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The Homeobox Gene *Caudal* Regulates Constitutive Local Expression of Antimicrobial Peptide Genes in *Drosophila* Epithelia

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In *Drosophila melanogaster*, although the NF-κB transcription factors play a pivotal role in the inducible expression of innate immune genes, such as antimicrobial peptide genes, the exact regulatory mechanism of the tissue-specific constitutive expression of these genes in barrier epithelia is largely unknown. Here, we show that the *Drosophila* homeobox gene product Caudal functions as the innate immune transcription modulator that is responsible for the constitutive local expression of antimicrobial peptides cecropin and drosomycin in a tissue-specific manner. These results suggest that certain epithelial tissues have evolved a unique constitutive innate immune strategy by recruiting a developmental "master control" gene.

The innate immune system is an essential means of host defense in all eukaryotes, and this system also plays an instructive role in the induction of adaptive immunity in vertebrates (32). The production of antimicrobial peptides (AMPs) is the key feature of innate immunity aimed at neutralizing microbial infections in all multicellular organisms inhabiting various microbial environments (17). In the past decade, our understanding of innate immune signaling pathways leading to AMP expression in *Drosophila melanogaster* has dramatically increased. Most studies focused on the inducible systemic AMP gene expression observed in response to bacterial injection in the hemocoel. Genetic evidence from *Drosophila* demonstrates the existence of at least two distinct regulatory mechanisms for AMP synthesis in systemic innate immunity: the Toll pathway and the *immune deficiency* (IMD) pathway. The Toll pathway, primarily involved in drosomycin (Drs) antifungal peptide expression, requires a hemolymph serine proteinase cascade for its activation. This cascade, initiated by soluble pattern recognition proteins, is required for the processing of the Toll ligand, spaetzle, and for the subsequent activation of the p65-like Rel protein, Dif (18, 25, 29, 33, 34, 44). The IMD pathway is more specifically implicated in the expression of antibacterial peptide genes (such as *Cecropin* [*Cec*] and *Diptericin* [*Dipt*]) than the Toll pathway and requires the sequential activation of membrane peptidoglycan recognition protein receptor, IMD, TAK1, dFADD, Dredd, IκB kinase, and the p105-like Rel protein, Relish (6, 11, 12, 14, 20, 27, 28, 31, 37, 42, 45, 47, 52). In addition to these NF-κB signaling pathways, the c-Jun NH_2-terminal kinase (JNK) and JAK-STAT pathways are also involved in other immune functions, such as cytoskeletal remodeling for wound healing in the case of the JNK pathway (3).

In *Drosophila*, all known AMP genes are synthesized by the fat body, a functional homologue of the mammalian liver, during a systemic immune response (15, 50). However, it is believed that the first line of defense of the organism consists of the local expression of AMPs in barrier epithelia (referred to as local innate immunity), which are in direct contact with microorganisms (5, 50, 53). The in vivo monitoring of AMP expression in transgenic flies, expressing green fluorescence protein (GFP), revealed the existence of two distinct types of local innate immunity: the so called inducible local AMP gene expression and constitutive local AMP gene expression. In inducible local AMP gene expression, most barrier epithelia express at least one AMP in an inducible tissue-specific manner, primarily through the IMD pathway (10, 38, 51). For example, *Drs* and *Dipt* are induced in the tracheae and the gut, respectively, via the IMD pathway in response to local infection by bacteria such as *Erwinia carotovora* (10, 51). In the midgut and the proventriculus, *Cec* expression is normally absent but is rapidly induced in response to local infection (51). This inducible local immunity is activated by natural local infection but not by bacterial injection into the hemocoel, used for the initiation of systemic immunity. The other important form of local AMP gene expression is the constitutive form. In this case, the AMP gene is expressed constitutively in a defined tissue and its expression is not up-regulated during microbial infection (10, 51). To date, the regulatory signaling pathway(s) controlling constitutive local AMP gene expression is unknown. The most intense constitutive expression of *Cec* is found in the reproductive organs, such as the male ejaculatory duct (51). For *Drs*, the strongest constitutive expression is found in the salivary glands and the female reproductive organs (51). Furthermore, it was demonstrated that this type of *Drs* expression was independent of NF-κB pathways (Toll and IMD pathways) (10, 51).

The high complexity of AMP regulation indicates that the gene promoters must be regulated by different types of *trans*-activators. The κB sites (found in all known AMPs) and Rel

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family transcription factors (Dif and Relish) are essential for inducible expression of all AMPs during systemic immune and inducible local immune responses. As for *Cec*, in addition to a κB site, a GATA site is necessary for fat body-specific and immune-inducible expression in vivo (38, 40, 48). As mentioned above, the molecular mechanism of infection-independent constitutive local expression of AMPs in the barrier epithelia that are in direct or indirect contact with the external environment is presently unknown. We suspect that other important *cis* elements and *trans*-activators are involved in the regulation of AMPs in certain epithelia. In this study, we provide evidence that Caudal (Cad), in addition to its role as a homeotic transcription factor for anteroposterior body axis formation, is involved in the constitutive expression of a subset of AMP genes in epithelia.

MATERIALS AND METHODS

Fly strains. Flies were maintained on standard cornmeal-agar medium at 25°C. *Oregon*^{R} flies were used as the standard wild-type strain. The *Cec-GFP*- and *Drs-GFP*-expressing flies were obtained from B. Lemaitre (CNRS, Gif-sur-Yvette, France) (51). The *Relish*^{E20}-expressing mutant flies were obtained from D. Hultmark (University of Umeå, Umeå, Sweden) (14). The *c729-GAL4*-expressing line was obtained from Y. Engström (University of Stockholm, Stockholm, Sweden) (40). The *Yolk-GAL4*-expressing line was obtained from D. Ferrandon (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) (11). The lines expressing *HS-GAL4*, *Da-GAL4*, and *cad*^{3} mutant [*b*^{1} *pr*^{1} *cad*^{3}/*In*(*2LR*)*Gla*, *wg*$^{Gla-1}$] were obtained from the Bloomington Stock Center.

Electrophoretic mobility gel shift assay (EMSA). The recombinant glutathione *S*-transferase (GST)-Cad DNA binding domain (amino acids 273 to 427) was expressed in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography according to the vendor's directions (Amersham Pharmacia Biotech). Nuclear extracts were prepared as described previously (20) from untreated and heat-shocked *UAS-Cad*/+; *HS-GAL4*/+-expressing transgenic flies. The binding reaction was performed for 30 min at room temperature by mixing 1 ng of purified ^{32}P-labeled probe, 10 μg of nuclear extracts (or 50 ng of GST-Cad), and 300 ng of poly(dI-dC) in the presence of a protease inhibitor cocktail (Complete; Roche Molecular Biochemicals) as described previously (20). Supershift analysis was performed by mixing nuclear extract with antiserum against the activation domain of Cad (amino acids 1 to 272) or preimmune serum for 15 min at room temperature prior to adding the ^{32}P-labeled probes.

Real-time quantitative PCR analysis. The total RNA was extracted with an RNAzol reagent. The first cDNA was synthesized by using a first cDNA synthesis kit (Roche) according to the manufacturer's instruction. Fluorescence real-time PCR was performed with double-stranded DNA dye SYBR Green (Perkin-Elmer) to quantify the amount of gene expression. Primer pairs for *Cec* (sense, 5′-ATG AAC TTC TAC AAC ATC TTC G-3′; antisense, 5′-GGC AGT TGC GGC GAC ATT GGC G-3′), *Drs* (sense, 5′-GCA GAT CAA GTA CTT GTT CGC CC-3′; antisense, 5′-CTT CGC ACC AGC ACT TCA GAC TGG-3′), *Cad* (sense, 5′-CCA TCG AAG CCG CCA TAC T-3′; antisense, 5′-TTT GCC TGG TTG TGG TTG TG-3′), *glucose dehydrogenase* (sense, 5′-GGA AGC CGC CGC GTA TTG TG-3′; antisense, 5′-GAT TCT CCG GAC CCG TGT TCT GC-3′), *B52* (sense, 5′-CAC CGG ACC GCA ATA ACG AGA GCA-3′; antisense, 5′-GAC GAG GCC CGA CAG TGG TGG ATT-3′), and the control *Rac2* (sense, 5′-CAG ACG ATC GAG AAG CTG AAG G-3′; antisense, 5′-GTG CCG CTT GGG TCC TCG AAC G-3′) were used to detect the target gene transcripts. SYBR Green analysis was performed on an ABI PRISM 7700 system (PE Applied Biosystems) according to the manufacturer's instruction. All samples were analyzed in triplicate, and the levels of messages detected were normalized relative to the control *Rac2* values. The normalized data were used to quantify the relative levels of a given mRNA according to the ΔCt analysis (26).

