Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6145–6149, July 1993
Biochemistry

Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1

(initiator-binding protein/glutathione *S*-transferase fusion proteins)

JENG-SHIN LEE*, KATHERINE M. GALVIN†, AND YANG SHI†‡

*Committee on Virology, and †Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115

Communicated by Howard Green, April 15, 1993

ABSTRACT **Two promoter elements are important for basal-level transcription, the TATA motif typically located 30 nucleotides upstream of the transcription initiation site and the initiator (Inr) element encompassing the start site. The mechanism of how Inr elements work is poorly understood, partly because very few proteins that bind to Inr elements have been identified and isolated. The recently cloned YY1 is such an Inr-binding protein. YY1 is able to direct transcription upon binding to its recognition sequence *in vitro*. The ability of YY1 to initiate transcription is augmented by the presence of a TATA motif or binding sites for transcription factor Sp1. To study the mechanism underlying the apparent functional cooperation between YY1 and Sp1, we explored the possibility of protein–protein interactions between these two transcription factors. We found that YY1 and Sp1 can form a physical complex. In addition, we identified domains within YY1 and Sp1 that mediate their interactions with each other. The physical interaction between YY1 and Sp1 may thus form the basis for the functional interplay observed previously.**

Evidence is accumulating that sequences at transcriptional initiation sites of many eukaryotic genes are important for transcription directed by RNA polymerase II (1–6). This class of cis element, the initiator (Inr) element, is involved in supporting basal transcription (1). The mechanism by which Inr elements direct basal-level transcription is not well understood. However, they are likely to be involved in the assembly of transcriptional preinitiation complexes. Many Inr elements have been identified and have been classified into five different groups based on their sequence homology (reviewed in ref. 7). One class is represented by the sequence surrounding the transcription initiation site of the adeno-associated virus *P5* promoter (AAV *P5*-Inr; ref. 6). A transcription factor termed YY1 that binds to the *P5*-Inr has been identified, and the gene encoding YY1 has been cloned (6, 8–10). Upon binding to its recognition sequence, YY1 is able to direct basal transcription *in vitro* in the absence of other promoter elements, including the TATA motif (11). YY1's ability to initiate transcription is enhanced by the presence of an adjacent TATA motif or of binding sequences for transcription factor Sp1 (11). This suggests a functional interaction between these binding sites and that of YY1. The interplay between YY1 and Sp1 is particularly intriguing because many TATA-less promoters possess an Inr and multiple adjacent Sp1 sites, both of which are required for efficient basal transcription. We wished to determine whether the functional interaction observed between YY1 and Sp1 was due to physical associations between these two proteins. Here we provide evidence that YY1 does indeed interact with Sp1. Further, we have identified a region encompassing 34 aa of YY1 that is necessary and sufficient to mediate its interaction with Sp1. Our initial attempt to locate YY1-interacting domains within Sp1 suggests the importance of the C-terminal 158 aa encompassing the zinc finger DNA-binding domain and the transcriptional activation domain D of Sp1.

MATERIALS AND METHODS

Plasmids. pGST-YY1 and pGST-WT1 were constructed by cloning full-length YY1 (6) and WT1 (12) cDNAs into the glutathione *S*-transferase (GST) expression vector pGEX-2TK (13). N- and C-terminal deletions of YY1 were generated by exonuclease III digestion (Erase-A-Base kit, Promega) and fused in frame to GST. pGST-Sp1Q1 and pGST-Sp1ZnF were constructed by cloning fragments from Sp1 cDNA (pSp1-fl, gift of R. Tjian, University of California, Berkeley) in frame into pGEX-2TK. pGST-Sp1Q1 and pGST-Sp1ZnF encode aa 1–262 and 620–778 of Sp1, respectively. pGAL4-Sp1 encodes the DNA-binding domain of GAL4 fused at its C terminus to the full-length Sp1 (gift from G. Gill, University of California, Berkeley). pGAL4E1BCAT contains five GAL4 binding sites inserted 5′ of the minimal E1B promoter that is linked to the chloramphenicol acetyltransferase (CAT) gene (gift of A. Levine, Princeton University). Plasmids pCMVYY1/VP16 and various YY1 deletion derivatives were constructed by joining the YY1 fragments in frame with the herpes simplex virus VP16 acidic activation domain (aa 413–490, gift of M. Green, University of Massachusetts), and the YY1/VP16 fragments were then cloned into a cytomegalovirus (CMV) promoter-driven expression plasmid (6). pCMV-VP16, expressing the activation domain of VP16, was constructed by placing VP16 under the control of the CMV early promoter containing translational initiation consensus sequence and a nuclear localization signal derived from the simian virus 40 large tumor antigen (PKKKRKV; ref. 14). All constructs were verified by sequence analysis.

