Head versus trunk patterning in the *Drosophila* embryo; *collier* requirement for formation of the intercalary segment

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SUMMARY

Whereas the segmental nature of the insect head is well established, relatively little is known about the genetic and molecular mechanisms governing this process. In this paper, we report the phenotypic analysis of mutations in *collier* (*col*), which encodes the *Drosophila* member of the COE family of HLH transcription factors and is activated at the blastoderm stage in a region overlapping a parasegment (PS0: posterior intercalary and anterior mandibular segments) and a mitotic domain, MD2. *col* mutant embryos specifically lack intercalary ectodermal structures. *col* activity is required for intercalary-segment expression both of the segment polarity genes *hedgehog*, *engrailed*, and *wingless*, and of the segment identity gene *cap and collar*. The parasegmental register of *col* activation is controlled by the combined activities of the head-gap genes *buttonhead* and *empty spiracles* and the pair-rule gene *even skipped*; it therefore integrates inputs from both the head and trunk segmentation systems, which were previously considered as being essentially independent. After gastrulation, positive autoregulation of *col* is limited to cells of anterior PS0. Conversely, heat-pulse induced ubiquitous expression of Col leads to disruption of the head skeleton. Together, these results indicate that *col* is required for establishment of the PS(-1)/PS0 parasegmental border and formation of the intercalary segment. Our data support neither a simple combinatorial model for segmental patterning of the head nor a direct activation of segment polarity gene expression by head-gap genes, but rather argue for the existence of parasegment-specific second order regulators acting in the head, at a level similar to that of pair-rule genes in the trunk.

Key words: COE transcription factor, *collier*, Head patterning, Intercalary segment, *Drosophila*, Autoregulation

INTRODUCTION

Extensive studies on the development of the *Drosophila* embryo have provided deep insight into the mechanisms determining the body pattern. An intricate cascade of hierarchical, cross- and autoregulatory interactions initiated by maternal morphogens, followed by activation of gap, pair rule and segment polarity genes, serially subdivides the trunk into reiterated units. Segment identity is conferred by the regionalised expression of homeotic selector genes from the *Antennapedia* (*ANTC*) and *Bithorax* (*BXC*) complexes, activated by combined action of gap and pair rule genes (reviews by Ingham, 1988, Pankratz and Jäckle, 1993). The number of segments composing the insect head has itself long been a matter of debate due to the diversity of form between, and within different taxa (Rempel, 1975). The pattern of expression of the segment polarity gene *engrailed* (*en*) during embryonic head formation in different insect orders revealed six Engrailed-accumulating segments, posterior to the labrum

(pre-antennal region): from anterior to posterior, two preoral – the ocular and antennal – segments and four postoral – the intercalary and gnathal (mandibular, maxillary and labial) – segments (Rogers and Kaufman, 1997). The mutant phenotypes and patterns of expression of the head gap-like genes *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*) (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Wimmer et al., 1993) led Cohen and Jürgens (1990) and Finkelstein and Perrimon (1991) to propose that a combinatorial input of head gap genes could be directly involved in partitioning the head anlage into a fixed number of segmental units with no need for second-level regulators, equivalent to pair rule genes in the trunk. The lack of an obvious homeotic phenotype associated with mutations in either *Deformed* (*Dfd*) or *labial* (*lab*) which are expressed in the maxillary and mandibular, and intercalary primordia respectively (Merrill et al., 1989; Mahaffey et al., 1989), led to the further suggestion that the combinatorial expression of head gap genes might also assign segmental identities (Cohen

and Jürgens, 1990). This model was recently questioned, however, by the results of inducible ectopic expression of either *btd* (Wimmer et al., 1997) or *otd* (Gallinato-Mendel and Finkelstein, 1998), which affected neither the head segment identities nor, or only marginally the pattern of segment polarity gene expression, suggesting that other mechanisms and factors were involved.

We report here the isolation and phenotypic analysis of mutations in *collier* (*col*), a gene specifically activated at the blastoderm stage in a region overlapping both a (cycle 14) mitotic domain, MD2, and a parasegment (PS0: posterior region of the intercalary segment and anterior region of the mandibular segment) (Crozatier et al., 1996). We show that *col* activity is required for expression of the segment polarity genes *hedgehog* (*hh*), *en*, and *wingless* (*wg*), and the segment-identity gene *cap and collar* (*cnc*; Mohler et al., 1995) in the intercalary segment. The parasegmental register of *col* activation in the head at the blastoderm stage integrates inputs both from the head and trunk segmentation systems. Together, our results indicate that *col* is a segment-specific patterning gene acting downstream of head-gap genes and required for establishing the PS(-1)/PS0 parasegmental border and formation of the intercalary segment.

MATERIALS AND METHODS

Fly strains

The bcd^{E1}, Dfd^{A325}, dl^{1310}, ems^{9Q84}, eve^{R13}, gt^{YB}, lab^4, prd^{IIB}, prd^{6L07}, otd^{YHB}, sal^{IIA}, sal^{IIB}, sna^{II6}, stg^{4B51} and stg^{7B69} strains were obtained from the Tübingen Stock Center (Tearle and Nüsslein-Volhard, 1987). The $slp^{\Delta34}$ deficiency was from U. Grossniklaus and W. Gehring, Basel, the btd^{XG81} and btd^{XA} strains from S. Cohen, Heidelberg, the cnc^{P2}, cnc^{VL70} and cnc^{VL110} strains from J. Mohler, New York and the $croc^{5F59}$ strain from H. Jäckle, Göttingen. The $D(f80850)$ deficiency were obtained from the Umea Stock Center.

Isolation of the *Df(2R)AN293* deficiency and *col* lethal mutations

To identify lethal mutations in the *col* chromosomal region (51C1-C3; see Fig. 1), we first generated a deficiency by removing this region. X-ray treatment was applied to the *(Pw+, AN34)* strain, which contains a *(Pw+, lacZ)* insert located at 51C and was provided by A. Nose. Irradiated males were mated to *Tft/CyO* virgin females. The F₁ progeny bearing white eyes were individually mated to *Tft/CyO-twist-lacZ* flies to establish *P*/CyO-twist-lacZ* stocks. A subsequent screen for loss of *col* expression by in situ hybridisation on whole-mount embryos with a mixture of *lacZ* and *col* probes allowed chromosomal rearrangements where *col* is not transcribed to be identified. From a screening of 2×10^5 irradiated chromosomes, a single deficiency was recovered, and designated *Df(2R)AN293*. We then used EMS as a mutagen and selected chromosomes that were lethal over *Df(2R)AN293* and viable over *Df80850* (51C1-51C3 region, Fig. 1A). From 6300 individual chromosomes tested, 50 mutations were isolated, representing 7 independent complementation groups.

