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Sp100 Interacts with ETS-1 and Stimulates Its Transcriptional Activity

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The cell nucleus is highly organized into distinct domains that spatially separate physiological processes. One of these domains, the Sp100-promyelocytic leukemia protein nuclear body (NB), is implicated in pathological processes, such as cancer and viral infection, yet its functions remain poorly understood. We show here that Sp100 interacts physically and functionally with ETS-1 and that NB morphology is affected by ETS-1. ETS-1 is a member of the ets family of transcription factors, which are key mediators of physiological and pathological processes. We have found that Sp100 interacts with two regions of ETS-1 (domains A+B and D+E+F). ETS-1 alters NBs while remaining localized throughout the nucleus, apparently by recruitment of the core component Sp100 away from the NBs. Sp100 strongly increases ETS-1 activation of natural and ets-focused promoters, through a mechanism involving the activation (C) domain of ETS-1 in addition to the interaction domains. Sp100 acts as a novel coactivator that potentiates the activator function of ETS-1. Our results provide an important new connection between nuclear structures and an important regulator of gene expression.

Physiological processes, such as transcription, DNA replications and pre-mRNA splicing, take place in defined nuclear domains. A part of the global nuclear architecture is the Sp100-PML nuclear body (NB, also called nuclear domain 10, and promyelocytic leukemia protein [PML] oncogenic domain). NBs appear to be involved in the pathogenesis of human diseases, including acute promyelocytic leukemia and viral infection, and appear to have physiological roles in the control of cell growth, differentiation and apoptosis (for reviews, see references 24, 38, 41, 56, and 64). NBs were initially identified by electron microscopy. They are immunologically defined as containing two major components, Sp100 and PML. Sp100 was first characterized as an antigen reactive with antibodies from patents with autoimmune disorders (67). PML was identified as part of a fusion protein with retinoic acid receptor α (PML-RARα) expressed in acute promyelocytic leukemia (for a review, see reference 38). NBs contain, in addition to their core components Sp100 and PML, transcription factors, chromosomal proteins, tumor suppressors, and proto-oncogenes (for a review, see reference 56).

Mammalians cells in general contain 5 to 20 NBs, ranging in size from 0.1 to 1 μm, that are spherical or torioidal in shape. NBs are highly dynamic structures. They are disrupted in acute promyelocytic leukemia cells by the PML-RARα fusion protein and reorganized by the physiological ligand of RARα, retinoic acid. NBs are disaggregated in some neurodegenerative diseases (for a review, see reference 56), during viral infection (for a review, see reference 40), and in the cell cycle (1). NBs increase in size in response to interferons, key inducers of cellular antiproliferative and antiviral responses that upregulate PML and Sp100 (18, 32, 62). NB structure is established and maintained through SUMO modification of PML (27, 45, 66, 80). In contrast, SUMO modification of Sp100 is not required for NB targeting (65).

Although the dynamics of NB structure have been extensively studied, their role as actual sites of regulation is not clear. NBs appear to function as a type of nuclear depot (40), in which proteins are sequestered to maintain an intranuclear homeostatic balance. The balance between storage and release is maintained by poorly understood mechanisms involving hormones, interferon, viral infection, and environmental stress (27, 42, 45, 81). Identifying the nuclear targets and functions of the released forms of NB components is important in order to understand their physiological role. Sp100 inhibits transcription when brought to DNA by heterologous DNA-binding domains (DBDs), through the recruitment of the heterochromatin component HP1 (33, 58). Sp100 does not bind to DNA alone but is probably recruited to DNA sites through interactions with specific DNA-binding proteins. Two potential recruiters for repression have been described: hHMG2/DSP1 (33) and Bright (82). However, there has also been a suggestion that Sp100 activates transcription through its cryptic activation domain (designated by AD) (77). We show here that Sp100 activates transcription through interactions with ETS-1.

ETS-1 is the founding member of the ets family of transcription factors. ets factors have important roles in oncogenesis, signal transduction and development (for reviews, see references 3, 5, 22, 26, 34, 35, 39, 43, 53, 59, and 78). They have a highly conserved ets DBD that has a winged helix-turn-helix structure and binds to DNA motifs containing the core sequence GGA. Ets-1 itself is expressed in the developing vasculature and lymphoid tissues in animals and is involved in the regulation of angiogenesis and differentiation of lymphoid lineages. Human ETS-1 (p54) can be subdivided structurally and functionally into six domains, A to F (Fig. 1A). D and F flank the ets domain E and inhibit DNA binding by forming a closed conformation through intramolecular interactions (20, 21, 29,

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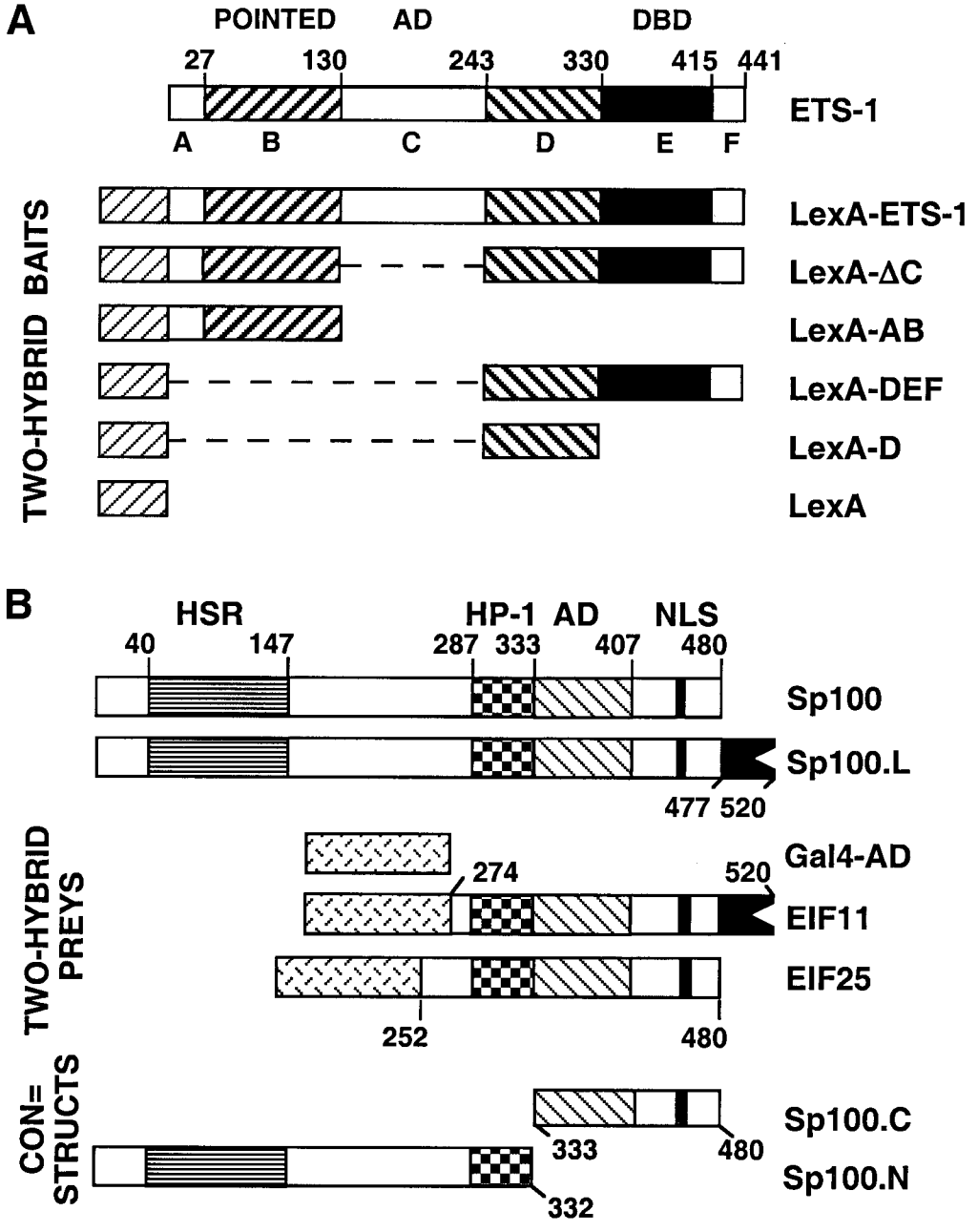


FIG. 1. Protein structures. (A) Human ETS-1 and the baits. ETS-1 can be subdivided into domains A to F; the last amino acid in each domain is indicated (21, 54, 70, 73). E is the highly conserved ETS DBD, D and F inhibit DNA binding, C is the transcriptional activation domain (AD), B is the Pointed domain which is conserved in a number of Ets proteins, and A has no known function. The baits are fusion proteins between LexA and ETS-1 deletion mutants. (B) Human Sp100, preys, and constructs. Sp100 is the first variant described (67). Sp100.L contains additional amino acids at the C terminus that were found in one of the preys and are common to the three Sp100 variants: Sp100B, Sp100-HMG, and Sp100C (14, 19, 57, 58, 77). HSR is involved in ND targeting and homodimerization (46, 65) and is the only region conserved in murine Sp100-rs that is encoded by an amplified gene that forms a homogeneously staining region (16, 49). The HP-1 region interacts with HP-1 (33, 58). AD is a transcription activation domain (77). NLS (positions 444 to 450) is a nuclear localization signal (65). The preys isolated by the yeast two-hybrid screen, EIF11 and EIF25, are fusion proteins between the Gal4 AD and the C-terminal parts of Sp100.L and Sp100, respectively. The constructs, Sp100.N and Sp100.C, are deletion mutants retaining sequences from 1 to 332 and from 333 to 480, respectively.

37, 48, 60, 73). Phosphorylation of the D domain through the Ca^{2+} signaling pathway further inhibits DNA binding (12, 53). C is the transcriptional activation domain (54). B, or the Pointed domain, is conserved in a subset of Ets proteins. The pointed domains of some of these Ets proteins, including Ets-1, Ets-2, and Pointed P2, contain a threonine residue (T38 in Ets-1 p54) that is phosphorylated through Ras signaling (for a review, see reference 78). The short A domain of ETS-1 has no known function. The longer unrelated A sequences of Ets-2 and Ets-1 p68 (an isoform of Ets-1 found in birds and reptiles) are activation domains (54).

Ets-1 activity is modulated by interactions with a number of

factors, including AP1, Sp1, c-Myb, GHF-1/Pit-1, Pax-5/BSAP, CBFα/AML1, MafB, and a multiprotein complex formed by CBFα/AML1, LEF1, and ATF/CREB on the T-cell receptor α enhancer (for a review, see reference 35). For example, Jun, a component of AP1, interacts with the Ets DBD (the E domain), whereas CBFα interacts with the activation domain (the C domain). Recently, Daxx/EAP1 has been shown to interact with the Pointed domain (36). Identification of cooperating factors is an important step toward understanding the physiological roles of Ets-1 and the interacting factor. We set out to identify cooperating factors that do not require DNA binding by using the yeast dual-hybrid screen. We found that Sp100 interacts physically and functionally with Ets-1. These results have significant implications for the roles of these previously unlinked physiologically important molecules.

