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Tissue-Specific Inducible Expression of Antimicrobial Peptide Genes in *Drosophila* Surface Epithelia

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Summary

The production of antimicrobial peptides is an important aspect of host defense in multicellular organisms. In *Drosophila*, seven antimicrobial peptides with different spectra of activities are synthesized by the fat body during the immune response and secreted into the hemolymph. Using GFP reporter transgenes, we show here that all seven *Drosophila* antimicrobial peptides can be induced in surface epithelia in a tissue-specific manner. The *imd* gene plays a critical role in the activation of this local response to infection. In particular, *drosomycin* expression, which is regulated by the Toll pathway during the systemic response, is regulated by *imd* in the respiratory tract, thus demonstrating the existence of distinct regulatory mechanisms for local and systemic induction of antimicrobial peptide genes in *Drosophila*.

Introduction

Insects defend themselves against infectious microorganisms by a sophisticated set of reactions that involve (1) phagocytosis and encapsulation by blood cells, (2) proteolytic cascades leading, in particular, to coagulation and melanization, and (3) secretion of a battery of potent antimicrobial peptides (reviewed in Khush and Lemaitre, 2000; Meister et al., 2000). This latter aspect of the insect host defense has been particularly well studied at the molecular level, using the fruit fly *Drosophila melanogaster* as a model, and has revealed several striking similarities with innate immunity in mammals (reviewed in Hoffmann et al., 1999).

Biochemical analysis of the hemolymph of many insect species has allowed the purification of several antimicrobial peptides, which, upon immune challenge, are synthesized by the fat body (a functional equivalent of the mammalian liver for many metabolic processes) and secreted into the hemolymph (Bulet et al., 1999, and references therein). In *Drosophila*, seven distinct antimicrobial peptides (plus isoforms) with distinct target specificities have been described. Although these molecules differ in size, amino acid composition, and three-dimensional structure, they can be grouped into three categories, based upon their biological activities. (1) Drosocin and defensin have antibacterial activities and are predominantly active against gram-negative and gram-positive bacteria, respectively. Attacins and diptericin have been identified in *Drosophila* through their cDNAs and are homologous to antibacterial peptides of other insect species; (2) drosomycin is a potent antifungal molecule, and, (3) lastly, metchnikowin and cecropins have both antibacterial and antifungal activities (reviewed in Meister et al., 2000). Interestingly, natural infection of *Drosophila* by the entomopathogen fungus *Beauveria bassiana* has been shown to trigger selective induction of peptides with antifungal activities, such as drosomycin and metchnikowin (Lemaitre et al., 1997). On the other hand, natural infection with the gram-negative bacteria *Erwinia carotovora* strongly induces the antibacterial peptide genes but has a less-marked effect on drosomycin expression (Basset et al., 2000). These data indicate that *Drosophila* can discriminate among various classes of microorganisms.

A genetic analysis has revealed that the antifungal and antibacterial responses are controlled by distinct intracellular signaling cascades, albeit with some cross-talk between these cascades (reviewed in Engstrom, 1999; Imler and Hoffmann, 2000). Indeed, the Toll pathway controls the antifungal response; expression of *drosomycin* by the fat body upon immune challenge is severely reduced in *Toll*-deficient flies and is constitutive in flies expressing the gain-of-function $Toll^{10b}$ allele. In contrast, expression of the antibacterial peptide genes *drosocin* and *diptericin* is normal in both $Toll^{-}$ and $Toll^{10b}$ flies (Lemaitre et al., 1996). Induction of these peptides, however, is strongly reduced in mutants of the *immune deficiency (imd)* gene (Lemaitre et al., 1995). Although the cloning of this gene has not yet been reported, several EMS mutations with immune phenotypes reminiscent of *imd* have recently been characterized, indicating that an Imd regulatory pathway controls the antibacterial reponse (Wu and Anderson, 1998; Elrod-Erickson et al., 2000; Leulier et al., 2000; Rutschmann et al., 2000). In addition, induction of antibacterial peptides has recently been shown to be severely reduced in mutant flies carrying a deletion in the gene *Relish*, indicating that the Rel transcription factor it encodes may be the nuclear target of the Imd pathway (Hedengren et al., 1999). Inducible systemic expression of *attacin*, *cecropin*, *defensin,* and *metchnikowin* appears to require an input from both the Toll and the Imd pathways (reviewed in Imler and Hoffmann, 2000). Importantly, the molecular characterization of the regulatory pathways involved in the control of antimicrobial peptide production has established *Drosophila* as a powerful model to study innate immunity. In particular, the discovery of the critical role played by the Toll receptor in *Drosophila* immunity was instrumental in promoting the search for related molecules in mammals. Two of these Toll-like receptors, TLR2 and TLR4, have recently been shown to play critical roles in nonself recognition. Analysis of knockout or

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mutant mice implicate TLR2 and TLR4 in peptidoglycan and lipopolysaccharide (LPS) signal transduction, respectively (reviewed in Means et al., 2000). In addition, several ex vivo studies using transfected cell lines point to a role for TLR2 in LPS signal transduction (e.g., Kirschning et al., 1998; Yang et al., 1998).

The production of antimicrobial peptides is also an important aspect of host defense in vertebrates. Similar to insects, mammals express multiple peptide antibiotics, such as the antibacterial defensins (α-, β-, and θ-) and cathelicidins and the antifungal histatins (reviewed in Lehrer and Ganz, 1999; Hancock and Scott, 2000). α-defensins were originally characterized as peptides produced in granules from polymorphonuclear neutrophils, which participate in the killing of phagocytosed microorganisms. More recently, two members of this family in humans, HD-5 and HD-6, were found to be constitutively produced by specialized secretory cells in small intestinal crypts, the Paneth cells (Mallow et al., 1996). HD-5 is also constitutively produced in the female reproductive tract (Quayle et al., 1998). Two human β-defensins have also been identified, which are expressed in many epithelia, either constitutively or in response to infection (Goldman et al., 1997; Bals et al., 1998a; Singh et al., 1998; Valore et al., 1998). The only cathelicidin identified thus far in humans, LL-37, is found in granules in myeloid cells but is also secreted onto the airway surface from epithelial cells of the lung (Bals et al., 1998b). Finally, histatins are secreted into the saliva (Edgerton et al., 1998). This situation contrasts with the systemic release of antimicrobial peptides into the hemolymph of *Drosophila*. Interestingly, epithelial expression of the antifungal peptide drosomycin has recently been described in the respiratory and reproductive tracts of *Drosophila* (Ferrandon et al., 1998). We have analyzed the expression domains of the six other *Drosophila* antimicrobial peptides, and we report that all barrier epithelia in this insect express at least one antimicrobial peptide in an inducible tissue-specific manner. We also show that this first-line epithelial immune reaction, which is probably present in most multicellular organisms, is dependent on the Imd pathway in *Drosophila*.