Assay of YY1 and Sp1 Interaction with GST-YY1 Fusion Proteins. pGST-YY1 and various C- and N-terminal deletion derivatives were induced with isopropyl β-D-thiogalactopyranoside to express the fusion proteins, which were bound to glutathione-Sepharose beads and purified as described (13). ^{35}S-labeled Sp1 was obtained by *in vitro* transcription using plasmid pSp1-fl (gift of R. Tjian) as template, with subsequent translation in rabbit reticulocyte lysate. The *in vitro* translation reactions were carried out following the protocol provided (Promega). Typically, 1 μg of fusion protein was incubated with either the *in vitro* translated Sp1 (1/10th of the translation reaction mix) or HeLa nuclear extract (about 500 μg of nuclear proteins). The bound ^{35}S-labeled Sp1 was visualized by autoradiography, while the bound Sp1 from HeLa nuclear extract was detected by Western analysis using 1:1000 diluted polyclonal antiserum against Sp1 (gift of R.

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Abbreviations: CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; Inr, initiator.

‡To whom reprint requests should be addressed.

Tjian) with the enhanced chemiluminescence method (Amersham).

Gel Shift Assay. Gel shift assays were performed with radioactively labeled YY1-binding-site oligonucleotides and 0.2 μg of purified, bacterially synthesized His_6-YY1 protein (6). For the "supershift" reactions, about 40 ng of purified Sp1 and AP2 proteins (Promega) were added. Conditions for the gel shift assays were described previously (6).

Transfection and CAT Assay. HeLa cells grown in 10-cm plates were transfected by a calcium phosphate precipitation procedure. Typically, 4 μg of the target GAL4 E1BCAT plasmid was used together with 1 μg of pGAL4-Sp1 and 2 μg of pCMV-YY1/VP16 DNA. Plasmid pSP72 (Promega) was used to adjust the total transfected DNA to 7 μg. Cells were harvested 48 hr after transfection. Whole cell extracts were prepared, and half of the extract from each plate was used for CAT assay (6). Each transfection experiment was repeated at least three times.

RESULTS

Physical Interactions Between YY1 and Sp1. Several different approaches were used to demonstrate interactions between transcription factors YY1 and Sp1. The first approach utilized YY1 protein affinity beads to capture Sp1. Full-length YY1 was fused to GST to create GST-YY1 fusion proteins, which were subsequently bound to glutathione-Sepharose to generate GST-YY1 protein affinity beads (13). The purified GST-YY1 beads were then incubated with *in vitro* translated, ^{35}S-labeled Sp1 proteins. Approximately 10% of input Sp1 was retained by the GST-YY1 beads, but not by GST alone (Fig. 1*A*, lanes 2 and 3). To further demonstrate the specificity of the interaction, we examined the ability of another transcription factor, WT1 (*W*ilms *t*umor *1* gene product) to bind Sp1. Like YY1, WT1 encodes a Cys_2His_2 ("C_2H_2")-type zinc-finger repressor protein (15) and was initially identified as a candidate gene for the Wilms tumor (12). GST-WT1 fusion protein failed to bind Sp1 (Fig. 1*A*, lane 4), strengthening the notion that the interaction detected between Sp1 and GST-YY1 is specific. To ascertain that YY1 also interacts with native Sp1 synthesized *in vivo*, the GST-YY1 beads were incubated with HeLa nuclear extract. The bound Sp1 protein was visualized by Western blot analysis using Sp1 antiserum (gift of J. L. Chen, University of California, Berkeley). Vaccinia virus-produced Sp1 was used as a positive control for the Sp1 antiserum (Fig. 1*B*, lane 1). Again, Sp1 was retained by GST-YY1 (Fig. 1*B*, lane 4) but not by GST alone (lane 3). Collectively, these experiments suggest that YY1 can specifically bind Sp1 synthesized both *in vitro* and *in vivo*.

