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The *Andropin* gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*

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In our study of the cecropin locus in *Drosophila* we have found a gene for a new peptide, andropin, with antibacterial properties. Transcripts from this gene, *Anp*, could be detected in newly eclosed males and reached steady-state levels after 1 day. Transcription was strongly induced in response to mating and is strictly confined to the ejaculatory duct of adult males. The deduced peptide sequence reveals a hydrophobic amino terminus with striking similarity to the signal peptide of the cecropins. The sequence of the predicted mature andropin shows no direct homology with the cecropins, but the two peptides may have similar secondary structures. We have synthesized the predicted gene product and shown it to be antibacterial. Crude extracts from male genital tracts show a potent bactericidal activity, and electrophoretic separation revealed at least three antibacterial components, one with the same mobility as the synthetic peptide. It appears that insects have evolved a mechanism for the protection of the seminal fluid and the male reproductive tract against microbial infections.

Key words: andropin/*Anp*/cecropin/*Drosophila*/male specific

Introduction

The humoral antibacterial defense system of insects has been relatively well characterized. As a response to infection, several antibacterial peptides and proteins are synthesized and exported into the circulation (reviewed in Dunn, 1986; Boman and Hultmark, 1987). Notable among the induced peptides are the cecropins, which attack the cell membrane and efficiently lyse a wide variety of bacteria. In *Drosophila*, the cecropin genes are found in a dense cluster of several genes and pseudogenes at 99E on the third chromosome, encoding at least two different types of cecropin (Kylsten *et al.*, 1990). The *Drosophila* cecropin genes are expressed mainly in the fat body and to some extent in hemocytes, and their antibacterial products accumulate in the hemolymph (Samakovlis *et al.*, 1990). In contrast to the situation in the blood, the defense mechanisms in other compartments of the insect body are largely unknown. Here we present a new aspect of the *Drosophila* defense system; that of the protection of the male reproductive tract and the seminal fluid.

In the seminal fluid of *Drosophila*, sperm is mixed with the secretions from two anatomically distinct glandular tissues—the accessory glands or paragonia and the ejaculatory duct. The male accessory glands are known to be an active tissue synthesizing many proteins that are secreted into the lumen and transferred to the female during copulation. Chen (1984) has observed >40 secreted polypeptides in the paragonial fluid. Transfer of these secretions to the female has been shown to elicit two major responses—elevation of oviposition and repression of sexual receptivity (Chen, 1984). Genomic and cDNA clones for some of the male-specific transcripts made in the paragonia have been isolated. However, the function of these gene products has been clearly demonstrated only in the case of the sex peptide (Chen *et al.*, 1988), which is transferred to the female and affects courtship response and egg-laying. The putative product of *mst 355a* (Monsma and Wolfner, 1988), another accessory gland-specific transcript, shows an interesting sequence homology to the egg-laying hormone from *Aplysia californica*. This suggests that *mst 355a* may also affect reproductive physiology and behavior. In comparison with the accessory gland secretions, the products of the ejaculatory duct have been studied in less detail. Two enzymes have been found in the ejaculatory duct lumen: glucose dehydrogenase (Cavener and MacIntyre, 1983) and esterase-6 (Richmond *et al.*, 1980); both activities are transferred to the female during mating and esterase-6 has been implied to affect female receptivity after mating.

In our study of the cecropin locus in *Drosophila* we found a closely linked gene that is specifically transcribed in the male reproductive tract. We here present a molecular study of this gene, and show that it encodes a bactericidal peptide. Since it is male specific and shows some similarity to the cecropins, we propose to call the peptide andropin and to designate the gene as *Anp*.

Results

Cloning of the andropin cDNA, structure and sequence of the gene

In a screen for transcribed genes from the *Drosophila* cecropin locus, we used a 7.8 kb genomic *Bam*HI fragment (Figure 1A) to probe a cDNA library from adult flies that had been injected with bacteria in order to induce cecropin transcription. This screen yielded 18 clones, nine corresponding to the cecropin gene A1, seven to A2 and one to B (Kylsten *et al.*, 1990). The remaining clone, k-9, contained a 279 bp insert. Sequencing revealed an open reading frame encoding a 57 amino acid peptide that was not a cecropin. We identified the 3′ end of this reading frame in the previously sequenced 4.4 kb *Hin*dIII–*Eco*RI genomic fragment that contained the three cecropin genes A1, A2 and B (Kylsten *et al.*, 1990). To obtain the sequence of the

