Expression and regulation of caudal in the lower cyclorrhaphan fly Megaselia

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Abstract The homeobox gene caudal (cad) regulates posterior development in Drosophila. In early embryos, the cad protein (CAD) is expressed in a posterior-to-anterior concentration gradient, which contributes polarity to the developing embryo. The CAD gradient is complementary to and dependent on the anterior pattern organizer Bicoid (BCD), which represses the translation of ubiquitous maternal cad transcripts in the anterior embryo through a direct interaction with the cad 3′ untranslated region (UTR). Here, we show that early embryos of the lower cyclorrhaphan fly Megaselia express the putative cad orthologue Mab-cad throughout the posterior three quarters of the blastoderm but lack maternal transcripts. In transgenic blastoderm embryos of Drosophila, Mab-cad cis-regulatory DNA drives the expression of a reporter gene in a similar pattern, while Mab-cad 3′ UTR fails to mediate translational repression of a ubiquitously transcribed reporter. For another lower cyclorrhaphan fly (Lonchoptera) and two related outgroup taxa of Cyclorrhapha (Empis, Haematopota), we report maternal cad expression in ovarian follicles. Together, our results suggest that BCD is not required for the translational repression of Mab-cad, and that maternal cad expression was lost in the Megaselia lineage.

Keywords Gene expression · Enhancer · Translational repression · Evolution · Brachycera

Introduction

The protein products of the homeobox gene cad (CAD) accumulate in a concentration gradient across early insect embryos and are an important factor of posterior patterning. cad-deficient Drosophila embryos exhibit severe defects in the abdomen and thorax (Macdonald and Struhl 1986; Wu and Lengyel 1998), and cad RNA interference (RNAi) in short-germ insects (Tribolium, Gryllus) yielded embryos that consist of the prothorax head only (Copf et al. 2004; Shinmyo et al. 2005). In Drosophila, the full complement of embryonic cad activity requires maternal and zygotic transcripts (Macdonald and Struhl 1986). Heterozygous cad embryos from heterozygous mothers only lack terminal structures including anal tufts, parts of the anal pads, and posterior sensory organs. In these embryos, maternal cad expression is sufficient for the proper formation of segments. Conversely, ectopic cad expression in the anterior embryo causes head defects (Mlodzik et al. 1990; Niessing et al. 1999; Niessing et al. 2000; Cho et al. 2005).

The CAD gradient of Drosophila forms by translational repression of ubiquitous early cad transcripts in the anterior embryo, and is mediated by the protein products of bicoid (BCD) and d4EHP (Dubnau and Struhl 1996; Rivar-Pomar et al. 1996; Cho et al. 2005). The current model of
this mechanism proposes that ubiquitous d4EHP binds to the 5' cap structure of cad mRNA and to BCD, which also directly binds to a BCD binding region (BBR) in the 3' UTR of cad mRNA, thereby circularizing the transcript and preventing translation initiation (Niessing et al. 2002; Cho et al. 2005). In other insects, a mechanism of CAD gradient formation has not been elucidated. In the parasitic wasp Nasonia, cad transcripts are localized at the posterior pole and a CAD protein gradient may be formed by diffusion (Olesnicky et al. 2006). In the silk moth, Bombyx, and in the red flour beetle, Tribolium, translational repression of ubiquitous maternal cad transcripts occurs in the anterior embryo but these insects, like Nasonia, lack a BCD orthologue (Xu et al. 1994; Schulz et al. 1998).

Here, we report data on the expression and regulation of a cad homologue (Mab-cad) from the lower cyclorrhaphan fly Megaselia abdita (Phoridae). We show that Mab-cad expression in the blastoderm is similar to the expression of cad in the higher cyclorrhaphan fly Drosophila. Unlike cad, however, Mab-cad is not expressed in ovarian follicles, and early embryos therefore lack maternal transcripts. A lacZ reporter construct containing ca. 8 kb of Mab-cad cis-regulatory DNA upstream of the first Mab-cad exon essentially recapitulates the endogenous early Mab-cad pattern in transgenic Drosophila, while the Mab-cad 3' UTR fails to suppress the anterior translation of a ubiquitously transcribed reporter gene. Finally, we report the expression of cad homologues in the ovarian follicles of three related dipterans (Lonchoptera, Empis, and Haematopota). Our results suggest the loss of maternal cad expression in the Megaselia lineage and that BCD is not required for the translational repression of Mab-cad.