Sequencing of the *col¹* mutation

Genomic DNA was isolated from hand-selected homozygote *col¹* mutant larvae to act as a matrix in PCR amplification experiments. The PCR amplification products were directly sequenced.

Transgenic lines and heat-shock treatment

The P[*col5*-lacZ] and Hscol constructs have been described by Crozatier and Vincent (1999). P[*col5*-cDNA] was constructed by subisuting the *col* cDNA for *lacZ* in P[*col5*-lacZ]. Males homozygous

for the *Hscol* transgene were crossed with w^- females, with w^- males used as controls. The embryos resulting from these crosses were allowed to develop for 3 hours at 25°C, before heat-shock treatment for 45 minutes at 37°C. Development was continued at 25°C until the embryos formed cuticles.

In situ hybridisation and antibody staining

Whole-mount in situ hybridisation to embryos, double labelling using several probes simultaneously, or double immunostaining and in situ hybridisation were performed as described by Crozatier et al. (1996), and references therein. RNA probes were synthesised from cDNA plasmids except for the *col* intronic probe (Crozatier and Vincent, 1999). Primary antibodies were used at the following dilutions: rabbit anti-phospho histone H3 from Upstate Biotechnology (2 µg/ml); home-produced rabbit anti-En (1/400), rabbit anti-Dfd (1/250), rabbit anti-Lb (1/100), mouse monoclonal anti-Wg (1/200). The antibody against Wg was kindly provided by S. Cohen, Heidelberg.

RESULTS

Genomic structure of the *col* gene; conserved domains split into multiple exons

The *col* gene maps to the chromosomal region 51C1,2 (Fig. 1A). In order to establish its molecular organisation, we isolated approx. 45 kb of overlapping genomic DNA covering the *col* transcription unit and sequenced the relevant regions. The *col* transcription unit consists of 12 exons and 11 introns spanning a genomic region of about 30 kb (Fig. 1B). Introns separate the coding regions for each Col functional domain, defined by biochemical dissection of EBF and sequence conservation during evolution (Hagman et al., 1993; Crozatier et al., 1996). These are the DNA binding domain (aa 59 to 288), the homodimerisation domain (aa 289 to 429), and a putative transactivation domain at its carboxy-terminal end (Fig. 1C). However, additional introns split the Col DNA binding and homodimerisation domains, despite their extensive primary sequence conservation in all COE proteins identified so far, from nematode to vertebrates (Bally-Cuif et al., 1998, for ref.). Within the homodimerisation domain, the helix-loop-helix (HLH) motif is encoded by a single exon, exon 9 (Fig. 1C). Finally, the genomic structure of *col* indicates that the two predicted Col embryonic protein isoforms, which differ in their carboxy-terminal protein coding region (Crozatier et al., 1996), result from alternative splicing of exon 11.

col mutants show specific defects in the embryonic head

In the absence of any known deficiency removing the *col* gene, we generated one by X-ray irradiation, using as a marker a P[*w+, lacZ*] insertion located at 51C (see Material and Methods). Approximately 2×10^5 irradiated chromosomes were first screened for loss of the w^+ marker. By searching for associated loss of *col* expression by in situ hybridization to *col* mRNA on whole-mount embryos, we were able to recover a deficiency removing the *col* gene, deficiency *Df(2R)AN293* (Fig. 1A). A subsequent screen for EMS-induced lethal mutations in the 51C1-C3 interval defined by *Df(2R)AN293* and *Df(2R)80850* (Fig. 1A) led to the isolation of 50 mutations representing 7 different complementation groups. As a first step to identify *col* mutations, cuticles from hemizygous mutant embryos were examined for head skeleton defects. All

three independent alleles of one complementation group (designated as *col¹*, *col²* and *col³*) displayed lethality at the late embryonic/first instar larval stage with specific defects in the head skeleton similar to those observed upon heat-shock induced expression of *col* antisense RNA in early gastrula embryos (Fig. 2A,B; Crozatier et al., 1996). These defects are a complete lack of the ventral arms (VA) and a strong reduction of the lateral gräten (LG), two structures thought to be derived from the intercalary/mandibular segment anlagen (Jürgens et al., 1986). The T-ribs in the floor of the pharynx and the antennal, maxillary or hypopharyngeal sensory organs (see Jürgens et al., 1986) are present, and a normal pattern of internal sensory structures, detected by 22C10 antibody staining (Schmidt-Ott et al., 1994), is observed (data not shown). The mutant embryos that hatch give rise to larvae that do not grow and tend to crawl out of the medium, suggesting that they are unable to feed. That these defects result from *col* mutations was further substantiated by the lack of a somatic dorsal muscle in which *col* is specifically expressed (Crozatier and Vincent, 1999). The same head and muscle phenotypes are observed for the 3 alleles in homozygous or hemizygous combinations, suggesting that they are strong hypomorphic or null alleles. Further support was provided by sequence analysis of the *col¹* allele, where