MATERIALS AND METHODS

Plasmids. For preparation of pLexA-ETS-1 (pHuE1-1), three ETS-1 cDNA fragments were ligated to pBTM116 linearized with *Eco*RI and *Sal*I: a PCR-generated *Eco*RI/*Bam*HI fragment containing the 5′ end of the coding region upstream from the *Bam*HI site, a 0.9-kb *Bam*HI/*Pvu*II cDNA fragment, and a PCR-generated *Pvu*II/*Sal*I fragment containing the sequence downstream from the *Pvu*II site. For pLexA-ΔC (pHuE1-2), three fragments from pLexA-ETS-1 (pHuE1-1) were ligated to remove the activation domain (domain C) between the *Pst*I and *Ava*I sites: a *Bam*HI/*Sal*I fragment containing pBTM116 sequence and the 5′ end (domain A and a part of domain B), a 0.2-kb fragment (a part of domain B) flanked with a *Bam*HI site and a blunt-ended *Pst*I site, and a 0.6-kb fragment flanked by a blunt-ended *Ava*I site and the *Sal*I site. For pLexA-AB (pHuE1-3), the largest *Pst*I fragment of pLexA-ETS-1 (pHuE1-1), containing pBTM116 and domains A and B, was self-ligated. For pLexA-DEF (pHuE1-4), pBTM116 linearized with *Eco*RI and *Sal*I was ligated with a 0.6-kb *Eco*RI/*Sal*I PCR fragment containing domains D, E, and F. For pLexA-D (pHuE1-5), pBTM116 linearized with *Eco*RI and *Sal*I was ligated with a 260-bp *Eco*RI/*Sal*I PCR fragment containing the D domain. For pSG5-ETS-1 (pSG5-HuETS-1.Ld), the 5′ leader upstream from the *Rsa*I site was deleted from pSG5-HuETS-1 (73) to increase the efficiency of expression by ligating the following three fragments: a 0.2-kb *Rsa*I/*Bam*HI fragment (the 5′ part of the coding region), a 1.5-kb *Bam*HI/*Hin*dIII fragment (the rest of the coding region and a part of the 3′ untranslated region), and a 4.4-kb *Hin*dIII/blunt-ended *Eco*RI fragment (the 3′ untranslated region and pSG5). For pSG5-CDEF (pSG5-HuETS-1.cdef), a PCR fragment encoding domains C, D, E, and F with flanking *Bam*HI (5′-end) and *Bgl*II (3′-end) sites was amplified from pSG5-ETS-1 (pSG5-HuETS-1.Ld) in the *Bam*HI site of pSG5-KOZ1, which has translation initiation sequences. For pSG5-ETS-2, we used the *Eco*RI insert from pBluescript-ETS-2 containing the full-length human ETS-2 coding sequence (from A. Giovane) in the *Eco*RI site of pSG5. For pSG5-Fli-1, we used the 1.7-kb *Eco*RI insert from pBB4-C4 (in pGEM7+) (4) in the *Eco*RI site of pSG5. For pSG5-Sp100, we used the 1.7-kb *Eco*RI insert from pSp65-Sp100.1722 in the *Eco*RI site of pSG5. Since this clone contains a splice variant that differs at the C terminus from the major form of Sp100 (C. Szostecki and H. Will, unpublished data), the 0.9-kb *Bam*HI fragment of pSp65-Sp100.1879, with the sequence of the C terminus and the 3′ untranslated region of the major form, was used to replace the corresponding sequence of the variant. For pSG5-Sp100.N (pSG5-Sp.N), the larger *Bam*HI fragment of pSp65-Sp100.1879 was self-ligated. For pSG5-Sp100.C (pSG5-Sp.C), we used the 0.9-kb *Bam*HI fragment of pSp65-Sp100.1879 in the *Bam*HI site of pSG5-KOZ1. For pSG5-Flag-ETS-1, -ΔC, -CDEF, and -DEF, we used *Bgl*II site-flanked PCR fragments, amplified from pLexA-ETS-1 (pHuE1-1) or pLexA-ΔC (pHuE1-2) and coding for the indicated proteins (Fig. 6A), in the *Bgl*II site of pSG5-Flag. For pSG5-Flag-Sp100, we used a *Bgl*II site-flanked PCR fragment, amplified from pSG5-Sp100 and coding for Sp100, in the *Bgl*II site of pSG5-Flag. To express Gal4-DBD, we used pG4MpolyII (75). For pGal4-ABCD, -ABC, -AB, and -DEF (pGhE 2, 3, 4 and 9), we used *Cla*I-*Bgl*II (5′-3′) site-flanked PCR fragments amplified from pSG5-ETS-1 (pSG5-HuETS-1.Ld) and coding for the indicated proteins (Fig. 7A) between the *Cla*I and *Bgl*II sites of pG4MpolyII. For pGal4-CDEF (pGhE10), we used the *Bam*HI/*Bgl*II insert of pSG5-CDEF (pSG5-HuETS1.cdef) in the *Bam*HI site of pG4MpolyII. For pGal4-Sp100.C (pGAL4-Sp.C), we used the 0.9-kb *Bam*HI fragment of pSp65-Sp100.1879 in the *Bam*HI site of pG4MpolyII. For pGal4-ΔC, we used a *Bgl*II site-flanked PCR fragment with ETS-1 ΔC coding sequences (1 to 130 and 243 to 441) in the *Bam*HI site of pG4MpolyII. To express Gal4-DBD fused to the VP16-AD, we used pGal4-VP16 (68). For pVP16-Sp100, we used an Asp718 site-flanked PCR fragment coding for Sp100 in the Asp718 site of pSG5NLSVP16F (68). For pVP16-Sp100.C, we used an Asp718 site-flanked PCR fragment coding for Sp100 amino acids 333 to 480 in the Asp718 site of pSG5NLSVP16F (68). For pBC-Sp100, we used Sp100 coding sequences flanked by *Eco*RV and *Kpn*I sites in the corresponding sites of pBC (9). The COLL-517/+63-Luc reporter contains the collagenase promoter in a luciferase reporter (72). For PALX8-Luc, we used eight copies of the palindromic ETS-binding sequence upstream from the TK promoter in pGL2 (13). The UAS-TK-Luc reporter contains five Gal4-binding sites in pGL2 (13). For PAL-CAT4, we used eight copies of the palindromic ETS-binding sequence upstream from the TK promoter in pBL-CAT4 (71). Cloned PCR fragments were verified by sequencing. The PCR primer sequences are available on request.

Library screening. The screening procedure was based on the protocol kindly provided by S. Hollenberg before publication (25). A few modifications were made. The genotype of L40 is *MAT***a** *his3Δ200 trp1-901 leu2-3,112 ada2 URA3*::$(lexAop)_8$-*lacZ LYS2*::$(lexAop)_4$-*HIS3*. To supplement the auxotropic deficiency, four 100× stocks were prepared: T (L-tryptophan, 2 mg/ml), H (L-histidine–HCl, 2 mg/ml), L (L-leucine, 3 mg/ml; L-isoleucine, 3 mg/ml; L-valine, 150 mg/ml), and A (adenine sulfate, 2 mg/ml). Minimal plates or media with, for example, adenine and histidine supplements were designated HA. The vector for the "bait" plasmids was pBTM116 containing the LexA gene and the TRP1 marker. HLA plates were used for transformation of pLexA-ΔC (pHuE1-2). A 100-ml culture of L40 containing pLexA-ΔC (pHuE1-2)was transformed with a mixture of 100 μg of cDNA library plasmids and 2 mg of denatured, sheared salmon sperm DNA by the lithium acetate method with dimethyl sulfoxide. The cDNA library purchased from Clontech is based on pACT, which has the LEU2 selection marker and sequences encoding the Gal4 activation domain that is fused to cDNA-encoded proteins. Prior to histidine selection, the yeast transformants were allowed to grow for 16 h in HA medium. For histidine selection, they were plated on minimal plates containing 40 mg of adenine per liter and 25 mM 3-aminotriazole. For cDNA plasmid preparations, yeast transformants were grown in THA medium for 2 days to cure them of pLexA-ΔC (pHuE1-2). Cells were pelleted, resuspended in lysis buffer (2.5 M LiCl; 50 mM Tris Cl, pH 8.0; 4% Triton X-100; 62.5 mM EDTA), and disrupted by vortexing in the presence of acid-washed glass beads. After two phenol-chloroform extractions, plasmids were precipitated twice with ethanol and resuspended in Tris-EDTA. The resulting plasmids were introduced into *Escherichia coli* EC350 (Trp^- Leu^-) by electroporation, and transformants were selected on M9 plates supplemented with Trp and ampicillin. The β-galactosidase (β-Gal) activity was measured by the filter or the quantitative liquid culture methods.

Tissue culture, CAT, and luciferase assays. Cells were grown in Dulbecco medium containing either 2.5% fetal calf serum and 2.5% calf serum (HeLa) or 10% fetal calf serum (COS-7). Confluent cells from one flask (75 mm^2) were split into 10 dishes (9 cm in diameter). After 2 to 6 h at 37°C, 20 μg of DNA in 1 ml of precipitate per 9-cm dish was added (10). The cells were washed the following day and harvested ca. 24 (luciferase assays) or 40 (chloramphenicol acetyltransferase [CAT] assays) hours after the wash. Cell extracts used for β-Gal and CAT assays were prepared in Solution A (15 mM Tris-HCl, pH 7.9; 60 mM KCl; 15 mM NaCl; 2 mM EDTA; 0.15 mM spermine; 1 mM dithiothreitol [DTT]; 0.4 mM phenylmethylsulfonyl fluoride). For luciferase assays, the cells were harvested in phosphate-buffered saline (PBS) plus 3 mM EDTA. Extracts, prepared by three sequential freeze-thaw cycles in 10 mM potassium phosphate (pH 7.8) plus 1 mM DTT, with vortexing between cycles, were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatants were assayed. Luciferase activity was measured in duplicate in 100 mM potassium phosphate (pH 7.8)–1 mM DTT–15 mM $MgSO_4$–5 mM ATP–0.2 mM luciferin by using a Monolight 2010 Luminometer. The LacZ control expression vectors were either ras-LacZ, which contains the β-Gal gene under the control of the ras promoter, or pSG5-LacZ. Transfections were repeated several times with different plasmid preparations for each construct.