**Materials and methods**

**Sample collection**

M. abdita Schmitz (Phoridae) was obtained from our laboratory culture. Adults of Empis livida L. (Empididae), Haematopota pluvialis L. (Tabanidae), and Lonchoptera lutea Panzer (Lonchopteridae) were caught in the surroundings of Göttingen (Germany).

**Cloning procedures**

Partial homeobox sequences of cad homologues were isolated by polymerase chain reaction (PCR) using the

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**Fig. 1** Characterization of Mab-cad. a Genomic organization of the Mab-cad locus. Restriction sites of CiaI (C), EcoRI (E), HindIII (H), PstI (P), SpeI (S), and XbaI (X) on two phage clones (Φ5, Φ2) are shown above alternative splice variants of Mab-cad. ORF sequence is shown as filled boxes. Mab-cad expression is shown at syncytial blastoderm (b), blastoderm cellularization (c), cellular blastoderm (d, d'), germband extension (e–f'), germband retraction (g, g') stage, and shortly before dorsal closure (h, h'). All embryos are shown in lateral view with anterior to the left. Embryos at later stages are also shown in dorsal view (d', e', f', g', h'). Amniotic expression (black arrows), posterior embryonic expression (white arrows), and posterior midgut expression (arrowhead) relative to the position of the unstained hindgut (white arrowheads) are indicated. i Northern Blot with poly (A) RNA from ovaries (ov), early (0–3 h) and late (3–17 h) embryos hybridized with Mab-cad probe (top) and subsequently with a Mab-rpL35 probe (bottom). RNA standard sizes in kb are shown at the left margin.
degenerate primer pair 5’ AAR ACS CGB ACS AAR GAY AA / 5’ VCG VCG RTY YTG RAA CCA RAT on genomic DNA. 5’ and 3’ rapid amplification of cDNA ends (RACE) reactions were performed using MARATHON and SMART cDNA Amplification Kits (Clontech) starting with ovariial (Empis, Haematopota), embryonic (Megaselia), and adult (Lonchoptera, Megaselia) poly(A+). A Megaselia riboprotein L35 gene fragment was accidentally recovered in a PCR with degenerate primers and used as a positive control in the Northern Blot analysis. All sequences have been deposited under EU295452–EU295458 in the GenBank database.

Two genomic phages, which include the Mab-cad locus, were obtained from a Lambda-Fix II (Stratagene) phage library of adult flies (unpublished). Genomic DNA for the reporter construct was PCR amplified using TaKaRa LA Taq polymerase (TaKaRa) on Lambda-Fix II phage clone Φ5 using 5’ TTT CGA TTG CAC TAA ATA A and M13 (−20). The product was cloned into pGEM-T Easy (Promega), excised with NotI and cloned into pCaSpeR-hs43-lacZ (Thummel and Pirrotta 1992). A clone with the insert in normal orientation relative to the transcription start was used for transgenic experiments.

To assay translational control in the Drosophila blastoderm, we generated a construct containing the Mab-cad open reading frame (ORF; 981 bp including start and stop codon) with an N-terminal hemagglutinin (HA)-tag (ATG TAC CCA TAC GAT GTG CCA GAT TAC GCC) as reporter gene. At the 5’ end, the reporter gene was flanked by the maternal hunchback promoter (nucleotides 1 to 1512 of GenBank accession Y00274; Tautz et al. 1987). At the 3’ end, AscI, MluI and FseI cloning sites and the SV40 small t antigen 3’ UTR (853 bp) were added. The cassette was cloned into the vector pCaSpeR4 (Thummel and Pirrotta 1992) using BclHI. Drosophila cad 3’ UTR (856 bp), truncated cad 3’ UTR downstream of the BBR (343 bp) and Mab-cad 3’ UTR (559 bp) were PCR amplified with the following primer pairs, respectively: 5’-GGC GCG CCA CGA CCA TTC CTG TTA TGC / 5’-GGC CGG CCG AGT TGC TTT ATC TAT GGT G, 5’-GGC GCG CCG TTA AAC ATA ATT TTA GTA CAA GC / 5’-GGC GCG CCG AGT TGC TTT ATC TAT GGT G, and 5’-GGC GCG CCA TAT TGG GAG AAC CAC GTC / 5’-GGC GCG CCG AAT GAT TAA AAA TTT TAT TTA CTC. These primers contain AscI (upper primers) or FseI (lower primers) endonuclease restriction sites that were used for directional cloning of the 3’ UTRs into the reporter cassette.