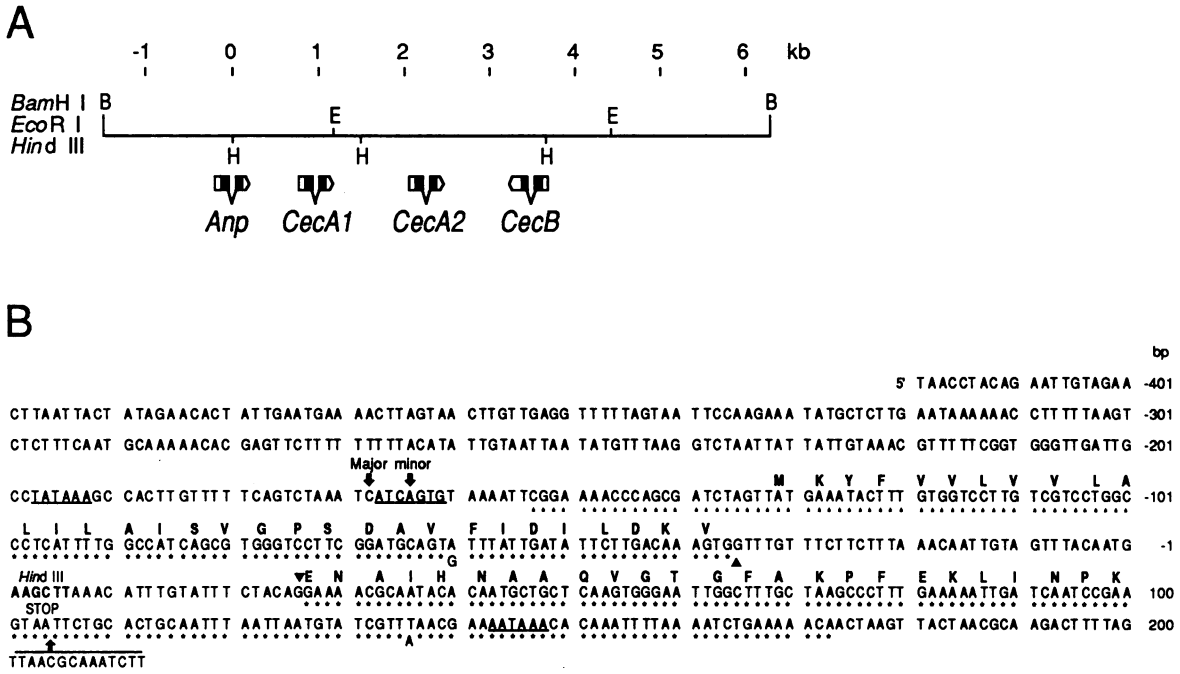

Fig. 1. The *Anp* gene. (**A**) Genomic map of the *Anp* region, including three cecropin-coding genes. Filled boxes indicate coding sequences. (**B**) Genomic sequence of *Anp*. The translated amino acid sequence is shown above the coding DNA. The sequence of the cDNA clone k-9 is indicated below the genomic sequence, the identity being indicated by an asterisk (*). Just 3′ of the stop codon there is a 12 bp insertion in the cDNA. TATA box, CAP site and AATAAA signal sequences are underlined, and the mapped major and minor transcription start sites are indicated. The genomic sequence to the right of the *Hin*dIII site (coordinates +1 to +200) is from Kylsten *et al.* (1990).