Results

Construction of Transgenic Reporter Strains

In order to study the expression pattern of the various antimicrobial peptides in *Drosophila*, we established transgenic strains expressing the green fluorescent protein (GFP) under the control of the promoters of the corresponding genes. The promoters from the *diptericin*, *drosocin*, *cecropinA1*, *metchnikowin*, and *drosomycin* genes have previously been characterized in transgenic flies (Engstrom et al., 1993; Meister et al., 1994; Charlet et al., 1996; Ferrandon et al., 1998; Levashina et al., 1998; Roos et al., 1998). The reporter genes (Figure 1) were injected into embryos, and several independent insertions were obtained and characterized for each transposon. The transgenic larvae and flies were pricked with a bacteria-soaked needle and examined under epifluorescent illumination with a dissecting scope, 6–48 hr later. As shown in Figure 1, all transgenic flies and larvae exhibited a diffuse fluorescence, which, upon dissection, corresponds to an induced expression in the fat body (Figure 1F and data not shown). This response was not observed in the absence of challenge (Figures 1B, 1C, and 1E). Only occasionally some fluorescence was seen in unstimulated flies or larvae (see below). Monitoring expression of the GFP mRNA and protein by Northern and Western blot analysis revealed similar kinetics of induction as the endogenous antimicrobial genes (data not shown), indicating that the transgenes used in this study are adequate reporters.

Tissue-Specific Activity of Antimicrobial Gene Promoters

Upon examination of a large number of unchallenged larvae (Figure 2) and adults (Figure 3), we detected a localized expression of GFP in some individuals. Interestingly, these larvae or flies showed an expression in tissues other than the fat body, following a complex pattern that was different for each promoter (Table 1). For example, fluorescence was often observed in tracheal epithelia of *drosocin-GFP* larvae (Figures 2A and 2B), similar to that reported for the *drosomycin-GFP* reporter (Ferrandon et al., 1998). In contrast, the *defensin-GFP* and *metchnikowin-GFP* reporters were detected in the oral region of the larvae, occasionally extending to the pharynx (Figures 2C–2F), and the *diptericin-GFP*, *defensin-GFP*, and *attacin-GFP* reporters were detected in the digestive tract (Figure 2G and data not shown). Fluorescence was also occasionally detected in the excretory system, namely, in the Malpighian tubules, of some larvae (Figure 2F). Importantly, HPLC and MALDI-TOF mass spectrometry analysis allowed us to detect endogenous drosocin and drosomycin peptides in dissected fluorescent fragments from larval tracheal trunks but not in nonfluorescent fragments, and the defensin peptide could be detected by MALDI-TOF analysis on dissected fluorescent preparations of the oral region of *defensin*-GFP larvae (S. Uttenweiler and P. Bulet, personal communication; Ferrandon et al., 1998). Thus, the GFP reporter system appears relevant to study epithelial expression of antimicrobial peptides.

In adults (Table 1), green fluorescence was frequently observed in the labelar glands (two small secretory organs that open at the beginning of the alimentary canal) of *defensin-GFP* and *metchnikowin-GFP* flies (Figures 3A and 3B). In the salivary glands, we exclusively observed fluorescence in transgenic adults carrying the *drosomycin-GFP* reporter, as previously reported (Ferrandon et al., 1998). Expression of the *diptericin-GFP* transgene was detected in the digestive tract (i.e., in the cardia) but also in the midgut (Figure 3D). This transgene, as well as the *cecropin-GFP* and *metchnikowin-GFP* transgenes, also yielded occasional fluorescence in the Malpighian tubules (Figure 3C). Finally, several promoters were active in the reproductive tract, as shown by fluorescence in the seminal receptacle and spermathecae for *cecropin-GFP*, *defensin-GFP*, *drosomycin-GFP*, and, to a lesser extent, *metchnikowin-* and *attacin-GFP* flies (Figures 3E and 3F). In addition, we detected

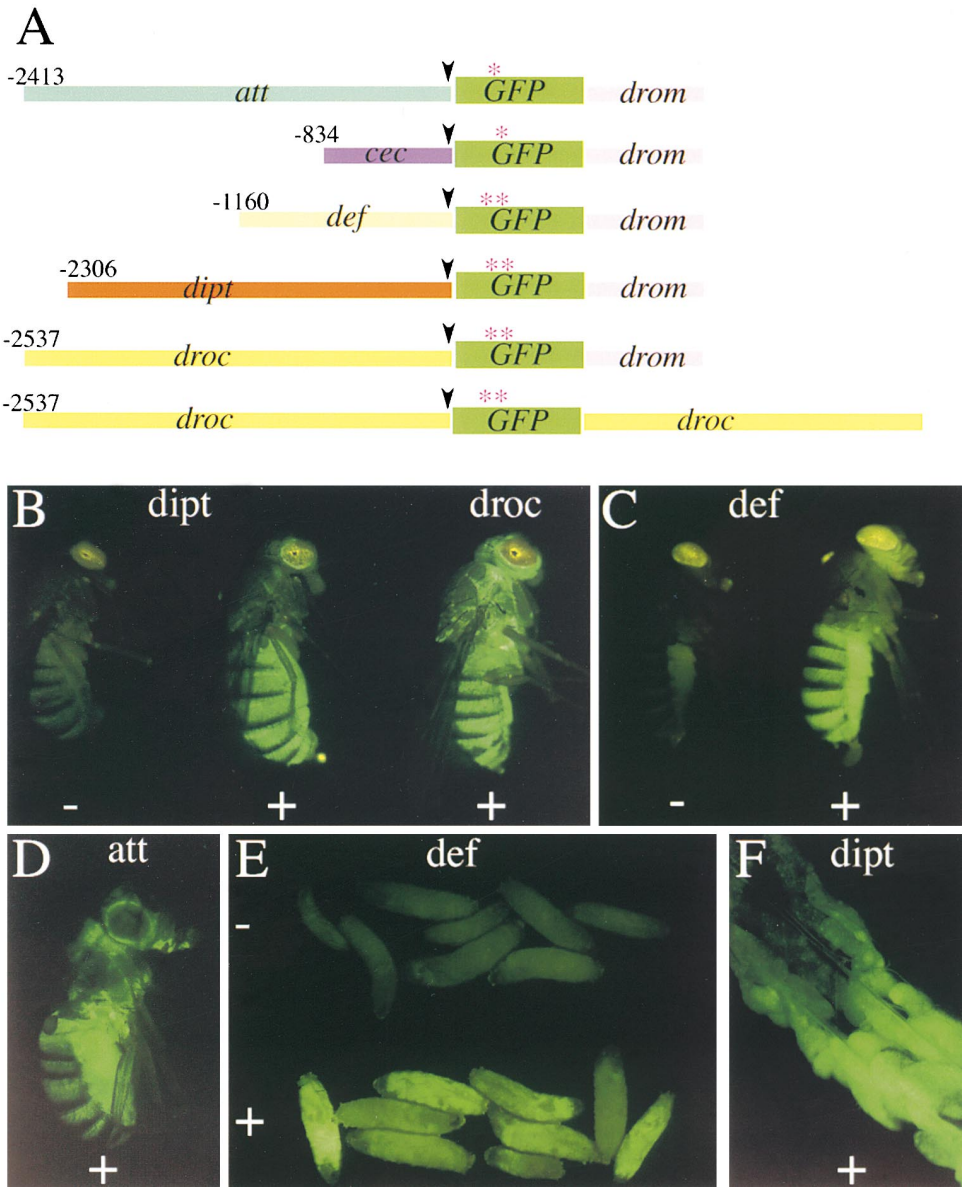

Figure 1. Construction of Reporter *Drosophila* Strains

(A) Schematic representation of the GFP expression cassettes. 780 bp of 3′ untranslated sequences from the *drosomycin* (drom) gene were used as transcription termination regulatory sequences for all promoters (Ferrandon et al., 1998; Levashina et al., 1998). In the case of the *drosocin* (droc) promoter, a second vector containing 2 kb of *drosocin* gene 3′ untranslated sequences (Charlet et al., 1996) downstream of the GFP coding sequences was constructed. Transgenic lines obtained with both vectors yielded similar results. The GFP activating mutations S65T and F64L/S65T are indicated by a single asterisk and double asterisks, respectively. Coordinates for the promoters are given, relative to the ATG (arrowhead), which serves to initiate translation of the GFP. Att, attacin A; cec, cecropin A1; def, defensin; dipt, diptericin.