We used a gel shift assay as another means to demonstrate the interaction between YY1 and Sp1. We reasoned that if Sp1 interacts with YY1, then Sp1 may cause further retardation ("supershift") of the YY1 DNA–protein complex. The DNA–protein complex formed between bacterially purified YY1 and its binding sequences was supershifted by the addition of purified Sp1 protein (Fig. 1*C*, lane 3) but not by purified transcription factor AP2 (lane 2). The purified Sp1 itself did not bind to the YY1 recognition sequence (lane 4). This experiment further suggests that YY1 and Sp1 can interact with each other. Moreover, since both purified YY1 and Sp1 were used in the assay, it is possible that YY1 and Sp1 can contact each other directly.

To determine whether YY1 and Sp1 interact *in vivo*, we asked whether Sp1-dependent transcription can be enhanced by YY1 carrying the acidic activation domain of the herpes simplex virus protein VP16 (16). If YY1 interacts with Sp1, then the added activation domain of VP16 in the form of YY1-VP16 fusion protein can be brought to the promoter through protein–protein interactions between YY1 and Sp1, resulting in enhanced transcription directed by Sp1. As a reporter, we used a plasmid containing the CAT gene under the control of adenovirus E1B promoter with five GAL4 sites upstream of the TATA box. The effector plasmid pGAL4-Sp1 contains the complete Sp1 coding sequence fused in frame to the DNA-binding domain of GAL4. YY1-VP16 was constructed by fusing the VP16 activation domain to the C terminus of YY1. GAL4-E1BCAT was activated by GAL4-Sp1 in HeLa cells (Fig. 1*D*, lane 4). Significantly, this Sp1-dependent transcriptional activation was increased 45-fold by cotransfection of a plasmid expressing the YY1-VP16 fusion protein (Fig. 1*D*, lane 6). This superactivation is dependent on binding of Sp1 to the promoter, since a target gene lacking the GAL4 sites was not responsive (Fig. 1*D*, lane 1). In addition, YY1/VP16 had no effect on the target gene when only the GAL4 DNA-binding domain was used (Fig. 1*D*, lane 3). These results suggested that YY1-VP16 was recruited to the promoter via its interaction with Sp1. Alternatively, YY1-VP16 could simply have increased the level of the effector protein GAL4-Sp1 driven by the simian virus 40 promoter. Since the YY1-VP16 hybrid protein does not activate the simian virus 40 promoter (data not shown), we consider the latter explanation unlikely. The VP16 activation domain itself or a truncated YY1 (aa 1–224) fused to the VP16 activation domain failed to superactivate Sp1-dependent CAT expression (Fig. 1*D*, lanes 5 and 7, 1.3- and 1.6-fold superactivation, respectively), indicating that the observed interaction was specific. Further, YY1/VP16 did not superactivate transcription directed by the GAL4 acidic activator (data not shown), strengthening the notion that YY1 interacted specifically with Sp1. This result, combined with those obtained with GST-YY1 fusion proteins and the gel shift assays, strongly argues that YY1 and Sp1 physically interact.

The First One and a Half Zinc Fingers of YY1 Interact with Sp1. YY1 associates with several cellular proteins (K.M.G. and Y.S., unpublished results). These interactions are probably mediated by discrete regions of YY1 and are likely to be important for the transcriptional activities of YY1. Mapping the domains of YY1 required for these various interactions will help us to understand how YY1 exerts different transcriptional activities. For this reason, we were interested in identifying Sp1-interacting domains within YY1. Localization of such a motif will also lend further support to the contention that the interaction between YY1 and Sp1 is specific. To identify Sp1-interacting domains, we constructed a series of GST-YY1 fusions with progressive deletions from either the N- or the C-terminal end of YY1 (Fig. 2*A*). and the fusion proteins were assayed for their ability to interact with Sp1. For the C-terminal deletion series, only the wild type and a GST-YY1 fusion protein lacking the C-terminal 84 aa (dl 331–414) were able to interact with Sp1 (Fig. 2*B*, lanes 3 and 4). Other GST-YY1 fusions with further deletions beyond aa 331 failed to bind Sp1 (Fig. 2*B*, lanes 5–8). This analysis places the C-terminal boundary of the Sp1-interacting domain at around aa 331. In the N-terminal series, deletion of YY1 sequence up to aa 260 had no effect on its binding to Sp1 (Fig. 2*C*, lanes 4–6). This result is in agreement with the finding that the truncated YY1-VP16 fusion protein containing aa 1–224 of YY1 was not able to superactivate Sp1-dependent transcription (Fig. 1*D*, lane 7). However, further deletion of YY1 beyond aa 260 abolished the ability of the fusion protein to bind Sp1 (Fig. 2*C*, lane 7). This delimits the N-terminal boundary of the Sp1-interacting domain at aa 260. Thus, aa 260–331 of YY1 are necessary for its interaction with Sp1. To determine whether this region of YY1 was sufficient to mediate interactions with Sp1, we constructed a GST fusion plasmid of this fragment [GST-YY1-(260–331)] and found that aa 260–331 of YY1 were capable of interacting with Sp1 (Fig. 3*B*, lane 4).