Reporter gene assay. *Drosophila* immunocompetent Schneider cells (ATCC CRL-1963) were maintained in Schneider medium (Sigma) as previously described (13). Transient transfections were carried out by the calcium phosphate method (9). All transfection mixtures contained 100 ng of pPacPL-LacZ as an internal standard, 3 μg of pPacPL-Cad, and 100 ng of the luciferase reporter constructs (*Drs*-luciferase construct [a 2.4-kb upstream fragment of the *Drs* promoter] or the *Cec*-luciferase reporter construct [bp −751 to +71]). In addition, various *Drs* and *Cec* reporter constructs with deletions and point mutations were also cotransfected. At 48 h after transfection, luciferase activity was measured according to the manufacturer's instructions (Promega). Unlike in vivo experiments, these experiments required, for unknown reasons, incubation of the Schneider cells with lipopolysaccharide (10 μg/ml) for 6 h in order to conduct the Cad-induced *Cec* reporter assay. Luciferase activity was normalized with respect to β-galactosidase activity to correct for variations in the transfection efficiency.

In vivo detection of reporter transgenes. GFP reporter-expressing flies and dissected organs were examined under a stereofluorescence microscope (Leica; MZFLIII). Histochemical analysis of β-galactosidase expression was performed as previously described (23). In the bacterial-challenge experiment, the flies were pricked with a fine needle previously dipped into a concentrated culture of *Escherichia coli* and *Micrococcus luteus*. Quantitative analysis of the GFP reporter was performed with protein extract (100 μg) of the ejaculatory ducts by using the spectrofluorophotometer (excitation wavelength, 488 nm; emission wavelength, 507 nm) according to the manufacturer's instructions (Shimadzu; RF-5301PC).

Plasmids and the generation of transgenic animals. The promoter region (3.1 kb) of *Cad* was generated by PCR and subcloned into the pCaSpeR-AUG-β-gal vector to obtain the Cad-LacZ construct. The open reading frame of the *Cad* cDNA was subcloned into the pUAST vector (4) to obtain the upstream activation sequence (UAS)-Cad construct. To obtain the *UAS-Cad-RNAi* construct, a 510-bp cDNA fragment encoding amino acids 24 to 193 of Cad was amplified by PCR and the head-to-tail inverted repeats were subcloned into the pUAST vector. Mutations of Cad binding sites (S2 and S5; see Fig. 1) on the *Cec A1* gene promoter construct (bp −751 to +71) were created by site-directed mutagenesis, and subsequently the *Drs-GFP* plasmid PJM802 was replaced with the *Nhe*I-*Spe*I fragment of the S2- and S5-mutated *Cec A1* promoter to yield the *Cecmut-GFP* construct. To yield the *Drsmut-GFP* construct, mutations of Cad binding sites (S1 to S4; see Fig. 2) on the *Drs* promoter construct were created by site-directed mutagenesis by using the *Drs-GFP* plasmid PJM802. To create the Cad repressor construct (*pUAST-Cad-En*) (19), the *Drosophila En* repressor domain (amino acids 1 to 296 of the *Drosophila En* protein) was amplified and inserted in front of the DNA binding domain (amino acids 273 to 427) of Cad to allow the production of an in-frame N-terminal fusion of the *En* repressor domain to the DNA binding domain of Cad. These constructs were then used to generate transgenic animals by P-element-mediated transformation (43). The construct was injected into *w*^{1118}-expressing embryos.

RESULTS

Identification of CDREs in *Cec* and *Drs* promoters. We have been interested in novel transcription factors involved in the innate immune response, and we performed the in silico identification of putative genomic binding sites of AMP genes and their transcription factors by using the MatInd and MatInspector systems (41). In this analysis, we found several *cis* elements (such as the κB motif, the GATA motif, and Cad binding motifs) commonly found in the promoter regions of all known AMPs. Transcription factors resulting from this analysis were systematically tested for their capacity to induce AMP genes in the immunocompetent Schneider cell line SL2 (13). In Schneider cells stably expressing Cad, the expression of all seven AMP genes was greatly enhanced, suggesting that Cad is a potential transcription regulator (data not shown). This result prompted our in-depth investigation into the in vivo role of Cad using two representative AMP genes (IMD pathway-controlled *Cec* and Toll pathway-controlled *Drs*). Because the *Cad* gene product contains a homeodomain, which indicates that the protein has a DNA-binding capability, we examined the *cis* elements responsible for Cad-induced *Cec* and *Drs* expression. To identify the *cis* elements responsible for Cad-induced *Cec* and *Drs* expression, we performed a luciferase reporter assay of various mutant constructs having deletions in the *Cec* promoter region in *Drosophila* Schneider cells. Cad-induced luciferase activity in cells transfected with the plasmid with a deletion from −751 to −484 bp was found to be almost invariant compared with that in cells transfected with the wild-type con-

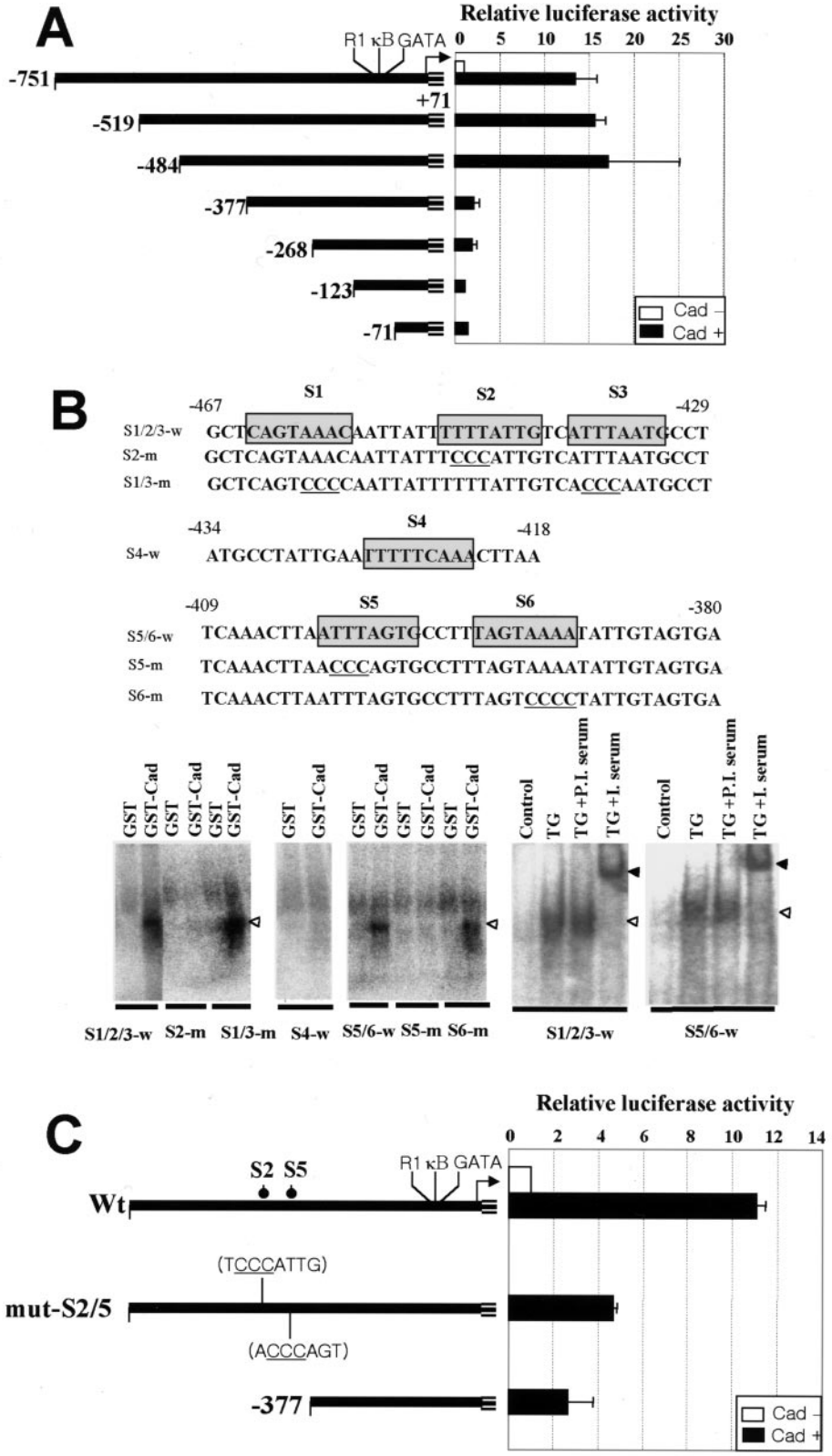

FIG. 1. Identification of Caudal protein DNA recognition elements in the *Cec* promoter. (A) Schematic structures of the transfected reporter plasmids are shown on the left. T bars, standard deviations (SD) of at least five independent experiments. Normalized luciferase activity in the absence of Cad expression was taken arbitrarily as 1, and results are presented as relative expression levels. (B) ^{32}P-labeled oligonucleotide probes (wild types and mutant types) and the sequence of the *Cec* promoter region, containing the putative Cad binding motifs (boxes S1 to S6). The mutant base pairs are underlined. DNA binding was carried out with GST-Cad or nuclear extract. Nuclear extracts were prepared from untreated

