

# Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism

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**The process of transcriptional activation in eukaryotes by site-specific DNA-binding proteins is a key step in gene regulation. Here we have examined the properties of four distinct activator domains of the human transcription factor Sp1. In vivo transient cotransfection assays with Sp1 show that templates bearing multiple Sp1 sites activate transcription with a high degree of synergism. However, there is no evidence of cooperative binding of Sp1 to adjacent sites. Using deletion mutants of Sp1 we have determined that the glutamine-rich activation domains A and B and the previously uncharacterized carboxy-terminal domain D are all required for Sp1 to activate transcription synergistically. Gel-shift, DNase footprinting, and chemical cross-linking experiments reveal a strong correlation between the ability of Sp1 mutants to form homomultimeric complexes and their ability to activate transcription synergistically when bound to multiple sites. We have also examined the process of superactivation, in which a molecule of Sp1 tethered to DNA via its zinc fingers can be transcriptionally enhanced by interacting directly with fingerless Sp1 molecules. The domains involved in superactivation appear to be a subset of those necessary to achieve synergistic activation. These findings suggest that different domains of Sp1 carry out distinct functions and that the formation of multimeric complexes may direct synergism and superactivation.**

[*Key Words:* Sp1; synergistic activation; transcription; multimerization]

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The regulation of transcription is accomplished through a cascade of events involving both protein–DNA and protein–protein interactions. The binding of site-specific regulators to promoter or enhancer elements of the DNA template constitutes one of the key steps governing transcriptional initiation. Once these promoter-selective factors become tethered to the template they most likely interact with each other, as well as with various non-DNA-binding transcription factors that constitute the basal initiation complex. Many sequence-specific activators have been identified, and much work has been focused on the structure and function of these activators. Elucidating the mechanism by which sequence-specific activators regulate transcription and how various components of the transcriptional machinery interact are key issues in understanding transcriptional control.

The eukaryotic transcription factor Sp1 provides a useful model for the study of these regulatory interactions. Human Sp1, like many transcription factors, was identified on the basis of its ability to bind DNA and activate transcription in a site-dependent manner (Dyanan and Tjian 1983; Briggs et al. 1986). Analysis of Sp1 structure and function by mutagenesis has revealed that the protein can be separated into discrete functional domains. The DNA-binding domain consists of three zinc fingers

that bind to a GC-rich consensus sequence, the GC box (Kadonaga et al. 1987). In addition, there are four separate domains that govern transcriptional activation (Courey and Tjian 1988). Two of these activation domains are glutamine rich, a motif found in several other transcription factors (Driever et al. 1989; Tanaka and Herr 1990). The other two activation domains of Sp1 differ in their amino acid makeup, with one being weakly basic and the other exhibiting no outstanding features or homologies.

The presence of at least four separate activation domains in Sp1 raises the intriguing question of what function(s) each of these protein surfaces carries out. In vitro studies of transcriptional activation suggest that Sp1 communicates with the general factor TFIID through coactivator proteins (Pugh and Tjian 1990). It seems likely, therefore, that one or more of the Sp1 domains are probably involved in this interaction with the coactivators and possibly with components of the basal transcriptional apparatus. Additional Sp1 domains may participate in interactions with other site-specific transcription factors such as the viral enhancer protein E2 (Li et al. 1991). Many transcription factors are known to multimerize to create an active species. For example, dimers of the leucine zipper proteins, c-Jun and CCAAT/enhancer-

binding protein (C/EBP), are necessary to form an active binding species (Jones 1990). Although DNA binding by Sp1 does not require dimers, it is possible that Sp1 uses some of its activation domains to form dimers and higher-order structures that are important for transcriptional activation.

Is there any evidence that Sp1 forms multimeric complexes that are important for transcriptional activation? In vivo studies have shown previously that Sp1 bound to distal enhancer regions can interact with Sp1 bound at sites proximal to the promoter and synergistically activate transcription (Courey et al. 1989). This synergism is thought to involve direct contact between Sp1 molecules bound at the two sites, thereby looping out the intervening DNA. Recently, electron microscopy analysis of Sp1/DNA complexes directly visualized what appear to be multimers of Sp1 bound at proximal and distal sites associating and forming looped DNA structures (Su et al. 1991; I. Mastrangelo and P. Hough, pers. comm.). Taken together, these studies suggest that at least one surface of each Sp1 promoter is likely to participate in direct Sp1–Sp1 interactions. Although Sp1 acts synergistically from these widely separated sites, paradoxically, Sp1 thus far has not been shown to work synergistically from adjacent sites. Most studies of Sp1 have focused on its role in the activation of the SV40 early promoter, which contains six neighboring Sp1 sites. However, analysis of the SV40 tandem GC boxes indicates that they work independently from one another (Barrera-Saldana et al. 1985; Gidoni et al. 1985). The absence of synergism between these sites could represent an intrinsic difference between Sp1 activation at closely spaced adjacent sites versus distantly separated sites. Alternatively, it is possible that the synergistic activation directed by Sp1 is dependent on the specific context of the promoter in which the site is located.

In this paper we have used cotransfection of wild-type and mutant Sp1 genes into a cell line lacking any endogenous Sp1 to assess the ability of different templates to direct synergistic activation by Sp1. In addition, we have used mutants of Sp1 to identify which domains of the protein are likely to participate in multimer formation and synergism. Our results identify distinct functions for the multiple activation domains of Sp1 and help distinguish among the different steps during activation at which synergism may occur.

## Results

### *Sp1 acts synergistically when bound at adjacent sites proximal to the promoter*

Traditionally, the transcriptional activity of Sp1 has been tested on natural templates such as the SV40 early promoter, which contains a complex array of *cis* elements including multiple binding sites for Sp1, as well as other factor-binding sites. We sought to create a simpler system to assess the activation properties of Sp1. First, we constructed test promoters fused to the chloramphenicol acetyltransferase (CAT) gene with one or more

high-affinity Sp1-binding sites (box III of the HTLVIII LTR; Jones et al. 1986) placed upstream of the E1b TATA box. The reporter constructs were cotransfected, along with an expression vector for Sp1, into *Drosophila* SL2 cells, which lack endogenous Sp1 activity. Studies of Sp1 have established previously that the activation domains of Sp1 function equivalently in *Drosophila* and mammalian systems (Courey and Tjian 1988).

