Regions of GAL4 critical for binding to a promoter *in vivo* revealed by a visual DNA-binding analysis

Akiko Mizutani¹ and Masafumi Tanaka^{1,2,3}

¹Unit Process and Combined Circuit, PRESTO, Japan Science and Technology Corporation (JST), Kawaguchi, Saitama 332-0012 and

²Department of Genetic Information, Division of Molecular Life Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan

³Corresponding author
e-mail: tanakamf@is.icc.u-tokai.ac.jp

Binding of transcriptional activators to specific sites on DNA, mediated by their DNA-binding domain, is a key regulatory point in transcriptional regulation. With a GFP-based microscopic assay, we investigated how the prototypical activator GAL4 effectively binds to a promoter in living yeast cells. We show that GAL4 relies on a previously unrevealed mechanism involving ‘DNA-binding enhancers’ (DBEs), the regions of GAL4 that assist DNA-binding domain association with DNA. GAL4 contains two DBEs, one, but not the other, physically overlapping the principal transcriptional activation domain. Either of the DBEs, however, can function independently of transcriptional activation, indicating a discrete mechanism responsible for DNA-binding enhancement. The effect of DBEs, while not limited to natural target promoters, is still not universal and can be profoundly affected by the binding-site context. The GAL4 DBEs can also enhance promoter binding of an unrelated DNA-binding domain, and possibly represent a new modular functional unit responsible for effective binding of diverse regulatory factors to DNA *in vivo*.

Keywords: activator/DNA binding/GFP/transcription/yeast

Introduction

To exert an effect on the transcription of a gene, transcriptional activators need to associate with their corresponding target DNA sequences near the gene (Ptashne, 1988). Binding of activators to DNA is a major determining step in transcriptional activation, as modulations in the efficacy of DNA binding have profound impacts on transcriptional outcomes. The mechanism by which transcriptional activators bind to DNA have been studied extensively, although mostly *in vitro*. These studies have led to identification of small regions of these activators, referred to as DNA-binding domains, that directly interact with DNA in a sequence-specific manner and are often sufficient for effectively binding to DNA *in vitro* (Ptashne, 1988; Pabo and Sauer, 1992). In some cases, binding of an activator to DNA is also influenced by specific protein–protein interactions, such as those between the activator and another protein that binds to DNA

cooperatively with the activator (Johnson and McKnight, 1989).

These DNA–protein and protein–protein interactions, defined *in vitro* through DNA-binding assays typically involving naked DNA, have been shown to play critical roles in transcriptional activation in cells through the analyses of transcriptional effects of activators *in vivo*. These studies have led to a general view of activator architecture as follows: transcriptional activators minimally bear two interaction surfaces: the first, the DNA-binding region, positions the protein onto specific sites on DNA so that the second, the activating region, can interact with some component(s) of transcriptional machinery to promote initiation of transcription (Ptashne, 1988; Ptashne and Gann, 1990, 1997). It has remained uncertain, however, whether the interactions that can position the activators to specific sites on DNA *in vitro* are also sufficient for doing so in living cells, where DNA is packaged into an organized nucleoprotein structure (i.e. chromatin; Workman and Kingston, 1998; Narlikar *et al.*, 2002; Orphanides and Reinberg, 2002).

Determining elements of activator binding to DNA has seldom been examined quantitatively in living cells. Nevertheless, a limited number of such *in vivo* studies have been reported, and have often suggested that the interactions observed under typical *in vitro* conditions are only a part of the interactions that effect activator binding to DNA in living cells. It was shown, for example, that multiple sites within a promoter can cooperatively recruit cognate factors *in vivo* (Giniger and Ptashne, 1988; Vashee and Kodadek, 1995; Tanaka, 1996; Vashee *et al.*, 1998). This possibly promiscuous cooperativity in DNA binding, which may be generated by competition with histones (Adams and Workman, 1995; Polach and Widom, 1995), could be a general basis for synergistic transcriptional activation by multiple transcriptional activators in eukaryotic cells (Giniger and Ptashne, 1988; Ptashne and Gann, 1997). We and others have also shown directly (Bunker and Kingston, 1996; Tanaka, 1996) and indirectly (Svaren *et al.*, 1994; Vashee and Kodadek, 1995; Stafford and Morse, 1997; Vashee *et al.*, 1998) that transcriptional activation domains can impact on activator binding to DNA in cells. A close correlation between the ability of activation domains to augment DNA binding and transcription (Tanaka, 1996) implied that the DNA-binding effect of activation domains is another general determinant of activator binding to DNA in cells, as previously suggested (Ptashne, 1988). These findings indicated that the step of activator binding to DNA plays a more substantial role than generally realized in sensing the promoter structure and activator quality/quantity to give rise to a specific transcriptional output. *In vivo* mechanisms of effective activator binding to DNA, however, are still poorly understood, with some basic issues remaining. It has been largely untested, for example, which regions of

natural transcriptional activators, other than their DNA-binding domains, are important for effective binding to DNA in cells.

In the present work, we extend our analyses on activator binding to DNA in living cells, using a green fluorescent protein (GFP)-based visual DNA-binding assay. Recent development in the use of GFP made it possible to visualize the association of DNA-binding proteins, such as the *lac* repressor, with their cognate sites in living cells (Robinett *et al.*, 1996). By using this technique, the association of a nematode transcriptional factor GATA with its own promoter (Fukushige *et al.*, 1999), and that of the glucocorticoid receptor with the mouse mammalian tumor virus promoter (McNally *et al.*, 2000), have been visualized in cells. Here we apply a GFP-based strategy to yeast and develop an *in vivo* visual DNA-binding assay for yeast GAL4, a prototypical and extensively studied transcriptional activator. With the visual DNA-binding assay, we functionally dissect GAL4 to examine which regions of GAL4 are important for effective binding to promoter DNA in living cells. Through these analyses, we uncover a new functional unit—the DNA-binding enhancer—of GAL4; GAL4 contains two DNA-binding enhancers, either of which can dramatically facilitate activator binding to a promoter *in vivo* without relying on transcriptional activation.

