

The fatty acid elongase NOA is necessary for viability and has a somatic role in *Drosophila* sperm development

Anita Jung^{*‡}, Martin Hollmann^{*} and Mireille A. Schäfer[§]

FB18 Zoologie/Entwicklungsbiologie, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany and III. Zool. Institut-Entwicklungsbiologie, Humboldtallee 34A, 37073 Göttingen, Germany

^{*}These authors contributed equally to this work

[‡]Present address: Department of Neurosciences and Pharmacology, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark

[§]Author for correspondence (e-mail: mirescha@uni-kassel.de)

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Summary

The essential gene *noa* (CG 3971; also known as *Baldspot*) encodes a very long chain fatty acid elongase which is most similar to the mammalian elongase ELOVL6. *noa* is expressed in the nervous system from embryogenesis on, in imaginal discs, the fat body, malpighian tubules and in the gonads of both sexes. Its function is dose dependent, since reduced levels of *noa* RNA lead to impaired motility and severely reduced viability. In testes, *noa* RNA is detected in the cyst cells during the postmeiotic phase of germ cell development. An RNAi construct selectively driven in cyst

cells leads to male sterility, demonstrating the necessity of *noa* function for male germline development and the interaction of the somatic cyst cells with the developing sperm.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/120/16/2924/DC1>

Key words: *Drosophila*, Fatty acid elongases, Spermatogenesis, Cyst cells, RNAi, Embryogenesis

Introduction

Male germ cell development in *Drosophila* is dependent on the close contact of the germ cells with the surrounding somatic cyst cells. This contact is established at the beginning of the process in the tip of the testis tubes. A single germ cell, a spermatogonium, becomes enclosed by two somatic cyst cells, thus forming a cyst. Within this cyst, germ cells synchronously go through the mitotic amplification phase, growth phase, meiosis and through differentiation until individualization. Several signals have been identified during the early phase of spermatogenesis that either are initiated in the cyst cells and influence the surrounded germ cells or are initiated in the germ cells and influence the cyst cells (reviewed by Renkawitz-Pohl et al., 2005). During spermatocyte maturation the transcription factors *eya* (*eyes absent*) and *so* (*sine oculis*) are required in the somatic cyst cells for correct development of the spermatocytes (Fabrizio et al., 2003). How this signal is transmitted to the developing spermatocytes remains unclear, however. Even less is known about the germ cell-cyst cell contact after meiosis. Although the two cyst cells are not distinguishable until shortly after meiosis, they then clearly behave differently. One of them differentiates into a head cyst cell into which all 64 spermatid heads are anchored, the other develops into a tail cyst cell that surrounds the spermatid tails of 1.8 mm length (Lindsley and Tokuyasu, 1980). The head cyst cell finally is engulfed by cells of the terminal epithelium to allow coiling of the spermatid bundles towards the testis base (Tokuyasu et al., 1972).

In an enhancer trap screen for genes expressed in cyst cells we identified the essential gene *neighbor of abl* (*noa*), which encodes the long-chain fatty acid elongase. In addition to

expression in various somatic tissues, in the testes *noa* is expressed in cyst cells and in cells of the terminal epithelium. *noa* expression is observed in the second half of sperm development, after the encased germ cells have completed meiosis. It is found in both the head and the tail cyst cells. Its function is required for male fertility since an RNAi construct selectively expressed in the cyst cells leads to male sterility.

Results

The enhancer trap lines exhibit a specific expression pattern in testes

By analyzing a collection of embryonic lethal enhancer trap lines (Spradling et al., 1999) for expression patterns in adult testes we identified two non-complementing P-element-insertion lines from genomic region 73B with β -galactosidase expression in the cyst cells, namely *l(3)01895* and *l(3)04106*. A third line, *l(3)02281*, was described in FlyBase (Drysdale et al., 2005) as non-complementing and found to exhibit the same expression pattern (for an overview see Fig. 1A). In remobilization experiments with the insertion lines *l(3)01895* and *l(3)04106* wild-type revertant flies were observed, indicating that the observed lethality and the P-element insertion are causally related.

The enhancer trap pattern is shown in Fig. 1A. Owing to the cloning strategy the *lacZ* gene in the P-element vector contains a nuclear localization signal. Thus, β -galactosidase activity is observed in the nuclei of the expressing cells. In adult testes (Fig. 1A) β -galactosidase expression is seen at the tip of the testis tubes (arrow), in scattered nuclei along the tubes that are located along the elongating spermatid tails – and thus

