Coordination of Hepatocyte Nuclear Factor 4 and Seven-up Controls Insect Counter-defense Cathepsin B Expression*  

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CmCatB, a cathepsin B-type cysteine protease, is insensitive to inhibition by the soybean cysteine protease inhibitor (scN). Cowpea bruchids dramatically induce CmCatB expression when major digestive proteases are inactivated by dietary scN, which is presumably an adaptive strategy that insects use to minimize effects of nutrient deficiency. In this study, we cloned the cowpea bruchid hepatocyte nuclear factor 4 (CmHNF-4) and demonstrated its involvement in transcriptional activation of CmCatB in the digestive tract of scN-adapted bruchids. Electrophoretic mobility shift assays demonstrated that CmHNF-4 binds to a CmCatB promoter region containing two tandem chicken ovalbumin upstream promoter (COUIP) sites, which is also the cis-element for Seven-up (CmSvp), a previously identified transcriptional repressor of CmCatB. Although CmSvp is predominantly expressed in unadapted insect midgut, CmHNF-4 is more abundant in adapted bruchids. When transiently expressed in Drosophila S2 cells, CmHNF-4 substantially increased CmCatB expression through COUIP binding. CmSvp inhibited CmHNF-4-mediated transcriptional activation even in the absence of its DNA-binding domain. Thus antagonism resulted, at least in part, from protein-protein interactions between CmSvp and CmHNF-4. Association of the two transcription factors was subsequently confirmed by glutathione S-transferase pulldown assays. Interestingly, anti-CmHNF-4 serum caused a supershift not only with nuclear extracts of scN-adapted insect midgut but with that of unadapted control insects as well. The presence of CmHNF-4 in unadapted insects further supported the idea that interplay between CmSvp and CmHNF-4 controls CmCatB transcription activation. Together, these results suggest that coordination between CmHNF-4 and CmSvp is important in counter-defense gene regulation in insects.

The battle between plants and herbivorous insects employs a variety of defense and counter-defense strategies. Insect feeding elicits synthesis of a wide array of plant-defense compounds, which in turn induce insect counteractive tactics to minimize the impact of plant defense. Analysis of these plant-pest interactions has become a research hotspot for understanding insect counter-defense mechanisms. Many insect survival mechanisms involve remodeling of the insect digestive system. Adjustments in cells lining the digestive tract allow pests to deal with a variety of plant toxins and anti-nutritional metabolites and are crucial for insect survival.

A number of studies on insect response to plant protease inhibitors indicate that one of the tactics used by insects is activation of inhibitor-insensitive digestive enzymes (1–7). Under regular growth conditions, these inhibitor-insensitive enzymes are often in low abundance, accounting for only a minor portion of the entire proteolytic activity. They can be strongly induced, however, and play an important role in protein degradation once the major digestive enzyme function is blocked. The cowpea bruchid (Callosobruchus maculatus) cathepsin B-like cysteine protease (CmCatB)2 has been shown to be such an enzyme. The cowpea bruchid is a grain storage insect pest; its larvae feed and develop inside seeds. Adults no longer feed after emergence from infested seeds but mate and lay eggs on the surface of cowpea seeds. Multiple generations of the insect per year lead to severe grain damage.

CmCatB, which is solely expressed in larvae (6), was identified as one of the midgut genes that was most highly induced by dietary scN in a cDNA microarray, although it was undetectable in insects feeding on regular diet (8). Expression of CmCatB protein revealed that it possesses cathepsin B-like cysteine protease activity. Interestingly, this proteolytic activity was unaffected by scN, in marked contrast to the major cathepsin L-like digestive enzymes that share similar tertiary structures with CmCatB (6, 9). Most likely, the occluding loop in CmCatB blocked access of inhibitor to the enzyme catalytic site, which has been demonstrated for its human ortholog (10, 11). Protease activity that is insensitive to scN no doubt helps to alleviate nutrient deficiency when major digestive enzymes are inhibited by scN, thus enabling insects to regain normal feeding and development (7).

The scN-induced expression pattern and scN-insensitive proteolytic activity suggest that CmCatB has a unique role in insect adaptation to the effect of dietary scN. In an effort to understand CmCatB transcriptional regulation, we cloned a CmCatB promoter and adopted electrophoretic mobility shift

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2 The abbreviations used are: CmCatB, C. maculatus cathepsin B-like cysteine protease; COUP, chicken ovalbumin upstream promoter element; COUP-TF, COUP-transcription factor; scN, soybean cysteine protease inhibitor; Svp, Seven-up; HNF-4, hepatocyte nuclear factor 4; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; DBD, DNA-binding domain; LBD, ligand-binding E domain; RACE, rapid amplification of cDNA ends; RT, reverse transcription; qRT, quantitative RT; CAT, chloramphenicol acetyltransferase; S2, Schneider 2 cell; GST, glutathione S-transferase.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EU545256.

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assays (EMSA) to identify potential regulatory elements using nuclear extracts from bruchid midgut tissue (12). We detected retarded bands of different mobility in control (scN-unadapted) and scN-fed (adapted) insects, which implies that different binding proteins might be interacting with the CmCatB promoter in the two insect groups. In unchallenged control insects, we demonstrated that specific association of a COUP-element and CmSvp, a homolog of the COUP-interacting transcription factor (COUP-TF), resulted in repression of CmCatB (12). Its high abundance in unadapted insects and reduced expression in adapted insects supported the repressor function. Identities of the cis- and trans-regulators in scN-adapted insect midgut remained to be elucidated.

Because CmSvp is apparently involved in negative regulation of the insect counter-defense gene, we speculated that the binding protein detected in nuclear extracts of scN-adapted insect midgut was an activator that promoted CmCatB expression. This hypothesis would be consistent with the observed high induction of CmCatB in scN-adapted bruchid midgut. To test this hypothesis and to obtain a more comprehensive understanding of insect adaptive mechanisms, we herein report on our investigation of this potential positive regulator, which appears to be a hepatocyte nuclear factor 4 (HNF-4). In addition, we have gained insight into the intimate relationship and coordinated activities of CmHNF-4 and CmSvp.