In situ and Northern hybridization and antibody staining

In situ and Northern hybridizations were done as described (Stauber et al. 2002; Rafiqi et al. 2008). Digoxigenin-labeled RNA probes were synthesized from 5’ (Empis, Lonchoptera), or 3’ cad RACE products (Megaselia, Haematopota) and from the complete ORF (lacZ). Transcripts were detected using anti-digoxigenin-AP antibody (Roche) and NBT/BCIP (Roche). HA was detected using anti-HA rat monoclonal antibody (Roche) and standard Drosophila protocols.

Results and discussion

Genomic organization and expression of a cad homologue from Megaselia

A homebox fragment of Megaselia caudal (Mab-cad) was obtained by degenerate PCR. This sequence was extended by 5’ and 3’ RACE experiments on cDNA templates from both embryos and adult flies. We obtained two transcripts, which differed in the presence of a small (54 bp) exon in

![Fig. 2](image-url) Expression of lacZ transcripts under the control of Mab-cad cis-regulatory DNA in transgenic Drosophila. Embryos are shown at a blastoderm, b early gastrulation, and c germ band extension. Anterior is left and dorsal up. The white arrow designates expression in the dorsal portion of the cephalic furrow and the black arrow expression in the posterior amnioserosa
the open reading frame of the embryonic transcript. Both *Megaselia* transcripts were mapped onto phage clones from a representative genomic library of *Megaselia*, revealing two alternative splice variants of the same locus (Fig. 1a; we refer to the sequence without the 54 bp exon as *Mab-cad* and to the sequence with this exon as *Mab-cad*). The position of the minixon is identical to the position of a minixon that has been reported as an alternative splice variant of the *Tribolium cad* transcripts *Te-cadA* and *Te-cadB* (Schulz et al. 1998). In *Drosophila*, this splice variant may have been lost. The predicted full-length proteins of *Mab-cad* and *Mab-cad* share 38% and 34% sequence similarity, respectively, with the predicted CAD protein of *Drosophila*. The homeodomains of both loci are orthologous to the *cad* locus of *Drosophila*. These data suggest that the *Mab-cad* locus is orthologous to the *cad* locus of *Drosophila*. The distributions of *Mab-CAD* and CAD are identical. These data support that the *Mab-cad* locus is orthologous to the *cad* locus of *Drosophila*.

Embryonic whole mount in situ hybridization experiments with *Mab-cad* probes against the 5′ exon (not shown) and the 3′ exon (Fig. 1b–h′) gave identical patterns. Zygotic expression starts one nuclear cycle prior to the onset of cellularization and occurs throughout the posterior 75% of the blastoderm. During blastoderm cellularization, *Mab-cad* expression changes rapidly and becomes restricted to the amnion anlage (black arrows in Fig. 1; Rafiqi et al. 2008) and a narrow posterior stripe (Fig. 1c–d′). During germ band extension, the posterior expression marks the end of the germ band, and the amniotic expression becomes restricted to a narrow stripe that invaginates with the proctodeum (Fig. 1e–g′). In addition, a new expression domain appears in the posterior midgut. This expression is strongest in the narrow posterior portion of the midgut, which bends dorsoanterior and connects to the hindgut, which does not express *Mab-cad*. In later embryos, we detected *Mab-cad* expression in this domain and in a ring around the posterior hindgut (Fig. 1h,h′). Unlike *cad* in *Drosophila* (Macdonald and Struhl 1986), *Mab-cad* is not expressed in the pole cells, malpighian tubules, and bilateral pairs of neurons. Also, we could not detect *Mab-cad* transcripts in ovaries and very early embryos, indicating that embryos lack zygotic transcripts. To confirm this result, we examined the expression of *Mab-cad* in a developmental Northern blot. This experiment revealed two transcripts in 0–3 h old embryos (Fig. 1i). The longer transcript was also detected at later stages and matches the predicted length of *Mab-cad* (with and without the mini exon). In ovaries, the detection of residual amounts of the longer *Mab-cad* transcript required a 16 times longer exposure time than the detection of much stronger bands in embryos (not shown), confirming the

