The bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo

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A gradient in concentration of the protein product of the bicoid *gene is a determinant of the anterior–posterior axis of* Drosophila *embryos. By binding upstream of the segmentation gene* hunchback *the bicoid protein controls its transcription, thereby translating maternal pattern-generating information into differential activation of zygotic gene expression.*

THE molecular link between the maternal genome and the activation of zygotic gene expression is realized by maternal factors deposited in the egg during oogenesis. Three groups of maternal genes specify distinct domains along the anteroposterior axis of the *Drosophila melanogaster* embryo[1]. In the anterior group the maternal gene *bicoid* (*bcd*) is crucial for the development of head and thorax[2]. *bcd* mRNA is localized at the anterior pole of the egg and early embryo[3,4] and the bcd protein comes to be distributed in a concentration gradient with a maximum at the anterior tip of the embryo[5]. The bcd protein gradient seems to determine position in the anterior half of the embryo in a concentration-dependent way[6]. The presence of a homoeodomain[7] in the bcd protein suggests that it binds to DNA in a sequence-specific way[8,9] and thereby regulates the spatially restricted expression of zygotic target genes. The earliest zygotic genes expressed in distinct spatial domains along the anterior-posterior axis of the embryo are the gap genes[10]. The phenotype of mutations in the gap gene *hunchback* (*hb*; deletion of gnathal and thoracic segments[11]), and the expression pattern of *hb* RNA in *bcd* mutant embryos[12] identify *hb* as a probable target for *bcd* regulation. Here, we show that the bcd protein binds to five sites upstream of the transcription start site of the zygotic gap gene *hunchback*. These binding sites define the consensus binding sequence: 5'TCTAATCCC3'. Transient expression assays in embryos as well as in tissue culture cells reveal that the three binding sites in the promoter region (−50 to −300) are necessary and sufficient for the activation of zygotic *hunchback* expression.

Mapping bcd protein binding sites

In the syncytial blastoderm *hb* expression occurs in two domains (ref. 13, see also Fig. 3*a*). The 2.9-kilobase (kb) transcript in the anterior domain is under the control of a separate promoter and is not expressed in *bcd* mutant embryos[12]. The regulatory region necessary for correct spatial expression of the zygotic 2.9 kb *hb* transcript has been mapped using p-transformation of *hb* β-galactosidase gene fusions[14] to a 700-base pair (bp) DNA fragment that includes the first intron and 300 bp upstream of the transcription start site.

Full-length bcd protein was expressed in *Escherichia coli* by generating a *Nde*I site at the start ATG of the longest open reading frame and cloning bcd-protein-coding sequences into the pAR3038 expression vector[15]. The same cDNA was also expressed in *Drosophila* Schneider cells using the actin 5c promoter (Fig. 1*a*). The protein derived from *E. coli* migrates slightly faster (lane 3, apparent relative molecular mass M_r = 53,000 (53K)) than that from Schneider cells (lane 2, 58K) or embryos (lane 1, 56–60K). Different electrophoretic mobilities can be explained by post-translational modification. To test whether the decrease in electrophoretic mobility is due to phosphorylation (see for example *engrailed* (*en*)[16], *Ultrabithorax* (*Ubx*), L. Gavis, personal communication), we immunoprecipitated bcd protein transiently expressed in Schneider cells which had been grown on [^{32}P] orthophosphate-containing media. The immunoprecipitate was analysed by SDS-PAGE. A ^{32}P-labelled protein migrated at the expected size of the bcd protein (Fig. 1*b*, lane 2). Treatment of immunoprecipitates from Schneider cells transiently expressing bcd protein with potato acid phosphatase results in a decrease in apparent M_r of the immunoprecipitated protein (lane 4, 5), which now co-migrates with the bcd protein expressed in *E. coli* (lane 3). In addition, multiple bands of intermediate apparent M_r are also visible. We conclude that the bcd protein in *Drosophila* Schneider cells, and probably also in the embryo, is subject to multiple phosphorylations.

We used an immunoprecipitation assay[17] to screen large regions of genomic *hb* DNA for bcd-binding sites (see Fig. 2). *E. coli*-derived full-length bcd protein was immunoprecipitated with polyclonal antibodies bound to fixed *Staphylococcus aureus* cells and the resulting immunoprecipitate was incubated with end-labelled restriction fragments from the *hb* promoter. Two restriction fragments from the *hb* gene were bound with high affinity: fragment A (−298 to −55) and B (−2,172 to −2,631) base pairs upstream from the transcription start site of the zygotic 2.9 kb *hb* transcript (Fig. 3*a*). A third fragment, C, from −4,242 to −3,890 base pairs upstream from the 2.9 kb transcription start site (or −1,020 to −672 upstream from the 3.2 kb transcript) was bound with relatively low affinity. Fragment A binds with the highest affinity as, even at the highest concentrations used, salmon sperm DNA had a negligible effect on the amount of fragment A bound. At this competitor concentration the binding of fragment B is reduced to about one-third and binding of fragment C is eliminated completely. The same fragments were also precipitated using bcd protein expressed in Schneider cells (data not shown), indicating that phosphorylation does not affect binding specificity in this assay.