struct (Fig. 1A). However, luciferase activity remained at the basal level in cells transfected with the plasmid having a deletion from bp −751 to −377 (Fig. 1A). These results suggest that the region from bp −484 to −377 of the *Cec* promoter is a candidate region for *Cad*-protein DNA recognition elements (CDREs). For *Drs*, we could also identify the region covering bp −1082 to −1008 as a candidate region for CDREs of *Drs* (Fig. 2A). Based on these results, we identified six putative binding sites (S1 to S6) with the consensus Cad binding motif (T > C/A > G)TTT(A > G > C)(T > G/A/C)(G > T/C/A)(A > G/T/C) (2) in the promoter region of *Cec* and *Drs* (Fig. 1B and 2B). To determine whether Cad possesses a DNA binding capability with these putative binding sites of the *Cec* promoter, we performed DNA-binding experiments with the recombinant Cad protein using wild-type probes and various mutant probes (Fig. 1B). The results showed that GST-Cad is able to bind to two Cad binding motifs, at the S2 and S5 sites. To further confirm this Cad binding activity, we performed an EMSA using a nuclear extract from Cad-expressing transgenic flies. Using CDREs as probes, we observed a faint nuclear Cad binding activity in the nuclear extract from control transgenic flies (*UAS-Cad*; *Heat Shock* [*HS*]*-GAL4*, without heat shock), which was greatly enhanced in the nuclear extract of Cad-expressing transgenic flies (*UAS-Cad*; *HS-GAL4*, with heat shock) (Fig. 1B). Furthermore, an immune serum directed against Cad supershifted the protein-DNA complex, whereas the addition of preimmune serum did not result in the formation of a CDRE-protein-antibody complex (Fig. 1B). Luciferase reporter analysis with a plasmid carrying double mutations in the putative binding sites (S2 and S5) revealed that these sites are essential for Cad-mediated *Cec* promoter regulation (Fig. 1C). We also employed similar methods to identify the CDREs for Cad-mediated *Drs* promoter regulation. The luciferase reporter assay with plasmids carrying point mutations in the putative CDREs together with the EMSA and supershift assay revealed that Cad is capable of directly regulating the expression of *Drs* via four CDREs (S1 to S4) found in its promoter (Fig. 2B and C). These results demonstrate the involvement of Cad in the regulation of AMP genes, providing yet another function for this homeotic transcriptional regulator, well known for its key role in anteroposterior patterning of the embryo (8, 35, 36).

Mutations affecting CDREs do not abolish inducible systemic AMP expression. The above results were obtained from in vitro-cultured cells. To analyze the contribution of CDREs to AMP gene expression in vivo, we generated GFP reporter-expressing transgenic flies carrying the *Drs* and *Cec* promoter, in which the CDREs (at the S1 to S4 sites for the *Drs* promoter and at the S2 and S5 sites for the *Cec* promoter) were mutated (*Drsmut-GFP* and *Cecmut-GFP*, respectively). We compared these reporter-expressing transgenic flies with transgenic flies carrying wild-type promoters: the *Drs* promoter fused to *GFP* (*Drs-GFP*) and the *Cec A1* promoter fused to *GFP* (*Cec-GFP*). To investigate whether CDREs are involved in the systemic expression of AMP genes after septic injury, *Drs-GFP*- and *Drsmut-GFP*-expressing flies were pricked with a bacterium-soaked needle. The result showed that mutation in the CDREs does not affect the systemic immune response: a strong diffuse fat body-derived fluorescence was observed in both lines of transgenic flies (Fig. 3A). No difference in fluorescence intensity between transgenic flies carrying *Drs-GFP* and those carrying *Drsmut-GFP* was observed. Similar results were obtained with both *Cec-GFP*- and *Cecmut-GFP*-expressing transgenic flies (data not shown). These results clearly indicate that the CDREs, in contrast to κB sites, are not required for the inducible expression of these genes.

Mutations affecting CDREs abolish constitutive local AMP expression. Previous studies have shown that, in addition to systemic expression of AMPs by the fat body, several epithelia can express AMPs (local expression of AMP genes) (5, 10, 38, 51). As occurs in the case of vertebrate epithelia, insect epithelial tissue specifically produces various AMPs that help maintain a steady state of natural microflora (5, 10, 16, 24, 38, 51). Some epithelial tissues constitutively express AMP genes even in the absence of infection. For *Drs*, such constitutive local *Drs* expression is mainly detected in the salivary glands and in the female reproductive organs (10, 51). Interestingly, *Drs* expression in these epithelial tissues is not dependent on the known NF-κB pathways (Toll and IMD pathways) (10, 51). As CDREs are not involved NF-κB-dependent inducible AMP expression, we questioned whether CDREs are involved in constitutive local *Drs* expression in these epithelia. Our results concerning in vivo *Cad* expression using *Cad-LacZ*-expressing transgenic flies showed that a high level of expression of LacZ is present in various *Drs*-expressing epithelial tissues, including the salivary glands and the spermathecas and seminal receptacles (Fig. 3B and 3C). We also checked the levels of endogenous *Cad* expression in salivary glands and spermathecas and seminal receptacles with regard to that in the intestine. Real-time PCR analysis showed that the expression levels of the salivary glands and of the spermathecas and seminal receptacles reached 88 and 22% of the intestinal *Cad* mRNA level, respectively (data not shown). This result and the existence of CDREs in the *Drs* promoter prompted us to further investigate whether CDREs are implicated in the constitutive expression of *Drs* in these tissues. The result showed that, in contrast to *Drs* expression in the salivary glands of transgenic flies carrying *Drs-GFP*, *Drs* expression in the salivary glands in our transgenic flies (12 independent transgenic lines) carrying *Drsmut-GFP* was almost completely absent (Fig. 3D). However, strong constitutive *Drs* expression in the female reproductive organs in the *Drsmut-GFP*-expressing flies and also in the *Drsmut-GFP*-expressing flies under a *Relish*$^{-/-}$ genetic background was not affected (Fig. 3E). This reporter analysis using GFP-

(lanes control) and heat-shocked (lanes TG) *UAS-Cad*/+; *HS-GAL4*/+-expressing transgenic flies. Immune serum against recombinant Cad (I. serum) and preimmune serum from the same animal (P.I. serum) were used for the supershift assay. Open arrowheads, protein-DNA bands; solid arrowheads, supershifted Cad-DNA complexes. (C) Schematic structures of the transfected reporter plasmids are shown on the left. T bars, SD of at least three independent experiments. Normalized luciferase activity in the absence of Cad expression was taken arbitrarily as 1, and the results are presented as relative levels of expression. The *Cec*-luciferase reporter carrying double mutations (at the S2 and S5 sites) on Cad binding motifs is indicated. Wt, wild type.

