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Regulation of *Drosophila spalt* gene expression

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Abstract

The region-specific homeotic gene *spalt* is involved in the specification of terminal versus trunk structures during early *Drosophila* embryogenesis. Later in development *spalt* activity participates in specific processes during organogenesis and larval imaginal disc development. The multiple functions of *spalt* are reflected in distinct spatio-temporal expression patterns throughout development. Here we show that *spalt* cis-regulatory sequences for region-specific and organ-specific expression are clustered. Their organization may provide the structural basis for the diversification of expression pattern within the *spalt/spalt related/spalt adjacent* gene complex. We also examined the transacting factor requirement for the blastodermal *spalt* expression domains. They are under the genetic control of maternal and gap gene products and we show that these products are able to bind to corresponding *spalt* cis-acting sequences in vitro. The results suggest that the transacting factors, as defined by genetic studies, functionally interact with the *spalt* regulatory region. In addition, we provide evidence that a zygotic gene product of the terminal system, Tailless, cooperates with the maternal gene product Caudal and thereby activates gene expression in the terminal region of the embryo. © 1997 Elsevier Science Ireland Ltd.

Keywords: *Drosophila*; Regulation; *spalt*

1. Introduction

The process of body patterning during *Drosophila* embryogenesis relies on the activity of maternal and zygotic genes. The maternal genes generate the positional information to establish the anterio-posterior and dorso-ventral coordinates of the embryo (for reviews, see Rivera-Pomar and Jäckle, 1996; St. Johnston and Nüsslein-Volhard, 1992). Zygotic gap genes interpret this information and provide the cues to spatially control the expression of the subordinate segmentation genes. The action of these genes ultimately constitutes the number and polarity of segments which become specified through the activity of the homeotic selector genes of the Antennapedia and Bithorax complexes (HOM-C genes; for reviews, see Akam, 1987; Hoch and Jäckle, 1993; Ingham, 1988).

In addition to the HOM-C genes, the homeotic genes *cap'n'collar* (*cnc*; Mohler et al., 1995), *teashirt* (*tsh*; Fasano et al., 1991), *spalt* (*sal*; Jürgens, 1988; Kühnlein et al., 1994) and *fork head* (*fkh*; Weigel et al., 1989) have been identified. *cnc* activity is required to control segment identity in the head, *tsh* activity is necessary for the diversification of a ground state within the trunk region and both *sal* and *fkh* act as region-specific homeotic genes both in the head and the tail region of the embryo.

Embryos lacking *sal* activity develop thoracic structures in the posterior head and abdominal structures in the anterior tail region, indicating that trunk structures are formed at the expense of terminal structures in *sal* mutants (Jürgens, 1988). In accordance with the mutant phenotype, *sal* expression was found in two broad domains covering the anlagen of posterior head segments and anterior tail structures. We refer to these domains as the *sal* anterior domain (salAD; parasegments 1–3) and the *sal* posterior domain (salPD; parasegments 14, 15 and part of the hindgut primordium), respectively. In addition, *sal* expression is also found in a 'horse shoe-shaped' domain (salHD) encompassing parts of the procephalic neurogenic region and anlagen of the acron (Kühnlein et al., 1994). The functional relevance of salHD is reflected by the lack of a derivative of the acron, the

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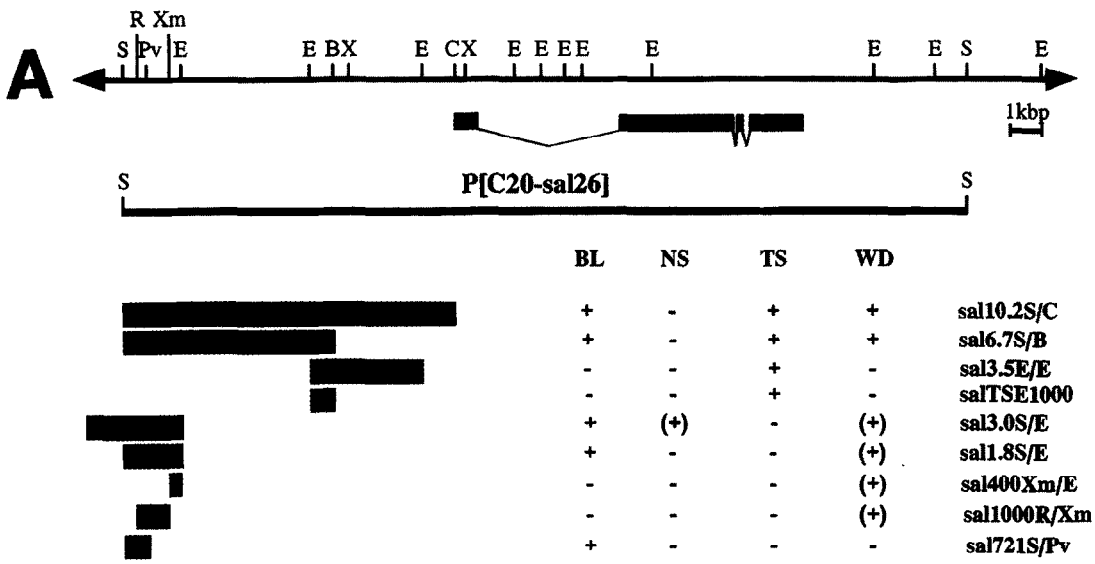
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P[C20-sal26]
S
BL NS TS WD
+ - + + sal10.2S/C
+ - + + sal6.7S/B
- - + - sal3.5E/E
- - + - salTSE1000
+ (+) - (+) sal3.0S/E
+ - - (+) sal1.8S/E
- - - (+) sal400Xm/E
- - - (+) sal1000R/Xm
+ - - - sal721S/Pv


B


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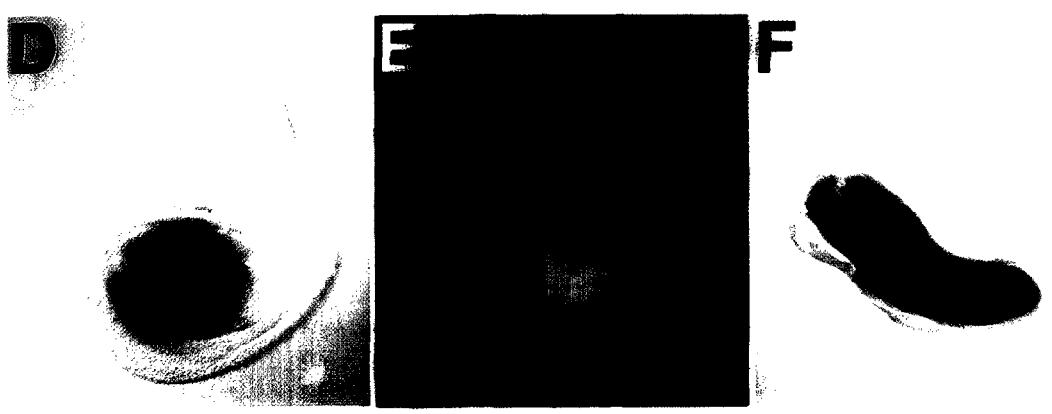
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dorsal bridge, in *sal* mutant embryos (Mohler et al., 1995).