Fig. 3 Translational repression assay. a Schematic overview of the translation assay cassette. TS, transcription start, TL, translation start. Transgenic *Drosophila* embryos containing the translation assay cassette with the complete *Drosophila cad* 3′ UTR (b–e), a truncated *Drosophila cad* 3′ UTR lacking the BBR (f–i), or the *Mab-cad* 3′ UTR (j–m) are shown. *Mab-cad* in situ hybridizations of pre-blastoderm (b, f, j) and blastoderm embryos (c, g, k) as well as two focal planes of early blastoderm embryos stained with a hemagglutinin (HA) antibody (d, e, h, i, l, m) are shown for each of the three transgenic lines. Note that in blastoderm embryos with the complete *Drosophila cad* 3′ UTR *Mab-cad* transcripts disappear from the anterior pole (e) coincident with translational repression of the reporter (d, e). The distribution of *Mab-cad* transcripts and protein remains uniform in embryos carrying the cassette with the truncated *Drosophila cad* 3′ UTR (g–i), or the *Mab-cad* 3′ UTR (k–m). In situ hybridizations with an SV40 anti-sense probe were negative, indicating that the polyadenylation signals (AAUAAA) of the added *cad* and *Mab-cad* 3′ UTR sequences are functional. All embryos are shown with anterior to the left and dorsal up
Regulation and evolution of Mab-cad expression

The lack of maternal Mab-cad expression suggests that in Megaselia, transcriptional regulation might be sufficient for the anterior repression of Mab-cad in the early blastoderm. To test this prediction, we cloned 8 kb immediately upstream of the identified Mab-cad transcript sequence into a lacZ reporter vector and examined the expression of lacZ under the control of Mab-cad cis-regulatory DNA in transgenic blastoderm embryos of Drosophila. Two independent transgenic lines expressed lacZ in patterns reminiscent of endogenous Mab-cad expression (Fig. 2a; a third transgenic line showed no expression). Unlike early zygotic transcripts of endogenous cad, which accumulate in two broad stripes (Schulz and Tautz 1995), the transcripts of the transgene accumulated evenly throughout the posterior blastoderm like Mab-cad transcripts in Megaselia. Compared to Mab-cad expression in Megaselia, the anterior expression boundary of the transgene was shifted by about 10% egg length towards posterior. In addition, the transgene was expressed in the dorsal portion of the cephalic furrow and in the posterior portion of the amnioserosa (Fig. 2b,c). These observations are consistent with the hypothesis that the anterior repression of Mab-cad activity in early Megaselia embryos is regulated at the transcriptional level, without BCD-dependent posttranscriptional regulatory input.

Surprisingly, BCD represses the translation of a ubiquitously expressed cad transgene from Tribolium (Wolff et al. 1998). The signal provided by the transcript of this cad homologue that mediates translational repression in transgenic Drosophila has not been identified, but the observation raises the question whether BCD can repress the translation of a broad range of cad homologues. To test the effect of BCD on the translation of maternally expressed Mab-cad, we generated a reporter cassette to test 3′ UTRs for their ability to mediate translational repression in early Drosophila embryos. The cassette contains the maternal promoter and 5′ UTR of the segmentation gene hunchback, the hemagglutinin (HA)-tagged Mab-cad open reading frame, and a cloning site for 3′ UTRs (Fig. 3a). To ensure the stability of reporter transcripts, SV40 3′ UTR was included downstream of the multiple cloning site. Using this cassette, we generated constructs containing the cad 3′ UTR from Drosophila, either with or without the Bicoid Binding Region (BBR; truncation 5′ of BBR) or the cad 3′ UTR from Megaselia, and tested their expression, both at the transcript and at the protein level in transgenic blastoderm embryos of Drosophila. In embryos containing the reporter construct with the complete 3′ UTR of Drosophila cad, early reporter transcripts were evenly distributed. However, the translation of

