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A CREB-binding site as a target for *decapentaplegic* signalling during *Drosophila* endoderm induction

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Decapentaplegic (Dpp) is an extracellular signal of the transforming growth factor-β family with multiple functions during *Drosophila* development. For example, it plays a key role in the embryo during endoderm induction. During this process, Dpp stimulates transcription of the homeotic genes *Ultrabithorax* in the visceral mesoderm and *labial* in the subjacent endoderm. Here, we show that a cAMP response element (CRE) from an *Ultrabithorax* enhancer mediates Dpp-responsive transcription in the embryonic midgut, and that endoderm expression from a *labial* enhancer depends on multiple CREs. Furthermore, the *Drosophila* CRE-binding protein dCREB-B binds to the *Ultrabithorax* CRE, and ubiquitous expression of a dominant-negative form of dCREB-B suppresses CRE-mediated reporter gene expression and reduces *labial* expression in the endoderm. Therefore, a CREB protein may act as a nuclear target, or as a partner of a nuclear target, for Dpp signalling in the embryonic midgut.
Keywords: CREB/Dpp signalling pathway/*Drosophila*/ midgut/transcription

Introduction

During animal development, cells often instruct each other by secreting signals. Transforming growth factor-β (TGF-β)-like growth factors such as activins and *Drosophila* Decapentaplegic (Dpp) are among the best studied extracellular signals that control development (Padgett *et al.*, 1987; reviewed by Jessel and Melton, 1992; Smith, 1994; Massagué, 1996). These signals act in many developmental contexts, e.g. they organize the embryonic dorsoventral pattern (Irish and Gelbart, 1987; Ferguson and Anderson, 1992) and patterning of adult appendages in flies (Zecca *et al.*, 1995; Lecuit *et al.*, 1996; Nellen *et al.*, 1996), and they function during mesoderm and endoderm induction in frogs and flies (Green and Smith, 1990; Smith *et al.*, 1990; Hemmati-Brivanlou and Melton, 1992; Bienz, 1994; Staehling-Hampton *et al.*, 1994; Frasch, 1995). In some of these events, the TGF-β-like signals have morphogenetic properties: they act at long range, and distinct and sharp cellular responses are elicited by multiple signalling thresholds (Green and Smith, 1990; Ferguson and Anderson, 1992; Green *et al.*, 1992; Gurdon *et al.*, 1994; Lecuit *et al.*, 1996; Nellen *et al.*, 1996; reviewed by Lawrence and Struhl, 1996). Ultimate decoding of these thresholds is likely to be achieved by transcription factors controlling expression of the signal target genes. None of these signal response factors has been identified as yet.

In the *Drosophila* embryo, *dpp* plays a key role during endoderm induction (reviewed by Bienz, 1994; Figure 1). Dpp is secreted from a localized source in the visceral mesoderm (VM) to stimulate transcription of the homeotic gene *labial* (*lab*) in the subjacent endoderm (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990; Neufeld *et al.*, 1996). As a result, different cell types of the larval gut are specified (Hoppler and Bienz, 1995), for example the copper cells whose development depends on *lab* (Hoppler and Bienz, 1994). However, Dpp also signals within the VM where it stimulates expression of three different genes (Figure 1): its own (Hursh *et al.*, 1993; Staehling-Hampton and Hoffmann, 1994; Yu *et al.*, 1996), expression of Wingless (Wg), an extracellular signal expressed in adjacent VM cells (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990), and of *Ultrabithorax* (*Ubx*), the homeotic gene expressed in the same VM cells as *dpp* (Panganiban *et al.*, 1990; Hursh *et al.*, 1993; Thüringer and Bienz, 1993). In turn, *dpp* expression is stimulated directly by Ubx (Sun *et al.*, 1995), and is also stimulated by *wg* (Yu *et al.*, 1996) which feeds back positively on *Ubx* expression as well (Thüringer and Bienz, 1993). Thus, *dpp* is part of an indirect autoregulatory loop by which *Ubx*, at the top of the inductive cascade, maintains its own expression (Thüringer and Bienz, 1993). Similar indirect autoregulatory feedback loops of cell fate-determining genes have been observed in vertebrate development, e.g. in the chick limb bud (Niswander *et al.*, 1994) and in the *Xenopus* embryo (Tada *et al.*, submitted). They may be designed to stabilize developmental decisions in groups of cells (Bienz, 1994).

Previously, we have characterized short enhancer fragments from *lab* and *Ubx* which confer the response to *dpp* signalling in the endoderm and in the VM, respectively (Tremml and Bienz, 1992; Thüringer *et al.*, 1993). Here, we identify the DNA target sequence in these enhancers which is necessary and to some extent sufficient for this response. In both cases, this sequence closely resembles the binding site for CREB (cAMP response element-binding protein, see below), and we present evidence to suggest that a *Drosophila* CREB protein may be a target transcription factor, or a dimerization partner of such a factor, for *dpp* signalling in the embryonic midgut.

Results

The Dpp response sequence in the Ubx midgut enhancer is a CRE

We previously have characterized a short *Ubx* enhancer, called B, which confers Wg- and Dpp-dependent