During later stages of embryonic development the *sal* activity is necessary for the directed outgrowth of specific tracheal cells which establish the main anterior-posterior trachea, the dorsal trunk (Kühnlein and Schuh, 1996). In addition, *sal* gene activity participates in vein patterning and cell growth of the adult wing. In this tissue *sal* and *spalt related* (*salr*), likely to represent a local gene duplication, carry an at least partly redundant function (de Celis et al., 1996). *salr* shares coding sequence similarity and a late embryonic and wing disc expression pattern with *sal*, but lacks the blastodermal expression domains. Those are common to *sal* and an other neighbouring gene, *spalt adjacent* (*sala*), which carries an unknown function (Barrio et al., 1996; Reuter et al., 1996). It has been suggested that the three genes and their cis-regulatory regions may have arisen through local DNA duplication and transposition events (Reuter et al., 1996).

sal sequence related genes have been isolated from *Xenopus* (Hollemann et al., 1996), *Medaka* (R. Köster and J. Wittbrodt, pers. commun.), mouse (Ott and Schütz, 1996) and human (Kohlhase et al., 1996). The function of these genes is not yet established, but they have common expression patterns in developing neural tissue, a feature that has also been noted with *Drosophila*. *sal* as well as the vertebrate homologues encode potential transcription factors. The *sal* protein (SAL) contains a characteristic structure of three widely spaced, sequence-related double zinc finger groups (Kühnlein et al., 1994). It is likely that *sal* acts as a repressor, since *sal* activity was shown to repress several homeotic genes during blastoderm, i.e. *sal* activity in the salHD prevents *cnc* expression (Mohler, 1993), salAD restricts *Ultrabithorax* expression in the head region (Casanova, 1989) and the activities of both the salAD and salPD confine *tsh* expression to the trunk region of the embryo (Röder et al., 1992). In addition, *sal* activity of both domains restrict the formation of tracheal placodes to the trunk region of the embryo by preventing tracheal cell fate in the corresponding epidermal cells (Kühnlein and Schuh, 1996).

Little is known about the regulation of *sal* gene expression during imaginal disc development. *sal* gene expression in antennal discs is controlled by cis-elements flanking the *sal* transcription start site and is repressed by *Antennapedia* in leg discs (Wagner-Bernholz et al., 1991). *sal* expression in the central territory of wing discs is activated in response to the local concentration of a secreted signalling molecule, the transforming growth factor-β (TGF-β) homologue Decapentaplegic (DPP; de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996). In contrast, *sal* gene regulation during embryogenesis and the organization of the *sal* cis-acting region have not been described. We therefore attempted to identify cis-acting requirements for *sal* expression and the trans-acting factors which are necessary for embryonic *sal* gene expression.

We show that the *sal* cis-regulatory region contains an array of regulatory modules that mediate the spatio-temporal aspects of *sal* gene expression. The cis-acting control region which mediates *sal* expression in the three blastodermal domains is localized within a 721 bp DNA fragment. The DNA fragment contains in vitro binding sites for the gene products of the maternal and gap genes *bicoid* (*bcd*), *hunchback* (*hb*), *Krüppel* (*Kr*), *tailless* (*tll*), *huckebein* (*hkb*) and *caudal* (*cad*). In addition, the activity of these genes mediate the genetic control of the three blastodermal *sal* expression domains, suggesting that their gene products control *sal* expression by their direct interaction with the 721 bp cis-acting control region. Our results also suggest that the modular organization of regulatory elements is the prerequisite for the generation of the divergent expression patterns of the genes of the *sal/salr/sala* gene complex.

2. Results

2.1. Cis-acting control elements of the spalt regulatory region

A transgene containing about 10 kb upstream and 15 kb transcribed DNA from the *sal* locus was previously shown to rescue the embryonic lethality of amorphic *sal* mutant embryos (Kühnlein et al., 1994). This DNA fragment therefore contains cis-regulatory sequences sufficient for *sal* expression during embryogenesis. We inserted the 10.2 kb genomic *sal* upstream sequences of the rescuing transgene into the P-element vector pHZ50PL generating sal10.2S/C

Fig. 1. *sal* enhancer-*lacZ* fusion gene constructs and their corresponding expression patterns. (A) The *sal* genomic region and its transcript structure (red) are shown schematically. The extension of the P(C20-sal26) rescue construct is indicated. Restriction enzyme sites are B, *Bam*HI; C, *Cla*I; Pv, *Pvu*II; E, *Eco*RI; R, *Rsa*I; S, *Sal*I; X, *Xho*I; Xm, *Xmn*I. Below are shown the spatial distribution (green bars) and the designation of *sal* enhancer-*lacZ* fusion gene constructs. The expression pattern of the various constructs during blastoderm stage (BL), in the central nervous system (NS), in the tracheal system (TS) and the wing disc (WD) is indicated as follows: wildtype like '+'; spurious '(+)'; lack of expression '–'. (B, C, G, H) Whole-mount in situ hybridization with an antisense *sal* (B) or *lacZ* (C, G, H) riboprobe of a stage 5 wildtype embryo (B), an embryo bearing sal10.2S/C (C), a stage 11 embryo bearing salTSE1000 (G) and a stage 5 embryo bearing sal721S/Pv (H). Anterior is to the left and dorsal is up. (I) Whole-mount antibody double staining of a sal3.0S/E embryo at stage 14 using anti-β-galactosidase (brown; cytoplasmic) and anti-SAL antibodies (blue; nuclear). (D) β-Galactosidase activity staining of a wing imaginal disc bearing sal10.2S/C. (E) Double staining of a wing imaginal disc bearing sal10.2S/C using anti-SAL antiserum (red, revealed with CY3-coupled secondary antibody) and anti-β-galactosidase antibody (green, revealed with fluorescein-coupled secondary antibody). Superimposition of the CY3 and fluorescein pattern (yellow) reveals coexpression of SAL and β-galactosidase in wing disc cells. (F) β-galactosidase activity staining of a wing imaginal disc bearing sal10.2S/C as well C765-Gal4 driver and UAS-tkv^{Q253D} effector constructs (see Section 4). Ectopic expression of the constitutively active receptor TKV in the wing disc causes ectopic expression of β-galactosidase throughout the wing pouch.

(Fig. 1A). Embryos carrying this transgene construct drive *lacZ* reporter gene expression in the blastoderm that is essentially indistinguishable from that of the endogenous *sal* expression (Fig. 1B,C). In addition, this construct shows *sal* expression-like *lacZ* transcript accumulation in other analysed organ systems as tracheal system, posterior spiracles and wing imaginal discs (see below).

