Unique establishment of procephalic head segments
is supported by the identification of cis-regulatory elements
driving segment-specific segment polarity gene expression
in Drosophila

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Abstract Anterior head segmentation is governed by
different regulatory mechanisms than those that control
trunk segmentation in Drosophila. For segment polarity
genes, both initial mode of activation as well as cross-
regulatory interactions among them differ from the typical
genetic circuitry in the trunk and are unique for each of the
procephalic segments. In order to better understand the
segment-specific gene network responsible for the proce-
phalic expression of the earliest active segment polarity
genes wingless and hedgehog, we started to identify and
analyze cis-regulatory DNA elements of these genes. For
hedgehog, we could identify a cis-regulatory element,
ic-CRE, that mediates expression specifically in the
posterior part of the intercalary segment and requires
promoter-specific interaction for its function. The interca-
lary stripe is the last part of the metameric hedgehog
expression pattern that appears during embryonic develop-
ment, which probably reflects the late and distinct estab-
lishment of this segment. The identification of a cis-
regulatory element that is specific for one head segment
supports the mutant-based observation that the expression
of segment polarity genes is governed by a unique gene
network in each of the procephalic segments. This provides
further indication that the anterior-most head segments
represent primary segments, which are set up independently,
in contrast to the secondary segments of the trunk, which
resemble true repetitive units.

Keywords Enhancer dissection · Enhancer–promoter
interaction · Head development · Metamerization ·
Segmentation

Introduction

Comparisons among distantly related insects suggest that
head and trunk segmentation may have followed different
paths during arthropod evolution (Minelli 2001; Tautz
2004). The mechanism of trunk segmentation found in the
vinegar fly Drosophila melanogaster represents a relatively
recent adjustment to its rapid embryonic development,
whereas the mode of head development has been proposed
to correspond to a phylogenetically more ancient mecha-
nism. Comparisons with vertebrates even suggest that the
origin of cephalization may be common for all Bilateria
(Reichert and Simeone 1999). However, due to complex
morphogenetic movements during embryonic head develop-
ment of the acephalic Drosophila maggot, understanding
head segmentation has not progressed as far as trunk
segmentation in this species.

The insect head is built by several segment primordia
that fuse to form the rigid head capsule with limbs that are
adapted to different roles in orientation and feeding. The
anterior head — the procephalon — consists of the ocular
(protocerebral) region whose segmental status is debated, the
antennal (deuterocerebral) and the intercalary (tritocerebral)
segment (Scholtz and Edgecombe 2006). The gnathoceph-
aeon — mandibular, maxillary, and labial segments —
constitutes the posterior portion of the insect head. In
Drosophila, the labial and maxillary gnathal segments are
patterned like the trunk segments according to the well-established hierarchical segmentation cascade involving maternal coordinate genes, gap, pair-rule, and segment polarity genes (St Johnston and Nüsslein-Volhard 1992), and segment identity specification is accomplished in these segments by the Hox genes (McGinnis and Krumlauf 1992). The mandibulovulvar development integrates inputs from both the head and the trunk patterning systems (Cohen and Jürgens 1990; Grossniklaus et al. 1994; Vincent et al. 1997). Anterior to the mandible, however, no pair-rule patterning is observed and the anterior-most expression of a gene of the Hox cluster is in the intercalary segment (Abzhanov and Kaufman 1999; Bucher and Wimmer 2005). For patterning of anterior head segments, Drosophila makes use of the so-called head gap-like genes orthodenticle (otd), empty spiracles (ems), and buttonhead (btd). They are expressed early in embryogenesis in broad and overlapping domains (Wimmer et al. 1997). Also their mutant phenotypes are overlapping and correspond roughly to the expression patterns (Cohen and Jürgens 1990; Cohen and Jürgens 1991). However, the functional analysis of the orthologs of these head gap-like genes in another insect — the red flour beetle Tribolium castaneum — revealed that only the late otd function seems similar, whereas ems and btd are not functionally conserved (Schinko et al. 2008). Moreover, another gap gene — knirps (kni) — which in Drosophila is not required for head segmentation is necessary for the development of the antennal and mandibular segments in Tribolium (Cerny et al. 2008).

The detected differences in gene function between Drosophila and Tribolium reflect a variation between insect species in the use of genes at the level of the first zygotically active genes, the gap genes. This might reflect the fact that early development of related species is more variable than development shortly after gastrulation. The stage of greatest similarity between the members of a phylum was termed “phylotypic stage” by Sander (1983). Earlier developmental stages are highly variable due to adaptations to particular modes of reproduction, and thereafter, development diverges toward the differently specialized postembryonic stages, which are again susceptible to adaptive changes. However, the phytotypic stage is not only by morphological criteria the most conserved between the different members of each phylum but represents also the stage with the most conserved gene expression patterns. The recognition that segment polarity genes like engrailed (en), which define the borders of segments in all arthropods analyzed to date, are expressed at this phytotypic stage indicates that the metamerization process of the arthropods is at least partially conserved (Patel 1994; Damen 2002; Chipman and Akam 2008). Very recently, the functional involvement of the segment polarity gene hedgehog (hh) has even been shown in the segmentation of an annelid (Dray et al. 2010) which indicates a common origin of metamerization for all protostomes. Also the metamerization of the arthropod head segments involves segment polarity genes (Rogers and Kaufman 1997; Pechmann et al. 2009); however, the exact mechanisms on how the head gap-like genes direct the metamic expression of the segment polarity genes in the anterior head is still unknown.

