# CrebA regulates secretory activity in the *Drosophila* salivary gland and epidermis

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*Accepted 14 April 2005*

Development 132, 2743-2758
Published by The Company of Biologists 2005
doi:10.1242/dev.01863

## Summary

**Understanding how organs acquire the capacity to perform their respective functions is important for both cell and developmental biology. Here, we have examined the role of early-expressed transcription factors in activating genes crucial for secretory function in the *Drosophila* salivary gland. We show that expression of genes encoding proteins required for ER targeting and translocation, and proteins that mediate transport between the ER and Golgi is very high in the early salivary gland. This high level expression requires two early salivary gland transcription factors; CrebA is required throughout embryogenesis and Fkh is required only during late embryonic stages. As Fkh is required to maintain late CrebA expression in the salivary gland, Fkh probably works through CrebA to affect secretory pathway gene expression. In support of these regulatory interactions, we show that CrebA is important for elevated secretion in the salivary gland. Additionally, CrebA is required for the expression of the secretory pathway genes in the embryonic epidermis, where CrebA had previously been shown to be essential for cuticle development. We show that zygotic mutations in several individual secretory pathway genes result in larval cuticle phenotypes nearly identical to those of CrebA mutants. Thus, CrebA activity is linked to secretory function in multiple tissues.**

Key words: CrebA, Fkh, *Drosophila*, Salivary gland, Secretory pathway

## Introduction

As tissues differentiate, they express a unique combination of genes encoding proteins controlling tissue structure and physiology. Deciphering how the genes are transcriptionally regulated is key to understanding the final stages of organogenesis and to providing molecular insight into how an organ becomes equipped to perform its specialized functions. For example, transcriptional regulation studies of genes crucial for liver function suggest that their regulation is relatively complex. In this tissue, the winged-helix transcription factors HNF3α and HNF3β, and the nuclear receptor superfamily member HNF4 converge on the regulation of the homeodomain-containing transcription factor HNF1 (Cereghini, 1996). HNF1 is directly involved in the transcriptional activation of terminal genes that are essential to liver function (Courtois et al., 1987; Cereghini et al., 1988). Functional binding sites for the transcription factors upstream of HNF1 have also been identified in the enhancer/promoter regions of the terminal genes. Furthermore, HNF3β and HNF3α (indirectly through HNF3β) are themselves regulated by the leucine-zipper transcription factor C/EBPα (Samadani et al., 1995). Thus, the regulatory mechanisms of liver gene expression appear to be both hierarchical and non-hierarchical, and include converging regulatory pathways.

In the case of pharyngeal development in the nematode *C. elegans*, transcriptional regulation appears to be simpler. In this organ, PHA-4, a winged-helix transcription factor homologous to mammalian HNF3β and *Drosophila* Fork head, is crucial for both pharynx specification and subsequent differentiation. The pharynx completely fails to form in *pha-4* mutants and ectopic expression of *pha-4* can activate at least one pharynx-specific gene in new places (Kalb et al., 1998). PHA-4-binding sites are present upstream of all tested pharyngeal genes, and temporal expression correlates with PHA-4-binding site affinity (Gaudet and Mango, 2002). In general, early genes have relatively strong binding sites for PHA-4, whereas later genes have relatively weak sites. Correspondingly, early in organ development, low levels of PHA-4 are present and are sufficient to activate the early-expressed genes. As levels of PHA-4 increase temporally, late genes are expressed. By using a temperature sensitive allele of *pha-4*, the same group confirmed that the late expression of *pha-4* is indeed important for proper organ development (Gaudet and Mango, 2002). Thus, the role of *pha-4* is not only to initiate a downstream regulatory cascade but also to function throughout the development of the *C. elegans* pharynx by directly regulating genes pivotal to its final form and function. Whether this single-tier mode of regulation is a recurring theme in organ development remains to be determined.

The *Drosophila* salivary gland is an excellent model for investigating how a tissue acquires specialized function, as much is already known with respect to how the gland is specified (reviewed by Andrew et al., 2000; Abrams et al., 2003). In addition, large-scale expression studies suggest that a significant number of genes are expressed to high levels in the salivary gland, and therefore access to a large pool of

potential downstream targets is available (http://www.fruitfly.org/EST/index.shtml). Salivary gland formation requires the homeotic transcription factors, Sex Combs Reduced (Scr), Extradenticle (Exd) and Homothorax (Hth); in loss-of-function mutants of any of these genes, salivary glands fail to form. Moreover, ectopic expression of *Scr*, the one component of the complex with limited spatial expression, leads to the formation of additional salivary glands (Panzer et al., 1992; Andrew et al., 1994). Although the Scr/Exd/Hth complex is required for the expression of every tested salivary gland gene, expression of *Scr* and *hth* and nuclear localization of Exd disappear shortly after the salivary gland initiates morphogenesis (Henderson and Andrew, 2000), suggesting that, unlike in the *C. elegans* pharynx, the genes that specify the Drosophila salivary gland cell fate are not involved in its terminal differentiation. Genes encoding three early transcription factors are known to be expressed in the early salivary gland under the control of Scr/Exd/Hth (Panzer et al., 1992; Andrew et al., 1994): *fork head* (*fkh*), which encodes the winged-helix transcription factor homologous to *C. elegans* PHA-4; *CrebA*, which encodes the Cyclic AMP Response Element Binding protein A; and *huckebein* (*hkb*), which encodes an Sp1/egr-like transcription factor. In the embryo, Fkh controls apical constriction of the salivary cells as they invaginate and promotes salivary cell survival by inhibiting apoptosis (Myat and Andrew, 2000b). During larval development, Fkh is required to activate expression of the *sgs* glue genes (Lehmann and Korge, 1996; Mach et al., 1996). The SGS glue proteins play a role in the adherence of the pupal case to a substratum. Thus, Fkh is similar to *C. elegans* PHA-4 in as far as it is important throughout salivary gland development and function. However, a significant number of salivary gland markers are still expressed in the uninvaginated salivary cells of *fkh* mutant embryos (Myat and Andrew, 2000b; Bradley and Andrew, 2001) (E. Grevengoed, U. Ng and D.J.A., unpublished), suggesting that Fkh is not an organ-specifying gene. *CrebA* has been shown to be important in cuticle patterning and in promoting the integrity of the larval cuticle (Andrew et al., 1997). Its role in the salivary gland, however, has been less clear, although a low percentage of *CrebA* embryos have crooked salivary glands (Andrew et al., 1997). Hkb is required for proper morphogenesis of the secretory tube. In *hkb* mutants, dome-shaped salivary glands form instead of elongated tubes because of a failure to generate and deliver sufficient apical membrane (Myat and Andrew, 2000a; Myat and Andrew, 2002). Thus, *fkh* and *hkb* have genetically defined roles in the morphogenesis of the salivary gland, whereas the role of CrebA in salivary gland development remains elusive.

To learn how the salivary gland is programmed for its primary function, secretion, we focused on the regulation of genes encoding early secretory pathway components. The secretory components crucial for targeting proteins to the endoplasmic reticulum (ER), for signal peptide processing and for vesicular trafficking between the ER and Golgi have been studied extensively in yeast and in mammalian tissue culture cells (reviewed by Harter, 1995; Kalies and Hartmann, 1996; Romisch, 1999; Wild et al., 2002; Barlowe, 2003a). Clear homologs of most of these proteins exist in flies based on sequence comparisons (Adams et al., 2000), although very few have been characterized to any extent (Valcarcel et al., 1999). Here, we show that genes encoding the *Drosophila* homologs to the yeast and/or mammalian early secretory pathway proteins are expressed at high levels in the *Drosophila* embryonic salivary gland and other secretory tissues, and that CrebA is essential for this high level salivary gland expression. Fkh is required for late expression of these genes and functions indirectly through maintenance of CrebA expression. Correspondingly, CrebA is required for enhanced secretory activity in the salivary gland. CrebA also activates SPCG expression in the embryonic epidermis, explaining the cuticle defects observed in *CrebA* mutant larvae.

## Materials and methods

### cDNA clones identification

cDNA clones were obtained either directly from BDGP (CK clones) or from Invitrogen (Huntsville, AL). CK cDNA clones expressed in the salivary gland were identified at the CK expression database at BDGP (Kopcyznski et al., 1998). Salivary gland expression of the following clones were identified through the BDGP embryonic expression study (http://www.fruitfly.org/EST/index.shtml): LD25651, RE14391, RE02772, GH04989, RE24638 and LD45288. The expression patterns of the remaining genes were determined in this study: Srp complex (RE23260, LD41750, LP10092 and AT23778); ER translocon (RE04612, RH61539, RE69515, RE23984, LD29171, LD27659 and GM12291); SPC (RH08585 and LD42119); COPII components (GH19061, RE35250, SD04292 and LD39266); and COPI components (GH18123, RE62270, RE38606 and LD24904).

### Fly strains

For *fkh* mutants, we used the null $fkh^6$ allele in an H99 background to prevent secretory cell apoptosis and to thus simplify the analysis (Myat and Andrew, 2000b). Accordingly, we performed the same experiments in H99 only lines as a control. For *CrebA* mutants, we used the protein null allele $CrebA^{wR23}$ (Andrew et al., 1997); for *hkb* mutants, we used $hkb^2$ (Bronner et al., 1994). *l(3)01031* (*sec13*), *l(3)j13C8* (*sec23*), *l(3)rK561* (*SrpRβ*) and *l(3)05712* (*sar1*) are all lethal P-element lines; both stocks and plasmid rescue data were obtained from FlyBase. $CrebA^{wR23}$, $fkh^6$ *H99* and $hkb^2$ were balanced over TM6B-Ubx *lacZ*. *l(3)j13C8* was balanced over TM3-ftz *lacZ*. The *lacZ*-containing balancers were used to distinguish the homozygous mutant embryos from siblings by staining with a *lacZ* probe.

### Identification of CrebA and SPCG enhancers

A 2.8 kb *Hin*dIII fragment that maps ~3.5 kb upstream of the CrebA transcription start site and drives β-gal expression in the salivary gland and amnioserosa has been identified previously (K. D. Henderson, PhD Thesis, Johns Hopkins University School of Medicine, 2000). The following primer pairs were used to further isolate the CrebA salivary gland enhancers: CrebA 1100 (5′GAATTCCTTCGCTGTC-ATGC and 3′GGATCCAGCGTCTTCTAGAGATAC), which amplify an 1100 bp sub-fragment of *Hin*dIII 2.8 containing six potential Fkh binding sites (Fig. 3); and CrebA 770 (5′GAATTCTAGAAGACGC-TGGCGAATG and 3′GGATCCCATCTTCGATCTGGC), which amplify a 770 bp sub-fragment of *Hin*dIII 2.8 containing four potential Fkh-binding sites (Fig. 3).

Six candidate SPCG genes, representing members of distinct pathway components, were selected and from 1.3 to 2.6 kb of upstream genomic sequence of each gene was amplified. The length was limited by the exclusion of *Bam*HI and *Eco*RI sites, which were needed for subcloning the resulting PCR fragments into the Casper β-Gal reporter plasmid (Thummel et al., 1988). The following genes and corresponding primer sets were used: *srp68*, 5′GAATTCGTTGTGT-GCTTCTCCAGATTGC and 3′AGTGTACGGCTATGGCGAAT; *p24,*

5′CAGGAACGTAAGCAGATCTCG and 3′TCCACGTAGACGATCTTGTGC; *srpRα*, 5′GAATTCTGAAGACAGGCTAGGCTGG and 3′GGATCCGCGATCGAAGTCGTAGTCAT; *ξ-cop*, 5′GAATTCGGAGTTGAGCACCTCGTTG and 3′GGATCCAGGCGCTTCTCAACGTTC; *spase25,* 5′GAATTCACACCATACACCGTACACC and 3′GTGCTTCACTGCGGATCCAT; and *sec61β*, 5′GAATTCCACCGAATCGAACGACTC and 3′CACACCAATGCCTCAACAAG.

