

Cap 'n' collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress Deformed homeotic function in the *Drosophila* head

Alexey Veraksa¹, Nadine McGinnis¹, Xuelin Li¹, Jym Mohler² and William McGinnis^{1,*}

¹Department of Biology, University of California, San Diego, La Jolla, CA 92093, USA

²Department of Biological Sciences, Barnard College, New York, NY 10027, USA

*Author for correspondence (e-mail: mcginnis@biomail.ucsd.edu)

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SUMMARY

The basic-leucine zipper protein Cap 'n' collar B (CncB) suppresses the segmental identity function of the Hox gene *Deformed* (*Dfd*) in the mandibular segment of *Drosophila* embryos. CncB is also required for proper development of intercalary, labral and mandibular structures. In this study, we provide evidence that the CncB-mediated suppression of *Dfd* requires the *Drosophila* homolog of the mammalian small Maf proteins, Maf-S, and that the suppression occurs even in the presence of high amounts of *Dfd* protein. Interestingly, the CncB/Maf-S suppressive effect can be partially reversed by overexpression of Homothorax (Hth), suggesting that Hth and Extradenticle proteins antagonize the effects of CncB/Maf-S on *Dfd* function in the mandibular segment. In embryos, multimers of simple CncB/Maf-S heterodimer sites are transcriptionally activated in response to CncB, and in tissue culture cells the amino-terminal domain of CncB acts as a strong transcriptional activation domain. There are no

good matches to CncB/Maf binding consensus sites in the known elements that are activated in response to *Dfd* and repressed in a CncB-dependent fashion. This suggests that some of the suppressive effect of CncB/Maf-S proteins on *Dfd* protein function might be exerted indirectly, while some may be exerted by direct binding to as yet uncharacterized *Dfd* response elements. We also show that ectopic CncB is sufficient to transform ventral epidermis in the trunk into repetitive arrays of ventral pharynx. We compare the functions of CncB to those of its vertebrate and invertebrate homologs, p45 NF-E2, Nrf and Skn-1 proteins, and suggest that the pharynx selector function of CncB is highly conserved on some branches of the evolutionary tree.

Key words: *Drosophila*, Cnc, Maf, Deformed, Hox, Homeotic, Pharynx

INTRODUCTION

Segmental diversification of the posterior head is a part of a conserved genetic system for anterior-posterior patterning of a metameric animal body, in which homeotic selector genes play a central role (Lawrence and Morata, 1994; Mann and Affolter, 1998; McGinnis and Krumlauf, 1992). In *Drosophila*, Hox genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*) and *Sex combs reduced* (*Scr*) are required for determining the identity of the head segments (Popadic et al., 1998). Their action is assisted by many other head-specific genetic functions, whose role is to elaborate on Hox signals and to specify the differentiation of head-specific structures (Rogers and Kaufman, 1997). One such function is encoded in the CncB protein isoform. In a previous study, we showed that the *cnc* locus encodes three transcript and protein isoforms. The *cncB* transcript is expressed in an embryonic pattern that includes the labral, intercalary and mandibular segments, while *cncA* and *cncC* are expressed ubiquitously (McGinnis et al., 1998; Mohler et al., 1991). Mutant embryos with reduced levels of

CncB protein have a duplicated set of mouth hooks and cirri in place of mandibular structures (Harding et al., 1995; McGinnis et al., 1998; Mohler et al., 1995). Mouth hooks and cirri derive from the maxillary segment and are specified by the *Dfd* segmental identity function. Transient ectopic expression of CncB can repress *Dfd* function in the maxillary segment, inducing a loss of normal maxillary structures, while having no effects on the functions of homeotic proteins in the trunk (McGinnis et al., 1998). The *cncB* loss-of-function phenotype has therefore been interpreted as a homeotic transformation in which the maxillary-promoting activity of *Dfd* is derepressed in the mandibular segment.

Lower levels of CncB function also result in other head defects, including the loss of the median tooth and dorsal pharynx (labrum-derived structures), loss of the floor of the pharynx (an intercalary segment-derived structure), and loss of the anterior lateralgraten and the base of the mouth hook. The lateralgraten and mouth hook base both originate in part from the posterior mandibular segment and both require *Dfd* and CncB function for normal development. Thus, in addition to

CncB's role in suppressing the maxillary-promoting function of Dfd, it is also required to promote the development of labral, intercalary and posterior mandibular structures (McGinnis et al., 1998; Mohler et al., 1995). The mechanisms by which CncB accomplishes these functions are poorly understood.

All three *Drosophila* Cnc protein isoforms contain a basic-leucine zipper region that is highly homologous to the p45 subunit of mammalian NF-E2 (Andrews et al., 1993a; Mohler et al., 1991). Numerous studies have demonstrated transcriptional activation properties of CNC-class b-Zip proteins from mammals: e.g. p45 NF-E2 (Amrolia et al., 1997; Bean and Ney, 1997; Deveaux et al., 1997; Nagai et al., 1998), TCF11/LCR-F1/Nrf1 (Johnsen et al., 1998), ECH/Nrf2 (Alam et al., 1999; Itoh et al., 1997, 1995; Wild et al., 1999) and Nrf3 (Kobayashi et al., 1999). Such activation has been shown to be dependent on heterodimerization of the CNC family members with small Maf subunits (reviewed in Blank and Andrews, 1997; Motohashi et al., 1997) and on the presence of specific heterodimer binding sites in target enhancers. In addition to NF-E2-like consensus sites, enhancers targeted by CNC family members contain other sites that are required for full activity and bound by cofactors such as GATA-1, Ets-like proteins and Sp1 (see Magness et al., 2000; Deveaux et al., 1997; Stamatoyannopoulos et al., 1995 and references therein). Only those CNC class proteins that contain BTB domains, such as Bach1 and Bach2, have been shown to function primarily as transcriptional repressors (Igarashi et al., 1998; Muto et al., 1998; Oyake et al., 1996).

In this study we have investigated the requirements for CncB function in *Drosophila* embryos, and find that it acquires sequence-specific DNA binding, and biological function, in combination with a *Drosophila* homolog of vertebrate small Maf proteins. We find that among the three Cnc isoforms, only CncB is involved in specification of embryonic head skeletal structures. The CncB protein contains a strong transcriptional activation domain and can activate simple response elements in vivo. We provide several lines of evidence suggesting that CncB-mediated repression on at least some Dfd response elements is likely to be indirect. Finally, we have found that CncB is sufficient for the development of ventral pharyngeal structures. Based on these results, we propose a model explaining the diverse functions of CncB in anterior-posterior patterning of the *Drosophila* embryonic head, and discuss possible links between genetic systems involving CNC family members in *Drosophila* and other organisms.

MATERIALS AND METHODS

EMS mutagenesis and identification of mutations by DGGE

Flies were reared on a standard medium at 25°C. To generate new *cnc* alleles, *ve st e ca* males were fed ethylmethanesulfonate (EMS), according to Grigliatti (1986). Mutagenized males were crossed en masse to virgin *TM3 Sb / TM6B Hu Tb* females, and *ve st e ca* * / *TM6B* males were then singly crossed to virgin *cnc^{VL110} / TM6B* females. Candidate *cnc* mutants were identified from these crosses by the absence of empty non-*Tb* pupal cases. A total of 8600 chromosomes were analyzed, and eight new lethals recovered.

Broad-range denaturing gradient gel electrophoresis (DGGE) was used to identify locations of the new *cnc* mutations, following the procedure described in Guldberg and Güttler (1994). All three Cnc

reading frames were covered by overlapping fragments of 300–600 bp that were PCR-amplified from genomic DNA extracted from heterozygous adults. The fragments were chosen using MacMelt software (BioRad). In each amplification reaction, one of the primers contained a 36-nucleotide 5' GC-clamp (Myers et al., 1985). PCR products were resolved using the D-GENE apparatus (BioRad) on a 0–80% denaturant 6% polyacrylamide gel run at 60°C according to the manufacturer's instructions. Candidate fragments containing mutations were sequenced on both strands along with parental lines to identify exact locations of mutations, which are summarized in Table 1.

Cuticular preparations, in situ and antibody staining

Heat-shock treatment: embryos were collected on apple juice/agar plates for 2–4 hours at 25°C, aged for 6 hours, then the plates were immersed in a 37°C water bath for 1 hour. After heat shock, embryos were immediately analyzed for mRNA expression or aged over 24 hours for cuticular preparations. Antibody and in situ staining and cuticular preparations were done as previously described (McGinnis et al., 1998).

Double stranded RNA interference (dsRNAi)

RNAs were synthesized from PCR products containing a T7 promoter sequence on each side, according to Kennerdell and Carthew (1998). The presence of double-stranded self-annealed products was verified on an agarose gel. RNAs were complementary to cDNA sequences spanning the following nucleotides: 22–464 for CncA (GenBank accession AF070062), 117–498 for CncB (GenBank accession AF070063), 61–994 for CncC (GenBank accession AF070064), 45–601 for Maf-S (GenBank accession AI238611). All three Cnc fragments contained 5' UTR sequences, while the Maf-S RNA covered the whole open reading frame plus parts of the 5' and 3' UTR. dsRNA was injected into the head region of precellular blastoderm embryos at a concentration of 0.5 µg/µl of the double-stranded product. Injected embryos were then aged for 26–28 hours at 25°C and analyzed for cuticular phenotypes.

Cnc/Maf binding site selection and electrophoretic mobility shift assays

Full-length CncA, CncB and Maf-S proteins were cloned into the pSP64ATG or pSP64Flag vectors (a gift from C. Murre). For site selection and EMSA, the proteins were produced using a TNT in vitro transcription/translation system (Promega). Site selection protocol followed the procedure of Neuteboom and Murre (1997), except the first four washes were performed using the salt concentration of 50 mM KCl/50 mM NaCl, and the final two washes used 50 mM KCl/250 mM NaCl. For site selection, Flag-tagged CncA or CncB were cotranslated with the untagged Maf-S. A 62-mer oligonucleotide used in site selection procedure was 5'-GTAAGCAGCGCCAGTGA-ATTCT(N)₁₄GTCTAGAGTGCTGACTGGGAAAAC, where (N)₁₄ indicates a core of 14 ambiguous positions. Primers A (5'-GTAAGCAGCGCCAGT) and B (5'-GTTTTCCAGTCAGCAC) were used for amplification of the 62-mer. After 6 cycles of binding, washes and amplification, the resulting pool of oligonucleotides was digested with *EcoRI* and *XbaI* and cloned into pBluescript (Stratagene). 20 clones were randomly chosen and sequenced.

Electrophoretic mobility shift assays were performed as in Neuteboom and Murre (1997). The double-stranded Cnc/Maf binding site oligonucleotide (CBS) was 5'-GGTCTGCTGAGTCATCGTG (consensus binding site is underlined), and the mutant Cnc/Maf binding site oligonucleotide (mCBS) was 5'-CTTCTTGACGGACTCGTGGAATT. To assay heterodimer binding, Cnc proteins and Maf-S were cotranslated, though we observed no decrease in binding when separately translated proteins were mixed.