In vivo protein-protein interactions. For glutathione *S*-transferase (GST)-tagged proteins, transfected COS-7 cells were washed with PBS and lysed on the plates with 1 ml of lysis buffer (0.1 M NaCl; 20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.5% NP-40) per 9-cm dish by gentle agitation for 15 min at 4°C. Cell lysates were cleared by centrifugation (10 min at 10,000 × *g*). Then, 900 μl was incubated for 2 h at 4°C with 40 μl of glutathione-agarose beads (Sigma; 50% suspension in 0.1% gelatin–0.01% sodium azide). The beads were washed twice with 1 ml of Ls buffer (50 mM Tris-HCl, pH 7.8; 0.1% NP-40; 250 mM NaCl) and resuspended in 20 μl of sodium dodecyl sulfate (SDS) loading buffer. For immunoprecipitations, lysates were prepared by direct freeze-thawing with 1 ml of IP buffer (44) per 9-cm plate, precleared with 50% Sepharose G-beads, rotated

with either Agarose Flag M2 beads (2.5 μl/500 μl of lysate; sIP in figures) or anti-Flag monoclonal antibodies (2FlB11, 2.5 μl/500 μl; IP in figures), followed by the addition of protein G-Sepharose for 90 min for each incubation, and subjected to four 5-min washes with SNNTE buffer (74); the pellets were then resuspended in SDS loading buffer. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Western blots. Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. After 1 h of saturation in PBSTM (PBS with 0.05% Tween 20 and 3% milk), the membranes were incubated either overnight at 4°C or for 1 h at room temperature with specific primary antibodies in PBSTM (0.5% milk), subjected to four 5-min washes with PBST, incubated 1 h at room temperature with secondary antibodies coupled to peroxidase (diluted 1/5,000) in PBSTM (0.5% milk), washed four more times with PBST for 5 min each time, and visualized with the ECL Detection kit (RPN 2106; Amersham).

Immunofluorescence. HeLa cells were cultured on coverslips, transfected, washed three times with PBS, fixed in 50% acetone–50% methanol for 20 min at −20°C, saturated for 40 min in PBS–3% bovine serum albumin (BSA) at room temperature, incubated for 60 min with primary antibodies (1/500) at 37°C, washed three more times for 10 min each time with PBS, incubated for 60 min at 37°C with Texas Red or fluorescein isothiocyanate (FITC)-labeled antibodies (Jackson) diluted 1/250 in PBS plus 0.5 mg of BSA/ml, and washed three times for 10 min each time in PBS. The coverslips were incubated for 1 min at room temperature in Hoechst dye (5 μg/ml in PBS), washed three times with PBS, and mounted by inversion on a drop of mounting solution (80% glycerol, 20% 1× PBS with 5% propylgallate) on a glass slide. The slides were stored in the dark at 4°C and visualized under a fluorescence microscope.

Antisense experiments. Antisense and sense ets-1 phosphorothionate oligonucleotides described previously (11, 47) were synthesized and purified by high-pressure liquid chromatography by the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) facility. Oligonucleotides were transfected with an expression vector for green fluorescent protein (GFP) by the calcium phosphate technique (10) in HepG2 cells in six-well plates containing coverslips (in Eagle medium plus 10% fetal calf serum). At 24 h after the wash the cells were fixed for 20 min at 4°C with 1.8% formaldehyde, permeabilized for 10 min at 4°C with 0.1% Triton X-100 in 0.1% sodium citrate, saturated for 30 min at 20°C with PBS–3% BSA, incubated 2 h at 37°C in a humid chamber with rabbit anti-Sp100 (Sp26) and mouse anti-ETS-1 (MAb94) diluted 1/400 in PBS–0.3% BSA, washed three times for 5 min each time with PBS, incubated for 2 h at 37°C with donkey anti-rabbit conjugated with Texas Red (1/250) and donkey anti-mouse antibody conjugated with FITC (1/75) in PBS–0.3% BSA, washed three times 5 min each time with PBS, incubated with 5 μg of Hoechst stain/ml for 1 min, and then mounted. The cells were observed by confocal and fluorescence microscopy. The numbers of NBs were counted in cells that were successfully transfected (GFP positive) or not (GFP negative), and in "normal" cells that had not undergone transfection. At least 200 cells each in different fields were counted, and the experiment was repeated five times. For Western blotting, the transfections contained pHOOK (3 μg per well of a six-well plate), and transfected cells were isolated with the Capture-Tec kit (Invitrogen) as described previously (74). Membranes were probed with rat anti-Sp100 (1/2,000; a generous gift from H. Will), rabbit anti-ETS-1 (1/200; Ets-1 N276; TEBU), and mouse anti-myc-tag (on the Hook protein; 1/1,000; 9E10.2; IGBMC facility) and revealed as described above.

RESULTS

Sp100 interacts specifically with ETS-1 in yeast. We screened for proteins that interact with ETS-1 by using the yeast dual-hybrid system. The baits and preys were expressed, respectively, from pBTM116 and pACT vectors in the L40 yeast strain that contains HIS3 and LacZ reporters (25). Interactions were detected by both growth in the absence of histidine and β-Gal activity. The bait was LexA-ΔC (Fig. 1A), a LexA–ETS-1 fusion protein that lacks the activation domain of ETS-1. LexA-ΔC did not give histidine prototrophy in the presence of 25 mM 3-aminotriazole, an inhibitor of the *HIS3* gene product glycerol phosphate dehydratase. LexA–ETS-1, containing full-length ETS-1 with the activation domain, stimulated growth under these conditions (Fig. 2A). We screened a cDNA library from Epstein-Barr virus-transformed peripheral blood B cells (Clontech) that expresses Gal4 activation domain-protein fusions. A total of 4×10^5 CFU were screened for both histidine prototrophy and β-Gal activity by the filter assay. Plasmids were purified from yeast that were positive in both assays, transferred into *E. coli*, purified, and analyzed by restriction digestion and sequencing. Sixty plasmids that were isolated from *E. coli* fell into 14 groups after restriction analysis and into 10 groups after sequencing. Three plasmids—pEIF11, pEIF23, and pEIF25 (Fig. 1B and data not shown)—were found to contain partial cDNAs for Sp100 by sequence comparisons with the NCBI database by using BLAST. pEIF11 and pEIF25 encode C-terminal sequences of Sp100L and Sp100, respectively (pEIF23 was not analyzed further). Sp100 was the first variant to be described (67), whereas Sp100.L contains additional amino acids at the C terminus that are common to the three Sp100 variants: Sp100B, Sp100-HMG, and Sp100C (14, 19, 57, 58, 77).

To ensure that the interactions are specific, pEIF11 and pEIF25 were reintroduced into yeast and compared with several unrelated preys for their ability to induce histidine prototrophy and β-Gal activity with different baits. EIF11 gave efficient growth in the absence of histidine with the original bait, LexA-ΔC, but not with seven different control baits (Fig. 2A). In contrast, the two control preys, the Gal4 activation domain and Gal4 activation domain fused to a nonspecific protein (HLA-DRγ), did not stimulate growth with pLexA-ΔC. Both EIF11 and EIF25 gave much higher levels of β-Gal activity than the control prey (Gal4 AD–HLA-DRγ) when the specific bait (LexA-ΔC) was used, whereas they all gave similar background levels of activity with the control bait (LexA-Lamin, Fig. 2B). These results show that Sp100 and ETS-1 sequences interact specifically in the yeast dual-hybrid assay. We then localized the sequences of ETS-1 that mediate the interactions and investigated whether the full-length proteins interact.

Baits containing various fragments of ETS-1 (Fig. 1A) were analyzed by both the histidine prototrophy and β-Gal assays. In the growth assay, specific interactions were detected with the domains A+B (LexA-AB) but not with either D+E+F (LexA-DEF) or D (LexA-D). In the more sensitive liquid β-Gal assay, interactions were detected with A+B (LexA-AB) and D+E+F (LexA-DEF). These results indicate that regions A+B and, to a lesser extent, D+E+F mediate interactions. Full-length ETS-1 in the bait (LexA–ETS-1) stimulated both growth and β-Gal activity more efficiently with the specific preys (Fig. 2), showing that full-length ETS-1 interacts with Sp100. Full-length Sp100 in the prey was found to be toxic to yeast, and the degree of toxicity depended upon the bait. However, interactions were detected with the bait containing domains A+B (LexA-AB [data not shown]), suggesting that full-length Sp100 can interact with ETS-1. These results show that full-length ETS-1 interacts specifically with Sp100. Furthermore, they have the opportunity to interact in human cells, since we found that Sp100 is coexpressed with ETS-1 in several T- and B-cell lines (data not shown).

Sp100 interacts specifically with ETS-1 in mammalian cells. Sp100–ETS-1 interactions were investigated in mammalian cells by using dual-hybrid, GST pull-down, and immunoprecipitation assays. In the dual-hybrid assays, the bait was the Gal4 DBD fused to ETS-1 lacking the activation domain (Gal4-ΔC, Fig. 3A). The prey was the VP16 activation domain linked to full-length Sp100 (VP16-Sp100). HeLa cells were

A

Bait \ Prey	Gal4-AD	Gal4-AD-HLA-DRγ	EIF11
LexA	-	-	-
LexA-Lamin	-	-	-
LexA-ETS-1	++	++	++++
LexA-ΔC	-	-	+++++
LexA-AB	-	-	+/-
LexA-DEF	-	-	-
LexA-D	+	+	+
LexA-Myc	-	-	-
LexA-Max	-	-	-
LexA-Bicoid	-	-	-
LexA-CyclinC	-	-	-
LexA-Cdc2	-	-	-

B

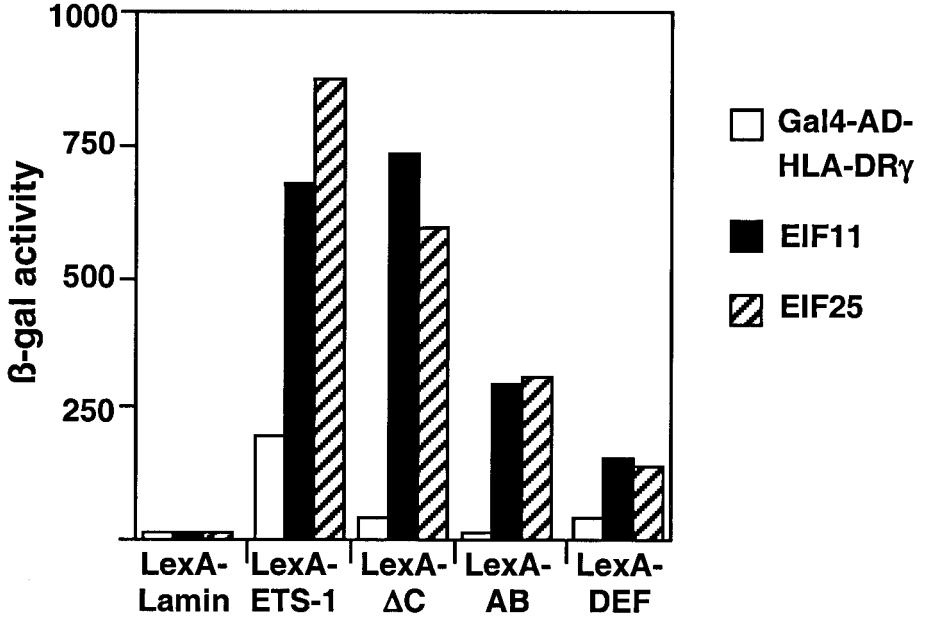


FIG. 2. Interaction of ETS-1 and Sp100 in yeast. (A) Histidine prototrophy test. Yeast transformants were streaked on histidine selection plates containing 25 mM 3-aminotriazole (an inhibitor of the histidine pathway) and scored after a 4-day incubation at 30°C. Growth is indicated as "±" to "+++++" based on the size of the colonies. The control baits were LexA, LexA-Lamin, LexA-Myc-Cterm, LexA-Max, LexA-Bicoid, LexA-Cdc2 (79), and LexA-cyclin C (69). Control inserts were transferred to pBTM116, in order to express the control and test ETS-1 baits from the same vector. The control preys were Gal4 AD and Gal4 AD–HLA-DRγ. (B). Liquid β-Gal assays. β-Gal activity was measured with the substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and is expressed in arbitrary units. Yeast extracts were adjusted for protein determined by the Bradford assay.