All of the resulting PCR fragments were subcloned into the Casper β-Gal reporter plasmid (Thummel et al., 1988) and used to transform $w^{1118}$ flies through standard methods (Spradling and Rubin, 1982). Individual lines were assayed for enhancer activity by staining with anti-β-Gal antibody.

### Site-directed mutagenesis

Point mutations known to affect the binding of Fkh to defined binding sites (E.W.A. and D.J.A., unpublished) were introduced into each of the six potential Fkh-binding sites contained within the CrebA 1100 enhancer using the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used (bold nucleotides indicate changes): site 1, ACTTCCTCCTATGAATGAATG**CC**TGTTATTGCGGGTTCCT; site 2, CGAACACATAAAGACTATA**GG**TATCTGCTACCACGGCTTAGAGCCG; site 3, CGCATTGAACAAAATTCTCTTG**CC**TAATGATATGCTATTATTA; site 4, CCCAGTTTCATCATTATCATCAC**CC**TGTCAAAAGCAGACTT; site 5, GCGACAGCAAAGTCAGACA**GG**TAAGTCAGCACTACGTTCT; and site 6, TAAATGATCTCATTGTTGACTG**CC**TGTTCTCCCTCAGGAC. The resulting mutant constructs were used to transform $w^{1118}$ flies and individual lines were assayed for enhancer activity by anti-β-Gal staining.

### In situ hybridization and antibody staining

In situ hybridization and antibody staining were performed as previously described (Lehmann and Tautz, 1994; Reuter et al., 1990). In each case, we compared the levels of expression in the homozygotes to the levels observed in their heterozygous siblings to control for experimental variation in staining levels. Antibody dilutions used in this study are as follows: α-β-galactosidase (1:5000), α-DSC73 (1:800), α-Pasilla (1:1000), α-PH4α-SG1 (1:5000) and α-En (1:200). α-PH4α-SG1 (E.W.A. and D.J.A., unpublished), α-Pasilla (Seshaiah et al., 2001) and α-DSC73 (D.J.A., unpublished) are polyclonal antisera made in rat. α-En is a polyclonal antibody made in rabbit that cross-reacts with unknown secretory products in the salivary gland (Myat and Andrew, 2002). Approximately 40 pairs of salivary glands were analyzed for each mutant in the En staining experiments to gauge changes in secretory granule levels. All biotin-conjugated secondary antibodies were used at a dilution of 1:500 (Vector Labs; Burlingame, CA) and all fluorescent secondary antibodies were used at a dilution of 1:400 (Molecular Probes; Eugene, OR). Confocal images were obtained with the Ultraview Confocal Microscope (Perkin Elmer) at the Johns Hopkins Microscope Facility. All other images were taken on a Zeiss Axiophot microscope with a Nikon Coolpix 4500 digital camera.

### Protein alignments and binding site searches

Homology searches of the *Drosophila* SPCGs to identify human proteins were performed using BLAST (http://www.ncbi.nlm.nih.gov). Identities/similarities were calculated using CLUSTALW (Combet et al., 2000). To identify conserved sequences upstream of the SPCGs, 500 nucleotides immediately upstream of the translation start sites of all 34 SPCGs was analyzed by MEME (http://meme.sdsc.edu/meme/website/) (Bailey and Elkan, 1994). The most conserved sequence (other than the runs of PolyA+ DNA) was a good match for the mammalian Creb-binding site consensus (http://www.cbrc.jp/research/dp/TFSEARCH.html/; threshold score 86.1). We subsequently looked for this site in the 2 kb region immediately upstream of the translation start sites of all of the SPCGs.

## Results

### Identification of salivary gland expressed secretory pathway component encoding genes (SPCGs)

A survey of the EST expression databases at the Berkeley Drosophila Genome Project (BDGP) revealed that several genes encoding components of the early secretory pathway are upregulated in the embryonic salivary gland (http://www.fruitfly.org/EST/index.shtml) (Kopcyznski et al., 1998). We identified *Drosophila* homologs to the remaining known components of the early secretory pathway through a search of the *Drosophila* genome database (Adams et al., 2000). The early secretory pathway components include the machinery that targets and translocates proteins into the ER, the complex that cleaves the N-terminal signal sequence, and the proteins involved in both anterograde and retrograde vesicle transport between the ER and Golgi (Fig. 1). We refer to these genes collectively as secretory pathway component genes (SPCGs; Table 1). All of these genes contain open reading frames with significant identity/similarity to their putative human counterparts (Table 1) and, with few exceptions, are expressed at much higher levels in the salivary gland than in other embryonic tissues (Fig. 2; see Figs S1, S2 in the supplementary material). Expression was observed from early stage 11 (Campos-Ortega and Hartenstein, 1997), just after the salivary cells begin to internalize (Myat and Andrew, 2000a), and continued throughout embryogenesis. In addition to high-level salivary gland expression, most of the SPCGs are also expressed in other embryonic tissues, including the epidermis, proventriculus, anterior and posterior midgut primordia, the hindgut and trachea (data not shown). The SPCGs that are not expressed to relatively high levels in the embryonic salivary gland are expressed to high levels in all tissues, including the salivary gland. These findings suggest that at least one aspect of salivary gland specialization into a secretory organ is mediated through transcriptional activation of genes encoding components of the early secretory machinery.

### Regulation of SPCGs involved in targeting and translocating proteins into the ER

Salivary gland formation requires the function of the homeotic gene *Scr* and its co-factor genes *exd* and *hth* (Henderson and Andrew, 2000). Correspondingly, we failed to detect expression of the small number of SPCGs we tested in *Scr* mutants in the cells that would normally form salivary glands in wild-type embryos (data not shown). As *Scr* and *hth* disappear in the salivary gland as soon as cells begin to internalize (Henderson and Andrew, 2000), just prior to when SPCG expression initiates, the Scr/Exd/Hth complex is unlikely to be controlling SPCG expression directly. Instead, SPCG expression is more likely to be controlled by the early transcription factors under the control of *Scr* such as Fkh, CrebA and/or Hkb. Thus, we examined expression of the SPCGs in embryos homozygous for null mutations of each of these transcription factor genes. An example of salivary gland morphology in *fkh*, *CrebA* and *hkb* mutants is shown in Fig. 2A using a salivary gland-specific marker, α-Pasilla (Ps), expression of which is not affected by these mutations (Myat and Andrew, 2000b).

The signal recognition particle (Srp) interacts with the N-

terminal signal peptide as it emerges from the ribosome. Subsequently, the Srp interacts with the Srp Receptor (SR) α-subunit, which is anchored to the cytoplasmic face of the ER through the transmembrane SR β-subunit (Fig. 1) (Legate et al., 2000; Song et al., 2000). The Srp complex is composed of a 7S RNA and six protein subunits, which are referred to as the 9, 14, 19, 54, 68 and 72 kDa Srp subunits (Fig. 1) (Wild et al., 2002). All of the known protein subunits of the Srp and the SR are expressed to very high levels in the embryonic salivary gland (Fig. 2B; see Fig. S1B,C in the supplementary material) and the levels are much higher than in other embryonic tissues, with the exception of Srp14 and Srp19, which are expressed to similarly high levels throughout the embryo (data not shown). Salivary gland expression of the Srp and SR genes is unaltered in *hkb* mutants, but is significantly diminished in later stages in both *fkh* and *CrebA* mutants (stage 13 and beyond; Fig. 2B; see Fig. S1B,C in the supplementary material). Interestingly, there are differences in the early expression of Srp and SR genes in *fkh* and *CrebA* mutants. Whereas in the early *fkh* mutants, expression levels are comparable with wild type, in the early *CrebA* mutants, expression is diminished to levels seen in surrounding, non-salivary gland tissues or was barely visible (Fig. 2D; Table 2; see Fig. S1B,C in the supplementary material). Thus, both *fkh* and *CrebA* are required to achieve persistent high-level expression of Srp and SR genes in the embryonic salivary gland; however, Fkh is required only to maintain and not initiate expression. *CrebA* mutants also had diminished Srp and SR gene expression in the late epidermis (where *CrebA* is also normally expressed), indicating a more general requirement for *CrebA* in the expression of these genes.

Translocation of nascent polypeptides into the ER is mediated by the sec61 complex, which is composed of sec61α, sec61β and sec61γ subunits (Fig. 1) (Schnell and Hebert,

