

# Activation of the innate immunity in *Drosophila* by endogenous chromosomal DNA that escaped apoptotic degradation

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Apoptotic cell death is accompanied by degradation of chromosomal DNA. Here, we established in *Drosophila* a null mutation in the gene for inhibitor of caspase-activated DNase (ICAD) by P-element insertion. We also identified a loss-of-function mutant in *Drosophila* for DNase II-like acid DNase. The flies deficient in the *ICAD* gene did not express CAD, and did not undergo apoptotic DNA fragmentation during embryogenesis and oogenesis. In contrast, the deficiency of *DNase II* enhanced the apoptotic DNA fragmentation in the embryos and ovary, but paradoxically, the mutant flies accumulated a large amount of DNA, particularly in the ovary. This accumulation of DNA in the *DNase II* mutants caused the constitutive expression of the antibacterial genes for dipterocin and attacin, which are usually activated during bacterial infection. The expression of these genes was further enhanced in flies lacking both *dICAD* and *DNase II*. These results indicated that CAD and DNase II work independently to degrade chromosomal DNA during apoptosis, and if the DNA is left undigested, it can activate the innate immunity in *Drosophila*.

[*Keywords:* apoptosis; DNA fragmentation; mutagenesis; antibacterial peptides; innate immunity]

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Apoptosis, or programmed cell death, is an evolutionarily conserved process that removes harmful or useless cells during development (Jacobson et al. 1997; Vaux and Korsmeyer 1999). The apoptotic process is accompanied by morphological changes, such as shrinkage and fragmentation of the cells and nuclei, and loss of microvilli from the plasma membranes (Wyllie et al. 1980). Genetic and biochemical studies revealed that caspases, a family of cysteine proteases, are activated during apoptosis (Thornberry and Lazebnik 1998; Earnshaw et al. 1999; Los et al. 1999). More than 100 cellular proteins have been identified in mammalian systems as caspase substrates, the cleavage of which brings about the morphological changes of the cells and nuclei, leading to the cell death (Stroh and Schulze-Osthoff 1998).

In addition to the morphological changes of cell and nuclei, the apoptotic process is often accompanied by the degradation of chromosomal DNA (Wyllie 1980; Nagata

2000). We and others have identified a DNase (caspase-activated DNase, CAD; also called DNA fragmentation factor, DFF40), that is activated in dying cells (Liu et al. 1997; Enari et al. 1998). CAD is complexed with its inhibitor (ICAD, inhibitor of CAD) in proliferating cells. When apoptosis is triggered, caspases—in particular caspase 3—cleave ICAD, and the CAD released from ICAD degrades the chromosomal DNA into nucleosomal units (Sakahira et al. 1998). Accordingly, cells that are deficient in the CAD system do not undergo apoptotic DNA fragmentation in vitro. However, DNA degradation is still observed in vivo in CAD-deficient mice. Because this DNA fragmentation is detected in apoptotic cells inside macrophages, we proposed that the DNA of apoptotic cells can be cleaved by lysosomal acid DNase (DNase II) after the cells are engulfed by macrophages (McIlroy et al. 2000).

Programmed cell death plays an important role in the development of *Drosophila melanogaster* (Abrams 1999). During embryogenesis, metamorphosis, and oogenesis, many cells die showing the characteristics of apoptosis. Therefore, *Drosophila* has been widely used to dissect the molecular mechanisms of apoptosis and to understand its physiological role (Bergmann et al. 1998). The programmed cell death in *Drosophila* is triggered by the expression of a set of genes: *reaper*, *hid*, and *grim*,

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which bring about the activation of a family of caspases. Loss-of function mutations of these genes impair the apoptotic process, and often prevent embryogenesis and/or lead to tumorigenesis (White et al. 1994; Song et al. 1997). The apoptotic cell death in *Drosophila* is also accompanied by DNA fragmentation (Nagano et al. 1998). Previously, we identified *Drosophila* homologs (dCAD and dICAD) for CAD and ICAD, and showed that the apoptotic DNA fragmentation in a BG-2 *Drosophila* neural cell line is mediated by the dCAD/dICAD system (Mukae et al. 2000; Yokoyama et al. 2000).

In this report, a *Drosophila* line that is deficient in *dICAD* was established by P-element-mediated mutagenesis. In the *dICAD*-null flies, neither the dICAD nor the dCAD protein was expressed. The apoptotic DNA fragmentation into nucleosomal units that normally occurs during embryogenesis and oogenesis was completely suppressed in the mutant flies. In addition, an ethyl-methyl-sulfonate (EMS)-induced mutant line in the *Drosophila* stock center was found to carry a loss-of-function mutant in lysosomal acid DNase (dDNase II). These dDNase II-deficient flies showed enhanced apoptotic DNA fragmentation, yet accumulated a large amount of DNA, particularly in ovaries, and constitutively expressed the genes for antibacterial peptides. This activation of the antibacterial peptide genes was enhanced in *Drosophila* mutants that lacked both dICAD and dDNase II. These results indicate that CAD and DNase II work independently to degrade chromosomal DNA during apoptosis, and this process plays an important role in maintaining the homeostasis of these animals.

## Results

### *Establishment of a dICAD-null fly by P-element mutagenesis*

To study the physiological roles of the CAD-ICAD system in *Drosophila*, a *Drosophila* line carrying a mutation in the *dICAD* gene was generated by a local hop of a nearby P-element. The dICAD gene is located on the 48E2 locus of the *Drosophila* chromosomes. A search of the FlyBase indicated that *Drosophila* line *l(2)k00617* carries a P-element 60 kb downstream of the *dICAD* gene. This P-element was mobilized in a stepwise manner into the *dICAD* gene locus (Fig. 1A). The movement of the P-element at each step was followed by a long PCR procedure, and confirmed by Southern hybridization (Fig. 1B). After repeating this local hop procedure three times, a fly line, *P(icad)*, which carries a P-element 17 bp upstream of the ATG initiation codon, was established.