a G to A transition (amino-acid position 228) eliminates a splice acceptor site. This lesion should result in the non-removal of intron 6 and the production of a truncated Col protein (Fig. 1C); indeed, no Col protein can be detected in *col¹* mutant embryos using anti-Col polyclonal antibodies directed against the divergent carboxy-terminal end of the protein (data not shown, and Crozatier and Vincent, 1999).
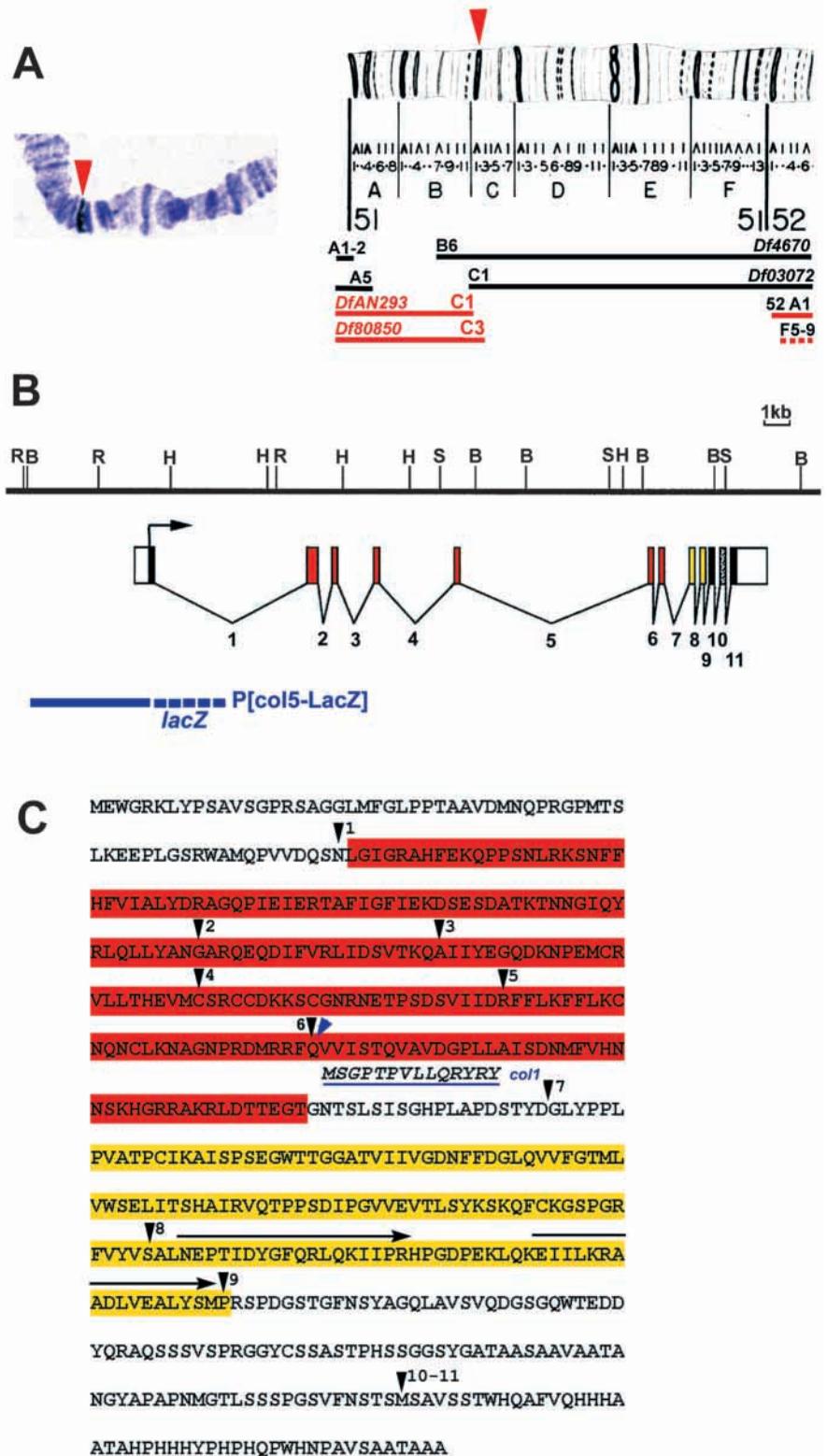
Fig. 1. Chromosomal location and structure of the *collier* transcription unit. (A) In situ hybridization of a *col* cDNA to polytene chromosomes indicating a cytological localisation in the region 51C1-2 (red arrowhead); breakpoints of various deficiencies in this region are indicated. *Df(2R)AN293* generated in our laboratory was used in combination with *Df(2R)80850* to isolate *col* mutations. (B) Composite genomic map of the *col* transcription unit reconstructed from overlapping clones. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I. The *col* transcription unit consists of 12 exons (boxes) spanning a region of about 30 kb The open reading frame is indicated by filled bars, with red bars corresponding to the DNA-binding domain, yellow bars to the homodimerisation region (see Hagman et al., 1993) and the stippled bar to an alternative exon (exon 11). The initiation codon ATG is indicated by the arrow, untranslated 5′ and 3′ regions by open bars. The position of the 5 kb *col* upstream region fused to *lacZ* in P[*col5-LacZ*] is indicated by a blue line. (C) Amino acid sequence of the Col protein product (see Crozatier et al., 1996) to show the position of introns (numbered black arrowheads) in the DNA binding domain (red) and homodimerisation domain (yellow). Horizontal arrows indicate the position of helices 1 and 2 of the HLH motif. The blue arrowhead indicates the position of the *col¹* mutation which removes a splice acceptor site and results in a truncated Col protein whose carboxy-terminal sequence is indicated in italics underlined in blue.

Fig. 2. Head defects in *col*[1] hemizygous embryos and *hs-col* embryos. Enlargement of the head region of the cuticle of a wild-type embryo (A), *col*[1] embryo (B) and *hs-col* transgenic embryo subjected to a 45 minutes heat treatment at stage 6-9 of development (C). The head skeleton structures, H piece (H), lateral-gräten (LG), ventral arm (VA), and posterior wall of the pharynx (ppw) are indicated. In *col* embryos, the VA are missing and the LG are reduced, while ubiquitous Col expression results in the lack of the H piece and abnormal LG.
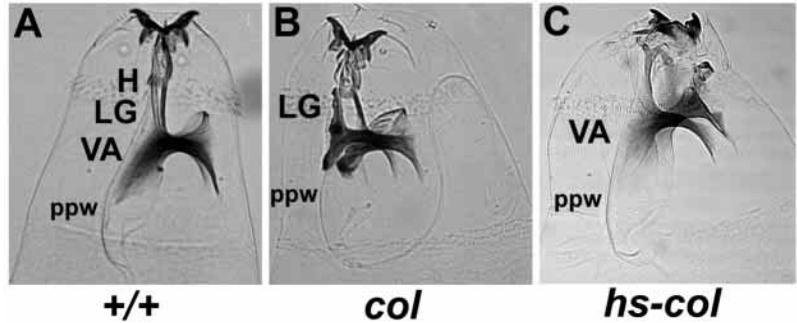
## Expression of a *col* cDNA in the head rescues *col* lethality

In order to confirm that our 3 putative *col* mutations were indeed affecting the *col* gene, we undertook to rescue this phenotype using a *col* transgene. The *cis*-acting regulatory region responsible for *col* expression in the head was identified by generating reporter transgenes expressing a Col/β-galactosidase fusion protein under the control of various *col* genomic DNA fragments. A reporter gene containing 5 kb of *col* upstream DNA (P[*col*5-lacZ], see Fig. 1B) showed a pattern of *lacZ* transcription that faithfully reproduced *col* transcription in MD2/PS0 from early cycle 14 (stage 5) up to stage 11 (see Fig. 7A and data not shown), and in the embryonic mesoderm (Crozatier and Vincent, 1999). We used this 5 kb upstream region to drive expression of a *col* cDNA in phenotypic rescue experiments (P[*col*5-cDNA] construct). A single transgenic copy of this construct rescued the embryonic/ larval lethality of *col*[1], *col*[2] or *col*[3] hemizygous embryos: about 80% of the rescued embryos hatched into wandering larvae and reached adulthood. Complete rescue of the head skeleton defects was observed in the cuticules of such embryos, confirming that only *col* function was affected in these embryos.

col controls the expression of segment polarity and segment identity genes in the intercalary segment