Regulatory element and protein expression constructs

Transgenic lines were established using standard methods (Spradling,

1986), and multiple lines were analyzed for each construct. All fragments made by PCR were verified by sequencing. To generate CncB^{Ala}, a part of CncB was amplified in a PCR reaction using a primer containing the nucleotide substitutions required to change the IRRRGKKNKV sequence in the DNA binding domain of CncB to IRAAGAAKV (Fig. 1E). Full-length CncB^{Ala} was then regenerated by substituting a wild-type part of the sequence with the mutated fragment. This cDNA was subcloned into pHSBJ-CaSpeR (Jones and McGinnis, 1993) for heat-shock induction in the embryos and into pSP64ATG for in vitro translation. *P{hs-cnc.A}* (HS-CncA) and *P{hs-cnc.B}* (HS-CncB) were described in McGinnis et al. (1998). Descriptions of published transgenic lines can also be found in FlyBase (The FlyBase Consortium, 1999). *P{UAS-hth.P}* (UAS-Hth) was a gift from Y. Henry Sun. A full-length Maf-S open reading frame is present in the clone GH14688 (GenBank accession AI238611), which is available from Research Genetics. *P{UAS-Dfd.L}* (UAS-Dfd) is described in Li et al. (1999a). GAL4 drivers are described in the following references: *P{GawB}69B* (69B-GAL4), Brand and Perrimon (1993); *P{GAL4-arm.S}* (arm-GAL4⁴), Sanson et al. (1996); *P{GAL4-Hsp70.PB}* (HS-GAL4), Brand et al. (1994).

To make *P{CBS Dfd-lacZ.4CE}* (4CE), a 20-bp oligonucleotide containing the Cnc/Maf binding site (CBS) was cloned into *PstI/EcoRI* sites of plasmid pB-E upstream of module E (Zeng et al., 1994) to generate pB-CE. The resulting CE cassette was quadruplicated in tandem to generate 4CE and cloned into the *XbaI/XhoI* sites of CZIII lacZ reporter vector (Zeng et al., 1994). *P{mCBS Dfd-lacZ.4ME}* (4ME) was created in the same way, except the mutant Cnc/Maf binding site (mCBS) was used in place of CBS. To make *P{CBS-lacZ.4C}* (4C), a 40-bp oligonucleotide containing two copies of CBS was cloned in tandem into the *EcoRI* site of pBluescript, and head-to-tail orientation was selected by sequencing individual clones. The final cassette was then cloned into *XbaI/XhoI* sites of CZIII.

To make a GAL4 DNA binding domain (DBD)::CncB-specific domain chimera, PCR-generated CncB-specific domain (amino acids 1-272) was ligated with GAL4 DBD in pBXG1 vector (Quong et al., 1993). This construct was cotransfected with the UAS-luciferase reporter into Bosc23 cells, and enzyme activity was measured as previously described (Li et al., 1999a).

P{UAS-cnc.B} (UAS-CncB) was created by subcloning an *EcoRV* fragment from a full-length CncB cDNA (McGinnis et al., 1998) into the pUAST vector (Brand and Perrimon, 1993). To make a *P{UAS-VP16::cnc.A}* (UAS-VP16-CncA) expression construct, CncA (common) region was ligated with the VP16 fragment from pGEX-VP16 (Li et al., 1999a) and inserted into the pUAST vector, so that VP16 is at the amino-terminal end. To test for coexpression effects of CncB and Dfd in *Dfd^{w21}* mutant background, the following chromosomes were prepared by recombination and combined by crossing appropriate lines: *UAS-Dfd Dfd^{w21}*, *arm-GAL4⁴ Dfd^{w21}* and *UAS-CncB UAS-Dfd Dfd^{w21}*. To test for coexpression effects of Cnc

proteins and Hth, *HS-CncA*; *HS-GAL4* or *HS-CncB*; *HS-GAL4* flies were crossed to *HS-CncA*; *UAS-Hth* or *HS-CncB*; *UAS-Hth*, respectively.

RESULTS

New *cnc* mutations and RNAi confirm the key role of the CncB isoform in head development

We have previously shown that the *cnc* locus encodes three transcript and protein isoforms: CncA (whose amino acid sequence is included in all Cnc protein isoforms), CncB and CncC (McGinnis et al., 1998). Based on our genetic and molecular analysis, only one isoform, CncB, was implicated in the suppression of Dfd function and formation of the intercalary and labral structures. However, available mutations in the *cnc* gene were limited to P element insertion and excision lines, disrupting the function of the whole locus (Mohler et al., 1995), and to three EMS alleles identified in a *Dfd* modifier screen that were not associated with sequence polymorphisms in the open reading frames of the Cnc isoforms (Harding et al., 1995; McGinnis et al., 1998). We therefore screened for additional EMS-induced lethals at the *cnc* locus to better understand the function of individual isoforms. Eight new lethal mutations were identified that failed to complement *cnc^{VL110}*, a deletion removing the common *cnc* exons. One of these mutations turned out to be a complex chromosomal rearrangement and was not analyzed further. The remaining seven chromosomes were analyzed for embryonic cuticular phenotype as well as for Cnc mRNA and protein levels as transheterozygotes over *cnc^{VL110}* (Table 1). The mutants were also analyzed for the presence of nucleotide changes in the open reading frames and adjacent intronic regions covering all three Cnc isoforms. We were able to identify the nature of mutations in four of these alleles by using PCR amplification, denaturing gradient gel electrophoresis, and sequencing of the candidate mutation-containing fragments (see Materials and Methods; schematic locations of mutations are shown in Fig. 1A).

Four of the new alleles (*cnc^{C5}*, *cnc^{C29}*, *cnc^{G15}* and *cnc^{K22}*) displayed a strong *cncB* mutant phenotype (Mohler et al., 1995; McGinnis et al., 1998), characterized by duplicated mouth hooks (Fig. 1B, arrow), loss of median tooth, shortened lateralgraten and loss of the posterior pharynx (Fig. 1B, arrowhead; compare to Fig. 1C). The mutations in *cnc^{C5}* and *cnc^{C29}* are predicted to result in truncated forms of all Cnc isoforms (Fig. 1A, Table 1),

Table 1. New *cnc* alleles: phenotypes, protein and mRNA levels, and molecular nature of mutations

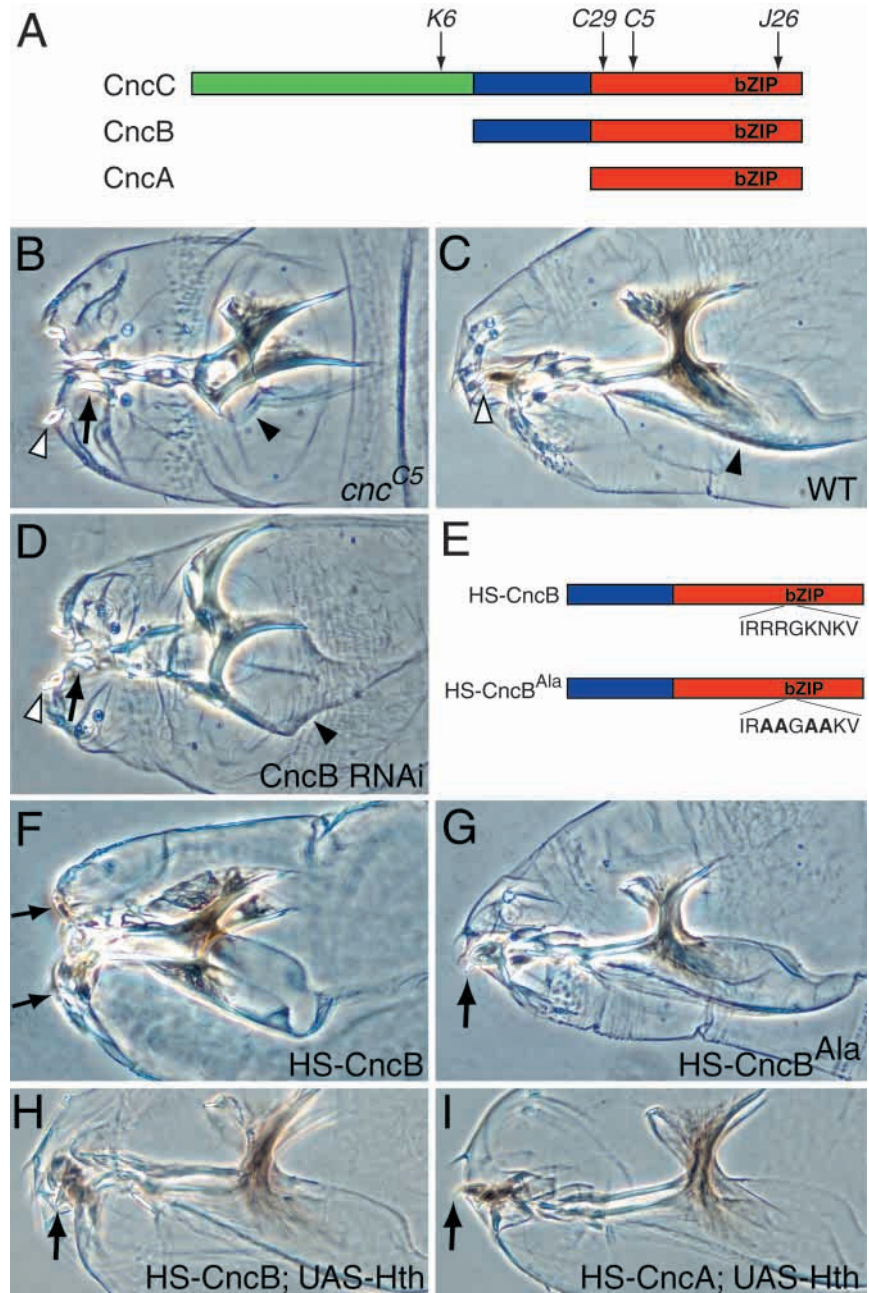
<i>cnc</i> allele	Embryonic cuticular phenotype over <i>cnc^{VL110}</i>	Cnc mRNA levels in mutants*	Protein levels in mutants*	Type of mutation
<i>C5</i>	Null	Slightly reduced	Absent	CAG(Gln881)‡ to TAG(Stop)
<i>C29</i>	Null	WT	Absent	Deletion with frameshift at Gly810
<i>G15</i>	Null	Strongly reduced	Absent	Not in ORF
<i>K22</i>	Null	Moderately reduced	Absent	Not in ORF
<i>C15</i>	Hypomorph	WT	WT	Not in ORF
<i>J26</i>	Hypomorph	WT	Slightly reduced	G to A in donor splice site at Gln1177
<i>K6</i>	WT	WT	WT	CAA(Gln471) to TAA(Stop)

WT, wild type.