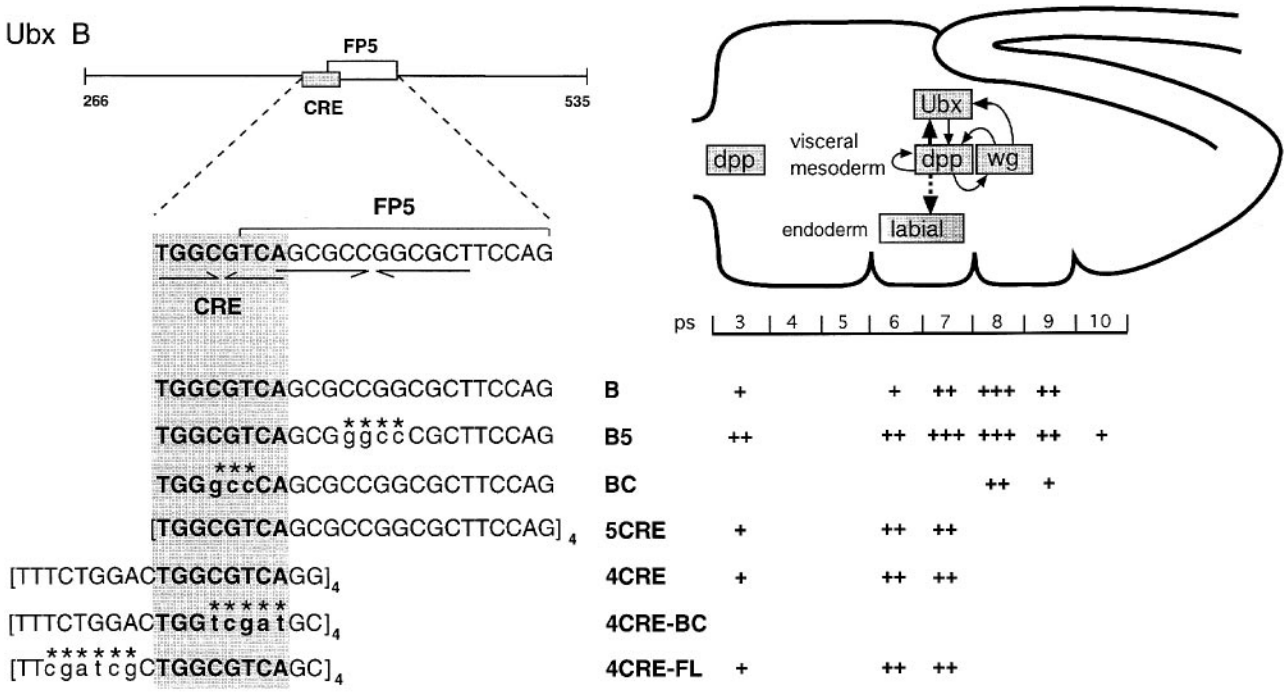

Fig. 1. The VM enhancer from *Ubx* and its mutants. Top left: outline of the CRE/FP5 region within the B enhancer from *Ubx* (numbers refer to residues from the*Eco*RI site at –3.1 upstream of the *Ubx* transcription start; Saari and Bienz, 1987). Underneath: sequence of CRE/FP5 (bold, CRE; the bracket indicates residues protected in footprint assays, see Materials and methods; palindromes within CRE and FP5 are marked by arrows; note the additional C residue within FP5 not present in the original sequence) in the wild-type B, in B5 and in BC (mutated residues in lower case letters, marked by asterisks; substitutions do not affect the palindromes), as well as oligomer sequences of 5CRE, 4CRE, 4CRE-BC and 4CRE-FL. Top right: schematic drawing of the embryonic midgut, with expression domains of *Ubx*, *dpp* and *wg* in the VM, and of *lab* in the endoderm, and the regulatory interactions between these genes [see text; the control of *lab* by *wg* (Hoppler and Bienz, 1995) is omitted from the diagram]. Underneath: expression mediated by wild-type and mutant constructs in individual midgut ps (aligned with midgut drawing); +/++/+++, levels of expression as estimated (expression due to B, B5 and BC is restricted to the VM, that due to 5CRE, 4CRE and CRE-FL is mostly in the endoderm). Note that B5 mediates stronger and wider, BC weaker and narrower expression than B, implying that CRE activates, whereas FP5 represses transcription.

β-galactosidase (*lacZ*) reporter gene expression in the VM. Staining mediated by *Ubx* B is in two stripes of cells in the VM, a wide prominent one in parasegments (ps) 6–9 and a narrow weak one in ps3 (Figures 1 and 2a; see also Thüringer *et al.*, 1993). Our previous dissection of *Ubx* B led us to conclude that the target sequences for Dpp and Wg signalling within this enhancer are separable (Thüringer *et al.*, 1993). To identify these signal target sequences, we carried out a footprint analysis of this *Ubx* enhancer, using crude nuclear protein extracts. We thus found eight distinct sequences to be protected by these extracts (to be described elsewhere in more detail; see also Figure 4a). We noticed that footprint 5 (FP5) partly overlaps a near palindromic sequence TGGCGTCA which closely resembles a typical cAMP response element (CRE) (TGACGTCA; Montminy *et al.*, 1986) (Figures 1 and 4a). To test the function of this sequence, and of the adjacent sequence covered by FP5, we introduced a 3 bp substitution into the former (mutant construct BC) and a 4 bp substitution into the latter (mutant construct B5). We then examined the *lacZ* expression patterns mediated by these mutant enhancers in stably transformed embryos and compared them with that mediated by *Ubx* B.

In the case of B5, the two stripes of *lacZ* expression are widened significantly and stain more strongly than those conferred by the wild-type B enhancer (Figures 1 and 2d). Conversely, in BC transformants, the wide stripe is narrowed to ps8/9 and stains only weakly, and the narrow stripe in ps3 is hardly detectable (Figures 1 and 2g). Thus, the sequence motif TGGCGTCA functions in the embryo to mediate transcriptional stimulation, whereas the adjacent FP5 sequence mediates transcriptional repression. We shall refer to the putative proteins which act positively or negatively through this region of the B enhancer as the CRE activator or the FP5 repressor, respectively.

dpp and *wg* synergize to stimulate *Ubx* expression in the VM (Thüringer *et al.*, 1993). We note that the loss of expression due to the BC mutation coincides with the two main sources of *dpp* expression (in ps7 and 3; cf. St Johnston and Gelbart, 1987; Bienz, 1994; Figure 1). Moreover, the residual BC expression in ps8/9 coincides with the main source of *wg* expression in the middle midgut (in ps8; van den Heuvel *et al.*, 1989; Figure 1). This suggests that BC still responds to Wg, but no longer to Dpp signalling. We tested this by monitoring the response of B, B5 and BC to ectopic expression of Dpp or Wg. In the case of B, ectopic Dpp or Wg each produces a slight widening of the *lacZ* stripes and an increase of their staining intensity; however, *lacZ* expression is still undetectable in certain midgut regions (e.g. in ps10/11; Figure 2b and c; Thüringer *et al.*, 1993). In the case of B5, lacZ staining is strongly increased under both conditions, and staining induced by either signal extends throughout the midgut VM (Figure 2e and f). In contrast, in the case of BC, there is some additional lacZ staining in response to ectopic Wg (Figure 2h), but there is no significant change of the normal BC pattern in response