The embryonic head phenotype of *col*[1] hemizygous mutant embryos indicates a loss of skeletal structures derived from the intercalary, and possibly mandibular, segments (Fig. 2A-B; Jürgens et al., 1986) without transformation towards another segment identity. To investigate this segmentation phenotype in more detail, we first compared *col* expression with that of the segment polarity genes *hh* and *wg*. At the blastoderm stage, the posterior limit of *col* expression is parasegmental (PS0/PS1), as it precisely abuts the mandibular stripe of *hh*-expressing cells (Fig. 3A). Whether its anterior limit is also parasegmental cannot be answered at this stage because the expression of segment polarity genes in pre-gnathal segments is not yet established. Examination of early stage 11 embryos shows that *col* expression overlaps the intercalary *hh* stripe and abuts the intercalary Wg spot, indicating a parasegmental anterior border for *col* expression. At this stage however, *col* expression has been lost from the posterior part of PS0, as it does not overlap mandibular Wg expression (Fig. 3C,D). The *cnc* gene, which codes for a b-ZIP transcription factor, has been postulated to act as a segment identity gene in the mandibular segment (Mohler, 1993; McGinnis et al., 1998). Consistent with *col* being expressed in PS0 *col* and *cnc* expression only

partly overlap, in the region corresponding to the anterior mandibular segment (Fig. 3B). Together, these data indicate a parasegmental register of *col* expression at the blastoderm stage, which is subsequently restricted to anterior PS0.

We then determined whether *col* mutations affect the expression of *wg* and En, which mark the anterior and posterior compartments of each segment, respectively (Schmidt-Ott and Technau, 1992). In *col*[1] hemizygous embryos, both the intercalary stripe of En and the spot of *wg* expression are missing (Fig. 4A). Since *col* expression does not overlap the intercalary Wg spot (Fig. 3D), the loss of this spot in *col* mutant embryos suggested that *col* does not regulate *wg* expression
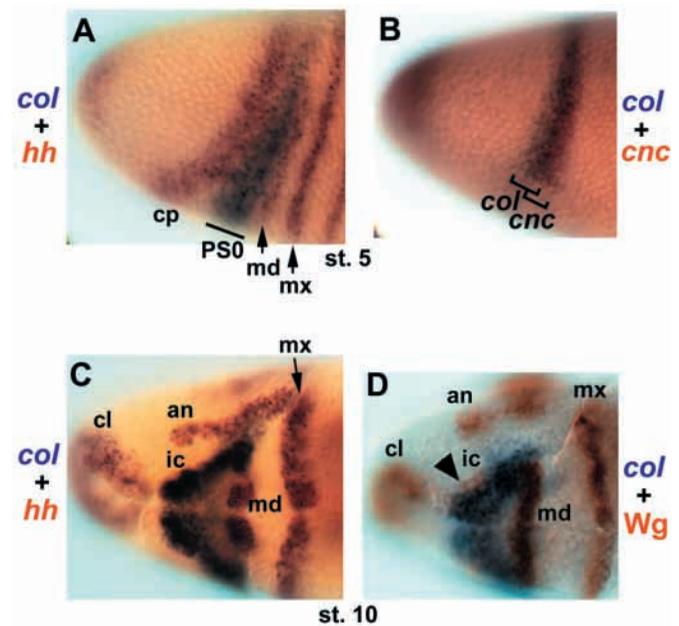
Fig. 3. The parasegmental register of *col* expression in the head. *col* mRNA expression (blue) in wild-type embryos at stage 5 (A,B) and stage 10-11 (C,D). *col* is activated in PS0 at the blastoderm stage, as determined by comparison with *hh* (A) and *cnc* (B) mRNA expression (brown). At stage 10, *col* expression is restricted to anterior PS0 cells, as determined by comparison with (C) *hh* mRNA and (D) Wg protein expression (brown). *col* expression overlaps the intercalary stripe of *hh*-expressing cells and abuts but does not overlap the 2-3 Wg-expressing cells of the intercalary segment (arrowhead). an, antennal; ic, intercalary; md, mandibular; mx, maxillary segments; cp, cephalic plate; cl, clypeolabrum. In all figures, embryos are oriented with the anterior pole to the left. A,B lateral views; C,D, ventral views. Stages are according to Campos-Ortega and Hartenstein (1985).

directly but possibly by an *hh*-dependent mechanism (Gallinato-Mendel and Finkelstein, 1997). We indeed found that in *col* mutant embryos, the intercalary stripe of *hh* is also absent, or much reduced (Fig. 4A). Together, these results show that *col* controls *hh*, *en* and *wg* expression in the intercalary segment and is required for establishing the PS(-1)/PS0 parasegmental border. The VA and LG, which are missing and reduced, respectively, in *col* mutant embryos, are also affected in two other head mutants, *crocodile* (*croc*), which codes for a forkhead-domain protein (Häcker et al., 1995) and *cnc*. These structures are also affected in embryos mutant for the homeotic genes *Dfd* and *lab*, which are expressed in the mandibular and maxillary segments, and in the intercalary segment, respectively (Diederich et al., 1991; review by Rogers and Kaufman, 1997). We first looked at *col* expression in embryos mutant for *croc*, *cnc*, *Dfd* or *lab*. In none of these embryos did we observe a change in *col* transcription. Conversely, no changes could be detected for *croc*, Dfd or Lb expression in *col*[1] hemizygous embryos, indicating that expression of each of these three genes is independent of *col* (not shown). In contrast, *col* is required for *cnc* transcription in the posterior intercalary segment at stage 9-10 (Fig. 4B). Because this region is anterior to the region of overlap between *col* and *cnc*

Fig. 4. En, *wg*, *hh* and *cnc* expression, and cell death in *col* mutant embryos. Compared expression of *en*, *wg*, *hh*, (A) and *cnc* (B) in wild type (left), and *col*[1] hemizygous (right) embryos. Note the specific loss of expression of all four genes in the presumptive intercalary segment (arrowheads) of *col*[1] embryos. (C) Accumulation of *rpr* mRNA in *col* mutant embryos reveals the programmed cell death in the invaginating floor of the pharynx (arrowhead). The asterisks indicate two sites of cells fated to undergo apoptosis in wild-type embryos.
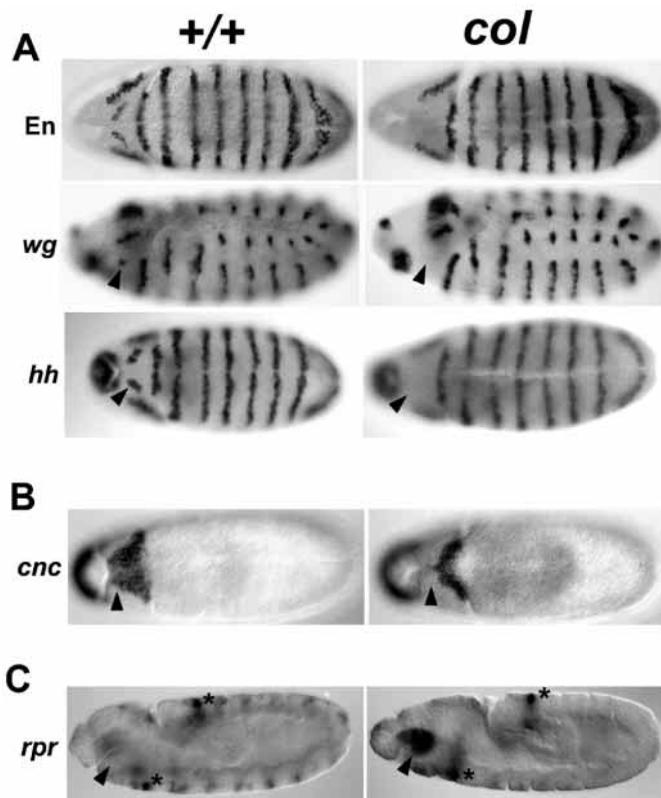
expression at the blastoderm stage (Fig. 3B), we conclude that it corresponds to a secondary site of *cnc* expression initiated at stage 9, under control of *col* activity. In *cnc* mutant embryos, intercalary *hh* expression is normal (data not shown), indicating that *hh* and *cnc* are regulated by *col* independently of each other.