Consensus sequence for bcd binding

The individual binding sites were analysed further by DNaseI footprint experiments[18]. The results are shown in Fig. 4 and summarized in Fig. 3. Fragment A contains three regions which are protected against DNaseI digestion by bcd protein, A1 (−280 bp), A2 (−170 bp) and A3 (−65 bp). A2 consists of two protected regions, one well protected (around −170) and one weakly protected (around −187). The A region is part of the 700 bp fragment shown to be sufficient for the correct spatial expression of the 2.9 kb *hb* transcript[14]. The B fragment consists of two binding regions, B1 (about −2,325) and B2 (about

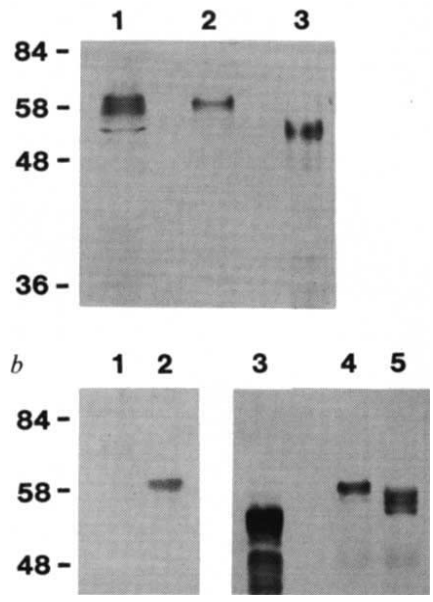

Fig. 1 *a*, Expression of bcd full-length protein in *E. coli* and *Drosophila* Schneider cell tissue culture. Western blot of extracts separated by PAGE, probed with anti-bcd antibodies. Lane 1, extracts from 2- to 3-hour-old *Drosophila melanogaster* embryos (500 μg protein); lane 2, extracts from the Schneider cell line S2/M3 that transiently expresses bcd under the control of the *Drosophila* actin 5c promoter (plasmid pPAcbcdEE, 100 μg protein applied); lane 3, extracts from *E. coli* that express bcd under the control of the T7 phi 10 promoter (plasmid pARbcdNB, 10 μg protein applied); M_rs are indicated on the left, in thousands. *b*, Modification of bcd protein by phosphorylation. The bcd protein was transiently expressed in Schneider cells grown in the presence of [^{32}P]orthophosphate. Immunoprecipitation from nuclear extracts with anti-bcd antibody and autoradiography of the precipitate resolved on SDS-PAGE demonstrates that in bcd-expressing cells (lane 2) but not in non-expressing cells (lane 1) a phosphorylated form of bcd protein is synthesized. Incubating bcd immunoprecipitates from extracts of Schneider cells transiently expressing bcd protein with potato acid phosphatase results in an increase of the electrophoretic mobility of bcd protein. The most rapidly migrating form is of the same size as the bcd protein expressed in *E. coli* (lane 3) as revealed from western blot analysis of the reaction products: bcd protein incubated without (lane 4), and with (lane 5) potato acid phosphatase.

Methods. The *bicoid* gene gives rise to a primary transcript that is subject to differential splicing. The alternatively spliced RNAs code for different protein products[3,5]. In this study we used only one protein species derived from cDNA c53.46.6 (ref. 3). Although it is not yet clear whether the different forms of bcd protein are functionally equivalent, RNA injections into early embryos reveal that this bcd protein species can rescue all aspects of the mutant *bicoid* phenotype (our unpublished data). For the protein expression constructs, *bcd* cDNA c53.46.6c was used (ref. 3; all the base pairs that differ from the genomic sequence and give rise to amino-acid substitutions were exchanged with genomic sequences: detailed protocol on request). Construction of pARbcdNB: the *Eco*RI-*Sal*I fragment from c53.46.6c was used to generate a *Nde*I site at the initiator ATG of the longest open reading frame by oligonucleotide site-directed mutagenesis (using the pMa/c 254 plasmid vectors, Stanssens and co-workers, unpublished results), the cDNA was reconstructed and a *Bam*HI site generated at the *Xma*I site 3′ to the open reading frame; the *Nde*I-*Bam*HI fragment was cloned into the expression vector pAR3038 (ref. 15). Construction of pAcbcdEE: the *Eco*RI fragment of cDNA c53.46.6c was blunt-end-ligated into the *Bam*HI site of the vector pPAc downstream of the actin 5c promoter and upstream of the actin 5c polyadenylation processing signals (vector pPAc constructed by H. Lipshitz using the *Drosophila melanogaster* actin 5c gene, ref. 28). bcd protein was expressed from pARbcdNB (ref. 15) in *E. coli* BL 21 DE3. *Drosophila* tissue culture and transfection techniques were as described[29]. Phosphate labelling was performed using 0.5 mCi ^{32}P-labelled NaH_2PO_4 per 5 ml of medium in a 6 cm petri dish. bcd protein was immunoprecipitated from cleared lysates of isolated nuclei sonicated in 20 mM Tris *p*H 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% NP-40, 2 mM phenylmethylsulphonylfluoride using polyclonal anti-bcd antibody prebound to *Staph. aureus* (Calbiochem). The immunoprecipitates where treated with 50 μg ml^{-1} DNaseI (Cooper), 10 mM $MgCl_2$ in the above buffer for 15 min on ice. Potato acid phosphatase treatment (20 μg per 100 μl) was as described[30]. Extract preparation from *Drosophila* embryos, electrophoresis and further processing were as previously described[5].