In order to test whether these *sal* regulatory sequences also respond to external cues, such as wild type *sal* gene expression, we analysed reporter gene expression in wing imaginal discs in more detail. During wing disc development it has been shown that *sal* expression in a broad stripe in the centre of the wing pouch is defined directly by the local concentration of the secreted morphogen DPP (Lecuit et al., 1996; Nellen et al., 1996). β-Galactosidase activity driven by the sal10.2S/C construct is detectable in the same region of the wing pouch as wild type SAL (Fig. 1D) and double labelling for SAL and β-galactosidase shows that both gene products are expressed in corresponding cells suggesting the same cis-acting control via the *dpp* mediated signaling pathway (Fig. 1E). In addition, ectopic β-galactosidase expression in the entire wing pouch is detectable after expression of the constitutively active, ligand-independent form of the DPP receptor Thick Veins (TKV) in corresponding regions of the wing disc (Fig. 1F). These results show that we have identified cis-regulatory sequences which respond to the local concentration of secreted *dpp* activity that is mediated via the receptor TKV.

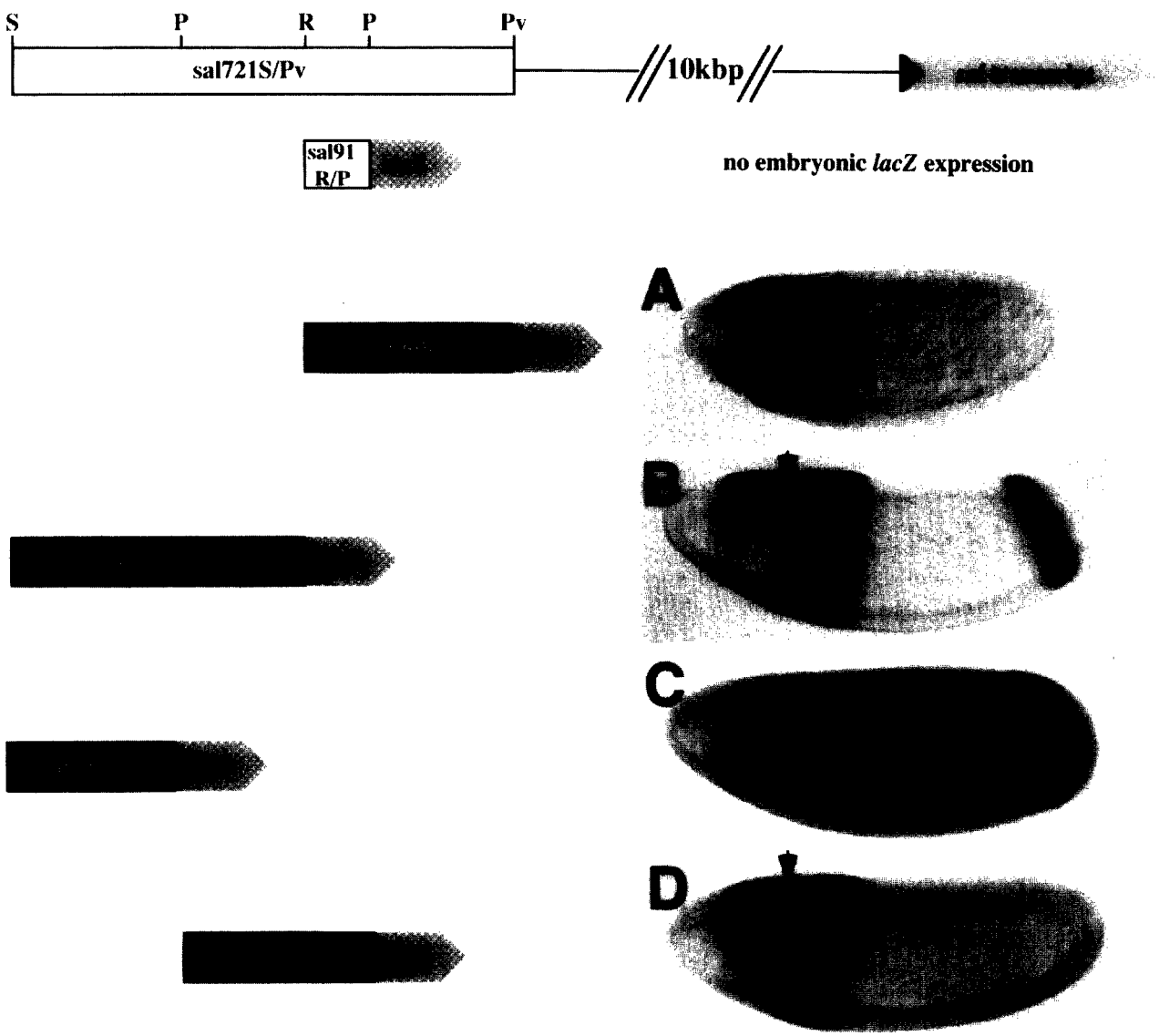


Fig. 2. *sal* blastodermal enhancer-*lacZ* fusion gene constructs and their expression pattern during embryogenesis. Map of the *sal* cis-regulatory fragment sal721S/Pv and its subfragments (below). Restriction enzyme sites are P, *Pvu*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sal*I. Whole-mount stage 5 in situ hybridization with an antisense *lacZ* riboprobe of wildtype embryos bearing sal300R/P (A), bearing sal242S/P (B), bearing sal272P/P (C) and bearing salBE421 (D). The arrows point to ectopic *lacZ* reporter gene expression between salHD and salAD.

To further delimit the 10.2 kb genomic DNA, subfragments of this control region were inserted into the P-Element vector pCaSpeR hs43 lacZ. The position and reporter gene expression of the subfragments within the *sal* upstream region is summarised in Fig. 1A. A 1 kb subfragment, salTSE1000 (*sal* *T*racheal *S*ystem *E*nhancer 1000), is the minimal cis-element which drives *sal* like β-galactosidase expression in the tracheal system and in the posterior spiracles (Fig. 1A,G). Rescue experiments using salTSE1000 indicate that these enhancer sequences are essential for *sal* function in the tracheal system (Kühnlein and Schuh, 1996). Interestingly, the cis-acting element that conducts gene expression in the three *sal* blastodermal domains is a compact module, confined to 721 bp cis-regulatory sequences (sal721S/Pv construct; Fig. 1A,H). Disparate *sal*-like wing disk reporter gene expression was detected with different transgene constructs, suggesting that the apparently coherent wing disc expression domain of *sal* is due to several separable cis-regulatory elements (Fig. 1A). Regulatory elements which mediate some aspects of *sal* expression in the central nervous system are located distal to the blastodermal enhancer (Fig. 1A,I). These results indicate that the cis-regulatory elements driving *sal* expression in different tissues and developmental stages are spread out over more than 10 kb, but they are organised in separate modules, each required to conduct a certain spatio-temporal aspect of *sal* gene expression (see also Discussion).