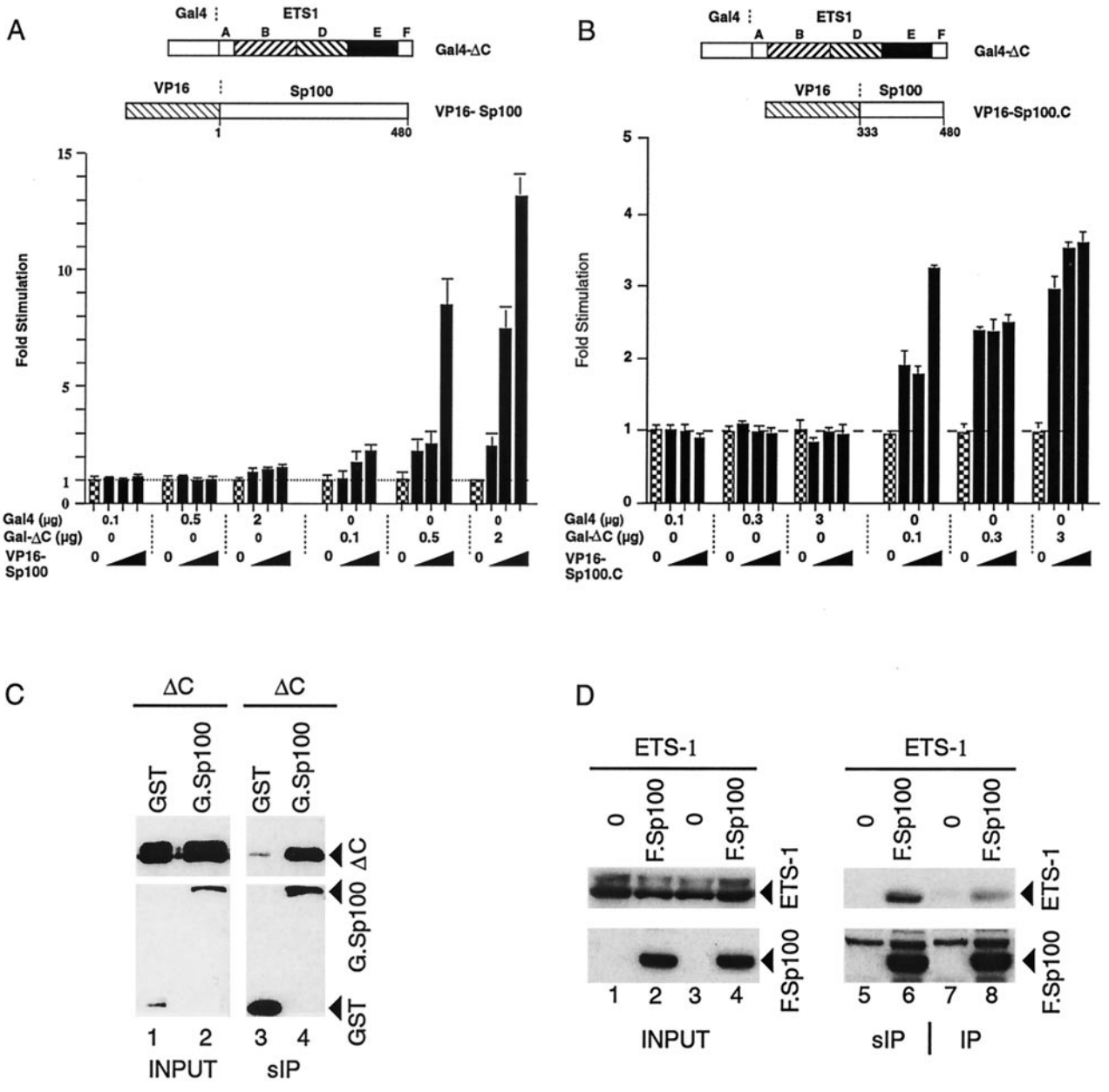


FIG. 3. Interaction of ETS-1 and Sp100 in mammalian cells. (A) Dual-hybrid assay with Sp100. HeLa cells were transfected with the expression vectors pGal4 (0, 0.5, and 2 μg), pGal4-ΔC (0, 0.5, and 2 μg), pVP16-Sp100 (0, 0.1, 0.5, and 3 μg), the reporter UAS-TK-Luc, and the internal control pSG5-LacZ (0.5 μg). Values from three independent experiments with at least two DNA preparations are plotted as the fold stimulation relative to the samples without VP16-Sp100. (B) Dual-hybrid assay with Sp100.C. The transfections were as in panel A except that pGal4 (0, 0.1, 0.3, and 3 μg), pGal4-ΔC (0, 0.1, 0.3, and 3 μg), and pVP16-Sp100.C (0, 0.1, 0.5, and 3 μg) were used. (C) In vivo GST pull-down assay. COS-7 cells were transfected with the expression vectors pSG5-Flag-ΔC (5 μg, lanes 1 to 4), pBC-Sp100 (10 μg, lanes 2 and 4), and pBC (5 μg, lanes 1 and 3). Cell extracts were incubated with glutathione-Sepharose beads, and immobilized proteins were analyzed by SDS-PAGE and Western blotting with antibodies against the Flag tag of ΔC (2Fl1B11 mouse monoclonal, upper panels) and GST (1D10, lower panels). The input (lanes 1 and 2) was 10% of the amount used for the solid-phase immunoprecipitation (sIP, lanes 3 and 4). (D) Coimmunoprecipitation. COS-7 cells were transfected with the expression vectors pSG5-ETS-1 (5 μg) and pSG5-Flag-Sp100 (5 μg). Cell extracts were incubated with immobilized Flag antibodies (anti-Flag M2 affinity gel, sIP, lanes 5 and 6) or Flag antibodies (2Fl1B11), followed by protein G (IP, lanes 7 and 8). The input (lanes 1 to 4) was 10% of the amount used for the immunoprecipitations. Western blots were probed with rabbit antibodies against Ets-1 (TEBU, N276, and sc-111), followed by protein A-peroxidase and with mouse anti-Flag monoclonal antibody (2Fl1B11), followed by goat anti-mouse κ-light chain peroxidase (Southern Biotechnology).

transfected with the reporter UAS-TK-Luc, the internal control pSG5-LacZ, and different quantities of expression vectors for Gal4-ΔC and VP16-Sp100. Luciferase activities were corrected for the internal control and expressed as the fold activation relative to transfections containing the control prey VP16 (black and white bars, Fig. 3A). VP16-Sp100 efficiently stimulated transcription in the presence of Gal4-ΔC in a dose-dependent manner. It had no significant effect with Gal4 alone.

These results indicate that ETS-1–ΔC interacts with Sp100 in mammalian cells, which is similar to the conclusions obtained with yeast. We also tested a prey with C-terminal Sp100 sequences (from positions 333 to 480) that contain the Sp100 activation domain and the nuclear localization signal (see Fig. 1B). VP16-Sp100.C activated in the presence of Gal4-ΔC but not with Gal4 alone (Fig. 3B), showing that the C-terminal sequences of Sp100 are sufficient for the interaction with ETS-1–ΔC.

For pull-down assays, Sp100 tagged with GST was coexpressed with ΔC in COS cells, extracts were incubated with glutathione-Sepharose beads, and retained proteins were detected by Western blotting (Fig. 3C). ΔC was found to be associated with GST-Sp100 (lane 4) but not significantly with the GST tag alone (lane 3). ΔC was expressed at comparable levels in the presence of GST or GST-Sp100 (lanes 1 and 2), and similar amounts of GST proteins were expressed and immobilized on the beads (lanes 1 to 4). In parallel experiments, Sp100 tagged with Flag was coexpressed with full-length ETS-1 in COS cells and interactions analyzed by either solid-phase immunoprecipitation with immobilized Flag antibodies or by conventional immunoprecipitation (Fig. 3D, sIP and IP, respectively). ETS-1 was found to be complexed with Sp100 (lanes 6 and 8) but not with Flag alone (lanes 5 and 7). ETS-1 was expressed at similar levels in the presence or absence of Sp100 (lanes 1 to 4) and F-Sp100 was efficiently immunoprecipitated (lanes 6 and 8). These results show that Sp100 interacts specifically with ETS-1 and ΔC in mammalian cells.

ETS-1 expression alters NBs. The effect of ETS-1 on NBs was investigated by confocal microscopy of immunostained HeLa cells. Endogenous Sp100 and PML colocalize in ca. 13 NBs (range, 6 to 20; Fig. 4A1, α-Sp100; Fig. 4B1, α-PML; Table 1) (24, 41, 56, 65). Exogenous Sp100, expressed at functionally significant levels (see below), formed enlarged NBs without increasing their number (Fig. 4A4, α-Sp100; Fig. 4B1, α-Sp100 plus α-PML; Table 1). Expression of ETS-1, also at functionally significant levels (see below), decreased the number of endogenous constitutive NBs to ca. 3 (range, 0 to 5, Table 1; Fig. 4A2, α-Sp100). Similarly, it decreased the number of Sp100-enlarged NBs while increasing the size of the remaining NBs (Fig. 4A5, α-Sp100; Fig. 4B2, α-Sp100 and α-PML; Table 1). The expressed ETS-1, detected with Flag antibodies, was distributed throughout the nucleus, suggesting that ETS-1 is not preferentially associated with NBs. A similar distribution was observed at all levels of ETS-1 expression tested (0.1 to 4 μg [data not shown]). Endogenous ETS-1 was not detectable with the available antibodies (data not shown). The morphological changes in NBs could possibly have resulted from changes in expression levels. However, Western blots of transfected cells showed that the levels of ETS-1 and Sp100 were not altered by coexpression (Fig. 4C), excluding that changes in the expression levels of Sp100 were involved. These results show that ETS-1 affects NBs but does not localize to these structures.

