The EMBO Journal Vol.17 No.20 pp.5998–6009, 1998

Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the *Drosophila* embryo

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The Bicoid morphogen directs pattern formation along the anterior–posterior (A–P) axis of the *Drosophila* embryo. Bicoid is distributed in a concentration gradient that decreases exponentially from the anterior pole, however, it transcribes target genes such as *hunchback* in a step-function-like pattern; the expression domain is uniform and has a sharply defined posterior boundary. A 'gradient-affinity' model proposed to explain Bicoid action states that (i) cooperative gene activation by Bicoid generates the sharp on/off switch for target gene transcription and (ii) target genes with different affinities for Bicoid are expressed at different positions along the A–P axis. Using an *in vivo* yeast assay and *in vitro* methods, we show that Bicoid binds DNA with pairwise cooperativity; Bicoid bound to a strong site helps Bicoid bind to a weak site. These results support the first aspect of the model, providing a mechanism by which Bicoid generates sharp boundaries of gene expression. However, contrary to the second aspect of the model, we find no significant difference between the affinity of Bicoid for the anterior gene *hunchback* and the posterior gene *knirps*. We propose, instead, that the arrangement of Bicoids bound to the target gene presents a unique signature to the transcription machinery that, in combination with overall affinity, regulates the extent of gene transcription along the A–P axis.

Keywords: anterior–posterior/Bicoid/cooperativity/*knirps*

Introduction

Pattern formation during embryogenesis requires the establishment of precise spatial domains of gene expression. These domains, with their characteristic sharp boundaries, arise through a complex set of interactions between transcription regulatory proteins and their *cis*-acting DNA binding sites. Regulatory proteins use at least three mechanisms to stimulate spatially correct target gene transcription. First, regulatory proteins are localized, thereby restricting their activity to broad regions within the embryo (Driever and Nüsslein-Volhard, 1988a; Roth *et al.*, 1989; Pankratz *et al.*, 1992). Second, regulatory proteins act in combination with other co-activators or repressors of transcription (Riddihough and Ish-Horowicz, 1991; Small *et al.*, 1991; Xue *et al.*, 1993; Sauer and Jäckle, 1995). Third, regulatory proteins interact with themselves in a cooperative manner to activate or repress transcription at subsaturating concentrations and to produce a sharp threshold response (Beachy *et al.*, 1993; TenHarmsel *et al.*, 1993; Wilson *et al.*, 1993). The *Drosophila* morphogen, Bicoid, which directs pattern formation along the anterior–posterior (A–P) axis in the developing embryo, is thought to employ all three mechanisms to establish spatially correct domains of target gene expression (Frohnhöfer and Nüsslein-Volhard, 1986; reviewed in Driever, 1993).

The first mechanism forms the basis for the classic morphogen model of Bicoid action. Bicoid is localized along the A–P axis into a protein concentration gradient that spans two to three orders of magnitude decreasing exponentially over the anterior half of the embryo (Driever and Nüsslein-Volhard, 1988a). The importance of Bicoid's localization in a gradient was shown by increasing or decreasing maternal *bicoid* gene dosage, which resulted in steeper or shallower concentration gradients, thus shifting the cephalic furrow towards the posterior or anterior region of the embryo, respectively (Driever and Nüsslein-Volhard, 1988b). Anterior regions that are exposed to higher levels of Bicoid express a different set of genes (e.g. *buttonhead*, *orthodenticle*) than more posterior regions where the Bicoid concentration is lower (e.g. *hunchback*, *Krüppel*). It is thought that the extent along the A–P axis to which a Bicoid target gene is expressed will depend on the affinity of Bicoid for its enhancer (i.e. the gradient-affinity model). Despite its vanishingly low concentrations at the posterior end of the embryo, Bicoid also activates posteriorly acting segmentation genes such as the abdominal gap gene *knirps* (Rivera-Pomar *et al.*, 1995) and the posteriormost stripe of the pair-rule gene *hairy* (La Rosee *et al.*, 1997). Thus, although Bicoid is localized in a gradient with a peak concentration at the anterior pole, it still functions as a transcriptional activator along the entire A–P axis of the *Drosophila* embryo.

The second mechanism by which Bicoid stimulates spatially restricted gene expression is by acting in combination with other transcription regulatory proteins. For example, the establishment of *even-skipped* (*eve*) stripe 2 results from competition between activators Bicoid and Hunchback, and repressors Giant and Krüppel, for overlapping sites within the *eve* stripe 2 regulatory element, and by a local repression mechanism termed quenching (Small *et al.*, 1991; Stanojevic *et al.*, 1991). In the posterior region of the embryo, Bicoid and the maternal homeodomain protein Caudal, which forms a posterior–anterior concentration gradient, act together in a partially redundant manner to activate the posterior gene *knirps* (reviewed in Rivera-Pomar and Jäckle, 1996). The regulatory region that mediates *knirps* gene activation contains two *cis*-

acting regulatory elements; one is activated in response to Caudal, the other, termed *kni*64, is a 64 bp enhancer that is activated in response to Bicoid (Rivera-Pomar *et al.*, 1995).

In the third mechanism, Bicoid monomers are thought to interact cooperatively with one another to bind DNA and stimulate target gene expression. Cooperative DNA binding by Bicoid has not been rigorously demonstrated, but has been widely proposed as the explanation for Bicoid's ability to regulate spatial transcription of *hunchback (hb)* and other genes (e.g. Ptashne, 1986; Driever, 1993). For example, cooperative binding by Bicoid to its sites in the *hb* upstream regulatory element is thought to result in a sharp posterior boundary of *hb* expression that specifies the position of the cephalic furrow.

Several indirect lines of evidence support the idea that Bicoid binds DNA cooperatively. First, reporter genes require multiple Bicoid binding sites to be activated in yeast (Hanes and Brent, 1989), cultured *Drosophila* cells (Driever and Nüsslein-Volhard, 1989) and transgenic *Drosophila* embryos (Driever *et al.*, 1989; Struhl *et al.*, 1989). Secondly, the minimal Bicoid binding site (TAAT-CCC; Hanes and Brent, 1991) does not define a unique target sequence; this 7 bp site is expected to be present ~10 000 times in the fly genome. However, an enhancer containing multiple sites would be comparatively rare; such a region would act as a local sink for Bicoid monomers. Thirdly, Bicoid-dependent transcription in yeast and *Drosophila* is sensitive to the spacing between binding sites (Hanes *et al.*, 1994) and to their relative orientation (G.Devasahayam, Burz & Hanes, unpublished), both hallmarks of proteins that bind DNA cooperatively (Mao *et al.*, 1994). Finally, *in vitro* evidence consistent with cooperative DNA binding has been reported (Ma *et al.*, 1996).

Here we demonstrate that Bicoid binds DNA cooperatively in yeast and *in vitro* and thus provide the first conclusive evidence for a mechanism proposed to regulate anterior patterning in the embryo (Driever *et al.*, 1989). Our experimental strategy was based on concepts drawn from the study of cI repressor of bacteriophage λ, in which the cooperative free energy of DNA binding is asymmetrically distributed between strong and weak sites in O_R (Ackers *et al.*, 1983, Beckett *et al.*, 1993). We show that Bicoid bound to a strong site promotes occupancy of an adjacent weak site by cooperative DNA binding. Without this cooperative coupling, Bicoid binding to these weak sites, which are similar to Antennapedia-class homeodomain sites, does not occur. Bicoid cooperativity should therefore increase the occupancy of weak, and otherwise unrecognized, sites, leading to a more concerted threshold response. Furthermore, we show that Bicoid cooperativity is pairwise, that is, DNA-bound Bicoid interacts with only one other Bicoid monomer at a time. This binding appears to be sequential: Bicoid binds DNA as a monomer (K_d 0.24 nM) and engages in cooperative interactions only when bound to DNA. Finally, we find that contrary to the explicit prediction of the gradient-affinity model for Bicoid action (Driever *et al.*, 1989), the posteriorly-expressed gene *knirps* did not have a higher affinity for Bicoid than the more anteriorly expressed gene *hunchback*. This result suggests that expression of Bicoid target genes at different positions along the A–P axis of the embryo can occur by a mechanism independent of DNA binding affinities.