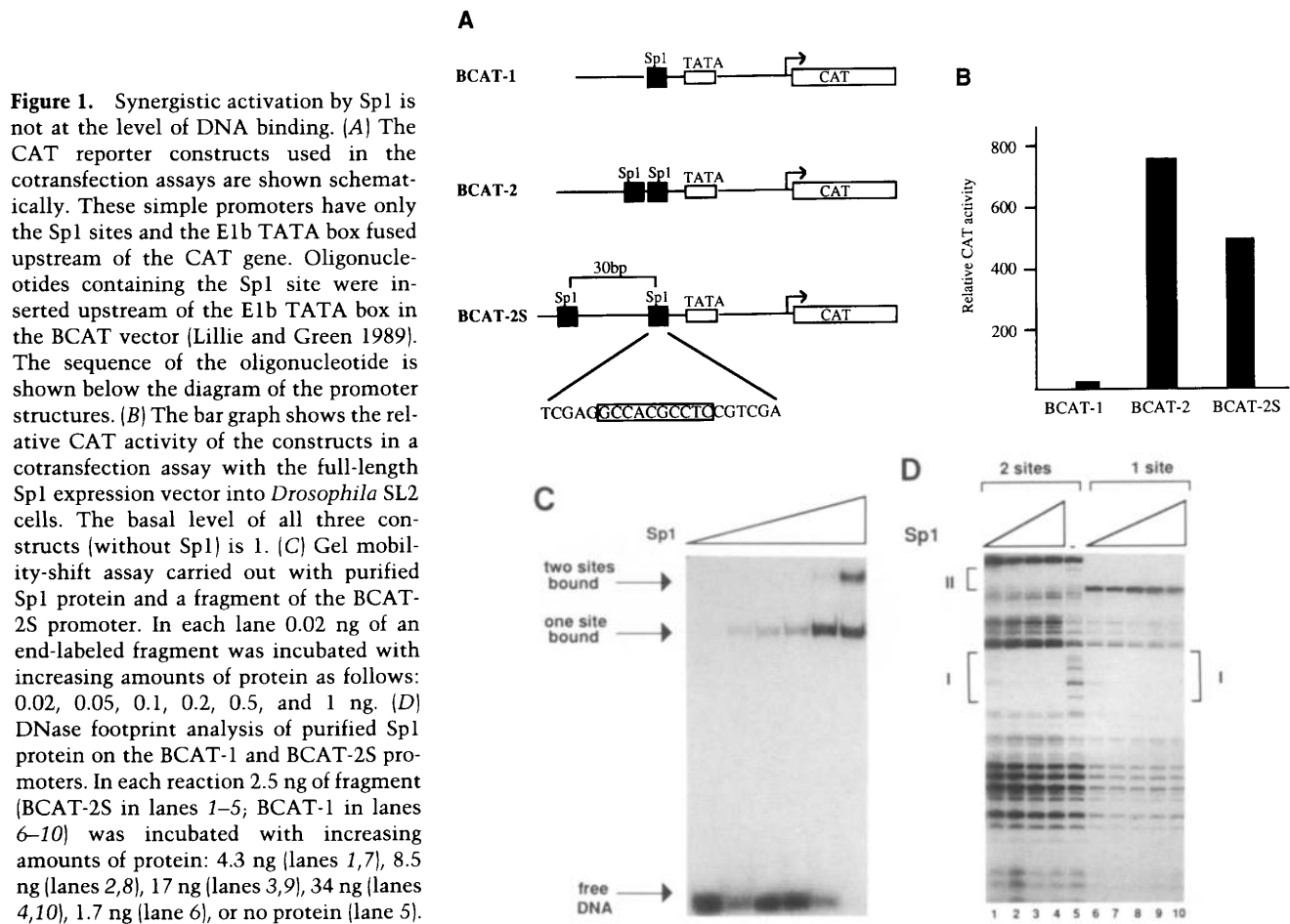
Consistent with previous results, Sp1 activated transcription at a modest level (two- to threefold) from the promoter with a single Sp1-binding site, BCAT-1. Surprisingly, the activation from the promoter with two Sp1-binding sites, BCAT-2, was 78-fold greater than the activation of BCAT-1 (Fig. 1A). This suggests that Sp1 bound to two sites interacts synergistically to activate transcription at a much higher level than the sum of two sites acting independently. However, increasing the number of binding sites above two does not seem to result in further synergistic effects because three Sp1-binding sites in BCAT-3 are only three times as active as BCAT-2 (data not shown). We also constructed BCAT-2S, which separated the two Sp1 sites by 30 bp. The activation of this construct was 50-fold greater than BCAT-1 (Fig. 1A), suggesting that the binding sites do not have to be adjacent for Sp1 to work synergistically.

### *Sp1 synergism is not at the level of DNA binding*

The dramatic enhancement of transcription directed by the template containing two Sp1 sites could be due to cooperative binding of Sp1 to adjacent sites or to synergism resulting from mechanistic steps occurring after DNA binding that directly effect activation. To help distinguish between these two possibilities, we carried out direct DNA-binding studies with templates containing multiple Sp1-binding sites. We used a gel-shift assay to assess the occupancy of sites on BCAT-2S (Fig. 1C). A titration of the Sp1 protein shows a single bound complex until most of the free template has been bound; a second bound complex then increases in appearance as the protein concentration is further increased. This result suggests that the binding of the two sites is independent. We also compared the occupancy of binding sites on BCAT-1 and BCAT-2S by DNase I footprinting (Fig. 1D). Both templates appear to require similar amounts of protein to achieve full occupancy of the binding sites. No increase in affinity of Sp1 for the BCAT-2 or BCAT-2S promoters was observed in any of the experiments performed. These results suggest that enhanced transcription is due to a synergistic effect in the process of activation rather than to cooperative DNA binding.

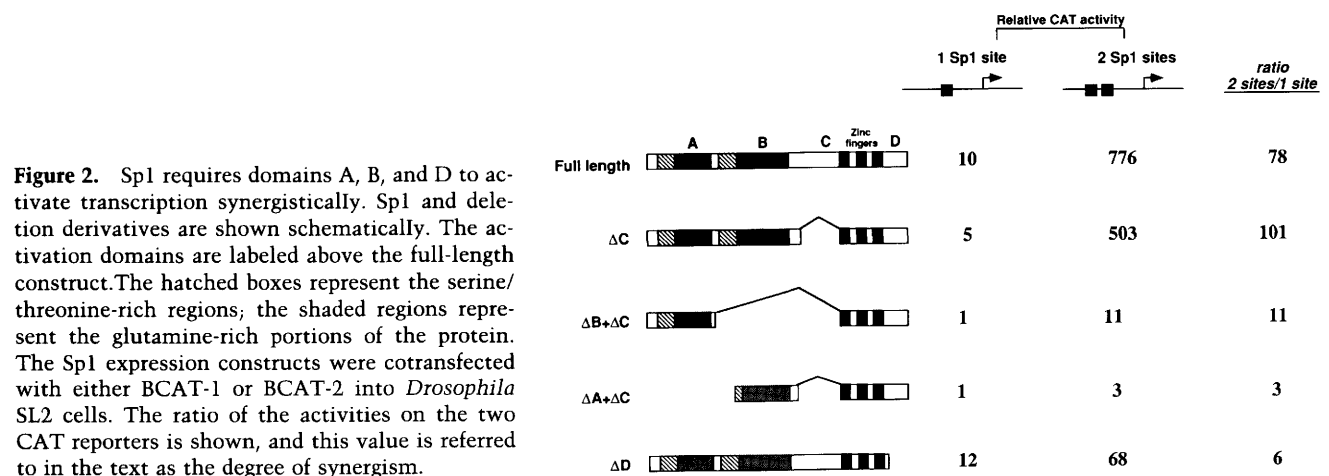
### *Domain D is required for Sp1 synergism*

Because our results suggest that cooperative DNA binding cannot account for the observed transcriptional synergism, we were prompted to look for synergistic effects involving the activation domains of Sp1. Deletion analysis of Sp1 has previously identified four regions that influence its ability to activate transcription (Courey and