ETS-1 could affect the structure of NBs through indirect effects on gene expression or through direct interactions. We tested whether ΔC, which lacks the activation domain, and a minimal heterologous factor, Gal4-AB, containing just an interaction domain for Sp100, affect NB morphology. ΔC and Gal4-AB expression decreased the number of constitutive and enlarged NBs and increased the diameter of the remaining enlarged NBs, similar to ETS-1 (Fig. 4A4, 6, and 7). These results indicate that the interaction of ETS-1 with Sp100 alters the morphology of NBs. Furthermore, these results are entirely consistent with the ability of ETS-1 to physically interact with Sp100 in vivo.

HeLa cells express low levels of ETS-1. In order to investigate the effects of decreasing endogenous ETS-1 on NBs, we used cell lines that express detectable levels of the protein (HepG2, CEM, Jurkat, and BJAB cells). We used antisense Ets-1 oligonucleotides that have been carefully characterized and shown to specifically decrease ETS-1 levels in various cell lines, including HepG2 (11, 28, 47). We showed that HepG2 cells contain two to four NBs per nucleus and a detectable level of ETS-1 that is dispersed throughout the nucleus (Fig. 4D, panels 1 to 4; Table 2). Transfection of the antisense Ets-1 oligonucleotide led to an increase in the number of NBs to 6 to 14 in the GFP-positive transfected cells, without affecting the number of NBs in nontransfected GFP-negative cells (Fig. 4D and Table 2). Transfection of the sense oligonucleotide did not alter the number of NBs. Similar results were obtained in five different experiments and with other cell lines (CEM, Jurkat, and BJAB cells [data not shown]). The levels of ETS-1 and Sp100 were investigated by Western blotting of transfected cells that had been purified with the Capture-Tec kit (Invitrogen). Ets-1 antisense did not affect the level of endogenous Sp100 but did decrease the level of ETS-1, as expected from previous studies (11, 28, 47). These results show that decreasing endogenous ETS-1 levels leads to an increase in the number of NBs. In contrast, increasing ETS-1 levels, in HeLa cells that express low levels of ETS-1, decreases the number of NBs.

Sp100 stimulates ETS-1 transactivation of both natural and focused promoters. The functional effects of the interaction between ETS-1 and Sp100 were investigated with the natural collagenase ETS-1-responsive promoter (72) and with a promoter containing a multimerized Ets-motif that focuses on the activity of ets factors (13). HeLa cells were transfected with the collagenase (−517/+63)-Luc reporter (Fig. 5A) or the PALx8-Luc focused reporter (Fig. 5B), expression vectors for ETS-1 and Sp100, and the internal control pSG5-LacZ. ETS-1 stimulated the collagenase reporter at the highest concentration tested (Fig. 5A, bars 1 to 4), whereas Sp100 alone had no effect. Sp100 strongly stimulated ETS-1 activity under conditions in which ETS-1 alone had no effect (compare bars 8 to 10 with 2 and 11 to 13 with 3). With the simplified ets-specific promoter, ETS-1 and Sp100 alone had little effect (Fig. 5B, bars 1 to 7). Sp100 synergistically increased ETS-1 activity (compare bars 8 to 10 with bar 2 and bars 11 to 13 with bar 3). The effects were not due to increased ETS-1 expression (Fig. 4C and data not shown). These results show that Sp100 stimulates ETS-1 activation of both natural and focused promoters.

We investigated whether Sp100 could also stimulate other heterologous or related factors. The 1-147 Gal4 fragment, containing the DBD and a weak activation domain, weakly stimulated the activity of the UAS-TK-Luc promoter in HeLa cells (Fig. 5C). Sp100 did not have an effect on this activity. Gal4 fused to the VP16 activation domain strongly activated the UAS-TK-Luc reporter (Fig. 5D). Under subsaturating conditions (0.1 μg, bar 2), Sp100 did not affect the activity of this heterologous transcription factor. Fli-1 is an ETS-1 related

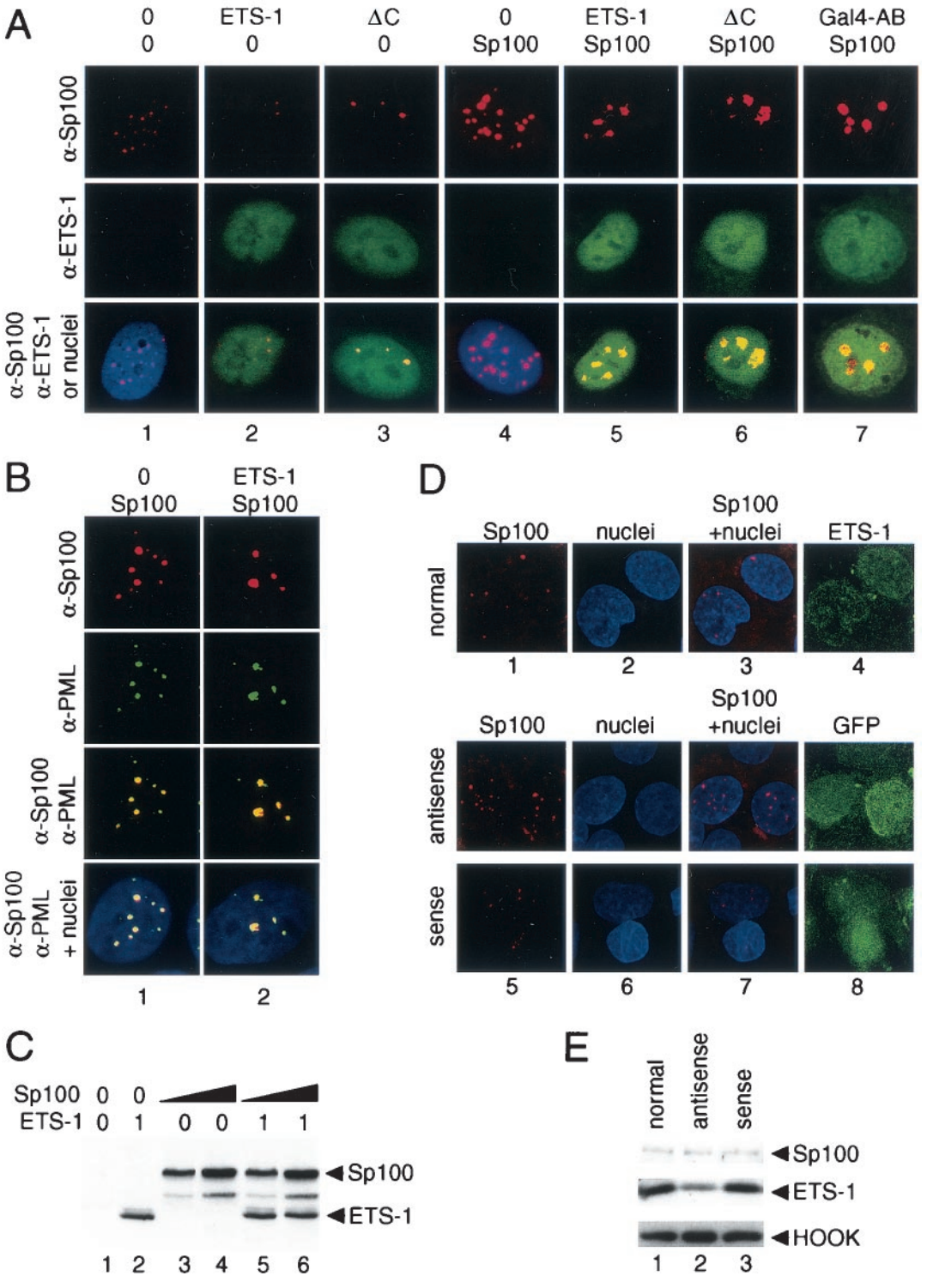


FIG. 4. ETS-1 levels alter the nuclear distributions of Sp100 (A and D) and PML (B). (A and B) HeLa cells were transfected with the following expression vectors: pSG5-Flag-ETS-1 (1 μg, columns 2 and 5), pSG5-Flag-ΔC (0.5 μg, columns 3 and 6), pGal4-AB (0.5 μg, column 7), and pSG5-Sp100 (0.5 μg, columns 4 to 7) (A) and pSG5-Flag-Sp100 (0.5 μg, columns 1 and 2) and pSG5-ETS-1 (0.5 μg, column 2) (B). The cells were fixed with acetone-methanol and treated with rabbit anti-Sp100 (α SP26, columns 1 to 7) and mouse anti-Flag (2Fl1B11, α-ETS-1, columns 1 to 6) or mouse anti-Gal4 (2GV3 plus 3GV2, column 7), followed by donkey anti-rabbit antibody coupled to Texas Red (α−Sp100) and donkey anti-mouse coupled to FITC (α-ETS-1) (A) or with mouse anti-Flag (2Fl1B11, columns 1 to 2) and rabbit anti-PML (494, columns 1 to 2), followed by donkey anti-mouse antibody coupled to Texas Red (α-Sp100) and donkey anti-rabbit antibody coupled to FITC (α−PML) (B). Nuclei were visualized with Hoechst stain. The cells were examined by confocal microscopy. (C) Expression levels in HeLa cells. The cells were transfected with the expression vectors pSG5-Flag-ETS-1 (1 μg, lanes 2, 5, and 6) and pSG5-Flag-Sp100 (0.5 μg, lanes 3 and 5; 1 μg, lanes 4 and 6). Cell extracts were analyzed by SDS-PAGE and Western blotting with mouse anti-Flag monoclonal antibodies (2Fl1B11), followed by goat anti-mouse

TABLE 1. NBs in HeLa cells

Protein	No.[a]		Size[b]	
	Sp100	PML	Sp100	PML
Endogenous	6–20	6–20	+	+
ETS-1	0–5	0–5	+	+
ΔC	0–5	0–5	+	+
Sp100	6–20	6–20	++	++
Sp100 + ETS-1	0–5	0–5	+++	+++
Sp100 + ΔC	0–5	0–5	+++	+++
Sp100 + Gal4-AB	0–5	0–5	+++	+++

[a] Number of NBs in greater than 70% of the cells.
[b] +, normal diameter; ++, two- to threefold increase; +++, three- to fourfold increase.

TABLE 2. NBs in HepG2 cells

Oligonucleotide	No. of NBs[a]:		
	Not transfected	Transfected	
		GFP positive	GFP negative
None (normal)	2–4	NR	NR
Antisense	NR	6–14	2–4
Sense	NR	2–4	2–4

[a] Number in more than 75% of the cells. NR, not relevant.

factor that has similar DBD and Pointed domains. Fli-1 had little effect on the focused PAL-CAT reporter in the presence or absence of Sp100 (Fig. 5E). In contrast, ETS-1 was activated by Sp100 in an ets-motif dependent manner (compare PAL-CAT4 with the pBL-CAT reporter that lacks uniquely the ets motifs). ETS-2 is more closely related to ETS-1 than Fli-1. ETS-2 stimulated the activity of the ets-specific promoter (PALX8-Luc), and Sp100 increased this activity more than twofold (Fig. 5F). These results show that Sp100 stimulates ETS-1 and ETS-2 activity but does not activate a number of other factors, showing that Sp100 is specific for several closely related ETS factors.