**EXPERIMENTAL PROCEDURES**

**Insect Treatment and Midgut Nuclear Extract—**Recombinant scn was expressed in *Escherichia coli* BL21 (DE3) and purified via Ni²⁺-chelating Sepharose affinity chromatography (Amersham Biosciences) as previously described (7). Cowpea bruchids were reared on artificial diet containing 0.2% scn until they reached the 4th instar larval stage (scN-adapted) when bruchids were reared on regular diet. Midguts were dissected, and gut contents were removed. Nuclear extracts were prepared using a Nuclear Extract kit (Active Motif, Carlsbad, CA). Briefly, freshly dissected midguts free from gut contents were placed in a pre-chilled, sterile Dounce homogenizer, and homogenized in cold hypotonic buffer with 1 mM DTT and 0.1% (v/v) detergent supplied by the manufacturer. For every five midguts, 100 µl of buffer was added. The homogenate was incubated on ice for 15 min prior to a 10-min centrifugation at 850 x g. The cell pellet was resuspended in 50 µl of hypotonic buffer, incubated on ice for 15 min, and then lysed by adding detergent to 1% (v/v) followed by vigorous vortexing for 10 s and centrifugation for 30 s at 14,000 x g. The nuclear pellet was resuspended in 30 µl of complete lysis buffer (1 mM DTT, 1% (v/v) protease inhibitor mixture, lysis buffer AM1). Nuclear proteins were then extracted by gently shaking on ice for 30 min. After centrifugation for 10 min at 14,000 x g, the supernatant (containing nuclear proteins) was transferred to a new tube in aliquots and stored at −80°C till use. All centrifugations were performed at 4°C.

**EMSA—**DNA probes for EMSA (Table 1) were radiolabeled either by PCR amplification (>60 bp probes) or using T4 DNA polynucleotide kinase (<60 bp probes). Probes P1 to P5 were randomly labeled with 1.5 µM [α-32P]dCTP by PCR amplification (94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 25 cycles).

| Probe | Primer sequence (5’ to 3’) | Position |
|-------|---------------------------|----------|
| P1    | Sense: G(360)GCTTATTCTGATCAAGGAGAG(470) | 493 to 244 |
|       | Antisense: A(470)GGCTTTATAGCGTTTATATAC(360) | 244 to 493 |
| P2    | Sense: A(324)GCTTGAGGAGAAGAGGAGG(420) | 302 to 41 |
|       | Antisense: C(420)GCCCTTCCAATCTCATGACAT(542) | 442 to 339 |
| P3    | Sense: G(430)GCTATTATATCTGAGTAAAG(510) | 439 to 339 |
|       | Antisense: C(510)GAAATTGATTTATAGGACATGC(379) | 379 to 430 |
| P4    | Sense: C(400)GAAATTGATTTATAGGACATGC(379) | 400 to 244 |
|       | Antisense: A(364)GAAAGAGTTTCTCTGGTCT(246) | 246 to 364 |
| P5    | Sense: G(400)GCTTTATAGCGTTTATATAC(360) | 439 to 339 |
|       | Antisense: C(339)AGAAGAATCTGAAAAAGGT(360) | 360 to 399 |
| P6    | Sense: C(400)GAAATTGATTTATAGGACATGC(379) | 400 to 378 |
| P7    | Sense: G(360)GCTTATTCTGATCAAGGAGAG(470) | 382 to 357 |

*The sequence of the top strand of the double-stranded oligonucleotides is shown.*

The PCR fragments were purified with a QiaQuick PCR purification kit (Qiagen). Probes P6 to P8 resulted from annealing two complementary oligonucleotides. These oligonucleotides were first end-labeled separately with 0.73 µM [γ-32P]ATP using T4 DNA polynucleotide kinase, then mixed in complementary pairs as previously described (12). After annealing, the double-stranded oligonucleotide probes were purified with QiaQuick Nucleotide Removal kit (Qiagen).

EMSA were performed by incubating 3 µg of midgut nuclear extract, or 2 µl of *in vitro* translated CmHNF-4, for 20 min with labeled probes (20,000 cpm per reaction) in binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 10 mM Tris-HCl, pH 7.5, 0.05 µg of poly(dI-dC)-poly(dI-dC)) at room temperature. Samples resolved on 4% native polyacrylamide gel were exposed to x-ray film. For competition assays, a 5-, 10-, or 50-fold molar excess of specific or nonspecific competitors was incubated with proteins for 20 min at room temperature prior to the addition of probe. AP2 was used as nonspecific competitor for P7 probe due to size similarity. Five nucleotides were added to each end of probe P7 to ensure that it was double-stranded. For longer probes, nonspecific DNAs of equivalent length to their corresponding competing probes were made as PCR products against cDNA fragments of a digestive protease CmCPA9. For supershift assays, 1 µl of anti-AaSvp or 2 µl of anti-CmHNF-4 serum (see below) was preincubated with proteins for 20 min at room temperature, followed by binding reactions. Anti-AaSvp serum (generously provided by A. Raikhel, University of California, Riverside, CA) was raised against a highly conserved region of mosquito AaSvp. Pre-immune serum (for anti-AaSvp) or synthetic CmHNF-4 peptide (see below) was used in EMSA to ensure antibody specificity.

Cloning of CmHNF-4 from Cowpea Bruchid Midgut and Quantitative RT-PCR—Total RNA was extracted from the midgut tissue of the scN-adapted cowpea bruchid 4th instar larvae using TRIzol reagent (Invitrogen), and reverse transcribed with Superscript™II reverse transcriptase (Invitrogen). Two pairs of nested degenerate primer sets were designed based on the highly conserved LBD region of HNF-4 family members (yet showed limited identity with CmSvp): primer 1, sense 5'-GATGTGTGGYAGKCTBTAAGG-3'; primer 2, antisense 5'-ATYGTYSRATCAYTYGCCAKG-3'; primer 3, sense 5'-GAAYCATBGAASARCAG-3'; and primer 4, antisense 5'-CATYTGCCAKTRATSWGCTG-3' (B = C, G, or T; Y = C or T; K = G or T; S = C or G; R = A or G; D = A, G, or T).
G, or T; and W = A or T). The primary PCR (95 °C for 30 s, 42 °C for 30 s, and 72 °C for 40 s for 35 cycles) was conducted with primers 1 and 2. The nested PCR (95 °C for 30 s, 42 °C for 1 min, and 72 °C for 1 min for 35 cycles) was then performed with primers 3 and 4. The PCR fragment was QIAquick gel-purified (Qiagen) and subcloned into a pCRII vector (Invitrogen), and DNA sequence was confirmed.