About the functional role of the segment polarity genes — e.g., en, hh, and wingless (wg) — in anterior head development, little is known despite the facts that they are compellingly conserved across the arthropods, and their expression patterns have been widely used to mark head segments. There is also not much known about the initial activation of the segment polarity genes in the anterior insect head region — where pair-rule genes are not functional. Moreover, in Drosophila, it has been shown that the cross-regulatory interactions among segment polarity genes in the anterior head region differ from the trunk and have been reported to be specific for each of the anterior head segments — ocular, antennal, and intercalary — while their interaction is identical in the mandibular and all posterior segments (Gallitano-Mendel and Finkelstein 1997). This also suggests a unique establishment of each of the anterior head segments in contrast to a common generation of the posterior segments. Interestingly, these different modes of regulation by segment polarity genes in anterior and posterior head segments may be due to their independent evolutionary origin. Classical embryology has revealed that the subdivision of the coelom — one important feature of segmentation — occurs in two different ways. Anterior segments arise by concomitant subdivision of one large coelom, giving rise to so-called primary or larval segments. Coelomic sacs of the more posterior segments, by contrast, are usually formed one by one from a posterior growth zone (Remane 1950). These latter segments are known as secondary segments. Such a bimodal segmentation is easily observed, for example, in some crustaceans: The nauplius larva typically forms three larval segments, namely first and second antennal segment (the latter corresponds to the intercalary/tritocerebral segment in insects) and the mandibular segment (Scholtz 2000). In this respect, the procephalic segments and the mandibular segment are correlates of larval segments, while the remaining gnathocoephalic and trunk segments are of the post-larval type. Differences in the regulation of segment polarity genes between anterior and posterior segments in the insect head could therefore reflect this ancestral subdivision in primary and secondary segmentation (Minelli 2001; Tautz 2004).

In order to identify novel components of the gene regulatory networks that govern anterior head metamerization, we started a bottom-up approach by detecting and functional dissecting cis-regulatory regions of the earliest expressed segment polarity genes, wg and hh, in the anterior head region (Mohler 1995). Such an approach can
lead to the identification of transcription factors directly involved in the gene networks patterning each of the anterior head segments. Here we present a functional dissection of wg and hh cis-regulatory regions which led to the isolation of the intercalary-specific enhancer element, ic-CRE. The functional isolation of such a segment-specific cis-regulatory element supports the theory of a unique mode of establishment of each of the procephalic head segments. Moreover, we provide evidence that the establishment of the intercalary segment is delayed in comparison to the other head segments as has been described for Tribolium (Posnien and Bucher 2010; Schaeper et al. 2010).

Materials and methods

Determination of hh transcription start site

The transcription start site of hh was determined at −353 relative to ATG by 5′ RACE PCR on 0–12 h embryonic cDNA 5′ RACE pool using the primer TTTGAGCG-CAGGGGAG (Roche, Mannheim, Germany). The cDNA 5′ RACE pool was synthesized using SMART PCR RACE cDNA Synthesis Kit (ClonTech, Heidelberg, Germany).