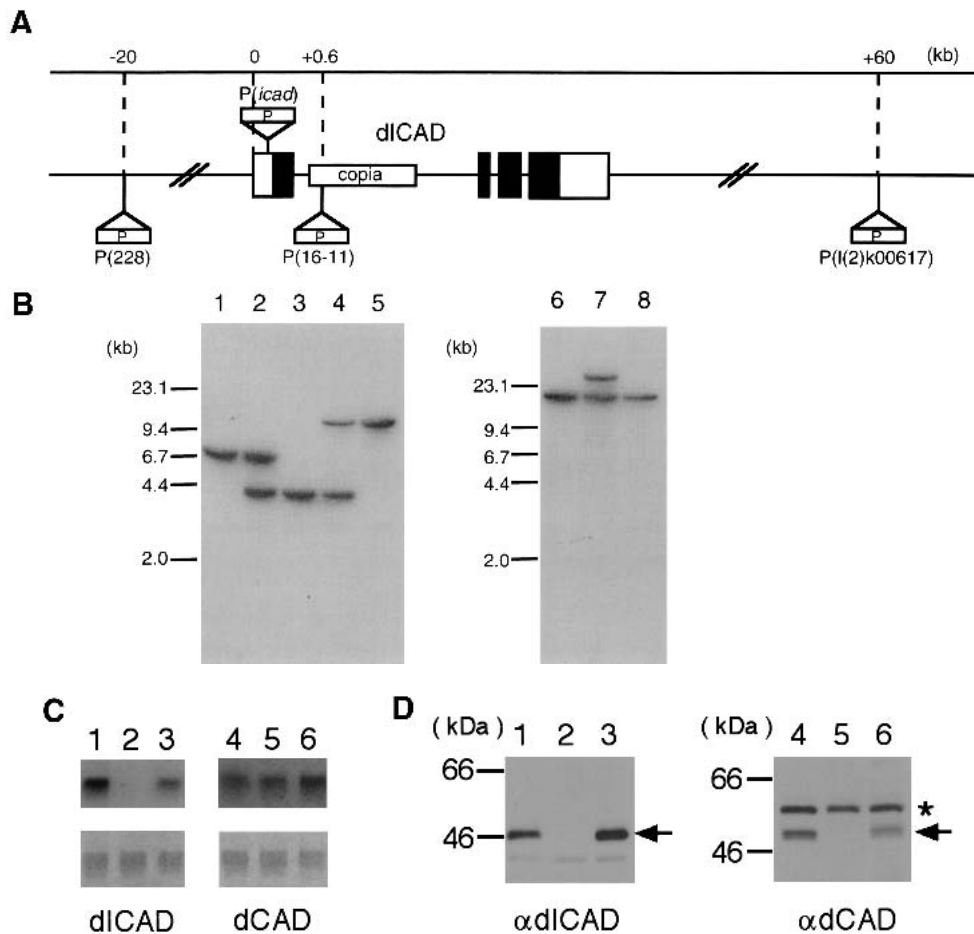
Northern hybridization analysis of the poly(A) RNA and Western blot analysis of the cell lysates from the adult flies indicated that the *P(icad)* flies expressed neither dICAD mRNA nor dICAD protein (Fig. 1C,D), indicating that *P(icad)* was a null allele for the *dICAD* gene. The excision of the P-element from the 5'-noncoding region of the *dICAD* gene (Fig. 1B) permitted the expression of dICAD mRNA and the dICAD protein, confirm-

ing the specific mutation of the *dICAD* gene in the *P(icad)* flies. The functional CAD protein cannot be properly folded without ICAD (Sakahira et al. 2000; Yokoyama et al. 2000). Accordingly, the 52-kD dCAD protein was not detected in the *dICAD* mutant flies, although they expressed the CAD mRNA as abundantly as did the wild-type flies (Fig. 1C,D). From these results, we concluded that the *P(icad)* allele works as a null allele not only for *dICAD* but also for *dCAD*. As reported for *ICAD* (*DF45*)-null mice (Zhang et al. 1998) and transgenic mice expressing a caspase-resistant form of ICAD (McIlroy et al. 2000), the *dICAD*-mutant flies were fertile, and there was no apparent adverse phenotype.

### *Identification of a Drosophila mutant of the DNase II gene*

An involvement of DNase II in the apoptotic process has been suggested for *Caenorhabditis elegans* (Wu et al. 2000) and mouse (McIlroy et al. 2000). Recently, Vernooij et al. (2000) reported that a gene called *CG7780* (FlyBase accession no. AY075328) in the *Drosophila* database codes for a protein related to mammalian DNase II. In fact, the amino acid sequence encoded by *CG7780* had 25% identity and 43% similarity with mouse or human DNase II (Fig. 2A). In particular, three histidine residues that may work as the active site for the enzymatic function of DNase II were well conserved. To confirm that the protein encoded by the *CG7780* gene had DNase II-like activity, the full-length cDNA for *CG7780* was isolated from adult flies by reverse-transcription polymerase chain reaction (RT-PCR). The cDNA was tagged with Flag at the C terminus, expressed in COS cells (Fig. 2B), and purified using an anti-Flag antibody. As shown in Figure 2C, the purified protein showed DNase activity under acidic conditions. Little DNase activity was observed under neutral conditions, and metal ions such as  $Ca^{2+}$  or  $Mg^{2+}$  were not required for the DNase activity (data not shown). Because these properties were similar to those of mammalian DNase II (Bernardi 1971), we concluded that the *CG7780* gene codes for dDNase II.

The *CG7780*, *dDNase II* gene is located at the 90D6 locus of the *Drosophila* chromosomes (FlyBase, <http://flybase.bio.indiana.edu>). A *Drosophila* mutant line (*DNase-1<sup>lo</sup>*) obtained by mutagenesis with EMS was reported to carry a low level of acid DNase activity, and this allele was mapped to the 90C2-91A2 locus (Grell 1976). Because this region includes the locus of the *dDNase II* gene, we examined the possibility that the *DNase-1<sup>lo</sup>* mutation is a mutation of dDNase II. The dDNase II cDNA was isolated by RT-PCR from adult *DNase-1<sup>lo</sup>* flies. A comparison of its sequence with the sequence of wild-type dDNase II showed that the cDNA from *DNase-1<sup>lo</sup>* had one point mutation. The AGC codon at position 223 was changed to AAC, causing a replacement of serine with asparagine (Fig. 2A). When this cDNA was expressed in COS cells, the recombinant protein showed very little DNase activity (Fig. 2C), indicating that the *DNase-1<sup>lo</sup>* allele is a loss-of-function allele for *dDNase II*. The flies carrying the *DNase-1<sup>lo</sup>* allele were fertile, but