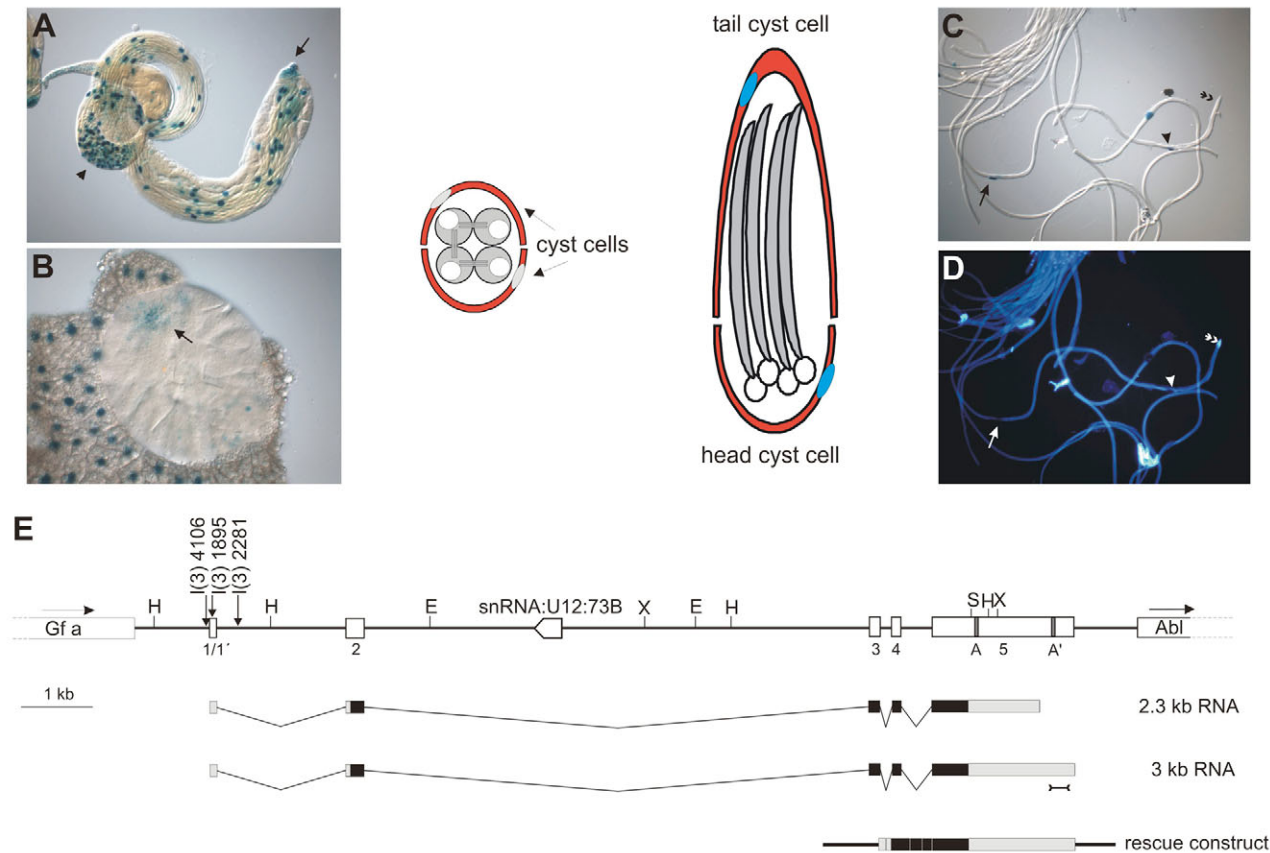


Fig. 1. Enhancer trap pattern and gene structure. (A,B) β -Galactosidase activity is demonstrated in adult testis tissue (A) and in larval testis anlagen (B). The terminal epithelium is indicated by an arrowhead in A. An arrow points to the apical expression seen at both stages (A,B). The relationship between germ cells and cyst cells is shown in the diagram with the cyst cells in red and the interconnected germ cells in grey. The situation is depicted for the premeiotic phase where the two cyst cells with their unstained nuclei are equivalent (as an example surrounding four germ cells) as well as for the postmeiotic phase (with four instead of the 64 elongated spermatids) in which the head and tail cyst cells with β -galactosidase-positive nuclei surround the germ cells in a directed manner. (C) Separated spermatid bundles show two stained nuclei (arrow and arrowhead) proving that both head and tail cyst cell are active. (D) The position of the spermatid nuclei is shown by Hoechst 33258 staining (double arrow). (E) The genomic organisation of the *noa* gene is shown (top). Exons 1-5 are shown as white boxes. The integration sites for the three P-element lines are indicated by arrows. Restriction sites within the gene are indicated for *EcoRI* (E), *HindIII* (H), *SacI* (S) and *XbaI* (X). In head RNA an alternative form of the first exon (1') was found which extends the first exon by 60 nt in 3' direction thus leading to an alternative splice donor site. The two bars (A,A') in the 3' UTR indicate poly(A) rich regions mentioned in the RNaseH experiment (see Fig. 2B). The two transcripts are outlined below the diagram. The exons show the transcribed sequences in grey and the coding region in black. The bracketed line corresponds to a PCR fragment that was used as probe to prove the existence of the long RNA species. The sequences contained within the rescue construct are given at the bottom (see Materials and Methods).

represent tail cyst cells (see diagram in Fig. 1) – and in a larger accumulation at the end of the tubes in the terminal epithelium in front of the seminal vesicles (arrowhead). As will be shown later, these nuclei belong to head cyst cells as well as to cells of the terminal epithelium. In the larval testes β -galactosidase activity is only observed in a small number of nuclei at one end of the gonad (Fig. 1B, arrow). Since the larval testes only contain germ cell stages up to the spermatocyte stage, this proves that the scattered nuclei in adult testes are from cyst cells around postmeiotic stages of germ cell development. To demonstrate clearly that both head and tail cyst cell show β -galactosidase activity isolated cysts were stained with Hoechst 33258. A bundle of elongating spermatids can be traced, on which two β -galactosidase-positive nuclei can be seen (arrow, arrowhead for the two cyst cell nuclei and double arrow for spermatid nuclei, Fig. 1C,D).

The *noa* gene generates two transcripts

We cloned P-element flanking genomic sequences in plasmid rescue experiments and with these as a probe identified three incomplete but overlapping cDNA clones from a testis cDNA library. From their analysis the gene structure was derived as depicted in Fig. 1E. The gene contains five exons, spans a genomic region of 12.5 kb (Fig. 1E) and is located in polytene region 73B on chromosome 3L. It is flanked at the 5' end by the gene for an alpha subunit of a G protein (*Gf alpha*) (Quan et al., 1993) and at the 3' end by the gene *Abl* (Segev et al., 1988). We, therefore, named the gene *neighbor of abl* (*noa*). In addition, the snRNA:U12:73B gene resides within the large second intron of *noa*. The three testis cDNAs add up to 2385 nucleotides and contain a potential polyadenylation signal roughly 300 nt before the 3' end of the cDNAs, indicating that longer transcripts should exist. Using the EST sequence in the

flybase database (Drysdale et al., 2005), the 5' end that extends furthest (LD 10431) was defined as the transcription start point of *noa*. The three P-element integration sites were determined by sequencing and reside 18 nucleotides (nt) in front of the transcription start site in the case of *l(3)04106*, 80 nt into the first exon in the case of *l(3)01895* and 360 nt into the first intron in the case of *l(3)02281*, respectively (Fig. 1E).

In northern blotting experiments two transcripts were detected (Fig. 2A left), which were found to be present in both sexes. The relative amounts of the two RNA species vary, however, between the sexes. Females contain more of the shorter transcript, which seems to be predominantly of ovarian origin (see Fig. 4B), whereas males always contain higher amounts of the larger transcript. The short transcript in males is mainly present in testes but less abundant than the larger transcript and thus is underrepresented in the RNA of whole flies (see Fig. 2A). The smaller species of 2.3 kb length corresponds to the size expected if the polyadenylation signal within the cDNA sequence is used. In some experiments this RNA species is resolved into two distinct bands; this heterogeneity could either be due to different degrees of polyadenylation and/or to the presence of a 60 nt longer variant, detected in head RNA (see legend to Fig. 1E). Three additional canonical polyadenylation signals can be found in the genomic sequence approx. 700 bp, 870 bp and 980 bp, respectively, downstream from the first polyadenylation signal mentioned above. A radioactively labelled PCR product from the 700 bp interval (see Fig. 1E) selectively hybridizes to the longer RNA species of 3 kb thus proving that one of the further 3' located polyadenylation signals is also used (Fig. 2A right).

To determine the exact size of the *noa* transcripts an RNaseH experiment was performed (Fig. 2B). Owing to incomplete

cleavage, intermediates are formed that can be accounted for by the following processes. The 3 kb RNA is initially reduced by 100 nt, which correlates well with polyadenylation signal 2 (see above). The RNA fragment at 2.5 kb results from an internal A-rich region within the longest transcript (A' in Fig. 1E) that binds oligo(dT) and thus is cleaved by RNaseH. The 2.3 kb RNA, which is generated from polyadenylation signal 1, is also initially reduced by about 100 nt. The shortest fragment at 1.4 kb finally results from cleavage at an A rich region at nt 1.372 of all transcripts (A in Fig. 1E, Fig. 2B left). Further digestion with RNaseH leads to almost complete cleavage to the 1.4 kb fragment, again supporting the above interpretation (Fig. 2B right).

NOA is a fatty acid elongase

The open reading frame extends from the second exon into the fifth and encodes a protein of 316 amino acids. It is common to all RNAs from the gene. In a computer search for known motifs the deduced protein shows similarity to a family of long chain fatty acid elongases. It contains all motifs that are found in fatty acid elongase (ELO) proteins (Fig. 3).

Searching the database with the NOA amino acid sequence reveals 20 similar proteins in the *Drosophila* genome, which reside in nine chromosomal regions (55E-F, 68A-B, 73B, 82B-C, 84F, 85E-F, 94B-C, 94D-E, 95D-E; supplementary material Table S1). For five of the genes no EST clones have been found so far. The ELO protein family is conserved among eukaryotic organisms and always consists of several members: yeast (3), *Caenorhabditis* (9), *Anopheles* (17), mouse (7), human (7), *Arabidopsis* (4), *Xenopus* (5), zebrafish (14) and *Dictyostelium* (8). The individual proteins differ in their N- and C-terminal ends, but are highly similar in their core region surrounding the histidine motif (see Fig. 3).

Among the *Drosophila* ELO proteins, NOA is the least conserved with a mean value of 18.3% amino acid identity to the other ELO proteins, which is in the same range as the conservation to the three yeast proteins SUR4 (also known as Elo3), FEN1 (Elo2) and Elo1 (19% identity). By contrast, the other *Drosophila* ELO proteins share 36.4% amino acid identity (mean value). In vertebrates the very long chain fatty acid elongase ELOVL6 shows the highest identity to NOA [45-46% in mouse (NP_569717), human (NP_076995) and in zebrafish (AAH59459); Fig. 3]. This value far exceeds the identities observed among the *Drosophila* proteins (see above), suggesting that NOA is the *Drosophila* homologue of ELOVL6.