Tjian 1988). Two of these regions, A and B, are glutamine rich, a motif found in several transcriptional activators (Driever et al. 1989; Tanaka and Herr 1990). We used a series of deletion mutants that removed one or more activation domains from Sp1 to determine which of these domains is required for synergistic activation. Expression plasmids of the Sp1 deletion mutants were

cotransfected with either BCAT-1 or BCAT-2 into SL2 cells. Deletion of either the A or B domains of Sp1 severely impaired activation on both BCAT-1 and BCAT-2 (Fig. 2). More importantly, the degree of synergism (ratio of activity on the two templates) directed by these two mutants is also greatly reduced. Interestingly, deletion of the carboxyl terminus D domain of Sp1 ( $\Delta$ D) also has a



severe effect on the ability of the protein to activate transcription synergistically. The activity of  $\Delta D$  is equivalent to the activity of full-length Sp1 on BCAT-1; however, the activity of  $\Delta D$  on BCAT-2 and on other multiple-binding-site constructs is greatly reduced (Fig. 2; data not shown). In contrast, deletion of the C domain does not affect the synergism, although the activity on BCAT-1 is slightly reduced. The C domain cannot substitute for either one of the glutamine-rich domains to regain synergistic activation (data not shown). These results indicate that Sp1 requires three of the activation domains, A, B, and D, to achieve synergistic activation on two adjacent binding sites. Domains A and B, which had been shown previously to participate in general activation by Sp1, are also required for full level activation on one or more adjacent sites. Moreover, the D domain also appears to play a key role in synergism as it is necessary for activation on multiple sites but not on a single site.

#### Requirement for synergism of Sp1 bound at proximal and distal sites

The synergistic activation of two adjacent Sp1 sites proximal to the promoter is reminiscent of the synergism seen when Sp1 sites are placed both upstream and distally (1.7 kb) downstream of the TK promoter (Courey et al. 1989). In this case, the downstream sites appeared to act through the proximal site to activate transcription synergistically. We wondered whether the synergy observed between adjacent proximal Sp1 sites and this long-distance effect were in some way related and whether both processes involved the same activation domains.

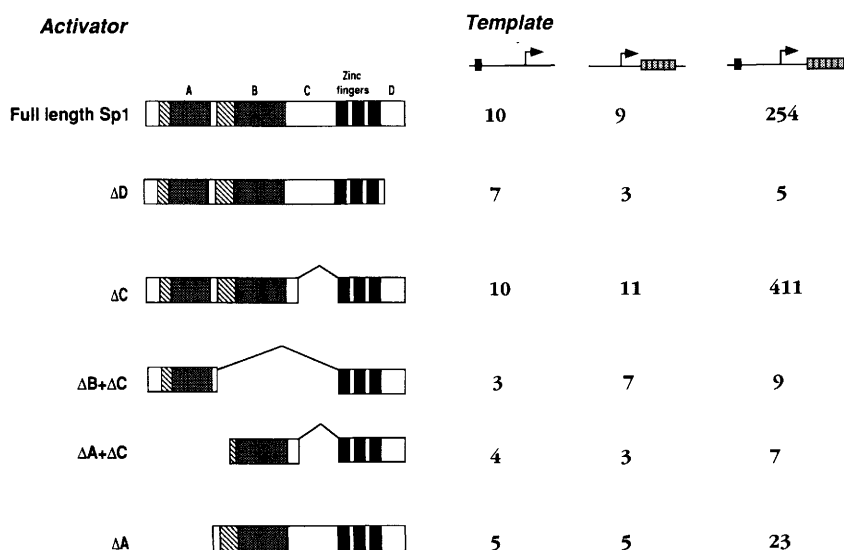
To address this issue, we tested the Sp1 deletions on the reporter CAT constructs containing either the proximal (-105tkCAT), distal (tkCAT15), or the proximal and distal sites together (-105tkCAT15). The full-length Sp1 activates the -105tkCAT15 promoter at a

level 25-fold greater than the promoter with either the proximal or distal sites alone (Fig. 3). The level of activation on -105tkCAT15 is therefore greater than the additive effect of the sites when considered alone (Fig. 3; Courey et al. 1989). As in the synergism with two adjacent sites, the synergism between the distal and proximal sites is lost if either of the two glutamine domains (A and B) is deleted. Significantly, deletion of the D domain also abolishes synergism. In contrast, deletion of the C domain actually slightly enhances the synergism. A deletion mutant of Sp1 that retains domains B and C but lacks A retains a low level of synergism on -105tkCAT15. Thus, the requirement for the domains A, B, and D in synergism of distal sites mirrors the requirements for the synergistic effect of two adjacent sites, suggesting that these three domains of Sp1 may be involved in related mechanistic steps in the processes of long-distance and short-distance synergism.

#### Superactivation by Sp1

The synergistic effects exhibited by Sp1 are thought to involve interactions between Sp1 promoters bound at adjacent and distal sites. Interestingly, it has been shown that a non-DNA-binding form of Sp1 can enhance transcriptional activation by the DNA-binding form of Sp1 (superactivation; Courey et al. 1989). The minimal DNA-binding form (the activator) that was tested previously consisted of the B domain attached to the DNA-binding and D domain. The minimal nonbinding (superactivator) form consisted of the A and B activation domains. Therefore, we have tested other deletion forms of Sp1 to compare the minimum domain requirements for superactivation with those requirements that we have identified for synergism.

We cotransfected various forms of Sp1 activators with a superactivator form consisting of the A, B, and C domains. The reporter plasmid consisted of the SV40 early promoter, with six GC boxes, fused to CAT. Although



**Figure 3.** Sp1 requires domains A, B and D to activate transcription synergistically from distal sites. Relative activity of full-length and deletion derivatives of Sp1 on promoters with upstream and/or downstream binding sites. Each Sp1 expression construct was cotransfected into SL2 cells with either -105tkCAT (one upstream site), tkCAT15 (six downstream sites), or -105tkCAT15 (one upstream and six downstream sites). The basal level of the reporter constructs (without Sp1) is 2, 3, 2, respectively.



