Signaling and transcriptional regulation of antimicrobial peptide genes in *Drosophila melanogaster*

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ABSTRACT

Insects rely solely on innate immune reactions for protection against infecting microbes in their environment. In *Drosophila*, one major defense mechanism is the production of a battery of antimicrobial peptides (AMPs). The expression of AMPs is primarily regulated at the level of transcription and constitutes both constitutive expression in a tissue-specific manner and inducible systemic expression in response to infection. The aim of my thesis has been to investigate the regulation of AMP gene expression at different levels. I have studied a novel cis-regulatory element, Region 1 (R1) found in the proximal promoter of all Cecropin genes in *Drosophila melanogaster*, as well as in other species of *Drosophila*. We found that the R1 element was important for the expression of *Cecropin A1* (*CecA1*) both *in vitro* and *in vivo*. A signaling-dependent R1-binding activity (RBA) was identified in nuclear extracts from *Drosophila* cells and flies. The molecular nature of the RBA, has despite considerable effort, not yet been identified. I also have studied the role of the JNK pathway in transcriptional regulation of AMP genes. The role of the JNK pathway in the regulation of AMP genes has long been elusive, however, in this study we showed that the pathway is directly involved in the expression of AMP genes. Analysis of cells mutant for JNK pathway components showed severely reduced AMP gene expression. Furthermore, over-expression of a JNK pathway-inhibitor also inhibited AMP gene expression. Lastly, I have studied transcription factors that have not previously been implicated in transcriptional regulation of AMP genes. In a yeast screen, three members of the POU family of transcription factors were identified as regulators of *CecA1*. Two of them, Drifter (*Dfr*) and POU domain protein 1 (*Pdm1*) were further characterized. We showed that *Dfr* was able to promote AMP gene expression in the absence of infection, suggesting it to play a role in constitutive expression of AMP genes. Indeed, down-regulation of *Dfr* expression using RNAi severely reduced the constitutive expression of AMP genes in the male ejaculatory duct. We also identified an enhancer element important for *Dfr*-mediated expression of *CecA1*. *Pdm1*, on the other hand, was shown to be important for the systemic expression of AMP genes. In *Pdm1* mutant flies, several AMP genes are systemically expressed even in the absence of infection, suggesting that *Pdm1* works as a repressor of those genes. However, at least on AMP gene, *Attacin A* (*AttA*) requires *Pdm1* for its expression, suggesting that *Pdm1* works as an activator for this gene. Upon infection, *Pdm1* was rapidly degraded, but, regenerated shortly after infection. We propose that the degradation of *Pdm1* is important for the activation of the *Pdm1*-repressed genes and that regeneration supports the expression of *AttA*.
List of papers included in the thesis

The thesis is based on the following articles, which will be referred to by their Roman numerals in the text.

I. Hanna Uvell and Ylva Engström

II. Joseph R. Delaney, Svenja Stöven, Hanna Uvell, Kathryn V. Anderson, Ylva Engström and Marek Mlodzik
“Cooperative Control of Drosophila Immune Responses by the JNK and NF-κB Signaling Pathways”
Resubmitted to EMBO J. after revision

III. Anna Junell, Hanna Uvell, Leslie Pick and Ylva Engström
“Isolation of regulators of Drosophila immune defense genes by a double interaction screen in yeast”
Manuscript

IV. Anna Junell*, Hanna Uvell*, Åsa Antonsson, Gunnel Björklund, and Ylva Engström
*These authors contributed equally
“The POU protein Drifter activates antimicrobial peptide gene expression in Drosophila.”
Manuscript

V. Hanna Uvell*, Anna Junell*, Åsa Antonsson, Rafael Cantera and Ylva Engström
*These authors contributed equally
“The Drosophila transcription factor Pdm1 inhibits antimicrobial peptide gene expression”
Preliminary manuscript
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<table>
<thead>
<tr>
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<th>Full Form</th>
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<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
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<tr>
<td>Anp</td>
<td>Andropin</td>
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<td>AP-1</td>
<td>Activator Protein-1</td>
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<tr>
<td>Att</td>
<td>Attacin</td>
</tr>
<tr>
<td>Cad</td>
<td>Caudal</td>
</tr>
<tr>
<td>Cec(A1)</td>
<td>Cecropin(A1)</td>
</tr>
<tr>
<td>Ddc</td>
<td>Dopadecarboxylase</td>
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<tr>
<td>Def</td>
<td>Defensin</td>
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<tr>
<td>Dfr</td>
<td>Drifter</td>
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<tr>
<td>Dpt</td>
<td>Diptericin</td>
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<tr>
<td>Dro</td>
<td>Drosocin</td>
</tr>
<tr>
<td>Drs</td>
<td>Drosomycin</td>
</tr>
<tr>
<td>E. cloaeae</td>
<td>Enterobacter cloaeae</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>G- bacteria</td>
<td>Gram negative bacteria</td>
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<tr>
<td>G+ bacteria</td>
<td>Gram positive bacteria</td>
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<tr>
<td>GNBP</td>
<td>Gram negative binding protein</td>
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<tr>
<td>Imd</td>
<td>Immune deficiency</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
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<tr>
<td>IL-1R</td>
<td>Interleukin receptor 1</td>
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<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LTA</td>
<td>Lipotechoic acid</td>
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<td>Lwr</td>
<td>Lesswright</td>
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<tr>
<td>M. luteus</td>
<td>Micrococcus luteus</td>
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<tr>
<td>MAPKKK</td>
<td>Map kinase kinase kinase</td>
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<tr>
<td>Mtk</td>
<td>Metchnikowin</td>
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<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>Pdm-1</td>
<td>POU domain protein-1</td>
</tr>
<tr>
<td>Pdm-2</td>
<td>POU domain protein-2</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
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<tr>
<td>PGRP</td>
<td>Peptidoglycan recognition protein</td>
</tr>
<tr>
<td>PO</td>
<td>Phenoleoxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PPO</td>
<td>Prophenoloxidase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>Psh</td>
<td>Persephone</td>
</tr>
<tr>
<td>R1</td>
<td>Region 1</td>
</tr>
<tr>
<td>RBA</td>
<td>R1-binding activity</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>SPE</td>
<td>Spätzle processing enzyme</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-Activated Kinase</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-Interleukin1-Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>wntD</td>
<td>wnt inhibitor of Dorsal</td>
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</table>
Introduction

Immunity

In mammals, immune reactions are manifested by two systems, the innate immune system and acquired or adaptive immune system. The innate immune system relies on germ-line encoded factors, which mount a fast and diverse response to infection. The acquired immune system is relatively slow, but very specific in its activity, and involved in the elimination of pathogens during the late phase of infection. The acquired immune system is also involved in the generation of immunological memory. Combination of the two immune systems gives us a very complex and efficient defense against infections [reviewed in (Medzhitov and Janeway, 1997a)].

The development of powerful mechanisms to counteract microorganisms has been a prerequisite for the evolution of the various phyla for billions of years since microorganisms have been present during the earliest stages of eukaryotic evolution. Innate immune reactions are found throughout the metazoan kingdom while acquired immune responses appeared in the ancestors of cartilaginous fish. All animals derived from these ancestral fish, i.e. all gnathostome vertebrates, have retained the innate immune mechanisms in parallel with the development of the acquired immune responses. Like all invertebrates, insects defend themselves through innate immune mechanisms [reviewed (Hoffmann and Reichhart, 2002)]. It is believed that innate immune reactions have been conserved through evolution. When studying innate immune reactions it is convenient to use a model system, which is devoid of an adaptive immune system. Drosophila constitutes one such model.
Innate immunity in *Drosophila*

*Drosophila* is known to be very resistant to microorganisms and the fly relies on innate immune reactions for protection against infections. In their natural habitat, *Drosophila* is threatened by infections from various bacteria, fungus, viruses and also from other insect species such as parasitoid wasps, laying their eggs inside *Drosophila* larvae. In the beginning of the 1970’s Hans G Boman’s research team was first to demonstrate that *Drosophila* exhibited an inducible defense system towards microorganisms (Boman et al., 1972). This response was later shown to encompass both cellular and humoral defense reactions. The cellular reactions involve encapsulation of the infecting agents as well as phagocytosis. The humoral responses are manifested by the production of antimicrobial peptides (AMPs), clot formation and melanization. For reviews see for example (Hoffmann et al., 1999; Tzou et al., 2002a). Below, I will discuss the various aspects of *Drosophila* immunity.

**First line of defense**

The main habitat for *Drosophila* is decomposing fruit. Since this environment is plentiful with yeast and bacteria, it is important for the fly to be protected from infections in this hostile milieu. As a first line of defense the fly has its cuticle. The cuticle of the insect is a matrix of carbohydrates and proteins secreted from the monolayer of epidermal cells, which covers the entire surface of the insect, including respiratory trachea, the anterior and posterior portion of the digestive tract as well as the reproductive ducts (Neville, 1975). The main constituent of the cuticle is a nitrogenous polysaccharide named chitin, which gives the cuticle its rigid structure. The cuticle serves as an efficient physical barrier to block microbes to penetrate and infect the fly (Schaefer et al., 1987; Wigglesworth, 1972). In addition to the physical barrier, there are also chemical barriers preventing microbes from infecting the fly. In barrier epithelia, constitutively expression of antimicrobial substances functions to limit infection of the fly. If a microbe succeeds to pass the physical and chemical barriers, however, the immune system is activated.
Cellular responses

Blood cells; phagocytosis and encapsulation

Insects possess an open circulatory system that contains hemolymph, the insect equivalent of blood. An organ called the dorsal vessel drives the circulation of the hemolymph [reviewed (Rizki, 1978)]. *Drosophila* larvae and adults contain a few thousand blood cells, which can be divided into three types on the basis of structural and functional properties:

1. **Plasmatocytes** are small rounded cells with phagocytic capacity and the most abundant cell type in the hemolymph. Phagocytosis is an evolutionary conserved process. In *Drosophila*, phagocytosis of microbial pathogens and apoptotic cells has been proposed to be dependent on a number of receptors including PGRP-LC, PGRP-SC1a, croquemort and the transmembrane protein Eater (Franc *et al*., 1996; Garver *et al*., 2006; Kocks *et al*., 2005; Rämet *et al*., 2002b). Phagocytosis of bacteria is an important feature of immunity in *Drosophila*. In *domino* mutant larvae, which are devoid of blood cells (Braun *et al*., 1997), the hemocoele contains numerous live bacteria, demonstrating that phagocytosis is important in non-immunized flies in order to keep the hemolymph free from microbes and to avoid infection (Braun *et al*., 1998).