2.2. *sal cis-control elements of the blastodermal enhancer region*

To further delimit the *sal* blastodermal cis-control we generated several expression constructs from subfragments of the 721 bp enhancer region (see scheme in Fig. 2). The sal300R/P construct mediates weak *lacZ* expression from 60%–100% EL during late stage 4 (data not shown) which retracts from the anterior pole during stage 5 (Fig. 2A). However, the salBE421 construct directs *lacZ* expression corresponding to salAD, salHD and salPD and, in contrast to wild type *sal* expression, it drives reporter gene expression between the two anterior expression domains (Fig. 2B). This observation indicates that sequences in the proximal region of the 721 bp regulatory region are needed for repression between the salAD and the salHD. Two adjacent subfragments of 242 bp and 272 bp, including the 421 bp *sal* enhancer region were used to generate the expression constructs sal242S/P and sal272P/P respectively. We found that the sal242S/P construct drives a *lacZ* transcript pattern like the salPD (posterior cis-regulatory element; Fig. 2C), while sal272P/P generates *lacZ* expression pattern like the salAD and salHD (anterior cis-regulatory element; Fig. 2D).

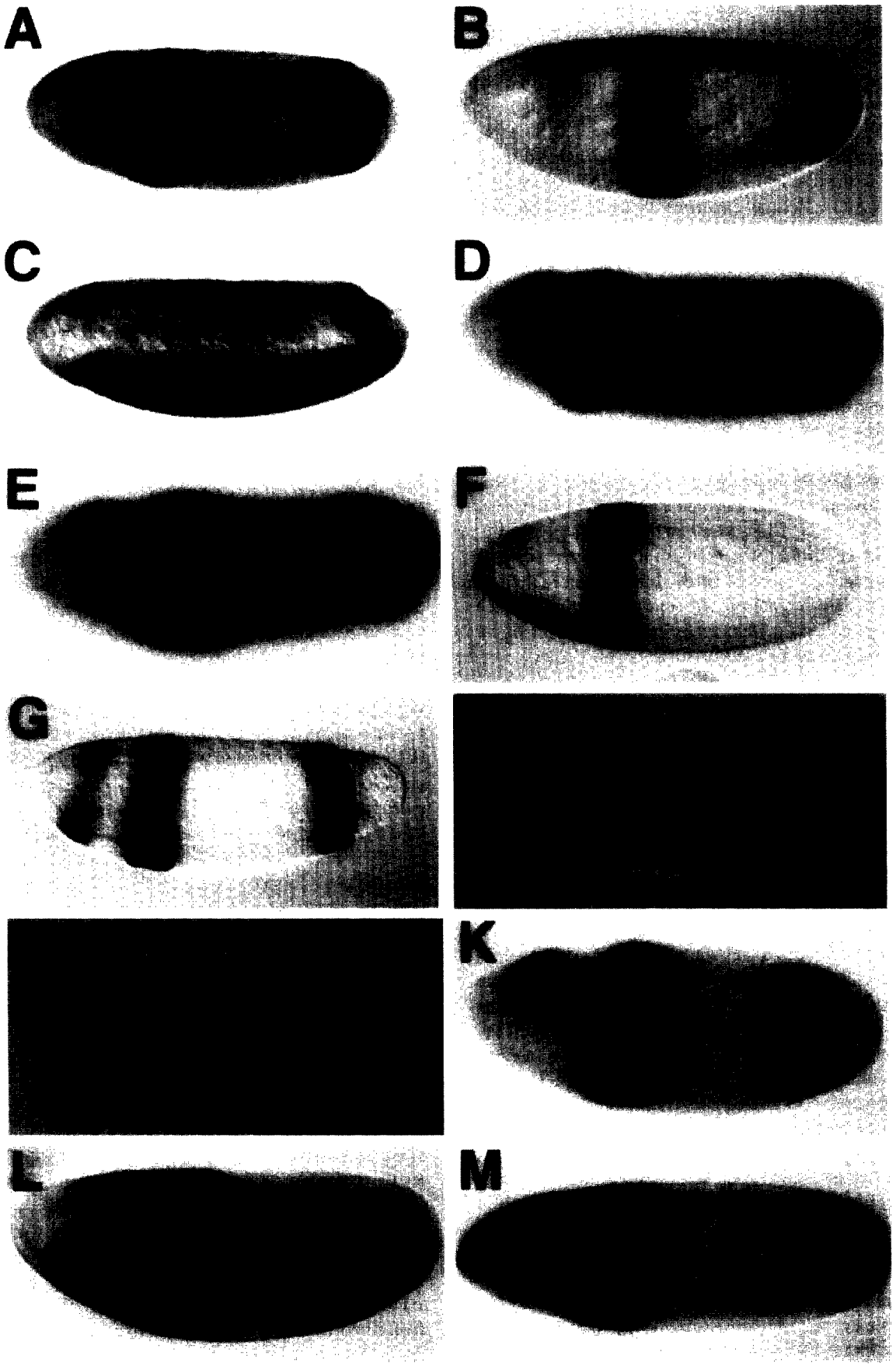
These results indicate that the cis-regulatory requirements, sufficient for *sal* expression during blastoderm, are confined within a 421 bp enhancer element which is located about 10 kb upstream of the *sal* transcription start site. Moreover, the element controlling salPD is separated from the elements which drive expression in salAD and salHD.

2.3. *Genetic control of sal expression during blastoderm*

In order to identify potential trans-acting factors which may be involved in the regulation of the three blastodermal *sal* domains we analysed *sal* expression in various mutant backgrounds.