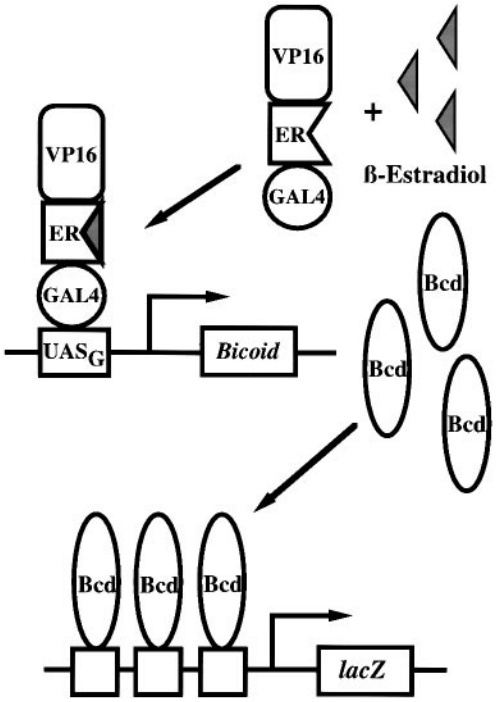

Fig. 1. An *in vivo* assay for studying cooperative gene activation in yeast. Yeast cells were co-transformed with plasmids that encode the indicated proteins. Hormone (β-estradiol) was added to mid-log phase cultures, where it entered cells and bound to the fusion protein GAL4–ER–VP16 to drive Bicoid expression in a concentration-dependent manner (Table I). Bicoid binds to different arrays of upstream sites to activate *lacZ* transcription, which is quantitated by assaying β-galactosidase activity.

Results

In vivo assay to study Bicoid cooperativity

To demonstrate that Bicoid activates gene expression cooperatively, we used the assay shown in Figure 1. In this assay, we measure Bicoid concentration-dependent activation of *lacZ* reporter genes in yeast. The *lacZ* reporters carry upstream Bicoid binding sites and their activation is quantitated by assaying β-galactosidase (β-gal) activity. To control the amount of Bicoid produced in cells, we used a fusion protein, GAL4–ER–VP16, which consists of the DNA-binding domain of Gal4, the ligand-binding domain of human estrogen receptor and the activation domain of VP16 (Louvion *et al.*, 1993). GAL4–ER–VP16 binds the *GAL1* promoter and drives expression of a *bicoid* cDNA in a hormone-dependent manner. By varying the amount of exogenous hormone (β-estradiol) added to yeast cultures, we can vary the intracellular Bicoid concentration by nearly three orders of magnitude, as assayed by Western analysis of yeast cell extracts (Table I). The ability to generate this broad range of Bicoid concentrations, combined with the use of *lacZ* reporters that carry particular arrays of Bicoid binding sites, allows us to study Bicoid cooperativity *in vivo*.

Increasing the number of Bicoid sites results in cooperative gene activation

We used our yeast assay to examine Bicoid-dependent *lacZ* activation as a function of increasing numbers of

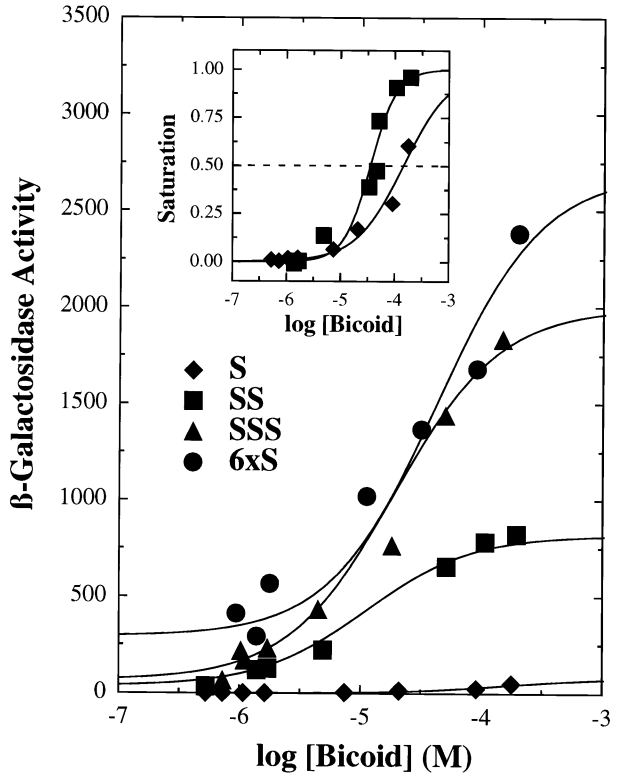

Fig. 2. Cooperative gene activation by Bicoid in yeast. Activation data were obtained using the assay shown in Figure 1. The magnitude and steepness of each activation curve increases with an increasing number of strong consensus sites (TCTAATCCC). S refers to the number of strong sites (1, 2, 3 or 6) present in the *lacZ* reporter. The curves represent the best fit to a simple concentration-dependent transition. Inset: normalized activation curves show that Bicoid binds more strongly to a two-site reporter than to a single site.

Table I. Hormone-induced Bicoid expression

Activator	[β-estradiol] (μM)	[Bicoid] (μM)[a]
GAL4–ER–VP16(mut)[b]	0	0.5–0.8
	0.0005	0.7–1.1
GAL4–ER–VP16	0	1.2–1.9
	0.0005	2.4–5
	0.0025	11–40
	0.01	25–85
	1	62–196

[a][Bicoid] is the total cellular concentration.
[b]GAL4–ER–VP16(mut) is mutated in the VP16 domain (F442P) to reduce the strength of activation.

strong (S) high-affinity consensus Bicoid sites (TCTAATCCC) derived from analysis of the *hunchback* promoter (Driever and Nüsslein-Volhard, 1989). At low and intermediate Bicoid concentrations the increase in β-gal activity between a one-site (S) and a two-site (SS) reporter is greater than 100-fold, with three sites (SSS) there is an additional two- to three-fold increase in β-gal activity, and with six sites (6 × S) there is a further 2-fold increase (Figure 2 and Table II). The greater-than-additive increase in *lacZ* expression with an increasing number of sites indicates cooperative gene activation. As expected, this effect is greater at lower concentrations at which the effects of cooperativity are most pronounced.

Cooperative gene activation by Bicoid might result

Table II. Bicoid-dependent reporter gene expression in yeast[a]

	Bicoid concentration[b]		
Reporter Gene[c]	Low	Medium	High
S S S	57	760	1830
S W S	41	613	1800
S S W	27	442	1350
S S	30	472	834
W S W	13	289	860
S W W	3	249	778
S X S	5	205	518
W S	0	108	427
S W	0	48	269
S	0	4	46
W W W	0	17	44
W W	0	0	3
W	0	0	0
6 × S	336	1060[d]	2381
kni64	251	1018	2240
Ψkni	417	1085[d]	2112
2Rkni	277	1169	2287
hb	322	1172	2499

[a]Data were obtained using the assay shown in Figure 1; results are given in units of β-gal activity.
[b]Low, medium and high Bicoid concentrations correspond to 0, 0.0025 and 1 μM β-estradiol (Table I).
[c]Target sites used: S = TCTAATCCC; W = TCTAATTCC; *kni*64 = *knirps* enhancer; Ψ*kni* = *knirps* enhancer with idealized (S) sites; 2R*kni* = idealized *knirps* enhancer with two sites reversed; 6 × S = enhancer containing six strong sites arranged head-to-tail; hb = *hunchback* enhancer.
[d]Samples were assayed on different days from others in this group (lower panel, Medium Bicoid Concentration); the values given are extrapolated from several independent experiments.

from either cooperative DNA binding or from cooperative interactions between DNA-bound Bicoid and the transcription machinery (i.e. transcriptional synergy, Carey *et al.*, 1990; Lin *et al.*, 1990). If cooperative DNA binding occurs, then the apparent midpoint of the normalized activation curve for a two-site reporter will shift to a lower concentration than that of a one-site reporter, reflecting occupancy of these sites at a lower overall Bicoid concentration. This is exactly what is observed (Figure 2, inset): there is an increase of about a half an order of magnitude in the affinity of Bicoid for the two-site reporter relative to that of a single site. By normalizing the data from 0 to 1, which correspond to completely vacant and fully occupied binding sites, we have taken into account the difference in activation potential of a single site versus two sites, allowing us to compare concentration-dependent curves directly, regardless of the number of sites. Finally, we note that the midpoint of the activation curves *in vivo* is several orders of magnitude greater than the binding affinities measured *in vitro* (see below). We believe that this is the result of the partitioning of total intracellular Bicoid, which we measured, into cytoplasmic and nuclear compartments. We do not know the actual nuclear concentration of Bicoid, but increasing the total Bicoid concentration ultimately results in saturation of transcription, a condition necessary for interpreting this experiment.