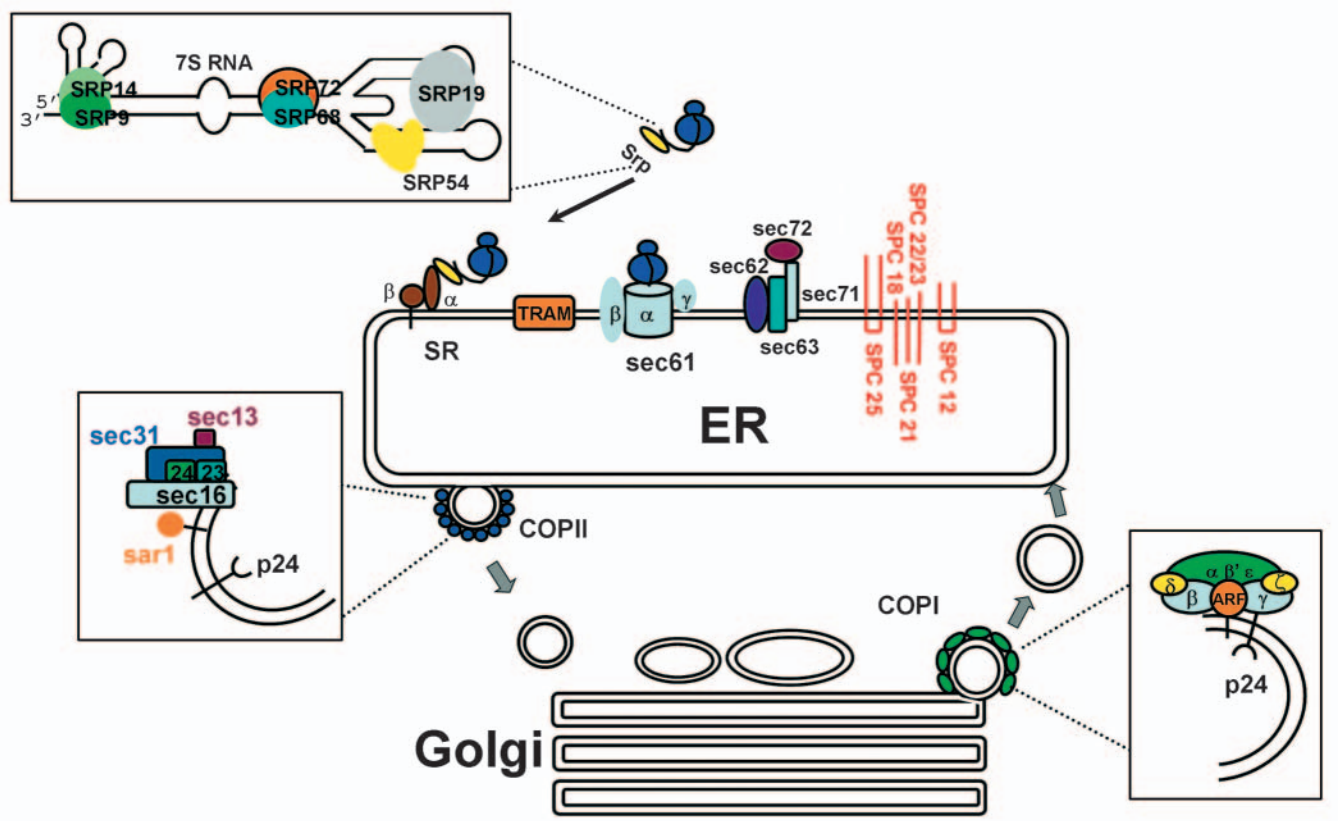


**Fig. 1.** The early secretory pathway. Transcripts encoding secretory/transmembrane proteins are targeted to the ER through interaction of the N-terminal peptide (black line), as it emerges from the ribosome (blue), with the Srp complex (yellow oval). The Srp complex is composed of a 7S RNA and six RNA-binding proteins, each of which is conserved in *Drosophila*. The Srp/signal peptide complex interacts with the α-subunit of the SR (brown). The ribosome is then transferred to the sec61 complex (light blue), which is composed of α-β-γ subunits (all are conserved in *Drosophila*). sec62, sec63, sec71 and sec72 are thought to be involved in Srp-independent (or post-translational) protein translocation. TRAM is required for efficient co-translational translocation and is important for incorporation of transmembrane proteins into the lipid bilayer of the ER. After the nascent peptide is translocated into the ER, the signal sequence is cleaved by the SPC. Four out of the five peptide subunits (red lines) are conserved in *Drosophila*, with one of the peptides showing homology to two of the mammalian proteins. The COPII coatamer is involved in the anterograde movement of vesicles from the ER to the Golgi (blue circles). It comprises sec13, sec16, sec23, sec24 and sec31 subunits. A sec16 homolog is not recognizable in the current annotated *Drosophila* genomic sequence. Sar1 (orange circle) is a small G-protein involved in the regulation of COPII assembly/disassembly. The p24 transmembrane family of proteins interact with soluble cargo destined to leave the ER. COPI-coated vesicles are involved in retrograde movement of secretory vesicles from the Golgi to the ER (green ovals). All of the COPI coatamer components, which include δ, ε, γ, ζ-cop and ARF-1, as well as other proteins involved in the retrieval of escaped resident proteins back to the ER are conserved in *Drosophila*. The Srp region of this figure was adapted from Wild et al. (Wild et al., 2002); the COPII region from Shaywitz et al. (Shaywitz et al., 1997); the SPC region from Kalies et al. (Kalies et al., 1996); the translocon region from Romisch (Romisch, 1999); and the COPI region from Wieland and Harter (Wieland and Harter, 1999).

2003). sec61α is expressed ubiquitously in the embryo and increased levels were detectable only in the salivary gland prior to internalization (data not shown). As the cells internalized, elevated expression of sec61α was restricted to the salivary gland cells that were still on the surface of the embryo (data not shown). In contrast to sec61α, high-level salivary gland expression of both sec61β and sec61γ persists throughout the remainder of embryogenesis. As with Srp and SR, salivary gland expression of sec61β and sec61γ is unaffected by mutations in *hkb* and is dramatically reduced in *CrebA* mutants at all embryonic stages (Fig. 2B; see Fig. S1D in the supplementary material). Similarly, mutations in *fkh* affect later but not earlier expression of both sec61β and sec61γ (Fig. 2D; data not shown). Epidermal expression of both genes is also diminished in *CrebA* mutants (Fig. 2B; data not shown).

The translocation-associating membrane protein (TRAM) is required for efficient co-translational translocation of proteins into the ER and is important for the incorporation of transmembrane proteins into the lipid bilayer (Schnell and Hebert, 2003). TRAM is highly expressed in the *Drosophila* salivary gland and epidermis. This expression appears normal in early *fkh* mutants and is attenuated but not completely removed in late *fkh* embryos (see Fig. S1D in the supplementary material). TRAM expression in both the

**Table 1. Secretory pathway component encoding genes (SPCGs) are upregulated in the embryonic salivary gland**

| Homology/function | CG number | cDNA* | Map position† | Human homology‡ |
|---|---|---|---|---|
| **Signal recognition particle (Srp)§** | | | | |
| Srp9 | CG8268 | RE23260[3] | 66A20 | 36 (73) |
| Srp68 | CG5064 | LD41750[3] | 66E5 | 40 (76) |
| Srp72 | CG5434 | LP10092[3] | 92F2 | 40 (73) |
| Srp54 | CG4659 | AT23778[3] | 64C9 | 77 (90) |
| Srp14** | CG5417 | RE48595[3] | 92F2 | 24 (49) |
| Srp19** | CG4457 | RH65975[3] | 65F5 | 46 (72) |
| **Srp receptor (SR)** | | | | |
| SrpR α subunit | CG2522 | LD25651[2] | 10A6 | 33 (68) |
| SrpR β subunit | CG5950 | CK02641[1] | 66D11 | 52 (80) |
| **ER translocon§,¶** | | | | |
| sec61-α | CG9539 | RE04612[3] | 26D7 | 90 (95) |
| sec61-β | CG10130 | RH61539[3] | 51B9 | 67 (89) |
| sec61-γ | CG14214 | RE69515[3] | 18D11 | 35 (41) |
| sec62 (Dtrp1) | CG4758 | RE23984[3] | 30F6 | 32 (70) |
| sec63 | CG8583 | RE14391[2] | 65F7 | 33 (55) |
| sec71 | CG7578 | LD29171[3] | 34D3 | 58 (80) |
| TRAM | CG11642 | LD27659[3] | 1D2 | 39 (71) |
| TRAPδ | CG9035 | GM12291[3] | 47F7 | 32 (69) |
| **Signal peptidase complex (SPC)** | | | | |
| Spase 22/23-subunit | CG5677 | CK02587[1] | 95F3 | 56 (73) |
| Spase 12-subunit | CG11500 | RE02772[2] | 99B1 | 39 (75) |
| SPase 18/21-subunit | CG2358 | RH08585[3] | 84A1 | 70 (91) |
| SPase 25-subunit | CG1751 | LD42119[3] | 10B15 | 33 (65) |
| **p24 family (cargo receptors)** | | | | |
| CHOp24 | CG3564 | GH04989[2] | 4C7 | 60 (87) |
| p24-related-1 | CG1967 | CK00398[1] | 10F1 | 42 (76) |
| p24-related-2 | CG9443 | CK01332[1] | 85E4 | 65 (85) |
| **COPII components¶** | | | | |
| sec31 | CG8266 | GH19061[3] | 44F3 | 32 (68) |
| sec13 | CG6773 | CK00043[1] | 94E13 | 50 (76) |
| sec23 | CG1250 | RE35250[3] | 83B7 | 74 (92) |
| sec24 | CG1472 | SD04292[3] | 46C1 | 59 (69) |
| sar1 | CG7073 | LD39266[3] | 94A5 | 70 (90) |
| **COPI components§ and KDEL-R** | | | | |
| ARF79F | CG8385 | LD24904[3] | 79F | 95 (99) |
| δ-Cop | CG14813 | GH18123[3] | B12 | 58 (85) |
| γ-Cop | CG1528 | RE62270[3] | 100C6 | 59 (86) |
| ζ-Cop | CG3948 | RE38606[3] | 73B6 | 60 (91) |
| ε-Cop | CG9543 | LD29885[3] | 26D9 | 36 (69) |
| Erd2 (KDEL-R) | CG5183 | CK00230[1] | 31E1 | 74 (94) |

*Original expression data obtained from [1]CK-EST database, [2]BDGP expression study and [3]this study.
†Cytological map positions determined by BDGP.
‡Percent identity (similarity).
§Srp14 and Srp19 are expressed ubiquitously at high levels (including the salivary gland).
¶sec72 and sec16 are not identifiable in the current annotated *Drosophila* sequence database.
**Non-salivary gland specific genes.

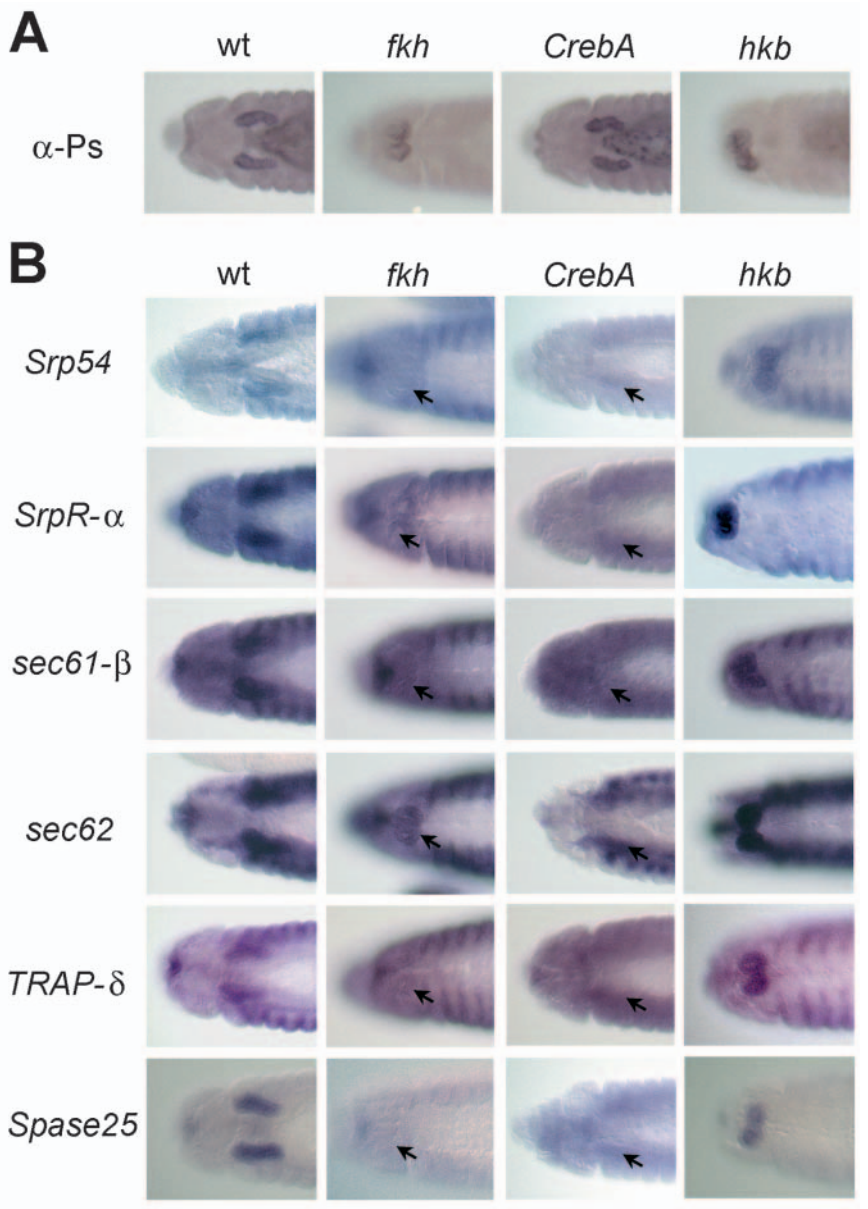


**Fig. 2.** Transcriptional regulation of early SPCGs in the salivary gland. α-Pasilla staining of stage 13-14 wild-type, *fkh*, *CrebA* and *hkb* mutant embryos reveals the morphology of the salivary gland (A). All embryos are ventral views, with anterior towards the left. This arrangement and positioning of wild-type and mutant embryos is also used for B and C. (D) Left panels are *CrebA* heterozygotes (*lacZ* expression in posterior regions), middle panels are *CrebA* homozygotes and the right panels are *fkh* homozygotes, all at early stages (~stage 11), oriented with anterior towards left and showing lateral views. Salivary gland SPCG expression is controlled by *fkh* (late) and *CrebA* (B-D; see Figs S1, S2 in the supplementary material). With a small subset of the genes, including sec62 and TRAPδ, expression can still be detected in *fkh* mutants (even late stage), albeit at reduced levels (B). The salivary gland expression of all the tested SPCGs in *hkb* mutants is comparable with that of wild-type salivary glands (B,C; see Figs S1, S2 in the supplementary material). Arrows indicate salivary gland expression in the mutants.