−2,280). Both are less well protected than the A sites. No footprint could be obtained with the C fragment (data not shown), but DNaseI hypersensitive sites could be identified around −4,100 and −3,998. Analysis of the sequences of fragment A and B revealed that each of the sites contains a sequence approximating a 9-bp consensus sequence TCTAATCCC (Fig. 3*b*). This sequence is best conserved in sites A2 and A3 whereas A1, B1 and B2 each contain mismatches with the consensus. Only the central part of the sequence, TAATC is conserved in all five sites. Using binding conditions of low stringency (no competitor DNA) hypersensitive sites could be detected around CTAAT or TAATC sequences (for example −4,100, −3,998) or even at TAAT alone (data not shown). In addition, within fragment A in the region −241 to −204 on the sense strand some hypersensitive sites do appear and one can detect weak protection of some bases on the antisense strand. Three sequence motifs in this region (TGCTAAGCT, −241 to −233; CGCTAAGCT, −225 to −217; and GATCATCC, −212 to −205) resemble the consensus sequence (underlined nucleotides) weakly. Notably, all three motifs are palindromic. The bcd protein seems to bind to DNA sequences that contain elements like TAAT, CTAA, CTAAT, TAATC, where the context of the surrounding nucleotides determines the affinity of binding, the best fit so far identified being the consensus TCTAATCCC.

For a few other *Drosophila* homoeodomain-containing proteins, sequence-specific binding has been demonstrated and the binding sites seem to lack palindromic character as does the bcd consensus sequence. Engrailed (en), fushi tarazu (ftz), zerknüllt (zen) and even skipped (eve) protein *in vitro* all bind *en* upstream sequences with the consensus TCAATTAAT[20,21]. In addition eve protein recognizes TCAGCACCG sequences in the *eve* upstream region[21]. Ubx protein binds to $(TAA)_n$ repeats (P. Beachy, M. Krasnow, L. Gavis and D. Hogness, personal communications). Analysing the 'contact amino acids' of the homoeodomain that are probably exposed to DNA (positions 1, 2, 5, 6 and 9 within the recognition helix of the helix-turn-helix motif[22], one finds that Ubx, ftz, zen and en are identical in all but the second amino acid, providing an explanation for their similar binding characteristics. In contrast, bcd differs in positions 1, 2 and 9 with respect to the above proteins. Further analysis will show if bcd has overlapping binding characteristics with other *Drosophila* homoeodomain proteins.

Embryonic expression assay

To investigate the influence of the bcd-binding sites on *hb* transcription, we used an embryonic transient expression assay (Fig. 5). The *hb* promoter fragments, including increasing numbers of bcd-binding sites, were fused to the chloramphenicol acetyltransferase (CAT) gene at position +107 of the 2.9 kb *hb* transcript. The plasmid constructs were injected into the anterior half of early cleavage embryos and the expression of the reporter gene CAT was measured at the onset of gastrulation. We expect that a small amount of the plasmid DNA ends up in a nuclear location during the rapid early cleavage cycles and comes to be expressed under conditions similar to those that lead to the expression of the chromosomal *hb* copies.

On injection of the *hb*-CAT constructs into early embryos, we do indeed observe a bcd-dependent expression of CAT. Figure 5 shows that with the longest upstream sequences CAT expression is about 50 times higher in embryos from wild-type females than in embryos derived from *bcd*$^-$ females. Elimination of the binding sites B1 and B2 did not significantly affect the level of expression. In contrast the successive elimination of the A sites results in a dramatic reduction of the bcd-dependent *hb*-CAT expression. If all binding sites (but not the TATA box) are removed, no significant expression is observed.

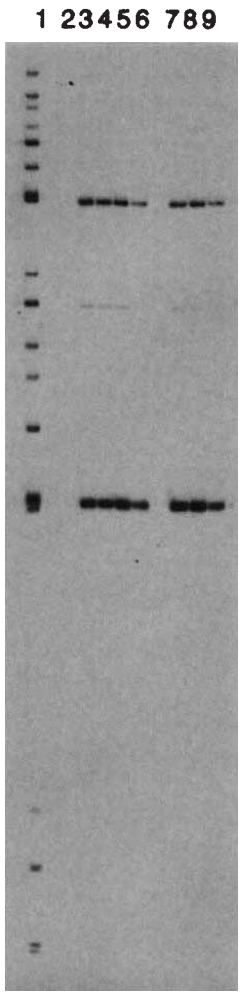

Fig. 2 Specific binding of bcd protein to restriction fragments from the *hb* gene. The genomic 7.5 kb *hb* *Bam*HI-*Eco*RI fragment contains the zygotic 2.9 kb transcript that is expressed in the anterior gap domain and about 4.5 kb of upstream sequences[12]. It was digested with a set of restriction enzymes to produce fragments of 100–1,000 bp and the fragments were end-labelled (lane 1). bcd protein expressed in *E. coli* under the control of T7 RNA polymerase (see Fig. 1), was used to immunoprecipitate fragments that bind specifically to the protein. Lane 2 shows a control reaction using anti-bcd immunoprecipitates from non-bcd expressing bacteria; no fragments were bound. In the immunoprecipitates with bcd protein (lanes 3–9), two fragments (A, a 243-bp *Sal*I-*Mlu*I fragment from −298 to −55 with respect to the transcription start site; B, a 459-bp *Hin*dIII-*Bgl*II fragment from −2,172 to −2,631) were bound with high affinity, one with relatively low affinity (C) a *Bam*HI-*Hin*dIII fragment from −4,242 to −3,890). To assess the specificity of binding, zero (lane 3), 0.25 (lane 4), 1 (lane 5) and 5 (lane 6) μg of competitor DNA (salmon sperm DNA, 1,000-fold excess over total labelled DNA, 40,000-fold excess over one specific labelled fragment, lane 6) were included in the binding reaction. Binding of fragments A and B is only slightly affected at the highest competitor concentration, fragment C is no longer bound under this condition. In the experiments shown in lanes 7, 8 and 9, after 45 min binding the immunoprecipitates were washed and competitor DNA was added for 10 min (0.2, 1 and 5 μg, respectively). The off rates for binding to fragment A and B are apparently rather low.