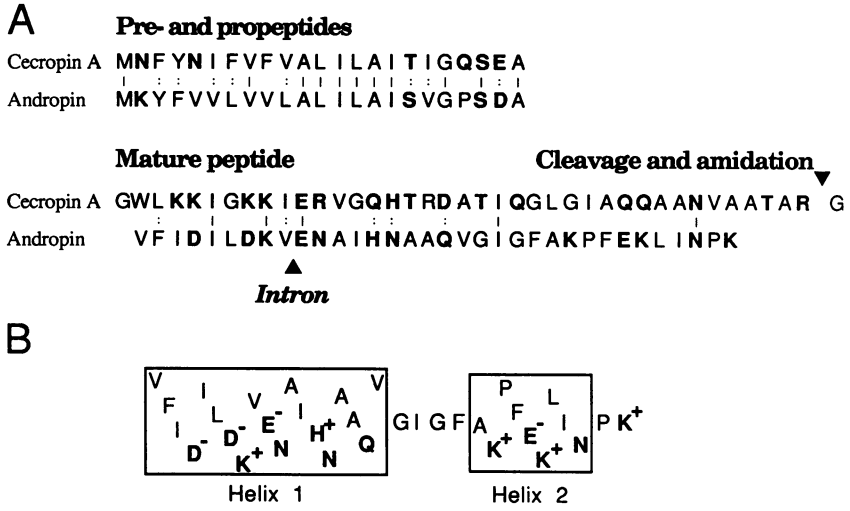

Fig. 2. Structure of the andropin peptide. (**A**) Primary structure. Alignment of the predicted primary translation products of the *Anp* and *CecA* genes. The mature peptides were aligned according to the position of the intron. This alignment also gives the maximum number of identical residues. The carboxy-terminal glycine residue in cecropin A is removed in the formation of an amidated terminus. Andropin lacks a C-terminal glycine and is probably not amidated. (**B**) Hypothetical model of the secondary structure of mature andropin. In the projections of the two predicted helices, all hydrophilic residues (shown in bold type) face one side of the helix.

remaining part of the gene, we extended the sequenced region upstream of the *Hin*dIII site. Figure 1(B) shows the genomic sequence and the deduced amino acid sequence of the andropin peptide. By primer extension we mapped one major and one minor transcription start site that are indicated in the figure. The exon−intron boundaries are also indicated and are deduced from a comparison to the cDNA sequence. The cDNA sequence differs from the genomic sequence by two nucleotide replacements and by an insertion of 12 bp as indicated in Figure 1(B). None of the differences affect the amino acid sequence of the peptide and they may be due to a different allele in the fly population that was used to construct the cDNA library (Kylsten *et al.*, 1990). Analysis of the amino acid sequence of the putative andropin product revealed several interesting features (Figure 2A). The amino-terminal part of the predicted peptide shows a remarkable degree of similarity to the signal peptides of the cecropins. Of the first 23 residues in the *Drosophila* cecropin A prepropeptide, 12 are identical in andropin and the majority of the remaining ones represent conservative replacements. A probable signal peptidase cleavage site (von Heijne, 1986) is also predicted immediately after this conserved region,

although alternative possible sites are found three and six residues closer to the N terminus. After this putative signal peptide, the similarity to the cecropins decreases abruptly, and if the predicted mature peptide is aligned with cecropin A as in Figure 2(A), with the introns in the same position, only five out of 34 residues are identical. Other alignments give equal or lower scores, and the improvement obtained by the introduction of gaps in the andropin sequence is limited. However, the secondary structure of andropin may still be very similar to that of the cecropins. *Hyalophora* cecropin A has been shown to form two amphipathic α-helices separated by a hinge region (Holak *et al.*, 1988). Using the rules of Chou and Fasman (1978), andropin is also predicted to form two helices, and as shown in Figure 2(B) all hydrophilic amino acids in the hypothetical helices would face the same side of the molecule, giving it a very strong hydrophobic moment. As for the cecropins, the amino-terminal helix is longer, and an intron is found in an equivalent position near the middle of this helix. Thus, although the predictive value of the methods used is limited, andropin clearly has the potential to fold in a manner very similar to the cecropins.

Andropin transcription is sex specific and strictly localized

The andropin gene is located very close to the cecropin genes. We have shown that transcription of all cecropin genes in *Drosophila* is strongly induced after injection of bacteria, in larvae, pupae and adults (Samakovlis *et al.*, 1990). In parallel, we used the same set of RNA preparations to investigate the expression of the andropin gene by RNase protection. Figure 3 shows that the andropin transcript is only detected in adult flies and that in contrast to the cecropin genes the level of transcription does not increase in response to bacterial infection. The andropin gene seems to be regulated independently and in a very different way from its closely neighboring genes. *In situ* hybridizations to whole body sections of injected and untreated flies localized the andropin transcript to a distinct tissue in the male abdomen (Figure 4). In order to confirm the sex specificity and identity of the hybridizing tissue, we separated males from females and dissected the male reproductive tract. RNA was prepared from these tissues and analyzed for the presence of andropin mRNA by RNase protection assay. Figure 5 (A) shows that the transcript is male specific and is made in the part of the reproductive tract that includes the accessory glands and the ejaculatory duct. A similar experiment in Figure 5(B) demonstrates that the ejaculatory duct is the site of andropin transcription, and that the accessory glands are inactive. We attribute the faint signal that can be seen in the lane with accessory gland RNA to contamination with ejaculatory duct fragments. By examination of serial sections, we can further conclude that the transcript is distributed throughout the length of the ejaculatory duct.