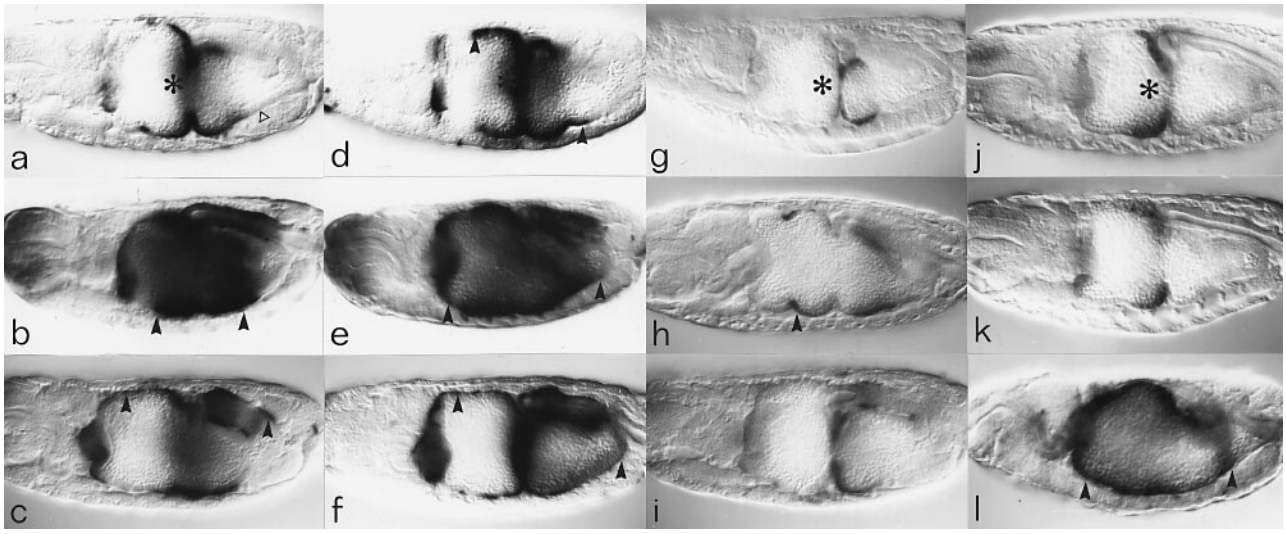

Fig. 2. The signal response in the midgut of wild-type and mutant *Ubx* constructs. Lateral views of 13–16-h-old embryos, transformed with B (**a**–**c**), B5 (**d**–**f**), BC (**g**–**i**) or 5CRE (**j**–**l**), and stained with lacZ antibody; top row, wild-type; middle row, ectopic Wg; bottom row, ectopic Dpp (see Materials and methods); heads to the left, dorsal up. Asterisks indicate the anteroposterior position of the Dpp source in VM ps7; the Wg source is posteriorly adjacent, in VM ps8; between the two signal sources is the middle midgut constriction (cf. Figure 1). Increased or ectopic lacZ staining in response to ectopic Dpp or Wg is indicated by arrowheads. Midgut staining mediated by the wild-type B enhancer (a) reflects *dpp*- and *wg*-mediated expression; lacZ staining due to this enhancer and its mutant versions is seen exclusively in the VM (a–i), whereas 5CRE-mediated lacZ staining is mostly endodermal (j–l). Note the staining abutting the Dpp source due to 5CRE (j) and its extensive response to ectopic Dpp (l), the reduced staining near the Dpp source due to BC (g) and its lack of a response to ectopic Dpp (i). Compare also the lack of lacZ staining in ps10/11 [open triangles in (a)] and gain of staining in this region (righthand arrowhead in d), revealing the function of FP5 in antagonizing the signal response of B (see text).

to ubiquitous Dpp (Figure 2i). As expected, B5-mediated lacZ staining, like B-mediated staining (Riese *et al.*, 1997), is substantially reduced in *dpp* mutants, whereas there is little change in the lacZ staining levels due to BC in these mutants (not shown). Most significantly, the BC mutation is the only one of 12 point mutations introduced into B (Riese *et al.*, 1997; J.Riese and S.Eresh, unpublished data) which causes complete loss of responsiveness to Dpp. We therefore conclude that the sequence TGGCGTCA acts as a Dpp response sequence (DRS) in the VM. Conversely, since B5 responds readily to Dpp and Wg, it is unlikely that the FP5 repressor is negatively regulated by either signal. Instead, it appears to be a constitutive repressor which antagonizes the stimulating effects of the two signals.

We asked whether the DRS might be sufficient to respond to Dpp in the midgut. We oligomerized four copies of the CRE flanked by residues from FP5 (5CRE) or from FP4 (4CRE) (Figure 1), and we placed these adjacent to a canonical TATA box. 5CRE (Figure 2j) and 4CRE transformants (not shown) both show conspicuous lacZ stripes in the midgut, in each case a wide and strongly staining one in ps6/7, and a weak narrow stripe in ps3. Each stripe is near a source of Dpp, which implies that 5CRE and 4CRE might respond directly to Dpp signalling. Indeed, while 5CRE expression is not changed in response to ectopic Wg (Figure 2k), this construct responds very clearly to Dpp in that lacZ staining is stronger and expanded through most of the midgut as a result of ectopic Dpp (Figure 2l). 4CRE also responds to ectopic Dpp, although less extensively than 5CRE. To ascertain that the midgut staining in these constructs is due to the CRE, we made two mutant versions of 4CRE: we introduced base substitutions into each CRE copy within 4CRE (4CRE-BC; Figure 1), or into the 5′ flanking sequences of the CREs (4CRE-FL; Figure 1). As expected, in 4CRE-BC transformants, we no longer observed any *lacZ* expression in the midgut, while the 4CRE-FL transformants showed a midgut expression pattern indistinguishable from that of 4CRE (not shown). Finally, 4CRE-mediated lacZ staining in the midgut is completely abolished in *dpp* mutant embryos (not shown). These results suggest that the DRS may be sufficient to mediate Dpp-responsive expression in the embryonic midgut.

Multiple functional CREs in the lab midgut enhancer

Curiously, lacZ staining mediated by 5CRE and 4CRE is mostly endodermal (Figure 2j), whereby the main stripe in each case roughly coincides with the region in which *lab* expression is induced by Dpp (Figure 1). Indeed, the shortest enhancer fragment from *lab* which confers robust *dpp*-dependent *lacZ* expression in the endoderm (HZ550; Tremml and Bienz, 1992) contains four sequences resembling the CRE consensus sequence TGACGTCA (cf. Materials and methods). Three of these are contained within a minimal 255 bp fragment (HZ255) which mediates a low level of *dpp*-dependent lacZ staining in the endoderm (Tremml and Bienz, 1992). We therefore asked whether these CREs are required for the endodermal response of the *lab* enhancers to Dpp.