Reporter constructs

turboGFP reporter [tgfp_SV40 952 bp sequence] was excised with AgeI (site T4 blunted)/AflII from pTGF_PRL (EVROGEN, Moscow, Russia) and inserted into pT4 plasmid/AflII of pSLaf1180af vector (Horn and Wimmer 2000) to generate pSLaf[tgfp]af.2. The promoter sequence of hh (∼120 bp) was isolated with primers CAACGCGGAATCGGGTTGGCTCGAC (XhoI Forward) and AACTAGTTAGCTCCTCGCCTCGAACAACCCT (SpeI Reverse) on Drosophila genomic DNA and subcloned into XhoI/Spel of pSLaf[tgfp]af.2 to result in construct pSLaf[hs43 promoter_tgfp]SV40. The promoter sequence of wg (∼159 bp around tsA) was isolated with primers CTCGCAGGGCGGATAATG (XhoI Forward) and ACTAGTTACTCGCCTCGACCTCGAG (SpeI Reverse). The promoter sequence of hh was excised from pSLaf[hs43 promoter][tgfp]SV40. The 156 bp hs43 promoter sequence was excised from pSLaf[hs43 promoter][tgfp]SV40 with XhoI/PstI (T4 blunted) and subcloned into XhoI/Spel (T4 blunt) of pSLaf[tgfp]af.2 to result in construct pSLaf[hs43 promoter]SV40. The 156 bp hs43 promoter sequence was excised from pSLaf[hs43 promoter][tgfp]SV40 with XhoI/PstI (T4 blunted) and subcloned into XhoI/Spel (T4 blunt) of pSLaf[tgfp]af.2 to result in construct pSLaf[hs43 promoter]SV40. pSLaf[hs43 promoter]af.2 was constructed by excising (HindIII/XhoI) a 4.4 kb [hs43 promoter]SV40 fragment from pCasper[hs43 lacZ] (Thummel and Pirrotta 1992) and ligating it into pSLaf1180af vector (Horn and Wimmer 2000). The DNA sequence spanning −6902 +265 bp of hh locus was amplified on genomic DNA template by long-range PCR (High Fidelity Enzyme, Fermentas, St. Leon-Rot, Germany) with primers CGAGCGCATTTGGAGGGTCACACTACA, forward and GACTTACTTCTCTGTGGGCA CAGAGCACGCT, reverse and cloned via T/A ligation in the PCRII vector (Invitrogen, Darmstadt, Germany) to result in pTAIL_Dm hh upstream(7.16). DNA sequence spanning −8,094 bp upstream of wg tsA to +193 bp downstream of tsB (i.e., +2,122 bp downstream of tsA) was amplified with primers CTGCAGCAGAACAACGA GAAGCGAGGAGTACT, forward and AGT CACTCCTCGCCTTGTTGCATGCAG, reverse and cloned in PCRII to result in pTAIL_Dm wg upstream(−8.1). Sequence spanning −16,212_−7,813 kb upstream of wg tsA was amplified with primers GCTGCTCCAGAT CATCACTTCGTGTGAC, forward and GAATCGGATTGAATTCGTCAG, reverse and cloned in PCRII to result in pTAIL_Dm wg upstream(−16.2_−7.8). The hh and wg upstream sequences were excised with NsiI (−6.43 kb) NotI (PCRII poly linker) from pTAIL_Dm hh upstream(7.16) and with EcoRI from pTAIL_Dm wg upstream(−8.1) and subcloned into the poly linker of pSLaf[wg lacZ]af to generate pSLaf[−6.43 kb hh upstream lacZ SV40] and pSLaf[−8.1 kb wg upstream lacZ SV40] respectively. pSLaf[wg lacZ]af was constructed by partially digesting the pSLaf[hs43 lacZ]af with PstI to remove the 197 bp hs43 fragment followed by ligation. Also the −8.1_−3.9 and −6.7_−3.8 kb hh upstream subfragments (resulting from EcoRI/Hpal and Xhol restriction, respectively) were subcloned into pSLaf[hs43 lacZ]af to generate pSLaf[−8.1_−3.9 kb hh upstream hs43 lacZ SV40] and pSLaf[−6.7_−3.8 kb hh upstream hs43 lacZ SV40] respectively. The −16,212_−7,813 kb (wg tsA) sequence was excised (KpnI_NotI) from pTAIL_Dm wg upstream(−16.2_−7.8) and subcloned into pSLaf[DM wg promoter tgfp]SV40 to generate pSLaf[−16.2_−7.8 wg upstream wg promoter tgfp]SV40. The 1,009 bp α fragment was isolated with primers TCGCGAGCTGATAGCACAATGGAC, forward and CTTCGAGCTTCTCCGCTGATGC, reverse and cloned in pSLaf[Dm hh promoter tgfp]SV40. Also the overlapping subfragments (γ1, 34, γ3, γ2, F6 R5, F3 R2, F5 R4) were isolated with proofreading PCR (primers are available upon request; Ntini 2009) and cloned in pSLaf[DM hh promoter tgfp]SV40 to generate the respective pSLaf[γ1/34/33/γ2/ F6 R5/F3 R2/F5 R4 hh promoter tgfp]SV40 constructs. γ1 and F5 R4 were also cloned into pSLaf[hs43 promoter]SV40 to generate pSLaf[γ1/hs43 promoter]SV40 and pSLaf [F5 R4 hs43 promoter]SV40, respectively. Cassettes consisting of [cis-regulatory region promoter]GFP]SV40 or [cis-
regulatory region_promoter_lacZ_SV40] were finally excised with AscI from the above pSLaf constructs and subcloned into pBac[3xP3_EGFPafm] (Horn and Wimmer 2000) to generate the respective piggyBac constructs. The ic-CRE γ1mF3-γ1mF6 subfragments (resulting from 5’ fragmentation of the γ1; primers are available upon request; Ntini 2009) were subcloned in pSLaf[DM hh promoter_tgfp_SV40] resulting into pSLaf[γ1mF3/F4/F5/F6 hh promoter_tgfp_SV40] and finally integrated via attB-attP site-specific recombination.

Transgenesis

For piggyBac-mediated transgenesis, the generated piggyBac constructs (please see “Reporter constructs”) were coinjected at 500–1,200 ng/μl (we observed that increasing the pBac construct concentration enhanced transformation efficiency of large constructs) with helper plasmid providing transposable activity (phspBac) at 300 ng/μl (Horn et al. 2000). For the piggyBac random insertions, at least two independent lines were analyzed to circumvent the possibility of position effects. For site-specific transgenesis using the attB-attP oC31-mediated integration system (Bischof et al. 2007), the complete 314 bp attB sequence was excised from pTA-attB (Calos MP, Stanford University, personal communication) with EcoRI; restricted ends were blunted with T4 DNA polymerase and subcloned into pBac[3xP3_EGFPafm] (Horn and Wimmer 2000) linearized with BglII (T4 blunted) generating the vector pBac_attB. The γ1mF3-γ1mF6 reporter cassettes were excised with Ascl from pSLaf[γ1mF3/F4/F5/F6 hh promoter_tgfp_SV40] and subcloned in the pBac-attB vector to generate the respective pB attB constructs (for example, pB_attB[γ1mF5 hh promoter_tgfp_SV40]). All the generated pB_attB constructs were injected and successfully assayed in the line bearing the attP landing site at position 96E of the third chromosome (Ac. Num EF362408). This is a combined line carrying on the X chromosome a codon-optimized oC31 integrase driven under the control of the vasa promoter (Bischof et al. 2007).