**Figure 1.** Establishment of a *dICAD* mutant by the insertion of a P-element. (A) The *dICAD* chromosomal gene locus and the position of P-element in each *Drosophila* strain are schematically shown. The *dICAD* gene consists of four exons and is depicted as boxes in which the open and filled areas represent the noncoding and coding regions, respectively. The *Drosophila l(2)k00617* line contains a *copia* element in intron 1 of the *dICAD* gene. Positions of the P-element are shown at the top in kb, starting from the 5' end of the *dICAD* gene. (B) Southern hybridization analysis of the *Drosophila* strains carrying the P-element. Genomic DNAs (10  $\mu$ g) from *P(l(2)k00617)* (lane 1), *P(l(2)k00617)-P(228)* (lane 2), *P(228)* (lane 3), *P(228)-P(16-11)* (lane 4), and *P(16-11)* (lane 5) were digested with *EcoRI* (lanes 1–5), and separated by electrophoresis on a 0.8% agarose gel. Hybridization was carried out with a  $^{32}$ P-labeled 1.0-kb DNA fragment carrying the P-element sequence as a probe. DNA was also prepared from *P(16-11)* (lane 6); *P(icad)*, which carries two P-elements at 16-11 and at the 5' noncoding region of the *dICAD* gene (lane 7); and the revertant of *P(icad)* (lane 8) in which the P-element at the 5' noncoding region was removed by excision. DNAs were digested with *XbaI* and subjected to Southern hybridization as above. (C) Northern hybridization analysis. Poly(A) RNA (2  $\mu$ g) prepared from adult flies of the wild-type (lane 1), *dicad* mutant (lane 2), and its excision revertant was analyzed by Northern hybridization with *dICAD* (left panel) or *dCAD* (right panel) cDNA as the probe. In the lower panels, the membranes used for hybridization were stained with methylene blue. (D) Western blot analysis. The cell lysates (30  $\mu$ g of protein) prepared from adult wild-type (lane 1), *dicad* mutant (lane 2), and its excision revertant (lane 3) flies were subjected to Western blot analysis using a rabbit anti-*dICAD* (left panel) or anti-*dCAD* (right panel) antibody. The relative molecular masses of the standard proteins are shown in kDa at left. The positions of *dICAD* and *dCAD* are indicated by arrows. The bands indicated by asterisks appeared to be nonspecific.

their egg production rate was significantly reduced (data not shown).

#### *The apoptotic DNA fragmentation in Drosophila development, and accumulation of DNA*

During *Drosophila* embryogenesis, cells die via the apoptotic process that is activated by reaper, grim, and hid (White et al. 1994; Abrams 1999). *Drosophila* oogenesis proceeds through asymmetrical division of a germ-line

stem cell, giving rise to a cluster of 16 cells. Of the 16 cells, one develops into the oocyte, while the other 15 work as nurse cells. At the late stage of oogenesis, the cytoplasmic contents of the nurse cells are rapidly transferred into the oocyte and their nuclei are degraded in a caspase-dependent manner (Cavaliere et al. 1998; McCall and Steller 1998). We examined the role of CAD and DNase II in the DNA fragmentation in these processes by the linker-mediated PCR method, which amplifies the 5'-phosphorylated DNA fragments (Staley et al.

**A** D. MRSLCFVLLFVWFFYQNEAKSKVSCCKDEAGNDVDWVHLYKLPKHYQHNDLGKDTSGLKLYLVTSQNYDTWQMSGKFI SDPLS 82  
M. MATLRSLLLAALLWVPAEALSCYGDSDGQPVDFWVYKLPKPAHSGSRDTPK-GLTYKYMDQNSDGWQDGVGYINSSEGAVG 78  
H. MIPLLLAALLCVFAGALTTCYGDSDGQPVDFWVYKLPALRSGSGEAAQRGLQYKYLDESSGGWRDRGRALINSPEGAVG 76

\* \* \*

D. LPAQT<sup>N</sup>LNPLNDDPSHTLLAAYNDQ<sup>Q</sup>PNGTVFSSG---GHAKGVVASDGETAIWIVH<sup>S</sup>VPK<sup>F</sup>PTI---PDYSYPTSGEQYAQS 158  
M. RSLQ<sup>L</sup>PLYRKNSSQLAFLL--YNDQ<sup>P</sup>PKSSSARDSTGHGHTKGVLLLDQEGGF<sup>W</sup>LVH<sup>S</sup>VP<sup>R</sup>FP<sup>P</sup>PPASSGAYTWPPNAQTFGQT 158  
H. RSLQ<sup>L</sup>PLYRSNTS<sup>L</sup>QLAFLL--YNDQ<sup>P</sup>PK<sup>S</sup>SKA<sup>Q</sup>DS<sup>S</sup>MRGHTKGVLLLDH<sup>D</sup>GGF<sup>W</sup>LVH<sup>S</sup>VP<sup>R</sup>FP<sup>P</sup>PPASSAAYS<sup>W</sup>PH<sup>S</sup>ACTY<sup>G</sup>QT 156

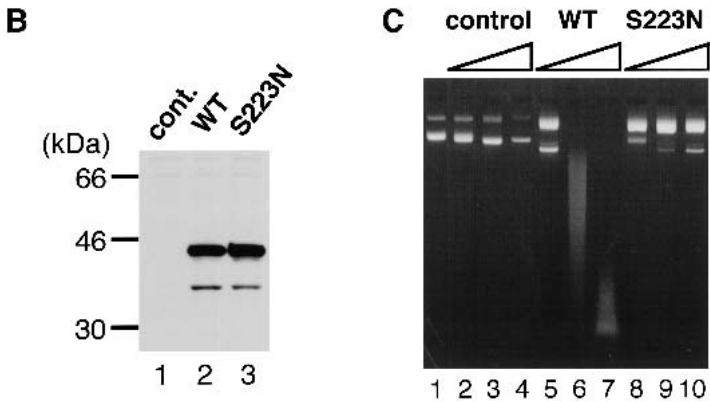
N

D. MLCVTLKGEDLEKVGQILVYNEPHFYQ<sup>R</sup>NPLATRSDEL<sup>F</sup>PSLERALH<sup>G</sup>QWR<sup>T</sup>ESPFQK<sup>D</sup>LEVR<sup>S</sup>LDG<sup>K</sup>KFR<sup>L</sup>FG<sup>K</sup>SGRANV 240  
M. LLCVSLPFTQFARIGKQLTYTYPLVYD<sup>H</sup>K--LEGFFAQKLPDLETVIK<sup>N</sup>QHV<sup>L</sup>HEP<sup>W</sup>NSSVIL<sup>T</sup>SQAGATFQ<sup>S</sup>FAK<sup>F</sup>FGKFGD 238  
H. LLCV<sup>S</sup>FPFAQ<sup>F</sup>SK<sup>M</sup>GKQLTYTYPWVYNYQ--LEGI<sup>F</sup>AE<sup>E</sup>FDLENV<sup>V</sup>KGH<sup>H</sup>VSQ<sup>E</sup>EP<sup>W</sup>NS<sup>S</sup>ITL<sup>T</sup>SQAGAVFQ<sup>S</sup>FA<sup>K</sup>FSK<sup>F</sup>FGD 236

