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The Caudal homeodomain protein activates *Drosophila* E2F gene expression

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ABSTRACT

The *Drosophila caudal* homeobox gene is required for definition of the anteroposterior axis and for gut development, and CDX1 and CDX2, human homologs, play crucial roles in the regulation of cell proliferation and differentiation in the intestine. Most studies have indicated tumor suppressor functions of Cdx2, with inhibition of proliferation, while the effects of Cdx1 are more controversial. The influence of *Drosophila* Caudal on cell proliferation is unknown. In this study, we found three potential Caudal binding sequences in the 5′-flanking region of the *Drosophila E2F* (*DE2F*) gene and showed by transient transfection assays that they are involved in Caudal transactivation of the *dE2F* gene promoter. Analyses with transgenic flies carrying an *E2F-lacZ* fusion gene, with and without mutation in the Caudal binding site, indicated that the Caudal binding sites are required for expression of *dE2F* in living flies. Caudal-induced E2F expression was also confirmed with a GAL4-UAS system in living flies. In addition, ectopic expression of Caudal with heat-shock promotion induced melanotic tumors in larvae. These results suggest that Caudal is involved in regulation of proliferation through transactivation of the *E2F* gene in *Drosophila*.

INTRODUCTION

Homeobox genes generally encode for transcription factors involved in developmental, proliferation and differentiation processes (1,2). The *Drosophila caudal* example is required for definition of the anteroposterior axis during early embryogenesis (3), and for gut development (4). When *caudal* activity is completely lacking, the hindgut and anal pads are almost absent.

In vertebrates, *Drosophila caudal*-related homeobox genes have also been characterized: *Xcad-1*, *Xcad-2* and *Xcad-3* in *Xenopus laevis*; *CdxA*, *CdxB* and *CdxC* in chicken; *Cdx-1*, *Cdx-2* and *Cdx-4* in mice; and *CDX1* and *CDX2* in the human (5). Cdx1 and Cdx2 are known to play crucial roles in the regulation of cell proliferation and differentiation in the intestine (6). Cdx1 is mainly expressed in undifferentiated crypt cells, while Cdx2 is expressed at a higher level in the differentiated villus tip (7). Most studies have indicated a tumor suppressor function of Cdx2, which inhibits proliferation (8,9). However, the effects of Cdx1 on cell proliferation are controversial and it has been reported to exert oncogenic potential by upregulating *ras* (10). Such *ras* activation is considered to be an early event in genesis of colorectal cancers and results in altered cell growth and differentiation (11,12). Recently, however, Cdx1 was also reported to inhibit proliferation of intestinal epithelial cells, by down-regulation of D-type cyclins (13).

Whether *Drosophila caudal* plays a role in the regulation of proliferation is unknown. Interestingly, we found three putative Caudal binding site-related sequences, A/CTTTATA/G (14), in the 5′-flanking region of the *Drosophila E2F* (*dE2F*) gene, encoding a transcription factor playing important roles in cell proliferation (15). Well known targets of E2F are critical for cell-cycle progression and DNA synthesis. These include cell-cycle regulators, such as cyclin E, cyclin A, cdc2, cdc25 and Myc, and DNA replication-related genes, such as DNA polymerase α, thymidine kinase, PCNA and ribonucleotide reductase (16). The basic unit of E2F is a heterodimer of E2F and DP. In mammals, at least, six E2F family members (E2F-1 to -6) and two dimer partners (DP1 and DP2) have been characterized (17–21). *Drosophila* contains two E2Fs, dE2F and dE2F2, and one dimer partner, dDP (22–24). It has been reported that promoters of *Drosophila* genes encoding DNA polymerase α, PCNA and Raf contain the E2F recognition sequence (5′-TTTCGCGC), which is essential for promoter activity (25–28).

