cDNA into the *Eco*RV and *Hind*III sites of pING14.1. Mutant *su(Hw)* zinc finger domains described previously (21) were amplified by PCR with a 5'-primer with an *Nco*I site and an initiation codon, and a 3'-primer with an *Eco*RV site, and the resulting DNA fragments were cloned upstream of the *Hind*III (blunted) to *Ssp*I *su(Hw)* cDNA fragment with the termination codon into the *Nco*I and *Bam*HI (blunted) sites of a pGEM3Z (Promega) vector modified to lack the polylinker *Hind*III site.

Sequencing of the *Chip*^{g96.1} Mutation. The *Chip*^{g96.1} ORF was amplified by PCR (using primers 5'-CGGAATTCAGTG-CATACACATACGCATG-3' and 5'-ATTAAGATCTGTGT-

GTAGAGTAGACGAC-3') from genomic DNA isolated from *y w; P[w⁺]57B *Chip*^{g96.1}/*Chip*^{k04405}* larvae according to Levis *et al.* (23), cloned into the *Eco*RI site of pBluescript (SK) (Stratagene), and sequenced using Sequenase II (U.S. Biochemicals).

Genetic interaction between *Chip*^{g96.1} and *bcd*^{E3}. *y w; P[w⁺]57B *Chip*^{g96.1}/+; *ru st bcd*^{E3} *ca e* females and sibling *y w; ru st bcd*^{E3} *ca e* females were crossed to *y w* males and allowed to lay embryos on apple juice agar plates at 25°C. After 48 hr, embryonic cuticles were prepared (1) and the segments counted under dark field illumination. Partially fused segments were counted as loss of one-half a segment. Calculation of standard errors and statistical significance was aided by the STATVIEW computer program (SAS Institute, Cary, NC).*

Effects of Chip on Activation by Bcd in Yeast. The *lacZ* yeast reporter plasmids and the Bcd and Gal4-ER-VP16 expression vectors are described by Burz *et al.* (24). The *Chip* expression vector was made by cloning the *Chip* cDNA as an *Msl*I to *Eco*RI fragment downstream of a *Kpn*I to *Nco*I (blunted) fragment with the yeast *ACT1* promoter (25) into the YCplac22 ARS-CEN vector (26). *Chip* mutants were made by substituting appropriate fragments from the pGEX2T-Chip mutant vectors described above into Ycplac22-Chip.

Yeast strain NLY2 (*MATα ura3 his3Δ200 leu2-1 lys- trp1Δ63 gal4- gal80-*) was sequentially transformed using LiCl (27) with the reporters and expression vectors and cultured in appropriate drop-out media with 2% glucose at 30°C. Mid-log cultures were diluted to an OD₆₀₀ of 0.2, and β-estradiol was added to induce Bcd expression. Four hours postinduction, extracts were assayed for β-galactosidase activity (27). All experiments were performed at least twice with four independent clones for each Bcd transformant and two independent clones for wild-type and mutant *Chip*.

Results

Chip Interacts with Diverse HD Proteins and with the Su(Hw) Insulator Protein.

The experiments of Fig. 1 demonstrate that *Chip* interacts *in vitro* with various HD proteins and with Su(Hw). To perform these experiments we prepared [³⁵S]-labeled HD and Su(Hw) proteins using *in vitro* translation, and tested their abilities to bind to Gst-*Chip* fusion protein, expressed in and purified from bacteria and attached to glutathione-bearing beads. Fig. 1*B* shows that full-length *Chip* interacted with the HD proteins Bicoid (Bcd) and Ftz, and with a fragment of the Su(Hw) insulator protein (lanes 3 and 10). Fig. 1*B* also shows, consistent with previous results, that *Chip* interacted with the Apterous LIM-HD protein and with itself (lanes 3 and 10; refs. 1, 12, 13, 15, and 16). In experiments not shown, we found that the HD protein *Otd* bound almost as efficiently as did Bcd and Ftz to *Chip*, but that the Eve HD protein bound poorly, a result possibly attributable to improper folding of the *in vitro*-translated protein. The depicted experiment with Su(Hw) was performed with a fragment lacking approximately one-third of its carboxyl residues [Su(Hw):ΔCTD]; full-length Su(Hw) also bound but less efficiently (not shown). As expected, Gst-*Chip* beads did not bind luciferase (Fig. 1*B*, lane 3), and none of the [³⁵S]proteins bound Gst control beads (Fig. 1*B*, lanes 2 and 9).

