Sp3 Is a Bifunctional Transcription Regulator with Modular Independent Activation and Repression Domains*

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Sp3 is a member of the Sp family of transcription factors and binds to DNA with affinity and specificity comparable to that of Sp1. We demonstrate that Sp3 is a bifunctional transcription factor that can both activate and repress transcription. Gene fusion experiments in mammalian cells demonstrate that the Sp3 activation potential is distributed over an extensive glutamine-rich N-terminal region, whereas the repressor activity has been mapped in a 72-amino acid region located at the 5′ of the zinc finger DNA-binding domain. We demonstrated that the repression activity is strictly dependent on the context of the DNA-binding sites bound by Sp3. We found that Sp3 represses transcription of promoters bearing multiple GAL4 DNA-binding sites, whereas it activates isogenic reporters containing a single GAL4-binding site. Transfection experiments in Drosophila cells that lack endogenous Sp activity demonstrated that Sp3 does not possess an active repression domain that can function in insect cells, rather it is a weak transcriptional activator of the c-myc promoter. Our results strongly suggest that Sp3 is a dual-function regulator whose activity is dependent upon both the promoter and the cellular context.

Sp1 is a ubiquitously expressed transcription factor that plays a major role in the regulation of a large number of gene promoters, including constitutive and inducible genes (1–3). Recently the existence of an Sp family of transcription factors has been documented (4, 5). Sp1, Sp3, and Sp4 proteins have similar structural features with highly conserved DNA-binding domains and they recognize GC and CT boxes with closely similar specificity and affinity (5–7). Moreover, it has been shown that both Sp1 and Sp3 proteins are ubiquitously expressed in many mammalian cell lines, whereas Sp4 expression appears to be restricted to certain cell types (5–7). Transfection experiments into mammalian and Drosophila SL2 cells have shown that Sp4, like Sp1, is a transcriptional activator of Sp1 responsive promoters, whereas Sp3 represses Sp1-mediated transcription (5–9). Clearly the existence of proteins similar to Sp1 suggests that gene regulation by Sp1 is more complex than previously assumed. We previously showed that transfer of the nonfingers region of the Sp3 protein to a heterologous DNA-binding domain confers repressor function to the fusion protein, allowing it to repress transcription from reporter promoters containing multiple DNA-binding sites. Our previous data suggest that repression by chimeric Sp3 proteins occurs via protein-protein interaction with components of the basal transcription complex (10).

In the present study we have embarked on a detailed analysis of the Sp3 function. To investigate the transcriptional properties of Sp3 in mammalian cells we have used gene fusion experiments to dissect the functional domains of Sp3. We found that Sp3 is a bifunctional protein containing independent modular repressor and activator domains. The activation potential of Sp3 is distributed over an extensive glutamine-rich N-terminal region. The negative regulatory function has been mapped 5′ of the zinc finger region. Moreover, we demonstrated that the Sp3-repression ability is strikingly dependent upon the context of the Sp3 DNA-binding sites present in the reporter promoters. Sp3 functions as a repressor when it is bound to the promoter through multiple DNA-binding sites. Conversely, Sp3 turns out to be an activator when it is targeted to the promoter via a single DNA-binding site. In addition, using the Drosophila Schneider SL2 cells that lack endogenous Sp activity, we demonstrate that Sp3 does not possess an active repression domain that can function in insect cells, but rather it is a weak transcriptional activator of the human c-myc promoter, and it functionally co-operates in vivo with the TATA-binding general transcription factor TBP.1

Our results suggest that Sp3 is a bifunctional transcriptional regulator and its predominant effect would depend on the context of Sp3 DNA-binding sites and on the nature of a co-repressor present in a particular cellular background.

**EXPERIMENTAL PROCEDURES**

**Reporter Plasmids**—The −839 HIV CAT reporter has been previously described (8), in which an oligonucleotide containing a single GC box was inserted into the XhoI site at −38 of the HIV promoter sequences. The pmyc-XAN is a reporter plasmid containing the human c-myc promoter sequences from the XhoI-NaeI fragment spanning from −93 to +54 of the P2 transcription start site, and the pmyc-XANmut is the isogenic plasmid in which the single Sp1/Sp3-binding site located at position −53 was mutated by changing the CT box in a sequence containing adjacent Sp1/Sp3 sites as described previously (9). The G5-XAN and G1-XAN contain five or a single GAL4-binding site inserted at the XhoI site of the pmyc-XAN reporter, respectively. The XAN-G5 and XAN-G1 reporters contain five or a single GAL4-binding site inserted in the Sp1/Sp3 sites present in the pmyc-XANmut. The 3×CT-XAN has been constructed by inserting in the Sp1/Sp3 sites present in the pmyc-XANmut, an oligonucleotide containing a duplicated CT box. The G5E1b (11) and G1E1b are CAT reporter plasmids containing five or one GAL4-binding site upstream from the E1b TATA box, respectively.