2. **Lamellocytes** are large flat cells, required for encapsulation of large foreign matters e.g. parasites. Lamellocytes are normally not found in circulation, but are differentiated from plasmatocytes upon a wasp infection (Meister, 2004). Encapsulation is a defense mechanisms against parasitic wasps depositing their eggs in the hemolymph of *Drosophila* larvae. The process of encapsulation is poorly understood. However, a picture is starting to emerge concerning the molecular mechanisms underlying lamellocyte-activation and the subsequent encapsulation. Recently, the Collier protein was shown to be important for the formation of lamellocytes after a wasp infection (Crozatier *et al*., 2004). Additionally, an integrin has been shown to be required for proper encapsulation (Irving *et al*., 2005).

3. **Crystal cells** are small cells carrying components required for the phenoloxidase (PPO) cascade. These cells constitute only about 5% of the cell population in the hemolymph. The PPO cascade will be discussed in greater detail below.
The classification of blood cells and their role in immunity is reviewed in (Lavine and Strand, 2002; Meister, 2004; Rizki, 1978; Rizki and Rizki, 1984).
Humoral responses

Coagulation and melanization

If wounded, it is important for *Drosophila* to quickly seal the lesion to avoid loss of body fluid and inhibit pathogens to enter the body cavity. Two systems work together in the formation of a clot: the coagulation system and the prophenoloxidase, PPO, activating cascade (Theopold *et al*., 2002). A first response to wounding in insects is hemolymph coagulation and initially, a soft clot is produced containing various proteins including lectins and ruptured blood cells. The soft clot is later hardened by precursors of melanin (derivates of tyrosine). Melanin is a product of the PPO activating cascade (Bidla *et al*., 2005; Li *et al*., 2002; Scherfer *et al*., 2004). Underneath the clot, epidermal cells are migrating to permanently seal the wound and the clot serves as a guide for proper cell migration (Galko and Krasnow, 2004).

The PPO activating cascade, results in the activation of phenoloxidase (PO) and the subsequent formation of melanin. The PPO activating cascade is triggered as a response to non-self, pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997b), by pattern recognition receptors (PRRs). PPO activation is reviewed in (Söderhäll and Cerenius, 1998; Theopold *et al*., 2002). The actual wounding likely also activates PPO, where damaged basal lamina is an activating signal (Gawa Bidla, personal communication). Melanization is, in addition to wound healing, also associated with encapsulation of parasites and microbes (Söderhäll and Cerenius, 1998). Intermediary compounds formed by the activated PPO cascade, quinones, are toxic to microorganisms, likely resulting in the killing of melanin-encapsulated microbes (Söderhäll and Cerenius, 1998). However, the direct killing of microbes by quinones is debated. One paper was recently published showing that absence of the PPO activating cascade do not make flies more susceptible to infection (Leclerc *et al*., 2006) and it has also been shown that bacteria trapped in a clot are not killed in a PPO dependent manner (Bidla *et al*., 2005).

The PPO cascade is a rapid and powerful system and to avoid self-destruction, inhibition is needed. Multiple inhibitors with specificity for various proteases in the PPO cascade have been identified. For example one serine proteinase inhibitor protein, Serpin-27A, has been shown to
inhibit the terminal protease PPO activating enzyme (De Gregorio et al., 2002). POs can also aggregate with other proteins and attach to surfaces nearby the wound in order to restrict the site of activation, protecting the insect from excessive melanization (Kanost, 1999).

**Antimicrobial peptides, systemic response**

A hallmark of the humoral responses in *Drosophila* is production of antimicrobial peptides (AMPs) in the fat body, a functional equivalent of the mammalian liver. Upon a systemic infection, AMPs are induced and synthesized in the fat body and secreted into the hemolymph where the invading microorganisms are effectively defeated. Fat body cells are found dispersed throughout the body in all life cycle stages of *Drosophila*. This makes the fat body well suited for providing a rapid increase in concentration of AMPs in the whole hemolymph, reviewed in (Engström, 1999; Hultmark, 1993). The induction of AMPs is fast and the peptides can be found in the hemolymph as soon as 2 to 4 hours after infection (Meister et al., 1997). AMPs can also be produced in epithelial tissues (Önfelt Tingvall et al., 2001; Tzou et al., 2000) and to some extent in hemocytes (Samakovlis et al., 1990). The induction of AMP genes is regulated at the level of transcription (Engström, 1999). Two signaling pathways have been identified as crucial for AMP gene expression, the Toll and the Imd pathways. In addition, the involvement of a third pathway, the JNK pathway, has also recently been shown.

The first AMPs were purified and characterized from insects in the beginning of the 1980s (Hultmark et al., 1980; Steiner et al., 1981) and today hundreds of AMPs have been isolated from insects. These small peptides are usually basic in character and are typically 20-40 amino acid residues. The peptides are effective in micro-molar concentrations, demonstrating that they are very potent [reviewed in (Otvos, 2000)]. The importance of AMPs for protection against microbial infections has been shown by ectopically expressed AMP genes in AMP-deficient flies, resulting in increased survival after infection (Tzou et al., 2002b). Although the exact mechanism of microbial destruction is not elucidated, AMPs act by interacting with the microbial cell membranes, causing stasis or lysis of the target microorganism. It is unlikely that the diverse group of AMPs has one single mechanism of action, but still disruption of membrane structures is believed to be an important feature for all the peptides (Epand and Vogel, 1999; Hancock and Scott, 2000; Lehrer and Ganz, 1999; Shai, 1999).

In *Drosophila*, seven different AMP families have been identified: Cecropin (Cec), Diptericin (Dpt), Defensin (Def), Attacin (Att), Drosocin
(Dro), Drosomycin (Drs) and Metchnikowin (Mtk). An additional AMP has been isolated from male *Drosophila*, Andropin (Anp), which is found in the reproductive organs (Samakovlis et al., 1991). For reviews see for example (Bulet et al., 1999; Hoffmann and Reichhart, 1997; Meister et al., 1997).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Number of genes expressed in <em>Drosophila melanogaster</em></th>
<th>Main biological activity</th>
</tr>
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<tbody>
<tr>
<td>Cecropin</td>
<td>4+2 pseudogenes (Kylsten et al., 1990; Tryselius et al., 1992)</td>
<td>G- and G+ bacteria (Samakovlis et al., 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fungi (DeLucca et al., 1997; Ekengren and Hultmark, 1999)</td>
</tr>
<tr>
<td>Dipterin</td>
<td>2 (Hedengren et al., 2000; Wicker et al., 1990)</td>
<td>G- bacteria</td>
</tr>
<tr>
<td>Defensin</td>
<td>1</td>
<td>Mainly G+ bacteria, by formation of voltage dependent channels (Cociancich et al., 1993; Matsuyama and Natori, 1988)</td>
</tr>
<tr>
<td>Attacin</td>
<td>4 (Åsling et al., 1995; Dushay et al., 2000; Hedengren et al., 2000)</td>
<td>G- bacteria</td>
</tr>
<tr>
<td>Drosocin</td>
<td>1 (Charlet et al., 1996)</td>
<td>G- bacteria (Bulet et al., 1993)</td>
</tr>
<tr>
<td>Drosomycin</td>
<td>7</td>
<td>Fungi (Fehlbaum et al., 1994)</td>
</tr>
<tr>
<td>Metchnikowin</td>
<td>1</td>
<td>G+ bacteria and fungi (Levashina et al., 1995)</td>
</tr>
</tbody>
</table>

Table 1. Antimicrobial peptides in *Drosophila melanogaster*. 
Antimicrobial peptides, local responses

As previously outlined AMPs can be produced in epithelial tissues. The epithelial AMP expression is referred to as local immunity. Local immunity can further be divided into two different types, induced or constitutive. The locally induced expression of AMPs is most likely a result of bacteria (or microbial elicitors) being in direct contact with surface epithelia and thereby promoting expression of AMPs. The expression of AMPs as a response to systemic infections has mainly been studied through the injection of bacterial suspensions into the body cavity of *Drosophila*. In order to study the expression of AMPs as a response to local infection, other infection methods have been employed, for example feeding of infectious bacteria and abrasion of the cuticle before exposure to bacterial suspension. Most surface epithelia produce at least one AMP upon local infection (Tzou et al., 2000). For example, after oral infection with bacteria *Drs* is expressed in trachea and *Dpt* is expressed in the digestive tract and malphigian tubules (McGettigan et al., 2005). Another example is the inducible expression of *CecA1* in epidermal cells underlining the cuticle in larvae and the perivitelline membrane in embryos (Önfelt Tingvall et al., 2001; Tingvall et al., 2001). Regulation of the local production of AMPs will be discussed in greater detail below.

In addition, AMPs are constitutively expressed in epithelial tissues. In the ejaculatory duct there is expression of the male specific peptide *Anp* as well as *CecA1* (Ryu et al., 2004; Samakovlis et al., 1991) Paper IV). *Drs* is constitutively expressed in female sperm storage organs and in salivary glands (Ferrandon et al., 1998; Ryu et al., 2004; Tzou et al., 2000). It has recently been published that in addition to the production of AMP there are oxidant-mediated antimicrobial responses in epithelial tissues. Those responses are also important for the elimination of microbial pathogens (Ha et al., 2005a; Ha et al., 2005b). Insect epithelial tissues were previously thought to be passive barriers of infection but it is now clear that they take an active part in immune defense. Interestingly, AMP production is found in mammalian epithelial tissues as well, where a systemic AMP response has not been identified (Bulet et al., 2004; De Smet and Contreras, 2005; Harder and Schroder, 2005; Selsted and Ouellette, 2005).

This might indicate that the innate immune system initially evolved in epithelial cells or possibly that the systemic responses have been down-regulated in the vertebrate lineage and diminished during the time of evolution.

Antimicrobial peptides, non-immune responses

In addition to induction by infection, the AMPs can also be induced by environmental and physical changes to the fly. Those responses are out of the
scope of this thesis, and will just be mentioned briefly. For example, the expression of AMP genes has been shown to vary in response to circadian rhythms (McDonald and Rosbash, 2001), aging (Zerofsky et al., 2005) and mating (Lawniczak and Begun, 2004; McGraw et al., 2004; Peng et al., 2005). This is important to keep in mind when analyzing experiments monitoring AMP gene expression.