The domains of *Chip* involved in homotypic and heterotypic interactions as deduced from our experiments, and from previous experiments with *Chip* and its homologs, are diagrammed in Fig. 1*A*. These include the LIM interaction domain (LID) and the self-interaction domain (SID) (this work and refs. 7, 8, 11, 15, 16, 28, 29). Consistent with the work of others, deletion of the LID reduced interaction with Apterous (*Chip*:Δ472–577, Fig. 1*B*, lane 7). That deletion, however, had no effect on interaction with Bcd, Ftz, Su(Hw):ΔCTD, or *Chip* (Fig. 1*B*, lane 7). In contrast, two other deletion mutants, *Chip*:Δ404–465 and *Chip*:Δ441–454, reduced binding to Bcd, Ftz, Su(Hw):ΔCTD,

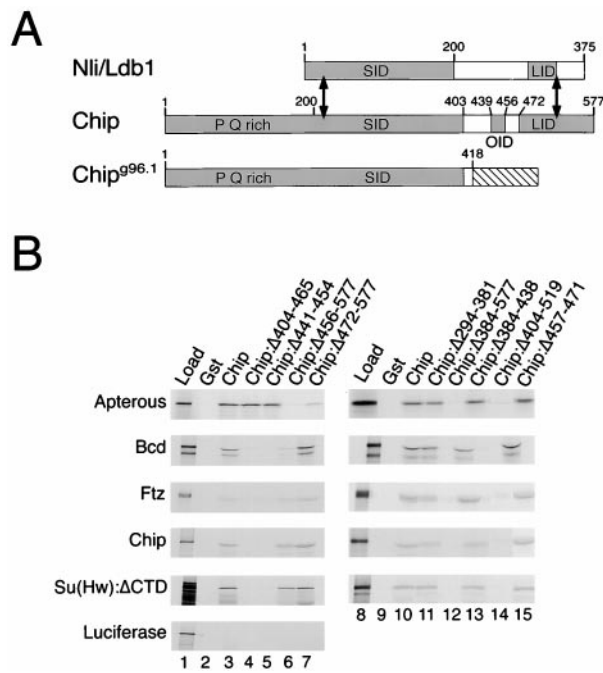


Fig. 1. Chip interacts with HD proteins and the Su(Hw) insulator protein. (A) The homologous region ($\approx 60\%$ identity) of Chip and mouse Nli (Ldb1) is between the double-headed arrows. The protein interaction domains of Chip and Nli are shaded. These are the SID, LID, and OID (this work and refs. 7, 8, 11, 15, 16, 28, and 29). The OID interacts with HD proteins, Chip and Su(Hw). The Chip^{g96.1} mutant contains the SID but lacks the OID and LID. (B) Autoradiograms of SDS-PAGE gels from representative affinity chromatography experiments with the Gst-Chip fusion protein beads indicated at the tops of the lanes and the *in vitro* translated [³⁵S]proteins indicated on the left. The left lanes (Load) contain the amount of input protein, and the second lanes (Gst) contain the amounts retained by beads with Gst alone. The other lanes contain the protein retained by the indicated fusion proteins. Residues deleted from the Chip mutants are indicated after the colons. Apterous is a LIM-HD protein; Bcd and Ftz are HD proteins. Su(Hw): Δ CTD is a Su(Hw) mutant truncated after residue 780, leaving the region containing 12 zinc fingers. Smaller products seen with some [³⁵S]proteins are assumed to result from incomplete translation or proteolysis. All experiments were reproduced at least three times. With all proteins except luciferase, a minimum of 10-fold more protein bound to Gst-Chip beads than to Gst control beads.

and Chip but had little effect on binding to Apterous (Fig. 1B, lanes 4 and 5). On the basis of this and additional deletion mutants (Fig. 1B, lanes 11, 13, and 15), we identified Chip residues 439–456 as the region that interacts with the HD proteins, Su(Hw), and with Chip itself. This region is labeled the other interaction domain (OID) in Fig. 1A.