*Probed with RNA antisense probe and antibody that are specific for the common (CncA) region. The levels indicated refer to head-specific expression of CncB.

‡Amino acid numbers are for the CncC isoform (McGinnis et al., 1998). See also Fig. 1A for schematic locations of identified mutations.

Fig. 1. Requirements for CncB function in head cuticle development. In all figures, anterior is to the left and dorsal is up, unless indicated otherwise. (A) Schematic locations of new EMS-induced mutations in *cnc* (see also Table 1). (B) Mutant cuticular phenotype of *cnc^{C5}* in which *cncA*, *B* and *C* functions are missing, i.e. equivalent to a deletion of the locus. The same phenotype is observed for *cnc^{C29}*, *cnc^{G15}* and *cnc^{K22}*. A pair of mouth hooks (Dfd-dependent structures) that derive from the maxillary segment is indicated by a white arrowhead. Ectopic mouth hooks arising from the mandibular segment are indicated by an arrow. A black arrowhead points to the missing pharynx. (C) Wild-type cuticular phenotype. Mouth hooks (white arrowhead) and pharynx (black arrowhead) are well formed. (D) dsRNA interference phenotype obtained with *cncB*-specific dsRNA. This phenotype resembles that observed in *cnc^{C5}* embryos (compare D and B), and is not seen when dsRNA specific to the *cncA* or *cncC* transcripts is injected. (E) Diagram of the heat-shock CncB construct (HS-CncB) and a version with mutated DNA binding residues (HS-CncB^{Ala}). (F) Heat-shock CncB phenotype. Embryos were heat shocked for 1 hour between 6 and 8 hours of development. Underdeveloped mouth hooks are indicated by arrows. Note also that head involution is incomplete and the head skeleton is misshapen. (G) Heat-shock CncB^{Ala} phenotype. Head skeletal elements develop normally (arrow points to fully developed mouth hooks). (H) A heat-shocked HS-CncB; HS-GAL4/UAS-Hth embryo. Overexpression of Homothorax largely rescues the repressive effects of CncB protein on Dfd function, as detected by nearly normal mouth hooks (arrow) and other head skeletal features (compare to F). (I) A heat-shocked HS-CncA; HS-GAL4/UAS-Hth embryo. This control embryo shows that overexpression of Hth does not lead to head defects (overexpression of CncA alone has no effect on head development; McGinnis et al., 1998).



while no mutations were detected in the open reading frames of the *cnc^{K22}* and *cnc^{G15}* alleles. In all four strong alleles, no protein is detected with an antibody specific for the Cnc common region (Table 1). The levels of CncB mRNA were normal or only slightly reduced in *cnc^{C5}* and *cnc^{C29}*, moderately reduced in *cnc^{K22}* and strongly reduced in *cnc^{G15}*. This suggests that *cnc^{G15}* has a mutation affecting a regulatory region required for the transcription of all *cnc* isoforms, and *cnc^{K22}* has a mutation affecting mRNA abundance as well as the accessibility of the mRNA to the translation apparatus.

The remaining three alleles (*cnc^{C15}*, *cnc^{J26}* and *cnc^{K6}*) are hypomorphs. *cnc^{C15}* and *cnc^{J26}* mutant embryos each show mild defects in lateralgraten and mouth hooks, but have normal median teeth and pharynx (data not shown). The mutation in *cnc^{C15}* is not in the open reading frame, whereas *cnc^{J26}* has a

sequence change that affects the donor splice site in the last intron of the common region, which is predicted to change the carboxy-terminal sequence of all three isoforms downstream of the leucine zipper. *cnc^{J26}* may affect translation efficiency or protein stability, as it results in slightly lower protein levels without any effect on mRNA levels (Table 1). *cnc^{K6}* homozygotes have normal embryonic morphology (data not shown). Interestingly, the molecular defect in *cnc^{K6}* is a nonsense mutation in the CncC-specific region, which is predicted to truncate CncC protein, without affecting CncA or CncB. The absence of cuticular defects in the *cnc^{K6}* mutant strongly suggests that the CncC isoform is not required for the development of embryonic head skeleton. Since *cnc^{K6}* is lethal over *cnc^{VL110}*, CncC is apparently required for some other essential function at later stages.

Since each Cnc transcript has at least one unique 5' exon (McGinnis et al., 1998), the method of double-stranded RNA interference (dsRNAi; Fire et al., 1998; Kennerdell and Carthew, 1998) allowed us to test the function of each isoform independently. Double-stranded RNA fragments specific for the 5' UTR of CncA, CncB or CncC (see Materials and Methods) were injected into the head region of precellular blastoderm embryos, and cuticular preparations were made to assess the extent of head skeleton development. For both CncA- and CncC-specific injections, the first instar larval hatch rate was comparable to control injection of buffer alone (40% of all injected embryos), and in the unhatched embryos, no consistent head defects were detected. The absence of any head phenotype and high survival rate of CncC-injected embryos agrees with the *cncC^{K6}* mutant data. The normal morphology of CncA-RNAi-injected embryos suggests that this isoform is not required for embryonic morphological development, but in the absence of CncA specific mutants, the lack of a dsRNAi-induced phenotype might also mean that dsRNAi was incapable of influencing CncA expression in our experiments.

The hatch rate for CncB-specific injections was significantly lower than in controls (less than 1%). Embryonic cuticles displayed partial or complete duplication of mouth hooks, and approximately 10% had a phenotype indistinguishable from strong mutations in *cncB* (Fig. 1D, compare to B). We also observed a similar phenotype when CncB dsRNA was injected into the posterior of the embryo. The percentage of severe head transformations in this case was slightly lower than for head-injected embryos. The results from the dsRNA interference experiments, combined with the analysis of the new *cnc* mutants, provide strong evidence that among the three Cnc isoforms, only CncB plays a role in specifying the development of the embryonic head skeleton, while CncA and CncC perform other functions.

DNA binding residues are required for the phenotypes produced by ectopic CncB

The presence of a basic-leucine zipper region in all three Cnc isoforms, including CncB, suggests that they function as DNA binding proteins. However, several experiments have indicated that p45 NF-E2, a mammalian protein that is closely related to the *Drosophila* Cnc isoforms, is involved in functional protein-protein interactions that do not require DNA binding (Cheng et al., 1997; Gavva et al., 1997). To test whether CncB activities are dependent upon DNA binding function, we generated a transgenic strain with a heat-shock-inducible form of CncB in which alanines were substituted for conserved residues in the basic domain (construct HS-CncB^{Ala}; Fig. 1E). Mutations of these residues abolish DNA binding in other b-Zip proteins (Carroll et al., 1997), as well as CncB (see Fig. 4C). Heat-shock-induced ubiquitous expression of wild-type CncB between 6 and 8 hours of development is lethal for 99% of embryos, and most lack mouth hooks and other Dfd-dependent structures in their defective heads (Fig. 1F, arrows) (McGinnis et al., 1998). When HS-CncB^{Ala} is expressed under the same conditions, about half of the embryos hatch, and the embryos that fail to hatch have normal head morphology (Fig. 1G). As measured by embryonic immunostaining and quantitative western blots, the mutant protein is present at the same level as wild-type protein, and is concentrated in nuclei (data not shown). Therefore, the residues in the b-ZIP region of CncB

that provide its DNA binding function are required both for the repression of Dfd-dependent structures as well as for the other cuticular defects resulting from ectopic expression.

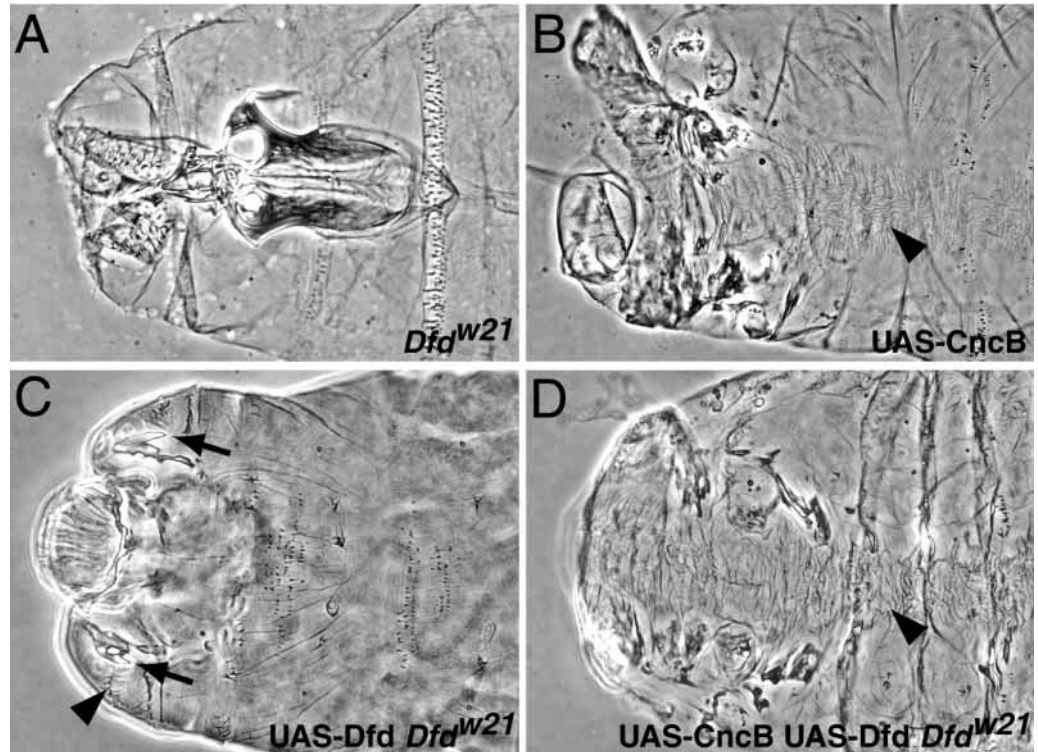
Overexpression of Homothorax rescues the ectopic CncB phenotype

Formation of maxillary structures such as mouth hooks depends on the activation of downstream target gene transcription by Dfd protein (Zhu and Kuziora, 1996). This activation has been shown to depend on parallel input from Extradenticle (Exd) and other cofactors (Li et al., 1999b; Pinsonneault et al., 1997; Zeng et al., 1994). A MEIS-like homeodomain protein Homothorax (Hth) is required for the normal expression levels and the nuclear localization of Exd, and has also been recently shown to participate in trimeric complexes with Hox proteins and Exd (Ryoo et al., 1999). Since Exd and Hth act in parallel to potentiate the transcriptional activation function of Dfd, and CncB appears to do the opposite, we asked whether increasing the levels of Hth and therefore of nuclear Exd would affect the ability of CncB to repress Dfd-dependent structures. When CncB and Hth are both ectopically expressed throughout the embryo, a reduction in severity of all aspects of the CncB ectopic phenotype is observed. Although overexpression of CncB and Hth still results in embryonic lethality, most head skeletal elements are close to wild type, and mouth hooks and other Dfd-dependent structures are seen in all embryos (Fig. 1H, compare to F). Control embryos that coexpress CncA (which has no effect on head development) and Hth under the same conditions show mild or no head defects (Fig. 1I). We conclude from this experiment that increasing the concentration and nuclear localization of Dfd activating cofactors such as Exd and Hth can partially rescue the repressive effects of CncB.