(B–D) Induction of the systemic response in *diptericin-GFP*, *drosocin-GFP* (B), *defensin-GFP* (C), and *attacin-GFP* (D) transgenic flies. Flies were pricked with a needle inoculated with bacteria (+) and examined 48 hr later.

(E) Induction of the systemic response in *defensin-GFP* larvae. Third instar larvae were naturally infected with *Bacillus megaterium* (+) and examined 24 hr later.

(F) Expression of GFP in the fat body of a *diptericin-GFP* larva 24 hr after septic injury.

fluorescence in the oviduct and calyx of *drosocin-GFP* female flies, confirming a previous report describing expression of a *drosocin-lacZ* transgene in these tissues (Charlet et al., 1996). We also observed fluorescence in the ejaculatory duct of *cecropin-GFP* transgenic males and, to a lesser extent, in *attacin-GFP* and *drosomycin-GFP* flies (Figure 3G).

Importantly, only a fraction of the flies or larvae exhibited fluorescence in surface epithelia, and the proportion of the GFP-expressing animals was variable from one culture vial to the next. In addition, fluorescence was rarely distributed thoughout the whole tissue and was limited to restricted areas of the epithelium, suggesting a local response to natural infection. The fact that we

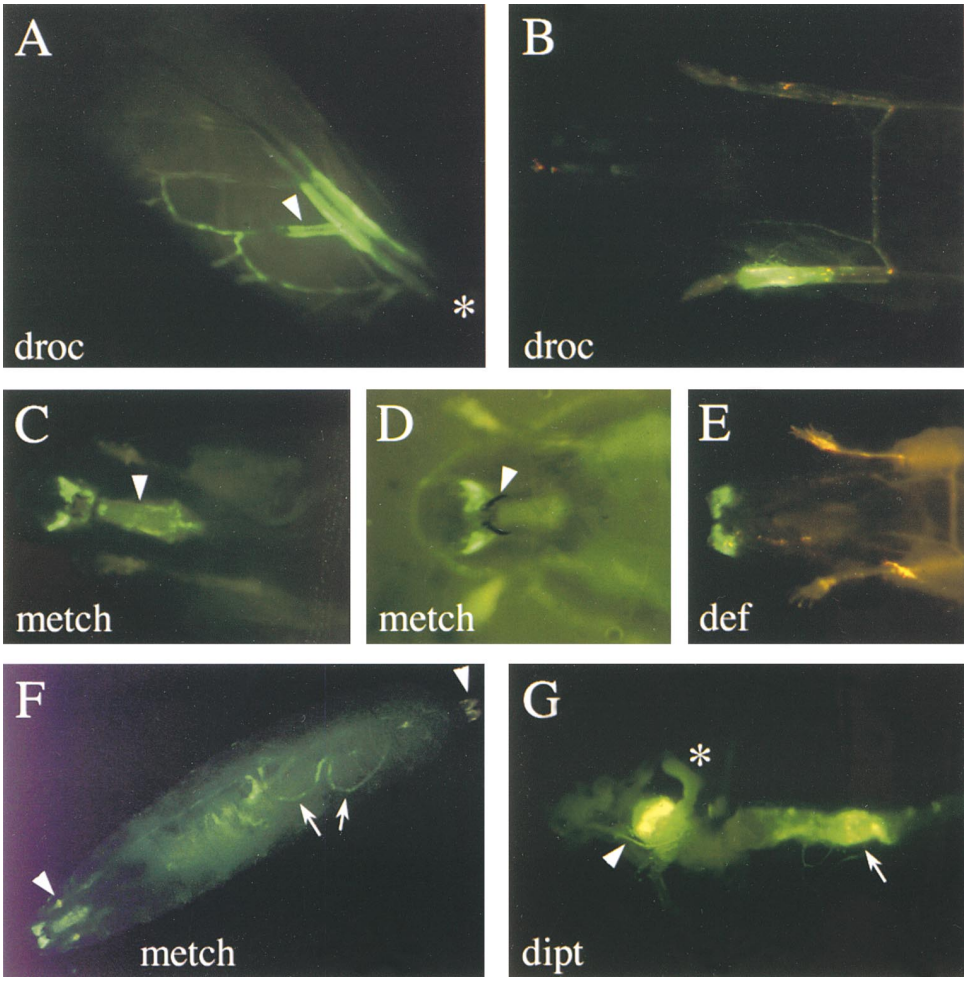

Figure 2. Localized Expression of the GFP Reporter Genes in Larvae

The larvae were not submitted to any experimental immune challenge unless otherwise specified.

(A) Expression of the *drosocin-GFP* reporter in the respiratory tract of a transgenic larva. Fluorescence is rarely seen in the whole tracheal system and usually only spans a portion of the respiratory tract, as illustrated here. Fluorescence often extends to lateral branches and originates from the tracheal epithelium that surrounds the tracheal cuticle (arrowhead). Position of the posterior spiracles is indicated by an asterisk.

(B) Anterior region of a drosocin-GFP transgenic larva, showing fluorescence in one of the two tracheal trunks.

(C) Expression of the *metchnikowin-GFP* reporter in the oral region in two groups of cells located dorsal to the mandibular hooks and corresponding to the ganglions of the antenno-maxillary organ. Fluorescence sometimes extends to the pharynx (arrowhead).

(D) Enlargement of the oral region of a *metchnikowin-GFP* transgenic larva, showing fluorescence in the ganglions of the antenno-maxillary organ. Arrowhead points to a mandibular hook.

(E) Expression of the *defensin-GFP* reporter in the antenno-maxillary organ.

(F) This *metchnikowin-GFP* transgenic larva expresses GFP in the antenno-maxillary organ and the pharynx, in the anterior and posterior spiracles (arrowheads), and in Malpighian tubules (arrows).