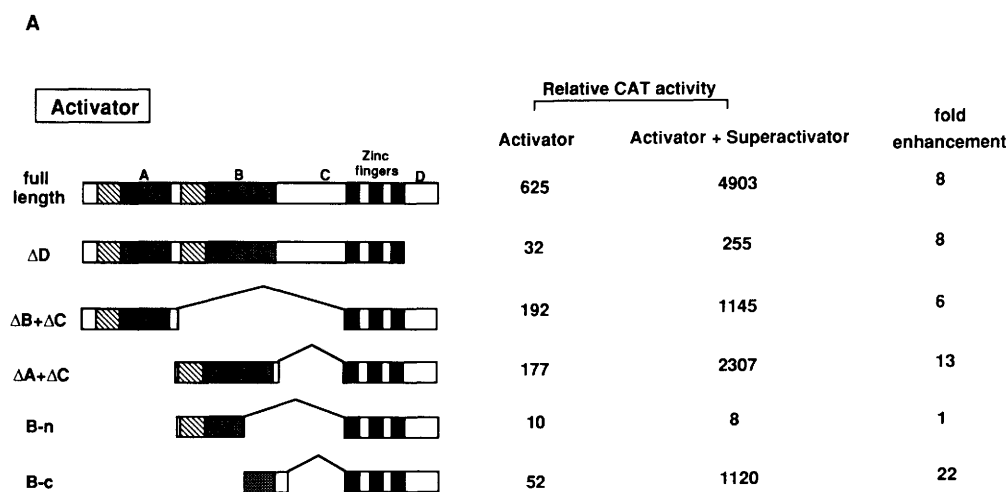
deletion of the D domain on the activator lowered the overall level of activation, surprisingly, it had no effect on superactivation, suggesting that the D domain influences the interaction of Sp1 bound at separate sites but not the interaction of DNA-bound and nonbound forms (Fig. 4A). Either the A or B domain alone fused to the DNA-binding and D domains is efficiently superactivated. Further deletion of the B domain indicated that the carboxy-terminal third of this region is sufficient to act as an activator for superactivation. This region of the B domain activates very poorly on its own but is superactivated >20-fold. These results suggest that the activator serves primarily to tether the superactivator to the DNA via protein-protein interactions and need not play a critical role in the activation function. The amino-terminal two-thirds of the B domain cannot be superactivated, suggesting that there is a specific portion of the protein that is necessary for activator-superactivator interactions.

We then tested the domain requirements for the superactivator by constructing variants consisting of only the A or B domains. These truncated molecules could superactivate full-length Sp1 but at a much lower level

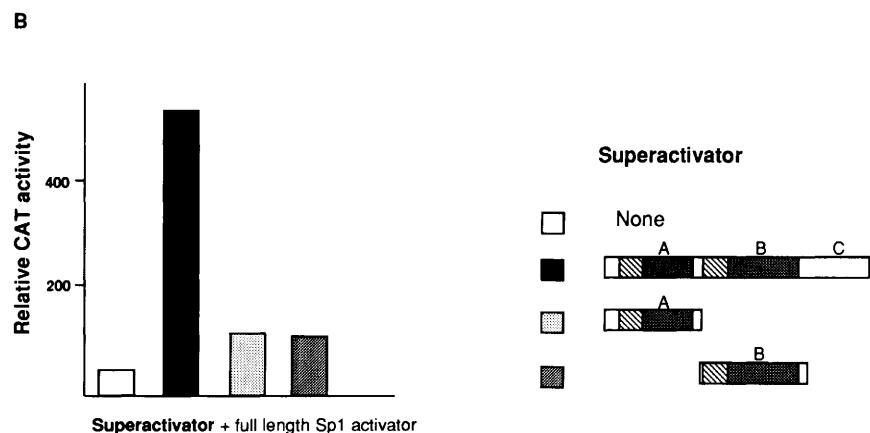
than the superactivator with both glutamine domains (Fig. 4B). One explanation for this finding is that one glutamine-rich domain serves to interact with the DNA-bound activator, and the other domain is necessary to participate in activation, presumably by interacting with components (coactivator and tethering factors) of the transcription machinery.

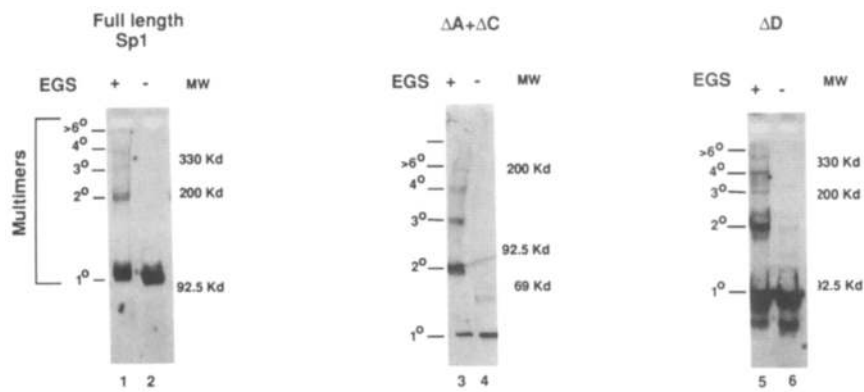
#### *Sp1 molecules can form multimers in solution*

The *in vivo* transfection studies of synergism and superactivation can be interpreted most simply if we assume that Sp1 forms multimeric structures during activation. To obtain direct evidence for such structures, we used the chemical cross-linking agent EGS [ethylene glycolbis-(succinimidylsuccinate)] to examine the multimerization properties of full-length and deletion derivatives of Sp1. Full-length Sp1 forms cross-linked dimers, trimers, and tetramers, as determined by SDS-PAGE (Fig. 5, lanes 1 and 2). It is unclear whether the full-length protein is able to cross-link to larger forms than tetramers because larger species were not resolved in this gel system or in others tried. We therefore used a smaller form of Sp1, the



**Figure 4.** Domains of Sp1 involved in superactivation. (A) The ability of full-length and deletion derivatives of Sp1 to be superactivated. Two nanograms of each expression vector containing the DNA-binding activator (shown schematically in the first column) was cotransfected with the reporter plasmid pUCSVCAT, which contains the SV40 early promoter (six Sp1 sites) upstream of CAT (Courey and Tjian 1988). The relative activity of each activator alone or with 200 ng of the superactivator expression vector (containing the A, B, and C domains of Sp1) is shown in the second and third columns, respectively. The fold enhancement achieved by the addition of the superactivator is shown in the last column. The basal level of SV40 early promoter without Sp1 is 7. (B) The bar graph depicts the enhancement of full-length Sp1 activity by variants of the superactivator. Each of the tested variants is shown schematically at right. In each case, 2 ng of full-length Sp1 expression plasmid and 200 ng of superactivator expression plasmid were cotransfected with the reporter plasmid pUCSVCAT.





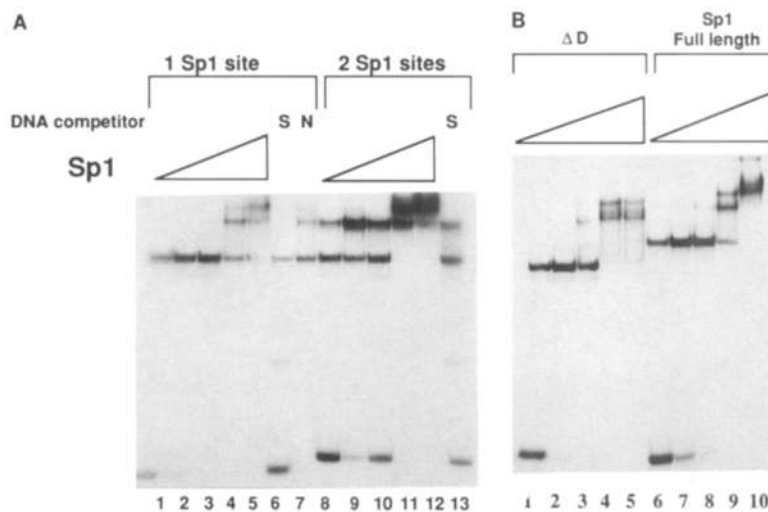
**Figure 5.** Sp1 forms multimers in solution. Partially purified proteins were incubated with (lanes 1,3,5) or without (lanes 2,4,6) the cross-linking reagent EGS. The products were then separated on 5% polyacrylamide gel and visualized by Western blotting (for details, see Materials and methods). The relative position of molecular mass markers is shown at right of each panel. The proposed multimerization state (monomer, dimer, etc.), on the basis of apparent molecular mass, is indicated at left of each panel. Cross-linking of purified or partially purified proteins gave the same results (data not shown).