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The abbreviations used are: TBP, TATA-binding protein; CAT, chloramphenicol acetyltransferase; TAF, TBP-associated factor; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; GR, glucocorticoid receptor.
**RESULTS**

**Sp3 is a Bifunctional Protein with Independent Modular Activation and Repression Activities**—Using transfection experiments into mammalian cells we and others have previously shown that enhanced expression of Sp3 did not have any significant effect on the activity of promoters containing Sp-DNA-binding sites (6, 8, 9, 15). However, a severe limitation of these experiments is due to the constitutively high level of expression of both Sp1 and Sp3 proteins in mammalian cells (6, 8). Moreover, it was quite difficult to find an appropriate control expression vector for normalization of transfection efficiency. All promoters that are employed to express indicator proteins have GC boxes and could be thereby influenced by ectopically expressed Sp3. Hence, we have used gene fusion experiments to analyze the Sp3 transcription properties. We have recently reported a study on the behavior of chimeric transcription factors composed of the nonfinger domain of Sp3 and heterologous DNA-binding domains. We have shown that such chimeric proteins were able to repress the activation of transcription by several types of activators, indicating that Sp3 contains a portable repressor domain that can function independently from the zinc finger DNA-binding domain (10). To elucidate the Sp3 domain(s) required for transcription regulation several coding regions of Sp3 were cloned in the expression vector pSG424, encoding the yeast DNA-binding domain (amino acids 1–147) of GAL4 (13). Resulting chimeras were tested for the ability to transactivate a reporter containing five GAL4-binding sites at position −93 relative to the P2 start site of human c-myc promoter (9). Results of gel shift assays with transiently transfected HeLa cell extracts ensured that all of the fusion proteins were expressed at comparable levels and were competent for DNA binding (data not shown). Accordingly with our previous

**Effector Plasmids**—The *Drosophila* expression vectors pPacSp1 and pPacSp3 were previously described (6, 8). The pPacSp3BD expressing the Sp3 DNA-binding domain was constructed by PCR amplification of the fragment encoding the Sp3 zinc-finger domain (amino acids 577–667) using primers containing BamHI (forward primer) and XhoI (reverse primer) sites, respectively. The PCR product was then cut with BamHI/XhoI and inserted in the pPacSp1 digested with BamHI/XhoI. The dTBP expression plasmid Act-TBP was described previously (12), and it was kindly provided by J. Manley. The GAL4–Sp3 (1–527) plasmid has been described (8). All the GAL4–Sp3 expression plasmids were constructed by PCR amplification of the relevant region of Sp3 using primers containing the EcoRI site. The PCR products were digested with EcoRI and cloned in the EcoRI site of pSG424 expressing the yeast DNA-binding domain (1–147) of GAL4 (13). All plasmids were analyzed by DNA sequencing to confirm correct construction. Fragments generated by PCR were sequenced in all cases to verify that no misincorporation had occurred during the reaction. Full details of each construction are available upon request.

**Transfection and CAT Assay**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfections were performed by calcium phosphate precipitation using subconfluent cell cultures using different amounts of reporter and effector plasmids. For normalization of transfection efficiencies a pCMVβ-gal expression plasmid (1 μg) was included in the co-transfections. *Drosophila melanogaster* SL2 cells were grown in Schneider’s medium supplemented with 10% heat-inactivated fetal calf serum, and transfected by calcium phosphate precipitation as described previously (8, 9). For normalization of transfection efficiencies the *copia* LTR-lacZ expression plasmid (14), kindly provided by J. Manley, was included in the co-transfections. CAT assays were performed with different amounts of extract to ensure linear conversion of the chloramphenicol with each extract and results are presented as the means ± S.D. of at least four duplicated independent transfection experiments. The CAT activity was quantified using the Molecular Dynamics PhosphorImager System™.