In the present study, since putative Caudal binding sites are located in the 5′-flanking region of the *dE2F* gene, we investigated involvement of the protein in transcriptional regulation. The results indicate that it can directly transactivate the *dE2F* gene promoter *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Oligonucleotides

The following double-stranded oligonucleotides containing a 6 bp linker sequence, recognized by *Bgl*II and *Bam*HI, were chemically synthesized. Sequences containing three Caudal sites or base substitutional mutants in the *E2F* promoter region were as follows: E2F-Cad1 wild-type (wt), 5′-gatcc-TTTGGGATATTTATGAGTACAAACa-3′ and 3′-gAAA-CCCTATAAATACTCATGTTTGtctag-5′; E2F-Cad1 mutant (mut), 5′-gatccTTTGGGATATGGCTGAGTACAAACa-3′ and 3′-gAAACCCTATACCGACTCATGTTTGtctag-5′; E2F-Cad2 wt, 5′-gatccACTTTTCTCTATAAAGTTGCAAATa-3′ and 3′-gTGAAAAGAGATATTTCAACGTTTAtctag-5′; E2F-Cad2 mut, 5′-gatccACTTTTCTCTAGCCAGTTGCAAATa-3′ and 3′-gTGAAAAGAGATCGGTCAACGTTTAtctag-5′; E2F-Cad3 wt, 5′-gatccGGCATAATTTTTATGATACGTA-GAa-3′ and 3′-gCCGTATTAAAAATACTATGCATCTtctag-5′; E2F-Cad3 mut, 5′-gatccGGCATAATTTGCCTGATACG-TAGAa-3′ and 3′-gCCGTATTAAACGGACTATGCATCTtc-tag-5′.

Double-stranded oligonucleotides for site-directed mutagenesis were as follows: E2F-Cad1-SM, 5′-GCGATTTT-GGGATATGGCTGAGTACAAACT-3′ and 3′-CACTTA-AACCCTATACCGACTCATGTTTGA-5′; E2F-Cad2-SM, 5′-CTAACTTTTCTCTAGCCAGTTGCAAATCTT-3′ and 3′-GATTGAAAAGAGATCGGTCAACGTTTAGAA-5′; E2F-Cad3-SM, 5′-TCAGGCATAATTTGGCTGATACGTAGA-AAT-3′ and 3′-AGTCCGTATTAAACCGACTATGCATCT-TTA-5′. Mutated bases are underlined and lower case letters indicate the linker sequences recognizable by *Bgl*II and *Bam*HI.

Plasmid construction

The promoter region of the *dE2F* gene was cloned by polymerase chain reaction (PCR) using *Drosophila* genomic DNA. Sequence analysis was performed to confirm the nucleotide sequence. The primers used, containing the linker sequences 5′-*Kpn*I and 3′-*Xho*I were as follows: 5′-GCGG-GTACCATGTGTTGGGCAAGGGAT-3′ and 3′-GCGGAG-CTCGCACTGCAGTCGTCAAGC-5′. An amplified 2401 bp fragment containing the *dE2F* promoter region (–2184 to +217) was inserted into the *Kpn*I and *Xho*I sites of pGL2-Basic (Promega) and the resulting reporter construct was designated as pE2F-Luc. To construct the plasmids pE2F-*lacZ* and pE2Fcadmut-*lacZ* for transgenic flies, the *dE2F* promoter region (–2184 to +217) with and without base-substituted mutations in the Caudal binding site were inserted into the *Kpn*I site of the plasmid pCaSpeR-AUG-βgal (29).

The expression plasmid pAc-cad containing the *Drosophila caudal* coding region (1419 bp) placed under control of the *Drosophila acin 5C* gene promoter was constructed by inserting the coding region fragment into the *Xho*I site of pAc5.1/V5-HisA (Invitrogen). In the case of the GST-cad fusion plasmid, a *caudal* DNA fragment containing a DNA binding region of 729–976 bp downstream of the transcription start site (30) was inserted into the pGEX 4T-2 vector with *Xho*I. For construction of the plasmid pUAS-cad, the *caudal* coding region (1419 bp) was inserted into the *Xba*I and *Eco*RI sites of the pUAST vector (31).