(G) Expression of the *diptericin-GFP* reporter in the dissected digestive tract of a transgenic larva after per os infection with *E. carotovora*. Fluorescence is seen in the proventriculus, a bulb-like structure that connects the oesophagus to the midintestine (arrowhead). Fluorescence sometimes extend to gastric caecae (asterisk). In addition, GFP is frequently expressed in the midgut (arrow) in these transgenic larvae. As illustrated here, fluorescence is, in most cases, limited to parts of the proventriculus or the midgut.

occasionally observed asymetric induction of fluorescence in paired structures (e.g., Figures 2B and 3B) further indicates that the expression of the reporter gene most likely results from a local exposure to microbes. The only exception was the constitutive activity of the *drosocin*, *cecropin*, and *drosomycin* promoters in various parts of the reproductive tract. The epithelial expression patterns recapitulated in Table 1 were never induced by septic injuries that trigger the systemic response.

Infection Triggers Induction of Antimicrobial Genes in Surface Epithelia

The above results suggest that promoters from antimicrobial peptide genes can be induced in surface epithelia to control local infections. We next performed natural infection of transgenic larvae and adults with the gram-negative bacteria *E. carotovora* (strain *Ecc15*) (Basset et al., 2000) and compared induction of the reporter genes in various tissues (Table 1). These bacteria have been found to infect both the digestive and the respira-

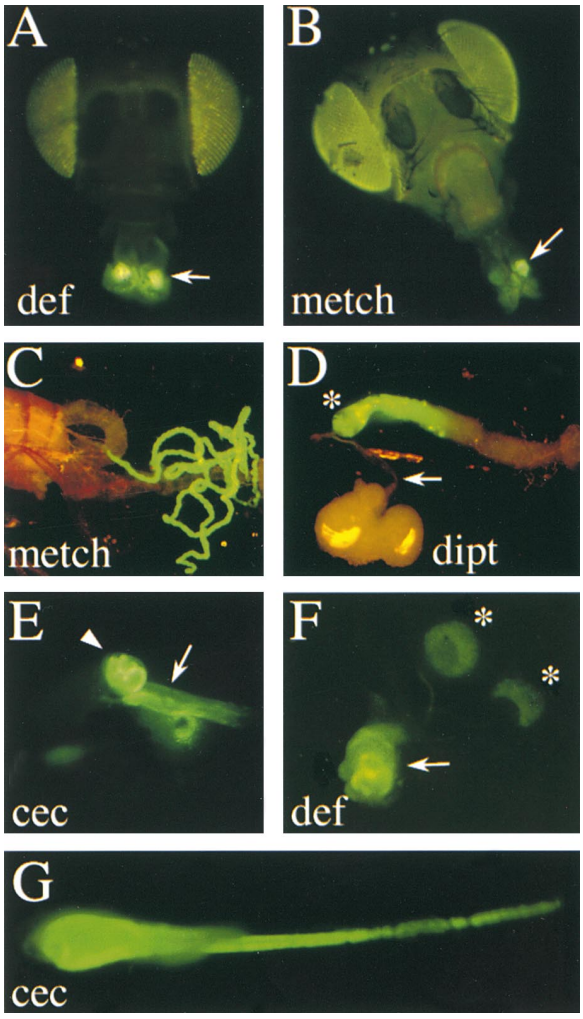

Figure 3. Localized Expression of the GFP Reporter Genes in Adults

The flies were not submitted to any experimental immune challenge unless otherwise specified.

(A and B) Expression of the *defensin-GFP* (A) and *metchnikowin-GFP* (B) reporters in the labellar glands (arrow), which are found inside the labellum and communicate with the alimentary canal.

(C) Expression of the *diptericin-GFP* reporter in the Malpighian tubules of a dissected transgenic fly, which has been infected per os with *E. carotovora*. In nonstimulated flies, fluorescence is rarely observed in Malpighian tubules and is restricted to small regions of the tubules.

(D) Expression of the *diptericin-GFP* reporter in the dissected digestive tract of a transgenic fly stimulated by per os bacterial infection. As illustrated here, fluorescence is often observed in the cardia (asterisk) and in the anterior region of the ventriculus, while the oesophagus (arrow) is negative. GFP synthesis is rarely observed in the digestive tract of nonstimulated flies.

(E) Constitutive expression of the *cecropin-GFP* reporter in the seminal receptacle (arrowhead) in the reproductive tract of a nonimmunized female fly. Fluorescence sometimes extends to the oviduct (arrow).

(F) Constitutive expression of the *defensin-GFP* reporter in the dissected spermathecae (asterisks) and seminal receptacle (arrow) of unstimulated females. This expression pattern (E and F) is not the result of an enhancer trap effect of the transposon insertion, since it was observed in all lines.

(G) Constitutive expression of the *cecropin-GFP* reporter in the ejaculatory duct of a nonimmunized male adult. All lines showed a similar pattern of expression, indicating that it is not the result of an enhancer-trap effect of the transposon insertion.

tory tract of *Drosophila* (Basset et al., 2000). β-galactosidase was occasionaly used as a reporter in these experiments, as it is more sensitive than GFP. As shown in Figure 4A, natural infection of the digestive tract with *Ecc15* triggered induction of the *diptericin* promoter in the proventriculus and part of the midgut but did not affect the *drosomycin* promoter in this tissue. Induction of the *attacin-GFP* transgene in the larval digestive tract by *Ecc15* could also be observed. When similar experiments were performed in adults, *Ecc15* infection of the digestive tract was found to induce *diptericin-GFP* in the cardia and midgut (Figure 3D) but also, to a lower extent, *attacin-GFP*, *drosocin-GFP*, *metchnikowin-GFP*, and *defensin-GFP*. When transgenic larvae were exposed to *Ecc15* bacteria for a long period of time, we observed a strong induction of the *drosomycin-GFP* reporter in tracheae (Figures 5A and 5B; Ferrandon et al., 1998). *Ecc15* also triggered activation of the *drosocin* and, to a lesser extent, *defensin* promoters in the respiratory tract. In adults, *Ecc15* infection was found to activate the *drosomycin* and *drosocin* promoters in the tracheae. Exposure of *drosomycin-GFP* flies to *Ecc15*, however, did not result in increased fluorescence in the salivary glands. Finally, natural infection by *Ecc15* was found to selectively activate the *diptericin*, *metchnikowin* (Figure 3C), and *cecropin* promoters in Malpighian tubules in adults. We conclude that local infection triggers induction of a subset of antimicrobial peptide genes in surface epithelia in a tissue-specific manner.

Regulation of Antimicrobial Genes in Surface Epithelia

Our data thus far indicate that all antimicrobial peptide genes can be activated in surface epithelia, in addition to the fat body, during the systemic response. This raises the question of the regulation of this local response. The *diptericin* promoter has been subjected to detailed molecular analysis, and we first monitored β-galactosidase activity in the fat body and proventriculus from transgenic *Drosophila* larvae in which *lacZ* expression is controlled by altered versions of this promoter (Figure 4A; Meister et al., 1994). As shown in Figure 4B, when point mutations were introduced in the two Rel binding sites located immediately upstream of the TATA box (*Dipt2.2mut*), induction was completely abolished in the fat body and in the proventriculus. Introduction of mutations separately in both Rel binding motifs revealed that the proximal site was critical for *diptericin* promoter inducibility in the fat body and the digestive tract, since no induction of the *Dipt2.2mut1* construct could be observed in either tissue (Figure 4B). These data strongly suggest that *diptericin* upregulation is mediated by a similar mechanism during systemic and local responses and that it involves a Rel protein.

