The CRE-Binding Protein dCREB-A Is Required for Drosophila Embryonic Development

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ABSTRACT

We have previously described the cloning of a cyclic AMP response-element (CRE)-binding protein, dCREB-A, in Drosophila melanogaster that is similar to the mammalian CRE-binding protein CREB. dCREB-A is a member of the bZIP family of transcription factors, shows specific binding to the (CRE), and can activate transcription in cell culture. In this report, we describe the gene structure for dCREB-A, protein expression patterns throughout development and the necessary role for this gene in embryogenesis. The 4.5-kb transcript is encoded in six exons that are distributed over 21 kb of DNA. There are seven start sites and no TATA consensus sequences upstream. The dCREB-A protein is expressed in the nuclei of the embryonic salivary gland, proventriculus and stomodeum. Late in embryogenesis, tracheal cell nuclei and specific nuclei within the segments show staining with anti-dCREB-A antibodies. In adult female ovaries, dCREB-A is expressed in the stage 9 through stage 11 follicle cell nuclei. Null mutations of the dCREB-A gene give rise to animals that no longer express dCREB-A protein and die late in embryogenesis before or at hatching. The absolute requirement of dCREB-A for embryogenesis demonstrates a nonredundant function for a CRE-binding protein that will be useful in studying the role of specific signal transduction cascades in development.

The signal transduction cascade that activates transcription through the induction of the second messenger cAMP is one of the most well-characterized pathways known. The protein kinase A (PKA) pathway itself is quite simple; the binding of hormones to receptors stimulates the production of cAMP that in turn causes the dissociation from the regulatory subunit and nuclear localization of the PKA catalytic subunit. Once in the nucleus, PKA activates transcription through a cAMP responsive element (CRE) by phosphorylating proteins that bind the CRE (CREB proteins) (reviewed by MEYER and HABENER 1993). Studies conducted over the last few years demonstrate that the transcriptional regulation of CRE elements by the family of CREB proteins is more complex. The arrangement of the CRE motif in the DNA and the composition of the flanking sequences is critical to the function of the enhancer. For example, the core glucagon CRE confers a cAMP induction of approximately eightfold. If the flanking sequence is increased from 9 to 14 bp, the cAMP response is completely abolished (DEUTSCH et al. 1988). CRE sequences have been found in the human T-cell lymphotrophic virus type I (HTLV I) where their regulation occurs through the viral protein p40tax and by adenovirus where they are involved in the regulation of the early genes by EIA (SASSONE-CORSI 1988; ZHAO and GIAM 1991). This diversity in CRE function can result from the differential regulation of CREB and other CREB family members that activate the enhancer.

The CREB proteins are members of the bZIP family of transcription factors and contain a basic DNA-binding domain adjacent to a leucine zipper dimerization domain (HOEFTLER et al. 1988). The amino-terminal activation domain contains phosphorylation sites for PKA, protein kinase C (PKC), glycogen synthase kinase-3 (GSK-3), casein kinase II, and calcium-calmodulin (CaM) kinases II and IV (GONZALEZ et al. 1989), suggesting that CREB proteins can mediate the effects of multiple signal transduction cascades. Recent evidence suggests that the Ca2+-dependent transcriptional regulation of CREB may involve activation by CaMK IV through the phosphorylation of Ser133 and repression through dual phosphorylation events at Ser133 and Ser142 by CaMK II (SUN et al. 1994). Nerve growth factor activates a ras-dependent protein kinase that activates the CREB-mediated transcription of c-fos (GINTY et al. 1994).

In addition to the differential regulation of CREB through various signaling pathways, the diversity of CRE activation can result from the heterodimerization of distinct CREB isoforms and family member proteins. For example, the CRE-binding protein CRE-BP1 can form a heterodimer with c-jun but not CREB. In this heterodimer, c-jun no longer recognizes its phorbol ester response element (TRE) and becomes a CRE-
binding protein (IVASHIV et al. 1990). Another CRE-binding protein, CREMa, may downregulate cAMP-stimulated CRE activity by forming heterodimers with CREB (FOULKES et al. 1991). Ultimately, we would like to understand how various signal transduction cascades regulate expression from a single enhancer element through the activation and repression of specific transcription factors.