Results

Visualization of GAL4 binding to a promoter in living yeast cells

The system we developed to visualize microscopically the GAL4 transcriptional activator binding to promoter DNA in living cells involves three components (Figure 1A): (i) a tandem array of a red fluorescent protein (RFP)-reporter cassette, containing a test promoter such as the yeast *GAL1* promoter; (ii) the GAL4–yellow fluorescent protein (YFP) fusion effector; and (iii) a chromosomal position marker, composed of a tandem array of the *lacO* sequence and the *LacI*–cyan fluorescent protein (CFP) fusion protein (Robinett *et al.*, 1996; Straight *et al.*, 1996). To visualize GAL4–YFP protein binding to the *GAL1* promoter in cells (note that the *GAL1* promoter contains four GAL4-binding sites; Bram and Kornberg, 1985; Giniger *et al.*, 1985), we initially tested one, three and six tandem copies of the *GAL1*–RFP reporter cassette, integrated into a chromosome. With the three- and six-copy reporter arrays, distinct GAL4–YFP foci of fluorescence were detected, with higher fluorescence from the six-copy reporter array, while the levels of RFP expression increased in a near linear fashion, depending on the number of reporter reiteration (data not shown). We therefore used the six-copy reporter array to visualize the YFP-fusion effectors binding to DNA in most of the analyses described below.

We first verified that the YFP fluorescent foci detected with the six-copy reporter array were indeed formed by the GAL4–YFP fusion proteins bound to their cognate sites, based on the positions of the YFP foci. Figure 1A shows that, as previously reported in other cases (Robinett *et al.*, 1996; Straight *et al.*, 1996), the *LacI*–CFP proteins concentrated into foci of fluorescence by binding to the *lacO* array (CFP images), which served as a chromosomal position marker. Six tandem copies of the *GAL1*–RFP

Fig. 1. Visualization of GAL4 binding to the *GAL1* promoter in living cells. Yeast cells derived from four different reporter strains were examined for YFP, CFP and RFP fluorescence. The reporter cassette contained the *GAL1* promoter (A and B), the *HIS4-a* promoter (C), bearing no GAL4 site, or the *HIS4-b* promoter (D), bearing four GAL4 sites. A six-tandem reporter array was integrated adjacent to the *lacO* array (A, C and D) or into a different chromosome (B). Six serial YFP and CFP images along the z-axis (A), a pair of YFP and CFP images, chosen for their highest focus intensities among a series of images along the z-axis (B), and a pair of YFP and CFP images at the same plane along the z-axis (C, D) are presented.

reporter cassette were integrated either adjacent to the *lacO* array (Figure 1A), or into a different chromosome (Figure 1B). A comparison of these two cases showed that, while the *GAL1* promoter was transcriptionally active in both cases (RFP images), the GAL4–YFP foci were formed in the vicinity of the *LacI*–CFP foci only in the former case. Accordingly, throughout the work presented here, we confirmed the identity of the YFP foci by their positions relative to the *LacI*–CFP/*lacO* position marker. We next verified the sequence specificity of GAL4–YFP foci formation by comparing a pair of artificial promoters: the *HIS4-a* and *HIS4-b* promoters. As shown in Figure 1C and D, the YFP foci were detected only with the latter, which contained the inserted GAL4 sites.

Identification of ‘DNA-binding enhancing regions’ of GAL4

With the visual promoter-binding assay *in vivo* available, we first asked a simple question: which regions of GAL4 are important for effective binding to the *GAL1* promoter in living cells? We first compared the wild-type GAL4 (GAL4 WT; Figure 2A) and its truncated form, bearing only the DNA-binding domain (GAL4 DB; Figure 2B), both fused to YFP and expressed at various concentrations from a copper-inducible promoter. To allow the growth of *GAL4*-deficient cells and furthermore to allow GAL4-mediated transcriptional activation, the cells were grown in the presence of either glucose and galactose (Figure 2), or raffinose and galactose (data not shown). In either case, GAL4 WT efficiently bound to the *GAL1* promoter; the YFP foci were readily detected even at concentrations at which free WT proteins were virtually undetectable (Figure 2A). In stark contrast, the GAL4 DB did not

Fig. 2. Identification of regions of GAL4 critical for efficient binding to the *GAL1* promoter. The wild-type GAL4 (A) and its truncations (B–D), fused to YFP, were examined for their binding to the *GAL1* promoter with the six-copy (6 $\times$) reporter array (see Materials and methods for details). For each effector, images of three representative cells are presented, with the results of quantitations on the right. The YFP and RFP images were processed as indicated in the gradient bars shown at the bottom. Small enhanced YFP images, labeled ‘Yen’, were obtained by setting the most intense signal of each image area to black. The scale bars represent 2 μ m. In the graphs, fluorescent intensities of the YFP foci and RFP, quantitated for individual cells, were plotted against levels of free YFP effector proteins. The YFP values in the graphs (i.e. YFP focus intensities and levels of effectors) are all comparable, and the value 1 was set so that the highest value does not exceed 1. Levels of RFP (i.e. RFP fluorescent intensities) of the graphs are also all comparable, and the value 1 was similarly set. The dotted lines represent the results with the WT protein.

show distinct YFP foci (Figure 2B). In this assay, therefore, GAL4 WT, but not its DNA-binding domain alone, is capable of binding to the *GAL1* promoter efficiently. The result predicts that a region(s) outside the DNA binding domain of GAL4 contributes significantly to promoter binding *in vivo*.

We divided GAL4 into three portions (Ma and Ptashne, 1987)—DB (DNA-binding domain; residues 1–94), CR (central region; residues 95–751) and AD (transcriptional activation domain; residues 752–881)—and examined the portion responsible for enhancing DNA binding in cells. The results described in Figure 2C show that, in spite of its inability to activate transcription detectably, the DB-CR

protein, bearing DB and most of CR (residues 95–692) but lacking AD, bound to the *GAL1* promoter as effectively as did the WT protein. A similar truncation (residues 1–94/95–751), bearing the DB and entire CR segments, although not expressed as readily as the DB-CR protein, also bound to the *GAL1* promoter as effectively (data not shown). Next we tested a complementary truncation DB-AD. This truncation, capable of weakly but significantly activating transcription (see data points at high effector concentrations), also readily bound to the *GAL1* promoter (Figure 2D). These results indicate that both the CR and AD segments have the ability to assist the GAL4 DNA-binding domain in associating with *GAL1* promoter DNA in cells. We refer to these two DNA-binding enhancing regions, revealed by the activity of the CR and AD segments, as DNA-binding enhancer (DBE) I and II, respectively.