CncB can suppress Dfd function in a way that is independent of its effects on Dfd transcription

Previous results showed that CncB acts as a selective suppressor of Dfd function, and can also act to repress *Dfd* transcription (McGinnis et al., 1998). It was still unclear, however, whether CncB-mediated repression of Dfd-dependent maxillary structures actually requires silencing *Dfd* transcription. To answer this question, we ectopically expressed CncB and Dfd proteins in *Dfd^{w21}* homozygous mutant embryos, which lack the endogenous Dfd protein (Zeng et al., 1994). As shown in Fig. 2A, *Dfd^{w21}* mutants lack mouth hooks, cirri and other Dfd-dependent structures (compare to Fig. 1C). When Dfd protein is provided in the *Dfd^{w21}* mutant embryos from a *UAS-Dfd* construct under the control of the *arm-GAL4⁴* driver, maxillary mouth hooks and cirri are restored to near-normal morphology, and some maxillary structures are produced in other segments (Fig. 2C). The rescue of the *Dfd^{w21}* mutant phenotype by Dfd protein under the control of the *arm* driver is suppressed when CncB protein is coexpressed with Dfd using the same driver (Fig. 2D) – mouth hooks, cirri and other Dfd-dependent structures are missing or severely underdeveloped. This is similar to the phenotype obtained when CncB is driven by *arm-GAL4⁴* in otherwise wild-type embryos (Fig. 2B). It is possible that CncB might interfere with Dfd expression from the GAL4-induced heterologous promoter by interfering with the activation of the UAS in the *UAS-Dfd* construct. We tested this by staining the

Fig. 2. Dfd modulator function of CncB. (A) *Dfd^{w21}* homozygous mutant embryo (ventral view). Dfd-dependent structures such as mouth hooks and cirri are missing (compare to Fig. 1C). (B) A ventrolateral view of the head of an *arm-GAL4/UAS-CncB* embryo. Note the loss of Dfd-dependent maxillary structures. Persistent expression of ectopic CncB obtained with the *arm-GAL4* driver has a much stronger effect on cuticular morphology than the transient treatment in HS-CncB embryos (compare to Fig. 1F). *arm-GAL4/UAS-CncB* embryos also display a ventral midline transformation (arrowhead), which is discussed later (see Fig. 8). (C) *Dfd^{w21}* mutant embryo in which Dfd is ubiquitously expressed using *arm-GAL4* (*arm-GAL4 Dfd^{w21}/UAS-Dfd Dfd^{w21}*). Mouth hooks (arrows) and cirri (arrowhead) are rescued (compare to A). Head involution is incomplete due to the effects of ubiquitous expression of Dfd in other head segments. (D) *Dfd^{w21}* mutant embryo in which Dfd and CncB are ubiquitously expressed using *arm-GAL4* (*arm-GAL4 Dfd^{w21}/UAS-CncB UAS-Dfd Dfd^{w21}*). Note that the rescue of mouth hooks and cirri by *UAS-Dfd* is inhibited by ectopic expression of CncB (compare to C). The ventral midline is also transformed (arrowhead; compare to B).



ectopic CncB, ectopic Dfd embryos, and found that Dfd protein is produced at similar levels and localized in nuclei, as seen in controls in which no ectopic CncB is present (data not shown).

Thus, even when Dfd protein is persistently expressed from a heterologous promoter, its maxillary-promoting function is inhibited if CncB protein is present in the same cells. The normal repression of *Dfd* transcription observed in anterior mandibular cells in wild-type embryos may be a secondary effect resulting from CncB dependent inhibition of Dfd protein function on the *Dfd* autoactivation circuit (Kuziora and McGinnis, 1988).

The function of CncB requires heterodimerization with a small Maf subunit

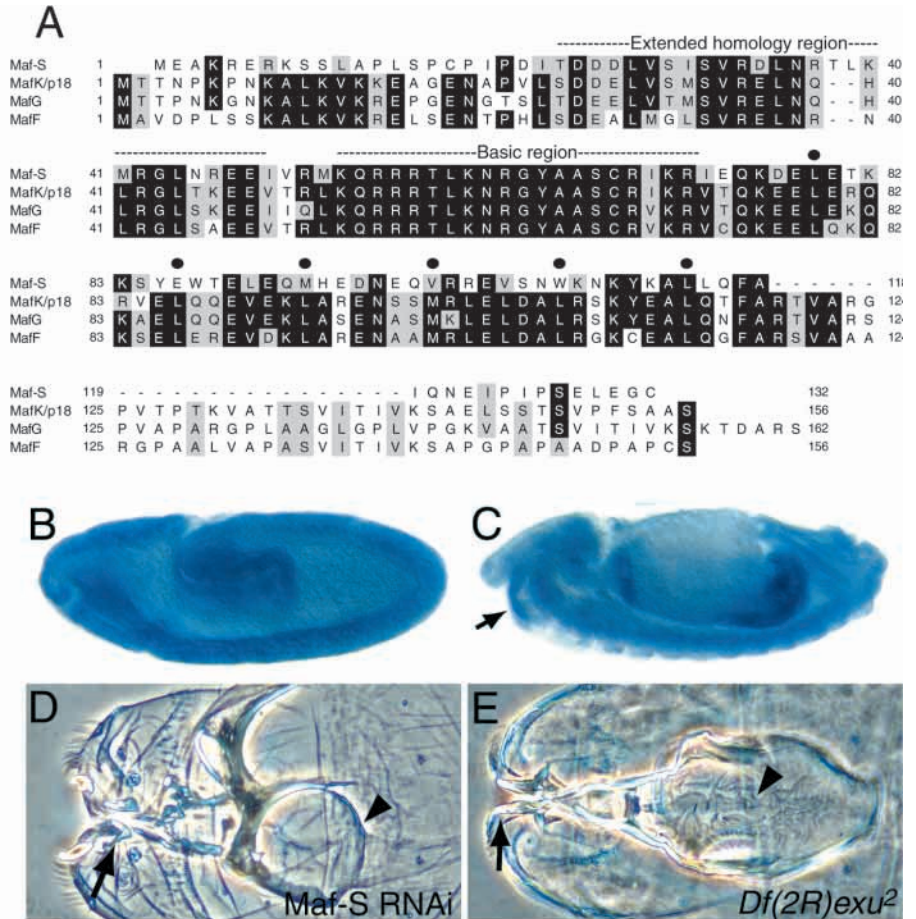
Mammalian homologs of Cnc (p45 NF-E2 and Nrf proteins) were shown to bind DNA as obligate heterodimers with small Maf proteins (MafK/p18, MafF and MafG) (Andrews et al., 1993b; Igarashi et al., 1994; Johnsen et al., 1996). An apparent *Drosophila* ortholog of the mammalian small Mafs was identified in a yeast two-hybrid screen for genes encoding peptides capable of interacting with the common b-Zip domain of Cnc proteins (J. M., unpublished results). BLAST searches with a corresponding cDNA sequence detect only one small *maf* gene in the near complete *Drosophila* genome (Adams et al., 2000) (FlyBase Genome Annotation Database (GadFly) identifier CG9954, <http://flybase.bio.indiana.edu/annot/>). We call the gene *Drosophila maf-S* (S for Small), and the protein Maf-S. In the basic region that contacts DNA, Maf-S shares a

domain of perfect identity with mammalian small Maf proteins (Fig. 3A), and partial identity in the leucine zipper and other protein regions. In situ hybridizations show that *maf-S* RNA is maternally deposited in the *Drosophila* egg, and the transcripts are present in all or nearly all embryonic cells throughout development, making the Maf-S protein available for potential interactions with all Cnc isoforms (Fig. 3B). In addition, there is an enrichment in *maf-S* transcripts in certain areas of the embryo, against the ubiquitous expression background. At stage 8, slightly higher levels are detected around cephalic furrow and in the mesoderm. At stage 11 and beyond, there is an enrichment in *maf-S* transcripts in the anterior and posterior midgut, in the nervous system, as well as in the involuting intercalary segment that will give rise to ventral pharynx (Fig. 3C, arrow).

We have used dsRNA interference in an attempt to test the phenotypic effects of loss of *maf-S* function. After injection of *maf-S* dsRNA into the head region of precellular blastoderm embryos, only 1.5% of embryos hatched as first instar larvae. The cuticles of injected embryos showed head defects that are remarkably similar to those found in *cncB* mutants (Fig. 3D): duplications of mouth hooks, shortened lateralgraten, missing or deformed median tooth and truncated ventral pharynx (compare Figs 3D and 1B). Immunostaining of *maf-S* dsRNA-injected embryos with Cnc-specific antibody revealed that the levels of CncB protein are not significantly different than wild type (data not shown). Results of the dsRNA interference experiment suggest that Maf-S is an obligate functional partner of CncB. Without the small Maf subunit, CncB is unable to

Fig. 3. Small Maf homolog in *Drosophila*.

(A) Alignment of the *Drosophila* small Maf protein (Maf-S) with three murine small Maf proteins: p18/MafK, MafG and MafF. Note the near identity in the basic regions that contact DNA, an extended Maf homology region, along with a highly divergent leucine zipper in Maf-S when compared to the mammalian small Mafs. Heptad repeat positions in the zipper are indicated by black ovals. Accession numbers for the proteins are: Maf-S, AI238611 (contains a full-length open reading frame, which is between nucleotides 92 and 490; new GadFly identifier CG9954); p18/MafK, A49391; MafF, NP_034885; MafG, NP_034886. Alignment was generated with the ClustalW module of MacVector (Oxford Molecular). (B) A stage-10 embryo showing ubiquitous expression of *maf-S* RNA. (C) At stage 13, *maf-S* RNA is present in all cells, although it appears to be at slightly higher levels in anterior and posterior midgut, nervous system, and the intercalary and mandibular cells (arrow). (D) *maf-S* mutant phenotype generated with dsRNA interference. Note resemblance to a strong *cnc* mutant phenotype (compare to Fig. 1B): duplicated mouth hooks (arrow) and severely reduced posterior pharynx (arrowhead). (E) A *Df(2R)exu²* homozygous mutant embryo (ventral view). This deficiency uncovers the *maf-S* gene. A defect in pharyngeal T-rib morphology is indicated by an arrowhead (compare to wild-type ventral pharynx shown in Fig. 8B). The arrow points to a near-normal pair of mouth hooks.



promote the development of intercalary, mandibular and labral head structures and to repress the function of Dfd.