In Drosophila, the regulatory processes that drive embryogenesis, oogenesis and disc development involve a number of signal transduction pathways, including ras-MAP kinase [reviewed by CHASAN and ANDERSON 1993; PERRIMON and DESPLAN 1994; ZIPURSKY and RUBIN 1994] and PKA-mediated cascades (JOHANNA et al. 1995). With the exception of the Drosophila jun homologue (BOHMANN et al. 1994) and the Ets proteins yan and pointed (O’NEILL et al. 1994) that mediate the sev-enless pathway during photoreceptor determination, not much is known of the transcription factors that respond to the different signaling cascades in flies. As a step toward understanding the complex regulation of the signal activated transcription factors, we have described two proteins homologous to the mammalian CREB proteins, dCREB-A and dCREB-B (SMOLIK et al. 1992; USUI et al. 1992). [The sequence for dCREB-A is also described by ABEL et al. (1992).] In this report we describe the gene structure of dCREB-A, its pattern of protein expression and the generation of dCREB-A null mutations. The null mutations are lethal at hatching, demonstrating that this transcription factor represents a nonredundant function that is required for Drosophila embryogenesis.

MATERIALS AND METHODS

Drosophila stocks and culture conditions: The two Pele-ment insertion strains, B204 (BIER et al. 1987) and l(3)3576 (KARPEN and SPRADLING 1992), are enhancer-trap lines from the G. RUBIN/C. GOODMAN and A. SPRADLING laboratories. B204 is an enhancer-trap insertion that carries the mini white gene as a marker. The l(3)3576 insertion is an enhancer-trap insertion that carries the ros gene as a marker. The large deletion, Df(3L)Bk10, includes the breakpoints 71C1;2/71F4 and was generated by LEICHT (1987). Df(3L)Y(78.3) (21A-41/71C1-26/100F-100A/40-60F;1A/71D4-E1-100F) was generated by W. GELBART (unpublished data) and Df(3L)Brd 659, which includes the breakpoints 71A-71C1, was generated by J. POSEKONY (LINDSEY and ZIMM 1992). These last three chromosomes were kindly provided by H. INACK and P. CHER-ERAS. The TMS, Sb P[ry+; Δ2-3]/Dr males, and the white eyed TMS, Sb Moi male progeny that lost the ry+ insertion were saved, and the mutant chromosomes balanced and kept in stock.

For the Pelement mutagenesis, l(3)3576/TM3, Sb ry males were crossed to Sb P[ry+; Δ2-3]/Dr females. Ten l(3)3576/TM3, Sb P[ry+; Δ2-3] females were mated to 10 ry e males in each of 10 bottles. The P element, P[ry+; Δ2-3], is inserted at position 99B on the third chromosome (ROBERT-SON et al. 1988). It provides constitutively active transposase to mobilize other P-elements but cannot itself transpose. The matings were allowed to proceed for 48 hr when the males were discarded. The brood times varied from 2 to 3 days. Male progeny that were l(3)3576/ry e and phenotypically ry+ were saved, and the mutant chromosomes balanced and kept in stock. One mutant from each bottle was used in the analysis.

Isolation and characterization of genomic dCREB-A clones: The 4.478-kb cDNA encoding the dCREB-A transcript (SMOLIK et al. 1992) was labeled with [α-32P]dCTP and used to screen an EMBL3 Drosophila genomic library (kindly provided by J. TAMUK). Approximately five genomes were screened by standard methods (SAMBROOK et al. 1989). Three overlapping clones representing 29.1 kb of genomic DNA were isolated. The introns and exons were mapped within the genomic DNA by Southern hybridization (SAMBROOK et al. 1989) and the intron/exon boundaries determined by se- quencing. Genomic intron/exon junction fragments were subcloned into pBluescript-KS+ (Stratagene) and sequenced as double-stranded DNA with α-32P-deoxycytosine triphosphates with the kit from Pharmacia and following their proto-col except that de-aza dGTP instead of dGTP was used.

Generation of dCREB-A antibodies: dCREB-A protein was expressed and purified as described (SMOLIK et al. 1992), then further purified for antibody production as follows. Ten milliliters of the dCREB-A protein extract was incubated at 72°C for 5 min, placed on ice for 5 min, centrifuged at 9000 rpm for 10 min and the supernatant removed and stored in 1-ml aliquots at −80°C. One milliliter of the dCREB-A protein extract was separated on a 7% SDS-polyacrylamide preparative gel. The protein bands were visualized by staining the gel in ice cold 0.5 M KCl and the band corresponding to dCREB-A was cut out. The dCREB-A gel fragments from 10 gels were pooled, crushed and placed into a dialysis bag along
with 8 ml of 1× Tris glycine buffer and the dCREB-A protein was electroeluted at 30 V overnight. The liquid from the dialysis bag was removed and the volume adjusted to 30 ml with PBS and concentrated in two centrif-prep concentrators (Amicon, Beverly, MA) until the total volume reached 1.5 ml. The purified dCREB-A protein was then used to prepare rat polyclonal antibodies at the Pocano Rabbit Farms (Pocano, New York) according to their protocols. The Western blot analysis was performed as described (SUMMERS et al. 1989). The three contaminating bacterial antibodies were removed by incubating the antiserum with acetone-treated bacterial extracts. (HARLOW and LANE 1988).