Accumulation of the andropin transcript after eclosion and induction after mating

The male reproductive system, except the testes, is a somatic tissue that derives from the genital imaginal disc. At the pupal stage the genital ducts and the external genitalia are formed and the connections to the testes are established (Bodenstein, 1950). We were interested to follow the level of andropin transcripts during the maturation of the adult fly. Figure 6(A)

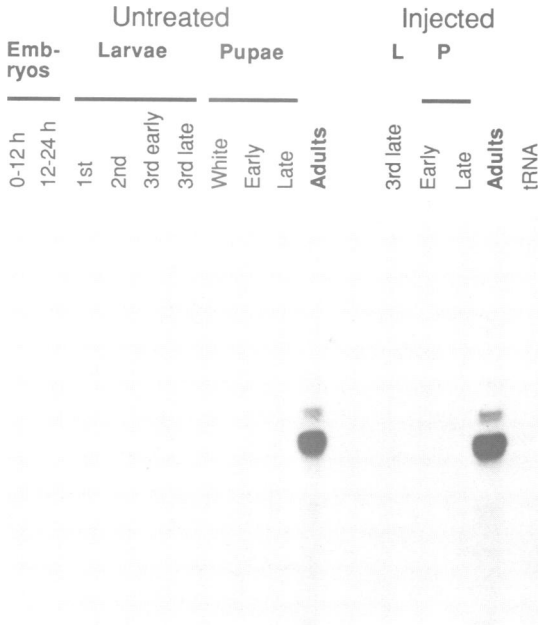

Fig. 3. Expression of the *Anp* gene during development, assayed by RNase protection of RNA from untreated and bacteria-injected animals. Early and late third instar larvae refers to feeding and wandering stages respectively; white, white perpupae; early pupae, up to 49 h after pupariation; late, 49 h until eclosion. Twenty micrograms of total RNA from untreated or vaccinated animals (6 h after injection) were used for each hybridization. Fifty micrograms of tRNA were used for the negative control lane. The integrity and quantity of the RNA in each lane was checked by Northern blotting, where the same volumes of RNA were blotted and hybridized to actin 5C (data not shown). Two major protected fragments of ~190 nucleotides were observed in this and other experiments, as compared to the size of the insert, which is 279 nucleotides. This may be explained by cleavage of the duplex at the 12 bp insert of the cDNA probe, and partial trimming at the ends.

shows that andropin mRNA is readily detectable in male adults 1 h after eclosion and that it reaches a steady-state level after 24 h. After this point the level of transcripts remains stable at least until day 5 of adult life. However, if 5-day old males are allowed to mate, transcription is induced again and the maximal level of transcripts is reached 12 h after mating (Figure 6B). Presumably the stored andropin is secreted and transferred to the female with the rest of the seminal fluid and needs to be replenished after mating.

Antibacterial activity of synthetic andropin

Because of the structural similarity of andropin to the cecropins it appeared likely to be an antibacterial peptide. We prepared the predicted gene product by chemical synthesis and compared its bactericidal activity to synthetic *Drosophila* cecropin A using the inhibition zone assay (Hultmark *et al.*, 1982). Table I shows that andropin has a moderate antibacterial activity against Gram-positive bacteria, but that in contrast to the cecropins it has little or no activity against Gram-negative bacteria. However, in experiments with buffered rather than standard plates, we found a significant activity of andropin against *Escherichia coli*. Figure 7 shows the strong effect of phosphate buffer concentration on the activity of andropin against this bacterium. Thus, at high buffer concentrations andropin becomes a potent bactericidal agent. This is probably an