The 5′- and 3′-cDNA ends were PCR-amplified (94 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 or 3 min for 35 cycles) using the BD SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA) with the following gene-specific primers: primer 5, sense 5′-GCTTTTACGGAAC-TGCAAACGATAGC-3′; primer 6, sense 5′-GACCGAGTAGCTTTGTTACGACACAT-3′; primer 7, antisense 5′-GAGAATGACATTGACCTTCTAGCTAATCC-3′; and primer 8, antisense 5′-AGCTTTTACGGGAAC-TGCAAACGATAGC-3′. The PCR fragments were subcloned into the BD SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA) with the following gene-specific primers: primer 9, sense 5′-GAATTCATTGACCTTCTAGCTAATCC-3′; and primer 10, antisense 5′-GAGATGCATTGACCTTCTAGCTAATCC-3′. The PCR product was then subcloned into pCRII vector, and the sequence of insert was confirmed by DNA sequence analysis.

For quantitative RT-PCR (qRT-PCR), total RNA was extracted from midguts of unadapted or adapted 4th instar larvae, pre-treated with RNase-free DNase (Qiagen) to avoid genomic DNA contamination, followed by reverse transcription. qRT-PCR (47 cycles of 95 °C for 15 s and 60 °C for 1 min following initial incubation at 50 °C for 2 min and 95 °C for 10 min) were conducted in SYBR Green Master Mix (Applied Biosystems, Gaithersburg, MD). Antigen-antibody complexes were detected using Supersignal West Femto Maximum Sensitivity Substrate (Pierce). To profile CmSvp abundance, the following antibodies were used: polyclonal chicken anti-AaSvp serum (1:50 dilution) and rabbit anti-chicken IgY serum (1:160,000 dilution) conjugated with horseradish peroxidase (Sigma). To ensure equivalent protein loading of midgut nuclear extracts, the membrane was incubated with rabbit anti-actin primary antibody (1:500 dilution, Sigma) and the same secondary antibody (anti-rabbit IgG (H+L) serum/horseradish peroxidase-conjugated) as above. Immune complexes were detected using Amersham Biosciences ECL Plus Western blotting Detection System (Amersham Biosciences).

### In Vitro Translation of CmHNF-4 Proteins—CmHNF-4 and its deletion mutant proteins were produced by coupled in vitro transcription and translation using the TnT T7 Quick for PCR DNA (Promega, Madison, WI). DNA templates were generated by PCR amplification (94 °C for 30 s, 50 or 56 °C for 30 s, and 72 °C for 1 or 2 min for 35 cycles), and PCR primers are listed in Table 2. Primers 1 and 2, 1 and 3, 2 and 4, 3 and 4, 1 and 5, 3 and 8 were used for amplification of the entire coding region of CmHNF-4, CmHNF-4ΔEF (see Fig. 10C), CmHNF-4ΔAB, CmHNF-4ΔAB/F, CmHNF-4ΔEF, CmHNF-4 (E), CmHNF-4 (ΔM), and CmHNF-4 (EΔEF-2), respectively. T7 promoter (underlined in Table 2), Kozak consensus (double underlined), and poly(A) tail sequences and translation start and stop codons (bold) were introduced into primers for efficient transcription and translation. The purified PCR fragment (0.3 µg) was incubated with TnT T7 PCR Quick Master Mix at 30 °C for 1.5 h. From this reaction, 2 µl of CmHNF-4 protein was used for EMSA and competition assays as described above to evaluate DNA-binding specificity of CmHNF-4.

### Expression Plasmid Construction—All the chloramphenicol acetyltransferase (CAT) reporter plasmids, pAc-CatB/CAT, pAc-CatBΔCOUP/CAT, pAc3075, and pAc1E1/CAT, as well as the expression plasmid pAc5-CmSvp, have been previously described (12). To make the pAc5-CmHNF-4 construct, the entire coding region of CmHNF-4 was amplified by PCR (95 °Cpolyvinylidene difluoride membrane for Western blot analyses.

### Polyvinylidene difluoride membrane Western blot analyses.

For CmHNF-4, the anti-CmHNF-4 serum was used as primary antibody (1:50 dilution), and the secondary antibody was goat anti-rabbit IgG (H+L) serum (1:200,000 dilution) conjugated with horseradish peroxidase (Kirkegard Perry Laboratories, Gaithersburg, MD). Antigen-antibody complexes were detected using Supersignal West Femto Maximum Sensitivity Substrate (Pierce). To profile CmSvp abundance, the following antibodies were used: polyclonal chicken anti-AaSvp serum (1:50 dilution) and rabbit anti-chicken IgY serum (1:160,000 dilution) conjugated with horseradish peroxidase (Sigma). To ensure equivalent protein loading of midgut nuclear extracts, the membrane was incubated with rabbit anti-actin primary antibody (1:500 dilution, Sigma) and the same secondary antibody (anti-rabbit IgG (H+L) serum/horseradish peroxidase-conjugated) as above. Immune complexes were detected using Amersham Biosciences ECL Plus Western blotting Detection System (Amersham Biosciences).

### TABLE 2

| Primer sequence (5′ to 3′) | Primer 1 | Primer 2 | Primer 3 | Primer 4 | Primer 5 | Primer 6 | Primer 7 | Primer 8 |
|---------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| 1. GCACAG AAGGCTT CATGAC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 2. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 3. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 4. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 5. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 6. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 7. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |
| 8. GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC | GCTTTT ACGGAAC-TGCAAAC GATAGC |

**Coordination of CmHNF-4 and CmSvp**

**Expression Plasmid Construction**—All the chloramphenicol acetyltransferase (CAT) reporter plasmids, pAc-CatB/CAT, pAc-CatBΔCOUP/CAT, pAc3075, and pAc1E1/CAT, as well as the expression plasmid pAc5-CmSvp, have been previously described (12). To make the pAc5-CmHNF-4 construct, the entire coding region of CmHNF-4 was amplified by PCR (95 °C...
for 30 s, 68 °C for 2 min for 35 cycles) using the following primers: sense, 5′-AAGCTGATTTCCAAAAAGATGCCTTCCTGCTT-3′; and antisense, 5′-GCTCACTGAGGAACTCATTATC-3′. EcoRI and XhoI (underlined) restriction sites were incorporated into primers for directional cloning. After restriction digestion, the PCR fragment was ligated to pAc5.1/V5-HisA expression vector (Invitrogen), and correct DNA sequence was verified.