The *maf-S* gene, which maps to chromosomal segment 57A, is uncovered by *Df(2R)exu²*. Homozygotes for this deletion, which lack the zygotic dose of *maf-S* and other nearby genes, exhibit mild head defects that include disruptions of the pattern of ventral pharyngeal transverse ribs (T-ribs) (Fig. 3E, arrowhead; compare to Fig. 8B). The weakness of this phenotype relative to the RNAi result may be due to the maternal contribution from *maf-S*, which is unaffected in the zygotic deletion mutants. Further genetic characterization of *maf-S* mutant embryonic defects is impossible with *Df(2R)exu²*, since it also uncovers *exuperantia (exu)*, and maternal mutations in *exu* are associated with severe head defects (Schupbach and Wieschaus, 1986).

DNA sequences recognized by cooperative Cnc/Maf heterodimer binding

Despite a high degree of primary sequence similarity between the DNA binding domains of CncB and NF-E2, the optimal recognition site for the Cnc isoforms in association with *Drosophila* Maf might be different. We sought to find CncA and CncB optimal binding sites by selecting specific sequences from a pool of degenerate oligonucleotides by Cnc/Maf-S heterodimers (see Materials and Methods). Flag-tagged full-length CncA or CncB proteins were cotranslated in vitro with Maf-S and bound to an oligonucleotide containing a core of 14

ambiguous positions. Four rounds of selection, washes, elution and PCR were performed under mild conditions (100 mM total salt concentration), after which the selected pools of oligonucleotides were tested in a gel-shift assay. CncA/Maf-S- and CncB/Maf-S-selected pools of oligonucleotides displayed indistinguishable specificity and strength of binding when bound by either CncA or CncB with Maf-S (data not shown). The CncA/Maf-S-selected pool of oligos was subjected to two more rounds of selection with stringent washes (300 mM total salt) and then cloned. 20 randomly chosen clones were sequenced. The resulting consensus binding site is quite homogeneous, with seven perfectly conserved nucleotides (shown in bold in Fig. 4A). All selected sites started from the same position (T, marked with an arrow in Fig. 4A), suggesting that this and nearby nucleotides influenced the selection process.

The sequence selected in our experiment by Cnc/Maf-S heterodimers (TGCTGAGTCAT) is identical to the preferred site selected by TCF11/MafG (Johnsen et al., 1998), and is very similar to the consensus binding site derived for p45 NF-E2/p18 MafK from gel shift competition experiments (Andrews et al., 1993a) (Fig. 4B). The TGCTGAG part of the site corresponds to the Maf-bound sequences in the sites for mammalian CNC family proteins, and also to the T-MARE (TRE[phorbol-12-O-tetradecanoate-13-acetate (TPA)-responsive element]-type Maf recognition element) consensus half-site for large Maf homodimers (Motohashi et al., 1997).

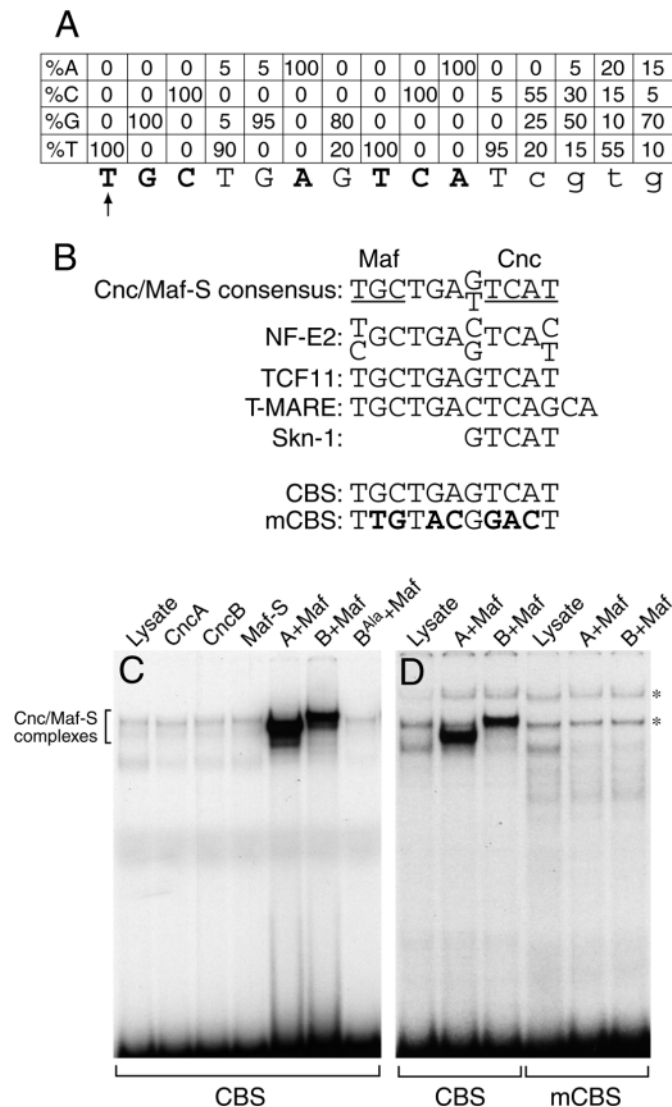


Fig. 4. DNA sequences bound by Cnc/Maf-S heterodimers. (A) A collection of binding sites selected after six rounds by CncA/Maf-S heterodimers (see Materials and Methods). Numbers in the table show the frequencies (percentage) of each of the four nucleotides for a given position in the sequenced pool of oligonucleotides. A consensus sequence is given below the table. Nucleotides encountered at a given position in all sites are indicated in bold. The arrow points to a position corresponding to an invariable nucleotide from the primer, which all selected sites contained. (B) Comparison of the Cnc/Maf binding site with the known binding sites for CNC family homologs: NF-E2, TCF11/LCR-F1/Nrf1, large Mafs (T-MARE site) and Skn-1. CBS (Cnc/Maf binding site) was used in electrophoretic mobility-shift assays (C,D) and in regulatory element constructs (Fig. 5). mCBS is a mutant version of CBS. (C,D) Electrophoretic mobility-shift assay with Cnc and Maf-S proteins produced in wheat germ lysates. (C) Only heterodimers of CncA and Maf-S (A+Maf) or CncB and Maf-S (B+Maf) form stable complexes with the Cnc/Maf binding site probe. Mutation in the DNA binding domain of CncB abolishes binding (B^{Ala}+Maf). (D) Mutation in the Cnc/Maf binding site (mCBS probe) abolishes specific heterodimer binding. Asterisks indicate non-specific complexes formed by lysate components.

The GTCAT half-site is contacted by Cnc homologs, and also is a perfect match to the binding site for the *C. elegans* Skn-1 protein that binds DNA as a monomer (Blackwell et al., 1994). We tested the consensus sequence (CBS, for Cnc/Maf binding site) in an electrophoretic mobility-shift assay (EMSA) with in vitro translated CncA, CncB and Maf-S proteins, along with a mutated site, mCBS (Fig. 4B). As shown in Fig. 4C, monomers of CncA or CncB do not appreciably bind to the Cnc/Maf consensus. Interestingly, in our assays, Maf-S does not bind as a homodimer either to this sequence or to the T-MARE consensus (Fig. 4C and data not shown). This property of the *Drosophila* small Maf protein is different from mammalian small Mafs, which form stable homodimer complexes on T-MARE-like sites (reviewed in Blank and Andrews, 1997; Motohashi et al., 1997). When CncA or CncB are cotranslated with Maf-S, abundant and stable complexes form on Cnc/Maf binding sites (Fig. 4C), suggesting that DNA binding is achieved only by Cnc/Maf heterodimers and that such binding is highly cooperative. CncA or CncB and Maf-S can be easily coimmunoprecipitated from separate in vitro translation reactions (data not shown), suggesting that stable heterodimers form in solution without DNA. Mutating DNA contact residues in the CncB protein abolishes protein/DNA binding (B^{Ala}+Maf, Fig. 4C), as does changing the nucleotides at critical positions in the Cnc/Maf binding site sequence (Fig. 4D, mCBS lanes). These data demonstrate that Maf-S and Cnc proteins can form specific heterodimer complexes on NF-E2-like sites, and that the presence of both protein subunits is required for complex formation.

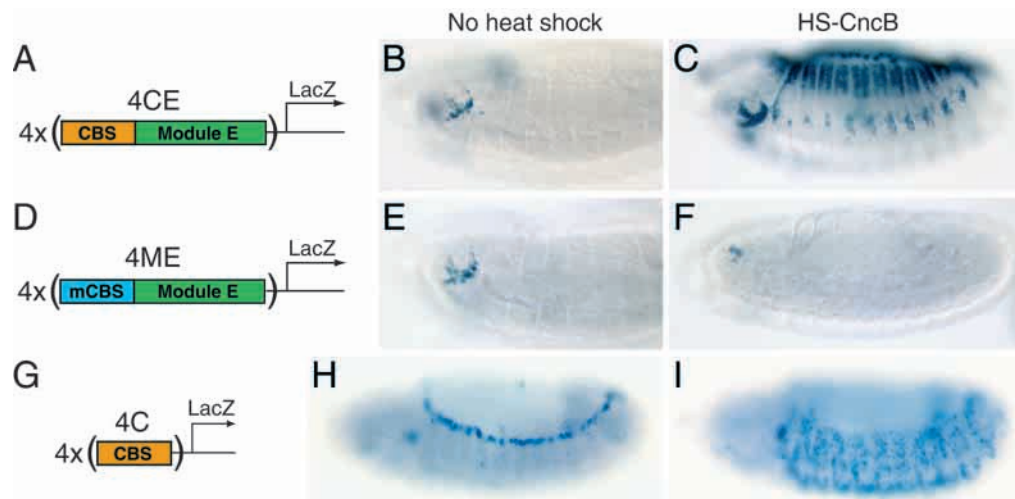
The requirement of both CncB and Maf-S for specific DNA binding agrees well with the similarity of the loss of function phenotypes generated with dsRNA interference for either gene alone. Taken together, our data suggest that the biological function of CncB is achieved via its association with specific sites in the genome, in heterodimer complexes with the small Maf subunit. With the knowledge of requirements for specific DNA recognition by CncB, we set out to investigate the mode by which CncB achieves *Dfd* suppression and promotes the development of labral and intercalary structures.