\*

D. ELYADVVA<sup>P</sup>TLDVSLFVEAWRDGAGNLPNSCDKSDKVLN<sup>V</sup>ESISNPELSVD-FKTTQ<sup>D</sup>H<sup>S</sup>KWAVSRPTGILIYHWRVGGGDW 321  
M. DLYSGWLAELALGTNLQVQFWQNSPGILPNSCNGAYQVLDV<sup>T</sup>QTGFPGPSRLTFSATEDH<sup>S</sup>KWCVAPQ-----GPW 308  
H. DLYSGWLAALALGTNLQVQFWHKT<sup>V</sup>GILPNSCSDI<sup>W</sup>QV<sup>L</sup>NV<sup>N</sup>QIAFP<sup>G</sup>PG<sup>A</sup>GPSFN<sup>S</sup>TE<sup>D</sup>H<sup>S</sup>KWCV<sup>S</sup>PK-----GPW 306

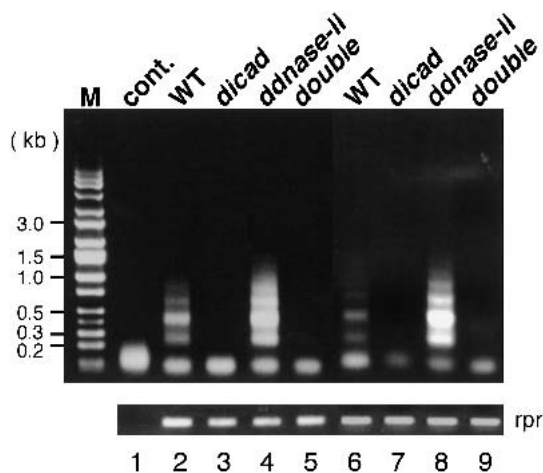
D. ICVGDINRQEGQLHRGGGTVCHKSARVSNLYRQLVTNYDKCAQQE 366  
M. ACVGD<sup>M</sup>NRNKAETHRGGGT<sup>V</sup>CTQLPSFWKAFQSLVKDWKPCIEGS 353  
H. TCVGD<sup>M</sup>NRN<sup>Q</sup>GEE<sup>Q</sup>RGGGT<sup>L</sup>CAQLPALWKA<sup>F</sup>QPLV<sup>K</sup>NYQPCNGMARKPSRAYKI 360



**Figure 2.** Identification of a *Drosophila* mutant in the *DNase II* gene. (A) The amino acid sequence of *Drosophila* DNase II and its mutant. The amino acid sequences of *Drosophila* DNase II (D), murine DNase II (M), and human DNase II (H) are aligned to give maximum homology by introducing several gaps (-). The amino acid residues that are conserved in all three proteins are shown in bold, and sets of three residues regarded as favored substitutions are indicated by underlines. Three histidine residues conserved in *Drosophila*, human, and mouse DNase II are indicated by asterisks. The serine residue (S) at amino acid position 223 of *Drosophila* DNase II was replaced with asparagine (N) in the *DNase-1<sup>lo</sup>* mutant. (B) Expression of dDNase II in COS cells. COS cells were transfected with the expression vector (lane 1), the expression vector carrying the Flag-tagged wild-type *Drosophila* DNase II cDNA (lane 2), or the Flag-tagged *S223N* mutant DNase II cDNA (lane 3). The cell lysates (40 μg of protein) from the transfected cells were separated by electrophoresis on a 10%–20% gradient polyacrylamide gel, and analyzed by Western blot using an anti-Flag antibody. Molecular masses of marker proteins are shown in kD at left. (C) Lack of DNase activity in the mutant DNase II. COS cells were transfected with the empty vector for the wild-type DNase II (lanes 5–7), or its *S223N* mutant (lanes 8–10). The cell lysates (2 mg of protein) were applied to M2 agarose beads (30 μL bed-volume), and the Flag-tagged DNase II was eluted with 90 μL of PBS containing 100 μg/mL Flag peptide. Using aliquots of 1.0 μL (lanes 2,5,8), 3.0 μL (lanes 3,6,9), and 9.0 μL (lanes 4,7,10) of the eluate, the acid DNase activity was determined with 1.0 μg of plasmid DNA as a substrate. In lane 1, the plasmid DNA was incubated in the assay buffer without the eluate.

1997), using the flies deficient in *ICAD* or *DNase II*. As shown in Figure 3, the DNA from the wild-type embryos and ovaries showed a clear DNA ladder. On the other hand, no DNA ladder was detected with the DNA from the *dICAD*-null flies, indicating an indispensable role for CAD in the apoptotic DNA fragmentation that occurs during embryogenesis and oogenesis. In contrast to the *dICAD* mutation, the mutation of the *dDNase II* gene did not impair the formation of the apoptotic DNA ladder (Fig. 3). Rather, the DNA ladder in the *DNase-1<sup>lo</sup>* flies was more pronounced than that observed in the wild-type flies, indicating an enhanced level of this type of degradation. The DNA ladder was not detected in double-mutant flies lacking both *ICAD* and *DNase II*. These results indicated that *CAD* and *DNase II* work

independently to degrade the chromosomal DNA during embryogenesis and oogenesis. Stone et al. (1983) previously detected Feulgen-positive materials in the egg chambers of the *Cy/M(2)S2<sup>lo</sup>* *Drosophila* strain, which they found to carry a hypomorphic allele of the gene affected by the *DNase-1<sup>lo</sup>* mutation, that is, *DNase II*. In fact, the ovaries of *DNase-1<sup>lo</sup>* flies showed materials that could be strongly stained with acridine orange in the dorsal appendage of the mature eggs (Fig. 4). By day eight, many acridine orange-positive vesicles were present near the lateral oviduct and these vesicles could be stained with Feulgen, suggesting that the DNA of the nurse cell nuclei were left undigested during oogenesis in the *DNase-1<sup>lo</sup>* mutant flies. A similar accumulation of acridine-positive mate-