**Figure 1.** Schematic overview of the *Drosophila* immune system. The cuticle provides a physical barrier from infecting agents. A local production of AMP genes is activated when microbes are in contact with barrier epithelia. If wounded, the PPO cascade is activated as well as coagulation and wound healing. If microbes enter the body cavity, they are recognized and a systemic AMP production is induced in the fat body. In the hemolymph, microbes are phagocytosed by plasmatocytes.
Transcriptional regulation of antimicrobial peptides

Pattern recognition and receptors
In order to initiate an immune reaction an intruding microorganism must be recognized as non-self. The current view is that receptors can recognize surface determinants (pathogen-associated molecular patterns, PAMPs) that remain invariant among microbes, but are not found within the host itself. Examples of PAMPs include structural molecules from the bacterial cell wall such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipoteichoic acid (LTA). Another example of a PAMP is β-1,3-glucan, which is a main component of the outer membranes of fungi (Medzhitov and Janeway, 1997b).

The PAMPs are recognized by germ-line encoded receptors (pattern recognition receptors, PRRs), which are found either circulating free in the hemolymph or on the surface of immune competent cells (Janeway, 1989; Medzhitov and Janeway, 1997b). After recognition of PAMPs, the receptors can stimulate immune responses by activating extracellular proteolytic cascades in the hemolymph and intracellular signaling pathways in immune-responsive tissue. It has been shown that Drosophila can distinguish different types of infection (Lemaitre et al., 1997). The discrimination between a Gram positive (G+) and Gram negative (G-) bacterial infections have recently been shown to rely on the recognition of specific forms of PGN (Leulier et al., 2003; Swaminathan et al., 2006). PGN is found, as a cell wall constituent, in both G+ and G- bacteria. However, the amino acid cross-linking the two stem peptides differs in PGN from G+ and G- bacteria. Generally, lysine is the cross-linking amino acid in G+ PGN, whereas meso-diaminopimelic acid (DAP) is the cross-linking amino acid in G- and some species of G+ bacterial PGN (Schleifer and Kandler, 1972). The different types of PGN will hereafter be referred to as Lys-type and DAP-type respectively. The molecular identities of recognition receptors in Drosophila are to a large extent unknown and so far two families of PRRs have been identified and characterized, the G- bacteria-binding proteins (DGNBPs) (Kim et al., 2000a).
and PGN recognition proteins (PGRPs) (Werner et al., 2000). DGNBP-1 was identified as a receptor for LPS and β-1,3-glucan, but studies on mutant DGNBP-1 have shown that the protein is important for the recognition of G+ bacterial infections (Gobert et al., 2003). DGNBP-1 will be discussed in greater detail below (The Toll pathway).

The Drosophila genome encodes at least thirteen distinct PGRP genes, which can be grouped into two classes: PGRP-S (short transcript) and PGRP-L (long transcript). The PGRP-S genes are predicted to encode extracellular proteins while the PGRP-L genes encode intracellular or membrane bound proteins. Several of the PGRPs are up-regulated after infection and the transcripts are found in immune-competent organs, such as the fat body and in hemocytes (Werner et al., 2000). In the process of characterizing the individual PGRPs two functional classes have emerged. One class is involved in microbial recognition and the subsequent activation of AMP genes and the other class has enzymatic activity (Mellroth et al., 2003) adjusting the effect of PGN in the immune responses. PGRPs are extensively reviewed in (Dziarski, 2004; Steiner, 2004). The characterized PGRPs and their role in immunity will be discussed in detail below (see The Toll pathway and The Imd pathway). The completion of the Drosophila genome project has greatly facilitated the search for pattern recognition receptors in the genome. Analysis of the Drosophila genome reveals a large number of genes with putative PAMP-recognition properties (Khush and Lemaitre, 2000). The molecular nature of recognition is just emerging and it will be interesting to follow what the future holds with respect to this area of innate immunity.

The Toll pathway

The Toll pathway is activated by Lys-type PGN and fungus

The Toll pathway was originally identified for its essential role in regulation of dorso-ventral polarity in developing Drosophila embryos (Anderson et al., 1985) and has later been shown to be a major regulator of the expression of AMPs (Lemaitre et al., 1996; Rosetto et al., 1995). The Toll pathway is homologous to NF-κB signaling in mammals (Wasserman, 1993).

G+ bacteria (Lys-type PGN) and fungi activate the Toll pathway and the antifungal peptide Drs is strongly expressed by the activation of this pathway. Toll-deficient flies are susceptible to fungal and G+ bacterial infections, but not to G- bacterial infection. (Lemaitre et al., 1996; Rutschmann et al., 2000a; Rutschmann et al., 2002)
Pattern recognition and extracellular signaling

As mentioned above, the Toll signaling pathway is activated by Lys-type PGN (G+ bacteria) and fungi, the two microbes are sensed by different mechanisms as illustrated by different mutants acting upstream of the transmembrane receptor Toll. G+ infections are identified in the hemolymph by at least four proteins, PGRP-SA (semelweiss) (Chang et al., 2004; Michel et al., 2001; Reiser et al., 2004), PGRP-SD (Bischoff et al., 2004), PGRP-SC1a (Garver et al., 2006) and DGNBP1 (osiris) (Gobert et al., 2003; Kim et al., 2000a; Pili-Floury et al., 2004). In addition to its role in recognition of G+ bacteria and the subsequent Toll signaling, PGRP-SC1a also harbors enzymatic activity important for phagocytosis of the G+ bacteria Staphylococcus aureus (Garver et al., 2006). Toll pathway activation, as a response to fungal infections is not impaired in either PGRP-SA, -SD or GNBP1 mutants indicating that there is another mechanism for activation as a response to fungal infections. Indeed, a mutation in the blood serine protease Persephone (Phs) leads to impaired induction of the Toll pathway as a response to fungal but not G+ bacterial infections. However, the receptor recognizing fungi has so far not been identified (Ligoxygakis et al., 2002b). Recognition of microbial substances in the hemolymph initiates an extracellular serine protease cascade, resulting in the subsequent cleavage and activation of the Toll ligand, Spätzle.

Spätzle is the only confirmed Toll-ligand in Drosophila and it has been demonstrated that the cleaved form of Spätzle directly interacts with the Toll receptor, resulting in subsequent signaling (Weber et al., 2003 and Gay, 2005 #313). Spätzle activates the Toll signaling pathway in both development and immunity (Lemaitre et al., 1996; Morisato and Anderson, 1994). The proteolytic cascade resulting in the cleavage and activation of Spätzle has been determined in detail in the developing embryo (LeMosy et al., 1999). However, genetic analysis have shown that the genes encoding the zymogens of the embryonic cascade (easter, snake, and gastrulation defective) are dispensable for the induction of a Toll-dependent immune response by septic injury (Lemaitre et al., 1996).

The protease cascade(s) needed for activation of Spätzle in response to infection is up to this point not fully elucidated. However, three proteins have been shown to be important for the regulation of Spätzle in response to infection, Spn43Ac, Persephone and Spätzle processing enzyme (SPE). (Jang et al., 2006; Levashina et al., 1999; Ligoxygakis et al.) Spn43Ac is encoded by the necrotic (nec) gene (Green et al., 2000) and is a blood serine protease inhibitor of the serpin family. It has been shown to negatively regulate the activation of Spätzle, in response to infection (Levashina et al., 1999). Persephone was identified as a suppressor of Spn43Ac and biochemical studies of Spn43Ac shows that it is likely to inhibit Persephone in vivo (Robertson et al., 2003). However, Persephone
is not the sole serine protease involved in Spätzle activation, since it is only involved in the response to fungal infection. Recently, a Spätzle processing enzyme (SPE), responsive to both fungal and G+ infections was identified (Jang et al., 2006). Moreover, in a large scale in vivo RNAi screen three additional Spätzle activating serine proteases were identified responding to both types of infection (Kambris et al., 2006). The identification of SPE and the three novel serine proteases indicates that the different mechanisms of sensing G+ and fungal infections have a common way of activating Spätzle.

Intracellular signaling

Toll is a transmembrane receptor (Hashimoto et al., 1988), its extracellular domain contains leucine-rich repeats and its intracytoplasmic region shows significant homology to the interleukin-1 receptor (IL-1R) and is referred to as the Toll-IL-1R (TIR) domain (Gay and Keith, 1991; Schneider et al., 1991). The signal from the activated Toll receptor is propagated through the cytoplasm via two adapter proteins, dMyD88 (a homologue of the human MyD88) (Charatsi et al., 2003; Horng and Medzhitov, 2001) and Tube (Letsou et al., 1991). dMyD88 interacts with the Toll receptor via a TIR domain (Tauszig-Delamasure et al., 2002) and Tube, in turn, interacts directly with MyD88 via a death domain (Sun et al., 2002). A serine/threonine kinase, Pelle (Shelton and Wasserman, 1993), is then recruited to the plasma membrane by Tube. Tube and Pelle form a heterodimer through their death domains. The heterodimerization of Tube and Pelle has been shown to be crucial for a subsequent phosphorylation cascade, which results in the phosphorylation and degradation of the inhibitory protein Cactus (Xiao et al., 1999). Cactus is not a direct target of Pelle phosphorylation and the kinase responsible for that reaction is currently not known (Shen and Manley, 1998). However, two proteins have been proposed to interact with Pelle, dTRAF2 and Pellino, possibly involved in the downstream signaling to Cactus (Grosshans et al., 1999; Shen et al., 2001). The protein Cactus is an IκB homologue (Geisler et al., 1992), which sequesters a dimer of the Rel protein Dorsal in the cytoplasm via ankyrin repeats in the C-terminus (Kidd, 1992). Upon phosphorylation, Cactus is broken down and Dorsal can then enter the nucleus and activate transcription (Gillespie and Wasserman, 1994; Whalen and Steward, 1993). Cactus expression is up-regulated upon infection and this is a plausible mechanism for shutting off the immune response after an infection (Nicolas et al., 1998).

For dorso-ventral patterning, Dorsal is the Rel protein activating transcription (Rushlow et al., 1989; Steward, 1987). In an immune reaction, however, another Rel protein, Dif (Ip et al., 1993), is the predominant target for Toll signaling (Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000a). Dorsal seems, however, to be involved in im-
munity as well. After an immune challenge, both Dif and Dorsal undergo rapid translocation to the nucleus (Ip et al., 1993; Petersen et al., 1995; Reichhart et al., 1993) and it has been shown that Dif and Dorsal can heterodimerize in vitro (Gross et al., 1996). As with Dorsal, Dif is also sequestered in the cytoplasm by Cactus. This interaction has been shown both in vitro and in vivo (Lehming et al., 1995; Stein et al., 1998; Tatei and Levine, 1995; Wu and Anderson, 1998). An inhibitor of Dorsal has been recently identified, wnt inhibitor of Dorsal (WntD). The Toll pathway regulates the expression of WntD and WntD is most likely involved in the termination of the Toll signaling as a response to infection as well as functioning as an inhibitor of Dorsal during embryogenesis (Ganguly et al., 2005; Gordon et al., 2005).