salivary gland and epidermis is significantly reduced at all stages in *CrebA* mutants and is unaffected by *hkb* (see Fig. S1D in the supplementary material; data not shown).

sec62, sec63 and sec71 encode three ER transmembrane proteins implicated in mediating post-translational translocation of proteins into the ER (Romisch, 1999) (Fig. 1). These genes are highly expressed in the salivary gland as well as in the epidermis (see Fig. S1D in the supplementary material). In contrast to the Srp and sec61 genes, but similar to TRAM, expression of sec62 and sec63 can still be detected in the salivary gland cells of *fkh* embryos beyond stage 13, albeit at lower than wild-type levels (Fig. 2B; see Fig. S1D in the supplementary material). As in the previous cases, the salivary gland and epidermal expression is greatly diminished in both early and late *CrebA* mutants (see Fig. S1D in the supplementary material; data not shown). With sec62, the segmental muscle staining, which in wild-type embryos is obscured by the epidermal staining, is now obvious (Fig. 2B). sec71 expression is affected similarly to TRAM, sec62 and sec63 in *fkh*, *CrebA* and *hkb* mutants.

**Table 2. Summary of salivary gland regulation data**

| Protein complex | Early Fkh | Late Fkh | *CrebA* | Hkb |
|---|---|---|---|---|
| Srp/SR | Normal/reduced | Absent | Very low | Normal |
| Sec 61β, γ | Normal | Absent | Very low/absent | Normal |
| Sec62, 63, 71, TRAM, TRAPδ | Normal | Reduced | Very low/absent | Normal |
| SPC | Normal/reduced | Absent | Very low | Normal |
| p24-related | Normal | Absent | Very low | Normal |
| COP II | Normal/reduced | Absent | Very low | Normal |
| COP I | Normal/reduced | Absent | Reduced/very low | Normal |
| KDEL-R | Normal/reduced | Absent | Reduced | Normal |

The translocation-associated protein (TRAP) complex is composed of four subunits, α, β, δ and γ, and is thought to be associated with the translocon machinery. Recently, the TRAP complex has been shown to be required for the translocation of proteins in a substrate-specific manner. For example, the secretory protein Prolactin is efficiently translocated into the ER in the absence of functional TRAP. However, prion protein (PrP), which enters the secretory pathway in three different topographical configurations, requires TRAP (Fons et al., 2003). Homologs to all four TRAP subunits exist in *Drosophila*, although the expression of only TRAP δ was analyzed in this study. TRAPδ is expressed at high levels in the salivary gland and epidermis. The salivary gland expression requires *fkh* late and *CrebA* throughout embryogenesis for wild-type expression in the salivary gland and epidermis (Fig. 2B,D).

Soluble nascent polypeptides have their N-terminal signal peptides cleaved off by the signal peptidase complex (SPC).

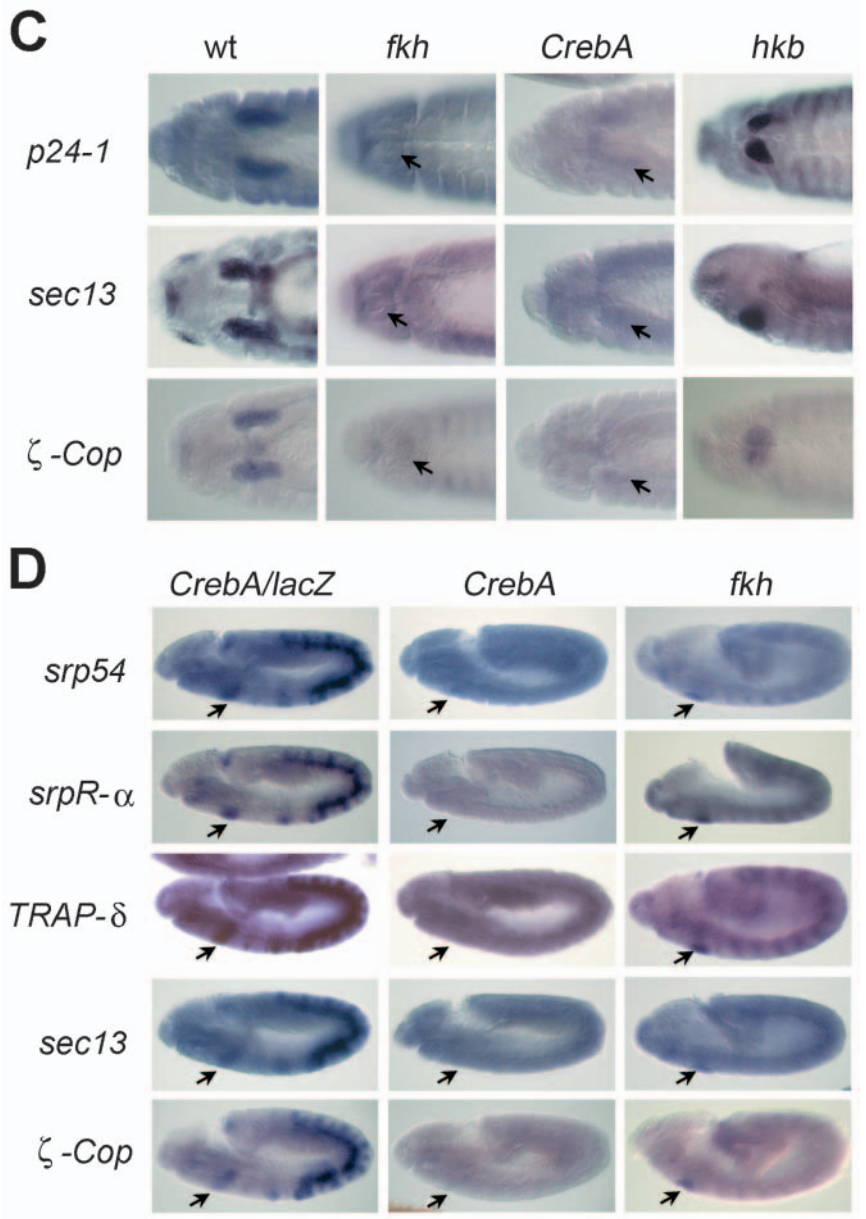


**Fig. 2C,D.** See previous page for legend.

The SPC is made up of five transmembrane subunits (SPases) with the following masses: 12, 18, 21, 22/23 and 25 kDa (Fig. 1) (Kalies and Hartmann, 1996). The gene encoding the 18 kDa homolog is not present in the *Drosophila* genome and is most related to the 21 kDa subunit by sequence comparison. The four recognizable *Drosophila* SPase genes require *fkh* for late but not early expression (Fig. 2B; see Fig. S1E in the supplementary material). *CrebA* is required for wild-type levels of SPase expression in the salivary gland at all stages. As with all the other genes discussed so far, *hkb* mutants have levels of SPase expression comparable with those in wild type (Fig. 2B; data not shown).

### Regulation of SPCGs involved in ER export and retrieval

Soluble proteins that are fully translocated, processed (e.g. signal peptide cleaved, glycosylated) and destined to proceed through the secretory pathway interact with proteins that recruit them to specialized domains (exit sites) in the ER (Aridor et al., 1998; Tang et al., 2001). For soluble proteins, this recruitment is thought to occur, in part, through interactions with cargo receptors of the p24 single transmembrane family of proteins (Fiedler et al., 1996; Dominguez et al., 1998). In *Drosophila*, expression of three p24 family members is upregulated in the salivary gland; this upregulated expression requires *CrebA* at all embryonic stages and *fkh* from stage 13 onwards (Fig. 2C; see Fig. S2A in the supplementary material; data not shown).

Once recruited to export sites in the ER, cargo molecules are packaged into transport vesicles. COPII coatomer molecules assemble onto vesicles involved in the anterograde movement of secretory products from the ER to the cis Golgi (Fig. 1) (Ellgaard et al., 1999). Transmembrane proteins destined to exit the ER contain cytoplasmic tails with exit signals, which interact directly with COPII subunits (Tang et al., 2001). The assembly of the coat from a cytoplasmic pool of soluble COPII subunits is thought to drive the actual budding of vesicles. This process is regulated by the small G protein Sarl, where hydrolysis of GTP-Sar1 to GDP-Sarl leads to coat disassembly shortly after vesicle formation (Barlowe, 2003b). In *Drosophila*, the genes encoding the COPII components Sec13, Sec31, Sec23, Sec24 and Sar1 are conserved and require *fkh* and *CrebA* for high levels of salivary gland expression (Fig. 2C; see Fig S2B in the supplementary material). However, as with the aforementioned SPCGs, expression of the COPII subunits appears to be relatively independent of *fkh* prior to stage 13 (Fig. 2D; data not shown).