**Figure 3.** The apoptotic DNA fragmentation in *Drosophila* embryos and ovaries. Genomic DNA was prepared from the 7–11 h embryos (lanes 2–5) or ovaries of day eight adult female flies (lanes 6–9) of the wild-type (CS; lanes 2,6), *dicad*-mutant (lanes 3,7), *ddnase II*-mutant of *DNase-1<sup>lo</sup>* (lanes 4,8), and the double mutant for *dicad* and *ddnase II* (double; lanes 5,9). An aliquot of DNA (40 ng) was subjected to LM-PCR, and the products were separated by electrophoresis on a 1.0% agarose gel. In lane 1, the LM-PCR was carried out without genomic DNA. The lower panel shows the control PCR for the *reaper* gene, confirming the equivalent amounts of DNA used for LM-PCR. The molecular mass size marker DNAs were run in lane M, and their sizes are indicated in kb at left.

rials was observed in the heterozygous flies that were generated by crossing *DNase-1<sup>lo</sup>* flies with a deficiency line of *Df(3R)P14*, indicating that this was a specific phenotype for the loss-of-function mutation of the *ddNase II* gene. The ovaries of the double mutants deficient in *dICAD* and *ddNase II* also accumulated a large amount acridine-positive material. In contrast, there was no abnormal disposition of acridine-positive materials in the ovaries of *dICAD* mutant flies.

#### Constitutive activation of antibacterial genes

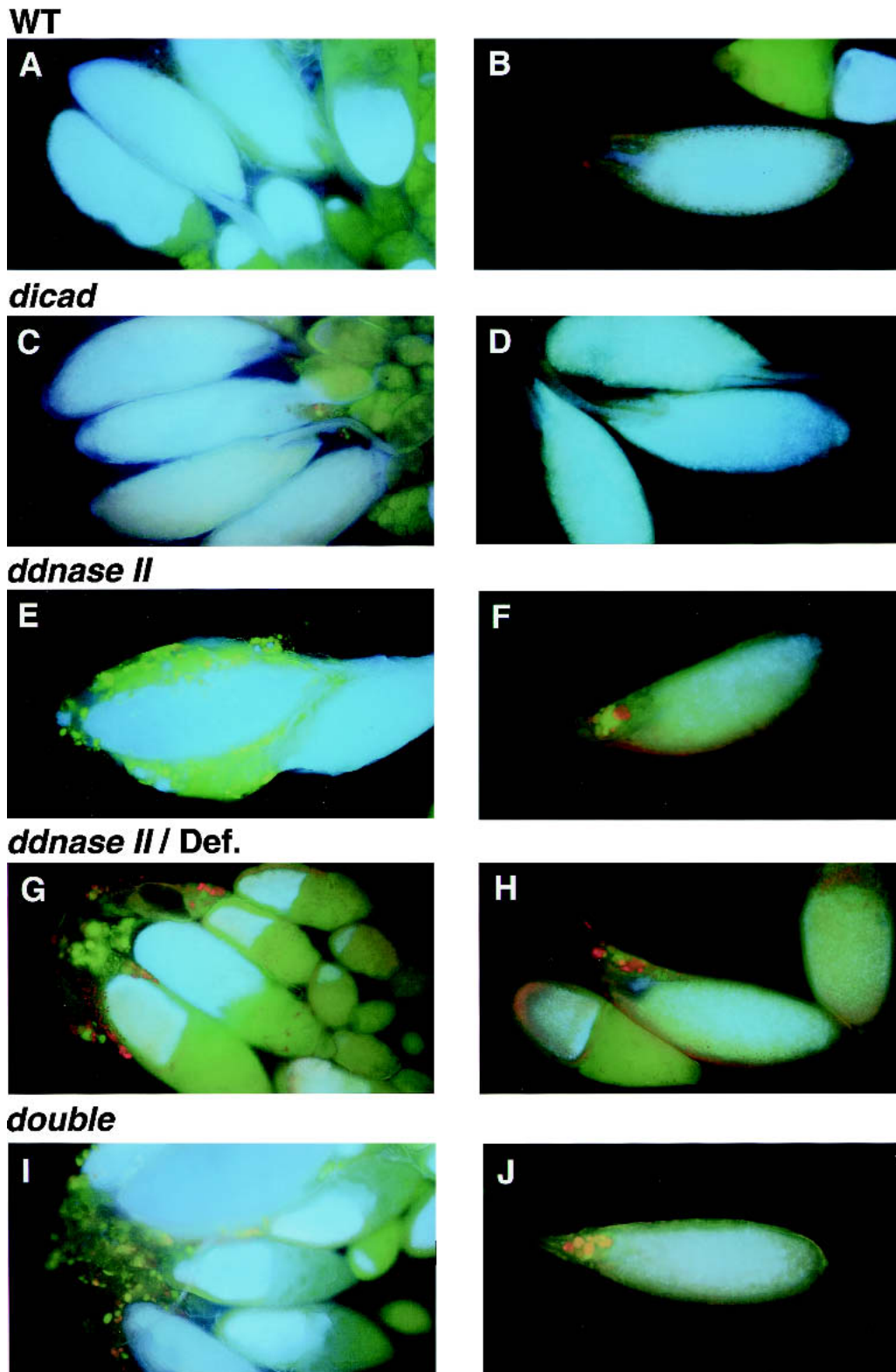
In mammals, nonmethylated CpG DNA activates the innate immunity through the toll-like receptor (TLR) in lysosomes (Hemmi et al. 2000; Leadbetter et al. 2002). Because the *ddNase II* mutant flies accumulated chromosomal DNA, we examined whether this accumulated DNA could activate the innate immunity in *Drosophila*. RNA was prepared from adult flies at day eight, and analyzed by Northern hybridization using as probes DNAs for antibacterial or antifungal peptides that are known to be induced during bacterial or fungal infection. As shown in Figure 5A, wild-type flies did not express the genes for the antibacterial peptides, dipteracin or attacin, and the mutation in the *dICAD* gene had little effect on the expression of these genes. In contrast, the mutation in the *ddNase II* gene caused the constitutive expression of the dipteracin and attacin genes. The expression level was enhanced in the double-mutant flies that were defi-

cient in both *dICAD* and *ddNase II*, but it was significantly lower than that observed by infection of flies with *Escherichia coli* (Fig. 5B). In contrast, the gene for the antifungal peptide drosomycin was weakly expressed in the wild-type flies as reported previously (Lemaitre et al. 1997), and its expression was little influenced by the mutation in *ddNase II* or *dICAD*. These results indicate that endogenous *Drosophila* DNA can activate a specific signaling pathway that leads to the induction of genes for antibacterial peptides if it is left undigested during the apoptotic process.