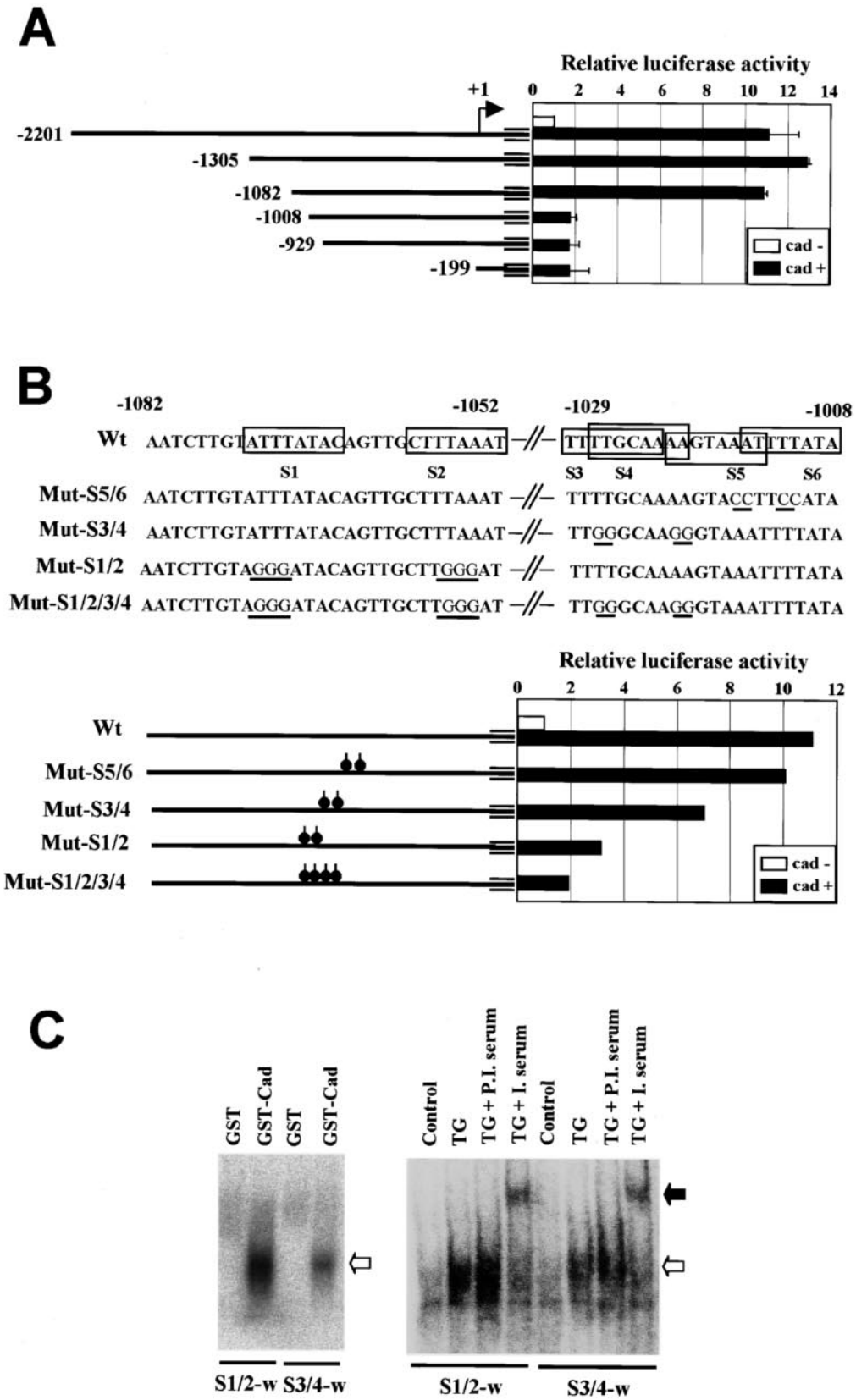

FIG. 2. Identification of Caudal protein DNA recognition elements in the *Drs* promoter. (A) Schematic diagram of the various *Drs* deletion constructs used in this study. The expression levels of the various *Drs* mutant constructs in the Schneider cells overexpressing the Cad protein were measured. T bars, standard deviations of at least three independent experiments. The normalized luciferase activity in the absence of Cad

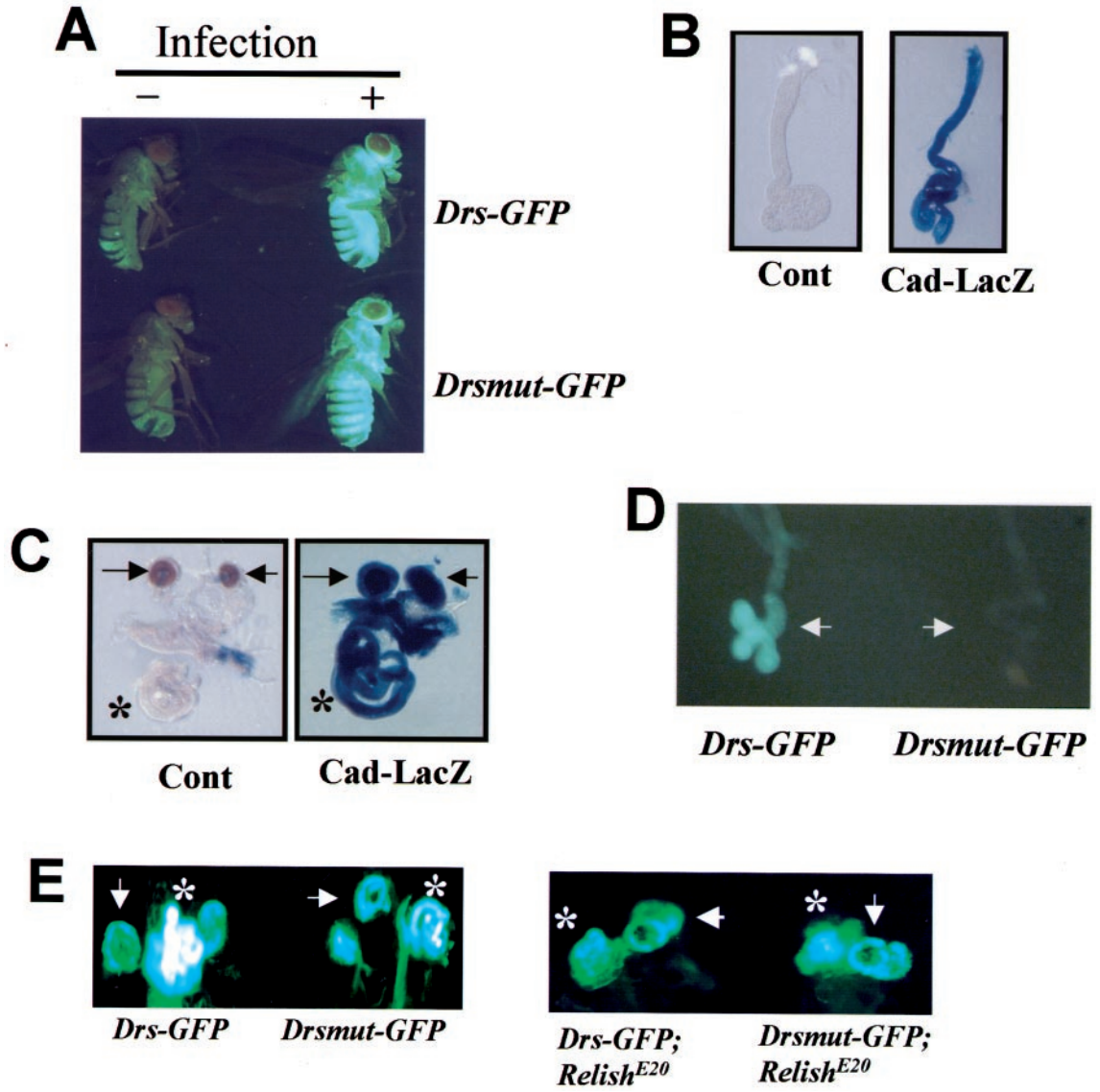

FIG. 3. Caudal protein DNA recognition elements are required for the constitutive local expression of *Drs*. (A) Cad protein DNA recognition elements are not involved in inducible systemic innate immunity. Transgenic flies carrying the wild-type *Drs* promoter fused to GFP (*Drs-GFP*) and transgenic flies carrying the mutant form of the *Drs* promoter (four Cad binding motifs, the S1 to S4 sites, were mutated by site-directed mutagenesis) fused to *GFP* (*Drsmut-GFP*) were pricked with a bacterium-soaked needle to induce a systemic immune response as described in Materials and Methods. A strong fat body-derived fluorescence was observed in both lines of transgenic flies. No difference of fluorescence intensity between transgenic flies carrying *Drs-GFP* and *Drsmut-GFP* was observed. (B and C) Histochemical staining of Cad-LacZ activity in the salivary glands (B), the spermathecas (C; arrows), and the seminal receptacles (C; asterisks) of the control adult flies (Cont) and the flies carrying *Cad-LacZ* (Cad-LacZ). (D) Transgenic flies carrying *Drs-GFP* exhibited a strong fluorescence in the salivary glands (arrow), whereas the transgenic flies carrying *Drsmut-GFP* did not. (E) Strong constitutive *Drs* expression in the female reproductive organs (spermathecas [arrows] and seminal receptacles [asterisks]) in the *Drsmut-GFP*-expressing flies and in the *Drsmut-GFP*-expressing flies under a *Relish*^{E20} genetic background.

expression was taken arbitrarily to be 1, and the results are presented as relative levels of expression. (B) Sequence of the *Drs* promoter region (−1082 to −1008) containing the Cad binding motifs (boxes S1 to S6). *Drs*-luciferase reporters carrying single or multiple mutations on the Cad binding motifs were generated. The numbers on the top denote the nucleic acid sequence numbers derived from the *Drs* promoter. The mutant base pairs are underlined. (C) The DNA binding was carried out with GST-Cad (left) or nuclear extract (right) as described in the legend of Fig. 1. The nuclear extracts were prepared from untreated (lanes control) and heat-shocked (lanes TG) *UAS-Cad*/+; *HS-GAL4*/+-expressing transgenic flies. Immune serum produced against recombinant Cad (I. serum) and the preimmune serum of the same animal (P.I. serum) were used for the supershift assay. The ^{32}P-labeled oligonucleotide probes were S1/2-w (containing wild-type S1 and S2 sites; 5′-ATCTTGTATTTATACAGTTG CTTTAAATAATCA-3′) and S3/4-w (containing wild-type S3 and S4 sites; 5′-ATTTTGCAAAAGTAAATTTTATATTGTTCA-3′). Open arrows, protein-DNA bands; solid arrow, supershifted Cad-DNA complex.