**Figure 2.** Schematic picture of the Toll pathway. The dotted circle around PGRP-SC1a, PGRP-SA/SA and DGBP1 indicates involvement in the recognition of G+ bacteria (Lys-type PGN) and not that they are found in a complex. PGRP-SC1a is also involved in phagocytosis of *Staphylococcus aureus* (G+). The result of the signaling is the translocation of the Rel proteins Dorsal and Dif into the nucleus and the subsequent transcription of target genes such as AMP genes.
Toll signaling in hemocyte proliferation

In addition to several developmental functions and the regulation of the antifungal response, the Toll pathway is also required for proper hemocyte proliferation. In gain-of-function Toll mutants the blood cell number is increased whereas in loss-of-function mutants the blood cell number is decreased (Lemaitre et al., 1995b; Qiu et al., 1998). Furthermore, Dif and Dorsal have been found to be negatively regulated by the lesswright (lwr) protein, a SUMOylating (small ubiquitin-related modifier) protein, in hematopoiesis. Consistent with the phenotype observed in Toll gain-of-function mutations there is over-proliferation of hemocytes in an lwr mutant (Huang et al., 2005).

Toll receptor homologues

Besides Toll, eight other Toll-like receptors have been identified in Drosophila, Toll-2 or 18-wheeler and Toll 3-9 (Tauszig et al., 2000; Williams et al., 1997). Their putative role in immunity is elusive. 18-wheeler is important for the expression of AMPs in the larval fat body, but is dispensable in the adult fly. Those results indicate that there is differential regulation of the immune systems at different developmental stages of Drosophila (Ligoxygakis et al., 2002a; Williams et al., 1997). Toll-9 is promoting high levels of drs expression in vitro and is thought be important for the constitutive expression of drs in larvae and flies (Ooi et al., 2002). Toll-5 has been shown to activate drs in vitro, but its function in vivo is still elusive (Tauszig et al., 2000). The role of the other Toll receptors in immunity has to be further investigated. Analysis of the expression pattern of Toll 3-9 in embryonic development suggests that many of the Tolls have a role in development (Kambris et al., 2002). Recently, 18-wheeler was shown to play a role in follicle cell migration and mutants showed morphological effects on the shape of the egg shape (Kleve et al., 2006).

Mammalian Toll signaling

Proteins with strong homology to the Drosophila Toll receptor have been identified in mice and humans. The proteins are called Toll-like receptors (TLRs) (Medzhitov et al., 1997). So far, eleven TLRs have been identified and the receptors sense a large spectrum of microbial patterns to mount a fast response to infection of various pathogens. The TLR interact with adaptor proteins (MyD88, TIRAP, TRAM or TRIF) essential to one or more individual TLR, to propagate the signal resulting in transcription of immune response genes. In contrast to the Drosophila Toll, the mammalian TLRs are pattern recognition receptors, for example PGN can bind to and activate TLR2 (Takeuchi et al., 1999), LPS is recognized by TLR4 (Poltorak et al.,
1998; Poltorak et al., 2000), double-stranded viral RNA is signaling through TLR3 (Alexopoulou et al., 2001) and TLR9 can detect bacterial DNA (Hemmi et al., 2000). Another discrepancy between the Drosophila and the mammalian Toll signaling is that in Drosophila, Toll has a dual role in immunity and development whereas no mammalian TLR has been shown to have a role in development. Toll signaling in mammals is reviewed in for example (Aderem and Ulevitch, 2000; Akira et al., 2001; Heine and Ulmer, 2005; Kimbrell and Beutler, 2001; Kopp and Medzhitov, 1999; Medzhitov and Janeway, 2000; Schnare et al., 2006; Takeda et al., 2003). Even though there are differences between the two systems, the similarities are striking and use of Toll pathways in innate immunity in both Drosophila and mammals is likely to reflect a common evolutionary origin. At this point it is too early to verify the evolutionary origin of Toll signaling, if it has evolved from one common ancestor or if the appearance and utilization are independent events in different evolutionary divergent species. In a recent paper the isolation and characterization of NF-κB and IκB homologues from a horseshoe crab, Carcinoscorpius rotundicauda, was presented. The horseshoe crab is the most ancient arthropod and the CrNF-κB and CrIκB homologues were shown to function in the immune responses and thus, implying that immune reactions, signaling through NF-κB molecules, are a conserved feature through evolution (Wang et al., 2006b).

The IMD pathway

The IMD pathway is induced by DAP type PGN

In contrast to the Toll pathway the Imd pathway has not been shown to play a role in development, however, it shows similarities to the human tumor-necrosis factor (TNF) receptor pathway. The Imd pathway is mainly activated by DAP type PGN (G- bacteria and a few G+ bacteria). Flies mutant for Imd pathway components are highly susceptible to G- infection but remain resistant to fungal and G+ infection (Lemaitre et al., 1995a). The Imd pathway is mainly regulating the expression of Dpt and Dro, whereas Cec, Def, Att and Mtk gene induction require an additional contribution from the Toll signaling pathway (Hedengren et al., 1999; Lemaitre et al., 1995a; Levashina et al., 1998).

Pattern recognition

Although abundant in G- bacteria, LPS does not seem to be an elicitor of the Imd pathway, as initially believed (Kaneko et al., 2004). Instead a PGRP, PGRP-LC, has been proven important for recognition of G- bacteria (DAP
type PGN) and subsequent signaling via the Imd pathway. PGRP-LC mutant flies are impaired in their synthesis of AMP after G- infection and therefore susceptible to G- infection (Choe et al., 2002; Gottar et al., 2002). PGRP-LC has been shown to bind PGN in vitro (Mellroth et al., 2005). It has been elusive to determine how the signal is propagated from PGRP-LC to an activated Imd pathway. However, it has recently been shown that PGRP-LC interacts directly with Imd to propagate the signal. (Choe et al., 2005). PGRP-LC has also been shown to be of importance for phagocytosis of G-, but not G+ bacteria (Rämet et al., 2002b).

PGRP-LC does not confer full activation of the Imd pathway, DAP type PGN is also recognized by PGRP-LE and it has been suggested that PGRP-LC and –LE work synergistically to activate the Imd pathway. In addition, PGRP-LE also activates the PPO cascade resulting in melanization (Takehana et al., 2002; Takehana et al., 2004).

Intracellular signaling

When a G- bacterial infection is recognized the signal from PGRP-LC is propagated to the intracellular Imd protein (Choe et al., 2005). The Imd gene has been cloned and the gene product is a 30 kDa protein containing a death domain similar to that of human tumor necrosis factor-α (TNF-α) receptor-interacting protein (RIP) (Georgel et al., 2001). The downstream signaling of the Imd pathway is complex. Activated Imd interacts with the adaptor protein dFADD and the caspase Dredd (Hu and Yang, 2000; Leulier et al., 2002; Naitza et al., 2002); (Elrod-Erickson et al., 2000; Leulier et al., 2000; Stöven et al., 2000). In parallel, activated Imd is also activating dTAK1, a Drosophila homologue of the mammalian MAP kinase kinase kinase (MAPKKK) TAK1 (Vidal et al., 2001). Activation of dTAK1 was recently shown to be dependent on an ubiquitin-ligase complex (Zhou et al., 2005). dTAK1 in turn confers a second branch point of the Imd pathway activating both the IKK complex and the JNK pathway (Silverman et al., 2003). The role of the JNK pathway in immunity will be discussed below. The IKK complex constitutes of two proteins dIKKβ (Ird5 or DLAK) (Kim et al., 2000b; Lu et al., 2001) and dIKKγ (Kenny) (Rutschmann et al., 2000b) and is important for signal dependent phosphorylation of Relish (Silverman et al., 2000). Imd pathway activation results in the translocation of the Rel protein Relish (Dushay et al., 1996; Hedengren et al., 1999). In contrast to Dif and Dorsal, Relish is not sequestered in the cytoplasm by Cactus, but it carries its own inhibitory sequences in the C-terminal end of the protein. This part of the protein shows homology to Cactus and contains several ankyrin repeat domains (Dushay et al., 1996). To become activated, Relish therefore needs to be endoproteolytically cleaved. Upon cleavage, the N-terminal Rel-homology domain is translocated into the nucleus and pro-
motes transcription of effector genes, whereas the inhibitory domain remains in the cytoplasm. (Cornwell and Kirkpatrick, 2001; Stöven et al., 2000). The activation and cleavage of Relish is dependent of the IKK complex as well as Dredd (Stöven et al., 2003). Recently, another level of complexity was added to the regulation of the Imd pathway. In an RNAi screen two proteins involved in Imd signaling were identified, IAP2 and TAB. When the expression of the two proteins were down-regulated by RNAi, Relish was cleaved in a signal dependent manner but was blocked in its nuclear translocation (Gesellchen et al., 2005; Kleino et al., 2005). The nuclear pore complex (NPC) is important for the translocation of proteins into the nucleus. Mutations in subunits of the NPC have been isolated, which selectively abolishes the nuclear translocation of the Rel proteins. (Bhattacharya and Steward, 2002; Uv et al., 2000).

**Figure 3.** Schematic picture of the Imd pathway in *Drosophila*. G- bacteria (Dap-type PGN) are recognized by PGRP-LC and –LE. The signal is propagated in the cytoplasm and results in the cleavage and translocation of Relish, which leads to the expression of AMP genes.
Down-regulation of the Imd pathway

Although a vast amount of information about positive regulation of Imd pathway has been obtained, not much is known about how the pathway is kept silent in the absence of infection and how the signaling is down regulated after infection. One study has shown that fly mutants in SkpA, the Skp1 component of *Drosophila* SCF ubiquitin ligases, makes the Imd pathway constitutively active. This indicates that the proteasome might be needed for negative regulation of the Imd pathway (Khush et al., 2002). Dredd is the protein presumably responsible for the cleavage of Relish. An inhibitor of Dredd, Dnr1, has been identified and suggested to be involved in the termination of Imd pathway signaling (Foley and O'Farrell, 2004). Another protein involved in negative regulation of the Imd pathway is PGRP-LB. The Imd elicitor, Lys-type PGN, is degraded by PGRP-LB, which has amidase activity, resulting in down-regulation of the Imd pathway (Kim et al., 2003; Zaidman-Remy et al., 2006). There are three PGRP-SCs’, -SC1a, -SC1b and SC2 and when they are collectively down-regulated by RNAi the Imd signaling is sustained for a longer period of time. The PGRP-SCs’ also have amidase activity and the results are consistent with the PGRP-LB study (Bischoff et al., 2006). As mentioned above, a previous study has however shown that PGRP-SC1a is involved in Toll pathway signaling and phagocytosis of G+ bacteria (Garver et al., 2006). The discrepancy between the studies might be explained by the fact that Bischoff and co-workers down regulated the expression of all three PGRP-SCs collectively using RNAi and did not analyze their individual contribution on the immune reactions. Even though a lot of information is accumulating concerning negative regulation of the Imd pathway, more work is needed to get a comprehensible picture.