The salAD covers a region of the embryo which is controlled by anterior maternal organiser activities. In embryos from homozygous females mutant for the gene *bicoid* (*bcd*), the key component of the anterior organiser system (Driever and Nüsslein-Volhard, 1988b; Frohnhöfer and Nüsslein-Volhard, 1986), the salAD expression is not detectable (data not shown). In embryos from females that contain multiple *bcd* gene copies, the salAD is shifted along the anterior-posterior axis in response to the different levels of *bcd* activity (Fig. 3A,B). In order to see whether this shift is due to the altered *bcd* activity concentration gradient exclusively or may depend on a synergistic interaction between *bcd* and *hb* (Simpson-Brose et al., 1994; Wimmer et al., 1995), we examined salAD expression in embryos lacking maternal and zygotic *hb* activity. Such embryos fail to express the salAD as has been observed with embryos lacking *bcd* activity (Fig. 3C). These results indicate that both *bcd* and maternal *hb* activities are necessary to synergistically activate salAD expression. *hb*-dependence of salAD is also seen in embryos lacking zygotic *hb* activity. In such embryos the salAD shifts about 3% of egg length (EL) in anterior direction and the posterior region of salAD narrows about 3% of EL (Fig. 3D). The anterior shift is either the result of lack of repression due to zygotic *hb* activity and/or due to the reduction of maternal *hb* activity to 50% in homozygous zygotic *hb* embryos that derive from heterozygous females. Conversely, the narrowing of the posterior region of salAD may be caused by the derepression of the gap gene *Kr* when zygotic *hb* activity shifts anteriorly in zygotic *hb* mutant embryos (Hülskamp et al., 1990). In fact the posterior border of the salAD domain is shifted posteriorly in *Kr* mutant embryos (Fig. 3E). This indicates that *Kr* indeed acts to locally repress *sal* expression. The anterior border of salAD forms in a region of the embryo where the gap genes *giant* (*gt*; Petschek et al., 1987) and *buttonhead* (*btd*; Cohen and Jürgens, 1990) are expressed. However no effect on *sal* expression is noted in the respective mutant embryos (data not shown). These results favour a model in which expression in salAD is activated by maternal *bcd* and *hb* activities and locally repressed by *Kr* activity which generates the posterior salAD border. High concentration of *bcd* and *hb* activities seem to repress *sal* expression and thereby establish the anterior border of salAD. Alternatively, the border could be formed by combined or redundant repression mediated by the head gap genes.

salHD expression is under the genetic control of three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maternal systems; the anterior, the terminal and the dorso-ventral system. In embryos derived from females homozygous mutant for *bcd* the salHD is absent and it shifts along the anterior-posterior axis of the embryo according to variations of the BCD morphogen gradient as observed for the salAD (see above). In embryos lacking *torso* (*tor*) gene activity, a key component for the terminal system (Klingler et al., 1988), salHD is reduced and shifted anteriorly (Fig. 3F). In embryos derived from females which are homozygous mutant for the gene *dorsal* (*dl*), the key gene of the dorso-ventral system, the salHD extends its expression to the ventral side of the embryo and hence appears as a ring (Fig. 3G). This suggests that in wild-type embryos *dl*-dependent repression prevents salHD expression on the ventral side of the embryo. However, regulation of salHD expression by maternal systems is of an indirect nature and mediated by the zygotic terminal gap genes *tll* (Pignoni et al., 1990; Steingrimsson et al., 1991) and *hkb* (Brönner and Jäckle, 1991). In wildtype embryos of cellular blastoderm stage, the salHD covers the anterior *tll* expression domain except for the anterior portion where *hkb* is expressed. In embryos homozygous mutant for *hkb* the salHD extends about 3% of EL to the anterior (Fig. 3H) and thereby fully overlaps the wild-type *tll* horseshoe-like expression domain (Pignoni et al., 1990). In embryos mutant for *tll* the salHD is not detectable (Fig. 3I), suggesting that *tll* acts as a genetic activator for salHD expression. The results indicate that the *tll*-dependent salHD activation is antagonized by dominant repression provided by *hkb* activity in the anterior part of the *tll* horseshoe-like expression domain.

The salPD expression is also controlled by the terminal maternal system, since no salPD is detectable in embryos from homozygous *tor* mutant mothers (Fig. 3F). The posterior terminal maternal information is mediated by the terminal gap gene activities *tll* and *hkb* in form of overlapping gradual posterior-anterior decreasing expression domains (Brönner and Jäckle, 1991; Weigel et al., 1990). In *tll* mutant embryos the salPD is absent (Fig. 3I) while the domain extends to the posterior pole in *hkb* mutant embryos (Fig. 3H). These observations indicate that salPD expression is activated by *tll* activity and repressed by *hkb* activity as observed for salHD expression. To exclude the possibility

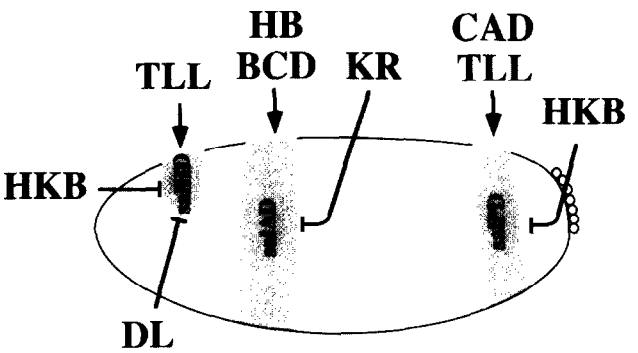

Fig. 4. Schematic representation of the transacting factors regulating blastodermal *sal* gene expression. Arrows indicate activating and bars repressing activities of the indicated gene products. For details see text.

that the absence of salPD in *tll* mutant embryos is due to the ectopic activation of the abdominal repressor gene *knirps* (*kni*) as it has been shown for *hairy* stripe 7 expression (La Rosée et al., 1997) we tested salPD expression in *tll/kni* double mutant embryos. In such embryos the salPD is also absent (data not shown), which favours a mechanism of salPD activation via *tll* (see also Discussion). Since salPD expression is also in the region of *cad* gene activity (Macdonald and Struhl, 1986; Rivera-Pomar et al., 1995) we analysed *sal* expression in embryos which lack zygotic and/or maternal *cad* activity. salPD expression is normal in embryos mutant for zygotic *cad* (data not shown) while the level of salPD expression is strongly reduced in embryos lacking both zygotic and maternal *cad* activity (Fig. 3K). These results suggest that *cad* acts in addition to *tll* as second necessary but not sufficient activator for salPD expression. The salPD expression is not affected in mutant embryos of genes that are regulated by the posterior gap genes *tll* and *hkb* such as zygotic *hb*, *gt* and *T-related gene* (Brönner and Jäckle, 1991; Casanova, 1990; Kispert et al., 1994; Steingrimsson et al., 1991; Weigel et al., 1990). These observations suggest that the regulatory input for salPD expression from the activators *tll* and *cad* as well as the repressor *hkb* may be direct.

The genetic network required for activation and regulation of the spatial limits of the three blastodermal *sal* expression domains are summarised in Fig. 4.