#### Discussion

The fragmentation of chromosomal DNA into nucleosomal units is regarded as a hallmark of apoptotic cell death (Earnshaw 1995; Nagata 2000), and the TUNEL staining that detects DNA fragments carrying 3'-hydroxyl ends is widely used to detect apoptotic cells in vitro and in vivo (Gavrieli et al. 1992). The recently developed linker-mediated PCR (LM-PCR) detects DNA fragments with 5'-phosphate ends, with high sensitivity (Staley et al. 1997). We reported previously that CAD, a DNase that produces DNA fragments with 5'-phosphate and 3'-hydroxyl ends (Widlak et al. 2000), is activated in apoptotic cells and can cleave the chromosomal DNA into nucleosomal units, giving rise to a DNA ladder that is typical for apoptosis (Enari et al. 1998). However, many other nucleases have been proposed as the nuclease that cleaves the chromosomal DNA during apoptosis (Li et al. 2001; Oliveri et al. 2001; Parrish et al. 2001; van Loo et al. 2001), and the contribution of CAD to apoptotic DNA fragmentation has been elusive, particularly in vivo. In this report, we established in *Drosophila* a *CAD*-null allele by disrupting the *ICAD* gene with a P-element insertion, and examined the role of the CAD system in the apoptotic processes associated with embryogenesis and oogenesis in *Drosophila*. In both of these processes, the apoptotic DNA fragmentation detected by LM-PCR did not occur in the mutant fly lacking the CAD system, suggesting that CAD is solely responsible for the apoptotic DNA fragmentation, at least in the embryogenesis and oogenesis of *Drosophila*. The contribution of other nucleases to this process, if any, would be small.

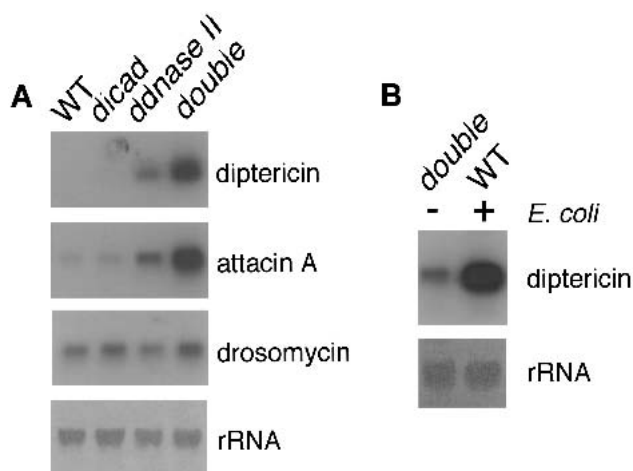
Despite the lack of DNA fragmentation in *CAD*-deficient flies, neither the embryos nor the ovaries apparently accumulated DNA. In *C. elegans*, a three-step process has been proposed for the degradation of DNA in programmed cell death (Wu et al. 2000). At least one of these steps is mediated by Nuc-1, a homolog of DNase II. We have proposed that in mammalian cells, the DNase II in the lysosomes of macrophages can degrade the DNA of apoptotic cells after macrophages engulf the apoptotic cells (McIlroy et al. 2000). However, because *DNase II*-null mice die prenatally due to severe anemia, the in vivo role of DNase II in the apoptotic process could not be evaluated in mice (Kawane et al. 2001). Here, we identified the *DNase-1<sup>lo</sup>* allele of *Drosophila* as a loss-of-function mutation of a DNase in lysosomes (Gaszner



**Figure 4.** Accumulation of DNA in ovaries. Ovaries from the adult flies (8 d) of wild-type (A,B), *dicad*-mutant (C,D), *ddnase II*-mutant (E,F), the heterozygous mutant line *DNase-1<sup>lo</sup>* (*ddnase II*)/*Df(3R)P14* (G,H), and the double mutant for *dICAD* and *ddNase II* (I,J) were stained with acridine orange as described in Materials and Methods.

and Udvardy 1991) that has significant homology to mammalian DNase II. The *DNase-1<sup>lo</sup>* mutants showed

enhanced CAD-dependent generation of apoptotic DNA fragments. Paradoxically, these mutants also accumu-



**Figure 5.** Expression of antibacterial peptide genes. (A) Constitutive activation of antibacterial peptide genes. Poly(A) RNA (3  $\mu$ g) from the adult *Drosophila* flies at day eight of the wild-type (WT), *ddicad*-mutant (*dicad*), *DNase-1<sup>lo</sup>* (*ddnase II*), and the double mutant for *dICAD* and *dDNase II* (double) was subjected to Northern hybridization using cDNA for dipterericin, attacin A, and drosomycin. At bottom, the filter was stained with methylene blue. (B) The endogenous DNA- or bacteria-induced expression of the *dipterericin* gene. The adult flies of the double mutant for *dICAD* and *dDNase II* (double) were kept uninfected, and the wild-type flies (WT) were infected for 16 h with *E. coli*. Total RNA (15  $\mu$ g each) was analyzed by Northern hybridization with dipterericin cDNA as a probe.