We also compared Bicoid binding to one- and two-site templates *in vitro* using gel-shift assays. The affinity of a

Table III. Bicoid homeodomain ($Bcd^{89–154}$) binding *in vitro*

Template	K_d (nM)	n_H	σ
W	1.14	1.0[a]	0.336
W W W	1.07	1.7	0.034
S	0.24	1.0[a]	0.125
S S	0.21	2.0[a]	0.123
S S S	0.17	2.1	0.020
S W S	0.21	3.0[a]	0.062
S X S	0.88	2.5	0.028
W S W	0.18	1.7	0.095
kni64	0.53	1.4	0.080
Ψkni	0.54	2.1	0.097
2Rkni	0.61	1.1	0.128
hb	0.98	3.0	0.031

n_H = Hill Coefficient; σ = standard deviation of fit.
[a]n_H was held fixed during analysis, as smaller values increased the standard deviation of the fit; all other values were resolved using the equation given in Materials and methods.

homeodomain-containing fragment of Bicoid ($Bcd^{89–154}$) for a two-site template (K_d = 0.21 nM) was slightly higher than that of a one-site template (K_d = 0.24 nM; Table III). This increase in affinity is in general agreement with our yeast assay; however, the difference observed *in vitro* is much smaller. The fact that the difference is so small is not unexpected because the magnitude of the cooperative free energy is smallest when the coupled sites are of identical affinity (Ackers *et al.*, 1983; see also below). Thus, in this example, only a small amount of the total cooperative gene activation appears to be due to cooperative DNA binding. In yeast (Figure 2, inset), the apparent difference in affinity might be artificially amplified due to unequal partitioning of Bicoid between the cytoplasm and nucleus, e.g. a 10-fold increase in total cellular Bicoid concentration might correspond to only a 2-fold increase nuclear concentration.

Bicoid bound to strong sites promotes Bicoid binding to weak sites

The binding of proteins to adjacent DNA sites of different affinities provides a dramatic demonstration of binding cooperativity. This is because the cooperative free energy is distributed asymmetrically, with most of the free energy contributed to binding at the weaker site (Ackers *et al.*, 1983). The result is that weak site saturation occurs at a lower total protein concentration. Therefore, if Bicoid binds DNA cooperatively, then at subsaturating concentrations, Bicoid bound to a strong site should promote occupancy of a nearby weak site. We tested whether this was true using *lacZ* reporter genes that carry different combinations of strong and weak Bicoid sites. The strong site (S) used in these experiments was the consensus Bicoid site (TCTAATCCC) described above. The weak site (W) contained a C:G to T:A change at position 7 in the Bicoid site (TCTAATTCC); the C:G base pair is required for specific recognition by Bicoid in yeast and in flies (Hanes and Brent, 1991; Hanes *et al.*, 1994). The W site resembles an Antennapedia (Antp) class homeodomain binding site that is recognized poorly if at all by Bicoid in yeast (Hanes and Brent, 1991). Gel-shift analyses using $Bcd^{89–154}$ confirm that the affinity of Bicoid for the W site is indeed lower than for the S site (Table III).

Control experiments show that reporter genes that carry three weak sites (WWW) are not activated, suggesting that Bicoid does not occupy these sites *in vivo* (Figure 3A). In contrast, reporters that carry three strong sites (SSS) are activated strongly, suggesting that Bicoid does occupy these sites well. If Bicoid binding is cooperative, then reporters that carry a mixture of sites (SWS) should be activated to levels comparable to that of a three strong-site reporter (SSS), rather than to reporters that carry only two strong sites. Indeed, this is what we observed at all Bicoid concentrations examined (Figure 3A). The level of transcription elicited by SWS was always greater than that of either SS (Table II) or SXS, where the X site lacks all base pairs required for recognition by homeodomains, but preserves the spacing between S sites. Activation of SWS was virtually identical to that of SSS, particularly at lower Bicoid concentrations. The simplest explanation for these results is that Bicoid bound to strong sites promotes Bicoid binding to a weak site by cooperative coupling. The effect of transcriptional synergy is ruled out because we compare reporter genes with identical numbers of sites; therefore, the maximum level of activity attainable in this experiment corresponds to three saturated sites, while lower activity reflects partial vacancy of these sites.

The level of β-gal activity obtained for SS is greater than that of SXS, confirming the previous observation that, in yeast, Bicoid activates gene expression better from closely spaced sites than from more widely spaced sites (Hanes *et al.*, 1994). Site-spacing preferences are often indicative of cooperative DNA binding; changes in site-spacing may disrupt or enhance protein–protein interactions that stabilize DNA-bound monomers, thereby affecting levels of gene activation (Mao *et al.*, 1994).

To confirm the idea of cooperative coupling between strong and weak sites, we compared *in vitro* binding of $Bicoid^{89–154}$ to SWS versus SSS and SXS using gel-shift assays. The results (Table III) indicate that binding to SWS (K_d = 0.21 nM) is much more similar to that of SSS (K_d = 0.17 nM) than to that of SXS (K_d = 0.88 nM), corroborating the results observed *in vivo*. Site loading, as detected by the formation of three distinct complexes, occurs at the same Bicoid concentration for SWS as for SSS, and these complexes are formed in the same relative proportions at a given Bicoid concentration (Figure 4). However, loading of SXS requires a Bicoid concentration about 10-fold higher than for SWS, and the complexes form at different relative proportions. For example, at the highest concentrations, the ratios of triply bound to doubly bound complexes (C3:C2) for SSS, SWS and SXS are 2.6:1, 2.5:1 and 1:1, respectively. These results indicate that the W site is readily occupied in SWS, despite the intrinsically low affinity of Bicoid for an isolated W site, or a W site flanked by other weak sites (WWW; Table III). Similar experiments using full-length Bicoid show that it also exhibits cooperative coupling, forming a triply bound complex with SWS and SSS, but not with SXS (data not shown). Thus, *in vitro* experiments demonstrate that Bicoid bound to strong sites helps Bicoid bind to a weak site, confirming the *in vivo* results.

Surprisingly, at the highest Bicoid concentrations the X

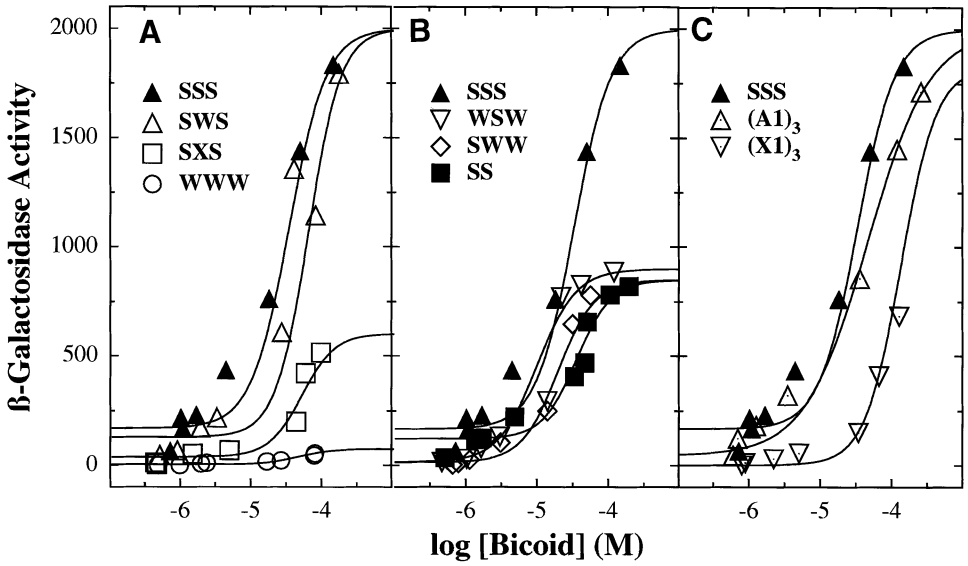

Fig. 3. Bicoid binds DNA in a cooperative, pairwise manner in yeast, and distinguishes between sites of different affinity from the *hunchback* enhancer. (**A**) Bicoid bound to strong (S) sites promotes binding to a weak (W) site. (**B**) Bicoid binds with pairwise cooperativity. (**C**) Cooperative interactions amplify differences between the intrinsic affinities of strong (A1) and weak (X1) sites. The data were generated as shown in Figure 1, and the curves represent the best fit of the data to a simple concentration-dependent transition. Reporter constructs contained the indicated sites inserted upstream of *lacZ*.