Whole mount embryo in situ hybridization

To generate Dig- or Fluor-labeled RNA probes for in situ hybridization, cDNA sequences of genes of interest were cloned in the PCRII vector (Invitrogen, Darmstadt, Germany) and antisense-RNA was generated from T7 or Sp6 promoter using the respective RNA polymerase (Roche, Mannheim, Germany). In vitro transcription was in the presence of 10% DIG-labeling or Fluor-labeling rNTP mix (Roche, Mannheim, Germany); 0–10.5-h embryo collections were dechorionated for 3 min in 50% chlorix and fixed in 2 ml heptane, 3.7% formaldehyde in 1.5 ml PEM (0.1 M PIPES, 1 mM MgCl2, 1 mM EGTA, pH 6.9) for 20 min at rt. Double in situ hybridization was as in Rehm et al. (2009), apart from an additional 30-min detergent treatment (1% SDS, 0.5% Tween-20, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl) before prehybridization. NBT/BCIP staining was in AP buffer, pH 9.5, and FastRed (Sigma-Aldrich, Munich, Germany) staining in AP buffer, pH 8.2.

Microscopy

Embryos stained after in situ hybridization were mounted in glycerol (~90%) and documented with a Zeiss Axioplan 2 microscope (~20 or ~40 planes) using the ImageProPlus software (Version 6.2; MediaCybernetics). Embryonic staging was after Campos-Ortega and Hartenstein (1997).

In silico analysis of DNA sequences

In silico analysis to identify putative transcription factor binding sites within cis-regulatory regions was performed using the MatInspector (Cartharius et al. 2005; http://www.genomatix.de/online_help/help_matinspector/matinspector.html; http://www.genomatix.de/cgi-bin/matinseqtor_prof/mat_fam.pl). Predictions were inspected manually, checked in correlation with literature reports, and filtered through phylogenetic conservation using the Drosophila EvoPrinter (http://evoprinter.ninds.nih.gov/evoprintpro gramHD/evphd.html ) or the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

Results and discussion

A distinct cis-regulatory element controls wg expression during foregut development at the anterior terminal pole

A cis-regulatory region, WLZ4.5L, spanning 4.8 kb upstream of the wg transcription start site A (tsA) had been previously assayed by Lessing and Nusse (1998). Their construct mediates reporter expression in the wg gnathal and trunk stripes from the early onset of the wg expression at the blastoderm stage and then during germ band extension stages but lacks the metameric expression pattern in the procephalon. In search for anterior head segment-specific cis-regulatory elements, we thus assayed two overlapping 5’-extended DNA fragments (~6.7–3.8 kb and ~8.1–3.9 kb) combined with an hs43 basal promoter (Thummel and Pirotta 1992; Fig. 1A). The larger fragment (~8.1–3.9 kb) mediates reporter expression in the anterior-most terminal region at the cellular blastoderm stage (Fig. 1H, I) overlapping the endogenous anterior-most wg expression domain (Fig. 1E, F). During germ band extension the reporter is expressed in the foregut anlage but not in the labral spot (Fig. 1J). Because the smaller
fragment (−6.7−3.8 kb) does not mediate expression in this region (Fig. 1G), we conclude that the region between −8.1 and −6.7 kb contains cis-regulatory elements essential for transcriptional control of wg foregut expression. Please note that the −6.7−3.8 kb fragment mediates “patched” reporter expression in the trunk stripes of stage 11 embryos, excluding the gnathal segments. This pattern seems wg-specific as it overlaps with endogenous wg expression and turns on when the WLZ4.5L-mediated expression fades (Lessing and Nusse 1998). The fact that the larger fragment (−8.1−3.9 kb) does not report this expression indicates a rather complex regulation during the maintenance phase of wg expression.

Procephalic and trunk metamerization employ separate cis-regulatory elements controlling wg expression

Lessing and Nusse (1998) used in their WLZ4.5L construct an endogenous wg promoter. To exclude the possibility that the reason we did not find any procephalic regulatory elements is based on the use of a heterologous promoter (hs43 basal element), we made a large construct that contains all the upstream regions so far analyzed plus both previously identified transcriptional promoters (tsA and tsB) of wg spanning the region −8.094 kb upstream of tsA to +195 bp downstream of tsB (NCBI reference sequences NM_078778.3 and NM_164746.1, respectively). This 10.216 kb fragment mediates reporter expression in the gnathal and trunk stripes, as well as in the labral spot, the foregut region, and in excess in the prospective hind gut region (similar to WLZ4.5L; Lessing and Nusse 1998), but still it lacks cis-regulatory information for the procephalic stripes (Fig. 2).

At the late blastoderm stage 5/6, the even-numbered reporter stripes come up delayed compared to the endogenous wg stripes (Fig. 2A, B). Again this is reminiscent of the expression pattern generated by WLZ4.5L (Lessing and Nusse 1998). On the contrary however, the reporter expression generated by that construct fades from the epidermis of late germ bands (stage 11) whereas the foregut region, and in excess in the prospective hind gut region — similar to WLZ4.5L; Lessing and Nusse 1998, but still it lacks cis-regulatory information for the procephalic stripes (Fig. 2).