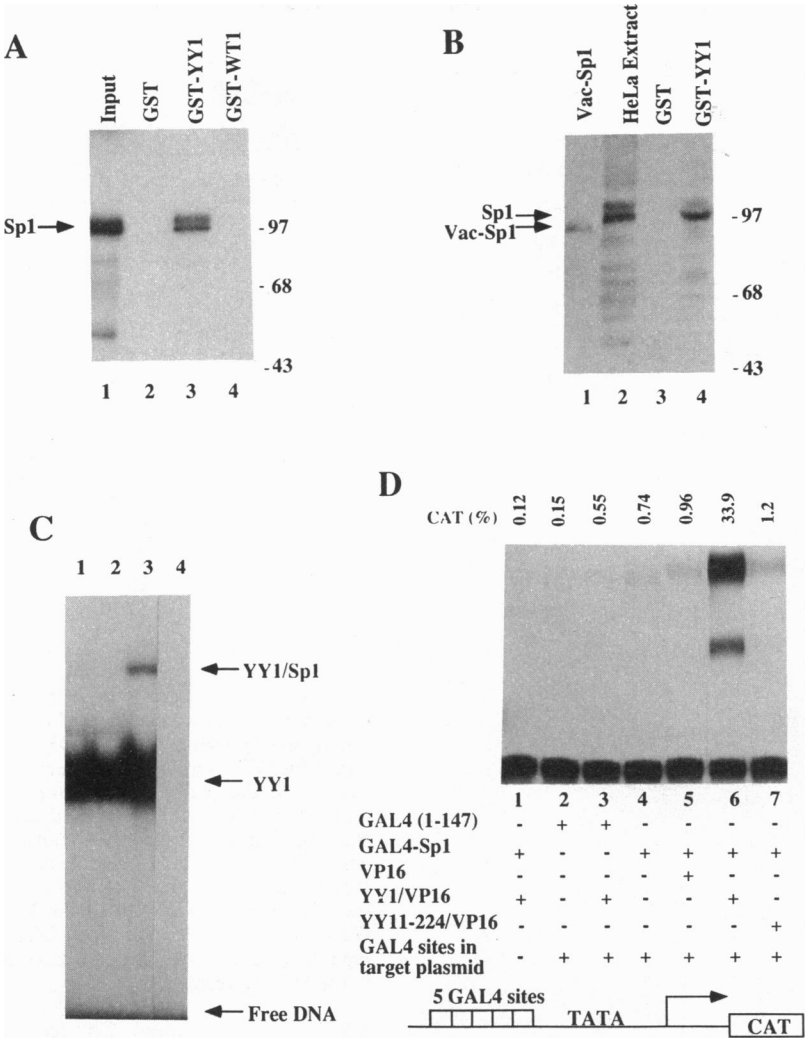

FIG. 1. The zinc-finger transcription factors YY1 and Sp1 interact with each other. (*A*) *In vitro* translated Sp1 binds to immobilized YY1. *In vitro* transcribed/translated ^{35}S-labeled Sp1 was incubated with 1 μg of either GST alone (lane 2) or GST-YY1 (lane 3) or GST-WT1 (lane 4) fusion protein bound to glutathione-Sepharose beads. The input lane (lane 1) was loaded with one-fifth the amount of Sp1 used in the binding reactions. The bound Sp1 was eluted and analyzed by PAGE as described in the text. The position of Sp1 in the gel is indicated. The sizes (kDa) of the molecular weight markers are indicated at right. (*B*) Native Sp1 proteins bind to immobilized YY1. Essentially the same experiment was performed as described in *A* except that crude HeLa nuclear extract was incubated with GST (lane 3) or GST-YY1 (lane 4). The Sp1 protein was identified by an anti-Sp1 polyclonal antibody and its position is indicated by an arrow on the left. Recombinant vaccinia virus-produced Sp1 (Vac-Sp1) was used as a positive control for the antibody used (lane 1). The input HeLa nuclear extract is shown in lane 2. (*C*) Sp1 supershifts a DNA–YY1 complex. ^{32}P-labeled YY1-binding-site oligonucleotide corresponding to sequence from −9 to +12 of the adeno-associated virus *P5* promoter (6) was used as substrate for binding with purified, bacterially synthesized His-YY1 protein (lane 1). Forty nanograms of purified AP2 (lane 2) or Sp1 (lane 3) was added to the reaction mixtures. Supershifted Sp1/YY1 complex is indicated by an arrow. Purified Sp1 was also incubated with labeled YY1 oligonucleotide in the absence of YY1 protein (lane 4). The positions of the YY1 DNA-protein complex as well as the free DNA are indicated. (*D*) Interactions between YY1 and Sp1 in HeLa cells. Four micrograms of GAL4-E1BCAT, 2 μg of GAL4-Sp1, and 2 μg of CMV-VP16, CMV-YY1/VP16, or CMV-YY1-(1-224)/VP16 were used in each transfection. The presence (+) or absence (−) of a particular plasmid in a given transfection is indicated. Percentage acetylation shown (CAT %) represents an average of two independent transfection assays.