lated acridine orange-positive material, particularly in the ovaries. By hybridizing a labeled polypyrimidine probe to DNA from *DNase-1<sup>lo</sup>* flies, Stone et al. (1983) detected a low-melt hybrid with low thermal stability in the ovaries and proposed a two-step model for the degradation of nurse cell nuclei. In the first step, DNA is degraded into AT-rich DNA fragments of <200 bp, and these fragments are further digested to oligo- or mononucleotides by acid DNase in the second step. Our results agree with this model and support a scenario in which the first step of DNA degradation is mediated by CAD in the dying cells. CAD is an endonuclease that cleaves naked DNA into oligonucleotides. However, early in the cell-death process, DNA is still wrapped around histones in nucleosomes and CAD can only cleave the spacer regions between the nucleosomes, producing DNA fragments of nucleosomal units (180 bp). When the apoptotic cells are engulfed by phagocytes, the proteins of nucleosomes are digested by lysosomal proteases, and the DNA is exposed to the lysosomal DNase for complete digestion. The fact that the DNA fragmentation is not necessary for phagocytes to recognize apoptotic cells (McIlroy et al. 2000; Hanayama et al. 2002) explains why no accumulation of abnormal DNA was seen in the *CAD*-deficient flies. This model apparently differs from the model proposed for the apoptotic DNA degradation in *C. elegans*, in which Nuc-1 works in the dying cells (Wu et al. 2000). Although we cannot rule out this possibility, we prefer the model in which DNase II functions in phagocytes because DNase II is rather spe-

cifically expressed in macrophages (Kawane et al. 2001). Mice deficient in either CAD or DNase II have recently been established (Kawane et al. 2001; K. Kawane, H. Fukuyama, and S. Nagata, unpubl.). The coculturing of CAD-null apoptotic cells with DNase II-null macrophages will clarify the relationship between CAD and DNase II in the apoptotic DNA degradation.

The physiological role of the DNA degradation in programmed cell death has been elusive. Here, we found that endogenous DNA that escaped from apoptotic degradation could activate the innate immunity in *Drosophila*. Infection of *Drosophila* with *E. coli* induced a significantly higher level of expression of the antibacterial peptide genes. This may be because bacterial peptidoglycan or lipopolysaccharides (LPS) in addition to DNA activate the antibacterial peptide genes (Gottar et al. 2002), or the cells responding to the endogenous DNA or to bacteria differ. It will be necessary to study when and which cells express the antibacterial peptide genes in DNase II-null flies. There are two signaling pathways for the innate immunity in *Drosophila* (Hoffmann and Reichhart 2002). In one, infection with gram-positive bacteria or fungi activates the gene for an antifungal peptide, drosomycin, via the Toll/MyD88 pathway. In the other, infection with gram-negative bacteria induces the expression of genes for antibacterial peptides such as dipterericin and attacin via a Toll-independent immune deficiency (IMD) pathway. The specific activation of dipterericin and attacin but not drosomycin in the DNase II-null flies indicates that the accumulated DNA activates the innate immunity in a Toll-independent manner. This finding seems to contradict the fact that nonmethylated CpG DNA activates the innate immunity through the Toll-like receptor 9 (TLR9)/MyD88 pathway in mammals (Hacker et al. 2000; Hemmi et al. 2000). To clarify this point, it will be necessary to study whether the activation of antibacterial peptide genes in the DNase II-null flies requires MyD88 or IMD signaling, and whether any TLR9-like receptor is involved in recognition of the accumulated DNA in *Drosophila*. The flies deficient in DNase II as well as the flies deficient in both DNase II and CAD showed no apparent adverse phenotype except for a reduction in egg production. On the other hand, the activation of the innate immunity in mammals is likely to have adverse effects on their homeostasis by producing inflammatory cytokines such as TNF and IL-6 (O'Neill 2002). Previously, we reported that mouse embryos lacking the *DNase II* gene accumulate a large amount of DNA in the fetal liver and die due to a defect in erythropoiesis (Kawane et al. 2001). Whether this defect is due to inflammation caused by deregulated innate immunity remains to be studied.

## Materials and methods

### Materials and antibodies

The rabbit anti-dICAD antibody was described previously (Mukae et al. 2000). A rabbit dCAD antibody was produced by immunizing rabbits with recombinant dCAD. In brief, SF9 cells

were infected with Baculovirus carrying a cDNA for His-tagged dCAD as described previously (Sakahira et al. 1999). The infected cells were disrupted by three cycles of freezing and thawing in buffer A [10 mM Hepes-KOH at pH 7.2, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 1 mM DTT, and 1 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (*p*APMSF)], and the lysates were spun for 20 min at 15,000 rpm. The precipitates containing dCAD were washed with buffer B (10 mM Tris-HCl buffer at pH 8.0 and 100 mM NaH<sub>2</sub>PO<sub>4</sub>) containing 4 M urea, and dissolved in buffer B containing 7 M guanidine-HCl. The His-tagged dCAD was then loaded onto a Ni-NTA agarose (QIAGEN), eluted from the beads with 10 mM Tris-HCl buffer (pH 4.5) containing 100 mM Na<sub>2</sub>PO<sub>4</sub> and 8 M urea, and used to immunize rabbits. The anti-dCAD antibodies were partially purified from the sera using a Protein A-Sepharose column. The anti-Flag antibody was purchased from Sigma.

#### Fly stocks, mutagenesis, and septic injury

All fly stocks were maintained at 25°C on standard fly food. Canton S was used as the wild-type strain. The *DNase-1<sup>lo</sup>* mutant line (Grell 1976) and *Df(3R)P14* deficiency line were obtained from the Bloomington Stock Center. The flies carrying transposase and balancer lines were provided by Drs. F. Matsuzaki (Laboratory of Cell Assymetry, Riken Center for Developmental Biology, Chuo-ku, Kobe, Hyogo, Japan) and M. Miura (Laboratory of Cell Recovery Mechanisms, Riken Brain Science Institute, Tsukuba, Ibaraki, Japan).

A fly line carrying a mutation in *dICAD* gene was generated by the stepwise transposition of a P-element (Tower et al. 1993). In brief, *P(1(2)k00617)/CyO* flies carrying a P-element at the *48E2* region were crossed to female flies carrying transposase [*w/w*; *+/+*; *Dr/TMS*,  $\Delta 2-3$ , *Sb*]. Mosaic male progeny [*w/Y*; *P(1(2)k00617)/CyO*; *+/TMS*,  $\Delta 2-3$ , *Sb*] were backcrossed to *w/w*; *Sp/SM1*; *+/+* virgins. Descendant male flies with more intensely red eyes were crossed to *w/w*; *Sp/SM1* and analyzed by a long PCR method. One line (line 228) was found to carry a new P-element insertion ~20 kb upstream of the *dICAD* gene. After removing the P-element at the original position, this line was used to further transpose the P-element, and a line (16-11-36) carrying a P-element in intron 1 of the *dICAD* gene was established. The local P-element hop was repeated once again to establish line 42-1 [*P(1)cad*], which carried a P-element at the 5' noncoding region of the *dICAD* gene. This P-element insertion was found to be a null allele for *dICAD*.