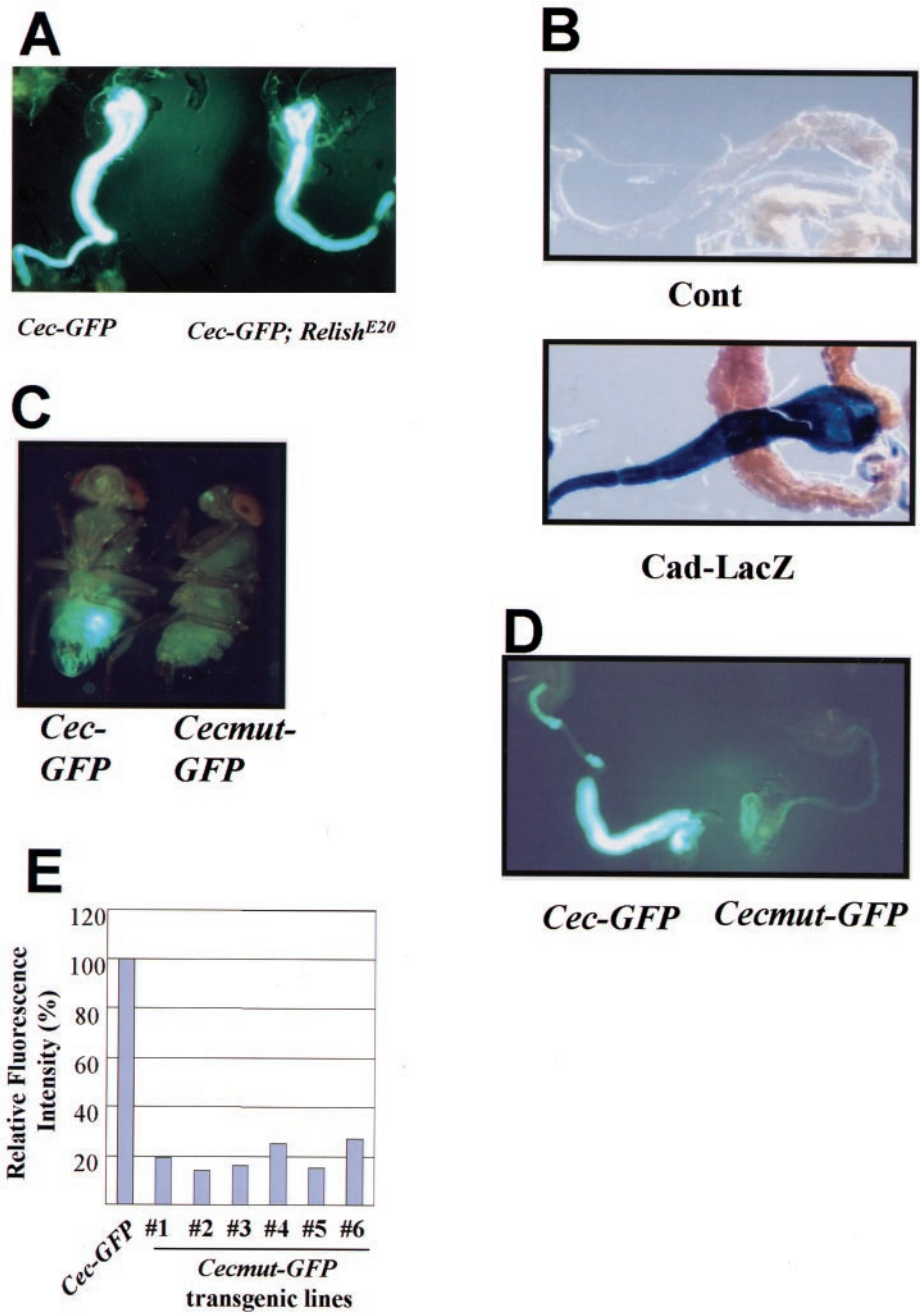

FIG. 4. Caudal protein DNA recognition elements are required for the constitutive local expression of *Cec*. (A) Relish is not required for *Cec* expression in the ejaculatory duct. Shown is constitutive Cec-GFP reporter activity in the ejaculatory ducts of the wild type and of a homozygous *Relish* mutant. (B) Histochemical staining of Cad-LacZ activity in the ejaculatory ducts of control flies (Cont) and flies carrying *Cad-LacZ* (Cad-LacZ). (C) Transgenic flies carrying the wild-type *Cec* promoter fused to *GFP* (*Cec-GFP*) exhibited strong fluorescence in the posterior region, whereas transgenic flies carrying the mutant *Cec* promoter (Cad binding motifs, S2 and S5 sites, were mutated by site-directed mutagenesis) fused to *GFP* (*Cecmut-GFP*) did not. (D) Upon dissection, this strong fluorescence was found in the ejaculatory ducts of male *Cec-GFP*-expressing flies. Note that the fluorescence of the ejaculatory duct in *Cecmut-GFP*-expressing flies is greatly diminished. (E) Quantitative measure of *GFP* reporter expression in the ejaculatory duct. The ejaculatory ducts from *Cec-GFP*-expressing transgenic flies and six independent *Cecmut-GFP*-expressing transgenic flies (*Cecmut-GFP* 1 to 6) were homogenized, and total lysates (100 μg) were subjected to spectrofluorometer analysis. Fluorescence activity in the ejaculatory ducts of *Cec-GFP*-expressing transgenic flies was taken arbitrarily as 100%, and the results are presented as relative levels of expression.

expressing transgenic flies clearly showed that the CDREs are absolutely necessary for the constitutive expression of *Drs* in the salivary glands, but not in other *Drs*-expressing tissues, such as female reproductive organs.

To determine whether CDREs are implicated in the local constitutive expression of AMP genes other than *Drs*, we tested their role in *Cec* expression. In *Drosophila*, the strongest local constitutive expression of *Cec* is observed in the male ejacula-

tory duct in unchallenged adults (51). The inducible local expression of *Cec* in midgut, proventriculus, and Malpighian tubules has been shown to be under the control of the IMD pathway-activated p105-like NF-κB, Relish (38, 51). However, the involvement of the IMD-Relish pathway in the expression of *Cec* in the ejaculatory duct is unknown. Therefore, we first investigated whether the high constitutive expression of *Cec* in the male ejaculatory duct is controlled by Relish. We analyzed the expression of *Cec* by using transgenic flies carrying *Cec-GFP* in a *Relish*$^{-/-}$ genetic background. The result showed the .constitutive expression of *Cec-GFP* in the male ejaculatory duct was not significantly affected in *Relish* mutant flies (Fig. 4A). This result suggested that the *Cec* expression in the ejaculatory duct is constitutive and that a transcription factor other than Relish is involved in this organ. To see whether *Cad* is normally expressed in the ejaculatory duct, we first examined in vivo Cad expression. The result showed that the Cad reporter is detected in this tissue by using transgenic flies carrying *Cad-LacZ* (Fig. 4B). We further checked the level of endogenous *Cad* expression in the ejaculatory duct with regard to that of the intestine. Real-time PCR analysis showed that endogenous *Cad* expression in the ejaculatory duct reached 45% of intestinal *Cad* mRNA level (data not shown).

To investigate whether the CDREs found in the *Cec* promoter are essential for the high constitutive expression level of *Cec* in the male ejaculatory duct, we examined *Cec* expression in transgenic flies carrying *Cec-GFP* and *Cecmut-GFP*. Male transgenic flies carrying *Cec-GFP* exhibited strong constitutive *Cec* expression in the abdominal region, whereas *Cec* expression was severely impaired in flies carrying *Cecmut-GFP* (Fig. 4C). Upon dissection, we observed that the *Cec* reporter activities in the ejaculatory ducts of male transgenic flies carrying *Cecmut-GFP* were significantly reduced (Fig. 4D). Quantitative analysis of GFP reporter expression showed that transgenic flies carrying *Cecmut-GFP* gained only 20% of the reporter activity in the ejaculatory ducts gained with transgenic flies carrying *Cec-GFP* (Fig. 4E). All six independent transgenic fly lines carrying *Cecmut-GFP* displayed reduced GFP reporter activity, showing that CDREs are needed for full activation of the *Cec* promoter. However, we still detected a low level of residual GFP expression in the ejaculatory ducts of flies carrying *Cecmut-GFP*, which suggested that other regulatory elements may also be involved in AMP signaling in this tissue.

Altogether our results demonstrate that CDREs are obligatory for the constitutive local expression of *Cec* and *Drs* in a subset of epithelial tissues where the expression of these genes is NF-κB independent.