CncB acts as an activator of transcription on simple response elements in vivo

We have previously shown that overexpression of CncB can partially repress *Dfd* response elements (McGinnis et al., 1998). However, none of the known *Dfd* response elements have close matches to the CncB/Maf-S consensus binding site (CBS, Fig. 4B). We have therefore positioned this site near a well-characterized *Dfd* response element, module E (Zeng et al., 1994), to test whether *Dfd*-dependent expression of module E would be influenced when ectopic CncB is supplied. Four copies of such a hybrid element (CE) were inserted in tandem into a lacZ reporter vector with a minimal promoter (construct 4CE, Fig. 5A). As a control, a mutated binding site (mCBS, Fig. 4B) was fused to module E with the same spacing and orientation (construct 4ME, Fig. 5D). Element CE was readily bound by CncB/Maf-S heterodimers in an EMSA, whereas no association was observed between element ME and CncB/Maf-S (data not shown).

Chimeric constructs 4CE and 4ME are expressed in posterior maxillary cells in an anchor-shaped pattern that is characteristic of module E (Fig. 5B,E). Such expression has

Fig. 5. Activation properties of CncB on simple response elements in vivo. (A) Schematic diagram of construct 4CE. CBS is a Cnc/Maf binding site (see Fig. 4B). Module E is a small Dfd response element (Zeng et al., 1994). (B,C) In situ localization of *lacZ* transcripts in embryos carrying the 4CE transgene and a heat-shock CncB expression construct. (B) In untreated embryos, 4CE is expressed in posterior maxillary cells similar to module E. (C) After a 1 hour heat shock, which results in ubiquitous production of CncB, extensive ectopic activation of the 4CE reporter is observed in the trunk segments, and the maxillary expression is also enhanced (compare to B). (D) Schematic diagram of construct 4ME. mCBS is a mutated Cnc/Maf binding site (see Fig. 4B). (E,F) In situ localization of *lacZ* transcripts in embryos carrying the 4ME transgene and the heat-shock CncB construct. (E) In untreated embryos, 4ME is expressed in posterior maxillary cells, as is 4CE, or the E element alone (Zeng et al., 1994). (F) After a 1 hour heat-shock treatment, CncB protein is ubiquitously activated. This does not induce ectopic expression of 4ME (compare to C), and maxillary reporter activity is repressed. (G) Schematic diagram of construct 4C, which consists of four copies of the Cnc/Maf binding site (CBS). (H) Expression of 4C in untreated embryos. The staining is concentrated in dorsal lateral epidermal cells surrounding the dorsal closure region. (I) Activation of 4C reporter expression in embryos carrying a heat-shock CncB construct, after a 1 hour heat shock. Extensive ectopic expression is observed in the trunk segments.



been previously shown to depend on direct binding and activation of this element by Dfd and other cofactors (Li et al., 1999b; Zeng et al., 1994). In addition, construct 4CE, but not 4ME, is expressed in the intercalary and labral cells that contain high levels of endogenous CncB protein (this aspect of 4CE expression is discussed in the next section). We tested whether ectopic expression of CncB from a heat-shock construct could influence the maxillary expression of 4CE and 4ME. After a 1-hour heat shock, construct 4CE is ectopically activated in many cells in stage-13 embryos, especially in the dorsal-lateral epidermal region (Fig. 5C). In the maxillary segment, expression is also stronger and occurs now in more cells than in the non-heat-shocked embryos. 4ME, on the other hand, does not provide any ectopic expression with HS-CncB (Fig. 5F). In fact, construct 4ME is repressed by overexpression of CncB, in agreement with previous data on the normal 4xE element (McGinnis et al., 1998). Furthermore, HS-CncB^{Ala} (see Fig. 1E) is incapable of activating ectopic expression of 4CE or repressing maxillary expression of 4ME (A. V. and W. M., unpublished observation). These results suggest that CncB may be a transcriptional activator, with activation occurring through interaction of CncB/Maf-S with heterodimer binding sites in the target element. These data also indicate that when a simple Cnc/Maf binding site is placed near a functional Dfd element (module E), CncB protein does not repress Dfd-mediated activation of the element, but instead enhances it. In addition, ectopic CncB is able to activate expression of the 4CE element in the trunk cells that do not have any Dfd protein.

To test for CncB activation potential in the absence of any influence from adjacent sequences, we tested reporter expression driven by a multimerized Cnc/Maf binding site

(construct 4C, Fig. 5G). No reporter expression from the 4C element is detected in embryonic labral, mandibular, or intercalary segments – regions where CncB is normally expressed. This suggests that a minimal sequence like 4C does

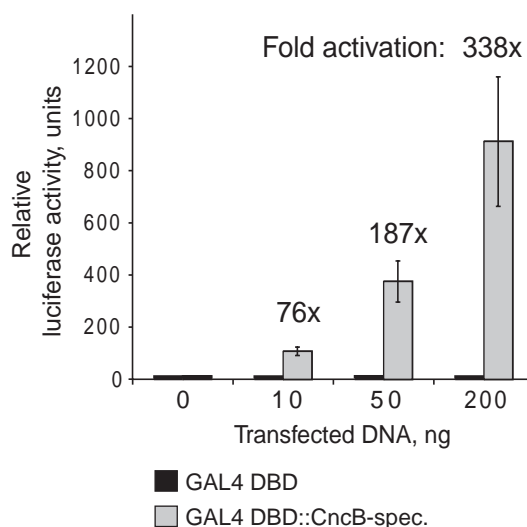


Fig. 6. CncB contains a strong transactivation domain. Bosc23 cells were transfected with constant amounts (0.33 μ g) of a UAS-luciferase reporter and increasing amounts of either GAL4 DNA binding domain (DBD) alone (black bars) or a GAL4 DBD::CncB-specific domain chimera (gray bars). Reporter expression is activated by the GAL4 DBD::CncB-specific domain chimeric protein in a dose-dependent manner. Relative luciferase activity was measured as previously described (Li et al., 1999a).

not contain the information required to provide an activation response to CncB when the protein is provided at wild-type levels. Ectopic expression of CncB using heat shock leads to extensive activation of the 4C element in the trunk epidermal

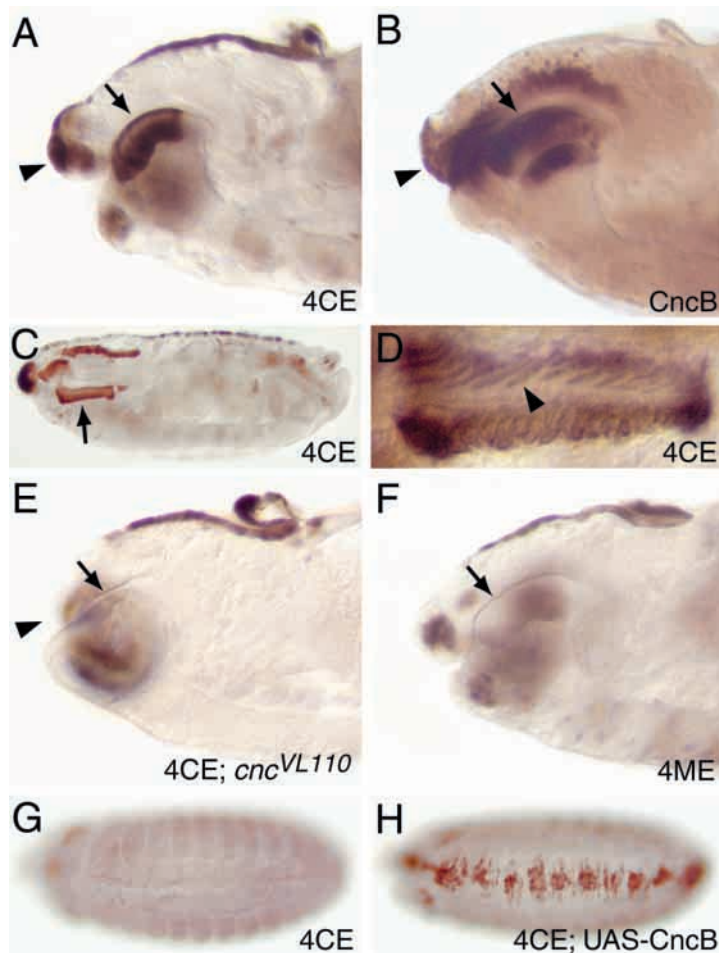


Fig. 7. Activation of CncB/Maf-S response elements in pharynx precursor cells. (A) Reporter expression from construct 4CE (see Fig. 5A) in the ventral pharynx (arrow) and clypeolabrum (arrowhead) of the stage-13 embryo, detected by immunostaining for β -galactosidase. (B) Expression pattern of CncB protein, detected with antibodies raised against the common Cnc protein domain. Note the concentration of the antigen in the pharynx (arrow) and clypeolabrum (arrowhead). (C,D) Reporter expression provided by 4CE in late embryos (stage 16). (C) 4CE is prominently expressed in the ventral pharynx (arrow). (D) A ventrolateral view of pharyngeal expression of 4CE, showing the outlines of the T-ribs (arrowhead). (E) Expression from 4CE in a null *cncB* mutant, *cnc^{VL110}*. Note the absence of pharyngeal (arrow) and labral expression (compare to A). Also note severe reduction in the size of clypeolabrum (arrowhead). A dark area of expression in the gnathal region corresponds to the maxillary segment, which is out of focus. (F) Expression of construct 4ME (see Fig. 5D) in the normal embryo. No pharyngeal (arrow) or epidermal labral expression is seen (compare to A). (G) Ventral view of a stage-13 wild-type embryo carrying a 4CE reporter construct, showing an absence of reporter expression in the ventral midline. (H) Ventral view of a stage-13 embryo carrying a 4CE reporter construct in a background in which ubiquitous CncB expression was driven by *69B-GAL4*. Note the domain of 4CE expression at the ventral midline and the elongated shape of the staining cells which give rise to T-rib-like pharyngeal structures (compare to control (G) and to cuticular preparation in Fig. 8C).

cells immediately after heat treatment (Fig. 5I), indicating that CncB possesses intrinsic activation properties that can be revealed on this element when CncB is provided at higher levels. However, even ectopic CncB protein is still incapable of activating the 4C element in the head region, indicating that a coactivator for CncB is localized in the trunk, and/or a corepressor for CncB is localized in the head. Assuming that a corepressor exists, its function can obviously be overcome on 4CE elements in the head region, as shown above. Ectopic CncA protein does not activate the 4CE, 4ME or 4C elements in embryos (data not shown).

From stage 12 onward, element 4C is activated in epidermal cells fated to become dorsal midline, cells that do not contain CncB protein (Fig. 5H). This 4C activity may be due to other factors such as Jun or Fos proteins, which can recognize the AP-1 core (TGANTCA) contained in the Cnc/Maf binding site. A control construct with mutated core nucleotides gave no detectable staining (data not shown).