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**Fig. 4** Homeodomain tree of CAD homologues and expression in ovarian follicles. (a) Quartet Puzzling analysis (Strimmer and von Haeseler 1996) with sequences from the dipters E. livida (Eli), H. pluvialis (Hpl), L. lutea (Llu), M. abdita (Mab) and Drosophila melanogaster (Dme; accession no. NP_476954), the silk moth Bombyx mori (Bmo; NP_001037514), Tribolium castaneum (Tca; NP_001034498), the nematode Caenorhabditis elegans (Cel; NP_001021210) and the mouse (Mmu; Cdx-1: AAA37412; Cdx-2: AAA19645). The tree has been constructed with the homeodomain sequences using the default settings of software on http://bioweb.pasteur.fr/. Support for internal branches is shown in percent. Branch lengths indicate the average number of amino acid changes per position (see scale). In situ hybridizations of ovarian follicles from L. luteu (b), Empis livida (c) and H. pluvialis (d), with anti-sense probes against the respective cad homologues. Expression is detected in the nurse cells (arrows) of Lonchoptera and Empis follicles, and in the nurse cells and the oocyte (arrowhead) of the Haematopota follicle. Note that the chorion may have prevented cad transcript detection in the oocytes of Lonchoptera and Empis. e Northern blot of poly(A) RNA from ovarian follicles of Haematopota hybridized with a radio-labeled Haematopota cad probe. RNA standard sizes are given in kb.
the reporter was repressed in the anterior quarter of the blastoderm, and in late blastoderm stages, the reporter transcript had disappeared from the anterior pole (4 independent lines; Fig. 3b–e). These observations match with the sequence of events in *Drosophila*, in which translational repression of ubiquitous *cad* transcripts at the anterior pole precedes the disappearance of which translational repression of ubiquitous independent lines; Fig. 3f)

transcript and protein throughout the blastoderm (three independent lines; Fig. 3j–m). These results suggest that, unlike the 3′ UTR of *Drosophila cad* expressed the reporter transcript and protein throughout the blastoderm, albeit at a lower level (three independent lines; Fig. 3j–m). These observations suggest that the occurrence of two independent lines; Fig. 3i). Likewise, the reporter construct with the truncated 3′ UTR of *Drosophila cad* expressed the reporter transcript and protein throughout the blastoderm (three independent lines; Fig. 3f–i). Likewise, the reporter construct with the 3′ UTR of *Mab-cad* expressed the reporter transcript and protein throughout the blastoderm, albeit at a lower level (three independent lines; Fig. 3j–m). These results suggest that, unlike the 3′ UTR of *cad*, the 3′ UTR of *Mab-cad* is insufficient to mediate BCD-dependent translational repression in *Drosophila*.

Finally, we tested whether the absence of maternal *cad* expression is the ancestral condition for cyclorrhaphan flies, i.e. a plesiomorphic trait of Cyclorrhapha. We cloned *cad* homologues from a second lower cyclorrhaphan fly (*L. lutea*, Lonchopteridae), and two paraphyletic lower brachyceran taxa (*H. pluvialis*, Tabanidae; *E. livida*, Empididae) (Fig. 4a), and tested ovaries for maternal expression by in situ hybridization (Fig. 4b–d). In all three species, the nurse cells of ovarian follicles expressed the respective *cad* homologue. In *Haematopota*, we detected *cad* transcripts also in the oocyte (Fig. 4d). Failure to detect *cad* transcripts in the oocytes of *Lonchoptera* and *Empis* follicles is most likely a staining artefact due to the developing eggshell. For *Haematopota*, we verified maternal expression in a Northern blot and detected two *cad* transcripts (Fig. 4e), like in *Megaselia* (Fig. 1i) and *Drosophila* (Mlodzik and Gehring 1987), suggesting that the occurrence of two *cad* transcripts is conserved in evolution. However, the developmental time course of their appearance is not. In *Drosophila*, alternative *cad* promoters express the shorter transcript (2.4 kb) during oogenesis and the longer transcript (2.6 kb) during blastoderm cellularization (Mlodzik and Gehring 1987), while in *Megaselia*, the two *cad* mRNAs are transcribed during early embryogenesis. We cannot exclude that a paralogue of *Mab-cad* provides maternal expression, but a very recent duplication of *Mab-cad* is unlikely because stringent hybridization of endonuclease digested genomic DNA with a *Mab-cad* probe in Southern blots is consistent with a single copy gene (not shown). Taken together, our results suggest that maternal *cad* expression was lost in the *Megaselia* lineage and that BCD is not required for the translational repression of *Mab-cad*.

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