Fig. 3. Genetic control of blastodermal *sal* gene expression. The *sal* gene expression was detected by RNA in situ hybridization using the 10.1 *sal* cDNA (A, D, E, K) or by anti-SAL antibody staining (B, C, F, G, H, I). (A) Wildtype embryo during cellular blastoderm stage (two maternal copies of *bcd*). (B) Embryo derived from a *bcd* fly stock with four copies of *bcd* in the genome. Posterior shift of salHD and salAD proportionally to the *bcd* concentration in the embryo. (C) Early gastrulating embryo lacking the entire *hb* gene function (maternal allele hb^{FB}; zygotic allele hb^{9Q}). salAD is absent. (D) Embryo homozygous mutant for hb^{9Q}. The arrow indicates a minor anterior shift of the salAD and the line indicates the narrowing of salAD. The salAD expression slightly shifts towards the anterior. (E) Embryo homozygous mutant for Kr^{2}. The salAD expression is broadened at the posterior border. (F) Embryo derived from mother homozygous for tor^{PM}. The salHD expression is reduced and the embryo lacks the salPD expression. (G) Embryo derived from mother homozygous for dl^{1}. The salHD is expressed as a circumferential ring. (H) Embryo homozygous mutant for hkb^{2}. The salHD expression is broadened and the salPD expression shifts towards the posterior pole. (I) Embryo homozygous mutant for tll^{g}. salHD and salPD expression is lacking. (K) Embryo lacking all *cad* gene activity (maternal allele cad^{2} and zygotic allele cad^{3}). salPD expression is reduced and broadened. (L, M) Whole-mount stage 5 in situ hybridization with an antisense *lacZ* riboprobe of an embryo homozygous mutant for tll^{g} bearing salBE421 (l) and an embryo homozygous mutant for hkb^{2} bearing salBE421 (M). Orientation of embryos; anterior is left, dorsal is up. Note: The *sal* expressing domains closely match the domains of the *sal* protein domains, except that the latter are seen slightly later (unpublished results). This indicates that blastodermal expression of *sal* is not controlled by post-transcriptional regulation.

To determine whether reporter gene expression is subject to the same genetic control as the endogenous *sal* gene we examined salBE421 directed expression in embryos lacking gene activities of the terminal and anterior system. *tll* mutant embryos lack salHD and salPD corresponding reporter gene expression patterns (Fig. 3L), while these domains expand towards the termini in *hkb* mutants (Fig. 3M). Furthermore, salAD-like reporter gene expression responds to *bcd* activity in the same way as endogenous salAD expression (data not shown). Thus, salBE421 enhancer sequences mediate the same responses to changes in the gene activities of the anterior and terminal system as does the wild-type *sal* regulatory region.

2.4. *sal cis-regulatory sequences contain binding sites for the regulation by maternal and zygotic gene products*

Genetic results described above have shown that *bcd*, *hb*, *tll* and *cad* activate while *Kr* and *hkb* repress *sal* transcription. All these genes encode proteins containing DNA binding motifs and in vitro DNA binding has been shown in the cases of the gene products BCD, HB, TLL, CAD and KR

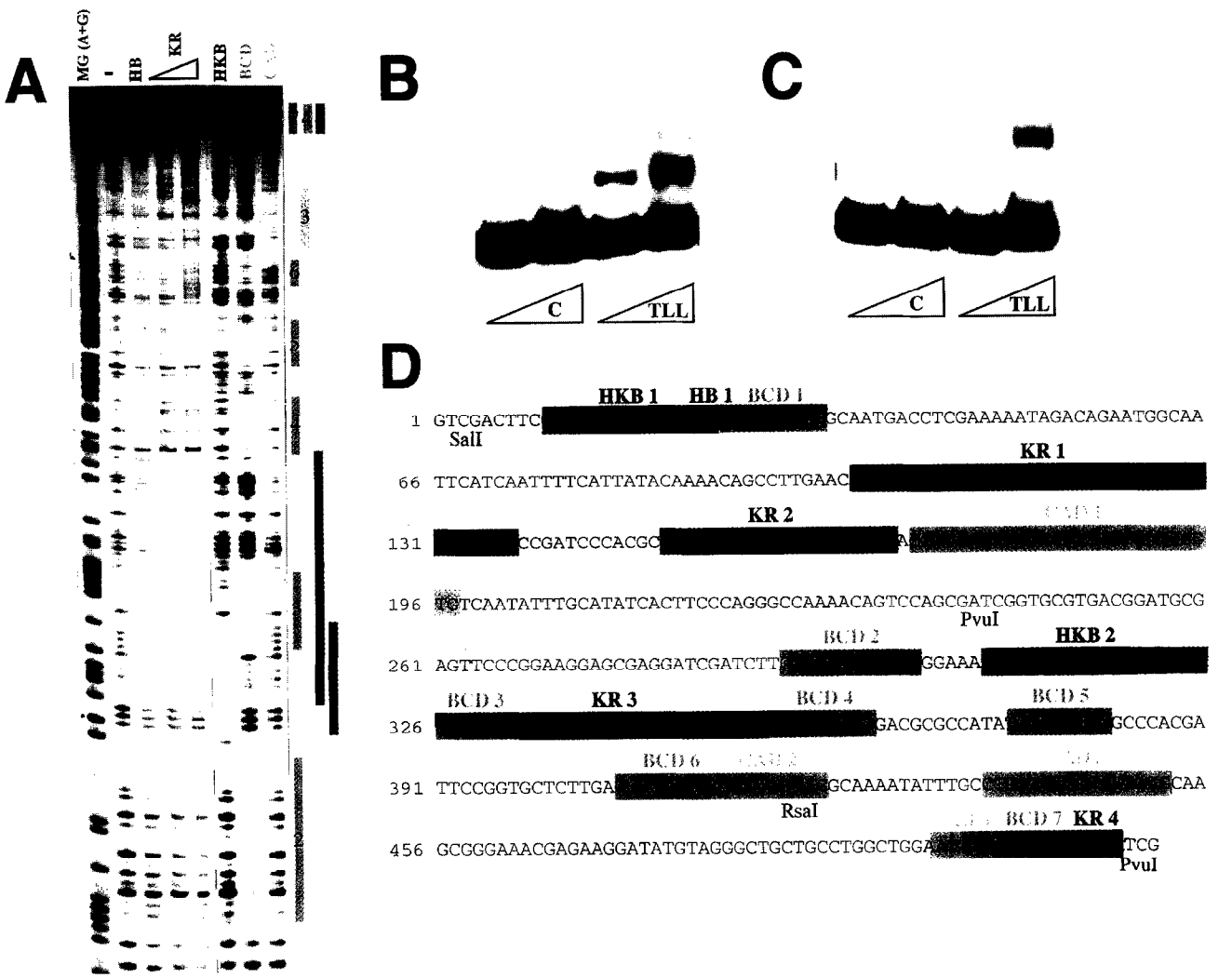

Fig. 5. in vitro binding of transacting factors to the *sal* blastodermal enhancer region. (A) Autoradiograph of a DNase I footprinting assay which was performed with bacterial extracts (see Section 4) of HB, KR, BCD, HKB and CAD on subfragment sal272P/P of the blastodermal *sal* cis-regulatory region. MG refers to a marker lane containing a Maxam-Gilbert reaction (A + G). For control, the fragment was incubated with no extract (indicated by -). On the right side of the autoradiogram the protected areas of the various proteins are indicated by the same colour code. Triangle indicate increasing protein amounts. The numbers refer to the binding sites as shown in D. Footprinting experiments were also performed with different subfragments and their corresponding (–) strands of the blastodermal cis-regulatory region. (B, C) Autoradiographs of gel mobility experiments using crude bacterial Tailless (TLL) extracts and DNA of the blastodermal *sal* cis-regulatory region (B: Nucleotides 1–242; C: Nucleotides 234–421). The control with crude bacterial extract lacking TLL is indicated by C. Triangles indicate increasing protein amounts. (D) Schematic representation of the in vitro binding sites within the *sal* blastodermal cis-regulatory element. The protected areas are shown by coloured bars using a specific colour code for the different proteins. Overlapping binding sites are indicated by overlapping bars. *sal* anterior regulatory element (sal242S/P): nucleotides 1–242; *sal* posterior regulatory element (sal272P/P): nucleotides 243–516. Diagnostic restriction sites: SalI; PvuI and RsaI. Note: D represents protected regions of the opposite DNA strand compared to A and were deduced from several independent footprint experiments.