We propose that the simple 4CE and 4C elements can be directly activated by CncB/Maf-S heterodimers in certain cells, but not by CncA/Maf-S. The difference between these two Cnc isoforms lies in the CncB-specific amino terminal domain, which is absent from the CncA isoform. We next asked whether this CncB-specific domain is sufficient to confer activation properties on a heterologous DNA binding domain. To that end, the 272 amino acid CncB-specific region was fused to the carboxy terminus of the GAL4 DNA binding domain (DBD). This chimeric protein was tested in mouse tissue culture cells (Bosc23) for its ability to activate a reporter element containing UAS sequences upstream of a basal promoter. We found that the GAL4 DBD::CncB-specific fusion protein strongly activates reporter expression in a dose-dependent manner (Fig. 6). Taken together, the response element expression in *Drosophila* embryos and in mouse cultured cells strongly suggests that the CncB/Maf-S complex can act as a sequence-specific transcriptional activator on simple regulatory elements.

CncB is required and sufficient for the development of the pharynx

As mentioned in the previous section, the hybrid 4CE element is strongly expressed in the intercalary segment and in the clypeolabrum, as well as the posterior maxillary segment. Ventral intercalary cells internalize during head involution (Fig. 7A, arrow) to form the floor of the pharynx (Fig. 7C, arrow). At stage 15 and beyond, the 4CE transgene is expressed in the ventral pharyngeal cells that produce the T-ribs (Fig. 7D, arrowhead). Clypeolabral expression corresponds to the future position of the median tooth (Fig. 7A, arrowhead). Both the labral and intercalary expression domains of 4CE correspond to cells that contain abundant levels of CncB protein (Fig. 7B). To test whether CncB was required for the 4CE driven reporter expression, we tested 4CE activity in the *cnc^{VL110}* null mutant background. In embryos homozygous for this deficiency, both hypopharyngeal and clypeolabral domains of 4CE

expression are absent (Fig. 7E), suggesting that CncB is required for activation of the element. Construct 4ME, which contains mutations in the Cnc/Maf binding site, provides no expression in the pharynx or median tooth primordia in wild-

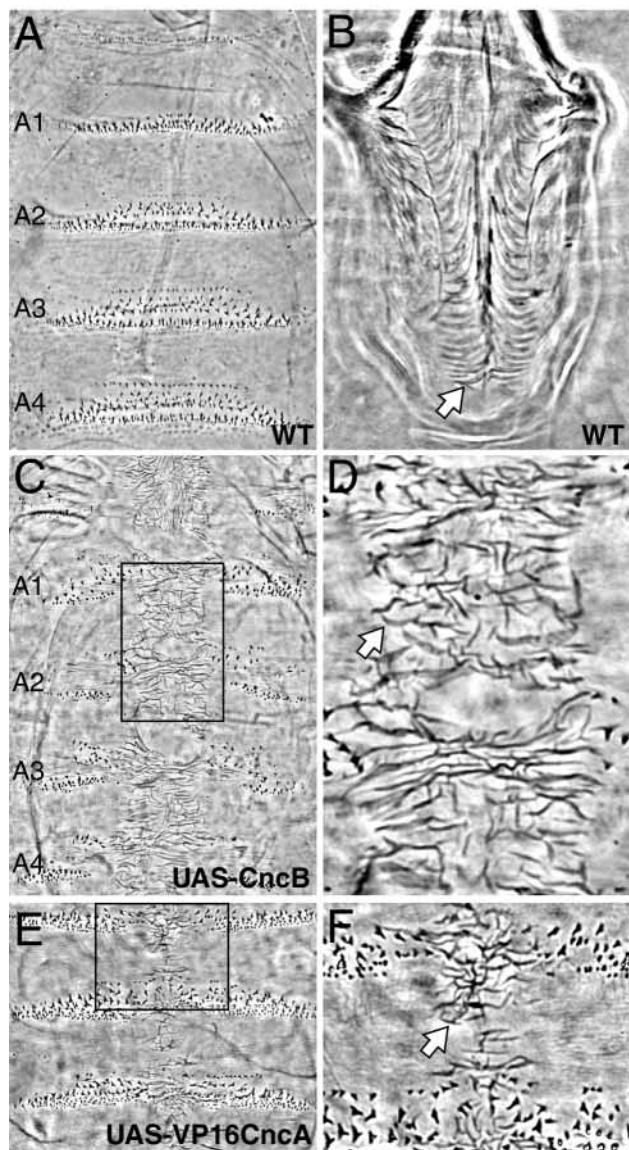


Fig. 8. CncB is sufficient to promote pharyngeal development in ventral epidermal cells. (A) A wild-type pattern of denticle belts on the ventral abdomen of an embryo at the end of embryogenesis. Anterior is up in all panels. A1-A4 denote abdominal segments 1-4. (B) Ventral view of the pharynx of a wild-type embryo. The white arrow points to one of an ordered array of transverse ribs (T-ribs). (C) Ventral abdomen of an embryo in which CncB was ubiquitously expressed using the *69B-GAL4* driver (compare to A). (D) An enlarged portion of the boxed area in C, showing that the ventral midline is transformed to a disorganized array of pharyngeal T-ribs (white arrow, compare to B). (E) A segment of the ventral abdomen of an embryo in which VP16-CncA chimeric protein was overexpressed using the *69B-GAL4* driver. Note the midline transformation that is similar to but weaker than the one observed with *69B-GAL4/UAS-CncB* (compare to C). (F) An enlarged portion of the boxed area in E. Note that the ventral epidermis is transformed towards T-rib identity (white arrow, compare to D).

type embryos (Fig. 7F). It is still possible that the loss of pharynx-specific expression of 4CE in *cnc^{VL110}* embryos is due to cell loss in the mutants. At present, we do not have a CncB-independent marker to confirm that pharynx precursor cells are still present at this embryonic stage in *cncB* mutants.

The absence of 4CE expression in the pharynx precursors in *cnc^{VL110}* embryos correlates well with the absence of ventral pharyngeal structures in strong *cncB* mutants. When CncB is ubiquitously expressed using the GAL4-UAS system (*69B* driver), the abdominal denticle pattern along the ventral midline of the embryo (Fig. 8A) is replaced with a band of incurved folds, which upon close examination resemble the T-ribs of the ventral pharynx (Fig. 8C,D, compare to B). An even stronger phenotype was observed when ectopic CncB expression is driven by *arm-GAL4⁴* (see Fig. 2B). No additional transformations are seen when CncB is coexpressed with Maf-S (data not shown), suggesting that localization of transformation to the ventral region is not due to the limiting levels of Maf-S. Interestingly, 4CE is strongly expressed in epidermal ventral midline cells in *69B-GAL4/UAS-CncB* embryos (Fig. 7H), whereas in the wild-type background such expression is absent (Fig. 7G). These 4CE-positive midline trunk cells elongate in transverse directions, similar to cells that form the T-ribs. This result, along with the formation of ectopic T-rib-like cuticle upon misexpression of CncB, suggests that persistent presence of this protein is not only necessary but sufficient for specification of the ventral pharynx.

An important question is whether the transcriptional activation function of the CncB-specific domain is sufficient

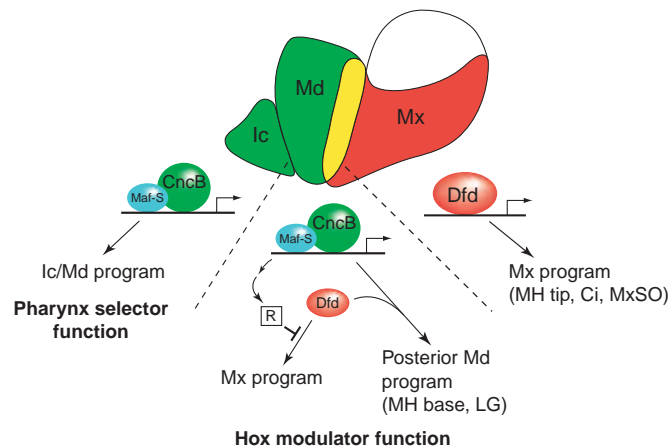


Fig. 9. A summary model for the functions of CncB in the development of gnathal segments. Only the intercalary (Ic), mandibular (Md) and maxillary (Mx) segments are shown for simplicity. CncB expression is shown in green, Dfd expression is in red, and their overlap in the posterior Md segment is in yellow. Anterior is to the left, dorsal is up. In the intercalary segment, CncB together with Maf-S selects pharyngeal development. In the mandibular segment, CncB suppresses the maxillary-promoting function of Dfd, at least in part through an indirect mechanism, possibly through an intermediate repressor molecule (R). In the posterior mandibular segment, both CncB and Dfd are required for the development of such structures as mouth hook base (MH base) and lateralgraten (LG). In the maxillary segment, CncB is absent, and Dfd protein specifies maxillary identity, resulting in the formation of the mouth hook tip, cirri (Ci), maxillary sense organ (MxSO) and other structures.

to provide the pharynx-promoting function of this isoform. We substituted the CncB-specific domain with the similarly sized VP16 activation domain (Triezenberg et al., 1988). This VP16-CncA fusion protein was tested in an ectopic expression experiment. Remarkably, when *UAS-VP16-CncA* is ectopically activated with the *69B-GAL4* driver, embryonic ventral cuticle is transformed towards pharyngeal identity, with a somewhat weaker expressivity than the one observed with wild-type CncB (Fig. 8E,F). In addition, *UAS-VP16-CncA* embryos have reduced maxillary structures and have general head defects that resemble those induced by CncB (data not shown). These data suggest that the CncB-specific domain encodes a general activation function, which can be partially mimicked by a heterologous activation domain. This activation function is required for the pharynx selector role of CncB as well as its function in suppressing Dfd-dependent maxillary development.

DISCUSSION

The role of CncB in segmental diversification

Previous genetic and molecular data have established that CncB has several functions in the developing *Drosophila* embryo: it is required for the formation of labral and intercalary structures such as median tooth and pharynx, and it is required to inhibit Dfd function in the mandibular segment (McGinnis et al., 1998; Mohler et al., 1995). Based on results presented in this study, we propose a summary model (Fig. 9) in which these functions are mediated in part by the transcriptional activator properties of CncB, and are achieved through specific DNA binding with Maf-S to an 11-bp CncB/Maf-S heterodimer binding site. In the intercalary and anterior mandibular segments of the embryo, heterodimerization of CncB with Maf can activate some downstream target genes, and the heterodimers are required to develop ventral pharyngeal structures (T-ribs and ventral arms). Furthermore, we find that CncB, in the context of the ventral epidermis of other head and trunk segments, is sufficient to promote pharynx identity, and thus *cncB* functions as a pharynx selector gene.