To obtain the expression plasmid pAc5-Gal4DBD, yeast Gal4DBD DNA fragment encoding the amino acid residues 1-147 was PCR-amplified (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 35 cycles) using the following primers: sense, 5′-AAGCTGTGATCCAAAGATGCGATCTGGTCTCTGCTT-3′; and antisense, 5′-GCTCAATGCAGATACCAGTCATTATC-3′. KpnI and EcoRI (underlined) restriction sites were incorporated into primers for directional cloning. After restriction digestion, the PCR fragment was ligated to pAc5.1/V5-HisA vector, and correct DNA sequence was confirmed.

To replace DBD of the CmSvp with Gal4DBD, the D/E/F region of CmSvp was PCR-amplified (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for 35 cycles) using sense primer 5′-AAGCTGATTTCCAAAAAGATGCCTTCCTGCTT-3′ and antisense primer 5′-GCTCACTGAGGAACTCATTATC-3′. EcoRI and XbaI (underlined) restriction sites were incorporated into primers for directional cloning. The chimeric expression construct pAc5-Gal4-CmSvp(D,E,F) was generated by cloning of the restricted PCR fragment into EcoRI and XbaI sites of pAc5-Gal4DBD, and correct DNA sequence was verified.

**Transient Transfection and CAT Assays**—Transfection experiments were performed as previously described (12). *Drosophila* Schneider 2 (S2) cells were maintained in Shieilds and Sang M3 insect medium (Sigma) with supplements at 27 °C. 1 h prior to transfection, the cells were seeded at a density of 1 × 106 cells per well on a 6-well titer plate. A mixture of 8 μg of pDNA plasmid, 0.5 μg or 1 μg of an expression plasmid, and 1 μg of pAc5.1/V5-HisA/lacZ (Invitrogen) was introduced to the cells by the calcium phosphate DNA precipitation method. After 24 h post-transfection, the cells were harvested and lysed by repeated freeze-thawing for CAT and β-galactosidase assays. pAc5.1/V5-HisA/lacZ was used as an internal standard. Appropriate amounts of pAc5.1/V5-HisA vector alone were added to the transfection reactions to ensure comparable total DNA amount in transfected S2 cells. pAc-IE1/CAT (CAT gene under control of the promoter of a baculovirus immediate-early gene) (13) was used to evaluate specificity of CmHNF-4 and CmSvp effects on CmCatB promoter. Transfection assays were carried out independently for three times or more. CAT assays were performed using chloramphenicol and [3H]acetyl-CoA. Enzymatic activity was measured by production of [3H]acetylated chloramphenicol using a Beckman LS6500 scintillation counter. β-Galactosidase activity in the cell extract was measured as previously described (12), and the values were used to normalize variations in transfection efficiency. A paired samples t test was used to evaluate the CAT activity data using SPSS for Windows 12.0.1.

**GST Pulldown Assay**—CmSvp and its deletion mutants were expressed in bacteria as GST fusion protein. Briefly, the full-length CmSvp and mutants were amplified by PCR (95 °C for 30 s, 68 °C for 2 min or 56 °C for 30 s, and 72 °C for 1 min, for 35 cycles) using primers listed in Table 3. Primer sets 1 and 2, 1 and 3, 2 and 4, and 5 and 6, 7 and 5, and 8, 9, and 10 amplify the entire coding region, CmSvpΔ390-419, CmSvpΔAB/C, CmSvp(E), CmSvp(EΔ359-408), and CmSvp(EΔ309-408), respectively. BamHI and XhoI sites (underlined in Table 3) were introduced into primers for directional cloning. The PCR-amplified fragment was cloned into a pGEX-6P-1 expression vector (Amersham Biosciences). After sequence confirmation, the construct pGEX-CmSvp was transferred into bacterial strain Rosetta-gami (DE3), and transformed bacteria were grown at 37 °C until A600 reached between 0.4 and 1.0. Expression of GST-CmSvp fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to 1 mM. Cells were grown overnight at room temperature and harvested. To improve protein solubility, GST-CmSvpΔ390-419, GST-CmSvp(E), GST-CmSvp(EΔ359-408), and GST-CmSvp(EΔ309-408) proteins were expressed at 5 °C for 48 h. Pelleted cells were resuspended in Dulbecco’s phosphate-buffered saline (8 mM Na2HPO4, 2 mM KH2PO4, 140 mM NaCl, 10 mM KCl, pH 7.4) containing 5 mM DTT and complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Cells were lysed by sonication (Model 250 Sonifier, Branson), and cell debris was removed by centrifugation at 31,000 × g for 20 min. The supernatant was incubated with immobilized glutathione-agarose beads (Pierce) pre-equilibrated in Dulbecco’s phosphate-buffered saline containing 5 mM DTT and complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Cells were lysed by sonication (Model 250 Sonifier, Branson), and cell debris was removed by centrifugation at 31,000 × g for 20 min. The supernatant was incubated with immobilized glutathione-agarose beads (Pierce) pre-equilibrated in Dulbecco’s phosphate-buffered saline containing 5 mM DTT and complete protease inhibitor at 4 °C for 30 min. Immobilized GST fusion protein was centrifuged at 1,250 × g for 5 min and washed first with 5 mM ATP in buffer (20 mM MgCl2, 50 mM Tris-HCl, pH 7.4, 50 mM KCl) to remove binding of *E. coli* chaperonins. Subsequently, protein was washed three times with Dulbecco’s phosphate-buffered saline buffer and analyzed by SDS-PAGE.