**Immunocytochemistry and X-Gal staining:** Embryos were collected and dechorionated in 50% bleach for 5 min. They were then rinsed in distilled water and fixed in a 1:1 mix of heptane and 4% paraformaldehyde in PBS for 10 min. The permeabilized and fixed embryos were transferred from the interphase to a depression slide, washed in PBS, 0.3% Triton X-100 and incubated in an X-Gal cocktail (5 mM NaHPO4/ Na2HPO4, [pH 8.0], 4 mM K4Fe(II)CN6, 4 mM K3FeIII-CN6, 0.2% Triton X-100 and 1.2 mg/ml X-Gal, diluted from a 20 mg/ml in dimethyl formamide solution) for 1–3 hr. The β-galactosidase expressed from the β-gal promoter can perdure through hatching and thus the homozygous mutant embryos can be identified throughout embryogenesis. After the X-Gal staining, the embryos were washed in PBS and incubated for 1 min in PBS, 0.5% TCA to weaken the vitelline membrane. The membranes were removed in a standard 1:1 mixture of heptane and 100% methanol. The embryos were rehydrated through a Tris-methanol series into PBS, 0.3% Triton X-100 and washed for at least 1 hr in 10% horse serum in PBS, 0.3% Triton X-100 (HS-PBT). The embryos were incubated overnight at 40° with dCREB-A antibody diluted 1:5000 in HS-PBT. After washing extensively in HS-PBT, the embryos were incubated for 1 hr at room temperature with a secondary biotinylated anti-rat antibody (Vector Laboratories) diluted 1:400 in HS-PBT. Again, after extensive washes, the embryos were incubated for 30 min in avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories) and washed in HS-PBT. The peroxidase was localized with 0.01% diaminobenzidine, 0.01% H2O2. The embryos were then dehydrated through an ethanol series, cleared in xylene and mounted in PRO-TEXX mounting medium (Lerner Laboratories).

Salivary glands, disks and brains from third instar larvae and ovaries from adult females were dissected in Drosophila ringers and fixed in 4% paraformaldehyde in PBS for 1 hr. They were washed extensively in PBS and stained for β-galactosidase activity or treated with anti-dCREB-A antibody as described above. Antibodies were preabsorbed with eggs and used in whole-mount staining of ovarian tissue. Preabsorption of the antibodies completely prevented any background staining of the chorion in the developing oocytes. After a final wash in PBS, the tissues were incubated for 10 min in 10, 25 and 50% glycerol before being mounted in 50% glycerol. All tissues were photographed with Nomarski optics. Embryo stages are assigned as described by CAMPOS-ORTEGA and HARTEINSTEIN (1985) and the stages of oogenesis as summarized by SPRADLING (1993).

**Mutant analysis:** DNA from Canton-S and the various mutant strains was isolated using standard methods (ASHBURNER 1989). The DNA was digested with the appropriate enzymes, separated on 0.7% agarose gels and transferred to Hybond-N (Amersham) paper. The B204 and I(3)3576 P-element insertions were mapped using [α-32P]dCTP-labeled genomic subclones as probes and using standard Southern hybridization conditions (SUMMERS et al. 1989). The I(3)3576 P-element insertion was localized with a 2.5 kb XhoI-EcoRI genomic fragment that lies adjacent to the second exon. The B204 P-element insertion was found to shift a 2.1 kb XhoI-SalI genomic fragment that lies 1.0 kb from the start site. These genomic fragments that are affected in the mutants were used to determine the nature of the mutations.

**Germ line transformation:** The fragment containing the entire dCREB-A cDNA was cloned into the transformation vector pUFST (BRAND and PERRISON 1993) and injected at a concentration of 500 mg/ml into w11; Δ2-3 embryos as described (SPRADLING 1986) and using p50Δ2-3 (kindly provided by J. POSAKONY) as a helper plasmid. The four independent transformants generated were mated in two generations to w11; TM3, Sb Ser/TM6B, Tb animals to eliminate the Δ2-3 chromosome. The two transformed lines, TbTr2 and TbTr1, that express the w11 gene most strongly were used in this study.