Upon examining the sequence of aa 260–331, we noticed that this region contains one and a half zinc fingers of YY1 (out of a total of four zinc fingers), as well as the portion of YY1 immediately preceding the zinc-finger region. To determine whether the Sp1-interacting domain included the zinc fingers, we divided the Sp1-interacting domain into the non-zinc-finger portion (aa 260–297) and the zinc-finger portion (aa 298–331) and fused each to GST (Fig. 3*A*). These constructs were then induced with isopropyl β-D-thiogalactopyranoside to synthesize GST fusion proteins, which were tested for their ability to bind Sp1. The GST-YY1 fusion protein containing one and a half zinc fingers of YY1 (aa 298–331) bound Sp1 (Fig. 3*B*, lane 6), albeit at a lower level than the wild-type GST-YY1. In contrast, the subdomain spanning aa 260–297 clearly did not bind Sp1 (Fig. 3*B*, lane 5). This allowed us to designate the first one and a half zinc fingers of YY1 as a potential Sp1-interacting domain.

Identification of a YY1-Interacting Domain Within Sp1. Several functional domains are present in Sp1, including the DNA-binding domain, which is composed of three zinc fingers and four transcriptional activation domains, A–D (17). To search for sequence motifs that might mediate Sp1 interactions with YY1, we first tested the ability of a C-terminally truncated Sp1 to bind YY1. This mutant Sp1 protein, lacking the C-terminal 120 aa, was obtained by *in vitro* transcription/translation using a truncated Sp1 cDNA as template. The mutant Sp1 failed to bind YY1 (Fig. 4*B*, lane 6). This suggested that the last 120 aa were necessary for Sp1 to bind