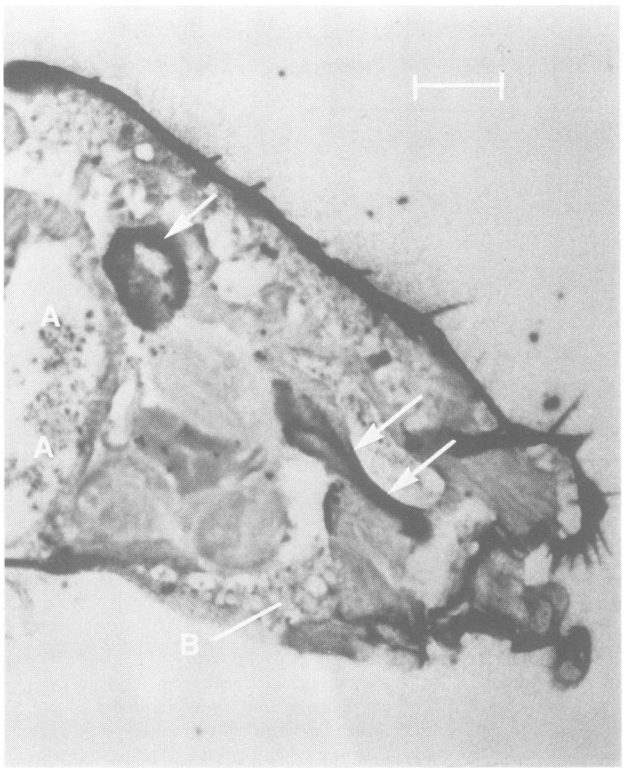

Fig. 4. Localization of the *Anp* transcript. Tissue sections of adult flies were hybridized with ^{35}S-labelled insert of the *Anp* cDNA clone k-9. This sagittal section shows the posterior half of a male abdomen with hybridization in a near terminal segment of the ejaculatory duct (double arrows) and in an anterior region of the same duct (single arrow). Immunized and untreated flies gave the same result. Anterior, left. Posterior, right; genital apparatus in lower corner. (**A**) Gut lumen. (**B**) Fat body. Scale bar = 0.1 mm.

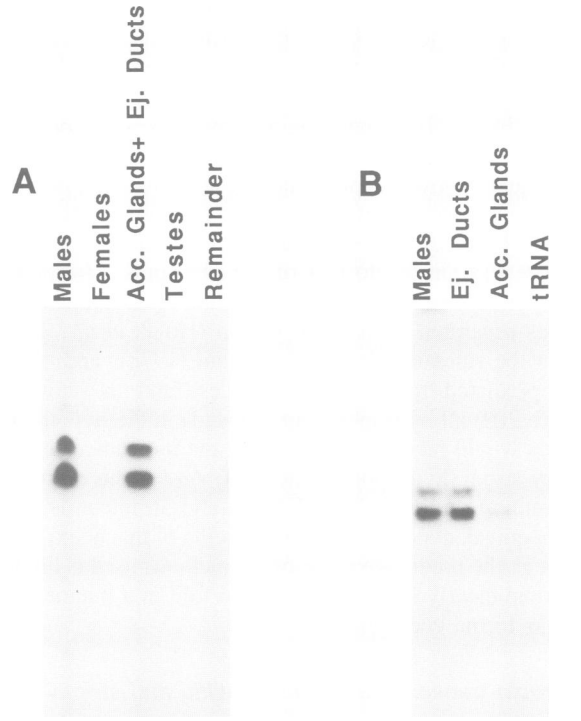

Fig. 5. Expression of the *Anp* gene in different tissues, assayed by RNase protection. RNA was extracted from whole flies or from dissected tissues, and for each lane an amount corresponding to five flies was used. Twenty micrograms of tRNA were used for the negative control lane.

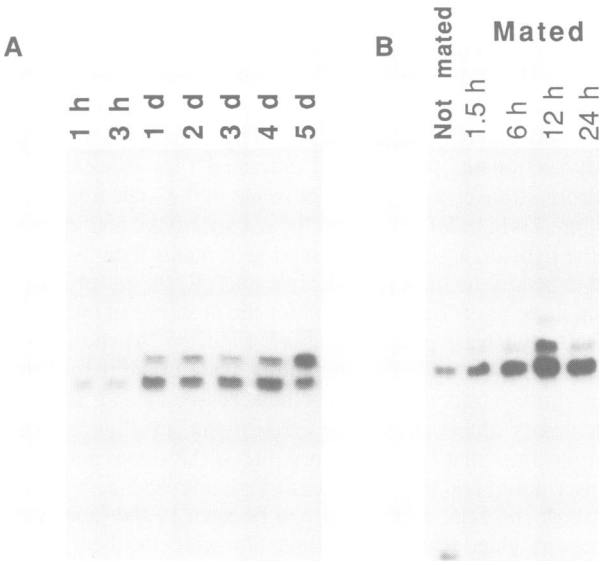

Fig. 6. Expression of the *Anp* gene in adult males, assayed by RNase protection. (**A**) Virgin males at different time points after eclosion. Film exposed for 18 h. (**B**) Induction of *Anp* transcript after mating. Five day old virgin males were mated and collected at different time points after mating. Film exposed for 8 h. The RNA samples in both experiments were checked by running aliquots on an agarose gel and staining with ethidium bromide.

effect of ionic strength rather than pH, since 0.2 M NaCl gives a similar effect to 0.05 M phosphate, whereas no difference was observed when pH was varied between 6.3 and 8.3 (in 0.2 M phosphate buffer, data not shown).