Previous studies suggested that the SID is sufficient for self-interaction of Chip, but as noted above, Fig. 1B shows that Chip self-interaction was reduced by deletions affecting the OID (Chip: Δ 404–465 and Chip: Δ 441–454, lanes 4 and 5) but unaffected by a deletion that removes much of the SID (Chip: Δ 294–381, lane 11). Further affinity chromatography experiments show that an isolated SID fragment (Chip: Δ 404–519) interacted with itself but did not interact well with intact Chip, whereas a Chip fragment lacking the SID (Chip: Δ 2–381) interacted both with itself and with intact Chip (not shown). The previous experiments showing interactions between the SID and intact Chip were performed by translating the two interaction partners together *in vitro* (16). Evidently, cotranslation permits an interaction not seen by affinity chromatography. We conclude that Chip interacts with itself through both the SID and the OID.

We mapped the portions of Bcd and Su(Hw) that interact with Chip to determine if the OID recognizes a common motif in its

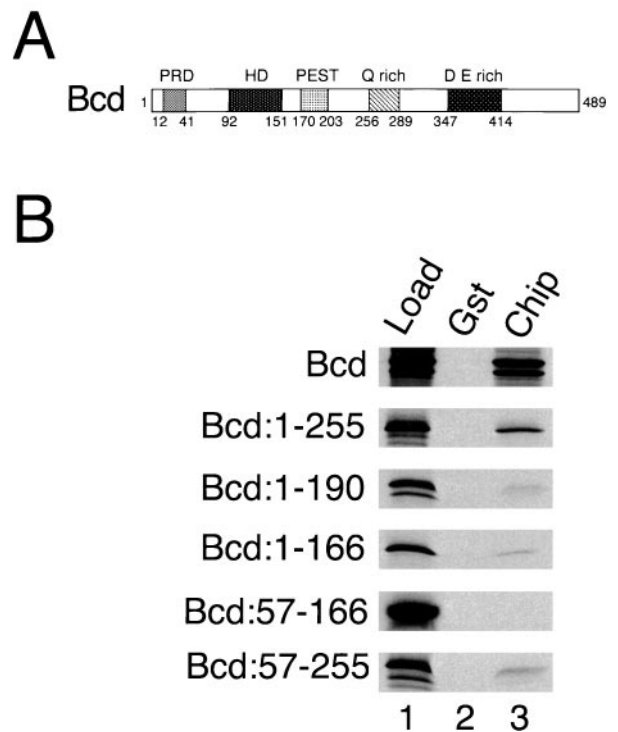


Fig. 2. Regions flanking the Bcd HD interact with Chip. (A) Bcd contains paired repeats (PRD), a HD, a PEST protein degradation motif, glutamine-rich (Q rich), and acidic (D E rich) regions. (B) Autoradiograms of SDS-PAGE gels from representative affinity chromatography experiments with Gst and Gst-Chip beads, and the [³⁵S]Bcd proteins containing the indicated residues on the left.

diverse interaction partners. The N-terminal half of Bcd (residues 1–255) contains the HD (Fig. 2A) and everything needed to rescue *bcd* mutants *in vivo* (30, 31). The N-terminal half of Bcd interacted with Gst-Chip (Fig. 2B, lane 3), whereas the C-terminal half (residues 246–489) did not (not shown). Smaller Bcd fragments containing the HD (residues 1–190, 1–166 or 57–255) bound more weakly than did the 1–255 fragment, and a fragment (residues 57–166) consisting mostly of the HD (residues 92–151) did not bind (Fig. 2B, lane 3). Thus, residues on both sides of the HD are required for strong binding. Similar results were obtained with the Otd HD protein (C.R., D.D., Frieder Schöck, and Herbert Jäckle, unpublished data). The region of Su(Hw) that contains 12 zinc fingers (residues 204–672) interacted with Gst-Chip, whereas the N-terminal region (residues 1–190) and the C-terminal region (residues 706–944) did not (not shown). Mutation of any one of the 12 zinc fingers (21) did not significantly affect binding to Chip (not shown). The regions of Bcd, Su(Hw), and Chip that interact with the Chip OID are not homologous at the primary sequence level.

Chip Supports Bcd HD Protein Activity in *Drosophila* Embryos. The interactions between Chip and HD proteins *in vitro*, as well as other considerations discussed above raised the question of whether Chip affects the activities of HD proteins *in vivo*. We chose to test the effect of Chip on Bcd activity in embryos because both Chip and Bcd are provided maternally and do not regulate each other's expression. Thus, any effect of Chip on Bcd is likely to be direct. The design of the experiment in Fig. 3 showing that, in embryos, reducing Chip activity decreases the activity of a partially defective Bcd protein, was guided by the following considerations.