Methods. The genomic 7.5 kb *Bam*HI-*Eco*RI fragment was isolated from the plasmid pE8-B1000A (obtained from D. Tautz, ref. 13), digested with a set of restriction enzymes and end-labelled with polynucleotide kinase (restriction enzymes: *Ava*II, *Bgl*II, *Mlu*I, *Hin*dIII, *Nde*I, *Sal*I and *Nco*I; no additional fragments were precipitated when the same experiments were performed using either a *Hinf*I or a *Taq*I digest). bcd protein was expressed in the bacterial strain BL21(DE3) as described in ref. 15. Induced cells were collected by centrifugation and resuspended in 1:200 volume of buffer Z (ref. 21), incubated for 15 min on ice and sonicated twice for 15 s. The lysate was spun clear and the supernatant taken as extract. Immunoprecipitation was carried out according to a modified procedure[9]. Fixed *Staph. aureus* cells (Calbiochem, 100 μl of 10% suspension) were washed twice with binding buffer BB (20 mM Tris *p*H 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 10% glycerol) and incubated with polyclonal anti-bcd antibody (20 μg in 500 μl BB) for one hour on ice. The suspension was washed twice with BB. Extracts (100 μl) from bacteria carrying pARbcdNB or pAR3038, respectively, were added in 1 ml BB, 2 mM PMSF and incubated for two hours on ice. The immunocomplexes were washed twice with BB, once with BB 0.2% NP-40 and resuspended in 100 μl BB. Binding reactions were in 50 μl total volume including 10 μl immunocomplex suspension, NaCl added to 170 mM, 5 ng end-labelled fragments and various amounts of sonicated salmon sperm DNA as competitor for 40 min on ice. For lanes 7–9, immunoprecipitates were washed once with BB and incubated for 10 min in the above reaction mixture with the indicated amounts of competitor DNA. Precipitates were washed four times with 1 ml BB 0.2% NP-40 each, resuspended in 100 μl 10 mM Tris pH 7.5, 1 mM EDTA, 5 μg sonicated salmon sperm DNA was added as carrier, the fragments phenol/chloroform extracted, precipitated and separated on a 4% denaturing polyacrylamide gel. Autoradiography was performed using Cronex 4 films.

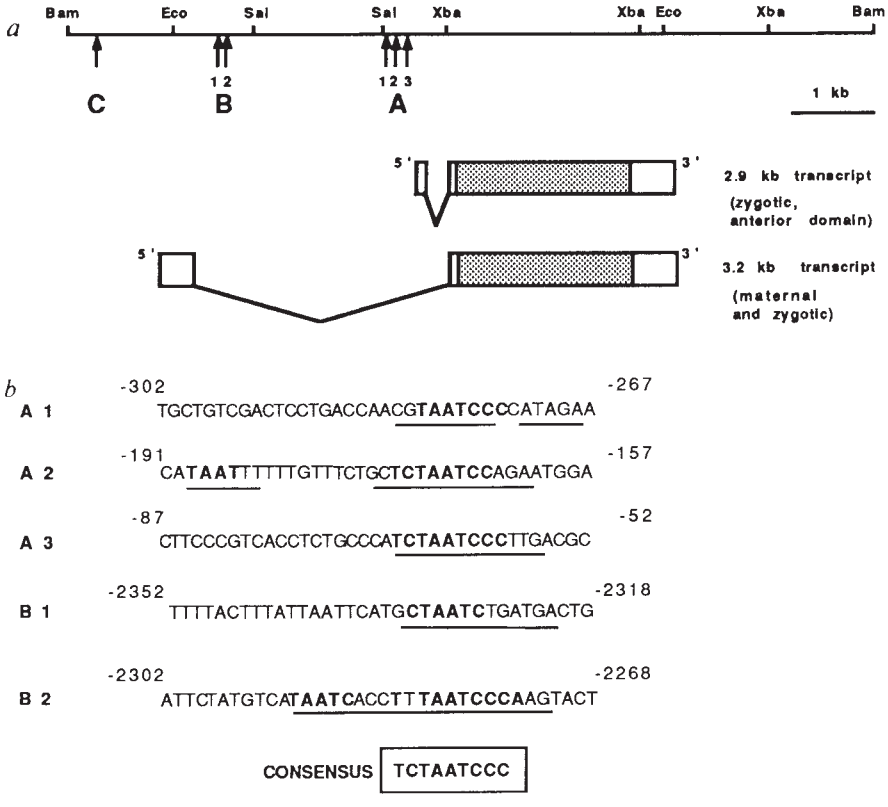

Fig. 3 *a*, Map of the *hb* gene indicating the locations of bcd-binding sites. During the syncytial blastoderm stage *hb* expression occurs in two domains which are under separate regulation. The 2.9 kb *hb* transcript is expressed in an anterior domain which extends from 55–100% egg length, whereas the 3.2 kb transcript (which is expressed maternally and zygotically) is localized to 0–25% egg length[13]. In addition, the 3.2 kb transcript can be detected in a narrow stripe at about 53% egg length shortly before the onset of cellularization. A, B and C are the fragments identified in the experiment shown in Fig. 2. *b*, Nucleotide sequences of the regions where bcd protein binds to *hb* regulatory regions. Base pairs protected against DNaseI digestion as referred from Fig. 4 are indicated by a bar below the sequence. Comparison of the protected sequence elements led to the identification of a consensus binding sequence. Bases in the protected regions that fit the consensus sequence are in bold.