The Imd pathway in local activation of AMP genes

As stated above, the Toll pathway is regulating the expression of *Drs* in the fat body. In epithelial tissues, however, the situation seems to be different. The regulation of *Drs* expression in the respiratory tract in larvae and adults has been shown to be dependent on the Imd rather than Toll pathway (Ferrandon et al., 1998; Tzou et al., 2000). In *imd* mutant larvae, the tracheal expression of *Drs* was abolished after natural infection with *E. carotovora* (Tzou et al., 2000). The *Drs* expression in fat body was, however, not affected in Imd mutants after natural *E. carotovora* infection (Lemaitre et al., 1996). Likewise, the Imd pathway was shown to be important for the local expression of *CecA1* in the embryonic and larval epithelia underlying the cuticle (Önfelt Tingvall et al., 2001).
The Imd pathway and apoptosis

In addition to its role in regulating the expression of AMP genes, the Imd pathway is believed to play a role in apoptosis. The Dredd caspase, which is important for cleavage of Relish, was first identified as an effector of apoptosis (Chen et al., 1998). Similarly, dFADD was first identified for its role in apoptosis (Hu and Yang, 2000). This indicates that the regulation of AMPs and apoptosis share certain proteins. It has also been shown that over-expression of Imd in flies can promote apoptosis and in particular induce the expression of the pro-apoptotic reaper gene (Georgel et al., 2001). Over-activation of the Imd pathway has deleterious effects on larval development and shows increased larval mortality after infection (Bischoff et al., 2006).

The JNK pathway

Jun N-terminal kinase (JNK) signaling has been implicated in diverse biological processes, such as stress responses, cell shape, apoptosis and immune responses, reviewed in for example (Goberdhan and Wilson, 1998). The JNK pathway is activated by various stimuli including LPS (Boutros et al., 2002; Sluss et al., 1996). As mentioned above dTAK1 has been suggested to have a dual role in activating the IKK complex and the JNK signaling pathway (Silverman et al., 2003). dTAK1 has previously been shown to function as a MAPKKK in the JNK pathway regulating cell shape and development (Mihaly et al., 2001; Takatsu et al., 2000). The model of dTAK1 having its function upstream of both the IKK complex and JNK is challenged by our findings, presented in Paper II. We show that Relish cleavage, nuclear localization and promoter binding is not impaired in a dTAK1 mutant. Thus, activation of AMP genes by Relish does not require dTAK1 (Paper II). Support for these findings has already been published in a paper by Anni Kleino and co-workers, where they showed that nuclear translocation of Relish is intact after down-regulating the expression of dTAK1 using RNAi (Kleino et al., 2005). In Paper II, we suggest that the function of dTAK1 in JNK signaling is required in parallel to Imd/Relish signaling to promote expression of AMP genes. The role of JNK signaling in transcriptional regulation of the AMP genes was proven by analysis of larval fat body clones mutant for JNK pathway components, showed severely decreased AMP expression. The AMP expression was also inhibited by over-expression of the JNK repressor Puckered (Paper II). These results are contradictory to previous publications though (Boutros et al., 2002; Kim et al., 2005; Park et al., 2004; Silverman et al., 2003). Those latter studies were done in vitro in S2 cells and might reflect a difference in regulation between blood cells and the systemic response in the fat body. Intriguingly, in a recent study,
RNAi towards JNK pathway components in S2 cells greatly reduced the expression of Atf, supporting the involvement of JNK signaling in transcriptional regulation of AMP genes (Kallio et al., 2005). The role of the JNK pathway in immunity is not restricted to transcription of AMP genes, it has also been shown to be important for efficient wound healing and cytoskeletal rearrangements after wounding (Boutros et al., 2002; Rämet et al., 2002a). More research is needed to fully explain the role of JNK signaling in immunity in general and in transcriptional activation of AMP genes specifically.

The JAK/STAT pathway

The Toll, Imd and JNK pathways are involved in the regulation of the transcriptional activation of AMP genes. However, they are not the only pathways involved in immune reactions in Drosophila. The Janus kinase/signal transducers and activators of transcription, JAK/STAT, pathway has, in addition to other processes, been postulated to also regulate immunity. The JAK/STAT pathway was first identified in mammals where it plays a critical role in hematopoesis and immune responses [reviewed in (Darnell, 1997)]. Four main components of the pathway have been identified in Drosophila melanogaster, the ligand unpaired (Udp) (Harrison et al., 1998), the receptor domeless (Dome) (Brown et al., 2001; Chen et al., 2002), one JAK, hopscotch (hop) (Binari and Perrimon, 1994; Perrimon and Mahowald, 1986), and one STAT, STAT 92E/Marelle (Hou et al., 1996; Yan et al., 1996). The JAK/STAT pathway is involved in multiple developmental events such as patterning of the embryo, wing formation and eye development. Another process, which, at least in part, is regulated by the JAK/STAT pathway in Drosophila, is the larval hematopoesis and the subsequent formation of plasmatocytes. Over-stimulation of the JAK/STAT pathway also results in an infection-independent proliferation of lamellocytes. The role of the JAK/STAT pathway is reviewed extensively in (Zeidler et al., 2000) and its role in immunity in (Agaisse and Perrimon, 2004). Larval hemolymph isolated from a gain-of-function mutant of hopscotch (hop^Tum^) contains substances, which enhances phagocytosis of yeast particles (Åsa Holm, unpublished results). This suggests that the JAK/STAT pathway is regulating the production of substances capable of opsonizing the prey. One candidate protein involved in opsonization is TEP-1 and its up-regulation is dependent on the Drosophila JAK protein hopscotch (Lagueux et al., 2000). This hypothesis is strengthened by a previous study in the mosquito Anopheles gambiae. The homologous protein, AgTEP-1, has been shown to bind and opsonize G-bacteria, resulting in phagocytosis of ditto (Levashina et al., 2001). The JAK/STAT pathway has also been shown to be involved in the systemic response to infection via activation of the cytokine-like factor Upd3 (Agaisse
et al., 2003). Recently it was also shown that the JAK/STAT pathway, at least in part, is regulating the immune responses elicited by viruses in *Drosophila* (Dostert et al., 2005). Taken together, although not involved in the transcriptional regulation of AMP genes, the JAK/STAT pathway plays a role in regulation of the *Drosophila* immune reactions, directly or indirectly.
Transcription factors

Rel proteins

In *Drosophila*, Rel proteins are known to activate transcription via κB motifs (Engström *et al*., 1993; Han and Ip, 1999; Kappler *et al*., 1993; Petersen *et al*., 1995). The three different *Drosophila* Rel proteins, Dorsal, Dif and Relish, have been described above. The Toll pathway regulates the nuclear translocation of Dorsal and Dif whereas the Imd pathway regulates Relish translocation. Other signal transduction pathways might modify the Rel proteins and thereby affecting their ability to form dimers, bind DNA and activate transcription etc. Rel proteins have been shown to specifically interact with the general transcription machinery. For example, the dTRAP80 mediator module has been shown to specifically interact with Dif and promote expression of *Drs* (Kim and Lis, 2005; Park *et al*., 2003). Additionally, a TATA-binding protein interacting helicase, Helicase89B, has also been identified for its role in specifically activating Rel target genes in *Drosophila* (Yagi and Ip, 2005). Thus, there is a link between the general transcription machinery and specific transcription of particular genes.

The Rel proteins show homology in the so-called Rel homology domain (RHD), which is important for dimerization and DNA binding. The C-terminal domain is varying and it is important for transcriptional activation. No further members of the Rel protein family have been identified even after the completion of the *Drosophila* genome-sequencing project (see: http://flybase.bio.indiana.edu/).

The different Rel proteins can form heterodimers in all various combinations in vitro and over-expression of various combinations of Dif, Dorsal and Relish results in differential levels of expression of the different AMP genes in a *Drosophila* cell line (Han and Ip, 1999). The differential expression of AMPs as a response to various stimuli during systemic infections is probably due to differences in concentration of the three Rel proteins.
The GATA factor Serpent

Many AMP genes have been shown to also harbor GATA motifs in their proximal promoters (Kadalayil et al., 1997; Senger et al., 2004). The GATA motif has been investigated for its role in immunity and in the larval fat body the GATA factor Serpent is indeed needed for expression of CecA1 (Petersen et al., 1999). Analysis of the Mtk promoter has similarly shown that the GATA motif is important for expression of the peptide (Senger et al., 2004). Serpent is constitutively nuclear and is thought to prime the gene for activation by Rel proteins. In embryos, Serpent has been shown to be crucial for expression of CecA1 in yolk, but dispensable for the local expression of CecA1 in epidermis (Tingvall et al., 2001). Since GATA motifs are found in the promoter region of other AMP genes, it is likely that this motif serves a similar function in the regulation of these genes.

Homeodomain proteins

Homeodomain proteins are key regulators in embryonic development and important for the differentiation of specific tissues (Gehring et al., 1994; Morgan, 2006). Recently, the homeodomain protein Caudal (Cad) was shown to be important for the constitutive expression of AMP genes. More specifically, Cad was shown to be important for the constitutive expression of CecA1 and Drs in the male ejaculatory duct and in the salivary glands respectively. Not surprisingly, the regulation was independent of Relish, as Relish is not activated in uninfected flies. This suggests a novel type of regulation of constitutively expressed AMPs compared to the locally induced expression (Ferrandon et al., 1998; Ryu et al., 2004; Tzou et al., 2000). Studies from our lab have identified another protein, the POU protein Drifter (Dfr), to also be involved in the constitutive CecA1 expression in the male ejaculatory duct (Paper IV). The POU-proteins belong to a subclass of the family of homeodomain proteins and in Drosophila five POU proteins have been characterized. POU proteins are characterized by an unique bi-partite DNA-binding domain, referred to as POU-domain first described in the mammalian transcription factors Pit1, Oct1/Oct2 and the Caenorhabditis elegans developmental control gene Unc-86 (Herr and Cleary, 1995) The bi-partite DNA-binding domain makes DNA binding very flexible and it is difficult to predict binding sites for POU proteins. Different POU genes exhibit distinct expression patterns varying from cell type-specific to ubiquitous.