COPI coats are typically involved in retrograde movement of transport vesicles, although in certain contexts they have been shown to also be involved in anterograde movement (Orci et al., 1997). The COPI coatomer component genes, α, β, β′, δ, ε, γ, and ζ-cop (Rothman and Orci, 1992) are all conserved in *Drosophila* (Adams et al., 2000). As with COPII, COPI coat formation is regulated by a small G-protein, ARF-1. Although each of the five *Drosophila* ARF-1 homologs is highly conserved (data not shown), ARF79F is the most homologous to human ARF1 (Table 1). Finally, the KDEL receptor (KDEL-R) has been shown in yeast and mammals to be important in the retrieval of escaped resident proteins back to the ER (Lewis et al., 1990; Lewis and Pelham, 1992). δ, ε, γ, ζ-cop, ARF79F and the KDEL-R genes are upregulated in the salivary gland and require *fkh* for their elevated expression from stage 13 and later (Fig. 2C; see Fig. S2C in the supplementary material). Although the expression of these genes is reduced in the salivary glands of *CrebA* mutants, both early and late expression of δ-cop, γ-cop and the KDEL-R

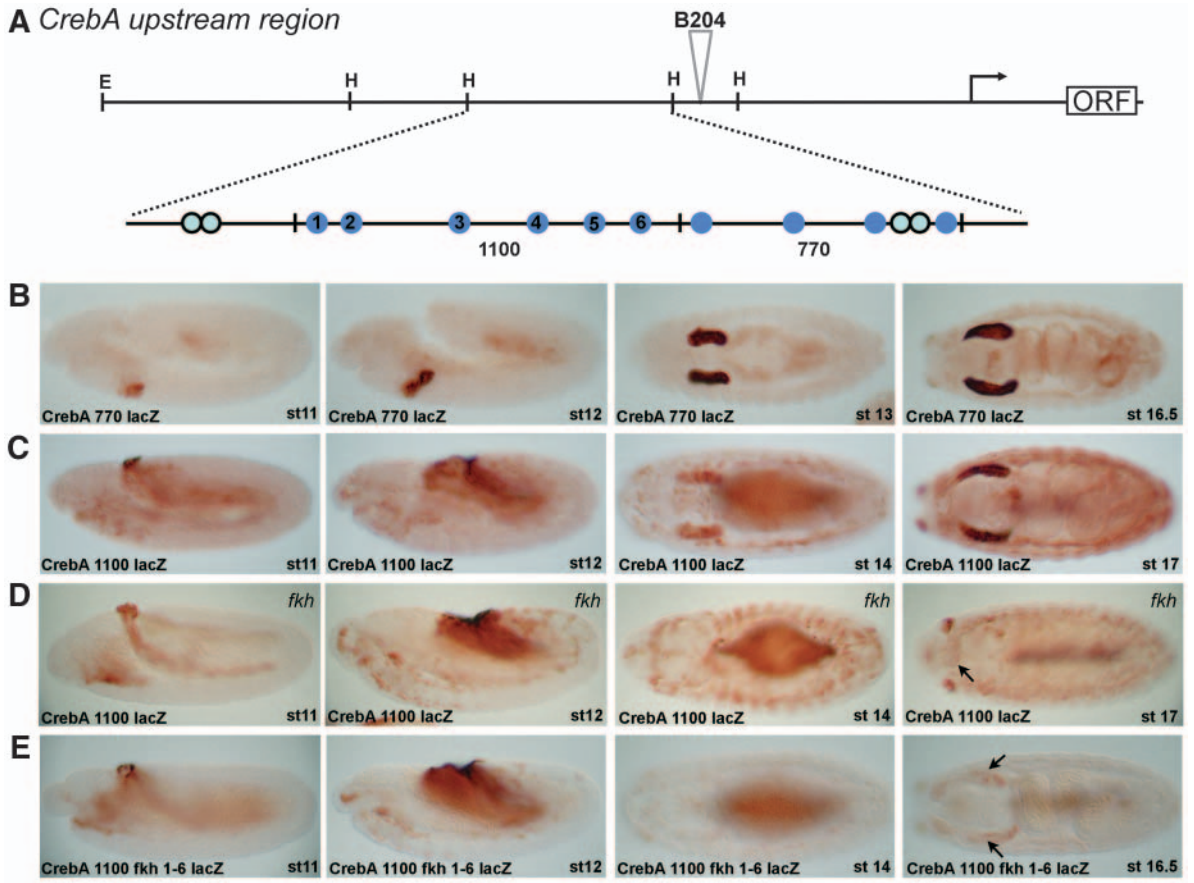


**Fig. 3.** Regulation of *CrebA* by Fkh. A map of the region upstream of the *CrebA* transcription unit showing the 2.8 kb enhancer fragment that drives reporter gene expression in the salivary gland (A). This fragment is just upstream of a P-element insertion (B204) that results in β-gal expression in only the salivary gland and amnioserosa. Two subfragments of 1100 and 770 bp also resulted in salivary gland expression of a *lacZ* reporter gene, with the CrebA 770 fragment driving salivary gland expression slightly earlier than the CrebA 1100 fragment (B,C). The CrebA 770 fragment has four Fkh consensus binding sites (A, blue circles) as well as two consensus Scr/Exd/Hth-binding sites (A, blue circles with black edges). The Creb 1100 site has six Fkh consensus binding sites and no consensus Scr/Exd/Hth-binding sites (A). The CrebA 1100 *lacZ* construct is not expressed to high levels in the salivary glands of *fkh* mutant embryos (D), with only some very low level expression detectable at late embryonic stages (D, right-most embryo, arrows). CrebA 1100 constructs with all six Fkh consensus sites mutated also show a loss of reporter gene expression specifically in the salivary gland (E), again with only some very low level expression detectable in late embryonic stages (E, right-most embryo, arrows).

gene are not as reduced as the other SPCGs (Table 2; Fig. S2C in the supplementary material; data not shown).

In summary, our expression studies reveal that the genes encoding components of the early secretory pathway are transcriptionally upregulated in the *Drosophila* embryonic salivary glands beginning shortly after the gland is specified and continuing, for the most part, throughout embryogenesis. This high level expression in the salivary gland requires *CrebA* function throughout embryogenesis and *fkh* function only after embryonic stage 13. *CrebA* is also required for the elevated expression of the early secretory pathway genes in the embryonic epidermis at late stages. These data suggest that Fkh maintains salivary gland expression of the SPCGs indirectly by maintaining expression of *CrebA*.

### Regulation of *CrebA* by Fkh is direct

To test whether regulation of *CrebA* by Fkh is direct, we identified a 2.8 kb fragment upstream of the *CrebA* transcription unit that could drive salivary gland expression of a *lacZ* reporter gene (Fig. 3) (K. D. Henderson, PhD Thesis, Johns Hopkins University School of Medicine, 2000). Two smaller fragments from this enhancer resulted in salivary gland expression of the *lacZ* reporter gene either only after invagination had begun and later (CrebA-1100) or prior to invagination and later (CrebA-770) (Fig. 3; top two rows of embryos). As the later expression pattern fitted the timeframe for Fkh-dependent salivary gland expression of *CrebA*, we further characterized the CrebA-1100 construct, which contains six consensus Fkh-binding sites (Kaufmann et al., 1994; Lehmann and Korge, 1996; Mach et al., 1996; Takiya et al., 2003). β-Gal expression in the salivary glands with the CrebA-1100 construct was significantly reduced in *fkh* homozygotes although expression in the amnioserosa was unaffected, indicating that we had identified a Fkh-dependent salivary gland enhancer of *CrebA* (Fig. 3, third row of embryos). We next transformed flies with a CrebA-1100 reporter construct in which all six consensus Fkh-binding sites were mutated (CrebA-1100 fkh1-6 *lacZ*). Both lines carrying

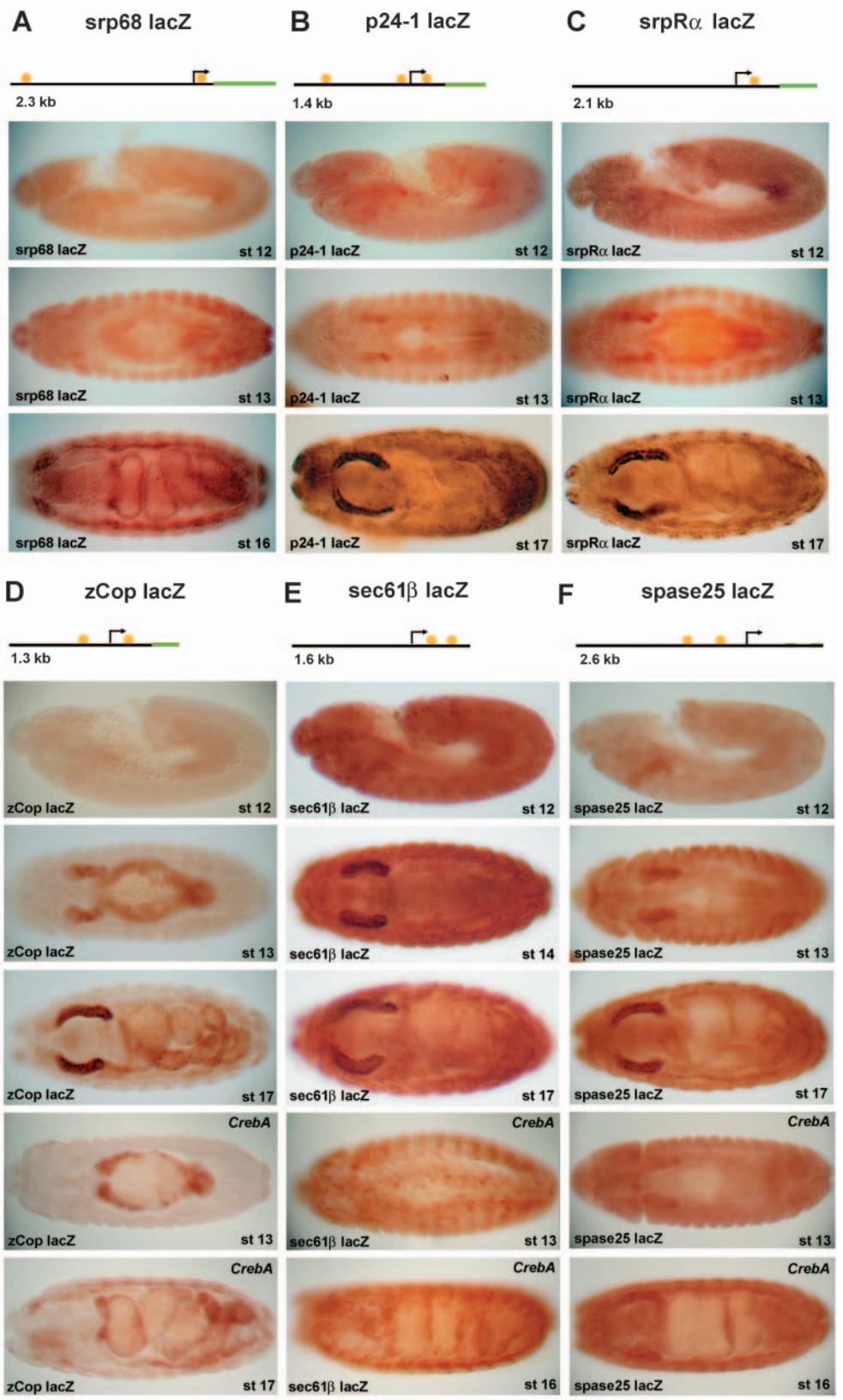


**Fig. 4.** Characterization of SPCG salivary gland enhancers identifies at least three CrebA-dependent enhancers**.** Reporter gene constructs were built for six of the 34 SPCGs analyzed in this study (A-F, diagrams at the top). Yellow circles of each set of embryos indicate the consensus motif found in SPCGs by MEME analysis. Arrows indicate putative transcription start sites based on the longest sequenced 5′ end cDNAs for each gene. Green lines indicate the open reading frames.Three independent lines carrying the *srp68 lacZ* construct did not have β-gal expression in the salivary gland (A). Multiple independent lines carrying *p24-1 lacZ*, *srpRα lacZ* and *zcop lacZ* constructs showed salivary gland β-gal expression beginning during embryonic stage 13 and continuing through the rest of embryogenesis (B-D). Independent lines carrying the *spase25 lacZ* (F) and *sec61β lacZ* (E) constructs had β-gal salivary gland staining from early stage 12 through the rest of embryogenesis (E,F). The *zcop* and *sec61β lacZ* constructs that gave salivary gland expression of β-gal in wild-type embryos did not express β-gal in the salivary glands of *CrebA* mutants (D,E, lower two rows). The *spase25 lacZ* constructs showed significantly reduced expression in the salivary glands of *CrebA* mutants (F, lower two rows).

the mutated construct had significantly diminished salivary gland expression of β-Gal, although βGal expression in other tissues, including the amnioserosa and hemocytes was unaffected (Fig. 3, bottom row). We conclude that Fkh functions directly to maintain late high-level expression of CrebA in the salivary gland.