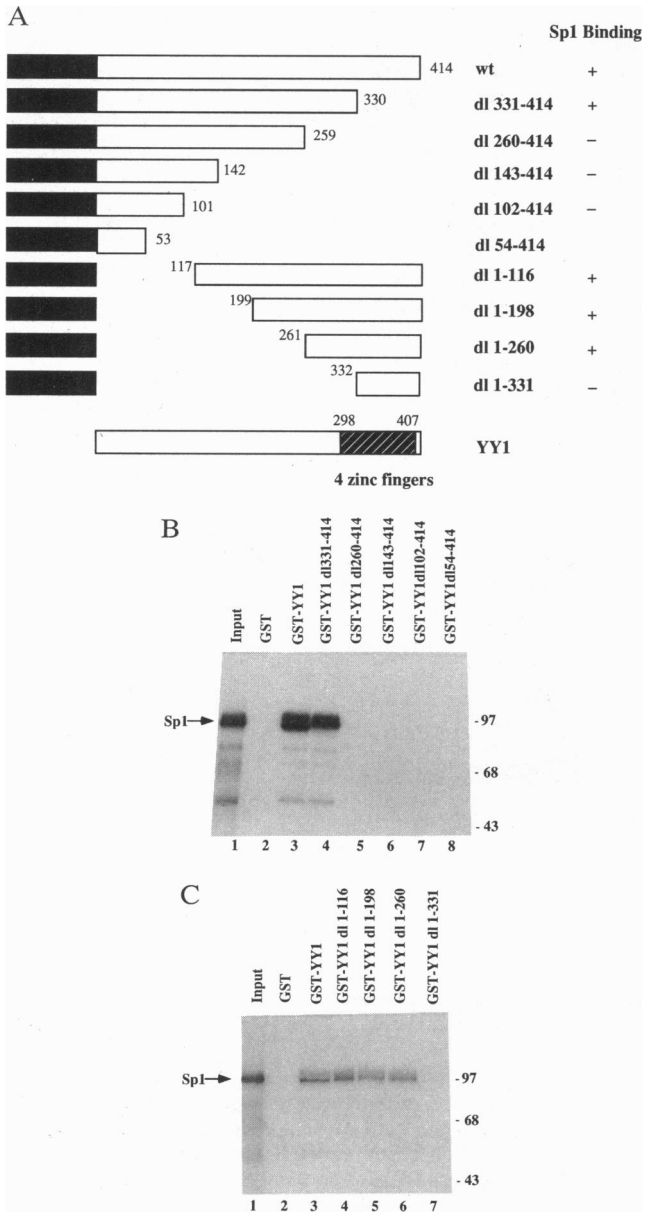

FIG. 2. Identification of an Sp1-interacting domain in YY1. (*A*) Construction of GST-YY1 fusion proteins with progressive C- and N-terminal deletion of YY1. Various YY1 cDNA fragments with C- or N-terminal deletions were cloned in frame into the pGEX-2TK vector (13) to generate the GST-YY1 fusion constructs. The black rectangles represent the GST binding domain (GST), and the open rectangles represent YY1. The position of the four zinc fingers of YY1 relative to the other deletion constructs is shown by a hatched rectangle. The ability of each GST-YY1 fusion protein to bind Sp1 is indicated (+ or −). The end point of each GST-YY1 deletion construct is shown. wt, Wild type. (*B*) Binding of *in vitro* translated ^{35}S-labeled Sp1 to C-terminal deletion series of GST-YY1 fusion proteins. *In vitro* translated Sp1 (5 μl) was incubated with 1 μg of GST (lane 2), wild-type GST-YY1 (lane 3), and various C-terminally deleted YY1 as schematized in *A* (lanes 4–8). The bound Sp1 was eluted and analyzed by PAGE. The input lane (lane 1) was loaded with one-fifth the amount of Sp1 used in the binding reactions. The position of Sp1 and the sizes (kDa) of the molecular weight markers are indicated. (*C*) Binding of *in vitro* translated ^{35}S-labeled Sp1 to N-terminal deletion series of GST-YY1 fusion proteins. The experiment was the same as that described in *B* except that the N-terminally deleted GST-YY1 fusion proteins were used. *In vitro* translated Sp1 was incubated with 1 μg of GST (lane 2); wild-type GST-YY1 (lane 3), and GST-YY1 fusion proteins with YY1 carrying different N-terminal deletions (lanes 4–7). The input lane (lane 1) was loaded with one-fifth the amount of Sp1 used in the binding reactions.