To confirm that there is a dramatic difference between the GAL4 WT and DB protein in their ability to bind to the *GAL1* promoter in cells, we employed dimethyl sulfate (DMS) methylation protection assay as an independent measure of the DNA-binding efficacy. Figure 3 shows the results of the assay on the endogenous *GAL1* promoter in its natural location. As effector proteins, we analyzed GAL4 WT and GAL4 DB, which were not fused to YFP in this experiment. In agreement with previously published results (Giniger *et al.*, 1985), the 5′-most guanine of the GAL4 site I and III in the *GAL1* promoter were readily protected by the GAL4 WT protein from modification by DMS (Figure 3). Protection of the corresponding guanine of GAL4 site II was also detectable but was less evident, because the same guanine, together with neighboring guanines, was protected in the absence of GAL4, presumably by the GRF2 (REB1) protein (Chasman *et al.*, 1990). In contrast to GAL4 WT, the DNA-binding domain alone (GAL4 DB) did not appreciably protect these site I and III guanines at low concentrations, and protected them only partially at high concentrations. When the experiment was repeated using the WT and DB proteins fused to YFP, results essentially identical to those of Figure 3 were obtained (data not shown). These results are consistent with those of the visual DNA-binding assay and verify that GAL4 relies on a region(s) outside its DNA-binding domain to effectively bind to the *GAL1* promoter. Furthermore, a quantitative analysis of the results revealed that GAL4 WT occupies the GAL4 sites in the *GAL1* promoter at concentrations ~15-fold lower than does DB.

Separation of the DNA-binding enhancing and transcriptional activating function

We and others previously reported that a transcriptional activation domain can effect activator binding to DNA in living cells (Introduction). Of the two DBEs described here, DBE I did not activate transcription detectably in the context of the DB-CR protein (Figure 2C). Furthermore, although a portion near the N-terminus of the CR has been shown to contain an activation domain (activating region I; residues 148–196; Ma and Ptashne, 1987), a deletion of a region encompassing the activating region I resulted in only a minor reduction in the DNA-binding efficacy (Figure 4; DB-CRA1). A further N-terminal deletion decreased, but did not abolish, the activity (DB-CRA2), while a small C-terminal deletion was deleterious

Fig. 3. *In vivo* site occupancy of the endogenous *GALI* promoter by GAL4 and its DNA-binding domain. Products of G-specific cleavages of DMS-methylated DNA of the *GALI*-promoter region are shown on the right, and expression of effector proteins are shown on the left. The top panel shows the result with naked DNA, methylated by DMS *in vitro*, with the positions of G-residues in the GAL4-site I/II/III region indicated above the panel. The G-residues previously described to be protected by GAL4 (Giniger *et al.*, 1985) are marked by filled triangles. The panel labeled 'None' shows the result with *GAL4*-deficient cells, and the third to sixth panels, labeled 'GAL4 WT', show the results with cells expressing GAL4 WT protein at four different concentrations, as monitored by immunoblot analysis shown to the left. The next four panels, labeled 'GAL4 DB', show the results with the GAL4 DNA-binding domain at four different concentrations. The panel at the bottom shows 2-fold serially diluted quantitation standards.

(DB-CRΔ3). The western blot analysis in Figure 5A shows that the YFP-fusion effectors were properly expressed at expected sizes. These results showed that the C-terminal portion of the CR segment, which does not contain a known transcriptional activation domain, can effect DNA binding and is responsible for the major activity of DBE I.

The C-terminal DBE II, on the other hand, overlaps the principal transcriptional activation domain (the activating region II; Ma and Ptashne, 1987). We also analyzed a smaller region of the activation domain, corresponding to the C-terminal 42 amino acid portion of GAL4 (Wu *et al.*, 1996; Ansari *et al.*, 1998), referred to here as AD42. Compared with the DB-AD protein, however, the DB-AD42 protein did not bind to DNA or activate transcription as effectively, or support the growth of yeast cells in galactose at comparable concentrations in our assay (data not shown). We therefore attempted to amplify the activities of AD42 by reiterating the segment

two and four times to give DB-2× AD42 and DB-4× AD42 activators. Additionally, we introduced a threonine-to-proline substitution in each AD42 copy of the DB-2× AD42 protein at a position corresponding to residue 860 in the GAL4 amino acid sequence. This mutation was described previously by Ansari *et al.* (1998) and was shown to have no deleterious effect on activation function, despite the fact that it presumably introduces a proline-mediated kink into the protein structure and indeed reduces sensitivity to GAL80-mediated inhibition.

The results of the DNA-binding and transcription assays of these activators are shown in Figure 6, and the results of western blot analysis, indicating proper expression of the YFP fusion activators, are shown in Figure 5B; note that the apparent heterogeneity of the DB-AD sample is likely to be caused by phosphorylation at residue 837 (Sadowski *et al.*, 1991), while the WT protein, which presumably is also phosphorylated and indeed displayed heterogeneous bands in different conditions, did not show heterogeneity here partly due to a high gel concentration. Under transcriptionally inducing conditions (i.e. in galactose; fluorescent images in Figure 6A on the top row, and data depicted by open symbols on graphs in Figure 6B), the 2× AD42 segment enhanced DNA binding and transcription nearly as effectively as did the 130 amino acid AD segment. The effect on DNA binding and on transcription were further enhanced by reiterating the AD42 segment four times (Figure 6; DB-4× AD42), and the effects were not diminished by the threonine-to-proline mutation (Figure 6; DB-2× T/P42). The AD42 segment is therefore capable of enhancing both DNA binding and transcription, and neither of these activities was diminished by the T/P mutation.