Pull-down experiments were performed following Kitstaki and Talianidis (14) with modifications. Briefly, 10 μl of glutathione-agarose beads containing ~10 μg of the fusion protein was incubated with 20 μl of 35S-labeled, in vitro translated CmHNF-4 and derivatives in 500 μl of interaction buffer (100 mM KCl, 20 mM HEPES, pH 7.9, 0.1% Nonidet P-40, 5 mM MgCl2, 0.2% bovine serum albumin, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C for 1.5 h with constant mixing. The beads were then washed five times with 500 μl of wash buffer (100 mM KCl, 20 mM HEPES, pH 7.9, 0.1% Nonidet P-40,
5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride), resuspended in 20 μl of 1× SDS sample buffer, and analyzed by 8 or 15% SDS-PAGE. The gel was dried, and the CmHNF-4 protein was assessed by exposure to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). The GST pulldown assay was performed independently three times.

RESULTS

COUP Is a Specific cis-Element for Nuclear Factors of Both scN-challenged and Control Bruchid Midgut—Previous EMSA experiments using overlapping probes covering the proximal 493-bp CmCatB promoter region as shown in Fig. 1A and midgut nuclear extracts of scN-fed versus control insects revealed DNA-protein complexes of differing mobility (12). We proposed that different nuclear proteins might interact with the promoter to control CmCatB expression in the two insect groups. The protein factor specific to control insects, i.e. the repressor CmSvp, has been reasonably defined, but the protein unique to scN-adapted insects, its corresponding cis-element, as well as potential interactions between the two nuclear proteins remained unknown. Because a DNA-protein complex was formed in scN-challenged insects where CmCatB was highly induced, it seemed reasonable to hypothesize that this nuclear protein could serve as a trans-activator that enhanced CmCatB expression through binding to target promoter elements. We also established that, just like CmSvp, this nuclear factor from scN-adapted insect midgut also interacted with the P1 probe (spanning nucleotides −493 to −244) rather than with the more proximal P2 probe (12).

To precisely locate the putative cis-regulatory element of adapted insects, we performed a series of gel shift and competition assays (Fig. 1). The specificity of nuclear extracts for P1 probe was verified by competition assays using unlabeled P1 or nonspecific DNA competitors (Fig. 1B). Overlapping DNA probes P3 and P4, covering the P1 region, both exhibited specific associations with nuclear extracts prepared from midgut of scN-adapted insects (Fig. 1C). This result suggested that the potential nuclear protein-binding region resided in the overlap between P3 and P4. Subsequent detection of the protein-binding complex using the P5 probe, corresponding to the region common to P3 and P4 probes, confirmed this assumption.
TFSEARCH, a cis-element-predicting program, identified three putative cis-elements in P5 for the known DNA-binding proteins corresponding to CdxA, COUP-TF, and CRE-BP (Fig. 1A). Therefore, synthetic probes P6, P7, or P8, each corresponding to one cis-element, were used in competition EMSAs. Although P6 (CdxA) and P8 (CRE-BP) did not interfere with P5-protein interaction, probe P7 (COUP-ele-
ment) on the other hand, could compete with P5 for protein binding (Fig. 1C).

This result was initially surprising, because this same cis-element was previously shown to mediate interaction between the CmCatB promoter and the transcription repressor, i.e., CmSvp, in insects that were not challenged by dietary scN (12). To eliminate the possibility that CmSvp was the COUP-binding protein in scN-adapted bruchid midgut, we used anti-AaSvp in the gel shift assay, in which midgut nuclear extract was from scN-challenged bruchids. Anti-AaSvp antibody was previously shown to abolish COUP-CmSvp interaction (12). The fact that the shifted band was unaffected by the antibody (Fig. 2), excluded CmSvp from being the binding protein in adapted insect midgut.

CmHNF-4 Is a COUP-binding Protein—This finding was intriguing, because it implied that CmCatB transcription is regulated by different factors interacting with the same cis-element. The trans-factor yet to be found most likely played a role opposite to CmSvp, activating CmCatB. Indeed, the P7 probe contains two sets of imperfect A/GGGTCA direct repeats, a response element that can be targeted by different members of the nuclear receptor superfamily, including HNF-4 (15), a transcription factor identified in vertebrates (16, 17), as well as some insects (18–20).

To illustrate the potential trans-activator function of CmHNF-4 in CmCatB expression, we cloned the putative transcription factor from scN-adapted cowpea bruchid midgut by a combination of RT-PCR and RACE. The PCR reaction using degenerate primers, designed from an LBD region conserved among vertebrate and invertebrate HNF-4 proteins, resulted in a 486-bp fragment. After sequence confirmation, gene-specific primers for 5′- and 3′-RACE were synthesized. A 1756-bp full-length cDNA sequence, namely CmHNF-4, was finally PCR-amplified, and revealed to encode a protein of 507 amino acid residues (Fig. 3A).

The open-reading frame of CmHNF-4 exhibited five functional domains, typical for nuclear receptors (Fig. 3B). The peptide used for anti-CmHNF-4 serum production is indicated by a double-headed arrow.

**FIGURE 3.** Cloning of CmHNF-4 and domain comparison with its homologs. A, amino acid sequence deduced from a full-length CmHNF-4 cDNA (GenBank™ accession number EU545256). The boundaries of the putative DBDs and LBDs are indicated by bent arrows. The zinc finger modules in the DBD are boxed, and eight highly conserved cysteine residues are marked by asterisks. The P and D boxes in the DBD are underlined. The signature motif of the LBD of nuclear receptors is also boxed. The double-underlined region highlights the AF-2 activation domain located at the C terminus of the LBD. The peptide used for anti-CmHNF-4 serum production is indicated by a double-headed arrow. B, CmHNF-4 shares high homology in protein sequence and domain structure with HNF-4 members from Bombyx mori (BmHNF-4a), Drosophila melanogaster (DmHNF-4), Aedes aegypti (AaHNF-4a), and Homo sapiens (HsHNF-4/1). Letters above the CmHNF-4 indicate conventional functional domains. Percentages indicate levels of identical amino acid residues shared between CmHNF-4 and respective HNF-4 proteins in functional domains. The length and beginning and ending amino acids of the DBD and the LBD are numbered.
C terminal of the LBD. This E domain presumably mediates nuclear hormone receptors (23). The AF-2 activation domain, which interact with the same cis-element but elicit opposite transcriptional response (24), was cloned under the control of the CmCatB promoter (12). Drosophila S2 cells were cotransfected with the reporter plasmid (pAc-CatB/CAT) and the CmHNF-4-expression construct (pAc5-CmHNF-4) and assayed for CAT activity. Transient expression of CmHNF-4 reproducibly caused a 3- to 4-fold increase in reporter gene activity, which was not seen with the control construct (Fig. 6B). Thus we conclude that CmHNF-4 promotes transcriptional activation of CmCatB.