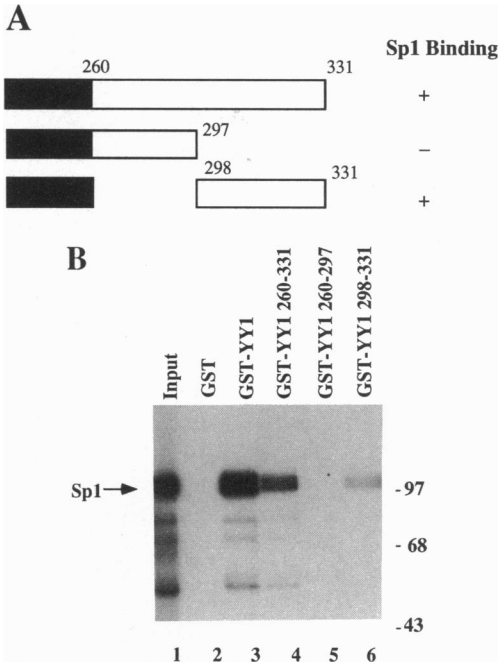

FIG. 3. The first one and a half zinc fingers of YY1 are sufficient to bind Sp1. (*A*) Construction of GST-YY1 fusion plasmids cDNA fragments representing aa 260–331, 260–297, or 298–331 were obtained by PCR, verified by sequencing, and subsequently cloned in frame into the pGEX-2TK vector. Black rectangles, GST; open rectangles, YY1. Ability to bind Sp1 is indicated (+ or −). (*B*) Binding of *in vitro* transcribed/translated Sp1 to the first one and a half zinc fingers of YY1. Binding assays were done as described for Fig. 1. The *in vitro* translated Sp1 was incubated with 1 μg of GST (lane 2), wild-type GST-YY1 (lane 3), GST-YY1-(260–331) (lane 4); GST-YY1-(260–297) (lane 5), or GST-YY1-(298–331) (lane 6). The input lane (lane 1) was loaded with one-fifth the amount of Sp1 used in the binding reactions.

YY1. The C-terminal 120 aa include the transcriptional activation domain D and a partial DNA-binding domain of Sp1 (two of the three zinc fingers). To determine whether this region was sufficient for interactions with YY1, we constructed a GST fusion plasmid (GST-Sp1ZnF/D) encoding the C-terminal 158 aa of Sp1. The fusion protein encompasses the complete DNA-binding domain as well as activation domain D of Sp1. GST-Sp1ZnF/D fusion protein bound YY1 (Fig. 4*B*, lane 10). The specificity of the interaction was suggested by the finding that the GST-Sp1 fusion protein containing the glutamine-rich activation domain A (aa 1–262) of Sp1 barely bound YY1 (lane 9). This result implies that the last 158 aa of Sp1 are necessary and sufficient to mediate its interactions with YY1.

DISCUSSION

Two zinc-finger transcription factors, YY1 and Sp1, can physically interact with each other both *in vitro* and *in vivo* (Fig. 1). The interaction between these two proteins is likely to be direct, since purified Sp1 can supershift a DNA–protein complex formed with purified YY1 (Fig. 1*C*). Previous studies demonstrated that Sp1 can form homomultimers (18). It can also directly interact with the site-specific DNA-binding protein E2 from bovine papilloma virus (19). Our finding that YY1 and Sp1 form a physical complex expands the repertoire of Sp1 partner proteins to cellular sequence-specific DNA-binding transcription factors. Most importantly, the physical association between YY1 and Sp1 may explain the functional interaction observed previously; i.e., Sp1 may facilitate binding of YY1 to the Inr (or vice versa),