**Heat shock regimen:** Females of the genotype w11; Tr21; Df(3L)BK10/MKRS or w11; Tr2; Df(3L)BK10/MKRS were mated to males of the genotype w11; hs-GALA4/CyO; P+;TM3; Sb Ser or w11; HS-GALA4/CyO; P+;TM3; Sb Ser. These matings were allowed to proceed for 3 days at 25°. The matings were transferred to fresh vials and allowed to lay eggs for 16 hr at 25°. These eggs were immersed in a 37° water bath for 1 hr and then placed at 25° to continue development. The 0–16-hr control egg lay from the same matings were left at 25° for the entire developmental cycle. Rescued animals were scored by the darker eye color, not C37 nor Sb phenotype.

**RESULTS**

**Structural analysis of the dCREB-A gene:** The structure for the dCREB-A gene is illustrated in Figure 1. Three genomic clones spanning ~30 kb of DNA were detected with the dCREB-A cDNA. The six exons are distributed over 21 kb and are defined by one large, 10.5-kb intron and four small introns ranging from 71 bp to 1.5 kb. It is interesting to note that the intron/exon boundary that separates the basic domain and leucine zipper from the activation domain is the same as that found in the mammalian CREB gene (RUPPERT et al. 1992). No obvious TATA box was found upstream of the 5′ most start site. A canonical CRE sequence, 5′TGAAGTCA3′, was found 61 bp upstream from the 5′-most start suggesting that, like a number of other transcription factors, dCREB-A may autoregulate in vivo.

**The temporal and spatial expression pattern of dCREB-A:** We have previously reported the spatial and temporal expression pattern for dCREB-A transcripts using RNA probes to whole mount embryos and adult sections (SMOLIK et al. 1992). The generation of dCREB-A antibodies allowed us to assess the expression pattern of dCREB-A protein throughout Drosophila development. In Western blots against full-length and truncated forms of dCREB-A protein expressed in bacteria, the antisera detects a 62 kD protein that comigrates with the recombinant protein. The antisera did not react with mammalian CREB. The preimmune serum did not react with bacterially expressed dCREB-A or mammalian CREB (data not shown). The antibody is specific for the dCREB-A gene product because mutants for dCREB-A do not stain with the anti-dCREB-
A antibody (Figure 5). The protein detected with this antiserum has a spatial and temporal expression pattern that is virtually identical to the one described for dCREB-A transcripts. The increased sensitivity of antibody staining defined dCREB-A expression patterns in addition to those detected by RNA probes (SMOLIK et al.). The dCREB-A protein is first seen in the salivary gland placodes of stage 11 embryos and continues to be expressed in the salivary gland nuclei throughout embryogenesis and in all larval stages (Figure 3, B–D and F). Anti-dCREB-A antibody staining is also seen in the nuclei of the proventriculus that forms from the anterior-most region of the midgut in stage 16 embryos (Figure 3D), the amnioserosa of stage 11 embryos (Figure 3, A and B) and the stomadeum. During germband retraction, the staining in the amnioserosa disappears. At stage 13, before dorsal closure is completed, dCREB-A protein is detected at high levels in the epithelial cell nuclei of the segmental boundaries and in two nuclei of each segment so that lateral stripes of staining are seen on each side of the embryo (Figure 3E). These lateral two nuclei may, in fact, be tracheal cell nuclei. Although they are seen at the surface with Nomarski optics, the staining is strong enough to be seen from
The 96G14 lane is a dCREl3-A protein truncated by 137 amino acid residues (calculated Mr of 45,000) with antidCREl3-A antibodies. Two microliters of heat-treated contaminants (arrows) provide an internal measure of the full-length dCREl3-A protein. The three bacterial extracts were loaded in each lane. The four bacterial A42 lane is the bZIP domain of dCREl3-A fused to glutathione and the LysE is a bacterial extract made in a LysE host. The lower molecular weight moieties expressed from the pETll promoter and all proteins are controlled by the pET11 promoter and all proteins are made in a LysE host. The lower molecular weight moieties observed in the first three lanes, but not in the control lanes, probably correspond to breakdown products generated in the heat treatment.

Immunostaining of whole mount ovaries demonstrates that the RNA and protein expression patterns are superimposable. dCREB-A is initially expressed in stage 9 follicle cell nuclei as they migrate posteriorly toward and surround the oocyte (Figure 4A). In stages 10A and 10B, dCREB-A is expressed uniformly in the nuclei of the columnar follicle cells surrounding the oocyte (Figure 4, B and C). This expression pattern lasts until stage 11 when only a few nuclei expressing the dCREB-A protein can be seen over the reduced nurse cell chamber. By the onset of stage 12, dCREB-A protein is no longer detected. The dCREB-A protein is also seen in the genital-disc derived, spermathecal cell nuclei (Figure 4D).