The above results suggest that DBE II physically overlaps the C-terminal activation domain. We therefore tested whether the DNA-binding enhancing activities of the 130 and 42 amino acid activation-domain segments are functionally separable from their transcriptional activity, by analyzing the same set of the activators under transcriptionally repressing conditions (i.e. in glucose; fluorescent images in Figure 6A on the bottom row, and data depicted by closed symbols on graphs in Figure 6B). In comparison to the results under transcriptionally inducing conditions, RFP reporter expression was decreased to nearly undetectable levels under repressing conditions for all the activators except for DB-4× AD42 (Figure 6B, graphs at the bottom); the DB-4× AD42 protein was also sensitive to repression, but to a lesser degree. In stark contrast, the degrees of promoter binding of these activators were not diminished in glucose (Figure 6B, graphs at the top). These results indicate that the 130 and 42 amino acid activation-domain segments are capable of eliciting their DNA-binding enhancing effect to full extents without effectively participating in transcriptional activation. Thus, although DBE II has not been physically separable from the C-terminal activation domain, its ability to augment DNA binding is functionally separable from that to stimulate transcription. The results also underscore the fact that a modest difference in expression levels is predominantly responsible for more efficient binding of GAL4 to promoter DNA in galactose (Griggs and Johnston, 1991; Dudley *et al.*, 1999).

Fig. 4. Delineation of DBE I. Truncations of the DB-CR protein, along with the WT, DB and DB-CR proteins, were analyzed for binding to the *GAL1* promoter. The 18-copy (18×), instead of the six-copy (6×), reporter array was used to allow detection of weak DB binding. The assay was performed and the results are presented as described in Figure 2. The solid and dotted lines in the graphs represent the results with WT and DB, respectively.

Effects of DNA and protein contexts on the effects of DBEs

In all of the analyses described above, DNA binding of GAL4 and its derivatives was examined with the *GAL1* promoter. We found, however, that neither DBE I nor DBE II relies on the precise sequence of the natural *GAL1* promoter in augmenting DNA binding *in vivo*. As shown in Figure 7A (panels labeled 'GAL4'), GAL4 WT, DB-CR and DB-AD bound more readily than the DNA-binding domain alone to an artificial promoter, which bears four GAL4 sites fused to an unrelated *HIS4* basal promoter. We next tested the activity of DBEs in a different protein context; DBE I and DBE II were individually fused to an unrelated DNA-binding domain (POU) derived from a mammalian transcriptional activator Oct-2. The results shown in Figure 7A (panels labeled 'POU') illustrate that either of the DBEs can enhance promoter binding of the POU DNA-binding domain, while only the POU-AD protein activated transcription detectably (data not shown). These results underscore the flexible nature of DBEs, and indicate that, like classical transcriptional activation domains, DBEs can function as modular units.

In contrast to these *in vivo* results, our preliminary *in vitro* DNA-binding analyses, using crude extracts that contained GAL4-YFP fusions and a DNA probe bearing four GAL4 sites, failed to show appreciable activity of DBE I or DBE II (Supplementary figure 1 available at *The EMBO Journal* Online). The activity of DBE I was also examined with partially purified preparations (effector protein contents being ~1%), giving rise to essentially the same results (data not shown). It is therefore unlikely that DBEs effect DNA binding by enhancing intrinsic ability of GAL4 to interact with DNA, for example, through direct interaction with DNA.

In the experiment shown in Figure 7B, we extended our analyses on the effects of the binding-site context by

Fig. 5. Expression of YFP fusion GAL4 derivatives in yeast cells. Sets of YFP fusion GAL4 derivatives used for the experiments in Figures 4 and 6 were examined by immunoblot analysis, as shown in (A) and (B), respectively.

comparing the following three cases: (i) four GAL4 sites were fused to a truncated *HIS4* promoter, and a reporter cassette containing this fusion promoter was reiterated six times for the DNA-binding assay (note that this six-copy reporter array is identical to the *HIS4* fusion reporter array in Figure 7A); (ii) 24 copies of the same GAL4 site were fused to the same truncated *HIS4* promoter, and a single copy of the reporter bearing this promoter was used for the assay; and (iii) identical to (ii), except that 80 tandem GAL4 sites were fused to the truncated *HIS4* promoter. Although a natural promoter is unlikely to contain such large numbers of clustered sites, the following results nevertheless indicate that the effect of GAL4 DBEs is not universal in living cells.

A comparison of the first two cases in Figure 7B shows that, although the overall number of GAL4 sites were identical (i.e. 24), and binding of WT was similar, binding of DB was appreciably more efficient in the second case than in the first case. In the third case, where the number of tandem GAL4 sites was increased to 80, the DNA-binding efficacy of WT does not appear to be significantly increased compared with the first two cases (Figure 7B); moderate increases in the relative focus intensities observed here can be explained by the increase in the overall number of the sites from 24 to 80. In contrast, the DNA-binding efficacy of DB substantially increased, and reached a level similar to that of WT (Figure 7B). In the third case, therefore, neither DBE I nor DBE II displayed appreciable activity to enhance DNA binding. These results illustrate that the DNA-binding effect of DBE is dependent on the context of the binding sites, and suggests that the GAL4 DNA-binding domain has the intrinsic potential to bind to DNA as effectively as does intact GAL4 activator in cells. The context of binding sites, however, influences the WT protein (bearing DBEs) and the DB protein (without DBE) differently, leading to well over a 10-fold difference in the DNA-binding efficiency or virtually no difference.

Discussion

Transcriptional activators are generally composed of two basic functional units: the DNA-binding and transcriptional activation domain (Ptashne, 1988). To exert its transcriptional effect, the activation domain needs to be positioned to specific sites on DNA through sequence-

Fig. 6. Separation of the DNA-binding enhancing and transcriptional activation function. Binding of YFP fusion GAL4 derivatives to the *GAL1* promoter was assayed with the six-copy (6 $\times$) reporter array. Examples of YFP, CFP and RFP images are shown in (A), and quantified results are shown in (B). In (A), the top five panels show the results in galactose, and the bottom six panels show the results in glucose. In (B), extents of promoter binding are shown in the top six graphs, and levels of reporter gene expression are shown in the bottom six graphs. Open and filled symbols in these graphs represent the results in galactose and in glucose, respectively. The dotted lines represent the results with WT in galactose.

specific interactions between the DNA-binding domain and DNA. In this report, we functionally dissected the prototypical yeast activator GAL4 in living yeast cells, and found that effective binding of GAL4 to the *GAL1* promoter relies on an additional mechanism that involves yet another functional unit, the DBE. GAL4 contains two DBEs (I and II), each with the ability to facilitate activator binding to DNA in cells.

