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'-TCTTCCGCGC), 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.
MATERIALS AND METHODS

Oligonucleotides

The following double-stranded oligonucleotides containing a 6 bp linker sequence, recognized by BglII and BamHI, 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′-gacct-TTGTGATTATATTAGACTAACAa-3′ and 3′-gAAA-CCTCATAATCTCATGTGttctag-5′; E2F-Cad1 mutant (mut), 5′-gacctTTGTGATTATATTAGACTAACAAa-3′ and 3′-gAAAACCCTCATAATCTCATGTGttctag-5′; E2F-Cad2 wt, 5′-gacctTCTTCTCTATAAGTTGCAAAtctag-5′ and 3′-gTGAAAGAGATATTCTCAACGTttctag-5′; E2F-Cad2 mut, 5′-gacctTCTTCTCTATAAGTTGCAATA-3′ and 3′-gTGAAAGAGATATTCTCAAAGTTGttctag-5′; E2F-Cad3 wt, 5′-gacctGCATAATTTTGATAGTACAAACT-3′ and 3′-gCGGTCAACGTTTAtctag-5′; E2F-Cad3 mut, 5′-gacctGCATAATTTTGATAGTACAAACT-3′ and 3′-gCGGTCAACGTTTAtctag-5′.

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 recognizable by BglII and BamHI, indicated the linker sequences recognizable by BglII and BamHI.

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 MgCl2 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 8.1, 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). 32P-labeled probes were incubated for 15 min on ice in a reaction mixture containing 20 mM HEPES pH 7.5, 10 mM MgCl2, 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 dimethylidioctadecyl 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 E2Fcrm729 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 Na₂HPO₄·2H₂O, 3 mM NaH₂PO₄·2H₂O, pH 7.5) containing 5% glutaraldehyde, washed in PBS, and immersed in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in staining buffer containing 6.1 mM K₄Fe(CN)₆, 6.1 mM K₃Fe(CN)₆·13 and 14, unlabeled competitor DNA with the wild-type E2F-Cad as probe (Fig. 2C). Unlabeled E2F-Cad3 mut did not. The complex was also detected when the assay was performed with the radiolabeled E2F-Cad2 mut. The complex was also detected 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.
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. β-Galactosidase expression in the wild-type (Oregon R) (A) and transgenic flies carrying dE2F-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.
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<sup>rm729</sup>/hs-GAL4 line by crossing E2F<sup>rm729</sup> 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<sup>rm729</sup>/hs-GAL4 line were heat shocked at 37°C for 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<sup>rm729</sup> larvae were processed in the same way (A).

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/CTTATA/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 differentiatted 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 esophageal 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 \textit{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 \( \alpha \), thymidine kinase, PCNA and ribonucleotide reductase (16). Therefore, our results indicate that \textit{Drosophila} Caudal may be involved in cell proliferation by regulating \textit{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 \textit{hs-GAL4/UAS-cad} was detected especially in the fat body (Fig. 6), which is responsible for immune response in \textit{Drosophila} (55). We have also found that \textit{caudal} is expressed in the fat body and is involved in the immune response of \textit{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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REFERENCES