col mutations provoke cell death in the forming hypopharynx

The normal pattern of Lab expression and morphology of the intercalary lobe (sometimes referred to as hypopharyngeal lobe; Rogers and Kaufman, 1997) at stage 11 indicated that lack of *hh* and *cnc* expression in *col*[1] hemizygous embryos was probably not linked to cell death. In order to address this question more directly, we looked at the distribution of *reaper* (*rpr*) mRNA, which marks cells fated to undergo apoptosis (White et al., 1993). In wild-type embryos at stage 11, there are two invariant sites of *rpr* expression: inside the epidermal layer of the gnathal region where the primordium of the salivary gland invaginates, and near the caudal tip of the extended germ band (Fig. 4C; Abrams et al., 1993). In *col*[1] embryos, an additional site of *rpr* expression is observed, which does not correspond to cells of the intercalary lobe but rather to cells of the hypopharyngial epithelium (floor of the forming pharynx). These cells have been previously shown to derive from the ventral side of the intercalary segment primordium (Jürgens and Hartenstein, 1993) and lineage tracing analysis has linked them to MD2 (Cambridge et al., 1997). This specific cell death is consistent both with the domain of *col* expression at the onset of gastrulation and the head skeleton phenotype of *col* mutant larvae.

col expression in PS0/MD2 interfaces the head and trunk segmentation mechanisms

col expression is first detected during the interphase of mitotic cycle 14 (stage 6), when expression of head-gap genes has already resolved from initial broad domains into defined stripes. The stripe of *col* expression is included in that of *btd*, overlaps that of *ems*, and is restricted both dorsally and ventrally to neuroectodermal cells (Crozatier et al., 1996 and Fig. 5A). Examination of *dorsal* (*dl*) mutant embryos showed that Dl is required for *col* repression in the mesodermal plate. The ectopic expression of *col* observed in *twist* (*twi*) and *snail* (*sna*) mutant embryos suggests that Dl target genes (review by Rushlow and Arora, 1990), rather than Dl itself, are involved (Fig. 5B and data not shown). Embryos lacking *ems* function also show a ventral derepression of *col* expression. Further, at stage 10, *ems* mutant embryos show an abnormal pattern of *col* mRNA accumulation, with a mandibular in addition to intercalary stripe of *col*-expressing cells (Fig. 5C). This suggests a second role for *ems* in regulating *col*. In *btd* mutant embryos, there is a complete loss of *col* expression, whereas there is no change in embryos lacking both *slp* (*slp1* and *slp2*) genes (data not shown), consistent with previous data establishing that *btd* but not *slp* is required for intercalary *en* and *wg* expression (Wimmer et al., 1993; Grossniklaus et al., 1994). Together, these results confirm that *col* acts downstream of head gap genes in the transcriptional cascade patterning the head. The fact that *col* is expressed in a parasegment

**Fig. 5.** Regulation of *col* expression by A/P and D/V patterning genes. (A) Diagrammatic representation of the head region of a *Drosophila* embryo at stage 6 with the segmental limits of the AN, IC and MD segments schematically drawn as black lines. Positions of parasegments PS1 and PS3 are indicated by horizontal bars. The domains of gene expression are colour coded, with *btd* red, *btd+ems* light purple, *eve* yellow, *btd+eve* orange and *col* blue; *otd* expression is represented by a black bar. (B) *col* mRNA expression in early gastrula embryos (stage 6-7), wild-type (+/+) or mutant for *dl*, *twi* and *ems*. In all three mutants, *col* is derepressed in the mesodermal plate. (C) Ectopic *col* expression is still observed in *ems* mutant embryos at the germ band extension stage (stage 10). Conversely, this expression is restricted to a few cells anterior to the abnormal cephalic furrow which forms in *prd* mutant embryos. In wild-type embryos at that stage, *col* is expressed in ventral cells which have invaginated (arrowhead).
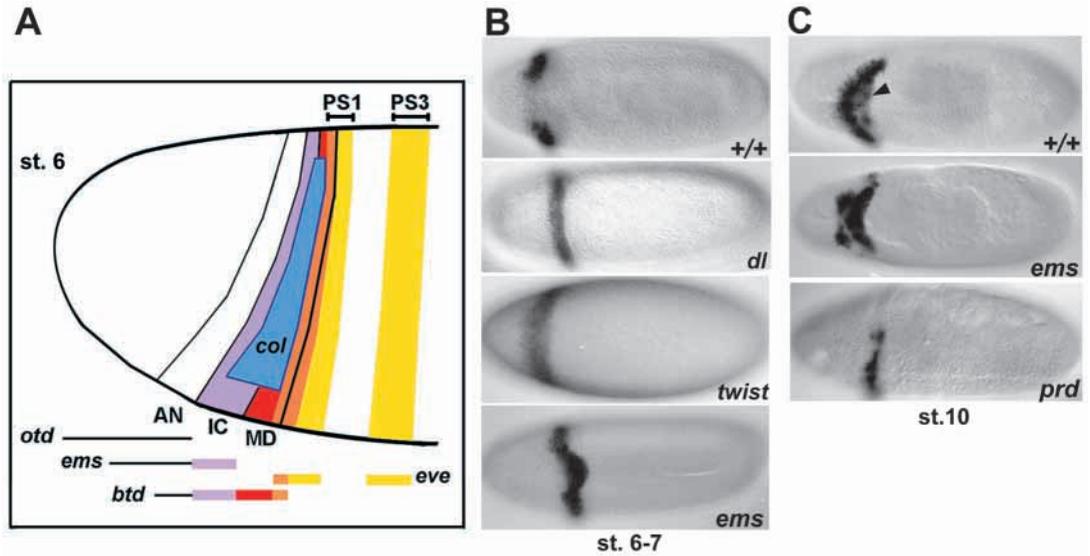
immediately anterior to the trunk, raised the possibility of a regulation by components of the trunk segmentation system such as pair rule genes. In *paired* (*prd*) mutant embryos, *col* expression starts normally but, soon after gastrulation, becomes restricted to a few cells located anterior to the abnormal cephalic furrow which forms in these embryos (Sander, 1980; Fig. 5C). *col* mis-expression might thus be a rather indirect effect of the *prd* mutation. In addition to *prd*, the only pair rule gene whose mutations appear to affect *col* expression is *eve*.