Of the two GAL4 DBEs described in this report, the principal activity of DBE I was identified within a region that does not contain a known transcriptional activation domain. On the other hand, DBE II was physically inseparable from the C-terminal activation domain. The physical overlap of the DBE and activation domain is reminiscent of previous findings that transcriptional activation domains can impact on DNA binding in both yeast and mammalian cells (Svaren *et al.*, 1994; Vashee and Kodadek, 1995; Bunker and Kingston, 1996; Tanaka, 1996; Stafford and Morse, 1997; Vashee *et al.*, 1998). In one of these experiments and in the present work, we were unable to separate the two activities by mutations introduced into activation domains (Tanaka, 1996).

The similar structural requirements for transcriptional activation and DNA-binding enhancing function appear to suggest that the DNA-binding and transcriptional activation effects of activation domains are functionally linked and possibly interdependent. In the case of GAL4 DBE II, however, the magnitude of DNA-binding

enhancement was similar in both transcriptionally inducing and repressing conditions. Therefore, although physically overlapping the activation domain, DBE II is able to effect on DNA binding without relying on transcriptional activation. GAL4 can therefore effectively bind to its target sites in living cells independently of, and, in principle, prior to processes that lead to transcriptional activation. We suggest that the physical overlap of GAL4 DBE II and the prototypical acidic activation domain of GAL4 is not simply a coincidence, but rather reflects similar, if not identical, structural requirements to impact on transcription and on DNA binding. Many other acidic activation domains would then also be able to enhance DNA binding in cells, as we have previously shown for multiple activation modules of the herpes simplex virus activator VP16 (Tanaka, 1996), and they may do so without relying on transcriptional activation, as does the GAL4 C-terminal activation domain.

In contrast to the results from analyses *in vivo*, neither DBE I nor DBE II elicited appreciable effects in our DNA-binding analyses *in vitro*. It is therefore likely that DBEs do not enhance DNA binding by augmenting intrinsic ability of GAL4 to interact with DNA. Kang *et al.* (1993) previously reported that GAL4 protein but not its DNA-binding domain alone, can cooperatively bind to GAL4 sites *in vitro*, consistent with the possibility that the central (i.e. DBE I) region mediates cooperative DNA binding. However, although our experiments *in vitro* do not exclude

Fig. 7. Effects of DNA and protein contexts on activator binding. The GAL4 and POU derivatives were examined for their binding to DNA. (A) Examples of YFP and CFP images, showing binding of GAL4 derivatives to GAL4 sites (top panels, labeled 'GAL4') or binding of POU derivatives to POU-binding sites (bottom panels, labeled 'POU'). A six-copy (6 $\times$) reporter array, which was composed of a *HIS4*-promoter fused to four tandem GAL4 sites, or an 18-copy (18 $\times$) reporter array, which was composed of a *HIS4*-promoter fused to eight tandem POU-binding sites, was used. (B) DNA binding of WT and DB proteins are compared among three different reporter configurations: (i) the six-copy (6 $\times$) *HIS4* reporter array also used in the experiment in (A), shown on the left; (ii) a single copy of a *HIS4* reporter bearing 24 tandem GAL4 sites, shown in the middle; and (iii) a single copy of a *HIS4* reporter bearing 80 tandem GAL4 sites, shown on the right. In the graphs, YFP focus intensities with the WT (open circles) and DB (small filled circles) effectors were plotted against levels of free effector proteins. The dotted lines in the graphs represent the result from the WT effector with configuration (i).

the possibility of DBE I-mediated cooperativity, such effects, even if present, did not significantly impact on the DNA-binding efficacy in our assay *in vitro*, and seem unlikely to be responsible for the profound effects of DBE I *in vivo*. Nevertheless, further analyses are required to fully clarify the relationship between the DNA-binding cooperativity reported by Kang *et al.*, the effects of DBE I described here, and also the effects of DBE II and promiscuous DNA-binding cooperativity *in vivo* (Vashee and Kodadek, 1995; Tanaka, 1996; Vashee *et al.*, 1998), and eventually to elucidate how these effects are combined to impact on the extents of GAL4 binding to a promoter in cells.

We have demonstrated the effects of DBEs in two different promoter contexts and in two different protein contexts. It appears, therefore, that GAL4 DBEs do not have strict requirements, in terms of DNA and protein structures, in exerting their effects. We have also shown, however, that the effects of DBEs are still not universal in cells. Comparisons of the 4-, 24- and 80-site promoters indicated that the DBE effects are less profound with more tandem sites, probably because the binding of the DNA-binding domain alone becomes more efficient with more tandem sites. The pattern of these differences suggests that the effects of DBEs and the cooperative effects of multiple sites are redundant, possibly because they act through a common pathway (i.e. by counteracting a common negative effect, for example). More importantly, these results indicate that DBEs impact on DNA binding depending on the context of binding sites (i.e. promoter architecture, including the presence or absence of binding sites for other regulators, and chromatin structure, including nucleosomes). Therefore, by responding differently to different contexts, DBEs would play a major regulatory role in determining the extent of activator binding to a promoter with a particular configuration under a particular environment.

It is plausible that DBEs, as well as the context of binding sites, impact on activator binding to DNA by influencing a higher order of DNA-protein structures in living cells. In this respect, it is intriguing that the four GAL4 sites in the *GAL1* promoter are believed to be maintained in a constitutively nucleosome-free region with or without GAL4 (Fedor *et al.*, 1988; Fedor and Kornberg, 1989). Consistent with this previous observation, the GAL4-site region of the *GAL1* promoter, but not the downstream region, was indeed highly accessible to restriction endonucleases *in vivo* (our unpublished results).

It has been reported that induction of *GAL1* transcription depends on a chromatin remodeling complex SWI/SNF during mitosis (Krebs *et al.*, 2000) and also on a chromatin-modifying complex SAGA (Spt-Ada-Gcn5-acetyltransferase; Dudley *et al.*, 1999). Under transcriptionally inducing conditions, however, effective GAL4 binding to the *GAL1* promoter does not require the activity of either complex (Burns and Peterson, 1997; Krebs *et al.*, 2000; Bhaumik and Green, 2001). Therefore, although it is still possible that one of the two functionally redundant GAL4 DBEs requires the activity of these complexes, it is likely that, because the GAL4 sites are nucleosome free, GAL4 is capable of binding to the *GAL1* promoter independently of these chromatin remodeling/modifying complexes (Burns and Peterson, 1997). Furthermore, it was shown that the SAGA complex is recruited to the *GAL1* promoter by GAL4 only under transcriptionally inducing conditions (Bhaumik and Green, 2001; Larschan and Winston, 2001), suggesting that under repressing conditions neither DBE I nor DBE II requires the activity of SAGA. These results raise an intriguing possibility that, in promoting binding to the *GAL1* promoter, GAL4 DBEs can function independently of nucleosomes and, therefore, independently of these chromatin remodeling/modifying activities, and suggest further that other, perhaps still unrevealed structural and contextual features of the *GAL1* promoter are important for the DNA-binding effects of GAL4 DBEs and the binding-site context.