Cotransfection experiments were also performed with pAc-CatBACOUP/CAT, the reporter construct without the cis-element. Enhanced CAT activity was diminished, as was the basal activity (Fig. 6C). Thus binding to the COUP site appears to be necessary for CmHNF-4 positive regulatory function. CmHNF-4 Activity Is Antagonized by CmSvp—To gain some insight into the relationship between the two transcription factors, which interact with the same cis-element but elicit oppo-
Coordinator of CmHNF-4 and CmSvp

A. CmHNF-4 peptide
Antisera to the CmHNF-4 peptide was incubated with nuclear extract prior to the binding reactions with P7, forming the immune complex. Preincubation of antisera with excess of the synthetic peptide abolished the supershift.

B. CmSvp enhanced CmCatB expression in Drosophila S2 cells. S2 cells were cotransfected with 8 μg of reporter plasmids and 0.5 μg of CmHNF-4 expression plasmid (black bars) or equivalent empty expression vector (white bars). The reporter plasmid pAc-IE1/CAT was used to determine the specificity of CmHNF-4 on the CmCatB promoter. CAT activity driven by the CmCatB promoter was significantly different (t test, p < 0.01) in the presence versus absence of CmHNF-4 expression plasmid, whereas no statistical difference was shown in control reporter plasmids of either no-promoter (pAc3075) or HNF-4 binding site-null (pAc-IE1/CAT).

C. CmHNF-4-mediated activation of CmCatB required binding at the COUP-element. Shown are schematic promoter structures in reporter plasmids, pAc-CatB/CAT, pAc-CatBΔCOUP/CAT, and pAc3075 that have no promoter. Numbering is relative to the transcription start point. Reporter plasmids (8 μg) were cotransfected with 0.5 μg of CmHNF-4-expressing plasmid (black bar) or 0.5 μg of non-expressing empty vector (white bar), respectively. The latter was used to ensure comparable total DNA amounts in transfected S2 cells. Only pAc-CatB/CAT report plasmid showed statistical significance (t test, p < 0.01) in the presence versus absence of CmHNF-4. Transfection and normalization of CAT activity was performed as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of four independent cotransfections.

FIGURE 6. CmHNF-4 is a potential transcription activator in scN-adapted insect midgut. A, CmHNF-4-mediated specific interaction between the nuclear extract of scN-adapted insect midgut and COUP element. Antisera to the CmHNF-4 peptide was incubated with nuclear extract prior to the binding reactions with P7, forming the immune complex. Preincubation of antisera with excess of the synthetic peptide abolished the supershift. B, CmSvp enhanced CmCatB expression in Drosophila S2 cells. S2 cells were cotransfected with 8 μg of reporter plasmids and 0.5 μg of CmHNF-4 expression plasmid (black bars) or equivalent empty expression vector (white bars). The reporter plasmid pAc-IE1/CAT was used to determine the specificity of CmHNF-4 on the CmCatB promoter. CAT activity driven by the CmCatB promoter was significantly different (t test, p < 0.01) in the presence versus absence of CmHNF-4 expression plasmid, whereas no statistical difference was shown in control reporter plasmids of either no-promoter (pAc3075) or HNF-4 binding site-null (pAc-IE1/CAT).

C. CmHNF-4-mediated activation of CmCatB required binding at the COUP-element. Shown are schematic promoter structures in reporter plasmids, pAc-CatB/CAT, pAc-CatBΔCOUP/CAT, and pAc3075 that have no promoter. Numbering is relative to the transcription start point. Reporter plasmids (8 μg) were cotransfected with 0.5 μg of CmHNF-4-expressing plasmid (black bar) or 0.5 μg of non-expressing empty vector (white bar), respectively. The latter was used to ensure comparable total DNA amounts in transfected S2 cells. Only pAc-CatB/CAT report plasmid showed statistical significance (t test, p < 0.01) in the presence versus absence of CmHNF-4. Transfection and normalization of CAT activity was performed as described under “Experimental Procedures.” Each bar represents the mean ± S.E. of four independent cotransfections.

site effects, we transiently expressed both CmSvp and CmHNF-4 in S2 cells and evaluated the combined effect on CmCatB promoter. Intermediate CAT activity indicated that CmHNF-4-mediated gene activation was inhibited by CmSvp (Fig. 7). In contrast, when the IE1-CAT plasmid, which does not contain the COUP-element, was cotransfected with CmSvp and CmHNF-4 expression constructs, no significant change in CAT activity was observed. This result suggests that the two transcription factors, potentially both involved in CmCatB regulation, antagonize each other’s effect when both are present.

CmHNF-4 and CmSvp Co-regulate CmCatB Expression—Several studies have shown that positive transcriptional regulation could sometimes be repressed by COUP-TF/Svp proteins (25–30). The inverse pattern of protein abundance of CmHNF-4 and negative CmSvp factors. One possible inhibitory mechanism of CmSvp in unchallenged insects could be simply through mutual exclusion by competing for the cis-element, in which case intact DBD would be necessary for the antagonistic effect. We thus replaced the N terminus of the CmSvp (consisting of A/B and the DBD) with Gal4 DBD. Gal4 DBD itself did not impact CmHNF-4 function (Fig. 8). When transfected into S2 cells, the chimeric protein Gal4-CmSvp(D,E,F) alone exhibited a basal level of CAT activity. However, it repressed CmHNF-4-mediated transactivation when the constructs were cotransfected (Fig. 8). Thus DBD did not appear to be essential for the inhibitory activity of CmSvp. Therefore, we hypothesized that, rather than impacting DNA binding of CmHNF-4, CmSvp might repress CmHNF-4 activity through protein-protein interaction. Such interaction may prevent CmHNF-4 from stimulating the general transcription machinery.