We next examined expression of the *diptericin-lacZ* transgene in the digestive tract of *imd* mutant larvae. As shown in Figure 4A, induction of the reporter by *Ecc15* was completely abolished in the digestive tract of *imd* mutant larvae. Activation of the *diptericin* promoter in the digestive tract and Malpighian tubules in adults was also found to be dependent on the *imd* gene (Table 1). Upregulation of the *metchnikowin* promoter in the oral region in *imd* larvae and in the labelar glands

Table 1. Local Expression of the GFP Reporters in Transgenic Larvae and Adult Flies

	ATT		CEC		DEF		DROC		DIPT			DROM			METCH		
	-	+[a]	-	+	-	+	-	+	-	+	*imd* +	-	+	*imd* +	-	+	*imd* +
Larvae																	
Antenno-maxillary organ	0	0	0	0	*	*	0	0	0	0	0	0	0	0	***	***	*
Proventr.+ midgut	*	**	0	*	*	*	0	0	*	**		0	0	0	*	*	0
Salivary glands	0	0	0	0	0	0	0	0	0	0	0	*	*	*	0	0	0
Tracheae	0	0	0	0	0	*	*	**	0	0	0	*	***	*	*	*	0
Adults																	
Labellar glands	0	0	*	*	**	***	0	*	0	0	0	*	*	*	**	***	**
Cardia+ midgut	0	**	*	*	0	*	0	**	**	***	*	*	*	*	0	**	*
Salivary glands	0	0	0	0	0	0	0	0	0	0	0	***	***	***	0	0	0
Malpigh. tubules	0	0	*	**	0	*	0	0	*	***	*	0	0	0	*	**	*
Tracheae	0	0	0	0	0	0	0	***	0	0	0	**	***	*	0	0	0
Female reprod. tract	*	*	***	***	***	***	***	***	0	0	0	***	***	***	*	*	*
Male reprod. tract	*	*	***	***	0	0	0	0	0	0	0	*	*	*	0	0	0

The frequency of each expression is scored semi-quantatively from * to *** (0= not detected; *=0-30%; **=30-70%; ***=70-100%; *,*,*=weak, intermediated and strong fluorescence). These should be considered only as indications, since the actual frequencies in the absence of experimental immune challenge vary considerably and are dependent on the vial culture conditions. ATT: attacin; CEC: cecropin; DEF: defensin; DROC: drosocin; DIPT: diptericin; DROM: drosomycin; METCH: metchnikowin.

[a] After natural infection with *E. carotovora*.

in *imd* adults was also severely reduced, and GFP expression triggered by *Ecc15* in the tracheae of *drosomycin-GFP* transgenic larvae was not observed in *imd* mutants (Figure 5; Table 1). However, the constitutive expression of *drosomycin-GFP* in the female spermathecae and seminal receptacle was not affected in *imd* mutants. Furthermore, adult *drosomycin-GFP* flies with strong fluorescence in the salivary glands were observed at a similar frequency in *imd* mutant and in wild-type flies, indicating that activation of the *drosomycin* promoter in this organ is not dependent on *imd*. Altogether, these results indicate that *imd* plays a critical role in the control of antimicrobial promoter inducibility in surface epithelia. Furthermore, we did not observe constitutive epithelial activity of any of the promoters in larvae or flies carrying the gain-of-function *Toll*^{10b} allele (data not shown), suggesting that the Toll pathway is not involved in epithelial immunity. This confirms previous data indicating that local expression of *drosomycin-GFP* is not eliminated in *Toll* loss-of-function mutants (Ferrandon et al., 1998).

Finally, we studied the local response in *domino* (*dom*) mutants, which have a general defect in cell proliferation and a significantly reduced number of hemocytes (Braun et al., 1998). It has recently been shown that induction of the systemic response upon natural infection with *E. carotovora* is reduced in *dom* mutants, suggesting that blood cells play a role in regulating the *Drosophila* antimicrobial response (Basset et al., 2000). In contrast, we observed expression of the *drosomycin* reporter in the respiratory tract (Figure 5D) and expression of the *diptericin* reporter in the gut (data not shown) of *dom* mutant larvae. This suggests that blood cells are not required for antimicrobial peptide production during local responses. Surprisingly, we observed a higher frequency of expression of the *drosomycin-GFP* reporter in the absence of immune challenge in *domino* mutants than in wild-type larvae in the fat body (47% of dom^1/dom^1 larvae versus 0% in the control heterozygous cohort), in salivary glands (79% versus 38%), and in the respiratory tract, mainly at the level of the spiracles (74% versus 13%; compare Figures 5A and 5D). This latter observation may result from a compensatory mechanism when the cellular arm of the immune response is defective or, alternatively, from an indirect effect of the *domino* mutation on the cuticle or the epithelial barrier, which facilitates exposure to microorganisms.

Discussion

Antimicrobial Peptides and Epithelial Host Defense

We show here that, in *Drosophila*, antimicrobial peptide genes are expressed in several epithelia, which are potentially in contact with the environment. These include the respiratory tract, the oral region and the digestive tract, the Malpighian tubules, and the male and female reproductive tracts (Figure 6A). These tissues are sites of major physiological functions, such as gas exchange, nutrient absorption, water conservation, and reproduction, which necessitate host–environment interaction,

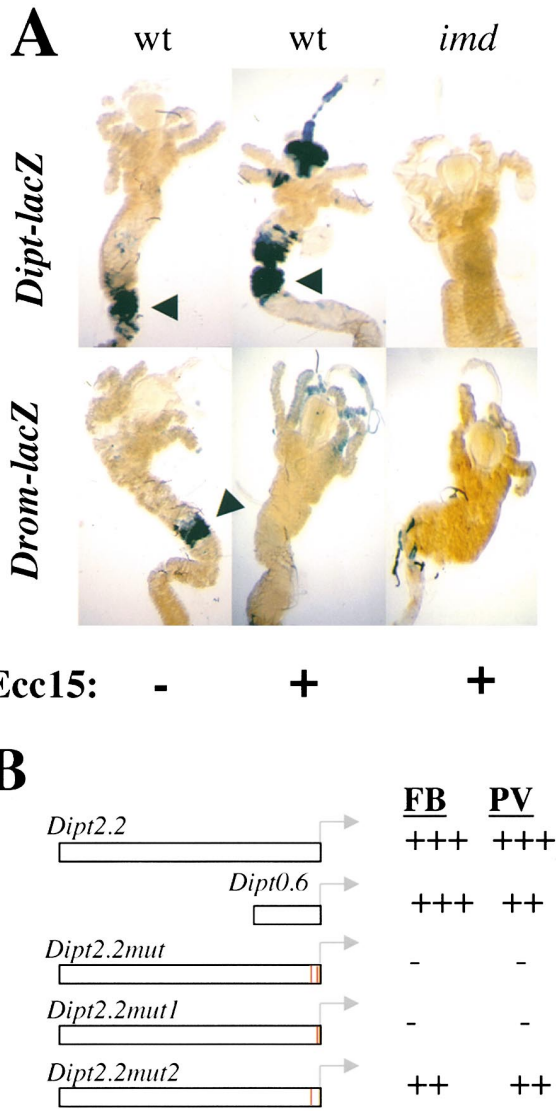

Figure 4. Regulation of Inducible Activation of the *diptericin* Promoter in the Digestive Tract in Larvae