To demonstrate a helping effect of Chip on Bcd activity, we

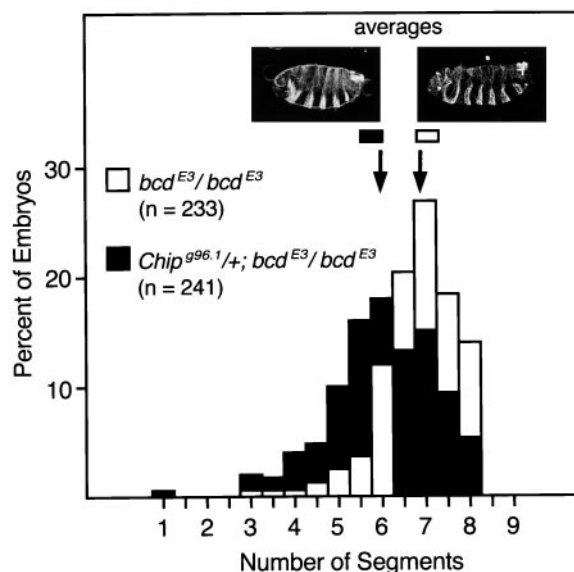


Fig. 3. Chip potentiates Bcd activity in embryos. The histograms show the percentage of embryos with the indicated number of segments produced by each maternal genotype. Embryos from $+/+; bcd^{E3}/bcd^{E3}$ mothers (white bars) crossed to $+/+; +/+$ males produced an average of 6.84 ± 0.06 segments (standard error, $n = 233$), whereas embryos from their $Chip^{96.1}/+; bcd^{E3}/bcd^{E3}$ sisters (black bars) crossed to $+/+; +/+$ males produced an average of 6.02 ± 0.08 segments ($n = 241$). This difference is significant ($P < 0.0001$, unpaired Student's t test), and virtually identical results were obtained in an independent experiment. Cuticles approximating the average phenotypes are shown for each maternal genotype.

could not simply eliminate maternal Chip because that manipulation results in a more severe segmentation defect than does elimination of Bcd itself (1, 32). Nor could we merely halve the dosage of maternal Chip because that change has no effect, even if the maternal Bcd level is also reduced by one-half (not shown). Moreover, zygotic Bcd makes no contribution to segmentation (32), and heretofore no effect has been seen on segmentation by changing the level or nature of zygotically expressed Chip (1). To detect an effect of Chip on Bcd activity, therefore, we reduced the activities of both Bcd and Chip to less than that provided by a single maternal dose of each. This was accomplished by producing doubly mutant mothers: these mothers were homozygous for the bcd^{E3} allele, which encodes a mutant with reduced DNA-binding activity (33), and were also heterozygous for the $Chip^{96.1}$ allele. This latter mutant allele encodes the SID fragment (Fig. 1A), which acts as a dominant negative, inhibiting, but not eliminating, maternal Chip activity. We deduced that the SID fragment inhibits maternal Chip activity from the observations that $Chip^{96.1}/Chip^{96.1}$ embryos produced by $Chip^{96.1}/+$ mothers die before reaching the larval stage (some display a mild segmentation defect), whereas all $Chip^{-}/Chip^{-}$ embryos produced by $Chip^{-}/+$ mothers segment normally and die as larvae. We further deduced that at least one maternal and two zygotic doses of the SID fragment are required to cause embryonic lethality from the fact that $Chip^{96.1}/+$ embryos from $Chip^{96.1}/+$ mothers segment normally and survive to adulthood. Presumably the SID fragment, produced in our experiment both maternally and zygotically, forms nonfunctional multimers with maternal wild-type Chip.