site can be loaded to form a triply bound complex. We suspect that cooperative interactions between Bicoid bound to the adjacent S sites promotes binding to the non-specific X site. Loading of the X site might also occur *in vivo*; we observe that the level of activation of an SXS reporter at very high Bicoid concentrations (higher than shown in Table III) exceeds that of SS (data not shown).

Bicoid cooperative interactions are pairwise

We tested whether Bicoid cooperativity is pairwise, that is, does Bicoid bound to a strong site interact cooperatively with only one additional Bicoid monomer at a time. To do this, we measured the level of β-gal activity resulting from a three-site reporter gene containing a strong site in the middle position (WSW). If Bicoid bound to the S site facilitated Bicoid binding to both W sites, then we would expect the level of gene expression to be comparable to that of SSS. Instead, we find that the level of activation by WSW is similar to that of SS, particularly at high Bicoid concentrations (Figure 3B, Table II), suggesting that only one or the other weak site is occupied, consistent with the idea of pairwise cooperativity. Our assay cannot determine whether a single weak site is being occupied, or whether both weak sites are occupied part of the time with equal probability; however, these two conditions are statistically equivalent. Likewise, the activity of SWW is similar to that of SS, again suggesting that only one of the weak sites is occupied. Taken together, these data support the idea that a Bicoid monomer bound to a strong site mediates predominantly pairwise cooperative DNA binding.

Pairwise cooperativity predicts that under certain conditions, discrete pairs of adjacently bound Bicoid monomers will dominate the binding process. Indeed, we found that the levels of activity elicited by SSW at low and intermediate Bicoid concentrations are similar to that of SS, implying that the W site is vacant at these concentrations. However, at the highest Bicoid concentrations examined, SSW stimulates more gene expression than SS, but still less than SSS (or SWS), which suggests that the W site in SSW is only partially occupied. Thus, most of the cooperative free energy is shared between monomers bound to the S sites, with little available to facilitate binding to the W site, which loads only at very high Bicoid concentrations.

Bicoid pairwise cooperativity is directional

If Bicoid cooperativity is strictly pairwise, then the maximum activity resulting from Bicoid binding to a strong and weak site (SW) should yield transcription activity comparable to that of SS. However, the maximum level of activity elicited by SW is 3 to 4-fold weaker than that of SS, although it is still 5-fold greater than that of a single S site (Table II). The reason for this is unclear. One explanation is that Bicoid cooperativity is directional. To test this idea, we reversed the orientation of the strong and weak sites (i.e. WS) and found that the activity of the WS reporter is more similar to that of SS (about twice that of SW) at all Bicoid concentrations (Table II). Gel-shift experiments gave similar results: WS sites are bound with slightly higher affinity than are SW sites (data not shown). Thus, a Bicoid monomer bound to a strong site prefers to interact with a Bicoid monomer positioned in the 5′ direction.

Yeast assay distinguishes between A1 and X1 sites in hunchback

Driever *et al.* (1989) identified two classes of Bicoid sites in the *hb* promoter region: strong sites (termed A1, A2 and A3) and weak sites (termed X1, X2 and X3), and a third uncharacterized class (termed B1, B2 and B3). However, *in vitro* measurements by others of Bicoid binding to the so-called strong and weak sites failed to resolve a difference in affinity between them (Ma *et al.*,

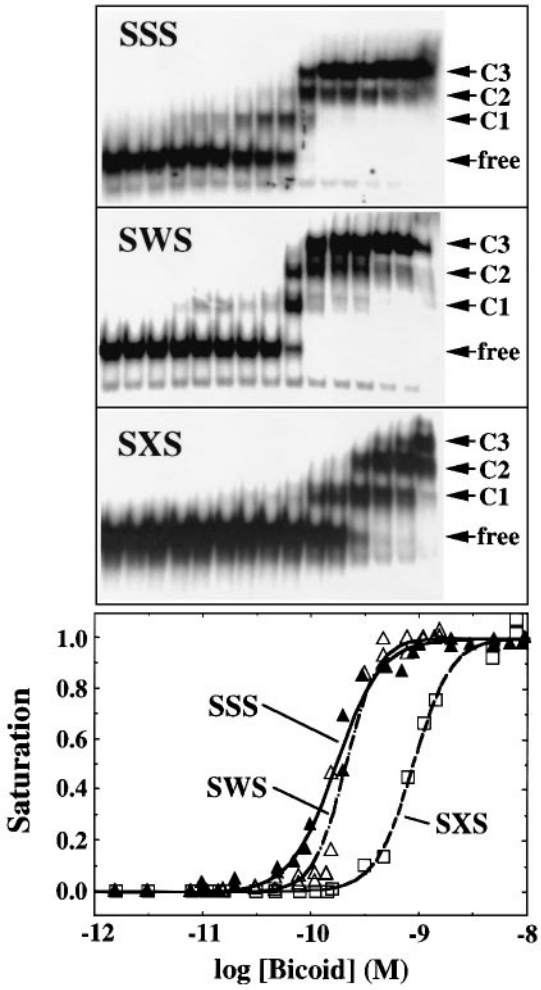

Fig. 4. Bicoid cooperative DNA binding shows strong-site/weak-site coupling *in vitro*. The upper three panels are gel-shifts of Bicoid$^{89–154}$ binding to oligonucleotide templates SSS, SWS and SXS. The lower panel shows isotherms resolved from data in the upper panels and data from other experiments. Note that the isotherm for SWS is nearly coincident with that of SSS, implying occupancy of the W site. Bicoid concentration ranges are the same for all three gels (10^{-12} M–10^{-9} M, with five steps per log unit). Free DNA, and singly (C1), doubly (C2) and triply bound (C3) complexes are indicated. The templates are identical to oligonucleotides used to make the reporter genes tested in Figure 3.