Identification of the andropin peptide in genital tract extracts

We wanted to test whether an antibacterial activity can also be found in the genital tract of the male fly. For this purpose we prepared acidic extracts from accessory glands and ejaculatory ducts. We could indeed observe bactericidal activity with the inhibition zone assay by applying on the plate the extract from as little as two genital tracts. This activity, and in addition its transfer to the female after mating, has also been observed by S.-L.Wu, M.Robertson, T.Landon, D.Grace, Y.-L.Yan and J.Postlethwait (in preparation). It is noteworthy that the antibacterial activity from the extract could be detected in unbuffered plates, whereas significant activity of the synthetic andropin could only be demonstrated in buffer plates. This indicated to us that the extract probably contains additional antibacterial components. To approach this question we fractionated male genital tract extracts on native acidic polyacrylamide gels and detected antibacterial peptides as clear bands in a bacterial lawn that was applied on the gel after electrophoresis. On the same gel we could also test whether our synthesized peptide corresponded to one of the natural antibacterial products of the ejaculatory duct. Figure 8 shows that the extract from male genital tracts contains at least three antibacterial components and one of them shows similar

Table I. Comparison of the antibacterial activity of andropin and cecropin

	Lethal concentrations (μM)	
	Andropin	Cecropin A[a]
Escherichia coli D21	140	0.3
Enterobacter cloacae β12	>300	0.4
Pseudomonas aeruginosa OT97	>300	2.2
Serratia marcescens Db11	>127[b]	93[b]
Serratia marcescens Db1140	>127[b]	>93[b]
Bacillus megatherium Bm11	11	2.6
Bacillus subtilis Bs11	17	16
Micrococcus luteus MI11	20	8.5

[a]Cecropin data are from Samakovlis *et al.* (1990).
[b]Values obtained by MIC assay (Samakovlis *et al.*, 1990).

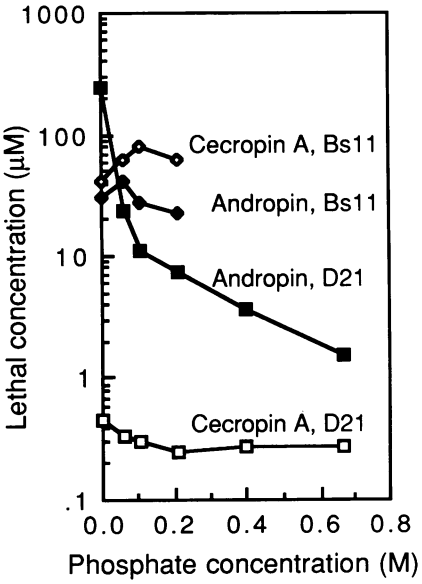

Fig. 7. Buffer dependence of andropin activity. The resistance of *Escherichia coli* D21 and *Bacillus subtilis* to andropin and cecropin was assayed in the presence of sodium phosphate, pH 7.4, at different concentrations. *B.subtilis* did not grow at buffer concentrations of 0.4 M and higher.

mobility to the synthetic andropin peptide. From this type of experiment we estimate that each ejaculatory duct contains ~10 ng andropin.

Discussion

Expression of the andropin genes

To our knowledge, *Anp* is the first gene to be identified as ejaculatory duct specific (an ejaculatory duct transcript from the cecropin locus that may derive from the *Anp* gene has been independently observed by S.-L.Wu, M.Robertson, T.Landon, D.Grace, Y.-L.Yan and J.Postlethwait, in preparation), and we used it as a probe to follow the maturation of this tissue in adults. We could first detect andropin transcript in males that had just eclosed from the puparium and the transcription rate reached its maximum 24 h after eclosion. The same accumulation kinetics have been reported for two accessory gland specific transcripts, *mst 355a* and *355b* (Chapman and Wolfner, 1988; Monsma and Wolfner, 1988). Both the accessory glands and the ejaculatory duct are somatically derived genital tissues and the similar kinetics

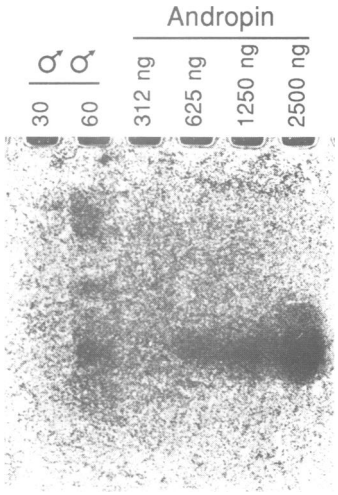

Fig. 8. Separation of antibacterial proteins from the male reproductive tract on an acidic polyacrylamide gel. Extracts from the indicated numbers of dissected reproductive tracts excluding testes, and known amounts of synthetic andropin were electrophoresed, and the antibacterial components were detected with a bacterial overlay.