The key result is shown in Fig. 3: on average, embryos from $Chip^{96.1}/+; bcd^{E3}/bcd^{E3}$ mothers (and wild-type fathers) produced nearly one segment less than did embryos from bcd^{E3}/bcd^{E3} mothers (6.02 ± 0.08 vs. 6.84 ± 0.06 , respectively). This effect is almost certainly an underestimate of the effect of Chip

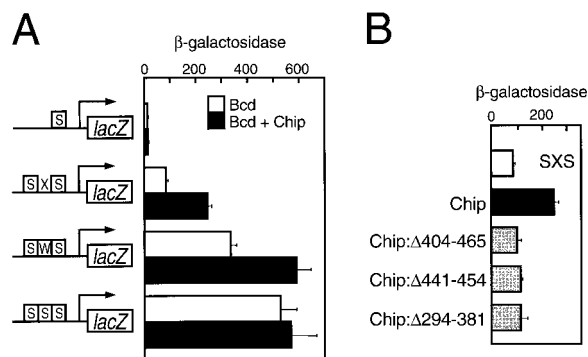


Fig. 4. Chip potentiates transcriptional activation by Bcd in yeast. (A) Effects of Chip on expression of $GAL1-lacZ$ reporter genes with Bcd-binding sites. The reporters are: S, single strong Bcd-binding site; SXS, two strong binding sites flanking a nonbinding sequence; SWS, two strong binding sites flanking a weak binding site; SSS, three tandem strong binding sites (24). The bars indicate the β -galactosidase activity (Miller units per A_{595} Bradford protein unit) produced by yeast containing the reporter genes in the presence of nonsaturating levels of Bcd (induced with 0.5 nM β -estradiol) and in the absence (white bars) or presence (black bars) of Chip. The β -galactosidase levels obtained with the S reporter are >10 -fold higher than the background activity. Error bars are standard errors. (B) The Chip SID and OID are required to increase activation of the SXS reporter. Bars indicate the β -galactosidase activity produced by the SXS $GAL1-lacZ$ reporter gene in the presence of Bcd and the indicated Chip proteins.

on Bcd activity because the embryos are zygotically of two different $Chip$ genotypes, $Chip^{96.1}/+$ and $+/+$, which cannot be distinguished by inspection. Presumably, the $Chip^{96.1}/+$ embryos have more SID fragment and display a larger effect than do the $+/+$ embryos. Moreover, embryos adapt to large changes in Bcd activity (34), and this adaptation may partly counteract the effects of reducing Chip activity.

We deduce that the $Chip^{96.1}$ mutation is solely responsible for the effect on segmentation observed in Fig. 3 because a wild-type $Chip$ gene introduced on a P element (1) completely rescues $Chip^{96.1}$ homozygous embryos (not shown). We cannot formally rule out the possibility that part of the effect of the $Chip^{96.1}$ allele shown in Fig. 3 is due to reduced activities of other HD proteins, even though Bcd was the only HD protein that was mutant in this experiment. If $Chip^{96.1}$ were to substantially reduce the activities of other HD proteins, however, we would expect that posterior segments would be affected. This expectation arises because in contrast to Bcd, many other HD proteins have effects on posterior segments, as does the complete loss of maternal Chip activity. Fig. 3 shows, however, that posterior segments were not affected. We conclude, therefore, that most, if not all of the effect of partially reducing maternal Chip activity observed in Fig. 3 reflects a decrease in Bcd E3 protein activity.

Chip Aids Transcriptional Activation by Bcd from Multiple Bcd-Binding Sites in Yeast.

The experiments of Fig. 4 provide additional evidence that Chip directly affects Bcd activity by showing that, in yeast, Chip can potentiate the transcriptional activating function of Bcd (30, 33) at reporter genes that have multiple Bcd-binding sites. Yeast do not contain endogenous Chip or Bcd homologs, and exogenously added Bcd activates a reporter bearing Bcd-binding sites without contributions from other activators and repressors (unlike the situation with the typical *Drosophila* gene). This experiment thus provides a direct assay of the effect of Chip on Bcd activity. We used various combinations of Bcd-binding sites driving a yeast $GAL1-lacZ$ reporter (Fig. 4A), and a vector producing Bcd under control of a β -estradiol sensitive activator (24). Increasing the β -estradiol concentration increases the amount of Bcd, which in turn

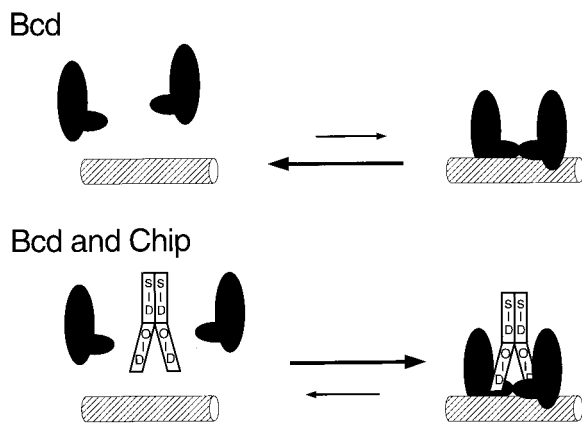


Fig. 5. Model for how Chip potentiates cooperative interactions between Bcd molecules. Bcd interacts cooperatively with itself through residues flanking the HD (*Upper*, ref. 36). We posit that a Chip dimer interacts with two Bcd molecules to fortify the Bcd interactions and increase DNA binding (*Lower*).