In the search for cis-regulatory elements governing expression of wg in the procephalic region, 8.4 kb of further upstream sequence was isolated, spanning region −16.212 to −7.813 kb relative to tsA and assayed in combination with a 280 bp endogenous promoter region surrounding tsA (−159 to +121 bp; Fig. 1A). This upstream region also mediates the metameric expression pattern of wg; however, the reporter trunk stripes do not come up at the early cellular blastoderm stage 5 but only at stage 6/7 (Fig. 3A). The reporter expression thus lacks the early onset of trunk expression at blastoderm stage as it is mediated by the −8.1 kb enhancer. In addition, this further upstream enhancer fragment mediates reporter expression in the cell stripe of the antennal segment primordium at stage 7/8 (Fig. 3B, C). This is the time point when the endogenous wg antennal expression domain first appears at the lateral procephalic ectoderm (Liu et al. 2006). Since the first detection of the antennal-specific reporter expression pattern coincides with formation of the endogenous wg antennal stripe, the −16.212 to −7.813 kb enhancer fragment probably contains cis-regulatory elements underlying the establishment of procephalic wg expression specific for the antennal segment. In contrast, the mediated reporter expression pattern does not overlap the anterior procephalic expression domain of wg at the blastoderm stage corresponding to the presumptive ocular region, the so-called head blob (Liu et al. 2006). Actually the first reporter expression in this ocular region is detectable only at stage 8 in the ventral-most cells of the head blob (Fig. 3C). Also during germ band extension, reporter expression is mediated only in the ventral-most part of the
ocular region (Fig. 3D–G). The mediated reporter expression pattern in the intercalary segment comes up later, at stage 11, overlapping the endogenous wg intercalary spot (Fig. 3G) which is already detected clearly during stage 10 (Figs. 2f and 3F; Gallitano-Mendel and Finkelstein 1997; Liu et al. 2006). The −16.212- to −7.813 kb enhancer fragment, therefore, does not mediate the onset of the endogenous wg expression in the intercalary segment.

This delay in the reporter expression implies that the set up of the intercalary-specific wg expression is under the...
control of separate cis-regulatory information and reflects that regulation of segment polarity gene expression can be divided into sequential phases of establishment and maintenance. The cis-regulatory elements mediating the intercalary expression included in this upstream enhancer seem to be rather involved in the maintenance of wg expression. This is consistent with the fact that also for the trunk segments this further upstream region mediates the maintenance phase of wg expression rather than the onset (Fig. 3A, G). Only for the antennal expression, this further upstream element seems to contain all cis-regulatory information for both establishment as well as maintenance of wg expression (Fig. 3B–G).

Transcriptional control of wg in the ocular region is mediated by distinct dorso- and ventral-specific cis-regulatory elements

In principle, the anterior-most procephalic expression domain of wg corresponds to a proposed segmental unit, i.e., the ocular segment (Schmidt-Ott and Technau 1992), based on data of the phylogenetically conserved expression pattern of en grailed (Schmidt-Ott et al. 1994; Urbach and Technau 2003). In more primitive insects, it splits into expression subdomains, namely the median protocerebral neuroectoderm expression domain (mpn), the dorsal protocerebral (dpn), and the ventral protocerebral neuroectoderm domain (vpn; Liu et al. 2006). On the contrary, in Drosophila, it remains intact constituting the “head blob” (Schmidt-Ott and Technau 1992). The vpn domain has been specifically lost in Drosophila, and as reported in Liu et al. (2006), the contiguous protocerebral neuroectoderm domain (pne) or head blob may be equivalent to (a) the mpn, (b) the dpn, or (c) the primordial yet non-dissociated protocerebral ectoderm domain of primitive insects. The 8.4 kb wg upstream sequence (~16.2 to -7.8 kb) contains cis-regulatory information that drives expression only in the ventral part of the head blob (Fig. 3D–G). These data indicate distinct
Dorsoventral differences in the regulation of segment polarity genes have also been reported in the context of the anterior head segment-specific cross-regulatory networks. For instance, **wg** represses **hh** expression in the dorsal part of the ocular and antennal segments, while it maintains **hh** expression ventrally within the same segments (Gallitano-Mendel and Finkelstein 1997). Thus, transcriptional control of segment polarity genes in the procephalic region may involve dorso- and ventral-specific **cis**-regulatory elements functional within the very same segmental unit. In a developmental context, this may reflect the response of segment polarity genes to distinct signals in different parts of the ocular and antennal segments.

**Identification of an intercalary-specific **cis**-regulatory element of **hh**

The transcription start site (tss) of **hh** was identified by 5′ RACE PCR to be located −353 bp relative to translation start site, which is different from the tss reported in Lee et al. (1992), at −385 bp. This may be due to a nucleotide polymorphism (T>C) that the strain we used carries at position −387. Moreover, the annotated EST, EK111112.5prime, starts at position −374 bp and is also affected by a polymorphism (C>G) right at this position. For the following constructs, the numbering refers to the transcription start site we identified being +1.

A 6.43 kb upstream sequence plus endogenous promoter and part of the 5′ UTR (−6.43 kb to +265 bp) were initially assayed (Fig. 4A–I). At blastoderm stage, this upstream sequence mediates expression of the reporter in an anterior domain broadly overlapping the early endogenous procephalic expression domain of **hh** (Mohler 1995; Fig. 4B, C), while it is not mediating any expression in the presumptive trunk. The odd-numbered reporter stripes appear at stage 8 (Fig. 4E) followed up with a delay by the even-numbered ones (Fig. 4F, G). During germ band extension, the reporter is also expressed in the procephalic head stripes (Fig. 4G–I). This 6.43 kb upstream region was then dissected into 5′ shortened fragments (Fig. 4A). The −4.08 kb fragment mediates expression in the intercalary segment and in some dorsal epidermal cells (Fig. 4J–L), while the −3.17 kb fragment does not retain expression in the intercalary segment (Fig. 4M). Therefore, the region between −4.08 and −3.17 kb (represented by the red bar in Fig. 4A) must contain **cis**-regulatory information essential for the transcriptional control of **hh** expression in the intercalary segment.