We introduced minimal base substitutions into each of the four CREs in HZ550 (mutant construct 550C), or into the three CREs in HZ255 (255C), and we compared the lacZ staining patterns of these with those produced by the corresponding wild-type fragments. We found that, while HZ550 mediates strong lacZ staining in the region of the endoderm in which *lab* is expressed (Figure 3a; Tremml and Bienz, 1992), 550C produces at most residual lacZ staining in some of the cells in this endodermal region

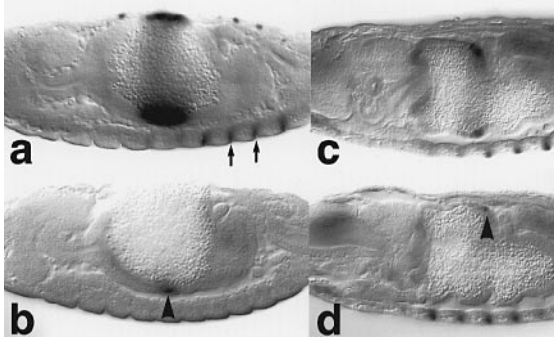

Fig. 3. Endodermal expression from wild-type and mutant *lab* enhancers. Lateral views of ~13- (a and b) or ~15-h-old embryos (c and d), transformed with HZ550 (**a**), 550C (**b**), HZ255 (**c**) or 255C (**d**) and stained with lacZ antibody (orientation as in Figure 2). lacZ staining in the endoderm is drastically reduced due to mutation of the CREs in HZ550 (compare b with a; residual staining indicated by arrowhead), and is also reduced due to mutation of the CREs in HZ255 (compare d with c; an endodermal cell with sporadic lacZ staining is indicated by the arrowhead in d). Note also that the lateral epidermal staining due to HZ550 (within or overlapping posterior compartments; indicated by arrows in b) is abolished in 550C transformants.

(Figure 3b). In two of the five 550C transformant lines, we saw even less endodermal staining (not shown). Also, the thin lacZ stripes in the lateral epidermis (within or overlapping posterior compartments; arrows in Figure 3a) are no longer visible in any of the 550C transformants (Figure 3b). Similarly, while the wild-type HZ255 construct mediates low but reproducible lacZ staining in the endodermal cells in which *lab* induction is maximal (Figure 3c; Tremml and Bienz, 1992), only one of the three 255C transformant lines showed any lacZ staining in the endoderm. This staining was very low and sporadic (Figure 3d; the ectodermal staining due to HZ255, different from that seen with HZ550, does not disappear in the 255C transformants). There was no detectable endodermal lacZ staining in the other two 255C lines (not shown). Thus, the CREs within the *lab* 550 enhancer are critical for this enhancer's activity.

Evidently, the *Ubx* CRE can mediate the response to *dpp* signalling in both cell layers of the embryonic midgut, in the VM and in the endoderm. This implies that other transcription factors act through the *Ubx* B enhancer to confer its tissue-specific response to Dpp in the VM. In our oligo constructs 5CRE and 4CRE, the *Ubx* CRE is detached from its normal enhancer context and thus avoids the constraints imposed by these factors. Supporting this notion, we find that an extended version of 4CRE (L-CRE, including a binding site for lymphocyte enhancer-binding factor 1, or LEF-1) produces Dpp-responsive *lacZ* expression not only in the endoderm, like 5CRE and 4CRE, but also in the VM (Riese *et al.*, 1997). This and additional evidence led us to conclude that the CRE needs to cooperate with the LEF-1-binding site to respond to the Dpp signal in the VM. Why the CRE should be apparently sufficient to respond to Dpp in the endoderm, we do not presently understand.

Binding of dCREB to the DRS

In order to find out which transcriptional activator might act through the DRS to confer the Dpp response, we asked whether any of the putative CRE-binding proteins known in *Drosophila* would bind to the *Ubx* CRE. Candidates for CRE-binding proteins include CREB (Hoeffler *et al.*, 1988) and CREB relatives, e.g. CREM (Foulkes *et al.*, 1991) or ATF protein (Hai *et al.*, 1989). CREB-like proteins belong to the large family of basic region/leucine zipper (bZIP) transcription factors which bind to DNA as dimers (reviewed by Lalli and Sassone-Corsi, 1994). Mammalian CREB-related proteins can also heterodimerize with AP1 proteins (e.g. Hai and Curran, 1991; Masquilier and Sassone-Corsi, 1992; van Dam *et al.*, 1993).

Two genes encoding CREB-like proteins are known in *Drosophila*, *dCREB-A* and *dCREB-B/dCREB-2* (Abel *et al.*, 1992; Smolik *et al.*, 1992; Usui *et al.*, 1993; Yin *et al.*, 1995); *dCREB-2* is closely related to, and may be an ancestral form of, mammalian *CREB* and *CREM* (Yin *et al.*, 1995). Like *CREM*, *dCREB-2* encodes multiple differentially spliced isoforms (Yin *et al.*, 1995) of which dCREB-B is one (called dCREB-2c by Yin *et al.*, 1995; note that all known isoforms of dCREB-2 have the same bZIP domain). *dCREB-B* is expressed uniformly and at moderately high levels throughout the embryonic VM and endoderm (not shown; see Materials and methods, and also Usui *et al.*, 1993), but there does not seem to be any *dCREB-A* expression in the midgut (Smolik *et al.*, 1992; Andrew *et al.*, 1994; our unpublished observations). There are also two *Drosophila* AP1 proteins, D-Jun and D-Fos (Perkins *et al.*, 1990), both of which appear to be expressed throughout the two cell layers of the midgut (Perkins *et al.*, 1990; Tremml, 1991; unpublished observations), but it is not known whether these AP1 proteins can heterodimerize with *Drosophila* CREBs. Interestingly, D-*fos* expression is elevated to high levels in the endoderm in the *lab* expression domain (Perkins *et al.*, 1990), reflecting induction by *dpp* independent of, and in parallel to, *lab* (Tremml, 1991; J.Riese, G.Tremml and M.Bienz, submitted). Based on their expression patterns in the embryonic midgut, we shall consider dCREB-2, D-Jun and D-Fos as candidate proteins which may act through the CRE to mediate the Dpp response.