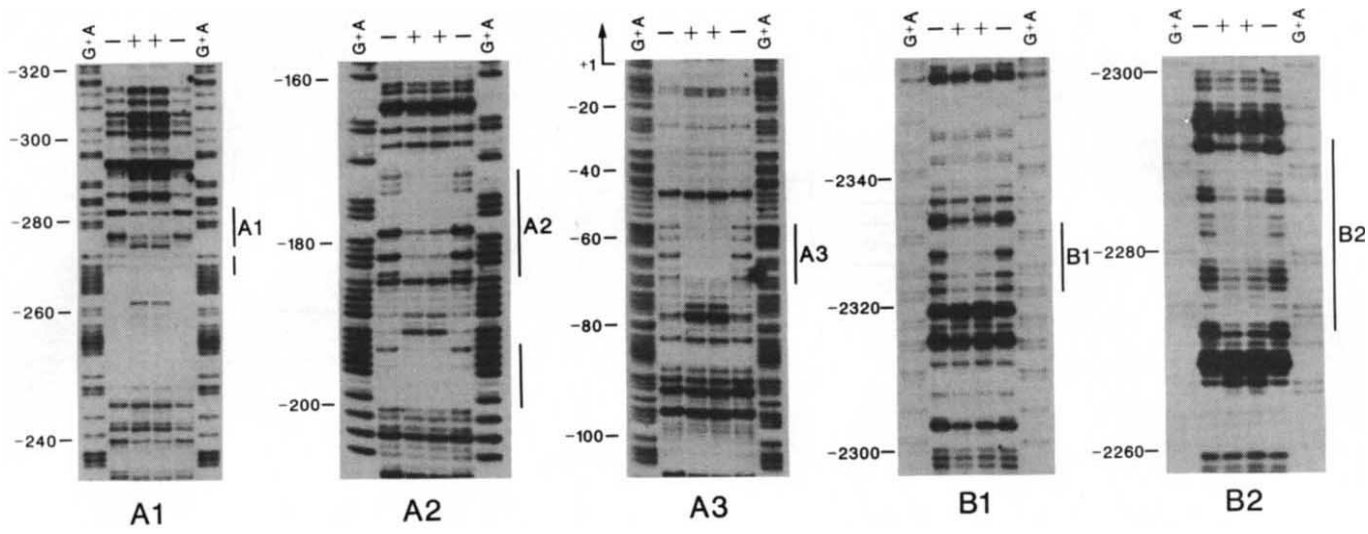

Fig. 4 Analysis of bcd protein binding to *hb* sequences by DNaseI footprinting. The DNA fragments identified in the immunoprecipitation assay, the sequences surrounding these fragments as well as the intron of the 2.9-kb transcript were analysed for bcd protein binding sites by DNaseI footprinting. Specific binding could only be detected in the same regions that were identified by the immunoprecipitation assay, regions A and B. Region A seemed to consist of three *bcd* binding sites, A1, A2 and A3, each spaced at about 100-bp intervals. Region B consists of two binding sites, about 40 bp distant from one another. Lanes show (G+A) sequence marker and footprint reactions with (+) and without (−) bcd protein. Positions are indicated on the left side of each panel in base pairs distance from the transcription start site. Protected regions are marked by a bar on the right side.

Methods. For DNaseI protection assays (ref. 31) bcd protein was affinity purified as described in Fig. 2 and resuspended in 100 μl FPBB[21] (110 mM KCL, 47 mM HEPES *p*H 7.8, 12 mM $MgCl_2$, 0.05 mM EDTA, 1 mM dithiothreiotol, 17% glycerol and 0.02% NP 40). Genomic fragments were asymmetrically labelled with Klenow and radioactive dNTPs (A1: labelled at *Mlu*I site −55, linearized at *Nsi*I site −434, sense strand; A2 and A3: labelled at an *Eco*RI site generated at *Sal*I−298 and linearized at *Xba*I+431, antisense strand; B1 and B2 labelled at an *Eco*RI site generated at the *Bgl*II site −2,170 linearized at *Hin*dIII −2,631, sense strand). Binding reactions were done with 4–8 ng of labelled fragment and 20 μl of immunocomplex in a total volume of 50 μl FPBB for 45 min on ice. 50 μl 10 mM $MgCl_2$/5 mM $CaCl_2$ were added to the reaction followed by 5 μl of freshly diluted DNaseI (Worthington) at a final concentration of 5 μg per ml reaction mix. After 5 min on ice, digestion was stopped by the addition of 110 μl 1% SDS, 20 mM EDTA, 200 mM KCl, 250 μg per ml yeast tRNA (65°C). After phenol/chloroform extraction and ethanol precipitation samples were electrophoresed on 8% polyacrylamide/7.5 M urea gels and visualized by autoradiography.