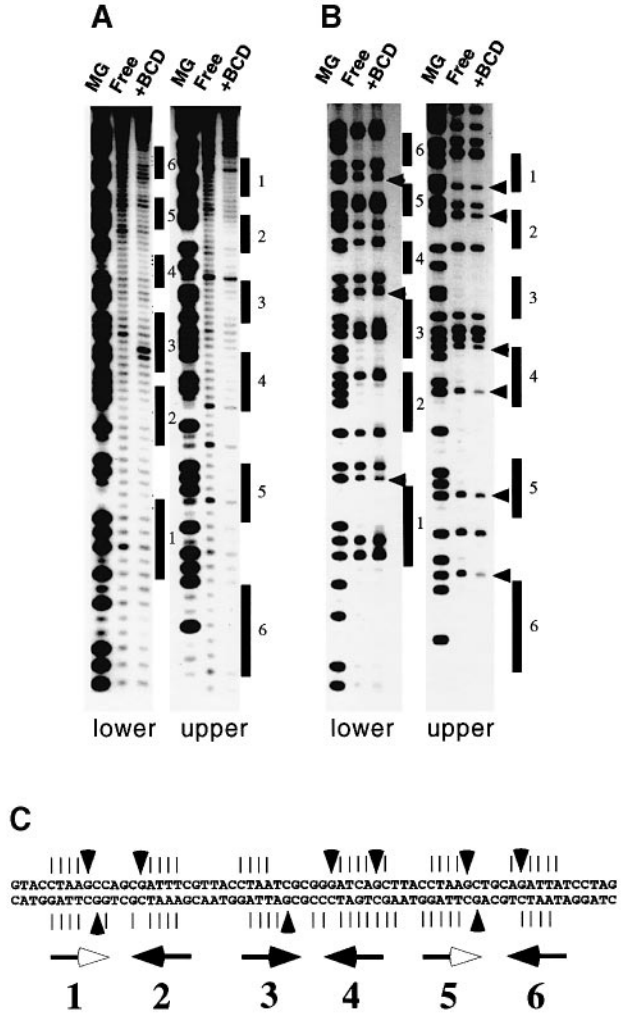

Fig. 5. Structural analysis of the *kni*64 element reveals three pairs of opposed Bicoid binding sites. (**A**) Hydroxyl radical footprints identify Bicoid binding sites (bars) on both strands of the *kni*64 element. MG: G + A cleavage of the Maxam and Gilbert reactions. Free: no protein added to the reaction. +Bcd: 1–10 pM Bicoid$^{89–154}$ added to the reaction. (**B**) DMS footprints of the same element; arrowheads indicate the protected bases. (**C**) Summary of the Bicoid binding sites present in the *kni*64 element deduced from the pattern of hydroxyl radical attack (lines) and the DMS-protected G residues (arrowheads). Filled arrows and open arrows identify predicted strong and weak sites, respectively.

1996). In contrast, our assay easily resolves differences between Bicoid recognition of A1 and X1 sites *in vivo*. For example, Bicoid activation of *lacZ* reporters containing three A1 sites is virtually identical to that observed for SSS, while the activation by reporters containing three X1 sites is substantially lower, suggesting a reduced level of occupancy (Figure 3C). These results are consistent with the gradient-affinity model and experiments in transgenic *Drosophila* embryos in which strong A sites have been shown to drive reporter gene expression further along the A–P axis than do weak X sites (Driever *et al.*, 1989; Struhl *et al.*, 1989). However, the model might not hold for all Bicoid target genes (see below).

Is cooperative gene activation necessary for knirps activation in the posterior of the embryo?

Expression of the gap gene *knirps* depends on a 64 bp enhancer known as *kni*64 which carries six potential Bicoid binding sites, some of which are predicted to be strong while others are predicted to be weak (Rivera-Pomar *et al.*, 1995). Biochemical analysis of this sequence by hydroxyl radical footprinting and dimethyl sulfate protection assays confirms that all six sites can be bound by Bicoid *in vitro* and that they are arranged in quasi-palindromic, opposed pairs (Figure 5). This clustering and unique arrangement of sites within a very short stretch of DNA (64 bp) suggested to us that cooperative interactions between Bicoid and *kni*64 might result in an extraordinarily high-affinity interaction. This could explain how *knirps* is expressed far down the Bicoid gradient in the posterior of the embryo. To test this idea, we examined the interaction between Bicoid and *kni*64, and derivatives of *kni*64 in transgenic *Drosophila* embryos, in yeast, and *in vitro*.

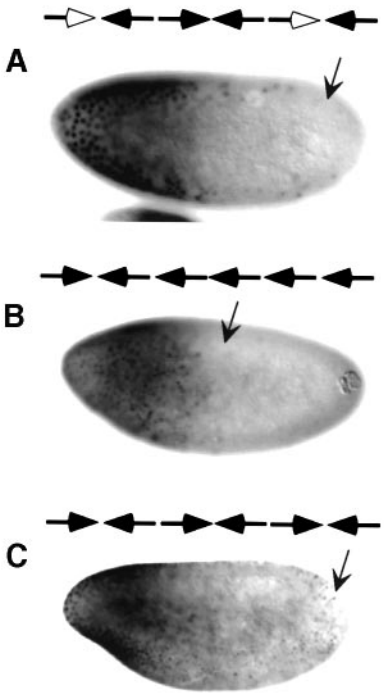

Fig. 6. Orientation of Bicoid binding sites is critical for posterior activation in embryos. *In situ* hybridization is used to detect *lacZ* reporter gene expression in transgenic, blastoderm-staged embryos. The arrow indicates the posterior limit of expression. Above each embryo the schematic indicates the array of Bicoid binding sites in the reporter transgene; filled arrows represent strong sites, open arrows predicted weak sites. (**A**) *LacZ* expression in an embryo carrying a *kni*64 reporter extends far into the posterior. (**B**) *LacZ* expression is restricted to more anterior positions when the orientation of two of the six Bicoid consensus sites are reversed in the 2R*kni* reporter. (**C**) *LacZ* expression using a reporter, Ψ*kni*, in which Bicoid consensus sites are arranged as in *kni*64, extends even further into the posterior than *kni*64.

Kni64 drives posterior expression and is dependent on the orientation of the Bicoid binding sites

We generated transgenic embryos containing reporter genes that carry the *kni*64 element and altered versions of it. In contrast to the Bicoid-site reporter genes used by Driever *et al.* (1989), Struhl *et al.* (1989) and Hanes *et al.* (1994), which are anteriorly restricted, *kni*64 can drive expression over the entire length of the embryo (Figure 6A). Expression in transgenic embryos starts early in development before nuclear cycle 13. Most of the sites of transcription appear as 'nuclear dots' consisting of two spheres of staining within the nuclei (Pritchard and Schubiger, 1996), with some cytoplasmic accumulation of mRNA. Note that these constructs also show strong anterior transcription, which contrasts with the normal expression pattern of *knirps*. This is because the reporter constructs we used lack the normal *cis*-acting repressor element that inhibits *knirps* expression in the anterior region (Rivera-Pomar *et al.*, 1995). Early activation is completely abolished in embryos derived from *bcd* mutant females, which demonstrates that the activation is Bicoid dependent (Rivera-Pomar *et al.*, 1995).

If cooperative DNA binding is critical for Bicoid-dependent gene activation driven by *kni*64 in embryos, then we would expect the arrangement of individual sites within the *kni*64 element to be important, since changes in geometry often disrupt cooperative interactions (Smith and Johnson, 1992). We tested this by reversing the orientation of two of the six sites within *kni*64 and testing this enhancer (2R*kni*) in transgenic embryos. Note that, in this experiment, all the Bicoid sites are idealized strong consensus sites. This reporter is not activated as far into the posterior region of the embryo (Figure 6B) as reporters that carry either the native *kni*64 element (Figure 6A) or an idealized *kni*64 element called pseudo-*knirps* (Ψ*kni*) in which all the Bicoid sites are consensus sites and are oriented in opposed pairs as in *kni*64 (Figure 6C). In the extreme case in which all six Bicoid sites are oriented in the same direction (head-to-tail), no activation is detected (Hanes *et al.*, 1994). These experiments demonstrate that the orientation of sites is critical for Bicoid-dependent gene activation in embryos, and suggests the possibility that the native *kni*64 element is organized so as to promote high affinity, cooperative DNA binding and/or cooperative gene activation (synergy) by Bicoid.

Analysis of the kni64 element in yeast and in vitro

Based on the results in *Drosophila* embryos, we tested the ability of *kni*64 and its derivatives to drive transcription of *lacZ* reporter genes in yeast and to bind Bicoid *in vitro*. In contrast to the embryo results, the orientation of individual sites was not critical; there was no significant difference between *lacZ* activation driven by *kni*64, 2R*kni* or the Ψ*kni* element (Table II). At very low Bicoid concentrations, the idealized element Ψ*kni* was slightly more effective than *kni*64, as expected, because all the Bicoid sites are strong consensus sites; whereas at higher Bicoid concentrations, activation by *kni*64 was indistinguishable from that of the idealized element Ψ*kni*, which suggests that cooperative coupling between strong and weak sites in *kni*64 overcomes any differences in their intrinsic affinities.

The inability to detect differences between the various *kni*64 derivatives in the yeast assay prompted us to compare their binding affinities for Bicoid *in vitro*. Using gel-shift assays we determined the overall affinity of $Bcd^{89–154}$ for each element and found no significant differences (Table III). Qualitatively similar results were obtained using full-length Bicoid (data not shown). Therefore, the differences in the posterior border of expression observed in embryos carrying *kni*64, 2R*kni* and Ψ*kni* reporters must be due to something other than differences in DNA binding affinity. For example, it is possible that the interaction of DNA-bound Bicoid with the transcription machinery in embryos is sensitive to the orientation of Bicoid monomers on the DNA.