P-element insertions lead to transcript reduction and to lethality

Using a balancer chromosome with an actin-GFP transgene we determined the lethal phase of the P alleles by screening for non-fluorescent individuals. In line *l(3)02281* homozygous third instar larvae were never observed. In this line a second P element resides at polytene region 61D. Thus, deleterious effects of both integrations could result in a more severe phenotype and could obscure dose-dependent quantitative effects in homozygotes. This is unlikely, however, since the lethal phenotype of *l(3)02281* is not complemented in crosses with a deletion in 73B (see Materials and Methods) or with the two other P-element lines for *noa* (see below) indicating that the lethality observed in *l(3)02281* is caused by the insertion

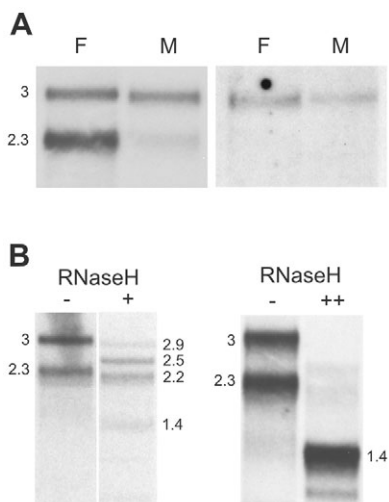
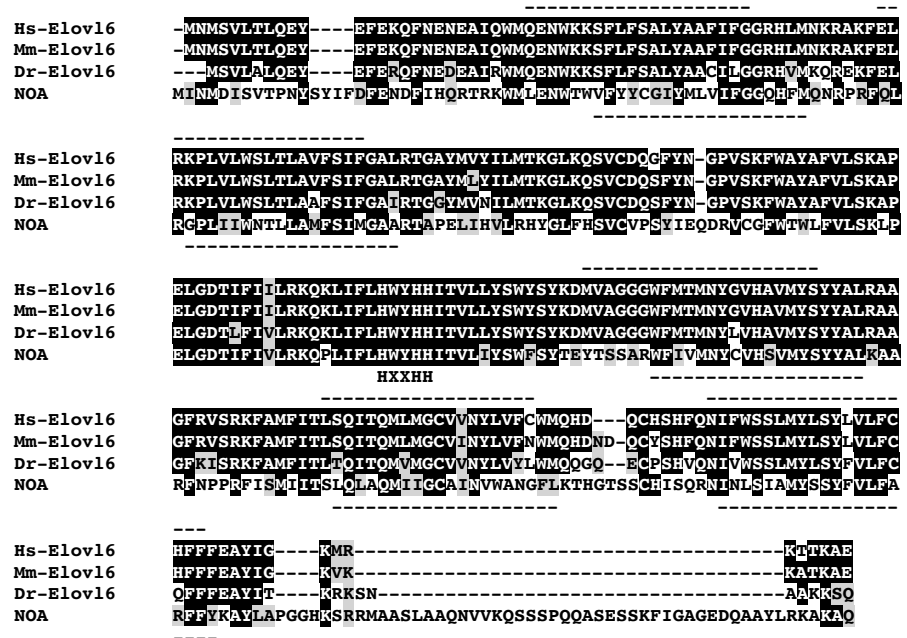


Fig. 2. Transcript characterization. (A) Northern blot showing the two sizes of *noa* mRNA of female (F) and male (M) OreR flies after hybridization with a *noa* cDNA next to a northern blot that was hybridized with the PCR fragment indicated in Fig. 1B. Transcript sizes are given in kb on the left. (B) RNaseH analysis. *OreR* mRNA was hybridized with oligo(dT), RNaseH treated to varying degrees (+, ++) and analyzed with a *noa* cDNA probe in parallel with untreated samples (-). The size of the different cleavage products is indicated in kb at the side of the blots.

Fig. 3. Amino acid alignment of NOA and its closest vertebrate relative ELOVL6. (Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Danio rerio*). Identical amino acids are shown on a black background, similar amino acids/conservative changes on grey background. The position of the transmembrane domains (Hofmann and Stoffel, 1993), which are common to all ELO proteins, is indicated by dashed lines above the sequences for the mammalian homologues and below the sequences for NOA. The conserved histidine motif HXXHH in the centre of the protein (aa 147-151) is indicated. It is a Fe-chelating ligand used for electron transfer in O₂-dependent redox reactions (Shanklin et al., 1994). A putative ER localization signal is found at the C terminus (di-lysine motif) (Gaynor et al., 1994; Schröder et al., 1995). Two potential N-glycosylation signals (NYS and NWT) at the N terminus suggest, that the N terminus resides in the ER and the C terminus on the cytoplasmic side as proposed for other ELO proteins (Tvrdik et al., 2000).



in *noa*. In addition, lethality could be rescued by the introduction of a wild-type copy of the *noa* gene (see below).

Homozygous *l(3)01895* individuals reached larval third instar and in the case of allele *l(3)04106* some homozygous individuals even survived to adulthood. These adult flies were severely impaired in motility and had a drastically reduced lifespan. Males proved to be sterile, but their testes showed no sign of a spermatogenesis defect and they contained motile sperm. Thus, their extremely impaired motility and vitality must be the reason for the observed sterility. Northern analyses demonstrated that all *noa* transcripts are synthesized in the mutant third instar larvae but at reduced levels compared with the wild type (Fig. 4A). More importantly, the extent of reduction parallels the effect on fitness and viability, i.e. RNA from homozygous *l(3)04106* larvae contained much higher levels of *noa* transcripts than RNA from *l(3)01895* larvae both in northern hybridizations (Fig. 4A) and in *in situ* hybridization experiments on larval tissue (data not shown). Compared with the constant levels of ribosomal protein L9 mRNA the amount of the 3.0 kb *noa* transcript was reduced to about half in *l(3)04106*, whereas the 2.3 kb transcript was almost undetectable. The latter is probably due to the fact that the 2.3 kb RNA is not synthesized in larval ovaries. In the case of *l(3)01895* even the 3.0 kb transcript was barely visible. Thus, the function of *noa* must be dose dependent. The analysis of viability in various allelic combinations supports the notion of a dose-dependent function of *noa*. The flies were just as affected as the occasional survivors mentioned above (data not shown).

To unequivocally correlate *noa* function with the observed phenotypes a rescue construct was generated (see Fig. 1E) and transformed lines carrying the *noa* transgene on the second or the first chromosome were established. To test whether this additional wild-type copy of the gene can restore viability, transgenic flies were crossed to the three different P-element-induced *noa* alleles. With seven of eight different integration lines homozygous *l(3)02281*, carrying one copy of the

transgene, survived to adulthood, although these flies still showed a reduced lifespan. Adult flies with a normal lifespan are observed when four copies of the *noa* transgene are introduced. This result is in agreement with our earlier interpretation that the encoded gene product has a dose-dependent effect and also indicates that the transgene does not support full wild-type activity.

noa is expressed in several tissues

As described above, two RNA species are synthesized from the *noa* gene (3.0 and 2.3 kb; Fig. 2A). In northern experiments

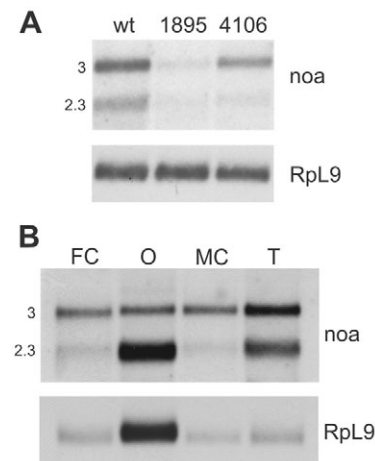


Fig. 4. Transcript levels in mutants and gonads. (A) RNA was isolated from wild-type (wt) third instar larvae and from larvae homozygous for the alleles *l(3)01895* (1895) or *l(3)04106* (4106). *Noa* transcripts were detected using a cDNA probe. (B) RNA was isolated from OreR ovaries (O) and testes (T) and separated next to RNA from female (FC) and male (MC) carcasses. RNA amounts in the samples of A and B were compared by hybridization to RpL9 mRNA (Schmidt et al., 1996).

these RNAs were shown to be present in both sexes, mainly in the gonads, but also to a lesser extent in the remaining parts of adult flies (Fig. 4B). Whereas the amount of the shorter transcript exceeds that of the larger transcript by far in ovaries, in testes – as in all other tissues – the larger transcript was still the predominant form of the *noa* RNA. In staged embryos the small RNA, of 2.3 kb, was found to be very prominent in 1-hour-old embryos but then quickly declined, in contrast to the increasing amounts of the 3 kb *noa* transcript (data not shown). It was also predominant in eggs laid by virgin females. Thus, the maternal store mainly consists of the small *noa* transcript. Apart from this difference both transcripts were found throughout development without significant qualitative or quantitative changes (data not shown).