For screening by long PCR, a set of 30-mer oligonucleotides carrying sequences derived from 10 kb upstream, downstream, and coding regions of the *dICAD* gene was prepared referring to the FlyBase (<http://flybase.bio.indiana.edu>) and used as a primer together with a 30-mer oligonucleotide (5'-GACGGGACCACACCTTATGTTATTTCATCATG-3') carrying a sequence in the LTR region of the P-element. PCR was carried out with rTth DNA polymerase (PE Applied Biosystems) using a Gene Amp<sup>R</sup> XLPCR kit. For plasmid rescue, *Drosophila* DNA was digested with *EcoRI* or *PstI*, ligated with T4 DNA ligase, and introduced into *E. coli*.

To generate a *Drosophila* line deficient in both the *dICAD* and *DNase II* genes, the lines *w/w*; *P(1)cad*, *P(16-11)/CyO*; *+/MKRS* and *w/Y*; *Sco/CyO*; *DNase-1<sup>lo</sup>/DNase-1<sup>lo</sup>* were established, and these were crossed to obtain *w/w*; *P(1)cad*, *P(16-11)/CyO*; *DNase-1<sup>lo</sup>/MKRS* flies. These flies were intercrossed to generate the double-mutant flies. The heterozygous flies carrying *DNase-1<sup>lo</sup>/Df(3R)P14* were generated by crossing the female flies *DNase-1<sup>lo</sup>/DNase-1<sup>lo</sup>* with *Df(3R)P14* flies *Df(3R)P14*, *sr[1]/T(2;3)ap[Xa],ap[Xa]*.

The septic injuries were done as described (Rutschmann et al. 2000). In brief, the adult wild-type flies were infected at 25°C by pricking with a tungsten needle that had been dipped in a concentrated culture of *E. coli* DH10B, and kept at 25°C for 16 h.

#### Cloning and expression of *dDNase II* cDNA

Total RNA was isolated by the acid-guanidine thiocyanate method using an Isogen kit from Nippongene. Two oligonucleotides (a sense primer, 5'-ACTTGAAGGTTGTACAATGCG-3', and an antisense primer, 5'-TTCTATTCTGTTGGGCGCAC-3') were designed from *Drosophila* EST clones (A1114173 and GH10876) and Gene *CG7780* in the FlyBase. RNA was reverse-transcribed using Superscript II reverse transcriptase (GIBCO-BRL), and the coding sequence of *dDNase II* cDNA was amplified by PCR using *Taq* polymerase. The PCR product of 1.1 kb was inserted into pGEM-T-Easy (Promega), and the nucleotide sequence was determined.

To express *dDNase II* in monkey COS cells, the coding sequence of *dDNase II* was tagged with a FLAG-epitope at the C terminus and inserted into the pEF-BOS-EX vector. COS cells in DMEM containing 10% fetal calf serum (GIBCO-BRL) were transfected with the *dDNase II* expression plasmid by electroporation. After culturing at 37°C for 48 h, the cells were suspended in lysis buffer (20 mM Tris-HCl at pH 7.5, 2 mM EDTA, 1% Triton X-100, 1 mM *p*APMSF, and 1  $\mu$ g/mL aprotinin), and disrupted by three cycles of freezing and thawing. After adding NaCl to a final concentration of 150 mM, the lysates were homogenized by grinding with a pestle, and the nuclei and large cell debris were removed by centrifugation. The FLAG-tagged *dDNase II* in the lysates was purified using anti-FLAG M2 agarose (Sigma).

The enzymatic activity of *dDNase II* was measured as described (Kawane et al. 2001). In brief, samples were preincubated on ice for 30 min in 20  $\mu$ L of 50 mM Citrate-NaOH buffer (pH 4.5) containing 10 mM EDTA and 50  $\mu$ g/mL RNase A. Plasmid DNA (1.0  $\mu$ g) was added to the mixture, followed by incubation at 30°C for 2 h. The DNA was analyzed by electrophoresis on a 1.5% agarose gel.

#### Acridine orange staining and linker-mediated PCR

Acridine orange staining of the ovary was conducted according to Foley and Cooley (1998). In brief, ovaries were dissected in buffer C (10 mM Hepes-HCl buffer at pH 6.9, 130 mM NaCl, 4.7 mM KCl, and 1.9 mM CaCl<sub>2</sub>), immersed for 3 min in the dark in a 1:1 mixture of 10  $\mu$ g/mL acridine orange in buffer C and heptane, and placed onto glass slides. After removing the excess dye by aspiration, samples were covered with halocarbon oil and examined with an Olympus IX70 fluorescence microscope using a UV-G excitation filter.

For linker-mediated PCR (LM-PCR), the genomic DNA prepared by the standard method was further purified by treatment with RNase A followed by precipitation with 7.5% polyethylene glycol. The DNA (40 ng) was subjected to an LM-PCR Ladder assay (Staley et al. 1997) using a kit from Clontech. The PCR was carried out for 26 cycles and the products were analyzed by electrophoresis on a 1% agarose gel.

#### Southern hybridization, Northern hybridization, and Western blotting

Southern and Northern hybridization were carried out under high stringency (Sambrook and Russell 2001) with <sup>32</sup>P-labeled fragment of *dICAD* or *dCAD* cDNA (Mukae et al. 2000; Yokoyama et al. 2000). The cDNAs for dipterin (Wicker et al.



1990), attacin A (Lee et al. 1983), and drosomycin (Fehlbaum et al. 1994) were isolated by RT-PCR from adult *Drosophila*, cloned in the pGEM-T-Easy vector, and used as probes for Northern hybridization.

For Western Blotting, adult flies were homogenized in the lysis buffer using a pestle. The homogenates were spun for 30 min at 30,000g, and the supernatants were used as the S-30 fraction. Proteins were separated by electrophoresis on a 10%–20% gradient polyacrylamide gel (Dai-ichi Pure Chemicals) and transferred to a PVDF membrane (Hybond P, Amersham Biosciences). The membranes were probed with 2000-fold diluted anti-dCAD or 1000-fold diluted anti-dCAD antibody followed by peroxidase-conjugated anti-rabbit IgG (DAKO). The bands were revealed by chemiluminescence using a Renaissance kit from PerkinElmer Life Sciences.

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