Test of the gradient-affinity model for Bicoid-dependent gene activation

According to the gradient-affinity model for Bicoid action, a posteriorly expressed gene should have a higher affinity for Bicoid than an anteriorly expressed gene (Driever *et al.*, 1989). Thus, the *kni*64 element from *knirps* should have a higher affinity for Bicoid than the enhancer element from the anterior gene, *hunchback*. We tested this directly by comparing the ability of *kni*64 and the 230 bp *hunchback* element (Driever and Nüsslein-Volhard, 1989) to drive expression in our yeast assay, and by comparing

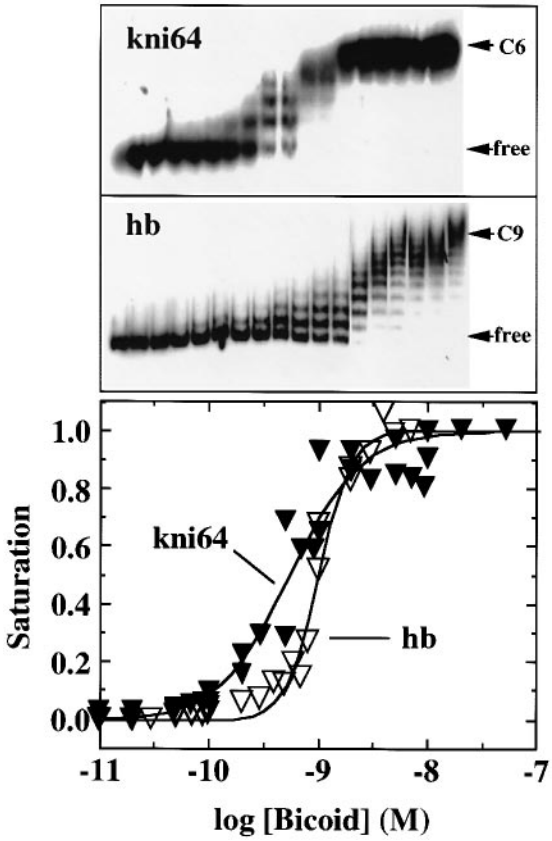

Fig. 7. The affinity of Bicoid for a posterior gene is comparable to that of an anterior gene. The upper two panels are representative gel-shifts of Bicoid$^{89–154}$ binding to *kni*64 and the 230 bp *hb* enhancer. The isotherms in the lower panel show that the K_ds for Bicoid binding to *kni*64 and the 230 bp *hb* enhancer are almost identical. However, the loading of *kni*64 occurs at a slightly lower Bicoid concentration and the isotherm for the *hunchback* element is steeper, implying a higher degree of cooperativity. Bicoid concentration ranges for both gels are ~10^{-11} M–10^{-8} M, with six steps per log unit. Free DNA and the maximally bound complexes are indicated.

their affinities for Bicoid *in vitro*. In yeast, the *kni*64 and the *hb* element were virtually indistinguishable at all Bicoid concentrations tested, and, if anything, the *hb* element was more active (Table II). Gel-shift assays showed that the overall affinity of Bicoid for *kni*64 was less than 2-fold greater than that of the *hb* element (Figure 7; Table III). Furthermore, while the Hill coefficient for each showed cooperative binding, the *hb* element (n_H = 3.0; nine sites) appears to be slightly more cooperative than *kni*64 (n_H = 1.4; six sites). These affinity measurements argue against the simple interpretation of the gradient-affinity model for Bicoid action.

Discussion

In this study we have shown that Bicoid binds DNA cooperatively *in vivo* and *in vitro*, and that this cooperativity results in the occupancy of sites that would otherwise not be recognized by Bicoid. Cooperative DNA binding lowers the concentration of Bicoid required to drive high levels of gene activation in yeast, and presumably in *Drosophila*. Our results help explain how, in *Drosophila* embryos, genes like *hunchback* are activated by Bicoid in a highly concerted manner, thus allowing the Bicoid gradient to activate its target genes in a step-function-like pattern. This on/off conversion helps establish precise domains of gene expression critical for proper segmentation. Our results also suggest that Bicoid cooperativity is likely to be important for activation of posterior genes such as *knirps* and *hairy* stripe 7 in the posterior region of the embryo, where the Bicoid concentrations are extremely low. However, in contrast to the gradient-affinity model for Bicoid action, much of this cooperativity might be contributed by a process other than DNA binding, for example, by transcriptional synergy. A model for how Bicoid activates genes throughout the entire embryo is discussed.

A yeast assay for studying cooperativity in vivo

We have developed a sensitive assay to study cooperative DNA binding by heterologous proteins in yeast. This assay should be broadly applicable to other DNA binding proteins. By generating a wide range of Bicoid concentrations and using reporter genes with appropriate combinations of strong and weak binding sites, we were able to infer the occupancy of these sites *in vivo*. A similar approach has been used in classic studies of bacteriophage λ repressor (Ptashne *et al.*, 1980) and in binding studies involving LacI (Lehming *et al.*, 1987), TrpR (Staacke *et al.*, 1990) and Gal4 (Xu *et al.*, 1995; Tanaka, 1996). Since binding takes place within an intracellular environment (physiological pH, ionic strength, etc.), the assay revealed features of Bicoid DNA binding that were not possible to detect *in vitro*. For example, this sensitivity allowed us to resolve easily the different affinities of the X1 and A1 sites of the *hunchback* upstream element for Bicoid. In this assay, full-length soluble protein is produced, rather than truncated proteins typically used for *in vitro* experiments; this leaves intact the domains that may be important for the subtle effects on binding-site recognition, cooperative DNA binding and protein–protein interactions. Finally, although both cooperative DNA binding and transcriptional synergy contribute to gene activation by Bicoid, a major accomplishment of this assay was the experimental uncoupling of these events to demonstrate cooperative DNA binding *in vivo*.

Cooperative gene activation Bicoid

As with many transcription activators, Bicoid-dependent gene activation increases in a more-than-additive fashion with increasing numbers of binding sites (Driever *et al.*, 1989; Hanes and Brent, 1989; Struhl *et al.*, 1989; this study, Figure 2). However, this fact alone does not necessarily indicate cooperative DNA binding, since non-additivity can arise from at least three different phenomena. The first is transcriptional synergy in which multiple DNA-bound proteins interact with members of the transcription machinery, either basal components or transcription co-activators (reviewed in Verrijzer and Tjian, 1996). Indeed, Bicoid has been shown to activate synergistically transcription *in vitro* (Sauer *et al.*, 1995) and possibly *in vivo* (Sauer *et al.*, 1996), by interacting with $TAF_{II}60$ and $TAF_{II}110$, and our yeast data (Figure 2) suggest that synergy is an important component of the high-level activation by reporters that carry more than one Bicoid site. The second is a statistical effect that results from the simultaneous loading of multiple binding sites of similar

affinities even in the absence of cooperative interactions (Cantor and Schimmel, 1980a). This effect results in a high degree of site occupancy and a steep threshold response that appears as non-additivity. However, the statistical effect does not change the overall binding affinity from that of the individual sites. The third is *bona fide* cooperative DNA binding as occurs, for instance, between lambdoid (cI or HK022) repressor dimers (Ackers *et al.*, 1982; Ptashne, 1986; Carlson and Little, 1993). This effect does increase the overall binding affinity, resulting in concerted loading of sites at a lower overall concentration of protein and a sharp transition response to a relatively small increase in activator concentration. For Bicoid, this was best demonstrated using strong-site/weak-site combinations.