At the beginning of cycle 14, the stripe of *col* expression is located immediately anterior to Even-skipped (Eve) stripe 1, these two domains becoming separated by a single row of cells during the process of cellularisation (Fig. 6A). We found that *col* is ectopically expressed in *eve* mutant embryos, in a region roughly corresponding to PS1, indicating that Eve acts
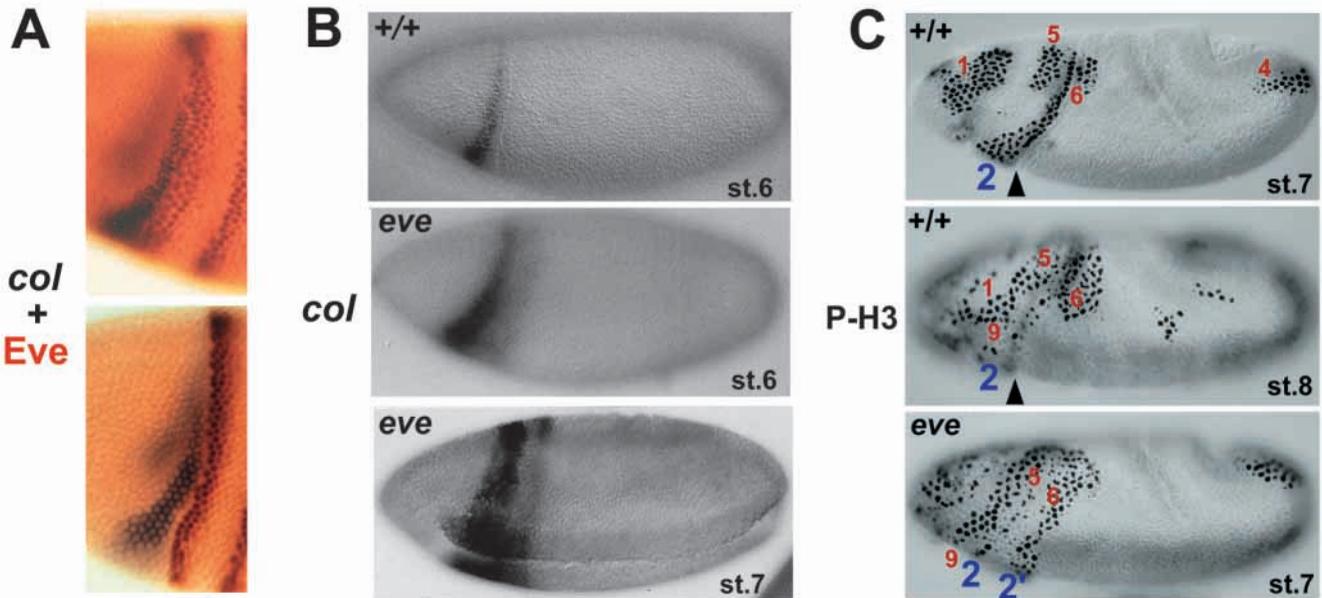
Fig. 6. Ectopic stripe of *col*-expressing cells and MD2 in *eve* mutant embryos. (A) Compared distribution of Eve protein (brown) and *col* mRNA (black) in wild-type embryos. Initially juxtaposed, the *col* stripe and Eve stripe 1 become separated by a single row of cells during the interphase of mitotic cycle 14 (stage 5). (B) Ectopic *col* expression in the presumptive PS1 of *eve* mutant embryos at the blastoderm stage. This ectopic expression evolves into a well separated second stripe of *col*-expressing cells during gastrulation. (C) Positions of mitotic domains 1, 2, 4-6 and 9 (coloured numbers) in wild-type and *eve* mutant embryos at stages 7-8, as visualised by the phosphorylation pattern of histone H3 (P-H3). An ectopic stripe of early mitotic cells (domain 2′) is observed at stage 8 in the presumptive PS1 of *eve* mutant embryos. Position of the cephalic furrow which forms in wild-type but not in *eve* mutant embryos is indicated by a black arrowhead.

as a repressor of *col* in this parasegment (Fig. 6B). During the interphase of cycle 14, the broad band of ectopic *col* activation resolves into a distinctive stripe, separated from the normal PS0 stripe by one to three cells going from ventral to dorsal (Fig. 6B). *col* expression precisely overlaps the expression of *string* (*stg*), in the region prefiguring MD2 (Foe, 1989; Edgar et al., 1994; Crozatier et al., 1996). Expression of *stg,* which triggers the G$_2$/M transition, is unchanged in embryos deficient for *col* and vice versa, arguing that MD2 cells undergo a concerted mitotic and differentiation programme, set upstream of both *col* and *stg* (data not shown). This led us to examine whether *eve* was also involved in defining the position of MD2, using antibodies against the phosphorylated form of histone H3 as a marker of mitosis (Fig. 6C). Like *col* transcription, MD2 expands posteriorly in *eve* mutant embryos at early cycle 14 to form a second, ectopic, stripe of mitotic cells at the beginning of gastrulation. Together, our results show that *col* expression and MD2 position integrate inputs from both the head and trunk segmentation systems, which were previously considered as being essentially independent.

Late control of *col* expression involves restriction to the anterior PS0 and positive autoregulation

At the onset of gastrulation (stage 7), *col* is expressed in the entire PS0 (Fig. 3A). At stage 10, the two separate patches of cells which keep expressing *col* correspond to lateral cells of the intercalary lobe and ventral cells invaginating within the atriopharyngeal cavity, respectively, indicating a restriction of *col* expression to anterior PS0 (Fig. 5C). To determine when this restriction occurs, we made use of the greater stability of the β-gal protein compared to *lacZ* (or *col*) mRNA and compared the two patterns in P[*col5-lacZ*] embryos. In order to follow *col* transcription rather than transcript accumulation, we used a *col* intron probe which labels nascent nuclear transcripts (Crozatier and Vincent, 1999). Before mitosis 14, the *lacZ* and *col* mRNA and β-gal protein patterns completely overlap (Figs 5B and 7A, and data not shown). After completion of mitosis 14, all the ectodermal cells derived from the MD2 domain can be visualised by β-galactosidase immunostaining. Only the most anterior cells continue to transcribe *col-lacZ* (or *col*) (Fig. 7A). This observation suggests that, at cycle 14, an asymmetric cell division occurs with respect to the maintenance of *col* transcription. After stage 11, *col* transcription is only maintained in a subset of the cells of the intercalary lobe. In *col* mutant embryos, this transcription is lost (Fig. 7B), indicating a direct or indirect, positive auto-regulation.