**Functional role of dCREB-A in embryogenesis:** While dCREB-A can activate transcription in cell culture, a null mutation in the gene is necessary to determine if this activity is required for the function and/or development of the tissues in which it is expressed. Because the dCREB-A protein may be part of a family of bZIP transcription factors, it is possible that its function is entirely redundant and a null mutation would have no consequence. Thus, a phenotype associated with deletions of the dCREB-A gene would suggest that at least some of its function is unique. To determine the function of dCREB-A, we analyzed null mutations in the gene. To generate these mutations, we mobilized two P-element insertions in the dCREB-A gene, B204 and l(3)3576, by γ-rays and by exposing the insertions to the Δ2-3(99B) transposase (Robertson et al. 1988). The B204 insertion is viable when homozygous. The lethality associated with the l(3)3576 chromosome is due to a lesion in the 71C/D region because animals heterozygous for l(3)3576 and either Df(3L)BK10 or Df(3L)878.3 (two deletions between 71C1,2 and D/E) are lethal and those heterozygous for l(3)3576 and Df(3L)BrdR15 (a deletion between 71A and C1,2) are viable. Although the two P elements did not define a previously known genetic locus, we determined that they were likely alleles of the dCREB-A gene because their patterns of β-galactosidase expression mimicked that of dCREB-A and each showed a predictable change in the banding pattern of the DNA in Southern hybridization analyses (Figure 7). Furthermore, l(3)3576/Df(3L)BK10 embryos show no immunohistochemical staining with the anti-dCREB-A antibody. The sites of insertion for the B204 and l(3)3576 P elements are shown in Figure 1, and the β-galactosidase expression patterns associated with these insertions are illustrated in Figure 6. The B204 insertion expresses β-galactosidase in the embryonic salivary glands, proventriculus and in the region surrounding the posterior spiracles; however there is no expression in larval structures or the adult ovarian tissue. The l(3)3576 insertion chromosomal expression β-galactosidase in the embryonic salivary gland nuclei but not the proventriculus, in the salivary gland of third instar larvae and in the nuclei of the columnar follicle cells of stage 9 and 10 egg chambers and in the spermathecal cell nuclei.

From the γ-ray mutagenesis we generated five large deletions that included the 71C/D region and one cytologically normal mutation (Figure 1). The l(3)3576 cytologically normal mutation, rR3-4, lost the γ+ function but retained the β-galactosidase activity. The rR3-4 allele is lethal when homozygous and when heterozygous for the deficiencies Df(3L)BK10 and Df(3L)878.3. Mobilization of the l(3)3576 insertion with the Δ2-3(99B) transposase generated three independent deletion mutations, rR7, rR9 and rR5, that are lethal when homozygous or heterozygous for Df(3L)BK10 or Df(3L)878.3 and at least four independent excision events that reverted the lethal phenotype to viability. Four lethal dele-
tions, wR11, wR23, wR83 and wR84, and 10 viable excision events were generated in a similar mutagenesis using the B204 insertion. Southern hybridization analysis of the insertion and deletion alleles shows that for three of the lethal B204 mutations, the P-element and flanking sequences are removed while the four viable B204 excision events analyzed either restore the wild-type restriction fragment pattern or delete ~100 bp flanking the B204 insertion site. One of the lethal alleles, wR83, retains some of the insertion sequences. The Southern analysis of three viable alleles and the four lethal alleles is shown in Figure 6. The B204 insertion is 3 kb from the dCREB-A transcriptional start sites, and the deletions, ranging from 100 to 200 bp, do not include coding sequences. For each of the lethal l(3)3576 alleles, at least some of the insertion DNA remains in place (Figures 1 and 7). The l(3)3576 insertion is located ~2.5 kb 5’ to the second exon and appears to act as a transcription disruption mutation. All of the lethal excision events retain some of the P-element sequences while two of the four viable excision events delete the P element and adjacent sequences and two

restore the wild-type restriction fragment pattern. The Southern analysis of three lethal alleles and one of the viable alleles is shown in Figure 7. The l(3)3576 insertion is under the control of the adult enhancers because it has the embryonic salivary gland and adult expression patterns of dCREB-A. None of the lethal B204 and l(3)3576 deletion alleles complement each other and therefore they define a single complementation group. They are lethal when heterozygous with the deficiencies Df(3L)BK10 and Df(3L)878.3 and viable when heterozygous with Df(3L)BrdR15. Homozygous mutant embryos or embryos heterozygous for each of the lethal alleles and Df(3L)BK10 do not express dCREB-A protein detectable with the anti-dCREB-A antibody (Figure 5).