## Regulation of SPCGs by CrebA

A second prediction of our model is that SPCG expression is controlled directly by CrebA. As a first step toward testing this possibility, we built and injected *lacZ* reporter constructs for six of the 34 SPCGs analyzed in this study. Each SPCG enhancer fragment spanned the 5′ end of the most 5′ cDNA for each gene and included ~1-2 kb of DNA further upstream (Fig. 4). Transformant lines generated from five of the six constructs resulted in embryonic salivary gland expression. The *srp68 lacZ* enhancer construct did not express in the embryonic salivary gland (Fig. 4A). Salivary gland *lacZ* expression from the *spase25* and *sec61β* enhancer constructs was detected from early stage 12 and throughout embryogenesis (Fig. 4E,F).

Salivary gland *lacZ* expression from the *p24-1*, *zCop* and *SrpRa* enhancer constructs was first detected during stage 13 and later (Fig. 4B-D). Expression of three of the constructs were examined in *CrebA* mutants; expression of β-Gal from both the *zCop-lacZ* and *sec61β-lacZ* constructs was completely absent in the salivary glands (Fig. 4D,E), whereas salivary gland β-Gal expression from the *spase25*-lacZ construct was significantly reduced (Fig. 4F). Thus, we have identified CrebA-dependent salivary gland enhancers for at least three of the SPCGs.

A search of the regions immediately upstream of the translation start sites of the SPCGs using MEME (http://meme.sdsc.edu/meme/website/) (Bailey and Elkan, 1994) revealed a motif that is an excellent match for a mammalian Creb-binding site (http://www.cbrc.jp/research/dp/TFSEARCH.html/; threshold score 86.1) and that is present within 2 kb upstream of 32 of the 34 SPCGs (Table 3). (The translation start site is used as a reference point since transcription start sites have not been mapped for any of the SPCGs.) Interestingly, of the two SPCGs that do not contain this consensus, one (*sec62*) is among the least affected by mutations in *CrebA* (Fig. 2B) and the other, *srp19*, is one of only two genes we examined that had ubiquitously high levels of expression in all tissues, including the salivary gland (Table 1). Even more compelling is the finding that 13/32 have the site within 100 bp, another 7/32 have the site within 200 bp and another 5/32 have the site within 500 bp of the translation start site. All of the SPCG reporter gene constructs we built contain this consensus site. Thus, not only do we predict that the site is important for salivary gland expression of the SPCGs, but that this could be the site through which CrebA acts to elevate transcription. The proximal location of these putative binding sites with respect to the start site of translation is consistent with the finding that mammalian Creb proteins bind close to the start of transcription (Mayr and Montminy, 2001). Also of relevance to these studies was the failure to discover consensus Fkh-binding sites conserved among the SPCGs through MEME analysis, further supporting an indirect role for Fkh in SPCG regulation.

## CrebA and enhanced levels of salivary gland secretory activity

Our discovery that *CrebA* is required to achieve high-level expression of all early secretory pathway genes in the salivary gland suggests a predominantly physiological rather than morphogenetic role for this transcription factor. Such a role in secretion is consistent with the previously reported salivary gland defects in *CrebA* mutants, where a relatively mild defect was observed: the salivary glands of stage 13 to 15 *CrebA* mutants were, on average, slightly more crooked than those of their wild-type siblings (Andrew et al., 1997). To determine directly if defects in salivary gland secretion occur with the loss of *CrebA* function, we stained collections of *CrebA* mutant embryos with a polyclonal Engrailed (En) antiserum that crossreacts with material found in both late secretory vesicles and the salivary gland lumen (Myat and Andrew, 2002). These embryos were simultaneously stained with an antibody to PH4αSG1, a resident ER protein (E.W.A. and D.J.A., unpublished). At early stages, the localization of these markers in the *CrebA* mutants was indistinguishable from wild type. By embryonic stage 15, however, far fewer En-positive vesicles were observed in the salivary gland cells of *CrebA* mutants compared with their wild-type and heterozygous siblings (Fig. 5A, arrows). The difference in vesicle accumulation in salivary gland cells was even more pronounced at embryonic stage 17, where the number of En-positive vesicles in wild-type embryos increased substantially relative to earlier stages, yet few could be detected in the stage 17 *CrebA* mutants (Fig. 5B, arrows and arrowheads). These results indicate that loss of *CrebA* function is associated specifically with a decrease in the secretory vesicle population in late embryos, supporting a role for *CrebA* in the increased secretory function associated with the salivary gland.

**Table 3. Consensus sequence motif within 2 kb upstream of SPCGs**

Consensus 

| Gene | Sequence | Distance from ORF |
|---|---|---|
| *srp9* | T G A C G T T G A | <100 bp |
| *srp68* | C G A C G T G T A | <100 bp |
| | T G A C G T G G T | |
| *srp72* | T G A C G T T G A | |
| *srp54* | C G A C G T G T C | <200 bp |
| | C G A C G T G G A | <200 bp |
| *srp14* | G G A C G T G T T | |
| *srp19* | No site within 2 kb | |
| *srpRα* | T G A C G T G G A | <100 bp |
| *srpRβ* | C G A C G T T G C | |
| | C G A C G T T G T | |
| *sec61α* | T G A C G T T T C | <200 bp |
| | C G A C G T G T C | <200 bp |
| *sec61β* | T G A C G T G G A | <200 bp |
| | T G A C G T G C T | <100 bp |
| *sec61γ* | T G A C G T T G C | <100 bp |
| | C G A C G T G G C | <100 bp |
| | C G A C G T G G A | ~500 bp |
| *sec62* | No site within 2 kb | |
| *sec63* | C T A C G T G T C | <500 bp |
| | T G A C G T T C T | |
| *sec71* | G G A C G T T C T | |
| *tram* | C G A C G T T C A | <200 bp |
| *trapδ* | T G A C G T G A C | <200 bp |
| *spase22/23* | C G A C G T G T C | <100 bp |
| *spase 12* | T G A C G T G C C | <100 bp |
| *spase18/21* | A T A C G T G T C | <200 bp |
| *spase 25* | T G A C G T T T C | <500 bp |
| | G G A C G T G G A | |
| *chop24* | C T A C G T G C T | <100 bp |
| *p24-rel1* | T G A C G T G T T | <200 bp |
| | T G A C G T G T C | <500 bp |
| *p24-rel2* | T G A C G T G T C | <100 bp |
| *sec31* | G G A C G T G A C | <100 bp |
| | T G A C G T G C G | |
| | G G A C G T G T A | |
| | T G A C G T T T A | |
| *sec13* | T G A C G T G G A | <100 bp |
| *sec23* | T G A C G T G G C | <100 bp |
| *sec24* | T G A C G T G C C | |
| | G G A C G T G T T | |
| *sar1* | C T A C G T G G T | ~500 bp |
| | C T A C G T G G A | |
| *arf79F* | C T A C G T G T A | |
| | G G A C G T G T A | |
| *δ-Cop* | T G A C G T T T T | <100 bp |
| | T G A C G T G T T | <200 bp |
| *γ-Cop* | T G A C G T G A A | <500 bp |
| *ζ-Cop* | T G A C G T G T A | <100 bp |
| | T G A C G T T G G | <500 bp |
| *ε-Cop* | C T A C G T G G T | |
| *erd2* | T G A C G T G G C | <500 bp |

We also tried to determine whether zygotic loss-of-function mutations in individual secretory pathway component genes had secretion defects similar to those observed with *CrebA* mutants. For these experiments, we obtained lethal P-element insertions in several early secretory genes: *srpRβ*, *sec13*, *sec23* and *sar1*. The P-elements had inserted in the coding region (*sec13*), the 5′ UTRs (*srpRβ* and *sec23*) or the first intron (*sar1*), and thus, would be expected to either eliminate or severely attenuate gene function. The accumulation of En-positive secretory vesicles was not altered detectably in any of the single secretory pathway mutants (Fig. 5C; data not shown). These observations suggest that residual zygotic function in combination with maternally supplied mRNAs are sufficient to support salivary gland secretory function of these single secretory pathway components during embryogenesis.

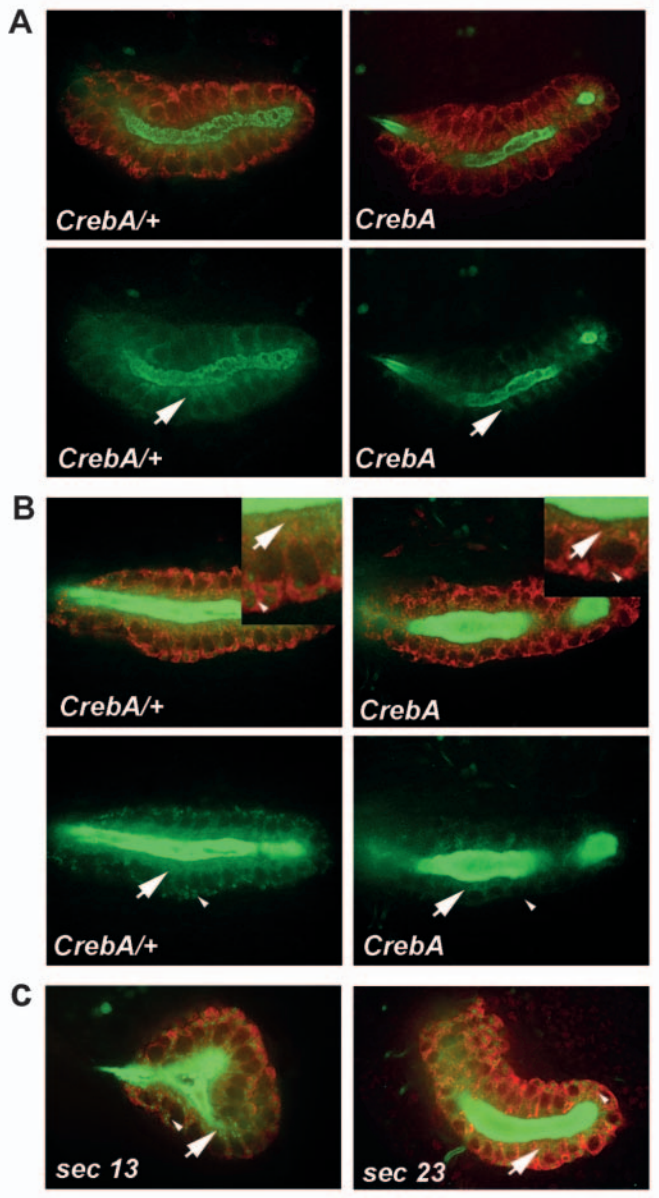


**Fig. 5.** *CrebA* mutations affect secretory activity in the salivary gland. (A,B) Stage 15 and 17 wild-type (*CrebA*/+) and *CrebA* mutant embryos were stained with α-PH4αSG1 (red) and α-En PAb (green). (A) At stage 15, Engrailed-positive secretory granules can be detected in *CrebA*/+, while very few were seen in *CrebA* mutant salivary glands (arrows). (B) Significantly more secretory granules were detected in stage 17 *CrebA*/+ salivary glands than in the stage 17 *CrebA* mutant salivary glands (arrows and arrowheads). The amount of lumenal content increases significantly from stage 15 to 17 in both *CrebA*/+ and *CrebA* salivary glands, but is not different in the wild-type versus *CrebA* mutant glands. (C) Stage 17 *sec13* and *sec23* salivary glands appear relatively normal with respect to secretory granule levels. The abnormal salivary gland shape in the *sec13* mutants is probably linked to other patterning defects observed when this P-element insertion is homozygous. Arrows indicate punctate staining near the apical surface. Arrowheads indicate larger vesiclar staining throughout the cell.