Dfr and Cad were shown to mediate CecA1 expression via the same promoter region, suggesting a tissue specific enhancer functioning for the constitutive CecA1 expression in the male ejaculatory duct (Ryu et al.,
2004) and Paper IV). Such a tissue-specific enhancer, utilized for constitutive AMP expression, has not been previously shown.

Another POU protein that has been shown to be involved in transcriptional regulation of AMP genes is POU protein-1 (Pdm1) (Paper V). Studies of Pdm-1 mutant flies suggest that it functions as a transcriptional repressor for some genes and a transcriptional activator for others. In contrast to Dfr, Pdm1 seems to be involved in the systemic expression of AMP genes in response to an infection, rather than the constitutive expression of AMP genes (Paper V).
Defense against viral infections

All data concerning the regulation of the immune mechanisms discussed above comes from studies where different bacteria or fungus, or purified cell wall components from ditto, have been used as infecting agents. *Drosophila* is also susceptible to viral infections but not so much is known about the viral defense mechanisms. However, in recent years some information has accumulated concerning the viral defense mechanisms in *Drosophila* (Cherry et al., 2005; Cherry and Perrimon, 2004; Roxstrom-Lindquist et al., 2004; Sabatier et al., 2003). In sharp contrast to abdominal injection of bacteria and fungus, injection of virus into flies does not cause expression of AMPs. This indicates that the antiviral responses are different from the antibacterial and anti-fungal responses (Sabatier et al., 2003). The Toll pathway has however, been suggested to play a role in the antiviral defense, but it is rather the cellular responses evoked by the pathway than the production of AMPs that is involved in eliminating the infecting viruses (Zambon et al., 2005). In addition, the JAK/STAT pathway has been suggested to regulate the antiviral defense. The pathway is up-regulated as a response to viral infection, and the transcription factor Marelle is important for the transcription of virus-induced genes (Dostert et al., 2005). In plants, the RNAi machinery has been shown to play an important role in the elimination of viral infections (for a review see for example (Vance and Vaucheret, 2001). Recently it was shown, by two independent groups, that the RNAi machinery is involved also in *Drosophilas’* defense against viruses *in vivo* (Galiana-Arnoux et al., 2006; Wang et al., 2006a). Thus, antiviral defense, at least in part, may have common features in such diverge eukaryotes as plants and insects.
The present study

Aim

The general aim of this project was to study the regulation of innate immune responses in *Drosophila*. During my thesis study, I have participated in research projects including the analysis of a novel *cis*-acting element (Paper I) and signaling transduction pathways important for AMP gene expression (Paper II). Furthermore, I have been involved in the identification of transcription factors previously not implicated in immunity, and their role in the activation of AMP genes (Paper III-V).

Results and discussion

R1, a novel *cis*-regulatory element (Paper I)

The *Drosophila* Cec locus has been cloned and characterized (Kylsten *et al.*, 1990). It is a tight gene cluster, about 10 kb, covering the four Cec genes (*CecA1, CecA2, CecB* and *CecC*), as well as the male specific Andropin gene (Figure 4A). It has been shown that a 760 base pair (bp) region upstream of the *CecA1* transcription start site contains all the *cis*-acting elements necessary for inducible and fat body-specific expression (Engström *et al.*, 1993). In the proximal upstream promoter, a 40 bp region conserved among all the *Drosophila* Cec genes, has been identified (Tryselius *et al.*, 1992). This conserved region can be divided into three distinct motifs: Region 1 (R1), the κB motif and the GATA motif (Figure 4B).
As previously reported, a large number of genes encoding AMPs in insects contain clusters of κB and GATA sites (Kadalayil et al., 1997; Sengers et al., 2004). We conducted a similar search for clustered κB and R1 sites and found that in addition to the Cec genes the Def gene contain such a cluster, as well as three Cec genes of Drosophila virilis (Zhou et al., 1997). The Defensin and Cecropin peptides are functionally related, as they possess strong activity against G+ bacteria. The Cecropins are also active against G- bacteria and fungi. We decided to investigate the function of the κB-R1 cluster in the promoter of CecA1.

In reporter gene studies it has previously been shown that deleting the κB-R1 cluster from the CecA1 promoter abolished expression of the gene, both in cell cultures and in transgenic larvae and flies (Roos et al., 1998). We carried out site-directed mutagenesis separately on the κB and R1 motifs. After infection, CecA1 expression was reduced to less than half in both mutants compared to wild type. CecA1 expression was analyzed both in transient transfection experiments and in transgenic flies, using β-galactosidase as a reporter gene and similar results were obtained in the two systems. We conclude that κB and R1 act in concert to gain high levels of CecA1 gene expression and that neither the κB, nor the R1 site, is sufficient for promoting efficient CecA1 expression from an otherwise intact upstream region.
The R1-binding activity

Using electrophoretic mobility shift assays (EMSAs), we set out to isolate an R1-binding activity (RBA). We found one specific RBA in nuclear extracts from mbn-2 cells (a *Drosophila* hemocyte cell line) and wild type flies after infection. The RBA was inducible, as it was not observed in uninfected cells or flies. Yeast, G+ and G- bacteria, as well as PGN and crude LPS, induced the RBA, although with somewhat different kinetics. Up to this end we have not been able to identify the molecular composition of the RBA. In our attempts to discover the R1-binding proteins(s) we have used three different approaches: 1) Qualified guesses and analysis of candidate transcription factors from two different screens; 2) yeast one-hybrid screening and 3) protein purification and identification of the purified proteins using mass spectrometry. The latter project was done in collaboration with Olga Loseva, Stockholm University. The different approaches and the outcome will be discussed in greater detail below.

1. Qualified guesses

The R1 motif shows some sequence homology to κB motifs (Figure 4B), which are known to bind Rel proteins. Therefore, we first investigated if Rel proteins might be components of the RBA. The RBA was neither super-shifted using antibodies directed towards any of the three Rel proteins, nor was the binding abolished in nuclear extracts from *dif, dorsal* or *relish* mutant flies. Those results suggested that none of the Rel proteins are major components of the RBA (Uvell and Engström, 2003). The R1 site is also somewhat reminiscent of an Activator Protein-1 (AP-1) binding site. Jun/Fos heterodimers are known to bind and affect transcription via AP-1 binding sites. As the RBA is found in mbn-2 cells, RNAi towards an RBA component (R1-factor) should result in a decrease in RBA when nuclear extract is analyzed using EMSA. The *CecA1* expression should also be reduced since mutations in the R1 site dramatically decreases the expression of *CecA1* reporters both *in vitro* and *in vivo*. As a proof of concept we performed RNAi towards Relish and analyzed the cells both using EMSA and RT-PCR. As shown in Figure 5A the κB binding activity is dramatically decreased after Relish-RNAi and the *CecA1* expression after infection is much reduced after Relish-RNAi (Figure 5B).
Figure 5. κB-binding and CecA1 expression is reduced after Relish-RNAi. A κB-binding was analyzed before (-) and after (+) Relish RNAi in EMSA. The binding is strongly reduced as a result of the RNAi. Relish has previously been shown to bind to the κB-sequence used in the EMSA (Stöven et al., 2000). B RT-PCR analysis of CecA1 before (-) and after (+) Relish RNAi showed a decrease of CecA1 as a response to the RNAi. Rp49 is used as a loading control.

An RNAi approach was employed to investigate if dFos or dJun were part of the RBA. However, we could not prove either dFos or dJun to be components of the RBA. In collaboration with Edan Foley, we analyzed four transcription factors, CG8484, CG8506, CG9398 and CG15636, that had been isolated as activators of Diptericin in a large-scale RNAi screen (Foley and O'Farrell, 2004). Again, an RNAi approach was taken to investigate if any of the transcription factors were part of the RBA. Unfortunately, none of the proteins analyzed were shown to be components of the RBA and had only minor effects on CecA1 expression. In a parallel project, we conducted a yeast screen to identify regulators of CecA1 expression (Paper III) and I analyzed four putative transcription factors, CG1960, CG7785, CG8664 and CG9775 for their possible involvement in the R1-binding complex. Again, none of the transcription factors analyzed could be shown to be part of the RBA.

2. Yeast one-hybrid screen

Yeast one-hybrid screens have successfully been used to identify DNA-binding proteins. I constructed a pentamer of the R1 site and used this as bait to screen an adult Drosophila cDNA library for R1-binding proteins. To our
disappointment, none of the isolated cDNAs showed any protein domains reminiscent of transcription factors and were not further analyzed.

3. Protein purification

We then took a different approach to identify the RBA; we followed a protein purification protocol shown to be successful for purification of transcription factors (Yaneva and Tempst, 2003). In short, nuclear extract from mbn-2 cells were separated on a P11 phosphocellulose column using FPLC, the fractions were analyzed with EMSA and the RBA positive fractions were pooled and concentrated. The concentrated fractions were then subjected to positive and negative selection using oligos containing wild type and mutant R1 concatemers respectively. The purified proteins were separated using SDS-PAGE and the individual proteins were analyzed using mass spectrometry. Three putative candidate proteins were identified using this method: 14-3-3ε, Tudor-SN and ypsilon schachtel. The RNAi approach was taken in order to investigate if 14-3-3ε, Tudor-SN or ypsilon schachtel are binding to and promoting expression of CecA1 via the R1 site. Unfortunately, none of the isolated proteins were proven to be part of the RBA.

Next, in trying to identify the R1 binding factor, we took a slightly different approach. Nuclear extract from mbn-2 cells was fractionated using a P11 phosphocellulose column. Again, fractions containing the RBA were concentrated but this time the concentrated proteins were separated on a preparative EMSA. The RBA was cut out of the gel and subjected to mass spectrometry and protein sequencing. This method has previously been successfully employed for the identification of RNA binding proteins (Stenger et al., 2004). 26 proteins with putative DNA binding capacity (Table 2) were identified and we are in the process of testing these proteins for being part of the RBA. Again, we are using an RNAi approach in combination with EMSA and RT-PCR to identify any of the proteins to be the R1 binding factor.