of transcript accumulation suggest that they may differentiate in parallel. The induction of transcription of male-specific genes after mating has been described by Yamamoto *et al.* (1988). They observed an increase of RNA and protein synthesis in dissected ejaculatory ducts and paragonial glands after copulation. An increase in enzymatic activity after mating was also described for glucose dehydrogenase in the ejaculatory duct of *Drosophila* by Cavener and MacIntyre (1983). We could show that the andropin gene is induced by mating and maximal induction is reached 12 h after copulation. Yamamoto *et al.* (1988) have also shown that juvenile hormone III has a stimulatory effect on the RNA synthesis of genital tracts *in vitro* and that a similar effect is observed *in vivo* after treatment of males with the juvenile hormone analog hydroprene. We tested both juvenile hormone III *in vitro* and its more stable analog methoprene *in vivo* as inducers of andropin transcription, but we could not detect any induction (data not shown). *Anp* seems not to belong to the group of male-specific genes that are induced by juvenile hormone.

The presence of the *Anp* gene at the cecropin gene cluster presents an interesting problem in terms of gene regulation. Although the *Anp* gene is situated only 600 bp upstream of the *CecA1* gene, the two genes show completely different patterns of expression. The *Anp* gene shows strong constitutive expression that is exclusively restricted to the male reproductive tract. Furthermore, it is unresponsive to bacterial injections, but is induced by mating. In contrast, *CecA1* is mainly expressed in the fat body and to some extent in hemocytes, and its transcription is dependent on induction by microbial products (Samakovlis *et al.*, 1990). A low level of constitutive expression is sometimes observed with the cecropin genes, and we reinvestigated this phenomenon to see if it may be due to a male-specific transcript. However, there was no difference between males and females in the basal level expression of any of the cecropin genes (data not shown). Thus, the short segment that separates *Anp* from the cecropin genes functions as a good insulator and does not allow the activation of one gene to interfere with the transcription from the other.

Origin of the andropin gene and biological function of its product

Although andropin differs considerably from the cecropins in its primary structure, it is hard to avoid speculating that the genes are phylogenetically related. The strongest evidence for a common origin is the striking similarity in the signal peptide sequences and the close proximity of the genes in the cecropin locus. It is conceivable that only the signal peptides share a common origin, and that they have been connected to unrelated genes, perhaps as a result of exon shuffling. For example, Kuchler *et al.* (1989) have found that several otherwise unrelated peptides in frog skin share such a common 'export ticket'. However, the predicted secondary structures of the mature peptides are very similar, and we think it is more likely that andropin is truly related to the cecropins. Also, the gene structures are related, with two exons separated by a short intron in the middle of the region that codes for the predicted amino-terminal helix.

If andropin is derived from a cecropin-like ancestor, it must have evolved very fast, faster than its signal peptide. This may be an adaptation to the special physical conditions in the seminal fluid, or to kill a particular pathogen. On the other hand, it may also be an indication that it has acquired an entirely new function. Its antibacterial activity would then only be a vestige of its original role in immune defense. The amphipathic character of andropin makes it a possible candidate for a signalling substance. Since products from the male reproductive tract are known to affect the behavior of females after mating (Chen, 1984), we injected synthetic andropin in virgin females but found no effect on courtship response or egg-laying, and we also failed to detect any effect of andropin on sperm motility *in vitro* (data not shown).

We have detected a potent antibacterial activity in extracts from the male reproductive tract that could be separated into several active components. We synthesized the andropin peptide based on the assumption that the signal peptide is removed by cleavage after residue 23 and that this is the only modification that occurs. Since the synthetic product co-migrates on acidic gels with one of the antibacterial products of the ejaculatory duct, we find it highly suggestive that it represents the true mature andropin and that its antibacterial activity reflects the physiological function in the fly. Andropin is the first example of a new group of defense molecules in the seminal fluid, identifying a new aspect of the insect immune system.

Materials and methods

Cloning and sequencing

The cDNA clone containing the andropin insert was isolated in the screen described in Kylsten *et al.* (1990). The Bluescript plasmid was excised from the clone k-9 as described in the Stratagene manual and the insert was sequenced on both strands. The 1.5 kb *Hin*dIII–*Bam*HI fragment was subcloned in the pTZ (Pharmacia) and the Bluescript vectors. Single-stranded DNA was prepared from these clones and sequenced with the M13 reverse primer for the pTZ subclone and an internal primer designed for primer extension experiments (see below) for the Bluescript subclone. All sequencing was performed with the chain termination method using the Sequenase kit (USB).