## Secretory function and *CrebA* cuticle defects

CrebA has a crucial role in larval cuticle development; *CrebA* mutant first instar larvae are only about 40% the length of wild-type larvae, and have a faint cuticle with frequent large dorsal holes (Andrew et al., 1997). A close examination of the ventral denticles and dorsal hairs reveal morphologies of structures typically found in more lateral positions in wild-type cuticle, suggesting a role for CrebA in controlling dorsal/ventral cuticle patterning. Our findings that CrebA is required for robust SPCG expression in the epidermis prompted us to ask if some or all of the cuticle defects observed in *CrebA* mutants could be explained by reduced SPCG expression. Thus, we performed cuticle preparations of larvae carrying the same P-element mutations discussed in the previous section. Mutations in *srpRβ*, *sec13*, *sec23* and *sar1* result in cuticle defects largely indistinguishable from those of *CrebA* mutant larvae. The cuticles were on average only 40-50% the length of wild-type cuticles and the cuticles were faint (Fig. 6A). The mouthparts and filzkörper were barely detectable in dark-field low magnification images of the *CrebA* and SPCG mutant larvae (Fig. 6A) and only faint outlines of these structures, with very little pigmentation, could be seen in higher magnification phase images (Fig. 6B). The SPCG mutant larvae exhibited defects in ventral denticle and dorsal hair morphology that were similar to those previously described for *CrebA* mutants (Fig. 6C). Ventral denticles were frequently missing and those that remained were smaller and less pigmented on average than wild-type denticles. Dorsal hairs were often completely absent and, when present, showed alternations between naked cuticle and thin hairs, similar to those observed in more lateral domains of the dorsal cuticle of wild-type larvae. Although all of the SPCG mutants analyzed had the same range of cuticle defects, the *srpRβ* and *sec23* phenotypes appeared somewhat milder than those of *CrebA*, *sec13* and *sar1*, perhaps reflecting either how much gene function remains in the P-element mutants or differential persistence of the maternally supplied components. Interestingly, larvae mutant for the individual SPCG genes did not show the large dorsal holes frequently observed in *CrebA* mutants.

The defects in the larval cuticles of *CrebA* and SPCG mutants support a crucial role for these genes in cuticle secretion. In wild-type animals, secretion begins at about 12 hours after egg laying (AEL) (Martinez-Arias, 1993), which roughly corresponds to embryonic stage 15 (Campos-Ortega and Hartenstein, 1997). To determine if secretory defects could be detected at this earlier stage, we stained *CrebA* and wild-type embryos with antibodies to DSC73, a secreted protein expressed to high levels in all cells that form the denticles and hairs of the larva (D.J.A.,

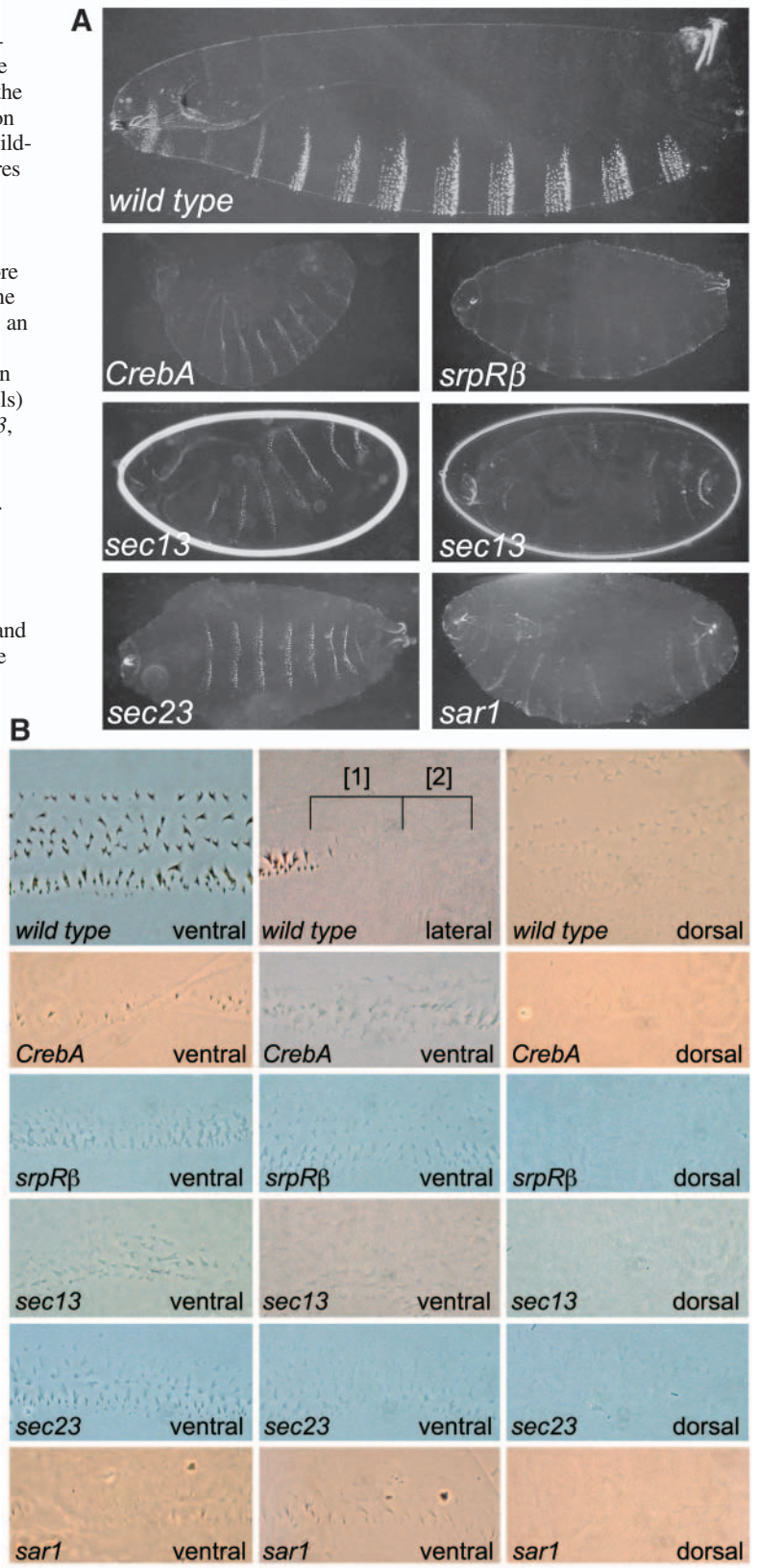


**Fig. 6.** Larval cuticle defects in *CrebA* and SPCG mutants. (A) Dark-field images are shown of cuticles prepared from wild-type, *CrebA*, *srpRβ*, *sec13*, *sec23* and *sar1* first instar larvae. The cuticles from each of the mutants were smaller and fainter than the cuticle prepared from the wild-type larva. (B) High-magnification images of the ventral, lateral and dorsal cuticular structures of wild-type larvae (top panels), and ventral and dorsal cuticular structures of *CrebA*, *srpRβ*, *sec13*, *sec23* and *sar1* first instar larvae (lower panels). The ventral cuticles of the mutants showed a loss of denticles and the denticles that were present have very light pigmentation, more consistent with the pigmentation seen in more lateral denticles of wild-type larvae [1]. The dorsal surfaces of the mutant larvae typically consisted of thin hairs and naked cuticle, an arrangement more typical of dorsal-lateral regions of wild-type cuticles (top panel, [2]). (C) High-power phase images are shown of the mouthparts (mp; left panels) and filzkörper (fk; right panels) from cuticles prepared from wild-type (wt), *CrebA*, *srpRβ*, *sec13*, *sec23* and *sar1* first instar larvae. Both the mouthparts and filzkörper are less robust and have reduced pigmentation than observed in the mouthparts and filzkörper of wild-type embryos. (D) DIC images of stage 15 embryos stained with α-DSC73 in combination with α-β-gal and α-CrebA to distinguish *CrebA* mutant embryos (bottom two panels) from their heterozygous (wild-type) siblings (top two panels). Left panels show lower magnification images of the same embryo shown in the middle and right panels to reveal CrebA staining in the salivary glands of the heterozygotes (wild type) and the loss of CrebA staining in the salivary glands of the *CrebA*-mutant embryos. DSC73 staining intensity in the denticle-producing cells was reduced in the *CrebA* mutants compared with that of wild-type embryos. Staining of dorsal hair-producing cells, however, was only mildly decreased in the *CrebA* mutants compared with wild-type embryos. The process of dorsal closure appeared to be lagging in the *CrebA* mutants compared with wild type; the epidermal cells that meet and fuse at the dorsal midline were relatively closer in the wild-type embryos compared with the *CrebA* mutants. This defect is probably linked to the dorsal holes observed in the cuticles of *CrebA* mutant larvae. Embryos were staged based on extent of head involution, gut invaginations and elongation of the proventriculus.

unpublished). Wild-type stage 15 and older embryos showed high-level DSC73 staining in all epidermal cells that form the denticles and hairs (Fig. 6D; top two rows). Such consistent and uniform DSC73 staining was not observed in the *CrebA* mutants. In the ventral cuticle precursors, *CrebA* mutants showed variable DSC73 staining, with levels ranging from almost wild type to barely detectable (Fig. 6D; bottom two rows). DSC73 levels in the dorsal cuticle were only slightly diminished, if at all, when compared with wild-type levels. DSC73 staining did reveal a significant lag in dorsal closure in the CrebA mutants, a defect likely to be linked to the dorsal holes frequently observed in the *CrebA* larval cuticles. Thus, defects in secretion in both the salivary gland and epidermis can be detected in *CrebA* mutants as early as 12 hours AEL, during embryonic stage 15.