Expression of GST fusion proteins and gel-mobility shift assays

Expression of GST-Cad fusion proteins in *Escherichia coli* was carried out as described previously (32). The *E.coli* were collected and suspended in a solution containing 25 mM HEPES pH 7.9, 1 mM EDTA, 0.02% 2-mercaptoethanol, 10% glycerol, 0.1% Tween-80 and 0.2 M KCl, sonicated and then centrifuged. The supernatant was loaded onto a glutathione–Sepharose column equilibrated with buffer A (20 mM Tris–HCl pH 7.8, 6% glycerol, 0.1 M KCl, 0.1 mM EDTA, 0.2% Tween-80 and 5 mM DTT) and washed with 20 ml of buffer A and 20 mM WE buffer (20 mM Tris–HCl pH 7.8, 2 mM $MgCl_2$ and 1 mM DTT). Then the proteins were eluted with 20 ml of buffer G (50 mM Tris–HCl pH 9.6 and 5 mM glutathione), concentrated using Centricon 20 (Amicon) and dialyzed against PC buffer (50 mM Tris–HCl pH 7.8, 1 mM DTT and 0.1 mM EDTA) containing 0.15 M KCl and 50% glycerol. The purified proteins were stored at –20°C. Gel-mobility shift assays were performed as described previously (33). ^{32}P-labeled probes were incubated for 15 min on ice in a reaction mixture containing 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.1 M KCl, 500 ng of sonicated herring sperm DNA and 500 ng of poly(dI–dC). For this step, unlabeled oligonucleotides were added as competitors. Then, 20 µg of purified GST fusion proteins were introduced followed by incubation for 10 min on ice. DNA–protein complexes were electrophoretically resolved on 6% polyacrylamide gels in 50 mM Tris–borate pH 8.3, 1 mM EDTA and 2.5% glycerol at 25°C. Gels were dried and then autoradiographed or analyzed with a BAS2000 (Fuji Film) imaging analyzer.

Cell culture, DNA transfection and luciferase assay

Drosophila S2 cells were grown at 25°C in M3 (BF) medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco BRL). Transfection of DNA mixtures into S2 cells was performed using dimethyldioctadecyl ammonium bromide (DDAB) (34) and the cells were harvested at 48 h thereafter. The luciferase assay was carried out by means of a Luciferase Assay System (Promega), as described previously (35). Normalized luciferase activities were calculated by determining the luciferase/β-galactosidase activity ratios.

Site-directed mutagenesis

To obtain mutant reporter plasmids carrying base-substitution mutations in the Caudal binding sites in the 5′-flanking region of the *Drosophila E2F* gene, mutagenesis reactions were carried out on double-stranded DNA of pE2F-Luc using a QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The reactions were set up essentially as recommended by the manufacturer. The mutations, as well as the fidelity of the rest of the DNA were confirmed by sequencing or enzymatic manipulation.

Establishment of transgenic flies and fly stocks

For ectopic expression experiments, the GAL4-UAS system was used with Hsp70-GAL4 flies (*hs-GAL4*) provided by the Bloomington Stock Center. Seven independent transgenic lines carrying *UAS-cad* were generated with the plasmid

pUAS-cad by P-element-mediated transformation (36). Although only data with the line carrying *UAS-cad* in the second chromosome are shown in this report, essentially the same results were obtained with the other lines. To examine the role of the Caudal binding sites for *dE2F* gene expression *in vivo*, three independent transgenic lines carrying *E2F-lacZ* and two independent lines carrying the *E2Fcadmut-lacZ* fusion gene were generated with pE2F-*lacZ* and pE2Fcadmut-*lacZ* constructs, respectively. The lines carrying the same fusion genes showed the same *lacZ* expression patterns. An $E2F^{rm729}$ line in which P-element-*lacZ* was inserted upstream of the initiation site of the *dE2F* gene in the same orientation as *dE2F* gene was also used (27).

Ectopic expression of Caudal by heat-shock induction

Females carrying homozygous *hs-GAL4* in the third chromosome were crossed with males carrying homozygous *UAS-cad* in the second chromosome. The progeny third larvae were heat shocked at 37°C for 30 min and then returned to 25°C.

RT–PCR

Total RNA from larvae was isolated with Trizol Reagent (Molecular Research Center, Inc.) according to the protocol furnished by the manufacturer and cDNAs were synthesized with M-MLV-RT (reverse transcriptase) (Promega). The RT–PCR products were analyzed on agarose gels stained with ethidium bromide.

X-gal staining

The tissues were dissected and fixed for 15 min in phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM $Na_2HPO_4{\cdot}2H_2O$, 3 mM $NaH_2PO_4{\cdot}2H_2O$, pH 7.5) containing 5% glutaraldehyde, washed in PBS, and immersed in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranocyde (X-gal) in staining buffer containing 6.1 mM $K_4Fe(CN)_6$, 6.1 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4. Incubation was in the dark at 37°C.