The function of CncB in suppressing the maxillary-promoting function of Dfd is carried out in the mandibular cells. Early in development CncB and Dfd are coexpressed throughout the mandibular segment, but by stage 13 Dfd expression retracts and becomes localized to a row of cells in the posterior of the segment (yellow region in Fig. 9). In these cells, CncB and Dfd are both required to specify lateralgraten and the base of the mouth hook (Mohler et al., 1995). Although Dfd function is required for the normal structures that derive from the posterior mandibular segment, the maxillary-promoting function of Dfd is inhibited in these cells (McGinnis et al., 1998). In the anterior mandibular segment, CncB represses both the function and expression of Dfd. Since Dfd protein activates its own transcription, inhibition of Dfd function inevitably results in silencing the endogenous transcription of the *Dfd* gene. Persistent expression of Dfd in the posterior mandibular cells is likely to be mediated by regulatory regions that are different from the autoactivation enhancers and are insensitive to CncB-mediated repression.

Based on the present data, we suggest that CncB-mediated repression of Dfd function is at least in part indirect (Fig. 9),

for several reasons. First, there are no close matches to the consensus CncB/Maf-S binding site in known Dfd response elements, despite the fact that these elements can be repressed by ectopic expression of CncB, e.g. modules C, E, and F from the 2.7 kb Dfd epidermal autoregulatory enhancer (McGinnis et al., 1998) (see also Fig. 5F). Second, the CncB-specific amino terminal region, crucial for the suppression of Dfd function, contains a strong transcriptional activation domain. Third, a CncA fusion protein with a simple heterologous transcriptional activation domain from VP16 can also partially repress Dfd-dependent maxillary structures upon overexpression. Fourth, chimeric Dfd response elements containing Cnc/Maf binding sites adjacent to Dfd binding sites are not repressed by overexpression of CncB, as might be expected if the action of CncB on Dfd were direct. Instead, as shown in Fig. 5C, the endogenous Dfd-dependent maxillary expression of 4CE is enhanced in HS-CncB embryos when compared to non-heat-shocked controls. Suppression of Dfd function by CncB may therefore be achieved by transcriptional activation of an intermediate gene encoding a repressor or corepressor that would interfere with the function of Dfd. Another possibility is that CncB might compete for a common cofactor or coactivator (such as Exd, Hth or CBP), or it might activate a specific Dfd modifying enzyme such as a kinase or phosphatase. We note however, that it is still possible that the suppressive effect that CncB protein exerts on Dfd function may involve CncB mediated transcriptional repression on as yet uncharacterized response elements. We have observed a binding interaction between Dfd and CncB proteins in GST pull-down assays, but the biological significance of this result is as yet unclear (X. L., unpublished observation).

Repression of Dfd function by CncB is analogous to the phenomenon of phenotypic suppression (also known as posterior prevalence; González-Reyes et al., 1990), which denotes the ability of more posterior Hox proteins to suppress the function of more anterior Hox proteins in the same cells. 'Anterior prevalence' of CncB over Dfd provides additional morphological diversity in the mandibular and maxillary segments. The indirect way by which CncB acts on Dfd maxillary function has a precedent in Hox phenotypic suppression. The inhibitory effects of posterior Hox proteins on Antennapedia (*Antp*) function can apparently be exerted via an indirect pathway involving phosphorylation of *Antp* protein by casein kinase II (Jaffe et al., 1997). Dfd protein is also phosphorylated in vitro by CKII (A. V., unpublished observation), but whether this phosphorylation is relevant to the suppression by CncB is unknown.

Maf proteins in *Drosophila* and mammals

Drosophila apparently has only one small *maf* gene. Ubiquitous expression of *maf-S* RNA suggests that Maf-S protein can potentially form heterodimers with all Cnc isoforms. The CncB/Maf-S heterodimer binds a consensus sequence (TGCTGAGTCAT) that is very similar to the binding site for NF-E2 and is the same as the sequence selected in vitro by TCF11(LCR-F1/Nfr1)/MafG heterodimers (Johnsen et al., 1998). Vertebrates possess at least three small Maf homologs: p18/MafK, MafF and MafG (Blank and Andrews, 1997; Motohashi et al., 1997), which are ubiquitously expressed and heterodimerize with vertebrate CNC-type proteins on DNA regulatory sites (Blank et al., 1997; Igarashi et al., 1994; Itoh

et al., 1995; Kobayashi et al., 1999). Single mutations in the *mafF* or the *mafK* genes do not lead to obvious phenotypic defects (Kotkow and Orkin, 1996; Onodera et al., 1999). *mafG* mutant mice are also viable and fertile, but have defects in megakaryopoiesis and neurogenesis (Shavit et al., 1998). Recently, *mafK/mafG* double mutant mice were characterized and found to have erythroid defects, as well as more severe defects in neurogenesis than those observed in *mafG* mutants alone (Onodera et al., 2000). Hematopoietic defects in mammalian small *maf* mutants resemble the phenotypes observed in mice mutant for NF-E2 or Nrf1 proteins, which suggests that in mammals small Mafs and CNC family proteins can cooperatively activate a set of common targets (Chan et al., 1998; Farmer et al., 1997; Shivdasani and Orkin, 1995). Here, we have shown by dsRNA interference that *Drosophila* embryos with lower levels of Maf-S have a phenotype that is very similar to strong *cncB* mutations. This, in combination with the heterodimer binding, suggests that Maf-S protein functions in parallel to CncB as a required cofactor.

We have found an important difference between the binding of the vertebrate Maf proteins and *Drosophila* Maf-S. Mammalian small Maf proteins were shown to homodimerize on NF-E2-like and T-MARE sites and to function as transcriptional repressors (Igarashi et al., 1994; Johnsen et al., 1998; Nagai et al., 1998; Wild et al., 1999). In contrast, we have observed no detectable binding of Maf-S to Cnc/Maf binding sites (Fig. 4C) or to the T-MARE binding site (data not shown). The leucine zipper region in the Maf-S protein is quite divergent from that of its mammalian counterparts (Fig. 3A). This may prevent homodimerization and therefore the binding of Maf-S to DNA.

In addition to *maf-S*, a gene for a large Maf protein is present in the *Drosophila* genome (GadFly identifier CG10034) (Adams et al., 2000) that encodes a *Drosophila* homolog of mammalian Kreisler/MafB and NRL (reviewed in Blank and Andrews, 1997; Motohashi et al., 1997). We suggest that this gene should be called *maf-L* (for Maf-Large). Binding tests of the *Drosophila* Maf-L protein show that it does not heterodimerize with CncB on the CncB/Maf-S binding site, but is capable of binding this site as a homodimer (data not shown). While the function of the large Maf in *Drosophila* is unknown, its ability to bind the CncB/Maf-S sites presumably adds to the complexity of regulatory interactions on these sites.

CncB as a transcriptional activator of artificial target elements in embryos

In the precursors of ventral pharynx and labrum, endogenous CncB is required to activate transcription from the CncB/Maf-S binding sites in element 4CE, and ectopic expression of CncB using a heat-shock construct results in significant ectopic activation of 4CE, as well as another simple reporter element, 4C. This activation is likely to be direct, since it requires the DNA binding function of CncB and the presence of Cnc/Maf binding sites in the target elements, and is observed immediately after heat-shock induction. Moreover, we have demonstrated that in tissue culture cells the CncB-specific domain acts as a strong transactivation interface. These results support the view that the CncB/Maf-S heterodimer can act as a transcriptional activator in vivo, at least on simple sites in certain cells.

The complex, natural enhancers through which mammalian

CNC family members act as activators include and require binding sites for a variety of cofactors, such as GATA-1, Ets-like factors, and Sp1, in addition to conserved NF-E2-like sites (see Magness et al., 2000; Deveaux et al., 1997; Stamatoyannopoulos et al., 1995 and references therein). Similarly, naturally evolved direct target elements for CncB/Maf-S heterodimers are likely to be quite complex. Hints of this are seen in the spatial responses of 4C and 4CE elements – neither element is activated in mandibular cells where CncB and Maf normally overlap, even when CncB is overexpressed. The 4C element is not activated in any head cells even under conditions where CncB levels are high. So CncB in the mandibular segment appears to have its activation function ablated when compared to cells in other body regions. It is possible that CncB is converted into a repressor on many targets in mandibular cells, although the present study provides no direct evidence for or against this idea.

Pharynx selector role of CncB and the functions of CNC family members

CncB is crucial for the development of the pharynx in *Drosophila*. In the nematode *C. elegans*, the Cnc homolog Skn-1 has been shown to be a factor required for the establishment of pharyngeal and midgut fates (Blackwell et al., 1994; Bowerman et al., 1992). GATA-like proteins have been shown to be important for the function of NF-E2 response elements, and it is interesting to note that two GATA-related factors, *Elt-2* and *End-1*, are required for midgut development in the nematode (reviewed in Labouesse and Mango, 1999). This evidence is consistent with the idea that nematodes and arthropods (and perhaps other ecdysozoans) conserve a similar genetic system involving Cnc-like and GATA-like factors to specify anterior midgut and pharyngeal fates. Other *Drosophila* proteins, such as *Knot/Collier* and *Crocodile*, are specifically expressed in, and required for, pharynx and anterior midgut development (Crozatier et al., 1999; Häcker et al., 1995; Merrill et al., 1989). *knot* is upstream of *cnc* in the pathway leading to formation of the pharynx (Seecoomar et al., 2000), while the regulatory relationship between *cnc* and *crocodile* is not known.

Cross-talk between the functions of CncB and Hox proteins such as *Dfd* may also be present in *Tribolium*, since *Tribolium* Cnc and *Dfd* homologs are expressed in patterns similar to those observed in *Drosophila* (Rogers and Kaufman, 1997). As yet there is no evidence suggesting the involvement of mammalian CNC homologs in Hox pathways. It has been shown that p45 NF-E2 and its Nrf paralogs map near the four Hox clusters in mammals, which suggests that a single ancestral CNC gene existed near the ancestral Hox cluster and was duplicated twice along with the clusters (Kobayashi et al., 1999). The lineage including *Drosophila* has apparently evolved a different means of diversifying the functions of Cnc proteins by evolving three isoforms generated by alternative promoters and splicing.

Numerous experiments have established a major function for mammalian CNC homologs in controlling the expression of genes involved in hematopoiesis. Recently, it has been shown that CNC homologs are also activated in response to xenobiotics, and play a role in activation of several phase II detoxifying enzymes (Alam et al., 1999; Itoh et al., 1997; Wild et al., 1999). It is possible that other Cnc isoforms, CncA or

CncC, or even CncB, may be involved in these processes. CncC is ubiquitously expressed and is essential for life, since *cnc^{K6}* mutants stop in development at some point after embryogenesis. It will be of interest to test whether this and other *cnc* alleles have any impairment in hematopoiesis or detoxification processes.

Target genes that are directly activated by CncB/Maf-S are still unknown, but their identification is crucial for advancing our understanding of the diverse functions of CncB. With the availability of a complete *Drosophila* genome sequence (Adams et al., 2000) that allows analysis of the structure of natural regulatory elements, and with the use of new techniques for gene expression profiling such as microarrays, it should be possible to identify candidate targets for CncB activation in the near future.

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