CmHNF-4 and CmSvp Complex in scN-unadapted Insect Midgut—To establish the in vivo relevance of potential protein-protein interactions, we reasoned that CmHNF-4 might not have to physically detach from the DNA element to be inactive and that its activity could be repressed by action of CmSvp, which is abundant in scN-unadapted insect midgut. To test this idea, anti-CmHNF-4 serum was incubated with the gel shift reaction between P7 (the COUP probe) and nuclear extract from unchallenged insect midgut. Reproducible retardation of the DNA-protein complex mobility (Fig. 9A) indicated that CmHNF-4 is a component of the regulatory complex in unadapted insects as well. The same COUP-nuclear factor complex was also disrupted by anti-AaSvp antibody (Fig. 9B).

Because anti-AaSvp serum did not affect the complex formed in adapted insect midgut (Fig. 2), CmSvp is only a regulatory component for CmCatB expression in unadapted insect midgut. Protein-protein interaction is thus a viable mechanism for CmSvp to repress the stimulatory capacity of CmHNF-4, when CmCatB function is not needed, i.e. when insects are not facing dietary challenge.

To better understand the antagonistic activity of the two transcription factors, we studied physical interactions between CmSvp and CmHNF-4 using GST pulldown assay. In vitro translated 35S-labeled CmHNF-4 retained substantial interac-
tion with recombinant GST-CmSvp fusion protein, but not with the GST tag alone (Fig. 10). To map the CmSvp domain required for interaction with CmHNF-4, pulldown analyses were performed with a series of truncated recombinant proteins (Fig. 10A). Removal of amino acid residues 390–419, known to contain the repression domain, pAc-IE1/CAT that lacks HNF-4 binding site was used as a control plasmid to demonstrate the specific effects of CmHNF-4 and CmSvp on CmCatB promoter.

Likewise, a series of deletion constructs narrowed the interacting region of CmHNF-4 to the E domain, between amino acid residues 267 and 374 in LBD. Deletion of AF2 activation domain from LBD was not sufficient to abolish CmSvp-CmHNF-4 interaction (Fig. 10, C and D). This result suggests that protein-protein interaction could play a crucial role in CmCatB transcriptional regulation.

**DISCUSSION**

HNF-4 is a transcription factor that belongs to the nuclear receptor superfamily. It is most abundant in liver, kidney, and small intestine in vertebrates and in the functionally analogous fat body, malpighian tubules, and midgut in insects. The conserved sequences and tissue preference from insects to mammals reflects its conserved function through evolution. HNF-4 activates a large number of genes that are crucial for development (18–20, 31–34). It is also a key regulator of metabolic pathways that involve lipid and glucose homeostasis (15). In addition, it modulates transcriptional expression of cytochrome P450 genes functional in detoxification of foreign chemical compounds (25, 35–37). In the current study, we explored the positive regulatory function of HNF-4 from cowpea bruchid midgut. Our results revealed a new aspect to the already widespread impacts of HNF-4. We found that HNF-4 contributes to reconfiguration of insect counter-defense genes in the midgut under dietary challenge, and that it does so in coordination with another transcription factor Svp.

We hypothesized that CmHNF-4 was a positive regulator of CmCatB, after the cis-element was identified, based on several factors. First, HNF-4 is known to regulate many genes essential for nutrient metabolism and chemical detoxification (15). Although not previously investigated for its regulatory action on proteases, we believe that CmHNF-4 could potentially target CmCatB. Induction of CmCatB facilitates food protein degradation and amino acid assimilation in insects that are under
dietary challenge, consistent with a metabolic role of the transcription factor. Second, the COUP-element identified in the CmCatB promoter exhibits the important features of an ideal binding site for HNF-4 (15). These features include 1) two direct repeats of a half-site are separated by one nucleotide, 2) triple adenosines forming the center of the site, and 3) a conserved guanine in the second positions of all half-sites (Fig. 1A). Third, HNF-4 is a positive regulator that shares closely related binding specificities with COUP-TF or Svp in insects. Several genes have been shown to be coordinately regulated by the two transcription factors (14, 25–28, 38–40).

Our previous study revealed that different transcription factors are involved in CmCatB regulation in nuclear extracts of scN-adapted and unadapted insect midgut (12). Here we showed that, although CmSvp inhibited CmCatB expression, CmHNF-4 promoted its transcription when transiently expressed in Drosophila S2 cells (Fig. 6). This activity, however, was repressed by CmSvp (Fig. 7). The inverse relationship of CmHNF-4 and CmSvp protein abundance in the two insect groups (Fig. 4) is in agreement with their antagonistic function in CmCatB regulation.

Competing for occupancy of the common binding sites has been the typical inhibitory mechanism of HNF-4-mediated transcriptional activation by COUP-TF/Svp (25–30). For instance, HNF-4 and COUP-TF bind to the C3P site of apolipoprotein CIII promoter in a mutually exclusive manner. Replacing the DNA-binding domain of COUP-TF resulted in the loss of antagonism of HNF-4 transactivation of a lipid-binding protein apolipoprotein CIII (28). Similarly, activation of the hypoxia-specific erythropoietin gene via HNF-4 is repressed by COUP-TF (26). The promoter and enhancer regions of rat ornithine transcarbamylase consist of four cis-elements that interact with both HNF-4 and COUP-TF, which have opposing effects (27). Balancing positive HNF-4 and negative COUP-TF regulators that bind the common cis-element was also considered a predominant mechanism regulating human P450 CYP2D6 gene expression (25). Cowpea bruchids may have adopted a similar mechanism of regulation; that is, in unchallenged insect midgut, when CmCatB is not needed, the COUP-

FIGURE 10. CmHNF-4 and CmSvp interacted with each other via their LBD domains. A and C, diagrams of CmSvp and CmHNF-4 and their deletion constructs. GST was fused with full-length and truncated CmSvp proteins. B and D, GST pull-down assays. In vitro translated, 35S-labeled CmHNF-4 proteins were incubated with bacterially expressed GST-CmSvp fusion proteins or GST protein alone immobilized on glutathione-agarose beads. Specific interactions were analyzed on 8 or 15% SDS-PAGE followed by exposure to a PhosphorImager screen. The input lane represents 5% of the total in vitro translated CmHNF-4 proteins used in the binding reaction. CmHNF-4 (EΔAF-2): deletion of 35 amino acid residues in the C terminus of LBD, including AF-2 AD. Numbers indicate amino acid residues.