Cooperative coupling between weak and strong sites in embryos

We have shown in yeast and *in vitro* that Bicoid bound to a strong site promotes occupancy of a nearby weak site (Figure 3A, Figure 4). Coupling between strong and weak sites is therefore likely to be important for regulation of Bicoid-dependent genes in *Drosophila* embryos. For example, the three so-called weak sites, X1, X2 and X3, within the 230 bp *hunchback* upstream element are predicted to be weaker than neighbouring A sites. Our results predict that the X1–X3 sites are important for Bicoid-dependent expression due to coupling with A sites, and are likely to be occupied even at moderate Bicoid concentrations. This cooperative coupling would enhance the sharp threshold reponse observed for Bicoid-dependent *hunchback* expression in embryos. The fact that the weak sites are conserved in the upstream region of *hunchback* from *Drosophila virilis* (Treier *et al.*, 1989) suggests that they are functionally important. Moreover, two of the six Bicoid sites in the *kni*64 element are predicted to be of low affinity, yet this element is extremely sensitive to Bicoid-dependent regulation (Rivera-Pomar *et al.*, 1995). This suggests that the *kni*64 element is optimally configured to respond to very low Bicoid concentrations through cooperative coupling between strong and weak sites.

The weak site used in our experiments contains a C:G to T:A base pair change that disrupts a critical contact between Lys50 of the Bicoid homeodomain and position 7 of the consensus Bicoid site. The T:A base pair at this position (TCTAATTCC) is characteristic of the binding consensus for homeodomain proteins of the Antennapedia class (Laughon, 1991). Cooperative coupling between Bicoid monomers is strong enough to compensate for the disruption of the Lys50-C:G contact so that Bicoid now binds an Antennapedia-like site. On their own, sites that contain a T:A at this position are not recognized well by Bicoid in yeast or in *Drosophila* embryos even at high Bicoid concentrations (Hanes and Brent, 1991; Hanes *et al.*, 1994). This finding has far-reaching implications regarding the combinatorial nature of gene regulation by homeodomain proteins, suggesting that they may use cooperative coupling to bind sub-optimal sites and/or to recruit other homeodomain proteins that would not ordinarily bind.

Pairwise cooperativity by Bicoid

Cooperative binding by Bicoid monomers appears to be pairwise (Figure 3B). Pairwise cooperativity has been shown to occur between DNA-bound dimers of cI repressor from bacteriophage λ and HK022 (Ackers *et al.*, 1982; Brenowitz *et al.*, 1986; Carlson and Little, 1993), both of which bind to three-site operators. In the case of λ cI repressor, pairwise interactions occur to either side of DNA-bound dimers, and differ in strength by only ~1 kcal (Burz and Ackers, 1994). This implies considerable flexibility in the DNA-bound protein and that different surfaces may mediate cooperative protein–protein contacts. We propose that Bicoid, like λ repressor dimers, is flexible when bound to the DNA, and can interact with an adjacent monomer on either side (as in WSW), but that these interactions differ in strength. This would explain Bicoid's apparent directional preference, i.e. why WS reporters are more active in yeast than SW reporters (Table II) and are bound better *in vitro* (data not shown). These observations underscore the idea that the arrangement of sites in an upstream regulatory element is critical for dictating the transcriptional response of that element to an activator protein.

Other homeodomain proteins are likely to bind DNA in a pairwise cooperative manner. For example, the *Drosophila* Paired homeodomain binds cooperatively to a dimeric site (Wilson *et al.*, 1993), suggesting that these interactions might be pairwise. Cooperative interactions also occur between heterologous homeodomain proteins **a**1 and α2 from yeast (Mak and Johnson, 1993; Phillips *et al.*, 1994), Extradenticle and HomC proteins from *Drosophila* (Lu and Kamps, 1996; reviewed in Mann, 1995; Wilson and Desplan, 1995) and between Pbx and Hox proteins from human (van Dijk *et al.*, 1995; Chang *et al.*, 1996; Peltenburg and Murre, 1997). It is possible that, like Bicoid, these proteins will show strong-site/weak-site cooperative coupling, and that these interactions will depend on the precise configuration (spacing, orientation) of their binding sites.

Bicoid binds DNA as a monomer

Bicoid appears to bind DNA as a monomer, engaging in cooperative protein–protein interactions only in the presence of DNA. In this model, Bicoid–Bicoid interactions occur only when at least one monomer is bound to DNA. This is similar to the model proposed for the prokaryotic repressors LexA (Kim and Little, 1992) and a mutant cI repressor (PT158) from bacteriophage λ (Burz and Ackers, 1996), both of which dimerize on sequence-specific DNA. The idea that Bicoid binds DNA as a monomer is supported by several lines of evidence: first, Bicoid binds to a single non-palindromic (S) site with a K_d of 0.24 nM. Secondly, Bicoid does not dimerize or oligomerize in sedimentation equilibrium experiments, up to a total protein concentration of ~1 μM (R.Rivera-Pomar and H.Jäckle, unpublished), nor does full-length Bicoid interact with full-length Bicoid in a two-hybrid assay (R.Hackett and S.D.Hanes, unpublished). Finally, when a single site is present, Bicoid activates gene expression weakly in yeast (this study) and in embryos (Driever *et al.*, 1989), suggesting that a monomer can bind *in vivo*.

Activation of hunchback might not require synergy with Hb^{mat}

Previous work of Simpson-Brose *et al.* (1994) reported that activation of zygotic *hunchback* required synergistic interaction between Bicoid and maternal Hunchback protein (Hb^{mat}) and suggested that the sharp posterior border of *hunchback* expression was due to this synergy. Their experiments relied on reporter genes that carried both Bicoid and Hunchback binding sites. In contrast, our work (Hanes *et al.*, 1994) shows that reporter genes that carried Bicoid-binding sites but lacked Hunchback-binding sites were strongly activated in embryos, and that the expression domain had a sharp posterior border. Thus, synergy with Hb^{mat} apparently is not required for strong Bicoid-dependent gene expression. Bicoid cooperativity as demonstrated in the present study provides a mechanism by which a sharp threshold response can be generated by Bicoid without invoking synergy with Hb^{mat}.

Bicoid activation throughout the embryo

The anterior morphogen model of Bicoid action (reviewed in Driever, 1993) posits that (i) the intensity of gene transcription and sharpness of the posterior border of anteriorly expressed genes depends on the number of Bicoid binding sites and (ii) that the extent to which Bicoid-regulated genes are expressed along the A–P axis depends on the affinity of their binding sites.

Our results provide a mechanism to explain hypothesis (i) above. Cooperative coupling between strong and weak sites promotes the occupancy of intrinsically weak sites, increasing the probability that more Bicoid sites will be occupied in a given enhancer. This coupling would increase the intensity of gene expression, as observed for the *hunchback* gene. Cooperative DNA binding also results in concerted loading of Bicoid sites, and thus steepens the transition between the on and off states for gene activation, sharpening the borders of the expression domains as observed for both *hunchback* and *eve* stripe 2.

Our results are not consistent with hypothesis (ii) above. In contrast to the simple prediction based on the Bicoid gradient-affinity model, the *knirps* enhancer element (*kni*64) did not show a significantly higher affinity for Bicoid than did the 230 bp *hunchback* enhancer element. Thus, while cooperative DNA binding will increase Bicoid's overall affinity for target genes like *knirps* and *hairy* stripe 7, it does not explain why they are expressed further down the Bicoid gradient than genes like *hunchback*. We suggest that for some genes, a mechanism other than DNA binding affinity can dictate the extent of their expression along the A–P axis. For example, it is possible that the tight clustering and unique arrangement of Bicoid sites within the *kni*64 element promotes highly cooperative interactions with the transcription machinery. If these interactions were more favorable than those occuring at the *hb* enhancer element, then this would explain how *knirps* is expressed more posteriorly than is *hunchback*.

Materials and methods

Yeast expression and reporter constructs

Bicoid was expressed in yeast from plasmid pDB1, which contains a full-length Bicoid cDNA inserted into the *Eco*RI site of pBC103 (gift of Barak Cohen and Roger Brent). Plasmid pDB1 (2μ, *LEU2*) expresses HA-1 epitope-tagged Bicoid from the *GAL1* promoter. Expression was driven using a GAL4–ER–VP16 fusion protein (Louvion *et al.*, 1993), or a mutated version, GAL4–ER–VP16(mut), which contains a point mutation (F442P) in the VP16 activation domain (Grace Stafford and Randy Morse, submitted) that lowers its activation potential (Regier *et al.*, 1993).