increases activation of the reporter. These constructs were previously used to demonstrate that activation of a gene with multiple Bcd-binding sites is cooperative, involving pairwise interactions between Bcd molecules (24).

Activation of a reporter containing three tandem, 11-bp strong Bcd-binding sites (SSS) was approximately half maximal at 0.5 nM β -estradiol, as measured by β -galactosidase activity. Fig. 4*A* shows that under these conditions, activation of a reporter bearing two strong Bcd-binding sites flanking a weak site (SWS) was $\approx 65\%$ of that obtained with the SSS reporter. The figure also shows that two strong sites flanking a nonbinding spacer (SXS) mediated $\approx 15\%$, and a single strong site (S) $\approx 2\%$ the activity observed with the SSS reporter. In the absence of either β -estradiol or the Bcd expression vector, there was $<0.2\%$ of the β -galactosidase activity obtained with the SSS reporter. These results confirm previous observations that activation increases with the strength and number of Bcd-binding sites (24).

Chip had little effect on activation of the SSS reporter by Bcd, but it increased activation of the SWS reporter to the level obtained with the SSS reporter (i.e., nearly 2-fold; Fig. 4*A*). Chip also increased activation of the SXS reporter 2.5- to 3-fold (Fig. 4*A*). Chip did not increase activation with a single binding site (S) (Fig. 4*A*). Chip mutants lacking the OID (Chip: $\Delta 404$ –465 and Chip: $\Delta 441$ –454) or the SID (Chip: $\Delta 294$ –381) did not increase activation by Bcd (Fig. 4*B*), although Western blots confirmed that they were present at levels similar to wild-type Chip (not shown).

Chip did not increase activation above the level that can be obtained with higher concentrations of Bcd alone. For instance, when higher levels of Bcd were induced (using 2.5 nM β -estradiol), expression of the SXS reporter was increased to maximal levels and Chip did not significantly further increase activation (not shown). These results, taken together, are consistent with the idea that the effect of Chip on Bcd activity in yeast is to promote binding of Bcd to multiple sites (Fig. 5).

Discussion

We have shown that Chip has a region, the OID, that interacts *in vitro* with diverse HD proteins (Bcd, Ftz, and Otd) and with the Su(Hw) insulator protein. We have also shown that Chip potentiates the activity of Bcd in embryos and in yeast. These results help explain the broad segmentation and gene expression defects displayed by *Chip* mutants. The interaction between Chip and Su(Hw) supports previous genetic data indicating that Chip and the Su(Hw) insulator protein are antagonists *in vivo*, and

therefore the notion that Chip facilitates enhancer–promoter communication.

Effects of Chip on HD Protein Activity. Our results suggest that Chip increases interactions between Bcd molecules (Fig. 5). Thus, in yeast with nonsaturating levels of Bcd, Chip increased activation by Bcd from two strong binding sites separated by a weak site or by a nonbinding spacer, but not from one or three contiguous strong sites. Moreover, Chip did not increase activation above levels that were achieved with high concentrations of Bcd itself. Bcd binds DNA cooperatively, mediated by interactions of regions overlapping those that interact with Chip (Fig. 5; refs. 24 and 35–37), and we suggest that Chip interacts with Bcd to amplify that cooperativity (Fig. 5). It is unlikely that Chip itself is a transcriptional activator. Previous experiments have shown that Chip does not activate when tethered upstream of yeast promoters but that it can induce activation by recruiting an activation domain fused to LIM domains (1).