We first tested whether any of these proteins could bind to the *Ubx* CRE, using bandshift assays. Indeed, recombinant dCREB-B binds to the wild-type *Ubx* CRE sequence (which is identical to CRE2 in the *lab* HZ550 enhancer; see Materials and methods), but not to the mutant sequence BC (Figure 4, lanes 2–6 and 9–13). As expected, the same is true for dCREB-2a (not shown). However, neither recombinant D-Jun nor D-Fos by themselves bind to the CRE (Figure 4, lanes 7 and 8). We also do not see any evidence for binding of either of these in combination with dCREB-B (Figure 4, lanes 10 and 11). However, these binding data do not rule out a low level of binding of a putative heterodimer between dCREB-B and D-Jun or D-Fos: the signal from a putative dCREB-B–D-Jun heterodimer might have been obscured by the signal due to the similarly sized dCREB-B homodimer, and a signal from a putative dCREB-B–D-Fos heterodimer might have been below detection levels because of the low binding activity of our D-Fos extracts (see Materials and methods). As a control, we tested the binding of these proteins to a consensus AP1-binding site. D-Jun clearly binds this site (Figure 4, lane 16), while D-Fos appears

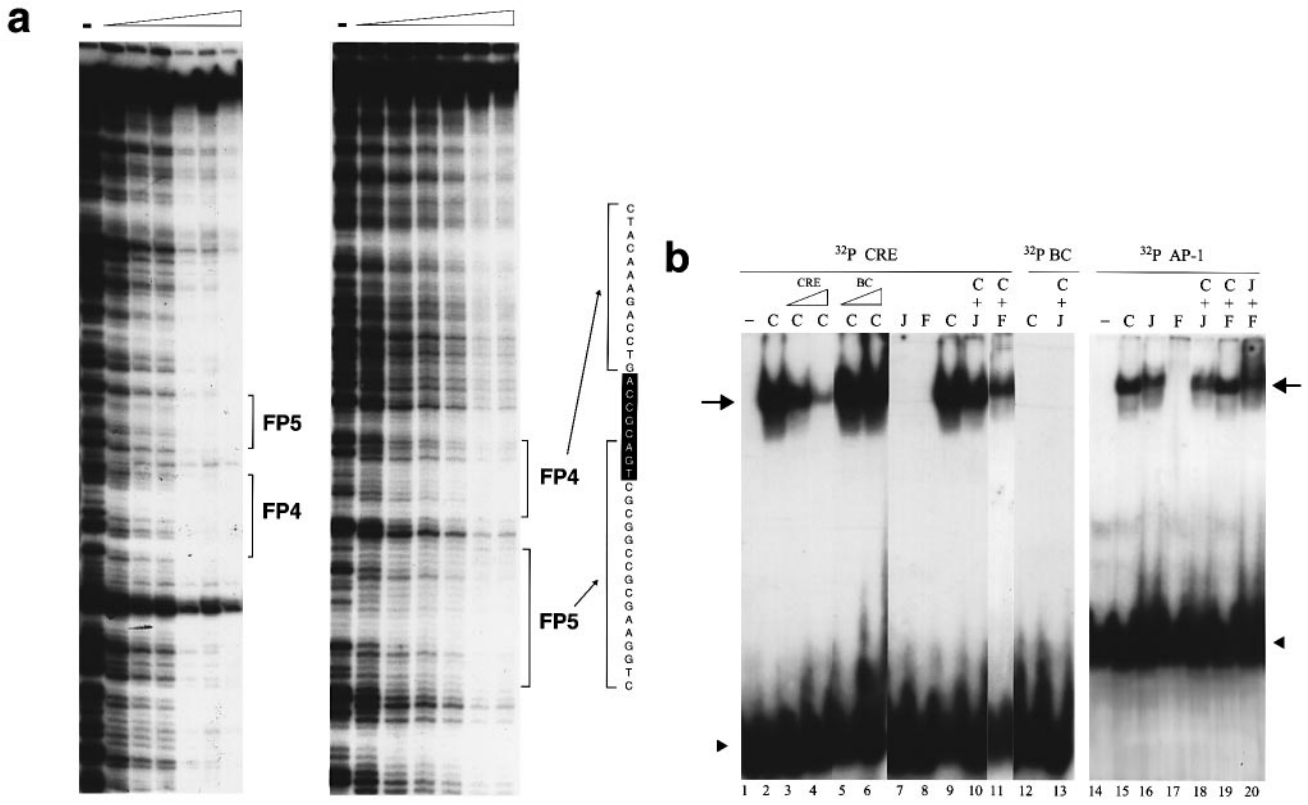

Fig. 4. Footprint analysis of the *Ubx* B enhancer and binding of CREB to the *Ubx* CRE. (**a**) Protection by crude embryonic nuclear extracts of the coding (left) and non-coding strand (right) of the *Ubx* B enhancer fragment (after incubation with increasing amounts of extracts; –, no protein added), with FP5 and the adjacent FP4 sequence on the non-coding strand bracketed (see also Figure 1; on the coding strand, the 3′-most eight residues of FP5 are only weakly protected, whereas six more residues are protected 5′ flanking to the sequence bracketed as FP4 on the right). Note that only the 3′-most three residues of the CRE are protected by the protein extract. FP4 contains a LEF-1-binding site (Riese *et al.*, 1996). (**b**) Bandshift assays, showing complexes (arrows) between radiolabelled wild-type CRE or AP1 oligomers with dCREB-B (C), D-Jun (J) or D-Fos (F); no such complexes are seen if these proteins are incubated with mutant CRE oligomer (BC); arrowheads at the bottom point to free probes. In lanes 3–6, competition for binding was done by adding unlabelled wild-type (CRE) or mutant (BC) oligomer (lanes 3 and 5, 10× molar excess; lanes 4 and 6, 50× molar excess). dCREB-B (lanes 2 and 9) and dCREB-2a (not shown) bind to the CRE, and also to the AP1-binding site (lane 15), but not to the mutated BC sequence (lane 12). Neither D-Jun nor D-Fos bind to the CRE alone (lanes 7 and 8) nor apparently in combination with dCREB-B (lanes 10 and 11) nor with dCREB-2a (not shown). As a control, the binding activity of D-Jun and D-Fos can be seen with a probe encoding an AP1-binding site (lanes 15–20; see also text and Perkins *et al.*, 1990).

to bind to it only in combination with D-Jun (see the additional smeary bands above the main band in Figure 4, lane 20, which we observe reproducibly if recombinant D-Fos is included in the binding reaction; but see also Perkins *et al.*, 1990). dCREB-B also binds to the AP1-binding site (Figure 4, lane 15). These binding data imply that dCREB-2 isoforms are good candidates, whereas D-Jun and D-Fos are poor candidates, for transcriptional activators acting through the *Ubx* CRE.