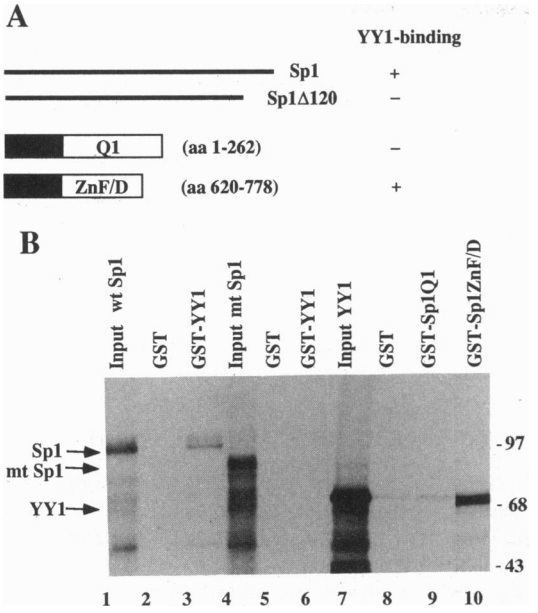

FIG. 4. The C terminus of Sp1 is important for binding to YY1. (*A*) Diagram of *in vitro* transcribed/translated Sp1 proteins and various GST-Sp1 constructs. The Sp1 proteins transcribed/translated *in vitro* are represented by solid lines. The mutant Sp1 lacking the C-terminal 120 aa was transcribed/translated from a truncated Sp1 template linearized with *Xmn* I. Black rectangles, GST; open rectangles, Sp1. Ability to bind YY1 is indicated (+ or −). (*B*) Assays of interactions between Sp1 and YY1. Binding assays were done as described for Fig. 1. Labeled wild-type and mutant Sp1 proteins were incubated with the GST protein alone (lanes 2 and 5) or GST-YY1 (lanes 3 and 6). Input lanes (1 and 4) were loaded with one-fifth the amount of the wild-type or the mutant Sp1 used in the binding reactions. Labeled YY1 was incubated with GST (lane 8), GST-Sp1Q1 (lane 9), or GST-Sp1ZnF/D (lane 10). The input lane (lane 7) was loaded with one-fifth the amount of YY1 used in the binding reactions. mt Sp1, mutant Sp1.

resulting in an increase in the level of correctly initiated transcripts (11). In addition to Sp1, YY1 also interacts with many other proteins, among them several general transcription factors such as the TATA-binding protein (TBP) (J.-S.L. and Y.S., unpublished result). That YY1 interacts with both Sp1 and TBP raises the intriguing possibility that YY1 may be a coactivator for Sp1 (20).

YY1 is a multifunctional protein with four zinc fingers at the C terminus serving as the DNA-binding domain. Previous studies established that in addition to being an Inr-binding protein involved in basal transcription, YY1 is also a sequence-specific DNA-binding repressor (6, 8, 10). The activating and repressing functions of YY1 may conceivably be carried out by discrete domains within the protein. The domain that mediates transcriptional repression has been mapped to the C-terminal 83 aa, corresponding to the last two and a half zinc fingers of YY1 (ref. 6 and unpublished data). Here we show that the first one and a half zinc fingers of YY1 contain an element necessary and sufficient for interaction with Sp1 (Figs. 2 and 3). Thus the Sp1-interacting domain seems distinct from the repressor domain of YY1. Since this putative Sp1-interacting domain bound Sp1 less well than wild-type YY1 (Fig. 3), it is possible that the optimal Sp1-binding motif in YY1 may involve amino acids N-terminal to the first zinc finger of YY1 (aa 298). Nevertheless, it is safe to suggest that we have identified a minimal sequence motif that can mediate interactions between YY1 and Sp1. Our mapping data support the hypothesis that YY1 zinc fingers are crucial not only as a DNA-binding motif but also as a functional domain for YY1's transcriptional activities.

Our initial characterization of domains within Sp1 that may mediate interactions with YY1 indicates the importance of the C-terminal 158 aa. It is interesting that this region of Sp1 contains two important functional domains: the three zinc fingers, constituting the DNA-binding domain, and transcriptional activation domain D. Domain D may be involved in the interactions needed for higher-order complex formation among Sp1 molecules (18). Functionally, domain D is important for the synergistic transcriptional activation of a promoter containing multiple Sp1 sites. Thus, it is conceivable that domain D may also be involved in interactions with other proteins such as YY1.

Protein–protein interactions among sequence-specific DNA-binding transcription factors suggest novel modes of regulation. The DNA-bound transcription factor can target the second factor to the promoter where binding sites for the latter are absent. Alternatively, the DNA-bound factor may increase the apparent affinity of the second factor to a weak recognition site via protein–protein interactions.

We thank A. Lassar, J. Flanagan, and members of the lab for critical reading of the manuscript and for their helpful comments. We thank T. Shenk and E. Seto for communicating results prior to publication. We are grateful for the gifts of GAL4-Sp1 plasmid, vaccinia virus-produced Sp1, and Sp1 antiserum from G. Gill and J.-L. Chen (lab of R. Tjian, University of California, Berkeley); pGEX-2TK vector from W. Kaelin (Dana Farber Cancer Institute, Boston); WT1 cDNA from F. Rauscher III (Wistar Institute, Philadelphia); and VP16 cDNA plasmid from M. Green (University of Massachusetts, Worcester). We thank R. Tung for help with the chemiluminescence assay. The excellent photographic service provided by S. Borack is acknowledged. This work was supported in part by the Milton Fund and a grant to Y.S. from the National Cancer Institute (CA58997-01).

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