<table>
<thead>
<tr>
<th>Gene name (CG#)</th>
<th>Molecular function (information from FlyBase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG8108</td>
<td>nucleic acid binding, zinc ion binding</td>
</tr>
<tr>
<td>lethal(2)k10201</td>
<td>nucleic acid binding, zinc ion binding</td>
</tr>
<tr>
<td>(CG 13951)</td>
<td></td>
</tr>
<tr>
<td>CG7518</td>
<td>DNA binding</td>
</tr>
<tr>
<td>CG5195</td>
<td>DNA binding, DNA-directed DNA polymerase activity, 3’-5’ exonuclease activity</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Molecular Function</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GATA (CG5034)</td>
<td>General RNA polymerase II transcription factor activity, transcription factor activity</td>
</tr>
<tr>
<td>Tudor-SN (CG7008)</td>
<td>Nucleic acid binding, transcription coactivator activity, nuclease activity</td>
</tr>
<tr>
<td>CG9273</td>
<td>DNA binding, single-stranded DNA binding</td>
</tr>
<tr>
<td>DDB1 (CG7769)</td>
<td>Damaged DNA binding, glyceraldehyd-3-phosphate dehydrogenase activity</td>
</tr>
<tr>
<td>RpA-70 (CG9633)</td>
<td>Single-stranded DNA binding</td>
</tr>
<tr>
<td>Karl (CG4139)</td>
<td>Molecular function unknown</td>
</tr>
<tr>
<td>Translin (CG11761)</td>
<td>DNA binding</td>
</tr>
<tr>
<td>Mutagen-sensitive-209 (CG9193)</td>
<td>DNA binding, nucleic acid binding, DNA polymerase processivity factor activity</td>
</tr>
<tr>
<td>CG13900</td>
<td>Poly(A) binding, damaged DNA binding</td>
</tr>
<tr>
<td>CG13597</td>
<td>Single-stranded DNA binding, nucleic acid binding, kinase activity</td>
</tr>
<tr>
<td>Translin associated factor X (CG5063)</td>
<td>Molecular function unknown</td>
</tr>
<tr>
<td>CG32473</td>
<td>Nucleic acid binding, zinc ion binding, membrane alanyl aminopeptidase activity, endonuclease activity, glutamyl aminopeptidase activity</td>
</tr>
<tr>
<td>CG10576</td>
<td>Nucleic acid binding, transcription regulatory activity, methionyl aminopeptidase activity</td>
</tr>
<tr>
<td>Modulo (CG2050)</td>
<td>DNA binding, mRNA binding, protein binding</td>
</tr>
<tr>
<td>CG8149</td>
<td>DNA binding</td>
</tr>
<tr>
<td>Suppressor of variegation 205 (CG8409)</td>
<td>Chromatin binding, mRNA binding, histone binding, transcriptional activator activity, transcriptional repressor activity, methylated histone residue binding</td>
</tr>
<tr>
<td>Ypsilon schachtel (CG5646)</td>
<td>DNA binding, mRNA binding, nucleic acid binding, transcription regulator activity</td>
</tr>
</tbody>
</table>
Table 2. List of the proteins identified by protein sequencing using tandem mass spectrometry. All proteins with putative DNA binding potential are listed. Frank Kjeldsen and Roman Zubarev, Uppsala University (BMMS) carried out the mass spectrometry and protein sequencing analysis. It remains to be proven if any of the proteins are part of the RBA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabeza (CG3606)</td>
<td>mRNA binding, general RNA polymerase II transcription factor activity</td>
</tr>
<tr>
<td>Belle (CG9748)</td>
<td>Nucleic acid binding, ATP binding, ATP-dependent helicase activity, ATP-dependent RNA helicase activity</td>
</tr>
<tr>
<td>Bancal (CG13425)</td>
<td>mRNA binding, transcription factor binding</td>
</tr>
<tr>
<td>CG7033</td>
<td>Nucleic acid binding, ATP binding, ATPase activity, ATP-dependent helicase activity</td>
</tr>
<tr>
<td>14-3-3ε (CG31196)</td>
<td>Transcription regulatory activity, diacylglycerol-activated phospholipids-dependent protein kinase C inhibitory activity, protein domain specific binding</td>
</tr>
</tbody>
</table>

**Conclusion**

In conclusion, our data strongly indicate that the R1 motif is a novel cis-acting element, which is required for expression of the CecA1 gene. We have ruled out the possibility of any of the Rel proteins, as well as dFos and dJun, to be part of the RBA.

Despite a considerable effort to identify the RBA we have not yet been successful. Hopefully, the RBA can be found among the proteins identified by mass spectrometry and protein sequencing (Table 2). When identified, a careful characterization of its function in transcriptional activation of AMP genes as well as its implication for the Drosophila immune system as a whole will be carried out.

**Future perspectives**

The primary goal for the continuation of this project is to isolate the transcription factor(s) binding and promoting transcription via the R1 site and investigate if the protein affects transcription of other genes beside CecA1. In the last decade, and especially after the sequencing and annotation of the Drosophila genome (Adams et al., 2000), a wealth of molecular and genetic tools has accumulated. For example, collections of complete cDNAs of virtually all genes are distributed by the Drosophila Genome Research Center.
(DGRC) and development of commercial shuttle vectors suitable for use in bacteria, yeast and *Drosophila* and for the expression of tagged proteins in *Drosophila* cell-lines, as well as in transgenic flies. Furthermore, large collections of fly mutants, nested deletions and P-element insertions (transposable elements) have been created, with targets in the majority of all genes. In addition, systems for over-expressing cDNAs and down-regulating genes by RNAi using tissue specific and inducible promoters enable manipulation of the expression of interesting genes. Consequently, once the identity of the R1-binding protein(s) has been determined many tools will be available to rapidly initiate an analysis of the function of the R1-protein in transcriptional regulation of AMP genes.

The role of dTAK1 in regulating AMP gene expression (Paper II)

The role of dTAK1 signaling in the transcriptional regulation of AMP genes has for a long time been enigmatic. In a collaboration with Joe Delaney in Marek Mlodzik group at the Mount Sinai School of Medicine, NY we set out to do an in depth investigation on the role of dTAK1 for the regulation of AMP genes. As mentioned above, dTAK1 has been suggested to activate both IKK and JNK signaling as a response to infection (Silverman *et al.*, 2003) and dTAK1 mutants show a severely reduced expression of Relish-dependent AMP genes (Vidal *et al.*, 2001).

In this study, several aspects of Relish activation appeared normal in infected dTAK1 mutant animals, including cleavage, nuclear localization and promoter-binding. In a recent paper from Mika Rämets group it was also reported that nuclear localization of Relish is unaffected in cells treated with dsRNA towards dTAK1 (Kleino *et al.*, 2005). Taken together, those results imply that dTAK1 is not important for IKK activation and the subsequent cleavage of Relish.

From previous reports, dTAK1 is known to function as a MAPKKK in JNK signaling (Igaki *et al.*, 2002; Mihaly *et al.*, 2001; Moreno *et al.*, 2002) and is activated via Imd as a response to infection (Vidal *et al.*, 2001). With the obvious connection to the JNK pathway, we set out to investigate if the reduced AMP gene expression in dTAK1 mutants was due to inhibited JNK signaling. Several lines of evidence indicate that JNK signaling is important for AMP gene expression: 1) Over-expression of hemipterous, a JNK kinase, signaling down-stream of dTAK1 (Glise *et al.*, 1995), rescued *Att* and *Dpt* expression in dTAK1 mutant animals. 2) Expression of Puckered (Puc) phosphatase, an inhibitor of JNK activity (Martin-Blanco *et al.*, 1998), suppressed the expression of AMP genes. 3) In JNK mutant clones in the larval fat body, expression of *Att, Dpt, Meth*
and *Drs* was reduced after infection. Again, results from Mika Rämets group are in line with our findings. They have shown that down-regulation of JNK signaling pathway components by RNAi greatly reduces the expression of *Att* and to some extent the expression of *Drs* (Kallio *et al.*, 2005). This clearly indicates that JNK signaling is important for expression of AMP genes.

We propose a model where the JNK and Imd/Relish signaling pathways are organized in parallel to promote transcription of AMP genes. Signals from both pathways are required for the transcription, as illustrated by the lack of AMP expression in Relish mutants (Hedengren *et al.*, 1999) and in JNK mutant clones in the fat body after infection. In addition, Imd seems to be important for the activation of both signaling pathways as a response to infection.

In Paper I, I showed that the expression of *CecA1* was almost abolished after infection, when the R1 site was mutated. This result indicates that Rel proteins are not the sole activators of *CecA1* expression after infection, but rather that additional transcription factors are needed. However, we could not prove either dFos or dJun to be part of the RBA. Consistently, the R1-binding was not compromised in nuclear extracts from dTAK1 mutants. Those results could imply that yet additional signaling is needed in parallel to promote transcription of AMP genes.

The actual transcription factor(s) activating transcription of the AMP genes, as a response to JNK pathway after infection is at this point not fully elucidated. dJun was shown to be binding to the *CecA1* promoter under all condition tested in this study, both before and after infection and also in dTAK1 mutants. This indicates that nuclear translocation and binding of dJun to the promoter is not dependent on signaling through dTAK1. However, one cannot rule out that additional signaling and subsequent modifications of dJun is dependent on dTAK1/JNK signaling and important for initiation of transcription. dJun could also require another protein partner for activation of transcription, for example dFos, whose translocation might be dependent on dTAK1 signaling. The translocation and binding of dFos to the *CecA1* promoter was not examined in this study. A third possibility for AMP gene expression via dTAK1/JNK is that dJun is not involved in the transcription, but rather that another transcription factor is activated as a result of signaling. Even though the exact mode of activation via dTAK1/JNK signaling is not presented in this study we have clearly shown that JNK signaling is important for AMP gene expression.

In humans, a parallel debate of the role of TAK1 in NF-κB and JNK signaling is going on. Similarly to the findings in *Drosophila*, human TAK1 is shown to activate both NF-κB and JNK signaling *in vitro* (Ninomiya-Tsuji *et al.*, 1999). Furthermore, *in vitro* studies of human cells have shown that down-regulation of TAK1 using RNAi reduces NF-
κB activation by TNFα and IL-1 stimulation (Takaesu et al., 2003). Analysis of different cell types from TAK1-deficient mouse cells revealed a complicated picture. Different cell types and stimuli gave differential activation of NF-κB and JNK signaling in TAK1-deficient cells. For example, TAK1-deficient B-cells were able to activate NF-κB but not JNK in response to B cell receptor stimulation, but failed to activate NF-κB and JNK, as a response to interleukin 1β, TNF and Toll-like receptor ligands (Sato et al., 2005). It might well be so that TAK1 regulation of the two different pathways differs from tissue to tissue, this has to be further investigated both in humans and Drosophila.