**Fig. 1. Sp3 contains modular independent activation and repressor domains.** Top, the schematic representation of the GAL4–Sp3 constructs and reporter plasmids G5E1b and G5-XAN. A, G5E1b reporter (5 μg) was co-transfected into HeLa cells with the indicated GAL4 expression vector (10 μg). B, G5-XAN reporter (5 μg) was transfected into HeLa cells together with indicated GAL4 vector (10 μg). Data are presented as fold activation relative to the sample without effector. Each histogram bar represents the mean of three independent transfections after normalization for the internal control β-galactosidase activity. Standard deviations are indicated. The basal levels of G5-XAN and G5E1b promoters were 10 and 0.4% of CAT activity/100 μg of cell extract, respectively.
report (10) the GAL4-Sp3(1–527) chimera did not activate the G5E1B reporter and repressed G5-XΔN transcription 10-fold in our assay. Interestingly, the GAL4-Sp3(1–358) activates transcription of both reporters. When this region was subdivided into amino acids 1–191 or 189–339, these segments function as well as the large intact domain, albeit the region 189–339 at lower efficiency. Conversely, all fusion proteins containing amino acids 455–527 failed to activate G5E1B and repressed to a similar level (about 10-fold) the G5-XΔN reporter. These effects were specific to reporter plasmids containing GAL4 sites with no activation or repression being observed on reporter plasmids lacking such sites (Ref. 10, and data not shown). From the results shown in Fig. 1 it appears that, in contrast to an extensive redundant activation domain (amino acids 1–358), Sp3 contains a repressor function that can be precisely localized in the 72-amino acids (from position 455 to 527) adjacent to the DNA-binding domain. These data strongly suggest that Sp3 is a bifunctional protein containing domains that can both activate and repress transcription in transient transfection assays, and both domains can function as independent modular components of the Sp3 transcription factor.

Sp3-mediated Repression Is Dependent on the Number of DNA-binding Sites—It has been reported that several transcription factors required reiteration of the cognate DNA-binding sites for prominent transcription activation (16). Moreover, BTEB, a transcription factor that binds to the same GC box as Sp1 and Sp3, exerts an opposite regulation of transcription depending on the number of BTEB DNA-binding sites (17). These observations led us to construct the G1-XΔN and G1E1b reporters. These reporters are isogenic to G5-XΔN and G5E1b except for the presence of a single GAL4 DNA-binding site, respectively. We then tested the response of these promoters to the GAL4-Sp3(1–527) fusion protein. As reported in Fig. 2, A and B, opposite different results were observed using promoters containing five or one GAL4-binding site, respectively. Dose-dependent GAL4-Sp3(1–527)-mediated repression occurred when the G5-XΔN served as reporter, and no significant effects were found with the G5E1b reporter, which has a very low basal level of expression. Unexpectedly, coexpression of GAL4-Sp3(1–527) resulted in a dose-dependent activation of both G1-XΔN and G1E1b reporters. Therefore, it appears that the GAL4-Sp3(1–527) acts as activator or repressor depending on the number of GAL4-binding sites present in the test promoter. Since the reporter pmyc-XΔN contains a single Sp1/Sp3 DNA-binding site at −53, it is very likely that the activity of the G5-XΔN and G1-XΔN reporters is affected by both the ectopic GAL4 protein and endogenous Sp1/Sp3 proteins. We therefore sought to determine the GAL4-Sp3-mediated regulation in the absence of the DNA-binding site for endogenous Sp1/Sp3 proteins. To this end we substituted the CT box at −53 with a single (XΔN-G1) or five (XΔN-G5) GAL4 DNA-binding sites. The XΔN-G1 and XΔN-G5 reporters were co-transfected into HeLa cells along with increasing amounts of GAL4-Sp3(1–527) expression vector. Although the basal transcription levels of XΔN-G1 and XΔN-G5 were 5-fold lower then the parental pmyc-XΔN promoter, likely due to the absence of the positive CT element present at −53, basal CAT activity of both reporters was easily detectable. Coexpression of GAL4-Sp3(1–527) repressed in a dose-dependent manner the XΔN-G5 activity. Conversely, the opposite result was obtained using the XΔN-G1 as reporter. An evident dose-dependent activation was observed in the presence of GAL4-Sp3(1–527) effector (Fig. 2C). Therefore, a similar behavior of GAL4-Sp3(1–527) was observed using three different reporters. In each case we found that the nonfinger region of Sp3 was acting as a repressor when bound to multiple DNA-binding sites, and it acted as an activator when tethered to the promoter via a single DNA-binding site. Since GAL4-Sp3(1–527) contains both the activating and repressor domains as defined in Fig. 1, we sought to analyze the effects of the activating domain (1–358) and repressor domain (337–527) on promoters bearing one or five GAL4 DNA-binding sites, respectively. As reported in Fig. 3A, the glu-
amine-rich Sp3 domain functions as an activator of promoter bearing either one or five GAL4-binding sites, albeit the activation ability was higher on the reporter with five GAL4 sites. The Sp3 domain (337–527) repressed the G5-XN, but it has only a small repression effect on the isogenic G1-XN reporter (Fig. 3B). Thus, it appears that Sp3 is a dual-function regulator acting as repressor when bound to a promoter bearing multiple binding sites, and it functions as an activator when tethered to a promoter via a single DNA-binding site.