To identify the cell types expressing *noa* three independent experiments were performed: (1) analysis of the β -galactosidase pattern generated by the enhancer trap function in the P-element insertion lines; (2) in situ hybridizations with *noa* cDNAs; and (3) analysis of the expression pattern of *noa* fusion genes in transgenic flies.

Whole-mount in situ hybridizations on embryos showed expression in the syncytial blastoderm (Fig. 5C), again demonstrating that *noa* transcripts are maternally provided (see above) since at this stage the embryonic genome is still transcriptionally inactive. In early cellular blastoderm, *noa* transcripts localized to the apical region of the newly forming cells (Fig. 5D,E black arrow) but the pole cells did not contain *noa* transcripts at all (Fig. 5E, white arrow). Later, transcript levels gradually decreased. They could be found at low levels in the involuting mesoderm as well as in the invaginating foregut and hindgut. During organogenesis, transcripts were again detectable and accumulated in the CNS (Fig. 5F) as well as in segmental groups of cells from the peripheral nervous system (PNS; Fig. 5G). The gene is still active in the CNS of third larval instar as judged by the persistence of β -galactosidase activity from a *noa-lacZ* reporter fusion (Fig. 5I). In addition, transcripts were present at low levels in all

imaginal discs (Fig. 5H). High expression levels were observed in the larval fat body by means of the enhancer trap function (see Fig. 1B and Fig. 8C).

In the adult fly *noa* was found to be transcribed in a variety of tissues such as regions with sensory organs, the fat body, the malpighian tubules, parts of the intestine (data not shown) and in specific cells of the gonads. In the ovary, hybridization occurred in the nurse cells as well as in the follicular epithelium that surrounds the growing follicle (Fig. 5B). In early stages of oogenesis germ cells showed β -galactosidase activity from the enhancer trap in all regions of the germarium prior to follicle formation (Fig. 5A). A clear cell-type-specific *noa* expression could be observed in adult testes. Transcripts accumulated around elongating spermatid bundles (Fig. 7E top). This is consistent with the observation that β -galactosidase expression in the enhancer trap lines occurs selectively in the cyst cells that surround each synchronously developing group of germ cells in postmeiotic stages (see below, Fig. 1A, Fig. 8A). Expression was also seen in the region of the terminal epithelium (Fig. 1A, Fig. 8A). In addition, a weak but reproducible expression was observed in the tip of the testis tubes (Fig. 7A).

A *noa-lacZ* reporter fusion containing the first 20 amino acids of the *noa* protein and a full length *noa*-GFP fusion protein displayed basically the same pattern as seen in the enhancer trap line and in in situ experiments (e.g. Fig. 7F and data not shown), indicating that the enhancer trap function faithfully represents the expression pattern of *noa*.

5' and 3' UTR are necessary for correct *noa* expression

To determine the amount of 5' flanking sequences necessary for *noa* regulation, promoter fusions to the *lacZ* gene were generated (Fig. 6). 204 nt and 493 nt of 5' flanking sequences together with 148 nt of the 5' UTR can no longer maintain *noa* expression when fused to a synthetic AUG in front of the *lacZ* gene (Fig. 6 top). If the entire *noa* 5' UTR (212 nt) with the translation start site is included in the construct, however,

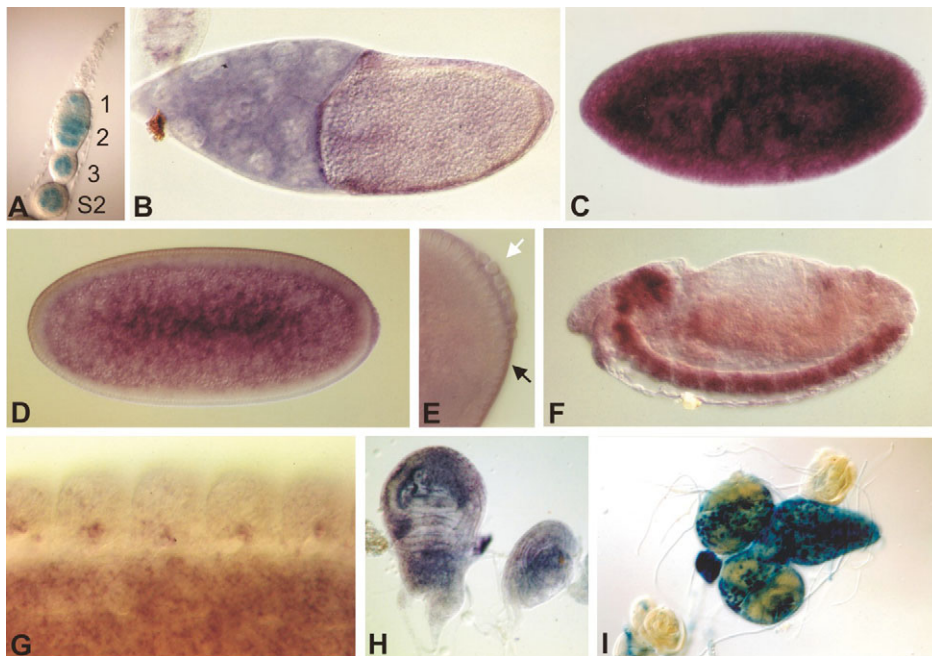


Fig. 5. Expression analysis.

(A) Expression pattern in the germarium at the beginning of oogenesis is shown via the enhancer trap function. Germ cells show β -galactosidase activity in all regions of the germarium (1-3) and in all follicle stages (e.g. S2). (B-H) In situ hybridizations to *noa* sequences are shown for a stage 10 follicle (B), an embryo at the syncytial blastoderm stage expressing maternal *noa* RNA (C), an embryo at the cellular blastoderm stage (D,E) with apical localization of the transcripts (black arrow in E) and pole cells without *noa* transcripts (white arrow, E), and an embryo around stage 8-9 with expression in the central nervous system (F) and in the PNS (G). Two imaginal discs (wing and leg) also contain *noa* transcripts (H). (I) Expression in a third instar larval brain is detected by X-Gal staining after expression of a *noa-lacZ* reporter gene.

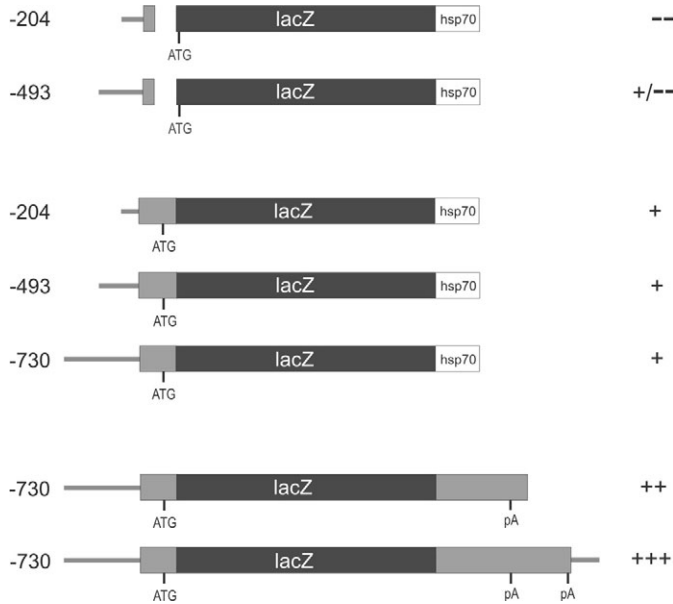


Fig. 6. Diagram of *noa-lacZ* reporter constructs. Grey lines represent flanking sequences of the *noa* gene. Their extent is indicated by the start nucleotide of the sequence. Grey boxes represent transcribed regions of the *noa* gene (for orientation pA signals are indicated in the 3' UTR). The *lacZ* coding region is shown in black. The top two constructs contain only a portion of the 5' UTR and a synthetic ATG in front of the *lacZ* coding region. Termination of transcription occurs in the *hsp70* 3' UTR (white box). The constructs in the middle contain the entire 5' UTR with the *noa* translation start codon together with varying sizes of the promoter region. The constructs at the bottom contain varying lengths of the *noa* 3' UTR. The relative intensities of resulting β -galactosidase activity are indicated on the right and refer to expression in all tissues.

correct expression is resumed (Fig. 6 middle). This may reflect a higher translation rate from the gene-specific translation start site or the presence of additional promoter elements in the 5' UTR.