The ETS-1 activation domain is required for Sp100 stimulation. The ETS-1 sequences required for Sp100 activation were investigated in HeLa cells by using deletion mutants of ETS-1 retaining the DBD and the focused reporter (PALx8-Luc, Fig. 6). Sp100 increased the activity of ETS-1 and CDEF but failed to activate ΔC and DEF, even though Sp100 can interact with both of these factors (see above). We further investigated the sequence requirements in the absence of the Ets DBD by using Gal4-ETS fusion proteins and the UAS-TK-Luc reporter (Fig. 7). Sp100 activated Gal4-ABCD and Gal4-ABC but did not activate either Gal4-AB or Gal4-DEF, which lack the C-domain. Sp100 fused to VP16 activated both Gal4-AB and Gal4-DEF, as expected from the ability of Sp100 to interact with both regions of ETS-1 (see above). Similar results were obtained in COS cells (data not shown). Sp100 did not alter the expression levels of the Gal4 proteins, as shown by Western blotting after transfection in COS cells (Fig. 7C). These results show that Sp100 activation of ETS-1 requires the ETS-1 activation domain as well as physical interactions. A heterologous activation domain fused to Sp100 can functionally replace the ETS-1 activation domain.

Transcription activity of Sp100 mutants. The C-terminal domain (from positions 333 to 480) of Sp100 is sufficient to interact with ETS-1 (Fig. 3B and data not shown). However, the C-terminal fragment was not sufficient to activate Gal4-ABCD (Fig. 7D) or ETS-1 (data not shown) in HeLa cells, even though it was localized in the nucleus (data not shown), as expected from the presence of a nuclear localization signal. The remainder of Sp100 (positions 1 to 332; Sp100.N) also did not activate Gal4-ABCD (Fig. 7D) or ETS-1 (data not shown). These results suggest that the interaction domain of Sp100 for ETS-1 is not sufficient for efficient activation. Sp100 has been reported to be an inhibitor of transcription (33, 57) and also to contain a transcription activation domain (77). We used Gal4-Sp100 fusion proteins to investigate the transcriptional activity of Sp100 under our conditions. Gal4-Sp100 inhibited transcription and the C-terminal fragment activated transcription very weakly (Fig. 7E). The Gal4-Sp100 N-terminal fragment had no detectable effect on transcription. These results show that Sp100 has different activities. In the presence of ETS-1 it behaves as a transcriptional activator. The effects on ETS-1 are at least quantitatively similar or even superior to the previously reported activities of Sp100.

DISCUSSION

We have found that Sp100 interacts with ETS-1 physically and functionally. Sp100 interacts with ETS-1 in vivo and in dual-hybrid, GST pull-down, and coimmunoprecipitation assays in yeast and mammalian cells. The cellular localization of Sp100 is affected by the expression of ETS-1 and the N-terminal domain, which is sufficient for physical interaction, a finding consistent with their ability to interact. ETS-1–Sp100 interactions involve both the A+B and D+E+F regions of ETS-1 and the C-terminal region of Sp100. Sp100 does not bind to DNA directly but inhibits transcription when brought to the DNA by a heterologous DBD (33, 58; the present study). Sp100 also contains a weak activation function that is unmasked by deletion of the N-terminal region (77). The activation domain of ETS-1 is weak. Sp100 activates ETS-1 efficiently, on both natural and ets-focused promoters. The

peroxidase. (D) HepG2 cells in six-well plates were transfected with antisense or sense Ets-1 phosphorothionate oligonucleotides (10 μM), an expression vector for GFP (0.5 μg of pXJ41-GFP-C1) and carrier DNA (3 μg of pBluescript). After 24 h they were fixed, permeabilized, stained with rabbit anti-Sp100 (α SP26) and mouse anti-ETS-1 (MAb94) antibodies, followed by donkey anti-rabbit antibody coupled to Texas Red and donkey anti-mouse antibody coupled to FITC. Nuclei were visualized with Hoechst stain. The cells were examined by confocal microscopy. Endogenous ETS-1, detected with FITC (green), is shown for cells that had not been subjected to transfection (normal). The GFP fluorescence of transfected cells is shown for the antisense- and sense-transfected cells. (E) Expression levels in HepG2. The cells in six-well plates were transfected with antisense or sense Ets-1 phosphorothionate oligonucleotides (10 μM) and 3 μg of pHook (Invitrogen). Tranfected cells were purified with the Capture-Tec kit (Invitrogen). Extracts were analyzed by SDS–8% PAGE and Western blotting with rat anti-Sp100 antibody (gift from H. Will), rabbit anti-ETS-1 antibody (TEBU Ets1 N276), and mouse anti-Myc tag (monoclonal antibody 9E10.2, to detect the Hook protein as an efficiency control). "Normal" refers to cells that had not been subjected to transfection.

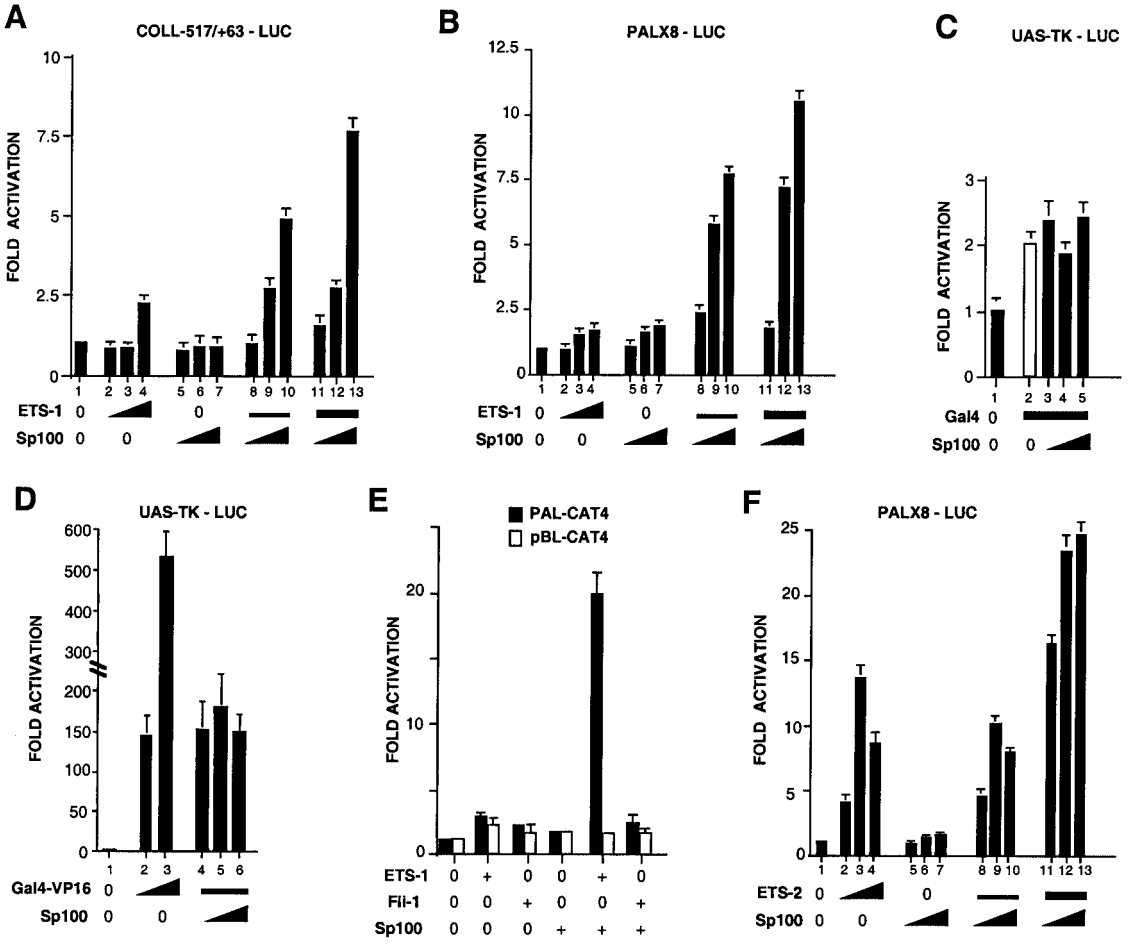


FIG. 5. Stimulation of ETS-1 and ETS-2 transcriptional activity by Sp100. HeLa cells were transfected with expression vectors, reporters and the internal control pSG5-LacZ (0.5 μg). Corrected luciferase (A to D and F) or CAT (E) activities were used to calculate the fold activation relative to transfections with empty expression vectors. The values are from three independent experiments with at least two DNA preparations. (A, B, and F) Reporters with the natural ets-reponsive collagenase promoter (A, 1 μg) and the ets-specific promoter (B and F; 1 μg). The tranfections contained pSG5-ETS-1 (A and B) or pSG5-ETS-2 (F) (0 μg, bars 1 and 5 to 7; 0.1 μg, bars 2 and 8 to 10; 0.5 μg, bars 3 and 11 to 13; 2 μg, bar 4) and pSG5-Sp100 (0 μg, bars 1 to 4; 0.1 μg, bars 5, 8, and 11; 1 μg, bars 6, 9, and 12; 2 μg, bars 7, 10, and 13). (C, D, and E) Sp100 does not stimulate the activity of weak (C, Gal4), strong (D, Gal4-VP16), and related (E, Fli-1) transcription activators. The transfections contained pSG5-Gal4 (C, 0.5 μg, bars 2 to 5), pSG5-Gal4-VP16 (D, 0.1 μg, bars 2 and 4 to 6; 0.5 μg, bar 3), pSG5-Sp100 (0.1 μg, bar 3 [C] and bar 4 [D]; 0.5 μg, bar 4 [C] and bar 5 [D]; 2 μg, bar 5 [C] and bar 6 [D]; 4 μg [E]), UAS-TK-Luc (1 μg [C and D]), PAL-CAT (4 μg [E]) and pBL-CAT4 (4 μg [E]), pSG5-ETS-1 (4 μg [E]) and pSG5-Fli-1 (4 μg [E]).

activation is more than expected from the sum of their individual activities. It is specific, in that Sp100 does not affect the activities of both unrelated weak and strong activators and a related ets factor (Fli-1), but it does increase the activity of the more closely related ETS-2. The physical interaction between ETS-1 and Sp100 is not sufficient for the functional interaction. Activation requires, in addition, the activation domain of ETS-1 and the N-terminal part of Sp100, but physical interactions do not. ETS-1 domains have been shown to have intramolecular effects on their physical and functional properties. A+B inhibits the activation domain, whereas D+F blocks DNA binding. All of these observations would fit into a model in which Sp100 acts as a novel coactivator which, through physical interactions with ETS-1, induces a conformational change that exposes the activation functions of the complex (Fig. 8).