RESULTS

Potential Caudal recognition sequences in the 5′-flanking region of the *Drosophila E2F* gene

We found three putative Caudal binding sites similar to the Caudal binding consensus sequences, A/CTTTATA/G in the 5′-flanking region of the *dE2F* gene (Fig. 1). They were named E2F-Cad1, E2F-Cad2 and E2F-Cad3. The E2F-Luc reporter plasmid containing the *dE2F* gene (–2184 to +217) was constructed as described in the Materials and Methods. To examine whether the Caudal protein can recognize the putative binding sites found in the *dE2F* promoter region, a gel-mobility shift assay was performed using recombinant GST-Cad fusion proteins. Oligonucleotides E2F-Cad1 wt, E2F-Cad2 wt and E2F-Cad3 wt, were used as probes. Binding of GST-Cad to the oligonucleotide E2F-Cad1 wt was detected and the DNA–protein complexes were diminished effectively by adding unlabeled oligonucleotides E2F-Cad 1 wt, but not oligonucleotide E2F-Cad1 mut carrying base substitutions in the Caudal core binding sequence (Fig. 2A). A complex between GST-Caudal and the oligonucleotide E2F-Cad2 wt was detected (Fig. 2B) and diminished by adding excess

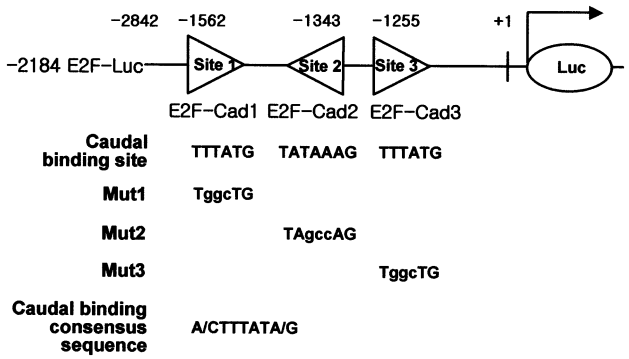

Figure 1. Potential Caudal homeodomain binding sites in the *Drosophila E2F* promoter. The three potential Caudal homeodomain binding sites in *Drosophila E2F* gene promoter, named E2F-Cad1, E2F-Cad2 and E2F-Cad3, are shown. The reporter construct used in the transfection assay contains a more upstream region, up to –2184, and a downstream region, up to +217. Lower case letters indicate mutated bases in the Caudal sites with numbering relative to the transcription initiation site.

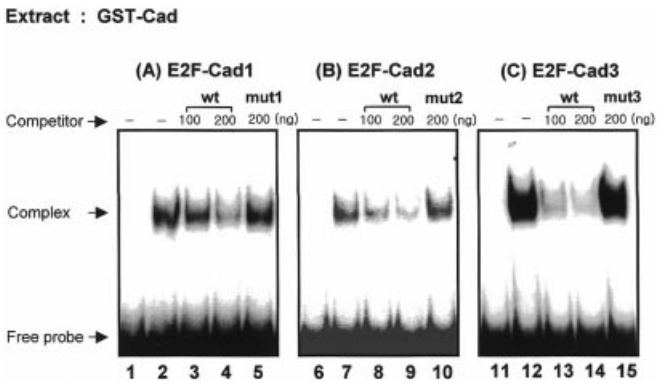

Figure 2. Complex formation between putative Caudal binding sites in the *dE2F* promoter and GST-Cad. Radiolabeled double-stranded E2F-Cad oligonucleotides were incubated with 20 µg of GST-Cad in the presence or absence of the indicated competitor oligonucleotides. Lanes 1, 6 and 11, GST only. Lanes 2, 7 and 12, binding without competitor. Lanes 3, 4, 8, 9, 13 and 14, unlabeled competitor DNA with the wild-type E2F-Cad sequences (wt). Lanes 5, 10 and 15, unlabeled competitor DNA with mutants having three base changes in E2F-Cad sequence (mut). E2F-Cad1, E2F-Cad2 and E2F-Cad3, oligonucleotides containing the Caudal sites 1–3 of the *dE2F* gene promoter.

amounts of unlabeled E2F-Cad2 wt. Such competition was not evident with E2F-Cad2 mut. The complex was also detected when the assay was performed with the radiolabeled E2F-Cad3 wt oligonucleotide as probe (Fig. 2C). Unlabeled oligonucleotide E2F-Cad3 wt competed with the formation of complex, whereas unlabeled E2F-Cad3 mut did not. The results indicate that GST-Caudal binds to E2F-Cad1, E2F-Cad2 and E2F-Cad3 binding sites with sequence specificity.