We also expressed the CAT constructs in Schneider cells. In this assay, there is a substantial expression of CAT even in the absence of *bcd*. Co-transfection (M. Krasnow and D. Hogness, unpublished results) with the *bcd* transcription unit under the control of the actin 5c promoter results in a fivefold stimulation of CAT expression using constructs that contain the whole A region (Fig. 5*d*). The strong reduction in expression when removing only the A1 site supports the idea that the three sites cooperatively activate *hb* expression. Deletion of A1 and A2 decreases the expression to background levels.

Discussion

The concerted action of three regulatory sites, each 100 bp apart, raises the question of how they interact with the distant transcription initiation complex. In prokaryotes, loop formation between promoter elements has been visualized on a molecular level[23]. In eukaryotes, the chromatin structure might influence the spatial arrangement of regulatory regions. The analysis of the *Drosophila* 26K heat-shock protein promoter[24] demonstrates that the positioning of a nucleosome might bring regulatory sequences together on its surface[25]. The spacing of bcd-binding sites A1–A3 might allow a similar mechanism. Remarkably, in *Drosophila virilis*, not only the bcd-binding sites, but also their spacing seem to be conserved (D. Tautz, personal communication). A close spatial proximity of the binding sites might be the basis for cooperativity in activation of transcription.

An interesting question is whether bcd protein provides only the DNA-binding function or also the transcription-activating functions that interact with the cellular transcription machinery. In yeast, transcriptional activators are characterized by acidic protein domains[26] and these acidic regions seem to function in other eukaryotic systems as well[27]. A good candidate for a transcriptional activator domain within the bcd protein is the region between amino acids 345 and 390 (see ref. 3 for protein sequence) that has 10 negative charges and in addition several putative phosphorylation sites. As noted above, the protein is phosphorylated in *Drosophila* Schneider cells. The level of phosphorylation might influence the strength of the transcriptional activator function of bcd.

The molecular interaction between the bcd protein and the *hb* promoter is probably one of the first events in gene regulation during embryogenesis and shows how a maternally derived regulatory protein, bcd, participates in initiating the process of pattern formation. A major question about the interactions between maternal coordinate genes and zygotic segmentation genes is how the information provided by the smoothly graded distribution of a maternal gene product like bcd is transformed into the more sharply defined gap gene domains. In the wild-type, the early posterior border of *hb* expression is at 55% egg length[12]. In this region of the embryo the bcd gradient is already very shallow, although the bcd protein is detectable up to 30% egg length[5]. Several models might explain how the transition from a shallow gradient to a sharp on/off decision is achieved. For example, other so far unidentified factors might compete for bcd-binding sites or inhibit bcd binding and thereby sharpen the posterior border of *hb* expression. So far there is no genetic evidence for the existence of genes that could function as competitors[1,2,6]. Our data support models that include cooperativity as an important mechanism to control the spatially restricted expression of zygotic target genes. A precedent for the role of cooperativity in gene regulation is the action of λ repressor (for review see ref. 19). The three bcd-binding sites identified in the