Dominant-negative effects of a truncated CREB protein in the midgut

In order to test whether dCREB-2 or AP1 proteins can act through the DRS *in vivo*, we generated truncated versions of dCREB-2, D-Jun and D-Fos, consisting in each case of the bZIP fragment (called Cbz, Jbz and Fbz; see Materials and methods). bZIP domains such as these are known to act dominant-negatively as they are able to dimerize and bind DNA without being able to stimulate transcription (Lloyd *et al.*, 1991; Bohmann *et al.*, 1994). We expressed these bZIP fragments ubiquitously in the embryo, using the yeast GAL4 system (Brand and Perrimon, 1993), to see whether any of them would affect reporter gene expression, or *lab* expression itself (we did not expect to see any effect on *Ubx* expression as lack of *dpp* signalling only mildly reduces *Ubx* expression in the VM; Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990).

We found that Cbz, if expressed with a strong hs.GAL4 driver line, virtually eliminated 5CRE expression in the endoderm (Figure 5b, compare with a). This effect was not seen if Cbz expression was limited to the VM, using the mesodermal driver line 24B.GAL4 (not shown), arguing that the effect of Cbz on endodermal 5CRE expression is autonomous and direct. Neither Jbz nor Fbz showed any reduction of 5CRE-mediated lacZ staining in the endoderm (though we did see a slight widening of endodermal 5CRE expression in the case of Jbz; this, however, appears to be caused indirectly as a similar widening is caused non-autonomously by Jbz expression in the VM). This lack of an effect of Jbz and Fbz on CRE-mediated expression is not due to inactivity or instability of these bZIP protein fragments since both bZIP constructs strongly interfere with proper eye development when expressed in the eye imaginal disc (D.B. and D.B.J., unpublished results). More significantly, Fbz interferes

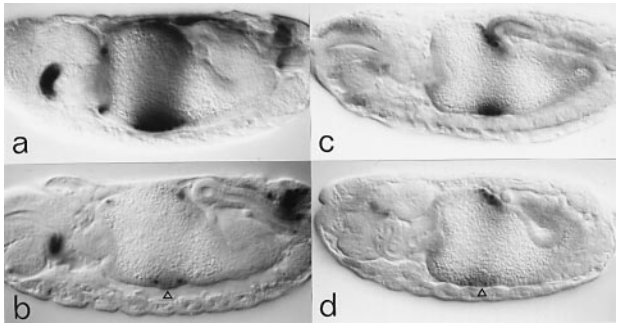

Fig. 5. Dominant-negative effects of Cbz in the midgut. Side views of 13–15-h-old embryos, bearing 5CRE as well as two copies of UAS.Cbz and of hs.GAL4 transposons, heat-shocked as described (see Materials and methods) and stained with lacZ (**a** and **b**) or lab (**c** and **d**) antibody (orientation as in Figure 2). Note the reduction in lacZ or lab staining (triangles in b and d) due to Cbz.

with copper cell development when expressed in the embryonic endoderm (J.Riese, G.Tremml and M.Bienz, submitted; see also below).

We also stained embryos expressing each of these bZIP constructs with lab antibody. We found that, in the case of ubiquitous Cbz, lab staining in the endoderm was significantly reduced, and even absent in some endodermal cells in the ps6/7 region (Figure 5d, compare with c). This reduction of staining was not seen after mesodermal expression of Cbz, or after ubiquitous expression of Jbz. Ubiquitous expression of Fbz caused a reduction of lab antibody staining similar to ubiquitous Cbz expression (not shown). Consistent with this, endodermal expression of Fbz leads to copper cell defects in the larval gut (J.Riese, G.Tremml and M.Bienz, submitted; recall that copper cells require continuous *lab* function in order to develop; Hoppler and Bienz, 1994). Note, however, that the suppressive effect of Fbz on *lab* expression and on copper cell development most probably is not mediated by the *lab* CREs since we cannot detect any effect on 5CRE-mediated lacZ staining under the very same conditions of expressing ubiquitous Fbz (see above; note that reporter gene expression is typically a more sensitive assay than expression of an endogenous gene; e.g. Tremml and Bienz, 1992; Riese *et al.*, 1997; Yu *et al.*, 1996). This result is fully consistent with our failure to detect binding of D-Fos to the *Ubx* CRE. We therefore presume that the suppressive effect of Fbz on *lab* expression is mediated through AP1-binding sites that are located outside the *lab* 550 enhancer (there are no AP1-binding sites in the *lab* 550 enhancer fragment; Tremml, 1991; Tremml and Bienz, 1992).

Taken together, our results strongly indicate that *Drosophila* CREB proteins are capable of activating transcription through the *Ubx* and *lab* CREs in the midgut. Furthermore, although D-Fos may have a function in stimulating *lab* expression in the endoderm, we found no evidence that either of the two AP1 proteins, D-Fos or D-Jun, can act through the *Ubx* CRE, the Dpp response sequence in the midgut.

Discussion

Our work identifies a CRE within the *Ubx* midgut enhancer as a target sequence for Dpp signalling in the embryonic midgut. Two lines of evidence implicate a *Drosophila* CREB protein in the response of midgut cells to Dpp: firstly, *Drosophila* CREB isoforms bind to the *Ubx* CRE and, secondly, expression of the DNA-binding bZIP domain of dCREB-2 in stably transformed embryos acts dominant-negatively to suppress expression from a DRS-containing reporter gene and to reduce *lab* expression. Taking into account their uniform expression in the embryonic midgut, dCREB-2 isoforms, rather than dCREB-A, are good candidates for transcription factors acting through the DRS. Finally, we have shown that the DRS mediates transcriptional activation, and we have not found any evidence for a repressor acting through the *Ubx* CRE. As dCREB-2a is the only dCREB-2 isoform known to be a transcriptional activator (Usui *et al.*, 1993; Yin *et al.*, 1995), dCREB-2a is currently the best candidate for a transcription factor involved in the response to Dpp in the embryonic midgut. Interestingly, dCREB-2a is the only CREB isoform known to be signal responsive (Yin *et al.*, 1995). However, we would like to point out that there may be additional dCREB-2 isoforms and additional CREB-like genes, unidentified as yet, that could be involved in this process.