To test whether this enhancer fragment is also sufficient to ensure intercalary-specific expression of **hh**, the sequence from −4.08 to −3.077 kb (named thereafter α fragment;
Fig. 4 Functional dissection of the hh upstream region. A Schematic representation of the fragments assayed for enhancer activity. B, C The −6.43 kb fragment mediates expression in an anterior domain broadly overlapping the endogenous anterior expression domain of hh at blastoderm stage. D–F The even-numbered stripes of the mediated reporter expression pattern come up after the odd-numbered ones have fully developed. G–I By the completion of germ band extension, the enhancer mediates expression in the trunk and procephalic stripes. J, K The −4.08 kb fragment mediates expression in the intercalary segment while M the −3.17 kb fragment does not. Thus, the −4.08−3.17 kb region (red bar in A) is an essential element for intercalary-specific transcriptional control of hh termed ic-CRE. For abbreviations, see Fig. 1.

Fig. 5A) was assayed in combination with an hs43 basal promoter (Thummel and Pirotta 1992) or with the endogenous hh promoter region (−120 to +99 bp). Expression of the reporter was specifically mediated in the intercalary segment (plus a few cells in the mandibular and maxillary segments) when the endogenous promoter was used (Fig. 5B–D) but not in combination with the heterologous promoter (data not shown). Therefore, the α fragment is essential and sufficient for the transcriptional control of the intercalary-specific expression of hh. This sequence and its functional subfragments are thus referred to as the intercalary-specific cis-regulatory element (ic-CRE). In an effort to further restrict the cis-regulatory element crucial for intercalary-specific transcriptional control of hh, we first performed a
phylogenetic conservation analysis (Bejerano et al. 2005; see “Materials and methods”) of the ic-CRE sequence to detect highly conserved sequence blocks (Fig. 5A; EN and EAW unpublished). Second, the 1 kb ic-CRE sequence (α fragment) was further dissected by assaying overlapping subfragments or 5′ truncated sequences that end at a common point, −3,465 bp (Fig. 5A), together with the endogenous hh promoter. During this dissection analysis, fragments were designed so that highly conserved sequence blocks were not disrupted. The 5′ truncation of the γl sequence was assayed at the same genomic integration site using the attP–attB site-specific recombination system (Bischof et al. 2007). This system was selected to overcome potential position effects by random integration resulting from piggyBac-based germ line transformation (Horn et al. 2000). For that reason, we examined at least two independent transgenic lines when using the piggyBac-mediated transformation system. The 335 bp sequence F5_R4 (−3,799 to −3,465 bp; Fig. 5A) was the minimum sequence assayed that still retains expression in the intercalary segment with an onset of expression at stage 10 (Fig. 5E–H) and with a partial and spotty metameric expression later in the trunk (Fig. 5H). In contrast, the 450 bp ic-CRE sequence termed γlM5F5 (−3,914 to −3,465 bp) covering all of and extending the 335 bp fragment (Fig. 5A) mediates expression specific to the intercalary segment already from stage 9 on (Fig. 5I–L). This fragment, however, still lacks the very early onset of endogenous hh expression in the intercalary segment anlage at stage 8, which is mediated by the 621 bp ic-CRE fragment γl (arrow in Fig. 5M, N) and the γlM3F3 subfragment (Fig. 5A, C). To further confirm the requirement of the ic-CRE to interact with the endogenous hh promoter, the 621 bp γl and the 335 bp F5_R4 subfragments were also assayed in combination with the hs43 basal promoter, again not showing the ic-CRE-mediated expression.

The ic-CRE requires promoter-specific interaction

During the functional dissection analysis, we observed that the 1 kb ic-CRE (α fragment) and its functional subfragments mediate the intercalary-specific expression pattern only in combination with the endogenous hh promoter region (−120 to +99 bp) but not with the hs43 TATA-box basal promoter (Thummel and Pirotta 1992). On the other hand, subfragments of the −8.1 kb upstream region of wg mediate specific reporter expression patterns when combined with the same hs43 basal promoter (Fig. 1A, G–J). These results indicate that an enhancer–promoter-specific interaction underlies transcriptional control of the intercalary-specific expression of hh or that the hs43 promoter lacks core promoter elements required to mediate the ic-CRE function. The endogenous hh promoter is TATA-less and contains instead a downstream promoter element (DPE; Butler and Kadonaga 2001; Lim et al. 2004) as one of its core elements. On the other hand, the wg endogenous promoter is also TATA-less but remarkably there is no detectable DPE sequence matching the consensus RGWYVV(T). The only detected core promoter element in the wg promoter sequence is the initiator element (consensus TCAKTY; Lim et al. 2004) in the case of tsA, which typically encompasses the transcription start site (the underlined A is +1). Enhancer–promoter specificity has been reported in several cases of transcriptional regulation and may depend on the activity of sequence-specific transcription factors which function as DPE-specific activators (Hsu et al. 2008; Juven-Gershon et al. 2008; Juven-Gershon and Kadonaga 2010). Interestingly, occurrence of the DPE motif in the Drosophila endogenous core-promoters is as common as the TATA-box (Kutach and Kadonaga 2000). Consistently, the reporter expression pattern mediated by the complete −6.43 kb hh upstream region appeared faded when the DPE was disrupted by a point mutation (data not shown), supporting a functional role for DPE activity in embryonic transcriptional control of hh expression.