(A) Selective induction of the *diptericin (Dipt)-lacZ* (Reichhart et al., 1992) reporter in the digestive tract of a transgenic larva by per os infection with *E. carotovora* (*Ecc15*). Infection triggers expression of β-galactosidase when the reporter gene is placed under the control of the *diptericin* promoter, but no induction is seen when it is replaced by the *drosomycin* promoter in *drosomycin(Drom)-lacZ* transgenic larvae (Manfruelli et al., 1999). No induction of β-galactosidase activity is seen when the *diptericin-lacZ* reporter is placed in an *imd* mutant background. Endogenous β-galactosidase activity observed in the posterior part of the midgut is indicated by an arrowhead. Wt, wild-type w^- backgound.

(B) The proximal Rel binding site in the *diptericin* promoter is required for induction in the fat body (FB) upon septic injury and in the proventriculus (PV) upon per os infection of transgenic larvae. Groups of ten transgenic larvae expressing *lacZ* under the control of the wild-type 2.2 kb *diptericin* promoter (*Dipt2.2*), a truncated version of 0.6 kb (*Dipt0.6*), or mutant versions in which the proximal (*Dipt2.2mut1*), the distal (*Dipt2.2mut2*), or both (*Dipt2.2mut*) Rel binding sites are mutated (Meister et al., 1994) were challenged with bacteria. 24 hr later, β-galactosidase activity in the proventriculus and the fat body was determined by histochemical staining. +, scattered weak activity; ++, staining in 80% of the cells; +++, strong staining in all cells.

and where cells are likely to encounter microorganisms. Interestingly, in most tissues that we examined, we could detect expression of at least two antimicrobial peptides with complementing spectra of activity; for example, in the oral region of larvae and adults, the antibacterial peptide defensin is coexpressed with the antifungal peptide metchnikowin, while, in the respiratory tract, the antibacterial peptide drosocin is coexpressed with the antifungal peptide drosomycin. The regulation of antimicrobial peptide concentration in these tissues may involve additional steps, such as mRNA stability, posttranslational modifications, and release of peptides from storage compartments. The simple structure of the epithelial tubes forming the Malpighian tubules and the tracheae, as well as the fact that antimicrobial peptide gene expression can be induced in the whole structure (see Figures 3C and 5B), indicates that all cells within these tissues are reactive. In contrast, immune inducibility in the digestive tract appears limited to the anterior part of the gut (the proventriculus in larvae and the cardia in adults) and restricted areas of the midgut (Figures 2G, 3D, and 4A), although bacteria are present throughout the midgut after per os infection (Basset et al., 2000). This points to the existence of local epithelial specialization within the gut epithelium in *Drosophila*, a finding supported by a previous study using enhancer trap lines (Murakami et al., 1994).

Expression of antimicrobial peptides has occasionally been reported in some epithelia of other insect species. In the *Bombyx mori* silkworm larva, *cecropin* mRNA is induced in the epithelial cells underlying the cuticle when it is lightly abraded in the presence of live bacteria (Brey et al., 1993). Although we did not observe expression of our reporter constructs in the integument of *Drosophila*, injury-induced integumental expression of β-galactosidase has been reported in transgenic larvae expressing *lacZ* under the control of a multimer of the Rel binding site from the *diptericin* promoter (Meister et al., 1994). Hence, it is likely that some antimicrobial peptides are expressed in the integument but to a level that does not allow detection with the GFP reporters. Production of two defensins, Smd1 and Smd2, has been documented in the midgut of the blood-sucking stablefly *Stomoxys calcitrans* (Lehane et al., 1997). Finally, the malaria parasite *Plasmodium berghei* elicits expression of defensin in the mosquito vector *Anopheles gambiae* in the midgut and salivary glands epithelia (Dimopoulos et al., 1998).

In larger animals and in plants, antimicrobial peptides also play a critical role in local responses to infection. In mammals, resident epithelial cells of the skin, the respiratory, alimentary, and genitourinary tracts synthesize and release antimicrobial peptides (reviewed in Huttner and Bevins, 1999; Lehrer and Ganz, 1999). Antimicrobial peptides present at epithelial surfaces are derived either from synthesis by resident epithelial cells or from storage granules in the cytoplasm of specialized white blood cells, such as neutrophils localized in the vicinity of the epithelium. As described here for *Drosophila*, it has been possible to show the presence of multiple peptides with overlapping spectra of antimicrobial activity in most mammalian epithelial tissues studied (see Huttner and Bevins, 1999; Figure 6B). In several plant species, roots, leaves, flowers, and seeds have also been shown to express cocktails of plant defensins

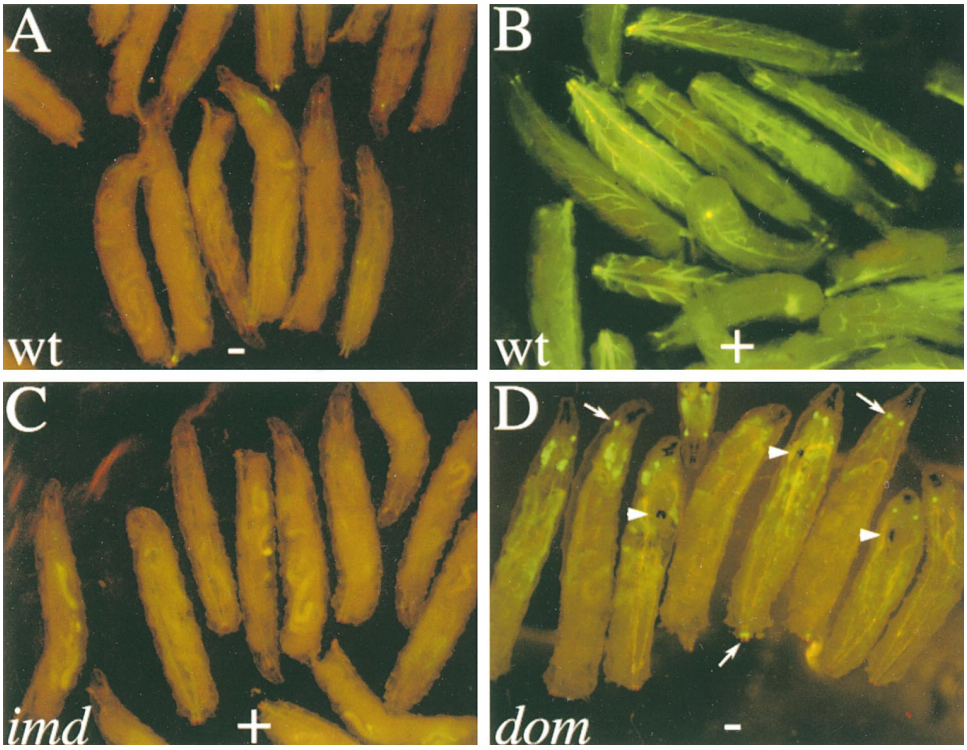

Figure 5. Regulation of the *drosomycin* Promoter in the Respiratory Tract in Larvae

Larvae of the indicated genotype were incubated for 30 min with crushed banana in the absence (A and D) or presence (B and C) of *E. carotovora*. Natural infection triggers expression of the *drosomycin-GFP* reporter in the tracheae in a wild-type w^- background (B) but not in an *imd* mutant background (C).