In this report, we have focused our *in vivo* DNA-binding analyses on GAL4, a prototypical and one of the best characterized transcriptional activators. We imagine, however, that GAL4 is not an exceptional or isolated example; in effectively binding to their cognate sites in living cells, other natural activators may also rely on mechanisms that depend on DBE-like units, are distinct from transcriptional activation and do not necessarily involve nucleosomes. Although such DBE-like units have not been described for those other activators, they would be conceivably revealed by quantitative analyses of their binding to DNA *in vivo*. The ability of DBEs to facilitate activator binding to its targets independently of and prior to transcriptional activation could contribute to establishing an activation-ready status of a promoter, and could also provide an additional pathway to regulate DNA binding of activators separately from transcriptional activation. The independence of GAL4 DBEs from transcriptional activation, furthermore, implies that DNA-binding enhancement by the DBE-like units does not have to be restricted to transcriptional activators. It is therefore possible that the context-dependent enhancement of DNA binding by DBE-like units is broadly involved in effective DNA binding of other factors that are involved in other aspects of transcription (e.g. repression) or in other nuclear processes (e.g. DNA synthesis).

Materials and methods

Yeast strains and plasmids

All the yeast strains used for the analyses were diploid, and were constructed by crossing an effector strain, expressing an effector protein, with a reporter strain, bearing a test promoter and the chromosomal position marker (i.e. the *lac* operator/repressor system; Robinett *et al.*, 1996). For each effector and reporter strains, we tested at least two independent transformants, which gave rise to indistinguishable results. The effector strains were constructed from a *GAL4*-deficient derivative of YPH499 (Sikorski and Hieter, 1989) by integrating an effector-expression cassette, fused to the *LEU2* gene, into the *TRP1* gene. The effector-expression cassette contained the *CUP1* promoter (position -459 to -23 relative to the *CUP1* initiation codon), followed by the *ADH1* 5' untranslated segment (*ADH1* 5'; -20 to -1), the ATG start codon, the HA epitope tag (SSYPYDVPDYASLGSP; Tanaka, 1996), a nuclear localization signal (NP nls; VKRPAATKKAGQAKKKKLD) of *Xenopus laevis* nucleoplasmin, a sequence encoding Topaz YFP (Tsien, 1998), derived from yEGFP3 (Cormack *et al.*, 1997) by introducing appropriate mutations, a sequence encoding GAL4 or its truncations, six histidines, and a stop codon. In the experiment shown in Figure 3, the WT and DB effectors that did not contain NP nls, YFP or six histidines were used. Each of the GAL4 truncations contained the following GAL4-derived segments: WT (residues 2-881; note that region 1-881 was also tested and gave rise to indistinguishable results), DB (residues 1-94), DB-CR (residues 2-692; note that region 1-692 was also tested and gave rise to indistinguishable results), DB-AD (residues 1-94 and 752-881), DB-CRA1 (residues 1-94 and 236-692), DB-CRA2 (residues 1-94 and 638-692), DB-CRA3 (residues 1-537), DB-2× AD42 (residues 1-94 and 2× 840-881), DB-4× AD42 (residues 1-94 and 4× 840-881), and DB-2× T/P42 (residues 1-94 and 2× 840-881; T860P). The YFP-POU fusions were constructed by placing the POU domain segment (Tanaka, 1996) in place of the GAL4 DB segment, except for POU-CR, which was constructed by fusing a GAL4 segment (residues 74-692) to the POU domain. The reporter strains were constructed from a *GAL4*-deficient derivative of YPH500 (Sikorski and Hieter, 1989). The *LacI* expression cassette contained a truncated *PGK3* promoter segment (-434 to -154) and the *DED1* promoter segment (-106 to -1), followed by *ADH1* 5', ATG, HA, a sequence encoding a mutant Lac repressor that forms dimers (Straight *et al.*, 1996), NP nls and a sequence encoding the enhanced CFP (Heim and Tsien, 1996), derived from yEGFP3 (Cormack *et al.*, 1997). In order to increase the intensity of fluorescent signals, the CFP sequence was reiterated six times in this construct. The *LacI*-CFP expression

cassette, together with the *TRP1* gene, was integrated into the *MET3* gene. A tandem array of a sequence (CGAGCACAAATTGTTATCCGCT-CACAATTCCACA), bearing the *lac* operator, was inserted together with the *HIS3* gene at the 3' region of the *ARG4* gene between position 1467 and 1468 relative to the *ARG4* initiation codon. Into the strain containing both the *LacI*-CFP cassette and the *lacO* array, a reporter array bearing up to 18 tandem copies of an RFP reporter cassette (see below) was integrated, together with the *URA3* gene, into the *ARG4* gene; upon this integration, position -39 to 997 of the *ARG4* gene was displaced. In one control strain, a reporter array was integrated into the *RAD16* gene (Figure 1B). While introducing the reporter array into the *ARG4* locus, the adjacent *lacO* array appears to have become unstabilized, and, thus, the reporter strains contained different numbers (between 60 and 80) of the *lacO* sequence, although the *lacO* array was sufficiently stable once the strains were established. All RFP reporter cassettes were constructed by combining a test promoter with *ADH1* 5', ATG and a sequence encoding RFP (dsRED; Clontech Laboratory, Inc.), followed by the 3' region of the *HIS3* gene (position 626-864). The entire reporter cassette was reiterated in an *Escherichia coli* plasmid up to six times. For further reiteration, appropriate fragments were ligated *in vitro*, and the ligated products were introduced into yeast cells. As a test promoter, we used either the *GAL1* promoter (-484 to -1) or a *HIS4* fusion promoter, bearing a *HIS4* upstream segment (-705 to -324) and the TATA-box segment (-144 to -7). The *HIS4*-b and *HIS4*-a fusion promoters were constructed by inserting either four copies of a GAL4-binding sequence (GAGCGGAA-GACTCTCTCCGTCTC) or eight copies of an unrelated sequence, respectively, between the *HIS4* upstream and TATA segments. In the experiment described in Figure 7, a derivative of the *HIS4*-b promoter, bearing 24 or 80 copies of the same GAL4-binding sequence, or eight copies of a POU-domain binding sequence (GAGTACATATGCA-AATCTATCAGTGATAGAGTAC), was used. The sequence of the endogenous *GAL1* promoter and that of the *GAL1* reporters are consistent with 'the sequence of *GAL1*-*GAL10* inducible promoter and genes' (DDBJ/EMBL/GenBank accession No. K02115.1), while the GAL4-binding sequence in the *HIS4* fusion promoters bears GAL4 site III of the *GAL1* promoter found in a different strain (DDBJ/EMBL/GenBank accession No. X76078.1). The genomic structures of the yeast strains were confirmed by Southern blot analysis. Detailed descriptions of the plasmids and yeast strains are available upon request.