Early onset of the ic-CRE-mediated expression pattern is ensured by a 30 bp sequence which possibly recruits HMG DNA-binding activity

During the 5′ fragmentation assay of the 621 bp γl fragment (Fig. 5A), we identified a 30 bp sequence from −4,014 to −3,985 bp which is required to ensure the early onset of the ic-CRE-mediated expression pattern in the intercalary segment anlage at stage 8, as this expression is mediated (Fig. 5A, C) by the fragment γlM3F3 (−4,014 to −3,465 bp) but not by the fragment γlM4F4 (−3,985 to −3,465 bp). This
sequence does not mediate early expression by itself but only in concert with sequences within the 335 bp element, since even the β3 fragment (−4,085 to −3,757 bp) does not mediate reporter gene expression. In silico analysis indicates that this short sequence consists of two highly conserved blocks GGATCACAAA and GTTGACAAAt separated by a 6 bp stretch (Fig. 5A; capitals represent 12 Drosophila species phylogenetic conservation). Both sequences resemble the binding motif of high mobility group (HMG) protein factors [WCAAAAS] (entry in the CDD Database of NCBI: cd01388 “SOX-TCF_HMG-box”; Love et al. 1995; Werner et al. 1995). In addition, they both conform to the consensus binding sequence of HMG-box proteins of the SOX family [WWCAAW] (Churchill et al. 1995; Lefèvre et al. 2007). In silico prediction in the 50 bp DNA sequence (−4,019 to −3,970 bp) using the MatInspector generates a hit in the first block GGATCACAAA, scoring the binding matrix of dTCF (Drosophila T-cell factor homolog or Pangolin) which is WTCAAAS (underlined are the four nucleotides of the core sequence used by MatInspector; Lee and Frasch 2000). The non-conserved A nucleotide which disturbs the conservation block does not match the matrix at the corresponding position 7 (S) (S stands for “strong nucleotide,” i.e., G/C). Still, the site strongly resembles the consensus binding

Fig. 6 Early onset of the intercalary-specific expression of hh. A, C The γ1mF3 enhancer fragment (see Fig. 5A) mediates the early onset of reporter expression in the intercalary segment anlage (arrow) at stage 8 (see also Fig. 5M, N). B, B′′, D, D′′ Early procephalic expression of hh at stage 8. B, B′′ The procephalic stripes (oc, an, ic) are detected at a different focal plane (B, B′′) than the mn stripe (D″). B, B′′ In this lateral view, although detected at the same focal plane, the cell stripe in the intercalary (ic) segment anlage (arrow) is discontinuous from the antennal (an) segment anlage which progressively delineates from the ocular (oc) one. D, D′′ In this ventrolateral view, the cell stripes in the an and oc segment anlagen are detected at the same focal plane (D″) while the ic is out of focus and vice versa. D, D′′ The slightly different focal plane of D′ compared to D allows the cell groups of both the ic anlagen to be detected. E–G′ Double in situ hybridization of hh (purple) and en (red). F Anterior to the mn stripe, the early procephalic expression domain of hh progressively splits into the antennal and ocular primordium during stage 7. The cells at the posterior margin of the early procephalic hh domain co-expressing en are precursors of cells of the antennal ectodermal stripe formed at the posterior procephalic margin at stage 8 (G). The open arrow depicts the precursor cells of the presumptive ic segment anlage. G, G′ Different focal planes of the same embryo at stage 8. hh but not en expression is detected in the ic segment anlage at stage 8 (arrow). For abbreviations, see Fig. 1.
sequence of dTCF determined by PCR-based binding site selection [GATCAAAGG] (van de Wetering et al. 1997) which matches well the canonical Lef1/TCF binding motif [WWTCAAAGG] (van de Wetering et al. 1991, 1993). Only the first block, but not the second one, scores in silico the binding matrix of dTCF, as it seems that a T residue in the (second) W position of the general HMG-box consensus WCAAAS (or WWCAAAS) is a prerequisite for specific recognition by the HMG-box of dTCF. Remarkably, one more putative HMG binding site (TACAAAC) lies 3′ juxtaposed to the isolated fragment matching the WCAAAS consensus (at position −3,984 to −3,978 bp, reverse complement). This sequence is filtered through 11 species phylogenetic conservation, with the Drosophila yakuba sequence being divergent.

In conclusion, although the enhancer fragment γImF5 (−3,914_−3,465 bp) mediates specific expression in the intercalary segment during stages 9–11, early onset at stage 8 is only ensured by an additional fragment (−4,014_−3,985 bp) which provides early temporal control. HMG DNA-binding activity is predicted in silico in that specific DNA sequence, and collectively the enhancer fragment (−4,014_−3,975 bp) consists of three highly conserved sequence blocks, all of which conform to the HMG DNA-binding consensus. The first block also scores with one mismatch the binding matrix of dTCF; the endogenous sequence, however, is not efficiently recognized by dTCF in vitro (not shown). Still, this cluster of three highly conserved HMG putative binding sites may recruit HMG DNA-binding activity in vivo necessary to ensure the early onset of the ic-CRE-mediated expression pattern. The functional, architectural role of HMG proteins within the context of chromatin environment is attributed to their strong DNA-bending properties, thereby facilitating DNA-binding of sequence-specific factors and the assembly and stabilization of transcriptional complexes (Giese et al. 1997; Dragan et al. 2004). HMG activity has been previously implicated in transcriptional control of other early embryonic developmental processes as well (reviewed in Dailey and Basilico 2001). In particular, members of the Sox protein family are expressed temporal and spatiotemporal and can interact with other sequence-specific transcription factors to control crucial aspects of developmental gene expression (Kamachi et al. 1998, 2001; Wilson and Koopman 2002; Kondoh and Kamachi 2010). In Drosophila, eight Sox genes have been characterized (Crémazy et al. 2001; McKinnie et al. 2005). Function of the fish-hook/Dichaete/Sox70D protein, containing an HMG domain homologous to that of the mammalian Sox2, was shown essential for segmentation in the early Drosophila embryo (Nambu and Nambu 1996; Russell et al. 1996; Sánchez-Soriano and Russell 2000). In mouse, the temporal-specific late onset of Sox9 expression correlates with the timing of neuron-to-glial switching, being thereby involved in cell-type specification (Stolt et al. 2003). Interestingly, it was recently shown in zebrafish that Sox factors crucially control the timing of biphasic target gene expression, since their activation threshold determines the onset of the second phase of specific target gene expression (Onichtchouk et al. 2010).