Independent of the extent of 5' sequences in the gene fusion the 3' UTR is also necessary for correct *noa* expression (Fig. 6 bottom). A 1.1 kb segment including the first polyadenylation signal 930 nt after the stop codon increased overall expression. Addition of the sequences to the last possible polyadenylation signal, approx. 2 kb after the stop codon, increased the expression level further. Altogether expression was increased several fold compared to the construct with the *hsp70* 3' UTR. Furthermore, expression in imaginal discs, which was undetectable with the shorter 3' UTR, could now be observed (data not shown). This must mean that regulatory elements reside in both parts of the 3' UTR and that the shorter transcripts also contain some of these elements. Whether the increase in expression level is due to higher transcriptional activity or to increased stability of the RNA or even to altered translational efficiency has not been determined.

Cell type specific expression in the testis

To identify the cell types expressing *noa* in the testis, in situ hybridizations were performed. Transcripts were detected close to elongated spermatid bundles (Fig. 7E top). In situ

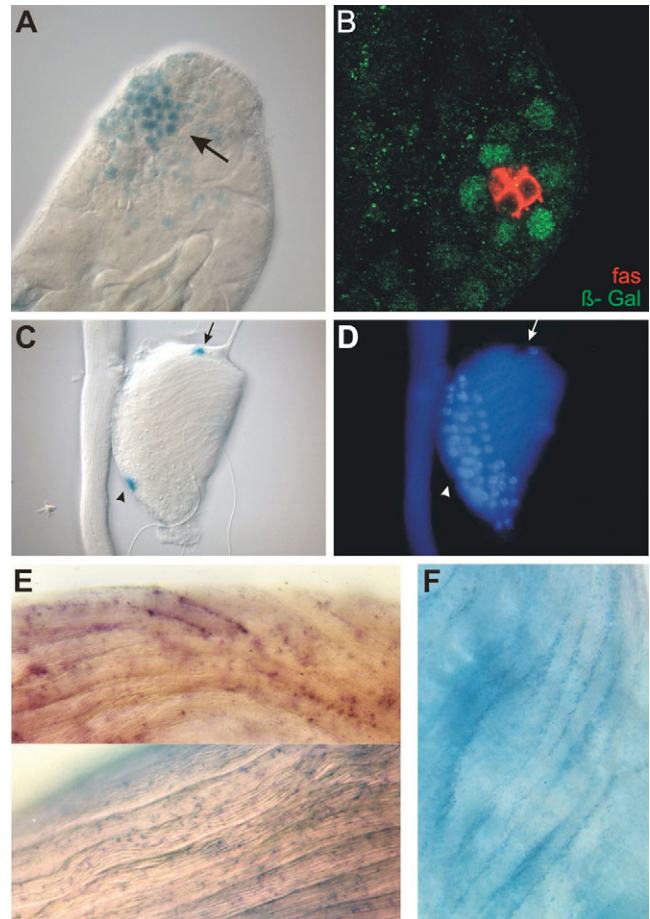


Fig. 7. *Noa* expression in the adult testis. (A) A group of cells in the testis tip of the enhancer trap line exhibits β -galactosidase activity. (B) Confocal microscopy after staining with anti-Fasciclin III antibody (to locate the hub cells, red) and anti- β -galactosidase antibody (green) proves close proximity of the two cell types. (C) Both head (arrowhead) and tail (arrow) cyst cell express β -galactosidase in an isolated cyst with elongating spermatids. (D) Nuclei are visualized using Hoechst 33258 staining. (E) In situ hybridizations on spermatid bundles are shown. The *noa* transcripts are found along the spermatid bundles, i.e. in the cyst cells (top). For comparison, the germ cell-specific transcripts of *Mst87F* are detected within the spermatid bundles (bottom). (F) NOA-*lacZ* fusion proteins are also detected around the spermatid bundles and not within the bundles.

hybridizations with a germ-cell-specific probe *Mst87F* (Kuhn et al., 1988a) that labels the entire bundle (Fig. 7E bottom), *noa* transcripts were seen at the periphery of the spermatid bundles, i.e. in the cyst cells. The staining along the elongating bundles must reflect *noa* activity in the tail cyst cells, whereas an intense staining in the region of the terminal epithelium could reflect activity in the head cyst cells and/or in the cells of the terminal epithelium itself (data not shown). This cell-type-specific expression was also observed using the enhancer trap properties of the residing P elements where *lacZ* expression was detected in nuclei of only those cyst cells that surround postmeiotic stages of sperm development (see also below, Fig. 1A,B and Fig. 8A,C). A *noa-lacZ* fusion and a *noa*-GFP fusion exhibited the same expression pattern (e.g. Fig.

7F). Thus, cyst cell expression of *noa* starts after the surrounded germ cells have completed meiosis and differentiation begins. Earlier cyst cell stages are free of *noa* RNA and protein.

In situ hybridizations in cyst cells are at the limits of detection because of the very thin cell body. Therefore, since the enhancer trap activity proved to faithfully reproduce the gene activity of *noa*, we used the enhancer trap function to clearly demonstrate expression in both cyst cells. Fig. 7C shows an isolated cyst containing early elongating spermatids surrounded by two cells with β -galactosidase-positive nuclei. Hoechst 33258 staining, to locate the spermatid nuclei, permits the identification of the head (arrowhead) and the tail cyst cell (arrow, Fig. 7D).

The cells that exhibit β -galactosidase activity in the testis tip were analysed by antibody staining. An antibody against Fasciclin III was used to identify the cells of the hub (Fig. 7B, red). Anti- β -galactosidase antibody then revealed that the *lacZ*-positive cells are clustered around the hub in a loose arrangement (Fig. 7B, green). As judged by their number (up to 20), counted in stacks using the confocal microscope, these cells should represent the cyst progenitor cells and not the stem cells of which there should be five to seven.

Cyst cell-germ cell communication is necessary for *noa* gene activity

Since cyst cells are postulated to communicate with germ cells the question arises of whether the development of cyst cells is coordinated with germ cell development, i.e. whether expression of *noa* is dependent on the development of the encased germ cells. This can be tested if enhancer trap alleles are crossed into mutants that block spermatogenesis (*benign gonial cell neoplasm; bgcn*) (Gateff, 1981) or do not contain any germ cells at all (*tudor; tud*) (Boswell and Mahowald, 1985) and alterations in the β -galactosidase pattern are monitored in comparison with the wild-type situation (Fig.