Cad regulates the constitutive expression of *Cec* and *Drs* in a subset of epithelial tissues. The results in the previous section suggest that Cad is involved in the regulation of constitutive local expression of *Cec* and *Drs* through CDREs. To test this hypothesis, we first examined whether the constitutive local expression of *Cec* and *Drs* is affected in a heterozygote *Cad* mutant (30), because homozygous expression of mutant *Cad* is embryonically lethal. We analyzed the expression of these genes by using transgenic flies carrying *Cec-GFP* or *Drs-GFP* in a *Cad*$^{+/-}$ genetic background. The result showed that the constitutive expression of *Cec-GFP* in the male ejaculatory duct and *Drs-GFP* expression in the salivary glands in heterozygote *Cad* mutant flies were not significantly affected (Fig. 5A). We then performed RNA interference (RNAi) experiment by generating transgenic flies carrying the *UAS-Cad-RNAi* construct in order to mimic the loss-of-function mutation. Using this method, we achieved a partial decrease of Cad activity in the ejaculatory duct after introducing *Cad-RNAi* using a line ubiquitously expressing *Daughterless* (*Da*)-*GAL4*. Real-time PCR analysis showed an endogenous *Cad* mRNA reduction of 37% and an endogenous *Cec* mRNA reduction of 40% (Fig. 5B). No Cad-RNAi effect was observed by using a control female fat body-specific *Yolk-GAL4* driver (11), which is not expressed in the ejaculatory duct (Fig. 5B). To see whether the general physiological functions of the ejaculatory duct were affected by the expression of Cad-RNAi, the expression of the ejaculatory duct-specific gene *Gld* (encoding glucose dehydrogenase) was examined (46). The result showed that the expression of *Gld* in the ejaculatory ducts of flies carrying *UAS-Cad-RNAi* and *Da-GAL4* was not affected (Fig. 5B). As *Cad* is known as a developmental gene, we set out to compare levels of expression of *Cec* and *Cad* in the male reproductive organs of two different developmental stages (larvae and adults). The result showed that a similar levels of *Cad* mRNA were detected in both the larval genital disk and the adult ejaculatory duct (Fig. 5C). However, the level of *Cec* mRNA is much more higher in the adult ejaculatory duct than in the larval genital disk (Fig. 5C). To completely inhibit endogenous Cad activity in the adult ejaculatory duct, we generated a dominant-negative construct of Cad by using the domain-swapping method. Accordingly, we removed the transcriptional activation domain of Cad and replaced it with the strong transcriptional repressor domain of the *Drosophila engrailed* (*En*) protein (Cad-En) (19). When this Cad repressor construct was cotransfected with Cad, Cad-induced *Cec* reporter activity in *Drosophila* immunocompetent Schneider cells was completely inhibited, confirming the dominant-negative effect of the Cad-En fusion protein (Fig. 5D). To inhibit endogenous Cad activity in vivo, we generated transgenic flies carrying *UAS-Cad-En*. These transgenic flies were crossed with flies carrying the *HS-GAL4* driver in order to inhibit endogenous Cad activity, in the presence of a *Cec-GFP* insertion. As was confirmed by using flies carrying *UAS-EGFP*, the adult flies carrying *HS-GAL4* expressed GAL4 ubiquitously, including expression in the ejaculatory duct after heat shock treatment (data not shown). The result showed that strong expression of the *Cec-GFP* reporter in the ejaculatory ducts of *Cec-GFP*/*UAS-Cad-En*; *HS-GAL4*/+-expressing flies was severely reduced when the flies were subjected to heat shock treatment, compared with that in control flies (untreated *Cec-GFP*/*UAS-Cad-En*; *HS-GAL4*/+-expressing flies or treated *Cec-GFP*/+; *HS-GAL4*/+-expressing flies) (Fig. 5E). These findings agree with the results for *Cecmut-GFP*-expressing flies presented in the previous sections and demonstrate that Cad activity is important for constitutive *Cec* expression in the ejaculatory duct in a Relish-independent manner.

Previous studies demonstrated that constitutive Drs-GFP expression is mainly observed in the adult salivary glands (10, 51). When we compared levels of expression of *Drs* and *Cad* in the salivary glands of flies at different developmental stages, we found that *Drs* and *Cad* expression in the adult salivary glands is higher than that in the larvae and prepupae (Fig. 6A). Thus,

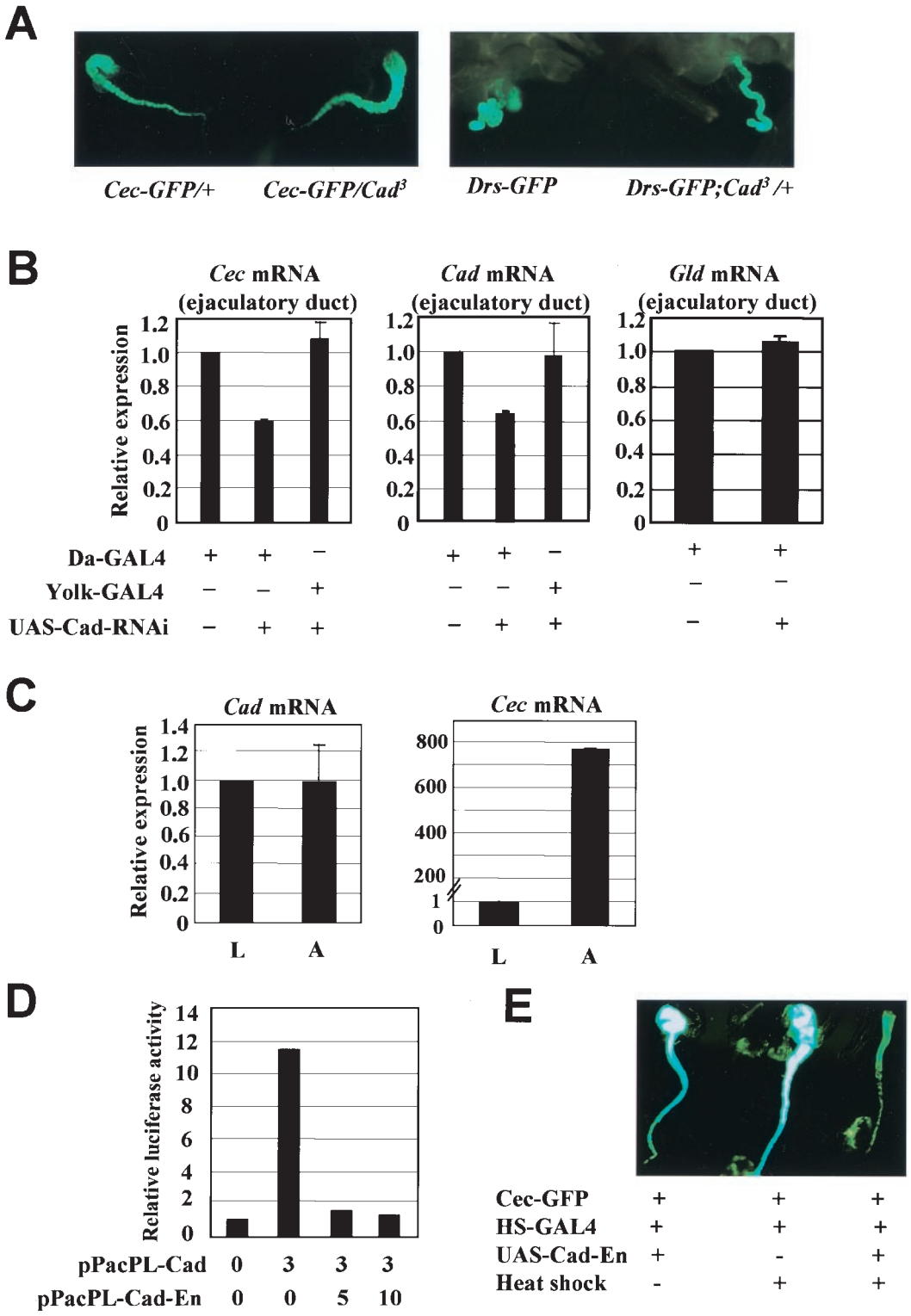

FIG. 5. Caudal regulates the constitutive expression of *Cec* through Caudal protein DNA recognition elements. (A) Constitutive *Cec* and *Drs* expression is not affected in a heterozygote *Cad* mutant. Constitutive Cec-GFP reporter activity in the ejaculatory ducts of the wild type and of a heterozygote *Cad* mutant and constitutive Drs-GFP reporter activity in the salivary glands of the wild type and of a heterozygote *Cad* mutant are shown. (B) High constitutive *Cec* expression is partially reduced in the ejaculatory ducts of the *Cad-RNAi*-expressing flies. Quantitative real-time PCR analysis of endogenous *Cec* gene transcription in the control flies carrying *Da-GAL4* alone and the flies carrying *UAS-Cad-RNAi*; *Da-GAL4* is presented. In this condition of Cad-RNAi, the expression of the control gene (ejaculatory duct-specific *Gld* gene) was also examined. In the GAL4 control experiment, the flies carrying *UAS-Cad-RNAi* combined with *Yolk-GAL4* were used. Gene expression (*Cec*, *Cad*, and *Gld* expression) in the flies carrying *Da-GAL4* alone was taken arbitrarily as 1, and the results are shown as relative levels of expressions. T bars,