The A+B and the D+E+F regions of ETS-1, which interact with Sp100, have important regulatory roles through intramolecular mechanisms and interactions with other proteins. Pointed (B) domains are found in 4 Ets proteins in *Drosophila* (26) and 14 proteins in vertebrates (including Ets-1, Ets-2, and Fli-1; see Fig. 3C in reference 17). The Pointed domain adopts a monomeric five-helix bundle structure (61) that resembles the *S*-adenosylmethionine (SAM) domains found in more than a hundred diverse proteins (55). SAM domains in general are involved in protein-protein interactions (63). The Pointed domain of ETS-1 interacts with EAP1/Daxx (36), and the related domains of other Ets proteins are involved in homodimerization and interactions with various proteins (2, 30, 51, 52; for a review, see reference 35). We did not detect homodimerization of the A+B region of ETS-1 in the yeast dual-hybrid assay (data not shown), indicating that it is rather involved in heterotypic interactions. The Pointed domains of ETS-1 and other Ets factors are functionally involved in transcription activation

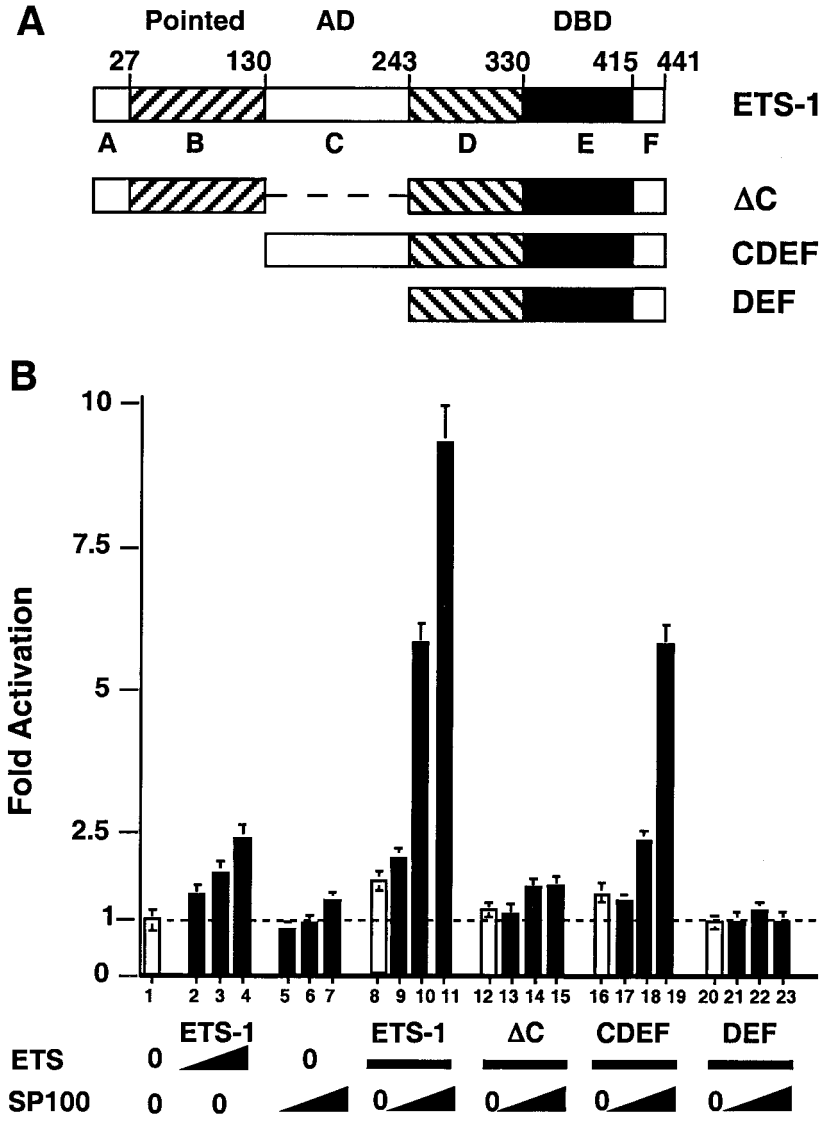
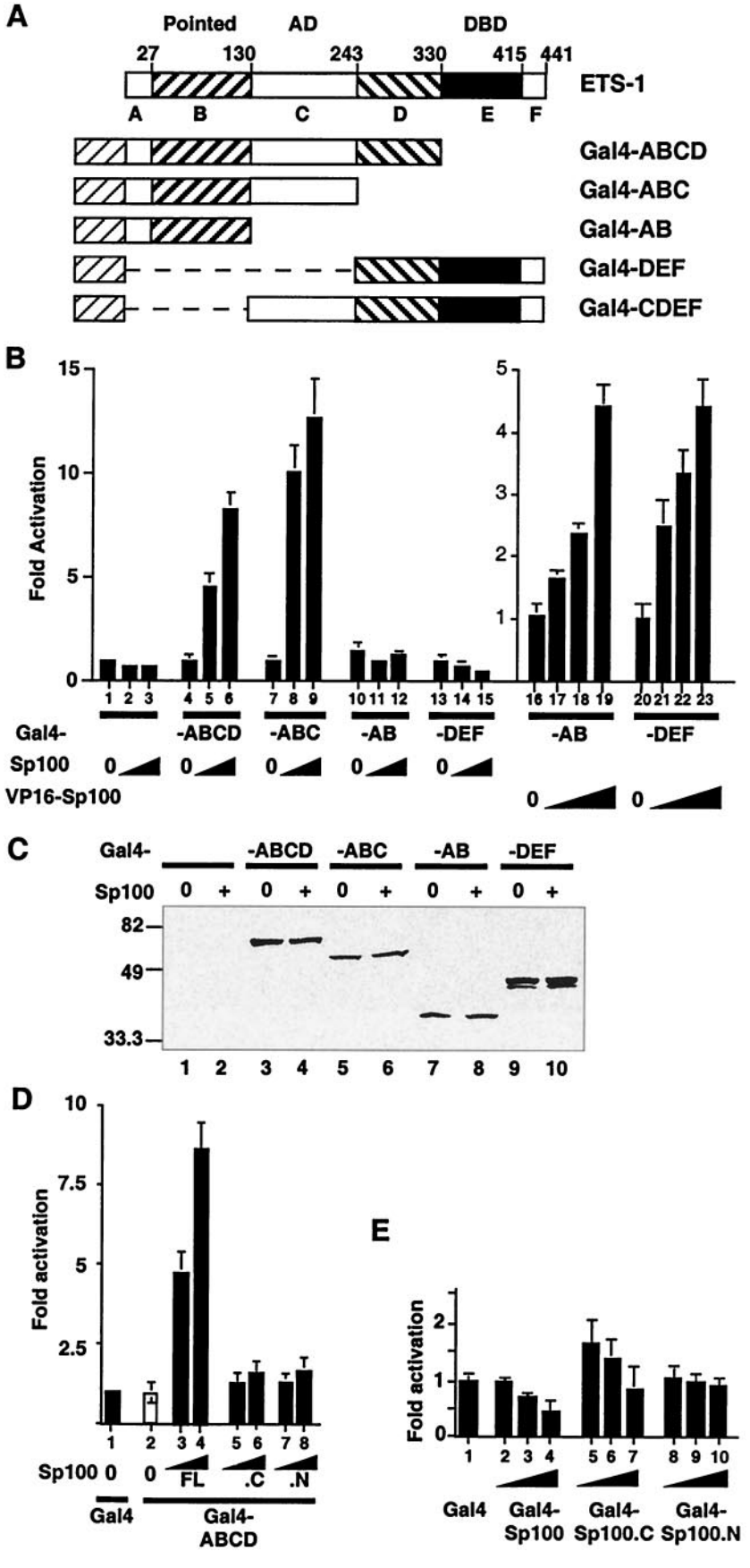


FIG. 6. The ETS-1 activation domain C is required for efficient stimulation of ETS-1 by Sp100. (A) Scheme of the ETS-1 mutants. (B) HeLa cells were transfected with the PALx8-Luc reporter (1 μg), the internal control pSG5-LacZ (0.5 μg), pSG5-ETS-1 (0.1 μg, bar 2; 0.5 μg, bars 3 and 8 to 11; 2 μg, bar 4), pSG5-Flag-ΔC (0.5 μg, bars 12 to 15), pSG5-Flag-CDEF (0.5 μg, bars 16 to 19), pSG5-Flag-DEF (0.5 μg, bars 20 to 23), and pSG5-Sp100 (0.1 μg, bars 5, 9, 13, 17, and 21; 0.5 μg, bars 6, 10, 14, 18, and 22; 1 μg, bars 7, 11, 15, 19, and 23). Corrected luciferase activities were used to calculate the fold activation relative to transfections lacking Sp100 (open bars). The values are from at least three independent experiments with at least two DNA preparations.

by the Ras–mitogen-activated protein kinase pathway 2; reviewed in reference 78) and transcription repression (for a review, see reference 43). Our data indicate that it is also involved in Sp100 activation. Sp100 activation is specific, insofar as it does not activate Fli-1, an ETS-1 related factor with a Pointed domain.

Sp100 activates ETS-1 apparently without itself binding to DNA, since it stimulates ETS-1 through a number of reporters that have no obvious homology, and has not been reported to bind specifically to DNA. It appears unlikely that Sp100 activation in the presence of ETS-1 results from simple recruitment of Sp100 to DNA. In fact, Sp100 inhibits transcription when brought to the DNA by fusion to a heterologous DBD (33, 58; the present study). Sp100 has a C-terminal activation domain (amino acids 333 to 407; Fig. 1B) that is active in the full-length protein in yeast (77) but not in mammalian cells and which is unmasked by deletion of the N terminus (77; the present study). The levels of activation and repression by Sp100 appear to be relatively low in the conditions described here, raising the possibility that they do not contribute significantly to the overall activity of Sp100 in the presence of ETS-1. Sp100 does not activate the transcription of ETS-ΔC, DEF, Gal-AB, and Gal-DEF, even though it interacts with these molecules. However, we cannot exclude that the transcription modulation properties of Sp100 are affected by interactions with full-length ETS-1. Sp100 activation requires the C activation domain of ETS-1. The ETS-1 A+B region has been reported to be a negative regulator of the flanking C activation domain (8, 54). Sp100 interactions with the A+B region may relieve this repression (Fig. 8).