Ubiquitous *col* expression specifically affects the head skeleton

To investigate further the role of *col* transcriptional regulation in head morphogenesis, we examined the functional consequences of ubiquitous expression of the Col protein, using *hs-col* transgenic lines. One 45 minutes heat shock was applied at different times during embryonic development. Whereas heat-shock treatment of hs-*col* embryos later than 5 hours after egg laying (AEL) has no apparent effect on embryonic development and viability (Crozatier and Vincent, 1999), heat-shock treatment between 3 and 5 hours AEL (stages 6 to 9) causes embryonic death. Cuticle preparations

of dead embryos display a normal segmental array of thoracic and abdominal denticles, but a disrupted head skeleton (Fig. 2C and data not shown). The H piece, which originates in part from the maxillary, and partly from the labial segment anlagen (Jürgens et al., 1986), is consistently missing and the LG are also affected. Because *col* activity is required for maintaining its own expression in the head ectoderm (Fig. 7B), we asked whether ubiquitous expression of the Col protein was altering endogenous *col* expression. In situ hybridization to *hs-col* embryos with a *col* intron probe which allows *hs-col* and endogenous *col* transcripts to be distinguished, revealed that *col* transcription is ectopically activated in mandibular cells (Fig. 7C). Whether this ectopic
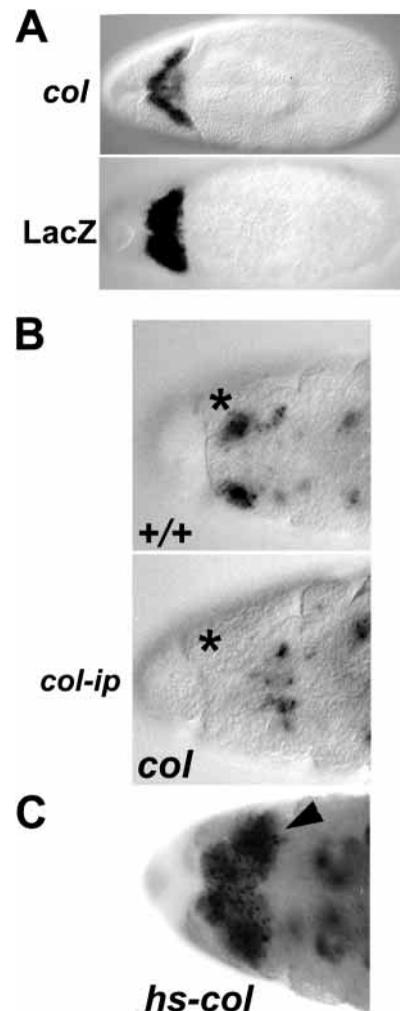
Fig. 7. Restriction of *col* expression to anterior PS0 and positive autoregulation. (A) Compared patterns of *col* mRNA and β-gal protein in P[*col5-lacZ*] embryos whose pattern of transcript accumulation reproduces that of endogenous *col* transcripts (Fig. 5B,C). The greater stability of β-gal allows visualisation of all the cells derived from PS0/MD2 at stage 10, indicating that *col* transcription has become restricted to anterior PS0 cells. (B) *col* expression in the intercalary lobe at stage 11 (asterisk) is lost in *col*[1] hemizygous embryos. (C) Ubiquitous expression of the Col protein results in ectopic transcription of the endogenous *col* gene in the mandibular lobe (arrowhead). In B and C a *col* intronic probe (*col-i.p*) was used to reveal the nascent *col* nuclear transcripts.

col transcription results from activation and/or maintenance in all MD2-derived cells after mitosis 14 remains to be established. No specific change in the expression pattern of En, *cnc*, Dfd and Lab could be detected, however, suggesting that ectopic activation of other Col targets is responsible for the induced phenotype.

DISCUSSION

The *col* gene was first suggested to be a second level regulator of head patterning, in the light of its expression pattern and the phenocopy produced by expressing antisense RNA (Crozatier et al., 1996). We report here the analysis of *col* mutations, which shows that *col* is a segment-specific patterning gene required for establishing the PS(-1)/PS0 parasegmental border and formation of the intercalary segment.

A conserved and stippled structure of the COE genes

The extensive amino acid sequence similarity between Col and rodent EBF/Olf-1 defined a new family of transcription factors highly conserved among metazoans (Crozatier et al., 1996 and references therein). The subsequent characterisation of mouse EBF paralogs and closely related orthologs in other vertebrates (see Bally-Cuif et al., 1998) and *C. elegans* (Prasad et al., 1998) confirmed the high degree of evolutionary conservation of these transcription factors, designated as COE factors. A helix-loop-helix (HLH) motif is the only region of significant homology to other families of transcription factors. The structure of the *col* gene shows that this motif is encoded by one of the 12 exons; it also reveals that the DNA-binding domain, although sharing between 74% and 92% sequence identity between all COE proteins (Bally-Cuif et al., 1998), is encoded by 6 exons scattered over 15 kb of genomic DNA. The same organisation in 12 exons is found in the orthologous nematode *unc3* gene and at least four introns are located at the same position in the DNA binding domain of *col* and *Olf-1* (Prasad et al., 1998). Whether the highly splintered structure of the *COE* genes is linked to their diversity of functions in different phyla (Lin and Groschedl, 1995; Dubois et al., 1998; Prasad et al., 1998; Crozatier and Vincent, 1999; this report), remains unknown. So far, alternatively spliced exons have been found in *Olf-1* (Wang et al., 1997) and *col* (Crozatier et al., 1996) but it is not clear whether these various splicing forms perform different functions.