Isolation of CecA1 regulators (Paper III)

The focus of the present studies has been to understand signaling and transcriptional regulation of the AMP genes. We have mostly used CecA1 as a model gene for the analysis of AMP expression. CecA1 is used for several reasons. First of all, it has been shown to be a target for Rel transcription factor activation via both the Toll/Dif/dl and the PGRP-LC/Relish pathways in systemic responses to infection (Petersen et al., 1995; Stöven et al., 2000). In addition, CecA1 has been shown to be expressed both constitutively and inducibly in a tissue-specific manner (Engström et al., 1993; Roos et al., 1998; Ryu et al., 2004; Tzou et al., 2000). Thus, using CecA1 as a model gene has enabled us to study various aspects of regulation of AMP genes.

As mentioned previously, Rel proteins (Petersen et al., 1995; Stöven et al., 2000) and the GATA factor Serpent (Petersen et al., 1999) are known to regulate the expression of AMP genes. Additional transcription factors downstream of JNK signaling are also involved in the regulation. (Paper II). Recently, a homeodomain protein, Cad, was found to be a regulator of tissue-specific expression of AMP genes (Ryu et al., 2004). In order to find additional regulators of AMP expression, we used CecA1 as bait in a yeast screen. This type of yeast screen, called double-interaction screen (DIS) (Yu et al., 1999), combined the features of a “one-hybrid” and a “two-hybrid” screen. It enables the isolation of three classes of cDNA products: 1) DNA-interacting factors, 2) DNA-protein interacting factors and 3) protein-protein interacting factors. Furthermore, the design of the DIS enables isolation of regulatory factors irrespectively whether such factors act as positive or negative regulators in their normal context or if they are nuclear or cytoplasmic regulators of signal transduction and gene expression. Another advantage of performing a yeast screen compared to an EMS screen in flies is that it allows for the identification of proteins that are important for development and causes embryonic lethality.

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In the screen, we isolated cDNA clones of 15 genes and 10 out of them were shown to be specific for activation via the CecA1 promoter region (Table 1, Paper III). Several cDNA clones of Relish were isolated in the screen, confirming that this screen could isolate true regulators of CecA1. As mentioned above, four clones (CG1960, CG7785, CG8664 and CG9775) were tested for being components of the R1 complex, but were negative. Neither were they shown to have any significant effect on CecA1 expression after over-expression in transfections studies nor after down-regulation by RNAi. Additionally, three cDNA products belonging to the POU family of transcription factors were isolated in the screen; Pdm1, Pdm2 and Dfr. The role of the three POU proteins in transcriptional regulation of AMP genes was analyzed by transient transfections in a Drosophila cell line. All three proteins activated AMP gene expression, although to a different extent (Figure 2, Paper III). We have analyzed two of the POU proteins further; Dfr and Pdm1, and the data concerning their involvement in transcriptional regulation of AMP genes will be presented below (Paper IV and V).

cDNAs for two cytoplasmic proteins were also picked up in the screen; the ribosomal protein, RpL12 and a serine protease, Jonah 99Fii. At this point they have not been analyzed for their involvement in the regulation of immune responses in Drosophila.

Characterization of two POU proteins in transcriptional regulation of AMP genes (Paper IV & V)

As mentioned above Dfr and Pdm1 were isolated in a yeast screen (Paper III). Dfr and Pdm1 belong to the family of POU transcription factors and are important during development. Dfr has previously been shown to regulate several processes, such as tracheal formation (Anderson et al., 1995; de Celis et al., 1995), proper commissure formation in the central nervous system (Anderson et al., 1996) and the expression of Dopa decarboxylase (Ddc) in the nervous system (Johnson and Hirsh, 1990). Dfr is essential during development and mutants are embryonic lethal. Pdm1 has been shown to be important for the development of the nervous system and the formation of the wings (Bhat et al., 1995; Billin et al., 1991; Kitamoto and Salvaterra, 1995; Neumann and Cohen, 1998; Ng et al., 1995). Pdm1 mutants are not embryo lethal, but have poorly developed wings and cannot fly. Neither Dfr, nor Pdm1 have previously been shown to play any role in the transcriptional regulation of AMP genes.
Drifter (Paper IV)

In this paper, we report a new function for Dfr, the involvement in transcriptional regulation of AMP genes. We found that over-expression of Dfr in cell cultures and in flies resulted in an infection-independent expression of AMPs. Analysis of Dfr immuno-stainings in uninduced larvae and flies showed that Dfr was localized to the nucleus in a number of tissues (Figures 4 & 5, Paper IV). Many of the Dfr-positive tissues have previously been shown to express AMPs in a constitutive manner (Tzou et al., 2000). The fact that Dfr can promote AMP gene expression in an infection-independent manner and that Dfr is found nuclear prior to infection, led us to investigate if Dfr could be involved in the constitutive expression of AMP genes. As Dfr mutants are lethal, we undertook an RNAi approach to down-regulate the level of Dfr protein in flies. We chose to study the constitutive expression of AMPs in the ejaculatory duct, as robust Dfr staining was co-localized with CecA1 expression in this tissue (Figure 6, Paper IV). We dissected the male reproductive organs, including the ejaculatory duct. RNAi of Dfr greatly reduced the expression of CecA1, as well the expression of AttA, AttB, and Drs, in the dissected tissue in uninfected flies. These results strongly indicate that Dfr is indeed needed for constitutive expression of the AMP genes.

We identified an approximately 100 bp region, located ~400 bp from the transcription start, to be important for Dfr-mediated expression of CecA1. We also showed that purified Dfr could directly bind to this region. This region has previously been shown to be important for Cad-mediated CecA1 expression in the ejaculatory duct (Ryu et al., 2004). In transfection studies, we showed that Dfr and Cad work synergistically to promote CecA1 expression. We propose that the 100 bp region in the distal promoter of CecA1 confers a tissue-specific enhancer, important for the constitutive expression of CecA1 in the ejaculatory duct. Furthermore, we propose that Dfr and Cad are working in concert to promote high levels of CecA1 in the absence of infection, since the two proteins are working synergistically in cell transfection studies (Figure 7, Paper IV). Cad was also shown to be important for Drs expression in the salivary glands, and it will be interesting to investigate if Dfr binds to the same enhancer as Cad in the Drs promoter. One way to investigate if there are other immune responsive genes that are regulated by Dfr and Cad would be to perform a micro-array analysis on RNA isolated from flies over-expressing the two proteins individually or in combination. In the study by Ryu et al. (2004) it was shown that the constitutive expression of Drs in the female reproductive tract was not dependent on Cad. In accordance with the enhancer hypothesis, we were not able to detect Dfr staining in these organs. Perhaps other homeodomain proteins regulate constitutive expression of AMP genes in different tissues. Homeodomain proteins and their importance in development have been conserved over time in
evolution (Gehring et al., 1994). It will be very exciting to see if the homeodomain proteins play a role in the immune defense, which is also evolutionary conserved. The mammalian POU protein Oct1 has been suggested to play a role in immunity, but the effects are vague and have lately been questioned (Wang et al., 2004a; Wang et al., 2004b). However, with our results on Dfr (Paper IV) and the results from Ryu and co-workers on Cad (Ryu et al., 2004) and their involvement in epithelial defense it would be very thrilling to examine the regulation of AMP expression in humans since many human AMP genes are constitutively expressed in epithelial tissues.

Pdm1 (Paper V)
In this paper we show that Pdm1 is a regulator of the transcription of AMP genes. In contrast to Dfr, Pdm1 seems to be involved in the systemic response to an infection. Analysis of nubbin (nub) flies, Pdm1 mutant, revealed a complicated picture. The expression of several AMP genes, CecA1, Dpt and AttB, were more strongly expressed compared to control flies both in the absence and presence of an infection, whereas the expression of AttA was repressed also after an infection in nub flies (Figure 1, Paper V). This suggests that Pdm1 can function both as a transcriptional repressor and activator of different genes. The localization of CecA1 repression in nub flies was analyzed and determined to the fat body (Figure 2, Paper V), indicating that Pdm1 is involved in the systemic response to an infection. After infection, Pdm1 is rapidly degraded in the larval fat body. However, Pdm1 is rapidly regenerated again (Figure 3, Paper V). This finding allows us to speculate that Pdm1 is needed for the repression of certain AMP genes (CecA1, Dpt and AttB) in the absence of infection. Upon infection the repression is removed allowing for rapid expression of those genes, in a Rel protein-independent manner. The regeneration of Pdm1 within hours after infection was not sufficient to suppress the Rel protein-dependent expression of CecA1, Dpt and AttB as those genes are strongly expressed at the time of Pdm1 regeneration (Lemaitre et al., 1997). However, regeneration of Pdm1 may then support the expression of AttA.

To explain how Pdm1 can act both in positive and negative regulation of different target genes one may hypothesize that Pdm1 interacts with different co-activators and co-repressors for its activity. It would therefore be interesting to isolate putative co-regulators of Pdm1. Another important question to answer is which signal transduction pathway(s) control Pdm1 degradation. Is Toll, Imd or JNK signaling important? The mechanism of degradation would also be interesting to study and one may easily imagine that the proteasome could play a role in this process.
Concluding remarks

The focus of my thesis study has been to investigate the regulation of AMP gene expression. I have looked at different levels of regulation ranging from promoter studies, analysis of transcription factors, to studies of the upstream signaling events. In all three areas, me and my co-workers have made substantial progress. First of all, we identified a novel cis regulatory element important for the in vivo expression of CecA1 and most likely other peptide genes. Second, we identified a family of transcription factors, POU proteins, to be important for the transcriptional regulation of AMP genes. The role of POU proteins in immune regulation have not previously been recognized. Last, we showed that JNK signaling is important for the expression of AMP genes in the larval fat body and that dTAK1 is activating JNK signaling as a response to infection. We further showed that dTAK1 was dispensable for Relish activation and promoter-binding.

Much of my work was done in vivo in Drosophila. Drosophila constitutes an excellent model system to study innate immune processes and with the wealth of molecular and genetic tools that have been developed during the last decade it is almost only your own imagination that sets the limit to which questions can be answered in vivo.
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