Activation of the *dE2F* gene promoter by Caudal

Since GST-Cad binds to the three potential Caudal binding sites in the 5′-flanking region of the *dE2F* gene, we investigated whether the Caudal homeodomain protein can regulate activity of the *dE2F* promoter, using a pE2F-Luc reporter plasmid containing the promoter region (–2184 to +217) of the *dE2F* gene fused with the luciferase reporter

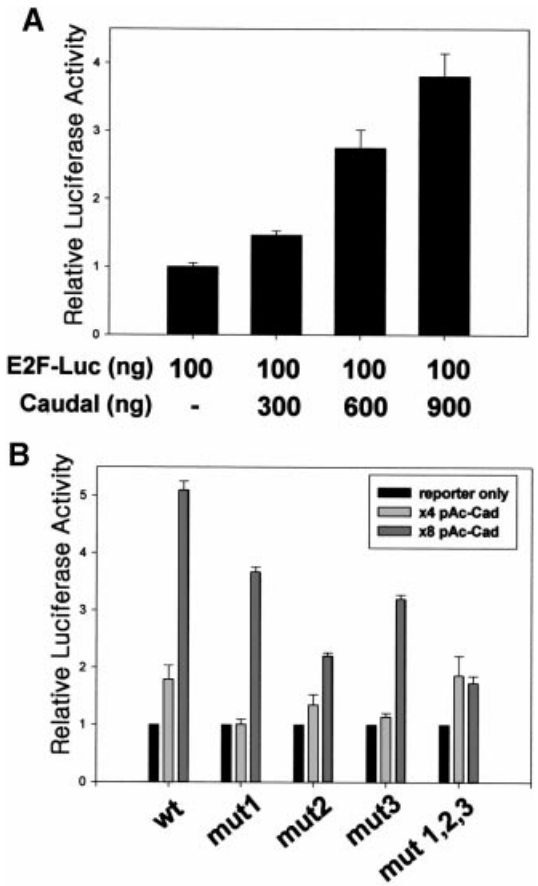

Figure 3. Effects of Caudal on luciferase expression driven by the *dE2F* promoter. Transfections were performed with *Drosophila* S2 cells and promoter activities measured as luciferase activity normalized to β-galactosidase activity at 48 h thereafter. (**A**) Each transfection mixture contained the E2F-Luc reporter plasmid, the pAc-lacZ internal control plasmid and the indicated amount of Caudal expressing plasmid, pAc-cad. (**B**) Constructs carrying a point mutation in Caudal binding sites in the *dE2F* promoter were cotransfected with the indicated amount of pAc-cad. The mean activities ±SE from three or four independent transfections are shown.