Microscopy and image processing

Yeast cells were grown to mid-log phase for 6-8 h in synthetic media containing glucose (2%), galactose (2%), or glucose plus galactose (1.5% each), and supplemented with CuSO₄ typically at eight different concentrations up to 100 μM to prepare cells expressing YFP-fusion proteins at various different levels. The cell samples that were to be compared (e.g. all the cell samples described in Figure 2) were cultured in parallel, and were simultaneously placed on ice prior to microscopic observations. YFP, CFP and RFP fluorescence, including foci fluorescence, of cells that were placed on ice were indistinguishable from those of growing cells, and were stable for at least a few hours, during which microscopic photographs were taken. The cells were visualized using an Olympus BX62 microscope equipped with a Hamamatsu C4742-98-24NR ORCA camera, and the images were acquired and processed using AquaCosmos software (Hamamatsu Photonics K. K.). Filters for YFP, CFP and RFP were obtained from Omega Optical, Inc. (XF104-2, XF114-2 and XF35, respectively). Yeast cells were sectioned in the z-axis at 400 or 500 nm intervals for over 6 μm. All YFP and RFP images of each set of experiments and CFP images in Figure 1A and E were processed in a comparable manner, while the other CFP images were individually processed to clarify the positions of *LacI*-CFP fluorescent foci. For quantitative analyses, we chose single-nuclear cells with moderately sized buds to minimize cell-to-cell fluctuations, because the results of cell cycle analyses showed that although the YFP foci are detectable throughout the cell cycle, their intensity is consistently high in those cells (Supplementary figure 2). We nevertheless confirmed that the results described in the present work are not limited to cells in specific cell cycle phases. To determine the fluorescence intensity of a YFP focus, a YFP image that displayed the most intense focus was chosen from serial images along the z-axis. From the chosen YFP image, fluorescent signals of an area of ~0.2 μm² containing the YFP focus was integrated, and background signal, calculated from fluorescence of the surrounding region, was subtracted. Where the YFP focus was not detectable, a YFP image corresponding to the CFP image showing the most intense CFP focus was chosen. The levels of YFP effector and RFP reporter proteins were determined by quantitating fluorescent signals from the images showing highest YFP and RFP signals, respectively, among serial images

along the z-axis; note that fluorescent signals of the YFP focus region was excluded in this quantitation to avoid complications caused by optical blurring effects of intense YFP foci. Quantitative microscopic analyses were performed at least twice for every sets of experiments, and the results of representative sets are shown.

DNA and protein analysis

In vivo DMS methylation protection assay was performed essentially as described previously (Mueller and Wold, 1989; Tanaka, 1996). In brief, yeast cells were grown to mid-log phase for 6 h in synthetic media supplemented with various concentrations of CuSO₄ (0–250 μ M). Portions of the cell samples were harvested for protein analysis. The remaining cells were treated with 60 mM DMS at 30°C for 4.5 min, followed by extraction of genomic DNA. The extracted DNA was subjected to piperidine treatment, primer extension and ligation-mediated PCR amplification, followed by an additional primer extension reaction with a fluorescent primer. The products were fractionated using ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Inc.). For immunoblot analyses, protein samples were prepared either from the cells harvested as described above (for the analysis described in Figure 3) or from cells grown to mid-log phase in synthetic media supplemented with CuSO₄ at 500 μ M (for the analysis described in Figure 5). The samples were fractionated on 12.5% SDS–polyacrylamide gel, followed by immunoblotting. The blots were probed with a high-affinity anti HA antibody (3F10; Roche Diagnostics), and the signals were detected by enhanced chemiluminescence (ECL-Plus; Amersham Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank B.P.Cormack and K.T.Arndt for providing the plasmids, M.Kimura, H.Inoko and Y.Oshima for their support, and H. Kawata for technical assistance. We also thank Y.Marahrens and N.Kikuchi for helpful comments on the manuscript. This work was supported by a PRESTO award from Japan Science and Technology Corporation (JST) to M.T.