A
Pointed
AD
DBD
27
130
243
330
415
441
ETS-1
A
B
C
D
E
F
Gal4-ABCD
Gal4-ABC
Gal4-AB
Gal4-DEF
Gal4-CDEF
B
Fold Activation
Gal4-
-ABCD
-ABC
-AB
-DEF
-AB
-DEF
Sp100
VP16-Sp100
C
Gal4-
-ABCD
-ABC
-AB
-DEF
Sp100
82
49
33.3
D
Fold activation
Sp100
FL
.C
.N
Gal4
Gal4-ABCD
E
Fold activation
Gal4
Gal4-Sp100
Gal4-Sp100.C
Gal4-Sp100.N

ETS-1 is also regulated intramolecularly by the D+F domains, which inhibit specific DNA binding by the Ets (E) domain (20, 21, 37, 48, 73). Inhibition results from the formation of a helical bundle between helices of the D, E, and F domains, which constrains the E domain from interactions with DNA (29, 48, 60). The transition between the closed and open conformations can be driven by DNA (21, 48), dephosphorylation (12, 23, 53), and interactions with proteins (15). The D+E+F domains of ETS-1 mediate physical and functional interactions with Sp100 and a number of other factors (for a review, see reference 35). Even though Sp100 interacts with the D+E+F region, it does not appear to stimulate the DNA-binding activity of ETS-1 in band shift experiments by using both strong and weak motifs as probes (data not shown). The D domain appears to be important for Sp100 activation, at least in the context of the natural splice variant (ETS-1ΔVII), which is not activated by Sp100 (data not shown).

A working model for the mechanism by which Sp100 activates ETS-1 can be proposed which takes into account our observations and the known properties of ETS-1 (Fig. 8). DNA-bound ETS-1 does not efficiently activate transcription because the C activation domain is weak and is inhibited by domains A+B. Sp100 interacts with ETS-1 through contacts with two regions, A+B and D+E+F, and induces a conformational change that exposes the C activation domain. The C and possibly the Sp100 activation domains activate transcription. The N-terminal domain of Sp100 may be required to induce the conformational change, although we cannot exclude that it contributes in other ways to transcription activation. A novel aspect of this mechanism is that Sp100 acts as a coactivator that exposes an activation domain in the transcription factor with which it interacts. Further more, it provides a scenario for activation of transcription, in contrast to the proposed models of inhibition. Evidence has been presented to suggest that DSP1 (hHMG2) represses transcription by recruitment of Sp100 or its variant Sp100B and in turn the heterochromatin protein HP1 (33, 58). Sp100 represses the activity of the transcription factor Bright by inhibiting DNA binding in vitro and relocalization to NBs. However, the involvement of HP1 was not excluded (82). Our results show that Sp100 can activate transcription, apparently acting as a coactivator. Interestingly, two other members of the Sp100 family, Sp110 and Sp140, also activate transcription. Sp110 has been suggested to function as a nuclear hormone receptor coactivator, and both Sp100 homologues activate transcription when recruited to DNA by heterologous DBDs (6, 7).

ETS-1 affects the size and number of NBs, as well as interacting with the classical component, Sp100. These observations have broad implications for understanding the function of this subnuclear domain. NBs can be considered to be flexible homeostatic regulators (for reviews, see references 41, 56, and 64). NBs appear to be built from a network of interacting proteins, with PML and Sp100 at their core. When they are sequestered in NBs they are segregated away from the rest of the nuclear compartment, but they remain available for rapid release and control of physiological processes. Increasing the level of ETS-1 decreases the number of NBs, detected with antibodies against PML or Sp100. In contrast, decreasing the level of ETS-1 increases the number of NBs. ETS-1 remains dispersed throughout the nucleus, with no evidence for preferential localization in NBs. A dispersed nuclear localization of ETS-1 has been described previously (50). Expression of a transcriptionally inactive form of ETS-1 (ΔC) or just an interaction domain (Gal4-AB) is sufficient to disrupt the NB structure. Furthermore, ETS-1 expression does not affect the expression levels of Sp100. These observations can be simply interpreted in the light of the current view that Sp100 is in equilibrium between the NB-bound and "free" forms in the nucleus. An increase in the number of binding sites for Sp100 in the nucleus, in the form of ETS-1, would be expected to dissociate Sp100 from the NB. A decrease in ETS-1 levels would shift the equilibrium toward Sp100 bound to NBs (Fig. 8). Sp100 associated with ETS-1 at promoters would then activate transcription. Importantly, NB disruption is observed in conditions that are optimal for transcription activation. Interestingly, the fact that shuttling between NBs and the rest of the nucleus could be involved in ETS-1 regulation raises the possibility that factors that regulate shuttling could also modulate ETS-1 activity. The morphology of NBs changes during the cell cycle, during viral infection, and in response to interferon. In addition, nucleation, turnover, modification, and the interaction of NB components with other factors have all been proposed to alter the composition of NBs. These are all possible modulators of the availability of Sp100 for ETS-1. Interestingly, ETS-1 has also been shown to be negatively regulated by the NB component Daxx, which, like Sp100, interacts with the Pointed domain. However, the connection with NBs was not considered in this study (36). This raises the interesting possi-

FIG. 7. The ETS-1 activation domain C is required for efficient activation of Gal4–ETS-1 fusion proteins by Sp100 (A to C) and transcription regulation by Sp100 mutants through ETS-1 domains ABCD (D) and directly (E). (A) Scheme of the Gal4–ETS-1 fusion proteins. (B) HeLa cells were transfected with the UAS-TK-Luc reporter (1 μg), expression vectors for the indicated Gal4 fusion proteins (0.5 μg), pSG5-Sp100 (0.1 μg, bars 2, 5, 8, 11, and 14; 0.5 μg, bars 3, 6, 9, 12, and 15), pSG5-VP16-Sp100 (0.1 μg, bars 17 and 21; 0.5 μg, bars 18 and 22; 1 μg, bars 19 and 23), and the internal control pSG5-LacZ (all bars). Corrected luciferase activities were used to calculate the fold activation relative to transfections lacking Sp100. The values are from at least three independent experiments with at least two DNA preparations. (C) Expression of Gal4–ETS-1 proteins. A total of 2 μg of pSG5-Gal4, pSG5-Gal4-ABCD, pSG5-Gal4-ABC, pSG5-Gal4-AB, and pSG5-Gal4-DEF was cotransfected without (−) or with (+) 4 μg of the Sp100 expression vector in COS-7 cells, and whole-cell extracts were prepared. Comparable amounts of extracts, based on protein concentration, were analyzed by SDS-PAGE and Western blotting with a mixture of monoclonal antibodies against Gal4 (2GV3 and 3GV2) at a 1:1,000 dilution. The sizes of the marker bands are indicated on the left (molecular weight [10^{-3}]). (D and E) HeLa cells were transfected with the reporter UAS-TK-Luc (1 μg), the internal control pSG5-LacZ (0.5 μg), and the expression vectors pSG5-Sp100 (D, 0.1 μg, bar 3; 0.5 μg, bar 4), pSG5-Sp100.C (D, 0.1 μg, bar 5; 0.5 μg, bar 6), pSG5-Sp100.N (D, 0.1 μg, bar 7; 0.5 μg, bar 8), pGal4 (pG4MpolyII; D and E, 0.5 μg, bars 1), pGal4-ABCD (D, 0.5 μg, bars 2 to 8), pGal4-Sp100 (E, 0.1 μg, bar 2; 0.5 μg, bar 3; 2 μg, bar 4), pGal4-Sp100.C (E, 0.1 μg, bar 5; 0.5 μg, bar 6; 2 μg, bar 7), and pGal4-Sp100.N (E, 0.1 μg, bar 8, 0.5 μg, bar 9, 2 μg, bar 10). Corrected luciferase activities were used to calculate the fold activation relative to the controls (D and E, bars 1). The values are from at least three independent experiments with at least two DNA preparations.

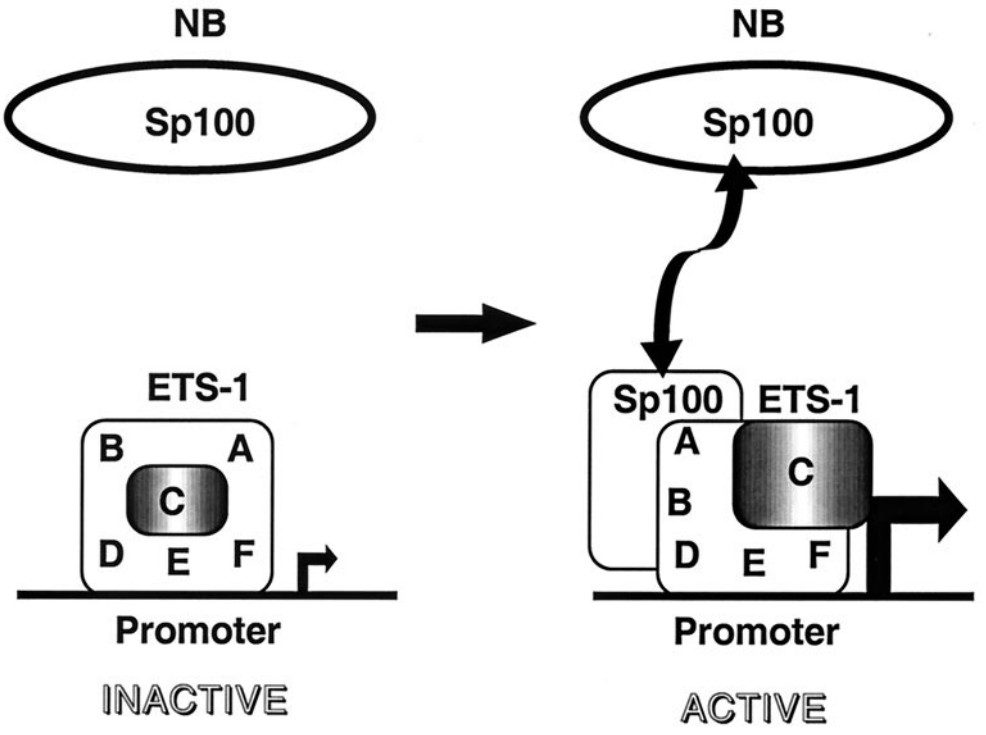


FIG. 8. Working model of Sp100 activation of ETS-1. Promoter bound ETS-1 is inactive, possibly due to the activation domain C being masked or incorrectly folded. Sp100 is mainly confined to NBs. Cellular events that increase the availability of Sp100 (e.g., release from NBs) result in complex formation between Sp100 and promoter-bound ETS-1. A conformation change in ETS-1 leads to the interaction of the activation domain with the transcription machinery and transcription activation.

bility that factors that differentially affect the segregation of Sp100 and Daxx to NBs would have profound effects on ETS-1 activity. Koken et al. (31) have also implicated NBs in ETS-1 regulation. They found that PML is upregulated in proliferative disorders, in locations similar to those of ETS-1 and ets responsive genes (76), and suggested that their functions may be related. Our results provide an important new connection between ETS-1, the structure of NBs, and the functions of Sp100 in transcription regulation and suggest new important pathways for the physiological role of NBs.

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