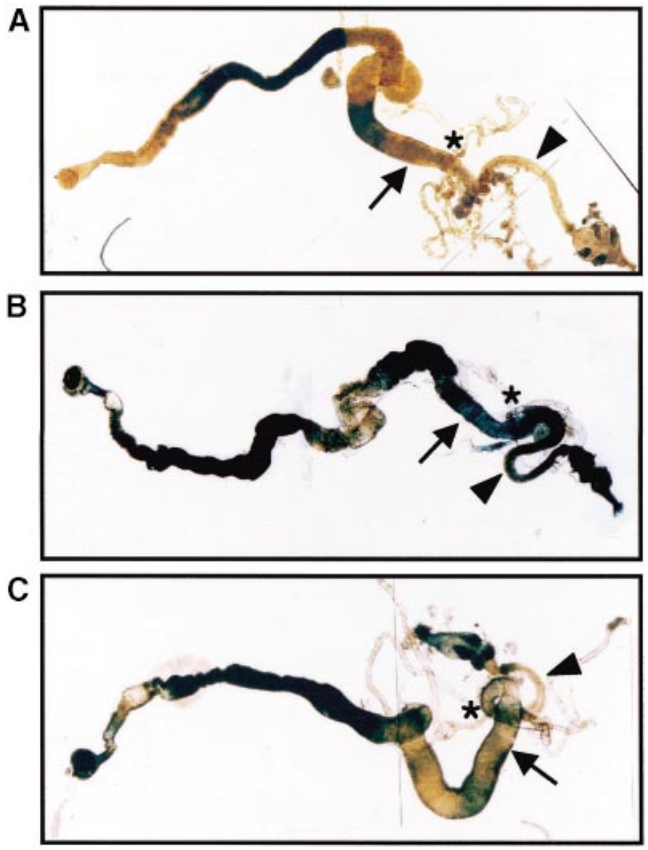

Figure 4. Effects of Caudal binding sites on *dE2F* expression *in vivo*. β-Galactosidase expression in the wild-type (Oregon R) (**A**) and transgenic flies carrying *E2F-lacZ* (**B**) and *E2Fcadmut-lacZ* (**C**) was detected by X-gal staining. The adult guts were dissected and stained with 0.2% X-gal solution in the dark. Reduced expression of the *E2Fcadmut-lacZ* is apparent in the posterior midgut and hindgut, compared with *E2F-lacZ*. The arrow indicates the posterior midgut and the arrowhead the hindgut. The asterisk indicates the Malpighian tubules.

gene. Effects of Caudal on *dE2F* gene promoter activity were examined in transient transfection experiments using pE2F-Luc reporter plasmids and a pAc-cad expression plasmid in the *Drosophila* S2 cell line. The wild-type *dE2F* promoter was transactivated depending on the amount, up to nearly 4- or 5-fold, of pAc-cad (Fig. 3A). For further confirmation of Caudal binding sites as targets of Caudal, the mutant reporter plasmids, pE2F-Cad1mut-Luc, pE2F-Cad2mut-Luc and pE2F-Cad3mut-Luc having the same mutation as those used for the gel-mobility shift assay, and the E2F-Cad1,2,3mut-Luc having base-substitution mutations in all three Caudal binding sites were constructed and used for reporter assays. As shown in Figure 3B, mutant reporter plasmids showed little activation with Caudal expression plasmids. A comparison between promoter activities of *dE2F* mutants and the wild type revealed no alteration in promoter activity (data not shown). These observations can be explained by the results of RT–PCR analysis in *Drosophila* S2 cells, by which *Drosophila caudal* mRNA was barely detectable (data not shown). Thus, Caudal apparently activates the *dE2F* promoter and this is mediated by the Caudal binding sites in the *dE2F* promoter.

Role of the Caudal binding sites in expression of *dE2F* in living flies

To investigate the role of the Caudal sites for expression of *dE2F* in living flies, we established transgenic flies carrying the *dE2F-lacZ* fusion gene, containing the *dE2F* promoter region (–2184 to +217) fused to *lacZ*, or *E2Fcadmut-lacZ* with base-substituted mutations in the Caudal binding sites of the *dE2F* promoter. *Drosophila caudal* is known to be expressed in the posterior midgut, Malpighian tubules and hindgut of the embryo (4) and in the anal plates and hindgut of adult flies (37). Therefore, we compared expression patterns of *lacZ* in the gut of adult flies carrying *E2F-lacZ* and *E2Fcadmut-lacZ* by X-gal staining. The *lacZ* staining signals of adult flies carrying the *E2Fcadmut-lacZ* were markedly decreased in the posterior midgut and the hindgut (Fig. 4B and C). Endogenous β-galactosidase expression was detected in the anterior part and the central region of the midgut (Fig. 4A). This result indicates that the Caudal binding sites are required for *dE2F* gene expression in the posterior midgut and the hindgut.

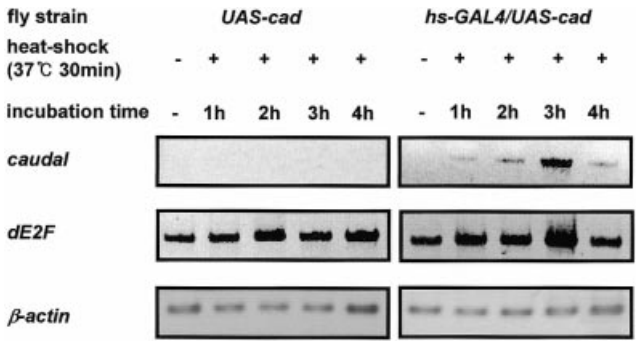

Figure 5. Expression of *caudal* and *dE2F* in *hs-GAL4/UAS-cad* third instar larvae. Total RNA was prepared from the third instar larvae carrying *hs-GAL4* and *UAS-cad* after heat shock at 37°C for 30 min and incubated at 25°C for various time periods. Then, RT–PCR was performed to measure *caudal* and *dE2F* mRNA levels. The line carrying *UAS-cad* was used as a control and processed in the same way as that carrying *hs-GAL4/UAS-cad*.