we wanted to confirm the role of Cad in *Drs* expression in the adult salivary glands. When *Cad-RNAi* was introduced in lines expressing *GAL4* in the salivary glands (*UAS-Cad-RNAi* combined with the *c729-GAL4* and the *Drs-GFP* reporter gene), expression of the *Drs-GFP* reporter in the salivary glands of these flies overexpressing the *Cad-RNAi* construct was nearly abolished (Fig. 6B). The control flies carrying *c729-GAL4* alone showed a normal Drs-GFP activity in this tissue (Fig. 6B). Similar results were obtained with transgenic flies carrying *UAS-Cad-RNAi* and with other *GAL4*-expressing lines (such as lines carrying *Da-GAL4* or *HS-GAL4*) (data not shown). In this condition, consistent with the result for transgenic flies carrying *Drsmut-GFP* (Fig. 3E), Cad inhibition by RNAi does not have an effect on *Drs* expression in the female reproductive organs (Fig. 6B). Real-time PCR analysis showed that, in the salivary glands of *Cad-RNAi*-expressing flies, endogenous *Drs* transcripts reached ~25% of the control level and that endogenous *Cad* transcripts reached ~35% of the control level (Fig. 6C). No Cad-RNAi effect was observed by using a control female fat body-specific *Yolk-GAL4* driver (Fig. 6C). To see whether the condition of Cad-RNAi could influence the expression of other genes unrelated to innate immunity, we examined the expression of *B52* (21), which is known to be expressed in the salivary glands and some other tissues. The result showed that the expression of *B52* in the salivary glands of flies carrying *UAS-Cad-RNAi* and *Da-GAL4* was not affected (Fig. 6C). Altogether, these results demonstrate that full Cad activity is essential for constitutive *Drs* expression in the local epithelial tissues such as the salivary glands.

DISCUSSION

The homeobox transcription factor Cad was originally found to regulate the anteroposterior body axis of *Drosophila* (8, 35, 36). During embryogenesis, the Cad expression level is tightly regulated in response to developmental signals. For example, a high level of Cad is needed in posterior structures to activate the segmentation genes *fushi tarazu* and *spalt*, which are involved in terminal specification (8, 22), whereas the development of the anterior part of the embryo is associated with a low Cad expression level (8, 22, 35). During postembryogenesis, Cad expression is primarily restricted to the intestine and to the Malpighian tubules and gonads (35). Cad expression in postembryonic life is known to be restricted to organs that display cell renewal or remodeling, such as the intestine (35). Our results show that the Cad-LacZ reporter (Fig. 3B and 4B) and endogenous *Cad* mRNA (Fig. 5 and 6) are also expressed in the salivary glands and ejaculatory duct, where AMP expression is constitutive. Vertebrate Cad homologues are well known to participate in early embryogenesis, the development of the intestine, and colon tumorigenesis (7, 49). However, apart from their developmental roles, the physiological functions and target genes of the Cad homeobox gene family are unknown. The observation that Cad regulates AMP gene expression in a subset of epithelia indicates a new function for this *trans*-activator in the local defense against microbial infection and/or maintenance of microbial flora. At present, the real in vivo function of AMP gene expression in local epithelia in *Drosophila* is not known (10, 51). In the local-infection experiment, we could not observe the enhanced mortality in the *Cad-RNAi*-expressing flies following short-term (1 h) bacterial feeding (J.-H. Ryu and W.-J. Lee, unpublished data). However, although local AMP expression is not directly related to the rate of survival of infection, the locally secreted AMPs may help to prevent the onset of infections.

It is well known that the Toll/NF-κB signaling pathway for dorsoventral body axis formation mainly regulates the inducible expression of the *Drs* gene during the systemic immune response (17, 25). Interestingly, this pathway has been well conserved during evolution and assists NF-κB activation via Toll-like receptors in the human innate immune system (1, 18). Our results show that, in the local epithelial immune system, NF-κB-independent, constitutive expression of *Drs* and *Cec* in the barrier epithelial tissues is mainly controlled by the homeobox gene *Cad*, a master controller of anteroposterior body axis formation. The developmental genes involved in specification of the fly body plan (dorsoventral and anteroposterior body axes) have been recruited for this evolutionally ancient first line of defense. Our results together with those of others further demonstrate a link between development and immunity.

The involvement of Cad in the constitutive local innate immunity illustrates the complexity of the tissue-specific regulation of AMP expression in *Drosophila*. To better visualize the complexity and dynamic of the innate immune response in *Drosophila*, we constructed a comprehensive scheme (Fig. 7). Experimental infection such as septic injury rapidly induces various AMPs, mainly in the fat body (known as systemic immunity), via two different NF-κB pathways (Toll and IMD pathways), whereas natural infection, such as local bacterial infection, activates the expression of AMPs via the IMD pathway only in a subset of epithelial tissues (known as inducible local innate immunity) (10, 38, 51). These two inducible innate immune systems in *Drosophila* are rather distinct because septic injury cannot activate the inducible local immune system (51). The third type of AMP regulation is the constitutive local

standard deviations (SD) of at least three independent experiments. (C) Endogenous *Cec* and *Cad* expression in the larval and adult reproductive organs. Quantitative real-time PCR analysis of *Cec* and *Cad* gene transcription was performed using the third-instar larva genital disk (L) and the adult ejaculatory duct (A). *Cec* and *Cad* expression in the larval genital disk was taken arbitrarily as 1, and the results are shown as relative levels of expression. T-bars, SD of at least three independent experiments. (D) Inhibition of Cad-induced *Cec* reporter activity by the overexpression of the Cad dominant-negative construct (Cad-En) in Schneider cells. A fixed amount of pPacPL-Cad (3 μg) was cotransfected with 5 or 10 μg of pPacPL-Cad-En construct together with 100 ng of *Cec*-luciferase and 100 ng of a β-galactosidase construct. Relative luciferase activity measurement was performed as described in Materials and Methods. Normalized luciferase activity in the absence of Cad expression was taken arbitrarily as 1. The reporter assay was repeated at least three times, and the results obtained were found to be highly reproducible. A representative experiment is shown. (E) Transgenic flies carrying *Cec-GFP*/*UAS-Cad-En*; *HS-GAL4*/+ after heat treatment exhibit strongly reduced *Cec* reporter activity in the ejaculatory duct. Transgenic flies carrying *Cec-GFP*/*UAS-Cad-En*; *HS-GAL4*/+ without heat treatment or flies carrying *Cec-GFP*/+; *HS-GAL4*/+ with heat treatment (37°C for 45 min) were used as controls.