## Discussion

This study demonstrates that the *Drosophila* salivary gland prepares soon after specification to generate the machinery for its high-level secretory activity. The machinery includes components of the early secretory pathway crucial for targeting and translocating proteins into the ER and for vesicle transport between the ER and Golgi. Thus, one way the gland

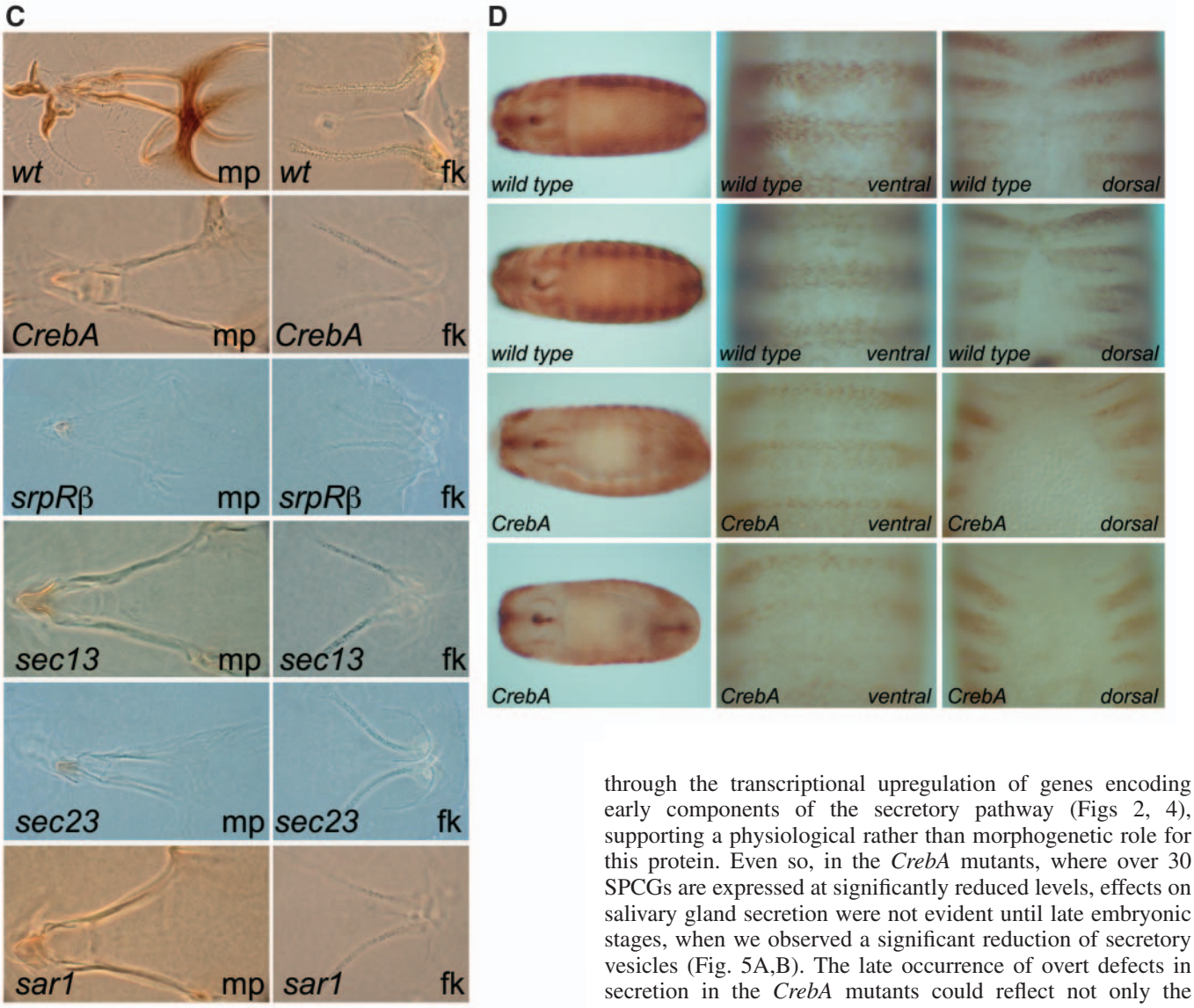


**Fig. 6C,D.** See previous page for legend.

distinguishes itself from surrounding tissues is to greatly increase the relative transcriptional levels of the secretory pathway component genes (SPCGs). The leucine zipper transcription factor CrebA has a crucial and probably direct role in activating increased levels of SPCG expression not only in the salivary gland, but also in the epidermal cells, which secrete the larval cuticle. Fkh, the *Drosophila* FoxA/PHA-4 homolog, is required to maintain SPCG expression in the salivary gland, but acts indirectly, by maintaining *CrebA* expression. Hkb, the other early transcription factor examined in this study, is not required for elevated SPCG expression.

### CrebA and secretory function

*CrebA* is expressed at very high levels in the early salivary gland and this high level expression persists throughout larval life. Nonetheless, embryonic salivary glands in *CrebA* mutant embryos are relatively normal, showing only a mildly crooked phenotype when compared with the salivary glands of wild-type embryos (Andrew et al., 1997). This study indicates a role for CrebA in mediating salivary gland secretory function through the transcriptional upregulation of genes encoding early components of the secretory pathway (Figs 2, 4), supporting a physiological rather than morphogenetic role for this protein. Even so, in the *CrebA* mutants, where over 30 SPCGs are expressed at significantly reduced levels, effects on salivary gland secretion were not evident until late embryonic stages, when we observed a significant reduction of secretory vesicles (Fig. 5A,B). The late occurrence of overt defects in secretion in the *CrebA* mutants could reflect not only the increased secretory load on these cells that occurs only at the later embryonic stages, but also some level of maternal rescue of secretory function, as *CrebA* is provided maternally (Smolik et al., 1992). Interestingly, loss-of-function mutations in single secretory pathway component genes did not show the same loss of secretory activity observed in *CrebA* mutant salivary glands. The residual function of each of the individual SPCGs, from either maternal supplies or the remaining function of the P-element insertional alleles, appears to suffice when all other components are present at wild-type levels, at least with regards to salivary secretion during late embryonic stages.

*CrebA* mutants have major defects in cuticular development (Fig. 6); the larval cuticles are smaller and weaker than the cuticles of their wild-type siblings, the mouthparts and filzkörper are poorly formed, and *CrebA* mutants frequently have large holes in the dorsal cuticle (Andrew et al., 1997). In addition, there appears to be a general defect in patterning of the cuticle, with dorsal and ventral structures appearing more lateralized (Andrew et al., 1997). Embryos mutant for individual SPCGs, whose epidermal expression is also dependent on *CrebA*, had nearly identical defects in the larval cuticle (Fig. 6). The similarity in *CrebA* and the individual

SPCG mutant cuticles suggests that *CrebA* defects could be entirely due to compromised secretory function in the epidermal cells that produce the cuticle. The lateralized appearance of the denticles and hairs could simply reflect compromised secretory function, which would limit the types of cuticular structures that form to the smaller, less pigmented structures that are characteristic of the lateral cuticle.

### Direct regulation of SPCGs by CrebA

Our expression studies of the SPCGs indicate that CrebA could directly activate their high level expression. Moreover, we have discovered a conserved motif upstream of the SPCGs that is not only a good fit with the mammalian Creb-consensus binding site (TGACGTG G/T C/A; Table 3), but that also matches the first six nucleotides of the sequence that was used to discover *CrebA* (TGACGTCAG) (Smolik et al., 1992). However, the experiments of Smolik et al. were designed to discover the *Drosophila* homolog of the cAMP-regulated Creb protein, which turns out to be what is now known as CrebB (Usui et al., 1993). Gel shift experiments (EMSAs) indicate that CrebA can bind the to the TGACGTCAG consensus but not with the same high affinity and specificity as the mammalian cAMP-regulated Creb protein (Smolik et al., 1992); thus, CrebA may bind instead with high affinity to the site discovered in our MEME motif search of the regions upstream of the SPCGs to regulate their expression. The CrebA-dependent SPCG enhancers we have characterized so far (for *z-cop*; *sec61β* and *spase25*) contain at least two copies of the consensus motif.

### A new and indirect role for the FoxA protein, Fkh, in the salivary gland

Fkh has several roles in salivary gland development and function, including mediating the cell shape changes of invagination (Myat and Andrew, 2000b), maintaining secretory cell viability (Myat and Andrew, 2000b) and transcriptional activation of the *sgs* genes in late larval life (Lehmann and Korge, 1996; Mach et al., 1996). In addition to these positive roles, FKH also represses the expression of salivary duct-specific genes in the secretory cells (Haberman et al., 2003). Here, we discover yet another role for *fkh* in the salivary gland: the maintenance of SPCG expression.

*fkh* is a direct transcriptional target of Scr and Exd (Ryoo and Mann, 1999) and the temporal expression of *CrebA* and the presence of consensus Scr/Exd-binding sites upstream of the gene suggest that *CrebA* may also be directly controlled by Scr and its co-factors. Late expression of *CrebA*, however, requires *fkh* (Myat and Andrew, 2000b), as does late expression of *fkh* itself (Zhou et al., 2001). Here, we show that Fkh functions directly to maintain *CrebA* expression in the salivary gland (Fig. 3). Based on the requirement for *CrebA* for expression of the SPCGs at all embryonic stages and the requirement for *fkh* only at late stages, our data support a model in which CrebA controls the expression of the SPCGs and Fkh is required only because of its role in maintaining *CrebA* expression (Fig. 7). A direct test of this model would be to express *CrebA* in the salivary glands of embryos missing *fkh* function; this experiment, unfortunately, could not be carried out because Fkh-independent drivers capable of providing high-level salivary gland-specific expression of *CrebA* are not yet available.

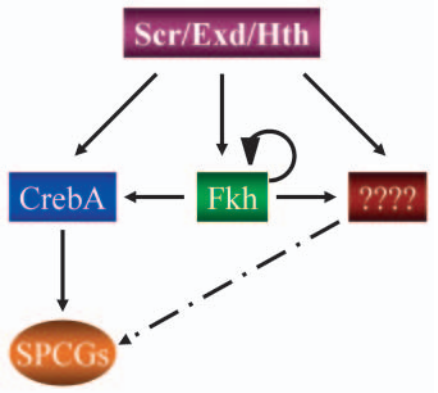


**Fig. 7.** Model of transcriptional regulation of early SPCGs. Fkh and CrebA are directly regulated by the Scr/Exd/Hth complex. Late expression of CrebA requires Fkh and Fkh is autoregulated. The expression of most of the SPCGs is greatly reduced in *CrebA* mutants at all embryonic stages, whereas only late (beyond stage 13) expression of all tested SPCGs is affected by the loss of *fkh* function. As late expression of the SPCGs was lower in late *fkh* mutants than in *CrebA* mutants, we propose the existence of another early transcription factor gene in SPCG regulation. As with CrebA, late but not early expression of this putative transcription factor would require Fkh.

As previously mentioned, a subset of the SPCGs that encode proteins required for retrograde vesicle transport from the Golgi to the ER were still expressed at low levels in *CrebA* mutants (Fig. 2D). However, in late but not early *fkh* mutants, expression of these genes was not above levels in surrounding tissues. We propose that the residual expression of the genes observed in the *CrebA* mutants would be controlled through other early transcription factor genes that, like *CrebA*, would require Scr and its co-factors for their initial expression and would require Fkh for maintaining late expression (Fig. 7). Taken together, our studies suggest that regulation of salivary gland genes does not fit the simple paradigm suggested by studies of the *C. elegans* pharynx. The genes that specify the salivary gland (Scr/Exd/Hth) are distinct from the genes that activate and maintain gene expression in the organ. Moreover, no single gene takes over for the organ-specifying genes as even Fkh, the PHA-4 homolog, is not required for expression of every salivary gland gene (Myat and Andrew, 2000b; Bradley et al., 2001). In cases where Fkh is required, it is often indirect, such as with the SPCGs. Fkh does appear to have direct roles, however, much later in development, as demonstrated by regulation studies involving the *sgs* glue genes (Lehmann and Korge, 1996; Mach et al., 1996). Thus, the involvement of Fkh in salivary gland development and function is complicated and more consistent with the complexity of gene regulation seen in the liver than that suggested for the *C. elegans* pharynx. The existence of a single ‘organ-specifying gene’ may be more the exception than the rule.

We thank C. Machamer for many helpful discussions. We thank A. Cheshire, L. Grevengoed, B. Kerman, C. Machamer, M. Vining and two anonymous reviewers for critical reading of the manuscript. We thank the Indiana Stock Center in Bloomington for the fly stocks and P. O’Farrell for the En polyclonal antiserum. This work was supported by an NIH grant to D.J.A. (NIH RO1 DE13899).

### Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/12/2743/DC1

### Note added in proof

Salivary gland expression of the *srpRα lacZ* construct shown in Fig. 4C is completely lost in a *CrebA* mutant background, indicating that at least four *CrebA*-dependent salivary gland SPCG enhancers have been identified.

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