8A,B). In *bgcn* mutant testes in which spermatogenesis stops during the mitotic proliferation phase and postmeiotic stages never develop β -galactosidase activity is seen in a tightly packed ring of nuclei towards the end of the testis tube (Fig. 8E). These nuclei are too close together to be cyst cell nuclei that surround germ cells. Rather they are nuclei of the terminal epithelium (the extent of which is shown in Fig. 8A,E,G,H in the region between the arrowhead and the arrow). This can be seen very clearly in *tudor* mutant testes which do not contain any germ cells at all (Fig. 8G). Here the seminal vesicle and the region of the terminal epithelium are well separated and β -galactosidase-positive nuclei are seen at the beginning of the terminal epithelium (black arrowhead in Fig. 8G). Thus, *noa* expression in cyst cells seems to be dependent on signals emanating from the germ cells or on the differentiation state of

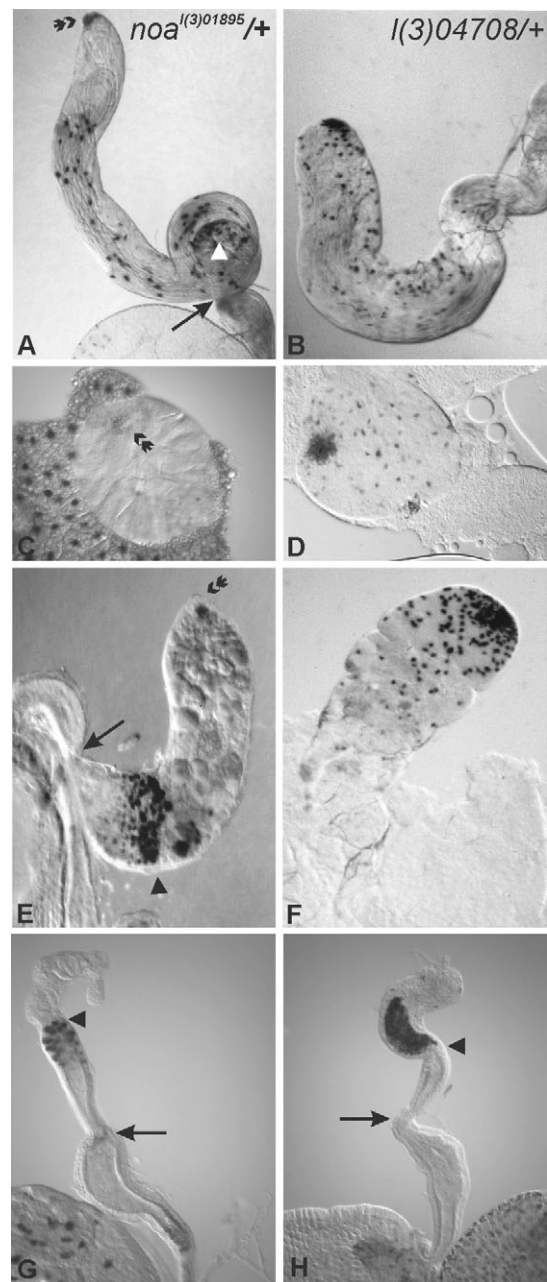


Fig. 8. *Noa* expression in cyst cells is dependent of germ cells. Expression patterns are compared for postmeiotic cyst cell expression in *noa*^{l(3)01895/+} testes (A,C,E,G) and premeiotic expression in *l(3)04708/+* testes (B,D,F,H). The normal enhancer trap pattern is shown in adult (A,B) and in larval testes (C,D). Apical expression is indicated by a double arrow in A,C and E. The larval testis anlagen demonstrate the postmeiotic expression in *noa*^{l(3)01895/+}, since they contain only premeiotic stages of spermatogenesis and show no staining in cyst cells. In addition, expression in the nuclei of the fat body cells is visible (C). Premeiotic expression, starting with the progenitor cells, is clearly visible in larval *l(3)04708/+* testes (D). The enhancer trap pattern is altered but β -galactosidase activity can still be observed in *bgcn* mutant testes (E,F) and in germ cell free *tudor* testes (G,H). The patterns are distinctly different and in the case of *l(3)04708/+* correlate well with premeiotic expression (F). The modification in the pattern is due to the altered testis shape resulting from lack of elongated stages. In the case of *noa*, expression at the testis base is apparently due to activity in the cells of the terminal epithelium (E,G), as can best be seen in G, where the various parts of the testis base are well extended. The beginning of the terminal epithelium is indicated by an arrowhead, the beginning of the seminal vesicle by an arrow in A,E,G,H. Note that enhancer trap activity is in the terminal epithelium in *noa*^{l(3)01895/+} testes, but clearly above that region in *l(3)04708/+* testes (G,H).

the cyst cells themselves. The β -galactosidase-positive nuclei at the tip of the testis (double arrow in Fig. 8A,C,E) remain. By contrast, in the enhancer trap line *l(3)04708* the cyst cell nuclei are labelled at the cyst cell progenitor stage and throughout the proliferation phase (Fig. 8B,D; M.A.S., unpublished). This enhancer trap line still shows β -galactosidase-positive nuclei in the tip region of the testes when crossed into a *bgen* mutant background (Fig. 8F). In the *tud* mutant testes β -galactosidase activity is observed in a region above that of the terminal epithelium, demonstrating that this activity is independent from a germline signal and that cyst cells are still present in agametic testes. In addition, the number of stained nuclei proves that the cyst progenitor cells have undergone mitoses even though cysts cannot be formed.

Cyst-cell-specific *noa* expression is required for normal spermatogenesis

To specifically affect *noa* transcripts in the cyst cells we generated a PPY-GAL4 line with the promoter of the cyst-cell-specific phosphatase PPY (Armstrong et al., 1995). This was used to activate a *noa* RNAi construct. Since at 25°C fertility was only partially affected, cultures were shifted to 29°C to enhance Gal4 expression levels. At this elevated temperature fertility is generally affected and OreR control males show almost no sperm motility whereas sperm development itself appears completely normal. By contrast, sperm cell development was specifically affected in the males expressing the PPY-GAL4 driven *noa* RNAi construct (Fig. 9). Many testes were smaller and contained fewer germ cells or cysts than the wild-type control (Fig. 9A,B). The number of individualisation cones (ICs) was drastically reduced. If ICs had formed they frequently appeared dispersed and overall morphology was distorted (Fig. 9F). In bundles with ICs next to their nuclei frequently some nuclei were dislocated and had no IC (Fig. 9D) whereas in wild-type testes the occasional mislocated nucleus would be accompanied by an IC, as in the rest of the bundle (Fig. 9C). Many elongated spermatid nuclei were observed scattered around in the massively enlarged region of the terminal epithelium. In addition, no individualized sperm could be found in the seminal vesicles (Fig. 9B,D). In general, the effect of RNAi was very variable, ranging from a severe phenotype (Fig. 9A) to a weak phenotype leaving testis morphology relatively normal (Fig. 9F). In an analogous experiment with the T80-GAL4 driver line, which induces expression in imaginal discs, complete lethality resulted, proving the functionality of the *noa* RNAi construct. Phosphatase PPY was shown to be weakly expressed also in spermatocytes (Armstrong et al., 1995). To formally rule out that the observed effects could result from RNA degradation in spermatocytes we raised the flies at 18°C, shifted them to 29°C and analyzed germ cell morphology 4–5 days later when the early postmeiotic stages had developed into individualizing sperm. Under these conditions the results were the same, thus proving that postmeiotic cyst cell expression is the cause for the defect.

Noa expression is not increased by lower temperatures

The first elongase described in the mammalian system (CIG30) (Tvrdik et al., 1997) was found to be involved in the recruitment of brown adipose tissue, and its transcription was increased upon cold treatment. In addition, a male-specific elongase in *Drosophila* has been described recently that