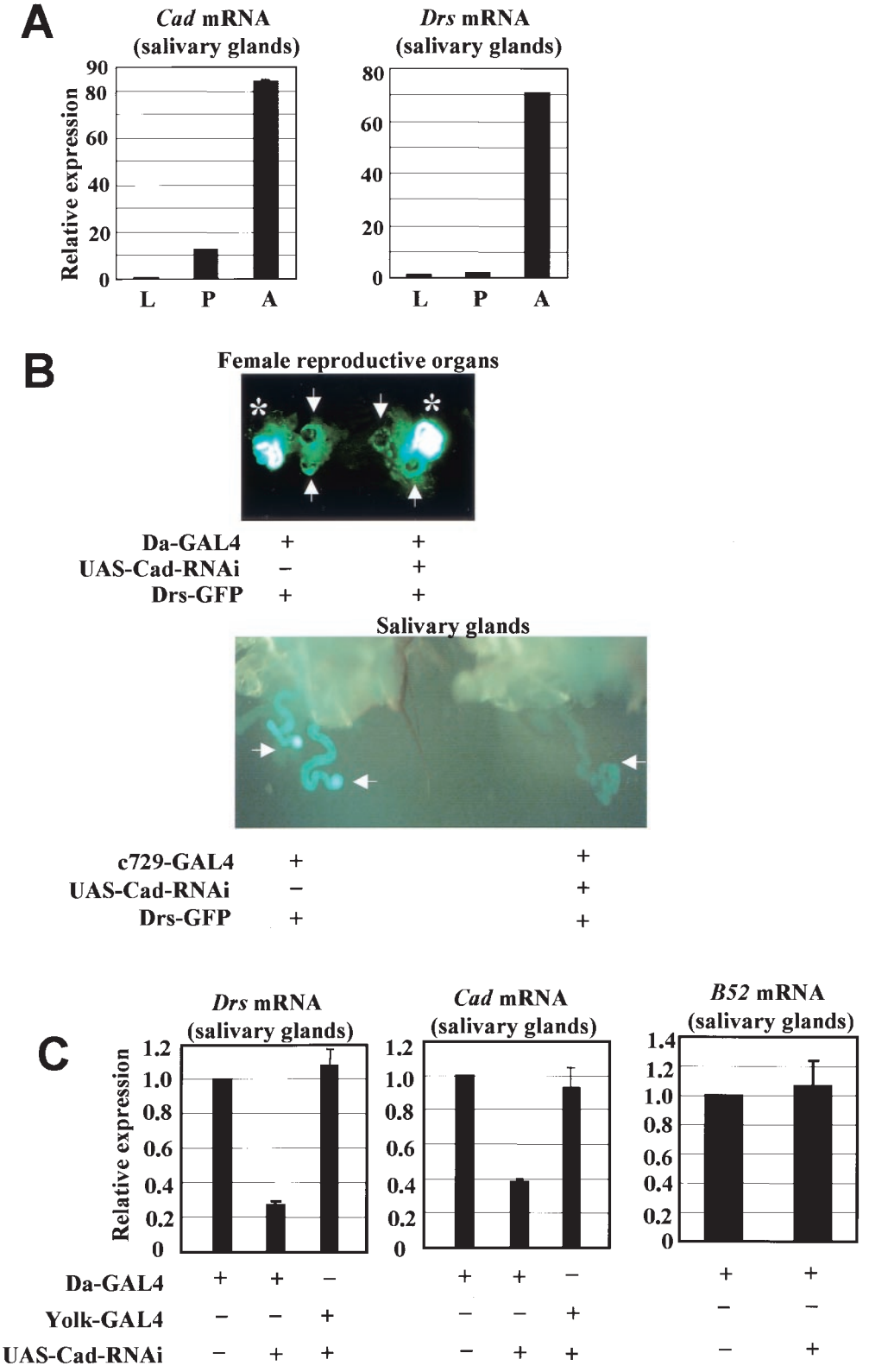

FIG. 6. Caudal regulates the constitutive expression of *Drs* through Caudal protein DNA recognition elements. (A) Endogenous *Drs* and *Cad* expression in the salivary glands of different developmental stages. Quantitative real-time PCR analysis of *Drs* and *Cad* gene transcription was performed using the third-instar larva (L), early prepupal (P), and adult (A) salivary glands. *Drs* and *Cad* expression in the larval salivary glands was taken arbitrarily as 1, and the results are shown as relative levels of expression. T bars, standard deviations (SD) of at least three independent experiments. (B) Cad is required for the constitutive expression of *Drs* in the salivary glands but not in the female reproductive organs. (Top) Transgenic flies carrying *Da-GAL4* and *UAS-Cad-RNAi* under a *Drs-GFP* insertion exhibit levels of *Drs* reporter activity in the female reproductive

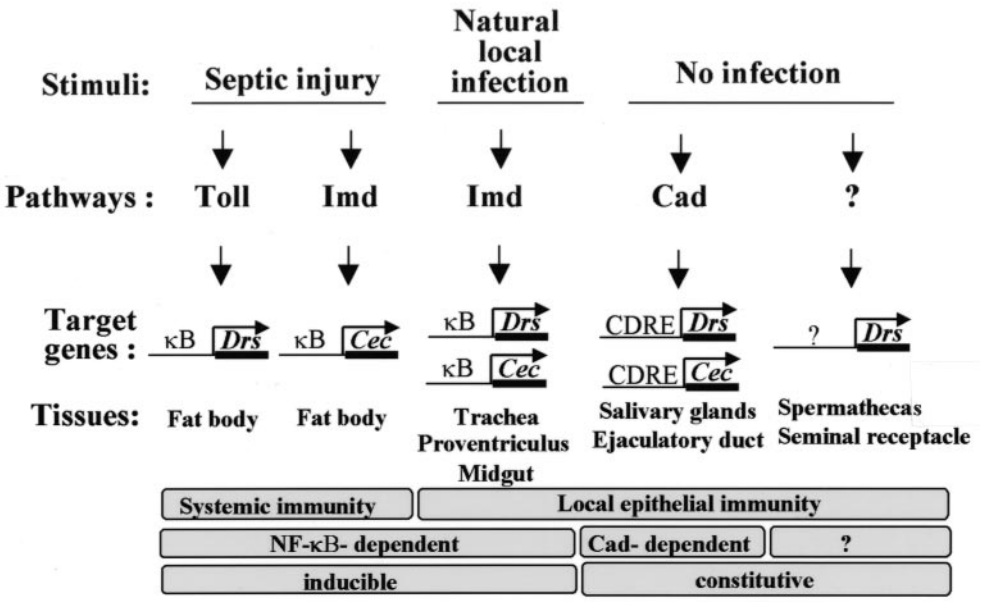

FIG. 7. Tissue-specific regulation of antimicrobial peptide genes in *Drosophila*. This model is based on previous studies (10, 38, 51) and the present study. Note that different types of infection can activate distinct innate immune pathways (systemic immunity versus local innate immunity; NF-κB-dependent inducible immunity versus Cad-dependent constitutive immunity) in a tissue-specific manner. See Discussion for additional details.

expression of AMPs in an NF-κB-independent manner in several epithelia (10, 51). This type of strategy is believed to be very ancient in evolution and may be very efficient in certain epithelia by avoiding chronic NF-κB activation where the contact with microbes is continuous.

In this study, we show that that Cad is capable of directly regulating *Cec* and *Drs* via CDREs found in the their promoters in *Drosophila* Schneider cells (Fig. 1 and 2). Furthermore, Cad binds in vitro to the CDREs found upstream of AMP genes in a gel shift assay (Fig. 1 and 2). These results demonstrate that Cad is a direct *trans*-activator of AMP genes. The in vivo reporter analysis demonstrates that mutations affecting CDREs do not abolish the inducible systemic *Drs* expression in the fat body (Fig. 3A). These results clearly indicate that the CDREs, in contrast to κB sites, are not required for inducible *Drs* expression in the fat body during a systemic immune response. In addition to the fat body, the trachea is involved in inducible *Drs* expression. This tissue, in which *Drs* expression is normally absent but rapidly induced in response to local infection by *Erwinia carotovora*, is known to be involved in inducible local immunity (51). Surprisingly, even though there is no appreciable role for CDREs in the fat body, we found that all 12 independent *Drsmut-GFP*-expressing fly lines (larvae and adults) exhibited spontaneous constitutive expression of *Drs* reporter activity in the trachea in the absence of local infection (J.-H. Ryu and W.-J. Lee, unpublished data). One may speculate that CDREs can also act as negative *cis* elements in some epithelial tissues such as the trachea, where they can maintain the silencing of *Drs* expression, and that this depends on the specific cell type. Further studies will be needed to understand the complete tissue-specific Cad signaling pathway for AMP regulation in all epithelial tissues. In contrast to κB-dependent inducible AMP expression, the constitutive local innate immunity employs Cad for the expression of AMPs through CDRE motifs rather than κB motifs (Fig. 3 and 4). Interestingly, for salivary glands, overexpression of the *Cad-RNAi* construct is sufficient to severely reduce *Drs* expression, indicating that constitutive local expression of *Drs* in salivary glands is greatly dependent on Cad (Fig. 6). For the ejaculatory duct, although partial reduction of Cad modestly reduces *Cec* expression (Fig. 5B), we can detect only minor expression (~20%) of the *Cec* reporter in flies carrying *Cecmut-GFP* (Fig. 4D and E), as well as flies overexpressing the dominant-negative form of Cad (Fig. 5E). This also indicates that *Cec* expression in this tissue is largely dependent on Cad. Interestingly, our study showed that not all constitutive local expression is dependent on Cad.

organs (spermathecas [arrows] and seminal receptacles [asterisks]) similar to those exhibited by control flies carrying *Da-GAL4* alone under a *Drs-GFP* insertion. (Bottom) Transgenic flies carrying *c729-GAL4* and *UAS-Cad-RNAi* under a *Drs-GFP* insertion exhibit strongly reduced *Drs* reporter activity in the salivary glands (arrows) compared to the control flies carrying *c729-GAL4* alone under a *Drs-GFP* insertion. (C) High constitutive *Drs* expression is greatly reduced in the salivary glands of the *Cad-RNAi*-expressing flies. Quantitative real-time PCR analysis of endogenous *Drs* gene transcription in the control flies carrying *Da-GAL4* alone and the flies carrying *UAS-Cad-RNAi*; *Da-GAL4*. In this condition of Cad-RNAi, the expression of the control gene (salivary gland expressing the *B52* gene) was also examined. In the GAL4 control experiment, flies carrying *UAS-Cad-RNAi* combined with *Yolk-GAL4* were used. Gene expression (*Drs*, *Cad*, and *B52* expression) in the flies carrying *Da-GAL4* alone was taken arbitrarily as 1, and the results are shown as relative levels of expression. T-bars, SD of at least three independent experiments.

The constitutive local expression in the female reproductive organs is completely CDRE independent (Fig. 3E and 6B), suggesting the existence of yet another unknown signaling pathway(s). Recently, *Drosophila* Toll-9, one of the Toll-related receptors, was found to trigger the constitutive expression of *Drs* in cultured cells (39). It is possible that Toll-9 may control constitutive *Drs* expression in certain epithelia. The studies on the in vivo function of Toll-9 should elucidate this issue.

The expression of various AMPs in analogous human epithelial tissues suggests that epithelial innate immunity is well conserved and that the careful regulation of AMP levels may be needed to maintain homeostasis in these tissues from *Drosophila* to humans (24). The presence of human Cad homologues, CDXs, raises interesting questions concerning their putative role(s) in human epithelial innate immune gene regulation.

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