deletion derivative  $\Delta A + \Delta C$ , to examine further multimerization to higher forms.  $\Delta A + \Delta C$  cross-links to multimers of two, three, and four molecules, as well as to larger species of hexamers and octamers (Fig. 5, lanes 3 and 4). The proteins appear to build from monomers to higher-order structures. This is consistent with scanning transmission electron microscopy data (I. Mastrangelo and P. Hough, pers. comm.), which shows that Sp1 is primarily a monomer at low concentrations of protein, becomes incorporated into specific complexes of 1–4 molecules as the concentration is increased, and forms even higher-order complexes of 8–12 molecules at higher concentrations. We also examined the multimerization properties of the  $\Delta D$  mutant. This form cross-linked at least to tetramers and possibly to higher-order structures, although large complexes were difficult to resolve (Fig. 5, lanes 5 and 6). In contrast, a truncated mutant containing only the DNA-binding domain and domain D ( $\Delta A + \Delta B + \Delta C$ ) did not multimerize in the cross-linking assay (data not shown). These results suggest that at least domain B is important for multimerization.

#### Multimerization of Sp1 when bound to DNA

The chemical cross-linking data suggest that full-length Sp1 and some mutant forms exhibit similar properties in

multimer formation in solution. However, we also wanted to examine the assembly of Sp1 multimers in the presence of DNA. Studies with proteins such as SV40 T antigen have indicated that interactions with DNA can change the multimerization properties of a protein (J. Hurwitz, pers. comm.). We therefore carried out gel-shift analysis with a DNA probe containing only one Sp1 site to examine complex formation. A titration of protein concentration in the gel-shift assay indicates that Sp1 initially binds to the DNA as a single (most likely monomer; see below) species until there is no free probe remaining (Fig. 6A). At concentrations of protein beyond this point Sp1 forms discrete slower-migrating complexes. In addition, the slower-mobility complexes could be competed by an oligonucleotide containing the Sp1 site but not an oligonucleotide of equivalent size with no Sp1 site (Fig. 6A, lanes 6 and 7). We reasoned that these complexes could represent either binding at cryptic Sp1 sites in the probe or multimers of Sp1 forming on a single site. To distinguish between these possibilities we performed DNase I footprinting analysis at various Sp1 concentrations. In the presence of excess Sp1, DNase footprinting revealed full occupancy of the single Sp1 site without protection of the ends of the DNA or cryptic sites (Fig. 1D). There was also no detectable decrease in the intensity of the DNase cleavage pattern over the



**Figure 6.** Complex formation of Sp1 when bound to DNA. (A) Gel mobility-shift assay of Sp1 when bound to one site (BCAT-1, lanes 1–7) and two sites (BCAT-2, lanes 8–13). Each lane contains 1 ng of labeled DNA fragment. Increasing amounts of protein were added to the reaction in lanes 1–5 as follows: 0.3, 0.7, 1.4, 5.7, and 11.3 ng. Lanes 8–12 are an identical titration of protein with the BCAT-2 fragment. Lanes 6, 7, and 13 each contain 5.7 ng of Sp1 and 4 ng of competitor DNA [(S) specific; (N) nonspecific]. The faint band in lanes 6 and 13 in the middle of the gel appears to be nonspecific and is not seen reproducibly. (B) A comparison of complex formation of full-length Sp1 and  $\Delta D$  proteins. Increasing amounts of protein,  $\Delta D$  (lanes 1–5) or full-length Sp1 (lanes 6–10), were incubated with the BCAT-1 promoter fragment as follows: 0.17 ng (lanes 1,6), 0.42 ng (lanes 2,7), 0.83 ng (lanes 3,8), 2.0 ng (lanes 4,9), 10 ng (lanes 5,10).

length of the probe, suggesting that Sp1 was not aggregating on the DNA nonspecifically (Fig. 1D). These findings indicate that the discrete slower-migrating complexes detected by gel-shift analysis likely represent higher-order multimers of Sp1 stacked on top of a monomer bound to the single site on the DNA. Consistent with the chemical cross-linking assay, Sp1 appears to form dimer, trimer, tetramer, and larger complexes when bound to DNA.

Although the gel-shift results clearly show that Sp1 binds to the probe and then forms larger complexes, an intrinsic weakness of the gel-shift assay is that the size of the complexes is difficult to determine. We have therefore carried out additional experiments to attempt a reasonable assignment of size to each of the complexes. We have determined the number of Sp1 molecules in a given complex by comparing the mobility of complexes formed with probes that contain one or two Sp1 sites (Fig. 6A). As expected, with both templates the singly bound probes migrate equivalently. When both sites of the two-site probe are bound, the complex (two Sp1 molecules) migrates with the slower-migrating species on the single site probe that corresponds to a dimer of Sp1 (Fig. 6A, cf. lanes 4 and 8). Pore exclusion limit analysis (Clos et al. 1990; data not shown) confirms that the singly bound species of both probes migrates at the correct molecular weight for a monomer of Sp1 and that the fully occupied two-site probe migrates at the molecular weight of two Sp1 molecules. These experiments therefore allow us to extrapolate that the slower-migrating complexes actually correspond to dimer, tetramer, and higher-order structures, confirming the results of the chemical cross-linking reactions (Fig. 5) and electron microscopy findings (I. Mastrangelo and P. Hough, pers. comm.).

#### Aberrant complex formation with the $\Delta D$ deletion

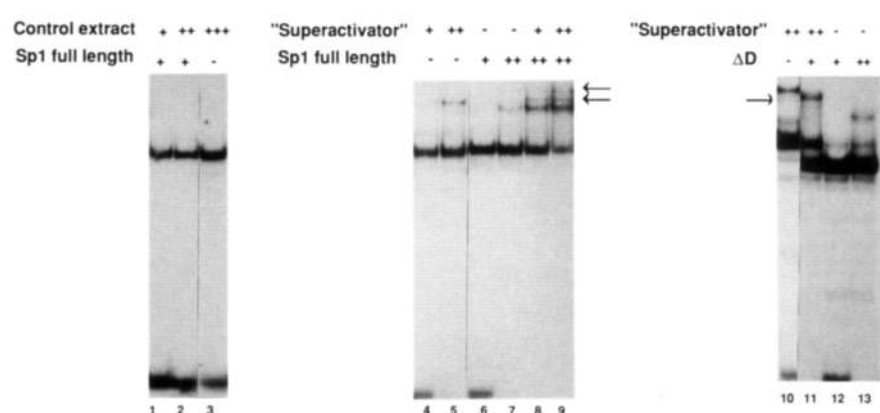
Because the  $\Delta D$  mutant differed greatly from wild type in its ability to activate transcription synergistically, we

have looked specifically at its ability to form oligomers by the gel-shift assay. A titration of  $\Delta D$  and full-length proteins shows that both species can form multimeric complexes on the DNA (Fig. 6B). However, the  $\Delta D$  protein appears to be arrested in complex formation so that even the highest concentrations of protein do not lead to the formation of larger complexes that the wild-type protein is capable of forming. This result suggests that the D domain may be directly involved in the interactions needed for higher-order complex formation on DNA, that may explain the inability of  $\Delta D$  to activate transcription synergistically.