References

- Adams,C.C. and Workman,J.L. (1995) Binding of disparate transcriptional activators to nucleosomal DNA is inherently cooperative. *Mol. Cell. Biol.*, **15**, 1405–1421.
- Ansari,A.Z., Reece,R.J. and Ptashne,M. (1998) A transcriptional activating region with two contrasting modes of protein interaction. *Proc. Natl Acad. Sci. USA*, **95**, 13543–13548.
- Bhaumik,S.R. and Green,M.R. (2001) SAGA is an essential *in vivo* target of the yeast acidic activator Gal4p. *Genes Dev.*, **15**, 1935–1945.
- Bram,R.J. and Kornberg,R.D. (1985) Specific protein binding to far upstream activating sequences in polymerase II promoters. *Proc. Natl Acad. Sci. USA*, **82**, 43–47.
- Bunker,C. and Kingston,R. (1996) Activation domain-mediated enhancement of activator binding to chromatin in mammalian cells. *Proc. Natl Acad. Sci. USA*, **93**, 10820–10825.
- Burns,L.G. and Peterson,C.L. (1997) The yeast SWI–SNF complex facilitates binding of a transcriptional activator to nucleosomal sites *in vivo*. *Mol. Cell. Biol.*, **17**, 4811–4819.
- Chasman,D.I., Lue,N.F., Buchman,A.R., LaPointe,J.W., Lorch,Y. and Kornberg,R.D. (1990) A yeast protein that influences the chromatin structure of UASG and functions as a powerful auxiliary gene activator. *Genes Dev.*, **4**, 503–514.
- Cormack,B.P., Bertram,G., Egerton,M., Gow,N.A., Falkow,S. and Brown,A.J. (1997) Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology*, **143**, 303–311.
- Dudley,A.M., Rougeulle,C. and Winston,F. (1999) The Spt components of SAGA facilitate TBP binding to a promoter at a post-activator-binding step *in vivo*. *Genes Dev.*, **13**, 2940–2945.
- Fedor,M.J. and Kornberg,R.D. (1989). Upstream activation sequence-dependent alteration of chromatin structure and transcription activation of the yeast *GAL1–GAL10* genes. *Mol. Cell. Biol.*, **9**, 1721–1732.
- Fedor,M.J., Lue,N.F. and Kornberg,R.D. (1988) Statistical positioning of nucleosomes by specific protein-binding to an upstream activating sequence in yeast. *J. Mol. Biol.*, **204**, 109–127.
- Fukushige,T., Hendzel,M.J., Bazett-Jones,D.P. and McGhee,J.D. (1999) Direct visualization of the elt-2 gut-specific GATA factor binding to a target promoter inside the living *Caenorhabditis elegans* embryo. *Proc. Natl Acad. Sci. USA*, **96**, 11883–11888.
- Giniger,E. and Ptashne,M. (1988) Cooperative DNA binding of the yeast transcriptional activator GAL4. *Proc. Natl Acad. Sci. USA*, **85**, 382–386.
- Giniger,E., Varnum,S.M. and Ptashne,M. (1985) Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell*, **40**, 767–774.
- Griggs,D.W. and Johnston,M. (1991) Regulated expression of the GAL4 activator gene in yeast provides a sensitive genetic switch for glucose repression. *Proc. Natl Acad. Sci. USA*, **88**, 8597–8601.
- Heim,R. and Tsien,R.Y. (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.*, **6**, 178–182.
- Johnson,P.F. and McKnight,S.L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.*, **58**, 799–839.
- Kang,T., Martins,T. and Sadowski,I. (1993) Wild type GAL4 binds cooperatively to the *GAL1-10* UAS_G *in vitro*. *J. Biol. Chem.*, **268**, 9629–9635.
- Krebs,J.E., Fry,C.J., Samuels,M.L. and Peterson,C.L. (2000) Global role for chromatin remodeling enzymes in mitotic gene expression. *Cell*, **102**, 587–598.
- Larschan,E. and Winston,F. (2001) The *S. cerevisiae* SAGA complex functions *in vivo* as a coactivator for transcriptional activation by Gal4. *Genes Dev.*, **15**, 1946–1956.
- Ma,J. and Ptashne,M. (1987) Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell*, **48**, 847–853.
- McNally,J.G., Muller,W.G., Walker,D., Wolford,R. and Hager,G.L. (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science*, **287**, 1262–1265.
- Mueller,P.R. and Wold,B. (1989) *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. *Science*, **246**, 780–786.
- Narlikar,G.J., Fan,H.-Y. and Kingston,R.E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell*, **108**, 475–487.
- Orphanides,G. and Reinberg,D. (2002) A unified theory of gene expression. *Cell*, **108**, 439–451.
- Pabo,C.O. and Sauer,R.T. (1992) Transcription factors: structural families and principles of DNA recognition. *Annu. Rev. Biochem.*, **61**, 1053–1095.
- Polach,K.J. and Widom,J. (1995) Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation. *J. Mol. Biol.*, **254**, 130–149.
- Ptashne,M. (1988) How eukaryotic transcriptional activators work. *Nature*, **335**, 683–689.
- Ptashne,M. and Gann,A.A. (1990) Activators and targets. *Nature*, **346**, 329–331.
- Ptashne,M. and Gann,A. (1997) Transcriptional activation by recruitment. *Nature*, **386**, 569–577.
- Robinett,C.C., Straight,A., Li,G., Willhelm,C., Sudlow,G., Murray,A. and Belmont,A.S. (1996) *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using *lac* operator/repressor recognition. *J. Cell Biol.*, **135**, 1685–1700.
- Sadowski,I., Niedbala,D., Wood,K. and Ptashne,M. (1991) GAL4 is phosphorylated as a consequence of transcriptional activation. *Proc. Natl Acad. Sci. USA*, **88**, 10510–10514.
- Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
- Stafford,G.A. and Morse,R.H. (1997) Chromatin remodeling by transcriptional activation domains in a yeast episome. *J. Biol. Chem.*, **272**, 11526–11534.
- Straight,A.F., Belmont,A.S., Robinett,C.C. and Murray,A.W. (1996) GFP tagging of budding yeast chromosomes reveals that protein–protein interactions can mediate sister chromatid cohesion. *Curr. Biol.*, **6**, 1599–1608.
- Svaren,J., Schmitz,J. and Hörz,W. (1994) The transactivation domain of Pho4 is required for nucleosome disruption at the *PHO5* promoter. *EMBO J.*, **13**, 4856–4862.
- Tanaka,M. (1996) Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 4311–4315.

- Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.*, **67**, 509–544.
- Vashee, S. and Kodadek, T. (1995) The activation domain of GAL4 protein mediates cooperative promoter binding with general transcription factors *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 10683–10687.
- Vashee, S., Melcher, K., Ding, W.V., Johnston, S.A. and Kodadek, T. (1998) Evidence for two modes of cooperative DNA binding *in vivo* that do not involve direct protein–protein interactions. *Curr. Biol.*, **8**, 452–458.
- Workman, J.L. and Kingston, R.E. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.*, **67**, 545–579.
- Wu, Y., Reece, R.J. and Ptashne, M. (1996) Quantitation of putative activator–target affinities predicts transcriptional activating potentials. *EMBO J.*, **15**, 3951–3963.

*Received May 28, 2002; revised February 18, 2003;
accepted March 13, 2003*