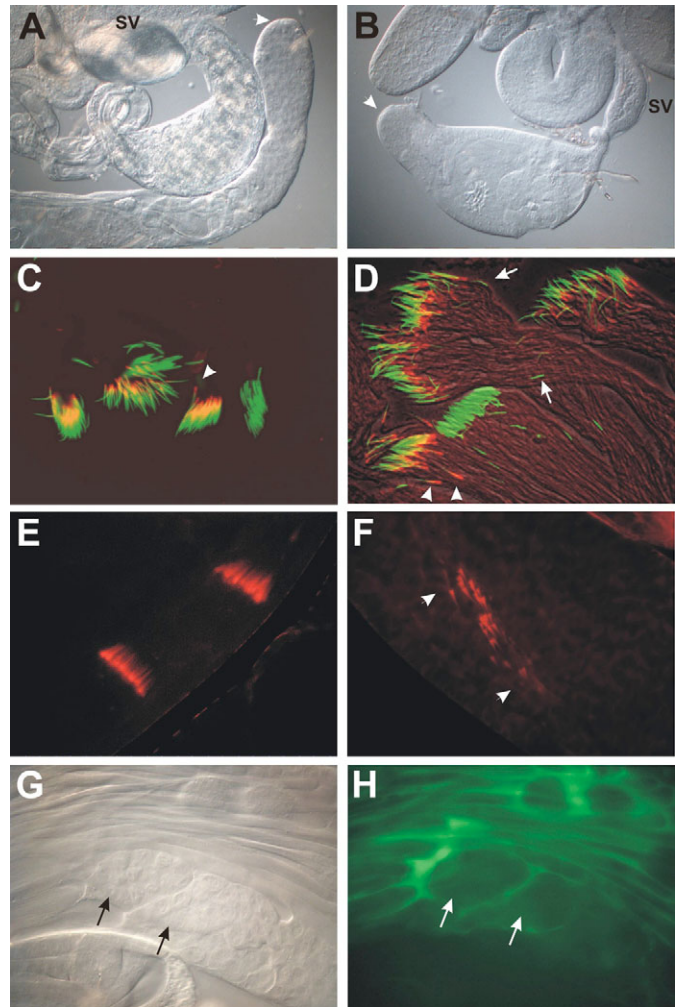


Fig. 9. *Noa* expression in cyst cells is necessary for sperm development. Wild-type testes (A,C,E) and testes from transgenic animals carrying a PPY-Gal4-driven UAS-*noa*-RNAi construct (B,D,F) were analyzed by microscopy. Testis shape is altered (testis tip is indicated by a white arrowhead) and the small seminal vesicle (sv) remains empty (compare A with B). The testis shown in B represents a severe case of the variable RNAi phenotypes. (C–F) Squash preparations of testes with Hoechst 33258 (green) and TRITC-coupled phalloidin (red) staining. (D) Testes with a weak phenotype showing displaced nuclei that do not show an individualization cone even though the rest of the cyst does (arrows in D). In the wild-type sometimes nuclei are also displaced but these then also contain an individualization cone (arrowhead in C). Individualization cones of the 64 spermatids in a cyst are found dispersed after staining with TRITC-coupled phalloidin (region between the white arrowheads in F) in contrast to the synchronized individualization complexes observed in wild-type cysts (E). The testis in F represents a weak phenotype after RNAi. The expression characteristics of the PPY-driver line are demonstrated by a UAS-GFP transgene (G,H). Cysts containing early stages of spermatogenesis (e.g. spermatocytes, arrows in G,H) are stained all around proving premeiotic expression in both cyst cells by the PPY driver.

showed higher transcript levels at 21°C compared with 24°C and 29°C (*elo68a*) (Chertemps et al., 2005). This prompted us to test whether *noa* transcript levels are also

temperature dependent. Newly eclosed adult flies were kept at 10°C for 1 or 3 days, before northern analyses were performed. There was no increase in any of the three transcripts at 29°C compared with the situation at 24°C. Transcript levels were also not increased in third instar larvae after the same cold treatment. In addition, if adult flies or third instar larvae were kept for 4 days at 21, 24 or 29°C, transcript levels again remained unaltered (data not shown).

Discussion

We have presented the cloning and expression pattern of *noa*, a gene encoding a long chain fatty acid elongase. P-element-induced *noa* mutant alleles are lethal or semilethal, depending on the level of *noa* expression in these alleles. As demonstrated by rescue experiments and precise excision of the residing P elements, loss or even only reduction of *noa* expression is the cause of lethality. Thus, *noa* is an essential gene and its function is dose dependent.

In *Drosophila* as in mammals and yeast the majority of fatty acids are composed of species with a maximum chain length between 14 and 18 carbon atoms. A minority, the so called 'very long chain fatty acids' consist of 20 or more carbon atoms. They result from further elongation by the very long chain fatty acid elongases (elo vl) and participate in many biological processes since they are the substrate for sphingolipid formation. Sphingolipids are important for cell growth (Wells and Lester, 1983; Hanada et al., 1992), contribute to the epidermal water barrier (Wertz, 1992) and play a role in cell recognition and adhesion (Hakomori and Igarashi, 1995). In addition, sphingolipids and their degradation products are involved in signal transduction (Hannun, 1996; Spiegel and Merrill, 1996; Testi, 1996) and formation of lipid rafts (Simons and Ikonen, 1997).

Data relating to the function of long chain fatty acid elongases have been gathered mostly for yeast [three genes, reviewed by Tehlivets et al. (Tehlivets et al., 2007)]. For mouse and human, seven genes have been reported so far (reviewed by Leonard et al., 2004). The first reported gene in mouse, *Elov13* (also known as Cig30) (Tvrdik et al., 1997) plays a role in recruitment of brown adipose tissue upon cold stimulation. In addition, it is important for the formation of neutral lipids that are necessary for normal skin function (Westerberg et al., 2004). In the human, a mutation in *ELOVL4* causes Stargardt-like macular dystrophy, a dominant form of juvenile macular degeneration (Edwards et al., 2001; Zhang et al., 2001). A high expression in testes is found for *ELOVL5* (also known as HELO1) (Leonard et al., 2000) as well as for *ELOVL6* (see below). In comparison with *Drosophila* (and other insects) the number of genes found in mouse and man is rather low. Comparable heterogeneity is generated in this system, however, by a greater variability in expression at the transcript and the protein level for most of the genes. For *ELOVL5*, for example, ten different transcripts are found, from which seven protein isoforms could be generated (AceView at NCBI).

NOA is the homologue of the mammalian ELOVL6, which is involved in the addition of 2-carbon units to C12-C16 fatty acids (LCE) (Moon et al., 2001). *Elov16* is highly expressed in liver, brown adipose tissue and also in testes. For this gene, too, five different transcripts have been found, which could generate four different protein variants. Tissue-specific expression, however, has not been described.

In *Drosophila* not much is known about long chain fatty acid elongases, although 20 genes can be identified by their homology to the three yeast genes *Elo1*, *FEN1* (Elo2) and *SUR4* (Elo3). For five of these genes the expression is unclear since EST clones have not been identified yet. The large number of elongases in the *Drosophila* genome could permit highly cell-type-specific expression. Thus, the various genes could cover different and complementing needs for long chain fatty acids. In this case reduction of activity for any of the genes should result in a phenotype. Alternatively, the large number of ELO genes could be expressed in largely overlapping patterns and one gene might be able to compensate for the loss of another gene. Recently one of these elongases, *elo68α*, has been reported to be expressed male specifically in cells of the reproductive tract (Chertemps et al., 2005).

Knockdown of *noa* activity in imaginal discs resulted in lethality proving that compensation by other elongases does not occur in this tissue. Also, reduction of *noa* activity in general leads to reduced viability or even lethality. Apparently none of the other elongases identified in the *Drosophila* genome can compensate for the decrease in *noa* function in any of the affected tissues, even though several genes are expressed in the same tissues as judged by the EST clones. Thus, *noa* should have a function that is distinct from that of other elongases in the *Drosophila* genome. Such a unique function may also be reflected by the low level of similarity to all other *Drosophila* elongases. By contrast, when the *noa* RNAi construct was selectively driven in the follicle cells, no phenotype was observed. Thus, in the follicle cells other fatty acid elongases must be able to compensate for the loss of *noa* activity.

The *noa* 3' UTR is unusually long (900 nt to polyadenylation signal 1 and 1.6 kb in the case of polyadenylation signal 2). This it is about half the size of the entire mRNA and suggests that regulatory elements reside within this gene segment. In accordance with this assumption, addition of the 3' UTR to *lacZ* fusions enhanced the overall expression level dramatically and allowed expression in imaginal discs to be observed. A very long 3' UTR was also reported for the NOA homologue in mouse ELOVL6 (also known as FACE) (Moon et al., 2001; Matsuzaka et al., 2002) and for ELOVL2 (also known as SSC2), which shows particularly high expression in testes (Tvrdik et al., 2000). By contrast, ELOVL1 (SSC1) contains a very short 3' UTR (Tvrdik et al., 2000). Sequence analyses gave no indication for an involvement of micro RNAs in the regulation via the long 3' UTR.