(D) Expression of the *drosomycin-GFP* reporter in the spiracles (arrows) and tracheae of unstimulated transgenic larvae in a *dom* backgound. The black dots indicated by arrowheads correspond to the melanized defective hematopoietic organs (lymph glands).

(Thomma and Broekaert, 1998; Figure 6C). The physiological relevance of antimicrobial peptide secretion by surface epithelia in mammals is underlined by recent studies in humans and mice. It has been proposed that the elevated salt concentrations in the lung fluids of cystic fibrosis patients diminish the antimicrobial properties of the β-defensin hBD-1, which is produced by the pulmonary epithelium (Goldman et al., 1997). In addition, disruption of the gene coding for matrilysin (a metalloprotease involved in the processing of α-defensins in the Paneth cells of the small intestine) results in mice with increased susceptibility to oral infection with *Salmonella typhimurium* (Wilson et al., 1999).

Genetic Regulation of Tissue-Specific Induction of Antimicrobial Peptide Genes

Our results indicate that the Imd pathway plays a central role in the regulation of antimicrobial genes in epithelia. This is most strikingly illustrated by the *drosomycin* and *metchnikowin* genes, which, in *imd* mutants, are not upregulated upon local infection in the tracheae and oral region, respectively, yet remain fully inducible in the fat body (Lemaitre et al., 1996; Levashina et al., 1998). In addition, our data showing that functional Rel binding sites are necessary for proper induction of the *diptericin* promoter in the digestive tract suggest that Relish regulates antimicrobial peptide expression in epithelia. The inducibility of the *drosomycin* gene by the gram-negative bacteria *E. carotovora* in tracheae contrasts with the weak inducibility of this gene by gram-negative bacteria in the fat body (Lemaitre et al., 1997) and raises the question of the function of drosomycin in the respiratory tract. One possibility is that, although devoid of antibacterial activity on its own, drosomycin can synergize with other molecules to increase their antibacterial power. Synergy between antimicrobial peptides has previously been reported (see Hancock and Scott, 2000). Alternatively, studies in vertebrates have revealed that, in addition to their microbicidal activities, antimicrobial peptides may acquire other functions and participate in the modulation of the inflammatory response, wound repair, cell division, and adaptive immune response (e.g., van den Berg et al., 1998; Lillard et al., 1999). In particular, the mammalian β-defensins have recently been shown to be chemotactic for dendritic and T cells and to recruit these cells to the site of microbial invasion (Yang et al., 1999). It will be interesting to investigate the possible role of *Drosophila* antimicrobial peptides in blood cell function.

In addition to Imd/Relish, other tissue-specific mechanisms control the inducibility of antimicrobial peptides in epithelia. Indeed, when bacteria are injected into the *Drosophila* body cavity, antimicrobial gene promoters are induced in the fat body but not in epithelia. This suggests that different receptors trigger these genes in the fat body and in epithelia. Induction of *drosomycin*

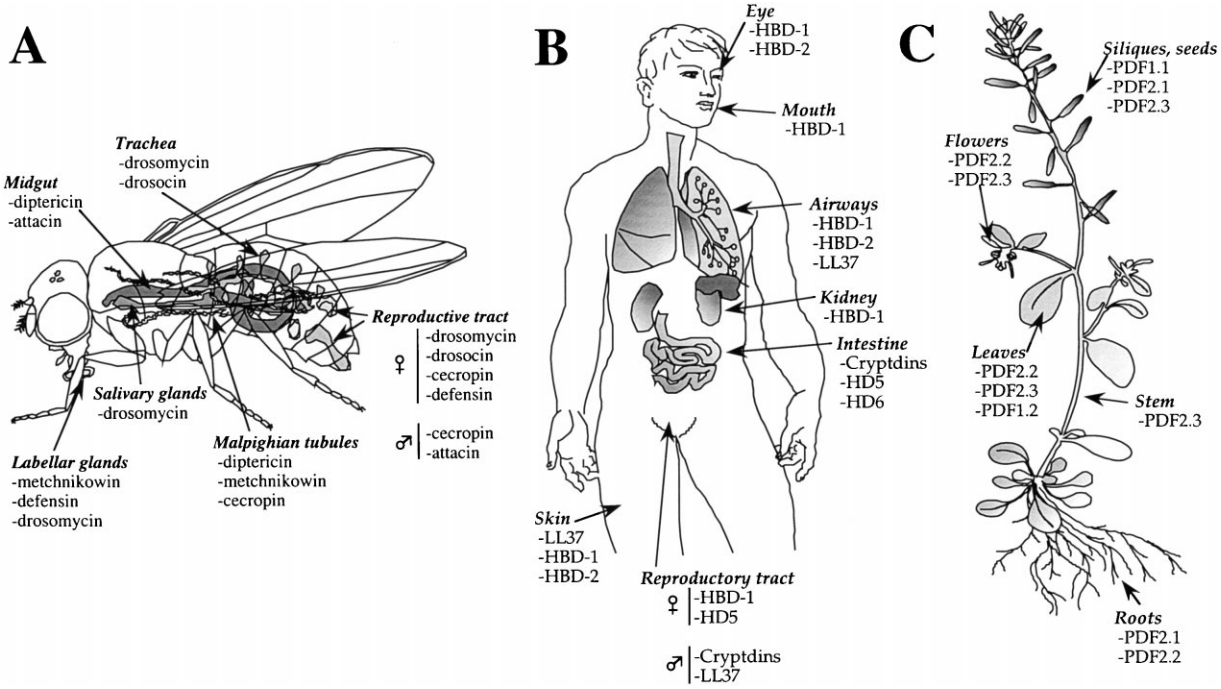

Figure 6. Tissue-Specific Expression of Antimicrobial Peptides in Multicellular Organisms

The main expression sites of the reporters in *Drosophila* (A), humans (B), and *Arabidopsis* (C) are indicated. HBD, human β-defensin; HD, human α-defensin; PDF, plant defensin. See the text for references.

in the fat body is thought to involve activation of a proteolytic cascade, which leads to processing of the cytokine Spaetzle and activation of the Toll receptor (Levashina et al., 1999). One intriguing possibility is that *drosomycin* expression in tracheae or salivary glands is triggered by one of the eight Toll-related receptors encoded by the *Drosophila* genome (Tauszig et al., 2000). Tissue-specific mechanisms of gene activation may also exist in mammals and could help to reconcile divergent hypotheses about the roles of TLRs in the immune response (see Hirschfeld et al., 2000; Means et al., 2000). In particular, TLR2, which has been suggested by studies based on transfection of cultured embryonic kidney cells to be involved in LPS signal transduction (Kirschning et al., 1998; Yang et al., 1998), may operate as such only in specific tissues, thus explaining why TLR2 knockout mice have an apparent normal response to LPS in macrophages and B cells (Takeuchi et al., 1999). Indeed, the recent demonstration that the upregulation of the β-defensin hBD2 by LPS in the tracheobronchial epithelium in humans requires CD14 and that this tissue expresses the mRNAs coding for TLR1–6, suggests that Toll-like receptors may be involved in the innate immune response of the respiratory epithelium (Becker et al., 2000).