(for review, see Jäckle and Sauer, 1993). The *hkb* gene encodes a Sp1/egr-related zinc-finger protein which contains a glutamine-rich region and an alanine-rich region corresponding to an activation and repressor domain, respectively. Based on this structural motifs it was suggested that the *hkb* protein (HKB) may act as a DNA-binding transcriptional regulator, although a direct protein-DNA interaction has not been shown (Brönner et al., 1994).

In order to elucidate whether the different maternal and gap proteins might directly interact with the early cis-regulatory region of *sal* we analysed their DNA binding ability by band-shift and in vitro footprinting techniques (Fig. 5A,B,C). Within the posterior regulatory element (sal242S/P) we could detect one binding site for TLL, HKB and CAD each. The anterior regulatory element (sal272P/P) contains six binding sites for BCD, two binding sites for KR and one for HKB and TLL each. The location, orientation and sequences of the binding sites within the *sal* cis-regulatory region are shown in Fig. 5D. The in vitro interaction of the two cis-regulatory elements with different sets of transacting factors is in agreement with the genetic dependence of the various blastodermal *sal* expression domains and indicates that *bcd, Kr, hkb, tll* and *cad* could mediate regulatory effects on *sal* regulation via direct DNA-binding of their gene products.

The alignment of the HKB1 and HKB2 binding sites (Fig. 5D) reveals a HKB consensus binding sequence (5′-G/AGGGCGTTA/C-3′) which is similar to binding sequences found for the SP1 transcription factor (consensus sequence: 5′-GGGGCGGGG-3′; Kadonaga et al., 1987; Wimmer et al., 1996). HKB contains like SP1 three adjacent zinc-finger motifs mediating DNA-binding and it has been shown that such zinc-finger domains recognize nine adjacent nucleotides of the target DNA-sequence. Within the zinc-finger motif defined amino acid positions are crucial for DNA binding specificity (El-Baradi and Pieler, 1991) and such amino acids are identical in the second and third zinc-fingers of HKB and SP1. This high homology is reflected by the nearly identical binding consensus sequences of the second and third zinc-fingers of both transcription factors. In summary, the two in vitro HKB binding sequences of the *sal* blastodermal regulatory element reveals a binding consensus which shows high homology to SP1 binding sites.

3. Discussion

We provide evidence that blastodermal *sal* expression is regulated by three pattern organiser systems which control body formation: the anterior system, the morphogen *dl* and the terminal system together with the *cad* gene function. in vitro studies led to the identification of BCD, CAD, HB, KR, HKB and TLL binding sites within the *sal* blastoderm enhancer, suggesting a direct transcriptional control by these gene products. The genetic and in vitro data provide the basis for our model of early *sal* regulation.

3.1. Regulation of sal expression in the anterior region of the blastoderm embryo

The region of salAD expression overlaps the intersection of two differently regulated parts of the embryo: the labial and the maxillary segment is under the consecutive control of maternal, gap, pair-rule and segment-polarity gene activities, while the pattern of the mandibular segment is established by the gap gene *buttonhead* (*btd*) lacking functional contribution of pair-rule gene activities (Cohen and Jürgens, 1990; Wimmer et al., 1995). The head gap-genes are activated by the anterior morphogen *bcd* independently from maternal *hb* activity. In contrast the salAD is activated by both *bcd* and *hb* activity.

The alterations of *sal* expression pattern in embryos containing different levels of BCD and the detection of six in vitro BCD binding sites in the *sal* anterior cis-regulatory element, strongly argues that BCD activates *sal* gene expression by interaction with this regulatory target sequence in vivo. The lack of in vitro HB binding sites in the *sal* anterior regulatory element argues against cooperativity by BCD and HB in salAD activation as shown for zygotic HB activation (Simpson-Brose et al., 1994). How *bcd*-dependent repression of salAD is achieved in the region of high BCD concentration and how this repression controls the anterior boundary of the salAD expression is unknown. However, the possibility that BCD may activate target genes like the gap gene *gt* and the gap-like gene *btd* which in turn repress *sal* transcription is unlikely, since none of these genes act as an repressor on *sal* expression as revealed by single mutant analysis.

The posterior border of salAD expression appears, on the basis of genetic evidence and of the detection of two in vitro KR binding sites in the *sal* anterior regulatory element to be established by direct interaction of KR with *sal* regulatory sequences. This would imply that KR acts as a repressor within the *sal* anterior regulatory element. This conclusion is consistent with tissue culture experiments showing that KR provides repression by dimer formation at high concentrations (Sauer and Jäckle, 1991) conditions existing at the posterior border of salAD. The finding that KR and BCD share overlapping binding sites may account for an additional or alternative mechanism, which may provide suppression through competition of activators and repressors at common binding sites as previously shown for *eve* stripe 2 and *Kr* expression (Stanojevic et al., 1991; Hoch et al., 1992; Small et al., 1992).

3.2. Regulation of sal expression in the posterior region of the blastoderm embryo

The *tll* gene codes for a member of the nuclear receptor superfamily which contains a nuclear receptor DNA-binding domain with two zinc fingers. It has been shown previously that *tll* acts as a genetic activator of genes expressed in terminal regions of the embryo like *fushi tarazu* (*ftz*), *fkh*

and *hb* (Casanova, 1990; Weigel et al., 1990). For *hb* regulatory sequences a direct molecular interaction with TLL has been described (Margolis et al., 1995).

The *hkb* gene encodes a Sp1/egr-related zinc finger protein (HKB) and genetic analysis suggests that *hkb* acts as a negative regulator of *ftz* and *hb* as well as a positive regulator of *fkh* (Brönner and Jäckle, 1991; Weigel et al., 1990). Still, it remains to be shown that the potential transcription factor HKB directly interacts with regulatory sequences of target genes.