Delayed and distinct establishment of the intercalary segment

The functional detection of an early active cis-regulatory control element underlying the onset of the intercalary-specific hh expression led us to address the developmental issue of formation of the intercalary segment. Therefore, we re-examined the mode of establishment of segment polarity gene expression in the intercalary segment anlage in comparison to the rest of the procephalic head segments (Fig. 6). The complex morphogenetic movements during the early gastrulation, marked by the formation of the cephalic furrow, makes it hard to clearly define the primordia of the procephalic segments and ascribe them back to the blastodermal fate map. Lateral embryonic views of stages 7/8 (Fig. 6B, B′) are misleading to assume that the ventrally located intercalary stripe, marked by the expression of hh (from stage 8 on) and en (from stage 10 on), arises from splitting from the more dorsal ectodermal antennal stripe (Gallitano-Mendel and Finkelstein 1997; Mohler 1995; de Velasco et al. 2006). However, from slightly twisted ventrolateral views (Fig. 6D–D′) it can be seen that the onset of hh expression in the intercalary segment anlage at stage 8 is detected at a focal plane which is different from detection of the antennal and ocular stripes. This indicates that formation of the intercalary hh stripe arises in a distinct and independent set of embryonic cells that are not in direct continuation to the antennal and ocular hh expressing cells. In addition, at stage 8, the hh antennal and ocular stripes are still in contact at their dorsal- and ventral-most ends (Fig. 6D′), suggesting that they arise from progressive separation (or splitting) of the early procephalic anterior wide expression domain of hh (Fig. 6F; Chang et al. 2001). The en co-expressing cells at the posterior margin of this domain (at stage 6; Fig. 6F) will subsequently belong to the antennal stripe formed and defined at the posterior margin of the procephalic ectoderm (Fig. 6G; Schmidt-Ott and Technau 1992). Since not only hh (stage 8) but also wg and en (both at stage 10) are expressed late in the intercalary segment compared to the other segments (Schmidt-Ott and Technau 1992), we postulate that the intercalary segment anlage is formed by the ventral ectodermal cells lying anterior to the blastodermal mandibular anlage (depicted by an open arrow in Figs. 2d, e;
3D; and 6F), at a region that is spatially distinct from the region covered by the early blastoderm expression patterns of hh (Fig. 6E, F) and wg (Fig. 2a, b). A developmentally delayed establishment of the intercalary segment has also been described for Tribolium (Posnien and Bucher 2010; Schaeper et al. 2010), thus reflecting a phylogenetically conserved mode of delayed intercalary segment establishment within at least the holometabolous insects.

Conclusions

Resulting from the functional enhancer dissection assays, the isolation of an intercalary-specific cis-regulatory element of hh supports the concept of a unique mode of establishment of each of the procephalic head segments, as it was initially devised based on the results of mutant analysis (Gallitano-Mendel and Finkelstein 1997). Functional data that add further support to this conclusion are the detection of a large cis-regulatory region of wg which specifically confers establishment of procephalic wg expression only in the antennal segment among the procephalic segments. The above results indicate that distinctive cis-regulatory information and thus segment-specific transcriptional gene networks underlie the metamORIZATION process in the procephalon. This supports the idea that establishment of the procephalic segments reflects the primary segmentation mode (Minelli 2001; Tautz 2004). Moreover, regulatory mechanisms comprising enhancer–promoter–specific interactions and the function of additional temporal cis-regulatory control elements contribute to the specificity of transcriptional regulation governing the segment polarity gene expression in the anterior head. The functional isolation of an intercalary-specific cis-regulatory element of hh can now lead to the identification and verification of direct, trans-acting sequence-specific binding factors and thus elucidate how the patterning information is molecularly transmitted from the head gap-like genes to segment polarity gene expression. Two candidates for such second order regulators acting on this particular ic-CRE are the helix-loop-helix transcription factor Collier (Crozatier et al. 1996, 1999) and the basic-leucine-zipper transcription factor Cap’n’collar B (Mohler et al. 1995; Veraksa et al. 2000). A detailed, molecular, and biochemical analysis on the direct interactions of these second order regulators with the ic-CRE will be published elsewhere (EN and EAW unpublished).

Finally, our results support that during the process of metamORIZATION of the anterior head region, the establishment of the intercalary segment — apart from having a morphogenetic independent origin — is also developmentally delayed in comparison to the rest of the procephalic segments. The latter also provides an indication for an evolutionary conserved mode of establishment of the insect intercalary segment (Posnien and Bucher 2010; Schaeper et al. 2010).

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