#### DNA-binding and nonbinding forms of Sp1 can interact

A further indication of the nature of Sp1–Sp1 interactions was investigated by using a non-DNA-binding form (the superactivator form) of Sp1 containing only domains A, B, and C of the protein. A mixture of full-length Sp1 and superactivator produces several new species revealed by gel-shift analysis that are not seen with the full-length protein alone. One of these species is found with superactivator alone, but it is significantly enriched when full-length Sp1 is added (Fig. 7, cf. lanes 4 and 8). This complex is most likely the result of interactions between the superactivator and full-length Sp1 protein because the extract containing the superactivator also contains some endogenous full-length HeLa Sp1 that copurifies with the superactivator expressed in vaccinia virus-infected HeLa cells (Fig. 7, lanes 4 and 5; Western analysis data not shown). The mixture of superactivator and full-length protein also produces a second, slower-migrating species that increases in concentration as more superactivator is added and as the monomeric full-length species decreases (Fig. 7, lanes 8 and 9). Complexes other than a single species of bound Sp1 are not seen with the control virus extract (Fig. 7, lane 3) alone or when mixed with the full-length form (Fig. 7, lanes 1

**Figure 7.** Formation of complexes between activator and superactivator forms of Sp1. Various combinations of proteins (purified from vaccinia virus-infected HeLa cells) were incubated together for 15 min at room temperature; 0.02 ng of labeled DNA fragment (promoter from BCAT-1) was then added, and the mixture was incubated for an additional 15 min before loading onto a 4% native gel. In lanes 4–9, full-length Sp1 (+ 0.2 ng, + + 0.5 ng) was incubated with partially purified superactivator protein (+ 0.5 ng, + + 1 ng). The superactivator extract contains some copurifying endogenous HeLa Sp1, and this accounts for the binding activity of the superactivator extract alone (lanes 4,5). As a control, extract from cells infected with a nonexpressing vaccinia virus, containing a similar amount of Sp1 to the superactivator extract, was mixed with full-length Sp1 (lanes 1,2) or added alone (lane 3). Lanes 10–13 show complexes formed when the superactivator protein (1 ng) is mixed with the  $\Delta D$  protein (+ 0.3 ng, + + 1.7 ng). The arrows indicate the novel species formed upon mixing.



and 2). These results suggest that Sp1 interacts directly with the superactivator to form complexes on the DNA. Thus, the superactivator may function by docking on top of the DNA-binding species and forming large heteromeric complexes that are able to enhance efficiently transcription directed by Sp1.

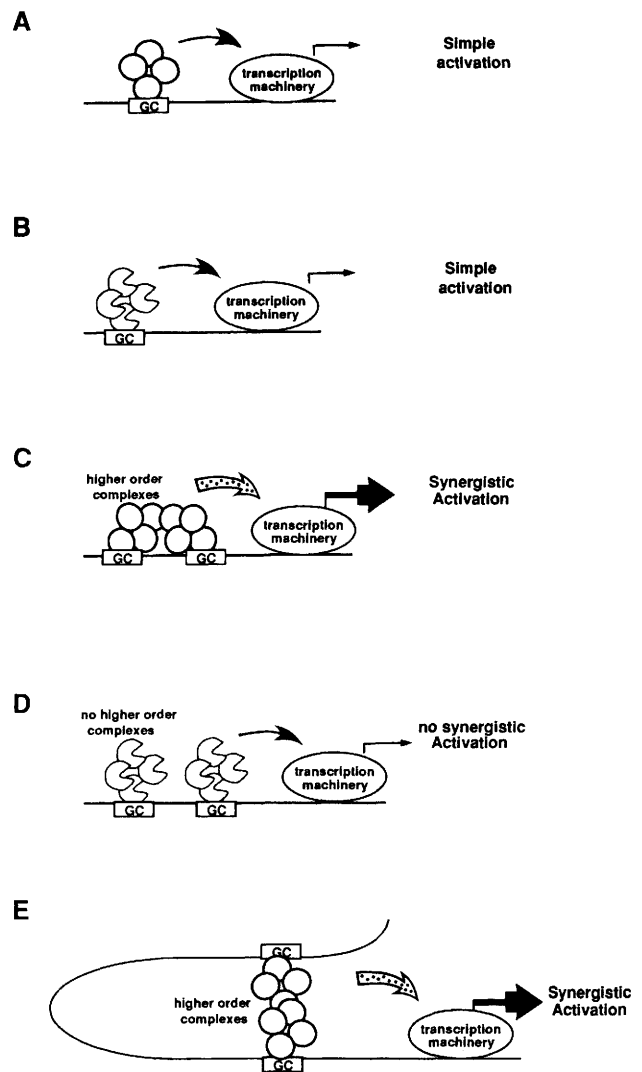
Our *in vivo* transfection assays suggest that  $\Delta D$  can interact with the superactivator. To test this hypothesis, we performed gel-shift reactions with a mixture of  $\Delta D$  and the superactivator forms. A novel complex is detected with the mixture of the two proteins that is not present in the reactions with each protein alone (Fig. 7, lane 11). No novel complexes are seen with the mixture of  $\Delta D$  and control extract (data not shown). It appears that the  $\Delta D$  protein contains the portions of Sp1 required for direct interaction with the superactivator but lacks the domains necessary to form large functional homomeric complexes that may be involved in synergism. These results are consistent with the observation that  $\Delta D$  can be superactivated, although it does not activate synergistically. These results also suggest that the nature of the activator–superactivator complexes differs from that of the large homomeric complexes that may participate in synergistic activation.

## Discussion

Using *in vivo* cotransfection of wild-type and mutant Sp1, we have assayed the function of individual activation domains at promoters with single and multiple GC box-binding sites. Three of the activation domains, A, B, and D, are essential for synergistic activation of transcription. The previously uncharacterized D domain appears to play a central role in mediating synergistic activation and is only needed for activation from multiple sites. *In vitro* analysis of Sp1–Sp1 complexes reveals a correlation between the ability of Sp1 to multimerize and its ability to activate transcription synergistically. On the basis of these studies we have developed a model (Fig. 8) to help explain the requirement of multiple activation domains and the synergistic transcriptional activation mediated by Sp1.