Sp3 Is a Positive Regulator in Drosophila Schneider Cells—We have recently shown that both Sp1 and Sp3 proteins bind to the same sites of c-myc promoter, and co-transfection experiments in Drosophila Schneider SL2 cells, which are devoid of endogenous Sp1-like activity, indicated that enforced expression of Sp3 represses Sp1-mediated activation of c-myc promoter (9). A likely interpretation of these results is that Sp3 lacks transactivation potential and it can suppress the Sp1 activity simply because it binds to, and thus competes for, the same DNA-binding sites. Alternatively, Sp3 may actively repress Sp1-dependent transcription due to the strong repressor domain located near the DNA-binding region. To test these possibilities we performed co-transfection experiments in Drosophila SL2 cells using as reporter the pmyc-XN promoter construct which contains a single Sp1/Sp3-binding site located 53 base pairs upstream from the P2 initiation site (9). The Drosophila expression vectors for Sp1 and Sp3 (pPacSp1 and pPacSp3) have been previously described, and both proteins are expressed at a comparable efficiency in transfected SL2 cells (6, 9). In addition, the pPacSp3BD plasmid expressing the Sp3 DNA-binding domain alone was constructed. As reported in Fig. 4A the Sp1-mediated activation of the c-myc promoter was repressed by both the complete Sp3 protein and the DNA binding alone. Thus, it appears that the Sp3 repression of Sp1-mediated activation is likely due to competition of both proteins for the same DNA-binding site present in the c-myc promoter, rather than an active repression domain present in the Sp3 protein. Moreover, using increasing amounts of pPacSp3 vector we found a dose-dependent activation of the reporter plasmid pmyc-XN (Fig. 4B). No transactivation was seen with the pmyc-XNmut reporter. In this promoter the single Sp1/Sp3 DNA-binding site at −53 was mutated and it has been previously shown that this promoter construct does not bind both Sp1 and Sp3 (9). Since the pmyc-XN construct contains a single Sp3-binding site, we wished to determine whether Sp3 activation was also occurring using an isogenic reporter containing multiple Sp3-binding sites. To this end we substituted the single CT box at −53 with three consecutive CT boxes. As reported in Fig. 5A, Sp3 was able to activate in a dose-dependent manner this reporter, albeit at a lower level compared to the Sp1-mediated activation. Finally, we used two HIV-based reporters bearing a single (GC-38HIV) and three GC boxes (−83HIV), respectively. The HIV long terminal repeat contains three GC-binding sites, and we have previously shown that Sp3 binds specifically to these GC sites (8). As reported in Fig. 5B, Sp3 alone has no significant effect on HIV basal transcription. Since the HIV promoter possesses a very low basal level of CAT expression in SL2 cells a potential negative regulation exerted by Sp3 would have been unscored. We therefore sought to increase the basal level of transcription by co-transfecting a vector expressing the ΔTBP basal transcription factor. Accordingly with previous studies, suggesting that TBP is rate-limiting for TATA box-containing promoters in SL2 cells (12), we found that an actin 5′C-driven Drosophila TBP cDNA was able to increase the basal transcription of both HIV reporters (Fig. 5B). However, coexpression of TBP in the presence of Sp3 results in a synergistic effect resulting in enhanced transcription from the HIV promoters bearing a single or three GC boxes.