The idea that Chip increases interactions between certain other proteins agrees with all previous observations on Chip and its homologs. In transient transfection experiments with mammalian cells, Chip homologs increased transcriptional activation by the combination of the P-Otx HD and the Lhx3 LIM-HD proteins from a promoter containing a single binding site for each molecule (9). The Chip homologs had little effect with P-Otx or Lhx3 alone, indicating that they aid P-Otx-Lhx3 interactions. Furthermore, the nuclear LIM interactor (Nli) homolog of Chip aids formation of different LIM-HD protein dimers *in vitro*, an effect requiring the Nli SID (11). Finally, an Apterous-Chip fusion protein, in which the LIM domains of Apterous are replaced by the Chip SID, can replace wild-type Apterous in *Drosophila* wings, suggesting that Chip aids formation of Apterous dimers *in vivo* (15, 16).

Effects of Chip on Segmentation Gene Expression. Our experiments demonstrate that Chip potentiates Bcd activity in the *Drosophila* embryo when the Bcd activity is low. This effect is consistent with previous studies on the expression of segmentation genes in embryos lacking maternal Chip activity. Embryos contain a gradient of Bcd protein, with a high concentration at the anterior end and a low concentration at the posterior end (38). Loss of maternal Chip strongly reduces all seven blastoderm stripes of *Eve* protein produced by the *eve* pair-rule gene (1). Many, if not all of these stripes are also regulated by Bcd, even though most occur in regions with low to intermediate Bcd concentrations (39). The *eve* stripes are activated by several remote enhancers located ≈ 1.5 –9 kb from the promoter (40, 41) and Bcd-binding sites are critical for activation by at least the stripe 2 enhancer (42, 43). It is likely, therefore, that Chip increases *eve* expression at least in part by increasing binding of Bcd to the enhancers.

Accumulation of the Hb protein encoded by the *hunchback* (*hb*) gap gene is not substantially affected by loss of maternal Chip (1) even though *hb* expression is dependent on Bcd and several Bcd-binding sites just upstream of the promoter (33, 44, 45). This lack of an effect of Chip is not unexpected, however, because *hb* is expressed in the anterior end where the Bcd concentration is the highest (38).

The effects of Chip on Bcd activity that we have demonstrated cannot fully explain the roles of Chip in segmentation because Chip affects all segments (1) whereas Bcd does not (32). Proper formation of every segment does, however, depend on multiple HD proteins, and we show here that Chip also interacts with Otd and Ftz, and it is likely that it interacts with other HD proteins as well. Potentiation of the activities of other HD proteins besides Bcd would explain many of the additional effects of Chip on segmentation.

Effects of Chip on Enhancer–Promoter Communication. Based on observations presented here and elsewhere, we suggest that Chip plays two roles in the regulation of gene expression. First, as discussed above, Chip is likely to aid binding of proteins to enhancers, and second, as previously proposed, Chip is also likely to function between enhancers and promoters to support enhancer–promoter communication (1–3). The *in vitro* interaction between Chip and the Su(Hw) insulator protein shown here is consistent with the notion that Su(Hw) is directly antagonistic to Chip activity as previously demonstrated genetically at the *cut* locus (2).

It remains to be seen how, if our speculations are correct, Chip facilitates enhancer–promoter communication and how that communication is disrupted by Su(Hw). It is believed that Su(Hw) blocks activation not by reducing the binding of proteins to enhancers, but rather by hindering enhancer–promoter communication (see reviews in refs. 3 and 6). For instance, an enhancer blocked in its interaction with one promoter by Su(Hw) can nevertheless activate a second promoter located on the opposite side of the enhancer from Su(Hw) (46, 47). Thus, although Su(Hw) is antagonistic to Chip, it is unlikely to affect binding of proteins to enhancers. It is also unlikely that Chip functions merely by preventing binding of Su(Hw) to gypsy because Chip is also important for the expression of several genes, e.g., *cut* and *eve*, in the absence of gypsy and Su(Hw) (1, 2)

It has been suggested that, *in vivo*, different HD proteins, including Bcd and Ftz, bind both to sites in the *eve* stripe enhancers and to many sites between the enhancers and the promoter (48, 49). It is conceivable that Chip aids binding of Bcd and Ftz to the sites between the enhancers and the promoter as well as to the sites in the enhancers. This could help form a series of loops that brings the enhancers and the promoter closer together, or help HD proteins bind near the promoter to serve as surrogate activators (3). The latter possibility is similar to the “linking” model put forth to explain long range activation of human β -globin genes by their locus control region (50). According to either scenario, Su(Hw) would block enhancer–promoter communication by interacting with the Chip molecules that function between the enhancer and the promoter and somehow interfering with their activities.

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