Yeast reporter plasmids were constructed by inserting Bicoid site oligonucleotides into the *Xho*I site of pLR1Δ1 (West *et al.*, 1984; Hanes and Brent, 1989). These plasmids (2μ, *URA3*) contain a *GAL1–lacZ* reporter gene whose expression is Bicoid dependent (the UAS_G is deleted). The oligonucleotides were of the general sequence 5′-TCGAC[(Bicoid site)TA]$_n$-3′ and varied only in the number and sequence of Bicoid binding sites. The sites were spaced 11 bp center-to-center and oriented head-to-tail (→ →) unless otherwise indicated. The strong (S) site used in these experiments was the Bicoid consensus site [TCTAATCCC] derived from the *hunchback* upstream element (Driever and Nüsslein-Volhard, 1989). The weak (W) Antennapedia-like site [TCTAATTCC] has a C to T change at position 7 (Hanes and Brent, 1991). The 'dead' (X) site [TCCACAGCC] lacks the consensus for homeodomain recognition and Bicoid specificity (Hanes *et al.*, 1994). Strong sites (A1) [CGTAATCCC] and weak sites (X1) [GCTAAGCTG] are as found in the *hunchback* upstream element (Driever *et al.*, 1989). The *kni*64 element was as described previously (Rivera-Pomar *et al.*, 1995). The sequences of Ψ*kni* and 2R*kni* are available upon request. The 230 bp *hunchback* enhancer (Driever *et al.*, 1989) was amplified from *Drosophila* DNA by PCR and inserted into the *Xho*I site of pLR1Δ1.

Expression and purification of proteins

To express an epitope-tagged Bicoid in *Escherichia coli* strain BL21 (DE3) (Studier and Moffatt, 1986) for use as a standard in Western analyses, we constructed plasmid pDBHABcd-HD, which expresses a 6× His-, HA-tagged fusion protein (Bicoid amino acids 56–170, which includes the homeodomain), and which carries 40 non-specific vector-encoded amino acids at its C-terminus, under the control of an inducible T7 promoter. The protein was purified using Ni^{2+} affinity chromatography (Novagen). The truncated Bicoid (Bicoid$^{89–154}$) used in binding assays, was expressed as described previously (Rivera-Pomar *et al.*, 1995). The concentration of HABcd-HD was estimated by averaging the contributions of Phe, Tyr and Trp to the intrinsic absorbance at 280 nm (Cantor and Schimmel, 1980b), while Bicoid$^{89–154}$ was quantitated according to the Trp absorption spectrum of the denatured protein. Full-length Bicoid was produced using a Baculovirus expression system (InVitrogen).

Yeast transformations, cell growth and β-galactosidase assays

Saccharomyces cerevisiae strain EGY48 (*MAT*α, *leu2, ura3, trp1, his3 lexAOp-LEU2*) (Gyuris *et al.*, 1993) was used throughout. Transformations were performed using the LiOAc procedure (Ito *et al.*, 1983). Transformants were grown in complete synthetic medium lacking uracil, leucine and histidine, to maintain the three plasmids used in the assay. Hormone (β-estradiol; Sigma) was added to induce Bicoid synthesis when the cells reached 0.12–0.15 OD_{600} (~2–3×10^7 cells/ml). Induced cultures were grown to mid-log phase (~0.5 OD_{600}; 1×10^8 cells/ml), and harvested for both β-gal assays and Western analyses. (Detailed procedures for cell growth, collection and assay are available upon request.) β-galactosidase assays were performed essentially as described previously (Hanes and Brent, 1989). For each clone, at least five independent transformants were assayed and values were averaged; errors were typically <10%.

Quantitation of Bicoid concentrations

Yeast cells were thawed in the presence of protease inhibitors and proteins separated by SDS–PAGE. Samples were diluted prior to loading, such that the Bicoid concentrations fell into the linear range of detection by Western analysis. The HA-1 epitope-tagged Bicoid standard (HABcd-HD) was mixed with control EGY48 cell extracts prior to SDS–PAGE, which was found to improve the blotting efficiency. Proteins were transferred to Immobilon P membranes (Millipore), and reacted with monoclonal antibody 12CA5 (HA-1 epitope; Amersham) and sheep anti-mouse Ig HRP-linked secondary antibody (Amersham). Chemiluminescent signals were generated using ECL reagents (Amersham) and exposed to X-Omat film (Kodak).

Quantitative densitometry of the luminescent signal was performed using an ImageMaster DTS (Pharmacia) and the data processed using ImageMaster software. The response was linear between 0.01 to 2.0 OD units; the peak ODs of all the data fell within this range. Bicoid

concentrations were determined by comparing the OD of the full-length Bicoid to a calibration curve generated using known molar quantities of HABcd-HD. This internal standard was included on every gel to eliminate errors due to variation in transfer efficiency, antibody reactivity and developing conditions.

Reporter constructs and P-mediated transformation

The *kni*64 element (Rivera-Pomar *et al.*, 1995) and synthetic reporter genes were subcloned into pBluescript KS(+) (Stratagene) and subsequently inserted into pCaSpeR-hs43 (Thummel and Pirrotta, 1992). Fragments were ligated into the shuttle vector in their original orientation with respect to the transcription start site. P element-mediated transformation was performed using standard procedures (Rubin and Spradling, 1982). The genetic marker, *white* (*w*), was used for insertion detection in either *w* or *yw* flies. At least three viable, independent homozygous lines representing P-insertions were analyzed. Reporter gene (*lacZ*) expression was examined by *in situ* hybridization using anti-sense RNA probes according to Klingler and Gergen (1993). The mutant stock used for genetic analysis, bcd^{E1}, is described in Fly Base (http://cbbridges.harvard.edu:7081/). To assess the correct mutant identity and avoid spurious phenotypes due to the P-insertion, the stocks of P-insertions were analyzed for segmentation defects by examination of cuticle preparations, prior to crossing into the appropriate mutant background.

In vitro binding assays

DNA-labeling reactions were performed by filling-in the recessed 3′ end using the Klenow fragment of DNA polymerase and α-[^{32}P]labeled dNTPs (Sambrook *et al.*, 1989). For gel shift assays, reactions were carried out in 25 mM HEPES K (pH 7.5) containing 0.1 M KCl, 12.5 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.1% NP-40 and 40% glycerol, and equilibrated at 4°C for 1 h, loaded onto polyacrylamide gels at 18 V/cm and electrophoresed at 12 V/cm. DNA concentrations were sufficiently low to assume that total protein equals free protein. Saturation (Y_{bar}) was determined by summing the fraction of each complex formed, multiplied by its stoichiometry (for a given protein concentration), and dividing by the total number of sites present. Quantitation of autoradiographic densities was performed using an ImageMaster DTS (Pharmacia) and the data were fitted to the equation $Y_{bar} = K^n[X]^n/1+K^n[X]^n$ to obtain estimates of the Hill coefficients and binding constants using the non-linear least-squares program, NONLIN (Johnson and Frasier, 1985). Footprinting reactions (Tullius and Dombrowski, 1986) and dimethyl sulfate (DMS) protection experiments (Rhodes, 1989) were performed as described previously (Rivera-Pomar *et al.*, 1995). Briefly, 1–10 pmol protein (purity >95%, as assessed by SDS–PAGE and silver staining) were incubated with 1–10 fmol labeled DNA for 5 min on ice in reaction buffer. After cleavage, the samples were extracted with phenol, ethanol precipitated and loaded onto a 10% polyacrylamide-8M urea gel. Quantitation of the extent of protection was performed by scanning autoradiographs or by using a PhosphorImager (Molecular Dynamics). Protected regions were identified using a G + A sequence ladder (Maxam and Gilbert, 1980).

Acknowledgements

We wish to thank Randy Morse for suggesting the use of and providing the GAL4–ER–VP16 expression plasmids. We also wish to thank Robin Hackett for technical assistance, Heike Tauber for embryo injections, the Wadsworth Center Core Facilities, Tim Moran, Matt Shudt and Gordon Dowe for oligonucleotide synthesis and sequencing, and Richard Lease and Vergine Madelian, for comments on the manuscript. This work was supported by the Alexander von Humboldt Society (R.R.-P.), the Company of Biologists Ltd. Traveling Fellowship (R.R.-P.), the Max-Planck Society (H.J., R.R.-P) and by Research Project Grant DB-128 from the American Cancer Society (S.D.H.).

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Received October 8, 1997; revised July 30, 1998; accepted August 25, 1998