The characterisation of TLL and HKB in vitro binding sites in the *sal* posterior regulatory element suggests a direct mediation of terminal information by TLL and HKB on salPD expression. Furthermore, the gradual distribution of *tll* and *hkb* gene products in overlapping terminal gradients suggests that the anterior border of salPD is determined by a critical TLL threshold concentration while the posterior border is established by a specific HKB repressor concentration or a defined activator (TLL) repressor (HKB) relation. The mechanism of salPD expression seems to be similar to the regulation of the posterior zygotic *hb* expression domain in the region of PS 13 and 14. In contrast to the identification of several strong, medium and weak TLL binding sites in the posterior *hb* enhancer we could only detect two TLL binding sites in the posterior *sal* regulatory element. Therefore, *sal* expression may become activated only by high TLL concentration which is in agreement with salPD expression posterior to PS 14. An alternative mechanism which explains the lack of salPD expression in *tll* mutants involves the known derepression of *kni* in *tll* mutant embryos. Such ectopic *kni* activity would then account for the repression of salPD expression in embryos lacking *tll* activity. This mechanism of the derepression of the repressor *kni* has been shown to be involved in the regulation of the hairy stripe 7 expression (La Rosée et al., 1997). However, repression of salPD by *kni* function is unlikely since embryos lacking both *kni* and *tll* also lack salPD expression. The salPD expression is also activated by *cad*, whose gene product (CAD) binds to the *sal* posterior enhancer as well. However, the mechanisms of salPD expression by the potential activators *cad* and *tll* is different: *tll* mediates transcriptional activation which is necessary for salPD expression while *cad* activity acts on top of the pre-activated basal level and adjusts it to a high expression level. We favour a model of the synergistic activation of salPD by the *tll* and *cad* gene products which is reminiscent of the zygotic *hb* activation by maternal *hb* and *bcd* gene activities in the anterior region of the embryo (Simpson-Brose et al., 1994).

3.3. Phylogenetic implications of the sal cis-regulatory region

The *sal* gene expression is controlled by an array of cis-regulatory modules which mediate tissue- or stage-specific aspects of *sal* expression. This is most obvious for the *sal* cis-control during blastoderm stage, which is confined to a 421 bp DNA fragment although the three *sal* domains are regulated by a variety of different transacting factors. In addition wing disc expression is mediated by sequences between tracheal and blastodermal enhancers while central nervous system expression is controlled by sequences downstream of the blastodermal regulatory module.

The modular organisation of the *sal* cis-regulatory region may represent different *sal* gene functions which have been adopted independently during evolution. The region-specific homeotic *sal* function mediates terminal versus trunk development and may have evolved as an integral function during early insect evolution for the integration of anterior trunk segments into posterior head segments (Jürgens, 1988). The cis-regulatory sequences necessary for this specific function are confined to the blastodermal enhancer. In contrast, the *sal* function which is necessary for the formation of the dorsal trunk, the main anterior-posterior connection of the tracheal system, may have originated during specialisation of high metabolic active insect species (Whitten, 1972). Furthermore, the *sal* expression in the CNS may represent the phylogenetic most conserved gene function, since *sal* homologous genes from *Xenopus*, mouse, *Medaka* and human are expressed in a complex but distinct CNS pattern (Hollemann et al., 1996; Kohlhase et al., 1996; Ott and Schütz, 1996).

The modular structure of the *sal* regulatory region may also account for the diversification of the expression patterns of the genes within the *sal/spalt related* (*salr*)*/spalt adjacent* (*sala*) gene complex (Barrio et al., 1996; Reuter et al., 1996). It has been suggested that the *sal* and *salr* genes have originated from a local gene duplication/transposition event. While *salr* shares *sal* coding sequences, function and cis-acting elements for late embryonic and wing disc expression it lacks blastoderm cis-regulatory sequences. The transposition event left behind the blastodermal regulatory module, which is then adopted by *sala*, a gene of unknown function (Reuter et al., 1996). The *sal* and *sala* expression during blastoderm is spatially and temporally identical and controlled by the same trans-acting factors. Therefore, it is tentative to speculate that the modular organisation of *sal* cis-regulatory elements in combination with duplication events provides the playground to adopt novel gene functions in a region-specific manner.

4. Experimental procedures

4.1. Drosophila strains

The following mutant fly lines were used in this study: bcd^{E1}; dl^{1}; tor^{PM}; cad^{2}; cad^{3}; hb^{9Q}; hb^{7M48};btd^{XG81}; fkh^{XT6}; hkb^{2}; Kr^{2}; tll^{8}; tll^{L49}; Df(1)62g18; Df(3L)vin4; Df(3L)vin6 (Lindsley and Zimm, 1992). Females containing additional copies of *bcd* were obtained from the strain $bcd^{+5}bcd^{+8}$/FM7

(Driever and Nüsslein-Volhard, 1988a). Embryos lacking maternal *hb* or *cad* activity were obtained as described (Rivera-Pomar et al., 1995).

4.2. Methods for DNA and protein analysis

Preparation, subcloning and restriction analysis of DNA were done by standard methods (Sambrook et al., 1989). DNA was sequenced by the dideoxynucleotide procedure. Whole-mount in situ hybridisations using digoxigenin-labelled antisense RNA probes were performed as published (Klingler and Gergen, 1993). Antibody staining with anti-β-galactosidase (Cappel) or anti-SAL (Kühnlein et al., 1994) antibodies to whole mount embryos were carried out as described (Macdonald and Struhl, 1986). UAS-tkv^{Q253D} driven by the GAL4 enhancer trap gene C765 was used to analyse β-galactosidase reporter gene expression in the wing disc under the control of constitutively active TKV receptor (Nellen et al., 1996). Wing discs from late third instar were fixed and stained as described (Zecca et al., 1995).

4.3. Reporter gene constructs and generation of transgenic fly lines

Different *sal* upstream DNA restriction fragments were cloned into the vectors pHZ50PL and pCaSpeR hs43 lacZ, respectively (Thummel and Pirrotta, 1992). The location and the restriction sites of the DNA fragments is shown in Fig. 1A (for detailed description see (Kühnlein, 1996). Transgenic fly lines of the recombinant plasmids were generated by P-element mediated germline transformation (Rubin and Spradling, 1983). For each construct several independent fly lines were generated and analysed.

4.4. Footprint and bandshift experiments

The following expression vectors were used to purify protein from bacterial extracts as described (Kadonaga et al., 1987): pET*bcd* ho (Hoch et al., 1991); pET*hkb*c7SmaI/DraI; pET3c*tll* (Hoch et al., 1992); pET*hb* (Hoch et al., 1991); pRS*cad*XP (Rivera-Pomar et al., 1995); pRSET*Kr* (La Rosée et al., 1997) DNaseI-footprint experiments and bandshift experiments were done as described by Kadonaga et al., 1987 and by Garner and Revzin, 1981, respectively.

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