In the male gonad *noa* expression is mainly observed in the cyst cells and in cells of the terminal epithelium. Cyst cell expression is restricted to the postmeiotic stages, when spermatid differentiation takes place. In earlier analyses it was demonstrated that the germ cells affected cyst cell fate during early spermatogenesis (Gönczy and DiNardo, 1996). In agametic testes, cyst cell proliferation continued and some cyst cells adopted hub cell fate. In agreement with these data, germ cell maturation seems necessary for correct onset of *noa* expression in the surrounding cyst cells. Its 'postmeiotic' expression is prevented if germ cells are either not present or never reach the corresponding maturation state. Only expression in the terminal epithelium can be observed. In the tumorous testes of the *bgen* mutant it cannot be excluded that the β-galactosidase-positive nuclei are composed of cyst cell

nuclei and nuclei of the terminal epithelium. In that case, however, cyst cells must have survived the degeneration process of the undifferentiated germ cells and become concentrated in the area of the terminal epithelium. By contrast, in *l(3)04708* flies cyst-cell-specific expression is maintained during the proliferation phase. In the case of the mutant *bgen* this could be due to the fact that expression is initiated early, when germ cell development is still correct. However, it is also initiated when germ cells are not present as in *tud* testes. Therefore, this activity is independent of germ cell development.

When activity of the specifically testis-expressed elongase *elo68a* in *Drosophila* was reduced to 1%, a 50% decrease of vaccenyl acetate levels was reported, but no effect on fertility or viability resulted (Chertemps et al., 2005). By contrast, a cyst cell-specific knockdown of *noa* did result in male sterility. The defect is observed at the end of spermatogenesis when sperm should individualize and enter the seminal vesicle. Instead they seem to separate before maturation and then disintegrate. If indeed *noa* function in cyst cells is part of a signal to the germ cells it could be possible that *noa* knockdown results in too low a signal to permit engulfment by cells of the terminal epithelium. Guidance cues might be missing and final maturation of sperm is prevented. Future experiments are aimed at identifying other components of this potential signalling pathway in which *noa* plays a role.

Materials and Methods

Fly strains and culture

A collection of embryonic-lethal enhancer trap lines (Spradling et al., 1999) induced by insertion of the PZ element (Mlodzig and Hiromi, 1992) was kindly provided by H. Jäckle. In this collection we identified *l(3)01895* and *l(3)04106* with cyst-cell-specific β -galactosidase expression.

Enhancer trap line *l(3)02281* was described in FlyBase as noncomplementing to *l(3)01895* and *l(3)04106*. We found that *l(3)02281* contains a second P insertion in polytene region 61B3. It is located in the short intergenic region between the divergently transcribed genes *E(bx)* and *mth114*.

Strain *l(3)02281* as well as the deficiency strains *Df(3L)st8P*, *In(3L)P*, *ry*TM6B* and *Df(3L)st4*, *gl[2]e[4]TM6* were obtained from Bloomington Stock Center. *bgen* mutant flies were kindly provided by E. Gateff and *tudor* mutant flies by R. Boswell.

All fly cultures and crosses were kept on standard fly medium at 25°C or 18°C.

Molecular biology

All methods followed standard protocols (Sambrook et al., 1989).

Poly(A)⁺ RNA was isolated directly from tissues and developmental stages using oligo(dT)-coated beads (Dynal and Novagen). RNAs were separated in formaldehyde-agarose gels, transferred to Nylon membrane (Hybond-N, Amersham) and crosslinked by UV light. Hybridization with α -³²P-labeled in vitro transcripts was performed as described previously (Kuhn et al., 1991). RNaseH experiments were performed as described in Hollmann et al. (Hollmann et al., 2002).

Plasmid rescue of P element flanking genomic sequences was performed as described in Steller and Pirrotta (Steller and Pirrotta, 1985). A 14.5 kb long genomic clone was isolated by screening a bacteriophage λ library (Stratagene) with the genomic plasmid rescue clone. cDNA clones were obtained by screening a custom made testis cDNA library (Stratagene) with genomic fragments. Sequencing of genomic and cDNA clones was done by the dideoxy method (Sanger et al., 1977). Database searches were performed with the BLAST program (Altschul et al., 1990) using the WWW server at NCBI and the WWW server of the Berkeley Drosophila Genome Project (BDGP). Alignments were done with the ClustalW program (Higgins et al., 1994) at the EMBL-EBI server and processed with the Jalview program (Clamp et al., 2004). Sequences were deposited under the accession nos. AF279257, AF279258 and AH009765. Sequence information on more than 50 ESTs was derived from BDGP.

For the rescue construct, a genomic clone was combined with an RT-PCR-derived fragment and one of the cDNA clones to include 727 bp upstream of the transcription start site. The 3' part of the gene was added from a genomic clone that extends 685 bp beyond the last polyadenylation signal. The combined fragments were cloned into pCasPer4 (Pirrotta, 1988).

For promoter *lacZ* fusions, PCR products of the desired length were amplified with primers containing suitable restriction sites (*Xba*I and *Kpn*I for pWATGlacl

and *Kpn*I for pWlac2). For constructs excluding part of the *noa* 5' UTR pWATGlacl (Kuhn et al., 1988b) was used, which contains a synthetic ATG codon. The *lacZ* vector pWlac2 (J. Weinert and U. Schäfer (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany), personal communication) was used for fusions of the *noa* 5' UTR (including the *noa* translation start site) to *lacZ*. To test the role of the 3' UTR, PCR-amplified 3' UTR fragments were ligated into a *Bam*HI site at the 3' end of the *lacZ* cassette in the existing pWlac2 constructs.

For a *noa*-GFP fusion construct a PCR fragment containing the *noa* promoter and the coding region was amplified using the rescue construct (see above) as a template. This fragment was cloned in frame to the GFP cassette in the pUAST-green vector (U. Renner, M.H. and M.A.S., unpublished). The UAS sequences of the vector remain in front of the gene's promoter and have no influence on GFP expression even if a GAL4 driver is crossed into the transgenic flies (M.H., unpublished).

For the RNAi construct the *noa* coding region was amplified and cloned back to back separated by a 400 bp GFP stuffer fragment into pUAST (Brand and Perrimon, 1993), as in Piccin et al. (Piccin et al., 2001). To drive the *noa* RNAi construct specifically in cyst cells, the PPY-Gal4 line was established. A 1.5 kb promoter fragment of PPY (Armstrong et al., 1995) was amplified, cloned into the vector p221-Gal4. All constructs were injected into *w¹¹¹⁸* embryos according to the standard protocol (Spradling, 1986).

Whole-mount in situ hybridizations with digoxigenin-labelled DNA probes were performed following the protocol of Tautz and Pfeifle (Tautz and Pfeifle, 1989) with modifications as described by Hollmann et al. (Hollmann et al., 2002).

β -Galactosidase staining was performed according to Glaser et al. (Glaser et al., 1986), using glutaraldehyde as fixative. To reduce nonspecific background staining 0.25% Triton X-100 was added to the staining solution. Isolated cysts were counterstained under the coverslip with 2 μ g/ml Hoechst 33258 using a filter paper to soak off the buffer and replace it with staining solution.

Testes of PPY-GAL4/*noa*-RNAi and OregonR wild-type flies as control were dissected in PBS, fixed for 20 minutes with 4% formaldehyde in PBS and washed twice in PBT. Staining for nuclei (1 μ g/ml Hoechst 33258) and individualization cones (0.25 μ g/ml TRITC-phalloidin) was for 10 minutes followed by three washes for 10 minutes each in PBS. Testes were mounted in PBS-glycerine (1:1) and analyzed by epifluorescence microscopy (Zeiss Axiophot).

For antibody stainings, testes were fixed for 20 minutes with 4% formaldehyde in PBS and then washed twice in PBT. Primary and secondary antibody incubations were performed overnight at 4°C with several washes in PBT at room temperature. Rabbit anti- β -galactosidase (Cappel) was used at 1:500; mouse anti-Fasciclin 1:50; Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes) 1:400; Cy3-conjugated sheep anti-mouse (Sigma) 1:200. Testes were mounted in PBS-glycerine (4:1) and analyzed by epifluorescence microscopy (Zeiss Axiophot) and by confocal microscopy (Leica TCS-SP).

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