Flies and antibacterial assays

Canton S flies were kept on autoclaved corn meal/yeast food at 25°C with a 10/14 h light/dark cycle. Flies were injected with bacteria as described in Samakovlis *et al.* (1990). For the detection of antibacterial components in the genital tracts, males were surface sterilized in ethanol and the reproductive tract was dissected in Ringer solution (Yamamoto *et al.*, 1988). Testes were removed and the accessory glands together with the ejaculatory duct were kept in an Eppendorf tube on ice. One microliter extraction buffer, 0.1 M trifluoroacetic acid, 0.2 M NaCl (Esch *et al.*, 1983), was added per three to five genital tracts; the tissues were mashed with a yellow tip on ice and the extract was kept frozen at −70°C until use. Extracts were loaded for electrophoresis in a 0.75 mm polyacrylamide gel, pH 4.0 (Hultmark *et al.*, 1980), and antibacterial components detected with a bacterial overlay. Approximately 5 ml log-phase *E.coli* D21/10^6/ml was applied on the surface of the neutralized gel, and after 1 min the excess was removed. The gel was incubated at 30°C overnight.

Antibacterial activity was determined by the inhibition zone assay; serial dilutions of the synthetic peptide were applied in wells on bacterial plates, and the lethal concentrations were calculated from the concentration dependence of the diameter of growth inhibition zones (Hultmark *et al.*, 1982). The plates contained 0.8% agarose in 5 ml LB medium, and where indicated they had been equilibrated by overlaying with 10 ml sodium phosphate buffer, pH 7.4, of the appropriate concentration in LB for 1 h. After application of the sample they were incubated at 30°C overnight, or until bacterial growth was visible. References for the bacterial strains used are given in Hultmark *et al.* (1982).

Probes

Probes were prepared from the Bluescript plasmid k-9 containing the andropin cDNA insert. The insert of the clone extends between the coordinates −154 and 173 given in Figure 1(B). RNA probes for the RNase protections were prepared by *in vitro* transcription from the T7 promoter as described by Gilman (1987). The template was linearized with *Bam*HI, which cleaves in the polylinker of Bluescript, 18 bp from the *Eco*RI cloning site. The probe contained ~95 nucleotides of vector sequence in addition to the whole insert.

DNA probes for tissue *in situ* hybridizations were prepared from gel-purified k-9 cDNA insert using a Pharmacia kit based on the random oligo-priming method of Feinberg and Vogelstein (1983).

RNA preparation and analysis

RNA was prepared from flies according to Kylsten *et al.* (1990). RNase protection assays were as in Gilman (1987) with ^{32}P-labelled probes; hybridizations were done overnight at 45°C. RNase digestions were done at 30°C for 45 min. The protected fragments were loaded on a 6% sequencing gel together with sequencing ladders as markers.

Primer extension

The T4 kinase labelled synthetic primer AAGGACCCACGCTGATGGCC (synthesized by SYMBICOM AB, Umeå, Sweden) was annealed at 25°C to 3 μg poly(A)$^+$ RNA overnight. Extension was done with 50 U M-MuLV reverse transcriptase (Kingston, 1987). DNA sequencing reactions primed with the same primer were used as markers.

In situ hybridization

Adult flies for tissue sections were quick frozen in OCT compound (Tissue Tek II). Eight micrometer sections were cut at −14°C, recovered onto subbed slides, and fixed in 4% paraformaldehyde. Pretreatment of sections was according to Hafen *et al.* (1983), except that after fixation the sections were acetylated as by Akam and Martínez-Arias (1985). The probe was the k-9 cDNA insert labelled with [^{35}S]dCTP according to the random oligo-priming method. Hybridizations (overnight, 37°C) and washing (7 h, 45°C) were according to Akam (1983), except that dextran sulfate (to 10%) was added to the hybridization mix, and dithiothreitol (up to 10 mM) was added to the hybridization mix and wash buffer. Autoradiography and staining were also according to Akam (1983). Slides were exposed for 5 days up to 6 weeks.

Synthetic peptides

The synthesis of *Drosophila* cecropin A by automated solid-phase synthesis has been described (Samakovlis *et al.*, 1990), and andropin was synthesized by the same method, using an Applied Biosystems (Foster City, CA) Model 430 A peptide synthesizer. Symmetric anhydrides were coupled twice and eventually remaining free amino groups after coupling were blocked with acetic anhydride. After final cleavage with HF the peptide was purified by reversed-phase chromatography and the purity of the final products was ascertained by ^{252}Cf plasma desorption time-of-flight mass spectroscopic analysis on a BIOION Model 20 spectrometer (Bio-ion Nordic AB, Uppsala, Sweden).

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