Recently, we have identified a LEF-1-binding site within the FP4 region of the *Ubx* midgut enhancer as the target sequence for Wg signalling (WRS) in the embryonic midgut (Riese *et al.*, 1997). We have shown that, in contrast to the DRS, the WRS is not sufficient to confer transcriptional stimulation on its own, but that it requires linkage to the DRS. These and additional results led us to propose that a *Drosophila* LEF protein mediates integration of Wg and Dpp signalling. Interestingly, mouse LEF-1 by itself is not a transcriptional activator, but functions in concert with other enhancer-binding proteins one of which is a CREB (Carlsson *et al.*, 1993; Giese and Grosschedl, 1993). This is an additional, and independent, indication that the protein acting through the DRS may be a *Drosophila* CREB protein.

We did not find any evidence that *Drosophila* AP1 proteins could act through the DRS: we failed to detect binding of D-Jun and D-Fos to the *Ubx* CRE *in vitro*, and we also failed to see dominant-negative effects of their bZIP domains on CRE reporter gene expression in the midgut. Interestingly, we did see a suppressive effect of D-Fos bZIP on *lab* expression, indicating a role for D-Fos in the transcriptional regulation of *lab* (J.Riese, G.Tremml and M.Bienz, submitted). However, our evidence does not support the idea that D-Fos takes part in the direct transcriptional response to Dpp signalling; rather, it suggests that D-Fos may act in parallel to Dpp signalling to stimulate *lab* transcription.

Our results raise the possibility that Dpp signalling may modify the activity of a CREB protein, or that of a CREB dimerization partner. Mammalian CREB is known to be phosphorylated, and thus activated, in response to cAMP (Gonzales and Montminy, 1989; Lee *et al.*, 1990). Protein kinase A (PKA) phosphorylates CREB at a critical serine residue (conserved in dCREB-2; Usui *et al.*, 1993; Yin *et al.*, 1995) which facilitates binding of CREB to the CREB-binding protein CBP, a step that is thought to contribute to target gene activation (Chrivia *et al.*, 1993). In the *Drosophila* midgut, we think it unlikely that PKA plays a significant role, as overexpression of a

constitutively activated PKA catalytic subunit (Jiang and Struhl, 1995; Li *et al.*, 1995) affects neither midgut morphology nor expression of *Ubx*, *lab* or their reporter genes (unpublished observations). However, CREB and CREM can also be phosphorylated by other kinases *in vitro* and *in vivo* (de Groot *et al.*, 1993), including a Ras-dependent CREB kinase (Ginty *et al.*, 1994), implying that CREB-like proteins are targeted by signals other than cAMP. Indeed, it has been reported that phosphorylation of CREB transfected into mammalian cells is increased after TGF-β stimulation of these cells (Kramer *et al.*, 1991). However, it remains to be seen whether Dpp signalling directly causes modification of a *Drosophila* CREB protein.

Recently, a gene called *schnurri* (*shn*) has been described which is required downstream of the Dpp signal in multiple developmental contexts including the embryonic midgut (Arora *et al.*, 1995; Grieder *et al.*, 1995). This led to the proposal that the *shn* product, a zinc finger protein, may be a target transcription factor of Dpp signalling (Arora *et al.*, 1995; Grieder *et al.*, 1995). However, preliminary results from *in vitro* DNA binding assays with individual shn zinc fingers suggest that these fingers bind neither to the *Ubx* CRE nor to the FP5 sequence with high affinity (M.Affolter, K.Arora and R.Warrior, personal communication). However, *lacZ* expression mediated by 5CRE is abolished in *shn* mutant embryos even if Dpp is resupplied with a heat-shock promoter (M.Affolter, personal communication). This raises the possibility that the requirement for *shn* in the response to Dpp signalling may be an indirect one.

Finally, what is the role of FP5, the sequence overlapping the CRE? Evidently, this sequence antagonizes the activating effects of Dpp and Wg signalling on the *Ubx* enhancer, and our results argue that the FP5 repressor is constitutively active and not controlled by either signal. The close physical linkage of FP5 and the CRE suggests that there may be competition for transcriptional activation of *Ubx* between the CRE-binding activator and the FP5 repressor at the level of DNA binding. As a consequence, the signal response of *Ubx* would be spatially limited. It is very common that *cis*-regulatory elements controlling the spatial expression of developmental regulators contain arrays of closely linked or overlapping binding sites for transcriptional activators and repressors (e.g. Small *et al.*, 1991). Such arrays constitute transcriptional switches that are eminently sensitive to small changes of repressor and/or activator availability (reviewed by Ptashne, 1986). A switch designed like CRE/FP5 is likely to confer a sharp response to signalling thresholds, and similar switches might account for the strikingly sharp responses to TGF-β-type signalling as observed for *Xenopus* embryonic cells (Green *et al.*, 1992). Thus, such switches would appear to be ideal targets for extracellular signals and morphogens.

Materials and methods

Fly strains

The following fly transformants were used: Bhz (Thüringer *et al.*, 1993); HZ550 and HZ255 (Tremml and Bienz, 1992); hs-wg (Noordermeer *et al.*, 1992); UAS.dpp and 24B.GAL4 (Brand and Perrimon, 1993; Staehling-Hampton *et al.*, 1994); and a strongly expressing hs.GAL4 line (Brand *et al.*, 1994). The dpp^{s4} allele (Immerglück *et al.*, 1990) was used to test *dpp* dependence of reporter gene expression. Mutant embryos were identified by their midgut morphology; note that the dpp^{s4} mutation selectively affects midgut expression of our reporter genes, but not their expression elsewhere, e.g. in the ectoderm (cf. Immerglück *et al.*, 1990).

Plasmids

B5 and BC substitutions (Figure 1) were generated by standard procedures, using mutator oligomers, and mutant constructs were generated analogously as the wild-type construct Bhz (Thüringer *et al.*, 1993). For CRE5, four copies of the 5CRE oligomer sequence (Figure 1; one copy in the 'non-coding' followed by three copies in the 'coding' orientation) separated by TCGA linkers were cloned into the *Sal*I site of Bluescript and subcloned as an *Xba*I–*Xho*I fragment into the transformation vector cut with *Xba*I and *Kpn*I (*Xho*I and *Kpn*I blunt-ended). The same was done for 4CRE, except that the linkers between individual oligomer copies were TTTC (between oligomer 1/2 and 3/4) and TCGACGGTATCGTCGAGGTCGA (between oligomer 2/3); the final orientation in the transformation vector was 'non-coding'. Two distinct mutant versions of 4CRE (4CRE-BC and 4CRE-FL) were generated by using oligomers with base substitutions as shown in Figure 1.