The dCREB-A null mutants die late in embryogenesis just before or at hatching and have no obvious developmental defects in the organs in which it is expressed (Figure 5). The role of dCREB-A in epidermal and tracheal development will be described elsewhere (Andrew et al. 1997).

Rescue of the embryonic lethality by germ line transformation with the dCREB-A gene: To ensure that the

FIGURE 3.—The embryonic expression pattern of dCREB-A. (A) Early stage 11 embryo showing dCREB-A expression in the flattened cells of the amnioserosa (as). (B) Late stage 11 embryo showing dCREB-A expression in the salivary gland placodes (s) and amnioserosa. (C) Stage 13 embryo showing dCREB-A expression in the invaginating salivary gland. (D) Stage 16 embryo showing dCREB-A expression in the salivary gland and newly formed proventriculus (p). (E) A higher magnification of the embryo in C showing the expression of dCREB-A in the epidermal nuclei outlining the segments (the two obliquely pointing arrows to the right) and defining a lateral stripe of segmental or tracheal cell nuclei (the four arrows pointing up and down on the left). (F) dCREB-A expression in the nuclei of third instar larval salivary gland. In all cases the embryos are oriented so that anterior is left. In A, C and E ventral is down. In B ventral is facing and in D, dorsal is facing.
Figure 4.—The ovarian expression pattern of dCREB-A. (A) Stage 9 egg chamber showing dCREB-A expression in the follicle cell nuclei migrating over the oocyte (arrows). (B) Stage 10A egg chamber showing dCREB-A expression in the follicle cells as they form a columnar epithelium over the oocyte. (C) Stage 10A and 10B egg chambers. (D) dCREB-A expression in the polyploid nuclei of the spermathecae (arrows). This organ is derived from the genital disc.

B204 and l(3)3576 deletion alleles were uniquely dCREB-A mutations, we rescued the embryonic lethality with the dCREB-A cDNA. Because the dCREB-A gene, including the 5' embryonic enhancer, is distributed along >21 kb of DNA we cloned the dCREB-A cDNA into the GAL4-UAS transformation vector, pUAST (MATERIALS AND METHODS). This construct was injected into Drosophila embryos homozygous for the Δ2-3 chromosome and the transformants generated were kept in stock (MATERIALS AND METHODS). When activated by a heat-shock regulated GAL4 gene, two of the independent transformed lines, Tr2 and Tr11, rescued the embryonic lethality of the rR3-4/Df(3R)BK1O and rR7/ Df(3L)BK1O heterozygotes. No animals with the rescued phenotype were detected in the controls that were not exposed to heat shock.

The heat shock regimen used was critical to the rescue. A number of heat shock regimes were used to rescue the dCREB-A null mutations and most of them did not allow survival. For example, if the animals were incubated for 30 min or 1 hr at 37°C each day of the developmental cycle, none of the hs-GAL4/Tr2 or hs-GAL4/Tr11 animals survived, even if they carried a wild-type allele of the dCREB-A gene. Only one scheme allowed partial rescue. A single 1-hr incubation of 0–16 hr embryos at 37°C allowed 15% of the expected rescued adults to eclose. These results suggested that the indiscriminate expression of dCREB-A in different tissues at different times could be disruptive to the normal developmental process. To assess this possibility further, we expressed the two UAS-dCREB-A insertions in a variety of GAL4 enhancer-trap lines and also asked if any specific GAL4 line would allow rescue of the dCREB-A mutant. None of the nine GAL4 insertions on the second and X chromosomes tested was able to rescue the rR3-4/Df(3R)BK1O mutant phenotype. The overexpression of dCREB-A in six of the lines (128, 323, 35, 113, 202 and 32B) tested was lethal early in development.

One possible explanation for the late embryonic lethality was that the salivary gland was unable to secrete factors necessary for hatching. To test this possibility we asked if dCREB-A expression in the salivary gland could rescue the lethal phenotype of the dCREB-A mutant. The GAL4 323 line expresses GAL4 in the embryonic salivary glands, the oenocytes of pharate adults, adult thoracic and abdominal muscles and a small cluster of cells in the adult brain. When this GAL4 line is used to drive the expression of the UAS-dCREB-A insertions, it cannot rescue the embryonic lethality of rR3-4/Df(3R)BK1O heterozygotes.

DISCUSSION

We have previously described the cloning and molecular characterization of two CRE-binding protein genes in flies, dCREB-A (SMOLIK et al. 1992) and dCREB-B (USUI et al. 1992). Both of these proteins are structurally similar to mammalian CREB protein and have an N terminal activation domain, basic DNA binding domain and a C terminal leucine zipper dimerization domain. The dCREB-B protein is more similar to the mammalian CREB than many members of the mammalian CREB family of transcription factors and the different isoforms can either activate or suppress transcription (USUI et al. 1992; YIN et al. 1995). However, no true nulls of this gene have yet been identified.