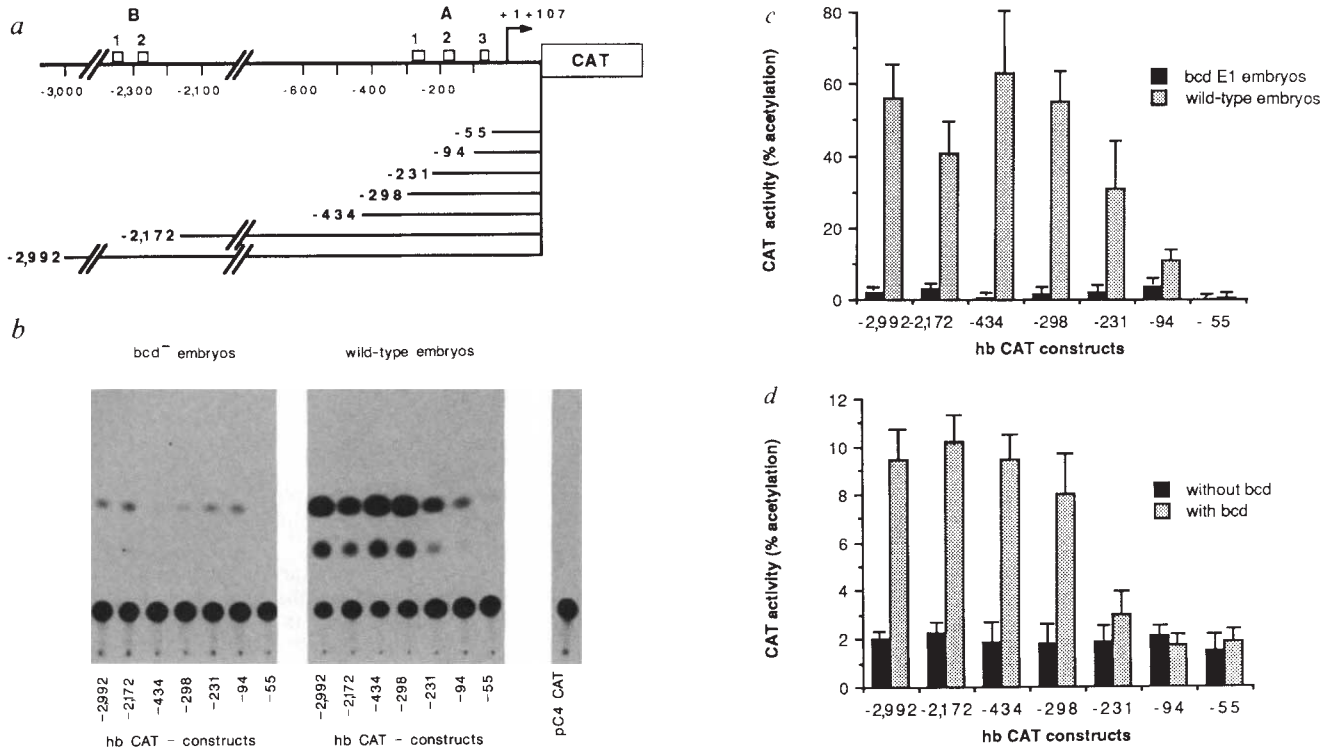

Fig. 5 Influence of *hb* upstream sequences on the expression of a reporter gene coding for CAT as assayed by transient expression in embryos and Schneider cell tissue culture. *a*, Map of *hb* CAT fusion genes used in this analysis. *hb* upstream sequences and the *hb* transcription start site plus 107 bp of the non-translated leader were fused to the CAT gene. Construct hbCAT-55 ends just downstream of the A3 binding site, hbCAT-94 includes the A3 binding site, hbCAT-231 includes A2 and A3, hbCAT-298 the whole A cluster. To analyse a possible function of the B cluster, we made constructs ending upstream and downstream of that cluster. *b*, Embryonic transient expression assay. hbCAT fusion gene plasmids were injected into early cleavage embryos and the embryos analysed for expression of CAT at the onset of gastrulation. The left part shows CAT assays using embryos from homozygous bcd^{E1} females (control with no bcd protein) and the right part those from wild-type embryos. pC4 CAT marks the control when just injecting the vector alone without any *hb* sequences. *c*, Quantitative analysis of the embryonic transient expression assay. Percentage of acetylated forms of total chloramphenicol were plotted for the expression of CAT in wild-type and bcd^{E1} embryos. The stimulation of expression in wild-type embryos compared to *bcd* mutant embryos is about 50-fold when all three sites of the A cluster are present, 10-fold with just A2 and A3 and 3-fold when only A3 is present. Comparing the construct that has no bcd-binding site (hbCAT-55) with the one that contains all three A sites (hbCAT −298) gives a 200-fold stimulation of expression. The presence or absence of the B cluster does not seem to have a significant influence on the expression starting from the zygotic 2.9 kb *hb* transcript start site *d*, Transient expression of *hb*-CAT fusion genes in *Drosophila* Schneider cells co-transfected with *bcd* under the control of the actin 5c promoter (pPAcbcdEE, see Fig. 1). Correction for variation in transfection efficiency was achieved by co-transfection of an actin 5c *LacZ* fusion gene and assay of β-galactosidase activity in the cell extracts. The graph shows activation of CAT expression when comparing transfections with or without bcd. All *hb*-CAT constructs showed a high background expression in the absence of bcd. The presence of bcd protein leads to a fivefold stimulation of CAT expression from constructs containing all three A sites. Removal of A1 results in a strong reduction of expression. CAT expression in the presence of just A3 is no more different from background levels. Northern analysis of RNA from cells transfected with similar *hb lacZ* fusion genes indicate an about fourfold increase in transcript level comparing the *hb*-55lacZ to the *hb*-298lacZ construct.

Methods. A *Bam*HI site was generated at the *Taq*I site within the zygotic 2.9-kb *hb* transcript (+107) by ligating a *Bam*HI linker to the filled in *Taq*I site. Genomic *hb* DNA was digested with enzymes cutting at the sites indicated in *a*, blunt-ended and then cut at the newly introduced *Bam*HI site (+107). The CAT reporter gene vector pC4 CAT (ref. 32) was linearized at the *Sal*I site in the polylinker, blunt-ended and cut with *Bam*HI. Isolated *hb* promoter fragments were cloned into the pC4 CAT vector by standard techniques. Each plasmid (5 pmol) was dissolved in 0.1 ml sterile water containing 10^6 c.p.m. ^{32}P dCTP. Injections into early cleavage embryos, 100 of each mounted on one coverslip and covered with Voltalef 10S oil, were according to standard procedures[2]. The embryos were left to develop at 18 °C until the onset of gastrulation, washed off the coverslip with heptane, washed carefully three times with heptane to remove last traces of oil and the heptane was evaporated completely with an air stream. Radioactivity injected into the embryos was determined and the embryos were frozen in liquid nitrogen. An average of 70 nl was injected into each 100-embryo batch (about 7% of the egg volume). Batches with injected amounts more than 30% different from the average were discarded. For each wild-type construct four groups of 100 embryos and for the bcd^{E1} control as well as the vector control two groups of 100 embryos were analysed. Modified CAT assays were used[33]. 100 μl of 0.25 M Tris *p*H 7.8 was added to each batch, the embryos sonified for 5 s, incubated at 65 °C for 5 min and insoluble material discarded after centrifugation. Aliquots (25 μl) of the extract were mixed with 54 μl 0.25 M Tris *p*H 7.8, 20 μl 4 mM acetylCoA and 1 μl [^{14}C]chloramphenicol (0.2 μCi). The reaction mixture was incubated for one hour at 37 °C, the chloramphenicol extracted with ethyl acetate (0.5 ml), the ethyl acetate evaporated and the acetylated forms of chloramphenicol separated by TLC. The acetylated and non-acetylated forms of chloramphenicol were visualized by autoradiography and quantitated by scintillation counting. Bars in *c* show mean values of the determinations and the standard error of the mean. *Drosophila* tissue culture and transfection techniques were used[29]. The pPAcLacZ plasmid was constructed by inserting the blunt ended *Sal*I-*Hin*dIII fragment from pCaSpeR AUGβGAL (ref. 32) containing the coding region for β-galactosidase and the SV40 polyadenylation site into a blunt-ended *Sal*I-digested pPAc actin 5c promoter vector (H. Lipshitz, unpublished results). Co-transfections were performed using 5 μg pPAcLacZ, equimolar amounts of the *hb*-CAT constructs (1–1.5 μg) with or without 10 μg of pPAcbcd. pUC 18 DNA was added to reach 20 μg total DNA per transfection. Cells from one 6-cm plate were washed in PBS and lysed in 200 μl 250 mM Tris *p*H 7.8 by sonication. Cleared lysates were assayed for β-galactosidase activity using *o*-nitrophenyl-βD galactopyranoside as substrate. The measured activities were used to correct the amount of extract used in the CAT assay (see above) for the variation in transfection efficiency. The graph shows mean values of two determinations and the mean errors.