Taken together, our data strongly suggest that Sp3 does not possess an active repression domain that can function in Drosophila Schneider SL2 cells.
The experiments described here demonstrate that Sp3 is a bifunctional protein containing domains that can both activate and repress transcription in transient transfection assays, and the predominant Sp3 function depends upon both the promoter and cellular context. We have found that Sp3 activation ability is distributed over an extensive redundant domain (amino acids 1-358). This region is strikingly similar to that of Sp1, thus both proteins have a N terminus glutamine-rich region that functions as a transferable activation domain. The Sp3 transcriptional repressor activity has been mapped in a small amino acid region located at the 5’ of the zinc finger DNA-binding domain, and this domain is sufficient to confer repression function when fused to a heterologous DNA-binding domain. Our gene fusion experiments have shown that all chimeric GAL4 proteins containing the Sp3 region from amino acid residue 455 to 527 function as transcriptional repressors when bound to a promoter bearing multiple DNA-binding sites. These results suggest that the repressor domain is able to mask the glutamine-rich activating domain. Accordingly, GAL4 fusions lacking the repressor domain function as transcriptional activators. Notably, the ability of the repression domain to overcome the glutamine-rich activating region is lost when the protein is targeted to a promoter via a single DNA-binding site. We found that when Sp3 is bound to a promoter via a single GAL4 DNA-binding site it acts as a positive factor. Therefore, it appears that Sp3 is a dual-function regulator whose predominant activity depends upon the number of DNA-binding sites present in the promoter. At present the molecular basis of this transition is unknown. Transcription factors that activate in one circumstance and repress in another have been documented, and the molecular basis for these transitions is quite diverse (18). Kruppel converts from an activator to a repressor in a dose-dependent manner (19, 20). However, the Sp3-mediated regulation does not appear to be modulated by changing the relative Sp3 concentration (Figs. 4 and 5). It is the context of DNA-binding sites that appears to determine the Sp3 mediated activity. A similar dual-function regulation has been described for the glucocorticoid receptor (GR). It has been reported that GR can activate or repress transcription depending on how it is tethered to a target gene. When bound to a single glucocorticoid response element, GR activates gene expression. In contrast, when tethered to DNA via composite glucocorticoid response elements, GR represses gene expression (21). More recently, it has been reported that the transcription activity of Ets-1 is modified upon DNA binding, and it has been suggested that allosteric changes may alter the structure of a transcription factor as a result of its interaction with DNA (22, 23).

Based on these considerations, we suggest that Sp3 may be modified by, or act in association with, a corepressor. However, different allosteric changes may occur depending upon the context of DNA-binding sites, allowing mutual exclusive interactions between diverse Sp3 domains and putative cofactors leading to a different transcription response. Biochemical approaches will be useful to decipher the molecular basis for the Sp3-mediated regulation.

Whatever is the molecular mechanism responsible for the dual-function of Sp3 it is clear that the repression ability is depending upon the cellular context. In fact, we have shown that in Drosophila SL2 cells Sp3 does not possess an active repression capability that can function in this cellular background. We found that Sp3 functions as DNA-binding dependent activator of the c-myc promoter, albeit at a lower level compared to Sp1. It is pertinent to note that using the HIV reporters we were unable to detect any significant Sp3-mediated transactivation. However, coexpression of TBP with Sp3 led to a super-activation likely due to a functional interaction between Sp3 and TBP, as part of the TFIIID complex. In vivo and in vitro cooperation between Sp1 and TFIIID has been well documented (24, 25). It has been demonstrated that the glutamine-rich activation domain of Sp1 can interact with the TBP-associated factor dTAF110 and TBP itself (24, 25). Moreover, Sp1 residues that are sensitive to mutations that abolish interaction with dTAF110 and transcription activation have been mapped (26). Interestingly, both Sp1 and Sp3 contain very similar hydrophobic patches. Having established that both Sp1 and Sp3 function in a similar fashion in the TBP superactivation assay in vivo, it seems likely that the homologous glutamine-rich domains of Sp1 and Sp3 share a common pattern of protein-protein interactions. In vitro binding studies with recombinant TBP, dTAF110, and Sp3 are currently being performed.

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