While the dCREB-A protein binds to the CRE specifically as evidenced by the fact that in gel shift experi-
start site suggesting that dCREB-A may autoregulate. However, a DNA fragment containing the presumptive 5’UT sequences, the CRE and sequences up to but not including the B204 insertion site is unable to drive β-galactosidase expression in transformed Drosophila lines (S. M. Smolik, unpublished observation). This result suggests that if the CRE is necessary for the regulation of dCREB-A transcription, it is not sufficient.

The embryonic enhancer appears to be ~3 kb upstream from the seven start sites. The β-galactosidase expression from the B204 insertion is seen only in embryonic salivary gland and proventriculus. β-galactosidase expression is not detected in the salivary glands of the third instar larvae nor is it detected in any adult structures. Embryonic, larval and adult enhancers control the β-galactosidase expression from the l(3)3576 insertion. This insertion lies within the 10 kb intron, ~2.5 kb 5’ to the second exon. This result suggests that the larval and adult enhancers may lie within the 10 kb intron. While it is possible that a second promoter regulates a second larval and adult dCREB-A transcript, this is unlikely because a Northern analysis of adult and larval transcripts identifies a single 4.47-kb transcript and not the 3.4-kb transcript predicted by a second promoter (Smolik et al. 1992).

The dCREB-A protein is first detected in the salivary gland placodes and amnioserosa of stage 11 embryos. The amnioserosa differentiates from a middorsal strip of cells during stage 8. During germ band extension, these postmitotic cells undergo a complex series of shape changes and become a deeply folded sheet that dips down into the proctodeal cavity at the end of the extended germ band. The dCREB-A protein is not detected in these cells until stage 11 when the amnioserosa is fully differentiated and its expression disappears during germ band shortening when the amnioserosa expands to cover the dorsal side of the embryo. In dCREB-A null mutants the development of the amnioserosa proceeds normally although function of the membrane may be perturbed in ways that are not easily detected.

The large, basophilic cells of the salivary gland placodes arise from precursor cells in parasegment 2 (PS2) (Panzner et al. 1992). By stage 11, the salivary gland premordia are clearly different from the surrounding cells and have completed most, if not all, mitotic cell divisions (Campos-Ortega and Hartenstein 1985). The dCREB-A protein is detected in the cytoplasm of the placode cells and does not appear to be localized to the nuclei. However, after invagination begins and throughout subsequent gland development, dCREB-A protein is seen only in the nucleus. A cascade of genetic interactions regulates the development of the salivary gland. Positive regulation by the homeotic gene Sex combs reduced (Scr) and negative regulation by genes that determine dorsoventral patterning such as decapentaplegic (dpp), dorsal (dl) and spitz, specify the location of the salivary gland placode to PS2 (Panzner et al. 1992).
The positional information established by these interactions activates the genes that differentiate the placode cells and stimulates the expression of genes such as *fork head (fkh)* that mediate the morphogenesis and growth of the salivary glands (PANZER et al. 1992). The *fkh* gene encodes a protein with homology to the mammalian hepatocyte nuclear factor, HNF-3, (WEIGLE and JACKLE 1990) and probably regulates the expression of the genes that elaborate salivary gland-specific characteristics. It is not required for the determination of the salivary glands because the salivary gland placodes form normally in fkh loss-of-function mutant embryos. However, this gene is important in the morphogenesis and maintenance of gland identity because in the fkh null mutants the placodes fail to invaginate and ultimately degenerate (JÜRGENS and WEIGLE 1988; WEIGLE et al. 1989). A gene homologous to the hepatocyte nuclear factor HNF-4 has also been identified in Drosophila (ZONG et al. 1993). While no null alleles of this gene have been described, embryos homozygous for a deletion that includes the HNF-4 gene develop salivary gland placodes that fail to invaginate and are reduced in size. The genes like *fkh* and the HNF-4 homologue control the expression of the genes that direct later steps in gland development and mediate the function of the differentiated cell state. Although fkh mutants express *dCREB-A* (S. BECKENDORF, personal communication), the phenotype of the *dCREB-A* mutants suggests that it may be a target gene for regulators like *fkh* and the HNF-4 homologue. It may also be regulated directly by *Scr* since ectopic expression of *Scr* results in ectopic expression of *dCREB-A* (ANDREW et al. 1994). Furthermore, *dCREB-A* expression in the salivary gland is not affected by any salivary gland gene so far examined (D.J. ANDREW, A. BAIG, P. BHANOT, S. M. SMOLIK and K. HENDERSON, unpublished data). In the *dCREB-A* null mutants, the placodes appear to develop and invaginate properly so that virtually wild-type salivary glands are seen in the late stage embryo. The one anomaly detected is a twisted lumen (ANDREW et al. 1997). This twisting may indicate a defect in the terminal steps of organogenesis or it may reflect the inability of *dCREB-A*-deficient glands to produce and secrete their products. In any event, it is clear that *dCREB-A* is not needed to establish or maintain the identity of the salivary gland tissue and may only affect terminal differentiation events.