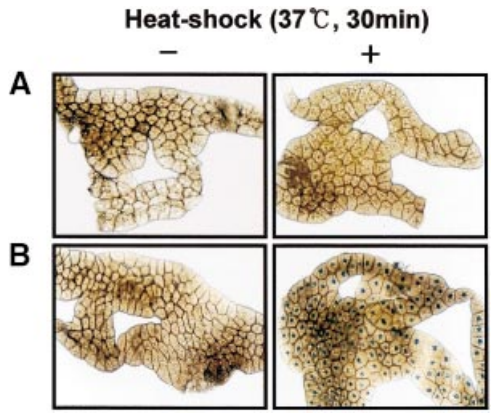

Figure 6. Effects of ectopic Caudal expression on *dE2F* gene expression *in vivo*. The third instar larvae of *UAS-cad/+;E2F^{rm729}/hs-GAL4* lines (**B**) were dissected after heat shock (37°C, 30 min) and incubated at 25°C for 8 h. They were then fixed in 5% glutaraldehyde and stained with 0.2% X-gal solution in the dark at 37°C. Tissues from *E2F^{rm729}* larvae were processed in the same way (**A**).

Effects of ectopic Caudal expression on the expression of *dE2F*

Ectopic expression of Caudal in living flies was performed with the GAL4-UAS system (31,38). The coding region of *caudal* was subcloned into the pUAST vector, and the resultant plasmid was designated pUAS-cad. We established transgenic flies for ectopic expression of Caudal with the pUAS-cad construct by P-element-mediated transformation (36). Transgenic flies carrying *UAS-cad* were crossed with transgenic flies carrying GAL4 cDNA placed under control of the *hsp70* gene promoter (*hs-GAL4*). Ectopic expression of Caudal in the transgenic larvae after heat shock was confirmed by RT–PCR (Fig. 5). The *caudal* mRNA in larvae carrying single copies of *hs-GAL4* and *UAS-cad* was detected at 1 h after heat shock and its level increased with time to reach a maximum at 3 h (Fig. 5). However, *caudal* mRNA in larvae carrying only *UAS-cad* was not detected at any time (Fig. 5). As mentioned above, we examined the *dE2F* expression in larvae carrying *hs-GAL4* and *UAS-cad* by RT–PCR. The expression level of *dE2F* was increased at 3 h after heat shock (Fig. 5).

To investigate the effects of ectopic Caudal expression on the *dE2F* gene expression *in vivo*, we made a *UAS-cad/+;E2F^{rm729}/hs-GAL4* line by crossing *E2F^{rm729}* flies, in which *lacZ* is inserted upstream of the initiation site of the *dE2F* gene, with transgenic flies carrying *UAS-cad* with the *hs-GAL4* line. The third instar larvae of the *UAS-cad/+;E2F^{rm729}/hs-GAL4* line were heat shocked at 37°C for 30 min and incubated at 25°C for 8 h. Then, they were dissected and X-gal staining was carried out. Expression of the *lacZ* gene in the heat-shocked *UAS-cad/+;E2F^{rm729}/hs-GAL4* larvae was increased dramatically in fat bodies by overexpressed Caudal (Fig. 6B).

Effects of the ectopic Caudal expression during development were also analyzed with transgenic flies carrying *hs-GAL4* and *UAS-cad*. Heat-shock induction of Caudal caused generation of melanotic tumors (Fig. 7), indicating that overexpression can result in abnormal proliferation and differentiation.