### *Sp1 forms tetramers on a single binding site*

The wild-type Sp1 protein binds to a single GC box as a monomer and then builds higher-order complexes (tetramers) by direct protein–protein interactions that do not appear to involve additional contacts with the DNA (Fig. 8A). However, there is no evidence to distinguish whether Sp1 actually functions as a monomer or as a multimer at a single site. Once bound to a single GC box, Sp1 directs a moderate level of transcriptional activity (simple activation) that is dependent on the glutamine-rich domains A and B but does not require domains C and D. Thus, the  $\Delta D$  mutant, lacking the carboxy-terminal 77 amino acids, also forms tetramers when bound to



**Figure 8.** Model for synergistic activation by Sp1. (A) Sp1 can bind to a single site and interact with the general transcription machinery to activate a low level of transcription. Although Sp1 is shown as a tetramer, our results do not distinguish between a monomer and/or a multimeric form as being the active complex on a single site. (B) The deletion of the D domain does not change the ability of Sp1 to activate from a single site. Therefore, the  $\Delta D$  mutant is shown forming the same complex and interactions as the full-length protein at a single GC box. (C) Tetramers of Sp1 bound at adjacent sites interact to form higher-order complexes. The formation of these complexes may generate an activation surface that can interact more efficiently with the general transcription machinery and thereby activate transcription to a higher level (synergistically). (D) The deletion of the D domain of Sp1 prevents the formation of higher-order complexes by adjacently bound tetramers; therefore,  $\Delta D$  does not activate transcription synergistically. (E) Sp1 bound to distal sites forms higher-order complexes, looping out the intervening DNA. These complexes can interact with the general transcription machinery in a manner similar to that of the complexes shown in C to activate transcription synergistically. (○) Full-length Sp1; (⊖)  $\Delta D$  mutant.



a single GC box and directs essentially the same low level of transcription (Fig. 8B).

*Synergistic activation mediated by Sp1 bound to multiple sites*

In stark contrast to templates bearing a single Sp1 site, templates containing two adjacent or separated GC boxes can lead to very high levels of transcriptional activation by Sp1 (synergistic activation), which are 50- to 80-fold greater than the activation at a single site. Thus, the activity of two sites is greater than the additive effect of two independent sites. This dramatic synergistic effect strongly suggests that Sp1 bound at adjacent sites interacts and that this interaction somehow enables Sp1 to activate transcription at a much higher level (Fig. 8C). Direct DNA-binding studies failed to detect any evidence of Sp1 binding cooperatively to the DNA that could account for the transcriptional synergism. Thus, we conclude that the synergistic effects of multiple Sp1 sites occur at steps following DNA binding. Not surprisingly, Sp1 bound at adjacent sites also formed tetramers and even higher-order structures.

The activation by Sp1 from two adjacent sites, like the single binding site case, requires the glutamine domains A and B and does not require the C domain. However, unlike the single-site situation, maximal activation from multiple sites requires domain D. This suggests that domain D may be important for interaction between molecules of Sp1 when tethered to multiple binding sites (Fig. 8C,D). In support of this hypothesis, *in vitro* analysis of protein complexes reveals that the  $\Delta D$  mutant is defective in the formation of complexes larger than tetramers on templates with either one or two binding sites. Taken together, the *in vivo* and *in vitro* data suggest that formation of a specific higher-order complex may be a necessary step in synergism and that the D domain somehow participates in the formation of this complex (Fig. 8C,D).

The synergism and complex formation on adjacent Sp1 sites is strikingly similar to enhanced activation observed from a promoter containing widely separated upstream and downstream Sp1 sites. As expected, the A, B, and D domains are essential for the synergism from the distal sites in the same way that they are needed for high levels of activation from adjacent sites. Recent electron microscopy studies have directly visualized Sp1–Sp1 interactions, revealing that molecules bound to the separated sites form a large protein–DNA complex, looping out the intervening DNA (Su et al. 1991). Furthermore, the Sp1 in the complex appears to consist of two or more stacked tetramers as determined by scanning transmission electron microscopy (I. Mastrangelo and P. Hough, pers. comm.). Thus, Sp1 acting on distal sites could undergo specific complex formation in a manner similar to complex formation on adjacent sites (Fig. 8E). In both cases (Fig. 8C,E), two bound Sp1 multimers interact to form a distinct protein interface that enhances the transcriptional activation synergistically.

How does this synergism occur? We propose that the

interaction of adjacent Sp1 complexes may generate a more effective activation surface to interface with components of the transcriptional machinery such as coactivators or basal factors. One possibility is that adjacent Sp1 complexes bind to the target (a component of the transcription machinery) cooperatively. Alternatively, the association of adjacent Sp1 complexes may present a novel, more potent activation surface. It is also possible that the interaction of adjacent multimers of Sp1 may allow contact with more than one factor in the transcription complex or even influence more than one step in the transcription reaction, thus leading to a synergistic response. Further investigation of the interactions of sequence-specific activators with the general transcription machinery and its associated coactivators will help to elucidate the detailed mechanism of synergism.

Our observation of transcriptional synergism directed by adjacent Sp1-binding sites is somewhat unexpected in light of earlier studies of Sp1 activities on the SV40 early promoter. The six tandem GC boxes of SV40 apparently operate independently to activate transcription (Barrera-Saldana et al. 1985; Gidoni et al. 1985). Although these results may appear inconsistent at first, they raise the intriguing possibility that synergistic activation by Sp1 may be promoter context dependent. By placing Sp1 sites upstream of different promoters, we have found that a subset of these promoters do not allow Sp1 to function synergistically (E. Pascal, unpubl.). The differences in these promoters may help to define further the interactions that govern the activity of Sp1.

*Superactivation by Sp1 occurs through a direct interaction of activator and superactivator proteins*

Previous studies have shown that a non-DNA-binding mutant of Sp1 can enhance the ability of a DNA-binding form of Sp1 to activate transcription (Courey et al. 1989). This process, called superactivation, was postulated to involve direct Sp1–Sp1 interactions. To substantiate this hypothesis, we have obtained evidence for direct interactions between the DNA-binding and nonbinding forms by gel-shift assays. We have examined the involvement of individual activation domains and found that unlike synergism, superactivation does not require the D domain. The mechanism by which superactivation occurs may be akin to the process of simple activation by Sp1. The addition of the superactivator merely increases the number of activation domains by forming multimers on the activator. The formation of these heteromeric complexes may be analogous to the homomeric complexes formed with high concentrations of wild-type Sp1 (Fig. 8A,C). Although the D domain is not necessary for the interaction of activator and superactivator, the ability of the heteromeric complex to activate transcription is enhanced by the D domain (Fig. 4A). This result suggests that interactions between activator–superactivator complexes bound at multiple sites further enhance the transcriptional activity in the same way that the interaction of homomeric activator complexes bound at multiple sites may lead to synergism (Fig. 8C). Thus, the Sp1–Sp1

complexes involved in superactivation may be a subset of the interactions used in synergistic activation.

Why are both glutamine-rich activation domains A and B required for synergism and superactivation? The correlation of complex formation with the two processes suggests that perhaps one of their functions may be participation in multimerization. However, a single glutamine domain, although sufficient for tetramerization and interaction with the superactivator (Fig 5; data not shown), is insufficient for synergism and superactivation (Figs. 2 and 4B). We propose that one of the glutamine-rich domains is needed for complex formation, and the second glutamine-rich domain may be needed to interact with the transcription machinery (coactivators and tethering factor).