Our data also point to the existence of tissue-specific transcription factors, which associate with Relish to trigger induction of a subset of antimicrobial peptides in a given epithelium. This would explain why peptides like diptericin and drosocin, which are both regulated by the sole Imd/Relish pathway in the fat body during the systemic response (Lemaitre et al., 1996; Hedengren et al., 1999), are induced in different epithelial locations (namely, the tracheae for drosocin and the digestive tract for diptericin). Several studies have indeed demonstrated that, in mammalian cells, NF-κB interacts with other transcription factors, which regulate positively or negatively its transcriptional activity (reviewed in Siebenlist et al., 1994). In *Drosophila*, a detailed molecular analysis of the *diptericin* and *cecropin* promoters has revealed the existence of nuclear factors binding to DNA sequences adjacent to the Rel binding sites (Engstrom et al., 1993; Georgel et al., 1993), and it has been suggested that the GATA transcription factor encoded by the gene *serpent* modulates the activity of the Rel proteins during the immune response in larvae (Petersen et al., 1999).

In conclusion, we have described the expression pattern of antimicrobial peptides in *Drosophila* epithelia. Epithelial expression of antimicrobial peptides appears to be a general feature of host defense in multicellular organisms. In contrast, systemic expression of antimicrobial peptides by fat body cells and secretion into the hemolymph appears to be restricted to the classes of insects undergoing metamorphosis. Thus, we propose that inducible expression of antimicrobial peptides in surface epithelia represents the ancestral system of host defense. Our data suggest that the machinery for immune recognition and subsequent signal transduction first appeared in epithelial cells isolating the multicellular organism from the environment and was later, during the course of evolution, recruited to more specialized cell types, such as the fat body in higher insects or the white blood cells in mammals. Surprisingly, the Toll pathway, which has been proposed to be an evolutionary ancient immune pathway, does not appear to be

involved in the control of antimicrobial peptide expression in *Drosophila* epithelia. Rather, the Imd/Relish pathway plays a critical role in the control of epithelial expression of antimicrobial peptides and may represent the most ancestral pathway.

Experimental Procedures

Plasmids

The construction of the pCasper transformation vectors in which the S65T version of GFP is fused to the *drosomycin* promoter (pJM802) and in which the F64L/S65T version of GFP is placed under the control of the *metchnikowin* promoter (pJM879) has been previously described (Ferrandon et al., 1998; Levashina et al., 1998). The *drosomycin* promoter in pJM802 was replaced by a NheI-SpeI fragment containing the *cecropin A1* gene upstream sequences from position −760 to +62 (Engstrom et al., 1993) to obtain the plasmid that contains the *cecropin-GFP* cassette (pJL164). Similarly, replacement of the *drosomycin* promoter in pJM802 by a SpeI-NheI fragment containing the *attacin A* promoter (J. L. I., unpublished data; nucleotides 149 339 to 146 924 of Genbank sequence no. AE003813) yielded the vector containing the *attacin-GFP* cassette (pJL166). The *metchnikowin* promoter in pJM879 was replaced (1) by a SalI-NheI fragment containing the *diptericin* gene upstream sequences from position −2266 to +42 (Reichhart et al., 1992) to construct the *diptericin-GFP* plasmid (pJM863), (2) by a HindIII-NheI fragment containing the *defensin* gene upstream sequences from position −1134 to +28 (Dimarcq et al., 1994) to yield the *defensin-GFP* reporter (pJM878), or (3) by a Hind III fragment containing the *drosocin* promoter (nucleotides −2505 to +34) (Charlet et al., 1996) to obtain the *drosocin-GFP* construct (pJM877). A second *drosocin-GFP* vector (pJM876) was constructed by replacing the *drosomycin* termination sequences in pJM877 by a 2 kb SacII-HindIII fragment of *drosocin* 3′ untranslated region. We did not observe any differences between the transgenic lines obtained with these two vectors (data not shown). Details about all constructions can be obtained upon request.

Fly Strains

All flies were maintained at 25°C on a standard cornmeal medium (65 g/l cornflour, 50 g/l sugar, 12 g/l baker yeast, 5 g/l agar, and 3 g/l methyl 4-hydroxybenzoate). The mutant strains used in this study have been described (Meister et al., 1994; Lemaitre et al., 1995, 1996; Braun et al., 1998). Transgenic lines were generated by P element transformation of a w^- strain. Standard crosses with flies carrying appropriate balancers were performed to establish stable heterozygous or homozygous lines, as well as to determine the chromosome carrying the insertion. We analyzed at least three independent lines for each construct. Northern blot and Western blot analysis showed that each endogenous gene and its corresponding reporter gene are expressed at similar levels with the same kinetics of accumulation upon immune challenge (data not shown).

Infection Experiments

The systemic response was triggered by pricking adult flies or third instar larvae with a thin needle, previously dipped in a concentrated culture of *Escherichia coli* and *Micrococcus luteus*. For natural infection, the gram-negative bacteria *Erwinia carotovora* (strain *Ec carotovora 15*), which causes potato blackleg disease, was used (Basset et al., 2000). Approximately 200 third instar larvae were placed in a 2 ml microfuge tube containing 200 μl of concentrated bacterial pellet from an overnight culture and 400 μl of crushed banana. The larvae, bacteria, and banana were thoroughly mixed in the tube, which was stoppered with a foam plug and incubated at room temperature for 30 min. Larvae were then transferred to standard cornmeal fly medium and incubated at 29°C for 24 hr before observation. For observation of GFP expression in tracheae, second instar larvae were infected as described above, and the infected larvae were incubated on standard medium at 18°C for 5–7 days before observation. For natural infection of adults, flies were placed in a tube containg pieces of paper saturated with a water solution containing 5% glucose and 30% of concentrated bacterial pellet ($DO_{600} = 200$) for 1 hr. Flies were then placed on standard medium at 25°C and observed after 3 or 4 days.

Microscopic Observations

Live flies and larvae were anesthetized with ether or on ice, respectively, and viewed under epifluorescent illumination (excitation filter, 480/40 nm; dichroic filter, 505 nm; and emission filter, 510 nm) with a Leica MZ12 or MZFLIII dissecting microscope. Dissections were performed in phosphate-buffered saline (PBS) under the dissecting scope and were viewed either using the dissecting microscope or a Leitz Diaplan epifluorescence microscope using a fluorescein isothiocyanate (FITC) filter set. Photographs were taken on a 400 ASA Fujicolor film or recorded with a charge-coupled device camera (Sony). Histochemical detection of β-galactosidase activity was performed as described in Charlet et al. (1996).

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