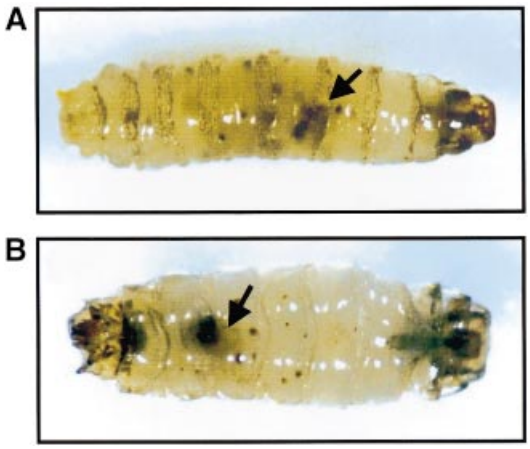

Figure 7. Formation of melanotic tumors with ectopic Caudal expression under the GAL4-UAS system. Melanotic tumors (arrow) are apparent in third instar larvae at 36 h (**A**) and 48 h (**B**) after heat shock. Most of the larvae had died by that time.

DISCUSSION

In this study, we found three Caudal binding sites in the 5′-flanking region of the *dE2F* gene (Fig. 1) and demonstrated their requirement for *in vivo* expression of the *dE2F* gene. Thus, the homeodomain protein Caudal positively regulates the cell proliferation-related gene *dE2F* at the transcriptional level.

Caudal-related homeodomain proteins bind to an AT-rich sequence of A/CTTTATA/G (14). *Drosophila caudal* is expressed in the posterior region of the embryo during early embryogenesis and activates the segmentation gene, *fushi tarazu*, involved in terminal specification (39). Maternal and zygotic *caudal* products are known to exert their function through activation of *folded gastrulation*, *fork head* and *wingless* (4). Caudal can induce *Distal-less* gene expression and internal analia development by activating the

brachyenteron and *even-skipped* genes (37). *Cdx1* and *Cdx2*, mammalian homologs of the *Drosophila caudal* gene, are mainly expressed in the intestine and colon where they appear to be involved in the regulation of cell proliferation and differentiation (40). *Cdx1* expression is restricted to the proliferative crypt compartment (41), while *Cdx2* is found at high levels in the differentiated cells of villus tip (42). The several targets of Cdx2 include the *Hox-C8* homeobox gene (43), the small intestinal genes encoding sucrase-isomaltase (44), lactase-phlorizin hydrolase (45), phospholipase-A (46), calbindin-D9K (47), the colonic gene for carbonic anhydrase I (48), and the genes encoding insulin and glucagon in the pancreas (49). These are all differentiation-related, supporting a tumor suppressor function of Cdx2 inhibiting proliferation (8,9). In the case of Cdx1, only *Hox-A7* has been shown as a direct target (50). Overexpression of Cdx1 in non-intestinal cells increases cell growth *in vitro* and promotes cell tumorigenicity in xenografts (2), which suggests oncogenic potential. Intestinal metaplasia in gastric and esophagal tumors is associated with ectopic expression of Cdx1 (51) and recently we reported CDX1 to have a pro-proliferative effect through transactivation of the PCNA promoter activity (52). Here, we demonstrated that Caudal directly transactivates the *dE2F* gene promoter activity. E2F is a key regulator of cell proliferation. The well known targets of E2F are critical for cell-cycle progression and DNA synthesis, such as cyclin E, cyclin A, cdc2, cdc25, Myc, DNA polymerase α, thymidine kinase, PCNA and ribonucleotide reductase (16). Therefore, our results indicate that *Drosophila* Caudal may be involved in cell proliferation by regulating *dE2F* gene expression so that its function is similar to that of Cdx1 rather than Cdx2.

Recently, it was reported that both E2F and DP mutant larvae exhibit development of melanotic tumors (53). Interestingly, we also observed that ectopic Caudal expression under the control of the heat-shock promoter leads to death in the developmental stage and generation of melanotic tumor in third instar larvae (Fig. 7). It has been proposed that two distinct classes of melanotic tumor mutations exist (54). Class 1 affect autoimmune responses or the normal immune system reaction to abnormal target tissue. Class 2, in contrast, cause obvious defects in the hematopoietic organs or hemocytes, resulting in aberrant immunity, contributing to melanotic tumor formation. In the present case, upregulation of E2F expression by ectopic Caudal induced under *hs-GAL4/UAS-cad* was detected especially in the fat body (Fig. 6), which is responsible for immune response in *Drosophila* (55). We have also found that *caudal* is expressed in the fat body and is involved in the immune response of *Drosophila* (M.-A. Yoo and W.-J. Lee, unpublished results). Further studies to investigate the roles of Caudal and E2F in the development of melanotic tumors are clearly warranted.

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