The *lab* HZ550 enhancer fragment contains four CRE-like sequences (Tremml, 1991) which match the CRE consensus sequence in 7/8 (CRE1, CRE2) or 6/8 positions (CRE3, CRE4): TCACGTCA (CRE1), TGGCGTCA (CRE2; same sequence as the *Ubx* CRE; Figure 1), TGTGGTCA (CRE3), GAACGTCA (CRE4). The following base substitutions (indicated by lower case letters) were introduced into these: TagtactA (CRE1), TGctcgag (CRE2), atgcGcaA (CRE3) and GAggGcCc (CRE4). Mutant constructs with these substitutions (255C, CRE2–4 mutated; 550C, each CRE mutated) were generated analogously to the corresponding wild-type constructs HZ550 and HZ255 (Tremml and Bienz, 1992; note that the HZ255 bp construct contains a *Nar*I–*Cla*I fragment which constitutes the 3′ portion of the 550 bp *Cla*I fragment contained in HZ550, instead of a *Bst*XI fragment from the central portion of HZ550 as indicated in Figure 1 of Tremml and Bienz, 1992; sequence available on request).

The Cbz, Jbz and Fbz constructs were generated using standard PCR-based methods. These constructs encompassed amino acids 183–289 of D-Jun, 252–337 of D-Fos (Perkins *et al.*, 1990) and 223–285 of dCREB-B (Usui *et al.*, 1993). A consensus translation initiation sequence (Cavener, 1987) was engineered at the 5′ end of these open reading frames. These constructs subsequently were cloned into the pUAST germline transformation vector (Brand and Perrimon, 1993). Full-length pUAST constructs were also made for dCREB-B (Usui *et al.*, 1993), dCREB-2a (Yin *et al.*, 1995), D-Fos and D-Jun (Perkins *et al.*, 1990; see also Bohmann *et al.*, 1994); details of constructs available on request.

P-element transformation and analysis of transformants

For each construct, 3–5 individual transformant lines were isolated and made homozygous for the transposon; *cn*; ry^{42} was used as a host strain for *lacZ* constructs, *y* w^{1118} for the bZIP constructs. Analysis of *lacZ* expression was done as described (using formaldehyde fixation and a monoclonal mouse antibody against lacZ; Busturia and Bienz, 1993; Thüringer *et al.*, 1993). The rat polyclonal antiserum against lab protein was generated by Tremml (1991). We used the same hs-wg strain and heat-shock procedure as described (Thüringer *et al.*, 1993), but we used the GAL4 system (UAS.dpp and 24B.GAL4, see above) to express *dpp* throughout the mesoderm since this produced a stronger and more reproducible Dpp response than the hs-dpp strain previously used. To produce clear and strong effects with good penetrance in the case of bZIP constructs, both the hs.GAL4 driver and the bZIP-encoding UAS transposon had to be homozygous (although the same effects were also observed with poor penetrance in the presence of just one copy each). The following heat-shock conditions were used: 4–8-h-old embryos were subjected to four consecutive heat shocks at 37°C (20 min each; plates immersed in a waterbath) separated by 2 h at 25°C. UAS constructs expressing the full-length dCREB-B, dCREB-2a, D-Jun and D-Fos proteins produced phenotypic effects in wings (to be described elsewhere); however, none of these produced any effects on reporter gene expression or on midgut morphology when ubiquitously expressed in the embryo as described.

Recombinant dCREB-B (Usui *et al.*, 1993) was purified by standard procedures by virtue of its His-Tag, and injected into rats to produce a polyclonal antiserum. This serum recognizes recombinant dCREB-B and dCREB-2a, but not dCREB-A on Western blots. Embryos stained with this antiserum show moderately high levels of stained nuclear antigen in most if not all embryonic cells, including VM and endodermal cells which show nuclear staining levels uniformly throughout the midgut, confirming earlier studies of dCREB-B transcript expression by Usui

et al. (1993). Uniform expression of D-Jun throughout the VM and endoderm was also observed using a polyclonal rabbit antiserum against D-Jun (Bohmann *et al.*, 1994).

Crude protein extracts and DNA binding assays

For the preparation of crude protein extracts from embryonic nuclei (0–24-h-old embryos) and subsequent DNase I footprinting, the protocols of Biggin and Tjian (1988) were followed. As competitor DNA, 1 μg of poly(dIdC) was added into binding reactions of 50 μl; increasing amounts of protein extract were added into these reaction as follows: 1.7, 3.4, 5.1, 6.8, 8.5 and 10.2 μg (see Figure 4a). To identify the footprint regions (Figures 1 and 4a), the protection patterns from various experiments with different extracts were averaged.

Crude protein extracts containing recombinant dCREB-B (Usui *et al.*, 1993), dCREB-2a (J.Yin, unpublished), D-Jun (Peverali *et al.*, 1996) and D-Fos (F.Peverali and D.Bohmann, unpublished) were prepared essentially as described by Studier *et al.* (1990), with the following modifications. Harvested bacterial cells were resuspended in 100 μl of lysing buffer [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol, 0.2 mg/ml lysozyme in phosphate-buffered saline pH 8.5], incubated for 20 min on ice followed by sonication on ice in 1% Triton X-100 (final concentration). After centrifugation, the supernatant was used as a crude protein extract. These extracts were incubated for 10 min on ice in binding buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.1% NP-40, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, 3 mg/ml bovine serum albumin) in a final volume of 20 μl. After addition of radiolabelled oligomer probe (15 000 c.p.m.), the mix was incubated for a further 20 min on ice. The resulting complexes were separated on 6% native polyacrylamide gels run in 0.5× Tris borate buffer. Oligomer probes were end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and reannealed according to standard procedures. The following oligomer sequences were used: wild-type CRE, GGGCTGGACTGGCGTCAGCGCCGG; BC mutant CRE, GGGCTGG-ACTGGgccCAGCGCCGG (base substitutions in lower case letters); AP1, GAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCAGG.

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