There are several other examples of enhanced or synergistic activation of transcription mediated by the interplay of multiple site-specific transcription factors. For example, Gal4 interaction with Gal11 (Himmelfarb et al. 1990; Nishizawa et al. 1990) and Oct-1 complexed with VP-16 (Triezenberg et al. 1988a,b; Kristie et al. 1989; Stern et al. 1989) both lead to a higher level of activation. Similarly, the association of HAP2, HAP3, and HAP4 results in enhanced transcription (Forsburg and Guarente 1989; Olesen and Guarente 1990). Recently, complexes of Sp1 and the bovine papillomavirus (BPV) E2 protein have been shown to augment transcription (Li et al. 1991). All of these cases are thought to work through direct and specific protein-protein contact between factors, forming higher-order complexes that presumably provide a more effective structure for assembling or triggering transcription initiation by the basal machinery. Thus, the general theme is that multimerization, either homomeric or heteromeric, leads to the formation of a structure specifically suited for activation.

## Materials and methods

### Plasmids

The reporter plasmids BCAT-1, BCAT-2, and BCAT-3 were created by cutting the vector BCAT (Lillie and Green 1989) with *Sall* and inserting one, two, or three copies of the oligonucleotide shown in Figure 1A (box III of the HTLVIII LTR; Jones et al. 1986). BCAT-2S was made by inserting an additional oligonucleotide into the *Xho*I site of BCAT-1, thereby separating the two oligonucleotides by 30 bp of polylinker sequence. pUCSV-CAT is described in Courey and Tjian (1988). All Sp1 expression vectors are based on the parent plasmid pPacU. The construction of those not mentioned below has been described previously (Courey and Tjian 1988; Courey et al. 1989). For the purposes of this paper, we have renamed some of the expression constructs according to which Sp1 domains have been deleted. For comparison with Courey and Tjian (1988) the conversions are as follows: full-length = pPacSp1,  $\Delta A = 516C$ ,  $\Delta C = \Delta int112$  (this is pPacSp1 with the same internal deletion as  $440C\Delta int112$ ),  $\Delta D = N619$ ,  $\Delta A + \Delta C = 440C\Delta int112$ ,  $\Delta B + \Delta C = \Delta int349$ . The superactivator containing A, B, and C domains has been referred to as N539. The Sp1 derivatives B-n and B-c were made by exonuclease III deletion mutagenesis as described in B.F. Pugh et al. (in prep). B-n contains amino acids

263–421 and the carboxy-terminal 168 amino acids of Sp1. B-c contains amino-acids 425–542 and the carboxy-terminal 168 amino acids of Sp1. The superactivator expression vectors pPacA and pPacB were made by subcloning the A domain (for pPacA, amino acids 82–262 of Sp1) or the B domain (for pPacB, amino acids 263–542) into pBluescript KS+ and inserting an *Xba*I 14-mer (stop codons in all three frames) into the *Eco*RV site. The fragment containing domain A or B and the stop codons was then inserted into pPacU.

### In vivo transfection and CAT assays

SL2 *Drosophila* tissue culture cells were transfected and assayed as described in Courey and Tjian (1988), with the following modifications. Cells were plated on 6-cm plates at a density of  $4.5 \times 10^6$  cells per plate. All cells received 2  $\mu$ g of reporter plasmid, 8–25 ng of expression plasmid (except where different amounts are noted in the figure legends), and pUC118 carrier DNA to bring the total to 5  $\mu$ g of DNA per plate. CAT assays were performed and quantitated as in Baichwal and Tjian (1990). The data presented are the averages of at least two and, in many cases, four to six transfections done in duplicate.

### Expression and purification of full-length and deletion derivatives of Sp1

All proteins were expressed by infecting HeLa cells with recombinant vaccinia viruses as described in Jackson et al. (1990). Full-length Sp1 (v-Sp1) and the superactivator expression virus (v-3'Sp1) are described in Jackson et al. (1990). The virus expressing  $\Delta D$  contains amino acids 83–701 of Sp1. The  $\Delta A + \Delta C$ -expressing virus contains amino acids 338–500 and 611–778 of Sp1. The methionine codon for expression from these two viruses was provided by inserting the oligonucleotide (top strand, 5'  $\rightarrow$  3') CTAGAACCCACCATGG (bottom strand, 5'  $\rightarrow$  3') GATCCCATGGTGGG, in-frame upstream of the coding sequence (the *Bam*HI site of N619, the *Sau*3A site of  $440\Delta int112$ ; Courey and Tjian 1988). The fused sequences were then inserted into the *Xba*I-cut (for  $\Delta D$ ) or the *Xba*I- and *Kpn*I-cut (for  $\Delta A + \Delta C$ ) pAbT4537 vector (kindly provided by Applied Biosystems, Inc.). The control virus used was NYCBH (kindly provided by Applied Biosystems, Inc.).

Full-length Sp1 and  $\Delta D$  proteins were purified by DNA affinity chromatography, followed by chromatography on wheat germ agarose resin (Jackson et al. 1990 and references therein). The  $\Delta A + \Delta C$  protein was purified by DNA affinity chromatography. The superactivator protein was purified by wheat germ agarose chromatography. The partially purified full-length and  $\Delta D$  proteins used in the EGS cross-linking were purified by DNA affinity column only. Concentration of purified proteins was quantitated by silver staining. The superactivator protein was quantitated by Western blot in comparison to a known amount of Sp1.

### Gel mobility-shift and DNase I footprinting assays

Labeled DNA probe for both the gel-shift and footprinting reactions was made from BCAT-1, BCAT-2, and BCAT-2S by digesting the plasmids with *Eco*RI, end-labeling with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ ]ATP, and digesting with *Xho*I. The labeled promoter fragment was gel purified.

For gel-shift assays, 0.02–1 ng of probe was mixed with variable amounts of purified protein (for amounts used in individual experiments, see figure legends) for 15–30 min at room temperature in a buffer consisting of 12.5 mM HEPES-KOH (pH 7.5), 6.25 mM  $MgCl_2$ , 10% (vol/vol) glycerol, 0.05% (vol/vol) NP-40,

5  $\mu\text{M}$   $\text{ZnSO}_4$ , 50 mM KCl, and 50  $\mu\text{g/ml}$  of BSA. The reactions were electrophoresed at room temperature on 4% native polyacrylamide gels containing 0.5 $\times$  TBE, 1 mM EDTA, and 0.05% (vol/vol) NP-40. The Sp1 oligonucleotide contained in the labeled probe (sequence shown in Fig. 1A) was used as specific competitor DNA. The nonspecific competitor used was an oligonucleotide with the random sequence: (top strand, 5'  $\rightarrow$  3') CTAGAACCCACCATGG; (bottom strand, 5'  $\rightarrow$  3') GATCCATGGTGGGTT.

DNase I footprinting reactions were carried out as described previously (Dyan and Tjian 1983) except that binding was carried out at room temperature for 20 min.

#### Chemical cross-linking analysis

Partially purified full-length and  $\Delta\text{D}$  proteins (10–20% pure) and purified  $\Delta\text{A} + \Delta\text{C}$  protein were incubated in the presence of 0.4 mM EGS (Sigma) and the buffer used in the gel-shift DNA-binding reactions for 15 min at room temperature. The reactions were stopped by the addition of D-lysine to a final concentration of 12.5 mM. The resulting products were separated on 5% PAGE and visualized by Western blotting. The blots were probed first with a rabbit polyclonal antibody (raised against full-length Sp1) and then with protein G conjugated to horseradish peroxidase (Bio-Rad). The blots were developed with the ECL detection system (Amersham).

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## Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism.

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