FIGURE 10. CmHNF-4 and CmSvp interacted with each other via their LBD domains. A and C, diagrams of CmSvp and CmHNF-4 and their deletion constructs. GST was fused with full-length and truncated CmSvp proteins. B and D, GST pull-down assays. In vitro translated, 35S-labeled CmHNF-4 proteins were incubated with bacterially expressed GST-CmSvp fusion proteins or GST protein alone immobilized on glutathione-agarose beads. Specific interactions were analyzed on 8 or 15% SDS-PAGE followed by exposure to a PhosphorImager screen. The input lane represents 5% of the total in vitro translated CmHNF-4 proteins used in the binding reaction. CmHNF-4 (EΔAF-2): deletion of 35 amino acid residues in the C terminus of LBD, including AF-2 AD. Numbers indicate amino acid residues.
element was occupied by repressor CmSvp due to its relatively high concentration (Fig. 4C), but in nuclear extracts from adapted insects where CmSvp is scarce, increased CmHNF-4 could replace CmSvp and activate CmCatB. Expression of CmCatB enables food protein degradation even in the presence of the inhibitor scN. However, the DNA binding domain was not required for CmSvp to repress CmHNF-4-mediated activation; even in the absence of CmSvp binding to COUP-site, protein-protein interaction between CmSvp and CmHNF-4 was able to repress transcription (Fig. 8). Thus competition for the same *cis*-element, a well known antagonistic mode of action between HNF-4 and COUP-TF/Svp, need not be the sole mechanism for CmCatB regulation. Protein-protein interaction presumably at least in part contributes to modulation of CmCatB promoter activity in cowpea bruchids (Figs. 8 and 10).

CmSvp repression of CmHNF-4 function through protein-protein interaction is supported by supershift assays using anti-CmHNF-4 antibody (Fig. 9A). Apparently, rather than being excluded from the *cis*-element, CmHNF-4 was a component of the DNA-protein complex in unchallenged insects. Protein association between COUP-TF/Svp and HNF-4 has so far only been known to enhance HNF-4 transactivation where COUP-TF acts as an auxiliary coactivator (14, 41). In these cases, COUP-TF itself did not bind to the promoters, but oriented HNF-4 through the HNF-4 LBD in a more efficient configuration to elicit synergistic transcriptional activity. In our experiment, protein-protein interaction apparently led to a silencing effect, opposite to these reported studies. *In vitro* experiments suggested that CmHNF-4 interacted with CmSvp through its LBD domain upstream AF2 activation domain (Fig. 10D). Helix H7 of human HNF-4, corresponding to amino acid residues 294–310 in CmHNF-4, has been shown to be important for recruitment of coactivators. Deletion of the Glu in H7 (Glu302 in CmHNF-4) abolished recruitment COUP-TF (41). The significance of Glu302 is not yet known, and more experiments are needed to locate the exact interacting residues in CmHNF-4.

It should be noted that, although protein contact alone is sufficient for CmSvp to repress CmHNF-4 function (Fig. 8), it does not exclude binding of CmSvp to the COUP site. Indeed, the presence of two adjacent COUP-elements renders the possibility of each transcription factor binding to one element, and the closeness of the two *cis*-elements could enable protein contact while binding. CmSvp without a COUP-binding domain, *i.e.* Gal4-CmSvp(D,E,F), exhibited basal CAT activity, whereas unaltered CmSvp showed further repression. Therefore, although the DNA binding domain was not essential for CmSvp to repress CmHNF-4-mediated activation, it certainly enhances the impact of CmSvp’s negative regulation of CmCatB. In the model shown in Fig. 11, we incorporated DNA-protein and protein-protein interactions to illustrate the molecular antagonistic mechanism; binding to the COUP site could help anchor CmSvp and stabilize its association with CmHNF-4. Such association likely masks the activation surface of CmHNF-4, resulting in transcription inhibition in unadapted insects. Binding to DNA near a DNA-bound activator and preventing transcription initiation, although unreported in HNF-4-Svp interactions, has been recognized as a transcription inhibitory mechanism (42). In adapted insects, decreased CmSvp abundance was insufficient to sustain this interaction, leading to transcriptional activation.

Although it is unclear how insects sense the presence of scN and how the signal of amino acid shortage was transduced, activation of CmCatB appears to be controlled by the intracellular balance of CmHNF-4 and CmSvp. The effect of upstream stress signals may be largely mediated by CmSvp. This reminded us of the control of gluconeogenesis in mammals. The key regulator of glucose synthesis PGC-1 works with HNF-4 to regulate downstream gluconeogenic enzymes (43). Similarly, the intrinsic activating role of CmHNF-4 may be altered by cofactors it partners with in the regulatory complex, even as its own expression is also adjusted. A balance between transcription activators and repressors enables achievement of the full regulatory potential for CmCatB transcription.

The fact that anti-AaSvp serum diminished the DNA-protein complexes formed between COUP probe and unadapted insect midgut nuclear extracts (Fig. 9B) but had no impact on the complex of adapted insects (Fig. 2) indicates that CmSvp is not a component in the activation machinery. The vacant COUP-site may be replaced by CmHNF-4, which is more abundant in adapted insects. Alternatively, other coactivators could also bind to the *cis*-element previously occupied by CmSvp, when insects were not under dietary challenge. It has been reported that co-factors are able to engage HNF-4 in a constitutively active configuration (24, 41, 43, 44). Such coactivators in CmCatB transcription regulation remain to be identified. In another scenario, because HNF-4 has been reported to cooperate with other transcription factors in regulating gene expression, CmHNF-4 could also serve as one of the coactivators facilitating another nuclear receptor, as in the case of human CYP3A4 (37). Such a factor could even influence the CmSvp-CmHNF-4 interactions.

Eukaryotic gene transcription is complicated not only because of the involvement of multiple transcription factors that control expression of genes of diverse function, but also because of the interplay of transcription factors possessing related structural motifs that recognize common target DNA.
sequences. We have demonstrated another facet of HNF-4 in metabolic processes particularly under nutrient stress; it is not only important for lipid and glucose homeostasis but also has a role in maintaining the supply of amino acids. By networking with various cofactors, HNF-4 orchestrates complex transcriptional responses. Such a regulatory mode of action helps us understand how insects readjust gene expression in the alimentary tract to mitigate the impact of dietary toxins and anti-nutritional factors, which is crucial for insect health and survival.

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