hb promoter might allow cooperative binding and thereby full activation of transcription above a low threshold of bcd concentration. Indeed, the level of *hb* expression is constant within the anterior 40% of the embryo[12], independently of the marked increase in bcd protein concentration towards more anterior positions. In addition, a cooperative mechanism can generate an on/off transition within a relatively small range of bcd protein concentrations. One would not expect that such a mechanism immediately generates a sharp border; indeed, initially the posterior border of *hb* expression is not clearly defined within a stripe two or three nuclei wide[12]. Subsequent regulatory interactions between *hb* and other zygotic genes might be involved in the establishment and maintenance of a border as sharp as that observed in later blastoderm stages[12].

Genetic analysis suggests that bcd regulates more than one zygotic target gene in a spatially restricted way[2,6]. Different spatial limits of target gene expression could be achieved if the affinities of their bcd-binding sites vary. Thus a reduced affinity for bcd-protein binding would restrict target gene expression to more anterior regions which contain higher levels of bcd protein. Analysis of further target genes will show how bcd protein fulfils its function as a morphogen at the molecular level.

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LETTERS TO NATURE

Strong variability of weak-core radio sources

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The variability of radio sources whose emission is dominated by single unresolved cores (core-dominated sources) is well established[1]. Evidence is also mounting that sources such as Cen A and 3C111, which are not core-dominated, also have varying cores[2–4]. Statistical studies of source fluxes using single-dish data and comparisons with known morphologies suggest that even in sources whose emission is swamped by that of the extended emission, the cores can be highly variable on timescales of weeks to years[5,6]. Here we present direct observational evidence for core variability of two lobe-dominated (weak-core) radio sources, GT1945+241 and GT0304+575. In the context of the beaming model, weak-core sources have little Doppler boosting and any residual variability is mainly intrinsic. The extreme nature of the core variability of the two sources described here suggests that the intrinsic variability can dominate beaming-related variability. If future studies reveal that this type of source is common, aspects of the beaming model, particularly in the context of the Unified Scheme, would be brought into question.

Confirmation of weak-core variability through direct measurements of the core fluxes bears directly on the relationship between the core-dominated sources and the weak-core sources. The advent of the beaming model[7,8] has led to the 'Unified Scheme'[9], in which the two classes of sources differ only because of geometry. Sources with major axes close to the line of sight beam towards us. The Doppler boosting associated with relativistic beaming amplifies the synchrotron radio emission. The beaming itself increases the degree of variability and, through time dilation effects, reduces the variability timescales[3]. Sources with major axes well away from the line of sight should show minimal beaming effects. These sources should therefore have fainter cores, fewer of them should be variable and the timescales of variability should be, on average, greater. A competing view holds that the two classes of sources are intrinsically different, as suggested by their differing spectra and polarization properties[10]. Unambiguous detection of strong, short-term variability in weak-core sources would argue against a straightforward interpretation of the Unified Scheme and in favour of the two-class scenario.

Here we present direct evidence for such variability in two specific sources, GT1945+241 and GT0304+575. These sources are taken from a sample of variable radio sources from the 6-cm galactic-plane survey of Gregory and Taylor[11]. The sources were selected solely on the basis of their variability, with no prior knowledge of their morphologies. Because the sources are all in the galactic plane it has not been possible to identify them optically. Distances are therefore not known and the possibility exists that the one or both of the sources discussed here are galactic. Statistical studies of source counts suggest, however, that ~90% of the survey sources are expected to be extragalactic[13]. Furthermore, recent H I absorption measurements (made by us) indicate degrees of absorption by foreground gas that are large enough to make an extragalactic origin likely. Once variability was established, follow-up observations on the Very Large Array (VLA) were used to determine the source structures. Observations were carried out at 6 and 20 cm in the A array with the resulting resolution of 0.3″ and 1.0″ respectively.