A modular promoter region correlates with multiple *col*-specific functions

We found that a 5 kb *col* upstream region reproduces expression of a reporter gene in PS0 at the blastoderm stage and a single somatic muscle precursor (muscle DA3; Crozatier and Vincent, 1999), but not in the embryonic PNS and CNS or the wing disc (see below). Correspondingly, P[*col*5-cDNA] fully rescues the *col* embryonic head and muscle phenotypes (this report and Crozatier and Vincent, 1999), but it does not rescue other *col* developmental functions. First, about 20% of rescued embryos do not reach adulthood and the adults that do emerge are weak and have a short life span. Second, these adults have wings lacking the central intervein region (Vervoort et al., 1999). *col* expression in the wing disc, in a stripe of cells

located anterior to the A/P compartment boundary is not reproduced by the P[*col*5-lacZ] transgene, providing an explanation for this wing phenotype. The isolation of *col* mutants has, so far, led us to identify three independent Col functions during development, each correlating with a tightly regulated site of expression: establishment of the intercalary segment in the embryonic head, patterning of the central part of the wing, and formation of a specific somatic muscle. *col* is also expressed in specific subsets of post-mitotic sensory neurons in the head and trunk, and in CNS neurons both in the brain and ventral nerve cord (Crozatier et al., 1996). Mutations of the *C. elegans COE* gene *unc-3* result in defects in the axonal outgrowth of some motor neurons and in dauer formation, a process requiring inputs from specific sensory neurons (Prasad et al., 1998). While a key role of COE proteins in vertebrate neuronal differentiation is also suggested by data obtained in mouse, *Xenopus* and zebrafish (Garel et al., 1977; Wang et al., 1997; Bally-Cuif et al., 1998; Dubois et al., 1998), the potential functions *col* expression in the nervous system remain to be explored.

Interactions between the head and trunk segmentation systems and D/V patterning genes define the limits of *col* expression

Activation of *col* expression in PS0 is dependent upon activity of the head-gap gene *btd*. *btd* is, however, expressed in a much larger domain than *col*, both posterior- and anteriorwards, indicating that *col* expression is restricted by other factors. The factor defining the anterior parasegmental border of *col* remains unknown. It is probably a target of Bicoid (Bcd), however, since *col* expression is progressively shifted anteriorwards by gradually decreasing the amount of maternal Bcd by means of *sry δ* mutations (Payre et al., 1994; data not shown). Posteriorly, the limit of *col* expression abuts Eve stripe 1. Each of the 7 pair rule stripes of Eve protein foreshadows the position of odd-numbered parasegments (PS1 to 13) (Frasch and Levine, 1987; Lawrence et al., 1987). We have recently shown that *btd* is required for Eve expression in PS1 (Vincent et al., 1997). We show here that *eve*, in turn, negatively regulates *col* expression in this parasegment and defines the posterior limit of the mitotic domain MD2. This cross-talk between the head and trunk segmentation systems thus represents a key component of the transcriptional cascade patterning the head.

Early *col* expression is repressed ventrally by the mesoderm-determining genes *snail* and *twist*, and by *ems*. Mutations in each of these three genes, causes a premature activation of *col* in the mesodermal plate. The D/V regulation of *col* expression differs from that of *stg*, – MD2 is either narrowed or lacking in *ems* mutant embryos, and fused in *snail* but not *twist* mutant embryos (Edgar et al., 1994) and *cnc*, whose expression is affected in neither *ems* nor *twist* mutant embryos (Mohler, 1993; McGinnis et al., 1998). Thus, different combinations of transacting factors appear to be involved in precisely defining the spatial expression of each of these genes in the head, along both the D/V and A/P axes.

The maintenance of *col* expression in intercalary cells after stage 11 requires the activity of *col* itself, suggesting a positive autoregulation. A similar loop operates in the formation of the embryonic muscle DA3 (Crozatier and Vincent, 1999). Further support for a positive autoregulation comes from the activation
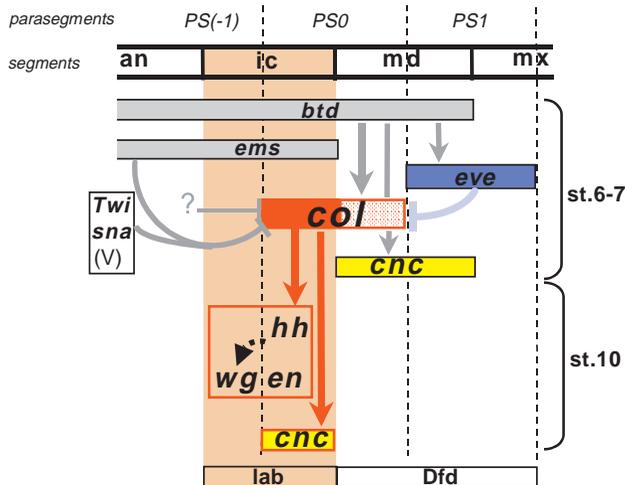
Fig. 8. Summary of the regulatory cascade controlling formation of the intercalary segment. The spatial domains of activating (→) and repressing (⊣) regulatory activities are indicated. Activation of *col* in PS0 at the blastoderm stage (stage 6) is strictly dependent upon *btd*. Col is repressed by *ems* and the mesodermal genes *sna* and *twi* ventrally (V), the trunk pair rule gene *eve* posteriorly and a yet unknown factor (?) anteriorly. At stage 10, *col* expression has become restricted to anterior PS0 (orange box), where it activates intercalary expression of *hh* and *cnc*, and possibly *en*. Loss of *hh* activity leads to the non cell-autonomous loss of *wg* expression (dashed arrow) in anterior adjacent cells (PS(-1) cells) (Gallinato-Mendel and Finkelstein, 1998). *btd* activation of *eve* in PS1 creates a regulatory loop allowing for differential gene expression between PS0 and PS1. *cnc* expression in the mandibular segment was previously reported to be dependent upon *btd* (Mohler, 1993); The domains of *lab* and *Dfd* expression have been re-drawn from Rogers and Kaufman (1997).

of endogenous *col* transcription, following ubiquitous expression of the Col protein. The observation that this activation is restricted to mandibular cells indicates the involvement or another factor cooperating with Col, whose expression is itself limited to these cells.

A transcriptional cascade of regulation specific for the intercalary segment

Recent experiments have questioned the existence of a simple combinatorial code of head-gap genes responsible both for direct activation of segment polarity genes and for assigning segment identity in the anterior head region. First, misexpression of *btd* in the anterior half of the blastoderm indicated that the spatial limits of *btd* expression are not instructive (Wimmer et al., 1997). Second, ectopic expression of *otd* results in variable changes in En and *wg* expression in different head segments, but does not change segment identity (Gallinato-Mendel and Finkelstein, 1998). The absence of intercalary En and *wg* expression in *col* mutant embryos is the first demonstration that activation of segment polarity genes does not result solely from the direct action of cephalic gap genes but involves intermediate regulators (Fig. 8). Contrasting with the trunk, the interactions between segment polarity genes in the head seem to be segment-specific (Gallinato-Mendel and Finkelstein, 1997). *col* requirement for intercalary *wg* expression, although the two genes are not expressed in the same cells, indicates that this requirement is non cell-

autonomous but probably relayed by Hh signalling (Fig. 8). *cnc* is another target of Col in the intercalary segment, correlating with the partly overlapping *col* and *cnc* head phenotypes (Mohler et al., 1995 and this report). Together, our results enable us to draw up a detailed cascade of transcriptional regulation specifically controlling the formation of the intercalary segment. How to integrate *lab* into this model and which of these interactions are direct at the molecular level are now issues to be addressed.

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