It has been proposed that the salivary glands produce the enzymes necessary for the first larval instar to hatch from the egg (BERENDES and ASHBURNER 1978) and *dCREB-A* may regulate the transcription of these enzymes. However, if this is the case, then the production of the enzymes, while it may be necessary, is not sufficient to rescue the *dCREB-A* lethality. Embryos deficient for *dCREB-A* do not survive when the *dCREB-A* protein is expressed solely in the salivary gland.

Late in embryogenesis, at stage 16–17, the anterior-most epidermal cells of the midgut migrate toward and overgrow the tip of the esophagus to form the proventriculus. The esophageal cells derived from the foregut form the interior of this specialized structure and do not stain with *dCREB-A* antibody. Only the midgut-derived cells, known to secrete the peritropic membrane, express *dCREB-A* protein. Because the *dCREB-A* mutants develop a morphologically wild-type proventriculus, it is likely that *dCREB-A* is not involved in the differentiation of the structure but is required to carry out the terminal functions of the epithelial cells.
In stage 13 embryos, at the end of germ band shortening and before dorsal closure, strong dCREB-A expression is detected in the nuclei of specific epidermal cells in each segment and perhaps two laterally positioned tracheal cell nuclei. The phenotypic consequences of dCREB-A loss of function in these cells is discussed elsewhere (Andrew et al. 1997).

In the adult female, dCREB-A is activated in the follicle cells of stage 9 egg cases as they begin to migrate over the growing oocyte. There is no expression in the nonmigrating follicle cells that remain to cover the nurse cells. It is during this time that vitellogenesis occurs, the vitelline membrane is deposited and the anterior-posterior and dorsoventral polarities are established in the growing oocyte. While the anterior-posterior axis is generated by the graded deposition of maternal products transported by the nurse cells to the oocyte, the dorsoventral pattern is generated by the combined action of the somatic follicle cells and germ-line products. It is tempting to speculate that the ubiquitously expressed dCREB-A factor can be activated by spatially restricted signal transduction systems to mediate the role of the follicle cells in the establishment of dorsoventral polarity in the egg.

We have demonstrated that dCREB-A is required for the viability of late stage embryos, presumably through the regulation of genes needed for development. It is expressed in a variety of physiologically distinct, differentiated tissues that have the ability to secrete cuticle in common. Thus, it is likely that the lethality of dCREB-A mutations arises from defects in the terminal differentiation steps of these tissues or their inability to make and secrete substances necessary for the development of the embryo. Cellular functions are sensitive to the expression of the dCREB-A gene because the ubiquitous expression of the protein during embryogenesis is lethal in otherwise wild-type flies and the ectopic expression of dCREB-A in discs causes dominant disc defects (S. M. Smolik, unpublished observation).

Most members of the mammalian CREB family, as well as the Drosophila dCREB-B protein, are ubiquitously expressed. A variety of protein kinases, activated in response to different signal cascades, can stimulate the activity of the mammalian CREB proteins. The specificity of CREB responses in different tissues may result from interactions with spatially restricted bZIP partners or the differential activation of the signaling cascades. The similarity in expression pattern between the mammalian CREB and the fly dCREB-B proteins may reflect a conserved function for these factors in highly diver-
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gent organisms. Unlike these proteins, dCREB-A has a tissue-specific expression pattern. Perhaps dCREB-A modulates a tissue-specific response to a given signal transduction pathway by interacting with a more ubiquitously expressed protein in this set of tissues. In addition, the dCREB-A protein structure is quite different from the mammalian CREB and Drosophila dCREB-B proteins. This observation implies that there may be a mammalian homologue to dCREB-A that has a longer hydrophobic dimerization domain and a more restricted pattern of expression. Further genetic and molecular studies of this gene and the proteins with which it interacts will allow us to determine how a single factor, expressed in functionally distinct tissues, is used to activate different sets of genes either in response to the same signal transduction pathway or to different pathways.

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LITERATURE CITED


